

Oxygenation of α - and β -Pinene by Selected Basidiomycetes

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Several strains of basidiomycetes were examined for their ability to transform α - and β -pinene in agitated submerged cultures. Four major metabolites of α -pinene (verbenol, verbenone, myrtenol, and *trans*-pinocarveol) and three main metabolites of β -pinene (1,4-cineol, myrtenol, and *trans*-pinocarveol) were isolated from the fermentation broth. The metabolic pathways included allylic oxidation, oxidative cleavage and further regioselective oxidation. *Ganoderma applanatum* was found to carry out the stereoselective allylic hydroxylation of α -pinene to verbenol, and of β -pinene to *trans*-pinocarveol in *trans* position to the C–C bridge. The optimal conditions of the bioreaction were established with respect to substrate concentration, incubation time and conversion time. Due to growth inhibition caused by elevated substrate concentration, the bioconversion of β -pinene required pre-grown cultures. Generally, mycelial pellet cultures were supplemented with the terpene substrate when a residual glucose content of 50% was reached. Depending on strain this point was reached after about 48 h. An incubation period of two to three days gave best yields. The transient accumulation of oxygenated products apparently reflected different reaction velocities of the successive catabolic steps.

Introduction

In organic chemistry, microbial transformations are an appreciated tool of semisynthesis for introducing chemical functions into inaccessible sites of molecules, and for preparing chemicals using their ability to catalyze reactions under mild conditions and with a high degree of regio- and/or enantioselectivity and -specificity (Einsele *et al.*, 1985). In natural product chemistry, microbial conversions are especially used for bioconversion of steroids to biologically active compounds and for conversions of some easily available monoterpene hydrocarbons to oxygenated products which might be of potential interest in the fragrance industry (Grayson, 1987). Due to the instability of certain isolated enzymes and their dependence of cofactors, hydroxylations, epoxidations and multienzyme controlled oxidations are still restricted to the use of intact microorganisms in the form of growing, resting or immobilized cultures. A number of microorganisms were studied for these abilities; however, basidiomycetes have not yet been investigated in detail, in spite of their potential to synthesize a range of different monoterpenoids

de novo (Abraham *et al.*, 1994), their good growth in various media, and their conceived ability to form enzymes that can act oxidatively upon terpene hydrocarbons.

The main conversion products of α -pinene by *A. niger* (NCIM 612) were D-*cis*-verbenol, D-verbenone and (+)-*trans*-sobrerol (Bhattacharyya *et al.*, 1960). *Acetobacter methynolicus* on methanol as the single carbon source was able to convert this substrate to (+)-verbenone, (+)-*trans*-verbenol, (+)-*trans*-sobrerol, and (+)-*cis*-verbenol as a side metabolite. Borneol, phellandrene acid, myrtenol and its acid were described as the major metabolites of the conversion by *Pseudomonas* (Shukla and Bhattacharyya, 1968). The conversion of β -pinene was studied to a lesser extent than α -pinene. *Aspergillus niger*, besides the oxidation to myrtenol, offers a preference for the oxidation in allylic position to pinocarveol and pinocaryone (Abraham *et al.*, 1985).

α - and β -pinene are widespread in nature and are available in larger quantities from essential oils. (+)- α -pinene is present in oil from *Pinus palustris* Mill. at a concentration up to 65%; oil from *Pinus pinaster* Soland, and American oil from *Pinus caribaea* contain 70% and 70–80%, respectively of the (–)-isomer. Reactions used in the fragrance industry are isomerization to β -pinene,

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hydrogenation to pinane, hydration with and without simultaneous ring opening to terpineol, *cis*-terpine hydrate, borneol and camphor. As a fragrance substance it is used to improve the odour of industrial products. Optically active and racemic β -pinenes are present in turpentine oils, although in smaller quantities than α -pinene. Pyrolytic cleavage to myrcene, the starting material for acyclic terpenes, is used on an industrial scale.

Materials and Methods

Microorganisms

The basidiomycetes originated from the "Pilzkultursammlung, Friedrich Schiller-Universität Jena" (Weimar), from the "Culture collection of basidiomycetes" (Prague, Czechia) and from the "Centraalbureau voor Schimmelcultures" (Baarn, The Netherlands). The organisms were maintained on agar slants at 25 °C. The medium consisted of 10 g glucose, 3 g malt extract, 5 g peptone and 3 g yeast extract dissolved in 1 l distilled water at a final pH of 6.0. These stock cultures were transferred to 500 ml liquid medium of the same chemical composition, but adjusted to pH 5.5. Immediately after transition to the stationary growth phase the pellets were homogenized (Ultra-Turrax, 4 s), and aliquots of 20 ml suspended cells were inoculated into the shake flasks for serial experiments.

Piptoporus betulinus, IX, Nr. 583, Bull.: Fr/P. Karst (Prague); *Mycena pura*, I, Nr. 817, Pers.: Fr/Kumm (Prague); *Marasmius alliaceus*, Nr. 413, Jacq.: Fr/Fr (Prague); *Kuehneromyces mutabilis*, K 01-6 (Weimar); *Pleurotus sapidus*, P 226-1 (Weimar); *Pleurotus sajor-caju*, P 225-3 (Weimar); *Pleurotus flabellatus*, P 127-2 (Weimar); *Pleurotus cornucopiae*, P 125-7 (Weimar); *Lentinula edodes*, A 20-5 (Weimar); *Pholiota squarrosa*, CBS 570.87; *Hericium erinaceus*, CBS 260.74; *Meripilus giganteus*, CBS 561.86; *Grifola frondosa*, CBS 480.63; *Ganoderma applanatum*, CBS 250.61; *Trametes hirsuta*, CBS 282.73.

Biotransformations and extraction of broth

The fungi were grown on a rotary shaker in 100 ml of the complex medium in 200 ml Erlenmeyer flasks for two to three days at ambient tem-

perature. The growth of the fungus was followed by measuring glucose consumption, pH and formation of biomass. After the addition of substrate, the fermentation was continued for an additional 72 h period. At the end of the respective incubation period, the broth was pooled and extracted three times with pentane/dichloromethane (2/1). The combined extracts were dried over anhydrous sodium sulphate, and concentrated on a water bath (40 °C) to a volume of 1 ml using a Vigreux column.

Identification of metabolites

The extracts were analyzed by gas chromatography (GLC), gas chromatography-olfactometry (GLC-O) and gas chromatography-mass spectrometry (GLC-MS). The metabolites were identified by comparing their Kovats index, their specific odour activity and their MS spectra with those of authentic samples. GLC was performed using a Carlo Erba HRGC 5300 Mega Series gas chromatograph equipped with a FID and a Shimadzu CR 5 A integrator. The column utilized was a CW20M (J&W Fisons) column, 0.32 mm i.d., 25 m length and the temperature program started at 40 °C, rising with a rate of 3 °C·min⁻¹ to 200 °C, which was maintained for 15 min; the carrier gas was H₂ at a pressure of 50 kPa to result in a flow of 3.8 ml·min⁻¹. Mass spectra were recorded by a Hewlett-Packard mass spectrometer 5989 A (Quadrupol), using electron ionization at 70 eV and He as the carrier gas. The enantioselective gas chromatography was carried out using the same GLC conditions as above, but isothermally, using an octakis(6-O-methyl-2,3-di-O-pentyl)- γ -cyclodextrin 50% in Polysiloxane PS086 column (König *et al.*, 1990).

Preparation of cell-free extract

The cells were harvested by centrifugation (5000×g, 20 min). The combined supernatant was used as the extracellular source of enzyme.

Identification of acids in mycelium and in medium

After centrifugation of the suspension medium, the medium was decanted, adjusted with NaHCO₃ to pH = 7.5 and extracted with pentane/dichloro-

methane. After adjustment with 1 N HCl to a pH of 1.5, the extraction was repeated. The mycelium was decomposed with a solution of 1 N KOH, and was extracted in the same way. The acids were determined as methyl esters ($\text{MeOH} \cdot \text{BF}_3$) by gas chromatography (Knapp, 1979) and were quantified using methyl decanoate as an internal standard.

Results and Discussion

An extended screening of more than 50 strains of basidiomycetes resulted in the selection of the above mentioned strains which gave the best yields of metabolites. The pinenes were added to the cells when a residual glucose content of about 50% was reached. This point of the growth cycle

correlate with dry weights reached in the range from 1.2 to $4.8 \text{ g} \cdot \text{l}^{-1}$ dependent on the strain used. It was found that the conversion rate correlated with a fast growth (increase of dry weight of $6 \text{ g} \cdot \text{l}^{-1}$ within 96 h). Moreover, the initial pH of the submerged culture was set not lower than 5.5 to suppress concurrent chemical conversions. During active growth the pH of all cultures ran through a minimum that coincided with the middle of the linear phase. Presentation of results will be restricted to *Ganoderma applanatum* (at the point of terpene addition: $5.2 \text{ g} \cdot \text{l}^{-1}$, pH = 4.0), *Marasmius alliaceus* ($1.2 \text{ g} \cdot \text{l}^{-1}$, pH = 4.7), *Pleurotus sapiidus* ($6 \text{ g} \cdot \text{l}^{-1}$, pH = 5.7) and *Pleurotus flabellatus* ($4.8 \text{ g} \cdot \text{l}^{-1}$, pH = 5.3) which were most suited for the bioconversion of pinenes.




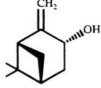



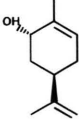
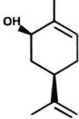
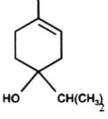
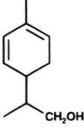
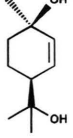

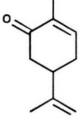
 α-pinene medicinal	Structure Nomenclature Odour			
 verbenol fruity	 myrtenol minty	 trans-pino-carveol sweet	 verbenone mentholic	 myrtenal spicy, sweet
 2,6,6-trimethylbicyclo[3.1.1.]heptane-3-one parfum	 trans-carveol minty	 cis-carveol minty	 1-isopropyl-4-methylcyclohex-3-ene-1-ol	
 p-mentha-1,5-diene-8-ol	 (E)-p-menth-2-ene-1,8-diol mentholic	 (Z)-p-menth-2-ene-1,8-diol mentholic	 carvone sweet, minty	

Fig. 1. Metabolites of the conversion of α -pinene by basidiomycetes.

Conversion of α -pinene

The autoxidation of α -pinene was reported to yield products such as verbenol, verbenone, pinene epoxids, *trans*-pinocarveol, *trans*-carveol and myrtenal (Moore *et al.*, 1956). In this study, the autoxidation products of α -pinene in a complex medium at pH 6 were borneol, verbenol and terpineol in trace concentrations. The respective concentrations of the chemically formed compounds were taken into account when evaluating their simultaneous biochemical formation. Upon addition of α -pinene to the fungal suspensions volatile metabolites were unambiguously identified as summarized in Fig. 1. Each of the strains formed verbenol, verbenone, myrtenol and *trans*-pinocarveol as the major metabolites. Acids and diols could not be identified. Verbenol and verbenone were products resulting from allylic oxidation, and myrtenol was formed by oxidation of the primary carbon of the substrate. Further oxidation to myrtenal was rarely observed. *Trans*-pinocarveol and 2,6,6-trimethyl-bicyclo[3.1.1.]heptane-3-one were found in smaller amounts as products resulting from the direct attack at the double bond. The basidiomycetes examined converted the separately added (+)- and (-)-enantiomers of α -pinene to the *trans* enantiomer of verbenol only. Thus, the introduction of the hydroxy group into the bicyclic system was highly stereoselective in *trans* position to the carbon bridge.

In cultures of *Pseudomonas* α -pinene was degraded to β -isopropyl-pimelinic acid, and β -isopropenyl-pimelinic acid by ring cleavage of the substrate (Hungund *et al.*, 1970). These catabolites were not found in cultures of basidiomycetes.

Conversion of β -pinene

Among the conversion products identified during the transformation of β -pinene by basidiomycetes 1,4-cineol, 1,8-cineol, exhibiting an eucalyptus odour, and myrtenol were the most important flavour compounds (Fig. 2). Corresponding to the experiments with α -pinene, the allylic oxidation was one of the most preferred metabolic pathway, and *trans*-pinocarveol was identified as a major product by each of the examined strains. Previous publications on the microbial conversion of β -pinene did not report 1,8-cineol, 1,4-cineol and fenchyl alcohol as products.

The chemical synthesis of 1,8-cineol commences with the addition of water at α -terpineol to terpine and is followed by acid dehydration to cineol, the major compound of eucalyptus oil (Habermehl and Hamman, 1992). Corresponding to the studies of the enantioselective introduction of a hydroxy group into the bicyclic system of α -pinene, the conversion of (-)- β -pinene was examined and again only the (-)-enantiomer of *trans*-pinocarveol was found.

The comparison of the conversion of α - and β -pinene clearly showed that an enzyme system introduced the hydroxy group preferably into the activated allylic position of the two substrates leading to the main products verbenol, verbenone, myrtenol and *trans*-pinocarveol. The good stability of the allylic cation in 3-position could explain the high concentrations of *trans*-pinocarveol, verbenol and myrtenol. The formation of the identified, monocyclic products carveol, perilla-alcohol, *p*-mentha-2,8-diene-1-ol, 1-isopropyl-4-methylcyclohex-3-ene-1-ol, as well as 1,8-cineol and 1,4-cineol could be due to a ring cleavage of the bicyclic cation to a double unsaturated cation, which could be isomerized and oxidized. Fig. 3 shows the possible metabolic pathways to the identified products. These proposed 1,2-hydrid and 1,3-hydrid transfers, as well as the Wagner-Meerwein rearrangements are in agreement with the general mechanisms described for the biogenesis of monoterpenes (Croteau, 1975).

Kinetics of the biotransformation of β -pinene and α -pinene

Time course experiments were conducted feeding α - and β -pinene to both *Pleurotus flabellatus* and *Marasmius alliaceus*. The kinetics were determined using a sample rate of 24 h over a time period of 240 h after substrate addition at a concentration of 0.15% v/v. A volume of 100 ml was extracted exhaustively by 250 ml of solvent, and the yields of metabolites were quantitatively determined by gas chromatography. The time course of the bioconversion of α -pinene in Fig. 4 shows an increase of verbenone during the first two days of incubation and a decrease starting after 120 h, corresponding to the approaching depletion of glucose. The maximum concentration of verbenol was reached already after one day of incubation

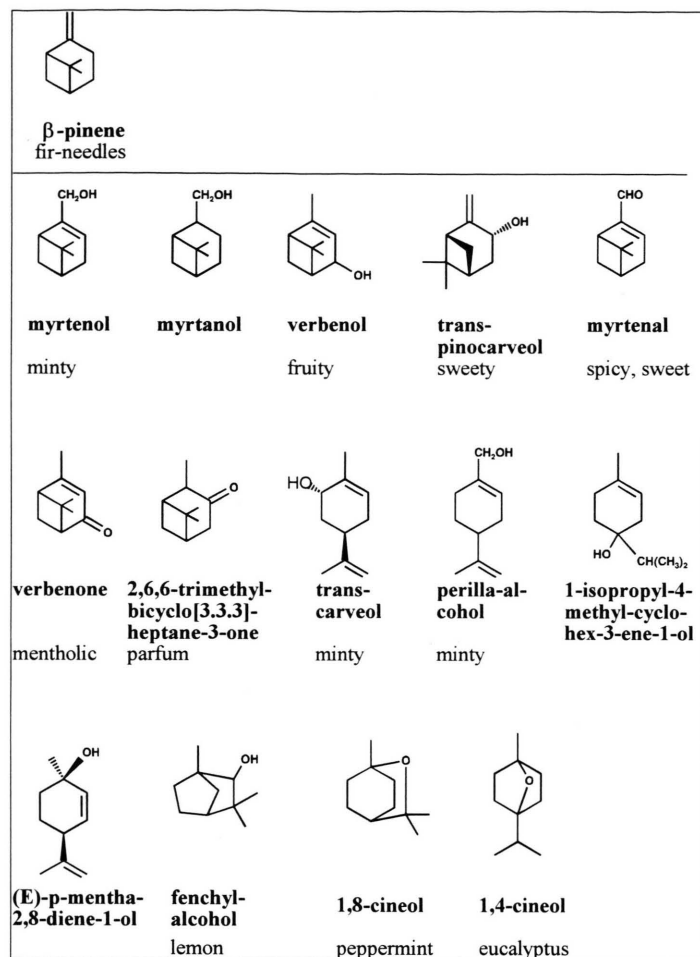


Fig. 2. Conversion products of β -pinene by basidiomycetes.

and decreased afterwards during further incubation. The substrate, α -pinene, was available during the whole fermentation period. The results of the conversion of β -pinene by *Marasmius alliaceus* are shown in Fig. 5. The transformation of β -pinene to 1,4-cineol and 1,8-cineol proceeded rapidly by both organisms. The cineols were detected by gas chromatography already after the first 24 h of incubation. After 72 h, the sensorically active substances decreased, and after 240 h, when glucose was exhausted, they completely disappeared, concurrent with a loss of the eucalyptus odour during the fermentation period. Analogous to the results of the conversion of α -pinene, the main metabolites showed their maximal concentration after 48 h of incubation and decreased with the consumption of glucose. Myrtanol appeared after 192 h of incubation.

At the beginning and at the end of the incubation period the acid composition in medium and in mycelium of *Marasmius alliaceus* was quantified for the incubation with and without β -pinene. 4-Methyl-3-pentenoic acid could be identified only in the medium of a culture incubated with β -pinene, showing a maximum in the concentration after 72 h and a decrease during the further bioreaction. 3-Methyl-2-butenic acid and 3-methyl-butanoic acid were found in the medium and in the mycel, showing maxima after 72 and 96 h, respectively. Consequently, a further decomposition of the substrate β -pinene to short chain and branched acids and subsequently to CO_2 and water can be assumed. The membrane toxicity of the terpene hydrocarbons was indicated by the identification of saturated and unsaturated C_{16} and C_{18} fatty acids in the mycelium, but also in the

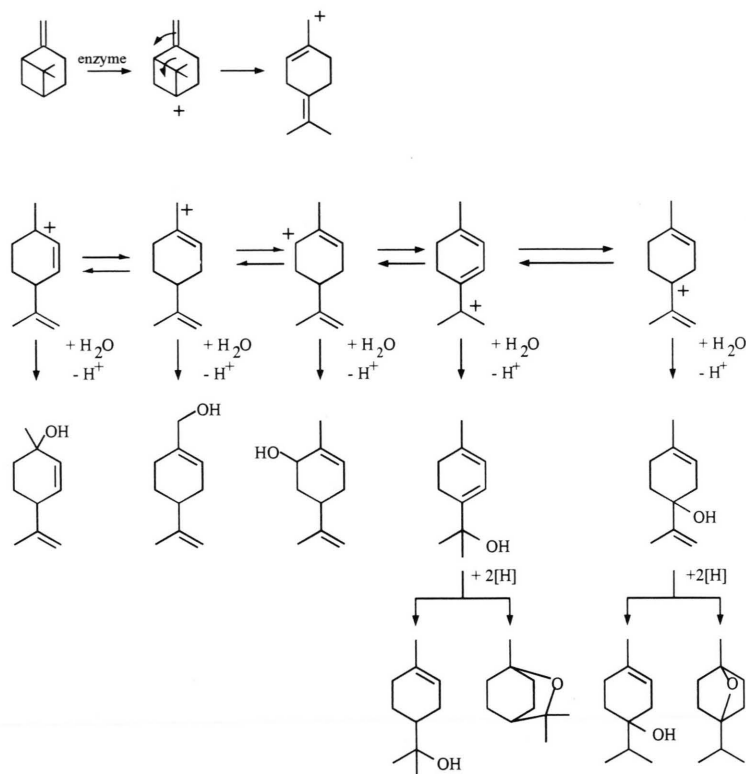


Fig. 3. Possible mechanism for the biogenesis of the monocyclic metabolites during conversion of β -pinene by basidiomycetes.

medium of a culture which was incubated with β -pinene. *Vice versa*, these fatty acids could not be identified in the media of cultures lacking the terpene substrate. Thus, the terpene substrate may have interacted with the membrane and lead to the release of these long chain fatty acids (Arota *et al.*, 1992).

Optimization of the conversion rate of β -pinene

The influence of the emulsifier Tween-80 on the bioconversion of β -pinene by *Marasmius alliaceus* and *Pleurotus flabellatus* was studied. Tween-80 in concentrations of 0.05 and 0.1%, respectively, was added together with the substrate through a sterile filter to three days old cultures, corresponding to 0 h in Fig. 4 and 5. After an additional incubation of three days the medium of *Marasmius alliaceus* (pH = 4.7) and of *Pleurotus flabellatus* (pH = 5.3) were extracted and analyzed. No effect of Tween-80 was found regarding to growth and pH, but obviously, the formation of an emulsion during the liquid/liquid extraction was increased in the pres-

ence of Tween-80. Moreover, the concentrations of both cineols, measured after 73 h of incubation, decreased with the use of the emulgator, but the concentrations of myrtenol and *trans*-pinocarveol increased.

A more pronounced effect on the conversion of β -pinene could be demonstrated during the investigation of the incubation of the monoterpene at different sections of the growth phase. To ensure that the viability of the organisms was not diminished by the toxic substrate, the addition was done continuously in small amounts. In order to get the maximum amount of products it was optimal to start the fermentation at a 0.2% v/v level of β -pinene with one subsequent replenishment of the hydrocarbon after 48 h. The total fermentation period was then extended to 120 h and the total amount of β -pinene added was 0.4% v/v. The incubation of β -pinene in an amount of 0.4% v/v to a 100 ml culture at once at the first day of the growth phase led to reduced growth, consumption of glucose and production of verbenone. A single addition of β -pinene at the third day of growth

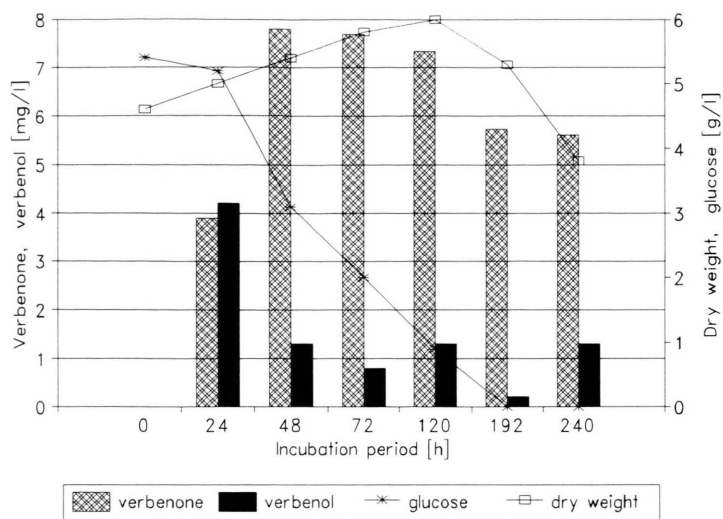


Fig. 4. Kinetics of verbenone and verbenol during conversion of α -pinene by *Pleurotus flabellatus*.

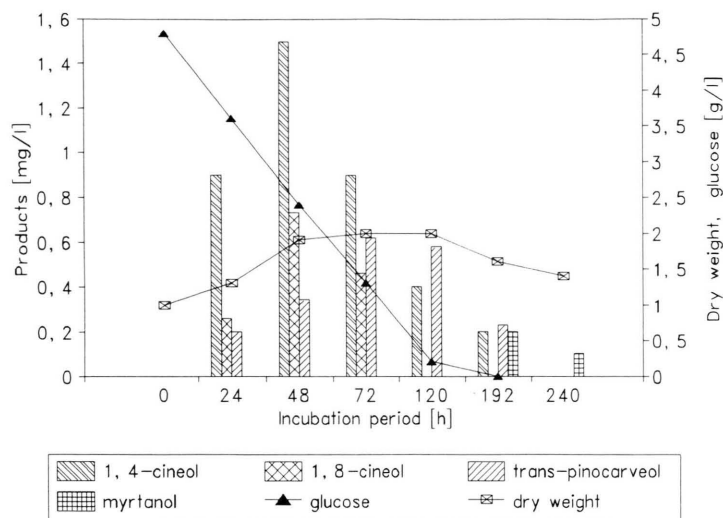


Fig. 5. Kinetics of the conversion of β -pinene by *Marasmius alliaceus*.

phase resulted in a higher growth rate and an increased conversion than upon substrate addition on the first day. A further increase of conversion could be observed by the stepwise incubation during the logarithmic growth phase at a rate of 24 h in portions of 0.1% v/v to a 100 ml culture. However, it was impossible to adapt one of the strains mentioned to pinenes as the sole C source.

To examine the substrate addition of α -pinene through the gas phase, a filter paper was attached at the inner side of the cotton wool plug at the top of the Erlenmeyer flask. This filter was soaked with 0.5 ml α -pinene. After an incubation period of three days, the concentrations of metabolites

were the same as after using the stepwise substrate addition procedure. Therefore, the continuous addition through the gas phase seems to be a feasible alternative for supplementing hydrophobic substrates to aqueous cultivation systems.

Experiment for locating responsible enzymes

Attempts for isolating hydroxylases from microorganisms and using them *in vitro* as pure biocatalysts have been largely unsuccessful. The problems in this field can be attributed to enzymes instabilities, the need for expensive cofactors with cell-free enzyme systems, and the multicomponent nature

of monooxygenase which leads to their inactivation during removal from the cell matrix. One of the enzymes identified in the course of the conversion of limonene is perilla-dehydrogenase (Dhavalikar and Bhattacharyya, 1966). In the field of basidiomycetes detailed studies are restricted to the identification of enzyme systems employed in the decomposition of lignins (Kannan and Oblisami, 1990).

Fermentation experiments with cell-free systems of *Ganoderma applanatum* showed no conversion products of β -pinene. It was, therefore, concluded that the oxidating activities are located at or in the cell membrane or intracellularly. Furthermore, the conversion of β -pinene by two

strains of basidiomycetes with and without peroxidase activity was compared. The maximum of the peroxidase activity of *Pholiota squarrosa* was found between the second and fourth day after inoculation. In this period the conversion of β -pinene was examined. It could be established that the peroxidase-free, fast growing cells of *Ganoderma applanatum* produced larger amounts of terpenic metabolites ($6.9 \text{ mg} \cdot \text{l}^{-1}$) than *Pholiota squarrosa* ($4.6 \text{ mg} \cdot \text{l}^{-1}$).

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