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Heterologous biosynthesis of (+)-nootkatone in unconventional yeast *Yarrowia lipolytica*

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ABSTRACT

Yarrowia lipolytica is an unconventional yeast that is regarded as safe. It is the potential platform for terpenoids production because it possesses the endogenous MVA pathway which can provide precursors for terpenoids synthesis. Herein, we constructed (+)-nootkatone, a sesquiterpenoid which is highly demanded in cosmetics and fragrance industries, synthetic pathway in *Yarrowia lipolytica* ATCC 201249. Heterologous production of (+)-nootkatone was achieved by co-expressing (+)-valencene synthase CnVS, codon-optimized (+)-nootkatone synthase opCYP706M1 and codon-optimized NADPH-cytochrome P450 reductase opAtCPR1. The initial (+)-nootkatone production was 45.6 μg/L. Fusion of opCYP706M1 and opt46AtCPR1 (opAtCPR1 with 46 amino acids truncated at N-terminal) increased (+)-valencene conversion efficiency to (+)-nootkatone and (+)-nootkatone production increased to 312.2 μg/L, nearly six times higher than the initial. Overexpression of the MVA pathway rate limiting enzymes 3-hydroxy-3-methylglutaryl-coenzyme A reductase tHMG1 and FPP synthase ERG20 improved the (+)-nootkatone production furtherly. The final engineered strain achieved a (+)-nootkatone titer of 978.2 μg/L, which was a 20.5-fold increase compared to those simply coexpressed CnVS, opCYP706M1 and opAtCPR1. This is the first report of heterologous biosynthesis of (+)-nootkatone in *Y. lipolytica*, which will provide a favorable reference for studies on heterologous production of other sesquiterpenoids and high-efficiency expression of P450 enzymes in *Y. lipolytica*.

1. Introduction

(+)-Nootkatone is a highly sought-after compound which belongs to sesquiterpenoids family and naturally exists in many plants like grapefruit (*Citrus paradisi*) [1] and pummelo (*Citrus grandis*) [2]. Due to its special odour characteristics and low odour threshold [3], (+)-nootkatone is widely used in fragrance, cosmetics and food industries. What's more, (+)-nootkatone shows repellent activity against termites, which makes it an effective insecticide [4,5]. Additionally, it was reported that (+)-nootkatone and its metabolites had anti-proliferative [6] and anti-platelet activities [7], making it valuable in pharmaceutical applications.

Natural (+)-nootkatone is extracted from plants like grapefruit, which suffers from low amounts and depends on annual harvest, so could not meet the industrial demand [8]. Most of the commercial (+)-nootkatone is chemically synthesized from the presumed precursor (+)-valencene, but the catalysts used in the oxidation reaction are toxic and harmful to the environment [9]. Biotransformation and enzymatic conversion of (+)-valencene to (+)-nootkatone have been described [10,11], but finding the regio-selective P450 enzymes for allylic oxidation of (+)-valencene is still the key problem. Recently, (+)-nootkatone *de novo* synthesis using microbial cell factories has come into our sights. Beekwilder's group firstly identified the valencene synthase CnVS and valencene oxidase CYP706M1 from Alaska cedar (*Callitropsis nootkatensis*), a plant that naturally produces (+)-nootkatone [12,13], and expressed these two enzymes in *Saccharomyces cerevisiae* successfully. Wriessnegger et al. [14] reported the production of (+)-nootkatone by metabolic engineering of *Pichia pastoris* and enhanced the titer to 208 mg/L through high cell-density fermentation.

Yarrowia lipolytica, generally regarded as safe (GRAS), is a non-conventional yeast that has attracted more and more interest because of its unique characteristics, such as non-pathogenicity

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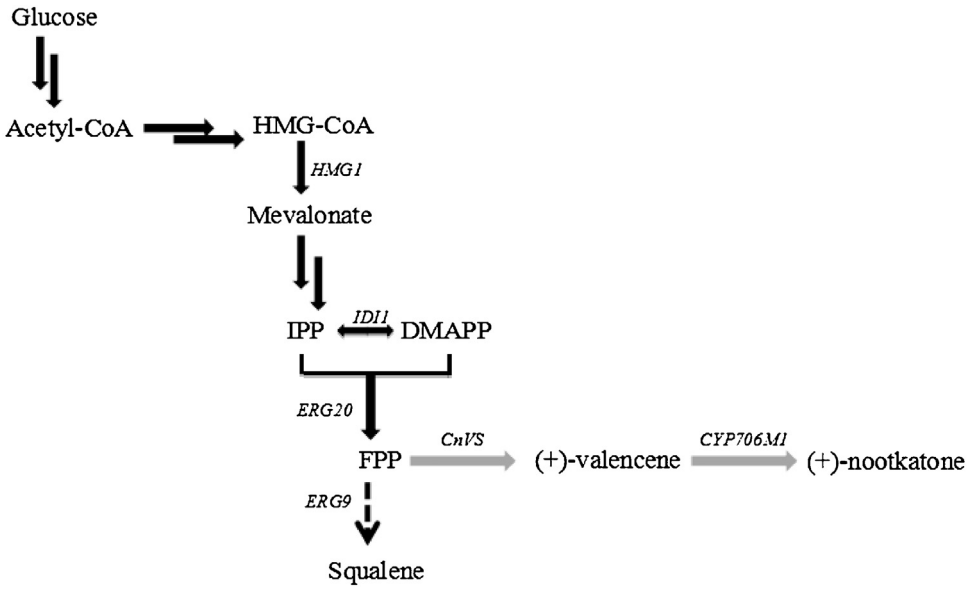

Fig. 1. Graphical depiction of (+)-nootkatone synthetic pathway in *Y. lipolytica*. Solid black arrows indicate the endogenous pathway in *Y. lipolytica*. Double black arrows represent multiple steps. Grey arrows represent the exogenetic steps introduced for (+)-nootkatone biosynthesis. Dashed arrows indicate FPP competing steps. Intermediates: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; CnVS, (+)-valencene synthase from *Callitropsis nootkatensis*; CYP706M1, (+)-nootkatone synthase from *Callitropsis nootkatensis*.

and suitability for protein expression [15]. As an oleaginous yeast, *Y. lipolytica* can use both hydrophobic and hydrophilic substrates as carbon sources to produce many important metabolites such as organic acids [16] and secreted proteins [17]. It has been successfully used for industrial applications by means of metabolic engineering in many fields, including biodiesel fuel, fatty acids and carotenoids [18] and some products such as omega-3 eicosapentaenoic acid and ricinoleic acid reached relatively high productions [19,20]. Stephanopoulos group engineered *Y. lipolytica* for lipid overproduction using different carbon sources and obtained engineered yeasts featured high yield, titer and productivity [21,22]. Blazeck et al. [23] produced itaconic acid in *Y. lipolytica* through heterologous expression of the itaconic acid synthesis enzyme and 4.6 g/L itaconic acid was produced in bioreactors. Completion of the genome sequencing and mature genetic tools have made it possible to be genetically modified for biotechnological applications [24]. Many DNA manipulation methods have been described for heterologous gene expression in *Y. lipolytica* [25,26]. Gao et al. [27,28] reported an efficient and time-saving method for one-step integration of multiple genes into the genome of *Y. lipolytica*. Schwartz et al. [29] developed a tool for markerless gene integration into the *Y. lipolytica* genome based on CRISPR-Cas9. Engineering of promotors in *Y. lipolytica* also give a chance to utilize this organism more efficient [30,31]. These results laid the foundation for metabolically engineering of *Y. lipolytica*.

In yeast, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) derived from mevalonic acid (MVA) pathway are the common building blocks for all terpenoids synthesis [32]. *Y. lipolytica* possesses the endogenous MVA pathway, making it suitable for production of terpene-related natural products [33]. Cao et al. [34] reported the heterologous production of a monocyclic monoterpene limonene by constructing the limonene synthetic pathway in *Y. lipolytica* and got the highest limonene production level at the time. α-Farnesene, a valuable sesquiterpene, was firstly synthesized in *Y. lipolytica*. The concentratuin reached 259.98 mg/L through MVA pathway engineering and process optimization [35]. Matthäus et al. [36] expressed the codon-optimized *crtB* and *crtI* genes in *Y. lipolytica* and produced lycopene with the highest yield of 16 mg/g (dry cell weight). Gao et al. [27,37] demonstrated heterologous biosynthesis of β-carotene in *Y. lipolytica* and improved the production to 4 g/L. All these studies showed the huge ability of *Y. lipolytica* to biosynthesize and produce terpenoids.

However, there is no report about the production of sesquiterpenoid (+)-nootkatone by *Y. lipolytica*. *Y. lipolytica* doesn't produce (+)-nootkatone in its natural state for lacking the (+)-nootkatone synthase, it only provides the precursor farnesyl diphosphate (FPP). In this study, we present a method for (+)-nootkatone heterologous biosynthesis in *Y. lipolytica* by introducing (+)-valencene synthase CnVS and (+)-valencene oxidase CYP706M1 (Fig. 1). (+)-Nootkatone titer was steply increased by means of protein fusion and metabolic engineering of MVA pathway. This study may contribute to recherches on heterologous biosynthesis of other sesquiterpenoids and high-efficiency expression of P450 enzymes in *Y. lipolytica* in the future.

2. Material and methods

2.1. Strains, medium and culture condition

The original strain *Y. lipolytica* ATCC 201249 and single copy plasmid pINA1269 carrying the *LEU2* gene were kindly provided by professor Yuan Yingjin (Tianjin University, China). All the engineered strains and plasmids constructed in this work were listed in Table 1. *Escherichia coli* Trasns1 T1 was used for construction and proliferation of plasmids. Screening of *Y. lipolytica* transformants was conducted with auxotrophic SC plates containing 10 g/L glucose, 6.7 g/L yeast nitrogen base, 16 g/L agar, and 2 g/L amino acid mixture lacking appropriate nutrients [35]. The YPD medium (20 g/L glucose, 10 g/L yeast extract and 20 g/L peptone) was used for *Y. lipolytica* growth and fermentation. 10% (v/v) dodecane was added to 30 ml YPD culture broth to capture the products (+)-nootkatone and (+)-valencene. Shake flasks were cultivated under 30 °, 220 rpm for 5 days.

2.2. Plasmid construction

The farnesyl diphosphate synthase gene *ERG20* was amplified from the genomic DNA of *Y. lipolytica* and the truncated