


REVIEW

Optimization of factors influencing enzyme activity and product selectivity and the role of proton transfer in the catalytic mechanism of patchoulol synthase

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Abstract

The patchoulol synthase (PTS) from *Pogostemon cablin* is a versatile sesquiterpene synthase and produces more than 20 valuable sesquiterpenes by conversion of the natural substrate farnesyl pyrophosphate (FPP). PTS has the potential to be used as a biocatalyst for the production of valuable sesquiterpenes such as (–)-patchoulol. The objective of the present study is to develop an efficient biotransformation and to characterize the biocatalytic mechanism of the PTS in detail. For this purpose, soluble PTS was prepared using an optimized cultivation protocol and continuous downstream process with a purity of 98%. The PTS biotransformation was then optimized regarding buffer composition, pH-value, and temperature for biotransformation as well as functional and kinetic properties to improve productivity. For the bioconversion of FPP, the highest enzyme activity was reached with the 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer containing 10% (v/v) glycerol and 10 mM MgCl₂ at pH 6.4 and 34°C. The PTS showed an unusual substrate inhibition for sesquiterpene synthases indicating an intermediate sesquiterpene formed in the active center. Deuterium experiments were used to gain further insights into the biocatalytic mechanism described in literature. Thus it could be shown that a second substrate binding site must be responsible for substrate inhibition and that further protonation and deprotonation steps are involved in the reaction mechanism.

KEYWORDS

biocatalysis, biocatalytic mechanism, patchouli oil, patchoulol synthase, sesquiterpenes

1 | INTRODUCTION

Terpenes are basic components of essential oils in plants and play a major role in the aroma and fragrance industry. Essential oils are primarily produced by steam distillation or extraction from blossoms, roots, or flowers of the plants. Most of these plants grow in developing countries and are among the cash crops.^{1–3} Patchouli is an aromatic plant

with essential oils that is cultivated in the subtropical regions of India, China, and Southeast Asia like Indonesia. Patchouli oil has immense export potential due to its low production costs and high demand in the perfume and cosmetics industry.^{4,5} Usually the production is done in small companies where the equipment does not meet European standards and there are less regulatory oversights. Therefore the quality of the essential oil may vary. Furthermore climatic crop failures usually

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cause the market price to fluctuate.^{6,7} Indonesia's patchouli oil world market price in 2016 was \$175 per kg.⁸

Main component responsible for the typical woody patchouli scent is the terpene alcohol (–)-patchoulol with a percentage of 40–50%.^{9–11} However, patchouli oil contains more than 20 other different terpenes that determine the complex composition of the oil.^{5,8,11} The contained sesquiterpenoids have functionalized cyclic structures such as (–)-patchoulol, which are difficult to synthesize by chemical synthesis.¹³ The formation of this broad variation of terpenes from the central substrate *E,E*-farnesyl pyrophosphate is catalyzed by a single enzyme, the patchoulol synthase (PTS).

The biosynthetic pathway for the conversion of farnesyl pyrophosphate (FPP) by PTS is not yet fully understood, but it is known that the reaction mechanism involves ring closures, hydride shifts and rearrangements.^{12,14,15} The presence of several aspartate-rich regions with the sequence DDXXD around the active center is characteristic for sesquiterpene synthases and coordinates magnesium ions, which enable the binding of *E,E*-FPP molecules.^{13,16,17} Similar to some other sesquiterpene synthases, the PTS also has another NSE/DTE motif, which is located in the active pocket opposite the DDXXD motif and possibly functions as an additional binding site. This structural motif might, according to the literature, be relevant for the broad product spectrum of PTS.^{13,18} After the dephosphorylation step of FPP by coordination of the diphosphate group to the magnesium ions, the formation of a reactive primary carbenium ion follows. This carbenium ion is the starting point for the construction of the complex carbon atom skeletons from which the reaction to the major products trans- β -caryophyllene and β -patchoulene via a 1,11-ring closure and the formation of (–)-patchoulol, guaia-5,11-diene and α -bulnesene via a 1,10-ring closure is determined.^{13,14,17,19,20}

The used PTS in this work is a cDNA variant of the plant PTS with 19 exchanged amino acids which was constructed in previous works.^{13,21} Previous GC-FID and GC-MS analyses have shown no effect of amino acid exchange on product spectrum, but possibly a minor effect on product selectivity (see Supporting Information). This PTS variant has a His6 tag at the N-terminal of the enzyme, which is useful for an easier chromatographic purification. A further difference to the plant-based variant is the lack of posttranslational modifications, such as glycosylation, which according to the literature are not critical for enzyme activity.¹² As a multi-product sesquiterpene synthase, the PTS with its broad active center is very versatile. Changes in the enzyme structure influence the formation of the end product and reaction routes could be variable by influencing the reaction parameters. Enzymatic reactions are mostly influenced by protons.^{22–24} Compared with previous studies, the propose of this work was to systematically analyze the enzymatic activity on different influencing factors such as buffer components using different biological buffers over a wide pH range. Based on this, the optimal reaction conditions were determined. Finally, studies were performed on the biocatalytic mechanism of PTS by monitoring the five main products, with the reaction carried out in an alternative medium with deuterated water. This research focused on the influence of protons

(deuterons) from medium to reveal its influence and contribution to the reaction mechanism.

2 | MATERIALS AND METHODS

2.1 | Microorganism

The PTS (E.C. 4.2.3.70) from *P. cablin* (GenBank: ABC87816.1, UNIPROT sequence accession number: Q49SP3) was expressed according to the method of Frister et al.¹³ successfully in the *Escherichia coli* strain BL21 (DE3). Cell transformation was carried out in an expression system based on the pET16b vector (pET16b::his-FXaPTS) which was constructed in previous works.^{21,25} The constructed vector has an ampicillin resistance, a factor Xa inhibitor to minimize the production of proteases and a His₆-tag to simplify the chromatographic purification via metal chelate chromatography.

2.2 | Cultivation and enzyme purification

The production and purification of the recombinant PTS was accomplished as reported by Brämer et al.²⁶ Therefore a starter culture (100 ml baffled flask) in 25 ml LB-Miller medium (lysogeny broth, 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl) mixed with Carbenicillin (100 μ M) was started by inoculating with 100 μ l cryo culture and incubating over night at 37°C and 180 rpm. The preculture was used to inoculate the 500 mL main culture (2 L baffled flask) in TB media (terrific broth, 12 g L⁻¹ tryptone, 24 g L⁻¹ yeast extract, 4 mL L⁻¹ glycerol, 100 mL L⁻¹ potassium phosphate buffer) with a starting OD₆₀₀ value of 0.1. The culture was grown at 37°C and 180 rpm until OD₆₀₀ ~0.5 following by induction with 0.5 mM IPTG at OD₆₀₀ = 0.5–0.8. For protein expression the temperature was cooled down to 20°C overnight. After centrifugation (4,700g, 4°C, 30 min) and washing the pellet with PBS buffer, the pellet was stored at –20°C.

Before enzyme purification, the harvested biomass was re-suspended in a ratio of 1:10 in binding buffer (50 mM MES pH 6.4, 10 mM MgCl₂, 10% (v/v) glycerol). The cell suspension was disrupted by sonication in 4 \times 45 s time intervals on ice (amplitude 100%, 100 W, 0.6 s cycle) following by centrifugation (10,000g, 4°C, 10 min) and sterile filtration with 0.2- μ m syringe filters.

The purification of the protein extract was conducted by the continuous chromatographic method described in Brämer et al.²⁵ after optimizing the batch purification process using a FPLC system. The sample flows through serially connected membrane adsorbers according to the principle of periodic counter-current chromatography.

The qualitative analysis of the purified PTS reveals a purity of >90% using SDS-PAGE gel electrophoresis and densitometry. For further experiments the protein quantification was determined by NanoDrop spectrophotometer (ND-1000; Peqlab Biotechnologie GmbH, Erlangen, Germany) using the calculated molar extinction coefficient for the PTS of $\epsilon = 90,190 \text{ L mol}^{-1} \text{ cm}^{-1}$ by Frister et al.¹³

2.3 | Analytical methods

2.3.1 | Temperature and pH optimization

Optimization of pH during bioconversion was tested by measuring product ratio with optimal activity buffer (50 mM MES, 10 mM MgCl₂, 10% (v/v) glycerol) at pH 5.6, 5.8, 6.0, 6.2, 6.4, and 6.6 and 38°C. After evaluation of optimal pH, the biotransformation was analyzed for optimal temperature with optimal activity buffer (50 mM MES, 10 mM MgCl₂, 10% (v/v) glycerol, pH 6.4) at 30, 32, 34, 36, 38, 40, 42, and 44°C. For each measuring point a glass vial was filled with activity buffer, substrate (FPP = 30 μM) and 0.5 μM enzyme to a total volume of 500 μl, overlaid with 150 μl of iso-octane and incubated for 10 min in a water bath. After stopping reaction by shaking for 30 s, reaction volume was centrifuged, 80 μl of the organic phase was transferred to a GC vial and measured in GC-FID (see Section 2.3.3).

2.3.2 | Analysis of enzyme kinetics

To identify kinetic parameters of purified PTS, master mixes of 2.5 ml total volume with activity buffer (see Section 2.3.3) and 0.5 μM enzyme was prepared. Master mixes were split to glass vials with 500 μl reaction volume each. To initiate the reaction different substrate concentrations (1, 5, 20, 30, 50, 80, 100, 150 μM) were added, reaction mixtures were overlaid with 150 μl of iso-octane and incubated at 34°C for 2, 4, 6, 8, and 10 min. To stop the reaction, the mixture was shaking for 30 s and centrifuged for 5 min at 14,000 rpm and room temperature. After centrifugation 80 μl of the organic phase containing the products were transferred to a 1.5 ml GC vial with a small insert (200 μl). Samples were measured in GC-FID.

2.3.3 | Bioactivity assay via GC-FID

Biotransformation of purified PTS was conducted by preparing a mixture of activity buffer, 30 μM FPP and 0.5 μM enzyme (equal to 0.13 μg PTS) in a single vial with a total volume of 500 μl. The assays were overlaid with 150 μl iso-octane and incubated for 15 min at 34°C. To stop the reaction, the mixture was shaken for 30 s and then centrifuged for 5 min at 14,000 rpm and room temperature. After centrifugation 80 μl of the organic phase containing the products were transferred to a 1.5 ml GC vial with a small insert (200 μl). Samples were measured in GC-FID.

2.3.4 | Product identification by GC-MS

Samples were prepared like described in Chapter 2.3. Substrates were transformed with purified PTS by preparing a mixture of the activity buffer (50 mM MES, 10 mM MgCl₂, 10% (v/v) glycerol, pH 6.4), substrate (FPP = 30 μM), and 0.5 μM enzyme (equal to 0.13 μg PTS) in a

single vial with a total volume of 3 ml. The assays were overlaid with 150 μl pentane and incubated for 15 min at 34°C. The reaction was stopped by shaking the mixture for 30 s and then centrifuged for 5 min at 14,000 rpm and room temperature. After centrifugation 80 μl of the organic phase containing the products were transferred to a 1.5 ml GC vial with a small insert (200 μl). Samples were measured in GC-MS.

2.3.5 | Product identification by nuclear magnetic resonance

The enzymatic reaction to convert the *E,E*-FPP with the PTS was realized in a 1 L scale. For the reaction solution, 1 L of the D₂O activity buffer (25 mM MOPS, 5 mM MgCl₂·6H₂O, pH 7.25) was mixed with 2 μM PTS. A solution of 150 μmol *E,E*-FPP dissolved in 1 ml 30 mM NH₄HCO₃ was added to the reaction solution. The reaction solution was sealed hermetically and the solution was heated to 30°C in a water bath with gentle stirring. After 1 hr another aliquot of 150 μmol *E,E*-FPP was added and incubated for further 17 hr.

After incubation, the reaction solution was transferred to a shaking funnel. To extract the patchouli oil, the D₂O solution was extracted three times with 150 ml pentane. The extraction success was checked using GC-FID. The combined organic phases were extracted with 200 ml saturated NaCl solution to remove finely dispersed water from the pentane solution. The organic phase was then reduced to 1 ml under nitrogen until a weakly yellow, oily supernatant with a woody scent was left. The supernatant was dissolved in 500 μl ethanol and the (–)-patchoulol was isolated by thin-layer chromatography and identified by GC-FID. A α -Cedrol Standard (Merck KGaA, Germany) was used as reference substance. α -Cedrol is a suitable reference substance because, like (–)-patchoulol, it is a tricyclic sesquiterpene with a tertiary alcohol group. The oil dissolved in ethanol was absorbed in 0.7 ml CDCl₃ and transferred to a nuclear magnetic resonance (NMR) tube. For comparison, a sample of undeuterated (–)-patchoulol isolated from the analytical standard as described before was measured. A ¹H, ¹³C, ¹³C HSQC (heteronuclear multiple quantum coherence), and ¹³C HMBC (heteronuclear multiple binding correlation) spectrum of both compounds as well as DQF-COSY (double-quantum-filtered correlation spectroscopy) and 1D NOEs (Nuclear Overhauser effects) with shaped pulses were measured. Spectra were recorded on a Bruker 500 MHz Ultrashield with DRX console and Triple Resonance Inverse CryoProbe (TCL) with z-gradients.

3 | RESULTS AND DISCUSSION

3.1 | Buffer optimization

Enzymatic reactions are known to be influenced by protons from the reaction medium.¹³ In comparison to previously published studies,^{13,14} it was intended to present a structured analysis of the reaction components and conditions for optimizing the catalytic reaction. The chemical component of the buffer plays a decisive role in the enzymatic reaction.

Changing the buffer system is therefore useful to increase and control product formation. Since enzymes are sensitive to pH changes, in enzyme assays a buffer solution to keep the pH constant should be used. To achieve the full buffer capacity, a variety of biological buffers with a pH close to their pK_a was selected. At the same time the effect of the buffer component on the enzymatic reaction could be investigated. After determining the buffer component with the highest patchoulol concentration, the pH effect can be examined in detail in the pH range of the selected buffer. In particular, the effects of biological buffer on enzymatic conversion by PTS were observed. Biological buffers are buffers which satisfy most requirements of biological buffers such as low interaction with proteins, high solubility, as well as metabolic and chemical stability.^{27,28} For buffer screening, biological buffer systems with pH-range 3–10 were selected: citric acid (pH 3.2), sodium citrate (pH 4.0), sodium acetate (pH 4.9), 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.1), 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.0), tris(hydroxymethyl)amino-methane (TRIS) (pH 7.4), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8.0), triethanolamine (TEA) (pH 8.4), and glycine (pH 9.8). Buffers contained 50 mM buffer salt, 10 mM $MgCl_2$ and 10% (v/v) glycerol.

In order to compare how different buffers affect enzyme activity, the five main products of Patchouli oil ((-)-patchoulol, α -bulnesene, guaia-5,11-diene, β -patchoulene, trans- β -caryophyllene) were considered. Highest enzyme activity was found in MES buffer at pH 6.1 and TEA buffer at pH 8.4. (-)-patchoulol is responsible for the characteristic scent of Patchouli oil with a proportion of 30–50%.

Since the PTS does not form only one but many different products, the sum of the GC-FID peak areas of the five main products was correlated with the concentration of the same five main products of a patchouli oil standard for quantification. The five main products make up about 80% of the total composition of patchouli oil, including about 35% (-)-patchoulol. The concentration obtained was given in $\mu g\ ml^{-1}$ and converted to a molar concentration in μM . A mean molar mass of $M = 210.69\ g\ mol^{-1}$ was used to calculate the concentration of the five main products and a molar mass of $M = 222.36\ g\ mol^{-1}$ to determine the concentration of (-)-patchoulol. If the total concentration of the five main products is considered, two apparent optima with the MES buffer (pH 6.1) and TEA buffer (pH 8.4) can be recognized. However, looking at the patchoulol concentration of these two buffers, the concentration of pure patchoulol in the TEA buffer is 45% lower. This demonstrates that product selectivity can change depending on the buffer component. Nevertheless, the focus of this study is on the optimization of patchoulol production. Therefore, the buffer with the highest yield of (-)-patchoulol was selected for further optimization. Highest (-)-patchoulol ratio was observed in MES buffer pH 6.1 (see Figure 1). Based on these results, recombinant PTS has also been purified continuously with membrane adsorbers.²⁶ As described by Brämer et al. this buffer is similar to the activity buffer of Munck et al. with less $MgCl_2$ and no dithiothreitol (DTT) and corresponds to the fact that the typical pH optimum of terpene synthases is between pH 6.5–7.5.^{17,26}

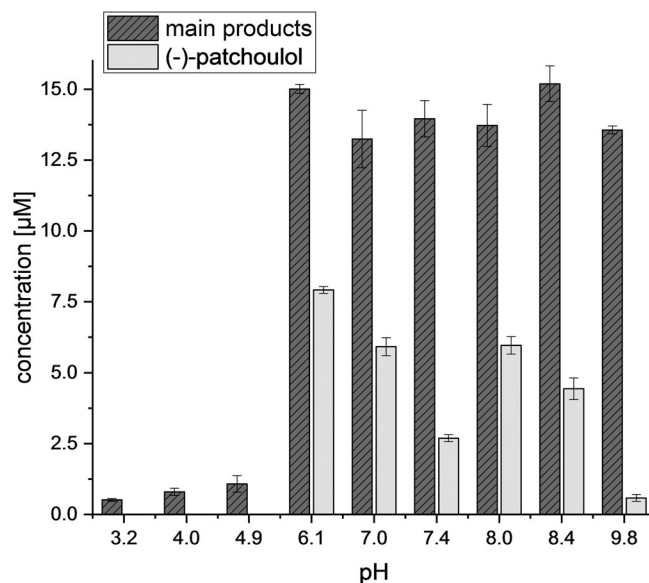


FIGURE 1 Results of screening the enzyme activity and (-)-patchoulol production with biological buffers in a pH 3.2–9.8

3.1.1 | Buffer additives

Up to now there are no studies on the effect of buffer additives such as DTT and NaCl on PTS activity available. Therefore, the influence of these buffer components on product formation was tested by comparing the product ratio of the five biocatalytic main products after biotransformation. For these experiments, the buffer with the highest (-)-patchoulol concentration was selected. In order to achieve a high enzyme activity, protein aggregation should be reduced. DTT, in particular, stabilizes the enzyme by preventing inter- and intramolecular disulfide bridges between cysteines and reduces protein aggregation.^{29,30} These reducing agents were used in literature and have been used in previous experiments and should be tested on their necessity in the activity buffer.^{12,13,26,31}

The experiments, summarized in Figure 2 showed a negative effect of NaCl (150 mM) and DTT (5 mM) on (-)-patchoulol formation as in further experiments²⁶ in the optimal chromatography buffers. Due to the fact that the PTS has only a few disulfide bridges and DTT has a negative influence on the PTS activity, DTT as reducing agent is not necessary.²¹ Without both components, the enzyme activity was higher and an optimal product ratio of the five main products was determined. Therefore these reducing agents were not used in further experiments.

3.2 | Kinetic studies

The product formation of the PTS as a multi-product sesquiterpene synthase is based on a cascade of carbocationic ring closure, rearrangement and interception reactions in the active pocket. The structural composition of the active pocket, which represents the

reaction space of these reaction cascades, is decisive for which sesquiterpene is formed. The exact three-dimensional structure of the active center of enzymes can be slightly changed by factors like pH and temperature, so that different reaction courses become favorable or less likely.

Buffer screening showed clearly that not only the pH-value is important for enzyme activity and product selectivity, but also the buffer components. In this case, the enzyme activity of PTS is noticeably increased from a pH value of 6. Looking at the selected biological buffers using the most stable pH value close to their pK_a value, the MES buffer, in contrast to the MOPS buffer from Frister et al., had a significantly positive influence on patchoulol production. Therefore, the product selectivity of the five main products should

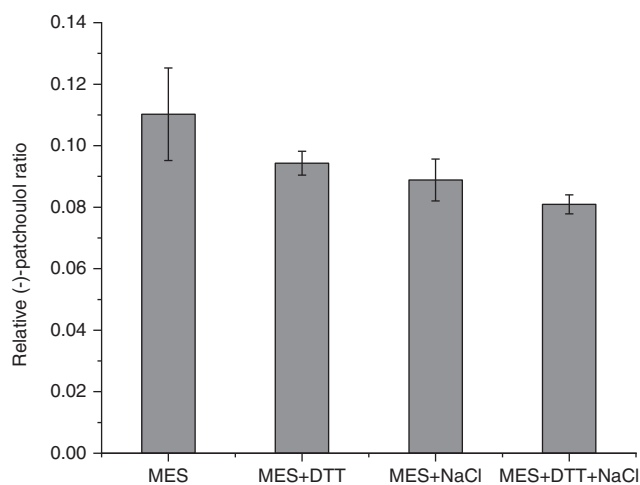
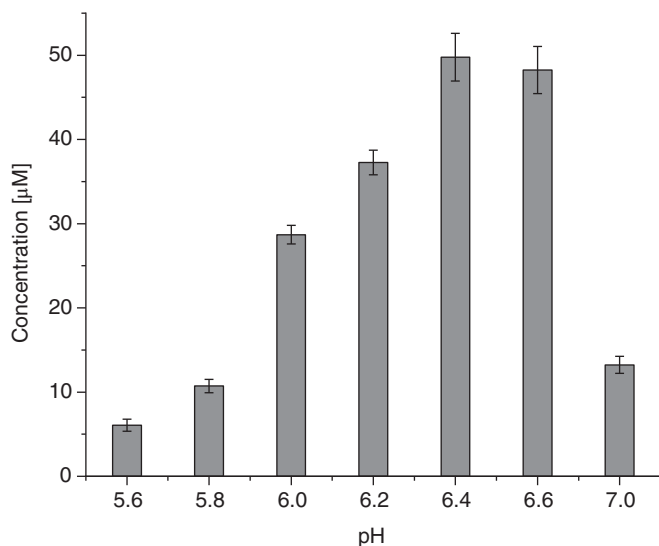


FIGURE 2 Results of the effect of the buffer components dithiothreitol (DTT) and NaCl on the (-)-patchoulol production. Reaction was performed in MES buffer at pH 6.4 and 34°C



also be evaluated in the useful pH-range of the MES buffer (pH 5.6–6.6). In order to test the optimum temperature in MES buffer, a temperature range of 30–44°C was examined. The results are shown in Figure 3.

It is noticeable that the concentration of the five main components of Patchouli oil in the MES buffer is with 49.5 μM the highest at pH 6.4 and 34°C. These parameters were used for further kinetic studies.

For the kinetic characterization of the relevant parameters K_M , v_{max} , and the related characteristic k_{cat} value, the reaction rate had to be determined as a function of the substrate concentration. For this purpose enzyme assays with different concentrations of *E,E*-FPP were performed at the previously determined optimal reaction conditions of 34°C and pH 6.4 in the MES buffer (see Section 2.3.2). The enzyme assay was prepared by adding 0.5 μM purified PTS and an *E,E*-FPP concentration between 1 and 200 μM to the reaction buffer (see Section 2.3.2). From preliminary tests with PTS, it is known that substrate inhibition is present.¹³ The substrate concentration was plotted against the molar concentration of the five main products per minute and a curve with the substrate inhibition model was generated. Based on a Michaelis–Menten kinetics, for the PTS purified a $K_M = 8.28 \pm 0.7 \mu\text{M}$ and $v_{max} = 5.81 \pm 0.5 \mu\text{M min}^{-1}$ were determined. This was used to calculate the enzyme activity with $k_{cat} = 0.0776 \text{ L s}^{-1}$ and $k_{cat}/K_M = 9,373.4 \text{ L mol}^{-1} \text{ s}^{-1}$.

3.2.1 | Effect of temperature and pH on product selectivity

It was observed from the experiments that the quantities of all main products change depending on the reaction conditions. Figure 4 shows the effect of the pH and temperature on the product selectivity during the enzymatic reaction. If the formation of the main products in the pH-range of the MES buffer and selected temperature range is

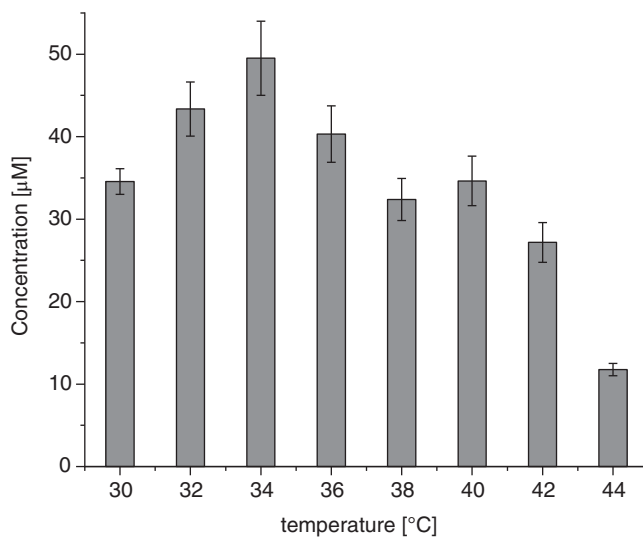


FIGURE 3 Results of testing the optimal pH and T on the enzyme activity and especially on (-)-patchoulol production. Reaction was performed in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer

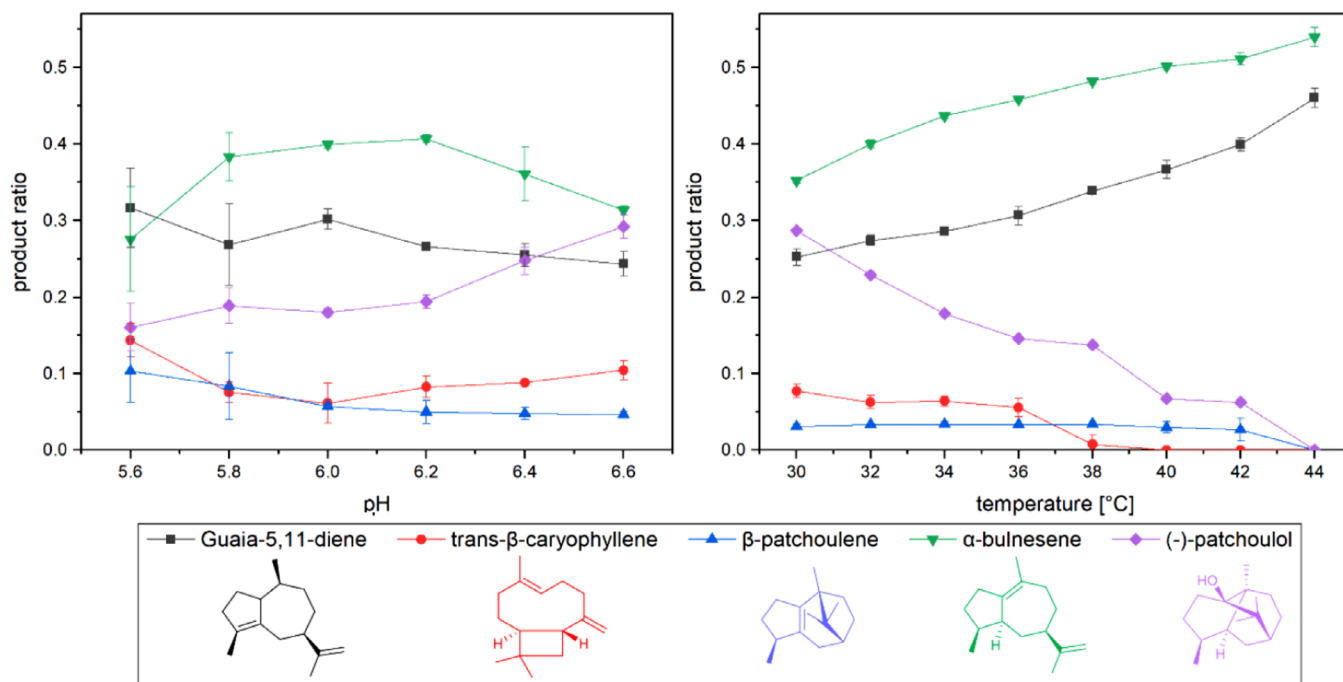


FIGURE 4 Results of pH and temperature dependence of product selectivity

considered in detail, it is clear that the pH-value as well as the temperature have an influence on product selectivity.

With increasing pH and decreasing temperature the formation of the three products β -patchoulene, trans- β -caryophyllene, and (-)-patchoulol increases. In contrast, the ratios of guaia-5,11-diene and α -bulnesene develop largely parallel to each other, with the pH-value rising slightly in the pH-range of the MES buffer. If the reaction cascade according to Faraldos et al. is considered, trans- β -caryophyllene and (-)-patchoulol have another reaction route as the two guaiene isomers, guaia-5,11-diene and α -bulnesene.¹⁴ With increasing temperature, the active pocket of the enzyme is modified in a way that the 1,11-cyclisation from which the trans- β -caryophyllene is formed as well as the proton transfer involved in the formation of (-)-patchoulol after the 1,10-cyclisation can no longer take place. The production of β -patchoulene is relatively temperature-independent with a proportion of around 3%, whereby no β -patchoulene is formed at very high temperatures such as 44°C. This effect is consistent with the results previously obtained by Frister et al. in the MOPS buffer. The trans- β -caryophyllene with an average proportion of about 6% is no longer formed at 40°C and above. However, the proportions of the guaiene isomers increase continuously with the temperature. In the range of 30–44°C, the proportions of guaia-5,11-diene and α -bulnesene grow by ~20%. (-)-patchoulol as the main fragrance component yielded 28.7% of the produced patchouli oil and decreased by about 22% with rising temperature up to 44°C. Compared with the experiments with MOPS buffer at pH 7.25, the PTS in MES buffer is more thermosensitive.¹³ This means that the product balance in MES buffer can be changed easier depending on the reaction temperature.

The pH dependence of the product selectivity is analogous to temperature dependence for all main products. The selectivity of β -patchoulene and trans- β -caryophyllene is more or less constant in comparison to the other products. While the proportion of (-)-patchoulol in the measured pH-range increases by about 13%, the ratio of the guaiene isomers decreases by about 8%. But this does not have such a strong effect on the smell of the patchouli oil, as the guaiene isomers contribute only little to the smell. Since (-)-patchoulol is the major fragrance component in patchouli oil, a higher proportion of (-)-patchoulol is desirable.²⁶

3.3 | Deuteration experiments

The reaction mechanism of (-)-patchoulol formation by the PTS has not yet been clarified in detail.³² More particularly, there is no proof in the literature on further reprotonation steps as postulated by Faraldos et al.¹⁴ Because the formation of PTS products is obviously influenced by the pH-value, it is possible that protons from the reaction solution are directly involved in the mechanism of terpene formation. Furthermore, PTS showed an unusual substrate inhibition for sesquiterpene synthases. This behavior might, according to literature, be explained by intermediary uncharged sesquiterpenes present in the mechanisms of product formation. Faraldos et al. used deuterated FPP for their experiments to establish the reaction mechanism.¹⁴ The use of deuterated water for the reaction buffer instead allows to study the influence of protons from the reaction medium in the reaction mechanism. Therefore, the enzymatic conversion was performed in a conversion buffer based on deuterated water and the reaction

products were examined for deuterated sesquiterpenes using GC–MS and various NMR techniques.

3.3.1 | Mass spectrometric analysis

The object of this analysis was to evaluate whether and how proton transfer by the reaction medium is initiated. The enzymatic conversion of *E,E*-FPP to patchouli oil was performed following the procedure described in Section 2.3.4, using a D₂O buffer. The resulting patchouli oil was analyzed for the deuteration of the sesquiterpenes using a GC–MS analysis.

The (–)-patchoulol produced in H₂O has a molar mass of 222 g mol^{−1} and thus a molecular ion with a mass-to-charge ratio (*m/z*) of 222 in the mass spectrum (MS). It is thereby expected that a (–)-patchoulol molecule produced in D₂O has as an ion a mass-to-charge ratio (*m/z*) of 223, as only the hydroxyl group should result from quenching in an aqueous medium. So a molecular ion with a higher *m/z* would indicate a proton exchange with the solvent during product formation.

The two mass spectra in Figure 5 show significant differences. In the mass spectrum of (–)-patchoulol formed in H₂O, the molecular ion has a *m/z* of 222, whereas the molecular ion of D-patchoulol has predominantly an increased *m/z* of 224, partly even 225. This pattern can

also be found in the other characteristic fragments of (–)-patchoulol. The same analysis was performed for the molecular ion of the other four main products of PTS. Since the other products do not contain a hydroxyl group, the molecular ion of the products formed in H₂O has a *m/z* of 204. For each of the products, the MS results can be used to determine how many hydrogen atoms in the molecule have been exchanged for deuterium atoms.¹⁴

In this process, it was observed that the degree of deuteration varies depending on the product. While *trans*- β -caryophyllene shows no deuteration at all, the isomers α -bulnesene and guaia-5,11-diene appear single and double deuterated. β -patchoulene is present in double and (–)-patchoulol in double and triple deuterated forms. It is particularly remarkable that *trans*- β -caryophyllene, which is the only main product of the intermediate humulyl cation (initial 1,11-cyclization), is not deuterated at all, while all other products (resulting from initial 1,10-cyclization) have at least minimum one deuteration. In addition, the *m/z* ratios of the two guaiene isomers, α -bulnesene and guaia-5,11-diene, have almost identical values. If these results are interpreted against the background of the known reaction mechanism, it is noticeable that the degree of deuteration depends on the reaction route of each product. Thus, the formation of the products of the 1,11-cyclisation is not influenced by protons from the reaction medium. To determine the exact position of the deuteration, NMR experiments are necessary. This allows conclusions

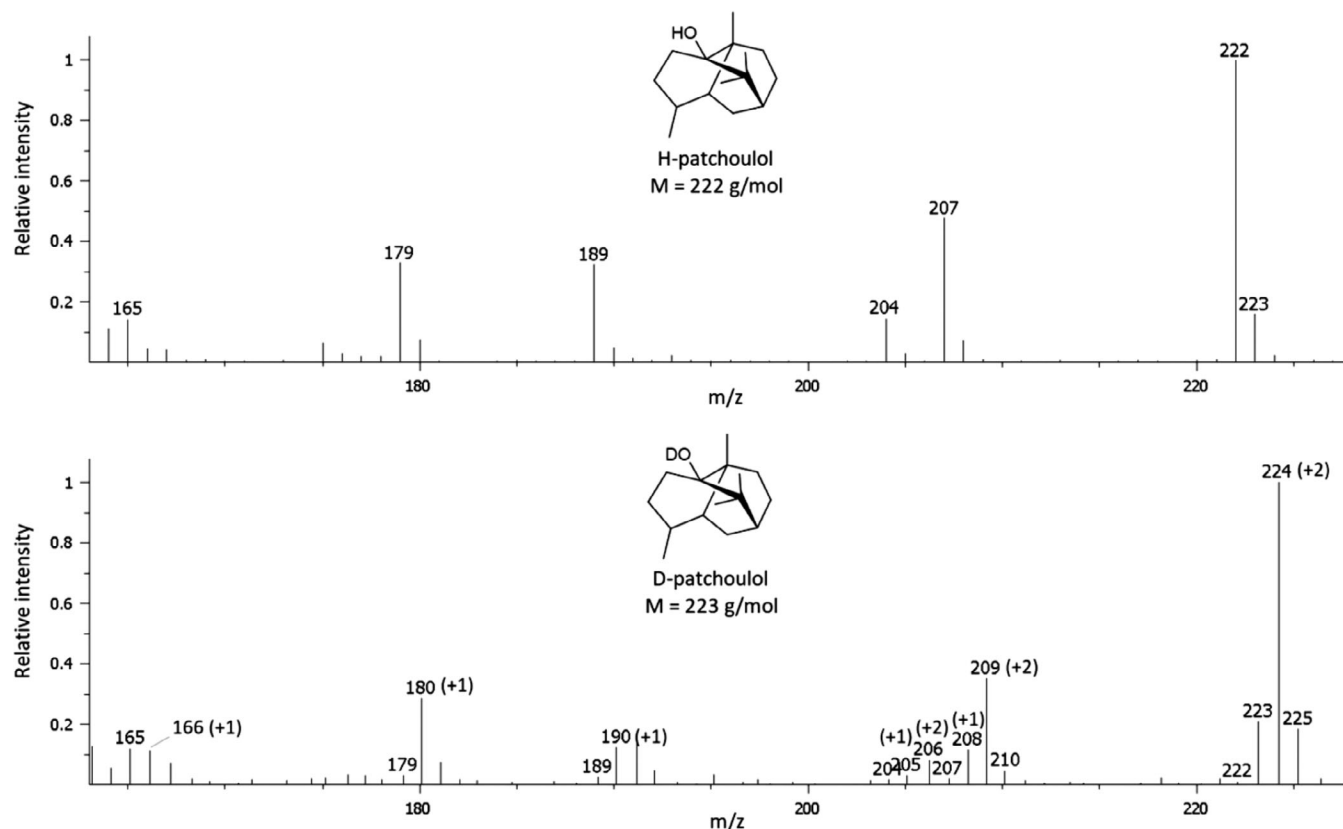


FIGURE 5 Cutout of the mass spectra in the molecular ion range of (–)-patchoulol from a conversion with patchoulol synthase (PTS) performed in H₂O compared with the D-patchoulol from a conversion with PTS in D₂O. The deviations of D-patchoulol from H-patchoulol are shown in brackets

about the position in the formation mechanism where the necessary protonation step for deuteration occurs.

3.3.2 | NMR studies

NMR analyses are necessary to determine the exact position of deuterium atoms in the molecule and to understand the reaction mechanism. From the NMR and MS results, it is possible to identify at which position in the formation mechanism the protonation step is located. For this purpose the conversion of the *E,E*-FPP with the PTS was performed with a D₂O activity buffer according to the experimental procedure described in Chapter 2.3.5.

TABLE 1 Degree of deuteration of the main products of patchoulol synthase (PTS) using D₂O buffer compared with H₂O buffer

Products	M ⁺	H ₂ O	D ₂ O	Deuteration
Trans- β -caryophyllene	204	71%	70%	-
	205	23%	27%	
	206	7%	3%	
β -patchoulene	204	84%	2%	Double
	205	14%	26%	
	206	1%	71%	
Guaia-5,11-diene	204	83%	12%	Single and double
	205	15%	76%	
	206	2%	12%	
α -bulnesene	204	85%	11%	Single and double
	205	13%	75%	
	206	1%	14%	
(-)-patchoulol	222	85%	1%	Double and triple
	223	13%	15%	
	224	2%	71%	
	225	0%	13%	

Note: Not all reaction products have the same number of deuterations. The degree of deuteration depends on the formation mechanism of the individual sesquiterpene. The bold numbers indicate the relevant degrees of deuteration.

The results of the NMR studies (see Table 1) showed two deuterated sites in the carbon skeleton of (-)-patchoulol (see Figure 6). The deuteration at C4 confirms the intermediate sesquiterpene postulated by Faraldos et al. and is not contrary to the published mechanism of (-)-patchoulol formation.^{12,14} However, the deuteration at one of the two methyl groups at C11 cannot be explained by any published mechanism.

Decisive for the presence of the uncompetitive substrate inhibition is the ability of the PTS to bind a second substrate molecule. From literature, no sesquiterpene synthase is known to have a second substrate binding site, for example, for regulating enzyme activity, in addition to the active center. The publication by Faraldos et al. investigated the reaction mechanism of (-)-patchoulol formation using ²H-labeled *E,E*-FPP.¹⁴ The partial double deuteration observed by Faraldos et al. is possible if the deuterium atom of one substrate molecule can be transferred to another. At this point two substrate molecules are bound to the PTS. The unknown sesquiterpene in the hydrophobic pocket is then the second substrate molecule that forms the substrate-substrate-enzyme complex in the model of uncompetitive substrate inhibition. This sesquiterpene can be returned to the reaction cascade after the transfer of a proton from another intermediate.

However, by using a medium with deuterated water, a further deuteration of one of the two methyl groups at C11 was observed. Previously, it could be shown that the pH-value has a significant influence on the product selectivity of the PTS. The reaction mechanism could depend on the proton flow from the medium. It is therefore possible that protons from the reaction solution are directly involved in the terpene formation mechanism. The triple deuteration proves that, independently of a second substrate, deuterated water molecules in the hydrophobic active pocket also support further proton exchange. A modified proposal for (-)-patchoulol biosynthesis was formulated on the basis of the obtained NMR data. The mechanistic approach is shown in Figure 7.

The difference between this mechanism and the ones published in Reference 14 has been the presence of two intermediate uncharged sesquiterpenes. It is supposed that a deprotonation results in the formation of a neutral compound in a hydrophobic pocket of the PTS. This neutral molecule could then be deuterated in a reprotonation step before returning to the reaction cascade. This is the closest possible explanation for the NMR-detected deuteration of the carbon skeleton.

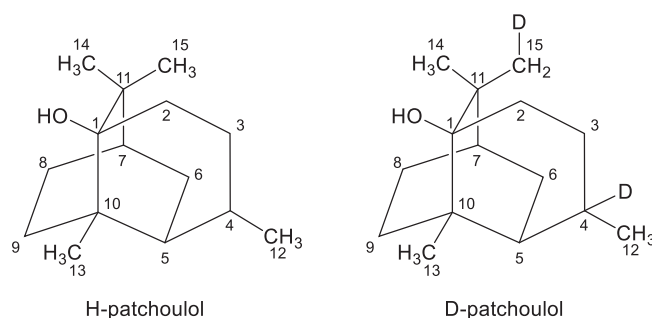


FIGURE 6 Deuteration of the (-)-patchoulol molecule resulting from the data of the ¹³C-nuclear magnetic resonance (NMR) spectra of H-patchoulol compared with D-patchoulol

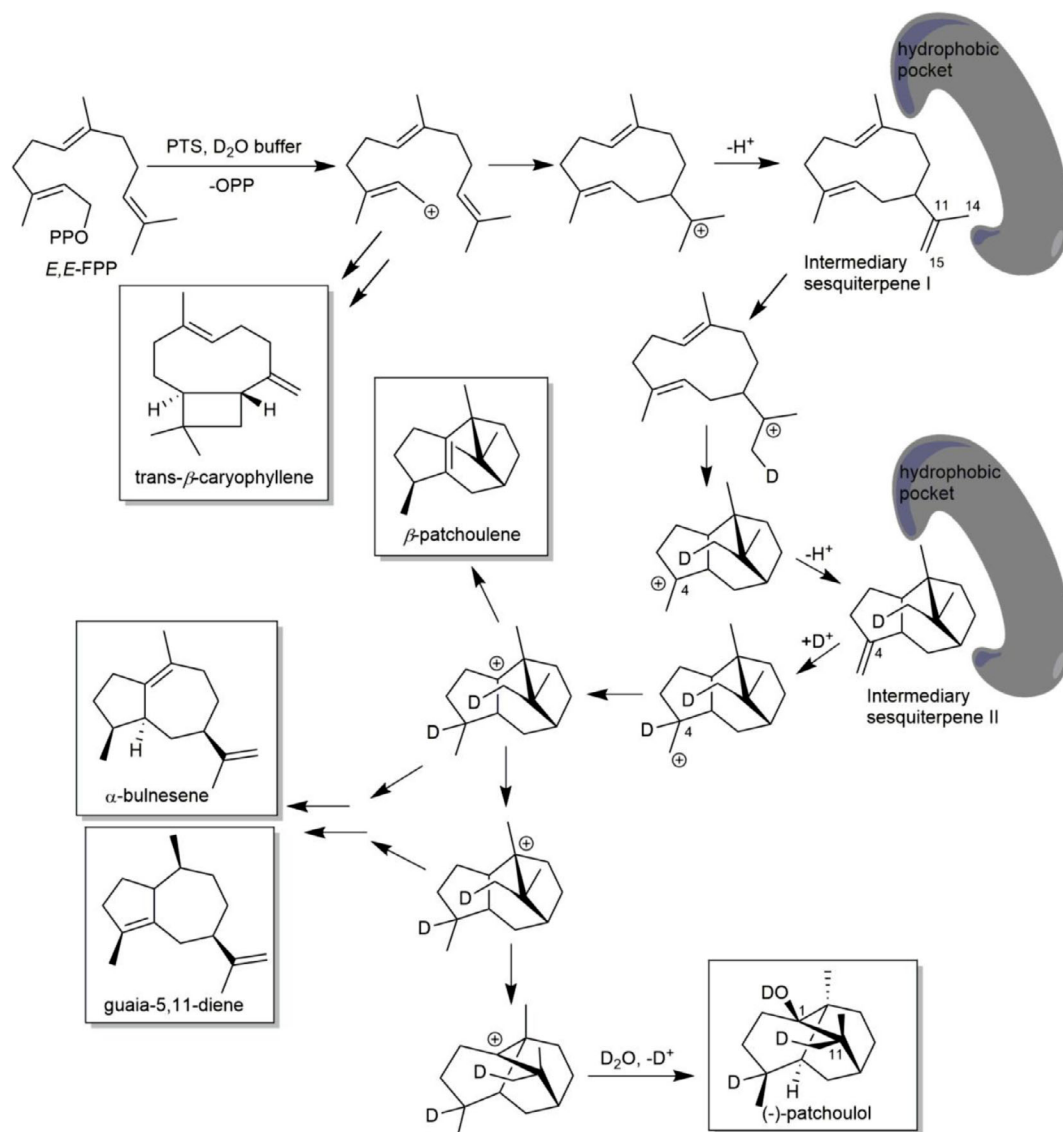


FIGURE 7 Based on the nuclear magnetic resonance (NMR) data postulated reaction mechanism of the (–)-patchoulol formation catalyzed by PTS

4 | CONCLUSION

Publications about PTS so far reported as optimal reaction parameters a pH-value between pH 7.0–7.5 and 38°C.^{13,17} The highest PTS activity with an optimum ratio of all five main products was observed by using a 50 mM MES buffer with 10% (v/v) glycerol and 5 mM MgCl₂ at pH 6.4 and 34°C. Compared with the previous data from Frister et al. with a maximum reaction rate of ~1.45 M min⁻¹, the use of this MES buffer increased the reaction rate by a factor of 3 to 4.95 μM min⁻¹.¹³ From the kinetic data a k_{cat} of 0.0776 L s⁻¹ was calculated, which is similar to the catalytic activity of other sesquiterpene synthases in literature.^{13,14,33,34}

Deuteration experiments on reaction mechanism of the PTS point out that the carbon skeletons of the reaction products were deuterated differently and the number of deuterations of the product molecules depends on the reaction route. The deuteration pattern of (–)-patchoulol

showed that the carbon skeleton is deuterated at two sites, which partly explains the multiple deuteration of (–)-patchoulol and the other main products of PTS based on the MS data. While the deuteration at the tertiary carbon atom C4 could still be explained by the mechanism postulated by Faraldos et al. assuming an intermediate uncharged sesquiterpene, there is no basis in the literature for the other deuteration at C14 or C15.¹⁴ Therefore, a new proposal for (–)-patchoulol biosynthesis was postulated on the basis of the knowledge obtained from the NMR data.

It is useful to evaluate the enzymatic conversion of other substrates, like monoterpene precursors or synthetic substrates as tested by Oberhauser et al. by the PTS, with different buffers to test whether the formation of different products can be detected.^{13,35} To complete these NMR based studies, enzymatic experiments like homology modeling of PTS and further deuteration tests on reaction mechanisms of the other main products could be essential.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

NOTATION

FPP	farnesyl pyrophosphate
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detector
GC–MS	gas chromatography-mass spectrometry
MES	2-(N-morpholino)ethanesulfonic acid
NMR	nuclear magnetic resonance
PTS	patchoulol synthase

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