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Research Article

Mitochondrial Compartmentalization Confers Specificity to the 2-Ketoacid Recursive Pathway: Increasing Isopentanol Production in *Saccharomyces cerevisiae*

Sarah K. Hammer, Yanfei Zhang, and José L. Avalos*

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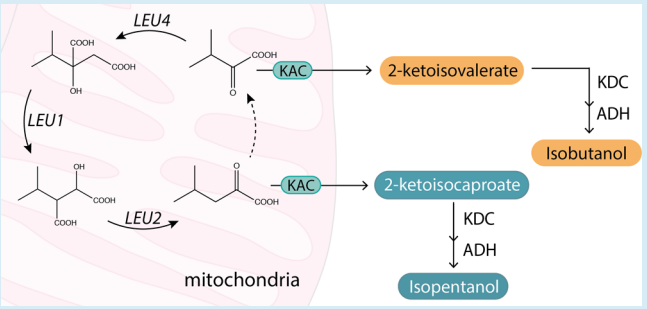
ABSTRACT: Recursive elongation pathways produce compounds of increasing carbon-chain length with each iterative cycle. Of particular interest are 2-ketoacids derived from recursive elongation, which serve as precursors to a valuable class of advanced biofuels known as branched-chain higher alcohols (BCHAs). Protein engineering has been used to increase the number of iterative elongation cycles completed, yet specific production of longer-chain 2-ketoacids remains difficult to achieve. Here, we show that mitochondrial compartmentalization is an effective strategy to increase specificity of recursive pathways to favor longer-chain products. Using 2-ketoacid elongation as a proof of concept, we show that overexpression of the three elongation enzymes—*LEU4*, *LEU1*, and *LEU2*—in mitochondria of an isobutanol production strain results in a 2.3-fold increase in the isopentanol to isobutanol product ratio relative to overexpressing the same elongation enzymes in the cytosol, and a 31-fold increase relative to wild-type enzyme expression. Reducing the loss of intermediates allows us to further boost isopentanol production to 1.24 ± 0.06 g/L of isopentanol. In this strain, isopentanol accounts for 86% of the total BCHAs produced, while achieving the highest isopentanol titer reported for *Saccharomyces cerevisiae*. Localizing the elongation enzymes in mitochondria enables the development of strains in which isopentanol constitutes as much as 93% of BCHA production. This work establishes mitochondrial compartmentalization as a new approach to favor high titers and product specificities of larger products from recursive pathways.



KEYWORDS: *recursive elongation, compartmentalization, mitochondria, isopentanol, branched-chain higher alcohols, Saccharomyces cerevisiae*

Cyclic pathways are a hallmark of natural metabolism. They include the tricarboxylic acid (TCA) cycle, the urea cycle, the pentose phosphate pathway, and β-oxidation. A subset of cyclic pathways involve recursive elongation, in which the functional group of the substrate that participates in new bond formation is reproduced with each cycle.[1] As a result, the product of one cycle can serve as the substrate for the next,[1] and with each cycle the chain length and molecular weight of the compound increases. There are four recursive carbon elongation pathways known in nature, which produce fatty acids, polyketides, isoprenoids, and 2-ketoacids.[1] Efforts to engineer these pathways for chemical production are often met with unique challenges specific to their recursive nature. One major challenge is overcoming substrate preferences of the termination enzymes that cause compounds to exit the recursive cycle, which may favor products of chain lengths different than desired.

Native 2-ketoacid biosynthetic pathways consist of either one or two elongation cycles, allowing for the production of alcohols with as many as five carbon atoms.[2,3] One elongation cycle is required for the production of 1-butanol or isopentanol, while a second elongation cycle enables 1-pentanol biosynthesis (Figure 1a). When these pathways are engineered to produce higher alcohols, a 2-isopropylmalate synthase (2-IPMS) is used as a generic 2-ketoacid C-acetyl transferase (2-KAT) to catalyze the first step in the conversion of a 2-ketoacid with n carbons to one with n + 1 carbons (Figure 1b). However, 2-KATs are often outcompeted by promiscuous 2-ketoacid decarboxylases (KDCs), which terminate the recursive pathway by converting the 2-ketoacid to an aldehyde. Ultimately, alcohol dehydrogenases (ADHs) convert the aldehydes to their corresponding alcohols (Figure 1b). For example, when the 2-ketoacid precursor to 1-propanol (2-ketobutyrate) is fed to *Escherichia coli*, over four times more 1-propanol is produced than 1-butanol.[3]

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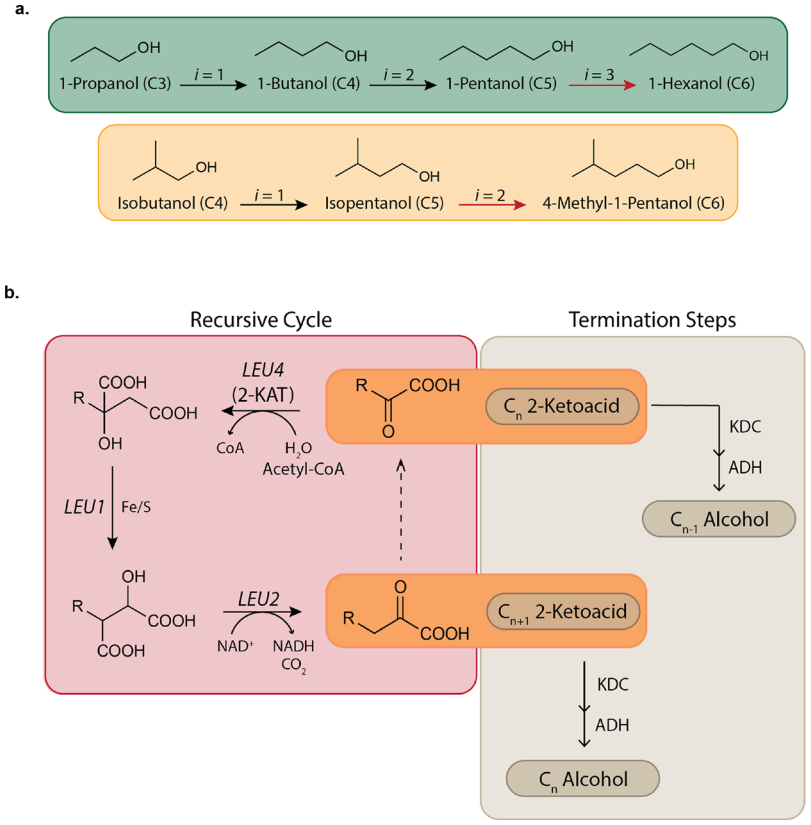

Figure 1. Schematics of 2-ketoacid elongation and the resulting alcohol products. (a) Trajectories to natural (black arrows) and non-natural (red arrows) four- to six-carbon (C4−C6) higher alcohols that could be achieved by means of 2-ketoacid elongation in *Saccharomyces cerevisiae*, starting with the 2-ketoacid derived from threonine (green) or valine (yellow). The *i* denotes the number of iterative elongation cycles needed to achieve a given alcohol product. (b) A general 2-ketoacid elongation scheme using acetyl-CoA to extend the carbon chain. The *S. cerevisiae* enzymes encoded by *LEU4*, *LEU1*, and *LEU2* catalyze the elongation of a 2-ketoacid by one carbon atom (shaded in red). The dashed line indicates how recursive behavior can be achieved through iterative action of a 2-ketoacid C-acetyl transferase (2-KAT)—here encoded by *LEU4*—on increasingly longer 2-ketoacids. The termination enzymes, ketoacid decarboxylases (KDCs) and alcohol dehydrogenases (ADHs), convert *n*-carbon ketoacids to their corresponding $(n - 1)$-carbon alcohols (shaded in beige). Orange boxes denote the 2-ketoacid substrates for which *LEU4* and KDCs compete.

The main strategy to manipulate product size specificity of recursive pathways has been to utilize enzymes that prefer specific substrates. One approach involves introducing heterologous termination enzymes with different natural substrate specificities. For example, introduction of heterologous plant thioesterases with preferences for specific fatty acyl-ACP (acyl carrier protein) substrates improves control of the chain-lengths of fatty acid ethyl esters (FAEEs) and fatty alcohols produced in *E. coli*.[4] Alternatively, site-directed or random mutagenesis have been utilized to engineer native enzymes to favor specific substrates. Various mutants of 2-IPMS and KDC have been obtained by these methods to expand their substrate binding pockets,[5−7] enabling more cycles of 2-ketoacid elongation in *E. coli* and production of alcohols with up to eight carbons. However, alcohol concentrations still tend to decrease with increasing chain-length due to premature decarboxylation of shorter chain-length 2-ketoacids.[6,7]

In *Saccharomyces cerevisiae*, isopentanol is naturally produced in small amounts as a degradation product of leucine.[8] However, isopentanol can also be synthesized from glucose by engineering the native 2-ketoacid elongation pathway. This pathway begins with 2-ketoisovalerate (KIV), which is produced from pyruvate by three mitochondrial enzymes, encoded by *ILV2*, *ILV3*, and *ILV5* (Supplementary Figure S1).[9−11] The 2-ketoacid carbon-chain elongation proceeds *via* 2-IPMS (encoded by *LEU4* or *LEU9*), which acetylates the 2-ketoacid. The addition of two carbon atoms to KIV results in 2-isopropylmalate (2-IPM), which is then isomerized by isopropylmalate isomerase (encoded by *LEU1*) to 3-isopropylmalate (3-IPM), and finally oxidized and decarboxylated by 3-IPM dehydrogenase (encoded by *LEU2*).[12] The end result of this cycle is a new 2-ketoacid (2-ketoisocaproate, KIC), with one more carbon than KIV, which could potentially accept another acetylation reaction from 2-IPMS (as a 2-KAT) to begin the next elongation cycle (Figure 1b). The 2-ketoacid elongation pathway can be terminated at each 2-ketoacid intermediate by KDCs and ADHs, resulting in an alcohol (Figure 1b, Supplementary Figure S1). If the pathway terminates at KIV ($n = 5$) the product is isobutanol; if it terminates at KIC ($n = 6$), the product is isopentanol (Figure 1). A similar series of linear alcohols can be produced if the 2-KAT acts on 2-ketobutyrate instead of pyruvate (Figure 1a).

While substantial efforts have been made to engineer microbes for the production of isobutanol,[13−16] isopentanol has received relatively little attention. Both are advanced biofuels and potential gasoline substitutes,[17,18] yet isopentanol has more favorable fuel properties than isobutanol, including lower solubility in water and higher energy density.[19] We previously showed that compartmentalizing the isobutanol biosynthetic pathway in yeast mitochondria alone is enough to increase isopentanol production.[20] Subsequent studies dem-

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onstrated that overexpressing the recursive enzymes of the 2-ketoacid elongation pathway in their natural compartments (Leu1p and Leu2p in cytosol, and Leu4p in both mitochondria and cytosol),[21,22] or exclusively in the cytosol,[23] can increase isopentanol production. However, in all these studies, isobutanol production is still a major byproduct (Supplementary Table S1) due to premature decarboxylation of KIV by KDCs before it can be elongated to KIC.

Compartmentalization of biosynthetic pathways within yeast subcellular organelles has received increasing attention as a metabolic engineering strategy in recent years.[24] We and others have found that compartmentalizing pathways in mitochondria can boost production of several chemicals, pharmaceuticals, and fuels including isobutanol and *n*-butanol.[20,24,25] In these studies, mitochondrial localization alleviates intracellular metabolite transport bottlenecks, increases concentrations of enzymes and intermediates, reduces byproduct formation, and improves enzymatic activity.[24] Here, we show that mitochondrial compartmentalization is also an effective way to prevent early termination in recursive elongation pathways and enhance product specificity. By localizing the three recursive enzymes responsible for 2-ketoacid elongation (Leu4p, Leu1p, and Leu2p) to mitochondria, separated from the cytosolic termination enzymes (KDC and ADH), a larger proportion of KIV can be converted to KIC. The result is a significant increase in the specificity of the pathway for isopentanol compared to colocalizing the recursive enzymes with the termination enzymes in their native compartment (the cytosol). With this approach alone, we can engineer strains where isopentanol accounts for as much as 93% of BCHA production. Further increasing flux through the pathway and minimizing loss of 2-IPM results in a strain that produces 1241 ± 55 mg/L isopentanol, while still corresponding to 86% of the total BCHAs produced by this strain. This work presents a novel application of mitochondrial compartmentalization as a strategy to improve product specificity in engineered recursive pathways.

RESULTS AND DISCUSSION

Experimental Strategy. In this work we employ mitochondrial compartmentalization to enhance specificity for isopentanol, the alcohol that results from the second 2-ketoacid (KIC) in the iso-branched-chain 2-ketoacid recursive pathway (Figure 1a, Supplementary Figure S1). We hypothesized that mitochondrial localization of the recursive pathway enzymes (Leu4p, Leu1p, and Leu2p) responsible for elongating KIV to KIC (Figure 1b, Supplementary Figure S1), while keeping the termination enzymes (KDC and ADH) in the cytosol, would prevent early decarboxylation of KIV, favoring isopentanol production (Figure 2). Thus, we altered the native enzyme localization in *S. cerevisiae* (Figure 2a) by targeting Leu4p, Leu1p, and Leu2p to mitochondria (Figure 2b).

Comparing Mitochondrial and Native Localization of Leucine Biosynthetic Genes. Leu4p, the main 2-IPMS in *S. cerevisiae*, is feedback-inhibited by leucine.[26] Accordingly, our first step toward expressing an active ketoacid elongation pathway was to incorporate a leucine-feedback-insensitive Leu4p mutant. When the regulatory domain of IPMS1 from *Leptospira biflexa* is truncated, the enzyme maintains high catalytic activity while eliminating sensitivity to leucine.[27] However, the truncated *LEU4* from *S. cerevisiae* at the equivalent location (amino acid 474) is inactive in the absence

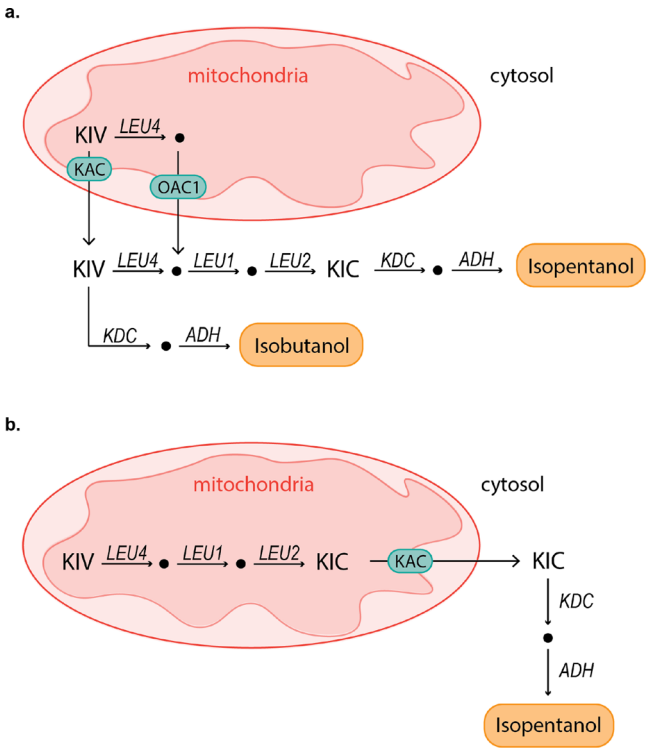

Figure 2. Mitochondrial context of the 2-ketoacid elongation pathway in *S. cerevisiae*. (a) Native localization of the isopentanol biosynthetic pathway in *S. cerevisiae*. (b) Mitochondrial compartmentalization strategy to increase product specificity toward isopentanol. Mitochondrial membrane transporters are teal; alcohols derived from 2-ketoacids are orange. KAC, unidentified mitochondrial 2-ketoacid carrier.

of the native *LEU4* or *LEU9* genes (Supplementary Figure S2). To avoid dependence on endogenous 2-IPMS enzymes, we chose to employ a leucine-insensitive mutant identified for its resistance to the leucine analogue 5,5,5-trifuoroleucine (TFL)[28] instead of a truncated Leu4p. Deleting a single amino acid (S547) from *LEU4* results in a mutant that retains 84% of its activity in the presence of 10 mM leucine,[28] and remains active even in the absence of endogenous *LEU4* and *LEU9* (Supplementary Figure S2b). In addition, the $LEU4^{S547\Delta}$ mutant performs better than the wild-type *LEU4* when both are targeted to mitochondria (C1 and C4, Supplementary Figure S2b). Accordingly, all engineered strains in this study constitutively express the $LEU4^{S547\Delta}$ mutant.

The next two enzymes required for 2-ketoacid elongation—Leu1p and Leu2p—are naturally located in the cytosol. However, previous work engineering *S. cerevisiae* for *n*-butanol production has shown that Leu1p and Leu2p are also active when localized to mitochondria.[25] We conducted initial experiments in the wild-type background to determine whether overexpression of $LEU4^{S547\Delta}$, *LEU1*, and *LEU2* (collectively referred to as the *LEU* genes) with their products targeted to mitochondria can increase isopentanol titers and specificity as compared to their products targeted to their native subcellular locations. Overexpressing the *LEU* gene products in their native subcellular locations (SHy128) increases isopentanol titers to 175 ± 33 mg/L (Figure 3a), a 2.6-fold increase over the wild-type control (SHy23). Yet, mitochondrial compartmentalization (see Methods) of the same enzymes (SHy74) has a much larger impact, boosting titers 7.3-fold relative to the

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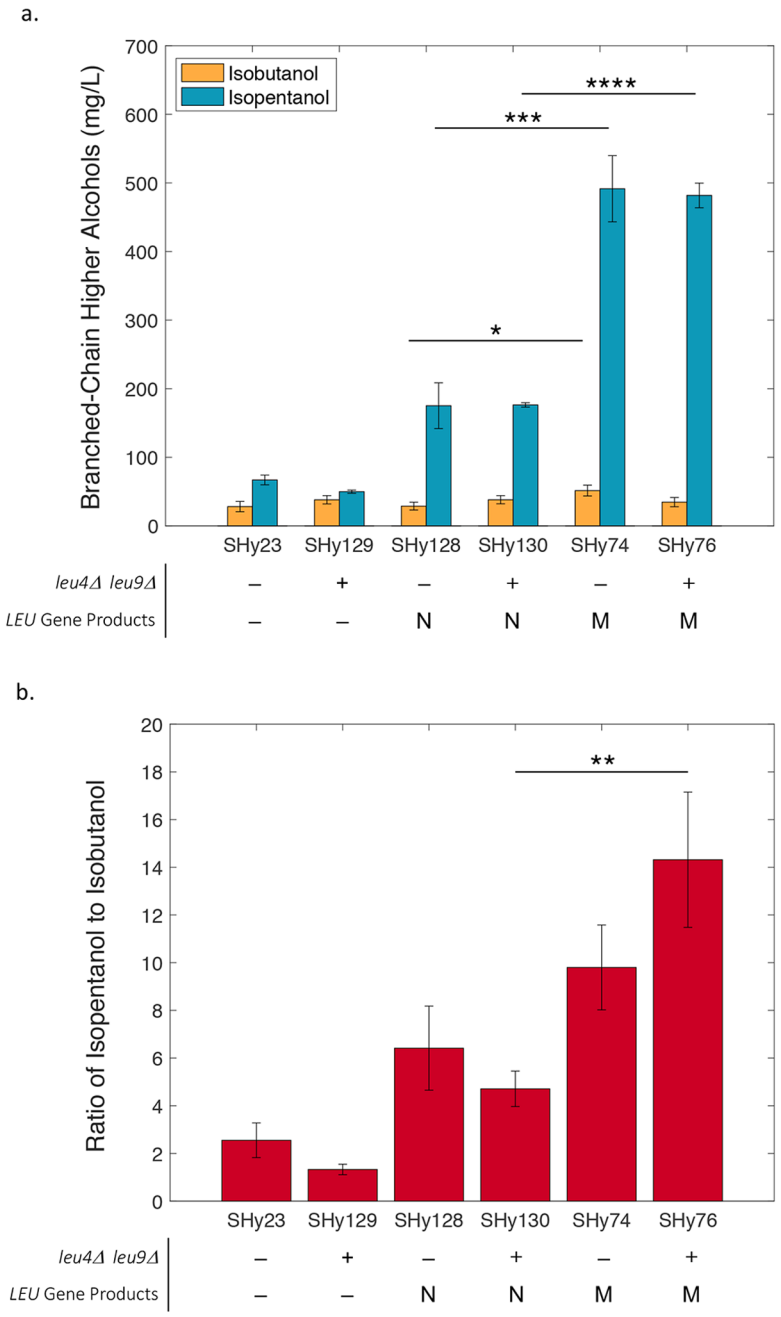

Figure 3. Effects of recursive pathway localization on isopentanol titers and specificity. BCHA concentrations were quantified in two different strain backgrounds, each harboring a 2 μ plasmid overexpressing $LEU4^{S547\Delta}$, *LEU1*, and *LEU2* in either their native locations (N) or in mitochondria (M). Control strains harbor empty 2 μ plasmids (−). (a) Isobutanol (orange) and isopentanol (teal) titers of wild-type and *leu4*Δ*leu9*Δ strains. BCHA titers were measured after 48-h high-cell-density fermentations in SC-Ura-Val medium containing 10% glucose. Error bars represent the standard deviation of at least three independent fermentations. (b) Ratios of isopentanol to isobutanol titers of the strains detailed in (a). Error bars represent the propagated error calculated using the standard deviations shown in (a). Two-tailed *t*-tests were used to determine the statistical significance of changes in ratios of isopentanol to isobutanol; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

wild type (SHy23) and 2.8-fold relative to the strain with *LEU* gene products in their native locations (SHy128), to 492 ± 48 mg/L (Figure 3a). Moreover, this strain (SHy74) produces 9.8-fold more isopentanol than isobutanol (Figure 3b), a 53% increase over the ratio achieved in the strain with native localization of the *LEU* genes (SHy128).

We repeated these experiments in a strain with the native *LEU4* and *LEU9* genes deleted to ensure that the effects seen in the wild-type background are independent of any interaction of the $LEU4^{S547\Delta}$ mutant with endogenous 2-IPMS enzymes. The *leu4*Δ*leu9*Δ strains produce similar isopentanol titers (Figure 3a, SHy130 and SHy76) compared to their analogues in wild-type backgrounds (Figure 3a, SHy128 and SHy74), which confirms that $LEU4^{S547\Delta}$ encodes a self-sufficient, active 2-IPMS. However, the specificity for isopentanol production is further enhanced by the double deletion. The *leu4*Δ*leu9*Δ strain with *LEU* gene products localized to mitochondria (SHy76) produces 14.3-fold more isopentanol than isobutanol (Figure 3b), representing a 3-fold increase over the ratio achieved in the strain with native localization of *LEU* gene

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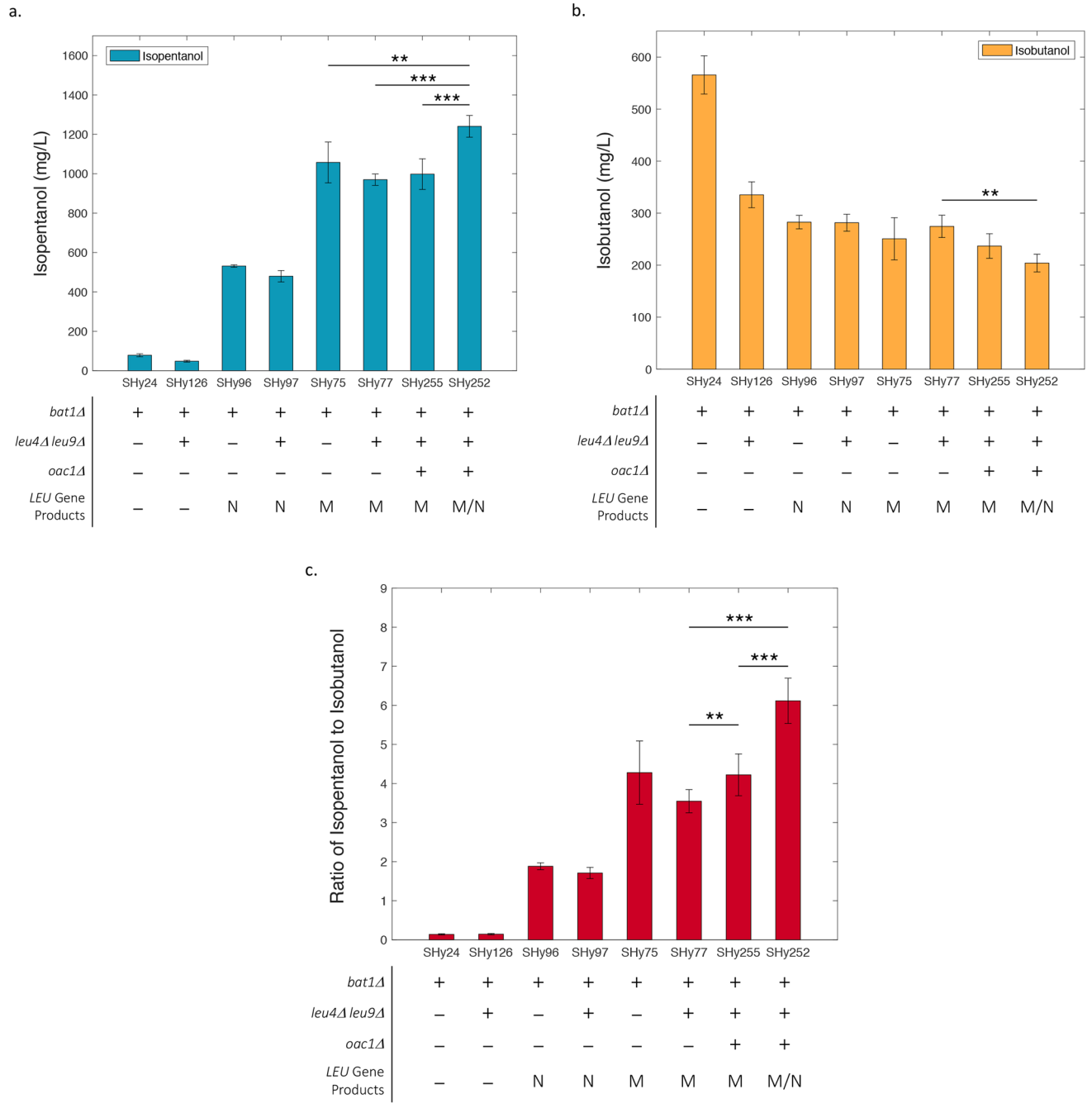

Figure 4. Effects of deleting *BAT1* and recovering cytosolic intermediates. (a) Isopentanol (teal) and (b) isobutanol (orange) titers of *bat1*Δ and *bat1*Δ*leu4*Δ*leu9*Δ strains with or without *OAC1* deleted. Each strain harbors a 2 μ plasmid overexpressing $LEU4^{S547\Delta}$, *LEU1*, and *LEU2* in either their native locations (N) or in mitochondria (M). Control strains harbor empty 2 μ plasmids (−). SHy252 has an additional single copy of $LEU4^{S547\Delta}$, *LEU1*, and *LEU2* integrated into the *HIS3* locus to overexpress these genes in their native locations (M/N). BCHA titers were measured after 48-h high-cell-density fermentations in SC-Ura-Val or SC-Ura-His-Val medium (as applicable) containing 10% glucose. Error bars in (a) and (b) represent the standard deviation of at least three independent fermentations. (c) Ratios of isopentanol to isobutanol titers of the strains detailed in (a) and (b). Error bars in (c) represent the propagated error calculated using the standard deviations shown in (a) and (b). Two-tailed *t*-tests were used to determine the statistical significance of changes in isobutanol titers, isopentanol titers, and ratios of isopentanol to isobutanol; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

products (SHy130). The isopentanol to isobutanol titer ratio of 14.3, or 93% isopentanol, achieved by compartmentalizing the elongation enzymes (Leu4p, Leu1p, and Leu2p) in mitochondria while keeping the termination enzymes KDC and ADH in the cytosol, represents the highest isopentanol specificity reported in *S. cerevisiae* (Supplementary Table S1), demonstrating for the first time that mitochondrial compartmentalization can improve product specificity in addition to boosting product titers of a recursive pathway.

Increasing Flux through Branched-Chain Amino Acid Biosynthesis. Experiments in the wild-type and *leu4*Δ*leu9*Δ strains demonstrate that mitochondrial localization of the *LEU*

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genes is superior to native localization for increasing isopentanol titers and specificity. However, regardless of whether the *LEU* genes are overexpressed in mitochondria or the cytosol (or not overexpressed at all) strains exhibit nearly constant isobutanol titers (Figure 3a). To test the capacity of our strategy to shift product specificity, we investigated whether mitochondrial compartmentalization could still achieve high-specificity isopentanol production in a strain in which metabolic flux through branched-chain amino acid biosynthesis is increased, enhancing isobutanol production and making it a stronger competitive byproduct in the recursive pathway.

We aimed to increase metabolic flux through the BCHA biosynthetic pathway by deleting *BAT1* and integrating multiple copies of *ILV2*, *ILV3*, and *ILV5* (collectively referred to as the *ILV* genes, Supplementary Figure S1), which we have shown boosts isobutanol production.[15,20] We first tested the conventional approach of overexpressing *LEU* genes in their native subcellular locations, resulting in nearly equal titers of isobutanol and isopentanol in *BAT1* deletion strains with the *ILV*s delta integrated (Supplementary Figure S3, SHy277). By comparison, mitochondrial compartmentalization of the *LEU* gene products (SHy228) substantially increases isopentanol production (Supplementary Figure S3a) and isopentanol specificity (Supplementary Figure S3b) in the same strain background. Yet, isobutanol production remains high—above 395 mg/L—in this strain, raising the possibility that increased mitochondrial pools of KIV caused by *ILV* gene overexpression[20] enhance KIV export *via* the mitochondrial KIV carrier (KIVC),[15] thereby maintaining isobutanol production rather than increasing flux toward isopentanol production. To test this hypothesis, we compartmentalized the *LEU* genes in a strain with *BAT1* deleted, but not overexpressing the *ILV* genes (SHy75). We found that this strain maintains isopentanol production, while substantially decreasing isobutanol production relative to SHy228, whose only difference is the additional overexpresses of the *ILV* genes (Supplementary Figure S3a). Accordingly, the ratio of isopentanol to isobutanol production increases 51% upon eliminating *ILV* gene overexpression in the *bat1*Δ strain background (Supplementary Figure S3b). This result supports our hypothesis that increased mitochondrial pools of KIV caused by *ILV* gene overexpression disproportionately enhance KIV export from the mitochondria, with mitochondrial $LEU4^{S547\Delta}$ struggling to compete with KIVC activity.

With the knowledge that deletion of *BAT1* alone is superior for enhancing isopentanol specificity when *LEU* genes are localized to mitochondria, we investigated how much of the branched-chain amino acid biosynthetic flux could be diverted to isopentanol in *bat1*Δ strains when the elongation enzymes are targeted to different subcellular locations. Relative to a strain harboring only a *BAT1* deletion (SHy24), which produces 566 ± 37 mg/L isobutanol and 79 ± 8 mg/L isopentanol (Figures 4a and 4b), overexpressing the *LEU* gene products in their native locations shifts some pathway flux toward isopentanol, boosting isopentanol production to 532 ± 6 mg/L (Figure 4a, SHy96); however, isobutanol still comprises 35% of BCHA production (Figure 4c). In contrast, when the *LEU* gene products are instead targeted to mitochondria, isopentanol titers are doubled to 1057 ± 104 mg/L (Figure 4a, SHy75) and the isobutanol content drops to only 19% of the total BCHA mixture. The isopentanol to isobutanol ratio of 4.3 achieved by this strain (Figure 4c, SHy75) represents a 2.3-fold improvement over that of SHy96. Thus, compartmentalizing the elongation enzymes in mitochondria results in the largest improvement (31-fold) over the 0.14 ratio of isopentanol to isobutanol observed in the parent strain (Figure 4c, SHy24). A similar trend is observed when the native *LEU4* and *LEU9* genes are deleted along with *BAT1* (Figures 4a–4c).

Even without additional optimization, our strains achieve improvements in titers comparable to previous efforts to localize metabolic enzymes to different yeast organelles. For example, mitochondrial localization of the two downstream enzymes (KDC and ADH) in isobutanol biosynthesis increased isobutanol titers 3.2-fold compared to overexpressing these two enzymes in their native, cytosolic location.[20] In a similar manner, targeting the mitochondrial alcohol acetyltransferase encoded by *EEB1* instead to lipid droplets increased the enzyme's activity 3-fold.[29] In addition to harnessing native yeast organelles, recent work has shown that clustering metabolic enzymes in synthetic organelles, or recruiting them to synthetic protein scaffolds, can improve titers and reduce byproduct formation.[30,31] For example, colocalizing the galactose and xylose permease (Gal2p) with the heterologous xylose isomerase (XI) using a synthetic protein scaffold increased production of the desired product, ethanol, at the expense of the byproduct, xylitol.[31] The molar ethanol to xylitol ratio improved as much as 3.7-fold over controls with no scaffold, comparable to the 3-fold increase in isopentanol to isobutanol ratio we observe from overexpressing the *LEU* genes in mitochondria (SHy76) rather than in their native locations (SHy130), but substantially less than the 11-fold increase we observe over wild-type gene expression (SHy129, Figure 3b).

Beyond achieving comparable improvements in performance as a result of enzyme localization to yeast organelles, our results demonstrate the novel application of mitochondrial compartmentalization to favor carbon-chain elongation over termination in a recursive pathway. Previously, fatty acyl-CoA reductase was targeted to yeast peroxisomes to intercept medium chain fatty acyl-CoAs in the catabolic beta-oxidation pathway, increasing medium-chain fatty alcohol production.[32] However, this is the first example of localizing anabolic enzymes to a yeast organelle in order to favor a recursive elongation cycle and avoid premature termination. In addition to mitochondria serving as vessels to contain the elongation enzymes (Leu4p, Leu1p, and Leu2p) separate from the cytosolic termination enzymes (KDCs and ADHs), promoting a cycle of 2-ketoacid elongation, Leu4p and Leu2p likely benefit from mitochondrial supplies of cofactors (acetyl-CoA and NAD^+ respectively),[33] while the synthesis of iron–sulfur clusters in mitochondria[34] is likely advantageous for Leu1p.

Exploring Loss of the Intermediate 2-Isopropylmalate from Mitochondria. To improve our control over the first cycle of the 2-ketoacid recursive pathway, it would be desirable to prevent mitochondrial export of all intermediates (KIV, 2-IPM, and 3-IPM). Mitochondrial carriers for KIV and 3-IPM have not been identified, and thus cannot be manipulated. However, we reasoned that deleting *OAC1*, the only known mitochondrial carrier for 2-IPM,[35] could prevent premature export of 2-IPM from mitochondria, which would increase its conversion to KIC within the organelle. Unfortunately, deletion of *OAC1* (SHy251) has no significant impact on isopentanol production or the ratio of isopentanol to isobutanol (Supplementary Figure S4a,b). It is possible that

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our intended effect is obscured by loss of Leu3p-mediated transcriptional activation of many genes in the branched-chain amino acid biosynthetic pathway,[26] which depends on 2-IPM binding to Leu3p (Supplementary Figure S1). To test this possibility, we overexpressed a constitutively active, truncated Leu3p,[36] previously shown to enhance isobutanol production,[21] in our *OAC1* deletion strains. However, we observed no significant improvement in isopentanol production relative to strains with wild-type *LEU3* expression (data not shown). This suggests that the lack of improvement in isopentanol production upon deleting *OAC1* is likely due to the presence of one or more additional mitochondrial carriers in yeast, besides *OAC1*, capable of exporting 2-IPM to the cytosol.

Reducing Loss of Intermediates in the Cytosol. Ideally, we would delete the mitochondrial carriers that export KIV, 2-IPM, and 3-IPM to the cytosol to prevent the loss of these intermediates from the mitochondrial recursive pathway. However, the genes encoding these carriers (including those probably complementing the *OAC1* deletion) have not yet been identified. Nevertheless, as a second recourse, we are still able to salvage some of these leaked intermediates by overexpressing cytosolic versions of the elongation enzymes in conjunction with their mitochondrial counterparts. Adding a single copy of constitutively expressed $LEU4^{S547\Delta}$, *LEU1*, and *LEU2*, whose gene products are targeted to their native locations, to a strain containing a mitochondrial recursive pathway, increases isopentanol production another 24% to 1241 ± 55 mg/L (SHy252 *vs* SHy255, Figure 4a). This is consistent with the hypothesis that KIV, 2-IPM, and potentially 3-IPM are prematurely exported from mitochondria, and that cytosolic Leu4p, Leu1p, and Leu2p can salvage some of these intermediates to produce isopentanol by competing with KDC and ADH for cytosolic KIV. Also consistent is the observation that overexpressing cytosolic *LEU* enzymes decreases isobutanol production to 204 ± 17 mg/L (SHy252, Figure 4b). The concerted changes in isopentanol and isobutanol production in SHy252 increase the isopentanol to isobutanol production ratio to 6.1 (or 86% isopentanol, Figure 4c), while also achieving the highest isopentanol titers and yields (from glucose) reported in yeast (Supplementary Table S1, Figure 4a). Because SHy255 lacks cytosolic Leu4p and Leu2p, it does not produce KIC in the cytosol, and the increase in isopentanol production in SHy252 can be attributed to the recovery of leaked intermediates in the cytosol. This means that 80% of isopentanol production in SHy252 is still derived from the recursive pathway compartmentalized in mitochondria.

While overexpressing a single copy of the *LEU* elongation enzymes in the cytosol is an effective strategy to recapture some of the KIV leaked from mitochondria, it is important to improve our understanding of the mitochondrial carriers responsible for transporting key metabolites in and out of this organelle in order to better control compartmentalized elongation. This includes identifying the genes encoding the carriers for KIV, 2-IPM, and 3-IPM, so they may be downregulated or deleted. Furthermore, understanding the molecular mechanisms of substrate recognition employed by these carriers would allow us to rationally engineer them to transport different metabolites with high specificity. For example, it is possible that the mitochondrial KIVC is also responsible for transporting KIC from mitochondria. If so, it would be desirable to engineer this transporter so that it preferentially transports KIC (required for isopentanol production) over KIV (responsible for isobutanol byproduct formation).

CONCLUSION

Previous efforts have shown that expanding the binding pocket of the first enzyme in the recursive catalytic cycle and the termination enzyme (KDC) can increase the number of 2-ketoacid elongation cycles in *E. coli*.[6,7] Yet this strategy has difficulties addressing alcohol product specificity, with lower concentrations often observed as chain length increases. From these studies, it has become generally accepted that the first step toward harnessing recursive catalytic cycles is enzyme engineering to enable the binding of larger substrates. It is assumed that directed evolution is then needed to also introduce specificity.[1] Our approach offers an alternative paradigm where organellar compartmentalization is used to direct specificity toward larger products by separating the elongation enzymes in mitochondria from the termination enzymes in the cytosol, thereby protecting elongation intermediates from early termination. Using this approach, we achieve not only the highest isopentanol to isobutanol product ratio, but also the highest isopentanol titer reported in *S. cerevisiae* (Supplementary Table S1). We envision that combining protein engineering (of enzymes and carriers) with subcellular compartmentalization will be a powerful approach to favor multiple elongation cycles in recursive pathways to produce larger, even non-natural products.

METHODS

Plasmid Construction. Plasmids developed in this study (Supplementary Table S2) were constructed from pJLA vectors,[20] which are derived from the pRS vector series.[37] Cloning for the construction of these plasmids was carried out in *Escherichia coli* strain DH5α. Endogenous *S. cerevisiae* genes (*ILV2*, *ILV3*, *ILV5*, *LEU1*, *LEU2*, *LEU4*) were PCR amplified with Phusion high-fidelity polymerase (NEB) from the genomic DNA of CEN.PK2−1C with primers appending NheI and XhoI sites to the 5′ and 3′ ends of the amplified gene, respectively. Addition of these restriction sites enables genes digested with NheI and XhoI to be inserted in the pJLA vectors. *S. cerevisiae* gene *LEU2* was PCR-amplified in a similar manner from the genomic DNA of FY4/5 due to the *LEU2* auxotrophy of CEN.PK2−1C. Plasmid pYZ33 was produced by inserting the ILV genes under constitutive promoters in the previously described pYZ23 vector[16] (Supplementary Table S2).

Before any modifications were made to *LEU4*, the internal NheI cut site (GCTAGC) encoded by amino acids 25 (Lysine, AAG), 26 (Leucine, CTA), and 27 (Alanine, GCC) was eliminated using QuikChange site-directed mutagenesis to mutate AAG to AAA, preserving the Lysine residue. QuikChange PCR was also used to mutate the second ATG start site (amino acid 31) within *LEU4* to ATT (isoleucine) in order to generate a putative exclusively mitochondrial Leu4p. The first 474 amino acids of this construct were amplified by PCR to yield the truncated $LEU4^{M31I/\Delta(E475\text{-}A619)}$. The *LEU4* leucine insensitive mutant $LEU4^{S547\Delta}$ was also generated using QuikChange PCR. In all of the above cases, the modified genes were sequenced by GENEWIZ to confirm the presence of the desired mutations. Previously described N-terminal localization signals from *COXIV*,[38,39] *CDC9*,[40] and *COXVI*[41] were used to target Leu4p, Leu1p, and Leu2p to mitochondria, respectively.

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Specifically, the first 26 amino acids from *COXIV*, the first 47 amino acids from *CDC9*, or the first 41 amino acids from *COXVI* were appended to the N-terminus of the corresponding open reading frame.

In addition to NheI-HF (NEB) and XhoI (NEB), XmaI (NEB), MreI (Thermo Fisher Scientific), and AscI (NEB) were used for cloning. *E. coli* DH5α was used to clone and amplify all plasmids. Colonies were screened by colony PCR, purified DNA was subjected to analytical digests, and final candidates were sequenced by GENEWIZ before transformation into yeast. PmeI (NEB) was used to linearize DNA (p553, p558, pYZ33, pSH132) before genomic integration into yeast.

Yeast Strains and Transformations. All of the *S. cerevisiae* strains used in this study were derived from CEN.PK2−1C (*MATa ura3−52 trp1−289 leu2−3, 112 his3Δ1 MAL2−8^{C}SUC2*). PCR-based homologous recombination was used to delete *BAT1, LEU4, LEU9*, and *OAC1*. Plasmids pAG26, pUG6, and pYZ84 (Supplementary Table S2) were used as PCR templates to amplify their drug resistance markers; primers were designed with 20 bases annealing to the template and 50 base pair overhangs homologous to the regions upstream and downstream of the gene of interest. These PCR products were used to transform CEN.PK2−1C, replacing *BAT1* with Hygromycin B (Thermo Fisher Scientific) resistance, *LEU4* with Geneticin (Thermo Fisher Scientific) resistance, and *LEU9* with Nourseothricin (Werner BioAgents) resistance. After YZy117 was obtained, Cre recombinase-based marker rescue[42] with p222 (Supplementary Table S2) was used to recover the *kanMX* and *natMX6* antibiotic resistances, allowing *OAC1* to be additionally deleted with Geneticin. Genotyping PCRs were used to confirm that the genes of interest were successfully deleted and that the antibiotic resistance markers were inserted in the correct loci.

Yeast cells were cultured in either YPD medium (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone, and 0.15 g/L tryptophan) or synthetic complete (SC) medium (20 g/L glucose, 1.5 g/L yeast nitrogen base without amino acids or ammonium sulfate, 5 g/L ammonium sulfate, 36 mg/L inositol, and 2 g/L of an amino acid mixture lacking amino acids or nucleotides as needed for selection). If all the leucine provided in the media were catabolized to isopentanol, a maximum of 147 mg/L of isopentanol would be produced. This accounts for isopentanol production in SHy23 (wild type CEN.PK2−1C with empty plasmid) and other strains without *LEU* gene overexpression, which is expected due to the *LEU2* auxotrophy of the CEN.PK2−1C background. All strains (Supplementary Table S3) were transformed using the standard lithium acetate protocol.[43] Transformants that acquired a 2 μ plasmid were isolated on SC agar plates containing 2% glucose, lacking uracil or leucine. For plasmids containing more than one gene construct, at least 12 colonies were screened in high-cell-density fermentations to identify the highest isopentanol producers. In order to integrate into the *HIS3* or *TRP1* loci, plasmids pSH132, p553, and p558 were linearized with PmeI and purified by gel extraction before transformation into yeast. After selection on SC agar plates containing 2% glucose and lacking tryptophan or histidine, 6 colonies were screened for isopentanol production. For delta integration of *ILV2*, *ILV3*, and *ILV5*, pYZ33 was linearized with PmeI and purified by gel extraction before transformation. Among the transformants selected on YPD plates containing 1200 μg/mL Zeocin (Thermo Fisher Scientific), 12 colonies were screened to identify the highest isopentanol producer.

Fermentations. All high-cell-density fermentations were performed in 24-well plates as follows. Cells were grown overnight in 1 mL SC media containing 2% glucose, lacking other amino acids or nucleobases as needed. After 19 h at 30 °C and 200 rpm agitation, plates were centrifuged at 1000 rpm for 5 min. After the supernatant was discarded, the cell pellets were resuspended in 1 mL SC media containing 10% glucose, lacking the amino acids or nucleobases absent from the overnight culture. With the exception of fermentations to evaluate *LEU4* variants (Supplementary Figure S2b), all fermentations were conducted in media lacking valine. The 24-well plates were covered in a SealPlate film to prevent evaporation and fermented for 48 h at 30 °C and 200 rpm agitation. After 48 h, plates were centrifuged at 1000 rpm for 10 min and 900 μL of supernatant were collected from each well for analysis.

Quantification of Branched-Chain Higher Alcohol Production. An Agilent 1260 Infinity instrument was used to perform high-performance liquid chromatography (HPLC) in order to quantify isobutanol and isopentanol concentrations present in fermentation samples. Fermentation samples were centrifuged in 1.5 mL Eppendorf tubes for 40 min at 13,300 rpm and 4 °C to remove residual debris, and then transferred to 2 mL glass vials for analysis. Samples were eluted with 5 mM H_2SO_4 through an Aminex HPX-87H ion-exchange column (Bio-Rad) at a rate of 0.6 mL/min for 50 min. Isobutanol and isopentanol concentrations were measured using a refractive index detector (RID) and calibration curves made from standard solutions.

Error Propagation and Statistical Analyses. After three or more high-cell-density fermentations (described above) were performed with each strain, ratios of isopentanol to isobutanol were calculated for each replicate by dividing isopentanol production by isobutanol production. The average of the ratios calculated for each replicate are reported, with the error bars representing the error propagated using the following formula, as is standard for division:

$$\delta R = |R| \cdot \sqrt{\left(\frac{\delta P}{P}\right) + \left(\frac{\delta B}{B}\right)}$$

where R is the average ratio of isopentanol to isobutanol, P is the average isopentanol titer, δP is the standard deviation in isopentanol titers, B is the average isobutanol titer, and δB is the standard deviation in isobutanol titers.

Statistical significance of differences in titers or ratios observed between some of our strains (when displaying nonobvious differences) were calculated using two-tailed Student's t-tests assuming equal variances. P-values less than 0.05 are denoted with asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00420.

Summary of previous efforts to engineer *S. cerevisiae* for isopentanol production; schematic of isobutanol and isopentanol biosynthesis in *S. cerevisiae*; screen of putative leucine insensitive Leu4p variants; effects of *ILV* gene overexpression on isobutanol and isopentanol

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production; effect of *OAC1* deletion on isobutanol and isopentanol production; plasmids and yeast strains used in the study (PDF)

AUTHOR INFORMATION

Corresponding Author

José L. Avalos − *Department of Chemical and Biological Engineering, Andlinger Center for Energy and the Environment, and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, United States;* orcid.org/0000-0002-7209-4208; Email: javalos@princeton.edu

Authors

Sarah K. Hammer − *Department of Chemical and Biological Engineering, Princeton University, Princeton, New Jersey 08544, United States*

Yanfei Zhang − *Department of Chemical and Biological Engineering, Princeton University, Princeton, New Jersey 08544, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.9b00420

Author Contributions

S.K.H. and J.L.A. designed experiments. S.K.H. conducted experiments. Y.Z. helped with vector design and cloning. S.K.H. and J.L.A. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Felnagle, E. A., Chaubey, A., Noey, E. L., Houk, K. N., and Liao, J. C. (2012) Engineering synthetic recursive pathways to generate non-natural small molecules. *Nat. Chem. Biol. 8*, 518−526.

(2) Mauricio, J. C., Moreno, J., Zea, L., Ortega, J. M., and Medina, M. (1997) The effects of grape must fermentation conditions on volatile alcohols and esters formed by Saccharomyces cerevisiae. *J. Sci. Food Agric. 75*, 155−160.

(3) Atsumi, S., Hanai, T., and Liao, J. C. (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature 451*, 86−89.

(4) Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., del Cardayre, S. B., and Keasling, J. D. (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature 463*, 559.

(5) Chen, G. S., Siao, S. W., and Shen, C. R. (2017) Saturated mutagenesis of ketoisovalerate decarboxylase V461 enabled specific synthesis of 1-pentanol *via* the ketoacid elongation cycle. *Sci. Rep. 7*, 11284.

(6) Marcheschi, R. J., Li, H., Zhang, K., Noey, E. L., Kim, S., Chaubey, A., Houk, K. N., and Liao, J. C. (2012) A synthetic recursive "+1" pathway for carbon chain elongation. *ACS Chem. Biol. 7*, 689−697.

(7) Zhang, K., Sawaya, M. R., Eisenberg, D. S., and Liao, J. C. (2008) Expanding metabolism for biosynthesis of nonnatural alcohols. *Proc. Natl. Acad. Sci. U. S. A. 105*, 20653−20658.

(8) Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., and Dickinson, J. R. (2008) The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism. *Appl. Environ. Microbiol. 74*, 2259−2266.

(9) Strassman, M., Thomas, A. J., and Weinhouse, S. (1955) The Biosynthesis of Valine. *J. Am. Chem. Soc. 77*, 1261−1265.

(10) Strassman, M., Shatton, J. B., and Weinhouse, S. (1960) Conversion of alpha-acetolactic acid to the valine precursor, alpha, beta-dihydroxyisovaleric acid. *J. Biol. Chem. 235*, 700−705.

(11) Wixom, R. L., Shatton, J. B., and Strassman, M. (1960) Studies on a dehydrase in valine biosynthesis in yeast. *J. Biol. Chem. 235*, 128−131.

(12) Ryan, E. D., Tracy, J. W., and Kohlhaw, G. B. (1973) Subcellular localization of the leucine biosynthetic enzymes in yeast. *J. Bacteriol. 116*, 222−225.

(13) Choi, Y. J., Lee, J., Jang, Y. S., and Lee, S. Y. (2014) Metabolic engineering of microorganisms for the production of higher alcohols. *mBio 5*, No. e01524-14.

(14) Generoso, W. C., Schadeweg, V., Oreb, M., and Boles, E. (2015) Metabolic engineering of Saccharomyces cerevisiae for production of butanol isomers. *Curr. Opin. Biotechnol. 33*, 1−7.

(15) Hammer, S. K., and Avalos, J. L. (2017) Uncovering the role of branched-chain amino acid transaminases in Saccharomyces cerevisiae isobutanol biosynthesis. *Metab. Eng. 44*, 302−312.

(16) Zhao, E. M., Zhang, Y., Mehl, J., Park, H., Lalwani, M. A., Toettcher, J. E., and Avalos, J. L. (2018) Optogenetic regulation of engineered cellular metabolism for microbial chemical production. *Nature 555*, 683.

(17) Li, Y.-l., Yu, Y.-c., Wang, Z.-w., and Wang, J.-f. (2015) Physical and chemical properties of isobutanol-gasoline blends. *Environ. Prog. Sustainable Energy 34*, 908−914.

(18) Yang, Y., Dec, J. E., Dronniou, N., and Simmons, B. (2010) Characteristics of Isopentanol as a Fuel for HCCI Engines. *SAE Int. J. Fuels Lubr. 3*, 725−741.

(19) Park, S., Chung, S. H., Lu, T., and Sarathy, S. M. (2015) Combustion Characteristics of C5 Alcohols and a Skeletal Mechanism for Homogeneous Charge Compression Ignition Combustion Simulation. *Energy Fuels 29*, 7584−7594.

(20) Avalos, J. L., Fink, G. R., and Stephanopoulos, G. (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. *Nat. Biotechnol. 31*, 335−341.

(21) Park, S. H., Kim, S., and Hahn, J. S. (2014) Metabolic engineering of Saccharomyces cerevisiae for the production of isobutanol and 3-methyl-1-butanol. *Appl. Microbiol. Biotechnol. 98*, 9139−9147.

(22) Yuan, J., Chen, X., Mishra, P., and Ching, C.-B. (2017) Metabolically engineered Saccharomyces cerevisiae for enhanced isoamyl alcohol production. *Appl. Microbiol. Biotechnol. 101*, 465−474.

(23) Yuan, J., Mishra, P., and Ching, C. B. (2017) Engineering the leucine biosynthetic pathway for isoamyl alcohol overproduction in Saccharomyces cerevisiae. *J. Ind. Microbiol. Biotechnol. 44*, 107−117.

(24) Hammer, S. K., and Avalos, J. L. (2017) Harnessing yeast organelles for metabolic engineering. *Nat. Chem. Biol. 13*, 823−832.

(25) Shi, S., Si, T., Liu, Z., Zhang, H., Ang, E. L., and Zhao, H. (2016) Metabolic engineering of a synergistic pathway for n-butanol production in Saccharomyces cerevisiae. *Sci. Rep. 6*, 25675.

(26) Kohlhaw, G. B. (2003) Leucine biosynthesis in fungi: entering metabolism through the back door. *Microbiol. Mol. Biol. Rev. 67*, 1−15.

(27) Zhang, Z., Wu, J., Lin, W., Wang, J., Yan, H., Zhao, W., Ma, J., Ding, J., Zhang, P., and Zhao, G. P. (2014) Subdomain II of alpha-isopropylmalate synthase is essential for activity: inferring a mechanism of feedback inhibition. *J. Biol. Chem. 289*, 27966−27978.

(28) Cavalieri, D., Casalone, E., Bendoni, B., Fia, G., Polsinelli, M., and Barberio, C. (1999) Trifluoroleucine resistance and regulation of

alpha-isopropyl malate synthase in Saccharomyces cerevisiae. *Mol. Gen. Genet. 261*, 152−160.
(29) Zhu, J., Schwartz, C., and Wheeldon, I. (2019) Controlled intracellular trafficking alleviates an expression bottleneck in S. cerevisiae ester biosynthesis. *Metab. Eng. Commun. 8*, No. e00085.
(30) Zhao, E. M., Suek, N., Wilson, M. Z., Dine, E., Pannucci, N. L., Gitai, Z., Avalos, J. L., and Toettcher, J. E. (2019) Light-based control of metabolic flux through assembly of synthetic organelles. *Nat. Chem. Biol. 15*, 589−597.
(31) Thomik, T., Wittig, I., Choe, J.-y., Boles, E., and Oreb, M. (2017) An artificial transport metabolon facilitates improved substrate utilization in yeast. *Nat. Chem. Biol. 13*, 1158−1163.
(32) Sheng, J., Stevens, J., and Feng, X. (2016) Pathway Compartmentalization in Peroxisome of Saccharomyces cerevisiae to Produce Versatile Medium Chain Fatty Alcohols. *Sci. Rep. 6*, 26884.
(33) Agrimi, G., Brambilla, L., Frascotti, G., Pisano, I., Porro, D., Vai, M., and Palmieri, L. (2011) Deletion or Overexpression of Mitochondrial NAD+ Carriers in Saccharomyces cerevisiae Alters Cellular NAD and ATP Contents and Affects Mitochondrial Metabolism and the Rate of Glycolysis. *Appl. Environ. Microbiol. 77*, 2239.
(34) Mühlenhoff, U., and Lill, R. (2000) Biogenesis of iron-sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria. *Biochim. Biophys. Acta, Bioenerg. 1459*, 370−382.
(35) Marobbio, C. M., Giannuzzi, G., Paradies, E., Pierri, C. L., and Palmieri, F. (2008) alpha-Isopropylmalate, a leucine biosynthesis intermediate in yeast, is transported by the mitochondrial oxalacetate carrier. *J. Biol. Chem. 283*, 28445−28453.
(36) Friden, P., Reynolds, C., and Schimmel, P. (1989) A large internal deletion converts yeast LEU3 to a constitutive transcriptional activator. *Mol. Cell. Biol. 9*, 4056−4060.
(37) Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene 110*, 119−122.
(38) Maarse, A. C., Van Loon, A. P., Riezman, H., Gregor, I., Schatz, G., and Grivell, L. A. (1984) Subunit IV of yeast cytochrome c oxidase: cloning and nucleotide sequencing of the gene and partial amino acid sequencing of the mature protein. *EMBO J. 3*, 2831−2837.
(39) Hurt, E. C., Pesold-Hurt, B., Suda, K., Oppliger, W., and Schatz, G. (1985) The first twelve amino acids (less than half of the pre-sequence) of an imported mitochondrial protein can direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix. *EMBO J. 4*, 2061−2068.
(40) Willer, M., Rainey, M., Pullen, T., and Stirling, C. J. (1999) The yeast CDC9 gene encodes both a nuclear and a mitochondrial form of DNA ligase I. *Curr. Biol. 9*, 1085−1094.
(41) Wright, R. M., Ko, C., Cumsky, M. G., and Poyton, R. O. (1984) Isolation and sequence of the structural gene for cytochrome c oxidase subunit VI from Saccharomyces cerevisiae. *J. Biol. Chem. 259*, 15401−15407.
(42) Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D., and Hegemann, J. H. (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res. 30*, No. e23.
(43) Gietz, R. D., and Woods, R. A. (1998) Transformation of Yeast by the Lithium Acetate/Single-Stranded Carrier DNA/PEG Method. In *Methods in Microbiology* (Brown, A. J. P., and Tuite, M., Eds.), pp 53−66, Academic Press.

https://dx.doi.org/10.1021/acssynbio.9b00420
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