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Screening of microorganisms for bioconversion of (+)-valencene to (+)-nootkatone

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(+)-Valencene (PubChem CID: 9855795)
(+)-Nootkatone (PubChem CID: 1268142)
Ethyl acetate (PubChem CID: 8857)
Glucose (PubChem CID: 53782692)
Sucrose (PubChem CID: 5988)
Tween 80 (PubChem CID: 5281955)
Sodium chloride (PubChem CID: 5234)
Potassium dihydrogen phosphate (PubChem CID: 516951)
Magnesium sulphate (PubChem CID: 24083)
Copper sulphate (PubChem CID: 24462)

ABSTRACT

The production of (+)-nootkatone, highly appreciated by fragrance and flavour industries, can be performed by whole-cell bioconversion from the sesquiterpene (+)-valencene, a compound readily available in orange essential oil. The aim of this work was to screen for microorganisms that convert (+)-valencene to (+)-nootkatone using different bioconversion systems. The screening was conducted using six different microorganisms, and bioconversion experiments were set up on surface culture using serological flasks containing PDA at 30 °C. It was observed that *Botryodiplodia theobromae* 1368, *Yarrowia lipolytica* 2.2ab, and *Phanerochaete chrysosporium* oxidised (+)-valencene to (+)-nootkatone, reaching (+)-nootkatone concentrations of 231.7 ± 2.1, 216.9 ± 5.8 and 100.8 ± 2.6 mg L^{-1}, respectively. Different bioconversion conditions were also tested—aqueous, organic, and biphasic—all resulting in similar (+)-nootkatone production. Both *B. theobromae* 1368 and *Y. lipolytica* 2.2ab showed substrate inhibition above 4.2 × 10^{-2} and 0.13 g of (+)-valencene (g of biomass)$^{-1}$, respectively, in aqueous phase experiments. Furthermore, *B. theobromae* 1368 and *Y. lipolytica* 2.2ab showed product inhibition when concentrations reached above 17.02 and 34.78 mg of (+)-nootkatone (g of biomass)$^{-1}$, respectively. The experimental method presented will be useful for ongoing studies on the selection and operation of the proper bioreactor at different bioconversion conditions.

1. Introduction

Flavours and aromas are highly valued in food, cosmetic, chemical, and pharmaceutical industries. Most of the aromatic compounds available are obtained by chemical synthesis or solvent extraction. However, consumers' growing aversion towards synthetic chemicals added to foods, cosmetics, and other household products has encouraged research efforts towards the production of aromatic and flavouring compounds using biological organisms (Maróstica & Pastore, 2007). The sesquiterpene molecules and their oxygenated derivatives are widely used in the aroma industry. Of particular interest is (+)-valencene ((2R)-8,8,8a-trimethyl-2-prop-1-en-2-yl-1,2,3,4,6,7-hexahydronaphthalene), the main bicyclic sesquiterpene found in orange peel oil (Elston, Lin & Rouseff, 2005). Sesquiterpenes are low-priced compounds commonly used as substrates for the bioconversion of various aromas (Misawa et al., 2011). The production of (+)-nootkatone has been conducted by chemical synthesis mainly from the sesquiterpene (+)-valencene, but also through the use of oxidising agents known to be unsafe to the environment, such as tert-butyl peracetate (Wilson & Shaw, 1978) or tert-butyl hydroperoxide in combination with metal catalysts supported on silica (Salvador & Clark, 2002). As such, the

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(+)-nootkatone molecules obtained through the mentioned methods may not be considered natural products, and therefore, do not satisfy the increasing demand for natural aromatic compounds. In order to meet this demand, many efforts are underway to find a biotechnological process using bacteria, fungi, or plants (Fraatz, Berger, & Zorn, 2009).

Microorganisms, plants, or animal cells can make these new biotechnological products in natural forms at a relatively low cost. Microorganisms are of particular interest because of the multitude of naturally-occurring strains and their great metabolic diversity for modifying and upgrading a variety of complex organic molecules; consequently, it is expected that one of the many native strains may be responsible for catalysing any specific reaction (Tan & Day, 1998). The use of diverse microorganisms as catalytic agents for the bioconversion of (+)-valencene to (+)-nootkatone (Fig. 1) has previously been studied (Kaspera, Krings, Nanzad, & Berger, 2005). Fraatz et al. (2009) used *Pleutorus sapidus* to obtain 600 mg of (+)-nootkatone L^{-1} in a fed-batch system. Sowden, Yasmin, Rees, Bell, and Wong (2005) used *Pseudomonas putida* and *Bacillus megaterium* for the same bioconversion process. Girhard et al. (2009) worked with enzymes from *Bacillus subtilis* inserted into *Escherichia coli* to convert 94.2 mg L^{-1} of the intermediate nootkatol and the product (+)-nootkatone. The yeast *Saccharomyces cerevisiae* (OGM) was also used as a catalyst for this bioconversion, producing a low concentration (10 mg L^{-1}) of (+)-nootkatone (Gavira et al., 2013). The aim of this work was to screen for microorganisms with the potential for the allylic oxidation of (+)-valencene using different bioconversion conditions.

2. Materials and methods

2.1. Chemicals

Sesquiterpenes (+)-valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity of >70% and >85%, respectively, were purchased from Fluka (Switzerland). Ethyl acetate (99.5%) was purchased from Quimex (México). Orange essential oil (*Citrus aurantium*, var. *amara*) was purchased from Cosmopolita Drugstore (Mexico DF) and used as the organic phase.

2.2. Microorganisms

The strains *Botryodiplodia theobromae 1368*, *Aspergillus tamari* V12307, *Phanerochaete chrysosporium*, *Yarrowia lipolytica* 2.2ab, *Kluyveromyces marxianus* NCYC1429, and *Rhyzomucor* sp. that were used came from the culture collection of the Solid State Fermentation Plant at the Autonomous Metropolitan University Iztapalapa. *Y. lipolytica* 2.2ab strain was previously sequenced in the Laboratory of Industrial Biotechnology of the National Polytechnic Institute (CBG-IPN), Mexico. The access in the National Centre for Biotechnology Information (NCBI) is EF643594.1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, identities 94% with *Y. lipolytica* strain LN-13. The strains were propagated in Potato Dextrose Agar plates (PDA, Bioxon-Dickinson, México) incubated at 30 °C for 3–7 days.

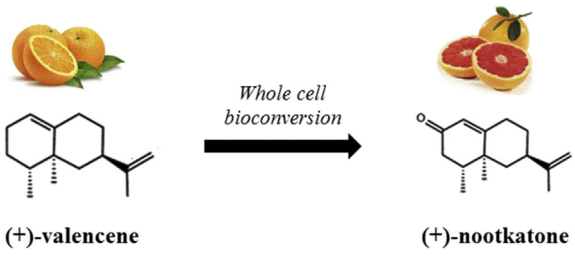

Fig. 1. Bioconversion of (+)-valencene to (+)-nootkatone using whole cells.

For the determination of substrate and product inhibition, microorganisms were grown in 250 mL baffled Erlenmeyer flasks containing 100 mL of culture medium. The culture medium for *Y. lipolytica* 2.2ab was composed of (in g L^{-1}): glucose, 30; Na_2MoO_4, 0.2; $MnSO_4$, 0.4; $FeCl_3$, 0.2; KI, 0.1; $CuSO_4$, 0.04; H_2BO_3, 0.5; $CaCl_2$, 0.1; NaCl, 0.1; $MgSO_4{*}7H_2O$, 0.5; KH_2PO_4, 1.0; $ZnSO_4$, 0.4; $(NH_4)_2SO_4$, 5; yeast extract, 4.004. Prior to sterilisation, the pH of the culture medium was adjusted to 5.5 with 2 M HCl (Tsigie et al., 2012). The culture medium for *B. theobromae* 1368 was composed of (in g L^{-1}): sucrose, 50; $NaNO_3$, 7.5; KH_2PO_4, 2.0; KCl, 0.3; $MgSO_4{*}7H_2O$, 0.6; $FeSO_4{*}7H_2O$, 0.6; $ZnSO_4{*}7H_2O$, 0.03; $MnSO_4{*}7H_2O$, 0.003; $CuSO_4{*}7H_2O$, 0.003; $Na_2MoO_4{*}2H_2O$, 0.003; yeast extract, 1.0. Prior to sterilisation, the pH of the culture medium was adjusted to 5.5 with 2 M HCl (Eng, Gutiérrez, & Favela, 1998). Flasks were then inoculated with 1×10^6 cells mL^{-1} and incubated for 3 days (*Y. lipolytica* 2.2ab) or 7 days (*B. theobromae* 1368) at 200 rpm and 30 °C. After incubation, a concentrated solution of (+)-valencene was added to provide different initial concentrations of (+)-valencene: 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.2, 4.5 and 6.4 g L^{-1}. To determine product inhibition on the bioconversion process, a concentrated solution of (+)-nootkatone was added in other experimental units to ensure different initial concentrations of (+)-nootkatone: 50, 100, 200, 300, 400, 600 and 1000 mg L^{-1}. The flasks were incubated for 4 days to carry out the bioconversion process, and samples (2.0 mL) were taken every 24 h. The concentrations of (+)-valencene and (+)-nootkatone were determined. Control experiments containing sterile broth without inoculum were also conducted. All the experiments were conducted in triplicate. Every day, three Erlenmeyer flasks were taken and centrifuged at 5000 rpm for 10 min. Biomass determination was carried out by measuring the dry weight of cells. Sesquiterpenes were extracted from the supernatant by adding 500 μL of ethyl acetate to 500 μL of sample. The mixture was vortexed twice for 20 s and allowed to settle until phase separation occurred, after which 1 μL of the ethyl phase was injected into a GC (Girhard et al., 2009; Sowden et al., 2005).

2.3. Partition coefficients determination

Partition coefficients of (+)-valencene and (+)-nootkatone between a buffer solution (aqueous phase) and orange essential oil (organic phase) were determined taking into account the phase ratio (1:1 v/v) and the initial (+)-valencene concentration conditions used in the serological flasks. During the bioconversion process, (+)-valencene is transferred from the organic phase to the aqueous phase, and (+)-nootkatone is transferred from the aqueous phase to the organic phase. Therefore, the aqueous phase was prepared as follows: 3.2 g L^{-1} of nootkatone was dissolved in a 0.1 M phosphate buffer adjusted to pH 7.5. Also, the organic phase was orange essential oil containing 3.2 g L^{-1} of (+)-valencene. Then, equal amounts of both solutions were mixed, yielding a final emulsion with a total volume of 6 mL. The emulsion was subjected to mechanical agitation for 6 h at 30 °C in order to reach thermodynamic equilibrium. The phases were then separated by centrifugation (+)-valencene and (+)-nootkatone were extracted from the aqueous and organic phases, respectively, using 500 μL of ethyl acetate. Both phases were analysed quantitatively by GC and the partition coefficient (*Kp*) was subsequently determined. The partition coefficient was calculated as the ratio of the solute concentration in the organic phase divided by the solute concentration