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1 **Valorisation of wheat bran to produce natural pigments using selected**
2 **microorganisms**

3 *Author names and affiliation.* Mathieu Cassarini, Ludovic Besaury, Caroline Rémond

4 Affiliation. Université de Reims Champagne Ardenne, INRAE, FARE, UMR A 614, Chaire AFERE,
5 51097 Reims, France

6 Emails. mathieu.cassarini@univ-reims.fr; ludovic.besaury@univ-reims.fr; [caroline.remond@univ-](mailto:caroline.remond@univ-reims.fr)
7 reims.fr

8 *Corresponding author.* Ludovic Besaury - Email : ludovic.besaury@univ-reims.fr; Université de Reims
9 Champagne Ardenne, INRAE, FARE, UMR A 614, Chaire AFERE, 51097 Reims, France

10

11 Abstract

12 Pigments are compounds with highly diverse structures and wide uses, which production is increasing
13 worldwide. An eco-friendly method of bioproduction is to use the ability of some microorganisms to
14 ferment on renewable carbon sources. Wheat bran (WB) is a cheap and abundant lignocellulosic co-
15 product of low recalcitrance to biological conversion. Microbial candidates with theoretical ability to
16 degrade WB were first preselected using specific databases. The microorganisms were *Ashbya*
17 *gossypii* (producing riboflavin), *Chitinophaga pinensis* (producing flexirubin), *Chromobacterium*
18 *vaccinii* (violacein) and *Gordonia alkanivorans* (carotenoids). Growth was shown for each on minimal
19 salt medium supplemented with WB at 5 g.L⁻¹. Activities of the main enzymes consuming WB were
20 measured, showing leucine amino-peptidase (up to 8.45 IU.mL⁻¹) and β-glucosidase activities (none to
21 6.44 IU.mL⁻¹). This was coupled to a FTIR (Fourier Transform Infra-Red) study of the WB residues
22 that showed main degradation of the WB protein fraction for *C. pinensis*, *C. vaccinii* and *G.*
23 *alkanivorans*. Production of the pigments on WB was assessed for all the strains except *Ashbya*, with
24 values of production reaching up to 1.47 mg.L⁻¹. The polyphasic approach used in this study led to a
25 proof of concept of pigment production from WB as a cheap carbon source.

26 Keywords

27 Pigment production; Biorefinery; Wheat bran; Microorganisms; Lignocellulosic enzymatic activities; FTIR

28 Abbreviations

29 AA: Auxiliary Activity
30 *A. goss.*: *Ashbya gossypii*
31 CAZy: Carbohydrate Active Enzyme database
32 CAZyme: Carbohydrate Active Enzyme
33 CBM: Carbohydrate Binding Module
34 CE: Carbohydrate Esterase
35 *C. pin.*: *Chitinophaga pinensis*
36 *C. vacc.*: *Chromobacterium vaccinii*
37 FTIR: Fourier Transform InfraRed (spectroscopy)
38 *G. alk.*: *Gordonia alkanivorans*
39 GH: Glycoside Hydrolase
40 GT: Glycosyltransferase
41 LAP: L-Leucine-7-amido-4-methylcoumarin hydrochloride
42 OD: Optical density
43 ORFs: Open Reading Frames
44 PL: Polysaccharide Lyase

45 SD: Standard deviation

46 WB : Wheat bran

47

48 **1. Introduction**

49 Pigments are often described as compounds having the ability to absorb and reflect a specific part of
50 the electromagnetic visible spectrum. With such a broad definition, pigments are ubiquitous in nature
51 and have a huge variety of chemical structures, properties and uses (Ramesh et al. 2019). They are
52 commonly used as food colorants, dyes in the textile or cosmetic industry, or for some specific
53 biological activities such as antioxidant, antibacterial, or anticancer (Lagashetti et al. 2019).

54 Nowadays, natural organic pigments are seen as less harmful for the planet and for the health, and the
55 global demand for these pigments is increasing. In 2018, the organic pigment market was estimated to
56 be worth USD 3.5 billion, and it could reach around USD 4.8 billion by 2024 (MarketResearchFuture
57 2020). When regarding the market of specific pigment categories such as carotenoids, synthetic
58 pigments are predominant (more than 80% of carotenoid syntheses is chemical) (Venil et al. 2020),
59 because those are cheaper to produce.

60 Production of natural organic pigments from plant and animal extraction has some limitations. Those
61 means of productions are dependent on seasonality (for plants), contents are low, and the extraction
62 processes can be polluting at times (Capolupo and Faraco 2016). The use of microbial fermentation as
63 a bioproduction process is increasing, as microbial growth is fast, independent on seasonality and
64 requires overall less energy than plants and animals (Jorissen et al. 2020). Numerous organisms
65 producing pigments have already been studied in yeasts (Johnson and Lewis 1979), fungi (Chiba et al.
66 2006), bacteria (Venil et al. 2013), and others (Sui et al. 2014).

67 A limiting factor for the fermentation approach is the important cost of growth media (Hong et al.
68 2011). Viable alternatives to lower this cost can be obtained by using the ability of some species to
69 grow on agro-industrial co-products that are cheap and abundant (Sansinenea and Ortiz 2011). There is
70 as a wide variety of pigments already being produced by microbial fermentation. In most of previous
71 studies, agro-industrial co-products or wastes are mixed with rich growing medium to study pigment
72 production (Silveira et al. 2008; Frengova and Beshkova 2009; Nguyen et al. 2020). Studies on the use
73 of saccharides and phenols for pigment production by microorganisms are scarce. Lignocellulose,

74 which is cheap and abundant, is generally viewed as a promising candidate for bioproduction (Diaz et
75 al. 2018; Sindhu et al. 2020).

76 The objective of this study was to evaluate the ability of various microorganisms from different
77 species to produce pigments using a cheap lignocellulosic biomass, wheat bran (WB), as carbon
78 source. Its production is estimated to be around 10⁸ tons a year (Reisinger et al. 2013). It contains
79 mainly starch (15-25%), cellulose (\approx 10%), arabinoxylans and glucans (10-25%) and proteins (\approx 15%)
80 (Apprich et al. 2014). This co-product is of interest for bioconversion as its poor content in lignin
81 makes it of low recalcitrance to biological conversion compared to other lignocellulosic compounds.
82 WB was notably described as an interesting carbon source for the microbial production of
83 hemicellulases with the bacterium *Thermobacillus xylanilyticus* (Rakotoarivonina et al. 2012;
84 Rakotoarivonina et al. 2016).

85 For this study, the microorganisms were screened from databases and literature with two selecting
86 criteria: (1) microorganisms already described as pigment producers, (2) microorganisms possessing
87 genes coding for enzymes involved in WB components fractionation.

88 Four microorganisms producing each a different category of pigment were then selected: the
89 bacterium *Gordonia alkanivorans* for carotenoids production (mostly canthaxanthin); the bacterium
90 *Chitinophaga pinensis* for the production of flexirubin; the bacterium *Chromobacterium vaccinii* that
91 produces violacein and deoxyviolacein (for readability, the production and quantification of both
92 pigments will be referred to as "violacein" in the rest of the article); and the fungi *Ashbya gossypii* that
93 produces riboflavin.

94 Historically, *A. gossypii* is the first organism used at the industrial scale for riboflavin production. It
95 was isolated as a plant pathogen from cotton bolls (Aguar et al. 2015). *C. pinensis* is a *Bacteroidetes*
96 that was isolated from pine forest leaf litter (McKee and Brumer 2015), and *C. vaccinii* was isolated
97 from cranberry roots and soil (*Vaccinium macrocarpon* Ait.) (Soby et al. 2013). Since those three
98 species were isolated from plants, and regarding their Carbohydrate Active enzyme (CAZyme) profile,
99 it was expected that they could be good candidates to valorise lignocellulose. The remaining strain of

100 interest, *G. alkanivorans*, was isolated from soils contaminated by tar and phenols and is mostly
101 known to degrade hydrocarbons (Kummer et al. 1999).

102 In this study, strains having the ability to degrade lignocellulose and produce pigments were chosen by
103 analysis of the CAZyme and MEROPS databases (dedicated to carbohydrate active enzymes and
104 proteases). The use of WB as carbon source was then studied by measuring specific enzyme activities
105 and analysing the WB composition with Fourier Transform InfraRed (FTIR) spectroscopy. Finally,
106 pigments were extracted according to already described protocols and their productions were
107 measured.

108 2. Materials and methods

109 2.1. Materials

110 Wheat bran (WB) in ground form (particle sizes: 0.8-2 mm) was kindly provided by BioWanze
111 (Belgium). The strains were obtained from DSMZ (Leibniz Institute - German collection of
112 microorganisms and cell cultures GmbH).

113 2.2. WB analysis

114 The sugar composition of WB was evaluated by HPAEC-PAD with a Dionex CarboPac PA-1 column
115 (4 x 250 mm, Thermo Fisher Scientific, Waltham, USA), after acid hydrolysis of WB as previously
116 described (Rémond et al. 2010). The quantification of starch was performed with the Total Starch HK
117 Assay kit (K-TSHK, Megazymes, Bray, Ireland) WB protein content was evaluated with the Dumas
118 method using a CHNS-O Elemental Analyser 'EA3000, Eurovector, Milan, Italia) of total nitrogen
119 measurement after catalytic combustion according to the corresponding ISO norm (ISO 2016).

120 2.3. Bioinformatic analyses

121 Full genomes were available at GenBank (www.ncbi.nlm.nih.gov/genbank/) under the accession
122 numbers [NC_005782.2](#) (*Ashbya gossypii*), [CP001699.1](#) (*Chitinophaga pinensis*), [CP017707.1](#)
123 (*Chromobacterium vaccinii*) and [NZ_BACI00000000.1](#) (*Gordonia alkanivorans*). Full theoretical
124 proteomes from the genomes were obtained using Augustus software (default parameters) installed on
125 usegalaxy.org (Stanke et al. 2006). For total CAZymes predictions, theoretical proteomes were
126 annotated using the dbCAN2 meta server (<http://bcb.unl.edu/dbCAN2/>, option HMMER) (Zhang et al.
127 2018). For the full proteasome, the BLAST MEROPS tool (Rawlings et al. 2009) for comparison to
128 the MEROPS peptidase database was used (www.ebi.ac.uk/merops/). For the secreted proteins, the
129 SignalP 5.0 server was used (<http://www.cbs.dtu.dk/services/SignalP> (Almagro Armenteros et al.
130 2019). Transmembrane regions were determined with the TMHMM Server v. 2.0
131 (<http://www.cbs.dtu.dk/services/TMHMM/>) (Krogh et al. 2001). The predicted secretome were then
132 annotated using dbCAN2 (see above) and the MEROPS database in order to obtain respectively the
133 secreted CAZymes and proteasome.

134 2.4. Strains and culture media

135 Four strains were used in this study: *Ashbya gossypii* DSM 3485, *Chitinophaga pinensis* DSM 2588,
136 *Chromobacterium vaccinii* DSM 25150 and *Gordonia alkanivorans* DSM 44369. Optimal growth
137 media were used for growth on agar-plate and optimal liquid cultures. Optimal growth media
138 compositions are available at the www.dsmz.de/ website. Optimal media were: Yeast medium
139 (medium 186, *A. gossypii*); Cy-broth (medium 67, *C. pinensis*); Luria-Bertani (medium 381, *C.*
140 *vaccinii*) and Trypticase Soy Broth (medium 535, *G. alkanivorans*). Minimal salt medium M3
141 (KH_2PO_4 , 1.9 g.L⁻¹; Na_2HPO_4 , 5.1 g.L⁻¹; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.L⁻¹; $(\text{NH}_4)_2\text{SO}_4$, 0.2 g.L⁻¹; pH 7.2) was
142 used for *A. gossypii* and *G. alkanivorans*. Minimal salt medium M9 (NH_4Cl , 1 g.L⁻¹; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$,
143 6 g.L⁻¹; KH_2PO_4 , 3 g.L⁻¹; NaCl , 0.5 g.L⁻¹; pH 7.2) was used for *C. pinensis* and *C. vaccinii*.

144 2.5. Growth conditions

145 Inocula were prepared from differentiated colonies grown on agar plate. One colony was inoculated
146 per erlenmeyer containing 50 mL of culture medium. Minimal media consisted of minimal salt
147 medium supplemented with WB at 5 g.L⁻¹. Wet autoclave protocol: the WB and the minimal salt
148 medium are mixed prior the autoclave program (20', 121°C, slow decrease in temperature and
149 pressure). Dry autoclave protocol: the WB is sterilized within an erlenmeyer (autoclave program: 20',
150 121°C, immediate decrease in temperature and pressure afterwards) and sterilized minimal salt
151 medium was added after. Agitation was of 100-150 rpm. Temperatures of growth were 30°C for *A.*
152 *gossypii* and *C. pinensis* and 22°C for *C. vaccinii* and *G. alkanivorans*. Growths were followed by
153 sampling 1 mL of culture medium and measuring the OD at 600 nm, except for *A. gossypii*. All data
154 were collected in biological triplicates, where three separate cultures were inoculated then treated of
155 the same manner.

156 2.6. Enzymes activities

157 2.6.1. Enzymes extraction

158 Enzymes extraction was achieved from the cultures in minimal medium. For α -amylase, xylanase and
159 β -glucosidase activities, 1 mL of culture was lysed in microtubes containing \approx 5 mm of zirconia-

160 coated silica-microbeads (11079101z, Biospec Products, Bartlesville, USA) with a vortex mixer for
161 10-20' at room temperature. Microtubes were then centrifuged and supernatants were collected. For
162 LAP activities, 1 mL of culture was centrifuged, and the supernatant was collected. Controls were
163 non-inoculated media incubated with the same conditions. All data for enzyme extraction were
164 collected in biological triplicates, with samples obtained from three separate cultures. Enzymatic
165 activity was expressed in international units (IU), where 1 IU is the quantity of enzyme required to
166 release 1 μ mole of reducing sugar per min. Temperature for the reactions were the same as the growth
167 temperatures of the corresponding microorganisms.

168 2.6.2. *α -amylase and xylanase activities*

169 Those activities were assessed by measuring the reducing sugars liberated from xylans or starch by the
170 xylanase or amylase activities, respectively. Done according to the Kidby-Davidson methodology as
171 previously described (Rémond et al. 2010). 100 μ L of enzyme extracts were incubated with 900 μ L of
172 either native potato starch (1259, Merck, Darmstadt, Germany) or xylan from beechwood (4414.4,
173 Roth, Karlsruhe, Germany) at 0.5 % (w/v) in Tris-HCl, 10 mM, pH 7.5. 100 μ L of the mix was added
174 to 1.5 mL of alkaline ferricyanide reactant after 0', 5' and 10' of reaction. Reactions were boiled 5
175 minutes, cooled down, and absorbances measured at 420 nm, 25°C.

176 2.6.3. *β -glucosidase activity*

177 This activity was assessed using *p*-nitrophenyl- β -D-glucopyranoside (*p*NP-Glc) (N7006, Sigma-
178 Aldrich, Darmstadt, Germany). 100 μ L of enzyme extracts were incubated with 900 μ L of *p*NP-Glc 10
179 mM in Tris-HCl, 10 mM, pH 7.5. The absorbance was measured at 401 nm for 15'.

180 2.6.4. *LAP activity*

181 This activity was assessed using L-Leucine-7-amido-4-methylcoumarin hydrochloride (LAP) (L2145,
182 Sigma-Aldrich, Darmstadt, Germany). Reaction were made with 1050 μ L of Tris-HCl 10 mM, pH 7.0;
183 300 μ L of LAP 200 μ M in ultrapure water; 150 μ L of enzyme extract. Reactions were incubated
184 between 10' and 3h in order to obtain significant activities without using all the LAP substrate.
185 Reactions were stopped with 10 μ L of NaOH 1M. 250 μ L of each reaction mix were dispatched 5

186 timed in a 96-well black microplate for fluorescence (MaxiSorp Nunc, Thermo Fisher Scientific,
187 Waltham, USA). Fluorescence was measured; (excitation: 365 nm, emission: 450 nm, room
188 temperature) (SpectraMax Gemini, Molecular Devices, San Jose, USA). Standard fluorescence curve
189 were obtained with 7-Amino-4-methylcoumarin (7-AMC) (A9891, Sigma-Aldrich, Darmstadt,
190 Germany).

191 **2.7. FTIR analyses**

192 FTIR analyses were performed on WB without fermentation (culture media were treated the same way
193 as a fermentation) and on WB residues after the stationary growth phase was reached. After
194 cultivation, supernatant was discarded, and the remaining WB was rinsed several times with water.
195 WB was then dried at 50°C for at least 2 days. Attenuated Total Reflectance - Fourier Transform
196 Infra-Red (ATR-FTIR) analyses were performed using a Nicolet 6700 FT-IR (Thermo Fisher
197 Scientific, Waltham, USA) with ATR-diamond crystal (Smart iTR, Thermo Fisher Scientific,
198 Waltham, USA). Each sample was measured at least 3 times (technical replicates). Spectra were
199 treated with the OMNIC 8 software. A mean spectrum of the three readings was calculated; the
200 baseline was corrected and the area of interest ($\approx 2990\text{-}800\text{ cm}^{-1}$) was normalized. The TQ analyst Z
201 software was used to retrieve the absorbances associated to each peak of interest (cf. Table 1). Ratios
202 of absorbances of fermented over non-fermented WB were calculated. All data were collected in
203 biological triplicates, with samples obtained from three separate cultures.

204 **2.8. Pigment extraction and quantification**

205 All data for enzyme extraction were collected in biological triplicates, with samples obtained from
206 three separate cultures grown in the same manner. Minimal media conditions were done with wet-
207 autoclaved media.

208 *2.8.1 Riboflavin* (Adapted from (Ledesma-Amaro et al. 2015))

209 Samples of cultures at 210h (optimal medium) and 70h (minimal medium) of growth were collected.
210 1:1 culture:HCl 0.1 M were added and boiled at 100°C for 30'. Each sample was sonicated (Sonicator
211 Vibra-Cell, 72410, Sonics, Newtown, USA) for 40', 20-40 Hz, with pulses of 4 seconds on/off, at

212 30°C. Lysates were centrifuged (room temperature) and supernatants collected. The lysates were
213 placed in a lyophiliser (Cosmos 20k, Cryotec, S aint-Gély-du-Fesc, France) for a 1-2 days at -20°C.
214 The solids were then resuspended in 2 mL of ultrapure water. Those 2 mL were centrifuged (room
215 temperature) and the supernatant was collected. Riboflavin fluorescence was measured in 96-well
216 black microplates (MaxiSorp Nunc, ThermoScientific, Waltham, USA) with a SpectraMax Gemini
217 spectrophotometer (Molecular Devices, San Jose, USA) (excitation: 450 nm; emission: 350-650 nm).

218 2.8.2 *Flexirubin* (Adapted from (Venil et al. 2015))

219 20 mL of cultures at 96h of growth were collected then centrifuged (10', 8.10³ rpm, 4°C). The
220 supernatant was discarded, and the pellet rinsed with deionised water, then discarded after another
221 centrifugation. 20 mL acetone 5% was added to the pellet and cells were sonicated (Sonicator Vibra
222 Cell, 72410, Sonics, Newtown, USA) for 40', 20-40 Hz, with pulses of 4 seconds on/off, at 30°C.
223 Lysates were centrifuged (room temperature) and supernatants were collected. Another sonication in
224 10 mL acetone 5% was performed and the supernatant collected. Butan-1-ol was added (ratio
225 lysate:butan-1-ol 11:2) and vortexed. After a centrifugation the organic phase was collected. The
226 butan-1-ol was then evaporated using a rotating evaporator (R-215, Büchi, Villebon sur Yvette,
227 France) at 50°C, 25 mbar, rotation at 85 rpm. The pigments were resuspended in pure acetone, the
228 optical density read at 450 nm and the concentration calculated with an extinction coefficient of
229 99.400 mL.μmol⁻¹.cm⁻¹ (Reichenbach et al. 1974).

230 2.8.3 *Violacein* (Adapted from (Kanelli et al. 2018))

231 Samples of cultures at 73h (optimal medium) and 73h (minimal medium) of growth were collected,
232 centrifuged (room temperature), rinsed with Tris-HCl, pH 7.0, 10 mM followed by another
233 centrifugation (room temperature) and the supernatant was discarded. 1 mL of DMSO (ACS reagent
234 grade) was added to each pellet and the mix was vortexed (2'). Supernatant was collected after
235 centrifugation (room temperature) then evaporated using a rotating evaporator (R-215, Büchi, Villebon
236 sur Yvette, France) at 50°C, 4 mbar, and rotation at 85 rpm. Content was resuspended in absolute

237 ethanol. Absorbance was measured at 575 nm and concentration calculated with an ϵ of 0.05601
238 mL. μg^{-1} .cm $^{-1}$ (Mendes et al. 2001).

239 2.8.4 Carotenoids (Adapted from (Fernandes et al. 2018))

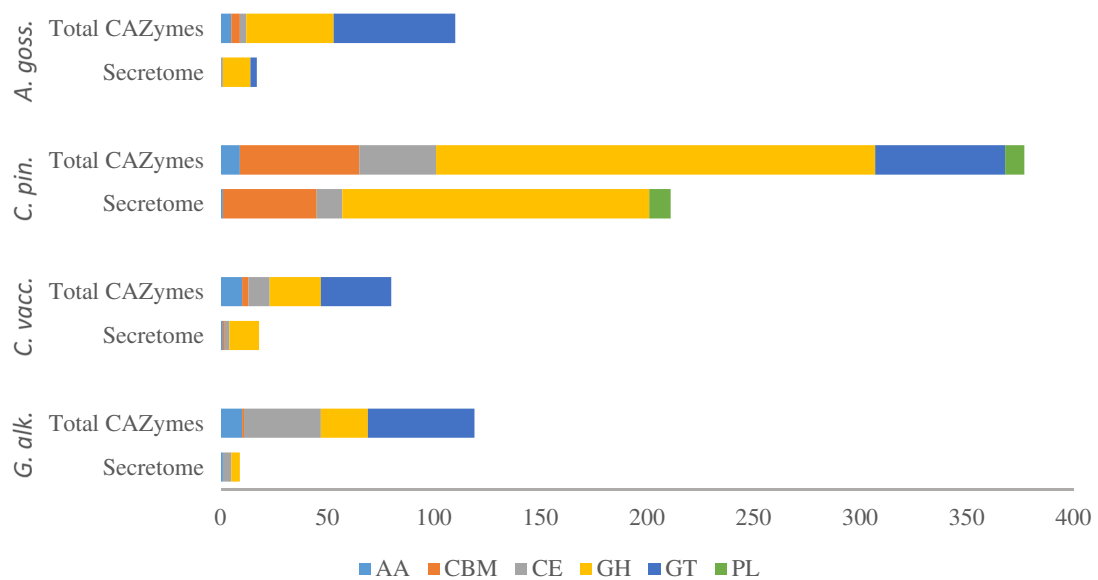
240 Samples of cultures at 168h of growth were collected. Cells were centrifuged (room temperature),
241 supernatant was discarded and 1.5 mL DMSO (ACS reagent grade) were added. The mix was
242 transferred into a microtube containing \approx 5 mm of zirconia-coated silica-microbeads (11079101z,
243 Biospec Products, Bartlesville, USA). Lysis was done by vortex mixer for more than 4h at room
244 temperature. The microtubes were then centrifuged (4°C) and supernatants were collected. Another
245 0.5 mL DMSO was added for the optimal medium conditions, treated as before and collected. A mix
246 of DMSO / Acetone / NaCl 20% / ethyl acetate (3/1/1/6) was done, mixed gently, the upper part
247 collected after 1h and absorption measured at 477 nm. Concentrations were calculated with an ϵ of
248 0.20676 mL. μg^{-1} .cm $^{-1}$ (477 nm, canthaxanthin in ethyl acetate) (made from canthaxanthine, DRE-
249 CA10947000, Dr Ehrenstorfer, Augsburg, Germany).

250

251 3. Results

252 3.1. CAZymes prediction

253 Strains for this study were selected in part on the theoretical enzymes arsenal required to degrade the
254 polysaccharides present in WB: notably starch, cellulose, glucans and xylans. For this, the search for
255 genes coding for CAZymes was performed. The CAZy database (Lombard et al. 2014) is often used to
256 find potential enzymes with an activity of interest in a genome (Aguiar et al. 2013), or to perform
257 transcriptome (Ben Ali et al. 2020) or metasecretome analyses (Jimenez et al. 2015).



258 **Figure 1** - Distribution of the CAZy classes predicted from the genomes of each selected pigment
259 producer. Total CAZymes represent the entirety of the CAZy classes predicted from a genome, while
260 the secretome corresponds to the predicted modules with signal peptides and no transmembrane
261 region. AA: Auxiliary Activity; CBM: Carbohydrate Binding Module; CE: Carbohydrate Esterase;
262 GH: Glycoside Hydrolase; GT: Glycosyl Transferase; PL: Polysaccharide Lyase.

263 Total amount of theoretical CAZymes in the genome represents 1.73% of the proteome for *C. vaccinii*,
264 2.24% for *A. gossypii*, 2.77% for *G. alkanivorans* and 4.64% for *C. pinensis*. CAZymes are classified
265 in different classes. The repartition of the *in silico* predictions between these classes is shown in
266 Figure 1. Glycosyl Transferases (GT) are highly predicted since they are generally involved in the

267 biosynthesis of the oligo- and polysaccharides (Breton et al. 2001). Glycoside Hydrolases (GH)
268 represent 18.5-54.6% of the total predicted CAZy modules and are the most predicted ones in *C.*
269 *vaccinii*. GHs mainly act on cell wall polysaccharides, and can have a role either in their biosynthesis
270 or degradation (Minic 2008). Carbohydrate Esterases (CE) and Polysaccharide Lyase (PL) classes are
271 mainly directed towards degradation of polysaccharides (Lombard et al. 2010; Biely 2012) and are
272 predominant in *G. alkanivorans* and *C. pinensis*, respectively. Carbohydrate binding modules (CBM)
273 are more present in *C. pinensis* (14.9% of the total modules) than in the other strains. CBMs are
274 responsible for the binding of a CAZyme to a carbohydrate, supposedly to its substrate (Bornscheuer
275 et al. 2014). Auxiliary Activity (AA) modules, usually active on non-carbohydrate substrates such as
276 lignins (Bornscheuer et al. 2014), are more present in *C. vaccinii* and *G. alkanivorans* (12.5 and 8.4%
277 of the total modules, respectively).

278 Since only secreted enzymes can act on exogenous lignocellulosic compounds, secreted CAZymes
279 were then studied (Table S1). Secreted enzymes were selected on the presence of a signal peptide
280 sequence and the absence of transmembrane regions.

281 In *A. gossypii*, families GH5, GH17, GH31 and GH32 are probably involved in hydrolysis of
282 carbohydrates (Chen et al. 2018). The others display wide specificities for polysaccharides. For *C.*
283 *pinensis*, the most present families have the ability to hydrolyse hemicelluloses (GH43, GH29, GH78).
284 This correlates with the high number of carbohydrate-binding modules (CBM) specific to
285 hemicelluloses. CAZymes AA10, GH8 and CH15-CBM20 from *C. vaccinii* act on cellulose, xylans
286 and starch, respectively. For *G. alkanivorans*, only the GH23 family has no probable lignocellulolytic
287 activity.

288 **3.2. Secreted peptidases analysis.**

289 In parallel to the carbohydrate degrading enzymes, the ability to use another major constituent of the
290 WB was screened. Since the protein fraction makes 15.5% of the dry mass of the WB (Apprich et al.
291 2014), strains were also selected onto their genetic potential of secreted peptidases that could be
292 involved in the hydrolysis of WB proteins during growth. Only the secreted peptidases were screened,

293 since peptidase activities are a main metabolic activity in any given cell. This study was done by
 294 interrogating the MEROPS database (Rawlings et al. 2009), specialized in peptidases.

295 **Table 1.** Predicted secreted peptidases families. The number of predicted proteins belonging to a
 296 family is shown in the second column, and the main function associated with the members of this
 297 family according to the MEROPS website data is given. Results are ordered according to the families
 298 having the highest number of predicted proteins. For *C. pinensis*, only results with occurrences (Occ.)
 299 >1 are shown.

<i>A. gossypii</i>	Occ.	Fonction	<i>C. vaccinii</i>	Occ.	Action
A01A	4	Pepsin	S53	3	Probable digestion of food proteins
S08A	2	Nutrition / Pathogenesis	C40	2	Bacterial cell wall
S10	2	Structural role	C82A	2	Bacterial cell wall
C13	1	Legumain	M23B	2	Bacterial cell wall
C44	1	Autolytic - Transferase	S09A	2	Degradation of biological active peptides
M14A	1	Various	S09D	2	Degradation of biological active peptides
M20F	1	Degradation of proteins into amino acids	S12	2	Bacterial cell wall
M28A	1	Various	C93	1	Unknown
M28E	1	Various	M09A	1	Precursors
<i>C. pinensis</i>			M09B	1	Precursors
S33	9	Use proline-rich substrates	M13	1	External polypeptides
S12	8	Bacterial cell wall	M14A	1	Various
M01	6	Aminopeptidases	M16B	1	Mitochondrium and other Bacterial cell wall (lysis of peptidoglycans)
M28F	5	Various	M23A	1	Various
M97	4	Unknown	M24B	1	Various
S09B	4	Degradation of biological active peptides	M35	1	Various
S09X	4	Degradation of biological active peptides	M48C	1	Various
S41A	4	Degradation of incorrectly synthesized prots	S01E	1	Various
M14X	3	Various	S09B	1	Degradation of biological active peptides
M93	3	Unknown	S13	1	Bacterial cell wall
S08A	3	Nutrition / Pathogenesis	S66	1	Bacterial cell wall
S09D	3	Degradation of biological active peptides	S82	1	Unknown
C01A	2	Lysosome	T03	1	Glutathione degradation
C40	2	Bacterial cell wall	<i>G. alkanivorans</i>		
C56	2	Various	S12	4	Bacterial cell wall
M13	2	External polypeptides	M23B	3	Bacterial cell wall
M20D	2	Degradation of proteins into amino acids	C82A	2	Bacterial cell wall
M23B	2	Bacterial cell wall	C40	1	Bacterial cell wall
M38	2	Detoxification	C44	1	Autolytic - Transferase
M57	2	Unknown	M15D	1	Bacterial cell wall
S09A	2	Degradation of biological active peptides	M24A	1	Remove initiating methionine
S15	2	Casein degradation	M28A	1	Various
S41B	2	Cytoplasmic proteolytic pathway	T03	1	Glutathione degradation
T03	2	Glutathione degradation			
...	<1	Not shown			

300 The most predicted family for *A. gossypii*, A1A, encodes for pepsin-like peptidases and has already
301 been found to be highly present in a lignocellulolytic organism (Aylward et al. 2013). Interestingly,
302 members of the family S8A (subtilisin family) are known for some of their pathogenicity in animals
303 and plants depending on the species (Segers et al. 1999).

304 Overall, there are more peptidases predicted in *C. pinensis* than in the three other strains of this study.
305 (104 peptidases for 48 families) (Table 1). The most predicted family, S33, is known to be active on
306 proline rich substrates, and therefore can have a nutritional role that gives selective advantages for the
307 strain. The S33 family has already been shown to be expressed differently on various lignocellulosic
308 biomasses in *Hypocrea jecorina* (Bengtsson et al. 2016). In the families predicted more than once,
309 there are no clear (M1, M28F, M14X) unknown (M97), or very specific biological roles (the other
310 classes). Only the S8A family has a known role in nutrition/pathogenesis. Regarding these results, a
311 moderate to high peptidase activity can be predicted for this strain.

312 The most predicted family (S53) in *C. vaccinii* has uncertain functions, but is believed to be part of the
313 "extracellular digestion of food proteins" (Table 1). This family was abundant in a lignocellulosic
314 organism and thought to potentially use plant proteins (Aylward et al. 2013). For the families only
315 predicted once, multiple candidates could be involved in the fractionation of proteins of the WB.
316 Those families have either unknown functions (C93 and S82), degrades small polypeptides (S9B), or
317 very diverse activities (M14A, M24B, M35, M48C, S1E). Therefore, no precise use of the protein
318 fraction of WB can be certainly predicted for *C. vaccinii*, but it still has interesting enzymes for such
319 activity.

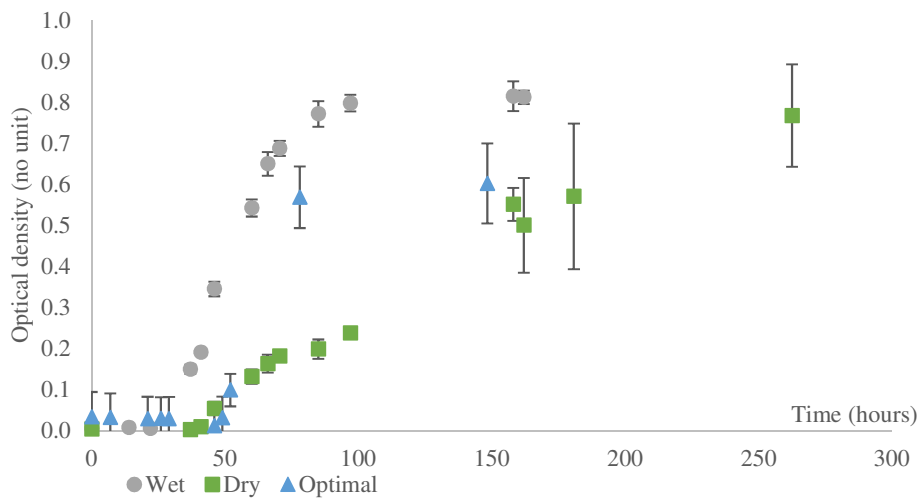
320 Only 15 proteins were predicted representing 9 families for *G. alkanivorans* (Table 1). All of them
321 have defined major functions in the cell that could theoretically not be used for the degradation of WB
322 proteins. Only one family has a diverse range of activity in its members (M28A), so its function in
323 *Gordonia* cannot be predicted.

324 According to these results, the valorisation of the protein fraction of WB is theoretically possible for
325 *A. gossypii*, *C. pinensis* and *C. vaccinii* and less feasible for *G. alkanivorans*.

326

327 **3.3. Growth kinetics of the different strains**

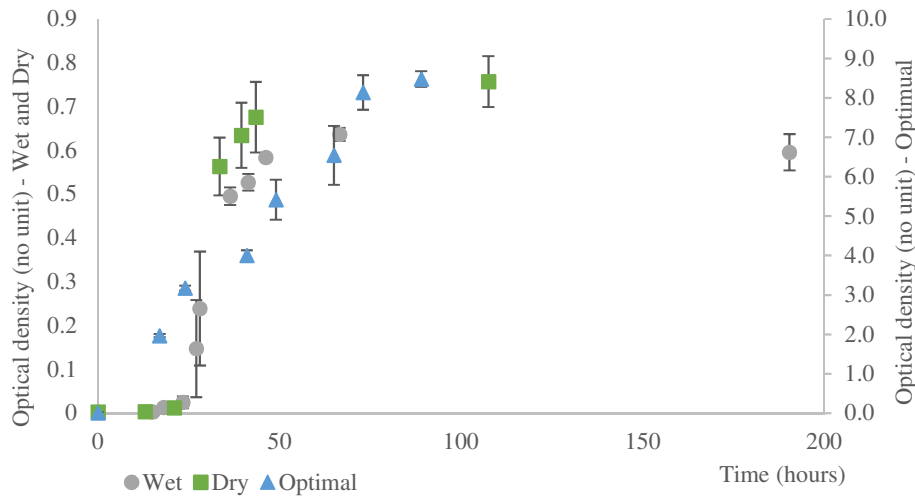
328 *A. gossypii* forms pellets during growth, showed to reach 1-2 mm diameter on WB (Figures S1).
329 Therefore it was not possible to follow OD at 600 nm, as it was the case for the other organisms, and
330 growth was visually assessed. Both wet autoclave and dry autoclave protocols gave similar results. In
331 its optimal medium *A. gossypii* grows beyond the formation of pellets and fills the entirety of the flask.
332 Stationary phases were reached during the second week of culture.



333

334 **Figure 2.1** - Growths of *C. pinensis* were done at 22°C, 100 rotations per minute (rpm). Optical
335 density was followed at 600 nm. Optimal medium was Cy-broth. n=3, brackets are SD (standard
336 deviation).

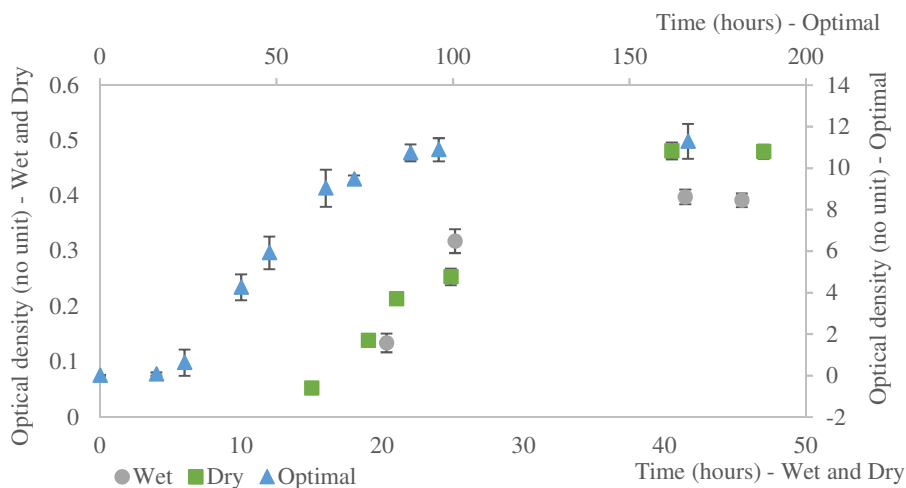
337 *C. pinensis* reached similar stationary phases for the three types of culture medium, but interestingly
338 OD was slightly more important for WB than for the optimal medium (max. absorptions : optimal
339 medium - 0.60 ± 0.03 ; dry autoclave - 0.77 ± 0.13 ; wet autoclave - 0.81 ± 0.02) (Figure 2.1). The
340 growth on dry-autoclaved WB was significantly slower than the one on wet-autoclaved WB
341 (stationary phases were reached at almost 150h and 100h, respectively), while the exponential phases
342 of wet autoclave and optimal medium are very similar. These findings show that the wet autoclaving
343 of WB might be necessary to improve *C. pinensis*' growth on this substrate.



344

345 **Figure 2.2** - Growths of *C. vaccinii* were done at 30°C, 100 rpm. Optical density was followed at 600
 346 nm. Optimal medium was LB. Ordinates were adapted depending on the growth medium for
 347 readability. n=3, brackets are SD.

348 Growth kinetics of *C. vaccinii* on WB were not impacted by the autoclave protocol (Figure 2.2) and is
 349 lower than optimal medium (max. OD : 8.48 ± 0.20 ; stat. phase reached at ≈ 70 h). During the kinetics,
 350 the WB was coloured in purple, showing that some violacein is secreted and binds to the substrate.



351

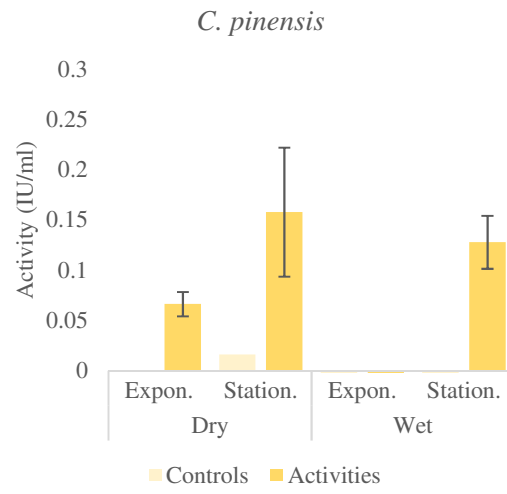
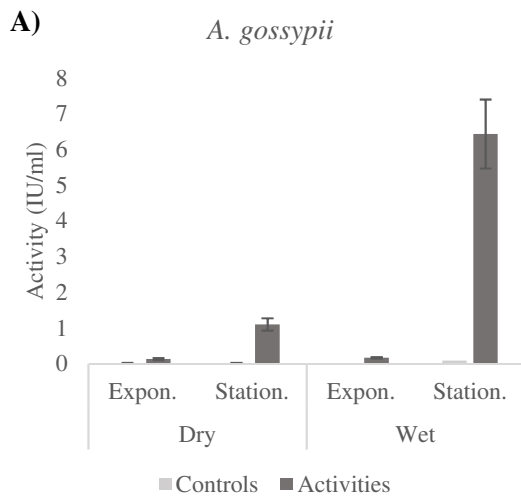
352 **Figure 2.3** - Growths of *G. alkanivorans* were done at 30°C, 100 rpm. Optical density was followed at
 353 600 nm. Optimal medium was Trypticase Soy Broth. Ordinates and abscissas were adapted depending
 354 on the growth medium for readability. n=3, brackets are SD.

355 *G. alkanivorans* growth was also possible on WB, but was much more lower than its optimal medium
356 (wet : 0.31 ± 0.03 ; dry : 0.48 ± 0.01 ; optimum : 0.48 ± 0.01)

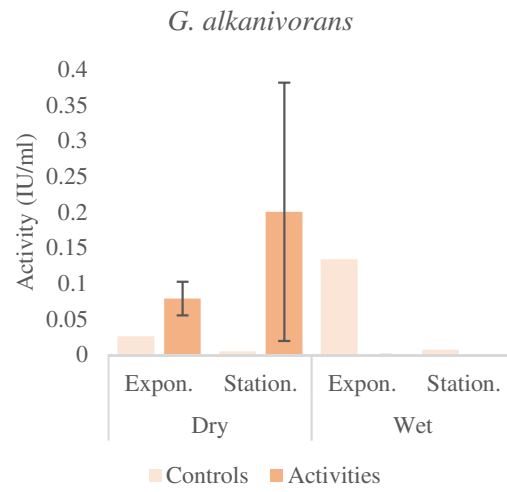
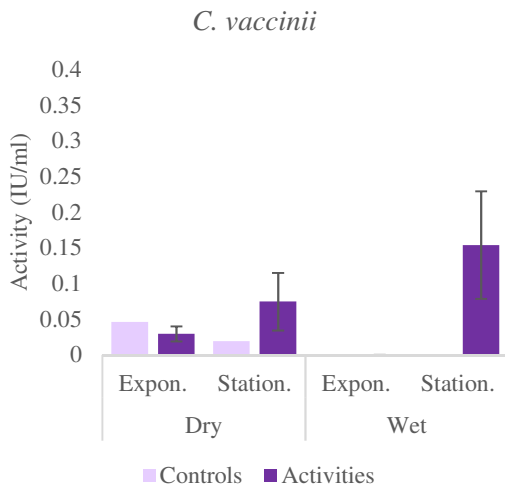
357 Overall, the type of autoclave has little impact on growth (except for *Chitinophaga*), and the
358 microorganisms reached early their stationary phase, which is quite low (ODs are always under 1). In
359 order to highlight which components from WB were used as carbon source by the strains, some
360 enzymatic activities produced by the strains during their growth were quantified, and the evolution in
361 composition of the WB was assessed by FTIR analysis.

362

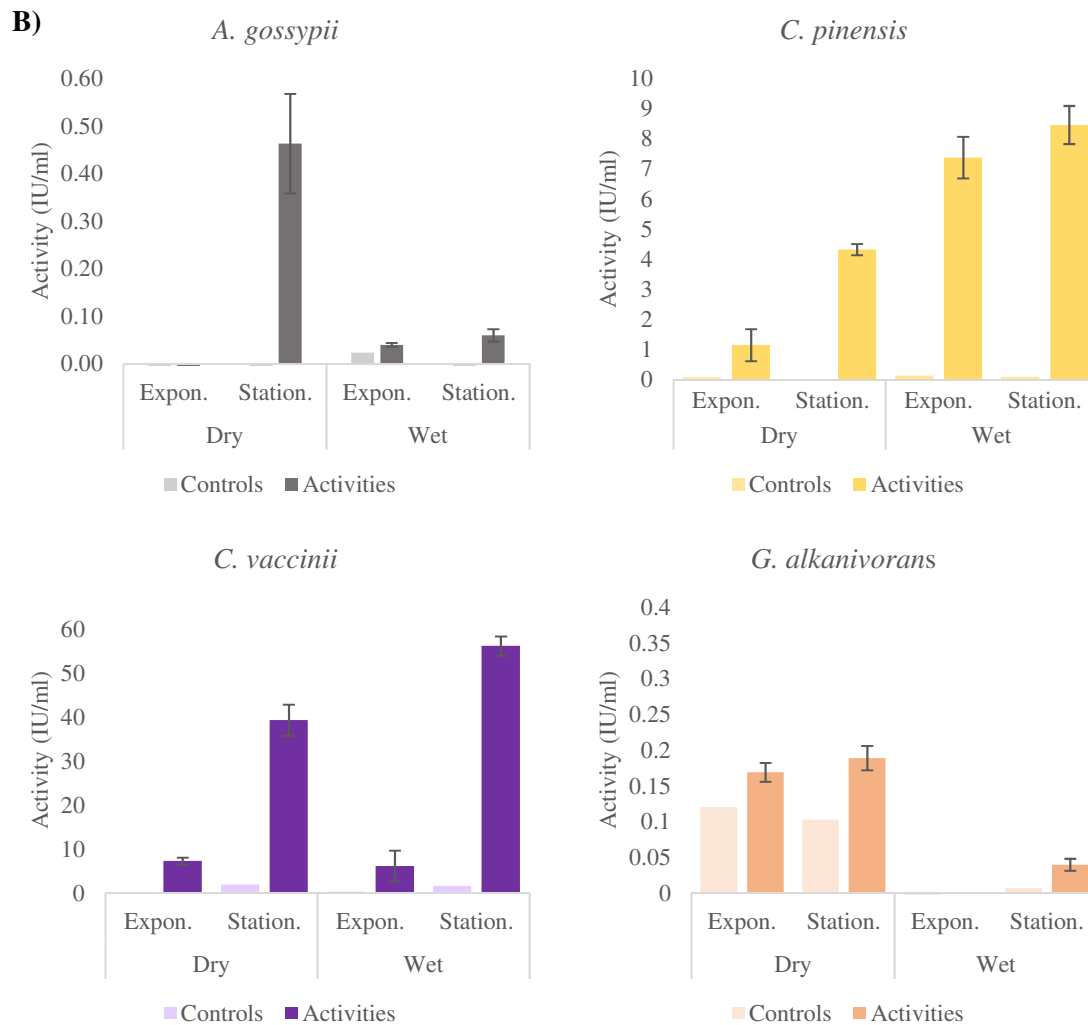
363 **3.4. Enzymatic activities**



364



365



366

367

368 **Figure 3** - Measured β -glucosidase and LAP activities for the four microorganisms. β -glucosidase was
 369 assessed for extra- and intra-cellular lysates combined. LAP was assessed for extracellular extracts
 370 only. Results are displayed according to the type of autoclave (dry or wet) and the growth phase
 371 (exponential or stationary). For a same condition, results obtained for controls (no microbial
 372 inoculation) are shown next to the results of inoculated cultures. n=3, brackets are SD. (A) - β -
 373 glucosidase activities. (B) - Extracellular LAP activities.

374 The saccharidic and protein content of the WB was precisely analysed according to the methods
 375 previously described. Its major components (in percent of dry weight) are glucose ($33.11\% \pm 6.7$),
 376 xylose ($21.63\% \pm 2.13$), protein (around 15.5%), arabinose ($12.12\% \pm 1.61$) and starch ($11.67\% \pm$
 377 0.38). Measured enzyme activities were chosen regarding this composition. Xylanase, α -amylase and

378 β -glucosidase activities were measured for the mixed intra- and extra-cellular enzymatic activities.
379 This allows an overview of the degradation mechanisms of WB displayed by the strains. Ligninolytic
380 activities were not measured here, as WB has poor contents in lignin (less than 5% DM).

381 3.4.1. α -amylase

382 Starch is a polymer of glucose units linked by α -1,4 and/or α -1,6 linkages that can be enzymatically
383 hydrolysed by the action of exo- and endo- α -amylases. No α -amylase activity was detected for the
384 four strains.

385 3.4.2. β -glucosidase

386 Almost a third of the dry weight mass composition is made of glucosidic units. In WB, glucose is a
387 constituent of cellulose, β -glucan and some hemicellulose. β -glucosidase enzymes are the final step for
388 a strain to produce D-glucose from such polymers. Results for the glucosidase activity assay (Figure
389 3A) showed no activity for *G. alkanivorans*, and results were low for *C. vaccinii* ($75 \text{ mIU.mL}^{-1} \pm 40$
390 for the dry-autoclave, stationary phase, with some controls having values of 46 mIU.mL^{-1}). Weak
391 glucosidase activity was visible for *C. pinensis* (up to $158 \text{ mIU.mL}^{-1} \pm 64$). and important activities
392 were measured for the stationary phases of *A. gossypii* ($6.44 \text{ IU.mL}^{-1} \pm 0.97$, wet autoclave).

393 3.4.3. Xylanase

394 The WB used in this study has important amounts of xylose and arabinose (21.63% and 12.12% of dry
395 weight, respectively), which are components of arabinoxylan, a highly present polymer in WB
396 (Ebringerová and Heinze 2000). No xylanase activity was detected for any of the four strains.

397 3.4.4. Leucine aminopeptidase

398 As mentioned earlier, WB contains a non-negligible amount of proteins (15.5%). Fermentation on
399 proteins is possible by some microorganisms (Lai et al. 2016), and proteolytic enzymes can be
400 produced on agricultural wastes (Oliveira et al. 2006; Mat'at'a et al. 2016). Leucine amino-peptidase
401 (LAP) is an ubiquitous enzyme that release N-terminal amino-acids of proteins, with a preference for
402 leucine, that is produced by numerous microbial strains (Gonzales and Robert-Baudouy 1996;

403 Wharam et al. 2008; Rahulan et al. 2012). LAP is secreted by each of the microorganisms in almost
404 every condition, except for *G. alkanivorans*, wet autoclave, exponential growth phase and *A. gossypii*,
405 dry autoclave, exponential growth phase (Figure 3B). *C. pinensis* and *C. vaccinii* showed high LAP
406 activities (maximums : $8.45 \text{ UI.mL}^{-1} \pm 0.63$ and $56.29 \text{ IU.mL}^{-1} \pm 2.18$, respectively). Their activities
407 were a bit lower when the microorganisms were grown on dry autoclaved WB. It was the opposite for
408 *A. gossypii* and *G. alkanivorans*, when an activity was measured.

409

410 **3.5. FTIR**

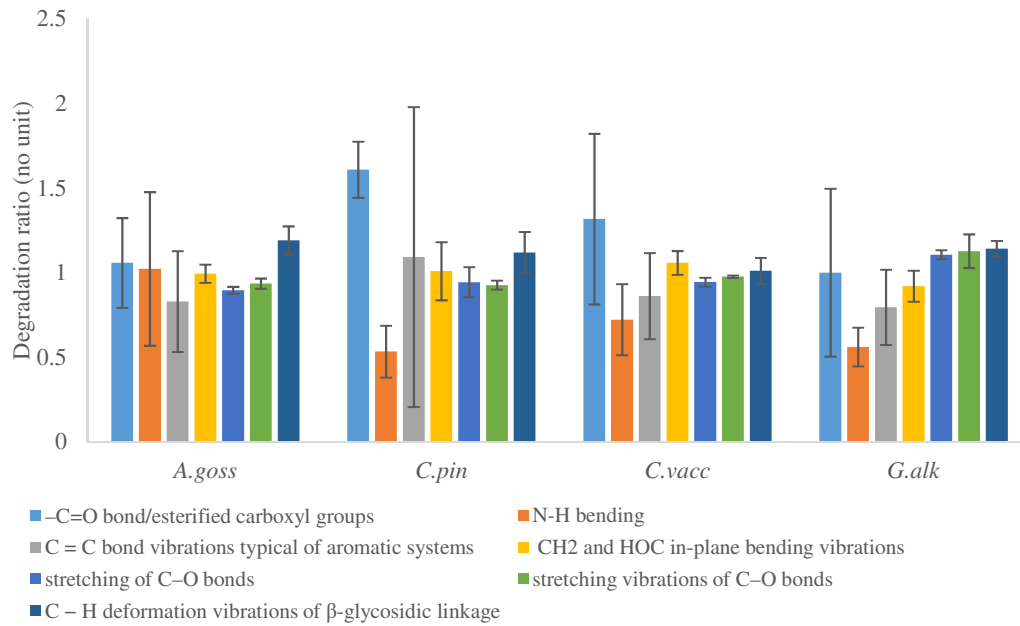
411 The use of FTIR is a relevant approach to highlight which components from lignocellulosic biomasses
 412 are modified after an enzymatic or microbial conversion (Sills and Gossett 2012; Waghmare et al.
 413 2014; Heidary Vinche et al. 2020). FTIR allows the association between some absorbances values
 414 with specific chemical liaisons. By extension, this technique can correlate absorbances with specific
 415 types of molecules constituted with these chemical liaisons. Absorbances used in this study and
 416 associated molecules are presented in Table 2.

417 **Table 2** - Associations of absorbances measured at specific wavelengths using FTIR with specific
 418 functional groups, and related wheat bran components (Fackler et al. 2011; Wang et al. 2013; Xu et al.
 419 2013; Lun et al. 2017; Heidary Vinche et al. 2020).

Wavelength (cm ⁻¹)	1732	1540	1510	1370	1100	1030	896
Functional groups	-C=O bond/esterified carboxyl groups	N-H bending	C = C bond vibrations typical of aromatic systems	CH ₂ and HOC in-plane bending vibrations	stretching of C-O bonds	stretching vibrations of C-O bonds	C - H deformation vibrations of β-glycosidic linkage
Associated molecules	Hemicellulose	Proteins (Amide II)	Lignin	Cellulose (mostly)	Phytate content	Polysaccharide	Cellulose (mostly)

420

421 Ratios of the absorbance values at a given wavelength of fermented WB over unfermented one were
 422 measured for each organism. Ratio values inferior to 1 usually indicate a consumption of the element
 423 during fermentation. Ratio values over 1 can either indicate a specific enrichment, more accessibility
 424 due to a degradation of surrounding components (Dong et al. 2019; Zhang et al. 2020), or (for proteins
 425 mostly) newly synthesised molecules



426

427 **Figure 4** - Degradation ratios determined by FTIR analyses and associated to specific type of
 428 molecular linkages. Each ratio is made with the Total Attenuated Reflectance value measured at a
 429 specific wavelength of dried WB and normalised. Ratios correspond to the value obtained with
 430 microorganisms growth over the value of WB treated with the same conditions but not inoculated with
 431 microorganisms. WB were wet- autoclaved. n=3, brackets are SD.

432 Overall, FTIR results indicate few changes in the composition of the WB after microbial growth
 433 (Figure 4). For *A. gossypii*, degradation ratios lower than 1 for stretching of C-O bonds (phytate
 434 content, 0.90 ± 0.05) and stretching vibrations of C-O bonds (polysaccharides, 0.93 ± 0.03) were
 435 observed. The C-H deformation ratio attributed to cellulose is increasing (1.23 ± 0.11) but the other
 436 ratios attributed to cellulose and hemicellulose do not variate. This means that no lignocellulose
 437 variation occurred from the microbial fermentation of WB. For *C. pinensis*, there is a moderate
 438 decrease in the stretching vibrations of C-O bonds (polysaccharides, 0.93 ± 0.03). Parallel to this
 439 decrease, the -C=O bound/esterified carboxyl group ratio is clearly increasing (hemicellulose, $1.61 \pm$
 440 0.17), indicating a change in the lignocellulose content. There is no evolution in the cellulose,
 441 hemicellulose, lignin and overall polysaccharide contents in *C. vaccinii*. An enrichment was measured
 442 for *G. alkanivorans* corresponding to the stretching of C-O bonds (phytate content, 1.11 ± 0.03).
 443 Another weak enrichment can be observed for one peak of cellulose (C-H deformation vibrations of

444 beta-glucosidic linkages, 1.14 ± 0.05) but not for the other (CH_2 and HOC in-plane bending
445 vibrations).

446 The 1540 cm^{-1} absorption associated to proteins is the most changing of FTIR values observed for all
447 strains in this study, except for *A. gossypii* for which there is no apparent variation (*C. pinensis*: $0.53 \pm$
448 0.15 ; *C. vaccinii*: 0.72 ± 0.21 ; *G. alkanivorans*: 0.56 ± 0.11). This clearly indicate a variation in the
449 protein content of the WB.

450

451 **3.6. Pigment production**

452 After reaching their stationary growth phases, pigments were extracted and concentration measured
 453 using specific protocols. Production of pigments was achieved for *C. pinensis*, *C. vaccinii* and *G.*
 454 *alkanivorans* on a lignocellulosic substrate, but not for *A. gossypii* (Table 3). Growth on their
 455 respective optimal medium led to pigment production, as it has already been demonstrated in the
 456 literature. As expected, pigment production was more important on optimal medium. Still, *C. pinensis*
 457 is not a strong flexirubin producer, and can produce almost half of its pigment on WB compared to the
 458 optimal medium (0.15 ± 0.09 compared to 0.27 ± 0.02). To assess the quality of the purification, an
 459 absorption spectrum was made on each extract. The observed molecular absorption coefficient (ϵ_{\max})
 460 were those expected, and the pics were isolated and characteristic of the pigments.

461 **Table 3** - Pigment concentration produced by the microorganisms and extracted with specific
 462 extractions and determined using absorbances at specific wavelengths. Concentrations are given in
 463 mg.L^{-1} . Results are compared between growth on optimal medium and specific minimal medium
 464 supplemented with WB at 5 g.L^{-1} . Mean values are given here. $n=3$.

	Riboflavin (<i>A. gossypii</i>)		Flexirubin (<i>C. pinensis</i>)		Violacein (<i>C. vaccinii</i>)		Carotenoids (<i>G. alkanivorans</i>)	
	Extracted (mg.L^{-1})	SD	Extracted (mg.L^{-1})	SD	Extracted (mg.L^{-1})	SD	Extracted (mg.L^{-1})	SD
Optimal medium	59.04	15.2	0.27	0.02	20.82	4,68	1.45	0.5
Min. medium with WB	0	-	0.15	0.09	1.47	0,29	0.07	0.02

465

466

467 **4. Discussion**

468 **4.1. CAZymes prediction and secreted peptidases analysis.**

469 All four genomes had CAZy families predicted to degrade starch, cellulose or hemicellulose (Table
470 S1). The number of total and secreted CAZymes predictions (Figure 1) could be compared to other
471 CAZy predictions. *Clostridia phytofermentas* is known for its good lignocellulolytic activities. It has
472 161 CAZymes predicted in total from its genome, which is in the same range as the organisms in this
473 study, and has a smaller proteome compared to them (3926 proteins, compared to 4259-6997 predicted
474 genes) (Tolonen et al. 2011). *C. pinensis* gives better predictions, with 325 theoretical total CAZymes
475 and 211 secreted. Another example can be found in the filamentous fungi *Pycnoporus cinnabarinus*,
476 which is known to have a great lignocellulosic activity and 315 predicted CAZymes (Levasseur et al.
477 2014). Even though predictions for *A. gossypii*, *C. pinensis*, *C. vaccinii* and *G. alkanivorans* are lower,
478 the analysis of their secreted CAZymes families showed potentially interesting lignocellulosic
479 activities.

480 Few studies on the fractionation of agro-industrial co-products focus on the fermentation on the
481 protein content. With this *in silico* study of secreted peptidases, this article propose an interesting new
482 approach for the selection of strains recycling plant biomasses. This, added to the types of pigments
483 that they were supposed to produce, encouraged the selection of these organisms for the rest of the
484 study.

485 **4.2 Growth kinetics of the different strains**

486 In the literature, when growth of *A. gossypii* is studied on a sole carbon source, it is usually on
487 monosaccharides such as glucose, fructose or mannose (Demain 1972). The use of wastes as carbon
488 sources is rare and is almost exclusively studied for lipids-rich wastes (corn oil, rapeseed oil, soybean
489 oil) (Demain 1972; Park et al. 2007) As expected, and regarding the size of the pellets that were
490 formed, growth on WB is not optimal and lower than those on pure monosaccharides. Thermal action
491 at high pressure under water vapour might induce a destructuration of the plant cell walls that could
492 improve their use as carbon source by this strain as it was described previously for other strains

493 (Biswas et al. 2015). This can explain the growth of *C. pinensis* depending on the type of autoclave.
494 However, this was not observed for the three other strains. Previous studies of the use of
495 polysaccharides as sole carbon source showed that *C. pinensis* was unable to grow on starch and
496 cellulose (McKee and Brumer 2015). This strain was also reported to use polysaccharides and
497 lignocellulosic biomasses such as glucomannans (maximum OD = 1.45), curdlan (maximum OD =
498 0.71), wheat arabinoxylan (maximum OD = 0.26) or spruce wood (maximum OD = 0.1) when added
499 to M9 medium (McKee et al. 2019).

500 As a relatively recent discovered species (Soby et al. 2013), no data are available for growth of *C.*
501 *vaccinii* on monosaccharides and polysaccharides. This study is the first to show its ability to grow on
502 a lignocellulosic biomass.

503 Growth of *G. alkanivorans* on alternative carbon source has been reported for sugar beet molasses
504 (Alves and Paixao 2014), artichoke juice (Pacheco et al. 2019) and recycled paper sludge (Alves et al.
505 2008). This study is the first demonstrating the use of lignocellulosic biomass for carotenoid
506 production by *G. alkanivorans*.

507 **4.3. Enzymatic activities**

508 As an element of the upcoming discussion, it has to be noted that there is no data available in the
509 literature for *C. vaccinii* regarding its consumption of starch, xylan, cellulose, as for any of the related
510 enzyme activities.

511 *4.3.1. α -amylase*

512 A previous study in *C. pinensis* showed its inability to hydrolyse starch (Sangkhobol and Skerman
513 1981). For *A. gossypii*, α -amylase has already been measured with an activity of ≈ 0.02 IU (dry weight
514 of cell)⁻¹ on a solidified agar-starch substrate (Ribeiro et al. 2013). In the article introducing *G.*
515 *alkanivorans* (Kummer et al. 1999), potato starch was hydrolysed by the strain, but no enzymatic
516 activity value was given. Interestingly, *C. vaccinii* has the potential to secrete a CAZyme combining
517 both a starch hydrolysing activity (GH15) and a module binding this substrate (CBM20). Therefore, it

518 is possible that WB is not sufficient to trigger some α -amylase activity in *A. gossypii*, *C. pinensis* and
519 *C. vaccinii*.

520 4.3.2. β -glucosidase

521 A semi-quantitative analysis for β -glucosidases (Kummer et al. 1999) showed a weak but positive
522 activity for *G. alkanivorans*, which contrasts with the results of this article. *C. pinensis* was previously
523 demonstrated to be unable to use cellulose (McKee et al. 2019). β -D-glucosidase activities in *C.*
524 *pinensis* could then act on oligosaccharides that might probably be derived from another polymer than
525 cellulose. *A. gossypii* was found early to be unable to degrade cellulose (Ribeiro et al. 2012), but β -
526 glucosidase activity was measured after growth on carboxymethylcellulose-agar (Ribeiro et al. 2013).
527 Since one of its predicted CAZymes (GH5) might specifically target cellulose in the crystalline state,
528 the state of cellulose could have a significant impact on the production for some of its enzymes.

529 4.3.3. Xylanase

530 For *G. alkanivorans*, it was predicted that xylose consumption does not occur regarding previous
531 articles (Kummer et al. 1999; Alves and Paixao 2014). *C. pinensis* is the only strain which was shown
532 to have the ability to grow on wheat arabinoxylan and wood glucuronoxylans (McKee and Brumer
533 2015). Growth on xylose led to a stationary phase at ≈ 0.4 (OD, no unit) [ibid.], which is a bit lower
534 than the mean 0.83 observed for the wet and dry autoclaved WB in this study.

535 4.3.4. Leucine Amino-Peptidase

536 *In silico* secreted proteasome analysis showed that all these strains had proteolytic potential, therefore
537 LAP activity, being ubiquitous, had definite chances to be observed. In the literature, no LAP
538 activities were previously measured for those four strains.

539 4.4. FTIR

540 For the four strains, no clear cellulolytic, hemicellulolytic or ligninolytic effect was visible
541 individually. Overall, the evolution of a single ratio does not have a clear effect on the others.
542 Decreases and increases in the FTIR absorbance values with fermentation seem more to indicate the

543 complex interaction between all the components of the WB. That is also why no clear correlation
544 between the *in silico* predictions and the FTIR results can be made.

545 Proteins are heterogeneously displayed in the different WB layers, with varying structures and
546 activities (Baladrán-Quintana et al. 2015). Thus, even if the interactions between the proteins of the
547 WB and its other components has not received major attention yet, multiple types of linkages can be
548 expected. This could explain the fact that the decreases in the 1540 cm^{-1} ratios does not have the same
549 effect on the other FTIR ratios depending on the strain. Ratios were unchanged for *C. vaccinii*, there
550 were slight specific enrichment for the 1100 cm^{-1} and 896 cm^{-1} bands in *G. alkanivorans*, and specific
551 enrichment only for 1732 cm^{-1} with *C. pinensis*. FTIR measurements seem to confirm that LAP is not
552 the main enzyme responsible for protein degradation of the WB, as the highest LAP activities for wet-
553 autoclaved WB (*C. vaccinii*) did not lead to the lowest 1540 cm^{-1} ratio. Also, LAP activity was
554 detected with a low level for *G. alkanivorans* and can probably not be responsible for the important
555 decrease in proteins contents within WB. Since LAP is not the only proteolytic activity in a given
556 organism, this activity in itself cannot be used to predict accurately the degradation of the protein
557 fraction of the WB. Though, it is still an interesting indicator of a general secreted proteolytic activity.
558 FTIR analysis confirms some changes in the state of the WB, with a clear indication of the use of its
559 protein content.

560 **4.5. Pigments production**

561 Concentrations in organic matter are quite important within the four optimal media (more than 15 g.L⁻¹)
562 ¹). In comparison, concentration of WB in minimal salt medium was set to 5 g.L⁻¹, mostly for
563 homogenisation reasons during cultures. The structure and the nature of the components are quite
564 different from those in an optimal medium. WB components are also less readily available for
565 consumption than those of an optimal medium. Therefore, yields of pigments were logically expected
566 to be lower on WB than optimal media. Nevertheless, it was demonstrated the possible use of a cheap
567 and abundant lignocellulosic co-product as carbon source for pigments production by some
568 microorganisms already known to produce them.

569 4.5.1. Riboflavin production

570 *A. gossypii* is known to be a strong producer of riboflavin (Kato and Park 2012). On a rich medium,
571 wild type strains can produce this pigment up to 5.2 g.L⁻¹ (Sugimoto et al. 2009). Riboflavin
572 overproduction is expected to appear when the culture reaches its stationary phase and the growth rate
573 declines (Kato and Park 2012). Since overproduction is known to be triggered by a variety of stresses
574 (nutritional, oxidative, ...) (Averianova et al. 2020), it can be hypothesized that the low amount of
575 microorganisms (Figure 3) at the end of the cultures was not enough to trigger these stresses.

576 4.5.2. Flexirubin production

577 Potent applications of flexirubin are its anti-cancer properties and treatment of tuberculosis (Venil et
578 al. 2016). This study is the first to our knowledge to quantify a flexirubin production by *C. pinensis*. In
579 comparison, the highest flexirubin production recorded in the literature was 521.64 mg.L⁻¹, obtained
580 by *Chryseobacterium artocarpi* CECT 8497 (Venil et al. 2015). Liquid pineapple waste has also been
581 used as a mean to reduce the overall cost of production for the same species, and yielded 200 mg.L⁻¹ of
582 flexirubin (Aruldass et al. 2016). Still, flexirubin quantification in the literature is often given in dry
583 weight of pigment after solvent evaporation so comparisons have to be done cautiously.

584 4.5.3. Violacein production

585 *C. vaccinii* is known to produce both violacein and deoxyviolacein by a process of quorum sensing
586 (Soby et al. 2013). Violacein has many interesting properties for bioproduction, as anticancer,
587 antibacterial, antifungal and anti-leishmanial agent (Kothari et al. 2017). A previous attempt to
588 quantify violacein production in *C. vaccinii* in Nutrient Broth medium gave 975 μg.L⁻¹ (Palaniswamy
589 and Vishnu 2016). *Chromobacterium violaceum* was used on immobilized sugar-cane bagasse in a
590 column system alone to produce 150 mg.L⁻¹ of violacein (Ahmad et al. 2012). The use of liquid
591 pineapple waste 10% (v/v) in rich Nutrient Broth medium for this strain led to an increase of 46 mg.L⁻¹
592 compared to NB alone (Aruldass et al. 2015).

593 4.5.4. Carotenoids production

594 Carotenoids are widely used as food colorants, and their consumption is linked to multiple health
595 benefits (Chandi and Gill 2011). Overall, *G. alkanivorans* gave poor results for carotenoid production
596 (Table 3). Carotenoid production by *G. alkanivorans* has not been studied on sole lignocellulosic
597 components. However, the *Gordonia* genera is well known for its carotenoid production (Arenskotter
598 et al. 2004). *G. alkanivorans* is one of the best producers of carotenoids, with one of its highest
599 production being of 2.5–3.1 mg.(g of dry cell weight)⁻¹ in a minimum salt medium containing glucose
600 and sulphate (Fernandes et al. 2018). Therefore, an efficient release of glucose from cellulose could
601 greatly impact carotenoid production. Also, it has to be noticed that since the use of light is important
602 for carotenoid production in *Gordonia* species (Fernandes et al. 2018), adjusting this factor can be
603 essential for improving productivity.

604

605 **6. Conclusion**

606 In the end, this study shows that known pigment producers from the literature can be rationally chosen
607 on their ability to use renewable carbon sources as a substrate. A precise knowledge of the
608 components in a substrate allows to interrogate specific enzyme databases to select potent candidates
609 able to degrade and feed on it. Structural studies of the substrate with FTIR combined to enzymatic
610 assays are interesting tools to analyse complex changes in the composition of the fermented substrate.
611 More importantly, this study contributes to the discovery of new methods for the renewable
612 production of molecules of interest. Best candidates from this study will be chosen for a deeper
613 analysis of their consumption of WB, in order to improve biomass recycling and pigment production.

614

615 **Author contributions**

616 The manuscript was written through contributions of all authors. All authors have given approval to
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625 **Declaration of competing interest**

626 The authors declare no conflict of interest.

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