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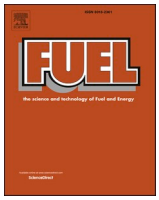

Full Length Article

Developing a high-rate ABE fermentation system for rice straw: CSTR with immobilization on granular-activated carbon with *ex situ* pervaporation

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ABSTRACT

This study assessed the performance of two continuous stirred tank reactors (CSTRs) to immobilize *Clostridium acetobutylicum* DSM 792 and *Clostridium beijerinckii* DSM 6422 on granular activated carbon. Dilution rates (D) from 0.05 to 0.60 h^{-1} were tested using a sugar mixture to mimic rice straw hydrolysate. Immobilization was shown to be a highly effective technique for processing high flows without cell washout. Both CSTRs demonstrated significantly higher butanol productivity than their free-cell counterparts, achieving the remarkable value of 2.29 g L^{-1} h^{-1} with *C. beijerinckii* at D = 0.60 h^{-1}. Rice straw hydrolysate was fed to the high-rate CSTR, reaching a butanol productivity of 2.07 g L^{-1} h^{-1} at D = 0.30 h^{-1}. A novel downstream configuration of *ex situ* gas stripping followed by two pervaporation cycles operated under rough vacuum was explored in discontinuous mode, that reduced the energy demand by 63 % of conventional distillation methods. This configuration holds great promise for the advance of the lignocellulosic biorefinery process.

1. Introduction

Biobutanol, a promising biofuel with gasoline-like properties, can be produced by fermenting solventogenic *Clostridium* spp., of which *Clostridium acetobutylicum* and *Clostridium beijerinckii* are among the most efficient producers of butanol by acetone-butanol-ethanol (ABE) fermentation [1]. The current high prices and the reliance on oil reserves, coupled with environmental awareness, have regained interest in the production of biofuels from renewable resources. Lignocellulosic biomass is the most abundant and cost-effective renewable resource on the planet and is postulated to be a key factor in the feasible production of biobutanol. Rice straw, wheat straw and sugarcane bagasse are some of the leading types of lignocellulosic biomass due to their great potential as fermentation substrates. Rice Straw (RS), in particular, is an interesting feedstock for ABE fermentation, as rice is one of the most widely grown crops in the world, with an estimated production of 1.140 $\times$ 10^6 tons of RS per year [2].

Research on the ABE fermentation process has been frequently carried out in batch mode, yielding typical butanol productivities of <0.5 g L^{-1} h^{-1} [3], even with advanced configurations such as fed-batch mode. Industrial ABE fermentation has been hindered by low solvent yield and productivity, along with the high cost of the downstream process [4]. For an industrial application, the productivity of bioprocesses intended to produce bulk chemicals must be >3.0 g L^{-1} h^{-1} to be profitable [5]. Developing a competitive ABE fermentation process is greatly influenced by the reactor configuration besides the operational strategy used. For example, butanol productivity can be increased by up to 1.0 g L^{-1} h^{-1} in free-cell continuous stirred tank reactors (CSTRs) [6]. Continuous configurations have additional advantages over batch reactors, such as reduced downtime and lower inoculum requirements [7], although biomass growth in the CSTR is coupled to the flow rate, which hinders operations at high dilution rates (D) due to cell washout. Several studies have shown that continuous culture systems with immobilized cells can significantly improve reactor productivity and stability by decoupling biomass growth from the D [8,9]. Granular activated carbon (GAC) has been proposed as a carrier for biomass immobilization as a promising and easy alternative for fixing *Clostridium* spp. due to its large porous surface area and non-toxicity [10]. Although it has been widely used as a carrier for hydrogen production via fermentation [11–13], it has seldom been applied to immobilizing *Clostridium* spp. in ABE fermentation. The successful immobilization on GAC of *C. acetobutylicum* [14,15] and *C. beijerinckii* [16] in batch fermentations has recently been reported,

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although its effectiveness as a biomass carrier in CSTR configurations has not yet been explored.

The huge energy demand for recovering butanol from the fermentation broth is a key challenge facing large-scale ABE fermentation. Butanol inhibition of the solventogenic *Clostridium* spp. at very low concentrations (<20.0 g L^{-1}), in addition to its high boiling point, make butanol recovery by conventional distillation a highly energy intensive technique [6]. For instance, Matsumura et al. [17] pointed out that the conventional distillation process requires 79.5 MJ kg^{-1} to separate butanol from the fermentation broth at a concentration of 5.0 g L^{-1}, which exceeds the heat of combustion of butanol (~36 MJ kg^{-1}), thus limiting the process's industrial sustainability. Vane [18] pointed out that the energy consumption of the distillation unit should be ≤1/3 of butanol's combustion heat. To achieve this target, Mariano and Filho [19] determined that butanol concentrations of 36.0 g L^{-1} in the fermentation broth can reduce the distillation energy consumption. Several purification techniques are now under investigation to reduce the downstream processes' energy consumption, such as gas stripping, liquid–liquid extraction, adsorption, pervaporation, and perstraction [20], of which gas stripping (GS) and pervaporation (PV) are promising techniques owing to their high butanol selectivity and simplicity of operation [21].

In this work, a novel ABE fermentation approach that combined advances in fermentation with downstream processes was studied to enhance the potential feasibility of lignocellulosic biorefining. ABE fermentation, consisting of a CSTR with cells immobilized on GAC was evaluated for *C. acetobutylicum* and *C. beijerinckii*. Hence, the novelty of this research lies in the use for the first time of GAC for the immobilization of solventogenic species in a CSTR system. The effect of D was assessed for both species and compared with their free-cell counterparts in pH-controlled fermentation, using a mixture consisting of glucose and xylose that mimicked the sugar composition of the RS hydrolysate. The best operational results achieved with the glucose:xylose mixture were also tested for alkaline-pretreated RS hydrolysate to assess the feasibility of this approach with real lignocellulosic feedstock. This is one of the few studies of continuous ABE fermentation systems using real lignocellulosic biomass hydrolysate, and the first using rice straw hydrolysate. Furthermore, a novel proposal for a discontinuous downstream process consisting of a GS followed by two PV cycles in rough vacuum was explored as an alternative for process intensification. The energy consumption of the downstream stage was assessed by simulating the process on SuperPro Designer® V12 software.

2. Materials and methods

2.1. Microorganisms and fermentation media

C. acetobutylicum DSM 792 and *C. beijerinckii* DSM 6422 were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) and individually preserved at −80 °C in Reinforced Clostridial Medium (RCM) with 20 % (v/v) glycerol. Each inoculum was prepared by statically growing the preserved strain at 37 °C in a modified RCM solution (19 g L^{-1} RCM supplemented with 10 g L^{-1} glucose). The fermentation medium contained: sugars (glucose:xylose mixture of 45:15 g L^{-1} or RS hydrolysate), 4.0 g L^{-1} yeast extract, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} K_2HPO_4, 2.2 g L^{-1} ammonium acetate, 0.001 g L^{-1} resazurin sodium salt, 0.01 % antifoam 204 and minerals. Mineral salt solutions composed of 0.20 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.01 g L^{-1} $MnSO_4{\cdot}7H_2O$ and 0.05 g L^{-1} $FeSO_4{\cdot}7H_2O$ or 0.09 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.001 g L^{-1} $MnSO_4{\cdot}7H_2O$ and 0.02 g L^{-1} $FeSO_4{\cdot}7H_2O$ were used for *C. acetobutylicum* and *C. beijerinckii*, respectively. The medium was sterilized by autoclave (121 °C for 10 min) and mineral solutions by filtering at 0.22 μm.

2.2. RS pretreatment and hydrolysis

The RS obtained from L'Albufera Natural Park (Spain) was milled to a particle size of between 0.1 and 2.0 mm and then dried at 45 °C in an oven. The RS contained approximately 36 % glucan, 15 % xylan, 14 % lignin, and 17 % ash. Details of its composition can be seen elsewhere [22]. Alkaline RS pretreatment was carried out with a biomass loading of 5 % (w/v) in a 0.75 % (w/v) NaOH solution at 134 °C for 40 min [23]. The solid fraction was recovered by centrifugation (2934 *g* for 6 min), dried for 24 h and enzymatically hydrolyzed at a solid loading of 8 % (w/v) with 20 FPU g-dw^{-1} of the commercial enzyme Cellic® CTec2 blend (Novozyme, Denmark). Enzymatic hydrolysis was performed at 50 °C, 150 rpm, and pH 5.2 with 50 mM sodium acetate buffer for 48 h [24]. RS hydrolysate was recovered by centrifugation (2934 *g* for 6 min), followed by filtration through 1.2 μm.

2.3. Continuous stirred tank bioreactors

Fermentation was carried out in two parallel CSTRs of 250 mL (100 mL working volume) seeded with *C. acetobutylicum* or *C. beijerinckii* with an inoculum size of 5 % (v/v) in each bioreactor. The temperature was maintained at 37 °C in a water bath (Digiterm S-150, J.P. SELECTA, Spain) and stirring was set at 150 rpm (bioMIXdrive 4, 2mag AG, Germany). The bioreactor volume was kept constant by equalizing the inlet and outlet stream flow rates by a multichannel peristaltic pump (Reglo ICC, Ismatec, Wertheim, Germany). Fermentation pH was controlled by LoggerPro software (Vernier, Beaverton, OR, USA) through a pH electrode (InLab Micro Pro-ISM, Mettler Toledo, Switzerland) coupled with NaOH addition. Prior to inoculation, oxygen was displaced with N_2 gas.

In the first set of experiments, CSTRs with free-cells were operated using a glucose:xylose mixture of 45:15 g L^{-1}. The CSTRs were set in batch mode for 24 h, after which continuous mode was started. Five D were tested: 0.05, 0.10, 0.15, 0.30 and 0.60 h^{-1}, each one operated for at least 6 residence times. CSTRs of both *Clostridium* spp. were maintained for 625 h. To avoid over-acidification, pH was controlled by on/off feedback strategy with NaOH to keep a minimum threshold ≃5.2 for *C. acetobutylicum* and ≃5.5 for *C. beijerinckii*. In the second stage, the use of GAC at a loading of 10 % (w/v, carrier material to bioreactor liquid) was evaluated to passively retain bacteria during continuous fermentation. GAC (Scharlau, Spain) of a particle size of 0.92 ± 0.02 mm and a specific surface area of 820 ± 10 m^2 g^{-1} was washed with deionized water, dried overnight and sterilized prior to use. The CSTRs with immobilized cells of the *Clostridium* spp. were operated at the same D as the free-cell counterparts using the same sugar feeding for >460 h to assess the effect of GAC as a biomass carrier on bioreactor productivity. For *C. acetobutylicum*, the pH was kept ≃5.2 and for *C. beijerinckii*, two pH values were tested (≃5.5 and ≃5.2). The operational conditions that provided the best results in terms of butanol productivity were then applied to the RS hydrolysate. In this case, the experiment was performed only for *C. beijerinckii*, the culture that showed the best performance. This test was narrowed using RS hydrolysate for 160 h at a pH ≃5.2 for D of 0.15 h^{-1} and 0.30 h^{-1}.

2.4. Downstream process

A discontinuous downstream process consisting of *ex situ* separation of butanol with a GS followed by two PV cycles was applied in the experiment performed with the RS hydrolysate. First, GS was carried out in the 2-L bioreactor outflow manifold, in which N_2 was sparged at 4 L min^{-1} by a vacuum gas pump (VP 86, VWR, Germany) during 24 h. The stripped solvents were recovered in a condenser at 2 °C through a refrigeration system (AD15R-30, VWR, USA). Then, the condensate from GS was concentrated in a first PV cycle using a setup that involved a 1-L bottle maintained at 65 °C connected to a PV module via a peristaltic pump. The PV module (STC-049, Pervatech, Netherlands) consisted of a commercial flat sheet PDMS membrane (Pervatech, Netherlands) with a

thickness of 1.5 μm and effective area of 76 cm^2. The module was connected to a vacuum pump (12991131, Fisherbrand, USA) to maintain an absolute pressure of ~150 mbar during 4 h. The permeate was recovered in a condenser at −13 °C with a refrigeration system (AD15R-30, VWR, USA). Finally, collected permeate from the first PV cycle was reconcentrated in a second PV cycle.

2.5. Energy evaluation

The process was simulated on SuperPro Designer® V12 software (Intellingen, Inc.; Scott's Plain, NJ, USA). The energy demand of the downstream process was evaluated to obtain grade fuel butanol (99.5 % wt) based on the experimental data obtained from the effluent. The simulations consisted of conventional distillation columns from the fermentation broth (Scenario A) and a system composed of GS, two PV cycles and distillation columns (Scenario B).

2.6. Analytical methods

Effluent samples of 1-mL were taken every few hours to monitor the fermentation process. Cell density (g-dw L^{-1}) was calculated from the optical density at 600 nm (OD_{600}) and measured using a UV–Vis spectrophotometer (SpectroFlex 6600, WTW, Germany). The correlation between OD_{600} and cell density was as follows *C. beijerinckii* (g-dw L^{-1}) = 0.2153·OD_{600} + 0.0689 (R^2 = 0.9907) and *C. acetobutylicum* (g-dw L^{-1}) = 0.2941·OD_{600} + 0.0331 (R^2 = 0.9908). Liquid samples were centrifuged (6800 *g* for 5 min) and filtered through a 0.22 μm filter prior to analysis. The glucose and xylose concentrations and fermentation products (acetone, butanol, ethanol, acetic acid, and butyric acid) were determined on an Agilent HPLC system (1100 Series, Agilent Technologies, USA) equipped with an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA) with a refractive index detector and diode array detector. The mobile phase (5 mM H_2SO_4) was set at 0.6 mL min^{-1}. Scanning electron microscope (SEM) S-4800 (Hitachi, Japan) was used to observe the *C. beijerinckii* DSM 6422 cells adsorbed on the GAC surface. The samples were coated with a mixture of gold and palladium prior to SEM imaging at an accelerating voltage of 10 kV.

3. Results and discussion

3.1. Free-cell continuous stirred tank reactors

Free-cell CSTRs of both strains were operated at different D values for 625 h. The time-course data of the sugars (glucose and xylose), suspended biomass, acids (acetic and butyric acid), and solvents (acetone, butanol, and ethanol) concentrations, are depicted in Fig. 1a for *C. acetobutylicum* and Fig. 1b for *C. beijerinckii*. The *C. beijerinckii* DSM 6422 species produces ethanol at very low concentrations <0.05 g L^{-1}. The start-up in batch mode was satisfactory for both strains, achieving near-complete depletion of glucose as well as butanol concentrations of up to 10.0 g L^{-1} in less than 24 h (Fig. 1). As expected, the consumption rate of glucose was higher over xylose for both microorganisms because of glucose preference [25]. The continuous mode was then started by setting the initial D at 0.05 h^{-1} after which (147 h) complete glucose depletion was achieved, while the xylose leakages were 5.0 g L^{-1} and 3.0 g L^{-1} for *C. acetobutylicum* and *C. beijerinckii*, respectively. The acetic and butyric acid peaks were above 6.0 g L^{-1} and 11.0 g L^{-1}, respectively, indicating that the acidogenic phase occurred at a faster rate than the solventogenic phase. A significant decrease in butanol production was in fact observed at the end of this stage, with values of 4.7 g L^{-1} and 6.3 g L^{-1} for *C. acetobutylicum* and *C. beijerinckii*. In the next stage, D was doubled to 0.10 h^{-1} and maintained for more than 16 residence times. Although both species showed complete glucose depletion, there were differences in xylose leakage under this hydraulic condition. For *C. acetobutylicum* there was a significant oscillation in xylose leakage from 15.0 g L^{-1} at 191 h to 5.3 g L^{-1} at 289 h, while *C. beijerinckii* reached a more stable value, 10.1 ± 0.8 g L^{-1}, from 265 h onwards. Both bioreactors also exhibited similar variations in butyric acid and butanol concentrations as follows: butyric acid oscillated between 7.0 g L^{-1} and 10.0 g L^{-1} and after reaching the peak, a subsequent reassimilation occurred, in which butanol concentration rose to values ~6–7 g L^{-1}, indicating that the butyric acid itself regulated the transient butanol

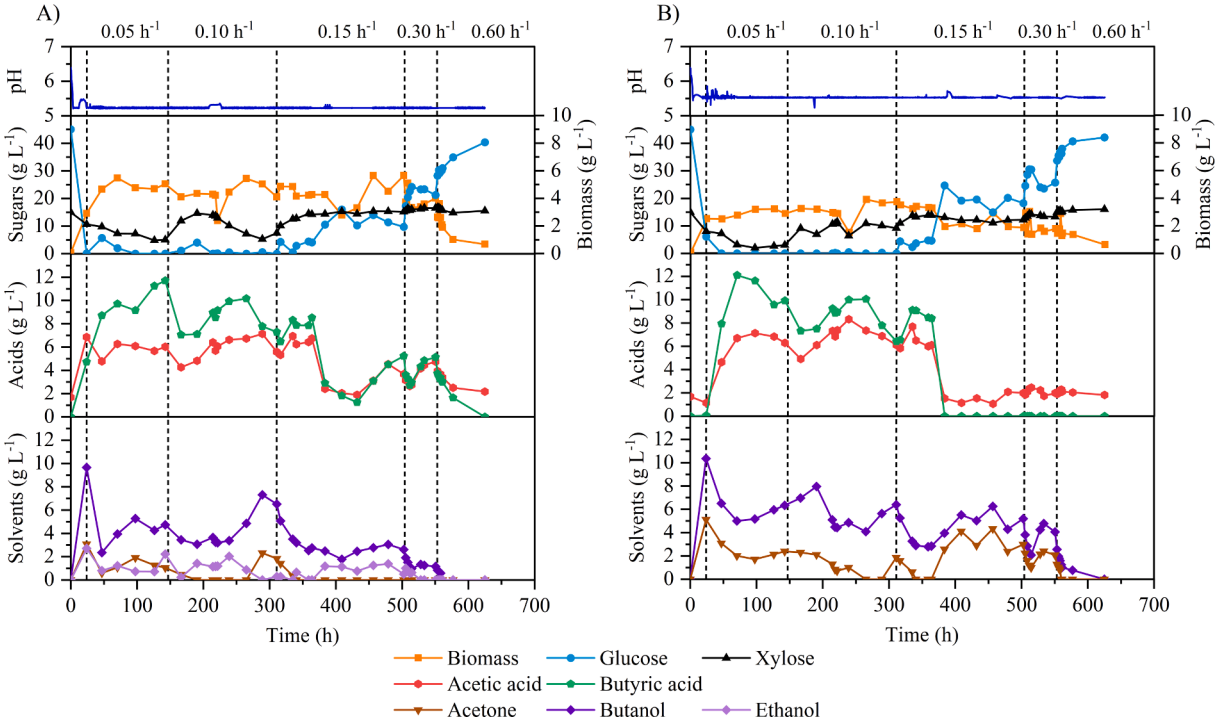

Fig. 1. Time-course data of the sugars, suspended biomass, acids, and solvents concentrations of the effluent in the free-cell CSTR at different dilution rates (0.05, 0.10, 0.15, 0.30 and 0.60 h^{-1}) for A) *C. acetobutylicum* and B) *C. beijerinckii*.

Table 1
Butanol yield and productivity at different D for the CSTRs with suspended-growth cells.

Microorganism	D (h^{-1})	Butanol yield (g g of consumed $sugar^{-1}$)	Butanol productivity (g L^{-1} h^{-1})
C. acetobutylicum	0.05	0.085 ± 0.011	0.23 ± 0.03
	0.10	0.117 ± 0.022	0.62 ± 0.13
	0.15	0.076 ± 0.013	0.41 ± 0.03
	0.30	0.060 ± 0.007	0.38 ± 0.03
	0.60	0	0
C. beijerinckii	0.05	0.107 ± 0.013	0.28 ± 0.03
	0.10	0.116 ± 0.013	0.53 ± 0.11
	0.15	0.181 ± 0.049	0.76 ± 0.12
	0.30	0.171 ± 0.046	1.31 ± 0.11
	0.60	0	0

concentrations during this stage. At 0.10 h^{-1}, *C. acetobutylicum* achieved a slightly higher butanol production than *C. beijerinckii,* 7.3 g L^{-1} versus 6.4 g L^{-1}.

D was later increased by 50 % to 0.15 h^{-1} and maintained for more than 29 residence times. The increased flow rate led to a glucose leakage for the first time, with values of ~10.0 g L^{-1} for *C. acetobutylicum* and ~20.0 g L^{-1} for *C. beijerinckii*. However, *C. beijerinckii* was able to use xylose (outlet concentration 12.0 g L^{-1}, corresponding to 20 % consumption) more efficiently than *C. acetobutylicum*, which showed no xylose removal (Fig. 1). Furthermore, product concentrations began to differentiate between the two species, for *C. acetobutylicum* the average butanol production fell, reaching values of 2.7 ± 0.2 g L^{-1} (359–456 h), which was directly linked to the reduced sugar consumption. With *C. beijerinckii*, butanol production notably increased from 2.8 ± 0.1 g L^{-1} (341–364 h) to 5.3 ± 0.7 g L^{-1} (409–503 h), accompanied by a full reassimilation of butyric acid, indicating that the rate-limiting step was the acidogenic phase, not the solventogenic phase. *C. beijerinckii* was thus taken to be a better candidate for processing sugars from lignocellulosic hydrolyzates to produce butanol at high flow rates. When the D was doubled to 0.30 h^{-1} only *C. beijerinckii* kept its ability to metabolize sugars to butanol, although the greater leakages of glucose and xylose (up to 23.0 g L^{-1} and 13.0 g L^{-1}, respectively) indicated that this flow rate was excessive for operating a free-cell bioreactor for ABE fermentation. When D was raised to 0.60 h^{-1}, both bioreactors clearly exhibited complete cell washout (Fig. 1).

Table 1 summarizes the butanol yield and productivity obtained at different D values for both strains. These data (average and standard deviation) were determined from values obtained during intervals exceeding ≥4 residence times. In general, higher butanol yields and productivities occurred with *C. beijerinckii* versus *C. acetobutylicum*, especially when working at high flow rates (D of 0.15–0.30 h^{-1}). Butanol productivity values for *C. acetobutylicum* varied between 0.23 and 0.62 g L^{-1} h^{-1}, which are within the range in the literature for free-cell continuous ABE fermentation (0.28–0.81 g L^{-1} h^{-1}, at D of 0.05–0.20 h^{-1}) [26–28]. For *C. acetobutylicum*, the maximum butanol yield and productivity were achieved at a relatively moderate D of 0.10 h^{-1}, while increasing the flow rate caused a clear decline in the bioreactor performance. In contrast, *C. beijerinckii* showed a very good performance at high D. A maximum butanol productivity of 1.31 g L^{-1} h^{-1} was achieved at 0.30 h^{-1} accompanied by a high butanol yield (0.171 g g of consumed $sugars^{-1}$). Data on free-cell CSTR with *C. beijerinckii* are still scarce and the reported values are much lower. For example, Lee et al. [8] reached a butanol productivity of 0.22 g L^{-1} h^{-1} when operating at 0.04 h^{-1} by feeding 30 g L^{-1} of glucose supplemented with 18 mM sodium butyrate. The maximum butanol productivity achieved in our work by operating a pH-controlled CSTR for *C. beijerinckii* is 2.1-fold higher than the best data observed herein for *C. acetobutylicum* (0.62 g L^{-1} h^{-1}) and much higher than the previous data reported in the literature for this species (0.81 g L^{-1} h^{-1} [28]). This result is of great interest for improving the overall cost of butanol production in lignocellulosic biorefineries.

3.2. CSTRs with immobilized cells on GAC

3.2.1. Glucose:xylose mixture as substrate

GAC was used to immobilize the strains in an attempt to prevent cell washout when treating high flow rates. Two CSTRs containing 10 % (w/v) GAC were inoculated and fed with the 45:15 g L^{-1} glucose:xylose mixture and operated at the same D values as their free-cell counterparts. The time-course data of the continuous fermentation with immobilized cells are depicted in Fig. 2a for *C. acetobutylicum* and Fig. 2b for *C. beijerinckii*. In this case, the start-up was also performed in batch mode, reaching almost complete glucose depletion for both strains after 24 h, whereas 13.4 g L^{-1} and 7.6 g L^{-1} of xylose remained unconsumed for *C. acetobutylicum* and *C. beijerinckii*, respectively. Notably, no butanol was produced in either bioreactor due to GAC's good butanol adsorption capacity [20]. These data are consistent with those of previously reported studies in batch mode with GAC as a carrier, which indicated a lower butanol concentration than expected [14,15]. Different sugar and product concentrations to the first D tested (0.05 h^{-1}) were observed in both strains in comparison to the free-cell counterpart. When GAC was used, both species were able to fully metabolize both glucose and xylose, showing the positive effect of attaching cells to the GAC surface. Butanol production of *C. acetobutylicum* was practically twice as high (8.7 ± 0.3 g L^{-1}) as that of its free-cell counterpart (4.7 g L^{-1}). Indeed, this species has a great ability to form biofilm structures by excreting exopolysaccharides that facilitate their immobilization and protect the cells from potentially harmful substances [29]. This explained the easy immobilization and the absence of inhibitory effects of adsorbed butanol in the microenvironment around the cells. Comparing both strains, *C. beijerinckii's* bioreactor showed increasing butanol concentrations of up to 3.8 g L^{-1} at the end of this stage (144 h) and reached only half of that produced with *C. acetobutylicum*. In fact, *C. beijerinckii's* butanol production was lower, although the remaining parameters (sugars, acids, and acetone concentrations) were very similar to those of their free-cell counterpart (Fig. 1b). As data from a previous experiment showed that *C. beijerinckii* DSM 6422 was immobilized on the microfibrous cellulose structures of alkaline pretreated RS without biofilm formation [30], our main assumption was that *C. beijerinckii* was passively attached on the GAC surface without forming a biofilm structure, resulting in slower immobilization than that of *C. acetobutylicum* in the early stages of operation.

For the next D (0.10 h^{-1}), *C. acetobutylicum* showed relatively low acid concentrations, while butanol remained the main fermentation product (6.9 ± 0.2 g L^{-1}), indicating that GAC improved the balance between the acidogenic and solventogenic phases, which is attributed to the higher cell concentration of *C. acetobutylicum* when immobilized than that of the free-cell bioreactor. A low affinity for xylose (leakage of

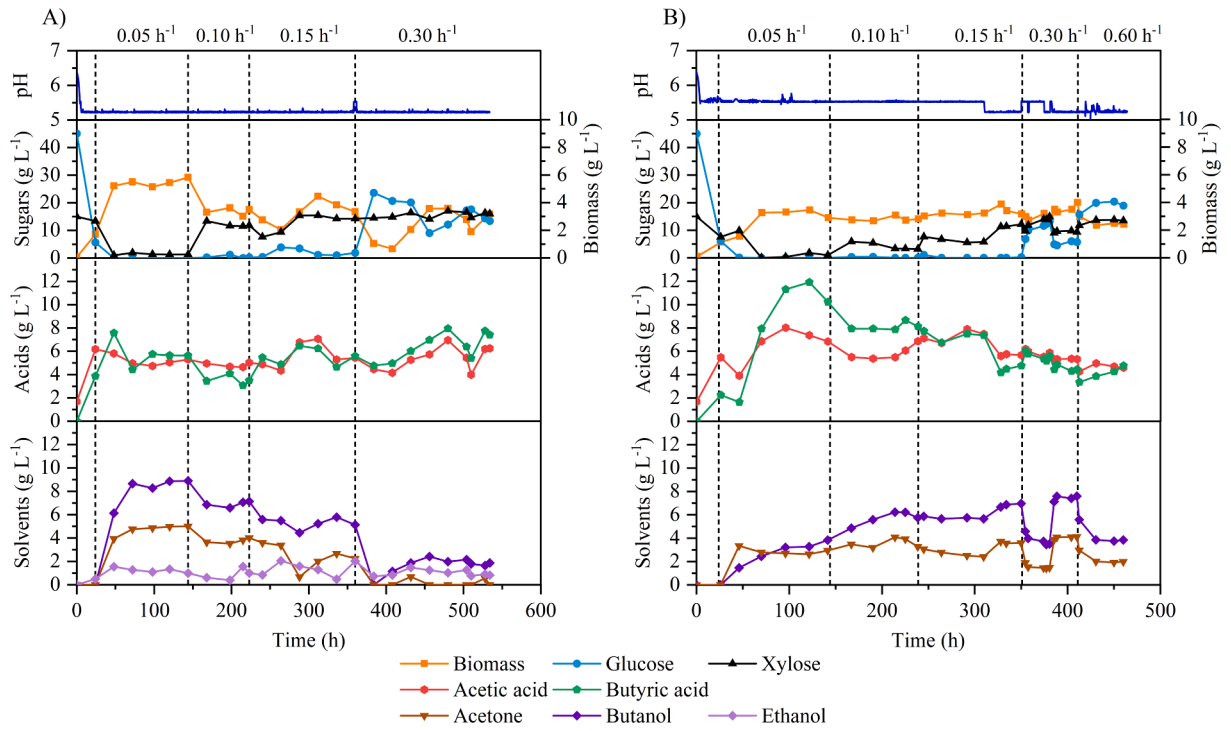

Fig. 2. Time-course data of the pH, sugars, suspended biomass, acids, and solvents concentrations of the effluent in the CSTR with immobilized cells on GAC at different dilution rates (0.05, 0.10, 0.15, 0.30 and 0.60 h^{-1}) for A) *C. acetobutylicum* and B) *C. beijerinckii*.

Table 2
Comparison of immobilization process at different D for both strains.

Microorganism	D (h^{-1})	Butanol yield (g g of consumed sugar^{-1})	Butanol productivity (g L^{-1} h^{-1})
C. acetobutylicum	0.05	0.148 ± 0.005	0.43 ± 0.01
	0.10	0.146 ± 0.004	0.69 ± 0.02
	0.15	0.116 ± 0.008	0.79 ± 0.07
	0.30	0.063 ± 0.008	0.57 ± 0.06
C. beijerinckii	0.05	0.058 ± 0.006	0.16 ± 0.02
	0.10	0.107 ± 0.005	0.59 ± 0.03
	0.15[a]	0.141 ± 0.005	1.02 ± 0.02
	0.30[a]	0.167 ± 0.002	2.22 ± 0.05
	0.60[a]	0.144 ± 0.004	2.29 ± 0.04

[a] Data obtained at a pH threshold of ≃5.2.

11.7 ± 0.2 g L^{-1}) was observed due to carbon catabolite repression. Prior studies showed that the genes involved in xylose metabolism in *C. acetobutylicum* are repressed when glucose is available and hinder its metabolism [31]. In contrast, *C. beijerinckii* was able to efficiently metabolize xylose, as shown by the significantly lower leakages of 3.2 ± 0.1 g L^{-1}. This strain's xylose metabolism appears to be free of repression by glucose, which could be explained by the presence of a large cluster of D-xylose pathway genes [32]. *C. beijerinckii* therefore has an attractive advantage over *C. acetobutylicum* for processing lignocellulosic feedstocks when the secondary sugar is xylose. The butanol concentration with *C. beijerinckii* increased during nearly the whole D (0.10 h^{-1}) until it reached a steady-state value of 6.0 ± 0.3 g L^{-1}, which was very similar to their free-cell test in the same hydraulic conditions (Fig. 1b). Comparing both strains, *C. acetobutylicum* had a slightly higher butanol production, while *C. beijerinckii* reached a higher level of sugar conversion. This difference can be attributed to the influence of the pH control, as lower values (pH ≃5.2) favor solvent production for *C. acetobutylicum* and higher values (pH ≃5.5) promote higher biomass growth and sugar consumption in *C. beijerinckii*.

When operating at a D of 0.15 h^{-1} both bioreactors achieved almost complete glucose depletion, demonstrating a substantial improvement over their free-cell counterparts, showing that GAC led to a larger operative flow for both strains. Butanol production was also improved, especially with *C. acetobutylicum*, reaching twice as high (5.3 ± 0.5 g L^{-1}; Fig. 2a) as in the free-cell bioreactor (2.7 ± 0.2 g L^{-1}; Fig. 1a), although xylose metabolism in *C. acetobutylicum* was fully suppressed at this stage. As *C. beijerinckii* was shown to be the best candidate, it was decided to test two pH thresholds at this flow rate (D = 0.15 h^{-1}): ≃5.5 (239–310 h) and ≃5.2 (310–351 h). Xylose leakage doubled from 6.4 ± 0.9 g L^{-1} to 11.6 ± 0.5 g L^{-1} when pH was reduced to ≃5.2. As expected, reducing the pH threshold also increased butanol production from 5.7 ± 0.1 g L^{-1} to 6.8 ± 0.2 g L^{-1} due to the promotion of acid reassimilation. Interestingly, comparing the performance of the two strains under the same D (0.15 h^{-1}) and pH control (≃5.2), *C. beijerinckii* showed better performance in terms of sugar consumption and butanol production than *C. acetobutylicum*, corroborating the positive effect of GAC in this species.

When further increasing D to 0.30 h^{-1}, *C. acetobutylicum* performance dropped sharply, with a clear reduction in butanol production to 2.0 ± 0.3 g L^{-1}. Indeed, the concentrations of sugars, acids and solvents

observed for immobilized bioreactor (Fig. 2a) were similar to those in the free-cell bioreactor (Fig. 1a). The *C. acetobutylicum* cells were spread between two different fractions, i.e. those immobilized within the GAC and the freely suspended cells in the fermentation broth. The excessive accumulation of inactive cells within the microporous GAC channels could thus explain the similar performance to that of the free-cell bioreactor. As the decline in performance at 0.30 h^{-1} suggested that operation at higher D values could worsen performance even further, the experiment for this species was stopped. In contrast, *C. beijerinckii* showed a good performance at 0.30 h^{-1}, regardless of the pH threshold (≃5.2 and ≃5.5). The lower pH level was found to better promote sugar conversion and higher butanol production (7.4 ± 0.2 g L^{-1}). The pH threshold of ≃5.2 was thus deemed more appropriate for immobilized *C. beijerinckii,* as it facilitated a better balance between the acidogenic and solventogenic phases. Comparing both strains, the absence of biofilm formation in *C. beijerinckii* appears to be a positive feature for performance in long-term operations as GAC allows its attachment without blockages. SEM images taken at the end of this stage further verified the large number of *C. beijerinckii* DSM 6422 cells attached to the GAC surface without a biofilm. The bioreactor with immobilized *C. beijerinckii* was then tested at a D of 0.60 h^{-1} using the minimum pH threshold of ≃5.2. Despite the high sugar leakage (up to 20.0 g L^{-1} for glucose and 14.0 g L^{-1} for xylose), the GAC avoided cell-washout, thus achieving a very stable butanol production of 3.8 ± 0.1 g L^{-1}.

Table 2 shows the butanol yield and productivity of both CSTRs with immobilized cells. In the case of *C. beijerinckii*, the data obtained at a pH threshold of ≃5.2 were used for D values from 0.15 h^{-1} to 0.60 h^{-1}. Both species exhibited significantly higher butanol productivities than in the free-cell test, showing that GAC made it possible to apply higher flow rates during continuous ABE fermentation, thus overcoming the limitations associated with cell washout. This result could have a positive impact on the overall cost of the process. For both *C. acetobutylicum* and *C. beijerinckii*, we have demonstrated the effectiveness of GAC as a carrier for continuous ABE fermentation in stirred tank reactors. The most remarkable advantage was found in *C. beijerinckii*, despite its slower immobilization than *C. acetobutylicum*. Indeed, *C. beijerinckii* exhibited an upward trend in butanol productivity as D increased, reaching a peak of 2.29 g L^{-1} h^{-1} at 0.60 h^{-1}. This finding is of great interest, as high butanol productivity is the keystone in the potential scale-up of ABE fermentation. Other advanced configurations, such as extractive fed-batch fermentation with immobilization resulted in considerably decreased butanol productivity (0.673 g L^{-1} h^{-1}) [33]. Similarly, vacuum fermentation with cells immobilized and operated in a repeated batch demonstrated a butanol productivity roughly 10-fold less (0.21 g L^{-1} h^{-1}) [34]. Thus, proving the competitive advantage of continuous processes, particularly immobilized continuous processes, to achieve scalable butanol productivity. The data in the literature on butanol productivities in CSTRs with immobilization using pure monomeric sugars range between 0.39 and 1.09 g L^{-1} h^{-1} when operating at D values between 0.05 and 0.70 h^{-1} [8,9,27,28]. As far as we know, this is the first study to show butanol productivities ≥2.0 g L^{-1} h^{-1} using a single-stage immobilized CSTR and only fixed bed reactors offer higher butanol productivities. For instance, a continuous packed bed reactor with Tygon® rings as a carrier for *C. acetobutylicum* DSM 792 achieved significantly higher butanol productivity (4.4 g L^{-1} h^{-1}) by feeding a solution mimicking cheese whey at a D value of 0.97 h^{-1} [35]. As CSTRs are comparatively easier to operate, while fixed bed reactors can suffer overpressure due to excessive cell growth, this result promises to reduce the cost of ABE fermentation by significantly increasing the operational flow rates.

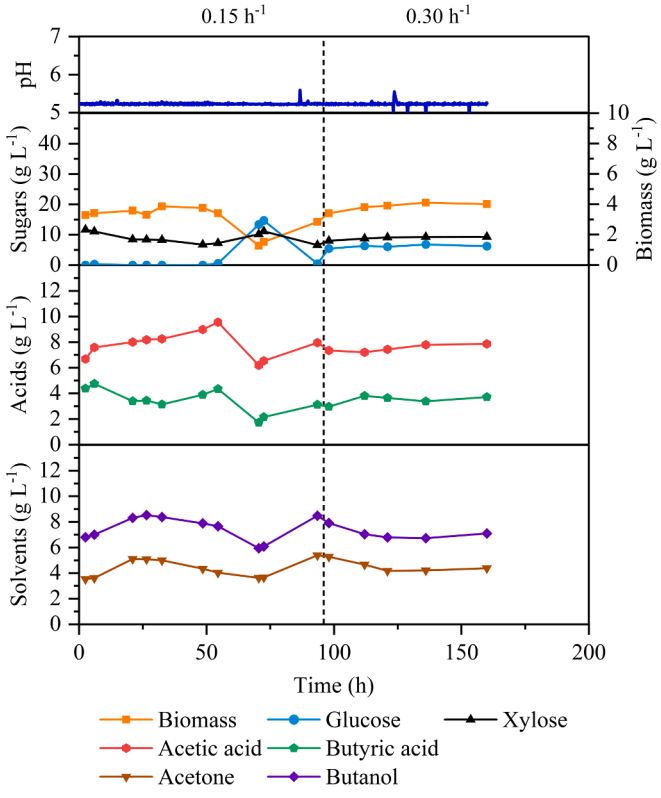

Fig. 3. Time-course data of the pH, sugars, suspended biomass, acids, and solvents concentrations of the effluent in the CSTR with immobilized cells of *C. beijerinckii* on GAC at dilution rates of 0.15 and 0.30 h^{-1} using RS hydrolysate (45 g L^{-1} of glucose; 15 g L^{-1} of xylose).

3.2.2. RS hydrolysate as substrate

To assess the feasibility of immobilized *C. beijerinckii* on GAC for efficiently processing lignocellulosic biomass, the mixture of pure monomeric sugars was replaced by alkali-treated RS hydrolysate after 464 h (Fig. 2b) without any further interruption (see time-course data in Fig. 3). The experiment lasted 160 h, and two D (0.15 h^{-1} and 0.30 h^{-1}) were tested with the minimum pH threshold of ≃5.2. The return to a lower hydraulic condition (0.15 h^{-1}) caused a sudden recovery of butanol production. Indeed, the average butanol production when using RS hydrolysate was slightly higher (7.5 ± 1.0 g L^{-1}; Fig. 3) compared with the glucose:xylose mixture (6.8 ± 0.2 g L^{-1}; Fig. 2b). Xylose conversion also improved, with fewer leakages (8.9 ± 1.9 g L^{-1}) than with pure sugars (11.6 ± 0.5 g L^{-1}). Moradi et al. [36] also reported higher butanol production when using alkali-treated RS hydrolysates versus the synthetic medium. The complex composition of the RS hydrolysate could explain the improved outcomes over those obtained with the pure monomeric sugar solution. However, butanol concentrations of up to 8.5 g L^{-1} caused local inhibition in the microenvironment around the cells in the GAC, which explains the oscillation at 0.15 h^{-1} (Fig. 3). A drop in butanol to 5.9 g L^{-1} was observed at 70 h, accompanied by a sharp reduction of biomass and noticeable sugar leakage. When bacterial strains are clustered at a relatively high density, local inhibition can occur with butanol concentrations as low as 3.0–6.0 g L^{-1} [37]. Solventogenic metabolism then resumed and again reached the inhibitory butanol level (8.5 g L^{-1}) at 94 h (Fig. 3). In contrast, by doubling the D to 0.30 h^{-1} there was no inhibition as butanol production dropped slightly to 7.1 ± 0.4 g L^{-1}. These results show the effectiveness of the pH-controlled CSTR with *C. beijerinckii* immobilized on GAC for high-rate fermentation of lignocellulosic feedstock. RS hydrolysate also proved to be an excellent candidate for butanol production in biorefinery platforms.

Other advanced configurations for butanol production from lignocellulosic feedstocks are compared in Table 3. Fed-batch fermentation with simultaneous saccharification and recovery [30] as well as repeated-batch fermentation with immobilization [38,39] achieved butanol productivities (0.03–0.34 g L^{-1} h^{-1}) and butanol yields (0.210–0.290 g g of consumed $sugar^{-1}$) which were similar to those

Table 3
Comparison of advanced configurations for butanol production via fermentation using different lignocellulosic hydrolysates.

Configuration	Feedstock	Microorganism	D (h^{-1})	Butanol productivity (g L^{-1} h^{-1})	Butanol yield (g g^{-1})	Ref.
Fed-batch SSRF[a]	Rice straw	*C. beijerinckii* DSM 6422	–	0.34	0.210	[29]
Immobilized repeated-batch	Sugarcane bagasse[b]	*C. saccharoperbutylacetonicum* DSM 14923	–	0.03–0.07	0.240	[34]
Immobilized repeated-batch	Cassava bagasse	*C. beijerinckii* ATCC 6014	–	0.23	0.290	[35]
Free-cell CSTR	Wheat straw	GBL- B	0.014	0.18	0.160	[36]
Free-cell CSTR	Rice bran	*C. acetobutylicum* YM1	0.020	0.14	0.240	[37]
Four-stage CSTR	Corn stover	*C. saccharobutylicum* DSM 13864	0.038[c]	0.29	0.279	[38]
Immobilized column reactor	Spruce wood chips[d]	*C. acetobutylicum* DSM 792	0.640	2.90[e]	–	[39]
Cell recycle CSTR	Pine wood	*AvapClo*™	0.736[e]	5.73[e]	–	[40]
Immobilized CSTR	Rice straw	*C. beijerinckii* DSM 6422	0.300	2.07	0.155	This study

[a] Simultaneous saccharification, fermentation and recovery.
[b] Non-detoxified sugarcane bagasse hemicellulose hydrolysates supplemented with crude molasses.
[c] Overall D from the four-stage CSTR with each stage at 0.15 h^{-1}.
[d] SO_2–ethanol–water spent liquor from fractionation of spruce wood chips.
[e] Estimated from published data.

achieved in free-cell CSTR systems reported in the literature [40–42]. Former configurations usually achieve low butanol productivities what hinder its industrial application. High cell density processes operated in continuous allow to achieve significantly higher butanol productivities overcoming this limitation. In comparison with our system, continuous ABE fermentations with free-cells were operated at low D values (0.014–0.038 h^{-1}) [40–42], achieving lower butanol productivity (0.136–0.29 g L^{-1} h^{-1}) and higher yield (0.160–0.279 g g of consumed $sugar^{-1}$). Hence, the CSTR with immobilization on GAC was able to operate at a significantly higher D (0.30 h^{-1}), leading to much higher butanol productivity (2.07 g L^{-1} h^{-1}). An alternative in immobilization systems is the use of packed bed bioreactors. For instance, a continuous column reactor packed with wood pulp achieved a slightly higher butanol productivity (2.90 g L^{-1} h^{-1}) [43]. Nevertheless, packed bed reactors are prone to clogging, thus reducing reactor's operation time. Only one study has been published using a CSTR configuration that achieved butanol productivity of ~5.73 g L^{-1} h^{-1} by using two membrane cell-recycle CSTRs with integrated *in situ* liquid–liquid extraction [44]. In any case, both GAC immobilization and cell-recycling improved butanol the productivity of a single CSTR by more than one order of magnitude, showing that high cell density techniques could play a key role in substantially reducing the cost of ABE biorefineries. As the cost of solvent recovery is highly dependent on its concentration in the fermentation broth, it is important to consider not only the productivity but also the final product concentration when evaluating ABE fermentation processes. One of the advantages of using biomass immobilization in the CSTR configuration is that high butanol concentrations can be achieved at very high flow rates. In our case, immobilization on GAC provided butanol productions of 7.1 ± 0.4 g L^{-1} when operating at D = 0.30 h^{-1} (Fig. 3), so that it was considered a good alternative for further downstream intensification.

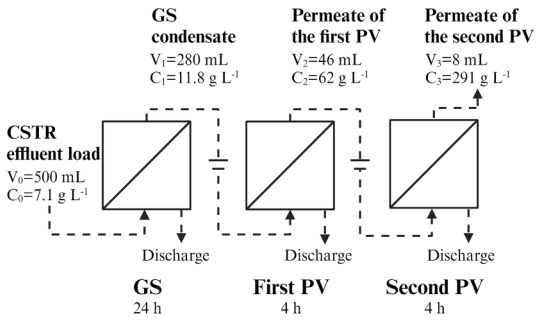

Fig. 4. Schematic diagram and results of discontinuous downstream process composed of an *ex situ* GS followed by two PV cycles. The letters V and C represents the loading volume and butanol concentration at the inlet/outlet from the units.

3.3. Downstream process of the effluent by ex situ GS plus two PV cycles

Downstream butanol purification processes are of the highest importance in terms of the economic feasibility of biorefineries. The conventional distillation process has a high energy consumption due to the low butanol concentration in the fermentation broth, as well as a higher butanol boiling point (117.7 °C) than water (100 °C). For this reason, process intensification is being studied focused on preliminary units to concentrate butanol prior to distillation (GS, PV, perstraction, adsorption, and liquid–liquid extraction). However, as these techniques either alone or in combination are unable to produce fuel-grade butanol a final distillation stage is always required. In this work we used the proposed discontinuous downstream process composed of an *ex situ* GS followed by two PV cycles operated in rough vacuum. Schematic diagram with the results is shown in Fig. 4. This system was applied to the effluent obtained from RS hydrolysate fermentation operated at D = 0.30 h^{-1}. Starting with a volume of 500 mL (V_0) from the CSTR effluent with an average butanol concentration of 7.1 g L^{-1} (C_0), the GS system recovered 93 % of the butanol in the fermentation broth after 24 h of operation, although a relatively low butanol concentration of 11.8 g L^{-1} (C_1) was achieved in the condensate owing to the large amount of water also recovered. Afterwards, a clean condensate of 280 mL (V_1), devoid of cells and non-fermentable solids, was fed to the PV system, showing that the drawbacks of membrane fouling can be avoided. The PV units reported in the literature generally operate at very low pressures (<5 mbar) that are seldom employed in industrial processes [45] and very low temperatures (from −70 °C to −196 °C) to ensure the recovery of all the permeate compounds. In this work, PV units were operated under mild conditions (150 mbar and −13 °C condensing temperature). The serial-PV units were operated sequentially for 4 h each. The butanol recovery ratio of the two-stage PV process was 74 %, providing 46 mL (V_2) of permeate with an average butanol concentration of ~62 g L^{-1} (C_2) in the first stage and about 8 mL (V_3) of permeate with ~291 g L^{-1} (C_3) in the second one. The butanol recovery ratio of the overall downstream system (GS + two PV cycles) was 69 %, while the concentration was 41 times higher than the CSTR effluent. No acetic or butyric acid was detected in the condensate, while the secondary solvent, acetone, was concentrated from ~4.4 g L^{-1} to ~122 g L^{-1}. The flow rate to the final distillation process was reduced by 98 %, indicating that a significantly lower volume of water must be separated in this

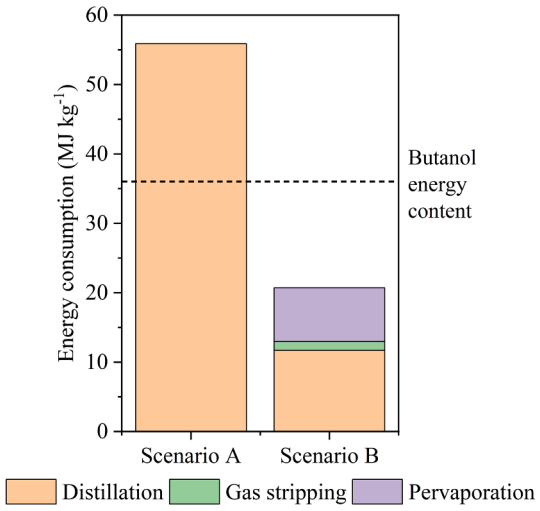

Fig. 5. Energy consumption of conventional distillation columns (Scenario A) and discontinuous downstream processing by *ex situ* GS followed by two PV cycles (Scenario B).

stage, thus lowering the energy demand. Other authors have tested a hybrid downstream configuration of GS plus PV, but with a single PV unit operated at the very low pressure of ~50 mbar [46] and <0.5 mbar [47]. Butanol concentrations of up to 521.3 g L^{-1} were achieved in the condensates but with very low pressures and condensing temperatures (−196 °C), which are easily applied on a lab scale but not feasible in industrial operations. Van Hecke and De Wever [40] found that these very low pressures and temperatures could lead to less accurate energy evaluations, since the same performance cannot be guaranteed in industrial conditions. The proposed system of GS + two PV cycles is a better approach for the potential scale-up of this technology prior to final distillation, since relatively high butanol concentration was achieved using permeate pressures and condensing feasible industrial cost-effective temperatures.

3.4. Energy assessment of downstream processes

The energy assessment of the proposed downstream process to obtain commercial-grade butanol was performed and compared with a conventional distillation train. The scenarios considered were conventional distillation columns in continuous mode from the fermentation broth (Scenario A) and a discontinuous downstream processing by an *ex situ* GS plus two PV cycles, followed by final distillation columns in continuous mode (Scenario B). The mass and energy balances of the downstream scenarios to separate 100 kg h^{-1} of effluent and obtain butanol 99.5 %wt were determined on SuperPro Designer® software. The scheme of the conventional distillation train proposed in Scenario A can be found in Cai et al. [47]. The main difference with other processes is that an ethanol column was not required, as *C. beijerinckii* produce this solvent in traces (<0.05 g L^{-1}). The total energy consumption for each downstream Scenario is shown in Fig. 5. The energy demand for purifying butanol in Scenario A was 55.9 MJ kg^{-1}, which was much higher than the energy content of the butanol itself (~36 MJ kg^{-1}). In this scenario, 82 % of the energy demand was related to the beer column (45.6 MJ kg^{-1}). Matsumura et al. [17] determined that conventional distillation downstream had an energy consumption of 79.5 MJ kg^{-1}. The absence of an ethanol purifying column appears to have advantages as it reduces the energy consumption of the process.

The Scenario B flowsheet incorporated the GS and two-stage PV. The total energy consumption was 20.6 MJ kg¯1, including that estimated from the distillation train (acetone, water and butanol columns), which is in the range reported in the literature (20–23 MJ kg^{-1}) for GS-PV-distillation [46,47]. The energy demand for the different stages was estimated at 1.3, 7.7, and 11.7 MJ kg^{-1} for GS, PV, and distillation columns, respectively. This is a promising result for the success of lignocellulosic ABE biorefinery applications, as Vane et al. [18] pointed out that the energy consumption when recovering butanol by distillation should be less than one-third of its combustion heat (~36 MJ kg^{-1}). Despite the significant reduction in the distillation energy requirements, it continues to be the unit operation with the highest energy demand, so that vacuum distillation combined with heat integration could reduce its energy demand to values as low as 5.3 MJ kg^{-1} [48]. The high-rate CSTR with GAC immobilization coupled with emerging downstream technologies enhances scalability and significantly reduces the energy requirements for producing commercial-grade butanol, showing its potential for successful and sustainable lignocellulosic biorefining.

4. Conclusions

Immobilization on GAC was shown to be an efficient technique for processing high flow rates on CSTR for butanol production. A notable butanol productivity of >2.00 g L^{-1} h^{-1} was achieved by processing RS hydrolysates at a high D (0.30 h^{-1}) in a single CSTR. The butanol content of the CSTR effluent was efficiently concentrated by a novel configuration composed of discontinuous GS, followed by two PV cycles operated in a rough vacuum suitable for industrial operations. The total energy consumption was 20.6 MJ kg^{-1}, which is a step forward in achieving profitable operations in ABE biorefinery.

CRediT authorship contribution statement

Carlos Silvestre: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miguel Capilla:** Data curation, Formal analysis. **Alejo Valles:** Conceptualization, Methodology. **Pau San-Valero:** Conceptualization, Methodology. **Carmen Gabaldón:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **F. Javier Álvarez-Hornos:** Conceptualization, Methodology, Investigation, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fuel.2023.130538.

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