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Characterisation of a Recombinant Patchoulol Synthase Variant for Biocatalytic Production of Terpenes

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Abstract The patchoulol synthase (PTS) is a multi-product sesquiterpene synthases which is the central enzyme for biosynthesis of patchouli essential oil in the patchouli plant. Sesquiterpene synthases catalyse the formation of various complex carbon backbones difficult to approach by organic synthesis. Here, we report the characterisation of a recombinant patchoulol synthase complementary DNA (cDNA) variant (PTS var. 1), exhibiting significant amino acid exchanges compared to the native PTS. The product spectrum using the natural substrate E,E-farnesyl diphosphate (FDP) as well as terpenoid products resulting from conversions employing alternative substrates was analysed by GC-MS. In respect to a potential use as a biocatalyst, important enzymatic parameters such as the optimal reaction conditions, kinetic behaviour and the product selectivity were studied as well. Adjusting the reaction conditions, an increased patchoulol ratio in the recombinant essential oil was achieved. Nevertheless, the ratio remained lower than in plant-derived patchouli oil. As alternative substrates, several prenyl diposphates were accepted and converted in numerous compounds by the PTS var. 1, revealing its great biocatalytic potential.

Keywords Terpene synthase · Sesquiterpenes · Biocatalysis · Patchoulol · Essential oil

Introduction

In nature, sesquiterpene synthases catalyse the key step in the biosynthesis of sesquiterpenes. The patchoulol synthase (PTS) from *Pogostemon cablin* (Indian Patchouli) is a multi-product sesquiterpene synthase and the key-enzyme in the biosynthesis of patchouli oil—an important essential oil for the perfume industry. Today, patchouli oil is mainly produced from the plant

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raw materials by steam distillation. Usually, it contains 30–50 % of the commercially relevant terpene alcohol patchoulol which embodies the main contributor to the typical patchouli scent [1].

Alongside patchoulol, the PTS produces at least 22 additional sesquiterpenes with different structural motives that are part of the complex composition of patchouli oil [2]. Such a broad product spectrum is uncommon and in contrast to this, the majority (>80%) of sesquiterpene synthases form only one single product [3]. All sesquiterpene synthases transform the structurally relatively simple substrate *E*,*E*-farnesyl diphosphate (FDP, C_{15} unit) into various sesquiterpenes including macrocyclic, bicyclic and tricyclic compounds [4]. The complex C_{15}-carbon backbones of sesquiterpenes combined with their limited number of functional groups make it difficult to produce substances such as patchoulol or *trans*-β-caryophyllene by organic synthesis approaches [5–7].

The large product spectrum of multi-product sesquiterpene synthases, such as the PTS, is caused by manifold formation mechanisms that are typical in terpene biosyntheses. Initial dephosphorylation of the FDP is followed by a carbocation reaction cascade including ring closures, rearrangements, hydrid shifts and protonation, as well as deprotonation steps [8]. The sequence of these reactions and the formation of particular intermediates and products is guided by the chemical appearance and the plasticity of the active site pocket which act as a template for the stereo electrical driven reactions [9]. With no crystal structure of the PTS being published so far, the only structural insights available in literature are based on in silico modelling [10, 11]. As in other sesquiterpene synthases, the DDXXD motif binds cofactor Mg^{2+} and ensures the right orientation of the FDP molecule in the active site pocket [12]. The NSE/DTE motif located in close proximity the DDXXD motif is assumed to be an additional binding site which could influence the carbocationic reaction cascade leading to an even more diverse product spectrum [11–13].

In nature, FDP is build up by the MEV and MEP pathway using glucose as substrate. Some studies have been performed cloning these complex pathways into production organisms in order to accomplish a whole cell biosynthesis of sesquiterpenes [14–16]. These attempts demonstrate the importance of sesquiterpene synthases in modern biotechnology. In some applications, the use of such pathway-engineered microorganisms might be limited due to their unspecificity and catalytic inefficiency of many enzymes involved in specialised pathways such as the MEV pathway which is essential for the initial biosynthesis of the substrate FDP, the central C_{15}-building block in the sesquiterpene formation [17].

In contrast to the extensive expression and optimisation of the whole MEP and MEV pathway, we present a more straight forward in vitro approach based on the recombinant PTS var. 1. The enzyme was produced heterologously in *Escherichia coli* applying a solubility enhancing strategy by using a codon optimised PTS gene in order to increase the yield of active PTS var. 1 [10]. In combination with a short organic synthesis of the prenyl diphosphate substrates using a modified method described by Keller et al. [18], sesquiterpene synthases are promising tools for in vitro biocatalysis to build up challenging sesquiterpene carbon backbones, including precursors for pharmaceutical active molecules such as artemisinin and zerumbone [19]. Furthermore, the in vitro application of those enzymes allows the use of alternative substrates that can extend the scope of accessible products [20, 21].

The patchoulol synthase variant used in this work demonstrates several amino acid mutations within and around the NSE/DTE motif [10]. The position and chemical properties of the amino acids within the active site of the enzyme are crucial for the particular constitution of the final sesquiterpene at the end of the biocatalytic process. As it is reported, even a few amino acid mutations can affect terpene synthases significantly; therefore, PTS var. 1 needs to

be characterised thoroughly [22]. The PTS var. 1 was characterised in terms of its product spectrum and selectivity, optimal reaction conditions, kinetic properties and substrate scope. The enzyme consists of 553 amino acids with a theoretical weight of 64.2 kDA and a pI of 5.2 [10, 23].

Materials and Methods

The patchoulol synthase (PTS, E.C. 4.2.3.70, UNIPROT sequence accession number: Q49SP3) is a sesquiterpene synthase originated from *P. cablin* (GenBank: ABC87816.1). In this work, a complementary DNA (cDNA) variant (GenBank: KF983531.1) of the enzyme (PTS var. 1) was used. The PTS var. 1 exhibits 19 amino acid substitutions compared to the native PTS. For the enzyme production, an *E. coli* strain BL21 (DE3) containing an IPTG-inducible expression system (pET16b::his-FXa-PTSopt) was constructed in previous works. To maintain reliable and high yield in production of the PTS in soluble form, the PTS var. 1 gene was previously optimised to the codon usage of *E. coli* [10]. The PTS var. 1 is equipped with a His_6-tag in order to enable easy and reliable purification.

All chemicals used in this study were purchased either from Sigma-Aldrich (Steinheim, Germany) or Carl Roth GmbH (Karlsruhe, Germany), unless indicated otherwise. *E*,*E*-, *E*,*Z*- and *Z*,*E*-farnesol isomers were kindly provided by Symrise AG (Holzminden, Germany). Aqueous solutions were prepared using double de-ionised water from an Arium® water purification system (Sartorius-Stedim Biotech, Göttingen, Germany). pH values were adjusted at 25 °C using a Professional Metre PP-15 (Sartorius-Stedim Biotech, Göttingen, Germany). Optical density was determined at 600 nm using an Orion 700 UV-meter (Thermo Fisher Scientific, Waltham, USA).

Cultivation

Cultivation for enzyme production was performed in TB-medium in a 2-L stirred tank reactor Biostat A plus (Sartorius-Stedim Biotech, Göttingen, Germany) equipped with a pH electrode (EasyFerm Plus K8 200; Hamilton, Reno, USA) and a dissolved oxygen (DO) sensor (OxyFerm FDA 225; Hamilton, Reno, USA). Oxygen saturation level for the DO-sensor calibration was achieved by applying air pressure at a stirrer speed of 1200 rpm. After inoculation with pre-culture to OD_{600} = 0.05, the bacteria grew at 30 °C and a DO level of 30 % achieved by 2 rvm air gassing with the stirrer in cascading mode as *p*H-stat culture at pH 7.2. At OD_{600} = 0.9, the temperature was lowered to 20 °C and expression of protein was induced by addition of IPTG to a final concentration of 500 μM in the culture broth. Thereafter, DO level was set to 15 % to slow down bacterial growth in order to increase yield of active PTS. Cultivation was stopped 12 h after induction, and the cells were harvested by centrifugation (8000×*g*, 4 °C, 30 min) and stored at −20 °C.

Enzyme Purification

For the purification of PTS, 15-g wet biomass were suspended in 200-mL lysis buffer (150 mM NaCl, 50 mM MOPS, 50 mM imidazole, 10 mM $MgCl_2$, 5 mM DTT, 10 % (*v*/*v*) glycerine, pH 7.4) at 4 °C. Cell lysis was achieved using a cell microfluidizer (M 110 L; Microfluidics, Newton, USA) at 9000 psi (fivecycles). The cell lysate was centrifuged

(8000×g, 4 °C, 30 min) to separate the cell debris from the lysate containing the soluble PTS. The purification of PTS was achieved by immobilised metal ion affinity chromatography (IMAC) using a HisTrap FF® (cv = 5 mL, GE Healthcare, Fairfield, USA) column functionalised with IDA ligands decorated with Ni^{2+}-ions connected to a chromatography system (BioLogic DuoFlow; Bio-Rad, Hercules, USA). Eluent A was the lysis buffer, eluent B was lysis buffer supplemented with 500 mM of imidazole and flow rate was 3 mL min^{-1}. Proteins were detected with a PDA detector at a wavelength of 260 nm. After loading 200 mL of the sample onto the column and washing with 50 mL eluent A, the elution was carried out by using a step gradient as follows: 10 % eluent B for 15 min to elute unspecific bound host cell proteins (HOP) of *E. coli* and 100 % eluent B for 15 min to elute PTS. To prevent inactivation of enzyme due to high imidazole concentrations, the PTS containing fraction was immediately applied onto a centrifugal concentrator with a cutoff of 10 kDa (Vivaspin 20; Sartorius-Stedim Biotech, Göttingen, Germany) in order to perform a buffer exchange as follows: centrifugation (8000×*g*, 4 °C, 30 min) and refill with PTS assay buffer (150 mM NaCl, 50 mM MOPS, 10 mM $MgCl_2$, 5 mM DTT, 10 % (*v*/*v*) glycerine, pH 7.4); this procedure was repeated until imidazole concentration was below 0.05 mM. The purified enzyme was stored at −20 °C.

Coomassie stained standard SDS-PAGE gel electrophoresis indicates a purity of PTS of 93 % using densitometry (Alpha Ease®FC version 6.0 Software, Alpha Innotech Co., San Leandro, USA). The purified enzyme was quantified by a NanoDrop spectrophotometer (ND-1000; Peqlab Biotechnologie GmbH, Erlangen, Germany) using the calculated molar extinction coefficient for the PTS of $\varepsilon = 90{,}190$ L mol^{-1} cm^{-1}. To confirm the enzyme concentration a Bradford assay was performed using a BSA standard calibration.

Synthesis of Alternative Substrates

Syntheses of prenyl diphosphates were performed using the corresponding terpene alcohols as starting material according to Keller et al. [18] The products were purified by flash chromatography (silica gel 60, 400-230 mesh; Carl Roth GmbH, Karlsruhe, Germany) and quantified by RP-HPLC using a VWR Hitachi Chromaster (Hitachi, Tokyo, Japan) equipped with a Jupiter Proteo 90 A column (205 × 4.6 mm, Phenomenex, Torrance, USA). The separation of prenyl phosphates was achieved by using a gradient of two eluents at a flowrate of 1 mL min^{-1} at a column-oven temperature of 25 °C. Eluent A was a NH_4HCO_3 solution (30 mM) and eluent B was MeCN/deionised H_2O (95:5). The gradient programme consisted of the following: decrease from 70 % A to 36 % A within 10 min to 10 % A within 1 min, this level was held for 2 min, increase to 30 % A within 1 min, this level was held for 4 min. Prenyl phosphates were detected by a PDA detector at an absorption wavelength of 214 nm.

GC-FID and GC-MS Analysis

Quantitative analytics of mono- and sesquiterpenes were performed on a GC-2010 plus (Shimadzu, Kyoto, Japan) gas chromatograph equipped with Zebron-WAXplus column (30 m length, 0.25 mm I.D., 0.25-μm film thickness, Phenomenex, Torrance, USA) using hydrogen as carrier gas. Two microlitres of sample were injected via autosampler (splitless mode, injector temperature 240 °C). Temperature gradient was as follows: 40 °C held for 20 s, raised to 210 °C (10 °C min^{-1}), held for 1 min, raised to 230 °C (40 °C min^{-1}) and held for 3 min. The FID was heated to 300 °C.

Sesquiterpenoid compounds were identified by GC-MS on a Fisions GC 8000 gas chromatograph (CE Instruments, Hindley Green, UK), equipped with Zebron-WAX column (30 m length, 0.25 mm I.D., 0.25-μm film thickness, Phenomenex, Torrance, USA) in on-column injection mode (1 μL) using helium as carrier gas. Temperature gradient as follows: 40 °C held for 3 min, raised to 230 °C (3 °C min^{-1}). The GC was connected to a Fisions MD800 mass selective detector (interface 230_C; ion source 200_C: quadropol1 100_C; EI ionisation 70 eV; scan range 33-330 amu). Mass spectra were compared to a digital library (Wiley08/NIST08, 2008) and to spectra extracted from standard compounds.

Assay for the Determination of the PTS var. 1 pH and Temperature Optimum

Enzyme assays to determine pH and temperature optimum were performed by measuring the reaction rate either at pH values of 6.5–8.5 at 37 °C or at temperatures of 32-44 °C at pH 7.25. For each pH value and temperature tested, 2.5 mL assay buffer adjusted to the particular pH was supplemented with 0.1 μM PTS and 50 μM FDP and aliquoted quickly into five 500 μL portions in 1.5-mL reaction vessels and overlaid with 200-μL iso-octane. After incubation at 37 °C in a water bath for 2, 4, 6, 8 and 10 min, the aliquots were mixed vigorously for 30 s on a vortex-mixer in order to stop enzymatic reaction and to extract sesquiterpenoid products from the aqueous phase. Thereafter, the reaction mixture was centrifuged (10,000×*g*, 10 s, RT) and the organic phase was transferred into a GC vial for the analysis of enzymatic products using GC FID. The enzyme assay experiments were repeated thrice, and the resulting values were averaged.

Enzyme Assay for Kinetic Studies

To determine kinetic parameters of PTS var. 1, the reaction rate at different substrate concentrations was examined. The reactions were performed in an aqueous buffer (50 mM MOPS, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 10 % (*v*/*v*) glycerin) at 37 °C and pH 7.4. At these conditions, the ionic strength of the buffer solution was calculated to be $I = 179$ mM.

For enzyme kinetic assays, 2.5-mL assay buffer, supplemented with 0.5 μM PTS var. 1 and different FDP (MoBiTec, Göttingen, Germany) concentrations between 1 and 100 μM, were aliquoted quickly into five 500-μL portions in 1.5-mL reaction vessels, and each was overlaid with 200 μL iso-octane containing 100 ppm α-cedrene as internal standard. After incubation in a water bath at 38 °C for 2, 4, 6, 8 and 10 min, the aliquots were shaken for 30 s in order to stop enzymatic reaction and trap terpenoid products. Phase separation was obtained by centrifugation for 10 s at 14,000×*g*. The organic layer was separated and subsequently analysed by GC-FID.

Bioconversion of Alternative Substrates

Biotransformation experiments were performed in a 5 mL glass vial equipped with a gas-tight Teflon-sealed screw cap in 3 mL of a buffer solution (50 mM MOPS, 10 mM $MgCl_2$, pH 7.4) supplemented with 100 μM of the alternative substrate and 1.0 μM PTS var. 1. The reaction mixture was overlaid with 300 μL pentane and incubated for 60 min at 30 °C. In order to extract terpenoid products, the reaction mixture was shaken vigorously and subsequently centrifuged to ensure phase separation. The extract was separated from the aqueous phase and analysed by GC-FID and GC-MS.

Results

Identification of Main Products

The patchoulol synthase is known to be a multi-product sesquiterpene synthase converting the universal substrate FDP into numerous polycylic sesquiterpenes including patchoulol. To this day, no crystal structure is known for this enzyme. Since the PTS var. 1 features 19 amino acid exchanges, it was monitored whether the product spectrum was altered in terms of sesquiterpene spectrum product ratios. The products resulting from the conversion of the natural substrate *E*,*E*-FDP were analysed by GC-FID and compared to commercial available patchouli oil from *P. cablin.* Additionally, the five main constituents were characterised by GC-MS (Fig. 1).

According to the GC-MS analysis, the major products of the PTS var. 1 beside the terpene alcohol patchoulol (RI = 2155) are β-patchoulene (RI = 1682), *trans*-β-caryophyllene (RI = 1609) and the guaienes α bulnesene (RI = 1694) and guaia-5,11-diene (RI = 1578). Apart from the particular percentage of the compounds, all identified sesquiterpenes are natural constituents in the essential oil of the plant *P. cablin.*

Evaluation of Optimal Reaction Conditions

The PTS var. 1 was characterised in terms of general parameters including pH and temperature optimum in order to compare the results with the data of the native patchoulol synthase available in literature. Furthermore, alternative divalent metal cations were tested for their acceptability as alternative cofactors replacing the natural cofactor Mg^{2+}.

To determine the optimal pH value, the reaction rate was measured by using a two-phase activity assay consisting of an aqueous phase hosting the enzymatic conversion and an isooctane phase to trap terpenoid products [24]. The enzyme activity assays were performed using

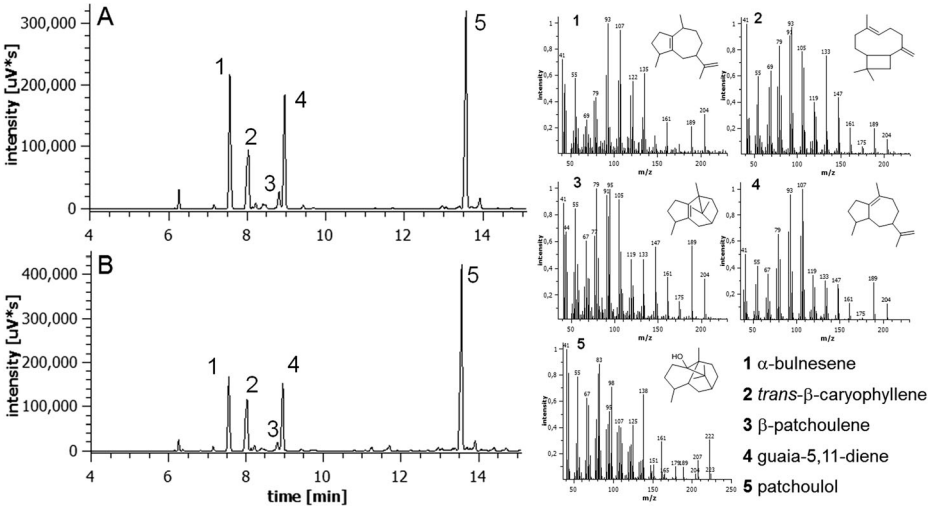


Fig. 1 Gas chromatogram and appendant MS spectra of the patchouli oil formed by the PTS var. 1 (**a**) in comparison with commercial available plant derived patchouli oil from *P. cablin* (**b**)

0.5 μM of the purified PTS var. 1 and 50 μM *E*,*E*-FDP as substrate. Reaction rates were determined by measuring the product formation by GC-FID. The pH value in the aqueous phase was varied in a range of pH 6.5–8.0. For the determination of the optimal reaction temperature, the reaction rate was varied from 30 to 44 °C (Fig. 2). According to literature, optimal reaction conditions for sesquiterpene synthases are usually found within those ranges [20, 23, 25–29].

The PTS var. 1 demonstrates its highest reaction rate at pH 7.25. At a slightly higher pH value of pH 7.50, the enzyme shows similar activities, whereas a shift to pH 7.00 results in a decline to 70 % activity. Higher as well as lower pH values of pH 6.50 and pH 8.00 cause a significant decrease of the PTS var. 1 activity to nearly 20 % residual activity compared to the activity at its pH optimum.

Highest activity of the PTS var. 1 was found to be at 36-40 °C with a maximum reaction rate at 38 °C. As temperatures exceeded 40 °C, the activity was reduced to an activity less than 60 %, and at 44 °C, an activity of only 50 % enzyme activity was monitored. The loss of activity towards lower temperatures was not as significant as at higher temperatures. At 30 °C, the PTS var. 1 exhibited still 65 % of the activity compared to the temperature optimum.

For the cofactor screening, Ca^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, $Co^{2+}$and Cu^{2+} were used instead of Mg^{2+} in a concentration of 10 mM as alternative cofactor in the enzyme activity assay. None of the tested divalent metal cations led to any PTS var. 1 activity.

PTS var. 1 Kinetics

To determine the kinetic parameters of the PTS var. 1, the reaction rate was monitored at different substrate concentrations by quantification of the formed sesquiterpenes by GC-FID. Such as in the case of natural patchouli oil, the products formed by PTS var. 1 enzyme are a complex mixture of numerous sesquiterpenes. Thus, the quantification of the reaction products was simplified by monitoring only the peak area of the five main constituents which constitute approximately 80 % of the total composition (Fig. 1). These combined values were compared to those of a calibration using commercial patchouli oil as analytical standard. To convert the resulting reaction rates that were given in $\mu g\, mL^{-1}\, min^{-1}$ into a molar concentration, the average molar mass of patchouli oil was calculated based on the five main products in the analytical patchouli oil standard. The standard contains 35.2 % patchoulol with

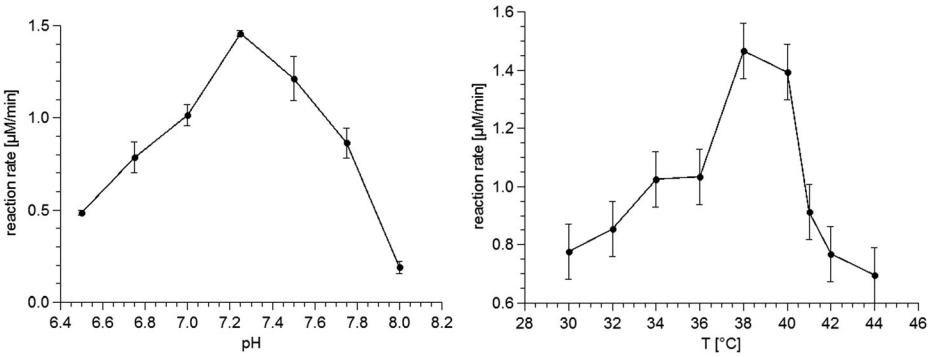


Fig. 2 Results of the determination of the optimal pH (*left graph*) value and reaction temperature (*right graph*)

M = 222.36 g L^{1} and 64.8 % other, unhydroxylated sesquiterpenes with M = 204.35 g mol^{-1}. Thus, the average molar weight of patchouli oil is 210.69 g mol^{-1}. Using this number for calculation of the molar reaction rate, a curve was fitted to the data using the Levenberg-Marquardt algorithm that is based on the method of partial least squares. The fit was applied by using the data analysis and visualisation software OriginPro 8.6 (OriginLab, Northampton, USA). Assuming a standard Michaelis-Menten kinetic, the PTS var. 1 has K_M = 8.0 ± 6.5 μM and v_{max} = 6.93 ± 1.33 μM min^{1}. Accordingly to that, enzyme activity was calculated to be k_{cat} = 0.072 s^{1} and K_{cat}/K_M = 9000.0 s^{1} mol^{-1}.

pH Dependency of Product Selectivity

In order to study the product selectivity of the PTS var. 1, its five main products were monitored. The change in product composition was analysed by comparing the ratio of sesquiterpenes formed during the enzymatic reaction. The product percentages were calculated based on the averaged peak areas taken from the 10 min GC FID measurements generated in the experiments for the evaluation of the pH optimum (Fig. 3). Reactions were performed as described above in a two-phase enzyme assay using the standard assay buffer supplemented with the purified enzyme and 50 μM FDP at 38 °C overlaid with iso-octane.

The selectivity of the main products formed by the PTS var. 1 was strongly influenced by the pH value of the assay buffer. Especially the ratio of the commercially relevant patchoulol is affected by the pH value during the reaction, exhibiting a maximum at pH 7.25. At this conditions, the two other major products α-bulnesene and guaia-5,11-diene have minimal ratios in the product spectrum. In line with that, at either higher or lower pH values, ratios of α bulnesene and guaia-5,11-diene increase while the patchoulol ratio declines. Interestingly, the ratio of α bulnesene increases not as much as that one of its isomer guaia 5,11 diene. At pH 8.5, both products are formed in almost equal parts. The product ratios of β patchoulene and *trans* β caryophyllene remain stable over the entire pH range tested in this study.

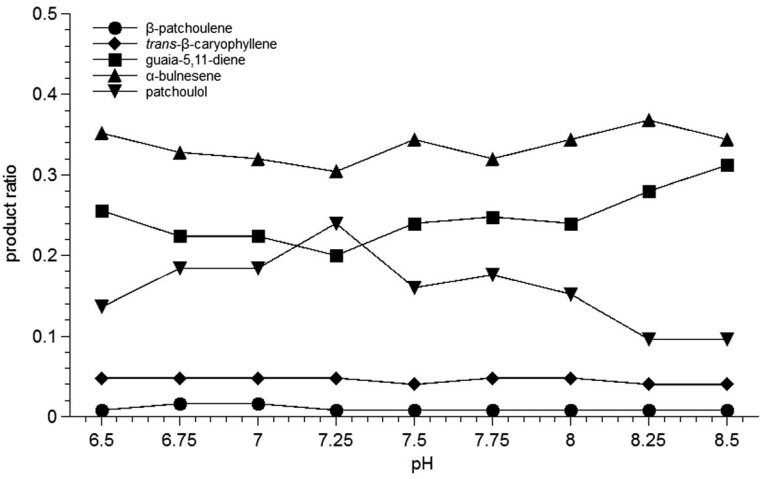


Fig. 3 Results of the pH dependency of the product selectivity. The curve of inverted triangle symbols illustrates the patchoulol ratio in the PTS var. 1 product spectrum with its maximum at pH 7.25

Temperature Dependency of Product Selectivity

As well as in the pH dependency studies, the influence of the reaction temperature on the product selectivity of the PTS var. 1 was studied. The conversions were performed as described above in standard assay buffer at pH 7.25 supplemented with the purified enzyme and 50 μM FDP overlaid with iso-octane (Fig. 4).

The temperature of the reaction mixture was found to be a strong parameter for product selectivity of PTS var. 1. Especially, the ratio of patchoulol shows a strong tendency towards lower values at increasing temperatures. At temperatures up to 36 °C, patchoulol is the main product with a ratio of 35-40 % in the product spectrum. With increasing temperatures, the patchoulol selectivity declines to 20 % at 44 °C. In line with that, the formation of α-bulnesene is extremely favoured at higher temperature. The α-bulnesene selectivity increases from 26 % to over 50 % in the temperature range tested, whereas the ratio of its isomer guaia-5,11-diene shows no clear trend and fluctuates between 19 and 27 %. Also, the ratio of *trans*-β-caryophyllene is not affected by the temperature alteration and remains on the same level. In contrast to that, the selectivity of the β-patchoulene isomers decrease from 2.4 % at 30 °C to 0.8 % at 44 °C.

Substrate Scope of PTS var. 1

In order to study the substrate scope of the PTS var. 1 enzyme, several other prenyl diphosphates including the *E*,*Z*- and *Z*,*E*-FDP isomers, geranyl diphosphate (GPP), neryl diphosphate (NPP) and the diterpene precursor geranylgeranyl diphosphate (GGPP) were tested in bioconversion experiments. Except GGPP which was purchased from Sigma-Aldrich, all prenyl diphosphates were synthesised according to the simple and reliable method by Keller et al. [18]. Since this approach is based on a stepwise esterification mechanism, the corresponding mono- and triphosphates are formed in the reaction as well and can be separated

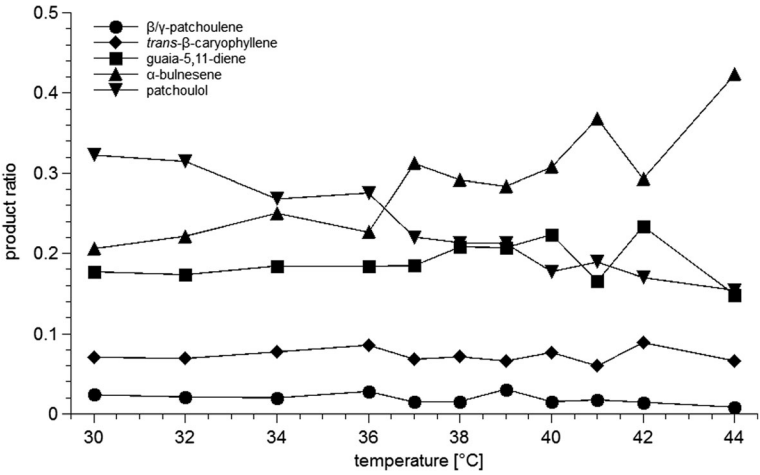
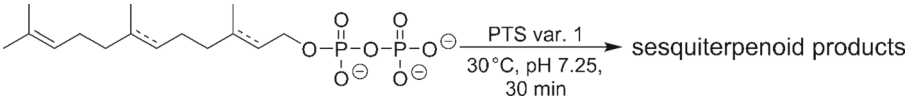


Fig. 4 Results of the temperature dependency of the product selectivity. The curve with the *inverted triangle symbols* shows a declining trend of the patchoulol ratio with decreasing temperatures. The α-bulnesene ratio represented by the triangles is increasing simultaneously

from the diphosphates by extended flash chromatography. The mono phosphate esters *E*,*E*-farnesyl and geranyl monophosphate (FMP, GMP) as well as the corresponding triphosphate esters (FTP, GTP) were also tested as alternative substrates.

Among the prenyl diphosphates tested in these experiments, only GGPP was not converted by the PTS var. 1. The monoterpene precursor GPP and NPP as well as the *E*,*Z*- and *Z*,*E*-FFP isomers were accepted as substrates and successfully converted into terpenoid products

Table 1 Reactions products formed in the conversion of the alternative sesquiterpene precursors



Substrate	RI[a]	Rel. %	MS Peaks[b]	Compound	CAS	Identification
***2Z,6E*-FDP**						
	1473	6.0	105[c], 119, 93, 161, 204[d]	ylangene	14912-44-8	[e], [30]
	1539	26.2	161[c], 105, 133, 91, 204[d]	α-gurjunene	489-40-7	[e], [31]
	1662	24.5	105[c], 91, 161, 105, 204[d]	γ-gurjunene	22567-17-5	[e], [31]
	1679	1.8	105[c], 93, 40, 133, 204[d]	longifolene	475-20-7	[e]
	1686	0.7	40[c], 189, 93, 105, 204[d]	β-chamigrene	18431-82-8	[e], [32]
	1706	4.0	161[c], 119, 105, 134, 204[d]	δ-cadinene	483-76-1	[e]
	1715	2.2	105[c], 161, 93, 40, 204[d]	α-muurolene	31983-22-9	[e]
	1738	3.9	67[c], 93, 107, 81, 204[d]	helmintho-germacrene	75023-40-4	[e]
	1748	1.8	161[c], 119, 105, 134, 204[d]	cadina-1,4-diene	483-76-1	[e], [33]
	1789	19.0	121[c], 93, 105, 41, 204[d]	germacrene B	15423-57-1	[e], [34]
	1982	1.3	43[c], 161, 105, 121, 222[d]	germacrene-D-4-ol	72120-50-4	[e]
	2070	2.9	43[c], 161, 105, 81, 222[d]	epiglobulol	552-02-3	[e], [35]
	2301	5.6	69[c],41, 81, 93, 222[d]	*2Z,6E*-farnesol	3790-71-4	[e], [36]
***2E,6Z*-FDP**						
	1670	3.1		γ-elemene	30824-67-0	[e] [37,38]
	1715	39.9		unknown, $C_{15}H_{24}$		
	1720	52.2	67[c], 93, 107, 53, 205[d]	(*1Z,-5E*)-8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	75023-40-4	[37]
	2322	4.9	69[c], 41, 81, 93, 222[d]	*2E,6Z*-farnesol	3879-60-5	[e], [39]

[a] Kovats retention index by using a normal alkane standard solution (C_8–C_{25}) time on a Zebron-WAX column (30 m length, 0.25 mm I.D., 0.25-μm film thickness) with hydrogen as carrier gas

[b] MS peaks, major fragmentation ions in decreasing order of relative abundance

[c] Base peak

[d] Molecular ion peak (M^+);

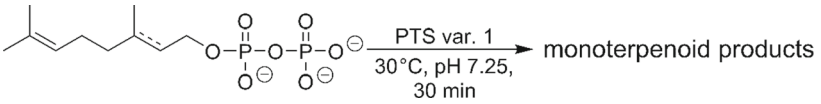
[e] Digital library (Wiley08/NIST08, 2008)

(Tables 1 and 2). Using the prenyl mono and triphosphate esters as substrates, no terpene formation was obtained.

Discussion and Conclusions

The sesquiterpenoid product spectrum produced by the PTS var. 1 converting the natural substrate *E*,*E*-FDP is overall consistent with the composition of plant-derived patchouli oil as well as the recombinant produced patchouli oil reported by Deguerry et al. [2]. The exact composition of the product spectrum, meaning the ratios of the formed products, can vary due to different genotypes in geographical cultivars. Even though terpene synthases are known to

Table 2 Reactions products formed in the conversion of the alternative monoterpene precursors



Substrate	RI[a]	Rel. %	MS Peaks[b]	Compound	CAS	Identification
GPP						
	1179	9.6	41[c], 69, 93, 53, 136[d]	β-myrcene	123-35-3	[e], [38]
	1206	17.2	39[c], 67, 93, 79, 136[d]	lemonene	138-86-3	[e], [40]
	1281	1.5	93[c], 41, 79, 53, 136[d]	sabinene	3387-41-5	[e]
	1690	22.5	59[c], 93, 121, 136, 154[d]	α-terpineol	98-55-5	[e], [40]
	1844	49.1	41[c], 69, 53, 93, 154[d]	geraniol	106-24-1	[e], [41]
NPP						
	1155	4.7	93[c], 69, 41, 79, 136[d]	β-mycrene	123-35-3	[e], [38]
	1165	1.3	40[c], 121, 44, 93, 136[d]	*Z*-ocimene	29714-87-2	[e]
	1185	39.3	39[c], 67, 93, 79, 136[d]	lemonene	138-86-3	[e], [40]
	1270	10.3	93[c], 121, 136[d], 91, 40	α-terpinolene	586-62-9	[e], [42]
	1452	3.4	40[c], 71, 93, 81, 154[d]	sabinene hydrate	17699-16-0	[e], [43]
	1539	3.7	71[c], 40, 93, 55, 154[d]	linalool	78-70-6	[e], [44]
	1690	32.6	59[c], 93, 121, 136, 154[d]	α-terpineol	98-55-5	[e], [40]
	1787	4.6	69[c], 41, 93, 53, 154[d]	nerol	106-25-2	[e], [45]

[a] Kovats retention index by using a normal alkane standard solution (C_8–C_{25}) time on a Zebron-WAX column (30 m length, 0.25 mm I.D., 0.25-μm film thickness) with hydrogen as carrier gas

[e] Digital MS library (Wiley08/NIST08, 2008)

[b] MS peaks, major fragmentation ions in decreasing order of relative abundance

[c] Base peak

[d] Molecular ion peak (M^+)

[e] Digital MS library (Wiley08/NIST08, 2008)

be sensitive for amino acid exchanges in close proximity to the DDXXD-motif at the active site, the product spectrum of PTS var. 1 was not affected significantly by the appearance of the 18 exchanges in its amino acid sequence [22].

In literature, the pH optimum of a patchoulol synthase isolated from a *P. cablin* plant was found to be pH 7.50 [23]. The only published data of a recombinant produced patchoulol synthase is a pH optimum of pH 7.0–7.5 and an estimated optimal reaction temperature of 40 °C which is consistent to the results obtained in this work [2].

Apart from Mg^{2+}, neither of the tested divalent metal cations have shown any activity in the conversion experiments. Since it is reported that Mn^{2+} ions are accepted as alternative cofactors at concentrations below 0.1 mM, but deploy an inhibiting effect at higher concentrations, the absence of activity is thereby explainable [2].

Recent kinetic studies of terpene synthases usually measure the reaction rate either by monitoring the decline of the substrate concentration using radiotracer assays with ^{31}P-labelled *E*,*E*-FDP, the recently developed malachite green assay for terpene synthases or due to the detection of product formation by GC-MS via total ion monitoring [24, 46, 47]. In this work, the reaction rate was determined by GC-FID quantification of the terpenoids reaction products. The K_M value of the PTS var. 1 ranks higher than both values of the PTS available in literature which could be caused by the differences in the amino acid sequence (Table 2).

The values of k_{cat} and the k_{cat}/K_M are close to the data reported by Munck et al. but significantly higher than those of the recombinant PTS reported by Dequerry et al. Compared to the plant derived PTS, the specific activity of the enzyme used in this work is almost a hundred times higher. This result is either caused by the modifications in the amino acid sequence, or more likely, by the more effective protein purification in this study that caused a higher yield of enzyme in active form. However, the kinetic parameters are common for sesquiterpene synthases [13]. The data show that the PTS var. 1 with its k_{cat} below 0.1 s^{-1} is a relatively slow enzyme and with its K_{cat}/K_M value several magnitudes below the diffusion limit (Table 3). These results are also consistent to a recently published statistical study that indicates that enzymes involved in the secondary metabolism usually exhibit significant lower activity compared to enzymes operating in the central metabolism [48].

The ability of many sesquiterpene synthases to accept GPP as an alternative substrate is well known [20, 21, 49, 50]. Therefore, the acceptance of its *Z*-isomer NPP leading to similar monoterpenoid products is not surprising. Both FDP isomers 2*Z*,6*E*-FDP and 2*E*,6*Z*-FDP tested were converted into sesquiterpenoid products that are very different to the products from the *E*,*E* FDP conversion. In case of the 2*Z*,6*E*-FDP, 13 products were obtained, whereas the 2*E*,6*Z* FDP was converted into only two products. These two substances are particularly interesting, because it was not possible to identify them definitely by GC-MS databases. Fragmentation patterns as well as Kovats retention indices indicate that the substances are

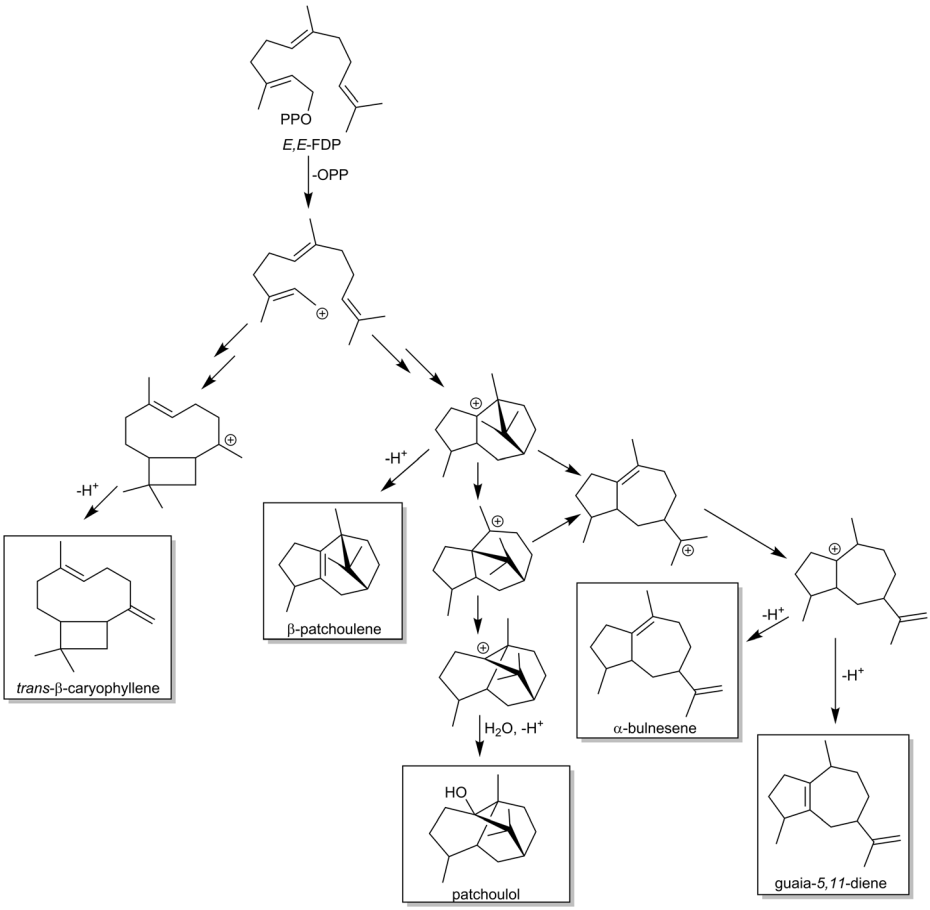
Table 3 Kinetic parameters of the PTS var. 1 in comparison with kinetic data of the native PTS as well as the recombinant PTS by Deguerry et al.

Enzyme	v_{max} [μM min^{-1}]	K_M [μM]	k_{cat} [s^{-1}]	K_{cat}/K_M [s^{-1} M^{-1}]	Spec. activity [U mg^{-1}]
PTS var. 1	6.93 ± 1.33	8.0 ± 6.50	0.072	9000.0	1.92
PTS isolated from *P. cablin* [23]		6.8 ± 0.50	0.030	4411.8	0.02
Recombinant PTS [2]		4.45 ± 0.56	0.00043	96.6	

sesquiterpenes. Since germacrenyl cations are important intermediates in the sesquiterpene biosynthesis [51], it is a logical assumption that the substances are germacrene isomers with an unusual double bond position.

Especially, the capability to accept different *E*,*Z*-FDP isomers as substrates is particular interesting, as the *E*,*Z*-isomers were originally isolated from synthetic farnesol, which is a mixture of isomers and has therefore a significantly lower price than the natural *E*,*E*-farnesol. Converting such a mixture of isomers into the corresponding diphosphates and using those as substrate for the PTS var. 1, the result is an essential oil with probably similar, but slightly different odour to plant-derived patchouli oil. In this way, the PTS var. 1 could be an interesting in vitro biocatalyst for the production of essential oils.

The product selectivity of the PTS var. 1 converting *E*,*E*-FDP is strongly depending on the pH value and the temperature during the reaction. By varying these substantial reaction parameters, the enzyme, which is in fact a patchouli synthase, can be virtually switched into



Scheme 1 The diversity of the product spectrum of the PTS is based on numerous cyclisations, rearrangements and quenching reactions. Presumably, these reactions can be influenced by pH and temperature leading to different product ratios at different reaction conditions. The shown mechanisms were adapted from Faraldos et al. [11]

a α-bulnesene synthase. Two possible reasons for this sensitivity to pH and temperature have to be considered: On the one hand, the influence of the reaction conditions could affect the sterical appearance of the enzyme-cavity which maintains the template for the product formations [9]. The proceeding cationic reaction cascades are guided by stereoelectric driving forces towards the different products and can therefore be affected by structural changes of the cavity. On the other hand, the immediate effect of the changed reaction conditions on the particular steps involved in the complex reaction mechanisms has to be considered as well. Several reaction types such as ring-closures, long-range hydrid-shifts and Wagner-Meerwein rearrangements are part of nature's toolbox of terpene formation which is required to build up the diverse and often complex carbon backbones of sesquiterpenoids [4, 12]. In these reactions, π-cation and hydrophobic interactions play an important role, whereas the role of the cleaved-off diphosphate is still unsolved [52, 53]. However, the stability of the numerous energetic often similar, transition states and intermediates is crucial for the selectivity of which particular product is formed by the multi-product terpene synthase and either under thermodynamic or kinetic control, which implies a temperature dependence [54]. Quenching reactions such as deprotonations in order to form double bonds or the addition of water leading to hydroxyl-functionalized terpenes are key-steps in the furnish of terpenes and are clearly pH dependent (Scheme 1).

Even though the enzyme-cavity which serves as the template for the terpene formations is commonly assumed to be a water-free reaction space [8], protons and in case of the PTS even water must be somehow able to leave and enter the cavity in order to fulfil their role in various reactions.

By combining the results from the determination of the optimal reaction conditions and the product selectivity studies, it was possible to identify the optimal reaction conditions in order to maximise the formation of patchoulol. This was achieved by lowering the reaction temperature to 30 °C. Concerning reaction conditions reported in literature, the ratio of the commercially relevant sesquiterpene patchoulol is below 20 % (Fig. 5).

The extensive characterisation of the PTS var. 1 terpene synthase in terms of product selectivity and substrate scope demonstrates the potential of sesquiterpene synthases as

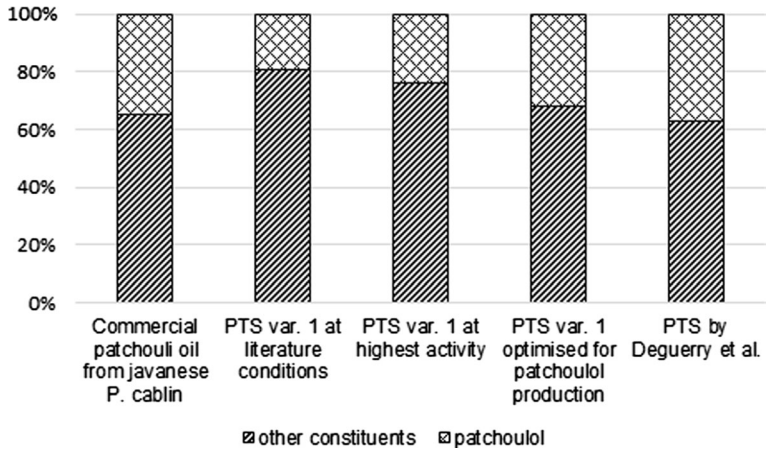


Fig. 5 Patchoulol ratio in patchouli oil formed by the recombinant PTS var. 1 at different reaction conditions, compared to values of plant-derived patchouli oil and the data by Deguerry et al. [2]

powerful biocatalysts. The flexibility to accept different substrates enables the PTS var. 1 to form various mono- as well as sesquiterpenoid products including the fragrance compounds patchoulol, α-bulnesene, longifolene and limonene as well as substances with medical potential such as germacrenes [55] and *trans*-β-caryophyllene [56]. Since the product selectivity of the PTS var. 1 is that pH and temperature sensitive, the enzyme can be remote controlled by the adjustment of the reaction conditions. In the future, the kinetic parameters of the enzyme need to be significantly improved, e.g. by directed evolution, in order to create a unique biocatalyst for the in vitro use.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

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