EFFECT OF NON-CONVENTIONAL CARBON SOURCES ON THE PRODUCTION OF TRITERPENOIDS IN SUBMERGED CULTURES OF *Pleurotus* MACROFUNGII

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ABSTRACT

Among the secondary metabolites of pharmacological, chemical and biotechnological interest, triterpenoidal compounds are widely distributed throughout the fungi kingdom. Triterpenoids produced by species of the genus *Pleurotus*, basidiomycetes characterized by their important biological actions and excellent nutritional quality, can be obtained by fermentation processes that stimulate the production of the bioactive compounds. This study evaluates the effect of using 12 non-conventional carbon sources (CS) on the production of triterpenoids from 3 *Pleurotus* species: *P. ostreatus*, *P. djamor* and *P. pulmonarius* obtained in submerged culture. The variations found among these species with regard to their quantities and structural differences were dependent on the CS as well as the species, and it was concluded that the cultivation of the mycelium of *P. pulmonarius* with wheat bran promotes the production of the compounds of interest. In addition no direct influence of the C/N ratio of the substrate on the biosynthesis of the triterpenoids was observed, as reported in the case of other secondary metabolites such as polysaccharides and statins.

Keywords: *Pleurotus*, triterpenes, cereals, liquid-state fermentation

INTRODUCTION

Members of the kingdom Fungi have been widely recognized as one of the world's most biodiverse natural resources. For ages both wild and cultivated mushrooms have been known for their nutritional and culinary values as well as viewed as tonics and used as medicines by humans. Certain mushrooms are abundant sources of a wide range of useful natural products. Indeed, various compounds including terpenoids, steroids, phenols, alkaloids and nucleotides, that have been isolated and identified from the fruiting body, culture mycelium and culture broth of mushrooms are shown to have promising biological effects, preventing a range of diseases such as hypertension, hypercholesterolemia, diabetes and cancer that are predominant in developed Western countries¹. The biosynthesis of these compounds is determined by the climatic conditions that surround them and the type of nutrients that form the substrate on which they develop. The compositions of both wild and cultivated mushrooms depend on various environmental factors, such as the pH and composition of the substrate, oxygenation, temperature, humidity, the size of the pellet and the stirring rate^{2,3}, making these species valuable biofactories of secondary metabolites and promising sources for new pharmaceuticals.

Among the secondary fungal metabolites, triterpenoids are characteristic macromycete fungi components that exhibit anti-infective, cytotoxic, immunomodulatory, antitumor⁴⁻⁷ hypocholesterolemic^{8,9}, antibiotic¹⁰, anti-inflammatory^{9,11}, antifungal¹², insecticidal, antimicrobial^{9,13-16}, antiviral¹³, cytostatic¹³, intestinal cholesterol absorption reducing¹⁷, and antioxidant¹⁸ activities, among others. Given that they are the most abundant metabolite in macromycetes and given the influence that the substrate has on their production by the fungus¹⁹, the study of triterpenoids is a research priority.

Among the macromycete fungi, the genus *Pleurotus* is well known for its many uses, which ranging from being a highly nutritious food^{16,20,21} to having organoleptic qualities^{22,23}, and being used for medicinal purposes. A large number of biological activities result in the production of metabolites, with antioxidant effects, which include the following: antimicrobial²⁴, hypocholesterolemic^{9,25}, antiallergenic¹³, anti-inflammatory²⁶, antifungal, and analgesic²⁷. In addition, the vast enzyme pool of these species allows them to grow on a wide variety of substrates²⁸. The aforementioned qualities have caused an exponential increase in the scientific community's interest in developing processes to obtain either the mycelium or the fruiting bodies more quickly and with improved culture efficiency.

Although fungi are usually grown by conventional cultures, their product quality and the productivity of the desired metabolites can be difficult to control. Fermentation in the submerged state, also known as liquid-state fermentation (LSF), is a promising technology with regard to efficient production of these valuable compounds. Studies have already been conducted to determine the factors affecting the production of fungal mycelium, including

broth composition, oxygenation levels, agitation rates, and different culture strategies²⁹⁻³². In addition, the cost of the medium is important and could limit the production. Recent studies have shown that using non-conventional CS such as some cereal flours, for the production of *Ganoderma lucidum* and *Grifola frondosa* can reduce traditional laboratory costs by as much as 98%, with a 3.8% increase in biomass production and a 36.02% increase in exopolysaccharides³³⁻³⁵.

This study evaluates the effects of 12 non-conventional CS on the triterpenoid production of 3 *Pleurotus* species obtained by liquid-state fermentation.

EXPERIMENTAL

Preparation of fungal material

Strains of *Pleurotus ostreatus* (BioVeg Fungi-002), *Pleurotus pulmonarius* (BioVeg Fungi-001), and *Pleurotus djamor* (BioVeg Fungi-005) from the culture collection of the Biotechnology Laboratory of the University of Antioquia were maintained on potato dextrose agar (PDA) and stored at 4°C. Pieces of agar of 0.5 to 1cm in diameter containing mycelial inocula were transferred to Petri dishes with of the following medium in g/L: CS, 30; yeast extract, 3; sucrose, 5; agar, 8. The pH was 5.5 ± 0.1. The following CS were used: barley flour (*Hordeum vulgare* L. BR), oatmeal (*Avena sativa* L. OM), wheat flour (*Triticum aestivum* L. WT), rice flour (*Oryza sativa* L. RC), bienestarina (vegetable mixture flour BT), corn flour (*Zea mays* L. CR), soybean flour (SY), wheat bran (WB), whole-wheat flour (WW), pinto corn flour (CP), seven-grain flour (SG), and yellow corn flour (YC). The cultures were incubated at 26°C in the dark for 15 days. Subsequently, 250ml flasks containing 62 mL of the following medium in mg/L were supplemented with different CS: NaNO₃, 80; MgSO₄·7H₂O, 20; KH₂PO₄, 30; KCl, 10. The pH of the medium was adjusted to 5.6 ± 0.1, and the flasks were autoclaved. One-gram pieces of agar (0.5 to 1cm in diameter) containing mycelia were used as the inoculum. Cultures were incubated at 100 rpm for 9 days at 25 ± 1 °C.

Determining the C/N ratio of carbon sources

The C and N contents were determined using a FLASH 2000 CHNS/O Analyzer (Thermo Scientific) which uses dynamic flash combustion (modified Dumas method) to determine the elements in a sample. The experimental data were then analyzed for multivariate using Statgraphics 5.1 software, and results were obtained in triplicate for each case.

Obtaining the *Pleurotus* extracts

The fermentation products were filtered, separating the mycelium from the broth. The broth was lyophilized, and fresh mycelia were extracted with EtOAc. The extracts were dried with anhydrous Na₂SO₄, evaporated to dryness in a rotary evaporator, and later characterized by GC-MS using a Hewlett Packard 6890 gas chromatograph with a DB5 capillary column (30 m, 0.33 mm ID, 25 µm) coupled to a 5973 mass spectrometer with a 70-eV ionization source under the following conditions: helium 4.5 to 1.1 mL/min; splitless

mode; injector temperature, 300 °C; and heating rate, 60 °C for 1 min, then increased by 7.4 °C/min up to 310 °C. The extract components were identified by analyzing the fragmentation patterns obtained from their mass spectra and comparing them with the equipment's software and literature reports.

Determination of triterpenoid and sterol content in the *Pleurotus* extracts

The Lieberman-Burchard colorimetric method was used to determine the sterols concentration in the EtOAc mycelia extracts³⁶ using the following standard solutions (prepared by dilution in CHCl₃): 0.071mg/ml, 0.14 mg/ml, 0.21mg/ml, 0.28mg/ml, 0.35mg/ml, 0.43mg/ml, 0.5mg/ml, 0.64 mg/ml and 0.71 of stigmasterol. The experimental data were then analyzed for multivariate using Statgraphics 5.1 software based on triplicates results for each case.

The relative proportions of all triterpenoidal compounds identified (triterpenes and sterols) were determined using the relative areas obtained from the GC analysis as a percentage of the total relative areas of the compounds identified.

RESULTS AND DISCUSSION

Studies of the triterpenoidal composition of the macrofungi species from the genus *Pleurotus* have allowed for the isolation and identification of a major series of compounds with ergostane, cholestane, and stigmastane skeletons. These compounds vary in the numbers and positions of the double bonds on their rings and side chains and in the presence of substituent groups, such as polyhydroxy, epidioxy, and keto groups on the hexahydronaphthalene moiety³⁷⁻⁴⁶. *P. ostreatus* has received more research attention than the other species, particularly compared to *P. djamora* (for which there is only one publication⁴⁶). For the first time, the present study describes the triterpenoidal composition of *P. pulmonarius* and the effect of the substrate on the production of triterpenoids by LSF using non-conventional CS in fungi of the genus *Pleurotus*.

Biotechnological product separation was performed in mycelium and broth to determine both the intra- and exo-metabolites. None triterpenoids were identified in the broth regardless of the CS or species tested, eliminating the possibility of excretion processes by the fungus, a peculiarity that has been observed in fungi of the genus *Ganoderma* cultivated by this type of process (unpublished data) and on *Rhizopus oligosporus* which ergosterol excreted in proportions close to 4%⁴⁷.

The compounds were identified using MS and an analysis based on diagnostic peaks (Table 1) as well as by comparison with the literature. Most of the compounds have already been reported for *Pleurotus* and other basidiomycetes and, in some cases, for lichens and plants (Table 2). Of the 14 triterpenes, 8 correspond to ergostane, 5 to stigmastane, and 1 to cholestane. With regard to their substituents, the compounds identified are classified as 2 triterpenes, 7 sterols, and 5 steroidal ketones. No reports have yet been published regarding the presence of 5 α -stigmast-3-one, ergostan-3-one, 6-hydroxystigmast-4-en-3-one and stigmast-4-en-3-one in fungi. There is, however, an interesting hypothesis regarding bacteria in which the formation of some steroidal ketones is generated by sterol oxidation. A predominant product formed in this way is sitosterol, which leads to the formation of stigmast-4-en-3-one via the oxidation of 3 β -OH to an oxo group and, later, a C4-C5 double-bond isomerization that leads to the formation of a 4-en-3-one derivative. This reaction is catalyzed by a bifunctional enzyme, cholesterol oxidase, that enables some non-pathogenic bacteria to use sterols as CS and enables certain pathogenic bacteria to infect host macrophages by altering the physical structure of the membrane lipids, oxidizing cholesterol to the keto derivative⁴⁸. The abovementioned sitosterol derivative is a promising candidate for the treatment of endocrine disorders⁴⁹. Identification of these steroidal ketones in *Pleurotus* species grown in LSF opens the field to research regarding the formation mechanism of this group of compounds in mushrooms.

Table 1. Characteristic fragmentations determined via mass spectrometry of compounds obtained from mycelia of the *Pleurotus* species.

COMPOUND	t _R (min)	M ⁺	CHARACTERISTIC FRAGMENTS
I	34.029	394	379 (M ⁺ -CH ₃), 376 (M ⁺ -H ₂ O), 361 (M ⁺ -CH ₃ -H ₂ O), 269 (M ⁺ -SCH), 251 (M ⁺ -SCH-H ₂ O), 209 (M ⁺ -CH ₃ -Fission ring D)
II	32.259	376	361 ((M ⁺ -CH ₃), 251 (M ⁺ -CL), 235 (M ⁺ -CL-CH ₃ -H), 225 (M ⁺ -Fission ring D), 209 (M ⁺ -Fission ring D-CH ₃), 156 (M ⁺ -Fission ring C-CH ₃), 141 (M ⁺ -Fission ring C-CH ₃).
III	32.677	374	359 (M ⁺ -CH ₃), 303 (M ⁺ -C ₅ H ₁₁), 275 (M ⁺ -C ₇ H ₁₅), 249 (M ⁺ -C ₉ H ₁₇), 233 (M ⁺ -C ₉ H ₁₇ -CH ₃ -H), 217 (M ⁺ -C ₉ H ₁₇ -2CH ₃ -H).
IV	34.409	396	381 (M ⁺ -CH ₃), 378 (M ⁺ -H ₂ O), 363 (M ⁺ -CH ₃ -H ₂ O), 337 -C ₄ H ₁₁), 271 (M ⁺ -SCH), 253 (M ⁺ -SCH-H ₂ O), 229 (M ⁺ -Fission ring D-H ₂ O), 211 (M ⁺ -CH ₃ -Fission ring D), 159 (M ⁺ -Fission ring C- H ₂ O), 143 (M ⁺ -Fission ring C- H ₂ O- CH ₃), 128 (M ⁺ -Fission ring C- H ₂ O-2CH ₃).
V	34.524	398	383 (M ⁺ -CH ₃), 380 (M ⁺ -H ₂ O), 365 (M ⁺ -H ₂ O-CH ₃), 300 (M ⁺ -C ₇ H ₁₄ -2H), 273 (M ⁺ -SCH), 271 (M ⁺ -SCH-2H), 253 (M ⁺ -SCH -2H-H ₂ O), 246 (M ⁺ -Fission ring D), 231 (M ⁺ -Fission ring D), 213 (M ⁺ -Fission ring D-H ₂ O), 160 (M ⁺ -Fission ring C-H ₂ O), 145 (M ⁺ -Fission ring C-H ₂ O-CH ₃).
VI	35.546	414	399 (M ⁺ -CH ₃), 396 (M ⁺ - H ₂ O), 381 (M ⁺ -CH ₃ -H ₂ O), 273 (M ⁺ - SCH), 255 (M ⁺ -SCH-H ₂ O), 246 (M ⁺ -Fission ring D), 213 (M ⁺ -Fission ring D-H ₂ O-CH ₃), 163 (M ⁺ -Fission ring C-CH ₃), 145 (M ⁺ -Fission ring C-H ₂ O-CH ₃).
VII	35.680	416	401 (M ⁺ -CH ₃), 398 (M ⁺ -H ₂ O), 383 (M ⁺ -CH ₃ -H ₂ O), 275 (M ⁺ - SCH), 273 (M ⁺ -SCH-2H), 257 (M ⁺ -SCH-H ₂ O), 233 (M ⁺ -Fission ring D-CH ₃), 215 (M ⁺ -Fission ring D-H ₂ O), 180, 147.
VIII	36.190	414	399 (M ⁺ -CH ₃), 273 (M ⁺ -SCH), 246 (M ⁺ -Fission ring D), 231 (M ⁺ -Fission ring D-CH ₃), 217 (M ⁺ -CO-H).
IX	39.752	428	413 (M ⁺ -CH ₃), 410 (M ⁺ -H ₂ O), 287 (M ⁺ -SCH), 245 (M ⁺ -Fission ring D-C ₃ H ₆), 227 (M ⁺ -Fission ring D- C ₃ H ₆ -H ₂ O).
X	34.438	392	377 (M ⁺ -CH ₃), 267 (M ⁺ -SCH), 265 (M ⁺ -SCH-2H), 293 (M ⁺ -CH ₃ -C ₆ H ₁₂), 251 (M ⁺ -CH ₃ -C ₆ H ₁₂ -C ₃ H ₆), 240 (M ⁺ -Fission ring D), 225 (M ⁺ -SCH-2H-Fission ring D), 223 (M ⁺ -SCH-2H-Fission ring D-2H).
XI	36.483	408	393 (M ⁺ -CH ₃), 390 (M ⁺ -H ₂ O), 375 (M ⁺ -CH ₃ -H ₂ O), 365 (M ⁺ -C ₃ H ₇), 297 (M ⁺ -SCH), 281 (M ⁺ -SCH-CH ₃ -2H), 271 (M ⁺ -Fission ring D-CH ₃), 253 (M ⁺ -Fission ring D-CH ₃ -H ₂ O), 191 (M ⁺ -Fission ring C), 185 (M ⁺ -Fission ring C-H ₂ O-2H).
XII	37.252	412	397 (M ⁺ -CH ₃), 370 (M ⁺ -C ₃ H ₆), 355 (M ⁺ - C ₃ H ₆ -CH ₃), 314 (M ⁺ -C ₃ H ₆ -CH ₃ -CO-CH), 271 (M ⁺ -SCH), 229 (M ⁺ -Fission ring D), 124.
XIII	35.146	400	385 (M ⁺ -CH ₃), 273 (M ⁺ -SCH), 246 (M ⁺ -Fission ring D), 231 (M ⁺ -Fission ring D-CH ₃), 217 (M ⁺ -Fission ring D-CO-H), 163 (M ⁺ -Fission ring C-CH ₃).
XIV	34.605	400	382 (M ⁺ -H ₂ O), 315 (M ⁺ -C ₆ H ₁₃), 367 (M ⁺ -H ₂ O-CH ₃), 273 (M ⁺ - SCH), 213 (M ⁺ -H ₂ O-CH ₃ -Fission ring D).

Table 2. Triterpenoidal compounds identified from mycelia of *Pleurotus* cultured in LSF using non-conventional CS.

NUMBER	COMPOUND	PREVIOUSLY IDENTIFIED IN:	REFERENCE
I	Ergost-5,7,9(11),22-tetraen-3 β -ol	<i>Pleurotus sajor-caju</i> cultured in LSF	⁵⁰ ENREF_12
II	Ergost-2,5,7,9(11),22-pentaene	<i>Pleurotus sajor-caju</i> cultured in LSF	⁵⁰ ENREF_12
III	Ergost-2,5,7,9(11),14,22-hexaene	<i>Suillus luteus</i> and native <i>Laetiporus sulphureus</i>	51, 52
IV	Ergost-5,7,22-trien-3 β -ol	<i>Pleurotus sajor-caju</i> cultured in LSF and native <i>P. ostreatus</i>	21, 37, 43, 50
V	Ergost-7,22-dien-3 β -ol	<i>Pleurotus sajor-caju</i> cultured in LSF and native <i>P. ostreatus</i>	21, 37, 43, 50
VI	Stigmast-5-en-3 β -ol	<i>Laetiporus sulphureus</i> and native <i>Inonotus obliquus</i>	53, 54
VII	Stigmastanol	<i>Pleurotus djamor</i> native	⁴⁶ ENREF_1
IX	6-Hydroxystigmast-4-en-3-one	Plants and cane bagasse	55, 56
X	Ergosta-4,6,8(14),22-tetraen-3-one	<i>Pleurotus ostreatus</i> and <i>Pleurotus sajor-caju</i> cultured in LSF	50, 57
XI	4,4-Dimethyl-cholesta-5,7,14,22-tetraen-3-ol	<i>Aphylllophoral</i>	58
XII	Stigmast-4-en-3-one	Lichens, plants and <i>Papulaspora immersa</i>	49, 59
XIV	Ergosta-5-en-3 β -ol	<i>Pleurotus sajor-caju</i> native.	²¹ ENREF_15

The total sterol content in the extracts depends on the species and the CS utilized (Figure 1). Quantification of the compounds (Figure 1) reveals clear differences. The use of WB produces more sterols on *P. djamor*. In the case of *P. pulmonarius*, the amounts obtained showed no statistically significant differences when WB and BR are used. In *P. ostreatus*, the highest sterol content was obtained using WB and YC. With respect to the relationship between species and amount of sterols obtained, *P. pulmonarius* produced a higher content than *P. ostreatus* and *P. djamor* using seven of the CS, followed by *P. djamor*, for which the contents obtained using 4CS are the largest, and finally by *P. ostreatus* using SY, for which the content showed no statistically significant differences compared to *P. pulmonarius*.

Figure 1. Sterols present in EtOAc extracts from *Pleurotus* mycelia obtained in LSF with different substrates.

BR barley flour, **OM** oatmeal, **WT** wheat flour, **RC** rice flour, **BT** bienestarina, **CR** corn flour, **SY** soybean flour, **WB** wheat bran, **WW** whole-wheat flour, **CP** pinto corn flour, **SG** seven-grain flour, and **YC** yellow corn flour. The results are given after multiple comparisons, based on significant differences, of results obtained via the CS for each species. The mean values obtained from 3 repetitions are indicated by letters **a** to **j**. Two mean values accompanied by the same letter indicate that these means were not significantly different from each other; otherwise, different letters indicate significant differences.

The use of different substrates to grow the fungi produced clear differences in the compounds obtained from the mycelium of *P. ostreatus* (Table 3). A large number of compounds (at low quantities) were identified in BR. No triterpenes production was confirmed in OM, WT, RC, or SY, and only small quantities were present in BT, CR, WW, and CP, for which there were no distinct structural variations compared to the compounds present in WB and YC (in which the triterpenes content reached 41.5 and 32.3%, respectively). SG produced only 12.8% stigmast-5-en-3 β -ol, the most common sterol in *P. ostreatus* extracts.

Table 3. Triterpenoidal compounds identified in *Pleurotus ostreatus* mycelium obtained in LSF by using non-conventional CS.

SUBSTRATE	TRITERPENOID ^a											
	I	II	IV	V	VI	VII	VIII	X	XI	XII	XIII	TOTAL
CB	NQ	NQ		NQ			NQ	NQC	NQ	NQ		NQ
OM												0
WT												0
RC												0
BT	NQ				NQ							NQ
CR					NQ		NQ					NQ
SY												0
WB		2.9			4.7		15.99			6.1	11.9	41.5
WW	NQ	NQ										NQ
CP					NQ		NQ			NQ		NQ
SG		NQ			12.8							12.8
YC	2.9	1.7	17.1		8.3	2.3						32.3
NQ: not quantifiable												
^a Percentages are calculated based on results from the total ion chromatogram (TIC).												

BR barley flour, OM oatmeal, WT wheat flour, RC rice flour, BT bienestarina, CR corn flour, SY soybean flour, WB wheat bran, WW whole-wheat flour, CP pinto corn flour, SG seven-grain flour, and YC yellow corn flour.

No triterpenes production was detected in *P. djamor* in BR, OM, WT, or WB, and no measurable quantities were found in RC or BT, while CR, WW, CP, SG and YC produced small quantities, with stigmast-5-en-3 β -ol being the most abundant compound in all cases. The WB substrate again favored the biosynthesis of products, with 46.3% of the total compounds identified in the extract (Table 4).

Table 4. Triterpenoidal compounds identified in *Pleurotus djamor* mycelium obtained in LSF by using non-conventional CS.

SUBSTRATE	TRITERPENOID ^a												
	I	II	III	IV	V	VI	VII	VIII	IX	XII	XIII	XIV	TOTAL
CB													0
OM													0
WT													0
RC						NQ							NQ
BT					NQ								NQ
CR	NQ					3.8							3.8
SY													0
WB	1.9	NQ	NQ	3.3	1.3	5.7	NQ	12.0	9.7	5.2	7.2	NQ	46.3
WW	NQ					5.1							5.1
CP	NQ					5.1							5.1
SG		NQ	NQ			7.4							7.4
YC						6.0	NQ					NQ	6.0
NQ: not quantifiable													
^a Percentages are calculated based on the results of the total ion chromatogram (TIC).													

BR barley flour, OM oatmeal, WT wheat flour, RC rice flour, BT bienestarina, CR corn flour, SY soybean flour, WB wheat bran, WW whole-wheat flour, CP pinto corn flour, SG seven-grain flour, and YC yellow corn flour.

In *P. pulmonarius*, BR and WB produced triterpenoids in significant quantities and with structural variations. However, despite the detection of different structures, no quantifiable quantities were produced in OM and CP. Low production occurred in BT, WW, and SG, while intermediate production levels were detected in WT, RC, and CR, the latter of which were primarily represented by ergosta-2,5,7,9(11),22-pentene, and ergosta-5,7,22-trien-3 β -ol. Finally, no production of triterpenes was detected in YC or SY (Table 5).

Table 5. Triterpenoidal compounds identified in *Pleurotus pulmonarius* mycelium obtained in LSF by using non-conventional CS.

SUBSTRATE	TRITERPENOID ^a									
	I	II	IV	V	VI	VIII	IX	XII	XIII	TOTAL
CB	1.2	1.6	21.7		2.4					26.9
OM		NQ	NQ		NQ	NQ		NQ		NQ
WT		1.5	6.0							7.5
RC		1.5	9.6		NQ					11.1
BT		NQ	4.1							4.1
CR		1.7	3.3		1.9					6.9
SY										0
WB	1.5	1.3	16.3	0.7	3.2	5.2	3.3	3.2	3.4	38.1
WW		2.8								2.8
CP	NQ				NQ		NQ		NQ	NQ
SG	NQ				1.5		NQ		1.5	3.0
YC										0
NQ: not quantifiable										
^a Percentage points based on the results obtained from the total ion chromatogram (TIC).										

BR barley flour, **OM** oatmeal, **WT** wheat flour, **RC** rice flour, **BT** bienestarina, **CR** corn flour, **SY** soybean flour, **WB** wheat bran, **WW** whole-wheat flour, **CP** pinto corn flour, **SG** seven-grain flour, and **YC** yellow corn flour.

These results demonstrate that the CS significantly influences the structures and quantities of triterpenoidal compounds biosynthesized by the organisms. In general, the use of WB generated the highest quantity of structures independent of the specie employed.

The objective of the work was to study the production of triterpenoid compounds by *Pleurotus* species using non-conventional substrates in LSF. Similar studies have been performed in *Pleurotus*; however, these studies centered on the production of polysaccharides, proteins, lipids, and mycelial biomass using different carbohydrates as CS⁶⁰, varying the nitrogen content of the substrates⁶¹, or evaluating the production of fatty acids and triterpenes to obtain fructifications in solid state fermentation (SSF) using industrial agricultural waste^{19, ENREF_62}.

The C/N ratio of the substrates used in SSF and LSF with fungi is known to be one of the variables that governs the production of primary and secondary metabolites⁶²⁻⁶⁵, culture yields, rates of substrate colonization, and primordia formation in SSF⁶¹. Determination of the C/N ratios for the CS used (Table 6) indicated that the group of OM, WT, WW, and SG and the group of CR, CP, and YC have similar C/N ratio values, ranging from 16.7 and 18.0 and from 23.9

and 24.9, respectively. However, no relationship has been detected between the C/N ratio of the CS and the production of compounds by the mushrooms. The production of triterpenes in the *pulmonarius* species is expected to be similar for OM, WT, WW, and SG, while the structures of the compounds identified in WW and SG were different despite the fact that similar quantities were produced, as shown in Table 5.

In OM and WT, the effect of the C/N ratio on the output is even less clear. The products obtained using OM were not quantifiable, although 5 different structures could be identified. In contrast, only 2 triterpenoids (7.5%) could be identified in WT. In SY, which marks the lower limit of the C/N ratio, no compounds of interest were produced, which might suggest a direct relationship between the C/N ratio and the biosynthesis of triterpenes. However, this hypothesis was rejected as a result of the behavior in WB, for which a low C/N ratio was detected and in which both the highest number of structures and the greatest quantity of triterpenoids were produced. Further evidence indicates no clear relationship between the C/N ratio and metabolite production in WB and BR, which exhibit the highest triterpene production in *P. pulmonarius* but have different C/N ratios.

Table 6. C/N ratios for the carbon sources used as a substrate to obtain *Pleurotus* in LSF.

SUBSTRATE	%C	%N	C/N
BR	40.3 ± 0.1 c	1.84 ± 0.02 b	22.1 ± 0.2 f
OM	42.3 ± 0.1 i	2.58 ± 0.04 d,e	16.7 ± 0.2 d
WT	39.8 ± 0.1 b	2.21 ± 0.01 c	17.7 ± 0.3 d,e
RC	39.4 ± 0.1 a	1.37 ± 0.13 a	28.7 ± 0.1 h
BT	40.4 ± 0.1 d	3.45 ± 0.02 f	11.2 ± 0.4 b
CR	40.2 ± 0.1 c	1.62 ± 0.02 b	24.7 ± 0.3 g
SY	46.7 ± 0.1 k	5.28 ± 0.02 g	8.84 ± 0.04 a
WB	41.5 ± 0.1 h	2.78 ± 0.05 e	15.2 ± 0.3 c
WW	40.8 ± 0.1 f	2.22 ± 0.04 c	18.0 ± 0.3 e
CP	41.2 ± 0.1 g	1.75 ± 0.02 b	23.9 ± 0.2 g
SG	42.6 ± 0.1 j	2.43 ± 0.02 c,d	17.5 ± 0.1 d,e
YC	40.6 ± 0.1 e	1.58 ± 0.05 b	24.9 ± 0.8 g

Results were obtained after multiple comparisons, and mean values are followed by a letter from 'a' to 'k' based on significant differences. If two means are accompanied by the same letter, no significant difference exists between them; those having different letters exhibit a significant difference ($p \leq 0.05$).

In addition to differences between the C/N ratio and the accompanying biosynthesis of triterpenes for each CS, species-dependent differences were also found. When cultured in WB, SG, and YC, *P. djamor* and *P. ostreatus* exhibited similar effects with regard to biosynthesis, but immeasurable to no production of metabolites of interest was observed when using SY, WT, OM, RC, and BT. Thus, it appears that the C/N ratio of the substrate has no real effect on the triterpenes production. A greater structural variety of triterpenes were produced in *P. pulmonarius*. Regardless of the species tested, the use of WB favors biosynthesis, with regard to both quantity and structural variation.

The most common metabolite found in the species studied was stigmast-5-en-3 β -ol (sitosterol). Although isolated from macromycetes⁵³, sitosterol is the most common sterol in the plant kingdom and is the predominant sterol, together with other sterols such as ergost-5-en-3 β -ol (campesterol) and stigmastanol (sitostanol), identified for most of the CS used to obtain biotechnology products. These sterols, along with sitosterol, have been reported as components of some cereals such as rice bran, wheat bran, wheat husk, oat bran, and fine corn fiber⁶⁶⁻⁶⁹. Given these findings, the presence of these sterols in the species obtained by LSF in the present study could correspond to the incorporation of substrate components by the fungi, a phenomenon reported by our group in the SSF of *Pleurotus*⁷⁰. To confirm this hypothesis, CS and process controls (salt mixture without mycelium) were extracted under the same conditions, and stigmast-5-en-3 β -ol (VI) was identified in all CS except RC and BT. This result supports the hypothesis but does not allow for generalization because, in some cases (such as with *P. pulmonarius* and *P. djamor* in RC and *ostreatus* in BT), the sitosterol appears to be biosynthesized by the fungus.

4. CONCLUSIONS

The present investigation determined that the type of cereal flour used as the CS impacts the production of triterpenoids. This influence depends on the cultured species and is reflected in the quantity and/or structural variety of such compounds. The C/N ratio of the CS does not directly affect the biosynthesis of triterpenoids and no obvious direct relationship was found between this ratio and overall production. In general, *Pleurotus pulmonarius* growth in wheat bran favor the production of a mycelium with a higher quantity and variety of triterpenoidal compounds. Of the 14 triterpenes identified, 8 correspond to ergostane, 5 to stigmastane, and 1 to cholestane. With In regard to their substituents, the identified compounds are classified as 2 triterpenes, 7 sterols, and 5 steroidal ketones. No reports have yet been published regarding the presence of 5 α -stigmast-3-one, ergostan-3-one, 6-Hydroxystigmast-4-en-3-one and stigmast-4-en-3-one in fungi.

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