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Review

Recent progress in hydrocarbon biofuel synthesis: Pathways and enzymes

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ABSTRACT

Biofuels derived from hydrocarbon biosynthetic pathways have attracted increasing attention. Routes to hydrocarbon biofuels are emerging and mainly fall into two categories based on the metabolic pathways utilized: Fatty acid pathway-based alkanes/alkenes and isoprenoid biosynthetic pathway based terpenes. The primary focus of this review is on recent progress in the application of hydrocarbon biosynthetic pathways for hydrocarbon biofuel production, together with studies on enzymes, including efforts to engineering them for improved performance.

1. Introduction

Environmental, economic, and geopolitical concerns over continued fossil-fuel dependence have spurred research into the conversion of renewable biomass to "drop-in" fuels. Biofuels derived from hydrocarbon biosynthetic pathways have attracted increasing attention. These fuels have great potential to replace petroleum-based liquid transportation fuels, as they have high-energy content and physicochemical properties comparable to fossil fuels, and hence are compatible with current engines, distribution systems and storage infrastructure. Routes to hydrocarbon biofuels are emerging and mainly fall into two categories based on the metabolic pathways utilized: Fatty acid pathway-based alkanes/alkenes and isoprenoid biosynthetic pathway based terpenes (Fig. 1). This review focuses on recent progress in the application of hydrocarbon biosynthetic pathways for hydrocarbon biofuel production, together with studies on enzymes, including efforts to engineering them for improved performance.

2. Fatty acid pathway-based alkanes/alkenes

2.1. Metabolic pathways and application to biofuel production

There are mainly three types of hydrocarbons derived from the fatty acid pathway: Alkanes, α-olefins and internal olefins. Alkanes are biosynthesized ubiquitously in nature where they function as waterproofing agents in plants' leaves [1] and birds' feathers [2], as pheromones in insects [3], and as energy storage molecules in algae [4]. Recently, an alkane biosynthesis pathway from cyanobacteria, comprising acyl-coA reductase (cACR) and aldehyde deformylating oxygenase (cADO), was identified through comparative genomics [5]. In this pathway, fatty acyl-ACP (CoA) is converted by cACR to fatty aldehydes, which are then converted by cADO to alkanes/alkenes through losing the aldehyde carbon as formate. This heterologous pathway was introduced into *E. coli*, leading to 300 mg/L alkane production over 40 h [5]. A similar hydrocarbon biosynthesis pathway was constructed using carboxylic acid reductase (CAR) from *Mycobacterium marinum* to convert fatty acids (C8–C16) into the corresponding aldehydes, which were further converted to alkanes (C7–C15) by cADO [6].

α-Olefins, hydrocarbons with terminal double bonds, can be blended with diesel fuels. In a recent effort to elucidate their biosynthesis, a terminal olefin-forming fatty acid decarboxylase from the bacteria *Jeotgalicoccus* was identified and named OleT

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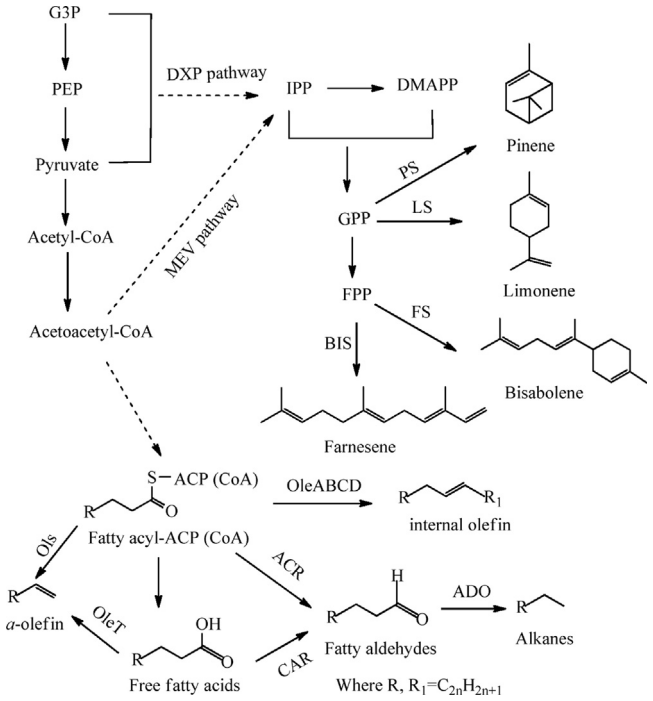

Fig. 1. Overview of the hydrocarbon biofuel biosynthetic pathways as reviewed here. *Abbreviations*: G3P: glyceraldehyde 3-phosphatel; PEP, phosphaenolpyruvate; ACP, acyl carrier protein; IPP, isoprenyl pyrophosphate; DMAPP, dimethyllallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; FS, farnesene synthase; PS, pinene synthase; LS, limonene synthase; BIS, bisabolene synthase.

[7]. OleT catalyzes the decarboxylation of free fatty acids to generate α-olefins and is a cytochrome P450 enzyme. The heterologous expression of OleT enabled *E. coli* to produce 1-pentadecene and 1,10-heptadecadiene at unreported titers. α-Olefins can also be formed from fatty acids by an elongation-decarboxylation mechanism, similar to that of a polyketide synthase. A gene named *ols* (olefin synthase) was identified to be involved in the α-olefin biosynthesis in cyanobacterium *Synechococcus* sp. PCC 7002 [8]. However, no α-olefin was detected when *ols* was introduced into *E. coli*, because it was found that the *ols* gene was not actively expressed [9].

Internal olefins can be generated through the head-to-head condensation of fatty acids catalyzed by a set of enzymes termed OleA, B, C and D. The heterologous expression of a three-gene cluster from *Micrococcus luteus* (Mlut_13230-13250) in *E. coli* led to the production of long-chain internal olefins, mainly C27:3 and C29:3 [10].

2.2. Enzymology and engineering of related enzymes

2.2.1. cADO

cADO catalyzes the conversion of fatty aldehydes to alkanes. In contrast to other decarbonylases from insects or plants, which are integral membrane proteins, cADO is soluble and can easily be expressed recombinantly in *E. coli*, making it more amenable to study. The crystal structure of cADO (pdb 2OC5) from *Prochlorococcus marinus* shows that cADO is a member of the nonheme di-iron family of oxygenases exemplified by enzymes such as methane monooxygenase (MMO), class I ribonucleotide reductase, and fatty-acyl-ACP desaturase. Interestingly, although the deformylation reaction is redox neural, cADO requires molecular oxygen and an external reducing system, either a protein reductive system (NADPH, ferredoxin, and ferredoxin reductase) [5] or a chemical reducing system (phenazine methosulfate and NADH) [11]. During the reaction, O_2 is completely reduced with one atom of oxygen incorporated into formate and the other into water [12]. The mechanism of cADO has been the subject of intense interest, the reader is referred to a recent review for more details [13]. However, the activity of cADO *in vitro* is extremely low. It has been reported that the *in vitro* activity of cADO is inhibited by hydrogen peroxide (H_2O_2) and interestingly, this inhibition can be relieved by fusing catalase to cADO and converting H_2O_2 to the cosubstrate O_2 [14]. Even so, the highest steady turnover number achieved is only $\sim$1 min^{-1}.

2.2.2. cACR

Despite the fact that it synthesizes highly insoluble products (fatty aldehydes), cACR appears to be a cytosolic enzyme. However, its tendency to form inclusion bodies when expressed in *E. coli* has hindered mechanistic studies on this enzyme. Recombinant cACR from *Synechococcus elongatus* has been characterized. The enzyme is specific for NADPH and catalyzes the reduction of fatty acyl-CoA (ACP) to the corresponding aldehydes, rather than to alcohols [15]. However, over-expression of cACR in *E. coli* resulted in the production of both even chain fatty aldehydes and fatty alcohols [5]. It is believed that the production of fatty alcohols was due to intrinsic alcohol dehydrogenases in *E. coli*. The enzyme was shown to function by a well-precedented mechanism involving the formation of an enzyme-thioester intermediate. cACR required divalent metal ions, *e.g.* Mg^{2+}, for activity and was stimulated significantly by K^+. The enzyme was active toward the reduction of acyl-CoA of chain lengths ranging from 12 to 20 carbon atoms, with the highest enzymatic activity toward stearoyl-CoA. Surprisingly, given the straightforward reduction chemistry involved, cACR exhibited very slow turnovers with k_{cat} = 0.36 $\pm$ 0.023 min^{-1}. Both cADO and cACR have slow turnover numbers, posing challenges for their use in biofuel application. Furthermore, the toxicity of the aldehyde products, if produced in high concentration in recombinant strains, would need to be considered and resolved.

2.2.3. CAR

CAR catalyzes the reduction of aromatic (including benzoic, vanillic, and ferulic acids) and C4–C18 carboxylic acids to their corresponding aldehydes in a reaction that requires ATP and NADPH [6,16]. For activity, CAR requires the prosthetic group 4′-phosphopantetheine, which is covalently bound through a phosphodiester bond to a serine residue [17]. This modification is carried out post-translationally by a separate phosphopantetheinyl transferase enzyme [17]. The reaction catalyzed by CAR involves three key steps: (i) adenylylation of the bound fatty acid substrate to form an AMP-fatty acyl complex and pyrophosphate, (ii) transfer of the activated fatty acid to the phosphopantetheine prosthetic group with the formation of a reactive thioester linkage, and (iii) reduction of the thioester intermediate to the aldehyde by NADPH.

2.2.4. OleT

OleT is a terminal olefin-forming fatty acid decarboxylase. OleT has 59% amino acid sequence identity to a P450 from *Macrococcus caseolyticus* and was assigned to the cyp152 family that comprises P450s from various bacteria. Although the function of most P450s in the cyp152 family is unknown, several members of the family, such as *Bacillus subtilis* P450 ($P450_{BS\beta}$) and *Sphingomonas paucimobilis* P450 ($P450_{SP\alpha}$), have been revealed to hydroxylate fatty acids at either the α- or β-position. Interestingly, it is observed that OleT also catalyzed the α- and β-hydroxylation of fatty acids as side reactions [7,18]. Moreover, OleT has a structure highly similar to $P450_{BS\beta}$ [19]. Therefore, there appears to be a mechanistic link between the ability of a P450 enzyme to hydroxylate fatty acids in the β-position and the ability to oxidatively decarboxylate fatty acids to the terminal olefin. In contrast to other P450s, which require O_2, NADPH, and redox