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Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized consortia: The consistency of the process and the microbial communities involved

Alejandra Cerda, Laura Mejías, Teresa Gea, Antoni Sánchez*

GICOM Research Group

Department of Chemical, Biological and Environmental Engineering

School of Engineering, Edifici Q

Universitat Autònoma de Barcelona

08193 Bellaterra (Barcelona, Spain)

* Corresponding Author:

Email: antoni.sanchez@uab.cat

Tel.: +34-935811019

Fax: +34-935812013

Abstract

Solid state fermentation is a promising technology however rising concerns related to scale up and reproducibility in a productive process**.** Coffee husk and a specialized inoculum were used in a 4.5L and then in 50L reactors to assess the reproducibility of a cellulase and hemicellulase production system. Fermentations were consistent in terms of cellulase production and microbial communities. The higher temperatures achieved when operating at 50L generated a shift on the microbial communities and a reduction of nearly 50% on 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 deviation coefficients of 16 and 19% for FPase and Xyl production. Gaseous emissions assessment revealed an emission factor of $2.6 \cdot 10^{-3}$ kg volatile organic compounds per Mg of coffee husk and negligible NH_3, CH_4 and N_2O emissions.

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

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

substrate) in a sequential batch operation mode with promising results towards a continuous and reproducible cellulase production (Cerda et al., 2017). There are very few other attempts in developing a continuous process for enzyme production (Astolfi et al., 2011; Cheirsilp & Kitcha, 2015) and even less to assess the SSF scaling up problem (Biz et al., 2016; Idris et al. 2017; Ortiz et al., 2017). In order to deliver a sustainable and low cost continuous SSF process it is necessary to consider the inherent variability of the organic wastes and the inoculum required for enzymatic production. Most of the reported SSF process are performed under sterile conditions and using specific microorganisms as seen in Table 1 (El-Bakry et al., 2015; Behera & Ray, 2016; Kuhad et al., 2016). In this sense, a multispecies SSF such as the proposed by our group (Cerda et al., 2017) has been only been suggested by one other author (Wang et al., 2016). In this SSF, these authors used autochthonous microbiota (without inoculation) which allowed them to guarantee the reproducibility of the process using a metagenomics approach. Taking this into consideration, it seems of great importance to assess the proper propagation of the specialized mixed inoculum at a large scale in order to achieve a standardized cellulase production process**.**

Besides the production of the targeted product, the environmental impacts of the SSF process must be taken into account when considering SSF benefits. This fact has been assessed in previous studies (Maulini-Durán et al., 2015), which reported gaseous emissions from the SSF for cellulases, proteases and lipases production at the pilot scale. This work remains, to our knowledge, the only reported research on this subject, although the issue of emissions from SSF is crucial for further environmental impact studies.

Considering the above, the main objectives of this work are i) to study the influence of using a specialized inoculum in the production of cellulases and xylanases operating a pilot scale SSF process, with a full characterization of the microbial communities ii) the assessment on the reproducibility of the process at laboratory and pilot scale and iii) the

complete characterization of the gaseous emissions of the process, including emission factors for ammonia, volatile organic compounds, methane and nitrous oxide. To our knowledge, there are no similar studies reported in literature.

2. Materials and methods

2.1 Raw material

Coffee husk (CH) was kindly provided by Marcilla S.A (Mollet del Vallès, Barcelona, Spain) and stored frozen at -20ºC until use. CH presented a general characterization of: moisture content 68.2±0.4% (wet basis), pH 6.5±0.1 and C/N ratio 13.3±0.5. Fiber content in CH was 25.7±0.2, 14.6±0.1 and 17.6±0.5% (dry basis) of cellulose, hemicellulose and lignin, respectively.

2.2 Specialized inoculum

The inoculum used in this work consisted on a specialized consortium of microorganisms able to produce cellulases using CH as sole substrate. This was previously obtained operating SSF by sequential batch (SB) using compost as starting inoculum. Main species identified by DNA sequencing were the bacteria *Pseudoxanthonomas taiwanensis* and *Sphingobacterium composti* and the yeasts *Cyberlindnera jardinii* and *Barnettozyma californica*, all of them previously reported as lignocellulose degraders (Cerda et al., 2017). The procedure to obtain this inoculum is described in Cerda et al. (2017). Briefly, compost was added in a 10% (w/w) ratio to a mixture of CH and wood chips as bulking agent in a 1:1 (v/v) ratio to provide the proper porosity to the mixture (Ruggieri et al., 2009). During the process there was no evidence suggesting wood chips degradation due to the short time of each cycle. Sequential batch operation was performed in cycles until stable cellulase

production of 9 ± 1 FPU g^{-1} DM (filter paper units per grams of dry matter). SB operation started with a 48h fermentation stage followed by a substrate exchange of 90% of volume every 48h. The process was operated for several cycles until stable operation was achieved and the final fermented solids were stored frozen for further use as specialized inoculum. The specialized inoculum presented a general characterization of: moisture content of 55.0±3.1% (wet basis), pH 9.1±0.1 and C/N ratio of 13.1±0.6. Fiber content in the specialized inoculum was 23.5±0.5, 13.3±0.5 and 18.3±0.6% (dry basis) of cellulose, hemicellulose and lignin, respectively.

2.3. Solid State Fermentation (SSF)

SSF was performed in a 50L pilot bioreactor in triplicate batches (R1, R2 and R3). Each batch was inoculated with fermented solids from a 4.5 L propagation reactor (P1, P2 and P3, respectively) inoculated with the thawed specialized consortium. Both propagation and pilot operations were non-isothermal, using thermally isolated reactors in all cases. Full description is presented below.

2.3.1. Inoculum preparation in propagation reactors

A propagation reactor was required to provide the proper amount of biomass to inoculate the pilot reactors. Inoculum was obtained by using an initial mixture containing CH and 10% of the mixed specialized inoculum with wood chips, which was used in a ratio of 1:1 (v/v), giving a total weight of 1.2 kg per batch. The process was carried out for 48h in 4.5 L air-tight packed bed reactors, working under non-isothermal conditions and oxygen controlled aeration (120-240 mL min^{-1}, oxygen setpoint 11.5% in air).

2.3.2 Pilot reactor operation

Three batches were performed in 50 L air-tight packed bed reactors. A schematic diagram of the pilot reactor and a detailed description can be found elsewhere (Puyuelo et al., 2010). Temperature, exhaust gas oxygen concentration and inlet airflow were monitored during the trials. The experiments were performed with forced aeration and airflow was manually adjusted to ensure that the oxygen content in the reactor remained above 10%, in order to provide full aerobic conditions (Puyuelo et al., 2010).

The mixtures were prepared by mixing CH and the specialized inoculum obtained from the propagation reactor in a 90:10 (w/w) ratio respectively. Wood chips were added as bulking agent in a volume ratio of 1:1 (v/v). The final weight of the mixture was 15.2 kg for each reactor. The first batch (R1) was performed to obtain a full profile of enzymatic activity production, collecting gaseous and solid samples at 0, 8, 16, 24, 35, 48, 58, 72 and 134 h. Also the process time for maximum cellulase activity was established. Two additional batches (R2 and R3) were performed in order to assess the reproducibility of the process and the fermentation was stopped at the moment of maximum cellulase activity.

2.3.3 Sampling

Gaseous samples were collected in 1-L Tedlar® bags for ammonia (NH_3), volatile organic compounds (VOC), nitrous oxide (N_2O) and methane (CH_4) content determination prior to opening the reactor. After the collection of the gaseous samples, the reactor was opened and the solid content was manually homogenized to obtain a representative solid sample for enzymatic activity determination. Filter paper activity (FPase) was measured for cellulase production. Xylanase (Xyl) production was followed for hemicellulase production.

In addition to the enzymatic measurements, the solid samples were analyzed in order to determine the neutral detergent fiber, acid detergent fiber and lignin content. These analysis were carried out by the method of Van Soest (1991) using the Ankom200 Fiber

Analyzer incubator (Ankom Technology, Macedon, NY), adding amylase and sodium sulphite solutions. Degradation percentage of cellulose, hemicellulose and lignin were calculated according to a mass balance and considering the weight evolution throughout the process.

2.3.4 Parameters monitoring

Temperature and oxygen content were continuously monitored and recorded in the reactors during the fermentations. Specific oxygen uptake rate (sOUR) was calculated according to the following:

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

where: sOUR is the Specific Oxygen Uptake Rate (mg O_2 g^{-1} DM h^{-1}); F, airflow into the reactor (mL min^{-1}); y_{O2}, is the oxygen molar fraction in the exhaust gases (mol O_2 mol^{-1}); P, pressure of the system assumed constant at 101325 (Pa); 32, oxygen molecular weight (g O_2 mol^{-1} O_2); 60, conversion factor from minute to hour; 10^3, conversion factor from mL to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, temperature at which F is measured (K); DW, initial dry weight of solids in the reactor (g); 10^3, conversion factor from g to mg. The area below the sOUR curve was also determined, which represents the cumulative oxygen consumption (COC) during the process (Ponsá et al., 2010).

2.4 Enzyme extraction

Fermented solids were mixed thoroughly on a magnetic stirrer for 30 min with 0.05M citrate buffer, at a pH of 4.8 and ratio of 1:15 (w:v) respectively. The extract was separated 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

provider's specifications. DNA samples were checked for concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware; USA). Bacterial 16S rRNA hypervariable regions V3-V4 and fungal ITS1-ITS3 were targeted. Later sequencing of the extracted DNA and bioinformatics were performed on MiSeq platform by Life Sequencing S.A (Valencia, Spain).

2.8 Routine methods

Moisture content, total and volatile solids and pH were determined according to standard procedures (U.S. Composting Council, 2001) in a representative solid sample, as explained above.

2.9. Statistical Analysis

Statistical analysis was performed with basic ANOVA techniques while pairwise comparisons were based on the Tukey test ($p<0.05$). Statistics were performed with MINITAB ™ V17.

3. Results and Discussion

3.1 Specialized inoculum preparation

The average initial moisture, pH and C/N ratio for propagation reactors were 67±1, 8.01±0.01 and 12.5±0.5, respectively. Figure 1 presents the sOUR and the temperature profiles of the three propagation reactors (P1, P2 and P3) for inoculum preparation. All three propagation reactors presented a similar trend. However, the heterogeneity of the materials led to a series of small differences during the processes despite of using the same specialized 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

Figure 2a presents the fermentation profile for a 134 h operation using a previously propagated inoculum. The fermentation presented a quick start-up with no lag phase as observed in the sOUR and thermophilic temperature profiles (Figure 2a). This can be attributed to the initial conditions of the inoculum, which was in its highest biological and enzymatic activity. Biological activity was found at its maximum during the first 30h of fermentation in a full thermophilic stage. Maximum sOUR and temperature were 3.6 mg O_2 g^{-1} DM h^{-1} and 71ºC, respectively, at 24h. Oxygen requirements reached a total COC of 243.2 mg O_2 g^{-1} DM. COC found at 24h of fermentation, at its highest sOUR, was 80.2 mg O_2 g^{-1} DM, which roughly represents 33% of the total oxygen consumption in 136h. In this context, sOUR obtained at pilot scale, was slightly higher than the obtained in the propagation reactors and the observed during the process of adapting the inoculum (Cerda et al., 2017), which was around 2.5-3.0 mg O_2 g^{-1} DM h^{-1}. The increase on the rate of degradation can be associated to the conditions of the inoculum in terms of temperature and sOUR. It is important to highlight the importance of choosing the exact moment to have the maximum enzymatic activity, which in occasions, can match with the maximum biological activity, reflected as sOUR (Abraham et al., 2013) or not (Cerda et al., 2016).

In addition, the pH reached an alkaline value of 9.22±0.01 and the C/N ratio was 13.0±0.2, which reflected organic matter degradation. These conditions remained until the end of the fermentation, achieving final values of 9.34±0.01 for pH and a C/N ratio of 11±1.

Final moisture value for propagation fermentations was 63±2% while pilot fermentations a moisture content of 62±2% was observed at 24h of fermentation. The differences of moisture content between propagation and pilot reactors, and even among replicates, showed no significant differences. Probably, the fact of working on close reactors and short times has result in this moisture conservation.

Cellulolytic and hemicellulolytic activities also showed their maximum during the most active stage of the fermentation (Figure 2b). FPase and Xyl reached their maximum 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} DM, respectively. FPase presented a local maximum, decreasing after this moment to negligible values. This can be attributed to several factors such as nutrient depletion or the inhibition of the enzymatic system due to the formation of by-products (Brijwani & Vadlani, 2011; Salgado et al., 2015) or non productive adsorption to lignin hydrolyzates (Gao et al., 2014). On the contrary, Xyl activity presented a dramatic decrease to almost zero at 72h of fermentation and increased to 9 ± 2 U g^{-1} DM by the end of the fermentation.

The measurements for cellulase and xylanase activity are currently performed using several different methods, some of them with many concerns on its reproducibility (Coward-Kelly et al., 2003). It is for this reason that the comparison between researches is complex; however, a summary of several enzyme production processes is presented in Table 1. All enzymatic activities are produced in a wide range of values, probably due to the variety of inocula and substrates used. FPase production in this work was found in the reported range of production, as seen in Table 1. As for Xyl production, the reported range of production (10.6 to 2601 U g^{-1} DM, according to Table 1) is wider than the reported for cellulase.

Considering cellulase and xylanase production, it is remarkable the fact that most of the production systems shown in Table 1 are carried out using small amounts of substrates (1-25g), using pure strains (species of the genera *Aspergillus* or *Trichoderma*) and long fermentation times (96-240h). In this sense, the results obtained in this work are promising, due to the significant cellulase and hemicellulase production in a short fermentation time (24h), therefore allowing the faster valorization of organic wastes and improving process 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

survive and colonize the propagation reactors, achieving a relative abundance of 14.7, 14,9 and 9.6% in P1, P2 and P3 respectively. *P. taiwanensis* has been widely related to cellulose degradation systems, for its β-glucosidase production and the potential enhancement of the growth of other cellulolytic bacteria (Eichorst et al., 2013).

As mentioned before, the results showed that the same families predominant on propagation reactors were predominant on the pilot SSF. In spite of the great resemblance, there is a shift on the predominant specie when comparing with the propagation reactors. *Sphingobacteriaceae* family became more relevant, with a 27.9, 28.9 and 22.9% of relative abundance for R1, R2 and R3, and 10.9, 12.2 and 18.8 for P1, P2 and P3, respectively. The harsh conditions generated during pilot SSF could have affected the development of the different microorganisms in the solid matrix. In this context, strong thermophilic and alkaline environments favored the growth of bacteria able to thrive at these conditions, among them *Sphingobacterium thermophillum* (15.8, 11.1 and 9.7% for R1, R2 and R3) and *Sphingobacterium arenae* (3.6, 7.8 and 6.8% for R1, R2 and R3). The new dominant species relegate *P.taiwanensis* to a specie with a minor presence in the solid matrix, with a relative abundance of 2.4, 2.7 and 8.2% for R1, R2 and R3, respectively. *S. thermophilum* and *S. arenae* have been reported to be present during the thermophillic stage of composting processes with a potential for β-glucosidase production (Yabe et al., 2013). In addition, it has been found that several cellulase obtained under these conditions can be halotolerant (Gladden et al., 2014), which is of great interest when it is considered their potential use in bioethanol production.

In general, the relative abundance at family level presented high similarities in propagation reactors and the pilot SSF triplicates. Also, it is interesting to highlight the fact that families with relative abundance below 1% (registered in Figure 5 as "others") are higher in the propagation reactors than in the pilot SSF. This indicates a further reduction on

biological diversity, as confirmed by the Shannon diversity index for propagation reactors P1, 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 respectively. Using a specialized consortium instead of a specific strain provided a robust performance of the fermentation when scaling up. The adaptive shift in microbial community allowed to keep a significant cellulase and xylanase production.

3.3.2 Fungi and yeasts

A total number of 21 fungal families have been identified in the final products of propagation reactors (P1, P2 and P3) and the pilot fermentations (R1, R2 and R3). The full composition is presented in Figure 6. *Phaffomycetaceae* was found as the dominant family in all the assessed samples. This family accounted for a total of 74.4, 64.5 and 48.2% for P1, P2 and P3, and 79.7, 61.9 and 77.8% for R1, R2 and R3, which represented most of the biological diversity of the process. This family was also found as predominant in the initial inoculum, with a relative abundance of 43.2% (Cerda et al., 2017). These results proved, at the family level, a consistent propagation and colonization of the specialized mycobiota present in the initial inoculum. Even more, when performing the pilot SSF, the biological diversity was further reduced, maintaining *Phaffomycetaceae* as the predominant family and increasing its relative abundance. Two species of this family appear as dominant in propagation reactors: *Cyberlidnera jardinii* with 64.3, 55.1 and 3.5% for P1, P2 and P3 and *Barnettozyma californica* with a 10.1, 9.4 and 43.6% for P1, P2 and P3 respectively. These two species were also found as dominant in the initial inoculum (Cerda et al., 2017), with a relative abundance of 34 and 9.1% for *C.jardinii* and *B. californica*, respectively. There is a clear difference on the relative abundance of these species in P3 when compared with P1 and P2. Both of these species are related to hemicellulose degradation with potential tolerance to grow in the presence of lignin hydrolyzates, which is of great interest for a potential use on bioethanol production (Morais et al., 2013; Nordberg et al., 2014).

As described, results for pilot reactors presented great similarities, at family level, with propagation reactors, although a major shift occurred at specie level. In pilot SSF the dominant specie was *B. californica* with a 72.6, 59.7 and 72.8% of relative abundance, leaving *C. jardinii* as a minor component of the mycobiota with an abundance below 3% in all cases. *B. californica*, as mentioned before, is related to hemicellulose degradation and an important xylanase producer; however, it has been also reported as an enzyme producer for lignin degradation (Martorell et al., 2012). This specie is a producer of tyrosinase, which is an enzyme able to oxidize phenolic compounds that can potentially enhance lignin hydrolizates degradation. The great abundance of this specie in the pilot SSF can, in a way, explain the high degradation of lignin in the reactors (above 11% of degradation).

In general, in the pilot SSF, most of the identified mycobiota presented high hemicellulose and lignin degradation potential. Improvements of degradation on these structures can make cellulose structure more accessible for the enzymes to act and, therefore, increase cellulase production. In addition, biological diversity obtained in the pilot reactors was reduced when compared with the propagation reactors. Shannon index for R1, R2 and R2 were 0.44, 0.62 and 0.50 and 1.02, 0.99 and 0.97 for P1, P2 and P3, respectively.

The most important result on these experiments relies on the fact that when the operational conditions of the reactors are similar, the biomass that is able to colonize the reactors is the same, even when the fermentation is carried out using non sterile substrates, thus demonstrating a robust and consistent process. When the scale or operational conditions such as temperature change, there is a shift in microbial communities ensuring cellulase and xylanase production, although lower still in the range of reported values (Table 1). It would be of great interest, as future research, to assess possible synergetic or antagonist behaviors 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.

524

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531
532

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681

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.

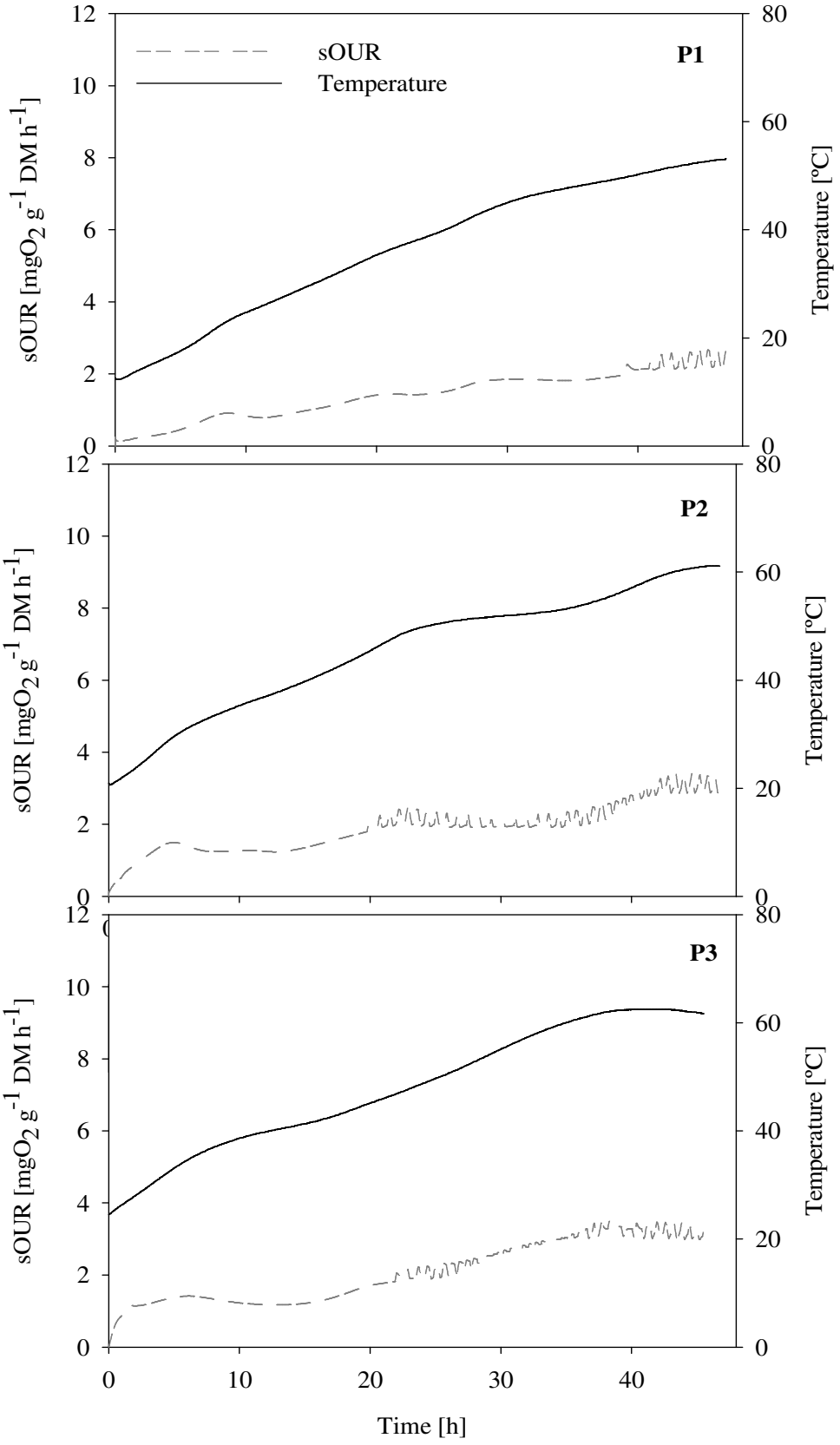
694

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".

Figure 6. Fungal distributions at the family level according to the ITS sequencing for the final products of propagation reactors (P1, P2 and P3) and pilot fermentations at highest cellulase activity (R1, R2 and R3). Only families with a relative abundance >1% in all samples are depicted. Families detected with a relative abundance < 1% in these samples are grouped as "others".



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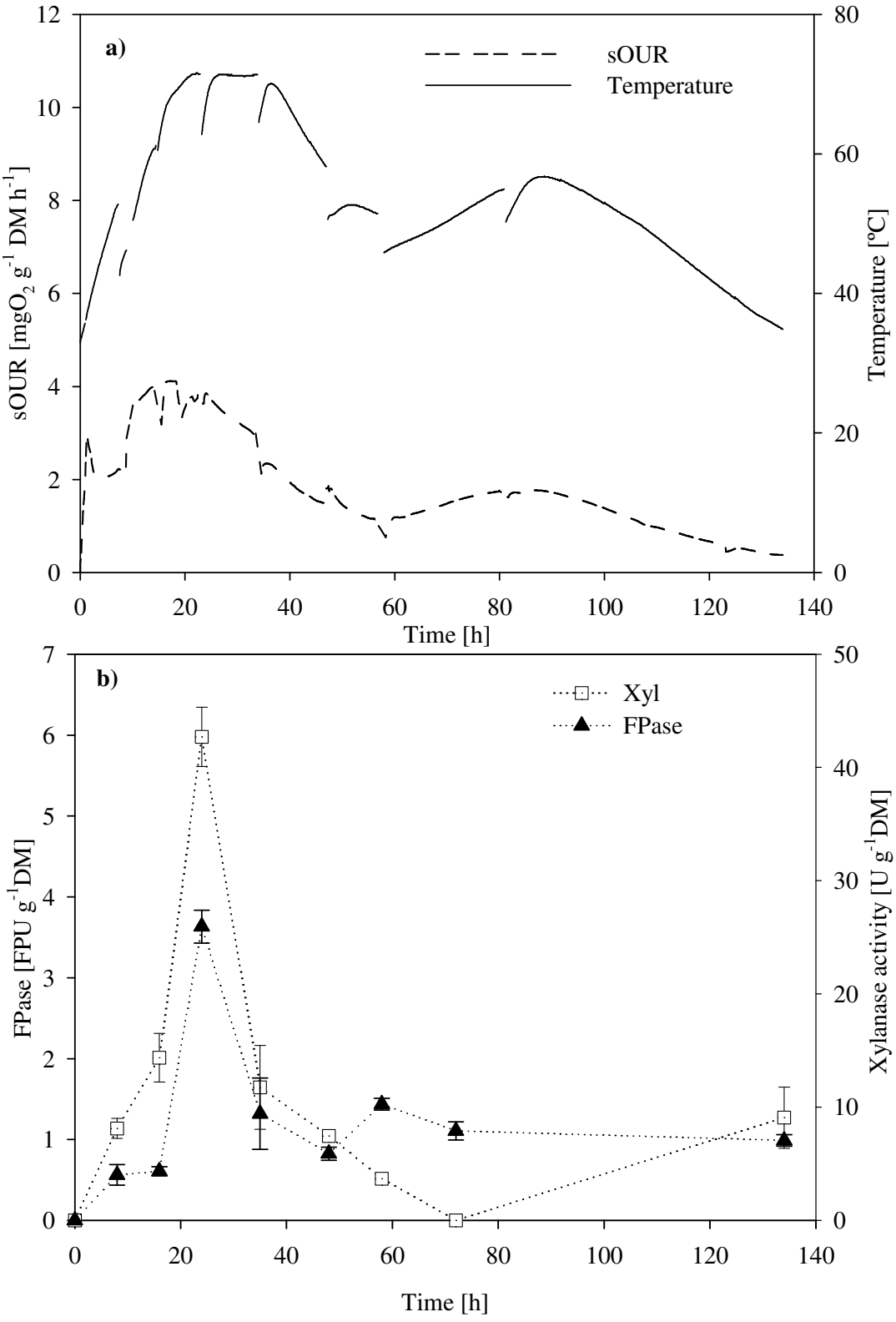
723 **Figure 1.**



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725 **Figure 2.**

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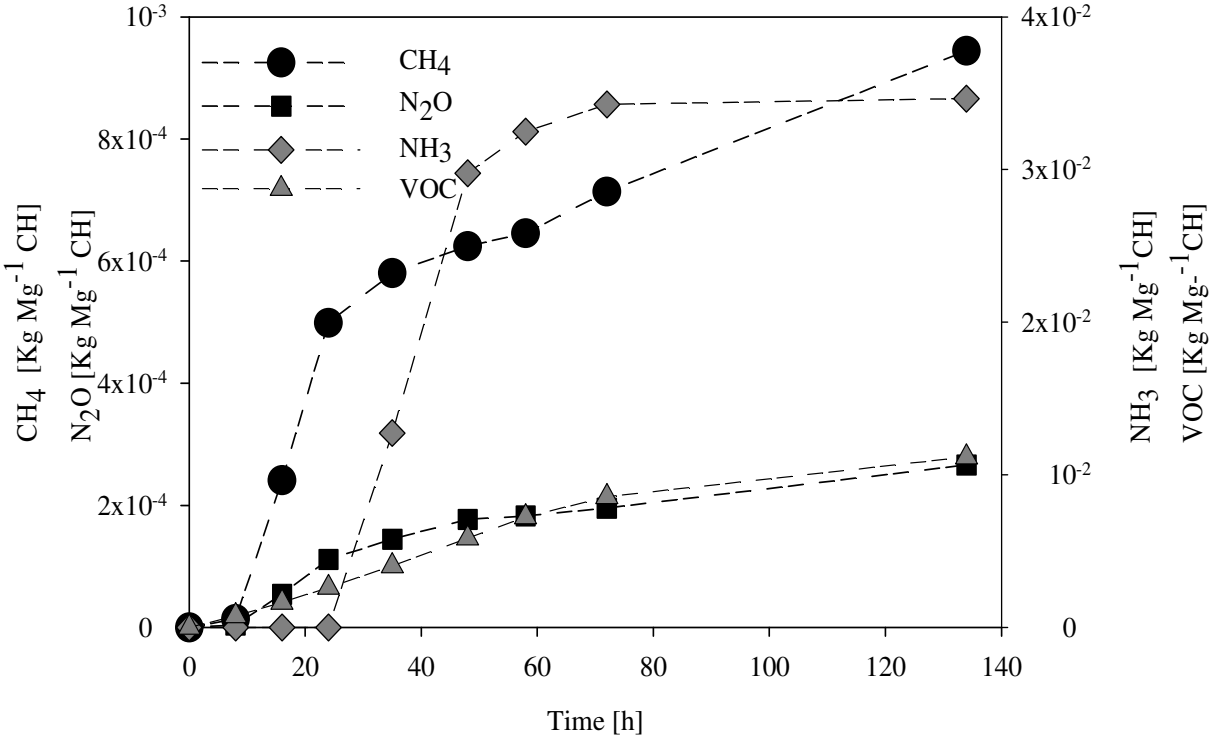

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728 **Figure 3.**

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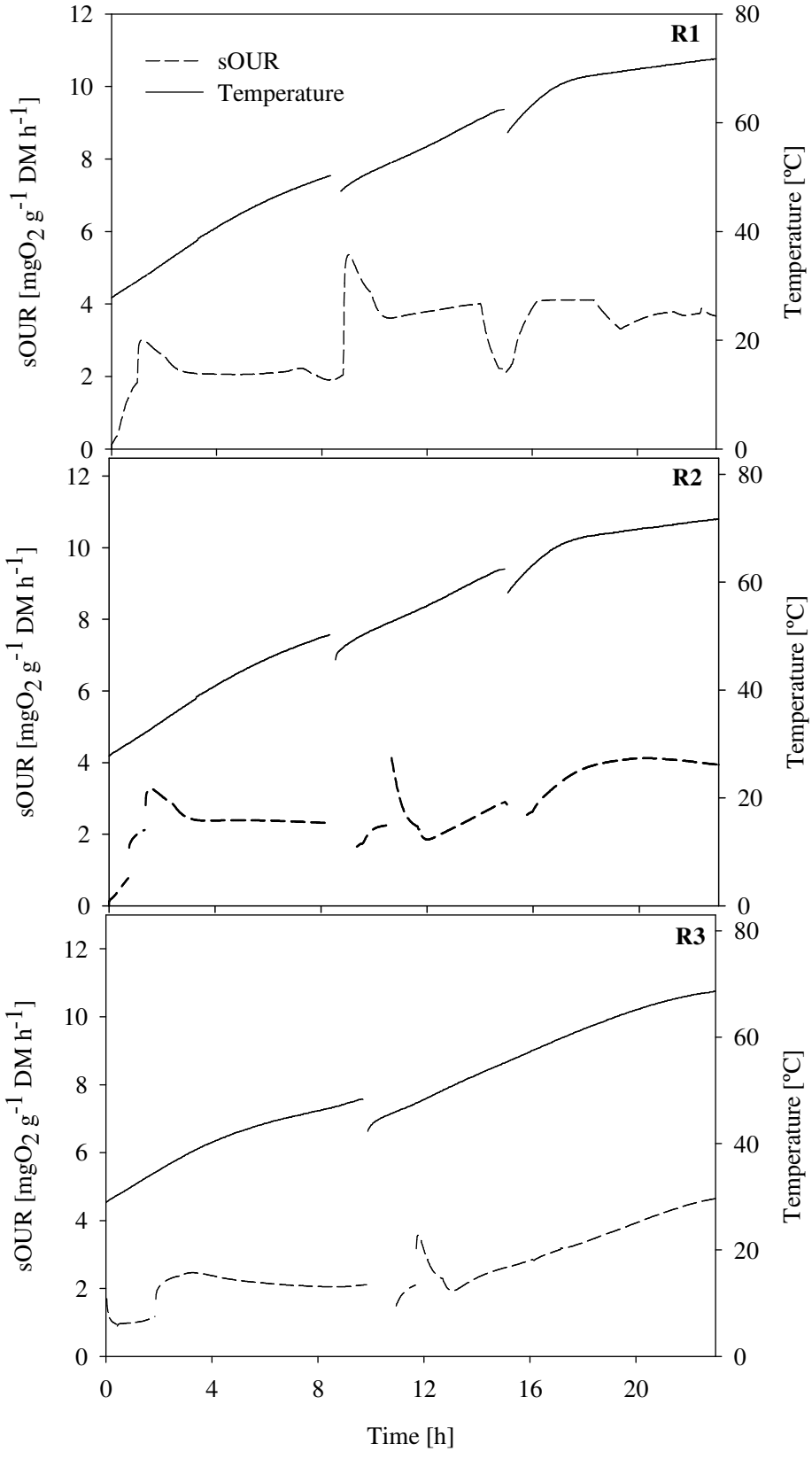

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736 **Figure 4.**

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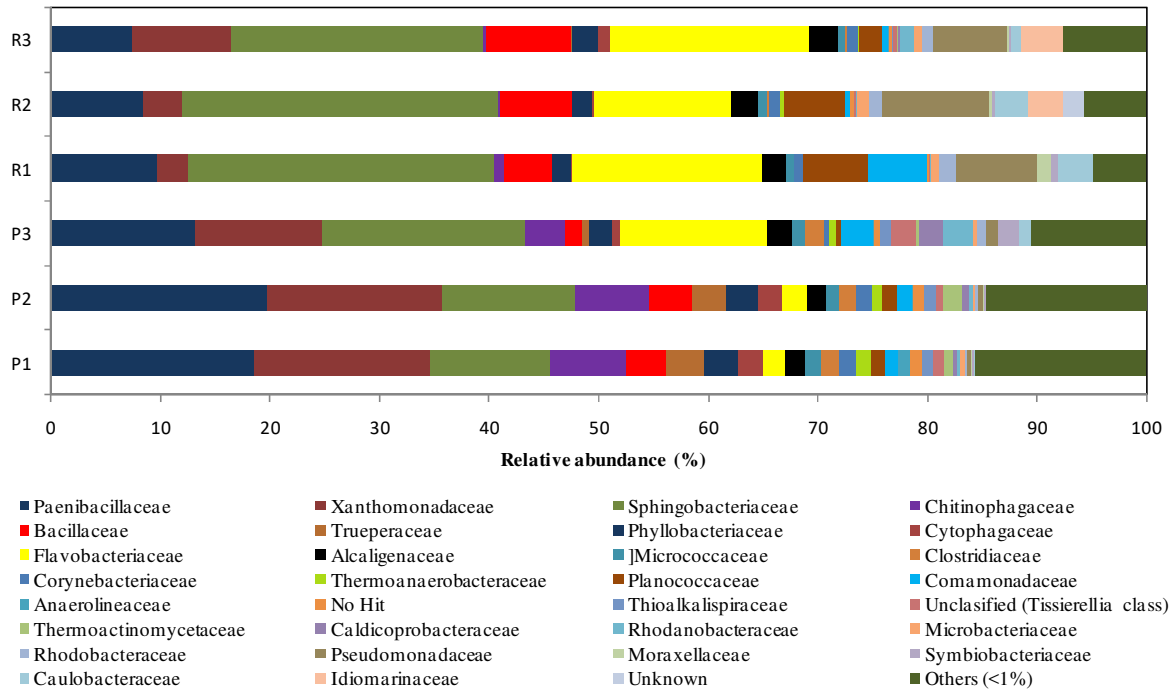

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739 **Figure 5.**

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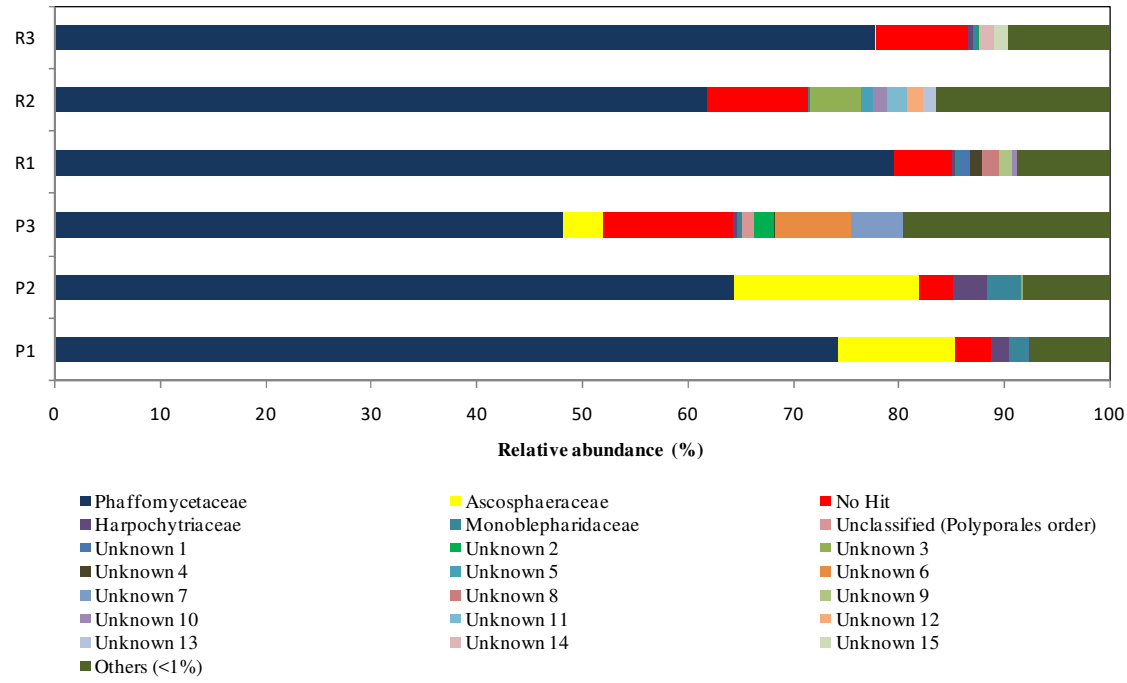
R3
R2
R1
P3
P2
P1
0
10
20
30
40
50
60
70
80
90
100
Relative abundance (%)
Phaffomycetaceae
Ascosphaeraceae
No Hit
Harpochytriaceae
Monoblepharidaceae
Unclassified (Polyporales order)
Unknown 1
Unknown 2
Unknown 3
Unknown 4
Unknown 5
Unknown 6
Unknown 7
Unknown 8
Unknown 9
Unknown 10
Unknown 11
Unknown 12
Unknown 13
Unknown 14
Unknown 15
Others (<1%)

Figure 6.

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%