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► **To cite this version:**

Nicolas Hardy, Frédéric Augier, Alvin W. Nienow, Catherine Béal, Fadhel Ben Chaabane. Scale-up agitation criteria for *Trichoderma reesei* fermentation. *Chemical Engineering Science*, 2017, 172, pp.158-168. 10.1016/j.ces.2017.06.034 . hal-01544606

HAL Id: hal-01544606

<https://agroparistech.hal.science/hal-01544606v1>

Submitted on 9 Mar 2018

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1 **Scale-up agitation criteria for *Trichoderma reesei* fermentation**

2

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13 **ABSTRACT**

14 Scale-up of aerobic fungal fermentation processes still remains a challenging issue for the
15 biotechnology industry. This difficulty arises due to the complex interactions between
16 operating conditions (agitation, aeration, etc.), the physicochemical state of the broth
17 (viscosity, the dissolved oxygen concentration, etc.) and the biology of fungi (growth,
18 production, morphology, etc.). Because of their size, filamentous fungi are affected by fluid
19 dynamic stresses but quantification of this complex parameter is a difficult task. In general,
20 indirect criteria are used for the effect of fluid dynamic stresses on scale-up (tip speed, power
21 draw or the energy dissipation/circulation function (EDCF)). In order to better understand the
22 impact of such criteria on the fermentation of the fungus *Trichoderma reesei*, a wide range of
23 agitation conditions has been explored. The morphology of *T. reesei* fungus, its specific
24 growth rate and the rheological properties of the broth have all been measured both at bench

25 scale (~ 2.5 L) and for the first time, at a typical commercial scale. These three aspects of the
26 fermentation at both scales were then compared with respect to tip speed, specific power and
27 EDCF. This work has shown that tip speed as a correlator of any of these parameters is totally
28 ineffective whilst the EDCF is clearly the best for extrapolating laboratory data to the
29 commercial scale.

30

31 **KEYWORDS**

32 *Trichoderma reesei*, scale-down, scale-up, morphology, rheology, filamentous fungus.

33

34 **1. INTRODUCTION**

35 Biofuels are candidates to substitute oil with a lower carbon footprint. Among them,
36 bioethanol is the most consumed biofuel in the world (Simbolotti 2007) and bioethanol from
37 lignocellulosic resources (also called second generation bioethanol) appears to be a good
38 candidate to reduce CO₂ production (Percival Zhang et al. 2006). The biotransformation of
39 lignocellulosic biomass involves the utilization of specific enzymes named cellulases
40 (Bischof et al. 2016), which are needed to degrade cellulose into simple and fermentable
41 sugars. However, the cost of these enzymes is one of the limiting factors of bioethanol
42 production from cellulolytic materials. To decrease the impact of the cost of cellulase on the
43 price of ethanol, different advances can be targeted. Firstly, the efficiency of the enzymatic
44 cocktail has to be improved in order to reduce the amount of enzymes needed during the
45 hydrolysis (Ayrinhac et al. 2011). Secondly, the production capacity has to be developed by
46 means of an efficient process optimization and scale-up strategy (Percival Zhang et al. 2006).
47 Nowadays, cellulase production is mainly performed with the filamentous fungus
48 *Trichoderma reesei* (Gusakov 2011). This microorganism is known for its high cellulase

49 secretion capacity and productivity (Gusakov 2011). An efficient protocol for cellulase
50 production (Pourquié et al, 1988) consists of two sequential phases: a batch phase followed
51 by a fed-batch phase. The fungus first grows during the batch phase, with sugar and nutrients
52 in excess, in order to produce a large concentration of cellular biomass. During this step, the
53 enzyme production keeps low and nutrients are mainly used for the growth of the fungi. The
54 latter has a hyphal structure exhibiting a highly viscous, non-Newtonian behaviour. In the
55 subsequent fed-batch phase, a limiting rate of sugar is fed continuously to the bioreactor to
56 favor the synthesis of the enzyme. During this phase, the hyphae break down and the
57 viscosity falls. At the same time, the oxygen requirement by microorganisms is also strongly
58 decreased. Thus, the most challenging phase with respect to the specific energy input
59 required for effective agitation is the first, as it requires the highest oxygen transfer rate
60 during the period of highest viscosity (Gabelle et al. 2012).

61

62 Furthermore, despite their high productivity, *T. reesei* fermentations are difficult to scale-up
63 due to the complex rheology of the broth during the biomass growth phase. The hyphal
64 development induces an increase of the broth viscosity with a shear thinning behaviour
65 (Gabelle et al. 2012; Hardy et al. 2015; Malouf et al. 2013). The shear thinning behaviour
66 induces changes in the hydrodynamics of the broth that tend to increase spatial
67 heterogeneities and may lead to caverns formation in the bioreactor (Amanullah et al. 2002;
68 Marten et al. 1996; Stocks 2013). In addition, *T. reesei* is strictly aerobic and the increase of
69 viscosity also strongly reduces the rate of oxygen mass transfer (Gabelle et al. 2012). To
70 ensure a sufficiently high rate, the power input has to be increased, which results in an
71 increase of the fluid dynamic stresses on the mycelial structures, which because of their size
72 and filamentous nature are prone to size reduction (Serrano-Carreón et al. 2015). Thus, the
73 fungus morphology impacts process parameters and the process conditions affect in return the

74 fungus morphology. Though significant advances in understanding these interrelations have
75 been made in the past (Amanullah et al. 2002; Jüsten et al. 1996), their impact on processes at
76 the full industrial scale is still not fully understood, especially because the magnitude of the
77 fluid dynamic stresses changes with scale (Stocks 2013). All those elements show that the
78 scale-up of the process is complex and that these changes in fluid dynamic conditions have to
79 be included in order to scale-up process involving filamentous fungus such as *T. reesei*. The
80 fluid dynamic stress that affects the microorganism morphology and possibly its productivity
81 in a bioreactor have often been called ‘shear stress’ (or just ‘shear’), but the precise nature of
82 the stress that causes such damage is not known in the complex three dimensional turbulent
83 or near turbulent flow field found in bioreactors with a very wide range of local specific
84 energy dissipation rates. Therefore, the term will not be used here. Whatever the mechanism
85 that damages microorganisms, it has generally been related to relatively simple and
86 quantifiable parameters, namely the impeller tip speed, the specific power input into the
87 bioreactor by the impeller (or mean specific energy dissipation rate), or to the concept of an
88 “energy dissipation/circulation” function, EDCF (Gabelle et al. 2012; Jüsten et al. 1996;
89 Sánchez Pérez et al. 2006). Indeed, it was suggested many years ago that the tip speed of the
90 impeller (V_{tip}) was a measure of the ‘maximum stress’ (Oldshue 1966). V_{tip} is given by Eq.1:

91

$$92 \quad \mathbf{V}_{tip} = \boldsymbol{\pi} \times \mathbf{D} \times \mathbf{N} \quad (1)$$

93

94 where D is the diameter of the impeller (m) and N the rotational speed (s^{-1}). This concept
95 appears to have received wide acceptance in the biochemical engineering community (Bailey
96 and Ollis 1986; Wang et al. 1979), though there seems to be little experimental evidence to
97 support it. Another approach is to use an estimate based on an average shear rate but this is
98 dependent on the flow regime. In the laminar flow regime, the Metzner-Otto approach

99 (Metzner and Otto, 1957) can be used. However, even at the bench scale, the Reynolds
 100 number (Re) $\gg 10$, thus much higher than that which defines laminar flow. In the turbulent
 101 regime, the average shear rate can be linked to the mean specific energy dissipation rate and
 102 kinematic viscosity for a Newtonian fluid. The power draw per unit of volume (P/V , $W.m^{-3}$)
 103 is equivalent to the mean specific energy dissipation rate (Sánchez Pérez et al. 2006) where
 104 power P can be determined from Eq. 2 (Stocks 2013):

105

$$106 \quad \mathbf{P} = \mathbf{Po} \times \boldsymbol{\rho} \times \mathbf{N}^3 \times \mathbf{D}^5 \quad (2)$$

107

108 where Po is the power number of the impeller (dimensionless) and ρ the density of the broth
 109 ($kg.m^{-3}$). Though here, at the bench scale, the flow is transitional ($Re < 2 \times 10^4$) and the fluid
 110 is non-Newtonian (shear thinning), it is certainly closer to turbulent flow than laminar, at
 111 least in the impeller region, where energy is mostly dissipated. As an approach based on the
 112 transitional regime is not available, turbulent flow relationships are generally assumed. A
 113 more sophisticated approach uses the “energy dissipation/circulation” function EDCF where
 114 again the assumption of turbulent flow is made. It was first proposed by Smith et al. (1990);
 115 here the potential for damage was related to the energy dissipated in the region of a Rushton
 116 turbine impeller and the frequency of the passage of the mycelium through that region. This
 117 basic concept was modified by Jüsten et al. (1996) to include an additional geometrical factor
 118 (k) by which the relevant volume depended on impeller type and a volume swept out by it as
 119 it rotated. In this way, different kinds of impeller could be compared. Thus Eq.3:

120

$$121 \quad \mathbf{EDCF} = \left(\frac{\mathbf{P}}{\mathbf{k} \times \mathbf{D}^3} \right) \left(\frac{\mathbf{1}}{\mathbf{t}_c} \right) \quad (3)$$

122

123 Here EDCF is in $\text{W}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$, k is a dimensionless constant and t_c the circulation time (s) that is
 124 obtained from Eq.4:

125

$$126 \quad t_c \approx \frac{V}{Fl \times N \times D^3} \quad (4)$$

127

128 where V is the liquid volume in the bioreactor (m^3) and Fl is the Flow number of the impeller
 129 (dimensionless). The power draw in the swept volume is used in Eq.3 to represent the energy
 130 ‘perceived’ by the fungus during its passage through the impeller. This concept relies on the
 131 Kolmogorov’s theory of isotropic turbulence that connects dissipation rate and shear rate or
 132 stress (Kolmogorov, 1941). Jüsten et al. (1996) showed that the EDCF concept can be
 133 correlated to the size reduction of the mycelia due to agitation with a wide range of impellers
 134 at bench and small pilot plant scale with *P. chrysogenum*; and Amanullah et al. (2000)
 135 showed its applicability to *A. oryzae*. Nevertheless, there are issues to be resolved such as
 136 how to determine the value of the parameter k for complex impellers. As an alternative to the
 137 determination of appropriate k values, it is proposed in the present study to use the maximum
 138 specific energy dissipation rate (ϵ_{\max}) in W/m^3 , instead of P/kD^3 , which leads to the definition
 139 of the new criteria, $\text{EDCF}_{\epsilon_{\max}}$:

140

$$141 \quad \text{EDCF}_{\epsilon_{\max}} = \frac{\epsilon_{\max}}{t_c} \quad (5)$$

142

143 Though the maximum local specific energy dissipation rate (ϵ_{\max}) is difficult to determine
 144 experimentally since laser based techniques such as PIV or LDA followed by complex data
 145 manipulation must be used (Gabriele et al. 2009), it has been shown by Grenville and
 146 Brown (2012) that it can be estimated from Eq.6:

147

$$\varepsilon_{max} = 1.04 \times \rho \times P \omega^{\frac{3}{4}} \times N^3 \times D^2 \quad (6)$$

149

150 There are also some other related studies in the literature. For example, Malouf et al. (2013)
151 explored the relationships between *T. reesei* morphology, rheology and biomass
152 concentration as a function of rotational speed. Other studies are focused on the impact of
153 agitation intensity and agitation devices on cellulase production (Marten et al. 1996; Patel et
154 al. 2009). However, to our knowledge, no work has been reported with such data for *T. reesei*
155 cultures from the laboratory scale to the industrial scale. In addition, as Quintanilla et
156 al. (2015) underlined, most of published studies on filamentous fermentation were made
157 under conditions that are quite different from industrial ones (low-producing strains, growth
158 with substrate and/or oxygen limitations, utilization of pure substrates, not optimized
159 processes, etc.).

160 The goal of this work is to identify scientifically based scale-up criteria for filamentous fungi
161 fermentations. To do so, the three representations of fluid dynamic stress discussed above
162 will be determined for a wide range of *T. reesei* fermentation conditions at laboratory scale.

163 The ability of these criteria to correlate with growth rate, fungal morphology and rheology is
164 discussed.

165 As, uniquely, some data are available from commercial scale, the three candidates as “stress”
166 criteria are also determined for the large scale conditions as a test of robustness for scale-up
167 considerations.

168 **2. MATERIALS, METHODS AND EXPERIMENTAL PREREQUISITES**

169 *Fungus strain*

170 The industrial strain of *Trichoderma reesei* Tr3002 (IFPEN culture collection) was used. It
171 derived from the strain *T. reesei* CL847 obtained by classical and molecular genetics (Durand

172 et al. 1988). It displayed an improved β -glucosidase gene, a hyperproducing cellulases
173 capacity and was glucose-derepressed (Ayrinhac et al. 2011). Conservation was made in
174 spores form at a concentration of 2×10^7 colony forming units per milliliter. Spores were
175 stored on frozen form in water added with 50 % glycerol, at -80°C .

176 *Culture media*

177 The preculture medium was composed of di-potassium phthalate 0.02 mol.L^{-1} ; (Na_2MoO_4 ,
178 $2 \text{ H}_2\text{O}$) 1.25 mg.L^{-1} ; (CuSO_4 , $5 \text{ H}_2\text{O}$) 2.4 mg.L^{-1} ; (MnSO_4 , H_2O) 5.12 mg.L^{-1} ; (ZnSO_4 ,
179 $7 \text{ H}_2\text{O}$) 6.72 mg.L^{-1} ; ($\text{Co}(\text{NO}_3)_2$, $6 \text{ H}_2\text{O}$) 7.2 mg.L^{-1} ; (FeSO_4 , $7 \text{ H}_2\text{O}$) 24 mg.L^{-1} ; (Na_2HPO_4 ,
180 $12 \text{ H}_2\text{O}$) 92 mg.L^{-1} ; (CaCl_2 , $2 \text{ H}_2\text{O}$) 0.48 g.L^{-1} ; (MgSO_4 , $7 \text{ H}_2\text{O}$) 0.48 g.L^{-1} ; (NH_4) $_2\text{SO}_4$
181 2.24 g.L^{-1} ; H_3PO_4 85% 2.4 mL.L^{-1} ; KOH 1.33 g.L^{-1} and corn steep solid 2 g.L^{-1} . pH was
182 adjusted to 5.25 with NaOH 7 mol.L^{-1} .

183 For bioreactor cultivation, the culture medium included (Na_2MoO_4 , $2 \text{ H}_2\text{O}$) 1 mg.L^{-1} ;
184 (CuSO_4 , $5 \text{ H}_2\text{O}$) 3.0 mg.L^{-1} ; (ZnSO_4 , $7 \text{ H}_2\text{O}$) 8.4 mg.L^{-1} ; (MnSO_4 , H_2O) 6.4 mg.L^{-1} ;
185 ($\text{Co}(\text{NO}_3)_2$, $6 \text{ H}_2\text{O}$) 9 mg.L^{-1} ; (FeSO_4 , $7 \text{ H}_2\text{O}$) 30 mg.L^{-1} ; (Na_2HPO_4 , $12 \text{ H}_2\text{O}$) 115 mg.L^{-1} ;
186 (CaCl_2 , $2 \text{ H}_2\text{O}$) 0.6 g.L^{-1} ; (NH_4) $_2\text{SO}_4$ 2.8 g.L^{-1} ; H_3PO_4 85% 3 mL.L^{-1} ; KOH 1.66 g.L^{-1} ; corn
187 steep solid 2 g.L^{-1} . Antifoam SB2121 from Strucktol (Hamburg, Deutschland) was added at
188 1 % (v/v) and pH was adjusted with NH_3 10.25 % (v/v) to 4.8.

189 All media were sterilized by autoclaving at 121°C for 20 min.

190 *Precultures*

191 A volume of 250 mL of preculture medium was prepared in a 2 L Fernbach flask by mixing
192 225 mL of preculture medium and 25 mL of a sterile glucose solution at 250 g.L^{-1} . The flasks
193 were seeded with 1 mL of thawed spores and incubated in a shaker Multitron II (Infors,
194 Bottmingen, Switzerland) at 30°C for 70 h. The agitation was set to 180 rpm with an orbital
195 of 50 mm.

196 *Cultivation at bench scale*

197 The preculture was used to inoculate a 3.5 L bioreactor (IFPEN, Rueil-Malmaison, France)
198 containing 2.5 L culture medium (1.75 L of culture medium and 500 mL of sterile industrial
199 grade glucose solution at 250 g.L⁻¹) as previously described by Gabelle et al.(2012).
200 Fermentations were performed in batch culture mode and each was repeated once (run in
201 duplicate). Temperature was set at 27°C, pH was maintained at 4.8 with NH₃ (10.25 %, v/v)
202 and gas flow rate was fixed at 1.25 L.min⁻¹ (0.5 VVM). Agitation was carried out by using
203 four different kinds of impeller (Fig. 1) that were characterized by their shape, their
204 dimensions, their number of blades, their power number and their flow number (Table 1).
205 Power and flow numbers were provided by the suppliers, except for the four-blade disk
206 paddle one, which was determined by CFD simulation. They were driven by an asynchronous
207 electric motor 1LA7063 4AB12 (Siemens, Munich, Germany) controlled by a variable speed
208 drive ATV31C018M2 (Schneider, Rueil-Malmaison, France) at four different stirring rates:
209 800 min⁻¹, 935 min⁻¹, 1250 min⁻¹ and 1700 min⁻¹. Dissolved oxygen (dO₂) concentration was
210 measured with an InPro 6860i optical oxygen sensor (Mettler-Toledo, Greifensee,
211 Switzerland) and controlled by the admission of a gas mix composed of nitrogen and
212 compressed air. The gas mix was achieved by two gas flow mass controllers EL-FLOW F-
213 201CV (Bronkhorst, Ruurlo, The Netherlands) that permitted the bioreactor gas flow rate to
214 be kept constant. dO₂ was set at 40 %, to be higher than the critical dissolved oxygen
215 concentration of ~15% at atmospheric pressure (Marten et al. 1996) so that the culture
216 performance should be independent of oxygen concentration. The constraint on the minimal
217 dissolved oxygen set a minimal P/V of 6 kW.m⁻³ below which the minimal value of 40% is
218 not reached. The minimal stirring rate of 800 RPM was able to meet this requirement for the
219 four tested impellers.

220 Seven fermentation experiments were used in the present study. All cultivation have been
221 duplicated. Fermentations were stopped before the end of the exponential growth phase, with
222 residual glucose concentration above than 1 g.L^{-1} . Samples were collected at least twice every
223 24 h for biomass and glucose concentration analyses. Rheological property measurements
224 and image analyses were made on samples collected with a biomass concentration between 4
225 and 8 g. kg^{-1} .

226 *Cultivation at industrial scale*

227 Uniquely, two experiments were conducted at a commercial scale with the same strain. One
228 was a full run in which growth rate, morphology and rheology were measured in exactly the
229 same way as at the bench scale. In an additional preliminary run, the growth rate was
230 measured in the same way but the rheology was measured at a slightly higher biomass
231 concentration before the method had been standardised. At that time, the new image analysis
232 technique had not been established. However, at this scale, there was no possibility of
233 duplicating the one full experiment because of the cost and time involved.

234 With respect to the bioreactor configuration, for reasons of commercial confidentiality, only
235 those aspects critical to the application of the three estimates of fluid dynamic stress can be
236 given. Similar operating conditions with respect to dO_2 , temperature and gas flow rate were
237 used. The total volume of the bioreactor was 220 m^3 and the liquid volume was between 80
238 and 130 m^3 . As usual at these scales, the vessel was equipped with multiple impellers, in this
239 case one high power number impeller at the bottom and several down-pumping hydrofoil
240 impellers of much lower power number above, each of the same diameter. The operating
241 conditions at industrial scales are constrained first by the performances of the existing stirring
242 device, which delivers a rather low specific power consumption compared to bench scale
243 bioreactors. But as similar VVM was applied at both scales to avoid any inhibition by the
244 dissolved CO_2 , the gas linear velocity is much higher at industrial scale, which enhances k_{La}

245 (van' t Riet and Tramper, 1991). At the same time, the static pressure also increases the
246 average dissolved oxygen concentration at large scale and the Reynolds number is higher
247 (Stocks, 2013). Consequently, the oxygen transfer rate is enhanced so that the specific power
248 requirement (P/V) was about 10 times lower than at bench scale for a similar oxygen uptake
249 rate.

250 Interestingly, in some preliminary lab-scale experiments operated with a specific power of ~
251 1 kW/m³ (the same specific power as at the industrial scale) satisfactory fermentations could
252 not be conducted due to dO₂ being too low, as also reported by Amanullah et al. (2002) with
253 *Aspergillus oryzae*. In addition, the biomass grew as one large pellet of fungi of completely
254 different morphology. On the other hand, the agitation conditions at the bench scale required
255 to give the desired dO₂ at obtainable sparge rates led to a minimum tip speed of the impellers
256 of 3.7 m.s⁻¹ at the bench scale, which matched approximately the tip speeds used at the
257 commercial scale.

258 The common use of multiple impellers at the commercial scale whilst typically only one is
259 used at bench scale generally poses specific problems for scale up as geometric similarity is
260 not maintained. Clearly if tip speed is being assessed, then since this parameter is
261 independent of the number of impellers, a direct comparison can be made as the impellers are
262 of the same size. If specific power, P/V, is under consideration, though the high power
263 number impeller clearly inputs more power, for the impeller spacing used here, the total
264 power can be evaluated by summing the power numbers (Nienow 1998) and using Eq.2.
265 Finally, for assessment of EDCF_{ε_{max}}, as the Po ratio of the high Po impeller to the low one
266 was > 3, the ε_{max} value is clearly much higher for the high Po impeller. Therefore, it should
267 be used for estimating the appropriate EDCF_{ε_{max}} value.

268 In addition, in order to estimate EDCF_{ε_{max}} at the commercial scale, there is also a problem in
269 estimating the circulation time, t_c for multiple impellers. At the bench scale, the flow number

270 of a single impeller is used to estimate it via the simple concept implied by Eq.4. Here, it was
271 decided to evaluate t_c from the mixing time, t_m using the long standing equation, Eq.7 (van't
272 Riet and Tramper 1991).

273

$$274 \quad t_m = 4 \times t_c \quad (7)$$

275

276 Therefore, the mixing time was measured by a pulse injection of 5 liters of a solution of
277 ammonia (25 % w/w) at the top of the bioreactor and the measurement of the pH at the
278 bottom (Singh et al., 1986). The time response of the pH probe was below 5 s and the method
279 was found to be robust with measurements being repeatable within approximately ± 3 s.
280 Thus, $\text{EDCF}_{\text{emax}}$ could be determined from Eq.6 and Eq.7.

281 In order to have confidence in the mixing time measurements, they have been compared to a
282 correlation available for multiple impeller configurations. Cooke et al. (1988) developed an
283 initial correlation for multiple radial flow impeller configurations (Rushton turbines). These
284 authors observed that in case of combination of radial and axial flow impellers, the mixing
285 times are reduced by a factor ~ 2 at the same mean specific energy dissipation rate. As
286 recommended by Nienow (1998), the correlation of Cooke et al. (1988) has been modified to
287 allow for this reduction and in that case, the values predicted are very close (within 10%) to
288 the present mixing time measurements. This consistency strengthens the use of the measured
289 mixing times to calculate $\text{EDCF}_{\text{emax}}$.

290

291 *Rheological property measurements*

292 It is well known that with increasing biomass concentration, the apparent viscosity increases.

293 For a given fungi, whilst this increase is dominated by biomass concentration, it has been

294 shown that it also depends on the size of the filamentous entities or clumps, the larger the

295 clumps the higher the apparent viscosity at a particular shear rate due to increasing
 296 entanglement between clumps (Riley et al. 2000, Wucherpfennig et al. 2010). In order to
 297 minimize the link between biomass and apparent viscosity, rheological property
 298 measurements were made on samples taken during the exponential growth phase with a
 299 biomass concentration between 4 and 8 g/kg so that difference between fermentations was
 300 very predominantly associated with changes in clump size due to different agitation
 301 conditions as all other parameters (initial media composition, pH, temperature, dO₂, etc.)
 302 were held constant.

303 A rotational rheometer AR2000 (TA Instruments, New Castle, Delaware, the United States)
 304 equipped with a helical rotor, was used to carry out rheological property measurements
 305 (Hardy et al. 2015) at 27°C under controlled shear rates of 1 to 100 s⁻¹. Two cycles were
 306 performed during which shear rate was increased (from 1 to 100 s⁻¹) and decreased (from 100
 307 to 1 s⁻¹) in 20 steps. Measurements of dynamic viscosity (η_a , Pa.s) were recorded when a
 308 steady state was achieved, i. e. when 3 consecutive values with less than 5 % variation were
 309 reached at each step. Results were considered with this method to have an uncertainty of 5%
 310 associated with the Couette analogy (Hardy et al. 2015).

311 In order to exclude possible errors due to secondary flow effects (Mezger 2014), only
 312 measurements with Taylor numbers < 1700 and Reynolds numbers < 53 were retained. Then,
 313 the Hershel-Bulkley (HB) model (Mezger 2014) was used to fit the rheograms and hence the
 314 fermentation broth apparent viscosity (Eq.8).

315

$$316 \quad \eta_a = \frac{\tau_{HB}}{\dot{\gamma}} + K_{HB} \times \dot{\gamma}^{n-1} \quad (8)$$

317

318 with η_a the dynamic apparent viscosity (Pa.s), τ_{HB} the yield stress (Pa), $\dot{\gamma}$ the shear rate (s⁻¹),
 319 K_{HB} the consistency index (Pa.sⁿ), and n the flow behaviour index. The parameter τ_{HB} was

320 used to estimate the potential cavern diameter relative to the diameter of the tank (Elson et al.
321 1986). For all experiments, the predicted diameter of the cavern was greater than the diameter
322 of the tank, so that no cavern formation issues were expected (Stocks 2013).

323 For comparing quantitatively the impact of agitation intensity associated with each impeller
324 type and agitator speed on broth apparent viscosity, in each case the latter were determined at
325 a reference shear rate of 10 s^{-1} . This shear rate is not the average shear rate present in the
326 bioreactors, that indeed varies between geometries and stirring rates. But as set out above, it
327 allows a simple and factual criteria for rheological property/apparent viscosity comparisons
328 as measure of the impact of agitation on structure of the filamentous mycelia at the different
329 scales.

330 *Biomass concentration determination*

331 The dry weight method was used to quantify the biomass concentration. A weighed glass
332 microfiber filter (Whatman GF/C filters) was used to filter a weighed sample of the culture
333 broth with particle retention of $1.2 \mu\text{m}$. One volume of the sample was washed with three
334 volumes of distilled water and oven-dried at 105°C until constant weight. The sample was
335 weighted after cooling in a desiccator to reach room temperature, thus allowing calculation of
336 biomass concentration (X , in g.kg^{-1}). The maximum specific growth rate was determined
337 during exponential phase by fitting (Eq.9) with weighted least squares method.

338

$$339 \quad X_t = X_0 \times e^{\mu_{max} \times t} \quad (9)$$

340

341 With X_t the biomass concentration at t time (g.kg^{-1}), X_0 the initial biomass concentration
342 (g.kg^{-1}), μ_{max} maximum the specific growth rate (h^{-1}) and t the considered time for which the
343 batch fermentation had been occurring (h).

344 *Glucose concentration analyses*

345 Glucose concentration was measured by high performance liquid chromatography (HPLC)
346 from Waters Corporation (Milford, Massachusetts, the United States). Separation was carried
347 out using a Varian Metacarb87P column 300 x 7.8 mm (Agilent Technologies, Santa Clara,
348 California, the United States), at 31 bar and 80°C. Mobile phase was composed of ultrapure
349 water with a flow rate of 0.4 mL.min⁻¹. Detection was achieved with a refractive index
350 detector (Waters 2414) and glucose concentration (g.L⁻¹) was quantified using a range of
351 calibration solutions.

352 *Staining and microscopy analyses*

353 Samples were diluted in 50 mmol.L⁻¹ phosphate-citrate buffer (pH 4.8) to reach a final
354 biomass concentration of 2 to 5 g.kg⁻¹ (Hardy et al. 2016). They were stained at a ratio 1:4
355 (v:v) with a commercial lactophenol-blue solution (Merck, Darmstadt, Germany) that was
356 half-diluted with a solution of lactophenol-bleu without cotton blue and without phenol.
357 Stained samples were fixed and sealed with nail varnish between a slide and a cover slip
358 before microscopic observations. A bright field microscope AxioImager M2pCarl Zeiss AG
359 (Oberkochen, Germany) bearing an N-Achroplan 20X objective (Carl Zeiss) was used. It was
360 equipped with a motorized stage in X-Y-Z axis (steps: 0.1 µm, 0.1 µm and 25 nm), with a
361 color camera 5 megapixels Axicam 105 Color through a video adapter 60N-C 2/3" 0.5x (Carl
362 Zeiss). After white balance, normalization of light intensity and calibration, stacks of
363 60 mosaics corresponding to a surface of 3.7 mm² and separated by 2 µm, were acquired
364 (Hardy et al. 2016).

365 *Characterization of fungus morphology by image analysis*

366 Image analysis was carried out using a dedicated method described by Hardy et al. (2016).
367 This method included an original extended depth-of-field approach, a specific segmentation
368 and both skeleton and topological analyses. It led to the determination of 31 morphological

369 criteria from the acquisition of about one thousand fungal images per sample. Among these
370 31 criteria, four were retained as most relevant in relation to size and to damage due to fluid
371 dynamic stress. For each fungal image, these four were the hyphal growth unit length (μm),
372 the surface area (μm^2), the total length (μm) and the number of holes (nH). With respect to
373 the latter, this advanced technique measures the three-dimensional clumped structure of each
374 fungus. Thus the larger it is, the more folded it becomes as it is held between the glass plates
375 used to help get clear images; and these holes relate to a combined effect of the size and
376 extent of folding. Hardy et al. (2016) showed that when the fungi were exposed to extreme
377 fluid dynamic stress levels, of the four criteria, the number of holes was the best measure of
378 size, i. e. the lower the agitation intensity, the greater the number of holes. The value of nH
379 recommended as a measure of size was the 90% quantile (q90) of the distribution of the
380 number of holes per image.

381 **3. RESULTS**

382 *Influence of bench scale stirring conditions on T. reesei cultures*

383 Initially, fermentations of *T. reesei* were conducted with the stirrer shown in Fig 1.c at the
384 bench scale at two stirring rates (800 rpm and 1700 rpm) to generate strongly different
385 agitation conditions, namely a tip speed (V_{tip}) of 3.4 and 7.1 $\text{m}\cdot\text{s}^{-1}$, a specific power input
386 (P/V) of 6.2 and 59.5 $\text{kW}\cdot\text{m}^{-3}$ and an energy dissipation circulation function considering the
387 maximum specific energy dissipation rate near the impeller ($\text{EDCF}_{\epsilon_{\text{max}}}$) of 94.2 and 1920.7
388 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$. The impact of these different conditions on *T. reesei* growth kinetics, on
389 fermentation broth viscosity and on the fungus morphology was determined. Fig. 2 shows the
390 growth curves of *T. reesei* including all of the data for both the duplicate runs for each of the
391 two stirring conditions. As can be seen, the data from the two runs cannot be separated and in
392 both cases for biomass concentrations below 8.2 $\text{g}\cdot\text{kg}^{-1}$, the exponential growth indicates that

393 there was no growth limitation related to substrate concentration. Similar data were obtained
394 in that biomass range in all fermentations undertaken. At 800 rpm, the maximum specific
395 growth rate was equal to $0.082 (\pm 0.003) \text{ h}^{-1}$ whereas at 1700 rpm it was $0.065 (\pm 0.004) \text{ h}^{-1}$, a
396 20 % reduction of μ_{max} .

397 The apparent viscosity over the whole shear rate range from samples taken during the
398 exponential growth phase when using the centripetal turbine are shown in Fig 3. At 800 rpm,
399 data are shown for a sample taken at the end of that phase (8.2 g/kg) and another a little
400 earlier (5.3 g/kg). Also shown are data from another experiment with agitation at 1700 rpm at
401 a biomass concentration of 7.4 g/kg. It can be seen that though there is a difference between
402 the apparent viscosities associated with the two biomass concentrations, there is a much
403 greater difference between those at different speeds with similar biomass concentrations, with
404 the apparent viscosity being ~ 5 times lower at the higher stirrer speed across the whole shear
405 rate range. For precise comparison showing the impact of agitator speed at a shear rate of 10
406 s^{-1} , η_a falls from $0.659 \pm 0.053 \text{ Pa.s}$ to $0.127 \pm 0.001 \text{ Pa.s}$.

407 The morphology of *T. reesei* is illustrated in Fig. 4 where at 800 rpm, the quantile value
408 $q_{90_{\text{NH}}}$ is $14.3 (\pm 1.3)$ while at 1700 rpm, it is $3.5 (\pm 0.5)$, a diminution 75 % of the number of
409 holes at high stirring rate. From these results, it is very clear that agitation speed affected the
410 growth kinetic of *T. reesei*, leading to changes in the morphology of the fungus and in the
411 apparent viscosity of the fermentation medium.

412

413 *Correlations for the effect of stirring at the bench scale on culture parameters*

414 As a second step, *T. reesei* cultures were carried out at lab-scale with all four of the impellers
415 shown in Fig. 1, at four different stirring rates (800, 935, 1250 and 1700 rpm), giving V_{tip}
416 values of 3.5, 3.9, 5.2 and 7.1 m.s^{-1} , six values for P/V (6.2, 8.4, 17.9, 23.7, 59.5 and
417 59.6 kW.m^{-3}) and six for $\text{EDCF}_{\text{E}_{\text{max}}}$ (94.2, 196.1, 521, 561.4, 829.3 and $1920.7 \text{ kW.m}^{-3}.\text{s}^{-1}$).

418 Based on the three mixing parameters (V_{tip} , P/V and $EDCF_{\epsilon_{max}}$) expressed as $\ln(X)$ as the
 419 independent variable and the three fermentation parameters (growth rate, apparent viscosity
 420 at 10 s^{-1} , and indicator of morphology q_{90}), as the dependent variable (Y), correlations were
 421 developed of the form:

422

$$423 \quad Y = a \times \ln(X) + b \quad (10)$$

424

425 where a and b were fitted constants based on a linear regression, and reported in Table 2. In
 426 Fig. 5, the fermentation parameters are maximum specific growth rate (μ_{max} , in h^{-1}), apparent
 427 viscosity at 10 s^{-1} after fitting the data with a Herschel-Bulkley model (η , in $\text{Pa}\cdot\text{s}$) and the
 428 quantiles 90 of the number of holes (q_{90}). The correlation coefficients (R^2) for each case are
 429 also shown, and it is seen to be the lowest when V_{tip} was used as the independent variable and
 430 the highest with $EDCF_{\epsilon_{max}}$ whatever the dependent variable under consideration. P/V
 431 emerges also as an interesting parameter, but its R^2 coefficients are systematically lower than
 432 $EDCF_{\epsilon_{max}}$ ones. However, the similarity between the P/V and $EDCF_{\epsilon_{max}}$ criteria is not
 433 surprising given the expression of ϵ_{max} (Eq. 6). The $\frac{3}{4}$ exponent on Po , not far from 1, makes
 434 the way the two variables change with agitation intensity somewhat similar. Furthermore, in
 435 general, flow numbers of impellers do not change as much as power numbers and that is also
 436 true here where Fl for the four impellers used only differs by a factor 2.6 between the two
 437 extremes, thus making the induced circulation times similar in value.

438 *Scale up of culture parameters*

439 With respect to the fermentation parameters in the preliminary and full run at the commercial
 440 scale, the specific growth rates were the same (0.12 h^{-1}). In both cases, the apparent viscosity
 441 was very high compared to the bench scale but, as explained earlier, in the preliminary run,
 442 the protocol for ensuring a like-for-like comparison with respect to biomass concentration

443 was not well established. As a result, the biomass concentration was ~ 15 % higher, giving an
444 apparent viscosity ~ 25% higher. Such a non-linear relationship is typical for such
445 suspensions (Hardy et al., 2015). Overall, the results from the two large scale runs are
446 essentially the same and very different from the bench scale. However, only the apparent
447 viscosity value obtained with the matching biomass concentration has been used in the
448 quantitative comparison of the large scale data with the bench scale in the discussion below.

449 Image analysis was only conducted on the full run.

450 Growth rate, viscosity and fungus morphology from the full run are reported in Fig. 5. The
451 relative error of the prediction of the large scale value from the correlations developed at
452 bench scale are reported on Fig. 5 (as “error”) and in Table 2 (% of the measured value). On
453 the other hand,, Table 3 reports the fitted parameters of the correlations in Eq.10 and R^2 when
454 both bench and commercial scale experimental results are used for the fitting procedure.
455 Finally, Table 3 also reports the relative error when the correlations, based on data from both
456 scales, are used to predict the large scale characteristics. Growth rate, morphology (number of
457 holes) and apparent viscosity are all higher at large scale than at bench scale, which shows
458 that these parameters are strongly coupled to hydrodynamic conditions. The error based on
459 the extrapolation of the V_{tip} correlation gave a noticeably higher error (error range from 34 %
460 to 65 %) for all criteria. The fact that the tip speeds at all scales are quite similar strongly
461 suggests that tip speed is not a good scale up parameter (as also found in the earlier, much
462 more limited scale-up study of Jüsten et al. (1996)). On the other hand, the predictions based
463 on P/V and $EDCF_{\epsilon_{max}}$ were much better (error range from 5.5 to 25.3 %) with those for the
464 latter being less than the former except for morphological parameter $q_{90_{nH}}$ (25.3 % for
465 $EDCF_{\epsilon_{max}}$, 16.5% for P/V). These errors following scale-up by $EDCF_{\epsilon_{max}}$ correlations are
466 very satisfying given that the range of values at the laboratory scale, which are used to fit the
467 correlations, were obtained at $EDCF_{\epsilon_{max}}$ values higher by a factor 100 than the value at the

468 industrial scale. In addition, as pointed out above, in every case, R^2 for the correlations of the
469 bench scale data was greatest with $EDCF_{\epsilon_{max}}$. This result remains valid when bench and large
470 scale experiments are used, as reported in Table 3, which shows the greater precision of the
471 $EDCF_{\epsilon_{max}}$ criteria.

472 A statistical t-test analysis gives P-values, reported in Table 4, that reinforce the consistency
473 of the correlations in Tables 2 and 3 based on $EDCF_{\epsilon_{max}}$. $EDCF_{\epsilon_{max}}$ always furnishes lower P
474 ($< 0.4\%$). P-values associated with P/V are slightly higher excepted for q90 ($P=2.5\%$), whilst
475 the correlations based on V_{tip} are not statistically significant ($P > 5\%$).

476 Variations coefficients have also been calculated, considering correlation parameters of Table
477 3. They represent the part of the experimental variability that is not explained by the linear
478 regressions. Variation coefficients are between 5% and 52% depending on the variable of
479 interest and the chosen input parameter. As expected, the highest variation coefficients are
480 associated with V_{tip} correlations, whereas the lowest ones are associated with $EDCF$
481 correlations (variations coefficients between 5% and 27%). As a conclusion, it can be stated
482 that the $EDCF$ correlations explain the major part (72% or more) of the experimental
483 variability, with the rest being linked with experimental uncertainty, including differences of
484 biomass concentrations between samples as reported in Fig. 3.

485 **4. DISCUSSION**

486 *On the effect of stirring intensity and choice of impeller at the bench scale*

487 The different stirring conditions (geometry of the impellers and stirring rates) used in this
488 study allowed very different fluid dynamic stress levels to be obtained. These stresses have
489 been characterized by three main parameters: V_{tip} , P/V and $EDCF_{\epsilon_{max}}$. In the literature, the
490 ranges of V_{tip} were limited to 0.9 to 2.7 $m.s^{-1}$ (Lejeune and Baron 1995; Malouf et al. 2013;
491 Marten et al. 1996; Patel et al. 2009) whereas here a much higher range of 3.5 to 7.1 $m.s^{-1}$

492 was obtained, more typical of those found on the commercial scale. For the parameters P/V
493 and $\text{EDCF}_{\text{emax}}$, no information is available with respect to *T. reesei*. However, similar ranges
494 of EDCF and P/V were explored with other filamentous fungi. With *Penicillium*
495 *chrysogenum* and *Aspergillus oryzae*, Amanullah et al. (2002) using the former organism
496 reported EDCF values based on the swept volume between 2 and 1000 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ and with
497 the latter, Jüsten et al. (1996) used values ranging from 2 to 2000 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$, both with P/V
498 values from 0.5 and 10 $\text{kW}\cdot\text{m}^{-3}$. In this study, higher P/V values were obtained of 6.2 to
499 59.5 $\text{kW}\cdot\text{m}^{-3}$, in order to avoid any oxygen transfer limitation. In addition, $\text{EDCF}_{\text{emax}}$ values
500 from 94.2 to 1920.7 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$, (corresponding to an EDCF (swept volume) of 244 to 4971
501 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$) extended the range used previously as a result of the relative small volume of
502 liquid used here at the bench scale.

503 Historically, impellers have often been described as either ‘low shear’ or ‘high shear’, where
504 the former have a low Po/Fl ratios and the latter, high ratios (Oldshue, 1983); and that
505 terminology is still in use today (Lightnin’, 2016), though its usefulness has been questioned
506 (Nienow, 2016). These terms imply that ‘low shear’ impellers would damage filamentous
507 organisms less than ‘high shear’ at similar specific power. However, Jüsten et al. (1996;
508 1998) found that the opposite, e.g. ‘high shear’ Rushton turbines caused less damage than
509 ‘low shear’ hydrofoil impellers with respect to morphology and productivity. In two of the
510 fermentations here using the ‘high shear’ 4-blade disc paddle (Po = 12; Fl = 1.6) at 935 rpm
511 and the ‘low shear’ profiled tri-blade Rayneri impeller (Po = 0.6; Fl = 0.87) at 1700 rpm, P/V
512 was approximately the same ($58 \pm 1.5 \text{ kW m}^{-3}$). However, the equivalent $\text{EDCF}_{\text{emax}}$ values
513 were 830 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ and 1920 $\text{kW}\cdot\text{m}^{-3}\cdot\text{s}^{-1}$ respectively. Under these conditions, the viscosity
514 was 0.128 ($\pm 0.003 \text{ Pa}\cdot\text{s}$) with the Rayneri and 0.258 ($\pm 0.020 \text{ Pa}\cdot\text{s}$) with the 4-blade disc
515 paddle. In addition, the specific growth rates were 0.061 (± 0.002) h^{-1} and 0.070 (± 0.003) h^{-1}
516 and the number of q90 number of holes were 25 (± 2.2) and 31.2 (± 2.5) respectively. Thus, all

517 the process parameters indicate more damage with the ‘low shear’ impeller than with the
518 ‘high’ one.

519 Another way to consider the effect of impeller type on $EDCF_{\varepsilon_{\max}}$ is to rearrange Eq. 7 using
520 Eq. 2, 4 and 6 to eliminate the impeller speed, N. In this way, $EDCF_{\varepsilon_{\max}}$ can be written as a
521 function of (P/V) to give:

522

$$523 \quad EDCF_{\varepsilon_{\max}} \propto (V / \rho)^{1/3} (P/V)^{4/3} D^{-5/3} (Fl / Po^{7/12}) \quad (11)$$

524

525 If, in addition, it is assumed that $V \propto D^3$,

526

$$527 \quad EDCF_{\varepsilon_{\max}} \propto (P/V)^{4/3} D^{-2/3} (Fl / Po^{7/12}) \quad (12)$$

528

529 Thus, at one scale, at constant P/V and D, so called “low shear” impellers (high Fl/Po ratios)
530 will have a higher $EDCF_{\varepsilon_{\max}}$ than “high shear” impellers and can therefore potentially induce
531 more damage to the filamentous fungi, as was shown in this study.

532 *Fluid dynamic stress affected the growth of T. reesei*

533 Comparison of the results obtained with the different stress conditions at the bench scale
534 showed that increasing levels negatively impacted the maximum specific growth rate of
535 *T. reesei*. This result confirms the observation of Lejeune and Baron (1995) who also showed
536 that an increase of agitation speed reduced the growth of *T. reesei*, as indicated by the
537 increase of the lag phase. However, these results disagree with those obtained by other
538 authors (Malouf et al. 2013; Marten et al. 1996; Patel et al. 2009) who observed a positive
539 impact of high agitation intensity on growth of *T. reesei*. This difference can probably be
540 explained by the presence of some oxygen transfer limitation during their cultures since

541 unlike in the present work, dO_2 was not controlled, so that the elevated agitation speed would
542 have increased it. There may also have been issues with substrate limitation as discussed
543 further below.

544 *Fluid dynamic stress affected the 'viscosity' of T. reesei fermentation broth*

545 The apparent viscosity of the fermentation broth was reduced by increasing the stress level.
546 This result agrees with that of Hardy et al.(2016). However, Marten et al.(1996) observed an
547 increased viscosity at higher agitation speed whilst Patel et al.(2009) found that agitation
548 speed did not affect viscosity, except for the lowest agitation speed that led to a lower
549 viscosity. These differences may be ascribed to the lower agitation intensities explored in the
550 earlier studies but again are more probably due to the fact that, at the agitation rates used,
551 oxygen limitations occurred in the work of Patel et al.(2009), which reduced the growth of
552 the fungus. On the other hand, as pointed out by the authors themselves, substrate limitations
553 took place in the work of Marten et al.(1996), which induces fungal fragmentation, which in
554 turn causes a reduction of the viscosity, as observed by Henaut et al.(2013). To avoid these
555 problems, in this work, all experiments were performed without carbon limitation, by using
556 directly fermentable substrates (lactose as a carbon source instead of cellulose) and a constant
557 dO_2 of 40 % by gas blending (as done in the earlier work of Amanullah et al., (2002)). These
558 conditions permitted the impact of agitation conditions to be dissociated from the effect of
559 substrate and oxygen limitations, whilst also achieving conditions that are desired at large
560 scale.

561 *Fluid dynamic stress affected the morphology of T. reesei*

562 The stress conditions modified the morphological parameters that characterized the fungus
563 during the growth phase. Notably, a decrease of 75 % of the number of holes inside the
564 fungus was measured when the stress was highest. As discussed above, in the image analysis
565 technique used in this study (Hardy et al. 2016), the larger the structure, the higher number

566 of holes. Thus, this reduction in the number of holes clearly shows that, in this work, high
567 stresses also induced a reduction of the size of *T. reesei* cellular structures. This result is in
568 agreement with those of Patel et al.(2009), but here it was obtained in conditions that avoided
569 carbon and oxygen limitations, thus being more clearly associated with the impact of the fluid
570 dynamic stresses (as well as being related to the desired large scale operating conditions)

571 *Use of the different stress criteria to predict T. reesei culture parameters at commercial scale*
572 *from bench scale studies*

573 Uniquely, in this study, *T. reesei* culture parameters were also measured at the commercial
574 scale. Thus it was possible to relate the maximum specific growth rate, the apparent viscosity
575 of the broth and the morphological characteristics of the fungus at this scale to those
576 predicted by three different criteria reflecting the fluid dynamic stresses arising from
577 agitation. However, the morphology of the fungus and the rheological properties of the
578 fermentation broth also depend on biomass concentration, in addition to agitation intensity
579 (Hardy et al. 2015; Malouf et al. 2013). So, in order to dissociate the effect of biomass from
580 the effect of the different stress parameters the rheological property measurements were made
581 over a narrow range of biomass concentrations (4 to 8 g.kg⁻¹). This approach was
582 complementary to that implemented by Malouf et al.(2013) who proposed correlations
583 between rheological and morphological parameters that directly integrated the biomass
584 concentration.

585 By comparing the three measured fermentation parameters with the three criteria of fluid
586 mechanical stress (agitation intensity) of tip speed, power input per volume or $EDCF_{\text{max}}$, this
587 study has demonstrated that the $EDCF_{\text{max}}$ criterion was the most suitable for predicting the
588 growth of *T. reesei*, the apparent viscosity of the broth and the morphology of the fungus at
589 the commercial scale. Considering the fungus morphology, this result is in agreement with
590 those of Jüsten et al. (1996) who showed that the EDCF concept is better able to correlate

591 morphological parameters than the other two criteria at different scales. Also as found by
592 Jüsten et al. (1998), the EDCF concept was the best fit to the fermentation productivity, here
593 in terms of maximum specific growth rate and in the earlier study, penicillin production. In
594 addition, to our knowledge, for the first time, it is also seen that the apparent viscosity of the
595 broth is best correlated and predicted by $EDCF_{\epsilon_{max}}$. It is particularly effective at
596 demonstrating that even though the tip speed at the commercial scale is similar to those at the
597 bench scale, the apparent viscosity is much higher, just as the $EDCF_{\epsilon_{max}}$ function predicts.
598 The interconnections between all these variables can be explained by the fact that they are all
599 dependent on the operating conditions (Quintanilla et al. 2015). The good result obtained
600 with $EDCF_{\epsilon_{max}}$ as independent variable is due to the integration of more phenomena than the
601 other representations of fluid dynamic stress. Notably, as introduced by Smith et al. (1990)
602 and Jüsten et al. (1996), it includes the notion of passage frequency of the fungi through the
603 high energy dissipation region near the impeller, coupled with the maximum local specific
604 energy dissipation rate in that region, both of which are important factors in determining the
605 fluid mechanical stress ‘perceived’ by the fungus in the bioreactor. According to
606 Stocks (2013), these two phenomena (frequency and intensity) may change in an opposite
607 way during scale-up: frequency decreases at large scale, whereas fluid dynamic stress if
608 defined by tip speed generally increases. This work clearly shows that V_{tip} is not an
609 appropriate measure of stress. On the other hand, whilst the P/V parameter is proportional to
610 the maximum specific energy dissipation rate, ϵ_{max} , the allowance for the frequency of
611 circulation through that region is found to improve the predictability.

612 The relative accuracy of the three different methods of predicting the three large scale
613 fermentation parameters can be seen in Fig 5. Using $EDCF_{\epsilon_{max}}$ as the independent variable to
614 predict the viscosity of the fermented medium and the maximum specific growth rate of
615 *T. reesei* at commercial scale led to error values lower than 5%, and 15% respectively,

616 whereas the error reached 25 % for the morphological criterion. Considering this information,
617 the predictions of the viscosity and the specific growth rate seemed good, thus validating the
618 use of $EDCF_{\text{emax}}$ as the best extrapolation factor for *T. reesei* cultures. The higher error in
619 assessing the morphology of the fungus could be explained by the fact that there are many
620 morphological criteria that could be used as explained by Hardy et al. (2016). Thus, those,
621 which are best at bench scale as found here and in earlier work, may not be the best when
622 extended to the commercial scale. Nevertheless, the trend is certainly correct.

623 5. CONCLUSIONS AND PERSPECTIVES

624 During *Trichoderma reesei* batch cultures at the bench and commercial scale, increasing fluid
625 dynamic stress under conditions of controlled dO_2 and without nutrient limitation, has been
626 shown to lower the apparent viscosity of the broth and the maximum specific growth rate and
627 reduced the size of the fungus (reduced the number of holes as measured by image analysis).
628 Three criteria (V_{tip} , P/V and $EDCF_{\text{emax}}$) were considered as measures of this stress and were
629 correlated with the fermentation parameters measured at the bench scale. It was found that
630 the $EDCF_{\text{emax}}$ function gave the best fit. These correlations were then used to predict the same
631 parameters in a commercial scale bioreactor. This extrapolation showed again that $EDCF_{\text{emax}}$
632 was the best parameter to predict these variables, whilst the use of V_{tip} was extremely
633 inaccurate. However, to draw out the difference in prediction performance between P/V and
634 $EDCF_{\text{emax}}$, it is interesting to data fit Eq. 10 to the data from the runs at both scales. Again, it
635 can be seen that the fit (Table 3) with the $EDCF_{\text{emax}}$ is the best in each case, giving further
636 support to its use for predicting performance across the scales as well as the impact of
637 different impellers.

638 Overall, this work has demonstrated that it was possible to make a scientifically based scale-
639 up to commercial scale in relation to fluid dynamic stresses from scale-down experiments

640 done at the bench. In the future, these correlations should be improved by taking account the
641 effect of biomass concentration or by expanding the range of $EDCF_{\text{emax}}$ values used to reach
642 lower values. In addition, as the industrial strain used in this work exhibited an unusual
643 morphology, it would be interesting to expand this study by considering a well-known strain
644 such as *T. reesei* RUT-C30, to facilitate the comparison with previous works. Finally, it is
645 planned to use the correlations and approach established in this work to the production of
646 cellulases.

647 **ACKNOWLEDGMENTS**

648 The authors acknowledge the Agence de l'Environnement et de la Maîtrise de l'Energie
649 (ADEME, Angers, France) for financial support of PhD of Nicolas Hardy and Dr. Claudio
650 Pereira Da Fonte for Computational fluid dynamics characterization of the paddle impeller
651 (Power number, flow number).

652

653 **NOMENCLATURE**

654	a, b	Fitted constants in Eq. 10
655	D	Impeller diameter (m)
656	dO ₂	dissolved oxygen concentration (% from saturation value at ambient pressure)
657	EDCF	Energy dissipation/circulation function (W.m ⁻³ .s ⁻¹)
658	EDCF _{εmax}	EDCF considering the maximum specific energy dissipation rate (W.m ⁻³ .s ⁻¹)
659	Fl	Flow number of the impeller
660	K _{HB}	consistency index (Pa.s ⁿ)
661	N	Rotation speed (s ⁻¹)
662	n	flow behaviour index (-)
663	nH	number of holes in a fungal image
664	Po	Power number of the impeller
665	P	Power draw (W)
666	q90	90 % quantile in the distribution of nH in the images analysed <i>t</i> time (h)
667	<i>t_c</i>	Circulation time (s ⁻¹)
668	<i>t_m</i>	Mixing time (s ⁻¹)
669	R ²	Correlation coefficient
670	V	Volume of broth (m ³)
671	V _{tip}	Impeller tip speed (m.s ⁻¹)
672	VVM	Aeration of the broth (in normal Volume of air per Volume of liquid per minute)
673	W	Impeller blade width (see Table 1) (m)
674	X	Biomass concentration (g.kg ⁻¹); and independent variable in Eq. 10.
675	Y	Dependent variable in Eq. 10.
676	ε _{max}	Maximum local specific energy dissipation rate (W.m ⁻³)
677	μ _{max}	Maximum specific growth rate (h ⁻¹)

678	η_a	Apparent viscosity (Pa.s)
679	ρ	Density (kg.m^{-3})
680	$\dot{\gamma}$	Shear rate (s^{-1})
681	τ_{HB}	Yield stress (Pa)

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790

791 **Table Captions**

792

793 Table 1: Mixing and geometric characteristics of the impellers used in the study. ⁽¹⁾ Data from
794 VMI the Mixing Company Rayneri (Montaigu, France); ⁽²⁾ Data obtained by Computational
795 Fluid Dynamics at IFPEN (Rueil-Malmaison, France) with the software ANSYS CFD from
796 Ansys (Cecil Township, Pennsylvania, U.S.A.) using the Moving Reference Frame method.

797

798 Table 2: Correlation coefficients (Eq.10) - Fit at Bench scale only

799

800 Table 3: Correlation coefficients (Eq.10) - Fit at Bench and Commercial scale

801

802 Table 4: P-values corresponding to t-test of Eq.10, based on correlation parameters of Tables
803 2 and 3.

804

805 **Figure Captions**

806 Fig. 1. Pictures of the impellers used in the study, with associated symbols. (a) Propeller; (b)
807 Profiled triblade; (c) Centripetal turbine; (d) 4-blade disc paddle; The scale bar in the upper-
808 left corner represents 10 cm.

809 Fig. 2. Growth curves characterizing duplicate batch fermentations of *T. reesei* under two
810 stirring conditions with data from duplicate runs at each speed included. The centripetal
811 turbine is used at a speed rate of 800 rpm (white) and 1700 rpm (black). Arrows point to time
812 when samples were taken for apparent viscosity and image analysis measurements.

813 Fig. 3. Dynamic apparent viscosity versus shear rate for culture samples of *T. reesei* fungi.
814 Samples were collected during exponential growth phase at 800 rpm using the centripetal
815 turbine, 5.3g/kg (white circles) and 8.2g/kg of biomass (white triangles), and at 1700 rpm and
816 7.4g/kg of biomass (black circles). Duplicates with two cycles per sample are presented for
817 each condition.

818 Fig. 4. Representative pictures of *T. reesei* morphology after cultures conducted at 800 rpm
819 (A and B) and 1700 rpm (C and D). The images are composed of mosaics of 16 subimages,
820 with a size of 10098 per 7538 pixels. They were obtained before segmentation (A and C) or
821 after segmentation (B and D). Holes are shown in red in the segmented images.
822 Width of images is 2 mm.

823 Fig. 5. Comparison of growth, rheological and morphological parameters obtained from
824 predictions based on three fluid dynamic stress criteria. V_{tip} : tip speed (in $m.s^{-1}$); P/V: power
825 input per volume (in $kW.m^{-3}$); EDCF(ϵ_{max}): EDCF considering the maximum specific energy
826 dissipation (in $kW.m^{-3}.s^{-1}$). Marker's sizes are proportional to biomass concentrations.
827 Propeller (square); Profiled triblade (inverted triangle); Centripetal turbine (circle); 4-blade
828 disc paddle (star); Commercial scale (black cross). 800 rpm (white); 935 rpm (light-grey);
829 1250 rpm (dark-grey); 1700 rpm (black). R^2 are the correlation coefficients for the linear

830 models in logarithmic scale; errors represent the difference (in %) between predictions of a
831 parameter and its measurement at commercial scale.

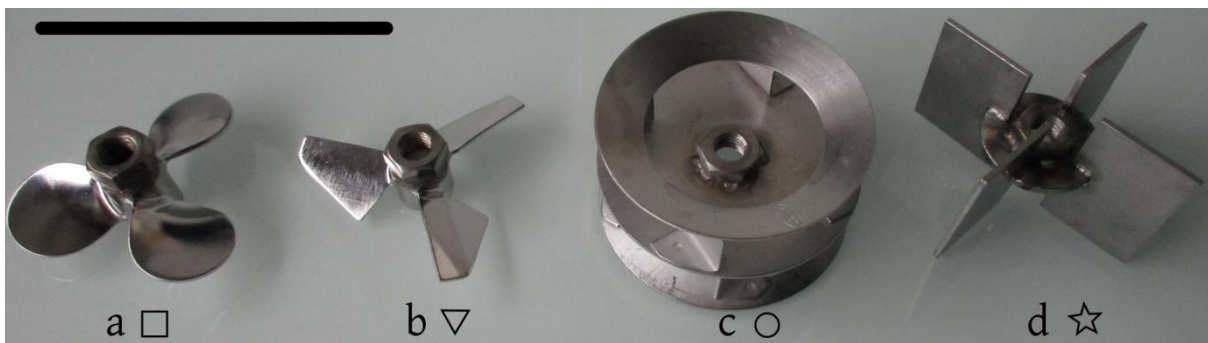
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833 **Figures**

834

835 Figure 1

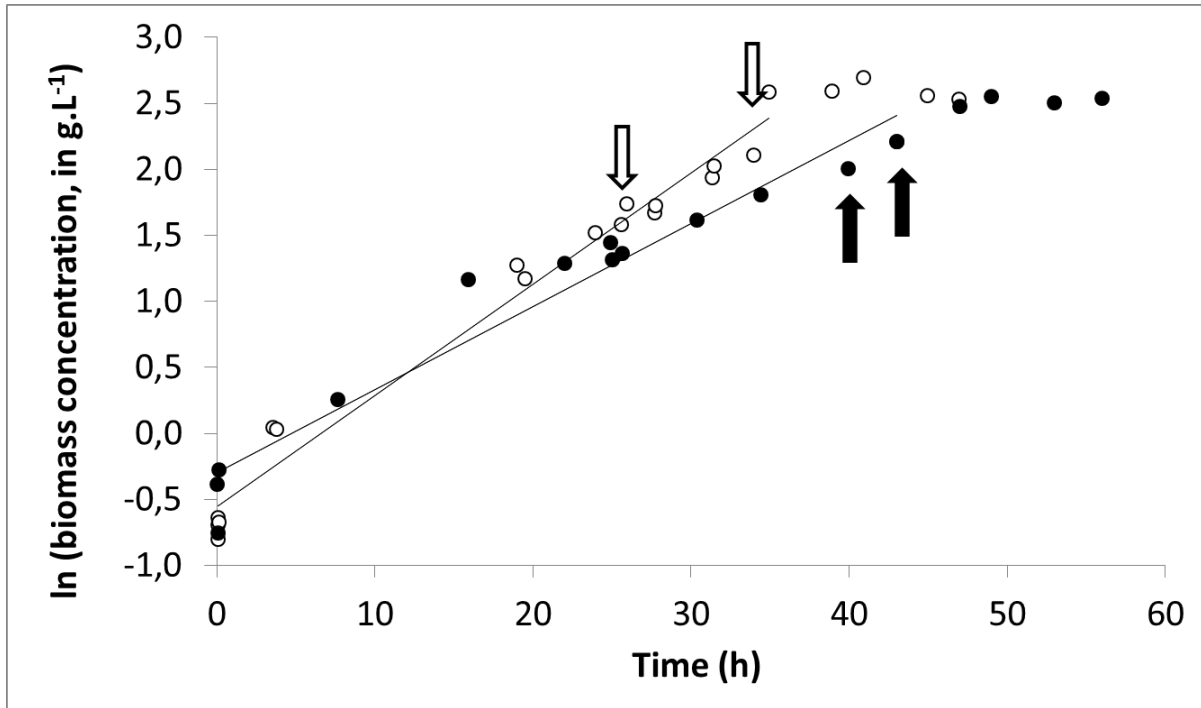
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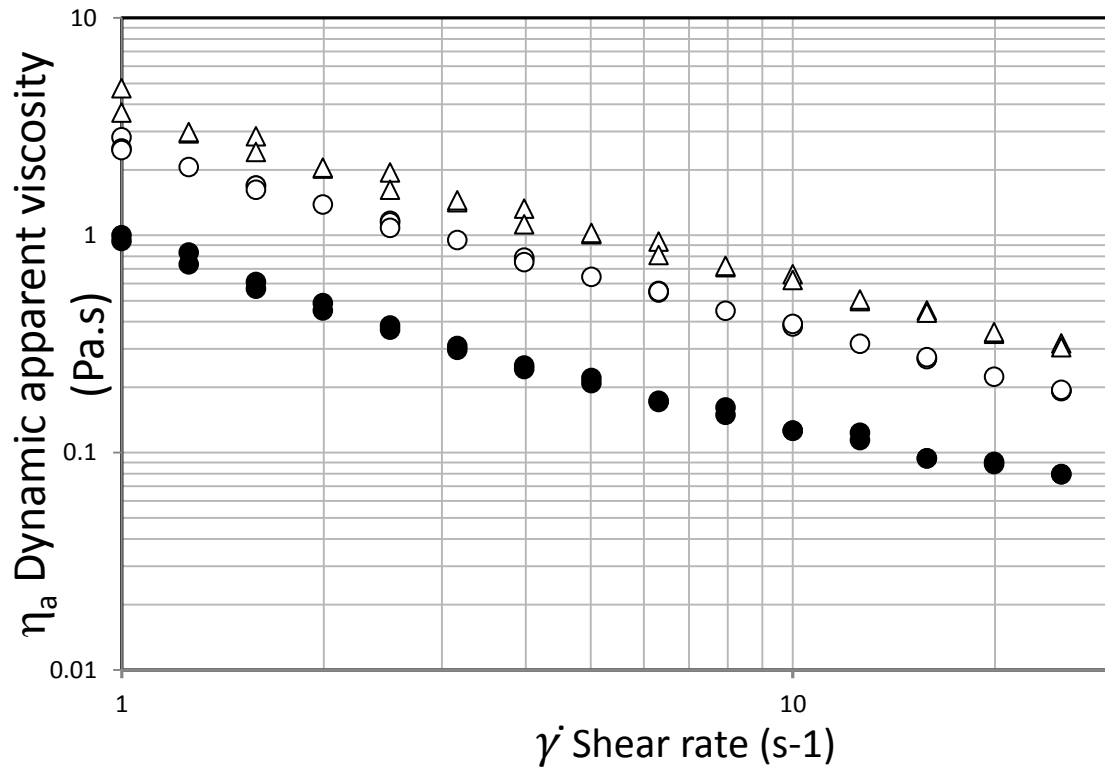
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846 *Arrows point to time when samples were taken for apparent viscosity and image analysis*
 847 *measurements.*

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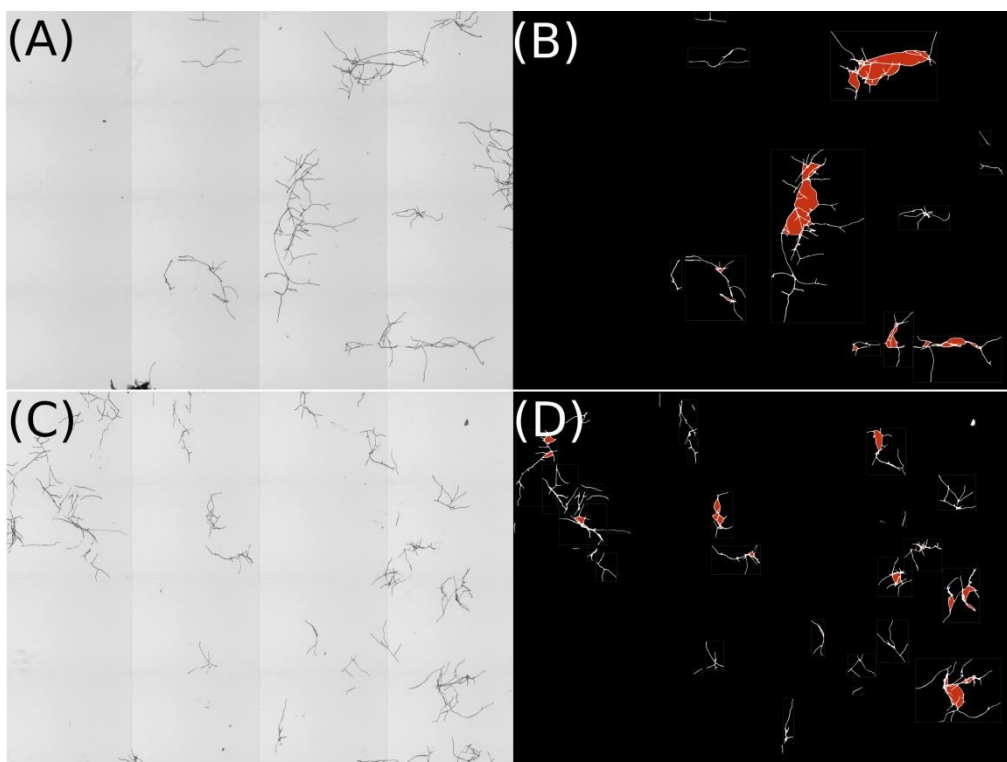
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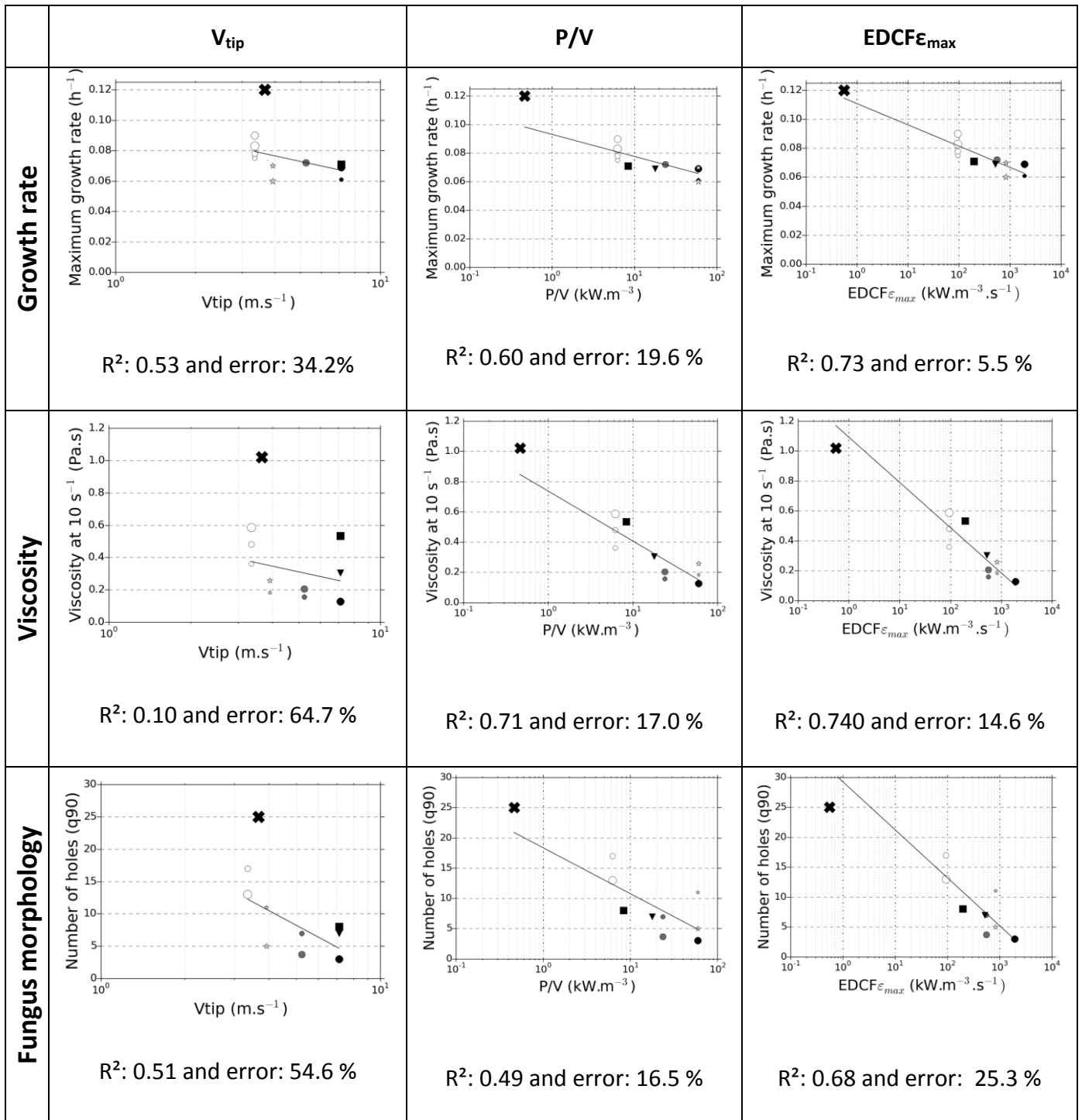
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