

Whole-cell biosensor of cellobiose and application to wood decay detection

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- 1 Research article
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- 3 Whole-cell biosensor of cellobiose and application to wood decay detection
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- 29

31 Abstract

Fungal biodegradation of wood is one of the main threats regarding its use as a material. So 32 far, the detection of this decaying process is empirically assessed by loss of mass, when the 33 fungal attack is advanced and woody structure already damaged. Being able to detect fungal 34 35 attack on wood in earlier steps is thus of special interest for the wood economy. In this aim, 36 we designed here a new diagnostic tool for wood degradation detection based on the bacterial whole-cell biosensor technology. It was designed in diverting the soil bacteria 37 Streptomyces CebR sensor system devoted to cellobiose detection, a cellulolytic degradation 38 39 by-product emitted by lignolytic fungi since the onset of wood decaying process. The conserved regulation scheme of the CebR system among Streptomyces allowed constructing 40 a molecular tool easily transferable in different strains or species and enabling the screen for 41 optimal host strains for cellobiose detection. The selected biosensor was able to detect 42 43 specifically cellobiose at concentrations similar to those measured in decaying wood and in a spruce leachate attacked by a lignolytic fungus, indicating a high potential of applicability to 44 45 detect ongoing wood decay process.

47 **1.** <u>Introduction</u>

Thanks to its intrinsic mechanical properties, wood is used since the Neolithic era as a 48 49 material for construction, furnishing, heating or wood-derived products such as paper. 50 Nowadays, there is also a regain of interest for this material as it is renewable and a source 51 of biomass for conversion into bio-ethanol [1]. As a material, wood is highly resistant to the 52 environmental conditions such as rain, sun or other abiotic stresses and the main threat 53 regarding its use comes mostly from the attack by lignolytic organisms. This wood biodegradation represents a major economic problem. For instance, in the US only, it costs 54 more than 5 billion USD each year to homeowners and almost 10% of the annual product of 55 forest is used to replace the degraded products [2]. Being able to detect the early wood bio-56 57 degradation is thus crucial. During storage, it would avoid to use contaminated wood for 58 construction. When the wood is already used as a material it would enable to apply early 59 curative measures before any significant loss of mechanical properties. However, to our 60 knowledge, there is no tool available to achieve this goal and wood degradation is so far only assessed by resistance testing and visual expertise only relevant and operating on wood 61 decayed at an advanced level. 62

63 Wood bio-degradation in forest ecosystems is mainly the fact of diverse fungi [3] classified 64 according to the type of decay they cause (e.g. white-rots, brown-rots, soft-rots, see for 65 review [4]). If rot-fungi have distinct specificities in term of ecology or degradation strategies, 66 they nevertheless all degrade and use as a carbon source the cellulose that represents 40% to 50% of the plant dry weight [5]. Two main degradation strategies exist during the wood 67 degradation by fungi: enzymatic pathways involving cellulases or oxidative mechanisms 68 69 using Fenton reaction and polysaccharide monooxygenases [6,7]. However, a canonical 70 degradation pathway is common for all of them where the cellulose fiber is shortened into simpler forms: the cellobiose (two β -1,4 linked glucose units) or sometimes into 71 72 cellodextrines (generally from three to six β -1,4-linked glucose units) [7,8]. These cellulose degradation by-products will be hydrolyzed later on into glucose by the action of β -73 74 glucosidases [8]. Thus, the initial release and presence of cellobiose and cellodextrines is a 75 common denominator and can be considered as a signature of the attack of wood by rot-76 fungi.

The *Streptomyces* are filamentous spore forming soil dwelling bacteria that are generally not able to degrade native wood, but are considered as essential to recycle biomass polymers in environment thanks to their large-enzymatic arsenal able to degrade wood by-products [9,10]. The detection of these compounds and the activation of the enzymatic pool to degrade them has been linked to the regulator CebR of the Lacl family in *Streptomyces*

griseus [11], Streptomyces reticuli [12], Streptomyces sp. ActE [13] and Streptomyces 82 scabies [14]. Since its characterization by Schlösser et al. [12], and Marushima et al. [11], it 83 is known that the CebR transcriptional repressor prevents gene expression from binding a 84 85 conserved 22 bp hairpin motif (cebR-box) found in the transcriptional region of its targets. In presence of inducer molecules such as cellobiose or in some cases cellodextrines or 86 cellulose, the CebR repression is alleviated and enables the expression of the controlled 87 88 genes. So far, the complete CebR regulome is not well-known. However, a transcriptomic analysis of the Streptomyces sp. ActE (Sirex) (a symbiotic strain that helps the pine-boring 89 90 woodwasp Sirex noctilio to deconstruct wood biomass) has shown that, in presence of wood 91 derived compounds, the most up-regulated genes were under the control of the CebR-92 system. These genes were mostly involved in the uptake (ABC transporter system) or in the production of cellulolytic and hemicellulolytic enzymes (β-glucosidases, cellulases, 93 cellobiohydrolases, mannosidases) [13]. Moreover, CebR could induce other functions like 94 the pathogenic factors of S. scabies in presence of cellobiose [15]. 95

Since cellobiose is a key product of cellulose hydrolysis and indirectly indicates fungal wood degradation, we designed and developed a *cebR*-box based biosensor expressed by *Streptomyces* in order to detect the presence of cellobiose, demonstrated its sensibility and specificity and showed its applicability in a case of wood degradation detection.

100

101 2. Material and Methods

102 2.1) Plasmids, strains and media

103 Plasmids and strains used in this work are presented in Table 1. Escherichia coli strains were grown in LB medium [16] at 37°C and Streptomyces at 30°C either on solid SFM 104 medium [20 g mannitol, 20 g soy flour and 20 g bacto-agar per liter] or in liquid modified HT 105 106 medium (HT*) [1 g yeast extract, 1 g beef extract, 5 g mannitol, 2 g bacto-tryptone and 0.02 g COCL₂ per liter, pH=7.3]. For the biosensor tests, cellobiose (Alfa Aesar, Karlsruhe, 107 108 Germany) and cellodextrines (Elicityl, Crolles, France) were added after autoclaving in liquid modified HT at the appropriate concentration. Avicel Ph-101 (Sigma, St. Louis, USA), a 109 crystalline form of cellulose, was added in liquid medium before autoclaving. Media were 110 111 supplemented with apramycin (50 µg.ml⁻¹) for the selection of strains transformed with the pIB139 derived plasmids or with ampicilline (100 µg.ml⁻¹) for the selection of the E. coli 112 transformed with pGemT-easy (Promega, Madison, USA). 113

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2.2) Wood infection, leachate preparation and cellobiose quantification

Wood degradation was performed according the European standard EN 113, 1996 [17] with 118 some modifications. Spruce (Picea abies L.) wood specimens (40x15x5 mm) were sterilized 119 120 at 103°C for 2 days. Then wood specimens were placed under sterile conditions in petri dishes containing one-week old mycelium of the lignolytic Poria placenta fungus. Wood 121 specimens were incubated at 22°C and 70 % relative humidity for 16 weeks. For leachate 122 preparation, *P. placenta* mycelium was carefully removed from the wood by hand scratching. 123 Eight wood specimens were impregnated under vacuum with 100 ml of PBS buffer pH 7.4 for 124 15 min. For cellobiose quantification, 20 µl of leachate were run on HPLC equipped with a 125 refractometer. Sugars were separated on a Shodex sugar KS-803 column (Waters SAS, 126 Guyancourt, France) at 80°C with a flow of 0.8 mL.min⁻¹ using HPLC grade water as solvent. 127 128 Cellobiose and glucose were detected after 12.4 min and 13.2 min of elution, respectively. 129 The cellobiose concentration present in the leachate was estimated by peak area integration and comparison with a cellobiose standard calibration curve. The cellobiose calibration curve 130 ranged from 2.92 mM to 29.3 µM. Specificity and detection of free glucose resulting from 131 wood cell wall degradation was detected by comparison with a calibration curve build from 132 133 glucose concentrations ranging from 5.56 mM to 55.6 µM.

134 2.3) DNA manipulations

Polymerase chain reactions were performed with the Dream Taq polymerase 135 (ThermoFisher Scientific, Waltham, USA) for fragments under 1.5 kb in size or Taq 136 137 polymerase Takara (Takara Bio Inc., Kusatsu, Japan) for larger fragments. PCR primers are 138 listed in Table S1. Ligation reactions were carried out with T4 DNA ligase (ThermoFisher 139 Scientific, Waltham, USA). DNA was digested and purified from gel matrix respectively with 140 restriction enzymes and the GeneJet Gel Extraction Kit purchased from ThermoFisher Scientific (Waltham, USA). The kits were used according to supplier's recommendations. 141 Alkaline lysis plasmid extractions, plasmid electroporations and DNA extractions were 142 performed as described in [18]. 143

144 2.4) Functional characterization of biosensors

Functional screenings of the biosensors efficiency were performed by streaking of 2 μ L of spore suspension (ca 10⁸-10⁹ spores/mL) of each biosensor on HT* plates supplemented with 5 g.L⁻¹ of cellobiose and incubation at 30°C for 2 days. Controls without cellobiose or with crystalline cellulose (Avicel) were performed in parallel. After growth, plates were sprayed with a 0.5 M catechol solution and incubated for 10 to 20 min in the dark. The visualization of a yellow color in the mycelium and in its surrounding medium attested the detection of cellobiose by the biosensor after confrontation with the control plates *i.e.* without cellobiose and supplemented with crystalline cellulose. A positive result in both latter cases would constitute false positives.

154 2.5) Biosensor microplate tests

Spore suspensions of the Streptomyces S4N27 biosensor strain were realized on SFM 155 complemented with apramycin [16] and stored in a 20% (v/v) glycerol solution at -20°C. To 156 perform biosensor qualitative and quantitative tests in presence of cellobiose, cellodextrines 157 or wood leachates, 5.10⁵ spores were inoculated into 3 ml of modified HT medium cultures 158 complemented with the sugars at the selected concentrations or with 1 ml of wood leachate. 159 Cultures were incubated at 30°C for 16-18 hours under agitation (200 rpm). Cells were 160 161 harvested by centrifugation at 4,500 rpm for 5 min, washed with 1 ml of 20 mM phosphate buffer pH 7.2 and suspended in 1 ml of sample buffer (100 mM phosphate buffer pH 7.5, 20 162 mM NaEDTA pH 8, 0.1% Triton X100, 10% acetone [v/v]). Cells were then lysed on ice by 163 164 sonication (three cycles of 20 s in Bioruptor Standard device, Diagenode, New Orleans, 165 USA). The cell lysate was harvested by centrifugation for 6 min at 7,000 rpm at 4°C and 20 µl 166 were mixed with 300 µl of assay buffer (100 mM phosphate buffer pH 7.5, 0.2 mM cathecol) 167 previously warmed at 30°C. Assay buffer was prepared just before use by adding the catechol from a 20 mM stock solution (in water) to the pre-warmed phosphate buffer [19]. 168

169 The yellow catechol degradation signal resulting from XyIE activity was quantified by 170 spectrometry at OD 595 nm (Bioteck, Winooski, USA). The catechol dioxygenase activity 171 was determined and normalized at 375 nm per min per milligram of protein and converted to 172 milliunits per milligram [16,19]. The protein concentration was estimated by the method of Bradford [20] by using bovine serum albumin as a standard. For each condition, experiments 173 were realized with at least three repeats. The result of each experiment was expressed as 174 the expression of XyIE in the assay relative to that observed in the controls (*i.e.*, in the 175 176 absence of cellobiose) systematically run in parallel. The significance of the relative signal of the assay versus the controls was statistically assessed with a *t*-student test. 177

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182 <u>3. Results</u>

183 3.1 In silico analysis to assess the potential of Streptomyces as biosensor 184 hosts

CebR prevents gene transcription in binding a specific sequence called the cebR-box and 185 186 this repression is alleviated by the recognition of the inducer molecules, *i.e.* the cellobiose 187 (Figure 1A). The CebR sensor system has been previously found and reported as a highly 188 conserved regulatory system in some Streptomyces species [11-13], notably in term of sequence identity of the cebR-box. The concept of the biosensor was to build a generic 189 190 biosensor cassette that could be regulated in different genetic backgrounds, *i.e.* in 191 Streptomyces strains potentially exhibiting different specificities or sensitivities towards the 192 target molecules. In order to assess the range of utilization of this construction within the 193 Streptomyces genus we investigated the distribution of the CebR system within the 42 194 available fully sequenced genomes. BlastP searches (NCBI) were done using as query the 195 CebR sequence of the fully characterized Streptomyces reticuli system CebR (CUW28692) [12] with a cut-off of 50% of identity in amino acid. Conserved regulatory motif 196 197 TGGGAGCGCTCCCA the so-called "cebR-box" was also searched. All genomes but five (Streptomyces albulus NK660, Streptomyces albulus ZPM, Streptomyces albus DSM41398 198 and Streptomyces sp. 769, Streptomyces lydicus A02) possessed of at least one CebR 199 200 homologue with amino acid identity ranging from 63% to 96% and the cebR-box motif as well 201 as conserved regulatory *cebR*-boxes. This analysis showed that the CebR-system is 202 widespread in this genus and conserved enough to recognize similar cebR-boxes. 203 Consequently, most of the *Streptomyces* strains (including presumably environmental ones) 204 are likely to be able to regulate a construction based on a conserved *cebR*-box through their 205 endogenous CebR protein and could be turned into biosensors.

206 207

3.2 Construction of the biosensor

The biosensor design is depicted in Figure 1B. Briefly, the concept consists to put a reporter gene under the transcriptional control of CebR. The *xyIE* gene from *Pseudomonas putida* which was successfully used in *Streptomyces* was chosen [16,19]. It codes for a catechol dioxygenase that converts the colorless substrate catechol to an intensely yellow hydroxymuconic semialdehyde. The genomic DNA of *Streptomyces coelicolor* KC900 which includes a *xyIE* gene was used as a PCR template with the primer couple consisting of the forward Biosens_F and the reverse Biosens_R primers (Table S1, Figure 1B.I). The

Biosens_F primer sequence includes a *cebR*-box and a ribosome binding site (RBS). The 215 amplification thus places the cebR-box and a RBS site upstream of the coding region of xy/E 216 (Figure 1B.I). The 955 bp PCR product was subcloned in the pGEM-T Easy vector, 217 introduced in *E. coli* DH5a and its sequence checked by sequencing. After releasing from 218 pGEM-T Easy by a double restriction digestion *Bam*HI-*Eco*RI present in the Biosens_F and 219 Biosens_R primer sequences respectively. This DNA fragment was further cloned 220 directionally into the multicloning site of the pIB139 vector also digested with the same 221 222 couple of endonucleases (Figure 1B.II). This cloning step resulted in the positioning of the 223 xy/E gene downstream the strong constitutive promoter PermE* [21,22]. This construct could 224 then be mobilized into *Streptomyces* cells by intergeneric conjugation using the intermediary 225 of E. coli S.17 host strain. The vector pIB139 was chosen for its site-specific integration 226 system targeting the bacteriophage Φ C31 attachment site [16]. The integration into the chromosome secures the stability of the construct. A single copy is expected to be inserted. 227

228 Nine Streptomyces species were chosen as receptors and the insertion of the construct was 229 selected by the presence of apramycin in the culture medium. The absence of endogenous 230 catechol dioxygenase activity was checked on plate in presence of catechol which is the XyIE substrate (data not shown). Three of them were reference strains (Streptomyces 231 ambofaciens ATCC 23877, Streptomyces coelicolor M145 and Streptomyces lividans TK23). 232 The seven others were environmental strains (Streptomyces sp. S9N29, S2N2, S6N6, 233 S9N14, S4N22, S1N3 and S4N27). These strains were chosen for their cellulolytic properties 234 and their representativeness of the taxonomic diversity of a forest soil [9]. The presence of 235 236 CebR was expected and highly likely considering the wide distribution of the CebR system in sequenced genomes (see above). After conjugation with E. coli S17.1 harboring the 237 biosensor construct, Streptomyces transconjugants were selected on SFM plates with 238 apramycin and nalidixic acid which counter selects E. coli. For each Streptomyces strain, 239 240 eight transconjugants were selected. The integration into the Φ C31 attachment site was 241 checked by PCR for S. ambofaciens ATCC 23877. For environmental strains, the insertion of 242 the construct was checked by PCR amplification of the xylE gene (data not shown).

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3.3 Screening for optimal host strain for the biosensor

In order to confirm the efficiency of the constructed strains as biosensors, they were functionally tested for their ability to emit a yellow color on plates complemented with cellobiose (5 g.L⁻¹) as an inducer molecule. In comparison with plates without cellobiose, all transformed clones for 5 strains (*S. ambofaciens* ATCC 23877, *S. coelicolor* M145, *S.lividans* TK24, *Streptomyces* S4N27 and *Streptomyces* S6N6) exhibited a bright yellow coloration of mycelium and in the surrounding solid medium, indicating that the cellobiose was able to

unlock the CebR repression and to activate the expression of xy/E. In order to choose the 250 optimal host strain among them, several screenings were performed (Table S2) and the 251 252 strain S4N27 was selected for the following reasons. First, it visually exhibited the highest yellow color on plate (Figure 2) suggesting an optimal production of the XylE protein. 253 254 Secondly, no detection signal was obtained in presence of crystalline cellulose (Avicel), indicating that the presence of none-degraded cellulose could not lead to false positive. 255 256 Further specificity and sensitivity experiments were carried out on mycelium grown in liquid 257 medium in order to ensure quantitative and reproducible assays. For that purpose, mycelium 258 samples were sonicated to release the intracellular XyIE content. Consequently, another 259 selection criterion was the ability to lyse the mycelium. One of our candidate environmental 260 Streptomyces strain, S4N27, was typified by a planktonic growth mode while other species grew as typical dense mycelium pellets difficult to lyse. For that reason, we finally selected 261 strain S4N27 as the best host biosensor for further tests (Table S2). 262

263

3.4 Sensitivity and specificity of cellobiose detection

Tests were performed in liquid cultures to enable the quantification of the XyIE activity of the 264 biosensor S4N27 in presence of cellobiose. Its sensitivity was tested with cellobiose 265 266 concentrations ranging from 1.5 µM to 15 mM and after 10 min of reaction. Controls without 267 cellobiose were performed for each experiment and exhibited a background signal 268 presumably corresponding to the basal xy/E expression from the construction as no 269 endogenous dioxygenase activity was observed in wild-type strains devoided to the 270 construction. As the latter signal was equivalent in all controls, the xylE activity of the assays 271 was expressed as a relative expression of this baseline and did not raise an issue regarding the interpretations of the results. A significant detection was observed starting from 15 µM of 272 cellobiose (equivalent to 5 mg.L⁻¹ concentration). This is in the same range than a leachate 273 produced from spruce wood in degradation (see below) as measured by HPLC. However, no 274 275 significant signal could be detected at 1.5 µM (Figure 3). An increasing signal was observed in response to cellobiose concentration to reach a plateau from 80 µM. The stability of the 276 277 colorimetric reaction was assessed every two minutes for 1 h at 80 µM of cellobiose. Since 4 minutes and until the end of the experiment, all the measured intensity ratios were statically 278 279 identical with a value close to 2.4 that showed there was no signal loss over time (data not shown). The chemical reaction was thus stable during this lap of time and biochemical 280 instability of the reaction could not technically impair the liability of the measures. 281

At the same 80 µM concentration of cellodextrines (triose, tetraose, pentaose and hexose),
no signal could be observed (data not shown) showing that cellobiose was the specific signal
sensed by the CebR system in the S4N27 strain.

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287 3.5 Application of the biosensor for wood degradation detection.

288 In order to test whether our biosensor was able to detect the presence of cellobiose in a context of wood degradation, spruce sticks were infected by the lignolytic fungi Poria 289 placenta during a period of 7 weeks to allow an effective fungal attack. Wood leachates were 290 291 extracted and tested on the biosensor along with control leachates collected from uninfected 292 spruce sticks. The xylE expression for infected spruce was 1.8 relative to the control in the 293 same range than the ratio observed using pure cellobiose as inducer (Figure 4). The 294 cellobiose presence was checked and its concentration estimated in leachates by HPLC. While no cellobiose was detected in the uninfected control, a concentration of 15 µM was 295 measured in the infected wood. This result confirmed that the biosensor was able to detect a 296 low cellobiose concentration in a complex wood leachate and consequently enabled the 297 298 detection of the fungal decaying process.

300 <u>4. Discussion</u>

301 The aim of this study was to create a biosensor dedicated to cellobiose detection. We based 302 its construction on a microbial cellobiose sensor: the Streptomyces CebR system. Since, its 303 characterization [10,11], it is known that the CebR transcriptional repressor prevents gene 304 expression from binding a conserved cebR-box. In presence of cellobiose it releases its action and enables the expression of the repressed genes such as genes coding cellulolytic 305 306 enzymes [11,12] or pathogenic factors in S. scabies [15]. The presence of this system has 307 been assessed in few Streptomyces so far [11–13,15], but thanks to an in silico prospection of the CebR-system, we showed that it was actually highly prevalent in this genus. We also 308 showed that the CebR harboring Streptomyces all had highly conserved cebR-boxes, 309 310 suggesting that their CebR regulators were prone to recognize similar ones.

We took advantage of this conserved genetic scheme to design a whole-cell biosensor. Most 311 312 microbial sensors are generally based on a specific promoter cloned upstream a reporting 313 gene [23,24]. Once integrated in the cell, the promoter initiates the transcription of the reporting gene when the cell comes in contact with the inducer signal. As CebR is a 314 315 transcriptional repressor, we adapted this strategy in consequence and cloned a canonical 316 cebR-box in the promoter region of a constitutively expressed reporter gene in order to 317 regulate its expression by CebR. The cebR-box was cloned between the promoter and the 318 transcription start of the gene as it is found in some Streptomyces [11]. Considering the 319 widespread distribution of CebR among Streptomyces, we decided to integrate the construct 320 in various Streptomyces and relied on the endogenous CebR for its regulation. As these CebRs were naturally present in the strains they had the advantage to be perfectly adapted 321 322 to the recipient strain and thus to offer an optimal regulation of the construct. The construct 323 was successfully transferred and integrated with the pIB139 vector in different Streptomyces both model strains and environmental isolates, and its good regulation in presence of 324 cellobiose was observed in one third of them. For the other strains, the absence of a visual 325 326 signal during this screening could be due to various reasons such as a low xylE expression, 327 a diffusion problem of the dioxygenase or non-recognition of cellobiose. All together, these results showed that our biosensor design was efficient and enabled to create a promiscuous 328 329 construct that could be easily transferred and regulated in most Streptomyces to turn them into potential biosensors. More generally, this strategy that consists in diverting without a 330 priori a conserved sensor system in different strains to control a same conserved molecular 331 332 construction could be probably applied to other systems and in other bacteria. A main 333 advantage is that it allows for a same construction the screening of different strains with 334 different sensitivity or sensibility towards the molecule of interest and might ease the 335 construction of whole-cell biosensors. A further development to our approach could be to

introduce the construction in a strain that does not harbor the CebR system (e.g. E. coli or a 336 cebR-free Streptomyces) along with a heterologously expressed cebR gene to control it. 337 Previous biosensors, principally amperometric enzyme-based, were able to detect cellobiose 338 339 [23–25]. They generally used cellobiose hydrolases that are catalytic enzymes able to 340 degrade cellobiose. However, these enzymes have a large spectrum of molecule recognition and the biosensors built with them were used to detect other compounds such as glucose, 341 342 lactose or catechol, but never specifically and directly the cellobiose [25]. Regarding the CebR system, some cross specificity for cellodextrines in addition to cellobiose has been 343 344 identified in S. griseus [11]. Thus we did test the specificity of the Streptomyces S4N27 345 biosensor strain towards these compounds and got no positive detection, indicating a 346 specificity of cellobiose recognition with our biosensor like in S. reticuli [12].

347 We designed the biosensor to detect wood degradation. Fungal attack is one of the main 348 threats regarding the use of wood as a material. Generally, such attack is empirically 349 discovered when the decaying process had already altered the wood mechanical properties. Even in the case of standardized tests aiming to assess the efficiency of protective 350 treatments against fungal attack, professionals rely on 4 month experiments after infection 351 where they measure the wood mass loss (EN 113) [17]. In order to assess whether our 352 353 biosensor could bring a quicker answer in comparison to such tests, we performed a similar 354 experiment as in the EN113 norm in infecting a spruce stick with *Poria placenta*, but for half 355 of the normal time of the test. The application of the biosensor in this real context was successful and enabled to detect cellobiose in the wood attacked by the xylophagous fungus, 356 357 but not in the uninfected control and by extension revealed the ongoing wood decaying process at this stage, dividing by two the time of the EN113 test. Such experiments are 358 359 preliminary and despite further experiments are still needed to insure the full applicability of the biosensor in that context, this study led to a patent (PCT/EP2016/052417) regarding the 360 361 exploitation of this biosensor. In future experiments, other types of wood-rots and kinds of 362 wood should be tested but according to the fact that cellobiose remains a common 363 denominator of fungal wood degradation process; it should be detected in most cases. 364 Another aspect to be tested will be the kinetic of degradation to assess at which stage the biosensor would be applicable. Yet, as cellobiose is produced since the beginning of the 365 decaying process, degradation of wood should be detected in the earlier steps. 366

In conclusion, we created here a whole-cell biosensor in diverting a widespread sensor of cellobiose in *Streptomyces*. According to the conservation of this system, we designed a construct optimized for being transferable and regulate by most *Streptomyces*. After selection of a biosensor strain, we have been able to detect cellobiose even in complex 371 solutions such as a wood leachate. The application of this biosensor to detect wood372 degradation appears thus very promising for the wood industry.

374 **<u>5. Reference:</u>** 375

- [1] Wang, L., Littlewood, J., Murphy, R.J., Environmental sustainability of bioethanol
 production from wheat straw in the UK, *Renew. Sustain. Energy Rev.* 2013, *28*, 715–725.
- Schultz, T.P., Nicholas, D.D., Introduction to Developing Wood Preservative Systems and
 Molds in Homes, Dev. *Commer. Wood Preserv*, American Chemical Society, 2008, pp. 2–8.
- 381
 382 [3] Rajala T., Peltoniemi M., Pennanen T., Mäkipää R., Fungal community dynamics in
 383 relation to substrate quality of decaying Norway spruce (*Picea abies* [L.] Karst.) logs in boreal
 384 forests, *FEMS Microbiol. Ecol.* 2012, *81*, 494–505.
- 385
 386 [4] Schwarze, F.W.M.R., Engels, J., Mattheck, C., Fungal Strategies of Wood Decay in
 387 Trees, Springer Science & Business Media, 2000.
- 388
 389 [5] Howard, R.L., Abotsi, E., Van Rensburg, E.J., Howard, S., Lignocellulose biotechnology:
 390 issues of bioconversion and enzyme production, *Afr. J. Biotechnol.* 2004, 2,602–619.
 391
- [6] Phillips, C.M., Beeson, W.T., Cate, J.H., Marletta, M.A., Cellobiose dehydrogenase and a
 copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by
 Neurospora crassa, ACS Chem. Biol. 2011, *6*, 1399–1406.
- In Superior State
 In State</li
- 398
 399 [8] Langston, J.A., Shaghasi, T., Abbate, E., Xu, F., et al., Oxidoreductive cellulose
 400 depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61,
 401 *Appl. Environ. Microbiol.* 2011, *77*, 7007–7015.
- 402
 403 [9] Bontemps, C., Toussaint, M., Revol, P.-V., Hotel, L., et al., Taxonomic and functional
 404 diversity of *Streptomyces* in a forest soil, *FEMS Microbiol. Lett.* 2013, *342*, 157–167.
 405
- 406 [10] Bruce, T., Martinez, I.B., Maia Neto, O., Vicente, A.C.P., et al., Bacterial community 407 diversity in the Brazilian Atlantic forest soils, *Microb. Ecol.* 2010, *60*, 840–849.
- 408
 409 [11] Marushima, K., Ohnishi, Y., Horinouchi, S., CebR as a Master Regulator for
 410 Cellulose/Cellooligosaccharide Catabolism Affects Morphological Development in
 411 Streptomyces griseus, J. Bacteriol. 2009, 191, 5930–5940.
- 412
 413 [12] Schlösser, A., Aldekamp, Schrempf, T., H., Binding characteristics of CebR, the
 414 regulator of the ceb operon required for cellobiose/cellotriose uptake in *Streptomyces reticuli*,
 415 *FEMS Microbiol. Lett.* 2000, *190*, 127–132.
- 416
- [13] Takasuka, T.E., Book, A.J., Lewin, G.R., Currie, C.R., et al., Aerobic deconstruction of cellulosic biomass by an insect-associated *Streptomyces*, *Sci. Rep.* 2013, *3*, 1030.
- 419 420 [14] E
- [14] Padilla-Reynaud, R., Simao-Beaunoir, A.-M., Lerat, S., Bernards, M.A., et al., Suberin
 Regulates the Production of Cellulolytic Enzymes in *Streptomyces scabiei*, the Causal Agent
 of Potato Common Scab, *Microbes Environ. JSME.* 2015, *30*, 245–253.
- 423
 424 [15] Francis, I.M., Jourdan, S., Fanara S., R. Loria, et al., The Cellobiose Sensor CebR Is the
 425 Gatekeeper of *Streptomyces scabies* Pathogenicity, *mBio.* 2015, *6*, e02018–14.
- 426

[16] Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., et al., Practical Streptomyces 428 429 Genetics, John Innes Center Norwich, The John Innes Foundation, 2000. 430 [17] CEN (1996) EN 113 Wood preservatives. Test method for determinating the protective 431 432 effectivness against wood destroying basidiomycetes. Determination of the toxic values. 433 European Committee for Standardisation, Brussels, Belgium 434 435 [18] Sambrook, J., Russell, D.W., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001. 436 437 438 [19] Ingram, C., Brawner, M., Youngman, P., Westpheling, J., xylE functions as an efficient 439 reporter gene in Streptomyces spp.: use for the study of galP1, a catabolite-controlled 440 promoter, J. Bacteriol. 1989, 171, 6617-6624. 441 442 [20] Bradford, M.M., A rapid and sensitive method for the quantitation of microgram 443 quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 1976, 72, 444 248-254. 445 [21] Bibb, M.J., J. White, J.M. Ward, G.R. Janssen, The mRNA for the 23S rRNA methylase 446 447 encoded by the ermE gene of Saccharopolyspora erythraea is translated in the absence of a conventional ribosome-binding site, Mol. Microbiol. 1994, 14, 533-545. 448 449 450 [22] Schmitt-John, T., Engels, J.W., Promoter constructions for efficient secretion expression in Streptomyces lividans, Appl. Microbiol. Biotechnol. 1992, 36, 493-498. 451 452 453 [23] Bereza-Malcolm, L.T., Mann, G., Franks, A.E., Environmental sensing of heavy metals 454 through whole cell microbial biosensors: a synthetic biology approach, ACS Synth. Biol. 2015, *4*. 455 456 [24] Park, M., Tsai, S.-L., Chen, W., Microbial Biosensors: Engineered Microorganisms as 457 the Sensing Machinery, Sensors. 2013, 13, 5777-5795. 458 459 460 [25] Ludwig, R., Harreither, W., Tasca F., Gorton, L., Cellobiose Dehydrogenase: A Versatile 461 Catalyst for Electrochemical Applications, ChemPhysChem. 2010, 11, 2674–2697. 462 463 [26] Hanahan, D., Studies on transformation of Escherichia coli with plasmids, J. Mol. Biol. 464 1983, 166, 557-580. 465 [27] Simon, R., Priefer, U., Pühler, A., A Broad Host Range Mobilization System for In Vivo 466 Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria, Nat. Biotechnol. 467 1983, 1, 784–791. 468 469 [28] Bruton C.J., Guthrie E.P., Chater K.F., Phage Vectors that Allow Monitoring of 470 Transcription of Secondary Metabolism Genes in Streptomyces, Nat. Biotechnol. 1991, 9, 471 472 652-656. 473 474 [29] Thibessard, A., Haas, D., Gerbaud, C., Aigle, B., et al., Complete genome sequence of 475 Streptomyces ambofaciens ATCC 23877, the spiramycin producer, J. Biotechnol. 214 (2015) 476 117-118. 477 [30] Rückert, C., Albersmeier, A., Busche, T., Jaenicke, S., et al., Complete genome 478 479 sequence of Streptomyces lividans TK24, J. Biotechnol. 2015, 199, 21-22. 480 481 [31] Wilkinson, C.J., Hughes-Thomas, Z.A., Martin, C.J., et al., Increasing the efficiency of heterologous promoters in actinomycetes, J. Mol. Microbiol. Biotechnol. 2002, 4, 417-426. 482

TABLE

Table 1. Bacterial strains and vector used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
<i>E. coli</i> DH5α	supE44 Δ lacU169(Φ 80lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-11 relA1	[26]
E. coli \$17.1	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7	[27]
S. coelicolor KC900	actl::KC900 hisA1 uraA1 strA1 pgl SCP1 SCP2	[28]
S. ambofaciens ATCC 23877	Wild-type strain	[29]
S. lividans TK24	Wild-type strain	[30]
S. coelicolor M145	SCP1' SCP2'	[15]
Streptomyces sp. S9N29	Environmental wild-type strain	[9]
Streptomyces sp. S4N27	Environmental wild-type strain	[9]
Streptomyces sp. S6N6	Environmental wild-type strain	[9]
Streptomyces sp. S2N2	Environmental wild-type strain	[9]
Streptomyces sp. S9N14	Environmental wild-type strain	[9]
Streptomyces sp. S1N3	Environmental wild-type strain	[9]
Streptomyces sp. S4N22	Environmental wild-type strain	[9]
plB139	oriT attP int aac(3)IV PermE*	[31]

502 Figure legends

503 Figure 1: Design and construction flowchart of the biosensor

504 A) Schematic illustration of the cellobiose biosensor system. The biosensor construction 505 consists in a Streptomyces conserved cebR-box sequence cloned between the constitutive 506 promoter *PermE*^{*} and the reporter gene *xyIE*. Once integrated in a *Streptomyces* genome harboring an endogenous CebR able to recognize the *cebR*-box, this transcriptional 507 repressor will bind it and prevents the *xylE* expression. The presence of cellobiose alleviated 508 509 the CebR binding on the cebR-box and enables the transcription of xvIE. In presence of 510 catechol, the XyIE protein will emit a yellow compound revealing the presence of cellobiose. B) Flowchart of the biosensor construction. I. For the construction of the biosensor, the 511 reporter gene xylE was amplified from Streptomyces coelicolor KC900 with the Biosens_F 512 and Biosens_R primers (955 bp). The primer Biosens_F was designed from 5' to 3' with a 513 BamHI site as a future cloning site in pIB139. The black box symbolizes the cebR-box, the 514 515 light grey box a Ribosome Binding Site (RBS) and the white arrow the beginning of the xylE gene. The reverse primer Biosens_R has from 5' to 3', an EcoRI site as future cloning site 516 517 with pIB139 and the white arrow the end of the xylE gene. II. After amplification, the PCR product was cloned in the pIB139 plasmid that enables the integration of the construction into 518 519 Streptomyces chromosome.

520 *PermE**: constitutive promotor; *ori_{pUC}*: replicative origin in *E. coli*; Apra^R: Apramycine gene 521 resistance; *oriT*: transfert origin, Int: integrase gene; MCS: Multiple cloning site.

522

523 Figure 2: Illustration of the detection of the cellobiose by the biosensor

The S4N27 biosensor strain was grown for 2 days on HT* plates without cellobiose in A) or with cellobiose at 5 g.L⁻¹ in B). After catechol addition the yellow color is only observed in presence of cellobiose.

527

528 Figure 3: Detection threshold of cellobiose by the biosensor.

The experiments were performed in HT* medium complemented with increasing cellobiose concentrations ranging from 1.5 μ M to 15000 μ M. For each concentration, the results are expressed as the ratio of the *xylE* activity measured in presence and in absence of cellobiose (background signal). An activity ratio of 1 (represented by the black horizontal line) corresponds to an activity equivalent to the background signal. Results for each concentration corresponded to three independent cultures. Error bars represent the standard 535 deviation. Significant differences of signal intensity between the different conditions and the 536 background signal are represented by asterisks and were determined with the Student's *t*-537 test (P < 0.05).

538

539 Figure 4: Detection of the wood degradation by a lignolytic fungus.

Wood leachates were obtained from a spruce wood stick infected for 7 weeks by *Poria placenta* (black box) and from a non-infected control (white box). The results correspond to five independent biosensor assays and are expressed as the relative expression with the non-infected condition. Thus, the activity ratio of the non-infected spruce wood condition has a value of one and corresponds to the background signal. The significant difference between the two experimental conditions was attested by a Student's *t*-test (P < 0.05) and is symbolized by an asterisk.

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603

604 **Conflict of interest.**

The authors declare no financial or commercial conflict of interest.





- cellobiose

В



+ cellobiose







