Optimization of alkali pretreatment to enhance rice straw conversion to butanol

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Abstract

The use of rice straw (RS) was enhanced to produce biobutanol as biofuel, for which the NaOH pretreatment was optimized by considering the butanol-biomass ratio that quantify the mass balance efficiency of the three sequential stages of the process: pretreatment, enzymatic hydrolysis and fermentation by *Clostridium beijerinckii*. The optimum point (solid loading of 5% w/v with 0.75% w/v NaOH at 134 °C for 20 min) of the best cost-wise option yielded an enhanced biomass use of 77.6 g kg RS^{-1}. A maximum butanol titer of 10.1 g L^{-1} was reached after 72 h of fermentation with the complete uptake of glucose and nearly complete uptake of xylose. The NaOH concentration was the most influential parameter. The appropriate dosage to maximize fermentable sugars instead of the mass balance efficiency of the three stages underestimated the biomass use by 13%, showing the importance of correctly selecting the variable response during optimization.

Keywords: alkaline pretreatment, biofuels, butanol, *Clostridium beijerinckii*, rice straw

1 Introduction

In 2020 the European Commission updated its First Circular Economy Action Plan launched in 2015 [1]. Among the actions derived from the EU's agenda for sustainable growth, the recast Renewable Energy Directive (EU) 2018/2001 laid down a target for advanced biofuels contributing 3.5% of the total transport sector energy. These biofuels must be produced from feedstocks like lignocellulosic biomass (bagasse, straw, or forestry waste), food cellulosic materials, animal manure or algae, among others. Rice straw (RS), mainly composed of cellulose and hemicellulose, is a promising feedstock with a global production in the range of 370 – 520 million tons per year [2]. Alcohols like methanol, ethanol, propanols, and butanols have been proposed as biofuel candidates. Although butanol has several advantages over shorter alcohols including higher volumetric energy content, it is less corrosive and is more compatible with conventional fuels [3].

ABE anaerobic fermentation is a sustainable method of producing biobutanol from rice straw. Clostridia species such as *Clostridium acetobutylicum* and *Clostridium beijerinckii* carry out ABE fermentation through biphasic metabolism: the organic acids produced in the acidogenic phase are re-assimilated later in the solventogenic phase to produce acetone, butanol and ethanol [4]. Although there are some shortcoming in batch fermentation (product inhibition, dead time periods required for medium preparation and sterilization) which result in lower butanol productivity, batch configuration has been conventionally selected for industrial ABE systems in Europe because of its easy operation and minimum control [5]. However, these Clostridia species are not able to hydrolyze lignocellulosic biomass efficiently [6], so that sugar monomers need to be obtained in an upstream pretreatment stage, followed by hydrolysis. The pretreatment alters the complex polymeric biomass structure by breaking the lignin seal, removing lignin and/or increasing its porosity [7]. The pretreatment should meet the following requirements: low energy demand and overall costs, efficient and rapid

release of sugars in the subsequent hydrolysis, reducing carbohydrate degradation and avoiding the formation of inhibitory compounds (e.g., acids, furans and phenols). Concerning pretreatment of rice straw, several methods have been proposed, including: alkaline pretreatment [8–10], acid pretreatment [8,10,11], steam explosion [11], organosolv pretreatment [12] and the recent microwave-assisted hydrothermolysis [13]. Alkaline pretreatment with sodium hydroxide, ammonium hydroxide, potassium hydroxide or calcium hydroxide is effective in biomass with low lignin content and has other benefits such as low sugar degradation and a non-corrosive nature [14]. This chemical method disrupts and removes the lignin-carbohydrate structure and causes the biomass to swell, which reduces polymerization and cellulose crystallinity and increases the internal surface area. Among the different alkaline reagents, sodium hydroxide leads to a more efficient straw delignification [15], as well as other effects such as alteration of the silica layers and the cuticle wax, so that NaOH pretreatment could be proposed as the best method of increasing enzyme accessibility to cellulose in RS biomass [16–18]. Indeed, Moradi et al. [8] obtained a higher butanol production and overall conversion from RS pretreating with NaOH instead of H_2SO_4.

The operational conditions of the pretreatment affect the overall performance of butanol production, since they govern the susceptibility of the substrate to hydrolysis and the subsequent fermentation of the liberated sugars [19]. The most common approach for optimizing pretreatment conditions such as temperature, time, reagent concentration and solid loading focuses on evaluating their effect on the hydrolysis performance or on the delignification degree. For example in RS pretreatment, Singh et al. [20] used reducing sugar concentration as a response variable, Kim and Han [16] employed glucose recovery from untreated biomass and Hosseini et al. [15] and Mukherjee et al. [17] adopted the quantity of lignin removed from the biomass. Nevertheless, RS hydrolysates with high glucose

concentration it can lead to reduced efficiency in terms of butanol production, butanol yield and overall butanol productivity [10].

Alternatively, the selection of a butanol to biomass ratio as response variable can be useful when pretreated hydrolysates are compared in terms of raw material conversion to butanol. The aim of this work was to optimize the alkaline pretreatment of RS considering the mass balance of the whole process from raw RS to butanol in order to assess the efficient use of lignocellulosic biomass. The global efficacy of the three serial steps: pretreatment, hydrolysis and ABE batch fermentation was defined by a single response variable. The effect of the temperature, time, NaOH concentration and solid loading was assessed on the (i) solid recovery, (ii) released sugars and (iii) produced butanol in order to integrate the efficiency of these three stages in the response variable, the butanol-biomass ratio (g butanol kg RS^{-1}). Pretreatment optimization was carried out by a preliminary fractional factorial design followed by a central composite design (CCD).

2 Materials and methods

2.1 Materials

RS was obtained from the Albufera Natural Park in Valencia (Spain). The raw material was milled and the particle size between 100 and 500 μm was selected by an ISO-3310.1 sieve (CISA, Spain). Before storing for further use, the biomass was dried in an oven at 45 ºC to reduce residual moisture content below 5% w/w. The chemical composition (% dry weight basis) of the RS was: cellulose 35.8 ± 2.1%, hemicellulose 17.5 ± 1.4%, acid soluble lignin 0.1 ± 0.0%, acid insoluble lignin 14.3 ± 0.4%, ash 16.7 ± 0.1% and others 15.6 ± 3.7%.

Saccharification of the pretreated biomass was carried out by a commercial Cellic® CTec2 enzyme blend (Novozymes, Denmark). The cellulase activity resulted in a value of

157 filter paper units (FPU) mL^{-1} according to the National Renewable Energy Laboratory method [21]. *Clostridium beijerinckii* DSM 6422 (NRRL B-592), obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was stored at -80 ºC in a Reinforced Clostridial Medium with 20% (v/v) glycerol. Before ABE fermentation the cells were grown following the procedure described elsewhere [13].

2.2 Alkaline pretreatment and enzymatic hydrolysis

Alkaline pretreatment was carried out in 500-mL glass bottles in which the RS was mixed with an NaOH solution (concentration ranging from 0 to 2% w/v) to achieve the appropriate solid loading (5 or 10% w/v), according to the experimental design described in Section 2.5. The bottles were then heated to 121 or 134 ºC for a reaction time between 10 and 60 min in an autoclave (MED20, J.P. Selecta, Spain). The slurry was centrifuged at 4000 rpm for 6 min (Centrifuge 5804, Eppendorf, Germany), the solid phase was washed four times with deionized water and pH was adjusted to 6.5. Finally, the pretreated RS was dried at 45 ºC for 24 h. The severity of the pretreatment conditions was estimated by the Severity Factor (SF) proposed by MacAskill et al. [22]:

$$SF = Log\left[time\ (min.) \times \exp\left[\frac{Temperature\ (°C) - 100}{14.75}\right]\right] \quad (1)$$

Enzymatic hydrolysis was conducted in 100-mL conical flasks (with a working volume of 45 mL) containing 8% w/v of the pretreated RS to avoid end-product inhibition which was previously reported at higher RS loadings [9]. The commercial enzyme blend Cellic® CTec2 was added with a load of 15 FPU $g\text{-}dw^{-1}$ based on the optimal configuration obtained in our previous study on simultaneous saccharification and fermentation of microwave-pretreated RS [13]. Hydrolysis was carried out at pH of 5.5 (50 mM acetate

buffer), 50 ºC and 200 rpm in an SI500 orbital shaker (Stuart, UK) for 72 h. After saccharification the samples were centrifuged at 4000 rpm for 10 min, filtered through 1.2 µm and stored at 4 ºC until ABE fermentation.

2.3 ABE fermentation

Batch fermentations of 26 mL of the RS hydrolysate were performed in a 50 mL serum bottles with a working volume of 30 mL. The medium was composed of: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4, 2.20 g L^{-1} NH_4Ac, 0.09 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $MnSO_4 \cdot H_2O$, 0.02 g L^{-1} $FeSO_4 \cdot 7H_2O$ and 4 g L^{-1} of yeast extract. The initial pH was adjusted to 5.8, oxygen was displaced and bottles were autoclaved for 10 min at 121 ºC. The bottles were inoculated with 2 mL (5% v/v) of *C. beijerinckii* DSM 6422, and incubated at 37 ºC and 150 rpm for a maximum of 144 h.

2.4 Analytical methods

The efficiency of the pretreatment was characterized by analysis of sugars and inhibitory compounds from 1.5-mL samples of the RS hydrolysate. The chemical composition of the pretreated RS was determined for each pretreatment condition. Structural carbohydrates, lignin and ash were measured following the National Renewable Energy Laboratory procedures [23]. Fermentation was monitored by analyzing pH, cell growth, products of acids and solvents, and sugar consumption from 1-mL samples withdrawn every 24 h. The pH was measured by a Minitrode electrode (Hamilton, USA). Cell density (g-dw L^{-1}) was determined from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer (SpectroFlex 6600, WTW, Germany) and using the linear correlation: g-dw $L^{-1} = 0.2153 \cdot OD_{600} + 0.0689$ (n = 10, $R^2 = 0.9907$).

153 Liquid samples were centrifuged at 10000 rpm for 5 min and filtered through 0.22 μm
154 before HPLC analysis. Sugars (glucose, xylose and arabinose), acids (acetic acid, butyric acid
155 and levulinic acid), solvents (butanol, acetone and ethanol) and other inhibitory compounds
156 (furfural and 5-HMF) were analyzed by a liquid chromatograph (Agilent HPLC 1100 Series,
157 Agilent Technologies, USA). An Aminex® HPX-87H column (300 mm × 7.8 mm, Bio-Rad
158 Laboratories Inc., USA) was operated at 50º C. The mobile phase was 5 mM H_2SO_4 at a flow
159 rate of 0.6 mL min^{-1}. Sugars, butyric acid, butanol and ethanol were analyzed by a refractive
160 index detector (RID). A diode array detector (DAD) was used to measure acetic, formic and
161 levulinic acids at 210 nm, while acetone, furfural and 5-HMF were analyzed at 280 nm. The
162 total phenolic compounds were determined by the Folin-Denis method [24], expressing
163 phenolic concentration as gallic acid equivalents (GAE).

164 For the evaluation of the enzymatic hydrolysis, the glucose yield was defined as:

165 $$Glucose\ yield\ (\%) = \frac{Glucose\ released\ (g\ L^{-1}) \times 0.9 \times 100}{[80\ (g\ L^{-1})/Solid\ recovery\ (\%)] \times Cellulose\ in\ raw\ RS(\%)} \quad (2)$$

166 The overall conversion of RS into biobutanol or ABE solvents was calculated as
167 follows:

168 $$Butanol\ (or\ ABE) - biomass\ ratio\ (g\ kg^{-1}) =$$

169 $$\frac{Butanol\ (or\ ABE)\ \ produced\ (g)/V_{hydrolisate\ fermented}\ (L)}{[0.08\ (kg\ L^{-1})/Solid\ recovery\ (\%)] \times 100} \quad (3)$$

170

171

172 2.5 Design of experiments and statistical analysis

173 The alkaline pretreatment of RS was optimized in two subsequent statistical analyses.
174 In both cases, the butanol-biomass ratio (g kg RS^{-1}), which indicates the mass balance

efficiency of the three serial stages (pretreatment, hydrolysis and ABE fermentation) was selected as the response parameter. First, the significant variables were selected in the overall conversion to butanol by fractional factorial design. These variables were then optimized by the response surface method using CCD. Design of experiments and data analysis were conducted using the MINITAB® v.2020.1.0 commercial software (Minitab Inc., USA). Analysis of variance (ANOVA) was performed at a confidence level of 95% (p-value < 0.05).

2.5.1 Fractional factorial design and data analysis

The significant factors affecting the butanol-biomass ratio at 72 h were screened by a 2^{4-1} fractional factorial design (resolution IV, 8 experiment runs). Table 1 summarizes the coded and real values of the four variables. Pretreatment temperature (X_1) and solid loading (X_4) were established as the categorical variables, while pretreatment time (X_2) and NaOH concentration (X_3) were the range variables.

2.5.2 Central composite design and data analysis

Based on the results of the fractional factorial design, a response surface method with CCD (composed of 13 experiments with 5 central point replications) was used to determine the optimal combination of the significant variables (pretreatment time and NaOH concentration). The established range for each factor was as follows: pretreatment time (from 20 to 60 min) and NaOH concentration (from 0 to 1% w/v). Finally, a validation step was carried out by three replicates using the optimized conditions for RS use.

3 Results and discussion

3.1 Screening key factors on the overall conversion to butanol

The influence of the pretreatment variables (temperature, time, NaOH concentration and solid loading) was initially assessed for maximizing the release of the fermentable sugar

198 and the butanol-biomass ratio. Table 2 summarizes the results of the fractional factorial
199 design, including the solid recovery from pretreatment (%), sugars released after 72 h of
200 enzymatic hydrolysis (g L^{-1}), butanol concentration (g L^{-1}), and butanol-biomass ratio (g kg
201 untreated RS^{-1}) obtained from 72 h ABE fermentation. The alkaline pretreatment provided
202 total solid recoveries ranging from 43.0 to 85.7%, with the highest values for the lowest
203 NaOH concentration (runs 1 – 4) despite the SF.

204 The influence of temperature, time and solid loading seems to be negligible on the
205 partial biomass solubilization and/or degradation. Figure 1 shows the chemical composition
206 (%) of raw and pretreated RS of the 8 runs. All pretreatment experiments with the highest
207 NaOH concentration led to pretreated RS enriched in cellulose (>49%, runs 5 – 8), while a
208 minimum time of 40 min was required to achieve some cellulose enrichment for the lowest
209 NaOH concentration (40 – 42%, runs 3 – 4). The cellulose enrichment came mostly from the
210 preferential removal of acid insoluble lignin rather than the hemicellulose solubilization,
211 except for run 3, which hardly altered the biomass structure. The highest biomass loading of
212 run 3 explains the lower release of sugars than in run 4 (4.9 vs. 33.4 g L^{-1}), these being
213 unsuitable operational conditions for butanol production. High degrees of delignification
214 (80.3 to 97.6%) were found in the experiments with the highest alkali concentration (2% w/v,
215 runs 5 – 8), while moderate delignification (57.0%) was achieved in run 4 with the lowest
216 alkali concentration (0.2% w/v).

217 The highest cellulose recovery (84.1%) was from the soft alkaline conditions (run 4),
218 which positively influenced the subsequent hydrolysis and fermentation processes. Alkaline
219 pretreatment is known to be able to remove lignin from RS by producing pores on its surface
220 [17]. Other pretreatments such as acid or microwave-assisted hydrothermolysis resulted in
221 lower delignification degrees, which could limit enzymatic sugar recovery to some extent.
222 Moradi et al. [8] obtained a 27% delignification from acid pretreatment (50 ºC, 30 min and

85% H_3PO_4) and Valles et al. [13] obtained 13.3% delignification by microwave-assisted hydrothermolysis at a ramp temperature from 100 °C to 200 °C for 40 min. The delignification achieved in this study was better than those obtained with other RS alkaline pretreatments. For example, Kim and Han [16] achieved less than 80% delignification working at a longer reaction time (60 min) and lower temperature (100 ºC). Mild alkaline conditions can also achieve high delignification but they require a high NaOH concentration. Moradi et al. [8] reported 76% delignification working at 0 °C, 3 h and 12% w/v NaOH.

The sugars released after 72 h of enzymatic hydrolysis of the pretreated samples confirmed the efficiency of alkaline RS pretreatment. The three samples (runs 1 – 3) with delignification degrees less than 20% provided the lowest sugar concentrations (< 18 g L^{-1}, Table 2), since lignin hinders cellulase access to cellulose fibers [16] and binds non-productively to cellulase [25]. The maximum sugar concentration was obtained in run 7, reaching a value of 65.3 g L^{-1} (50.5 g L^{-1} glucose, 13.5 g L^{-1} xylose and 1.3 g L^{-1} arabinose) although this experiment did not lead to the highest delignification. This could be attributed to the lower hemicellulose content of this sample (16.0%, Figure 1), as hemicellulose can inhibit hydrolysis since acetyl groups from xylan cause steric hindrance of enzymes [26]. Nevertheless, these results showed that non-specific adsorption of the enzyme to the remained lignin do not play an adversely effect. The results of glucose yield (Figure 1) showed that by increasing SF and reducing the solid loading the consumption of NaOH can be reduced to achieve similar values (runs 4 and 5 resulted in glucose yields ~40%). Hydrolysates were measured to quantify the potential inhibitory compounds. The analysis outcomes showed that the viability of the subsequent fermentation would not be negatively affected by the presence of inhibitory compounds. Levulinic acid and furfural were not detected in any of the samples and HMF concentration was negligible (< 0.01 g L^{-1}). Total phenolic compound concentration ranged from 0.14 to 0.32 g L^{-1}, below the inhibitory level (0.71 g L^{-1}) for *C. beijerinckii* DSM

6422 [27]. The acetic acid in the hydrolysate was mainly formed during alkaline pretreatment by hydrolysis of the acetyl groups of hemicellulose [28]. The concentration of acetic acid ranged from 2.58 to 4.45 g L^{-1}, which could be beneficial for ABE fermentation as it has been reported that 3 g L^{-1} of acetic acid enhanced solvent production by *C. beijerinckii* DSM 6422 [29].

The hydrolysates were fermented by *C. beijerinckii* DSM 6422 to assess the efficiency of the whole process. As can be seen in Table 2, maximum concentration (10.6 g L^{-1}) was obtained for the maximum released sugars (65.3 g L^{-1}, run 7). In terms of sugar conversion to butanol, run 4 achieved similar values to run 7 (0.16 g butanol g released $sugars^{-1}$) in spite of its lower sugar concentration. The lower butanol production of runs 5, 6 and 8 was related to poor sugar conversion. After 24 h of fermentation, undissociated acids were higher in those samples than in run 7 (acetic and butyric acid: 23.0 ± 1.3 mM in runs 5, 6 and 8; 16.0 mM in run 7). Undissociated acids can pass across the *Clostridium* cell membrane, while their subsequent dissociation at the neutral internal pH of the cell can result in growth inhibition [4]. To keep the undissociated acid concentration at < 16 mM, initial pH was set at 5.8 for further experiments. RS-to-butanol conversion was analyzed to consider the loss of solids along with the sugar conversion (Table 2). The highest RS use was achieved in run 7 with a butanol-biomass ratio of 51.9 g kg untreated RS^{-1}, followed by run 4 with a value of 42.9 g kg untreated RS^{-1}. Both experiments had the lowest solid loading (5% w/v) but different NaOH requirements. Valles et al. [13] pretreated rice straw by microwave assisted hydrothermal hydrolysis, obtaining 51 g kg untreated RS^{-1} as the best value, showing that further optimization of alkaline pretreatment would be promising for efficient RS use.

The ANOVA regression model of the butanol-biomass ratio at 72 h (Table 2) was statistically significant with a p-value lower than 0.05 (95% confidence, p-value of 0.0479). Among the four variables included in the 2^{4-1} fractional factorial design, the solid loading (p-

273 value of 0.0205) and the interaction between temperature and NaOH concentration (p-value of
274 0.0218) were found to be significant with a negative effect on the butanol-biomass ratio (g kg
275 RS^{-1}). The good accuracy of the model was due to the coefficient of determination (R^2:
276 0.9805) and adjusted coefficient of determination (Adj. R^2: 0.9319) values. Considering the
277 negative effect of the solid loading (categorical parameter) on the process efficiency the value
278 of 5% w/v was selected for further optimization. For this solid loading, a model prediction
279 was carried out to select the temperature level (categorical parameter) and the range of NaOH
280 concentration and time to define the CCD. Figure 2 shows the cube plot of fitted means of the
281 butanol-biomass ratio, in which the vertices of the cube display the model results of the
282 predicted values for all the combinations of the low and high levels of three parameters
283 (temperature, NaOH concentration and time). As can be seen, there are two sets of
284 temperature-NaOH conditions with RS-to-butanol conversion values higher than 40 g kg RS^{-1}.
285 The two vertices lie on the plane intersecting the cube at 40 min corresponding to the pairs:
286 134 °C with 0.2% w/v NaOH and 121 °C with 2.0% w/v NaOH. Due to the potential saving
287 in the reagent cost, it was decided to use 134 °C as the working temperature. From the edge at
288 40 min and 134 °C, it was decided to limit the maximum NaOH amount to 1% w/v.

289

290 3.2 Optimization of butanol-biomass ratio

291 Once the temperature and solid loading were set at 134 °C and 5% w/v from the
292 results of the preliminary fractional factorial design experiment, a response surface method
293 with full factorial CCD was carried out. The maximum butanol-biomass ratio was found from
294 the best NaOH concentration and time conditions. To confirm the goodness of the
295 optimization approach, the model was validated by running an experiment (3 replicates) in the
296 optimum conditions.

3.2.1 Response surface methodology

The RS-to-butanol conversion was maximized through a five-level, two-factor CCD, followed by linear regression analysis to adjust the experimental values to a second-order model. The experimental design is shown in Table 3 and includes the coded and real values of the range factors (NaOH concentration: Z_1; time: Z_2) for the 13 experimental runs, including five central point replications to assess the experimental variability. The central point conditions (0.5% w/v of NaOH concentration and 40 min pretreatment time) were selected from previous results. The axial point conditions ($\alpha = 1.4142$) were set to extend the NaOH concentration from 0 (hydrothermal equivalent) to 1% w/v with time in the range ± 20 min. As temperature was set at 134º C, a narrower SF variation (2.30 – 2.78) was used than in the previous factorial design (1.62 – 2.60).

Table 3 also summarizes the response for the butanol-biomass ratio (g kg untreated RS^{-1}) along with the results of the three serial processes: (i) solid recovery (%) from alkaline pretreatment; (ii) released sugars (g L^{-1}) and glucose and xylose yields of untreated RS (%) in enzymatic hydrolysis; and (iii) butanol produced (g L^{-1}) after 72 h of ABE fermentation. Good reproducibility of the representative parameters was obtained for the three stages of the process for the central point replicates (run 9 – 13; solid recovery: 50.6 ± 0.0%; released sugars: 51.9 ± 1.3 g L^{-1}; butanol production: 8.8 ± 0.5 g L^{-1}). A broad solid recovery range was achieved from the experimental results (37.9 – 75.9%), which negatively correlated with the cellulose content of the samples. Of the two tested parameters, NaOH concentration had a strong effect, with RS pretreatment being less sensitive to time changes. This is in agreement with the results that Mukherjee et al. [17] obtained from alkaline pretreatment with a wider range of NaOH concentrations (0.73 – 12.73% w/v), time (39.55 – 140.45 min) and temperature (16.45 – 133.86 °C). They found that alkali concentration was more important in RS delignification during pretreatment than the other two factors. Especially when alkali

concentration was as low as 0.15% w/v (runs 1, 3) or when alkali was not used (run 5) small solid losses were achieved, thus indicating the inefficiency of mild alkali usage for increasing the sugar released from RS. Indeed, these samples had a similar lignin content (13.2 – 16.9%) to the raw material. Insoluble lignin was extensively removed for 0.5% w/v NaOH or higher, while the content in the pretreated solid fractions varied from 0.7 to 6.4% (runs 2, 4, 6, 7 – 13). These findings show that similar RS delignification can be achieved by limiting NaOH concentration to a maximum of 1% w/v as by using 2% w/v NaOH (factorial fraction screening, Figure 1).

All the pretreated solid fractions without any further detoxification were enzymatically hydrolyzed. As can be seen in Table 3, the concentration of the reducing sugars in the hydrolysate increased mainly with NaOH concentration, reaching the maximum value of 64.4 g L^{-1} (46.2 g L^{-1} glucose, 16.5 g L^{-1} xylose and 1.7 g L^{-1} arabinose) for the highest NaOH concentration (1% w/v, run 6), and the minimum value of 8.6 g L^{-1} (4.8 g L^{-1} glucose, 3.7 g L^{-1} xylose and 0.1 g L^{-1} arabinose) in the absence of NaOH (run 5). When low lignin removal was obtained (runs 1, 3 and 5), glucose yield varied from 11.6 to 31.7% and xylose yield varied from 20.7 to 43.7%. In the experiments with a high degree of delignification (runs 2, 4, 6, 7 – 13) more sugars were recovered from raw RS (53.3 – 59.7% for glucose and 46.4 – 56.8% for xylose). Although the experiments with high delignification lost more sugars, higher yields were obtained as enzymatic digestibility was improved. The glucose/xylose ratio in these experiments (2.6 ± 0.1) was substantially higher than the samples with NaOH concentration ≤0.15% w/v (1.6 ± 0.2). Solubilisation of hemicellulose during pretreatment occurred at low NaOH doses. However, compared with the results of the fractional factorial screening (Table 1), it was confirmed that 1% w/v NaOH would be enough to reach the maximum concentration of fermentable sugars (~65 g L^{-1}).

346 The hydrolysates were subsequently fermented and the butanol production was
347 evaluated. The low sugar content of run 5 made solvent production unfeasible as the bacteria
348 metabolism did not have enough carbon sources to assimilate the acids produced during the
349 acidogenic phase. In the rest of the samples, the ABE fermentation was not negatively
350 impacted by the potentially inhibitory compounds produced during pretreatment. Levulinic
351 acid and furfural were not detected in the hydrolysate and the concentrations of HMF (< 0.01
352 g L^{-1}), while total phenolic compounds (0.31 ± 0.08 g L^{-1}) and acetic acid (3.95 ± 0.80 g L^{-1})
353 were below the inhibitory threshold level. Interestingly, the use of 0.15% w/v NaOH (runs 1
354 and 3) provided the highest butanol conversion of the released sugars (0.20 g butanol g
355 released $sugars^{-1}$). Zhang et al. [30] reported that an acid insoluble lignin content of 8.55% in
356 alkaline-pretreated corn stover by twin-screw extrusion released up to 0.74 g L^{-1} of soluble
357 lignin compounds which inhibited ABE fermentation by *C. acetobutylicum* ATCC 824. In
358 contrast, our results indicate that the lower lignin removal rate during RS pretreatment does
359 not seem to have a negative impact on the Clostridia metabolism. The lack of by-product
360 inhibition in our work could be attributed to the differences in the lignocellulosic biomass
361 combined with the differences in the alkaline application. All the samples with a high lignin
362 removal rate (runs 2, 4, 6 – 13) from the abundant carbon source in the hydrolysates (>50 g L^{-1})
363 produced high butanol concentrations (8.1 – 10.3 g L^{-1}), although the higher sugar
364 concentration was not accompanied by higher sugar-to-butanol conversion. Although run 6
365 achieved the maximum sugar concentration (64.4 g L^{-1}), the sugar-to-butanol conversion was
366 the lowest (0.16 g butanol g released $sugars^{-1}$), due to the lower xylose uptake than in the
367 samples with sugar concentrations in the range of 51 – 57 g L^{-1}. This could be attributed to the
368 greater impact of the carbon catabolite repression phenomena for glucose levels of ~65 g L^{-1}
369 [10]. These results show the importance of assessing the effectiveness of the pretreatment

process by not only evaluating the amount of fermentable sugar released after hydrolysis but also the butanol conversion from fermentation.

The three-stage butanol-biomass ratio was therefore selected as the variable response for maximizing RS use. Regardless of the pretreatment reaction time, this ratio varied from 55.9 to 69.2 g kg untreated RS^{-1} when NaOH concentration was 0.5% w/v or higher (runs 2, 4, and 6 – 13), which is a considerable improvement on the previous fractional factorial screening (maximum value of 51.9 g kg untreated RS^{-1}, Table 1). The quadratic model obtained for the butanol-biomass ratio versus NaOH concentration (Z_1) and time (Z_2) is described by equation 4.

$$Butanol - biomass\ ratio = -2.9 + 212.8Z_1 - 0.00Z_2 - 132.5Z_1^2 + 0.0071Z_2^2 - 0.871Z_1Z_2 \quad (4)$$

The ANOVA and the coded regression coefficients of the quadratic model are summarized in Table 4. The results of the statistical analysis showed that the model was highly significant at the confidence levels (95%, p-value = 0.0004), whereas the lack-of-fit was not significant (p-value = 0.0777). The value of the coefficient of determination (R^2) was 0.9389, indicating that the experimental results had a good correlation with the predicted ones (Table 3), in which only 6.11% of the total variations were not explained by the fitted model. The value of the adjusted coefficient of determination (Adj. R^2: 0.8953) confirmed that equation 4 can adequately describe the effect of NaOH concentration and time on the butanol-biomass ratio obtained from RS after 72 h. The NaOH concentration had a much higher influence than time on the response. Both the linear (Z_1) and the quadratic (Z_1Z_1) coefficient were found to be significant with the same p-value (0.0002) but with opposite effects, while neither the linear (Z_2, p-value of 0.4584) or the quadratic (Z_2Z_2, p-value of 0.5725) coefficients of time were significant. The interaction effect between both factors was also found to be insignificant (Z_1Z_2, p-value of 0.2028).

393 The model was used to plot the three-dimensional response surface and the associated
394 two-dimensional contour to map the optimal combination of the evaluated factors (Figure 3a).
395 The butanol-biomass ratio increases as NaOH concentration rises from 0 to 0.5% w/v, until
396 reaching a saddle-shaped region. From 0.8% w/v NaOH, the response decreases as alkali
397 concentration increases. The contour plot shows the weak interaction between NaOH
398 concentration and time. Indeed, there are two opposing time values that led to the highest RS-
399 to-butanol conversion: ~20 to 25 min or ~55 to 60 min for very similar NaOH concentrations
400 (0.6 – 0.8 % w/v). Of these two zones, the shorter time is the best cost-wise option, since the
401 additional reagent is negligible relative to the extra time required. The model estimated the
402 maximum butanol-biomass ratio at 72 h of fermentation (71.9 g kg untreated RS^{-1}) with
403 0.75% w/v NaOH and 20 min pretreatment time.

404 The combined effect of NaOH concentration and time on solid recovery after
405 pretreatment (Figure 3b), sugars released in enzymatic hydrolysis (Figure 3c) and butanol
406 produced in fermentation (Figure 3d) were also plotted. Solid losses increase gradually with
407 NaOH concentration due to the higher solubilization of the biomass in harsh alkaline
408 conditions (Figure 3b). The amount of fermentable sugar in the hydrolysate predicted to
409 increase with NaOH concentration in the pretreatment rises to the optimal value (Figure 3c),
410 which was estimated by the model to be 61.7 g L^{-1} for an NaOH concentration of 0.88% w/v
411 (and a pretreatment time of 39 min). As expected, both contour plots show very flat profiles
412 versus time. The response surface plot for butanol production (Figure 3d) shows a saddle
413 shape similar to that of the butanol-biomass ratio (Figure 3a), with two optimal regions near
414 the minimum and maximum tested times ~20 – 23 min and ~55 – 60 min, but for higher
415 NaOH dose (~0.9% w/v). Indeed, the butanol production quadratic model predicted a
416 maximum butanol concentration of 10.6 g L^{-1} using 0.88% w/v NaOH and 21 min alkaline
417 pretreatment. The discrepancies in alkali severity between butanol titer and RS-to-butanol

conversion are attributed to the different extension of the solubilization of the structural components of the biomass during pretreatment. Other authors have also found that larger amounts of butanol and ABE can lead to lower mass balance efficiency [12,31]. Our results show the importance of selecting the right variable response during process optimization. The alkali dosage which maximizes fermentable sugars correlates well with the maximum butanol titer, but underestimates RS use by 13%. To improve the mass balance efficiency of ABE fermentation from the lignocellulosic biomass, the efficiency of all three pretreatment, saccarification and fermentation stages should be jointly considered when optimizing the operational parameters of the pretreatment process.

3.2.2 Model validation

The validation of the optimum value for the second-order model of the butanol-biomass ratio was carried out in triplicate using 5% w/v RS pretreated with 0.75% w/v NaOH at 134 °C for 20 min. The following profiles are depicted in Figure 4: pH, cell density, sugar concentration (glucose, xylose and arabinose), acid concentration (acetic and butyric acid) and solvent concentration (acetone and butanol, ethanol was not detected). A ~12 h lag phase was observed, after which the *Clostridium* culture started to grow by consuming glucose, indicating its adaptation to the nutritional environment. The exponential phase of cell growth was reached at ~24 h, although there was almost no acid accumulation or a notable change in pH shift in the first 24 hours.

The absence of a substantial drop in pH during sugar consumption (acidogenesis stage) was related to the initial amount of acetic acid from the RS hydrolysate (4.9 ± 0.2 g L^{-1}). The acetic acid enhanced the early development of a mixed population of solvent and acidogenic cells with the coupled production of acids and solvents [32,33]. In this case, the early production of butanol and acetone as an acid consumer step caused a slight increase in

pH from 24 to 33 h. Interestingly, 87% of the butanol (8.9 ± 0.1 g L^{-1}) and ABE solvents (14.5 ± 0.1 g L^{-1}, butanol:acetone mass ratio of 1.58) were produced in 48 h, accompanied by almost complete glucose depletion and about two-thirds of the xylose uptake, giving maximum butanol and ABE productivities of 0.185 ± 0.001 g L^{-1} h^{-1} and 0.302 ± 0.002 g L^{-1} h^{-1} respectively. Maximum butanol (10.1 ± 0.2 g L^{-1}) and ABE (16.7 ± 0.1 g L^{-1}, butanol:acetone mass ratio of 1.54) concentrations were obtained at the end of fermentation (72 h), with a butanol yield of 0.24 ± 0.01 g g^{-1} and ABE yield of 0.39 ± 0.01 g g^{-1}. In contrast to our results, production of similar butanol concentrations from non-detoxified NaOH-pretreated lignocellulosic biomass hydrolysates is related to an extensive fermentation time. For example, Cai et al. [34] achieved 9.4 g L^{-1} of butanol and 12.2 g L^{-1} of ABE from corn cob after 60 h of fermentation by *C. acetobutylicum* ABE 1301. Fernández-Delgado et al. [35] reported 11.3 g L^{-1} of butanol and 14.5 g L^{-1} of ABE after 96 h of fermentation from brewer's spent grain by *C. beijerinckii* DSM 6422. Only Gao and Rehmann [36] achieved high solvent titers (12.3 g L^{-1} of butanol and 19.4 g L^{-1} of ABE) in short fermentation times (36 h) with NaOH-pretreated corn cob using *C. saccharobutylicum* DSM 13864. Our results indicate that RS alkaline pretreatment without further detoxification can achieve high butanol production accompanied by a high productivity. These results are promising for a further scale-up, as butanol productivity and not butanol concentration is the variable response of interest for in-situ butanol recovery processes [37]. In these integrated processes enhancing butanol productivity is critical to improving the butanol removal rate [38].

A butanol-biomass ratio of 77.6 ± 1.1 g kg untreated RS^{-1} was achieved at the end of fermentation with a solid recovery after pretreamtent of 53.0 ± 0.1%. The observed value was slightly higher than the predicted one (71.9 g kg untreated RS^{-1}), validating the proposed model on butanol-biomass ratio. The discrepancy between the experimental and predicted values was 7.3%, which nearly matched the predicted deviation from the value of R^2 (6.1%),

and was even better than that predicted from Adj. R^2 (adjusted coefficient of determination, 11.5%). Valles et al. [13] pretreated the same waste by microwaves and obtained a butanol-biomass ratio one-third lower (51 g kg untreated RS^{-1}) due to biomass delignification being less efficient than alkaline pretreatment. Few studies to date have assessed the overall lignocellulosic biomass conversion to butanol. Neither are there any previous reports on using the global mass balance efficiency of the three sequential steps: pretreatment, hydrolysis and fermentation, as the variable response in optimization. The RS converted to butanol achieved in the present study was quite similar to that obtained by Amiri et al. [12], 80 g butanol kg untreated $RS^{-1}$with an ethanol organosolv pretreatment, while a higher value (96 g butanol kg untreated RS^{-1}) was reported by Chi et al. [39] with NaOH-pretreatment. However, the high butanol-biomass ratios of these studies were not accompanied by high butanol titers (about 5 – 6 g L^{-1}). Our butanol production was nearly double and also achieved higher maximum productivity. It is important to note that the butanol stripping rate increases when the butanol titer in the reactor increases due to the enhanced butanol mass transfer associated with its higher driving force [40]. The promising mass balance efficiency, butanol titer and productivity achieved here with alkaline-pretreated RS shows promise for further research on reducing the cost by combining several stages in a single reactor (simultaneous saccharification and fermentation; simultaneous saccharification, fermentation and an *in-situ* recovery process).

4 Conclusions

Pretreating rice straw with NaOH was optimized to enhance rice straw conversion to butanol in batch ABE fermentation. Using the butanol-biomass ratio as the response variable has been identified as the key decision, since maximizing the sugar release without taking fermentation into account leads to underestimating RS use. By selecting the optimal conditions, the RS-to-butanol conversion was 77.6 g kg untreated RS^{-1} and a butanol titer as high as 10.1 g L^{-1} was

492 achieved. In future studies, simultaneous saccharification and fermentation coupled with
493 integrated recovery process will be carried out to increase the butanol productivity and final
494 titer.

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500

501 **Supplementary material**

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

503 **6 References**

504 [1] European Commission, A new Circular Economy Action Plan For a cleaner and more
505 competitive Europe, (2020) COM/2020/98.

506 [2] N. Van Hung, M.C. Maguyon-Detras, M.V. Migo, R. Quilloy, C. Balingbing, P.
507 Chivenge, M. Gummert, Rice Straw Overview: Availability, Properties, and
508 Management Practices, in: Sustain. Rice Straw Manag., Springer International
509 Publishing, 2020: pp. 1–13. https://doi.org/10.1007/978-3-030-32373-8_1.

510 [3] T. Schubert, Production routes of advanced renewable C1 to C4 alcohols as biofuel
511 components – a review, Biofuels, Bioprod. Biorefining. 14 (2020) 845–878.
512 https://doi.org/10.1002/bbb.2109.

513 [4] D.T. Jones, D.R. Woods, Acetone-butanol fermentation revisited, Microbiol. Rev. 50

(1986) 484–524.

[5] C.A. Vees, C.S. Neuendorf, S. Pflügl, Towards continuous industrial bioprocessing with solventogenic and acetogenic clostridia: challenges, progress and perspectives, Springer International Publishing, 2020. https://doi.org/10.1007/s10295-020-02296-2.

[6] S. Maiti, G. Gallastegui, G. Suresh, S.J. Sarma, S.K. Brar, P. Drogui, Y. LeBihan, G. Buelna, M. Verma, C.R. Soccol, Hydrolytic pre-treatment methods for enhanced biobutanol production from agro-industrial wastes, Bioresour. Technol. (2018). https://doi.org/10.1016/j.biortech.2017.09.132.

[7] V. García, J. Päkkilä, H. Ojamo, E. Muurinen, R.L. Keiski, Challenges in biobutanol production: How to improve the efficiency?, Renew. Sustain. Energy Rev. 15 (2011) 964–980. https://doi.org/10.1016/j.rser.2010.11.008.

[8] F. Moradi, H. Amiri, S. Soleimanian-Zad, M.R. Ehsani, K. Karimi, Improvement of acetone, butanol and ethanol production from rice straw by acid and alkaline pretreatments, Fuel. 112 (2013) 8–13. https://doi.org/10.1016/j.fuel.2013.05.011.

[9] S. Zhu, Y. Wu, Z. Yu, J. Liao, Y. Zhang, Pretreatment by microwave/alkali of rice straw and its enzymic hydrolysis, Process Biochem. 40 (2005) 3082–3086. https://doi.org/10.1016/j.procbio.2005.03.016.

[10] T. Zhao, Y. Tashiro, J. Zheng, K. Sakai, K. Sonomoto, Semi-hydrolysis with low enzyme loading leads to highly effective butanol fermentation, Bioresour. Technol. 264 (2018) 335–342. https://doi.org/10.1016/j.biortech.2018.05.056.

[11] A. Ranjan, V.S. Moholkar, Comparative study of various pretreatment techniques for rice straw saccharification for the production of alcoholic biofuels, Fuel. 112 (2013) 567–571. https://doi.org/10.1016/j.fuel.2011.03.030.

[12] H. Amiri, K. Karimi, H. Zilouei, Organosolv pretreatment of rice straw for efficient acetone, butanol, and ethanol production, Bioresour. Technol. 152 (2014) 450–456. https://doi.org/10.1016/j.biortech.2013.11.038.

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

[14] N. Vivek, L.M. Nair, B. Mohan, S.C. Nair, R. Sindhu, A. Pandey, N. Shurpali, P. Binod, Bio-butanol production from rice straw – Recent trends, possibilities, and challenges, Bioresour. Technol. Reports. 7 (2019) 100224. https://doi.org/10.1016/j.biteb.2019.100224.

[15] S.M. Hosseini, H.A. Aziz, A. Mojiri, Enhancement of Rice Straw Biodegradability by Alkaline and Acid Thermochemical Pretreatment Process: Optimization by Response Surface Methodology (RSM), Casp. J. Appl. Sci. Res. 1 (2012) 8–24.

[16] I. Kim, J.-I. Han, Optimization of alkaline pretreatment conditions for enhancing glucose yield of rice straw by response surface methodology, Biomass and Bioenergy. 46 (2012) 210–217. https://doi.org/10.1016/j.biombioe.2012.08.024.

[17] A. Mukherjee, S. Banerjee, G. Halder, Parametric optimization of delignification of rice straw through central composite design approach towards application in grafting, J. Adv. Res. 14 (2018) 11–23. https://doi.org/10.1016/j.jare.2018.05.004.

[18] S. Imman, J. Arnthong, V. Burapatana, V. Champreda, N. Laosiripojana, Influence of alkaline catalyst addition on compressed liquid hot water pretreatment of rice straw, Chem. Eng. J. 278 (2015) 85–91. https://doi.org/10.1016/j.cej.2014.12.032.

560 [19] P. Bajpai, Pretreatment of Lignocellulosic Biomass, in: Pretreat. Lignocellul. Biomass
561 Biofuel Prod., Springer, Singapore, 2016: pp. 17–70. https://doi.org/10.1007/978-981-
562 10-0687-6.

563 [20] R. Singh, S. Tiwari, M. Srivastava, A. Shukla, Microwave Assisted Alkali Pretreatment
564 of Rice Straw for Enhancing Enzymatic Digestibility, J. Energy. 2014 (2014) 1–7.
565 https://doi.org/10.1155/2014/483813.

566 [21] C. Ververis, K. Georghiou, D. Danielidis, D.G. Hatzinikolaou, P. Santas, R. Santas, V.
567 Corleti, Cellulose, hemicelluloses, lignin and ash content of some organic materials and
568 their suitability for use as paper pulp supplements, Bioresour. Technol. 98 (2007) 296–
569 301. https://doi.org/10.1016/j.biortech.2006.01.007.

570 [22] J.J. MacAskill, I.D. Suckling, J.A. Lloyd, M. Manley-Harris, Unravelling the effect of
571 pretreatment severity on the balance of cellulose accessibility and substrate
572 composition on enzymatic digestibility of steam-pretreated softwood, Biomass and
573 Bioenergy. 109 (2018) 284–290. https://doi.org/10.1016/j.biombioe.2017.12.018.

574 [23] a. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker,
575 NREL/TP-510-42618 analytical procedure - Determination of structural carbohydrates
576 and lignin in Biomass, Lab. Anal. Proced. (2012) 17.
577 http://www.nrel.gov/docs/gen/fy13/42618.pdf.

578 [24] O. Folin, W. Denis, On phosphotungstic-phosphomolybdic compounds as color
579 reagents, J. Biol. Chem. 12 (1912) 239–243.

580 [25] T.Q. Lan, H. Lou, J.Y. Zhu, Enzymatic saccharification of lignocelluloses should be
581 conducted at elevated pH 5.2-6.2, Bioenergy Res. 6 (2013) 476–485.
582 https://doi.org/10.1007/s12155-012-9273-4.

583 [26] F. Kong, C.R. Engler, E.J. Soltes, Effects of cell-wall acetate, xylan backbone, and
584 lignin on enzymatic hydrolysis of aspen wood, Appl. Biochem. Biotechnol. 34 (1992)
585 23–35. https://doi.org/10.1007/BF02920531.

586 [27] J.C. López-Linares, M.T. García-Cubero, S. Lucas, G. González-Benito, M. Coca,
587 Microwave assisted hydrothermal as greener pretreatment of brewer's spent grains for
588 biobutanol production, Chem. Eng. J. 368 (2019) 1045–1055.
589 https://doi.org/10.1016/j.cej.2019.03.032.

590 [28] L.J. Jönsson, B. Alriksson, N.-O. Nilvebrant, Bioconversion of lignocellulose:
591 inhibitors and detoxification, Biotechnol. Biofuels. 6 (2013) 16.
592 https://doi.org/10.1186/1754-6834-6-16.

593 [29] C. Bellido, C. Infante, M. Coca, G. González-Benito, S. Lucas, M.T. García-Cubero,
594 Efficient acetone-butanol-ethanol production by Clostridium beijerinckii from sugar
595 beet pulp, Bioresour. Technol. 190 (2015) 332–338.
596 https://doi.org/10.1016/j.biortech.2015.04.082.

597 [30] Y. Zhang, T. Hou, B. Li, C. Liu, X. Mu, H. Wang, Acetone-butanol-ethanol production
598 from corn stover pretreated by alkaline twin-screw extrusion pretreatment, Bioprocess
599 Biosyst. Eng. 37 (2014) 913–921. https://doi.org/10.1007/s00449-013-1063-7.

600 [31] J.C. López-Linares, M.T. García-Cubero, S. Lucas, M. Coca, Integral valorization of
601 cellulosic and hemicellulosic sugars for biobutanol production: ABE fermentation of
602 the whole slurry from microwave pretreated brewer's spent grain, Biomass and
603 Bioenergy. 135 (2020) 105524. https://doi.org/10.1016/j.biombioe.2020.105524.

604 [32] I.S. Maddox, E. Steiner, S. Hirsch, S. Wessner, N.A. Gutierrez, J.R. Gapes, K.C.
605 Schuster, The cause of "acid crash" and "acidogenic fermentations" during the batch

606 acetone-butanol-ethanol (ABE-) fermentation process, J. Mol. Microbiol. Biotechnol. 2
607 (2000) 95–100.

608 [33] W.H. Chen, Y.C. Chen, J.G. Lin, Evaluation of biobutanol production from non-
609 pretreated rice straw hydrolysate under non-sterile environmental conditions,
610 Bioresour. Technol. 135 (2013) 262–268.
611 https://doi.org/10.1016/j.biortech.2012.10.140.

612 [34] D. Cai, P. Li, Z. Luo, P. Qin, C. Chen, Y. Wang, Z. Wang, T. Tan, Effect of dilute
613 alkaline pretreatment on the conversion of different parts of corn stalk to fermentable
614 sugars and its application in acetone-butanol-ethanol fermentation, Bioresour. Technol.
615 211 (2016) 117–124. https://doi.org/10.1016/j.biortech.2016.03.076.

616 [35] M. Fernández-Delgado, P.E. Plaza, M. Coca, M.T. García-Cubero, G. González-
617 Benito, S. Lucas, Comparison of mild alkaline and oxidative pretreatment methods for
618 biobutanol production from brewer's spent grains, Ind. Crops Prod. 130 (2019) 409–
619 419. https://doi.org/10.1016/j.indcrop.2018.12.087.

620 [36] K. Gao, L. Rehmann, ABE fermentation from enzymatic hydrolysate of NaOH-
621 pretreated corncobs, Biomass and Bioenergy. 66 (2014) 110–115.
622 https://doi.org/10.1016/j.biombioe.2014.03.002.

623 [37] T.C. Ezeji, N. Qureshi, H.P. Blaschek, Bioproduction of butanol from biomass: from
624 genes to bioreactors, Curr. Opin. Biotechnol. 18 (2007) 220–227.
625 https://doi.org/10.1016/j.copbio.2007.04.002.

626 [38] C. Moon, C.H. Lee, B.-I. Sang, Y. Um, Optimization of medium compositions favoring
627 butanol and 1,3-propanediol production from glycerol by Clostridium pasteurianum,
628 Bioresour. Technol. 102 (2011) 10561–10568.

https://doi.org/10.1016/j.biortech.2011.08.094.

[39] X. Chi, J. Li, S.-Y. Leu, X. Wang, Y. Zhang, Y. Wang, Features of a Staged Acidogenic/Solventogenic Fermentation Process to Improve Butanol Production from Rice Straw, Energy and Fuels. 33 (2019) 1123–1132. https://doi.org/10.1021/acs.energyfuels.8b03095.

[40] C. Xue, G.-Q. Du, J.-X. Sun, L.-J. Chen, S.-S. Gao, M.-L. Yu, S.-T. Yang, F.-W. Bai, Characterization of gas stripping and its integration with acetone-butanol-ethanol fermentation for high-efficient butanol production and recovery, Biochem. Eng. J. 83 (2014) 55–61. https://doi.org/10.1016/j.bej.2013.12.003.

Table 1. 2^{4-1} fractional factorial design of 4 variables.

Independent variables		Coded and real values	
		Level -1	Level +1
X_1	Temperature (°C)[a]	121	134
X_2	Time (min)	10	40
X_3	NaOH concentration (% w/v)	0.2	2.0
X_4	Solid loading (% w/v)[a]	5	10

[a] Categorical

Table 2. 2^{4-1} fractional factorial design matrix along with the values of SF, solid recovery (%), released sugars after 72 h-enzymatic hydrolysis (g L^{-1}), butanol production (g L^{-1}) and butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h of ABE fermentation.

Run	Real values				SF	Solid recovery	Released sugars	Butanol	Butanol-biomass ratio
	X_1[a]	X_2[b]	X_3[c]	X_4[d]		(%)	(g L^{-1})	(g L^{-1})	(g kg untreated RS^{-1})
1	121	10	0.2	5	1.62	78.4	17.3	2.8	24.7
2	134	10	0.2	10	2.00	83.4	10.4	1.7	16.4
3	121	40	0.2	10	2.22	85.7	4.9	0.3	2.5
4	134	40	0.2	5	2.60	72.3	33.4	5.2	42.9
5	121	10	2	10	1.62	55.4	43.8	4.6	29.3
6	134	10	2	5	2.00	44.0	61.2	4.4	22.2
7	121	40	2	5	2.22	43.0	65.3	10.6	51.9
8	134	40	2	10	2.60	48.6	46.1	2.1	11.4

[a] X_1: temperature (°C); [b] X_2: time (min); [c] X_3: NaOH concentration (% w/v); [d] X_4: solid loading (% w/v).

Table 3. CCD experimental matrix along with the values of solid recovery (%), released sugars (g L^{-1}) and glucose and xylose yield (%, referred to untreated RS) after 72 h-enzymatic hydrolysis, butanol production (g L^{-1}) at 72 h and the observed and predicted values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Run	Coded values		Real values		Solid recovery (%)	Released sugars (g L^{-1})	Glucose yield (%)	Xylose yield (%)	Butanol (g L^{-1})	Butanol-biomass ratio (g kg untreated RS^{-1})	
	Z_1[a]	Z_2[b]	Z_1[a]	Z_2[b]						Observed	Predicted
1	-1	-1	0.15	26	71.8	17.0	23.0	32.9	3.3	34.2	27.4
2	+1	-1	0.86	26	44.4	56.9	55.6	50.4	10.3	66.0	67.4
3	-1	+1	0.15	54	68.4	24.4	31.7	43.7	4.8	46.9	39.5
4	+1	+1	0.86	54	41.7	57.3	53.3	46.4	10.2	61.3	62.1
5	-α[c]	0	0.00	40	75.9	8.6	11.6	20.7	0.0	0.0	8.9
6	+α[c]	0	1.01	40	37.9	64.4	54.9	46.7	10.2	55.9	53.1
7	0	-α[c]	0.50	20	51.4	51.1	55.7	56.8	8.4	62.1	64.7
8	0	+α[c]	0.50	60	47.6	54.8	57.7	51.8	9.6	66.1	69.6
9	0	0	0.50	40	50.6	50.8	56.5	52.8	8.1	58.9	64.4
10	0	0	0.50	40	50.6	53.6	59.7	54.0	9.5	69.2	64.4
11	0	0	0.50	40	50.6	51.1	56.7	53.3	8.7	63.6	64.4
12	0	0	0.50	40	50.5	53.1	58.2	55.3	9.0	65.6	64.4
13	0	0	0.50	40	50.6	51.1	56.0	53.7	8.9	64.7	64.4

[a] Z_1: NaOH concentration (% w/v); [b] Z_2: time (min); [c] $\alpha = 1.4142$.

Table 4. ANOVA of the CCD model for butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h.

Source	Degrees of freedom	Sum of squares	Mean square	*F* value	*p*-value Prob > F	Coefficient[a]
Model	5	4088.61	817.72	21.52	0.0004	
Linear	2	1985.18	992.59	26.12	0.0006	
Z_1: NaOH concentration	1	1961.79	1961.79	51.63	0.0002	15.66
Z_2: Time	1	23.39	23.39	0.62	0.4584	1.71
Square	2	2028.43	1014.21	26.69	0.0005	
Z_1Z_1	1	1938.68	1938.68	51.02	0.0002	-16.69
Z_2Z_2	1	13.32	13.32	0.35	0.5725	1.38
2-way interactions	1	75.00	75.00	1.97	0.2028	
Z_1Z_2	1	75.00	75.00	1.97	0.2028	-4.33
Error	7	265.98	38.00			
Lack-of-fit	3	209.72	69.91	4.97	0.0777	
Pure error	4	56.26	14.07			
Total	12	4354.58				
Standard Deviation, S					6.1642	
R^2					0.9389	
Adj. R^2					0.8953	

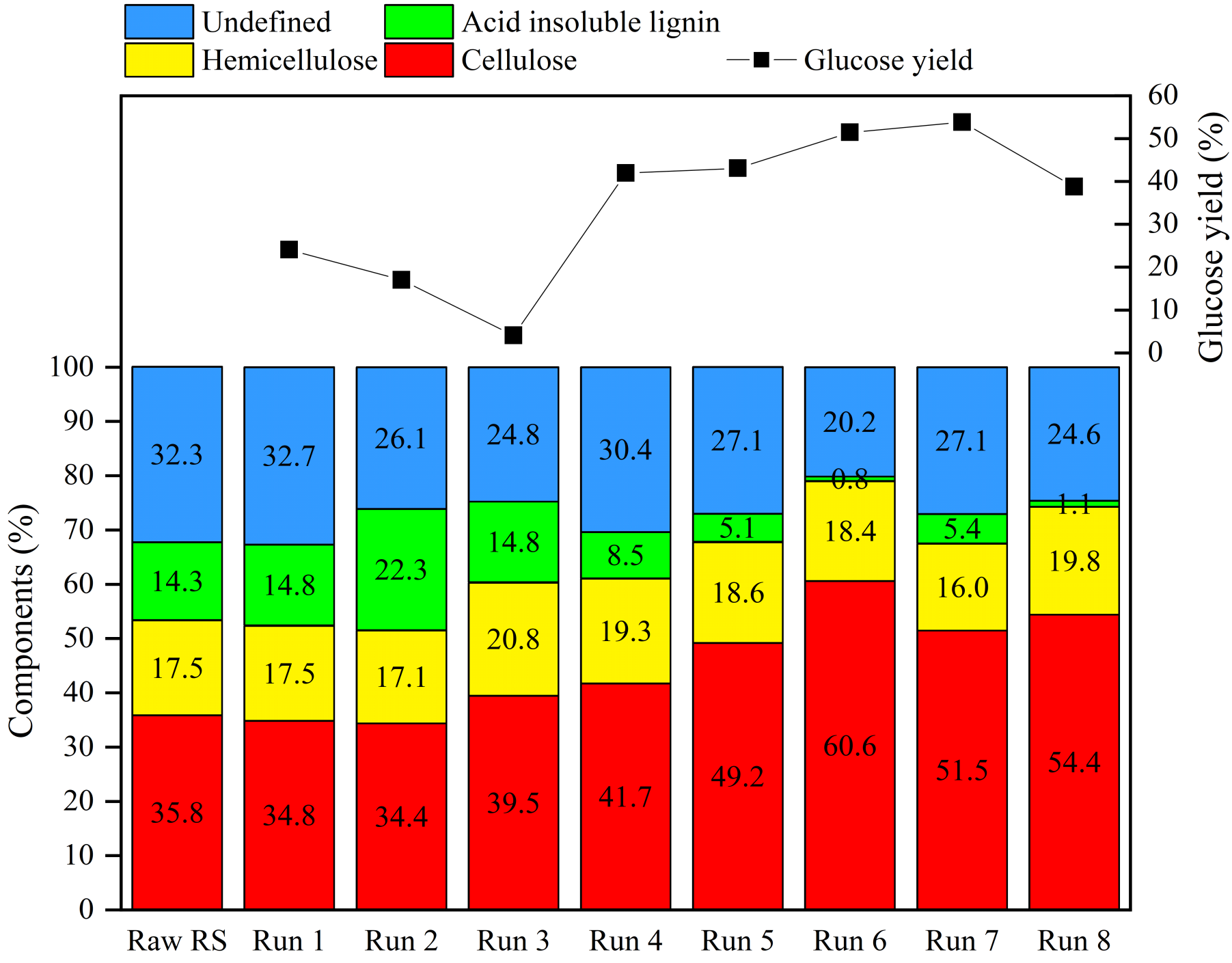
[a] For coded variables.

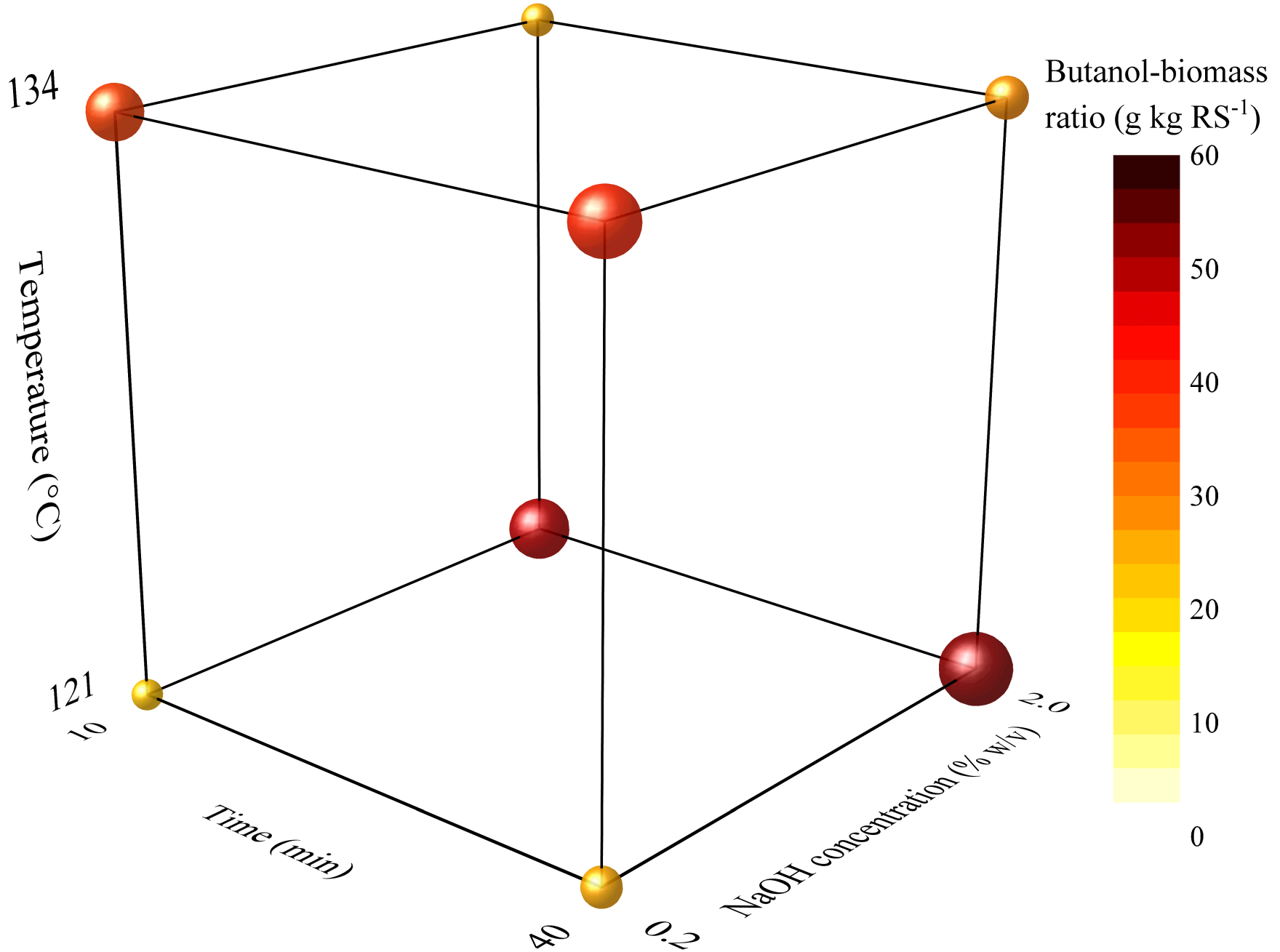
Figure 1. Chemical composition of raw RS and RS after the different pretreatments included in the 2^{4-1} fractional factorial design with glucose yield (%, referred to untreated RS) after 72 h of enzymatic hydrolysis.

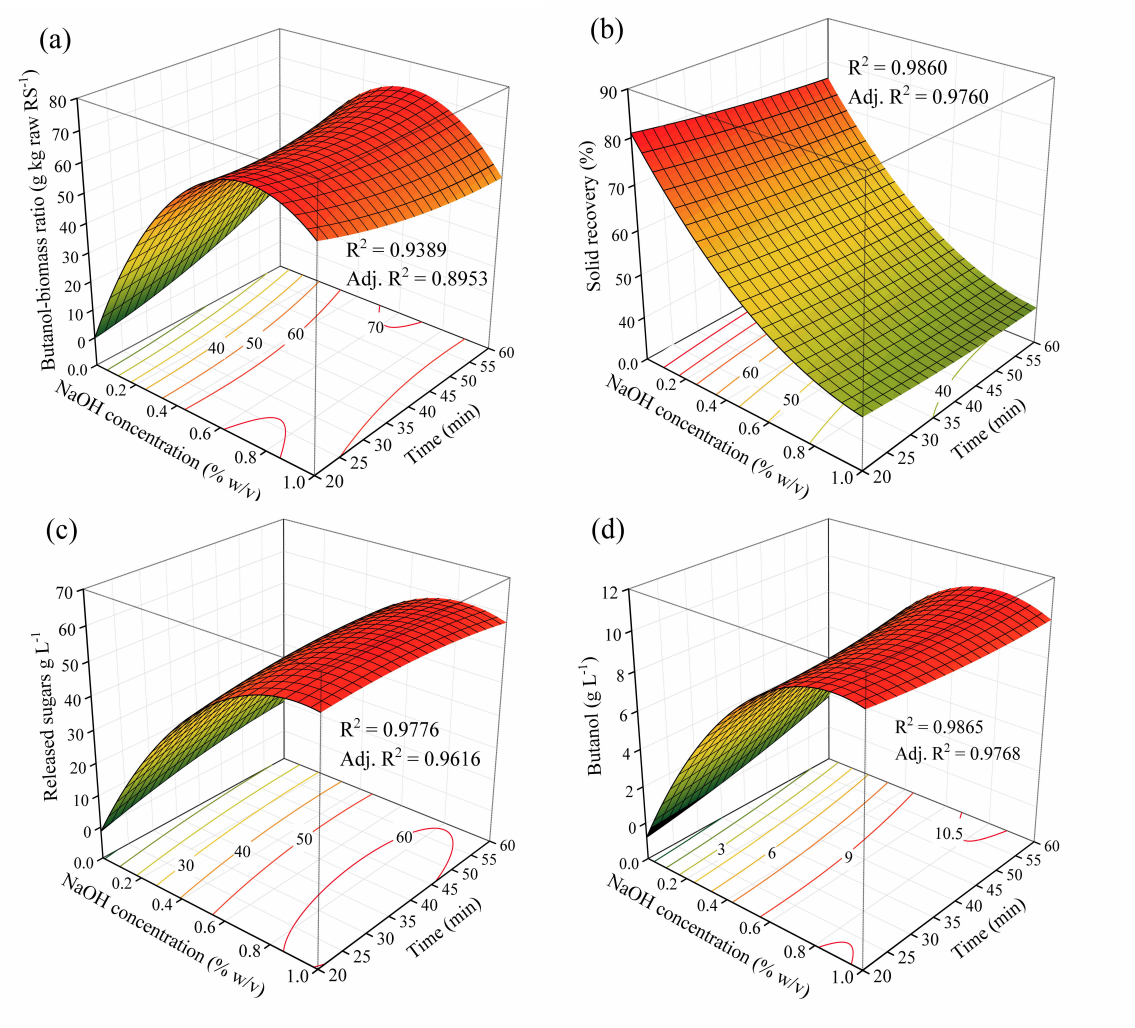
Figure 2. 2^{4-1} fractional factorial cube plot using predicted model values of butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h. Solid loading = 5 % w/v.

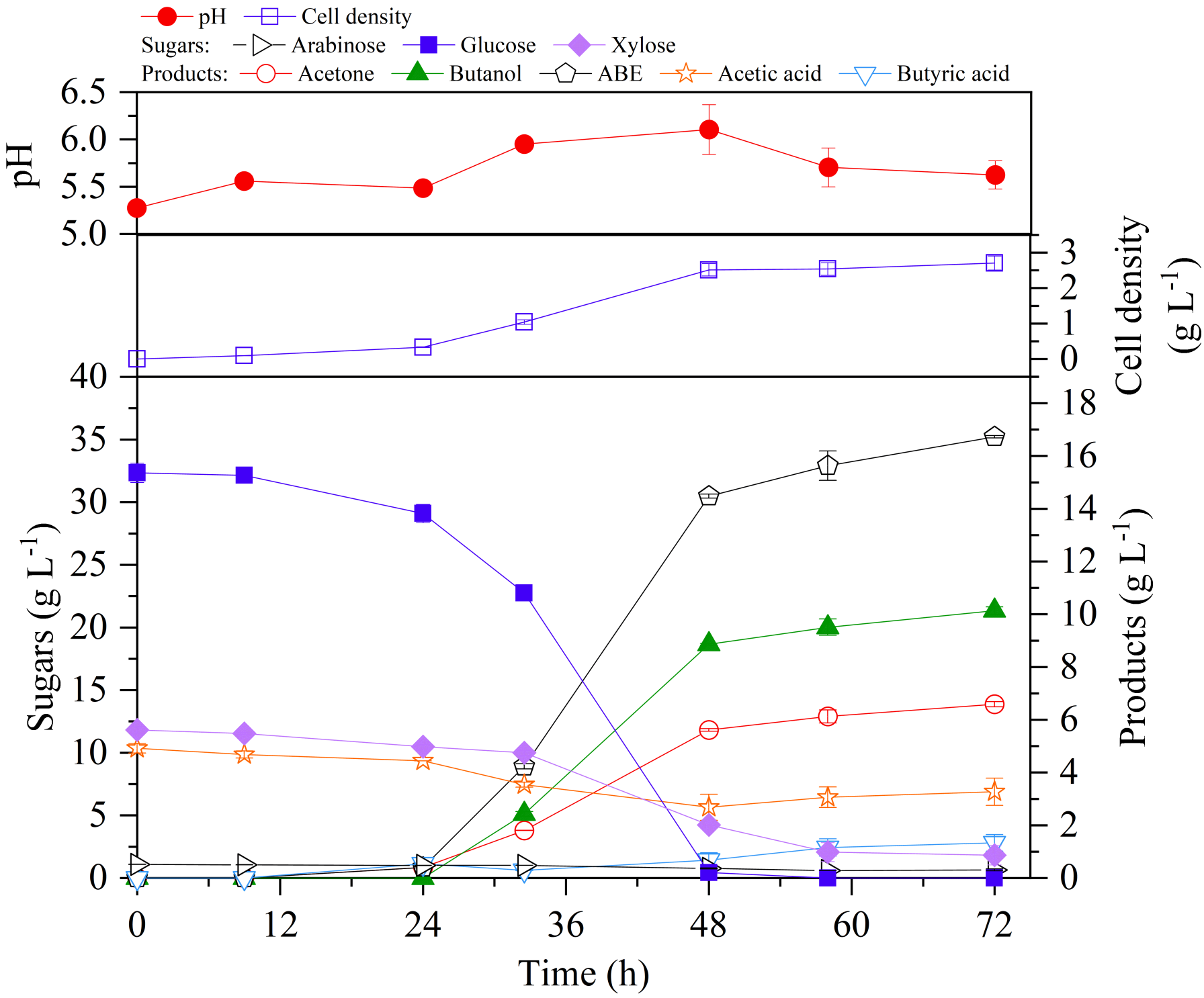
Figure 3. Response surface and corresponding contour plot for (a) butanol-biomass ratio (g kg untreated RS^{-1}) at 72 h, (b) solid recovery (%), (c) released sugars (g L^{-1}) after 72 h-enzymatic hydrolysis and (d) butanol production (g L^{-1}) at 72 h: combined effect of NaOH concentration (% w/v) and time (min).

Figure 4. CCD model validation at the predicted optimum conditions.


Undefined
Acid insoluble lignin
Hemicellulose
Cellulose
Glucose yield
Components (%)
Glucose yield (%)
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
Raw RS
Run 1
Run 2
Run 3
Run 4
Run 5
Run 6
Run 7
Run 8
32.3
14.3
17.5
35.8
32.7
14.8
17.5
34.8
26.1
22.3
17.1
34.4
24.8
14.8
20.8
39.5
30.4
8.5
19.3
41.7
27.1
5.1
18.6
49.2
20.2
0.8
18.4
60.6
27.1
5.4
16.0
51.5
24.6
1.1
19.8
54.4


Butanol-biomass ratio (g kg RS-1)
60
50
40
30
20
10
0
Temperature (°C)
134
121
Time (min)
10
40
NaOH concentration (% w/v)
0.2
2.0


(a)
Butanol-biomass ratio (g kg raw RS^{-1})
R^2 = 0.9389
Adj. R^2 = 0.8953
NaOH concentration (% w/v)
Time (min)
(b)
Solid recovery (%)
R^2 = 0.9860
Adj. R^2 = 0.9760
NaOH concentration (% w/v)
Time (min)
(c)
Released sugars g L^{-1}
R^2 = 0.9776
Adj. R^2 = 0.9616
NaOH concentration (% w/v)
Time (min)
(d)
Butanol (g L^{-1})
R^2 = 0.9865
Adj. R^2 = 0.9768
NaOH concentration (% w/v)
Time (min)


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