The combined effect on initial glucose concentration and pH control strategies for acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* DSM 792

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Abstract

The use and depletion of fossil fuels raised the interest in biofuels like biobutanol. *Clostridium acetobutylicum* DSM 792 is capable of producing biobutanol through ABE fermentation. Butanol production can be influenced by low sugar concentrations, like those obtained after hydrolysis of pre-treated lignocellulosic biomass. This study aimed to evaluate the influence of the initial glucose concentrations (33, 66 and 100 g L^{-1}) and pH control strategies on biobutanol production and glucose consumption. Uncontrolled pH fermentation exhibited low butanol production due to either glucose exhaustion (33 g L^{-1}) or the phenomenon of acid crash (66 and 100 g L^{-1}), which was alleviated by the use of any of the minimum pH set-points (4.8; 5.0; 5.1 and 5.5). Fermentation at a pH_{min} of 5.1 gave the best performance in butanol production and glucose consumption rate. Fermenting with a pH_{min} of 5.1 with 33 g L^{-1} of initial glucose caused acidogenic fermentation, while the use of 66 and 100 g L^{-1} of glucose led to a ~1.5 and ~1.7-fold increase in butanol concentration over their counterparts without pH control respectively. By controlling the pH after the acidogenic phase 15.8 g L^{-1} of butanol was obtained with 100 g L^{-1} of glucose, which was ~2-fold higher than without pH control. Different initial glucose concentrations therefore require different pH strategies to optimize butanol production.

Keywords: ABE fermentation; Acid Crash; Biobutanol; *Clostridium acetobutylicum*; Glucose; pH control.

1. Introduction

The extensive use of fossil fuels has led to a series of problems, such as climate change, an energy crisis and health problems. The importance of these issues has revived the interest of governments in the production of greener renewable energy. Biofuels generated biologically will become viable alternatives to oil and other derivate products when the price of biofuels becomes competitive [1]. Several types of biofuel have recently gained interest, including biomethanol, bioethanol and biobutanol. Of these, biobutanol appears especially likely to become widely used due to its physicochemical properties [2]. In addition, biobutanol has a higher heating value and lower vapour pressure than bioethanol and can be transported and distributed via existing pipelines and service stations reducing the investment cost in infrastructures [3,4].

Solventogenic *Clostridium* strains can produce biobutanol by Acetone-Butanol-Ethanol (ABE) fermentation. These *Clostridium* strains (*Clostridium acetobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium beijerinckii* or *Clostridium sporogenes*) metabolize monosaccharides (pentoses and hexoses) from different sources, such as starch or lignocellulosic biomass to produce ABE [5,6]. Among the raw materials currently available for ABE production, lignocellulosic biomass appears as an ideal candidate in terms of the circular economy, as it can be obtained from a wide range of residues such as rice straw, sweet sorghum stalk or sago hampas. Unlike the first generation of biofuels obtained from feedstocks (sugarcane, starch, molasses, etc.), lignocellulosic biomass does not interfere with the food chain supply [7].

Lignocellulosic biomass is composed mainly of cellulose, hemicellulose and lignin. However, it requires a pre-treatment and enzymatic hydrolysis to release the fermentable sugars prior to fermentation, which affects the final composition. Researchers have recently applied different pre-treatments (acid, alkali, ammonium sulphide, organic solvents or ionic liquids) to diverse lignocellulosic materials

(softwood, apple pomace or rice and wheat straw) resulting in a range of 22 to 52 g L^{-1} of total sugars after hydrolysis depending on their conditions [5,8–11]. Unfortunately, low sugar concentrations can result in low butanol production, such as ~2 g L^{-1} of butanol with 20 g L^{-1} of glucose [12]. Novel strategies have been developed to concentrate the lignocellulosic hydrolysate, including evaporation or vacuum. However, these approximations increase the pretreatment cost [13,14].

ABE fermentation is characterised by two phases: an acidogenic phase followed by a solventogenic phase. In the acidogenic phase, biomass, acids (butyric and acetic) and gases (CO_2 and H_2) are produced, accompanied by a drop in pH. During the solventogenic phase, the acids are re-assimilated producing butanol, acetone and ethanol with pH recovery [15]. The bacteria selected and the external conditions, such as medium composition, temperature and pH, regulate the end products obtained [16,17]. *Clostridium acetobutylicum* is a widely-known bacterium used for ABE fermentation [18–20], although there are still some issues that should be evaluated for efficient production of solvents [21]. In this regard, proper pH control is a key factor in ABE fermentation. High pH in the medium is related to more biomass and acids concentration in the reactors [22,23], while the reduced pH due to acid production is needed to produce solvents [24]. In fact, a minimum undissociated butyric acid concentration is necessary to initiate solvent production [25]. Moreover, the relation of the dissociated and undissociated species of acids, which are directly linked to the pH, plays an important role in the fermentation process [23]. Excessive acid production and low pH can create the conditions to cause acid crash as the undissociated acid concentration rises. As a consequence, acid crash causes the cease of glucose consumption and butanol production resulting in an ABE production failure [26]. Chemical control is now being applied in ABE fermentation, especially in continuous reactors, as a way of regulating the medium pH [27]. The pH regulation allows the study of the effect of the substrate on the butanol production. For example, butanol

production was ~2.3-fold higher when xylose was used instead of lactose by keeping the pH controlled at 4.7 under continuous ABE fermentation with *C. acetobutylicum* DSM 792 [28,29]. In batch mode, Guo et al. [30] demonstrated that the best pH set-point range was between 4.5 and 4.9 when fermenting 60 g L^{-1} of glucose and *C. acetobutylicum* XY16. Alternatively, by implementing pH control exclusively during the acidogenic phase Luo et al. [31] obtained ~8.1-fold higher butanol production by avoiding acid crash with 60 g L^{-1} of glucose and *C. acetobutylicum* ATCC 824. With this pH control strategy these authors reported an increase in butanol production from 9.42 to 11.52 g L^{-1} by adding trace concentrations of inhibitors such as the phenolic compounds. Implementing of a pH control strategy would thus be beneficial for improving ABE productivity from lignocellulosic hydrolysates. Despite this, more systematic studies are required on ABE fermentation to evaluate the combined effect of the initial substrate concentration and the pH control strategy.

The aim of this study was thus to systematically evaluate the combined effect of the initial sugar concentration and the pH control strategy in ABE fermentation by *C. acetobutylicum* DSM 792 using batch reactors and glucose as the model substrate. For this, we compared the performance of the process with three different levels of initial glucose concentration (33, 66 and 100 g L^{-1}) either without pH control or with two pH control strategies: firstly, by limiting the minimum pH over the fermentation time course, and secondly by spontaneous acidogenesis without pH control followed by limiting the minimum pH during solventogenesis.

2. Material and methods

2.1 Microorganism and chemical reagents

C. acetobutylicum DSM 792 was obtained from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). The cultures were maintained in 20% glycerol at -80 ºC. Prior to the experiments, the stock was cultured statically at 37 ºC in 19 g L^{-1} of Reinforced Clostridial Medium (RCM) supplemented with 10 g L^{-1} of glucose as a seed

inoculum. Chemicals were purchased from VWR, except for antifoam 204 (Sigma-Aldrich), $CaCO_3$ (Merk) and yeast extract (Alfa Aesar).

2.2. Experimental set-up

The fermentations were carried out in a modified 1 L bottle (total volume: 1.1 L) with a working volume of 0.8 L (stirred tank reactor, STR). The medium contained (g L^{-1}): glucose, 60 (Solution I); Yeast Extract, 5; NH_4Cl, 2; KH_2PO_4, 0.5; K_2HPO_4, 0.5; Antifoam 204, 0.01 %; resazurin sodium salt, 0.001 (Solution II); $MgSO_4.7H_2O$, 0.2; $MnSO_4.7H_2O$, 0.01; $FeSO_4.7H_2O$, 0.05 (Solution III) and $CaCO_3$, 5. All the components were sterilized prior to inoculation. Solutions I and II were autoclaved at 121 ºC for 10 and 20 min, respectively. Solution III was filter-sterilized by 0.22 μm. $CaCO_3$ was autoclaved (121 ºC, 20 min) inside the reactor without any liquid. The media was flushed with nitrogen for 30-35 min before inoculation to create anaerobiosis. The fermentation was carried out at 37 ºC and 120 rpm with an inoculum size of 5%. The fermentations were monitored by a Tris-compatible flat pH sensor with LoggerPro software (Vernier, USA). When needed, the pH was controlled with NaOH (3M). The pH probes were sterilized with 50% ethanol solution for at least 12 h. Figure 1 shows the experimental setup for the fermentation. Liquid samples from the fermentations were taken at appropriate time-points for the analysis of cell growth, glucose and products (butyric acid, acetic acid, butanol, acetone and ethanol).

<*Figure 1*>

2.3 Experimental plan

Four sets (runs) of experiments were planned (Figure 1) to study the behavior of *C. acetobutylicum*. Run 1 was buffered media with 5 g L^{-1} of $CaCO_3$ without active pH control. The effect of three different initial glucose concentrations (33, 66 and 100 g L^{-1}) was evaluated in terms of butanol production and yield. A pH control strategy was applied in the following runs (run 2, 3 and 4). The pH control strategy for run 2 and run 3 consisted of natural acidification by acid production until a minimum pH set-point was

142 reached, after which the pH was not allowed to fall by automatically adding NaOH
143 solution. The base dosing pump was activated as many times as needed by the pH
144 probe readings until the pH value increased 0.1 of the set-point. The spontaneous rise
145 of pH was not controlled, allowing spontaneous pH recovery as the solventogenesis
146 stage advanced. Run 2 was performed as the screening of several minimum pHs (4.8,
147 5.0, 5.1 and 5.5) to find the best pH for butanol production and glucose consumption
148 with the intermediate initial glucose concentration (66 g L^{-1}). From these results, a pH
149 of 5.1 was selected to evaluate the ABE fermentation profiles of the three initial glucose
150 concentrations (33, 66 and 100 g L^{-1}) in run 3. A hybrid pH strategy with the three
151 levels of initial glucose concentrations was then carried out in run 4, which consisted of
152 natural acidification up to the spontaneous minimum pH from the acidogenesis stage,
153 followed by a sudden increase of pH to the set-point (5.1). The pH set-point was kept
154 as the minimum value by pH control until the end of the fermentation.
155 2.4. Analytical methods
156 Biomass concentration (gDM L^{-1}) was calculated from the optical density at 600 nm
157 (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany) as
158 gDM L^{-1} = $0.2949 \cdot OD_{600} + 0.0596$ (n=8, R^2=0.995). The liquid samples were
159 centrifuged at 10000 rpm for 5 min and filtered by 0.22 μm for glucose and products
160 analysis. Glucose was analysed using an ionic chromatograph (Basic IC Plus 833,
161 Metrohm, Switzerland) with an amperometric detector (945 Professional Detector Vario
162 IC Amperometric Detector, Metrohm, Switzerland). The eluent was 100 mM NaOH and
163 3 mM sodium acetate with a flow of 0.5 ml min^{-1} and the temperature detector was set
164 at 35 ºC. Fermentation products were analysed by a gas chromatograph (7890A GC-
165 System, Agilent Technologies, USA) equipped with a flame ionization detector (FID)
166 and an Rtx-VMS column (Restek, USA), using helium as the carrier gas with a flow rate
167 of 1.1 mL min^{-1}. The oven programme was: 100 ºC for 7 min; 30 ºC/min until 210 and
168 210 ºC for 5 min. The temperatures of the injector and the detector were set at 190 ºC

and 240 ºC, respectively. Cell growth was measured by an UV-Vis spectrophotometer (SpectroFlex 6600, WTW) at 600 nm. Undissociated acid concentration was calculated by the Henderson-Hasselbalch Equation and the pKa of acetic acid (4.76) and butyric acid (4.82).

2.5. Determination of the glucose consumption rate and the specific growth rate

Glucose consumption and maximum growth rates were calculated during the exponential growth phase by Eqs. (1) and (2):

$$-q_{glucose} = \frac{S_{t2} - S_{t1}}{t_2 - t_1} \quad (1)$$

$$\mu_{max} = \frac{\ln(X_{t2}) - \ln(X_{t1})}{t_2 - t_1} \quad (2)$$

where $-q_{glucose}$ is the glucose consumption rate (g L^{-1} h^{-1}), S_{t1} and S_{t2} are the glucose concentrations at the beginning and end of the exponential growth phase (g L^{-1}), t_2 and t_1 are the initial and end time of the exponential growth phase (h), μ_{max} is the specific growth rate (h^{-1}), X_{t1} and X_{t2} are the biomass concentrations at the beginning and end of the exponential growth (gDM L^{-1}).

3. Results and discussion

3.1. $CaCO_3$ buffered ABE fermentation (uncontrolled pH)

Three different initial glucose concentrations (33, 66 and 100 g L^{-1}) were used as the substrate for the ABE fermentation under 5 g L^{-1} $CaCO_3$ as a buffering agent. These initial sugar concentrations were chosen within the typical range of sugar production after pre-treatment and enzymatic hydrolysis of lignocellulosic biomass. Sugar concentrations below ~45 g L^{-1} are usually obtained, although concentrations techniques may be applied to increase them [3]. The fermentation profiles are shown in Figure 2. The $CaCO_3$ was able to buffer the fermentation media and keep the minimum pH around 4.6 in all the experiments. The maximum production of acetic and butyric acids was between 5 and 6 g L^{-1} in all the tests. In fact, high acid concentration can be

196 related to the presence of $CaCO_3$. Ren et al. [32] obtained ~6-fold more acetic and
197 ~1.4-fold more butyric when culturing *C. acetobutylicum* ATCC 824 in P2 media
198 supplemented with $CaCO_3$ than supplementing with P2 media only. $CaCO_3$ could
199 stimulate the acid production by the stabilization of the cell membrane proteins due to
200 the presence of divalent ions (Mg^{+2} and Ca^{+2}) [33]. The three experiments had almost
201 the same biomass production. The glucose consumption rate and maximum growth
202 rate were nearly the same as the average values of 2.09 ± 0.15 g L^{-1} h^{-1} and 0.197 ±
203 0.008 h^{-1} respectively. However, the test with the lowest initial glucose experiment (33
204 g L^{-1}) showed some differences in the ABE fermentation profile and product yields.
205 <*Figure 2*>
206 In the experiment with 33 g L^{-1} of initial glucose, nearly all the glucose was depleted in
207 the first 24 h, accompanied by increased butanol production (~2.64 g L^{-1} at 28 h).
208 However, the rapid glucose depletion limited, to some extent, the assimilation of butyric
209 acid to butanol, giving a final butanol concentration of 3.35 g L^{-1} (ABE concentration:
210 3.99 g L^{-1}). Butyric acid remained at a high concentration (~6.0-6.6 g L^{-1}) from 15 h, and
211 glucose depletion kept the butyric acid assimilation rate lower than its production rate.
212 At the glucose level of 33 g L^{-1}, maximum butanol and ABE yields of 0.099 and 0.118 g
213 g^{-1}, respectively, were obtained (Table 1). Similar solvent yields have been reported in
214 previous studies. For example, Ibrahim et al. [12] obtained a butanol (ABE) yield of
215 0.11 (0.15) and 0.13 (0.22) with 20 and 40 g L^{-1} of glucose with *C. acetobutylicum*
216 ATCC 824 without pH control. In Long et al. [34], who tested gradually increasing
217 glucose concentration, the *C. acetobutylicum* P262 strain produced ~1, ~4, ~12 and
218 ~13 g L^{-1} of ABE with 20, 30, 40 and 60 g L^{-1} of glucose, respectively, indicating that
219 >40 g L^{-1} of glucose is required to achieve ABE yields >0.20. However, other
220 *Clostridium* species may behave differently under the same glucose concentrations.
221 For example, *C. beijerinckii* P260 produced 8.0 (11.5) g L^{-1} of butanol (ABE) with 29.3 g
222 L^{-1} of initial glucose concentration without pH control [8]. Our results are thus important

for adequate planning the conversion of butanol from lignocellulosic waste using *C. acetobutylicum*.

<*Table 1*>

However, the reactors with 66 and 100 g L^{-1} achieved butanol (ABE) concentrations of 7.47 (10.50) and 8.01 (11.59) g L^{-1} respectively (Table 1). These titers gave a ~2.3 (~2.8)-fold increase in butanol (ABE) production when compared with 33 g L^{-1} of initial glucose concentration. In addition, the butanol (ABE) yields were 0.155 (0.217) and 0.151 (0.220) g g^{-1} for 66 and 100 g L^{-1} respectively (Table 1), an increase of ~54% (~85%) versus the 33 g L^{-1} of the initial glucose experiment. A minimum pH of ~4.65 was achieved at ~16-18 h, with an acid production (acetic plus butyric) of 9.63 (at 18 h) for 66 g L^{-1} of initial glucose and 8.54 g L^{-1} (at 21h) for the 100 g L^{-1} reactor. Also, ~2 g L^{-1} of butyric acid was reassimilated in both reactors from the acid peak concentration until the end of the fermentation. The better performance of these two reactors than the lowest initial glucose experiment is associated with the residual glucose remaining when the minimum pH was reached (near the end of the acidogenic stage). A glucose concentration higher than 40 g L^{-1} (at ~16-18 h) was sufficient to develop the solventogenic stage in both reactors. However, the glucose was not completely depleted in either reactor, with only 74% of the glucose consumed in the 66 g L^{-1} experiment and 54% in the 100 g L^{-1}. Glucose consumption sharply declined after 12 h from the point at which the butyric acid peaked (Figure 2). This could be associated with the high undissociated acid concentrations reached when the acids peaked (63 and 53 mM for 66 and 100 g L^{-1} of initial glucose reactors, respectively). Accordingly to Maddox et al. [26], acid crash phenomenon can occur when the upper limit of total undissociated acid concentration (57 to 60 mM) is exceeded. Other authors have obtained better butanol production for this strain in similar environmental conditions, but with lower initial glucose concentrations. For example, Yang et al. [23] reported a butanol production of 10.8 g L^{-1} (butanol yield: 0.216 g g^{-1}) with 50 g L^{-1} of initial

glucose with the same minimum pH (4.7) using *C. acetobutylicum* ATCC 824 and 5 g L^{-1} of $CaCO_3$ as a buffer. The lower butanol production and yield obtained in our experiment seems to indicate that the spontaneous ABE fermentation without pH control could facilitate conditions for acid crash when the initial glucose concentrations is higher than 60 g L^{-1}. The undissociated form of the acid is more toxic than the dissociated, as the former species can diffuse through the plasma membrane to the cytosol, where it dissociates and reduces the intracellular pH [35]. An active pH control could thus help to limit the concentration of undissociated acid species as a strategy to improve butanol production.

3.2. Fermentations under active pH control

3.2.1. Screening of the set-point for minimum pH

In order to avoid acid crash, a pre-screening experiment was carried out to evaluate the fermentation profiles under different minimum controlled pHs and using 60 g L^{-1} of initial glucose. The minimum pH tested were 4.8; 5.0; 5.1 and 5.5. The chosen pH_{min} control strategy was selected to optimize both butanol production and glucose consumption. The results indicate that controlling the pH_{min} led to reduced total undissociated acid concentration in the reactors. The maximum concentrations (total acetic and butyric acids) were 50.61, 42.03, 37.64 and 39.46 mM for the reactors with the minimum pH of 4.8, 5.0, 5.1 and 5.5 respectively. Controlling the pH thus kept the undissociated acid values below the levels related to acid crash. In addition, complete glucose depletion occurred in all the conditions tested, even though increasing the pH_{min} from 4.8 to 5.5 led to a higher glucose consumption rate, from 1.90 to 3.47 g L^{-1} h^{-1} (Table 2). The maximum growth rates were close within any of the pH studied, with an average value of 0.202 ± 0.006 h^{-1}, similar to the reactors with no active pH control (Table 1). When pH_{min} was 5.5, the main fermentation product was butyric acid (15.88 g L^{-1}), indicating acidogenic fermentation. In contrast, solventogenesis failed to occur and butanol production was almost residual (2.01 g L^{-1}, yield: 0.031 g g^{-1}) (Table 2). In

277 fact, the acidogenic fermentation could have been related to the residual glucose
278 concentration of 6 g L^{-1} at 20 h, which may be below the limit for developing
279 solventogenesis. The reactors at 5.1, 5.0 and 4.8 pH reported similar butanol (ABE)
280 productions of 11.22 (16.12), 11.42 (16.67) and 11.57 (18.27) g L^{-1} respectively. ABE
281 yield was slightly higher at pH of 4.8 (0.301 g g^{-1}) as more ABE was produced. A ~1.5-
282 fold increase in butanol production was obtained over the same initial glucose
283 concentration without pH control. Little data is available in the literature on comparing
284 the effect of different values of pH on *Clostridium* species. Jiang et al. [36] evaluated a
285 set of pH values from 4.9 to 6.0 for *C. beijerinckii* IB4 with 60 g L^{-1} of initial glucose.
286 This strain showed an optimum pH of 5.5 for butanol production and glucose
287 consumption rate. Other pH may be set for ABE fermentation of other *Clostridium*
288 species. For example, a value of 4.8 was successfully stablished for *C. beijerinckii*
289 DSM 6423 [37] and a value of 5.5 for *C. saccharoperbutylacetonicum* N1-4 [38]. Our
290 results showed that when pH_{min} was controlled, solvent production and glucose
291 consumption rate outperformed the uncontrolled pH experiment. A pH_{min} of 5.1 was
292 therefore selected for the next experiments as this value showed the highest glucose
293 consumption rate (~3.0 g L^{-1} h^{-1}) among similar butanol titers (~11 g L^{-1}).
294 *<Table 2>*
295 *3.2.2. Combined effect of initial glucose concentration and the minimum pH control*
296 Batch fermentations keeping pH_{min} at 5.1 were performed with glucose concentrations
297 of 33, 66 and 100 g L^{-1} to evaluate the combined effect of controlled pH and the initial
298 glucose concentration on the fermentation process. The profiles of these fermentations
299 are shown in Figure 3 and the main representative parameters are given in Table 3.
300 The use of a pH control clearly modified the pH profiles as compared to their respective
301 uncontrolled pH fermentations (Figure 2). At 5.1, the maximum undissociated acid
302 concentration obtained in the 33, 66 and 100 g L^{-1} of the initial glucose reactors were
303 43.88; 37.64 and 36.15 mM, respectively, a reduction of about 30-40% in comparison

with the non-controlled pH experiments. None of the controlled reactors therefore reached the value of 57-60 mM of total undissociated acids in the media that could cause an acid crash. The maximum biomass concentration in the two reactors with the highest initial glucose concentration (66 and 100 g L^{-1}) were ~1.5-fold higher than the reactor with 33 g L^{-1} of initial glucose. As expected from the pre-screening experiment, active pH_{min} control resulted in higher glucose consumption rate values (average: 3.25 ± 0.21 g L^{-1} h^{-1}) than the uncontrolled reactors (average: 2.09 ± 0.15 g L^{-1} h^{-1}). A similar growth rate was observed in previous experiments with a maximum average growth rate of 0.200 ± 0.009 h^{-1} (Table 3). No significant differences were obtained between the three sets of experiments (uncontrolled, pre-screening and pH_{min} at 5.1) (ANOVA p-value: 0.82), indicating that the maximum growth rate value may be used as a checkpoint to determine whether the culture has developed correctly during a sequential series of experiments. In any case, as maximum growth rates are culture dependent they must be determined *in situ*. For example, Liao et al. [39] and Buendia-Kandia et al. [40] found values of ~0.18 and ~0.26 h^{-1} respectively, when *C. acetobutylicum* ATCC 824 was grown in different media formulations.

<*Figure 3*>

<*Table 3*>

The ABE fermentation profile of 33 g L^{-1} initial glucose showed a failure in solvent production (Figure 3). A redistribution of the carbon flux was observed as the main fermentation product was butyric acid (9.43 g L^{-1}) and the glucose was exhausted at ~17 h. In fact, maximum butyric acid concentration increased by ~1.5-fold over the uncontrolled reactor. Butanol (ABE) production was 1.09 (1.59) g L^{-1} and butanol (ABE) yield was 0.032 (0.047) g g^{-1} (Table 3). These solvent productions were on average ~0.4-fold less butanol, acetone and ABE concentration than the uncontrolled pH assay, although ethanol production increased from 0.18 to 0.26 g L^{-1}. The yields with 33 g L^{-1} of initial glucose were almost identical to those obtained in the controlled fermentation

331 at pH 5.5, in which the glucose was completely exhausted (Screening pH Section,
332 Table 2). The low residual glucose in the early stages of fermentation could explain the
333 failure to switch to solventogenesis and the acidogenic fermentation. *C. acetobutylicum*
334 DSM 792 may not be the most suitable when working at low sugars concentrations,
335 although, Dolejš et al. [41] were able to produce 2.7 g L^{-1} of butanol with 5.0 pH and 20
336 g L^{-1} initial glucose concentration using *C. acetobutylicum* DSM 1731.
337 A minimum glucose concentration may be necessary to properly develop ABE
338 fermentation with pH_{min} at 5.1. Butanol production in the reactors with 66 and 100 g L^{-1}
339 initial glucose was 11.22 and 13.83 g L^{-1}, respectively. These values were a ~1.5 and
340 ~1.7-fold increase over their counterparts with no pH control. Indeed, acetone and
341 ethanol increased an average of ~1.6-fold and ~1.8-fold, respectively, when pH control
342 was applied. This indicates the feasibility of limiting the pH_{min} to 5.1 to improve solvent
343 titers when sufficient carbon source is available for *C. acetobutylicum* DSM 792. When
344 a similar strategy was recently applied by Luo et al. [31], they obtained 9.42 g L^{-1} of
345 butanol with pH set at 5.0 during acidogenesis using 60 g L^{-1} of initial glucose with *C.*
346 *acetobutylicum* ATCC 824. Our results confirm that a suitable pH control strategy can
347 increase the butanol titer in ABE fermentation. Butanol and ABE yields were almost
348 identical, with 66 and 100 g L^{-1} of initial glucose concentration (Table 3), although more
349 butanol was produced when using 100 g L^{-1} of glucose. In fact, maximum butyric acid
350 was 6.89 g L^{-1} (at ~18 h) for the 66 g L^{-1} reactor and 7.11 g L^{-1} (at ~21 h) for the 100 g
351 L^{-1}, these concentrations represent an average ~1.3-fold increase over uncontrolled pH
352 control fermentations. Furthermore, pH was recovered in both reactors as butyric acid
353 was assimilated. A similar butyric acid concentration of ~2.60-2.80 g L^{-1} remained in
354 both reactors at the fermentation end point, indicating a ~2.1-fold increase in butyric
355 acid reassimilation with pH control over the uncontrolled pH experiment. Complete
356 glucose depletion was achieved in the fermentation with 66 g L^{-1} of initial glucose, while
357 the fermentation with 100 g L^{-1} of initial glucose consumed 84.9 g L^{-1} (87% of total

358 glucose). Therefore, an increase of 42% and 59% in glucose consumption with 66 and
359 100 g L^{-1} of initial glucose, respectively, was achieved versus the no pH control assays.
360 High initial glucose concentrations may induce substrate inhibition in the *Clostridium*
361 species. For example, Qureshi and Blaschek [42] found substrate inhibition for *C.*
362 *beijerinckii* with 158 g L^{-1} of initial glucose. In our study, no substrate inhibition was
363 found at glucose concentrations as high as 100 g L^{-1}, as similar biomass was obtained
364 in reactors with 66 and 100 g L^{-1} of initial glucose concentration. Due to the higher
365 butanol production in the study with 100 g L^{-1} of initial glucose, a concentration step for
366 sugars in lignocellulosic hydrolysates could be a suitable option to enhance the
367 economic viability of ABE fermentation.
368 3.3. Hybrid pH strategy
369 A hybrid pH control strategy was proposed and tested for the three initial glucose
370 concentrations (33, 66 and 100 g L^{-1}). This strategy consisted of spontaneous pH
371 evolution until reaching minimum pH, followed by a sudden pH step control up to 5.1 at
372 ~21-24 h, and from then on keeping pH_{min} at 5.1. In our reactors with 33 and 66 g L^{-1} of
373 initial glucose, butanol production with the hybrid pH strategy was lower than either
374 uncontrolled pH (2.56 g L^{-1}) or pH_{min} at 5.1 (9.08 g L^{-1}). On the other hand, when higher
375 initial sugars concentrations were used, better fermentation results were found by
376 implementing this pH strategy. This type of pH modification strategy may rely on culture
377 conditions such as growth phase or carbon source availability at the modification time.
378 The results of the fermentation of 100 g L^{-1} of initial glucose under the hybrid pH control
379 strategy are shown in Figure 4. The butanol and ABE production for the assay with 100
380 g L^{-1} of initial glucose was 15.80 and 23.68 g L^{-1} respectively. An increase of ~1.1 and
381 ~1.2-fold in butanol and ABE production over to the experiment carried out at 5.1 pH_{min}
382 with the same initial glucose. Although butanol inhibition could occur when more than
383 14 g L^{-1} were present in the media when using *C. acetobutylicum* ATCC 824 [43], our
384 data indicate that a delay in cell death may allow this limit to be exceeded.

385 *<Figure 4>*

386 The maximum butyric acid of 6.25 g L^{-1} was obtained at ~18 h with a pH of 4.57. At this
387 time-point the total undissociated acids concentration was 71.73 mM, which could have
388 caused an acid crash. Even though the pH was increased to 5.1 at ~21 h, the
389 fermentation was able to avoid the acid crash. Indeed, butyric acid was reassimilated
390 during solventogenesis with a final concentration as low as 1.68 g L^{-1} (~1.2-fold
391 increase over the reactor with pH_{min} at 5.1). In this assay 85.9 g L^{-1} of glucose (83% of
392 total) was consumed, thus showing no improvement in carbon source uptake over the
393 experiment with pH_{min} at 5.1. The glucose consumption rate was 2.55 g L^{-1} h^{-1}, an
394 intermediate value between the no pH control and the pH_{min} control at 5.1. The
395 maximum growth rate was 0.223 h^{-1} which matched the values previously obtained.
396 The highest biomass concentration was achieved in these conditions, reaching a
397 concentration of 3.95 g L^{-1} at ~31 h. A delay of 6-8 h in the maximum concentration
398 was found, which was attributed to the activation of the pH control at 21 h (time point
399 when biomass nearly peaked in previous experiments, Figure 3). The time-point
400 recommended for the sudden pH step may thus be at the mid-final exponential growth
401 phase when the minimum pH is reached. These results show that fermentation pH has
402 a direct impact on butanol biosynthesis and glucose uptake, however, this pH strategy
403 might be difficult carry out as it is dependent on time and acid concentration. Indeed,
404 glucose availability could be a key factor in developing a proper pH control strategy.
405 For example, Yang et al. [23] carried out a time dependent (12, 24 and 36 h) pH control
406 procedure for 50 g L^{-1} of glucose and butanol production was only improved (~2.1-fold
407 higher) when the pH was modified at 12 h. Our results indicate that for glucose levels
408 of ~50-70 g L^{-1}, using pH_{min} is a more robust strategy than implementing a time
409 dependent pH control set-points.
410 This work has shown that the best pH control strategy depends on the initial glucose
411 level (low, medium and high). At the low level (33 g L^{-1} of initial glucose) uncontrolled

pH was more efficient, while at the medium level (66 g L^{-1} of initial glucose) using controlled pH_{min} was the best alternative. Lastly, at the high level (100 g L^{-1} of initial glucose) the hybrid pH strategy provided the highest butanol production. These promising results might be validated under more realistic conditions by checking the effect of other sugars or potential inhibitors on product yields. Future research will therefore focus on testing these pH control strategies with lignocellulosic wastes hydrolysates.

4. Conclusion

This study aimed to determine the most favourable pH control strategy for maximizing butanol production and glucose consumption by *C. acetobutylicum* DSM 792 within a broad range of initial glucose concentrations. For an initial glucose concentration of 33 g L^{-1}, formulating the media with an alkalinity buffer was shown to be the best alternative to enhance butanol production. For the experiments with 33 g L^{-1}, lower butanol concentration was achieved, indicating that this strain needs a higher sugar concentration to compete with other strains such as *C. beijerinckii*. Otherwise, when enough glucose is available (>66 g L^{-1}), the results indicate the superiority of controlling minimum pH as a way of increasing solvent production in ABE fermentation. When high sugar concentrations are present (100 g L^{-1}) a more sophisticated pH control strategy coupled with the acid production pattern can substantially improve the butanol titer. These results are of practical interest for establishing *ad-hoc* strategies in ABE fermentation from lignocellulosic waste, as the best pH control strategy has been shown to depend on initial sugar concentration. The experimental process proposed here can also be extended to other *Clostridium* species.

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References

[1] A. Pugazhendhi, T. Mathimani, S. Varjani, E.R. Rene, G. Kumar, S.H. Kim, V.K. Ponnusamy, J.J. Yoon, Biobutanol as a promising liquid fuel for the future - recent updates and perspectives, Fuel. 253 (2019) 637–646. https://doi.org/10.1016/j.fuel.2019.04.139.

[2] P. Dürre, Biobutanol: An attractive biofuel, Biotechnol. J. 2 (2007) 1525–1534. https://doi.org/10.1002/biot.200700168.

[3] M.F. Ibrahim, N. Ramli, E. Kamal Bahrin, S. Abd-Aziz, Cellulosic biobutanol by Clostridia: Challenges and improvements, Renew. Sustain. Energy Rev. 79 (2017) 1241–1254. https://doi.org/10.1016/j.rser.2017.05.184.

[4] B. Bharathiraja, J. Jayamuthunagai, T. Sudharsanaa, A. Bharghavi, R. Praveenkumar, M. Chakravarthy, Y. Devarajan, Biobutanol – An impending biofuel for future: A review on upstream and downstream processing tecniques, Renew. Sustain. Energy Rev. 68 (2017) 788–807. https://doi.org/10.1016/j.rser.2016.10.017.

[5] L.D. Gottumukkala, B. Parameswaran, S.K. Valappil, K. Mathiyazhakan, A. Pandey, R.K. Sukumaran, Biobutanol production from rice straw by a non acetone producing *Clostridium sporogenes* BE01, Bioresour. Technol. 145 (2013) 182–187. https://doi.org/10.1016/j.biortech.2013.01.046.

[6] H.G. Moon, Y.S. Jang, C. Cho, J. Lee, R. Binkley, S.Y. Lee, 2016. One hundred years of clostridial butanol fermentation, FEMS Microbiol. Lett. 363, fnw001. https://doi.org/10.1093/femsle/fnw001.

[7] R.K. Prasad, S. Chatterjee, P.B. Mazumder, S.K. Gupta, S. Sharma, M.G. Vairale, S. Datta, S.K. Dwivedi, D.K. Gupta, Bioethanol production from waste lignocelluloses: A review on microbial degradation potential, Chemosphere. 231

(2019) 588–606. https://doi.org/10.1016/j.chemosphere.2019.05.142.

[8] Q. Jin, N. Qureshi, H. Wang, H. Huang, Acetone-butanol-ethanol (ABE) fermentation of soluble and hydrolyzed sugars in apple pomace by *Clostridium beijerinckii* P260, Fuel. 244 (2019) 536–544. https://doi.org/10.1016/j.fuel.2019.01.177.

[9] G. Qi, D. Huang, J. Wang, Y. Shen, X. Gao, Enhanced butanol production from ammonium sulfite pretreated wheat straw by separate hydrolysis and fermentation and simultaneous saccharification and fermentation, Sustain. Energy Technol. Assessments. 36 (2019) 100549. https://doi.org/10.1016/j.seta.2019.100549.

[10] L.T.P. Trinh, Y.J. Lee, J.W. Lee, W.H. Lee, Optimization of Ionic Liquid Pretreatment of Mixed Softwood by Response Surface Methodology and Reutilization of Ionic Liquid from Hydrolysate, Biotechnol. Bioprocess Eng. 23 (2018) 228–237. https://doi.org/10.1007/s12257-017-0209-x.

[11] A. Valles, F.J. Álvarez-Hornos, V. Martínez-Soria, P. Marzal, C. Gabaldón, 2020. Comparison of simultaneous saccharification and fermentation and separate hydrolysis and fermentation processes for butanol production from rice straw, Fuel. 282, 118831. https://doi.org/10.1016/j.fuel.2020.118831.

[12] M.F. Ibrahim, S. Linggang, M.A. Jenol, P.L. Yee, S. Abd-Aziz, Effect of buffering system on acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum* ATCC 824 using pretreated oil palm empty fruit bunch, BioResources. 10 (2015) 3890–3907. https://doi.org/10.15376/biores.10.3.3890-3907.

[13] S.A. Survase, R. Zebroski, C. Bayuadri, Z. Wang, G. Adamos, G. Nagy, V. Pylkkanen, Membrane assisted continuous production of solvents with integrated solvent removal using liquid-liquid extraction, Bioresour. Technol. 280 (2019) 378–386. https://doi.org/10.1016/j.biortech.2019.02.024.

[14] C. Lu, J. Zhao, S.T. Yang, D. Wei, Fed-batch fermentation for n-butanol

492 production from cassava bagasse hydrolysate in a fibrous bed bioreactor with
493 continuous gas stripping, Bioresour. Technol. 104 (2012) 380–387.
494 https://doi.org/10.1016/j.biortech.2011.10.089.
495 [15] F. Monot, J.R. Martin, H. Petitdemange, R. Gay, Acetone and butanol production
496 by *Clostridium acetobutylicum* in a synthetic medium, Appl. Environ. Microbiol.
497 44 (1982) 1318–1324. https://doi.org/10.1128/aem.44.6.1318-1324.1982.
498 [16] C.L. Cheng, P.Y. Che, B.Y. Chen, W.J. Lee, L.J. Chien, J.S. Chang, High yield
499 bio-butanol production by solvent-producing bacterial microflora, Bioresour.
500 Technol. 113 (2012) 58–64. https://doi.org/10.1016/j.biortech.2011.12.133.
501 [17] R. Díez-antolínez, M. Hijosa-valsero, A.I. Paniagua-garcía, Effect of Nutrient
502 Supplementation on Biobutanol Production from Cheese Whey by ABE (
503 Acetone – Butanol – Ethanol) Fermentation, Chem. Eng. Trans. 49 (2016) 217–
504 222. https://doi.org/10.3303/CET1649037.
505 [18] H. Bahl, W. Andersch, G. Gottschalk, Continuous production of acetone and
506 butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited
507 chemostat, Eur. J. Appl. Microbiol. Biotechnol. 15 (1982) 201–205.
508 https://doi.org/10.1007/BF00499955.
509 [19] N. Qureshi, M.M. Meagher, J. Huang, R.W. Hutkins, Acetone butanol ethanol
510 (ABE) recovery by pervaporation using silicalite-silicone composite membrane
511 from fed-batch reactor of *Clostridium acetobutylicum*, J. Memb. Sci. 187 (2001)
512 93–102. https://doi.org/10.1016/S0376-7388(00)00667-0.
513 [20] M. Mirfakhar, M.A. Asadollahi, H. Amiri, K. Karimi, 2020. Co-fermentation of
514 hemicellulosic hydrolysates and starch from sweet sorghum by *Clostridium*
515 *acetobutylicum*: A synergistic effect for butanol production, Ind. Crops Prod. 151,
516 112459. https://doi.org/10.1016/j.indcrop.2020.112459.
517 [21] H. Luo, P. Zheng, M. Bilal, F. Xie, Q. Zeng, C. Zhu, R. Yang, Z. Wang, 2020.
518 Efficient bio-butanol production from lignocellulosic waste by elucidating the

519 mechanisms of *Clostridium acetobutylicum* response to phenolic inhibitors, Sci.
520 Total Environ. 710, 136399. https://doi.org/10.1016/j.scitotenv.2019.136399.
521 [22] F. Raganati, A. Procentese, G. Olivieri, M.E. Russo, P. Gotz, P. Salatino, A.
522 Marzocchella, Butanol production by *Clostridium acetobutylicum* in a series of
523 packed bed biofilm reactors, Chem. Eng. Sci. 152 (2016) 678–688.
524 https://doi.org/10.1016/j.ces.2016.06.059.
525 [23] X. Yang, M. Tu, R. Xie, S. Adhikari, Z. Tong, 2013. A comparison of three pH
526 control methods for revealing effects of undissociated butyric acid on specific
527 butanol production rate in batch fermentation of *Clostridium acetobutylicum*,
528 AMB Express. 3, 3. https://doi.org/10.1186/2191-0855-3-3.
529 [24] S.Y. Li, R. Srivastava, S.L. Suib, Y. Li, R.S. Parnas, Performance of batch, fed-
530 batch, and continuous A-B-E fermentation with pH-control, Bioresour. Technol.
531 102 (2011) 4241–4250. https://doi.org/10.1016/j.biortech.2010.12.078.
532 [25] F. Monot, J.M. Engasser, H. Petitdemange, Influence of pH and undissociated
533 butyric acid on the production of acetone and butanol in batch cultures of
534 *Clostridium acetobutylicum*, Appl. Microbiol. Biotechnol. 19 (1984) 422–426.
535 https://doi.org/10.1007/BF00454381.
536 [26] I.S. Maddox, E. Steiner, S. Hirsch, S. Wessner, N.A. Gutierrez, J.R. Gapes, K.C.
537 Schuster, The Cause of “Acid Crash” and “Acidogenic Fermentations” During the
538 Batch Acetone-Butanol-Ethanol (ABE-) Fermentation Process Fermentation, J.
539 Mol. Microbiol. Biotechnol. 2 (2000) 95–100. www.caister.com/bacteria-plant.
540 [27] T. Millat, H. Janssen, G.J. Thorn, J.R. King, H. Bahl, R.J. Fischer, O.
541 Wolkenhauer, A shift in the dominant phenotype governs the pH-induced
542 metabolic switch of *Clostridium acetobutylicum* phosphate-limited continuous
543 cultures, Appl. Microbiol. Biotechnol. 97 (2013) 6451–6466.
544 https://doi.org/10.1007/s00253-013-4860-7.
545 [28] A. Procentese, F. Raganati, G. Olivieri, M.E. Russo, P. Salatino, A.

Marzocchella, Continuous lactose fermentation by *Clostridium acetobutylicum* - Assessment of solventogenic kinetics, Bioresour. Technol. 180 (2015) 330–337. https://doi.org/10.1016/j.biortech.2015.01.008.

[29] A. Procentese, F. Raganati, G. Olivieri, M.E. Russo, P. Salatino, A. Marzocchella, Continuous xylose fermentation by *Clostridium acetobutylicum* - Assessment of solventogenic kinetics, Bioresour. Technol. 192 (2015) 142–148. https://doi.org/10.1016/j.biortech.2015.05.041.

[30] T. Guo, B. Sun, M. Jiang, H. Wu, T. Du, Y. Tang, P. Wei, P. Ouyang, Enhancement of butanol production and reducing power using a two-stage controlled-pH strategy in batch culture of *Clostridium acetobutylicum* XY16, World J. Microbiol. Biotechnol. 28 (2012) 2551–2558. https://doi.org/10.1007/s11274-012-1063-9.

[31] H. Luo, P. Zheng, F. Xie, R. Yang, L. Liu, S. Han, Y. Zhao, M. Bilal, Co-production of solvents and organic acids in butanol fermentation by: C*lostridium acetobutylicum* in the presence of lignin-derived phenolics, RSC Adv. 9 (2019) 6919–6927. https://doi.org/10.1039/c9ra00325h.

[32] C. Ren, Y. Gu, S. Hu, Y. Wu, P. Wang, Y. Yang, C. Yang, S. Yang, W. Jiang, Identification and inactivation of pleiotropic regulator CcpA to eliminate glucose repression of xylose utilization in *Clostridium acetobutylicum*, Metab. Eng. 12 (2010) 446–454. https://doi.org/10.1016/j.ymben.2010.05.002.

[33] A. El Kanouni, I. Zerdani, S. Zaafa, M. Znassni, M. Loutfi, M. Boudouma, The improvement of glucose/xylose fermentation by *Clostridium acetobutylicum* using calcium carbonate, World J. Microbiol. Biotechnol. 14 (1998) 431–435. https://doi.org/10.1023/A:1008881731894.

[34] S. Long, D.T. Jones, D.R. Woods, Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262, Appl. Microbiol. Biotechnol. 20 (1984) 256–261. https://doi.org/10.1007/BF00250635.

[35] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition, Bioresour. Technol. 74 (2000) 25–33. https://doi.org/10.1016/S0960-8524(99)00161-3.

[36] M. Jiang, J.N. Chen, A.Y. He, H. Wu, X.P. Kong, J.L. Liu, C.Y. Yin, W.F. Chen, P. Chen, Enhanced acetone/butanol/ethanol production by *Clostridium beijerinckii* IB4 using pH control strategy, Process Biochem. 49 (2014) 1238–1244. https://doi.org/10.1016/j.procbio.2014.04.017.

[37] S.A. Survase, G. Jurgens, A. Van Heiningen, T. Granström, Continuous production of isopropanol and butanol using *Clostridium beijerinckii* DSM 6423, Appl. Microbiol. Biotechnol. 91 (2011) 1305–1313. https://doi.org/10.1007/s00253-011-3322-3.

[38] M. Gao, Y. Tashiro, Q. Wang, K. Sakai, K. Sonomoto, High acetone–butanol–ethanol production in pH-stat co-feeding of acetate and glucose, J. Biosci. Bioeng. 122 (2016) 176–182. https://doi.org/10.1016/j.jbiosc.2016.01.013.

[39] Z. Liao, Y. Suo, C. Xue, H. Fu, J. Wang, Improving the fermentation performance of *Clostridium acetobutylicum* ATCC 824 by strengthening the VB1 biosynthesis pathway, Appl. Microbiol. Biotechnol. 102 (2018) 8107–8119. https://doi.org/10.1007/s00253-018-9208-x.

[40] F. Buendia-Kandia, E. Rondags, X. Framboisier, G. Mauviel, A. Dufour, E. Guedon, 2018. Diauxic growth of *Clostridium acetobutylicum* ATCC 824 when grown on mixtures of glucose and cellobiose, AMB Express. 8, 85. https://doi.org/10.1186/s13568-018-0615-2.

[41] I. Dolejš, V. Krasňan, R. Stloukal, M. Rosenberg, M. Rebroš, Butanol production by immobilised *Clostridium acetobutylicum* in repeated batch , fed-batch , and continuous modes of fermentation, 169 (2014) 723–730. https://doi.org/10.1016/j.biortech.2014.07.039.

[42] N. Qureshi, H.P. Blaschek, Recent advances in ABE fermentation: Hyper-

butanol producing *Clostridium beijerinckii* BA101, J. Ind. Microbiol. Biotechnol. 27 (2001) 287–291. https://doi.org/10.1038/sj.jim.7000114.

[43] K. Gao, Y. Li, S. Tian, X. Yang, Screening and characteristics of a butanol-tolerant strain and butanol production from enzymatic hydrolysate of NaOH-pretreated corn stover, World J. Microbiol. Biotechnol. 28 (2012) 2963–2971. https://doi.org/10.1007/s11274-012-1107-1.

608 **Figure Captions**

609 Figure 1. Experimental setup and plan for ABE fermentations.

610 Figure 2. Effect of initial glucose concentration on butanol production under
611 uncontrolled pH by *C. acetobutylicum* (a, 33; b, 66; c, 100 g L^{-1} of initial glucose).

612 Figure 3. Effect of initial glucose concentration on butanol production with pH_{min} at 5.1
613 by *C. acetobutylicum* (a, 33; b, 66; c, 100 g L^{-1} of initial glucose).

614 Figure 4. Batch fermentation on butanol production under hybrid pH control with 100 g
615 L^{-1} of initial glucose concentration by *C. acetobutylicum.*

$C_{glucose}$ ($g\ L^{-1}$)	$Butanol_{max}$ ($g\ L^{-1}$)	ABE_{max} ($g\ L^{-1}$)	$Y_{B/S}$ ($g\ g^{-1}$)	$Y_{ABE/S}$ ($g\ g^{-1}$)	μ_{max} (h^{-1})	$-q_{glucose}$ ($g\ L^{-1}\ h^{-1}$)	Glucose conversion (%)
33	3.35	3.99	0.099	0.118	0.188	2.27	100
66	7.47	10.50	0.155	0.217	0.202	2.00	74
100	8.01	11.59	0.151	0.220	0.202	2.02	54

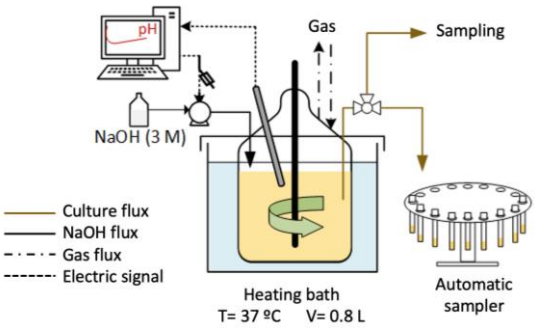
Table 1. Production and kinetics parameters under uncontrolled pH (natural evolution of pH). Yields were calculated with the maximum solvent concentration. Glucose conversion refers to the end of fermentation.

pH	$Butanol_{max}$ ($g\ L^{-1}$)	ABE_{max} ($g\ L^{-1}$)	$Y_{B/S}$ ($g\ g^{-1}$)	$Y_{ABE/S}$ ($g\ g^{-1}$)	μ_{max} (h^{-1})	$-q_{glucose}$ ($g\ L^{-1}\ h^{-1}$)
5.5	2.01	2.92	0.031	0.046	0.204	3.47
5.1	11.22	16.12	0.163	0.235	0.205	3.02
5.0	11.42	16.67	0.179	0.256	0.194	2.61
4.8	11.57	18.27	0.186	0.301	0.206	1.90

619 Table 2. Influence of the minimum controlled pH on production and kinetics
620 parameters. Initial concentration of glucose: 60 $g\ L^{-1}$. Yields were calculated with the
621 maximum solvent concentration.

$C_{glucose}$ ($g\ L^{-1}$)	$Butanol_{max}$ ($g\ L^{-1}$)	ABE_{max} ($g\ L^{-1}$)	$Y_{B/S}$ ($g\ g^{-1}$)	$Y_{ABE/S}$ ($g\ g^{-1}$)	μ_{max} (h^{-1})	$-q_{glucose}$ ($g\ L^{-1}\ h^{-1}$)	Glucose conversion (%)
33	1.09	1.59	0.032	0.047	0.205	3.35	100
66	11.22	16.12	0.163	0.235	0.205	3.02	100
100	13.83	19.55	0.163	0.232	0.190	3.34	87

Table 3. Production and kinetics parameters under controlled pH (at $pH_{min} \geq 5.1$). Yields were calculated with the maximum solvent concentration. Glucose conversion refers to the end of fermentation.



Run	glucose, g L^{-1}	pH control	set-point
1	33, 66, 100	No	
2	66	Yes	pH ≥ 4.8, 5.0, 5.1, 5.5
3	33, 66, 100	Yes	pH ≥ 5.1
4	33, 66, 100	Yes	pH ≥ 5.1 from ~24 h

Figure 1

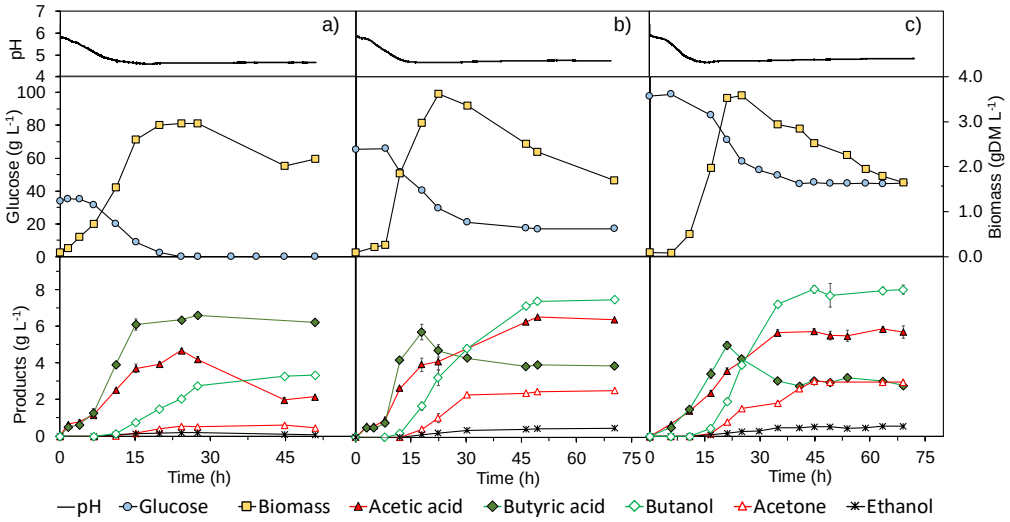

a)
b)
c)
pH
Glucose (g L-1)
Biomass (gDM L-1)
Products (g L-1)
Time (h)
pH
Glucose
Biomass
Acetic acid
Butyric acid
Butanol
Acetone
Ethanol

Figure 2

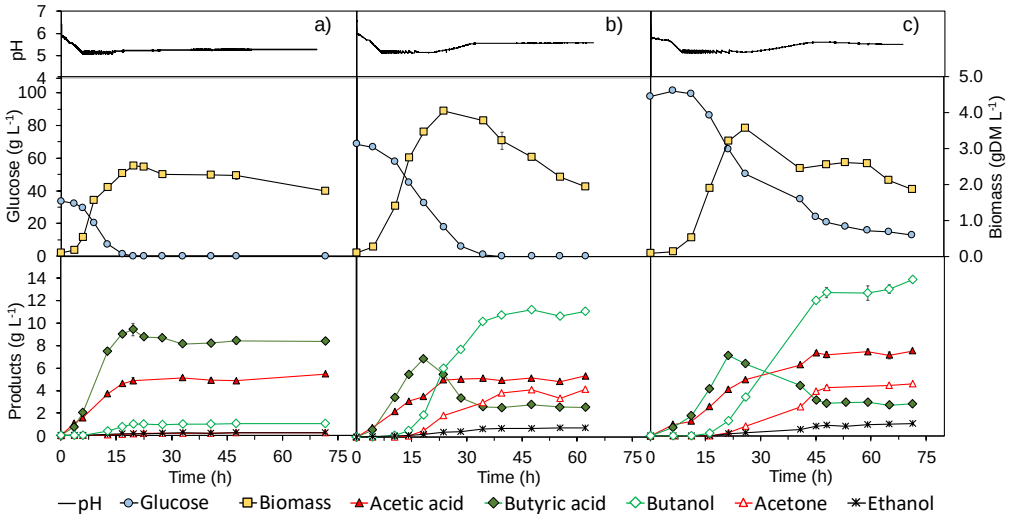

a)
b)
c)
pH
Glucose (g L-1)
Biomass (gDM L-1)
Products (g L-1)
Time (h)
pH
Glucose
Biomass
Acetic acid
Butyric acid
Butanol
Acetone
Ethanol

Figure 3

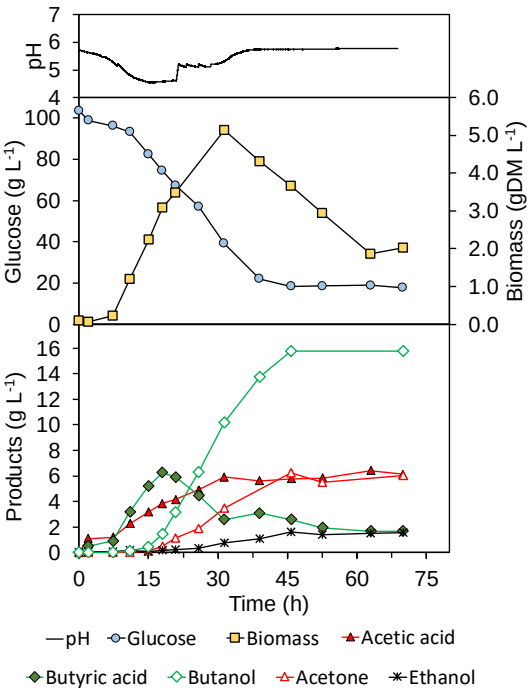

pH
Glucose (g L-1)
Biomass (gDM L-1)
Products (g L-1)
Time (h)
pH
Glucose
Biomass
Acetic acid
Butyric acid
Butanol
Acetone
Ethanol

Figure 4