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Carotenoid-based phenotypic screen of the yeast deletion collection reveals new genes with roles in isoprenoid production

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ABSTRACT

Beside their essential cellular functions, isoprenoids have value as pharmaceuticals, nutriceuticals, pesticides, and fuel alternatives. Engineering microorganisms for production of isoprenoids is relatively easy, sustainable, and cost effective in comparison to chemical synthesis or extraction from natural producers. We introduced genes encoding carotenoid biosynthetic enzymes into the haploid yeast deletion collection to identify gene deletions that improved isoprenoid production. Deletions that showed significant improvement in carotenoid production were further screened for production of bisabolene, an isoprenoid alternative to petroleum-derived diesel. Combining those deletions with other mevalonate pathway modifications increased production of bisabolene from 40 mg/L to 800 mg/L in shake-flask cultures. In a fermentation process, this engineered strain produced 5.2 g/L of bisabolene.

1. Introduction

Isoprenoids are a large and diverse group of organic compounds found in all domains of life (Holstein and Raymond, 2004). They carry out essential roles as sterols, electron transporters, photosynthetic pigments, toxins, attractants, or protein targeting compounds (Gershenzon and Dudareva, 2007). In most eukaryotes and some prokaryotes, isoprenoids are synthesized through the mevalonate pathway (Lombard and Moreira, 2011). The mevalonate pathway condenses three acetyl-CoAs to form hydroxyl-methyl-glutaryl-CoA (HMG-CoA), which is further reduced to mevalonate by the action of a rate-limiting enzyme, HMG-CoA reductase (HMGR) (Basson et al., 1988; Polakowski et al., 1998). Consecutive phosphorylations and decarboxylation of the mevalonate result in isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are further condensed by prenyl transferases to synthesize geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranylgeranyl diphosphate (GGPP, C20). A variety of terpene synthases use GPP, FPP, and GGPP to synthesize monoterpenes, sesquiterpenes, and diterpenes, respectively (Tholl, 2006).

Beside their essential cellular functions, isoprenoids have great industrial value as pharmaceuticals (e.g., artemisinin, taxol) (Ro et al., 2006; Ajikumar et al., 2010), nutriceuticals (e.g., carotenoids) (Nelis and De Leenheer., 1991), and pesticides (Katsuda, 2012). Traditionally, these commercially valuable isoprenoids are extracted from plants through laborious and expensive procedures (Ishida and Chapman, 2009; Lapkin et al., 2006; Mattina and MacEachern, 1994). Most of these compounds accumulate to minute concentrations in their host organisms and are difficult, if not impossible, to chemically synthesize. Metabolic engineering of microorganisms for production of commercially valuable isoprenoids is a relatively inexpensive and fast route to produce these compounds, and often it can yield purer products than those obtained either by chemical synthesis or by extraction from plants (Keasling, 2010; Herrero et al., 2008; Farhi et al., 2011). The budding yeast, *Saccharomyces cerevisiae*, is the organism of choice for many industrial processes due to its favorable physiological properties. It is also very amenable to genetic modification with established molecular biology tools making it an ideal organism for engineering metabolic pathways (Hong and Nielsen, 2012; Nevoigt, 2008).

In particular, yeast has been used widely for the production of biofuels, namely ethanol, and is being explored for production of advanced biofuels. Recently, we identified fully hydrogenated bisabolene as a diesel D2 alternative and demonstrated its microbial production by expressing the codon optimized version of the bisabolene synthase gene from *Abies grandis* (*AgBIS*) (Peralta-Yahya et al., 2011). Previous efforts to engineer yeast for high titer production of valuable isoprenoids have mostly focused on manipulating the mevalonate pathway (Ro et al., 2006; Asadollahi et al., 2010; Westfall et al., 2012, Scalcinati

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et al., 2012). For efficient production of isoprenoids, these studies demonstrated that it is essential to over-express the catalytic domain of *HMG1*, the primary HMGR in yeast (Donald et al., 1997). Repressing squalene production and over-expressing FPP synthase (*ERG20*) increased isoprenoid production further, presumably by increasing the FPP flux to the isoprenoid synthase. When *AgBIS* was expressed in an engineered yeast strain carrying a combination of these modifications, 5 mg of bisabolene was produced per gram of glucose. The maximum theoretical yield (Li et al., 2010) is estimated to be about 250 mg of bisabolene per gram of glucose, suggesting that there is significant room for improvement.

Though metabolic pathways are often treated as independent entities when they are engineered or introduced into cells, they are actually highly inter-connected with the rest of cellular metabolism and tightly regulated (Szappanos et al., 2011). Therefore, seemingly irrelevant genes can have significant and unexpected effects on any given pathway. We sought to identify genes not directly involved in the mevalonate-based isoprenoid biosynthetic pathway that might improve pathway activity. Unfortunately, many useful isoprenoids can only be measured using low throughput methods like liquid and gas chromatography. Carotenoids provide a visual phenotype for fast and easy measurement of isoprenoid production levels (Alper et al., 2005; Park et al., 2008; Scaife et al., 2012). To identify additional genes that affect isoprenoid synthesis in yeast, we used carotenogenic genes from *Xantophyllomyces dendrorhous* (Verwaal et al., 2007) to screen the yeast deletion collection (Winzeler et al., 1999). Three gene deletions that showed an almost four-fold increase in total carotenoid production were further tested by replacing the carotenogenic genes with bisabolene synthase. Combinations of these deletions and other pathway modifications increased titers of bisabolene more than 20-fold.

2. Materials and methods

2.1. Yeast strains, media and transformation

The yeast strains used in this study were isogenic to BY4741 (derivative of S288C) and CEN.PK2 (Table 1). The yeast ORF knockout collection was purchased from Open Biosystems (Catalog number YSC1053). Carotenoid-producing plasmids were kind gifts from Verwaal et al. (2007). The plasmids used in this study are listed in Table 2.

Detailed explanation of the strain and plasmid construction is included in the supplementary information. Information about all the strains and plasmids have been deposited in the public instance of the JBEI Registry (https://public-registry.jbei.org/).

For bisabolene production, an overnight pre-culture grown in SC-Leu liquid medium supplemented with 2% dextrose was inoculated into the 5 ml induction medium at an OD_{600} of 0.05 (except for the experiment in Fig. 5C where the cultures were 30 ml using 250 ml shake flasks). Induction medium was a modified version of minimal medium that included 6.7 g/L YNB, 5.5 g/L of SC-Leu amino acid mix (Sunrise Science Products), 0.1 M sodium phosphate buffer (pH 6), and 0.2% dextrose and 1.8% galactose as a carbon source (2% dextrose in strains deleted for *gal80*). Modified lithium acetate transformation was used as described (Becker and Lundblad, 2001).

To transform the yeast deletion collection with *BTS1/YB/I*, frozen yeast stocks were first grown on YPD-agar medium for 2 day. Grown strains were inoculated into 1 ml of YPD and grown overnight at 30 °C. Next day, cultures were pelleted and washed twice with 1 ml of lithium acetate/TE solution. 50% PEG (molecular weight 3350), salmon sperm DNA (100 μg/ml), and plasmid (1 μg/ml) were mixed, and each pellet was resuspended in 350 μl of this PEX mixture. Samples were first incubated at 30 °C for 1 h and then heat-shocked for 30 min at 42 °C. After discarding the PEG mixture, pellets were resuspended in 50 μl dH_2O, and 20 μl of each was spotted onto selective medium agar plates (SC-URA). When transformants were grown, carotenoid-synthesizing colonies were picked, inoculated into 600 μl SC-URA medium, and grown overnight. Next day, about 5 μl of this culture was spotted onto SC-URA plates. After 2 day of growth at 30 °C, carotenoid levels were scored.

Gene deletions and genomic integrations were done using one-step integration of PCR-amplified knockout cassettes (Goldstein and McCusker, 1999; Longtine et al., 1998) and confirmed by PCR and phenotypic validation.

Oligonucleotide sets used for PCR, cloning, tagging, and knockouts in this study are included in the Supplementary information.

Table 1
Yeast strains used in this study.

Strain name	Genotype	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3,112 his3Δ 1 MAL2-8^C SUC2*	Euroscarf
JBEI-4716	CEN.PK2-1C *NATMX-P_{CYC1}_ERG9*	This study
JBEI-4717	JBEI-4716 *rox1::hph*	This study
JBEI-4718	JBEI-4716 *yjl064w::hph*	This study
JBEI-4719	JBEI-4716 *ypl062w::hph*	This study
JBEI-4720	JBEI-4716 *prb1::hph*	This study
JBEI-4721	JBEI-4716 *rox1::hph yjl064::HIS5*	This study
JBEI-4722	JBEI-4716 *yjl064w::hph ypl062w::HIS5*	This study
JBEI-4723	JBEI-4716 *rox1::hph ypl062w::HIS5*	This study
JBEI-4724	JBEI-4716 *rox1::hph yjl064::HIS ypl062w::KAN-MX*	This study
JBEI-4725	JBEI-4716 *gal80::HIS5*	This study
JBEI-4726	*YPRCδ15::KANMX-P_{GAL1}-C.O.BisSyn∼ERG20/P_{GAL10}-tHMG gal80::HIS5*	This study
JBEI-4727	JBEI-4726 *rox1::hph*	This study
JBEI-4728	JBEI-4726 *ypl064w::hph*	This study
JBEI-4729	JBEI-4726 *yjl064w::hph*	This study
JBEI-4730	JBEI-4726 *rox1::hph ypl062w::Ca.URA3*	This study
JBEI-4731	JBEI-4726 *rox1::hph yjl064w::Ca.URA3*	This study
JBEI-4732	JBEI-4726 *ypl064w::hph yjl064w::Ca.URA3*	This study
JBEI-4733	JBEI-4726 *rox1::hph yjl064w ypl062w::Ca.URA3*	This study
JBEI-4927	JBEI-4730 *TRP1*	This study
JBEI-4928	JBEI-4731 *TRP1*	This study
JBEI-4734	JBEI-4732 *TRP1*	This study

Table 2
Plasmids used in this study.

Plasmid name	Description	Reference
YB/I	*YEplac195 TDH3p-crtYB-CYC1t; TDH3p-crtI-CYC1t*	Verwaal et al. (2007)
YB/I/BTS1	*YEplac195 TDH3p-crtYB-CYC1t; TDH3p-crtI-CYC1t; TDH3p-BTS1-CYC1t*	Verwaal et al. (2007)
YB/I/E	*YEplac195 TDH3p-crtYB-CYC1t; TDH3p-crtI-CYC1t; TDH3p-crtE-CYC1t*	Verwaal et al. (2007)
JBEI-2922 (*pRS-AgBIS*)	*pRS425-Leu2d-P_{GAL1}.AgBIS*	Peralta-Yahya et al. (2011)
JBEI-4738 (*B*)	*pESC-Leu2d-P_{GAL1}-AgBIS*	This study
JBEI-4739 (*H/B*)	pESC-*Leu2d-P_{GAL1}-AgBIS/P_{Gal10}-tHMG1*	This study
JBEI-4740 (*H/B/E*)	pRS425-*Leu2d-P_{GAL1}-AgBIS/P_{Gal10}-tHMG1/P_{GAL10}-ERG20*	This study
JBEI-4742 (*H/B∼E*)	*pESC-Leu2d-P_{GAL1}-AgBIS∼ERG20/P_{GAL10}-tHMG1*	This study
JBEI-4743 (*B-myc*)	*pESC-Leu2d-P_{GAL1}-AgBIS-myc*	This study
JBEI-4744 (*H/B∼E-myc*)	*pESC-Leu2d-P_{GAL1}-AgBIS∼ERG20-myc*	This study
JBEI-4745 (*H/B-myc*)	*pESC-Leu2d-P_{GAL1}-AgBIS-myc/P_{GAL10}-tHMG1*	This study

2.2. Qualitative and quantitative evaluation of the carotenoid levels

The deletion collection transformed with the carotenoid-producing plasmid was first visually analyzed. A scale of −5 to +5 was developed as follows. Each 96-well transformation plate included the parent wild-type strain. The color of the carotenoid-expressing, wild-type, parent strain was labeled as 0. Strains colored darker than the wild type parent strain were graded at a scale of +1 to +5, +5 being the darkest color. Those strains lighter than the parent strain were graded −1 to −5, −5 being the strains that are completely white with no visible carotenoid color.

For spectrophotometric analysis, carotenoids were extracted as described previously (Verwaal et al., 2007) with the following modifications. About 8–9 ml of hexane was used until all the carotenoids were extracted from the cell pellets. Absorbance was measured at 449 nm.

2.3. GC–MS analysis of metabolites

Cultures were overlayed with 10% dodecane to reduce evaporation of bisabolene, and the amount of bisabolene dissolved in the dodecane layer was determined using gas chromatography–mass spectrometry (GC–MS). To determine the amount of bisabolene dissolved in dodecane, 10 μl of dodecane from the cultures was mixed with 990 μl of ethyl acetate to analyze GC–MS. Samples were run using a previously described method (Martin et al., 2001) with some differences: The GC oven temperature program used a 100 °C for 0.75 min, followed by a ramp to 300 °C at 50 °C/min.

To measure the mevalonate levels, 200 μl of culture medium (not the dodecane phase) was mixed with 50 μl of 2 M HCl and vortexed for 15 min. Ethyl acetate (250 μl) spiked with caryophyllene (internal standard) was added and vortexed for an additional 5 min. The tubes were centrifuged for 1 min at the highest speed and the top layer was acquired for further GC–MS analysis.

2.4. Protein analysis

Yeast whole-cell extracts were precipitated using 20% trichloroacetic acid (TCA) and solubilized in SDS loading buffer. Alkaline phosphatase-conjugated, anti-myc antibody (Invitrogen) was used to detect myc-tagged Bisabolene Synthase and AgBis∼Erg20 proteins.

2.5. Plasmid maintenance assay

At the end of the six-day production run about 100–200 cells were spread on SC and SC-Leu agar media. After 2 day of incubation at 30 °C, the number of colonies was counted and the percent plasmid maintenance was calculated by dividing the number of colonies growing on SC-Leu with number of colonies on SC and multiplying by 100.

2.6. Bioreactor media

The media used for this work were based on media described previously (van Hoek et al., 2000). The vitamin solution contained calcium pantothenate (0.4 g/L), inositol (2 g/L), niacine (0.4 g/L), *p*-amino benzoic acid (0.2 g/L), pyridoxine hydrochloride (0.4 g/L), and thiamine (0.4 g/L). The mineral solution contained boric acid (0.5 g/L), $CuSO_4 \times 5H_2O$ (0.62 g/L), KI (0.1 g/L), $FeCl_3 \times 6H_2O$ (0.33 g/L), $Mn_2SO_4 \times 7H_2O$ (0.91 g/L), $Na_2MoO_4 \times 2H_2O$ (0.23 g/L), $ZnSO_4 \times 7H_2O$ (0.7 g/L).

Two different batch and fed batch media were prepared for each strain. For the JBEI4734 strain, the batch culture medium (0.7 L) contained glucose (15 g/L), anhydrous $(NH_4)_2SO_4$ (10 g/L), anhydrous KH_2PO_4 (8 g/L), anhydrous $CaCl_2$ (0.5 g/L), anhydrous $MgSO_4$ (4 g/L), NaCl (0.8 g/L), galactose (5 g/L), vitamin solution (10 mL/L), mineral solution (10 mL/L) and 0.5 mL Antifoam 204 (Sigma). 100 mL of dodecane was included to capture the product. The glucose-fed-batch medium contained glucose (500 g/L), anhydrous KH_2PO_4 (15 g/L), anhydrous $CaCl_2$ (0.5 g/L), anhydrous $MgSO_4$ (4 g/L), NaCl (0.8 g/L), galactose (5 g/L), vitamin solution (10 mL/L), and mineral solution (10 mL/L). The pH control was made with 7 M NH_4OH. For the control strain (JBEI4716) which requires amino acids supplementation, both batch media (0.7 L) and the fed-batch media were further added with uracil (0.32 g/L) and tryptophan (1.12 g/L).

2.7. Bioreactor studies

The seed cultures for fermentation were prepared by inoculating a scoop of frozen cells in 20% (vol/vol) glycerol into a 250-mL shake flask containing 20-mL medium. The seed culture was grown for about 24 h at 30 °C and reached a final OD of about 4. The entire 20-mL seed culture was used to inoculate 0.7 L initial batch medium in the bioreactor.

The bioreactor studies were carried out in a 2-L Satorius Biostat-B plus, and the pH, which was maintained at 5, was controlled automatically by addition of a 7 M NH_4OH. The temperature was maintained at 30 °C, and airflow was supplied at a rate of 0.7 L/min.

The bioreactor was inoculated and the cells were grown until all the initial glucose in the batch media was consumed. During the batch phase, the initial glucose is converted to biomass and ethanol. When the initial glucose is depleted, the cells consume the ethanol produced during batch phase. When all the glucose and the ethanol are consumed, the pH rises and triggers the start of the fed-batch phase, in which the feed rate was constant for the remainder of the experiment.

3. Results

3.1. Screening the yeast deletion collection for improved carotenoid production

Introducing a desaturase (*crtYB*) and a cyclase (*crtI*) from the red yeast *Xanthophyllomyces dendrorhous* is sufficient for carotenoid production in *S. cerevisiae* (Verwaal et al., 2007) (Fig. 1). Three different plasmids were previously constructed by Verwaal et al. with different levels of carotenoid production (Fig. 2A). The native GGPP synthase (*BTS1*) is the limiting enzyme in the pathway, therefore expression of an additional copy of *BTS1* or its homolog from *X. dendrorhous* (*crtE*) is necessary for visibly orange colonies. We chose the plasmid *YB/I/BTS1* to screen the yeast deletion collection as it produced average levels of carotenoid compared to the other plasmids, giving us the chance to detect those deletion strains with decreased or increased carotenoid levels.

The haploid yeast deletion collection constituting more than 5000 strains with individual deletion of non-essential genes (Winzeler et al., 1999) was transformed with *YB/I/BTS* in 96-well plates and qualitatively screened for color changes by plating the transformants on selective agar media plates. The color of the wild-type strain (harboring plasmid *YB/I/BTS1*) was assigned the score of 0 and all the strains in the collection were evaluated respectively on a -5 to $+5$ scale (Fig. 2B). Color scores for all the strains can be found in Supplementary Information. The transformation was unsuccessful for 329 strains, 151 of which were petites that would not be suitable for further genetic engineering or high-titer production of isoprenoids due to growth restrictions. The rest of the deletion strains showed a normal distribution in color score with about half of them showing almost identical coloring to the wild-type. About 1300 strains showed some reduction in color compared to the wild-type strain and 60 of those were white with no visible orange color. Color loss could be either a direct effect of the gene deletion or could be due to loss of the carotenogenic genes from these strains since these vectors are reported to be structurally unstable (Verwaal et al., 2007). Therefore, we focused our efforts on those gene deletions that increased carotenoid levels. The functional categories of genes whose deletions enhanced colony color (scored higher than 2) are plotted in Fig. 2C. About $1/5^{th}$ of these genes are unknown, and $1/5^{th}$ have metabolic functions. The majority of the remaining genes had roles related to gene expression (histone modifications, transcription factors, RNA regulation, ribosome) or protein regulation (protein degradation, ER/Golgi, kinase/phosphatase). Out of these gene deletions, we focused on 156 strains that showed significant color enhancement (scored 3, 4, or 5) (Supplementary File). These strains were orange to dark orange in color and presumably had higher levels of lycopene (red) in addition to increased levels of β-carotene (yellow) (Verwaal et al., 2007). They were screened again by transforming individually and scoring the colony color of the transformants to confirm the results from the first round of transformation. Twenty-four of these strains with consistent and very strong carotenoid production (scoring 4 or 5 in both rounds of transformation) were selected for further evaluation.

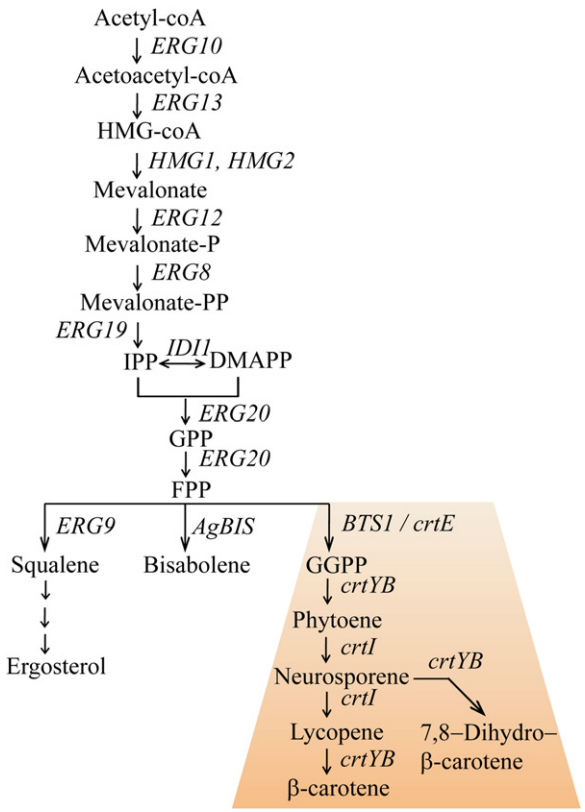

Fig. 1. Mevalonate pathway in yeast and the carotenoid synthesis pathway of *X. dendrorhous*.

3.2. Quantitation of total carotenoid levels and bisabolene production in high producing deletion strains

Twenty-four deletion strains that had the darkest orange color and had growth rates similar to the wild-type parent strain were grown in liquid culture and harvested after 3 day. Carotenoids were extracted from the cell pellets using hexane. Total carotenoid levels were determined by measuring absorbance at 449 nm (Fig. 3). All the selected deletion strains showed significantly higher carotenoid levels than the wild-type parent. Strains *arp6*, *erg24*, *ser33*, *ydr215c* and *ypl062w* had four-fold more carotenoids than the parent.

Increased carotenoid levels suggested that the levels of precursors to isoprenoids, namely FPP and GPP, were higher in these deletion strains. We tested these gene deletions to determine if they also improved the production of bisabolene, an isoprenoid with diesel-like properties (Peralta-Yahya et al., 2011). The engineered version of bisabolene synthase gene from *Abies grandis* (*AgBIS*) was over-expressed in these twenty-four deletion strains. Surprisingly, all of the strains produced less bisabolene than the wild-type strain (Supplementary Fig. 1). The catalytic efficiency of the AgBIS ($38.21\ M^{-1}\ s^{-1}$) (McAndrew et al., 2011) is about 200-fold lower than that of Bts1 ($7.8\times10^{3}\ M^{-1}\ s^{-1}$) (Chang et al., 2006), which could lead to substrate accumulation. Hmg1 is regulated by a negative-feedback system that may be activated by excess accumulation of mevalonate or mevalonate pathway intermediates (Dimster-Denk et al., 1994). To circumvent this negative feedback, we over-expressed a truncated version of *HMG1* (*tHMG1*). To ensure substrate abundance, we also over-expressed an additional copy of the FPP synthase gene (*ERG20*) along with the *AgBIS* (Fig. 3). Despite the pronounced increase in carotenoid production in all these strains, bisabolene production was enhanced modestly in only four of the strains, namely *rox1*, *prb1*, *yjl064w*, and *ypl062w*.

3.3. Constructing a better parent strain for bisabolene production

The deletion strains used in this study are descendents of S288C, a well-established lab strain with a fully annotated genome (Cherry et al., 1997). Although S288C is favored for lab-scale experiments, the physiological properties of CEN.PK2, another *S. cerevisiae* strain,

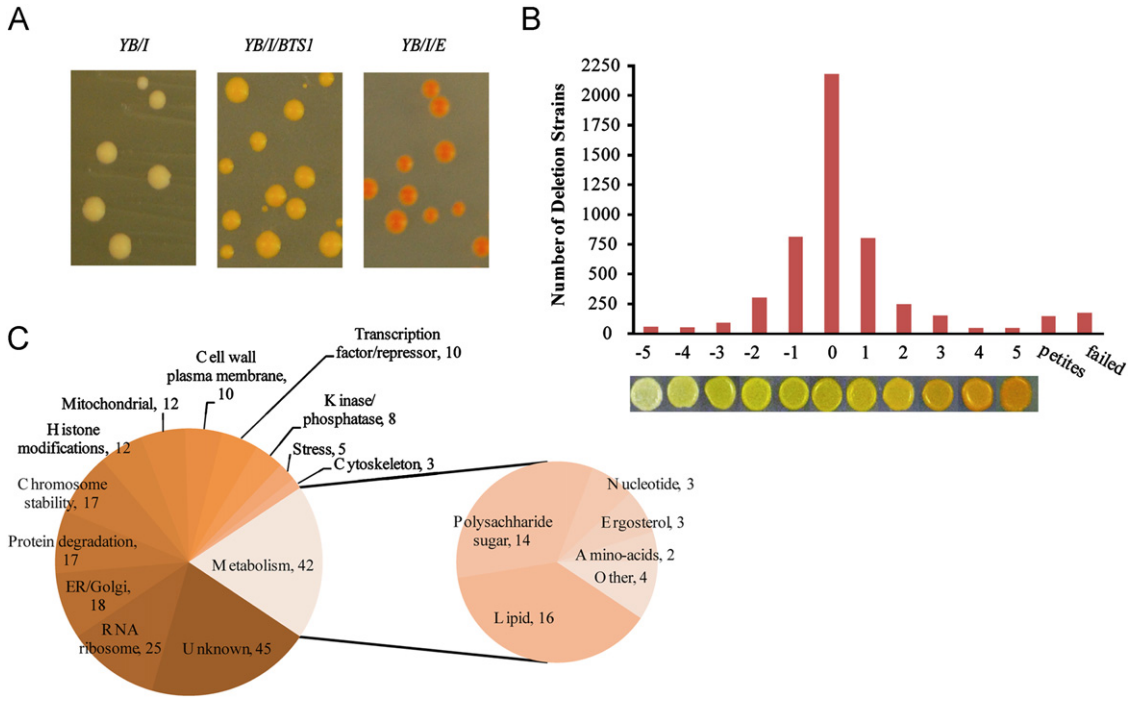

Fig. 2. Screening the yeast deletion collection. (A) Yeast colonies harboring different carotenoid-producing plasmids. (B) Color scale and color distribution of the yeast deletion collection strains harboring the carotenoid-producing plasmid *YB/I/BTS1*. Carotenoid producing strains were grown in liquid medium overnight and spotted onto solid medium. Pictures were taken after 2 day of growth on solid medium. (C) Functional categories of gene deletions with improved carotenoid production (scored 3 and higher).

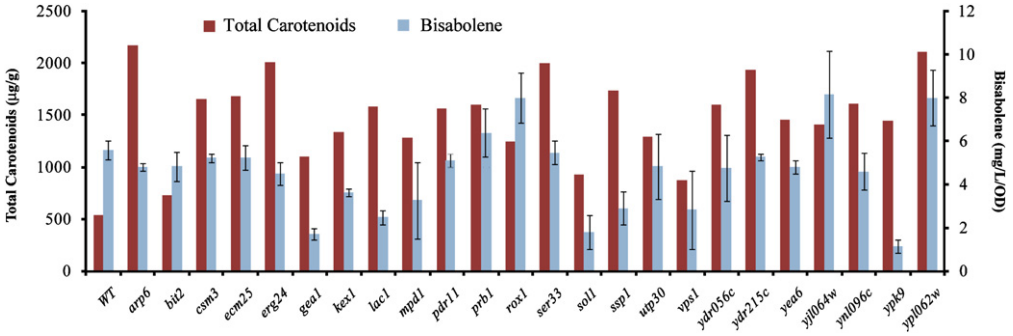

Fig. 3. Re-screening the deletion strains that produced the highest levels of carotenoids. Carotenoid levels as measured by absorbance at 449 nm in the parent strain and the 24 deletion strains selected using the visual screen (red bars). Data are an average of two independent experiments. Bisabolene production in the same deletion strains transformed with *H/B/E* plasmid. Bisabolene levels were measured six days after galactose induction using GC–MS (blue bars). Bisabolene data are average of 3 biological replicas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

makes it a better strain for industrial fermentation (van Dijken et al., 2000; Pereira et al., 2010). It has higher stress resistance with better sugar uptake compared to S288C. CEN.PK2 also has a higher ergosterol content and higher expression of *HMG1* than S288C (Otero et al., 2010). Therefore, we decided to test the characterized gene deletions in this strain background.

We first introduced additional copies of the enzymes known to be flux limiting to determine how much they contributed individually. The addition of *tHMG1* alone increased the production of bisabolene by 2-fold. Expressing an additional copy of *ERG20* further increased the production by 25% (Fig. 4A).

Previous work (Tokuhiro et al., 2009) showed that fusing *ERG20* to *BTS1* increased geranyl geraniol production. Hoping that we could achieve similar increases in flux to bisabolene, we fused *ERG20* to *AgBIS* using a flexible 4 amino acid (GGGS) linker. The C-terminal fusion of *ERG20* to bisabolene (*B*∼*E*) produced almost two-fold more bisabolene than the separate genes (Fig. 4A). Surprisingly, the fusion protein expressed significantly better than the unfused bisabolene synthase (Fig. 4B). On the other hand, cloning an additional gene under the control of the divergent *GAL* promoters negatively affected the bisabolene synthase levels.

To divert the FPP flux from ergosterol synthesis in CEN.PK2, we down-regulated the squalene synthase, *ERG9*, by changing its promoter to that of *CYC1*, a constitutive, weak promoter (JBEI4716) (Mumberg et al., 1995). The combination of fusing *AgBIS* to *ERG20* and down-regulating *ERG9* increased the bisabolene production two-fold (Fig. 4A).

3.4. Testing the gene deletions in CEN.PK2 background

We introduced the four gene deletions that were characterized for high bisabolene production in S288C background to the CEN.PK2 strain with down regulated *ERG9* promoter (JBEI4716) individually and in combination (Fig. 5, strains JBEI 4720–4724). After introducing the plasmid that expresses *tHMG1* along with the *AgBIS*∼*ERG20* fusion (*H*/*B*∼*E*) into these strains, bisabolene

production was measured. Deletion of *prb1* had little effect on bisabolene production, whereas the *rox1* deletion had the greatest effect, increasing titers from 200 mg/L to almost 400 mg/L. Although the combination of these deletions gave rise to higher bisabolene levels than the single deletions, they did not show a true synergistic effect and the bisabolene levels did not increase beyond 450 mg/L. This could be due to the fact that the plasmid maintenance decreased with each additional gene deletion introduced. Mevalonate levels in the same strains were also measured (Fig. 5). Mevalonate accumulated in the medium up to an average of 35 mg/L in those strains with high bisabolene production. These results showed that our carotenoid screen identified useful gene deletions that also increased the bisabolene production as much as 2.5-fold.

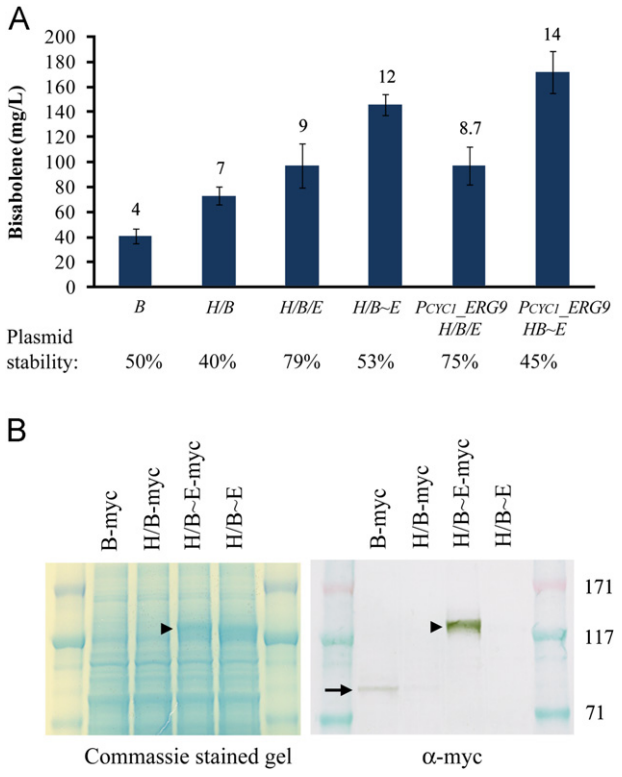

Fig. 4. Fusing bisabolene synthase to FPP synthase encoded by *ERG20*. (A) Bisabolene production in CEN.PK2 and P_{CYC1}*_ERG9* (JBEI4716) strains with plasmids indicated on the *x*-axis. Respective plasmid stabilities are indicated as percent values where the standard deviation is less than 10% in each case. Data are average of 3 biological replicas, repeated at least three times. (B) Immunoblotting for *myc*-tagged versions of *AgBIS* and its fusion with *ERG20* in strain JBEI4716. Arrow points to the AgBIS (85 KDa) and arrowhead indicates the AgBIS~Erg20 (126 kDa) fusion protein. Numbers on top of the bars in the graphs indicate the specific bisabolene and mevalonate production (mg L^{-1} OD^{-1}).

3.5. Preparing a robust strain for large scale production

Bisabolene synthase and the additional copies of the rate limiting enzymes introduced into the parent strain were placed under the control of inducible *GAL* promoters. Expression from *GAL* promoters required galactose, an expensive sugar that is also consumed during the growth. To circumvent galactose consumption and decrease the cost of industrial scale production we deleted *GAL80*, the repressor gene for the *GAL* promoters, making the promoters constitutive. Deletion of *GAL80* did not affect the production when cells were grown in glucose medium (Fig. 6A).

To address the decreased plasmid maintenance in the deletion strains (Fig. 5), we chromosomally integrated the *GAL1*/*GAL10* divergent promoters expressing *tHMG1* and *AgBis*~*ERG20* fusion into the *YPRcδ15* locus, a chromosomal location that was shown to highly express integrated heterologous genes (Flagfeldt et al., 2009). This strain (JBEI4726) produced about 4-fold less bisabolene than the one harboring bisabolene synthase on a high-copy plasmid, suggesting that a single copy of one or more of these integrated genes was not sufficient. Next, we systematically introduced additional copies of the chromosomally-integrated genes using plasmids to determine whether production levels were restored (Fig. 6A). An additional copy of bisabolene synthase on a multi-copy plasmid restored the bisabolene titer to that of the strain harboring only the plasmid-borne gene. Having additional copies of *ERG20* or *tHMG1*, however, did not enhance the production further. The plasmid maintenance in JBEI4726 was greater than 95% (Fig. 6B).

Next we deleted *ROX1*, *YJL064W*, and *YPL062W* individually and in combination in the strain JBEI4726, creating strains JBEI4727 to JBEI4733. The plasmid *pRS-AgBIS* was better maintained in these strains than the plasmid H/B/E in strains JBEI4716 to JBEI4724. Strains with double gene deletions (JBEI4730, JBEI4731, and JBEI4732) showed higher production than the single gene deletions (JBEI4727, JBEI4728, JBEI4729), though the amounts of bisabolene were not additive (Fig. 6B). Mevalonate levels in cultures of these strains were also different from those

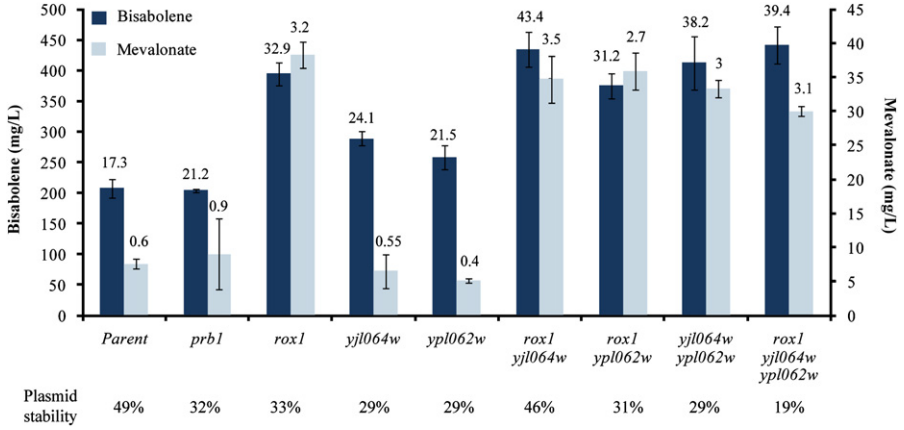

Fig. 5. Testing the characterized gene deletions in the CEN.PK2 background. Bisabolene production (dark blue bars) in *H/B*~*E* transformed strains: Parent (JBEI4716), *prb1* (JBEI4720), *rox1*(JBEI4717), *yjl064w* (JBEI4718), *ypl062w* (JBEI4719), *rox1 yjl064w* (JBEI4721), *rox1 ypl062w* (JBEI4722), *yjl064w ypl062w* (JBEI4723), *rox1 yjl064w ypl062w* (JBEI4724). Mevalonate levels in the same strains (light blue bars). Numbers on top of the bars in the graphs indicate the specific bisabolene and mevalonate production (mg L^{-1} OD^{-1}). Data are average of 3 biological replicas, repeated at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

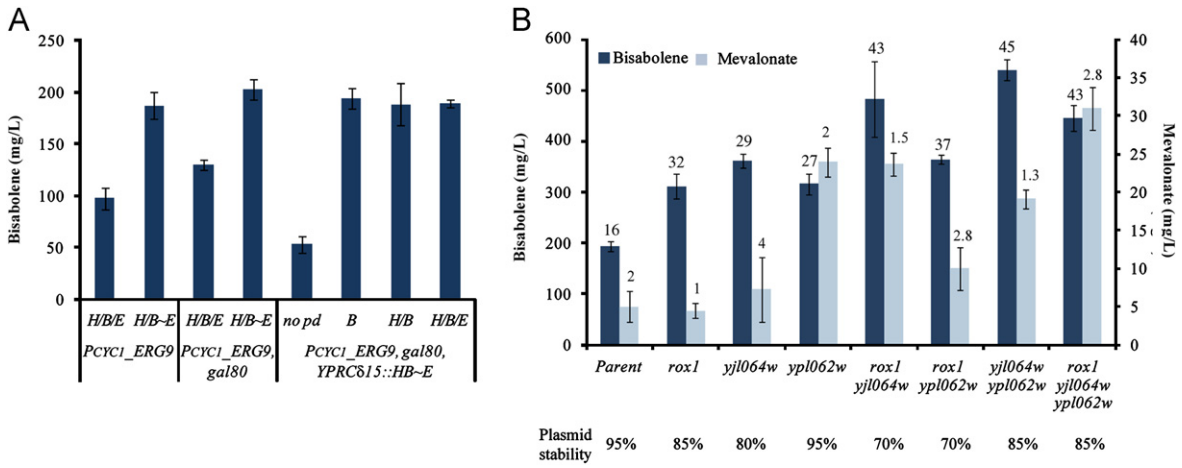

Fig. 6. Preparing the strains for bioreactor studies. (A) Bisabolene production by P_{CYC1}_ERG9 (JBEI4716) with the *gal80* deletion (JBEI4725) and introduction of the *H/B*~*E* genes into the genome (JBEI4726). Name of the plasmid in each strain is indicated on the *x*-axis. 'No pd' stands for no plasmid. (B) Bisabolene (dark blue bars) and mevalonate (light blue bars) production by JBEI4726 with the indicated gene deletions: *rox1* (JBEI4727), *yjl064w* (JBEI4729), *ypl062w* (JBEI4728), *rox1 yjl064w* (JBEI4731), *rox1 ypl062w* (JBEI4730), *yjl064w ypl062w* (JBEI4732), *rox1 yjl064w ypl062w* (JBEI4733). All strains harbored the plasmid *pRS-AgBIS*. Percent plasmid maintenance for each strain is indicated below the *x*-axis. Data are average of 3 biological replicas, repeated at least three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

strains in the JBEI4716 background (Fig. 6). Deletion of *ROX1* did not result in accumulation of mevalonate nor did it increase the bisabolene levels as much as it did in the JBEI4716 background. Once again, the double and triple deletions showed similar levels of bisabolene production. However, both the total production and the specific production were higher in this strain background than the strains in JBEI4716 background. Strain *yjl064w ypl062w* (JBEI4732/*pRS-AgBIS*) produced the highest titer of bisabolene with an average of 540 mg/L. Therefore, combination of these two gene deletions identified by our screen led to almost three-fold increase in bisabolene production.

3.6. Testing the strains for production in bioreactors

To select the strain best suited for production in a bioreactor, we tested the high-producing strains in shake flask cultures and measured bisabolene levels through six days of production (Fig. 7A and B). These growth conditions gave rise to higher levels of production resulting in bisabolene titers as high as 800 mg/L. All the strains produced similar levels of bisabolene, as observed when these strains were grown in culture tubes (Fig. 6B). Nevertheless, the strain deleted for *yjl064w* and *ypl062w* (JBEI4732) showed persistently good production, good plasmid maintenance (85%), high cell density at the end of production (Fig. 7A), and moderate levels of mevalonate (Fig. 6), suggesting that the mevalonate was neither limiting nor excessive. Therefore we picked this strain to test in the bioreactor.

Strain JBEI4732 is prototrophic for all amino acids except for tryptophan. To avoid amino-acid supplementation during fermentation and hence to decrease the production cost, we selected TRP revertants of this strain (JBEI4734). Once transformed with a plasmid-borne copy of the bisabolene synthase (*pRS-AgBIS*), this strain produced fairly similar levels of bisabolene to JBEI4732 in the absence of supplemented amino acids (Supplementary Fig. 2). Next, we tested JBEI4734 in a glucose-limited, fed-batch bioreactor study (Fig. 7C and D). This strain grew steadily without accumulation of much ethanol or glucose (Supplementary Fig. 3). Since the bisabolene production peaks during stationary phase in shake-flask experiments (Fig. 7A and B), the bioreactor culture was continued for additional days in the absence of carbon feed. This increased bisabolene levels from 3.2 g/L to 5.2 g/L during the first 24 h. However, longer incubations resulted in decreased bisabolene levels suggesting that there was a significant loss of bisabolene by evaporation throughout the experiment even though dodecane was added into the bioreactor to trap the bisabolene (Newman et al., 2006).

4. Discussion

Screening the yeast deletion collection for carotenoid-producing strains resulted in more than a hundred deletion strains with only four of them showing a modest increase in the bisabolene levels (Fig. 3). The carotenoids measured include phytoene, neurosporene, lycopene, and β-carotene, which are produced at different steps of the carotenoid synthesis pathway (Fig. 1). Therefore, combinatorial increase in these products could be more than a single product, bisabolene. It is also possible that some of the gene deletions only affected the carotenogenic genes, especially those pertaining to mRNA and protein stability (e.g., *arp6* or *kex1*). However, there were several gene deletions that seemed to have down-regulated other metabolic pathways, such as lipid synthesis (*lac1*), amino acid synthesis (*ser33*), or ergosterol synthesis (*erg24*). Since both bisabolene and carotenoid synthases use the same building block, FPP, and have the same energy and co-factor requirements, we originally anticipated these deletions to have similar effects on the production of bisabolene and carotenoids. Surprisingly, while they enhanced carotenoid production almost four-fold, they had either no effect on bisabolene levels (*ser33*) or decreased bisabolene production (*erg24*, *lac1*). Deletions of these metabolic genes are likely to result in major changes to metabolic pathways (Szappanos et al., 2011) affecting the redox balance of the cell. Under such conditions, carotenoid production may be more favorable than bisabolene production due to the antioxidant properties of carotenoids (Stahl and Sies, 2003; Palozza, 2005). Moreover, additional functions of carotenoids in cell cycle regulation and induction of detoxifying enzymes can give these strains an edge in production as well (Stahl et al., 2002). Despite these discrepancies between the carotenoid and bisabolene production, our screen was successful in identifying gene deletions that improved bisabolene production.

When these gene deletions were introduced into a different *S. cerevisiae* background (CEN.PK2), their effects on bisabolene production were reproduced except for *prb1*. Prb1 is a vacuolar proteinase and its deletion may increase flux through the pathway

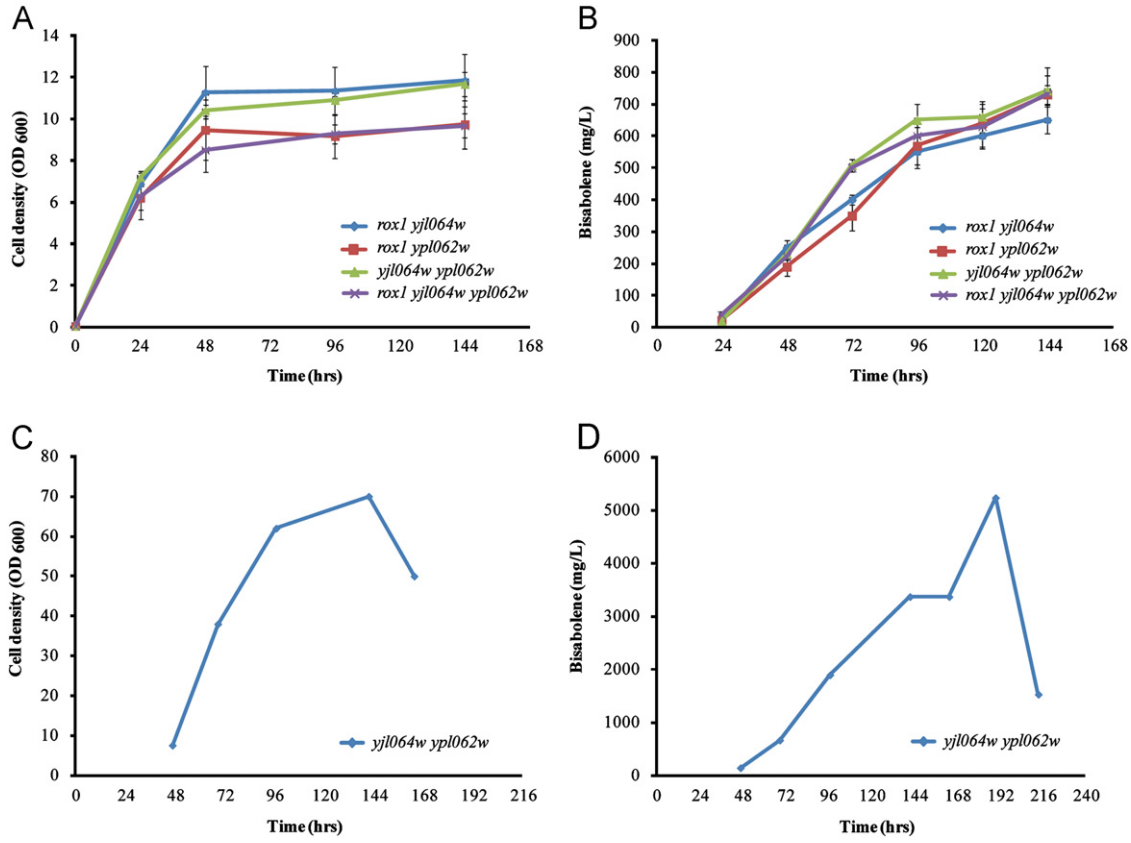

Fig. 7. Testing the strains in bioreactors. (A) Growth and (B) bisabolene production profile of the top four producing strains (JBEI4730–JBEI4733 harboring *pRS-AgBIS*) in shake flask. Data are average of 3 biological replicas. (C) Growth and (D) bisabolene production of *yjl064w ypl062w* (JBEI4732 harboring *pRS-AgBIS*) in bioreactor.

by stabilizing certain proteins in only the BY4741 strain background. It is also possible that heterogeneity in this particular strain background contributed to the final increase in bisabolene levels, independent of the *prb1* deletion.

Two gene deletions, *yjl064w* and *ypl062w*, significantly increased bisabolene production in both strain backgrounds. These genes do not have any previously characterized functions. However, since they increased the production of both bisabolene and carotenoids, we believe they increased the flux through the pathway rather than acting through the bisabolene synthase or carotenogenic enzymes. The *ypl062w* mutant was previously shown to have lower levels of glycogen (Giaever et al., 2002). In this mutant, less carbon is sequestered in the form of glycogen, presumably making more acetyl-coA available for other metabolic pathways, including the mevalonate pathway as suggested by the increased mevalonate levels in this mutant (Fig. 6B).

The other impactful gene deletion identified by our screen was *rox1*. Rox1 is a transcription factor that represses many enzymes in the mevalonate pathway and ergosterol synthesis (Henry et al., 2002), including *HMG2*, the other HMGR in yeast (Deckert et al., 1998). Deletion of *ROX1* increased the mevalonate levels in cells and subsequently increased bisabolene production two-fold (Fig. 5). However, *rox1* deletion did not result in mevalonate accumulation in strains engineered for bioreactor studies even though bisabolene production was enhanced (Fig. 6B). This discrepancy was not specific to the *rox1* strain. The bisabolene and mevalonate levels showed differences between the galactose-inducible strains (Fig. 5) and the strains engineered for bioreactor (Fig. 6B). This could be due to the differences in galactose and glucose metabolisms and how they affect the mevalonate pathway. Additionally, different gene deletions can act differently when the promoters of the engineered genes are regulated. In both cases though, double and triple deletion strains produced high levels of both bisabolene and mevalonate suggesting that the flux through the pathway was strong in all these strains and not all of the pathway intermediates were converted to bisabolene. Like most terpene synthases, AgBIS is a very slow enzyme and does not express as a soluble protein very well (Peralta-Yahya et al., 2011). Fusing it to *ERG20* increased its expression level, which in turn increased bisabolene production by two-fold. Further engineering of AgBIS to improve its catalytic rate is crucial for additional improvement of bisabolene production.

We picked the double deletion strain *yjl064w ypl062w* (JBEI4734) to test in bioreactors. A typical shake flask experiment is performed in batch, where all the growth medium components are added initially, and no additional growth medium is added throughout the growth. Under such conditions, the majority of the bisabolene production occurred during stationary phase when all the glucose and ethanol were exhausted. In the fed-batch bioreactor studies, however, glucose was added gradually to achieve high cell density and to avoid accumulation of ethanol and other byproducts. Therefore, the cells in the fed-batch fermentation never experienced stationary phase. To simulate the stationary phase observed in shake-flask experiments, we continued the bioreactor run for two more days after all the carbon was exhausted. To our surprise the titers increased almost two-fold in the first day. However, additional days of incubation showed decreased levels of bisabolene, presumably due to evaporation. Further improvement in product recovery both from the culture broth and vapor phase is critical to harvest the entire amount of bisabolene produced in the bioreactor. Additional process development is also necessary to determine the optimal conditions to produce high titers of bisabolene. Nonetheless, the bioreactor experiment showed that the engineered strain would continue to produce high levels of bisabolene at industrial scales.

The results of our system-level screen showed that yeast has great potential for metabolic engineering to improve isoprenoid production further. However, we will not be able to utilize this potential fully until after we engineer terpene synthases with better catalytic properties.

Author contributions

B.O., H.B., T.S.L., and J.D.K. designed the experiments. B.O. and H.B. performed the experiments. B.O., H.B., T.S.L. and J.D.K. wrote the manuscript.

Competing financial interests

JDK has financial interest in Amyris Biotechnologies and LS9 Inc. The remaining authors declare no competing financial interest.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.07.010.

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