Enhancing microbial production of biofuels by expanding microbial metabolic

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Abstract

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bab.1529.

Fatty acid, isoprenoid and alcohol pathways have been successfully engineered to produce biofuels. By introducing three genes, *atfA*, *adhE* and *pdc*, into *Escherichia coli* to expand fatty acid pathway, up to 1.28 g/L of fatty acid ethyl esters can be achieved. The isoprenoid pathway can be expanded to produce bisabolene with a high titer of 900 mg/L in *Saccharomyces cerevisiae*. Short- and long-chain alcohols can also be effectively biosynthesized by extending the carbon chain of ketoacids with an engineered "+1" alcohol pathway. Thus, it can be concluded that expanding microbial metabolic pathways has enormous potential for enhancing microbial production of biofuels for future industrial applications. However, some major challenges for microbial production of biofuels should be overcome in order to compete with traditional fossil fuels: lowering production costs, reducing the time required to construct genetic elements and to increase their predictability and reliability, and creating reusable parts with useful and predictable behavior. To address these challenges, several aspects should be further considered in future: mining and transformation of genetic elements related to metabolic pathways, assembling biofuel elements and coordinating their functions, enhancing the tolerance of host cells to biofuels, and creating modular sub-pathways that can be easily interconnected.

Key words

Microbial production; Biofuels; Fatty acid-derived pathway; Isoprenoid-derived pathway; Alcohol-derived pathway

1.Introduction

Biofuels have been produced from fatty acids of plant and animal oils for more than a century.

However, the increasing demand of biofuels has currently resulted in an enormous competition with food supply and heavily economic and ethical problems owning to the use of a large number of crops[1-4]. Biofuels are renewable, non-toxic and biodegradable compared to traditional fossil fuels[5-7]. They can be used alone, or in combination with traditional fossil fuels, to reduce the emission of harmful gases and particulate matters[6]. The first-generation biofuels whose main components are fatty acid methyl- or ethyl-ester derive from soybean, rapeseed or palm oil[8, 9]. The extra chemical or biological transesterification is required during their production, leading to a high cost and a complex

process[10-12]. Molecular structures of produced biofuels rely on acyl moieties of triglycerides from raw materials. It is therefore difficult to carry out further transformation to improve the production and performance of biofuels[13]. Researchers have devoted to exploring new catalytic methods and processes[14-22], looking for efficient transesterases [13, 15, 23-27], optimizing the operation conditions [28-36], and searching for new raw materials[33-35, 37, 38] to resolve above problems. Although some successes have been achieved in recent several years, a series of energy-intensive processes can not still be removed [39].

Biofuels can be produced by oleaginous microorganisms, such as yeast, fungi and bacteria [40-43]. Traditional microbial strains for industrial applications have been developed by the random mutagenesis and the screening processes[44]. However, these approaches result in unknown genotypic/phenotypic changes and can cause unwanted alterations in the genome that may become problematic when the fermentation conditions need to be modified. Recent advances in microbial engineering also offer opportunity to convert renewable resources into biofuels[45]. After the chemical treatment of feedstocks, cellulosic biomass is decomposed into simple sugars, which are metabolized by microorganisms and converted into biofuels. Although some microorganisms can naturally produce certain biofuels[46, 47], native microorganisms often suffer from low growth rates, intolerance of toxic biofuel products, and

incomplete utilization of carbon sources. For example, many microorganisms cannot metabolize xylose, which comprises approximately 30% of plant cellulosic biomass.

With the development of metabolic engineering, the microbial production of biofuels by expanding microbial metabolic pathways has gradually become possible[48-55]. In comparison to other techniques, metabolic engineering has the following advantages: renewability, shorter production cycle, less labor requirement, less influence of venue, season and climate, and easier to being scaled up[56-62]. It allows successful development of microorganisms that are capable of producing different biofuels, including bioethanol, biobutanol, 1,3-propanediol, 2,3-butanediol, alkane, and even hydrogen[63-71]. By introducing promiscuous enzymes into engineered strains, a powerful platform with a scalable, controllable and economic route for the production of advanced biofuels can be established[72-75]. Different metabolic engineering strategies for biofuel production include the modulation of redox metabolism, in silico gene insertion, gene knockout, and the expression of different gene combinations, etc. The advanced biofuels produced by metabolic engineering have similar properties as traditional fuels[76-78], and the comparison of them with traditional fuels is listed in Table 1. The challenge of using wild-type microorganisms to produce advanced biofuels is to resolve endogenous regulations pertinent to metabolic pathways of the biofuel production in order to achieve high yields. Expanding or reconstruction of biofuel-producing pathways in heterologous hosts is possible, including

Escherichia coli and *Saccharomyces cerevisiae*[79, 80]. However, some challenges must be solved in order to maximize the metabolic flux, such as the overexpression of plant- derived enzymes and the balance of their activities[81]. Genetically intractable hosts with the capacity to convert non-sugar substrates or with a high solvent tolerance are attractive alternatives when different raw materials are used. In the past ten years, the production of biofuels directly from abundant and cost-effective renewable resources using microorganisms by expanding their metabolic pathways has achieved great progresses[58, 82-85]. For example, the substitutes for gasoline and diesel, bioethanol and biodiesel, respectively, have been marketed [86-88]. Herein, we review the latest advances in enhancing microbial production of biofuels by expanding microbial metabolic pathways.

2.Fatty acid-derived pathways

Fatty acids are an important source of energy and adenosine triphosphate for many organisms. Excessive fatty acids, glucose and other nutrients can be stored as fat. Triglycerides yield more than twice as much energy as do carbohydrates or proteins. Fatty acid-derived pathways in cells are used to biosynthesize basic components of cell membrane, and are also necessary for the cell growth and survival.

2.1 Fatty acid metabolic pathway and its regulation

Main catalytic enzymes of the fatty acid pathway include acetyl-CoA carboxylase (*ACC*), fatty acid synthase and thioesterase. *ACC* and fatty acid synthase are divided into I- and II-type ones. Catalytic centers of I-type *ACC* from *S. cerevisiae* are located in a polypeptide chain, whereas ones of II-type *ACC* from the model organism *E. coli* exist in the form of separate proteins [89, 90]. Activities of enzymes related to the fatty acid pathway have been studied systematically, and even their crystal structures have been resolved in detail[91-93].

Fatty acid metabolic pathway in *E. coli* is illustrated in Fig. 1[94-96]. Malonyl-CoA is formed by *ACC* with acetyl-CoA as the substrate, and then fatty acyl carrier protein is synthesized by fatty acid synthase with malonyl-CoA and acetyl-CoA as starting units and with malonyl-CoA as a prolonging unit. Most of fatty acyl groups are transferred from acyl carrier proteins by glycerol -3 -phosphate transferase to form lipids, main raw materials for synthesizing cell membrane. Minor of fatty acyl groups are converted to lipid A and lipoic acid. Free fatty acids are released from excessive fatty acyl *ACP*s by thioesterase. Excessive fatty acids are degraded quickly by β-oxidative pathway, and eventually generate acetyl-CoA[97]. All genes from fatty acid pathway are tightly regulated by *FabR* and *FadR* proteins at transcriptional level[98, 99], but the long-chain fatty acyl *ACP* has an obvious feedback inhibition to activities of *ACC, FabI* and *FabH* at protein level[100-102]. Due to

above regulations, *E. coli* cannot accumulate fatty acids under natural conditions, and this must be solved if biofuels are produced in *E. coli* by fatty acid pathway.

2.2 Modification of the fatty acid pathway

2.2.1 *ACC* is a key rate-limiting enzyme

The first-step reaction of the fatty acid pathway catalyzed by *ACC* is rate-limiting. The activity of *ACC* has an obvious impact on the rate of the entire fatty acid pathway. Cronan *et al*. [103] cloned the genes coding four subunits of *ACC* from *E. coli*, *accB, accC, accD* and *accA*, into a plasmid under the control of T_7 promoter, and this obviously improved the content of malonyl- CoA in transformed strain. However, enhancing the content of *ACC* only is not adequate for enhancing the production of fatty acids. The high expression of *ACC* and the introduction of thioesterase can weaken the feedback inhibition of the long-chain fatty acyl *ACP* to the entire fatty acid pathway, which can increase the concentration of malonyl-CoA by 100-fold[104]. Moreover, the yield and productivity of fatty acids can reach 4.0 g/(L.d) and 0.04 g/(h.gDCW, dry cell weight) by introducing thioesterase from plant seed into *E. coli*, respectively[105].

2.2.2 Inhibiting the β-oxidative pathway for the accumulation of fatty acids

The β-oxidative pathway of fatty acids has to be inhibited, or even be interrupted when biofuels are produced by fatty acids or their metabolic intermediates in microbial cells.

Steen *et al*. [106] reported that the content of fatty acids was improved by 4-fold when *fadE*, a gene encoding a fatty acyl-CoA dehydrogenase of the β-oxidative pathway, was knocked out, and was up to 1.2 g/L when a thioesterase gene was expressed simultaneously. Lu *et al*. [107] reported that the content of fatty acids was improved by 3-fold when *fadD*, a gene coding a fatty acyl-CoA synthase in β-oxidative pathway, was knocked out. Moreover, in the *fadD* deleted strain, the coexpression of three genes, two thioesterases from *Cinnamomum camphorum* and *E. coli*, as well as ACC from *E. coli*, resulted in 20-fold enhancement of the total fatty acid production in shake flasks. 2.5 g/L of fatty acids were produced by this engineered strain in fed-batch cultivation, but no cultivation optimization was further conducted. With further efforts done to increase fatty acid production by targeting the genes in the fatty acid biosynthetic pathway, the productivity was increased to 4.5 g/L/d [105]. The reversal of the β-oxidative pathway was also engineered as a metabolic platform to synthesize fatty acids in *E. coli* with various chain lengths above 10 carbons at a high titer of 7 g/L and a yield of 0.28 g/g glucose[108].

2.2.3 Enhancing the production of fatty acids by metabolic engineering

Overall, enhancing the content of fatty acids by expanding microbial metabolic pathways is suggested as follows (Fig. 2): (1) overexpression of *ACC* in *E. coli* to increase the content of the substrate malonyl-CoA, (2) overexpression of thioesterase containing no leader peptide in *E. coli* to weaken the inhibition of fatty acyl *ACP* to the entire fatty acid pathway, (3)

heterologous expression of thioesterase with a medium-chain fatty acyl *ACP* as the most appropriate substrate to regulate product components, and to remove the inhibition of fatty acyl *ACP* to the entire fatty acid pathway, (4) knock-out of *fadD*, a gene coding a fatty acyl CoA synthase in the β-oxidative pathway, to increase the accumulation of free fatty acids, (5) heterologous expression of *ACC*, (6) expression of acyl carrier protein, (7) increasing the concentration of acetyl-CoA, and (8) regulating the concentration of thioesterase in *E. coli*. Keasling *et al*. (2009) obtained a mutated *E. coli* strain with a yield of 1.2 g/L of fatty acids by the knockout of *fadD* and *fadE* and the expression of thioesterase. Zhang *et al*. found that *FadR* could enhance the fatty acid production by globally tuning the expression levels of many genes to optimal levels. By tuning the expression of *FadR* in *E. coli*, the fatty acid titer was increased by 7.5-fold to 5.2 ± 0.5 g/L, with the yield accounting for 73% of the theoretical yield[109]. Moreover, some strategies for the enhancement of the accumulation of fatty acids in *E. coli* by expanding its metabolic pathways and their yields are listed and compared in Table 2.

2.3 Production of biofuels by fatty acids and their metabolic intermediates

Generally, fatty acids and their metabolic intermediates are not the final products. The production of biofuels by fatty acid-derived pathways can be divided into two stages: (1) a lot of fatty acids or their metabolic intermediates are produced by modifying their biosynthetic

pathways; (2) the final biofuels can be obtained by the introduction of promiscuous enzymes to modify fatty acids or their metabolic intermediates.

2.3.1 Production of fatty acid ethyl esters

Kalscheuer *et al*. [110] found a key enzyme for the biosynthesis of biofuels—wax ester synthase/diacylglycerol acyltransferase (*WS/DGAT*) from *Acinetobacter baylyi* with a wide substrate selectivity. It can catalyze the reaction between fatty acyl-CoAs with different chain lengths and ethanol to generate fatty acid ethyl esters (FAEEs). The introduction of *atfA* (a gene coding *WS/DGAT*) and two critical genes of the ethanol production from *Zymomonas mobilis*, *adhE* and *pdc*, into *E. coli* can make the concentration of fatty acid ethyl esters reach 1.28 g/L and 25.4% of the cell dry weight by the combination with optimizing fermentation conditions. Furthermore, to eliminate the need to feed ethanol, FAEEs derived from endogenously produced fatty acids and ethanol were produced in *E. coli* by directly expressing the *Z. mobilis* genes *pdc* and *adhB* [106]. Overexpression of acetyl-CoA carboxylase in *E. coli* was employed to increase the pool of fatty acyl-CoA and up to FAEEs titer of 922 mg/L was finally reached under optimized fermentation conditions[64].

An effective *de novo* biosynthetic pathway for the biosynthesis of fatty acid ethyl esters in *E. coli* can be constructed as follows: (1) overexpression of *accBCDA* genes to improve the content of fatty acids, (2) knockout of *fadE* to interrupt the β-oxidative pathway, (3) overexpression of thioesterase to release more free fatty acids, (4) removal of the inhibition

of fatty acid pathways, (5) overexpression of *fadD* to improve the yield of fatty acyl-CoA, (6) overexpression of *adhE* and *pdc* genes to construct the biosynthetic pathway of ethanol, and (7) overexpression of *atfA* gene to make fatty acids and ethanol combine to form biofuels. 0.92 g/L of fatty acid ethyl esters can be obtained by the optimization of culture conditions. Fatty acid ethyl esters can also be produced in *E. coli* using different raw materials, such as cellulose and hemicellulose, etc. by constructing different *de novo* biosynthetic pathways (Fig. 3)[111-116].

2.3.2 Production of fatty alkenes/alkanes

Besides fatty acid ethyl esters, fatty acids can be converted to fatty alkenes/alkanes (Fig. 3), a more ideal substitute for fossil diesels[117]. Lennen *et al*. [118] constructed a mutated *E. coli* strain with a 6-fold high yield of fatty acids by the knockout of *fadD*, the expression of four subunits of *ACC* and the introduction of acyl-acyl carrier thioesterase from California laurel (*Umbellularia californica*). Huber *et al*. [119] obtained liquid alkanes from sugars derived from cellulose by the chemical conversion. The heterologous expression of the alkane operon in *E. coli* led to the production and secretion of C13 to C17 mixtures of and alkenes. Alkane titers were over 300 mg/L and more than 80% of hydrocarbons were found outside the cells[120]. The direct production of fatty alkanes by engineered *E. coli* has currently been still at an initial stage. Moreover, fatty alkanes produced often contain a certain proportion of alkenes [120]. Further studies on the catalytic mechanisms and

regulations of biosynthetic pathways of fatty alkanes from different microorganisms will be helpful to modify them to improve their yields and to control the proportion of product components[121, 122]. Current strategies for the production of biofuels by expanding the fatty acid biosynthetic pathways are listed and compared in Table 3.

3.Isoprenoid-derived pathways

Isoprenoids are the most functionally and structurally diverse group of plant metabolites, and are present in almost all organisms but are especially abundant in plants. Over 30,000 isoprenoid compounds have been reported so far[123, 124]. Many isoprenoids play an important role in membrane architecture (phytosterols), protein modification (dolichol, prenyl groups), respiration (ubiquinones), photosynthesis (carotenoids, chlorophylls, plastoquinones), and regulation of cell growth and development (gibberellins, abscisic acid, cytokinins, and brassinosteroids). The largest number of isoprenoids, however, are secondary metabolites that function in protecting plants against herbivores and pathogens, attracting pollinators and seed-dispersing animals, and affecting competition among plant species[124].

3.1 General overview of the isoprenoid pathway

Isoprenoid-like chemicals can be synthesized from the same basic units-isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are combined by prenyltransferase to form geranyl diphosphate (GPP, C10 precursor to monoterpenes),

farnesyl diphosphate (FPP, C15 precursor to sesquiterpenes and triterpenes), and geranylgeranyl diphosphate (GGPP, C20 precursor to diterpenes) (Fig. 4). Many chemicals, such as α- and β- farnesene, sabinene, γ-humulene and longifolene, can be produced based on the above pathway. Isoprenoid-like chemicals with a chain length of more than 30 carbon atoms may be used as biofuels[125, 126].

3.2 Expanding the isoprenoid pathway for the production of biofuels

Biofuels can be produced by expanding the isoprenoid pathway[127](Fig. 5). A critical problem is how to produce two general precursors, IPP and DMAPP. The two substances can be produced by either the mevalonate pathway or the non-mevalonate pathway under natural conditions. The latter is a main way to produce them in *E. coli*, using glyceraldehyde-3-phosphate and pyruvate as starting materials. Yields of IPP and DMAPP can be significantly improved by optimization of the non-mevalonate pathway in *E. coli*, or by the introduction of the exogenous mevalonate pathway into *E. coli*[128, 129]. IPP and DMAPP can be further converted to geranylflavanone pyrophosphate (C10), farnesyl pyrophosphate (C15) and geranylflavanone-geranylflavanone pyrophosphate (C20) according to a head-tail connecting mechanism. These metabolites can be further converted to a variety of products by different terpene synthases or terpene-modified enzymes. For example, the introduction of an isoprene pyrophosphate phosphatase from *Bacillus subtilis* into *E. coli* can

convert isoprene pyrophosphate to isoamyl enolase[130]; the introduction of a terpene synthase can convert geranylflavanone pyrophosphate to terpenoids with different structures, among which pinene, sabinene and terpinene are considered as the potential components of the next-generation aviation fuel[131, 132]; the introduction of a phosphatase from yeast and a terpene synthase from plant can convert farnesyl pyrophosphate to farnesol and farnesene, respectively.

Farnesane as a high-performance advanced biofuel has been produced based on isoprenoid- derived pathways[133]. The Amyris company located in Emeryville, California, USA, has used the industrial strain *S. cerevisiae* PE-2 to produce farnesene, which was then further reduced to farnesane by the chemical method. Bisabolene, a precursor of bisbolane considered as a potential biofuel, has also been succefully overproduced in *E. coli*. Five plant-derived isoprenoid synthases that can efficiently convert farnesyl diphosphate to bisabolene were screened, and their genes were optimized based on the codon bias of *E. coli* for obtaining the maximal production of isoprenoid synthases and bisabolene. Bisabolene could also be produced in *S. cerevisiae*, and its maximal yield was over 900 mg/L[134]. Isopentanol and pinene dimers can be chemically obtained from pinene and isopentenol[135]. Pinene could be produced in *E. coli* by a pinene synthase and a geranyl diphosphate synthase to reroute IPP and DMAPP. Isopentenol can be obtained by a phosphatase to dephosphorylate isopentenyl diphosphate[136, 137]. The introduction of pinene-specific enzymes into *E. coli*

that can metabolize both cellulose and xylose results in 1.5 mg/L of pinene[138]. Recently, a flux-controlling enzyme, the bisabolene synthase that can accelerate the determination of the crystal structure of the most efficient bisabolene synthase from *Abies grandis*, has been found, which assists in the expanding of isoprenoid-derived pathways to improve the production of bisabolene[139]. Additionally, the expanding of the mevalonate pathway produced 27 g/L of the C15 amorphadiene in *E. coli* [140] and 40 g/L in *S. cerevisiae*[141].

4.Alcohol-derived pathways

Short- and long-chain alcohols are desirable alternative fuels that may be mixed with the conventional gasoline, or may be used as a drop-in replacement. By mimicking the Ehrlich pathway, Atsumi *et al*. [142] developed a new strategy to produce C3-C5 alcohols. In their design, amino acid precursors 2-keto acids were converted to aldehydes by 2-keto acid decarboxylase (KDC), and then to long-chain alcohols by alcohol dehydrogenase (ADH). To construct the artificial pathways in the amenable *E. coli* host, *Adh*2 from *S. cerevisiae* was selected as the alcohol dehydrogenase, and five KDCs from different organisms were screened for their abilities to produce alcohols. Fermentation results demonstrated that Kivd from *Lactococcus lactis* is the most capable decarboxylase, which enabled the synthesis of 1-propanol, 1-butanol, isobutanol, 2-metyl-1-butanol, 3-methyl-1-butanol, and

2-phenylethanol. Increasing the 2-keto acid flux could obviously enhance the specificity and production levels of alcohols. In particular, the preliminary optimization of the isobutanol production by deleting genes corresponding to the byproduct formation and replacing *ilvl*H gene with *als*S (*B. subtilis*) led to 22 g/L of isobutanol after 112 h with a yield of 0.35 g/g glucose (86% of the theoretical maximum).

Longer-chain (C6-C8) alcohols can also be biosynthesized by extending the carbon chain of ketoacids with an engineered "+1" metabolic pathway. Zhang *et al*. [85] expanded branched amino acid pathways to produce non-natural C5-C8 alcohols by engineering 2-isopropylmalate synthase (LeuA, enzyme for carbon chain elongation) and 2-ketoisovalerate decarboxylase (KIVD) (Fig. 6). They demonstrated an approach via constructing the isoleucine biosynthesis pathway to generate 2-keto-3-methylvalerate and assuming that *Leu*A*BCD are promiscuous enough for 2-keto-3-metylvalerate to go through the chain elongation reaction to produce 2-keto -4-methylhexanoate with the addition of acetyl-CoA. Followed by Ehrich pathway, 2-keto -4-methylhexanoate could then be converted to 3-metyl-1-pentanol (C6) by engineered KIVD and alcohol dehydrogenase VI (ADH6). By engineering the selectivity of KIVD and reliving the steric hindrance of LeuA, the level of (*S*)-3-methyl-1-pentanol increased from 6.5 mg/L (WT LeuA, WT KIVD) to 793.5 mg/L (G462D/S139G *Leu*A*, F381/V461A KIVD). Due to the larger binding pocket of *Leu*A*, several non-natural and enantiomerically pure alcohols accumulation could be

observed: 1-pentanol (C5), 1-hexanol (C6), 4-metyl-1-pentanol (C6), 4-methyl-1- hexanol (C7), and 5-methyl-1-heptanol (C8), demonstrating the ability to build the artificial metabolism and the possibility of producing these biofuel candidates from renewable resources.

5. Comparison and analysis of novel biofuels from fatty acid- and isoprenoid-derived pathways

Many biofuels and their precursors can be produced by fatty acid- and isoprenoid-derived pathways. Farnesane produced after hydrogenation of farnesene derived from isoprenoid pathway, and fatty alkanes or fatty acid esters, such as pentadecane and ethyl palmitate, are a good substitute for traditional fuels. Compared to the traditional short-chain alcohols, they have a low solubility in water and can be effectively separated from fermentation broth by centrifugation if they can be secreted into the extracellular broth, which reduces a large amount of energy consumption due to the use of original distillation[143, 144]. Meanwhile, they are ideal substitutes for fuels because they have a high combustion value and are easy to being transported[133].

The octane rating and antioxidant stability of farnesene are low due to having a plurality of unsaturated double bonds. Thus, farnesene has been developed as a biofuel precursor.

Farnescene must be converted to farnescane with a high octane rating and a good low-temperature fluidity by chemical hydrogenation. However, this process will increase the production cost and cause the decrease in the yield.

In contrast, the octane rating and low-temperature fluidity of fatty acid esters derived from fatty acid-derived pathways can be regulated by introducing different modifying enzymes to alter their compositions. The compositions of the first-generation biofuels are difficult to alter because their fatty acid moieties derive from plant oil, which is regarded as a major obstacle of artificially designed diesels[145]. Free fatty acids with different carbon chain lengths may be produced by expressing different thioesterases, which was then converted to fatty acid esters with different carbon chain lengths[146]. The degree of unsaturation of fatty acid esters may be regulated by manipulating key regulatory genes of the biosynthetic pathways of unsaturated fatty acids[147]. The branched chains can be introduced into the main carbon chain by expressing enzymes related to their biosynthesis[148]. Overall, fatty acid ester chemicals with different structures can be produced by microorganisms through metabolic engineering[143].

Compared to fatty acid ethyl esters, pentadecane from fatty acid-derived pathway has higher octane rating. Although the synthesis of pentadecane includes decarbonylation which decreases the carbon utilization, it is entirely composed of carbon and hydrogen, and has a moderate carbon chain length, a very similar structure with existing fossil diesels, higher

combustion value and better fluidity. Compared to farnesene, FAEEs and pentadecane have a similar combustion value, but the theoretical productivity of aliphatic hydrocarbons is higher. From a structural point of view, farnesene contains some branched chains and unsaturated double bonds. Although this can improve its fluidity, its cetane rating is decreased. Thus, the extra chemical hydrogenation is needed to ensure that its cetane rating can reach the use standard of biofuels.

6. Research status of biofuels by expanding microbial metabolic pathways

The first-generation biofuels, such as bioethanol fermented from corn and biodiesels esterified from edible vegetable oils or animal fats, dominant 90% of the current biofuel market[149]. Using the first-generation biofuels to replace the traditional petroleum fuels requires a large number of crops for the biofuel production, which will compete with food supply and lead to heavily economic and ethical problems[150]. A large amount of water, fertilizers, and pesticides were used when cultivating food crops for producing biofuels, and this leads to environmental burden[151, 152]. Furthermore, ethanol is difficult to being distilled from fermentation broth because it contains about 70% of the energy content of gasoline and is miscible with water[84]. Advanced biofuels can be produced from cellulosic biomass, a non-food raw materials including wastes including proteins, wheat straw,

switchgrass and orange peel waste by expanding microbial metabolic pathways[22, 153-155]. These raw materials are low-cost agricultural wastes or easily cultivated and fast-growing crops. They provide more abundant cellulosic biomass and reduce the use of water and fertilizer. Advanced biofuels produced from cellulosic biomass have very similar properties as the current transportation fuels, such as storage, energy content and transportation[156, 157].

The development of synthetic biology and metabolic engineering enable expanding heterologous microbial metabolic pathways in well-studied microbial hosts, including *E. coli* and *S. cerevisiae*, for producing biofuels from the entire sugar biomass[158-160]. These microorganisms have been used for industrial-scale production for many years, can be engineered to tolerate toxic biofuels[161] and metabolize a range of carbon sources[162, 163]. Their charaters and a lot of genetic manipulation tools not only enable heterologous metabolic pathways to improve the production titers and yields, but also extend the choices of biofuels.

In addition, the biosynthetic pathways engineered recently in *E. coli* or *S. cerevisiae* have enormously expanded the candidate repertoire of biofuels. The microbial production of alkanes and alkenes whose biosynthetic pathways have been elucidated in a few recent studies is of very interest[120, 164]. Each engineering endeavor, a particular target molecule in a given selected host, comes with its own set of advantages and challenges, and guides us

in the construction of biosynthetic pathways in the future. It is beneficial to consider to use native high flux pathways to provide key precursors when a target compound of interest is produced by constructing a pathway in a highly engineerable host. Promiscuous enzymes, either designed by protein engineering or found in nature, can be expressed to convert these precursors into the target compounds. The heterologous pathway enzymes chosen preferably will be orthogonal to the host native pathways, implying few heterologous intermediates will be used in native cellular metabolism. Moreover, metabolic flux towards products can be increased through limiting the use of precursors by competing pathways and creating irreversible steps in the engineered pathway. Other factors affecting the overall yield and productivity, including redoxbalance, ATP, and cofactor requirement, etc, should also be considered.

Currently, neither a fuel candidate nor a single microbial host has completely supplanted the need for development of any other biofuels. The efficiency that converts a cellusolic biomass into the desirable biofuel can have numerous impact on the economic feasibility of the fuel production. Fortunately, with the development of genomics, transcriptomics, proteomics, metabolomics, and fluxomics, it is feasible to mine genomes for potential pathways, to design, construct and characterize host strains and to interate through the process again quickly[165]. In recent years, a steady process have been made towards

engineering microbially derived biofuels by expanding microbial metabolic pathways. This will reduce our dependence on petroleum and the emission of carbon dioxide.

7. Conclusions and perspectives

Metabolic engineering is an emerging field that aims to bring engineering principles into the manipulation of genes in microorganisms so that engineering an organism for biofuel production is relatively easy. Biofuels can only be competitive when the production costs are less than or equivalent to refining petroleum, and thus the viability of biofuels depends absolutely on reducing production costs. In order to achieve this goal, any extraneous cellular processes that reduce the production cost must be systematically controlled. To do this, technologies that enable rapid prototyping, testing, and optimization of pathways and the host organisms are crucial.

A challenge of metabolic engineering are to reduce the time required to make genetic constructs and to increase their predictability and reliability. The construction of many pathway variations can be more efficiently handled by better assembly techniques rather than through synthesis of each element[166]. Another challenge of metabolic engineering is the creation of reusable parts that have useful and predictable behavior. The development of a diverse array of regulatable expression systems that can be used to fine-tune expression is very useful in engineering metabolic pathways[167, 168]. The creation of modular sub-pathways

that can be easily interconnected is an area of active research with much potential. To achieve this goal, a strong framework for characterization and standardization is being sought. Recent efforts in standardizing measurements are encouraging, but further refinements of the methods by which these parts are tested and characterized as well as industry-wide agreement on those methods is necessary[169].

The production of biofuels by expanding microbial metabolic pathways has been realized, but their yields are not enough high to reach the level of the industrial production. It is a future direction to further construct high-yield strains. Several aspects must be considered: (1) mining and transformation of elements pertinent to metabolic pathways. A lot of efficient genes and enzymes remain to be excavated from abundant resources of nature[170, 171]. Mining efficient gene elements by designing high-throughout screening strategies may provide materials for the transformation of metabolic pathways. Emerging technologies of metagenomics and comparative genomics become good strategies for screening biological elements. For example, multiple cellulases have been found from termites or bovine stomach by metagenomic technologies, providing materials for constructing efficient cellulose-utilizing strains [23, 172]. Moreover, the activity and selectivity of mined elements from nature are sometimes not good when they are expressed in heterologous host cells. Thus, it is essential for further modifying them by protein or enzyme engineering, or designing them to be newer and more efficient elements based on their functions[173]. (2)

assembling biofuel elements and coordinating their functions. The DNA assembling technique provides a good program for the quick assembling of metabolic pathways[174]. The prospect of assembling new metabolic pathways by simply putting together sub-pathways into microorganisms is is very exciting. The rapid advances in synthetic biology have brought new tools for assembly and control that move the entire field closer to this goal. The University of California scholars succeeded in anchoring enzymes of metabolic pathways to a protein scaffold containing a variety of structural domains expressed in *E. coli*. This technology made the metabolic flux coordinated, and the productivity of the target product was increased by 77-fold[175]. However, it has been still unclear if this technology is suitable for eukaryotes or other heterologous hosts so far, and so it is necessary to develop other new techniques to regulate the metabolic flux. (3) enhancing the tolerance of host cells to biofuels. The toxicity of biofuel molecules directly affect the cell growth and metabolism, which limits further improvement of the production of target chemicals[176-179]. Thus, it is necessary to increase the tolerance of host cells to biofuel molecules in order to obtain high-yield target chemicals. The traditional domesticated strategy can get well-tolerated strains, but it is time-consuming and labor-intensive, and the genetic traits of obtained strains are not enough stable[180]. In recent years, a stable and high ethanol-tolerate strain was obtained by the transformation of transcriptional factors by the global transcriptional engineering[161]. The tolerance of *E. coli* to butanol was improved to 1.5% through artificial transcriptional factor

technology[181]. (4) Given that *E. coli* is a general host strain that is the most easily chosen for the production of biofuels because of its many advantages, enhancing activities of some enzymes, increasing the metabolic efficiency or introducing exogenous pathways often makes its metabolic system dysfunction. The expression of some heterologous genes in *E. coli* may run out of raw materials or some precursors, and may increase its survival pressure. Moreover, although the introduction of exogenous metabolic pathways can overcome some regulatory restrictions related to original metabolic pathways in *E. coli*, it easily results in lack or imbalance of enzymes in its original metabolic pathways. This diminishes the carbon flux and the accumulation of toxic intermediates that are harmful to a normal operation of the entire metabolic pathway.

Acknowledgements

This study is supported by the National Natural Science Foundation of China (No. 31171658). We thank professor Hujun Xie and professor Xuan Zhu for proofreading this article.

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Figure captions

Figure 1. Fatty acid metabolic pathway in *E. coli*. Products in red brackets are biofuel molecules. Abbreviations: ACC, acetyl coenzyme A carboxylase; BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, transcarboxylase; MAT, malonyl CoA:ACP transacylase; KS, ketoacyl- ACP synthase; KR, ketoreductase; ER, enol reductase; HD, dehydratase; ACP, acyl carrier protein; TE, thioesterase; FadD, E, fatty acid metabolism regulator protein; FAR, fatty acid reductase.

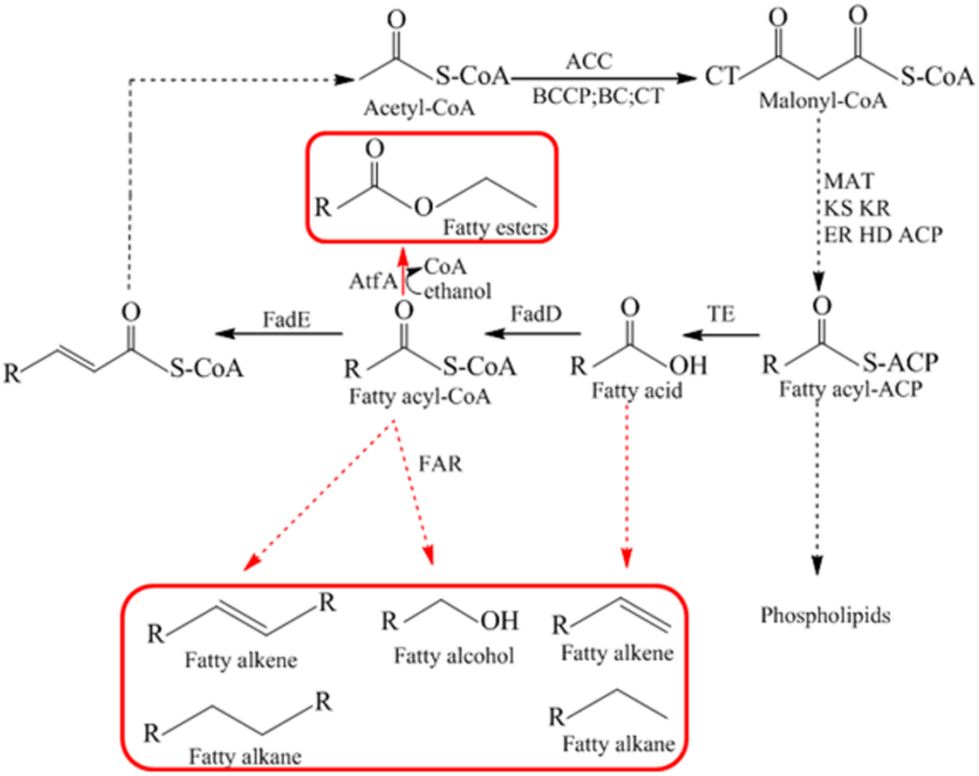

Figure 1

Figure 2. Biofuel production by fatty acid pathway. Solid arrows and crosses represent modified steps that can enhance the yield of fatty acids, whereas dashed arrows and crosses are reverse.

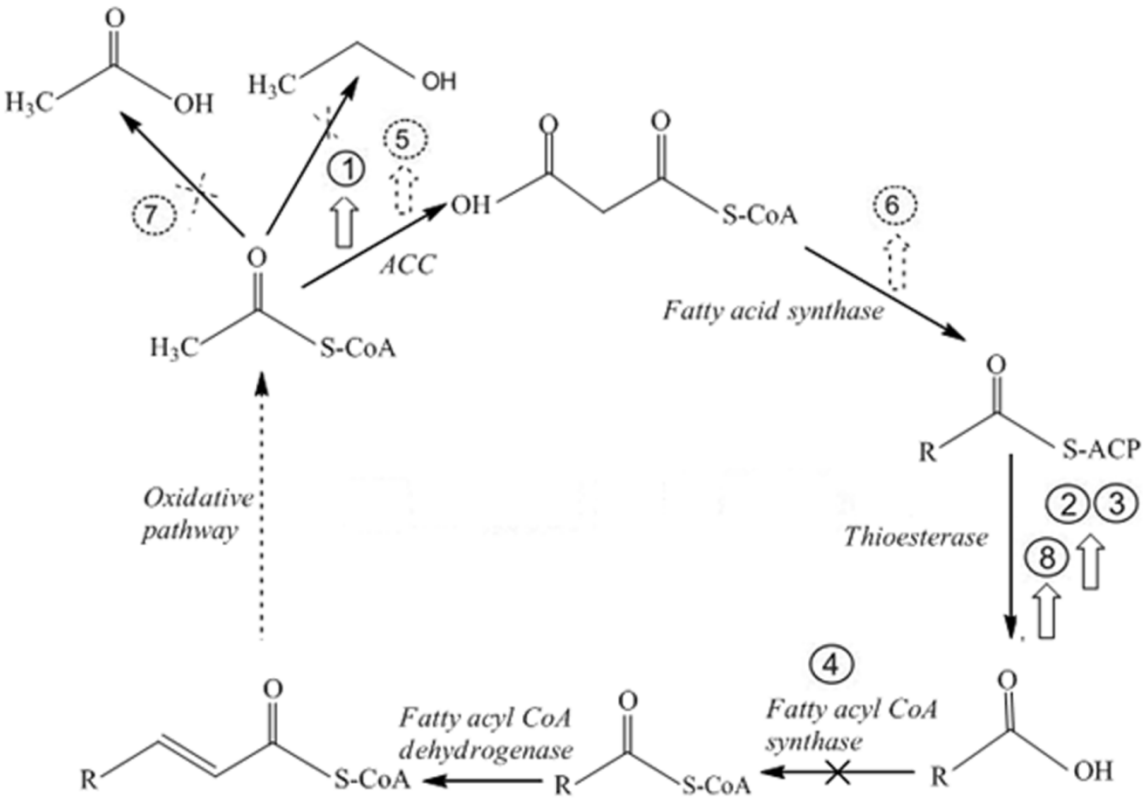

Figure 2

Figure 3. *de novo* biosynthetic pathway for the production of fatty acid esters in *E. coli*. Dashed lines represent multienzyme catalytic processes. Solid lines represent monoenzyme catalytic processes. Single arrows represent metabolic pathways in *E. coli*. Double arrows represent introduced metabolic pathways. The products in red brackets represent biofuel molecules.

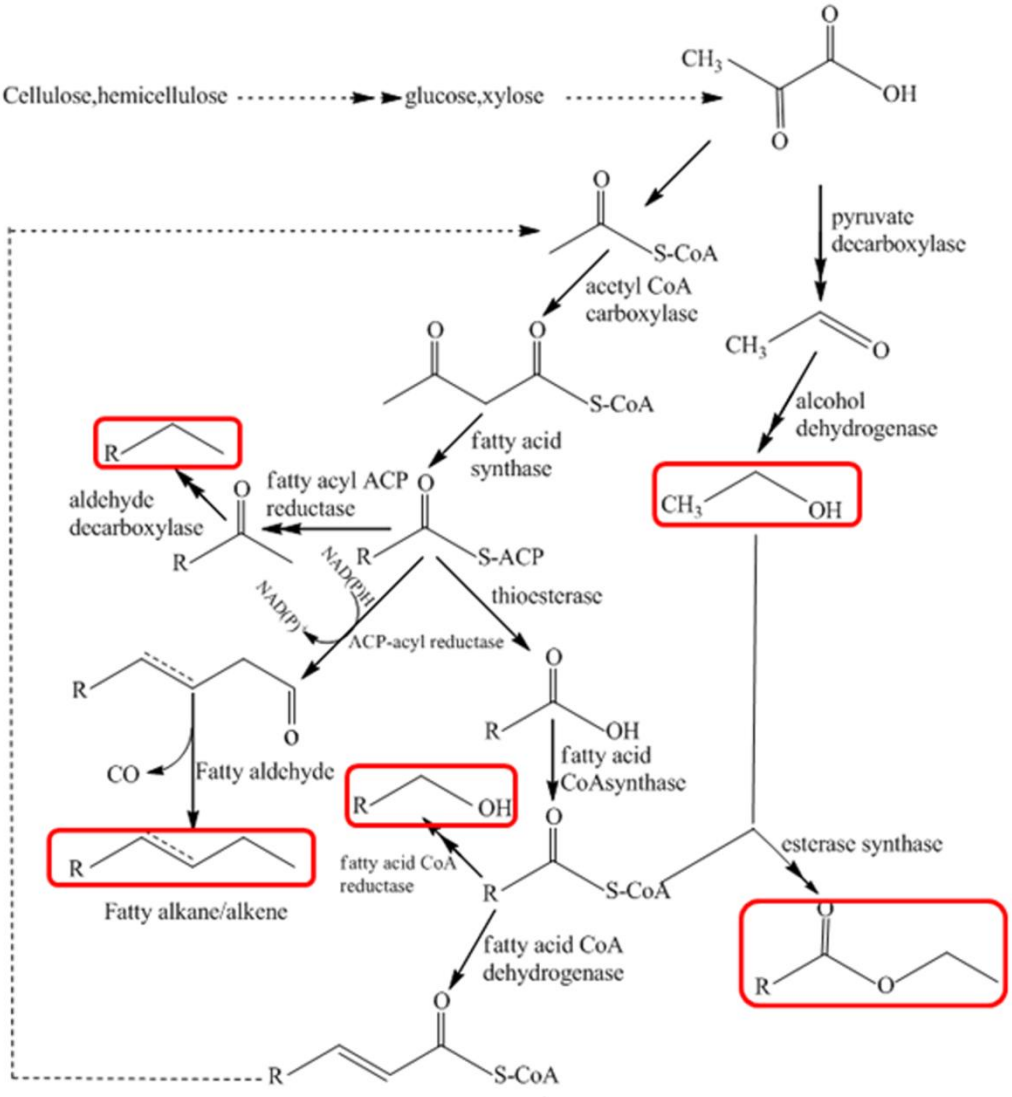

Figure 3

Figure 4. General overview of isoprenoid pathway. Prenyltransferases catalyze the conversion of IPP and DMAPP to GPP and FPP.

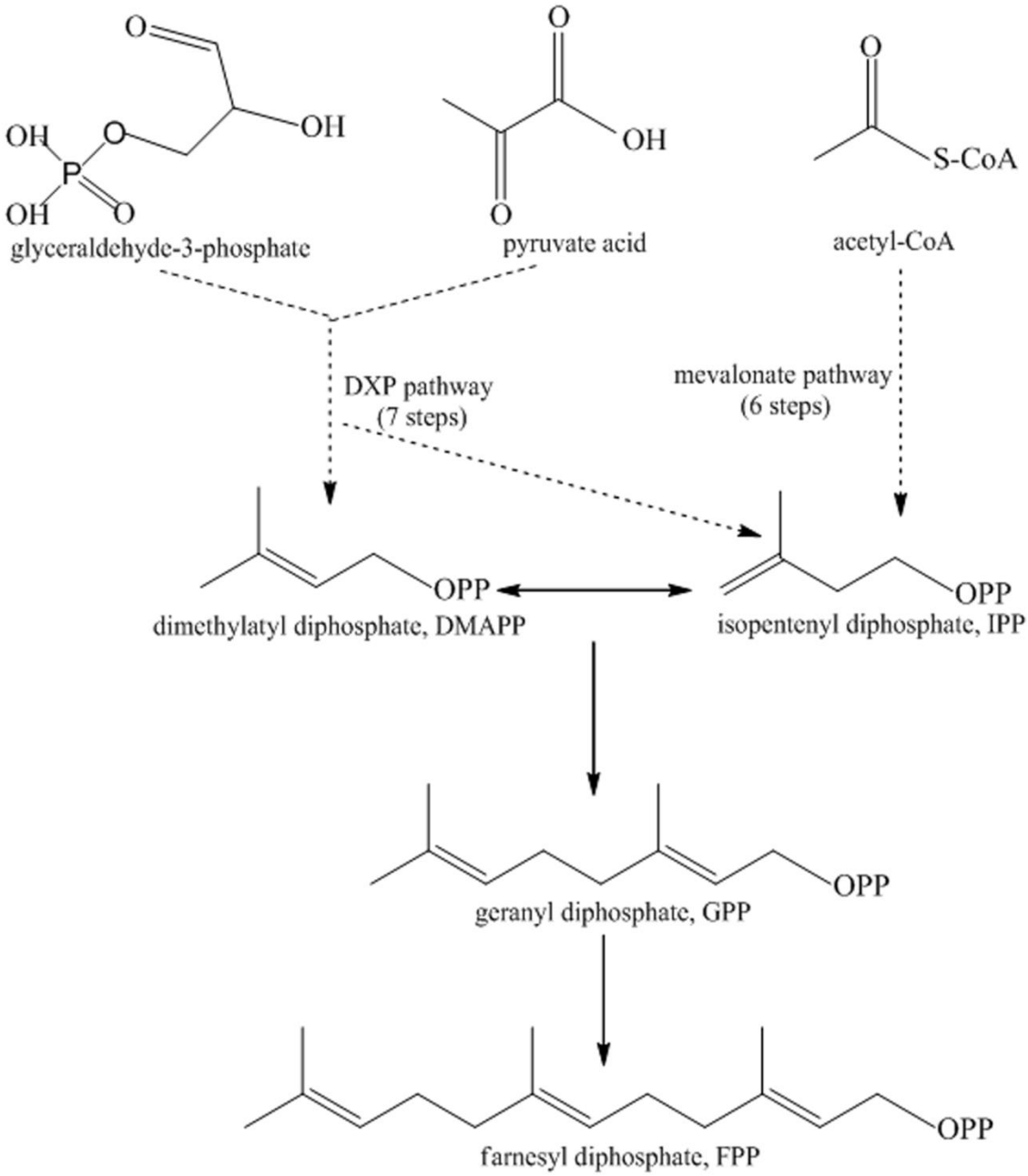

Figure 4

Figure 5. Expanding isoprenoid pathway for the production of biofuels. Products in red brackets represent biofuel molecules.

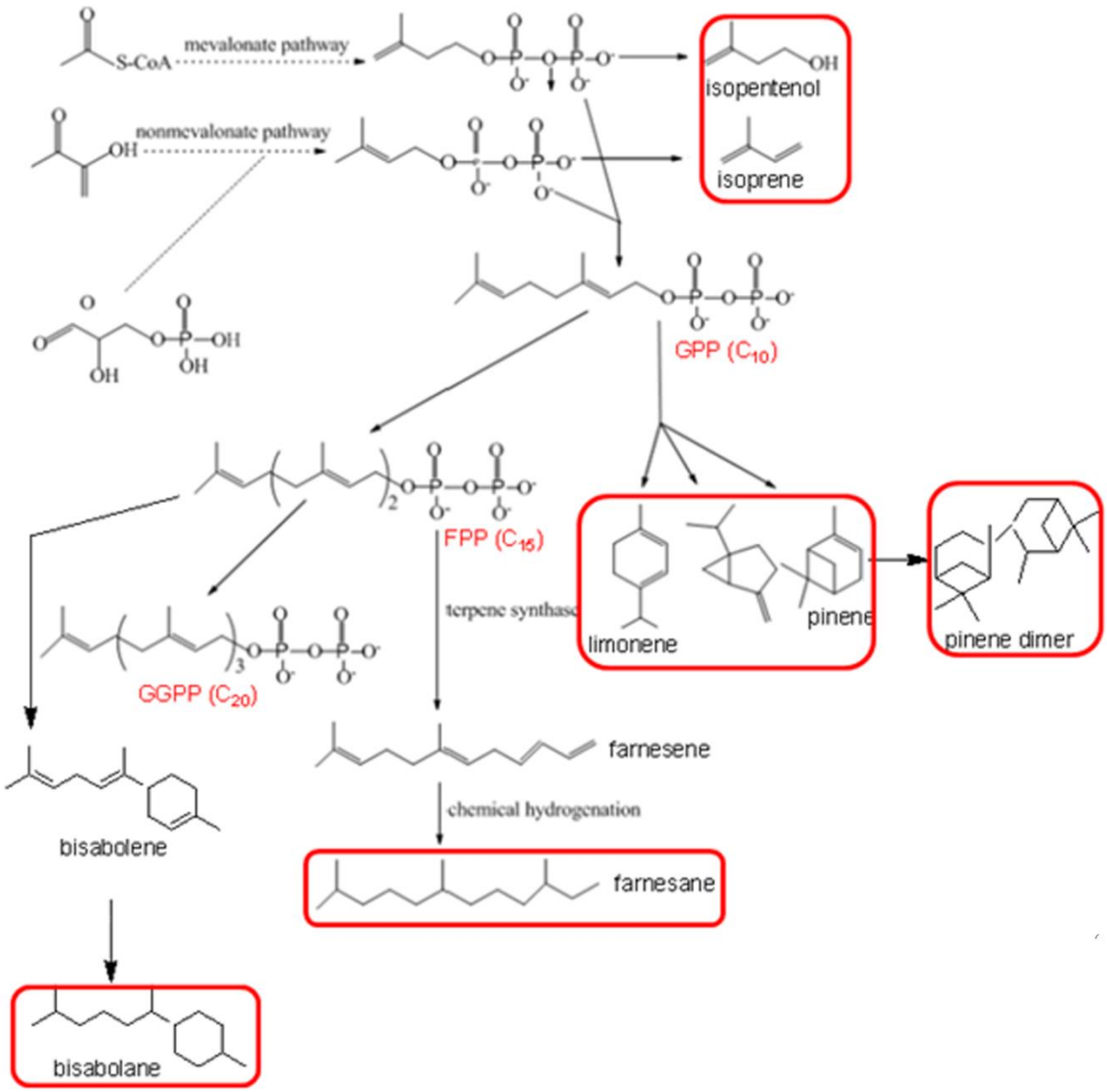

Figure 5

Figure 6. (a) Artificial pathways expanded from 2-keto acids by combinatorial enzyme screening or engineering. Decarboxylation is catalyzed by 2-ketoacid decarboxylase. Reduction is catalyzed by alcohol dehydrogenase and oxidation is enabled by aldehyde dehydrogenase. Chain elongation is facilitated by engineered LeuABCD ‘‘+1’’ module. (b) Examples of C4, C5, and C6 compounds produced in *E. coli* with the platform pathways.

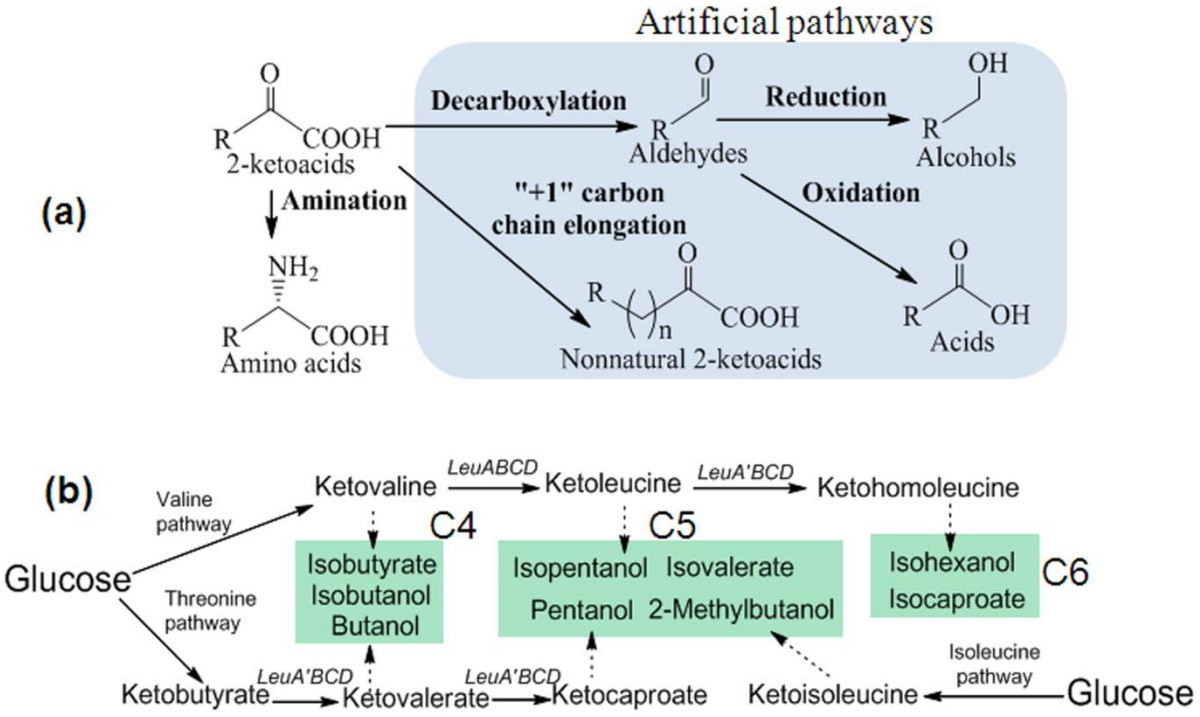

Figure 6

Table 1 Comparison of three fuels

Names	Sources	Production process	Renewable	Products
Fossil fuels	Petroleum	transesterification	No	mixture of alkanes and aromatic compounds
First-generation biofuels	plant oil	biosynthesis in combination with transesterification	Yes	fatty acid ethyl esters or methyl esters
New-type biofuels	cellulose/solar energy or CO_2	Biosynthesis	Yes	fatty acid ethyl esters or methyl esters/fat alkanes/ farnesene

Table 2 Current strategies and comparison of metabolic engineering for the production of fatty acids in *E. coli* by expanding its metabolic pathways

Strains	Strategy	Results	References
E. coli K12Ymel	Expression of ACC and plant acyl-ACP thioesterases from *U. californica*, eliminating β-oxidation	7-fold increase	[182]
E. coli BL21 star (DE3)	Coexpression of thioesterases from *C. camphorum* and *E. coli* and ACC from *E. coli*, knockout of *fadD*	2.5 g/L	[183]
E. coli XL100	Coexpression of endogenous ACC and TesA and plant TE	4.5 g/L/d	[105]
E. coli MG1655	Engineered reversal of the β-oxidative cycle	7 g/L	[108]
E. coli DH1Δ*fad*E	Tuning the expression of *FadR*	5.2 g/L	[109]
E. coli BL21 (DE3)	Expression of *Tes*A and knockout of *fad*L, on-line extraction	4.8 g/L of extracellular fatty acid	[184]

Table 3 Current strategies and comparison for the production of novel biofuels by expanding fatty acid biosynthetic pathways

Strains	Product	Strategy	Results	References
E. coli Top10	FAEEs	Expression of *Z. mobilis* PDC and AdhE and acyltransferase from *A. baylyi* strain ADP1	1.28 g/L	[65]
E. coli C41 (DE3)	FAEEs	Expression of *AtfA* and *FadD* and *TesA*	400 mg/L	[106]
E. coli BL21 (DE3)	FAEEs	Expression of acetyl-CoA carboxylase, PDC, AdhB and acyl-CoA: diacylglycerol acyltransferase	922 mg/L	[64]
E. coli C41(DE3)	Fatty alcohol	Overexpressing *tesA*, *fadD*, and *acr1*, knockout of *fadE*	60 mg/L of medium chain fatty alcohol	[106]
E. coli BL21 (DE3)	Fatty alcohol	Coexpression of genes *BTE*, *fadD* and *acr1*	449.2 mg/L of C12/14 alcohol	[185]

E. coli BL21 (DE3)	Alkane	Coexpression of genes *tesA*, *fadD* and fatty acyl-CoA reductase gene *Far*	101.5 mg/L of C16/C18 alcohol	[109]
E. coli MG1655	Alkane	Expression of the alkane operon	300 mg/L of alkane	[120]