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RESEARCH ARTICLE

Microbial transformation of β-caryophyllene and longifolene by *Wolfiporia extensa*

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Abstract

β-Caryophyllene and longifolene are sesquiterpenes with characteristic aroma properties, which are primary constituents of essential oils and important ingredients commonly used in food, perfumery, cosmetics, detergents and pharmaceuticals, which create a worldwide market. β-Caryophyllene and longifolene were converted through microbial biotransformation by using *Wolfiporia extensa* to a mixture of products over seven days at 25 °C to be evaluated as potential aroma and antimicrobial agents. The characterization of transformation products was carried out by comparison of their GC-MS spectra and retention indices with that of published data (Wiley, NIST and ADAMS databases). As a result, microbial transformation reactions produced various volatile compounds mainly consisting of ketones, aldehydes and alcohol-bearing derivatives of β-caryophyllene and longifolene.

Keywords: Aroma substances, GC-MS, microbial transformation, terpenoids, *W. extensa*

Introduction

The production of aroma or flavour substances is carried out by 90 % extraction from plants and chemical synthesis methods. Obtaining aroma compounds from plants is expensive due to tedious isolation protocols. Chemical synthesis serves as a good alternative for the synthesis of aroma substances, but they are not preferred because of sometimes harmful effects to the health and environment. This directed attention for the development of environmentally friendly processes. In recent years, the utilisation of biotechnological processes on the production of flavour compounds appeared to be the first choice. However, only less than 10 % of aroma compounds is derived from bioprocesses (Berger, 2007; Wright, 2010).

β-caryophyllene, a natural sesquiterpene, is a major plant volatile found in the essential oils of many different spices and food condiments (Fitjer et al., 1995; Schmitt, Levy, & Carroll, 2016). It has been used in a wide variety of prepared foods; baked goods, frozen dairy, meat products, commercially used as a food additive and a natural flavouring compound. It has been also used in cosmetics (Andersen et al., 2010; Sharma et al., 2016; Younis & Mohamed, 2019).

Longifolene is a natural tricyclic olefin and occurs to the extent of 5-10 % in the Indian turpentine oil. Longifolene produced commercially from the oleoresin of Himalayan pine, *Pinus longifolia* Roxb and its structure was established in 1953, but still continues to attract attention as a novel substrate for transformations, generating interesting chemistry and widely used in many different fields (Cao et al., 2019; Dev, 1981; Ford, Api, & Letizia, 1992)

As already mentioned β-caryophyllene and longifolene substrates are common widespread sesquiterpenes found in essential oils. The new biotransformations derivatives formed thereof can be used as potential flavour and fragrance ingredients.

The aim of the present study was to biotransform the substrates β-caryophyllene and longifolene with the help of the fungus *Wolfiporia extensa* (Peck) Ginns (syn. *Poria cocos* F. A. Wolf, Polyporaceae) for the production of new natural aroma compounds, which was the most promising and substrate yielding biocatalyst.

Materials and Methods

Microorganisms and chemicals

Wolfiporia extensa (Strain no:279.55) was purchased from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). β-caryophyllene and longifolene were purchased from Sigma-Aldrich, Germany. Cyclohexanol (≥ 99 %; Merck-Schuchardt, Hohenbrunn, Germany) and cyclopentanol (99 %; Sigma-Aldrich, Steinheim, Germany) were used as analytical standards in gas chromatography.

Conditions of cultivation

Small scale biotransformation studies with substrates were carried out by different microorganisms. Among the evaluated, the fungi *W. extensa* produced at least three polar compounds according to chromatographic analyses, which urged further preparative scale biotransformation studies for 7 days at 25 °C.

Pre-cultures (100 mL medium in 250 mL flasks) were prepared by homogenisation a 10 × 10 mm agar plug with mycelium using an Ultra Turrax (Miccra D-9, Art, Mühlheim, Germany). Afterwards, the fungi were grown submerged in modified standard nutrient liquid medium (SNL) by mixing *D*-(+)-glucose monohydrate (30 g), *L*-Asparagine monohydrate (4,5 g), yeast extract (3 g), KH_2PO_4 (1,5 g), $MgSO_4$ (0,5 g) and trace element solution [0.08 g/L $FeCl_3$ x 6 H_2O, 0.09 g/L $ZnSO_4$ x 7 H_2O, 0.03 g/L $MnSO_4$ x H_2O, 0.005 g/L $CuSO_4$ x 5 H_2O, 0.4 g/L EDTA disodium salt dehydrate] in distilled water (1 L). Prepared medium was adjusted to pH 6.0 with 1 M NaOH and autoclaved at 121°C and 1 bar for 20 min. Flask were grown aerobically at 24 °C and 150 rpm on an orbital shaker (Multitron, Infors, Bottmingen, Switzerland).

Biotransformation of the substrates

Biotransformation was started 24 h after incubation by adding 15 mM of β-caryophyllene and longifolene. The structures were examined by means of gas chromatography and mass spectrometry (GC/MS).

Extraction of the volatile metabolites

Liquid culture was extracted in a separating funnel three times or *via* continuous liquid/liquid extraction with pentane/diethyl ether [1:1.12 (v/v)] (P/E). The organic layers were dried over Na_2SO_4 and concentrated with a Vigreux column at 40 °C, brought to a final volume of about 1 mL by fractionated distillation.

External standard was cyclopentanol, whereas internal standard (determination of the recovery) was cyclohexanol. The flavour fractionated distillation extracts were analysed by means of gas chromatography (GC).

Characterization of transformation products

About 1 µL was injected into GC and quantification was performed according to the internal standard and external standard.

Gas chromatography-mass analysis

The GC/MS analysis was carried out using an Agilent 5975 GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was

kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 450.

Gas chromatography-FID analysis

The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300 °C. To obtain the same elution order with GC/MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts (%) of the separated compounds were calculated from FID chromatograms.

Identification

Identification of the essential oil components in the biotransformation mixture was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (Johnston, 1989; Koenig & D. Hochmuth, 2004) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (Adams, 1995; Jennings & Shibamoto, 1980; Joulain & Konig, 1998; Kondjoyan & Berdagué, 1996; Rychlik et al., 1998), were also used for the identification.

Results and Discussion

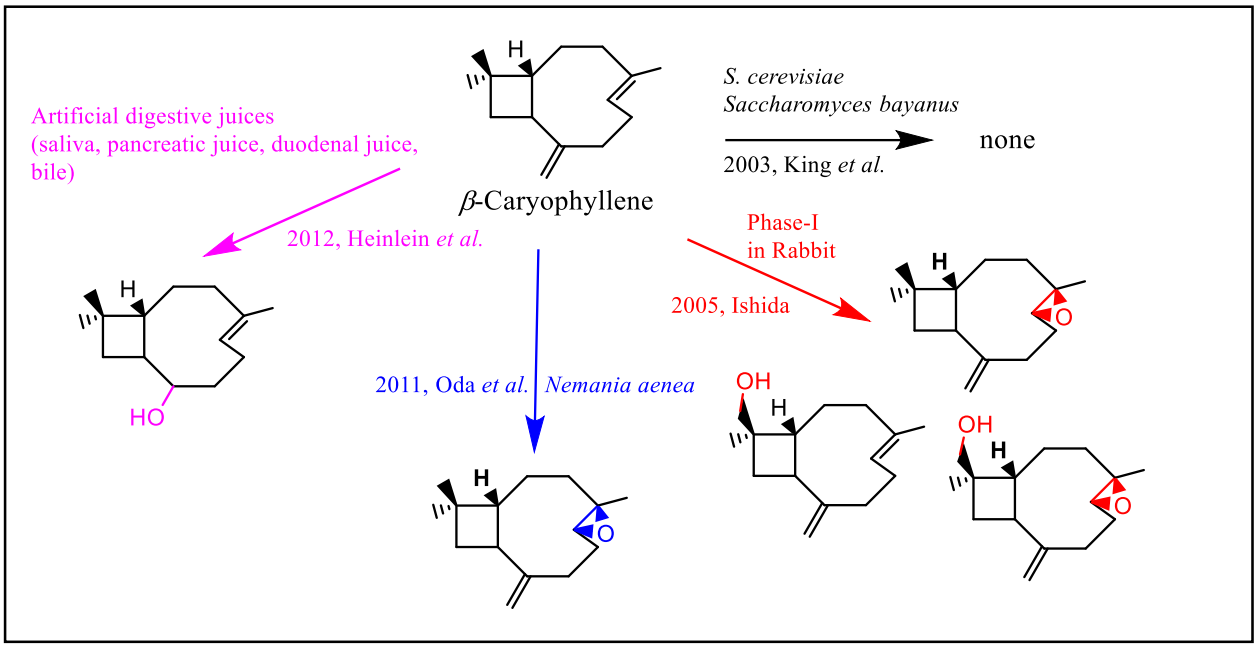
The biotransformations of β-caryophyllene (Scheme. 1) and longifolene (Scheme. 2) were carried out using *W. extensa.* Overall, 15 volatile compounds were present for two substrates in library, but three of them not identified.

The spectral of the three major transformation ketones-aldehydes products, Table 1 (7), (8) and Table 2 (7) resulted in the detection of so far unknown natural compounds.

Further minor oxidation products, especially the corresponding alcohol-bearing derivatives, caryophylladienol-I (4), caryophyllenol-I (5) in Table 1 and salviadienol (3), spathulenol (4), torilenol (5), cedranol (6) in Table 2 were identified. Besides, a metabolite named as "4,4,8-trimethyltricyclo [6.3.1.0(1,5)] dodecane-2,9-diol" (Winmain) was obtained from the biotransformation of β-caryophyllene. There were two similar mass spectra in the literature (Choudhary, Siddiqui, Nawaz, & Atta ur, 2006; Noma, Hashimoto, Uehara, & Asakawa, 2010). It was thought that β-caryophyllene was first converted to caryopyllene oxide and then to clovandiol without the microorganism. Therefore, no caryophyllene was detected.

As a result, substrates were converted by *W. extensa* over 7 days at 25 °C to new metabolites. Biotransformation reactions produced various volatile compounds mainly consisting of ketones, aldehydes and alcohol-bearing derivatives of β-caryophyllene and longifolene. The formed metabolite structures were identified by comparison of their GC-MS spectra and retention indices with that of published data (Wiley, NIST and ADAMS databases). The compounds identified in the biotransformation mixture are shown in Tables 1 and 2. The results showed that the enzymatic reactions were highly regio- and enantioselective.

Scheme 1. Biotransformation of β-caryophyllene (Heinlein & Buettner, 2012; Ishida, 2005; King & Dickinson, 2003; Oda, Fujinuma, Inoue, & Ohashi, 2011).



Scheme 2. Biotransformation of longifolene (Abraham, Hoffmann, Kieslich, Reng, & Stumpf, 1985; Asakawa, Ishida, Toyota, & Takemoto, 1986; Bhattacharyya, Prema, Dhavalikar, & Ramchandran, 1963; Devi, 1979; Ishida, 2005; Joglekar, Vora, Dhere, & Dhavilkar, 1968; Khan, Atif, & Al-Aboudi, 2014).

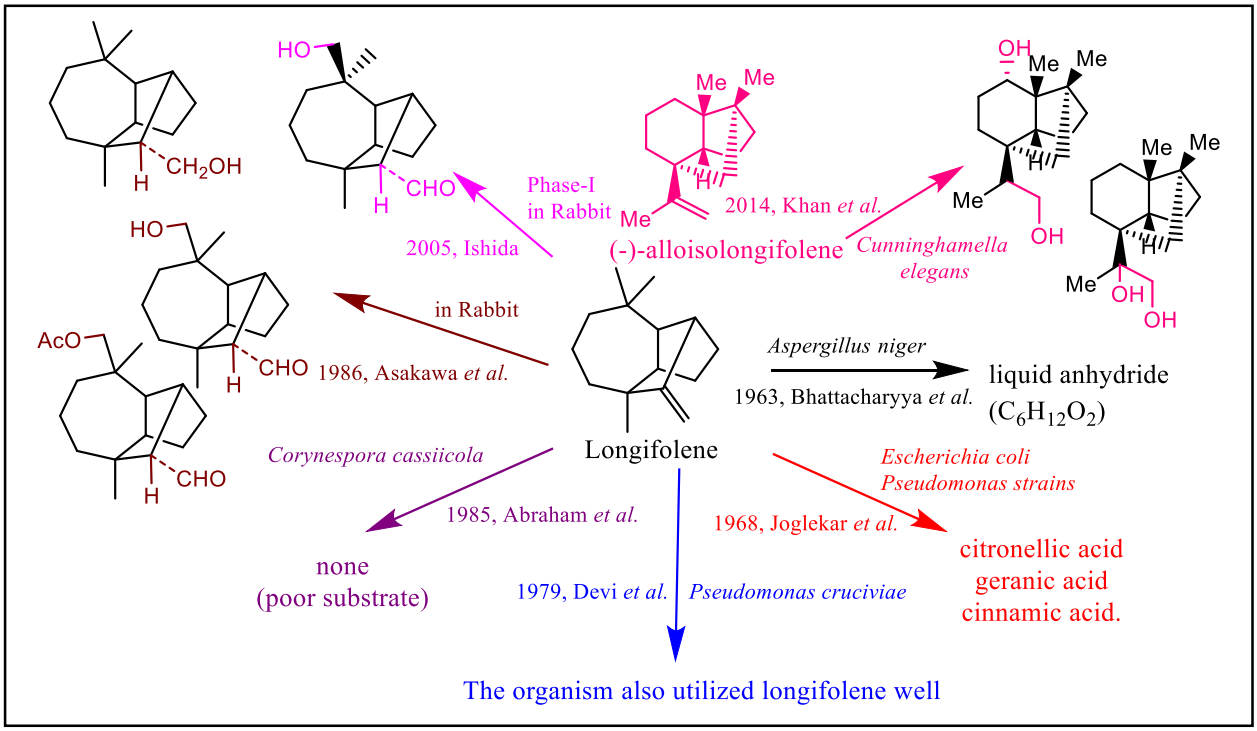


Table 1. β-caryophyllene transformation products from cultures of *W. extensa* identified in this study

Peak no:	R.R.I.	%	Compound
1	1612	15.1	β-caryophyllene (substrate) (Mw: 204)
2	1687	1.8	*α*-caryopyllene= Humulene (substrate) (Mw: 204)
3	2071	*tr*	Humulene epoxide-II (Mw: 220)
4	2316	0.8	Caryophylladienol-I (Mw: 220)
5	2389	1.0	Caryophyllenol-I (Mw: 220)
6	2563	2.0	4,4,8-trimethyltricyclo [6.3.1.0(1,5)] dodecane-2,9-diol (Mw: 238)
7	2628	6.6	M1 (220, 205, 187, 177, 162, 147, 140, 135, 128, 121, 112, 107, 98, 93, 84, 79, 72, 67, 61, 55, 43)
*n.i**			
8	2716	9.8	M2 (220, 202, 194, 187, 179, 168, 161, 153, 147, 139, 133, 123, 117, 109, 103, 95, 89, 81, 75, 69, 55, 45, 39)
*n.i**			

RRI: Relative retention indices calculated against *n*-alkanes; % percentages calculated from FID data; *tr:* Trace (<0,1 %); *n.i**: non identified.

Scheme 3. β-caryophyllene transformation products (Table 1)

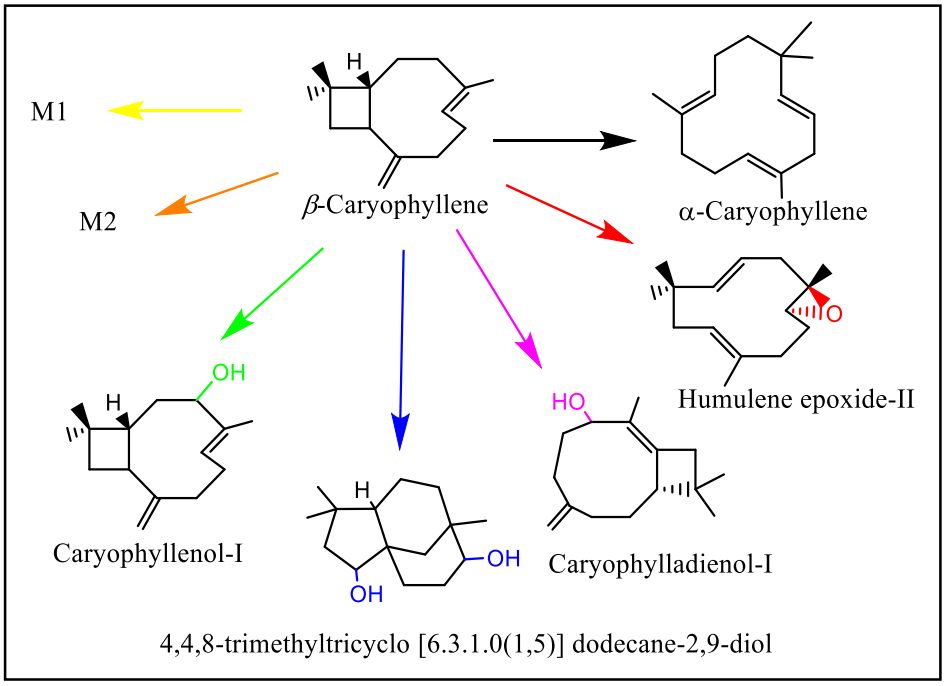
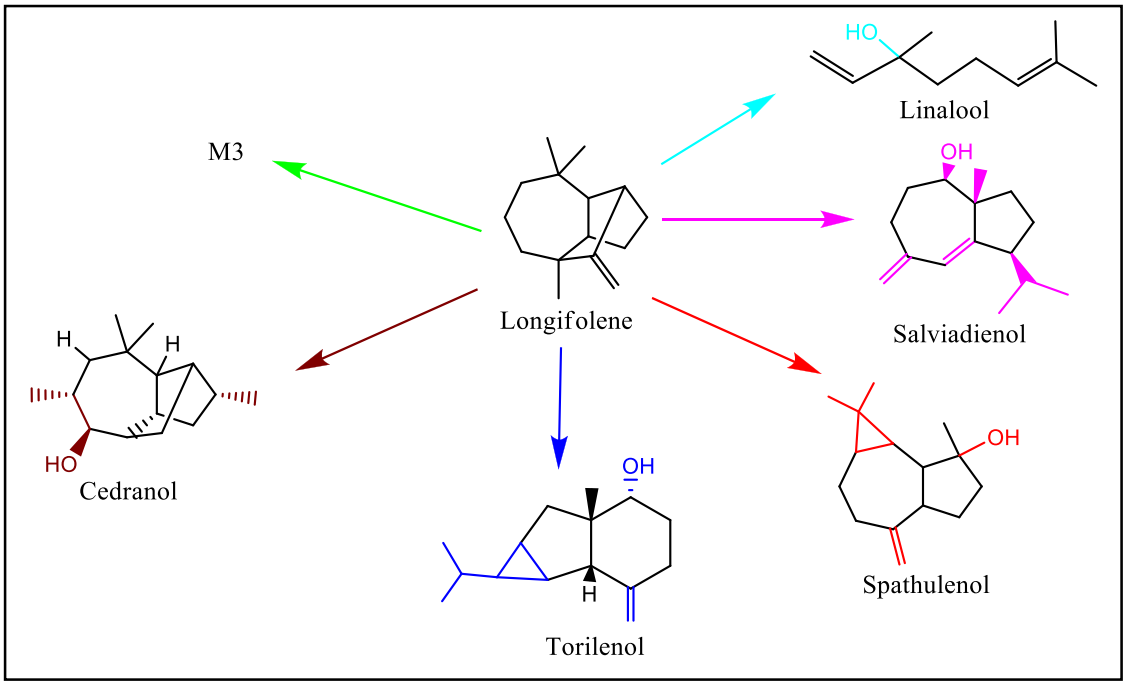


Table 2. Longifolene transformation products from cultures of *W. extensa* identified in this study

Peak no:	R.R.I.	%	Compound
1	1553	0.28	Linalool (Mw: 154)
2	1583	0.92	Longifolene (Substrate) (Mw: 204)
3	2130	1.27	Salviadienol (Mw: 220)
4	2144	3.38	Spathulenol (Mw: 220)
5	2278	3.31	Torilenol (Mw: 220)
6	2736	5.59	Cedranol (Adams, 1995) (Mw: 222)
7	2930	14.97	M3 (236, 218, 203, 189, 181, 175, 163, 157, 147, 136, 128, 121, 115, 109, 99, 93, 82, 73, 67, 55, 41)
*n.i**			

RRI: Relative retention indices calculated against *n*-alkanes; % percentages calculated from FID data; *n.i**: non identified.

Scheme 4. Longifolene transformation products (Table 2.)



As conclusion, the fungi *W. extensa* produced also several polar compounds according to chromatographic analyses, which could not be identified in the present study. Further studies are needed to evaluate the three of metabolites which are not identified.

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