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1   **Effect of hydraulic retention time on the modelling and optimization of joint 1,3 PDO and**

2   **BuA production from 2G glycerol in a chemostat process**

3

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13
14   **ABSTRACT**

15   This study investigated the possibility to perform statistical optimization of an enriched mixed microbial

16   consortium, MMC, fed in continuous with a highly inhibiting 2G glycerol, for the joint production of 1,3

17   propanediol (1,3 PDO) and butyric acid (BuA). Key variables taken into consideration were HRT, pH

18   and feed concentration. The optimized process reached a glycerol consumption rate of 137 g/L/d and a

19   maximum predicted production rate of 82.61 g/L/d and 21.13 g/L/d for 1,3 PDO and BuA, respectively.

20   This corresponded to a 2.2 and 1.9-fold increase compared to the non-optimized process. The model was

21   able to satisfactorily predict the joint maximum production of 1,3 PDO and BuA (22.6 and 8.1 g/L

22   respectively) of the steady state also in non-sterile conditions, implying thus that MMC can be a robust

23   and reliable biological production platform.

24

25   **Key words:** HRT, continuous fermentation, statistical optimization, joint production, mixed microbial consortia,

26   2G crude glycerol.

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28

29   **INTRODUCTION**

30   In the frame of a Circular Economy there is an increasing need for valorizing by-products/waste streams

31   and developing so-called "markets of secondary products" [1]. However, in order to create such

32   "secondary markets" we need to develop more efficient processes that can utilize new and renewable

33   feedstocks and become competitive compared to consolidated technologies based on non-renewable

1

1   resources. In this context, the valorization of crude glycerol derived from the biodiesel industry into

2   green chemicals and biofuels is considered of paramount importance [2] and has been largely

3   investigated in the last decade, due to its increased availability. In fact, the production of crude glycerol is

4   expected to surpass the commercial demand for purified glycerol [3], thus negatively affecting the

5   economic viability of the small-medium sized biodiesel industries [4,5]. Consequently, crude glycerol is

6   becoming a cheap and abundant feedstock, which can be used as a platform for biobased higher-value

7   products. Nevertheless, valorization of crude glycerol derived from second generation (2G) biodiesel

8   and, in particular, bioconversion of crude glycerol originated from the processing of animal fat has not

9   been sufficiently investigated yet [6]. Bioconversion of such a feedstock could represent several

10   advantages, especially due to its lower costs.

11   Clearly, a major challenge in the biological exploitation of low-grade feedstocks is the development of

12   microbial strains and/or communities able to withstand the inhibiting compounds, such as organic

13   solvents, long chain fatty acids or salts, present in the crude glycerol [7]. A major advantage, on the other

14   hand, is represented by the possibility to convert this substrate into a large number of very different

15   compounds, including 1,3 propanediol, 2,3 butanediol, ethanol, butanol, volatile fatty acids, lactic acid,

16   succinic acid, dihydroxyacetone, polyesters, acrolein, hydrogen, methane, polyhydroxyalkanoates,

17   trehalose, vitamin B12, ß-carotene, etc. [8–11].

18   So far, most studies have investigated the use of pure strains, which are easier to control, and often lead

19   to high yields. Glycerol-fermenting species that can be typically found in literature comprise for instance

20   *Citrobacter freundii , Klebsiella pneumonia , K. oxytoca, K. planticola, Enterobacter agglomerans,*

21   *Enterobacter aerogenes, Clostridium pasteurianum, C. butyricum, C. acetobutylicum, Escherichia coli,*

22   *Lactobacillus sp., Bacillus sp*., etc. [12–18]. In most cases, their fermentation pathways have been

23   explored and the processes optimized. On the other hand, an increasing number of studies are suggesting

24   that exploring the available diversity in nature to developing mixed microbial consortia (MMC) is a

25   promising alternative approach, that can provide interesting results (for instance in terms of conversion

26   efficiencies, reduced substrate and operating costs due to unnecessary substrate pretreatment or

27   sterilization, etc.), particularly in the case of industrial waste feedstock containing compounds of

28   undefined composition [19–22]. In fact, the ability of the enriched MMC to create synergistic effects can

2

1   help degrading complex substrates with different grades of impurities, such as i.e. different

2   concentrations of salts, ashes, soaps, methanol or long chain fatty acids that can be found in different

3   crude glycerol types (which depend on the characteristics of the initial feedstock and the

4   transesterification process used) [6]. The exploitation of such synergies can thus represent an important

5   strategy in the conversion of waste to bioproducts, where the combination of biological and chemical

6   pathways leads to the development of a so-called "carboxylation platform" (with MMC

7   processes being used to generate a mixture of carboxylates as intermediate platform chemicals towards

8   generation of complex chemicals and fuels [19]).

9   Besides their ability to metabolize a wider range of carbon sources, MMC further offer the advantage of

10   cheaper inocula generation and maintenance, higher robustness against contaminations, as well as a more

11   cost effective alternative to genetically engineered strains [23,24]. This is of particular importance in the

12   case of 2G crude glycerol derived from meat processing waste, which is known to have increased

13   inhibiting effect in comparison to crude glycerol types derived from plant and/or cooking oils [25].

14   Nonetheless, the successful development of this type of bioprocesses depends not only on the availability

15   of robust strains and/or communities, but also on the optimization of the process parameters of the

16   fermentation, which will improve the efficiency and stability of the process itself [26,27].

17   So far, most optimization studies for bioprocesses have focused on fermentations using pure cultures, to

18   guarantee stability and easier process control. A previous work by the authors showed the possibility to

19   apply statistical optimization also to MMC, when working with a properly enriched and stable functional

20   consortium (*sensu* Adav [28]), that can be considered as a "superorganism" acting in a stable and

21   reproducible way, from a metabolic/functional point of view [27].

22   Statistical optimization is often applied for the improvement of biotechnological processes [26,29,30]. It

23   is typically performed in batch mode, allowing for statistical independent experiments [27], higher

24   amounts of replicates and faster operation times. Moreover, it allows to evaluate the interaction among

25   the different variables, which cannot be determined when changing one variable at a time [31], and

26   provides mathematical models that can describe both, chemical and biochemical processes [32]. There is

27   a large number of studies dedicated also to the use of statistical optimization of fermentation processes.

28   Most of them were used to optimize the medium composition and/or main key performance variables of

1   batch processes [33–37]. In some cases the authors have subsequently validated the results in continuous

2   mode [23]. However, optimal conditions obtained in batch operations are not necessarily corresponding

3   to the best ones in continuous mode, since they do not take into consideration important factors of

4   continuous processes, such as the dilution rate/hydraulic retention time (HRT) and, more importantly, its

5   effect on the other parameters. Interestingly, the use of continuous processes might offer several

6   advantages in terms of future industrial applications, also in the case of glycerol valorization processes

7   [32,38]. Such advantages may include higher volumetric production rate (with an important impact on

8   the reduction of the reactor size and capital investment), as well as a more intensive use of the equipment,

9   while facilitating operations from a control standpoint [7,39,40]. Nonetheless, only extremely few studies

10   have addressed statistical optimization in continuous mode, mainly focusing on chemical reaction

11   processes [41,42]. Moreover, there are not many studies concerning continuous operations for glycerol

12   fermentation processes [7,43–46] and to the best of our knowledge none regarding statistical optimization

13   including the HRT, which has a fundamental effect on the fermentation process. The study by Silva and

14   colleagues [47] is, to our knowledge, the only one investigating the effect of HRT and pH on glycerol

15   fermentation for H_2 production by MMC and applied a first-order regression model (so rather a screening

16   design) on the experimental data to predict yields at different pH and HRT values. However, the focus of

17   the study was on the discussion of microbial diversity, so the authors did not perform a complete

18   statistical optimization that would consider the interactions among variables. Dubey and colleagues [48],

19   on the other hand, performed a complete statistical optimization to a bioprocess in CSTR (not involving

20   glycerol), without considering the effect of HRT though. Since one of the major advantages of growing

21   microbes in chemostats over batch cultures is exactly the ability to experimentally control the growth rate

22   of cells [49] through the HRT, it would be important to take this into consideration when working in

23   continuous operation.

24   In a previous study the authors have selected different MMC and tested their stability in continuous

25   processes [6,7]. The overall idea was to develop a two-stage process in which glycerol fermentation

26   would be coupled to the production of polyhydroxyalkanoates (PHA) through the selective consumption

27   of butyric acid, while leaving intact the 1,3 PDO, thus facilitating its recovery [50] (or eventually through

28   the conversion of 1,3 PDO and butyric acid to PHA [51]). Therefore, the joint production of 1,3 PDO and

1 butyric acid is of particular interest for the full exploitation of the crude glycerol as a carbon source (in

2 fact butyric acid is considered as a preferred substrate for PHA production [52]), leading to the

3 production of two high-value compounds. In such a two-stage approach, maximizing the production of

4 these specific metabolites (1,3 PDO and butyric acid) is thus a necessary prerequisite to obtain the

5 highest amount of high-value products. Moreover, the co-production of 1,3 PDO and butyric acid has

6 concrete industrial application potentials in itself. In fact, in May 2017 the industrial biochemicals

7 company METEX, which is specialized in fermentation processes, announced that its strategic priority

8 was to become the "market leader of natural butyric acid for animal feed and benchmark producer of

9 GMO-free 1,3 PDO for cosmetic applications". METEX is planning to conclude the construction of their

10 facilities in 2018 and reach a production capacity of 24 Kt (with 20 Kt of 1,3 PDO and 4 Kt of butyric

11 acid) [53].

12 The aim of the present study was to significantly contribute to creating new knowledge and to develop

13 the first step of this process by 1) applying statistical optimization to the continuous process, taking into

14 consideration the effect of the HRT on the substrate concentration and pH; 2) using MMC grown on non-

15 purified 2G crude glycerol; and 3) increasing the productivities and product concentrations of both, 1,3

16 PDO and butyric acid.

17

18 **MATERIAL AND METHODS**

19 **2.1 Inoculum**

20 The MMC was previously enriched from heat-treated anaerobic sludge, collected from the Municipal

21 Wastewater Treatment plant in Lundtofte (DK), as described in [6]. It was chosen among a total of 8

22 different MMC tested, based on its better performance in terms of stability, kinetics and production [7].

23 The community was mainly characterized by *Clostridium,* representing by far the main genus (with an

24 average relative abundance of 34.25%), followed by *Blautia* (26.33%), *Unclassified genera* (6.21%),

25 *Klebsiella* (6.07%), *Pseudomonas* (5.93%) and *Ruminococcus* (5.50) [7]. For statistical reasons, to

26 guarantee the same activation conditions throughout the statistical experiments, a large amount of MMC

27 was prepared beforehand and frozen into aliquots, to be used as inoculum for the pre-activation.

28 **2.2 Inoculum storage and activation**

1   Inoculum samples were stored in the freezer at -18°C. Prior to use, the frozen mixed culture was

2   transferred to the refrigerator at 4°C, for 2 hours, and then for an additional hour at room temperature,

3   before being inoculated. 125 mL serum vials were used for batch experimentation. 45 mL of synthetic

4   growth medium (described in the following paragraph) were flushed for 5 minutes with a mixture of 80%

5   N_2 and 20% CO_2, in order to obtain anaerobic conditions, prior to inoculation, and incubated at 37°C and

6   150 rpm. Gas and liquid samples were collected regularly. Batches at 24 h fermentation (exponential

7   phase) were used as inoculum for continuous experiments. In all experiments, 10% v/v inoculum was

8   used to start up the continuous fermentation.

9   **2.3 Growth Medium**

10   BA medium was prepared from the following stock solutions (chemicals in g/L of double distilled

11   water): (A) NH_4Cl, 100; NaCl, 10; $MgCl_2 \cdot 6H_2O$, 10; $CaCl_2 \cdot 2H_2O$, 5; (B) $K_2HPO_4 \cdot 3 H_2O$, 200; (C) trace

12   metal and selenite solution: $FeCl_2 \cdot 4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCI_2 \cdot 2H_2O$, 0.038; $MnCl_2 \cdot 4H_2O$,

13   0.05; $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05; $NiCl_2-6H_2O$, 0.092;

14   ethylenediaminetetraacetate, 0.5; concentrated HCl, 1 mL; $Na_2SeO_3 \cdot 5H_2O$, 0.1; (D) $NaHCO_3$ 52 g/L; (E)

15   vitamin mixture according to Wolin and colleagues [54]. The following stock solutions were added to

16   974 mL of redistilled water: A, 10 mL; B, 2 mL; C, 1 mL; D, 50 mL; E, 1 mL [55].

17   Crude glycerol was provided by Daka Biodiesel (Denmark) and was used as the only carbon source. This

18   crude glycerol was obtained from the transesterification of animal fats and butchery waste (based on

19   animal fat categories 1 and 2 according to the EU regulation numbers 1069/2009 and 142/2011) and

20   represented a highly inhibiting matrix for non adapted/pure strains. The main characteristics of this type

21   of crude glycerol have been presented elsewhere [6].

22   **2.4 Continuous Experiments**

23   Continuous experiments were run in two 1L INFORS HT Multifors Reactors (using a working volume of

24   0.5 L), with the software IRIS loaded on the relevant controllers. Offgas flow was measured through a

25   Ritter MilliGas counter (Type MGC-1). pH was controlled through the addition of alkali (KOH 4 M) and

26   the temperature was kept at 37 °C. The reactor was flushed for 20 minutes with a mixture of 80% N_2 and

27   20% CO_2 to obtain anaerobic conditions prior to inoculation (10 % v/v). The feed vessels were also

28   flushed with 80% N_2 and 20% CO_2 and maintained under the same atmosphere throughout the

1 experiments, to secure anaerobic conditions. The medium was sterilized in 20L blue cap bottles, in order

2 to avoid the introduction of non-controlled variability, due to eventual microbial growth (which would

3 have unpredictable effects on the substrate composition). Tubes and media were changed every 4-5 days.

4 The sterilization in the 20L blue caps caused a certain loss of volume, due to water evaporation, that

5 determined up to 10% increase of the initial glycerol concentration. The outlet vessel was changed

6 regularly and was connected with a vessel filled with water, to discharge pressure and to prevent air inlet

7 [7]. HRT values were calculated based on the average volume of liquid discharge every day in the

8 effluent. Oscillations from the set value were observed, due to typical imprecision of small peristaltic

9 pumps and the occasional growth of biofilm inside the tubes. Substrate consumption rate (R_{Gly}) during

10 steady state was calculated according to the following equation:

11 $R_{Gly} = (Gly_0-Gly) \cdot D$ Eq. 1

12 Where D is the dilution rate (h^{-1}), Gly_0 is the glycerol concentration in the feed and Gly the concentration

13 of glycerol in the reactor at steady state. For a data consistency check, a carbon recovery (Rc) was

14 calculated [7] using the average values of the metabolites obtained at each steady state (expressed as

15 carbon moles), divided by the carbon moles of glycerol consumed.

16 **2.5 Statistical Optimization**

17 Based on the previous experiments in continuous mode [7], three design variables were chosen to be

18 investigated: pH (range 5-7), HRT (range 6h-18h) and glycerol concentration (10-50 g/L). The response

19 variables were: butyrate production rate (g/L/d), butyrate concentration (g/L), 1,3 PDO production rate

20 (g/L/d) and 1,3 PDO concentration (g/L). The ranges were chosen (based on preliminary results) in a way

21 to avoid experimental conditions that would lead to cell washout [7]. Statistical optimization was

22 performed by applying an Inscribed Central Composite (ICC) Design, using the software Design Expert

23 9 (Stat-Ease, USA). The software defined 17 experimental conditions (runs), with three replicates at the

24 central point, which were used to evaluate the variability of the system and perform the ANOVA and

25 response surfaces. Each run was performed for a period of 20 HRTs, in order to guarantee the

26 establishment of steady state and the ability to maintain it [7]. The average values of the responses

27 obtained during the steady state (corresponding to an operation period of approximately 6 HRTs with less

28 than 30% deviation) were used for the statistical model. Response Surface Methodology (RSM) was thus

7

1   applied to investigate the experimental region and predict the optimal conditions, according to the

2   second-order polynomial function:

3    $y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$ Eq. 2

4   Where y is the predicted response, β_0 a constant, β_i the linear coefficients, β_{ii} the squared coefficients,

5   and β_{ij} the cross-product coefficients. The joint maximization of 1,3 propanediol and butyric acid was

6   evaluated using the "desirability" (D) concept for the optimization of dual goals, according to Oddone

7   and colleagues [56], where the desirability for an experimental response value can range between 0 (for

8   the lowest value) and 1 (in the case of the highest observed value).

9   **2.6 Analytical Methods**

10   Detection and quantification of glycerol, ethanol, 1,3 PDO, lactic acid and VFAs were obtained with a

11   Shimadzu HPLC equipped with a Aminex HPX-87H column (BioRad) operated at 60°C, as previously

12   described [6,27]. In brief, a solution of 12mM H_2SO_4 was used as eluent at a flow rate of 0.6 mL/min.

13   Samples were diluted 1:5 with deionized H_2O, acidified with a 10% w/w solution of H_2SO_4, centrifuged

14   for 10 min at 10000 rpm and filtered with a 0.22 mm membrane, before injection. The concentrations and

15   standards used for the calibration curves are reported in Table S1. The HPLC method and reproducibility

16   of results were tested before starting this study, to evaluate the potential effect of instrumental variability.

17   Microbial biomass was estimated through the determination of Total Suspended Solids (TSS), according

18   to standard methods [57]. Absorbance of samples was measured every day at an optical density of 600

19   nm (OD600) and microbial biomass concentration was calculated based on the correlation curve of TSS

20   concentration with OD.

21   The yields were calculated as the ratio between the product concentration (expressed in g) and the

22   glycerol consumed (in g), while productivities were obtained by dividing the average concentration,

23   measured during the steady state, by the HRT.

24

25   **3 RESULTS AND DISCUSSION**

26   **3.1 Statistical Optimization**

8

1   The main results of the experimental runs of the ICC Design are reported in Table 1, while additional

2   information about the concentration of the main metabolites and the cell biomass can be found in Table 2

3   (carbon recovery is shown in Table S2). The best productivities were obtained with a glycerol (feed)

4   concentration of 41.9 g/L, HRT of 8.4 h and pH of 6.6 (Run 17), which showed a rate of up to 60 g/L/d

5   and 15 g/L/d for 1,3 PDO and BuA, respectively, while reaching a maximum glycerol consumption rate

6   of 137 g/L/d. It is worth noting that this condition represented the best compromise for joint maximized

7   productivity and concentration of products, also reaching one of the highest biomass concentrations (of

8   around 2 g/L; Table 2). Run 15 (41.9g/L;15.6h HRT; pH 6.6), which had a higher HRT, reached the

9   highest 1,3 PDO and BuA concentrations, with 20.7 g/L and 6.2 g/L respectively. High concentrations

10   were also obtained in Run 6, using 50 g/L glycerol, 12h HRT and pH = 6.

11   **[Table 1]**

12   Low biomass, together with high residual glycerol concentration in the reactor, seemed to be associated

13   to low pH (and high feed concentration), as can be seen in run 7, run 14 and 16 (Table 2). Run 7 in

14   particular, showed a very poor performance for all responses, with low concentrations and production

15   rates, together with a very low biomass concentration (0.43 g/L). The experiment was performed at the

16   lowest pH (set at 5) and led to a high residual substrate concentration (23.5 g/L compared to a feed

17   concentration of 30 g/L) in the fermentation broth (Table 2), despite the relatively high HRT (12h). A

18   similar situation was observed in the case of the experiment 16, run at a pH 5.4 (41.9 g/L and 8.4 h

19   HRT), which showed the lowest BuA concentration (0.02 g/L) as well as the lowest cell concentration

20   (with only 0.24 g/L) and yield (see Table S3). At the same time, this condition also led to the highest

21   residual glycerol concentration in the reactor, reaching more than 31 g/L. A similar situation was

22   observed in the case of Run 14, which was performed at the same conditions, but with a higher HRT.

23   **[Table 2]**

24   It must be underlined that at low pH, the effective maximum growth rate (μ_{max}) may be much smaller

25   than for instance at pH=7 [40]. This might be due to higher energy requirements for maintenance and

26   might explain why the combination of low pH and low HRT led to a very low cell concentration, while at

27   higher pH the same HRT did not have the same effect. The inhibitory effect of pH on glycerol

28   consumption and cell growth was also observed in a batch study by Yang and colleagues [32], who

9

1 reported a rapid increase in glycerol consumption in the pH range from 4.0 to 7.0, and no cell growth

2 inhibition in the range from 6.5 to 8.

3 **3.2 Statistical Model**

4 Four responses were calculated for each experimental run, namely butyric acid production rate ($Y_{BuA\,Rate}$),

5 butyric acid concentration ($Y_{BuA\,Conc}$), 1,3 PDO rate ($Y_{PDO\,Rate}$) and 1,3 PDO concentration ($Y_{PDO\,Conc}$).

6 Multiple regression analysis was applied to the experimental data, in order to model the four responses. A

7 second-order polynomial function (based on Eq.2) was therefore fitted to correlate the relationship

8 between the design variables and the responses. Quadratic equations for the significant terms are shown

9 below (factors having $p > 0.05$ were excluded; for the complete ANOVA see also Table S4):

10

11 $Y_{PDO\,Conc} = 12.5 + 5.7\,A + 6.1\,C + 7.3\,AC - 6.0\,A^2$ Eq.3

12 $Y_{PDO\,Rate} = 26.6 + 12.3\,A - 14.9\,B + 13.4\,C - 5.7\,AB + 15.3\,AC - 10.2\,BC - 12.0\,A^2 + 6.2\,B^2 - 2.7\,C^2$ Eq.4

13 $Y_{BuA\,Conc} = 3.7 + 0.9\,A + 0.8\,B + 1.8\,C + 3.0\,AC - 2.5\,A^2$ Eq.5

14 $Y_{BuA\,Rate} = 5.5 + 1.9\,A - 2.8\,B + 3.9\,C + 5.9\,AC - 1.4\,BC - 2.97\,A^2 + 3.9\,B^2 + 2.2\,C^2$ Eq.6

15

16 where A, B and C are the coded factors of pH, HRT (h) and glycerol concentration (g/L), respectively.

17 Analysis of variance ANOVA showed that the fitting models were highly significant for all responses (p-

18 value < 0.0003). The lack of fit (LOF) was not significant (> 0.05), suggesting that the models were able

19 to represent adequately the data within the experimental region [58]. The coefficient of determination (R-

20 square) was higher than 0.94, thus explaining more than 94 % of the total variability in all cases (Table

21 3), with a Predicted R-square ranging between 0.89 and 0.96 (a comparison between the Observed versus

22 Predicted values for all four responses is shown in Table S5).

23 **[Table 3]**

24 Glycerol concentration (C) and pH (A), as well as their interaction (AC), showed a significant positive

25 effect (p-value < 0.05) on all responses. Higher concentrations of the feed, together with a higher pH,

26 were thus beneficial for the glycerol conversion into 1,3 PDO and BuA. The quadratic effect of pH (A^2),

27 on the other hand, was always negative. With the exception of $Y_{PDO\,Conc}$, HRT was also significant and

28 showed a negative effect in the case of $Y_{PDO\,Rate}$ and $Y_{BuA\,Rate}$, meaning that lower HRT favoured higher

29 productivities, within the studied range. On the contrary, HRT showed a positive effect on $Y_{BuA\,Conc}$, thus

1   implying that an increase in the HRT would favour BuA production (as confirmed by the sensitivity

2   analysis, which showed an 80% increase; Fig. S6), in good agreement with Papanikolaou and colleagues

3   [46].

4   The three-dimensional response surfaces for all responses are shown below. They are based on the

5   equations 3-6 and display the influence of the variables on the four responses, keeping one variable

6   constant at its optimum level, while changing the other two within the experimental range. The response

7   surfaces show the interactions between HRT and pH (Fig. 1 A-D) and between glycerol concentration

8   and pH (Fig. 2 A-D).

9   **[Fig 1 A-D]**

10   The model predicted a maximum 1,3 PDO production rate of 82.6 g/L/d (at pH 6.9, and HRT of 6.2 and a

11   glycerol concentration of 46.9 g/L), and a maximum butyric acid production rate of 21.1 g/L/d (with pH

12   6.9, 6.1h HRT and a glycerol concentration of 45.1 g/L). This corresponds to a 2.2 and 1.9-fold increase

13   in the production rates of 1,3 PDO and BuA, compared to the non-optimized process [7]. Moreover, a

14   maximum 1,3 PDO concentration of 25.1 g/L was predicted by the model (with pH 6.8, 11.1 h HRT and

15   49.7 g/L glycerol), while the maximum predicted butyric acid concentration was 8.0 g/L (with pH 6.4,

16   17.8h HRT and a glycerol concentration of 48.6 g/L).

17   **[Fig 2 A-D]**

18   Comparable results were reported by Chatzifragkou et al., who reached a maximum productivity of 45

19   g/L/d 1,3 PDO (with an HRT of 12.5 h), obtaining, however, significant amounts of residual glycerol

20   inside the chemostat [43]. Similar productivities were also observed by Papanikolaou and colleagues

21   when growing a pure strain of *C. butyricum* on crude glycerol, using single-stage continuous cultures in

22   comparable conditions: 1,3 PDO production rate reached around 80 g/L/d (3.3 g/L/h) with a feed of 60

23   g/L [46]. Even though the authors also reported higher feed concentrations, it is important to bear in mind

24   that the feedstock used in the present study was crude glycerol from animal fat-based biodiesel

25   production, while most of the literature studies focus on plant oils and/or cooking oils [6,27,46].

26   Therefore, a straight-forward comparison is very difficult. In fact, it has been reported that impurities (i.e.

27   (high amount of sulfur and LCFA, very low pH, etc) in crude glycerol (CG) derived from meat

28   processing waste cause increased inhibition in comparison to impurities in CG derived from pure

11

1   substrates [59,60]. In a previous study, for instance, a hexane-extraction step was applied in order to

2   reduce the concentration of lipids and (long chain) fatty acids present in the 2G crude glycerol and

3   evaluate its potential inhibitory effect on the microbial growth [6]. The assumption was that high

4   concentration of LCFA could have a negative interaction with the cell membranes of Gram positive

5   anaerobic bacteria of the anaerobic sludge. It is known that adapted MMC have the ability to create

6   synergistic effects which can improve the degradation of complex substrates (also containing different

7   impurities), both in sterile and non-sterile conditions [6], thus making them of particular interest in

8   fermentation processes [61,62]. For this reason, the use of an adapted MMC was of particular importance

9   in the present study, since preliminary tests had shown inhibition of pure cultures already with 10 g/L of

10   this type of crude glycerol [63], and here effective fermentation of up to 50 g/L glycerol succeeded.

11   Similarly, the study by Khanna and colleagues showed that the percentage of unutilized glycerol by the

12   pure strain *Clostridium pasteurianum* was 89.5% (with an initial glycerol concentration of only 5 g/L)

13   and increased up to 93.9% when using 25 g/L of glycerol [64].

14   It is worth noting that the response surfaces did not show a clear peak (see also [23,48,65,66]), which

15   could suggest that the optimum points were rather at the border of the design boundaries. This might

16   represent a drawback in the application of statistical optimization using chemostats, since the curve of

17   productivity will always lead to a dramatic drop when the dilution rate reaches the critical value (causing

18   cell washout). This means that the shape of the response surface will not reach a clear peak, when the

19   system approaches the maximum growth rate. It might therefore be questioned whether a more extended

20   range of the parameters would not have allowed to reach an even higher performance, even though

21   preliminary results with higher feed concentration (60 g/L) did not improve product concentration or

22   rates (while lower HRT, with a feed concentration of only 35 g/L, was already causing a biomass loss of

23   30%) [7]. It is also worth emphasizing that in the present study, run 16 (41.9g/L;8.4h HRT; pH 5.4)

24   represented already a limit condition, leading to a very low biomass concentration (0.239 g/L, compared

25   to 2.18 g/L of run 1) and a high residual glycerol concentration, which was approaching the feed

26   concentration (thus suggesting the approaching of the wash out dilution [40]). The critical dilution rate

27   was most probably affected by a combination of low HRT, together with the low pH and high glycerol

28   concentration (see Table 2). This experimental condition (run 16) was repeated twice in order to exclude

1   eventual experimental mistakes, but it confirmed to represent the limit of the experimental boundaries,

2   and had to be excluded from the model calculation (since it was causing a decrease of LOF < 0.05 in the

3   case of Y_{BuA}).

4   **3.3 Validating the model by maximizing the joint production of 1,3 propanediol and butyric acid**

5   When developing statistical models it is important to validate them by testing an experimental condition

6   that was not used to build the model itself. Since the scope of the present work was to optimize the

7   fermentation step of crude glycerol (with the idea to use BuA to stimulate production of PHA and

8   recover of 1,3 PDO, in a further step), the validation was run in a way to maximize both, the production

9   of BuA and 1,3 PDO at the same time, within the conditions used for the optimisation. The reason behind

10   this, was that maximizing the titers would allow to achieve the highest amount of high-value products

11   (1,3 PDO and PHA) in a two-stage process (see also [50]), with butyric acid being used as a preferred

12   carbon source for the PHA production [52]. The mathematical model predicted that maximum joint

13   production would be reached under the following conditions: pH = 6.4, HRT = 17.6 and 49.3 g/L of

14   glycerol. Therefore, two reactors were set-up and run in parallel under such conditions. One of the

15   reactors was run in non-sterile conditions to evaluate the robustness of the model as well as the selected

16   MMC, which was able to convert animal fat derived 2G crude glycerol also without sterilization [7].

17   Figure 3 (A-B) shows the distribution of the main metabolites (over a time of 264 h) of the two reactors.

18   Steady state results were calculated from the concentration of 120h to 264h, corresponding to

19   approximately 8 HRTs. The average results of the validation test are reported in Table 4:

20   **[Fig 3 A-B]**

21   The experimental results of the concentrations reported in Table 4 (actual values) represent the average

22   of the two experiments (in sterile and non-sterile conditions) and showed a difference of 9-10% between

23   the predicted and experimental values, which confirms the validity of the models [67,68]. This is

24   considered a satisfactory result, especially when modelling a process with mixed cultures and non-sterile

25   conditions, which can lead to reduction in operational costs [69]. It is worth noting that one of the main

26   drawbacks of the use of CSTR in fermentation process, that is the risk of contamination, was clearly not

27   an issue, since the process operated under non-sterile conditions did not show significant variations from

28   the predicted results. This represents an important advantage of using adapted MMC, where the

13

1 synergistic mechanisms and the adaptation to this substrate makes the community not only better tolerate

2 inhibition, but also outcompete non-selected strains that can be present, due to the lack of sterilization

3 (with significant reduction of operating costs).

4 Statistical optimization provided thus important information to improve the fermentation of a 2G glycerol

5 and can be used as a decision-making tool [70], especially in the view of managing multi-stage processes,

6 due to the possibility to maximize different (individual or joint) responses at the same time. To our

7 knowledge this is the first study to model the interaction among HRT, pH and substrate concentration for

8 the joint production of BuA and 1,3 PDO from a fat-derived (non purified) crude glycerol.

9 **[Table 4]**

10

11 **4 CONCLUSIONS**

12 The results presented in this study confirmed the possibility to perform statistical optimization of an

13 enriched MMC fed in continuous with a highly inhibiting (non-purified) 2G glycerol and model

14 satisfactorily the interactions between HRT, pH and feed concentration. The adapted MMC reached a

15 glycerol consumption rate of 137 g/L/d. Maximum predicted production rates were 82.6 g/L/d and 21.1

16 g/L/d for 1,3 PDO and BuA, respectively. This corresponded to a 2.2 and 1.9-fold increase compared to

17 the non-optimized process. The model was able to predict the joint maximum production of 1,3 PDO and

18 BuA (22.6 and 8.1 g/L respectively) also in non-sterile conditions, implying also that MMC can actually

19 be a robust and reliable biological production platform to work with. Despite the challenge of modelling

20 a continuous process when approaching the critical (wash out) dilution, the experimental design proved

21 to be a valuable and flexible decision-making tool.

22

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CAPTIONS

Tables

Table 1. ICC Design: effect of pH, HRT and glycerol concentration on the responses and the substrate consumption rate.

Table 2. Concentrations of glycerol, cells and main soluble metabolites in the reactor.

Table 3. ANOVA results for the quadratic equations modelling the four responses.

Table 4. Validation of the statistical model showing the difference between the predicted and the experimental results for joint 1,3 PDO and BuA production.

Figures

Figure 1. Three-dimensional response surface for the maximum 1,3 PDO concentration a), butyric acid concentration b), 1,3 PDO rate c), and butyric acid rate d), keeping glycerol concentration constant it its optimal value (for 1,3 PDO conc: 49.7 g/L; 1,3 PDO rate: 46.9 g/L; BuA conc:48.6 g/L; BuA rate: 45.1 g/L), while changing pH and HRT.

Figure 2. Three-dimensional response surface for the maximum 1,3 PDO concentration a), butyric acid concentration b), 1,3 PDO rate c), and butyric acid rate d), keeping HRT constant it its optimal

value (for 1,3 PDO conc: 11.1 h ; 1,3 PDO rate: 6.2 h; BuA conc: 17.8 h; BuA rate: 6.1 h) while

changing pH and glycerol concentration.

Figure 3. Concentration of the main metabolites and substrate concentration in 2 reactors run for

264 h for the validation tests in sterile a) and non-sterile conditions b). The reactors were started up

as a batch (with 20 g/L glycerol) and after 24 h they were shifted to continuous operation with a

feed of around 50 g/L.

Table 1.

Runs	Set Parameters			1,3 PDO	BuA	$R_{1,3\ PDO}$	R_{BuA}	R_{Gly}
	pH	*HRT*	*Gly*	g/L	g/L	g/L/d	g/L/d	g/L/d
1	**6.0**	**18.0**	**30.0**	11.9	5.5	16.2	7.1	42.3
2	**6.0**	**12.0**	**10.0**	5.0	1.7	10.5	3.7	25.1
3	**6.0**	**12.0**	**30.0**	13.3	3.5	27.1	5.6	62.9
4	**7.0**	**12.0**	**30.0**	11.9	2.0	25.6	4.2	61.5
5	**6.0**	**6.0**	**30.0**	13.1	3.1	48.2	11.8	113.8
6	**6.0**	**12.0**	**50.0**	18.5	6.1	36.0	11.8	94.4
7	**5.0**	**12.0**	**30.0**	1.2	0.4	2.3	0.9	8.7
8	**6.0**	**12.0**	**30.0**	13.3	3.3	25.5	5.3	60.7
9	**5.4**	**15.6**	**18.1**	6.6	2.5	10.7	3.9	25.5
10	**6.0**	**12.0**	**30.0**	12.7	2.9	27.3	5.9	64.4
11	**6.6**	**15.6**	**18.1**	7.8	1.6	11.1	2.0	29.9
12	**5.4**	**8.4**	**18.1**	5.5	2.2	16.2	6.5	47.1
13	**6.6**	**8.4**	**18.1**	8.1	1.5	24.9	5.2	53.9
14	**5.4**	**15.6**	**41.9**	6.8	2.5	9.0	3.4	23.10
15	**6.6**	**15.6**	**41.9**	20.7	6.2	31.1	9.7	65.1
16	**5.4**	**8.4**	**41.9**	6.2	0.0	18.3	0.1	36.8
17	**6.6**	**8.4**	**41.9**	19.0	4.8	59.3	15.0	137.4

R_{Gly} = glycerol consumption rate; $R_{1,3\ PRO}$ = 1,3 propanediol production rate; R_{BuA} = butyric acid production rate.

Table 2.

	Set conditions	Residual Glycerol g/L	1,3 PDO g/L	Butyric acid g/L	Lactic acid g/L	Acetic acid g/L	Cell biomass g/L
Run1	30 g/L; 18h HRT;pH 6	0.43 ± 0.23	11.88 ± 0.44	5.45 ± 0.32	2.69 ± 1.43	0.41 ± 0.05	2.18 ± 0.22
Run2	10 g/L; 12h HRT; pH 6	0.11 ± 0.07	4.96 ± 0.25	1.71 ± 0.37	0.24 ± 0.28	0.25 ± 0.17	1.18 ± 0.28
Run3	30g/L; 12h HRT; pH 6	2.39 ±1.39	13.26 ± 0.89	3.48 ± 0.23	2.98 ± 0.56	1.02 ±0.08	1.37 ± 0.40
Run4	30g/L; 12h HRT; pH 7	0.81 ± 0.60	11.86 ± 1.06	2.01 ± 0.35	1.82 ± 0.34	0.523 ± 0.20	1.41 ± 0.17
Run5	30g/L; 6h HRT; pH 6	1.65 ± 1.72	13.11 ± 0.96	3.01 ± 0.74	2.00 ± 1.12	0.94 ± 0.23	1.70 ± 0.18
Run6	50g/L; 12h HRT; pH 6	3.96 ± 1.47	18.48 ± 1.04	6.13± 0.87	4.55 ± 0.66	0.92 ± 0.20	2.04 ± 0.19
Run7	30g/L; 12h HRT; pH 5	24.92 ± 0.58	1.17 ± 0.20	0.43 ± 0.12	0.76 ± 0.10	0.21 ± 0.02	0.43 ± 0.10
Run8	30g/L;12h HRT;pH 6	2.09 ± 0.65	13.26 ± 0.78	3.27 ± 0.46	3.68 ± 0.68	1.08 ± 0.25	1.32 ± 0.29
Run9	18.1g/L;15.56h HRT; pH 5.4	2.44 ± 032	6.60 ± 0.27	2.49 ± 0.13	1.48± 0.20	0.37 ± 0.02	1.06 ± 0.19
Run10	30g/L;12h HRT;pH 6	2.06 ± 0.53	13.73 ± 1.09	2.93 ± 0.38	3.36 ± 0.85	1.17 ± 0.27	1.48 ± 0.17
Run11	18.1g/L;15.56h HRT; pH 6,59	0.08 ± 0.03	7.77 ± 0.22	1.55 ± 0.12	0.06 ± 0.06	0.23 ± 0.01	1.44 ± 0.11
Run12	18.1g/L;8,43h HRT; pH 5.4	3.97 ± 1.34	5.50 ± 0.57	2.15 ± 0.29	1.07 ± 0.15	0.39 ± 0.01	0.93 ± 0.03
Run13	18.1g/L;8,43h HRT; pH 6,59	0.19 ± 0.08	8.10 ± 0.35	1.49± 0.22	1.81 ± 0.33	0.34 ± 0.19	0.89 ± 0.11
Run14	41,89g/L;15,56h HRT; pH 5.4	30.92 ± 1.34	6.75 ± 0.85	2.51 ± 0.24	1.79 ± 0.20	0.36 ± 0.07	048 ± 0.14
Run15	41,89g/L;15,56h HRT; pH 6,59	0.19 ± 0.08	20.70 ± 0.68	6.22 ± 0.34	0.79 ± 0.36	0.37 ± 0.04	1.77 ± 0.15
Run16	41,89g/L;8,43h HRT; pH 5.4	31.18 ± 2.11	6.12 ± 0.05	0.02 ± 0.01	0.21 ± 0.14	0.28 ± 0.01	0.24 ± 0.03
Run17	41,89g/L;8,43h HRT; pH 6,59	0.71 ± 0.27	18.99 ± 0.71	4.81 ± 0.14	3.55 ± 0.46	0.474 ± 0.03	1.98 ± 0.37

Table 3.

	p-value	R-square	Adjusted R-square	Pred R-Squared	LOF
PDO Conc	< 0.0001	0.983	0.968	0.893	0.071
PDO Rate	< 0.0001	0.996	0.991	0.964	0.305
BuA Conc	0.0003	0.940	0.888	0.784	0.166
BuA Rate	< 0.0001	0.994	0.984	0.942	0.231

Table 4.

	Predicted Values g/L	**Actual values** g/L	**C.V.** %
$Y_{PDO\ Conc}$	22.6	20.7 ± 1.2	9.1
$Y_{BuA\ Conc}$	8.1	7.4 ± 0.8	10.3

C.V. = coefficient of variability

Figure 1.

a) 1,3 PDO concentration (g/L)

b) Butyric acid concentration (g/L)

c) 1,3 PDO productivity (g/L/d)

d) Butyric acid productivity (g/L/d)

Figure 2.

a) 1,3 PDO concentration (g/L)

b) Butyric acid concentration (g/L)

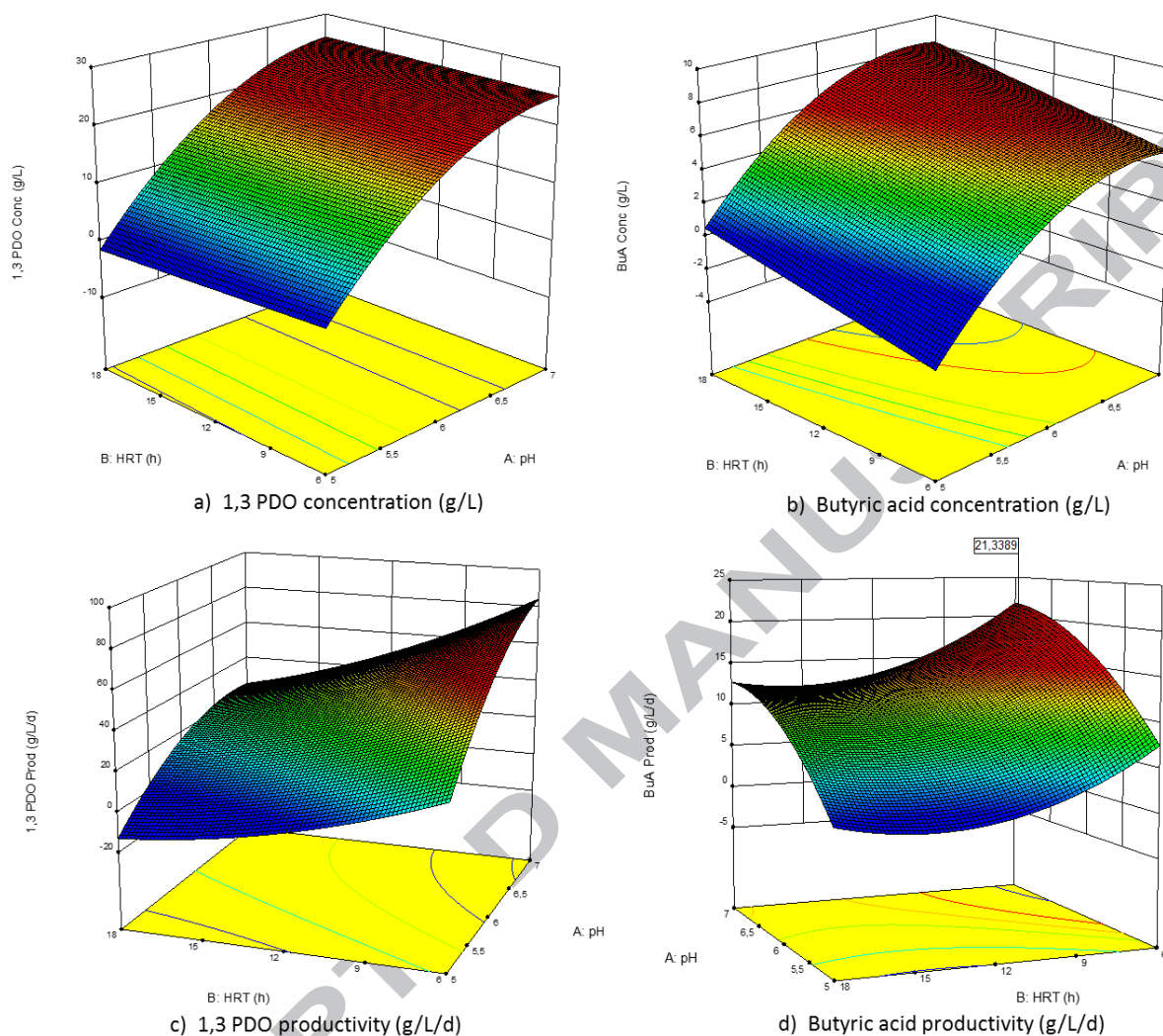c) 1,3 PDO productivity (g/L/d)
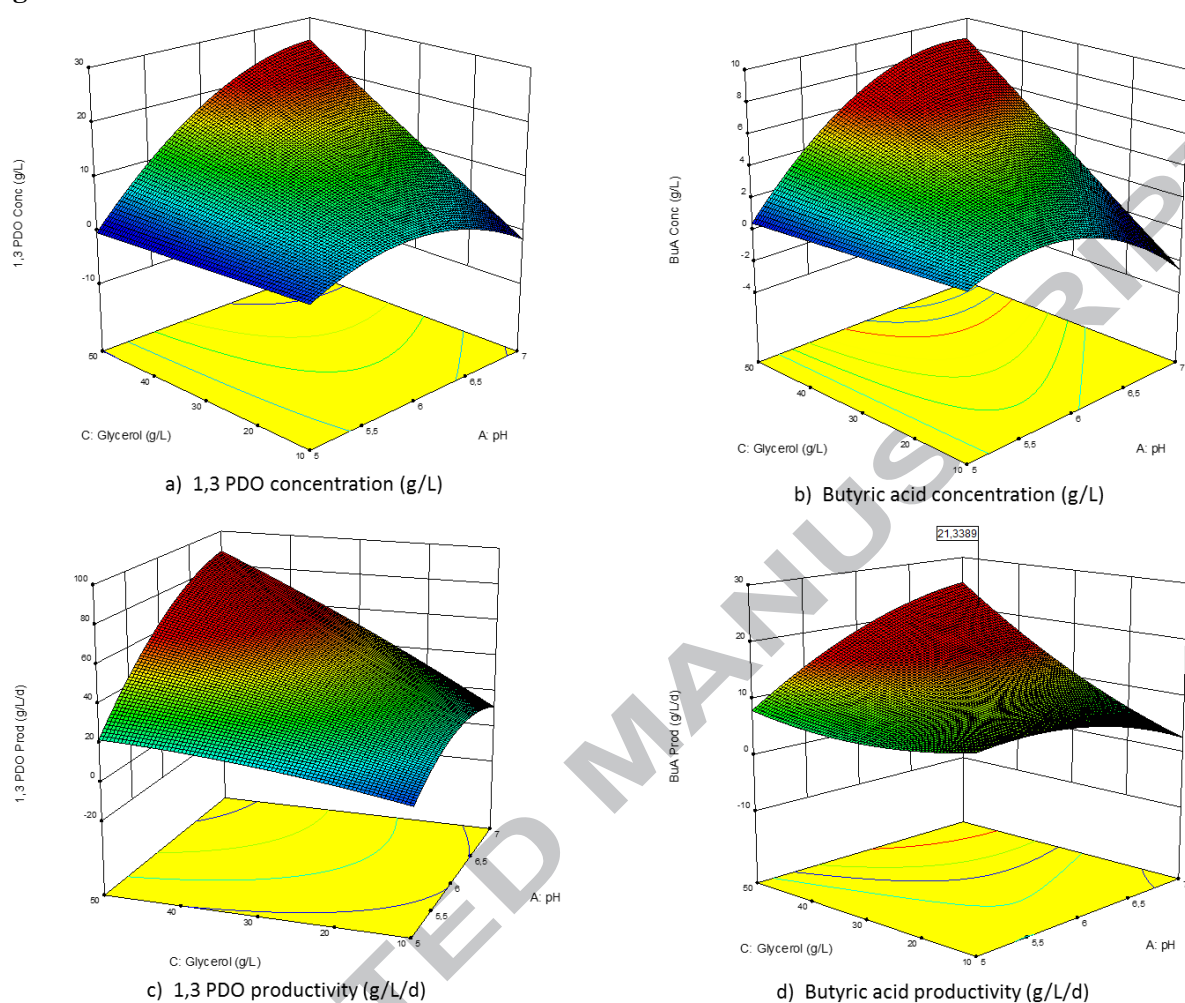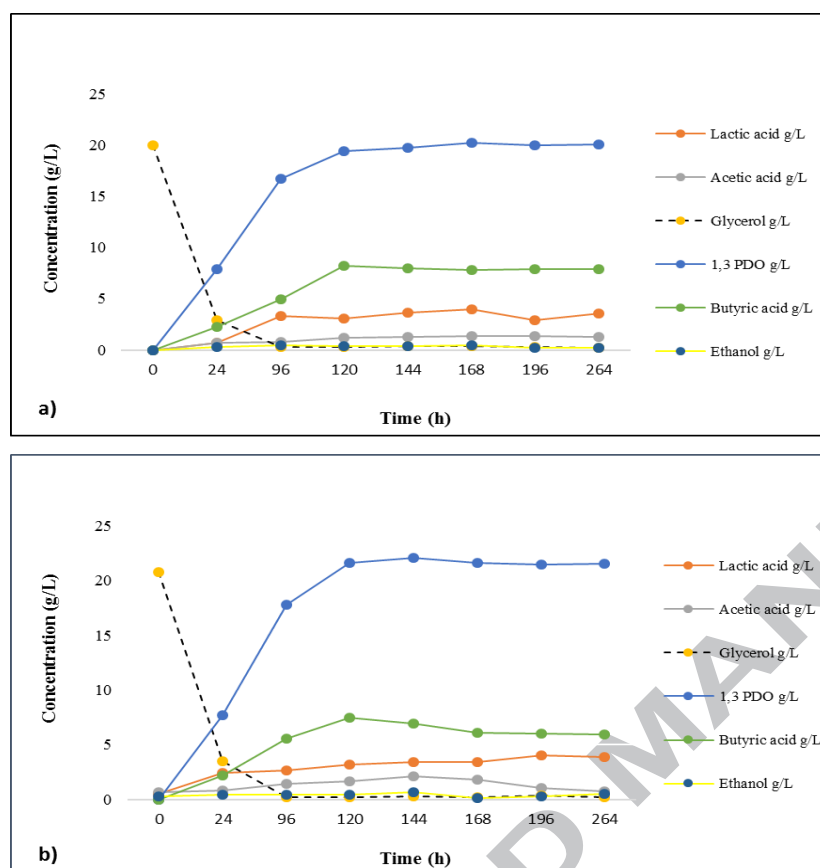
d) Butyric acid productivity (g/L/d)

Figure 3.

HIGHLIGHTS:

- **Statistical optimization of a continuous fermentation process involving HRT**

- **Experimental design using mixed microbial consortia**

- **Validation of model also in non-sterile conditions**

- **High substrate degradation and productivities using an animal-fat derived (2G) crude glycerol**

GRAPHICAL ABSTRACT