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1 **The impact of fluid-dynamic stress in stirred tank bioreactors on the**
2 **synthesis of cellulases by *Trichoderma reesei* at the intracellular and**
3 **extracellular levels**

4

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15

16 **Abstract**

17

18 Cellulases for bioethanol production are mainly made by fed-batch fermentation using a
19 filamentous fungus, *Trichoderma reesei*. Agitation at different scales impacts on
20 morphology, rheology and growth rate and can be correlated by $EDCF_{\epsilon_{max}}$. Typically,
21 $EDCF_{\epsilon_{max}}$ is much smaller at commercial scale and fungal size, viscosity and growth rate are
22 greater. Here, to increase understanding, continuous culture in 2.5 L bioreactors using two
23 $EDCF_{\epsilon_{max}}$ values. The higher $EDCF_{\epsilon_{max}}$ decreased the cellulase production (concentration, 21
24 %; specific production rate, 24 %; protein yield, 20 %) whilst proteomic analysis showed, at
25 an intracellular level, a decrease of cellulase and hemicellulase synthesis. An increase of
26 stress proteins also occurred, which may help cells to limit the impact of fluid dynamic
27 stress. Also, cellulase production during continuous culture at the bench varied with
28 $EDCF_{\epsilon_{max}}$ similarly to that between bench and commercial scale during fed-batch culture.

29

30 **Key-words:** *Trichoderma reesei*; cellulases; fluid dynamic stress; scale-up/scale-down;
31 proteomic analysis.

32

33 **Highlights**

34

- 35 • Higher values of $EDCF_{\epsilon_{max}}$ reduce production of extracellular cellulases
- 36
- 37 • This reduction is linked to a lowering of intracellular synthesis of cellulases
- 38
- 39 • Higher fluid dynamic stress leads to the appearance of stress proteins
- 40
- 41 • Intracellular stress proteins induce moderate changes in q_p from large ones in $EDCF_{\epsilon_{max}}$
- 42

43 1. Introduction

44 Second-generation (2G) bioethanol from lignocellulosic materials, is a good
45 candidate to replace conventional fuels. Indeed, 2G bioethanol allows a reduction of about
46 85% of greenhouse gas emissions compared to fossil fuels (Morales et al., 2015). Also, 2G
47 bioethanol uses lignocellulosic waste from the agri-food and forestry and does not compete
48 with food. However, production costs remains high compared to ethanol from starch. This
49 increase is mainly due to the high price of cellulases, the enzymes which hydrolyze
50 lignocellulosic biomass into simple sugars. The 2G bioethanol price can be reduced if
51 cellulases production costs are lowered (Hardy et al., 2017). Economy of scale is a way of
52 doing so, requiring the use of bioreactors of several hundred cubic meters, a huge size
53 compared with that at which the process is developed. With increasing scale, the medium in
54 the bioreactor experiences increasing spatial heterogeneities (Amanullah et al., 2004; Lara
55 et al., 2006) and the fluid dynamic stresses change (Hardy et al., 2017); and it is important to
56 know what their impact will be.

57 Cellulolytic enzymes are mainly produced at industrial scale by the filamentous
58 fungus *Trichoderma reesei* because of its high cellulases secretion capacity (Ferreira et al.,
59 2014; Soni et al., 2018). As enzyme production is dissociated from growth, industrial
60 cultures are often carried out in two steps: a batch phase for growth of the fungus followed
61 by a fed-batch phase for the production of cellulases (Jourdier et al., 2013). During growth,
62 *T. reesei* can use different carbon sources, but glucose is the most common (Hardy, 2016).
63 As the *T. reesei* grows, it develops hyphae which form complex structures (Hardy et al.,
64 2017b).. The complex morphology and high concentrations at the end of growth lead to a
65 high viscosity medium with shear thinning behavior (Gabelle et al., 2012; Hardy et al., 2015;
66 Quintanilla et al., 2015).

67 The production phase starts when the growth stops by limiting the carbon source to
68 induce the production of cellulases (dos Santos Castro et al., 2014). In this phase, soluble
69 sugars such as sophorose, cellobiose and lactose are used to induce the secretion of
70 cellulases. In order to avoid further growth, an inductive substrate is fed continuously at an
71 optimal limiting rate (Jourdier et al., 2013; Hardy et al., 2017). As *T. reesei* is a strictly
72 aerobic microorganism, the dissolved oxygen concentration (dO_2) must be above the critical
73 value, 15 % dO_2 (1.2 mg of O_2/L) (Marten et al., 1996) for the strain RUT-C30. However, the
74 high viscosity at the end of the batch phase reduces mass transfer rates, and oxygen
75 transfer (Albaek et al., 2012; Gabelle et al., 2012). To ensure $dO_2 > 15\% dO_2$ at this time, the
76 power input has to be increased (Gabelle et al., 2012), enhancing the fluid dynamic stress on
77 the mycelia.

78 There are many ways of characterizing fluid dynamic stress with changes in agitation
79 intensity and with scale (Amanullah et al., 2004). Until the 1990s, the two most common
80 were tip speed or specific power input, P/V ($W \cdot m^{-3}$). However, in 1996, it was shown with
81 the fungus, *P. chrysogenum*, that whilst tip speed increased with scale at constant specific
82 power, damage to the organism was reduced (Jüsten et al., 1996). However, a new function
83 which reduced with increasing scale, the energy dissipation/circulation function, *EDCF*, was
84 able to correlate fungal breakage and various growth parameters, better than P/V (Jüsten et
85 al., 1996). In essence, the *EDCF* concept is that fungus break to an equilibrium size
86 dependent on the maximum specific energy dissipation rate, ϵ_{max} ($W \cdot m^{-3}$) in a region close
87 to the impeller and the frequency with which the organism passes through that region, $1/t_c$
88 (s) where the circulation time, $t_c = V/(Fl \cdot N \cdot D^3)$. Here, V (m^3) is the volume of broth in
89 the bioreactor, N ($rev \cdot s^{-1}$) the agitator speed, D (m) its diameter and Fl (dimensionless), its
90 flow number. Because the circulation time increases with increasing scale even at constant

91 P/V , $EDCF$ generally decreases with scale, sometimes markedly. There are a number of
92 ways of assessing ε_{max} and a recently developed definition by Grenville et al. (2017) is
93 $\varepsilon_{max} = 1.04 \cdot x \cdot \rho \cdot Po^{\frac{3}{4}} \cdot N^3 \cdot D^2$. Here, x is the ratio of impeller diameter to trailing vortex
94 diameter, a dimensionless characteristic of the impeller being used, as is Po , the power
95 number; and ρ ($\text{kg} \cdot \text{m}^{-3}$) is the density of the broth. In 2017, we used this definition of
96 ε_{max} to develop (Hardy et al., 2017) an improved $EDCF$ function, $EDCF_{\varepsilon_{max}}$. We then used
97 $EDCF_{\varepsilon_{max}}$ to correlate the impact of the fluid dynamic stress associated with a variety of
98 impellers on various process parameters during the initial batch growth phase of *T. reesei*
99 fermentations at the bench scale and also with a multiple impeller combination at the
100 industrial scale.

101 Other filamentous organisms to which the $EDCF$ has been applied are *T. harzianum* (Rocha-
102 Valadez et al., 2007), *A. oryzae* (Amanullah et al., 2002) and *P. ostreatus* (Fernández-
103 Alejandro et al., 2016), where it was related to the transition of the organism from clumps
104 to pellets. With *T. reesei* (Hardy et al., 2017), higher values of $EDCF_{\varepsilon_{max}}$ reduced the mycelial
105 size and specific growth rate (by up to 20 %) at the bench scale whilst at the commercial
106 scale, as explained above, though the tip speed increased, $EDCF_{\varepsilon_{max}}$ decreased leading to
107 larger mycelia, higher viscosity and higher growth rates. However, as the bench runs were
108 limited to the growth phase, cellulases were not produced; so the impact of fluid dynamic
109 stresses on their production could not be investigated.

110 Previous work on the proteome of *T. reesei* has been studied in order to better
111 identify the enzymes involved in lignocellulosic biomass degradation and the protein
112 secretion profile under different environmental conditions (Herpoël-Gimbert et al., 2008).
113 Apart from the common proteins involved in general metabolism, it mostly consists of two
114 cellobiohydrolases (CBHI and CBH2), five endoglucanases (EGI, EGII, EGIII, EGIV and EGV)

115 and two β -glucosidases (BGLI and BGLII) that act in synergy to degrade lignocellulosic
116 materials (Herpoël-Gimbert et al., 2008). Proteomic studies have investigated the secretome
117 and/or intracellular proteins synthesized by filamentous fungi (Peterson and Nevalainen,
118 2012) by combining one-dimensional (1D) or two-dimensional (2D) gel electrophoresis with
119 protein identification by liquid chromatography/ mass spectrometry. This technique allows
120 the analysis of the multicomponent cocktail of cellulolytic enzymes secreted by *T. reesei* in
121 culture media (Herpoël-Gimbert et al., 2008; Jun et al., 2013; Kubicek, 2013), and to
122 investigate the differential protein synthesis at intracellular level (Bianco and Perrotta,
123 2015). The influence of carbon source (Jun et al., 2013; dos Santos Castro et al., 2014;
124 Peciulyte et al., 2014), pH (Adav et al., 2011), light intensity (Stappler et al., 2017) and
125 agitation intensity (Mukataka et al., 1988; Lejeune and Baron, 1995) on extracellular enzyme
126 secretion has also been studied. However, except for Jun et al., 2013, which compared its
127 intracellular proteome with various carbon sources; and Arvas et al., 2011 that studied the
128 intracellular effects of growth rate and cell density in chemostat culture, the impact of
129 culture conditions on intracellular protein synthesis by *T. reesei* remains poorly investigated.
130 To our knowledge, the impact of fluid dynamic stress (here expressed as $EDCF_{\epsilon_{max}}$) on the
131 synthesis of intracellular proteins by *T. reesei* has never been reported.

132 The objective of this work therefore was to characterize the effects of fluid dynamic
133 stress on the production of both intracellular proteins and extracellular cellulases by
134 *T. reesei*.

135 2. Material and Methods

136 2.1. Strain, preculture and culture media

137 The *T. reesei* strain used and the composition of the preculture medium and of the
138 batch-culture medium were fully described previously (Hardy et al., 2017). For the
139 production phase, the medium had the same composition as the batch-culture medium but
140 it was supplemented with 0.83 g.L⁻¹ yeast extract instead of powdered corn steep liquor.

141 A 150 mL preculture was prepared in a 2 L Fernbach flask by mixing 135 mL of
142 preculture medium and 15 mL of a sterile glucose solution at 250 g L⁻¹. The flasks were
143 seeded with 1 mL of conidia to give a seeding rate of 10% and held in a shaker incubator
144 (Multiron II, Infors, Bottmingen, Switzerland) for 72 hours at 30°C and 180 rpm with an orbit
145 of 50 mm before being added into the bioreactor culture.

146 2.2. Bioreactor cultures using two different fluid dynamic stress conditions

147 This work is a follow on to that reported in Hardy et al. (2017). In that that study,
148 the initial batch production of *T. reesei* was studied at the bench scale of 2.5 L working
149 volume and at commercial scale. At the bench scale, a Rayneri centripetal turbine impeller
150 (VMI-mixing, Montaigu, France) (see Fig 1) was used along with 3 others. All of the changes
151 in process performance brought about by fluid dynamic stress for each impeller were
152 successfully related to changes in $EDCF_{emax}$. In order to move to continuous culture, a
153 different bioreactor had to be selected and one of a similar size was available which used a
154 Rayneri impeller. Since that impeller had fitted in well with the others in the earlier work,
155 in order to have bench scale data in a similar sized bioreactor to aid comparison between
156 the different aspects of the overall study, the present configuration was chosen.

157 A 3.0 L, dished bottomed, stirred bioreactor (ezControl BioBundle, Applikon Biotechnology,
158 Foster, CA, USA) with a working volume of 1.5 L, diameter $T = 126$ mm with a broth height,
159 H mm such that $H/T = 1$ and without baffles but with sufficient probes inserted to provide a
160 significant baffling effect. The Rayneri centripetal turbine impeller was 0.08 m diameter,
161 height, 0.045 m with $Po = 2.0$ and $Fl = 1.3$. The temperature was controlled at 27 °C, pH at
162 4.8 and dissolved oxygen concentration at 70 % by introducing a blend of air and nitrogen
163 (thereby altering the driving force for mass transfer) through a sparger. The total flow rate
164 was held constant at 0.8 L/min so that neither the dO_2 or flow rate changed during the
165 experiment (Hardy et al., 2017). However, as in earlier work (Herpoël-Gimbert et al., 2008),
166 to control to this dO_2 at the bench scale even with gas blending required a high P/V (Hardy
167 et al., 2017) of at least $6 \text{ kW} \cdot \text{m}^{-3}$, which needed a minimum speed of 800 rpm. Such speeds
168 led to very high $EDCF_{\text{max}}$ compared to the commercial scale, where the circulation time is
169 significantly longer and the specific power was kept as low as possible for economic reasons.

170 Two complete runs, each consisting of 3 parts, were undertaken. In each, there was
171 an initial batch phase during which the fungus was grown at an agitation speed of 800 rpm
172 until a fungal biomass concentration of $\sim 7.9 \pm 0.2 \text{ g} \cdot \text{kg}^{-1}$ was reached. Then, the continuous
173 phase of the run was begun, thereby commencing cellulase production. That time is marked
174 as 'start of continuous feed' on Fig 1 and from then, it was held at 800 rpm for another 150
175 hrs which allowed time for approximately 5 volume changes so that a steady state should
176 have been reached (Macauley-Patrick and Finn, 2008). The concentration of biomass and
177 the production of cellulases also indicated an approximate steady state for 50 hrs. At that
178 point, the speed was increased to 1700 rpm to give a higher fluid dynamic stress and then
179 kept constant with the same feed and discharge rate. This condition was held constant for
180 another approximately 140 hrs, equivalent to approximately 4 volume changes, sufficient to

181 approach another steady state and allow the impact of the change on biomass and
182 intracellular and extracellular proteins to be assessed. The experiment was duplicated and
183 samples were withdrawn at regular intervals for biomass and protein concentration
184 measurements and those taken at the times noted on Fig 1 were used for proteomic
185 analysis.

186 In the batch phase, the bioreactor contained 0.75 L 2N culture medium, 0.3 L water
187 and 0.3 L glucose solution (150 g.L^{-1}) and it was inoculated with 0.15 L preculture to give an
188 initial fungal concentration in the medium of 0.8 g.kg^{-1} . During the whole of the continuous
189 phase, the constant feed and discharge rates of two solutions were lactose (180 g.L^{-1}) at
190 6 mL.h^{-1} and the enriched 2N medium at 39 mL.h^{-1} giving a dilution rate, D (0.03 h^{-1}) which
191 since $D = \mu$, the growth rate, is also 0.03 h^{-1} .

192 **2.3. Determination of concentrations in the culture medium**

193 Biomass was quantified as described previously (Hardy et al., 2017) as was lactose
194 following the method used for glucose. The concentration of the cellulases produced was
195 quantified on filtered broth samples according to the Lowry method, by using the Protein
196 Assay DC_{TM} kit (Biorad, Hercules, CA, USA) and a range of bovine serum albumin (BSA)
197 concentrations from 0 to 1.5 g.L^{-1} as standards (Hardy, 2016). β -glucosidase activity was
198 measured as described by Jourdir et al. (2013). All analyses were performed in duplicate.

199 **2.4. Proteomic analyses**

200 The proteomic analyses of intracellular proteins were carried out in 3 steps: protein
201 extraction, two-dimensional (2D) electrophoresis on sodium dodecyl sulfate polyacrylamide
202 gel (SDS PAGE) and identification of proteins by mass spectrometry coupled to liquid
203 chromatography.

204 The cytoplasmic proteins were first extracted by cell grinding from a biomass cake
205 obtained by filtration as previously described. Initially, 0.2 g of biomass cake was washed
206 and re-suspended as described previously (Wang et al., 2011). The cells were lysed with 0.6
207 g of glass beads (0.3 g of diameter 0.1 mm and 0.3 g of diameter 0.5 mm, Biospec Products,
208 Bartlesville, OK, USA) in a FastPrep FP 120 equipment (Bio 101, Savant Instruments,
209 Holbrook, AZ, USA), for four times with cycles of 30 s at an intensity of 6.5. After grinding
210 the cells, the samples containing solubilized cytoplasmic proteins were centrifuged, purified
211 with 60 U endonuclease (Biorad, Hercules, CA, USA) and precipitated, and their protein
212 concentration was assayed using the colorimetric method of Bradford (1976) using bovine
213 serum albumin as a standard in order to ensure 300 µg of proteins were present (Wang et
214 al., 2011).

215 The separation of the extracted proteins was achieved by successive separation
216 according to their isoelectric point by isoelectric focusing (IEF) and to their molecular weight
217 by SDS-page electrophoresis. All samples from the two conditions were analyzed in triplicate
218 from three different extractions. The cell extracts containing 300 µg of proteins were diluted
219 in 350 µL of extraction buffer [31]. To perform IEF, the diluted samples were loaded onto
220 Immobiline DryStrip gels (pI 4-7, 17 cm, Biorad) that were submitted to electric focusing at
221 20°C on an IEF Cell (Biorad, Hercules, CA, USA) to reach 73,000 Wh for about 24 hours.

222 After equilibration of the DryStrip gels, the second dimension separation was
223 performed in SDS-polyacrylamide gels as described by Wang et al. (2011). Proteome image
224 analysis was carried out with PDQuest 2-D software (Biorad) to compare the proteins
225 density on the gels obtained from different conditions. Spots only present on one gel
226 condition or showing a difference in density greater than a factor of 2 were considered as
227 different between two conditions and retained for identification.

228 The spots corresponding to the differentially synthesized proteins were collected,
229 washed separately with 50 mM ammonium hydrogen carbonate and 50 % acetonitrile and
230 then dried. After digestion with 0.5 μg of trypsin (Promega, Madison, WI) and 50 mM
231 ammonium hydrogen carbonate for 12 h at 37 $^{\circ}\text{C}$, the supernatant containing peptides were
232 used for protein identification by tandem mass spectrometry coupled to liquid
233 chromatography (LC-MS / MS) using a LTQ-XL mass spectrophotometer (ThermoFinnigan,
234 Waltham, MA, USA) (PAPPSO, Gif-sur-Yvette, France). The sequences were identified using
235 the Joint Genome Institute databases (JGI, Walnut Creek, CA USA).

236 **3. Results and Discussion**

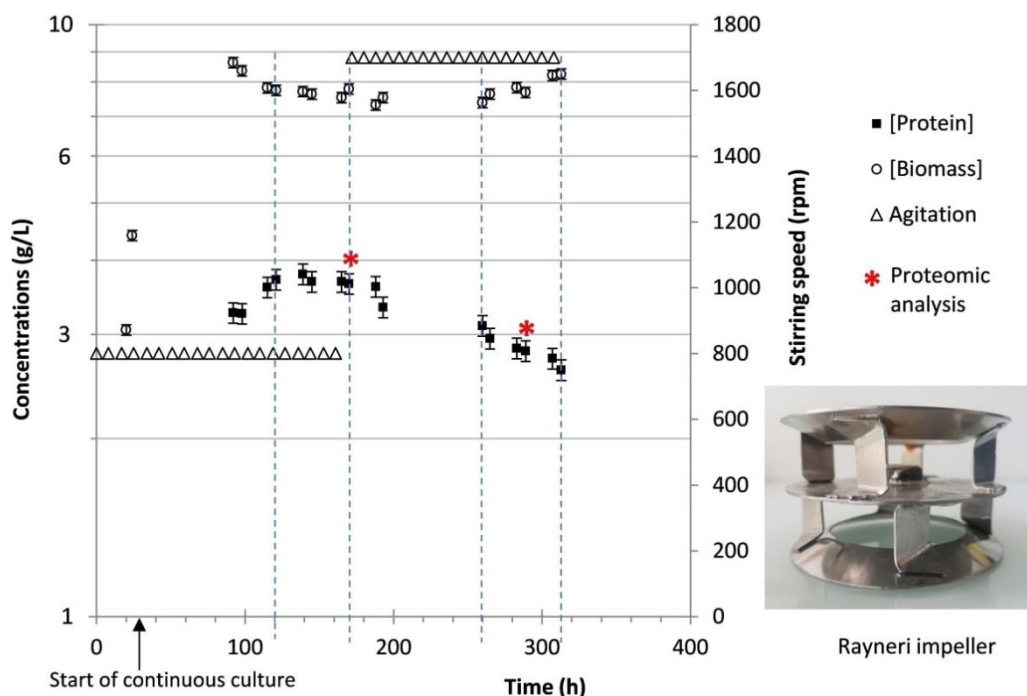
237 **3.1. Estimation of the fluid dynamic stress, $EDCF_{\epsilon_{max}}$**

238 In order to estimate ϵ_{max} (see Section 1), a value of x is required. The values (Grenville
239 et al., 2017) for different impellers range from 12 to 17 but one is not available for the
240 Rayneri impeller, which is a very different shape to others (see Fig 1). However, it
241 produces radial flow as does a Rushton turbine which has an x value of 12 and this
242 value is initially assumed here. Thus $EDCF_{\epsilon_{max}}$ was 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$
243 respectively, a little higher than used previously (Hardy et al., 2017) because though
244 similar speeds were used, here the bioreactor volume was smaller.

245 At the commercial scale, multiple impellers were used but the Rushton turbine
246 provided most of the power input (Hardy et al., 2017). Thus, both the Po value and x
247 of the Rushton turbine were chosen for the determination of $EDCF_{\epsilon_{max}}$ for the
248 commercial scale, which as explained earlier was very much lower at $\sim 6.65 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$
249 (Hardy et al., 2017). Overall, $EDCF_{\epsilon_{max}}$ was the best correlator of hyphal size, broth
250 rheology and growth rate for all the impellers and across these scales.

251 **3.2. Effect of fluid dynamic stress, $EDCF_{\epsilon_{max}}$ on the production of cellulases**

252 The details of the runs are given in Section 2.2. Figure 1 shows the biomass and
 253 protein concentration throughout the culture whilst Table 1 gives the mean biomass and
 254 cellulase concentrations at the first steady state (the lower $EDCF_{\epsilon_{max}}$) and the near-steady
 255 state at the higher $EDCF_{\epsilon_{max}}$. Table 1 also gives the value of a range of other process
 256 parameters obtained for these two conditions and p values from ANOVA tests for the results
 257 for the time periods indicated by the dotted lines on Fig 1. The variables describing the
 258 biomass (biomass concentration, specific growth rate, growth yield) were not influenced by
 259 the agitation rate (p-values higher than 0.05). The variables that characterize the protein
 260 production (protein concentration, specific production rate, production and conversion
 261 yields and β -glucosidase activity) were significantly affected by the $EDCF_{\epsilon_{max}}$ value (p-values
 262 lower than 0.05).



263 **Figure 1:** Biomass and protein concentrations during continuous culture agitated by a
 264 Rayneri centripetal turbine impeller (embedded image) at initially 800 rpm increasing to
 265 1700 rpm ($EDCF_{\epsilon_{max}}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively). Dotted lines indicate time
 266 periods used for ANOVA tests. Error bars represent the standard deviation.
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Table 1: Measured values of culture parameters and associated p-values at the two different speeds and $EDCF_{\varepsilon_{max}}$ values

Stirring speed	800 rpm	1700 rpm	p-value
$EDCF_{\varepsilon_{max}}$ (kW. m ⁻³ . s ⁻¹)	1884	38400	na
Biomass concentration, X (g. kg ⁻¹)	7.7 ± 0.2	7.6 ± 0.2	0.538
Specific growth rate, μ (gX. gX ⁻¹ . h ⁻¹)	0.028 ± 0.01	0.028 ± 0.03	0.064
Protein concentration, P (g. kg ⁻¹)	3.7 ± 0.1	2.9 ± 0.1	0.001
Specific protein (cellulases) production rate, q_p (mg. gX ⁻¹ . h ⁻¹)	13.5 ± 0.5	10.3 ± 0.8	0.0002
Specific substrate consumption rate, q_s (mg. gX ⁻¹ . h ⁻¹)	0.084 ± 0.002	0.077 ± 0.002	0.0001
β -Glucosidase activity (IU/mL)	129.0 ± 1.7	107.9 ± 1.2	0.001
$Y_{X/S}$ (g. g ⁻¹)	0.35 ± 0.02	0.36 ± 0.04	0.471
$Y_{P/S}$ (g. g ⁻¹)	0.17 ± 0.01	0.13 ± 0.01	0.005
$Y_{P/X}$ (g. g ⁻¹)	0.48 ± 0.02	0.37 ± 0.02	0.002

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The data presented corresponds to the mean values for each condition (800 and 1700 rpm) for the period shown on Figure 1 and their standard deviations. Legend as in Nomenclature ; na: not applicable.

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The specific growth rate remained unchanged since a constant value was imposed by the constant dilution rate, D . However, constant biomass concentration is globally determined as cell dry weight. Ideally, the biomass concentration should also be subdivided into the percentage of active and inactive/damaged cells. However, reliable techniques for quantitatively measuring the viability of filamentous fungi are not available whether by fluorescent dyes, because of sampling issues, or by flow cytometry, because of the filamentous structure of the fungi which tends to block the capillaries. On the other hand, the constant biomass implies that it should all be active.

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Table 1 and Figure 1 show that the specific production rate decreased by 24 % and the protein production yields in relation to sugar and to biomass concentration decreased by 20 % and 23 % respectively with p values again showing statistical significance. These results agree with previous studies on the effect of fluid dynamic stress on secondary metabolites production by filamentous fungus in stirred bioreactors. Jüsten et al. (1998) observed increase in $EDCF$ with different impellers decreased penicillin production during fed-batch fermentations of *P. chrysogenum*. Lejeune and Baron (1995) and Mukataka et al.

289 (1988) found a lower production of cellulases at higher speeds with *T. reesei* cultures but did
290 not analyze the impact of different impellers or quantify hydrodynamic stress beyond
291 agitator speed. Reese and Ryu (1980) determined the effect of ‘shear’ on the stability of a
292 crude cellulase preparation of *T. reesei* and found that it caused deactivation of the enzyme
293 exoglucanase cellobiohydrolase (CBH), which slowed down cellulose digestion in a cellulosic
294 biomass hydrolysis. Ganesh et al. (2000) observed similar inactivation with increasing speed
295 in stirred reactors. These studies confirm that higher fluid dynamic stresses lead to a
296 decrease in cellulases production by *T. reesei* and to a deactivation of components of its
297 cellulolytic cocktail.

298 **3.3. Effect of higher fluid dynamic stress on the synthesis of intracellular proteins**

299 Scanned pictures of the two SDS-PAGE gels from proteomic analysis of cells are given
300 in Figure 1 (Supplementary Material). The gels indicate intracellular proteins that were
301 differently synthesized at the two $EDCF_{\epsilon_{max}}$ values of 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$
302 respectively. Image analysis of these gels allowed these proteins to be collected and
303 identified. In total, 30 spots, which corresponded to 24 different proteins, had their
304 synthesis modified as $EDCF_{\epsilon_{max}}$ increased. They were identified by tandem mass
305 spectrometry coupled with liquid chromatography (LC-MS/MS) and compared with *in silico*
306 data from sequence libraries as described in Section 2. At the higher $EDCF_{\epsilon_{max}}$, six proteins
307 were newly-synthesized (NW1 to NW6 in Table 2); six proteins were increasingly-
308 synthesized (up-regulated) by times 2 or more (UP1 to UP6, Table 3); and 18 proteins were
309 under-synthesized (down-regulated) by times 2 or more (UN1 to UN18, Table 4).

310 An analysis of Table 2 shows the increase in $EDCF_{\epsilon_{max}}$ only produced five new
311 proteins, as two of the 6 spots are isoforms of the same protein. Of the others, three assist

312 in protein quality control (including one chaperonin and two ubiquitin dependent
313 proteasomes) and are recognized as stress proteins. From Table 3, three up-synthesized
314 proteins are involved in the central carbon metabolism as they are related to
315 oxidoreductase activity (dihydrolipoyl dehydrogenase and FAD/NAD(P) domain-containing
316 protein) and Acetyl CoA biosynthesis (acetate kinase). The four others are involved in carbon
317 metabolism (adenosylhomocysteinase), proteolysis (glutamate carboxylpeptidase) and GDP-
318 mannose biosynthesis (phosphomannomutase). Finally, of the under-synthesized proteins
319 (Table 4), there are a number of isoforms and only 12 different ones are identified from 18
320 spots of which four relate to cellulolytic (glucosidases, endoglucanase and
321 cellobiohydrolases) and hemicellulolytic (xyloglucanases) activities. Of the eight others, one
322 involved putrescine biosynthesis (putative agmatine deiminase), two to transferase activity
323 (transketolase and uracil phosphoribosyl transferase) plus one isomerase (UDP-glucose-4-
324 epimerase), one kinase (diphosphomevalonate decarboxylase), one oxidoreductase
325 (NAD(P)H-dependent-D-xylose reductase), one involved in Ca^{2+} homeostasis (SGL domain-
326 containing protein) and one implicated in proteolysis (Zn-dependent exopeptidases).
327 Overall, two groups of proteins can be highlighted: under the higher $EDCF_{emax}$, stress
328 proteins were over-synthesized (up-regulated) and cellulases were under-synthesized (down
329 -regulated).

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Table 2: Proteins newly-synthesized by the increase from 800 to 1700 rpm ($EDCF_{\epsilon_{max}}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively)

Spot	Protein ID*	Protein description	MW (KDa) in the 2D gel	IP in the 2D gel	Coverage** (%)	Functional category
NW1	135423	Chaperonin HSP60	63.1	5.25	75	Chaperone molecular family
NW2	26797	20S proteasome subunit alpha type (homologs PSMA4 / PRE9)	26.2	6.12	73	Threonine endopeptidase activity - Ubiquitin-dependent protein
NW3	99350	20S proteasome alpha type (homologs PSMA6 / SCL1)	26.3	6.30	73	
NW4	80225	Uroporphyrinogen decarboxylase	39.2	5.99	71	Porphyrin biosynthesis
NW5 & NW6***	39524	Malate dehydrogenase	35.5	5.92	37	Oxidoreductase activity - Carbohydrate metabolism
			35.5	6.22	49	

331

*MW: molecular weight; IP: isoelectric pH; *Protein ID were obtained from JGI database; **Coverage corresponds to the percentage of the protein sequence*

332

*covered by identified peptides; ***Spots that correspond to isoforms of the same protein*

333

334

335

Table 3: Proteins over-synthesized from the increase from 800 to 1700 rpm ($EDCF_{max}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively)

Spot	Protein ID*	Protein description	MW (KDa) in the 2D gel	IP in the 2D gel	Coverage** (%)	Log2 fold change	Functional category
UP1	78299	Dihydrolipoyl dehydrogenase	55.3	6.47	77	1.9	Oxidoreductase activity - Cell redox homeostasis
UP2	25190	FAD/NAD(P)-binding domain-containing protein	59.5	6.07	78	1.4	
UP3	97186	Phosphomannomutase	28.1	5.38	71	1.3	GDP-mannose biosynthetic process
UP4	137356	Glutamate carboxypeptidase	57.8	5.69	82	2.5	Proteolysis
UP5	99242	Acetate kinase	46.6	6.49	81	1.3	Acetyl-CoA biosynthesis
UP6	142425	Adenosylhomocysteinase	46.0	6.22	68	1.5	Nucleic acid and amino-acid metabolism

336

*MW: molecular weight; IP: isoelectric pH; *Protein ID were obtained from JGI database; **Coverage corresponds to the percentage of the protein sequence covered by identified peptides*

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Table 4: Proteins under-synthesized by the increase from 800 rpm to 1700 rpm ($EDCF_{Emax}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively)

Spot	Protein ID*	Protein description	MW (KDa) in the 2D gel	IP in the 2D gel	Coverage** (%)	Log2 fold change	Functional category
UN1	122470	Exoglucanase II (1,4- β -cellobiohydrolase)	63.5	5.22	67	-2.3	
UN2	124438	Endo- β -1,4-glucanase	22.8	5.81	71	-2.3	
UN3 to UN7***	136547	β -D-glucoside glucohydrolase I	94.8	5.98	66	-1.5	Hydrolase activity – Cellulases
			91.8	6.15	65	-1.5	
			74.9	6.15	67	-2.8	
			74.5	6.34	69	-2.6	
			75.7	6.49	71	-2.8	
UN8 & UN9***	111943	Xyloglucanase	96.5	4.97	61	-2.4	Hydrolase activity – Hemicellulase
			92.1	4.87	63	-1.5	
UN10	108605	Uracil phosphoribosyl transferase	25.4	5.82	85	-1.2	Transferase activity - Nucleoside metabolism
UN11	111063	Peptide hydrolase	67.5	4.60	58	-2.3	Proteolysis
UN12 & UN13***	102903	Putative agmatine deiminase	47.0	4.55	84	-1.5	Putrescine biosynthesis
			46.8	4.65	40	-2.6	
UN14	99640	SMP30/gluconolactonase / LRE-like protein	33.7	5.12	75	-1.2	Ca ²⁺ homeostasis and signal transduction
UN15	101957	Diphosphomevalonate decarboxylase	36.0	5.63	45	-1.1	Kinase activity - ATP binding - Phosphorylation
UN16	137982	UDP-glucose 4-epimerase	37.0	6.15	39	-1.3	Coenzyme binding - Isomerase activity - Cellular metabolism
UN17	94809	NAD(P)H-dependent D-xylose reductase	24.4	5.08	53	-1.6	Oxidoreductase activity
UN18	110941	Transketolase	74.7	6.39	72	-2.9	Transferase activity – Metal ion binding - Carbohydrate metabolism

341 MW: molecular weight; IP: isoelectric pH; *Protein ID were obtained from JGI database; ** Coverage corresponds to the percentage of the protein sequence

342 covered by identified peptides; ***Spots that correspond to isoforms of the same protein

343 3.3.1. *Effect of higher fluid dynamic stress on the synthesis of stress proteins*

344 It is known that environmental stresses cause protein denaturation by aggregation
345 and misfolding leading to loss of biological functions and cell apoptosis (Roller and Maddalo,
346 2013; Tiwari et al., 2015). Table 2 and Figure 1 (Supplementary Material) show the higher
347 speed ($EDCF_{\epsilon_{max}}$) produces the chaperonin protein HSP60 and the ubiquitin-proteasome
348 system which are known to reduce cell damage by acting on post-translational processes
349 (Tiwari et al., 2015), correcting protein folding, helping refolding and stabilizing them under
350 stress (Pickart, 1999). Specifically, HSP60 has a crucial role in synthesis, transportation,
351 folding and degradation of proteins (Chen et al., 1999) and its over-expression under heat
352 (Raggam et al., 2011; Tiwari et al., 2015) and heavy metal (Enjalbert et al., 2006) stress has
353 been reported in fungal pathogens. Kashyap et al., 2016 observed its expression under
354 saline stress in halotolerant fungus *P. clavariiformis*. The ubiquitin-proteasome system
355 performs a similar function (Shang and Taylor, 2011; Kashyap et al. (2016) with various
356 fungi; for example, nitrogen deprivation (Shang and Taylor, 2011), heat shock (Staszczak,
357 2008) and exposure to cadmium (Goller et al., 1998).

358 The present study clearly shows for the first time that the cells response to higher
359 fluid dynamic stress is similar to that with other environmental stresses in order to protect
360 themselves against irreversible damage.

361 3.3.2. *Effect of fluid dynamic stress on the synthesis of cellulases*

362 Figure 1 (Supplementary Material) and Table 4 show that at the higher $EDCF_{\epsilon_{max}}$, the
363 synthesis of four intracellular cellulases decreased: glucosidases (β -D-glucoside
364 glucohydrolase I), endoglucanases (endo-1,4- β -glucanases), cellobiohydrolases (exo-1,4- β -
365 glucanases) and xyloglucanases. The first three make up the *T. reesei* cellulolytic cocktail

366 (Jourdier et al., 2013). Cellobiohydrolases attack the cellulose chains from the ends,
367 releasing cellobiose molecules. Endoglucanases hydrolyze cellulose chains randomly,
368 generate new ends accessible to cellobiohydrolases. β -glucosidase has the role of
369 hydrolyzing into glucose the cellobiose released by the action of endoglucanases and
370 cellobiohydrolases. This synergistic action is essential for hydrolysis of lignocellulosic
371 biomass by the fungus (Kumar et al., 2008) as are xyloglucanases (Herpoël-Gimbert et al.,
372 2008) for improving hydrolysis efficiency as the hemicellulolytic activity of this enzyme
373 increases the surface area (Benkő et al., 2008).

374 The fact that the synthesis of cytoplasmic cellulases is negatively affected by higher
375 fluid dynamic stress is strongly linked to the reduction of extracellular cellulases (Figure 1
376 and Table 1). This result indicates that the profile of extracellular proteins is directly linked
377 to the synthesis of intracellular proteins, as demonstrated above from the proteomic
378 analysis. This finding is original because, although some authors have reported the negative
379 effects of 'shear' stress on the secretion of cellulases by *T. reesei* (Mukatana et al., 1988;
380 Lejeune and Baron, 1995), the literature has not made any connection with the effect of
381 fluid dynamic stress on the intracellular synthesis of cellulases.

382 **3.4. Effect of scale on the production of cellulases**

383 For this study in chemostat culture, $EDCF_{\epsilon_{max}}$ was $1884 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ and $38400 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$
384 and the proteomic analysis showed that, as a result, a change took place at the
385 intracellular level to protect the cells and partially maintain the production of cellulases. As
386 a result of this protective action, though $EDCF_{\epsilon_{max}}$ increased substantially, the fall in cellulase
387 production was relatively small, from 13.5 to $10.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$.

388 In the batch cultures previously reported (Hardy et al., 2017) (i.e. during the growth
389 phase of the fermentation), $EDCF_{\epsilon_{max}}$ was a little smaller at the bench scale than here
390 because a larger bench scale bioreactor was used. As usual, it was also much lower than
391 here at the commercial scale ($6.65 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$) for the reasons given earlier (Hardy et al.,
392 2017) and in Section 3.1. The lower $EDCF_{\epsilon_{max}}$ was mirrored by increases in the growth rate,
393 the viscosity of the broth and the size of the fungi.

394 At the commercial scale (Hardy et al., 2017), since the aim was to produce as much
395 cellulase as possible, fed batch culture was used for the production phase to give a q_p of 17
396 $\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. In bench scale work (Hardy, 2016) related to this continuous culture study and the
397 earlier batch study (Hardy et al., 2017), two fed batch cultures were also undertaken. These
398 fed-batch studies were done in the same 3.5 L bioreactor (Hardy et al., 2017) with the
399 Rayneri centripetal impeller at 1000 rpm to give an $EDCF_{\epsilon_{max}}$ of $2760 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$. The
400 biomass concentration was controlled to 3.5 g/kg or 12 g/kg but the impact on the
401 production of cellulases was not statistically significant ($q_p = 11.9 \pm 3.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). Clearly,
402 the scatter was large (Hardy, 2016). It is interesting to compare the small change in q_p for
403 cellulase production with the very different $EDCF_{\epsilon_{max}}$ values in the continuous culture
404 conditions reported here and those arising in fed-batch at the two very different scales. It is
405 also convenient to assess whether the choice of $x = 12$ for the Rayneri impeller is an
406 appropriate one.

407 Following the comparison of the various definitions of fluid dynamic stress in relation
408 to process parameters in the initial batch production stage of *T. reesei* fermentation (Hardy
409 et al., 2017), which showed the effectiveness of $EDCF_{\epsilon_{max}}$, a similar relationship

410

$$q_{p_{cont}} = A \cdot \ln(EDCF_{\epsilon_{max}})_{cont} + B \quad (1)$$

411 has been used to correlate the continuous culture results from the two $EDCF_{\epsilon_{max}}$ conditions.
 412 Using the q_p value at the two $EDCF_{\epsilon_{max}}$ used, gives $A = -1.05$ and $B = 21.4$. If Eq (1 is now
 413 applied to the fed-batch case at the bench (subscript fb1) and commercial scale (subscript
 414 fb2) to give Eq (2,

$$q_{p_{fb2}} = q_{p_{fb1}} - A \cdot \ln \left(EDCF_{\epsilon_{max_{fb1}}} / EDCF_{\epsilon_{max_{fb2}}} \right) \quad (2)$$

415 where $A = -1.05$ and it is assumed that though different fermentation methods (batch, fed-
 416 batch and continuous) give different product yields, the impact of fluid dynamic stress is the
 417 same for the particular organism. Thus,

$$q_{p_{fb2}} = 11.9 + 1.05 \cdot \ln(2760/6.65) = 18.3 \quad (3)$$

418 This predicted value of q_p ($18.3 \text{ g.kg}^{-1}\text{h}^{-1}$) at the commercial scale is impressively close to the
 419 actual one ($17 \text{ g.kg}^{-1}\text{h}^{-1}$) considering the large difference in the scales used for these two
 420 fed-batch runs and that other factors can come into play at the commercial scale such as
 421 increased levels of inhomogeneity due to the greater mixing time and $p\text{CO}_2$ and $d\text{O}_2$ due to
 422 the static head.

423 The question regarding the impact of the value of x (the ratio of impeller diameter to
 424 trailing vortex diameter) chosen for the Rayneri impeller can also be addressed in relation to
 425 this type of analysis. Grenville et al. (2017) give a range of x values from 12 to 17 with 12
 426 being the value for the Rushton turbine and chosen for the Rayneri because, though very
 427 different from most impellers, they are both radial flow. If the other extreme, $x = 17$, is
 428 chosen to test the range of possible impacts on this analysis, then the $EDCF_{\epsilon_{max}}$ values of the
 429 continuous cultures reported in Table 1 are multiplied by $17/12$, the B parameter of Eq.1
 430 becomes 21.9 instead of 21.4, and the A parameter remains unchanged. Applying the same
 431 x value of 17 for the bench scale fed batch run, $EDCF_{\epsilon_{max}}$ becomes $3910 \text{ kW.m}^{-3}.\text{s}^{-1}$. If this

432 value is used in Eq 3 then, since $EDCF_{\epsilon_{max}}$ for the large scale where the Rushton turbine
433 dominates remains the same, the predicted value of q_p for the commercial scale becomes
434 $18.6 \text{ g.kg}^{-1}.\text{h}^{-1}$. In this case, the choice of x clearly is not a significant factor in the analysis of
435 the impact of fluid dynamic stress on the production of cellulases. Therefore given the
436 similarity in flow pattern between the Rayneri impeller and the Rushton turbine, $x = 12$
437 remains the value of choice.

438 Overall, it can be concluded that not only can the use of the $EDCF_{\epsilon_{max}}$ function
439 enable bench scale data to predict hyphal size, broth rheology and growth rate at the
440 commercial scale (Hardy et al., 2017), it is also a powerful tool in aiding scale-up in relation
441 to predicting the production of cellulases. Indeed, the production of cellulases at the
442 commercial scale should not be compromised by fluid dynamic stresses. More importantly,
443 the agitation intensity chosen should be based on ensuring it is sufficient to meet the
444 demands of oxygen mass transfer and carbon dioxide stripping along with an adequate
445 blending strategy, including sub-surface addition of nutrients and pH control chemicals
446 (Amanullah et al., 2004). In addition, economic considerations must be taken into account.

447 **4. Conclusion**

448 *T. reesei* was cultivated in continuous cultures at two $EDCF_{\epsilon_{max}}$ values, the lower one
449 ($1884 \text{ kW.m}^{-3}.\text{s}^{-1}$) until a steady state was reached and the higher ($38400 \text{ kW.m}^{-3}.\text{s}^{-1}$) until a
450 near-steady state, sufficient to show a significant fall in protein production linked to
451 measureable changes in intracellular proteins. This method of defining fluid dynamic stress
452 has already been successful in correlating its impact in the batch growth phase of *T. Reesei*
453 at the bench and commercial scale (Hardy et al., 2017). Here, the use of $EDCF_{\epsilon_{max}}$ provided a
454 link between this bench scale continuous culture study with that batch work (Hardy et al.,

455 2017) and also related fed-batch culture to produce cellulases at the bench (Hardy, 2016)
456 and commercial scale ($\sim 100 \text{ m}^3$) (Hardy et al., 2017). It is worthy of note, that a recent
457 extensive review of scale-up methods concluded that the *EDCF* function was the best way of
458 defining agitation intensity in bioprocessing (Böhm et al., 2019).

459 At the higher $EDCF_{\epsilon_{max}}$ at the bench scale, the concentration of the cellulases in the
460 medium as well as the production yield and the specific production rate decreased by 21 %,
461 20 % and 24 % respectively. A proteomic analysis provided further insight that helped to
462 explain these observations. Firstly, at the higher $EDCF_{\epsilon_{max}}$ the intracellular synthesis of all
463 enzymes involved in cellulosic activity was decreased. On the other hand, stress proteins
464 such as the HSP60 chaperone protein and the ubiquitin-proteasome system whose role is to
465 protect the cells from the effects of fluid dynamic stress appeared. These findings
466 demonstrate that at higher fluid dynamic stress, the fungus adapts itself by triggering
467 protection and damage repair mechanisms at the intracellular level and by favoring its
468 central metabolism to the detriment of other less essential functions, the combination of
469 the two explaining the small decrease of cellulase biosynthesis even at much higher
470 $EDCF_{\epsilon_{max}}$.

471 The higher extracellular cellulase production (q_p) at the lower $EDCF_{\epsilon_{max}}$ in continuous
472 culture compared to that at the higher $EDCF_{\epsilon_{max}}$ is quantitatively similar to the increase in q_p
473 during fed-batch fermentation at the commercial scale compared to that at the bench scale.
474 Perhaps more importantly for the commercial aim of producing cellulases, the sensitivity to
475 fluid dynamic stress is relatively small as the proteomic analysis explains.

476

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483

484 **Nomenclature**

D	dilution rate (h^{-1}) or impeller diameter (m)
dO_2	dissolved oxygen concentration (% of saturation value at ambient pressure)
$EDCF_{\varepsilon_{max}}$	EDCF based on the maximum specific energy dissipation rate ($\text{W}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$)
Fl	flow number of the impeller (dimensionless)
IP	isoelectric pH (dimensionless)
MW	molar weight (kDa)
N	rotation speed ($\text{rev}\cdot\text{s}^{-1}$)
P	protein concentration ($\text{g}\cdot\text{kg}^{-1}$) or power input from impeller (W)
Po	power number of the impeller (dimensionless)
q_p	specific protein production rate ($\text{mgP}\cdot\text{gX}^{-1}\cdot\text{h}^{-1}$)
q_s	specific substrate consumption rate ($\text{mgS}\cdot\text{gX}^{-1}\cdot\text{h}^{-1}$)
t	time (s in ε_{max} and $EDCF_{\varepsilon_{max}}$ or h in Table 1)
t_c	circulation time (s^{-1})
V	volume of broth (L in Table 1 and m^3 in P/V)
x	ratio of impeller diameter to trailing vortex diameter
X	biomass concentration ($\text{g}\cdot\text{kg}^{-1}$)
$Y_{X/S}$	biomass yield in relation to substrate ($\text{g}\cdot\text{g}^{-1}$)
$Y_{P/S}$	protein yield in relation to substrate ($\text{g}\cdot\text{g}^{-1}$)
$Y_{P/X}$	protein yield in relation to biomass ($\text{g}\cdot\text{g}^{-1}$)
ε_{max}	maximum local specific energy dissipation rate ($\text{W}\cdot\text{m}^{-3}$)
ρ	density ($\text{kg}\cdot\text{m}^{-3}$)
μ	specific growth rate (h^{-1} or $\text{gX}\cdot\text{gX}^{-1}\cdot\text{h}^{-1}$)
Subscripts	
fb	fed-batch
$cont$	continuous culture

485

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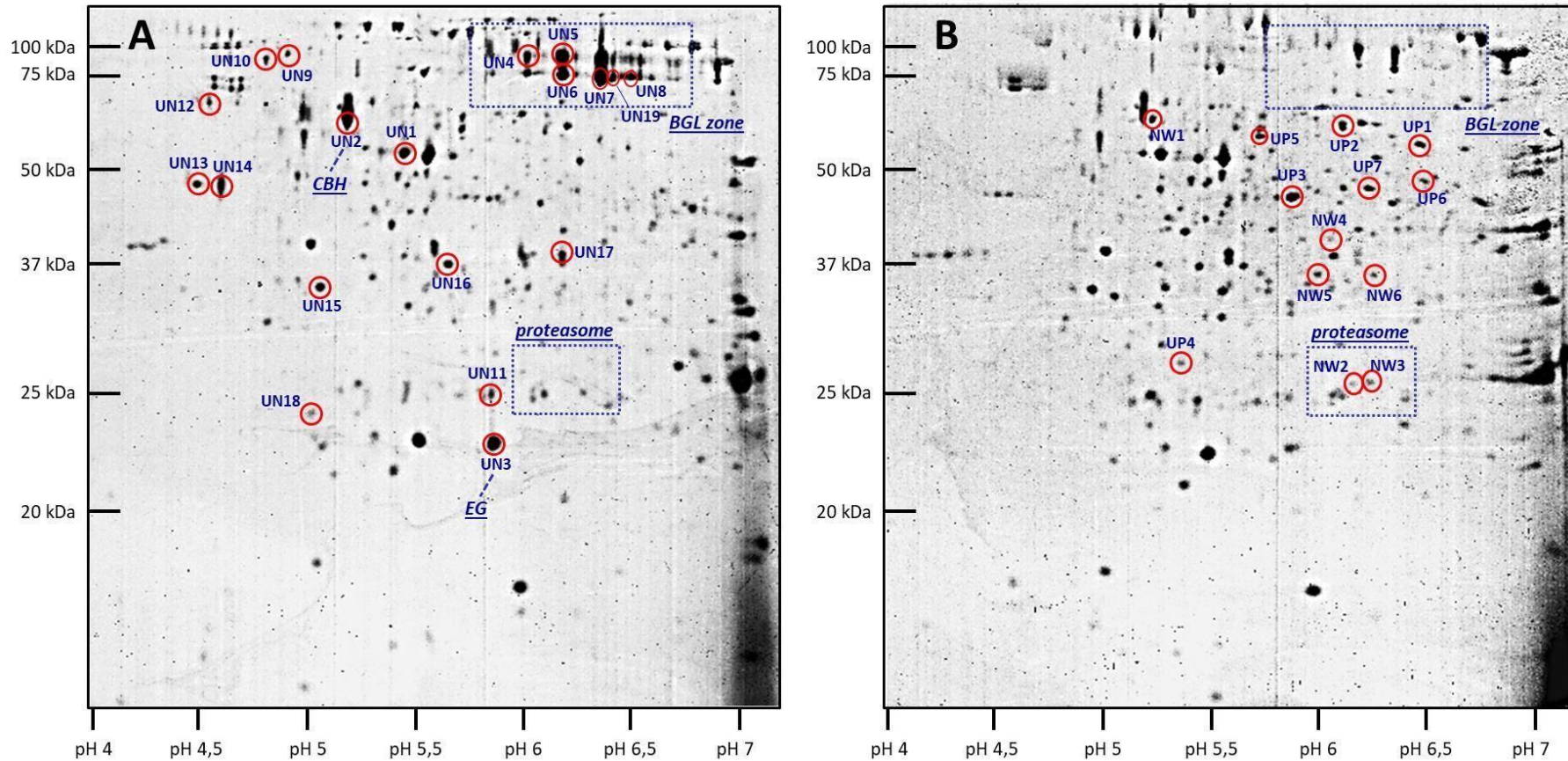
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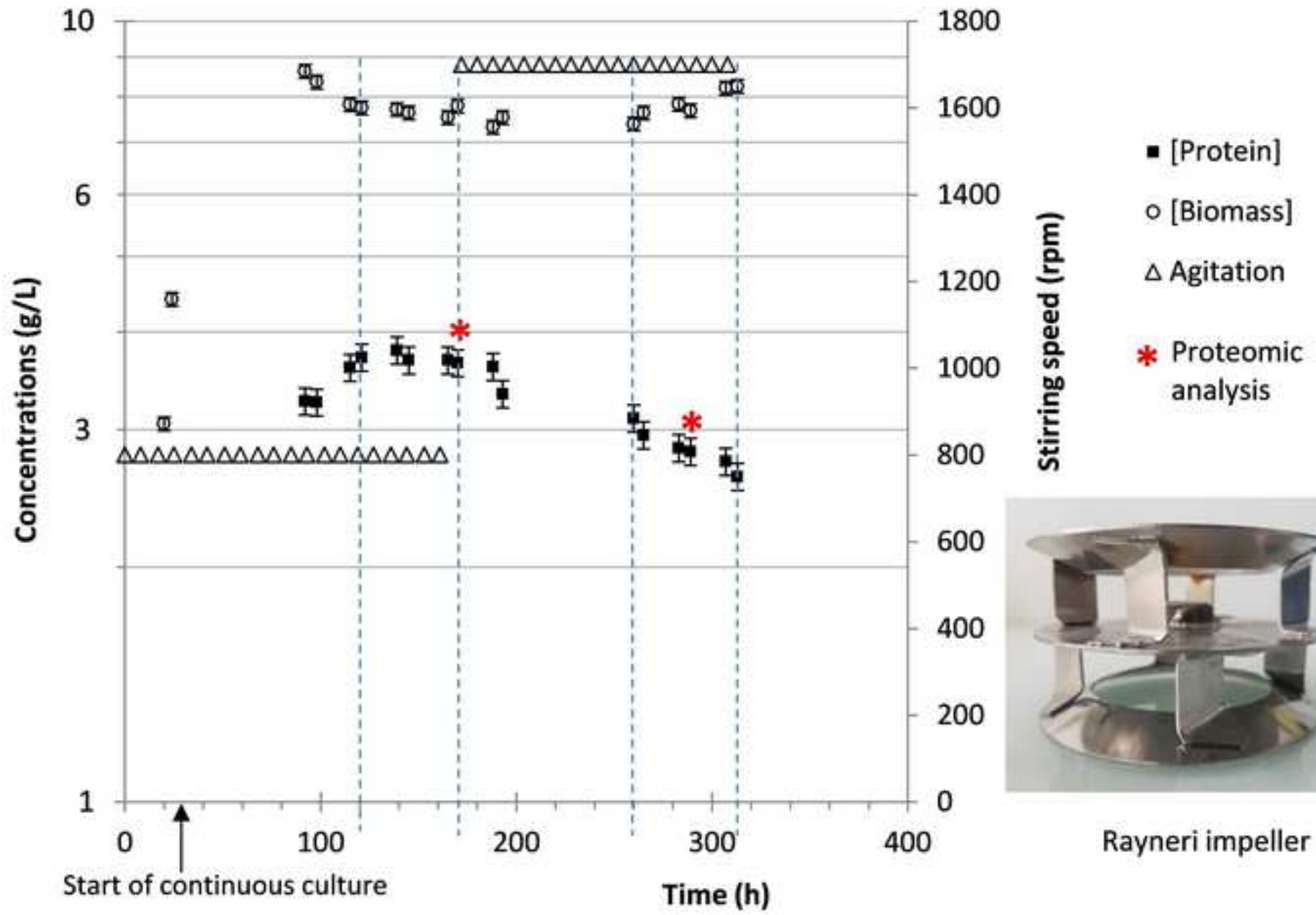
624 **Supplementary material**

625 **Figure 1** : Proteomic analysis of *T. reesei* cells: A) 800 rpm ($EDCF_{\text{emax}} 1884 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$); B) 1700 rpm ($EDCF_{\text{emax}} 38400 \text{ kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$); UN1-UN19:
626 under-synthesized proteins; UP1-UP7: over-synthesized proteins; NW1-NW6: newly-synthesized proteins.



627

Figure 1
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Rayneri impeller

Table 1[Click here to download Table: Table 1.docx](#)**Table 1: Measured values of culture parameters and associated p-values at the two different speeds and $EDCF_{emax}$ values**

Stirring speed	800 rpm	1700 rpm	p-value
$EDCF_{emax}$ (kW. m ⁻³ . s ⁻¹)	1884	38400	na
Biomass concentration, X (g. kg ⁻¹)	7.7 ± 0.2	7.6 ± 0.2	0.538
Specific growth rate, μ (gX. gX ⁻¹ . h ⁻¹)	0.028 ± 0.01	0.028 ± 0.03	0.064
Protein concentration, P (g. kg ⁻¹)	3.7 ± 0.1	2.9 ± 0.1	0.001
Specific protein (cellulases) production rate, q_p (mg. gX ⁻¹ . h ⁻¹)	13.5 ± 0.5	10.3 ± 0.8	0.0002
Specific substrate consumption rate, q_s (mg. gX ⁻¹ . h ⁻¹)	0.084 ± 0.002	0.077 ± 0.002	0.0001
β -Glucosidase activity (IU/mL)	129.0 ± 1.7	107.9 ± 1.2	0.001
$Y_{X/S}$ (g. g ⁻¹)	0.35 ± 0.02	0.36 ± 0.04	0.471
$Y_{P/S}$ (g. g ⁻¹)	0.17 ± 0.01	0.13 ± 0.01	0.005
$Y_{P/X}$ (g. g ⁻¹)	0.48 ± 0.02	0.37 ± 0.02	0.002

The data presented corresponds to the mean values for each condition (800 and 1700 rpm) for the period shown on Figure 1 and their standard deviations.

Legend as in Nomenclature ; na: not applicable.

Table 2: Proteins newly-synthesized by the increase from 800 to 1700 rpm ($EDCF_{\epsilon max}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively)

Spot	Protein ID*	Protein description	MW (KDa) in the 2D gel	IP in the 2D gel	Coverage** (%)	Functional category
NW1	135423	Chaperonin HSP60	63.1	5.25	75	Chaperone molecular family
NW2	26797	20S proteasome subunit alpha type (homologs PSMA4 / PRE9)	26.2	6.12	73	Threonine endopeptidase activity - Ubiquitin-dependent protein
NW3	99350	20S proteasome alpha type (homologs PSMA6 / SCL1)	26.3	6.30	73	
NW4	80225	Uroporphyrinogen decarboxylase	39.2	5.99	71	Porphyrin biosynthesis
NW5 & NW6***	39524	Malate dehydrogenase	35.5	5.92	37	Oxidoreductase activity -
			35.5	6.22	49	Carbohydrate metabolism

*MW: molecular weight; IP: isoelectric pH; *Protein ID were obtained from JGI database; **Coverage corresponds to the percentage of the protein sequence covered by identified peptides; ***Spots that correspond to isoforms of the same protein*

Table 3: Proteins over-synthesized from the increase from 800 to 1700 rpm ($EDCF_{\epsilon_{max}}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively)

Spot	Protein ID*	Protein description	MW (KDa) in the 2D gel	IP in the 2D gel	Coverage** (%)	Log2 fold change	Functional category
UP1	78299	Dihydrolipoyl dehydrogenase	55.3	6.47	77	1.9	Oxidoreductase activity - Cell redox homeostasis
UP2	25190	FAD/NAD(P)-binding domain-containing protein	59.5	6.07	78	1.4	
UP3	97186	Phosphomannomutase	28.1	5.38	71	1.3	GDP-mannose biosynthetic process
UP4	137356	Glutamate carboxypeptidase	57.8	5.69	82	2.5	Proteolysis
UP5	99242	Acetate kinase	46.6	6.49	81	1.3	Acetyl-CoA biosynthesis
UP6	142425	Adenosylhomocysteinase	46.0	6.22	68	1.5	Nucleic acid and amino-acid metabolism

*MW: molecular weight; IP: isoelectric pH; *Protein ID were obtained from JGI database; **Coverage corresponds to the percentage of the protein sequence covered by identified peptides*

Table 4: Proteins under-synthesized by the increase from 800 rpm to 1700 rpm ($EDCF_{emax}$ 1884 and 38,400 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively)

Spot	Protein ID*	Protein description	MW (KDa) in the 2D gel	IP in the 2D gel	Coverage** (%)	Log2 fold change	Functional category
UN1	122470	Exoglucanase II (1,4- β -cellobiohydrolase)	63.5	5.22	67	-2.3	
UN2	124438	Endo- β -1,4-glucanase	22.8	5.81	71	-2.3	
			94.8	5.98	66	-1.5	Hydrolase activity – Cellulases
UN3 to UN7***	136547	β -D-glucoside glucohydrolase I	91.8	6.15	65	-1.5	
			74.9	6.15	67	-2.8	
			74.5	6.34	69	-2.6	
			75.7	6.49	71	-2.8	
UN8 & UN9***	111943	Xyloglucanase	96.5	4.97	61	-2.4	Hydrolase activity – Hemicellulase
			92.1	4.87	63	-1.5	
UN10	108605	Uracil phosphoribosyl transferase	25.4	5.82	85	-1.2	Transferase activity - Nucleoside metabolism
UN11	111063	Peptide hydrolase	67.5	4.60	58	-2.3	Proteolysis
UN12 & UN13***	102903	Putative agmatine deiminase	47.0	4.55	84	-1.5	Putrescine biosynthesis
			46.8	4.65	40	-2.6	
UN14	99640	SMP30/gluconolactonase / LRE-like protein	33.7	5.12	75	-1.2	Ca ²⁺ homeostasis and signal transduction
UN15	101957	Diphosphomevalonate decarboxylase	36.0	5.63	45	-1.1	Kinase activity - ATP binding - Phosphorylation
UN16	137982	UDP-glucose 4-epimerase	37.0	6.15	39	-1.3	Coenzyme binding - Isomerase activity - Cellular metabolism
UN17	94809	NAD(P)H-dependent D-xylose reductase	24.4	5.08	53	-1.6	Oxidoreductase activity
UN18	110941	Transketolase	74.7	6.39	72	-2.9	Transferase activity – Metal ion binding - Carbohydrate metabolism

*MW: molecular weight; IP: isoelectric pH; *Protein ID were obtained from JGI database; ** Coverage corresponds to the percentage of the protein sequence covered by identified peptides; ***Spots that correspond to isoforms of the same protein*