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1 **Co-elicitation of lignocelluloytic enzymatic activities and metabolites production in an**
2 ***Aspergillus-Streptomyces* co-culture during lignocellulose fractionation**

3

4 Julian Detain[1], Caroline Rémond[1], Carine Machado Rodrigues[2], Dominique Harakat[2], Ludovic
5 Besaury*[1]

6 Correspondance : Ludovic Besaury ; ludovic.besaury@univ-reims.fr

7 [1]Université de Reims Champagne Ardenne, INRAE, FARE, UMR A 614, chaire AFERE, 51097 Reims,
8 France

9 [2] Université de Reims Champagne Ardenne, CNRS, ICMR UMR 7312, 51097 Reims, France.

10 Keywords : Lignocellulose ; co-culture ; Sreptomyces ; comparative genomics ; CAZymes

11

12 ABSTRACT

13 Lignocellulose, the most abundant biomass on Earth, is a complex recalcitrant material mainly
14 composed of three fractions: cellulose, hemicelluloses and lignins. In nature, lignocellulose is
15 efficiently degraded for carbon recycling. Lignocellulose degradation involves numerous
16 microorganisms and their secreted enzymes that act in synergy. Even they are efficient, the
17 natural processes for lignocellulose degradation are slow (weeks to months). In this study, the
18 objective was to study the synergism of some microorganisms to achieve an efficient and rapid
19 lignocellulose degradation. Wheat bran, an abundant co-product from milling industry, was
20 selected as lignocellulosic biomass. Mono-cultures and co-cultures involving one *A.niger* strain
21 fungi never sequenced before (DSM 1957) and either one of three different *Streptomyces* strains
22 were tested in order to investigate the potentiality for efficient lignocellulose degradability.
23 Comparative genomics of the strain *Aspergillus niger* DSM 1957 revealed that it harboured the
24 maximum of AA, CBM, CE and GH among its closest relative strains. The different co-cultures
25 set-up enriched the metabolic diversity and the lignocellulolytic CAZyme content. Depending
26 on the co-cultures, an over-expression of some enzymatic activities (xylanase, glucosidase,
27 arabinosidase) was observed in the co-cultures compared to the mono-cultures suggesting a
28 specific microbial cross-talk depending on the microbial partner. Moreover, metabolomics for
29 each mono and co-culture was performed and revealed an elicitation of the production of
30 secondary metabolites and the activation of silent biosynthetic cluster genes depending on the

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31 microbial co-culture. This opens opportunities for the bioproduction of molecules of interest
32 from wheat bran.

33 # INTRODUCTION

34 Biorefining is dedicated to produce energy, molecules and materials from renewable feedstocks
35 [1]). The use of lignocellulosic biomass such as agricultural and agro-industrial co-products
36 does not compete with plants dedicated for food. The use of cheap and abundant co-products
37 as substrates for fermentation allows to reduce the costs of the bioprocesses [2]. Microbial
38 cultures using lignocellulosic biomass as carbon sources are extensively studied for the
39 production of enzymes and of various molecules of interest [3, 4].

40 "Top-down" approaches, based on the study of natural ecosystems, where the
41 degradation of lignocellulose is significant such as forest soils, compost, have enabled advances
42 in the description and the multiple functions played by multiple microorganisms [5, 6]. On the
43 opposite, "bottom-up" approaches consisted in the implementation of co-cultures by a limited
44 number of microorganisms. The use of synthetic microbial co-cultures has also been
45 implemented in order to mimic natural processes and distribute the different functions to
46 specific populations. These microbial co-cultures based on mutualistic relationships, besides
47 the ability to perform different functions, have several advantages such as: the ability to prevent
48 a nutritional deficiency due to the diversity of metabolic pathways present, the ability to
49 exchange metabolites within community [7, 8]; stability and robustness within the microbial
50 community (Zuroff et al; 2013). The proof of concept in co-culture has been demonstrated
51 through various applications such as human health [9] or pollution control [10] and has made it
52 possible to increase the services provided compared to mono-cultures. The use of co-cultures
53 increases the production of enzymes. Several explanations exist: (1) a greater diversity of
54 enzymes is produced allowing a more efficient and more complete degradation of the substrate,
55 this is the enzymatic synergy [11], (2) a chemical interaction (interaction molecules , elicitors,
56 secondary metabolites), as well as the sharing of metabolic pathways allows emulation of
57 microbial development, this is the growth synergy [12].

58 In this study, co-cultures were performed between one fungal strain (belonging to the
59 species *Aspergillus niger*) and either one of three different actinobacteria (belonging to the
60 *Streptomyces* genus; *S. avermitillis* ATCC 31267, *S. coelicolor* A3(2) and *S. griseorubens* DSM
61 40160). Naturally, the fungi plays a role in the carbon cycle through extracellular hydrolytic
62 and oxidative enzymes. This fungus is used in the fermentation industry to produce citric acid,
63 cellulolytic enzymes by fermentation in solid media [13], or even enzymatic cocktails to pre-
64 treat lignocellulosic biomass [14]. Enzyme cocktails are easily recovered, since the fungus will

65 secrete the enzymes in the extracellular environment. *A.niger* enzymes panel includes
66 cellulases, endo/exoglucanases, β-glucosidases, xylanases [13]. Actinobacteria are Gram-
67 positive bacteria characterized by a genome with high G + C ratio and are numerous and widely
68 distributed group of soil microbes, constituting to about 10 to 50% of the soil microflora
69 community and important producers of diversified secondary metabolites which can have
70 several functions such as antifungal and antibacterial activities [15]. *Streptomyces* genus has
71 produced approximately 67% of the natural antibiotics among the actinobacteria and could
72 produce approximately around 7600 bioactive compounds [16]. The genus *Streptomyces*, very
73 widespread in soils, is a major player in the degradation of organic matter and lignocellulose
74 [17] . This microbial genus has a large enzymatic arsenal encoding carbohydrate esterases,
75 polysaccharide lyases, glycoside hydrolases and enzymes with auxiliary activities [18, 19].
76 They act in the catabolism of complex molecules and substances like lignocellulose, xylan,
77 cellulose, and lignin, which are important in soil organic matter catabolism [20]. The ability of
78 *Streptomyces* to deconstruct lignocellulose has been studied in several ecosystems such as
79 intestinal tracts of insects [18], grassland [21] and moreover, those bacteria can also act as
80 promotor and increase rice-straw composting by other microorganisms [22]. Due to their origin
81 (mainly superficial layers of soil), *Aspergillus* and *Streptomyces* can present several interactions
82 together. Indeed, the strain *Streptomyces leeuwenhoekii C58* has been used to trigger and elicit
83 secondary metabolites production of *Aspergillus fumigatus MR2012* [23].

84 In the present study, we report on the setup and the co-cultivation of one *A.niger* strain
85 DSM 1957 and three bacterial strains belonging to the actinomycetes for degrading wheat bran.
86 The genomic sequencing of *A. niger* DSM 1957 allowed to evaluate the genomic potential of
87 this strain for the production of enzymes such as CAZymes (Carbohydrate Active enZymes)
88 involved in lignocellulose degradation. Genomic comparisons were performed between the four
89 strains in order to highlight their CAZyme and secondary metabolites production potential. The
90 interactions between the strains were first investigated with classical cultivation on Petri dishes.
91 The efficiency of wheat bran degradation was investigated by quantifying the various enzymatic
92 activities produced by the microorganisms and also by analyzing the various metabolites
93 produced during the microbial growth onto wheat bran.

94 # MATERIAL AND METHODS

95 **Substrate preparation**

96 Wheat bran (WB) (0.5-2 mm) was provided by the ARD society (https://www.a-r-d.fr/). The
97 WB contained 16 % of dry matter (DM) of arabinose, 19 % DM of glucose, 26 % DM of xylose,
98 1.1% DM of galactose, 11.6% DM of starch, 15% DM of protein and presents a lignin content
99 of 5% DM". The % of glucose can be attributed to cellulose and mixed β-glucans present in
100 WB.**Growth media and inoculation of the microbial partners**

101 In order to characterize the ability of the strains to grow on wheat bran, 250 mL flasks
102 containing 50 mL of M3 media 1X (KH_2PO_4 1.9 g/L, Na_2HPO_4 5.1 g/L, $MgSO_4.7H_2O$ 0.1 g/L,
103 $(NH_4)_2SO_4$ 0.2 g/L) [24] supplemented with dry wheat bran up to 5 g/L were used. After
104 sterilization, 1 mL of a $Ca(NO_3)_2.4H_2O$ solution at 25 g/L and 1 mL of a trace element solution
105 were added. One pellet (2mm)of actinobacteria (10^8 cells) and 10^6 spores of *A. niger* DSM
106 1957 were inoculated into the flasks. Controls were performed using only one carbon source as
107 glucose supplemented up to 40 mM. All flasks were shaken for 144 h at 100 rpm at 30°C. The
108 experiments were performed in triplicate.

109 **Defining parameters of the co-cultures**

110 The physico-bio-chemical parameters of the co-cultures were determinated by taking into
111 account several conditions: 1) the biological interactions between the partners (competition,
112 mutualism or neutralism) and, 2) the inoculation parameters of individual partner (simultaneous
113 or shifted inoculation). In order to test a potential antagonism between microorganisms, the
114 interaction between microorganisms was observed on solid medium. Each interaction was
115 carried out on WB + agar medium in duplicate. A first interaction was tested by spreading the
116 *Streptomyces* all over the Petri dish and inoculating 10^6 spores of the fungi into the center of
117 the Petri dish (**Supplementary Figure 1**). Concerning the time of inoculation, different
118 strategies of inoculations were tested (simultaneous or shifted) (**Supplementary Figure 2**): the
119 parameters selected were based on the observation of two microbial partners at the end of the
120 incubation.

121 **Enzymatic activities of the mono and co-cultures**

122 To obtain intra- and extracellular enzymes, 1 mL of culture was collected and then centrifuged:
123 1) the supernatant corresponding to the extracellular enzymes was collected, 2) the pellet
124 obtained was then resuspended in Tris-HCl (pH7, 25 mM) and cells were lysed using FastPrep

125 and lysing matrix B at 6.5 G during 4 times * 40s (MpBiomedicals, France). All the
126 lignocellulolytic activities were measured in triplicate at the end of the growth curve (144 h)
127 except for the xylanolytic which was measured all along the growth curve. Protein
128 concentrations were determined by the Bradford method using BSA as a standard [25] in order
129 to normalize the enzymatic activities through time and the different samples. The intracellular
130 activities were expressed as mIU of enzymatic activity /mg of protein. Xylanolytic activity was
131 evaluated as previously described [26] with 0.1 mL of extracted proteins mixed with birchwood
132 xylan (Sigma Aldrich France) at 0.5% w/v in 50 mM phosphate buffer, pH 7.5 at 30°C for 10
133 min. Enzymatic activity was expressed in international milliunits (mIU), where 1 IU is defined
134 as the quantity of enzyme required to release 1 µmole of reducing sugar per min.

135 Total phenol-oxydase/peroxydases activities were evaluated as in [27]; for this, 0.1 mL
136 of extracted proteins was mixed in a total volume of 2 mL with 8 mM of pyrogallol, 1 mM of
137 EDTA and 0.5 mM of H_2O_2 at 30°C during 10 min and measured at 420 nm.

138 Arabinofuranosidase activity by determining the hydrolysis rate of *p*-nitrophenyl α-L-
139 arabinofuranoside (0.5 mM) and xylosidase activity was measured by quantifying the rate of
140 hydrolysis of of *p*-nitrophenyl-β-xylopyranoside (0.5 mM) in the same buffer in 1 mL reaction
141 containing 900 µL of buffer (50 mM sodium phosphate buffer, pH 7.5) and 0.1 mL of culture
142 supernatant. β-D-glucosidase activity were measured in the same conditions using as substrate
143 *p*-nitrophenyl -β-D-glucopyranoside. The extinction coefficient of *p*NP in the measurement
144 conditions was 15,850 $M^{-1}.cm^{-1}$. Experiments were measured using the absorbance at 401 nm
145 for 5 min at 30 °C and using recording spectrophotometer (Uvikon 933). All the enzymatic
146 activities will be presented in mIU/mg of protein.

147 **Genomic annotation: metabolic pathways and CAZYme**

148 The obtained genome sequence were finally annotated by using the NCBI Prokaryotic Genome
149 Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) in order to get
150 deposited at GenBank. All the *Streptomyces* genomes studied in that manuscript were also
151 annotated using the Microbial Genome Annotation & Analysis Platform
152 (https://mage.genoscope.cns.fr/) which provided information about : 1) the metabolic profiles
153 present in the genomes using the Metacyc database [28], 2) the core and pan-genome shared by
154 the strains using SILIX software [29], 3) the presence of syntenic pathways, and 4) the
155 distribution of Cluster of Ortholog groups (COGs). The presence and diversity of the
156 Carbohydrate Active enZyme was demonstrated by using the online resource dbCAN

157 (http://csbl.bmb.uga.edu/dbCAN/annotate.php).The CAZyme secretome of the fungi was
158 analysed by using the SignalP software in order to identify the proteins with a secretion signal
159 to the extracellular medium. The TMHMM server to identify the presence or absence of
160 transmembrane domains in the sequence of these proteins. Only the sequences with no
161 TMHMM topology were obtained and finally submitted to the dbCAN meta server in order to
162 determine the CAZy secretome [30, 31].
163

164 **UHPLC/Q-TOF HRMS analysis**

165 Samples extracted from the different cultures on WB and glucose were analyzed by UHPLC/Q-
166 TOF HRMS (Ultra High Performance Liquid Chromatography/ Quadrupole Time-of-Flight
167 High-Resolution Mass Spectrometry) method. The UHPLC–MS analysis was performed on a
168 Waters Acquity UHPLC system coupled with a Waters SYNAPT G2-Si High Resolution Mass
169 Spectrometry equipped with electrospray ionization (ESI) source (Waters Corp., Manchester,
170 UK). Chromatographic separation was carried out on an Acquity UHPLC BEH C18 (50 mm ×
171 2.1 mm, 1.7 μm) column at 40°C with a flow rate of 0.6 mL/min. The mobile phase A consisted
172 of water containing 0.1% formic acid, while the mobile phase B was acetonitrile. The linear
173 gradient profile started from 10% B, followed by a linear climb to 90% B over 7 min and held
174 for 2 min, and finally returned to10% B for reequilibration for 2 min. Mass detection was
175 conducted in negative ion mode, with the source temperature at 120°C, capillary voltage and
176 cone voltage were set at 2 KV and 40 V. The desolvation gas was optimized to 900 L/h, the
177 cone gas flow of 50 L/h and the scan range was from 50 to 2500 m/z. Mass was corrected during
178 acquisition using external reference (Lock-Spray) consisting of a 1 ng/μL solution of leucine
179 encephalin at a flow rate of 5 μL/min, in order to make sure the accuracy and reproducibility
180 during the MS analysis. All data collected were acquired using MassLynx™ (V4.1) software
181 in centroid mode.

182 **Statistical analysis**

183 The values given in this report are the means of triplicates, with error bars corresponding to the
184 standard deviation of this mean. Significance was tested with Student's t-test for two-sided
185 distribution and unpaired samples. All statistical calculations were performed on Microsoft
186 Excel.

187 **Nucleotide sequence accession numbers.**

188 The accession numbers of the whole genome of the strain *A.niger* DSM 1957 (VYXZ00000000)
189 was deposited at GenBank.

RESULTS AND DISCUSSION

Defining the parameters of the co-cultures

The growth of the two microbial partners was successfully observed whatever the condition culture used (liquid or solid). For the solid culture type, fungal growth was observed all over the Petri dish after inoculation of the spores in the middle; growth of the bacterial pellet was observed in the same area than the fungal mycelium **(Supplementary Figure 3)**. For the liquid culture type, fungal growth was characterized by the presence of a fungal pellet which could encompass the lignocellulosic substrate and potentially the typical bacterial pellet which confirms that the 2 partners are not antagonistic (**Supplementary Figure 4)**. Concerning the time of inoculation, when the fungi was inoculated prior to the bacteria either 6h, 24h30 or 30H, no growth of the bacteria was observed. On the opposite, when the bacteria was inoculated either in the same time of before compared to the fungi, both were able to develop and grow in the flasks. However, for the later times of the fungal inoculations (24h30 and 30h), a lower amount of fungal mycelium was observed. The results were comparable for a fungal inoculation after 6h or in the same time than the bacterial strains (data not shown). For a practical matter, a simultaneous inoculation was performed.

Genomic comparison of *A.niger* DSM 1957

The strain *A.niger* DSM 1957 was firstly studied by [32] in order to describe the sulfur and trace-element nutrition among one strain of the *A.niger* species. This strain was later described for its ability to express xylanases and cellulases during growth onto different lignocellulosic subtrates [33, 34].

The sequencing of the strain *A.niger* DSM 1957 generated 15452965 reads with an ultrasmall percentage of error (0.03%) with a Q30 of 91.4%. The assembly consisted into 138 (> 1000 bp) with a N_{50} of 544125 bp. The number of N was low in our contigs with a number of N's per 100 kbp of 1.84.

In order to analyze the strain *A.niger* DSM 1957, we compared it to relatively closed genome from the strains *A.niger* An76, *A.niger* ATCC 1015, *A.niger* ATCC 13496, *A.niger* CBS 513.88, *A.niger* CBS 101883 and *A.niger* ATCC 64974 N402. The strains have between 10373 and 13359 genes encoding proteins, the *A.niger* strain CBS 101883 has the most SCP. The length of *A.niger* DSM 1957 was 35.6 Mbp and its GC content was 47% and was in the range of the compared *A.niger* strains.

The genome content was annotated according to their CAZyme content. The diversity of structures, compositions and bonds of components forming lignocellulose has led microorganisms, during evolution, to produce large panels of enzymes capable of degrading it [35]. CAZymes are classified according to the CAZy database into five classes [36]: glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE) and anxilliary activities (AA). GHs (EC 3.2.1.*) hydrolyze the glycosidic bonds between two carbohydrates, or between a carbohydrate and a non-carbohydrate residue. GTs (EC 2.4.*.*) are involved in the biosynthesis of saccharide chains and have debranching activities. PLs (EC 4.2.2.*) mainly cleave bonds between acids and polysaccharides. The CEs catalyze the hydrolysis of the carbohydrate esters. AAs group together enzymes that act on lignins and polysaccharides (LPMO or Lytic Polysaccharides MonoOxygenases) through redox mechanisms. Another class exists, that of proteins consisting in binding modules (CBM or Carbohydrate Binding Module), which are not enzymes, but increase the efficiency of the latter. The strain *A.niger* DSM 1957 has the greatest number of hypothetical CAZymes (581 enzymes), it should be noted that *A.niger* 1957 and *A.niger* ATCC 64974 N402 which nevertheless have a smaller number of coding sequences have the same number of CAZymes. The strain with the best CAZymes /Single-coding proteins ratio is *A.niger* CBS 513.88, its CAZymes representing 5.43% of its total proteins. The strain *A.niger* 1957 has the most AA, CBM, CE and GH numbers among the compared dataset. The number of CAZymes in *A.niger* DSM 1957 was the most important (like in *A.niger* CBS 513.88 and *A.niger* CBS 101883) with a total of 581 CAZymes (**Table 1**).

Table 1: Genomic characteristics and CAZyme content of the *A.niger* DSM 1957 strain compared to other *Aspergillus* strains (GH= Glycoside Hydrolase, CE= Carbohydrate Esterase, CBM= Carbohydrate Binding Module, PL= Polysaccharide lyase, GT= Glycoside Transferase, AA= Auxiliary activity)

Strain	Length (MBp)	Single-coding proteins	CAZymes number	CAZymes (%)	AA	CBM	CE	GH	GT	PL
DSM 1957	35.6	10798	581	5.38	106	17	88	262	99	10
An76	34.6	10373	551	5.31	97	15	86	246	97	10
ATCC 1015	34.9	10950	565	5.16	104	17	81	256	97	10
ATCC 13496	35.7	12194	576	4.72	103	16	86	261	101	9

CBS 513.88	34	10609	576	5.43	104	15	83	249	107	18
CBS 101883	35.9	13359	581	4.35	106	15	88	262	100	10
ATCC 64974	35.5	11236	581	5.17	105	17	87	259	99	14

245

246 Among the 121 panCAZymes families present in the 7 fungal genomes analysed, a large
247 common core was shared between them; indeed 115 families were present among all the strains.
248 The strain *A.niger* DSM 1957 had 119 CAZymes families present. Among those families for
249 that genome (with over 15 occurrences), the most important ones were CE10 (59 enzymes),
250 AA7 (42), AA3 (33), GH13 (21), GH28 (21), GH3 (19), GT2 (18) and GH18 (14). Among the
251 GH13 present, those enzymes encoded for several alpha-amylases and glucanotransferases. The
252 GH28 encoded for several rhamnogalacturonases, exo-xylogalacturonan hydrolases and
253 exopolygalacturonases. The GH3 encoded for β-glucosidases and β-xylosidases. The GH18
254 played a role into chitin degradation. The xylanase activity was carried by the GH10 and GH11.
255 The GT2 were mainly involved in the chitin synthase process. The AA7 encoded for either
256 glucooligosaccharideoxidase, chitooligosaccharide oxidase, cellooligosaccharide
257 dehydrogenase and the AA3 for cellobiose dehydrogenase or glucose 1-oxidase. This result
258 shows that the strain *A.niger* harbours a wide variety of genes encoding for xylanases, endo-
259 glucanases and cellulases.

260 The potential CAZyme secretome of the strain *A.niger* DSM 1957 was studied; 272
261 among the 581 CAZymes were potentially secreted. Among those secreted CAZymes, 51 AA
262 among the 106 were present, 41 CE among the 88 present, 162 GH among the 262 present
263 which represented more than 46% of each family. The PL were all secreted. On the opposite,
264 only 4 GT and 4 CBM among the 99 and 17 are present in the secretome.

265 **<u>Genomic comparison of the actinobacterial strains</u>**

266 **Table** 2: Genomic characteristics and CAZyme content of the actinobacterial strains

	Length (Mbp)	Single-coding proteins	CAZymes number	CAZymes (%)	AA	CBM	CE	GH	GT	PL
S.avermitillis	10.5	10003	335	3,35	20	53	32	149	69	12
S.coelicolor	9.1	8128	336	4,13	15	57	37	156	59	12

S.griseorubens	7.7	6841	255	3,73	15	34	34	115	52	5

267

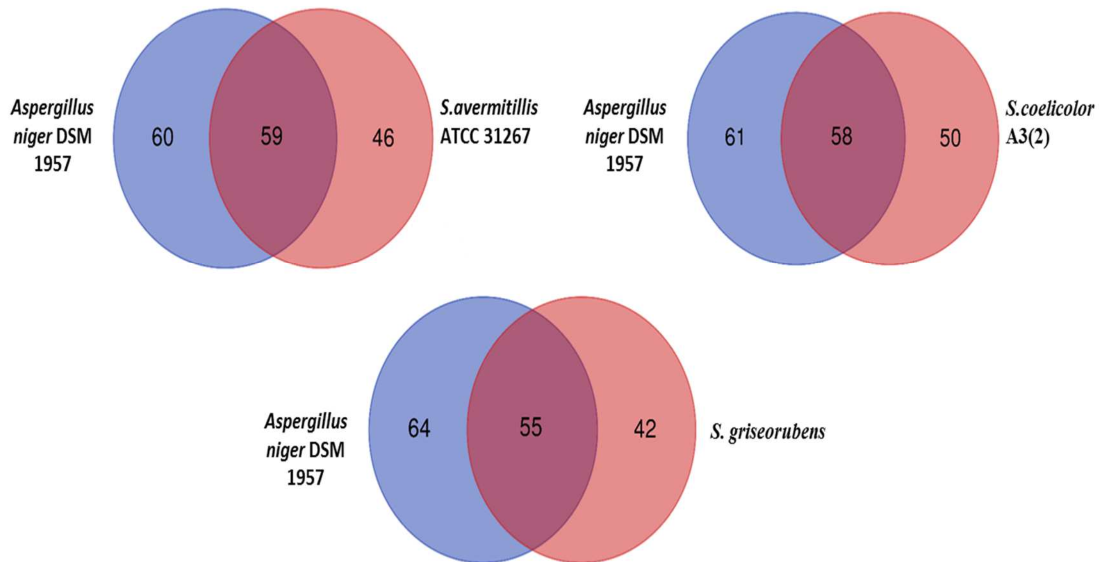
268 In comparison to the *Aspergillus* genome, the actinobacterial strains have a shorter
269 genome. The absolute number of CAZymes was lower compared to the fungal genomes
270 (minimum of 255 and 336 respectively for *S. griseorubens* and *S. avermitilis*) so do their
271 relative abundance in CAZymes (between 3.35 and 4.13%). The main difference between the
272 bacterial and fungal CAZyme content was the higher proportions of CBM (Carbohydrate-
273 Binding-Module) in the bacteria (average of 48 per genome) and fungi (16 per genome) (**Table**
274 **2**).

275 A Venn diagram (**Supplementary Figure 5**) presents the common CAZymes genes that
276 are present among the three *Streptomyces* analyzed. A large common core is shared between
277 the 3 genomes with 80 CAZymes families similar. The strain *S.avermitillis* carried 13 specific
278 CAZymes families which were *S.avermitillis* (AA6, CBM11, CBM50, CBM61, GH110,
279 GH145, GH27, GH53, GH85, GH88, GH89, PL29, PL4). Among the GH CAZymes only
280 present in *S. avermitillis*, the GH53, GH110, GH88 were mainly involved in hemicellulose
281 degradation. For the *S.coelicolor* CAZome, 10 specific CAZymes families were present
282 (CBM12, GH101, GH106, GH117, GH125, GH158, GH50, GH93, PL34, PL6) and involved
283 in the hemicellulose and mannose degradation. The *S.griseorubens* had only 5 specific
284 CAZymes families (GH136, GH81, GH84, GH97, GT84). The *Streptomyces* strains were thus
285 well equipped in order to fractionate lignocellulose.

286 **CAZyme comparison of the co-cultures**

287 A Venn diagram (**Figure 1**) describes the richness and diversity of the CAZymes
288 brought by each organism during the mono and co-culture. To do so, CAZyme families with
289 several iterations (such as the AA7 in *A.niger* which was present 42 times) were only considered
290 once (reducing then the number of CAZymes in the genomes). For the co-culture *S. avermitilis*
291 and *A.niger* DSM 1957, the fungal partner brought 60 unique CAZyme family into the genetic
292 pool, whereas the bacteria partner brought 46; 59 were shared in common by the 2 partners.
293 Similar results were obtained for the 2 other co-cultures. Among the CAZyme families only
294 brought by *A.niger* DSM 1957, AA9 (lytic polysaccharide monooxygenases), AA1 (Laccase-
295 like multicopper oxidase), GH71 (β-1,3-glucanosyltransglycosylase) and GH28
296 (polygalacturonase) were the most represented. Among the CAZyme families only brought by
297 the actinobacteria, AA10 (laccase-like multicopper oxidase), GH23 (chitinase) and GH42 (β-

298 galactosidase) were the most represented. The Venn diagram represents perfectly that diverse
299 CAZymes were carried by each of the microbial partner and thus that the metabolic CAZymes
300 diversity is drastically increased when a co-culture between *Aspergillus* and one of the three
301 actinobacteria is performed.



302

303 **Figure 1**: Venn diagram representation of the CAZyme family content of each co-
304 culture fungi-bacteria performed

305 **Secondary metabolite prediction and production**

306 Among the secondary metabolites predicted by fungal antismash
307 (https://fungismash.secondarymetabolites.org/#!/start), *A.niger* harboured 66 regions encoding
308 for secondary metabolites encoding majoritary for 19 T1PKS, 14 NRPS-like, 3 T1PKS/NRPS
309 and 2 terpene. *A.niger* DSM 1957 was able to produce naphthopyrone, pyranonigrin E, clavaric
310 acid and nidulanin. A majority of the secondary metabolites produced by this strain remains
311 unknown and might represent thus a new source of secondary metabolites.

312 For the actinobacteria, the strains *S. coelicolor* [37], *S. griseorubens* and *S. avermitillis*
313 encoded respectively for 29, 22, 36 regions of secondary metabolite predicted by the Antismash
314 software (https://antismash.secondarymetabolites.org). Among those secondary metabolites,
315 terpenes, NRPS, siderophores, T1PKS and lassopeptides were the most predominant. Among
316 the main secondary metabolites produced, the strain *S.avermitillis* was able to produce,
317 albaflavenone, avermitilol, carotenoid, citrulassin D, ectoine, filipin, geosmin, informatipeptin,
318 melanin, oligomycin and pentalenolactone. The strain *S.coelicolor* was able to produce

319 coelichelin, ectoine, melanin, desferrioxamine B, actinorhodin [38], albaflavenone, curamycin,
320 undecylprodigiosin, geosmin, hopene and germicidin [39]. For the strain *S. griseorubens*,
321 albaflavenone, alkylresorcinol, ectoin, antimycin and rhizomide were produced
322 (**Supplementary Table 1**).

323 After UHPLC/Q-TOF HRMS analysis, a principal component analysis (**Figure 2**) was
324 performed in order to describe the differential secondary metabolite expression by different
325 mono and co-cultures when grown on WB. The results showed that the replicates from each
326 group clustered together showing the reproducibility of the experiment. The groups hardly
327 separate among the principal component axes (PC1 and PC2 axe had percentages of 18.9 and
328 15.3 %). The results of the pcoA strain showed that the actinobacterial strains during their
329 growth on wheat bran separated well from their homolog during their growth on glucose. This
330 result is due do the large number of secondary metabolites produced by those actinobacterial
331 strains and the co-culture *A.niger/S.avermitilis* on wheat bran compared to the other conditions.
332 Indeed, the strain *S.griseorubens* was able to produce between 13 and 18 secondary metabolites
333 when grown on wheat bran; however when grown on glucose, a production of 6 to 8 secondary
334 metabolites was observed. For *S.coelicolor,* between 12 and 16 when grown on WB depending
335 on the replicates; on the opposite, only 8 secondary metabolites were produced. For
336 *S.avermitilis*, 17 secondary metabolites were produced whatever the number of replicate
337 whereas only 2 or 3 were produced were grown when grown on wheat bran. For the *A.niger*
338 strain, only one secondary metabolite was detected when growth was perfomed on glucose
339 whereas 5 were detected whatever the replicate when growth was done on glucose
340 (**Supplementary Table 1**). By comparing those number of secondary metabolites depending
341 on the carbon source, the results showed a lower production when grown on glucose; that result
342 can be due to the carbon catabolite repression which guarantees the sequential utilization of
343 carbon sources when more than one is simultaneously present in the culture media and would
344 thus activate more biosynthetic clusters genes [40]. For the strain *S.coelicolor*, several known
345 compounds were produced and identified by mass spectrometry: coelibactin (m/z= 481, elution
346 time at 3.57), nogalamycin (m/z= 788, elution time at 4.87) and actinorhodin which was clearly
347 detected with a blue pigmentation (m/z= 636, elution time at 3.19). For *S.avermitilis* and
348 *S.griseorubens*, none of the predicted secondary metabolites were identified by mass
349 spectrometry suggesting. For *A.niger*, one compound was produced with a predicted mass
350 identified by mass spectrometry (fumonisin B1, m/z= 722, elution time of 1.1) when grown on
351 wheat bran. The principal components analysis showed that the diversity of secondary

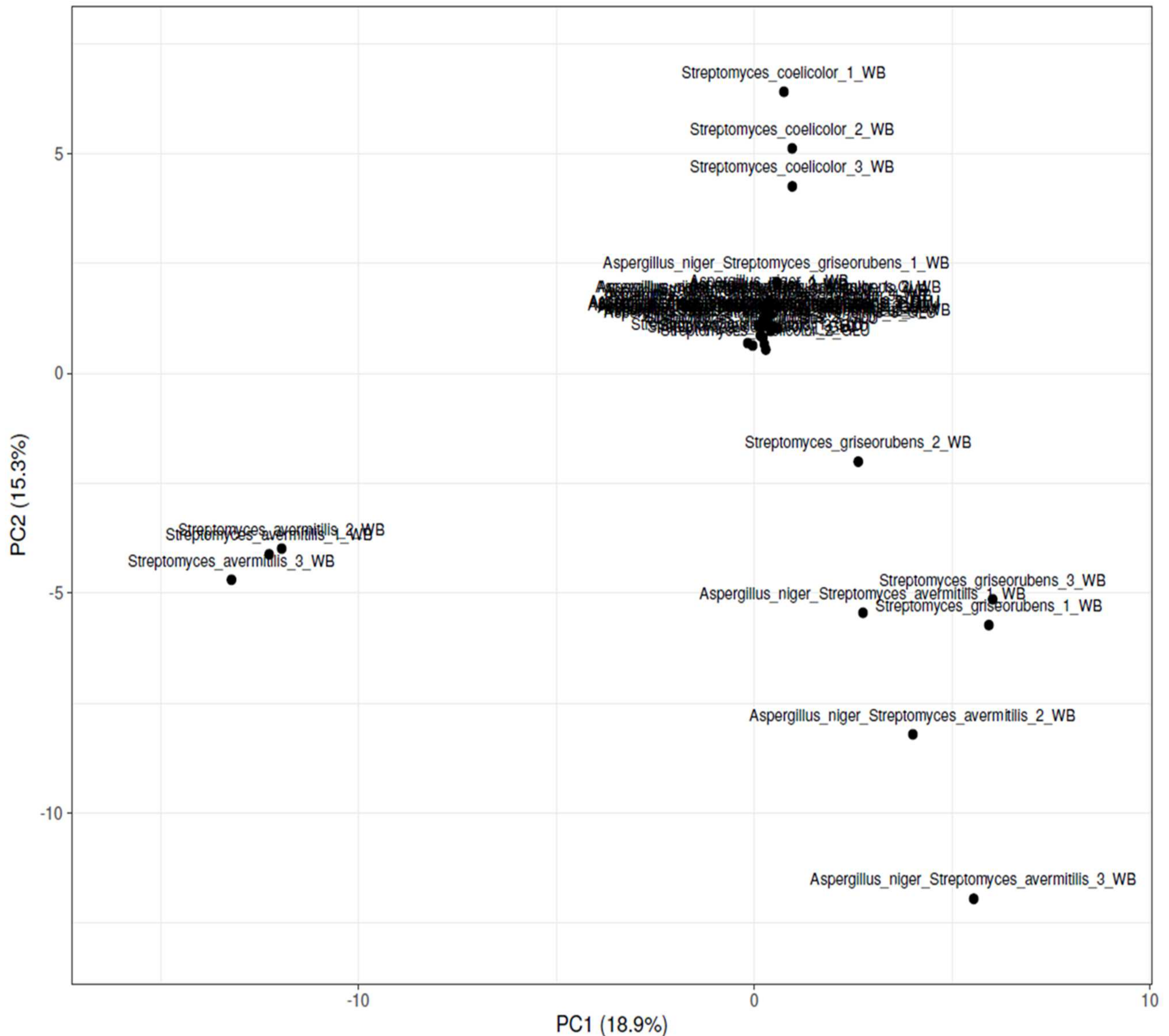
352 metabolites obtained for the different conditions (growth on wheat bran or glucose) is different
353 whatever the mono and co-culture; indeed few secondary metabolites were shared in common
354 during the growth on wheat bran and glucose. Overall, this suggests that some components from
355 WB (carbohydrates, proteins or lignin) could activate some silent biosynthetic cluster genes
356 and will provide a new fingerprint of secondary metabolite production.

357 Regarding the co-cultures, the *A.niger/S.griseorubens* co-culture, the number of
358 secondary metabolites produced varied between 5 and 7 secondary metabolites (on WB) which
359 were lower compared to the number of obtained for each member alone. By comparing with *A.*
360 *niger/S. griseorubens* co-culture on glucose, less secondary metabolites were produced with a
361 different diversity obtained compared to the one on WB. In the *A.niger/S.griseorubens* co-
362 culture, the number of secondary metabolites produced varied between 5 and 7 secondary
363 metabolites (on WB) which is lower compared to the number of obtained for each member
364 alone. By comparing with the same co-culture on glucose, less secondary metabolites were
365 produced with a different diversity obtained compared to the one on WB. The same trend was
366 observed for the *A.niger/S.coelicolor* with a lower number of secondary metabolites produced
367 compared to the growth on wheat bran (3 produced) to glucose (2 produced) and none shared
368 together in the different carbon conditions (**Supplementary Figure 6**).

369 Contrary, the strain *S.avermitilis* was able to produce 17 compounds where grown in
370 mono-culture. In the co-culture *A.niger/S.avermitilis*, 13 compounds in average were produced;
371 2 were in common with the *S.avermitilis* alone (at the elution times of 5.3 and 5.91 min) and
372 none of the ones produced *by A. niger* alone was found in the co-culture. By comparing with *A.*
373 *niger/S. griseorubens* co-culture on glucose, less secondary metabolites were produced with a
374 different diversity obtained compared to the one on WB. Eleven new secondary metabolites
375 which were not present in the mono-cultures were detected in the co-culture; among those
376 eleven new secondary metabolites, only 2 were recovered in the coculture with *A. niger/S.*
377 *avermitilis* grown on glucose which suggests thus an elicitation and activation of silent
378 biosynthetic cluster genes. None of the molar masses detected in the co-cultures were close to
379 the masses of the secondary metabolites predicted by the Antismash [41] and MIBIG algorithms
380 [42] Previous co-cultures of *Aspergillus* fungi and *Streptomyces* showed a suppression of the
381 production of the fungal metabolites [23]. The activation of silent of those silent biosynthetic
382 cluster genes in a second microorganism may be stimulated through microbial crosstalk and
383 may be interpreted as a defense mechanism triggered in response to a chemical signal from the
384 other microorganism [23]

In our study, it is not possible to confirm which of the microbial partners was able to produce those new secondary metabolites in the co-culture. In order to prove which one the microbial partners is able to produce those new secondary metabolites, 1) either a spiking of the supernatant of one of the microbial partner could be added to the culture of the remaining one, 2) or an elicitation with one of the microbial lysate or the microbial cell components [43].

Overall, the results of the metabolomic analysis showed a different relationship and crosstalk between the fungal strain and the different actinobacteria. In summary, an inhibition of the secondary metabolites produced by *S.griseorubens* and *S.coelicolor* was observed when grown with *A.niger* whereas when that fungal strain was grown with *S. avermitilis*, an activation and a possible dual elicitation was observed whatever the carbon source (WB or glucose). However, the diversity of the secondary metabolites tend to be different between the growths on the carbon source type revealing the potential role of lignocellulose as elicitor of biosynthetic cluster genes.



401 **Figure 2**: Principal component analysis of the metabolomics analysis for the different mono
402 and co-cultures when grown on wheat bran (WB) and glucose (GLU).

403 **Lignocellulolytic enzymatic activities during mono and co-cultures**

404 The enzymatic activities were quantified from the mono and co-cultures from wheat
405 bran and not on glucose; indeed, previous preliminary experiments performed at the laboratory
406 showed no lignocellulolytic enzymatic activity produced by the microorganism when grown on
407 glucose on the opposite to wheat bran. Debranching intracellular enzymatic activities were
408 measured for the 3 actinobacterial strains and *A.niger* in mono and co-cultures (**Figure 5**). The
409 results showed that the intracellular β-D-glucosidase and β-D-xylosidase enzymatic activities
410 were the most important with values higher than 1IU/mg of protein; on the opposite, α-L-
411 arabinosidase enzymatic activities were lower and never reached more than 200 mIU/mg of

protein. In all the experiments performed in mono-culture, enzymatic activities were always higher for the fungi compared to the other actinobacteria. The enzymatic activities measured showed different patterns depending on the strain added in the co-culture: 1) more enzymatic activities were detected in the co-cultures with *S.avermitilis* and *S.coelicolor* 2) less enzymatic activities were detected in the co-culture with *S.griseorubens*. Indeed, for the monoculture, intracellular β-D-glucosidase activity was 371.74 ± 3.09 mIU/mg of protein whereas it was lower than 10 mIU/mg of protein for the other actinobacteria; in the co-cultures, β-D-glucosidase activities were 928.7 ± 648.6, 1082.4 ± 812.8 mIU/mg of protein respectively for *A.niger/S.avermitilis* and *A.niger/S.coelicolor*. None of the statistical tests were significant. On the opposite, the β-D-glucosidase activity was lower in the *A.niger/S.griseorubens* co-culture with a value of 205.9 ± 90.9 mIU/mg of protein (p-value<0.05). The same patterns were also observed for the intracellular xylosidase activity: indeed, the activity quantified for the *A.niger* was 365.5 ± 112.3 mIU/mg of protein whereas it was much lower for the other actinobacterial strains. A decrease of the β-D-xylosidase activity was observed for the co-culture *A.niger/S.griseorubens* (303.4 ± 136.9 mIU/mg of protein) and an increase for the others co-culture with respectively 630.3 ± 392.4, 1720.6 ±1309.2 mIU/mg of protein respectively for *A.niger/S.avermitilis* and *A.niger/S.coelicolor*.

Regarding the α-L-arabinosidase activity, the enzymatic activities were less important compared to the β-D-xylosidase and β-D-glucosidase. The differences were less important between the activity produced by the fungi (83.25± 44.25 mIU/mg of protein) and the other actinobacteria respectively (26.9±12.1, 23.9±22.6 and 11.2 ± 2.2 *S.avermitilis, S.griseorubens* and *S.coelicolor* respectively*)* compared to the other activities measured. In the *A.niger/S.avermitilis* and *A.niger/S.coelicolor* co-cultures, the measured α-L-arabinosidase enzymatic activities were 107.7 ± 58 and 100 ±76.5 mIU/mg of protein and were superior to the enzymatic activity obtained for the fungi. On the opposite, the value observed for the *A.niger/S.griseorubens* co-culture was 24.9 ± 15.9 mIU/mg of protein.

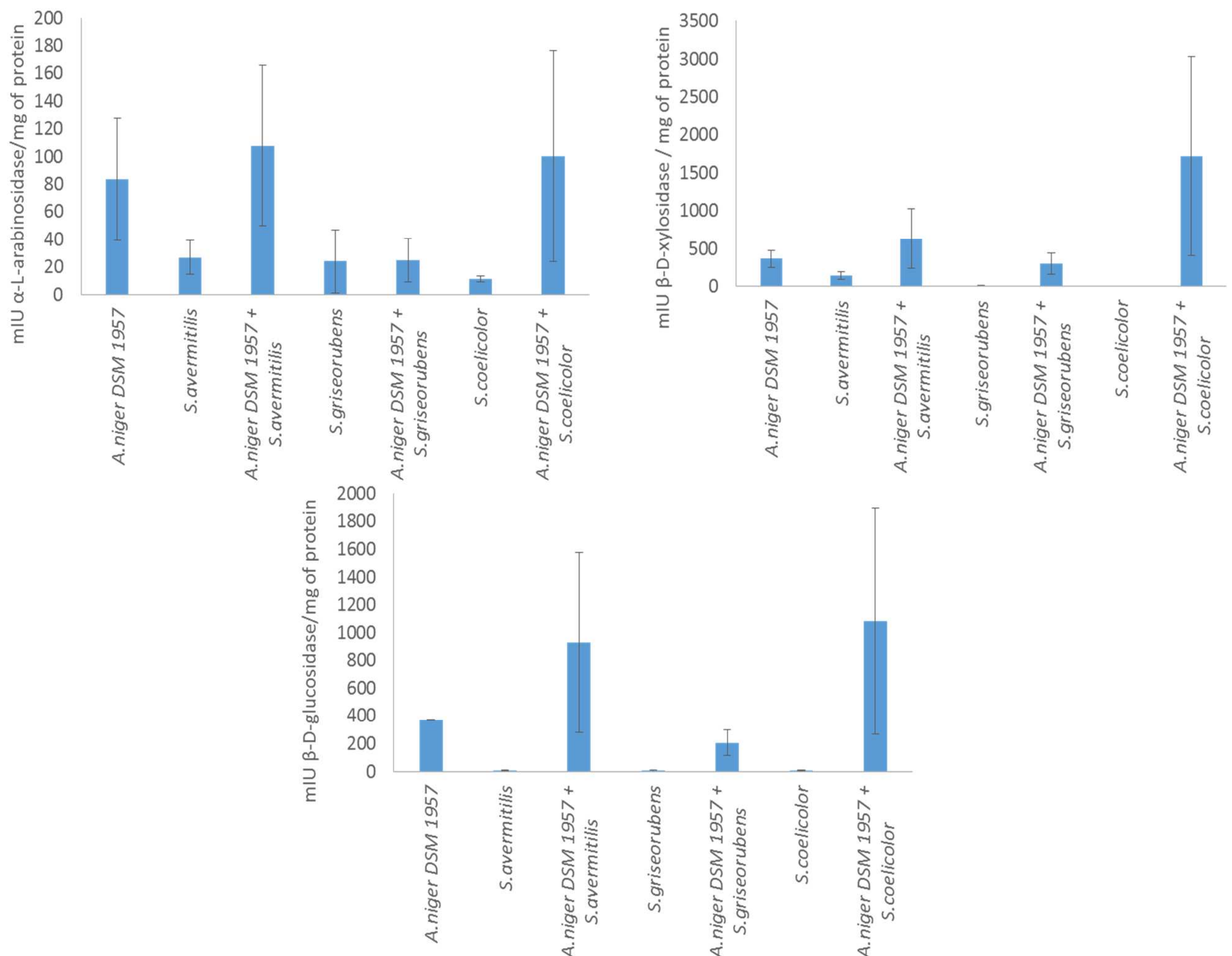
In all the co-cultures (exception of the α-L-arabinosidase for *A.niger/S.avermitilis*) between *A.niger* with *S.avermitilis* or *S.coelicolor,* the enzymatic values obtained were superior to the sum of the enzymatic activity produced by each microbial partner analyzed in its own mono-culture (excepted for the α-L-arabinosidase activity in *A.niger/S.avermitilis*. Indeed, an overproduction up 1351 mIU/mg of protein was observed in the coculture *A.niger/S.coelicolor* (1720.6 mIU/mg of protein) for the xylosidase activity compared to the expected activity by the addition of each microbial partner (365.5 ± 4.07 respectively).

445 For all those debranching activities, extracellular activities were also measured
446 (**Supplementary Figure 7**). For the strains *A.niger* and *S.avermitilis*, elicited enzymatic
447 activities were observed for the arabinosidase and glucosidase. Indeed, the arabinosidase
448 activity was 353 ± 31 mIU/mg of protein in the co-culture whereas they were respectively 7.6
449 and 34 mIU/mg of protein for *S.avermitilis* and *A.niger*. For the glucosidase activity in that
450 same co-culture, the values reached up to 927± 198 mIU/mg of protein in the co-culture whereas
451 they were respectively 97 and 60 mIU/mg of protein for *S.avermitilis* and *A.niger*.

452 For *A.niger/S.coelicolor* coculture*,* higher enzymatic activities were observed for the xylosidase
453 and glucosidase. For the xylosidase activity in that same co-culture, the values drastically
454 reached up to 1610 ± 101 mIU/mg of protein in the co-culture whereas they were respectively
455 9 and 57 mIU/mg of protein for and *A.niger*. A lower elicitation was observed for the
456 glucosidase activity; indeed the activity was 968 ± 400 mIU/mg of protein in the co-culture
457 whereas they were respectively 92 and 60 mIU/mg of protein *S.coelicolor* for *A.niger*.

458 For the *A.niger/S.griseorubens* coculture, a decrease of the enzymatic activities was observed
459 (except for the arabinofuranosidase) overall for the extracellular activities.

460

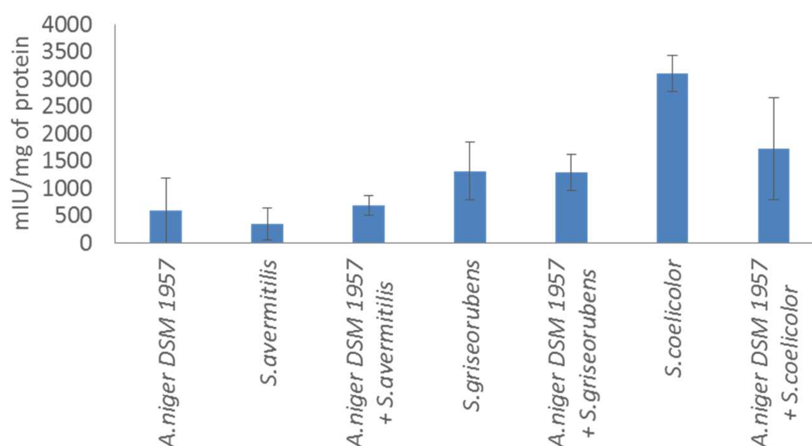


461

462 **Figure 5** : Intracellular debranching activities of the mono and co-cultures at 144 h

463 Intracellular peroxidase activities were also measured during mono and co-cultures (**Figure 6**).
464 The peroxidase activities mainly responsible of the lignin degradation [44]. Peroxidases
465 catalyze the oxidation of lignin in the presence of hydrogen peroxide as electron acceptor and
466 can involve cytochrome c peroxidase [44]. On opposite to the other enzymatic activities before,
467 all the peroxidase payload was not performed majoritary by the fungi but also by the bacteria.
468 Indeed, *A.niger* DSM 1957 showed a peroxidase activity that reached 588 mIU/mg which
469 superior only to the one observed in *S.avermitilis* (347 mIU/mg) whereas higher activities were
470 present for the 2 remaining bacteria which were *S.griseorubens* and *S.coelicolor* (1314 and
471 3091 mIU/mg of protein respectively). Among all the co-cultures tested, no one showed a
472 superior peroxidase activity compared to each microbial partner separately. This could be due
473 to the low abundance of lignin in wheat bran which would explain this absence of difference.
474 The utilization of more lignified agro-resources would maybe allow differences in term of
475 peroxidase activity. A small decrease was observed for the *A.niger/S.avermitilis* and

476 *A.niger/S.griseorubens* co-cultures whereas the decrease was more important for the
477 *A.niger/S.coelicolor* (1726 mIU/mg of protein the co-culture compared to the sum of each
478 microbial partner individually (3680 mIU/mg of protein).



479

480 **Figure 6 :** Intracellular peroxidase activity of the mono and co-cultures at 144 h

481 Xylanases catalyse the hydrolysis of xylans and have been widely studied in filamentous fungi
482 such as Aspergillus [45, 46]. *Aspergillus* xylanases have been [47] widely used in several
483 industrial processes such as paper pulp biobleaching [48]. Waste management programs make
484 use of xylanases so as to hydrolyze xylan found in industrial and municipal wastes [49]. Due to
485 the importance of xylanase in the lignocellulolytic, secretion system present in *Aspergillus*, a
486 dynamic study of the extracellular xylanase activity (at 48, 96 and 144 h) was performed on the
487 compared to the other enzymatic activities (**Figure 7**). The results showed that xylanolytic
488 activity load was carried by *A.niger* and not by the *Streptomyces* bacteria; indeed, the maximum
489 xylanase activity for one of the *Streptomyces* member was at 144 h for *S.coelicolor* (3513 ± 980
490 mIU/mg of protein). For *A.niger*, xylanolytic activity increased continuously from 48 h to 144
491 h reaching up 49251 ± 28763 mIU/mg of protein. The same increasing trend was observed for
492 the other actinobacteria through time.

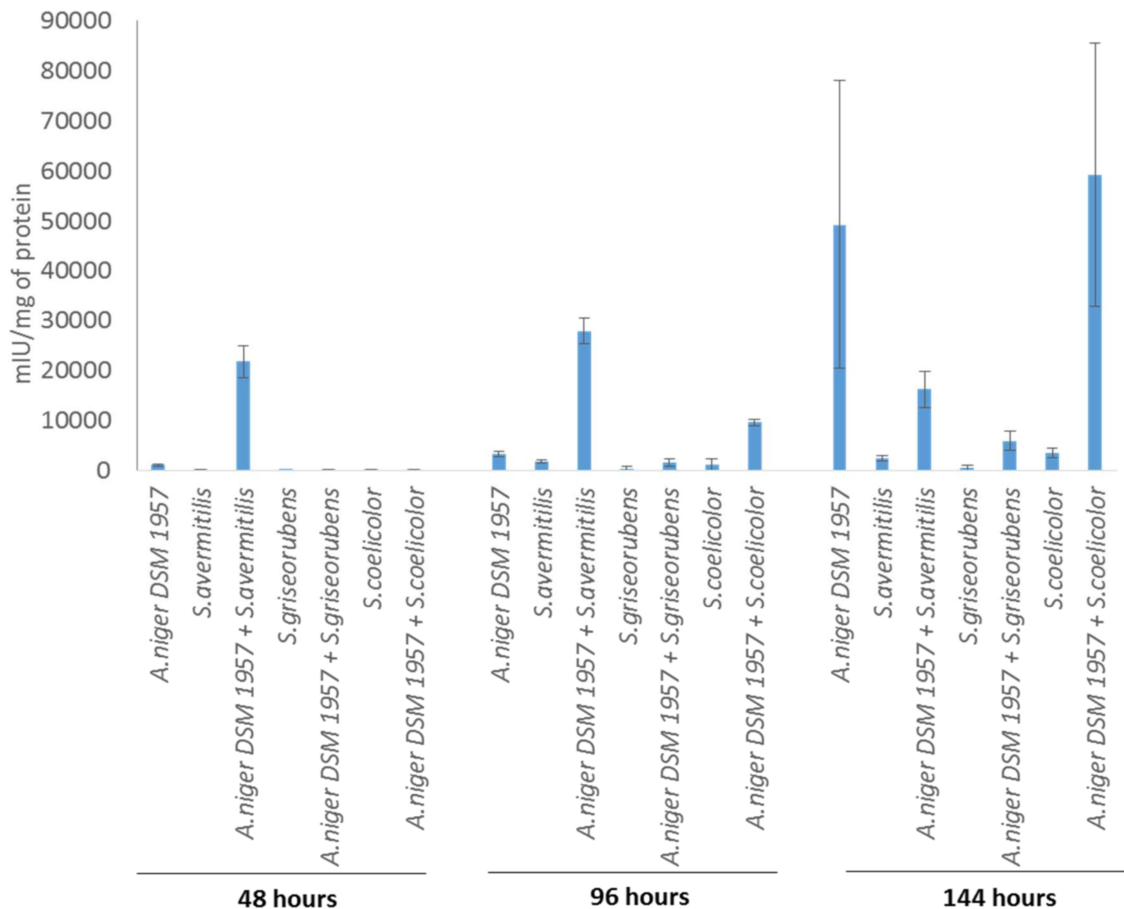
493 The trend of dynamic xylanalytic activity of the co-culture *A.niger/S.griseorubens* confirmed
494 the trends obtained from the others enzymatic activities depicted previously. Indeed, the
495 enzymatic activity was 3.5 more time less important for the co-culture *A.niger/S.griseorubens*
496 compared to the mono-culture of *A.niger* after 144 h.

497 On the opposite, for the other actinobacterial strains, an increase factor up to 147% and 229%
498 was found between *A.niger* and the co-cultures*, A.niger/S.avermitilis* and *A.niger/S.coelicolor*
499 respectively. A different dynamic was observed between those two previous co-cultures; 1)

500 indeed, the maximum xylanase activity was observed for *A.niger/S.avermitilis* was already
501 important at 48h reaching 21804 ± 3265 mIU/mg (then slowly increased at 96h and decreased
502 at 144h), 2) on the opposite, the xylanalytic activity of *A.niger/S.coelicolor* was low at 48 and
503 96 h but reached a peak at 144h up to 59226 ± 26239 mIU/mg. The dynamic lignocellulolytic
504 enzymatic activity was studied for all the co-cultures and showed that through time the xylanase
505 was always over-expressed compared to the other enzymatic activities. Xylanase activity is thus
506 necessary through all the growth and xylan represents consequently the main carbon and energy
507 source of the microbial partners. Those results are correlated to previous studies regarding the
508 dynamic secretion of *A.niger* An-76 where the expression of xylanase was detected on
509 hydrolysates of lignocellulose polysaccharide at 24h of inoculation until the end of growth
510 (144h) [50]. Debranching activities were detected at the final time point signifying that these
511 enzymes play a crucial role for the hydrolysis of the oligosaccharides produced by the xylanases
512 and other endo-enzymes. Overall, for all the enzymatic activities measured in that manuscript,
513 some standard deviation could be due to the growth type of the microbial partners involved in
514 those co-cultures. Indeed, those microorganisms form pellets of different size with different
515 amount of cells and subsequent protein content. Despite those standard deviations, all the results
516 confirm the different patterns where elicitation is observed between *A.niger* and *S.coelicolor* or
517 *S. avermitilis* and a possible inhibition between *A.niger* and *S.griseorubens*. The co-culture
518 growth observation was only conducted in a qualitative manner visually by assessing the
519 presence of the two microbial partners depending on their morphological shape. The main point
520 of our study was to describe the possible elicitation of enzymatic activities and production of
521 secondary metabolites at a final time point when grown on lignocellulose. It is acknowledged
522 that the metabolic profiles into a co-culture will depend on the distribution of the two partners
523 [51, 52]. The morphological patterns of the two microbial partners which form pellets do not
524 allow a spectrophotometric quantification and thus as future experiments, we will develop an
525 approach in follow-up studies about the metabolomic profiles obtained in those co-cultures at
526 different sampling points allowing to generate data for both microorganisms. Here, the
527 enzymatic activities were analyzed as mIU/mg of protein in order to normalize throughout the
528 different samples (through time and the different consortia). The similar enzymatic activities
529 and metabolomics profiles obtained showed that the experiments were reproducible and that
530 the distribution was comparable among the replicates. In our experiment, the precise
531 mechanism behind that microbial interaction is not clear and has not been investigated. The
532 activation of those enzymatic activities can be due to several hypothesis: the presence of
533 signaling molecules or direct contact between the two microbial partners. In the case of

534 signaling molecules, the activation of enzymatic activities in *Aspergillus* can be due to several
535 factors ; indeed the activation can be due to the presence of different types of molecule : 1)
536 previous study revealed indeed that 3 mM Cu^{2+} supplementation in recombined xylanase *A.*
537 *niger* US368 enhanced its activity by 54% [53]; 2) the presence of secondary microbial
538 metabolites which can over-express xylanase activity by 40% [54]. For the direct contact,
539 previous experiments proved that this was necessary between bacteria/fungi to observe
540 activation of cryptic metabolic pathways [55]. In order to prove that hypothesis of signaling
541 molecules, further experiments could be performed 1) either by spiking only the secondary
542 metabolites produced by the co-cultures fungi/actinobacteria to another fungal culture the fungi
543 is grown alone, 2) use membrane reactors which would only allow the transfer of the secondary
544 metabolites to each partner without direct contact between them. An accurate description of
545 those molecules will be performed in the future which could be used for the improvement of
546 fractionation by other microorganisms into other biotechnological processes by simple spike.
547 Those activators which would improve lignocellulolytic activities would be then ready-to-use
548 and less expensive compared to other approaches involving genome editing per example [56].
549 Overall, the results showed that the consortia tend to have a hemicellulolytic strategy compared
550 to the cellulolytic one. This is related to the chemical structure and diversity of wheat bran
551 which is mainly constituted of arabinoxylans (16 % DM of arabinose and 26 % DM of xylose)
552 compared to cellulose (19 % DM of glucose) which could hypothesis that more energy and
553 carbon source could be available for the consortia from the hemicellulose and notably the
554 arabinoxylans. Moreover, the cellulose degradation pathway requires the expression of more
555 enzymes in *A.niger* (the main degrader in our consortia) compared to the xylan degradation
556 pathway which could thus represent an energy save in the fungi metabolism [57].

557



558 **Figure 7**: Dynamic secreted xylanalytic activity of the mono and co-cultures at 48, 96 and 144
559 h

560 The obtained results in that study are promising with: 1) an easy set-up of the co-cultures from
561 2 different microorganisms from different domains in the "Tree of life" resulting into a greater
562 and more diverse metabolic and lignocellulolytic content, 2) an over expression of several key
563 enzymatic activities, 3) an elicitation of some specific biosynthetic cluster genes observed only
564 in the co-culture experiment, 4) a specific microbial crosstalk and interaction observed at the
565 species level between the 3 *Streptomyces* and the fungi leading to a specific of lignocellulolytic
566 enzyme and secondary metabolite production. Further experiments will be performed in order
567 to: 1) decipher the regulatory and expression mechanisms at the gene level over-expressed in
568 the co-culture, 2) describe the interaction type (chemical or physical) between the 2 microbial
569 partners, 3) identify the secondary metabolites produced during the co-culture experiments.

570 **CONFLICTS OF INTEREST**

571 The authors declare that there are no conflicts of interest.

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577 # AUTHOR STATEMENT

578 Authors : Julian Detain (JD), Caroline Rémond (CR), Carine Machado Rodrigues (CMR),
579 Dominique Harakat (DH), Ludovic Besaury (LB)

580 Conception and design of study: LB,

581 Acquisition of data: JD, CMR, DH, LB

582 Analysis and/or interpretation of data: JD, CMR, DH, LB

583 Drafting the manuscript: DH, LB

584 Revising the manuscript critically for important intellectual content: CR, CMR, DH, LB

585 Approval of the version of the manuscript to be published (the names of all authors must be
586 listed): Julian Detain (JD), Caroline Rémond (CR), Carine Machado Rodrigues (CMR),
587 Dominique Harakat (DH), Ludovic Besaury (LB)

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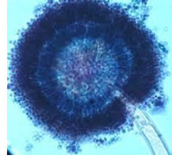
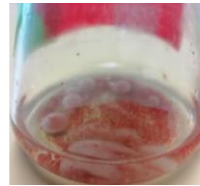
Aspergillus niger DSM 1957



Streptomyces spp.

Co-culture = Increase of lignocellulolytic metabolic potential



Set-up of co-culture on lignocellulosic biomass

Elicitation of lignocellulytic enzymatic activities and lignocellulose fractionation

Elicitation of production of new secondary metabolites