



Heterologous expression of pentalenene synthase (*PSS*) from *Streptomyces UC5319* in *Xanthophyllomyces dendrorhous*

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ABSTRACT

For the first time, the pentalenene synthase (*PSS*) gene from *Streptomyces UC5319* was expressed in *Xanthophyllomyces dendrorhous*, a native producer of astaxanthin. For the expression of the gene and the concurrent knock out of the native *crtE* or *crtYB* genes, two new vectors were engineered and used for the transformation of the wild-type strain of *X. dendrorhous*. The transformations resulted in white colonies, showing a complete shutdown of the carotenoid production. Furthermore, an additional vector was constructed for the insertion of the *PSS* gene in the rDNA of the yeast. All the mutant strains produce the sesquiterpene pentalenene and show no difference in growth when compared to the wild-type strain. In this report, we demonstrate that *X. dendrorhous* is a suitable host for the expression of heterologous terpene cyclases and for the production of foreign terpene compounds.

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1. Introduction

Xanthophyllomyces dendrorhous belongs to the phylum of the basidiomycota and shows a characteristic red pigmentation (Kucsera et al., 2000) given by the carotenoid astaxanthin, produced by the yeast in response to oxidative stress (Schroeder and Johnson, 1995). Since the market size of astaxanthin in 2007 reached nearly 220 million US dollars (Schmidt et al., 2011), the improvement of the production of the tetraterpene in *X. dendrorhous* has been the main aim of the research performed on this yeast. Screening of mutants (Baeza et al., 2009), genetic engineering (Verdoes et al., 2003) and optimization of medium and growth conditions (Jacobson et al., 1999; Liu et al., 2006) represented the most common approaches chosen to increase the yield of astaxanthin from *X. dendrorhous*. Integrative plasmids and a transformation protocol have been developed for the study of the carotenoid pathway. The biosynthetic pathway for the production of carotenoids in *X. dendrorhous* has been elucidated (Visser et al., 2003) and some of the genes have been transferred to *Saccharomyces cerevisiae* (Verwaal et al., 2007). The genes *crtE*, *crtI*, *crtYB*, *crtR* and *crtS* were identified, overexpressed and/or deleted in *X. dendrorhous*, yielding different phenotypes caused by the modified concentrations and ratios of the carotenoids in the yeast (Alcaino et al., 2008; Breitenbach et al., 2011; Ojima et al., 2006; Verdoes et al., 2003).

The production of carotenoids by *X. dendrorhous* implies the presence of an upstream mevalonate (MEV) pathway that can provide the precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), for the formation of terpenes (Muntendam et al., 2009). IPP and DMAPP constitute the building blocks for several different compounds, like volatile scented monoterpenes (Colby et al., 1993) sesquiterpenes with antimalarial or antibacterial properties (Hartmann et al., 1978; Wallaart et al., 2001), sterols for membrane fluidity (Landolfo et al., 2010) and diterpenes with antineoplastic characteristics (Ajikumar et al., 2010). While some of the terpenes are widely available in nature, e.g. limonene and menthol, some others are produced by the native host in low amounts or the extraction from the natural source is too inefficient. Thus, the need arises for alternative methods of production for such precious terpenoid drugs.

One of the potential strategies is genetic engineering of heterologous hosts for the production of molecules of interest or precursors that can be then easily chemically modified (Tsuruta et al., 2009; Wang et al., 2011; Williams et al., 2000). The metabolic engineering approach has been extensively applied, mostly to *Escherichia coli* and *S. cerevisiae*. While both models share several advantages, like the availability of the complete genome sequence and advanced genetic engineering tools, they may show a low natural terpene flux (Muntendam et al., 2009). A faster and less invasive solution might come from genetically engineering organisms that already have a higher level of terpene precursors, for example, *X. dendrorhous*.

We decided to use the pentalenene synthase (*PSS*) gene in our new expression vector as a proof that *X. dendrorhous* can

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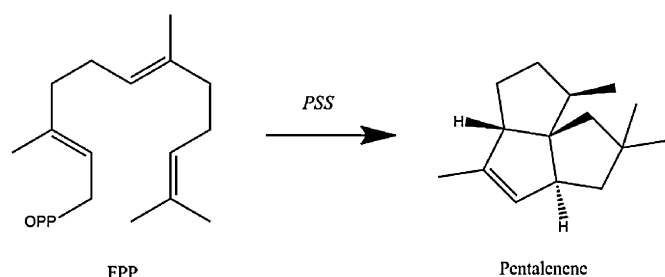


Fig. 1. Conversion of FPP towards pentalenene catalyzed by pentalenene synthase gene, *PSS*.

functionally express a heterologous terpene cyclase. *PSS* is a prokaryotic sesquiterpene cyclase that catalyzes the formation of pentalenene using farnesyl diphosphate (FPP) as substrate (Cane et al., 1994) (Fig. 1). The gene is relatively short (~1 kb) and it was isolated from *Streptomyces UC5319*.

For the expression of the cyclase, two different approaches were chosen. In the first strategy, the gene was inserted in one of the two new vectors engineered using pPR2TN as a template (Verdoes et al., 1999) allowing the insertion of the expression cassette into the genes *crtE* or *crtYB*. As second approach, a modified pPRcDNA1*crtE* vector (Breitenbach et al., 2011) was used for the integration of the *PSS* gene in the rDNA sequence.

2. Materials and methods

2.1. Strains and culture conditions

E. coli DH5 α strain was used for the cloning steps; bacteria were grown in LB (1% trypton, 0.5% yeast extract, 1% NaCl) with 100 mg/ml ampicillin in a shaking incubator at 37 °C, 250 rpm.

The wild type strain *X. dendrorhous* CBS 6938 (ATCC 96594) was used as control and for all transformation procedures. YPD (1% yeast extract, 2% peptone and 2% glucose) medium with and without 40 μ g/ml geneticin (G-418 Sulphate, Gibco) was used for the yeast growth in a shaking incubator at 21 °C, 180 rpm. The ratio between medium and air space in the flasks was always kept at 1:10.

2.2. Engineering of recombination vectors and transformation of *X. dendrorhous*

pGEMT vector was used as a backbone for the engineering of all the vectors in this study. The cassette for the resistance against

geneticin was amplified from the plasmid pPR2TN (Verdoes et al., 1999).

The expression cassette was created by fusing the *GPD* promoter to the *GPD* terminator sequence from pPR2TN. The product presented a small multiple cloning site between the promoter and the terminator sequences with the unique restriction sites for *NheI*, *HpaI*, *AvaI* and *XhoI*, and was flanked by restriction sites for *SpeI* and *BamHI*. The complete cassette was ligated into pGEMT; the construct was then digested with *NdeI* and *SpeI* and ligated to the resistance cassette that had been cut with the same restriction enzymes, resulting in one complete vector, pMCS.

The genes used as recombination sequences for the vectors were amplified from genomic DNA extracted from *X. dendrorhous*. The *crtE* gene, flanked by the sequence recognized by *EcoRI*, was amplified and ligated into pGEMT (pCrtE). Part of the *crtYB* gene was amplified and a *BamHI* site was inserted in the middle of the fragment by overlapping PCR; the gene was then ligated into pGEMT (pCrtYB).

The *PSS* gene (GenBank ID: U05213.1) was a kind gift of Prof. Claudia Schmidt-Dannert from University of Minnesota. After amplification and ligation in pGEMT, the gene was excised with *NheI* and *XhoI* and ligated into the multiple cloning site of the pMCS, which had been previously digested with the same enzymes, resulting in the new construct pPSS. The plasmid was digested with *BamHI* and ligated in pCrtE and pCrtYB, yielding the two final constructs pCrtE-PSS and pCrtYB-PSS, respectively (Fig. 2a and b).

The pPRcDNA1*crtE* plasmid (Breitenbach et al., 2011) was a kind gift of Prof. Sandmann, Goethe Universität, Frankfurt. The *crtE* gene was excised from the plasmid using *EcoRI* and *SacI* and replaced with the restriction sites for *NheI* and *XhoI* that were then used to clone the *PSS* gene giving the new vector pPR-PSS. pPR-PSS was linearized with *SwaI* (Fig. 2c), purified and concentrated.

Transformation of *X. dendrorhous* was performed according to the protocol of Wery et al. (1997). After the transformations with pCrtE-PSS and pCrtYB-PSS, only white colonies were chosen and grown again on selective medium with geneticin.

2.3. GC-MS analysis of organic layer

Dodecane was added to 10 ml of cultures in the concentration of 5% and hexadecane had a final concentration of 37.7 μ g/ml of medium. Cultures taken at different time points were pelleted for 10 min at 3000 $\times$ g; the upper dodecane layer was isolated and diluted 1:10 in ethyl acetate. The diluted dodecane extract was analyzed in total ion scan using a ZB-1ms dimethylpolysiloxane column (Phenomenex 0.25 mm inner diameter, 0.25 μ m thickness,

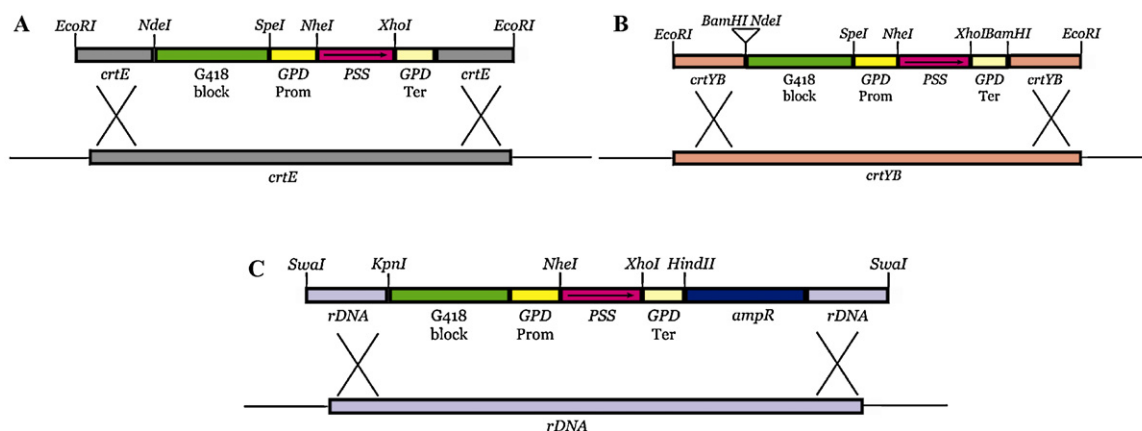


Fig. 2. Scheme of the *EcoRI* fragment of pCrtE-PSS (a) and pCrtYB-PSS (b) vectors and of the linearized pPR-PSS vector (c) used for the transformation of *X. dendrorhous*. The crosses indicate the double crossing over events with the genomic DNA that will have to occur in order to obtain the stable mutants.

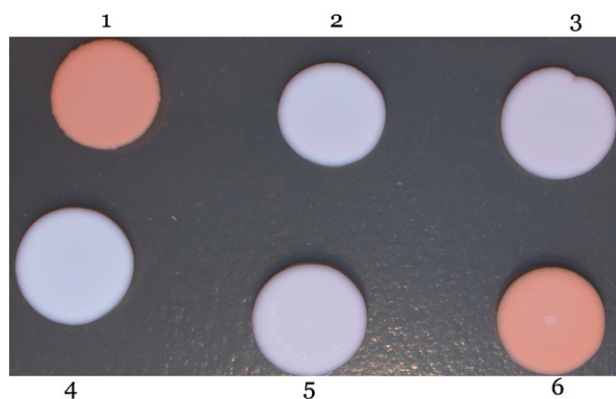


Fig. 3. Phenotype of wild type and mutant colonies. Strains: 1, WT; 2, $\Delta crtYB$; 3, $\Delta crtE$; 4, $\Delta crtYB$ -PSS; 5, $\Delta crtE$ -PSS; 6, pPR -PSS. YPD medium was used to resuspend colonies grown on plates; approximately 10 μ l of the solutions were spotted on a new YPD plate and let grow at 21 °C for 3 days.

15 m length) on a Shimadzu GCMS-QP5000. Two microliters of solution were injected splitless in the GC using helium as carrier gas. The injector temperature was 250 °C; the oven initial temperature was 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 solvent cut-off was of 10 min and 30 s due to high dodecane content.

3. Results

3.1. Mutants in the carotenoid pathway

The transformations of *X. dendrorhous* resulted in two different mutants in the terpenoid pathway. The geranyl-geranyl diphosphate synthase (GGPPS) gene *crtE* was the target for the creation of the first mutant, $\Delta crtE$, while the second mutated strain ($\Delta crtYB$) was characterized by the insertion of the resistance cassette in the gene *crtYB*, coding for the protein acting both as phytoene synthase and as lycopene cyclase. Both $\Delta crtE$ and $\Delta crtYB$ showed a white colony phenotype in contrast to the pink color of the wild type, as can be seen in Fig. 3. This clear lack of carotenoids, in addition to the ability to grow on selective medium, was a strong proof that the integration of foreign DNA had occurred. The presence of the resistance cassette in the genomic DNA was confirmed by PCR (data not shown).

In order to establish whether the lack of carotenoids in the mutants was affecting the growth of the yeast, a time course experiment was performed (Fig. 4). No significant changes in the growth rate could be detected.

3.2. *X. dendrorhous* strains transformed with PSS gene

The vectors for the expression of the pentalenene synthase (PSS) gene in *X. dendrorhous* were engineered starting from the constructs used to create the mutants $\Delta crtE$ and $\Delta crtYB$. The new expression cassette was introduced in the vector, flanking the antibiotic resistance region (Fig. 2a and b). To be able to transform *X. dendrorhous*, both $pCrtE$ -PSS and $pCrtYB$ -PSS were digested with *EcoRI* and the fragment was purified and concentrated. Two mutants lacking carotenoids (white) but containing the PSS gene were isolated after transformation of the wild-type strain and were named $\Delta crtE$ -PSS and $\Delta crtYB$ -PSS. The new strains showed the same color phenotype (Fig. 3) and the same growth curves (Fig. 4) as the respective mutants without the PSS gene.

In order to evaluate the effect of the production of carotenoids on the pentalenene concentration, we constructed the vector pPR -PSS (Fig. 2c) and used it for the transformation of the wild type

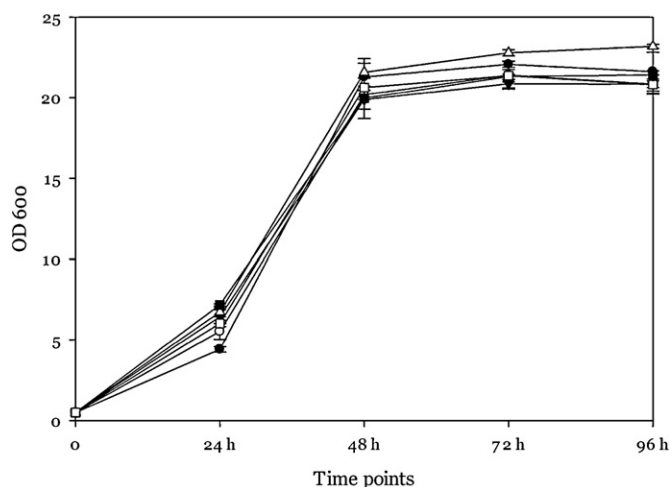


Fig. 4. Growth curves of wild type (closed circles), $\Delta crtYB$ (open circles), $\Delta crtE$ (closed triangles), $\Delta crtYB$ -PSS (open triangles), $\Delta crtE$ -PSS (closed squares) and pPR -PSS (open squares) strains grown in YPD medium, with geneticin where necessary. Overnight cultures were diluted in 10 ml of medium to an OD of 0.05 for each culture and each time point. Experiments were performed in duplo.

X. dendrorhous resulting in PSS expression without interference with the carotenoid pathway. As shown in Fig. 3, this mutant (pPR -PSS) exhibits similar pigmentation as the wild type strain, and the growth curve is comparable to the one obtained with the non-transformed yeast (Fig. 4).

3.3. Production of pentalenene in $\Delta crtE$ -PSS, $\Delta crtYB$ -PSS and pPR -PSS

The dodecane layer, expected to harbor the pentalenene, was isolated from the cultures after 24, 48, 72 and 96 h cultivation. The chromatograms of the diluted dodecane from $\Delta crtE$ -PSS, $\Delta crtYB$ -PSS and pPR -PSS showed a peak at about 11.5 min that was absent in the wild type strain, $\Delta crtE$ and $\Delta crtYB$ (Fig. 5). The fragmentation pattern of the peak (Fig. 6) matched the pentalenene pattern as reported in the literature (Fig. 7, adapted from Agger et al. (2009)) and presented two characteristic peaks for sesquiterpenes (m/z 204 and m/z 189) in the percentage of 68% and 47.6%, respectively, when considering the base peak (m/z 105.1) as 100%. Other major peaks could be found at m/z 175.1 (13.1%), 161.2 (41.1%), 147.2 (73.7%), 119.2 (57.2%) and 91.1 (91.3%). In order to be able to compare the production among the different strains, hexadecane was added at known concentration to the cultures as internal standard together with the dodecane. The concentration of pentalenene in the cultures was increasing with the optical density of the cultures as shown in Fig. 8, which is in concert with the constitutive regulation reported for the *GPD* promoter we have here used.

4. Discussion

X. dendrorhous has been the target of studies focusing on the biosynthesis and production optimization of the dye astaxanthin. Experiments involving genetic engineering of this yeast have converged toward the identification of the genes involved in the carotenoid pathway and the overexpression or deletion of those genes to analyze the differences in carotenoids production. In spite of the presence of tools for the transformation of the yeast, no effort has been put into expressing heterologous genes in *X. dendrorhous*, except for the sequences conferring resistance against geneticin and hygromycin (Alcaino et al., 2008; Wery et al., 1997).

Here we report for the first time the production of a heterologous terpene cyclase in *X. dendrorhous*. We designed and engineered

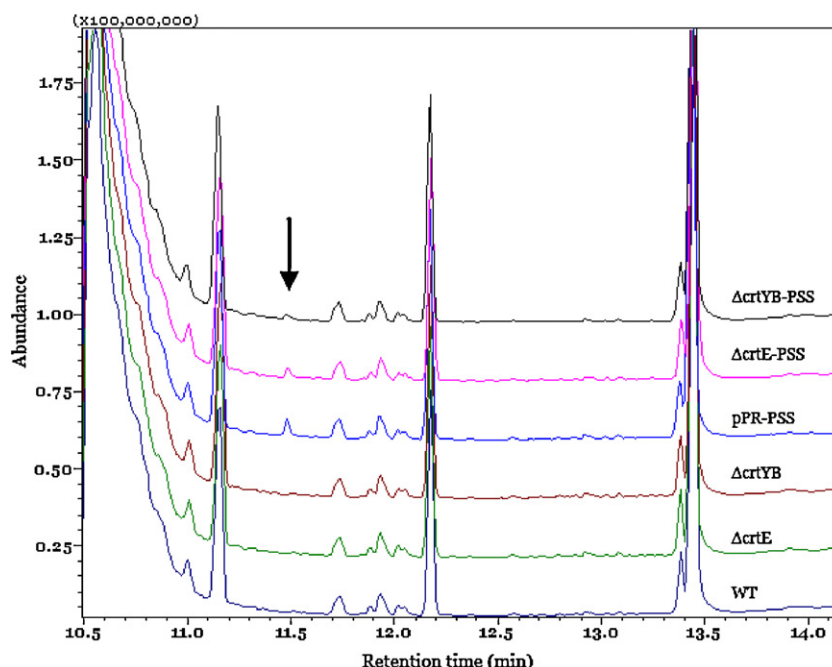


Fig. 5. Production of pentalenene in $\Delta crtE$ -PSS, $\Delta crtYB$ -PSS and pPR -PSS. The pentalenene peak (indicated by the arrow) was found at 11.5 min from injection time. The peak at about 13.5 min is the hexadecane used for standardization of the analysis. The other peaks are impurities coming from the dodecane added to the cultures.

two new vectors for the functional expression of the heterologous *PSS* and the concurrent knocking out of one of the host's genes. The mutants obtained after selection on antibiotic showed a clear change in phenotype, which is due to the insertional inactivation of *crtE* or *crtYB*. The strains switched from the wild-type orange pigmentation to a completely white color, indicating a complete lack of astaxanthin and of any other coloring carotenoid. Carotenoids, like astaxanthin, are known to be produced by the yeast in response to oxygen reactive species produced under high oxygen conditions or during cell aging (Schroeder and Johnson, 1995); we demonstrated that the absence of carotenoids did not seem to affect the growth of the mutants since they followed the same growth curve and reached the same final OD as the wild type strain (Fig. 4).

The production of pentalenene could easily be revealed by culturing the yeast in presence of dodecane, which trapped the volatile compounds produced by the yeast. Detection by GC-MS did not

show other terpenoids as by products, therefore we assume that pentalenene was the only biosynthesized terpenoid produced by the yeast that could be captured in the dodecane layer or at least detected.

The yield of pentalenene in the mutant strains ranged from 0.25 to 0.68 mg/L when compared with hexadecane as internal standard. The *pPR*-PSS strain showed a higher production compared to the other two mutant strains. The reason for this difference in production may be the integration of multiple copies of the gene in the genomic rDNA sequences. To support this theory, preliminary results in our lab show that low production of pentalenene in integration strains is probably caused by a low gene expression. When *pPR*-PSS mutants were selected on higher concentrations of geneticin only colonies with a higher copy number of the genes could grow, and a screening of those colonies showed that the production of the sesquiterpene could reach 4.8 mg/L, which corresponded to 0.37 mg of pentalenene per gram of dried yeast.

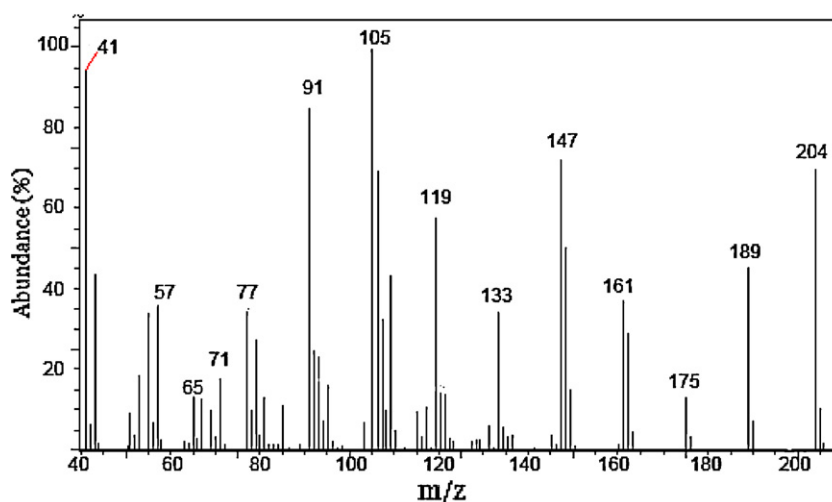


Fig. 6. Fragmentation pattern obtained for the peak corresponding to pentalenene at 11.5 min.

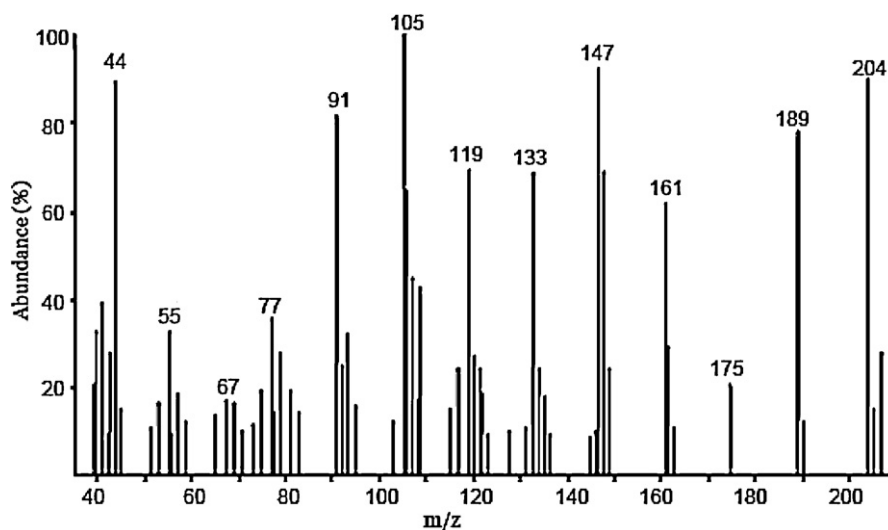


Fig. 7. Published pentalenene fragmentation pattern (modified from Agger et al., 2009).

The expression of a terpene cyclase in *X. dendrorhous* opens a new path toward the utilization of this yeast as a potential platform organism for the production of terpene based drugs. The possibility to knock out the carotenoid pathway and, thus, to channel the upstream precursors towards the production of new metabolites provides an additional advantage over the organisms that have been exploited until now for the terpene production. Breitenbach and colleagues have calculated that the wild type strain of *X. dendrorhous* grown in a poor medium can produce an average of 694 μg of total carotenoids per gram of yeast dry weight (Breitenbach et al., 2011). Furthermore, concentrations of astaxanthin of over 6.6 mg per gram of dry yeast weight could be obtained growing *X. dendrorhous* with different media, light and oxygen conditions (Jacobson et al., 1999).

These results confirm that the production of foreign terpenes in *X. dendrorhous* is not limited by the concentration of precursors, but rather by the gene expression driven by a weak regulating sequence. With a stronger promoter and with a codon optimized gene, the pentalenene production could reach even higher yields.

The maximum concentration of pentalenene we obtained in *X. dendrorhous* is still 20 times less than the maximum concentration

of astaxanthin isolated from the wild-type strain, showing that the potential of this yeast is still far from being completely exploited.

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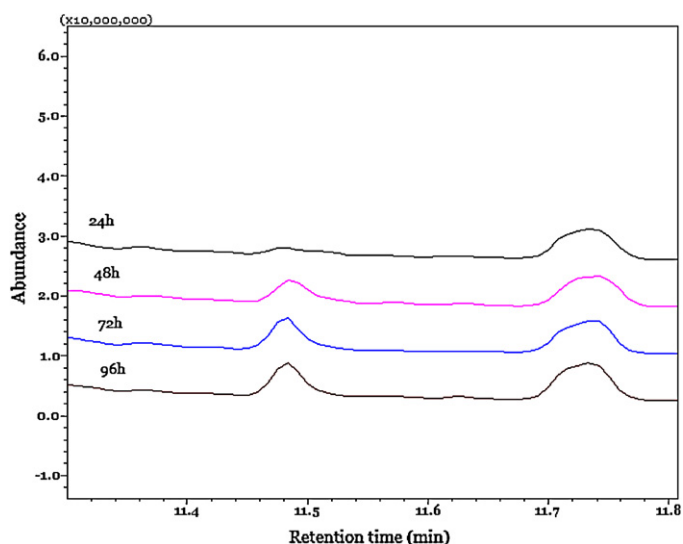


Fig. 8. Pentalenene production by pPR-PSS after 24, 48, 72 and 96 h from inoculation point. The retention time of the pentalenene is 11.48 min.

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