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# Metabolic Engineering of Biosynthetic Pathway for Production of Renewable Biofuels

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**Abstract** Metabolic engineering is an important area of research that involves editing genetic networks to overproduce a certain substance by the cells. Using a combination of genetic, metabolic, and modeling methods, useful substances have been synthesized in the past at industrial scale and in a cost-effective manner. Currently, metabolic engineering is being used to produce sufficient, economical, and eco-friendly biofuels. In the recent past, a number of efforts have been made towards engineering biosynthetic pathways for large scale and efficient production of biofuels from biomass. Given the adoption of metabolic engineering approaches by the biofuel industry, this paper reviews various approaches towards the production and enhancement of renewable biofuels such as ethanol, butanol, isopropanol, hydrogen, and biodiesel. We have also identified specific areas where more work needs to be done in the future.

**Keywords** Metabolic engineering · Ethanol · Butanol · Biodiesel · Biofuels

## Introduction

An increasing demand for fuels has emerged globally while the planetary reservoir of fossil fuels is significantly depleting [1]. There is a pressing need to address the future energy requirements without adversely impacting the environment. Recently, in some countries,

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renewable biofuels have been mass produced and used by automobile industry. Biofuels offer advantages as viable gasoline substitutes due to their higher energy density and lower hygroscopicity. Several research groups around the world have focused their attention on the production and enhancement of biofuels—the source of which can be sugar and starch crops, lignocellulosic material from wood, crop residues, and energy crops such as hybrid poplar and switchgrass.

Brazil globally leads in the production and consumption of sugarcane-based ethanol followed by the USA where ethanol production from maize showed an 11 times increase from 1996 (4 million liters) to 2005 (44 billion liters). In 2008, ethanol production by fermentation reached 9.2 billion gallons in the USA [2]. Ethanol is the most consumed biofuel and reached production of 13.2 billion gallons in 2010 [3]. The world's largest biodiesel producer is the European Union, accounting for 53 % of all biodiesel production in 2010. In comparison to gasoline, ethanol is hygroscopic nature, shows 65 % energy density of gasoline, and incompatible with current gasoline infrastructure [4].

Scientists have used metabolic engineering approach to produce various types of biofuels and increase their production significantly. Though researchers have focused on ethanol, a number of other biofuels offer advantages that include high energy density, low freezing point, and compatibility with the existing fuel storage and distribution infrastructure [5–7]. In contrast, there is an issue of cultivating food crops for biofuel production that consume large amounts of water, fertilizers, and pesticides which are burden to the environment [8].

As shown in Fig. 1, wastage biomass is a potential untapped resource for the production of biofuels. Bioconversion of lignocellulose into ethanol could make use of the abundant and largely untapped renewable resources such as agricultural wastes (corn stover, sugarcane bagasse, wheat and rice straw, etc.) and forestry residues [9]. Lignocellulose degradation is a major scientific challenge that can be used by engineered microbes to degrade the mass into pentose and hexose sugars [10]. *Saccharomyces cerevisiae* is unable to efficiently ferment both hexoses (glucose) and pentoses (xylose) [11]. Thus, metabolic engineering is required to find a solution for biodegradation through the genetic modification which is an effective way to manipulate the metabolic capabilities of microorganisms [12] such as *Escherichia coli* [13], *Klebsiella oxytoca* [14], *Zymomonas mobilis* [15], and *S. cerevisiae* [16]. Figure 2 shows a schematic representation of the process to produce biofuels from renewable feed stocks. A number of microorganisms have been engineered to convert simple sugars into several types of biofuels such as alcohols, fatty acid alkyl esters, alkanes, and terpenes with high titers and yields [17]. Both isopropanol and butanol have been produced in various strains of *Clostridium* [18].

Furthermore, isopropanol production in *E. coli* has surpassed the capabilities in *Clostridium* by artificial assembly of the pathway of acetone production [19, 20]. The production of 1-butanol has proven to be more difficult using *E. coli* as a host [21], but a novel strain containing a single construct has resulted in twofold production [22]. In addition to *E. coli*, 1-butanol production has been reported in *Pseudomonas putida*, *Bacillus subtilis*, and *S. cerevisiae* [23, 24]. In the present review, we highlight the production of renewable fuels by genetic engineering and metabolic engineering approaches.

## Metabolic Engineering for the Production of Ethanol

Increasing oil prices and environmental degradation caused by the use of fossil fuels has forced people to look for alternative sources to meet energy requirements. The recent scientific advances have renewed the hope that microbial biofuels have potential to compete against

**Fig. 1** Different types of biomass used in production of biofuels

fossil-based fuels [25]. There is an urgent need to support biofuel research given that the global energy consumption is expected to increase 44 % in the next 20 years [26].

Currently, ethanol, isopropanol, and 1-butanol are the only naturally produced alcohol-based biofuels. Isopropanol can be used directly as a fuel supplement to gasoline or as a feedstock for the transesterification of fats into biodiesel [27]. Microbes engineered for biofuel production are summarized in Table 1.

Figure 3 shows biosynthetic pathways of microbes used for the production of biofuels. Using native pathways are advantages to generate immediate precursors for biofuel production. In *E. coli* amino acid, biosynthesis pathways have been used to create 2-keto acid precursors and converted into alcohols by a single heterologous reaction. The production of isobutanol has touched the 20 g/L level after the host genetic edits and optimization through metabolic engineering. Likewise, efforts have been made to engineer production of 1-propanol and 1-butanol, 2-methyl-1-butanol, and 3-methyl-1-butanol [28].

Using microbial system for production of biofuels comes with a certain limit of tolerance of alcohol that is secreted in the medium, making yeast a better platform for alcohol production.

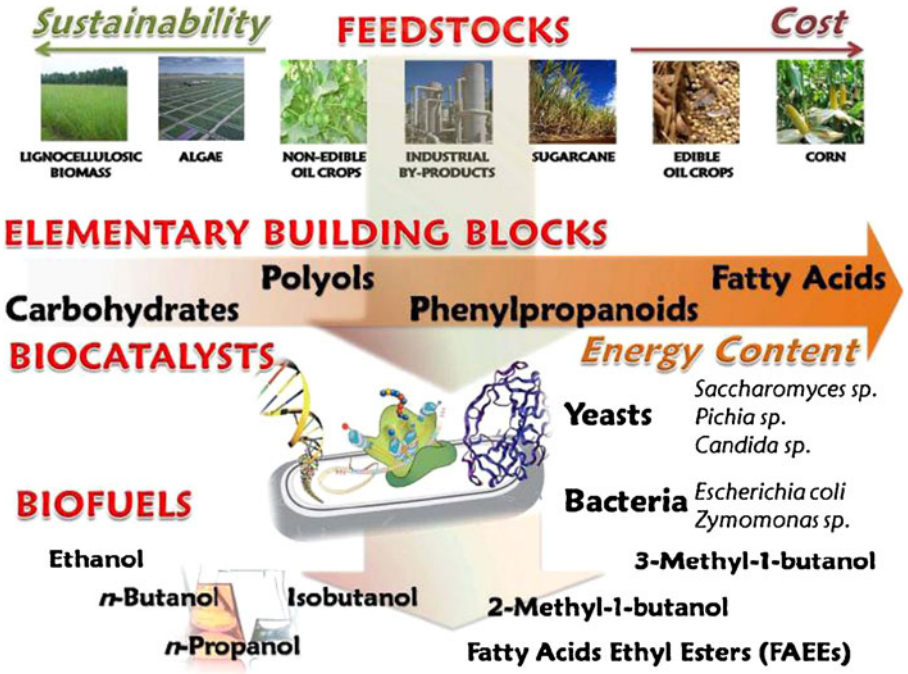


**Fig. 2** Schematic representation for the production of fuels from renewable feedstocks [64]

The yeast *Pichia pastoris* has been widely used for production of more than 400 recombinant proteins. However, *P. pastoris* has a strong preference for respiratory metabolism. It grows at high cell densities without accumulation of high concentrations of ethanol. Similarly, *S. cerevisiae* is regarded as an industrially safe for ethanol production [29] and has been used extensively for commercial production of alcohol. However, the community needs a commercially successful strain that can ferment lignocellulosic hydrolysate for efficient utilization of hexoses and pentoses [30].

Cyanobacteria are currently being explored as a commercial biofuel platform as it mainly uses sunlight, water, and atmospheric carbon dioxide ($CO_2$) as food and is therefore inexpensive to maintain. As shown in Fig. 4, the schematic representation of biosynthetic pathway and "tunable ethanol producing gene circuits" in cyanobacteria that use solar light and $CO_2$. The lacI gene is inserted under the control of constitutive promoter. Thus, lacI binds with the operator region of Ptrc_lac promoter stops the transcription of *pdc* and *adh*. There is no production of ethanol in its original state, but when isopropyl β-D-1-thiogalactopyranoside (IPTG) is added to culture, lacI forms a complex with IPTG leading to a controllable ethanol production.

## Metabolic Engineering for the Production of Butanol

Metabolic engineering has allowed redesign of microbes towards production of ethanol, butanol, alkane, biodiesel, and even hydrogen. It is expected that metabolic engineering can be employed as an essential strategy for the development of microbial strains for industrial applications [25]. It is possible to add two missing genes alpha-ketoisovalerate decarboxylase

**Table 1** Summary of biofuels production in engineered microorganisms [17]

| Biofuels | Strain | Key enzymes expressed and pathway | Titer (g/L) | Yield (%) | Fermentation time | Reference |
|---|---|---|---|---|---|---|
| Isobutanol | *E. coli* | AlsS, IlvCD, KDC, and Adh | 22 | 86 | 110 h | [21] |
| Isopropanol | *E. coli* | Thl, AtoAD, Adc, and Adh | 143 | 67 | 240 h | [65] |
| 3-Methyl-1-butanol | *E. coli* | IlvIHCD, Kivd, and ADH2 | 0.56 | na | 18 h | [41] |
| 2-Methyl-1-butanol | *E. coli* | MetA and Tdh | 1.25 | 0.17 | na | [41] |
| Isopropanol | *E. coli* | Thl, AtoAD, Adc, and Adh | 0.41 | 43 | 30.5 h | [19] |
| Ethanol | *S. cerevisiae* | Phosphoketolase pathway for increased $NAD^+$ during xylose metabolism | na | 0.42 | na | [66] |
| Pentenol | *E. coli* | NudF | 0.11 | 0.006 | na | [43] |
| 1-Pentanol | *E. coli* | LeuA and Kivd | 0.75 | 0.038 | na | [42] |
| Butanol | *E. coli* | AtoB, Hbd, Crt, Ter, and AdhE2 | 30 | 70–88 | 7 days | [67] |
| FAEE | *E. coli* | TesA, FadD, AtfA, Pdc, and AdhB | 0.43 | 9.4 | 72 h | [44] |
| Farnesol | *S. cerevisiae* and *E. coli* | Mevalonate pathway and IspA | 0.13 | nd | 48 h | [68, 69] |
| Fatty acids | *E. coli* | na | 0.2 | 9.7 | na | [59] |
| Fatty alcohols | *E. coli* | TesA, FadD, and Acr1 | 0.06 | 0.7 | 72 h | [44] |
| Pentanol | *E. coli* | Deletion of competing pathways; overexpression of leucine biosynthetic genes | | 0.11 | na | [28] |
| Terminal alkenes | *E. coli* | TesA and OleTJE | na | na | na | [70] |
| Alkanes | *E. coli* | AAR and ADC | 0.3 | 3.5 | 40 h | [71] |

*na* not available

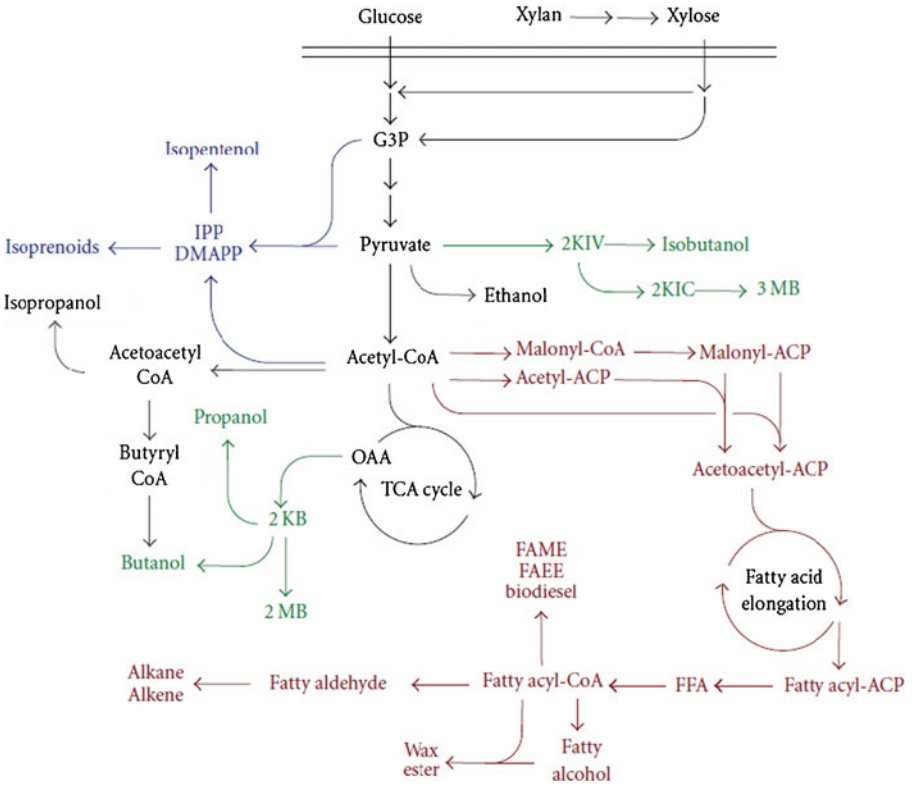


**Fig. 3** Metabolic schematic for biofuel production. Biofuel production pathways for traditional fermentative processes (*gray*), nonfermentative higher chain alcohols (*green*), isoprenoid fuels (*blue*), and fatty acid fuels (*red*) from central metabolism. *2 KB* 2-ketobutyrate, *2 KIV* 2-ketoisovalerate, *2KIC* 2-ketoisocaproate, *2MB* 2-methyl-1-butanol, *3MB* 3-methyl-1-butanol, *ACP* acyl carrier protein, *CoA* coenzyme A, *DMAPP* dimethylallyl diphosphate, *FAEE* fatty acid ethyl ester, *FAME* fatty acid methyl ester, *FFA* free fatty acid, *G3P* glyceraldehyde-3-phosphate, *IPP* isopentyl diphosphate, and *OAA* oxaloacetate [28]

(*kivd*) and alcohol dehydrogenase (*adhII*) to *Synechocystis* for the production of butanol. The branched-chain alcohols have higher octane numbers as compared with their straight-chain counterparts.

The metabolic engineering approach has been previously used in *E. coli* to produce higher alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol from glucose, a renewable carbon source. The high yield and specificity of isobutanol from glucose has been achieved recently [31]. It is now feasible to produce ethanol, propanol, and butanol from volatile fatty acids (VFAs). VFAs such as acetic, propionic, and butyric acids have been reduced by mixing microbial cultures with a headspace of 1.5 bar of hydrogen. Alcohol concentrations have been recorded as 3.69±0.25 mM of ethanol, 8.08± 0.85 mM of propanol, and 3.66±0.05 mM of *n*-butanol [32].

The lignocellulosic biomass consists of complex mixtures of different fermentable sugars that contain inhibitors and salts which affect the performance of microbes. The performance of six industrially relevant microbes like *E. coli*, *Corynebacterium glutamicum*, *S. cerevisiae*, *Pichia stipitis*, *Aspergillus niger*, and *Trichoderma reesei* have been compared for their ability to utilize the corn stover, wheat straw, sugarcane bagasse, and willow wood. The ability of

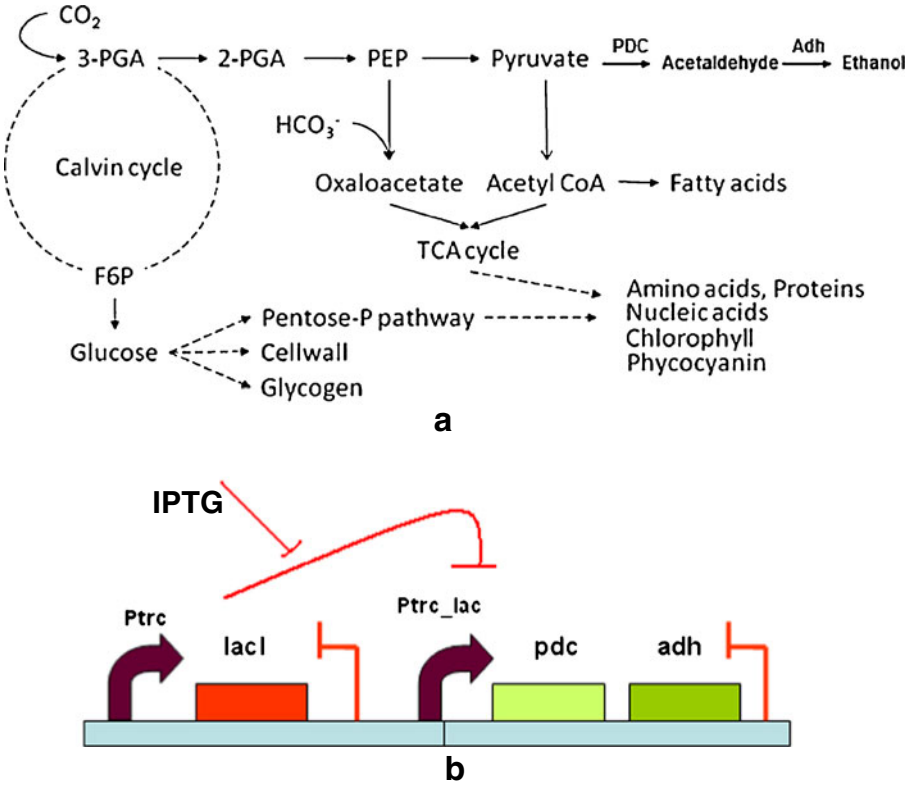


**Fig. 4** **(a)** Schematic representation of extension of ethanol biosynthetic pathway in cyanobacteria and **(b)** proposed tunable genetic circuit.

hosts is utilized for consuming waste glycerol from biodiesel industry. *P. stipitis* and *A. niger* were found to be most versatile, and *C. glutamicum* and *S. cerevisiae* have been shown to be least adapted to renewable feed stocks [33].

Advancements in metabolic engineering have led to the ability to efficiently engineer the *E. coli* as a biocatalyst for the production of biofuels from several biomass sources [34]. The hope is to utilize carbohydrates, carbohydrate mixtures, and noncarbohydrate carbon sources using fermentative and nonfermentative pathways for producing higher carbon biofuels [34].

Several attempts have been made for the conversion of cellulosic biomass into simple sugars followed by biofuels. Microorganisms have been engineered to convert simple sugars into alcohols, fatty acid alkyl esters, alkanes, and terpenes with high titers and yields [17]. Currently, butanol production is not economically competitive compared to petrochemicals. It has a major drawback such as high cost of the feedstocks, low butanol concentration in the fermentation broth, and the production of low-value by-products acetone and ethanol [35]. However, lignocellulosic biomass is recognized as potential sustainable source for production of power, biofuels, and variety of commodity chemicals [36].

In the future, it is expected that biofuels produced from engineered microbes will find a major inroad into transportation systems [37]. Protein engineering has been used to improve the performance of lignocellulose-degrading enzymes involved in biofuel synthesis [38]. Degradation of lignocellulose for biofuel production is a difficult process which is limited by recalcitrance of lignocellulose and biological toxicity of the products [39].

A number of biofuels are known to reduce the cell viability by damaging cell membrane interfering with essential physiological processes. To overcome this, cells trade off biofuel production and survival that reduces the yields. Efforts have been made toward the bioengineering of strains for biofuel tolerance. Engineering biofuel export systems, heat shock proteins, membrane modifications, more general stress responses, and approaches that integrate multiple tolerance strategies have been attempted [40]. *Clostridia* are recognized as promising butanol producers for industrial-scale production. However, the difficulty in genetically manipulating *Clostridia* strain has been rather slow [25].

*S. cerevisiae* is the most popular fermentation yeast used for the scale up of bioconversion, but it cannot metabolize pentose, xylose, and arabinose sugars. Therefore, biological engineering is required for the integration of pentose metabolic pathways for efficient bioconversion of these substrates. Genetically modified microbes produce butanol at maximum capacity of approximately 20 g/L. The fuel-producing hosts and pathways need to be engineered and optimized for enhancing production levels [7]. A number of knockout strains have been tested for improved 2-methyl-1-butanol production. Knockout strains leading to increased flux of threonine and 2 KB (*metA* and *tdh*) showed significant improvement [41]. The best strain tested achieved 1.25 g/L of 2-methyl-1-butanol with a yield of 0.17 g/g glucose [41].

Protein engineering is a classic approach for improving the enzyme/protein activity. The two promiscuous enzymes LeuA and Kivd have been developed for the production of 1-pentanol. First, the removal of leucine-induced feedback inhibition of LeuA allowed 1-pentanol to be detected, and an enlargement of the Kivd substrate-binding pocket allowed 1-pentanol production in *E. coli* up to 750 mg/L [42]. The screening of a genomic library for *B. subtilis* enzymes IPP and DMAPP helped lower toxic levels of farnesyl diphosphate, a product of IPP and DMAPP polymerization. The expression of the *nudF* gene from *B. subtilis* allowed the production of about 110 mg/L isopentenol [43]. Pentanol is an isomer of 2-methyl-1-butanol and 3-methyl-1-butanol which is a useful class of chemical with potential application as biofuels. Its production titer and yield is too low for practical applications. To fill in the need, metabolic engineering of microbes strain has been employed for increased 1-pentanol and pentenol [41].

## Metabolic Engineering for Production of Biodiesel

Biodiesel is a derivative of fatty acid such as methyl, ethyl, or propyl esters (fatty acid methyl esters (FAME), FAEE, and FAPE) and is currently consumed up to approximately 2 billion gallons per year. A FAEE-producing *E. coli* strain was engineered based on the above-listed fatty acid production strain [44]. Biodiesel can be made by several methods, but is most commonly synthesized by the transesterification of oils and fat triglycerides with methanol to make FAME. It is a major component of cell membrane. Fatty acids are synthesized in high flux and converted into phospholipids. The long hydrocarbon, fatty acyl chain is energy rich, making it an ideal precursor for biofuels.

The use of biodiesel has grown noticeably during the last few years and is expected to increase in the near future. It is a renewable biofuel and alternative diesel, but the first generation of biodiesel has less optimal properties and in production methods. The fatty acid biosynthesis pathway and isoprenoid pathway have been used to produce second generation of advanced biodiesel with better properties and quality [45]. Biodiesel production by the use of microbial systems has marked a turning point in the field of biofuels. It is emerging as an attractive alternative to the conventional technology [46]. The acid and enzymatic methods

have been developed for the hydrolysis of biomass and for transesterification of plant oils. The yeast metabolic engineering has resulted in the production of ethanol from biomass [47].

LS9 has been developed to convert the cellulosic hydrolysates into the hydrocarbon product as a replacement for diesel with a hope to mitigate issues with the current petroleum-based economy [48]. During fatty acid biosynthesis, fatty acids are activated as thioesters with coenzyme A (fatty acyl-CoAs) or acyl carrier protein [49] that can be used for enhancement of biofuels.

As shown in Fig. 5, combining the natural fatty acid synthetic ability with new biochemical reactions has been achieved. *E. coli* has been engineered to produce structurally tailored fatty esters (biodiesel), fatty alcohols, and waxes directly from simple sugars. The biodiesel-producing strains express hemicellulase towards producing these compounds directly from hemicellulose, which is a major component of plant-derived biomass [44].

*E. coli* has been engineered to produce FAEEs directly from glucose and ethanol. Expression of the gene encoding a wax-ester synthase (*atfA*) with expression of *fadD* and *'tesA* and the addition of ethanol (2 %) resulted in the production of approximately 400 mg $L^{-1}$ of FAEEs in 48 h which ranged from $C_{12}$ to $C_{18}$. The overexpression of *fadD* is a first step in the fatty acid degradation process and deletion of *fadE*. In the second step of fatty acid degradation, there is a marked increase in the FAEE production and other fatty acid products [44]. By expressing *acr1* instead of *atfA* (used in the FAEE-producing strains), medium chain fatty alcohols up to 60 mg $L^{-1}$ have been produced, whereas *FadD* improved the synthesis of all products; the lower level of alcohol as compared to FAEEs (400 mg $L^{-1}$) suggests that *Acr1*

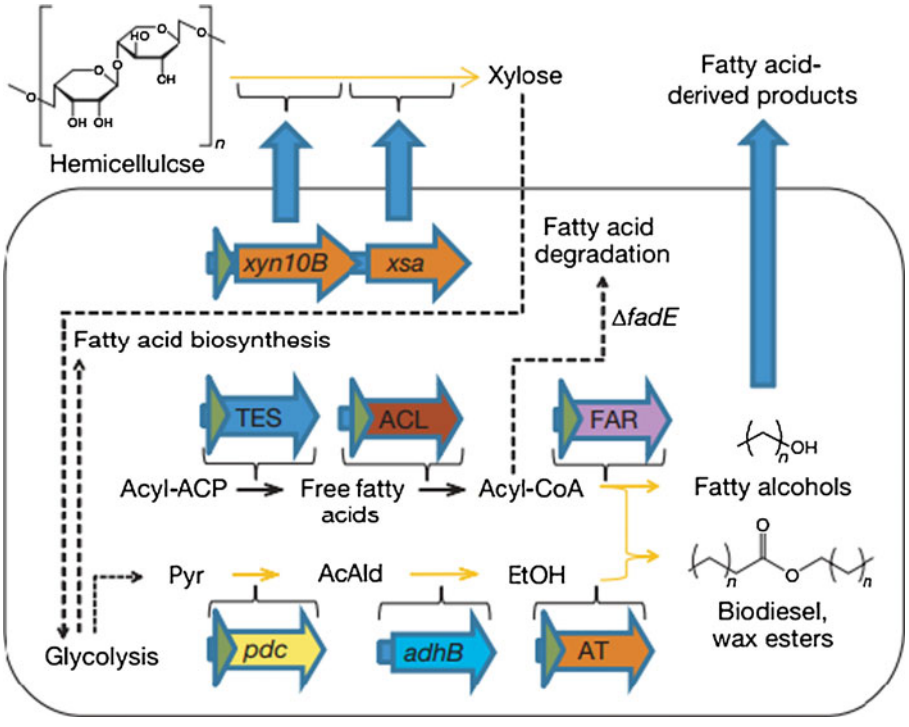


**Fig. 5** Engineered pathways for production of fatty acid-derived molecules from hemicelluloses or glucose and depiction of the synthetic operons [44]

may be limiting in this pathway [44]. The fatty acyl-CoAs or fatty acyl-ACPs are the starting materials for biosynthesis of fatty acid-derived fuels. Biodiesel belongs to this class of fuels. It consists of FAMEs manufactured by chemical transesterification of plant oils. The production of biodiesel has faced several problems such as limited supply and land yield, inconsistent performance, and challenging economics [50].

In addition to microbes, algae are alternative eco-friendly source for the production of biodiesel. Algal-based biodiesel is a second-generation biofuel [51]. Algal lipids are good candidates for biodiesel production because of their higher lipid content, shorter time growth cycle, and need for less land [52]. High biomass and lipid production under heterotrophic conditions have been achieved with *Chlorella protothecoides* by employing different carbon sources. *Chorella* accumulates the lipid as high as 55 % of the cell dry weight after 6 days of cultivation by feeding corn powder hydrolysate in fermentors [53, 54].

The prices of feedstock and operation process are two major components of biodiesel production. The value of feedstock accounts for 60–70 % of the total cost of the biodiesel [55]. Although the free fatty acids cannot be used as fuel directly, their derivatives including fatty alcohols, fatty acid alkyl esters, fatty acid-derived alkanes, and alkenes. These are good biofuel targets because of their low water solubility, high energy density, and low toxicity to the production hosts [56]. The fatty acid biosynthesis and regulation have been extensively characterized that provided the information for metabolic engineering [57]. The endogenous *fadD* gene was overexpressed together with a wax-ester synthase gene (*atfA*) to activate free fatty acids to acyl-CoAs and esterify to FAEEs [58]. The well-studied industrial microorganism *E. coli* is ideally suited for this purpose. *E. coli* is about 9.7 % lipid that produces fatty acid metabolites at the productivity of 0.2 g $L^{-1}$ $h^{-1}$ per gram of cell mass. It can be achieved the product-dependent mass yields of 30–35 % by genetic manipulation [59].

## Metabolic Engineering for Production of Hydrogen

Hydrogen ($H_2$) is an attractive alternative fuel for the future due to its nonpolluting nature and unlimited supply. Improving $H_2$ production capabilities of microorganisms is a challenging issue. Photosynthetic microorganisms are attracting considerable interest within these efforts due to their relatively high photosynthetic conversion efficiencies, diverse metabolic capabilities, superior growth rates, and ability to store or secrete energy-rich hydrocarbons. Relative to cyanobacteria, eukaryotic microalgae possess several unique metabolic attributes of relevance to biofuel production. The accumulation of significant quantities of triacylglycerol is synthesized as storage starch (amylopectin and amylose), which is similar to that found in higher plants and the ability to efficiently couple photosynthetic electron transport to $H_2$ production [60]. The cultivation and harvesting of cyanobacteria and microalgae for producing biofuels and coproducts is challenging [61]. The elimination of enzymes and carbon pathways interfering or competing with $H_2$ production needs to be addressed. Recently, a significant improvement in the yield and rate of $H_2$ production have been achieved [62].

The improvement of biosynthetic pathways has been resulted in increased lipid, carbohydrate, and $H_2$ yields [60]. The wild type and engineered cyanobacteria have been extensively used in metabolic engineering and synthetic biology. Cyanobacteria are considered model organisms to examine, demonstrate, and develop photobiological $H_2$ production. The production of carbon-containing solar biofuels like ethanol, butanol, and isoprene has been attempted. Cyanobacteria generating a portfolio of solar fuels such as hydrogen, alcohols, and isoprene have been reported [61, 63].

## Conclusion and Future Perspective

Metabolic engineering is an approach to improve the product formation or cellular properties through the genetic modification or introduction of novel pathways. In addition to deleting or overexpressing the genes that encode for metabolic enzymes, the current focus is to target the regulatory networks in a cell with a hope to amplify expression. The biosynthetic pathways have been redirected towards improvement of cellular activities by manipulation of enzymatic, transport, and regulatory function of host. In the future, the improvement of metabolic models will provide a better description of the physiological behavior of the cells. The efficiency of converting a feedstock into the desired biofuels can have considerable impact on the economic feasibility. Thus, there is a need to engineer or re-engineer/rewire/create the biosynthetic pathway using metabolic engineering, protein engineering, and synthetic biology for overproduction of biofuels.

Most of genetic manipulation has been achieved by plasmid based engineering of the host. The stability of plasmids in host cells is a matter of concern. Can biosynthetic pathway for production of biofuels be integrated in genome of microbes? What kind of microbes will be required for convenient production and tolerance of biofuels? Until 2012, already second and third generation biofuels are available at a mass scale. What would happen in coming years in the biofuel industry and what would be the fourth and fifth generations biofuels like? What kind of biosynthetic parts, devices, and pathway inventory required when fossils-based fuels are finished? What are the ethical issues in releasing engineered microbes in the environment? All these challenges and issues must be addressed for any good commercial application of synthetic biology to be absorbed and supported by the community.

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