Fed-batch simultaneous saccharification and fermentation including *in-situ* recovery for enhanced butanol production from rice straw

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Abstract

This paper describes a study of fed-batch SSFR (simultaneous saccharification, fermentation and recovery) for butanol production from alkaline-pretreated rice straw (RS) in a 2-L stirred tank reactor. The initial solid (9.2% w/v) and enzyme (19.9 FPU g-dw^{-1}) loadings were previously optimized by 50-mL batch SSF assays. Maximum butanol concentration of 24.80 g L^{-1} was obtained after three biomass feedings that doubled the RS load (18.4% w/v). Butanol productivity (0.344 g L^{-1} h^{-1}) also increased two-fold in comparison with batch SSF without recovery (0.170 g L^{-1} h^{-1}). Although fed-batch SSFR was able to operate with a single initial enzyme dosage, an extra dosage of nutrients was required with the biomass additions to achieve this high productivity. The study showed that SSFR can efficiently improve butanol production from a lignocellulosic biomass accompanied by the efficient use of the enzyme.

Keywords:

Butanol; fed-batch; gas stripping; rice straw; simultaneous saccharification and fermentation

1 Introduction

Global warming, one of the greatest challenges facing world, is accelerating the transformation of the energy system. The current EU policy includes the production of biofuels from biomass wastes as one of the systems to achieve a successful climate-neutral transition (European Union: European Commission, 2020). Compared to other liquid biofuels such as ethanol, biobutanol has a higher energy density and is less hygroscopic and corrosive (Schubert, 2020). Butanol production by ABE fermentation from agricultural waste (rice straw, wheat straw or sugarcane bagasse among others) has been explored in the last decade (Abo et al., 2019; Vees et al., 2020). Although these lignocellulosic residues are an abundant and low-cost feedstock, they require pretreatment and saccharification prior to fermentation. In the case of rice straw (RS), which has a much higher ash and silica content than other agricultural lignocellulosic by-products (Satlewal et al., 2017), the pretreatment method should be selected considering that these components hinder accessibility to inner cellulose microfibers in enzymatic hydrolysis. Imman et al. (2015) reported the destruction of the RS silica layers after alkaline-catalyzed liquid hot water pretreatment with a 0.25% NaOH solution. Mukherjee et al. (2018) found that NaOH pretreatment reduced the percentage of silica by favoring delignification due to silica links with lignin. Together with the efficient removal of lignin and silica, the low degradation of sugars and its non-corrosive nature make alkaline pretreatment one of the most suitable methods to use on RS (Vivek et al., 2019). In a previous study, RS delignification by NaOH pretreatment has provided adequate saccharification, recovering ~60% of reducing sugars from the original carbohydrates (Valles et al., 2021).

Integrated bioprocessing, in which multiple processing steps are combined in a single operation, is an attractive approach for industrial-scale butanol production, for which

simultaneous saccharification and fermentation (SSF) and fermentation with *in situ* product recovery (ISPR) are two of the most promising strategies (Ibrahim et al., 2018). In SSF, hydrolysis and fermentation take place together in the same vessel. This process could avoid the glucose inhibition of hydrolytic enzymes because sugars are simultaneously released and consumed by the bacteria. Also, SSF of microwave-pretreated RS has been shown to be more efficient than separate hydrolysis and fermentation (SHF) in terms of butanol production and productivity (Valles et al., 2020). ISPR reduces the high cost of downstream recovery processing, which is one of the major challenges in the commercialization of biobutanol (Abo et al., 2019). At the same time, it improves fermentation performance by alleviating butanol inhibition, which occurs at concentrations > 10 g L^{-1} (Ahlawat et al., 2019; Rochón et al., 2017). ISPR techniques include pervaporation, liquid extraction, gas stripping and perstraction. Of these, gas stripping is one of the simplest and most economic processes since it does not require either a membrane or chemicals and does not harm the culture (Li et al., 2020). By combining SSF with ISPR, the advanced SSFR (simultaneous saccharification, fermentation and recovery) configuration could markedly reduce the capital and operational costs of producing butanol from lignocellulosic biomass and food waste (Qureshi et al., 2020). Qureshi et al. (2006) carried out SSFR with *C. acetobutylicum* P260 from corn fiber arabinoxylan using gas stripping and reported that the full utilization of sugar and acids in SSFR, compared to SSF, increased ABE production (from 9.60 to 24.67 g L^{-1}) and productivity (from 0.20 to 0.47 g L^{-1} h^{-1}).

Combining SSFR with a fed-batch strategy, which allows large solid loadings without substrate or product inhibition is another method of drastically improving the cost-effectiveness of biobutanol production. Up to now, fed-batch has been considered to enhance ABE fermentation in SHF configurations (López-Linares et al., 2021; Rochón et al., 2017; Wen et al., 2018), but not in SSF, although fed-batch SSF was recently suggested as a

promising alternative to be explored (Ibrahim et al., 2018). Feeding sterile substrate into the reactor is still a technical challenge to be overcome, although several studies on ethanol production by *Saccharomyces cerevisiae* have demonstrated the feasibility of feeding a sequential biomass to the reactor (Shengdong et al., 2006; Wang et al., 2013). For example, Shengdong et al. (2006) carried out fed-batch SSF in which an extra 10% (w/v) of pretreated RS was aseptically added in addition to a 10% (w/v) initial substrate concentration. This increased the reaction time by 144 h and achieved a much higher ethanol concentration (57.3 g L^{-1}) than that obtained in the single batch process (29.1 g L^{-1}). Fed-batch SSFR could thus solve some of the major challenges in ABE fermentation.

In the present work, a novel ABE fermentation approach was evaluated that consisted of fed-batch SSF with ISPR by gas stripping for butanol production from alkaline-pretreated RS. First, the effect of solid and enzyme loading on production was assessed in a batch SSF configuration using a central composite design (CCD). Based on the optimal values of solid and enzyme loading, fed-batch SSFR was then conducted with or without an additional enzyme dosage or medium compounds (buffer, yeast extract and minerals) in the subsequent feed cycles to further improve the economic viability of the process.

2 Materials and methods

2.1 Materials

RS from the Albufera Natural Park (Spain) was milled. The size fraction ranged from 100 to 500 μm was dried at 45 °C and stored. Its chemical composition (dry weight) was: glucan 35.6 ± 0.6%, xylan 17.6 ± 0.5%, arabinan 2.1 ± 0.2%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 10.4 ± 0.7%, ash 12.3 ± 0.7% and extractives 13.0 ± 1.3%. The Cellic® CTec2 commercial enzyme blend (Novozyme, Denmark) was used for enzymatic hydrolysis. A cellulase activity of 193 filter paper units (FPU) mL^{-1} was determined following the

National Renewable Energy Laboratory (NREL) method (Adney and Baker, 1996) and additional information of the commercial enzyme blend can be found elsewhere (Aramrueang et al., 2017; dos Reis et al., 2013; Yang et al., 2017). *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was purchased from DSMZ (Germany) and stored at -80 °C in a Reinforced Clostridial Medium (RCM) with 20% (v/v) glycerol. The pre-culture was statically grown for 24 h in 19 g L^{-1} RCM with 10 g L^{-1} glucose.

2.2 RS pretreatment

Alkaline pretreatment was conducted according to the protocol and the optimal conditions derived from a previous study (Valles et al., 2021). In brief, a solid loading of 5% (w/v) of RS was mixed with 0.75% (w/v) of NaOH solution and heated at 134 °C for 20 min. The solid fraction was then separated by centrifugation at 4000 rpm for 6 min (Mega Star 3.0, VWR, Germany) and washed several times with deionized water with a final pH adjustment to 6.5. The pretreated RS, which was previously dried at 45 °C for 48 h, was stored at -20 °C. After pretreatment a solid recovery of 48.19 ± 1.97% was obtained. The chemical composition of the alkaline-pretreated RS (dry weight) was: glucan 51.9 ± 0.6%, xylan 21.6 ± 0.3%, arabinan 3.8 ± 0.1%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 9.2 ± 0.5% and ash 7.4 ± 0.3%, indicating enriched carbohydrates with the removal of 57.5% acid insoluble lignin and 71.1% ashes.

2.3 Batch SSF

The SSF process was first optimized in 50-mL serum bottles in which solid (3.8 – 12.2% w/v) and enzyme loading (3.7 – 26.3 FPU g-dw^{-1}) were tested following the experimental design shown in Section 2.6. The medium (40 mL) contained: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} $C_2H_7NO_2$, 4 g L^{-1} of yeast extract, 0.09 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $MnSO_4 \cdot H_2O$ and 0.02 g L^{-1} $FeSO_4 \cdot 7H_2O$. After oxygen displacement, the sealed bottles were

autoclaved (121 °C for 10 min). Minerals (filter-sterilized by 0.22 μm) and enzyme were added before inoculation with 5% (v/v) of pre-culture. Incubation was conducted at 37 ºC and 150 rpm for 72 h in an orbital shaker (SI500 model, Stuart, UK). Saccharification control without bacterial cells was conducted in triplicate with a solid loading of 8% (w/v) and an enzyme loading of 15 FPU g-dw^{-1}.

Once solid and enzyme loading were optimized, the process was scaled up in a 2-L stirred tank reactor (STR) using 500 mL of the above-mentioned medium with an RS loading of 9.2% (w/v). Start-up was similar to that performed with the serum bottles, but nitrogen was sparged after sterilization. After adding 4.73 mL of the enzyme blend (19.9 FPU g-dw^{-1}), the reactor was inoculated (5% v/v) and fermentation lasted 72 h at 37 ºC and 120 rpm.

2.4 Fed-batch SSF coupled with *in-situ* gas stripping

The fed-batch SSF with ISPR by gas stripping took place in the 2-L STR. Figure 1 shows a schematic diagram of the integrated reactor set-up. Gas stripping was performed by intermittently bubbling the fermentation gas (CO_2 and H_2) through the fermentation broth at 4 L min^{-1} by a vacuum gas pump (VP 86, VWR, Germany). The stripped solvents were recovered in a condenser at 4 ºC with a cooling system (AD15R-30, VWR, USA). O_2-free distilled water was pumped by a peristaltic pump to keep constant the reactor volume at 500 mL. The condensate was periodically transferred for volume measurement and solvent analysis. The same start-up and operational conditions (37 ºC and 120 rpm) described in Section 2.3 were used, ensuring an anaerobic environment by flushing the reactor headspace with nitrogen during feeding. Gas stripping started after 20 h and ended at 50 h. Dry alkaline-pretreated RS was added in three portions: 50% of the total quantity at the beginning of fermentation, 25% after 20 h and 25% after 30 h, thus doubling the solid loading from 9.2 to 18.4% (w/v). Three experiments were planned with different enzyme dosage and medium

158 components together with the solid additions. In run 1, the enzyme blend Cellic® CTec2 was
159 added to keep the enzyme loading constant at 19.9 FPU g-dw^{-1} throughout the entire process,
160 while in runs 2 and 3 no extra enzyme was added to assess the Cellic® CTec2 cellulase
161 activity over time. In runs 1 and 2, the fermentation was reinforced with medium components
162 to keep the same ratio of buffer, yeast extract and minerals with feed solids, while in run 3 no
163 additional medium components were used in order to assess the potential nutrient recycling
164 from dead *C. beijerinckii* cells.

165 2.5 Analytical methods

166 The raw and pretreated RS were analyzed to determine the chemical composition
167 according to NREL protocols (Sluiter et al., 2008). For all ABE fermentations, samples of 1 –
168 2 mL were periodically taken from the broth and, afterwards, were centrifuged (10000 rpm
169 for 5 min) and filtered by 0.22-μm. A Minitrode electrode (Hamilton, USA) was used to pH
170 measurements. The concentration of sugars (arabinose, glucose and xylose), inhibitory
171 compounds (furfural, 5-HMF and levulinic acid) and fermentation products (acetone, butanol,
172 ethanol, acetic acid and butyric acid) was determined by an Agilent HPLC (1100 Series,
173 Agilent Technologies, USA) equipped with a refractive index detector, a diode array detector
174 and an Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories Inc., USA).
175 The mobile phase (5 mM H_2SO_4) was set at 0.6 mL min^{-1}. The total phenolic compounds,
176 expressed as gallic acid equivalents, was measured by the Folin-Denis method (Folin and
177 Denis, 1912). A scanning electron microscope (SEM) S-4800 (Hitachi, Japan) was used to
178 observe the *C. beijerinckii* DSM 6422 cells absorbed on the surface of the pretreated RS.
179 Samples were coated with a gold and palladium mixture prior to imaging under SEM at an
180 accelerating voltage of 20 kV.

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182

183 2.6 Statistical design of experiments

184 Batch SSF was optimized by a CCD-based response surface method with concentration
185 of butanol produced at 24 h (g L^{-1}) as the response variable in a total of 13 experiments with 5
186 central point replications. Solid loading (from 3.8 to 12.2% w/v) and enzyme loading (from
187 3.7 to 26.3 FPU g-dw^{-1}) were evaluated as the independent variables and their coded and real
188 values are showed in Table 1. The statistical analysis was done on MINITAB® 19 software
189 (Minitab Inc., USA). The optimal levels of the solid and enzyme loading predicted by the
190 mathematical model were validated in triplicate. To obtain the product yield, a
191 saccharification control was carried out in triplicate with a solid loading of 9.2% (w/v) and an
192 enzyme loading of 19.9 FPU g-dw^{-1}.

193 **3 Results and discussion**

194 3.1 Batch SSF: optimization

195 The optimum values of the solid and enzyme loading to maximize butanol production
196 were assessed in batch SSF. For this, a five-level CCD was carried out using 50-mL serum
197 bottles. The CCD experimental matrix with the real values of both independent variables is
198 summarized in Table 2, along with the butanol production and sugar concentration in the
199 culture broth at 24 h. The butanol titer at the end of fermentation (72 h) is also given along
200 with the percentage of consumed sugars. These percentages were determined from the
201 potential final sugar concentration estimated for each solid loading based on the sugar
202 released by saccharification control (without inoculation).

203 At 24 h of fermentation, the butanol concentration ranged from 6.13 to 10.07 g L^{-1},
204 obtaining the minimum value from the lowest solid loading (3.8% w/v, run 5) and the

maximum value from the solid loading of 11.0% (w/v, run 4). The central point replicates (run 9 – 13) show the low experimental variability of the parameters studied (butanol production: 9.95 ± 0.35 g L^{-1}; residual glucose: 1.33 ± 1.03 g L^{-1}, xylose: 5.02 ± 0.18 g L^{-1} and arabinose: 1.04 ± 0.05 g L^{-1}). After 72 h of fermentation, the maximum butanol concentration of 12.06 g L^{-1} was obtained with 8.0% (w/v, run 8), whereas higher solid loadings had a negative effect on the sugars converted to butanol with the consumption of reducing sugars below 70%. The negative impact of high biomass loading on ABE-SSF due to mass transfer limitations or the accumulation of inert components such as ashes, among other factors, was previously reported (Guan et al., 2016; Razali et al., 2018). In this work, the hydrolysis process seems not adversely impact by high solid loadings, due to the fact that a noticeable accumulation of glucose was observed for run 4 (16.73 g L^{-1}) and run 6 (25.58 g L^{-1}); both experiments corresponding to the combination of high solid loadings (≥11%) and high enzyme loadings (≥15 FPU g-dw^{-1}). Meanwhile, low acid concentrations were observed at 24 h for the whole set of experiments (acetic acid ranging from 0.95 to 1.38 g L^{-1}, butyric acid ranging from 0.60 to 1.89 g L^{-1}, total free acid concentration < 6 mM) which were accompanied by butanol concentrations higher than 6 g L^{-1}. Thus, indicating a quick transition from acidogenesis to solventogenesis metabolism, being acidogenesis the limitation step at high solid loadings. These results corroborated the importance of the solid loading for an efficient biomass processing on simultaneous saccharification and fermentation.

Regarding the effect of enzyme dosing, increasing enzyme loading from 7.0 (run 1) to 23.0 FPU g-dw^{-1} (run 3) led to an improvement of 14% in butanol production at 24 h (from 6.70 to 7.64 g L^{-1}) when 5.0% (w/v) RS was used. Nevertheless, with the same degree of enzyme increase (7.0 to 23.0 FPU g-dw^{-1}) but with 11.0% (w/v) RS, 24-h butanol production slightly improved from 9.34 (run 2) to 10.07 g L^{-1} (run 4). At the end of the fermentation, butanol concentration in run 4 reached 11.74 g L^{-1} with around 68% of the glucose and xylose

consumed, so that the combination of high solid and enzyme loading did not achieve the best use of the biomass. 10 g L^{-1} is most likely a sub-lethal concentration of butanol for *C. beijerinckii* DSM 6422 that slows down the consumption of the sugar released when a high enzyme loading is used. Although it was not observed in these experiments, several authors have found that large amounts of enzyme in SSF processes can reduce butanol production and productivity by sugar inhibition and cellulase stress (Dong et al., 2016; Razali et al., 2018). Interestingly, ~9 g L^{-1} butanol was produced at 72 h from 8.0% (w/v) of pretreated RS with only 3.7 FPU g-dw^{-1} (run 7), showing alkaline-pretreated RS could be saccharified with low enzyme consumption. The results obtained from 8.0% (w/v) of solid showed that the use of 15.0 FPU g-dw^{-1} (run 9 – 13) is enough to achieve the same butanol production (1% of difference) at 24 h when almost twice the amount of enzyme is used (26.3 FPU g-dw^{-1}, run 8). The differences between both enzyme doses at the end of the fermentation shows a 10% variation in butanol production. This evidence is extremely important since the economic viability of the process is guaranteed by not adding extensive amounts of enzyme.

The results of the integrated SSF (Table 2) show that at least 79% of the final butanol production was reached at 24 h, thus indicating the fast fermentation profile obtained. With the aim of maximizing butanol production prior to developing the fed-batch SSFR alternative, instead of 72 h, butanol production at 24 h was therefore selected as the response variable in the optimization. After fitting the experimental data by means of a linear regression analysis, the following second-order model was obtained:

$$Butanol\ production\ (24\ h) = -4.77 + 2.630X_1 + 0.307X_2 - 0.1413X_1^2 - 0.00718X_2^2 - 0.0023X_1X_2 \quad (1)$$

Where X_1 is the solid loading (% w/v) and X_2 is the enzyme loading (FPU g-dw^{-1}). Table 3 shows the analysis of variance (ANOVA) and the coded regression coefficients of the above

252 quadratic model. At a confidence level of 95%, while the model was significant (p-value =
253 0.0006), as the lack-of-fit was not significant (p-value = 0.1466), Eq (1) could accurately
254 predict the effect of solid and enzyme loading on butanol production at 24 h. The good
255 agreement between the observed and predicted data was indicated by the high values of the
256 coefficient of determination (R^2: 0.9315) and the adjusted coefficient of determination (Adj.
257 R^2: 0.8826). According to R^2, only ~7% of the total disparity was not explained by the model.
258 Furthermore, a standard deviation of 0.4887 g L^{-1} denoted the small difference between the
259 experimental and fitted data in terms of units of the response. As can be seen in Table 3, for
260 both the solid (X_1) and the enzyme (X_2), the p-values of linear (X_1 = 0.0006, X_2 = 0.0113) and
261 quadratic effects (X_1X_1 = 0.0002, X_2X_2 = 0.0422) were lower than 0.0500, so that all the
262 effects of the two factors evaluated were found to be significant. No interaction was found
263 between solid and enzyme loading (X_1X_2, p-value = 0.8310). The relative importance of the
264 variables, based on the coded coefficients, was as follows: solid loading (X_1 = 1.01, X_1X_1 = -
265 1.27) > enzyme loading (X_2 = 0.59, X_2X_2 = -0.46).

266 The 3D response surface plot derived from the regression model and the corresponding
267 2D contour plot are shown in Figure 2. According to the ANOVA results, these plots indicate
268 that the variation of solids has a greater impact than that of the enzyme on the response and
269 show the non-interaction between both factors. The maximum butanol production of 10.32 g
270 L^{-1} at 24 h from 9.2% (w/v) solid and 19.9 FPU g-dw^{-1} enzyme loadings was estimated by the
271 model. Butanol concentration rises to a peak value by increasing solid loading to 9.2% (w/v),
272 since more fermentable sugars are released. From 9.2% to 12.2% RS (w/v), the response
273 decreases because the metabolism of *C. beijerinckii* DSM 6422 seems to be adversely
274 impacted by the buffering effect of some RS compounds such as ash. Regarding enzyme
275 loading, Figure 2 shows a flat area (~13 to 26 FPU g-dw^{-1}) around the optimum value in

276 which varying enzyme loading would reduce butanol production by less than 3%, so that the
277 model confirmed that neither sugar inhibition nor cellulase stress occurred.

278 3.2 Batch SSF: model validation

279 The butanol production model at 24 h predicted by the CCD was validated through three
280 identical assays performed on 50-mL serum bottles. Based on the optimal settings, 9.2% (w/v)
281 alkaline-pretreated RS was hydrolyzed and fermented simultaneously by 19.9 FPU g-dw^{-1} of
282 enzyme. Figure 3a depicts the time fermentation profile of the products (acetone, butanol,
283 acetic acid and butyric acid) and sugars (glucose, xylose and arabinose). Ethanol was not
284 detected as it has been previously reported by others authors using strain *Clostridium*
285 *beijerinckii* DSM 6422 (Plaza et al., 2017; Valles et al., 2021, 2020). At 12 h, before
286 *Clostridium* metabolism was entirely active, 23.78 ± 4.67 g L^{-1} of sugars (17.74 ± 3.50 g L^{-1}
287 glucose, 5.19 ± 1.02 g L^{-1} xylose and 0.85 ± 0.16 g L^{-1} arabinose) remained in the medium.
288 From the subsequent uptake of monosaccharides, 9.27 ± 0.87 g L^{-1} of butanol and 15.52 ±
289 1.19 g L^{-1} of ABE (butanol:acetone mass ratio = 1.48) were produced at 24 h. A slightly lower
290 production (~10%) was obtained by comparing the model's predicted response (10.32 g L^{-1}).
291 This small discrepancy was due to a minor delay in fermentation, as it only takes 12 h to
292 reach 10.67 g L^{-1} (Figure 3a). When fermentation finished at 72 h, 11.89 ± 0.49 g L^{-1} of
293 butanol and 19.42 ± 1.46 g L^{-1} of ABE (butanol:acetone mass ratio = 1.58) were obtained,
294 thus giving a butanol productivity of 0.165 ± 0.007 g L^{-1} h^{-1} and an ABE productivity of 0.270
295 ± 0.020 g L^{-1} h^{-1}. The butanol productivity increases to 0.386 ± 0.036 g L^{-1} h^{-1} when
296 considering 24 h, in which time 78% of the butanol had already been produced. The butanol-
297 biomass ratio as parameter to assess the mass balance of the whole process from raw RS to
298 butanol was calculated to be 62.6 ± 2.6 g-butanol kg-raw RS^{-1}. In the control assay conducted
299 under optimal conditions but without inoculation, sugar concentration reached 60.00 ± 5.93 g
300 L^{-1} (44.50 ± 2.19 g L^{-1} glucose, 13.36 ± 3.55 g L^{-1} xylose and 2.14 ± 0.19 g L^{-1} arabinose) at

301 72 h. From these values, butanol and ABE yields of 0.253 ± 0.020 and 0.412 ± 0.020 g g of
302 consumed sugar^{-1} were obtained. In comparison with those in the literature, the final butanol
303 concentration was within the greatest values (10.2 – 13.0 g L^{-1}) reported for SSF systems
304 from cellulosic material and different species of *Clostridium* (Dong et al., 2016; Guan et al.,
305 2016; Qi et al., 2019). Whereas higher values of butanol-biomass ratio (80 – 110 g-butanol
306 kg-raw RS^{-1}) have been reported for other cellulosic materials fermented in SSF systems
307 (Guan et al., 2016; Qi et al., 2019; Razali et al., 2018), butanol-biomass ratio obtained in this
308 study, 62.6 ± 2.6 g-butanol kg-raw RS^{-1}, improves the rice straw conversion to butanol in
309 23% from SSF process (Valles et al., 2020). Results are comparable not only in terms of
310 butanol production but also in productivity to Dong et al. (2016), who achieved 13.0 g L^{-1} of
311 butanol in 48 h using 9% (w/v) of alkaline-pretreated corn stover and *C. saccharobutylicum*
312 DSM 13864. In contrast, SSF with *C. acetobutylicum* ATCC 824 (Guan et al., 2016; Qi et al.,
313 2019) showed a slower fermentation rate and low butanol production at 24 h (< 4 g L^{-1}) and
314 requiring between 120 and 144 h to obtain the above-mentioned final concentrations. *C.*
315 *beijerinckii* DSM 6422 therefore seems to be a good candidate for SSF together with *C.*
316 *saccharobutylicum* DSM 13864. In comparison with the previous results of *C. beijerinckii*
317 DSM 6422 on SHF of hydrolyzates from 8% (w/v) of alkaline-pretreated RS (Valles et al.,
318 2021), the overall butanol productivity (considering both hydrolysis and fermentation time)
319 was 2.4 times higher in the one-step (SSF, 0.165 g L^{-1} h^{-1}) than two-step process (SHF, 0.070
320 g L^{-1} h^{-1}). This confirms that one of the SSF's great advantages over SHF is the time savings,
321 which reduces operational costs, along with a reduced risk of glucose contamination and
322 enzyme inhibition.

323 The optimized batch SSF was carried out in a 2-L STR with a working volume of 500
324 mL to evaluate the feasibility of the process in a bench-scale bioreactor. Scale-up was
325 successful as no relevant differences were found between the sugar and product profiles of

both experiments (Figure 3). The butanol concentration at 24 h (9.06 g L^{-1}) and 72 h (12.24 g L^{-1}) in the 2-L reactor differed by less than 3% with respect to the values observed in the serum bottles, corresponding to a final conversion of raw RS to butanol of 64.4 g-butanol kg-raw RS^{-1}. The final production of ABE was 8% higher (21.09 g L^{-1}, butanol:acetone mass ratio = 1.38) and the productivity was 0.170 g L^{-1} h^{-1} for butanol and 0.293 g L^{-1} h^{-1} for ABE. At 72 h, 8.27 g L^{-1} of sugars (3.29 g L^{-1} glucose, 4.17 g L^{-1} xylose and 0.81 g L^{-1} arabinose) remained unused in the medium. Taking into account the sugar released in the control experiments without inoculation, 86% of the sugars were consumed, thus resulting in butanol and ABE yields of 0.237 and 0.408 g g of consumed $sugar^{-1}$, respectively. The maximum concentrations of total phenolic compounds (0.42 – 0.45 g L^{-1}) were just about half the inhibitory concentration (0.71 g L^{-1}) for *C. beijerinckii* DSM 6422 (López-Linares et al., 2019). Furfural, 5-HMF and levulinic acid were not detected and the maximum concentration of undissociated acids (acetic and butyric) was 12.51 mM, below the inhibitory level (16 mM, Valles et al., 2021). The end of butanol production and the observed increase in the concentration of residual sugars from 48 to 72 h thus suggests that fermentation was inhibited by butanol at a concentration of 12.24 g L^{-1}. Ahlawat et al. (2019) found 12.56 g L^{-1} as the threshold concentration of butanol, at which the growth of *C. acetobutylicum* MTCC 11274 and sugar consumption stopped. The inhibitory concentration for *C. acetobutylicum* DSM 792 was 10.5 g L^{-1} (Rochón et al., 2017), showing that tolerance depends, among other factors, on the bacteria strain. To further improve RS butanol production and productivity, the optimized operational conditions (9.2% (w/v) of solid and 19.9 FPU g-dw^{-1} of enzyme loadings) were selected as the initial conditions of the fed-batch SSFR configuration.

3.3 Fed-batch SSF coupled with in-situ gas stripping.

A novel fed-batch SSF with ISPR by gas stripping was evaluated to assess the feasibility of using high amounts of biomass in ABE fermentation while avoiding both substrate and

351 butanol inhibition. From an initial alkaline-pretreated RS loading of 9.2% (w/v), two
352 additional biomass feedings were performed (as indicated by the arrows in Figure 4) to avoid
353 inefficient mixing or low water activity associated with the high amount of solids to be
354 processed (18.4% w/v). The first additional feeding (25% of the whole) was done at 20 h,
355 before expecting complete glucose depletion (Figure 3b). This model helps to maintain
356 biological activity once the solventogenesis has started. The second (25% of the whole) was
357 carried out 10 h after the first, when solubilization of the previously added solid was ensured.
358 Gas stripping was turned on at 20 h with the first biomass feeding to avoid butanol inhibition.
359 The application time of gas striping was planned to keep the butanol concentration in the
360 fermentation medium well below 12 g L^{-1}. It was turned off at 50 h when the concentration
361 was less than 5 g L^{-1}. Three experimental runs were carried out to assess the recycling of
362 enzyme and medium compounds (buffer, yeast extract and minerals) in the process.

363 The fermentation profile of run 1, where the final enzyme loading (19.9 FPU g-dw^{-1}) and
364 the nutrient ratio over solids were the same as in the batch SSF, is depicted in Figure 4a. As
365 shown, the pH and sugar profile in the first 20 h was very similar to those of the batch SSF
366 (Figure 3b). pH then remained stable at 5.74 ± 0.17 until the end of fermentation due to the re-
367 assimilation of acids into solvents, with low concentrations of acetic acid (0.76 – 2.98 g L^{-1})
368 and butyric acid (0.00 – 0.40 g L^{-1}) since 20 h. The maximum observed concentration of
369 undissociated acids (10.92 mM) was below the inhibitory value (16 mM). Regarding other
370 potential inhibitors, only total phenolic compounds were found at the end of the process, but
371 at a non-inhibitory concentration (0.70 g L^{-1}). The fed-batch strategy allowed processing in 72
372 h a total RS loading of 18.4% (w/v), which is equivalent to a gradual feeding of 120 g L^{-1} of
373 sugars (89.00 g L^{-1} glucose, 26.72 g L^{-1} xylose and 4.28 g L^{-1} arabinose) according to the
374 saccharification control where 60 g L^{-1} of sugars were obtained when half of the solid loading
375 was used. As fed-batch SSF successfully avoided product inhibition of saccharification, with

this approach the low sugar yield typically found in SHF when high sugar concentrations are processed (Zhu et al., 2005) is completely eliminated. While glucose accumulation stopped after 25 hours, the xylose concentration remained at 5.59 ± 1.77 g L^{-1} from 25 hours to 72 hours because a balance was established between hydrolytic enzyme activity and bacterial metabolism. *C. beijerinckii* DSM 6422 consumed 97% of the released sugars (100% of glucose and arabinose and 85% of xylose). After analyzing the condensates from gas stripping, a total volume of 150 mL was recovered at the end of the process with an average butanol concentration of 32.21 ± 3.91 g L^{-1} and ABE of 45.14 ± 4.52 g L^{-1}. The cumulative concentrations of solvents were calculated by dividing the mass of the solvents in the reactor plus those recovered in the condenser by the reactor volume. Cumulative ABE production at 72 h was thus 38.36 g L^{-1} (butanol:acetone mass ratio = 1.73), giving an ABE productivity of 0.533 g L^{-1} h^{-1}. Of the ABE solvents, 24.33 g L^{-1} were butanol, resulting in a butanol productivity of 0.338 g L^{-1} h^{-1} lower than the average butanol stripping rate (0.562 g L^{-1} h^{-1}) enabling the decrease on the butanol concentration in the reactor. The butanol selectivity, defined elsewhere (Qureshi et al., 1992), was calculated to 4.96 ± 0.97. In comparison with butanol selectivity (between 4 and 22.57) reported by other gas stripping studies (Qureshi et al., 2014, 2006), the selectivity of this experiment is in the lower range, so that it could be improved decreasing the gas flow rate or the cooling temperature as it has been suggested by Xue et al. (2014). Compared with the butanol (12.24 g L^{-1}) and ABE (21.09 g L^{-1}) production in the batch SSF (Figure 3b), the values obtained in run 1 nearly doubled, as did the productivity, as the fermentation time was the same (72 h), improving slightly the butanol-biomass ratio up to 64.0 g-butanol kg-raw RS^{-1}. Considering the results obtained in the system without product recovery, the butanol yield fell by 11% (from 0.237 to 0.210 g g of consumed $sugar^{-1}$) and that of ABE by 19% (from 0.408 to 0.330 g g of consumed $sugar^{-1}$). The moderate reduction in butanol and ABE yield could be associated with the fact that the cells

401 could increase their maintenance energy expenditure when they are continuously exposed to a
402 sub-lethal butanol concentration (Branska et al., 2018). SEM images showed a high amount of
403 long-chain *C. beijerinckii* DSM 6422 cells adhered to the altered surface of the RS at 20 h.
404 The micro-fibrous cellulose structures were used as an immobilization carrier, although no
405 biofilm was found. Unlike other *Clostridium* strains such as *C. acetobutylicum*, *C. beijerinckii*
406 does not form biofilms as it has a weak cell-to-cell communication system (Liu et al., 2018).
407 At 50 h, SEM images showed a small number of cells on the biomass support and some were
408 identified as mother cells for spores, since they showed a swollen clostridial shape. The broth
409 became viscous at this time, probably due to the autolysis promoted by sporulation as a
410 natural survival strategy for the long-term (Branska et al., 2018). A third feeding of RS was
411 added at 72 h but the fermentation did not progress further (data not shown). From SEM
412 images it could be seen that the outer surface of the rice straw has been significantly affected
413 by the enzymatic degradation after 72 h. Compared with the reported ABE-SSF studies, the
414 final solid loading used (18.4% w/v) was much higher than those typically found in the
415 literature, which range from 7.4 to 13% (Dong et al., 2016; Gallego et al., 2015; Guan et al.,
416 2016; Qi et al., 2019; Razali et al., 2018). Only Li et al. (2016) were able to efficiently use a
417 similar amount of lignocellulosic feedstock (17.5% w/w of steam-exploded corn straw) by
418 means of an intensification method such as periodic peristalsis.

419 Once it had been shown that fed-batch SSFR could efficiently produce butanol from
420 lignocellulosic waste, two additional experiments were planned with the aim of improving the
421 process's economics (runs 2 and 3). In run 2 (Figure 4b), the medium compounds ratio over
422 solids was kept as in run 1, but no extra doses of enzyme were incorporated, which halved the
423 final enzyme loading (9.95 FPU g-dw^{-1}). The same cumulative butanol (24.80 g L^{-1}) and ABE
424 (38.47 g L^{-1}, butanol:acetone mass ratio = 1.81) production were attained by using half the
425 enzyme dosing, giving a butanol productivity of 0.344 g L^{-1} h^{-1}, an ABE productivity of 0.534

426 g L^{-1} h^{-1} and a butanol-biomass ratio of 65.3 g-butanol kg-raw RS^{-1}. No remarkable
427 differences were found in the profiles of pH, products and sugars between run 1 (Figure 4a)
428 and run 2 (Figure 4b). These results suggest that the activity of cellulase, β-glucosidase and
429 hemicellulose combined in the Cellic® CTec2 blend lasted for the whole fed-batch SSFR
430 without further enzyme addition, which is a great economic saving. It is important to consider
431 that, depending on the selected substrate, the hydrolytic enzyme can account for over 12% of
432 operational costs in an integrated process such as SSFR (Qureshi et al., 2020). Simultaneously
433 repeated hydrolysis and fermentation (SRHF) is another strategy for enzyme recycling, where
434 sequential cycles of both stages are conducted in parallel in separate vessels (Zhao et al.,
435 2019). Unlike cellulases and xylanases, β-glucosidase without a cellulose-binding module
436 does not bound to lignocellulosic substrates (Várnai et al., 2011), so that β-glucosidase is
437 gradually lost in SRHF, notably reducing the glucose concentration over the saccharification
438 cycles (Zhao et al., 2019). One of the advantages of the process of this work is thus the use of
439 a sole dosage of enzyme blend to hydrolyze sequential biomass additions. The fermentation
440 profile in run 3, in which neither extra medium compounds nor enzymes were added after
441 inoculation, is shown in Figure 4c. While in previous experiments pH was recovered from 30
442 h (second biomass addition) to the end of fermentation, in this case pH decreased from 5.41 to
443 4.81, so that not adding ammonium acetate adversely impacts the buffering capacity, as this
444 compound was used as a solvent precursor. Although under these pH conditions the
445 undissociated acids did not reach inhibitory concentrations, 25% of the sugars released from
446 the RS remained unused, showing that nutrients were limited. The incomplete uptake of
447 sugars by bacterial cells led to a 20% reduction in the cumulative production of butanol
448 (18.67 g L^{-1}), ABE (30.30 g L^{-1}, butanol:acetone mass ratio = 1.60) and butanol-biomass ratio
449 (49.1 g-butanol kg-raw RS^{-1}) at 72 h compared to previous experiments. The results of this
450 work confirm that the nitrogen, vitamins, amino acids and minerals supplied by the initial

yeast extract dosage were not enough. In a previous study on SSF optimization from microwave-pretreated RS, the regression model predicted a 17% reduction in butanol production by *C. beijerinckii* DSM 6422 (from 5.28 to 4.37 g L^{-1}) when yeast extract was halved from 4 to 2 g L^{-1} (Valles et al., 2020).

Results show that the fed-batch SSF configuration previously investigated to produce ethanol from biomass (Shengdong et al., 2006; Wang et al., 2013) is also a feasible configuration for butanol production from lignocellulosic waste. By reducing the overall process time by combining saccharification, fermentation and product recovery, high butanol productivities with one enzyme dosage were achieved. Indeed, higher productivities than the alternative configurations for improving butanol productivity such as fed-batch SHF or continuous fermenters were obtained. For example, fed-batch SHF with product recovery has been used to ferment sugarcane-sweet sorghum juices, obtaining a productivity of 0.13 g L^{-1} h^{-1} (Rochón et al., 2017), while suspended-growth cell continuous processes reported productivities ranging from 0.18 – 0.23 g L^{-1} h^{-1} with lignocellulosic waste (Al-Shorgani et al., 2019; Van Hecke and De Wever, 2017). Fed-batch SSFR is in fact a promising configuration for improving ABE productivity, as it avoids the strain degeneration problem of a continuous process and SHF substrate inhibition. However, further research is required to avoid autolysis and extend the operational time by implementing, for instance, a fermenter bleeding strategy.

4 Conclusions

An advanced configuration based on simultaneous saccharification and fermentation combined with gas stripping (SSFR) was shown to be a suitable configuration to produce butanol from lignocellulosic waste. SSFR operated in fed-batch quickly processed high solid

loadings while avoiding both substrate and product inhibition, allowing butanol productivity as high as 0.344 g L^{-1} h^{-1} (titer of 24.80 g L^{-1}) by processing an RS loading of 18.4% (w/v) in the short operational time (hydrolysis+fermentation) of 72 h. The enzyme was efficiently used, as no additional enzyme dosing was necessary, thus improving the process's economic viability.

5 *Appendix A. Supplementary data*

E-supplementary data of this work can be found in online version of the paper.

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Table 1. 5-Level CCD of 2 independent variables. α = 1.4142.

Independent variables		Coded and real values				
		Level -α	Level -1	Central point (0)	Level +1	Level +α
X_1	Solid loading (% w/v)	3.8	5.0	8.0	11.0	12.2
X_2	Enzyme loading (FPU g-dw^{-1})	3.7	7.0	15.0	23.0	26.3

Table 2. CCD experimental matrix along with the values of butanol production (g L^{-1}) at 24 and 72 h, remaining sugars (g L^{-1}, glucose, xylose and arabinose) at 24 h and consumed sugars (%, glucose and xylose) at 72 h.

Run	Real values[a]		24 h				72 h		
			Butanol (g L^{-1})	Sugars (g L^{-1})			Butanol (g L^{-1})	Consumed sugars (%)[b]	
	X_1	X_2		Glucose	Xylose	Arabinose		Glucose	Xylose
1	5.0	7.0	6.70	0.25	1.92	0.33	8.49	96.4	85.6
2	11.0	7.0	9.34	4.17	4.47	1.00	10.56	59.6	63.7
3	5.0	23.0	7.64	0.38	1.96	0.14	8.35	97.2	75.9
4	11.0	23.0	10.07	16.73	5.77	1.41	11.74	68.2	68.7
5	3.8	15.0	6.13	0.29	1.08	0.00	6.61	96.9	84.4
6	12.2	15.0	8.23	25.58	7.09	1.44	10.37	50.4	62.7
7	8.0	3.7	7.74	0.19	2.96	0.38	8.86	76.0	72.7
8	8.0	26.3	9.88	6.00	4.43	0.99	12.06	88.8	72.7
9 – 13	8.0	15.0	9.95 ± 0.35	1.33 ± 1.03	5.02 ± 0.18	1.04 ± 0.05	10.87 ± 0.46	94.2 ± 0.6	60.6 ± 2.8

[a] X_1: solid loading (% w/v); X_2: enzyme loading (FPU g-dw^{-1}).

[b] Final sugar concentration of saccharification control (solid loading of 8% w/v and an enzyme loading of 15 FPU g-dw^{-1}): 39.95 ± 0.23 g L^{-1} glucose, 13.35 ± 0.16 g L^{-1} xylose and 1.86 ± 0.00 g L^{-1} arabinose.

Table 3. ANOVA of the CCD model for butanol production (g L^{-1}) at 24 h.

Source	Degrees of freedom	Sum of squares	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Model	5	22.74	4.55	19.04	0.0006	
Linear	2	10.87	5.44	22.76	0.0009	
X_1: solid loading (% w/v)	1	8.10	8.10	33.90	0.0006	1.01
X_2: enzyme loading (FPU g-dw^{-1})	1	2.77	2.77	11.61	0.0113	0.59
Square	2	11.86	5.93	24.82	0.0007	
X_1X_1	1	11.25	11.25	47.09	0.0002	-1.27
X_2X_2	1	1.47	1.47	6.15	0.0422	-0.46
2-way interactions	1	0.01	0.01	0.05	0.8310	
X_1X_2	1	0.01	0.01	0.05	0.8310	-0.05
Error	7	1.67	0.24			
Lack-of-fit	3	1.18	0.39	3.18	0.1466	
Pure error	4	0.49	0.12			
Total	12	24.41				
Standard Deviation, S					0.4887	
R^2					0.9315	
Adj. R^2					0.8826	

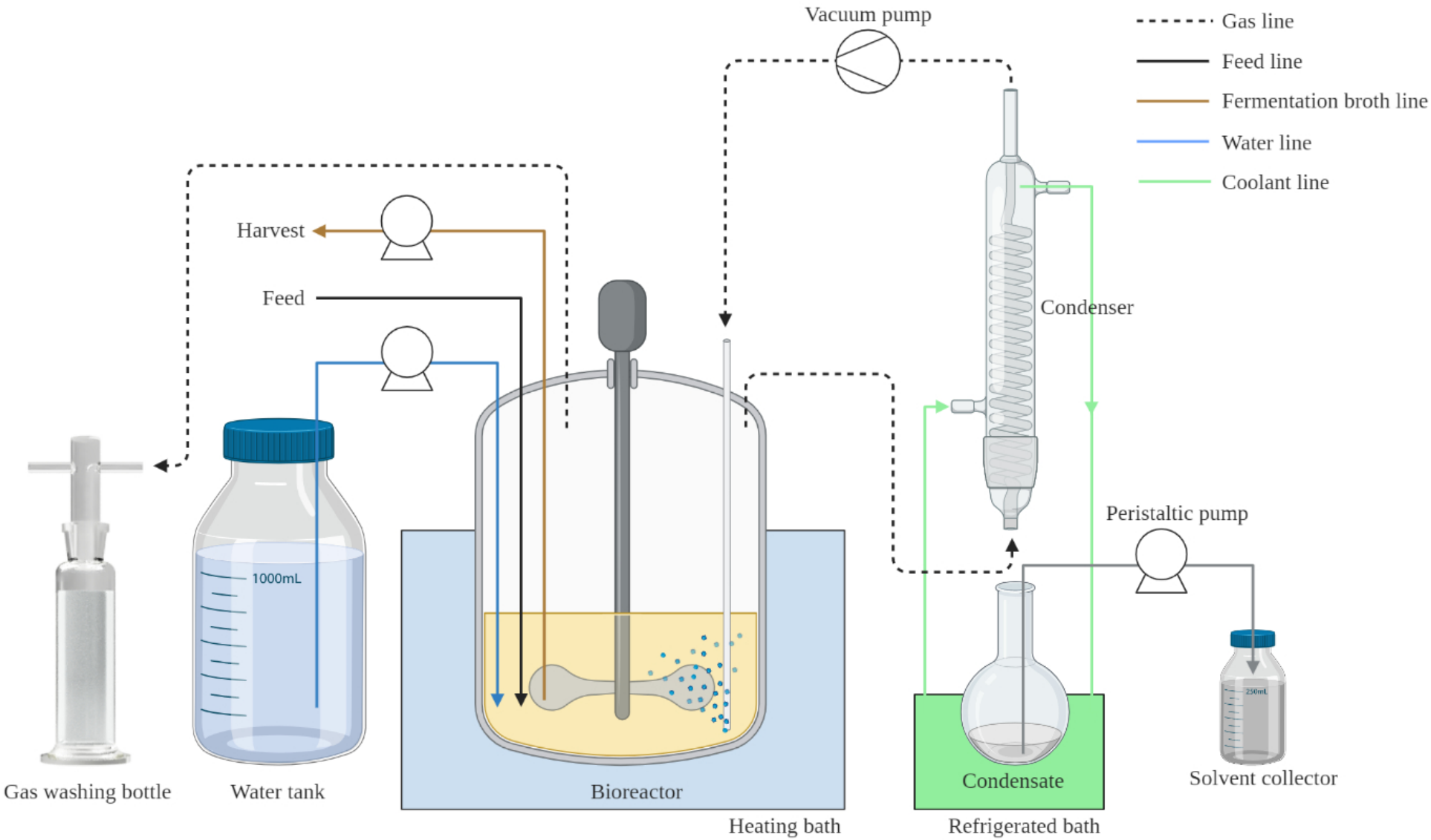
[a] For coded variables.

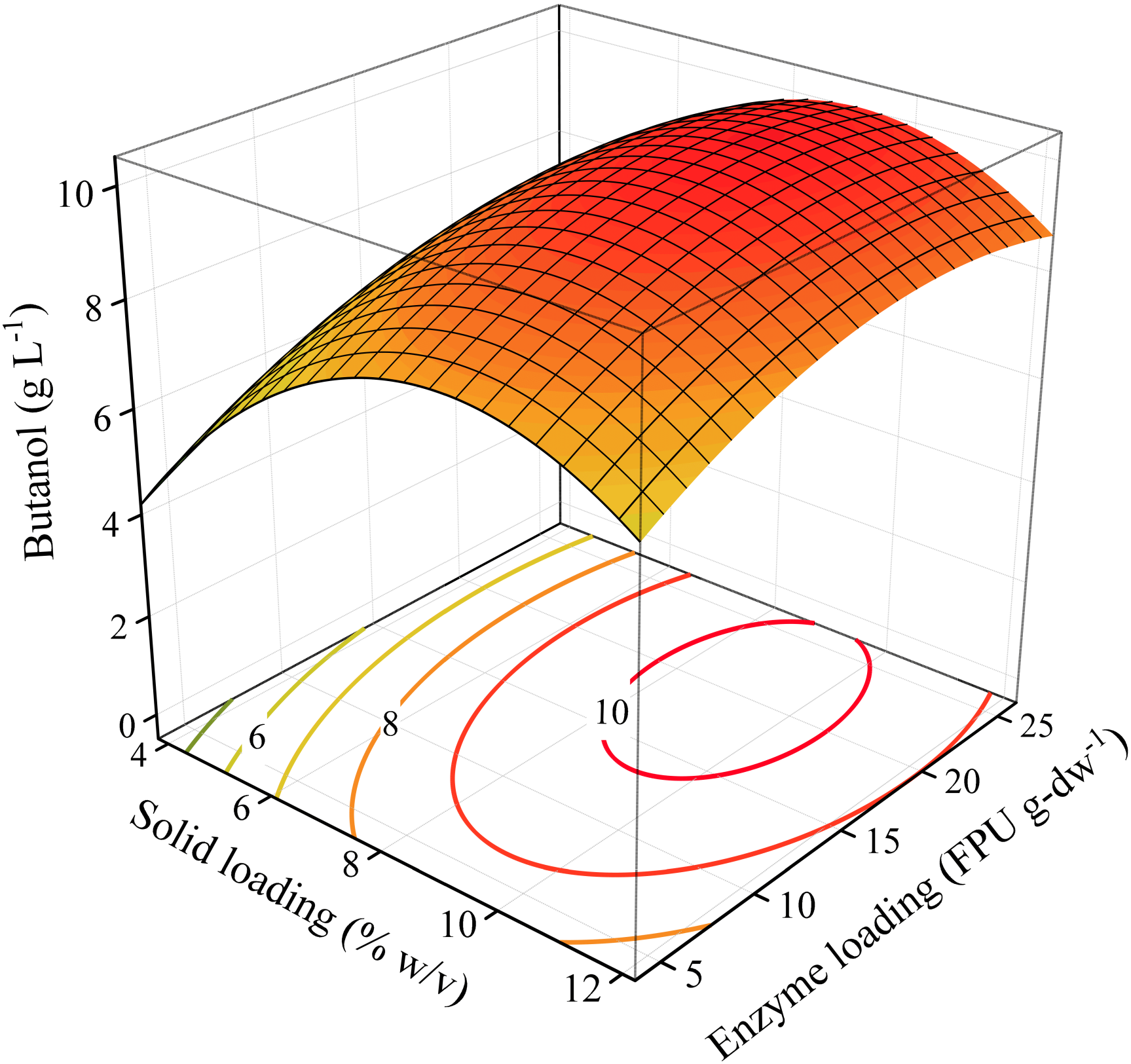
Figure 1. Schematic diagram of fed-batch SSF with *in-situ* product recovery by gas stripping (SSFR). Created with BioRender.com.

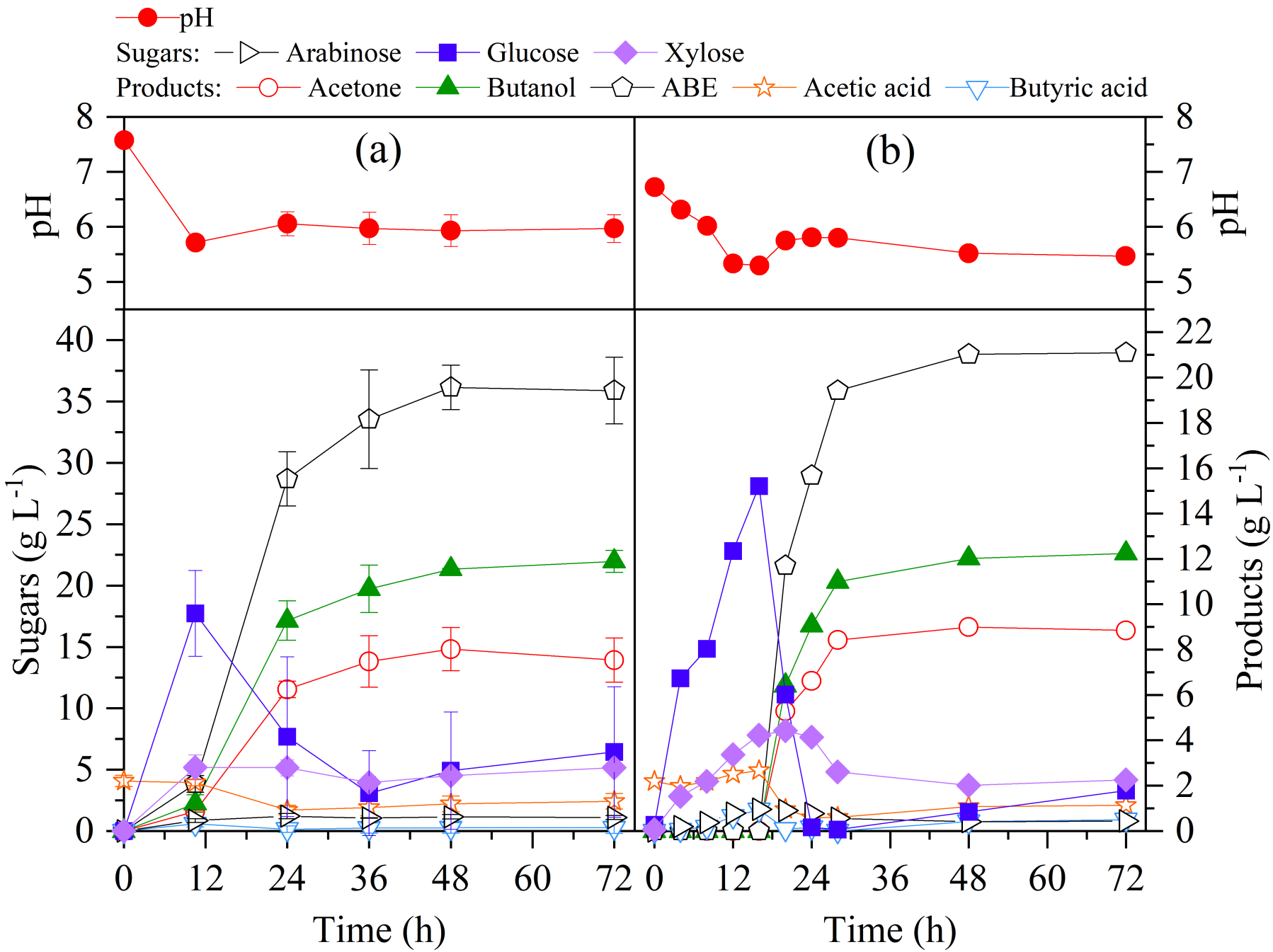
Figure 2. Response surface and corresponding contour plot for butanol production (g L^{-1}) at 24 h: combined effect of solid loading (% w/v) and enzyme loading (FPU g-dw^{-1}).

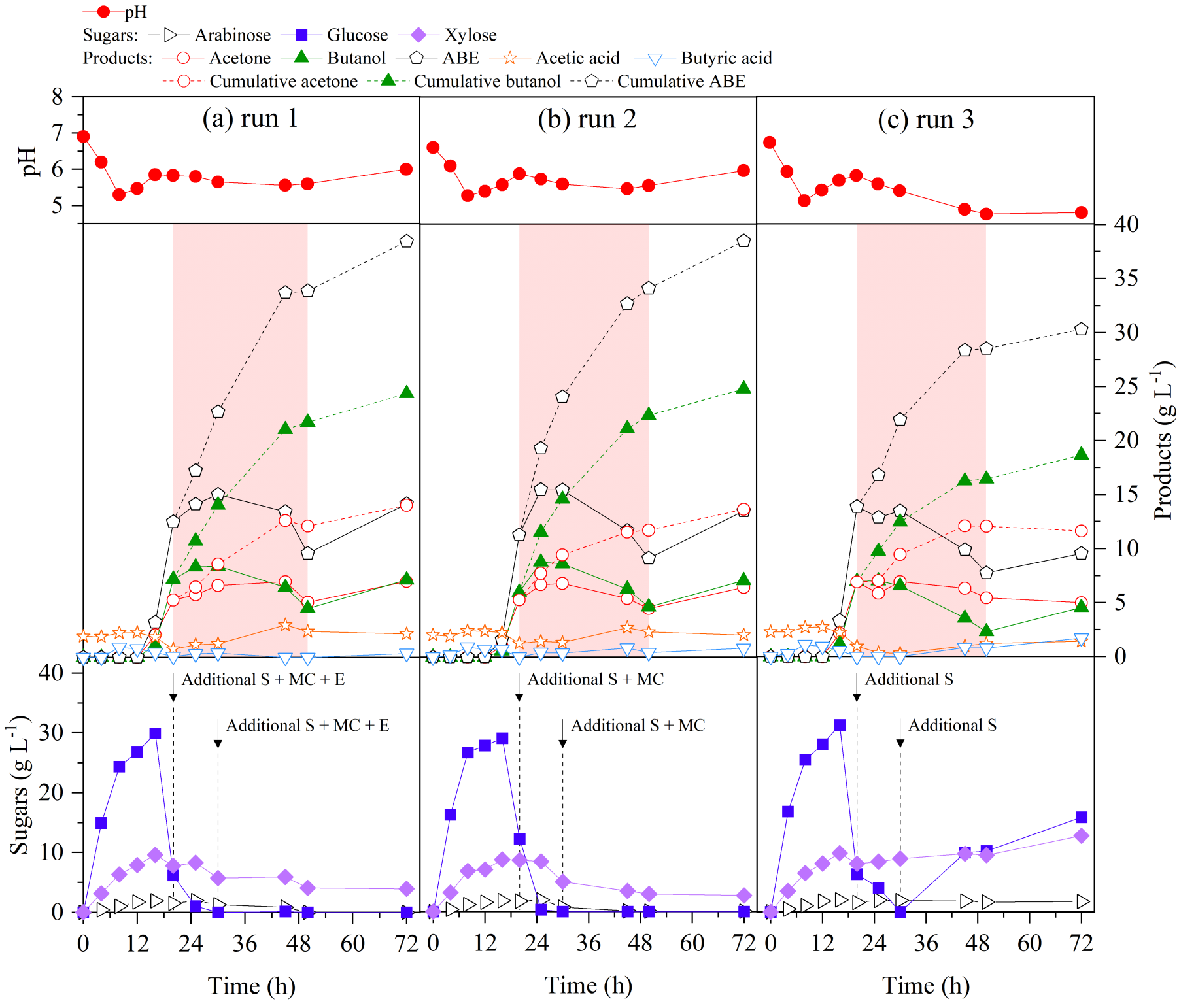
Figure 3. CCD model validation. (a) Batch SSF in a 50-mL serum bottles. (b) Scale-up of the batch SSF to a 2-L reactor.

Figure 4. Time course of the fed-batch SSFR in a 2-L reactor. Additional solid (S) was added with medium compounds (MC) and enzyme (E) in run 1, with MC but without E in run 2 and without MC and E in run 3. Red shaded region indicates the application period of gas striping.


Vacuum pump
Gas line
Feed line
Fermentation broth line
Water line
Coolant line
Harvest
Feed
Condenser
Peristaltic pump
1000mL
Gas washing bottle
Water tank
Bioreactor
Heating bath
Condensate
Refrigerated bath
Solvent collector


Butanol (g L-1)
0
2
4
6
8
10
Solid loading (% w/v)
4
6
8
10
12
Enzyme loading (FPU g-dw-1)
5
10
15
20
25
6
8
10


pH
Sugars:
Arabinose
Glucose
Xylose
Products:
Acetone
Butanol
ABE
Acetic acid
Butyric acid
(a)
(b)
pH
Sugars (g L-1)
Products (g L-1)
Time (h)
Time (h)


pH
Sugars: Arabinose Glucose Xylose
Products: Acetone Butanol ABE Acetic acid Butyric acid
Cumulative acetone Cumulative butanol Cumulative ABE
(a) run 1
(b) run 2
(c) run 3
pH
Products (g L-1)
Sugars (g L-1)
Additional S + MC + E
Additional S + MC
Additional S
Time (h)