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Biotransformation of (-)-α-pinene and geraniol to α-terpineol and p-menthane-3,8-diol by the white rot fungus, *Polyporus brumalis*

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In this study, the monoterpenes, α-pinene and geraniol, were biotransformed to synthesize monoterpene alcohol compounds. *Polyporus brumalis* which is classified as a white rot fungus was used as a biocatalyst. Consequently α-terpineol was synthesized from α-pinene by *P. brumalis* mycelium, after three days. Moreover, another substrate, the acyclic monoterpenoids geraniol was transformed into the cyclic compound, p-menthane-3, 8-diol (PMD). The main metabolites, i.e., α-terpineol and PMD, are known to be bioactive monoterpene alcohol compounds. This study highlights the potential of fungal biocatalysts for monoterpene transformation.

Keywords: biotransformation, monoterpene, α-pinene, geraniol, *Polyporus brumalis*

Introduction

Monoterpenoids are important flavor and fragrance compounds that have pleasant odors (van der Werf *et al.*, 1997) and are produced by branched-chain C-10 hydrocarbons formed from geranyl pyrophosphate (GPP). Similar to major terpenoids, monoterpenoids are generally extracted from plants, such as herbs and higher plants. However, the isolation process is difficult and expensive due to high purities in natural sources, and monoterpenoids are recovered at low concentrations. Although many inexpensive synthetic monoterpene compounds are available, consumers still prefer compounds that are derived from natural sources. Therefore, biotransformation technology that utilizes biocatalysts to produce natural products is an attractive method for the preparation of chemical derivatives of natural compounds. The biotransformations of terpenes have been a research topic of insert due to mild conditions required for such reactions and the definition of the biotransformed products as "natural" (De Carvalho and Da Fonseca, 2006).

In the present study, microbial transformations of monoterpene compounds were performed using mycelia of wood rot fungi. White rot fungi are a type of wood rotting fungi that are able to efficiently degrade lignin using a combination of extracelluar ligninolytic enzymes. The important roles of fungal extracelluar ligninolytic enzymes involve their abilities to oxidize a variety of aromatic substrate. The aim of this work was to study the biocatalytic transformation of the monoterpene hydrocarbon, α-pinene into monoterpene alcohol. Essential oil containg monoterpene alcohols exhibit good antiseptic, anti-viral, and anti-fungal properties. α-Pinene is classified as a monoterpene hydrocarbon, and monoterpene hydrocarbons are occasionally used as starting compounds for microbial transformations due to their simple structures and availability in large quantities as by-products of the pulp industry, e.g., turpentine. α-Pinene has been recovered with yields of 60–70% from the crude sulfate turpentine that results from the Kraft companies processing of pinewood by fractional distillation (Sathikge, 2008). Structurally, α-pinene is bicyclic, unsaturated alkene that is, highly reactive and amenable to modification via chemical synthesis.

The majority of monoterpene alcohol compounds can be derived from monoterpene precursor, geranyl pyrophosphate (GPP). GPP is the precursor of monoterpenoids with acyclic structures that can be converted into diverse monoterpenoids by cyclization, rearrangement and oxidation. Geraniol is the, active form of GPP and is, one of the intermediates in the terpene metabolic pathway. The natural form of geraniol occurs in geranium oil, which is widely used as a substitute for the creation of floral rose scents in the perfume and cosmetic industries (Gomes *et al.*, 2007). Therefore, we used two monoterpene substrates, i.e., the most common monoterpene hydrocarbon structure and intermediate of diverse monoterpene alcohol. The transformed products were analyzed with GC MS and GC FID.

Materials and Methods

Fungi and fungal suspension

In this study, six white rot fungi and six brown rot fungi to identify a suitable biocatalyst were used (Table 1). These were provided by the Korea Forest Research Institute and the American Type Culture Collection. The provided fungi were pre-inoculated in PDA (Potato Dextrose Agar) medium and maintained in a stationary incubator at 28°C for seven days. After seven days, the mycelium had fully grown

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Table 1. White rot fungi used in the biotransformation screening test

White rot fungi	Strain number	Cultivation
Heterobasidion annosum	KUC 8320	PDA[b], 28°C
Ceriporiopsis subvermispora	KUC 8904	PDA, 28°C
Lentinus edodes	KFRI[a] 784	PDA, 28°C
Polyporus brumalis	KFRI 20912	PDA, 28°C
Trametes versicolor	KFRI 100	PDA, 28°C
Pleurotus eryngii	KFRI 405	PDA, 28°C

[a] Korea Forest Research Institute
[b] Potato Dextrose Agar

and reached the edge of the Petri dishes and the layer of mycelium covering the agar medium was directly separated from the medium using platinum wire. Then, the mycelium was put into the container of a homogenizer with 20 ml of distilled water on a clean bench. The contents of the container were ground in the homogenizer was grind for 2 min. Finally, the mycelium of the fungi was obtained as fungal suspension. The dry weight of 1 ml of fungal suspension was calculated after 3 h on a drying machine at 121°C.

Biotransformation experiment

The biotransformation process was conducted in three steps; preparation of the substrates and biocatalyst, reaction upon substrate addition to the fungal culture and identification of the transformed metabolites. α-Pinene (Catalog number 147524) and geraniol (Catalog number 163333) were used as the substrates and were purchased from Sigma-Aldrich Korea. The reaction was carried out in a 500-ml Erlenmeyer flask with liquid medium to support metabolite extraction. The elements of medium are as follows; 1% glucose ($C_6H_{12}O_6$), 0.02% ammonium tartrate ($C_4H_4O_6$), 0.01% monopotassium phosphate (KH_2PO_4), 0.05% magnesium sulfate ($MgSO_4$), and 0.01% calcium chloride ($CaCl_2$). SM medium was modified from SSC (Shallow Stationary Culture) medium which was proposed by Kirk *et al*. (1987) for the activation of specific enzymes, such as ligninolytic enzymes.

Inoculation of fungi on the medium was performed by adding 1 ml of the fungal suspension (dry cell weight: 5 mg/ml). The flasks were incubated in a stationary incubator at 28°C for 5 days, to avoid toxic effects of substrates to the whole cells. After five days, 25 mg of each substrate, α-pinene and geraniol were directly added to growing cells in separate SM cultures. Then, the flasks were sealed with a rubber stopper and placed in a shaking incubator at 26°C and 80 rpm. Every five days, 5-ml solution was extracted by ethyl acetate using a shaking extractor with sodium chloride.

Chemical analysis

The transformed products were analyzed by gas chromatography (GC). Qualitative and quantitative analyses were performed using the FID and MS detectors. The stationary phase of the GC MS was a DB-5 column (dimension 30 m × 0.25 mm, coating thickness of 0.25 μm) and the carrier gas was He at a flow rate of 1 ml/min. The working conditions were injection and detector temperatures of 300°C and 250°C, respectively. The oven temperature was increased from 40 to 280°C at 5°C/min, with initial and final holding times of 10 min. A split ratio of 5:1 was used, and the mass range was from 50 to 800 m/z. Peak identification was based upon comparison of the mass spectra with the NIST 08 (National Institute of Standard and Technology) library and with the spectra of injected standards. The qualitative analysis of the retention indices of individual compounds was based on comparison of their relative retention times with an n-alkane (C_8-C_{30}) mixture using a DB-5 column.

The quantitative analysis of the terpene compounds was by GC FID with external standards. α-Pinene, geraniol, α-terpineol, and (-)-trans-p-menthane-3,8-diol were used as external standards for the analysis of concentration of substrates and transformed products. The calibration range, calibration curve equation and correlation values for each compound are displayed in Table 2. The correlation values (R^2) of α-pinene, geraniol, α-terpineol, and PMD ranged from 0.96–0.99. The concentrations of the substrate and the transformed products during biotransformation were being calculated based on these results.

Results and Discussion

GC MS analysis of the transformed products of α-pinene from *P. brumalis*

Six strains of white rot fungi were used for the biotransformation of α-pinene with the goal of identifying a suitable biocatalyst for the transformation of terpene compounds. Among the tested fungi, *P. brumalis* transformed α-pinene into other products, and the other fungi did not exhibit catalytic transformation. In this study, parallel control experiments were performed using substrate-free cultures because monoterpene hydrocarbons can be transformed by reactions with themselves. No transformation products were observed in the biocatalyst-free cultures. Figure 1 shows the transformed products of α-pinene produced by *P. brumalis* cultures as assessed with by GC MS analysis. After five days, α-terpineol (35.85%), fenchol (5.54%), borneol (8.59%) were identified. Specifically, relative proportion of α-terpineol (35.85–39.05%) was consistently high for 20 days, which indicates that no further transformation occurred. α-Terpineol is an important monoterpenoid with a lilac odor (Tan and Day, 1998). Terpineol is among the 30 most commonly used flavor compounds (Welsh *et al*., 1989) and is

Table 2. Calibration curve equation and correlation values (R^2) of the compounds according to GC FID analyses

Substrate	Calibration level (mg/ml)	Regression equation	Correlation coefficient (R^2)
α-Pinene	0.03–0.5	y = 2.5548x + 0.9739	0.9944
Geraniol	0.03–0.5	y = 15.087x + 0.9989	0.9603
α-Terpineol	0.03–0.5	y = 30.672x+ 0.1917	0.9987
(-)-Trans-p-menthane-3,8-diol	0.03–0.5	y = 7.672x + 0.5478	0.9957

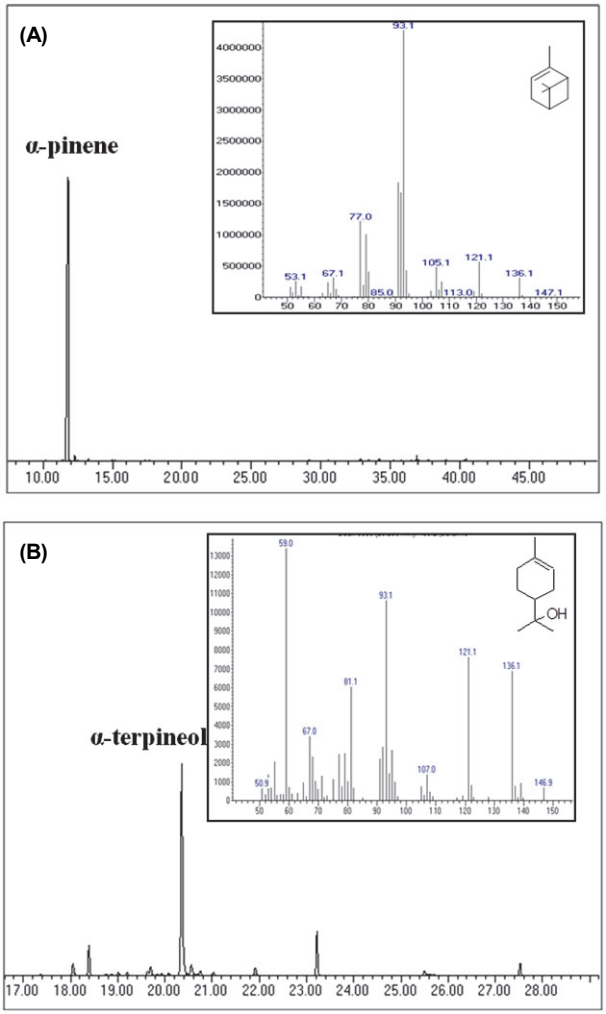

Fig. 1. Total Ion Chromatogram (TIC) of the substrate (α-pinene) and transformed products of α-pinene by GC MS analysis. (A) TIC of α-pinene before the reaction (B) and of the products of the biotransformation by *P. brumalis*, after five days.

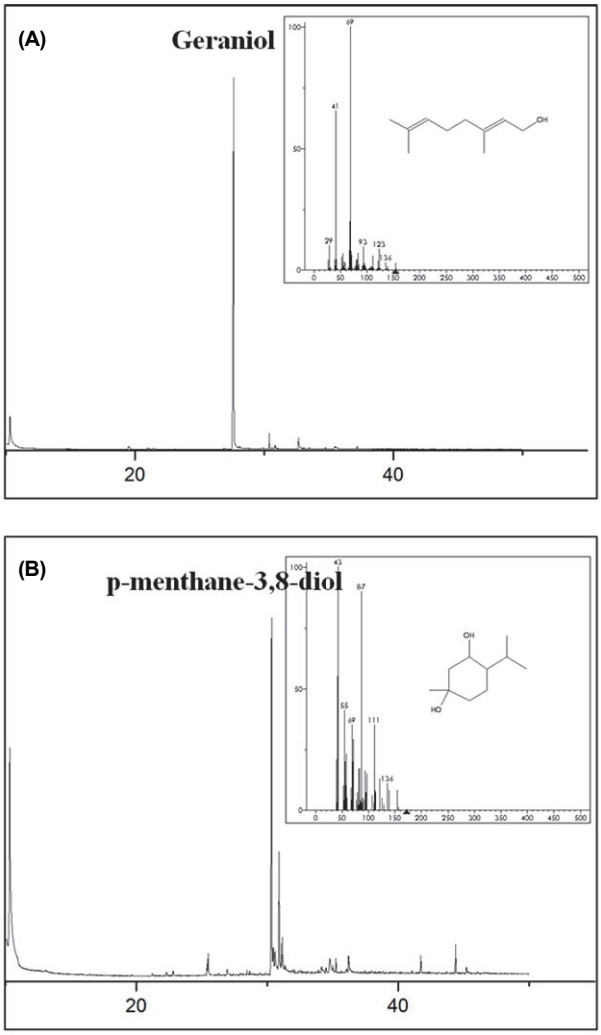

Fig. 2. TICs of geraniol and PMD by GC MS analyses. (A) TIC of geraniol before the reaction (B) and after biotransformation by *P. brumalis*.

listed by the Food and Drug Administration (FDA) as a synthetic flavoring substance that can be directly added to food for human consumption (Cording *et al.*, 2000). α-Terpineol is commonly used in soap and, in cosmetic and flavor preparations (Bauer *et al.*, 2008). However, α-terpineol in essential oils account for under 0.1–5.8% of the natural compounds. Therefore, α-terpineol is commonly produced by acid-catalyzed chemical synthesis from α-pinene or turpentine oil. The biotransformation of α-pinene into α-terpineol has been described in the literature (Rottava *et al.*, 2011). *Candida tropicalis* which is a species of yeast in the *Candida* genus that can transform of α-pinene into α-terpineol within fourdays and with a 0.5 g/L yield (Chatterjee *et al.*, 1999). A strain of the bacterium *Serratia marcescens* can oxidize the terpene hydrocarbon, α-pinene, to produce α-terpineol as the major oxidation product (Wright *et al.*, 1986).

Structurally, *P. brumalis* catalyzes the oxidation of a double bond and the hydration of α-pinene (Fig. 1). As described in the above literature review, oxidoreductases, such as cytochrome P450, can catalyze oxidation or hydration. In previous studies, oxidation has been shown to be catalyzed by cytochrome P450 and laccase in fungi. However, no attempts to verify the existence of these enzymes in *P. brumalis* have yet been reported. α-Pinene and α-terpineol are synthesized from geranyl pyrophosphate in plants. Additional transformations of monoterpene precursors were performed using *P. brumalis* with the goal of biosynthesizing more diverse terpenoids.

Products of the transformation of geraniol by *P. brumalis* by GC MS analysis

Geraniol (2-trans-3,7-dimethyloca-2,6-dien-1-ol) is one of the most important substrates in the biotransformation of monoterpenes. The biotransformation of geraniol was performed under the same conditions as the α-pinene biotransformation. The transformed products, linalool, citronellol, isopulegol and p-menthane-3,8-diol (PMD) were converted from geraniol after 15 days compared with the controls (Fig.

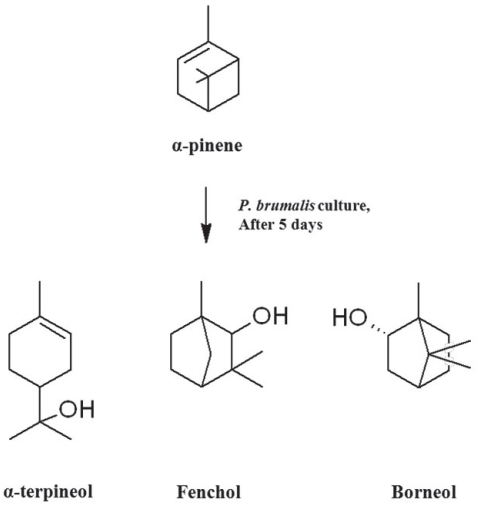

Fig. 3. **Biotransformation of *P. brumalis* after five days.**

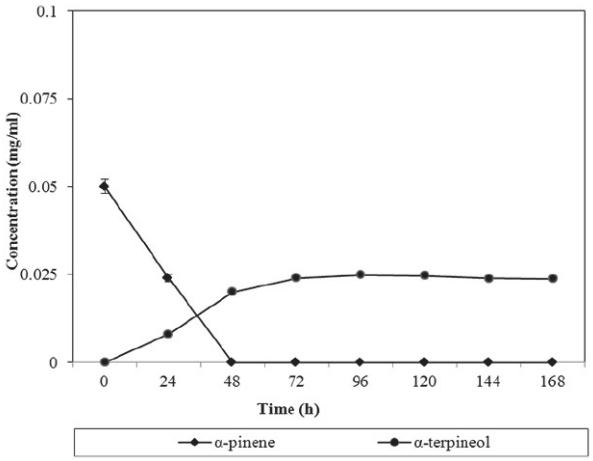

Fig. 5. **Substrate (α-pinene) consumption rate and production (α-terpineol) rate determined after cultivation.**

2). Two of these products, linalool and citronellol, are known to be monoterpene alcohols and are isomers of geraniol that are produced by ionization and reduction. Linalool is present in the essential oils of rosewood, bergamot, rose, jasmine, and lavender (Sköld *et al.*, 2002). Linalool and citronellol also have a sweet floral tastes and are, often used as ingredients in perfumes and, pesticides, and for the chemical synthesis of vitamins A and E (Sugawara *et al.*, 2000). Structurally, isopulegol (2-isopropenyl-5-methyl-cyclohexanol) and PMD are classified as having ρ-menthane skeletons that contains one ring (Fig. 4). These results suggest that *P. brumalis* catalyzed the cyclization of acyclically structured geraniol into monocyclic forms with ρ-menthane skeletons.

Isopulegol ($C_{10}H_{20}O$) is an important intermediate in the manufacture of menthol ($C_{10}H_{20}O$), and possesses characteristic peppermint odor and cooling effect (Chuah *et al.*, 2001). Isopulegol has also been used in the synthesis of natural products, such as pheromones (Zardoost *et al.*, 2012). PMD has a smell that is similar to menthol and also elicits a cooling sensation. PMD has been registered as an active ingredient by the US and can be synthetically manufactured from citronellal into the monocyclic PMD. Sulfuric acid is used for the cyclization of the acyclic starting compound, citronellal, to monocyclic PMD. Despite the advantages of heterogeneous catalytic systems for the cyclizaiton of citronellal, these methods involve the use of hazardous solvents, heating, a high molar ratio of the catalyst, or expensive catalysts or require special treatment for its activation (Jacob *et al.*, 2003). Furthermore, studies have found that repellents containing synthetic PMD mixtures are not as effective as naturally derived PMD (Carroll and Loye, 2006). Therefore, the synthesis of PMD by *P. brumalis* could be used to prepare 'natural PMD'.

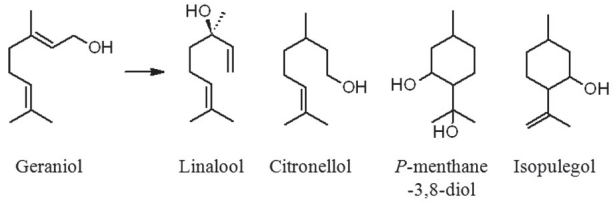

Fig. 4. **Biotransformation of geraniol by *P. brumalis* after 15 days.**

Quantitative analysis of main transformed products, α-terpineol and PMD by GC FID

Each of the α-pinene and geraniol substrates was added to cultures at a concentration of 0.05 mg/ml. After five days, the main transformation products of α-pinene, i.e., α-terpineol, exhibited the highest yield of 0.024 mg/ml (Fig. 3) according to GC FID analysis with an external standard. The α-pinene has completely disappeared at five days. In organic synthesis, the hydration of α-pinene catalyzed by zeolite produces mainly to monocyclic terpenes and alcohols, and α-terpineol is the primary product (up to 48%).

The major product, produced by *P. brumalis* was a-terpineol, which was present at a low concentration relative to

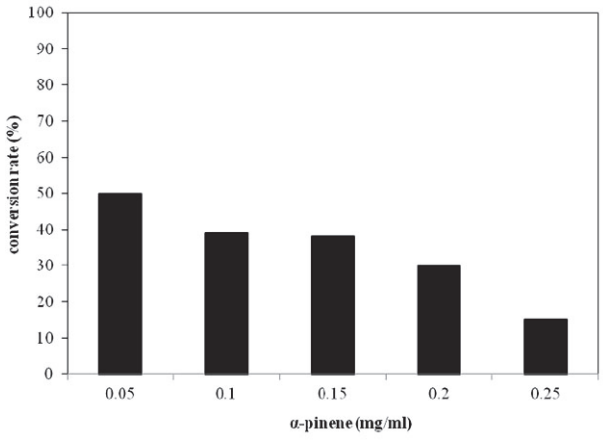

Fig. 6. **The conversion rate of α-terpineol depends on the α-pinene concentration after five days of cultivation.**

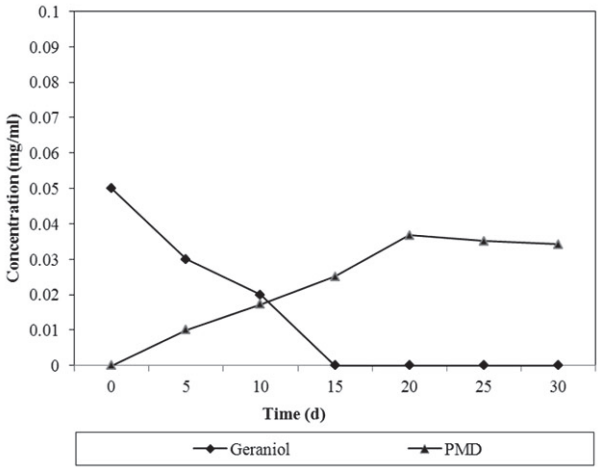

Fig. 7. Substrate (geraniol) consumption rate and production (PMD) rate determined after cultivation.

the α-pinene substrate. These results might have been affected by the high volatility of the substrate. Specifically, monoterpene hydrocarbons, such as α-pinene, are highly volatile, which causes serious problems during large-scale bio-production. In this study, the reaction was performed in an Erlenmeyer flask with a rubber stopper in a shaking incubator because the most interesting biotransformation reactions are oxygen-dependent. However, the conditions of this reaction, including vigorous aeration, may have resulted in substrate and product losses.

As shown Fig. 6, the concentrations of the substrate increased from 0.05 mg/ml to 0.10, 0.15, and 0.20 mg/ml. After five days, the conversion rate of α-pinene to α-terpineol decreased according to the substrate concentration. At the specific concentration of 0.25 mg/ml, the reaction might have been inhibited by the toxicity of the substrate to the fungus. The toxic effects of substrates and products have been correlated with low solubility and volatility problems in the transformation process. High concentrations of terpene substrates can result in the cell lysis of some bacteria and fungi (Andrews *et al.*, 1980; Sikkema *et al.*, 1995).

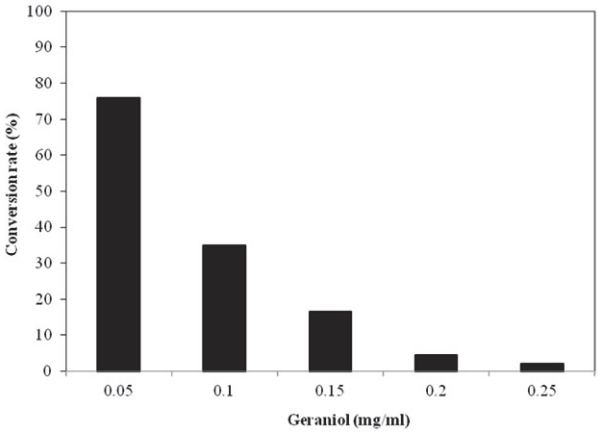

Fig. 8. The conversion rate of PMD depends on the geraniol concentration after 20 days of cultivation.

In contrast, 0.05 mg/ml of geraniol was transformed into PMD at the concentration of 0.038 mg/ml after 20 days (Fig. 7). These results indicated a higher conversion rate than that observed in the α-pinene experiment. However, with increases in the substrate concentration to 0.10, 0.15, and 0.2 mg/ml, the conversion rate to PMD rapidly decreased (Fig. 8). Additionally, the mycelia in the cultures containing higher concentrations of geraniol did not grow in contrast to the α-pinene and substrate-free controls. Therefore, despite the lower volatility of geraniol, its toxic effects on *P. brumalis* necessitated the use of a low concentration of substrate. Although α-terpineol and PMD were produced, the volatility and toxicity of the monoterpene substrates represent challenges to the biotransformation process.

Conclusion

We utilized white rotting fungi as a biocatalyst to synthesize monoterpene alcohols. We observed that the fungus, *P. brumalis* catalyzed the transformations of α-pinene and geraniol into two important monoterpenoids, i.e., α-terpineol and p-menthane-3,8-diol. These transformed products are known to be useful monoterpenoids in the flavor and fragrance industries. Structurally, *P. brumalis* catalyzed the hydration of α-pinene and the cyclization of geraniol. However, α-terpineol can be synthesized by acid catalysts via chemical synthesis. Terpineol produced by organic synthesis is commercially available at a relatively inexpensive price. Thus, the implementation of a microbial process would require higher conversion rates to be competitive with organic synthesis. The advantages of biotransformation by *P. brumalis* are required mild-reaction conditions, neutral cultures, and moderate temperature of 28°C. The biotransformations of terpene compounds by non-toxic microorganisms could be applied in response to the demand for 'green chemistry'. However, the volatility and toxicity were interfered with the transformation of monoterpene. Therefore the optimum conditions for increasing the yields of the transformed products should be further investigated. Further studies to increase our understanding of the oxidative systems of the white rot fungus are being conducted at the transcripts levels.

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