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β-Carotene production by *Saccharomyces cerevisiae* with regard to plasmid stability and culture media

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Abstract A recombinant *Saccharomyces cerevisiae* strain was used for the production of β-carotene. The episomal plasmid YEplac195YB/I/E was extended by a gene coding for the mevalonate kinase (*mvaK1*) from *Staphylococcus aureus*. The *adh1* promoter was chosen for constitutive expression of *mvaK1*. The recombinant strain *S. cerevisiae* G175 (YEplac-CaroSA) synthesised β-carotene by expressing the carotenogenic genes of *Xanthophyllomyces dendrorhous* together with the *mvaK1* gene. Cells of this strain were investigated for their carotenoid contents in YNB and YPD media. A corresponding *mvaK1* transcript in the recombinant yeast host was verified. Growth experiments of a specific *erg12* deletion mutant showed that the mevalonate kinase (MvaK1) was able to complement the function of the deleted native mevalonate kinase (Erg12) from *S. cerevisiae* in the MVA pathway under control of the constitutive *adh1* promoter. Cells of *S. cerevisiae* G175 (YEplac-CaroSA) exhibited high plasmid stability under either selective or non-selective cultivation conditions. Time course experiments demonstrated high plasmid stability even over extended cultivation periods. Carotenoid production was therefore also stable in larger culture volumes. Due to the stability of the plasmid, cultivation of the cells in complex YPD medium was possible, and 14.3 mg β-carotene per litre and a cell density of 9 g cell dry matter (CDM) per litre were achieved. The highest amount of 3,897 μg β-carotene per gramme CDM at a cell density of 1 g CDM per litre was measured after cultivation of the cells in YNB medium with glucose as sole carbon source.

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Introduction

Isoprenoids constitute one of the largest and most structurally diverse class of natural compounds. They are classified according to the number of carbon atoms: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}). Terpenoid biosynthesis starts with a condensation of the two universal C5 building blocks isopentenyl-diphosphate (IPP) and dimethylallyl-diphosphate (DMAPP) which can be synthesised by two different pathways. The mevalonate (MVA) pathway (Fig.1) (Lynen 1967) occurs mainly in eukaryotes, but exceptions within the prokaryotes were found (for example, *Staphylococcus aureus*). Six enzymes synthesise the formation of IPP from acetyl-CoA, and an isomerase converts IPP into DMAPP. Since mevalonate is the central metabolite of this pathway, it is referred to as the mevalonate pathway. In this pathway, mevalonate is phosphorylated two times by mevalonate kinase (Erg12) and phosphomevalonate kinase (Erg8). In nearly all prokaryotes and in the plastids of plants, the mevalonate-independent methylerythritol-4-phosphate (MEP) pathway (Fig.1) is common. In this pathway, glyceraldehyde-3-phosphate and pyruvate are converted by seven enzyme reactions into IPP and DMAPP in a ratio of 5:1 (Kirby and Keasling 2009). The pathway was first described by Rohmer et al. (1993), and its name is referring to the central metabolite methylerythritol-4-phosphate.

The next steps in terpenoid biosynthesis are the condensation of IPP and DMAPP and the successive

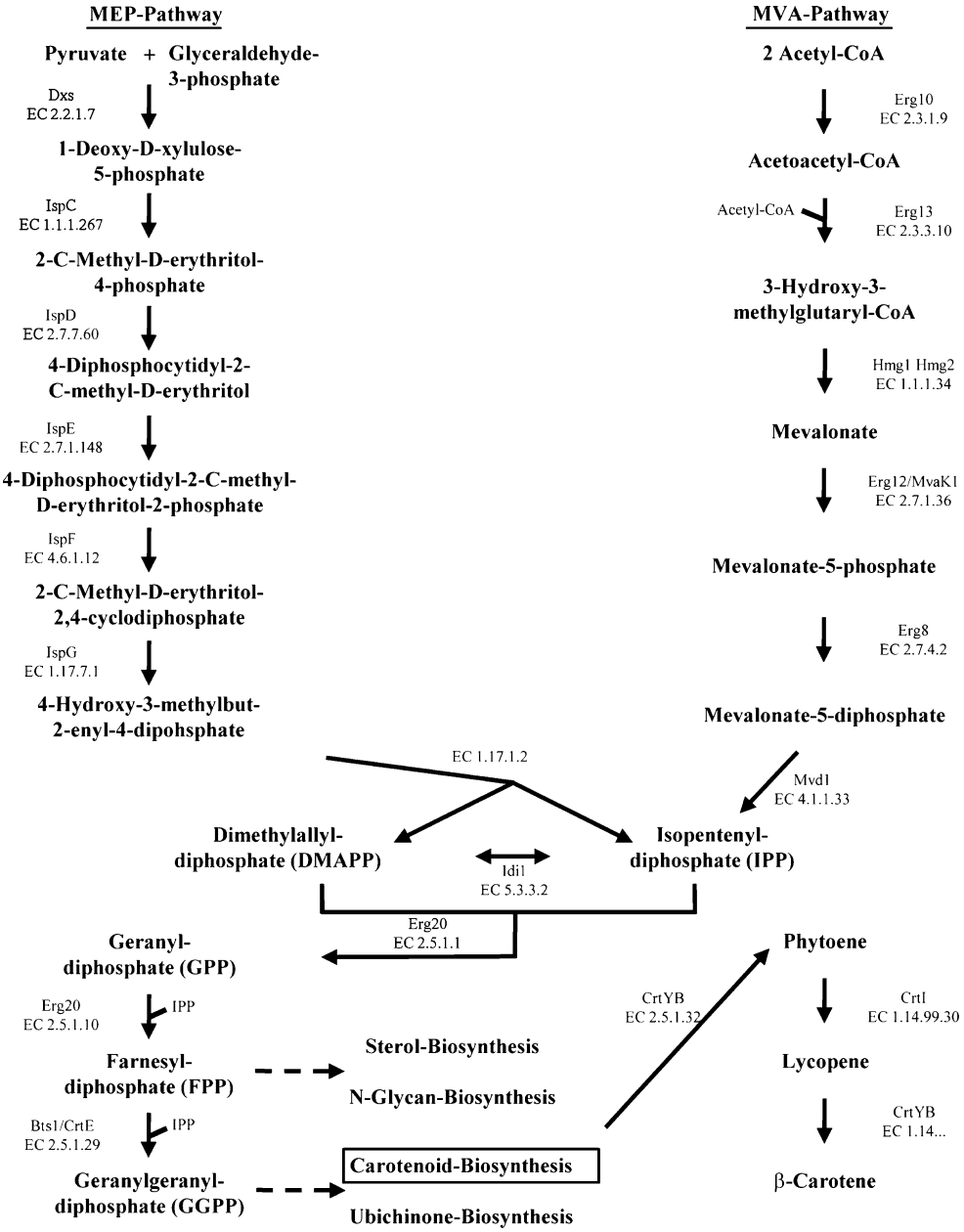

Fig. 1 Overview of the two pathways MVA and MEP for biosynthesis of IPP and DMAPP and subsequent steps required for biosynthesis of tetraterpenoids and, in particular, carotenoids. The abbreviations of the proteins in case of the MVA pathway are corresponding to those of the library of the *Saccharomyces* genome database (SGB, http://www.yeastgenome.org/), whereas the proteins of the MEP pathway correspond to the Kyoto encyclopaedia of genes and genomes (KEGG, http://www.genome.jp/kegg). The carotenoid biosynthesis genes *CRTE*, *CRTI* and *CRTYB* derived from *X. dendrorhous* (Verwaal et al. 2007), whereas *mvaK1* originated from *S. aureus* (Voynova et al. 2004)

additions of IPP units which are catalysed by short-chain-prenyltransferases (Erg20, Bts1/CrtE) (Fig.1). The final reaction links two molecules of geranylgeranyl-diphosphate (GGPP), thereby yielding the C_{40} backbone structure of the tetraterpenoids. After desaturation and cyclisation, the well-known carotenoids lycopene and subsequently β-carotene are formed (Armstrong et al. 1989, 1990; Misawa et al. 1990). Finally, the biosynthesis pathway is completed by the conversion of these compounds to their oxygenated derivatives, the xanthophylles (e.g. lutein, astaxanthin or zeaxanthin). More than 700 naturally synthesised carotenoids are known so far (Britton et al. 2004). They are widely spread in nature and are mainly produced in the plastids of plants via the MEP pathway (Lichtenthaler 1999). However, there are some coloured yeasts, phototropic bacteria and microalgae capable of producing carotenoids in acceptable amounts. One example is *X. dendrorhous* which produces astaxanthin at a concentration of 120 μg/g cell dry matter (CDM) (Ukibe et al. 2009). Animals and humans are not able to synthesise carotenoids; they rely on the diet to cover the demand for precursors of vitamin A synthesis. Therefore, and because of its colouring and the antioxidant function and possible tumour-inhibiting activity, carotenoids are of great interest for the industry (Frengova and Beshkova 2009). The global market for carotenoids was $766 million in 2007 with the largest share of β-carotene valued at $247 million ("The global market for carotenoids 2008"—report by BCC Research, Wellesley, USA).

The molecular formula $C_{40}H_{56}$ for one of the first known carotenoids, β-carotene, was unraveled in 1907 (Willstätter and Mieg 1907 cited in Britton et al. 1995). Its name originated from the carrots (*Daucus carota*) where high amounts of β-carotene are accumulated in the roots (Britton et al. 1995). β-Carotene is mainly produced by chemical synthesis and partially also by extraction from plants; furthermore, there are examples for the biotechnological production of β-carotene by microorganisms (Liu et al. 2009; Yoon et al. 2007, 2009; Misawa et al. 1991; Yamano et al. 1994; Verwaal et al. 2007). Hitherto, 49.3 mg/g CDM can be produced by metabolic engineered recombinant *Escherichia coli* strains (Yoon et al. 2007). Some attempts were also made to use *Saccharomyces cerevisiae* as production strain; however, only a concentration of 5.9 mg/g CDM was achieved (Verwaal et al. 2007).

Because of its genetics, biochemistry, physiology, large-scale fermentation performance and the GRAS (generally recognized as save) status *S. cerevisiae* is one of the most frequently used microorganism in biotechnology. More incidentally, this unicellular budding fungus started its career thousands of years ago as brewing and baking yeast. Nowadays, *S. cerevisiae* is mainly targeted at the industrial factory to produce large amounts of cell matter for compressed or dried yeast used by the bakery industry and as starter culture for ethanol production. In addition, a variety of compounds are produced for the health-care sector (Walker 1998; Huang et al. 2008). Since 1996, the knowledge of the yeast genome (Goffeau et al. 1996; Mewes et al. 1997; Cherry et al. 1997) further opened the application of recombinant DNA technology to engineer the genetic background. Different types of yeast vectors are available including integrative and episomal vectors as well as high- and low-copy number vectors; they have all in common of being *S. cerevisiae–E. coli* shuttle vectors (Zhang et al. 1996). The stability of these plasmids is one important aspect that has to be very closely considered when achieving an effective large-scale production process (Futcher and Cox 1984). Unfortunately, yeast cells often exhibit structural or segregational plasmid instability. Whereas structural instability is caused by deletion, insertion, recombination or other events, the segregational instability is mainly caused by uneven partitioning of plasmids during cell division (Primrose and Ehrlich 1981). To avoid plasmid instability of episomal vectors, addiction systems exploiting the complementation of specific auxotrophic mutations within the genome of the host strains were developed. In this case, the expression vector carries the deleted gene as a selectable marker for plasmid carrying cells (Walker 1998; Kroll et al. 2010). Nevertheless, selection pressure has to be applied by the use of defined media lacking the supplement; otherwise, it will be recovered from the media and the plasmid will be lost.

In this study, the production of the orange pigment β-carotene with the non-carotenogenic yeast strain *S. cerevisiae* was investigated with the aim to obtain this carotenoid in large amounts. For complementation experiments with an *erg12* deletion mutant and for the enhancement of the mevalonate pathway, an alternative mevalonate kinase from *S. aureus* was chosen, which should be deregulated in *S. cerevisiae*. This mevalonate kinase was encoded on an episomal plasmid, and the transfected yeast was cultivated in selective and in non-selective media.

Materials and methods

Strains, plasmids and growth conditions

All strains and plasmids used in this study are listed in Table 1. Recombinant strains of *E. coli* Mach1^{TM}-T1^{R} were used for plasmid maintenance and propagation and were incubated in Lysogeny broth (LB) medium supplemented with ampicillin (75 mg/l) at 37°C overnight and agitated at 120 rpm. Yeast strains were incubated in either complex medium YPD (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone and 2% (*w*/*v*) glucose) or in selective YNB medium (0.67% (*w*/*v*) amino acid-free Difco yeast nitrogen base, 2% (*w*/*v*) glucose). The YNB medium was supplemented as required with the amino acids L-leucine (100 mg/l), L-histidine (20 mg/l) and/or L-tryptophan (20 mg/l). For preparation of solid media, 2% (*w*/*v*) agar was added. Yeast strains were grown aerobically in 250 to 2,000-ml baffled Erlenmeyer flasks for 48 to 96 h at 30°C and agitated at 120 rpm. Cell growth was measured photometrically in a Klett-Summerson photometer (Manostat) using filter no. 54 (520–580 nm).

Construction of the plasmids and the linear deletion fragment

All molecular biological techniques were done according to the protocols of Sambrook et al. (1989). PCRs were performed with the Platinum® Pfx DNA polymerase according to the protocol of the manufacturer Invitrogen (Carlsbad, CA, USA). PCR fragments were purified from agarose gels using the peqGOLD gel extraction kit (peqlab, Erlangen, Germany) by following the instructions of the manufacturer. For plasmid isolation, the Roti®-Prep Plasmid MINI kit was used (Carl Roth, Karlsruhe, Germany).

For cloning the gene of the mevalonate kinase (EC 2.7.1.36, *mvaK1*; from *S. aureus*, sequence optimized for *E. coli* codon-usage), the vector YEplac195 YB/I/E, a generous gift of Rene Verwaal (Verwaal et al. 2007), was restricted with *Ssp*DI and dephosphorylated with FastAP^{TM} according to the protocol of Fermentas (St. Leon-Rot, Germany). The gene of the mevalonate kinase was amplified

Table 1 Strains, plasmids and oligonucleotides used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli MACH1^{TM}-1^{R}	F^{-} Φ80(*lacZ*)ΔM15 Δ*lacX74 hsdR*(r_K^- m_K^+) Δ*recA1398 endA1 tonA*	Invitrogen (Carlsbad, USA)
S. cerevisiae G175	*MATα ADE2 MET his3 leu2 ura3 trp1* TAG$^+$ SE$^+$	ScanBi (Alnarp, Sweden) Sandager et al. 2002
S. cerevisiae G175Δ*erg12*::*LEU2* (YEplac-CaroSA)	Derivative of *S. cerevisiae* G175 with deleted *erg12* gene and *mvaK1* carrying Caro-plasmid YEplac-CaroSA	This study
Plasmids		
YEplac195YB/I/E	Ap^r; episomal expression plasmid for *S. cerevisiae* carrying the genes *crtE*, *crtI* and *crtYB* for the recombinant production of β-carotene	Verwaal et al. 2007
YIplac211YB/I/E*	Ap^r; integrative expression plasmid for *S. cerevisiae* carrying the genes *crtE**, *crtI* and *crtYB* for the recombinant production of β-carotene	Verwaal et al. 2007
YEplac-CaroSA	Ap^r; derivative of YEplac195YB/I/E with the additional gene of the mevalonate kinase from *S. aureus (mvaK1)*	this study
pCOLADuet-1::MVA1-5	Km^r, T7/lac-promoter, carrying the gene *mvaK1*	Kroll et al. 2009
PADNs*β*AS1	Ap^r; episomal expression plasmid for the production of β-amyrin carrying a 1,448 bp fragment of the promoter region of *adh1* from *S. cerevisiae*	Scholz et al. 2009
pUG73	Ap^r; *URA3*, bacterial plasmid for multiple gene deletions in yeast hosts	Gueldener et al. 2002
Oligonucleotides		
PmvaK1-5′SspDI	5′-AAAAGGCGCCGAAGAAATGATGGTAAATG-3′	
PmvaK1-3′SspDI	5′-AAAAGGCGCCTTAGCCGCCCAGGTTCTC-3′	
PmvaK1fusion5′	5′-**GCATACAATCAACTCC**ATGACCCGTAAAGGTT-3′	
PmvaK1fusion3′	5′-AACCTTTACGGGTCAT**GGAGTTGATTGTATGC**-3′	
ura3-erg12KO-fw	5′-AGTCCTAACTTGCAAATTTATATCTACGTATAGAAAACTGTCAATTTAGGTG ACACTATAGAAC-3′	
ura3-erg12KO-rv	5′-TTAAGATTAAAAACAACTAATAAATAATGCCTATCGCAAATTAGCGCATAGG CCACTAGTGGATCTG-3′	
leu2_down	5′-GAAACCATCAAGACTGAGTTC-3′	
erg12ko	5′-AGAATGCGTACACATTTATG-3′	
RT-mvaK1-fw	5′-TACGATGCACCGGATCACC-3′	
RT-mvaK1-rv	5′-AGCCAGAGCTTCGAAATTG-3′	
RT-erg12-fw	5′-AGGCTCAACAAGCCACCGATG-3′	
RT-erg12-rv	5′-AGACCTTGGAATTCTAGTATAG-3′	
RT-tdh3-fw	5′-TGTTCAAGTACGACTCCACTC-3′	
RT-tdh3-rv	5′-CCGGTGGAGGATGGGATGATG-3′	

Sequence annealing to promoter sequence are in bold; sequence annealing to bacterial gene are underlined

[a] *Ap^r* ampicillin resistance, *TAG$^+$* TAG accumulation, *SE$^+$* sterol ester accumulation

from plasmid pCOLADuet-1:MVA1-5 (Kroll et al. 2009) as template using the oligonucleotides PmvaK1fusion5′ and PmvaK1-3′SspDI (Table 1). For constitutive expression, a 1,448-bp promoter fragment upstream of the *adh1* gene from *S. cerevisiae* was amplified in a second PCR with the oligonucleotides PmvaK1-5′SspDI and PmvaK1fusion3′ (Table 1) using plasmid PADNsβAS1 (Scholz et al. 2009) as template. Subsequently, the two PCR fragments were combined in a fusion PCR using the primers PmvaK1-5′ SspDI and PmvaK1-3′SspDI (Table 1). The obtained promoter-gene fragment (SspDI-P$_{adh1}$*mvaK1*-SspDI) was then ligated into YEplac195 YB/I/E yielding plasmid YEplac-CaroSA. The sequence of P$_{adh1}$*mvaK1* was verified by DNA sequencing (Seqlab, Göttingen, Germany).

A linear fragment for chromosomal deletion of the *erg12* gene was generated by PCR. The vector pUG73 was used as template encoding the *LEU2* gene for complementation in leucine auxotrophic *S. cerevisiae* strains. Two oligonucleotides with flanking regions homologous to the *erg12* gene were constructed for homologous recombination of the fragment during cell division (ura3-erg12KO-fw and ura3-erg12KO-rv; Table 1).

Transfer of DNA and selection conditions for the transformants

Competent cells of *E. coli* were prepared by the protocol of Sambrook et al. (1989). Yeast strains were transformed with plasmid or linear DNA applying the polyethyleneglycol-mediated lithium acetate method (Gietz and Woods 2002). Transformants were selected on YNB medium containing glucose and the appropriate amino acids as supplements.

The genes present on the plasmid and the chromosomal deletion were verified by PCR of total DNA isolated from the transgenic yeasts. For this, the oligonucleotides PmvaK1-5′SspDI and PmvaK1-3′SspDI (Table 1) were used to demonstrate the presence of the *mvaK1* expression cassette, whereas the insertion of the deletion fragment was proven by the oligonucleotides leu2_down and erg12ko (Table 1). The carotenoid genes were amplified with primers according to Verwaal et al. (2007).

Isolation of RNA and RT-PCR

Cells of 5 ml of YPD yeast liquid culture were harvested (5 min; 13,000 rpm) followed by a drying step of the cell pellet and mechanical disruption with glass beads in a bead mill (type MM301, Retsch, Haan, Germany). RNA isolation was done by using the RNeasy mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The remaining DNA was hydrolysed by DNaseI (Roche Diagnostics, Mannheim, Germany) for 45 min at 37°C. Afterwards, RT-PCR was applied to verify the transcription of *erg12* and *mvaK1* in transgenic yeasts by using the Qiagen OneStep RT-PCR kit (Hilden, Germany) following the protocol of the manufacturer using specific oligonucleotides for *mvaK1* (RT-mvaK1-fw and RT-mvaK1-rv; Table 1), *erg12* (RT-erg12-fw and RT-erg12-rv; Table 1) and *tdh3* (RT-tdh3-fw and RT-tdh3-rv; Table 1) as transcription control.

Isolation of carotenoids

For the isolation of carotenoids, cells of transgenic yeasts were collected in 50 ml portions, sedimented by 5 min centrifugation at 13,000 rpm and 4°C and were washed twice with 0.9% (*w*/*v*) NaCl. Approximately 100 mg of the freeze-dried cells were used for the following extraction step: 1 g of glass beads and 10 ml acetone–methanol (80:20, *v*/*v*) were added to the pulverised cell pellet and vortexed for 3 min. After 5 min centrifugation at 13,000 rpm and 4°C, the organic phase was removed and stored in the dark at 4°C. This procedure was repeated mostly five times until the extract did not show any strong yellow/orange colour. The appropriate extracts were combined and evaporated, and the resulting coloured dry material was resolved in a defined volume of acetone/methanol (80:20, *v*/*v*) with high-performance liquid chromatography (HPLC) grade.

Analysis and quantification of carotenoids

The carotenoid extract was analysed by HPLC. HPLC separation and quantification were done on a Nucleosil 100–5 C18, 250×4 mm, column (Knauer, Berlin, Germany) eluted isocratically with an acetonitrile–methanol–2-propanol mixture (85:10:5, *v*/*v*/*v*) at a flow rate of 0.8 ml min^{-1}. The carotenoids were detected with a dionex ultimate 3000 photodiode array detector (Dionex, Germany) and recorded online. For quantification, a standard calibration curve with at least seven solutions of defined amounts of β-carotene or lycopene (Sigma Aldrich GmbH, Germany) was done. The content of the carotenoids was calculated in microgrammes per extracted gramme CDM.

Analysis of plasmid stability

To investigate the plasmid stability, the number of plasmid-carrying cells was determined after cultivation. For this, time course cultures in series of six cultures were performed. At the end of every exponential growth phase, the next culture was inoculated, and an aliquot was diluted appropriately and spread on an YPD agar plate. After incubation, the number of colonies (CFU) was counted. Cells from 100 of these colonies were placed simultaneously on a non-selective YPD plate and a selective YNB plate lacking uracil and/or leucine. After incubation for 48 h at 30°C, the number of plasmid-carrying cells was calculated by comparing the CFU on YPD plates with the CFU on YNB plates.

Results

Construction of the expression plasmid YEplac-CaroSA and the addiction-system

In this study, a mevalonate kinase was co-expressed together with the β-carotene biosynthesis enzymes to support the MVA pathway during cultivation of the yeast host. Mevalonate kinases are a group of enzymes well characterised concerning their structure, reaction type and regulation events (Miziorko 2011). Due to the latter, which affects the activity of the native mevalonate kinase (Erg12) from *S. cerevisiae*, an alternative mevalonate kinase (MvaK1) from *S. aureus* was chosen to avoid regulation events (Voynova et al. 2004). This gene has been previously in vitro synthesised considering the codon usage of *E. coli* (Kroll et al. 2009) and had no nucleotide similarity to *erg12*. For construction of an expression plasmid, *mvaK1* and a DNA fragment encoding the promoter of the alcohol dehydrogenase isoenzyme 1 (*adh1*) from *S. cerevisiae* were amplified in two independent PCRs. Based on the complementary regions constructed within the sequence of the fusion primers, the two DNA fragments annealed together in a final PCR yielding P_{adh1}*mvaK1*. The fusion fragment was ligated into the *Ssp*DI cleavage site of the vector YEplac195YB/I/E into an area where the plasmid sequence had no function. The resulting plasmid YEplac-CaroSA (Fig. 2) carried both, the β-carotene production genes *CRTYB*, *CRTI* and *CRTE* from *X. dendrorhous*, which could be constitutively expressed by

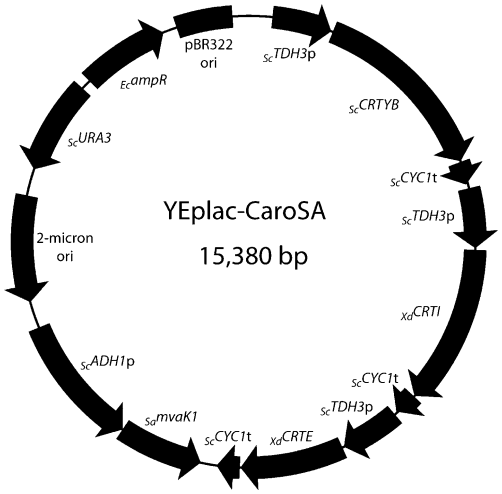

Fig. 2 Map of the episomal plasmid YEplac-CaroSA. Genes for β-carotene production: *CRTE,* geranylgeranyl diphosphate synthase; *CRTI,* phytoene desaturase; *CRTYB,* bifunctional enzyme phytoene synthase and lycopene cyclase; *mvaK1*, mevalonate kinase; *Xd*, *X. dendrorhous*; *Sc*, *S. cerevisiae*; *Sa*, *S. aureus*; *Ec*, *E. coli*; prokaryotic genes written in *small letters*

the *TDH3* (glyceraldehyde-3-phosphate dehydrogenase isozyme 3 from *S. cerevisiae*) promoter and the mevalonate kinase gene from *S. aureus* which was also constitutively expressed. The backbone of the episomal vector YEplac195 mediated uracil prototrophy for selection in YNB medium. *S. cerevisiae* G175 (Table 1) was chosen as host for β-carotene production and was transformed with YEplac-CaroSA yielding a transgenic yeast strain exhibiting uracil prototrophy (Table 1).

For construction of a medium-independent addiction system with MvaK1 encoded by the episomal plasmid replacing the native mevalonate kinase Erg12, a deletion fragment for *erg12* on chromosome XIII was amplified by PCR. This fragment mediated leucine prototrophy for selection of the transformants. Simultaneously, the episomal plasmid and the deletion cassette were transformed into *S. cerevisiae* G175, thereby generating the transgenic yeast *S. cerevisiae* G175 Δ*erg12*::*LEU2* (YEplac-CaroSA) (Table 1). This yeast strain exhibited uracil and leucine prototrophy for selection. Defined PCR products were obtained by using total DNA of the transgenic yeasts for verification of the chromosomal deletion and the presence of the genes encoded by the plasmid (data not shown).

Transcription of *mvaK1* and complementation of the mevalonate kinase in the transgenic yeasts

As the mevalonate kinase (*mvaK1*) from *S. aureus* was never expressed in *S. cerevisiae* before and because of the differing codon usage of both microorganisms, the expression of this gene was investigated. To verify the transcription of *mvaK1* under control of the *adh1* promoter, RNA was isolated and RT-PCR was performed. For this, cells of the transgenic yeast *S. cerevisiae* G175 (YEplac-CaroSA) were cultivated in 5 ml YNB medium containing 2% (*w*/*v*) glucose but lacking uracil. After 48-h incubation, the cells were harvested, and RNA was isolated. The subsequent RT-PCR showed the expected fragments (Fig. 3) of the reverse transcribed mRNA of *ERG12*, *mvaK1* and *TDH3* as a control.

To see if the enzyme MvaK1 could take over the function of the natural mevalonate kinase Erg12 in the essential mevalonate pathway, the chromosomal deletion of *erg12* in the transgenic yeast host was done. After transformation of the episomal plasmid together with the deletion cassette, colonies were grown on YNB plates selecting for uracil and leucine but lacking any ergosterol, because *erg12* deletion mutants were auxotrophic for ergosterol. The results showed that MvaK1 was able to fulfil the function of Erg12 and that it was replaced in the MVA pathway of *S. cerevisiae* G175 Δ*erg12*::*LEU2* (YEplac-CaroSA).

Stability of the plasmids within the transgenic yeasts

Plasmid instability is an undesired but frequently observed occurrence during cultivation processes. Especially, if the

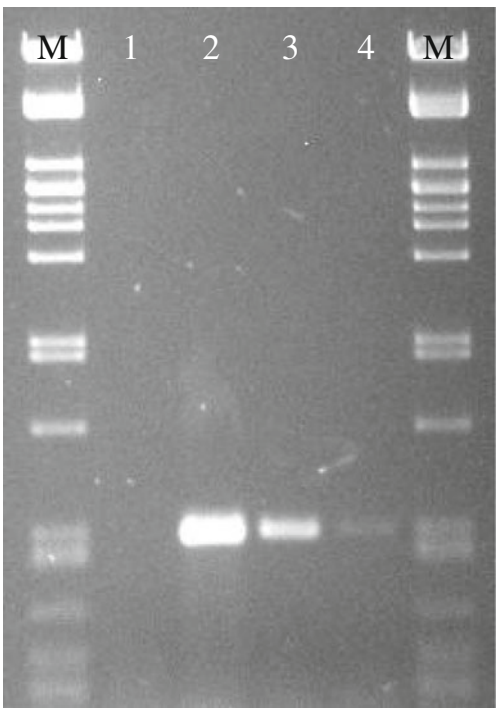

Fig. 3 Reverse transcription analysis of *TDH3*, *mvaK1* and *ERG12*. Cells of *S. cerevisiae* G175 harbouring YEplac-CaroSA were grown in YNB medium containing 2% (*w*/*v*) glucose and appropriate amino acids. Total RNA was isolated and RT-PCR was performed. PCR fragments were applied to a 1.5% (*w*/*v*) agarose gel. *1*, control for DNA contamination; *2*, *TDH3* from *S. cerevisiae*; *3*, *mvaK1* from *S. aureus*; *4*, *ERG12* from *S. cerevisiae*; M, Lambda DNA/*Pst*I Marker (Fermentas, Germany)

medium did not provide any selection pressure on the system, the cells get rid of the additional metabolic burden the plasmid may cause, and production processes may become ineffective. To see how the transgenic yeasts from this study behaved, the stability of the plasmids in the constructed yeast strains was analysed. After cultivation of the transgenic yeasts in a series of six liquid YPD or YNB cultures, aliquots were placed on agar plates to achieve single colonies. To determine the segregational loss, colonies were placed on selective and non-selective agar plates (Fig. 4a–d). By comparing the numbers of orange and white colonies, the structural plasmid loss was verified. After cultivation in YNB medium, the control strain *S. cerevisiae* G175 (YEplac195 YB/I/E) exhibited a segregational plasmid loss at an average of 56%, and the remaining cells exhibited structural plasmid loss. When using YPD medium, 99% of the cells had lost the plasmid, and the remaining cells lost the ability to synthesise carotenoids (structural plasmid instability, data not shown).

In contrast, *S. cerevisiae* G175 (YEplac-CaroSA) carrying *mvaK1* on the episomal plasmid gave totally different results regarding plasmid stability tests. After six cultivations, no segregational loss could be observed with this strain; thus, all colonies could grow on selective agar plates (Fig. 4d). Hence, segregational plasmid loss was not recognized either in YPD or in YNB medium, and all investigated clones were still harbouring the plasmid. Only about 1% of the clones previously cultivated either in YPD or in YNB remained white indicating structural plasmid instability. This demonstrates that the episomal plasmid YEplac-CaroSA was much more stable in the yeast host than the original plasmid YEplac195YB/I/E.

S. cerevisiae G175 Δ*erg12*::*LEU2* (YEplac-CaroSA) was constructed to verify the activity of MvaK1 (see above), but this strain also represents a novel medium-independent anabolism-based addiction system. The chromosomal deletion forces the cells to retain the plasmid to possess the indispensable complete MVA pathway; with this plasmid, also the genes for β-carotene production are retained. For this strain, segregational plasmid loss was not observed, but the structural plasmid loss for the production of carotenoids was 100%; hence, no orange colonies appeared on YPD plates after cultivation (data not shown).

Whereas episomal vectors offer autosomal replicating sequences like ARS or 2-micron, integrative vectors missing these motives can only maintained within the cell after integration into the chromosome. To compare the plasmid stability of the described episomal plasmid YEplac-CaroSA with the plasmid stability of an integrative plasmid, strain *S. cerevisiae* G175 (YIplac211 YB/I/E*) was generated (Table 1). This transgenic yeast strain carried the genes for carotenoid biosynthesis in the *URA3* gene locus on chromosome V. Plasmid stability tests revealed that the integrative plasmid remained in the cell, and a structural instability of about 4% after cultivation in YPD as well as in YNB medium was observed (data not shown).

Growth behaviour and cell dry matters of the carotenogenic yeasts

After transformation of the plasmids into the yeast host, growth behaviour of the resulting transgenic yeasts *S. cerevisiae* G175 (YEplac195 YB/I/E) and *S. cerevisiae* G175 (YEplac-CaroSA) was analysed. For this, 500 ml of YPD or YNB medium were inoculated with 5% (*v*/*v*) of 48-h old precultures. The OD was measured with a Klett-Summerson colorimeter (Manostat Corporation, USA) over a period of 96 h (Fig. 5 a,b). In YPD medium, both strains finally yielded an OD of about 610 Klett units after 96 h; however, the *mvaK1* harbouring strain reached the stationary phase earlier than the control strain (Fig. 5a). In YNB medium (Fig. 5b) with glucose as sole carbon source and in the absence of uracil to exert a selection pressure, *S. cerevisiae* G175 (YEplac-CaroSA) exhibited a shorter lag phase and, therefore reached the stationary growth phase earlier than the control. Also a higher OD of about 580 Klett units was measured in comparison to the control which exhibited an OD of only 440 Klett units. Figure 5 also summarised the data for the cell density in gramme CDM per litre and the β-carotene content of *S. cerevisiae* G175 (YEplac-CaroSA) cells. Cells of the control strain *S. cerevisiae* G175 (YEplac195 YB/I/E) did not produce any carotenoids in larger volumes.

Table 2 lists the data of the cell densities at the end of the cultivation process for both strains as means of three aliquots from three different cultures. Much more cell material (measured as CDM) was obtained for both strains in complex YPD medium than in YNB medium. In comparison to the control strain *S. cerevisiae* G175 (YEplac195 YB/I/E) (6.2 g/l), the cell density of strain *S. cerevisiae* G175 (YEplac-CaroSA) (9.1 g/l) was increased about 1.5-fold when cultivating the cells in YPD medium. No significant differences of the cell densities were measured during cultivation of either strain in YNB medium.

Analysis of β-carotene and lycopene in cells of transgenic yeast strains

During cultivation experiments some differences were observed between the transgenic yeasts. Cultures of *S. cerevisiae* G175 (YEplac-CaroSA) appeared intensively yellow independently on the volume of the culture; whereas the control strain *S. cerevisiae* G175 (YEplac195 YB/I/E) exhibited an orange colour but only in small liquid cultures of about 5–50 ml.

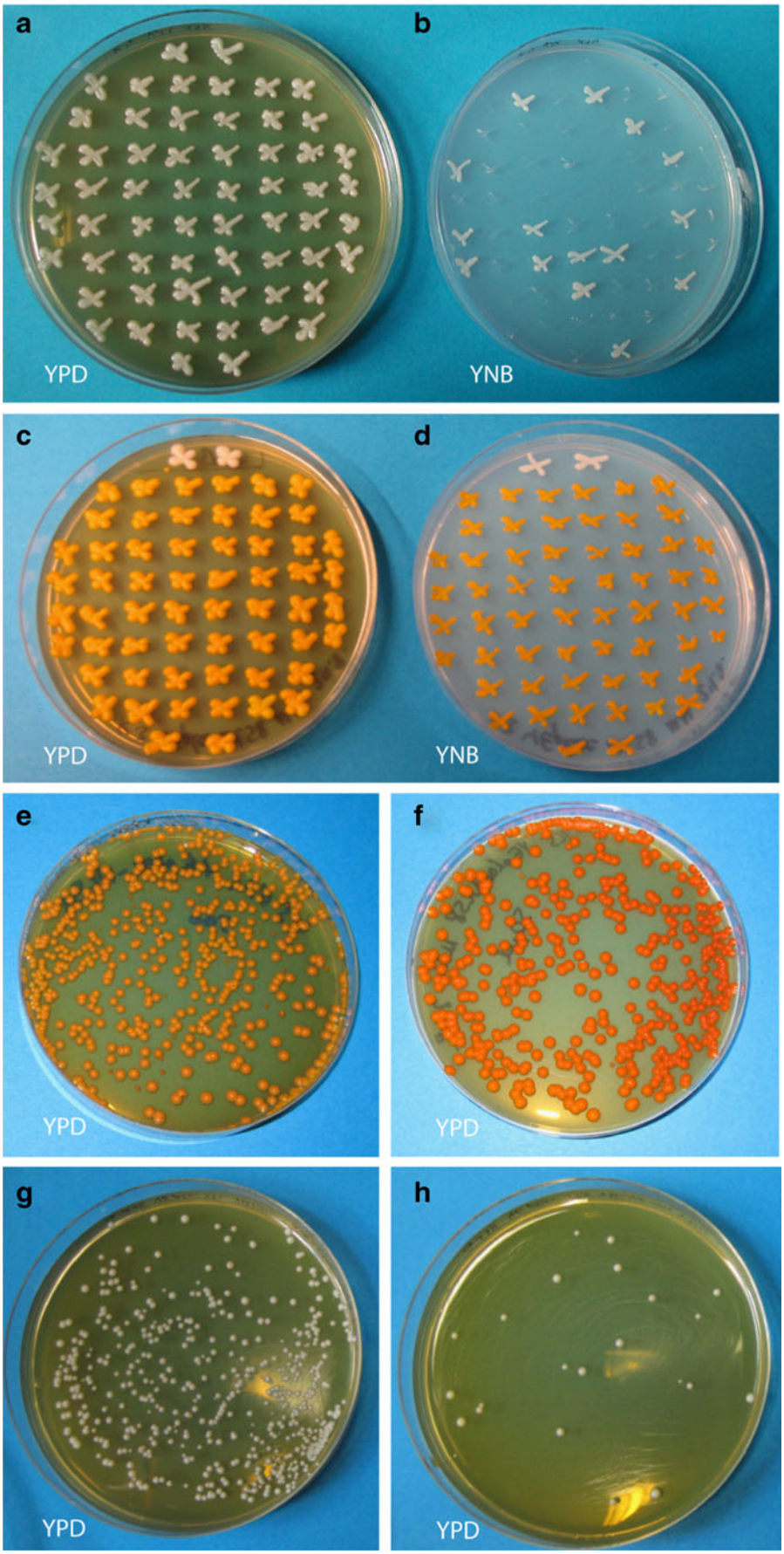

Fig. 4 Growth and phenotype of the transgenic yeast strains. **a**, **b**, **g** control strain *S. cerevisiae* G175 (YEplac195YB/I/E) cultivated in YPD; **h** control strain *S. cerevisiae* G175 (YEplac195YB/I/E) cultivated in YNB; **c**, **d**, **e** *S. cerevisiae* G175 (YEplac-CaroSA) cultivated in YPD; **f** *S. cerevisiae* G175 (YEplac-CaroSA) cultivated in YNB. All strains were grown for 48 h at 30°C in the appropriate liquid medium. YNB medium was used with 2% (*w*/*v*) glucose as sole carbon source but lacking uracil for selection. **a**–**d** Cells from colonies were transferred to selective and non-selective agar plates. **e**–**h** Aliquots of the culture broth were plated on agar plates. The used medium of the agar plates is indicated in the figure. For monochrome printout: light cells are white and grey cells are orange

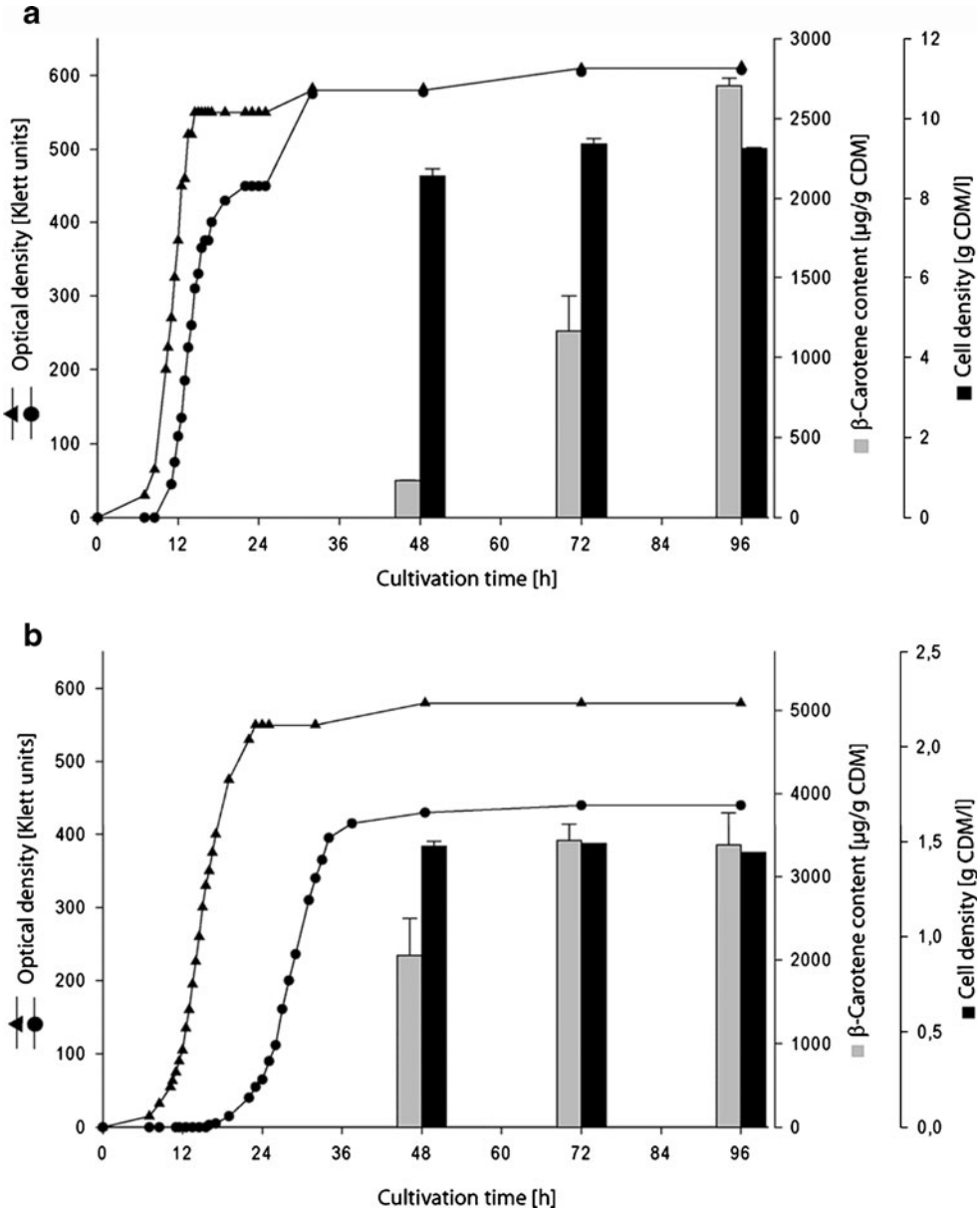

Fig. 5 Growth of recombinant *S. cerevisiae* strains and analyses of cell dry weight and β-carotene contents. *S. cerevisiae* G175 (YEplac195 YB/I/E) (*black circles*) and *S. cerevisiae* G175 (YEplac-CaroSA) (*black triangles*) were cultivated and monitored over a period of 96 h at 30°C in a 2-l Erlenmeyer flasks equipped with baffles containing 500 ml liquid media. **a** YPD medium; **b** YNB medium with 2% (*w*/*v*) glucose but lacking uracil. Cell dry weights and β-carotene contents were only shown for *S. cerevisiae* G175 (YEplac-CaroSA). The cell densities were calculated after freeze drying of three 50 ml aliquots of the cultures, which had been washed twice with 0.9% (*w*/*v*) NaCl and are given in gramme CDM per litre. β-Carotene contents of the cells were determined after extraction of three 100 mg freeze-dried cell samples and quantified with HPLC

The carotenoid contents of β-carotene and lycopene within the transgenic yeasts are summarised in Table 2. Three samples of freeze-dried cells from three independent cultures (except for the control strain, where only one sample was analysed) were used for a series of acetone/methanol (80:20) extractions. All extracts of each extraction were combined and evaporated.

Table 2 Cell dry matter and carotenoid content of different recombinant yeast strains

Strain	Medium	Cell density [g/l][a]	β-Carotene content[b] [µg/g CDM][a]	Lycopene content[b] [µg/g CDM][a]	β-Carotene content [mg/l]
S. cerevisiae G175 YEplac195YB/I/E	YPD	6.2 (±0.2)	263 (±61)	582 (±161)	1.6
	YNB	1.1 (±0.1)	208 (±66)	2,290 (±259)	0.2
S. cerevisiae G175 YEplac-CaroSA	YPD	9.1 (±0.2)	1,570 (±124)	n.d.	14.3
	YNB	1.4 (±0.02)	3,897 (±411)	n.d.	5.5

n.d. not detected

[a] Standard deviations were put in parenthesis

[b] The values are the means from three independent cultures. Whereas three samples of 50 ml aliquots of each 500 ml culture were analysed in case of *S. cerevisiae* G175 (YEplac-CaroSA), the whole culture of 50 ml was used in case of the control strain *S. cerevisiae* G175 (YEplac195 YB/I/E)

The control strain *S. cerevisiae* G175 (YEplac195 YB/I/E) produced 263 μg/g CDM of β-carotene and more than twice as much of lycopene (582 μg/g CDM) in YPD medium. After cultivation in YNB medium, the β-carotene content (208 μg/g CDM) reached nearly the same value, whereas lycopene occurred at an about four-fold higher concentration in the cells (2,290 μg/g CDM).

In contrast to the control strain, the analysis of the carotenoid content in the *mvaK1* harbouring strain *S. cerevisiae* G175 (YEplac-CaroSA) revealed only β-carotene at high concentrations independently of the medium used. The content was six times higher in YPD medium (1,570 μg/g CDM) and eighteen times higher in YNB medium (3,897 μg/g CDM). The data for this strain also indicated an earlier start of the β-carotene production when cultivating in YNB medium instead of in YPD medium (Fig. 5). More than two third of the β-carotene content was already obtained within 48 h, whereas in YPD medium, the β-carotene contents of the cells reached only a small portion of this high value.

Discussion

S. cerevisiae is one of the most frequently used microorganisms in biotechnology. For every product "fabricated" by the cells, the host needs own requirements concerning genetic conditions and process optimisations. In this study, a recombinant *S. cerevisiae* strain for β-carotene production was engineered. The expression of an alternative mevalonate kinase from *S. aureus* in *S. cerevisiae* and the possibility of cultivating this yeast in undefined complex YPD medium to produce β-carotene were achieved for the first time.

Verwaal et al. (2007) noticed that their yeast strain harbouring the carotenogenic episomal plasmid YEplac195 YB/I/E lost the plasmid even under selective cultivation conditions, and consequently, lower carotenoid amounts were obtained. The control strain *S. cerevisiae* G175 (YEplac195YB/I/E), which was constructed in this study harbouring the same plasmid, showed also high plasmid instability. To enhance the stability, an alternative mevalonate kinase was used in the present study, to support the MVA pathway and with this the β-carotene production. The mevalonate kinase MvaK1 from *S. aureus* was used with a modified nucleotide sequence together with the promoter of the alcohol dehydrogenase isoenzyme 1 (Adh1p) from *S. cerevisiae* (Fig. 2) which was reported to be a weak constitutive promoter (Partow et al. 2010). RT-PCR experiments (Fig. 3) showed for the first time the transcription of *mvaK1* in *S. cerevisiae*. Furthermore, a complementation experiment with an *erg12* deletion mutant of *S. cerevisiae* confirmed the activity of MvaK1 instead of Erg12 in this mutant. Hence, the *adh1* promoter constitutively expressed MvaK1 in sufficient quantity to support the mevalonate pathway of *S. cerevisiae* and with this maintained metabolic pathways afterwards like ergosterol biosynthesis (Fig. 1). Induction of a biotechnological process often caused high costs and is an unwanted certainty most of the time. Therefore, all genes on the production plasmid YEplac-CaroSA were expressed constitutively and no additional inductor or special media constitution was necessary.

Plasmid stability tests of *S. cerevisiae* G175 (YEplac-CaroSA) revealed high stability of the plasmid even without the addiction system. Whereas the control strain lost all plasmids either by segregational or structural events and would not be useful for cultivation experiments at a larger scale, the expression of *mvaK1* highly supported plasmid stability. This stability might be due to an unknown effect of the additional mevalonate kinase on the mevalonate pathway. Two active mevalonate kinases in a yeast strain could enhance the level of IPP or of other metabolites which might be beneficial for the cells. Therefore, the costs for plasmid maintenance and gene expression seem to be expended by the cells.

Ugolini et al. (2002) reported that a cell of *S. cerevisiae* could harbour up to 80 copies of an episomal plasmid. Therefore, it is assumed that this recombinant strain *S. cerevisiae* G175 (YEplac-CaroSA) might possess a higher genetic potential for the production process concerning plasmid copy number than the control strain even although both plasmids are using the same uracil auxotrophy. This effect of higher plasmid copy number might explain the total carotenoid composition. Whereas the carotenoid yield of the control strain was a mixture of lycopene and β-carotene, *S. cerevisiae* G175 (YEplac-CaroSA) goes the complete pathway to β-carotene, and no lycopene was left after 96 h (Table 2). There has to be an influence on the cyclisation step catalysed by the *crtYB* gene products. This enzyme is not directly influenced through the MVA pathway, but higher copy numbers of this expression cassette could allow the efficient enzyme reaction and substrate conversion to β-carotene. These results show that the production process applied with *S. cerevisiae* G175 (YEplac-CaroSA) was completed after a shorter cultivation period than by the control strain. Shorter cultivation periods and easier purification processes, in which the product (e.g. β-carotene) needs not to be separated from their precursor (lycopene), will be of great interest for the industry to lower the production costs.

Analyses of the β-carotene yields revealed an increasing production level of *S. cerevisiae* G175 (YEplac-CaroSA) investigated in this study (Table 2). So far, β-carotene contents of 85 μg/g CDM (Verwaal et al. 2007) and 103 μg/g CDM (Yamano et al. 1994) were published with *S. cerevisiae* strains as production host and with episomal plasmids. In both studies, the yeast cells were cultivated in defined YNB medium. In this study, the yield of β-carotene

by using *S. cerevisiae* G175 (YEplac-CaroSA) could be increased by about 37-fold from 103 μg/g CDM up to 3,897 μg/g CDM in comparison to the values presented by Yamano et al. (1994). Until now, the use of an undefined complex medium has not yet been reported for β-carotene production. The use of YPD medium as an example for a complex medium was described in this study, and stable β-carotene production was observed with the strain *S. cerevisiae* G175 (YEplac-CaroSA) harbouring *mvaK1*. Whereas the control strain produced only 263 μg/g CDM in small YPD culture volumes, the β-carotene production could be increased about six-fold to 1,570 μg/g CDM by cultivation of the new strain in YPD medium. This strain was also able to produce β-carotene in larger YPD culture volumes and during the time-course experiments, which were done during plasmid stability tests, the strain exhibited stable β-carotene production over a period of at least six cultures. This enhanced β-carotene production might be due to several effects. First of all, the wild-type strain *S. cerevisiae* G175 seems to have increased metabolism activity; therefore, the control strain already accumulates more β-carotene in comparison to the strain used by Verwaal (Verwaal et al. 2007) with the same plasmid. Furthermore, it is suggested that *S. cerevisiae* G175 (YEplac-CaroSA) might possess higher plasmid copy numbers which could effectively enhance the entire carotenoid biosynthesis. At last, the probably deregulated mevalonate kinase from *S. aureus* may force the MVA pathway to synthesise more IPP, thereby yielding higher β-carotene contents. All these factors might positively push the β-carotene production.

S. cerevisiae G175 (YEplac-CaroSA) grew to higher cell densities in YPD medium than in YNB medium; hence, the highest total β-carotene content was calculated to be 14.3 mg/l for the cultivation conditions in YPD medium used in this study (Table 2). Yoon et al. (2009) published the highest content of β-carotene ever achieved with microorganisms using metabolically engineered *E. coli* strain. Unfortunately, the β-carotene yield was only mentioned as a total amount of 465 mg/l without providing data for the cell density. The possibility to have a stable yeast strain in undefined complex medium allows the use of industrial waste products, like molasses or corn steep liquor as carbon sources. With these substrates, high cell density production processes were evaluated. For example, Vu and Kim (2009) reported a very high cell density of more than 180 g CDM per litre.

With regard to these facts concerning undefined medium composition, this study also described the deletion mutant *S. cerevisiae* G175 Δ*erg12*::*LEU2* (YEplac-CaroSA) which was constructed for MvaK1 complementation experiments. It also represents an addiction system in which the biosynthesis of β-carotene is coupled with the complementation of a chromosomal deletion. Most of the vector systems for *S. cerevisiae* are based on amino acid or nucleotide auxotrophic markers for plasmid maintenance in defined media (Walker 1998; Kroll et al. 2010); therefore, complex media are not useful for cultivation.

The aim of this addiction system was the development of high plasmid stability via complementation of the chromosomal deletion by the plasmid-encoded mevalonate kinase and with this the application to cultivation processes in complex undefined media. High plasmid stability for the β-carotene plasmid was achieved when cultivating the cells in all media used; however, the strain did, unfortunately, not produce any lycopene or β-carotene. It could be estimated that the activity of MvaK1 seems to be sufficiently high to sustain the ergosterol metabolism of *Saccharomyces*, but adequate additional amounts of IPP and subsequent metabolites for carotenoid production were not left when competing with sterol biosynthesis. The mevalonate pathway is well-regulated (Goldstein and Brown 1990). The flux of IPP to the carotenoid pathway lowered the IPP concentration for sterol biosynthesis which is crucial for the survival of the cells; hence, regulatory events like modifications of the gene or the promoter sequences on the plasmid might occur to stabilize sterol biosynthesis and thereby preventing cell death. Further investigations would be required to overcome the competition between ergosterol and carotenoid pathways. A suitable promoter for the enhanced expression of *mvaK1* might be useful to increase IPP level and thereby enabling *S. cerevisiae* G175 Δ*erg12*::*LEU2* (YEplac-CaroSA) to become a useful production strain for β-carotene. The usage of the native mevalonate kinase Erg12 or different mevalonate kinases from different species for the addiction system could be an alternative. Finally, this system might be applied to an expression cassette which is not dependent on the mevalonate pathway to adapt this system to production processes.

In summary, *S. cerevisiae* G175 (YEplac-CaroSA) has a high potential of being a candidate for the fermentative production of β-carotene at larger scale. Further experiments could be done concerning the volume of the culture media and its composition to achieve high cell density together with a high production yield of β-carotene. Furthermore, the influence of the *mvaK1* in the mevalonate pathway of *S. cerevisiae* might be studied in detail.

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