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Research review paper

Designing artificial pathways for improving chemical production

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ABSTRACT

Metabolic engineering exploits manipulation of catalytic and regulatory elements to improve a specific function of the host cell, often the synthesis of interesting chemicals. Although naturally occurring pathways are significant resources for metabolic engineering, these pathways are frequently inefficient and suffer from a series of inherent drawbacks. Designing artificial pathways in a rational manner provides a promising alternative for chemicals production. However, the entry barrier of designing artificial pathway is relatively high, which requires researchers a comprehensive and deep understanding of physical, chemical and biological principles. On the other hand, the designed artificial pathways frequently suffer from low efficiencies, which impair their further applications in host cells. Here, we illustrate the concept and basic workflow of retrobiosynthesis in designing artificial pathways, as well as the most currently used methods including the knowledge- and computer-based approaches. Then, we discuss how to obtain desired enzymes for novel biochemistries, and how to trim the initially designed artificial pathways for further improving their functionalities. Finally, we summarize the current applications of artificial pathways from feedstocks utilization to various products synthesis, as well as our future perspectives on designing artificial pathways.

1. Introduction

Metabolism is the collection of chemical reactions which makes life possible and thus regarded as one of the most complicated activities existed in nature (Hatzimanikatis et al., 2005). Natural metabolism exhibits huge diversity. Even within an *Escherichia coli* cell, the most extensively studied microorganism, more than 2000 different metabolic reactions can simultaneously occur (Erb, 2019). This diversity of metabolism has inspired scientists to harness microorganisms as living cell factories for producing a variety of valuable chemicals (Choi et al., 2020; Ko et al., 2020b; Nielsen and Keasling, 2016; Pontrelli et al., 2018).

Metabolic engineering aims at optimization of catalytic and regulatory processes within cells to increase the production of a certain product (Bailey, 1991; Nielsen and Keasling, 2016; Pontrelli et al., 2018; Stephanopoulos and Sinskey, 1993). Through genetic modifications, metabolic engineers can greatly improve the production performance of engineered strains. The introduction, knock-out, or fine-tuning of gene encoding enzymes in metabolic networks can create new cells that convert inexpensive raw materials into valuable desired products (Cao et al., 2022b; Gao et al., 2022; Li et al., 2021; Srinivasan and Smolke, 2020; Zhang et al., 2022b). Currently, most metabolic engineering strategies mainly focus on overexpressing and optimizing key enzymes within the product synthesis pathway, removing the feedback inhibition from pathway intermediate and/or end-product, eliminating by-product formation pathways and so on (Choi et al., 2019; Jiang et al., 2021; Ko et al., 2020b; Li et al., 2020).

Although pathways existing in nature are important resources for metabolic engineering, they are frequently inefficient and suffer from a series of drawbacks for biomanufacturing, including complicated regulation, slow kinetics and carbon/energy inefficiencies (Clomburg et al., 2019; Dellomonaco et al., 2011; Nielsen and Keasling, 2016; Tan et al., 2020; Wu et al., 2016). This is not unexpected given the role of natural metabolic pathways in providing advantages for cell's survival during evolution rather than serving as dedicated routes for high-efficient biomanufacturing of target products (Firn and Jones, 2009; Scossa and

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Fernie, 2020). Current metabolic engineering works, however, are still limited to existing reactions and pathways, which compromises the accessibility, solution space, and efficiency of chemicals to be produced (Erb, 2019).

The ideal production means of a target chemical should not be restricted by the randomicity and constraints of natural evolution, but be rational design (Erb, 2019). In contrast to the time- and labor-consuming engineering of natural metabolism and having to deal with their inherent inefficiencies, redesigned artificial pathways aim at overcoming the drawbacks of naturally evolved pathways, thus outcompeting naturally evolved metabolism to achieve the synthesis of desired chemicals at high titer, rate and yield (Biz et al., 2019; Erb et al., 2017; Martin et al., 2009). This way metabolic pathways are no longer just a gift from natural evolution, but the product of do-it-yourself approaches (Fig. 1A).

One key approach aimed to achieving this goal needs the successful assembly of reactions in a bottom-up manner to yield the full metabolic pathway (de Lorenzo, 2018; Schwille, 2011; Schwille et al., 2018). However, this requires a comprehensive and deep understanding of physical, chemical and biological principles necessary for designing, realizing and optimizing multi-step pathways *de novo*. As Richard Feynman's famous quote said: "What I cannot create, I do not understand" (Ijas and Koskinen, 2021), while the concept of "artificial pathway" does work in certain cases, the realization of such artificial pathways is still in its infancy. This is mostly because of our limitations in theoretical design, enzyme engineering and pathway optimization, the three components required to successfully build artificial pathways. Here we will discuss the basic procedure, experience from successful examples, as well as perspectives of the future of designing artificial pathways.

2. Novel pathway design

2.1. Pathway design: human intelligence or artificial intelligence?

A complete biosynthetic pathway often consists of multiple reactions, each of which corresponds to a transformation between chemical structures, catalyzed by different enzymes. The biosynthesis of target chemicals begins with simple substrates converted to more complex products through a series of interconnected biocatalytic reactions. Retrobiosynthesis has been developed as a promising method for novel pathway design, which builds on the retro-evolution hypothesis that was first proposed by geneticist Norman Horowitz in 1945 (Bachmann, 2010; Hadadi et al., 2016; Hadadi and Hatzimanikatis, 2015; Kim et al., 2021a). Briefly, its basic design concept is first defining a target chemical and then 'walking' backwards through the known chemical transformations to key precursors (Bachmann, 2010; Hadadi and Hatzimanikatis, 2015) (Fig. 1A). Currently, there are two main methods used for pathway design.

The first one is the knowledge-based artificial pathway design, which highly relies on human intelligence and rich experience of researchers, and several successful examples have appeared so far (Bogorad et al., 2013; Clomburg et al., 2019; Dellomonaco et al., 2011; Tan et al., 2020). For example, with the goal of avoiding carbon loss during acetyl-CoA

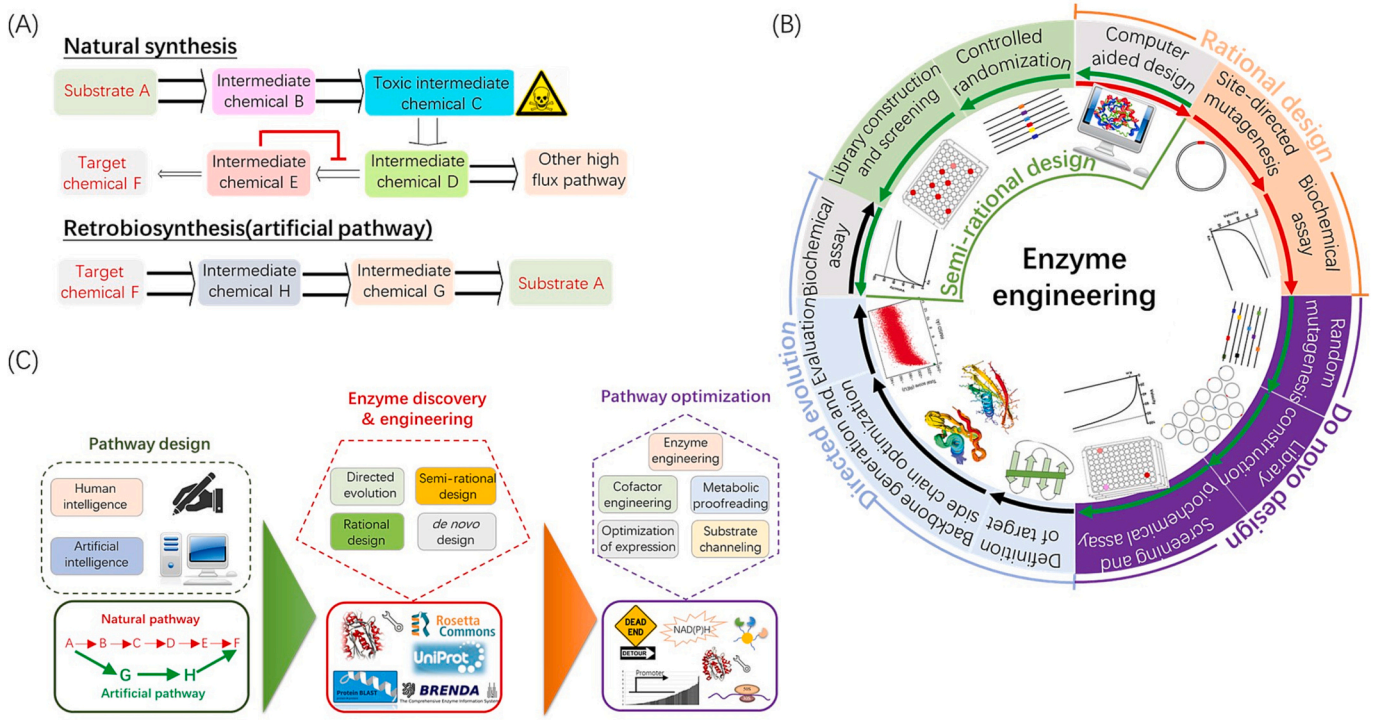

Fig. 1. Design, realization and optimization of artificial pathways. (A) Comparison of engineering naturally existing pathways and building artificial pathways through retrobiosynthesis. (B) Overview of four distinct enzyme engineering approaches for obtaining enzymes catalyzing desired reactions. During rational design, the protein structural information is required and employed for identification of interesting regions which will likely result in the desired outcome. Directed evolution does not require protein structural information, but generates a large protein mutant library and thus heavily relies on high-throughput screening technique by which the desired mutants can be screened for. Semi-rational design uses protein structural information to restrict the regions of interest and minimizes the size of the mutant library. Compared with directed evolution, this method relies on efficient screening technique to a lesser extent. *De novo* protein design workflow, adapted from (Marcos and Silva, 2018). This method begins with the definition of the target protein topology, which includes all length combinations to be explored. Then, the most suitable backbone generation method is selected to generate models that are compatible with the target topology followed by side chain optimization. Next, the most promising candidates will be further assessed by their sequence-structure compatibility followed by further experimental test (Marcos and Silva, 2018). (C) Overall scheme for artificial pathways construction. The basic construction process includes pathway design, enzyme discovery and engineering, as well as pathway optimization.

formation from glycolysis, Bogorad and Liao proposed the non-oxidative glycolysis (NOG) pathway as an alternative route that allows acetyl-CoA production from sugars without carbon loss (Bogorad et al., 2013) (Fig. 2A). Our research group also designed several artificial pathways in the past decade. For instance, Dellomonaco et al. demonstrated that the β-oxidation of fatty acid degradation can be operated in reverse and thus designed the reversal of the β-oxidation cycle (r-BOX) (Dellomonaco et al., 2011) (Fig. 2B). Distinct from natural fatty acid biosynthesis, r-BOX directly uses acetyl-CoA rather than malonyl-CoA as two-carbon units for fatty acyl chain elongation, and avoids ATP consumption and carbon loss, thus enabling the synthesis of products such as alcohols and carboxylic acids at maximum carbon and energy efficiency (Dellomonaco et al., 2011). Clomburg et al. proposed an artificial pathway for *de novo* synthesis of isoprenoids isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Clomburg et al., 2019). Opposed to the key intermediates 2-*C*-methyl-D-erythritol 4-phosphate existing in MEP pathway and mevalonate in MVA pathway, this artificial pathway centered around the synthesis and phosphorylation of (iso) prenol, and was thus termed as isoprenoid alcohol (IPA) pathway (Fig. 2C). In practice, a functional lower IPA pathway for converting (iso)prenol to isoprenoid pyrophosphate intermediates was first established. Then, the upper IPA pathways which forms (iso)prenol from the central carbon metabolite acetyl-CoA was developed. When the lower and upper pathways were combined in *E. coli*, a full IPA pathway was developed (Clomburg et al., 2019). Naturally, synthesis of polyketide backbones is catalyzed by polyketide synthases (PKSs) through iterative decarboxylative Claisen condensation reactions with malonyl-CoA as extender units (Jiang et al., 2008). Recently, Tan et al. proposed an artificial polyketoacyl-CoA thiolases (PKTs) pathway for synthesis of polyketide backbone, *i.e.*, through iterative non-decarboxylative Claisen condensation reactions with acetyl-CoA as extender units (Tan et al., 2020) (Fig. 2D). Compared with PKSs, PKTs have the simpler architectures, carbon and ATP-conserving and less competitions with essential metabolisms, which provides a synthetic and more efficient alternative route to PKSs.

Although knowledge-based artificial pathway design has achieved great successes, this approach might suffer from two potential shortcomings. First, the researchers themselves must possess wide knowledge, deep understanding of biochemical reaction mechanisms, along with rich experience in discovery or engineering of catalytic enzymes and metabolic pathways. Second, despite effectiveness, manual design cannot guarantee to generate all possibilities and pathway candidates, and thus fails to screen the most promising ones. In some cases, the manually designed pathways did not work very well or easily feasible due to the presence of many non-natural reactions (Clomburg et al., 2019; Lu et al., 2019; Siegel et al., 2015; Tan et al., 2020). Therefore, computer-based pathway design based on artificial intelligence has emerged and displayed unique advantages in rationality and

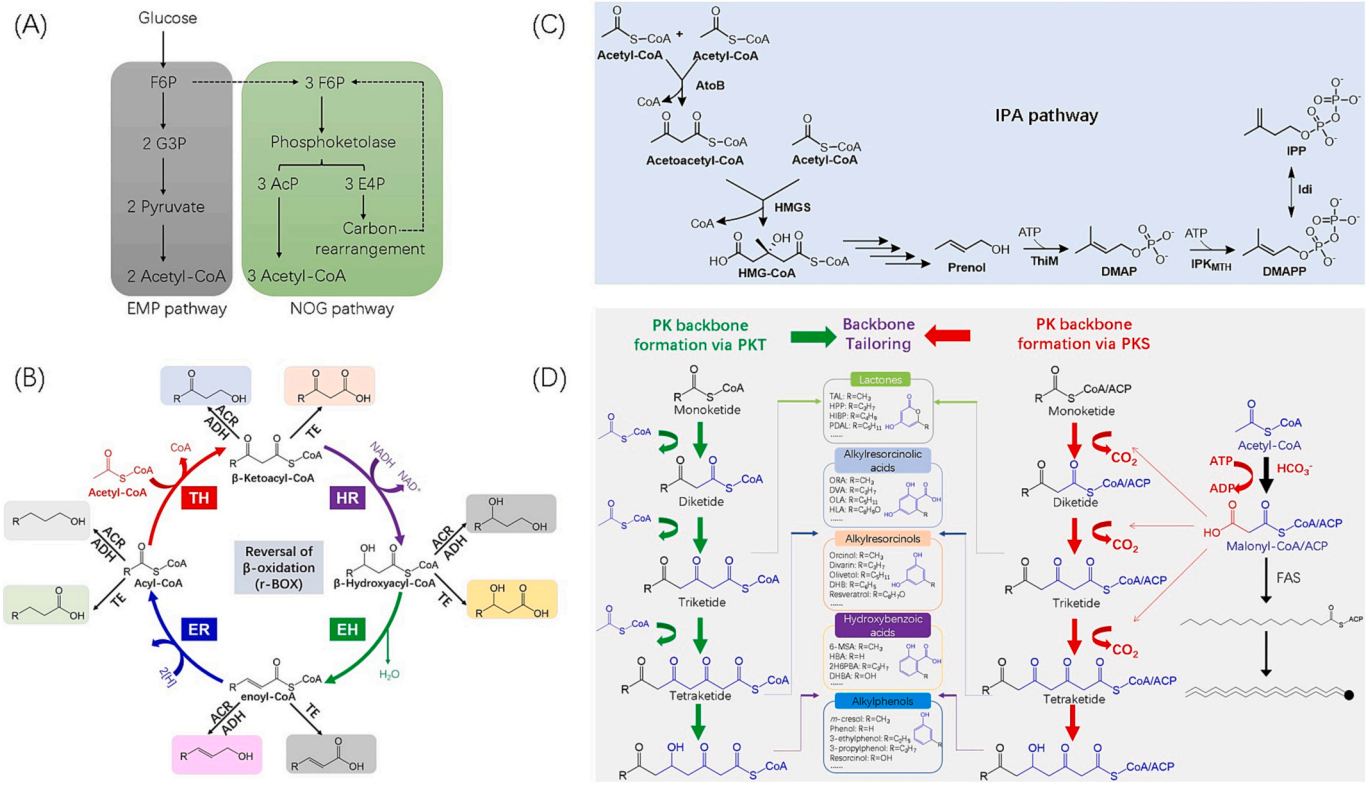

Fig. 2. Knowledge-based artificial pathways design. (A) Designing non-oxidative glycolysis (NOG) for acetyl-CoA production (Bogorad et al., 2013). F6P, fructose 6-phosphate; AcP, acetyl phosphate; E4P, erythrose 4-phosphate; G3P, glyceraldehyde 3-phosphate. (B) Exploring the reversal of the β-oxidation (r-BOX) cycle for the production of alcohols and carboxylic acids (Dellomonaco et al., 2011). The r-BOX cycle contains four enzymes: TH, thiolase; HR, 3-hydroxyacyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; and ER, trans-enoyl-CoA reductase. The entire r-BOX cycle contains four acyl-CoA intermediates, which can be either hydrolyzed by thioesterases (TE) to yield corresponding carboxylic acids, or reduced by reductases (ACR) and/or alcohol dehydrogenases (ADH) to yield final alcohols. (C) Designing isoprenoid alcohol (IPA) pathway for the generation of DMAPP and IPP precursors through retrobiosynthesis (Clomburg et al., 2019). Prenol was employed as the key intermediate linking the upstream and downstream pathways. DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; ThiM, hydroxyethylthiazole kinase; IPK, isopentenyl phosphate kinase. Idi, IPP isomerase; AtoB, acetyl-CoA acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase. (D) Exploring a ploketoacyl-CoA thiolase (PKT)-based pathway (left) for the synthesis of polyketide (PK) backbones and corresponding PKs (Tan et al., 2020). These backbones can be converted to lactones, alkylresorcinolic acids, alkylresorcinols, hydroxybenzoic acids, and alkylphenols. Distinct from PKS (right) that utilizes a decarboxylative condensation with malonyl-CoA/ACP as the extender unit and releases CO_2, PKT (left) utilizes a non-decarboxylative condensation with acetyl-CoA as the extender unit. Besides, PKS also competes for malonyl-CoA/ACP with essential metabolisms such as phospholipids biosynthesis.

comprehensiveness (Bar-Even et al., 2010; Erb, 2019; Kim et al., 2021b; Trudeau et al., 2018). Besides providing novel pathways, these prediction tools can also compare different pathway candidates according to a set of physicochemical properties, *e.g.* thermodynamic feasibility, kinetic efficacy, resources consumption, toxicity and hydrophobicity of intermediates, to screen for the most efficient pathways (Carbonell et al., 2014; Ding et al., 2020; Jörg et al., 2016; Yuki et al., 2010).

Numerous computational prediction tools have been developed to identify biosynthetic pathways, and have been reviewed elsewhere (Kenji et al., 2018; Van Raaphorst et al., 2012; Wang et al., 2017a). Basically, all of these tools harness biochemical reaction databases and the related enzymes to yield potential pathway candidates which connect the input compound and output product (Caspi et al., 2016; Kenji et al., 2018; King et al., 2016) (Table 1). In general, these tools can be categorized into two classes depending on whether they are host-related or not (Ding et al., 2020). The first class of tools mainly focusses on building novel pathways between two target chemicals without host considerations. For example, BNICE (Biochemical Network Integrated Computational Explorer) utilizes bond-electron matrix (BEM) to define non-bonded valence electrons and bond orders to predict novel pathways (Hatzimanikatis et al., 2005; Wang et al., 2017a). ATLAS is a database that includes all theoretical biochemical reactions based on known biochemical principles and compounds (Hadadi et al., 2016), and currently contains more than 150,000 reactions, 96% of which are new reactions linking two or more metabolites that have never occurred in living organisms (Hafner et al., 2020). SimPheny is a commercial software tool developed by Genomatica to enumerate and evaluate enzyme-catalyzed reaction networks to identify new pathways for the production of desired chemicals (Yim et al., 2011). PathPred is a web-based server that predicts possible pathways starting from a query compound to final product, based on local RDM pattern matching and global chemical structure alignment against the reactant pair library (Yuki et al., 2010). RDM pattern represents KEGG atom type changes at the reaction center (R), the difference region (D), and the matched region (M) for each reactant pair (Yuki et al., 2010). ReactionMiner is a java-based package that can predict a series of biochemical transitions connecting starting compound with final product based on subgraph mining (Aravind et al., 2017). Moreover, Kotera et al. developed a recursive supervised method referred to as “multistep reaction sequence likeness”, to predict the number of reactions that connect two metabolites (Masaaki et al., 2014). In contrast, the second class of tools can predict novel pathways to produce desired targets within a specified host. For instance, the reprime and novoStoic method developed by Kumar et al. is an optimization-based *de novo* path design framework that seamlessly integrates existing and new reaction rules (Kumar et al., 2018). GEM-path can predict artificial pathways by integrating retrosynthetic algorithms based on Biochemical Reaction Operators (BROs) and filtering procedures with GEMs (genome-scale metabolic models) at each iteration step. Furthermore, GEM-path also includes a novel reaction promiscuity analysis that is based on similarities of known reaction substrates (Campodonico et al., 2014). RetroPath2.0 is an automatic open-source workflow for retrosynthesis through an efficient and well-controlled protocol (Delépine et al., 2018). XTMS is a web-based pathway analysis platform that provides synthetic pathways through applying an Extended Metabolic Space modeling framework (Carbonell et al., 2014). Recently, a user-friendly web server, novoPathFinder, has been developed to predict novel pathways (Ding et al., 2020). This tool not only can design novel pathways for outputs from inputs but also provides heterologous novel pathways when engineering *E. coli* or yeast chassis cells without providing inputs (Ding et al., 2020).

These computational prediction tools have been already applied to the identification and construction of novel pathways (Koch et al., 2020; Lawson et al., 2021; Segler et al., 2018). For example, Yim and colleagues applied the SimPheny tool to construct a 1,4-butanediol biosynthetic pathway (Yim et al., 2011). More than 10,000 different pathway candidates to synthesize 1,4-butanediol from common central carbon intermediates were obtained and ranked based on multiple attributes. Introduction of this artificial pathway into *E. coli* finally produced up to 18 g/L of 1,4-butanediol (Yim et al., 2011). Fehér et al. used the RetroPath algorithm to predict pathways for the synthesis of flavanone pinocembrin (Fehér et al., 2014). In practice, RetroPath predicted and ranked 11 pathways which link endogenous metabolites to pinocembrin in *E. coli* (Fehér et al., 2014; Kenji et al., 2018). The top ranked pathway (No. 1.1) still contained 8.8 million constructs of possible enzyme combinations, which were further narrowed to 12. When experimentally implemented into *E. coli*, the best engineered strain synthesized ~24 mg/L of pinocembrin (Fehér et al., 2014). By combining 73 aldolase reactions from ATLAS database with ~6500 natural reactions from MetaCyc database, Yang et al. proposed several new C1 assimilation pathways without ATP consumption, with the best pathway carbon yield reaching up to 88% (Yang et al., 2019).

2.2. Realization: enzyme discovery and engineering for novel biochemistries

After establishing the artificial pathway, the next step is to recruit individual enzymes for pathway realization (Fig. 1). Currently, The UniProt Knowledgebase (UniProtKB) has deposited approximately 200 million proteins. Moreover, ~84,000 enzymes have been biochemically characterized and the related experimental data has been deposited to specialized databases, *e.g.*, BRENDA (Burgener et al., 2020b). Furthermore, with the development of sequencing technology, information of

Table 1
Different retrosynthesis prediction tools.

Name	Database	Reaction rules	Pathway ranking	Reference
BNICE	KEGG, ATLAS	Bond-electron matrix (BEM)	Pruning criteria assessment (thermodynamics, pathway length, *etc.*)	Hatzimanikatis et al., 2005
SimPheny	BiGG	Third EC number level	Pathway length, thermodynamics, product yield, number of known metabolites/enzymes, and existence of reaction operators	Yim et al., 2011
PathPred	KEGG	RDM patterns	Compound similarity and pathway score	Yuki et al., 2010
ReactionMiner	KEGG	Reaction signatures	A equation as the ranking function	Aravind et al., 2017
Multistep reaction sequence likeness	KEGG	KEGG reaction modules	Chemical structure similarity between the candidate and start compounds,the chemical structure similarity between the candidate and goal compounds	Masaaki et al., 2014
rePrime/novoStoic	MetRxn	BEM, RDM and SMIRKS	Enzyme performance, toxicity of intermediate metabolites	Kumar et al., 2018
GEM-path	BiGG	SMARTS	Thermodynamics and product yield	Campodonico et al., 2014
RetroPath2.0	MetaCyc, BioCyc	RetroRules	Enzyme promiscuity	Delépine et al., 2018
XTMS	MetaCyc, BioCyc KEGG, BioModels	SMARTS	Weighted sum of the terms corresponding to gene score, toxicity, yield and Gibbs energy	Carbonell et al., 2014
NovoPathFinder	Rhea, BiGG , KEGG, ChEBI	SMIRKS	Thermodynamic feasibility, enzyme promiscuity penalty score, Synthetic Complex Score, pathway length, overall stoichiometric conversions and theoretical yield	Ding et al., 2020

genomes and transcriptomes of different organisms have been published and deposited to all kinds of databases. Currently, more than 71,000 genome sequences are available in Genbank database. All of these databases provide "gold mines" to search for desired enzymes that hold specialized activity.

One of the most used approaches for new enzyme discovery is based on the primary sequence search such as Basic Local Alignment Search Tool (BLAST) (Johnson et al., 2008). Novel enzymes in the database possessing better catalytic activities can be discovered and identified by using a known and characterized enzyme. A good example here is the identification of geranylpyrophosphate:olivetolate geranyltransferase (GOT) from *Cannabis* transcriptomes by using several previously reported GOTs but with low activities as queries for BLAST search. One of the mined GOT candidates (CsPT4-T) that exhibited high geranyltransferase activity was then identified and employed for *de novo* synthesis of plant cannabinoids in yeast (Luo et al., 2019).

However, for many theoretically feasible reactions, there is no appropriate natural enzyme for direct use. Herein, the reversibility of enzymatic catalysis provides great potential for developing non-natural biosynthetic reactions (Erb et al., 2017). For instance, the traditional decarboxylase KdcA exhibits about half-maximal activity in the reverse carboxylation reaction, which has been employed for CO_2 fixation (Martin et al., 2018). In addition, for β-oxidation of fatty acids, three enzymes including thiolases, hydroxyacyl-CoA dehydrogenases (HACDHs) and enoyl-CoA hydratases (ECHs), were found to be reversible and catalyze non-natural anabolic reactions (Dellomonaco et al., 2011) (Fig. 2B).

Besides native substrates, enzymes are also capable of recognizing non-native substrates, *i.e.*, enzyme promiscuity (Gupta, 2016). Even in the model and simple microorganism *E. coli*, from a genome-scale model analysis, at least 37% of enzymes can recognize other non-native substrates (Nam et al., 2012). Thus, besides reversibility, enzyme promiscuity offers another powerful opportunity for developing non-natural reactions. For example, the ThiM kinase from *E. coli*, for which the native substrate is hydroxyethylthiazole, was found to also have isopentenol kinase activity, which has been harnessed to construct the artificial IPA pathway for isoprenoid biosynthesis (Clomburg et al., 2019). Besides conventional substrates of linear fatty acyl-CoAs, several thiolases can also recognize polyketoacyl-CoA as substrates and thus catalyze the Claisen condensation reaction with acetyl-CoA for synthesis of polyketides (Tan et al., 2020).

Such side activities are usually relatively low compared to the activity associated with the primary function of an enzyme and hence unsuitable for the desired reaction. To this end, enzyme engineering has proven its success in improving the catalytic activities of enzymes for non-native substrates or even non-natural reactions (Chen and Arnold, 2020; Katsimpouras and Stephanopoulos, 2021). Given the extent of knowledge of structure and function of the target enzyme, three different enzyme engineering approaches have been developed, *i.e.*, rational design, directed evolution and semi-rational design (Giessel et al., 2022) (Fig. 1B). The rational design approach depends on an in-depth knowledge of the active site structure and its role in performing catalysis (Victorino da Silva et al., 2022). Based on structure-function relationships, potential residue candidates locating in the active site are selected for site-directed mutagenesis (Reetz, 2022). In enzyme engineering, site-directed mutagenesis methods are employed to yield proteins with inserted, deleted or substituted residues. To do this, mutations are formed by PCR using a pair of primers which cover the mismatching nucleotides at their center (Edelheit et al., 2009). For instance, the wild-type NphB shows low geranylpyrophosphate:olivetolate geranyltransferase activity and thus cannot be directly used for effective cannabinoids biosynthesis. According to the crystal structure of NphB-substrate complex (Kuzuyama et al., 2005), several important residues which involve hydrogen bond formation in the active site were selected for site-directed mutagenesis, and the obtained NphB G286S mutant significantly improved catalytic activity with k_{cat} value increasing by greater than 10-fold (Qian et al., 2019).

Inspired by the process of natural evolution, Arnold and coworkers first reported the groundbreaking work of directed evolution in 1993 (Chen and Arnold, 1993). Distinct from rational design, a deep understanding of the structure-function relationships is not indispensable for directed evolution, and it also enables relatively faster engineering of enzymes (Arnold, 2018; Chen and Arnold, 2020). Since then, many enzymes have been modified *via* directed evolution strategies to enhance their activity, specificity, and stability (Martinez and Schwaneberg, 2013; Pourmir and Johannes, 2012; Ren et al., 2019; Sachsenhauser and Bardwell, 2018; Scheiblbrandner et al., 2017). Tan et al. reported that an intriguing mutant M32 of fucosyltransferase was successfully obtained through multiple rounds of directed evolution, with catalytic efficiency (k_{cat}/K_M) increasing ~6–14 fold (Tan et al., 2019). It should be noted that directed evolution often requires high-throughput screening (HTS) technologies from large variant libraries.

Currently, flow cytometric and chip-based microfluidic screening approaches are the most powerful HTS tools for identifying the desirable variant from large variant libraries, with a throughput of $>10^6$ h^{-1}(Markel et al., 2020). These HTS technologies often require a signal that corresponds to the activity of the enzyme variant engineered. Among different signals, fluorescence represents the most commonly used one. Thus, in order to develop efficient HTS platforms, establishing the link between enzyme activity and fluorescent signal is indispensable. Compartmentalization techniques play essential roles in building this link. Among compartments, cells are the most natural ones as cellular membrane is a powerful, selective separator. Besides cells, emulsions are also efficient, non-natural compartments (Markel et al., 2020).

Semi-rational design combines the benefits of rational design and directed evolution (Qu et al., 2021). In this method, promising residues from rational analysis are selected for directed evolution, thus greatly reducing the size of libraries for screening, as well as improving the efficiency (Chen et al., 2012; Chica et al., 2005; Joshi et al., 2019; Korendovych, 2018; Lutz, 2010; Yin et al., 2020). Specifically, *in silico* calculation and semi-rational mutagenesis were employed to engineer the wild-type fluoroacetate dehalogenase for synthesis of (*R*)-α-fluorocarboxylic acids and (*R*)-α-hydroxylcarboxylic acids. The two best mutants, W185N and W185T, showed significantly improved performance, and synthesized the corresponding acids on a gram scale (Zhang et al., 2020).

In addition to engineering naturally existing enzymes, *de novo* protein design is another promising approach for obtaining desired enzymes (Baek and Baker, 2022; Baker, 2019; Cao et al., 2022a; Huang et al., 2016) (Fig. 1C). Specifically, David Baker and colleagues built the Rosetta software that contains algorithms to model and analyze protein structures (Baek and Baker, 2022; Baek et al., 2021; Cao et al., 2022a; Hiranuma et al., 2021; Kuhlman, 2019; Yang et al., 2020a). *De novo* protein design is based on the theory that proteins fold into the lowest energy states based on their amino-acid sequences and the force to drive protein folding is the burial of hydrophobic amino acid residues in the protein's core (Huang et al., 2016). As the method for computing the energy of a protein chain and the method for sampling the space of possible protein structures and sequences are increasingly accurate, *de novo* design of protein becomes possible. Successful examples include the design and creation of an artificial globular protein Top7 with a novel fold (Kuhlman et al., 2003), multipass transmembrane proteins (Lu et al., 2018), fluorescence-activating β-barrel (Dou et al., 2018) and self-assembling helical protein filaments (Shen et al., 2018a).

2.3. Pathway optimization

When an artificial pathway is preliminarily realized by recruiting individual enzymes, it often suffers from a series of shortcomings such as low activity, formation of dead-end metabolites, pathway imbalance, and thus need to be further optimized for improving functionality (Claassens et al., 2019; Erb et al., 2017). Pathway optimization is often

operated in a "design-build-test-learn (DBTL)" iterative manner, in which a synthetic pathway is built, its actual performance is then tested, with potential drawbacks identified and learned from for the next round of design (Burgener et al., 2020b). According to the types of shortcomings, the corresponding optimizations can be listed as below (Fig. 1C).

A. Metabolic proofreading/detoxifying

Under most cases, although been engineered, enzymes of artificial pathway are still not as specific as assumed. Apart from desired substrates, they very frequently act on other undesirable substrates, resulting in abnormal metabolites that may be toxic if accumulated (Erb et al., 2017). Many tools have been developed to predict the promiscuity of enzymes. For instance, Pablo Carbonell and Jean-Loup Faulon proposed to use molecular signatures to build a tool to predict enzyme promiscuity by means of graph kernel support vector machines SVM (Carbonell and Faulon, 2010). Basically, key residues determining enzyme promiscuity would be identified and engineered for minimizing the undesirable activity.

In addition, several of these abnormal metabolites can be reconverted to normal metabolites by repair enzymes, the process of which is metabolic proofreading/detoxifying (Schaftingen et al., 2013). Specifically, this metabolic proofreading strategy often involves the addition of other auxiliary enzymes to fix the issues arisen from the core synthetic pathway. For example, a proofreading enzyme (Mct) was proposed to avoid the formation of the dead-end metabolite malyl-CoA, which significantly improved the pathway efficacy (Schwander et al., 2016). 4-hydroxybutyryl-CoA is a key intermediate of the 1,4-butanediol artificial biosynthesis pathway, however, it would undergo a spontaneous damage reaction to form γ-butyrolactone. To address this issue, a heterologous γ-butyrolactonase was employed to hydrolyze the unwanted γ-butyrolactone to 4-hydroxybutyrate, which drove it back to the 1,4-butanediol biosynthesis pathway (Sun et al., 2017).

B. Optimization of expression levels

The accumulation of intermediates within the synthetic pathway is undesirable as these intermediates either are toxic to the hosts or compromise the pathway efficacy (Fordjour et al., 2022; Kemble et al., 2020; Rinaldi et al., 2022). Moreover, excessive overexpression of the pathway enzymes will also impose huge metabolic burdens to the host cells, which is detrimental to both cell growth and production of desired chemicals (Li et al., 2022; Tsoi et al., 2018). Herein, optimization of the enzyme expression levels within and beyond pathway is crucial. Many effective expression-controlling tools such as controlling gene copies of pathway enzymes (Jones et al., 2000), promoter libraries (Alper et al., 2005), RBS libraries (Salis, 2011) and multivariate modular metabolic engineering strategy (Ajikumar et al., 2010; Tan et al., 2016) have been developed to address these limitations. For example, Shen et al. established an artificial pathway in *E. coli* for direct biosynthesis of arbutin. The pathway starts from 4-HBA, which is converted to hydroquinone (HQ) by 4-hydroxybenzoate 1-hydroxylase (MNX1) and subsequently to arbutin by glucosyltransferase (AS). Initial introduction of the artificial pathway into *E. coli* produced only ~0.05 g/L of arbutin from glucose. The toxicity of 4-HBA and HQ was deemed as potential limiting factor for arbutin production. Therefore, additional copies of *MNX1* and *AS* genes were further introduced, with the titer of arbutin rapidly increasing to 2.34 g/L (Shen et al., 2017). Besides static engineering, dynamic control is another rapidly developing and powerful tool which alleviates these imbalances through the introduction of genetically dynamic control systems which enable cells to autonomously turn on, turn off, turn up and turn down expression levels of pathway enzymes according to external and internal signals (Hartline et al., 2021; Liu et al., 2018).

C. Substrate channeling

Substrate channeling will bring higher local enzyme concentrations, improve availability of intermediates, minimize loss of intermediates to other competing pathways, and improve efficacy of artificial pathways (Kummer et al., 2021). In practice, spatial organization of synthetic pathway enzymes into multi-protein complexes and compartmentalization of full synthetic pathway are two commonly used methods for achieving substrate channeling (Dueber et al., 2009; Lee et al., 2012; Srinivasan and Smolke, 2020). Currently, spatial organization can be implemented using protein scaffold based on a variety of interaction mechanisms such as domain-domain or aptamer-adaptor interactions (Dueber et al., 2009). Compartmentation can be achieved by co-locating pathway enzymes to subcellular organelles or by encapsulating them within protein shells (Zhu et al., 2021).

D. Cofactor engineering

Cofactors such as NAD^+, NADH, $NADP^+$ and NADPH, are important participants in many biochemical reactions. These cofactors are expensive and direct addition is not economically feasible (Partipilo et al., 2021). This becomes more obvious if the pathway is operated *in vitro*. Therefore, cofactor regeneration is highly desirable in order to operate the synthetic pathway at high efficiency (Lee et al., 2022). So far, glucose dehydrogenase (GDH) and formate dehydrogenase (FDH) are the most used enzymes for cofactor regeneration, and other candidates including phosphite dehydrogenase (PDH), alcohol dehydrogenase (ADH), glucose 6-phosphate dehydrogenases (G6DH), and hydrogenases have been only used on a small scale (Wang et al., 2017b). Besides cofactor regeneration, altering NADH and NADPH availability in the cell (Tan et al., 2016), converting an enzyme's cofactor specificity into the desired characteristic (Solanki et al., 2017) and tuning the activity of transhydrogenases that can convert NADH into NADPH (*e.g.*, *E. coli* PntAB) or NADPH into NADH (*e.g.*, *E. coli* SthA) (Sauer et al., 2004), are all widely used strategies for cofactor engineering, either at the individual enzyme level or at the cellular biocatalyst level.

3. Applications of artificial pathways

3.1. Utilization of one-carbon feedstocks

Using various sugars as substrates, microbial fermentation has achieved great successes in synthesis of various chemicals such as bulk chemicals, biofuels, biomaterials and bioactive chemicals. However, the sustainability and cost of sugars gradually become major limitations for large-scale biomanufacturing. In contrast, due to high abundance and low cost, one-carbon (C1) feedstocks such as methane, methanol, formaldehyde, formate, CO and CO_2 have recently received great attentions for biomanufacturing (Cai et al., 2021; Chen et al., 2020a; Hu et al., 2022; Liew et al., 2022; Luo et al., 2022; Zhang et al., 2022a). Nevertheless, despite the potential of biotechnological processes for C1 bioconversion, efficient C1 biocatalysts have not been developed yet. To address this bottleneck, different artificial pathways have been built in the past decade (Fig. 3).

In 2015, Siegel et al. reported a formolase (FLS) that condenses three formaldehyde molecules into one C3 molecule dihydroxyacetone (Siegel et al., 2015). On this basis, a new C1 utilization pathway (formolase pathway) which consists of acetyl-CoA synthase, acetaldehyde dehydrogenase and FLS was built (Fig. 3). Compared with naturally existing pathways, the formolase pathway uses less steps to convert the C1 feedstocks into central carbon metabolites. Moreover, it does not require particular conditions and can function under full aerobic conditions.

In nature, the CBB cycle existing in plants, algae and some microorganisms can fix more than 90% of the CO_2. However, the CBB cycle suffers from a series of intrinsic shortcomings. Specifically, the key ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) shows slow activity and high side reaction with O_2, leading to the loss of both fixed carbon and ATP. Therefore, design of high-efficient synthetic

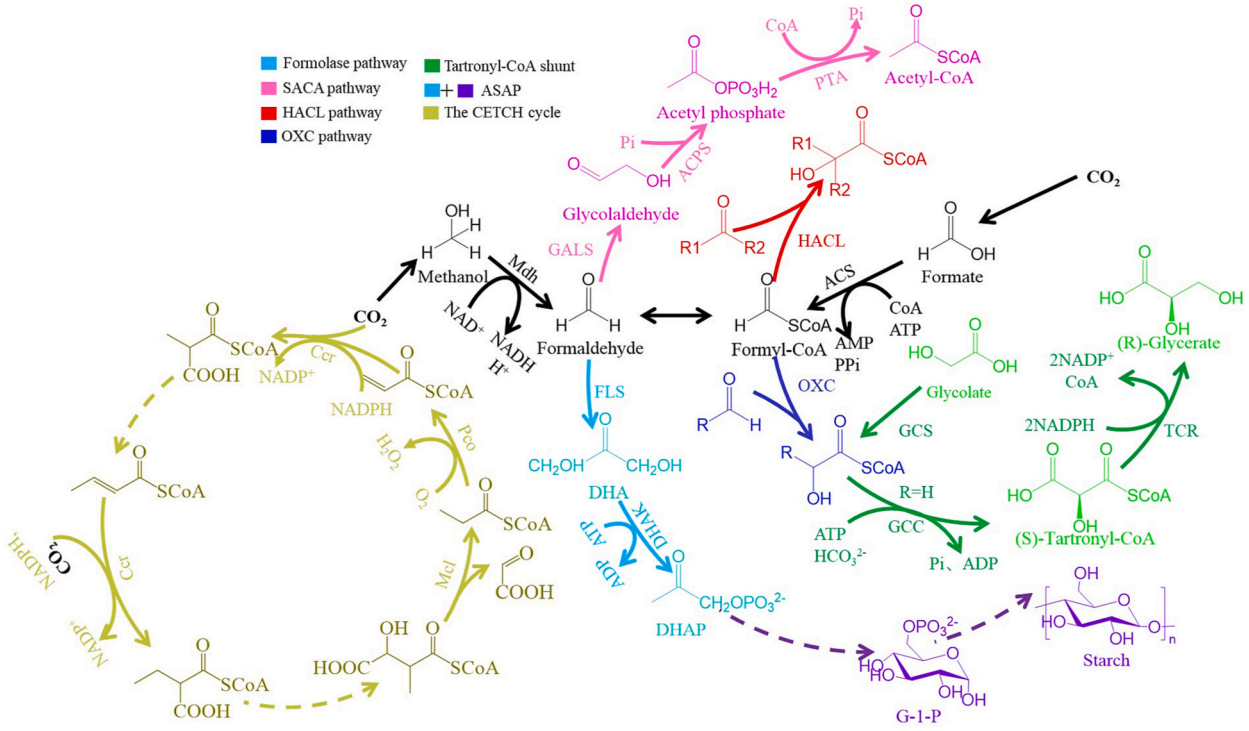

Fig. 3. Different artificial pathways designed for utilization of one-carbon feedstocks. The left circle represents the synthetic CETCH cycle for CO_2 fixation. Two molecules of CO_2 will be converted by the CETCH cycle per turn into one molecule of glyoxylate, which can be further condensed with acetyl-CoA to yield the final product malate. Mct was used to avoid the formation of the dead-end metabolite malyl-CoA. Pco, propionyl-CoA oxidase; Ccr, crotonyl-CoA carboxylase/reductase; Mcl, β-methylmalyl-CoA lyase; Mas, malate synthase; Mct, malyl-CoA thioesterase. The right part contains other artificial pathways developed for utilization of one-carbon feedstocks, as described in 3.2 section. FLS, formolase; DHAK, dihydroxyacetone kinase; GALS, glycolaldehyde synthase; ACPS, acetyl-phosphate synthase; PTA, phosphate acetyltransferase; HACL, 2-hydroxyacyl CoA lyase; OXC, oxalyl-CoA decarboxylase, GCS; glycolyl-CoA synthetase; GCC, glycolyl-CoA carboxylase; TCR, tartronyl-CoA reductase; Pco, propionyl-CoA oxidase; Mcl, β-methylmalyl-CoA lyase; Ccr, crotonyl-CoA carboxylase/reductase; Mdh, Methanol dehydrogenase; ACS, acetyl-CoA synthetase.

pathways for CO_2 fixation is highly desirable. In 2010, several synthetic pathways were proposed for CO_2 fixation (Bar-Even et al., 2010). However, these artificial pathways were not experimentally verified. In 2016, Schwander et al. demonstrated a crotonyl–coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle which converted CO_2 into desired products at a rate of 5 nmol min^{-1} mg^{-1} of protein (Fig. 3). Compared with the naturally evolved CBB cycle, the synthetic cycle displays obvious advantages such as reduced the number of reaction steps, less NAD(*P*)H and ATP consumption (Schwander et al., 2016). In 2020, the same group coupled the CETCH cycle (for the dark reactions) to the thylakoid membranes from spinach (for the light reactions), and encapsulating them in cell-like droplets to result in the artificial chloroplasts, of which showing a CO_2 fixation rate 100 times faster than any previously reported synthetic-biological approaches (Miller et al., 2020). In 2021, Cai and coworkers reported a cell-free chemoenzymatic pathway for converting CO_2 and hydrogen to starch (Cai et al., 2021). The artificial starch anabolic pathway (ASAP) was conceived by computational design, divided into four different modules, and optimized by engineering of key enzymes including the key FLS enzyme mentioned above (Fig. 3). Finally, the ASAP can synthesize starch at a rate of 22 nanomoles of CO_2 min^{-1} mg^{-1} of protein, which is ~8.5-fold higher than the rate in plants (Cai et al., 2021).

In 2018, Trudeau et al. proposed a novel pathway termed tartronyl-CoA (TaCo) shunt as a direct pathway for fixing CO_2 through the carboxylation of glycoyl-CoA to tartronyl-CoA (Trudeau et al., 2018) (Fig. 3). However, the corresponding enzyme of glycolyl-CoA carboxylase (GCC) does not exist in nature. In 2021, Scheffen et al. obtained the desired GCC activity through rational design of propionyl-CoA carboxylase (PCC) from *Methylorubrum extorquens*. Finally, the catalytic efficacy of the engineered GCC increased by three orders of magnitude and is comparable to naturally evolved CO_2-fixing enzymes, which enabled the successful realization of the entire TaCo pathway (Scheffen et al., 2021).

Many C1 feedstocks such as formaldehyde and formate can be easily converted to formyl-CoA, and using formyl-CoA as a C1 donor to yield interesting chemicals thus attracted increasing attentions. Chou et al. revealed that 2-hydroxyacyl CoA lyase (HACL) is capable of catalyzing the condensation of formyl-CoA with carbonyl-containing compounds to generate 2-hydroxyacyl-CoAs (Chou et al., 2019) (Fig. 3), which has been illustrated for converting formaldehyde and formaldehyde+acetone into glycolate and 2-hydroxyisobutyrate, respectively (Chou et al., 2019). It is worthy to note the artificial pathway shows high orthogonality, *i.e.*, has little undesirable overlapping with the host's inherent metabolic network. On this basis, the authors further built a formyl-CoA elongation (FORCE) framework for C1 continuous utilization. In this framework, different C1 feedstocks including formate, formaldehyde and methanol can be efficiently converted into a variety of products such as glycolate, ethylene glycol and glycerate (Chou et al., 2021). Besides HACL, Burgener and Erb revealed that ThDP-dependent oxalyl-CoA decarboxylase (OXC) can also add C1 unit to aldehydes to yield 2-hydroxyacyl-CoAs, and an enzyme cascade including engineered OXC, oxalyl-CoA synthetase, and 2-hydroxyacyl-CoA thioesterase (Fig. 3) is capable of converting substrates of oxalate and aromatic aldehydes into corresponding (*S*)-α-hydroxy acids with ee value up to 99% (Burgener et al., 2020a). Later, they further engineered the OXC enzyme to obtain the desired glycolyl-CoA synthase (GCS) activity which can directly condense formyl-CoA with formaldehyde, and the resulting OCX4 variant outcompetes all other C1–C1 condensing enzymes by at least 40-fold in catalytic efficiency (Nattermann et al., 2021).

Acetyl-CoA is a hub metabolite in metabolism networks of all life

forms, as well as a key intermediate to synthesize a wide variety of chemicals (Krivoruchko et al., 2015; Nielsen, 2014; Zhang et al., 2019). Nature has evolved several different pathways towards acetyl-CoA formation, *e.g.*, glycolysis pathway, phosphoketolase (PK) pathway, serine cycle pathway and Wood-Ljungdahl (WL) pathway (Lu et al., 2019; Nielsen, 2014; Zhang et al., 2019). However, such natural pathways also suffer from many intrinsic limitations, such as carbon losses, ATP consuming and oxygen-dependent, which impair the effectiveness of engineering efforts. To this end, Lu et al. designed and built a synthetic acetyl-CoA (SACA) pathway by recruiting and engineering glycolaldehyde synthase (GALS) and acetyl-phosphate synthase (ACPS) (Lu et al., 2019) (Fig. 3). In practice, they engineered the GALS which condenses two molecules of formaldehyde into one glycolaldehyde, with catalytic activity improving more than 70-fold. Next, the new-to-nature activity of ACPS, namely conversion of glycoaldehyde to acetyl phosphate, was obtained by engineering an existing phosphoketolase. Finally, the feasibility of SACA pathway was experimentally validated both *in vitro* and *in vivo*. Notably, this artificial pathway is the shortest acetyl-CoA formation pathway with additional advantages such as ATP and carbon-saving, which lays foundation for efficiently synthesizing acetyl-CoA derivatives from C1 feedstocks (Lu et al., 2019).

3.2. Production of bulk chemicals

3.2.1. Cis,cis-Muconic acid

Cis,cis-Muconic acid (MA) is an important C6 dicarboxylic acid that can be used for production of a wide variety of polymers and drugs (Khalil et al., 2020). The turnover of the MA market is expected to be more than US$ 110 million in 2024 (Khalil et al., 2020). In 1994, Draths and Frost reported the first pathway (pathway 1) for biosynthesis of MA. By recruiting the heterologous 3-dehydroshikimate (DHS) dehydratase, protocatechuate (PCA) decarboxylase, as well as the catechol 1,2-dioxygenase, the engineered *E. coli* strain successfully produced ~2.4 g/L of MA from 10 g/L of glucose. However, this pathway has inherent limitations in reducing cell viability and thus expensive aromatic amino acids have to been added to support cell viability, which greatly increases the cost for MA production.

Given such limitations, great efforts have been made for building artificial pathways for *de novo* MA biosynthesis from cheap carbon sources (Fig. 4). In addition to DHS, the possibility of deploying other intermediates in the shikimate pathway such as chorismite for MA production was considered. In practice, the pathway 2 for MA production deploys the chorismite as precursor, and recruits isochorismate

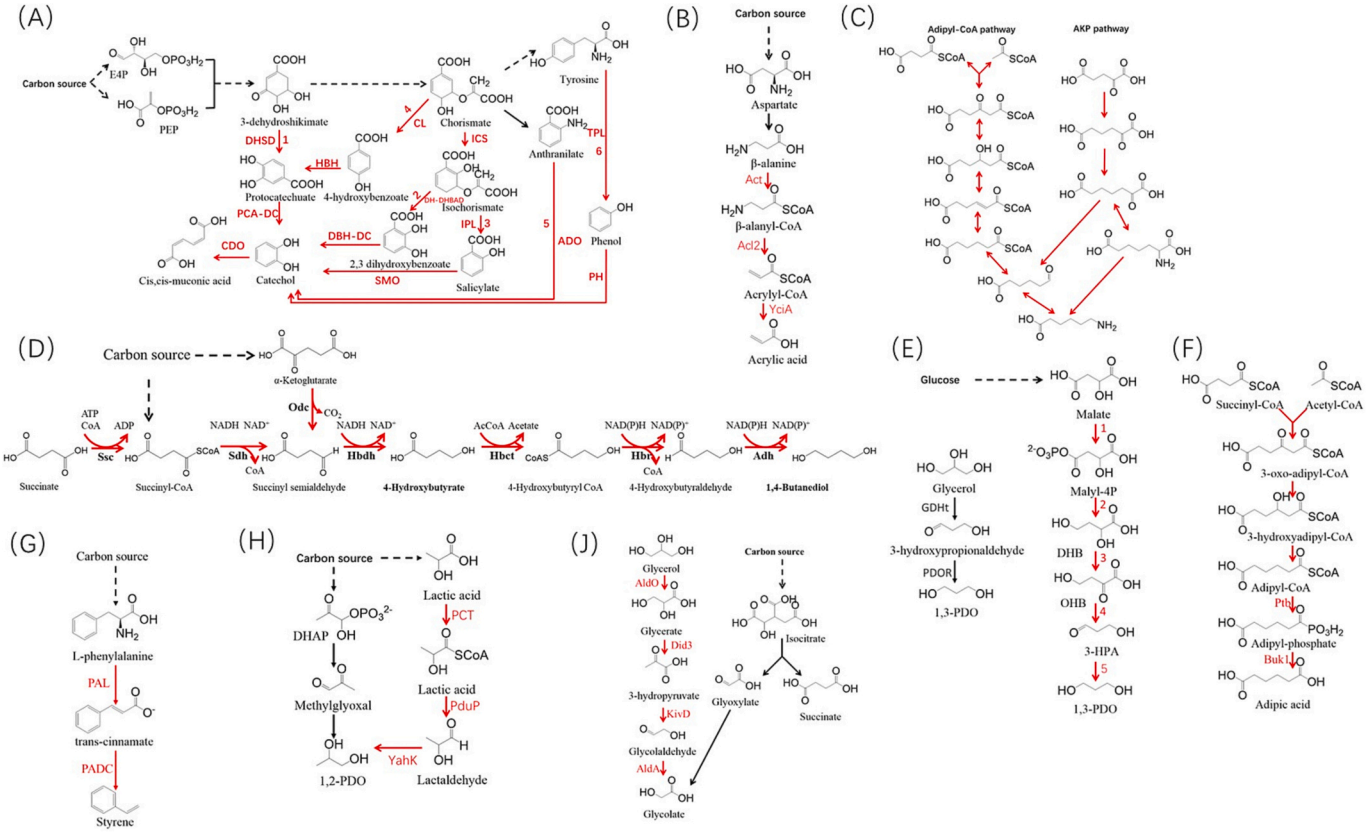

Fig. 4. Exploring the artificial pathway for production of bulk chemicals. (A). Artificial pathways for cis,cis-muconic acid (MA) biosynthesis. DHSD, 3-dehydroshikimate dehydratase; PCA-DC, protocatechuate decarboxylase; ICS, isochorismate synthase; DH-DHBAD, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase; CDO, catechol 1,2-dioxygenase; SMO, salicylate 1-monooxygenase; IPL, isochorismate pyruvate lyase; ADO, anthranilate 1,2-dioxygenase; TPL, tyrosine phenol lyase; pH, phenol hydroxylase; CL, chorismate pyruvate-lyase; HBH, p-hydroxybenzoate hydroxylase; DBH-DC, 2,3-dihydroxybenzoate decarboxylase. (B) Artificial pathways for acrylic acid biosynthesis. Act, β-alanine CoA transferase; Acl2, β-alanyl-CoA:ammonia lyase; YciA, CoA thioesterase. (C) Artificial pathways for 6-aminocaproic acid biosynthesis. (D) Artificial pathways for 1,4-butanediol (1,4-BDO) biosynthesis. Ssc, succinyl-CoA synthetase; Odc, 2-oxoglutarate decarboxylase; Sdh, succinate semialdehyde dehydrogenase; Hbdh, 4-hydroxybutyrate dehydrogenase; Hbct, 4-hydroxybutyryl-CoA transferase; Hbr, 4-hydroxybutyryl-CoA reductase; Adh, alcohol dehydrogenase. (E) Artificial pathways for 1,3-propanediol (1,3-PDO) biosynthesis. GDHt, glycerol dehydratase; PDOR, 1,3-PDO oxidoreductase.1, malate kinase;2, malate semialdehyde dehydrogenase; 3, malate semialdehyde reductase; 4, DHB dehydrogenase; 5, OHB decarboxylase and aldehyde reductase. (F) Artificial pathways for adipic acid biosynthesis. Detailed biosynthesis pathway can be seen in Fig. 2B (r-BOX). Ptb, phosphate butyryltransferase; Buk1, butyryl kinase. (G) Artificial pathways for styrene biosynthesis. PAL, phenylalanine ammonia lyase; PADC, phenylacrylate decarboxylase. (H) Artificial pathways for 1,2-propanediol (1,2-PDO) biosynthesis. PCT, lactoyl-CoA transferase; YshK, lactaldehyde reductase; PduP, aldehyde dehydrogenase. (J) Artificial pathways for glycolate biosynthesis. AldO, alditol oxidase; Did3, 2-hydroxyglutarate-pyruvate transhydrogenase; KivD, 2-ketoisovalerate decarboxylase; AldA, glycolaldehyde dehydrogenase.

synthase, isochorismatase, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, 3-DHB decarboxylase and catechol 1,2-dioxygenase. The engineered *E. coli* strain finally produced ~605 mg/L of MA (Sun et al., 2014; Wang and Zheng, 2015). The design of pathway 3 is similar to pathway 2, and the main difference is the key shikimate pathway intermediates. In pathway 2, it is 3-DHB while in pathway 3 it becomes salicylate. An engineered *E. coli* strain harboring pathway 3 produced ~1.5 g/L of MA (Lin et al., 2014). Pathway 4 was designed to produce MA from chorismite through intermediates PCA and 4-hydroxybenzoate, and the final engineered *E. coli* strain produced ~170 mg/L of MA (Sengupta et al., 2015). In designing pathway 5, the authors proposed that MA can be alternatively synthesized from anthranilate, the first branch intermediate during tryptophan biosynthesis. When introduced the heterologous anthranilate 1,2-dioxygenase (ADO) and catechol 1,2-dioxygenase (CDO) into *E. coli* strain, ~400 mg/L of MA was finally produced (Sun et al., 2013). Pathway 6 for MA synthesis starts from tyrosine, which is acted upon by tyrosine phenol lyase to result in the intermediate phenol. After hydroxylation by phenol hydroxylase, phenol is converted to MA. Nearly 186 mg/L of MA was finally produced from *E. coli* with this artificial pathway (Thompson et al., 2018).

3.2.2. Alcohols and carboxylic acids

In nature, the β-oxidation of fatty acids serves as an important source of acetyl-CoA formation (Kim et al., 2016). Opposed to focusing on its catabolic role, Dellomonaco et al. demonstrated that the reversal of β-oxidation (r-BOX) can be engineered as an anabolic platform for synthesizing carboxylic acids and alcohols (Dellomonaco et al., 2011) as discussed in the section 2.1. This synthetic pathway consists of three reversible reactions catalyzed by thiolase, hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and one irreversible reaction catalyzed by enoyl-CoA reductase (Fig. 2B) (Cheong et al., 2016; Dellomonaco et al., 2011). Notably, Cheong et al. further exploited the potential of r-BOX platform for synthesis of alcohols and carboxylic acids. They added 7 different primers, 3 different extender units into the r-BOX platform equipped with different termination pathways, and successfully synthesized up to 18 alcohols and carboxylic acids from 10 product classes, including 8 compounds that had not been previously produced through microbial biosynthesis, which illustrates the capability of r-BOX for accessing new products with different functionalities (Cheong et al., 2016).

Besides the most used workhorse of *E. coli*, r-BOX platform can also be widely established in other industrial microbial chassis such as *S. cerevisiae* (Lian and Zhao, 2015) and *C. glutamicum* (Shin et al., 2021), which demonstrates the easy transferability and host-independent capability of this artificial pathway (Tarasava et al., 2022). Furthermore, the native r-BOX pathway of *Clostridium* species were also engineered to expand its substrate diversity including lactate, acetate and propionate (Tarasava et al., 2022). More intriguingly, r-BOX can also be established in methylotrophs, which contributes to both synthesis of chemicals and utilization of C1 feedstocks. In addition to the *in vivo* applications, r-BOX platform has been further explored in the cell-free system. Recently, Vögeli et al. optimized the r-BOX with a high-throughput *in vitro* prototyping workflow, screened more than 700 different combinations using the cell-free system, and finally identified the desirable enzyme sets with improved activity and product selectivity (Vogeli et al., 2022). Later, implementation of these pathways in *E. coli* produced the highest titers of C6 acid (3.06 g/L of hexanoic acid) and alcohol (1.0 g/L of 1-hexanol) (Vogeli et al., 2022), which demonstrates the great potential of r-BOX for biomanufacturing.

3.2.3. Styrene

Styrene represents a versatile and bulk chemical for supporting the synthesis of numerous polymers (Zhao et al., 2019). However, the styrene biosynthetic pathways had not been reported. McKenna et al. described the first *de novo* design of an artificial styrene biosynthetic pathway (McKenna and Nielsen, 2011). The artificial pathway consists of phenylalanine ammonia lyase (PAL) and phenylacrylate decarboxylase (PADC), and utilizes *L*-phenylalanine as precursor (Fig. 4). Specifically, PAL converts *L*-phenylalanine to the intermediate compound of trans-cinnamate, and PADC then catalyzes the decarboxylation of trans-cinnamate to styrene. In practice, over-expression of PAL2 from *Arabidopsis thaliana* and FDC1 from *S. cerevisiae* in an *L*-phenylalanine overproducing *E. coli* strain enabled production of ~0.26 g/L of styrene (McKenna and Nielsen, 2011). As styrene is toxic to microbial hosts, tolerance engineering of host is desirable. To this end, Liang et al. constructed a transcription regulator library targeting 54 genes, and the best styrene-tolerant *E. coli* strain produced a 3.45-fold increase in styrene (330 mg/L of styrene in shake flasks) compared to the starting strain (Liang et al., 2020). Moreover, an *E. coli* cell-free system was established to address styrene toxicity and volatility issues, and finally produced up to 4.2 g/L of styrene *in vitro* (Grubbe et al., 2020).

3.2.4. 6-Aminocaproic acid

Nylon represents a class of most used synthetic polymers (Ouellette and Rawn, 2015). The global market for Nylon is estimated to grow to 10.4 million Tons by 2027 (Global Nylon 6 & 66 Market Research Report 2020). 6-aminocaproic acid (6-ACA) is the basic building block for synthesis of Nylon-6 (Fedorchuk et al., 2020). However, 6-ACA is a non-natural compound and there was no report about its biosynthetic pathways (Lin et al., 2019). To this end, Turk et al. used a retro-synthetic approach to identify two potential biosynthetic pathways for synthesis of 6-ACA (Turk et al., 2016) (Fig. 4). Both pathways require unreported novel reactions. Specifically, one of the biosynthetic pathways starts from α-ketoglutarate and contains several bioconversions from the ketoacid elongation pathway originally from methanogenic archaea. Subsequent implementation of this pathway in *E. coli* enabled 2 g/L of 6-ACA under lab-scale fed-batch fermentations (Turk et al., 2016).

3.2.5. 1,3-Propanediol

1,3-Propanediol (1,3-PDO) is a useful bulk chemical which can serve as a monomer to synthesize polymers including polyesters, polyurethanes and polyethers (Biebl et al., 1999; Saxena et al., 2009). Exploring synthetic pathways for 1,3-PDO production has received increasing attention. For example, an artificial pathway enabling 1,3-PDO biosynthesis from C6 sugars was built in *E. coli* by engineering the aspartate-homoserine pathway (Soucaille and Boisart, 2014). However, 1,3-PDO titers obtained with this synthetic pathway were still low (only mg/L level), which might be due to kinetic limitations in homoserine deamination (Soucaille and Boisart, 2014). Furthermore, another synthetic pathway for 1,3-PDO production which starts from the TCA cycle intermediate of malate, was also developed (Frazao et al., 2019). This pathway consists of malate kinase, malate semialdehyde dehydrogenase, malate semialdehyde reductase, DHB dehydrogenase, OHB decarboxylase and aldehyde reductase (Fig. 4). Through co-culture of one *E. coli* strain harboring the malate to DHB module with the second one bearing the DHB to 1,3-PDO module, approximately 300 mg/L of 1,3-PDO was produced (Frazao et al., 2019).

3.2.6. 1,2-Propanediol

Besides 1,3-PDO, 1,2-propanediol (1,2-PDO) is another industrially valuable commodity chemical for synthesis of various polymers (Fiori et al., 2004). Currently, it is mainly produced from petroleum-based feedstocks. Microbial biosynthesis of 1,2-PDO has attracted great attentions in the past decade, and several microorganisms such as *T. thermosaccharolyticum* and *Clostridium sphenoides*, have been shown to be natural producers of 1,2-PDO. The natural pathway starts from DHAP, the intermediate of glycolysis pathway. Methylglyoxal synthase converts DHAP into methylglyoxal, which will be further converted into 1,2-PDO by either aldehyde oxidoreductase and glycerol dehydrogenase or methylglyoxal reductase and 1,2-propanediol reductase (Cameron and Cooney, 1986). Such natural pathway has been transferred to *E. coli* heterologous host for 1,2-PDO production (Clomburg and Gonzalez,

2011; Altaras and Cameron, 2000), and the highest titers of 4.9 g/L and 5.6 g/L were obtained from glucose and glycerol, respectively (Clomburg and Gonzalez, 2011).

However, the natural pathway has its own limitations, including the severe cytotoxicity of methylglyoxal even at millimolar levels (Booth et al., 2003; Totemeyer et al., 1998). To address this limitation, Niu et al. proposed a novel 1,2-PDO biosynthetic pathway which eliminates the use of toxic methylglyoxal (Niu and Guo, 2015). This artificial pathway uses lactate as starting substrate and consists of lactoyl-CoA transferase, CoA-dependent aldehyde dehydrogenase and lactaldehyde reductase (Fig. 4). When expressed in *E. coli*, 1.5 g/L of R- or 1.7 g/L of S-1,2-PDO were obtained with supplies of D- or L- lactic acid under shake-flask conditions (Niu and Guo, 2015). For minimizing the additional supply of lactic acid, complete biosynthesis of 1,2-PDO from glucose was further performed (Niu et al., 2019). After a series of genetic manipulations and culture conditions optimization, the engineered *E. coli* strains bearing the artificial pathway finally produced 17.3 g/L (R-) and 9.3 g/L (S-) of 1,2-PDO from glucose respectively through fed-batch culture (Niu et al., 2019).

3.2.7. 1,4-Butanediol

1,4-Butanediol (BDO) is a valuable platform bulk compound for synthesis of various polymers used as plastics, elastic fibers and films (Philp and Atlas, 2017). Currently, it is mainly synthesized chemically by using petroleum feedstocks (Eriksen et al., 2013), leading to high energy consumption and environmental pollution. While biosynthesis of BDO is a better alternative to conventional chemical synthesis, there was no report on its complete biosynthetic pathway. To this end, Yim et al. reported the first example for direct biosynthesis of BDO from biorenewable feedstocks (Yim et al., 2011) (Fig. 4). They used the SimPheny software to predict all possible pathways that connect common central carbon metabolites with BDO. Among them, the pathway centers around the synthesis and subsequent conversion of 4-hydroxybutyrate (4-HB) was identified as the highest priority (Fig. 4). By integration of the upstream module that converts glucose into 4-HB and downstream module that converts 4-HB into BDO, the engineered in *E. coli* strain with full pathway produced up to 18 g/L of BDO using glucose as sole carbon source (Yim et al., 2011). This work demonstrated an approach of computational prediction-based artificial pathway for biosynthesis of commodity chemicals that are not naturally produced in nature.

3.2.8. Adipic acid

Adipic acid is an important organic acid and also platform chemical in industry (Skoog et al., 2018). Biosynthesis of adipic acid has attracted increasing attentions due to its advantages over traditional petrochemical method. In 2014, Yu et al. firstly proposed the artificial pathway for adipic acid biosynthesis (Yu et al., 2014) (Fig. 4). Basically, this pathway is analogous to the r-BOX mentioned above, and starts with condensation of acetyl-CoA and succinyl-CoA. After subsequent reduction, dehydration and reduction reactions, adipyl-CoA was formed. For conversion of adipyl-CoA to adipic acid, two enzymes of phosphate butyryltransferase (Ptb) and butyryl kinase (Buk1) were employed. Finally, the engineered *E. coli* strain produced approximately ~0.64 mg/L of adipic acid (Yu et al., 2014). Moreover, Cheong et al. further advanced this concept and the engineered *E. coli* produced 2.5 g/L of adipic acid under controlled bioreactor conditions (Cheong et al., 2016). One of the big differences is that Cheong et al. utilized an acyl-CoA transferase (Act), *i.e.*, Acot8 from *Mus musculus* that preferentially hydrolyzes longer dicarboxylyl-CoAs, *e.g.*, glutaryl-CoA, adipyl-CoA, suberyl-CoA, sebacyl-CoA, and dodecanedioyl-CoA (Westin et al., 2005) instead of Ptb-Bulk1 adopted by Yu et al. for hydrolysis of adipyl-CoA to adipic acid, which may explain why adipic acid titer is much higher.

3.2.9. Glycolate

Glycolate is a significant C2 α-hydroxy acid with a broad range of industrial applications (Tao et al., 2010; Zhu et al., 2019). Chemical synthesis is still the predominant method for glycolate production but suffers from high energy demand and environmental pollution. Using renewable feedstocks for glycolate production has drawn increasing attentions in recent years (Deng et al., 2018; Koivistoinen et al., 2013; Pereira et al., 2016; Stephens et al., 2007). Glycerol is formed as a byproduct during biodiesel production and shows great potential as feedstock for synthesis of various chemicals (Tan et al., 2018). Zhan et al. proposed a novel synthetic pathway to produce glycolate from glycerol (Zhan et al., 2020). The pathway starts from glycerol and consists of four enzymes, including alditol oxidase (AldO), 2-hydroxyglutarate-pyruvate transhydrogenase (Did3), 2-ketoisovalerate decarboxylase (KivD) and glycolaldehyde dehydrogenase (AldA) (Fig. 4). When introduced into *E. coli*, this artificial pathway enabled glycolate titers of 0.64 g/L in shake flasks and 4.74 g/L in fed-batch condition (Zhan et al., 2020).

3.2.10. Acrylic acid

Acrylic acid (AA) is a significant organic acid that can be used in several fields such as superabsorbent polymers and acrylate esters (Beerthuis et al., 2015). Although some biosynthetic pathways have been reported to produce AA, most suffer from thermodynamically unfavorable and insufficient driving force (Straathof et al., 2005). Therefore, developing novel and efficient pathways for AA biosynthesis is highly desirable. To this end, Ko et al. reported an artificial pathway for AA biosynthesis in *E. coli* through the β-alanine (BA) route (Ko et al., 2020a). This synthetic AA pathway starts from the TCA cycle intermediate fumarate and consists of two modules, including the upper BA-forming module and lower AA-forming module (Fig. 4). The upper module consists of aspartase and aspartate 1-decarboxylase to convert fumarate to BA, and the lower module consists of β-alanine CoA transferase, β-alanyl-CoA:ammonia lyase and CoA thioesterase which converts BA into AA. When implemented in *E. coli*, the engineered strain produced 55.7 mg/L of AA in shake flasks and 237 mg/L of AA under fed-batch condition, which represents the highest AA titer achieved so far (Ko et al., 2020a).

3.3. Production of fine chemicals

3.3.1. Hydroxytyrosol

Hydroxytyrosol (HT) is an important phenolic compound possessing intriguing antioxidant and pharmaceutical activities (Bertelli et al., 2020; Hu et al., 2014). The first reported HT biosynthetic pathway in *E. coli* started from tyrosine. Specifically, after sequential catalysis by tyrosine hydroxylase (TH), L-Dopa decarboxylase (LPDC), tyramine oxidase and native dehydrogenases, HT was formed. With pathway optimization, the engineered *E. coli* strain produced ~12 mg/L of HT from glucose (Satoh et al., 2012). However, for cost-effective production of HT, this pathway suffered from low activity. Opposed to working within the optimization of this reported pathway, Li et al. proposed a novel pathway for HT biosynthesis (Li et al., 2018). This pathway started from 4-hydroxyphenylpyruvate (4-HPP) instead of tyrosine. Through the sequential reactions catalyzed by ketoacid decarboxylase (KDC), alcohol dehydrogenase (ADH) and 4-hydroxyphenylacetic acid 3-hydroxylase (HpaBC), HT was finally formed (Fig. 5). Integration of the full pathway in *E. coli* produced ~0.65 g/L of HT from glucose and glycerol, which increased by ~50-fold over the first reported HT biosynthetic pathway (Li et al., 2018). The higher HT might be due to the fact that KDC, ADH and HpaBC enzymes were all from microbes while the two first enzymes (TH and LPDC) in the first reported pathway were from mammals. Compared with mammalian enzymes, enzymes from microbes do not require complicated intracellular membrane structures for correct folding, as well as post-translational modifications (Li et al., 2018).

3.3.2. Vanillyl alcohol

Vanillyl alcohol represents an important phenolic alcohol and has

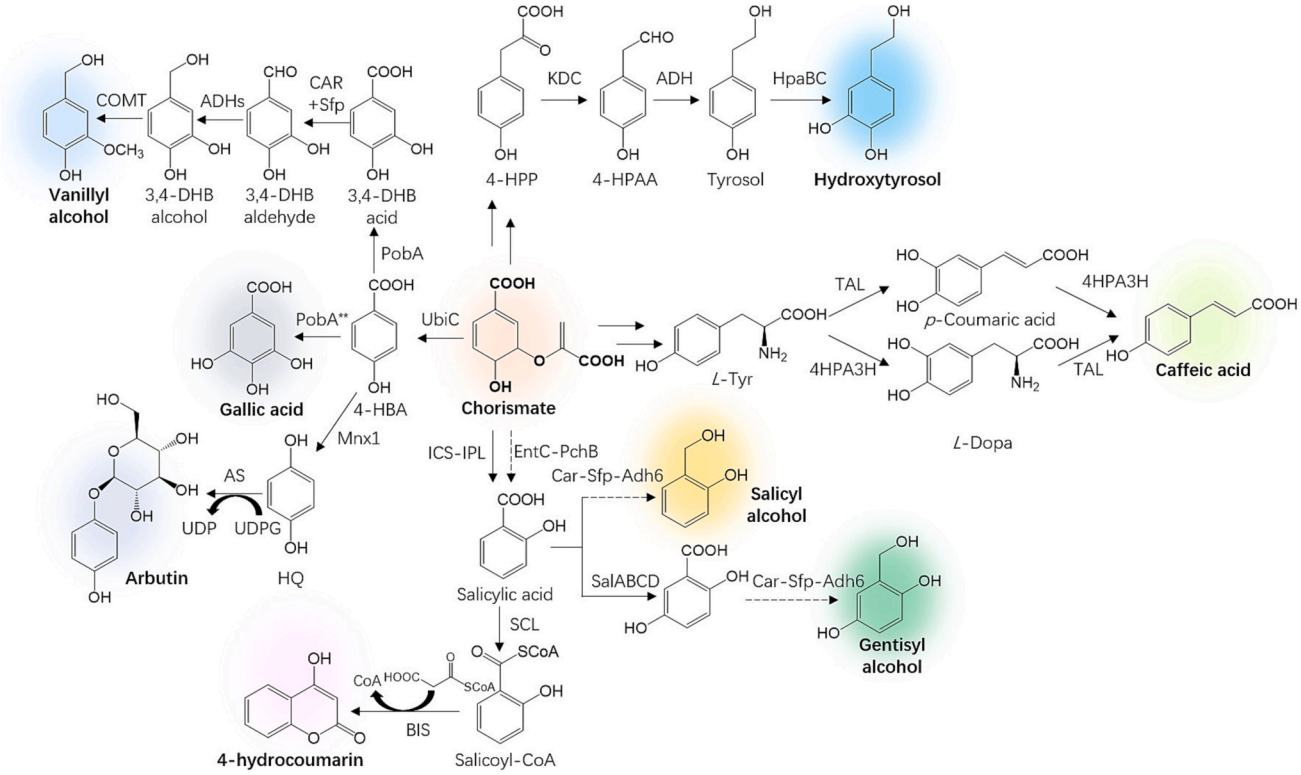

Fig. 5. Exploring artificial pathways for biosynthesis of fine chemicals. 4-HPP, 4-hydroxyphenylpyruvate; 4-HPAA, 4-hydroxyphenylacetaldehyde; 4-HPA, 4-hydroxyphenylacetic acid; KDC, ketoacid decarboxylase; ADH, alcohol dehydrogenase; HpaBC, 4-hydroxyphenylacetic acid 3-hydroxylase; UbiC, chorismate lyase; PobA, p-hydroxybenzoate hydroxylase; PobA**, p-hydroxybenzoate hydroxylase with Y385F and T294A mutations; CAR, carboxylic acid reductase; Sfp, the CAR maturation factor phosphopantetheinyl transferase; ADHs, alcohol dehydrogenases; COMT, caffeate *O*-methyltransferase; EntC, isochorismate synthase; PchB, isochorismate pyruvate lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; Car, carboxylic acid reductase; SalABCD, anthranilate 5-hydroxylase; 4HPA3H, 4-hydroxyphenylacetate 3-hydroxylase; TAL, tyrosine ammonia lyase; MNX1, 4-hydroxybenzoate 1-hydroxylase; AS, arbutin synthase; SCL, salicylate: CoA ligase; BIS, biphenyl synthases.

been widely used as flavoring agent in foods and beverages (Liu et al., 2002; Ong et al., 2015). Currently, direct extraction from various plants imcluding *Gastrodia elata* Blume (Ong et al., 2007) and *Vanilla planifolia* (Shyamala et al., 2007) is still the main method for vanillyl alcohol production, but suffers from the supply of raw material, high cost and low yield. To address such problems and realize the synthesis of vanillyl alcohol from bionewable sugars, Chen and coworkers proposed an artificial vanillyl alcohol pathway (Chen et al., 2017b). The pathway uses the 4-hydroxybenzoic acid, a metabolite of chorismate pathway, as substrate. Besides the endogenous alcohol dehydrogenase (ADH), three heterologous enzymes including p-hydroxybenzoate hydroxylase (PobA), carboxylic acid reductase (CAR) and caffeate *O*-methyltransferase (COMT) are required for the complete conversion of 4-hydroxybenzoic acid to vanillyl alcohol (Fig. 5). Finally, the final engineered *E. coli* strain produced 0.24 g/L of vanillyl alcohol, the highest titer reported through microbial biosynthesis (Chen et al., 2017b).

3.3.3. Gallic acid

Gallic acid (GA) represents a naturally existing chemical with strong antioxidant and antibacterial activities (Aruoma et al., 1993; Yen et al., 2002). Traditionally, the major way for GA production highly depends on hydrolytic degradation of tannins by acids, bases or microbes (Beniwal et al., 2010; Kar et al., 1999; Treviño-Cueto et al., 2007), which is costly and causes environmental pollution. Since there is no known natural pathway for GA production from biorenewable feedstocks, Chen et al. reported an artificial GA biosynthetic pathway from sugars (Chen et al., 2017a). Through expression of a *Pseudomonas aeruginosa* p-hydroxybenzoate hydroxylase (PobA) Y385F/T294A mutant in *E. coli*, they achieved bioconversion of 1 g/L of 4-HBA into 1.1 g/L of GA. On this basis, *de novo* production of GA was achieved by enhancing the concentration of 4-HBA within *E. coli* cells (Fig. 5). Further improvement of precursor supply through overexpression of key enzymes in shikimate pathway produced 1.2 g/L of GA from simple carbon sources in shake flasks (Chen et al., 2017a).

3.3.4. Salicyl alcohol and gentisyl alcohol

Salicyl alcohol and gentisyl alcohol are two valuable phenolic alcohols with appealing biological and pharmaceutical activities (Alfaro et al., 2003; Sumit et al., 2014). Since production of those two alcohols in microbes had not been reported yet, Shen et al. reported novel pathways for synthesis of salicyl alcohol and gentisyl alcohol from simple carbon sources (Shen et al., 2018b). Both pathways start from salicylic acid, and the salicyl alcohol can be directly generated from salicylic acid by carboxylic acid reductase (CAR). For formation of gentisyl alcohol, salicylic acid is firstly converted into 2,5-dihydroxy benzoic acid (2,5-DHBA) by salicylic acid 5-hydroxylase, then the 2,5-DHBA is catalyzed by CAR to yield the final product (Fig. 5). When these pathways were assembled in *E. coli*, ~0.6 g/L of salicyl alcohol and ~ 0.03 g/L of gentisyl alcohol were produced (Shen et al., 2018b).

3.3.5. Caffeic acid

Caffeic acid (3,4-dihydroxycinnamic acid) and its derivatives such as caffeic acid phenethyl ester (CAPE) exhibit appealing pharmaceutical and health-protection activities (Celik et al., 2009; Chao et al., 2009; Mori and Iwahashi, 2009; Prasad et al., 2011; Ikeda et al., 2011), and thus have received great attention in the past decade. Currently, their production process mainly relies on extraction from plants such as coffee beans. In contrast, microbial biosynthesis provides a good alternative

approach for caffeic acid production, but it also suffers from low yield and high cost associated with the feeding of expensive precursors such as tyrosine or p-coumaric acid (Choi et al., 2011; Sachan et al., 2006; Yan et al., 2005a, 2005b). Therefore, Lin and Yan built an artificial dual pathway mediated by 4-hydroxyphenylacetate 3-hydroxylase (4HPA3H) and tyrosine ammonia lyase (TAL) which use L-tyrosine as substrate for caffeic acid biosynthesis (Lin and Yan, 2012) (Fig. 5). Expression of this pathway in *E. coli* produced 50.2 mg/L caffeic acid (Lin and Yan, 2012).

3.3.6. 4-Hydroxycoumarin

4-Hydroxycoumarin (4HC) type anticoagulants such as warfarin have been used for treatment of thromboembolic diseases. 4HC serves as direct precursor for synthesis of these anticoagulants (Beinema et al., 2008; Ivanov et al., 1990; Melnikova, 2009). Although 4HC was found as a naturally existing compound, its biosynthetic pathway still remains unknown. Lin and colleagues designed an artificial biosynthetic pathway for achieving microbial biosynthesis of 4HC (Lin et al., 2013). This pathway centers around salicylic acid and can be divided into upper and lower modules. The upper module consists of isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL), and is responsible for converting chorismate into salicylic acid. The lower module consists of salicylate:CoA ligase (SCL) and biphenyl synthases (BIS), which converts salicylic acid to salicoyl-CoA intermediate and then to 4HC (Fig. 5). When the upper and lower modules were assembled in *E. coli*, ~500 mg/L of 4HC was produced from glycerol (Lin et al., 2013).

3.3.7. Lactams

Lactams are valuable compounds which can be directly polymerized into polyamides. However, no known natural biosynthetic pathways have been reported for direct production of lactams. Researchers from different groups separately developed *E. coli* strains for producing lactam through distinct biosynthetic pathways (Chae et al., 2017; Zhang et al., 2016). Zhang et al. propose an artificial 2-pyrrolidone biosynthetic pathway consisting of two-steps which uses glutamate as substrate. In this design, glutamate is firstly converted to γ-aminobutyrate (GABA) by glutamate decarboxylase, and butyrolactam synthase subsequently catalyzes the ring closing of GABA into 2-pyrrolidone. Although enzymes for catalyzing the first step are known, enzymes for the second step remain unclear. Zhang et al. successfully identified a butyrolactam synthase from *Streptomyces aizunensis* for catalyzing the second step. By co-expression of glutamate decarboxylase and butyrolactam synthase, the resulting strain produced ~1.1 g/L of butyrolactam from glutamate (Zhang et al., 2016), which illustrates the feasibility of this artificial pathway. In contrast, Chae et al. proposed a different pathway for producing various lactams with four-, five- and six-carbons. This pathway relies on a key β-alanine-CoA transferase which activates ω-amino acids to corresponding ω-amino acyl-CoAs followed by spontaneous cyclization to final lactams. Finally, overexpression of the alanine-CoA transferase in different *E. coli* strains for producing 4-aminobutyric acid, 5-aminovaleric acid, and 6-aminocaproic acid enabled the production of 54.1 g/L of butyrolactam, 1.2 g/L of valerolactam and 79.6 μg/L of caprolactam, respectively (Chae et al., 2017). Besides *E. coli*, biosynthesis of β-lactams was also achieved in yeast. By combining natural and artificial enzymes, Yang et al. constructed an artificial pathway in yeast for *de novo* synthesis of several β-lactams including 6-amino penicillanic acid (~5 mg/g DCW), 7-amino cephalosporanic acid (~6 μg/g DCW), and 7-amino desacetoxy cephalosporanic acid (~2 mg/g DCW) (Yang et al., 2022).

3.3.8. Didanosine

Didanosine represents an off-patent inhibitor of HIV-1 reverse transcriptase (Dziuban et al., 2015), and has been widely used as antiviral and anticancer drug in clinical treatment (Aschacher et al., 2012; Damaraju et al., 2003; Lembo and Cavalli, 2010; Shi et al., 2016). Given the high cost of current didanosine manufacturing (Pinheiro et al., 2006), developing a low-cost and high-efficient alternative approach for didanosine production is highly desirable. Birmingham et al. harnessed the bioretrosynthesis approach and proposed an artificial pathway for didanosine biosynthesis (Birmingham et al., 2014). In practice, the available substrate of 2,3-dideoxyribose is phosphorylated by ribokinase (RK) to generate 2,3-dideoxyribose 5-phosphate, which is subsequently catalyzed by 1,5-phosphopentomutase (PPM) to form 2,3-dideoxyribose 1-phosphate. Then, a hypoxanthine is added to the 2,3-dideoxyribose 1-phosphate precursor to result in the final didanosine, which is catalyzed by purine nucleoside phosphorylase (PNP). Through both structure-based rational engineering as well as directed evolution, activities and selectivity of key pathway enzymes were boosted. Finally, after these efforts, didanosine production increased by 50-fold (Birmingham et al., 2014). This work shows the huge potential of bioretrosynthesis for building non-natural biosynthetic pathways.

3.4. Production of biofuels

3.4.1. Higher alcohols

In contrast to the traditional biofuel ethanol, higher alcohols especially the branched-chain ones possess additional advantages such as higher energy density and lower hygroscopicity, which make them better gasoline substitutes (Cho et al., 2010). However, using native organisms to synthesize these alcohols is still not economically feasible. To this end, Atsumi and Liao proposed a novel metabolic pathway in *E. coli* which combines the active endogenous amino acid biosynthetic pathway for synthesizing 2-keto acid intermediates with compatible heterologous pathway for diverting 2-keto acid intermediates to final alcohols (Atsumi et al., 2008). When implemented in *E. coli*, several higher alcohols such as isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol are efficiently produced from glucose. Specifically, approximately 22 g/L of isobutanol was produced in an engineered *E. coli* strain under microaerobic conditions (Atsumi et al., 2008).

Branched five carbon (C5) alcohols represent attractive advanced biofuels. Traditional biosynthesis of isopentenols relied on the dephosphorylation of IPP. However, IPP is toxic to microbial host cells. To this end, Kang et al. designed two artificial IPP-bypass MVA pathways for C5 alcohol production by exploiting the promiscuities of phosphomevalonate decarboxylase (PMD) and phosphatase (AphA). Compared with traditional route, these bypass pathways required less ATP energy, a smaller number of enzymes, and got rid of the IPP toxicity issue (Kang et al., 2016). Clomburg et al. also developed a novel pathway for isopentenols production. This pathway started from acetyl-CoA. This pathway utilized the condensation of two acetyl-CoA, followed by the condensation of acetoacetyl-CoA and acetyl-CoA to yield the intermediate 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). Subsequently, HMG-CoA was catalyzed through dehydration, decarboxylation and reduction to yield the final prenol. Different from the use of traditional pathway leads to a mixture of C5 alcohols, recombinant *E. coli* with this novel pathway selectively produced nearly 2 g/L prenol in 48 h (Clomburg et al., 2019).

3.4.2. Biodiesels

Biodiesels, including fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs), are promising alternatives to the current diesel fuel (Rahman et al., 2019). Traditionally, FAMEs/FAEEs are obtained by transesterification reaction between methanol/ethanol and fats from plants, animals and microbes. With the goal of producing biodiesel at low-cost, it is desirable to engineer microbes that could grow on lignocellulose-derived sugars to directly produce FAMEs/FAEEs (Rahman et al., 2019). However, wild type *E. coli* is not capable of synthesizing FAMEs/FAEEs and the biggest challenge lies in the transesterification reaction. To address this limitation, a non-native pathway was constructed in *E. coli* to generate FAEEs (Kalscheuer et al., 2006). This pathway relies on a heterologous acyltransferase from

Acinetobacter baylyi with broad range of substrates. In combination with the other ethanol synthesis module from *Z. mobilis*, the final engineered *E. coli* strain produced 1.28 g/L of FAEEs (Kalscheuer et al., 2006). Polycyclopropanated fatty acid methyl esters (POP-FAMEs) are excellent fuels with higher energy density than currently used aerospace fuels. In 2022, Cruz-Morales et al. identified a potential fuelimycins iterative polyketide synthase. When expressed in in *Streptomyces coelicolor*, polycyclopropanated fatty acids (POP-FAs) were produced (Cruz-Morales et al., 2022).

3.4.3. Biogasoline

Besides biodiesel, biosynthesis of gasoline also has received growing attentions in the past decades. Gasoline is a mixture of straight- and branched-chain C4-C12 alkanes. Choi and Lee reported the utilization of *E. coli* as host for producing short-chain alkanes through a pathway that converts fatty acyl-ACPs to fatty acids then to fatty acyl-CoAs (Choi and Lee, 2013). Specifically, an engineered thioesterase was employed for hydrolysis of short-chain fatty acyl-ACPs to free fatty acids. Next, the authors recruited *E. coli* fatty acyl-CoA synthetase, *Clostridium acetobutylicum* fatty acyl-CoA reductase and *Arabidopsis thaliana* fatty aldehyde decarbonylase for complete conversion of free fatty acids to short-chain alkanes. Finally, the engineered strain produced 0.58 g/L of short-chain alkanes (Choi and Lee, 2013).

3.5. Synthesis of natural products

3.5.1. Isoprenoids

Currently, more than 65,000 structures of isoprenoids have been found in nature (Agatonovic-Kustrin and Morton, 2018). All of these chemicals are synthesized from the C5 diphosphate building blocks IPP and DMAPP. Naturally, IPP and DMAPP are yielded by either MVA pathway or MEP pathway (Drummond et al., 2019), both of which have been manipulated for synthesis of various isoprenoids with a wide variety of applications (Liu et al., 2019; Niu et al., 2017; Vavitsas et al., 2018; Vickers et al., 2014; Volke et al., 2019; Wang et al., 2018; Ward et al., 2018). Nevertheless, as both pathways intrinsically suffer from energy and carbon inefficiencies, as well as complicated regulation, isoprenoids biosynthesis at high levels remain challenging (Clomburg et al., 2019). Therefore, metabolic engineers turned to exploring non-natural pathways such as the isoprenoid alcohol (IPA) pathway (Clomburg et al., 2019) (Fig. 2C). the alcohol-dependent hemiterpene (ADH) pathway (Lund et al., 2019) and the isopentenol utilization pathway (IUP) (Chatzivasileiou et al., 2019) for synthesis of IPP and DMAPP. These synthetic pathways address energy and carbon inefficiencies, as well as complicated regulation, which provide good alternatives to naturally evolved MEP/MVA pathways for isoprenoids biosynthesis.

Besides the upstream section of isoprenoids biosynthesis (*i.e.*, C5 diphosphate building blocks formation), artificial pathways were also developed towards the downstream section of isoprenoids biosynthesis, *i.e.*, conversion of natural isoprenoids to non-natural isoprenoids. In particular, Huang et al. achieved the biosynthesis of a non-natural terpenoid cyclopropyl limonene in *E. coli* by combing limonene biosynthesis and the catalysis by an artificial metalloenzyme Ir-CYP119 (ArM) (Huang et al., 2021). These new artificial pathways broaden the scope of chemicals produced by organisms.

3.5.2. Polyketides

Polyketides are a large class of secondary metabolites with intriguing biological and pharmaceutical activities (Hertweck, 2009; Ma et al., 2009). Naturally, synthesis of polyketide backbones is catalyzed by polyketide synthases (PKSs) through iterative decarboxylative Claisen condensation reactions with malonyl-CoA as extender units (Jiang et al., 2008). However, this process is restricted by complex architecture of PKSs, carbon and energy losses, tight regulation, and direct competition with cellular essential metabolisms (Chan et al., 2009; Huo et al., 2019; Keatinge-Clay, 2012). To overcome these limitations, Tan et al. proposed an artificial PKT pathway for synthesis of polyketide backbone as mentioned in the Section 2.1 (Tan et al., 2020). As proof-of-concept examples, they showed that PKTs can synthesize a variety of backbones for representative lactone (triacetic acid lactone), alkylresorcinolic acid (orsellinic acid), alkylresorcinol (orcinol), hydroxybenzoic acid (6-methylsalicylic acid) and alkylphenol (*m*-cresol) polyketide families (Tan et al., 2020) (Fig. 2D). More recently, PKTs have been further employed for synthesis of bioactive compounds. For instance, a variety of α-pyrone derivatives including 4-hydroxy-6-styryl-α-pyrone, 4-hydroxy-6-phenethyl-α-pyrone and 4-hydroxy-6-pentyl-α-pyrone, have been successfully produced in *E. coli* by combing different PKTs and CoA ligases (Huang et al., 2022).

3.5.3. Phenylpropanoids

Phenylpropanoids include a wide variety of secondary metabolites with great potential for pharmaceutical and nutraceutical applications (Manach et al., 2004; Spatafora and Tringali, 2012). The core steps for phenylpropanoids production are deamination of L-tyrosine (or *L*-phenylalanine) catalyzed by *L*-phenylalanine ammonia lyases (PAL) (or L-tyrosine ammonia lyases, TAL) to yield 4-coumaric acid (or cinnamic acid), as well as a subsequent reaction catalyzed by 4-coumarate-CoA ligases (4CL) to form cinnamic acid 4-coumaroyl-CoA (or cinimoyl-CoA) (Jangaard, 1974; Liu et al., 2017). From 4-coumaroyl-CoA, a broad range of phenylpropanoids including lignols, (iso)flavonoids, stilbenes, catechin and naringenin, can be formed (Vogt, 2010). However, 4-coumaroyl-CoA formation suffers from low activity of PAL and 4CL, which becomes rate-limiting for efficient synthesis of phenylpropanoids (Philana et al., 2015). To this end, Kallscheuer et al. developed an alternative strategy to supply 4-coumaroyl-CoA which uses 4-hydroxybenzoic acid (4-HBA) as substrate (Kallscheuer et al., 2017). First, the CoA ligase is responsible for conversion of 4-HBA to 4-hydroxybenzoyl-CoA. Next, similar to the first three steps of r-BOX, 4-hydroxybenzoyl-CoA will be catalyzed by thiolase, 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, to yield 4-coumaroyl-CoA. This pathway was experimentally verified in *Corynebacterium glutamicum* with stilbene synthase (STS) for resveratrol production (Kallscheuer et al., 2017). Finally, the engineered strain produced 4.8 mg/L of resveratrol, indicating the feasibility of this artificial pathway for 4-coumaroyl-CoA formation independent from aromatic amino acid metabolism (Kallscheuer et al., 2017).

3.5.4. Alkaloids

Alkaloids are natural products including basic nitrogen atoms. Most alkaloids have been revealed to possess intriguing analgesic, sedative, and anti-cancer activities (Chen et al., 2020b; Yang and Stockigt, 2010). Among those alkaloids, the synthetic pathway of benzylisoquinoline alkaloids (BIAs) in plants remains unclear. Although the pathway from L-tyrosine to dopamine was proposed, not all of the enzymes have been identified as yet. Therefore, Nakagawa et al. proposed an artificial pathway for reticuline synthesis and built a microbial platform for synthesis of plant alkaloids (Nakagawa et al., 2011). The engineered strain produced up to ~46.0 mg/L of (*S*)-reticuline using glycerol as sole carbon source, indicating the feasibility of such microbial platform for cost-effective synthesis of alkaloids. In addition, Yang et al. reported an artificial pathway to synthesize plant tetrahydroisoquinoline alkaloids (THIQAs) from dihydroisoquinoline through the catalysis of imine reductase (IRED) and *N*-methyltransferase (NMT) (Yang et al., 2020b). By combining IRED, NMT, and glucose dehydrogenase (GDH) in one reaction, two artificial biosynthetic pathways were effectively built in *E. coli* and successfully applied to the synthesis of five (*S*)-THIQAs, three of whose natural biosynthetic pathways have not been elucidated (Yang et al., 2020b).

3.5.5. Other natural products

The C_7N aminocyclitols of valienamine and β-valienamine are precursors for synthesis of natural glycosidase and β-glycosidase inhibitors

for treatment of lysosomal storage diseases (Cumpstey et al., 2008; Ogawa et al., 1983). Chemical synthesis of C_7N aminocyclitols remains challenging because of the presence of multi-chiral centers in their structures (Mahmud, 2003; Ogawa et al., 2007). Cui and coworkers designed novel pathways for direct biosynthesis of valienamine and β-valienamine in *Streptomyces hygroscopicus* (Cui et al., 2020; Cui et al., 2016). In practice, different heterologous aminotransferases with stereospecificity were mined, identified and engineered. Specifically, they revealed that the BtrR aminotransferase from *Bacillus circulans*, was capable of converting valienone to β-valienamine. When introduced BtrR into *S. hygroscopicus*, the engineered strain produced 20 mg/L of β-valienamine (Cui et al., 2016). They further mined the aminotransferases and the aminotransferase WecE enzyme from *E. coli* was identified with the activity for conversion of valienone to valienamine. After enzyme engineering through directed evolution, a better variant (VarB) with activity improving by 32.6-fold was successfully obtained and introduced into *S. hygroscopicus* host. Finally, the related engineered strain produced ~0.52 mg/L valienamine (Cui et al., 2020). These studies demonstrated the feasibility of developing simplified artificial pathways for valienamine and β-valienamine production.

4. Conclusions and future perspectives

Classical metabolic engineering efforts are partial to employing the traditional "copy, paste, tuning" strategies and approaches to realize greater substrate utilization and synthesis of various products in either native or heterologous hosts. Given the inherent limitations of natural metabolic pathways, these approaches do not explore the full potential of biosynthesis. To this end, the rational design of non-natural pathways can lead to realizing true synthetic metabolism. With recent advances in computational biology, enzyme design, and synthetic biology, metabolic engineers are no longer restricted by the "given but not good enough" naturally existing pathways but can obtain more effective metabolic pathways by artificial design.

Currently, artificial pathways have been widely used in several areas, including utilization of one-carbon feedstocks and biosynthesis of a verity of target products including bulk chemicals, fine chemicals, biofuels and natural products, as reviewed systematically in this study (Fig. 6). However, as the most promising method for designing artificial pathways, retrobiosynthesis prediction analysis, often suffers from inherent drawbacks. One of the biggest drawbacks is that the false positive of prediction is excessively high, which impairs the acceptance and application of these tools. This might be due to that the core algorithms of these prediction tools cannot fully simulate the actual matching state between artificial pathways and chassis cells. Mismatching events that inability to accurately evaluate the toxicities of all intermediates of artificial pathway to the specific chassis cells, and the ignored effect of cellular structures (*e.g.*, compartmentalization) on performance of artificial pathway, often occur. Besides, most prediction tools and databases still lack enough data about multiple metrices of enzymes, including but not limited to specific activities, promiscuities and stabilities under different circumstances.

In future, fully leveraging artificial pathways will require additional developments. The first is further optimization of computational tools for pathways prediction and ranking. Besides currently used physicochemical metrics such as theoretical yield, thermodynamic feasibility and kinetic proficiency, other metrics such as synthetic complex score, availability of intermediates, toxicity of intermediates and cofactor consumption should be also considered when computational tools are developed in future. Secondly, artificial pathways should be considered to match well with native metabolism of chassis cells. As different chassis cells such as *E. coli*, *S. cerevisiae*, *B. subtilis* and *Streptomyces* possess respective advantages and disadvantages, performances of artificial pathways will vary or even contrary. As the proverb of "One man's meat is another man's poison" said, the potential restrictions and regulations from chassis cells should be also considered when designed and applied the artificial pathways.

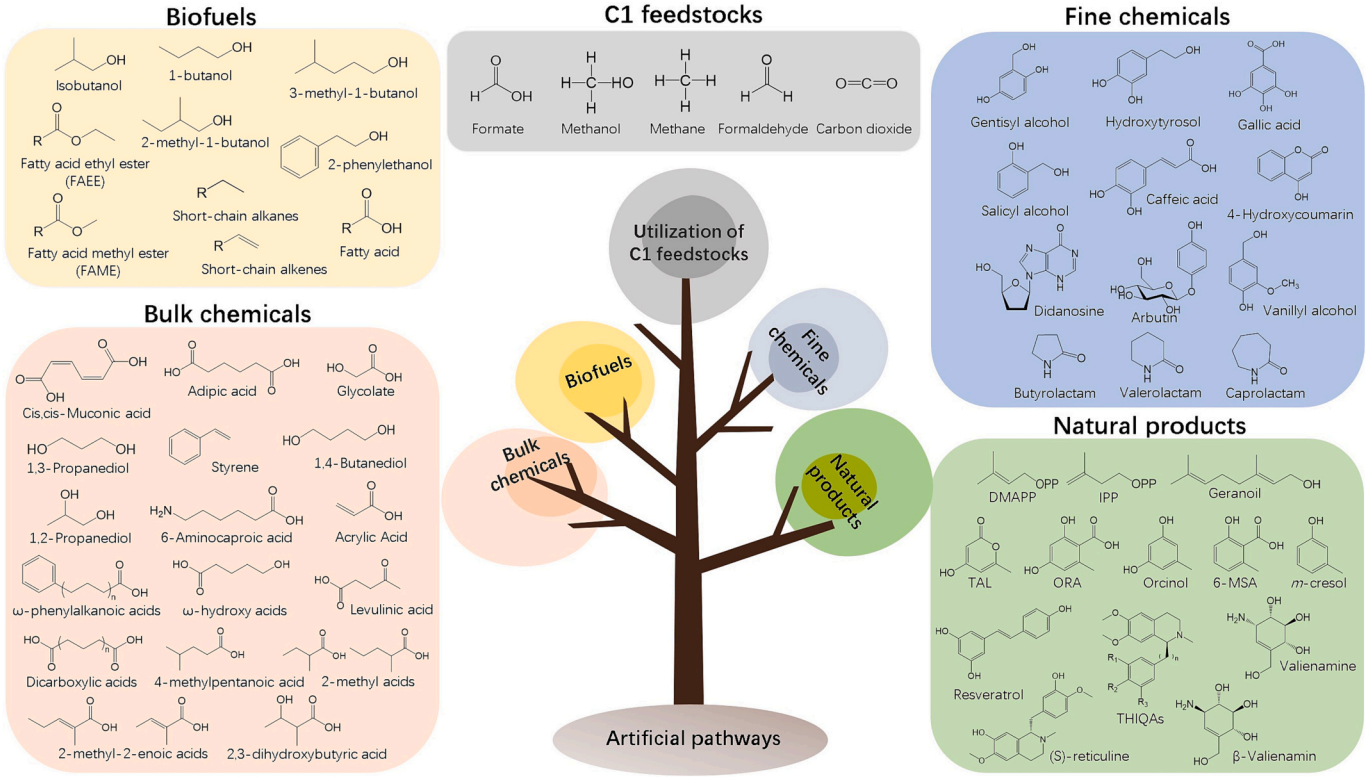

Fig. 6. Summary of application of artificial pathways. Artificial pathways have been widely applied to one-carbon feedstocks utilization, as well as biosynthesis of bulk chemicals, fine chemicals, biofuels and natural products.

Declaration of Competing Interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

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