Production of α-cuprenene in *Xanthophyllomyces dendrorhous*: a step closer to a potent terpene biofactory

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Abstract

**Background:** The red yeast *Xanthophyllomyces dendrorhous* is a natural producer of the carotenoid astaxanthin. Because of its high flux, the native terpene pathway leading to the production of the tetraterpene is of particular interest as it can be redirected toward the production of other terpene compounds. The genetic tools for the transformation of the yeast with the concurrent knock-out of genes involved in the astaxanthin biosynthesis are made available and here we show that the production of the sesquiterpene α-cuprenene is possible in mutant strains of *X. dendrorhous* transformed with the *Cop6* gene originating from the fungus *Coprinus cinereus*. For the evaluation of the production levels, we chose to express the same gene and analyze the accumulation of α-cuprenene in *Escherichia coli* and *Saccharomyces cerevisiae*, as well. Here we propose that *X. dendrorhous* is a candidate in the search for the potential platform organism for the production of terpenes.

**Results:** All three *X. dendrorhous* mutants functionally express the *Cop6* gene and accumulate α-cuprenene. The production of α-cuprenene in the red yeast reached 80 mg/L, which represents a far higher concentration compared to the levels obtained in the *E. coli* and *S. cerevisiae* mutants. At this expression levels the pool of terpene precursors has not become a limiting factor in the *X. dendrorhous* mutants since the expression of the *Cop6* gene in the genomic rDNA of the yeast allows production of both α-cuprenene and astaxanthin without affecting the growth or the accumulation levels of both compounds.

**Conclusions:** We have shown that *X. dendrorhous* can produce α-cuprenene, and the results here presented, next to the capability of accumulating at least two more non-native sesquiterpenes, demonstrates the high potential of this yeast to become an interesting terpene-based drugs producer.

**Keywords:** *Xanthophyllomyces dendrorhous*, α-cuprenene, Metabolic engineering, *Escherichia coli*, *Saccharomyces cerevisiae*, Terpene cell factory

Background

Since ancient times, microorganisms have been used to produce bread, wine and dairy products in order to improve the quality of food and its nutrients. Today, microbes are utilized for the manufacture of a wide variety of fine or bulk chemicals including antibiotics [1,2], vitamins [3], biofuels [4,5], biodegradable and biocompatible plastics from waste [6] and terpene-based drugs [7,8]. Originally, the choice for a production host was dictated by the ability of the organism to produce the desired compounds, but in most cases the concentration of the chemicals of interest was not sufficient to cover the market demand.

With the development of genetic engineering, new tools became available to overcome these obstacles. The possibility of transferring single genes or even complete pathways to other microorganisms led to improved yields or easier bioprocessing conditions. Furthermore, metabolic engineering allowed the enhancement of the yields in the native hosts by finely tuning the metabolic networks of the cells towards the optimized production of the specific compound. More recently, the combination of synthetic biology and metabolic engineering...
has resulted in the creation of hosts capable of producing a non-native compound with high efficiency obtainable only by optimizing the native pathways of the cells [9].

The majority of the yeast or bacterial strains used by industry have been selected for the several advantages they deliver: they are easy to cultivate, they can grow on cheap media, they are generally regarded as safe (GRAS status) and their metabolic pathways are easy to modify via genetic engineering.

*Xanthophyllomyces dendrorhous*, a red basidiomycetous yeast, represents one of the microbial strains already used in industry and shares all the aforementioned advantages [10]. Today, *X. dendrorhous* is grown at industrial scale for its native capability to produce the valuable carotenoid astaxanthin.

Carotenoids, together with several other pharmaceutically important compounds, like artemisinin and Taxol, belong to the natural compounds class of the terpenes [11]. Several efforts have been put in the engineering of a platform organism for the production of industrially important terpenes [7,8].

We hypothesize that, since *X. dendrorhous* can produce high levels of astaxanthin, which shares the same precursors with all other terpenes, it can also utilize those same precursors for the production of any other terpenoid compound.

The red yeast was already shown to be able to functionally express the pentalenene synthase from a *Streptomyces* strain involved in the biosynthesis of the antibiotic pentalenolactone [12]. In order to further evaluate the potential of the red yeast as a platform organism for terpenes, we expressed the sesquiterpene cyclase *Cop6* in *X. dendrorhous* mutant strains. The protein *Cop6*, originating from the fungus *Coprinus cinereus*, produces the cyclized sesquiterpene α-cuprenene, which is the basic structure for the formation of lagopodin A, an antimicrobial sesquiterpene quinone [13]. We also compared the accumulation levels of α-cuprenene with two of the most industrially utilized microbial strains, *E. coli* and *S. cerevisiae*.

**Results**

**Production of cuprenene in *E. coli***

Isolation of volatile terpenoids in *E. coli* via addition of a dodecane organic phase to the liquid cultures has been shown to be extremely efficient [14]. We have decided to adopt the same strategy to capture the α-cuprenene produced by the *E. coli* strains transformed with the *Cop6* gene. In order to be able to compare the levels of α-cuprenene at the different time points and from different organisms we added hexadecane in known concentrations, as an internal standard, to the dodecane.

The wild type strain and the transformed one exhibited the same growth curve and biomass accumulation; twenty hours after induction of the expression, the cells reached the highest density and then started dying after 30 hours. After 48 hours the concentration of cuprenene, based on the internal standard, in the modified strain reached approximately 0.25 mg/L of culture, a slight increase compared to the concentration at 20 hours (Figure 1).

**Growth curves and α-cuprenene production from *S. cerevisiae* and *X. dendrorhous* in rich medium**

After the separate transformations of *X. dendrorhous* wild-type strain with the vectors pCrtE-Cop6, pCrtYB-Cop6 and pPR-Cop6, one colony from each transformation plate was chosen to be grown and analyzed. As expected, since the astaxanthin pathway was disrupted (Figure 2), on the plates used to select ΔE-Cop6 and ΔYB-Cop6, the mutant colonies presented a white phenotype. In contrast, XdCop6 colonies, transformed with pPR-Cop6, in which the carotenoid pathway was not modified (Figure 2), shared an orange pigmentation with the wild type strain.

The *S. cerevisiae* mutant, ScCop6, was isolated after transformation of the wild type strain of *S. cerevisiae* with the plasmid p426GPD-Cop6, which allows constitutive expression of the *Cop6* gene.

A time course analysis was performed on the ScCop6 and on the three *X. dendrorhous* mutant strains, XdCop6, ΔE-Cop6 and ΔYB-Cop6. The strains were grown in YPD medium in order to obtain a high growth in shake flasks. The time course consisted of four sampling times after the inoculation in fresh medium; we chose 24 h, 48 h, 72 h and 96 h to be able to observe all the steps of the growth curve.

![Figure 1 Cuprenene production during time course with *E. coli* pHis8Cop6.](http://www.microbialcellfactories.com/content/12/1/13)
With respect to the OD$_{600}$ and to the cell dry weight, the *S. cerevisiae* and *X. dendrorhous* mutants exhibit similar curves compared to the respective wild type strains.

Figure 3 compares the growth curves obtained during the time course analysis from the mutant strains of both yeasts. In spite of the fact that *S. cerevisiae* cells reach a higher optical density, they accumulate a lower biomass compared to all *X. dendrorhous* strains.

The diluted dodecane solutions from the *S. cerevisiae* strains and from the wild type and mutant *X. dendrorhous* were analyzed by GCMS, and a single peak appeared in the chromatograms from the mutants at 12.8 minutes (Figure 4). The fragmentation pattern of the peak was compared to the pattern corresponding to α-cuprenene produced in *S. cerevisiae* (Figure 5). The mass and the relative ratio of the fragment peaks matched between the two patterns, allowing us to confirm that the only sesquiterpene produced by Cop6 in *X. dendrorhous* is α-cuprenene.

The time-course production of α-cuprenene in the four strains is represented in Figure 6. The level of the sesquiterpene in *S. cerevisiae* sharply increased after 24 hours to reach a maximum of 6.6 mg/L on the second day of culturing and then appeared to decrease during the following two days.

The α-cuprenene production in the three *X. dendrorhous* strains showed ten times higher levels with a major increase between 24 and 48 hours, as well. In contrast to *S. cerevisiae*, XdCop6, ΔE-Cop6 and ΔYB-Cop6 constantly produced α-cuprenene during the complete time course. The accumulation of the sesquiterpene appeared to be consistent in all the three *X. dendrorhous* strains and was directly proportional to the cell mass, in particular in the first three days after inoculation. The highest yield was obtained after 96 hours with the XdCop6 strain and corresponded to almost 80 mg/L of culturing medium. ΔE-Cop6 could produce up to 70 mg of α-cuprenene per liter of medium and ΔYB-Cop6 74 mg/L, both after growing for 96 hours.

**Growth curves and α-cuprenene production from *S. cerevisiae* and *X. dendrorhous* in minimal medium**

The ScCop6 mutant was selected on medium lacking uracil in order to allow only the colonies containing the plasmid to grow. The rich YPD medium contains all the necessary nucleotides, thus the selective pressure on the *S. cerevisiae* mutant strain grown in this medium was inexistent. For this reason, we decided to grow all the strains in a minimal medium lacking uracil which would
allow a more accurate comparison between the ScCop6 and the three X. dendrorhous mutants. The same settings chosen for the time course in rich medium were applied for the growth and α-cuprenene production analysis in minimal medium.

An overall lower cell mass and OD₆₀₀ compared to rich medium was observed for all the strains and no difference in growth could be discerned between the wild type strains and the mutant strains, both for S. cerevisiae and X. dendrorhous.

S. cerevisiae strains started growing already after 24 hours while XdCop6, ΔE-Cop6 and ΔYB-Cop6 showed a lag phase between 0 and 24 hours and a log growth between 24 and 48 hours (Figure 7). In minimal medium ΔE-Cop6 exhibited a slightly reduced cell growth compared to XdCop6 and ΔYB-Cop6.

The accumulation of α-cuprenene in the three X. dendrorhous strains was significantly lower than the concentration obtained in YPD reaching a maximum of 20 mg/l (Figure 8) for ΔYB-Cop6. When ScCop6 was grown in minimal selective medium, the sesquiterpene production increased nearly to 12 mg of α-cuprenene per liter of medium.

Microscopy analysis of ScCop6 and XdCop6

Figures 3 and 7 indicate that S. cerevisiae and X. dendrorhous are characterized by a different growth rate and optical density. For a better understanding of the
differences between the two yeasts, we performed a microscopy analysis on the strains ScCop6 and XdCop6.

A Burke chamber was used to count the yeast cells. In spite of the macroscopic red pigmentation of XdCop6, the cells appeared white under the microscope white light. After counting samples in duplo from ScCop6 and XdCop6 and calculating the cell numbers, we observed that one OD$_{500}$ unit in *S. cerevisiae* corresponded to $3 \times 10^7$ cells per milliliter, consistent with the data available in literature [15]. In contrast, the number of *X. dendrorhous* cells counted in one milliliter of culture with the same optical density was $4.8 \times 10^6$.

To further ascertain the morphological differences between ScCop6 and XdCop6, we compared the average cells sizes. Figure 9 compares the pictures of the two strains after four days of growth; the same magnification was used to visualize the cells. Cells from XdCop6 are round shaped and have a granular appearance, while ScCop6 cells, although showing a similar round morphology, show a more homogeneous cytosol. On average, the size of *X. dendrorhous* cells was 10 μm in diameter; *S. cerevisiae* cells were smaller with a size ranging between 7 and 8 μm. Not only ScCop6 cells were on average smaller than *X. dendrorhous*, but they also never had a diameter bigger than 10 μm, in contrast with the red yeast cells.

**Discussion**

*E. coli* and *S. cerevisiae* are two model organisms that also serve as industrial cell factories for the production of a wide variety of compounds ranging from pharmaceutically active substances to food ingredients and biofuels.

In spite of the fact that *X. dendrorhous* has not been studied as extensively as *E. coli* or *S. cerevisiae*, it shows...
great potential to become a platform organism for terpene production [12]. In order to assess the value of the red yeast as a cell factory, we expressed the Cop6 gene in the three X. dendrorhous mutants and compared the production of α-cuprenene with E. coli and S. cerevisiae strains expressing the same gene.

The cDNA from the Cop6 gene was expressed by all X. dendrorhous mutants, whereas the genomic version of the gene, when transferred to the red yeast, did not result in α-cuprenene accumulation (data not shown) indicating that X. dendrorhous cannot correctly splice the gene from C. cinereus.

When grown in rich medium, all X. dendrorhous strains, including the wild type, showed very similar growth rates: they reached OD600 values of 20 and produced a maximum of 12 grams of cell dry weight per liter of culture. Similarly, when looking at the α-cuprenene production, the three strains XdCop6, ΔE-Cop6 and ΔYB-Cop6 did not show big differences among each other, with levels of the sesquiterpene ranging from 70 to 80 mg of compound per liter of medium.

Comparing Figures 3 and 7, it is clear that, differently from the experiments in the rich medium, the three X. dendrorhous strains shared an altered growth behavior in the minimal medium. While XdCop6 and ΔYB-Cop6 reached a maximum cell dry mass of nearly 5 g/L, similar to the one obtained with the wild type strain, ΔE-Cop6 could not produce more than 3.5 g of dry cells per liter of medium. In 2008 Niklitschek and colleagues have reported the difficulty to isolate a X. dendrorhous strain
in which both \(crtE\) alleles had been knocked out [16], suggesting an important role of this protein in the yeast growth. In the light of the results shown in this study, we can conclude that difference in growth between the \(\Delta E\)-Cop6 mutant and all the other \(X.\ dendrorhous\) strains in the minimal medium could be explained by a lack, in this particular medium, of compounds important for the yeast growth produced directly or indirectly by the CrtE protein.

Concomitantly with the reduced growth in the minimal medium, the concentration of \(\alpha\)-cuprenene in the dodecane was also affected, reaching values ranging from 15 to 21 mg/L of culture. The decrease in sesquiterpene accumulation can partly be explained by the reduced cell mass and partly by the lower concentration of nutrients in the minimal medium which would induce the cells to minimize the energy consumption by shutting down unnecessary pathways.

When comparing cell mass accumulation and \(\alpha\)-cuprene production in all \(X.\ dendrorhous\), \(E.\ coli\) and \(S.\ cerevisiae\) strains, the prokaryote showed the lowest values. The low biomass in the bacterium is most likely to be ascribed to a lack of glucose in its growth medium, while the limited sesquiterpene production is due to the lower terpene flux in \(E.\ coli\) compared to the two eukaryotes.

The differences in growth curves and dry weight between the \(X.\ dendrorhous\) and the \(S.\ cerevisiae\) strains seem to have morphological reasons. \(X.\ dendrorhous\) cells are on average bigger than \(S.\ cerevisiae\) ones [17] and at the same optical density \(S.\ cerevisiae\) cell counts

![Figure 8 Production of \(\alpha\)-cuprenene in minimal medium.](image)

![Figure 9 Microscopy photographs. (A) ScCop6 and (B) XdCop6.](image)
are almost 10 times higher than in *X. dendrorhous* cultures, meaning that the same OD<sub>600</sub> value corresponds to more *S. cerevisiae* cells than it does for *X. dendrorhous*. Since, at the beginning of the time course experiments, the initial OD<sub>600</sub> for all the strains was set at 0.05, the number of cells initially transferred to the fresh medium was higher in *ScCop6* than in all the *X. dendrorhous* mutants. This would explain the delay in growth we observed for *XdCop6, ΔE-Cop6* and Δ*YB-Cop6* compared to *ScCop6*. Additionally, the higher cell mass accumulation observed in the *X. dendrorhous* strains compared to *S. cerevisiae* may be due to the red yeast’s bigger sized cells rather than to a higher number of cells.

The highest α-cuprenene production levels were obtained with the *X. dendrorhous* strains both in the rich and in the minimal medium experiments. Remarkably, in the YPD medium the gap in sesquiterpene accumulation between the red yeast and the *S. cerevisiae* strain was far more pronounced. We assume that, since the complete medium does not allow selective pressure on *ScCop6*, which was isolated by its ability to grow on minimal medium lacking uracil, the strain might have undergone a reduction in plasmid copy number.

While *ScCop6* mutants contain an average of 20 copies of *Cop6*, the *X. dendrorhous* white mutants possess just one copy of the gene since the recombination of the constructs can occur only once in the single *crtE* or *crtYB* genes. In order to obtain a mutant with a higher number of integrations of the gene in the genomic rDNA, we transformed the *X. dendrorhous* wild type strain with a higher concentration of the DNA fragment from the pPR-Cop6 vector and selected the transformants on YPD medium containing a concentration of geneticin 5 times higher than normal, hoping for gene amplification. Unfortunately, no colony grew after this transformation and we could not evaluate the effect of more gene copies on the α-cuprenene accumulation.

Nevertheless, we can safely assume that the concentration of the precursors is not a limiting factor in the sesquiterpene production in *X. dendrorhous*, since the strain *XdCop6* can easily sustain the production of both α-cuprenene and astaxanthin, especially when grown in the YPD rich medium. The production of both terpene compounds in *XdCop6* confirms the hypothesis that a higher gene copy number would positively influence the α-cuprenene production in *X. dendrorhous*.

In conclusion, *X. dendrorhous* shows great promise since it has the GRAS status, it grows at room temperature in minimal media, and it has already been used by industry for the production of astaxanthin. We discovered that it can produce at least three non-native sesquiterpenes, pentalenene [12], α-cuprenene and cubebol (data not shown). Furthermore, *X. dendrorhous* is the best microorganism, among the ones we have analyzed, to be used for the production of α-cuprenene. A better understanding of the molecular biology of this yeast will prove useful for the identification of stronger promoters for a higher gene expression.

In light of the aforementioned advantages and of the provided results, *X. dendrorhous* is an interesting candidate for being used as a cell factory for the production of terpenes.

**Methods**

**Strains and culture conditions**

The *E. coli* strain DH5α was used for the cloning processes, while the strain BL21 (DE3) was transformed and cultured for the time course experiments. *S. cerevisiae* MRG 5 #502 (MATα, ura3-52, leu2-Δ1, trp1-Δ36, his3-Δ200, Dade2) was used for the transformation and time course analysis. *X. dendrorhous* wild type strain (CBS 6938) was used for the transformations and as negative control for all experiments. Both *E. coli* strains were grown in LB (10 g/L Trypton, 5 g/L Yeast Extract, 10 g/L NaCl) with 30 mg/ml kanamycin. The rich medium for *S. cerevisiae* and *X. dendrorhous* was YPD (10 g/L Yeast Extract, 20 g/L Peptone and 20 g/L Dextrose) with additional 40 mg/ml geneticin (G-418 Sulphate, Gibco) only for the selection and growth of the *X. dendrorhous* mutants. The minimal medium for *S. cerevisiae* consisted of 13.4 g/L Yeast Nitrogen Base without amino acids, 20 g/L Dextrose, 100 mg/L leucine, 40 mg/L histidine, 40 mg/L tryptophan and 40 mg/L of uracil. The same concentrations of Yeast Nitrogen Base and Dextrose were kept for the minimal medium for *X. dendrorhous* and geneticin was added to the medium for the culturing of the mutants.

**Construction of the *E. coli* strain EcCop6 and time course analysis**

The plasmid pHis8Cop6 was a kind gift of Prof. Claudia Schmidt-Dannert from University of Minnesota and it contains the cDNA sequence of the *Cop6* gene from *Coprinus cinereus* under the control of the T7 promoter. *E. coli* BL21 (DE3) colonies containing pHis8Cop6 were selected on LB plates with kanamycin.

One transformed colony, EcCop6, was chosen for the time course analysis and a seed culture was started over night in LB plus kanamycin. The fresh cultures were inoculated and grown to OD<sub>600</sub> 0.5 and then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for the induction of expression of the *Cop6* gene. The time course was performed in duplo and consisted of six 100-ml flasks with 10 ml of LB medium plus kanamycin and 500 μl of dodecane mixed with the internal standard, heptadecane (68 μg/ml of medium), grown in a shaking incubator at 250 rpm at 37°C for 48 hours. Two flasks were removed from the incubator at each sampling point.
Construction of the _S. cerevisiae_ strain ScCop6 and time course analysis

pHis8Cop6 was used as a template for the amplification of the gene to be cloned in the p426GPD episomal vector for the expression of _Cop6_ in _S. cerevisiae_. The gene was amplified with primers flanked with the restriction sites for EcoRI and _Sc_Cop6 gene is flanked by the restriction sites for _NheI_ and _Sall_. The fragment was then cloned in the previously digested vector and the complete construct was used to transform the _S. cerevisiae_ wild type strain to obtain ScCop6. Positive colonies were isolated for the ability to grow on selective medium with geneticin. From the transformations with pCrtE-ΔCop6, pCrtYB-ΔCop6 and pPR-ΔCop6, respectively.

Isolation and characterization of _X. dendrorhous_ mutants

The plasmids pCrt-E-PSS, pCrtYB-PSS and pPR-PSS [12] were used as backbone for the creation of the new plasmids pCrtE-Cop6, pCrtYB-Cop6 and pPR-Cop6, respectively. _Cop6_ was amplified from the mutated gene used for the expression in _S. cerevisiae_ and was flanked by the restriction sites for _NheI_ and _Sall_. The fragment was then cloned in the digested vector and used for the transformation of _X. dendrorhous_. The positive colonies were isolated for the ability to grow on selective medium with geneticin. From the transformations with pCrtE-Cop6, pCrtYB-Cop6 and pPR-Cop6, the three new mutant strains ΔE-Cop6, ΔYB-Cop6 and XdCop6 were obtained, respectively (Figure 2). The three strains were grown at 21°C at 200 rpm for the analysis of production of α-cuprenene in time following the same conditions used for the time course for ScCop6.

GC-MS analysis

The dodecane solutions isolated from the different cultures at different time points were diluted 1:10 in ethyl acetate and run on GCMS to reveal and quantify the α-cuprenene production. A Shimadzu GCMS-QP5000 provided with a ZB-1 ms dimethylpolysiloxane column (Phenomenex 0.25 mm inner diameter, 0.25 μm thickness, 15 m length) was used for the analysis. Two microliters of diluted dodecane were injected splitless and analyzed in total ion scan using helium as carrier gas. The GCMS program consisted of an oven initial temperature of 50°C with an increment of 5°C/min up to 105°C and then up to 200°C with an increase of 30°C/min. The quantitation of the α-cuprenene was based on the hexadecane peak which had a known concentration.

Microscopy analysis and cell counting

Aliquots of ScCop6 and XdCop6 cultures were taken after 96 hours of growth, diluted to OD_{600} 0.15, approximately, and were then transferred to a Bürker counting chamber (Bright line, Labor Optik). The number of cells counted in a surface of 0.0025 mm^2 was multiplied by 10^4 and divided by the OD_{600} values of the cultures to obtain the number of cells per OD_{600} unit.

A Leica DM 6000B microscope provided with a 40x magnification objective and the LAS AF program was used for the visualization and to measure the size of the cells with the 10 μm bar provided by the LAS AF program.

**Competing interest**

The authors declare that they have no competing interests.

**Authors’ contributions**

EM conceived, designed and performed all the experiments. RS contributed to the microscopy analyses. WQ and OK supervised the research. All the authors read and approved the manuscript.

**Acknowledgements**

The authors wish to thank Prof. Claudia Schmidt-Dannert and Grayson Wawrzyn from University of Minnesota for providing the genes from _Coprinus cinereus_ and for the useful discussions. The Ubbo Emmius programme of the University of Groningen and the EUROCORES SYNMET programme (09-EuroSYNBIO-FP-023) have sponsored this research.

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Received: 9 October 2012 Accepted: 28 January 2013

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