

Review Article

The Role of Synthetic Biology in the Design of Microbial Cell Factories for Biofuel Production

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Insecurity in the supply of fossil fuels, volatile fuel prices, and major concerns regarding climate change have sparked renewed interest in the production of fuels from renewable resources. Because of this, the use of biodiesel has grown dramatically during the last few years and is expected to increase even further in the future. Biodiesel production through the use of microbial systems has marked a turning point in the field of biofuels since it is emerging as an attractive alternative to conventional technology. Recent progress in synthetic biology has accelerated the ability to analyze, construct, and/or redesign microbial metabolic pathways with unprecedented precision, in order to permit biofuel production that is amenable to industrial applications. The review presented here focuses specifically on the role of synthetic biology in the design of microbial cell factories for efficient production of biodiesel.

1. Introduction

Global warming and the continued depletion of nonrenewable fuel resources are two major problems that entangle our planet today and demand immediate solutions [1]. The extensive use of fossil fuels has caused greenhouse gas emissions and damage to the environment, and has also led to the current instability of oil supplies and continuous fluctuations in prices. These factors, which revolve around economic, environmental and geopolitical issues, are central to the continued interest seen in renewable energy sources [2]. An entire branch of biotechnology, referred to as “white biotechnology” [3], centers on the bioproduction of fuels and chemicals from renewable sources. For biofuels, delicate optimization, and fine tuning of these processes to maximize productivity and yield is of particular concern, as the viability of any biofuel process is extremely sensitive to factors related to both raw material supply and production costs [4].

About 90% of the current biofuel market is represented by biodiesel and bioethanol. However, bioethanol is not seen as an ideal biofuel for the future because of its low energy density and incompatibility with the existing fuel infrastructure [5, 6]. On the contrary, biodiesel is

already better established [7] and is preferable to petrodiesel in terms of several characteristics, such as environmental friendliness, renewability, reduced emissions, higher combustion efficiency, improved lubricity, and higher levels of safety [8]. Chemically, biodiesel comprises a mixture of fatty acid alkyl esters (FAAEs). The most commonly used method to produce biodiesel is the *in vitro* transesterification process, where triacylglycerides (TAGs) of vegetable oils are combined with methanol to form fatty acid methyl esters (FAMEs) and the byproduct glycerol (Figure 1). Alkalies (e.g., sodium hydroxide, potassium hydroxide, sodium metoxide, and potassium metoxide) [9–12], acids (e.g., sulfuric acid) [13], or enzymes can be used to catalyze this reaction [14]. However, issues related to high cost and limited availability of vegetable oils have become growing concerns for large-scale commercial viability of biodiesel production [15]. Also, the *in vitro* transesterification reaction presents some unresolved issues, such as the need to use large amounts of toxic compounds (sodium hydroxide, sulfuric acid, or methanol) and the high cost of isolation and immobilization of enzyme catalysts [16, 17]. Various approaches to addressing these problems have been explored. First, increasing interest in developing microbial processes

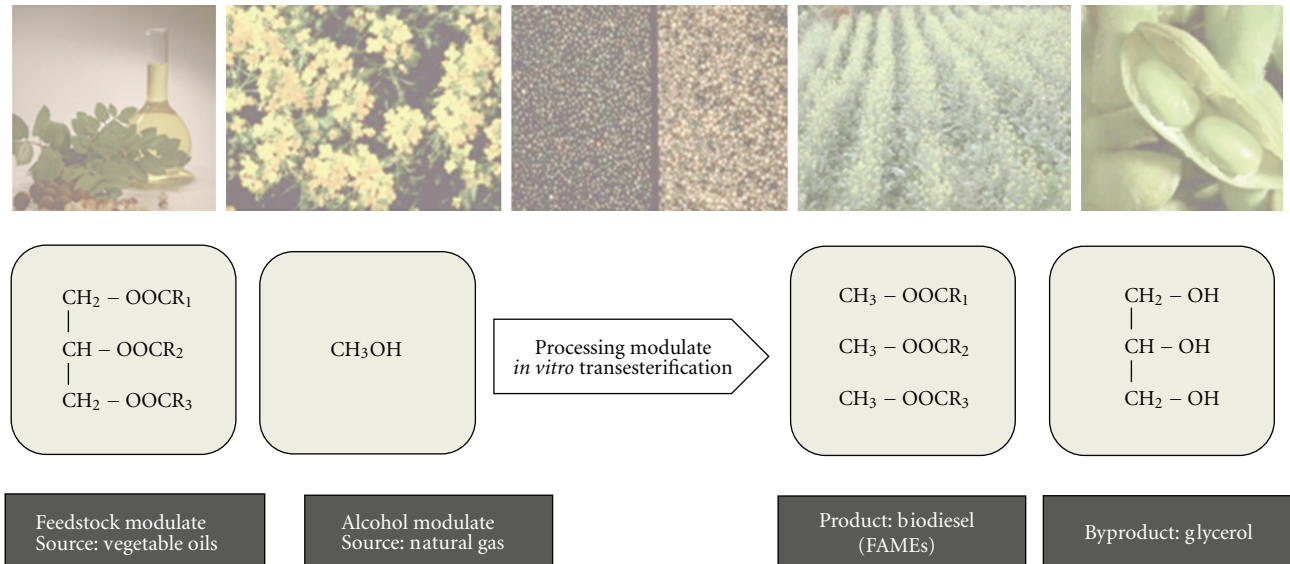


FIGURE 1: Conventional process for biodiesel production.

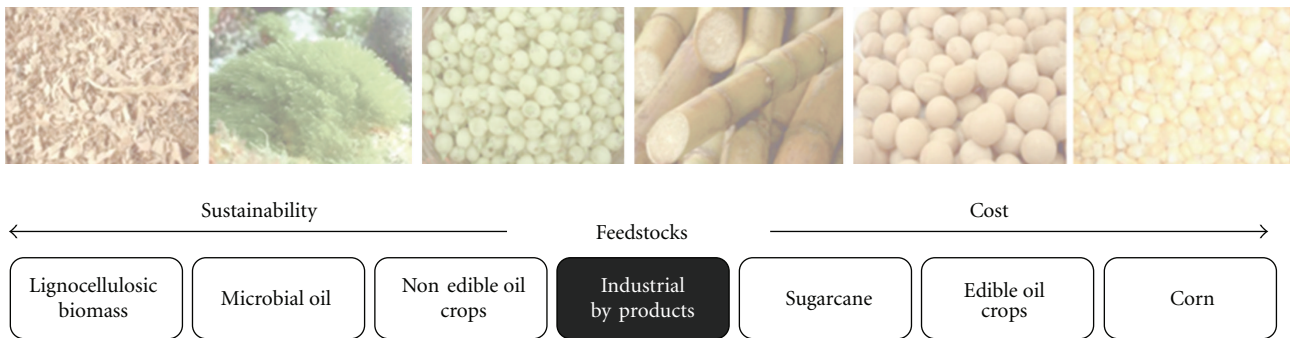


FIGURE 2: Different feedstocks for biofuel generation arranged according to their levels of environmental and economical sustainability.

for the production of biodiesel from a wide range of other raw materials may represent a promising alternative to the vegetable oils. Second, technologies now exist that use living cells to synthesize products that are more easily biodegradable, require less energy, and create less waste during production than those obtained by chemical synthesis. In order for a fermentation process to compete with existing petroleum-based processes, the target molecule must be produced at high levels of yield, titer, and productivity. These goals can be difficult to attain with naturally occurring microbes. While metabolic engineering has enabled extraordinary advances in the redesign of pathways for efficient target molecule production, including biofuels [5, 18–20], tools from synthetic biology make it possible to create new biological functions that do not exist in nature. Essentially, this is achieved either by heterologous expression of natural pathways or design of *de novo* pathways. This paper reviews approaches to microbial synthesis of biodiesel, focusing on the role of synthetic biology as an enabling technology in the design of optimal cell factories.

2. Biofuel Feedstocks: Potential Contributions of Synthetic Biology

Because of its abundance and renewable nature, biomass has the potential to produce extensive supplies of reliable, affordable, and environmentally sound biofuels to replace fossil fuels. Many biomass feedstocks, which include lignocellulosic agricultural residues as well as edible and nonedible crops, can be used for the production of biofuels. In Figure 2, biofuel feedstocks are listed according to their levels of environmental and economical sustainability.

More than 95% of global biodiesel production now begins from virgin edible vegetable oils [21], which account for about 80% of the total production cost. However, the socioeconomic impacts of large-scale biodiesel production from edible feedstocks can be significantly lowered by the use of alternative feedstocks such as nonedible oils or lignocellulosic biomass [22]. The use of nonedible vegetable oils is especially significant for biodiesel production in developing countries [9, 23, 24], because of the tremendous demand for

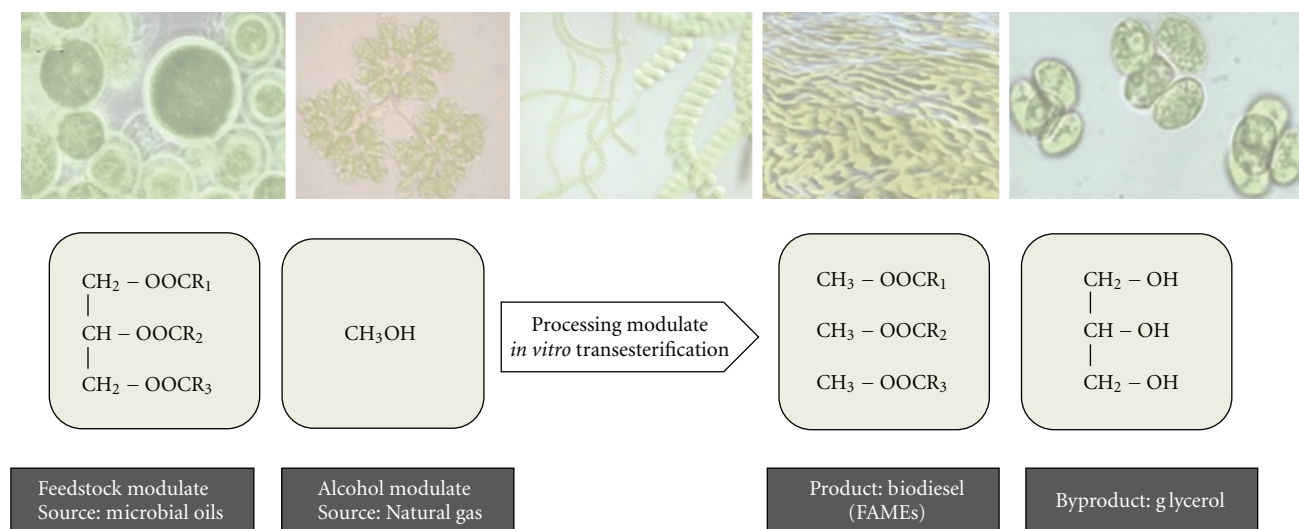


FIGURE 3: Synthesis of biodiesel from microbial oils.

edible oils as food. Increasing attention is also now being given to the use of microbial oils as biodiesel feedstock, which are produced by certain oleaginous microorganisms [25]. Lignocellulosic biomass, on the other hand, is the largest known renewable source of carbohydrates. It generally consists of about 25% lignin and 75% carbohydrate polymers (cellulose and hemicellulose) [22]. These polymers, upon complete hydrolysis, yield a mixture of hexose (glucose, galactose, and mannose) and pentose (arabinose and xylose) [26]. Synthetic biodiesel can be produced from this renewable carbon source using the Fischer-Tropsch process [27]. Although conversion of lignocellulose into biofuels appears simple in theory, the techniques used in this field are not fully established. The main reason for this lag is the recalcitrant nature of cellulose [28] and the toxic nature of the products of lignin degradation. Several prokaryotes and eukaryotes that have cellulolytic properties and are tolerant to the toxic products of lignin degradation have been identified [5, 29]. However, the yield and productivity of biofuels synthesized in this way are not sufficient to meet current energy demands. An efficient cellulolytic organism should be able to hydrolyze lignocellulose completely, ferment all sugars of lignocellulosic hydrolysate simultaneously, and tolerate toxic compounds of lignin without compromising productivity [30]. Therefore, for cost-effective production of biofuels, the fuel-producing hosts must be designed.

In terms of ethanol production, starches (maize, wheat, barley, etc.) and sugar-rich biomass (grasses, maize leaves, beets, sugar cane, etc.) have been the feedstocks most commonly used for their bioconversion [31]. However, advances in metabolic engineering and synthetic biology have provided new tools for creating desirable phenotypes for the production of ethanol from lignocellulosic biomass [32–36]. Since ethanol is one of the substrates used for *in vivo* synthesis of biodiesel, advances in terms of maximization of two-carbon alcohol production from the most economically viable feedstocks will be discussed later in this review.

3. Synthetic Biology and Biodiesel Production

Synthetic biology emerged around the year 2000 as a new biological discipline, and many different definitions have been applied to this field. However, one commonly used way to describe synthetic biology is as the design and construction of new biological functions that are not found in nature [37]. Synthetic biology is a discipline encompassing contributions from many fields [38–40], but this review places particular emphasis on the design of microbes, either by modification of existing pathways or by heterologous expression of natural pathways, in order to allow efficient production of biodiesel. In this connection, the synthesis of biodiesel using microbes is currently a highly promising alternative to conventional technologies. Microbial biodiesel production has been approached from two different angles: (1) by indirect synthesis from microbial oils, which are produced and harvested for use in the conventional *in vitro* transesterification process, and (2) by direct biodiesel synthesis using redesigned cell factories to increase production of alcohols and/or FFAs, which are subsequently used for *in vivo* synthesis of biodiesel. In the following sections, both approaches are reviewed.

3.1. Indirect Synthesis of Biodiesel from Microbial Oils. It is well known that many microbes, including certain types of microalgae, bacteria, filamentous fungi, and yeasts, can accumulate intracellular lipids, primarily TAGs, with these representing a large proportion of their biomass [41–53]. Oils derived from these oleaginous microbes represent promising raw materials for biodiesel production through transesterification using the plant-based process [54, 55] (Figure 3).

The use of microbial oils offers several advantages when compared to plant-derived oils [41]. However, oleaginous microbes have varying prospects in the biodiesel industry. For example, microalgae are photoautotrophic microorganisms that can convert CO_2 directly to lipids, which

can then be used for biofuel production, particularly for biodiesel [42, 56, 57]. The oil content of microalgae usually ranges between 15 and 70% by weight of the dry biomass [41–45]. Scaling-up process for autotrophic microalgae is complex, however, since light is needed during cultivation. Although it is known that algae could be grown in dedicated artificial ponds for generating biodiesel [58], the harvesting of miles and miles of algae growth is required in order to generate substantial amounts of biodiesel. Thus, while the microbiological aspects of this approach are extremely promising, the engineering aspects pose the greatest challenge. Tools from synthetic biology have been effectively used to convert certain autotrophic microalgae into heterotrophic microorganisms [59]. Essentially, this consists of the introduction of nonnatural metabolic pathways into the autotrophic microalgae, thereby, allowing cultivation using an organic carbon source instead of photosynthesis from sunlight.

3.2. Direct Synthesis of Biodiesel Using Cell Factories. Methanol, conventionally used as part of *in vitro* transesterification, is largely derived from nonrenewable natural gas and is also both toxic and hazardous. On the contrary, ethanol can be naturally produced from renewable resources, while exhibiting low levels of toxicity and a higher degree of biodegradability. Ethanol produced endogenously can therefore be used for *in vivo* synthesis of fatty acid ethyl esters (FAEEs) with exogenously added FFAs. Similarly, microbial FFAs can be used as feedstock for *in vivo* production of biodiesel, instead of TAGs from vegetable oils. Therefore, critical aspects of metabolic engineering and synthetic biology used for maximizing biosynthesis of ethanol and FFAs, and subsequent generation of biodiesel from these, are discussed next.

3.2.1. Biosynthesis of Ethanol. The yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* have long been used in the brewing industry to produce ethanol from 6-C sugars (glucose, galactose, and mannose), but these microorganisms are unable to ferment 5-C sugars (arabinose and xylose). On the other microorganisms such as the bacteria *Escherichia coli* and *Klebsiella oxytoca*, as well as the yeast *Pichia stipitis*, are in fact able to produce ethanol from pentose sugars, but they cannot do so naturally at sufficient yields and productivity levels. However, tools from metabolic engineering and synthetic biology have been effectively used to enhance ethanol production, through the use of two different strategies. The first essentially consists of the expression of pentose catabolic pathways in ethanologenic microorganisms such as *S. cerevisiae* [33, 34] and *Z. mobilis* [60]. Two natural enzymatic pathways for xylose catabolism are known, and both of these have been independently transferred to *S. cerevisiae*. In one pathway, the conversion of D-xylose to D-xylulose is performed by a bacterial heterologous xylose isomerase (XYLA) (Figure 4). However, this approach generally failed because xylose isomerase is strongly inhibited by xylitol, favoring an isomerization equilibrium towards xylose formation. A second natural pathway for xylose catabolism, which involves a xylose re-

ductase (XR) and a xylitol dehydrogenase (XDH), has been found in certain fungi and yeast. Introduction of this pathway from the xylose-fermenting yeast *P. stipitis* by engineering [61, 62], enables *S. cerevisiae* to consume xylose [63] (Figure 4). This strategy, however, was unsuccessful since this process also results in the excretion of xylitol, again favoring xylose formation [64].

Arabinose is another pentose sugar obtained through decomposition of biomass. Bacterial and fungal pathways for arabinose catabolism exist in nature, and both pathways have been independently expressed in *S. cerevisiae* [65, 66]. Recombinant strains engineered with the heterologous fungal pathway showed only low levels of growth on L-arabinose [66]. However, after introduction of the bacterial pathway for arabinose utilization from *Lactobacillus plantarum*, mutant yeast produced an ethanol yield of 0.43 g per g of carbohydrate consumed, and a specific ethanol production rate of 0.29 g/g/h.

As mentioned, *Z. mobilis* is an ethanologenic bacterium that is able to produce ethanol only from 6-C sugars. However, xylose metabolism was introduced into *Z. mobilis* CP4 (pZB5) by expression of *E. coli* genes encoding for xylose isomerase (XYLA) and xylulokinase (XYLB) (Figure 4). This resulted in xylose catabolism with an ethanol yield of 86% [60].

Arabinose metabolism was also introduced in *Z. mobilis* ATCC39676 (pZB206) by expression of *E. coli* genes encoding for L-arabinose isomerase (ARAA), L-ribulokinase (ARAB), and L-ribulose-5-P-4-epimerase (ARAD) (Figure 4). This results in growth on arabinose with an ethanol yield of 98% [67]. Xylose or arabinoses are both first converted to xylulose-5-P. Xylulose-5-P then, by means of the pentose phosphate pathway, yields D-glyceraldehyde-3-P, which is an intermediate product in the Embden-Meyerhof-Parnas (EMP) pathway (Figure 4). However, the recombinant *Z. mobilis* can produce ethanol only at low pentose concentrations, which limits its potential industrial applications [68].

A second strategy to enhance ethanol biosynthesis by fermentation is based upon construction of synthetic pathways in hosts that are naturally able to utilize pentose. Wild-type *E. coli* can make use of a wide range of substrates, including all of the lignocellulosic sugars [22]. However, ethanol yield is low because under fermentative conditions *E. coli* produces lactic, acetic, formic, and succinic acids [68]. Cleavage of pyruvate into acetyl CoA and formic acid by pyruvate formate lyase (PFL) begins the pathway to ethanol production for *E. coli* (Figure 4). Reduction of acetyl-CoA to ethanol proceeds by means of the multienzyme protein and requires two NADHs [69] while glycolysis to pyruvate only requires one NADH in conversion of glyceraldehyde-3-P to 1,3-bisphosphoglycerate. Under such circumstances, production of ethanol is balanced by that of other more oxidized products such as acetic acid, which no NADH consumes. To circumvent the redox limitation of the endogenous ethanol pathway, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) enzymes from *Z. mobilis* were expressed in *E. coli* (Figure 4), via a plasmid bearing an artificial *pet* operon containing the *pdC* and *adhB* genes [70]. This has resulted in an ethanol yield of 95%.

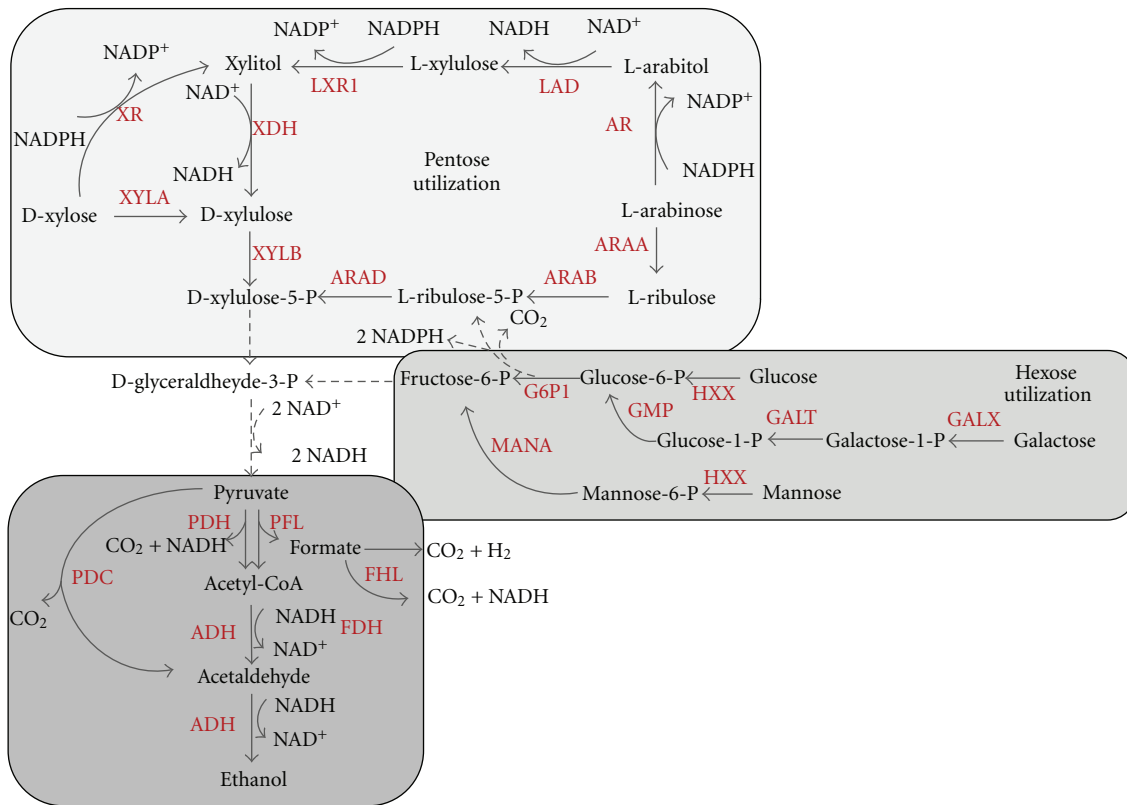


FIGURE 4: Redesigned pathways for microbial production of ethanol from carbohydrates. The dashed lines indicate multiple steps. Abbreviations: ADH: alcohol dehydrogenase; AR: aldose reductase; ARAA: L-arabinose isomerase; ARAB: L-ribulokinase; ARAD: L-ribulose-5-P-4-epimerase; FDH: formate dehydrogenase; FHL: formate hydrogen lyase; LAD: L-arabitol 4-dehydrogenase; LXRI: L-xylulose reductase; PDC: pyruvate decarboxylase; PDH: pyruvate dehydrogenase; PFL: pyruvate formate lyase; XDH: xylitol dehydrogenase; XR: xylose reductase; XYLA: xylose isomerase; XYLB: xylulokinase.

On the other hand, Kim et al. [32] reported homoethanol fermentation from xylose and glucose with yields of up to 82%, by combining the activity of pyruvate dehydrogenase and the alcohol dehydrogenase from native *E. coli* genes.

3.2.2. Biosynthesis of Fatty Acids. Most of the fatty acids produced in a microbial cell are utilized for the synthesis of phospholipids and TAGs, but some are also degraded through the β -oxidation pathway. Because of this, an improvement in the production of FFAs could be achieved by elimination of β -oxidation, along with overexpression of several genes related to fatty acid biosynthesis [71–75]. To this end, the *fadE* gene was initially disrupted in the *E. coli* strain, which was then incapable of degrading fatty acids and fatty acyl-CoA (Figure 5). The enforced fatty acid synthesizing ability was then accomplished through the overexpression of several genes encoding enzymes including thioesterase (*tesA*), acyl-CoA synthase (*fadD*), acetyl-CoA carboxylase (*accABCD*), and fatty acid synthase (*fabH*, *fabD*, *fabG*, and *fabF*) (Figure 5). To further enhance fatty acid production, expression of genes *aceEF* has been suggested for a production host, accompanied by the attenuation of glycerol-

3-phosphate dehydrogenase (*gpsA*), glycerol-3-phosphate O-acyltransferase (*plsB*), phosphate acyltransferase (*pta*), and acetate kinase (*ackA*) (Figure 5). The chain length of the fatty acids can also be made to vary from C8 to C18 by alternative expression of thioesterase in the bacterial cytoplasm [76]. Along with these modifications, the expression of the acyl-acyl carrier protein (ACP) thioesterase from the plant *Umbellularia* resulted in the production of medium chain fatty acids [75].

3.2.3. Biosynthesis of Fatty Acid Ethyl Esters (Biodiesel). The development of *in vivo* biodiesel synthesis using endogenously produced alcohols has marked a turning point in the field of biofuel production during the last decade. Two model organisms, *E. coli* and *S. cerevisiae*, have been effectively used to develop this approach. Direct synthesis of biodiesel from microbes produces primarily FAEEs, which show better performance as a fuel than FAMES. There are therefore multiple benefits associated with the production of FAEEs by using redesigned microbial cell factories. An important discovery related to this was a novel bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase (WS/DGAT) isolated from *Acinetobacter baylyi* strain ADP1.

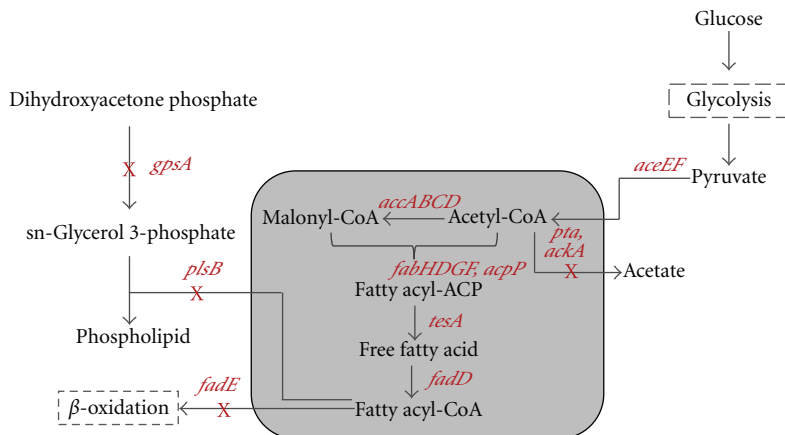


FIGURE 5: Metabolically engineered *Escherichia coli* strain for microbial production of free fatty acids. Deleted or attenuated genes are indicated by crosses. Gene/enzyme names: *fadE* (genes encoding for β -oxidation pathway); *tesA* (thioesterase); *fadD* (acyl-CoA synthase); *accABCD* (acetyl-CoA carboxylase); *fabH*, *fabD*, *fabG*, *fabF* (fatty acid synthase); *aceEF* (pyruvate dehydrogenase and pyruvate formate lyase); *gpsA* (glycerol-3-phosphate dehydrogenase); *plsB* (glycerol-3-phosphate O-acyltransferase); *pta* (phosphate acyltransferase); *ackA* (acetate kinase).

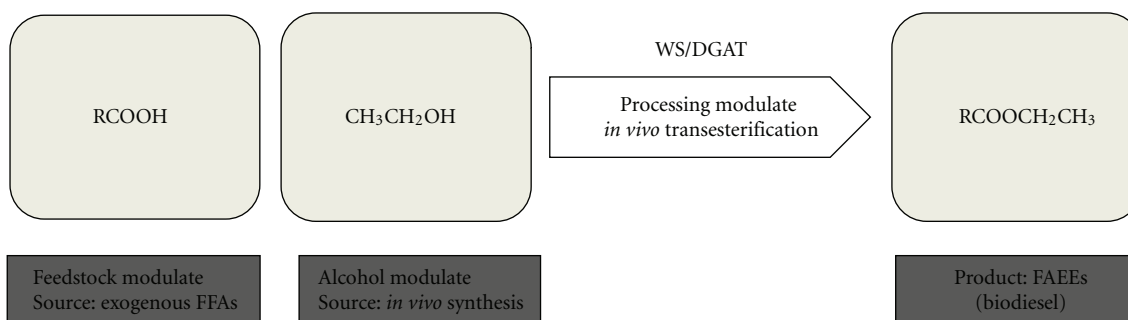


FIGURE 6: *In vivo* fatty acid ethyl esters synthesis using redesigned *Escherichia coli*. WS/DGAT: bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase.

This enzyme can synthesize wax esters from alcohols and fatty acids [77]. The idea of wax ester production was first applied effectively by Kalscheuer et al. [78], who reported on *in vivo* biodiesel synthesis from redesigned *E. coli*. Heterologous coexpression of the WS/DGAT gene (*atfA*) from *Acinetobacter baylyi* strain ADP1 with the ethanol synthesis pathway from *Z. Mobilis* (Figure 6) led to the production of 1.28 g/L of FAEEs after the addition of exogenous FFAs. More recently, Steen et al. [79] have reported on their engineering of *E. coli* to produce FAEEs, fatty alcohols, and waxes directly from simple sugars.

Given that ethanol is not naturally produced by *E. coli*, heterologous expression of a synthesis pathway for two-carbon alcohol is a prerequisite for any *E. coli* biodiesel producer. Because of this, a far better choice for a microbial cell factory for industrial production of biodiesel would be the yeast *S. cerevisiae*, which is a well-known organism already used in the production of ethanol through the fermentation of glucose [80]. Applying the same principle as used for *E. coli*, it has been reported that novel lipids, including FAEEs and fatty acid isoamyl esters, can be produced in *S. cerevisiae* H1246 after heterologous expression of the

gram-negative bacterium *Acinetobacter calcoaceticus* ADP1 bifunctional WS/DGAT enzyme [81]. *In vitro* studies have confirmed that WS/DGAT is capable of utilizing a broad range of alcohols as substrates, including long-chain fatty alcohols like hexadecanol as well as short-chain alcohols like ethanol or isoamyl alcohol. These studies have demonstrated the highly unspecific acyltransferase activity of WS/DGAT from *A. calcoaceticus* ADP1, thereby indicating the broad biocatalytic potential of this enzyme for *in vivo* biotechnological production of a large variety of lipids in both prokaryotic and eukaryotic expression hosts.

A patent application by Schmidt-Dannert and Holtzapfle [82] describes also a method for the production of biodiesel and wax esters, through heterologous expression of wax synthase (WS2) from *Marinobacter hydrocarbonoclasticus* in *S. cerevisiae*, after exogenous addition of fatty acids. Compared to the *A. baylyi* bifunctional WS/DGAT enzyme, WS2 shows higher wax synthase activity for ethanol and also does not have DGAT activity. It is important to point out that the WS2 does not catalyze the synthesis of TAGs from fatty acids, which could function as a competitive pathway for biodiesel production. Redesigned *S. cerevisiae* led to

the successful production of 62 mg/L of ethyl oleate after exogenous addition of oleic acid. It is therefore envisioned that the WS2 from *M. hydrocarbonoclasticus* could be very suitable for the specific purpose of producing biodiesel.

In terms of endogenously produced fatty acids, an *E. coli* strain was manipulated not only for improvement of the biosynthesis of FFAs but also for derivatives thereof, including fatty esters [71, 72, 74]. Knothe [83] reported that by adding different types and/or amounts of alcohols to a medium containing the engineered fatty-ester-producing *E. coli* strain, it was possible to successfully change the properties of the biodiesel obtained, including cetane number, cloud point, viscosity, and lubricity.

4. Conclusions and Future Perspectives

As noted throughout this review, increasing interest in the development of microbial processes for the efficient production of biodiesel has emerged in recent years. Metabolic engineering and synthetic biology, as the latest approaches, have been essential for allowing new technologies to be developed, as evident in the design and refinement of microbial cell factories amenable to industrial applications. The research cited in this review clearly demonstrates the feasibility of direct production of biodiesel by microbes. The possibility of developing a synthetic host for efficient target-molecule production presents great opportunities for further biofuel research. However, investment of significant amounts of time and effort is still required in order to produce a better host, carrying novel metabolic pathways to lead to satisfactory biofuel production. This is because the complexity of the intertwined metabolic pathways creates substantial limitations. Development of a recombinant host that possesses a well-defined metabolic pathway, but which is also devoid of any competing pathways to hinder the production of a specific metabolite, can be seen as the most desirable goal. However, mere engineering of all of the genes needed to produce the desired pathway is not sufficient to confer a novel characteristic to a recombinant cell, since there are always many other unexplored pathways as well. Even a simple biocatalyst like *E. coli* is a complex system involving an estimated 4,603 genes, 2,077 reactions, and 1,039 unique metabolites [69, 84]. Fortunately, it appears likely that the formal integration of functional genomics and systems biology with synthetic biology and metabolic engineering will lead to enhancement of lipid accumulation and improved engineered pathways for *in vivo* biodiesel synthesis. Microorganisms may therefore become an ideal platform for the production of biodiesel in the future.

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