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Original Article

Biosynthesis of Eudesmane-type Sesquiterpenoids by The Wood-rotting Fungus, *Polyporus brumalis*, on Specific Medium, including Inorganic Magnesium Source[1]

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ABSTRACT

Fungi, such as the wood-rotting *Polyporus brumalis*, are excellent sources of pharmaceutically interesting natural products such as sesquiterpenoids. In this study, we investigated the biosynthesis of *P. brumalis* sesquiterpenoids on modified medium. Ten additional species of white rot fungi were inoculated in medium containing nutrients such as $C_6H_{12}O_6$, $C_4H_{12}N_2O_6$, KH_2PO_4, $MgSO_4$, and $CaCl_2$ at 28℃ for 25 days. After 10 days of incubation, eudesmane-type sesquiterpenes, β-eudesmane and β-eudesmol, were only synthesized during the growth phase of *P. brumalis*. Experiments excluding one nutrient at a time were conducted to determine the effects of inorganic nutrients on sesquiterpene biosynthesis. In conclusion, GC-MS analysis showed that biosynthesis of sesquiterpenes was differentially regulated by inorganic nutrients such as $MgSO_4$, $C_4H_{12}N_2O_6$, and KH_2PO_4. We found $MgSO_4$ supplementation to be vital for eudesmane-type sesquiterpene biosynthesis in *P. brumalis*; nitrogen ($C_4H_{12}N_2O_6$) and phosphate (KH_2PO_4) inhibited the synthesis of *P. brumalis* metabolites. Magnesium is a known cofactor of sesquiterpene synthase, which promotes β-eudesmol synthesis. To mechanistically understand eudesmane-type sesquiterpene biosynthesis in *P. brumalis*, further research into the genes regulating the dynamics of such biosynthesis is warranted.

Keywords : wood rot fungi, *Polyporus brumalis*, sesquiterpene biosynthesis, β-eudesmol

1. INTRODUCTION

Sesquiterpenoids are defined as C_{15} compounds derived from farnesyl pyrophosphate (FPP), and their complex structures are found in the tissue of many diverse plants (Degenhardt *et al*., 2009). FPP's long chain length and additional double bond enables its conversion to a huge range of mono-, di-, and tri-cyclic structures. A number of cyclic sesquiterpenes with alcohol, aldehyde, and ketone derivatives have key biological and medicinal properties

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(Fraga, 1999). For example, potent anticancer, antitumor, cytotoxic, antiviral, antibiotic, and anti-herbivory properties have been reported in addition to the characteristic flavors of cyclic sesquiterpenes (Asadollahi *et al*., 2008). Sesquiterpenoids such as taxol (Rowinsky *et al*., 1992) and artemisinin (Dondorp *et al*., 2009) are commercialized products utilized in the treatment of cancer and malaria, respectively.

Sesquiterpenes are mostly synthesized by plants to protect themselves against microbes, insects, and herbivores. However, fungi are known to be remarkable organisms that produce a wide range of natural products in the form of secondary metabolites. Particularly, wood-rotting fungi, along with other fungi, produce volatile organic compounds such as terpenes, alcohols, aldehydes, ketones, and various aromatic compounds through their secondary metabolic pathways (Morath *et al*., 2012). The secondary metabolites of wood-rotting fungi, unlike other fungi such as *Ascomycota*, *Aspergillus*, and *Penicillium*, have received little to no attention. Some di-terpenes, produced via terpene synthases, have been identified and isolated from fungal species *Ganoderma lucidum* and *Poria cocos*. In this study, we specifically aimed to investigate the synthesis of bio-active sesquiterpenes in the wood-rotting fungus *Polyporus brumalis* as well as the effects of cultivation conditions on such biosynthesis. The synthesis of secondary metabolites is affected by the presence and/or absence of certain nutrients. For example, Harper *et al*. described that production of secondary metabolites often synthesized after fungal growth ceased afterwards due to nutrient limitations (Harper *et al*., 1991). Thus, it is crucial to explore the effects of different nutrients on sesquiterpene biosynthesis in wood-rotting fungi.

2. MATERIALS and METHODS

2.1. Fungi

Screening tests were conducted to identify a suitable fungus that could synthesize terpenoids. The fungi presented in Table 1 were provided by the American Type Culture Collection and Korea Forest Research Institute.

2.2. Preparation of fungal materials for cultivation

The strains were pre-inoculated in PDA (Potato Dextrose Agar) medium on Petri dishes and maintained in a stationary incubator at 28℃. After seven days, the mycelium had fully grown and reached the edge of the Petri dishes, and the layer of mycelium covering the agar medium was separated from the medium using a platinum wire. Then, under aseptic conditions, the mycelium was placed into a container with a homogenizer along with 20 mℓ of distilled water. The contents were homogenized for 2 min. The mycelium then became a liquid suspension, and the dry weight of 1 mℓ of fungal suspension was calculated after 3 h in a drying machine at 121℃.

Table 1. Fungal strains of wood-rotting fungi for identifying a secondary metabolites

Classification	Provided by	Scientific name
White rot fungi	Korea Forest Research Institute	*Heterobasidion annosum* *Phanerochaete chrysosporium* *Coriolus consors* *Ganoderma applanatum* *Ceriporiopsis subvermispora* *Cystidiodoniia isabellina* *Lentinus edodes* *Polyporus brumalis* *Stereum hirsutum* *Trametes versicolor* *Pleurotus eryngii*

Table 2. Nutrients of the media used for *P. brumalis* growth and sesquiterpene biosynthesis

Source	Media					
	PDB	(control)	N free	Mg free	P free	Ca free
Carbon	Dextrose	glucose	glucose	glucose	glucose	glucose
Nitrogen		$C_4H_{12}N_2O_6$	-[a]	$C_4H_{12}N_2O_6$	$C_4H_{12}N_2O_6$	$C_4H_{12}N_2O_6$
Magnesium		$MgSO_4$	$MgSO_4$	-[a]	$MgSO_4$	$MgSO_4$
Phosphate		KH_2PO_4	KH_2PO_4	KH_2PO_4	-[a]	KH_2PO_4
Calcium		$CaCl_2$	$CaCl_2$	$CaCl_2$	$CaCl_2$	-[a]

[a]: nutrient not added to the medium

2.3. Cultivation

Potato dextrose broth (PDB, Difco^{TM}) medium and five types of modified media were used in this study. The nutrient elements of each medium are described in Table 2. PDB mainly contains carbon sources, 4 g potato infusion, and 20 g dextrose. The modified media have diverse nutrient sources such as 10 g $C_6H_{12}O_6$, 0.2 g $C_4H_{12}N_2O_6$, 2 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $CaCl_2 \cdot 2H_2O$. To verify the effect of each nutrient on secondary metabolite production, media that excluded one inorganic nutrient each, were also used in this study. Homogenized fungal suspension (4 mℓ; cell dry weight: 0.01 mg/mℓ) was inoculated in 200 mℓ of each medium type in a 500 mℓ flask. Each flask was sealed with a rubber stopper and placed on a shaking incubator at 26℃ and 80 rpm. After 5, 7, and 12 days, the medium (200 mℓ) was separated from the mycelium by centrifugation. Then, the pH and total carbohydrate content (TCC) of the aqueous medium were measured. The phenol-sulfuric acid method used to estimate TCC during fungal cultivation. This method detects virtually all classes of carbohydrates, including mono-, di-, oligo-, and poly-saccharides. Glucose is

commonly used to create the standard curve, and the absorption is measured at 490 nm. After calculating pH and TCC, the medium was extracted by ethyl acetate for GC-MS analysis. Mycelium separated from the medium was dried in a 121℃ oven to calculate the biomass weight.

2.4. Chemical analysis

Transformed products were analyzed by gas chromatography-mass spectrometry (GC-MS). Particularly, qualitative and quantitative analyses were carried out using FID and MS detectors. For the qualitative analysis, the stationary phase of GC-MS was a DB-5 column (dimensions: 30 m × 0.25 mm; coating thickness: 0.25 μm), and the carrier gas was He at 1 mℓ/min. The working conditions were the following: injection at 300℃ and detection at 250℃. The oven temperature was increased from 40 to 280℃ at 5℃/min, with an initial holding time and a final holding time of 10 min, respectively. The split ratio was 5 : 1, and the mass range was from 50 to 800 m/z. Peak identification was based upon comparing our mass spectra with the NIST 08 (National Institute of Standards and Technology) library's spectra of injected standards. The identification of the retention index of individual compounds was based upon comparing their relative retention times with an n-alkane (C_8-C_{30}) mixture in the DB-5 column. Additionally, quantitative analysis of compounds was performed with an Agilent 6890A GC instrument equipped with a split injector and FID detector. The stationary phase was a DB-5 column (dimensions: 30 m × 0.25 mm; coating thickness: 0.25 μm), and the carrier gas was ultra-pure He at 1 mℓ/min. The working conditions were similarly carried out as per the qualitative GC-MS analysis. Lastly, calibration was conducted via external standards (β-eudesmol; Sigma-Aldrich, Korea) of substrate and product.

3. RESULTS and DISCUSSION

3.1. Metabolites analysis of fungi by GC-MS

Fungal suspensions of 11 white rot species were inoculated in modified medium containing $C_6H_{12}O_6$, $C_4H_{12}N_2O_6$, KH_2PO_4, $MgSO_4$, and $CaCl_2$ for 20 days. Cultivation was stopped by solvent extraction via separation of the mycelium. Fig. 1 shows the GC-MS chromatograms of media extractives of four different species. Interestingly, sesquiterpene compounds were detected in the medium of *P. brumalis* after 12 days of incubation. The metabolites were identified as follows: propionic acid (1), mevalonic acid lactone (2), β-eudesmane (3), and β-eudesmol (4), respectively (Fig. 1). The main peaks of β-eudesmane and β-eudesmol, which were indicative of sesquiterpene structures, were consistently detected for 5, 7, 12, and 15 days (Fig. 2). These results demonstrated the existence of terpene metabolism in the mycelium of *P. brumalis*. Thus, our data further corroborates that basidiomycetes can synthesize diverse terpenoids that may

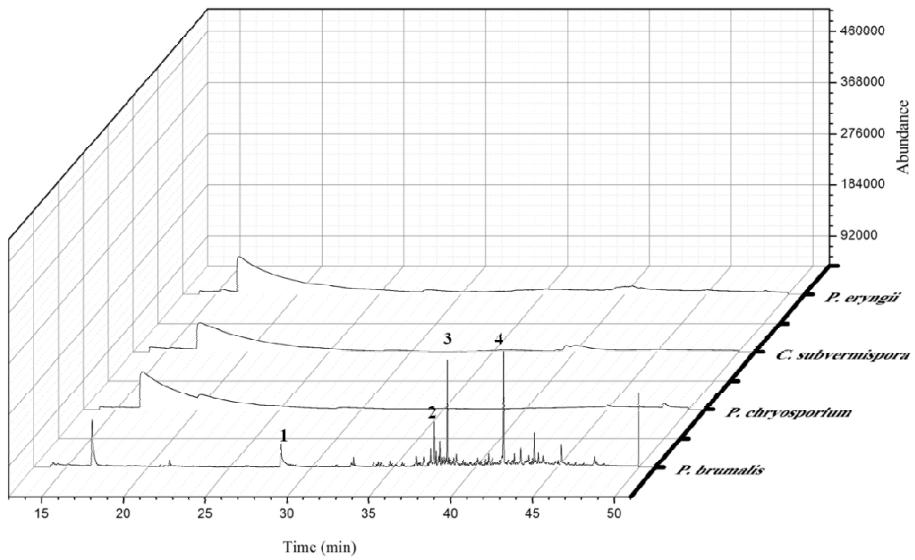

Fig. 1. TIC of extracts for four different fungal species. Peaks were specifically identified in *P. brumalis*: peak 1: propionic acid, peak 2: mevalonic acid lactone, peak 3: β-eudesmane, and peak 4: β-eudesmol.

potentially be used as pharmaceuticals. Sesquiterpenes of several structures, farnesene, caryophyllene, drimane, guaiane, and protoilludane, were previously isolated from *Lactarius spp*., which is one of the largest fungal species in the Agaricales order (Daniewski and Vidari, 1999). Additionally, a few species of wood-rotting fungi such as *Piptoporus betulinus*, *Fomitopsis pinicola*, *Gloeophyllum odoratum*, and *Trametes suaveolens* have been shown to synthesize monoterpene and sesquiterpenes. However, GC-MS analysis in this study did not detect any terpenes in the 10 other fungal species. *Polyporus spp*. are known to generate flavor components such as methyl 2,4-dihydroxy-3,6-dimethyl benzoate; 2-hydroxy-4-methoxy-6-methyl benzoic acid; 3-hydroxy-5-methyl phenol; and 3-methoxy-2,5-dimethyl phenol in submerged cultures (Hoffmann and Esser, 1978). Drimanes of sesquiterpenes were reported as metabolites from *P. arcularius* and shown to exhibit antimicrobial activity against Gram-positive bacteria such as *Staphylococcus aureus* (Fleck *et al*., 1996).

The main metabolites of *P. brumalis*, β-Eudesmol and β-eudesmane, were categorized as eudesmane-type sesquiterpene structures. The eudesmane skeleton could be biosynthesized from FPP-derived IPP, and approximately 1,000 structures have been identified in plants as essential oils. The biosynthesis of eudesmol from *P. brumalis* may thus be an important tool for the production of useful natural compounds as presumed from its identified potent bioactivity in plants. Essential oils comprising eudesmane-type sesquiterpenoids have been previously and extensively researched (Wu *et al*., 2006). β-Eudesmol is a well-known and important eudes-

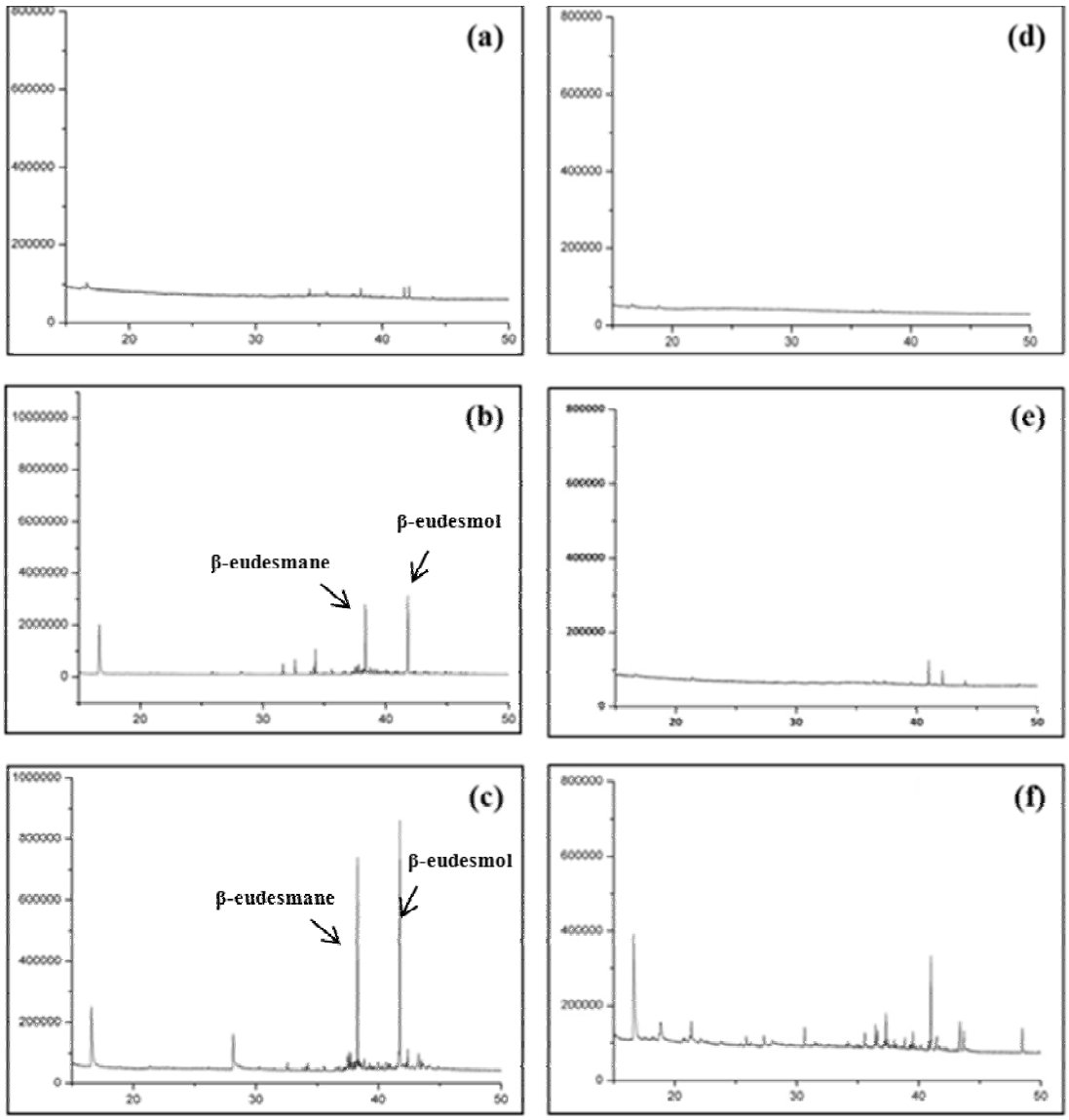

Fig. 2. TIC of *P. brumalis* extracts identified in two different media conditions. (a), (b), and (c) are data from *P. brumalis* grown on modified medium at 14, 18, and 22 cultivation days; (d), (e), and (f) are data from *P. brumalis* grown on PDB medium at 14, 18, and 22 cultivation days.

mane alcohol with an anticholinergic effect in the vascular endothelium (Tsuneki *et al.,* 2005). Additionally, recent studies demonstrated that β-eudesmol acts as a channel blocker for nicotinic acetylcholine receptors at the neuromuscular junction, and it can inhibit angiogenesis *in vitro* and *in vivo* by blocking the mitogen-activated protein kinase (MAPK) sig-

Table 3. Concentration (ppm) of β-eudesmol in different media across various cultivation days. Control medium included all nutrients

Source	Cultivation days				
	6	10	14	18	22
Control	-[a]	15.02	19.48	24.54	23.07
N-free medium	-	16.29	22.47	33.87	33.26
Mg-free Medium	-	-	-	-	-
P-free medium	-	20.27	27.48	48.87	47.55
Ca-free Medium	-	-	15.52	18.99	17.05

[a]: β-eudesmol was not identified via quantitative analysis using standard β-eudesmol

naling pathway (Seo *et al*., 2011).

3.2. Effect of cultures on sesquiterpenoids synthesis in the cultures

Variation of nutrients was conducted to determine an optimum condition for the biosynthesis of sesquiterpenes by *P. brumalis*. Genes encoding terpene synthases, which are crucial to the terpene synthesis pathway, generally respond to environmental factors such as pH, temperature, and available nutrients (Hoffmeister and Keller, 2007; Yu and Keller, 2005). Calvo *et al*. described the effect of major nutrients, carbon and nitrogen, on the synthesis of secondary metabolites (Calvo *et al*., 2002). Additionally, hemiterpenes from *Lentinus crinitus* are synthesized in different carbon sources (Abrahanr and Abateb, 1995). Firstly, to determine the effect of carbon source, PDB medium was additionally introduced into the cultivation of *P. brumalis*. PDB medium used for fungal growth was then modified to contain additional carbon, nitrogen, phosphate, magnesium, and calcium. Fig. 2 shows the TIC of medium extracts of *P. brumalis* in the PDB medium. Some constituents were detected after six days in PDB medium; the peaks had low matching qualities with the GC-MS library (National Institute Standard Technology 2011). *P. brumalis* did not prefer to synthesize sesquiterpenes under all growth conditions. Results of differences in metabolites observed in *P. brumalis* grown in PDB and modified medium highlighted the potential effect inorganic sources such as $C_4H_{12}N_2O_6$, KH_2PO_4, $MgSO_4$, and $CaCl_2$ on sesquiterpene synthesis. To verify the effects of each inorganic nutrient in the modified medium, each nutrient was sequentially eliminated in the modified medium.

Table 3 shows the concentration of β-eudesmol in *P. brumalis* across varying nutrient-free medium during 22 days of cultivation. β-eudesmol was apparent during cultivation except for when *P. brumalis* was grown on $MgSO_4$-free medium. These results demonstrated that $MgSO_4$ can specifically control the biosynthesis of β-eudesmol. Magnesium has been reported as a cofactor that binds to sesquiterpene synthase (Agger *et al*., 2008). Specifically, the Mg^{2+} ions bind to two con-

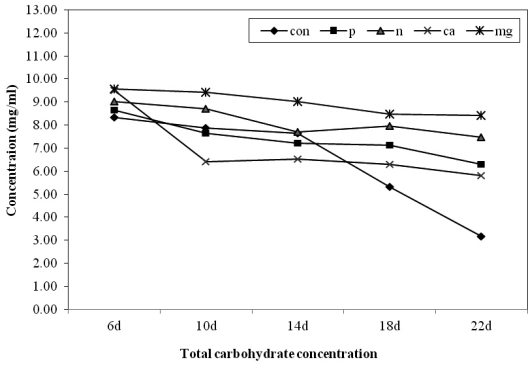

Fig. 3. TCC values of *P. brumalis* grown in either control (con) media or four differentially modified media, each excluding one nutrient (p, phosphate; n, nitrogen; ca, calcium; and mg, magnesium) for 6, 10, 14, 18, or 22 cultivation days.

served metal-binding motifs. These metal ions complex to the substrate pyrophosphate, thereby promoting the ionization of the leaving groups of FPP and resulting in the generation of a highly reactive allylic cation. Effect of magnesium source on the sesquiterpene biosynthesis was also identified via analysis of the concentration of total carbohydrates. In magnesium-free medium, the TCC decreased from 9.58 to 8.42 mg/mℓ (Fig. 3). There was little change compared with the control, which was medium including all nutrients. Precursors, isoprenoids and FPP, of β-eudesmane might be synthesized from glycolysis of glucose. The results of TCC and GC-MS analyses demonstrated that magnesium-free medium induced the terpene metabolism of the mycelium. To note, higher concentrations were identified on the N and P-free medium. The highest concentration was detected in phosphate-free medium at 18 days. Furthermore, mycelium of *P. brumalis* consumed the carbohydrates similarly to the control. In our study, the nitrogen and phosphate sources inhibited sesquiterpene synthesis of the mycelium. Nitrogen and phosphate are important major elements for the growth of microorganisms. Effect of nitrogen on terpene synthesis has been reported in a previous study. Particularly, high nitrogen concentration enhanced synthesis of linalool and citronellol in the yeast, *Saccharomyces cerevisiae*. Additionally, ammonia inhibited biosynthesis of di-terpene gibberellins in the fungus *Gibberella fujikuroi*. Our current study offered further insight that fungal sesquiterpene biosynthesis can be controlled by nutrients.

3.3. Sesquiterpene synthesis in growth curve of mycelium

To profile the metabolites of *P. brumalis*, the cultures were extracted based on the growth curve. Despite metabolites produced during mycelia growth, there was difficulty in detecting significant changes in metabolite production, especially those at low concentrations. These compounds may be of interest in understanding their synthetic mechanisms in *P. brumalis*. At day 5 of the lag phase, propionic acid (CH_3CH_2COOH), which is a fundamental fatty acid, was detected as a major product (Table 4). Previous research has consistently shown that environmental factors may influence the lipid content and fatty acid composition of fungi (Olennikov *et al*., 2014). Particularly, at low oxygen conditions, anaerobic metabolites such

Table 4. Metabolites identified in *P. brumalis* growth phase via solvent extraction and GC-MS analysis

RI [a]	5 days	7 days	12 days	15 days
702	Propionic acid (79.4[b])	Propionic acid (61.4)	Propionic acid (69.4)	Propionic acid (30.4)
785	Butyl acetate (2.10)	Butyl acetate (1.10)	Butyl acetate (4.16)	Butyl acetate (5.10)
936			Butyl oxitol (5.98)	Butyl oxitol (5.98)
1156		Mevalonic acid lactone (7.54)		
1398			β-Elemene (0.49)	
1424			Caryophyllene (0.71)	
1440			δ-Cadiene (0.30)	
1469			β-Eudesmane (3.17)	
1522				Elemol (2.90)
1597				Globulol (1.24)
1626				γ-Eudesmol (2.68)
1627			β-Eudesmol (4.18)	β-Eudesmol (17.46)
1685		Drimenol (5.10)		
1710		Farnesol (3.25)		

[a]: Retention index as determined by the Kovats retention index
[b]: Compounds and relative portions are presented in parenthesis

as ethanol, methanol, acetic acid, lactic acid, and propionic acid have been found in basidiomycetes (Schmidt, 2006).

The synthesis of terpene compounds began during the growth phase at day 7. Sesquiterpene synthesis occurred after growth was complete. At day 7, drimenol, farnesol, and mevalonic lactone (or mevalonic acid lactone) were identified. Mevalonic acid lactone is the precursor of the mevalonic pathway, and particularly, it is a precursor for a number of biologically important lipids, including cholesterol hormones (Buckley *et al*., 2002). Farnesol is the precursor of sesquiterpenoids. Drimenol compounds, bi-cyclic-sesquiterpene alcohols, can be synthesized from trans-trans farnesol via cyclization and rearrangement (Polovinka *et al*., 1994). They have also been identified in the basidiomycota *Lentinus lepideus* as secondary metabolites.

After 12 days in the growth phase, β-elemene caryophyllene, δ-cadiene, and eudesmane were detected with β-eudesmol. The data showed the synthesis of sesquiterpene hydrocarbons with bi-cyclic structures. These compounds can be synthesized from FPP by cyclization. Cyclic terpenoids are synthesized through the formation of a carbon skeleton from linear precursors by terpene cyclase, which is followed by chemical modification by oxidation, reduction, methylation, etc. Sesquiterpene cyclase is a key branch-point enzyme that catalyzes the complex intermolecular cyclization of the linear prenyl diphosphate into cyclic hydrocarbons (Toyomasu *et al*., 2007).

After 15 days in stationary phase, the oxy-

genated structures eudesmol, elemol, and caryophyllene oxide were detected. Thus, after growth, sesquiterpenes were identified. Per these results, we showed that terpene metabolism in wood-rotting fungi occurs in the stationary phase. We also showed that such metabolism can be controlled by magnesium supplementation in the growth medium. In conclusion, we identified *P. brumalis* as a wood-rotting fungus that can produce sesquiterpenes.

4. CONCLUSION

This study showed that biosynthesis of eudesmane-type sesquiterpenes in *P. brumalis* was regulated by modified cultivation media. Production of sesquiterpenes by microorganisms can provide an alternative means for the production of these pharmaceutically valuable compounds. However, the yield of this product observed in this may present a dilemma with regards to providing industrial-level quantities. Thus, research investigating factors critical to terpene metabolism of *P. brumalis* is needed to enhance production of eudesmane-type sesquiterpenes. Our data demonstrated the effect of nutrients on the cultivation conditions of *P. brumalis* and thus their important role on sesquiterpene biosynthesis. Magnesium, phosphate, and nitrogen differentially regulate sesquiterpene synthesis in *P. brumalis*. However, pH and other conditions need to be explored to holistically understand sesquiterpene metabolism. Additionally, genomic analyses will be essential to provide clarity on what genes are necessary for the *de novo* biosynthesis of sesquiterpenes.

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