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# Elimination of biosynthetic pathways for L-valine and L-isoleucine in mitochondria enhances isobutanol production in engineered *Saccharomyces cerevisiae*

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ABSTRACT

*Saccharomyces cerevisiae* has a natural ability to produce higher alcohols, making it a promising candidate for production of isobutanol. However, the several pathways competing with isobutanol biosynthesis lead to production of substantial amounts of L-valine and L-isoleucine in mitochondria and isobutyrate, L-leucine, and ethanol in cytosol. To increase flux to isobutanol by removing by-product formation, the genes associated with formation of L-valine (*BAT1*), L-isoleucine (*ILV1*), isobutyrate (*ALD6*), L-leucine (*LEU1*), and ethanol (*ADH1*) were disrupted to construct the *S. cerevisiae* WΔGBIALA1_2vec strain. This strain showed 8.9 and 8.6 folds increases in isobutanol concentration and yield, respectively, relative the corresponding values of the background strain on glucose medium. In a bioreactor fermentation with a gas trapping system, the WΔGBIALA1_2vec strain produced 662 mg/L isobutanol concentration with a yield of 6.71 $mg_{isobutanol}/g_{glucose}$. With elimination of the competing pathways, the WΔGBIALA1_2vec strain would serve as a platform strain for isobutanol production.

## 1. Introduction

Ethanol is the most common biofuel that can be produced by microbial strains, especially by *Saccharomyces cerevisiae*. However, bioethanol has several drawbacks to be used as a transportation fuel due to its high hygroscopicity, low energy density, and high water content. Higher or branched alcohols have received great attention as an alternative fuel (Savage, 2011). In particular, biobutanol has preferable properties such as low hygroscopicity and high energy density, which is comparable to that of gasoline (Ranjan and Moholkar, 2012). While isobutanol has the same fuel properties as other butanol isomers, its branched structure makes it less toxic and having higher octane values than its straight-chain counterpart (Blombach and Eikmanns, 2011). In addition, isobutanol can be used as a building block for production of other chemicals (Weber et al., 2010).

Bacterial strains including *Escherichia coli* (Baez et al., 2011), *Corynebacterium glutamicum* (Blombach and Eikmanns, 2011), and *Bacillus subtilis* (Qi et al., 2014) have been engineered to produce isobutanol by introducing the Ehrlich pathway. In the Ehrlich pathway, 2-ketoisovalerate is converted to isobutanol by ketoacid decarboxylase (*KDC*) and alcohol dehydrogenase (*ADH*) (Hazelwood et al., 2008). In these host strains, deletion of competing pathways and cofactor engineering resulted in isobutanol production near the theoretical maximal yield (Atsumi et al., 2010; Yamamoto et al., 2013).

*S. cerevisiae* is a workhorse strain for cost-effective production of biofuels and biochemicals, and it is tolerant to high concentrations of alcohols such as ethanol and n-butanol (Fischer et al., 2008). In *S. cerevisiae*, isobutanol is produced via the L-valine biosynthetic pathway and Ehrlich pathway (Buijs et al., 2013) (Fig. 1). In the mitochondria, acetolactate synthase (*ALS*), ketolacid reductoisomerase (*KARI*), dihydroxyacid dehydratase (*DHAD*), and branched-chain amino acid aminotransferase (*BAT*) synthesize L-valine from pyruvate. Therefore, 2-ketoisovalerate must be transported into the cytosol for the production of isobutanol via the Ehrlich pathway. To overcome low production yield of isobutanol caused by this different compartmentalization of pathways, we previously re-localized the L-valine biosynthetic pathway into the cytosol by deleting the mitochondria targeting sequences of ALS, KARI, and DHAD (Lee et al., 2012). Even with the above approach, the isobutanol yields and titers were still low. A recent study reported that the engineered *S. cerevisiae* strain with deleting *BAT1* coding for

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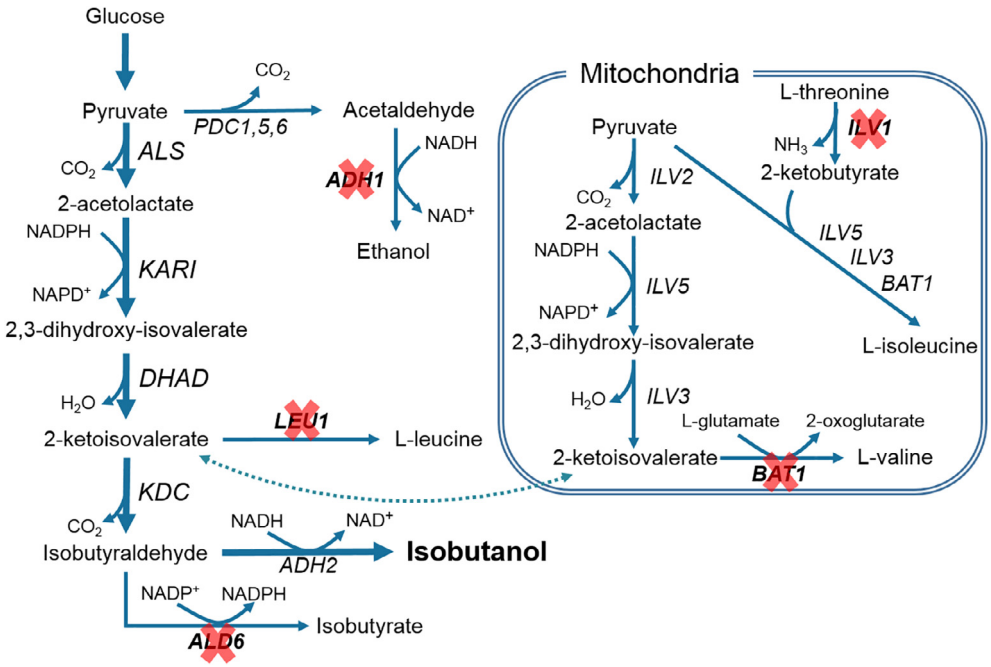


**Fig. 1.** Isobutanol biosynthetic pathway and target genes for eliminating competitive pathways. Fluxes in the isobutanol biosynthetic pathway were amplified by overexpressing *alsS* from *B. subtilis* for ALS activity, M.*ilvC* from *E. coli* for KARI activity, *ilvD* from *L. lactis* for DHAD activity, and *kivd* from *L. lactis* for KDC activity.

**Table 1**
*S. cerevisiae* strains and plasmids used in this study.

| Name | Description | Reference |
|---|---|---|
| Strains | | |
| D452-2 | *MATα*, *leu2*, *his3*, *ura3*, and *can1* | (Hosaka et al., 1992) |
| WΔG | D452-2 Δ*GRX3*::*CFD1* | This study |
| WΔGB | D452-2Δ*GRX3*::*CFD1*Δ*BAT1* | This study |
| WΔGBI | D452-2Δ*GRX3*::*CFD1*Δ*BAT1*Δ*ILV1* | This study |
| WΔGBIA | D452-2Δ*GRX3*::*CFD1*Δ*BAT1*Δ*ILV1*Δ*ALD6* | This study |
| WΔGBIAL | D452-2Δ*GRX3*::*CFD1*Δ*BAT1*Δ*ILV1*Δ*ALD6*Δ*LEU1* | This study |
| WΔGBIALA1 | D452-2Δ*GRX3*::*CFD1*Δ*BAT1*Δ*ILV1*Δ*ALD6*Δ*LEU1*Δ*ADH1* | This study |
| WΔG_3vec | WΔG, p423TDH3_M.*ilvC*, p425TDH3_*alsSkivd*, p426TDH3_*ilvD* | This study |
| WΔGB_3vec | WΔGB, p423TDH3_M.*ilvC*, p425TDH3_*alsSkivd*, p426TDH3_*ilvD* | This study |
| WΔGB_2vec | WΔGB, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd* | This study |
| WΔGBI_2vec | WΔGBI, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd* | This study |
| WΔGBIA_2vec | WΔGBIA, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd* | This study |
| WΔGBIAL_2vec | WΔGBIAL, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd* | This study |
| WΔGBIALA1_2vec | WΔGBIALA1, p423TDH3_M.*ilvCilvD*, p426TDH3_*alsSkivd* | This study |
| Plasmids | | |
| p423TDH3 | *LEU2*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, $Amp^R$ | (Mumberg, et al. 1995) |
| p425TDH3 | *URA3*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, $Amp^R$ | (Mumberg, et al. 1995) |
| p426TDH3 | *LEU2*, *TDH3* promoter, *CYC1* terminator, 2 μ origin, $Amp^R$ | (Mumberg, et al. 1995) |
| p423TDH3_M.ilvC | p423TDH3 harboring modified *ilvC* from *E. coli* | In this study |
| p425TDH3_alsSkivd | p425TDH3 harboring *alsS* from *B. subtilis* and *kivd* from *L. lactis* | In this study |
| p426TDH3_ilvD | p426TDH3 harboring *ilvD* from *L. lactis* | In this study |
| p423TDH3_M.ilvCilvD | p423TDH3 harboring modified *ilvC* from *E. coli* and *ilvD* from *L. lactis* | In this study |
| p426TDH3_alsSkivd | p426TDH3 harboring *alsS* from *B. subtilis* and *kivd* from *L. lactis* | In this study |
| pAUR_Cas9 | p414-TEF1p-Cas9-CYC1t, modified Cas9 expression plasmid | (Lee et al., 2017) |
| p42H_g*LEU2* | $Hyg^R$, 2 μ origin, $Amp^R$, $P_{SNR52}$_g*LEU2*_$T_{SUP4}$ | (Zhang et al., 2014) |
| p42H_g*BAT1* | $Hyg^R$, 2 μ origin, $Amp^R$, $P_{SNR52}$_g*BAT1*_$T_{SUP4}$ | In this study |
| p42H_g*ILV1* | $Hyg^R$, 2 μ origin, $Amp^R$, $P_{SNR52}$_g*ILV1*_$T_{SUP4}$ | In this study |
| p42H_g*ALD6* | $Hyg^R$, 2 μ origin, $Amp^R$, $P_{SNR52}$_g*ALD6*_$T_{SUP4}$ | In this study |
| p42H_g*LEU1* | $Hyg^R$, 2 μ origin, $Amp^R$, $P_{SNR52}$_g*LEU1*_$T_{SUP4}$ | In this study |
| p42H_g*ADH1* | $Hyg^R$, 2 μ origin, $Amp^R$, $P_{SNR52}$_g*ADH1*_$T_{SUP4}$ | In this study |

branched-chain amino acid transaminases located in the mitochondria and overexpressing the genes involved in the isobutanol biosynthetic pathway in the mitochondria produced 1.25 g/L isobutanol with a yield of 12.45 mg isobutanol/g glucose (Hammer and Avalos, 2017). The highest isobutanol production titer and yield in a yeast system was achieved by engineering *Pichia pastoris*. The engineered *P. pastoris* strain overexpressing the genes for keto-acid degradation pathway along with the genes for the endogenous L-valine biosynthetic pathway was able to produce 2.22 g/L isobutanol with a yield of 22.2 mg isobutanol/g glucose (Siripong et al., 2018). However, the isobutanol production titer and yield were still low compared with those from engineered bacterial strains, which can produce more than 20 g/L of isobutanol near the theoretical maximal yield (Atsumi et al., 2008). To maximize the fermentation performances of isobutanol production in *S. cerevisiae*,