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Engineering *Escherichia coli* for selective 1-decanol production using the reverse β-oxidation (rBOX) pathway

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ABSTRACT

1-Decanol has great value in the pharmaceutical and fragrance industries and plays an important role in the chemical industry. In this study, we engineered *Escherichia coli* to selectively synthesize 1-decanol by using enzymes of the core reverse β-oxidation (rBOX) pathway and termination module with overlapping chain-length specificity. Through screening for acyl-CoA reductase termination enzymes and proper regulation of rBOX pathway expression, a 1-decanol titer of 1.4 g/L was achieved. Further improvements were realized by engineering pyruvate dissimilation to ensure the generation of NADH through pyruvate dehydrogenase (PDH) and reducing byproduct synthesis via a tailored YigI thioesterase knockout, increasing 1-decanol titer to 1.9 g/L. The engineered strain produced about 4.4 g/L 1-decanol with a yield of 0.21 g/g in 36 h in a bi-phasic fermentation that used a dodecane overlay to increase 1-decanol transport and reduce its toxicity. Adjustment of pathway expression (varying inducer concentration) and cell growth (oxygen availability) enabled 1-decanol production at 6.1 g/L (0.26 g/g yield) and 10.05 g/L (0.2 g/g yield) using rich medium in shake flasks and bioreactor, respectively. Remarkably, the use of minimal medium resulted in 1-decanol production with 100% specificity at 2.8 g/L (0.14 g/g yield) and a per cell mass yield higher than rich medium. These 1-decanol titers, yields and purity are at least 10-fold higher than others reported to date and the engineered strain shows great potential for industrial production. Taken together, our findings suggest that using rBOX pathway and termination enzymes of proper chain-length specificity in combination with optimal chassis engineering should be an effective approach for the selective production of alcohols.

1. Introduction

1-Decanol is a 10-carbon saturated fatty alcohol used in the manufacture of plasticizers, lubricants, surfactants, solvents, agricultural chemicals, dairy/food flavor and cosmetics. Its ability to permeate the skin has led to it being investigated as a penetration enhancer for transdermal drug delivery as well (Kanikkannan and Singh, 2002). Its dicarboxylate, ω-hydroxy, and α, ω-diol derivatives are also important downstream products (Clomburg et al., 2015). Additionally, given its high energy density, 1-decanol can be used as fuel, including in the production of jet fuel (He et al., 2018; Zhou et al., 2018). The current global fatty alcohol demand is over 2 million tons and is expected to exceed 4.7 million tons by 2025 due to a compound annual growth rate (CAGR) of 4.5% from 2020 to 2025 (Sharma and Yazdani, 2021). The growth in the market is attributed to the increasing prices of petrochemicals and a shift in the trend toward sustainable chemicals. As an important ingredient in the chemical and fragrance industries, 1-decanol deserves special interest for its value. 1-Decanol is typically produced either from natural sources by the hydrogenation of related fatty acids from plant oils (coconut oil) and waxes or synthesized by a Ziegler-Alfol process (Krishnan et al., 2020; Mudge, 2005). These processes are not sustainable since they involve the use of non-renewable petroleum-based feedstocks and contribute to deforestation, underscoring the need for alternative production methods.

Microbial cells produce fatty acids for their growth and energy storage and therefore the production of fatty acid derivatives using microbes is one of the potential options among various forms of renewable energy (Zhang et al., 2011). Likewise, the production of fatty alcohols in engineered microorganisms from renewable carbon sources presents an attractive alternative to current methods. There are two primary pathways to produce fatty alcohols in microbes: the natural fatty acid biosynthesis (FAB) pathway and the engineered reversal

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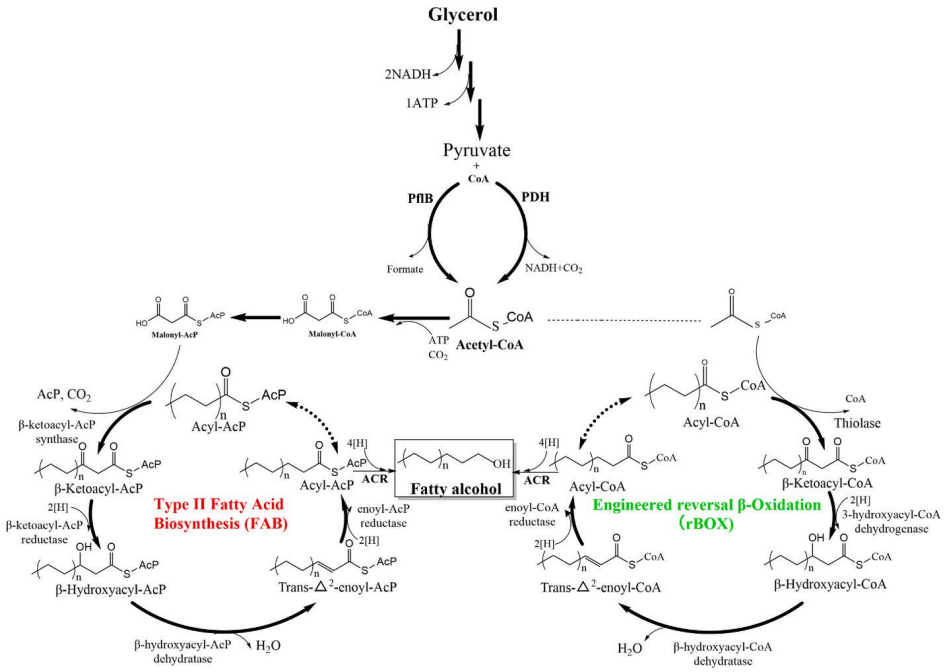


Fig. 1. Synthesis of fatty alcohols via type II fatty acid biosynthesis (FAB) and engineered β-oxidation reversal (r-BOX). PDH: pyruvate dehydrogenase, 2[H]: NADH or NADPH, ACR: bi-functional, alcohol-forming acyl-CoA/acyl-ACP reductases.

Table 1
Production of medium-to-long-chain alcohols in engineered *E. coli* and yeast.

Host	Pathway	Termination enzyme	Media	Product (alcohol)	1-decanol titer	1-decanol specificity[a]	Reference
E. coli	rBOX	YiaI (native)	MM with glucose	C6–C10	160 mg/L	40%	(Dellomonaco et al., 2011)
E. coli	rBOX	MaACR (Marinobacter aquaeolei)	LB with glucose	C6–C16	500 mg/L	30%	(Mehrer et al., 2018)
E. coli	rBOX	Maqu2507 (*Marinobacter aquaeolei*)	MM with glycerol	C6–C10	40 mg/L	13.3%	(Kim et al., 2015)
Saccharomyces cerevisiae	FAB	MmCAR (*Mycobacterium marinum*) *and* ADH/ALR (native)	MM with glucose	C6–C12	45 mg/L	25%	(Hu et al., 2020)
Yarrowia lipolytica	FAB	FAR (*Arabidopsis thaliana*)	RM with glucose	1-decanol	500 mg/L	90%	(Rutter and Rao, 2016)
E. coli	rBOX	Maqu2507 (*Marinobacter aquaeolei*)	RM with glycerol	1-decanol	5500 mg/L	91%	this study
E. coli	rBOX	Maqu2507 (*Marinobacter aquaeolei*)	MM with glycerol	1-decanol	2370 mg/L	100%	this study
E. coli	rBOX	Maqu2507 (*Marinobacter aquaeolei*)	RM with glycerol (fed-batch)	1-decanol	10050 mg/L	90%	this study

rBOX, reverse β-oxidation pathway; FAB, fatty-acid biosynthesis pathway, MM, minimal media; RM, rich media.
[a] 1-decanol specificity was calculated as the ratio of grams of 1-decanol per grams of total C6-12 fatty acids and fatty alcohols and expressed on percentage basis.

β-oxidation (rBOX) pathway (Fig. 1). While the FAB pathway has been widely exploited, it has inherent limitations to the product yield due to carbon and energy inefficiencies associated with the use of malonyl-AcP as extender units, low flux, and complex intrinsic regulation (Tarasava et al., 2022). The rBOX pathway can circumvent many of these challenges by using non-decarboxylative Claisen condensation reactions catalyzed by 3-ketoacyl-CoA thiolases (hereafter referred to as thiolases) to condense acyl-CoA substrates with acetyl-CoA as extender unit and hence providing a carbon- and energy-efficient way to manufacture important molecules of commercial interest (Cheong et al., 2016; Dellomonaco et al., 2011; Tarasava et al., 2022) (Fig. 1). Whether the FAB or rBOX pathway is used, the choice of termination enzyme(s) allows tailoring the product carbon chain length and class/functionality. Previous studies have demonstrated the production of fatty alcohols using both FAB and rBOX pathways (Table 1). However, these methods typically produce a mixture of different chain length alcohols in the C4–C16+ range due to the inherent promiscuity of both rBOX and termination enzymes toward acyl-CoA intermediates of different chain lengths. One of the current challenges is identifying strategies for the selective synthesis of products with specific chain lengths. For these reasons, 1-decanol is typically not the primary product in these mixtures, with titers in the 60–200 mg/L range (Henritzi et al., 2018; Hu et al., 2020; Kim et al., 2015; Li et al., 2022; Liu et al., 2013; Mehrer et al., 2018; Youngquist et al., 2013). Only one study, conducted in *Yarrowia lipolytica* and using the FAB pathway, has reported 1-decanol as the primary product from glucose achieving up to 500 mg/L, along with small amounts of 1-hexadecanol and 1-octadecanol, and yield of 4.5% on C-mole basis (Rutter and Rao, 2016).

Given that the rBOX presents a more energy efficient metabolic route for carbon chain elongation, in this work we seek to exploit this pathway for selective production of 1-decanol. We selected core rBOX enzymes with broad chain-length specificity and evaluated termination enzymes that when combined had the potential to yield 1-decanol as the primary product. Episomal expression of rBOX enzymes BktB, FadB and tdTER and termination by *Marinobacter aquaeolei* Maqu_2507 yielded up to 1.4 g/L of 1-decanol from glycerol in 48-h shake flask fermentations. By improving the intracellular NADH supply and reducing by-products, the 1-decanol titer was further increased by 40%. Through further optimization of fermentation conditions and the use of an n-dodecane overlay for *in situ* extraction of 1-decanol, the final strain produced about 6.1 g/L

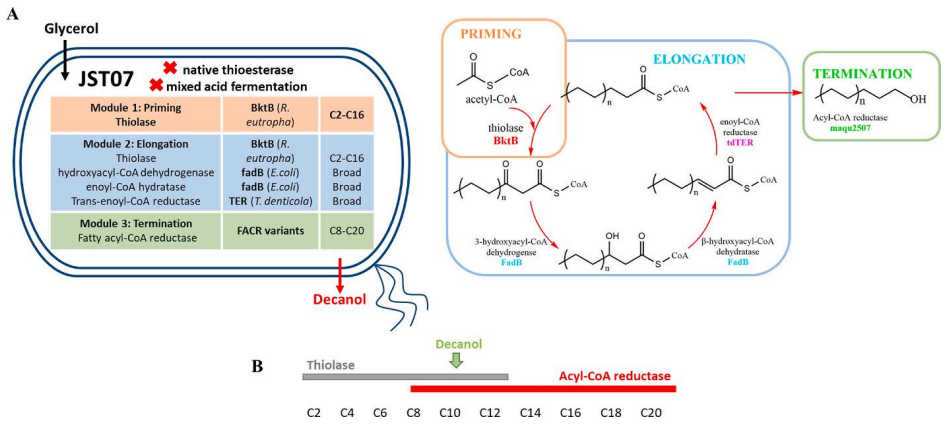


Fig. 2. Conceptual design and strategy for the selective synthesis of 1-decanol via rBOX. (A) Chassis strain background and selection of rBOX and termination enzymes with overlapping chain-length specificity. (B) Engineering strategy for the selective production of 1-decanol.

of 1-decanol with a yield of 0.26 g/g in rich medium, a titer 12-fold higher than previously reported. When minimal medium was used, this strain can produce over 2.8 g/L 1-decanol with a yield of 0.14 g/g and without any C6–C12 by-products, suggesting great potential for industrial application. These results demonstrate that the rBOX can be used for selective synthesis of 1-decanol as the primary product (91% of the total C6–C12 alcohols in rich medium and 100% of the total C6–C12 alcohols and acids in minimal medium) in *E. coli* at commercially significant titers.

2. Results and discussion

2.1. Pathway design and enzyme selection for 1-decanol production

Within the context of an engineered reversal β-oxidation (rBOX) pathway, the condensation (thiolase) and termination enzymes represent two critical steps that have been found to provide control points to determine specificity towards both chain length and acid versus alcohol functionalities (Tarasava et al., 2022). Consequently, selection of the chassis strain and specific enzymes for the rBOX pathway and termination reaction (s) are expected to play a key role in achieving desirable 1-decanol titer and yield. As the *E. coli* chassis, we selected a mixed acid fermentation- and thioesterase-deficient strain (strain JST07), which reduces the flow of carbon to native fermentation products and minimizes the hydrolysis of acetyl-CoA and off target CoA thioester intermediates in the rBOX pathway, respectively (Kim et al., 2015). The latter provides advantages for screening fatty acyl-CoA reductase (ACR) variants as termination enzymes for selective 1-decanol synthesis. Strain JST07 was transformed with two vectors for episomal expression of rBOX (rBOX vector) and termination (termination vector) enzymes, the latter providing an exit from the cycle and facilitating product formation (Fig. 2A).

The framework for expression of rBOX priming and elongation modules includes four functional units: 1) thiolase, used for initial priming and subsequent chain elongation by addition of acetyl-CoA to the acyl-CoA intermediate; 2) 3-hydroxyacyl-CoA dehydrogenase (HACD) which uses NADH for the first reduction; 3) enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydratase; and 4) trans-enoyl-CoA reductase (TER) which performs the second reduction step using another NADH molecule and generating a 2-carbon elongated acyl-CoA intermediate (Fig. 2A). Because 1-decanol production requires multiple turns of the rBOX cycle, these enzymes should have a broad chain length specificity to accommodate C4–C10 intermediates. Previous works have shown that *Ralstonia eutropha* BktB can catalyze the condensation of C2 to C10+ acyl-CoA chains with acetyl-CoA (Clomburg et al., 2015; Kim et al., 2014). The native *E. coli* FadB can act as a multi-functional enzyme with 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydratase functions for a broad range of chain length substrates. Finally, *Treponema denticola* TER (tdTER) was selected as the trans-enoyl-CoA reductase having been previously demonstrated to be effective on the

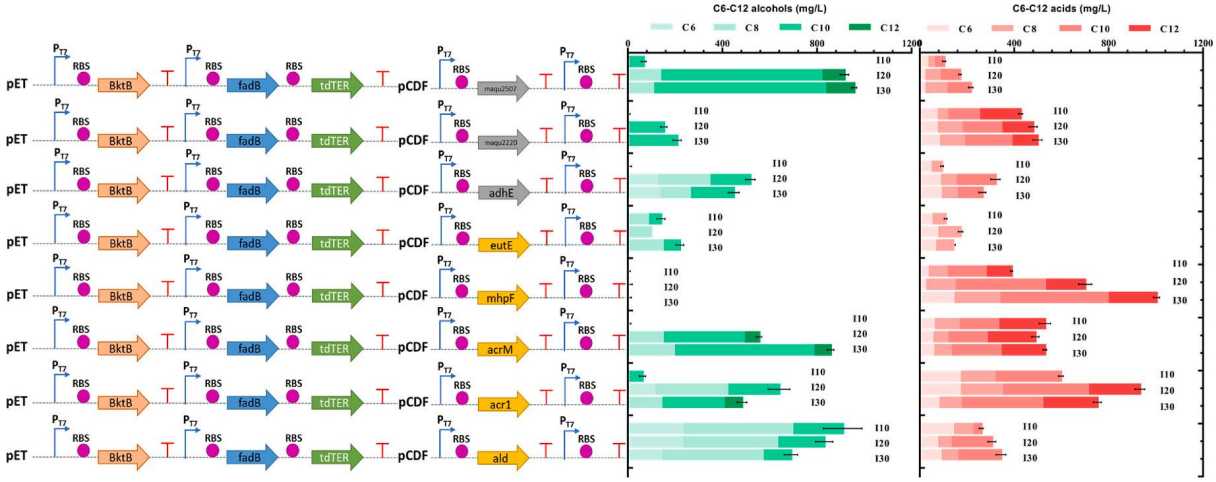
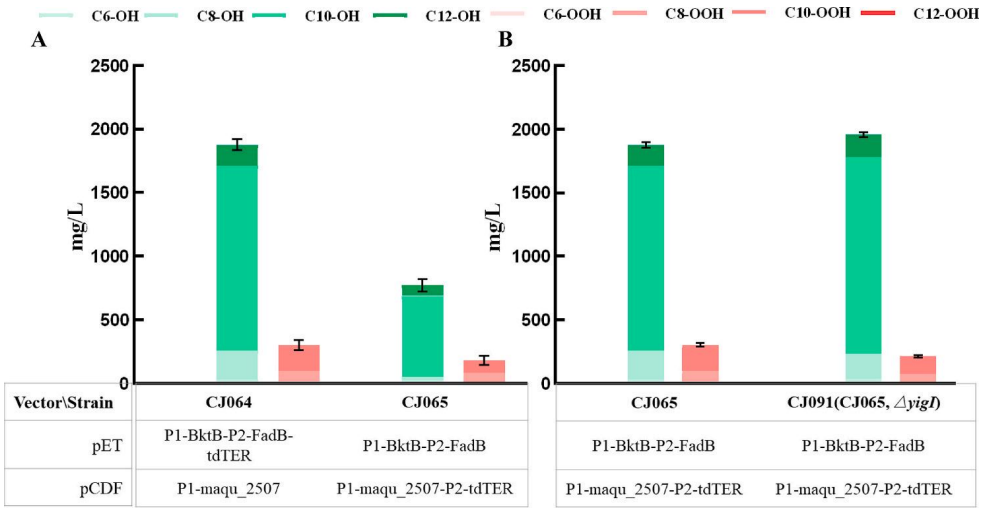


Fig. 3. Production of fatty acids (red bars) and fatty alcohols (green bars) in strain JST07 with episomal overexpression of rBOX enzymes (pET vector) and different termination enzymes (pCDF vector). Both bifunctional, alcohol-forming acyl-CoA reductases (shown in grey) as well as aldehyde-forming acyl-CoA reductases (shown in yellow) were evaluated as termination enzymes. Three concentrations of inducer IPTG were evaluated, and product concentrations are shown for biological replicates (n = 3) with error bars representing standard deviation.



Vector\Strain	CJ064	CJ065	CJ065	CJ091(CJ065, △*yigI*)
pET	P1-BktB-P2-FadB-tdTER	P1-BktB-P2-FadB	P1-BktB-P2-FadB	P1-BktB-P2-FadB
pCDF	P1-maqu_2507	P1-maqu_2507-P2-tdTER	P1-maqu_2507-P2-tdTER	P1-maqu_2507-P2-tdTER

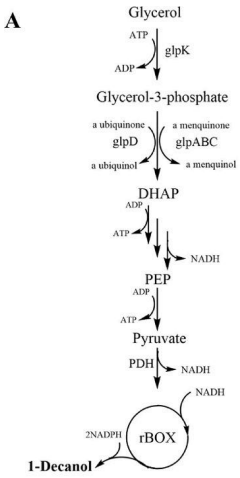
Fig. 4. Increasing pathway flux and decreasing byproduct synthesis by improving expression of trans-enoyl-CoA reductase tdTER (A) and deletion of thioesterase YigI (B). Concentrations are shown for biological replicates (n = 3) of 48-hour fermentations with error bars representing standard deviations.

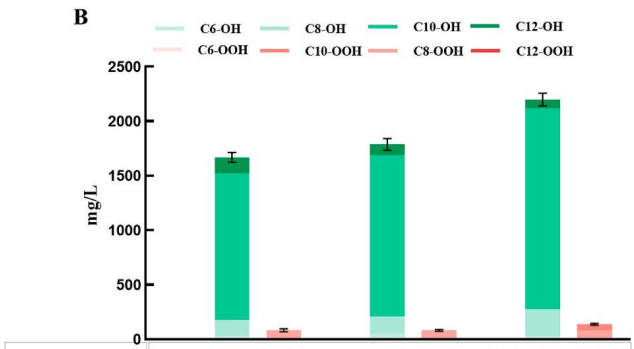
second reduction step. The above 3 genes were expressed from the rBOX vector under the control of an IPTG inducible *T7* promoter (Fig. 2A).

The other crucial component of the rBOX pathway design is the selection of termination enzyme(s). There are two types of ACRs, fatty aldehyde-forming ACR and bi-functional fatty alcohol-forming ACR (Fillet and Adrio, 2016; Krishnan et al., 2020; Willis et al., 2011). The first catalyzes a two-electron reduction of active forms of fatty acids to fatty aldehydes, whereas the second catalyzes a four-electron reduction of active forms of fatty acids to fatty alcohols. The use of aldehyde forming reductases for alcohol production necessitates their combination with an aldehyde reductase, a function that can be fulfilled by endogenous activities. Bi-functional fatty alcohol forming ACR can catalyze both the aldehyde formation and the alcohol dehydrogenase steps. The ideal ACR candidate would possess high specificity towards decanoyl-CoA and good reaction kinetics for both enzymatic steps. In order to identify the optimal termination enzyme, 8 different ACR enzymes were co-expressed under the control of IPTG-inducible *T7* promoter in the termination vector along with rBOX vector which contained selected "core" rBOX enzymes that should be able to generate decanoyl-CoA from acetyl-CoA. We hypothesized that the use of core and termination enzymes of overlapping chain-length specificities would favor the synthesis of products of certain chain lengths, such as 1-decanol.

Of the 8 ACRs tested, only MhpF couldn't produce 1-decanol with all displaying different levels of activity and specificity for 1-decanol production (Fig. 3). The results showed that Maqu2507 (ACR from *Marinobacter aquaeolei* VT8) (Willis et al., 2011) and Ald (aldehyde dehydrogenase from *Clostridium beijerinckii*) produced the highest amount of total C6–C12 alcohols. In addition, the main alcohol product by Maqu2507 was 1-decanol, while the main alcohol produced by Ald was 1-octanol, suggesting that Maqu2507 has a higher specificity towards decanoyl-CoA (Fig. 3). The strain CJ064 carrying Maqu2507 as termination enzyme produced about 700 mg/L 1-decanol, representing about 60% of the total C6–C12 alcohols and acids produced. Beyond that, the lowest titer fatty-acids by-product served as an important advantage of using Maqu2507.

To investigate the effect of different inducer concentrations on the expression of rBOX and termination enzymes, IPTG was varied between 10 and 30 μM. We observed a significant increase in total C6–C12 fatty alcohols and fatty acids for most tested ACR variants when the IPTG concentration changed from 10 to 20 μM, but no significant increase at 30 μM IPTG. (Fig. 3). Fatty alcohol and fatty acid production with EutE was very low and no significant impact varying IPTG concentrations was observed, suggesting that it may have low specificity and activity towards C6–C12 acyl-CoAs (Fig. 3). A similar behavior was observed with AcrM. A unique trend was observed for fatty alcohol production with Ald, as fatty alcohol titer decreased along with the increase in IPTG concentration from 10 to 30 μM, suggesting that a lower expression level could be beneficial.





Vector\Strain	CJ091 (CJ065, Δ*yigI*)	CJ083 (CJ065, Δ*yigI*, Δ*pflB*)	CJ118 (CJ065, Δ*yigI*, Δ*pflB*, *pM1-93-R1-aceEF*)
pET	P1-BktB-P2-FadB		
pCDF	P1-maqu_2507-P2-tdTER		

Fig. 5. Improving 1-decanol production by favoring pyruvate dissimilation via the pyruvate dehydrogenase complex (PDHC). (A) Generation and consumption of reducing equivalents during conversion of glycerol to 1-decanol. Pyruvate dissimilation via PDHC favors generation of NADH when compared to pyruvate formate-lyase (PFL). (B) Effects of PFL deletion and PDHC overexpression on 1-decanol production. Concentrations are shown for biological replicates (n = 3) of 48-hour fermentations with error bars representing standard deviation.

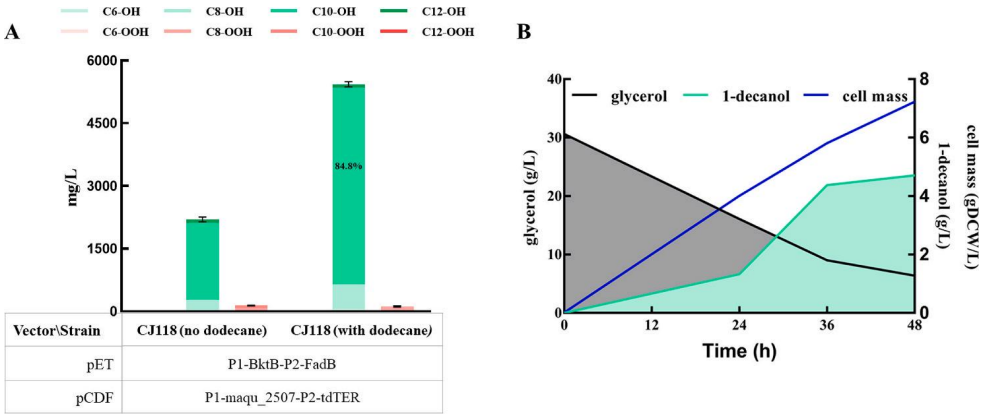


Fig. 6. Effect of organic solvents on 1-decanol production. (A) Effect of dodecane (15% v/v) layer on product titer in strain CJ118. (B) Time course of a representative fermentation of strain CJ118 with a 15% dodecane layer. Concentrations are shown for biological replicates (n = 3) of 48-hour fermentations with error bars representing standard deviation.

2.2. Increasing pathway flux and decreasing byproduct synthesis

Having constructed a plasmid-based 1-decanol producing strain, we set out to further optimize the entire pathway for improving 1-decanol titer and selectivity. Given the importance of tdTER/trans-enoyl-CoA reductase catalyzing the irreversible step in the rBOX cycle, we hypothesized that its location behind *EcfadB* rather than directly under the control of the *T7* promoter may reduce its expression level and the flux through the entire pathway. Two new vectors were constructed to evaluate the impact of increasing *tdTER* expression, the first vector contained *maqu2507* and *tdTER*, with each gene driven by its own *T7* promoter, and a second vector containing *ReBktB* and *EcfadB*, both under the control of a single *T7* promoter. Strain JST07 carrying these two new vectors is referred to as CJ065 (Fig. 4A). Consistent with our hypothesis, better *tdTER* expression using this approach resulted in a 2-fold increase in 1-decanol titers from 700 mg/L to 1350 mg/L (Fig. 4A).

While the 1-decanol titer achieved by CJ065 is 2.7-fold higher than the best reported in the literature, 1-decanol selectivity was limited due to synthesis of several byproducts, especially decanoic acid which compete for the decanoyl-CoA pool and compromise 1-decanol yield. Since strain CJ065 produced over 200 mg/L decanoic acid and a recent study identified YigI as a thioesterase responsible for decanoic acid production in *E. coli* (Schmidt et al., 2022), we knocked out *yigI* in strain CJ065. The resulting strain, named CJ091, displayed a 30% decrease in decanoic acid and 10% increase in 1-decanol titer when compared to CJ065 (Fig. 4B).

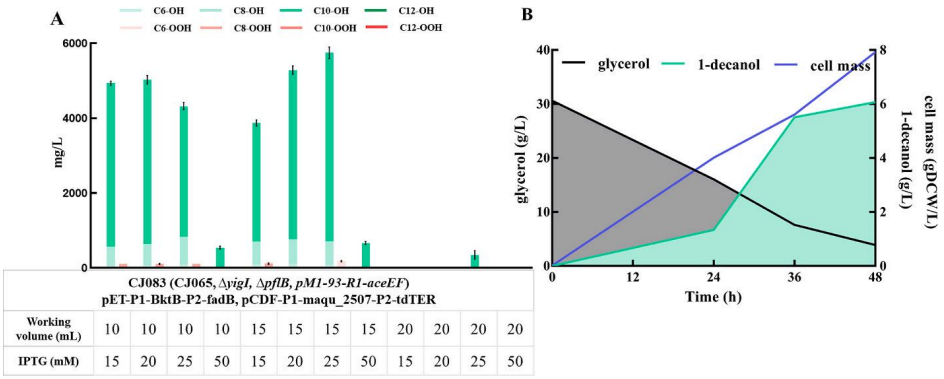
Despite the above improvements, about 10 g/L glycerol remained unmetabolized in the medium hence limiting the achievable 1-decanol titer. Given the high demand of reducing equivalents for the synthesis of 1-decanol (8 NADH and 2 NADPH per molecule of 1-decanol), we evaluated the impact of increasing NADH supply on 1-decanol production. To this end, we knocked out the *pflB* gene in strain CJ091, to ensure conversion of pyruvate into acetyl-CoA through PDH, thus generating additional NADH (Fig. 5A). The resulted strain named CJ083 produced 1478 mg/L decanol, which represents no significant increase compared with strain CJ091. Given that the OD of 1-decanol strain was over 20 in the late fermentation period, the fermentation condition was close to microaerobic or anaerobic. This condition is expected to generate a high NADH/NAD^+ ratio that would inhibit PDH activity (Kim et al., 2008), thus limiting acetyl-CoA generation and hence operation of the rBOX. In previous reports, the activity of PDH under anaerobic conditions has been improved by increasing the expression of *aceEF* (Zhou et al., 2008, 2010). Therefore, we overexpressed *aceEF* by using the *M1-93* promoter and a specific RBS sequence to obtain a high PDH activity under microaerobic conditions (the late period of fermentation). According to the results, the deletion of *pflB* in combination with overexpression of *aceEF* resulted in 40% improvement in decanol titer to 1900 mg/L (Fig. 5B). These results proved that the additional NADH supply can be an effective method to increase the titer of products whose synthesis require multiple turns of the rBOX cycle.

2.3. Biphasic fermentation with dodecane overlay

Given the toxicity of medium-chain fatty alcohols to *E. coli* and the low solubility of 1-decanol (37 mg/L at 20 °C), we hypothesized that product inhibition and transport issues could be limiting 1-decanol production. This was partially supported by the observation of a non-aqueous phase/layer after 48 h of fermentation in strains CJ083/CJ091/CJ118, which we speculated corresponds to insoluble fatty alcohols. In previous reports, the inclusion of an organic phase, like dodecane, has been shown to improve the production of decanoic acid and other low-solubility fatty acids/alcohols (Kim and Gonzalez, 2018; Vogeli et al., 2022). To explore if the addition of an organic phase can improve 1-decanol separation, we designed a biphasic fermentation using a 15% v/v dodecane overlay. Organic overlay fermentations were carried out and analyzed alongside the standard fermentation conditions controls (see Materials and Methods). When cultivated in the presence of a 15% v/v n-dodecane overlay, strain CJ118 produced 1-decanol at a level of 4.7 g/L (2.5-fold increase compared to no organic phase), which was also accompanied by an approximately 1.5-fold increase in cell growth and glycerol utilization (Fig. 6A). The inclusion of an organic phase significantly decreased the synthesis of byproducts as well (Fig. 6B). The fermentation profile of this strain shows that during the first 24 h it consumed 15 g/L of glycerol and produced approximately 1300 mg/L of 1-decanol (Fig. 6B). According to the fermentation results, we also observed that strain CJ118 produced 4400 mg/L 1-decanol with a yield of 0.21 g/g in 36 h, meaning CJ118 had an extremely high decanol productivity during the 24–36 h. After 48 h fermentation, strain CJ118 produced 4707 mg/L 1-decanol with a yield of 0.20 g/g and a selectivity of 85%, suggesting the productivity and yield of 1-decanol decreased gradually with the increase in fermentation time after 36 h (Fig. 6B).

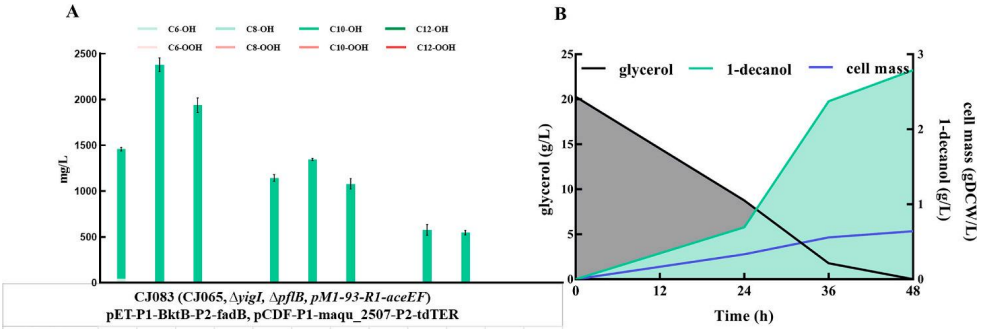
2.4. Evaluating the effect of fermentation conditions on product profile

Following the establishment of the best combination of enzymatic components and achieving the optimal pathway for 1-decanol production, we attempted to further improve product synthesis in strain CJ118 by evaluating different fermentation conditions. This included an assessment of the impact of working volumes and inducer concentrations on 1-decanol production. Prior reports showed that excessive IPTG addition can be toxic to *E. coli* cells and will cause formation of inclusion bodies due to excessive protein synthesis, resulting in inhibition of enzymatic activities and thus decreased product synthesis (Baneyx,



Working volume (mL)	10	10	10	10	15	15	15	15	20	20	20	20
IPTG (mM)	15	20	25	50	15	20	25	50	15	20	25	50

Fig. 7. Effect of different fermentation conditions on 1-decanol production in rich medium. (A) Effect of IPTG concentrations (15/20/25/50 μM) and working volumes (10/15/20 mL). (B) Representative time course for a strain CJ118 fermentation under optimal IPTG concentration (25 μM) and working volume (15 mL). Concentrations are shown for biological replicates (n = 3) of 36-hour fermentations with error bars representing standard deviation.



Working volume (mL)	10	10	10	10	15	15	15	15	20	20	20	20
IPTG (mM)	15	20	25	50	15	20	25	50	15	20	25	50

Fig. 8. Effect of different fermentation conditions on 1-decanol production in minimal medium. (A) Effect of IPTG concentrations (15/20/25/50 μM) and working volumes (10/15/20 mL). (B) Representative time course for a strain CJ118 fermentation under optimal IPTG concentration (20 μM) and working volume (10 mL). Concentrations are shown for biological replicates (n = 3) of 36-hour fermentations with error bars representing standard deviation.

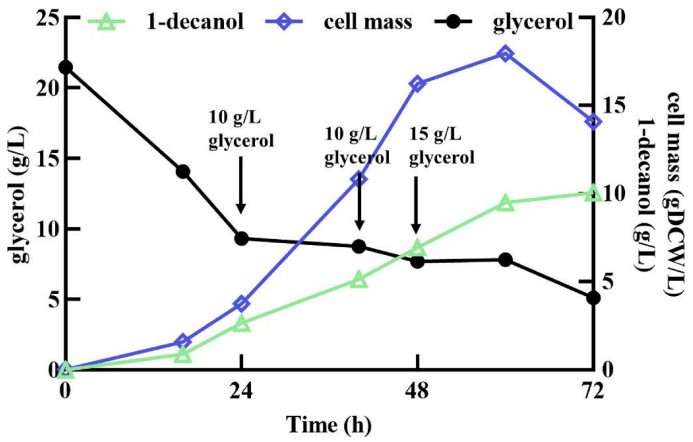


Fig. 9. 1-Decanol fermentation in bioreactor with controlled conditions. The fermentation was performed using 0.3 L LB + MOPS medium with 20 g/L glycerol in a 0.5 L bioreactor. Cultures were grown at 37 °C with an initial OD550 of 0.1, 25 μM IPTG and 15% (v/v) dodecane was added when OD550 reached 0.4−0.8. Furthermore, 10, 10 and 15 g/L glycerol were added at 24, 40 and 48 h, respectively. The pH was maintained at 7.0 by using 5 M NaOH and the dissolved oxygen level was also monitored.

1999). To this end, strain CJ118 was cultured under various working volumes and IPTG concentrations. The results showed that both the working volume and IPTG concentration had great effect, causing a 5-fold difference on 1-decanol production. When strain CJ118 was cultured under the optimal conditions (15 mL working volume and 25 μM IPTG), it produced over 6000 mg/L of 1-decanol in 48 h at a yield of 0.26 g/g (Fig. 7).

To further explore 1-decanol production by this strain, we used glycerol minimal medium, which is desirable due to lower cost of medium components and simpler downstream processing. Strain CJ118 produced 2788 mg/L of 1-decanol in minimal medium in 48 h at a yield of 0.14 g/g under the optimal conditions (10 mL working volume and 20 μM IPTG). Remarkably, no other C6–C12 fatty acids or fatty alcohols was produced, thus resulting in 1-decanol production at an unprecedented purity and selectivity (Fig. 8). Notably, the per cell mass yield of 1-decanol in minimal medium was 992 mg/gDCW which was 30% higher than in rich medium, indicating that strain CJ118 has a competitive 1-decanol production capacity in minimal medium. Our strategy of 1-decanol production succeeded in minimal medium as well, indicating great potential for industrial application.

2.5. 1-Decanol fed-batch fermentation in bioreactor

To demonstrate the scalability and potential higher titers of 1-decanol, we conducted a fed-batch fermentation in a 500 ml bioreactor with parameter control using engineered strain CJ118 and the identified optimal fermentation conditions (Fig. 9). The dissolved oxygen was controlled at 40% by air flow with the culture reaching a maximum OD_{550} of approximately 50 (corresponding cell mass ~ 16 gDCW/L). Glycerol was added at different times during the fermentation process to maintain its concentration at around 10 g/L. During the first 24 h, strain CJ118 consumed 12 g/L of glycerol and produced approximately 2 g/L of 1-decanol. The highest 1-decanol productivity was about 0.2 g/L/h, obtained during the 24–60 h. After 60 h, the OD_{550} decreased

Table 2
Strains and plasmids used in this study.

Plasmids/Strains	Genetic characteristics	Source
Plasmids		
pCDFDuet-1	pBR322 origin with T7 promoter, Sm^R	Novagen
pETDuet-1	pBR322 origin with T7 promoter, Amp^R	Novagen
pCDF-P1-maqu_2507	pCDFDuet-1 carrying *maqu_2507* from *Marinobacter auqaeolei*	This study
pCDF-P1-maqu_2220	pCDFDuet-1 carrying *maqu_2220* from *Marinobacter auqaeolei*	This study
pCDF-P1-adhE	pCDFDuet-1 carrying *adhE* from *Clostridium acetobutylicum*	This study
pCDF-P1-eutE	pCDFDuet-1 carrying *eutE* from *Salmonella typhimurium*	This study
pCDF-P1-mhpF	pCDFDuet-1 carrying *mhpF* from *E. coli*	This study
pCDF-P1-acrM	pCDFDuet-1 carrying *acrM* from *Acinetobacter* sp.	This study
pCDF-P1-acr1	pCDFDuet-1 carrying *acr1* from *Acinetobacter calcoaceticus*	This study
pCDF-P1-ald	pCDFDuet-1 carrying *ald* from *Clostridium beijerinckii*	This study
pCDF-P1-maqu_2507-p2-tdTER	pCDFDuet-1 carrying *maqu_2507* from *Marinobacter auqaeolei* and *TER* from *Treponema denticola*	This study
pETDuet-P1-BktB-P2-fadB-tdTER	pETDuet-1 carrying *BktB* from *Ralstonia eutropha*, *fadB* from *E. coli* and *TER* from *Treponema denticola*	This study
pETDuet-P1-BktB-P2-fadB	pETDuet-1 carrying *BktB* from *Ralstonia eutropha* and *fadB* from *E. coli*	This study
***E. coli* strains**		
JC01	MG1655, ΔldhA::FRT ΔpoxB::FRT Δpta::FRT ΔadhE::FRT ΔfrdA::FRT	Clomburg et al. (2012)
JST07	JC01 ΔyciA::FRT ΔybgC::FRT ΔydiI::FRT ΔtesA::FRT ΔfadM::FRT ΔtesB::FRT ΔfadE::FRT	Kim et al. (2015)
Maqu2507	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-maqu_2507	This study
Maqu2220	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-maqu_2220	This study
AdhE	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-adhE	This study
eutE	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-eutE	This study
mhpF	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-mhpF	This study
acrM	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-acrM	This study
acr1	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-acr1	This study
Ald	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-ald	This study
CJ064	JST07 with pETDuet-P1-BktB-P2-fadB-tdTER and pCDF-P1-maqu_2507	This study
CJ065	JST07 with pETDuet-P1-BktB-P2-fadB and pCDF-P1-maqu_2507-P2-tdTER	This study
CJ091	CJ065, *ΔyigI*	This study
CJ083	CJ065, *ΔyigI, ΔpflB*	This study
CJ118	CJ065, *ΔyigI, ΔpflB*, pM1-93-RBS::*aceEF*	This study

significantly and the titer of 1-decanol increased very slowly. Finally, CJ118 produced 10.05 g/L of 1-decanol in 72 h at a yield of 0.2 g/g. To our knowledge, this is the highest 1-decanol titer achieved in any wild type or engineered microorganism.

3. Conclusions

Given the high value of fatty alcohols and fatty acids, their production through carbon elongation pathways such as fatty acid biosynthesis (FAB) and the engineered β-oxidation reversal (rBOX) has been thoroughly investigated. The iterative nature of these pathways, however, often results in a mixture of products with various chain lengths as opposed to the selective synthesis of a product with the chain length of interest. To overcome this challenge and achieve the selective production of 1-decanol, we used rBOX enzymes able to generate acyl-CoA thioesters up to the target chain lengths (C10) and screened for termination enzymes with chain-length specificities overlapping with the selected rBOX enzymes.

Using this approach, we selected thiolase BktB, 3-hydroxyacyl-CoA dehydrogenase & 3-hydroxyacyl-CoA dehydratase FadB and enoyl-CoA reductase tdTER as the rBOX enzymes and identified a bifunctional ACR Maqu2507 from *Marinobacter aquaeolei VT8* as the termination enzyme. Through the optimization of rBOX enzyme expression, reduction of by-products and improvement of NADH supply, the 1-decanol titer was significantly improved. In addition, a biphasic fermentation strategy was employed by using dodecane as the extraction agent for in-situ 1-decanol removal. This avoided 1-decanol toxicity and increasing its transport thus achieving a 2.5-fold increase in titer. Finally, upon optimization of working volume and inducer concentration, 1-decanol production reached 6070 mg/L at a yield of 0.26 g/g with the 1-decanol specificity of 91% in rich medium. Remarkably, the use of minimal medium resulted in production of 2788 mg/L 1-decanol at 100% specificity and a per cell mass yield higher than rich medium. We also used 500 mL bioreactor to scale up the fermentation and obtained a higher 1-decanol titer of 10.05 g/L in 72 h. These are the highest 1-decanol titers, yields and purity reported to date. Taken together, our work suggests that combination of rBOX pathway and proper termination enzyme should be an effective approach for selectively producing fatty acids or fatty alcohols. While 1-decanol production with a combination of rBOX pathway and proper termination enzyme approach was demonstrated here, further identification and characterization of both thiolases and termination enzymes can lead to improved titer, yield, selectivity, and productivity of 1-decanol production. In this context, fine-tunning the expression of core rBOX enzymes as well as protein engineering of rate-limiting enzymes could be instrumental as well.

4. Materials and methods

4.1. Strains, plasmids and genetic methods

All strains used in this study are listed in Table 2. *E. coli* JST07 (DE3), was employed as the host strain. Plasmid based gene overexpression was achieved by cloning the desired gene(s) into either pETDuet-1 or pCDFDuet-1 (Novagen, Darmstadt, Germany) digested with appropriate restriction enzymes utilizing In-Fusion PCR cloning technology (Clontech Laboratories, Inc., Mountain View, CA). Cloning inserts were created via PCR of ORFs of interest from their respective genomic or codon-optimized DNA with Phusion polymerase (Thermo Scientific, Waltham, MA). The resulting In-Fusion products were used to transform *E. coli* Stellar cells (Clontech Laboratories, Inc., Mountain View, CA) and PCR identified clones were confirmed by DNA sequencing. All resulting plasmids used in this study are listed in Table 2 and the primers used in their construction are listed in Table 3.

The deletion of *pflB* gene and overexpression of *aceEF* gene were created using a CRISPR-Cas9 based system developed for *E. coli*. Plasmids pCas and pTargetF were gifts from S. Yang (Addgene plasmids nos. 62225 and 62226, respectively). Strains used in this study are listed in Table 2 and the primers used in their construction are listed in Table 3. For the overexpression of *aceEF*, the native promoter of *aceEF* gene was replaced by *M1-93* promoter with a particular RBS sequence.

4.2. Culture medium and cultivation conditions

Luria−Bertani (LB) medium was used for culturing *E. coli* cells for plasmid construction. The "LB-like" MOPS medium used for JST07 (DE3) strains contains 125 mM MOPS, supplemented with 20 g/L glycerol (or 30 g/L), 10 g/L tryptone, 5 g/L yeast extract, 2.78 mM Na_2HPO_4, 5 mM $(NH_4)_2SO_4$, 30 mM NH_4Cl, 100 μM $FeSO_4$. When necessary, ampicillin, spectinomycin, kanamycin and chloramphenicol were added at final concentrations of 100, 50, 50 and 34 mg/L, respectively. Minimal medium fermentations utilized the same medium

Table 3
Primers used in this study.

Primers	Sequence	Source
Maqu2507-up-Nco1	AGGAGATATACCATGAACTACTTTCTGACCGGT	This study
Maqu2507-down-EcoR1	GCCGAGCTCGAATTCGTTACCAGTAAATGCCACGCA	This study
Maqu2220-up-Nco1	AGGAGATATACCATG	This study
Maqu2220-down-EcoR1	GCCGAGCTCGAATTCG	This study
adhE-up-Nco1	AGGAGATATACCatgAAAGTTACAAATCAAAAAGAACTAAAACA	This study
adhE-down-EcoR1	GCCGAGCTCGAATTCGttaAAATGATTTTATATAGATATCCTTAAGTTCACTTA	This study
eutE-up-Nco1	AGGAGATATACCatgAATCAACAGGATATTGAACAGG	This study
eutE-down-EcoR1	GCCGAGCTCGAATTCGttaaACAATGCGAAACGCAT	This study
mhpF-up-Nco1	AGGAGATATACCATGatgAGTAAGCGTAAAGTCGC	This study
mhpF-down-EcoR1	GCCGAGCTCGAATTCGtcaTGCCGCTTCTCCTGC	This study
acrM-up-Nco1	AGGAGATATACCatgaacgccaaactgaaaaaac	This study
acrM-down-EcoR1	GCCGAGCTCGAATTCGtcaccaatgttcacccgga	This study
acr1-up-Nco1	AGGAGATATACCatgaataaaaaattggaagcattatttagaga	This study
acr1-down-EcoR1	GCCGAGCTCGAATTCGttaccaatgctctccaggg	This study
ald-up-Nco1	AGGAGATATACCATGAATAAAGACACACTAATACCTACAAC	This study
ald-down-EcoR1	GCCGAGCTCGAATTCGTTAGCCGGCAAGTACACAT	This study
BktB-up-Nco1	AGGAGATATACCATGACGCGTGAAGTGGTA	This study
BktB-down-EcoR1	GCCGAGCTCGAATTCGTCAGATACGCTCGAAGATGG	This study
tdTER-up-Nde1	GAAGGAGATATACATATGATTGTTAAGCCGATGGT	This study
tdTER-down-EcoRV	TGGCCGGCCGATATCTTAGATGCGGTCAAAACGTT	This study
fadB-up-Nde1	GAAGGAGATATACATATGCTTTACAAAGGCGACAC	This study
aceE-up-homo-arm-F	CAGAACTTCGAATTGCTCTATTCG	This study
aceE-up-homo-arm-R	GGGTTATTCCTTATCTATCTAATAACGTTG	This study
M1-93-RBS-oL-aceE-R	ATTTGGGAAACGTTCTGACATGGTGTGCCTCCTGGTTTAAACGTACATGC	This study
aceE-up-F	ATGTCAGAACGTTTCCCAAAT	This study
aceE-inner-R-366	AAAGCACACATCATAAATGGTTG	This study
aceE-inner-R-507	TTCCTGACGGAAGTTATCCAG	This study

described above without the addition of rich nutrients (tryptone and yeast extract).

Fermentations for 1-decanol production were conducted in 25 mL flasks. 25 mL Pyrex Erlenmeyer flasks (Corning Inc., Corning, NY) were filled with 10 mL of the above culture medium (or otherwise specified working volume) and sealed with foam plugs. Overnight precultures in LB medium with appropriate antibiotics were used as the inoculum into the fermentation medium with initial OD_{550} of 0.05. After inoculation, flasks were incubated at 37 °C and 200 rpm until an optical density of 0.3–0.5 was reached, at which point IPTG at specified concentration (and n-15% v/v dodecane for biphasic fermentations) were added. Flasks were then incubated under the same conditions for 48 h post-induction unless otherwise stated.

Fed batch fermentations for 1-decanol production were conducted in a 500 mL bioreactor (Infors) at 37 °C using 300 mL LB + MOPS medium with 20 g/L glycerol. An overnight seed culture was used to inoculate the bioreactor to an of $OD_{550} \sim 0.1$ and when the OD_{550} reached 0.4−0.8, 25 μM IPTG and 15% (v/v) dodecane were added. 10, 10 and 15 g/L glycerol were added at 24, 40 and 48 h, respectively. The pH was maintained at 7.0 by using 3 M sodium hydroxide (NaOH) as base solution. The air flow rate was set at 50 mL/min, stirring speed was set at 500 rpm. The dissolved oxygen (DO) level was set at 40% during the whole fermentation period.

4.3. Extraction of fatty alcohols and fatty acids

For aqueous-phase fermentations, 2 mL samples from shake flasks were transferred to 5 mL glass vials. A total of 50 mg/L of heptanol was added as an internal standard and 2 mL of hexane-MTBE (1:1) was added for extraction. The bottles were sealed with Teflon-lined septa (Fisher Scientific, Pittsburg, PA), secured with caps, and vigorously vortexed. The samples were then centrifuged for 2 min at 2375×*g* to separate the aqueous and organic layers. After centrifugation, 1 mL of the top organic layer was transferred from the upper organic layer to GC vials (Fisher Scientific) for GC analysis. After transferring, 50 μL of pyridine and 50 μL of BSTFA (N,O-bis[trimethylsilyl]trifluoroacetamide) were added and the mixture were incubated at 70 °C for 1 h. Derivatized samples were transferred to GC vials for GC analysis.

For two-phase fermentations, the samples were firstly centrifuged and separated into two parts upper/organic layer and lower/aqueous layer. The extracting method of aqueous layer was the same as above description. The upper organic phase was collected and transferred 20 μL to GC vials (Fisher Scientific), the samples were next diluted into 1 mL of hexane-MTBE (1:1) with 50 mg/L of heptanol as an internal standard. After vortexing, 50 μL of pyridine and 50 μL of BSTFA (N,O-bis [trimethylsilyl]trifluoroacetamide) were added and the mixture were incubated at 70 °C for 1 h. Derivatized samples were transferred to GC vials for GC analysis.

4.4. Analysis

Optical density (OD) was measured at 550 nm using a Thermo Spectronic Genesys 20 (Thermo Scientific, Waltham, MA) and used as an estimate of cell mass (1 O.D.550 nm = 0.34 g dry weight/L). The concentrations of glycerol, ethanol and organic acids were determined via ion-exclusion HPLC using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a refractive index detector and an HPX-87H organic acid column (Bio-Rad, Hercules, CA) with operating conditions to optimize peak separation (0.3 mL/min flowrate, 30 mM H_2SO_4 mobile phase, column temperature 42 °C). Quantification of C6–C12 fatty acids and fatty alcohols were conducted via GC-FID analysis using an Agilent 7890 B gas chromatograph equipped with an Agilent 5977 mass spectroscope detector (Agilent) and an HP-5 ms capillary column (0.25 mm internal diameter, 0.25 μm film thickness, 30 m length; Agilent). A total of 1 μL was injected into the GC at a 4:1 split ratio using helium as the carrier gas with a flow rate of 1.5 mL/min. The injector and detector temperature were 250 and 350 °C, respectively. The oven temperature was initially held at 50 °C for 3 min and then raised to 270 °C at 20 °C min^{-1} and held for 6 min.

Author contributions

R.G. conceptualized the research and supervised the project. J.C. and R.G. designed the methodology. J.C. performed experiments. J.C. analyzed the data. J.C. and R.G. prepared the manuscript.

Declaration of competing interest

R.G. is the sole proprietor of RBN Biotech LLC, which holds rights to several r-BOX patents. All other authors declare no competing interests.

Data availability

Data will be made available on request.

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