A Comparison of ergosterol and PLFA methods for monitoring the growth of ligninolytic fungi during wheat straw solid state cultivation

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HIGHTLIGHTS

- Ergosterol measurements were found to correspond with increased fungal biomass
- Amount of ergosterol produced differed depending on fungal species.
- Total PLFA and linoleic acid could be used to quantify *Postia placenta.*
- In the other fungi tested the measurement of fatty acids did not correspond with ergosterol values.

ABSTRACT

Ergosterol, total phospholipid fatty acid (PLFA) and linoleic acid (18:2n-6) have all been used to determine fungal growth. This paper compares these methods to assess the growth of four different saprotrophic fungal species during solid state cultivation using a wheat straw substrate. Ergosterol production appeared to track the mycelia growth well but its production differed considerably between fungi. This means that a specific conversion factor needs to be determined and applied for any given fungus. In comparison, measurements of total PLFA and linoleic acid only showed promise for determining the growth of *Postia placenta* due to the positive correlation with ergosterol measurements. In contrast, the other fungi tested (*Phanerochaete chrysosporium*, *Serpula lacrymans* and *Schizophyllum commune*) showed either no correlation or in some cases a negative correlation using this assay. The findings highlight the variation in fungal fatty acid between species, culture conditions and durations of incubation; suggesting that measurement of linoleic acid may be useful only in specific cases. These findings provide important consideration for the study of fungi growing in solid substrates and suggest that the use of PLFA bias diversity indices.

Keywords : ergosterol; phospholipid fatty acid; fungal biomass; linoleic acid; solid state cultivation; wheat straw

1. Introduction

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

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

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

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

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

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

2. Materials And Methods

2.1. Microorganisms and culture preparation

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

2.2. Solid state cultivation preparations

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

2.3. Ergosterol assay

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

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

different acid washed glass beads (10 mg of 212-300 µm diameter and 10 mg of 710-1180 µm diameter). The different aliquots of mycelium were resuspended in 2 ml ethanol, vortexed for 10 seconds in a 20 ml scintillation vial and placed into a basket in an orbital shaker for approximately 1 hour at 25ºC; 350 rpm in darkness. The samples were allowed to sediment for 15 minutes before a 1.5 ml aliquot was removed into 2 ml microfuge tube and centrifuged for 10 minutes at 11.000 rpm and 4ºC. The supernatant was filtered (0.2 µm) and the filtrate transferred into 1ml dark vials before being loaded into an auto sampler for HPLC analysis. Ergosterol was quantified using a LiChrosphere (5µm) C_{18} reverse column (Merck Millipore, United Kingdom), with UV detection at 282 nm (diode array detector; Agilent 1100 series G1315B). Methanol (HPLC grade; Fischer Scientific, United Kingdom) was used as the mobile phase with a flow rate of 1.5 ml min^{-1}, a column pressure of $1.15x10^7$ Pa, and a column temperature 25°C. Ergosterol content was calculated as microgram per gram of fungal mycelium. The amount of ergosterol was then compared with the standard (Sigma-Aldrich) and a conversion factor derived with which to estimate biomass for each fungus.

2.4. Extraction and analysis of PLFA

Straw cultures were squeezed through muslin to extract excess water. The solid biomass remaining was placed into centrifuge test tubes and centrifuged at 11,000 rpm (approximately 13,000g) for 5 minutes. 200 ml 3:1 dichloromethane (CH_2Cl_2) and ethanol was added to the biomass cake, samples were left overnight in the fume cupboard on an orbital shaker (Heidolph-Unimax) at 100 rpm and filtered with a 7 µm Whatman GF/A (Grade Filter A). The solvent filtrate was transferred to a rotary evaporator-water bath, this was achieved by setting to 70°C, 125 rpm and 100 ml of

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

2.5. Statistical analysis

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

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

3. Results

3.1. Ergosterol and fungal biomass

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

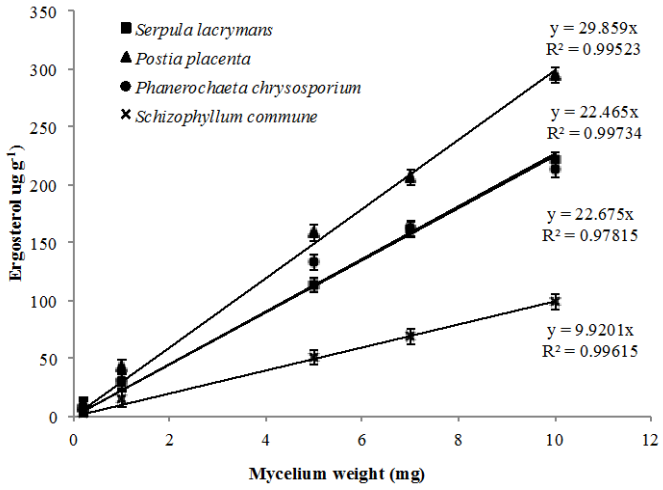

Figure 1. The relationship between ergosterol concentration and the amount of mycelium for the fungi *P. chrysosporium, P. placenta, S. commune* and *S. lacrymans* in malt extract liquid medium. Error bars represent LSD ($P<0.05$), each point is an average from 3 replicates

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

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

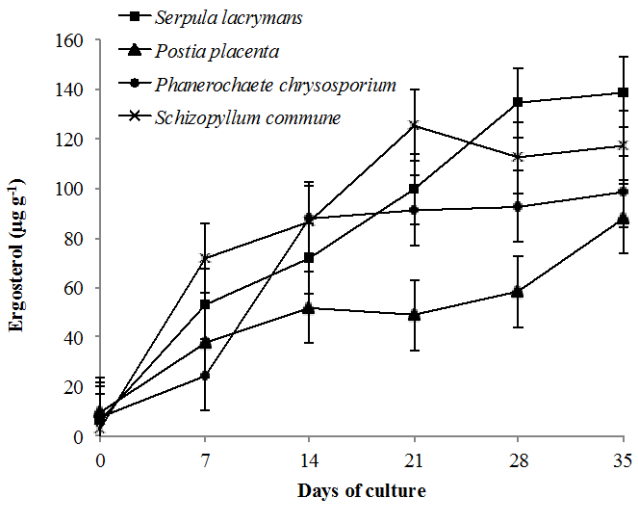

Figure 2. The amount of ergosterol produced by *P. chrysosporium, P. placenta, S. commune* and *S. lacrymans* during the fungal growth on wheat straw solid state cultivation (35 days) Error bars represent LSD (P<0.001), each point is an average from 3 replicates

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

Table 1. Predicted fungal biomass content (mg) in solid media culture (wheat straw) in mg g^{-1} as calculated from ergosterol measurements.

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

3.2. PLFA patterns in white and brown rot fungi

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

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

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

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

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

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 167.46-541.37µg g^{-1}
259 (*P. chrysosporium*); 186.92-345.78 µg g^{-1} (*S. lacrymans*); 188.49-506.15 µg g^{-1} (*P.*
260 *placenta*) and 66.35-288.93 µg g^{-1} (*S. commune*). The four fungi exhibited different
261 patterns of production of linoleic acid during the culture period (Figure 3b).

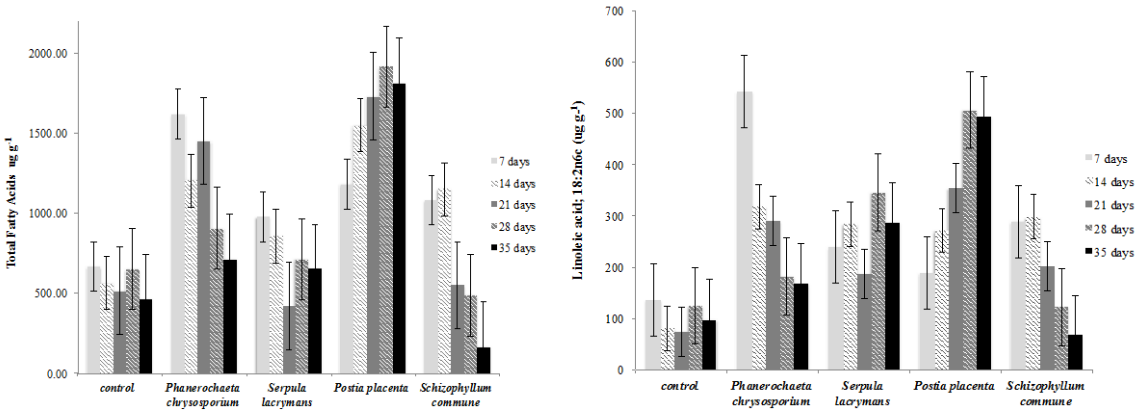

Figure 3. The amount of total fatty acids (3a) and linoleic acid (18:2n-6) (3b) extracted from wheat straw solid state cultivation (35 days) of four different fungi. Error bars represent LSD ($P<0.05$), each point is an average from 3 replicates

262 Multivariate analysis/canonical variance analysis (CVA) was performed to test
263 for correlation between ergosterol and abundance of 18:2n6c (Figure 4).

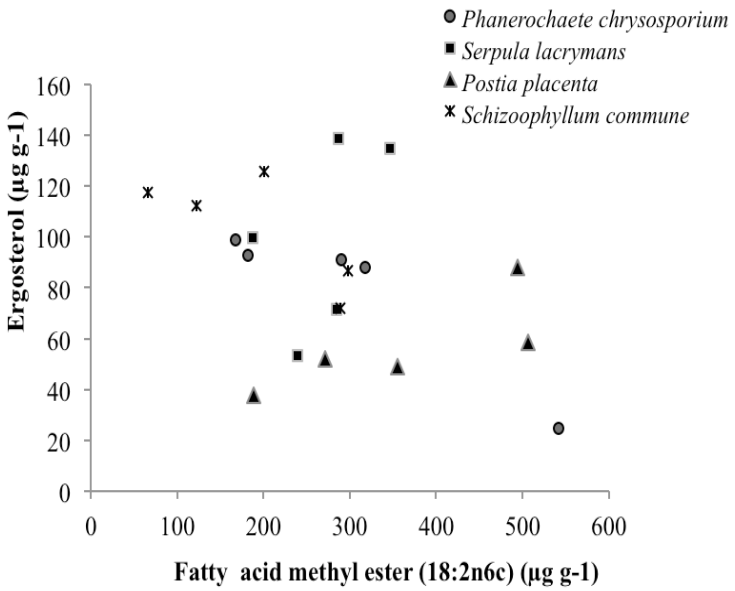

Figure 4. Correlation between ergosterol (µg g^{-1}) and PLFA (18:2n6c) (µg g^{-1}) content from wheat straw solid state cultivation. Each point is an average from 3 replicates.

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

4. Discussion

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

282 days at 24ºC 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).

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

5. Conclusion

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

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