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Towards a competitive solid state fermentation: cellulases production from coffee husk by sequential batch operation and role of microbial diversity.

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Abstract

The cost of cellulases is the main bottleneck for bioethanol production at commercial scale. Solid-state fermentation (SSF) is a promising technology that can potentially reduce cellulases cost by using wastes as substrates. In this work, a SSF system of 4.5L bioreactors was operated continuously by sequential batch operation using the fermented solids from one batch to inoculate the following batch. Coffee husk was used as lignocellulosic substrate. Compost was used as starter in the first batch to provide a rich microbiota. Two strategies were applied: using 10% fermented solids as inoculum in 48h batches (SB90) and using 50% solids in 24h batches (SB50). A consistent and robust production process was achieved by sequential batch operation. Similar cellulase activities around 10 Filter Paper Units per gram of dry solids were obtained through both strategies. Microbial diversity in the starting materials and in the final fermented solids was characterized by next generation sequencing. Microbial composition of both fermented solids was similar but the relative abundance of families and species was affected by the operation strategy used. Main bacteria in the final solids came from compost (families *Sphingobacteriaceae, Paenibacillaceae* and *Xanthomonadaceae*), while main fungi families came from coffee husk (families *Phaffomycetaceae, Dipodascaceae* and two unidentified families of the class of *Tramellomycetes*). There was a high presence of non-identified mycobiota in the fermented solids. Main identified species were the bacteria *Pseudoxanthonomas taiwanensis* (12.3% in SB50 and 6.1% in SB90) and *Sphingobacterium composti* (6.1 % in SB50 and 2.6% in SB90) and the yeasts *Cyberlindnera jardinii* and *Barnettozyma californica* (17.8 and 4.1% respectively in SB50 and 34 and 9.1% in SB90), all four previously described as lignocellulose degraders. The development of these operational strategies and further biological characterization of the end product could eventually benefit the process economics

by providing a standard and specialized inoculum for a continuous SSF for cellulases production.

Keywords: solid-state fermentation, cellulases, sequential batch reactor, microbial diversity, organic wastes.

1.Background

Increasing global demand for energy has resulted in the need for new and renewable energy forms such as bioethanol. In this sense, the second and third generation of bioethanol has gained great attention over the last years, due to the potential use of lignocellulosic biomass as raw material, avoiding the use of pure substrates such as starch (Behera et al., 2016, Kuhad et al., 2016). The use of agricultural by-products or wastes presents great advantages in comparison with pure substrates from the points of view of the energy balance and the environmental impact (Olofson et al., 2008).

In general, bioethanol production from lignocellulosic materials consists of a pretreatment to hydrolyse lignin and hemicellulose-based materials, followed by an enzymatic hydrolysis of cellulose and a final yeast-based fermentation of the released free sugars. From these stages, enzymatic hydrolysis appears as the main bottleneck for the production of bioethanol at commercial scale. This is mainly due to the cellulases production process, which accounts up to 40% of total costs (Arora et al., 2015). Moreover, some studies performed on the life cycle assessment of this process conclude that cellulase production requires a higher energy input than cellulose to ethanol conversion itself (Heather and Sabrina, 2009).

Cellulases used to break down cellulose into fermentable sugars are commercially produced by submerged fermentation mainly from pure substrates (McMillan et al., 2011). Despite the fact that the use of commercial cellulases potentially provides high sugar yields, its use is far of being a cost-effective alternative. The enzymatic preparations must be purchased continuously and its production process is complex and energy intensive (Lever, 2005). Submerged fermentation normally includes sterilization systems, control and monitoring of different parameters such as temperature, pH and dissolved oxygen, which implies the use of high amounts of energy in the process (Tolan and Foody, 1999). Thus, cellulases production should be optimized to avoid the high cost of commercial enzymes, and

to make the bioethanol production a sustainable process. Many aspects have been studied for the improvement of cellulases production, such as substrate (El-Barky et al., 2015), inoculum selection (Eichorst et al., 2013, Mihajlovski et al., 2015) and alternative fermentation strategies such as solid-state fermentation (Lever, 2005, Olofsson et al., 2008), among others.

Solid state fermentation (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 (Kuhad et al., 2012). However, its application at the industrial scale appears to be hindered by technology issues, such as reactor design, heat transfer issues or sterilization costs (Mitchell et al., 2006, Pessoa et al., 2016). To overcome some of these constrains, our research group have proposed to work in a composting-like process, under near adiabatic conditions using the native microbiota for the production of hydrolytic enzymes such as proteases (Abraham et al., 2013). However, a successful strategy for enzymatic production includes a proper selection of microorganisms to improve productivity coupled with these engineering aspects of SSF (Kuhad et al., 2016). In this context, we have proved in the same SSF configuration that thermophilic strains inoculated in non-sterile wastes were able to compete with autochthonous microbiota and significantly increase proteases activity (El-Bakry et al., 2016). Furthermore, it has been proven that it is possible to select and adapt compost microorganisms to use cellulosic materials as substrate, which could potentially increase the cellulolytic degradation capacity (Eichorst et al., 2013). These facts suggest the possibility to adapt a complex inoculum to degrade lignocellulosic materials in an easily scalable process to provide a standardized cellulase production process. The obtained enzymatic pool or even the fermented solids obtained by SSF can be potentially used in the hydrolysis step of the bioethanol production as suggested by some authors (Farinas, 2015). Pensupa et al. (2013) found a higher glucose release using cellulase obtained by SSF than commercial preparations

while Lever et al., (2010) successfully applied crude lignocellulosic extracts for bioethanol production.

Coffee husk (CH) is a lignocellulosic waste obtained in the roasting process of coffee. More than eight million tons of coffee beans are produced annually world-wide, 18% of which is obtained as CH (Murthy and Nadiu, 2012). Currently, coffee husk is disposed as an industrial waste and managed through incineration, landfill or composting in the best case. Shemekite et al. (2014) demonstrated the suitability of the native populations in coffee husk for biodegradation, correlating several enzymatic activities with the fungi communities. The current change of paradigm into the circular economy framework leads to an increasing interest in biomass as feedstock for new processes. Coffee husk has attracted interest of researchers worldwide as the substrate for solid-state fermentation and other processes to obtain valuable products (Mussatto et al., 2011; Narita and Inouye, 2014).

Despite all of the above advances, still the setting up of a long term SSF operation in continuous or semi-continuous regime remains a challenge with few attempts reported at lab scale using restrictive conditions, such as the use of pure strains under initial sterile conditions (Cheirsilp et al., 2015, Astolfi et al., 2011). Lately, a novel and more suitable approach has been assessed for enzymatic production by SSF using a sequential batch operation with non-sterile wastes, showing promising applications (Cerda et al., 2016).

The aims of this work are: i) to assess two different operational strategies for the development of a continuous SSF using coffee husk as a model substrate and ii) to produce a specialized inoculum for cellulases production and assess its microbial biodiversity. This process of optimization for cellulases production will provide a standardized and cost-effective technology with evident benefits for bioethanol production.

2. Materials and methods

126 2.1 Raw material

127 Coffee husk (CH) was kindly provided by Marcilla S.A (Mollet del Vallés, Barcelona,
128 Spain). Compost (C, from source-selected organic fraction of municipal solid waste) was
129 obtained from the municipal solid waste treatment plant Ecoparc de Montcada (Montcada,
130 Barcelona, Spain). All materials were stored frozen (-18ºC) until use. The full
131 characterization of both materials is presented in Table 1.

132

133 2.2 Solid-State Fermentation

134

135 2.2.1 Preliminary experiments at the lab scale

136 Preliminary SSF experiments were performed in order to assess the inoculum size and
137 the time for maximum cellulase production. Non sterile coffee husk was mixed with compost
138 as inoculum in 0, 10, 20, 50 and 100% (w/w) ratio. Wood chips were added in a 1:1 (v/v)
139 ratio as bulking agent. Fermentation was carried out in triplicates for 4 days at 37ºC in 500
140 mL Erlenmeyer flasks with 90 g of the mixture CH and C using continuous aeration of 20 mL
141 min^{-1} and oxygen monitoring.

142

143 2.2.2 Sequential batch operation in 4.5 L bioreactors

144 Fermentations were performed in 4.5L air-tight packed-bed reactors (adapted Dewar®
145 vessels), thermally isolated to work under near-to-adiabatic conditions (Dewar® vessels
146 provide excellent thermal isolation, minor heat exchange with the surroundings take place
147 through the airflow (in and out) and partially through the lid) . Experimental set-up is
148 presented in Figure 1. A self-made acquisition and control system was used based on
149 Arduino® and self-made software. Air was continuously supplied to the reactors by means of
150 a mass flow controller (Bronkhorst, Spain). Airflow was automatically adjusted by a

151 feedback controller (aeration range 15.8 – 31.6 L kg^{-1} DM h^{-1}, set point 11.5% oxygen in
152 exhaust gas). Airflow, temperature and oxygen content were continuously monitored.

153 The initial mixture for the first batch in both strategies contained 90% (wb) of fresh
154 coffee husk as substrate and 10% (wb) of compost as mixed inoculum. Compost was added in
155 order to provide active biomass and mainly for the potential incorporation of diverse
156 communities able to degrade lignocellulosic materials, as reported in recent studies (Lopez-
157 Gonzalez et al., 2014). Wood chips were added as bulking agent in a ratio of 1:1 (v/v) in
158 order to provide enough porosity to promote proper oxygen transfer (Ruggieri et al., 2009).
159 Fermentations were performed with a total weight of 1.2 kg per batch.

160 Maximum cellulases activity was achieved in 48h and thus the solid retention time
161 (SRT) was established in 48h. After this first 48h batch, two fermentations were performed in
162 parallel as sequential batches by means of two different operation strategies to be compared::

163 I. Strategy SB90: 90% of the wet fermented solids were removed from the reactor at
164 the end of the fermentation (every 48h) and used as product for final analysis. The remaining
165 10% of the fermented solids acted as inoculum to start a new batch, using 90% fresh coffee
166 husk. Six sequential batches (12 days) were performed to allow the microbial community to
167 develop and to assess the reproducibility of the cellulases production.

168 II. Strategy SB50: 50% of the wet fermented solids were removed from the reactor
169 every 24h and used as product for further analysis. In this case, the remaining 50% of the
170 fermented solid was used as inoculum to start a new batch, with the addition of 50% of fresh
171 coffee husk. SB50 operated for 18 days.

172 Both strategies performed in parallel until steady operation resulting in 12 days for
173 SB90 and 18 days for SB50. Sampling was always performed after a complete manual
174 homogenization of the fermented solids to obtain a full representative sample and prior to
175 feeding the reactor with fresh substrate.

176

177 2.2.3 Oxygen Uptake Rate and Cumulative Oxygen Consumption

178 Specific oxygen uptake rate (sOUR) was calculated on-line for continuous monitoring
179 in order to provide more accurate information on biological activity (Garcia-Ochoa et al.,
180 2010), according to:

181 $$sOUR = F \cdot (0.209 - y_{O_2}) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW \cdot 10^3}$$ Equation (1)

182 where: sOUR is the specific Oxygen Uptake Rate (mg O_2 g^{-1} DM h^{-1}); F, airflow (mL min^{-1});
183 yO_2, is the oxygen molar fraction in the exhaust gases (mol O_2 mol^{-1}); P, pressure of the
184 system assumed constant at 101325 Pa; 32, oxygen molecular weight (g O_2 mol O_2^{-1}); 60,
185 conversion factor from minute to hour; 10^3, conversion factor from mL to L; R, ideal gas
186 constant (8310 Pa L K^{-1} mol^{-1}); T, temperature at which F is measured (K); DW, initial dry
187 weight of solids in the reactor (g); 10^3, conversion factor from g to mg. Moreover, the area
188 below the sOUR curve was also determined, which represents the cumulative oxygen
189 consumption (COC) during the process (Ponsá et al., 2010). These parameters will provide
190 information on the overall biological activity and one of the main energy requirements for
191 cellulases production.

192

193 2.3 Microbial characterization

194 Identification of the microbial population was performed in the raw materials and in
195 the final samples from both SB50 and SB90 operational strategies using next generation
196 sequencing. The aim of this analysis was to determine the potential variation on biodiversity
197 and to characterize the specialized biomass obtained during sequential batch operations.

198 Total DNA was extracted and purified using PowerSoil™ DNA Isolation Kit (MoBio
199 Laboratories, USA) according to provider's specifications. DNA samples were checked for

200 concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop
201 Technologies, Wilmington, Delaware; USA) (Lopez-Gonzalez et al., 2014).
202 Bacterial 16S rRNA hypervariable regions V3-V4 and Fungal ITS1-ITS3 were
203 targeted. Later sequencing of the extracted DNA and bioinformatics were performed on
204 MiSeq platform by Life Sequencing S.A (Valencia, Spain).
205 Shannon-Wiener (H') and qualitative (I_S) and quantitative (I_{Squant}) Sorensen´s-Dice
206 biodiversity indices were estimated according to the following equations:

207 $$H' = -\sum p_i log_2 p_i$$

208 where $pi = n_i/N$, n_i= relative abundance of the ith species and $N = \Sigma n$, *and*

209 $$I_S = \frac{2c}{a+b} \quad ; \quad I_{Squant} = \frac{2pN}{aN+bN}$$

210 where a = number of species in sample 1, b = number of species in sample 2, c = number of
211 species shared by the two samples, pN = sum of the lower of the two abundances recorded for
212 species found in the two samples, aN = number of individuals in sample 1, bN = number of
213 individuals in sample.

214

215 2.4 Analytical methods

216

217 2.4.1 Enzyme extraction

218 Cellulases were extracted by adding 150 mL of citrate buffer (0.05 M, pH 4.8) to 10 g
219 of fermented solids in a 250 mL Erlenmeyer flask and mixing thoroughly on a magnetic
220 stirrer for 30 min at room temperature. The mixture was separated by centrifugation at 10000
221 rpm for 10 min, followed by filtration with a 0.45 µm filter. The remaining supernatant was
222 used for cellulases activity determination (Dhillon et al., 2012).

223

224 2.4.2 Enzymatic activity

225 The cellulase activity was measured according to the IUPAC filter paper assay
226 (Ghose, 1987). The reducing sugars were determined by the dinitrosalicylic (DNS)
227 colorimetric method (Miller, 1959). One filter paper unit (FPU) was expressed as equivalent
228 to the enzyme that releases 1µmol of reducing sugars under the assay conditions. The
229 cellulases production has been expressed with respect to the dry matter content, i.e, FPU g^{-1}
230 DM.

231

232 2.4.3 Routine methods

233

234 Moisture content, total and volatile solids, pH and electrical conductivity were
235 determined according to standard procedures (U.S Composting Council, 2001). Cellulose,
236 hemicellulose and lignin content were determined by the method of Van Soest et al., (1991)
237 using the Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY), adding
238 amylase and sodium sulphite solutions.

239

240 2.5. Statistical Analysis

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

244

245 3. Results and discussion

246

247 3.1 Preliminary experiments at lab scale

248 Figure 2a shows the profiles of the cellulase activity obtained when using different
249 inoculum sizes. Maximum cellulase activity was found in the first day of operation in all
250 cases. It can be noticed that the same trend is observed in all experiments; a rapid increase in
251 cellulase activity in day one followed by a gradual drop until the end of the fermentation.
252 The maximum cellulase activity was statistically similar for samples with 10 and 20% added
253 compost, reaching 7.0±0.3 and 7.5±0.5 FPU g^{-1} DM respectively. After day one, cellulase
254 production decreased achieving values around 0.18 FPU g^{-1}DM by the end of the
255 fermentation. Unexpectedly, adding 50% compost dramatically reduced cellulases production
256 until almost negligible values below values obtained with only CH or only C. A possible
257 explanation is that there was some diluting effect of the different types of biochemical
258 substrates and microbiota and the more complex composition of the mixture did not enhance
259 microbial activity.
260 In previous studies, the maximum enzymatic activity of amylases or proteases was not
261 related to the maximum biological activity measured as sOUR in similar systems (El-Bakry
262 et al., 2016, Cerda et al., 2016). However, for cellulases the opposite pattern was observed.
263 The maximum cellulase production was found on the peak of biological activity, i.e. during
264 maximum sOUR for all the assessed inoculum ratios (data not shown). As an example, Figure
265 2b shows a full sOUR profile for the sample using 10% of compost as inoculum. In this
266 Figure, it can be observed that the maximum cellulase activity was detected during the most
267 active biooxidative stage of the process, as other authors have also stated (Jurado et al.,
268 2014). Similar results were observed in batch experiments performed in 4.5L bioreactors
269 where a process time of 48h was fixed for maximum sOUR and cellulase production.
270
271 3.2 Sequential batch operation

272 The operation of bioreactors working under SB90 and SB50 conditions is presented in
273 Figures 3 and 4 respectively. In both cases, an initial 2-day fermentation process was
274 performed until the maximum sOUR was achieved adding compost in 10% ratio as a mixed
275 inoculum. Final average sOUR and cellulases activity in these initial fermentations for both
276 SB50 and SB90 strategies were 2.84 ± 0.66 mgO_2 g^{-1}DM $h^{-1}$and 7.6 ± 3.0 FPU g^{-1}DM
277 respectively. A similar temperature profile was obtained, with a maximum of 62ºC in both
278 cases. The characterization of the initial and final fermented solids for both tested strategies is
279 presented in Table 1.
280 Six sequential batches were performed (12 days of operation) for the SB90 strategy as
281 presented in Figure 3. Operation was consistent and maximum sOUR and final cellulase
282 activity remained stable among the different batches, with an average sOUR of 2.6 ± 0.1
283 mgO_2 g^{-1}DM h^{-1} and 9.0 ± 0.8 FPU g^{-1}DM. No statistical differences were observed for
284 cellulase activity among the six batches. The average COC was 87 ± 6 mgO_2 g^{-1}DM per
285 batch (48h).
286 For SB50 strategy the fermentation was performed for a total process time of 18 days
287 (Figure 4). After the first substrate change the sOUR achieved at the end of the batch (24h)
288 was 68% lower although cellulase activity was statistically similar to that of the initial batch.
289 After two batches, maximum sOUR performed consistently to an average value of 3.4 ± 0.2
290 mgO_2 g^{-1}DM h^{-1} (alterations in days 11 and 15 correspond to failures in the aeration system
291 and the process quickly recovered from these). The average COC was 58 ± 6 mgO_2 g^{-1}DM
292 per batch (24h). Contrary to the sOUR dynamics, cellulase production was not consistent.
293 Cellulase activity of final extracts dropped gradually to 2.1 ± 1.0 FPU g^{-1}DM on day 10, to
294 increase after day 13 to final values of 10 FPU g^{-1}DM on day 18, statistically similar to the
295 yields obtained with the SB90 strategy.

One of the main goals while developing these strategies was to achieve a steady cellulase production and biological activity (sOUR). The SB90 system was able to reach a steady maximum sOUR and cellulase production in only 12 days (6 sequential batches) while SB50 took 18 days. For SB50 it is likely that the sampling carried out in daily basis and the higher amount of the substrate exchanged generated an impact on the biomass which could delay the stabilization of the system.

Partial cellulose degradation of 3.1% and 3.2% was found for SB50 and SB90 in 24 and 48h respectively, which is in line with the values found in literature. Hemicellulose degradation of nearly 2% was observed in both SB50 and SB90. Lignin degradation was 2.5% and 0.8% for SB50 and SB90. These results are comparable to those reported by Umasaravanan et al. (2011) that observed only a 6% cellulose reduction and 3.1% lignin reduction in 21 days using sugarcane bagasse and rice straw. Other authors reported high cellulose degradation in SSF of lignocellulosic materials, for instance, 18% in 3 days using olive wastes (Salgado et al., 2015) or 48% degradation in 23 days using high-cellulose paper waste (Das et al., 1998). Whether the goal was to degrade and stabilize the lignocellulosic material in such a process as composting then a longer process time would be required. However in this case the main goal was to extract the maximum cellulase activity and thus these percentages of degradation are acceptable.

Lack of standardized cellulase activity determination makes difficult to provide a proper comparison with reported results. Even more, the most common substrate used for cellulase activity, filter paper, generates great concerns on reproducibility and accuracy, especially in mediums with low β-glucosidase production (Coward et al., 2003). However, cellulase activity obtained in this work is located in the range of reported researches, between 1-25 FPU g^{-1}DM for small bioreactors under sterile and mesophilic conditions (Behera et al., 2016).

As it can be observed in Fig. 3 and 4, similar final cellulase yields were obtained for both strategies. Also in both cases sOUR profile was displaced and the maximum was reached sooner in comparison with the two first batches with compost. As previously discussed, the maximum cellulase activity coincided with the maximum sOUR when using compost as inoculum.

According to sOUR profiles, a reduction in lag phase was accomplished and global biological activity was higher with the specialized biomass obtained at the end of the sequential operation fermentation (final batches) compared to the first batch using compost as inoculum. In general terms, a reduction of the lag phase and an increase in biological activity were expected as a result of inoculation and specialization of biomass (Jurado et al., 2015). This suggests growth stimulation of the microbial communities due to the inoculation of well adapted microbiota. Moreover, it has been stated that inoculation of mixed inoculums not only improved the process on its physical chemical parameters, but also stimulated microbiota growth and microbial diversity during the process (Ishii et al., 2000, Liu et al., 2011).

An enhancement of cellulases production was expected due to biomass specialization. Cellulase production is regulated by an inhibition system, in which cellobiose and glucose inhibits β-glucosidase (Kuhad et al., 2016). These readily metabolizable sugars released to the medium are easily consumed by the microorganisms present in the fermentation, which is one of the advantages of working with SSF. In this sense, it seems reasonable to assume that cellulase production was affected by other circumstances, such as the release of inhibitory compounds generated as the results of lignin degradation (Brijwani et al., 2011), non-productive adsorption to lignin hydrolysates (Akimkulova et al., 2016) or even to depletion of different mineral sources (Salgado et al., 2015).

345 A single research has been found on sequential batch in SSF for lipids and cellulases
346 production by oleaginous fungi (Cheirsilp et al., 2015), but this study was performed in a
347 different scale (<1g) and under sterile conditions. However, the authors found that at
348 different substrate exchange ratio (>50%), cellulases production could be sustained in values
349 ranging 1.81-2.25 FPU g^{-1}DM, although no increment on its activity was found, in
350 accordance with the findings described in this work.
351
352 3.3 Microbial characterization
353
354 3.3.1 Bacteria
355 A total of 22 bacterial families were detected in SB50 and SB90 with an abundance above
356 1% as presented in Figure 5. Only those families present in C and CH that were also found in
357 SB50 and SB90 final products are depicted for C and CH in order to better illustrate the
358 origin of the bacterial community present in the final products. Other families that did not
359 thrive along the fermentations have been ignored for clarity purposes. The complete
360 characterization of C and CH is presented in Table S1. SB50 and SB90 presented a uniform
361 distribution, with roughly 15 and 20% of less than 1% of relative abundance, and a relative
362 high biodiversity as indicated by Shannon-Wiener index, which reached levels greater than 3
363 for this taxonomic level (3.43 and 3.07, respectively). Taking into account the values found
364 for both raw materials (3.75 for C and 1.73 for CH), bacterial communities in two final
365 products seem to be more influenced by compost, despite its lower percentage at the initial
366 mixture. Sorensen´s-Dice indices, qualitative and quantitative (Figure 6), strengthen this
367 assessment, since higher values and, consequently, greater similarities were obtained when
368 comparisons were established between either both final products and C. According to these
369 results and those obtained from preliminary assay at lab scale (Section 3.1), CH would be a

proper and more efficient substrate to promote cellulase activity on account of its high cellulose content, but the bacterial cellulolytic community in compost dominates the structure of the final products. Compost is the result of a complex process characterized by the presence of recalcitrant and non-readily degradable substrates. From a biological point of view, this results in a very strong competitive selection of the microorganisms able to carry out all the necessary transformations. Thus, a concentration effect takes place throughout the process (Lopez-Gonzalez et al., 2014) that promotes the presence of a more specialized microbiota in the final product, adapted to those specific nutritional conditions that are typical in composting, among them, cellulolytic microorganisms.

The lower similarity between the bacterial population associated to CH and both final products is clearly evidenced by the fact that the most abundant families in CH, *Pseudomonadaceae* (33.7%), *Leuconostocaceae* (22.6%), *Enterobacteriaceae* (17.7%) and *Enterococcaceae* (16.3%) (Table S1), were sparingly represented in the latter. Thus, only the latest was found with a relative presence over 1% in SB50. On the other hand, in C nearly 80% of all families were a part of *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla, which have been found to be predominant in compost from lignocellulosic materials (Zhang et al., 2016). *Clostridiaceae* was the most predominant family in C present in 7.7%, which was present in both SB50 and SB90. It is important to point out that *Pseudomonaceae* and *Rhodospirillaceae* families were more abundant in the CH and C respectively, however their species were not able to thrive at the fermentations conditions of SB50 and SB90. Amounts of these families on final fermentation products were below 1%.

SB50 and SB90 resulted in similar bacterial distribution, in which compost influence as inoculum is clearly reflected. *Sphingobacteriaceae, Paenibacillaceae* and *Xanthomonadaceae* families together were present in a discrete 2.2% in C, however, they represented a 47.2 and 33.1% in SB50 and SB90 respectively. From these families, several

species have been identified during the early stage of lignocellulosic material composting process (Lopez-Gonzalez et al., 2015a).

From the dominant families, *Pseudoxanthonomas taiwanensis* and *Sphingobacterium composti* appeared as most abundant identified species in SB50 and SB90. Relative abundances of these species were 12.3 and 6.1% for SB50 and 6.1 and 2.6% for SB90 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 bacterias (Eichorst et al., 2013). On the other hand, *S. composti* has been related to lignin degradation and the production of acids in aerobic conditions (Karadag et al., 2013). The presence of these two species confirmed the results obtained in both sequential batch operations, which presented significant lignin and cellulose degradation. Furthermore, from *Paenibacillaceae* family *Paenibacillus* and *Thermobacillus* genera were the most abundant. Several species from both genera have been widely reported as strong cellulose degraders such as *P.chitinolyticus* (Mihajlovski et al., 2015), which was found on SB50 and SB90 in near 2% of relative abundance. Also *T.composti* has been found in 4 and 2.5% in SB50 and SB90 respectively. This specie is associated with cellobiase and xylanolytic activity during thermophilic stage in composting processes (de Gannes et al., 2013). Lately it has been characterized as halotolerant, which is of great interest for its bioethanol potential using ionic liquids (Watanabe et al., 2007).

3.3.2 Fungi and yeasts

A total of 12 families were detected for C, CH, SB50 and SB90 as presented in Figure 7. First of all, it is possible to notice that in all samples there is a great amount of non-identified DNA, described only as Eukariota, representing 25.7, 5.5, 44.8 and 5.8% of mycobiota from C, CH, SB50 and SB90 respectively. This relatively high rate of unidentified

fungi may be consequence of the still scarce and sometimes confusing information available on data base concerning molecular characterization of fungi, especially in comparison with bacteria, which now limits the potential for elucidating the structure of the mycobiota associated to different environments (Langarica-Fuentes et al., 2014).

Remarkably, most of the fungi and yeasts in SB50 and SB90 come from CH where four families account for 94.4% of all mycobiota. This similarity was particularly notable for SB50, with remarkable differences as expressed by the values of the Sorensen´s-Dice index associated to both comparisons, SB50 *v*. C and SB50 *v*. CH (Figure 6). Fungal biodiversity increased in the final materials compared to CH as the Shannon indices obtained were 1.63, 0.98, 1.39 and 1.33 for C, CH, SB50 and SB90. Predominant families were *Phaffomycetaceae*, *Dipodascaceae*, and two unidentified families of the class of *Tramellomycetes*. Distribution in SB50 and SB90 was similar in content but not in proportion, resulting in higher qualitative homology in contrast to quantitative. *Phaffomycetaceae* family was present in CH in 7.8% and in negligible amounts in C. This family thrived on both reactors, achieving a relative abundance of 21.9 and 43.2% in SB50 and SB90 respectively. In this family two yeasts species were identified. The more abundant specie was identified as *Cyberlindnera jardinii*, (17.8 and 34.0% in SB50 and SB90 respectively), which is able to metabolize pentoses and tolerates lignin by-products, which makes it a suitable specie for the treatment of lignocellulosic wastes (Nordberg et al., 2014, Lopez-Gonzalez et al., 2015b). The second specie was *Barnettozyma californica* (4.1 and 9.1% in SB50 and SB90 respectively), a xylose-fermenting and xylanase producer yeast (Morais et al., 2013). In this sense, in SB90, most of fungal/yeast population is focused on hemicellulases metabolism which, in a way, could increase cellulose availability by relaxing the rigid lignocellulosic matrix, which would result in an easier accessibility to this macropolymer, and an increase in the cellulases production. Another difference between both

445 final fermented solids is the high abundance of *Dipodascaceae* family only in SB50, with a
446 21.9%. in comparison with 0.15% of SB90. *Dipodascus australiensis* and *Galactomyces* sp.
447 are the most representative yeasts of this family and has been proven to be xylanase producer
448 and the latter, cellulose degrader with mild halotolerant characteristics (He et al., 2016).
449 Finally in *Tremellomycetes* class, *Tremellales* order, 3 different genera were detected as
450 *Trichosporonales* sp. LM659, uncultured *Tremellales* and uncultured *Trichosporon* which all
451 together account as 85.5, 10.3 and 46.5% of CH, SB50 and SB90 respectively. No species
452 were identified in this order, however some reports on different species of this order has been
453 proven to be unconventional due to absence of diauxic effect, i.e fermenting hexoses and
454 pentoses while also effectively utilizes xylose and *N*-acetylglucosamine, which are building
455 blocks of lignocellulosic materials (Kourist et al., 2015).
456
457 3.3.3. Overall performance of the process and the microbial communities involved
458 In general, solids obtained at the end of SB50 and SB90 fermentations, provided a
459 strongly specialized mixed inoculum for lignocellulosic materials degradation. A slightly
460 higher microbial diversity (expressed as the Shannon index) in SB50 compared to SB90
461 probably means a lower specialization degree of the biomass. This biomass could be
462 associated to the degradation of other components of the material, such as proteins or
463 hemicellulose as in the case of yeasts characterization as explained above. Cellulose
464 degrading activity was mainly provided by bacterial populations; however it is important to
465 remark that cellulolytic bacteria do not always express the full cellulose degradation system
466 (Behera et al., 2016). On the other hand, hemicellulose degradation potential was provided by
467 fungal/yeasts species. In spite of the abundance of hemicellulose degraders, hemicellulose
468 degradation was negligible (Table 1). It is possible that this biomass was not active during the
469 process, or the activity was low in comparison with cellulose or even lignin degraders, or that

the hemicellulolytic microorganisms act partially on the heteropolymer, promoting relaxation but not degradation in an extensive rate.

Considering these results it is likely that synergistic or complementary actions take place in the total microbiota of the reactors. Degradation of cellulose does not occur in isolation conditions. As a constituent of the lignocellulosic matrix, the action of cellulases is not the only requirement for the transformation of this glucose polymer (Kanokratana et al., 2015). Thus, the biodegradation process is the result of different cooperative actions that first promote the relaxation of the enmeshed structure that characterize lignocellulose. This initial phase-stage allows the access of enzymes to the site of action and, consequently, is one of the key steps in order to achieve the depolymerisation, not just of cellulose but of all macropolymers in lignocellulose too (Duarte et al., 2012). From a microbial point of view, the complexity of the process acts as a pressure factor that promotes the selection for a microbiota metabolically adapted to the nutritional demands associated to lignocellulosic environments. This is true for artificial hábitats in particular, such as the case described in this work, in which microbiota shifts from a metabolically diverse community to a specialized one (López-González et al., 2014). In this sense, it would be highly improbable that lignocellulosic and lignocellulosic-inhibiting populations coexisted during such a selective process. Nevertheless, it cannot be excluded some other inhibitory effects associated to the products resulting from the action of cellulases and other lignocellulosic enzymes, as glucose, cellobiose or phenolic compounds, although microorganisms that consume these inhibitory compounds use to be members of the lignocellulosic community (Wongwilaiwalin et al., 2013).

In light of the experimental data presented in this work, it is possible to remark that the use of fermented solids as inoculum in a sequential batch operation is very successful. The proposed operation offers a way to reduce costs in inoculum requirements and potentially to

495 the reduction of solid wastes generation as suggested by Farinas (2015). The studies on
496 microbial diversity on non-sterile SSF processes are scarce. In this process a specialized
497 biomass provides the generation of an enzymatic pool, containing different enzymatic
498 activities related to lignocellulose degradation. The positive effects of using a multi-
499 enzymatic preparation have been reported by Melikoglu et al., (2013). These authors were
500 able to improve bioethanol production by producing several enzymes by SSF. Furthermore, it
501 has been proven that the use of these enzymatic pools without any additional processing steps
502 provides better results than commercial preparations, reducing operational costs (Lever et al.,
503 2005). In summary, the proposed process presents the following economic advantages: the
504 use of organic wastes instead of pure substrates; saving the investment and the operating
505 costs related to sterilization; saving the costs of producing inoculum for each batch, since
506 fermented solids from one batch are re-used to inoculate the following batch; the potential
507 use of the multienzymatic extracts without additional purification steps. Further economic
508 assessment should confirm the cost effectiveness of the process.
509
510 4. Conclusion
511 Operation of SSF of coffee husk in sequential batches has been proven as a suitable
512 strategy for cellulases production using a mixed inoculum. SB50 and SB90 strategies
513 provided a sustained fermentation for 18 and 12 days respectively and cellulase production
514 stabilised at around 10 FPU g^{-1}DM. 48h batches in the strategy SB90 provided a more
515 consistent operation while SB50 required more time to reach a pseudo-steady state. Both
516 strategies obtained cellulase activity in the reported range of production. The sequential
517 process allowed the enrichment of cellulose and hemicellulose degraders, eliminating the
518 requirements of fresh inoculum for each batch. Bacterial communities obtained at the end of
519 both processes came from compost with great cellulose degradation potential. Fungal and

yeasts communities came mostly from coffee husk, with high hemicellulose degradation potential. The development of these operational strategies and further biological characterization of the end product could eventually benefit the process economics by providing a standard and specialized inoculum for a continuous solid state fermentation for cellulases production. Further research will focus on the improvement of cellulase production, the process scale-up and the optimization of cellulase extraction by comparing different downstream processing strategies.

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Tables

Table 1. Characterization of coffee husk (CH), compost (C), initial mixtures and final fermented solids for SB50 and SB90.

Parameter	Coffee Husk	Compost	SB50 initial	SB90 initial	SB50 final	SB90 final
Moisture % (wb)	60.8 ± 0.4	34.9 ± 0.3	61.2 ± 1.1	62.0 ± 1.3	55.0 ± 1.1	55.0 ± 3.1
Organic matter (%, db)	90.2 ± 0.1	82.5 ± 0.1	nd	nd	nd	nd
pH	6.51 ± 0.01	7.22 ± 0.01	6.45 ± 0.01	6.43 ± 0.01	9.11 ± 0.01	9.07 ± 0.01
Cellulose (%, db)	25.7 ± 0.2	9.8 ± 1.2	23.4 ± 0.8	27.9 ± 0.4	28.6 ± 0.5	23.5 ± 0.4
Hemicellulose (%,db)	14.6 ± 0.1	10.2 ± 0.1	13.2 ± 0.5	13.3 ± 0.3	13.2 ± 0.5	13.3 ± 0.5
Lignin (%, db)	17.6 ± 0.5	13.6 ± 1.2	20.4 ± 0.6	21.8 ± 0.6	16.5 ± 0.7	18.3 ± 0.6

wb: wet basis; db: dry basis; nd: not determined.

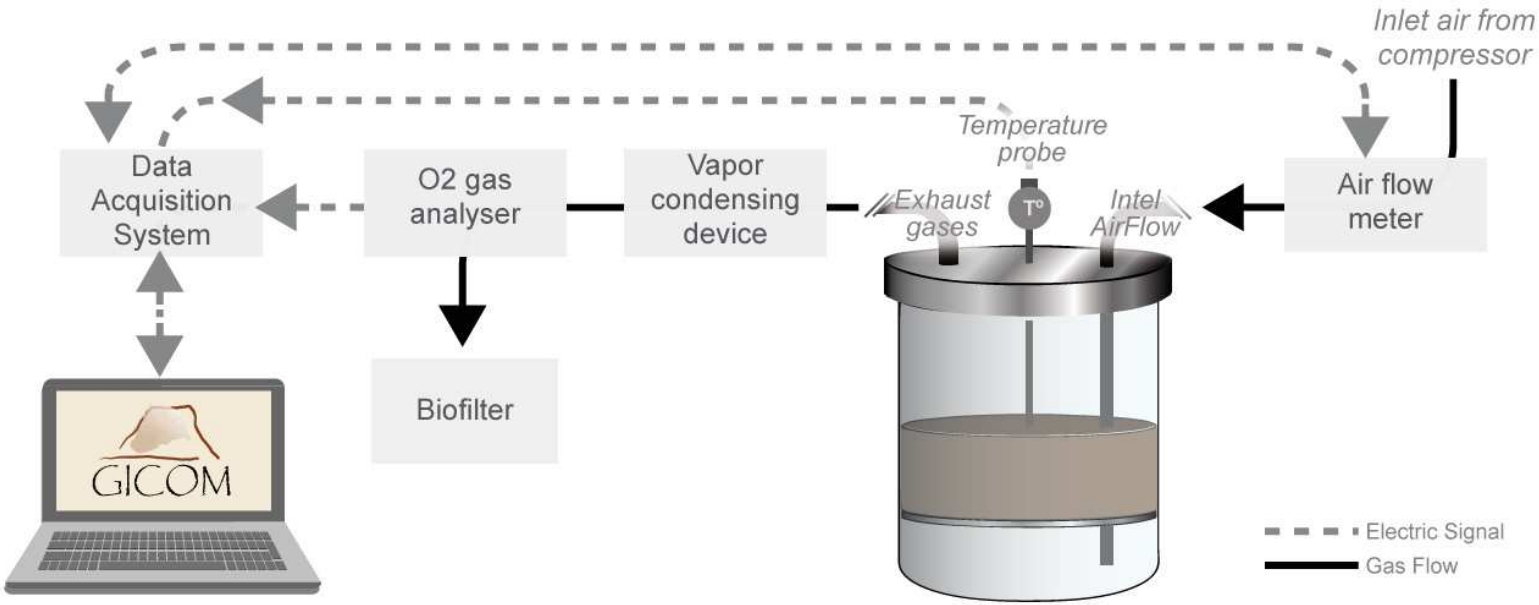

Figure 1. Experimental set-up of the solid-state fermentation system.

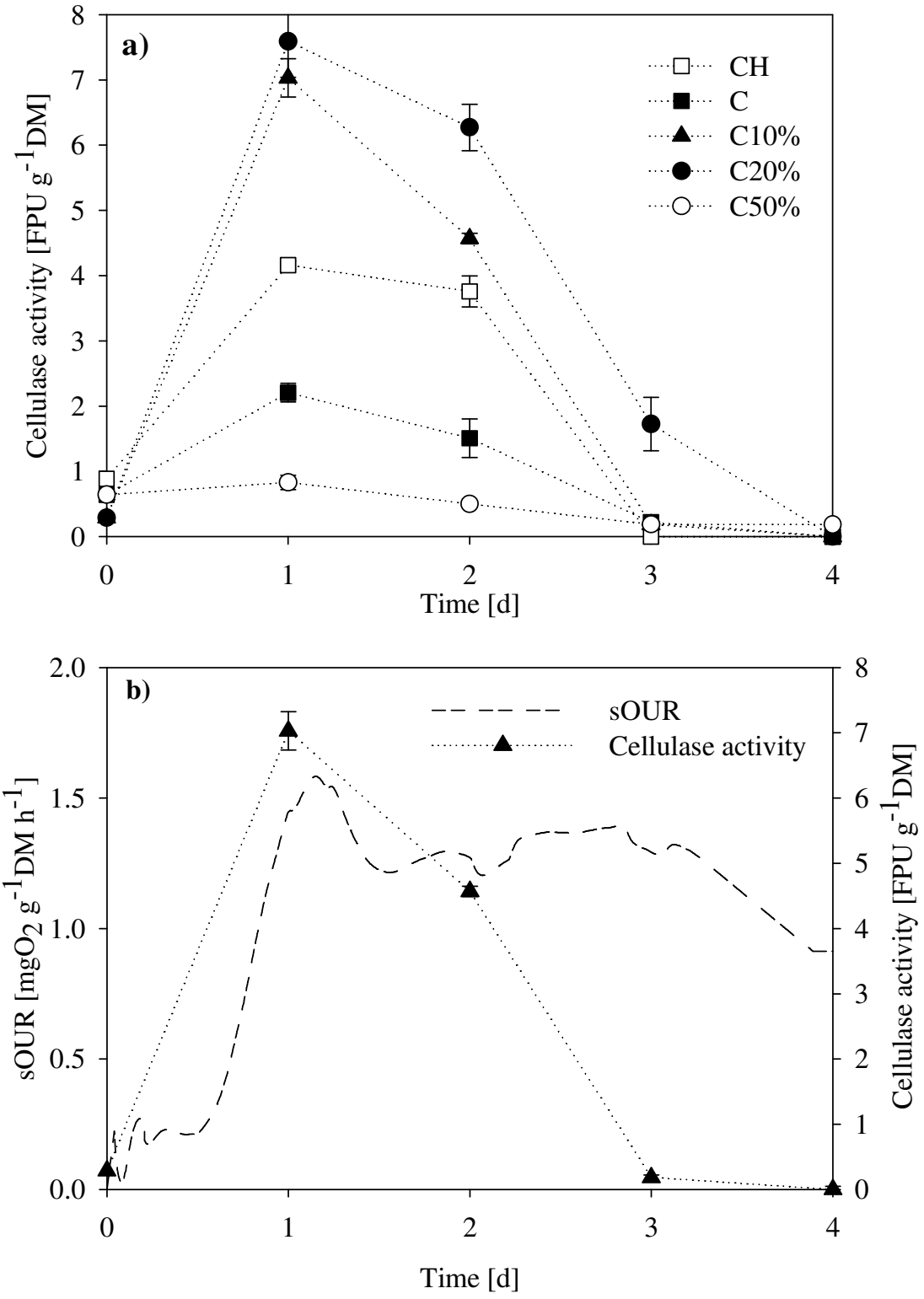

Figure 2. SSF of coffee husk using compost as inoculum at lab scale and 37ºC during 4 days. a) Profile of cellulase production using different inoculum sizes and b) profiles of sOUR and cellulase production using 10% of inoculum (CH: coffee husk; C:compost).

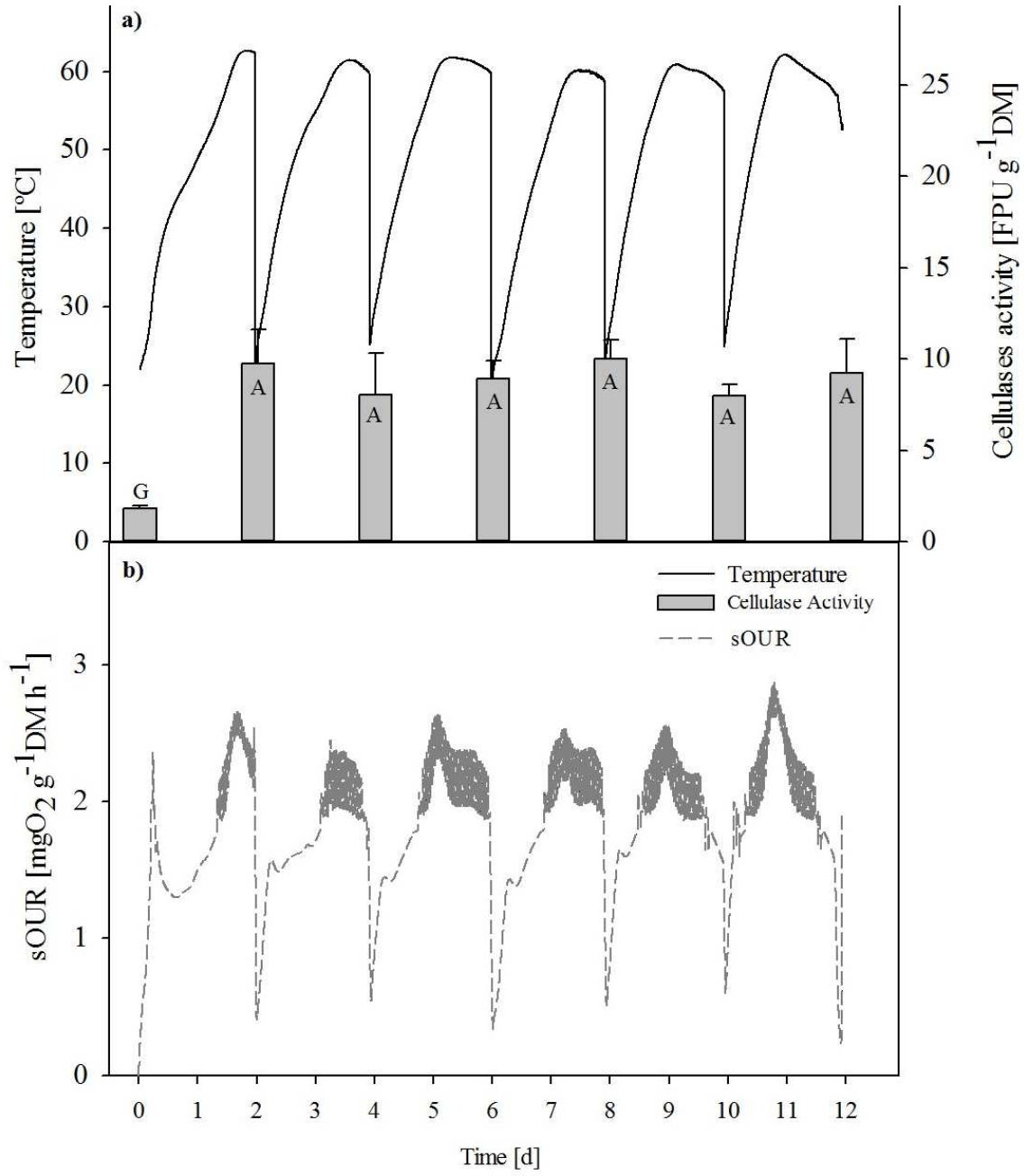

Figure 3. Process follow up of sequential batch operation SB90 of coffee husk for cellulase production. In a) temperature and cellulase activity and b) sOUR profiles are presented. Bars that do not share a letter are significantly different.

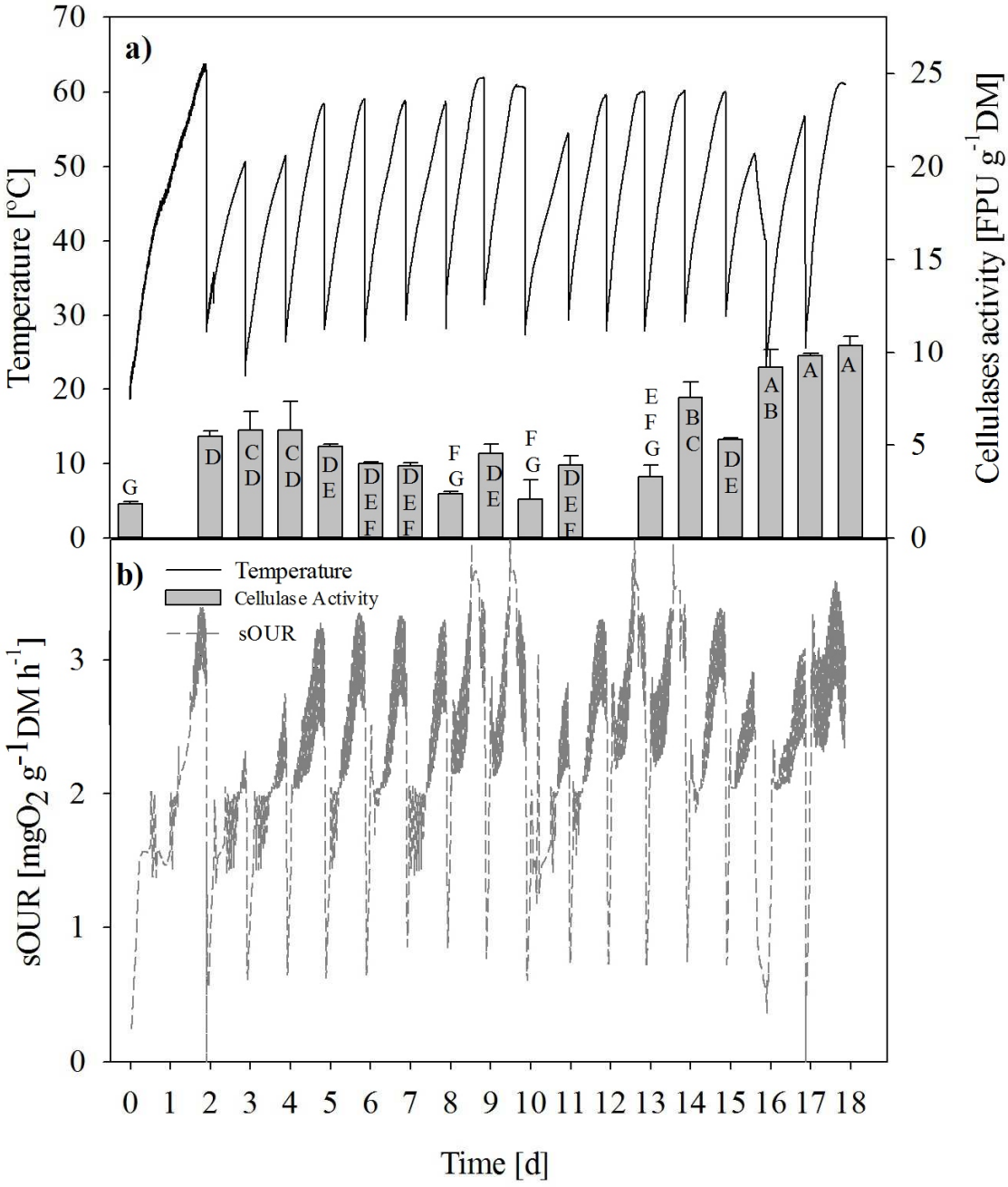

Figure 4. Process follow up of sequential batch operation SB50 of coffee husk for cellulase production. In a) temperature and cellulase activity and b) sOUR profiles are presented. Bars that do not share a letter are significantly different.

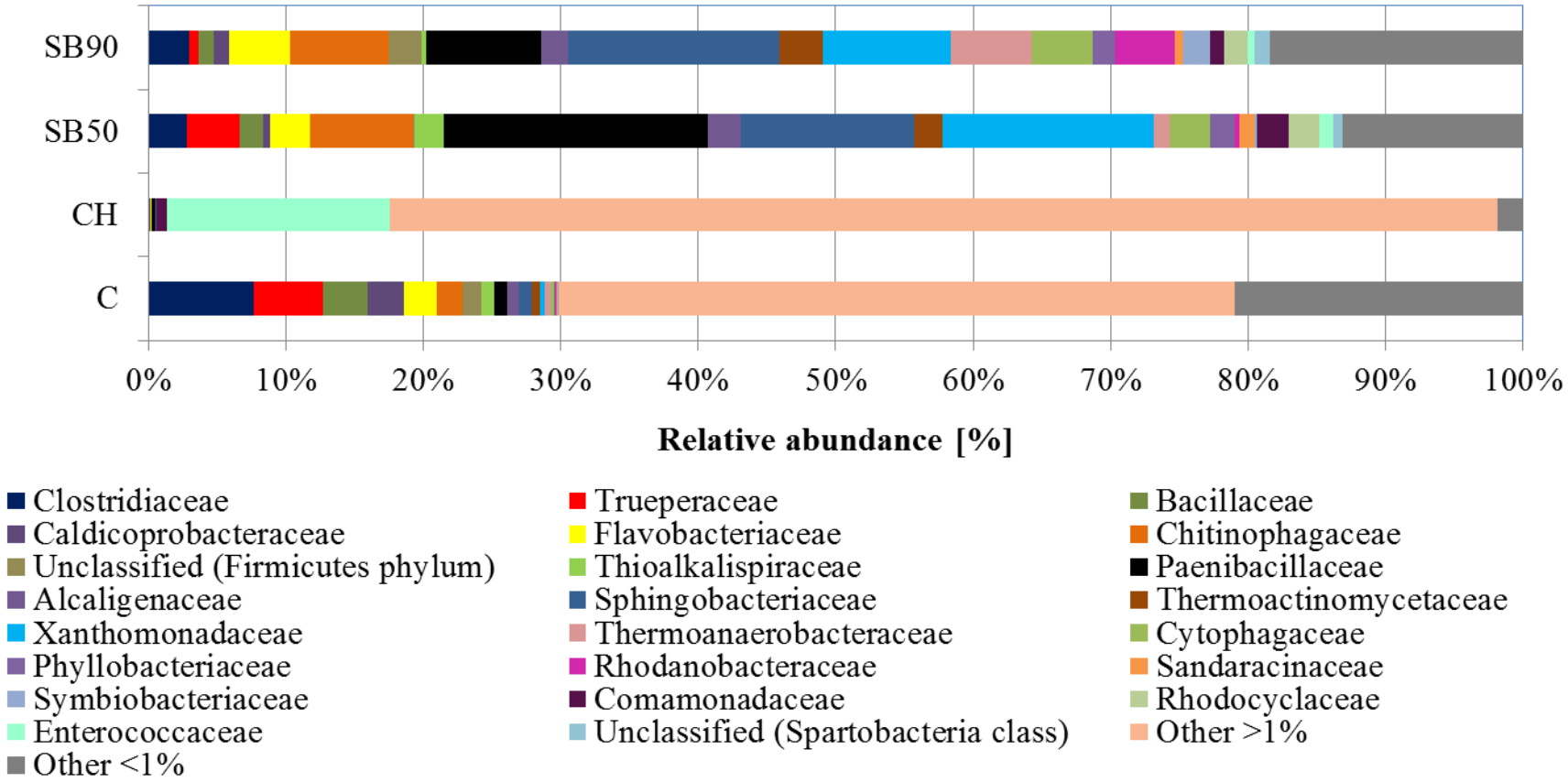

Figure 5. Bacterial distributions at the family level according to the16s sequencing for Compost (C), Coffee Husk (CH) and the final product of reactors SB50 and SB90. Only families with a relative abundance >1% in either SB50 or SB90 are depicted. Families detected with a relative abundance < 1% in these two samples are grouped as "others".

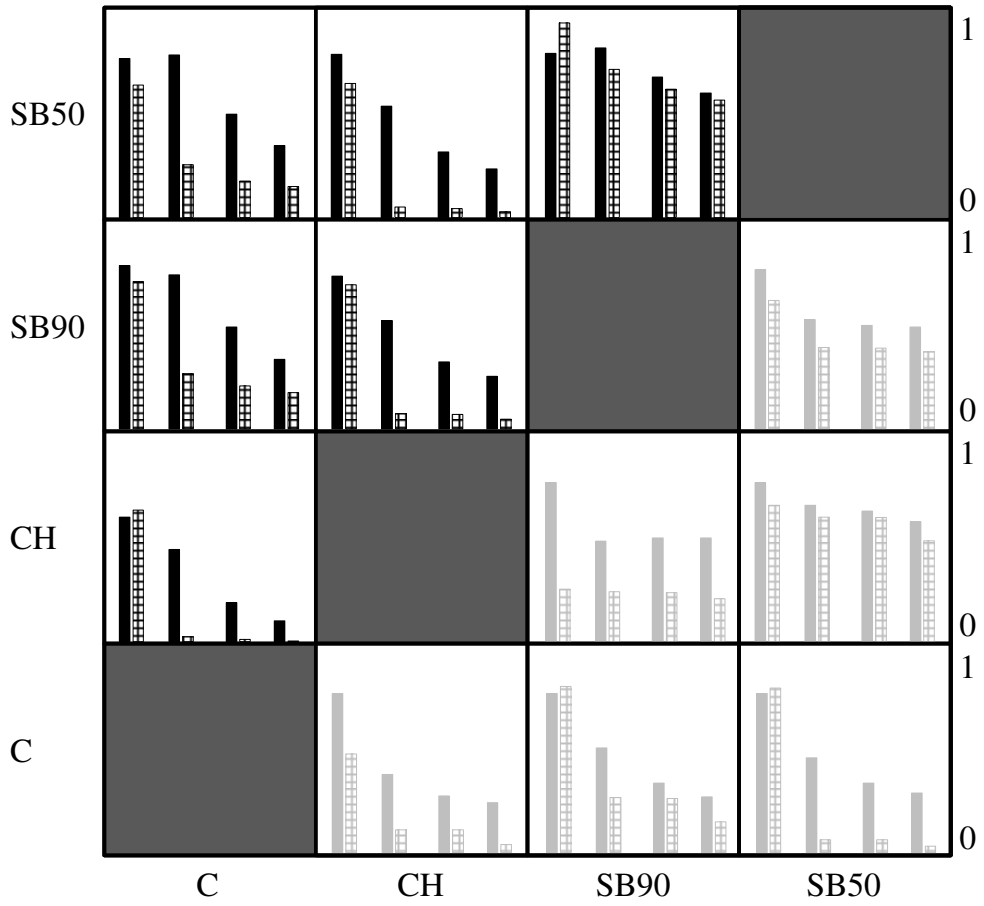

Figure 6. Sorensen´s-Dice indices for prokaryotic (black) and eukaryotic (grey) microbiota associated to raw materials and final products (Compost (C), Coffee Husk (CH) and the final product of reactors SB50 and SB90). Every quadrant shows both qualitative (■, ■) and quantitative (▦, ▦) indices for all taxonomic levels (from left to right: phylum, family, genus and specie).

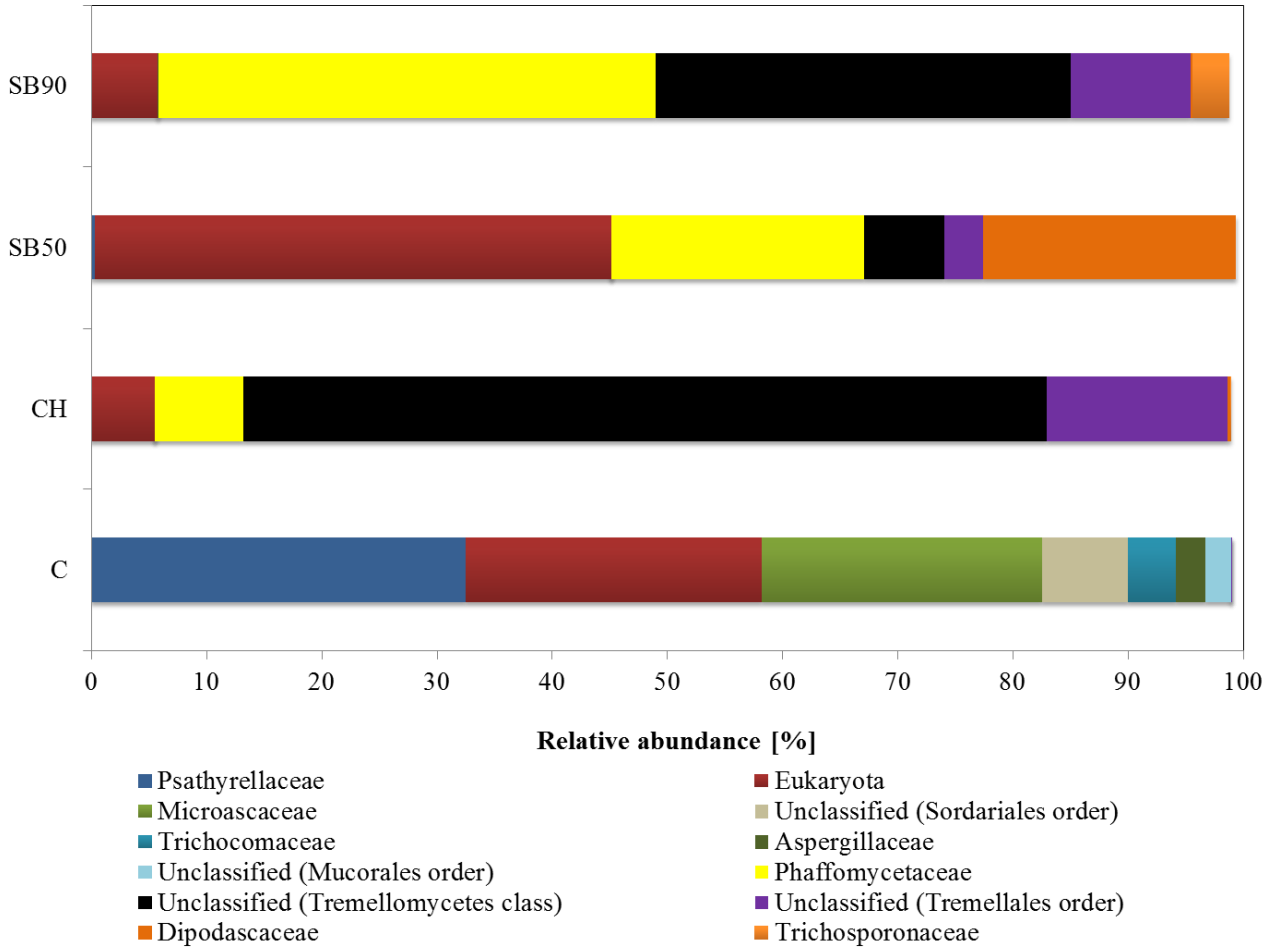

Figure 7. Identified fungal and yeasts families in Compost (C), Coffee Husk (CH) and the final product of reactors SB50 and SB90.