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Multifactorial optimization enables the identification of a greener method to produce (+)-nootkatone

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ABSTRACT

The natural aroma compound (+)-nootkatone was obtained in selective conversions of up to 74 mol% from inexpensive (+)-valencene substrate by using a comparatively greener biocatalytic process developed based on modifications of the previously published Firmenich method. Buffer identity and concentration, pH, temperature and downstream work-up procedures were optimized to produce a crude product in which >90 % of (+)-valencene had been converted, with high chemoselectivity observed for (+)-nootkatone production. Interestingly, the biotransformation was carried out efficiently at temperatures as low as 21 °C. Surprisingly, the best results were obtained when an acidic pH in the range of 3–6 was applied, as compared to the previously published procedure in which it appeared to be necessary to buffer the pH optimally and fixed throughout at 8.5. Furthermore, there was no need to maintain a pure oxygen atmosphere to achieve good (+)-nootkatone yields. Instead, air bubbled continuously at a low rate through the reaction mixture *via* a submerged glass capillary was sufficient to enable the desired lipoxygenase-catalyzed oxidation reactions to occur efficiently. No valencene epoxide side-products were detected in the organic product extract by a standard GCMS protocol. Only traces of the anticipated corresponding α- and β-nootkatol intermediates were routinely observed.

1. Introduction

The natural product (+)-nootkatone, present in grapefruit (Furusawa et al., 2005) and pomelo (Yin et al., 2023) amongst several other plant species, is a valuable sesquiterpenoid in the flavor, fragrance, pharmaceutical and agricultural industries. Its excellent organoleptic and medicinal properties render nootkatone a highly desirable natural product with numerous applications. Unfortunately, sustainable extraction of nootkatone from plants is not commercially feasible due to the high process costs and the low yields obtained in this way (Meng et al., 2020, Li et al., 2022). Chemical synthesis of nootkatone makes use of hazardous oxidizing agents, e.g. *tert*-butyl peracetate (Wilson and Shaw, 1978) and *tert*-butyl hydroperoxide (Davies, 2009), and such procedures have been carried out on supported silica metal catalysts (Salvador and Clark, 2002). Firstly, the corresponding α- and β-acetate derivatives are produced as major products, followed by α- and β-nootkatol as intermediates, which are both subsequently reacted with highly hazardous and toxic type 1 carcinogen chromic acid (DesMarias and Costa, 2019) to afford nootkatone. The disadvantages of such chemical syntheses are the use of hazardous reagents and in general, low overall yields. Furthermore, the resultant nootkatone is not considered marketable as "natural" (Palmerín-Carreño et al., 2016). Hence, we desired to produce nootkatone industrially in a "greener", cost-effective manner.

Biological processes for nootkatone synthesis have been explored and developed (Wriessnegger et al., 2014, Li et al., 2021), including the commercial BASF Isobionics fermentation-based process for nootkatone synthesis. Biocatalytic production of nootkatone is highly favored because it focuses on the use of renewable resources, non-toxic reagents, mild reaction conditions and more cost-effective methods with less waste being created. Nootkatone that is produced by biocatalytic processes is considered "natural" since the starting materials and the biocatalyst are from natural sources (Heshof et al., 2016). However, using biocatalysis to produce nootkatone presents challenges of low yield, expensive enzymes, low chemoselectivity, low enzyme recovery, as well

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as lengthy and costly enzyme purification procedures. Heshof *et al.* have reviewed these challenges and the various strategies that have been attempted to overcome them.

A promising biosynthetic approach to nootkatone entails the application of soy bean lipoxygenase isozymes (LOX) and relatively inexpensive (+)-valencene that is isolated from citrus, to produce nootkatone in an environmentally benign and green process (Fig. 1), as described in the now expired 1998 Firmenich patent by Muller et al. (1998). The catalytic mechanism of this reaction involves the co-oxidation of (1*Z*, 4*Z*)-pentadiene unsaturated fatty acids [e.g. linoleic (18:2) and linolenic (18:3) acids] into hydroperoxides that then effectively co-oxidize other olefin substrates (e.g. valencene) to produce ketone product (e.g. nootkatone) concomitantly. Mono-unsaturated fatty acid co-substrates, e.g. oleic acid, may also be used for this purpose. Side-products such as α- and β-nootkatol can be further converted into nootkatone (Feussner and Wasternack, 2002; Sigel et al., 2006; Nikolaiczyk et al., 2022). A definite preference was identified for this allylic oxidation reaction to occur on substrates containing endocyclic double bonds, e.g. valencene (Nikolaiczyk et al., 2022).

Although the Firmenich process (Muller et al., 1998) is itself considered "green", there are aspects of it that present drawbacks in implementation and, if improved, could result in a simpler, greener process. The process delivered a molar yield of *ca.* 37 mol% of nootkatone from valencene, under optimized reaction conditions of relatively high temperature (60 °C) and pH *ca.* 8, over a 24 h period of stirring in a pure oxygen atmosphere. Significant amounts of nootkatol and valencene epoxide were observed as side-products. One consideration is the possibility of adverse effects caused by high temperatures on enzyme half-life due to thermal denaturation that, in turn, can adversely affect nootkatone yield.

Another possible drawback is the use of phosphate buffer for LOX extraction and for buffering the biocatalytic reaction itself. Phosphate buffers are generally avoided owing to adverse effects, such as the general inhibition of enzyme activity (Good et al., 1966; Lee and Wang, 1968). Furthermore, the process as described in the patent was not completely characterized with respect to the pH of the reaction medium. The detailed process examples were performed at neutral pH and above, with an optimum pH found at *ca.* 8–8.5. However, from tabulated data in the patent it appeared that a higher nootkatone: nootkatol product ratio could be obtained when the reactions were conducted at lower pH (6.5–7.5), albeit that the lower pH reduced product yields.

To our knowledge, three major LOX isozymes have been isolated from soy bean to date, with pH optima ranging from 6.5 to 9.0 (Chikere et al., 1998; Lorenzi et al., 2006). Furthermore, several reports of acidic pH optima for LOX isozymes are found in literature. For example, for isolated green pea seeds LOX, the optimum pH was reported in the acidic range of 5.0–7.0 (Szymanowska et al., 2009). Incredibly, the lowest optimum pH activity for an isolated LOX enzyme ever reported was pH 3.6, for CsLOX1 from tea leaves (Liu. and Han, 2010). We therefore sought also to investigate the performance of our reaction system at pH values lower than the reported optimum pH ranges for soy bean LOX and, unlike the patented process, without the use of a pH-stat device.

Finally, Muller *et al.* also reported using Cu powder and various Cu salts in an effort to promote the LOX oxidation yields, with varying success. Instead, we wanted to test whether the addition of Fe^{2+} and Mn^{2+} salts could promote the conversion of valencene into nootkatone, seeing that such salts have more recently been shown to act as activators of LOX enzymes (Marvian-Hosseini and Asoodeh, 2017; de Melo et al., 2021). However, in excess they may also inhibit the activity of LOX (Marvian-Hosseini and Asoodeh, 2017).

Our efforts to investigate the above-mentioned issues and to incorporate newer ideas and knowledge about the behavior of crude soy bean LOX isozymes extracts culminated in a simpler, greener and more efficient (increased yield and chemoselectivity) variation of the process, which is described here.

2. Materials and methods

2.1. General

2.1.1 All chemicals and analytical standards used were purchased from Sigma-Aldrich or Merck (South Africa), unless otherwise stated. Nootkatone analytical standard (>98 % purity) was supplied by Evolva (USA). Solvents used were HPLC grade methanol, ethyl acetate, hexane, and diethyl ether. Hexane and ethyl acetate were distilled before use, and others were used without any further purification. For the reactions, valencene, of minimum purity >65 % v/v, was obtained from Evolva (USA). Fresh whole soy beans were obtained from Seeds for Africa (South Africa). Column chromatography was performed using Macherey-Nagel silica gel (particle size, 0.063–0.200 mm). Aluminum-backed Merck silica gel (60 F254) plates were visualized under UV light or stained using potassium permanganate. Reaction pH was monitored using universal pH strips.

2.1.2 Gas chromatography (GC) analysis was performed on an Agilent 7890 GC with an auto injector, coupled with a Leco Pegasus time-of-flight (TOF) mass spectrometer. The primary column (29.687 m × 0.25 mm × 0.25 μm Rxi-5Sil; Restek) and secondary column (1 m × 0.25 mm × 0.25 μm Rxi-17Sil; Restek) made of 5 % diphenyl/dimethyl polysiloxane was used to separate the compounds in the queued sample. The spectra acquisition rate, flow rate, and temperature program were adjusted to help optimize the analyte separation and detection methods. Due to the modification made above, the method below was follows:

A 1 μL splitless injection with carrier gas helium at 1.4 mL/min continuous flow rate whereby the GC primary oven was programmed at 50 °C (0.2 min), then increased by 20 °C/min to 280 °C (1 min) which was the target temperature, and the secondary oven program was +5 °C offset with respect to the primary oven. The transfer line and front inlet temperatures were 280 °C and 200 °C respectively. The run time was 14 minutes. The temperature of the ion source was 200 °C while the modulator was off. The mass spectrometer was operated in electron impact mode using an ionization voltage of 70 eV, and the ion source temperature was 200 °C. The mass spectrometer acquisition rate was 10 spectra per second at a set mass range of 25–450 *m/z*. The Leco ChromaTOF software and NIST library were used for data processing and peak identification

2.1.3 Proton (1 H) and carbon (13 C) Nuclear Magnetic Resonance (NMR) data was recorded on 300, 400 and 500 MHz Bruker spectrometers.

2.1.4 The ultraviolet-visible (UV–VIS) spectrophotometer used was Cary 100 UV–VIS from Agilent Technologies and the processing software was Cary WINUV. The optical system was a double beam, and the light source was tungsten halogen.

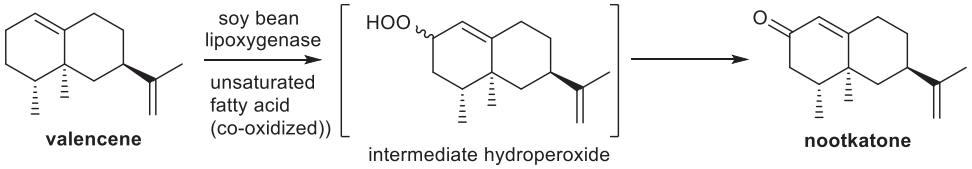


Fig. 1. LOX-catalyzed biotransformation of valencene into nootkatone.

2.2. LOX enzyme extract and sample preparation procedures

2.2.1. Enzyme extraction

The soybeans were prepared by crushing into a coarse meal using a mortar and pestle, or into flour by using a domestic coffee grinder. This coarse meal or flour (20 g) was subsequently suspended in a buffer (100 mL) and left in a refrigerator (4 °C) overnight, with no form of agitation applied. For smaller scale extractions, 2 g pre-made soya flour portions were soaked in buffers (10 mL volumes) at varying pH values and molarity. The soaked suspensions were placed on a shaker for 30 min at room temperature and allowed to cool for 45 min at 4 °C.

All soaked mixtures were filtered through cheesecloth and the supernatant was stored at −20 °C, prior to use, as crude LOX extracts.

2.2.2. Sample preparation

After extraction solvent (EtOAc) removal *in vacuo* by roto-evaporation, each oily sample obtained was dissolved in HPLC grade MeOH to make concentrations of 100–500 ppm, then diluted as needed. After GCMS analysis as described in 2.1.2, standard curves of valencene and nootkatone were plotted in the range of 10–100 ppm analyte concentration for both (see supporting information). The LOD (Limit Of Detection) for valencene by this method was 5.80 ppm, LOQ (Limit Of Quantification) was 17.40 ppm, whilst for nootkatone LOD was 11.66 ppm and LOQ was 33.78 ppm. Each oily product was dissolved in $CDCl_3$ prior to analysis by NMR. See Figures S4 and S5 (Supporting Information).

2.3. LOX enzyme activity (or methylene blue bleaching) assay

We used the methylene blue bleaching reaction as a rapid LOX activity test (Suda et al., 1995), as developed by Toyosaki (1992). The mechanism involves the hydroperoxide, formed during the lipid peroxidation reaction, which donates a hydrogen to the methylene blue and thereby reducing it to the bleached form (Toyosaki, 1996). In the Fenton type reaction, which involves an iron catalyst and peroxide (Kruid et al., 2017), the constituents are similar to those inthis LOX reaction (an iron metalloenzyme that generates peroxides).

A stock solution of 100 μM reagent was prepared by dissolving 3.2 mg of methylene blue in deionized water at room temperature. In an Eppendorf tube, the reagent was diluted to 5 μM and 10 μL of the substrate solution was added. The sample was centrifuged for 5 min at 10,000 x g and allowed to bleach for 30 min. Subsequently, the sample was analyzed for absorbance at 664 nm. The calibration curve was prepared by dilution of the stock solution to concentrations of 0.5–5 μM. See Figure S6 (Supporting Information).

2.4. Biotransformations

2.4.1. General procedure example

On a hotplate stirrer with an oil bath was placed a clamped 3-neck round bottom flask containing a magnetic stirrer bar and a tightly fitted thermometer was placed through one of the flask's necks. An air pump was connected onto the flask's second neck, *via* PVC tubing housing a glass capillary that was submerged into a mixture of valencene (11.5 g, 56.28 mmol) and oleic acid (5.6 g, 19.83 mmol). A glass reflux condenser was inserted into the flask's third neck, and cooled by flowing tap water through it. The mixture was rapidly stirred and heated to a constant temperature of 35°C. Crude LOX extract (pH 8.1, 100 mM sodium borate buffer, 56 mL), together with $FeSO_4.7\ H_2O$ (0.125 g, 0.44 mmol) and $MnCl_2.4\ H_2O$ (0.125 g, 0.64 mmol), were added to the reaction flask. Stirring and heating was continued and sampling took place every 24 h. After 24 hours the pH was approximately 3–4. The work-up procedure as described below in Section 2.5.1 was followed. The crude product was isolated as a brown oil with a yield of 68 % mass of crude product/mass of starting material valencene (m/m).

2.4.2. Buffer variation

Borate buffer was replaced by using either Tris (100 mM) or phosphate (100 mM) buffer only. The work-up procedure as described in Section 2.5.1 was followed. The Tris buffer isolated crude product was a yellow oil with m/m yield 28 %, and the phosphate buffer crude product was a colorless clear oil with m/m yield of 17 %.

2.4.3. Substrate concentration variation

The valencene quantity added was approximately halved to 29.4 mmol. After 24 hours, the pH was again approximately 3–4. The work-up procedure as described in Section 2.5.1 was followed and the isolated crude product was a clear red color, with a m/m yield of 86 %.

2.4.4. Varying the addition of Fe^{2+} and Mn^{2+} salts

The combined salts added was doubled to $FeSO_4.7\ H_2O$ (0.25 g, 0.88 mmol) and $MnCl_2.4\ H_2O$ (0.25 g, 1.28 mmol). The work-up procedure as described in Section 2.5.1 was followed. The isolated clear product oil had a lower mass yield (40 % m/m) as compared to the case when half the amount of Fe^{2+} and Mn^{2+} salts was used (68 % m/m).

2.5. Reaction work-up procedures

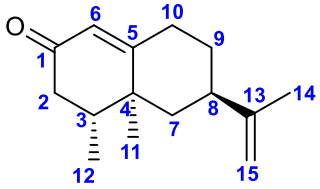
2.5.1. General

In a separatory funnel, the reaction suspension was added, together with 100 mL of EtOAc as extraction solvent, to a mixture of 100 mL 1 M NaOH and 100 mL saturated NaCl (brine). The top organic layer was washed again 3 times with the same NaOH and NaCl aqueous mixture, in order to remove any unwanted products and reagents from the organic layer. Anhydrous $MgSO_4$ was added to the organic layer before filtering and evaporating the supernatant *in vacuo* by roto-evaporation. Once concentrated, the solvent free oily sample was kept at 4°C until analysis was performed by GCMS.

2.5.2. Variation of bases and salts used in the work-up procedure

Three variations of the work-up procedure described in 2.5.1 were tested, i.e. the aqueous NaOH concentration was increased to 2 M, the NaOH solution was replaced with saturated aqueous K_2CO_3 (prepared with 100 g K_2CO_3 in 200 mL water), and the NaOH solution was replaced by sequential addition to the reaction suspension of 20 mL saturated K_2CO_3, 80 mL brine, and finally 120 mL saturated $NaHCO_3$ (prepared with 100 g $NaHCO_3$ in 200 mL water).

2.6. General optimized procedure for the production of nootkatone, including characterization data



In a 3-neck reaction flask, valencene (11.5 g, 56.28 mmol) and oleic acid (2.80 g, 11.06 mmol) were added. The air pump and reflux condenser were also connected as described in 2.4.1. The crude LOX (pH 8.1 or 7.5, sodium borate buffer, 56 mL), together with $FeSO_4.7\ H_2O$ (0.125 g, 0.44 mmol) and $MnCl_2.4\ H_2O$ (0.125 g, 0.64 mmol), were added to the reaction flask. The temperature of the reactions and buffer molarities, i.e. the aqueous buffer solution used both for LOX extraction and as reaction medium, were varied as described in section 3.4.1 Samples (24–96 h) were taken, and worked-up according to the procedure described in 2.5.2 C. In one preparative scale experiment, the crude product was purified by SiO_2 gel chromatography by using 5 % EtOAc/hexane as mobile phase. Eluted fractions were monitored by TLC; (+)-nootkatone Rf (20 % EtOAc/Hexane) = 0.26 and (+)-valencene Rf (20 % EtOAc/Hexane) = 0.94. The identity of (+)-nootkatone was satisfactorily confirmed in this way by GCMS and NMR: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.77

(1 H, s, C6H), 4.75 (2 H, d, C15 H2), 2.51 (1 H, ddd, C8H), 2.25–2.34 (4 H, m, C2 H2, C10 H2), 1.92–2.02 (4 H, m, C7 H2, C9 H2), 1.74 (3 H, s, C14 H3), 1.21–1.28 (1 H, m, C3H); 1.12 (3 H, s, C11 H3), 0.97 (3 H, d, C12 H3). 13 C NMR (100 MHz, CDCl3): δ 199.70 (C1), 170.75 (C5), 149.10 (C13), 124.69 (C6), 109.25 (C15) 43.92 (C7), 42.08 (C2), 40.46 (C8), 40.32 (C3), 39.33 (C4), 33.04 (C10), 31.62 (C9), 20.84 (C14), 16.86 (C12), 14.92 (C11). The data corresponded well with literature (de Melo et al., 2021). See also Figures S7 to S10 (Supporting Information).

3. Results and discussion

3.1. Soy bean processing

Preliminary experiments (data not included here) indicated that soybean fine flour (prepared using the coffee grinder) provided a higher active LOX content than that of soybean course meal (prepared by the mortar and pestle), as supported by both higher methylene blue (MB) bleaching results obtained in assays and increased valencene to nootkatone conversions measured by GCMS. Hence, the fine flour method was adopted for subsequent enzyme preparations.

3.2. Buffer variation

According to literature, three major soybean LOX isozymes are together active in a pH range of 6.5–9.0 (Chikere et al., 1998; Lorenzi et al., 2006). Therefore, for the activity assay, the crude LOX was extracted with different buffers within this pH range in order to determine which buffer afforded the best performing crude LOX extract. The three buffers tested were sodium borate (at pH 6.5, 7.6, 8.1), Tris (at pH 7.0, 7.5, 8.1) and phosphate (at pH 6.7, 7.7, 8.3). Buffer molarities tested were 10, 50, 100, 150 and 200 mM, respectively. The results are summarized in Fig. 2. The highest concentration in each sample was 5 μM, that was expected to bleach to as low as 0.5 μM. Tris buffer showed the highest concentration of bleached MB), i.e. 40 % of the initial MB concentration, at 2 μM between pH 7.0 (10, 50, 100 mM) and 7.5 (150 mM). The maximum amount observed for Tris buffer was at 1.6 μM (pH 8.1) and the lowest concentrations were as low as 0.6 and 0.4 μM (at pH 7.5), respectively, at concentrations 10 and 50 mM. Phosphate buffer showed the lowest LOX activity amongst the three buffers. The highest concentration of bleached MB was just under 1.5 μM at pH 6.7 (10 and 50 mM). At pH 7.7 (50 and 100 mM) there was no activity observed at all.

Amongst the three buffers, borate buffer displayed the best activity with the highest concentration of bleached MB (3.3 μM), as observed at a concentration of 150 mM and pH 8.1. This was followed by 2.9 μM at 10 mM at the same pH. However, at pH 6.5 the concentration decreased to less than 1.5 μM. This methylene blue LOX assay we developed here was a success as it is simple to perform and avoids the use of relatively expensive linoleic or linolenic acid (Suda et al., 1995). Oleic acid (a monounsaturated fatty acid) would display a low rate of being oxidized because the rate increases with increase in the degree of unsaturation of the fatty acid co-substrate (Franootkatoneel, 1980). However, it performed efficiently as co-substrate in our assay.

In conclusion, the best buffer to extract crude soybean LOX with was borate at pH 8.1 (10 and 150 mM), followed by Tris at pH 7.0 (50 and 100 mM) and lastly the buffer employed by Muller et al. (1998), i.e. phosphate, at pH 6.5 (10 and 50 mM). To extract LOX at a pH lower 7.5 and still retain the activity of the enzyme, Tris was the best option. However, at pH of 8.1, borate showed relatively very high activity. Borate buffer at 200 mM showed the highest activity yielding 2 and 2.3 μM bleached MB at pH 7.6 and 8.1, respectively. Optimal conditions observed for each of the buffers tested were: sodium borate, pH 8.1, 150 mM; phosphate, pH 6.7, 50 mM; Tris, pH 7.0, 50 and 100 mM.

3.3. Substrate and Fe^{2+} and Mn^{2+} salts concentrations

In our hands, reducing the substrate concentration below that described in the general and optimized procedures (see Section 2.4.1 and 2.6) failed to improve the conversion (mol%) of valencene into nootkatone. For example, when the substrate concentration was halved and the reaction was conducted at 37 °C for 48 h, approximately 70 % of valencene was converted into nootkatone, similar to the values found at higher substrate concentrations. Furthermore, no improvements were observed with increased Fe^{2+} and Mn^{2+} salts addition. In fact, when the concentrations of $FeSO_4.7\,H_2O$ and $MnCl_2.4\,H_2O$ salts were both approximately doubled (and the reaction again was conducted at 37 °C, pH measuring *ca.* 3–4 throughout), <1 % of valence to nootkatone conversion had occurred by 48 h. This was extremely low, indicating either enzyme inhibition or increased non-volatile/water-soluble side-product formation.

3.4. Work-up procedure

A surprising challenge encountered during this project was the development of a facile extraction method to separate nootkatone and residual valencene, in order to analyze and isolate the products at scale. Various extraction methods were tested, including those by Firmenich (Muller, 1998), but none of these worked satisfactorily in our hands. We found that, as non-defatted soya flour was used in all experiments during this project, emulsification during the attempted liquid-liquid product extraction was a major issue, which prevented efficient recovery of the

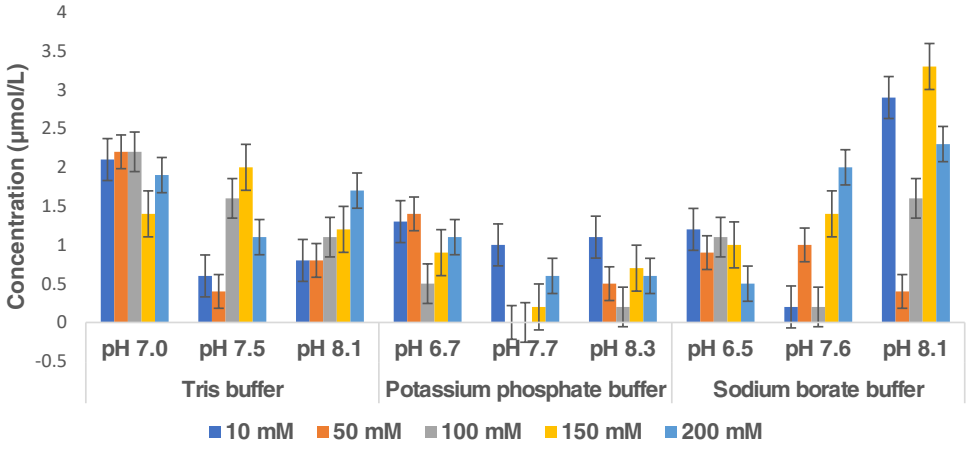


Fig. 2. Methylene blue (MB) bleached by crude LOX extracted into Tris (pH 7.0, 7.5, 8.1), phosphate (pH 6.7, 7.7, 8.3) and borate (pH 6.5, 7.6, 8.1) buffers, at molarities of 10, 50, 100, 150, 200 mM. Reactions were run in triplicate.

valencene-nootkatone crude product mixtures. After several optimization attempts, we discovered that the combination of saturated K_2CO_3 and $NaHCO_3$ solutions (to remove oleic acid into the aqueous layer) together with added saturated NaCl was most efficient in extracting the product mixture from the aqueous phase into EtOAc. The advantages were no emulsified cream, turbidity or multiple layers formed. This DSP improvement enabled us to proceed with multifactorial optimization in the preferred sodium borate buffer medium, at initial buffer pH of 8.1 and 7.5, respectively.

3.5. Biotransformation

3.5.1. Crude LOX in sodium borate buffer (pH 8.1)

The reactions were conducted at various temperatures (21, 30 and 35°C), and at two buffer strengths (100 and 150 mM), whilst recording reaction pH at 24 hour intervals. The parameters varied were buffer molarity and temperature (Fig. 3). However, observed temperature effects were minimal.

It is important to note that we added oleic acid in neutral form. It is obvious that the pH of the medium will naturally drop to much lower levels than the initial buffer pH, once a relatively large amount of the co-substrate fatty acid is added in its neutral, protonated form. Buffer capacity is overridden by the excess fatty acid added and the pH drops, hence our reaction pH was found to be in the range of 4–6. The best conversion into nootkatone was indeed observed in this pH range (i.e., when the sodium borate buffer had a starting pH of 8.1), and at 150 mM buffer concentration. The reaction duration was optimal for 24 h, with a rapid loss of product occurring after 72 h.

In all of the samples tested, converted valencene was found to be greater than 90 mol%. This was remarkable because it showed high enzyme activity, even though other side products, presumably due to overoxidation and/or polymerization (Dong et al., 2018) were also produced from valencene. These were either non-volatile and hence were not observed in the GC chromatograms or identified by the NIST library, or they were water soluble and not extracted into the GC-analyzed organic layer during processing, indicating that they are likely to be polymers of oxidized products, and/or water-soluble oxidized side-products. Good conversions of >70 mol% nootkatone from valencene were observed and these may be further optimized. The MB activity colorimetric assay, as described in Section 2.3, was validated at this stage because the conversion results (GCMS) correlated well with the enzyme assay results (MB assay) obtained after LOX extraction. Sodium borate buffer (150 mM) at pH 8.1 showed the highest concentration of bleached MB, which was also the case for % conversion into nootkatone when the reaction was carried out using the corresponding LOX extract as catalyst. In summary, the highest LOX activity was observed at pH 8.1 in 150 mM sodium borate buffer, for both the LOX assay and the nootkatone product concentration from the biocatalytic reaction. Extended reaction times beyond 72 h were highly counter-productive. We postulate that extended reaction periods cause

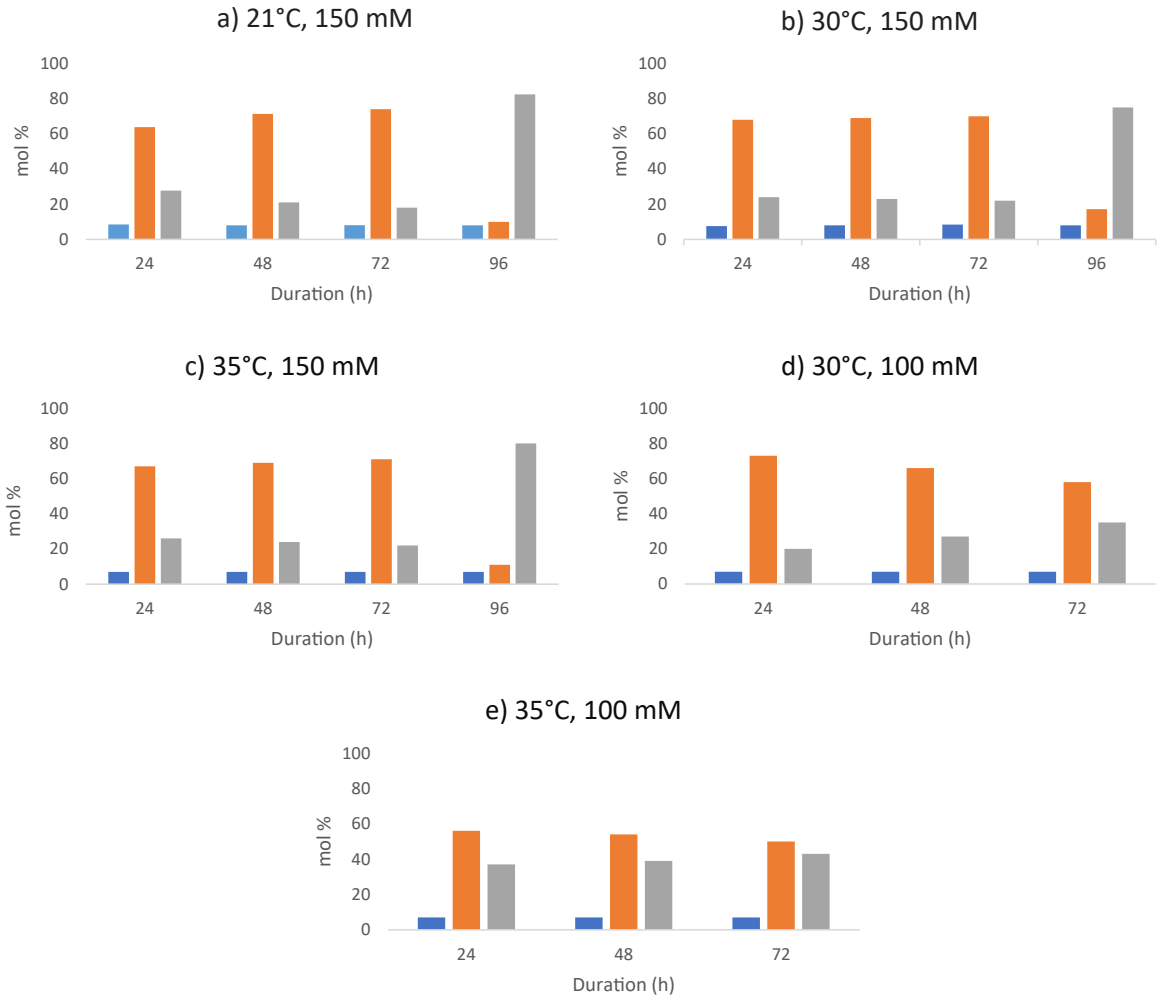


Fig. 3. Concentration (mol%) vs reaction duration (h) for unreacted valencene (blue), nootkatone (orange; i.e. the mol% of valencene converted into nootkatone), and other products (gray; i.e. the mol% of valencene converted into other products, including α- and β-nootkatol), in sodium borate buffer, at pH 8.1. See also Table S2 (Supporting Information).

either chemically or enzymatically catalyzed nootkatone over-oxidation and/or polymerization (Dong et al., 2018) to form side-products. The lower reaction temperature of 21 °C provided slightly better conversion (as determined by mol% conversion) due to less over-oxidation (and/or polymerization) occurring, as compared to 30 °C and 35 °C. The effects of buffer strength were proven to be much less significant.

3.5.2. *Crude LOX in sodium borate buffer (pH 7.5) and at low buffer strengths*

Similar to the reactions described in Section 3.4.1, as seen in Fig. 4, reaction pH and buffer molarity play a role in the efficient conversion of valencene into nootkatone. Final pH for the 100 mM sodium borate reaction mixture (at initial pH of 7.5) was again found to have dropped to 4–6. Subsequently, a further investigation was launched in order to find out whether the LOX extract was capable of converting valencene into nootkatone efficiently at a reaction pH lower than 4. The reaction was conducted at a temperature of 30 °C with low molarity, i.e. initial pH 7.5 (10 and 15 mM, respectively). At 10 mM, the reaction pH plummeted to 2. These were extremely acidic conditions yet, surprisingly, LOX isozymes were active and able to convert valencene into nootkatone. After 24 h, nootkatone concentration was measured to be 49 mol% and it increased to 51 mol% after 72 h. At 15 mM, the reaction pH remained constant at pH 3 for 96 h and, within 24 h the concentration of nootkatone was recorded to be 61 mol%. Again, converted valencene was observed to be *ca.* 90 mol% at both 10 mM and 15 mM buffer strength, and the resultant nootkatone was observed to degrade and/or polymerize rapidly after the 72 h mark, i.e. much reduced nootkatone concentrations as compared to that measured after 24 h. A reaction pH of 2 yielded an average of 50 mol% nootkatone and, at pH 3 it was 60 mol%. The good conversion to nootkatone at very low pH was an exciting result as, to our knowledge to date, LOX from soy bean has previously been reported to be active only within the pH range of 6.5–9.0 (Muller et al., 1998; Chikere et al.,1998; Lorenzi et al., 2006).

3.6. *GCMS analysis*

The identities of valencene and nootkatone were qualitatively verified by MS detection and comparison to the NIST library database, as well as by comparison with commercially available analytical standards of valencene and nootkatone. A typical GCMS chromatogram, as encountered throughout this study, is shown in Fig. 5. It can be noted that the only side-products we observed by this GCMS method and by employing the NIST database of MS spectral data were α- and β-nootkatol, yet no valencene epoxide, as reported in the Firmenich patent (Muller et al., 1998) in which it was a significant side-product, was observed. There is no clear explanation for this difference in results. It is

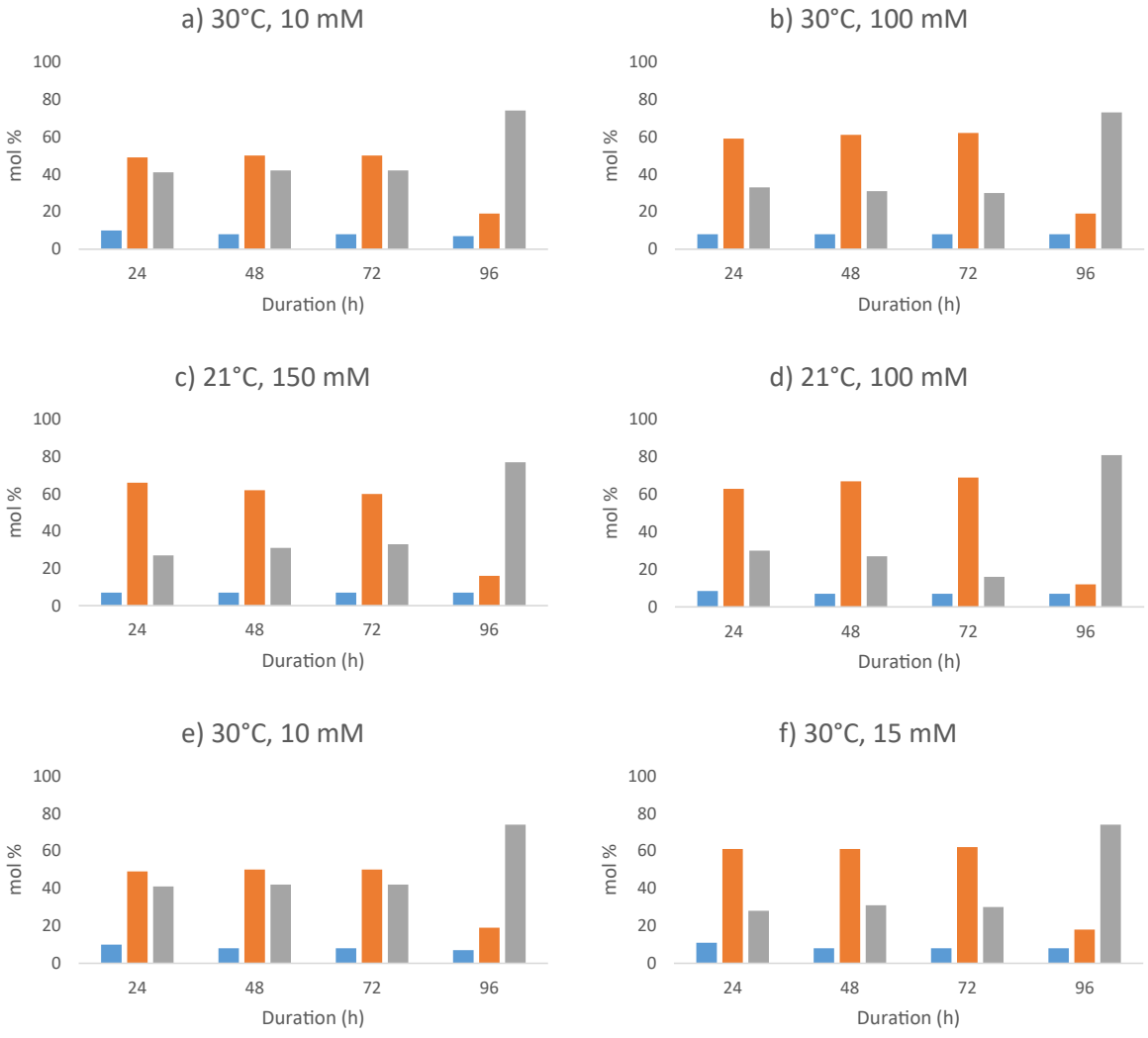


Fig. 4. Concentration (mol%) vs reaction duration (h) for unreacted valencene (blue), nootkatone (orange; i.e. the mol% of valencene converted into nootkatone), and other products (gray; i.e. the mol% of valencene converted into other products, including α- and β-nootkatol), in sodium borate buffer, at pH 7.5. See also Table S1 (Supporting Information).

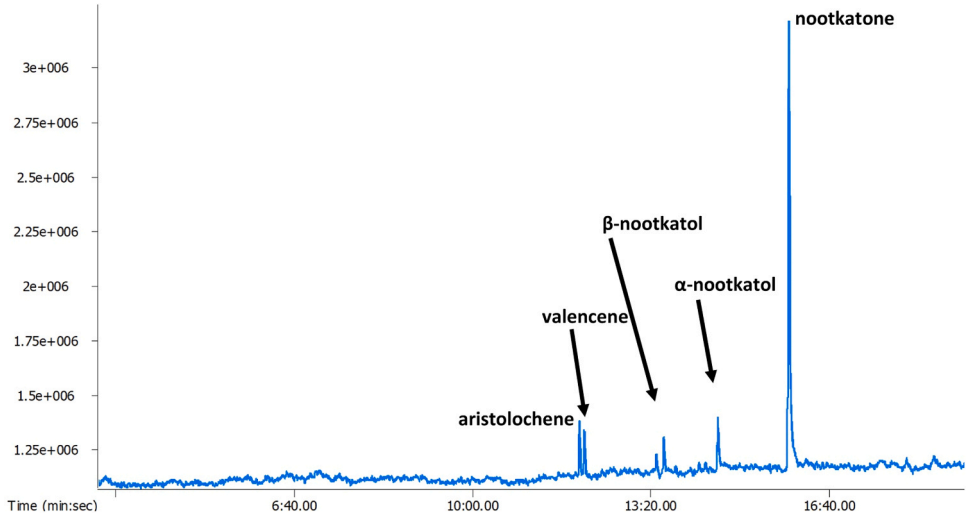


Fig. 5. Example of a typical GCMS chromatogram obtained in this study. No valencene epoxide (by comparison with NIST library reference spectra) nor fatty acid hydroperoxides were observed. See also Figs. S1 to S3 (Supporting Information).

possible that any formed valencene epoxide, containing a highly reactive functional group prone to ring-opening reactions, is being more readily polymerized and/or converted into the relatively involatile side-products that we are convinced are being produced during our procedure (and more so, over extended reaction times). However, it appears that our optimized method in general produces nootkatone significantly more chemoselectively from valencene.

4. Conclusion

Much progress has been made towards the development of a green industrial process to produce nootkatone from valencene. After highly successful multifactorial optimization experiments, through the use of borate buffer medium, we managed to obtain highly LOX-active crude extract, capable of producing up to 74 % mol nootkatone after 72 h, at the unprecedented temperature of only 21 °C. In contrast to previously published findings, no valencene epoxide side-product was observed, only the α- and β-nootkatol intermediates. Excellent >90 % valencene conversions could be readily achieved in many of the test reactions performed. Furthermore, the slow bubbling of atmospheric air at a pressure of 1 atm through the reaction mixture sufficed to deliver enough oxygen to promote efficient LOX isozymes activity. Very surprisingly, we discovered that, under optimal conditions crude soybean LOX functioned best to catalyze the production of nootkatone in an unprecedented final reaction pH range of 4–6. Hence, it was highly beneficial to simply add oleic acid in its protonated neutral form without no need to use a pH stat device to control the pH of the reaction medium. Future work includes extensive investigations into the effects of substrate-, co-substrate- and added trace metal concentrations, on nootkatone yield.

CRediT authorship contribution statement

Ida M. Makhubela: Methodology, Investigation, Data curation. **Daniel Pienaar:** Writing – original draft, Supervision, Project administration, Methodology, Investigation. **Dean Brady:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Alexander Zawaira:** Writing – review & editing, Supervision, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2024.07.008.

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