Comparison of simultaneous saccharification and fermentation and separate hydrolysis and fermentation processes for butanol production from rice straw

Alejo Valles, F. Javier Álvarez-Hornos, Vicente Martínez-Soria, Paula Marzal, Carmen Gabaldón*

*Address correspondence to Carmen Gabaldón, carmen.gabaldon@uv.es

Research Group GI2AM, Department of Chemical Engineering, Universitat de València, Av. De la Universitat S/N, 46100, Burjassot, Spain.

Phone: +34 96 354 34 37

22 **Abstract**

23 Rice straw (RS) is one of the lignocellulosic wastes with the highest global production. The
24 main objective of this study was to maximise the butanol production by *Clostridium beijerinckii*
25 DSM 6422 from RS pretreated by microwave-assisted hydrothermolysis. Two different
26 fermentation strategies were compared: separate hydrolysis and fermentation (SHF, two-step
27 process) and simultaneous saccharification and fermentation (SSF, one-step process). In
28 parallel, the variables that significantly affected the butanol production were screened by using
29 fractional factorial designs. Butanol concentration and productivity at 48 h were, respectively,
30 8% and 173% higher in SSF than in SHF. A one-step process was more efficient than a two-
31 step process, especially considering the time savings derived from much higher productivity.
32 From these results, SSF was further optimised by response surface methodology with central
33 composite design over the key factors on the butanol production at 48 h: initial pH, enzyme
34 loading and yeast extract concentration. The optimum point yielded a butanol productivity of
35 0.114 g L^{-1} h^{-1}, with a butanol-biomass ratio of 51 g kg^{-1} of raw RS (ABE-biomass ratio of 77.0
36 g kg^{-1} of raw RS). The parameter with the greatest effect was enzyme loading, with an optimal
37 value of 13.5 FPU g-dw^{-1}. This study showed that microwave-processed RS has great potential
38 as a substrate for the butanol production from ABE fermentation when combining process
39 stages by SSF.

40

41 **Keywords:** Butanol, lignocellulosic waste, microwave thermohydrolysis, rice straw,
42 simultaneous saccharification/fermentation

43

44 ## 1 Introduction

45 The expected increase of the world population by more than 30% in the next 40 years, the
46 depletion of resources, external energy dependence and climate change are altering the way in
47 which biological resources in Europe are managed. In this context, interest in biomass as a
48 source of carbon and energy has increased [1]. Lignocellulosic material is the most abundant
49 and economical biomass on the planet [2]. Numerous raw materials, such as agricultural
50 residues, forestry wastes, industrial and municipal wastes, and bioenergy crops, are available
51 for the production of biofuels, including biobutanol [3]. However, a pretreatment is necessary
52 to alter the lignocellulosic structure and to remove and/or alter lignin, generally followed by an
53 enzymatic or acid hydrolysis stage to obtain sugar monomers [4].

54 Biobutanol is mainly produced by *Clostridium acetobutylicum* or *C. beijerinckii* in
55 acetone-butanol-ethanol (ABE) fermentation by a pathway consisting of two metabolic phases:
56 acidogenesis, followed by solventogenesis [5]. During acidogenesis bacterial growth occurs
57 with the production of acids, hydrogen and carbon dioxide; whereas in the solventogenesis stage
58 the production of solvents and endospore formation occurs [6]. These gram-positive and
59 anaerobic bacteria belong to the only genus capable of producing this solvent as a major
60 metabolite [7]. Butanol has some benefits as a fuel in terms of energy density, handling,
61 transport and storage [8]. Despite these advantages, its production by fermentation cannot
62 compete economically with the butanol obtained in the petrochemical industry due to, among
63 other causes, lower development of bioprocesses, long fermentation times, high cost of the
64 substrate, low yields and high cost of product recovery [9]. Strategies developed to enhance
65 cellulosic biobutanol production include strain improvement by genetic engineering,
66 optimisation of the medium formulation and combination of ABE fermentation stages [4]. To
67 screen and optimise the effect of medium conditions and process parameters on ABE

68 productivity, statistical techniques such as fractional factorial design and response surface
69 methodology (RSM) are often used [10].

70 The processes derived from the combination of ABE fermentation stages are
71 simultaneous saccharification and fermentation (SSF), consolidated bioprocessing (CBP),
72 separate hydrolysis and fermentation with *in-situ* recovery (SHFR) and simultaneous
73 saccharification and fermentation with *in-situ* recovery (SSFR) [4]. SSF was developed by
74 Gauss et al. [11] and combines enzymatic hydrolysis and ABE fermentation in one step,
75 increasing the butanol yield and productivity compared to separate hydrolysis and fermentation
76 (SHF). SSF could potentially reduce operational costs and the risk of contamination. In
77 addition, the SSF process minimises glucose inhibition on cellulases and β-glucosidase because
78 bacteria consume sugars as soon as they are released [4]. For example, Qi et al. [12] observed
79 that butanol production was higher in SSF (12.64 g L^{-1}) than in SHF (11.25 g L^{-1}) by fermenting
80 ammonium sulfite-pretreated wheat straw with *C. acetobutylicum* ATCC 824, despite
81 decreasing the biomass loading from 10.5 to 9% (w/v). Not only was SSF more efficient in
82 terms of butanol production and time, but enzyme loading was reduced by one-half, thereby
83 conferring an economic advantage. However, Shao and Chen [13] obtained a shorter
84 fermentation time and a higher butanol concentration by the same bacterial strain from
85 *Amorphophallus* konjac waste in SHF, suggesting that the most appropriate process depends
86 on factors such as the feedstock type and the strain of bacteria used.

87 One of the most abundant lignocellulosic wastes in the world is rice straw (RS), with an
88 estimated annual production of 731 million tons [14]. Unlike other straws, RS is not generally
89 used as animal feed due to its low digestibility and, apparently, it has a low value for social
90 benefit, so it is burnt openly in the field, causing air pollution [15]. There are numerous reported
91 pretreatments (physical, chemical, physicochemical and biological) to enhance ABE
92 fermentation of RS [16]. Despite the low lignin content in RS [17], these methods must face

93 other limiting factors, such as the presence of accumulated silica [18] and high cellulose
94 crystallinity [19]. Among these pretreatment options, dielectric heating by microwave
95 irradiation is used on lignocellulose as an alternative to convection heating [20]. Indeed, Ma et
96 al. [21] noticed that microwave pretreatment could improve the enzymatic accessibility of
97 cellulose by partially breaking the lignin-hemicellulose structure and the waxy structure of
98 silicon, increasing solubility. Furthermore, Zhu et al. [19] determined that, compared to the
99 alkali-alone process, microwave-assisted alkali pretreatment eliminates more hemicellulose and
100 lignin from RS, consequently obtaining a hydrolysate with more glucose and less xylose after
101 enzymatic hydrolysis. One of the limitations for the production of biobutanol is the generation
102 during pretreatment of compounds that inhibit microbial growth, such as acetic acid, 5-
103 hydroximethylfurfural (HMF) and furfural [16]. After pretreating RS with dilute acid, Hsu et
104 al. [17] observed a correlation between the generation of these compounds and pretreatment
105 severity. Indeed, Fonseca et al. [22] demonstrated that detoxification of rice improved the
106 ethanol productivity from RS hydrolysate with dilute acid. Another alternative to overcome
107 the toxicity derived from chemical pretreatment is the use of non-catalysed methods such as
108 microwave irradiation. This strategy can avoid problems of inhibition by these compounds,
109 saving at the same time the cost derived from chemicals. Although SSF processes have been
110 reported for butanol production by ABE fermentation using other agricultural waste such as
111 wheat straw [8,12,23], corn stover [24] or corncob [25], among others, there is no literature data
112 on the effect of using SSF to produce butanol from RS.

113 The scope of this work is to evaluate the SSF process for butanol production by *C.*
114 *beijerinckii* DSM 6422 from RS previously treated by microwave-assisted hydrothermolysis.
115 SSF configuration was compared with SHF in terms of butanol productivity by evaluating the
116 effect of the following parameters: type of buffer (citrate or acetate) and enzyme loading for
117 enzymatic hydrolysis; and initial pH, yeast extract concentration and iron concentration in the

118 fermentation broth in two sets of fractional factorial design experiments. In a later stage, SSF
119 was further optimised using RSM with central composite design (CCD) over variables with
120 statistically significant effects.

121 **2 Materials and methods**

122 2.1 Materials

123 RS was obtained from local farmers of L'Albufera located near Valencia (Spain). The
124 biomass was dried for 24 h at room temperature, cut into fragments of ~2 cm and milled. Particle
125 size between 100 and 500 μm was selected by ISO-3310.1 sieve (CISA, Spain), afterwards it
126 was dried in an oven at 45 ºC until the residual moisture content was less than 5% (w/w), and
127 it was then stored for further use. The commercial enzyme blend Cellic® CTec2 (Novozyme,
128 Denmark) was employed for hydrolysis of the pretreated RS. The cellulase activity of the
129 enzyme was measured according to the method of the National Renewable Energy Laboratory
130 (NREL) [26], resulting in a value of 119 filter paper units (FPU) mL^{-1}.

131 2.2 RS pretreatment

132 Microwave-assisted hydrothermal hydrolysis was performed in an ETHOS One
133 microwave digestion system (Milestone, Italy). The microwave had a maximum power of 1500
134 W and was controlled via a microprocessor with a capacity of 10 TFM vessels (an internal
135 temperature sensor was installed in a reference vessel). The RS was pretreated at 10% (w/v)
136 using 3 g of dry biomass in 30 mL of deionized water. The microwave was heated using the
137 following ramp of temperature: an initial increase to 100 ºC at a rate of 15 ºC min^{-1}, which was
138 then increased at 6 ºC min^{-1} until 160 ºC and then to 4 ºC min^{-1} until 200 ºC, holding at 15 min
139 [27]. Once the heating was finished, the vessels were cooled at room temperature. The slurry
140 was centrifuged at 10000 rpm for 5 min (centrifuge 5804, Eppendorf, Germany), and the solid

141 phase was washed with deionized water and pH was adjusted to 6.5. Finally, the pretreated RS
142 was dried at 45 ºC.

143 2.3 Microorganism and inoculum preparation

144 The bacterial strain *Clostridium beijerinckii* DSM 6422 (NRRL B-592) was supplied by
145 the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
146 (Braunschweig, Germany). The strain was stored at -80 ºC in a Reinforced Clostridial Medium
147 (RCM) with 20% (v/v) glycerol. Before fermentation, the cells were grown in 50-mL serum
148 bottles containing 40 mL of modified RCM (19 g L^{-1} RCM supplemented with 10 g L^{-1} glucose)
149 under anaerobic conditions by sparging pure nitrogen in the medium. The inoculum was
150 statically incubated at 37 ºC for 24 h. The media used in the cryopreservation and the inoculum
151 preparation were sterilized in an autoclave for 21 min at 121 ºC.

152 2.4 ABE fermentation

153 2.4.1 ABE fermentation by SHF

154 Pretreated RS was hydrolysed prior to fermentation in a separate vessel using the
155 commercial enzyme blend Cellic® CTec2. Enzymatic hydrolysis was carried out in a 100-mL
156 conical flask (with 50 mL of working volume) in a SI500 orbital shaker (Stuart, UK). The
157 hydrolysis process was performed at 50 ºC and 150 rpm for 72 h with a biomass loading of 10%
158 (w/v) and an enzyme dosing of 4.1 FPU g-dw^{-1}. The buffer employed was citrate (50 mM) or
159 acetate (50 mM), whose effects on ABE fermentation were assessed using the fractional
160 factorial design of the experiment described in section 2.6. The initial pH was adjusted to 5.0
161 by NaOH and HCl. After enzymatic hydrolysis, the samples were centrifuged (6 min, 4000
162 rpm), filtered by 1.2 μm and stored at 4 ºC for a maximum of 12 h before fermentation. A
163 volume of 34.6 mL of the enzymatic hydrolysate was fermented in 50-mL serum bottles with a
164 working volume of 40 mL. The concentration of the buffer and the minerals was based on a

165 modified P2 medium introduced by Monot et al. [28]: 0.50 g L^{-1} KH_2PO_4, 0.50 g L^{-1} K_2HPO_4,
166 2.20 g L^{-1} NH_4OAc, 0.09 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.001 g L^{-1} $MnSO_4 \cdot H_2O$. The resazurin
167 concentration was set to 1 mg L^{-1}. $FeSO_4 \cdot 7H_2O$ and yeast extract were added in concentrations
168 of 0.01 or 0.02 g L^{-1} and 2 or 4 g L^{-1} respectively, and the initial fermentation pH was adjusted
169 to 6.4 or 7.4, according to the fractional factorial design of the experiment. Beforehand, the
170 sealed bottles were autoclaved for 10 min at 121 ºC; the oxygen was displaced by sparging pure
171 nitrogen. The inoculation was carried out with 2 mL (5% v/v) of actively growing cells, and the
172 serum bottles were incubated at 37 ºC and 150 rpm for 72 h.

173 2.4.2 ABE fermentation by SSF

174 In this configuration, the pretreated RS was simultaneously hydrolysed and fermented
175 in a 50-mL serum bottle (working volume of 40 mL) with a biomass loading of 9% (w/v). The
176 medium for conducting the SSF experiments was the same as that for the SHF experiments,
177 except that no hydrolysis buffer (50 mM citrate or acetate) was added as the fermentation media
178 contained 28.5 mM of acetate. The effect of the same media parameters (iron and yeast extract
179 concentrations) as in SHF was assessed by the fractional factorial design of the experiment. In
180 this case, the initial reaction pH was set to 5.2 or 6.2 as representatives of optimum values for
181 saccharification or fermentation respectively. The oxygen was displaced by sparging pure
182 nitrogen before autoclaving for 10 min at 121 ºC. Afterwards, the enzyme was added along with
183 the inoculum. A loading of 4.1 or 12.4 FPU g-dw^{-1} of Cellic® CTec2 was used in the fractional
184 factorial design experiments in order to assess its influence in SSF. The inoculation was carried
185 out with 2 mL (5% v/v) of actively growing cells. The SSF bottles were incubated at 37 ºC and
186 150 rpm for 120 h. Additionally, two independent replicates of a control experiment (without
187 inoculation) were carried out with the maximum enzyme loading (12.4 FPU g-dw^{-1}) at the
188 minimum pH (5.2) in order to evaluate the maximum release of monosaccharides from the

189 pretreated RS. From results obtained as described herein, CCD was used for further
190 optimisation of the SSF results.

191 2.5 Analytical methods

192 The structural carbohydrates, lignin and the moisture content of the RS were determined
193 according to the National Renewable Energy Laboratory (NREL) procedures [29]. The
194 characterisation of the fermentation was carried out by the analysis of pH, cell growth,
195 production of acids and solvents, and sugar uptake from 1-mL samples collected at appropriate
196 times. The pH was measured by a Minitrode electrode (Hamilton, USA). Cell density (g-dw L^{-}
197 ^{1}) was calculated from the optical density at 600 nm (OD_{600}) measured in a spectrophotometer
198 (SpectroFlex 6600, WTW, Germany). The correlation between OD_{600} and cell density was
199 determined as follows: g-dw L^{-1} = 0.2153·OD_{600} + 0.0689 (n = 10, R^2 = 0.9907). Samples were
200 centrifuged at 10000 rpm for 5 min, and the supernatant was filtered by 0.22 µm before
201 chromatographic analysis. Acids (acetic acid and butyric acid) and solvents (butanol, acetone
202 and ethanol) were analysed in a gas chromatograph (TRACE GC Ultra, Thermo Scientific,
203 USA) equipped with a Teknokroma TRB-FFAP capillary column (30 m × 0.25 mm × 0.25 µm),
204 with helium as carrier gas at a flow rate of 1 mL min^{-1}. One microliter of acidified samples was
205 injected at 250 ºC (10:1 split ratio), and the compounds were detected in a flame ionization
206 detector at 250 ºC. The oven temperature was held at 50 ºC for 4 min, increased at 30 ºC min^{-1}
207 until 80 ºC (hold time 3 min), and increased at 20 ºC min^{-1} until 210 ºC (hold time 5 min).
208 Sugars (glucose, xylose and arabinose) were analysed by an ion chromatograph (883 Basic IC
209 plus, Metrohm, Switzerland) equipped with an amperometric detector and a Metrosep Carb 2
210 anion exchanger column (150 mm × 4 mm × 5 µm). The mobile phase (20 mM NaOH) was set
211 at a flow rate of 0.5 mL min^{-1}. Data are the mean of, at least, two technical replicates.

212 For the evaluation of the process performance, the following parameters were used:

213 $$Butanol\ (or\ ABE) - biomass\ ratio\ (g\ kg^{-1}) = \frac{Butanol\ (or\ ABE)\ \ produced\ (g)/V_{hydrolisate\ fermented}\ (L)}{[Biomass\ loading\ (kg\ L^{-1})/Solid\ recovery\ (\%)]\times 100} \quad (1)$$

214 $$Butanol\ (or\ ABE)\ productivity\ (g\ L^{-1}\ h^{-1}) = \frac{Butanol\ (or\ ABE)\ concentration\ (g\ L^{-1})}{Total\ reaction\ time\ (h)} \quad (2)$$

215 $$\text{Butanol (or ABE) yield (g g}^{-1}) = \frac{\text{Butanol (or ABE) concentration (g L}^{-1})}{\text{Sugar consumed (g L}^{-1})} \quad (3)$$

216 To compare the reaction time of the SHF process with that of SSF, the total reaction
217 time of hydrolysis plus fermentation was assessed. Solid recovery refers to biomass recovered
218 after pretreatment expressed as a percentage.

219 ## 2.6 Design of experiments and statistical analysis

220 In this work, SSF was first assessed and compared with SHF, by fractional factorial
221 designs. As the SSF process performed better than the SHF process, the significant variables
222 for SSF were further optimised by RSM using CCD. The response variable in all cases was the
223 concentration of butanol produced at 48 h. The commercial software MINITAB® v.18.1 (LEAD
224 Technologies, Inc.) was used for the design of experiments, regression analysis and analysis of
225 variance (ANOVA) at a confidence level of 95% (p-value < 0.05).

226 ### 2.6.1 Fractional factorial design and data analysis

227 A 2^{4-1} fractional factorial design (resolution IV, 8 experiment runs) was used to identify
228 the significant factors affecting butanol production at 48 h both in SHF and SSF processes. The
229 effects of three variables (low level and high level) – yeast extract concentration (2 and 4 g L^{-1}
230), $FeSO_4 \cdot 7H_2O$ concentration (0.01 and 0.02 g L^{-1}) and initial fermentation pH (6.4 and 7.4 for
231 SHF, and 5.2 and 6.2 for SSF, respectively) – were evaluated in both processes. For SHF, the
232 fourth variable was the buffer employed for enzymatic hydrolysis (50 mM acetate and 50 mM
233 citrate), whereas for SSF it was the enzyme loading (4.1 and 12.4 FPU g-dw^{-1}).

234 ### 2.6.2 Central composite design and data analysis

235 After identification of significant factors, an RSM with CCD was used in the SSF
236 process to determine the optimal combination of enzyme loading, initial pH and yeast extract
237 concentration for maximising butanol production. The established range for each factor was as
238 follows: enzyme loading (from 5.4 to 19.1 FPU $g\text{-}dw^{-1}$), initial pH (from 5.6 to 7.2) and yeast
239 extract concentration (from 0.5 to 5.5 $g\ L^{-1}$). Table 1 summarises the coded and real values of
240 the three variables used in CCD, which comprised a total of 20 experimental runs with 6 central
241 point replications. Finally, a validation step was carried out by three replicates using the
242 optimised conditions for butanol production.

243 **3 Results and discussion**

244 3.1 Pretreatment of RS

245 Microwave-assisted hydrothermolysis was selected as RS pretreatment since it presents
246 short reaction times, uniform and rapid heating of biomass, lower generation of inhibitory
247 compounds, higher removal of acetyl groups in hemicellulose, and lower costs in comparison
248 with acid or alkaline pretreatments [30]. The chemical compositions of the raw and pretreated
249 RS are presented in Figure 1. The untreated dried material consisted of 35.8 ± 2.1% glucan,
250 14.8 ± 1.6% xylan, 2.7 ± 0.4% arabinan, 0.1 ± 0.0% acid soluble lignin, 14.3 ± 0.4% acid
251 insoluble lignin and 16.7 ± 0.1% ash. This composition is in the typical value range found for
252 RS of different sources [31,32]. Recently, Passoth and Sandgren [33] reported that the typical
253 values for the three major polymers ranged from 29.2 to 34.7% for cellulose, 12.0 to 29.3% for
254 hemicellulose, and 17.0 to 19.0% for lignin, being silica the major ash component.

255 The pretreatment resulted in a solid recovery of 80.5% of the raw RS with different
256 degrees of degradation among carbohydrate fractions. For example, the glucan percentage
257 increased from 35.8 to 39.4%, although the total percentage of carbohydrates remained almost
258 stable at ~53% due to the loss of hemicellulose. During pretreatment only 11.5% of glucan was

259 lost, but greater degradation of arabinan and xylan was observed, with losses of 50.6% and
260 34.5% respectively. These results indicated that some hemicellulose was removed in the
261 microwave pretreatment of RS; phenomena also observed by Zhu et al. [19] at lower irradiation
262 powers. Higher values of polysaccharides were recovered from raw RS (80.1%) when
263 compared to previous studies on this type of biomass. For example, Amiri et al. [34] obtained
264 a recovery of 76.2% after organosolv pretreatment with a 75% (v/v) ethanol and 1% (w/w)
265 sulfuric acid solution at 150 ºC during 30 min, and Moradi et al. [35] found a value of 51.9%
266 after 3 h of alkaline pretreatment at 0 ºC with a 12% (w/v) NaOH solution. The microwave
267 irradiation at the tested conditions resulted in a 13.3% delignification and removed 13.7% of
268 ashes (silica content), thus improving the RS digestibility. However, the delignification degree
269 obtained in this study could impact the saccharification of the waste, since lignin binds non-
270 productively to cellulase due to its hydrophobic nature [36]. On the other hand, the remaining
271 silica in the pretreated RS could also act as a physical barrier, protecting for enzymatic
272 hydrolysis [37].

273 3.2 Comparison of ABE fermentation by SHF and SSF: screening of key factors

274 The 2^{4-1} fractional factorial design was conducted for the SHF and SSF processes to
275 evaluate the influence of the selected parameters on butanol production. The experimental
276 design and the response results for both processes are shown in Table 2. The analysis of
277 variance (ANOVA) of the outcomes, with the estimated coefficients and significant levels for
278 the regression model and the evaluated variables for the SHF and SSF processes are shown in
279 supplementary material. In both cases, after 72 h of fermentation the concentrations of butanol
280 and ABE increased less than 1% from 48 h. Therefore, butanol production at 48 h was
281 considered as the response variable. The fast rate of solvent production demonstrated the
282 successful balance between the acidogenic and solventogenic metabolic phases of *Clostridium*
283 *beijerinckii* DSM 6422 using both the appropriate operational conditions and the adequate

284 biomass pretreatment. Models for SHF and SSF were statistically significant, with p-values
285 lower than 0.05. In addition, the values of the coefficient of determination (R^2: 0.9996 and
286 0.9997 for SHF and SSF, respectively) and the adjusted coefficient of determination (Adj. R^2:
287 09975 and 0.9976 for SHF and SSF, respectively) were close to 1.0, indicating the goodness of
288 fit of the models.

289 From the two-step process (SHF) results, among the four variables screened, only the
290 type of buffer used during enzymatic hydrolysis was found to be significant (p-value of 0.0120).
291 The linear coefficient of the buffer factor (low-high level: acetate-citrate) was lower than zero,
292 indicating that the use of citrate buffer during the saccharification step negatively affected
293 butanol production. Citrate buffer at 50 mM is widely used to maintain a pH around 5.0 during
294 enzymatic hydrolysis [34,35,38]. Furthermore, Xue et al. [39] showed that 60 mM citrate buffer
295 was optimum for ABE fermentation of Jerusalem artichoke stalk with *C. beijerinckii* CC101,
296 lower and higher values decreased solvent production. Contrarily, Liu et al. [40] observed that
297 *C. beijerinckii* NCIMB 8052 did not grow with 50 mM citrate; whereas when acetate was used
298 as a buffer ABE fermentation was not inhibited. In our study, butanol concentrations ranged
299 from 2.72 to 3.16 g L^{-1} by using citrate buffer, while a minimum of 4.68 g L^{-1} was obtained in
300 the experiments with acetate as hydrolysis buffer. Our results corroborated that the use of citrate
301 buffer provokes a negative effect on ABE fermentation of pretreated RS by *C. beijerinckii* DSM
302 6422. The yeast extract concentration did not show a significant effect in the tested range of 2
303 to 4 g L^{-1}. Contrarily, a significant impact on the production of butanol by *C. acetobutylicum*
304 MTCC 481 from RS hydrolysate was previously observed, with an optimal concentration of 3
305 g L^{-1} [41]. Thus, this demonstrates the importance of the preliminary screening of the media
306 composition for each specific lignocellulosic waste and bacterial strain. The non-significant
307 effect of iron on butanol production from RS hydrolysate indicates that the quantity containing
308 the raw material along with the amount from the minimum yeast extract concentration supplied

309 to the fermentation broth is sufficient. Gottumukkala et al. [38] determined that the
310 improvement in solvent production by *C. sporogenes* BE01 after removing mineral
311 supplementation from RS hydrolysate could be due to the presence of these minerals in the raw
312 material. Ranjan et al. [41] also found that iron concentration had no impact on ABE
313 fermentation of RS with *C. acetobutylicum* MTCC 481 supplemented with 3 g L^{-1} of yeast
314 extract. Furthermore, the initial fermentation pH was incorporated into the experimental design
315 since it would affect the biochemical and biophysical characteristics of the solventogenic
316 *Clostridium* spp. [42]. Fermentation pH, together with the rate of acid production, is one
317 potential key factor in the concentration of undissociated acids that can inhibit a correct shift
318 towards solventogenesis [43]. In contrast, fermentation pH was found to be non-significant,
319 likely because the sugar concentration released from enzymatic hydrolysis was not sufficiently
320 high enough to unbalance the rate of acid production.

321 In the case of the one-step process (SSF), two variables were found to be statistically
322 significant. The initial pH had a great effect on butanol production (p-value of 0.0164).
323 Furthermore, the enzyme loading was also significant (p-value of 0.0277). Based on the coded
324 coefficients of the linear effects, the order of importance was as follows: initial pH (1.2041) >
325 enzyme loading (0.7130). These results show that, for SSF, it is better not to use a value near
326 to the optimum for the saccharification of cellulosic materials as the initial pH. Although
327 enzymatic hydrolysis will proceed slowly, solventogenic shift would be favoured. Even though
328 no interaction between initial fermentation pH and enzyme loading had a significant effect, the
329 need to use a pH above the optimum for enzymatic hydrolysis could explain the higher enzyme
330 loading required in SSF (12.4 FPU g-dw^{-1}) to achieve butanol concentrations above 4 g L^{-1}
331 compared with the SHF process (4.1 FPU g-dw^{-1}). Furthermore, the in-situ ABE products in
332 SSF can be linked to the higher enzyme requirements, as they have been shown as inhibitors of
333 the cellulolytic and hemicellulolytic enzyme activity [44]. Contrarily, the fermentation

334 temperature selected (37 ºC) has been reported as a more suitable temperature in comparison to
335 50 ºC for better cellulase and xylanase activities in the presence of ABE products [44].

336 Both configurations were compared in terms of process efficiency. Table 3 summarises
337 the experimental data obtained in the runs with the highest butanol production at 48 h for each
338 configuration (run 7-SHF, run 8-SSF). The values of released sugars (g L^{-1}), butanol and ABE
339 production (g L^{-1}), butanol and ABE yield (g g^{-1}), butanol and ABE-biomass ratio (g kg RS^{-1}),
340 and butanol and ABE productivity (g L^{-1} h^{-1}) are included. After 72 h of enzymatic hydrolysis
341 (SHF), the concentrations obtained of glucose, xylose and arabinose were 17.68, 6.10 and 0.39
342 g L^{-1} respectively. In order to evaluate the maximum sugars released in the SSF processes, two
343 control saccharification assays without inoculum (initial pH = 5.2, enzyme loading = 12.4 FPU
344 g-dw^{-1}) were carried out, with average glucose, xylose and arabinose concentrations of 18.92,
345 6.91 and 0.64 g L^{-1} respectively. Regardless of the process used, the concentrations of sugars
346 released by the enzyme blend Cellic® CTec2 from the pretreated RS were very similar (~50%
347 sugar recovery). Thus, corroborating the idea that the need for higher enzyme dosing in SSF
348 than in SHF relies on the enzymatic inhibition by ABE products and/or on the impossibility of
349 performing the saccharification at the optimum pH. The delignification (13.3%) combined with
350 the ash removal (13.7%) achieved after microwave pretreatment limited, to some extent, the
351 sugar recovery from enzymatic hydrolysis. Concerning the SHF configuration, the butanol
352 concentration at 48 h was 4.85 g L^{-1} with an ABE concentration of 7.95 g L^{-1} (butanol:acetone
353 mass ratio of 1.6:1, ethanol was not detected). All the released sugars were consumed at the end
354 of the fermentation, resulting in a butanol (ABE) yield of 0.245 g g^{-1} (0.402 g g^{-1}). The RS
355 exploitation was evaluated with the butanol or ABE-biomass ratio, with observed values of 44.6
356 g of butanol and 73.1 g of ABE per kg of raw RS. In the SSF process, the butanol concentration
357 at 48 h of reaction time (5.24 g L^{-1}) increased by 8% of that observed in SHF and, in turn, the
358 butanol-biomass ratio rose to 48.2 g kg RS^{-1}; whereas the ABE concentration (butanol:acetone

359 mass ratio of 1.8:1, ethanol was not detected) increased only by 3.7%. The total concentration
360 of sugars in the fermentation broth was < 1.5 g L^{-1} and the butanol (ABE) yield resulted in
361 0.217 g g^{-1} (0.341 g g^{-1}) considering the maximum sugar concentration released in the two
362 control experiments. It should be noted that both glucose and xylose were nearly completely
363 consumed by the microorganisms, thus, maximum utilisation of the sugars released in the
364 saccharification step was reached. Guan et al. [45] also pointed out that the SHF process showed
365 higher ABE yields than those obtained in the SSF process from fermenting Kraft paper mill
366 sludge by *C. acetobutylicum* ATCC 824.

367 The most remarkable difference between the one-step and two-step processes was found
368 in the overall butanol productivity. A productivity of 0.040 g L^{-1} h^{-1} was achieved in the SHF
369 process, while a value 2.7-fold higher (0.109 g L^{-1} h^{-1}) was reached in the SSF process. This
370 greater productivity is related not so much to the increase (by 8%) in the final butanol
371 concentration but instead to the lower operation time needed to carry out the valorisation
372 process of the RS. The SHF process needed a total of 120 h (72 h of enzymatic hydrolysis
373 followed by 48 h of fermentation), while in the SSF process only 48 h were required to complete
374 the butanol production at the same or even slightly higher levels than in the two-step process.
375 Furthermore, the SSF process showed greater exploitation of the RS with a higher butanol-
376 biomass ratio. Other authors compared the simultaneous process to the conventional SHF in the
377 production of butanol from wheat straw [8,12], showing that SSF was more efficient and time-
378 saving than SHF. Our results corroborated previous findings, revealing the potential of SSF to
379 be less expensive than SHF in butanol production from the hydrolysate of straw. The greater
380 efficiency of the SSF process could imply a reduction in equipment investment (only one vessel
381 is necessary) and operational costs (lower production times, less contamination risk) in the
382 production of butanol from RS.

383 3.3 Optimization of butanol production by SSF

384 Based on the above results, a RSM with full factorial CCD was performed for the SSF
385 process to maximise butanol production by optimising three factors: enzyme loading, initial pH
386 and yeast extract concentration. The model was validated by performing an experiment, with 3
387 replicates, at the optimum conditions.

388 ### 3.3.1 Response surface methodology

389 The response surface methodology approach consisted of a five-level, three-factor CCD
390 (Table 1) and subsequent linear regression analysis to fit the experimental data with a second-
391 order model. Three independent variables were selected for the determination of the main
392 effects and their interactions on butanol production. Enzyme loading (Z_1) and initial pH (Z_2),
393 were found to be significant in the fractional factorial design of the SSF process, whereas yeast
394 extract concentration (Z_3) was included for further study by enlarging its variation range. Based
395 on the previous results, $FeSO_4 \cdot 7H_2O$ concentration was set to 0.02 g L^{-1}. Table 4 shows the
396 CCD experimental matrix with variables in real terms and the observed and predicted values of
397 butanol concentration after 48 h obtained from each condition. A total of 20 experimental runs
398 were carried out, including 6 central point replications to check the experimental variability.
399 The experimental results showed that the one-step process succeeded in producing butanol after
400 48 h within the ranges of the independent variables, achieving butanol concentrations from 1.06
401 to 5.31 g L^{-1}. Data of run 15 was not included due to oxygen contamination detected by a
402 resazurin indicator. The greater butanol production (5.31 g L^{-1}) was obtained in run 14 with an
403 ABE concentration of 8.48 g L^{-1} and a butanol:acetone mass ratio of 1.7:1 (ethanol was not
404 detected). Furthermore, a butanol productivity of 0.111 g L^{-1} h^{-1} was reached with a butanol
405 yield of 0.298 g g of consumed $sugar^{-1}$ and a butanol-biomass ratio of 48.8 g per kg of raw RS
406 (ABE-biomass ratio of 78.0 g per kg of raw RS). The second-order model obtained for the
407 concentration of butanol (g L^{-1}) in terms of actual factors was as follows:

$$Butanol\ concentration = -41.9 + 1.018Z_1 + 10.69Z_2 + 3.26Z_3 - 0.0641Z_1^2 - 0.804Z_2^2 - 0.1304Z_3^2 + 0.0783Z_1Z_2 + 0.0039Z_1Z_3 - 0.324Z_2Z_3 \quad (4)$$

408

409 The analysis of variance (ANOVA) and coded regression coefficients of the second-
410 order model for butanol production are presented in Table 5. The model was highly significant
411 at the 95% significance level, with a p-value of 0.0015, whereas the lack-of-fit was not
412 significant (p-value of 0.0500). The low standard deviation (SD) value of 0.5037 g L^{-1},
413 measured in the units of the response variable indicates that the data values are not far from the
414 fitted values. The coefficient of determination (R^2) value was 0.9004, showing a good
415 correlation between the experimental results and the predicted values, in which only 9.96% of
416 the total variations were not explained by the model. The goodness of the predictions was also
417 confirmed by the adjusted coefficient of determination (Adj. R^2: 0.8008), suggesting that this
418 model could properly predict the effect of enzyme loading, initial pH and yeast extract on
419 butanol production after 48 h from RS by SSF. As can be seen from the ANOVA of the model,
420 only the linear coefficients of enzyme loading (Z_1) and yeast extract concentration (Z_3) were
421 found to be significant (p-value of 0.0037 and 0.0009, respectively), whereas initial pH was not
422 significant (p-value of 0.3531). Unlike in other SSF processes [46], the variation of the initial
423 pH in the range studied was not crucial on the response, because the effect of this factor depends
424 on the strain, raw material and type of pretreatment [39]. The coded coefficients of the
425 significant linear effects showed the degree of importance of the factors on the response: yeast
426 extract (0.6671) > enzyme (0.5290). The p-value of the quadratic effect of enzyme (Z_1Z_1) was
427 0.0002, indicating that this variable had the greatest effect on butanol production. The rest of
428 quadratic and two-way interaction effects were found to be not significant.

429 3.3.2 Conjugated effect of enzyme and yeast extract

430 The response surface plot of the final model equation is shown in Figure 2, where the
431 combined effect of enzyme loading and yeast extract on butanol production at a constant initial
432 pH of 6.4 (central point in the CCD) is presented. In this figure, the three-dimensional surface
433 and the two-dimensional contours for the butanol concentration after 48 h are plotted. The
434 surface plot shape shows the great effect of the enzyme loading in comparison with the effect
435 of yeast extract concentration. In addition, the rounded shape of the contour plots reflects,
436 besides ANOVA outcomes, that the interaction effect between both factors was weak. As it can
437 be seen, there is a maximum on the butanol concentration within the range of the variables
438 established in the experimental design. According to the second-order model, the optimal
439 conditions of the significant factors needed to achieve a butanol concentration of 5.43 g L^{-1}
440 were an enzyme loading of 13.5 FPU g-dw^{-1} and a yeast extract concentration of 4.7 g L^{-1}. It
441 should be noted that an enzyme loading higher than 16.3 FPU g-dw^{-1} caused a sudden decrease
442 in the butanol concentration. In the one-step process, apart from increasing the operational
443 costs, a large enzyme load could be counterproductive by inhibiting bacterial growth, as other
444 authors have already pointed out [46]. Yeast is essential for ABE fermentation from bacteria
445 such as *C. acetobutylicum* DSM 792, unlike other sources of nitrogen such as NH_4Cl and
446 $NaNO_3$ [47]. Bacteria use nitrogen in the formation of nucleic acids, proteins and cell wall
447 components [48], so the increase in yeast extract concentration is usually related to the
448 improvement of growth, which would lead to an increase in sugar consumption and a greater
449 butanol production [49]. However, Al-Shorgani et al. [50] observed that an excessive reduction
450 of the C/N ratio inhibits butanol production despite favouring the growth of *C. acetobutylicum*
451 YM1.

452 3.3.3 SSF model validation

453 The validation of the predicted optimal conditions from the CCD results was carried out
454 in three replicates by using an enzyme loading of 13.5 FPU g-dw^{-1}, a yeast extract concentration

455 of 4.7 g L^{-1} and an initial pH of 6.4. The variation with time of the solvent concentration
456 (acetone and butanol; ethanol was not detected), acid concentration (acetic and butyric acid),
457 sugar concentration (glucose, xylose and arabinose) and pH are plotted in Figure 3a. Butanol
458 concentration at 48 h (5.49 ± 0.09 g L^{-1}) only differed by 1.09% from the value estimated from
459 the model (5.43 g L^{-1}), suggesting the goodness of model fit to predict the butanol concentration.
460 Butanol yield and productivity were obtained as 0.306 ± 0.004 g g of consumed $sugar^{-1}$ and
461 0.114 ± 0.002 g L^{-1} h^{-1} respectively. No increase in butanol concentration was observed after
462 48 h. Interestingly, 93% of the maximum value was already reached at 24 h, giving a
463 productivity of 0.212 ± 0.004 g L^{-1} h^{-1}. The production of solvents resulted in 8.00 ± 0.10 g L^{-1}
464 of total ABE at 24 h, when the concentration of acetone reaches its highest value (2.92 ± 0.04
465 g L^{-1}), and 8.40 ± 0.15 g L^{-1} at 48 h. Ethanol was not detected in significant concentrations
466 throughout the study, which is positive for further downstream. Glucose and xylose
467 accumulation were observed during the first 12 h, then decreased rapidly, indicating that
468 enzyme hydrolysis was not the rate-limiting step unlike bacterial metabolism. This reversed
469 after 48 h, when a slight increase in sugars was observed in the fermentation broth. For
470 comparison purposes, the run 7 of the 2^{4-1} fractional factorial design of the SHF processes
471 (highest butanol production for this configuration) was included in Figure 3b. One of the main
472 observable differences is that in order to achieve the maximum butanol production (4.85 g L^{-}
473 ^{1}), 72 extra hours are required compared to the one-step process.

474 A comparison of the results of this study with those derived from the SHF and SSF
475 processes reported in the literature is summarised in Table 6. Among the studies presented,
476 different species of the genus *Clostridium* and different lignocellulosic substrates were used.
477 When comparing butanol and ABE production, the achieved concentrations (5.5 and 8.4 g L^{-1},
478 respectively) were within the published values (4.0-12.6 g L^{-1} for butanol and 7.4-19.8 g L^{-1} for
479 ABE), although they were in the lower range due to the low sugar concentration derived from

480 the hydrolizated RS. The yield values of butanol and ABE found in this study (0.31 g g^{-1} and
481 0.47 g g^{-1}) were much higher than those achieved in the literature (0.16-0.20 g g^{-1} and 0.26-0.30
482 g g^{-1}), thus corroborating the notion that solvent production was limited not due to the capacity
483 of the bacterial strain but rather to the limited release of sugars from the lignocellulosic material
484 (22.98 g L^{-1}). This restriction is also indicated by the butanol (51 g kg RS^{-1}) and ABE-biomass
485 ratio (77 g kg RS^{-1}); parameters reflecting the overall conversion from raw RS to solvents that
486 need to be increased for a large-scale production. Besides the high yield, the productivity of
487 butanol (0.11 g L^{-1} h^{-1}) and ABE solvents (0.18 g L^{-1} h^{-1}) was higher than that previously
488 reported for the SSF process, where it takes between 72 to 144 h to reach the maximum
489 concentration of butanol, unlike the 48 h required in our study. Compared with SHF from the
490 literature, the values were even better, as reported productivities do not take into account the
491 required time for the biomass saccharification (48–72 h more). This is of great interest, as high
492 productivities are necessary to ensure an adequate butanol removal rate in in-situ product
493 removal processes [52]. Further study is necessary in order to increase the release of sugars
494 from the RS by enhancing the pretreatment method. The use of large concentrations of biomass
495 can lead to problems such as inappropriate energy efficiency in microwave pretreatment [18],
496 decrease of mass transfer [53] and decrease of substrate conversion due to enzymatic inhibition
497 [54]. Therefore, investigations will be focus on improving the delignification and ash removal
498 rather than to increase the biomass loading.

499 ## 4 Conclusions

500 The serious environmental problems arising from the consumption of fossil fuels are
501 increasing interest in producing biobutanol from lignocellulosic waste as a promising
502 alternative energy source. In this study we demonstrated the feasibility of using hydrolysed rice
503 straw by microwave irradiation as a substrate. By an adequate selection of operational
504 conditions, fermentation time was reduced to 48 h with nearly total consumption not only of

505 glucose, but also of xylose, resulting in high productivity which is a great advantage for scaling-
506 up. Besides, the SSF process was shown to be a favourable configuration with the potential
507 capability to reduce substantially the production cost when compared with a conventional SHF
508 process. From these promising results, further research on pretreatment conditions in order to
509 improve the release of sugar concentrations from saccharification are of great interest to
510 increase the butanol-biomass ratio prior to scale-up.

511 **5 Acknowledgements**

512 Financial support was obtained from the FEDER/Ministerio de Ciencia, Innovación y
513 Universidades – Agencia Estatal de Investigación/Project CTM2017-88042-R (Spain). A.
514 Valles acknowledges to the Generalitat Valenciana (Spain) and the Fondo Social Europeo for
515 the ACIF/2017/390 contract.

516 **6 References**

517 [1] European Union: European Commission. Communication from the Commission to the
518 European Parliament, the Council, the European Economic and Social Committee and
519 the Committee of the Regions. Innovating for sustainable growth: a bioeconomy for
520 Europe. COM(2012) 60 Final 2012.

521 [2] Birgen C, Dürre P, Preisig HA, Wentzel A. Butanol production from lignocellulosic
522 biomass: revisiting fermentation performance indicators with exploratory data analysis.
523 Biotechnol Biofuels 2019;12:167. https://doi.org/10.1186/s13068-019-1508-6.

524 [3] Bajpai P. Background and general introduction. Pretreat. Lignocellul. biomass biofuel
525 Prod., SpringerBriefs in Molecular Science. Springer, Singapore; 2016, p. 1–6.
526 https://doi.org/10.1007/978-981-10-0687-6.

527 [4] Ibrahim MF, Ramli N, Kamal Bahrin E, Abd-Aziz S. Cellulosic biobutanol by Clostridia:

528 Challenges and improvements. Renew Sustain Energy Rev 2017;79:1241–54.
529 https://doi.org/10.1016/j.rser.2017.05.184.

530 [5] Abo BO, Gao M, Wang Y, Wu C, Wang Q, Ma H. Production of butanol from biomass:
531 recent advances and future prospects. Environ Sci Pollut Res 2019;26:20164–82.
532 https://doi.org/10.1007/s11356-019-05437-y.

533 [6] Jones DT, Woods DR. Acetone-butanol fermentation revisited. Microbiol Rev
534 1986;50:484–524.

535 [7] Jiao S, Zhang Y, Wan C, Lv J, Du R, Zhang R, et al. Transcriptional analysis of
536 degenerate strain *Clostridium beijerinckii* DG-8052 reveals a pleiotropic response to
537 $CaCO_3$-associated recovery of solvent production. Sci Rep 2016;6:38818.
538 https://doi.org/10.1038/srep38818.

539 [8] Qureshi N, Saha BC, Hector RE, Hughes SR, Cotta MA. Butanol production from wheat
540 straw by simultaneous saccharification and fermentation using *Clostridium beijerinckii*:
541 Part I-Batch fermentation. Biomass and Bioenergy 2008;32:168–75.
542 https://doi.org/10.1016/j.biombioe.2007.07.004.

543 [9] García V, Päkkilä J, Ojamo H, Muurinen E, Keiski RL. Challenges in biobutanol
544 production: How to improve the efficiency? Renew Sustain Energy Rev 2011;15:964–
545 80. https://doi.org/10.1016/j.rser.2010.11.008.

546 [10] Mariano AP, Costa CBB, de Angelis D de F, Maugeri Filho F, Atala DIP, Wolf Maciel
547 MR, et al. Optimisation of a continuous flash fermentation for butanol production using
548 the response surface methodology. Chem Eng Res Des 2010;88:562–71.
549 https://doi.org/10.1016/j.cherd.2009.11.002.

550 [11] Gauss WF, Suzuki S, Takagi M. Manufacture of alcohol from cellulosic materials using

551 plural ferments. US Pat 3,990,944, 39909, No Issue 610731 1976.

552 [12] Qi G, Huang D, Wang J, Shen Y, Gao X. Enhanced butanol production from ammonium
553 sulfite pretreated wheat straw by separate hydrolysis and fermentation and simultaneous
554 saccharification and fermentation. Sustain Energy Technol Assessments 2019;36.
555 https://doi.org/10.1016/j.seta.2019.100549.

556 [13] Shao M, Chen H. Feasibility of acetone-butanol-ethanol (ABE) fermentation from
557 *Amorphophallus konjac* waste by *Clostridium acetobutylicum* ATCC 824. Process
558 Biochem 2015;50:1301–7. https://doi.org/10.1016/j.procbio.2015.05.009.

559 [14] Kim S, Dale BE. Global potential bioethanol production from wasted crops and crop
560 residues. Biomass and Bioenergy 2004;26:361–75.
561 https://doi.org/10.1016/j.biombioe.2003.08.002.

562 [15] Sarkar N, Aikat K. Kinetic study of acid hydrolysis of rice straw. ISRN Biotechnol 2013.
563 https://doi.org/10.5402/2013/170615.

564 [16] Vivek N, Nair LM, Mohan B, Nair SC, Sindhu R, Pandey A, et al. Bio-butanol
565 production from rice straw – Recent trends, possibilities, and challenges. Bioresour
566 Technol Reports 2019;7:100224. https://doi.org/10.1016/j.biteb.2019.100224.

567 [17] Hsu TC, Guo GL, Chen WH, Hwang WS. Effect of dilute acid pretreatment of rice straw
568 on structural properties and enzymatic hydrolysis. Bioresour Technol 2010;101:4907–
569 13. https://doi.org/10.1016/j.biortech.2009.10.009.

570 [18] Ma H, Liu W-W, Chen X, Wu Y-J, Yu Z-L. Enhanced enzymatic saccharification of rice
571 straw by microwave pretreatment. Bioresour Technol 2009;100:1279–84.
572 https://doi.org/10.1016/j.biortech.2008.08.045.

573 [19] Zhu S, Wu Y, Yu Z, Liao J, Zhang Y. Pretreatment by microwave/alkali of rice straw
574 and its enzymic hydrolysis. Process Biochem 2005;40:3082–6.
575 https://doi.org/10.1016/j.procbio.2005.03.016.

576 [20] Amiri H, Karimi K. Pretreatment and hydrolysis of lignocellulosic wastes for butanol
577 production: Challenges and perspectives. Bioresour Technol 2018;270:702–21.
578 https://doi.org/10.1016/j.biortech.2018.08.117.

579 [21] Ma H, Liu WW, Chen X, Wu YJ, Yu ZL. Enhanced enzymatic saccharification of rice
580 straw by microwave pretreatment. Bioresour Technol 2009;100:1279–84.
581 https://doi.org/10.1016/j.biortech.2008.08.045.

582 [22] Fonseca BG, Mateo S, Moya AJ, Roberto IC. Biotreatment optimization of rice straw
583 hydrolyzates for ethanolic fermentation with Scheffersomyces stipitis. Biomass and
584 Bioenergy 2018;112:19–28. https://doi.org/10.1016/j.biombioe.2018.02.003.

585 [23] Wang Z, Cao G, Jiang C, Song J, Zheng J, Yang Q. Butanol production from wheat straw
586 by combining crude enzymatic hydrolysis and anaerobic fermentation using *Clostridium*
587 *acetobutylicum* ATCC824. Energy and Fuels 2013;27:5900–6.
588 https://doi.org/10.1021/ef4010658.

589 [24] Qureshi N, Singh V, Liu S, Ezeji TC, Saha BC, Cotta MA. Process integration for
590 simultaneous saccharification, fermentation, and recovery (SSFR): Production of
591 butanol from corn stover using *Clostridium beijerinckii* P260. Bioresour Technol
592 2014;154:222–8. https://doi.org/10.1016/j.biortech.2013.11.080.

593 [25] Boonsombuti A, Tangmanasakul K, Nantapipat J, Komolpis K, Luengnaruemitchai A,
594 Wongkasemjit S. Production of biobutanol from acid pretreated corncob using
595 *Clostridium beijerinckii* TISTR 1461: Process optimization studies. Prep Biochem

596 Biotechnol 2016;46:141–9. https://doi.org/10.1080/10826068.2014.995810.

597 [26] Adney B, Baker J. Measurement of cellulase activities: Laboratory Analytical Procedure
598 (LAP). Natl Renew Energy Lab Golden, CO 1996.

599 [27] García-Puchol M, Poy H, Martínez-Soria V, Loras S, Gabaldón G. Pretratamiento
600 hidrotérmico de la paja de arroz para su uso en la producción de biobutanol. Proceedings
601 of the 1st Ibero-American Congress on Chemical Engineering; 2019 Jun 19-21; Santander,
602 Spain.

603 [28] Monot F, Martin J-R, Petitdemange H, Gay R. Acetone and butanol production by
604 *Clostridium acetobutylicum* in a synthetic medium. Appl Environ Microbiol
605 1982;44:1318–24.

606 [29] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, et al. Determination of
607 structural carbohydrates and lignin in biomass: Laboratory Analytical Procedure (LAP).
608 Natl Renew Energy Lab Golden, CO 2008.

609 [30] López-Linares JC, García-Cubero MT, Lucas S, González-Benito G, Coca M.
610 Microwave assisted hydrothermal as greener pretreatment of brewer's spent grains for
611 biobutanol production. Chem Eng J 2019;368:1045–55.
612 https://doi.org/10.1016/j.cej.2019.03.032.

613 [31] Zhao T, Tashiro Y, Zheng J, Sakai K, Sonomoto K. Semi-hydrolysis with low enzyme
614 loading leads to highly effective butanol fermentation. Bioresour Technol
615 2018;264:335–42. https://doi.org/10.1016/j.biortech.2018.05.056.

616 [32] Singh R, Tiwari S, Srivastava M, Shukla A. Microwave assisted alkali pretreatment of
617 rice straw for enhancing enzymatic digestibility. J Energy 2014.
618 https://doi.org/10.1155/2014/483813.

619 [33] Passoth V, Sandgren M. Biofuel production from straw hydrolysates: current
620 achievements and perspectives. Appl Microbiol Biotechnol 2019;103:5105–16.
621 https://doi.org/10.1007/s00253-019-09863-3.

622 [34] Amiri H, Karimi K, Zilouei H. Organosolv pretreatment of rice straw for efficient
623 acetone, butanol, and ethanol production. Bioresour Technol 2014;152:450–6.
624 https://doi.org/10.1016/j.biortech.2013.11.038.

625 [35] Moradi F, Amiri H, Soleimanian-Zad S, Ehsani MR, Karimi K. Improvement of acetone,
626 butanol and ethanol production from rice straw by acid and alkaline pretreatments. Fuel
627 2013;112:8–13. https://doi.org/10.1016/j.fuel.2013.05.011.

628 [36] Lan TQ, Lou H, Zhu JY. Enzymatic saccharification of lignocelluloses should be
629 conducted at elevated pH 5.2-6.2. Bioenergy Res 2013;6:476–85.
630 https://doi.org/10.1007/s12155-012-9273-4.

631 [37] Řezanka T, Sigler K. Biologically active compounds of semi-metals. Phytochemistry
632 2008;69:585–606. https://doi.org/10.1016/j.phytochem.2007.09.018.

633 [38] Gottumukkala LD, Parameswaran B, Valappil SK, Mathiyazhakan K, Pandey A,
634 Sukumaran RK. Biobutanol production from rice straw by a non acetone producing
635 *Clostridium sporogenes* BE01. Bioresour Technol 2013;145:182–7.
636 https://doi.org/10.1016/j.biortech.2013.01.046.

637 [39] Xue C, Zhang X, Wang J, Xiao M, Chen L, Bai F. The advanced strategy for enhancing
638 biobutanol production and high-efficient product recovery with reduced wastewater
639 generation. Biotechnol Biofuels 2017;10:148. https://doi.org/10.1186/s13068-017-
640 0836-7.

641 [40] Liu K, Atiyeh HK, Pardo-Planas O, Ramachandriya KD, Wilkins MR, Ezeji TC, et al.

642 Process development for biological production of butanol from Eastern redcedar.
643 Bioresour Technol 2015;176:88–97. https://doi.org/10.1016/j.biortech.2014.10.148.

644 [41] Ranjan A, Mayank R, Moholkar VS. Development of semi-defined rice straw-based
645 medium for butanol production and its kinetic study. 3 Biotech 2013;3:353–64.
646 https://doi.org/10.1007/s13205-013-0120-x.

647 [42] Millat T, Winzer K. Mathematical modelling of clostridial acetone-butanol-ethanol
648 fermentation. Appl Microbiol Biotechnol 2017;101:2251–71.
649 https://doi.org/10.1007/s00253-017-8137-4.

650 [43] Maddox IS, Steiner E, Hirsch S, Wessner S, Gutierrez NA, Gapes JR, et al. The cause of
651 "acid crash" and "acidogenic fermentations" during the batch acetone-butanol-ethanol
652 (ABE-) fermentation process. J Mol Microbiol Biotechnol 2000;2:95–100.

653 [44] Qi B, Chen X, Yi S, Wan Y. Inhibition of cellulase, β-glucosidase, and xylanase
654 activities and enzymatic hydrolysis of dilute acid pretreated wheat straw by acetone-
655 butanol-ethanol fermentation products. Environ Prog Sustain Energy 2014;33:497–503.
656 https://doi.org/10.1002/ep.11789.

657 [45] Guan W, Shi S, Tu M, Lee YY. Acetone-butanol-ethanol production from Kraft paper
658 mill sludge by simultaneous saccharification and fermentation. Bioresour Technol
659 2016;200:713–21. https://doi.org/10.1016/j.biortech.2015.10.102.

660 [46] Razali NAAM, Ibrahim MF, Bahrin EK, Abd-Aziz S. Optimisation of simultaneous
661 saccharification and fermentation (SSF) for biobutanol production using pretreated oil
662 palm empty fruit bunch. Molecules 2018;23:1944.
663 https://doi.org/10.3390/molecules23081944.

664 [47] Raganati F, Olivieri G, Götz P, Marzocchella A, Salatino P. Butanol production from

665 hexoses and pentoses by fermentation of *Clostridium acetobutylicum*. Anaerobe
666 2015;34:146–55. https://doi.org/10.1016/j.anaerobe.2015.05.008.

667 [48] Rehm N, Burkovski A. Engineering of nitrogen metabolism and its regulation in
668 *Corynebacterium glutamicum*: influence on amino acid pools and production. Appl
669 Microbiol Biotechnol 2011;89:239–48. https://doi.org/10.1007/s00253-010-2922-7.

670 [49] Magalhães BL, Grassi MCB, Pereira GAG, Brocchi M. Improved n-butanol production
671 from lignocellulosic hydrolysate by *Clostridium* strain screening and culture-medium
672 optimization. Biomass and Bioenergy 2018;108:157–66.
673 https://doi.org/10.1016/j.biombioe.2017.10.044.

674 [50] Al-Shorgani NKN, Shukor H, Abdeshahian P, Kalil MS, Yusoff WMW, Hamid AA.
675 Enhanced butanol production by optimization of medium parameters using *Clostridium*
676 *acetobutylicum* YM1. Saudi J Biol Sci 2018;25:1308–21.
677 https://doi.org/10.1016/j.sjbs.2016.02.017.

678 [51] Plaza PE, Gallego-Morales LJ, Peñuela-Vásquez M, Lucas S, García-Cubero MT, Coca
679 M. Biobutanol production from brewer's spent grain hydrolysates by *Clostridium*
680 *beijerinckii*. Bioresour Technol 2017;244:166–74.
681 https://doi.org/10.1016/j.biortech.2017.07.139.

682 [52] Moon C, Lee CH, Sang B-I, Um Y. Optimization of medium compositions favoring
683 butanol and 1,3-propanediol production from glycerol by *Clostridium pasteurianum*.
684 Bioresour Technol 2011;102:10561–8. https://doi.org/10.1016/j.biortech.2011.08.094.

685 [53] Bellido C, Infante C, Coca M, González-Benito G, Lucas S, García-Cubero MT.
686 Efficient acetone-butanol-ethanol production by *Clostridium beijerinckii* from sugar beet
687 pulp. Bioresour Technol 2015;190:332–8.

688 https://doi.org/10.1016/j.biortech.2015.04.082.

689 [54] Puri DJ, Heaven S, Banks CJ. Improving the performance of enzymes in hydrolysis of
690 high solids paper pulp derived from MSW. Biotechnol Biofuels 2013;6:107.
691 https://doi.org/10.1186/1754-6834-6-107.

692

Table 1. 5-Level CCD of 3 variables for the SSF process. α = 1.68.

Independent variables		Coded and real values				
		Level -α	Level -1	Central point (0)	Level +1	Level +α
Z_1	Enzyme loading (FPU g-dw^{-1})	5.4	8.2	12.2	16.3	19.1
Z_2	Initial pH	5.6	5.9	6.4	6.9	7.2
Z_3	Yeast extract (g L^{-1})	0.5	1.5	3.0	4.5	5.5

Table 2. 2^{4-1} fractional factorial design and experimental results for the SHF and for the SSF processes.

SHF Process					
Run	Real values				Response
	Yeast extract (g L^{-1})	$FeSO_4 \cdot 7H_2O$ (g L^{-1})	Initial pH	Saccharification buffer	Butanol 48 h (g L^{-1})
1	2	0.01	6.4	50 mM acetate	4.68
2	2	0.02	6.4	50 mM citrate	3.13
3	2	0.02	7.4	50 mM acetate	4.78
4	2	0.01	7.4	50 mM citrate	2.72
5	4	0.02	6.4	50 mM acetate	4.84
6	4	0.01	6.4	50 mM citrate	3.16
7	4	0.01	7.4	50 mM acetate	4.85
8	4	0.02	7.4	50 mM citrate	3.05
SSF Process					
Run	Real values				Response
	Yeast extract (g L^{-1})	$FeSO_4 \cdot 7H_2O$ (g L^{-1})	Initial pH	Enzyme loading (FPU g-dw^{-1})	Butanol 48 h (g L^{-1})
1	2	0.01	5.2	4.1	1.55
2	2	0.02	5.2	12.4	1.53
3	2	0.02	6.2	4.1	2.80
4	2	0.01	6.2	12.4	4.98
5	4	0.02	5.2	4.1	1.00
6	4	0.01	5.2	12.4	0.58
7	4	0.01	6.2	4.1	1.28
8	4	0.02	6.2	12.4	5.24

Table 3. Comparison of SHF and SSF processes after 48 h of fermentation.

Method	Released Sugars (g L^{-1})[a]		Butanol (ABE) Production (g L^{-1})	Butanol(ABE) Yield (g g^{-1})	Butanol (ABE)-biomass Ratio (g kg RS^{-1})	Butanol (ABE) Productivity (g L^{-1} h^{-1})
	Glucose	Xylose				
SHF	17.68	6.10	4.85 (7.95)	0.245 (0.402)	44.6 (73.1)	0.040 (0.066)
SSF	18.92	6.91	5.24 (8.24)	0.217 (0.341)	48.2 (75.8)	0.109 (0.172)

[a] Sugars obtained after 72h of hydrolysis time. In the case of SSF, sugars released from two abiotic controls.

Table 4. CCD experimental matrix along with the observed and predicted values of the response for the SSF process.

Run	Real values			Butanol (g L^{-1})	
	Z_1	Z_2	Z_3	Observed	Predicted
1	8.2	5.9	1.5	2.62	2.22
2	16.3	5.9	1.5	2.52	2.96
3	8.2	6.9	1.5	2.79	2.70
4	16.3	6.9	1.5	3.68	3.99
5	8.2	5.9	4.5	4.21	4.00
6	16.3	5.9	4.5	4.62	4.82
7	8.2	6.9	4.5	3.83	3.50
8	16.3	6.9	4.5	4.38	4.88
9	5.4	6.4	3.0	1.06	1.72
10	19.1	6.4	3.0	4.31	3.50
11	12.2	5.6	3.0	4.14	4.17
12	12.2	7.2	3.0	4.80	4.62
13	12.2	6.4	0.5	3.11	3.01
14	12.2	6.4	5.5	5.31	5.25
15	12.2	6.4	3.0	n.a.[a]	4.96
16	12.2	6.4	3.0	4.58	4.96
17	12.2	6.4	3.0	5.19	4.96
18	12.2	6.4	3.0	5.10	4.96
19	12.2	6.4	3.0	4.81	4.96
20	12.2	6.4	3.0	5.10	4.96

[a] n.a.: non available

Table 5. ANOVA of the second-order model for butanol production by SSF process.

Source	Degrees of freedom	Sum of squares	Mean square	F value	p-value Prob > F	Coefficient[a]
Model	9	20.6456	2.2940	9.04	0.0015	
Linear	3	10.1421	3.3807	13.33	0.0012	
Z_1: Enzyme loading	1	3.8221	3.8221	15.07	0.0037	0.5290
Z_2: Initial pH	1	0.2432	0.2432	0.96	0.3531	0.1334
Z_3: Yeast extract	1	6.0768	6.0768	23.95	0.0009	0.6671
Square	3	9.8683	3.2894	12.97	0.0013	
Z_1Z_1	1	9.4120	9.4120	37.10	0.0002	-0.8304
Z_2Z_2	1	0.5516	0.5516	2.17	0.1744	-0.2010
Z_3Z_3	1	1.1750	1.1750	4.63	0.0598	-0.2934
2-way interactions	3	0.6353	0.2118	0.83	0.5079	
Z_1Z_2	1	0.1591	0.1591	0.63	0.4488	0.1410
Z_1Z_3	1	0.0035	0.0035	0.01	0.9086	0.0210
Z_2Z_3	1	0.4726	0.4726	1.86	0.2054	-0.2431
Error	9	2.2832	0.2537			
Lack-of-fit	5	2.0244	0.4049	6.26	0.0500	
Pure error	4	0.2588	0.0647			
Total	18	22.9288				
Standard Deviation, SD					0.5037	
R^2					0.9004	
Adj. R^2					0.8008	

[a] For coded variables.

Table 6. Comparison of ABE fermentation through SHF and SSF processes from different feedstocks.

Substrate	Pretreatment	Fermentation method	Pretreated biomass loading	Enzyme loading	Microorganism	Butanol (ABE) production ($g\ L^{-1}$)	Butanol (ABE) yield ($g\ g^{-1}$)	Butanol (ABE)-biomass ratio ($g\ kg\ RS^{-1}$)	Butanol (ABE) Productivity ($g\ L^{-1}\ h^{-1}$)	Reference
Brewer´s spent grain	Dilute acid hydrolysis	SHF	10% (w/w)	Celluclast 1.5L (15 FPU $g\text{-}dw^{-1}$), Novozyme 188 (15 IU $g\text{-}dw^{-1}$)	*C. beijerinckii* DSM 6422	6.1 (8.2)	0.20 (0.26)	28 (38)	0.06 (0.08)**	[51]
Rice straw	Ethanol organosolv	SHF	8% (w/w)	Celluclast 1.5L (25 FPU $g\text{-}dw^{-1}$), Novozyme 188 (40 IU $g\text{-}dw^{-1}$)	*C. acetobutylicum* NRRL B-591	7.1 (10.5)	-	70 (103)	0.10 (0.15)**	[34]
Paper sludge	None	SSF	5% (w/v)	Cellic CTec2 (15 FPU g $glucan^{-1}$)	*C. acetobutylicum* ATCC 824	8.5 (14.5)	0.18 (0.30)	92 (157)	0.07 (0.12)	[45]
Oil palm empty fruit bunch	Alkaline	SSF	5% (w/v)	Acremonium cellulase (15 FPU $g\text{-}dw^{-1}$)	*C. acetobutylicum* ATCC 824	4.0 (7.4)	0.16 (0.30)	80 (148)*	0.03 (0.06)	[46]
Wheat straw	Ammonium sulfite	SSF	9% (w/v)	Cellulase (5 FPU $g\text{-}dw^{-1}$), Xylanase (10 IU $g\text{-}dw^{-1}$)	*C. acetobutylicum* ATCC 824	12.6 (19.8)	-	110 (173)	0.09 (0.14)	[12]
Rice straw	Microwave assisted hydrothermolysis	SSF	9% (w/v)	Cellic CTec2 (12 FPU g $glucan^{-1}$)	*C. beijerinckii* DSM 6422	5.5 (8.4)	0.31 (0.47)	51 (77)	0.11 (0.18)	This study

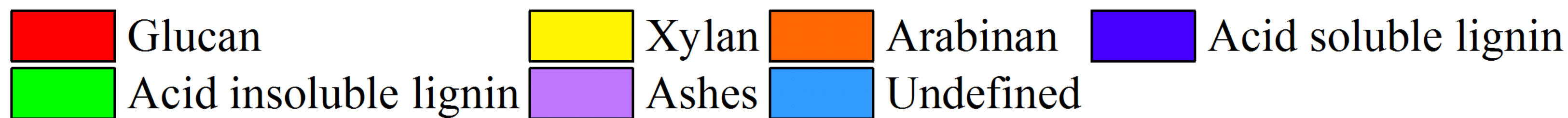
*The butanol and ABE-biomass ratio was calculated considering that the solid recovery was 100%.

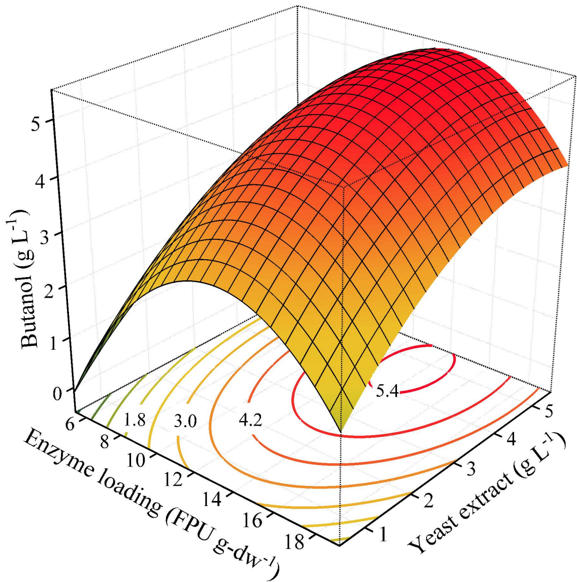
**The butanol and ABE productivity was calculated without considering the enzymatic hydrolysis time.

Figure 1. Chemical composition of raw and pretreated rice straw.

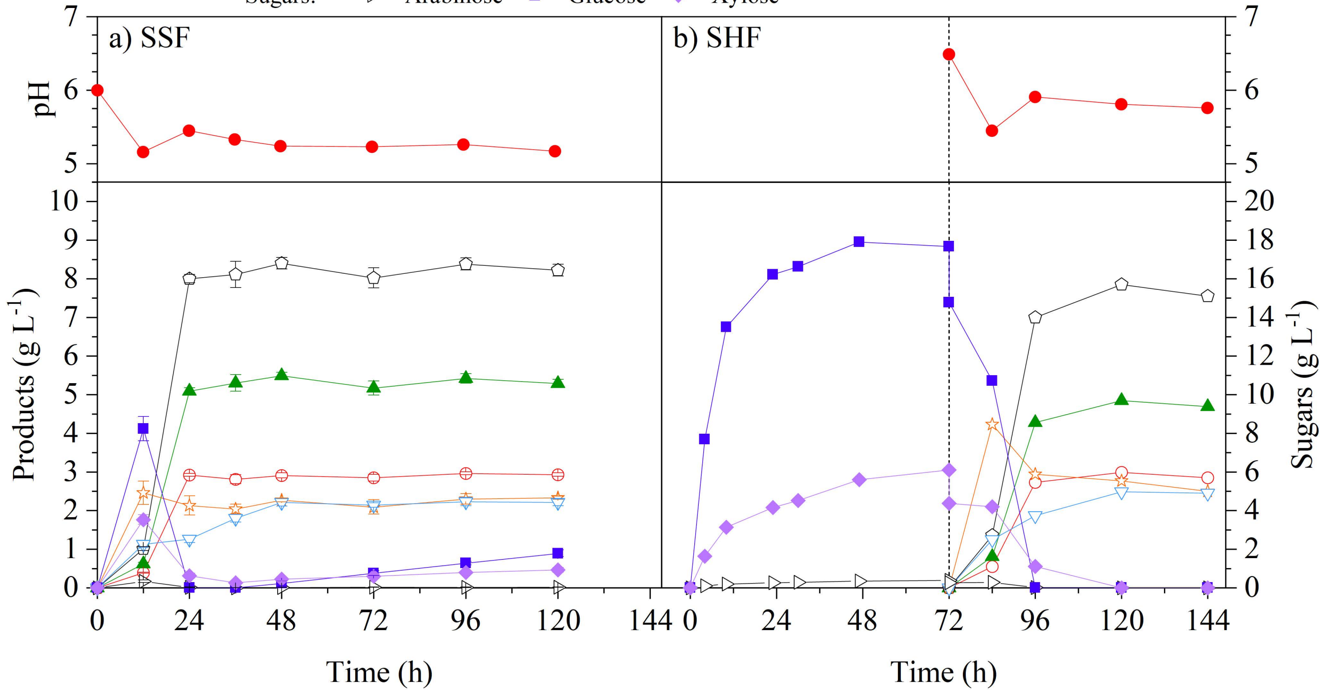
Figure 2. The response surface and the corresponding contour plot for butanol production (g L^{-1}) at 48 h in the SSF process: combined effect of enzyme loading (FPU g-dw^{-1}) and yeast extract concentration (g L^{-1}). Initial pH = 6.4.

Figure 3. Comparison of SSF and SHF processes. (a) SSF: CCD model validation at the predicted optimum conditions. Standard bar errors from three replicates; (b) SHF: Best results achieved, single run 7 of the 2^{4-1} fractional factorial design.


Glucan
Xylan
Arabinan
Acid soluble lignin
Acid insoluble lignin
Ashes
Undefined
Components (%)
100
90
80
70
60
50
40
30
20
10
0
15.6
16.7
14.3
14.8
35.8
13.6
17.9
15.4
12.0
39.4
Raw RS
Pretreated RS



Butanol (g L-1)
0
1
2
3
4
5
1.8
3.0
4.2
5.4
Enzyme loading (FPU g-dw-1)
6
8
10
12
14
16
18
Yeast extract (g L-1)
1
2
3
4
5


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