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BIOENERGY AND BIOFUELS

Metabolically engineered *Saccharomyces cerevisiae* for enhanced isoamyl alcohol production

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Abstract Higher chain alcohols have gained much attention as next generation transport fuels because of their higher energy density and low moisture absorption capacity compared to ethanol. In the present study, we attempted to engineer *Saccharomyces cerevisiae* for the synthesis of isoamyl alcohol via de novo leucine biosynthetic pathway coupled with Ehrlich degradation pathway. To achieve high-level production of isoamyl alcohol, two strategies are used in the current study: (1) reconstruction of a chromosome-based leucine biosynthetic pathway under the control of galactose-inducible promoters; (2) overexpression of the mitochondrial 2-isopropylmalate (α-IPM) transporter to boost the transportation of α-IPM from mitochondria to the cytosol. We found engineered yeast cells with a combinatorially assembled leucine biosynthetic pathway coupled with the Ehrlich degradation pathway resulted in high-level production of isoamyl alcohol; however, there was still a significant amount of isobutanol co-formed during the fermentation process. Further introducing an α-IPM transporter not only boosted the isoamyl alcohol biosynthetic pathway activity but also reduced isobutanol to a much lower level. Taken together, our work represents the first study to construct a chromosome-based leucine biosynthetic pathway for isoamyl alcohol production. Furthermore, the utilization of the mitochondrial compartment coupled with the transporter engineering serves as an effective approach to minimize the by-product formation and to improve the isoamyl alcohol production.

Keywords Leucine biosynthetic pathway · Ehrlich degradation pathway · Isoamyl alcohol · Pathway assembly · 2-isopropylmalate transporter · *Saccharomyces cerevisiae*

Introduction

The advanced biofuel, due to its sustainability and renewability, has been considered as an appealing solution to the global energy crisis and environmental issues (Buijs et al. 2013; Peralta-Yahya and Keasling 2010; Savage 2011). Recently, branched-chain alcohols have gained significant interests because of their low vapor pressure, lower hygroscopicity, low water solubility, high energy density, and larger octane numbers than their straight-chain counterparts (Atsumi et al. 2008; Blombach and Eikmanns 2011; Wang et al. 2012). Microbial synthesis of branched-chain alcohols has been extensively carried out in model organisms such as *Escherichia coli* (Atsumi et al. 2008; Cann and Liao 2008; Connor et al. 2010; Connor and Liao 2008; Smith and Liao 2011). By introducing the Ehrlich degradation pathway into *E. coli*, intermediates of keto acids involved in the branched-chain amino acid biosynthesis can be sequentially converted to aldehydes and alcohols. However, *E. coli* is a non-native host for the alcohol production and lacks high tolerance to alcohols (Smith et al. 2010). Thus, to explore other microbial hosts that

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have high tolerance to alcohols is beneficial for the microbial production of branched-chain alcohols.

The budding yeast *Saccharomyces cerevisiae* has been regarded as an appealing microbial host for producing higher chain alcohols due to its tolerance to high concentrations of alcohols and various harsh fermentation conditions. The fully sequenced genome and well-characterized metabolic pathways enabled versatile genetic tools to manipulate its metabolic pathways. The yeast *S. cerevisiae* can naturally produce very low amounts of branched-chain alcohols via the catabolism of branched-chain amino acids. Recently, a number of studies have been carried out to improve the production of isobutanol in *S. cerevisiae*. Current efforts on engineering *S. cerevisiae* still heavily rely on conventional approaches such as overexpressing pathway-specific genes using the plasmid-based system (Chen et al. 2011), deleting competing pathways (Kondo et al. 2012), improving the cofactor (NADH and NADPH) generation (Matsuda et al. 2013), and utilizing alternative substrates such as glycine (Branduardi et al. 2013). In addition, relocalizing the complete metabolic pathway into mitochondria or the cytosol to avoid the transportation of metabolites between mitochondria and the cytosol was also explored to improve the isobutanol production (Avalos et al. 2013; Brat et al. 2012). Nevertheless, there are only a few reports on engineering *S. cerevisiae* for the production of other branched-chain alcohols such as isoamyl alcohol. Recently, Park et al. examined isoamyl alcohol production in *S. cerevisiae* by manipulating the transcriptional factor and some pathway components, and isoamyl alcohol yield reached 7.66 mg per gram glucose (Park et al. 2014). As Westfall, P. J. et al. reported that overexpression of the entire mevalonate pathway offers a better way for improving amorpha-4,11-diene production over the conventional approach of manipulating the transcriptional factor (Westfall et al. 2012); therefore, there will be a significant room for improving the isoamyl alcohol production in *S. cerevisiae*.

In the present study, we attempt to overproduce isoamyl alcohol in *S. cerevisiae* via de novo leucine biosynthetic pathway coupled with the Ehrlich degradation pathway. To enable high-level production of isoamyl alcohol, two strategies are used: (1) reconstruction of a chromosome-based leucine biosynthetic pathway under the control of galactose-inducible promoters; (2) overexpression of the mitochondrial α-IPM transporter to boost the transfer of α-IPM from mitochondria to the cytosol.

Materials and methods

Strains, culture media, and reagents

E. coli strain DH5α was used for general plasmid constructions, and the strain was cultivated at 37 °C in Luria-Bertani medium with 100 μg/mL ampicillin. *S. cerevisiae* BY4741 was used as the parental strain for all genetic modifications. This strain was cultured in rich YPD medium. Engineered strains with different auxotrophic selection markers were grown in synthetic complete (SC) medium with different dropouts. For induction of genes under the control of galactose-inducible promoters, *S. cerevisiae* strains were grown in 3.6% galactose plus 0.4% glucose. Restriction enzymes, Taq polymerase, alkaline phosphatase (CIP), and T4 ligase were purchased from New England Biolabs (Beverly, MA). iProof HF polymerase and iScript™ Reverse Transcription Supermix were obtained from Bio-Rad (Hercules, CA). Gel extraction kit, PCR purification kit, plasmid purification kit, and RNeasy mini kit were purchased from QIAGEN (Hilden, Germany). FastStart Essential DNA Green Master Mix was purchased from Roche (Singapore, SG). All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Plasmid construction and yeast transformation

Oligonucleotides used for the plasmid construction are listed in Table 1. For constructing the entire leucine biosynthetic pathway into yeast chromosomes, the previously developed δ-integration platform was used for one-step assembly of a multi-gene pathway. To create the genome integration cassette, a series of plasmids was constructed as follows. Firstly, *ILV2*, *ILV5*, *ILV3*, *LEU9*, *LEU1*, and *LEU2* were PCR amplified from the genomic DNA of *S. cerevisiae* BY4741. The internal *Bam*HI restriction site in the *LEU2* gene was removed using overlap extension PCR. All PCR products were digested using *Bam*HI/*Xho*I, and inserted into plasmid pδBLE2.0, to yield plasmid pδBLE2.0-ILV2/ILV5, pδBLE2.0-ILV3/LEU9, and pδBLE2.0-LEU2/LEU1. Notably, all leucine biosynthetic genes were put under the control of strong galactose-inducible promoters. Next, these plasmids were used as template for the PCR amplification of genome integration cassettes using the primer pair Delta_IntF and Delta_IntR. Pathway integration cassettes were next transformed into the yeast following the electroporation method described in a previous study.

In the present study, we did not attempt to integrate *ARO10* and *ADH7* gene into yeast chromosomes as the abovementioned strains will be used for screening KDCs and ADHs with better substrate preference towards KIC. For the plasmid-based expression of Ehrlich degradation pathway, *ARO10* and *ADH7* gene were PCR amplified from the genomic DNA of *S. cerevisiae* BY4741. The PCR product of *ARO10* gene was digested with *Bam*HI/*Xho*I and inserted into pESC-URA derivative with a modified version of multiple cloning sites, and the resulting

Table 1 Oligonucleotides used for constructing plasmids and qRT-PCR studies

Name	Description
F_ARO10_*Bam*HI	CGGGATCCATGGCACCTGTTACAATTGAAAAG
R_ARO10_*Xho*I	ACACACGCTCGAGCTATTTTTTATTTCTTTTAAGTGCCG
F_ADH7_*Bgl*II	GAAGATCTATGCTTTACCCAGAAAAATTTC
R_ADH7_*Xho*I	ACACACGCTCGAGCTATTTATGGAATTTCTTATCATAATC
F_ILV2_*Bam*HI	CGGGATCCAAAACAATGATCAGACAATCTACGCTAAAAAAC
R_ILV2_*Xho*I	ACACACGCTCGAGTCAGTGCTTACCGCCTGTAC
F_ILV5_*Bam*HI	CGGGATCCAAAACAATGTTGAGAACTCAAGCCGCC
R_ILV5_*Xho*I	ACACACGCTCGAGTTATTGGTTTTCTGGTCTCAACTTTC
F_ILV3_*Bam*HI	CGGGATCCAAAACAATGGGCTTGTTAACGAAAGTTGC
R_ILV3_*Xho*I	ACACACGCTCGAGTCAAGCATCTAAAACACAACCG
F_LEU9_*Bam*HI	CGGGATCCAAACAATGGTAAAACATTCGTTCATAG
R_LEU9_OE	TATCCTTTGGgTCCAATGGT
F_LEU9_OE	ACCATTGGAcCCAAAGGATA
R_LEU9_*Xho*I	ACACACGCTCGAGTTACTCTGCCAGTAGAAC
F_LEU1_*Bam*HI	CGGGATCCAAAACAATGGTTTACACTCCATCCAAG
R_LEU1_*Xho*I	ACACACGCTCGAGCTACCAATCCTGGTGGAC
F_LEU2_*Bam*HI	CGGGATCCAAAACAATGTCTGCCCCTAAGAAGATC
R_LEU2_*Xho*I	ACACACGCTCGAGTTAAGCAAGGATTTTCTTAAC
F_OAC1_*Bam*HI	CGGGATCCAAAACAATGTCATCTGACAACTCTAAAC
R_OAC1_*Xho*I	ACACGCTCGAGTTAATTATGGCCTAAAACTC
F_pGAL10Scr	GAAAATTCGAATTCAACCCTC
R_tADH1Scr	CAAACCTCTGGCGAAGAATTG
F_pGAL1Scr	CGTCAAGGAGAAAAAACCCC
R_tCYC1Scr	CTTTTCGGTTAGAGCGGATC
Delta_IntF	AAACGGAATGAGGAATAATCG
Delta_IntR	TGAGATAATTGTTGGGATTCC
Primer for qRT-PCR study	
F_ACT1_q	tccgtctggattggtggt
R_ACT1_q	tgagatccacatttgttggaag
F_ILV2_q	tctgggcaaaatgatgtcaa
R_ILV2_q	tgagcaaaccattcagacctc
F_ILV5_q	gccattgttgaccaagggta
R_ILV5_q	tccttgaagactggggagaa
F_ILV3_q	ggtccactaatgctgttttgc
R_ILV3_q	acaacttgacacccgcaga
F_LEU9_q	ggtctgtatttctacgcattgtca
R_LEU9_q	gcctgcaagcatacctaattct
F_LEU1_q	cacctgctaaacctagctcttcc
R_LEU1_q	ggtgcgctaataccttccaa
F_LEU2_q	tgggaggtatttactttggtaagag
R_LEU2_q	ggtgtattgttcactatcccaagc

plasmid was designated as plasmid pRS426-ARO10. Next, the PCR product of *ADH7* gene was digested with *Bgl*II/*Xho*I and inserted into pRS426-ARO10 cut with *Bam*HI/*Xho*I. The resulting plasmid was designated as plasmid pRS426-ARO10/ADH7. The plasmid was transformed into the yeast using the standard lithium acetate approach.

For the plasmid-based expression of the α-IPM transporter, the *OAC1* gene was PCR amplified from the genomic DNA of *S. cerevisiae* BY4741, digested with *Bam*HI/*Xho*I, and inserted into pSH62 to yield plasmid pRS413-OAC1. The plasmid was transformed into the yeast using the standard lithium acetate approach.

PCR verification of genome integration events

For the yeast cell lysis, appropriate amounts of yeast cells were resuspended in 10 μL H_2O containing 20 mM NaOH and incubated in 100 °C water bath for 15 min. PCR program was set as follows: 1 cycle of 95 °C for 5 min; amplification, 30 cycles of 95 °C for 15 s, 50 °C for 30 s, and 68 °C for 90 s; 1 cycle of 68 °C for 3 min. Universal primer pair of F_pGAL10Scr and R_tADH1Scr was used for the PCR verification of *ILV2*, *ILV3*, and *LEU2*. Universal primer pair of F_pGAL1Scr and R_tCYC1Scr was used to verify the genome integration events of *ILV5*, *LEU9*, and *LEU1*. Only colonies with three-band pattern with size corresponding to each gene were considered to have the entire leucine biosynthetic pathway assembled into yeast chromosomes.

RNA extraction and quantitative real-time PCR

Yeast cells were harvested at the early log phase and a total amount of 1×10^7 cells was used for the total RNA extraction using the RNeasy mini kit (QIAGEN, Germany). Approximately 500 ng of RNA was converted to complementary deoxyribonucleic acid (cDNA) using the iScript™ Reverse Transcription Supermix from Bio-Rad (Hercules, CA).

The gene-specific primers for *IVL2*, *ILV5*, *ILV3*, *LEU9*, *LEU1*, *LEU2*, and *ACT1* were designed using the ProbeFinder (https://www.roche-applied-science.com), and oligonucleotides used for the qRT-PCR experiment are listed in Table 2. Quantitative PCR analysis was carried out using the LightCycler 96 real-time machine with FastStart Essential DNA Green Master Mix (Roche) according to the manufacturer's instructions. Each 20 μL reaction contained 50 ng of total cDNA, 10 μL FastStart Essential DNA Green Master Mix, and 0.5 μM of each primer. Thermal cycling conditions were set as follows: preincubation, 1 cycle of 95 °C for 10 min; amplification, 45 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s. *ACT1* was chosen as a reference housekeeping gene, and results were presented as ratios of gene expression between *IVL2*, *ILV5*, *ILV3*, *LEU9*, *LEU1*, *LEU2*, and the reference gene, *ACT1* (Pfaffl 2001).

Isoamyl alcohol production in engineered yeast cells

To enable high-level isoamyl alcohol production, yeast strains with an entire leucine biosynthetic pathway were further transformed with plasmid pRS426-ARO10/ADH7. The isoamyl alcohol production in engineered yeast cells was carried out under shake-tube conditions. Specifically, 14-mL tubes containing 2 mL SC-URA medium (3.6% galactose + 0.4% glucose) were inoculated with fresh cell cultures to an initial OD_{600} of 0.05. All tubes were immediately supplemented with 20% (vol/vol) dodecane after seeding to perform two-phase fermentation and harvest isoamyl alcohol. To investigate the effect of OAC1 on the isoamyl alcohol production, strains were further transformed with pRS413-OAC1. The experiments were carried out in SC-URA-HIS medium. Each time, 100 μL of cell culture was taken for measuring OD_{600} by microplate reader (Synergy H1, BioTek, USA), and 50 μL dodecane layer was sampled and diluted in 450 μL dodecane for the identification of isoamyl alcohol using gas chromatography-mass spectrometry (GC-MS). For GC-MS analysis, 1 μL of diluted sample was injected into Shimadzu QP2010Ultra system equipped with a DB-5 ms column (30 m × 250 μm × 0.25 μm thickness) (Agilent Technologies, USA). Split ratio was set with 15:1. Helium (ultra purity) was used as carrier gas at a flow rate 1.0 mL/min. GC oven temperature was initially held at 40 °C for 2 min, ramped with a gradient of 5 °C/min until 45 °C, and held for 4 min. And then, it was raised with a gradient 15 °C/min until 230 °C and held for 4 min. Both injector and detector were maintained at 225 °C. Scan mode was used to detect the mass range 40–120 m/z.

Results

Isoamyl alcohol production in the engineered *S. cerevisiae* with the entire leucine biosynthetic pathway overexpressed under the control of strong promoters

Isoamyl alcohol is produced as a by-product in the breakdown of leucine via the Ehrlich degradation pathway (Hazelwood et al. 2008). As shown in Fig. 1, it can also be produced directly from pyruvate in a biosynthetic pathway that recruits the leucine biosynthetic pathway (Avalos et al. 2013). The upstream pathway from pyruvate to α-ketoisocaproate (KIC) comprises of acetolactate synthase (encoded by *ILV2*), ketol-acid reductoisomerase (encoded by *ILV5*), dihydroxyacid dehydratase (encoded by *ILV3*), 2-isopropylmalate synthase (encoded by *LEU4* or *LEU9*), isopropylmalate isomerase (encoded by *LEU1*), and 2-isopropylmalate dehydrogenase (encoded by *LEU2*). The downstream Ehrlich degradation pathway from KIC to isoamyl alcohol comprises α-ketoacid decarboxylase (KDC, encoded by *ARO10*) and alcohol dehydrogenase (ADH, encoded by *ADH7*).

As shown in Fig. 1, the native leucine biosynthetic pathway in budding yeast is subjected to subcellular compartmentalization, in which the upstream part from pyruvate to α-IPM is confined inside mitochondria, while the downstream part of pathway from α-IPM to KIC is confined in the cytosol (Hazelwood et al. 2008). Since valine, isoleucine, and leucine biosynthetic pathways share the same enzymatic reactions (ILV2, ILV5, and ILV3), relocalizing the entire isoamyl alcohol biosynthetic pathway into the cytosol did reduce the 2-methyl-butanol formation but still leads to isobutanol as the

Table 2 List of plasmids and strains used in the present study

Name	Description	References
Plasmid name		
pSH62	Plasmid harboring *Cre* gene under the control of P_{GAL1}. This plasmid contains the centromeric replication origin and the *HIS3* selection marker.	(Gueldener et al. 2002)
pESC-URA	Plasmid for protein overexpression under galactose-inducible promoter. This plasmid contains 2-micron replication and the *URA3* selection marker.	Agilent technologies
pRS426-ARO10/ADH7	pESC-URA derivative with *ARO10* and *ADH7* under galactose-inducible promoter.	This study
pRS413-OAC1	pSH62 derivative with P_{GAL1}-*OAC1*-T_{CYC1}.	This study
pδBLE2.0	Plasmid for cloning genome integration cassettes.	
pδBLE2.0-ILV2	pδBLE2.0 derivative contains *ILV2* gene.	This study
pδBLE2.0-ILV3	pδBLE2.0 derivative contains *ILV3* gene.	This study
pδBLE2.0-LEU2	pδBLE2.0 derivative contains *LEU2* gene.	This study
pδBLE2.0-ILV2/ILV5	pδBLE2.0-ILV2 with an insertion of *ILV5* gene.	This study
pδBLE2.0-ILV3/LEU9	pδBLE2.0-ILV3 with an insertion of *LEU9* gene.	This study
pδBLE2.0-LEU2/LEU1	pδBLE2.0-LEU2 with an insertion of *LEU1* gene.	This study
Strain name		
MC1~MC6	Six representative strains derived from BY4741 with a chromosome-based leucine biosynthetic pathway under the control of strong galactose-inducible promoters.	This study
MC1a~MC6a	Strain MC1~MC6 derivative transformed with plasmid pRS426-ARO10/ADH7.	This study
MC1b~MC6b	Strain MC1a~MC6a derivative transformed with plasmid pRS413-OAC1.	This study

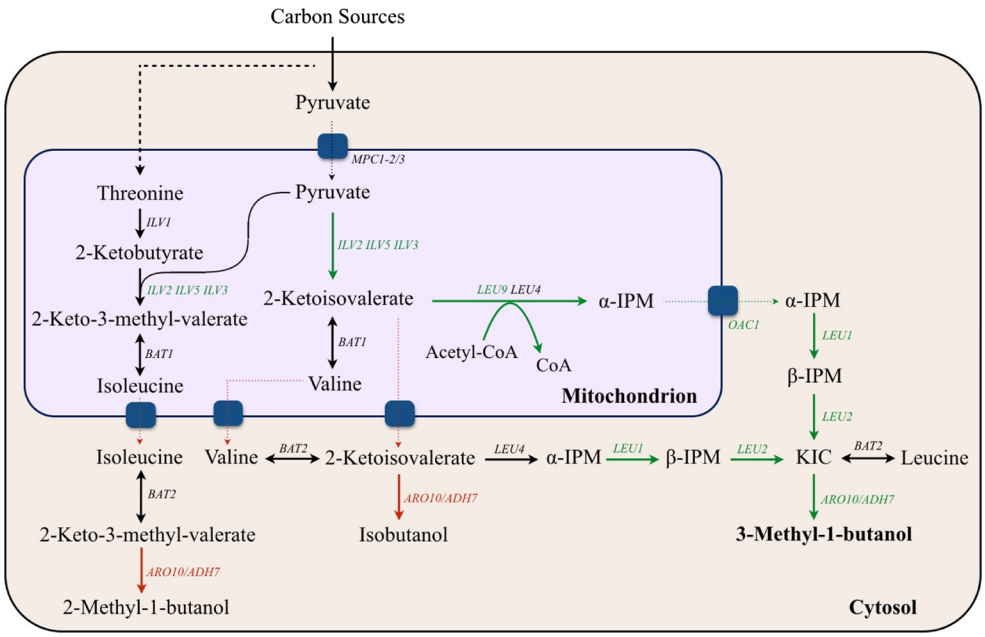

Fig. 1 Schematic diagram of isoamyl alcohol biosynthetic pathway in *S. cerevisiae*. *ILV2*, encoding acetolactate synthase; *ILV5*, encoding acetohydroxyacid reductoisomerase; *ILV3*, encoding dihydroxyacid dehydratase; *LEU4*/*9*, encoding α-IPM synthase; *LEU1*, encoding isopropylmalate isomerase; *LEU2*, encoding β-IPM dehydrogenase; *ARO10*, encoding ketoacid decarboxylase; *ADH7*, encoding alcohol dehydrogenase; *MPC1–2/3*, encoding mitochondrial pyruvate carrier; *ILV1*, encoding threonine deaminase; *OAC1*, encoding mitochondrial α-IPM transporter; *BAT1*, encoding mitochondrial branched-chain amino acid aminotransferase; *BAT2*, encoding cytosolic branched-chain amino acid aminotransferase. The pathway intermediates α-IPM, β-IPM, and KIC are defined as 2-isopropylmalate, 3-isopropylmalate, and α-ketoisocarpoate, respectively. The *dotted line* indicates the transportation of metabolites between mitochondria and cytosol

main product (unpublished data), and compartmentalizing the entire pathway into mitochondria would result in high amounts of both isobutanol and 2-methyl-1-butanol side products. To minimize the formation of side products, we decided to utilize the native compartmentalization of leucine biosynthetic pathway and the cytosolic expression of the downstream Ehrlich degradation pathway for the isoamyl alcohol overproduction. Here, the mitochondrial compartment serves as a barrier to KIV and 2-keto-3-methyl-valerate (KMV) and reduces the accessibility of downstream cytosolic Ehrlich degradation enzymes to these intermediates. This approach also takes the advantage of the mitochondrial environment for the optimal activity of branched-chain amino acid biosynthetic pathways (Avalos et al. 2013).

In the present study, the isoamyl alcohol pathway was divided into two modules: a chromosome-based leucine biosynthetic pathway and a plasmid-based expression of the downstream Ehrlich degradation pathway. More specifically, the upstream section of leucine biosynthetic pathway contains enzymes confined into mitochondria (ILV2, ILV3, ILV5, and LEU9), which catalyze the conversion of pyruvate to α-IPM in the mitochondrion. The downstream leucine biosynthetic pathway contains *LEU1* and *LEU2* that catalyze the conversion of α-IPM to KIC (Fig. 1). The entire leucine biosynthetic pathway was integrated into yeast chromosomes using the previously established δ-integration platform. Notably, *LEU9* was chosen for constructing the leucine biosynthetic pathway because the LEU9 is resistant to the feedback inhibition, whereas the LEU4 is subject to a strong feedback inhibition (Lopez et al. 2015). As shown in Fig. 2, all six randomly isolated variants (MC1a to MC6a) showed significant amounts of isoamyl alcohols, with the product titer ranging from 290 to 480 mg/L under shake-tube conditions. Surprisingly, there were still high amounts of isobutanol and 2-methyl-1-butanol produced in these variants, indicating KIV and KMV are capable of transporting outside of mitochondria and subjected to the Ehrlich degradation pathway (Fig. 2). In comparison, the reference strain BY4741 with a plasmid-based expression of *ARO10* and *ADH7* only produced less than 20 mg/L of branched-chain alcohols. As shown in Fig. 2, the best producer MC5a yielded approximately 467.83 mg/L isoamyl alcohol, together with 438.59 mg/L isobutanol and 181.70 mg/L 2-methyl-1-butanol, which is the highest branched-chain alcohol yield (per gram sugar) reported in budding yeast. Notably, there were different patterns of alcohol productions between MC1a~MC6a, indicating the combinatorial effect introduced by the δ-integration-mediated pathway assembly technique.

Although colony PCR verification confirmed the fully assembled leucine biosynthetic pathway before subjected to the characterization of isoamyl alcohol productions, we decided to further validate whether the leucine biosynthetic pathway genes were differentially overexpressed in our engineered yeast strains. As shown from Fig. 3, further quantitative real-time PCR (qRT-PCR) analysis in the best producer (MC5a) and the worst producer (MC3a) revealed that all leucine biosynthetic pathway genes highly expressed in the engineered strains when compared to that of a reference strain as previously reported (Yuan and Ching 2015a). Moreover, the mRNA abundance of each gene was differentially expressed in these engineered strains, confirming the combinatorial effect introduced by the method developed by a previous study (Yuan and Ching 2015a). In addition, both LEU1 and LEU2 were expressed to a much higher level in strain MC5a than

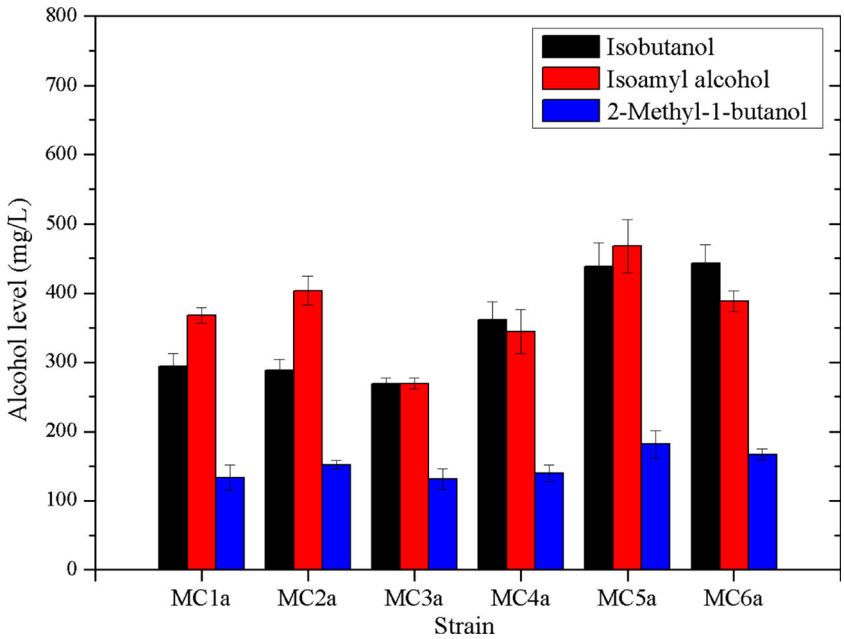

Fig. 2 Isoamyl alcohol production in engineered yeast cells with a chromosome-based leucine biosynthetic pathway and plasmid-based Ehrlich degradation pathway. Alcohol production by different strains. Strains MC1~MC6 were transformed with plasmid pRS426-ARO10/ADH7 to yield MC1a-MC6a. All strains were cultivated in 14-mL tubes supplemented with 2 mL SC medium with additional valine, leucine, isoleucine, and uracil dropped out. The alcohol production in engineered strains was measured using GC-MS after 72 h of cultivation. Data represented the average and standard deviation of three independent experiments

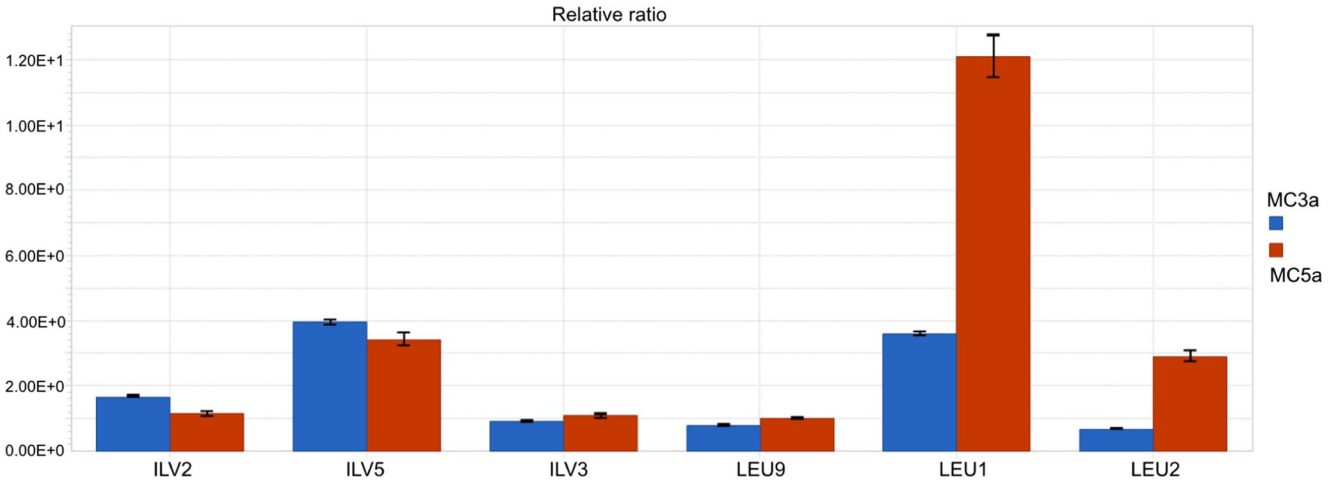

Fig. 3 Quantitative real-time PCR analysis of the gene expression profile in the engineered yeast cell. Two representative strains MC3a and MC5a were subjected to qRT-PCR analysis. Fresh overnight cultures were inoculated in SC-URA media at initial OD_{600} of 0.05 and harvested during early log phase for qRT-PCR analysis. The results are presented as the relative abundances of *ILV2*, *ILV5*, *ILV3*, *LEU9*, *LEU1*, and *LEU2* in each strain with respect to that of *ACT1*. Data represented the average and standard deviation of three independent experiments

that of strain MC3a, which also explains the higher isoamyl alcohol production in strain MC5a might be caused by more α-IPM be pulled towards isoamyl alcohol production due to LEU1 and LEU2 overexpression. In the future, variants with a better isoamyl alcohol productivity might be further isolated from the library, and qRT-PCR analysis of these strains would shed some light on the rational pathway engineering in the future.

Effects of the α-IPM exporter on the production of isoamyl alcohol

Based on the observation that significant amounts of isobutanol and 2-methyl-1-butanol were produced by variants with the entire leucine biosynthetic pathway overexpressed, we next proceeded to improve the isoamyl alcohol production in the abovementioned variants by introducing the transporter engineering technique. The α-IPM transporter is well studied in budding yeast and is encoded by *OAC1* gene (Marobbio et al. 2008). Moreover, it was reported that the OAC1 transporter not only exports α-IPM from mitochondria to the cytosol but also imports the oxaloacetate (OAA) from the cytosol to mitochondria as part of the pyruvate shunt (Marobbio et al. 2008). Therefore, increasing the expression level of the α-IPM transporter in our engineered yeast strains not only pulls α-IPM across the mitochondrial membrane to the cytosol but also pushes the pyruvate substrate into mitochondria for the synthesis of α-IPM.

Introducing an additional copy of *OAC1* in strain MC1a~MC6a increased the isoamyl alcohol production by additional 19.9~34.4%, whereas the isobutanol level was reduced by approximately 11.9~39.5% and 2-methyl-1-butanol showed only a slight change (Fig. 4a). As shown in Fig. 4a, the distribution of branched-chain alcohol profile had a noticeable change upon the OAC1 overexpression. There is a clear increase of the peak area for isoamyl alcohol in strain MC5b upon the additional overexpression of *OAC1* gene (Fig. 4b). The best producer MC5b reached a titer of 561.21 mg/L isoamyl alcohol, 321.64 isobutanol, and 160.82 mg/L 2-methyl-1-butanol after 72 h cultivation. Notably, the isoamyl alcohol level showed more than 78-fold improvement over the reference BY4741 strain only expressing *ARO10* and *ADH7*. These findings confirmed that overexpression of *OAC1* gene is effective in channeling more carbon flux towards the isoamyl alcohol production.

Discussion

The traditional plasmid-based expression has been widely used for a proof of concept genetic engineering of biosynthetic pathways in the yeast *S. cerevisiae*. However, the plasmid burden and stability during the cell division significantly reduce the yield of desired products (Karim et al. 2013). Moreover, the plasmid-based system suffers poor control of gene copy number to achieve the optimal gene expression level. For industrial applications, it is desirable to directly assemble metabolic pathways into yeast chromosomes for stable expressions. Recently, our group has successfully developed antibiotic-assisted δ-integration method for assembling multi-gene pathway in one-step fashion (Yuan and Ching 2014; Yuan and Ching 2016), which significantly reduces the amount of time required to construct metabolic pathways when compared to conventional genome integration approaches (Paddon et al. 2013; Sadowski et al. 2007; Yuan and Ching 2015b).

For high-level production of isoamyl alcohol in budding yeast, we sought to reconstruct a chromosome-based leucine

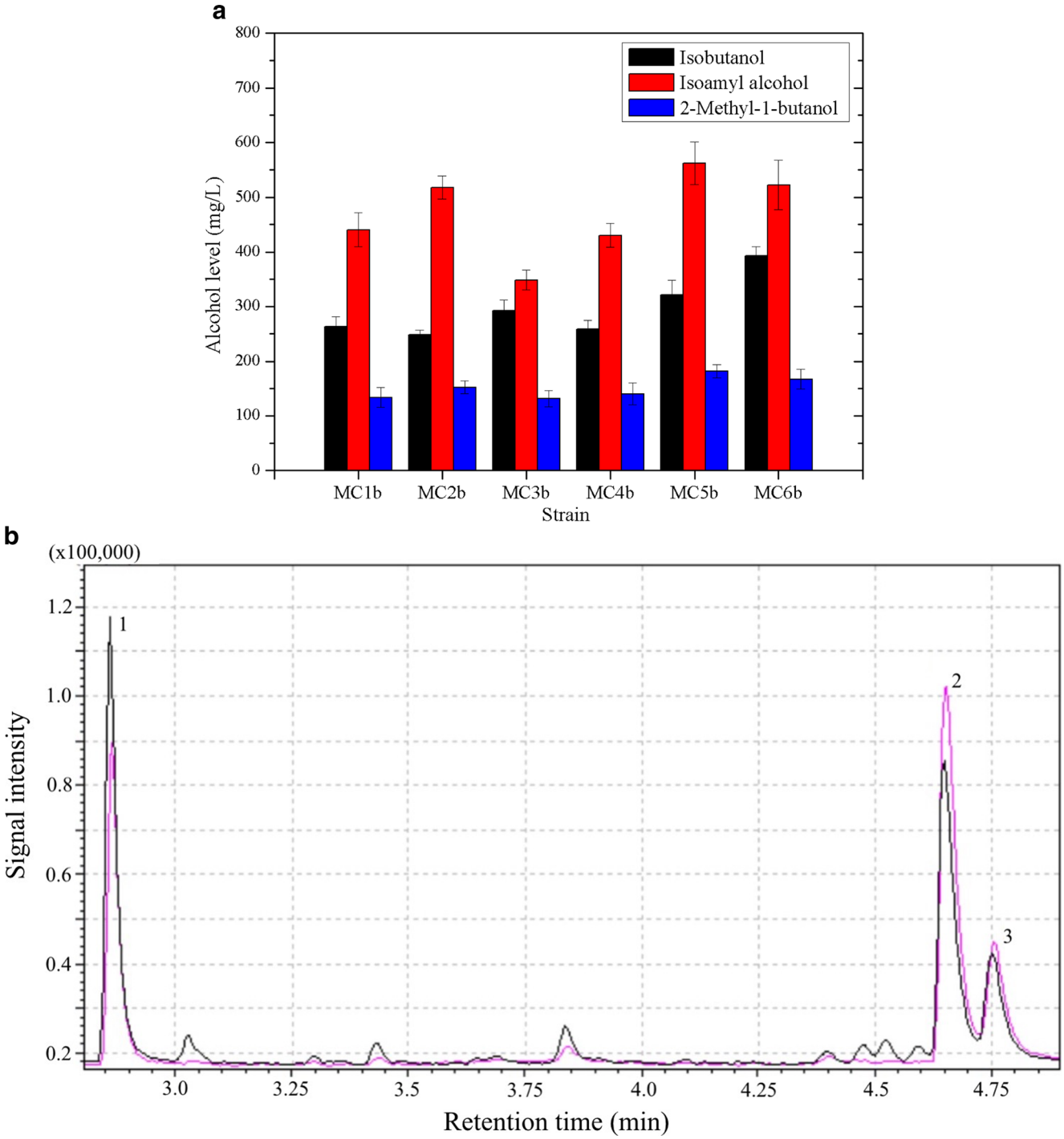

Fig. 4 Effect of α-IPM transporter on isoamyl alcohol production in the engineered yeast**. a** Branched-chain alcohols produced by different strains. Strains MC1a~MC6a were further transformed with plasmid pRS413-OAC1 to yield MC1b-MC6b. All strains were cultivated in 14-mL tubes supplemented with 2 mL SC medium with additional valine, leucine, histidine, isoleucine, and uracil dropped out. The alcohol production in engineered strains was measured using GC-MS after 72 h of cultivation. Data represented the average and standard deviation of three independent experiments. **b** Representative gas chromatogram of alcohols produced by strains MC5a and MC5b. Peak 1 (retention time, 2.86 min): isobutanol. Peak 2 (retention time, 4.65 min): isoamyl alcohol. Peak 3 (retention time, 4.75 min): 2-methyl-1-butanol. *Black line* indicates the gas chromatogram from strain MC5a. *Pink line* indicates the gas chromatogram from strain MC5b

biosynthetic pathway under the control of strong promoters in *S. cerevisiae*. When compared to conventional genetic engineering approaches such as manipulation of transcriptional factors to improve pathway activities, rewiring the entire leucine biosynthetic pathway under the control of strong promoters would not provoke the expression of unwanted genes

that commonly occurred during the manipulation of transcriptional factors (Park et al. 2014; Ro et al. 2006). As can be inferred from Fig. 2, strains with a combinatorially assembled leucine biosynthetic pathway resulted in different levels of branched-chain alcohol productions, indicating that different combinations of gene integration copy numbers would have beneficial effects on improving pathway activities. By simply assembling the leucine biosynthetic pathway into yeast chromosomes, one variant yielded approximately 467.83 mg/L isoamyl alcohol, together with 438.59 mg/L isobutanol and 181.70 mg/L 2-methyl-1-butanol from 3.6% galactose plus 0.4% glucose after 72 h.

To further increase the pathway activity, we next proceeded with the transporter engineering to avoid the limiting factor of translocating α-IPM across the mitochondrial membrane by overexpressing the α-IPM transporter encoded by *OAC1* gene. In addition, the OAC1 is also found as an OAA transporter that is capable of transporting the OAA generated in the cytosol to mitochondria (Palmieri et al. 1999). In the cytosol, the OAA is produced from pyruvate through the "pyruvate shunt", gluconeogenesis by the pyruvate carboxylase (Marobbio et al. 2008). Once imported inside mitochondria, the OAA can be converted to malate by the mitochondrial malate dehydrogenase (MDH) and then to pyruvate by the mitochondrial malic enzyme (MAE1) (Marobbio et al. 2008). Initially, we thought that the application of the OAC1 transporter as an exchanger of OAA and α-IPM would serve as a push and pull strategy to divert the cytosolic pyruvate flux towards the mitochondrial α-IPM synthesis and consequently enhance the isoamyl alcohol production. However, we did not observe any pushing effect as we did not observe an increased level for 2-methyl-1-butanol and isobutanol upon the expression of OAC1.

As can be seen from Fig. 4a, there was a clear trend of enhanced levels of isoamyl alcohol after overexpressing *OAC1* gene in the engineered strains with the entire leucine biosynthetic pathway. More importantly, there was a noticeable decrease of isobutanol peak upon the OAC1 overexpression, suggesting OAC1 is effective to channel more α-IPM across the mitochondrial membrane and improve the isoamyl alcohol production. The best producer MC5b reached the titer of 561.21 mg/L isoamyl alcohol, 321.64 mg/L isobutanol, and 160.82 mg/L 2-methyl-1-butanol after 72 h cultivation. The conversion rate of 14.18 mg per gram sugar into isoamyl alcohol is twice as high as that of the CEN.PK2-1C derived strain described in a previous report (Park et al. 2014).

Considering the fact that there are still significant amounts of isobutanol and 2-methyl-1-butanol co-formed in the engineered yeast cells, there is no doubt that further transporter engineering by knocking down transporters involved in the translocation of KIV, KMV, valine, and isoleucine would facilitate the engineered yeast cells to exclusively produce isoamyl alcohol. *S. cerevisiae* encodes approximately 40 proteins belonging to the mitochondrial carrier family, but two thirds of which are still poorly understood (Ferramosca and Zara 2013; Kuan and Saier 1993; Kunji 2004). Unfortunately, such transporters involved in transporting KIV, KMV, valine, and isoleucine are currently not identified. In the future, we will attempt to identify these transporters and examine whether utilization of the mitochondrial compartment coupled with the transporter engineering technique could lead to the engineered yeast cells to exclusively produce isoamyl alcohol. In addition, designing KDCs with a better substrate specificity towards KIC would offer an alternative solution for exclusively producing isoamyl alcohol in budding yeast.

In summary, our work represents the first study to construct a chromosome-based leucine biosynthetic pathway for the isoamyl alcohol production. Moreover, the utilization of the mitochondrial compartment coupled with the transporter engineering serves as an effective approach to minimize the by-product formation and improve the isoamyl alcohol production.

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Authors' contributions JY conceived the study, designed the experiments, performed the experiments, analyzed the data, and drafted the manuscript. XC participated in the experiments, analyzed the data, and helped to revise the manuscript. PM participated in the experiments, analyzed the data, and helped to revise the manuscript. CBC supervised the project and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Ethical approval This study does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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