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Bioprocess intensification for acetone-butanol-ethanol fermentation from brewer's spent grain: Fed-batch strategies coupled with *in-situ* gas stripping

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ABSTRACT

A fed-batch ABE fermentation process coupled with *in-situ* gas-stripping to mitigate butanol toxicity was investigated. Two feeding strategies were compared: pulses of sugars and continuous feeding of the liquid released in the dilute acid pretreatment of brewer's spent grain. The concentrations of butanol (13.2 g L^{-1} total and 50 g L^{-1} average in the condensates for glucose pulse feeding) were higher than those obtained under batch conditions, showing that *in-situ* gas-stripping can relieve butanol toxicity. The continuous feeding of the fed-batch reactor produced similar butanol concentrations (10.2 g L^{-1} total and 65 g L^{-1} in the condensates), more stable concentrations of solvents in the condensates and enhanced monosaccharides uptake (99.1%) in comparison with the pulse feeding strategy. The efficient utilization of the enzymatic and pretreatment hydrolysates in the same fed-batch reactor is an integrated approach that could reduce capital and operating costs. The mathematical model proposed showed good performance to predict concentrations in the fermentation broth.

1. Introduction

Butanol is a bulk chemical used in the production of paints, rubbers and resins and can be considered an advanced biofuel [1]. Butanol presents some advantages over ethanol, such as a higher calorific value (29 vs 21 MJ L^{-1}) similar to gasoline (32 MJ/L), among others [2]. The butanol market was estimated at 4560 million USD in 2020, with a prevision of 7150 million USD for 2028 [3].

Butanol can be obtained by means of chemical synthesis from petroleum derived products [4] or through fermentation processes. Nowadays, the emphasis is focused on the use of renewable raw materials from low cost lignocellulosic residues from agricultural and agro-industrial processes. The biochemical route is based on the use of microorganisms from the *Clostridium* genus [5]. These microorganisms produce acetone, butanol and ethanol at different ratios, depending of the strain used and the fermentation conditions; although it is frequent to find a mass ratio of 3:6:1 acetone:butanol:ethanol (ABE) [6]. *C. beijerinckii* was used in this study due to its capacity for pH auto-regulation and lack of strain degeneration compared to other *Clostridium* strains [7,8].

The performance of ABE fermentation can be affected by several factors, such as the initial pH, the substrate concentration [9], the presence of toxic compounds [10] and inhibition by solvents production [11]. The majority of these factors can be solved through detoxification processes and the conditioning of the substrate, except the strong inhibition caused by solvents. It has been demonstrated that product inhibition, especially by butanol, is the main reason for the low concentrations of solvents in ABE fermentation and one of the principal limitations for the industrial production of butanol [12]. Solvent production ceases, due to the hydrophobic nature of butanol, when the butanol concentration reaches values between 8 and 15 g L^{-1}, depending on the microorganism and strain [13].

Overcoming product inhibition is one of the main challenges to make the separation process energetically competitive. Considering the low butanol titers and the high boiling point of butanol (118 °C), reducing the energy consumption of the separation process is a critical point, as the purification of butanol through conventional distillation requires large quantities of energy (79.5 MJ kg^{-1} butanol [14]), making the

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Table 1
Composition of BSG, pretreated BSG, pretreatment liquid and enzymatic hydrolysate.

Solids	BSG (% w/w DM)	Pretreated BSG (%w/w DM)
Glucan	17.3 ± 0.3	23.2 ± 0.9
Xylan	14.0 ± 0.3	10.6 ± 0.5
Arabinan	6.6 ± 0.1	2.6 ± 0.3
Acid insoluble lignin	18.9 ± 0.6	26.2 ± 0.4
Total lignin	25.5 ± 0.6	30.6 ± 0.6
Total ash	3.7 ± 0.0	3.8 ± 0.1
Detoxified Liquids	Pretreatment (g L^{-1})	Enzymatic hydrolysate (g L^{-1})
Glucose	6.9 ± 0.4	38.3 ± 1.5
Xylose	15.3 ± 0.9	5.1 ± 0.3
Arabinose	8.9 ± 0.6	n.d
Acetic acid	0.9 ± 0.0	0.6 ± 0.0
Furfural	n.d.	n.d.
HMF	n.d.	n.d.
Phenolic compounds	0.6 ± 0.0	0.6 ± 0.0

n.d.: not detected.

process energetically non-viable. The integration of *in-situ* product recovery processes with ABE fermentation continuously removes solvents from the broth, reducing butanol toxicity. The recovered product has a higher butanol concentration, which reduces downstream processing costs. Integrated recovery processes must minimize costs, not affect microorganisms negatively, have a high affinity, selectivity, robustness and ease of implementation [15]. Some of the more commonly investigated technologies are liquid-liquid extraction [16], pervaporation [17], and perstraction [18]. Although these alternatives can reach high concentrations of solvents at low or moderate energy consumptions, they present some operational problems that made the gas-stripping separation process more attractive.

The gas-stripping separation strategy removes volatile components from the fermentation broth without harming the microorganisms [19], while improving the product yield, solvent productivity and also reducing waste stream volumes [2]. In this process, the fermentation off-gas is fed into the bioreactor to drag the volatile components, which are later recovered in a condenser. Total ABE solvents concentrations can reach 32 g L^{-1} using *Clostridium acetobutylicum* DSM 792 [9]. Thus, a concentrated condensate is obtained, which could reduce downstream energy consumption to 14–31 MJ kg^{-1} butanol [16]. ABE fermentation, coupled with *in-situ* gas-stripping, can be carried out in batch mode [20], but the solvents production is limited as the microorganism depletes the monosaccharides without reaching product inhibition. In this case, a fed-batch operation strategy can assure longer term operation, as it can avoid both the substrate and product inhibition, enabling the operation time to be extended and increasing productivity. The feeding is usually carried out through pulses with high concentrations of monosaccharides [21].

The objective of this study was to analyze different feeding strategies for the fed-batch ABE fermentation process coupled with *in-situ* gas-stripping to alleviate butanol toxicity and obtain condensates concentrated in butanol. Feeding with pulses of concentrated sugar solutions and the continuous feeding of the liquid fraction obtained after dilute acid pretreatment of brewer's spent grain (BSG) have been compared to select the most suitable to increase butanol production. The enzymatic hydrolysate and the pretreatment liquid fraction obtained from a lignocellulosic agro-industrial residue, such as BSG, were used as substrates to investigate an efficient strategy for integrating the whole process hydrolysates and reducing waste streams. Moreover, a mathematical model was developed to describe the integrated fermentation-recovery process. This work compares different fed-batch feeding strategies in an ABE fermentation process with in-situ solvent recovery by gas-stripping, such as pulse feeding of monosaccharide solutions (glucose, xylose and a mixture of both glucose and xylose) and continuous feeding of the liquid produce in the pretreatment step in order to increase butanol titer. To the best of our knowledge, there are no previous published articles analyzing this continuous feeding strategy that allows the integration of the different process hydrolysates.

2. Materials and methods

2.1. Hydrolysates to be fermented from BSG

The hydrolysates used in the ABE fermentation process were obtained through a dilute sulfuric acid pretreatment and further enzymatic hydrolysis of BSG. BSG was kindly donated by a local brewery. The fresh BSG was stored at −20 °C before use. Prior to the experimental runs, the BSG was dried in an oven at 45 °C. The BSG was used unmilled in the whole process. The chemical composition of BSG is shown in Table 1.

The BSG was pretreated under dilute acid conditions (15% w/w solids load, 0.05 g H_2SO_4 g^{-1} DM, 121 °C, 30 min), as established in previous works [22]. This pretreatment produced a liquid hydrolysate (Table 1) rich in monosaccharides that can be valorized by feeding the fed-batch ABE fermentation process. The enzymatic hydrolysis was performed on a Labfors Biofors HT (Infors, Switzerland) bioreactor using the pretreated BSG, type II water and a commercial enzyme cocktail composed by Cellic CTec2 (120 FPU mL^{-1}, cellulases and β-glucosidases). The enzyme was kindly provided by Novozymes (Denmark). The

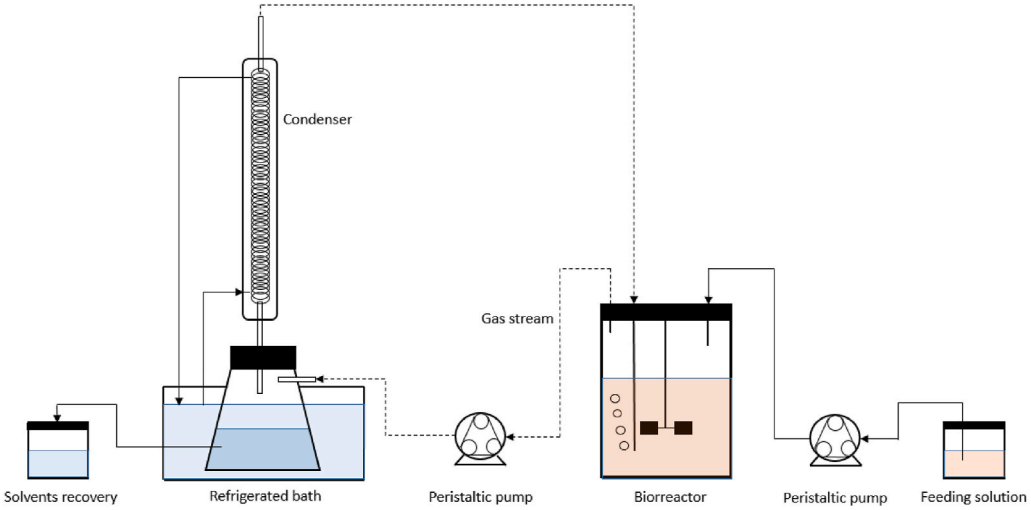

Fig. 1. Schematic diagram of the fed-batch fermentation process coupled with *in-situ* gas-stripping.

experimental conditions (15% w/w DM and 15 FPU g^{-1} DM, 50 °C, pH 4.8, 80 rpm) were determined in a previous work [10]. The enzymatic hydrolysis produced a monosaccharide rich liquid stream that was later used as substrate for the ABE fermentation process.

According to previous results [10], the pretreatment liquid and the enzymatic hydrolysate were subjected to a detoxification process. The detoxification was carried out with activated charcoal at a solid/liquid ratio of 1.5% w/v on a rotary shaker at 35 °C, 135 rpm for 1 h. The mixture was then vacuum filtered, the pH was adjusted to 5.5 and the detoxified liquids were used for the fermentation process. All experiments were carried out in triplicate.

2.2. Microorganism

The microorganism *Clostridium beijerinckii* DSM 6422 was obtained from the German collection of microorganisms (DSM, Leibniz, Germany). The strain was maintained on Reinforced Clostridial Medium, RCM (Fluka, Sigma-Aldrich, Spain) in Hungate tubes (18 × 150 mm), in spore form and cold stored at 4 °C under anaerobic conditions. The inoculum was grown as previously explained in other studies [22].

2.3. Fed-batch fermentation coupled with in-situ gas-stripping

The fed-batch fermentation coupled with *in-situ* gas-stripping (Fig. 1) was carried out in a 2 L bioreactor (Biostat Bioplus), containing 0.7 L of BSG enzymatic hydrolysate (total monosaccharides concentration about 43 g/L) as substrate for ABE fermentation by *C. beijerinckii* DSM 6422. After sterilization subjecting the substrate to 90 °C during 20 min inside the reactor, a vitamin solution (0.001 g L^{-1} PABA and 0.00001 g L^{-1} biotin), a salt solution (0.40 g L^{-1} $MgSO_4$, 0.015 g L^{-1} $MnSO_4$, 0.05 g L^{-1} $FeSO_4$ and 0.01 g L^{-1} NaCl, 0.06 g L^{-1} KCl) and acetate buffer solution (0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4 and 2.20 g L^{-1} ammonium acetate) were added to the medium and the preculture was then inoculated at 10% (v/v) and flushed with free O_2 nitrogen. The pH was adjusted to 6.3 ± 0.1. The temperature was selected at 35 °C and the stirring was set at 50 rpm. Prior to the fermentation, the gas-stripping equipment (condenser and gas lines) was flushed with O_2 free nitrogen. The fermentation process was started in batch mode for 32 h using the enzymatic hydrolysate as substrate. At this moment, the gas-stripping started, at a flowrate of 1.0 vvm, by recycling the fermentation off-gas (a mixture of CO_2 and H_2) using a peristaltic pump (Masterflex Quickload) and 18 size Tygon pump tubing (Cole-Parmer). The vapors from the gas-stripping process were cooled in a condenser at 0 °C, using a glycerol-water 30% v/v solution in a refrigerated circulating bath (Fisher Scientific Isotemp). The condensed solvents were collected in a flask immersed inside the refrigerated circulating bath. Samples were taken periodically from the reactor for monosaccharides, organic acids, solvents and optical density analysis and from the condenser of the gas-stripping process for solvent concentration analysis.

The integrated fermentation process was carried out with two different feeding strategies to compare their performances. In the first set of experiments, concentrated monosaccharide solutions (one experiment was performed with a solution of 600 g L^{-1} of glucose, another with a solution of 600 g L^{-1} of xylose and a third with a mixture of both sugars with 450 g L^{-1} glucose and 150 g L^{-1} xylose) were added in pulses to elevate the sugar concentration without significantly increasing the volume inside the reactor. In the second set of experiments, the pretreatment liquid, rich in pentose sugars, was used for continuous feeding to valorize the pentoses in this hydrolysate. The continuous feeding rate (10.6 mL h^{-1}) was adjusted to compensate for the uptake rate of monosaccharides by the microorganism. The feeding solution was added with a peristaltic pump (Watson Marlow 520S). An antifoam (Antifoam 204, Sigma) was added to control foam formation, if necessary.

Two different yields and productivities have been evaluated at 120 h: the first one was based on the total concentration of solvents produced, considering the solvents collected in the gas stripping condensate and the solvents remaining in the fermentation broth, while the second one was evaluated taking into account only the solvents collected in the gas stripping condensate. All experiments were carried out in triplicate.

2.4. Analytical methods

The chemical composition of the BSG was analyzed as described in a previous work [22]. The concentration of monosaccharides, solvents, organic acids, and potential inhibitors in the liquids were measured by HPLC as described in a previous work [22]. The biomass concentration was determined through the optical density analyzed using a spectrophotometer (Hitachi U-2000) at 600 nm.

2.5. Data analysis

An ANOVA variance analysis was performed to determine statistical differences at a confidence level of 95% ($p < 0.05$). A Tukey multiple range test was performed using Statgraphics Centurion XVIII.

2.6. Process modeling of the continuous fed-batch fermentation

A mathematical model was proposed to describe the fed-batch fermentation under continuous feeding coupled with *in-situ* gas-stripping. The model was based on Monod kinetic considering a term of product inhibition and cell death [9]. Individual mass balances for the concentration of cells (X), glucose (S_G), xylose (S_X), arabinose (S_A) and butanol (P) in the bioreactor are described by the following equations:

$$\frac{dX}{dt} = \left(\frac{\mu_{mG}S_G}{K_{SG}+S_G} + \frac{\mu_{mX}S_X}{K_{SX}+S_X} + \frac{\mu_{mA}S_A}{K_{SA}+S_A}\right)*X*\left(1-\frac{P}{K_p}\right)^a - k_d*X - \frac{Q_{in}}{V}*X \quad (1)$$

$$\frac{dS_G}{dt} = \frac{Q_{in}}{V}\left(S_{G,in}-S_G\right) - \left(\left(\frac{\mu_{mG}S_G}{K_{SG}+S_G}\right)*\left(1-\frac{P}{K_p}\right)^a*\frac{X}{Y_{X/S_G}}\right) \quad (2)$$

$$\frac{dS_X}{dt} = \frac{Q_{in}}{V}\left(S_{X,in}-S_X\right) - \left(\left(\frac{\mu_{mX}S_X}{K_{SX}+S_X}\right)*\left(1-\frac{P}{K_p}\right)^a*\frac{X}{Y_{X/S_X}}\right) \quad (3)$$

$$\frac{dS_A}{dt} = \frac{Q_{in}}{V}\left(S_{A,in}-S_A\right) - \left(\left(\frac{\mu_{mA}S_A}{K_{SA}+S_A}\right)*\left(1-\frac{P}{K_p}\right)^a*\frac{X}{Y_{X/S_A}}\right) \quad (4)$$

$$\frac{dP}{dt} = \left(1-\frac{P}{K_p}\right)^a*X*\left[\left(\frac{\mu_{mG}S_G}{K_{SG}+S_G}\right)\left(\frac{Y_{P/S_G}}{Y_{X/S_G}}\right) + \left(\frac{\mu_{mX}S_X}{K_{SX}+S_X}\right)\left(\frac{Y_{P/S_X}}{Y_{X/S_X}}\right) + \left(\frac{\mu_{mA}S_A}{K_{SA}+S_A}\right)\left(\frac{Y_{P/S_A}}{Y_{X/S_A}}\right)\right] - - k_S a*P - \frac{Q_{in}}{V}*\mathrm{P} \quad (5)$$

$$\frac{dV}{dt} = Q_{in} \quad (6)$$

In Eqs. (1) to (6), X is the cell concentration in the bioreactor (dry weight, g L^{-1}); μ_m is the maximum specific growth rate (h^{-1}) for glucose (μ_{mG}), xylose (μ_{mX}) and arabinose (μ_{mA}); S (g L^{-1}) is the substrate

concentration in the fermentation broth for glucose (S_G), xylose (S_X) and arabinose (S_A); S_{in} (g/L) is the substrate concentration in the hydrolysate fed for glucose ($S_{G,in}$), xylose ($S_{X,in}$) and arabinose ($S_{A,in}$). K_S (g L^{-1}) is the substrate saturation parameter for glucose (K_{SG}), xylose (K_{SX}) and arabinose (K_{SA}). P is the butanol concentration in the fermentation broth (g L^{-1}); K_p is the product concentration at which no cell growth occurs (g/L); α is the degree of product inhibition and k_d is the specific growth rate (h^{-1}). $Y_{X/S}$ (g g^{-1}) is the cell yield coefficient for glucose ($Y_{X/SG}$), xylose ($Y_{X/SX}$) and arabinose ($Y_{X/SA}$). $Y_{P/S}$ (g g^{-1}) is the butanol yield coefficient corresponding to glucose ($Y_{P/SG}$), xylose ($Y_{P/SX}$) and arabinose ($Y_{P/SA}$). k_sa (h^{-1}) is the butanol removal rate parameter, evaluated from batch stripping experiments, considering that the rate of removal of solvents, r_P follows the equation:

$$r_P = \frac{dP}{dt} = -k_s a \cdot P \quad (7)$$

The variation of the reaction volume (V) due to gas-stripping was considered negligible in comparison to the inlet flow rate (Q_{in}).

The software EcosimPro© was used to estimate the kinetic parameters that produce the best fit between the experimental data and the results predicted by the model. The parameter estimation was carried out by dynamic optimization using Sequential Quadratic Programming (SQP). The objective function to be minimized considered the sum of square residuals (differences between the measured and the predicted concentrations by the model).

3. Results and discussion

In order to obtain hydrolysates rich in monosaccharides to be fermented to butanol, BSG was subjected to a dilute acid pretreatment and the pretreated BSG was subjected to enzymatic hydrolysis. The pretreated BSG presented a composition of 23.2 ± 0.9% w/w DM glucan, 10.6 ± 0.5% w/w DM xylan and 2.6 ± 0.3% w/w DM arabinan and a total lignin content of 30.6 ± 0.6% w/w DM (Table 1). The pretreatment produced an hydrolysate with a content of monosaccharides after detoxification of 6.9 ± 0.4 g L^{-1} glucose, 15.3 ± 0.9 g L^{-1} xylose, 8.9 ± 0.6 g L^{-1} arabinose and a low concentration of inhibitors (0.9 ± 0.0 g L^{-1} acetic acid, 0.6 ± 0.0 g L^{-1} phenolic compounds). The enzymatic hydrolysis was carried out at a solids load of 15% w/w DM in order to obtain a hydrolysate more concentrated in monosaccharides [10]. After detoxification, the monosaccharides content was still high (38.3 ± 1.5 g L^{-1} glucose, 5.1 ± 0.3 g L^{-1} xylose), and the concentration of phenolic compounds (0.6 ± 0.0 g L^{-1}) is lower than that can produce inhibition of the fermentation process [10] (Table 1).

3.1. Fed-batch fermentation coupled with gas-stripping and pulse feeding

In this first set of experiments, fed-batch ABE fermentation runs coupled with gas-stripping and pulse feeding were carried out using *C. beijerinckii* DSM 6422. The enzymatic hydrolysate from pretreated BSG was used as a substrate for the microorganism. The pulse feeding consisted of a solution of glucose, xylose or a mixture of both at a high concentration (600 g L^{-1}). The gas-stripping system was started at 32 h, when the butanol concentration in the bioreactor reached 5–6 g L^{-1} to avoid product inhibition [9]. The pulses were added when the concentration of monosaccharides inside the reactor reached values between 5 and 15 g L^{-1}, with the aim of increasing the concentration to about 30–40 g L^{-1} and extending the operation time of the process. The experiments were conducted for 168 h at a gas flow rate of 1.0 vvm, selected from previous experimental runs (data not shown). There was no pH control in these experiments, as the microorganism can regulate the pH. The initial pH was around 6.3 ± 0.1 and the final pH was around 4.6 ± 0.2. The results are shown in Figs. 2–4 and Tables 2 and 3.

The monosaccharides uptake was high for the three feeding strategies (85.3% for the glucose pulses, 66.2% for the xylose pulses and 64.4% for the pulses of the solution of glucose and xylose). The pulse feeding strategies based on the addition of xylose, or the solution of glucose and xylose, presented lower sugar consumptions than the glucose pulse feeding. It can also be observed that the microorganism showed a strong preference for glucose, leaving xylose unconsumed when the pulses of glucose were fed (Fig. 2A). Glucose was consumed at a higher rate when the mixture of glucose and xylose was fed (leaving 70% of the xylose unconsumed, Fig. 4A). These results are probably due to catabolite repression [23], stressing the fact that feeding xylose at the same time as glucose had a moderate effect over the performance of the process. On the other hand, the microorganism showed that, in the absence of glucose in the feeding, it can metabolize xylose as substrate, reaching a good production of solvents (11.2 g butanol L^{-1} and 16.3 g ABE L^{-1}), yields and productivities (Tables 2 and 3). In all the experiments, the uptake of monosaccharides ceased between 120 and 168 h, leaving sugars unconsumed in the fermentation broth. This can be due to the lack of nutrients, since they were not introduced in the feeding pulses [9], to culture degeneration caused by inhibitors [24] and/or the accumulation of metabolites [25]. The production of organic acids, mainly butyric and acetic acid, was low throughout these experiments. Butyric acid was not detected whereas acetic acid reached maximum concentrations of 0.8 ± 0.1 g L^{-1}.

The concentration of butanol in the reactor declined when the gas-stripping started at 32 h in the three cases (Figs. 2B, 3B and 4B), reducing product inhibition and reaching total concentrations between 11.2 and 13.2 g butanol L^{-1} and between 16.3 and 19.3 g ABE L^{-1}. Compared with the results reached under batch fermentation (6 g butanol/L and 8 g ABE/L [22]), fed-batch fermentation coupled with *in-situ* gas-stripping considerably improved the solvent concentrations, because product toxicity was relieved. Acetone and ethanol concentrations were similar throughout the three experiments (concentrations of acetone and ethanol about 5 g L^{-1} and 1 g L^{-1}, respectively), producing mainly butanol under these conditions. The results agreed with data provided by the scientific literature (Table 3). Lu et al. [25] reported similar butanol and ABE concentrations (total concentrations of 13.5 g butanol L^{-1} and 17.7 g ABE L^{-1}) by batch fermentation coupled with *in-situ* gas-stripping using wood pulping hydrolysate as fermentation substrate and an adaptive mutant strain of *C. beijerinckii* (*C. beijerinckii* CC101) with a higher butanol tolerance. Wechgama et al. [26] analyzed a batch fermentation process by *C. beijerinckii* TISTR 1461 coupled with *in-situ* gas-stripping, reporting total concentrations of 14.1 g butanol L^{-1} and 18.9 g ABE L^{-1} using sugarcane molasses as substrate. Cai et al. [24] obtained total concentrations of 18.6 g butanol L^{-1} and 28.3 g ABE L^{-1}, with a fed-batch strategy, introducing pulses of corn stover bagasse (CSB) hydrolysate (210 g L^{-1} glucose, 78 g L^{-1} xylose) to increase the concentrations of monosaccharides in the fermentation broth from 5 to above 30 g L^{-1}. CSB hydrolysates were vacuum concentrated before feeding. The microorganism used by Cai et al. [24], *C. acetobutylicum* ABE-P 1201 derived from ATCC 824 by evolutionary engineering, also showed a preference for glucose over xylose, evidencing catabolite repression.

It should be noted that ABE mass ratios at 120 h for total concentrations were 2.4:7.2:0.4 for the glucose pulse feeding run, 2.8:6.9:0.4 for the xylose pulse feeding experiment and 3:6.1:0.9 for the glucose and xylose pulse feeding. Results showed that the pulse feeding strategies did not significantly improve the butanol mass ratio in comparison with the results obtained under batch fermentation (2.9:7.0:0.2 [22]).

The concentration of solvents in the liquids recovered from the condenser of the gas-stripping equipment (Figs. 2C, 3C and 4C) showed the concentration declined with time, obtaining less concentrated condensates with the time course of stripping and observing a sharp decline between 120 and 168 h, which coincided with the cease in the uptake of monosaccharides. Acetic and butyric acids were not detected in the condensates, as the gas-stripping process only removes acetone, butanol, ethanol and water. The solvent concentrations observed at 48 h were high for the three strategies (between 50.5 and 71.5 g of butanol L^{-1} and between 68.9 and 97.9 g of ABE L^{-1}) with very low concentrations of

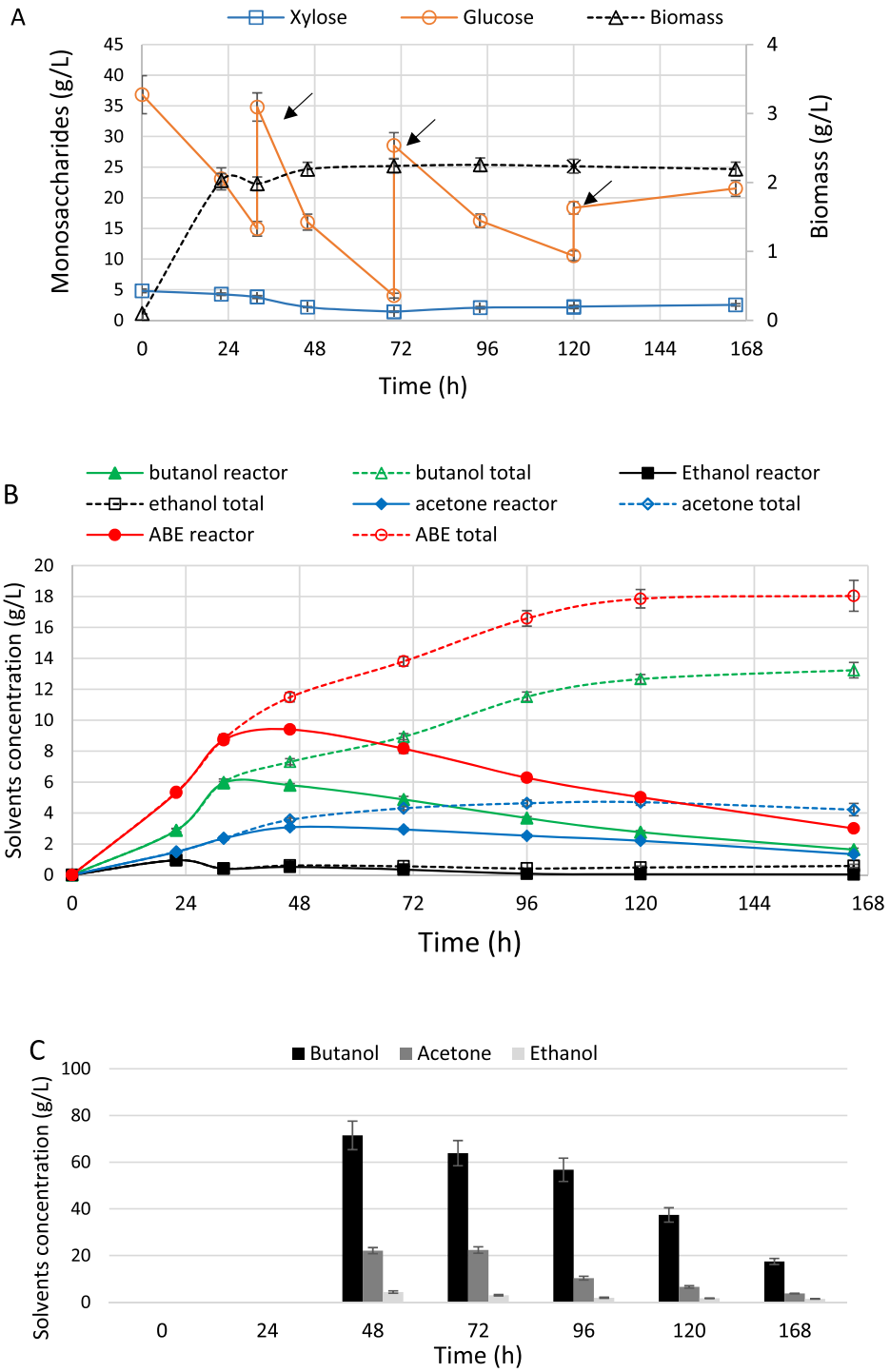

Fig. 2. Results for pulse feeding with glucose solution (600 g L^{-1}). A: Monosaccharides uptake and biomass production. B: Production of solvents. C: Concentration of solvents in the liquid recovered from the gas-stripping condenser. Figure B: Total: Total concentration (continuous line), Reactor: Concentration inside the reactor (dashed line). Feeding pulses pointed out with arrows on figure A.

ethanol. However, butanol concentrations were not high enough to observe phase separation, which can occur when the butanol titer is higher than its solubility in water, about 77 g of butanol L^{-1} at 20 °C [19]. Phase separation results in a more energy-efficient butanol recovery process [27]. Lu et al. [25] obtained maximum concentrations in condensates of 78 g butanol L^{-1} and 97 g ABE L^{-1} (not reaching phase separation), declining to 39 g butanol L^{-1} and 53 g ABE L^{-1} at the end of the process (75 h) using a gas flow rate of 0.25 vvm. Rochón et al. [9] obtained condensates with a concentration high enough for phase separation using a gas flow rate of 0.4 vvm, which allows higher concentrations but lower volumes to be obtained. Under these conditions, an organic phase with 444.8 g butanol L^{-1} and 499.9 g ABE L^{-1} and an aqueous phase with 79.1 g butanol L^{-1} and 126.2 g ABE L^{-1} were obtained at the end of the process. Cai et al. [24] reached concentrations of 135 g butanol L^{-1} and 220 g ABE L^{-1} in the first condensate, declining to 75 g butanol L^{-1} and 110 g ABE L^{-1} in the final condensate after 210 h

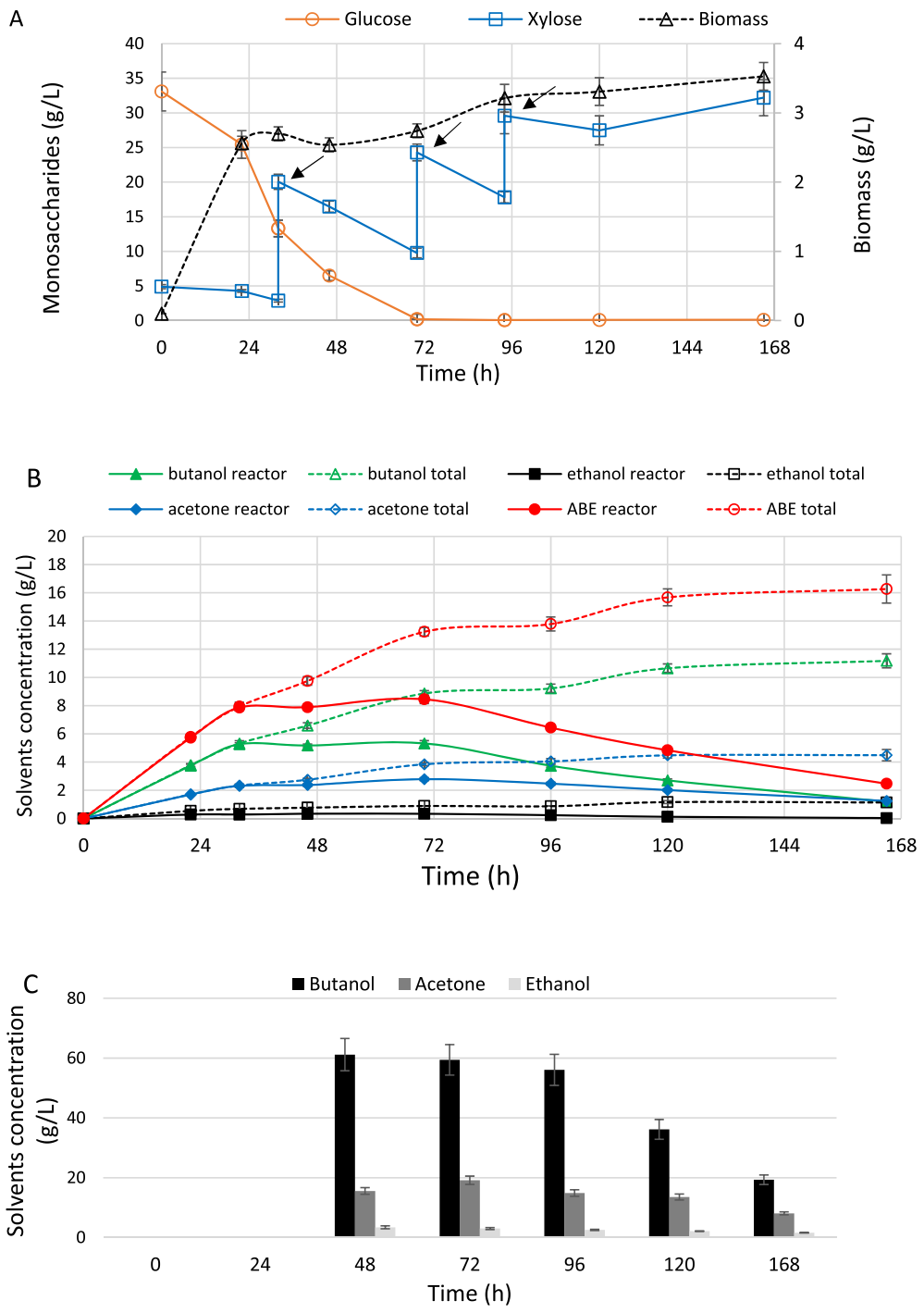

Fig. 3. Results for pulse feeding with xylose solution (600 g L^{-1}). A: Monosaccharides uptake and biomass production. B: Production of solvents. C: Concentration of solvents in the liquid recovered from the gas-stripping condenser. Figure B: Total: Total concentration (continuous line), Reactor: Concentration inside the reactor (dashed line). Feeding pulses pointed out with arrows on figure A.

using a gas flow rate of 1.3 vvm. Although the solvent concentration is much higher, the descending trend observed in the condensate concentrations was similar to that observed in this study. The concentration of solvents in the condensate is highly dependent on the concentration of solvents in the reactor [24,25]. In Figs. 2–4, it can be clearly observed that the concentrations of solvents in the condensates decrease as the concentration of solvents in the fermenter diminished. It should be noted that the composition of the condensates presents a higher proportion of butanol than the total concentrations, indicating a higher selectivity for butanol than acetone and ethanol in the gas-stripping process. The ABE mass ratios in the condensates (calculated at 120 h) were 1.9:7.7:0.3 for the glucose pulse feeding, 2.3:7.3:0.4 for the xylose pulse feeding and 2.6:7.0:0.4 for the pulse feeding of glucose and xylose. The higher proportion of butanol showed that the gas-stripping process is more selective towards butanol. The *in-situ* gas-stripping process was capable of recovering large quantities of solvents from the reactor. The removal of solvents in the reactor results in recoveries of butanol between 61.5 and 72.9 g in condensates/100 g total produced and ABE between 58.0

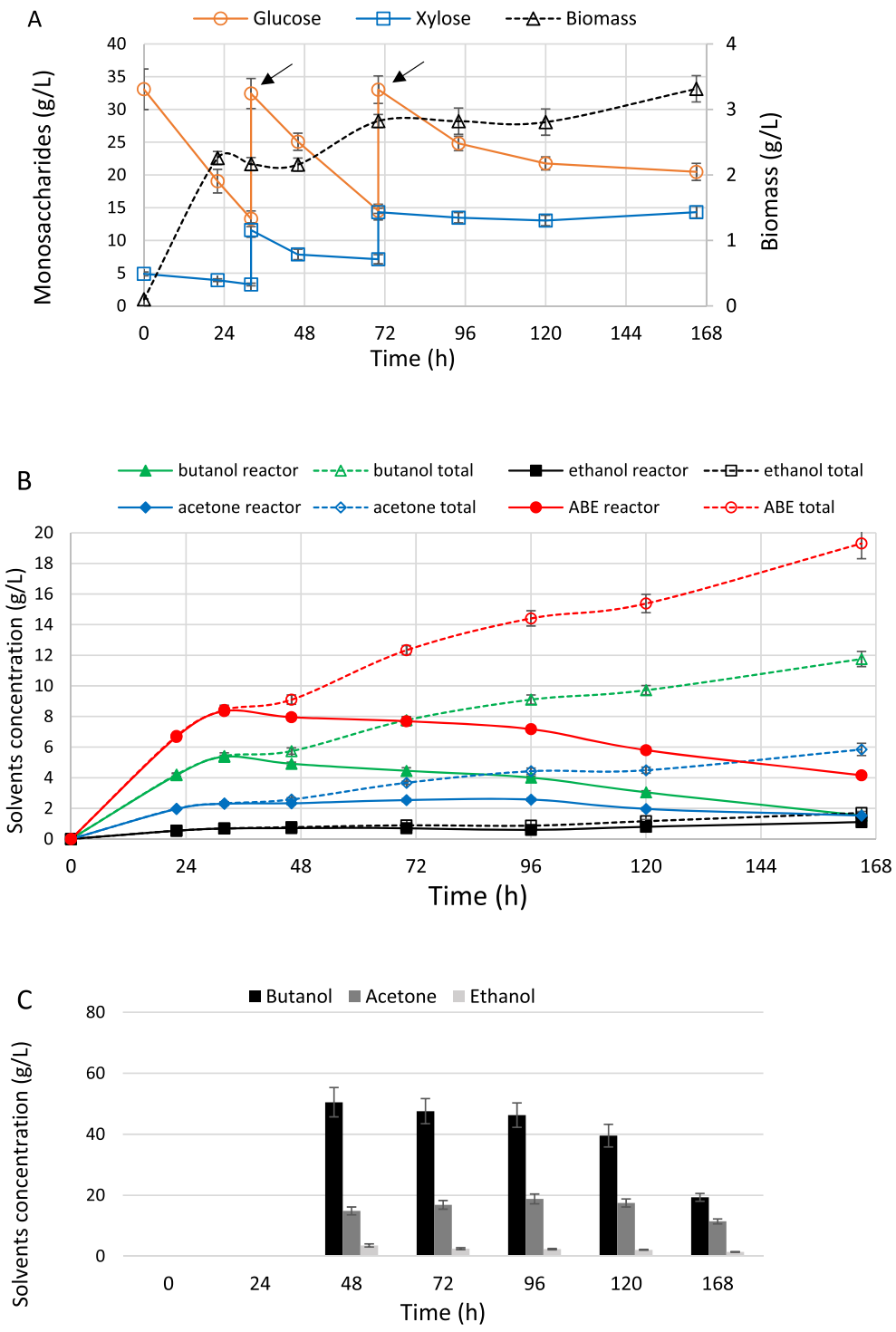

Fig. 4. Results for pulse feeding with mixture of glucose and xylose (75% glucose, 25% xylose, 600 g L^{-1}). A: Monosaccharides uptake and biomass production. B: Production of solvents. C: Concentration of solvents in the liquid recovered from the gas-stripping condenser. Figure B: Total: Total concentration (Continuous line), Reactor: Concentration inside the reactor (Dashed line). Feeding pulses pointed out with arrows on figure A.

and 69.1 g in condensates/100 g total produced (Table 2). Other authors [25] obtained somewhat lower recoveries (48% butanol, 39% acetone, 23% ethanol) at a lower gas recycled rate than that used in our study.

Two yields and productivities were calculated from the experimental data: considering the total concentrations of solvents and from the concentration of solvents in the condensates. The results were very similar for the three pulse feeding strategies. The yields calculated from the total concentrations (Table 3) were similar to those reported by other authors such as Rochón et al. [9] (0.16–0.18 g g^{-1} for butanol) and lower than those obtained by Lu et al. [25] (0.23 g g^{-1} for butanol and

Table 2
Influence of the feeding strategy on yields, productivities and recovery of solvents. Parameters calculated at 120 h.

Feeding strategy	$Y_{butanol}$ (g g^{-1})	Y_{ABE} (g g^{-1})	$Q_{butanol}$ (g $L^{-1}h^{-1}$)	Q_{ABE} (g $L^{-1}h^{-1}$)	Butanol recovery (g in condensates/100 g total produced)	ABE recovery (g in condensates/100 g total produced)
Pulses of glucose	0.76	0.98	0.46	0.60	61.5	62.3
Pulses of xylose	0.93	1.27	0.42	0.57	72.9	69.1
Pulses of glucose + xylose	0.81	1.16	0.38	0.55	65.1	58.0
Continuous feeding of pretreatment liquid	0.95	1.16	0.55	0.67	49.6	50.8

Butanol and ABE yields ($Y_{Butanol}$, Y_{ABE}) expressed as g g^{-1} sugars consumed; butanol and ABE productivities ($Q_{Butanol}$, Q_{ABE}) expressed as g $L^{-1}h^{-1}$. Yields and productivities calculated using the concentration of solvents in condensed liquids from gas-stripping.

Table 3
ABE fermentation coupled with in-situ gas-stripping. Comparison of solvent concentrations, yields and productivities. Parameters calculated at 120 h.

Substrate	Operation mode	Type of feeding	Microorganism	Total butanol (g L^{-1})	Total ABE (g L^{-1})	$Y_{butanol}$	Y_{ABE}	$Q_{butanol}$	Q_{ABE}	Reference
Wood pulping hydrolysate[a]	Batch		*C. beijerinckii* CC101	13.5	17.7	0.23	0.33	0.13	0.17	[25]
Sugarcane-sweet sorghum juices[a]	Batch		*C. acetobutylicum* DSM 792	10.5	17.5	0.18	n.a.	0.10	0.13	[9]
	Fed-batch	Pulses of concentrated sugarcane-sweet sorghum juices	*C. acetobutylicum* DSM 792	18.6	31.8	0.16	n.a.	0.13	0.22	
Surgacane molasses[a]	Batch		*C. beijerinckii* TISTR 1461	14.1	18.9	0.39	n.a.	0.29	n.a	[26]
Corn stover bagasse[b]	Fed-batch	Pulses of concentrated corn stover bagasse	*C. acetobutylicum* ABE-P 1201	18.6	28.3	0.19	0.29	0.09	0.13	[24]
P2 medium[a]	Batch		*C. acetobutylicum* JB200	19.8	31.8	0.25	0.40	0.41	0.66	[16]
Brewer's spent grain[a]	Fed-batch	Pulses of concentrated glucose	*C. beijerinckii DSM 6422*	13.2	18.0	0.17	0.24	0.11	0.15	This study
	Fed-batch	Pulses of concentrated xylose	*C. beijerinckii DSM 6422*	11.2	16.3	0.20	0.29	0.09	0.13	
	Fed-batch	Pulses of concentrated glucose and xylose	*C. beijerinckii DSM 6422*	11.8	19.3	0.20	0.27	0.08	0.13	
	Fed-batch	Continuous feeding of pretreatment liquids	*C. beijerinckii DSM 6422*	10.2	13.7	0.14	0.20	0.08	0.12	

Butanol and ABE yields ($Y_{Butanol}$, Y_{ABE}) expressed as g g^{-1} sugars consumed, calculated using the total production of solvents.
Butanol and ABE productivities ($Q_{Butanol}$, Q_{ABE}) expressed as g $L^{-1}h^{-1}$, calculated using the total production of solvents.
[a] Continuous stripping.
[b] Intermittent stripping; n.a.: not available.

0.33 g g^{-1} for ABE). The productivities reached lower values than those obtained by Rochón et al. [9] using *C. acetobutylicum* DSM 792 and sugarcane-sweet sorghum industrial juices (0.13 g butanol $L^{-1}h^{-1}$ and 0.22 g ABE $L^{-1}h^{-1}$). The yields referring to the concentrations in the condensates (Table 2) were 0.76–0.93 g g^{-1} for butanol and 0.98–1.27 g g^{-1} for ABE at 120 h. The productivity referring to the condensates reached values of 0.38–0.46 g $L^{-1}h^{-1}$ for butanol and 0.55–0.60 g $L^{-1}h^{-1}$ for ABE. These values are much higher than those obtained under batch conditions (0.04–0.07 g $L^{-1}h^{-1}$ for butanol and 0.06–0.09 g $L^{-1}h^{-1}$ for ABE) [22] due to the high concentration of solvents in the condensed liquids.

To sum up, the three pulse feeding strategies produced higher concentrations of solvents than those obtained in batch fermentation processes. Catabolite repression was observed in all the experiments, as the microorganism metabolizes glucose before xylose. In the experimental run where pulses of xylose were fed, the microorganism consumed xylose at a higher rate only when the glucose was depleted. Therefore, in the absence of glucose, *C. beijerinckii* DSM 6422 can metabolize xylose, reaching an adequate production of solvents, yields and productivities, demonstrating that pulse feeding of xylose is as beneficial for butanol production as pulse feeding of glucose. This result opens the door to fed-batch feeding of hemicellulose hydrolysates, which are rich in xylose and arabinose.

3.2. Fed-batch fermentation coupled with gas-stripping and continuous feeding of the hemicellulosic hydrolysate

The liquid obtained in the dilute acid pretreatment of BSG was used to continuously feed the bioreactor during the gas-stripping process. The objective of this strategy is to valorize the liquid fraction of the pretreatment by the production of butanol in the same bioreactor used to ferment the enzymatic hydrolysate, hence reducing capital costs. The fermentation of this stream, rich in pentoses, increases the global yields of the process [10]. For the industrial implementation of the ABE process, the hemicellulosic hydrolysate stream should be integrated with the enzymatic hydrolysate to be converted into butanol. There is no pH control used in these experiments, as the microorganism regulates the pH itself. The initial pH was around 6.3 ± 0.1 and the final pH was around 4.6 ± 0.2. The results are summarized in Fig. 5 and Tables 2 and 3

The concentration of monosaccharides in the pretreatment liquid (Table 1) is low for pulse feeding, as higher concentrations are required.

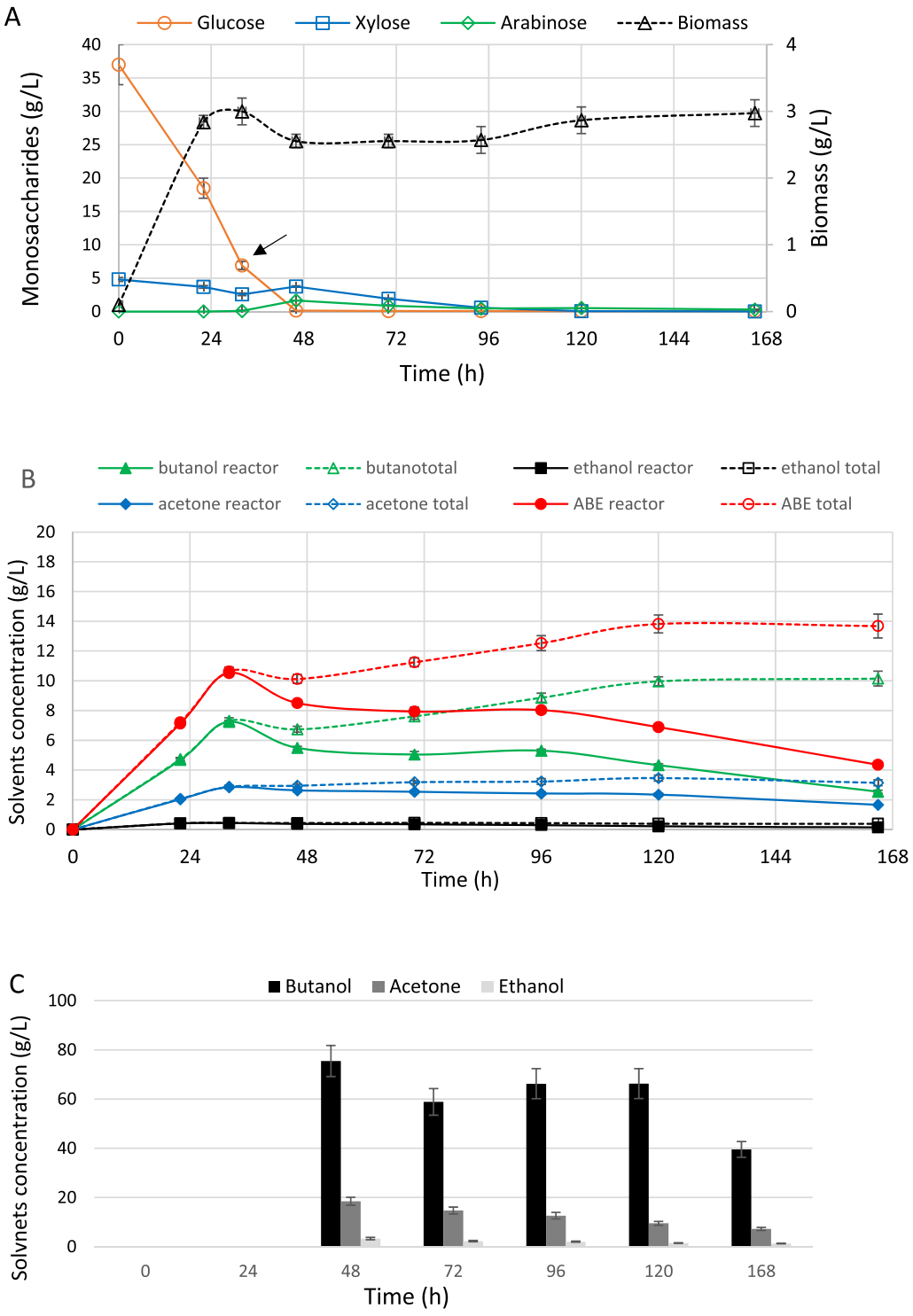

Fig. 5. Results for continuous feeding with pretreatment liquids. A: Monosaccharides uptake and biomass production. B: Production of solvents. C: Concentration of solvents in the liquid recovered from the gas-stripping condenser. Figure B: Total: Total concentration (Continuous line), Reactor: Concentration inside the reactor (Dashed line). Continuous feeding start pointed out with arrow in figure A.

One alternative is the vacuum concentration of this stream in order to make it suitable for pulse feeding [24]. However, this option would increase the energy consumption of the process, counteracting the energy savings of the gas-stripping process. Instead of this, a continuous feeding strategy was used, feeding a flow equivalent to the quantity of monosaccharides consumed by the microorganism per hour. The continuous feeding and the gas-stripping were started at 32 h when the butanol concentration was about 7 g L^{-1}. The feeding flow rate (10.6 mL h^{-1}) was calculated through batch fermentation experiments coupled with *in-situ* gas-stripping (data not shown). The results show that the co-fermentation strategy improved monosaccharides uptake to 99.1%. The feeding was stopped at 120 h because the maximum functional

volume of the reactor was reached. Although the microorganism showed a preference for glucose, all the monosaccharides fed into the reactor were consumed between 72 and 96 h (Fig. 5A), showing that when the monosaccharides were scarce inside the reactor, the microorganism was forced to consume all of them. This is an important advantage in comparison with the pulse feeding strategy as monosaccharide uptake was considerably improved. The production of organic acids, mainly butyric and acetic acid, was low throughout these experiments. Butyric acid was not detected, and acetic acid reached maximum concentrations of 1.1 ± 0.1 g L^{-1}.

The gas-stripping process reduced the solvents concentration in the bioreactor, as was observed in the pulse feeding experiments. Maximum concentrations of total butanol of 10.2 g L^{-1} and 13.7 g ABE L^{-1} (Fig. 5B) were obtained. The total concentrations of acetone (3.1 g L^{-1}) and ethanol (0.4 g L^{-1}) were significantly lower ($p < 0.05$) than those obtained in the glucose pulse feeding experimental run, as well as the total butanol concentration (10.2 g L^{-1}). It should be highlighted that the results were promising, as the feeding was carried out with the hemicellulosic hydrolysate from the dilute acid pretreatment of BSG instead of with the model solutions of sugars used in the pulse feeding runs. The concentration of solvents in the condensates (Fig. 5C) showed a more stable behavior than in the pulse feeding runs. The concentration stayed at around 65 g butanol/L until 120 h (around 5 g butanol L^{-1} in the reactor). At this time, the feeding was stopped and the concentrations of butanol at 168 h dropped, both in the reactor (from 5 to 3 g L^{-1}, Fig. 5B) and in the condensates (from 65 to 40 g L^{-1}, Fig. 5C). As in the previous experiments, no phase separation was observed in the condensates, probably due to the concentration of butanol in the bioreactor. Xue et al. [28] reported that it is necessary to conduct gas-stripping at a butanol concentration in the fermentation broth higher than 8 g L^{-1} to obtain a condensate with a butanol concentration which could result in phase separation. However, 8 g L^{-1} butanol is often inhibitory to the bacteria [7]. The condensates presented a higher proportion of butanol than the total concentrations. The ABE mass ratio was 2.3:7.4:0.3 for total concentrations, whereas for the condensate the ratio was 1.6:8.2:0.2 at 120 h. The proportion of butanol is higher than those obtained in the previous pulse feeding experiments, showing a higher selectivity of gas-stripping for butanol. This is probably a consequence of the more stable concentration of butanol inside the reactor throughout the time (about 5 g/L; Fig. 5B), which promotes a higher butanol concentration in the condensates of the gas-stripping (Fig. 5C). From the experimental concentrations, butanol and ABE recoveries were 49.6 g in condensates/100 g total produced, and ABE 50.8 g in condensates/100 g total produced, similar to the values reported by Lu et al. [25] using resin-detoxified wood pulping hydrolysate (53 g butanol recovered/100 g butanol produced).

The yields and productivities referred to the total concentrations (0.14 g g^{-1} and g $L^{-1}h^{-1}$ for butanol and 0.20 g g^{-1} and 0.12 g $L^{-1}h^{-1}$for ABE), which were similar to those obtained in the previous pulse feeding experimental runs (Table 2). Wechgama et al. [26] obtained higher yields (0.39 g butanol g^{-1}) and productivities (0.29 g butanol $L^{-1}h^{-1}$) using *C. beijerinckii* TISTR 1461 and sugarcane molasses as substrate for the fermentation process. The yield referred to the condensed liquids (Table 2) was 0.95 g g^{-1} for butanol and 1.16 g g^{-1} for ABE at 120 h. The productivity of the condensed liquids reached values of 0.55 g $L^{-1}h^{-1}$ for butanol and 0.67 g $L^{-1}h^{-1}$ for ABE. Values are much higher than those obtained in a batch fermentation process using BSG hydrolysates as substrate (yields of 0.20 g butanol g^{-1} and 0.28 g ABE g^{-1}; productivities of 0.06 g butanol $L^{-1}h^{-1}$ and 0.08 g ABE $L^{-1}h^{-1}$ [22]). These results showed that removing butanol by *in-situ* gas-stripping alleviated butanol toxicity and enhanced butanol yields and productivity compared to batch fermentation.

In comparison to data found in the literature about ABE fermentation coupled with gas-stripping (Table 3), other authors reached higher concentrations of total solvents using modified microorganisms in a batch process [16,25], pulses of concentrated juices [9] or both strategies (modified microorganism and pulses of concentrated juices) [24]. One of the possible reasons for obtaining lower concentrations of solvents could be the effect of the catabolite repression. The pretreatment liquid used in the continuous feeding is composed of glucose, xylose and arabinose. The microorganism shows a preference for glucose, which could affect the production of solvents [22]. Another possible reason could be the lower concentration of monosaccharides inside the reactor. Other authors, such as Rochón et al. [9], increased the concentration of monosaccharides up to 50 g L^{-1} with pulses, which helped to obtain total concentrations of 18.6 g butanol L^{-1} and 31.8 g ABE L^{-1}.

On the basis of the experimental data summarized in Fig. 5 and the model described in Eqs. (1)–(6), the kinetic parameters were estimated. The coefficient for the butanol removal term ($k_S a$), 0.02 h^{-1}, was calculated from batch stripping experimental runs, modifying the gas flowrate, according to the procedure described in section 2.6 (data not shown). Cell and butanol yields were calculated from the experimental data ($Y_{X/SG} = Y_{X/SX} = Y_{X/SA} = 0.07$; $Y_{P/SG} = Y_{P/SX} = Y_{P/SA} = 0.14$). Table 4 summarizes the values of the estimated parameters. As it can be observed, the predicted results fitted reasonably well to the experimental data (Fig. 6). Standard deviation of residuals (RSD) varied from a minimum value of 0.27 (arabinose) to a maximum of 2.21 (glucose). This showed that Eqs. (1)–(6) were suitable for describing cell growth, substrate consumption and butanol production and removal. Kinetic parameters were of the same order of magnitude as the values reported for *C. acetobutylicum* with glucose as substrate [9]. It should be noted that the model estimated the kinetic parameters for the different fermentable sugars present in the bioreactor (glucose, xylose and arabinose), showing that the maximum specific growth rate is higher for glucose than for xylose or arabinose. Discrepancies between the experimental and predicted values may be caused by the kinetic model considered to describe cell growth. Other authors considered inhibition by both product and substrate or cell maintenance [29]. Also, the variation of the broth volume due to water stripping could be considered [30].

The results showed that the continuous feeding strategy using the pretreatment liquid is an effective way to force the uptake of the totality of the monosaccharides inside the reactor and obtain a more stable concentration of butanol in the condensates. It also represents an integrated strategy to valorize the liquid obtained during the dilute acid pretreatment of the lignocellulosic material by co-fermenting it with the enzymatic hydrolysate, which reduces capital costs. The fed-batch ABE fermentation with *in-situ* gas-stripping and continuous feeding is a promising strategy that needs further studies for its optimization and effective implementation. Mathematical modelling could be a useful tool to optimize the fermentation process.

Table 4
Estimated parameters of the kinetic model for fed-batch fermentation with *in-situ* gas-stripping and continuous feeding of the hemicellulosic hydrolysate.

Parameter	Unit	Value
μ_{mG}	h^{-1}	0.134
μ_{mX}	h^{-1}	0.013
μ_{mA}	h^{-1}	0.014
K_P	g L^{-1}	9.3
K_{SG}	g L^{-1}	0.350
K_{SX}	g L^{-1}	0.503
K_{SA}	g L^{-1}	1.440
α	–	1.106
k_d	h^{-1}	0.006

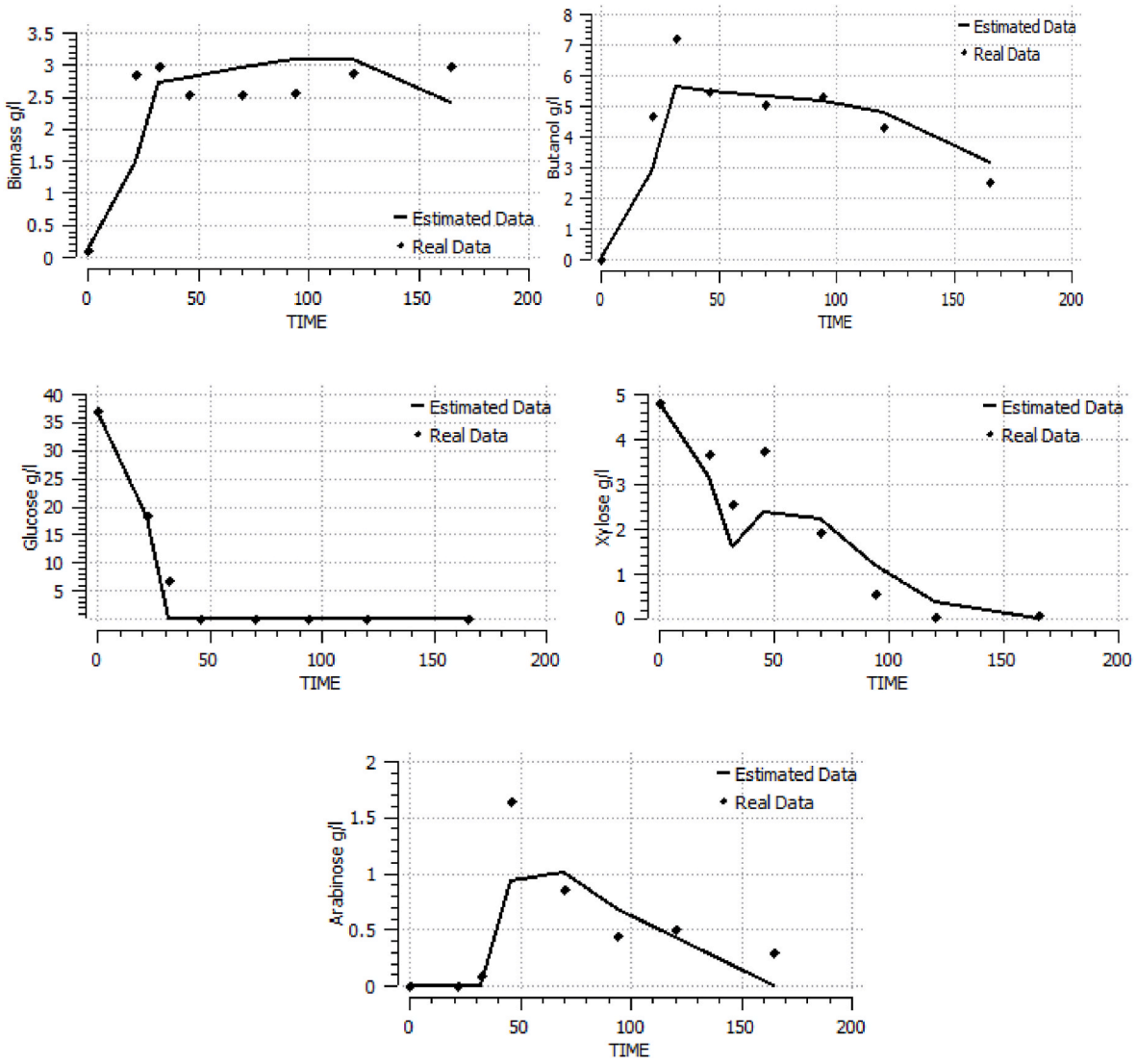

Fig. 6. Fed-batch fermentation with *in-situ* gas stripping and continuous feeding of the hemicellulosic hydrolysate. Concentration profiles of experimental data and predicted values for biomass, butanol, glucose, xylose and arabinose.

4. Conclusions

This study shows that fed-batch ABE fermentation coupled with *in-situ* gas-stripping could mitigate butanol inhibition during the fermentation process. Two feeding strategies were compared: pulses of concentrated monosaccharide solutions of glucose and/or xylose and continuous feeding of the liquid produced in the dilute acid pretreatment of BSG. Both strategies improved the fermentation process. However, the continuous feeding of the pretreatment liquid produced more stable solvent concentrations both in the bioreactor and the butanol condensate. Further research should be carried out to optimize the main operating conditions and the feasibility of the continuous operation to reduce capital and energy costs.

CRediT authorship contribution statement

Pedro E. Plaza: Investigation, Methodology, Writing - original draft. Mónica Coca: Conceptualization, Formal analysis, Supervision, Writing - review & editing. Susana Lucas Yagüe: Methodology, Writing - review & editing. Gloria Gutiérrez: Process modelling conceptualization, Methodology, Writing - review & editing. Eloísa Rochón: Conceptualization, Investigation, Methodology. M. Teresa García-Cubero: Conceptualization, Supervision, Writing - review & editing, Project administration, Funding Acquisition.

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