Research Article

Received: 23 September 2024 Revised: 2 December 2024 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jctb.7799

Enhanced biobutanol production from rice straw with a two-stage packed-bed fermenter coupled with in-line gas stripping

Carlos Silvestre,[a] Lukas Niedermeier,[b] Miguel Capilla,[a] Carmen Gabaldón[a] and Javier Álvarez-Hornos[a*]

Abstract

BACKGROUND: This study describes the operation of continuous packed-bed reactors containing *Clostridium beijerinckii* DSM 6422 attached to granular activated carbon to improve butanol productivity and sugar consumption from rice straw hydrolysate.

RESULTS: A single-stage fermenter, which was evaluated at dilution rates ranging from 0.3 to 1.8 h^{-1}, achieved a maximum butanol productivity of 4.44 g L^{-1} h^{-1} at a dilution rate of 1.2 h^{-1}, corresponding to a sugar consumption of 40%. The performance of the two-stage fermentation plant with in-line gas stripping and effluent recycling was studied at a dilution rate of 1.2 h^{-1} for each bioreactor, resulting in a butanol productivity of 4.16 g L^{-1} h^{-1} in the first stage and 0.98 g L^{-1} h^{-1} in the second. Nutrient supplementation was identified as an effective method of enhancing butanol productivity by up to 50% in the second stage. The overall sugar consumption of the two-stage packed-bed reactor with in-line gas stripping was 69%.

CONCLUSION: These findings show considerable promise for the future development of lignocellulosic biorefineries, as they demonstrate a potential to achieve economically profitable butanol production.

Supporting information may be found in the online version of this article.

Keywords: butanol; gas stripping; granular activated carbon; packed bed; rice straw; two-stage fermentation

INTRODUCTION

Using lignocellulosic waste to produce bio-based bulk chemicals is a key priority in the EU's policy for achieving climate neutrality by 2050.[1] Although the current production of bio-based chemicals represents only 0.3% of the EU chemical market,[2] it could account for 36% of organic chemical production by 2040[3] and 75% by 2050.[4] Rice straw (RS) is a plentiful, renewable, and inexpensive agricultural waste currently burned by the growers, with a detrimental impact on the environment and results in wasting valuable lignocellulosic resources.

Biobutanol is a particularly promising bio-based chemical with the potential to significantly contribute to the demand for next-generation biofuels due to its remarkable similarity to gasoline.[5] One of the principal obstacles to the cost-effectiveness of producing butanol by acetone–butanol–ethanol (ABE) fermentation is that butanol severely inhibits the solventogenic *Clostridium* spp.[6] This inhibition at a very low butanol concentration (<13 g L^{-1}) clearly has an impact on the productivity of ABE fermentation. Butanol productivity in batch fermenters is restricted to a narrow range of 0.2–0.4 g L^{-1} h^{-1},[7] while cell concentrations are typically less than 4.0 g L^{-1} and butanol-diluted broths require further energy-intensive downstream processes.[8] Although higher butanol productivity can be achieved by continuous stirred-tank reactors (CSTRs), this operation is restricted to dilution rates that do not exceed the maximum growth rate. This limits butanol productivity to approximately 1.0 g L^{-1} h^{-1},[9] which is below the minimum benchmark (≥3.0 g L^{-1} h^{-1}) required for commercially viable bulk chemical production.[10]

The possible ways of overcoming the limited productivity of suspended-growth CSTRs include several strategies, such as packed-bed reactors (PBRs), which can achieve cell densities as high as 40–75 g L^{-1}.[11,12] The formation of dense bacterial clusters thus enables PBRs to operate at relatively high dilution rates with productivities above the benchmark set-point,

* *Correspondence to: J Álvarez-Hornos, Research Group GI²AM, Department of Chemical Engineering, Universitat de València, Av. De la Universitat S/N, 46100 Burjassot, Valencia, Spain. E-mail: francisco.j.alvarez@uv.es*

a *Research Group GI²AM, Department of Chemical Engineering, Universitat de València, Valencia, Spain*

b *Department of Chemistry, Technical University of Munich, Munich, Germany*

although substrate utilization is lower than the suspended-growth alternatives.[13] The literature on PBRs for continuous ABE fermentation operations reveals that only 20–50% of the glucose fed to the system is consumed, while the remainder is lost in effluent.[14-16] As processing lignocellulosic raw materials to produce sugar-rich streams is associated with an energy-intensive pretreatment, it is of the utmost importance to achieve high sugar utilization in the fermentation step to obtain a positive energy output for the entire process (i.e., pretreatment, fermentation, and downstream). Some studies have shown that it is possible to improve substrate utilization by means of a multiple-stage PBR. For example, Badr *et al*. developed a continuous four-stage PBR with ceramic beads as carriers containing immobilized cells of *C. acetobutylicum* P-262 and fed with defibered sweet potato slurry composed of 39.7 g L^{-1} starch, reaching a butanol productivity of 0.71 g L^{-1} h^{-1} and total starch consumption.[17] However, this type of intense fermentation technology requires *in situ* product recovery (ISPR) to maintain the butanol concentration below inhibitory values. ISPR techniques are distinguished by their low energy consumption and improved fermentation performance by alleviating butanol toxicity.[18] Several ISPR techniques have been coupled in different PBR configurations. For instance, Ennis *et al*.[19] developed a continuous two-stage PBR using bone char as a carrier for *C. acetobutylicum* P262 incorporating different between-stage ISPR techniques: gas stripping (GS), adsorption, and molecular sieve. Bankar *et al*.[20] carried out a continuous two-stage PBR with *C. acetobutylicum* DSM 792 immobilized on wood pulp fiber with an integrated solvent recovery via liquid–liquid extraction between stages 1 and 2. Wang *et al*.[7] established a continuous single-stage PBR to immobilize *C. acetobutylicum* ATCC824 on bricks that was coupled with *in situ* extraction–gas stripping while, more recently, Liu *et al*.[21] immobilized *C. acetobutylicum* CICC 8012 on bagasse within a PBR that was coupled with pervaporation but working in fed-batch. Of these, gas stripping (GS) is particularly advantageous in terms of its simplicity as it does not require membranes or additional chemicals and does not remove nutrients.[22] With regard to two-stage PBR configurations, the incorporation of an ISPR subsequent to the first stage does not fully leverage the potential of this technique, given that the solvents generated in the second stage are not recovered and, indeed, the application of such techniques between stages with a recirculation strategy in the second stage has not yet been explored.

The aim of this study was thus to develop an advanced ABE fermentation configuration to efficiently bioprocess lignocellulosic biomass hydrolysates. The approach is based on a continuous PBR system containing granular activated carbon (GAC) to immobilize *C. beijerinckii* DSM 6422. The impact of dilution rate on butanol productivity and sugar consumption was evaluated in a single-stage PBR using alkaline-pretreated RS hydrolysate. The best hydraulic condition was then evaluated in a novel two-stage PBR configuration coupled with in-line GS and effluent recirculation as promising configurations for overcoming the economic constraints that hinder the development of large-scale ABE fermentation. The impact of the second PBR nutrient supplementation was also evaluated. The major innovative features of a two-stage PBR include the combination of GS between stages, plus effluent recirculation and nutrient supplementation to the second PBR for maximizing sugar utilization from a real lignocellulosic hydrolysate.

MATERIALS AND METHODS

RS hydrolysate production

RS from the L'Albufera Natural Park (Spain) was milled and sieved to a particle size between 0.1 and 2.0 mm and dried at 45 °C before an alkaline pretreatment of 0.75% (w/v) NaOH solution with a solid loading of 5% (w/v). The mixture was heated to 134 °C and held for 40 min, as described elsewhere.[23] The pretreatment slurry was then centrifuged (2934 × *g* for 6 min) and the resulting solid fraction was washed, dried at 45 °C for 24 h, and stored at 4 °C. The pretreated RS was saccharified at a solid loading of 8% (w/v) using the commercial enzyme blend Cellic CTec2 (Novozymes, Bagsvaerd, Denmark) at a dosage of 20 FPU g^{-1} dry weight. Saccharification was performed using a 50 mmol L^{-1} sodium acetate buffer at 50 °C and 150 rpm for 48 h according to the procedure in Capilla *et al*.[24] RS hydrolysate was centrifuged (2934 × *g* for 6 min), filtered, and stored at 4 °C until further use.[24] No detoxification was required as no inhibitory substances were detected.[23]

Bacterial strain and fermentation media

The bacterial strain *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and stored at −80 °C in Reinforced clostridial medium (RCM) with 20% (v/v) glycerol. Prior to fermentation, the inoculum was cultivated under static conditions at 37 °C in serum bottles containing 40 mL modified RCM (19 g L^{-1} RCM supplemented with 10 g L^{-1} glucose). The inocula were grown overnight.

The fermentation media consisted of: 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, 1.0 mg L^{-1} resazurin sodium salt, and 0.01% antifoam 204, sterilized by autoclaving at 121 °C for 20 min. Mineral salt solution (0.09 g L^{-1} $MgSO_4.7H_2O$, 0.001 g L^{-1} $MnSO_4.7H_2O$, and 0.02 g L^{-1} $FeSO_4.7H_2O$) was filter-sterilized using a 0.22 µm filter. The substrate was a glucose–xylose mixture (45:15 g L^{-1}) or alkali-pretreated RS hydrolysate (45.5 ± 1.8 g L^{-1} glucose, 15.8 ± 0.8 g L^{-1} xylose, 0.8 ± 0.1 g L^{-1} cellobiose, 1.5 ± 0.1 g L^{-1} arabinose, and 3.9 ± 0.4 g L^{-1} acetic acid), both sterilized by autoclaving at 121 °C for 10 min. Beforehand, anaerobiosis of the media was guaranteed by sparging N_2.

Continuous fermentation in PBRs

Single-stage fermentation

The bioreactor contained a vertical glass column with an inner diameter of 10 mm and a bed height of 150 mm packed with GAC (Scharlau, Barcelona, Spain). The bioreactor's void volume was 10 mL, representing a void fraction of 85%. The GAC (0.92 ± 0.02 mm size, specific surface area 820 ± 10 m^2 g^{-1}) was previously washed with deionized water and dried overnight. The bioreactor was sterilized by autoclaving at 121 °C for 20 min and N_2 was passed through the packed column overnight. The system was maintained at 37 °C by a circulating water bath (Digiterm S-150, JP SELECTA, Barcelona, Spain). The bioreactor was connected to a 1-L bottle containing the fermentation medium with a glucose–xylose mixture (45:15 g L^{-1}) as carbon source. The medium was inoculated with 50 mL (5%, v/v) of an actively growing *C. beijerinckii* culture. The bioreactor was seeded by transferring the growing culture from the 1-L bottle using a multichannel peristaltic pump (Reglo ICC, Ismatec, Wertheim, Germany) in a closed-loop flow operation for 1 day. Following the 24-h batch period, the seeded bottle was removed and the

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

continuous mode was initiated at a dilution rate of 0.30 h^{-1} for 5 days using a glucose–xylose mixture (45:15 g L^{-1}). RS hydrolysate was then used as a substrate for 12 days, during which four dilution rate values (0.3, 0.6, 1.2, and 1.8 h^{-1}) were evaluated. Each dilution rate was maintained for a minimum of 20 residence times to isolate the effect of each dilution rate and to ensure that the effects of the previous dilution rate did not influence the subsequent one.

Two-stage fermentation with in-line GS

The experimental setup was composed of two identical bioreactors (each with 10 mL of void volume) as described above and a single between-stages intermediate tank (100 mL working volume) where GS was carried out. Figure 1 is a diagram of the proposed configuration. The first stage was designated R1 and the second stage R2. Seeding of both bioreactors (R1 and R2) was carried out in a closed-loop flow operation between the inoculated medium in the intermediate tank and both PBRs during 24 h without GS action. The two fermenters were then placed sequentially in continuous operation. Using a multi-channel peristaltic pump (Reglo ICC, Ismatec) fresh medium was continuously fed to R1 (operated as a flow-through system) and its effluent was discharged to the intermediate tank. R2 (operated with effluent recirculation) was continuously fed by fermentation broth from the intermediate tank where the R2 effluent was discharged. GS was used to jointly remove the solvents generated in R1 and R2 as a prior step to feeding R2. The fermentation gas (CO_2 and H_2) was sparged through the fermentation broth at a rate of 4 L min^{-1} using a vacuum gas pump (VP 86, VWR, Darmstadt, Germany). GS was operated intermittently every hour using an on/off strategy. The stripped solvents were condensed at 4 °C using a refrigeration system (AD15R-30, VWR, Radnor, PA, USA). The condensate was extracted regularly for both volume measurement and solvent quantification. The system's outflow was adjusted to maintain the volume of the intermediate tank at 100 mL considering the net inflow of the intermediate tank (R1 effluent) and the outlet extracted by GS.

The dilution rate that yielded the most promising results in terms of butanol productivity in the experiments of the single-stage PBR was applied to each stage of the two-stage PBR configuration. The system was started up by feeding R1 with a synthetic glucose: xylose mixture at a dilution rate of 0.6 h^{-1} (influent volumetric flow rate of 0.1 mL min^{-1}) for 3 days, followed by 1.2 h^{-1} (influent volumetric flow rate of 0.2 mL min^{-1}) for 8 days, while R2 was continuously operated at the same dilution rate as R1 (i.e., recirculating flow rate in R2 was kept equal to the influent volumetric flow rate of R1). Therefore, when each stage was operated at a dilution rate of 0.6 h^{-1}, the dilution rate of the whole system was 0.3 h^{-1}. Each bioreactor was then operated at a dilution rate of 1.2 h^{-1} (dilution rate of the whole system of 0.6 h^{-1}), and R1 was fed with alkaline-pretreated RS hydrolysate for 8 days. In a second series of experiments, the nutrient supplement to R2 was evaluated with the aim of enhancing glucose and xylose utilization and productivity when using ISPR by GS. For this experiment, each bioreactor was operated at a dilution rate of 1.2 h^{-1}. A nutrient solution, comprising yeast extract, KH_2PO_4, K_2HPO_4, ammonium acetate, and a mineral salt solution in concentrations ten times higher than those in a standard fermentation medium was continuously supplied to the R2 inlet stream at a rate of 10% (v/v).

Analytical methods

Samples were collected periodically to monitor the fermentation process. Liquid samples were centrifugated (6800 × *g* for 5 min) and filtered through a 0.22 µm filter before analysis. Glucose and xylose concentrations and fermentation products (acetone, butanol, acetic and butyric acids) were quantified using a high-performance liquid chromatograph (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector and diode array detector. The system used an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA, USA) operated at 35 °C, while the mobile phase, consisting of 5 mmol L^{-1} H_2SO_4, was maintained at a flow rate of 0.6 mL min^{-1}.

RESULTS AND DISCUSSION

Single-stage PBR

The performance of the PBR with *C. beijerinckii* immobilized on CAG was monitored to evaluate butanol production from alkali-treated RS hydrolysates at different dilution rates. Figure 2(a) depicts the time-course data for the concentrations of sugars (glucose and xylose), acids (acetic and butyric acids), and solvents (acetone and butanol). As has been previously reported by other authors using the strain *C. beijerinckii* DSM 6422, ethanol was

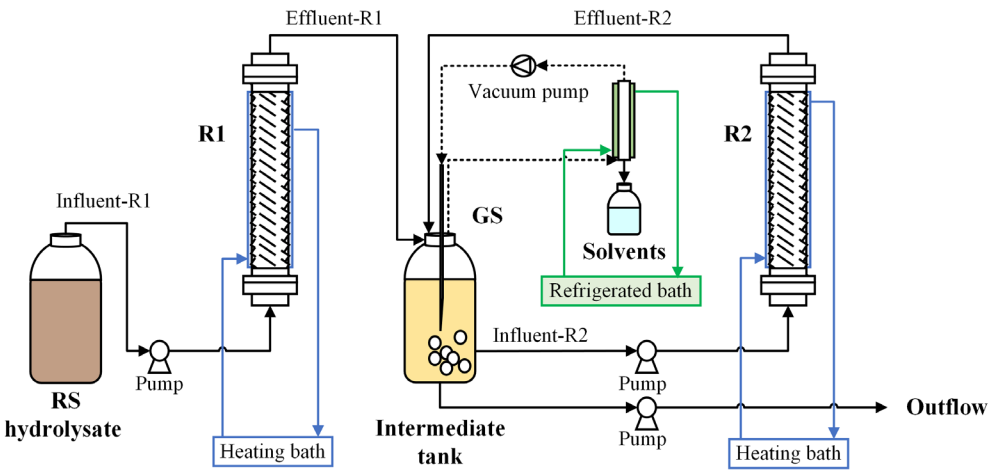

Figure 1. Schematic diagram of two-stage PBR configuration with in-line GS.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

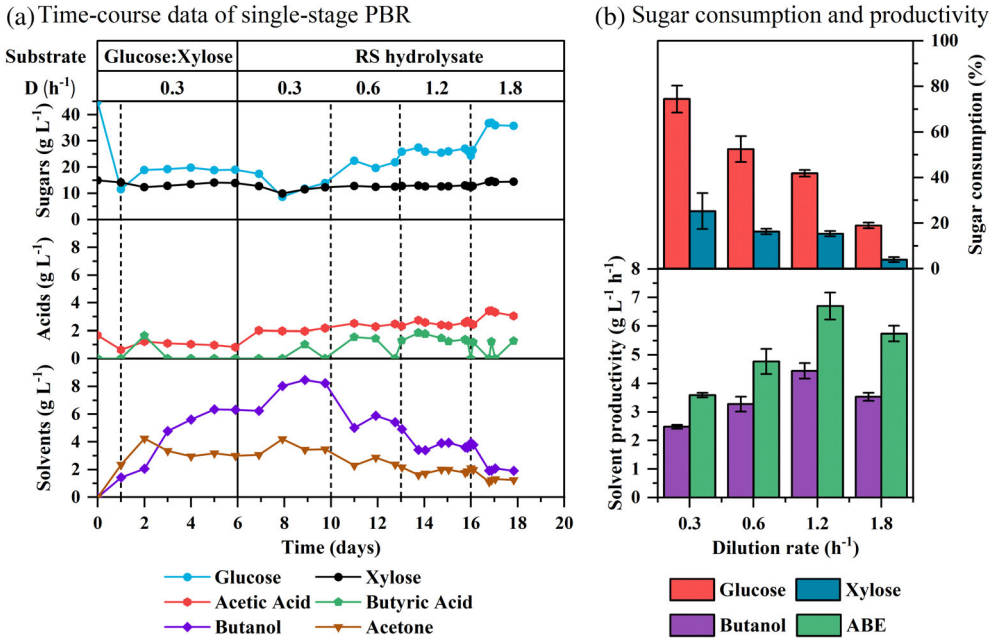

Figure 2. (a) Time-course data of the concentration of effluent sugars (glucose and xylose), acids (acetic and butyric), and solvents (acetone and butanol) in single-stage PBR at dilution rates (*D*) of 0.3, 0.6, 1.2, and 1.8 h^{-1}. (b) Influence of dilution rate on sugar consumption and solvent productivity of single-stage PBR. Data were obtained for each dilution rate as the average value obtained over a minimum of 13 residence times.

not detected.[25-27] The PBR was initiated in batch mode on the first day to establish a viable cell population attached to the GAC. Glucose consumption was found to be 74% after 24 h (remaining glucose concentration of 11.6 g L^{-1}) of seeding the bioreactor by transferring inoculated media in close-loop operation with a 1-L bottle. In the first 24 h xylose consumption was negligible due to carbon catabolite repression by glucose. Many microorganisms, including *C. beijerinckii*, exhibit a strong preference for glucose over xylose.[28,29] The low concentrations of butanol (1.4 g L^{-1}) and acetone (2.4 g L^{-1}) were attributed to the strong adsorption capacity of fresh CAG. After 24 h, continuous mode was started by feeding a synthetic glucose–xylose mixture at a dilution rate of 0.30 h^{-1} for 5 days to increase the amount of active biomass in the GAC prior to using the RS hydrolysate. During this period (days 1–6; Fig. 2a), average glucose and xylose concentrations in the effluent were 19.2 ± 0.4 g L^{-1} and 13.4 ± 0.7 g L^{-1}, respectively, thus indicating successful seeding of the bioreactor. Butyric acid was not detected at this dilution rate, except on day 2 (Fig. 2a), thus indicating that the acidogenic phase, was the rate-limiting step rather than the solventogenic phase. Working at a dilution rate of 0.3 h^{-1}, butanol concentration gradually increased until a stable value of 6.3 ± 0.1 g L^{-1}. The improvement in butanol concentration could be explained by the gradual accumulation of biomass attached to the GAC during the early days of operation, as confirmed by visual inspection of the bioreactor.

Glucose–xylose feeding was replaced by RS hydrolysate on day 6 without further interruptions. The experiment lasted 12 days (days 6–18; Fig. 2a), during which four dilution rate values were tested: 0.3, 0.6, 1.2, and 1.8 h^{-1}. By operating at a dilution rate of 0.30 h^{-1}, the change to real hydrolysate notably improved the bioreactor performance. The sugar concentration in the effluent fell to 11.5 ± 2.7 g L^{-1} for glucose and 11.3 ± 1.2 g L^{-1} for xylose, providing better sugar metabolism than that of the synthetic mixture, while butanol concentration increased to 8.3 ± 0.2 g L^{-1}. Similar findings were observed by Jin *et al.*, who noted faster sugar depletion during ABE fermentation of apple pomace residue than in pure sugar solution fermentation, which was attributed to its mineral content.[30] Capilla *et al.* demonstrated that batch fermentation of alkali-pretreated RS hydrolysate enhanced butanol concentration over a pure sugar medium.[24] The superior performance can thus be related to the type of substrate, as the richer mineral composition of the RS hydrolysate positively influenced *Clostridium* fermentation. When dilution rate was doubled to 0.6 h^{-1} (days 10–13; Fig. 2a), there was a notable increase in the sugar concentration in the effluent (21.3 ± 1.4 g L^{-1} for glucose and 2.6 ± 0.2 g L^{-1} for xylose). Along with the incomplete conversion of intermediate metabolites (acetic acid and butyric acid), this reduced butanol concentration to 5.4 ± 0.4 g L^{-1}. On day 13, the dilution rate was adjusted to 1.2 h^{-1} (days 13–16; Fig. 2a). Doubling the flow rate resulted in an even higher effluent glucose concentration (26.1 ± 0.9 g L^{-1}), while the affinity for xylose remained quite low, as indicated by its effluent concentration of 12.6 g L^{-1}. The poorer performance in terms of sugar metabolism further reduced butanol concentration to 3.7 ± 0.2 g L^{-1}. On raising dilution rate to 1.8 h^{-1}, the bioreactor performance declined (days 16–18; Fig. 2a), which was related to a sudden rise in the effluent glucose concentration and the almost complete suppression of xylose metabolism, and reduced butanol concentration to 2.0 ± 0.1 g L^{-1}.

Figure 2(b) gives the average consumption of glucose and xylose sugars and solvent productivity using RS hydrolysate at the four dilution rates tested. As can be seen, the increased flow rate was accompanied by a gradual reduction in terms of sugar utilization. In particular, glucose consumption fell from 71% at 0.3 h^{-1} to 19% at 1.8 h^{-1}, while xylose utilization was markedly lower, with values ranging from 22% to 3% over the same period. However, the bioreactor achieved notable butanol productivity with values over 2.0 g L^{-1} h^{-1} for all the dilution rates. This shows that the GAC-based PBR is a promising alternative for processing

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

high flow rates of RS hydrolysate combined with high butanol productivity, a key factor in industrial-scale ABE fermentation. In fact, butanol productivity showed an upward trend as dilution rate increased to 1.2 h^{-1}, although butanol productivity was reduced when dilution rate was raised to 1.8 h^{-1}. The maximum butanol productivity achieved by using the single PBR without GS was 4.44 ± 0.27 g L^{-1} h^{-1} at a dilution rate of 1.2 h^{-1}; and the total solvent productivity (acetone + butanol) was 6.70 ± 0.47 g L^{-1} h^{-1}. However, at the maximum solvent productivity, the consumption of glucose and xylose was only 42% and 15% respectively, with a large portion remaining unconsumed and lost through the effluent, with a negative impact on economic feasibility. Interestingly, butanol yield exhibited a different trend as it decreased from 0.220 ± 0.022 g g^{-1} of consumed $sugars^{-1}$ at a dilution rate of 0.3 h^{-1} to 0.175 ± 0.009 g g^{-1} of consumed $sugars^{-1}$ at 1.2 h^{-1}, correlating with the increased dilution rate and reduced sugar consumption. However, at the highest dilution rate (1.8 h^{-1}), butanol yield rebounded to 0.216 ± 0.015 g g^{-1} of consumed $sugars^{-1}$. This rebound can likely be attributed to the significantly lower substrate consumption at high flow rates, which reduced the extent of metabolic byproduct formation, thereby favoring butanol production.

These results are compared with others in the literature on immobilized ABE fermentation in Table 1. Very few studies have focused on using hydrolysates derived from lignocellulosic feedstocks, especially in CSTR configurations, as the stirring shear forces have a negative impact on biomass attachment, thus limiting the dilution rate (0.04–0.30 h^{-1}) to values only slightly above the maximum specific growth rate.[31,32] For example, in a previous study using GAC immobilization in a CSTR configuration at a dilution rate of 0.3 h^{-1}, we obtained a butanol productivity of 2.07 g L^{-1} h^{-1}.[32] By operating at the same dilution rate (0.30 h^{-1}), the favorable GAC-based PBR performance led to a 20% increase in butanol productivity (2.48 ± 0.07 g L^{-1} h^{-1}). Instead of using complex media, most of the studies on immobilization have been carried out in PBRs using hexose-based substrates,[7,15,33,34] and other studies have been focused on the use of cheese whey[35,36] (mainly composed of lactose) or food waste[37] (mainly composed of starch), with butanol productivities ranging from 2.38 to 8.55 g L^{-1} h^{-1} for dilution rates between 0.39 and 1.9 h^{-1}. Only the data sets 9 (butanol productivity: 6.30 g L^{-1} h^{-1}; Wang *et al.*[7]) and 11 (butanol productivity: 8.55 g L^{-1} h^{-1}; Survase *et al.*[34]) demonstrated a notable increase in productivity when compared to our maximum value (4.44 g L^{-1} h^{-1}; set 14). It is noteworthy that both experiments employed glucose as the sole substrate. It is well documented that glucose is the preferred sugar for butanol production by clostridia. Glucose is degraded via the preferential Embden–Meyerhof–Parnas metabolic pathway, while pentoses are degraded via the less efficient pentose phosphate pathway.[38] Sets 5 (butanol productivity: 2.90 g L^{-1} h^{-1}; Survase *et al.*[39]) and 10 (butanol productivity: 3.38 g L^{-1} h^{-1}; Survase *et al.*[16]) were performed using a lignocellulosic-based composition with hexoses and pentoses. In this case, slightly higher butanol productivities were achieved in both, with relatively moderate dilution rates (set 13: 3.27 g L^{-1} h^{-1} *vs.* set 5: 2.9 g L^{-1} h^{-1} at ~0.6 h^{-1}) and high dilution rates (set 15: 3.53 g L^{-1} h^{-1}at 1.8 h^{-1} *vs.* set 10: 3.38 g L^{-1} h^{-1} at 1.5 h^{-1}). This shows that PBR processing of lignocellulosic waste substantially exceeds the minimum benchmark productivity (3.0 g L^{-1} h^{-1}). Despite the promising results in terms of butanol productivity, one of the key challenges faced by PBR systems is to achieve high sugar conversion rates, as it is crucial to focus on converting the sugars produced in the pretreatment to butanol during the fermentation stage to achieve an energy-competitive biobutanol production system. It was therefore hypothesized that implementing a second PBR in

Table 1. Comparison of advanced configurations for butanol production via fermentation using immobilization techniques

Set	Bioreactor	Carrier	Substrate	Microorganism	*D* (h^{-1})	Butanol productivity (g L^{-1} h^{-1})	Reference
1	CSTR	PVA	Glucose + butyrate	*C. beijerinckii* NCIMB 8052	0.04	0.40	31
2	CSTR	GAC	Rice straw hydrolysate	*C. beijerinckii* DSM 6422	0.30	2.07	32
3	PBR	CAG	Food waste	*C. saccharoperbutylacetonicum deltptabuk*	0.39	2.38[a]	37
4	PBR	Tygon rings	Cheese whey	*C. acetobutylicum* ATCC 824	0.54	2.66	36
5	PBR	Wood pulp	Wood chips hydrolysate	*C. acetobutylicum* DSM 792	0.64	2.90[a]	39
6	PBR	Fibrous matrix	Glucose + butyrate	*C. acetobutylicum* ATCC 55025	0.90	4.60	15
7	PBR	Tygon rings	Lactose	*C. acetobutylicum* DSM 792	0.97	4.43	33
8	PBR	Bone char	Cheese whey	*C. acetobutylicum* P262	1.00	4.28[a]	35
9	PBR	Brick	Glucose	*C. acetobutylicum* ATCC824	1.00	6.30	7
10	PBR	Wood pulp	Sugar mixture[b]	*C. beijerinckii* DSM 6423	1.50	3.38[a]	16
11	PBR	Wood pulp	Glucose	*C. acetobutylicum* DSM 792	1.90	8.55[a]	34
12	PBR	GAC	Rice straw hydrolysate	*C. beijerinckii* DSM 6422	0.30	2.48	This study
13	PBR	GAC	Rice straw hydrolysate	*C. beijerinckii* DSM 6422	0.60	3.27	This study
14	PBR	GAC	Rice straw hydrolysate	*C. beijerinckii* DSM 6422	1.20	4.44	This study
15	PBR	GAC	Rice straw hydrolysate	*C. beijerinckii* DSM 6422	1.80	3.53	This study

[a] Estimated from published data.
[b] Glucose: 7.9 g L^{-1}, mannose: 22 g L^{-1}, galactose: 4.1 g L^{-1}, arabinose: 2.3 g L^{-1}, xylose: 8.7 g L^{-1}.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

combination with an ISPR technique would tackle the problem of butanol toxicity and permit further use of the hydrolysate sugars in the effluent from the first PBR.

Two-stage PBR coupled with in-line GS

An advanced configuration comprising a two-stage PBR with in-line GS was evaluated with the aim of improving butanol production and substrate utilization (Fig. 1). For this we selected the best dilution rate (1.2 h^{-1}) in terms of butanol productivity in the previous experiment with the single PBR. Fresh media was fed to R1 and its effluent was carried to an intermediate tank. R2 operated in a closed-loop mode, using the sugars that had not been consumed in R1 to produce solvents. The intermediate tank was equipped with in-line GS, which removed a significant quantity of solvents, thus mitigating the inhibitory effects of butanol on R2. Using closed-loop recirculation in R2 enables the operation of the entire system with a single GS, which eliminates the requirement for a dedicated R2 solvent recovery system and reduces operating costs. The time-course data for the concentration of sugars (glucose and xylose), acids (acetic and butyric), and solvents (acetone and butanol) in the R1 and R2 effluents are shown in Fig. 3(a) and Fig. 3(b), respectively. Initially, a synthetic glucose–xylose mixture was used (days 0–11; Fig. 3a,b). As can be seen, the fermentation profiles of both bioreactors showed clearly different trends from the beginning. It should be emphasized that R1 was operated on a flow-through system with fresh media, while R2 was operated with a recirculating flow rate from R1 and R2 effluents, so direct performance comparisons can be challenging due to the different operating principles. R1 obtained a relatively stable performance throughout the experiment, which was similar to the single-stage PBR when both operated with RS hydrolysate at 1.2 h^{-1}, thus confirming reproducible results with different previous operating conditions. The very similar effluent concentrations (single-stage PBR: 26.1 ± 0.9 g L^{-1} glucose, 12.6 g L^{-1} ± 0.2 xylose, and 3.7 ± 0.2 g L^{-1} butanol; R1 of two-stage PBR: 28.6 ± 0.3 g L^{-1} glucose, 12.8 ± 0.8 g L^{-1} xylose, and 3.5 ± 0.1 g L^{-1} butanol) provided almost the same butanol productivity (single-stage PBR: 4.44 ± 0.27 g L^{-1} h^{-1}; R1 of two-stage PBR: 4.16 ± 0.20 g L^{-1} h^{-1}) and yield (single-stage PBR: 0.175 ± 0.009 g g^{-1} of consumed sugars; R1 of two-stage PBR: 0.165 ± 0.019 g g^{-1} of consumed sugars).

R2 performance unfortunately deteriorated in terms of sugar conversion until reaching null xylose metabolism (<1.6%) and very low glucose consumption (8.9%). R2 sugar consumption remained low, despite removing butanol from its influent by GS. Low sugar utilization was accompanied by very low butanol concentration (2.3 ± 0.1 g L^{-1} from days 9 to 18). R2 butanol productivity (0.98 ± 0.17 g L^{-1} h^{-1}) was four times lower than R1 (4.16 ± 0.20 g L^{-1} h^{-1}), which could be attributed to nutrient deficiencies and lower sugar concentration of R2 influent due to the recycling setup. Besides, butanol yield of R2 (0.182 ± 0.039 g g^{-1} of consumed sugars) was, in fact, slightly higher than that for R1 (0.165 ± 0.019 g g^{-1} of consumed sugars). This indicates that when there is a lower substrate input and subsequently lower substrate consumption, there is a predominance of butanol production over acetone production (as previously mentioned, ethanol is not produced by this strain). Ennis *et al.* developed a two-stage continuous fermentation process with different between-stage recovery techniques – that is, GS and adsorbent resin XAD-16.[19] While stage 1 reported ABE productivities ranging from 2.26 to 2.76 g L^{-1} h^{-1}, those of stage 2 had ABE productivities of 0.62 g L^{-1} h^{-1} after GS and 0.24 g L^{-1} h^{-1} after adsorbent resin XAD-16. The authors' overall conclusion was that adsorbents remove some essential nutrients from the broth in

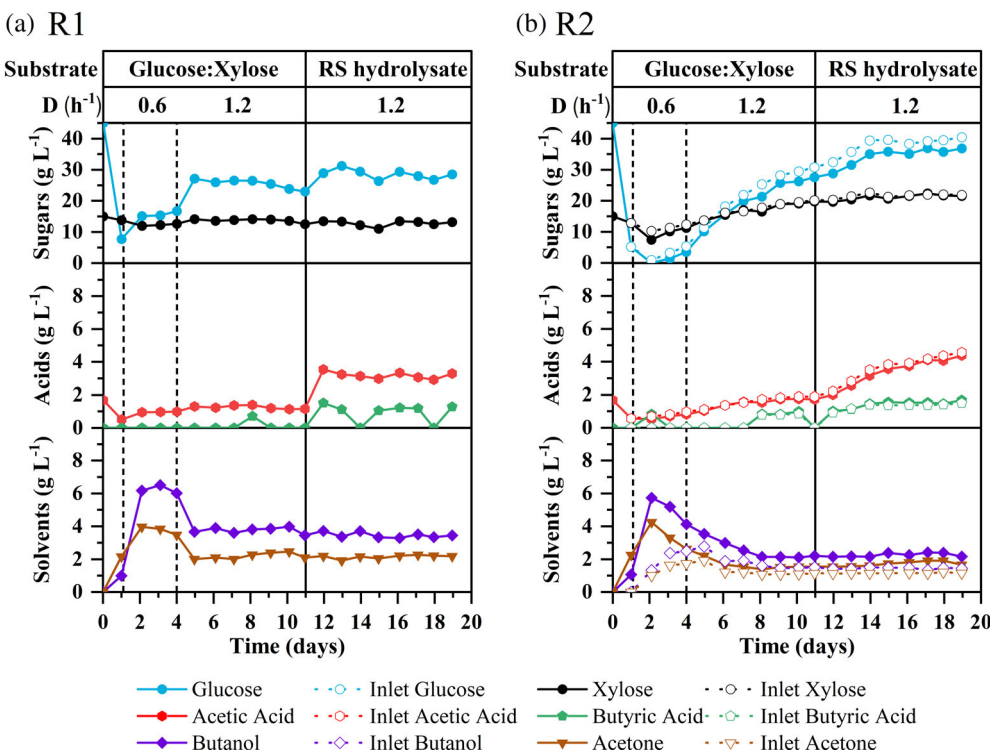

Figure 3. Time-course data of the concentration of influent and effluent sugars (glucose and xylose), acids (acetic and butyric), and solvents (acetone and butanol) in the two-stage PBR without nutrient supplementation at different dilution rates (*D*): (a) R1; (b) R2.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

addition to solvents. According to this study, an additional experiment was carried out to elucidate the performance of R2 with an extra supplementation of nutrient media.

Effect of nutrient supplementation on second stage PBR

To enhance R2 sugar consumption and the system's butanol productivity, a nutrient solution was continuously dosed at 10% (v/v) to the R2 inlet stream (influent-R2, Fig. 1). The time-course data for the glucose and xylose, acetic and butyric acids, and the acetone and butanol solvents in the effluent of R1 are depicted in Fig. 4(a) along with those of R2 (Fig. 4b). The glucose–xylose mixture (days 0–8; Fig. 4a,b) was fed for 8 days when the system was operated in batch mode for the first 24 h, followed by continuous mode at two dilution rates: 0.6 and 1.2 h^{-1} for each PBR. Thereafter, the alkali-treated RS hydrolysate was fed for 4 days working at a dilution rate of 1.2 h^{-1}. The R1 performance with RS hydrolysate was comparable to those previously observed (Fig. 2a, single-stage PBR; Fig. 3a, two-stage PBR). The glucose–xylose concentration in the R1 effluent was 24.9 ± 0.1 g L^{-1} and 12.5 ± 0.3 g L^{-1}, respectively. Butanol concentration in R1 was 3.4 ± 0.3 g L^{-1}, corresponding to a butanol productivity of 4.08 ± 0.31 g L^{-1} h^{-1}, while butanol yield was 0.135 ± 0.011 g g^{-1} of consumed sugars.

Additional nutrient supplementation in the R2 effluent caused a notable change in its performance (Fig. 3b *vs*. Fig. 4b) and avoided sugar accumulation. For example, working at a dilution rate of 0.6 h^{-1}, the input glucose concentration of R2 was 6.2 ± 1.8 g L^{-1}, achieving a net glucose consumption of 4.7 g L^{-1}. Butanol concentration was 3.5 ± 0.1 g L^{-1}, while the net concentration was 1.3 ± 0.1 g L^{-1}. When dilution rate was doubled to 1.2 h^{-1}, there was an increase in the effluent R2 glucose concentration from 1.5 g L^{-1} on day 5 to 9.8 g L^{-1} on day 8, with an approximately stable butanol concentration of 2.6 ± 0.1 g L^{-1} (net concentration of 1.4 ± 0.4 g L^{-1}). When RS hydrolysate was used as a substrate, pseudo-stable sugar concentrations were seen in R2, with 14.0 ± 0.1 g L^{-1} for glucose and 11.4 ± 0.2 g L^{-1} for xylose in the effluent. Net R2 butanol concentration was 1.2 ± 0.1 g L^{-1} for R2, corresponding to a butanol productivity of 1.47 ± 0.08 g L^{-1} h^{-1}, which was 50% higher than the non-supplemented test under the same hydraulic condition (0.98 ± 0.17 g L^{-1} h^{-1} at a dilution rate of 1.2 h^{-1}). Interestingly, butanol yield of R2 was 0.380 ± 0.09 g g^{-1} of consumed sugars, which was significantly higher than that of R1 and close to the maximum stoichiometric yield of butanol from glucose (0.411 g g^{-1} of consumed sugars).[9,40] To conclude, the overall butanol productivity of the two-stage PBR with in-line GS when operating each stage at a dilution rate of 1.2 h^{-1} (overall dilution rate of 0.6 h^{-1}) was 2.79 ± 0.21 g L^{-1} h^{-1}.

Figure 5 outlines the mass balance of both glucose and xylose for the proposed two-stage PBR configuration with nutrient supplementation. Two control volumes were used: Control volume 1 was R1 while Control volume 2 included R2 + GS. The mass flows were determined as the average glucose and xylose concentrations when operating each reactor at a dilution rate of 1.2 h^{-1} (days 8–12; Fig. 4a,b) multiplied by the flow rate of each stream. The R1 glucose and xylose consumption (streams 1–2) were 47.5 ± 0.3% and 17.6 ± 1.7%, respectively, while in R2 alone (streams 3–4) it was 13.7 ± 1.2% for glucose and 10.9 ± 0.7% for xylose. In comparison to the non-supplemented case, nutrient supplementation doubled the glucose consumption and increased xylose utilization sevenfold. The higher sugar consumption of R2 in the supplemented case kept sugar concentrations low in the intermediate tank (stream 6) and reduced sugar losses through the outflow. The mass balance of Control volume 2 (streams 2–6) showed glucose and xylose consumptions of 56.9% and 32.0%, respectively. It is important to highlight that

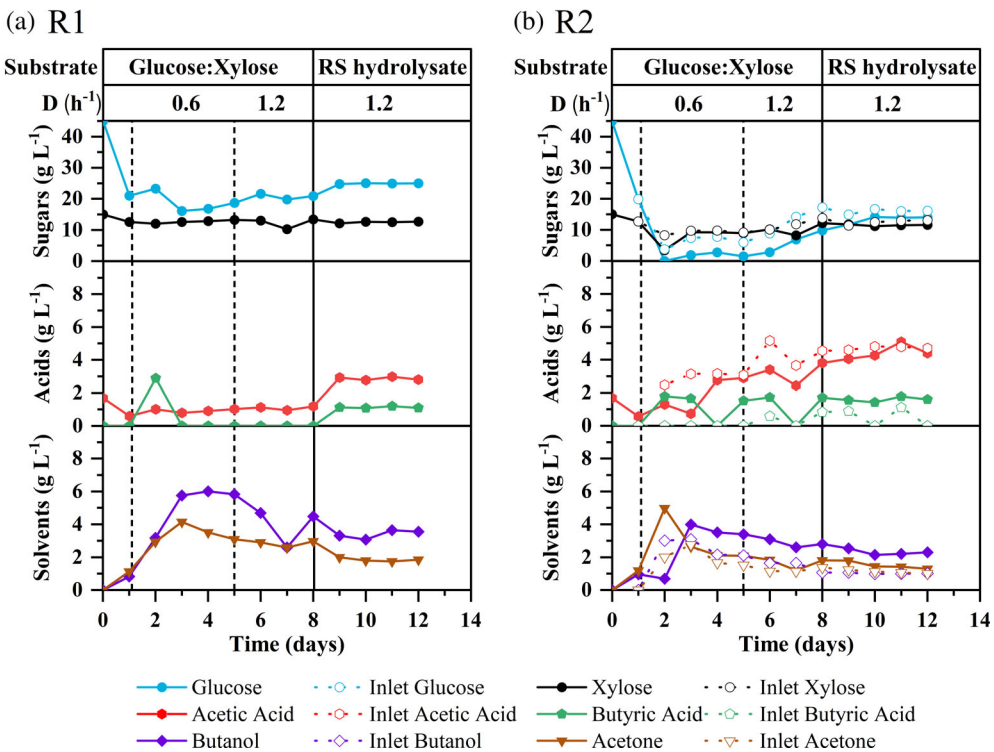

Figure 4. Time-course data of the concentrations of influent and effluent sugars (glucose and xylose), acids (acetic and butyric), and solvents (acetone and butanol) in the two-stage PBR for the supplemented case at different dilution rates (*D*): (a) R1; (b) R2.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

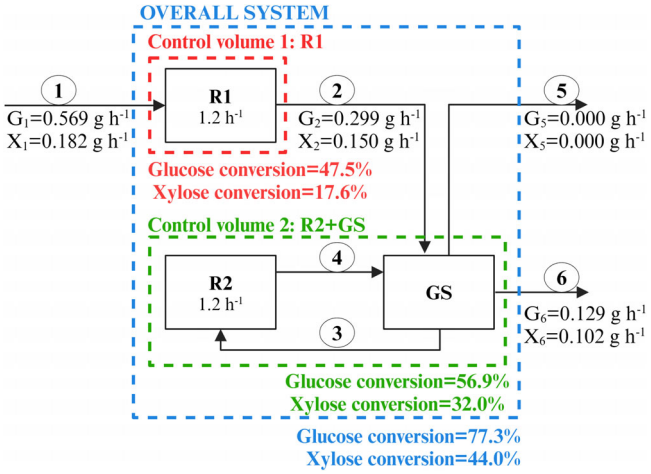

Figure 5. Schematic diagram of the system's sugar mass balances comprising a first PBR (Control volume 1, R1) followed by a closed-loop recirculating PBR integrated with an in-line GS (Control volume 2, R2 + GS). Streams are designated as follows: 1, system influent; 2, effluent from R1 to GS; 3, influent to R2; 4, recycled effluent from R2 to GS; 5, condensate of GS; and 6, system effluent. The letters G and X represent the mass flow of glucose and xylose, respectively, for each stream. Sugar consumption of both control volumes is calculated as a percentage.

the implementation of a recirculation strategy combined with a GS system when nutrient supplementation was applied resulted in a higher percentage of substrate consumption compared to the first control volume. The overall system consumption was calculated as the difference between the sugars derived from the RS hydrolysate entering R1 at a dilution rate of 1.2 h^{-1} (stream 1) and the sugar leakage due to system outflow (stream 6). The overall sugar consumption of the two-stage PBR with *in situ* GS (Control volume 1,2) was therefore 77.3 ± 0.5% for glucose and 44.0 ± 1.3% for xylose. These results show that supplementing R2 with nutrients was effective in activating bacterial metabolism and using the unconsumed sugars from the effluent of the first-stage sugars for additional butanol production. Ennis *et al.* demonstrated that supplementing the second-stage bioreactor with yeast extract promoted the acidogenic phase in preference to the solventogenic phase.[19] In this case, the promotion of acidogenesis proved advantageous for R2, as this phase is associated with cell growth and facilitates cell division in the GAC, as shown by the greater cell growth in the supplemented R2, as demonstrated by visual inspection of R2 during this period. The findings thus indicate that enhancing R2 performance by dosing nutrients to the media is relatively straightforward. Although the butanol productivity of R1 itself reached a significantly higher value (4.08 ± 0.31 g L^{-1} h^{-1}) with total utilized sugars of 40%, the overall butanol productivity (2.79 ± 0.21 g L^{-1} h^{-1}) obtained at an overall dilution rate of 0.6 h^{-1} with 69% sugar consumption paves the way for this technology to become a cost-effective means of producing butanol from lignocellulosic feedstocks. By considering the losses involved in the alkaline pretreatment and enzymatic saccharification steps of RS, as well as the yield of the fermentation step comprising a first PBR followed by a second PBR operating in close loop with an in-line GS, the butanol-to-biomass ratio is calculated to be 36.7 g butanol per kilogram of raw RS (see Fig. S1 in the Supporting Information).

Continuous two-stage fermentation of solventogenic clostridia was first proposed by Bahl *et al.* to stabilize biphasic fermentation in chemostats by operating an acidogenic first stage and a solventogenic second.[41] ISPR techniques for ABE fermentation have been reported in continuous two-stage chemostats; for example, Van Hecke *et al.* reported a two-stage CSTR coupled with a pervaporation unit using *C. acetobutylicum* ATCC 824 that raised solvent productivity by 300% to 0.30 g L^{-1} h^{-1} by alleviating solvent toxicity.[42] Studies can also be found on multiple-stage CSTRs with ISPR and cell immobilization. For instance, Bankar *et al.* developed a two-stage chemostat with immobilization on sugarcane bagasse combined with liquid–liquid extraction, reaching a butanol productivity of 1.6 g L^{-1} h^{-1}.[43] However, few studies have been published on multi-stage continuous ABE fermentation using the cell immobilization technique in PBR systems. Park *et al.* developed a two-stage PBR using polyester sponges to immobilize *C. acetobutylicum* ATCC 824, which provided a butanol productivity of 2.4 g L^{-1} h^{-1} at a dilution rate of 0.272 h^{-1}.[44] Bankar *et al.* used wood pulp as carrier in a two-stage PBR with solvent recovery by liquid–liquid extraction operated at an overall dilution rate of 0.2 h^{-1} by feeding a synthetic sugar mixture, in which the maximum butanol productivity was 2.71 g L^{-1} h^{-1}, with an overall sugar consumption of 93.7%.[20] Both of these authors operated continuous two-stage PBR with dilution rate values similar to the maximum specific growth rate of *Clostridium* spp., so that these systems were operated close to batch mode conditions. In comparison, our system was operated at a significantly higher overall dilution rate (0.6 h^{-1}), showing that the present proposal can effectively process high flow rates.

This proposed configuration entails a two-stage PBR fermentation process coupled with cell immobilization in GAC and *in situ* product recovery that operates at relatively higher dilution rates. The system is in fact a promising configuration for full-scale ABE fermentation and is the first two-stage PBR configuration that can efficiently deal with high hydrolysate flow rates from real lignocellulosic feedstocks.

CONCLUSIONS

This study is one of the few carried out on continuous ABE fermentation using real hydrolysates and represents a pioneering application in two-stage PBR with ISPR for converting lignocellulosic wastes. A single-stage PBR with GAC has been shown to be a promising pathway for intense ABE fermentation. The butanol productivity achieved (4.4 g L^{-1} h^{-1}) is the highest reported value using lignocellulosic-based substrates with PBRs. Adding a second PBR with in-line GS increased sugar consumption from 40% to 69% and achieved a notable butanol productivity of 2.8 g L^{-1} h^{-1}, thus paving the way for a feasible RS biorefinery to butanol.

ACKNOWLEDGEMENTS

Grant PID2021-122454OB-I00 was funded by MICIU/AEI/10.13039/501100011033 and by ERDF/EU. This research also received funding from Conselleria d'Innovacio, Universitats, Ciencia i Societat Digital, Generalitat Valenciana, Spain (AICO/2021/121). In addition, Carlos Silvestre acknowledges the Spanish Government for their PhD contract (FPU20/04982) from the Ministerio de Universidades (Spain). The authors would like to thank Marcos Poquet (granted by INVESTIGO contract, INVEST/2022/178, Generalitat Valenciana, Spain) for his technical support in producing the hydrolysate and in high-performance liquid chromatograph maintenance.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

DATA AVAILABILITY STATEMENT

Research data are not shared.

AUTHOR CONTRIBUTIONS

Carlos Silvestre: conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft, validation. Lukas Niedermeier: investigation, methodology. Miguel Capilla: investigation, methodology. Carmen Gabaldón: conceptualization, methodology, writing – review and editing, supervision, funding acquisition. Javier Álvarez-Hornos: conceptualization, methodology, investigation, supervision, writing – review and editing, funding acquisition, project administration.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 European Commission, Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. The European Green Deal 2019. Report No.: COM(2019) 640 final.
2 Spekreijse J, Lammens T, Parisi C, Ronzon T and Vis M, Insights into the European market of bio-based chemicals. Analysis based on ten key product categories (2019).
3 Többen JR, Distelkamp M, Stöver B, Reuschel S, Ahmann L and Lutz C, Global land use impacts of bioeconomy: An econometric input–output approach. *Sustainability* **14**:1976 (2022).
4 Schipfer F, Kranzl L, Leclère D, Sylvain L, Forsell N and Valin H, Advanced biomaterials scenarios for the EU28 up to 2050 and their respective biomass demand. *Biomass Bioenergy* **96**:19–27 (2017).
5 Green EM, Fermentative production of butanol—the industrial perspective. *Curr Opin Biotechnol* **22**:337–343 (2011).
6 Maddox IS, The acetone-butanol-ethanol fermentation: recent progress in technology. *Biotechnol Genet Eng Rev* **7**:189–220 (1989).
7 Wang YR, Chiang YS, Chuang PJ, Chao YP and Li SY, Direct in situ butanol recovery inside the packed bed during continuous acetone-butanol-ethanol (ABE) fermentation. *Appl Microbiol Biotechnol* **100**: 7449–7456 (2016).
8 Ezeji T, Qureshi N and Blaschek HP, Bioproduction of butanol from biomass: from genes to bioreactors. *Curr Opin Biotechnol* **18**:220–227 (2007).
9 García V, Päkkilä J, Ojamo H, Muurinen E and Keiski RL, Challenges in biobutanol production: how to improve the efficiency? *Renew Sustainable Energy Rev* **15**:964–980 (2011).
10 Van Dien S, From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. *Curr Opin Biotechnol* **24**:1061–1068 (2013).
11 Qureshi N, Schripsema J, Lienhardt J and Blaschek HP, Continuous solvent production by Clostridium beijerinckii BA101 immobilized by adsorption onto brick. *World J Microbiol Biotechnol* **16**:6–382 (2000).
12 Qureshi N, Paterson AHJ and Maddox IS, Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of Clostridium acetobutylicum immobilized by adsorption onto bonechar. *Appl Microbiol Biotechnol* **29**:323–328 (1988).
13 Mariano AP, Ezeji TC and Qureshi N, Chapter 4. Butanol production by fermentation: efficient bioreactors, in *Green Chemistry Series [Internet]*, ed. by Snyder SW. Royal Society of Chemistry, Cambridge, pp. 48–70 (2015) Available from: http://ebook.rsc.org/?DOI=10.1039/9781782622444-00048.
14 Qureshi N, Lai LL and Blaschek HP, Scale-up of a high productivity continuous biofilm reactor to produce butanol by adsorbed cells of clostridium Beijerinckii. *Food Bioprod Process* **82**:164–173 (2004).
15 Huang WC, Ramey DE and Yang ST, Continuous production of butanol by Clostridium acetobutylicum immobilized in a fibrous bed bioreactor. *Appl Biochem Biotechnol* **113**:12 (2004).
16 Survase SA, Van Heiningen A and Granström T, Wood pulp as an immobilization matrix for the continuous production of isopropanol and butanol. *J Ind Microbiol Biotechnol* **40**:209–215 (2013).
17 Badr HR, Toledo R and Hamdy MK, Continuous acetone–ethanol–butanol fermentation by immobilized cells of Clostridium acetobutylicum. *Biomass Bioenergy* **14**:119–132 (2001).
18 Rochón E, Ferrari MD and Lareo C, Integrated ABE fermentation-gas stripping process for enhanced butanol production from sugarcane-sweet sorghum juices. *Biomass Bioenergy* **98**:153–160 (2017).
19 Ennis BM, Qureshi N and Maddox IS, In-line toxic product removal during solvent production by continuous fermentation using immobilized Clostridium acetobutylicum. *Enzyme Microb Technol* **9**:672–675 (1987).
20 Bankar SB, Survase SA, Ojamo H and Granström T, The two stage immobilized column reactor with an integrated solvent recovery module for enhanced ABE production. *Bioresour Technol* **140**:269–276 (2013).
21 Liu J, Fan S and Xiao Z, Enhanced coproduction and trade-off of the hydrogen and butanol in the coupled system of pervaporation and repeated-cycle fixed-bed fermentation. *Ind Crops Prod* **161**: 113172 (2021).
22 Li S, Huang L, Ke C, Pang Z and Liu L, Pathway dissection, regulation, engineering and application: lessons learned from biobutanol production by solventogenic clostridia. *Biotechnol Biofuels* **13**:39 (2020).
23 Valles A, Capilla M, Álvarez-Hornos FJ, García-Puchol M, San-Valero P and Gabaldón C, Optimization of alkali pretreatment to enhance rice straw conversion to butanol. *Biomass Bioenergy* **150**:106131 (2021).
24 Capilla M, Valles A, San-Valero P, Álvarez-Hornos FJ and Gabaldón C, Solvent production from rice straw by a co-culture of Clostridium acetobutylicum and Saccharomyces cerevisiae: effect of pH control. *Biomass Convers Biorefinery* **14**:5561–5573 (2024).
25 Plaza PE, Gallego-Morales LJ, Peñuela-Vásquez M, Lucas S, García-Cubero MT and Coca M, Biobutanol production from brewer's spent grain hydrolysates by Clostridium beijerinckii. *Bioresour Technol* **244**: 166–174 (2017).
26 Valles A, Álvarez-Hornos J, Capilla M, San-Valero P and Gabaldón C, Fed-batch simultaneous saccharification and fermentation including in-situ recovery for enhanced butanol production from rice straw. *Bioresour Technol* **342**:126020 (2021).
27 Bellido C, Loureiro Pinto M, Coca M, González-Benito G and García-Cubero MT, Acetone–butanol–ethanol (ABE) production by Clostridium beijerinckii from wheat straw hydrolysates: efficient use of penta and hexa carbohydrates. *Bioresour Technol* **167**:198–205 (2014).
28 Birgen C, Dürre P, Preisig HA and Wentzel A, Butanol production from lignocellulosic biomass: revisiting fermentation performance indicators with exploratory data analysis. *Biotechnol Biofuels* **12**:15 (2019).
29 Ezeji T, Qureshi N and Blaschek HP, Butanol production from agricultural residues: impact of degradation products on Clostridium beijerinckii growth and butanol fermentation. *Biotechnol Bioeng* **97**: 1460–1469 (2007).
30 Jin Q, Qureshi N, Wang H and Huang H, Acetone-butanol-ethanol (ABE) fermentation of soluble and hydrolyzed sugars in apple pomace by Clostridium beijerinckii P260. 2019;9.
31 Lee SM, Cho MO, Park CH, Chung YC, Kim JH, Sang BI *et al*., Continuous butanol production using suspended and immobilized Clostridium beijerinckii NCIMB 8052 with supplementary butyrate. *Energy Fuel* **22**:3459–3464 (2008).
32 Silvestre C, Capilla M, Valles A, San-Valero P, Gabaldón C and Javier Álvarez-Hornos F, Developing a high-rate ABE fermentation system for rice straw: CSTR with immobilization on granular-activated carbon with ex situ pervaporation. *Fuel* **360**:130538 (2024).
33 Napoli F, Olivieri G, Russo ME, Marzocchella A and Salatino P, Butanol production by Clostridium acetobutylicum in a continuous packed bed reactor. *J Ind Microbiol Biotechnol* **37**:603–608 (2010).
34 Survase SA, van Heiningen A and Granström T, Continuous bio-catalytic conversion of sugar mixture to acetone–butanol–ethanol by immobilized Clostridium acetobutylicum DSM 792. *Appl Microbiol Biotechnol* **93**:2309–2316 (2012).
35 Qureshi N and Maddox IS, Reactor design for the ABE fermentation using cells of Clostridium acetobutylicum immobilized by adsorption onto bonechar. *Bioprocess Eng* **3**:69–72 (1988).
36 Raganati F, Olivieri G, Procentese A, Russo ME, Salatino P and Marzocchella A, Butanol production by bioconversion of cheese

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

whey in a continuous packed bed reactor. *Bioresour Technol* **138**: 259–265 (2013).
37 Jin Q, An Z, Damle A, Poe N, Wu J, Wang H *et al.*, High acetone-butanol-ethanol production from food waste by recombinant clostridium saccharoperbutylacetonicum in batch and continuous immobilized-cell fermentation. *ACS Sustainable Chem Eng* **8**:9822–9832 (2020).
38 Gheshlaghi R, Scharer JM, Moo-Young M and Chou CP, Metabolic pathways of clostridia for producing butanol. *Biotechnol Adv* **27**:764–781 (2009).
39 Survase SA, Sklavounos E, Jurgens G, Van Heiningen A and Granström T, Continuous acetone–butanol–ethanol fermentation using SO2–ethanol–water spent liquor from spruce. *Bioresour Technol* **102**:10996–11002 (2011).
40 Lee J, Yun H, Feist AM, Palsson BØ and Lee SY, Genome-scale reconstruction and in silico analysis of the Clostridium acetobutylicum ATCC 824 metabolic network. *Appl Microbiol Biotechnol* **80**:849–862 (2008).
41 Bahl H, Andersch W and Gottschalk G, Continuous production of acetone and butanol by clostridium acetobutylicum in a two-stage phosphate limited chemostat. *Eur J Appl Microbiol Bioteachnol* **5**: 15–205 (1982).
42 Van Hecke W, Vandezande P, Claes S, Vangeel S, Beckers H, Diels L *et al.*, Integrated bioprocess for long-term continuous cultivation of Clostridium acetobutylicum coupled to pervaporation with PDMS composite membranes. *Bioresour Technol* **111**:368–377 (2012).
43 Bankar SB, Survase SA, Singhal RS and Granström T, Continuous two stage acetone–butanol–ethanol fermentation with integrated solvent removal using clostridium acetobutylicum B 5313. *Bioresour Technol* **106**:110–116 (2012).
44 Park C, Okos MR and Wankat PC, Acetone–butanol–ethanol (ABE) fermentation in an immobilized cell trickle bed reactor. *Biotechnol Bioeng* **34**:18–29 (1989).

