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Production of β-ionone by combined expression of carotenogenic and plant CCD1 genes in Saccharomyces cerevisiae

Javiera López1, Karen Essus1, Il-kwon Kim2,3, Rui Pereira2,4, Jan Herzog1, Verena Siewers2,5, Jens Nielsen2,5,6 and Eduardo Agosin1*

Abstract

Background: Apocarotenoids, like the C13-norisoprenoids, are natural compounds that contribute to the flavor and/or aroma of flowers and foods. They are produced in aromatic plants—like raspberries and roses—by the enzymatic cleavage of carotenes. Due to their pleasant aroma and flavour, apocarotenoids have high commercial value for the cosmetic and food industry, but currently their production is mainly assured by chemical synthesis. In the present study, a Saccharomyces cerevisiae strain that synthesizes the apocarotenoid β-ionone was constructed by combining integrative vectors and high copy number episomal vectors, in an engineered strain that accumulates FPP.

Results: Integration of an extra copy of the geranylgeranyl diphosphate synthase gene (BTS1), together with the carotenogenic genes crtYB and crtI from the ascomycete Xanthophyllomyces dendrorhous, resulted in carotenoid producing cells. The additional integration of the carotenoid cleavage dioxygenase gene from the plant Petunia hybrida (PhCCD1) let to the production of low amounts of β-ionone (0.073 ± 0.01 mg/g DCW) and changed the color of the strain from orange to yellow. The expression of the crtYB gene from a high copy number plasmid in this former strain increased β-ionone concentration fivefold (0.34 ± 0.06 mg/g DCW). Additionally, the episomal expression of crtYB together with the PhCCD1 gene in the same vector resulted in a final 8.5-fold increase of β-ionone concentration (0.63 ± 0.02 mg/g DCW). Batch fermentations with this strain resulted in a final specific concentration of 1 mg/g DCW at 50 h, which represents a 15-fold increase.

Conclusions: An efficient β-ionone producing yeast platform was constructed by combining integrative and episomal constructs. By combined expression of the genes BTS1, the carotenogenic crtYB, crtI genes and the plant PhCCD1 gene—the highest β-ionone concentration reported to date by a cell factory was achieved. This microbial cell factory represents a starting point for flavor production by a sustainable and efficient process that could replace current methods.

Keywords: Metabolic engineering, Carotenoids, Apocarotenoids, Saccharomyces cerevisiae

Background

Terpenoids or isoprenoids are the largest and most diverse group of natural compounds found in nature [1]. Their biochemical role in cells is diverse ranging from cell membrane components through functions in subcellular targeting and regulation to plant defense, communication, and pigmentation [2, 3]. Terpenoids have attractive commercial applications as biofuels, antiseptics, flavour and fragrances, and medical agents, among others, which has recently raised the interest for their commercial production [4–6]. In plants and yeast, isoprenoids can be produced through the mevalonate pathway, which condenses acetyl-CoA to produce the universal isoprene building unit (C5), isopentenyl diphosphate (IPP) [1]. Successive condensations of IPP, and its isomer dimethylallyldiphosphate (DMAPP),
result in isoprenoid precursors of different length: geranyl diphosphate (GPP) for monoterpenes (C10), farnesyl diphosphate (FPP) for sesquiterpenes (C15), geranylgeranyl diphosphate (GGPP) for diterpenes (C20), 2 units of FPP for triterpenes (C30) and 2 units of GGPP for tetraterpenes (C40) [1, 7].

Apocarotenoids are a subclass of isoprenoids, which are highly appreciated in the flavoring industry due to their characteristic aromatic notes [8]. In plants, these compounds are produced by the cleavage of carotenoids (C40) by the enzymatic action of CCDs (carotenoid cleavage dioxygenases), a family of oxidative enzymes that specifically cleaves double bonds [9]. Between the different apocarotenoids, β-ionone is a prominent scent and aromatic molecule present in many flowers and fruits, such as blackberries, peaches and apricots, among others [10]. In odorant terms, ionones (α and β) are associated with violet scent, but β-ionone has also a woody odor character. Despite their low concentration in plants (in the order of ng/kg fresh weight), these compounds have the potential to strongly impact the flower aroma due to their significantly low odor threshold (7 ppt in water), only comparable to the rose-like aroma molecule, β-damascenone [11]. In nature, β-ionone is obtained by specific cleavage of β-carotene [10, 12]. This reaction is catalyzed by the action of CCD1, which cleaves carotenoids at the 9,10 position and the 9',10' position in the presence of oxygen [13]. Currently, β-ionone is used in the food and cosmetic industry due to its pleasant aroma and its contribution to flavor, but it is also a key intermediate in the synthesis of vitamins A, E and K and therefore has an annual production of several hundreds of tonnes [14].

The extraction of aroma compounds from their natural source is an expensive and arduous task, strongly dependent on agriculture and all the factors surrounding it [8]. Biotechnology represents a very attractive alternative for the sustainable production of flavors and fragrances that can still be considered as “natural” [15]. With the increasing development of genetic engineering, it became possible to produce heterologous products in microbial cell factories that are normally found only in small amounts in nature [16].

Central to any genetic manipulation is the vector used to transform DNA into the host. Vectors that can be integrated into the host chromosome are widely used because of their mitotic stability without the need of selection pressure. A series of site-specific integrating vectors has recently been designed making possible to transfer up to 22 genes in *Saccharomyces cerevisiae* [17]. This plasmid collection ensures stable expression, as all genes are genomically integrated in sites separated by essential genes minimizing the risk of homologous recombination between multiply used promoters or terminators. Furthermore, marker recycling can eliminate all markers in the host strain. Episomal vectors on the other hand, like multiple-copy plasmids, are still broadly used to ensure high-level expression of exogenous or endogenous genes for protein production and synthetic pathway optimization [18]. This type of expression is also useful in evaluating possible bottleneck enzymes in a pathway or the role of a particular protein under certain conditions.

To date, co-expression of the plant CCD1 enzyme together with the *Xanthophyllomyces dendrorhous* carotenoid enzymes in *Escherichia coli* and *S. cerevisiae*, respectively, has led to the proof-of-principle biotechnological production of β-ionone. A β-carotene overproducing *E. coli* strain, together with the episomal expression of the CCD1 gene from *Petunia hybrida*, was used to demonstrate the activity of the CCD1 enzyme [10]. For β-ionone production by *S. cerevisiae*, a *crtYB/crtI/ctrE* polycistronic episomal construct with the three genes necessary for the synthesis of β-carotene from FPP was expressed together with the CCD1 gene from raspberry (Figure 1). However, the low translational efficiency of this system, limited β-ionone production to a final titer of 0.22 mg/L [19].

In the present study, an alternative *S. cerevisiae* platform was constructed to synthesize β-ionone by combining two genetic engineering approaches to increase protein expression: USER cloning-compatible integrative vectors and high copy number episomal expressions systems. By overexpressing a truncated version of the *HMG1* gene (*tHMG1*) and the endogenous GGPP synthase gene *BTS1*, together with the *crtYB* and the *crtI* genes from *Xanthophyllomyces dendrorhous* and the CCD1 gene from *P. hybrida* (*PhCCD1*) in an FPP overproducing strain (SCGIS22) [20], we generated a β-ionone producing microbial cell factory, reaching a maximal concentration of 0.63 ± 0.02 mg/g biomass in shake flask cultures. In 2 L batch bioreactors, a final concentration of 1 mg/g biomass was reached, equivalent to a 15-fold increase. This corresponds to a titer of more than 5 mg/L, far exceeding the earlier study, and representing a starting point for flavor production by a sustainable and efficient process that could also replace current methods [21, 22].

**Results**

**Integration of the *tHMG1* gene and the β-ionone pathway**

The C13-norisoprenoid β-ionone was synthesized in the FPP-overproducing *S. cerevisiae* strain SCGIS22. This strain combines several strategies to overproduce isoprenoid precursors. It overexpresses *ERG20* (responsible for the production of FPP from IPP and DMAPP) and since FPP is the precursor of many essential compounds in yeast, the *ERG9* gene (whose gene product synthesizes squalene from FPP) was downregulated. Additionally, the
López et al. Microb Cell Fact (2015) 14:84

LPP1 and DPP1 genes were deleted in order to minimize farnesol formation—one of the major alternative pathways from FPP.

In this strain we integrated the cassette pIRP01 carrying a truncated version of the HMG1 gene, called tHMG1, which encodes the mevalonate pathway enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) lacking the trans-membrane region [23]. This new strain was called SCIGS22a (Figure 1).

In the SCIGS22a strain, the integration of the cassettes pIJL01 (BTS1 and crtYB genes) and pIJL02 (crtI gene) resulted in orange cells (strain JLS01) (Tables 1, 2), indicating the synthesis of the colored carotenoids lycopene and β-carotene. On the other hand, the integration of pIJL01 together with pIJL03 (crtI and PhCCD1 genes) resulted in yellow cells with a faint, pleasant violet flavor (strain JL02) (Figure 2). Transformants were grown on SC-URA plates without any color loss over time, indicating the genetic stability of the cells. When these strains were grown in a two-phase shake flask culture (with 10% dodecane), final biomass measurements were similar to the original engineered strain (SCIGS22a), indicating that carotenoids and β-ionone production, at least at these concentrations, did not affect cell growth (data not shown).

Carotenoid production reached 212 µg/g DCW and 103 µg/g DCW for strains JLS01 and JLS02, respectively. In strain JL02, almost all carotenoids were in the form of β-carotene (77%) and a small percentage in the form of lycopene (2%) (Table 3). Additionally, we confirmed β-ionone production (0.073 ± 0.01 mg/g DCW) in this strain by GC-FID analysis (Table 4). It is worthy to note than when β-ionone production was compared after growth at different temperatures, at 20 or 30°C, a 30% higher content was achieved at lower temperature [24].

Transformation with episomal vectors
Accumulation of carotenogenic intermediates (lycopene and β-carotene), concomitant with limited production of β-ionone when one copy of each gene was integrated,
suggested that there might be a limitation in channeling the different intermediates into the targeted flow direction [25]. To overcome possible bottlenecks in the pathway, a series of episomal vectors was constructed to generate new transformants, capable of increasing β-ionone production.

The transformation of strain JLS02 with episomally expressed \( \text{crtYB} \), \( \text{crtI} \) or \( \text{PhCCD1} \) genes generated the strains JLS03, JLS04 and JLS05, respectively (Table 2).

Strain JLS03 (with episomal \( \text{crtYB} \)) resulted in yellow cells with a strong violet aroma. This strain reached optical densities similar to those reached by the strain JLS02 after 48 h of cultivation (3.3 OD\(_{600}\) vs 3.2 OD\(_{600}\), respectively) (Table 4), suggesting that the introduction of this episomal vector did not affect cell growth nor resulted in any metabolic burden by vector replication. After 48 h of fermentation, most of the carotenoids were in the form of β-carotene (83.7% of total carotenoids) (Table 3) and the β-ionone specific concentration reached 0.34 ± 0.06 mg/g DCW (Table 4), almost five times higher than for strain JLS02 (Figure 3).

Transformation with the \( \text{crtI} \) (JLS04) or \( \text{PhCCD1} \) (JLS05) carrying plasmids resulted in strains with lighter color and with a lower final biomass (Figure 2; Table 4). Carotenoid and β-ionone quantification was not always possible, given the low biomass concentration reached after 48 h of incubation; these strains were therefore not considered in further analyses.

Two co-expression vectors with the genes \( \text{crtYB} \) and \( \text{crtI} \) (pEJL07) and \( \text{crtYB} \) and \( \text{PhCCD1} \) (pEJL08) under control of identical promoters and terminators were then constructed. The transformation with these vectors resulted in strains JLS06 and JLS07, respectively.

Both strains achieved higher β-carotene concentrations than JLS02. Nevertheless, no significant differences were observed when compared with JLS03. A different result was observed for β-ionone. Both JLS06 and JLS07 achieved higher β-ionone production when compared

| Table 1 List of \( \text{S. cerevisiae} \) strains used in this study |
|-----------------|----------------|------------------|------------------|
| Strain          | Genotype       | Plasmid          | References       |
| SCIGS22         | \( \text{MATa MAL2-8' SUC2 ura3-52 lpp1 \Delta : loxP dpp1 \Delta : loxP \text{P}_{\text{ERG22}}} \) | None             | [20]             |
| SCIGS22a        | SCIG22 + \( \text{P}_{\text{TEF1}} \cdot \text{tHMG1} \) | None             | This study       |
| JLS01           | SCIG22a + \( \text{P}_{\text{TEF1}} \cdot \text{BTS1} \cdot \text{P}_{\text{PhCCD1}} \cdot \text{crtYB} \cdot \text{P}_{\text{TEF1}} \cdot \text{crtI} \) | None             | This study       |
| JLS02           | SCIG22a + \( \text{P}_{\text{TEF1}} \cdot \text{BTS1} \cdot \text{P}_{\text{PhCCD1}} \cdot \text{crtYB} \cdot \text{P}_{\text{TEF1}} \cdot \text{crtI} \) | JLS02            | This study       |
| JLS03           | JLS02          | P426 \( \text{P}_{\text{GPD}} \cdot \text{crtYB} \)         | This study       |
| JLS04           | JLS02          | P426 \( \text{P}_{\text{GPD}} \cdot \text{crtI} \)         | This study       |
| JLS05           | JLS02          | P426 \( \text{P}_{\text{GPD}} \cdot \text{PhCCD1} \)       | This study       |
| JLS06           | JLS02          | P426 \( \text{P}_{\text{GPD}} \cdot \text{crtYB} \cdot \text{P}_{\text{GPD}} \cdot \text{crtI} \) | This study       |
| JLS07           | JLS02          | P426 \( \text{P}_{\text{GPD}} \cdot \text{crtYB} \cdot \text{P}_{\text{GPD}} \cdot \text{PhCCD1} \) | This study       |

| Table 2 Plasmid used in this study |
|-----------------|-------------------------------|------------------|
| Plasmid name    | Plasmid description           | Reference        |
| pSP-GM2         | \( \text{URA3} \)-based expression plasmid carrying a bidirectional \( \text{P}_{\text{TEF1}} \cdot \text{P}_{\text{PGK1}} \) promoter |                  |
| pXI-5           | \( \text{KlURA3} \)-based integration plasmid carrying regions for homologous recombination |                  |
| pXI-3           | \( \text{KlURA3} \)-based integration plasmid carrying regions for homologous recombination |                  |
| pX-2            | \( \text{KlURA3} \)-based integration plasmid carrying regions for homologous recombination |                  |
| P426 GPD        | \( \text{URA3} \)-based expression plasmid carrying a \( \text{P}_{\text{GPD}} \) promoter |                  |
| pIRP01          | \( \text{P}_{\text{TEF1}} \cdot \text{tHMG1} \) | This study       |
| pJIL01          | \( \text{P}_{\text{TEF1}} \cdot \text{BTS1} \cdot \text{P}_{\text{PhCCD1}} \cdot \text{crtYB} \) | This study       |
| pJIL02          | \( \text{P}_{\text{TEF1}} \cdot \text{crtI} \) | This study       |
| pJIL03          | \( \text{P}_{\text{TEF1}} \cdot \text{crtI} \cdot \text{P}_{\text{PhCCD1}} \cdot \text{crtI} \) | This study       |
| pEJL04          | \( \text{P}_{\text{GPD}} \cdot \text{crtYB} \) | This study       |
| pEJL05          | \( \text{P}_{\text{GPD}} \cdot \text{crtI} \) | This study       |
| pEJL06          | \( \text{P}_{\text{GPD}} \cdot \text{PhCCD1} \) | This study       |
| pEJL07          | \( \text{P}_{\text{GPD}} \cdot \text{crtYB} \cdot \text{P}_{\text{GPD}} \cdot \text{crtI} \) | This study       |
| pEJL08          | \( \text{P}_{\text{GPD}} \cdot \text{crtYB} \cdot \text{P}_{\text{GPD}} \cdot \text{PhCCD1} \) | This study       |
higher expression of the \textit{crtYB} gene than strain JLS02, with an increment of almost 18-fold. For JLS04 and JLS05, increased expression of \textit{crtI} and \textit{PhCCD1} genes were detected, but for \textit{PhCCD1} the increment in expression was lower compared to \textit{crtYB} and \textit{crtI} expression in strains JLS03 and JLS04, respectively (only a 3-fold increase compared to 18-fold for \textit{crtYB} and 7-fold for \textit{crtI}). Finally, for the high copy number vectors with double genes, both strains JLS06 and JLS07 resulted in clearly increased expression of \textit{crtYB}, surprisingly almost no expression increase of \textit{crtI} (JLS06) and a slight increase in expression of \textit{PhCCD1} (JLS07) (Figure 5).

\textbf{Discussion}

A \beta-ionone producing yeast platform was constructed by expressing the \textit{tHMG1} and \textit{BTS1} genes, the carotenogenic genes \textit{crtYB} and \textit{crtI} from \textit{X. dendrorhous}, to JLS02 that additionally expressed the \textit{P. hybrida} gene \textit{PhCCD1}. No significant differences in biomass concentration were found between strains JLS-02, -03, -06 and -07 (Figure 3; Table 4). Finally, production kinetics of JLS02 and JLS07 strains up to 72 h indicated that the maximal \beta-ionone concentration was reached after 48 h of cultivation in shake flask (Figure 4).

\textbf{\beta-ionone production in 2 L bioreactor}

Figure 6 shows that growth dynamics of strain JLS07 in batch mode in a 2 L aerated bioreactor consisted of an initial glucose-consuming growth, followed by ethanol consumption. Ethanol was produced due to the Crabtree effect in \textit{S. cerevisiae}, and later consumed. For the glucose-consuming phase, the specific growth rate reached a $\mu_{\text{max}} = 0.106$/h; and for the ethanol consuming phase, $\mu_{\text{max}} = 0.05$/h.

At stationary phase (after 51 h of cultivation) an $\text{OD}_{600} = 10.6$ was reached; neither glucose nor ethanol were found in the culture. 609.6 µg/g of carotenoids were determined intracellularly. The extracellular \beta-ionone concentration reached 5 mg/L.

\textbf{Influence of the expression system on \beta-ionone production}

The large number of genes required for the heterologous synthesis of \beta-ionone complicates a gene expression strategy exclusively based on plasmids, given the number of genetic markers needed for selection or the large plasmid size if expressed from a single vector. For
the integration of the target genes, two vectors from a USER cloning compatible plasmid collection were used. The advantage of using this collection is that the recombination sites are strategically positioned in the *S. cerevisiae* genome (between essential genes), making the engineered strain stable over time, with minimal risks of gene loss by recombination [17]. In our study, no white colonies were observed when we integrated these genes, strongly indicating the high efficiency of this technique.

Furthermore, the color was stable over generations, suggesting that the recombinant strains are genetically stable.

Nevertheless, engineering a metabolic pathway by expressing heterologous enzymes normally suffers from flux imbalance, as they typically lack the regulatory mechanisms present in the native metabolism [23]. For determining—and alleviating—possible bottlenecks of the pathway, we additionally expressed selected pathway genes from derivatives of the native multi-copy 2 μm plasmid in the original JLS02 recombinant strain, reaching significantly higher β-ionone concentrations compared to only integrative expression systems. Additionally, we observed coloration of the colonies when *crtYB* (alone or with *PhCCD1*) was expressed from an episomal vector (JLS03 or JLS07 vs. JLS02). We confirmed an increase in total carotenoids—mostly β-carotene—by HPLC analysis.

We can further improve our platform, at least in terms of expression, by integrating a higher number of copies of the target genes. Verwaal et al. [26] reported a fivefold increase in carotenoids in *S. cerevisiae* using the integrative expression of the *crtYB, crtI* and *BTS1* genes as compared to episomal expression. A similar result was

Table 3  Carotenoid biosynthesis by different strains after 48 h cultivation

| Carotenoids       | Carotenoid concentration (µg/g DCW) (% distribution) |
|-------------------|------------------------------------------------------|
|                   | JLS02 | JLS03 | JLS04 | JLS05 | JLS06 | JLS07 |
| Lycopene          | 1.6 (2) | – | – | – | – | – |
| β-carotene        | 62.53 (77.2) | 181.7 (83.4) | 7.37 | 13.7 | 140 (86.1) | 164.7 (86.1) |
| Torulene          | 7.77 (9.6) | 19.6 (9) | – | – | – | 8.78 (5.4) |
| Other carotenes   | 8.91 (11) | 16.55 (7.6) | – | – | – | 12.05 (7.4) |
| Total             | 81 ± 8.97 | 217.9 ± 4.42 | 7.37 ± 12.73 | 13.7 ± 13.86 | 162.6 ± 24.03 | 191.30 ± 44.25 |

Values represent the mean of three independent cultures after 48 h of cultivation.
– not detected.

Table 4  β-ionone production by different strains after 48 h cultivation

| strain | OD at 600 nm | β-ionone (ppm) | β-ionone (mg/g DCW) | Increase fold* |
|--------|--------------|----------------|---------------------|----------------|
| JLS02  | 3.33 ± 0.14  | 0.14 ± 0.02    | 0.073 ± 0.01        | –              |
| JLS03  | 3.22 ± 0.14  | 0.63 ± 0.13    | 0.34 ± 0.06         | 4.7            |
| JLS04  | 1.43 ± 0.07  | 0.0            | 0.0                 | –              |
| JLS05  | 1.36 ± 0.08  | 0.03 ± 0.03    | 0.04 ± 0.04         | –              |
| JLS06  | 2.76 ± 0.13  | 0.74 ± 0.06    | 0.49 ± 0.04         | 6.84           |
| JLS07  | 3.06 ± 0.3   | 0.96 ± 0.08    | 0.62 ± 0.05         | 8.5            |

Values represent the mean ± SD of five independent cultures after 48 h of cultivation.
* Increase fold with respect to strain JLS02.
obtained in *E. coli* where the production of β-carotene was higher using low-copy plasmids as compared to high-copy plasmids [27]. The major problem with the use of high copy number vectors is associated with the resulting high metabolic burden. To avoid unnecessary enzyme synthesis, the identification of bottlenecks and enzyme efficiency is a key aspect to express genes accordingly. Considering that the plasmid collection used has 11 integrative vectors, with a total capacity to integrate 22 genes in different yeast chromosomes, similar expression levels to those reached by episomal vectors could in principle be obtained, with the additional benefits of stability, and no necessity for selection pressure. Nonetheless, considering that the *S. cerevisiae* SCIGS22 strain has only uracil auxotrophy, each transformation required the removal of the previously used *URA3* marker gene. Given that the 5-FOA elimination method is laborious and time consuming, successive integration—and marker recycling—resulted in an arduous and cumbersome transformation.

**Figure 4** Kinetics of β-ionone production and cell growth of the two β-ionone producing strains JLS02 and JLS07 up to 72 h cultivation. β-ionone production (dashed line) and biomass (continuous line) dynamics of JLS02 (red) and JLS07 strains (blue) for 72 h shake-flask cultures with a second phase of dodecane. Values represent the mean of three independently grown cultures.

**Figure 5** qPCR analysis of the carotenogenic *crtYB* and *crtI* genes, and the plant *PhCCD1* gene in the different β-ionone producing strains. Three cultures of each strain were inoculated in SC-URA medium with 2% glucose (w/v) and 10% dodecane (v/v), at the same optical density. 48 h later, samples were processed for cDNA synthesis. qPCR experiments were performed to determine the abundance of *crtYB*, *crtI* and *PhCCD1* in each strain relative to *TEF1*, which was used as internal control. Results from strains JLS03–JLS07 were compared to JLS02 as a calibrator sample. The data represent the average and the standard deviation of three independently grown cultures.

**Figure 6** Batch culture dynamics of JLS07 strain. The strain was fermented in a two-phase culture at 20°C for 68 h. Samples were collected every 4 h for kinetics parameters and metabolite measurements.
process. The development of a new microbial cell factory containing several auxotrophies as selection markers will allow constructing strains overproducing terpenoids easier and faster. Alternatively, the application of targeted genome editing using engineering nucleases, like the clustered, regularly interspaced, short palindromic repeats (CRISPR) technology, can be used to insert a desired sequence through recombination of exogenous DNA with a specific locus, without marker selection [28].

Influence of gene expression levels

Four terpenoid pathway genes were expressed in \textit{S. cerevisiae} in order to achieve \(\beta\)-ionone biosynthesis. We first expressed an extra copy of the endogenous \textit{BTS1} gene under the strong promoter \textit{TEF1}. The \textit{BTS1} gene is normally expressed at low levels in wild type cells [26], because the \textit{P<sub>TS1</sub>} promoter is one of the weakest constitutive promoters related to terpenoid biosynthesis [29]. Then, we expressed the \textit{crtYB} and \textit{crtI} genes from the ascomycete \textit{X. dendrorhous} to produce \(\beta\)-carotene. Both genes encode bifunctional enzymes, commonly employed for \(\beta\)-carotene and astaxanthin production in microorganisms [30]. Finally, the \textit{CCD1} (\textit{PhCCD1}) gene from \textit{P. hybrida} was expressed to cleave \(\beta\)-carotene to \(\beta\)-ionone, since the respective enzyme has low catalytic efficiency. The low growth of the \textit{JLS05} strain may indicate the accumulation of inhibitory compounds or the use of other substrates, e.g. lipids, even though the \textit{PhCCD1} expression was not dramatically increased, as compared with the \textit{JLS02} strain (Figure 5). In carotenoid accumulating microorganisms, the maize \textit{CCD1} enzyme does not exclusively cleave the 9,10 and 9’,10’ double bonds of carotenoids, but also the 5,6 and 5’,6’ bonds [31], indicating that \textit{CCD1} might not exclusively produce \(\beta\)-ionone. Given that the \textit{PhCCD1} over-expressing strain showed low growth—and neither carotene nor volatiles were detected—it was not possible to identify the accumulation of toxic or other intermediate compounds. Nevertheless, strain \textit{JLS07} (\textit{PhCCD1-crtYB}) reached optical densities similar to strain \textit{JLS02}, indicating that the overexpression of \textit{PhCCD1} together with \textit{crtYB} did not produce the same effect in the cells.

Expression of \textit{CCD1} from different organisms or the use of other enzymes—like \textit{CCD4} that can also produce \(\beta\)-ionone from \(\beta\)-carotene—may further increase \(\beta\)-ionone production in yeast. Other possible strategies include the construction of fusion proteins—to reduce the access of enzymes to other substrates—or the use of an inducible promoter, in order to favoring cell growth and \(\beta\)-carotene accumulation at beginning of the fermentation before inducing \(\beta\)-ionone production.

\(\beta\)-Ionone titer

Beekwilder et al. [19] recently reported the production of \(\beta\)-ionone in \textit{S. cerevisiae} by heterologous expression of a polycistronic construct, an alternative way to synchronously express a heterologous multigene pathway in \textit{S. cerevisiae}. The maximum concentration reached with this strategy was 0.22 mg/L. The \textit{S. cerevisiae} \textit{JLS07} strain developed here increased the \(\beta\)-ionone production sevenfold, achieving a maximal titer of 1.5 mg/L after 72 h of cultivation in flask cultures, and a 23-fold increase if we consider the 5 mg/L achieved after 50 h in 1.5 L bioreactors.

Even though the constructed strains overproduce the precursor for the \(\beta\)-ionone pathway, low optical densities were reached, probably due to the high-level expression of several genes. However, the product yield (\(Y_{\text{sp}}\)) reached
for strain JLS07 in flask cultures was five times higher than the \( Y_w \) obtained by the polycistronic system.

Batch fermentation in bioreactors differed from flask cultures. The OD\(_{600}\) reached a threefold increase and the \( \beta \)-ionone concentration was eightfold higher after 50 h of cultivation compared to flask cultures. The higher \( \beta \)-ionone production during the stationary phase can result from the accumulation of this compound in the dodecane layer. Moreover, the lower expression of the \( PhCCD1 \) gene, compared to the expression of \( crtYB \) in this strain, could be related with a slow cleavage of \( \beta \)-carotene (compared to its synthesis) since at 100 h of fermentation in this strain, could be related with a slow cleavage of \( \beta \)-carotene (compared to its synthesis) since at 100 h of cultivation compare to flask cultures. The higher expression of the \( HXT1 \) gene under the \( ERG9 \) promoter, favoring the accumulation of FPP and the carotenogenic pathway towards \( \beta \)-ionone synthesis. qPCR analysis of the expression of \( ERG9 \) and carotenogenic genes, together with metabolomics during the fermentation, might help to elucidate this hypothesis.

Conclusions
In this work, we constructed a yeast platform for \( \beta \)-ionone production by differential expression of the carotenogenic genes \( crtYB \) and \( crtI \) and the plant gene \( PhCCD1 \). The fine-tuning of multi-gene expression, as demonstrated in this study for \( \beta \)-ionone biosynthesis, can expand our yeast platform towards the synthesis of other isoprenoid-derived compounds.

Methods
Plasmid construction
The genes coding for CrtYB, CrtI and PhCCD1 proteins were synthesized by Genscript (Piscataway, NJ, USA) (Additional file 1). All the sequences were codon optimized for expression in \( S.\ cerevisiae \). The catalytic domain of the HMG-CoA reductase gene (\( thMG1 \)) together with the \( TEF1 \) promoter was PCR amplified using genomic DNA from strain SCIGS23 [20] as template and the \( BTS1 \) gene was PCR amplified from genomic DNA of strain CEN.PK113-5D. Primers used for all amplifications are provided in Additional file 2.

We constructed four plasmids to integrate the \( thMG1 \) gene and the four genes needed for \( \beta \)-ionone production, into the yeast genome, using the USER cloning technique [32]. PCR amplification of the DNA fragments was carried out in 35 PCR cycles using the proofreading PfuTurbo Cx Hotstart polymerase (Agilent Technologies, Santa Clara, CA, USA) or PfuX7 [33], following the manufacturer’s instructions. The USER vector pXI-5 was amplified by PCR using primer pair 1/2, and the USER vectors pXI-3 and pX-2 were amplified by PCR using primer pair 3/4 followed by digestion with the Nb.Bsml nicking endonuclease for 1 h. The catalytic domain of the HMG-CoA reductase gene (\( thMG1 \)) [GenBank NM_001182434] together with the \( TEF1 \) promoter was amplified using primer pair 5/6. The \( BTS1 \) gene [GenBank NM_001183883] was amplified using primer pair 7/8. The genes \( crtYB \) [protein ID AAO53257], \( crtI \) [protein ID AAO47570] and \( PhCCD1 \) [protein ID AAT68189] were amplified using primers pair 9/10, 11/12 and 13/14, respectively. The bidirectional promoters (\( TEF1/PGK1 \)) used for the expression of the genes were amplified from the plasmid pSP-GM2 as a template using the primers 15 and 16. All PCR products were treated with DpnI enzyme to eliminate original vector residues. Purified digested vector (100 ng) was mixed at a molar ratio of (1:1) with purified PCR products amplified depending on their length. The DNA fragments were mixed with 1 µL of 10 × TE buffer (100 mM Tris–HCl, 1 mM EDTA; pH 8.0), 1 U of USER enzyme mix (New England BioLabs) and Milli-Q purified water until 10 µL. The mixture was incubated for 20 min at 37°C, followed by 20 min at 25°C. Finally, the reaction mix was used to transform chemically competent \( E. coli \) cells. The resulting plasmids were designated pIRP01 (with \( thMG1 \) under the \( TEF1 \) promoter), pJL01 (with \( BTS1 \) under the \( TEF1 \) promoter and \( crtYB \) under the \( PGK1 \) promoter), pJL02 (with \( crtI \) under the \( TEF1 \) promoter) and pJL03 (with \( crtI \) under the \( TEF1 \) promoter and \( PhCCD1 \) under the \( PGK1 \) promoter) Since the strain used is auxotroph for uracil (\( ura3 \)), all vectors contained the \( Klyuyveromyces\ \text{leucis} (K\text{I})\ URA3 \) gene flanked by direct repeats (in order to be able to recycle this marker for future transformations).

We also constructed a series of plasmids for episomal expression using the Gibson assembly technique.

All PCRs to obtain DNA fragments suitable for Gibson assembly were carried out in 35 PCR cycles using Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Gibson assembly was performed as previously described [34] with pairs of primers for each fragment to be assembled containing segments of about ~40 bp homologous to the adjacent fragment to be linked. The episomal yeast expression vector p426GPD (Addgene, Cambridge, MA, USA) was amplified with the primer pairs 17/18, 19/20 or 21/22 depending on the gene cloned. CrtYB, \( crtI \) and \( PhCCD1 \) were amplified using primers 23/24, 25/26 and 27/28, respectively. All PCR products were treated with DpnI enzyme to eliminate original vector residues and purified by gel extraction using the Qiaquick Gel Extraction kit from Qiagen according to the manufacturer's instructions. The purified genes fragments and vectors were mixed based on their molar ratios in a final volume of 5 µL containing...
100 ng of total DNA. This DNA mix was added to 15 μL of 1.33X master mix (5X isothermal mix buffer, T5 exonuclease 1U/μL, Phusion DNA polymerase 2U/μL, Taq DNA ligase 40 U/μL and Milli-Q purified water) and the reaction mixture was incubated at 50°C for 1 h. Finally, 10 μL reaction mix were used directly to transform chemically competent E. coli cells. The resulting plasmids were designated pEJL04, pEJL05 and pEJL06, containing crtYB, crtI and PhCCD1 genes, respectively.

For the construction of two gene-containing plasmids—pEJL07 and pEJL08—the pEJL04 plasmid was used as backbone and amplified using primers 29/30, including crtYb gene. The crtI and PhCCD1 genes were amplified from pEJL05 and pEJL06, respectively, including in each case the GPD promoter and CYC1 terminator using primer 31/32. Maps of all the plasmids constructed are in Additional file 3.

All plasmids were verified by sequencing (Macrogen Inc., Seoul, Korea).

Yeast strain construction

The S. cerevisiae strain SCIGS22 used in this work has a CEN.PK background with extra modifications in the genome for the overproduction of FPP [20].

All S. cerevisiae strains constructed from this strain are listed in Table 1.

Strain SCIGS22a carrying the truncated version of the HMG1 gene (tHMG1) encoding 3-hydroxy-3-methylglutaryl-CoA reductase lacking the trans-membrane region, was created from strain SCIGS22 by transformation with the cassette from plasmid pIRP01. The plasmid was digested with enzyme NotI (New England BioLabs, Ipswich, MA, USA), and the fragment purified and transformed into strain SCIGS22, finally called SCIGS22a. The transformation was performed using the standard lithium acetate/single-stranded DNA carrier/PEG procedure [35] and transformants were selected using SC-URA plates. Correct cassette integration into the pXI-2 locus was tested by PCR using primers 33/34. The transformants were grown in YPD medium at 30°C for 48 h and then directly spread onto 5-fluoroorotic acid (5-FOA) plates (50 mg/L) for the recycling of the KIURA3 marker. Colonies grown on 5-FOA plates were examined by colony-PCR using primers 35/34.

JLS01 carrying the genes BTS1, crtYB and crtI was created from strain SCIGS22a by transforming the strain with the cassettes from plasmids pJIL01 and pJIL02. For this purpose, the plasmids pJIL01 and pJIL02 were restricted with enzyme SwaI (New England BioLabs) and the fragments isolated from vector backbones were used for yeast transformation, one cassette at a time. Correct cassette integration into the pXI-3 locus was tested by PCR using primers 36/37. After the KIURA3 marker recycling, the cassette from plasmid pJIL02 was used for yeast transformation and again the KIURA3 marker was recycled for future transformation with episomal plasmids. The correct integration of the cassette in the pX-2 locus was tested by PCR using primers 38/39. Strain JLS02 carrying the genes BTS1, crtYB, crtI and CCD1 was created in the same way, using plasmids pJIL01 and pJIL03.

Strains JLS03, JLS04, JLS05, JLS06 and JLS07 were obtained transforming the strain JLS02 with the high copy number plasmid pEJL04, pEJL05, pEJL06, pEJL07 and pEJL08, respectively (Table 2) containing the URA3 gene from S. cerevisiae as selection marker and the genes crtYB, crtI and/or PhCCD1 under control of the strong constitutive promoter GPD (Table 1). For all comparative analyses, strains JLS01 and JLS02 were transformed with an empty vector p426GPD.

Strain maintenance

For long term storage of the strain, a yeast suspensions containing 25% (v/v) sterile glycerol was stored in cryovials at −80°C. Working stocks were maintained on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar. Plasmid carrying strains were maintained on synthetic dextrose medium (SC) agar plates lacking uracil containing 6.9 g/L yeast nitrogen base without amino acids (BD Difco™, BD and Co, Sparks, MD, USA), 0.77 g/L complete supplement mixture without uracil (CSM-URA) (Sunrise Science Products Inc., San Diego, CA, USA), 20 g/L glucose and 20 g/L agar (BD Difco™ BD and Co.).

Growth conditions

Single colonies were inoculated in 3 mL pre-cultures in SCD media without uracil. Then, cultures were grown in 250 mL shake flasks at 20°C and 180 rpm in a horizontal shaking incubator, with a culture volume of 50 mL with a second phase of dodecane (10%, v/v). All shake-flask cultures were inoculated from pre-cultures grown on the same medium, to an initial OD<sub>600</sub> of 0.1.

β Ionone quantification

Culture samples were centrifuged for 2 min at 6,000 rpm. The organic phase was dried over anhydrous sodium sulfate. Quantitation was performed by means of a gas chromatography system HP 5890 coupled to a flame ionization detector using a DB-FFAP capillary column (60 m × 0.25 mm id, 0.25 μm film thickness) (J&W Scientific, Agilent Technologies). Injection of the samples was performed in splitless mode at 250°C. The oven program started at 80°C for 1 min, then the temperature was raised up 10°C/min to 120°C and then 3°C/min until 240°C. Concentrations of β-ionone were calculated
by using a calibration curve in the range of 0.1–50 mg/L using 4-isopropyl-3-methylphenol 4IP3MP as internal standard (Sigma-Aldrich, St. Louis, MO, USA). Additionally, mass spectra were obtained using a HP5890A gas chromatograph connected to a HP 5975 C mass spectrometer in electron impact (EI) mode at 70 eV.

Carotenoid analysis
Carotenoid extraction was carried out from cellular pellets according to the acetone extraction method [36], with some modifications, using 50 mL culture volume. The cell pellet was washed once with deionized water and then the cells were broken with 500 µL of 0.5-mm glass beads with 1 mL of acetone for 1 min in a cooling Bead Beater (Bio Spec Products, Bartlesville, OK, USA). After breakage, the bead-cell mixture was centrifuged at 14,000 rpm for 5 min, and the clear acetone supernatant was poured off the cell pellet. This extraction procedure was repeated until the cell pellet was white. The acetone extracts were combined with 1/5 volume of petroleum ether and stirring to separate the two phases to finally centrifuge at 14,000 rpm for 5 min. The petroleum ether extract was collected and used for the total carotenoid quantification.

The total carotenoid composition was calculated by using the 1% extinction coefficient = 2,100 by the formula:

\[
\text{Total carotenoid (µg/g of yeasts)} = \frac{(\text{ml of petrol})(A450)(100)}{(21)(\text{yeast dry weight})}
\]

The analyses were performed in triplicate, and pigments were normalized relative to the dry weight of the yeast. Carotenoids were separated by RP-HPLC using a reverse phase C18 column with acetonitrile:methanol:isopropyl (85:10:5 v/v) as mobile phase, with a 1 mL/min flux, measured every 4 h for kinetics parameters and metabolite measurements. Glucose and ethanol were measured by HPLC as described Sanchez et al. [38]. The organic layer was collected at the same times in the fermentation for β-ionone quantification by GC-FID.

Quantitative real-time PCR
For the gene expression analysis, 2 mL of each culture sample were centrifuged at 4°C for 5 min and the pellet was kept in a liquid nitrogen bath for freezing and then stored at −80°C for the next RNA extraction step. RNA isolation was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions, and stored at −80°C to prevent nucleic acid degradation. Isolated RNA was treated with DNaseI, to remove residual genomic DNA. The purity and integrity of RNA was evaluated by electrophoresis in an agarose gel and measuring the A260/A280 ratio and concentration in a Nanodrop spectrophotometer. Total RNA (2 µg) was reverse transcribed with a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific) following the manufacturer’s instruction. The qPCR was realized in a StepOne plus Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA, USA) using the reagent Fast SYBR Green Master Mix (Applied Biosystems) and specific primers for each gene (Additional file 2). For each strain, three clones were analyzed and three technical replicates were done for each qPCR measurement.

The cycle threshold (CT) values and efficiency values obtained were used for further analysis and calculation of relative expression levels. Each sample was normalized using TEF1, as internal control, and then the results from samples JLS03–JLS07 were compared to those in JLS02, as a calibrator sample.

Batch fermentation
Batch cultures were conducted in a 2 L working volume of a 2.5 L aerated stirred bioreactor, BioFlo 11c (New Brunswick Scientific). The medium contained 5 g/L of (NH₄)₂SO₄, 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 0.77 g/L CSM-Ura and 20 g/L glucose. After autoclaving (121°C, 20 min), a filtered-sterilized vitamin solution, prepared according to van Hoek et al. [37], was added to the medium as well as 10% (v/v) dodecane for in situ recovery of β-ionone. The fermenter was inoculated with an adequate aliquot of a pre-culture grown in shake flasks prepared in the same medium as described above to give an initial OD₆₀₀ of 0.1.

During the cultivation, the broth was kept at 20°C, 60 rpm agitation and an air flow rate of 1.0 L/min. A 20% (w/v) solution of NaOH was employed to maintain the culture pH automatically at 5.0. Samples were collected every 4 h for kinetics parameters and metabolite measurements. Glucose and ethanol were measured by HPLC as described Sanchez et al. [38]. The organic layer was collected at the same times in the fermentation for β-ionone quantification by GC-FID.

Additional files

Additional file 1: Codon optimized nucleotide sequences.
Additional file 2: Primers used in the study.
Additional file 3: Maps of plasmids construct in this study.

Authors’ contributions
EA, JN and JL participated in the design of the study. EA and VS supervised the project. JL performed the experimental work. KE and IK assisted in the molecular biology experiments. RP contributed to the construction of strain SCGGS22a. JH and KE work in the batch fermentations. JL analyzed the data and together with EA wrote the manuscript. All authors read and approved the final manuscript.
Author details
1 Department of Chemical and Bioprocess Engineering, School of Engineer‑
ing, Pontificia Universidad Católica de Chile, Av Vicuña Mackenna 4860, Santiago, Chile. 2 Department of Biology and Biological Engineering, Chalmers University of Technology, Kemivägen 10, 412 96 Gothenburg, Sweden. 3 Bio R&D Center, Paikkhwang Industrial Co. Ltd., S7 Oehang-4-gil, Gunsan, Korea. 4 Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, Braga, Portugal. 5 Novo Nordisk Foundation Center for Biostability, Chalmers University of Technology, 412 96 Gothenburg, Sweden. 6 Novo Nordisk Foundation Center for Biostability, Technical University of Denmark, 2970 Harsholm, Denmark.

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Compliance with ethical guidelines

Competing interests
The authors declare that they have no competing interests.

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References
1. Wang G, Tang W, Bidigare R (2005) Terpenoids as therapeutic drugs and pharmaceutical agents. In: Zhang L, Demain AL (eds) Natural products: drug discovery and therapeutic medicine. Humana Press, Totowa, pp 197–227
2. Lange BM, Rujan T, Martin W, Croteau R (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci USA 97(24):13172–13177
3. Cheng A, Lou Y, Macoy, Lu S, Wang L, Chen X (2007) Plant terpenoids: biosynthesis and ecological functions. J Integr Plant Biol 49(2):179–186
4. Gershenzon J, Dudareva N (2007) The function of terpene natural prod‑
ucts in the natural world. Nat Chem Biol 3(7):408–414
5. Miziorko H (2011) Enzymes of the mevalonate pathway of isoprenoid
biosynthesis. Arch Biochem Biophys 505(2):131–143
6. Kirby J, Keasling JD (2009) Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annu Rev Plant Biol 60:335–355
7. Mizorko H (2011) Enzymes of the mevalonate pathway of isoprenoid biosynthesis. Arch Biochem Biophys 505(2):131–143
8. Rodríguez-Bustamante E, Sánchez S (2007) Microbial production of C13-norisoprenoids and other aroma compounds via carotenoid cleav‑
age. Curr Rev Microbiol 33(3):211–230
9. Auldridge M, McCarty D, Klee H (2006) Plant carotenoid cleavage oxygenases and their apocarotenoid products. Curr Opin Plant Biol 9(3):315–321
10. Simkin A, Underwood B, Auldridge M, Loucas H, Shibuya K, Schmelz E et al (2004) Circularization of the PhCCD1 carotenoid cleavage dioxygenase controls emission of b-ionone, a fragrance volatile of petu‑
nia flowers. Plant Physiol 136:3504–3514
11. Baldwin EA, Haven W, Scott JW, Shewmaker CK, Street F, Schuch W (2000) Flavor trivia and tomato aroma: biochemistry and possible mechanisms for control of important aroma compounds. Hort Sci 35(6):1013–1022
12. Schwartz S, Qin X, Zeevaart J (2001) Characterization of a novel carotenoid cleavage dioxygenase from plants. J Biol Chem 276(27):25208–25211
13. Schwab W, Huang F, Molnár P (2013) Carotenoid cleavage dioxygenase genes from fruit. In: Winterhalter P, Eberle S (eds) Carotenoids cleavage dioxygenase products. Oxford University Press, Washington, pp 11–19
14. Lakso J, Laczynski A, McGinty D, Bhatia S, Letizia C, Api M (2007) Fra‑
grance material review on beta-ionone. Food Chem Toxicol 45:5241–5247
15. Berger R (2009) Biotechnology of flavours—the next generation. Biotech‑
nol Lett 31(11):1651–1659
16. Keasling J (2012) Synthetic biology and the development of tools for metabolic engineering. Metab Eng 14(3):189–195
17. Mikkelsen M, Buron L, Salomonsen B, Olsen C, Hansen B, Mortensen U et al (2012) Microbial production of indolylglicosinolate through engi‑
eering of a multi-gene pathway in a versatile yeast expression platform. Metab Eng 14(2):104–111
18. Chen Y, Partow S, Scaliniati G, sewers V, Nielsen J (2012) Enhancing the copy number of episcopal plasmids in Saccharomyces cerevisiae for improved protein production. FEMS Yeast Res 12(5):598–607
19. Reekwilder J, van Rossum H, Koopman F, Sonntag F, Buchhaupt M, Schrader J et al (2014) Polyclonistastic expression of β-carotene biosyn‑
thetic pathway in Saccharomyces cerevisiae coupled to β-ionone produc‑
tion. J Biotechnol 192:383–392
20. Scaliniati G, Partow S, Sievers V, Schall M, Davlet I, Nielsen J (2012) Combined metabolic engineering of precursor and co-factor supply to increase α-santalene production by Saccharomyces cerevisiae. Microb Cell Fact 11(1):117
21. Winterhalter P, Rouseff R (2001) Carotenoid-derived aroma compounds; an introduction. In: Rouseff R, Winterhalter P (eds) Carotenoid-derived aroma compounds. Oxford University Press, Washington, pp 1–17
22. Vani P, Chida A, Srivinasan R, Chandrasekharam M, Singh A (2001) Synthes‑
is of β-ionone. Synth Commun Int J Rapid Commun Synth Org Chem 32(2):219–224
23. Hampton R, Rine J (1994) Regulated degradation of HMVG-Coa reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J Cell Biol 125(2):299–312
24. Shi F, Zhan W, Li Y, Wang X (2014) Temperature influences β-carotene production in recombinant Saccharomyces cerevisiae expressing carote‑
nogenic genes from Phaffia rhodoszyma. World J Microbiol Biotechnol 30(1):125–133
25. Dueber J, Wu G, Malmichegini G, Moon T, Petzold C, Ullal A et al (2009) Synthetic protein scaffolds provide modular control over metabolic flux. Nat Biotechnol 27(8):753–759
26. Verwaal R, Wang J, Meijnen J-P, Visser H, Sandmann G, van den Berg J et al (2007) High-level production of beta-carotene in Saccharomyces cerevisiae by successive transformation with carotenogenic genes from Xan‑thophyllomyces dendrorhous. Appl Environ Microbiol 73(13):4342–4350
27. Kim S-W, Kim J-B, Jung W-H, Kim J-H, Jung J-K (2008) Over-production of beta-carotene from metabolically engineered Escherichia coli. Biotechnol Lett 28(12):897–904
28. Sander J, Joung J (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32(4):347–355
29. Xie W, Ye L, Lv X, Xu H, Yu H (2015) Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in Saccharo‑myces cerevisiae. Metab Eng 28:8–18
30. Verwaal R, Jiang Y, Wang J, Daran J, Sandmann G, van Den Berg J, van Ooyen A (2010) Heterologous carotenoid production in Saccharomyces cerevisiae induces the pleiotropic drug resistance stress response. Yeast 27:983–998
31. Vogel J, Tani B, McCarty D, Klee H (2008) The carotenoid cleavage dioxy‑
genase 1 enzyme has broad substrate specificity, cleaving multiple carot‑
enoids at two different bond positions. J Biol Chem 283(17):11364–11373
32. Nour-Eldin H, Geu-Flores F, Halkier B (2010) USER cloning and USER fusion: the ideal cloning techniques for small and big laboratories. In: Fett-Neto AG (ed) Plant secondary metabolism engineering, methods in molecular biology, vol 643. Humana Press, Totowa, pp 185–200
33. Nørholm M (2010) A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. BMC Biotechnol 10:21
34. Gibson D, Young L, Chaung R, Venter J, Hutchinson C, Smith H (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6(5):12–17
35. Gietz B, Woods R (2002) Transformation of yeast by lithium acetate/sin‑
edeslamidate carrier DNA/polyethylene glycol method. Methods Enzymol 35.4:83–96
36. An G, Schuman DB, Johnson EA (1989) Isolation of Phaffia rhodoszyma mutants with increased astaxanthin content. Appl Environ Microbiol 55(3):116–124
37. van Hoek P, van Dijken J, Pronk J (1998) Effect of specific growth rate on fermentative capacity of baker’s yeast. Appl Environ Microbiol 64(11):4226–4233

38. Sánchez B, Pérez-Correa J, Agosin E (2014) Construction of robust dynamics genome-scale metabolic model structures of Saccharomyces cerevisiae through iterative re-parameterization. Metab Eng 25:159–173