

## Regular Article

# Metabolic engineering of *Saccharomyces cerevisiae* for the overproduction of short branched-chain fatty acids



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## ABSTRACT

Short branched-chain fatty acids (SBCFAs, C4–6) are versatile platform intermediates for the production of value-added products in the chemical industry. Currently, SBCFAs are mainly synthesized chemically, which can be costly and may cause environmental pollution. In order to develop an economical and environmentally friendly route for SBCFA production, we engineered *Saccharomyces cerevisiae*, a model eukaryotic microorganism of industrial significance, for the overproduction of SBCFAs. In particular, we employed a combinatorial metabolic engineering approach to optimize the native Ehrlich pathway in *S. cerevisiae*. First, chromosome-based combinatorial gene overexpression led to a 28.7-fold increase in the titer of SBCFAs. Second, deletion of key genes in competing pathways improved the production of SBCFAs to 387.4 mg/L, a 31.2-fold increase compared to the wild-type. Third, overexpression of the ATP-binding cassette (ABC) transporter *PDR12* increased the secretion of SBCFAs. Taken together, we demonstrated that the combinatorial metabolic engineering approach used in this study effectively improved SBCFA biosynthesis in *S. cerevisiae* through the incorporation of a chromosome-based combinatorial gene overexpression strategy, elimination of genes in competitive pathways and overexpression of a native transporter. We envision that this strategy could also be applied to the production of other chemicals in *S. cerevisiae* and may be extended to other microbes for strain improvement.

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## 1. Introduction

Short branched-chain fatty acids (SBCFAs) are carboxylic acids with four to six carbon atoms and a methyl branch on one or two carbons. They are versatile platform chemicals with high market demands because they are important precursors for the production of a wide range of valuable products in the manufacturing, pharmaceutical and food industries. For example, isobutyric acid (IBA) is used for the production of plasticizers, surfactants, textile auxiliaries, fragrances and flavorings (Lang et al., 2014; Zhang et al., 2011). IBA is also used to synthesize methacrylic acid, which is an industrially important monomer for producing plastics (Millet, 1998; Nagai, 2001). The menthyl ester of isovaleric acid (IVA) is a sedative (Suerbaev et al., 2012) as well as a fragrance (Ahmad et al., 2000), while the amide derivative of IVA is an anticonvulsant

drug (Eadie, 2004). Esters of 2-methylbutanoic acid (2MBA) have applications as a flavoring (Kwon et al., 2000) and in the syntheses of pharmaceutical drugs (e.g. mevastatin, pravastatin and lovastatin) (Weitz-Schmidt, 2002). At present, SBCFAs are produced predominantly by chemical synthesis. For instance, IVA and 2MBA are synthesized industrially via Koch reaction by reacting alkanes or alkenes with strong mineral acids (e.g. H<sub>2</sub>SO<sub>4</sub>, HF or BF<sub>3</sub>) and carbon monoxide (Charles, 1970). These methods are highly dependent on petrochemicals as starting materials and rely on toxic chemicals that may contribute to environmental pollution. With mounting concerns over finite fossil reserves and petrochemical costs, a growing need for clean and renewable sources of chemicals and materials is apparent. With the rapid developments in the fields of metabolic engineering and synthetic biology, sustainable production of bio-based chemicals using microbial cell factories is increasingly becoming a promising alternative to chemical synthesis (Chen et al., 2015; Gronenberg et al., 2013; Nielsen et al., 2013; Peralta-Yahya et al., 2012; Shin et al., 2013; Yu et al., 2014; Zhang et al., 2015a, 2015b). Thus, in this study, we explored

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microbial biosynthesis as an economical and environmentally friendly route for the production of SBCFAs.

Towards our objective of developing high-level microbial SBCFA biosynthesis, we targeted Baker's yeast *Saccharomyces cerevisiae* as the production host for two main reasons. Firstly, *S. cerevisiae* is highly robust and has well-established genetic tools for strain engineering, thus making it an important platform microorganism in industrial biotechnology and metabolic engineering (Le Borgne, 2012; Nevoigt, 2008). Secondly, *S. cerevisiae* possesses a native Ehrlich pathway that enables it to produce three types of SBCFAs, namely isobutyric acid (IBA), isovaleric acid (IVA) and 2-methylbutyric acid (2MBA), via catabolism of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine, respectively (Dickinson et al., 1998; Hazelwood et al., 2008). This eliminates the need to introduce heterologous pathways and avoids any complications associated with expression of heterologous genes including inactive and/or insoluble enzymes. Thus, *S. cerevisiae* provides an ideal platform for metabolic engineering to biosynthesize SBCFAs. However, natural production levels of SBCFAs are very low in *S. cerevisiae* (Lilly et al., 2006). Therefore, in order to develop *S. cerevisiae* into a highly productive microbial cell factory for SBCFA production, we optimized the endogenous Ehrlich pathway for SBCFA biosynthesis by metabolic engineering.

The biosynthesis of IBA, IVA and 2MBA from catabolism of BCAAs in *S. cerevisiae* via the Ehrlich pathway (Hazelwood et al., 2008) is illustrated in Fig. 1. The production of these SBCFAs through the Ehrlich pathway requires three reaction steps, i.e. transamination, decarboxylation and oxidation. *BAT1* and *BAT2* encode transaminases known to convert BCAAs, such as valine, leucine and isoleucine (Lilly et al., 2006), to their respective  $\alpha$ -keto acids. *ARO10*, *THI3*, *PDC1*, *PDC5* and *PDC6* encode decarboxylases that can transform  $\alpha$ -keto acids to short branched-chain aldehydes (Vuralhan et al., 2003). *ALD2*, *ALD3*, *ALD4*, *ALD5* and *ALD6* encode aldehyde dehydrogenases that can oxidize the aldehyde intermediates to the target SBCFA (Pigeau and Inglis, 2007). By overexpressing these 12 genes in the Ehrlich pathway, the flux towards

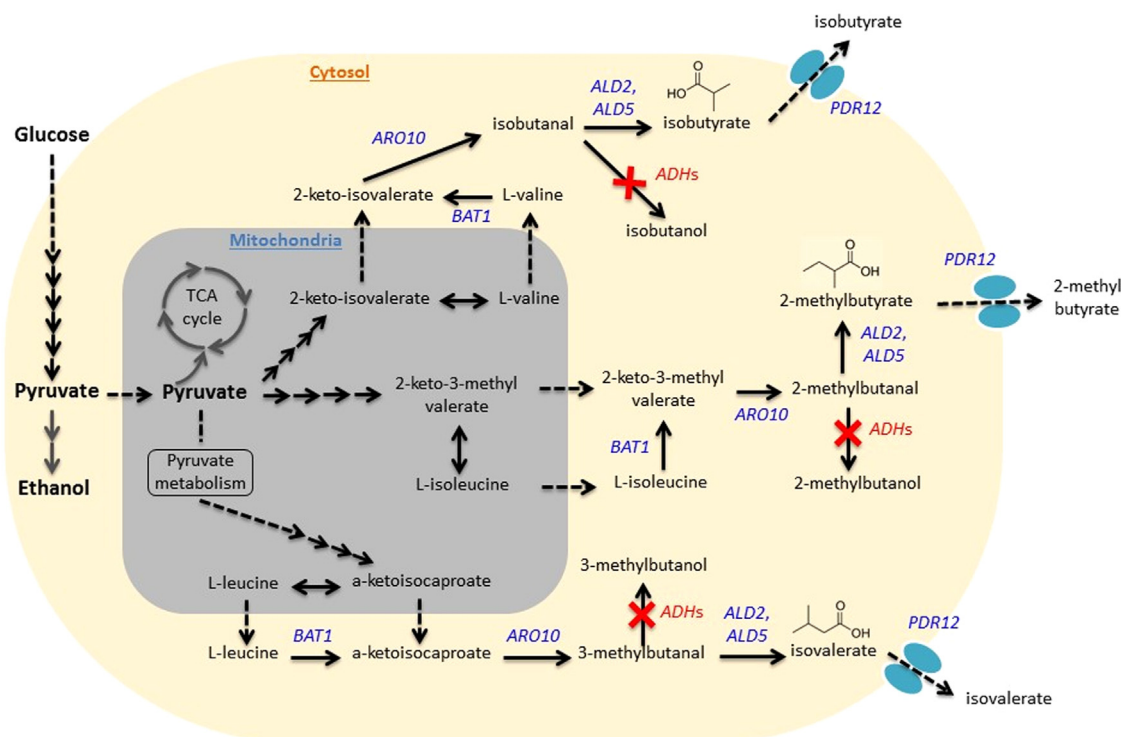
SBCFAs can potentially be increased, thus improving the SBCFA titer.

Successful examples of combinatorial expression optimization of pathway enzymes for improvement of product titer have been reported (Latimer et al., 2014; Xu et al., 2013, 2014; Zhang et al., 2015a, 2015b). Herein, we systematically investigated the influence of overexpressing the 12 aforementioned genes in the Ehrlich pathway on SBCFA production to identify bottlenecks in the SBCFA biosynthesis pathway. Subsequently, genes identified to be crucial in the SBCFA production pathway were combinatorially overexpressed and the genes in competitive pathways were knocked out, which resulted in a 31.2-fold increase in SBCFA production titer. Finally, a native transporter was overexpressed in the best SBCFA overproducer to export the SBCFAs produced, thus facilitating product secretion into the growth medium. The outcome of this study shows that our engineered yeast can serve as a platform strain for future metabolic engineering efforts to biosynthesize valuable SBCFA-derived chemicals.

## 2. Materials and methods

### 2.1. Chemicals, reagents and oligonucleotides

The iProof high-fidelity DNA polymerase, iScript cDNA Synthesis Kit and SsoFast EvaGreen Supermix Kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Restriction enzymes, T4 DNA ligase and PCR reagents were purchased from New England Biolabs (Beverly, MA, USA). QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit and RNeasy Mini Kit were purchased from Qiagen (Valencia, CA, USA). Peptone was purchased from Oxoid Ltd., (Basingstoke, Hampshire, UK). Oligonucleotide primers (listed in Supplementary Table S1) were synthesized by Integrated DNA Technologies (Singapore). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.



**Fig. 1.** Metabolic map of SBCFA biosynthesis in *S. cerevisiae*. Catabolism of BCAAs (isoleucine, leucine and valine) leads to the formation of 2-methylbutyric acid (2MBA), isovaleric acid (IVA), isobutyric acid (IBA) through Ehrlich pathway in *S. cerevisiae*. These SBCFAs are exported into the medium by the ABC transporter encoded by *PDR12*.

## 2.2. Strains, media and culture conditions

The yeast strain *S. cerevisiae* BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) was used for this work. Yeast cells were routinely grown at 30 °C in yeast extract–peptone–dextrose (YPD) medium consisting of 1% yeast extract, 2% peptone and 2% dextrose. YPD medium containing 200 μg/mL hygromycin B (YPDH) was used for the selection of hygromycin B-resistant colonies. YPD medium containing 1 mg/mL 5-fluoroorotic acid (5-FOA) was used for negative selection of *Ura<sup>-</sup>* strains. Synthetic complete medium lacking uracil (SC-Ura) containing 0.67% yeast nitrogen base, 1.92% uracil-deficient amino acid dropout mixture and 2% carbon source (glucose or raffinose) were used for the selection of *Ura<sup>+</sup>* transformants. *Escherichia coli* TOP10 was used for gene cloning and routinely cultured in Luria–Bertani medium (LB) containing 100 μg/mL ampicillin at 37 °C. All solid media plates contained 2% agar.

## 2.3. DNA manipulation, plasmid construction and strain generation

Plasmid pUG72 (carrying *loxP*–*URA3*–*loxP*) was used as template for generating promoter replacement and gene deletion cassettes (Guedener et al., 2002). The plasmid pSH69 (carrying *P<sub>GAL1</sub>*–*CRE*) which contains a hygromycin B resistance selection marker was used for marker rescue (Hegemann and Heick, 2011).

### 2.3.1. Plasmid construction of pUG72-TEF1

The strong constitutive *TEF1* (translation and elongation factor 1) promoter was amplified by PCR from genomic DNA of *S. cerevisiae* BY4741 strain using primers 1 and 2 (Table S1). Primer 1 was designed to flank the restriction site *SpeI* and Primer 2 contained *SacII* flanking restriction site. The *TEF1* promoter amplicon was purified and treated with *SpeI* and *SacII*, and then ligated to the *SpeI/SacII* sites of pUG72 to yield pUG72-TEF1 (Fig. S1). pUG72-TEF1 was then employed as the template for generating promoter replacement cassettes.

### 2.3.2. Generation of promoter replacement cassettes, gene deletion cassettes and yeast transformation

Promoter replacement cassettes containing a *TEF1* promoter and a *URA3* selection marker flanked by *loxP* sites were obtained from pUG72-TEF1 by PCR using primers 3–28, which contain 42 bp homology on both sides of each target integration site (Fig. S2). The primers used for the PCRs are listed in Table S1. The purified PCR fragments were transformed into BY4741 competent cells by the lithium acetate/polyethylene glycol/single-stranded carrier DNA transformation method (Gietz and Schiestl, 2007). Following yeast transformations, positive colonies were selected on SC-Ura plates. The transformants were further evaluated by PCR with primers 29–42 to verify the replacement of native promoters with the *TEF1* promoter. The forward primer 29 was designed to anneal to the sequence located within the *TEF1* promoter region. The reverse primers 30–42 were designed to be located inside the coding region of the genes. After confirmation of promoter replacement by PCR, all the correct strains were transformed with the *CRE*-expressing plasmid pSH69 and grown on YPDH plates to select for hygromycin B resistant cells. The *URA3* marker gene between the *loxP* sites in the promoter replacement cassettes was removed by the expressed Cre recombinase after induction with galactose. The resulting yeast cells were grown overnight on YPD medium containing 5-FOA to select for colonies with the *URA3* marker removed and to cure the cells of the pSH69 plasmid. The removal of *URA3* marker enabled subsequent rounds of targeted promoter replacement. In addition, a set of gene disruption cassettes were amplified from pUG72 with primers 43–58 (Table S1), which also contain 42 bp homology on each side of the targeted

locus for homologous recombination. The yeast knockout strains were subsequently generated using the same procedure as described for promoter replacement. The primers 59–67 used for PCR verification of gene deletions are listed in Table S1. To determine the genetic stability of the engineered strains, five consecutive passages were carried out. The strains were further evaluated by genomic DNA PCR using the same primers.

## 2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA extraction from yeast cell cultures during exponential growth phase was performed using the RNeasy Mini Kit as recommended in the manufacturer's protocol. Residual genomic DNA contamination was removed by an RNase-Free DNase I treatment after RNA purification. The iScript cDNA synthesis kit was used to synthesize the first-strand cDNA by reverse transcription PCR following the manufacturer's instructions. The mRNA levels of the corresponding genes were measured by qRT-PCR using Bio-Rad CFX Connect real-time PCR detection system. The qRT-PCR was carried out using SsoFast EvaGreen Supermix Kit according to the manufacturer's recommendations. Specific primers for the analysis of gene expression were designed and used in qRT-PCR. Primer sequences are given in Table S1. Relative expression level of mRNAs was calculated using the comparative  $C_T$  method, and all the data obtained were analyzed with the iQ5 Optical System Software (version 2.0). In brief, the analysis was performed as follow. Gene expression in experimental samples was normalized to that in control samples. The housekeeping gene  $\beta$ -actin (*ACT1*) was used as the reference gene.

## 2.5. Cultivation of engineered *S. cerevisiae* strains for SBCFA production

YPD medium was used to cultivate yeast strains for shake flask fermentation. Fresh single colonies of the yeast strains were inoculated into tubes containing 5 mL medium for overnight growth. The yeast cells were cultivated at 30 °C and shaken on a rotary shaker at 225 rpm. The overnight precultures were inoculated to an initial  $OD_{600}$  of 0.05 in 50 mL fresh YPD and grown under the same conditions in 250 mL shake flasks. The cultures were harvested after 72 h of cultivation for primary analysis of SBCFA production. For time course experiments, culture samples were collected every 12 h and analyzed to determine the SBCFA levels. Growth of the yeast strains was monitored by measuring the  $OD_{600}$  on a spectrophotometer (BioPhotometer Plus, Eppendorf). Analysis of SBCFAs was performed as described in Section 2.6. *S. cerevisiae* growth assays were also performed to determine the toxic effects of the SBCFAs. The growth medium (YPD medium) was supplemented with different concentrations of IBA, IVA and 2MBA, respectively. The concentrations of IBA, IVA and 2MBA in YPD medium were 0.5, 1.0 and 5.0 g/L respectively. Cell growth was monitored by  $OD_{600}$  measurement at every 2 h over 24 h.

## 2.6. GC/MS analysis of biosynthesized SBCFAs in the engineered yeast strains

Yeast cells were harvested by centrifuging 50 mL culture at 6000 rpm for 10 min. For quantitative determination of extracellular SBCFAs, 2 mL 10% hydrochloric acid–methanol (v/v) was vortexed with 10 mL culture supernatant for 2 min and incubated at 62 °C for 3 h to methylate the SBCFAs. After cooling to room temperature, the resulting fatty acid methyl esters were subsequently extracted from the supernatant by vortexing for 2 min with 2 mL hexane. For quantification of intracellular SBCFAs, the cell pellets were washed twice with 20 mL deionized water. Cell disruption was then performed by adding 1.5 mL concentrated



hydrochloric acid and incubation for 1 h at 70 °C. Following cell lysis, the 1.5 mL 10% hydrochloric acid–methanol (v/v) was added to the cell lysates followed by 2 min of vortexing. After incubation at 62 °C for 3 h, the reaction mixture was cooled to room temperature and the methyl ester derivatives of SBCFAs were extracted by vortexing for 2 min with 2 mL hexane. After centrifugation at 10,000 rpm for 15 min, the top hexane layer from the methylation reaction mixtures of both culture supernatants and lysates were then collected. An internal standard (heptadecanoic acid, C17:0) was used to quantify SBCFA concentration by comparing peak areas. The organic extracts were then subjected to gas chromatography/mass spectrometry (GC/MS) analysis using an HP 7890B GC system with an Agilent 5977A MSD and equipped with a HP-5MS column. The program used for GC analysis was as follow: initial hold at 45 °C for 3 min; ramp to 50 °C at 10 °C/min and hold for 3 min; ramp to 280 °C at 50 °C/min and hold for 5 min. Helium was used as the carrier gas and ran at a constant pressure of 13.8 psi. The injector was maintained at 250 °C and the ion source temperature was set to 230 °C. The injection volume was 1.0 µL in splitless mode. Relevant GC peaks were identified by comparing with the retention times and mass spectra of fatty-acyl methyl ester standards. Data analysis was performed using Agilent Enhanced Data Analysis software. Due to the structural similarity of 2MBA and IVA, the two 5-carbon SBCFAs could not be resolved and were quantitated as a 2MBA/IVA mixture.

### 3. Results and discussion

#### 3.1. Effects of individual gene overexpression on the biosynthesis of SBCFA in *S. cerevisiae*

To improve the production of the SBCFAs IBA, IVA and 2MBA, the catabolism of the BCAAs valine, leucine and isoleucine, respectively, was first increased in the Ehrlich pathway by overexpressing the genes involved. Twelve genes consisting of transaminases (*BAT1* and *BAT2*), decarboxylases (*ARO10*, *THI3*, *PDC1*, *PDC5* and *PDC6*) and aldehyde dehydrogenases (*ALD2*, *ALD3*, *ALD4*, *ALD5* and *ALD6*) were selected for overexpression. To determine the key genes for SBCFA biosynthesis through the Ehrlich pathway, the effects of the 12 encoded enzymes on overproduction of SBCFAs were first investigated by overexpressing the genes individually. To this end, we chose to chromosomally replace the native promoters of these genes with the strong constitutive *TEF1* promoter by active homologous recombination in *S. cerevisiae* (Fig. S3). Chromosomal replacement of the promoters has an advantage over plasmid-based overexpression because modifications of chromosome ensure genetic stability of the host strain without the need for selection pressure (Nevoigt et al., 2006). This strategy has already been successfully implemented in *S. cerevisiae* (Kaufmann and Knop, 2011; Nevoigt et al., 2006; Verstrepen and Thevelein, 2004).

Individual overexpression of the selected genes did not cause adverse effects on cell growth. qPCR analysis confirmed increased expression levels of all the 12 genes (ranging from 5 to 32 folds) over those of the wild-type controls (Fig. S4). Compared to the wild-type, the titers of SBCFAs after 72 h of cultivation were markedly increased by the engineered strains overexpressing *BAT1*, *ARO10*, *ALD2* or *ALD5* individually (Fig. 2). IBA production increased between 2.0- and 3.9-fold to achieve titers of 11.9–19.7 mg/L (Fig. 2A) and 2MBA/IVA production improved by 7.0- to 10.2-fold to attain titers of 64.3–89.5 mg/L (Fig. 2B). The highest total SBCFA titer at 109.0 mg/L was obtained by overexpressing *ALD5*, which was an 8.1-fold enhancement over the wild-type. Thus, our results indicate that the key transaminase and decarboxylase in the Ehrlich pathway towards SBCFA biosynthesis are

*Bat1p* and *Aro10p*, respectively, while *Ald2p* and *Ald5p* are crucial aldehyde dehydrogenases for producing SBCFAs. These four key enzymes were thus chosen to direct our subsequent metabolic engineering efforts to boost SBCFA production.

#### 3.2. Effects of combinatorial overexpression of key Ehrlich pathway genes for further enhancement of SBCFA production

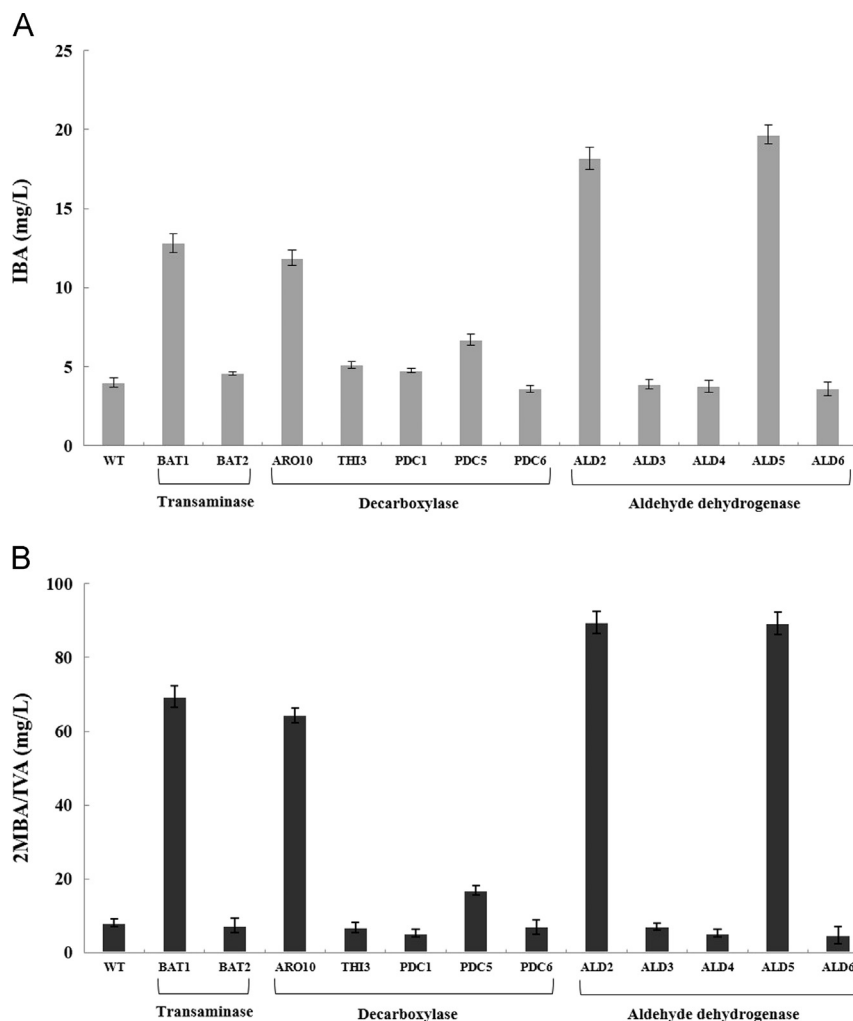
Upon identification of *BAT1*, *ARO10*, *ALD2* and *ALD5* as the key genes in the Ehrlich pathway for SBCFA biosynthesis, we hypothesized that combinatorial overexpression of these four genes will further enhance SBCFA production. To verify this hypothesis, different combinations of the four genes were overexpressed by promoter replacement (Fig. S3). First, *BAT1*, *ARO10*, *ALD2* and *ALD5* were overexpressed in pairs, hence strains overexpressing combinations of (*ALD2*, *ALD5*), (*ALD2*, *BAT1*), (*ALD2*, *ARO10*), (*ALD5*, *BAT1*), (*ALD5*, *ARO10*) and (*BAT1*, *ARO10*) were obtained. Compared to the *ALD5*-overexpressing strain, which is the best single-gene overexpressing SBCFA overproducer, five of the six double-gene overexpressing strains had higher SBCFA titers (Fig. 3). Notably, the highest SBCFA titer was attained by overexpressing *BAT1* and *ARO10* concurrently. Relative to the *ALD5*-overexpressing strain, IBA production was improved from 19.7 mg/L to 48.2 mg/L, and total 2MBA/IVA production increased from 89.3 mg/L to 227.6 mg/L. These titers correspond to 11.0-fold and 27.5-fold enhancement in IBA and 2MBA/IVA production, respectively, over the wild-type. These results validated our hypothesis that combinatorial overexpression of *BAT1*, *ARO10*, *ALD2* and *ALD5* can boost SBCFA production.

To further enhance SBCFA production, overexpression of triple gene combinations (*ALD2*, *ALD5*, *BAT1*), (*ALD2*, *ARO10*, *ALD5*), (*ALD2*, *ARO10*, *BAT1*) and (*ALD5*, *ARO10*, *BAT1*) was performed. The SBCFA production of the strain simultaneously overexpressing *ALD5*, *ARO10* and *BAT1* stood out from the other triple-gene overexpressing strains, which further improved IBA and 2MBA/IVA titers to 55.4 mg/L (12.8-fold increase compared to wild-type) and 249.7 mg/L (30.2-fold increase compared to wild-type), respectively (Fig. 3). This strain overexpresses a transaminase gene (*BAT1*), a decarboxylase gene (*ARO10*) and an aldehyde dehydrogenase gene (*ALD5*), thus demonstrating the synergistic effect of overexpressing a gene from each of the three reaction steps in the Ehrlich pathway on enhancing SBCFA titer.

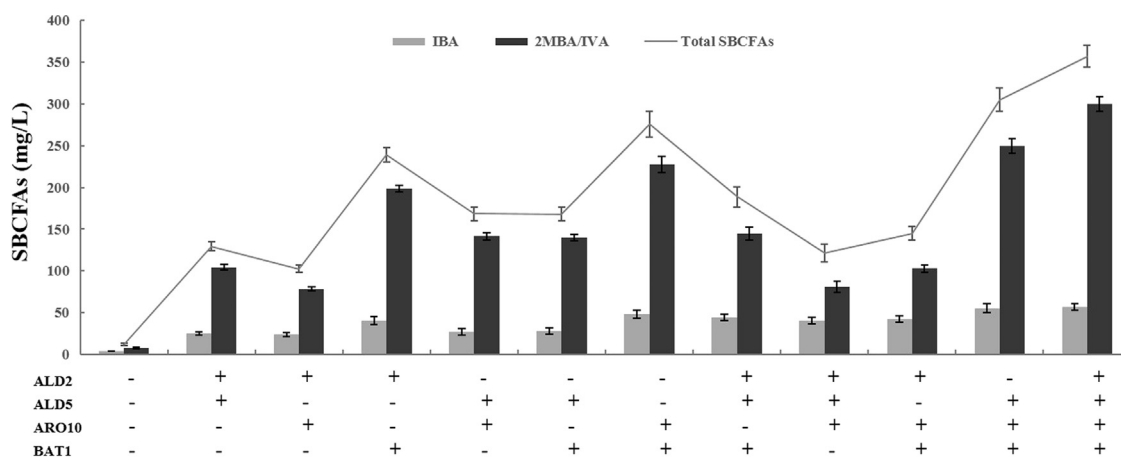
Finally, by overexpressing all the four key genes *BAT1*, *ARO10*, *ALD2* and *ALD5* concurrently, we obtained the best SBCFA overproducer (hereafter named 4G) amongst our engineered strains to reach titers of 56.8 and 300.0 mg/L for IBA and 2MBA/IVA, respectively. This corresponds to 13.2- and 36.5-fold improvement in IBA and 2MBA/IVA titers, respectively, and a 28.7-fold increase in total SBCFA titer over the wild-type (Fig. 3). The growth profiles of all the engineered strains were similar to that of the parental strain, indicating that overexpression of the four genes had no deleterious effect on cell growth. The lack of growth inhibition is consistent with the fact that the total SBCFA titer produced in the engineered strains was below the SBCFA concentrations required for growth retardation to be apparent (Fig. S5).

#### 3.3. Effect of *ADH* deletions on the biosynthesis of SBCFA in *S. cerevisiae*

In the Ehrlich pathway, SBCFAs are produced via oxidation of aldehyde intermediates by aldehyde dehydrogenases such as *Ald2p* and *Ald5p*. However, there are many native alcohol dehydrogenases (ADHs) present in *S. cerevisiae* that are capable of reducing aldehydes to alcohols (Fig. 1). Thus, elimination of ADHs may increase the accumulation of the aldehyde intermediates by preventing their reduction to alcohols, hence increasing the



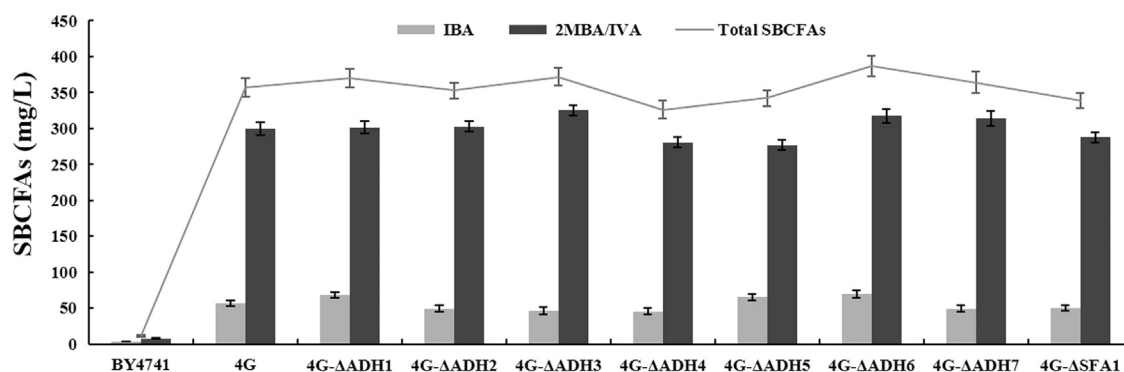
**Fig. 2.** Effects of single-gene overexpression of genes in Ehrlich pathway on SBCFA production in *S. cerevisiae*. Twelve genes in the Ehrlich pathway, including those that encode transaminases, decarboxylases and aldehyde dehydrogenases, were overexpressed individually and titers of IBA (A) and 2MBA/IVA (B) were quantified after 72 h of cultivation in shake flasks with YPD media. The BY4741 strain was cultivated in parallel as control. All values presented are the mean of three biological replicates  $\pm$  standard deviation.



**Fig. 3.** SBCFA production in strains overexpressing combinations of *BAT1*, *ARO10*, *ALD2* and *ALD5*. Double-, triple- and quadruple-gene overexpressing strains were cultivated for 72 h in shake flasks with YPD media. Wild type BY4741 and *ALD5* single-gene overexpression strains were cultivated in parallel as control. The IBA and 2MBA/IVA compositions of all strains were determined by GC/MS after methylation. All values presented are the mean of three biological replicates  $\pm$  standard deviation.

production of SBCFAs. To evaluate this hypothesis, we deleted native ADHs to block competing pathways that can lead to the formation of branched-chain alcohols, i.e. isobutanol, 2-methylbutanol and 3-methylbutanol (Fig. 1), which will compete

for precursors for the biosynthesis of IBA, 2MBA and IVA, respectively. Our best SBCFA overproducer strain, 4G, was further engineered to individually disrupt eight *S. cerevisiae* genes for alcohol dehydrogenase, namely *ADH1-7* and *SFA1*. These gene deletions



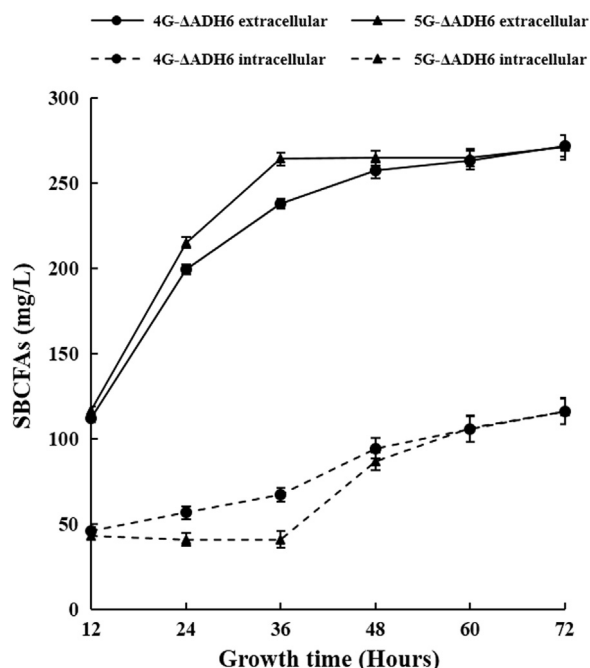
**Fig. 4.** SBCFA production in strains with various ADHs deleted from 4G. After 72 h of cultivation in shake flasks with YPD media, the IBA and 2MBA/IVA compositions of ADH-deleted 4G strains were determined by GC/MS. Strain 4G was cultivated in parallel as control. All values presented are the mean of three biological replicates  $\pm$  standard deviation.

did not affect cell growth rates. Among these ADH knockout strains, the best SBCFA-producing strain is 4G- $\Delta$ ADH6, which has ADH6 deleted from 4G. This strain achieved a titer of 387.4 mg/L total SBCFAs after 72 h of cultivation, which was about 31.2-fold higher than that of the wild-type and a further 8.6% increase in SBCFA titer over the 4G strain (Fig. 4). No marked enhancement in SBCFA titers observed after deletion of the other ADH genes. The lack of improvement in SBCFA production upon a single ADH deletion may be due to the broad substrate specificities of ADHs in *S. cerevisiae* (De Smidt et al., 2008), which allows other functional ADHs to continue reducing aldehyde intermediates to alcohols. This is consistent with reports that *S. cerevisiae* requires only one ADH present out of Adh1p-Adh5p or Sfa1p to be able to produce 3-methylbutanol (Dickinson et al., 2003). Thus, all ADHs may have to be inactivated to significantly improve SBCFA production.

#### 3.4. Effect of overexpressing native transporters on the secretion of SBCFAs in *S. cerevisiae*

It is always beneficial that the biochemicals produced within cell factories are exported into the extracellular environment in industrial applications. The accelerated efflux can reduce cell toxicity associated with the biochemicals produced and also ease recovery of the target compounds. Work has been reported on the use of transporter in engineered microorganisms to increase the efflux efficiency of biochemicals (Chen et al., 2013; Doshi et al., 2013; Dunlop et al., 2011; Fisher et al., 2013; Foo et al., 2014; Foo and Leong, 2013; Ling et al., 2013; Wang et al., 2013). The export of biochemicals has also been shown to successfully enhance production of target compounds (Dunlop et al., 2011; Wang et al., 2013). We therefore hypothesized that upregulation in the expression of an *S. cerevisiae* transporter may increase SBCFA secretion and hence SBCFA titers. In *S. cerevisiae*, Pdr12p is a plasma membrane ATP-binding cassette transporter that is able to export SBCFAs (Hazelwood et al., 2008, 2006), and was thus considered a suitable candidate to be studied for accelerating secretion of SBCFAs.

To investigate the effects of Pdr12p on SBCFA secretion and production, we overexpressed the *PDR12* gene in 4G- $\Delta$ ADH6 to generate strain 5G- $\Delta$ ADH6. After confirmation of *PDR12* overexpression by qPCR (Fig. S4), we conducted a time-course study on intracellular and extracellular SBCFA production in the 5G- $\Delta$ ADH6 strain. Fig. 5 shows that *PDR12* overexpression led to higher titer of extracellular SBCFAs than that of 4G- $\Delta$ ADH6 between 12 h and 60 h. Correspondingly, the intracellular SBCFA titer in 5G- $\Delta$ ADH6 strain was lower than that of the 4G- $\Delta$ ADH6 control within the same time period. This is consistent with increased secretion of SBCFAs by the overexpressed Pdr12p transporter, given that the



**Fig. 5.** Time-course study of the effect of *PDR12* overexpression on SBCFA production. The *PDR12*-overexpressing strain 5G- $\Delta$ ADH6 was cultured in shake flasks with YPD media. Periodically, the intracellular and extracellular SBCFA titers were quantified. The 4G- $\Delta$ ADH6 strain was cultivated in parallel as control. All values presented are the mean of three biological replicates  $\pm$  standard deviation.

levels of total SBCFAs in cells with and without *PDR12* overproduction were the same.

Upon *PDR12* overexpression, the extracellular SBCFA titer plateaued at 36 h, which is 24 h earlier than that of 4G- $\Delta$ ADH6. In contrast, the intracellular SBCFA titer of the *PDR12*-overexpressing strain stabilized at 60 h, which is comparable to that of 4G- $\Delta$ ADH6. This observation suggests that active secretion of SBCFAs was diminished as the cells approached stationary phase at 36 h (Fig. S6). It is noteworthy that the overexpression of *PDR12* did not lead to further increase in the production of total SBCFAs compared to 4G- $\Delta$ ADH6 (Fig. 5). This might be because SBCFAs at the current titer are not toxic enough to *S. cerevisiae* (Fig. S5) for product efflux to have a beneficial effect on SBCFA production, which was also consistent with our observation that none of our SBCFA overproducing strains exhibited growth inhibition. Nevertheless, the functional export of SBCFAs by Pdr12p demonstrates a feasible route for secretion of the products for easy recovery. Through this study, we have also verified that the 5G- $\Delta$ ADH6 strain was genetically stable after five rounds of consecutive

passage despite having multiple TEF1 promoters, which suggests its potential use for scaled up production of SBCFAs.

#### 4. Conclusions

Hitherto, no microorganisms have demonstrated the capacity to naturally generate significant amounts of SBCFAs. In previous studies, synthetic metabolic pathways were constructed in *E. coli* (Zhang et al., 2011) and *Pseudomonas* sp. (Lang et al., 2014) for biosynthesis of IBA. The branched-chain amino acid catabolic pathway has been successfully engineered to enhance the production of branched-chain fatty acids (Choi et al., 2014; Haushalter et al., 2014; Mustafi et al., 2012; Shi et al., 2013) and alcohols (Cao et al., 2015; Kondo et al., 2012; Shi et al., 2013, Si et al., 2014) in different host strains. To our knowledge, this study is the first report on improved SBCFA biosynthesis by engineering the branched-chain amino acid catabolic pathway–Ehrlich pathway in *S. cerevisiae*. Concurrent overexpression of four key Ehrlich pathway genes using a chromosome-based promoter replacement strategy greatly improved the production levels of total SBCFAs. Upon deletion of ADHs in competing pathways, SBCFA titer was further increased. Finally, enhanced secretion of the produced SBCFAs was achieved by overexpression of the native Pdr12p transporter. We hereby demonstrated the implementation of a combination of strategies to develop SBCFA overproducing yeast strains that can serve as platform strains for biosynthesizing valuable derivatives of SBCFAs. We have also presented insights into further manipulation possibilities of the Ehrlich pathway to facilitate future efforts to exploit this pathway for production of other high value added biochemicals. Improved understanding of the key components of the Ehrlich pathway and their regulation obtained from this study will open the way for improved SBCFA titers through systematic strain engineering in the future.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2015.12.005>.

#### References

- Adams, Charles R., 1970. Carbonylation with acid catalysts koch reaction, in: Carbon Monoxide in Organic Synthesis. Springer, Heidelberg Berlin, pp. 123–146.
- Ahmad, A., Aggarwal, K.K., Srivastava, S.K., Ahmad, J., Kumar, S., 2000. Carbon-13 and proton NMR shift assignments and physical constants of some L-menthol derivatives. *J. Essent. Oil Res.* 12, 775–780.
- Cao, Y.X., Xiao, W.H., Liu, D., Zhang, J.L., Ding, M.Z., Yuan, Y.J., 2015. Biosynthesis of odd-chain fatty alcohols in *Escherichia coli*. *Metab. Eng.* 29, 113–123.
- Chen, B., Lee, D.Y., Chang, M.W., 2015. Combinatorial metabolic engineering of *Saccharomyces cerevisiae* for terminal alkene production. *Metab. Eng.* 31, 53–61.
- Chen, B., Ling, H., Chang, M.W., 2013. Transporter engineering for improved tolerance against alkane biofuels in *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* 6, 21.
- Choi, K.Y., Wernick, D.G., Tat, C.A., Liao, J.C., 2014. Consolidated conversion of protein waste into biofuels and ammonia using *Bacillus subtilis*. *Metab. Eng.* 23, 53–61.
- De Smidt, O., Du Preez, J.C., Albertyn, J., 2008. The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. *FEMS Yeast Res.* 8, 967–978.
- Dickinson, J.R., Eshantha, L., Salgado, J., Hewlins, M.J.E., 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 278, 8028–8034.
- Dickinson, J.R., Harrison, S.J., Hewlins, M.J., 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 25751–25756.
- Doshi, R., Nguyen, T., Chang, G., 2013. Transporter-mediated biofuel secretion. *Proc. Natl. Acad. Sci. USA* 110, 7642–7647.
- Dunlop, M.J., Dossani, Z.Y., Szmids, H.L., Chu, H.C., Lee, T.S., Keasling, J.D., Hadi, M.Z., Mukhopadhyay, A., 2011. Engineering microbial biofuel tolerance and export using efflux pumps. *Mol. Syst. Biol.* 7, 487.
- Eadie, M.J., 2004. Could valerian have been the first anticonvulsant? *Epilepsia* 45, 1338–1343.
- Fisher, M.A., Boyarskiy, S., Yamada, M.R., Kong, N., Bauer, S., Tullman-Ercek, D., 2013. Enhancing tolerance to short-chain alcohols by engineering the *Escherichia coli* AcrB efflux pump to secrete the non-native substrate n-butanol. *ACS Synth. Biol.* 3, 30–40.
- Foo, J.L., Jensen, H.M., Dahl, R.H., George, K., Keasling, J.D., Lee, T.S., Leong, S., Mukhopadhyay, A., 2014. Improving microbial biogasoline production in *Escherichia coli* using tolerance engineering. *MBio* 5 e01932-14.
- Foo, J.L., Leong, S.S.J., 2013. Directed evolution of an *E. coli* inner membrane transporter for improved efflux of biofuel molecules. *Biotechnol. Biofuels* 6, 81.
- Gietz, R.D., Schiestl, R.H., 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* 2, 31–34.
- Gronenberg, L.S., Marcheschi, R.J., Liao, J.C., 2013. Next generation biofuel engineering in prokaryotes. *Curr. Opin. Chem. Biol.* 17, 462–471.
- Gueldener, U., Heinisch, J., Koehler, G., Voss, D., Hegemann, J., 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res.* 30, e23.
- Haushalter, R.W., Kim, W., Chavkin, T.A., The, L., Garber, M.E., Nhan, M., Adams, P.D., Petzold, C.J., Katz, L., Keasling, J.D., 2014. Production of anteiso-branched fatty acids in *Escherichia coli*; next generation biofuels with improved cold-flow properties. *Metab. Eng.* 26, 111–118.
- Hazelwood, L.A., Daran, J.M., van Maris, A.J., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl. Environ. Microbiol.* 74, 2259–2266.
- Hazelwood, L.A., Tai, S.L., Boer, V.M., De Winde, J.H., Pronk, J.T., Daran, J.M., 2006. A new physiological role for Pdr12p in *Saccharomyces cerevisiae*: export of aromatic and branched-chain organic acids produced in amino acid catabolism. *FEMS Yeast Res.* 6, 937–945.
- Hegemann, J.H., Heick, S.B., 2011. Delete and repeat: a comprehensive toolkit for sequential gene knockout in the budding yeast *Saccharomyces cerevisiae*. *Methods Mol. Biol.* 765, 189–206.
- Kaufmann, A., Knop, M., 2011. Genomic promoter replacement cassettes to alter gene expression in the yeast *Saccharomyces cerevisiae*. *Methods Mol. Biol.* 765, 275–294.
- Kondo, T., Tezuka, H., Ishii, J., Matsuda, F., Ogino, C., Kondo, A., 2012. Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae*. *J. Biotechnol.* 159, 32–37.
- Kwon, D.Y., Hong, Y.J., Yoon, S.H., 2000. Enantiomeric synthesis of (S)-2-methylbutanoic acid methyl ester, apple flavor, using lipases in organic solvent. *J. Agric. Food Chem.* 48, 524–530.
- Lang, K., Zierow, J., Buehler, K., Schmid, A., 2014. Metabolic engineering of *Pseudomonas* sp. strain VLB120 as platform biocatalyst for the production of isobutyric acid and other secondary metabolites. *Microbiol. Cell Fact.* 13, 2.
- Latimer, L.N., Lee, M.E., Medina-Cleghorn, D., Kohnz, R.A., Nomura, D.K., Dueber, J.E., 2014. Employing a combinatorial expression approach to characterize xylose utilization in *Saccharomyces cerevisiae*. *Metab. Eng.* 25, 20–29.
- Le Borgne, S., 2012. Genetic engineering of industrial strains of *Saccharomyces cerevisiae*. *Methods Mol. Biol.* 824, 451–465.
- Lilly, M., Bauer, F.F., Styger, G., Lambrechts, M.G., Pretorius, I.S., 2006. The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. *FEMS Yeast Res.* 6, 726–743.
- Ling, H., Chen, B., Kang, A., Lee, J.M., Chang, M.W., 2013. Transcriptome response to alkane biofuels in *Saccharomyces cerevisiae*: identification of efflux pumps involved in alkane tolerance. *Biotechnol. Biofuels* 6, 95.
- Millet, J.M.M., 1998. FePO catalysts for the selective oxidative dehydrogenation of isobutyric acid into methacrylic acid. *Catal. Rev.* 40, 1–38.
- Mustafi, N., Grünberger, A., Kohlheyer, D., Bott, M., Frunzke, J., 2012. The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids. *Metab. Eng.* 14, 449–457.
- Nagai, K., 2001. New developments in the production of methyl methacrylate. *Appl. Catal. A: Gen. 221*, 367–377.
- Nevoigt, E., 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 72, 379–412.
- Nevoigt, E., Kohnke, J., Fischer, C.R., Alper, H., Stahl, U., Stephanopoulos, G., 2006. Engineering of promoter replacement cassettes for fine-tuning of gene



- expression in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 72, 5266–5273.
- Nielsen, J., Larsson, C., van Maris, A., Pronk, J., 2013. Metabolic engineering of yeast for production of fuels and chemicals. Curr. Opin. Biotechnol. 24, 398–404.
- Peralta-Yahya, P.P., Zhang, F., Del Cardayre, S.B., Keasling, J.D., 2012. Microbial engineering for the production of advanced biofuels. Nature 488, 320–328.
- Pigeau, G., Inglis, D., 2007. Response of wine yeast (*Saccharomyces cerevisiae*) aldehyde dehydrogenases to acetaldehyde stress during Icewine fermentation. J. Appl. Microbiol. 103, 1576–1586.
- Shi, A., Zhu, X., Lu, J., Zhang, X., Ma, Y., 2013. Activating transhydrogenase and NAD kinase in combination for improving isobutanol production. Metab. Eng. 16, 1–10.
- Shin, J.H., Kim, H.U., Kim, D.I., Lee, S.Y., 2013. Production of bulk chemicals via novel metabolic pathways in microorganisms. Biotechnol. Adv. 31, 925–935.
- Si, T., Luo, Y., Xiao, H., Zhao, H., 2014. Utilizing an endogenous pathway for 1-butanol production in *Saccharomyces cerevisiae*. Metab. Eng. 22, 60–68.
- Suerbaev, K.A., Chapaikin, E.G., Zhaksylykova, G.Z., 2012. Hydro-menthoxy-carbonylation of isobutylene in the presence of Tetrakis (triphenylphosphine) palladium-based catalyst systems. Petrol. Chem. 52, 422–425.
- Verstrepen, K.J., Thevelein, J.M., 2004. Controlled expression of homologous genes by genomic promoter replacement in the yeast *Saccharomyces cerevisiae*. Methods Mol. Biol. 267, 259–266.
- Vuralhan, Z., Morais, M.A., Tai, S.L., Piper, M.D., Pronk, J.T., 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 69, 4534–4541.
- Wang, J.F., Xiong, Z.Q., Li, S.Y., Wang, Y., 2013. Enhancing isoprenoid production through systematically assembling and modulating efflux pumps in *Escherichia coli*. Appl. Microbiol. Biotechnol. 97, 8057–8067.
- Weitz-Schmidt, G., 2002. Statins as anti-inflammatory agents. Trends Pharmacol. Sci. 23, 482–486.
- Xu, P., Gu, Q., Wang, W., Wong, L., Bower, A.G., Collins, C.H., Koffas, M.A., 2013. Modular optimization of multi-gene pathways for fatty acids production in *E. coli*. Nat. Commun. 4, 1409.
- Xu, P., Li, L., Zhang, F., Stephanopoulos, G., Koffas, M., 2014. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc. Natl. Acad. Sci. USA 111, 11299–11304.
- Yu, A.Q., Juwono, N.K.P., Leong, S.S.J., Chang, M.W., 2014. Production of fatty acid-derived valuable chemicals in synthetic microbes. Front. Bioeng. Biotechnol., 2.
- Zhang, H., Pereira, B., Li, Z., Stephanopoulos, G., 2015a. Engineering *Escherichia coli* coculture systems for the production of biochemical products. Proc. Natl. Acad. Sci. USA 112, 8266–8271.
- Zhang, J., Kang, Z., Chen, J., Du, G., 2015b. Optimization of the heme biosynthesis pathway for the production of 5-aminolevulinic acid in *Escherichia coli*. Sci. Rep. 5, 8584.
- Zhang, K., Woodruff, A.P., Xiong, M., Zhou, J., Dhande, Y.K., 2011. A synthetic metabolic pathway for production of the platform chemical isobutyric acid. ChemSusChem 4, 1068–1070.