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1 **ENZYMATIC HYDROLYSIS AT HIGH LIGNOCELLULOSIC CONTENT:**
2 **OPTIMIZATION OF THE MIXING SYSTEM GEOMETRY AND OF A FED-BATCH**
3 **STRATEGY TO INCREASE GLUCOSE CONCENTRATION**

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7 **ABSTRACT**

8 Working at high values of lignocellulosic Dry Matter (DM), as wheat straw, increases the reaction
9 medium viscosity, making the mixing inefficient with the traditional agitators. Batch and fed-batch
10 tests were conducted using different impellers: i) inclined blades, ii) marine impeller, iii) anchor, iv)
11 paravisc and v) double helical impeller. Inclined blades appeared an inadequate device for batch
12 and fed-batch tests. On contrary, double helical impellers and anchor gave optimal performances.
13 An alternative to improve the reactor's rheology is the modification of the feeding strategy. A
14 particular fed-batch strategy allowed keeping low the reaction medium viscosity by a gradual
15 increasing of the DM content in the reactor. In this way, three main benefits were achieved: i) a
16 very good performances in terms of glucose concentration (85 g/L), ii) a strong reduction of the
17 energetic consumption compared to batch test and iii) the adoption of a simple mixing devise.

18 **KEYWORDS:** Bioethanol; High Dry Matter; Mixing; Enzymatic hydrolysis; Fed batch;
19 Lignocellulosic materials

1. Introduction

The conversion of lignocellulosic materials in glucose by enzymatic hydrolysis is a consolidated process in the biotechnological field [1] but requires improvements to be more economically advantageous, when the reactor works at high Dry Matter (DM) concentration. High DM content contributes to reduce the working volume, process water, capital costs and the energy demand for the following biological steps, i.e. the fermentation and distillation ones in bioethanol production [2], [3]. Previous studies on enzymatic hydrolysis of lignocellulosic materials at high DM content demonstrated that mass and heat transfer is inhibited in reactors operating with the most common impellers, i.e. Rushton or inclined blades [3], [4]. It depends on the recalcitrant nature of lignocellulosic polymers, which comports the increasing of the reaction medium's apparent viscosity. In particular Battista et al. [5] investigated on the correlation between the lignocellulosic physical characteristics and the viscosity within the reactor. They demonstrated that high porosity substrates, such as wheat straw (WS), have an elevated water adsorption tendency which, at high DM content, causes the increasing of reaction medium viscosity. Ghorbanian et al. [3] and Dasari and Berson [6] used no conventional reactor for the enzymatic hydrolysis at high DM concentration, adopting a Horizontal Rotating Reactor (HRR) rotating at very low speed to provide motion. They recorded a lower energetic power for the mixing than conventional stirred tank reactor and an adequate heat transfer. Despite these advantages, HRRs provided good mixing only in the angular direction of the motion, while in the case of enzymatic biomass processing, multi-direction mixing and transport was needed to disperse enzymes and optimize sugars productions [7], [8]. As consequence, at the end of the enzymatic hydrolysis the sugars concentration was low [9], [10], [11]. In addition, HRR configuration was not adapted for big scales, such as industrial ones [12]. Thus, improving the rheology of stirred tank reactors represents the most convenient way to work at high DM lignocellulosic content.

The mixing performances of a stirred tank reactor are affected by several factors: the tank dimensions and its geometry (the Height/Diameter (H/D) ratio, the tank bottom morphology, the

46 presence and number of baffles) [12]. The rotational speed of the impeller, which is strictly linked
47 to the power supplied by the motor, is another important parameter to consider. Anyway, the
48 impeller geometry (its shape and its dimensions) represents probably the most impacting factor on
49 the mixing performance. Different geometries influence the intensities of the radial and axial flows
50 changing deeply the reactor rheology. Anchor and helicoidal impellers, for example, are indicated
51 for viscous reaction medium, while Rushton and inclined blades recorded very low performance in
52 these conditions [12]. Finally, Mondebach and Nokes (2013) [13] also showed that the substrates
53 feeding's strategy was a way to improve the rheology of the bioreactor. They demonstrated that fed-
54 batch offers advantages in the enzymatic hydrolysis over the batch mode: the initial substrates
55 quantity fed into the reactor was lower, so diffusion and mixing limitations can be minimized. In
56 addition, fed-batch strategy permitted to the enzymes to better liquefy the recalcitrant
57 lignocellulosic polymer.

58 The aim of this work was the investigation of the influence of the mixing systems geometry and of
59 the reactor's feeding strategy on the enzymatic hydrolysis performances at high DM content.
60 Impellers, having different diameter sizes and shapes, have been tested in batch and fed-batch
61 mode. In addition, a fed batch strategy, the Fed Batch Gradual Addition (FBGA), has been
62 implemented in order to simplify the reactor mixing device and, at the same time, to have a high
63 glucose concentration and a reduction of the energetic consumptions for the mixing.

64 **2. Materials and Methods**

65 **2.1 Characterization of the substrates and of the enzymatic cocktail**

66 Pretreated WS has been used for the tests. The pretreatment, conducted by an external company,
67 consisted into the cutting of straws in 2 mm fibers, the soaking in an acid solution and the steam
68 explosion process at 200 °C and 13.4 bar for 7.5 minutes. Pretreated WS had a DM concentration

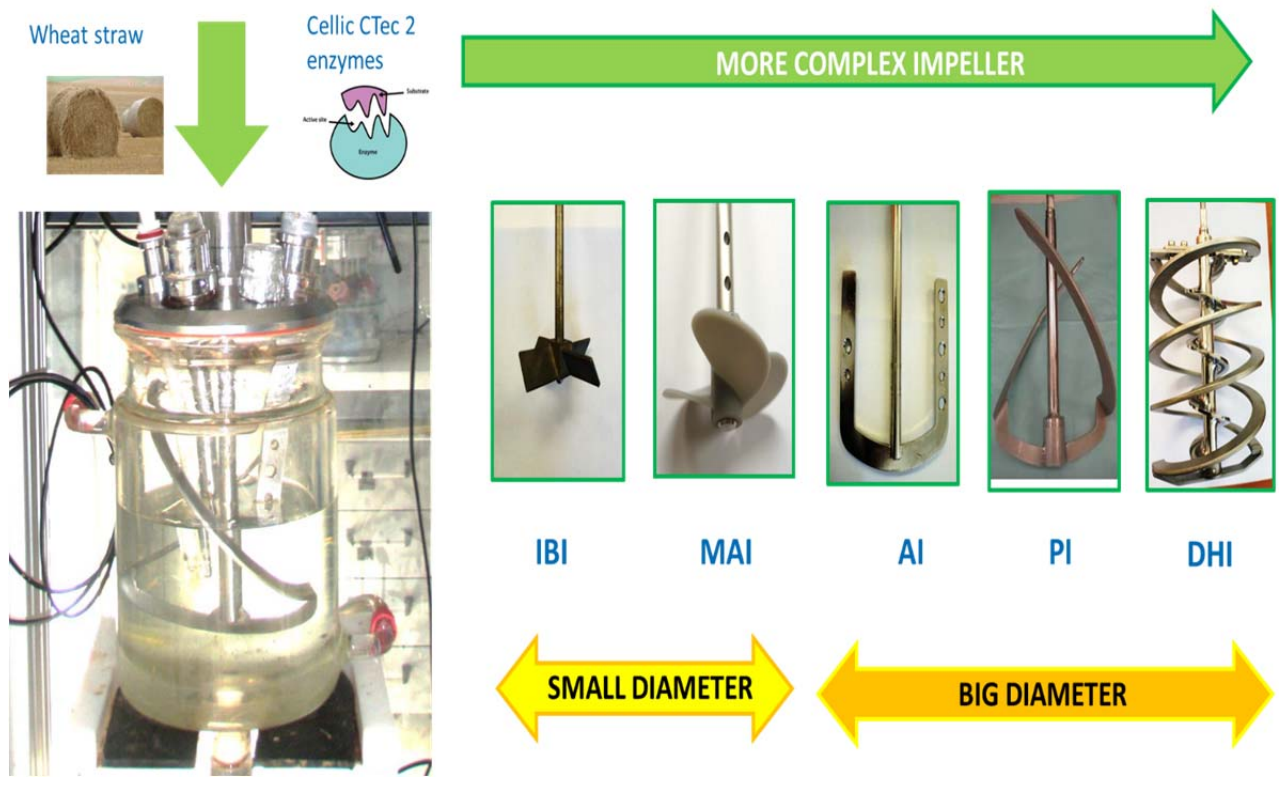
69 of 70.79 % w/w and a cellulose content of 42.20 ± 2.07 % w/w. Cellulose content in the pretreated
70 WS has been determined by acid hydrolysis method [14].

71 Cellic CTec-2 (Novozymes) cellulase blend was used for all enzymatic hydrolysis tests. The
72 enzymes concentration and the density of the enzymatic cocktail are 217.20 mg protein/g and 1.21
73 kg/L, respectively. The amount of the enzymatic cocktail was of 44 mL and has been determined
74 through the methods by McIntosh et al. [15].

75 **2.2 The equipment**

76 The enzymatic hydrolysis of WS has been conducted in a 3L reactor (height 175 mm, diameter base
77 150 mm) (Figure 1), equipped with a torque meter Kistler 4503A measuring torque till a value of 2
78 Nm and with a data detection frequency variable from 1 to 10 Hz. The reactor was also equipped
79 with a water-heater and with temperature and pH control sensors.

80 In order to demonstrate the mixing system geometry's influence on the enzymatic hydrolysis at high
81 DM conditions, five different impellers have been adopted for the tests (Figure 1). Two impellers
82 were characterized by small diameter (90 mm): the Inclined Blades Impeller (IBI) and the Marine
83 Impeller (MAI). IBI was made in stainless steel and the blades was 20 mm high. An alternative
84 version of IBI, the PLASTIC IBI, was realized in plastic by a 3D printer, and was adopted only for
85 the group tests described in 2.3.2 paragraph. MAI was realized in plastic by a 3D printer too. Three
86 different impellers with a big diameter (140 mm) have been also considered for the tests: the
87 Anchor Impeller (AI), the Paravisc Impeller (PI) and the Double Helicoidal Impeller (DHI), all
88 realized in stainless steel. The characterization of the impellers has been reported in Table 1. All
89 the mixing systems have been located at 30 mm from the bottom of the reactor.



90

91 Figure 1. The reactor and the impellers used for the tests

92

Impeller	Diameter (mm)	Height of the blades (mm)	Number of blades
IBI	90	20	4
PLASTIC IBI	90	40	4
MAI	90	20	3
AI	140	100	2
PI	140	150	2
DHI	Internal helice: 50 external helice: 140	Internal helice: 155 external helice: 160	2

93 Table 1. Characterization of the impellers used for the tests

94 **2.3 Description of the tests**

95 Two groups of tests have been conducted along this work. The first group consisted in Batch and
 96 Fed-Batch 50 tests, with the aim to investigate the influence of the impeller geometry on the
 97 enzymatic hydrolysis. The second group has been realized to implement a fed-batch strategy, the
 98 FBGA, considering only the small diameter impellers: the MAI, the IBI and the PLASTIC IBI,

99 which resulted inadequate in the previous Batch and Fed-Batch 50 tests. All tests have been
 100 conducted in triplicate, to ensure their repeatability, at optimal operative conditions of 50°C, pH in
 101 the range of 5.0 – 5.5. pH was adjusted using a NaOH (2 N) solution. The abbreviations and the
 102 descriptions of the tests have been reported in Table 2.

103

Labels	Description of the tests
B-IBI	Batch test with inclined blades impeller
B-MAI	Batch test with marine impeller
B-AI	Batch test with anchor impeller
B-PI	Batch test with paravisc impeller
B-DHI	Batch test with double helicoidal impeller
FB50-IBI	Fed batch test with 50% of the total mass of the reaction medium loaded at the beginning with inclined blades impeller
FB50-MAI	Fed batch test with 50% of the total mass of the reaction medium loaded at the beginning with marine impeller
FB50-AI	Fed batch test with 50% of the total mass of the reaction medium loaded at the beginning with anchor impeller
FB50-PI	Fed batch test with 50% of the total mass of the reaction medium loaded at the beginning with paravisc impeller
FB50-DHI	Fed batch test with 50% of the total mass of the reaction medium loaded at the beginning with double helicoidal impeller
FBGA-IBI	Fed batch with gradual addition of WS. Metallic inclined blades as impeller
FBGA-PLASTIC IBI	Fed batch with gradual addition of WS. Plastic inclined blades as impeller
FBGA-MAI	Fed batch with gradual addition of WS with marine impeller

104 Table 2. Abbreviations and descriptions of the tests

105

106 2.3.1 Batch and Fed-Batch 50 tests

107 Batch and fed-batch 50 tests (Table 2) have been prepared in order to have a constant DM
 108 concentration of 20% *w/w*, along all the test duration.

109 For batch tests the reactor was fed with 2.8 kg of WS-water mixture (0.85 kg of WS). These batch
 110 tests, where the entire reaction medium has been charged at the beginning of the enzymatic
 111 hydrolysis, represented the extreme situation for the mixing. In Fed batch 50 tests only the 50% of
 112 the 2.8 kg WS-water mixture was fed at the beginning of the tests. The rest of the mass has been
 113 added 10, 30, 60, 105 and 120 minutes after the beginning of the test in equal parts. At these times
 114 the apparent viscosity dropped under 150 cP for all the tests, a good mixing was observed allowing

115 new WS addition. This criterion was not applicable for FB50-IBI which did not demonstrate
116 improvements in mixing. The duration of each test has been established at 5 hours, while the mixing
117 devices operated at 80 rpm.

118 2.3.2 Fed Batch Gradual Addition (FBGA) tests

119 FBGA tests (Table 2) have been conducted using only the small diameter size impellers (IBI,
120 PLASTIC IBI and MAI). Compared to the previous fed batch strategy, where the DM concentration
121 was always constant at 20 % *w/w*, the FBGA tests contemplated a gradual increasing of DM content
122 during the test. 1,950 g of water and 250 g of WS have been charged in the reactor at the beginning
123 of the test, then the remaining 600 g of WS have been fed after 1.0, 2.5, 3.5, 19.0 and 24.0 hours, to
124 reach 20% DM *w/w*. In this way, a gradual liquefaction of the WS particles was possible, without
125 overcoming the critical apparent viscosity, which has not allowed an adequate mixing with IBI and
126 MAI in batch tests. The choice to adopt these small impellers does not constitute a contradiction. In
127 fact, one of the major goal of this research is to demonstrate how feeding strategy can influence the
128 reactor's rheology and allow the mixing system simplification. Therefore the positive performances
129 of FBGA strategy with simple impellers are surely valid with the big diameter ones, which achieved
130 good performance in batch test, where harder rheological conditions were applied.

131 To find the FBGA best operative conditions, three preliminary tests have conducted with IBI, which
132 recorded the worst performances in the previous Batch and Fed-batch 50 tests:

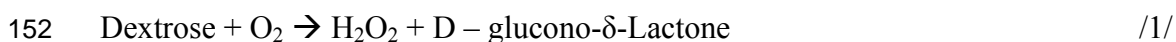
- 133 a) To determine the FBGA ideal duration, a five days test has been realized, monitoring the
134 energetic consumption to assure the mixing for gram of glucose produced. This test has been
135 conducted at 80 rpm, in order to be compared with the previous ones
- 136 b) To verify the mechanical stress influence on the enzymatic activity, a test at high (200 rpm)
137 rotational speed was conducted;
- 138 c) Tests with different enzymes strategy: i) Zero Enzymes test (ZE), where the 44 ml of the
139 enzymatic cocktail have been added at the beginning of the test, and ii) Gradual Enzymes

140 (GE) test, where the 44 ml of the enzymatic cocktail have been divided in 6 equal parts and
141 added simultaneously with WS.

142 **2.4 Analytical methods**

143 The DM content of WS has been determined according to standard methods described in literature
144 [16]. DM represents the content of solids present in the substrates, including the inert materials and
145 the degradable ones [17].

146 The apparent viscosity of the WS-water mixtures have been determined at 20% DM *w/w* before the
147 beginning of the enzymatic hydrolysis. The equipment used for the apparent viscosity measurement
148 and data recollection was the viscometer DV-II-PRO by Brookfield provided with a cross rotating
149 spindle working at 80 rpm. The glucose concentration has been quantified by an enzymatic reaction
150 using the GLUCOSTAT YSI2700. D-glucose determination was possible by the glucose-oxydase
151 enzyme, immobilized in the Dextrose YSI membrane. The occurring reaction was the following:



153 The oxygen water produced by the previous reaction was oxidised by a silver electrode: the released
154 electrical current was proportional to the glucose concentration.

155 **2.5 Definition of the parameters used for the evaluation of the tests**

156 The evaluation of the performances has been realised by three different parameters considering the
157 most affecting factors all the bio-technological processes: the reaction medium fluid-dynamic, the
158 mixing energy consumption and the glucose concentration.

159 **2.5.1 The Mixing Time**

160 Mixing time (*t_m*) is the characteristic parameter used to investigate the performance of stirred tank
161 reactors and it is often used as an indication of impeller effectiveness [18]. The shorter the mixing
162 time the more effective the blending [19]. The mixing time was determined by the pH pulse method
163 [20]: 10 mL of NaOH (2 N) solution will be put in the reaction medium and the mixing time was
164 estimated as the time required for the pH to reach 95 % of its final value. The mixing time

165 determination has been conducted at the beginning of the tests, when the adjustment of the acid
166 reaction medium is necessary to reach the operative pH value of 5.5, and at the end of the
167 hydrolysis, before the discharging of the reactor.

168 2.5.2 Power Input required by the mixing system

169 The power consumption was determined from the torque meter values. Because of the friction
170 factor, the torque generated by the motor (M_m) is not fully transmitted by the impeller to the
171 reaction medium [21]. The corrected torque value M_c was calculated by Equation 2:

$$172 M_c \text{ (Nm)} = M_m - M_r \quad /2/$$

173 where M_m is the measured torque and M_r is the residual torque, determined by measuring the
174 torque at 50 rpm in the empty vessel. The values of M_m were recorded each second by the torque-
175 meter for all the duration of the test. An average value of M_c has been calculated by Excel each 15
176 minutes (Δt) and used for the following calculation of the power (P) and mixing energy
177 consumptions (E):

$$178 P \text{ (W)} = M_c 2\pi N \quad /3/$$

179 Where N is the rotational speed. Finally, the mixing energy consumption is given by the equation:

$$180 E \text{ (J)} = \sum P_i \Delta t_i \quad /4/$$

181 Where P_i is the power consumption for the i -th time range Δt_i of 15 minutes (900 s).

182 2.5.3 Glucose concentration

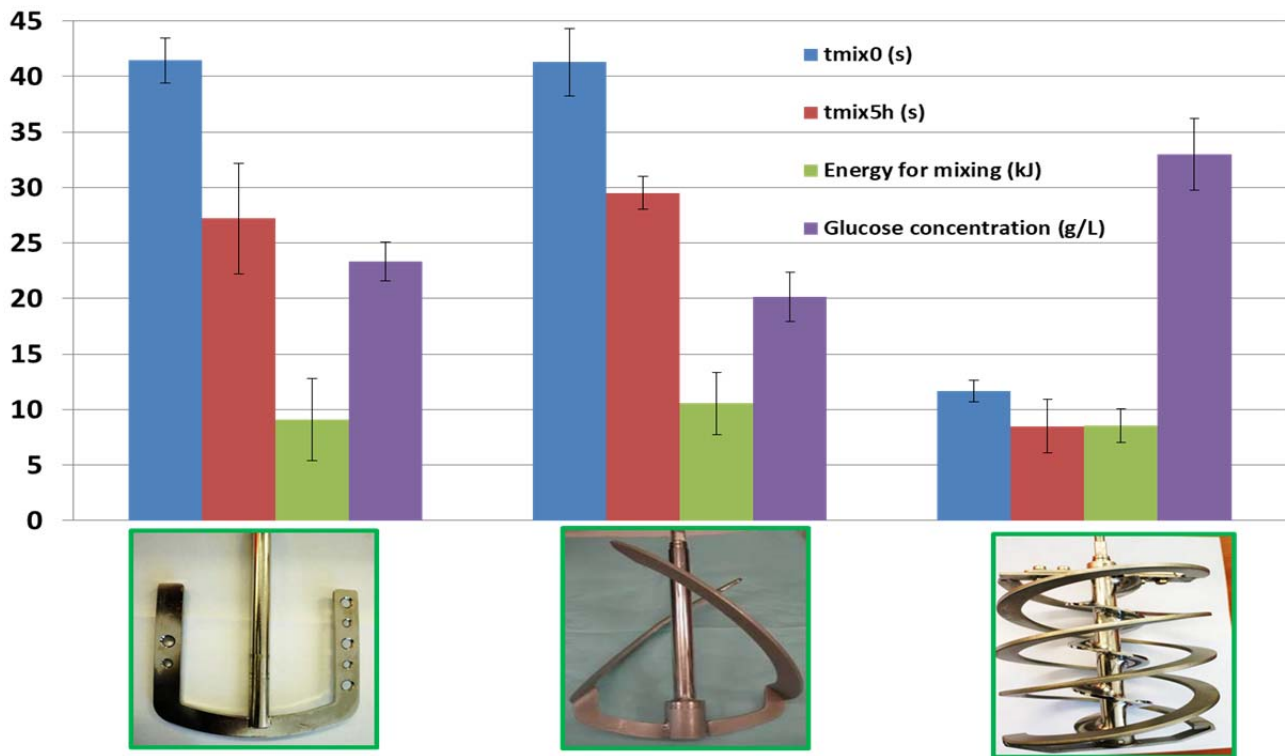
183 To evaluate the performance of the enzymatic activity, the cellulose conversion into glucose can be
184 used [22]. The glucose concentration was measured at the end of each test.

185 3. Results and Discussions

186 3.1 The influence of the impellers' geometry: Batch and Fed-Batch 50 tests

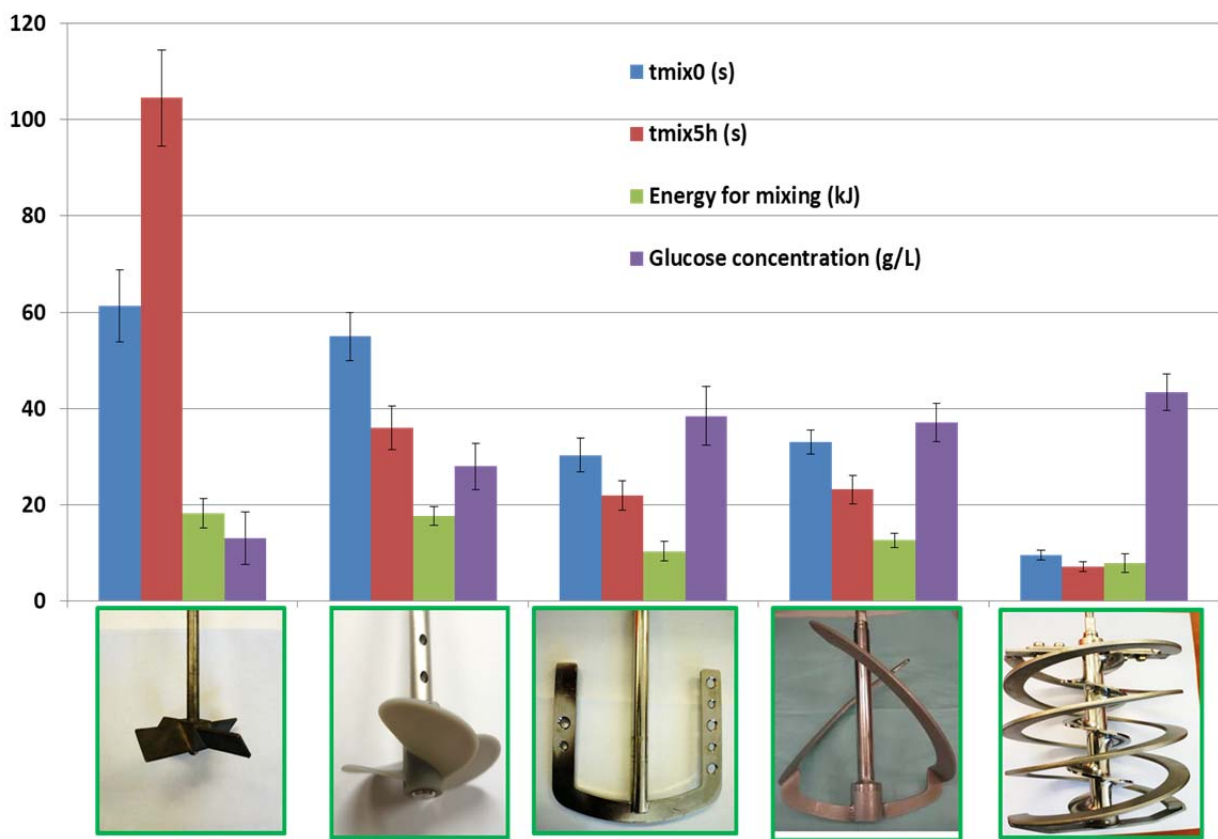
187 Figure 2 summarizes the performances of the big diameter impellers during the batch test. The
188 small devices IBI and MAI performances were not reported because the high reaction medium

189 apparent viscosity (about 350 cP), which has not permitted an adequate mixing at 20% w/w DM.
 190 MAI, in fact, assured the mixing only when the impeller rotational speed was increased to 250 rpm.
 191 Du et al. [23] demonstrated that increasing the rotational speed of the mixing device caused a
 192 reduction of the cellulose conversion into glucose, mainly when the DM concentration was higher
 193 than 10% w/w.



194
 195 Figure 2. Mixing time, energy consumption for mixing and glucose concentration of the batch tests
 196 Instead, AI, PI and DHI, characterized by tall and big diameter blades, were able to supply a
 197 sufficient torque to guarantee the reaction medium mixing at 80 rpm. AI and PI achieved similar
 198 results (Figure 2): the mixing time at the end of the test were 27 and 29 s respectively, the energetic
 199 consumption for the mixing of 9.12 and 10.50 kJ and the glucose concentration of 23.36 g/L and
 200 20.12 g/L. Anyway, the mixing was not perfect with AI: a 15 mm layer of sedimented particles was
 201 present at the bottom of the reactor. Nagata [24] reported that AI is suitable for the mixing of
 202 viscous liquids, but is not recommended for completely uniform mixing. More recently, this
 203 concept has been confirmed by Patel et al. [25], who explained that AI primarily generates a strong
 204 radial boost, but is not able to assure an axial movement, indispensable to avoid particles

205 sedimentation. For these reasons, a combination of AI with another impeller is recommended, to
 206 guarantee also some axial boots to the fluid [26].
 207 The best performances with the batch mode have been achieved by DHI, the most complex impeller
 208 used along this work (Table 1). The DHI dimension and shape allowed assuring adequate radial and
 209 axial boots to all the mass of the reaction medium. The good mixing performances were certified
 210 by smaller levels of mixing time and energetic consumption of 8.5 s and 8.54 kJ, respectively. The
 211 rheological improvements allowed the glucose concentration increasing to 33 g/L, confirming that
 212 the correlation between bioconversion activity and mixing [27].



213
 214 Figure 3. Mixing time, energy consumption for mixing and glucose concentration of the fed-batch
 215 50 tests

216 Figure 3 shows the performances of the fed-batch 50 tests. The first relevant fact is that all the
 217 impellers were able to assure the mixing at 80 rpm. It depended to the feeding strategy: only the
 218 50% of the total reaction medium was fed at the beginning of the test. In this way, the liquefaction
 219 of the particle of this first WS addition reduced the apparent viscosity from about 350 cP to 80 cP,

220 permitting the following WS additions. Fed-batch strategy, in fact, offers advantages in the
221 enzymatic hydrolysis over the batch mode: the initial substrates quantity fed into the reactor is
222 lower, so diffusion and mixing limitations can be minimized [13]. Anyway, the different impellers
223 have not worked at the same way. The worst performances were obtained with the IBI, which was
224 the unique case where mixing time increased during the enzymatic hydrolysis: it passed from 61 to
225 104 s. The high value of mixing time at the beginning of FB50-IBI demonstrated that the mixing
226 was not good. In addition, the liquefaction of the first 50% of the total WS mixture was not
227 complete when the following additions occurred, giving a further increasing of the reaction medium
228 apparent viscosity. Consequentially, the energetic consumption was the highest of all the fed-batch
229 tests (18 kJ), while the glucose concentration the lowest (13 g/L). B-IBI and FB50-IBI tests
230 demonstrated that IBI was inadequate for the mixing of Non-Newtonian and viscous fluids, because
231 it transmitted radial and axial boosts of weak intensity to the reaction medium, which can involve
232 only the region close to the impeller blades, leaving stagnant conditions in the other regions of the
233 reactor [25].

234 MAI achieved better performances: the mixing time decreased during the FB50-MAI test from 55 s
235 to 36 s, and glucose concentration reached the 28 g/L. This improvement is explicable considering
236 the MAI shape, designed to transmit a strong axial boots [28], which assured the mixing at the
237 whole reaction volume [17]. Anyway, the energetic consumption remained high of 17 kJ,
238 confirming that MAI was not the ideal configuration for high viscous fluid mixing.

239 The performances of fed-batch 50 tests achieved by big diameter size impellers (AI, PI and DHI)
240 have followed the same trends of batch tests (Figure 3). AI and PI obtained similar results, with
241 marginal sedimentation phenomena with AI. DHI confirmed the best performances, with very low
242 mixing time and energetic consumptions of 7.2 s and almost 8 kJ and a very high glucose
243 concentration value of 43.4 g/L after only 5 h of enzymatic hydrolysis. Feb-batch 50's better
244 performances can be also justified considering that the reaction medium gradual addition allow to
245 minimize the enzymes deactivation phenomena. Some works, in fact, claim for inhibition of

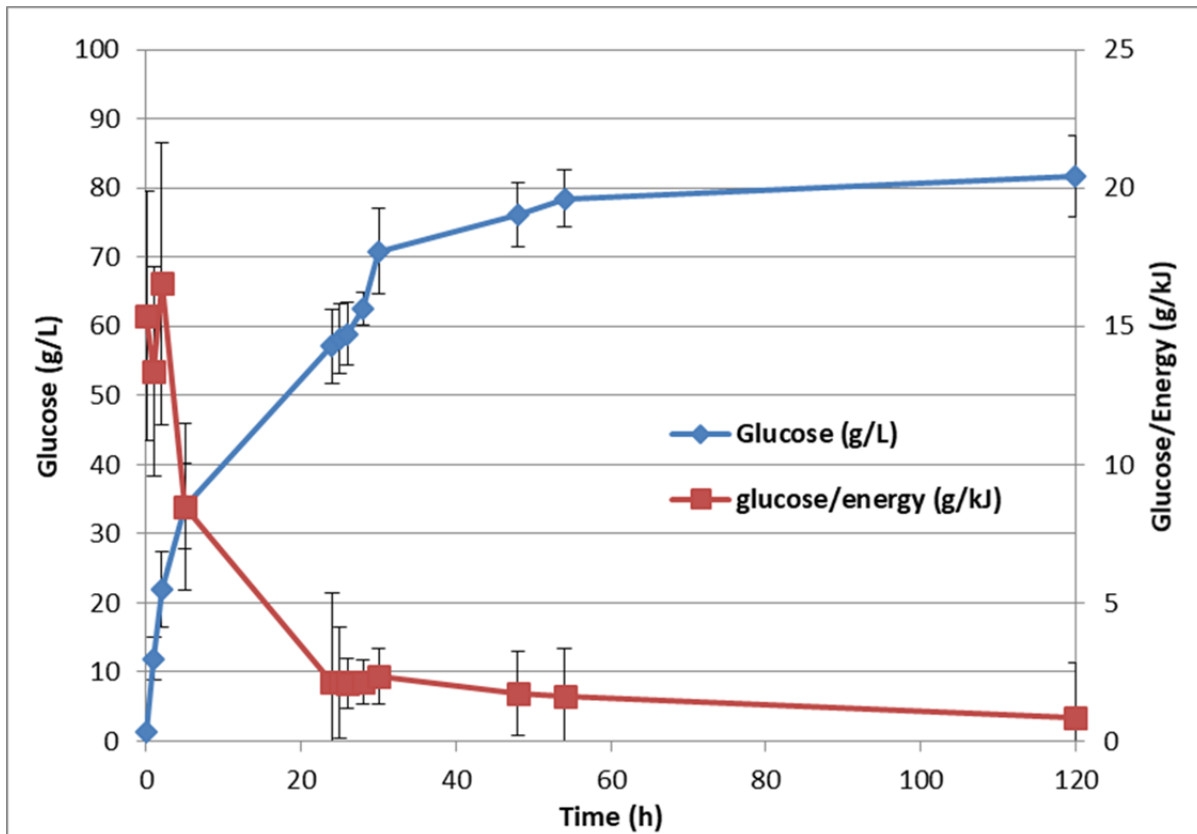
246 endoglucanases and exoglucanases when large concentrations of cellobiose and glucose, are present
247 in the reaction medium. Cellobiose can be consider an intermediate product in enzymatic reactions
248 which permit the cellulose degradation in glucose. Cellobiose is able to more affect exoglucanases
249 and endoglucanases, whilst the action of β -glucosidases could be more affected by glucose when its
250 concentration is more than 40 g/L [29], [30].

251 **3.2 FBGA tests**

252 The rheology of the reactor are not dependent univocally by the geometry of the mixing device, but
253 also by the strategy of the substrates addition, as remarked by Mondebach and Nokes (2013) [13].
254 In order to demonstrate the feeding strategy importance, the FBGA has implemented using simpler
255 and smaller impellers.

256 3.2.1 Operative conditions to optimize FBGA strategy

257
258 As previously reported, three preliminary tests have been conducted to find the operative conditions
259 which permitted to optimize FBGA strategy. Figure 4 shows the glucose concentration in the
260 reaction medium and the amount of glucose produced for kJ of electricity used to assure the mixing.
261 The glucose had an exponential increasing in the first 24 hours of the test, then the growth was
262 slowest. 50-70 g glucose were produced for kJ during the first two hours of the test. The production
263 was 10-15 g/kJ after 24 h, and it declined under 10 g/kJ after 48h from the beginning of the test.



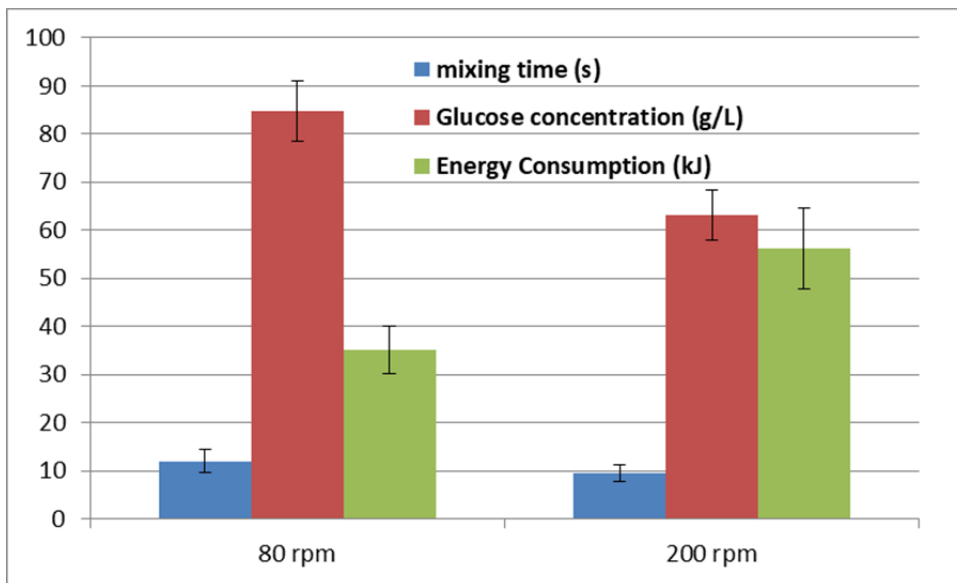
264

265 Figure 4. Trends of glucose and of glucose/energy ratio during enzymatic hydrolysis

266 For these reasons, the ideal duration for FBGA strategy has been established at 48h.

267 The impeller rotational speed was another important operative condition. The influence of low (80
 268 rpm) and high (200 rpm) speed on the enzymatic hydrolysis was investigated (Figure 5). On one
 269 hand a slightly improvement of the mixing time, from 12 s to 9.5 s, has been found at 200 rpm. On
 270 the other hand, this not sensible reduction, was payed by a strong energetic increasing in the
 271 energetic consumption for mixing, whose value passed from 35 kJ at 80 rpm to 56.5 kJ. Lastly,
 272 working at high rotational speed reduced the glucose concentration of about 25%, from almost 85
 273 g/L to 62 g/L. This phenomena is explicable considering the enzymes chemical nature. They are
 274 protein whose structure is stabilized by weak forces, where the free energy difference between the
 275 native and completely denatured state is often of few kcal/mol. Thus, a multitude of physical and
 276 chemical parameters can cause of perturbations of the geometrical and chemical structure of the
 277 protein, whit concomitant reduction in activity [12]. In this case, mechanical stress, due to the high
 278 rotational speed of the impeller, was the factor reducing the enzymatic activity and consequentially

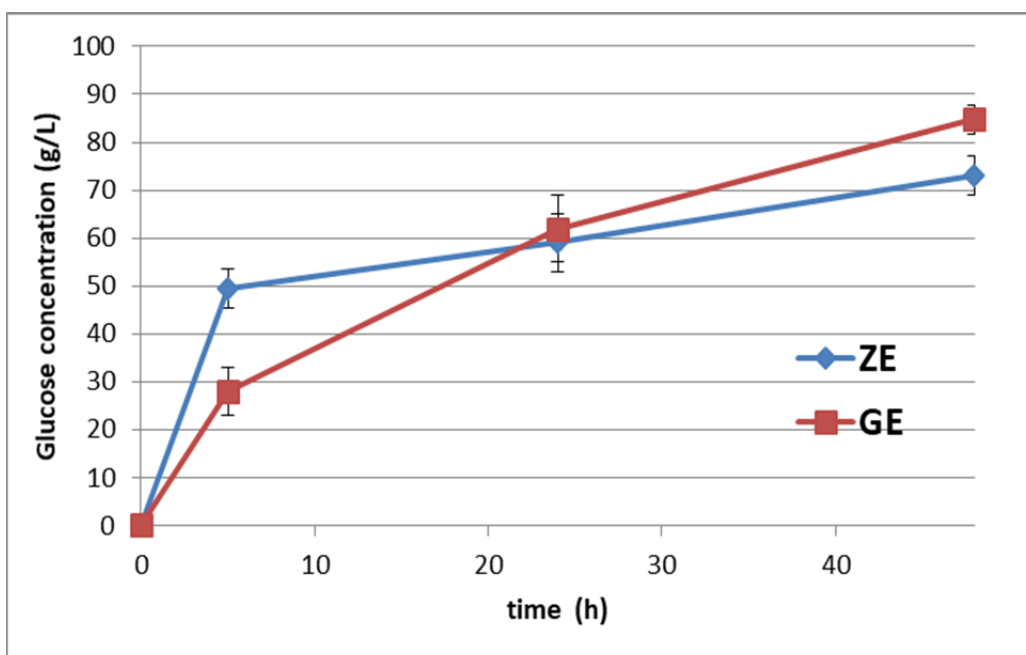
279 the glucose concentration of the test. The mechanical stress' removal usually comports a new
280 increasing of the enzymatic catalysis but at lower levels than the original ones [31].
281 It was so demonstrated that FBGA was able to reach better performances in term of glucose
282 concentration and energetic consumption at low rotational speed and 80 rpm was selected as
283 operative speed for FBGA strategy.



284

285 Figure 5. Effect of the rotational speeds on the enzymatic hydrolysis

286 Finally, the adoption of the best enzymes addition strategy has been investigated. Figure 6 shows
287 the difference in glucose concentration between ZE and GE strategies.



288

289 Figure 6. Effect of the enzymes addition strategy on enzymatic hydrolysis
290 The glucose growth was fast in ZE, where all the amount of enzymes was immediately available for
291 the cellulose conversion. By the time, these too rapid glucose accumulation in the reaction medium,
292 caused the inhibition of the enzymes. As previously described, it was demonstrated that cellobiose
293 accumulation negatively affected exoglucanases and endoglucanases enzymes, while the β -
294 glucosidases enzymes were more affected by a high glucose concentration [32]. Instead, the gradual
295 addition of the enzymes in GE test permitted to replace the inhibited ones. The glucose increasing
296 was slowest than ZE along the first 24h of the test, but a higher glucose concentration was achieved
297 at the end of the test (Figure 6). GE strategy was adopted for the FBGA tests.

298 3.2.2 FBGA tests with small diameter impellers

299
300 The optimized FBGA gave very good performances: MAI, IBI and PLASTIC IBI achieved a
301 glucose concentration of 80 -85 g/L, with a low energetic consumption between 35-40 kJ in 48 h of
302 enzymatic hydrolysis. Three considerable goals were achieved by FBGA strategy: i) the improving
303 of the cellulose conversion into glucose, ii) the simplification of the mixing system and iii) the
304 reduction of the energetic consumption. These results demonstrate that viscosity problem is not
305 present with FBGA strategy because it allows keeping the viscosity values always under the critical
306 value beyond which the mixing was not efficient with small diameter impeller in batch and fed-
307 batch tests, allowing the use of simple mixing devices.

308 **Conclusions**

309 The influences of the impellers geometry and of the feeding strategy on the enzymatic hydrolysis at
310 high DM lignocellulosic content were studied. It was demonstrated that small and simple diameter
311 were inefficacy in batch condition, while big diameter and complex impellers, especially DHI, had
312 good performances in batch and fed-batch 50 modes.
313 Instead, FBGA strategy, optimized opportunely for high DM content, achieved the adoption of
314 simple and small diameter, a very high glucose concentration and low energetic consumption for

315 the mixing by a gradual DM increasing within the reactor. This allows to demonstrate that the
316 rheology is influenced both by the impeller's geometry and by the reactor' feeding strategy.

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401 **LIST OF CAPTIONS**

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