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Enhanced isoprene biosynthesis in *Saccharomyces cerevisiae* by engineering of the native acetyl-CoA and mevalonic acid pathways with a push-pull-restrain strategy

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ABSTRACT

To explore the capacity of isoprene production in *Saccharomyces cerevisiae*, a rational push-pull-restrain strategy was proposed to engineer the mevalonic acid (MVA) and acetyl-CoA pathways. The strategy can be decomposed into the up-regulation of precursor supply in the acetyl-CoA module and the MVA pathway (push-strategy), increase of the isoprene branch flux (pull-strategy), and down-regulation of the competing pathway (restrain-strategy). Furthermore, to reduce the production cost arising from galactose addition and meanwhile maintain the high expression of Gal promoters, the galactose regulatory network was modulated by Gal80p deletion. Finally, the engineered strain YXM10-*ispS*-*ispS* could accumulate up to 37 mg/L isoprene (about 782-fold increase compared to the parental strain) under aerobic conditions with glycerol-sucrose as carbon source. In this way, a new potential platform for isoprene production was established via metabolic engineering of the yeast native pathways.

1. Introduction

With the development of industrial revolution, the demand for rubber has rapidly increased since the beginning of the 20th century. The current global production of rubber is approximately 10 million tons per annum (IRSG, 2010), 60% of which is derived from synthetic rubber (SR). As an important monomer for SR synthesis, isoprene (C_5H_8) is mainly derived from C5 petroleum distillate. However, a number of fatal drawbacks in this petroleum-based production process obstruct the sustainable development of the isoprene industry, such as high energy consumption, high refining cost, increasing price of the raw material, and rather low yield (less than 1.7%). Currently, great advances have been achieved in metabolic engineering and synthetic biology (Ajikumar et al., 2010; Keasling, 2012; Leonard et al., 2010), which makes the engineering of microorganisms for isoprene biosynthesis a potential alternative to the chemical route.

Over the past years, studies on microbial isoprene biosynthesis have mainly focused on bacteria such as *Escherichia coli*, *Bacillus subtilis* and *cyanobacteria* (Lindberg et al., 2010; Miller et al., 2001; Xue and Ahring, 2011; Zhao et al., 2011). Improvement has been achieved through regulation of the native methyl-erythritol-4-phosphate (MEP) pathway, such as overexpressing the native key enzymes 1-deoxyxylulose-5-phosphate synthase (DXS), 1-deoxyxylulose-5-phosphate reductoisomerase (DXR), and isopentenyl diphosphate (IPP) isomerase (IDI) (Lv et al., 2013). Moreover, heterogenous mevalonic acid (MVA) pathway has been introduced into *E. coli* to further enhance the precursor supply (Yang et al., 2012; Zurbriggen et al., 2012). However, up to now, little progress has been reported in the biosynthesis of isoprene in eukaryotes.

Saccharomyces cerevisiae is a eukaryotic model organism for genetic engineering and a promising cell factory for biotechnological applications (Nevoigt, 2008). It employs MVA pathway to generate essential isoprenoids such as geranyl, farnesyl and geranylgeranyl derivatives (Jackson et al., 2003; Oswald et al., 2007; Shiba et al., 2007). As an important member of isoprenoids, ergosterol could be accumulated to as high as 5% of the dry cell mass in *S. cerevisiae* (Lamacka and Sajbidor, 1997). Besides, metabolic engineering of *S. cerevisiae* has allowed production of up to 18 mg/L carotenoids, 488 mg/L miltiradiene and 40 g/L amorpha-4,11-diene (Dai et al., 2012; Reyes et al., 2014; Westfall et al., 2012). Therefore, eukaryotic yeast has a high inherent capacity for production of isoprenoids precursors and can be used as a candidate for isoprene biosynthesis. It was reported that heterologous transformation of *S. cerevisiae* with *ispS* of *Kudzu vine* resulted in stable and constitutive production of isoprene hydrocarbons, however, the production was rather low (500 μg/L) (Hong et al., 2012). To enhance the

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isoprene production in yeast, strategies for metabolic regulation and optimization need to be explored.

At present, the most common strategies for improving isoprenoids production in yeast mainly focus on engineering of the MVA pathway: (1) increasing precursor supply by over-expression of the limiting enzyme 3-hydroxyl-3-methylglutaryl-CoA reductase (tHMG1) (Kirby et al., 2008; Shimada et al., 1998); (2) over-expression of upc2-1 (a global transcription factor regulating sterol biosynthesis) (Engels et al., 2008; Ro et al., 2006); (3) down-regulation of the competing metabolic branches. For example, in carotenoid biosynthesis, the squalene and ergosterol fluxes were repressed by adding ergosterol biosynthesis inhibitors, or down-regulated by deleting the squalene synthase gene (*ERG9*) or decreasing *ERG9* gene expression with MET (methionine metabolism) promoter (Asadollahi et al., 2010; Miao et al., 2011; Sun et al., 2007). However, most of the down-regulation strategies proposed for isoprenoids synthesis have focused on balancing the farnesyl pyrophosphate (FPP) pool. Few studies have been conducted to balance the IPP and geranyl diphosphate (GPP) nodes for hemiterpene and monoterpene synthesis (Fischer et al., 2011).

As the precursor of the MVA pathway, the sufficient supply of acetyl-CoA is also important for the accumulation of isoprenoids. To our knowledge, only a few studies on the engineering of acetyl-CoA supply were reported, with main focus on the regulation of acetyl-CoA synthetase (Chen et al., 2010; de Jong-Gubbels et al., 1998). Yun Chen et al. has engineered the acetyl-CoA metabolism in the cytosol of yeast and achieved a four-fold increase in α-santalene production (Chen et al., 2013). However, the rate-limiting step of the acetyl-CoA synthesis pathway is still unclear. Furthermore, the effect of acetyl-CoA pathway engineering on isoprene production remains to be explored.

Targeting at solving the above mentioned problems and enhancing the isoprene production capacity of yeast, a rational push-pull-restrain strategy was proposed for regulation of the native acetyl-CoA and MVA modules in an *ispS*-expressing *S. cerevisiae* strain (Fig. 1). First, the MVA flux was enhanced by over-expression of *tHMG1*, then the isoprene branch flux was up-regulated by over-expression of IPP isomerase gene (*IDI1*) and *ispS*, and meanwhile the undesirable downstream flux was down-regulated by weakening the promoter strength of farnesyl pyrophosphate synthetase gene (*ERG20*). In addition, the acetyl-CoA metabolic pathway was engineered to further enhance the precursor supply by a push strategy and the consequent influence on isoprene production was investigated. Finally, to reduce the production cost and meanwhile maintain the high expression of Gal promoters, the galactose regulatory network was engineered by deletion of GAL80 (a transcriptional repressor involved in transcriptional regulation in response to galactose) and the carbon sources were optimized.

2. Materials and methods

2.1. Strains and media

The *S. cerevisiae* strains used in this study were derived from the wild-type *S. cerevisiae* strain BY4741 (Brachmann et al., 1998). *E. coli* DH5α (Novagen, USA) was used for gene cloning. LB (Luria-Bertani broth) medium with antibiotics (100 μg/mL of ampicillin or 50 μg/mL of kanamycin) was used for cultivation

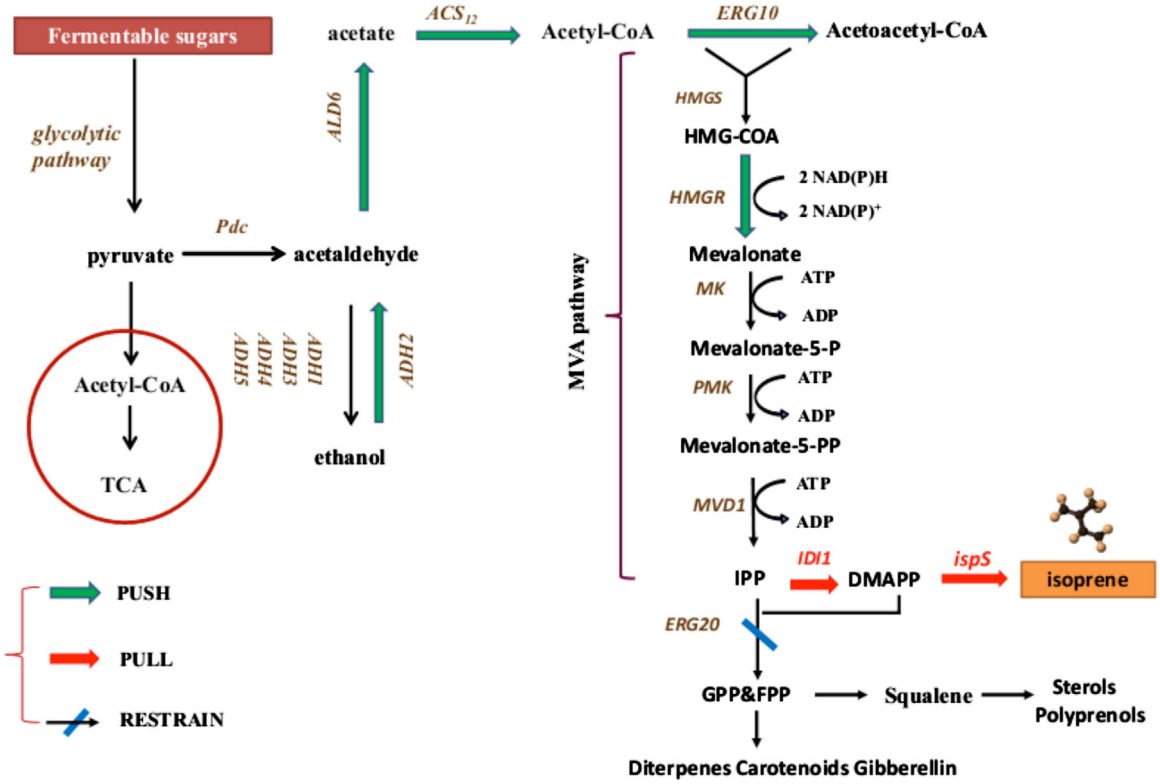

Fig. 1. Schematic representation of biosynthetic pathway for production of isoprene and illustration of the strategies applied for improving production. The green arrows represent the push steps, the red arrows represent the pull steps, the blue bar represents the restrain step and the double blue bar represents the block step. Genes of enzymes: *ADH*1/2/3/4/5, alcohol dehydrogenases; *ALD6*, aldehyde dehydrogenase; *ACS*, acetyl CoA Synthetase; *ERG10*, acetoacetyl-CoA thiolase. *HMGS*, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; *HMGR*, HMG-CoA reductase; *MK/ERG12*, Mevalonate kinase; *PMK/ERG8*, Phosphomevalonate kinase; *MVD1*, Mevalonate pyrophosphate decarboxylase; *IDI1*, IPP isomerase; *ispS*, isoprene synthase; *ERG20*, Farnesyl pyrophosphate synthetase; *BTS1*, Geranylgeranyl diphosphate synthase. Intermediates: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl diphosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
List of primers used in this study.

Primers	Sequence (5′-3′)
ispS-F1	CGCGGATCCATGTGTTCTGTTAGCACT
ispS-R1	CCCAAGCTTTTAACGTTCAAACGGCAGG
ispS-F2	AAATATGCGGCCGCAAAAAATGTGTTCTGTTAGCACT
ispS-R2	GGAAGATCTTTAACGTTCAAACGGCAGG
IDI-F	CGCGGATCCATGACTGCCGACAACAATAGTAT
IDI-R	CTAGCTAGCTTATAGCATTCTATGAATTTGCCT
tHMG1-F	CCCACTAGTAAAAATGGACCAATTGGTGAAAACTGAAG
tHMG1-R	CCCGTCGACTTAGGATTTAATGCAGGTGACG
P_{ERG20}-UP-F	GAAGACAAAGTCGACAATCATTACCACAAGATGAAC
P_{ERG20}-UP-R	AGCT*GGCC*TTGTAGGCCGCTTAGAATACCTCACACTG
P_{ERG20}-Down-F	GAAGAAGCGGCCGCATGGCTTCAGAAAAAGAAATTAGG
P_{ERG20}-Down-R	GTAATGATTGTCGACTTTGTCTTCAGGTGC
ADH2-F	GAAGAAGCGGCCGCATGTCTATTCCAGAAACTCAAAAAG
ADH2-R	ATCGCGAGCTCTTATTTAGAAGTGTCAACAACGTATC
ALD6-F	CGCGGATCCATGACTAAGCTACACTTTGACACTGC
ALD6-R	CCGCTCGAGTTACAACTTAATTCTGACAGCTTTT
ERG10-F	GAAGAAGCGGCCGCATGTCTCAGAACGTTTACATTGTATCG
ERG10-R	ATCGCGAGCTCTCATATCTTTTCAATGACAATAGAGGA
ACS2-F	CGCGGATCCATGACAATCAAGGAACATAAAGTAGT
ACS2-R	CCGCTCGAGTTATTTCTTTTTTTGAGAGAAAAATTG
PMRI-35-ΔLPP1-F	CCCAGAAACTCAAAAAGGTGTTTCTAGATGTAGGCCTCTTCGCTATTACGC
PMRI-35-ΔLPP1-R	CCTTGTAGACGGTGATACCAGCGAATTCGCTTTTGTTCCCTTTAGTGATCCGT
GAL80::LEU-F	ATTTACCGGCGCACTCTCGCCCGAACGACCTCAAAATGTCTGTGCGGTATTTCACACCG
GAL80::LEU-R	TTTATAACGTTCGCTGCACTGGGGGCCAAGCACAGGGCAAGATTGTACTGAGAGTGCAC

of recombinant *E. coli*. YPD (1% yeast extract, 2% peptone and 2% D-glucose) was used for cultivation of BY4741 and YXM derived strains (YXM1-10). YPD medium containing 200 μg/mL geneticin (G418) was used for selection of the yeast strains with *KanMX* marker. SD-LEU (synthetic complete drop-out medium with 2% glucose without leucine) was used for selection and cultivation of recombinant strains harboring LEU marker. Synthetic complete drop-out medium without uracil and with different carbon sources were used for cultivation of recombinant strains harboring pESC-URA (Stratagene) derived plasmids. The media and the corresponding carbon sources used in this work are listed as following: SD-URA (2% glucose), SG-URA (2% galactose), Ss-URA (2% sucrose), Sg-URA (2% glycerol), SDg-URA (1% glucose and 1% glycerol) and Ssg-URA containing different ratios of sucrose and glycerol (2% sucrose, 1% sucrose + 1% glycerol, 0.8% sucrose + 1.2% glycerol, 0.6% sucrose + 1.4% glycerol, or 0.4% sucrose + 1.6% glycerol). Referring to SD-, SG, Ss-, Sg-, SDg-, Ssg-URA medium, S indicates Synthetic complete drop-out medium, D indicates Dextrose (glucose), G indicates Galactose, s indicates sucrose, g indicates glycerol. In all media, the concentration of carbon source

Table 2
List of strains and plasmids.

Strain	Genotype	Plasmids*	Description	Source
BY4741	*MAT*a, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*	None		Brachmann et al. (1998)
YXM01	BY4741 (HMG1)::*tHMG1*	a: PMRI-11-*tHMG1*	P_{GPD}-*tHMG1*-T_{CYC1}	In this study
YXM02	YXM01 ΔP_{ERG20}::P_{HXT1}	a: PMRI-28-*ERG20*	EGR20^{up}-loxp-PBR322-kanMX-loxp-P_{HXT1}–*EGR20*$_{down}$	In this study
YXM03	YXM01 Δ P_{ERG20}::P_{BTS1}	a: PMRI-29-*ERG20*	EGR20^{up}-loxp-PBR322-kanMX-loxp-P_{BTS1}–*EGR20*$_{down}$	In this study
YXM07	YXM03ΔLPP1::*ADH2-ALD6*	a: PMRI-35-*ADH2-ALD6*	LPP1^{up}-loxp-PBR322-kanMX-loxp–T_{TPS1}-*ALD6*-P_{TEF1}-P_{HXT7}-*ADH2*-T_{PGK1}-LPP1$_{down}$	In this study
YXM08	YXM03ΔTy4::*ERG10-ACS2*	a:PMRI-40-*ERG10-ACS2*	Ty4^{up}-loxp-PBR322-kanMX-loxp–T_{CYC1}-*ACS2*-P_{TEF1}-P_{HXT7}-*ERG10*-T_{ADH1}-Ty4$_{down}$	In this study
YXM09	YXM02 Ty4::*ERG10-ACS2*	a: PMRI-40-*ERG10-ACS2*	Ty4^{up}-loxp-PBR322-kanMX-loxp–T_{CYC1}-*ACS2*-P_{TEF1}-P_{HXT7}-*ERG10*-T_{ADH1}-Ty4$_{down}$	In this study
YXM10	YXM09 Δ*GAL80*::LEU2	a:Δ*GAL80*::LEU2	*GAL80^{up}*-LEU2-*GAL80^{down}*	Our lab
BY4741-*ispS*	BY4741	b: pESC-URA-*ispS*	P_{GAL1}-*ispS*-T_{CYC1}	In this study
YXM01-*ispS*	YXM01	b: pESC-URA-*ispS*	P_{GAL1}-*ispS*-T_{CYC1}	In this study
YXM01-*ispS-IDI*	YXM01	b: pESC-URA-*ispS-IDI*	T_{ADH1}-*ispS*-P_{GAL10}-P_{GAL1}-*IDI*-T_{CYC1}	In this study
YXM01-*ispS-ispS*	YXM01	b: pESC-URA-*ispS-ispS*	T_{ADH1}-*ispS*-P_{GAL10}-P_{GAL1}-*ispS*-T_{CYC1}	In this study
YXM02-*ispS-ispS*	YXM02	b: pESC-URA-*ispS-ispS*	T_{ADH1}-*ispS*-P_{GAL10}-P_{GAL1}-*ispS*-T_{CYC2}	In this study
YXM03-*ispS-ispS*	YXM03	b: pESC-URA-*ispS-ispS*	T_{ADH1}-*ispS*-P_{GAL10}-P_{GAL1}-*ispS*-T_{CYC3}	In this study
YXM07-ispS-ispS	YXM07	b: pESC-URA-ispS-ispS	T_{ADH1}-ispS-P_{GAL10}-P_{GAL1}-ispS-T_{CYC1}	In this study
YXM08-ispS-ispS	YXM08	b: pESC-URA-ispS-ispS	T_{ADH1}-ispS-P_{GAL10}-P_{GAL1}-ispS-T_{CYC1}	In this study
YXM09-ispS-ispS	YXM09	b: pESC-URA-ispS-ispS	T_{ADH1}-ispS-P_{GAL10}-P_{GAL1}-ispS-T_{CYC1}	In this study
YXM10-ispS-ispS	YXM10	b: pESC-URA-ispS-ispS	T_{ADH1}-ispS-P_{GAL10}-P_{GAL1}-ispS-T_{CYC1}	In this study

*a: Used for chromosomal integration, b: used as adherent vectors.

was consistent (2%). All reagents, media components, and antibiotics were purchased from Sangon Biotech (Shanghai, China).

2.2. Plasmid and strain construction

Restriction enzymes, DNA polymerase, DNA ligase and primers used in cloning were purchased from Sangon Biotech (Shanghai, China). Genomic DNA from yeast transformants was prepared using Fungus Genomic DNA Extraction kit (Bioflux Corporation, Tokyo, Japan). Primers used are listed in Table 1. Plasmids and strains constructed are described in Table 2.

Detailed information for plasmids construction is presented in Fig. S1. All these plasmids (used for chromosomal integration) were linearized with either *Sfi*I or *Sal*I and integrated into the yeast genome by electroporation to generate the recombinant strains YXM01-10. Transformants were plated on YPD medium containing G418 (or SD-LEU medium) and the genotypes were verified by PCR using specific primers (Fig. S2, Table. S1). The procedures for the loop out of the KanMX expression module were as described previously (Guldener et al., 1996). The elimination of kan^r marker gene can ensure the repeated use of the disruption cassette (present in all the above plasmids) for several gene disruptions in one strain.

To endow the yeast strains with the property of isoprene production, plasmids pESC-URA-*ispS*, pESC-URA-*ispS-IDI1*, and pESC-URA-*ispS-ispS* were constructed and transformed into recombined strains YXM01-10.

2.3. Analysis of isoprene by GC-MS

Vapor samples from the sealed headspace of transformant strains were examined by GC-MS. The system consisted of a 6890 N network GC system (Agilent Technologies) and a 5973 network mass selective detector (Agilent Technologies). An HP-5 column (30 m × 0.25 mm; 0.25 μm film thickness; Agilent Technologies) was used, with helium as the carrier gas. The following oven temperature program was carried out: the initial temperature was 40 °C, then increased from 40 °C to 100 °C at 5 °C/min, and finally programmed from 100 °C to 290 °C at 20 °C/min. The injector was maintained at 250 °C.

2.4. Quantification of squalene and acetate

Intracellular squalene was extracted following the extraction procedure of Tokuhiro et al. (2009) with modifications: 2 mL of culture was harvested by centrifugation (4000 rpm for 10 min) and 700 μL of 3 M hydrochloric acid was added to the cell pellet followed by vortexing for 5 min at room temperature. Then the mixture was heated for 3 min at 100 °C to lyse the cells and then cooled in ice-bath. After centrifugation, the cell pellet was washed with water and extracted in 2 mL of acetone for 15 min with intermittent vortexing. Finally, all the extracts were pooled and filtrated into new tubes. The analysis of squalene was performed by HPLC (LC 20AT, SHIMADZU) equipped with a Kromasil C18 column (4.6 mm × 150 mm, AKZO NOBEL) and the UV signal was detected at 195 nm. Acetonitrile was used as the mobile phase with a flow rate of 1 mL/min at 40 °C (Lu et al., 2004). For acetate quantification, 2 mL of culture was centrifuged and the supernatant was prepared for test. The same HPLC equipment was applied for acetate analysis. The mobile phase was acetonitrile-0.1 M KH_2PO_4 buffer (pH 2.5) (10:90) with a flow rate of 1 mL/min at 35 °C and the UV signal was detected at 215 nm.

2.5. Isoprene production by recombinant strains in sealed vials

Single colonies of different yeast strains were picked, respectively into 3 mL of SD-URA medium and grown at 30 °C with shaking (200 rpm). Then about 2% of the overnight-grown seed broth was inoculated into sealed vials (17 mL) containing 5 mL fresh SG-URA, SD-URA medium or Ss-, Sg-, SDg-, Ssg-URA to an initial OC_{600} of 0.05 and incubated under the same conditions for 72 h. The sealed vials of cultures were heated to 37 °C with shaking (200 rpm) for 10 min to vaporize isoprene (boiling point 34 °C).

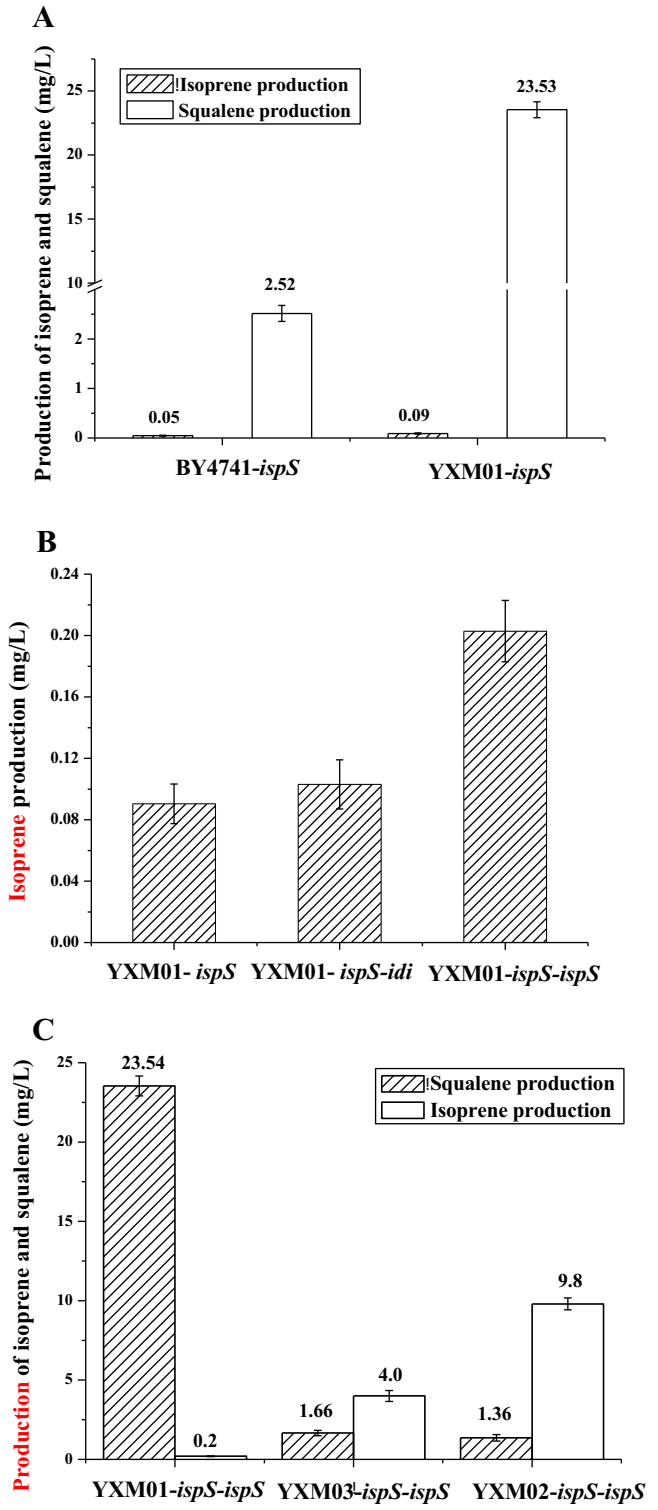

Fig. 2. Enhancement of isoprene production by engineering the MVA pathway with a push-pull-restrain strategy. (A) Over-expression of *tHMG1* (push); (B) over-expression of *IDI1* and *ispS* from vector pESC-URA (pull); (C) down-regulation of ERG20 (restrain). The strains were cultured in SG-URA medium in sealed vials for 72 h. Error bars represent standard deviations from the means of three measurements.

Then 0.8 mL of the gas sample was collected from the headspace of sealed cultures with a gas-tight syringe, and analyzed by a GC system equipped with a flame ionization detector (FID) and an HP-FFAP column (30 m × 0.25 mm, 0.25 μm film thickness). The temperature of oven, injector and detector was held at 80 °C, 180 °C and 180 °C, respectively. The peak area was converted to isoprene concentration using a calibration curve, which was prepared by adding various amounts of isoprene standard (Aladdin, Shanghai, China) into 5 mL SG-URA medium (calibration range: 24 μg–20 mg/L) under the same conditions for cultivation.

2.6. Isoprene production in batch fermentations under aerobic and microaerobic conditions

To investigate the effect of oxygen on isoprene production and yeast growth, batch fermentations were performed with strain YXM10-*ispS*-*ispS* under aerobic and microaerobic conditions. For aerobic fermentation, the airflow was 100 L/h and was sterilized by filtration. For microaerobic fermentation, the strains were cultured in an enclosed bioreactor without airflow supply. SD-URA and Ssg-URA (containing 0.6% sucrose and 1.4% glycerol) were used for seed preparation and fermentation, respectively. Batch fermentations were carried out in 5 L fermentor containing 3 L fermentation medium. The temperature was controlled at 30 °C and the pH was maintained at 5.5 by automatic addition of ammonium hydroxide. The stirring speed was set at 400 rpm. The isoprene concentration in the off-gas was determined by GC every three hours.

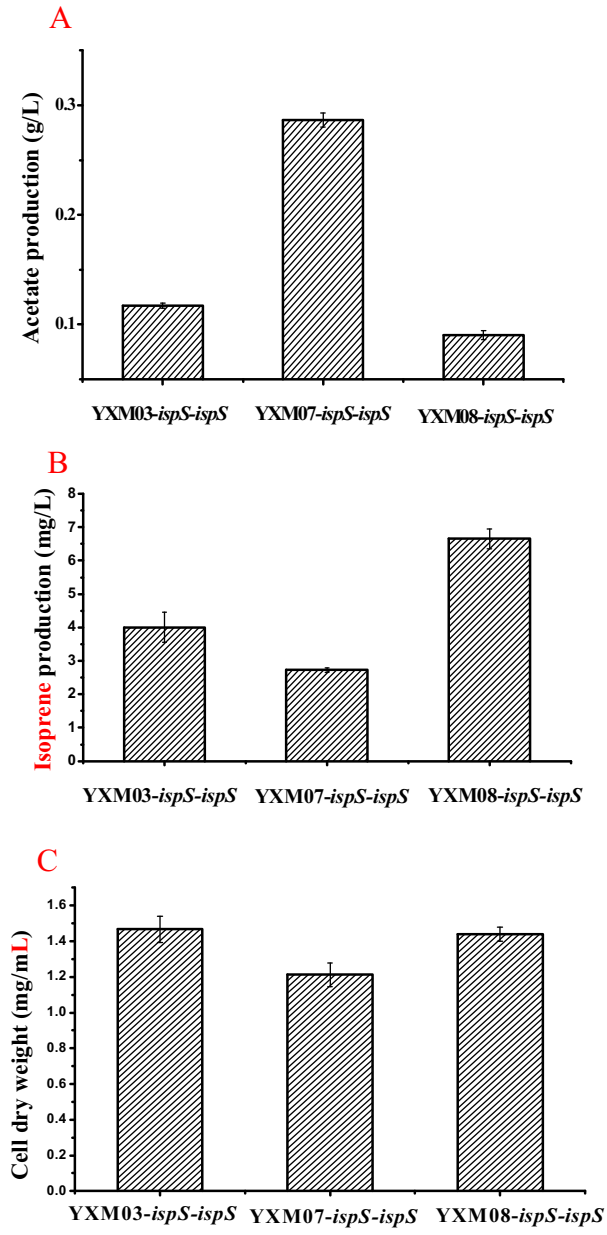

Fig. 3. Effects of genetic modification about acetyl CoA metabolic pathway on acetate (A), isoprene production (B) and cell growth (C). The strains were cultured in SG-URA medium in sealed vials for 72 h. Error bars represent standard deviations from the means of three measurements.

3. Results and discussion

3.1. Construction of an isoprene-producing S. cerevisiae strain

The *ispS* gene from *Populus alba* was introduced into *S. cerevisiae* for isoprene production. Headspace gas from the recombinant strain was examined by GC-MS. The GC spectra showed three peaks with retention times of about 3.93, 4.39 and 4.67 min (Fig. S3. A). Peaks with retention time of 3.93 and 4.39 min were identified as CO_2 and ethanol, respectively, and the 4.67 min peak with dominant mass peaks of 53, 67 and 68 was attributed to isoprene. In addition to the GC-MS analysis, GC-FID measurement was conducted for quantify the isoprene production from BY4741 and BY4741-*ispS*. During microaerobic incubation, BY4741-*ispS* was proven to accumulate 47 μg/L of isoprene within 3 d, while the control strain had no isoprene production (Fig. S3. B). The results confirmed the successful construction of the isoprene synthesis pathway in *S. cerevisiae*.

3.2. Enhanced metabolic flux of MVA pathway through over-expression of tHMG1

To maximize and balance the supply of the crucial metabolic intermediate IPP/DMAPP in the MVA pathway, a rational push-pull-block strategy (Fig. 1) was designed. As the first step, the rate-limiting gene *tHMG1* was over-expressed in BY4741-*ispS*, resulting in the strain YXM01-*ispS*. As shown in Fig. 2A, under microaerobic cultivation in sealed vials, the isoprene production in YXM01-*ispS* was improved by only 91% (90 μg/L vs. 47 μg/L) after over-expression of *tHMG1*, while the production of squalene was increased by 8.4-fold (23.5 mg/L for YXM01-*ispS* vs. 2.5 mg/L for BY4741-*ispS*). It indicated that the competitiveness of the isoprene synthesis branch was very weak compared with the FPP metabolic branch, probably due to the low activity of ISPS and the strong transcriptional regulation of squalene synthesis. Even though the precursor supply of MVA pathway can be enhanced by the over-expression of *tHMG1* (Pitera et al., 2007), the accumulated mevalonic acid might be lost to the competing metabolic pathway. Therefore, the next step was to reorient the enhanced MVA flux towards the target isoprene metabolic pathway. For this purpose, the pull and block strategies composed of enhancing the isoprene branch flux and weakening the competing ERG20 metabolic flux, respectively were employed as following.

3.3. Enhanced metabolic flux of the isoprene synthesis branch by over-expression of IDI1 and ispS

The synthesis of isoprene from IPP is a two-step process: IPP is first isomerized to DMAPP by *IDI1*, and then further catalyzed by *ispS* to generate isoprene. To pull the IPP flux more towards the synthesis of isoprene, *IDI1* and *ispS* were separately over-expressed. It turned out that over-expression of *IDI1* in YXM01-*ispS*-*IDI* did not improve isoprene production, while double-copy insertion of *ispS* in pESC-URA resulted in a 122% improvement (0.2 mg/L

in YXM01-*ispS-ispS* vs. 0.09 mg/L in YXM01-*ispS*) (Fig. 2B). These results indicated that the production of isoprene was limited by the expression of *ispS* rather than *IDI1*.

3.4. Restrained squalene synthesis by down-regulation of ERG20

In *S. cerevisiae*, as an important metabolic node, IPP/DMAPP does not only serve as precursor for isoprene, but can also be converted to FPP by ERG20. To eliminate flux loss to the competing isoprenoid pathway, ERG20 should be down-regulated. In a pretest, we have investigated the strength of a series of promoters for enzymes involved in isoprenoids biosynthesis and glycometabolism using carotene as an indicator, and found that P_{HXT1} and P_{BTS1} were weaker than the native promoter of *ERG20* (Xie et al., Unpublished results). The subsequent replacement of P_{ERG20} with P_{HXT1} and P_{BTS1} by homologous recombination (as shown in Fig. S4. A) resulted in strains YXM02-*ispS-ispS* and YXM03-*ispS-ispS*, respectively. The effects of promoter replacement on isoprene production and cell growth were investigated. As shown in Fig. S4. B, the growth of both mutants was decreased. This was probably caused by the fact that the decrease of FPP repressed the synthesis of isoprenoids such as sterols, dolichols and geranylgeranyl diphosphate, which play important roles in membrane structure, cell-wall synthesis and protein prenylation (Oswald et al., 2007; Szkopinska et al., 1997). Notably, after replacing the promoter of ERG20 with P_{BTS1} and P_{HXT1}, the production of squalene dropped to about 1/14-1/17 (23.54 mg/L for YXM01-*ispS-ispS*, 1.66 mg/L for YXM03-*ispS-ispS*, 1.36 mg/L for YXM02-*ispS-ispS*), while the production of isoprene accumulated to 4.0 and 9.8 mg/L (increased by 20 and 49 folds), respectively (Fig. 2C). The results indicated the FPP pool was successfully restrained and down-regulation of ERG20 can be regarded as an effective means for hemiterpene synthesis.

3.5. Further enhanced isoprene production by increasing acetyl-CoA supply

As the upstream building block of the MVA pathway, sufficient supply of acetyl-CoA is essential for accumulation of IPP and DMAPP so as to improve isoprenoids production. In the acetyl-CoA pathway, acetaldehyde is catalyzed by *ALD6* to generate acetate. To orient more acetaldehyde towards acetate, *ALD6* (cytosolic aldehyde dehydrogenase gene) was co-expressed with *ADH2* (alcohol dehydrogenases) under the control of the constitutive *TEF1* promoter, generating the strain YXM07. It was shown that the 3-day acetate production of YXM07-*ispS-ispS* was increased to 2.4 folds of that of YXM03-*ispS-ispS* (Fig. 3A), while both the isoprene production (Fig. 3B) and the biomass (Fig. 3C) declined. A previous study reported that overproduction of *ALD6* increased the acetate level and resulted in a 30–40% decrease in amorphadiene production (Shiba et al., 2007). From both results, it can be concluded that the increase of acetate level does not necessarily lead to accumulation of downstream isoprenoids. One possible explanation might be that the downstream pathway was limited and the increased acetate could thus not be converted into acetyl-CoA.

To test whether the metabolism of acetate was restrained or not, *ACS2* (acetyl CoA synthase gene) and *ERG10* (acetoacetyl-CoA thiolase gene) were co-overexpressed under the control of P_{TEF1} and P_{HXT7} and then integrated into the chromosome of YXM03, resulting in YXM08. As shown in Fig. 3, the production of acetate decreased in YXM08-*ispS-ispS* (Fig. 3A) while isoprene accumulated to 6.65 mg/L, showing a 60% improvement over YXM03-*ispS-ispS* (4.0 mg/L) (Fig. 3B). Similarly, *ACS2* and *ERG10* were also co-expressed and integrated into the chromosome of YXM02, resulting in the strain YXM09-*ispS-ispS*. The production was increased to 11.14 mg/L. These results suggested that the utilization of acetate was the main limiting step in the acetyl-CoA metabolic pathway

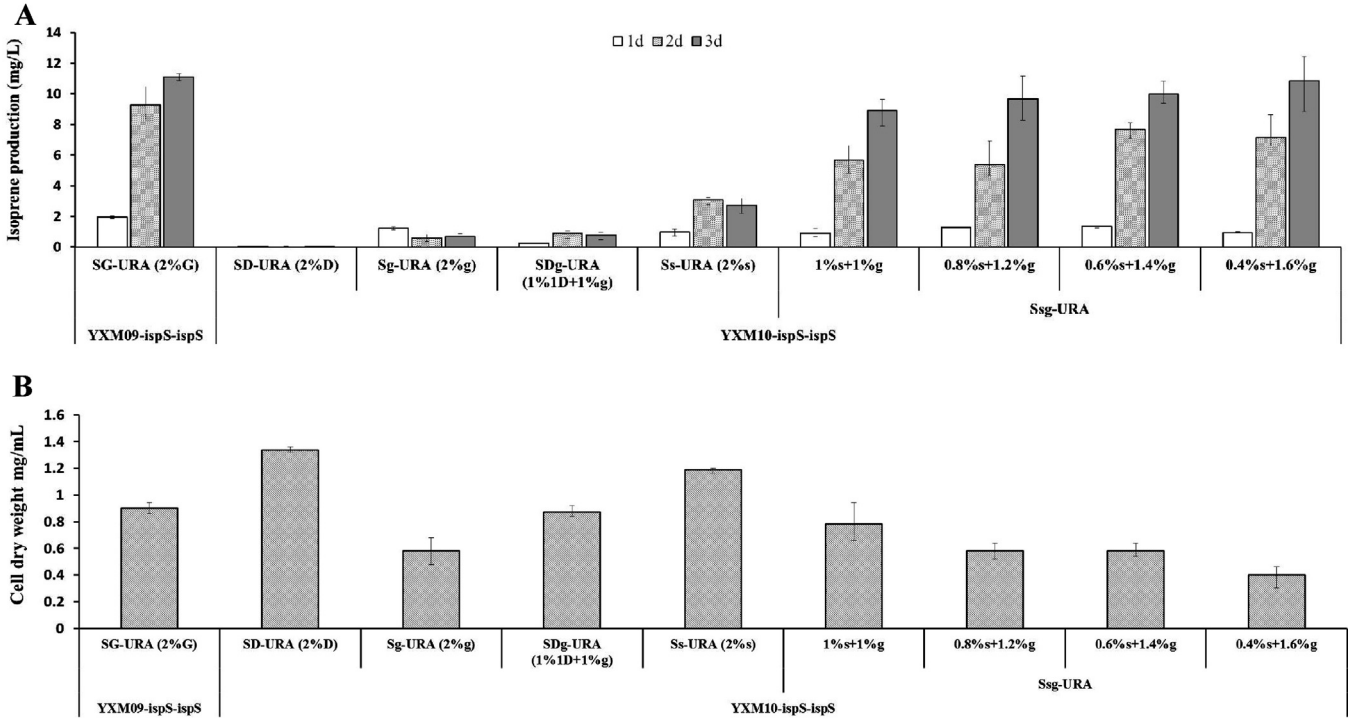

Fig. 4. Effects of GAL80 deletion and carbon source optimization on isoprene production (A) and cell growth (B). YXM09-*ispS-ispS* was cultured in SG-URA medium. YXM10-*ispS-ispS* was modified from YXM09-*ispS-ispS* by knocking out GAL80 and cultured in different carbon sources. SD-URA: synthetic complete drop-out medium with 2% glucose without uracil; SG-URA: synthetic complete drop-out medium with 2% galactose without uracil; Ss-, Sg-, SDg-, Ssg-URA: the first S indicates Synthetic complete drop-out medium, D indicates Dextrose (glucose), s indicates sucrose, g indicates glycerol. In all media, the concentration of carbon source was consistent (2%). The strains were cultured in sealed vials for 72 h.

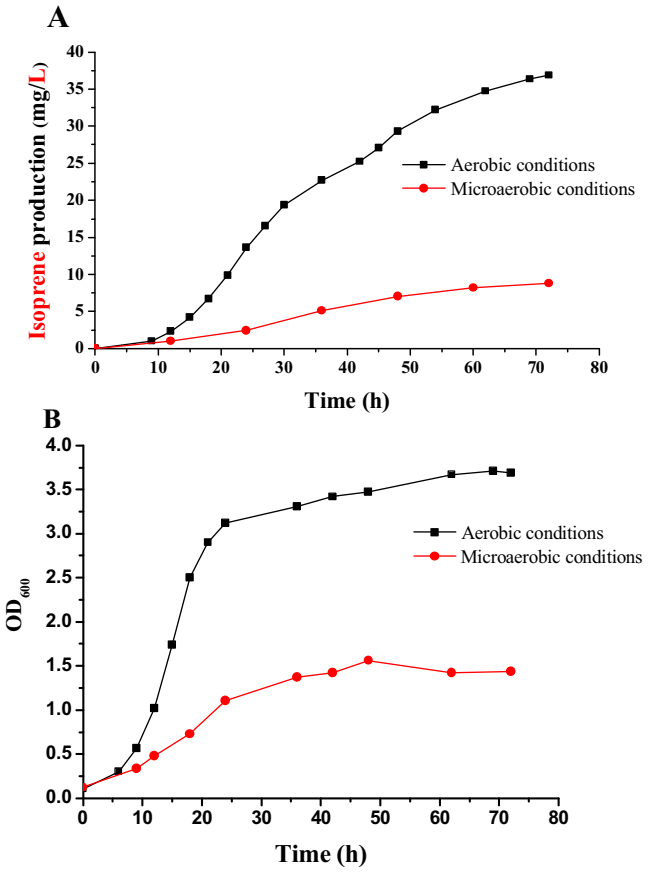

Fig. 5. Time courses of isoprene production (A) and growth (B) of the strain YXM10-*ispS-ispS* during batch fermentation under aerobic conditions and microaerobic conditions. This strain was cultivated in Ssg-URA (0.6%S + 1.4%g) for 3 days. Experiment conditions were described in Section 2.7.

and that the sufficient supply of acetyl-CoA in the cytoplasm was essential for the accumulation of isoprene.

3.6. Carbon source optimization towards reduced cost

Galactose was used as the carbon source and inducer for Gal promoter in order to maintain high expression of ISPS at the beginning of the study. The high cost of galactose is a fatal drawback for this process. To eliminate this disadvantage, we tentatively replaced GAL promoters with the strong constitutive promoters (P_{HXT7} and P_{TEF1}). It was found that the isoprene production in both YXM08-*ispS-ispS* (P_{HXT7}-P_{TEF1}) and YXM09-*ispS-ispS* (P_{HXT7}-P_{TEF1}) decreased (Fig. S5), which might be resulted from the relatively low strength of P_{HXT7}-P_{TEF1} compared with the inducible GAL promoters. Another contributor to the sharp decrease of isoprene production in YXM09-*ispS-ispS* (P_{HXT7}-P_{TEF1}) was the failure in down-regulation of ERG20 since P_{HXT1} was kept active by the high concentration of glucose in SD-URA (Mosley et al., 2003).

The expression of GAL genes is regulated by Gal4p, a transcriptional regulator involved in galactose metabolism. In the absence of galactose, Gal80p, encoding a transcriptional repressor involved in transcriptional regulation in response to galactose, binds to the transcription factor Gal4p in the form of a dimer, which consequently prevents Gal4p from activating GAL gene expression (Peng and Hopper, 2002; Pilauri et al., 2005). After deletion of GAL80, the genes under control of P_{Gal} could be expressed in galactose-free medium. Besides, Gal4p is also regulated by glucose metabolism. When cells are grown with glucose as carbon source, GAL4 transcription is reduced to roughly 1/5 (Griggs and Johnston, 1991). Moreover, glucose-limited conditions can also repress the activation of P_{HXT1} (Mosley et al., 2003). Based on these, GAL80 gene was knocked out in YXM09-*ispS-ispS* to generate YXM10-*ispS-ispS* and glucose-limited culture media were optimized.

As shown in Fig. 4A, glycerol and sucrose were found to be better carbon sources than glucose in terms of isoprene production. Different ratios of sucrose and glycerol (1% s + 1% g, 0.8% s + 1.2% g, 0.6% s +1.4% g, 0.4% s + 1.6% g) were therefore investigated. As a result, YXM10-*ispS-ispS* produced similar amount of isoprene in Ssg-URA medium as compared to that of YXM09-*ispS-ispS* in SG-URA medium (Fig. 4A). On the other hand, the strain showed good growth in sucrose and glucose, while addition of glycerol reduced the cell growth (Fig. 4B). To balance cell growth and isoprene production, Ssg-URA containing 0.6% sucrose and 1.4% glycerol was chosen as the optimal medium.

3.7. Effect of oxygen supply on isoprene production in batch fermentations

Oxygen supply was found to be an important influencing factor for squalene production (Fig. S6). To check whether it also affects the production of isoprene, batch fermentations were carried out under microaerobic and aerobic conditions using the recombinant strain YXM10-*ispS-ispS*. It turned out the isoprene production

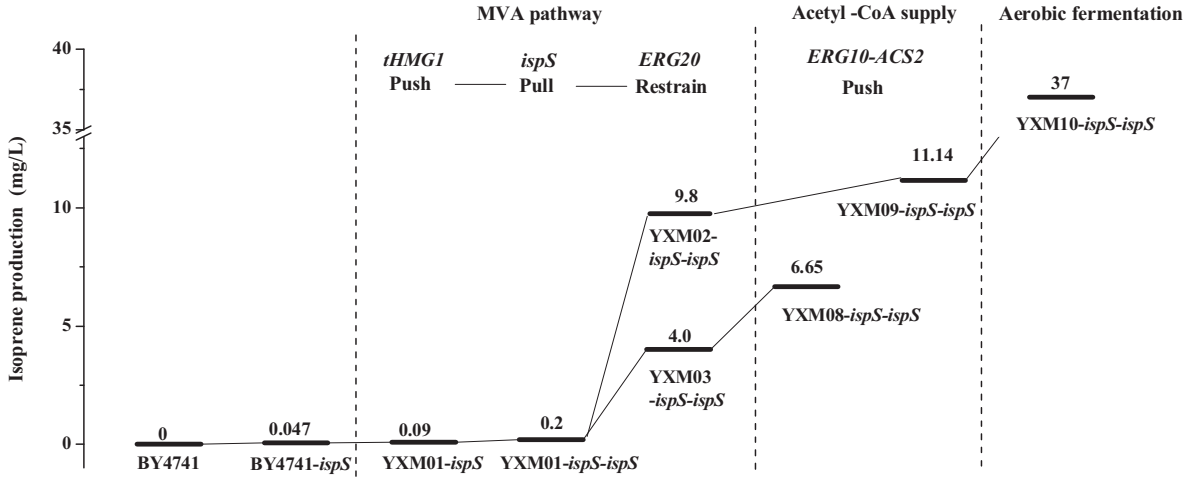

Fig. 6. Improvement of isoprene production in recombinant strains by engineering the MVA and acetyl-CoA pathways using the push-pull-restrain strategy and batch fermentation under aerobic conditions.

under aerobic conditions accumulated to 37 mg/L (Fig. 5A), about 3–4 folds of that in microaerobic conditions, indicating that oxygen was important for the synthesis of isoprenoids. The reason might be: on one hand, microaerobic conditions resulted in low growth rate (Fig. 5B); on the other hand, more ATP, NADH and NADPH were released under aerobic conditions, which were essential for enzyme-catalyzed reactions and thus affected the accumulation of metabolites.

In conclusion, in this work we applied a push-pull-restrain strategy for MVA pathway and acetyl-CoA pathways to improve the isoprene production (Fig. 6). As a result, the production of isoprene was increased from 0.047 mg/L to 37 mg/L (782-fold improvement), demonstrating the effectiveness of these strategies. In recent years, studies on isoprene biosynthesis in bacteria have achieved a lot of success. The production of isoprene in heterologous *Escherichia coli*, *Bacillus subtilis* and *cyanobacteria* has been improved up to about 180 mg/g, 15 μg/g and 250 μg/g dry cell weight, respectively (Bentley et al., 2014; Xue and Ahring, 2011; Yang et al., 2012). Our results suggest yeast can also be used as a potential platform for isoprene synthesis, even though the production (25 mg/g dry cells) at the moment is still lower than that in *E. coli*. ISPS modification towards higher catalytic activity and fed-batch fermentation for increased biomass might be additional approaches to improved isoprene production aside from metabolic engineering.

In addition to product yield, cost of raw material is also a critical factor for commercial isoprene production. The combination of sucrose and glycerol was found to be the optimal carbon source for isoprene production in the yeast platform established in this study. To make bioisoprene more economically competitive and attractive, cheap and renewable resources such as molasses from the sugar industry and waste glycerol from the soap industry can be employed.

4. Conclusions

A push-pull-restrain strategy was proposed and employed for engineering the native MVA pathway and acetyl-CoA pathways in *Saccharomyces cerevisiae* to improve isoprene production. Down-regulation of *ERG20* was found to play an important role in MVA pathway regulation for improved isoprene production, and acetate utilization was identified as the main limiting step in acetyl-CoA metabolism. Besides, GAL80 knockout strains were constructed for cost-effective biosynthesis in *S. cerevisiae*. Finally, the isoprene production was improved by 782 folds. This is for the first time that metabolic regulation of isoprene biosynthesis was reported in *S. cerevisiae*, demonstrating its feasibility as an isoprene production platform.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.06.024.

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