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Review

Engineering for life in toxicity: Key to industrializing microbial synthesis of high energy density fuels

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ABSTRACT

With the growing demand for air transportation combined with global concerns about environmental issues and the instability and lack of renewability of the oil market, microbial production of high energy density fuels for jets (bio-jet fuels) has received more attention in recent years. Bio-jet fuels can be derived from both isoprenoids and fatty acids, and, additionally, aromatic hydrocarbons derived from expanded shikimate pathways are also candidates for jet fuels. Compared to fatty acid derivatives, most of isoprenoids and aromatic hydrocarbons used for jet fuels have higher density energies. However, they are also highly toxic to host microbes. The cytotoxicity induced during the synthesis of isoprenoid or shikimate pathway-derived biofuels remains one of the major obstacles for industrial production even though synthetic and systems biology approaches have reconstructed and optimized metabolic pathways for production of these bio-jet fuels. Here, we review recent developments in the production of known and potential jet fuels by microorganisms, with a focus on alleviating cytotoxicity caused by the final products, intermediates, and metabolic pathways.

1. Introduction

Air transportation is a massive global business supporting one third of global trade by value in 2018 [1]. Similarly, the jet fuel market consisted of 106 billion gallons in 2019 and is projected to increase to 230 billion gallons in 2050 [2]. However, the combustion of jet fuel produces a large amount of CO_2, which accounts for about 2–3% of global carbon emissions and contributes to climate change [3]. The aviation industry aims to reduce net carbon emissions by half of the quantities in 2005 by the year 2050 [4]. Alternative jet fuels produced by microorganisms is a critical part of decoupling economic growth from carbon emissions and is crucial for environmental, energy, and economic security [5–7]. Jet fuel is a mixture of C8–C16 hydrocarbons of linear and branched alkanes and cycloalkanes at less than 25% (v/v) of aromatic compounds. It is the same fuel designed for gas turbines and has a high energy density and low freezing point performance (Table 1), both of which are primary jet fuel performance properties [8,9]. Biosynthetic alternative jet fuels must meet the standard specifications for civilian aviation in regards to energy content, low freezing point, and combustion quality [10,11]. Potential bio-jet fuels that could supplement or replace conventional jet fuels include isoprenoid and fatty acid-based biofuels, as well as some aromatics derived from expanded shikimate pathways (Fig. 1).

The monoterpenes (C10) and sesquiterpenes (C15) derived from isoprenoids are most suitable candidates for high-performance jet fuels for two reasons: 1) their carbon number is consistent with the carbon distribution of conventional jet fuel and 2) they have a comparable high energy density (Table 1). Monoterpenes (C10) and sesquiterpenes (C15) derived from isoprenoids are composed of C5 isoprenoid units, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), and synthesized via both the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Fig. 1). Additionally, farnesane, hydrogenated farnesene, has been approved to be used in a 10% blend with petroleum-derived jet fuel, but not used in fuel application due to the cost of production [2]. Moreover, medium and long chain fatty acid-derived compounds including fatty acid ethyl esters (FAEEs), fatty alcohols and alkanes are used as jet fuels (Fig. 1). Likewise, HEFA, hydroprocessed esters and fatty acids, was approved to be used in an up to a 50% blend with petroleum-derived jet fuel [2]. In addition, some aromatic compounds derived from expanded shikimate pathways, such as aromatic amino acid biosynthesis pathway, are excellent high-density fuel precursors. For example, 2-phenylethanol (Table 1) has relatively high energy density of 37.32 MJ/L. Most of isoprenoids and aromatics used for jet fuels have higher energy densities than fatty acid-derived compounds (Table 1). However, these compounds are also highly toxic to host organisms compared with fatty acid-derived compounds [19], which hinders their development and industrial production. Therefore, in this review, we focus on the synthesis of jet fuel replacements derived from isoprenoids and aromatic compounds in engineered microorgan-

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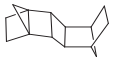
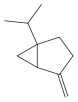
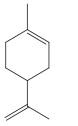
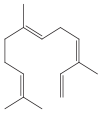
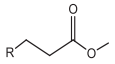
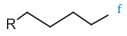
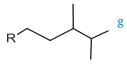
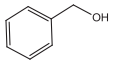
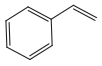
Table 1
Physical properties of conventional and biosynthetic alternative bio-jet fuels.

	Density (kg/L)[a]	Freezing point (°C)[b]	Flash point (°C)[c]	Energy density (MJ/L)	Blend ratio(%)[e]	Structure	Molecular family	Ref.
Jet A	0.775-0.840	-40	51.1	35.3				[10]
Jet A-1	0.775-0.840	-47	42.0	34.7				[10]
JP-8	0.775-0.840	-47	38.0	34.8				[11]
JP-9	0.935-0.955	-54	21.0	39.6				[11]
JP-10	0.935-0.943	-79	55.0	39.4			cyclo-alkanes	[11]
RJ-5	1.08	0	_	44.9			cyclo-alkanes	[12]
Sabinene	0.842	-40	37.0	36.25			cyclo-alkanes	[13]
α-Pinene	0.858	-60	32.0	37.03			cyclo-alkanes	[13]
β-Pinene	0.860	-60	34	37.43			cyclo-alkanes	[13]
Limonene	0.842	-40	48.3	36.10			cyclo-alkanes	[13]
Farnesene	0.844-0.879	-90	43	36.0[d]	10		iso-alkanes	[2,14]
FAME	_	_	78.9	32.6	5		*n*-alkanes	[2,15]
HEFA	0.762	-40	38	33.3	50	 [f]	*n*-alkanes	[2,16]
Bio-SPK	0.749	-57	46.5	33.2	10	 [g]	iso-alkanes	[2,17]
2-Phenylethanol	1.020	_	102	37.32			alkyl aromatics	[18]
Styrene	0.906	_	31	_			alkyl aromatics	
Phenol	1.071	_	79	_			alkyl aromatics	

FAME, fatty acid methyl ester; HEFA, hydroprocessed esters and fatty acids; Bio-SPK, Biologically derived synthetic paraffinic kerosene.
[a] The density at 15°C of conventional jet fuels, HEFA and Bio-SPK fuels. The density at 25°C of terpenes and aromatics and the data are from https://www.chemicalbook.com/.
[b] The freezing point is below the indicated temperature.
[c] The flash point of terpenes and aromatics are from https://www.chemicalbook.com/.
[d] The value is approximately 36.0 MJ/L in the reference paper.
[e] The blending components are hydrogenated products.
[f] Hydroprocessed esters and fatty acids leading to the *n*-alkane products.
[g] The Bio-SPK fuel is a mixture consisting of ~90% iso-alkanes and ~10% (wt%) n-alkanes.

isms as a means to produce high quality renewable jet fuel with high yield and quality when toxicity tolerance has been engineered.

Substantial research on the synthesis of the compounds used as bio-jet fuels has been performed and various metabolic engineering strategies and synthetic biology approaches have been developed to increase the titres, productivities, and yields (TPY) of products in engineered bacteria and fungi, including *Escherichia coli* and *Saccharomyces cerevisiae*. The sesquiterpene farnesene (C15) titre reached 130 g/L in engineered *S. cerevisiae* at industrial fermentation scales [20]. Recently, aromatic amino acids (L-phenylalanine, L-tyrosine, and L-tryptophan) derived from the expanded shikimate pathway were efficiently produced in *E. coli* with various metabolic engineering strategies [21–23]. These high production rates of farnesene or aromatic amino acids indicates that the isoprenoids or aromatics produced via the MVA pathway or shikimate pathways are capable of high metabolic flux. However, some isoprenoid compounds, especially monoterpenes, as well as compounds derived from aromatic amino acids are still synthesized at low levels with low efficiency in production. Furthermore, although these compounds are produced via different metabolic pathways, the microbial synthesis of bio-jet fuels has shared challenges, in particular the high cytotoxicity of target products is the major obstacle in achieving high-titre production in engineered microbes. Therefore, addressing cytotoxicity is a critical challenge for achieving the large-scale production of bio-jet fuels.

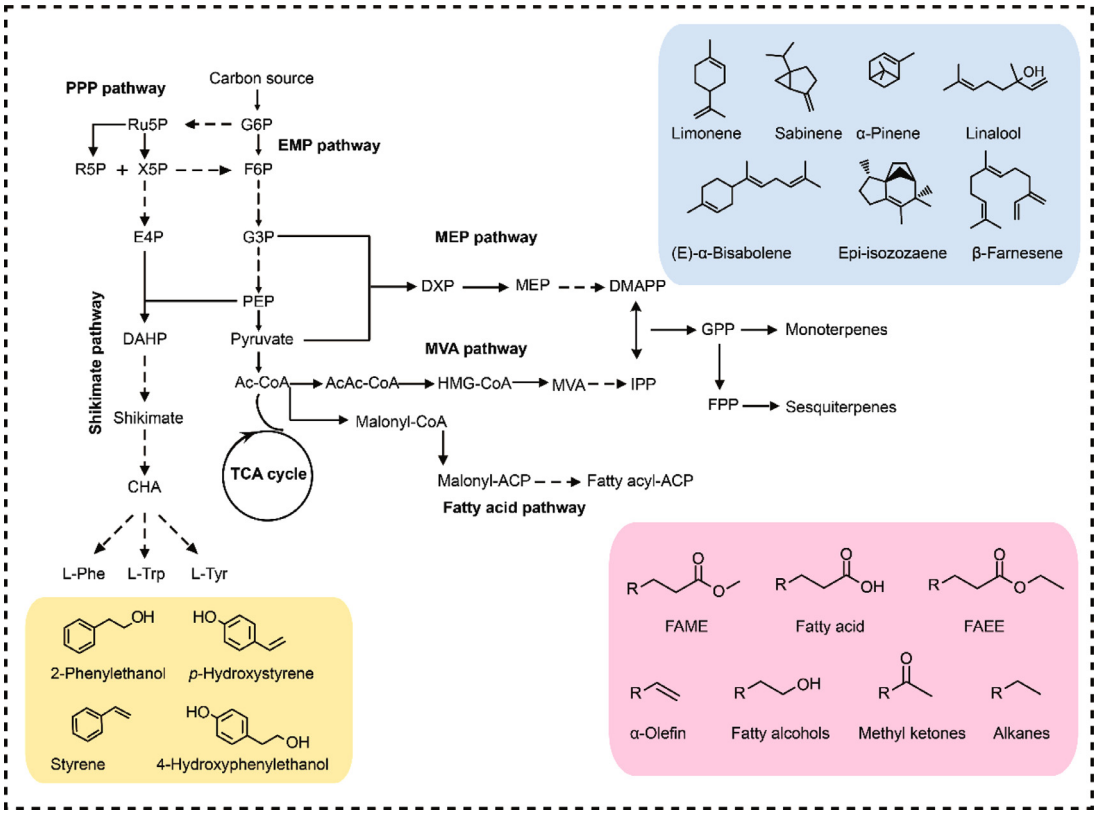


Fig. 1. Metabolic pathways of bio-jet fuels production. Metabolic pathway abbreviations, pathway names: EMP pathway, Embden-Meyerhof-Parnas pathway, also known as glycolysis; PPP pathway, Pentose phosphate pathway; MVA pathway, Mevalonate pathway; MEP pathway, Methylerythritol phosphate pathway; TCA cycle, Tricarboxylic acid cycle. Metabolite abbreviations: Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; CHA, chorismate; L-Phe, L-phenylalanine; L-Trp, L-tryptophan; L-Tyr, L-tyrosine; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; Ac-CoA, acetyl-CoA; DXP, 1-Deoxy-D-xylulose-5-phosphate; DMAPP, Dimethylallyl diphosphate; IPP, Isopentenyl diphosphate; GPP, Geranyl diphosphate; FPP, Farnesyl diphosphate; AcAc-CoA, Acetoacetyl-CoA; HMG-CoA, 3-Hydroxy-3-methylglutaryl-CoA; ACP, acyl carrier protein; FAME, fatty acid methyl ester; and FAEE, fatty acid ethyl ester. Dashed arrows indicate multiple steps while solid arrows indicate one single step.

In this paper, we aim to provide an overview of toxicity engineering which can be used in bio-jet fuels production with a focus on recent advances in toxicity engineering strategies and tools to improve the TPYs of target products. We discuss the modular and dynamic control of pathways, compartmentalization, efflux pumps, tolerance engineering, and fermentation control to alleviate the cytotoxicity caused by metabolic pathways, intermediates, and final products (Table 2). Additionally, related toxicity engineering strategies, including not only strategies used in the production of bio-jet fuels but also novel and promising approaches from other biofuel and chemical syntheses, were also evaluated. Furthermore, the challenges and perspectives for future bio-jet fuel production are also discussed.

2. Toxicity engineering

Most of the compounds used as jet fuels are highly cytotoxic to the host microbe, such as sabinene, pinene, limonene, linalool, aromatics 2-PE, and styrene (Table 3), and the accumulation of final products often inhibit host growth and limit the overall titre of target compounds [6,19]. Moreover, the intermediates of the metabolic pathways or even the themselves are often toxic to the host [6]. Therefore, metabolic engineering strategies and bioprocess optimization methods are critical to enhance the titres of these target products which are limited by cell toxicity. Alleviating the cytotoxicity caused by metabolic pathways, intermediates, and final products as well as improving the tolerance of host to these compounds are both key to increasing the production of desired compounds in microorganisms.

2.1. Modular and dynamic control of metabolic pathways

Overexpression of native or non-native pathways often affect the intrinsic regulatory mechanisms of the host, resulting metabolic imbalances, such as imbalanced consumption of precursors and resources, imbalance in intracellular redox, and accumulation of toxic intermediates or products, leading to impacts on cell fitness, growth, and yield of the target product [24,38,55]. Therefore, dynamic and specific control of each gene in metabolic pathways with autonomous adjustment to the flux in real time by sensing external and internal metabolic signals [56] is essential to optimizing metabolic pathways for efficient biosynthesis, and a myriad of approaches have been implemented to address these challenges (Fig. 2).

Modular pathway control, in which multiple genes are grouped into modules and coordinate the expression levels of each module, is a powerful strategy to fine-tuning control of metabolic pathways [35–37,57,58]. MMME (multivariate modular metabolic engineering) [59,60], MCE (modular co-culture engineering) [28], and spatiotemporal and integrative genome-scale metabolic modeling [61] have been developed to optimize pathways and increase the production of a broad variety of chemicals derived from isoprenoids and shikimate pathways. For example, partitioning the taxadiene-forming pathway into two modules, an upstream MEP pathway and a downstream terpenoid-forming pathway, and combining the promoter and gene copy-number screening into the engineered taxol precursor-produced *E. coli* host, exhibited a 15,000-fold increase in taxadiene production compared to the control (1.02 ± 0.08 g/L) in fed-batch bioreactor fermentations [35]. An *E. coli*-

Table 2
Examples of recently reported research on the microbial production of bio-jet fuels or chemicals via toxicity engineering strategies.

Strategies	Target	Host	Description	Product	Production increase	Ref.
Modular control of pathway	Metabolic pathways	*E. coli*	A native MEP pathway and a heterologous terpenoid–forming pathway; Modulating multivariable expression of two pathways using different promoter strength and gene/plasmid copy number.	Taxadiene	15,000-fold	[35]
		E. coli	Three modules: the upstream MVA pathway, the downstream MVA pathway, the linalool pathway; linalool synthase (*nls*) from *Agrocybe aegerita*; three modules were controlled by 3 different strength promoters and *nls* and *GPPS* were controlled by different RBSs of different translational efficiencies.	Linalool	28-fold	[36]
		E. coli and *S. cerevisiae*	Distributing the metabolic pathway into *E. coli* (taxadiene-producing) and *S. cerevisiae* (oxygenated taxanes-producing) respectively.	Oxygenated taxanes	_	[37]
		E. coli	*E. coli-E. coli* modular co-culture system; Distributing the upstream module of the MVA pathway and the downstream module of the TIGR-mediated gene cluster of *A. grandis GPPS*^{Mut} and *P. taeda Pt1*^{MUT} into pinene tolerance strain *E. coli* YZFP.	Pinene	1.9-fold	[28]
Dynamic control of pathway	Metabolic intermediates; Metabolic pathways.	*E. coli*	QS system from *Vibrio fischeri*; Sensor plasmid contains the *luxI-luxR* genes; Response plasmid carries MVA pathway for FPP synthesis driven by P_{luxI} promoter, and the gene *Bis* to convert FPP to bisabolene, driven by a continuously active P*trc* promoter.	Bisabolene	44%	[24]
		E. coli	Stress-response promoters; FPP-responsive promoters P_{gadE} and P_{rstA} which are downregulated and upregulated by FPP, respectively; FPP synthetic pathway is under the control of P_{gadE}; The amorphadiene synthase gene is under the control of P_{rstA}.	Amorphadiene	2-fold	[38]
		S. cerevisiae	Dynamic control of *ERG9* expression using different ergosterol-responsive promoters ($P_{ERG1/2/3/11}$) .	Amorpha-4,11-diene	2 ~ 5-fold	[39]
		Bacillus subtilis	A bifunctional and modular Phr60- Rap60-Spo0A QS system, based on two native promoters, P_{abrB} (down-regulation by Spo0A-P) and P_{spoiiA} (up-regulation by Spo0A-P).	Menaquinone-7	40-fold	[40]
		S. cerevisiae	The expression of genes in carotenoid pathway, MVA pathway, and competitive squalene pathway was sequentially controlled in response to the variation of glucose concentration; Combining a modified GAL regulation system and a HXT1 promoter-controlled squalene synthetic pathway.	Carotenoid	2-fold	[41]
		E. coli	An Esa QS system from *Pantoea stewartii*; The *ARO10-feaB* genes were controlled by P_{esaR} promoter.	4-Hydroxyphenylacetic acid	46.4%	[42]
Compartmentalization	Metabolic intermediates; Final products.	*S. cerevisiae*	Targeting the geraniol biosynthetic pathway to the mitochondria.	Geraniol	6-fold	[43]
		S. cerevisiae	Combined targeting of t34SabS1 in the cytosol and mitochondria; N-truncated t34SabS1 from *Salvia pomifera*.	Sabinene	2-fold[a]	[27]
		S. cerevisiae	Reconstructing a full MVA pathway in the peroxisome.	(*R*)-(+)-Limonene, (*S*)-(-)-Limonene, α-Pinene, Sabinene, and Camphene.	125-, 105-, 22-, 17- and 15-fold	[44]
		S. cerevisiae	Compartmentalization of toxic truncated norcoclaurine synthase (tNCS) from *Coptis japonica* in the peroxisome.	(*S*)-Norcoclaurine	54%	[45]
		E. coli	Localising tagged enzymes for 1,2-propanediol synthesis (P18-GldA, P18-DhaK, D18-MgsA and D18-FucO) to a recombinant empty Pdu BMC system.	1,2-Propanediol	245%	[46]
Efflux pumps	Final products	*E. coli*	Expressing an pump (YP_692684) from *Alcanivorax Borkumensis* in limonene producing strain.	Limonene	79%[a]	[47]
		E. coli	Overexpression of *yddG* gene.	L-Phenylalanine	2-fold	[48]
		E. coli	Overexpression of *ttgB from Pseudomonas putida* KT2440.	Pinene	25%[a]	[28]
		E. coli	YhcP	*p*-Hydroxybenzoic acid [b]	_	[49]
		E. coli	Overexpression of *marA* gene.	Geraniol[c]	_	[50]
		S. cerevisiae	Heterologous expression of *GcABC-G1* from *Grosmannia clavigera*.	(+)-3-Carene, (+)-Limonene, β-Pinene[d]	_	[51]
Tolerance engineering	Final products	*E. coli*	Improvement of sabinene tolerance of *E. coli* using ALE under sabinene stress.	Sabinene	8.43-fold	[25]
		E. coli	Improvement of pinene tolerance by ALE under pinene stress after atmospheric and room temperature plasma mutagenesis.	Pinene	31%	[28]
		E. coli	Overexpression of the mutated *flgFGH*.	Pinene	31%	[52]
		E. coli	*yceI* was identified by screening transgenic library from the hydrocarbon-degrader *Marinobacter aquaeolei* and was overexpressed in *E. coli*.	Pinene[e]	_	[53]
In situ product recovery	Final products	*E. coli*	Two-phase culture; isopropyl myristate.	Styrene	125%	[33]
		E. coli	Gas-stripping with three consecutive bottles containing n-dodecane.	Styrene	1.65-fold[a]	[34]
		S. cerevisiae	Solvents: dibutyl phthalate, dioctyl phthalate, isopropyl myristate, and farnesene.	Limonene[f]	_	[54]

[a] The value is obtained by calculations based on the papers's published data.
[b] Alleviating the toxic effect of *p*-hydroxybenzoic acid to *E. coli*.
[c] Overexpression of *marA* significantly enhanced the tolerance of *E. coli* against geraniol.
[d] Heterologous expression of *GcABC-G1* in *S. cerevisiae* improved the survival of yeast cells in the presence of some monoterpenes.
[e] Heterologous expression of *yceI* in *E. coli* improved the growth rates of cells in the presence of 0.15% (v/v) pinene.
[f] Solvents significantly reduced limonene toxicity in yeast cells.

Table 3
Cytotoxicity of monoterpenes and aromatics.

Compound	Toxcity[a] (% v/v)	Mechanism	Efflux Pumps	Tolerance engineering	Ref.
Sabinene	0.5 g/L		_	*ybcK, ygiZ, scpA*.	[25,26]
α-Pinene[b]	0.2%	Cell membrane disruption.	TtgB, AcrB, AcrAB, YceI.	tTcb3p$^{1\text{-}989}$	[28,">47,53,112,113,119]
Limonene	0.025%	Cell wall damage; Oxidative stress.	AcrAB and a pump from *Alcanivorax borkumensis*.	YALI0F19492p, tTcb3p$^{1\text{-}989}$, AhpC^{L177Q}.	[47,87,113–115,120]
Linalool	1.5 g/L		_	_	[31]
Geraniol	0.05%		AcrB, MarA overexpreesion, AcrR deletion.	RecA	[47,50,85,116]
2-Phenylethanol[b]	2.0 g/L	Cell membrane disruption; DNA damage.	_	_	[32,121]
Styrene	0.4 g/L		AcrB	LexA^{E45I}	[33,117,118]

[a] The lowest dose that completely inhibits the growth of *E. coli*.
[b] The toxic dose that partially inhibits the growth of *E. coli*.

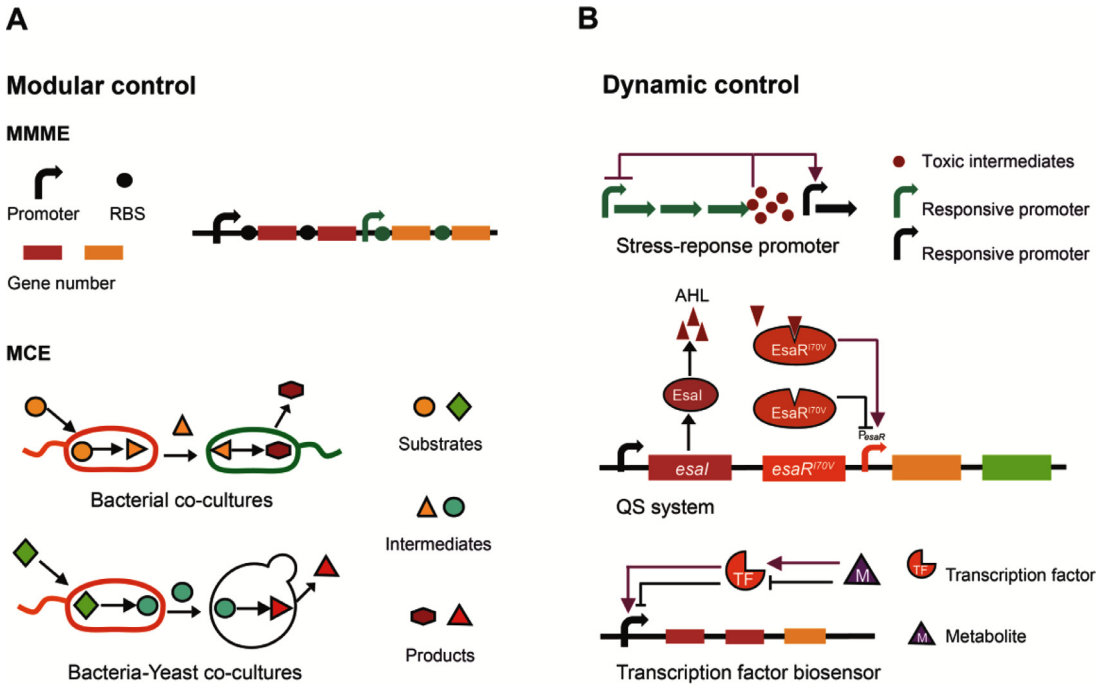


Fig. 2. Strategies for the modular and dynamic control of metabolic pathways. (A) Multivariate modular metabolic engineering (MMME) and modular co-culture engineering (MCE) pathway are two major strategies utilized for modular control pathways which address pathway burden of intermediates and products. Expression of several pathways using promoters of different strengths, RBSs of different translational efficiencies, and varied gene/plasmid copy number are applied in MMME strategies for functional optimization of the complete pathway. MCE is a spatial pathway modularization approach which distributes the different pathway modules in different strains derived from the same or different species. (B) Stress-response promoters, quorum sensing (QS) systems, and transcription factor-based biosensors are three strategies used in the dynamic control of pathways to support tolerance of the toxicity of intermediates and the metabolic burden. AHL, 3-oxohexanoyl-homoserine lactone.

E. coli modular co-culture system which modularized the heterologous MVA pathway and pinene-forming pathway, was engineered to improve pinene production by 1.9-fold compared to the mono-culture approach [28]. Recently, computational genome minimization has opened new paths to optimize microbial pathways in a modular synthetic community [62].

Although modular engineering is an effective approach for optimizing metabolic pathways for efficient biosynthesis, it can cause the over-exploitation of cellular resources (such as ATP and NADPH) and place metabolic burdens on the microbial host, which are in turn detrimental to cell fitness and product yield. However, dynamic control is a rapidly developing field that is addressing the challenges of metabolite toxicity and metabolic imbalance and is a powerful approach to achieve high TPY in metabolic engineering [56]. In recent years, several dynamic control systems have been successfully applied in biosynthetic pathway optimization and resolved intermediate compounds' toxicity, such as stress-response promoters [38,39], transcription factor-based biosensors [63], and quorum sensing (QS) systems [24,40,42]. To enhance the production of terpenoids and shikimate pathway derived aromatics, dynamic control has been widely used to avoid the accumulation of toxic intermediates [38,64], balance the synthesis of both essential terpenoids and final products [39–41,65], and block or repress a competing pathway [42,66,67]. To address the toxicity caused by FPP (Farnesyl diphosphate) accumulation in terpenoid production, dynamic control of the pathway in amorphadiene production was developed using the FPP-responsive promoters P_{gadE} and P_{rstA} which were downregulated and

upregulated by FPP, respectively. The FPP synthetic pathway under the control of P_{gadE} was inhibited while the expression of amorphadiene synthase under the control of P_{rstA} was increased with FPP accumulation. This strategy resulted in the production of amorphadiene reaching 1.6 g/L, which was over two-fold higher than production with inducible or constitutive promoters [38]. In another example, researchers improved menaquinone-7 production 40-fold (from 9 to 360 mg/L) by using a bifunctional Phr60-Rap60-Spo0A quorum sensing system to adjust gene regulation of genes in the menaquinone-7 biosynthesis pathway by maximizing carbon flux towards the pathway [49].

Dynamic control has been used to improve the yield of a variety of products via optimization of metabolic pathways, including mitigating effects of the accumulation of toxic metabolites and unbalanced pathway flux. However, this approach has not yet been broadly applied because of several limitations. First, this approach requires sensitive and specific sensors which can detect and respond to the specific metabolites and intermediates, such as responsive promoters, but many of these promoters are unknown. Secondly, there is a lag time between sensing and action, which may burden the cell metabolism. Furthermore, the balancing flexibility and control are important to the dynamic control of metabolic pathways, and additionally finding the optimal time point for activating the dynamic regulatory systems is labor intensive and time consuming.

2.2. Compartmentalization

In recent years, compartmentalization, which physically separates biological reactions from the cytosol has been used to overcome the challenges of toxic pathway intermediates, modular optimization, and competing metabolic reactions [68,69]. Microorganisms naturally perform compartmentalization: for example, organelles in eukaryotes [70,71] and bacterial microcompartments (BMCs) in prokaryotes [72] (Fig. 3).

In yeast, several organelles (such as mitochondria, peroxisomes, endoplasmic reticulum, and vacuoles) provide specific conditions which have been successfully exploited to compartmentalize different metabolic pathways for the efficient production of a variety of chemicals [71,73]. For example, peroxisomes have excellent detoxification function which has been extensively harnessed to produce isoprenoids [27,71,74], fatty acid derivatives [75,76], expanded shikimate pathway derivatives of alkaloids,[45] and prodeoxyviolacein [77]. Furthermore, peroxisomes have been shown to be effective in the production of monoterpenes, including α-pinene, limonene, sabinene, camphene, and geraniol [27,44]. Reconstructing the entire momoterpene-synthesizing pathway in the yeast peroxisome improves the production of several monoterpenes compared to cytosolic production with the same enzyme. With this method, the titres of camphene, α-pinene, sabinene, (*S*)-(-)-limonene, and (*R*)-(+)-limonene were improved 15-, 22-, 17-, 105-, and 125-fold, respectively [67]. Bacteria also have organelles such as bacterial microcompartments (BMCs) which have strong potential to compartmentalize flux, control cytotoxic metabolic intermediates, avoid cross-talk of metabolic pathways, and thus enhance metabolic efficiency [78]. The strategy of heterologous expression of BMCs in *E. coli* has been successfully used in metabolic engineering for chemical production [46,79]. In addition, synthetic protein scaffolds are also used to reduce metabolic load and fine-tune pathway flux for isoprenoid production [30,80,81] (Fig. 3). Organelles and BMCs can also sequester toxic metabolites and reduce the deleterious effects of these toxic intermediates on the host cell thus increasing productivity. However, the physiochemical conditions of various organelles (such as pH or redox state) must be carefully considered to avoid negative effects on enzyme activity and folding of relevant targets. In addition, expression of BMCs and enzymes with BMC targeting peptides may increase the metabolic burden on the host cell and compromise enzyme activity.

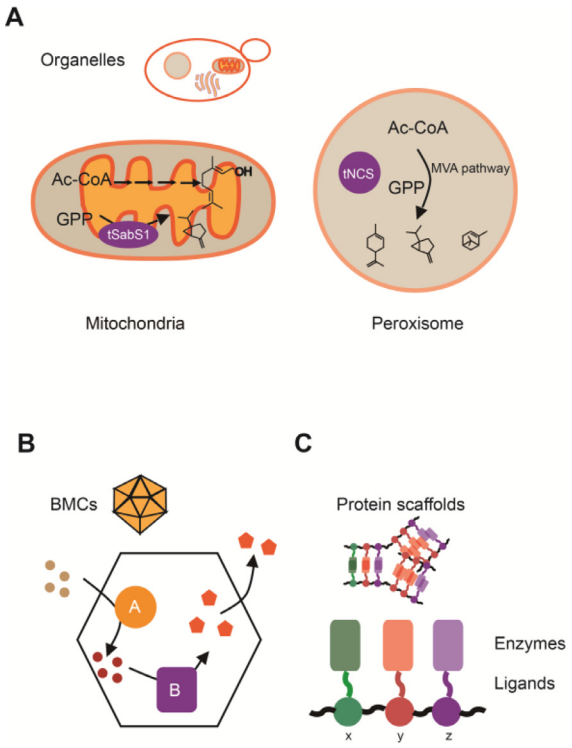


Fig. 3. Strategies of compartmentalization in toxicity engineering. (A) Strategies for compartmentalization using yeast organelles. For example, the N-truncated tSabS1 or the geraniol biosynthetic pathway occuring in the mitochondria produces sabinene and geraniol; the complete MVA pathway occurring in and Ac-CoA being used in the peroxisome to produce monoterpenes or express toxic enzymes (tNCS) relieves the related cytotoxicity. (B) Strategies of using bacterial microcompartments (BMCs) to cope with toxicity of intermediates or final products. Localized tagged enzymes in a recombinant empty BMC system (such as Pdu). (C) Strategies for compartmentalization using synthetic protein scaffolds. Modular protein domain–ligand interactions are used to co-localize enzymes and form large complexes. The enzyme stoichiometry can be controlled by changing the numbers (x, y, z) of each of the scaffold domains.

2.3. Efflux pumps

The intracellular accumulation of the final products, particularly of high toxicity compounds often disrupts cell physiology and leads to suboptimal production. Efflux pumps consisting of transporters can export the products outside of the cells and thus provide a direct and efficient route for relieving toxicity while also facilitating biosynthesis by creating pull on the bioconversion pathway [47,49,50]. Various efflux pumps (such as AcrAB-TolC, TtgB, AaeAB, MsbA, and MdlB) have been applied in the production of several terpenes (pinene, limonene, geraniol, β-carotene, isopentenol) and aromatics (pHBA, phenylalanine) [28,47,48,82–84] (Fig. 4). Overexpression of the *yddG* gene, which encodes an inner membrane protein and is a homolog of known amino acid exporters RhtA and YdeD, in *E. coli* enhances the L-phenylalanine tolerance of *E. coli* cells and thus increases the production of L-phenylalanine two-fold [77]. In addition, some uncharacterized pumps from various microorganisms could be employed to improve the tolerance and production of biofuels. For example, the uncharacterized pump (YP_692684) from *Alcanivorax borkumensis* was found to improve bacterial tolerance of limonene, which also enhanced the production titre [47].

Certain regulators (such as MarA and AcrR) which can activate or inactivate the expression of efflux pumps have also been engineered to enhance cell export of target products [50,85] (Fig. 4). For example, overexpression *marA* in *E. coli* enhances the tolerance and exportation of geraniol; intracellular geraniol concentration in *E. coli* with *marA*

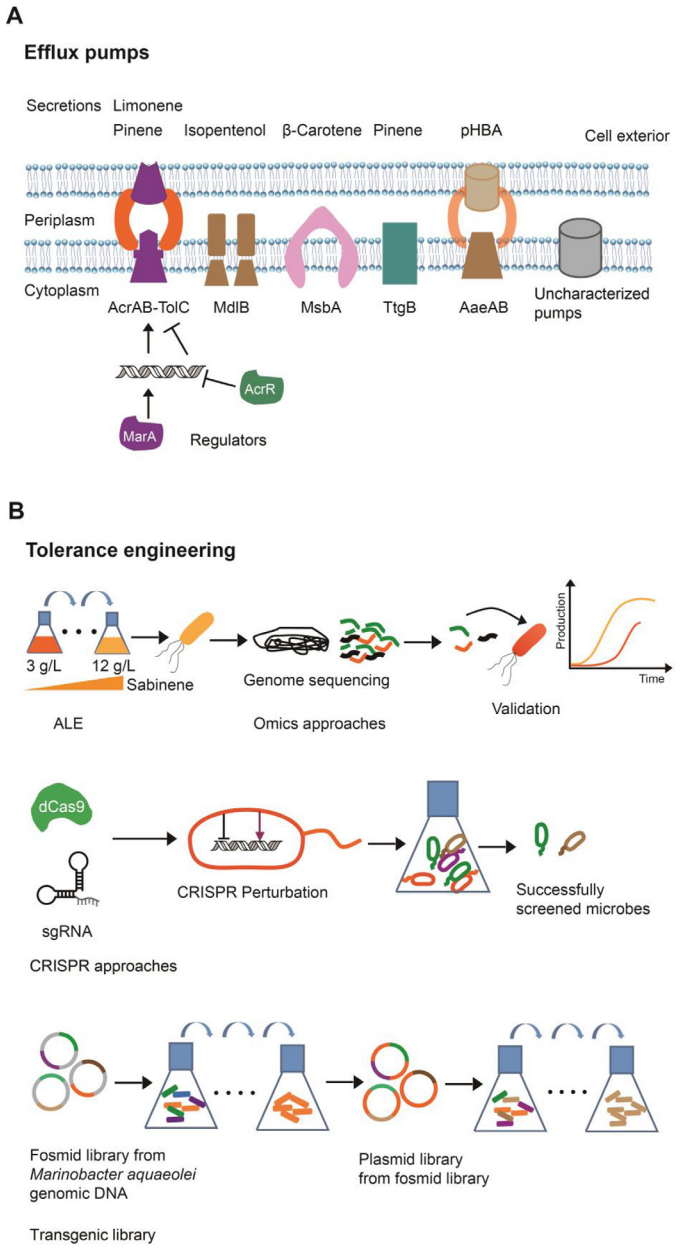


Fig. 4. Efflux pumps and tolerance engineering strategies for mitigating the toxicity of final products in microbial production of bio-jet fuels. (A) Several approaches in efflux pump engineering include overexpression of efflux pumps (such as AcrAB and TtgB), control of the expression of efflux pump regulators (such as MarA and AcrR), and identification of new efflux pumps. pHBA, *p*-hydroxybenzoic acid. (B) The tolerance engineering strategies and tools include adaptive laboratory evolution (ALE), omics, CRISPR, and transgenic library approaches.

overexpression was two-fold lower than that of wild type *E. coli* in a broth culture of geraniol, and similarly on plates with geraniol a 104-fold increase in colony forming efficiency was observed [74]. Unlike bacteria, there are no reported native efflux pumps for monoterpenes in *S. cerevisiae.* Therefore, heterologous expression of efflux pumps for transporting monoterpenes in *S. cerevisiae* is a promising approach to increasing the cell tolerance to monoterpenes [51]. Currently, identification of functional efflux pumps for specific products is a labor intensive and time consuming process presenting a significant technological challenge.

2.4. Tolerance engineering

Tolerance engineering, which improves the host tolerance to toxic products first requires an understanding of how microbes are affected by toxic product accumulation and how microbes naturally respond adaptively to counteract and mitigate these harmful effects. Although different compounds elicit distinct stress responses [86], the major types of toxicity caused by terpenoids and aromatics used as bio-jet fuels, which are mainly small hydrophobic compounds, have been studied and include membrane disruption, cell wall damage, protein misfolding, DNA damage, and oxidative stress [19,87–89]. Although specific mechanisms for diverse chemicals have been exploited to engineer for increased tolerance, engineering a tolerant strain, such as solvent tolerance, requires coordinated and fine-tuned expression of multiple genes. Therefore, adaptive laboratory evolution (ALE) and omics-driven approaches have been extensively applied in tolerance engineering to increase production [25,28,90] (Fig. 4B). For example, Wu *et. al* obtained a sabinene-tolerant strain by serial passaging of cultures in a medium supplemented with gradually increased concentrations of sabinene, and achieved a 8.43-fold higher production of sabinene compared with the parental BL21 (DE3) strain. Furthermore, Wu *et. al* identified several genes: *ybcK, ygiZ*, and *scpA,* which are important in sabinene tolerance [25]. Although ALE offers an efficient approach to improve host tolerance to specific targets, the improvements in tolerance does not always show an increase production as well. For example, overexpression of *AcrBDFa* (YP_692684) from *A. borkumensis* or *MexF* (NP_745564) from *P. putida* KT2440 improved pinene tolerance but did not improved pinene production [28]. Moreover, the stabilization of evolved strain phenotypes is also the challenge in the application of this method. Additionally, CRISPR-driven approaches, transgenic library, and semisynthetic stress response system methods, have also been applied in tolerance engineering research [52,53,91,92] (Fig. 4). For example, two uncharacterized genes, *yjjZ* and *yehS,* with strong potential for improving tolerance to n-butanol and n-hexane were identified using CRISPR-based perturbations of gene expression [91]. In addition, a single gene *yceI,* was isolated from the hydrocarbon-degrader *M. aquaeolei* using a sequencing strategy with a fosmid library followed by a plasmid library, thus improving pinene tolerance when expressed in *E. coli* [53].

2.5. In situ *product recovery*

ISPR (*in situ* product recovery), which can remove toxic target products by various physical approaches such as evaporation, product immobilization, phase extraction, perstraction, and gas stripping *in situ* during fermentation processes is widely applied in biochemical production [34,93,94]. Among these techniques, two-phase extractive fermentation with a solvent is a traditional and effective method to improve the production of terpenes and aromatics [54,95–98] (Fig. 5). For example, the production of styrene reached concentrations of 836 ± 64 mg /L with the addition of bis(2-ethylhexyl)phthalate extraction, which was a 320% improvement over single-phase cultures [95]. Sometimes, to minimize the loss of target product in the off-gas, particularly for volatile compounds, consecutive bottles containing solvent were connected to the air outlet of the bioreactor to capture the product with gas stripping during the fed-batch cultivation (Fig. 5B). In addition to reducing the cytotoxicity of the product, ISPR technology also has other advantages, such as increasing yield by alleviating product feedback inhibition and reducing evaporation loss [99]. A large variety of solvents (such as dodecane, dibutyl phthalate, isopropyl myristate) have been chosen to implement ISPR technology for microbial production of bio-jet fuels based on the criteria of biocompatibility, high product distribution coefficients, maximum product recovery, and low cost [29,33,34,54]. However, the cost and potential inhibition of the host cell by the solvent must be carefully considered in the application of solvent extraction. Additionally, the downstream purification process is also an important challenge, especially in large-scale production. Moreover, other ISPR techniques such

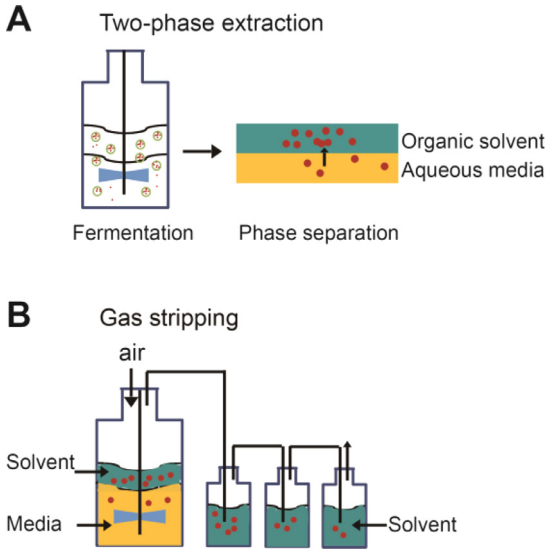


Fig. 5. Two major ISPR techniques to reduce the toxicity of final products in biofuel production. (A) Two-phase extraction is a physical approach used to mitigate final product toxicity *in situ* during fermentation using an extractive solvent. (B) Gas stripping is a simple method used to reduce final product toxicity and by recovering the product from the fermentation broth using exterior extraction modules (ie several consecutive bottles containing solvent).

as solvents containing microcapsules have been used for the recovery of 2-phenylethanol [100].

2.6. Alternative strategies

In addition to the approaches discussed above, some alternative strategies are emerging in the toxicity engineering, including utilization of naturally tolerant strains, cell free systems, and unnatural pathways or enzymes (Fig. 6).

The development of molecular biology and tools for genetically manipulating microorganisms make it practical to use non-model microorganisms with high tolerance to toxic inhibitors. Several examples of non-model hosts exhibiting solvent tolerance or resistance to other conditions have been reported and applied to produce toxic aromatic compounds and terpenes. For example, *Pseudomonas putida* strains exhibit high tolerances to a wide range of toxic organic solvents and have been used to produce many types of toxic aromatic compounds such as 2-phenylethanol, phenol, and *p*-hydroxystyrene [101–103]. *Corynebacterium glutamicum, P. ananatis, Zymomonas mobilis*, and *Candida glycerinogenes* also show inherent tolerance to certain biofuel targets [29,104–106]. The cell-free systems are alternatives to address cytotoxicity in metabolic engineering of living cells. A cell-free system with 27 enzymes was successfully designed for monoterpene production and conversion yeilding >95% and titres >15 g/L were obtained [107]. Some novel pathways that can bypass the toxic intermediates have been established to avoid the accumulation of inhibitors and enzymes which can resolve feedback inhibition have been developed to alleviate cytotoxicity in the microbial production of bio-jet fuels, such as IPP-bypass pathways and feedback-resistant enzymes of M5K and AroG variants [108–111]. For example, in one of the IPP-bypass pathways, the MVA can be directly converted into isoprenol by fatty acid decarboxylase ($OleT_{JE}$) from *Jeotgalicoccus* species. Subsequently, it was transformed to isoprene by oleate hydratase ($OhyA_{EM}$) in *Elizabethkingia meningoseptica* [110]. A range of feedback inhibition-resistant variants of AroG have been identified, and the expression of variant $AroG^{A202T/D146N}$ in *E. coli* confers a 116% improvement in phenylalanine production [111].

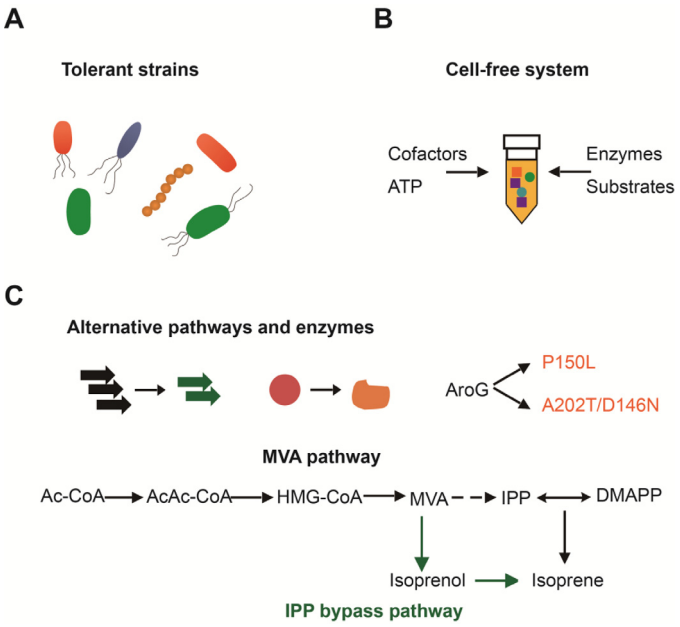


Fig. 6. Alternative strategies to address the toxicity of bio-jet fuel production to microorganisms. (A) Tolerant strains can be used in the production of toxic compounds, such as *Pseudomonas putida, Corynebacterium glutamicum, Zymomonas mobilis*, and *Candida glycerinogenes*. (B) Cell-free systems can address cytotoxicity in engineered living cells. (C) Alternative pathways and enzymes can bypass toxic intermediates and feedback inhibition respectively; for example, the IPP bypass pathway and AroG mutants with feedback-resistance.

3. Perspectives

With more efficient approaches being developed in microbial tolerance engineering for production of biofuels and in developing naturally tolerant hosts, the most successful of these technologies will combine tolerant phenotypes, strain robustness, and yield to achieve the optimal combination for production. Furthermore, the main challenge for the commercialization of bio-jet fuels is to attain sufficient production and economic efficiency. For example, farnesane (hydrogenated farnesene) has been produced commercially, but the cost of production hinders the application in fuels [2]. Currently, the cost of most microbial production of jet fuels is high compared to petroleum-derived fuels. To reduce this cost, more types of feedstocks (such as cellulosic biomass and municipal waste) and fermentation processes (such as open fermentation) should be applied in future production. For example, halophilic microorganisms grow rapidly in seawater, which may enable fermentation to be conducted under unsterile (open) conditions, and reduce the costs of energy and water, eventually leading to a competitive end-product price [122,123]. However, the raw materials are generally require pretreatment to produce fermentable sugars or other targeted precursors, which often results in the generation of toxic by-products. In addition, unconventional fermentation processes often require microorganisms with strong tolerances to one or several growth stressors in order to grow normally and to reach high yield and productivity in fermentation. Therefore, the toxicity engineering for special feedstocks utilization and fermentation process is another research focus. Finally, the performance of phenotypes with improved tolerance in large-scale industrial production should be explored and further efforts should be devoted to develop robust strains for industrial production with toxicity engineering. We can anticipate that advances in biotechnology and synthetic biology and the demand for sustainable biofuels will bring commercialization for an increasing number of bio-jet fuels' production in the near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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