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MINI-REVIEW

Microbial host engineering for sustainable isobutanol production from renewable resources

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Abstract

Due to the limited resources and environmental problems associated with fossil fuels, there is a growing interest in utilizing renewable resources for the production of biofuels through microbial fermentation. Isobutanol is a promising biofuel that could potentially replace gasoline. However, its production efficiency is currently limited by the use of naturally isolated microorganisms. These naturally isolated microorganisms often encounter problems such as a limited range of substrates, low tolerance to solvents or inhibitors, feedback inhibition, and an imbalanced redox state. This makes it difficult to improve their production efficiency through traditional process optimization methods. Fortunately, recent advancements in genetic engineering technologies have made it possible to enhance microbial hosts for the increased production of isobutanol from renewable resources. This review provides a summary of the strategies and synthetic biology approaches that have been employed in the past few years to improve naturally isolated or non-natural microbial hosts for the enhanced production of isobutanol by utilizing different renewable resources. Furthermore, it also discusses the challenges that are faced by engineered microbial hosts and presents future perspectives to enhancing isobutanol production.

Key points

- *Promising potential of isobutanol to replace gasoline*
- *Engineering of native and non-native microbial host for isobutanol production*
- *Challenges and opportunities for enhanced isobutanol production*

Keywords Isobutanol · Microbial hosts · Genetic engineering · Biofuel production · Synthetic pathway

Introduction

Limited fossil fuel resources and their environmental concerns have diverted human interest toward producing sustainable and renewable fuels (Bumrungtham et al. 2022). Today, ethanol is the most commonly produced biofuel worldwide, obtained from cane sugar or corn starch via fermentation technology (Aziz et al. 2023; Coimbra et al. 2023). Despite being a renewable resource, ethanol is not considered an advanced biofuel because of its subpar fuel properties. These include a low energy density, a high vapor pressure, and an incompatibility with the existing fuel infrastructure, the latter of which can be attributed in large part to ethanol's high hygroscopicity and corrosiveness (Ni et al. 2023). This situation provides an opportunity to find alternative fuels. As a result, there has been a lot of interest in producing advanced biofuels that would possess properties similar to those fuels generated from petroleum, such as higher alcohols (Choi et al. 2014; Gupta et al. 2022). Isobutanol and 1-butanol are the two examples of these higher alcohols, which have high energy densities and low vapor pressure than ethanol (Jawed et al. 2020; Zhang et al. 2019b). They can be mixed with gasoline in any ratio and can also be used as replacements for gasoline because they can perform better in conventional gasoline engines (Yousif and Saleh 2023). Isobutanol and its derivatives also have numerous applications in various chemical industries; for instance, these can be used as solvents, additives in paints,

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ink ingredients, and extractants for organic compounds (de Lima et al. 2023; Wang et al. 2012). In addition, isobutanol is comparatively less toxic and has superior energy density than its straight-chain counterparts (Siripong et al. 2018; Veettil et al. 2016). Thus, these properties make isobutanol more attractive than other butanol isomers.

Isobutanol can be produced naturally by branched-chain amino acid biosynthesis through five enzymatic steps (Kobayashi et al. 2022). In the isobutanol pathway, the two molecules of pyruvate are first converted into 2-acetolactate by the acetolactate synthase (AHAS). In the second reaction, 2-acetolactate is reduced to 2,3-dihydroxy isovalerate; this reaction is catalyzed by acetohydroxy-acid reductoisomerase (AHAIR). In the third reaction, 2,3-dihydroxyisovalerate is converted into 2-ketoisovalerate catalyzed by dihydroxyacid dehydratase (DHAD). Finally, isobutanol can be synthesized via the Ehrlich pathway from 2-ketoisovalerate using two more reaction steps; the enzymatic steps are catalyzed by ketoacid decarboxylases (KDCs) and alcohol dehydrogenase (ADH), respectively (Fig. 1) (Felpeto-Santero et al. 2015; Hasegawa et al. 2020; Hazelwood et al. 2008; Novak et al. 2020). Only a few microorganisms can naturally produce isobutanol in a meager amount, such as *Lactococcus lactis*, *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Candida* sp. (Derrick and Large 1993; Liu et al. 2021b; Smit et al. 2004). With rapidly evolving techniques and quick access to metabolic engineering tools and taking advantage of the increasing genomic information, considerable progress has been made to improve isobutanol production in native microbial hosts (Siripong et al. 2018; Wess et al. 2019). However, the titer of isobutanol produced by the native microbial host is still considerably below the levels needed for industrial purposes. For this reason, various non-native microbial hosts, such as *Escherichia coli*, *Bacillus subtilis*, *G. thermoglucosidasius*, *Corynebacterium glutamicum*, *Clostridium cellulolyticum*, *Ralstonia eutropha*, and *Synechococcus elongatus*, have been engineered for isobutanol production using a diverse range of feedstocks (Fig. 2) (Atsumi et al. 2009; Higashide et al. 2011; Huo et al. 2018; La et al. 2017; Li et al. 2011; Lin et al. 2014; Lu et al. 2012; Smith et al. 2010). Several approaches have been successfully employed to enhance isobutanol production in these microorganisms, such as deleting the competitive pathways, overexpressing the key enzymes of the isobutanol synthesis pathway, cofactor engineering, and improved robustness of microbial hosts (Acedos et al. 2021; Lu et al. 2012; Matsuda et al. 2013). All the native and non-native microbial-hosts used for isobutanol production are compiled in Table 1.

A wide range of reviews addressed biofuel production in microorganisms (Mainguet and Liao 2010; Meadows et al. 2018; Nawab et al. 2020; Xin et al. 2019); nevertheless, a comprehensive review enlisting all genetically engineered microbial hosts for the production of isobutanol is still not available. Therefore, a comprehensive review showing a full picture of engineered microbial hosts for isobutanol production is highly required. Here, we have summarized different engineered microbial hosts for isobutanol production and discussed different engineering strategies for enhancing the production of isobutanol. This review also explains the problems plaguing modern isobutanol manufacturing and suggests some approaches for addressing them. So, we conclude that this study will assist researchers in overcoming

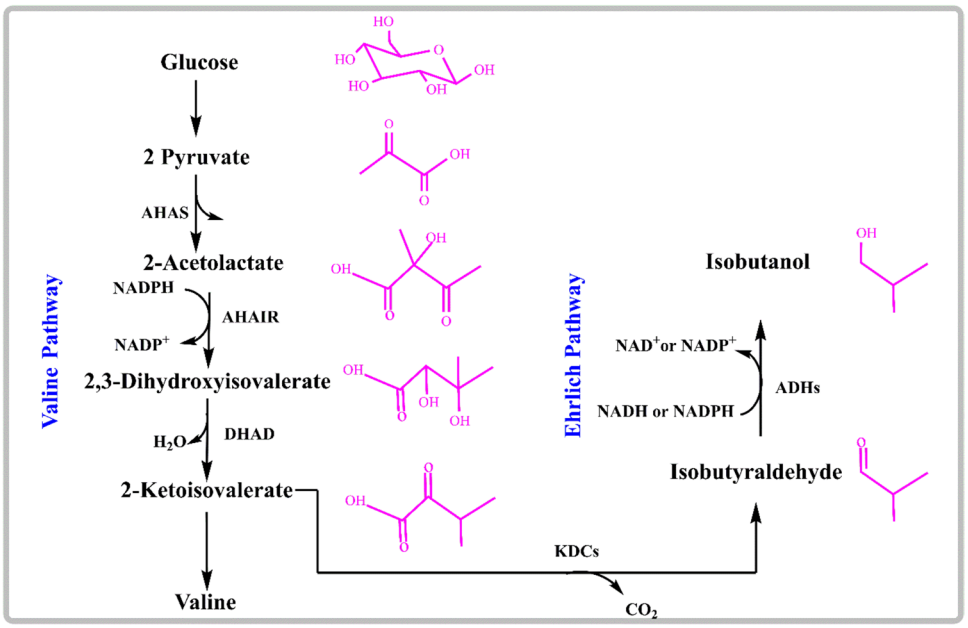

Fig. 1 Pathway used for production of isobutanol in microbial hosts. Figure modified from Felpeto-Santero et al. (2015)

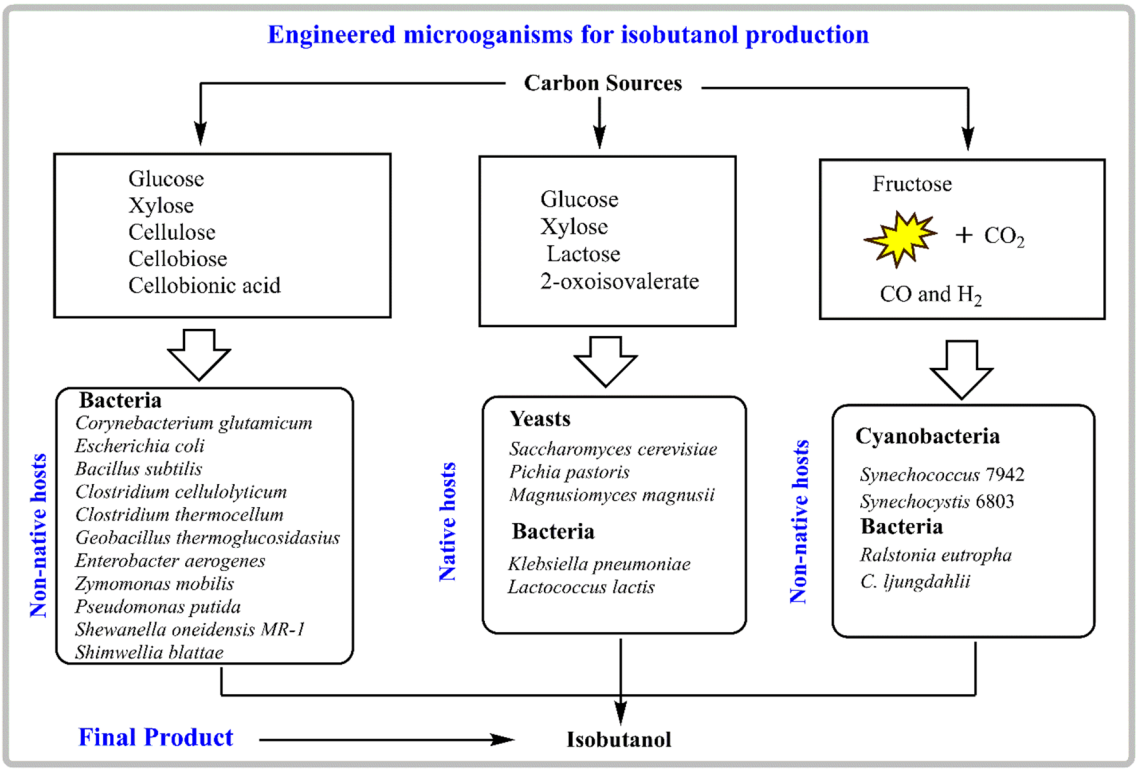

Fig. 2 Schematic representation of the microbial production of isobutanol from various feedstocks

the problems associated with engineering microbial hosts for producing isobutanol and developing efficiently engineered strains to fulfil the world's growing energy demands.

Engineering native microbial hosts for isobutanol production

Few microbial hosts are capable of producing isobutanol naturally, i.e., *S. cerevisiae*, *Pichia pastoris*, and *Lactococcus lactis* (Gambacorta et al. 2022; Priyadharshini et al. 2015; Siripong et al. 2018). However, these microorganisms produce a very minute amount of isobutanol. The production range of isobutanol varies from 0.01 to 0.44 g/L (Kurylenko et al. 2020; Priyadharshini et al. 2015). In order to increase isobutanol synthesis, scientists have designed a number of metabolic engineering strategies to modify the native-host organisms' isobutanol biosynthetic pathways.

Engineering *Saccharomyces cerevisiae* for isobutanol production

The *S. cerevisiae* (baker's yeast) is used to produce bioethanol and is considered a biofuel industry's workhorse (Coyle Diane Publishing 2010). *S. cerevisiae* has been modified for enhanced production of several advanced biofuels, including isobutanol and the sesquiterpenoids farnesene, bisabolene, and amorphadiene (Peralta-Yahya et al. 2011; Westfall et al. 2012). Due to the nature of this review, we shall restrict our attention to the isobutanol manufacturing process. The modified pathways constructed for the production of isobutanol in *S. cerevisiae* are depicted in Fig. 3 (Brat et al. 2012; Lee et al. 2012; Matsuda et al. 2013).

As mentioned before, yeast naturally produces a minute quantity of isobutanol; therefore, to enhance isobutanol production in *S. cerevisiae*, the genes involved in valine metabolism (*ILV2*, *ILV5*, and *ILV3*) were overexpressed to direct the metabolic flux toward 2-ketoisovalerate. The modified strain produced 0.97 mg of isobutanol for every gram of glucose. Isobutanol production was further increased twofold by the additional overexpression of the *BAT2* gene. Moreover, the combined over-expression of the *ILV2*, *ILV5*, *ILV3*, and *BAT2* genes in *S. cerevisiae* resulted in a 13-fold increased isobutanol production than the parental strain (Chen et al. 2011). In another study, isobutanol production was improved 13-fold by deleting *PDC1* and overexpression of *ILV2*, *kivd*, and *ADH6*. Deleting the *PDC1* gene shifted the metabolic flux from ethanol towards isobutanol synthesis (Kondo et al. 2012). To improve the production of isobutanol in a genetically modified strain of *S. cerevisiae*, an additional improvement

Table 1 Summary of the engineered microbial hosts used for isobutanol production

Host	Substrate	Genes overexpressed	knockout genes	Promoter	Isobutanol titer	References
Synechocystis sp. PCC 6803	CO_2	*kivd* and *adhA*	None	P*tac*	90 mg/L	(Varman et al. 2013)
Synechocystis sp. PCC 6803	CO_2	*ilvBN, ilvC, ilvD, kdc,* and *adh*	None	P*cpcG2*	238 mg/L	(Kobayashi et al. 2022)
S. elongatus PCC7942	CO_2	*alsS*, *ilvC*, *ilvD*, *kivd*, *yqhD, adhA* and *ADH2*	None	P*trc* and $P_L lacO_1$	450 mg/L	(Atsumi et al. 2009)
S. elongatus PCC7942	CO_2	*alsS*, *ilvC*, *ilvD*, *kivd* and *yqhD*	Δ*glgC*	P*trc* and $P_L lacO_1$	550 mg/L	(Li et al. 2014)
C. ljungdahlii	CO and H_2	*kivd, adh, kor,* and *AdhE*	*ilvE (inactivation)*	P*pta – ack*	2.4 mM	(Weitz et al. 2021)
R. eutropha H16	Fructose	*ilvB, ilvH, ilvC, ilvD*, *kivd* and *adh*	Δ*phaCAB*, Δ*ilvE*, Δ*bkdAB* and Δ*aceE*	P*phaC*	270 mg/L	(Lu et al. 2012)
R. eutropha H16	CO_2	*alsS*, *ilvC*, *ilvD*, *kivd* and *yqhD*	Δ*phaCAB*	P*phaC*	536 mg/L (Bioreactor)	(Li et al. 2012a)
R. eutropha H16	Fructose	*ilvB, ilvH, ilvC, ilvD* and *kivd*	Δ*phaCAB*, Δ*ilvE*, Δ*bkdAB*, Δ*aceE,* Δ*acrA,* and Δ*acrA6*	P*phaC*	260 mg/L	(Bernardi et al. 2016)
R. eutropha H16	Fructose	*phaJ*, *sbm1*, *phaA*, *phaB1*, *ter*, *bldh*, and *yqhD*	None	P_{CAT} and P*phaC*	32 mg/L	(Black et al. 2018)
S. cerevisiae	Glucose	*kivd*, *adh6* and *ilv2*	Δ*PDC1*	P*pgk1*	143 mg/L	(Kondo et al. 2012)
S. cerevisiae	Glucose	*ILV2*, *ILV5, ILV3*, *adh6*, *kivd, MAE1*, *MDH2*, and *PYC2*	Δ*LPD1*	P*pgk1*, P*tdh3* and *Padh1*	1.6 g/L	(Matsuda et al. 2013)
S. cerevisiae	Glucose	*ILV2*, *ILV5, ILV3*, and *kivd*	None	P*gpd*	151 mg/L	(Lee et al. 2012)
S. cerevisiae	Glucose	*ILV2*, *ILV5, ILV3*	Δ*ILV2*, Δ*ILV5*, and Δ*ILV3*	P*pgk1*, P*tdh3* and *Padh1*	630 mg/L	(Brat et al. 2012)
S. cerevisiae	Glucose	*ILV2*, *ILV5, ILV3*, *ARO10* and *adh2*	Δ*ILV2*, Δ*ILV5*, and Δ*ILV3*	P*hxt7*, P*fba1* and P*pfk1*	635 mg/L	(Avalos et al. 2013)
S. cerevisiae	Glucose	*ILV2*, *ILV5,* and *ILV3*	Δ*ILV2*, Δ*BDH1*, Δ*BDH2*, Δ*LEU4*, Δ*LEU9*, Δ*ECM31*, Δ*ILV1*, Δ*ADH1*, Δ*GPD1*, Δ*GPD2,* and Δ*ALD6*	P*hxt7*, P*fba1* and P*pfk1*	2.09 g/L	(Wess et al. 2019)
S. cerevisiae	Glucose	*alsS, ILV2, ILV5, and ILV3*	*BAT1* AND *ALD6*	P*adh1,* P*cup1* and P*tdh3*	263.2 mg/L	(Park and Hahn 2019)
S. cerevisiae	Xylose	*XI, XR, XDH, ILVs, KDC,* and *ADH*	*BAT1*, *ALD6*, and *PHO13*	P*tdh3*, P_{TEF1}, P_{PGK1}, P_{ADH1}	3.10 g/L	(Zhang et al. 2019b)
S. cerevisiae	Glucose	*ILV3, ILV2, ILV5,* and *ARO10*			4.20 g/L	(Zhang et al. 2019a)
P. pastoris X33	Glucose	None	None	None	0.065 g/L	(Nor and Roshanida 2015)
P. pastoris	Glucose	*kivd*, *ADH7, ILV5, ILV3, ILV6, ILV2* and *ATF1*	None	P*gap*	2.22 g/L	(Siripong et al. 2018)

Table 1 (continued)

Host	Substrate	Genes overexpressed	knockout genes	Promoter	Isobutanol titer	References
M. magnusii	Glucose	*None*	None	None	0.44 g/L	(Kurylenko et al. 2020)
M. magnusii	Glucose and 2-oxoisovalerate	*ILV2*	None	P_{TEF1}	0.62 g/L	(Kurylenko et al. 2020)
S. blattae	Glucose	*adhA*, *pntAB, alsS*, *ilvC*, *ilvD,* and *kdc*	None	P*trc*	5.98 g/L	(Acedos et al. 2021)
S. blattae	Glucose	*alsS, ilvC*, *ilvD, and kivD*	None	P*tac* and P*trc*	6 g/L	(Felpeto-Santero et al. 2015)
K. pneumonia	Glucose	*ipdC*	Δ*budA,* and Δ*ldhA*	P*bud*	2.45 g/L	(Gu et al. 2017)
K. pneumonia	Glucose	*Kivd*	Δ*budA,* and Δ*ldhA*	P*bud*	3.19 g/L	(Gu et al. 2017)
K. pneumoniae	Glucose	$ipdC^{T290L}$	Δ*budA,* Δ*ldhA*, and Δ*ipdC*	P*tac*	5.5 g/L	
L. lactis	Lactose	None	*None*	None	O.01 g/L	(Priyadharshini et al. 2015)
G. thermoglucosidasius	Glucose	*Kivd*, *alsS*, *ilvC*, *ilvD,* and *adhA*	None	P*ldh*	3.3 g/L	(Lin et al. 2014)
S. oneidensis MR-1	Lactate, pyruvate, and N-acetyl glucosamine	*kivD,* and *adh*	Δ*mtrA*, and Δ*mtrB*	P*lac*	19.3 mg/L	(Jeon et al. 2015)
C. cellulolyticum	Cellobiose	*kivd, alsS, ilvC, ilvD, and yqhD*	None	P*fdx*	0.364 g/L	(Higashide et al. 2011)
C. cellulolyticum	Cellulose	*kivd, alsS, ilvC*, *ilvD, and yqhD*	None	P*fdx*	0.660 g/L	(Higashide et al. 2011)
C. thermocellum	Cellulose	*ilvB*, *ilvN*, *ilvC,* *ilvD*, and *kivd*	None	P*pck*	5.4 g/L	(Lin et al. 2015)
B. subtillis	Glucose	*alsS*, *ilvC*, *ilvD*, *kivD*, and *adh2*	None	P43	2.62 g/L	(Li et al. 2011)
B. subtillis	Glucose	*alsS*, *ilvC*, *ilvD*, *kivD*, and *adh2*	Δ*ldh* and Δ*pdhC*	P43	5.5 g/L	(Li et al. 2011)
B. subtillis	Glucose	*alsS*, *ilvC*, *ilvD*, *kivD*, *adh2, zwf, pntBA*, and *udhA*	Δ*ldh*, Δ*pdhC* and Δ*pgi*	P43	6.12 g/L	(Qi et al. 2014)
B. subtillis	Glucose	*alsS*, *ilvC*, *ilvD*, *kivD* and *adhA*	143 kb DNA non-essential region deletion	P*grac* and PmanP	201.7 mg/L	(Tian et al. 2022)
P. putida	Glucose	*alsS, ilvC, ilvD,* and *kivD*	Δ*bkd,* Δ*ilvE,* Δ*leuA,* Δ*panB,* and Δ*pycAB*	P_{T7}	43 μM	(Lang et al. 2014)
P. putida	Glucose	*alsS, ilvCD, Kivd, kdcA, adhA,* and *yqhD*	Δ*sthA* and Δ*gcd*	P*bad*	22 mg/g_{glc}	(Nitschel et al. 2020)
C. glutamicum	Glucose	*alsS, ilvC, ilvD, kivd,* and *adhA*	Δ*pyc* and Δ*ldh*	P*eftu*	4.9 g/L	(Smith et al. 2010)
C. glutamicum	Glucose	*ilvBNCD, pntAB, kivd,* and *adhA*	Δ*aceE,* Δ*pqo*, Δ*ilvE,* Δ*ldhA,* and Δ*mdh*	P*tac*	12.97 g/L	(Blombach et al. 2011)
C. glutamicum	Glucose	*ilvBNCD,, kivd,* and *adhP*	Δ*ldhA*	P*tac,* P*ldhA*, and P*gapA*	25.3 g/L	(Yamamoto et al. 2013)

Table 1 (continued)

Host	Substrate	Genes overexpressed	knockout genes	Promoter	Isobutanol titer	References
C. glutamicum	Glucose	*ilvBN, ilvC*™, *ilvD, kivd, adhA, gapA*, *pgk*, *tpi*, *pfkA*, *pgi*, *zwf*, *edd*, and *eda*	Δ*pckA*, Δ*ppc,* Δ*ldhA,* and Δ*ilvE*	P*lac*(AA), P*lac*(GA)	20.8 g/L	(Hasegawa et al. 2020)
Z. mobilis	Glucose	*alsS*, *ilvC*, *ilvD*, *kdcA* and *adhA*	None	P*tet* and P*gap*	4.01 g/L	(Qiu et al. 2020)
E. aerogenes	Glucose	*budB, ilvC, ilvD, kivd* and *adhA*	Δ*ldhA*, Δ*budA,* and Δ*pflB*	PLtetO and P_{BAD}	4.3 g/L	(Jung et al. 2017)
E. aerogenes	Glucose and xylose	*budB, ilvC, ilvD, kivd* and *adhA*	Δ*ldhA*, Δ*budA,* Δ*pflB* and Δ*ptsG*	PLtetO and P_{BAD}	5.6 g/L	(Jung et al. 2018)
E. coli	Glucose	*alsS*, *ilvC*, *ilvD*, *kivd* and *adh2*	None	$P_L lacO_1$	22 g/L	(Atsumi et al. 2008)
E. coli	Glucose	*alsS*, *ilvC*, *ilvD*, *kivd*, *adhA*, *zwf*, *pgl*, *edd,* and *eda*	Δ*pgi,* Δ*gntR,* Δ*gnd*, Δ*pflB* and Δ*ldhA*	P*trc*, $P_L lacO_1$ and $P_A lacO_1$	15 g/L	(Noda et al. 2019)
E. coli	Cellobiose	*alsS*, *ilvC*, *ilvD*, *kivd, adhA* and *bglC*	None	$P_L lacO_1$ and $P_L tetO_1$	7.64 g/L	(Desai et al. 2014)
E. coli	Cellobionic acid	*alsS*, *ilvC*, *ilvD*, *kivd, adhA* and *ascB*	None	$P_L lacO_1$ and $P_L tetO_1$	2.7 g/L	(Desai et al. 2015)
E. coli	Glucose	*alsS*, *ilvC*, *ilvD*, *kivd* and *adhA*	Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pta*, and Δ*pflB*	P*fumA*	5.9 g/L	(Jin et al. 2019)
E. coli	Glucose	*alsS*, *ilvC*, *ilvD*, *kivd, LeuDH* and *yqhD*	Δ*araBAD*, Δ*mcrA*, Δ*endA*, Δ*recA* and Δ*mcrBC-hsdSMR-mrr*	$P_L lacO_1$	5.76 g/L	(Wang et al. 2020a)

Different exogeneous and homogeneous genes expressed/overexpressed, knockout genes in the microbial production of isobutanol and their corresponding enzymes are as follows: *kivd* and *kdcA*, 2-ketoisovalerate decarboxylase; *adh*, *adhA*, *adh1*, *adh2*, *adh6*, *adh7* and *yqhD*, alcohol dehydrogenases; *glgC,* glucose-1-phosphate adenylyltransferase; *budB*, *alsS* and *ilv2*, acetolactate synthase; *ilvBH* and *ilvBN*, acetohydroxyacid synthase; *ilv5* and *ilvC,* acetohydroxy acid isomeroreductase; *ilv3* and *ilvD,* dihydroxy-acid dehydratase; *phaA*, β-ketothiolase; *phaB*, acetoacetyl-CoA reductase; *phaC*, PHB synthase; *ilvE*, branch-chain-amino-acid transaminase; *bkdAB*, branched-chain keto acid dehydrogenase complex; *aceE*, pyruvate dehydrogenase complex; *acrA* and *acrA6*, components of a multidrug efflux pump; *mtrA* and *mtrB*, genes responsible for membrane transport systems; *ter*, trans-enoyl-CoA reductase; *pdc1,* pyruvate decarboxylase; *lpd1*, pyruvate dehydrogenase complex; *ARO10*, phenylpyruvate decarboxylase; *PYC2,* pyruvate carboxylase; *mdh* and *MDH2*, malate dehydrogenase; *MAE1*, malic enzyme; *BDH1* and *BDH2*, butanediol dehydrogenases; *LEU4* and *LEU9*, 2-isopropylmalate synthases; *GPD1* and *GPD2*, glycerol-3-phosphate dehydrogenase; *ALD6,* aldehyde dehydrogenases; *BAT1*, branched-chain amino acid aminotransferase; *ILV6*, small unit of acetolactate synthase; *ATF1*, alcohol acetyltransferase; *ipdC,* indole-3-pyruvate decarboxylase; *budA*, α-acetolactate decarboxylase; *ldhA*, lactate dehydrogenase; *pdhc*, pyruvate dehydrogenase complex; *zwf*, glucose-6-phosphate dehydrogenase; *pgi*, glucose-6-phosphate isomerase; *udhA* and *pntAB*, transhydrogenases; *bkd*, 2-keto acid dehydrogenase; *leuA*, 2-isopropylmalate synthase; *panB*, ketopantoate hydroxymethyl transferase; *pycAB*, pyruvate carboxylase; *sthA*, transhydrogenase; *gcd*, glucose dehydrogenase; *aceE,* E1p subunit of the pyruvate dehydrogenase complex; *pqo,* pyruvate:quinone oxidoreductase; *gapA*, glyceraldehyde- 3-phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *tpi*, triosephosphate isomerase; *pfkA,* fructose-6-phosphate kinase; *edd*, 6-phosphogluconate dehydratase; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase; *pckA*, phosphoenolpyruvate carboxykinase; *ppc*, phosphoenolpyruvate carboxylase; *gntR*, DNA-binding transcriptional repressor; *gnd*, 6-phosphogluconate dehydrogenase; *pflB*, pyruvate formate lyase; *bglC*, beta-glucosidase; *ascB*, 6-phospho-beta-glucosidase; *adhE*, aldehyde-alcohol dehydrogenase; *frdBC*, fumarate reductase; *fnr*, fumarate and nitrate reductase; *pta*, phosphate acetyltransferase; *leuDH*, leucine dehydrogenase; *mcrA*, 5-methylcytosine-specific restriction enzyme A; *endA*, DNA-specific endonuclease; *ptsG*, glucose-specific PTS enzyme IIBC component; *araA*, L-arabinose isomerase, *araB*, ribulokinase; *araD*, L-ribulose-5-phosphate 4-epimerase

was achieved by employing multiple strategies, such as the deletion of the *lpd1* gene, overexpression of *ILV2*, *ILV5, ILV3*, *ADH6*, *kivd*, and overexpression of some genes encoding transhydrogenase-like shunts such as *PYC2*, *MDH2*, and *MAE1* to solve the cofactor deficiency. After several engineering rounds, the recombinant strain accumulated 1.6 g/L of isobutanol in a batch fermentation from 100 g/L of glucose after 24 h (Matsuda et al. 2013).

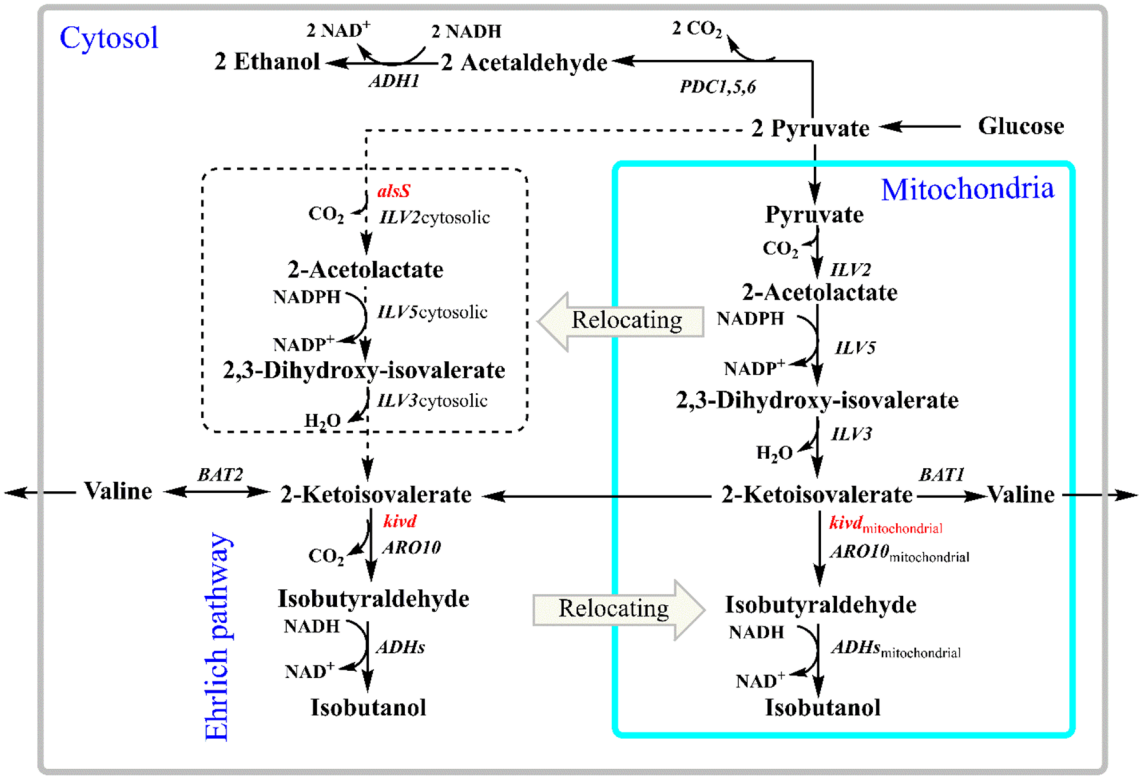

Fig. 3 Engineered pathways used for production of isobutanol in yeast cells. Figure modified from Lee et al. (2012) and Wess et al. (2019). Native genes are shown in black, and heterologous genes in red

Moreover, in *S. cerevisiae*, the enzyme for the isobutanol pathway is localized into two cellular components: mitochondria and cytosol. Thus, shifting the entire Ehrlich pathway to one component could improve the titer of isobutanol. For example, Lee et al. (2012) overexpressed three mitochondrial enzymes (*ILV2*, *ILV3*, and *ILV5*) in the cytosol by eliminating mitochondrial targeting sequences and introducing the *kivd* gene from *L. lactis*. The engineered strain accumulated almost sevenfold (151 mg/L) more isobutanol than its parent strain without deleting three native mitochondrial genes (Lee et al. 2012). Brat et al. (2012) employed a similar strategy by relocating the three mitochondrial enzymes (*ILV2*, *ILV3*, and *ILV5*) into the cytosol by eliminating mitochondrial targeting sequences and expressing *ARO10* and *ADH2* genes. The engineered strain with a combination of codon-optimized truncated *ILV* gene expression and *ILV2* gene deletion in mitochondria increased isobutanol titers to 630 mg/L. The titer of isobutanol was approximately 32-fold higher than the original strain (Brat et al. 2012).

Recently, in another approach to increasing the metabolic flux towards isobutanol production in engineered *S. cerevisiae*, non-essential competing metabolic pathways were deleted by the elimination of multiple (*bdh1*, *bdh2*, *leu4*, *leu9*, *ecm31*, *ilv1*, *adh1*, *gpd1*, *gpd2,* and *ald6*) genes (Wess et al. 2019). The engineered *S. cerevisiae* produced 200-fold more isobutanol (2.09 g/L) than its parent strain after the expression of codon-optimized three endogenous enzymes (*IlV2*, *ILV3*, and *ILV5*) in the cytosol and deletion of the *ilv2* gene to disrupt mitochondrial valine biosynthesis. This is the highest reported titer of isobutanol in *S. cerevisiae* (Wess et al. 2019). In another report, Park and Hahn (2019) engineered the strain of *S. cerevisiae* by expressing *alsS* from *B. subtilis* utilizing a copper-inducible *CUP1* promoter and overexpressing two endogenous mitochondrial genes, *ILV5* and *ILV3*, in the cytosol. The recombinant strain of *S. cerevisiae* accumulated 263.2 mg/L of isobutanol, which was almost 3.3 times higher than the control strain expressing the three native genes *ILV2*, *ILV5*, and *ILV3* on a plasmid utilizing a strong constitutive promoter (Park and Hahn 2019). These results demonstrated that blocking the competitive pathways, overexpressing the endogenous genes, and increasing cofactor availability effectively enhanced isobutanol production. However, the production of isobutanol by *S. cerevisiae* is still very low. Therefore, further engineering efforts are needed to identify novel enzymes for the isobutanol pathway with higher catalytic activities.

Engineering *Pichia pastoris* for isobutanol production

P. pastoris, a methylotrophic yeast, has been used as a production host for various high-value chemicals. The wild strain of *P. pastoris* accumulates a very low amount of isobutanol (Kharkwal et al. 2009; Nor and Roshanida 2015). Therefore, to improve isobutanol production in *P. pastoris* KM71, Siripong et al. (2018) overexpressed *KDC* and *ADH* genes from different candidates. Three candidates for *KDC* genes have been tested: *ARO10* and *THI3* from *S. cerevisiae*, *kivd* from *L. lactis*, and *ADH*, ADH6, and ADH7 from *S. cerevisiae*. Different combinations of *KDC-ADH* genes were employed to analyze isobutanol levels under the use of the additive 2KIV (4 g/L), the isobutanol precursor. The highest titer of 48 mg/L of isobutanol was accumulated with the expression of *kivd* (*L. lactis*) and *ADH7* (*S. cerevisiae*) (Siripong et al. 2018). To improve the titer, endogenous L-valine biosynthetic pathway genes (*Ilv6*, *Ilv2*, *Ilv5*, and *Ilv3*) were overexpressed. As a result, the engineered strain (PP300) produced nearly 0.90 g/L of isobutanol from glucose directly. Additional improvement was achieved by the introduction of an additional copy of the *ILV6* and *ILV2* genes by placing them on the episomal plasmid; the engineered strain (PP403) accumulated a titer of 2.22 g/L of isobutanol, which was almost a 43-fold improvement over the original strain (Siripong et al. 2018).

In another study, Kurylenko et al. (2020) reported that the wild-type strain of multinuclear yeast *M. magnusii* produces 0.44 g/L of isobutanol (10 to 20-fold higher than *S. cerevisiae*). To further enhance the production of isobutanol in *M. magnusii*, the exogenous *ILV2* gene of *S. cerevisiae* was overexpressed in *M. magnusii* under the control of the constitutive P_{TEF1} promoter. The recombinant strain accumulates 0.62 g/L of isobutanol, a 1.4-fold increase over the wild-type strain (Kurylenko et al. 2020). This result showed that the wild strain of *M. magnusii* performs better than the wild strains of *S. cerevisiae* and *P. pastoris* regarding isobutanol production. However, the genetic toolbox available for this species is not as sophisticated as that for *S. cerevisiae*. Therefore, implementing a better genetic toolbox would be a promising strategy to accelerate the process of pathway design in *M. magnusii* for isobutanol production.

Engineering *Klebsiella pneumoniae* for isobutanol production

K. pneumoniae is an indispensable industrial bacterium that can accumulate 2,3-butanediol naturally by utilizing glucose or other sugars as a substrate (Chen et al. 2015). Gu et al. (2017) demonstrated the presence of an isobutanol production pathway in wild *K. pneumonia* (Gu et al. 2017). However, this pathway did not lead to isobutanol production in the wild-type strain, while a very high amount of 2,3-butanediol (17.3 g/L) was detected in the wild-type strain (Gu et al. 2017). Thus, deletions of *budA* (encoded by α-acetolactate decarboxylase) and *ldhA* genes diverted the carbon flux from 2,3-butanediol and lactic acid to the valine pathway, respectively. As a result, the engineered strain accumulated 157 mg/L of isobutanol and a high amount of 2-ketoisovalerate (12.7 g/L), a precursor for isobutanol. Titers were further increased to 2.45 g/L in *ipdC* (encoding an indole-3-pyruvate decarboxylase) complementation strain after 24 h of fermentation, which is an almost 15.5-fold increase compared to *K. pneumoniae* Δ*budA*-Δ*ldhA* strain (Gu et al. 2017). It was found that the *ipdC* gene is responsible for making isobutyraldehyde from 2-ketoisovalerate in *K. pneumoniae*. However, a high amount of 2-ketoisovalerate was detected in the fermentation broth of *K. pneumoniae*, which showed that the main bottleneck in the isobutanol pathway is the decarboxylation of 2-ketoisovalerate. Another bottleneck for isobutanol production in *K. pneumoniae* is the catalytic activity of the *ipdC* gene towards pyruvate, which reduces the available precursor pyruvate for isobutanol production. Therefore, to further improve isobutanol production, the *ipdC* gene of *K. pneumoniae* was engineered through site-specific mutations to generate different variants of Kp-IpdC. Among the different variants of Kp-IpdC, one of the variants T290L accumulated 5.5 g/L of isobutanol, which was a 1.2-fold improvement over the control strain *K. pneumoniae* Δ*budA*–Δ*ldhA*–Δ*ipdC*–*ipdC* (IpdC) (Shu et al. 2022). These results showed that the decarboxylation of 2-ketoisovalerate is the main bottleneck in the isobutanol pathway in *K. pneumoniae*. Future studies should focus on modifying the existing enzymes through protein engineering or identifying alternative enzymes to enhance isobutanol production.

Engineering *Lactococcus lactis* for isobutanol production

L. lactis is a lactic acid-producing bacterium found in dairy products, vegetables, leaves, and animal skin (Li et al. 2020; Maślak et al. 2022; Perveen et al. 2023; Suzuki and Suzuki 2021). *KivD* and *kdc* are the native genes in *L. lactis* that convert 2-ketoisovalrate into isobutyraldehyde, and *kivd* is the most widely employed gene in the isobutanol synthetic pathway in microbial hosts. Additionally, *L. lactis* contains all the essential genes for the synthesis of isobutanol, including aminotransferase, decarboxylase, and alcohol

dehydrogenase. Priyadharshini et al. (2015) examined the viability of isobutanol production by *L. lactis* for the first time (Priyadharshini et al. 2015). After culturing the wild-type strain of *L. lactis* using an M17 medium, isobutanol was detected in the supernatant after 5 days of fermentation (0.01 g/L). However, the titer of isobutanol was lower than that of *E. coli* and the co-culture system of *L. lactis* and *E. coli*. This result showed *L. lactis* produces a very low amount of isobutanol. One reason for the low yield is that there are multiple competing pathways that utilize the same substrate. Therefore, blocking competing pathways and over-expressing the isobutanol synthesis genes could be a promising strategy for improving isobutanol production in *L. lactis*.

Engineering non-native microbial hosts for isobutanol production and its advantages

Engineering of lignocellulolytic and thermophile microorganisms

Heterologous expression of cellulolytic enzymes has been found challenging; therefore, engineering native lignocellulolytic organisms is of great interest to researchers for the production of biofuels. Higashide et al. (2011) constructed a hybrid valine biosynthesis pathway in *C. cellulolyticum* by expressing the *kivd*, *alsS*, *ilvCD*, and *yqhD* genes from *L. lactis*, *B. subtilis*, and *E. coli*, respectively. The engineered strain produced 0.66 g/L isobutanol growing on cellulose after 192 h and 0.346 g/L isobutanol from cellobiose in 90 h of fermentation (Higashide et al. 2011).

In industrial biotechnology applications, thermophilic organisms offer various benefits over mesophilic species. High temperature facilitates the removal of volatile products and reduces the risk of contamination (Cann et al. 2020; DiGiacomo et al. 2022; Irdawati et al. 2023; Vavitsas et al. 2022). *G. thermoglucosidasius* is an important facultative anaerobic, rod-shaped, endospore-producing thermophile bacteria; its metabolic products are lactate, formate, acetate, and ethanol (Cripps et al. 2009), and it was genetically engineered for the production of non-native products (i.e., isobutanol) at elevated temperatures. Genes were selected from two microorganisms to engineer the thermophile *G. thermoglucosidasius* toward isobutanol production. The *kivd* gene was selected from the *L. lactis*, which converts 2-ketoisovalerate into iso-butyraldehyde. The *alsS* gene was introduced from *B. subtilis* along with the expression of its native *ilvC* gene. The engineered strain produced 3.3 g/L of isobutanol after 2 days of fermentation at 50 °C using glucose as a substrate (Lin et al. 2014). Another cellulolytic thermophilic bacteria (*C. thermocellum*) has been engineered to produce isobutanol. The engineered thermophile accumulated 5.4 g/L isobutanol using cellulose at 50 °C after 75 h of fermentation (Lin et al. 2015).

Engineering autotrophic microorganisms for isobutanol production

One of the advantages of autotrophic organisms is that they can utilize CO_2 directly for biofuel production, eliminating the requirement for solid feedstock (i.e., glucose, glycerol, cellulose) (Purdy et al. 2022). To fix CO_2 into biofuels, the energy is provided by renewable resources, either sunlight or electricity (Wang et al. 2020b). In autotrophs, cyanobacteria is an excellent microbial cell factory that converts atmospheric CO_2 into useful products utilizing sunlight as an energy source (Fig. 2). Moreover, cyanobacteria can grow quickly, and its genetic modification is simple (Fathima et al. 2018; Gao et al. 2021; Lehmann et al. 2021). Owing to their advantages over heterotrophs, cyanobacteria are endowed with an isobutanol pathway to accumulate sustainable and environmentally friendly biofuels.

Atsumi et al. (2009) engineered *S. elongatus* for the first time to produce autotrophic isobutanol. The engineered strain was developed by expressing *B. subtilis alsS*, *E. coli ilvCD*, *L. lactis kivd*, and three different *S. cerevisiae ADHs*. Six days of fermentation resulted in 450 mg/L of isobutanol from the engineered strain (Atsumi et al. 2009). Further improvement in isobutanol production was achieved by deleting the *glgC* gene (encoding glucose-1-phosphate adenylyltransferase); the resulting Δ*glgC* strain accumulated 550 mg/L of isobutanol during 8 days of fermentation (Li et al. 2014). Another study reported the accumulation of 90 mg/L isobutanol in *Synechocystis* sp. strain PCC 6803 by overexpressing the two genes *kivd* (*L. lactis*) and *adhA* (*L. lactis*) without the addition of any inducers or antibiotics (Varman et al. 2013); the titers were fivefold higher compared to the previously engineered strain *Synechococcus* 7942 expressing the same enzymes (Atsumi et al. 2009).

In recent years, another facultative autotrophic bacterium *R. eutropha* has drawn the attention of researchers for its ability to fix CO_2 into value-added products utilizing non-photosynthetic energy sources such as H_2 or formate (Fig. 2) (Kim et al. 2022; Nangle et al. 2020; Wu et al. 2022). It can also use a wide range of feedstocks, such as glycerol, fructose, organic acids, and plant oil (Ingram and Winterburn 2022; Kajla et al. 2022; Strittmatter et al. 2022). Therefore, *R. eutropha* H16 was engineered to produce 270 mg/L of isobutanol and 40 mg/L of 3-methyl-1-butanol (at 48 h fermentation) from fructose. This was achieved after the deletion of carbon-consuming pathways (*phaCAB*, *ilvE*, *bkdAB*, and *aceE*) and by overexpression of the native valine biosynthetic pathway genes and alcohol dehydrogenase (*adh*) in combination with keto-isovalerate decarboxylase (*kivd*) from

L. lactis (Lu et al. 2012). In another study, the recombinant *R. eutropha* H16 strain accumulated 536 mg/L of isobutanol from CO_2 using electrolysis-generated H_2 as the sole energy source; the recombinant strain was established by deleting polyhydroxybutyrate synthesis (PHB) genes and expressing isobutanol biosynthesis genes (Li et al. 2012a). Moreover, to improve isobutanol production in *R. eutropha*, an isobutanol tolerant strain (Re2445) was developed by deleting the a*crA* gene. The recombinant strain (Re2445) expressing isobutanol biosynthesis genes produced 260 mg/L isobutanol within 72 h of fermentation. The titers were almost 1.3 times higher than their parental strain (Bernardi et al. 2016). Another recent study described the production of 4 mg/L isobutanol utilizing fructose as the sole carbon source in a minimal media, using the CoA-dependent pathway along with the overexpression of native isobutyryl-CoA mutase (*sbm1*) in a recombinant strain of *R. eutropha* H16. Isobutanol production was further enhanced to 32 mg/L after supplementation with vitamin B12, an almost eightfold increase compared to no vitamin B12 supplementation (Black et al. 2018). These results demonstrated that deleting carbon-consuming pathways and overexpressing native genes significantly enhanced the productivity of isobutanol. However, the titers accumulated by the autotrophic microorganisms are still very low, so additional strain engineering would be necessary to improve the isobutanol productivity.

Engineering solvent-tolerant microorganisms for isobutanol production

The researchers have been interested in engineering those organisms to produce isobutanol, which has excellent potential to tolerate higher isobutanol concentrations (Goyal et al. 2019; Tian et al. 2022; Ullah et al. 2023). One major limiting variable that prevents microbial biofuel production is solvent toxicity (Liu et al. 2021a). Therefore, considering the solvent tolerance factor of *B. subtilis*, it was engineered to produce isobutanol. In unbaffled shake-flask fed-batch fermentation, the engineered strain BSUL03 accumulated 2.62 g/L of isobutanol by expressing its native *alsS* and *ilvCD* genes of *C. glutamicum* in combination with the *kivD* gene of *L. lactics* and the *adh2* gene of *S. cerevisiae* (Li et al. 2011). Further, improvements were achieved by the inactivation of *ldh* and *pdhC* genes. The *ldh*- and *pdhC*-deficient strain BSUL05 resulted in 2.3-fold more isobutanol (5.5 g/L) than its parental strain BSUL03 in fed-batch fermentations (Li et al. 2012b). Further optimization of the *B. subtilis* strain was done via deactivating glucose-6-phosphate isomerase (*pgi*), overexpressing glucose-6-phosphate dehydrogenase (encoding *zwf*), and transhydrogenase (*udhA*) in the *ldh*- and *pdhC*-deficient strain BSUL05 to adjust the intracellular redox state; the resulting strain BSULO8 accumulated 11% more isobutanol (6.12 g/L) than its parental strain BSULO5 (Qi et al. 2014). Recently, Tian et al. (2022) constructed a mutant strain of *B. subtilis* with deletion of 134.4 kb non-essential region from the genome. The deletion mutant (GI12) accumulated 201.7 mg/L isobutanol in a shaking flask, 2.4-fold higher than the wild-type strain (Tian et al. 2022).

Similarly, another solvent-tolerant strain, *P. putida*, was engineered for isobutanol production. The overexpression of a single gene (*kivd*) of *L. lactis* in *P. putida* enabled the engineered strain to produce isobutanol (43.1 µM) directly from glucose via the valine synthesis route (Lang et al. 2014). In another study, additional improvement in isobutanol from *P. putida* was achieved by applying multiple strategies such as avoiding product and precursor degradation, deactivation of the *sthA* gene, overexpressing the native *ilvC* and *ilvD* genes, and the introduction of *alsS*, *kivd*, and *yqhD* from *B. subtilis*, *L. lactis*, and *E. coli*, respectively. *P. putida*-engineered strain Iso2 produced 22 mg/g_{glc} isobutanol under aerobic conditions (Nitschel et al. 2020).

Engineering *Escherichia coli* for isobutanol production

E. coli is considered a suitable host for producing various high-value chemicals due to its several advantages over other microbial hosts, such as fast growth, the availability of well-established genetic tools, and utilizing different carbon sources under both anaerobic and aerobic conditions (Olavarria et al. 2019; Pasini et al. 2022; Tong et al. 2021; Wang et al. 2011). Owing to these distinct advantages, *E. coli* has been engineered for various biofuels, including isobutanol (Abdelaal et al. 2019; Hosseini et al. 2020; Sathesh-Prabu et al. 2020). For example, to engineer *E. coli* for isobutanol production, the metabolic flux was directed to 2-ketoisovalerate by introducing the *alsS* gene from *B. subtilis* and overexpressing the endogenous *ilvC* and *ilvD* genes. The accumulated ketoacid was then converted into isobutanol with the expression of the *kivD* and *adh2* genes from *L. lactis* and *S. cerevisiae*, respectively. The engineered strain accumulated the highest titer of isobutanol at 22 g/L, increasing the theoretical yield to 86% (Atsumi et al. 2008). Additional optimization of isobutanol production in laboratory-scale fermenters with in situ product removal improved isobutanol titer to 50 g/L in *E. coli* (Baez et al. 2011).

It was found that high isobutanol concentration is toxic to microbial hosts and impairs microbial growth (Wilbanks and Trinh 2017). Chong et al. (2014) constructed an isobutanol-tolerant strain to alleviate this issue by modifying the global regulator cAMP receptor protein (CRP) via error-prone PCR. The recombinant strain produced 12 g/L of isobutanol without appreciable cell loss (Chong et al. 2014). Further improvements were achieved by engineering the ED pathway by deactivating the *pgi* and *gnd* genes, along with the deletions in the Embden-Meyerhof pathway (EMP), to divert

the metabolic flux towards isobutanol. The engineered strain accumulated 15 g/L of isobutanol as the final titer using glucose as a substrate (Noda et al. 2019).

E. coli expressing β-glucosidase could accumulate isobutanol from cellobiose at a titer of 7.64 g/L, representing 28% of the theoretical yield (Desai et al. 2014). Additionally, a strain of *E. coli* has been engineered to grow on cellobionic acid, which produced 2.7 g/L isobutanol after 24 h of fermentation, yielding productivity of 0.11 g/L/h and 65% of the maximum theoretical yield (Desai et al. 2015). In another study, a promoter engineering strategy was utilized to boost the production of isobutanol by changing the upstream sequence of the promoter. The recombinant strain accumulated 5.9 g/L of isobutanol, almost tenfold higher than its parental strain after 72 h of fermentation (Jin et al. 2019). In another approach, Wang et al. (2020a, b) developed a robustly engineered strain JW128, which outperforms the original *E. coli* MG1655 strain regarding transformation efficiency and plasmid stability. The engineered strain JW128 was generated by substituting *araBAD* with tet^r, eliminating *mcrBC-hsdSMR-mrr*, and deactivating the *mcrA*, *endA*, and *recA* genes. After introducing the isobutanol synthetic pathway into engineered strain JW128, the engineered strain JW128 accumulated 5.76 g/L isobutanol, which was 40-fold greater than the original strain of *E. coli* K12-MG1655 (Wang et al. 2020a).

Engineering *Corynebacterium glutamicum* for isobutanol production

The amino-acid-producing bacterium *C. glutamicum* was engineered to produce isobutanol owing to its potential for using amino-acid precursors for alcohol production and its greater tolerance against alcohol toxicity than *E. coli* (Smith et al. 2010). A titer up to 4 g/L of isobutanol was successfully obtained after the overexpression of native alcohol dehydrogenase (*adhA*), *ilvC*, and *ilvD* from *C. glutamicum*, *kivd* from *L. lactis*, and *alsS* from *B. subtilis*. Further optimization was carried out by knocking out the *pyc* and *ldh* genes to direct the metabolic flux toward the isobutanol. As a result, the recombinant strain produced 1.2-fold more isobutanol (4.9 g/L) than the strain without any gene deletion (Smith et al. 2010). In another study, to improve isobutanol production from *C. glutamicum*, the carbon flux was directed toward KIV by overexpression of *ilvBNCD* genes and elimination of *ilvE*, *aceE*, and *pqo* genes. Subsequently, under oxygen deprivation, efficient conversion of KIV to isobutanol was achieved by deleting the *ldh* and *mdh* genes, heterologous expression of the *kivd* gene from *L. lactis* and the *pntAB* genes from *E. coli*, and overexpression of the native *adhA* gene. In fed-batch fermentation, the engineered strain accumulated 12.97 g/L of isobutanol (Blombach et al. 2011). In another study, a titer up to 25 g/L of isobutanol was achieved under oxygen deprivation conditions in an IBU5 strain (*C. glutamicum* deficient in the *ldh* gene) expressing the *kivd* and *adhP* genes from *L. lactis* and *E. coli* under the control of the *ldhA* and *gapA* promoters, respectively, and overexpressing its native *ilvBNCD* genes (Yamamoto et al. 2013).

The expression levels of isobutanol-producing enzymes (encoded by *ilvBN*, *ilvC*™, *ilvD*, *kivd*, and *adhA*) have been recently adjusted by Hasegawa et al. (2020) by using several promoters in different combinations, inactivating alternative carbon-consuming pathways by the elimination of the *pckA*, *ppc*, *ldhA*, and *ilvE* genes and overexpressing glycolytic genes encoded by *gapA*, *pgk*, *tpi*, *pfkA*, and *pgi* to enhance glycolysis and glucose uptake. As a result, the final engineered strain accumulated a titer of 20.8 g/L of isobutanol during 24 h of fermentation under oxygen-limited conditions (Hasegawa et al. 2020).

Engineering *Zymomonas mobilis* for isobutanol production

Z. mobilis is a natural ethanologenic, facultative anaerobic α-proteobacterium bacterium. This bacterium has attracted the attention of researchers as a potential platform microorganism, well suited for producing chemicals and fuels from lignocellulosic sugars (Wang et al. 2018). Therefore, the *Z. mobilis* Z4 strain was engineered for isobutanol production by introducing the *kivd* and *adhA* genes from *L. lactis*. The engineered strain accumulated a very low titer of isobutanol (He et al. 2014). In another study, isobutanol production was improved by introducing a codon-optimized *kdcA* gene regulated by the tetracycline-inducible *Ptet* promoter. As a result, the engineered strain of *Z. mobilis* accumulated 150 mg/L isobutanol. The production of isobutanol was further improved by diverting the metabolic flux from ethanol to isobutanol synthesis through the expression of its native genes (*ilvC* and *ilvD*) and the exogenous *alsS* gene. This engineering led the recombinant strain to accumulate more than 1 g/L of isobutanol. Additional optimization was done by overexpressing the synthetic operon, *alsS-ilvC-ilvD*, and *kdcA* genes driven by *Ptet* and a strong constitutive *Pgap* promoter. The engineered strain accumulated the highest isobutanol titer of 4.01 g/L after 49 h of fermentation using glucose as a substrate (Qiu et al. 2020).

Engineering *Enterobacter aerogenes* for isobutanol production

E. aerogenes naturally synthesizes 1,3-propanediol and 2,3-butanediol (de Oliveira Paranhos and Silva 2020; Wu et al. 2021). It can consume numerous carbon sources, including glucose, xylose, sucrose, fructose, arabinose, and glycerol, and can survive at low pH (de Oliveira Paranhos and Silva 2020; Ewing et al. 2022; Sunarno et al. 2020).

Acetolactate, an intermediate of the valine and 2,3-butanediol pathways, can also serve as a precursor for isobutanol production. For this reason, this bacterium has attracted researcher's attention for isobutanol production through genetic engineering.

For example, Jung et al. (2017) developed an isobutanol-producing strain EMY-01DA by introducing *kivd* and *adhA* from *L. lactis* into EMY-01 (a *ldhA*-deficient strain of *E. aerogenes*). The recombinant strain EMY-01DA accumulated approximately 44 mg/L of isobutanol. Further, to enhance isobutanol production, the metabolic flux was directed to the valine synthesis pathway by knocking out the *budA* gene. As a result, the recombinant strain EMY-33DA produced 16-fold more isobutanol. Further optimization was carried out by knocking out the *pflB* gene and the expression of *ilvC*, *ilvD*, and *budB* genes from *K. pneumoniae* to direct the metabolic flux towards isobutanol. Finally, the recombinant strain EMY-333DCBDA accumulated almost 4.3 g/L isobutanol under semianaerobic conditions, including nitrate and formate in the medium (Jung et al. 2017). In addition, isobutanol production needs to be boosted in *E. aerogenes*; the *ptsG* gene was deleted in a parent strain to eliminate the preference for carbon utilization and to consume a cheaper carbon source like glucose and xylose derived from lignocellulosic biomass at the same time. After deleting the *ptsG* gene, strain EMY-340 was constructed, which simultaneously utilized glucose and xylose. Via the introduction of isobutanol pathway enzymes into strain EMY-340, strain EHM02 was constructed. The EHM02 strain could accumulate 5.6 g/L isobutanol after 24 h of fermentation, approximately 1.3-fold higher than its previously constructed strain EMY-333DCBDA (Jung et al. 2018). These accomplishments highlight the potential of *E. aerogenes* as a promising host for isobutanol production. Further improvements in isobutanol production via more novel and efficient synthetic techniques offer hope of achieving high isobutanol productivity in *E. aerogenes*.

Current problems and possible solutions for isobutanol production

Feedback inhibition of the enzymes

Isobutanol synthesis in microorganisms is limited by feedback inhibition of the enzymes by their products (Fig. 4a) (Zhang et al. 2022). A possible solution to eliminating the feedback inhibition created by substrates or products is using alternative enzymes or their engineering. Thus, alternative or engineered enzymes should be employed for high productivity to mitigate feedback inhibition problems. In the isobutanol synthetic pathway, an acetohydroxy acid synthase (AHAS) or an acetolactate synthase (ALS) enzyme used in branched-chain amino acid biosynthesis can catalyze acetolactate synthesis from pyruvate. AHAS enzyme consists of a large catalytic subunit and a small regulatory subunit and is normally regulated by the end products. However, the enzyme ALS is a single subunit and is highly specific for acetolactate synthase activity. Unlike most AHAS enzymes, ALS is not inhibited by the end products. For example, Atsumi et al. (2008) expressed the *alsS* gene of *B. subtilis* instead of its native biosynthetic enzymes (encoded by the *ilvIH* gene), which resulted in an increased isobutanol titer of 1.7-fold in *E. coli* (Atsumi et al. 2008).

Additionally, Smith et al. (2010) also confirmed that the expression of *B. subtilis alsS* is beneficial for the production of isobutanol in *C. glutamicum*, by confirming its activity in the crude extract with *alsS* (6,700 U/mg) and without *alsS* expression (100 U/mg) (Smith et al. 2010). Moreover, the *S. cerevisiae* AHAS enzyme is composed of two subunits, the catalytic subunit ILV2 and the regulatory subunit ILV6, thus deleting the ILV6 gene improved isobutanol titer up to 2.2-fold as compared to the wild strain (Hammer and Avalos 2017). These investigations demonstrated that removing feedback inhibition of AHAS enzymes could be a promising and viable technique for increasing isobutanol synthesis in microbial hosts.

Screening isobutanol overproducers

Various engineering strategies have been used to increase biofuel production in microbial hosts. Microbial mutation breeding was a basic method used for genome modification, including random mutagenesis by alkylating agents, ethyl methanesulfonate (EMS), ultraviolet (UV), atmospheric and room temperature plasma (ARTP), and nitrosoguanidine (NTG) (Elshobary et al. 2022; Roy and Dahman 2023; Su et al. 2021). These artificial mutagenesis were successfully employed to improve the mutation rate and screen the beneficial strains. For example, a high biobutanol-producing mutant, *Clostridial fusants*, was constructed using different mutagens, e.g., UV and EMS; the mutated strain accumulated 5.8% more biobutanol than the wild-type strain (Roy and Dahman 2023). The methods for screening the large strain libraries after mutagenesis for beneficial varieties are based on traditional screening methods, and traditional screening methods rely on high-performance liquid chromatography (HPLC) or gas chromatography (GC), which are expensive and laborious. Therefore, high throughput screening methods are required to screen large strain libraries, which can rapidly identify the strains that produce the target compounds in high amounts. Biosensor-based screening strategies have drawn much attention, as they are cost-efficient and quicker to run than traditional chemical analysis for biofuels based on HPLC and GC methods (Bahls et al. 2022). For example, Yu et al. (2019) developed a biosensor based on the transcription factor BmoR, which responded

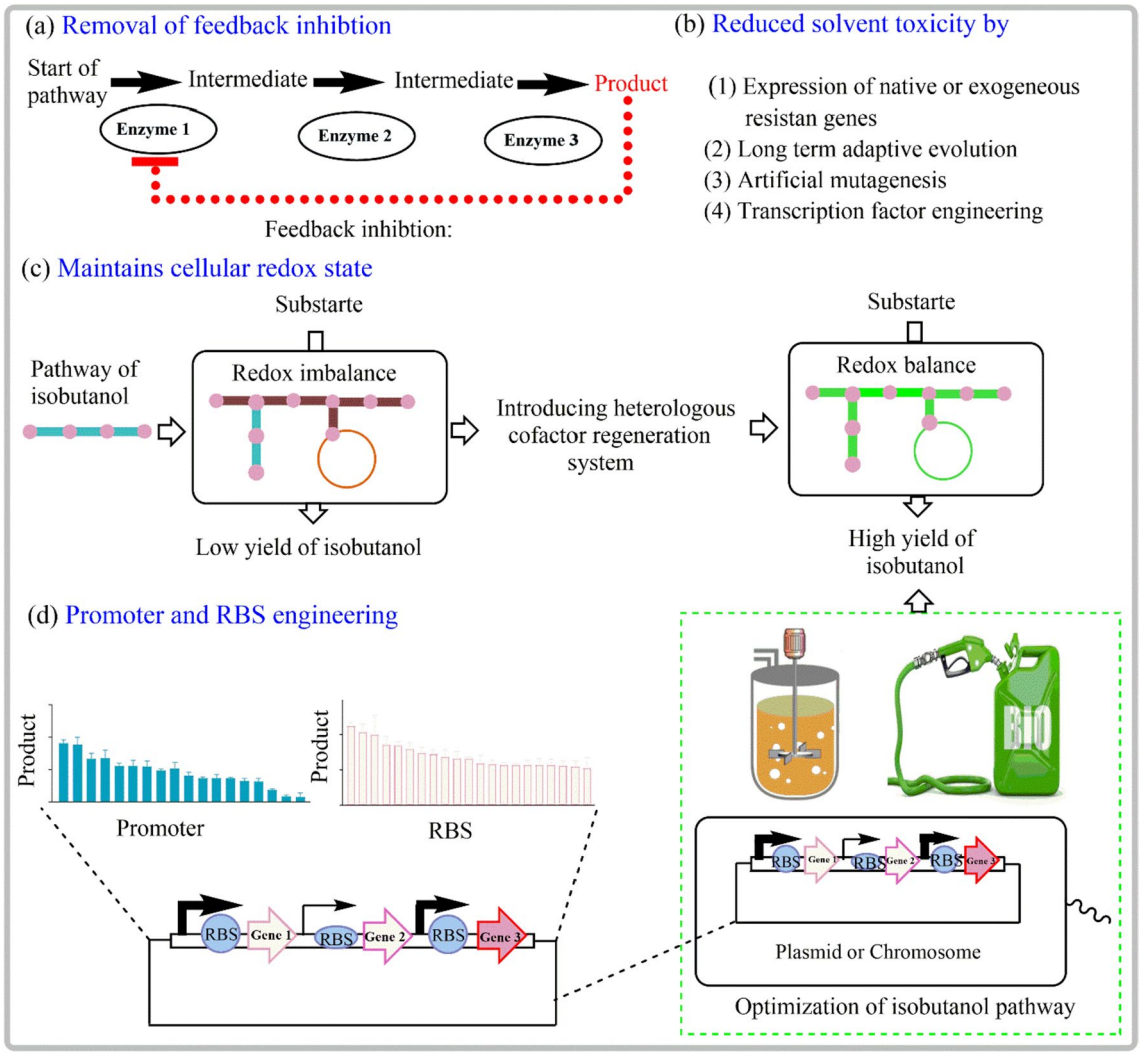

Fig. 4 Schematic illustration of challenges and possible solutions to improve isobutanol production in microbial hosts: **a** Feedback inhibition, **b** solvent toxicity, and **c**, **d** pathway imbalance

to intracellular isobutanol in *E. coli.* They used this biosensor to isolate an isobutanol-producing strain from an ARTP mutagenesis library. The isolated mutant accumulated two-fold more isobutanol (1597.6 mg/L) than the wild-type strain (Yu et al. 2019).

Solvent toxicity

High-value-added chemicals and biofuels (butanol and isobutanol) are harmful to microbial hosts; these chemicals disrupt the properties of the membrane, causing the leakage of cellular metabolites. As a result, cells cannot maintain cellular function (Kanno et al. 2013). Solvent toxicity is a significant limiting factor that impedes biofuel production during fermentation (Xu et al. 2021). Several engineering techniques have been employed to enhance the tolerance of microbial hosts to biofuels, such as the overexpression of well-characterized native or exogenous resistance genes, artificial mutagenesis, long-term adaptive laboratory evolution (ALE), and the introduction of engineered transcription factors (Fig. 4b). For example, Zhang et al. (2019a, b) successfully enhanced the tolerance of *S. cerevisiae* towards higher glucose and isobutanol concentrations by EMS mutagenesis followed by ALE. The evolved strain EMS39 performed better in higher concentrations of isobutanol (16 g/L) and glucose (100 g/L). After the expression of *ILV2*, *ILV3*, *ILV5*, and *ARO10* genes in the *S. cerevisiae* evolved strain EMS39, it produced 30% more isobutanol than the control strain (Zhang et al. 2019a).

In another approach, the tolerance of *E. coli* for isobutanol was enhanced by modifying its transcription factor cAMP protein (CRP). The modified strain exhibited a better growth rate (0.18 h^{-1}) compared to the control strain (0.05 h^{-1}) in the presence of 1.2% isobutanol (9.6 g/L) (Chong et al. 2014).

Recently, another study found that the overexpression of PHB synthesis genes improved the tolerance of *E. coli* towards isobutanol and efficiently enhanced the isobutanol titer. The titer of isobutanol was almost 1.6-fold higher than the control strain after 72 h of fermentation (Song et al. 2020). Thus, these overall results demonstrated that reducing the toxicity of the solvent should be a promising strategy to enhance the production capability of the host strain.

Pathway imbalance

The metabolic pathway for isobutanol production has been engineered for native and non-native microbial hosts. The non-natural metabolic pathway for isobutanol production has been constructed by combining multiple genes from various species. Nevertheless, imbalances in the metabolic pathways are usually observed due to cofactor imbalance and imbalanced gene expression, limiting the titer and productivity of the target compounds. Therefore, to maximize the production of the desired compound and maintain optimal cell growth, cofactor balancing and balanced expression of genes are indispensable strategies (Fig. 4c, d) (Park and Hahn 2019; Shi et al. 2013). For example, NADPH is an essential cofactor for the reaction catalyzed by *ILV5* and *ADH6* in the *S. cerevisiae* isobutanol pathway (Matsuda et al. 2013). In contrast, ethanol synthesis requires the utilization of NADH, which is generated during glycolysis. Therefore, an imbalance between NADH and NADPH occurs due to the stimulation of isobutanol production. The cofactor imbalance was adjusted by overexpressing some genes encoding transhydrogenase-like shunts, such as *MDH2*, *PYC2*, and *MAE1*. Therefore, the activation of transhydrogenase-like shunts improved NADPH supply and thus increased the titer of isobutanol up to 1.84-fold compared with its base strain BSW4 after 48 h of fermentation (Matsuda et al. 2013).

Another way to increase the titer of the target compounds is through balanced gene expression. Promoter engineering, RBS engineering, and gene copy number are engineering strategies that control balanced gene expression in endogenous and heterologous pathways (Fig. 4d) (Park and Hahn 2019). Promoters are the significant genetic elements that control gene expression. Thus, selecting the appropriate promoter is essential for achieving high productivity of the target compounds and ensuring smooth growth. For example, Lin et al. (2015) expressed the isobutanol synthetic pathway genes under different promoters to construct several recombinant strains. The amount of isobutanol among the different recombinant strains of *C. thermocellum* varied approximately from 0.05 to 0.6 g/L. The best recombinant strain accumulated 0.6 g/L of isobutanol after 24 h of fermentation, which was almost a 12-fold increase compared to the inferior strains (Lin et al. 2015). In another approach, isobutanol production was increased to 2.85-fold by balancing isobutanol pathway enzymes expressing the *B. subtilis alsS* gene under the control of a copper-inducible *CUP1* promoter and improving the translational efficiency of the *ILV5* and *ILV3* genes by the addition of Kozak sequence (Park and Hahn 2019). These studies demonstrated that cofactor balancing and balanced gene expression in the isobutanol pathway play an indispensable role in obtaining a high titer of biofuels.

Future perspectives

There are several promising strategies that can be used to enhance the production of isobutanol. First, addressing the challenges mentioned earlier, such as solvent toxicity, feedback inhibition of the enzymes, and pathway imbalance, can be potential approaches to improve the isobutanol titer. Solvent toxicity is a major issue hindering the productivity of isobutanol in engineered microbial hosts. This issue can be addressed by either using a solvent-tolerant strain or enhancing the tolerance of engineered microbial hosts through genetic engineering. Another approach is continuously removing the accumulated product. Redox imbalance is another factor leading to lower isobutanol production. Therefore, achieving a balanced redox state in engineered microbial hosts by expressing heterologous cofactor regeneration systems is a promising strategy for improving isobutanol production. Improving the catalytic activity of key metabolic enzymes through protein engineering and enhancing cellular robustness through transcription factor engineering are also promising approaches to increase the titer of isobutanol. Moreover, extensive research is required to identify novel strains that efficiently utilize cost-effective carbon sources and accumulate substantial quantities of isobutanol. Expanding the substrate spectrum for isobutanol production is another key challenge. Future research should focus on engineering microbial hosts that can utilize a diverse range of renewable feedstocks for isobutanol production.

Conclusions

Isobutanol, a valuable biofuel that has gained global interest in various industries, has been the focus of extensive research in recent years. The goal of these efforts has been to enhance the efficiency of microorganisms in producing isobutanol. In this review, we provided a comprehensive review of different engineered microbial hosts that can be used for isobutanol production. In the past decade, significant advancements in synthetic biology and metabolic engineering have led to a noticeable improvement in the biological production of isobutanol. However, the amount of isobutanol that can be accumulated by native microbial hosts is still insufficient to meet the demands of industrial applications. Another drawback is

that these native hosts rely on costly sugars like lactose and glucose as carbon sources. To overcome these limitations, researchers have turned to non-native microbial hosts such as *E. coli* and *C. glutamicum*, which have been genetically engineered to accumulate higher amounts of isobutanol compared to their native counterparts. However, these non-native hosts also require expensive sugars as carbon sources, and isobutanol itself poses safety hazards. In an effort to address these challenges, researchers have worked on engineering cellulolytic, photosynthetic, chemolithoautotrophic, and solvent-tolerant microorganisms for isobutanol production. Despite the potential of these non-native microbial hosts, their current production of isobutanol still falls short of that required for commercial purposes.

Author contribution SN and YCY conceived the topic; SN reviewed the literature and prepared the first draft manuscript; ZY, MWU, AFD, SBS, and MUR revised the manuscript; YCY and ZY reviewed and edited the content. All authors read and approved the final manuscript.

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Data availability Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

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

Conflict of interest The authors declare no competing interests.

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