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46 1. Introduction

47
48 The measurement of fungal growth on solid substrates is difficult and a number
49 of methods have been developed to try and address the problem (Baldrian et al., 2013;
50 De Ruiter et al., 1993). Ergosterol measurement is a widely recognised biomarker for
51 assessing fungal biomass in solid media or soil (Niemenmaa et al., 2008). Ergosterol is
52 the main sterol in fungal cell membranes, but is only a minor component of plants (de
53 Ridder-Duine et al., 2006; Nielsen and Madsen, 2000; Pasanen et al., 1999). It is a
54 major component of mycelia, spores, and vegetative cells (Newell, 1992; Pasanen et al.,
55 1999) and plays a role in membrane fluidity, cation permeability and cell growth
56 (Hippelein and Rugamer, 2004). Ergosterol has been recommended for quantifying
57 fungal growth, as there is a good relationship between ergosterol content and hyphal
58 length (Pasanen et al., 1999; Schnürer, 1993).

59 The use of a conversion factor (250 mg biomass mg⁻¹ ergosterol), to calculate
60 fungal biomass from ergosterol concentration was suggested by Montgomery et al.,
61 (2000) in a study of 6 fungi including *Penicillium* and *Trichoderma spp.* However, the
62 amount of ergosterol can vary depending on the fungal species, period of culture, stage
63 of development, and growth conditions (Newell, 1994; Pasanen et al., 1999; Schnürer,
64 1993) and therefore the use of a standard conversion factor for all fungi is inappropriate
65 (Klamer, 2004). Despite the limitation of ergosterol as an indicator of fungal biomass, it
66 has been applied to a wide range of environments such as soil (Gong et al., 2001;
67 Ruzicka et al., 2000, 1995), building material (Hippelein and Rugamer, 2004), indoor
68 environment (Flannigan, 1997), house dust (Saraf et al., 1997), grain (Börjesson et al.,
69 1990), seeds (Richardson and Logendra, 1997), plant litter (Gessner and Newell, 2002),
70 plant material (Newell, 1992), agar media and wood (Niemenmaa et al., 2006).

71 Measuring ergosterol has been reported to be more accurate than other fungal
72 biomass estimation methods that measure the production of molecules such as chitin or
73 adenosine triphosphate (ATP) (Klamer, 2004). Phospholipid fatty acid (PLFA)
74 quantification is another method that has been suggested as a complementary approach
75 to quantify fungal biomass (de Ridder-Duine et al., 2006; Klamer, 2004). Total PLFA
76 content has been shown to positively correlate with bacterial or fungal biomass, and it
77 can simultaneously distinguish the fingerprint of microbial communities (Frostegard
78 and Baath, 1996). The PLFA linoleic acid (18:2n-6) in particular has been identified as
79 a biomarker of fungal biomass since it has been estimated to constitute up to 45% of
80 fungal dry mass (Federle, 1986) and is absent in bacteria.

81 Total PLFA assay has been used to measure *P. chrysosporium* biomass during
82 cultivation in potato dextrose agar (PDA) (Klamer, 2004) and on rice straw (Yu et al.,
83 2009). The relationship between the specific PLFA (18:2n-6) and ergosterol content
84 was calculated to be linear and to give a conversion factor of 1.47 μmol 18:2n-6 to 1
85 mg ergosterol using 12 different fungal species in PDA culture (Klamer, 2004). In pure
86 culture (Eiland et al., 2001) recorded a value of 2.1 μmol 18:2n-6 to 1 mg ergosterol.
87 PLFA (18:2n-6) depending on growth and the type of ecosystem (e.g soil and
88 compost). However, Lechevalier and Lechevalier (1988) reported that PLFA does not
89 provide a good taxonomic marker, as there is a possibility of obtaining similar fatty
90 acid fingerprints for both Ascomycete and Basidiomycete fungi, and it will not
91 distinguish different species.

92 The measurement of biomass diversity and soil composition is becoming
93 increasingly important. The strengths and weaknesses of many of the techniques used
94 in forest soils was reviewed by Wallander et al., (2013). This review highlighted the

95 use of conversion factors for some species, it did not however consider how fungal
96 species variation may bias the overall findings. Measurement of phospholipid fatty
97 acid concentration has been widely used to study microbial community structure (Amir
98 et al., 2008; Daquiado et al., 2013; Francisco et al., 2016; Frostegård et al., 2011;
99 Klamer and Baath, 1998). However a lack of comprehension of the production of
100 PLFAs by different microorganism can result in misinterpretation and consequently
101 flawed diversity indices. During our studies to measure lignocellulose (wheat straw)
102 conversion by decay fungi we aimed to quantify the increase in biomass during solid
103 state cultivation. As a consequence we were able to show differences in accuracy of
104 the published biomarkers to determine biomass accrual of the different fungi tested.
105 Here we describe the most effective methodological approach to quantify fungal
106 biomass in solid state cultivation. A multivariate statistical analysis was also employed
107 as a powerful tool to distinguish the PLFA fingerprint for each fungal type.

108

109

110 **2. Materials And Methods**

111

112 **2.1. Microorganisms and culture preparation**

113

114 Members of three Basidiomycota class Agaricomycetes taxonomic orders were
115 tested and compared: two species from the Polyporales (*Phanerochaete chrysosporium*
116 and *Postia placenta*), *Schizophyllum commune* from the Agaricales and *Serpula*
117 *lacrymans* from the Boletales. Pure cultures of four species of fungal mentioned above
118 were supplied by the Warwick Life sciences collection and retrieved from a cold room
119 (4°C) and were grown on malt extract agar (MEA). Agar plugs of mycelia were added
120 to rye grain and grown to produce inocula (grain spawn) for the solid state cultivation.

121

122

123 **2.2. Solid state cultivation preparations**

124 Wheat straw was obtained locally from Warwick Life Sciences farm and
125 chopped into small pieces (about 1-2 cm length), 10 g was placed into honey jars (250
126 ml) with 13 ml distilled water and autoclaved twice (121°C for 1 hour). The prepared
127 straw was inoculated with 1 g of grain spawn of the appropriate fungal species and
128 incubated at the optimal temperature for each fungus (*P. chrysosporium* at 37°C; *S.*
129 *commune* and *P. placenta* at 25°C; while *S. lacrymans* at 20°C). Every 7 days post
130 inoculation 3 jars were harvested. For the ergosterol measurement 1g of homogenized
131 sample was used.

132

133 **2.3. Ergosterol assay**

134 Ergosterol levels must be shown to correlate with fungal biomass in pure liquid
135 culture before it can be used as a biomarker in solid state cultivation experiments
136 (Messner, 1998). The four fungi were initially cultured in liquid culture using 250 ml
137 malt extract liquid medium (Sigma Aldrich-70146) and cultivated for 35 days at 20°C
138 for *S. lacrymans*; 25°C for *S. commune* and *P. placenta* and 30°C for *P. chrysosporium*,
139 without agitation. The goal for culturing the fungi in liquid media was to collect fungal
140 mycelium and link biomass accrual to ergosterol content and standard curves of each
141 fungal species were performed. Montgomery et. al, (2000) suggested to use conversion
142 factor calculated from fungal biomass and ergosterol. By modifying that approach, the
143 conversion factor to biomass was derived from liquid culture of four different fungi.

144 Mycelia were harvested and freeze-dried for 24 hours before biomass
145 quantification and the measurement of ergosterol using a modified method developed
146 by Gong et al., (2001) which involved physical disruption of the mycelium using two

147 different acid washed glass beads (10 mg of 212-300 μm diameter and 10 mg of 710-
148 1180 μm diameter). The different aliquots of mycelium were resuspended in 2 ml
149 ethanol, vortexed for 10 seconds in a 20 ml scintillation vial and placed into a basket in
150 an orbital shaker for approximately 1 hour at 25°C; 350 rpm in darkness. The samples
151 were allowed to sediment for 15 minutes before a 1.5 ml aliquot was removed into 2 ml
152 microfuge tube and centrifuged for 10 minutes at 11.000 rpm and 4°C. The supernatant
153 was filtered (0.2 μm) and the filtrate transferred into 1ml dark vials before being loaded
154 into an auto sampler for HPLC analysis. Ergosterol was quantified using a
155 LiChrosphere (5 μm) C₁₈ reverse column (Merck Millipore, United Kingdom), with UV
156 detection at 282 nm (diode array detector; Agilent 1100 series G1315B). Methanol
157 (HPLC grade; Fischer Scientific, United Kingdom) was used as the mobile phase with a
158 flow rate of 1.5 ml min⁻¹, a column pressure of 1.15x10⁷ Pa, and a column temperature
159 25°C. Ergosterol content was calculated as microgram per gram of fungal mycelium.
160 The amount of ergosterol was then compared with the standard (Sigma-Aldrich) and a
161 conversion factor derived with which to estimate biomass for each fungus.

162

163 **2.4. Extraction and analysis of PLFA**

164

165 Straw cultures were squeezed through muslin to extract excess water. The solid
166 biomass remaining was placed into centrifuge test tubes and centrifuged at 11,000 rpm
167 (approximately 13,000g) for 5 minutes. 200 ml 3:1 dichloromethane (CH₂Cl₂) and
168 ethanol was added to the biomass cake, samples were left overnight in the fume
169 cupboard on an orbital shaker (Heidolph-Unimax) at 100 rpm and filtered with a 7 μm
170 Whatman GF/A (Grade Filter A). The solvent filtrate was transferred to a rotary
171 evaporator-water bath, this was achieved by setting to 70°C, 125 rpm and 100 ml of

172 dichloromethane was added to re-dissolve the precipitate. This solution was then
173 transferred to glass vials, and left to evaporate over 24 hours until a dry precipitate
174 remained. 0.5 mg of precipitate was extracted and 10 μ L 15:0 TAG (triacylglycerol)
175 internal standard (25 mg ml⁻¹ tripentadecanoin (Sigma T4257)) was added. Each sample
176 was mixed with 500 μ L 1 N HCl/MeOH vortexed and transferred to an oven at 80°C
177 and left to incubate for 10 hours. To release the fatty acids, 250 μ L 0.9% KCl was
178 added followed by the addition of 800 μ L hexane and vortexed. The resulting layers
179 were allowed to separate for 10 minutes. Approximately 500 μ L of the upper hexane
180 layer was transferred to a fresh vial and stored at 4°C until used for fatty acid methyl
181 ester measurement. An automatic sampler unit of the GC/MS (Agilent technology 6850
182 network GC system) was used to transfer and inject the samples into A GC column
183 (BPx70-0.2 μ m x 10m x 0.1mm). The following conditions were used: carrier gas:
184 nitrogen, constant flow mode, 30ml/min; oven temperature program 150°C (0.1 min),
185 15°C/min to 240°C (6 min); detector: flame ionization detector 240°C; injection
186 volume: 1 μ L.

187

188 **2.5. Statistical analysis**

189 All results were analysed using analysis of variance (ANOVA) in (Gentstat-
190 Release 11, VSNI-UK) to determine the significance of differences between each
191 samples in ergosterol, fungal biomass, total PLFA and linoleic acid analysis. Before
192 statistical analyses, the normality of the results were checked and plotted in two-
193 dimensional graphs. Where appropriate least square difference (LSD) was used to
194 determine any significant differences between treatments. MANOVA was used for
195 determining the significance difference (P<0.05) in FAME (Fatty Acid Metil Ester) in

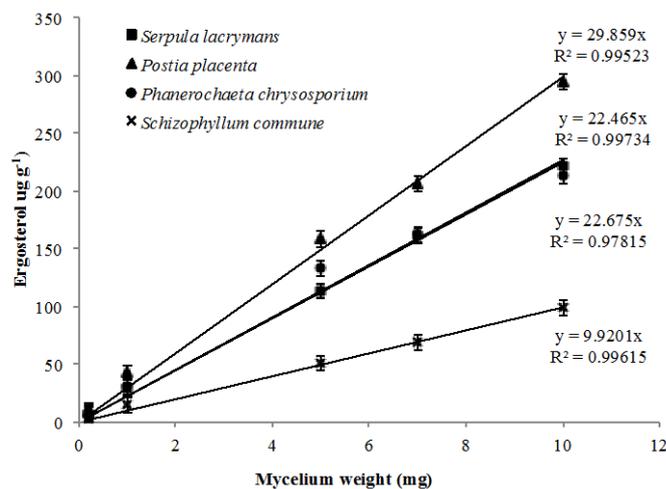
196 each treatment. Canonical Variate Analysis (CVA) was used to examine the effect of
197 treatments on the structure of the microbial community. 95% confidential intervals were
198 used to determine the significance of differences between treatments. The results were
199 plotted in 2 dimensional graphs where the first axis (X) represented 41% of variation
200 and the second axis (Y) accounted for 19%.

201 3. Results

202

203 3.1. Ergosterol and fungal biomass

204 The ergosterol content per mg mycelium was found to differ significantly
205 ($P < 0.001$, $LSD = 6.67$) between the fungi tested in malt extract liquid medium. *P.*
206 *placenta* liquid medium cultures contained the highest amount of ergosterol at $294.7 \mu\text{g}$
207 g^{-1} ergosterol per dry weight of mycelium. Mycelia from *P. chrysosporium* and *S.*
208 *lacrymans*, contained similar amounts of ergosterol at approximately $200 \mu\text{g g}^{-1}$, while
209 *S. commune* was the lowest at $98.4 \mu\text{g g}^{-1}$ (Figure 1).



210

211 Figure 1. The relationship between ergosterol concentration and the amount of
212 mycelium for the fungi *P. chrysosporium*, *P. placenta*, *S. commune* and *S. lacrymans* in
213 malt extract liquid medium. Error bars represent LSD ($P < 0.05$), each point is an average
214 from 3 replicates

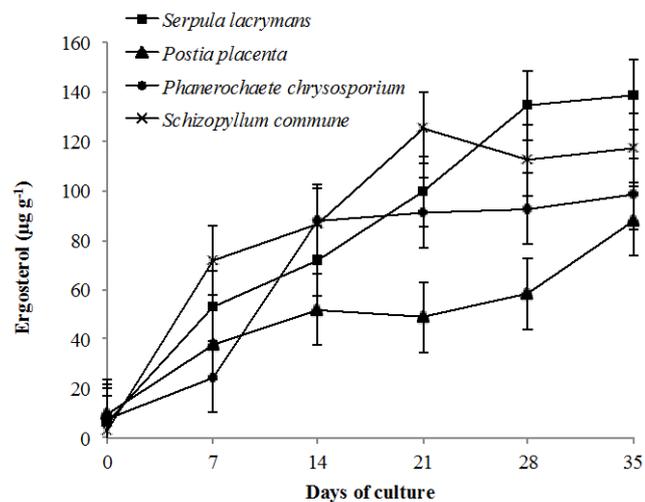
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217 Based on these values, a conversion factor allowing an estimation of mycelium
218 weight from total ergosterol was derived for each of the four different fungi (Table 1).
219 These were used to monitor the growth of each fungus in wheat straw solid state
220 cultivation over the course of 35 days.

221 The amount of ergosterol produced by all fungi increased during growth on
222 wheat straw solid state cultivation. The ergosterol content of *P. chrysosporium*
223 increased to 98.6 $\mu\text{g g}^{-1}$ wheat straw dry weight after 14 days then levelled off. *S.*
224 *lacrymans* values continued to increase throughout the duration of culture peaking at
225 138.70 $\mu\text{g g}^{-1}$ at day 35 *S. commune* produced the highest amount of ergosterol 125.361
226 $\mu\text{g g}^{-1}$ over 21 days but this subsequently plateaued. The lowest yield of ergosterol
227 during the fungal growth (35 days) was found with *P. placenta*, which reached a peak of
228 87.79 $\mu\text{g g}^{-1}$ (Figure 2).

229



230

231 Figure 2. The amount of ergosterol produced by *P. chrysosporium*, *P. placenta*, *S.*
232 *commune* and *S. lacrymans* during the fungal growth on wheat straw solid state
233 cultivation (35 days) Error bars represent LSD (P<0.001), each point is an average from
234 3 replicates

235

236

237 Using the conversion factors calculated in Figure 1, the fungal biomass in
 238 wheat straw cultures for each time point was estimated (Table 1).

239 Table 1. Predicted fungal biomass content (mg) in solid media culture (wheat straw) in
 240 mg g⁻¹ as calculated from ergosterol measurements.

Fungal Species	Conversion factor	7 days	14 days	21 days	28 days	35 days
<i>Serpula lacrymans</i>	22.46	2.36	3.18	4.43	5.99	6.17
<i>Postia placenta</i>	29.85	1.26	1.73	1.63	1.94	2.94
<i>Phanerochaete chrysosporium</i>	22.67	1.08	3.88	4.01	4.08	4.35
<i>Schizophyllum commune</i>	9.92	7.21	8.73	12.63	11.31	11.81

242

243 3.2. PLFA patterns in white and brown rot fungi

244 Phospholipid fatty acid quantification was carried out on the upper layer of
 245 solvent extraction on samples taken over the duration of culture (0, 7, 14, 21, 28 and 35
 246 days). Monitoring of the total PLFA in the four fungi tested indicated that only for one
 247 fungus (*Postia placenta*) was a significant increase detected (Figure 3a).

248 The composition of the total PLFA was also determined and changes in the
 249 relative abundance (%) of each FAME were measured from 0 to 35 days after
 250 incubation for each of the fungi. The most abundant fatty acid methyl esters were found
 251 to be 16:0 (palmitic acid), 18:2n-6 (linoleic acid), and 18:1n9c (oleic acid) although the
 252 proportions of each varied depending on the fungus and time of culture (Table 2).

253

254 Table 2. The amounts of the predominant fatty acids produced by as a percentage of the
 255 total fatty acid during 35 days incubation.

Fungus	Fatty acid	(%) of total fatty acid	Substrate
<i>P. chrysosporium</i>	16:0	19.36	Wheat straw solid state cultivation (35 days)
	18:2n6c	21.40	
	18:1n9c	8.78	
<i>S. commune</i>	16:0	25.39	Wheat straw solid state cultivation (35 days)
	18:2n6c	44.54	
	18:1n9c	16.99	
<i>S. lacrymans</i>	16:0	19.45	Wheat straw solid state cultivation (35 days)
	18:2n6c	49.97	
	18:1n9c	10.38	
<i>P. placenta</i>	16:0	22.25	Wheat straw solid

18:2n6c	26.90	state cultivation (35
18:1n9c	6.73	days)

256

257 The amounts of 18:2n-6 in cultures of the four fungi sampled at different times
 258 of incubation are shown in Figure 3b. The linoleic acid range was 167.46-541.37 $\mu\text{g g}^{-1}$
 259 (*P. chrysosporium*); 186.92-345.78 $\mu\text{g g}^{-1}$ (*S. lacrymans*); 188.49-506.15 $\mu\text{g g}^{-1}$ (*P.*
 260 *placenta*) and 66.35-288.93 $\mu\text{g g}^{-1}$ (*S. commune*). The four fungi exhibited different
 261 patterns of production of linoleic acid during the culture period (Figure 3b).

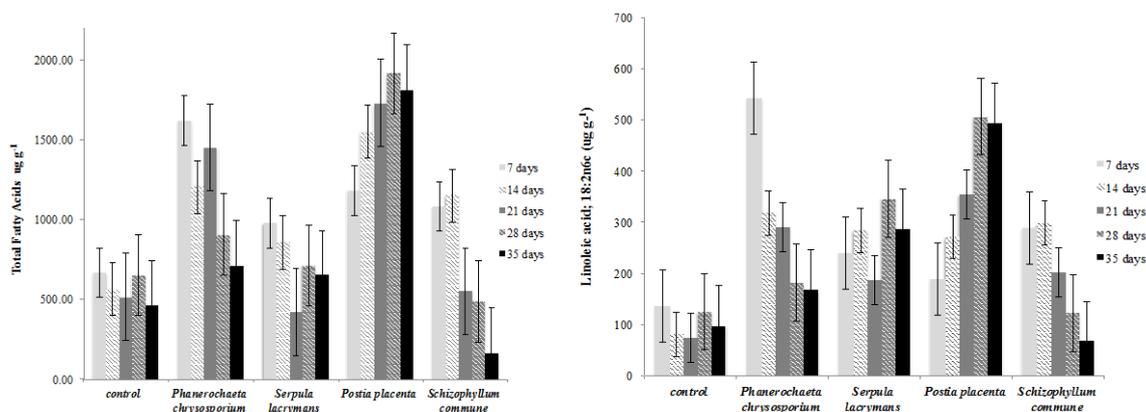


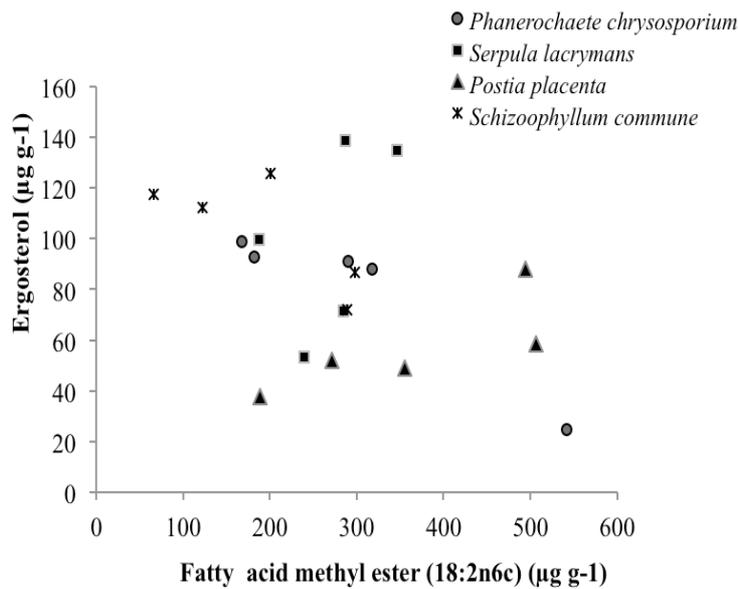
Figure 3. The amount of total fatty acids (3a) and linoleic acid (18:2n-6) (3b) extracted from wheat straw solid state cultivation (35 days) of four different fungi. Error bars represent LSD ($P < 0.05$), each point is an average from 3 replicates

262

Multivariate analysis/canonical variance analysis (CVA) was performed to test

263

for correlation between ergosterol and abundance of 18:2n6c (Figure 4).



264

265 Figure 4. Correlation between ergosterol ($\mu\text{g g}^{-1}$) and PLFA (18:2n6c) ($\mu\text{g g}^{-1}$) content
 266 from wheat straw solid state cultivation. Each point is an average from 3 replicates.
 267

268 The correlation for *P. placenta* differed compared to the other fungi as a positive
 269 correlation could be seen, whilst no separation was found between *P. chrysosporium*
 270 and *S. lacrymans*. *S. commune* fatty acid was separated from the other fungi through the
 271 second axis (Y). The relationship between ergosterol and fatty acid methyl ester varies
 272 between fungal type hence could not be generalized. Each fungus behaves differently.
 273 The only strong correlation was found with *P. chrysosporium* (-0.94), which displayed a
 274 negative correlation (Figure 4).

275

276 4. Discussion

277 Applying the calculated ergosterol conversion factors (Table 1) to the cultures
 278 after 28 days in liquid culture gave estimations of mycelia dry weight biomass from
 279 98.4 to 294.7µg depending on the species. These values are higher than some ergosterol
 280 measurements obtained previously e.g. *S. commune* and *P. chrysosporium* grown on
 281 Potato Dextrose Agar (PDA) and Carboxymethyl Cellulose Agar (CMC) for 12 and 24

282 days at 24°C under dark conditions, in which ergosterol measurements ranged between
283 1-24 mg g⁻¹ dry weight mycelium (Klamer, 2004). In this study, the amount of
284 ergosterol in wheat straw solid state cultivation (98.6 µg g⁻¹) was higher than the
285 ergosterol content found in fungi growing on wood substrates, which was 23.0 µg g⁻¹
286 for *P. chrysosporium* (Niemenmaa et al. 2006). However they are lower than previous
287 measurements of 210(±26) µg g⁻¹ reported on ADMS (asparagine ammonium nitrate
288 dimethylsuccinate) media for the fungi used in this study (Niemenmaa et al., 2008).
289 Nutritional mode has been suggested to influence the level of ergosterol recorded, for
290 example the brown rot fungus *Gloeophyllum trabeum* produced 3.9 mg g⁻¹ in liquid
291 medium (Niemenmaa et al., 2008). In the same study, the brown rot *P. placenta* yielded
292 1.37 mg g⁻¹ ergosterol which was similar to the white rot, *P. chrysosporium* at 1.39 mg
293 g⁻¹ (Niemenmaa et al., 2008), suggesting the phylogenetic relationship of being in the
294 Polyporales, rather than difference in nutritional mode, may be a greater influence on
295 ergosterol levels.

296 These findings are consistent with the ergosterol contents reported in this study,
297 i.e. the highest ergosterol content in liquid medium was detected in *P. placenta*,
298 followed by *P. chrysosporium* and *S. lacrymans*, whilst the lowest was found in *S.*
299 *commune*. The differences between all the studies emphasize the impact of different
300 media and growth conditions on ergosterol levels. This means that transferring the
301 conversion factors to mycelia grown in different substrates may lead to inaccuracies.
302 This is highlighted in the application of the technique in this study during the
303 measurement of *S. commune* on wheat straw solid state cultivation. With *S. commune*,
304 the biomass conversion factors from liquid media appeared to be too low and
305 consequently the estimation of the biomass growth on straw was higher than we would

306 expect based on observed growth. All four fungi showed an increase in ergosterol
307 amounts during culture indicating that it is a reasonable proxy for measuring fungal
308 growth. The pattern of production for all the fungi was broadly similar showing little
309 statistical difference suggesting that all were capable of utilizing straw despite their
310 perceived prevalence for wood.

311 The difficulty in comparing relative growth between fungi because of potential
312 discrepancy caused by inaccurate conversion values led to the testing of PLFA as an
313 alternative to assay fungal biomass in solid state cultivation. The composition of PLFA
314 in the fungi and the dominant fatty acids in each sample were evaluated and described
315 (Table 2). Total PLFA was only found to increase over the culture period for *P.*
316 *placenta*. The other fungi showed a decline in total PLFA suggesting that this is not a
317 suitable measurement of fungal biomass accrual in the system tested. The major PLFAs
318 identified within the four Basidiomycetes were similar. Most studies utilised fatty acid
319 profiling of Basidiomycetes in liquid medium (Klamer, 2004; Müller et al., 1994; Stahl
320 and Klug, 1996). However, data from solid medium and information on sequential
321 production of fatty acids over the duration of fungal culture is limited. While this study
322 revealed the contribution and change of each PLFA during solid state cultivation, our
323 data suggest that it is not a good proxy measurement of fungal biomass accrual when
324 compared with ergosterol quantification.

325 The relationship between linoleic acid (18:2n-6) corresponded with the pattern
326 of total PLFA production. Only *P. placenta* recorded an increase in total PLFA and
327 linoleic acid in relation to time in culture, indicating a potential link to fungal biomass.
328 *S.lacrymans* showed no clear pattern of production of these fatty acids while a negative
329 correlation was found with both *S. commune* and *P. chrysosporium* (Figure 4).

330 Previously, a correlation between PLFA 18:2n6c and fungal biomass was demonstrated
331 using fungus grown in liquid medium (Eiland et al., 2001; Klamer, 2004), however few
332 studies have been tested its potential using solid medium (Liu et al., 2017; Yu et al.,
333 2009). It has also been used to study fungi in soil (Frostegard and Baath, 1996). The
334 findings of this experiment do not support the previous research by Klamer, (2004),
335 who reported that there was a linear correlation ($R^2 = 0.782$), between ergosterol and
336 PLFA 18:2n6c among 11 species of fungi using agar media (e.g, CMC, PDA). Our
337 results indicate that the use of PLFA 18:2n6c as an indicator of fungal biomass may not
338 be appropriate. Consequently its use in studying soils may bias the diversity findings
339 due to the observed production within some fungi versus utilisation within others.

340

341 **5. Conclusion**

342 Assessment of fungal biomass accrual is important when determining the rate of
343 decomposition of lignocellulosic material for example in forest leaf litter or in a solid
344 state biorefinery. This study highlights how reliance on some methods developed for
345 liquid laboratory cultures may not be fully applicable to field scenarios due to the
346 variability in metabolites produced depending on the media the fungi utilise.
347 Application of both ergosterol and measurement of PLFA may give erroneous results
348 due to the difference in production and utilisation of these metabolites by different
349 fungi. Despite this overall measurement of ergosterol proved to be consistent and
350 allowed an estimation of fungal biomass to be made. Some inaccuracies due to
351 differences in the conversion factors used may still arise.

352

353

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355

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359

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361

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