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Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*

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ABSTRACT

The sesquiterpenoid (+)-nootkatone is a highly demanded and highly valued aroma compound naturally found in grapefruit, pummelo or Nootka cypress tree. Extraction of (+)-nootkatone from plant material or its production by chemical synthesis suffers from low yields and the use of environmentally harmful methods, respectively. Lately, major attention has been paid to biotechnological approaches, using cell extracts or whole-cell systems for the production of (+)-nootkatone. In our study, the yeast *Pichia pastoris* initially was applied as whole-cell biocatalyst for the production of (+)-nootkatone from (+)-valencene, the abundant aroma compound of oranges. Therefore, we generated a strain co-expressing the premnaspirodiene oxygenase of *Hyoscyamus muticus* (HPO) and the *Arabidopsis thaliana* cytochrome P450 reductase (CPR) that hydroxylated extracellularly added (+)-valencene. Intracellular production of (+)-valencene by co-expression of valencene synthase from *Callitropsis nootkatensis* resolved the phase-transfer issues of (+)-valencene. Bi-phasic cultivations of *P. pastoris* resulted in the production of trans-nootkatol, which was oxidized to (+)-nootkatone by an intrinsic *P. pastoris* activity. Additional overexpression of a *P. pastoris* alcohol dehydrogenase and truncated hydroxy-methylglutaryl-CoA reductase (tHmg1p) significantly enhanced the (+)-nootkatone yield to 208 mg L^{-1} cell culture in bioreactor cultivations. Thus, metabolically engineered yeast *P. pastoris* represents a valuable, whole-cell system for high-level production of (+)-nootkatone from simple carbon sources.

1. Introduction

(+)-Nootkatone was first isolated from the Nootka cypress tree (*Callitropsis nootkatensis*, aka Alaska yellow cedar) (Erdtman and Hirose, 1962) and trace amounts were later also identified in grapefruit (*Citrus paradisi*) (MacLeod and Buigues, 1964) or pummelo (*Citrus grandis*) (Ortuno et al., 1995). Like many other sesquiterpenoids, (+)-nootkatone exhibits unique odor characteristics, rendering it a highly sought-after flavor and fragrance compound for the food and cosmetics industries. Pharmaceutical industry has become interested in the application of (+)-nootkatone as it has been shown to be an effective repellent of insects (Dietrich et al., 2006; Flor-Weiler et al., 2011; Jordan et al., 2012; Zhu et al., 2001). Recently, interesting therapeutic activities of (+)-nootkatone and its metabolites have been reported, such as anti-platelet aggregation effects in rats (Seo et al., 2011), anti-proliferative activity towards cancer cell lines (Gliszczyńska et al., 2011) and enhancement of energy

Abbreviations: HPO, *Hyoscyamus muticus* premnaspirodiene oxygenase; CPR, cytochrome P450 reductase; ValS, valencene synthase; ADH, alcohol dehydrogenase; AOX1, alcohol oxidase; CDW, cell dry weight; YNB, yeast nitrogen base; HRP, horse radish peroxidase

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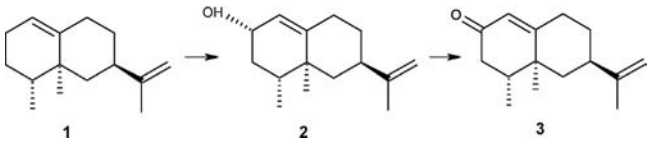

Fig. 1. Conversion of (+)-valencene (1) to trans-nootkatol (2) and (+)-nootkatone (3).

metabolism through AMP-activated protein kinase activation in skeletal muscle and liver (Murase et al., 2010).

Extraction of (+)-nootkatone from natural sources, *e.g.* citrus fruits, typically suffers from inadequate yields owing to slow biomass accumulation, low overall (+)-nootkatone concentrations and annual harvest fluctuations. Thus, chemical methods for (+)-nootkatone synthesis have been applied to satisfy the high industrial demand. As chemical synthesis often involved toxic heavy metals, highly flammable compounds or strong oxidants (Salvador and Clark, 2002; Wilson and Shaw, 1978), more attention is being paid to environment-friendly and safe methods for (+)-nootkatone synthesis. Several approaches for *de novo* (+)-nootkatone synthesis or the biotransformation of the abundantly available (+)-valencene to the rare (+)-nootkatone (Fig. 1) have been reported (Fraatz et al., 2009a). Whole-cell systems employing bacteria (Dhavlikar and Albroscheit, 1973; Girhard et al., 2009; Okuda et al., 1994; Sowden et al., 2005), fungi (Cankar et al., 2011, 2014; Kaspera et al., 2005; Krügener et al., 2010) and plants (Drawert et al., 1984; Furusawa et al., 2005; Sakamaki et al., 2005) or applications of cell extracts and/or purified proteins as biocatalysts (Bouwmeester et al., 2007; De Kraker et al., 2003; Gavira et al., 2013; Muller et al., 1998; Takahashi et al., 2007a; Zelena et al., 2012) have been described. Many of these biotransformation reactions are catalyzed by enzymes of the cytochome P450 monooxygenase (CYP) superfamily, although the screening and identification of efficient and regio-selective P450 enzymes for commercial applications is still challenging (Urlacher and Schmid, 2006). A number of limitations have restricted the use of CYPs in industrial processes including narrow substrate specificity, association with membranous structures, the need for co-expression of cytochrome P450-reductases, cofactor regeneration issues, overall low activity and process stability (Urlacher and Girhard, 2012). Recently, a comparative study involving several different yeast species and *Escherichia coli* has indicated that the methylotrophic yeast *Pichia pastoris* is an excellent host for the functional expression of membrane-associated cytochrome P450 enzymes (Geier et al., 2012). *P. pastoris* features additional advantages over the well-studied yeast *Saccharomyces cerevisiae*, like the ability to grow to very high cell densities in simple media and the availability of the strong and tightly regulated alcohol oxidase (*AOX*1) promoter (Cereghino and Cregg, 2000; Cregg et al., 2000; Macauley-Patrick et al., 2005; Ramón and Marín, 2011). Interestingly, *P. pastoris* has been described as host system for production of carotenoids like β-carotene, lycopene and astaxanthin (Araya-Garay et al., 2012a, 2012b).

S. cerevisiae has been used in several metabolic engineering approaches for enhanced production of industrially relevant terpenoids (Asadollahi et al., 2010; Chang and Keasling, 2006; Dumas et al., 2006; Muntendam et al., 2009; Nevoigt, 2008; Siddiqui et al., 2012; Takahashi et al., 2007b). For example, a plant cytochrome P450 enzyme, *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO), was characterized for the regio- and stereo-specific oxidation of various sesquiterpenes including (+)-valencene (Takahashi et al., 2007b). Engineering of the substrate recognition site of HPO enhanced trans-nootkatol formation, but HPO still failed to synthesize (+)-nootkatone in bioconversion reactions using *S. cerevisiae*. Relying on our experience with *P. pastoris* for recombinant expression of cytochrome P450 enzymes (Geier et al., 2012), we aimed at the development of a *P. pastoris* whole-cell biocatalyst for high-level production of (+)-nootkatone employing the engineered HPO variant V482I/A484I (Takahashi et al., 2007b). Co-expression of HPO and cytochrome P450 reductase (CPR) from *Arabidopsis thaliana* in *P. pastoris* cells led to the hydroxylation of externally added (+)-valencene to trans-nootkatol. Moreover, we identified and overexpressed an endogenous *P. pastoris* ADH leading to highly efficient trans-nootkatol to (+)-nootkatone conversion. In order to eliminate phase-transfer problems triggered by the external addition of (+)-valencene, we introduced a valencene synthase from Nootka cypress to produce (+)-valencene *in vivo* (Beekwilder et al., 2013). Thus, we created *P. pastoris* strains that can form industrially interesting quantities of (+)-valencene, trans-nootkatol and (+)-nootkatone, upon cultivation in two-phase systems using *n*-dodecane for trapping the produced terpenoid compounds.

2. Materials and methods

2.1. Chemicals and media

Restriction enzymes were purchased from Thermo Scientific, St. Leon-Rot, Germany. Monoclonal anti-FLAG® M2 antibody produced in mouse and anti-c-*myc* antibody produced in rabbit were obtained from Sigma-Aldrich®, Vienna, Austria. Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ peptone and Bacto™ yeast extract were obtained from Becton Dickinson and Company, Schwechat, Austria. Zeocin™ was purchased from InvivoGen (Eubio), Vienna, Austria. Kanamycin monosulfate, geneticin sulfate (G418S) and hygromycin B were purchased from Formedium™ (Norfolk, United Kingdom). Sterile water was acquired from Fresenius Kabi, Graz, Austria. Standard laboratory reagents were purchased from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Terpenoid standards were supplied by DSM Innovative Synthesis B.V., Geleen, The Netherlands.

Pichia cultures were either grown in YPD (1% yeast extract, 2% peptone and 2% glucose) or buffered complex glycerol medium, BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol). BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% methanol) was used as induction medium. Minimal dextrose (MD) plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose) were used for selection of strains containing the p*Pp*HIS4 expression vector. *E. coli* was cultivated in LB medium (Lennox) purchased from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Media for plates were solidified by addition of agar to 1.5%.

2.2. Vector and strain construction

E. coli TOP 10F' from life technologies (Vienna, Austria) (F'[lacI^q Tn10(*tet*^{R})] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(*Str*^{R}) endA1 λ^-) was used for all cloning experiments and propagation of expression vectors. The *P. pastoris* strain CBS7435 *his*4 *ku*70 was used as host strain for all further strain constructions (Näätsaari et al., 2012). Plasmids employed for strain constructions in *P. pastoris* have been described in the same work. Phusion® High Fidelity DNA polymerase (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany) was used for gene amplification according to the recommended PCR protocol. Native and codon optimized gene variants of *HPO* (*H. muticus* premnaspirodiene oxygenase, GenBank number of native gene: EF569601.1) and *A. thaliana CPR* (Cytochrome P450 reductase, GenBank number of native gene: NM_118585.3) were designed manually by applying the *Pichia*

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