1 **Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee**

2 **husk using specialized consortia: The consistency of the process and the microbial**

3 **communities involved**

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18   **Abstract**

19   Solid state fermentation is a promising technology however rising concerns related to

20   scale up and reproducibility in a productive process**.** Coffee husk and a specialized inoculum

21   were used in a 4.5L and then in 50L reactors to assess the reproducibility of a cellulase and

22   hemicellulase production system. Fermentations were consistent in terms of cellulase

23   production and microbial communities. The higher temperatures achieved when operating at

24   50L generated a shift on the microbial communities and a reduction of nearly 50% on

25   cellulase production at pilot scale. In spite, an overall enzymatic production of 3.1 ± 0.5 FPUg^{-1}DM and 48 ± 4 Ug^{-1}DM for FPase and Xyl activities was obtained, respectively, with low

27   deviation coefficients of 16 and 19% for FPase and Xyl production. Gaseous emissions

28   assessment revealed an emission factor of $2.6\cdot10^{-3}$ kg volatile organic compounds per Mg of

29   coffee husk and negligible NH_3, CH_4 and N_2O emissions.

30

31   *Keywords*: cellulase, coffee husk, specialized inoculum, solid-state fermentation, xylanase.

32

1. Introduction

Cellulases and xylanases are hydrolytic enzymes related to the biodegradation of lignocellulosic material, with great interest in the bioethanol production. Cellulase purchase has been reported as the most expensive point in the entire chain of bioethanol production, accounting up to 40% of the total cost (Arora et al., 2015). For this reason, many efforts have been made to obtain a low cost cellulase, using different technologies and substrates. In this context, agroindustrial wastes appear as ideal substrates for microbial fermentation, due to their rich content of organic components, low cost and wide availability (El-Bakry et al., 2015). Furthermore, the use of agricultural by-products presents a better energy balance and a lower environmental impact than those of pure substrates (Olofsson et al., 2008).

Solid state fermentation (SSF) is a suitable strategy for producing low cost enzymatic products (El-Bakry et al., 2015). SSF of lignocellulosic biomass presents important advantages over conventional submerged fermentation such as reduced energy requirements, high productivity and less inhibitory effects for enzymatic production (Chen, 2013). Also, SSF allows the use of organic wastes for biotransformation into bioproducts enabling the production of a concentrated product, due to the low amounts of water used in the process (Mitchell et al., 2006). Despite these great advantages, the main challenges to overcome in SSF processes are the scaling up and the development of a standard and continuous process at large scale (Kuhad et al., 2016; Mitchell et al., 2006). Our research group has operated SSF as a multispecies self-heating thermophilic batch (similar to the composting process), which demonstrated to be successful for the production of proteases (Abraham et al., 2013), amylases (Cerda et al., 2016) and cellulases (Cerda et al., 2017) using non-sterile wastes as substrate.

Specifically, cellulase production, using coffee husk as substrate and a specialized consortium as inoculum, was successfully carried out at a representative scale (>1 kg

58   substrate) in a sequential batch operation mode with promising results towards a continuous

59   and reproducible cellulase production (Cerda et al., 2017). There are very few other attempts

60   in developing a continuous process for enzyme production (Astolfi et al., 2011; Cheirsilp &

61   Kitcha, 2015) and even less to assess the SSF scaling up problem (Biz et al., 2016; Idris et al.

62   2017; Ortiz et al., 2017). In order to deliver a sustainable and low cost continuous SSF

63   process it is necessary to consider the inherent variability of the organic wastes and the

64   inoculum required for enzymatic production. Most of the reported SSF process are performed

65   under sterile conditions and using specific microorganisms as seen in Table 1 (El-Bakry et

66   al., 2015; Behera & Ray, 2016; Kuhad et al., 2016). In this sense, a multispecies SSF such as

67   the proposed by our group (Cerda et al., 2017) has been only been suggested by one other

68   author (Wang et al., 2016). In this SSF, these authors used autochthonous microbiota

69   (without inoculation) which allowed them to guarantee the reproducibility of the process

70   using a metagenomics approach. Taking this into consideration, it seems of great importance

71   to assess the proper propagation of the specialized mixed inoculum at a large scale in order to

72   achieve a standardized cellulase production process.

73   Besides the production of the targeted product, the environmental impacts of the SSF

74   process must be taken into account when considering SSF benefits. This fact has been

75   assessed in previous studies (Maulini-Durán et al., 2015), which reported gaseous emissions

76   from the SSF for cellulases, proteases and lipases production at the pilot scale. This work

77   remains, to our knowledge, the only reported research on this subject, although the issue of

78   emissions from SSF is crucial for further environmental impact studies.

79   Considering the above, the main objectives of this work are i) to study the influence of

80   using a specialized inoculum in the production of cellulases and xylanases operating a pilot

81   scale SSF process, with a full characterization of the microbial communities ii) the

82   assessment on the reproducibility of the process at laboratory and pilot scale and iii) the

83 complete characterization of the gaseous emissions of the process, including emission factors

84 for ammonia, volatile organic compounds, methane and nitrous oxide. To our knowledge,

85 there are no similar studies reported in literature.

86

87 **2. Materials and methods**

88 *2.1 Raw material*

89 Coffee husk (CH) was kindly provided by Marcilla S.A (Mollet del Vallès, Barcelona,

90 Spain) and stored frozen at -20ºC until use. CH presented a general characterization of:

91 moisture content 68.2±0.4% (wet basis), pH 6.5±0.1 and C/N ratio 13.3±0.5. Fiber content in

92 CH was 25.7±0.2, 14.6±0.1 and 17.6±0.5% (dry basis) of cellulose, hemicellulose and lignin,

93 respectively.

94 *2.2 Specialized inoculum*

95 The inoculum used in this work consisted on a specialized consortium of

96 microorganisms able to produce cellulases using CH as sole substrate. This was previously

97 obtained operating SSF by sequential batch (SB) using compost as starting inoculum. Main

98 species identified by DNA sequencing were the bacteria *Pseudoxanthonomas taiwanensis*

99 and *Sphingobacterium composti* and the yeasts *Cyberlindnera jardinii* and *Barnettozyma*

100 *californica*, all of them previously reported as lignocellulose degraders (Cerda et al., 2017).

101 The procedure to obtain this inoculum is described in Cerda et al. (2017). Briefly, compost

102 was added in a 10% (w/w) ratio to a mixture of CH and wood chips as bulking agent in a 1:1

103 (v/v) ratio to provide the proper porosity to the mixture (Ruggieri et al., 2009). During the

104 process there was no evidence suggesting wood chips degradation due to the short time of

105 each cycle. Sequential batch operation was performed in cycles until stable cellulase

106   production of 9 ± 1 FPU g^{-1} DM (filter paper units per grams of dry matter). SB operation

107   started with a 48h fermentation stage followed by a substrate exchange of 90% of volume

108   every 48h. The process was operated for several cycles until stable operation was achieved

109   and the final fermented solids were stored frozen for further use as specialized inoculum. The

110   specialized inoculum presented a general characterization of: moisture content of 55.0±3.1%

111   (wet basis), pH 9.1±0.1 and C/N ratio of 13.1±0.6. Fiber content in the specialized inoculum

112   was 23.5±0.5, 13.3±0.5 and 18.3±0.6% (dry basis) of cellulose, hemicellulose and lignin,

113   respectively.

114   *2.3. Solid State Fermentation (SSF)*

115   SSF was performed in a 50L pilot bioreactor in triplicate batches (R1, R2 and R3).

116   Each batch was inoculated with fermented solids from a 4.5 L propagation reactor (P1, P2

117   and P3, respectively) inoculated with the thawed specialized consortium. Both propagation

118   and pilot operations were non-isothermal, using thermally isolated reactors in all cases. Full

119   description is presented below.

120   2.3.1. Inoculum preparation in propagation reactors

121   A propagation reactor was required to provide the proper amount of biomass to inoculate the

122   pilot reactors. Inoculum was obtained by using an initial mixture containing CH and 10% of

123   the mixed specialized inoculum with wood chips, which was used in a ratio of 1:1 (v/v),

124   giving a total weight of 1.2 kg per batch. The process was carried out for 48h in 4.5 L air-

125   tight packed bed reactors, working under non-isothermal conditions and oxygen controlled

126   aeration (120-240 mL min^{-1}, oxygen setpoint 11.5% in air).

127   2.3.2 Pilot reactor operation

128   Three batches were performed in 50 L air-tight packed bed reactors. A schematic

129   diagram of the pilot reactor and a detailed description can be found elsewhere (Puyuelo et al.,

130   2010). Temperature, exhaust gas oxygen concentration and inlet airflow were monitored

131   during the trials. The experiments were performed with forced aeration and airflow was

132   manually adjusted to ensure that the oxygen content in the reactor remained above 10%, in

133   order to provide full aerobic conditions (Puyuelo et al., 2010).

134   The mixtures were prepared by mixing CH and the specialized inoculum obtained

135   from the propagation reactor in a 90:10 (w/w) ratio respectively. Wood chips were added as

136   bulking agent in a volume ratio of 1:1 (v/v). The final weight of the mixture was 15.2 kg for

137   each reactor. The first batch (R1) was performed to obtain a full profile of enzymatic activity

138   production, collecting gaseous and solid samples at 0, 8, 16, 24, 35, 48, 58, 72 and 134 h.

139   Also the process time for maximum cellulase activity was established. Two additional

140   batches (R2 and R3) were performed in order to assess the reproducibility of the process and

141   the fermentation was stopped at the moment of maximum cellulase activity.

142   2.3.3 Sampling

143   Gaseous samples were collected in 1-L Tedlar® bags for ammonia (NH_3), volatile

144   organic compounds (VOC), nitrous oxide (N_2O) and methane (CH_4) content determination

145   prior to opening the reactor. After the collection of the gaseous samples, the reactor was

146   opened and the solid content was manually homogenized to obtain a representative solid

147   sample for enzymatic activity determination. Filter paper activity (FPase) was measured for

148   cellulase production. Xylanase (Xyl) production was followed for hemicellulase production.

149   In addition to the enzymatic measurements, the solid samples were analyzed in order

150   to determine the neutral detergent fiber, acid detergent fiber and lignin content. These

151   analysis were carried out by the method of Van Soest (1991) using the Ankom200 Fiber

152   Analyzer incubator (Ankom Technology, Macedon, NY), adding amylase and sodium

153   sulphite solutions. Degradation percentage of cellulose, hemicellulose and lignin were

154   calculated according to a mass balance and considering the weight evolution throughout the

155   process.

156   2.3.4   Parameters monitoring

157   Temperature and oxygen content were continuously monitored and recorded in the

158   reactors during the fermentations. Specific oxygen uptake rate (sOUR) was calculated

159   according to the following:

160
$$sOUR = F \cdot (0.209 - y_{O_2}) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW \cdot 10^3}$$ Equation (1)

161   where: sOUR is the Specific Oxygen Uptake Rate (mg O_2 g^{-1} DM h^{-1}); F, airflow into the

162   reactor (mL min^{-1}); y_{O2}, is the oxygen molar fraction in the exhaust gases (mol O_2 mol^{-1}); P,

163   pressure of the system assumed constant at 101325 (Pa); 32, oxygen molecular weight (g O_2

164   mol^{-1} O_2); 60, conversion factor from minute to hour; 10^3, conversion factor from mL to L; R,

165   ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, temperature at which F is measured (K); DW,

166   initial dry weight of solids in the reactor (g); 10^3, conversion factor from g to mg. The area

167   below the sOUR curve was also determined, which represents the cumulative oxygen

168   consumption (COC) during the process (Ponsá et al., 2010).

169   *2.4 Enzyme extraction*

170   Fermented solids were mixed thoroughly on a magnetic stirrer for 30 min with 0.05M

171   citrate buffer, at a pH of 4.8 and ratio of 1:15 (w:v) respectively. The extract was separated

172   by centrifugation at 10000 rpm during 10 min followed by a filtration with a 0.45 μm filter.

173 The supernatant was used for the cellulase and xylanase activity assay (Ang et al., 2013;

174 Dhillon et al., 2012b).

175 *2.5 Enzyme assay*

176   Total cellulase activity was measured using the filter paper activity assay (FPase)

177 recommended by IUPAC (Ghose, 1987). Final products were measured using dinitrosalicylic

178 acid (DNS) reagent (Miller, 1959). One unit of FPase (FPU) was expressed as equivalent to

179 the amount of enzyme that releases 1 μmol of reducing sugars from Whatman filter paper in

180 0.05M citrate buffer (pH 4.8) under assay conditions. Xylanase activity (Xyl) was measured

181 according to the reported by Ang et al. (2013). The final products were also measured using

182 dinitrosalicylic acid (DNS) reagent (Miller, 1959). One unit of Xyl (U) activity was

183 expressed as 1 μmol of xylose released from xylan birch wood under assay conditions.

184   The enzyme yields are expressed in terms of activity units per grams dry matter, i.e,

185 FPU g^{-1} DM and U g^{-1} DM for FPase and Xyl, respectively.

186 *2.6 Determination of gaseous emissions*

187   VOC, CH_4 and N_2O analysis were performed by means of gas chromatography

188 (Agilent Tech. 6890 N Network GC system, Madrid, Spain). NH_3 concentration was

189 measured using an ammonia sensor (Industrial Scientific sensor iTX-T82). All these

190 measurements were performed as described elsewhere (Maulini-Duran et al., 2015).

191 2.7 *Microbial characterization*

192   Identification of the microbial population was performed in the final products from

193 the propagation reactors and in the fermented solids obtained from the pilot reactors at the

194 highest cellulase production, using next generation sequencing. Total DNA was extracted and

195 purified using PowerSoil ™ DNA Isolation Kit (MoBio Laboratories, USA) according to

196 provider's specifications. DNA samples were checked for concentration and quality using the

197 NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware;

198 USA). Bacterial 16S rRNA hypervariable regions V3-V4 and fungal ITS1-ITS3 were

199 targeted. Later sequencing of the extracted DNA and bioinformatics were performed on

200 MiSeq platform by Life Sequencing S.A (Valencia, Spain).

201 *2.8 Routine methods*

202   Moisture content, total and volatile solids and pH were determined according to

203 standard procedures (U.S. Composting Council, 2001) in a representative solid sample, as

204 explained above.

205 *2.9. Statistical Analysis*

206   Statistical analysis was performed with basic ANOVA techniques while pairwise

207 comparisons were based on the Tukey test ($p < 0.05$). Statistics were performed with

208 MINITAB™ V17.

209

210 **3. Results and Discussion**

211 *3.1 Specialized inoculum preparation*

212   The average initial moisture, pH and C/N ratio for propagation reactors were 67±1,

213 8.01±0.01 and 12.5±0.5, respectively. Figure 1 presents the sOUR and the temperature

214 profiles of the three propagation reactors (P1, P2 and P3) for inoculum preparation. All three

215 propagation reactors presented a similar trend. However, the heterogeneity of the materials

216 led to a series of small differences during the processes despite of using the same specialized

217 consortium.

218 P2 and P3 started at temperatures of 21 and 25ºC respectively, which was notably higher than

219 initial P1 temperature (12ºC). This difference was attributed to the preparation process of the

220 substrate, which includes a defrosting stage. Major differences were found during transition

221 to thermophilic stage after 24h of operation. After reaching 45ºC the automatic control started

222 to act according to the oxygen requirements of the fermentation, which can be clearly

223 observed in the oscillation of sOUR profiles of P2 and P3. In this sense, at the end of the

224 fermentation sOUR obtained for P1, P2 and P3 were 2.6, 3.0 and 3.1 mg O_2 g^{-1} DM h^{-1},

225 respectively.

226 Additionally, COC resulted in very similar with values of 65.3, 86.1 and 94.8 mg O_2

227 g^{-1} DM for P1, P2 and P3 respectively. It is highly possible that initial temperature affected

228 the performance of P1, in detriment of the global biological activity (Sundh & Rönn, 2002). It

229 is for this reason that, in order to improve reproducibility of the process, in terms of

230 biological and enzymatic activity, the initial temperature of the process must be set on proper

231 values to reduce the lag phase of microbial communities that will grow on the reactors.

232 The average cellulase activity produced in the three reactors was 8.3±0.2 FPU g^{-1} DM.

233 These values are in accordance to the obtained during the sequential batch operation for the

234 production of the specialized inoculum (9±1 FPU g^{-1} DM), which was carried out in the same

235 configuration and scale (Cerda et al., 2017). Then, it is remarkable the fact that it is possible

236 to carry out a consistent and reproducible SSF for the production of cellulases using a mixed

237 inoculum without compromising productivity.

238 *3.2 Pilot reactor operation*

239 3.2.1 Process follow up

240   Figure 2a presents the fermentation profile for a 134 h operation using a previously

241 propagated inoculum. The fermentation presented a quick start-up with no lag phase as

242 observed in the sOUR and thermophilic temperature profiles (Figure 2a). This can be

243 attributed to the initial conditions of the inoculum, which was in its highest biological and

244 enzymatic activity. Biological activity was found at its maximum during the first 30h of

245 fermentation in a full thermophilic stage. Maximum sOUR and temperature were 3.6 mg O_2

246 g^{-1} DM h^{-1} and 71ºC, respectively, at 24h. Oxygen requirements reached a total COC of 243.2

247 mg O_2 g^{-1} DM. COC found at 24h of fermentation, at its highest sOUR, was 80.2 mg O_2 g^{-1}

248 DM, which roughly represents 33% of the total oxygen consumption in 136h. In this context,

249 sOUR obtained at pilot scale, was slightly higher than the obtained in the propagation

250 reactors and the observed during the process of adapting the inoculum (Cerda et al., 2017),

251 which was around 2.5-3.0 mg O_2 g^{-1} DM h^{-1}. The increase on the rate of degradation can be

252 associated to the conditions of the inoculum in terms of temperature and sOUR. It is

253 important to highlight the importance of choosing the exact moment to have the maximum

254 enzymatic activity, which in occasions, can match with the maximum biological activity,

255 reflected as sOUR (Abraham et al., 2013) or not (Cerda et al., 2016).

256   In addition, the pH reached an alkaline value of 9.22±0.01 and the C/N ratio was

257 13.0±0.2, which reflected organic matter degradation. These conditions remained until the

258 end of the fermentation, achieving final values of 9.34±0.01 for pH and a C/N ratio of 11±1.

259   Final moisture value for propagation fermentations was 63±2% while pilot

260 fermentations a moisture content of 62±2% was observed at 24h of fermentation. The

261 differences of moisture content between propagation and pilot reactors, and even among

262 replicates, showed no significant differences. Probably, the fact of working on close reactors

263 and short times has result in this moisture conservation.

264   Cellulolytic and hemicellulolytic activities also showed their maximum during the

265   most active stage of the fermentation (Figure 2b). FPase and Xyl reached their maximum

266   production in the first 24h of operation with values of 3.6±0.2 FPU g^{-1} DM and 42.7±2.6 U g^{-1}

267   ^{1} DM, respectively. FPase presented a local maximum, decreasing after this moment to

268   negligible values. This can be attributed to several factors such as nutrient depletion or the

269   inhibition of the enzymatic system due to the formation of by-products (Brijwani & Vadlani,

270   2011; Salgado et al., 2015) or non productive adsorption to lignin hydrolyzates (Gao et al.,

271   2014). On the contrary, Xyl activity presented a dramatic decrease to almost zero at 72h of

272   fermentation and increased to 9 ± 2 U g^{-1} DM by the end of the fermentation.

273   The measurements for cellulase and xylanase activity are currently performed using

274   several different methods, some of them with many concerns on its reproducibility (Coward-

275   Kelly et al., 2003). It is for this reason that the comparison between researches is complex;

276   however, a summary of several enzyme production processes is presented in Table 1. All

277   enzymatic activities are produced in a wide range of values, probably due to the variety of

278   inocula and substrates used. FPase production in this work was found in the reported range of

279   production, as seen in Table 1. As for Xyl production, the reported range of production (10.6

280   to 2601 U g^{-1} DM, according to Table 1) is wider than the reported for cellulase.

281   Considering cellulase and xylanase production, it is remarkable the fact that most of

282   the production systems shown in Table 1 are carried out using small amounts of substrates (1-

283   25g), using pure strains (species of the genera *Aspergillus* or *Trichoderma*) and long

284   fermentation times (96-240h). In this sense, the results obtained in this work are promising,

285   due to the significant cellulase and hemicellulase production in a short fermentation time

286   (24h), therefore allowing the faster valorization of organic wastes and improving process

287   economics.

288   During the most active stage of the process at 24 h of operation, partial degradations

289   of cellulose, hemicellulose and lignin were of 4.9, 13.4 and 4.1%, which are in accordance

290   with reported literature (Salgado et al., 2015). At the end of the fermentation a final cellulose

291   degradation was 24.1%. In addition, final lignin hydrolysis was 11.25%, which is higher than

292   the observed by other researches in short solid-state fermentations (Umasaravanan et al.,

293   2011). The most interesting result was obtained for hemicellulose hydrolysis. A final

294   degradation of 34.9% was achieved in spite of the relatively low Xyl values as compared with

295   literature (Table 1).

296   This process was performed using a non-sterile substrate, which provides a complex

297   dynamic process, involving different metabolisms. For this reason, enzymatic (or not

298   enzymatic) products cannot be properly correlated to operational parameters, but only

299   expressed as the net result of these different metabolisms. This might be the reason for not

300   finding correlations among cellulases enzymatic complex and xylanase activities production

301   with their respective substrates. Even more, no correlation was found with any of the

302   monitoring parameters. This is probably due to the fact that parameters like sOUR or

303   temperature, even though useful, provide an extremely simplified overview of the process.

304   For this reason, there is the need to seek for different parameters or techniques to properly

305   correlate the enzymatic production profile. In this sense, the identification of microbial

306   communities appears as an attractive alternative that enables the potential direct correlation

307   among substrate consumption and enzyme production.

308   3.2.2 Gaseous emissions

309   Figure 3 presents the evolution of the cumulative emissions of CH_4, N_2O, VOCs and

310   NH_3 throughout the SSF process carried out during the first pilot batch (R1).

311   Ammonia was emitted in higher amounts than the rest of the gases analyzed, and was

312   mainly released after the thermophilic stage at the highest biological activity condition as

313   reported by other authors (Maulini-Duran et al., 2015). Ammonia emission factor was $3.5 \cdot 10^{-2}$

314   kg Mg^{-1} CH, obtained at 134 h. Ammonia emission is mainly related to nitrogen compounds

315   content such as ammonium from proteins, which can be stripped out as ammonia through the

316   exhausted gas outlet. VOCs, on the other hand, were also found as one of the main

317   contributors to emissions with a total emission factor of $1.1 \cdot 10^{-2}$ kg Mg^{-1} CH. In spite of this

318   value, when it is considered a productive process for cellulase and xylanase, the SSF should

319   take only 24 h, where the emission factor was $2.6 \cdot 10^{-3}$ kg Mg^{-1} CH.

320   Previous studies reported VOC emission factors 1000-fold higher than the obtained in

321   this work (Maulini-Duran et al., 2015), using orange peels (rich in limonene and other

322   volatile compounds) as substrate for cellulase production in a 5 day fermentation. VOCs

323   emitted during the current process presented an almost linear trend, indicating a nearly

324   continuous degradation of the carbonaceous material as suggested in other studies (Pagans et

325   al., 2006b).

326   In contrast to NH_3 and VOC emissions, CH_4 and N_2O are not directly related to

327   composition but to the appearance of anaerobic zones in the solid matrix of the reactor,

328   especially at larger scales than those reported in SSF literature. In the case of N_2O emissions,

329   these are reported to be associated either to anaerobic or anoxic conditions by means of the

330   heterotrophic denitrification and nitrifier denitrification processes during ammonia

331   degradation as reported by some authors Wunderlin et al., (2013). In this study, CH_4 and

332   N_2O emissions were only observed in very low amounts mainly during the most active stage

333   of the fermentation. In this context, the 134 h process presented total cumulative emission

334   factors of $9.4 \cdot 10^{-4}$ and $2.6 \cdot 10^{-4}$ kg Mg^{-1} CH for CH_4 and N_2O, respectively. Even more, when

335   considering the 24 h productive process, these factors were even lower, achieving values of

336 $4.9 \cdot 10^{-4}$ and $1.1 \cdot 10^{-4}$ kg Mg^{-1} CH for CH_4 and N_2O, respectively. Other studies presented

337 emissions of these pollutants obtained during the highest biological activity in similar

338 processes (Maulini-Duran et al., 2015), which are in agreement with this work. However, the

339 obtained emission factors were 10-fold higher than those observed in that process. The

340 absence of published data on the emissions of pollutant gases from SSF unable the

341 comparison of the obtained values, although in any case they are lower than those of similar

342 processes, such as composting (Pagans et al., 2006a; Pagans et al., 2006b).

343 The complete emission profile was performed in order to fully assess the potential

344 environmental impact and its correlation with enzyme production. However, if it is

345 considered the period of time when maximum enzymatic production is achieved, only VOC

346 emissions are relevant due to NH_3 is only produced after 32h of fermentation. This is of great

347 importance, when considering a potential gas treatment.

348 The operational measurements presented in Figures 2 and 3 and fibers degradation

349 were performed in order to fully understand the process dynamics and environmental impact,

350 however, in terms of cellulase and xylanase production, the process should be stopped at 24

351 h.

352 3.2.3 Replicates and consistency

353 In Figure 4 it is presented the first 24 h of operation of the first SSF (see Figure 2 for

354 the complete profile) and two replicates of this 24 h fermentation.

355 Average of the initial moisture, pH and C/N ratio for the triplicates were 68±2,

356 8.32±0.01 and 13.3±0.5 respectively. Initial temperature in the three pilot reactors were 27.8,

357 27.8 and 29.2ºC for fermentations R1, R2 and R3, respectively. The optimal initial conditions

358 of the inoculum along with these temperature values allowed a rapid start-up of the

359   fermentation, obtaining a very similar profile during the first 8h. It is during this period that

360   the thermophilic stage started in all replicates with an average temperature of 47-48ºC. After

361   this period differences on the sOUR profile appeared in R1 (Figure 4a) in comparison with

362   R2 and R3. It is possible that the initial conditions of the inoculum could have affected the

363   performance of the pilot fermentations. Final sOUR and COC obtained at 24 h of

364   fermentation for the three processes were 3.6 mg O_2 g^{-1} DM h^{-1} and 77.6 mg O_2 g^{-1} DM for

365   R1, 3.9 mg O_2 g^{-1} DM h^{-1} and 65.5 mg O_2 g^{-1} DM for R2 and 4.9 mg O_2 g^{-1} DM h^{-1} and 71.8

366   mg O_2 g^{-1} DM for R3, respectively. Also the most interesting difference between the

367   propagation and pilot fermentations is the maximum temperature reached during the

368   thermophilic stage, which was above 70ºC. It is likely that the higher temperature could

369   affect the microbial diversity of the process and therefore the enzymatic productivity (Ortiz et

370   al., 2017; Idris et al., 2017).

371   Enzymatic activities obtained at 24 h of fermentation are presented in Table 2. FPase

372   activity obtained in R2 and R3 presented no significant differences, as well as Xyl activity for

373   R1 and R3. Differences among the enzymatic yields can be attributed to the complexity of the

374   substrate and the nature of cellulolytic enzymes.

375   Additionally, Table 2 presents the average of enzymatic activities of the three

376   fermentations with their respective variation coefficient. FPase and Xyl production were

377   3.1±0.5 FPU g^{-1} DM and 45±8 U g^{-1} DM with a variation coefficient of 16 and 19%,

378   respectively. These variation coefficients can be considered relatively high; however, when

379   working with organic wastes the situation is different. Variation coefficients only from

380   proximal composition of the solid wastes can range between 10 to 50% (Leroy et al., 1992),

381   therefore it is likely that any process that includes solid wastes has an intrinsic high variation.

382   Moreover, using mixed enzymatic compounds obtained by SSF in highly controlled

383   processes can achieve variation coefficients of nearly 10% (Martínez-Ruiz et al., 2008). In

384   this sense, the variation coefficients obtained in this work can be considered as acceptable,

385   taking into account that it is a complex process using non sterile material in a pilot scale.

386   In spite the reproducibility of the process between replicates is acceptable, there is a

387   reduction on cellulase production of nearly 50% when it is compared with the obtained in the

388   propagation reactors, and previous experiments (Cerda et al., 2017). As mentioned before,

389   this can be attributed to the higher temperature observed at pilot scale (>70ºC) in comparison

390   with the propagation reactors (60ºC). This fact has been also observed by Idris et al., (2017)

391   when working in the scale up of a cellulase producing system from 1 to 50g of substrate.

392   These authors observed an increase in the temperature of the SSF, which generated a

393   reduction of more than 50% in cellulase production even at this small scale. Moreover, Ortiz

394   et al., (2015) also observed an increase of the temperature during the pilot trials of a cellulase

395   producing SSF, with its consequent drop in enzymatic productivity and viable cells. As a

396   measure to overcome this difficulties, these authors increased the aeration rate to decrease the

397   temperature of the reactor, recovering enzymatic production and viability of the

398   microorganisms. These authors concluded that further research must be performed to improve

399   the reproducibility of the results obtained at lab scale.

400   In light of these results, it seems that the specialized biomass was able to adapt to the

401   changing conditions in pilot reactors, still yielding adequate FPase and Xyl activities (Table

402   1). Even though the enzymatic productivities were found in the low-middle range of the

403   reported literature it is remarkable that, even after the process scale up, enzymatic production

404   was still adequate. This subject is not often studied because of the complexity of the process

405   and the difficulties of working with complex solid wastes.

406   Differences in the temperature at the beginning and during the process will determine

407   the performance of the pilot fermentations with a potential influence on the development of

408   microbial communities as it is discussed in the next section.

409   *3.3 Microbial characterization*

410   3.3.1 Bacteria

411   A total of 30 bacterial families were identified in the final products of propagation

412   reactors (P1, P2 and P3) and the pilot fermentations (R1, R2 and R3). The full composition is

413   presented in Figure 5. There are three main dominant families in all assessed samples:

414   *Paenibacillaceae*, *Xanthomonadaceae* and *Sphingobacteriaceae;* these are also the main

415   families identified in the original inoculum (Cerda et al., 2017). The sum of these families

416   account for a 45.6, 47.9 and 43.4% for P1, P2 and P3 and 40.5, 40.9 and 39.4% for R1, R2

417   and R3, respectively. In this context, the addition of the specialized inoculum to a

418   propagation reactor and then as inoculum for a pilot SSF generated great similarities among

419   the bacterial diversity at family level. Regarding the samples obtained from the propagation

420   reactors, P1 and P2 presented similar relative abundance of the microbial populations;

421   however, P3 showed slight differences. For instance, P1 and P2 showed low presence of the

422   family *Flavobacteriaceae* (2.1 and 2.4% for P1 and P2), while P3 showed significant

423   abundance of this family (13.4%). The main specie found in this family was *Flavobacterium*

424   *anatoliense,* which is a strict aerobic bacteria isolated from several environments that has

425   been reported as unable to grow on cellulose or to hydrolyze complex polysaccharides

426   (Kacagan et al., 2013). Thus, this specie must grow on other CH components or metabolites

427   produced by other species. In spite of the differences, the predominant specie found in all

428   propagation reactors was *Pseudoxanthomonas taiwanensis*. This bacterium was found

429   predominant in the original specialized inoculum (Cerda et al., 2017) and it was able to

430 survive and colonize the propagation reactors, achieving a relative abundance of 14.7, 14,9

431 and 9.6% in P1, P2 and P3 respectively. *P. taiwanensis* has been widely related to cellulose

432 degradation systems, for its β-glucosidase production and the potential enhancement of the

433 growth of other cellulolytic bacteria (Eichorst et al., 2013).

434 As mentioned before, the results showed that the same families predominant on

435 propagation reactors were predominant on the pilot SSF. In spite of the great resemblance,

436 there is a shift on the predominant specie when comparing with the propagation reactors.

437 *Sphingobacteriaceae* family became more relevant, with a 27.9, 28.9 and 22.9% of relative

438 abundance for R1, R2 and R3, and 10.9, 12.2 and 18.8 for P1, P2 and P3, respectively. The

439 harsh conditions generated during pilot SSF could have affected the development of the

440 different microorganisms in the solid matrix. In this context, strong thermophilic and alkaline

441 environments favored the growth of bacteria able to thrive at these conditions, among them

442 *Sphingobacterium thermophillum* (15.8, 11.1 and 9.7% for R1, R2 and R3) and

443 *Sphingobacterium arenae* (3.6, 7.8 and 6.8% for R1, R2 and R3). The new dominant species

444 relegate *P.taiwanensis* to a specie with a minor presence in the solid matrix, with a relative

445 abundance of 2.4, 2.7 and 8.2% for R1, R2 and R3, respectively. *S. thermophilum* and *S.*

446 *arenae* have been reported to be present during the thermophillic stage of composting

447 processes with a potential for β-glucosidase production (Yabe et al., 2013). In addition, it has

448 been found that several cellulase obtained under these conditions can be halotolerant

449 (Gladden et al., 2014), which is of great interest when it is considered their potential use in

450 bioethanol production.

451 In general, the relative abundance at family level presented high similarities in

452 propagation reactors and the pilot SSF triplicates. Also, it is interesting to highlight the fact

453 that families with relative abundance below 1% (registered in Figure 5 as "others") are higher

454 in the propagation reactors than in the pilot SSF. This indicates a further reduction on

455   biological diversity, as confirmed by the Shannon diversity index for propagation reactors P1,

456   P2 and P3 were 2.6, 2.4 and 2.2 and for pilot SSF were 1.8, 1.7 and 1.9 for R1, R2 and R3

457   respectively. Using a specialized consortium instead of a specific strain provided a robust

458   performance of the fermentation when scaling up. The adaptive shift in microbial community

459   allowed to keep a significant cellulase and xylanase production.

460   3.3.2 Fungi and yeasts

461   A total number of 21 fungal families have been identified in the final products of

462   propagation reactors (P1, P2 and P3) and the pilot fermentations (R1, R2 and R3). The full

463   composition is presented in Figure 6. *Phaffomycetaceae* was found as the dominant family in

464   all the assessed samples. This family accounted for a total of 74.4, 64.5 and 48.2% for P1, P2

465   and P3, and 79.7, 61.9 and 77.8% for R1, R2 and R3, which represented most of the

466   biological diversity of the process. This family was also found as predominant in the initial

467   inoculum, with a relative abundance of 43.2% (Cerda et al., 2017). These results proved, at

468   the family level, a consistent propagation and colonization of the specialized mycobiota

469   present in the initial inoculum. Even more, when performing the pilot SSF, the biological

470   diversity was further reduced, maintaining *Phaffomycetaceae* as the predominant family and

471   increasing its relative abundance. Two species of this family appear as dominant in

472   propagation reactors: *Cyberlidnera jardinii* with 64.3, 55.1 and 3.5% for P1, P2 and P3 and

473   *Barnettozyma californica* with a 10.1, 9.4 and 43.6% for P1, P2 and P3 respectively. These

474   two species were also found as dominant in the initial inoculum (Cerda et al., 2017), with a

475   relative abundance of 34 and 9.1% for *C.jardinii* and *B. californica*, respectively. There is a

476   clear difference on the relative abundance of these species in P3 when compared with P1 and

477   P2. Both of these species are related to hemicellulose degradation with potential tolerance to

478   grow in the presence of lignin hydrolyzates, which is of great interest for a potential use on

479   bioethanol production (Morais et al., 2013; Nordberg et al., 2014).

480   As described, results for pilot reactors presented great similarities, at family level,

481 with propagation reactors, although a major shift occurred at specie level. In pilot SSF the

482 dominant specie was *B. californica* with a 72.6, 59.7 and 72.8% of relative abundance,

483 leaving *C. jardinii* as a minor component of the mycobiota with an abundance below 3% in

484 all cases. *B. californica*, as mentioned before, is related to hemicellulose degradation and an

485 important xylanase producer; however, it has been also reported as an enzyme producer for

486 lignin degradation (Martorell et al., 2012). This specie is a producer of tyrosinase, which is an

487 enzyme able to oxidize phenolic compounds that can potentially enhance lignin hydrolizates

488 degradation. The great abundance of this specie in the pilot SSF can, in a way, explain the

489 high degradation of lignin in the reactors (above 11% of degradation).

490   In general, in the pilot SSF, most of the identified mycobiota presented high

491 hemicellulose and lignin degradation potential. Improvements of degradation on these

492 structures can make cellulose structure more accessible for the enzymes to act and, therefore,

493 increase cellulase production. In addition, biological diversity obtained in the pilot reactors

494 was reduced when compared with the propagation reactors. Shannon index for R1, R2 and R2

495 were 0.44, 0.62 and 0.50 and 1.02, 0.99 and 0.97 for P1, P2 and P3, respectively.

496   The most important result on these experiments relies on the fact that when the

497 operational conditions of the reactors are similar, the biomass that is able to colonize the

498 reactors is the same, even when the fermentation is carried out using non sterile substrates,

499 thus demonstrating a robust and consistent process. When the scale or operational conditions

500 such as temperature change, there is a shift in microbial communities ensuring cellulase and

501 xylanase production, although lower still in the range of reported values (Table 1). It would

502 be of great interest, as future research, to assess possible synergetic or antagonist behaviors

503 among the indentified bacteria and fungi. This could provide interesting information of the

504   microorganisms interaction and the potential of the biomass to produce an enzymatic cocktail

505   for lignocellulosic biomass hydrolysis in a scale close to industrial conditions.

506   As a final remark, it has to be stated that there is no previous research performed on

507   the reproducibility of pilot scale SSF. Many aspects considered as SSF drawbacks have been

508   undertaken, resulting in the development of a more robust and sustainable process. The use of

509   a standard inoculum using wastes as substrate makes this technology more consistent for its

510   application at larger scales, with the additional economic benefit of using organic wastes as

511   raw material.

512

513   4. Conclusion

514   This work demonstrates the use of a specialized consortium developed from compost

515   as inoculum for cellulase production by SSF using non-sterile substrates. Consistent

516   propagation of the specialized inoculum was obtained in 4.5L bioreactors, with enzyme

517   production and microbial diversity in accordance with the initial mixed inoculum. The

518   predominant cellulolytic microorganisms are bacteria (*Pseudoxanthomonas taiwanensis* and

519   *Sphingobacterium composti*) and some yeasts (*Cyberlindnera jardinii* and *Barnettozyma*

520   *californica*). Synergic effects have been also discussed. Experiments developed in 50L

521   bioreactors reached a high temperature. This modified the microbial communities and lead to

522   a lower enzymatic productivity. Additionally, the developed process presented low gaseous

523   emissions, therefore, it can be considered as a robust and environmentally friendly process.

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682 **Figure captions**

683

684 **Figure 1**. Operation profile of sOUR and temperature of the propagation reactors P1, P2 and

685 P3 (4.5L) of a specialized inoculum using coffee husk as substrate.

686

687 **Figure 2**. Operation profile of a) sOUR and Temperature and b) FPase and Xyl production

688 profile in a pilot reactor (50L) using a propagated specialized inoculum and coffee husk as

689 substrate, during 134 h. Xyl activity was expressed as U g^{-1}DM and FPase was expressed as

690 FPU g^{-1}DM.

691

692 **Figure 3.** Cumulative gaseous emissions generated during pilot scale solid-state fermentation

693 using a specialized inoculum and coffee husk as substrate.

694

695 **Figure 4.** Operation profile of sOUR and temperature of the three replicates of the pilot solid-

696 state fermentation R1, R2 and R3 (50L) using a propagated specialized inoculum with coffee

697 husk as substrate.

698

699 **Figure 5.** Bacterial distributions at the family level according to the16s sequencing for the

700 final products of propagation reactors (P1, P2 and P3) and pilot fermentations at highest

701 cellulase activity (R1, R2 and R3). Only families with a relative abundance >1% in all

702 samples are depicted. Families detected with a relative abundance < 1% in these samples are
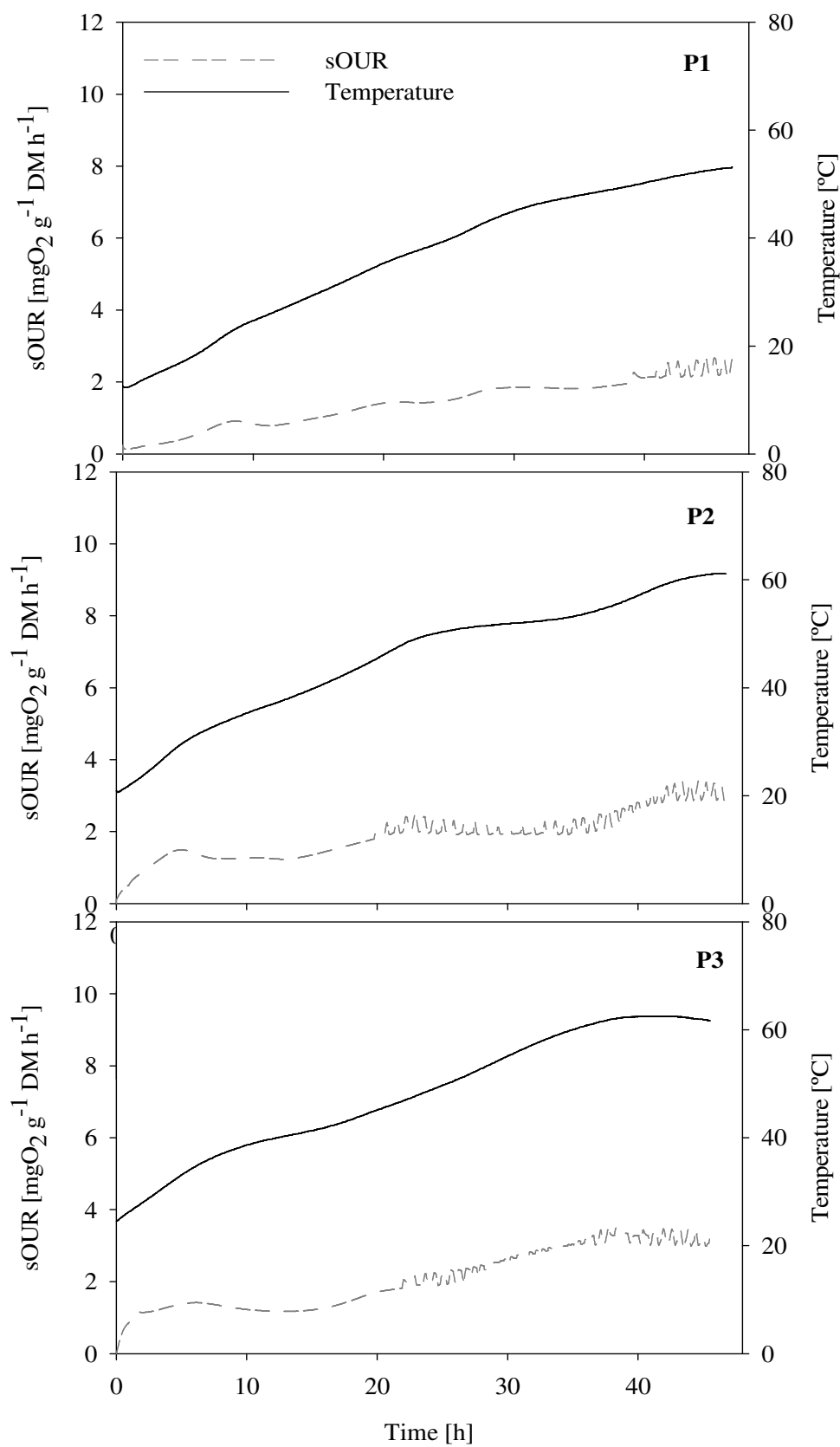
703 grouped as "others".

704   **Figure 6**. Fungal distributions at the family level according to the ITS sequencing for the

705   final products of propagation reactors (P1, P2 and P3) and pilot fermentations at highest

706   cellulase activity (R1, R2 and R3). Only families with a relative abundance >1% in all

707   samples are depicted. Families detected with a relative abundance < 1% in these samples are

708   grouped as "others".

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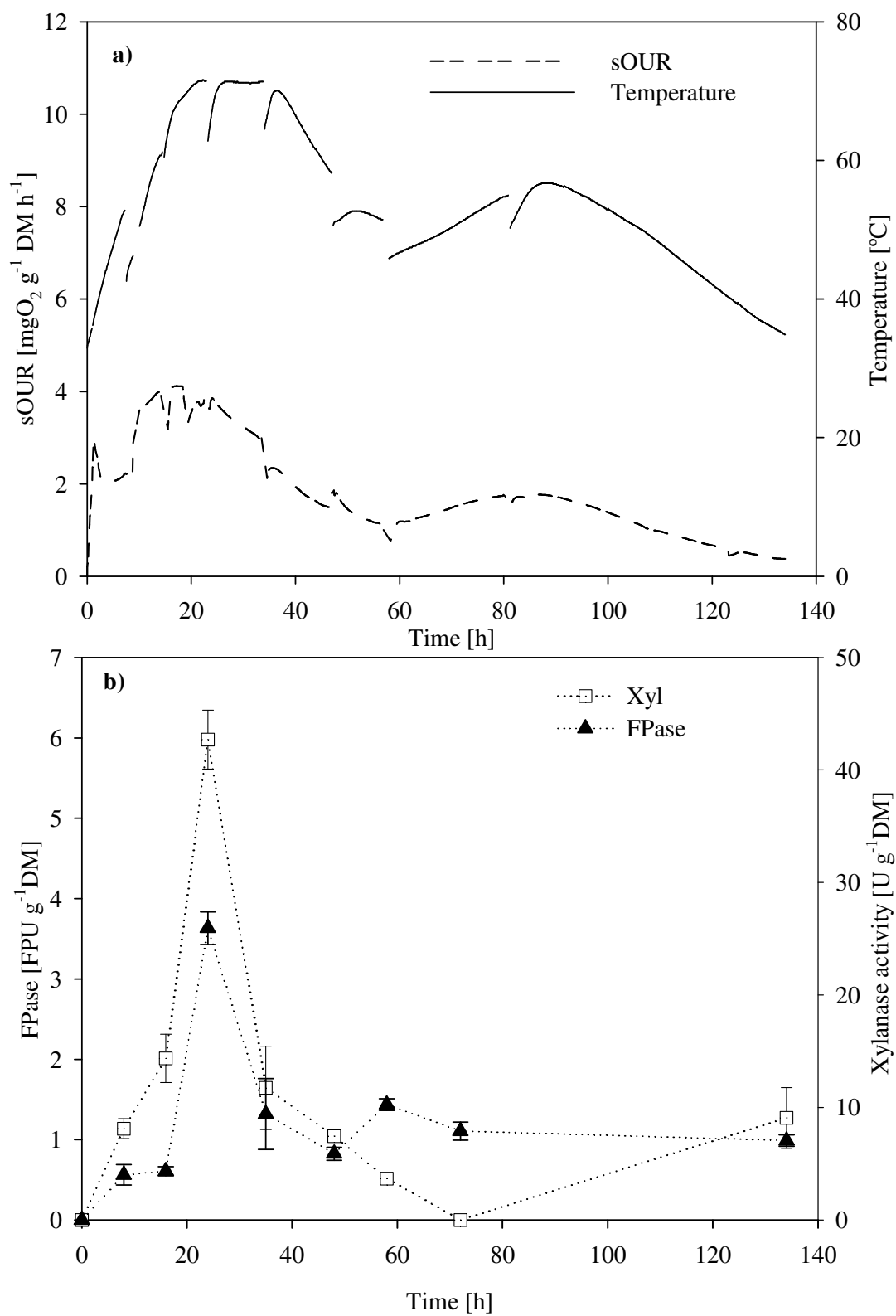711

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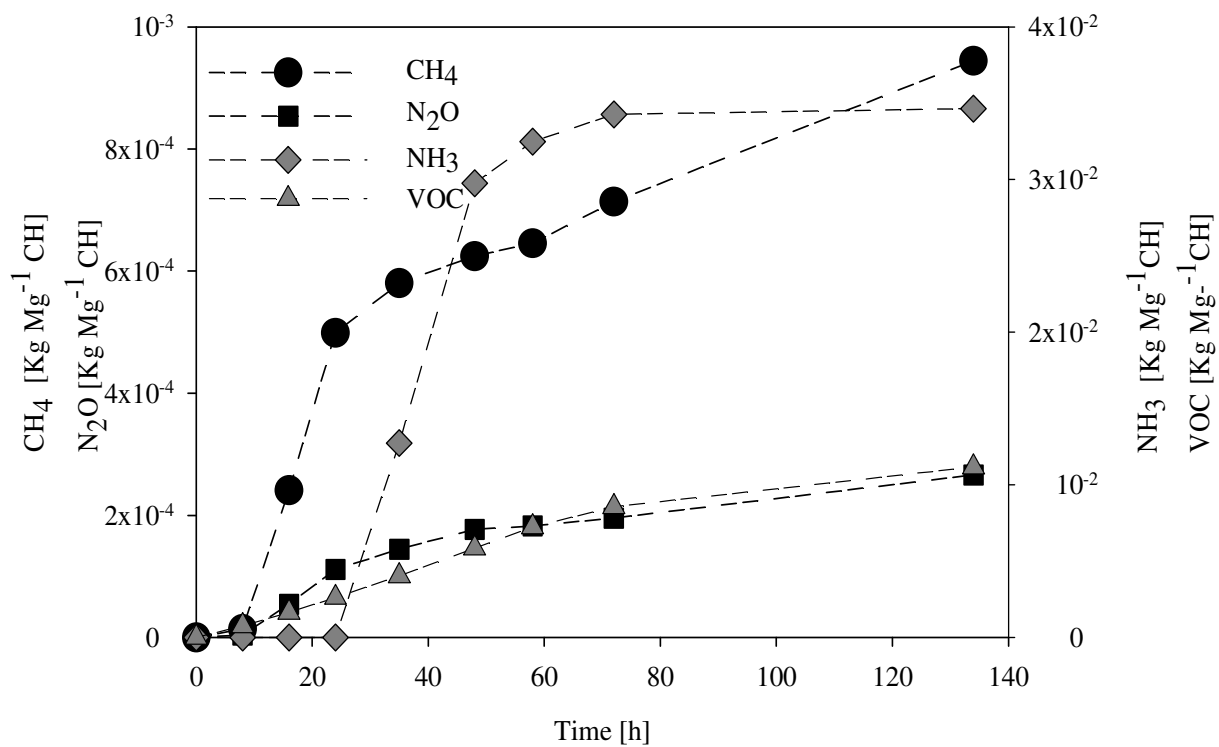
Figure 1.

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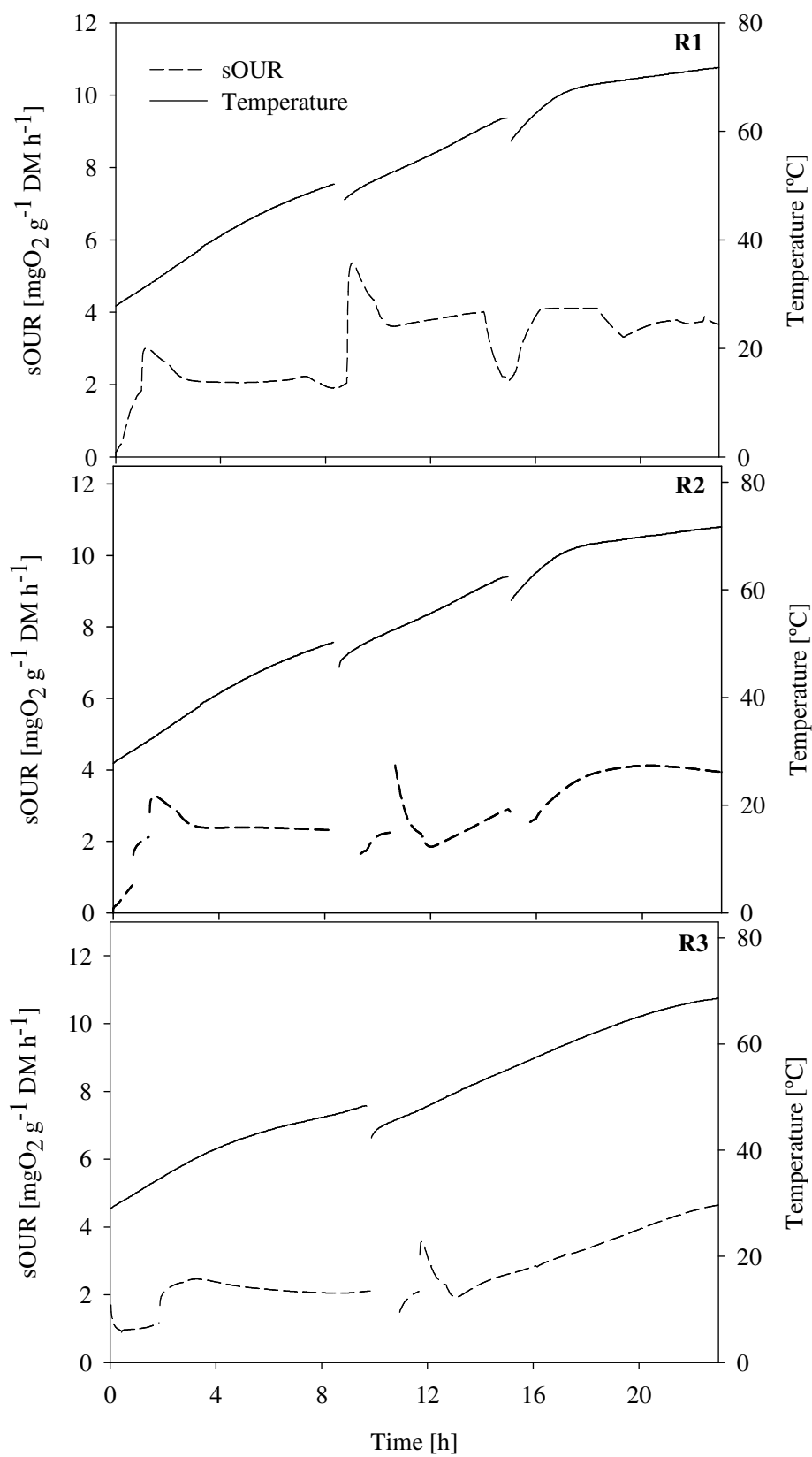
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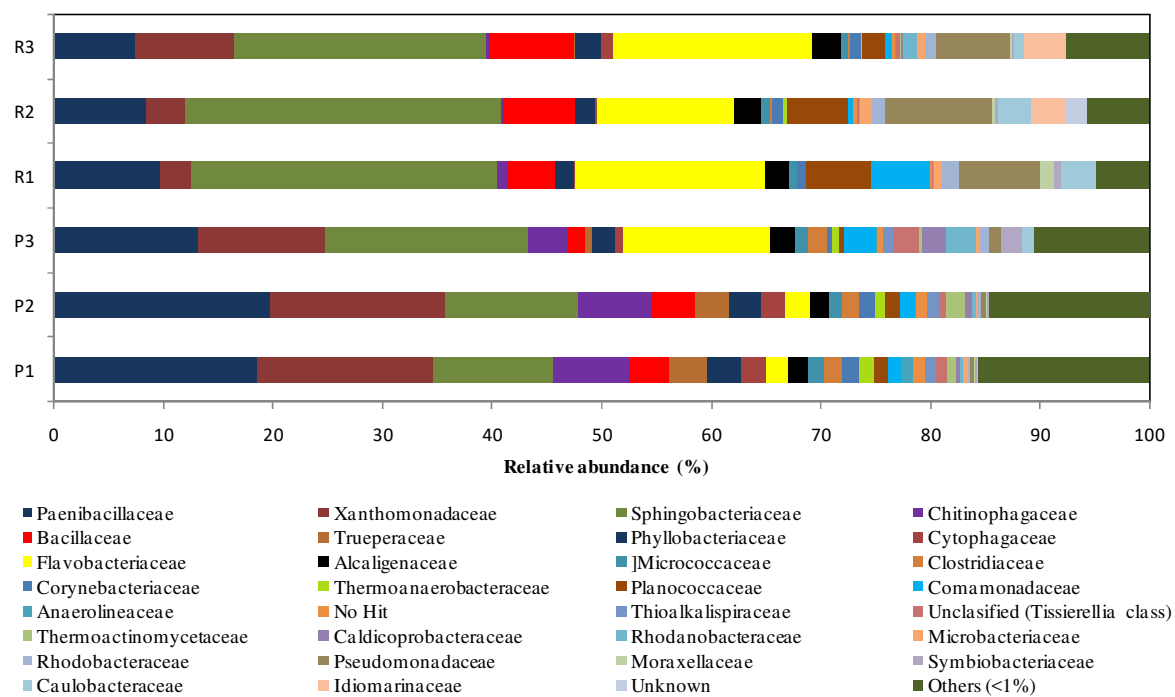
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Figure 4.

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739   **Figure 5.**
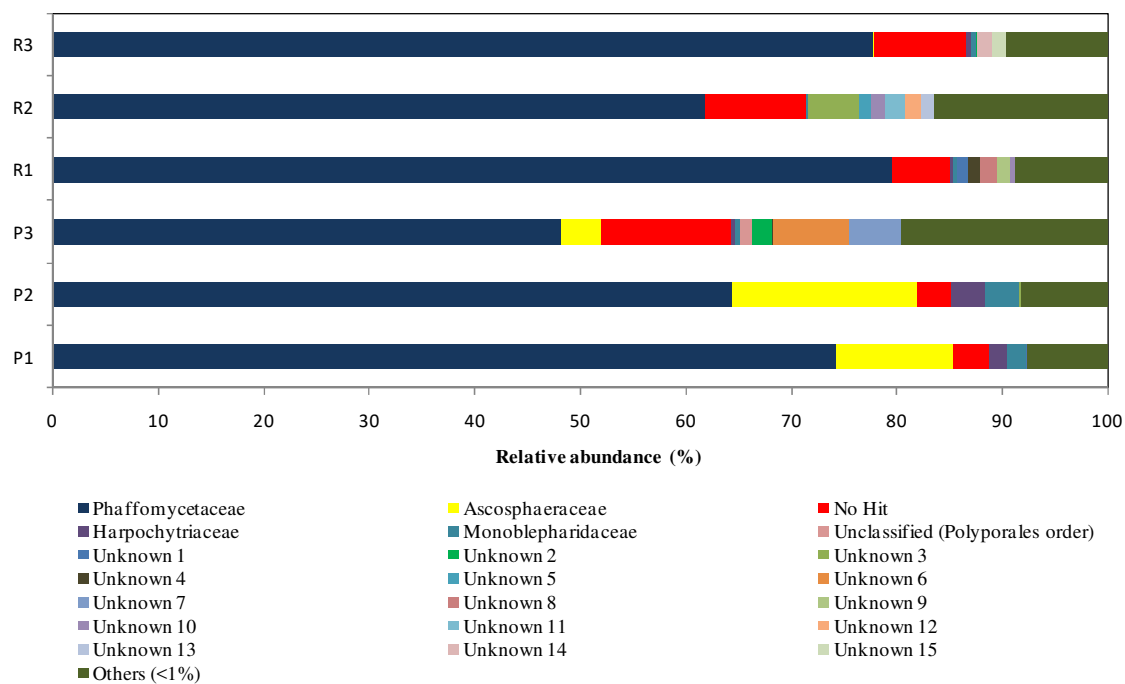
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751   **Figure 6.**

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Table 1. Summary of maximum cellulase and xylanase activities production (FPase and Xyl) obtained by SSF.n.r: not reported.

Substrate	Amount (g)	Inoculum	SSF time (h)	FPase (FPU g^{-1}DM)	Xyl (U g^{-1}DM)	References
Wheat straw	25	*T. reesei*	240	1.2	n.r	(Lever et al., 2010)
Soybean hulls/Wheat bran	100	*A. oryzae*	96	10.8	504.9	(Brijwani & Vadlani, 2011)
Rice straw	10	*A. níger*	96	9.0	936.1	(Dhillon et al., 2011)
Sugarcane bagasse	5	*P. ostreatus*	72-120	0.25	11.0	(Membrillo et al., 2011)
Wheat bran	10	*A. níger*	96	13.6	2601	(Bansal et al., 2012)
Apple pomace		*A. níger*	96	383.7	4868	(Dhillon et al., 2012a)
Rice husk	5	*A. níger*	96	3.1	n.r	(Bansal et al., 2012)
Orange peels	5	*A. níger*	96	1.9	n.r	(Martorell et al., 2012)
Wheat straw	5	*A. níger*	96	13.6	n.r	(Martorell et al., 2012)
Palm oil mill waste	1	*A. turbingensis*	120	2.4	11.8	(Bahera et al., 2016)
Wheat bran/soybean bran	5	*A. fumigatus*	120	5.0	10.6	(Delabona et al., 2013)
Wheat bran	1,000	*T. reesei*	96	8.2	n.r	(Ortiz et al., 2015)
Coffee husk	1,200	*Specialized consortium*	48	8-9	n.r	(Cerda et al., 2017)
Coffee husk	15,200	*Specialized consortium*	24	3.08	44.51	This work

Table 2.Cellulase and xylanase yields obtained in the three replicates of the pilot solid-state fermentation. Values of each enzymatic activity that do not share a letter are significantly different. n.m not measured.

Replicate	FPase (FPU g^{-1}DM)	Xyl (U g^{-1}DM)
R1	3.6±0.2(A)	43±3(F)
R2	2.9±0.3(B)	(n.m)
R3	2.9±0.4(B)	48±5(F)
Average ± s.d	3.1±0.5	45±8
Variation coefficient (%)	16%	19%