

# Genetic engineering of microorganisms for biodiesel production

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Biodiesel, as one type of renewable energy, is an ideal substitute for petroleum-based diesel fuel and is usually made from triacylglycerides by transesterification with alcohols. Biodiesel production based on microbial fermentation aiming to establish more efficient, less-cost and sustainable biodiesel production strategies is under current investigation by various start-up biotechnology companies and research centers. Genetic engineering plays a key role in the transformation of microbes into the desired cell factories with high efficiency of biodiesel production. Here, we present an overview of principal microorganisms used in the microbial biodiesel production and recent advances in metabolic engineering for the modification required. Overexpression or deletion of the related enzymes for de novo synthesis of biodiesel is highlighted with relevant examples.

## Introduction

Energy is essential for life while the industry development and the global economy actually run on energy. The application of fossil fuels as energy sources is unsustainable due to depleting limited resources and also due to the accumulation of greenhouse gases in the environment.<sup>1</sup> The current consumption of crude oil is about 11.6 million tons per day, from which we can conclude that the entire resources will suffice for a rather short time period only.<sup>2,3</sup> Without appropriate alternatives to crude oil, the global economy will suffer a dramatic collapse by reason of exploding oil prices as the demand will continuously rise.<sup>4</sup> It is inevitable to resolve the dependence to crude oil and the increasing impairment of the environment by establishing a sustainable and competitive alternative which is based on renewable and abundant feedstock like biomass<sup>5,6</sup> or on other regenerative sources. Environmental concerns and the depletion of oil reserves have resulted in research promotion on environmentally friendly and sustainable biofuels.<sup>7</sup>

Among all the biofuels, biodiesel has been receiving perhaps the most attention, due to the similarity with conventional diesel in terms of chemical structure and energy content. Additionally, no modification of the diesel engine is required, as biodiesel is compatible with existing engine models and has been

commercially blended with diesel as a transportation fuel in a number of countries including Germany, Italy and Malaysia.<sup>8</sup> Biodiesel is mainly obtained by the transesterification of fat and vegetable oils in the presence of a catalyst by an alcohol leading to a fatty acid methyl esters (FAMEs) or a fatty acid ethyl ester (FAEEs).<sup>9</sup> Unlike petroleum diesel, biodiesel is biodegradable and non-toxic, and it significantly reduces toxicant and other emissions when burned as a fuel.<sup>10</sup>

The traditional methods of biodiesel production include chemical catalysis and enzymatic catalysis. Chemical catalysis is of prime importance for the current industrial production of biodiesel,<sup>11</sup> while enzymatic catalysis is also an attractive approach for its mild reaction conditions, easy product separation and so on.<sup>12,13</sup> However, the current biodiesel production faces several problems: deficiency of a stable, sufficient feedstock supply system, inconsistent performance, and challenging economics.<sup>14</sup> The microbial production has the potential to overcome these challenges due to some advantages such as short producing period, little labor required, easy to scale up, and regardless of venue, season, climate change and other factors.<sup>15</sup> Making full use of microorganism for biodiesel production has a very large potential. Here, we present a simple overview of principle microorganisms used in the biodiesel production, important metabolism pathways related with biodiesel synthesis and potential routes for construction efficient and less-cost biodiesel production systems.

## Principal Microorganisms for Biodiesel Production

Microorganisms, usually one-celled organisms, could not only provide the biodiesel synthesis with the substrates such as fatty acids sources and alcohols, but also be used as a catalyst in this process.

**Grease microorganisms to supply fatty acid sources.** Grease microorganisms, also called oleaginous microorganisms, is frequently applied and studied in the current biodiesel production to supply fatty acids source for transesterification. Grease microorganisms, coming from a rich microorganism resource, can utilize or convert various agro-industrial materials (e.g., plant biomass) to cellular lipids.<sup>16</sup> The fatty acid composition of the lipids extracted from grease microorganisms is generally similar to vegetable oils, which can be converted into biodiesel.<sup>17</sup> Grease strains could be found in the species of bacteria, yeasts, molds and algae.<sup>17</sup> The key characteristics could be summarized as follows: (1) more than 50% of lipids should be accumulated in the

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cells; (2) the strains can be used in industrialization culture with simple apparatus; (3) the strains should have fast growth speed and anti-pollution ability; and (4) the oil is easy to be extracted.

In recent years, microalgae are attracting considerable interest as an economically viable oil feedstock due to its obvious superiority of easily culture, big group, diverse metabolic capabilities and high fatty acid content.<sup>18</sup> Many autotrophic microalgae capable of oil accumulation have been found, such as *Chlorella vulgaris*, *Botryococcus braunii*, *Navicula pelliculosa*, *Scenedesmus acutus*, *Cryptocodinium cohnii*, *Dunaliella primolecta*, *Monallanthus salina*, *Neochloris oleoabundans*, *Phaeodactylum tricornutum* and *Tetraselmis sueica*.<sup>19</sup> The average oil content of microalgae varies from 1 to 70% and would significantly affected by the cultivation conditions and algae species.<sup>20</sup> Khozin-Goldberg and Cohen<sup>21</sup> observed that TAG content increased in *Monodus subterraneus* under phosphate limitation conditions. Xin et al.<sup>22</sup> showed that microalga *Scenedesmus* sp LX1 yielded the highest amount of lipid with a content of 35.7% when only the cultivation temperature was 20°C. Microalgae have been considered to be the inevitable development trend of future biodiesel. However, several barriers, such as low growth rate, strict breeding condition and large upfront investment, need to be overcome before microalgae can be used as an economically viable biofuel feedstock.<sup>18</sup>

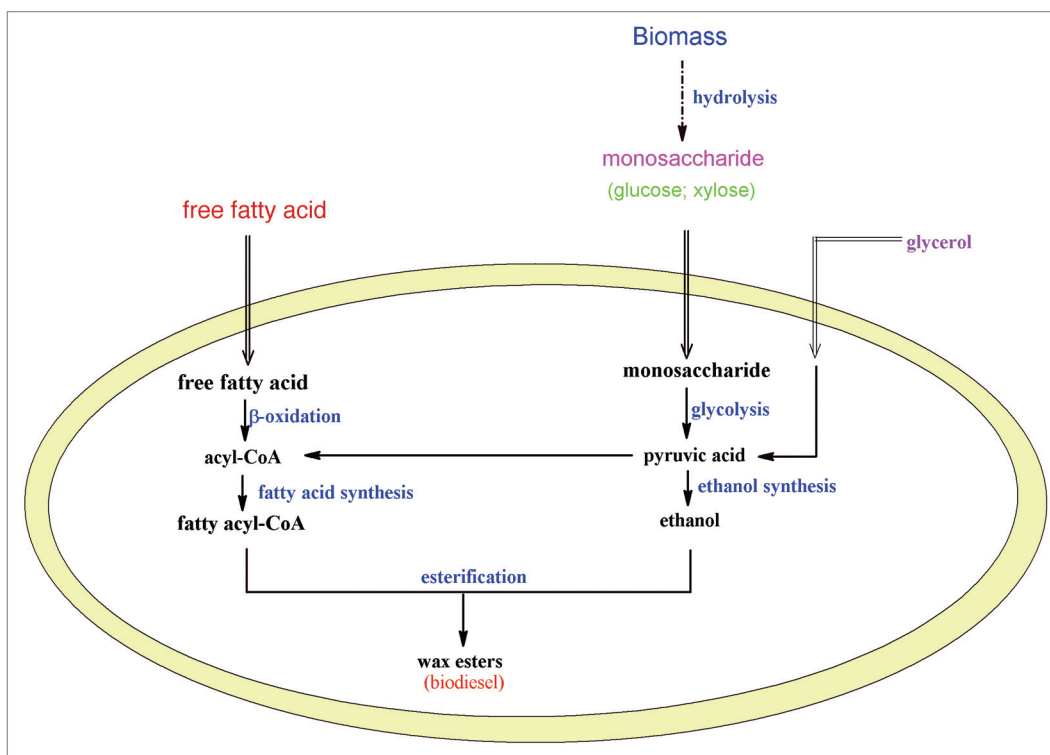
**Ethanol producers to supply alcohol sources.** Alcohol is another substrate in the transesterification reaction to generate the corresponding fatty acid ester. Methanol or ethanol is the most frequently used acyl acceptor, while FAMES is cheaper, more reactive and volatile than FAEEs. However, ethanol is less toxic and is considered more renewable. Ethanol can be easily produced from renewable sources by microbial fermentation,<sup>10</sup> which has been demonstrated by many reports and reviews.<sup>23,24</sup> Owing to so much effort from many researchers, the yield of ethanol can even reach the theoretical value.<sup>25,26</sup> The most commonly used microbe for ethanol production is yeast, among which, *Saccharomyces cerevisiae* is the preferred one producing ethanol to give concentration as high as 18% of the fermentation broth.<sup>23</sup> *S. cerevisiae* has been widely used as a key cell factory platform for future biorefineries.<sup>27</sup> *Zymomonas mobilis* is another well studied ethanol-producing strain with published genome sequence.<sup>28</sup> *Z. mobilis* has higher ethanol yields and faster specific rates compared with yeast, although it has a limited carbon substrate range of simple C6 sugars glucose, fructose and sucrose. The genetic manipulation systems in *Z. mobilis* have been well established, while many researches have been performed on its metabolic engineering focused on extending its substrate range for ethanol production.<sup>29</sup> In the study of Hideshi Yanase et al.<sup>25</sup> *Z. mobilis* was engineered to convert the wood hydrolysate containing glucose, mannose and xylose as major sugar components to ethanol by introducing genes encoding mannose and xylose catabolic enzymes. The yield of ethanol reached 89.8% of the theoretical yield within 72 h. Ethanol production could also be achieved by genetic engineering of *E. coli* strains, which was one of the early successful applications of metabolic engineering.<sup>30</sup>

**Recombinant microorganisms capable of de novo biosynthesis of biodiesel.** Pathway manipulation by genetic engineering allows the transformation of microbes into “cell factories”.<sup>16</sup>

De novo biosynthesis of biodiesel has been realized in some reports by metabolic engineering.<sup>31-34</sup> Figure 1 illustrates a common de novo biosynthetic pathway of biodiesel like FAEEs in the microbes. Several specific pathways such as ethanol production, fatty acid metabolism and transesterification are involved in this pathway.

*E. coli* is an excellent host for genetic engineering to produce high-value chemicals given its plethora of sophisticated genetic tools as well as the wealth of information available on its metabolism.<sup>35,36</sup> The world's first report of de novo biosynthetic pathway of biodiesel is showed in *E. coli* by the simultaneous overexpression of the ethanol production genes from *Z. mobilis* and the wax ester synthase/acyl-CoA-diacylglycerol acyltransferase (WS/DGAT) gene from the *Acinetobacter baylyi* strain ADP1.<sup>37</sup> By this approach, ethanol production in the cells was combined with subsequent esterification when they were cultivated under aerobic conditions in the presence of glucose and oleic acid. It is notable that biosynthesis of FAEEs in Kalscheuer's work<sup>37</sup> relied on the supplementation of exogenous fatty acids. The uptake of exogenous fatty acids from the medium and their activation to the corresponding acyl-CoA thioesters becomes a factor limiting FAEEs accumulation in the report of Kalscheuer. Yangkai Duan and his group members<sup>32</sup> further modified the fatty acids pathway of *E. coli* to increase the content of endogenous fatty acids, which supplied as acyl moieties of coenzyme A thioesters of fatty acids for transesterification. To decrease the process cost, Steen et al.<sup>33</sup> engineered the substrate metabolism pathway of the *E. coli* with FAEEs producing capability. This newly engineered strain was able to accumulate FAEEs utilizing hemicellulose as raw materials. Figure 2 illustrated the detailed genetic manipulations successfully used in *E. coli* to produce FAEEs directly from lignocellulose biomass without the addition of expensive enzymes.

In addition to *E. coli*, *S. cerevisiae* may also be a potential candidate for genetic manipulation. *S. cerevisiae* is a good ethanol producer and is able to accumulate fatty acids with a chain length of mainly 16 or 18 carbon atoms.<sup>38</sup> In *S. cerevisiae*, ethanol is accumulated at a high concentration and hence biodiesel production is not limited by ethanol supply.<sup>39,40</sup> The FAEEs synthesis in the *S. cerevisiae* would initiated only when acyltransferase is introduced, which synthesize fatty acid esters from alcohols and fatty acyl coenzyme A thioesters. On the other hand, comprehensive modifications in more and more functional microorganisms to produce less-cost biofuel will also be possible with advances in genetic transformation methods and increased knowledge regarding expression systems. For example, genes for microbial biodiesel production could be expressed in some of the oleaginous microorganisms independent from fatty acid or lipid feeding, since the fatty acyl-CoA supply in such microorganisms is sufficient.<sup>34</sup> The more and more fundamental understanding on the metabolism pathways and genetic manipulation technologies provided in oleaginous microorganisms, such as *Rhodococcus opacus*<sup>41</sup> and *Yarrowia lipolytica*,<sup>42</sup> suggests their potential application in microbial biodiesel production. Another promising functional microorganisms as the candidate for engineering is cellulosic microorganisms, which have multiple cellulase enzyme systems for complete biomass digest. Establishing the plasmid



**Figure 1.** Representative pathways in microorganisms could be engineered for de novo biosynthetic process of biodiesel (e.g., fatty acid ethyl esters).

for FAEs production in cellulosic microorganisms used as an expression host, such as cellulose-degrading or hemicellulose degrading strains, may help to decrease the cost from both raw materials and production process. In addition, microalgal is also recognized as a good model for biodiesel production though manipulation, although the application of genetic engineering in eukaryotic microalgae is still in its infancy.<sup>43</sup>

### Genetic Engineering in Fatty Acids Pathway

Understanding microbial fatty acid metabolism is of great interest for biodiesel production. Both the quantity and the quality of biodiesel precursors from a specific strain are closely linked to how fatty acid metabolism is controlled.

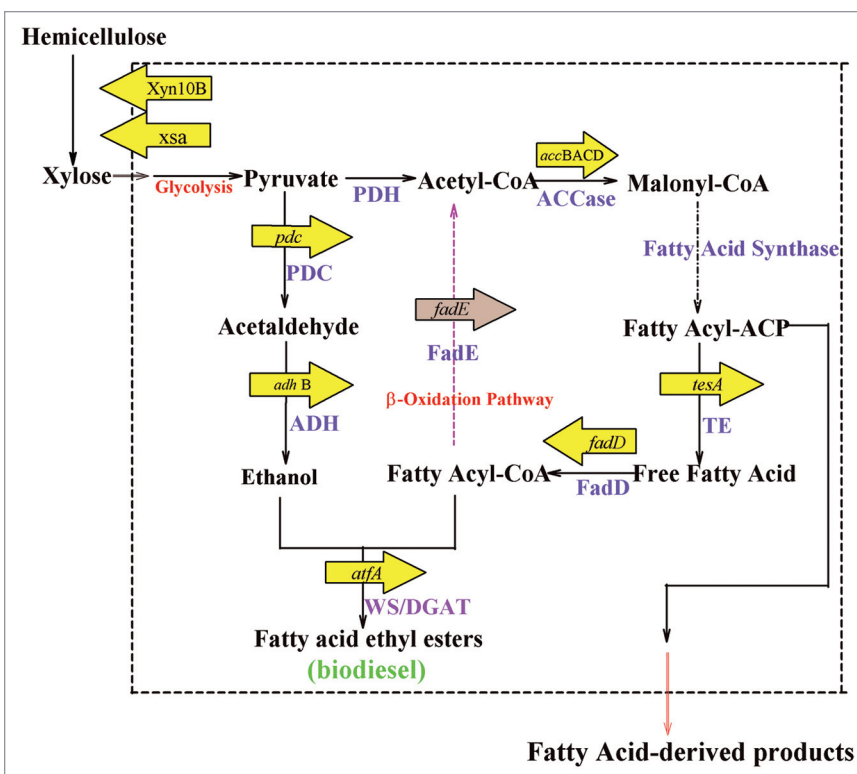
**Overview of fatty acids pathway.** Cellular fatty acids are derived from three different sources: external supply, endogenous lipid (protein) turnover, and de novo synthesis and elongation.<sup>38</sup> The major supply of fatty acids during cellular growth depends on de novo synthesis. **Figure 3** illustrates the biochemical reactions involved in fatty acid metabolism. In essence, the fatty acids biosynthesis of microbes is similar with that of animals and plants, both of which are performed by the fatty acid synthetase (FAS) and requires the constant supply of acetyl-CoA and malonyl-CoA.<sup>44</sup> In non-oleaginous microbes, acetyl-CoA is usually produced from the glycolysis pathway by breaking down pyruvate and from the cytosolic pyruvate-acetaldehyde-acetate pathway.<sup>45</sup> Additional acetyl-CoA could be provided from the cleavage of citrate by ATP citrate lyase (ACL) in oleaginous microorganisms.<sup>46</sup> Acetyl-CoA is then catalyzed to form malonyl-CoA by

acetyl-CoA carboxylase (ACC), which is the important committing step of the fatty acid synthetic pathway.<sup>47</sup> In *E. coli*, ACC is a protein containing four subunits with a biotin carboxyl carrier protein, a biotin-carboxylase subunit and a carboxyl-transferase subunit joined together into a heterotrimeric complex.<sup>38</sup> In contrast, the ACC in the eukaryotic cells is a multi-domain single polypeptide, having all the functions of the ACC.<sup>38,47</sup> Once malonyl-CoA is synthesized, it is condensed with acetyl-CoA catalyzed by the fatty acid synthetase (FAS) for fatty acid elongation. This resulting compound is transformed via three successive reactions, i.e., reduction, dehydration and reduction, and then condensed with another malonyl-CoA. This cycle is repeated until the saturated chain of a palmitic (16:0) or a stearic acid (18:0) is formed. Type II FAS, a multi-subunit protein in which each individual peptide is dissociable and can catalyze an enzymatic transacetylation reaction, is the major enzyme responsible for this process in bacteria and plant.<sup>48,49</sup> In yeast, this enzyme is found to be the type I FAS, a multifunctional protein.<sup>45</sup> To obtain longer or unsaturated chains, elongases and desaturases are required, which are usually located in endoplasmic reticulum membrane and mitochondria. During the fatty acids biosynthesis, NADPH is one of the important substrates required for two reduction steps in the fatty acid elongation cycle, which is mainly produced by malic enzyme (ME) and the pentose phosphate pathway.<sup>50</sup> Fatty acid produced in the cell will then be transformed to various fatty acid esters or be degraded.  $\beta$ -oxidation is the principal metabolic pathway responsible for the degradation of fatty acids, which is taking place in the peroxisome,<sup>51</sup> although the transport mechanism remains unclear.<sup>45</sup>

**Improving the fatty acids biosynthesis.** The biochemical approach refers to the strategy of enhancing fatty acids accumulation by controlling the nutritional or cultivation conditions to channel metabolic flux into lipid biosynthesis.<sup>43</sup> There is apparently a dilemma in the biochemical strategy, i.e., the conditions that stimulates fatty acids esters accumulation in cells may result in severely impeded cell growth and hence lowered overall lipid productivity.<sup>52</sup> It is likely that the dilemma could be solved by employing metabolic engineering approaches aiming at enhancing the metabolic flux into fatty acids biosynthesis. Numerous studies have been carried using the genetic strategy to enhance the lipid accumulation in different species.

As an important enzyme catalyzing the first committed step in fatty acid biosynthesis in many organisms, ACC has traditionally been thought to exert a strong control on the metabolic flux of fatty acid synthesis. Overexpression of ACC did enhance the fatty acid synthesis in various organisms, including plant, bacteria, yeast, and so on.<sup>53-55</sup> Ruenwai et al.<sup>55</sup> isolated a multi-domain ACC from an oleaginous fungus *Mucor rouxii*. Overexpressing the *M. rouxii* ACC in a non-oleaginous yeast *Hansenula polymorpha* resulted in a 40% increase in the total fatty acid content. By the overproduction of ACC in *E. coli*, 100-fold increase in the malonyl-CoA pool was obtained accompanying by a 6-fold increase in fatty acid production.<sup>53</sup> Similarly, Klaus et al.<sup>56</sup> achieved a 30% increase in total fatty acid content in *Solanum tuberosum* (potato) by overexpressing the ACC from Arabidopsis. Nevertheless, the low increase ratios in the above-mentioned reports indicate that overexpression of ACC alone may not be adequate to affect the whole fatty acids biosynthesis significantly. Actually, ACC was also successfully overexpressed in the diatoms *C. cryptica* and *N. saprophila*. However, no significant increase of lipid accumulation was observed in the transgenic diatoms.<sup>57,58</sup>

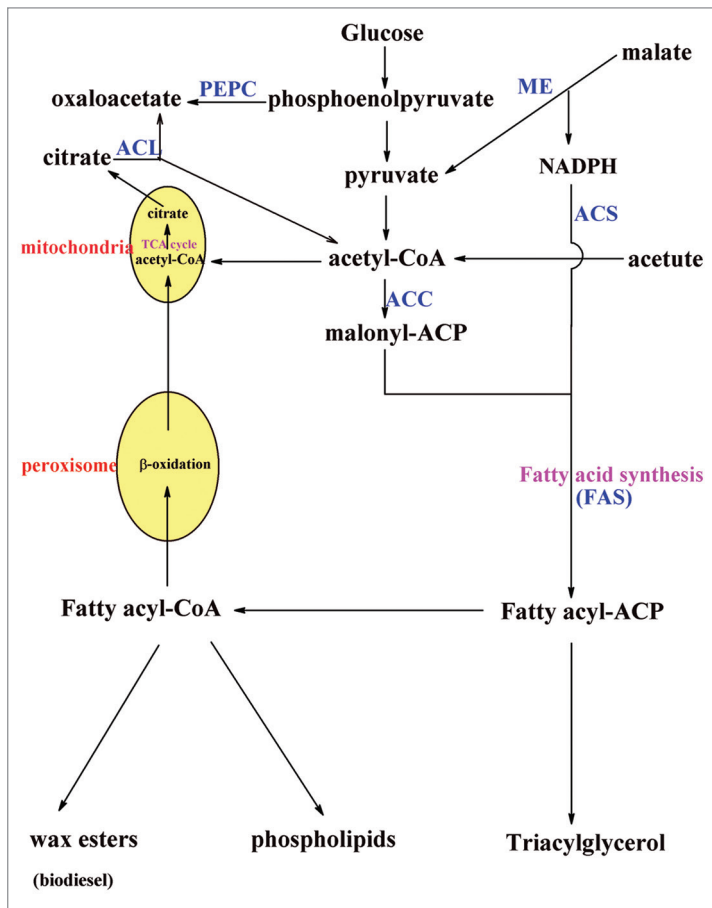
Subsequent steps in fatty acid biosynthesis can also limit fatty acid flux. There are also other enzymes presenting a strong correlation with the fatty acid biosynthesis.<sup>50,59</sup> The ATP: citrate lyase (ACL) catalyzes the conversion of citrate into acetyl-CoA and oxaloacetate, and represents a source of acetyl-CoA for fatty acid biosynthesis. An increased amount of fatty acids by 16% was obtained in tobacco by overexpression of a rat ACL gene leading to a 4-fold increase in the total ACL activity.<sup>60</sup> Similar enhancement in the fatty acid synthesis could also be achieved by overexpression of acyl-CoA synthetases (ACSSs), a family of enzymes that catalyze the thioesterification of fatty acids with coenzymeA to form activated intermediates.<sup>61</sup> It was observed by Lin et al.<sup>62</sup> that overexpressing the *acs* gene in *E. coli* resulted



**Figure 2.** The successfully engineered pathways in *E. coli* for de novo biosynthesis of biodiesel. An endoxylanase catalytic domain (*Xyn10B*) from *Clostridium stercorarium* and a xylanase (*Xsa*) from *Bacteroides ovatus* were introduced to hydrolyse the hemicellulose to xylose. Ethanol-forming pathway was introduced by overexpression of *pdc* gene and *adhB* gene, coding pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), respectively. Flux through the *E. coli* fatty acid pathway was increased to improve production of free fatty acids and acyl-CoAs by knockout of the *fadE* gene coding acyl-CoA dehydrogenase (FadE), by overexpression of acetyl-CoA carboxylase (ACCase), thioesterases (TE) and fatty acyl-CoA synthase (FadD). The *atfA* gene from *A. baylyi* coding the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase (WS/DGAT) were introduced for fatty acid ethyl esters formation.

in a significant increase of the assimilation of acetate from the medium and contributed to an increase in the rate of fatty acid synthesis. ME, an enzyme catalyzes the conversion of malate into pyruvate and simultaneously reduces a NADP<sup>+</sup> molecule into NADPH, is suggested to be a reducing power supplier for lipogenic enzymes such as ACC, FAS and ACL.<sup>63</sup> Overexpressing ME has been demonstrated as an effective method to enhance lipid accumulation in some strains, such as *Mucor circinelloides*<sup>59</sup> and *S. cerevisiae*.<sup>50</sup> Thioesterases is responsible for the release of fatty acid chains from the acyl carrier protein and the production of free fatty acids. Overexpression of thioesterases is also able to increase fatty acid production substantially in many strains such as *E. coli* and cyanobacteria.<sup>64-67</sup>

Blocking the lipid degradation pathway and other metabolic pathways would also lead to the accumulation of fatty acid sources. For example, starchless mutant of *Chlorella pyrenoidosa* has been shown to have elevated polyunsaturated fatty acid content.<sup>68</sup> Besides,  $\beta$ -oxidation is one of the major pathways responsible for metabolizing fatty acids, which consists of a set of four reactions operating at the carbons 2 or 3 of acyl-CoA esters



**Figure 3.** Simplified overview of fatty acid metabolism in microbes. ACL, ATP citrate lyase; ACS, acyl-CoA synthetases; ACC, acetyl-CoA carboxylase; ME, malic enzymes; PEPC, phosphoenolpyruvate carboxylase; FAS, fatty acid synthetase.

and shortening of the acyl-chain.<sup>69</sup> It is found that knocking out genes involved in  $\beta$ -oxidation not only can lead to increased amounts of intracellular free fatty acids but also result in extracellular fatty acid secretion in some cases.<sup>70-72</sup> For example, deletion of the *fadD* gene (encoding the fatty acyl-CoA synthetase) in *E. coli* could result in an enhanced fatty acids accumulation.<sup>32</sup> The same positive effect on the fatty acids accumulation achieved by knocking out of the *fadE* gene coding acyl-CoA dehydrogenase in *E. coli*.<sup>32</sup> However, cells would rely on the  $\beta$ -oxidation of fatty acids for cellular energy under certain physiological conditions. As a result, knocking out lipid catabolism genes may have deleterious effects on cellular growth and proliferation.<sup>73</sup> Cao et al.<sup>74</sup> demonstrated that using an indirect method, i.e., inhibiting the acetyl-CoA transportation system required for coupling the  $\beta$ -oxidation in peroxisome and the TCA cycle in mitochondria but not any enzyme of the  $\beta$ -oxidation, is capable of inhibiting the  $\beta$ -oxidation of *Candida tropicalis*. The third competitive pathway is the conversion of phosphoenolpyruvate to oxaloacetate, which is catalyzed by the phosphoenolpyruvate carboxylase (PEPC). Significantly enhanced lipid contents could be obtained by breaking the PEPC activity.<sup>75,76</sup> By expressing antisense PEPC in *Brassica napus*, Chen et al.<sup>77</sup> achieved a 6.4–18% increase

in oil content, which suggests that reduced PEPC activity enhanced the fatty acid esters accumulation.

Recently, more and more researchers present a comprehensive modification suggestion of engineering more than one enzyme relevant to the fatty acids accumulation.<sup>43</sup> One example of the comprehensive modification is shown in *E. coli*, which resulted in a 20-fold increase in free fatty acid production.<sup>66</sup> Genetic engineering of the transcription factor, which affects up or downregulation of genes responsible for the global fatty acid pathways, would also be a potentially promising strategy among various comprehensive modifications.<sup>78-80</sup> Reik et al.<sup>80</sup> overexpressed a zinc-finger protein transcription factors that binds a DNA sequence within the promoter in microalgae and led to an enhanced lipid synthesis.

**Modifying the fatty acids profiles.** In addition to engineering microorganisms for the increased production of fatty acid esters, it is also reasonable to attempt to improve the quality of the fatty acid esters, with regard to suitability as a diesel fuel feedstock. The carbon chain length and degree of unsaturation of the fatty acids can affect the cold flow and oxidative stability properties of a biodiesel fuel which is derived from this feedstock.<sup>18</sup>

The chain lengths of fatty acids are mainly determined by acyl-ACP thioesterases, which can release the fatty acid chain from the FAS. There are several acyl-ACP thioesterases from a variety of organisms that are specific for certain fatty acid chain lengths. Transgenic overexpression of thioesterases are able to change fatty acid chain length.<sup>81,82</sup> As in most other organisms, the major end products of the plant and *E. coli* FAS are usually 16- or 18-carbon fatty acids.<sup>83,84</sup> However, certain plant species synthesize in developing seeds large amounts of predominantly medium-chain (C8 to C14) fatty acids, which are deposited in TAGs for long-term carbon storage.<sup>85</sup> It is proposed that transgenically overexpression of shorter chain length specific acyl-ACP thioesterases would lead to the enhancement of shorter chain fatty acids. Actually, transformation and expression of a 12:0-biased thioesterase from *Umbellularia californica* in *E. coli* changed its lipid profiles drastically, with a great increase in the production of lauric acid.<sup>65</sup> Besides, the production of myristic acid was found to be greatly increased by the expression of a 14:0-biased thioesterase from *Cinnamomum camphorum*.<sup>86</sup> The similar phenomenon has also been shown in microalgae<sup>87</sup> that the heterologous expression of two thioesterases, biased toward the production of lauric (C12:0) and myristic acid (C14:0), led to an increased accumulation of shorter chain length fatty acids in the eukaryotic microalgae *Phaeodactylum tricoratum*.

There are other enzymes having significant effect on the fatty acid modification reaction. Overexpression of the FabH protein, which is one of three  $\beta$ -ketoacyl-ACP synthases (KAS) in *E. coli* that catalyze the elongations necessary for fatty acid biosynthesis, influenced the cell by producing more shorter-chain fatty acids.<sup>88</sup> Blocking the degradation of fatty acyl-CoA in *E. coli* through the deletion of the *fadE* gene encoding acyl-CoA dehydrogenase caused significant changes in the FAEEs composition and major

products were ethyl palmitate (16:0) and ethyl oleate (18:1) rather than ethyl myristate (14:0) after its deletion.<sup>32</sup>

### Genetic Engineering for Extracellular Secretion of Lipophilic Compounds

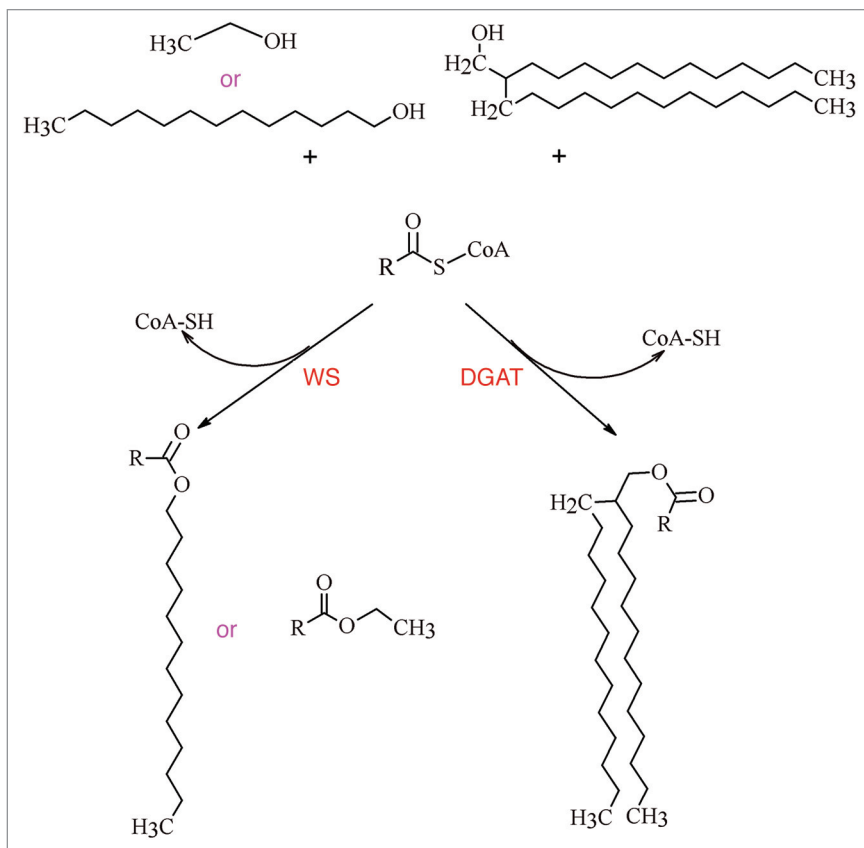
Prior to the use in the biodiesel industry, fatty acid esters or TAGs have to be separated from cells through a series of energy-intensive steps such as cell harvest, drying and solvent extraction. The cost of the energy-intensive processes usually accounts for more than 80% of total cost of biofuel production.<sup>89</sup> Secretion of such lipophilic products into the culture medium rather than intracellular accumulation can significantly reduce the costs of product recovery by skipping these separation processes. Besides, extracellular production of lipophilic compounds would not be directly limited by cell density or cell volume. Indeed, the localization of neutral lipids in organisms is not restricted to the cell cytoplasm, as extracellular lipid deposition has been shown in various strains. There are many strains of bacteria presenting the ability of extracellular TAGs production, like *Alcanivorax*,<sup>90</sup> *Acinetobacter*,<sup>91</sup> *Alcaligenes* and *Pseudomonas*.<sup>92</sup> It was also reported that similar secretion of fatty acids or TAGs can be achieved by metabolic engineering and mutagenesis, not only in bacterium *E. coli*, but also in some yeast strains and microalgae.<sup>71,93,94</sup>

Diffusion by concentration gradient and transportation by carrier proteins are the two ways for lipophilic compounds exporting.<sup>95-97</sup> It is suggested that manipulation that allows yeast cells to accumulate high levels of intracellular fatty acids or that allows yeast to change the fatty acids length may be favorable for extracellular secretion of free fatty acids.<sup>18,98</sup> Inactivation of genes involved in  $\beta$ -oxidation is one of the important manipulations that performed for enhancing fatty acid secretion. ACSs has been recognized as an intensively used gene in such cases.<sup>93</sup> As a family of enzymes that catalyze the thioesterification of fatty acids with coenzyme A to form activated intermediates, ACSs plays a fundamental role in lipid metabolism, which are also necessary for fatty acid import into cells through the process of vectorial acylation.<sup>61</sup> ACSs increases the concentration gradient between extracellular and intracellular fatty acid by converting fatty acid into acyl-CoA and contributes to the uptake of fatty acid.<sup>95</sup> Thus, the deletion of ACSs gene might increase the concentration of free fatty acid in the cytosol and enhance fatty acid export out of cells. Scharnewski et al.<sup>93</sup> demonstrated that in a mutant strain of *S. cerevisiae* deficient in ACSs, which could secrete fatty acid out of cells with a maximum titer of 200  $\mu\text{mol L}^{-1}$ . There is also ample evidence in *E. coli* proving fatty acid export out of cells is relevant with ACSs.<sup>95,98</sup> Nevertheless, it was recently suggested that the deletion of ACSs genes is unable to improve the extracellular fatty acid production when the cell associated fatty acid titer was high. It is clear that fatty acid secretion was not only determined by the concentration gradient, but also related with other carrier proteins or some undetermined mechanisms.<sup>65,98</sup> Thioesterase is also known to influence the fatty acids secretion. For instance, expression of acyl-ACP thioesterase from *Umbellularia californica* in the fatty acid-degradation mutant *E. coli* led to the secretion of free fatty acid into medium.<sup>65</sup> In

the study of Liu et al.,<sup>98</sup> an engineered *E. coli* was obtained with enhanced extracellular fatty acids production through the cytosolic expression of an engineered *E. coli* thioesterase (a "leadless" version of thioesterase without the NH<sub>2</sub>-terminal 26 amino acid residues). The similar phenomenon was also found in the cyanobacterium *Synechocystis* sp PCC 6803. By introducing acyl-acyl carrier protein thioesterases, the cyanobacterium *Synechocystis* sp PCC 6803 were able to overproduce fatty acids (C10-C18) and secrete them into the medium at an efficiency of up to  $133 \pm 12 \text{ mg L}^{-1}$  of culture per day at a cell density of  $1.5 \times 10^8 \text{ cells mL}^{-1}$  (0.23 g of dry weight per liter). Transportation with the help of carrier proteins is proposed to be another type for fatty acid export out of cells. The process for fatty acid transport is inducible and commensurate with the expression of specific sets of proteins thought to participate in the secretion.<sup>99,100</sup> FadL is outer membrane protein in *E. coli*, which has been found to be specifically involved in the binding and translocation of the long-chain fatty acids across the outer membrane of the cell. A tight linkage exists between FadL and fatty acyl CoA synthetase, indicating that these two proteins must act in concert in the facilitated transport and activation of these hydrophobic compounds.<sup>96,98,101,102</sup> As another recently discovered lipid transporter, MsbA is the ATP-binding cassette (ABC) transporter and is implicated in the export of lipopolysaccharides and phospholipids in *E. coli*.<sup>103,104</sup> The fatty acid transport apparatus in yeast is quite distinct from that defined in *E. coli* but may emulate that being defined in higher eukaryotic cells. In *S. cerevisiae*, a protein Fat1p (encoded within the *fat1* gene) was identified as a plasma membrane-bound long-chain fatty acid transporter,<sup>105</sup> which acted with other two proteins (i.e., Faa1p and Faa4p) to facilitate the transport and activation of exogenous long-chain fatty acids.<sup>93,105,106</sup> Besides, the secretion yields of fatty acid or lipid could also be increased by weakening the S-layer and peptidoglycan layers to facilitate fatty acids secretion.<sup>98,107</sup>

### Genetic Engineering for Intracellular Transesterification

Enzymatic transesterification by lipases is a method extensively used for FAMES or FAEEs production.<sup>9</sup> Fatty acids sources and short chain alcohols are essential substrates in the transesterification processes with lipases as biocatalyst, while most of the catalyzed processes are performed in organic solvents in the absence of water.<sup>12</sup> Over the past few years, much effort has been devoted to constructing intracellular transesterification systems for microbial biodiesel production. Acyltransferases, a class of enzymes which are responsible for wax ester or TAG biosynthesis in vivo (Fig. 4), present the potentiality to be used as an alternative for lipase-catalyzed acylation in the biodiesel production.<sup>108-111</sup> Long-chain fatty alcohols or diacylglycerols and fatty acid coenzyme A thioesters (acyl-CoA) is used as substrates for the transesterification of acyltransferases for wax ester or TAG biosynthesis.<sup>112</sup> The biotechnologically relevant synthesis reactions are performed in aqueous systems, while the enzymes are only active with CoA-activated fatty acids.<sup>113</sup> The dependence of acyltransferases on CoA esters suggests that the acyltransferase-catalyzed acylation



**Figure 4.** In vivo reaction catalyzed by acyltransferases for the synthesis of wax ester and triacylglycerol (TAGs). WS, Wax ester synthase; DGAT, diacylglycerol acyltransferase.

is the native reaction under physiological conditions. Thus, the acyltransferase mediated biodiesel synthesis would preferably be performed in vivo. In vitro applications using acyltransferase as biocatalyst in biotechnological processes will not be economic in analogy to lipase catalyzed esterifications. However, the acyltransferase mediated biodiesel production is the possible development of processes that can utilize bulk materials, such as sugars, or better starch, cellulose, and hemicellulose as substrates, which are not dependant on oilseed crops. This will not only significantly extend the substrate range that can be used for fuel production, but will also use resources that do not compete with food and feed production.<sup>113</sup>

There are three unrelated families of acyltransferases found in higher plants, mammals and bacteria, which might be suitable for biodiesel synthesis. The first identified acyltransferase of plants is jojoba embryo wax ester synthase, which did not show activity when heterologously expressed in *S. cerevisiae*.<sup>114</sup> The second group of identified acyltransferases was from bacteria, which is a bifunctional enzyme functioning as wax ester acyltransferases (WS) and as acyl-CoA:diacylglycerol acyltransferase (DGAT). The third group of acyltransferases is from mammals such as the acyltransferase from *Mus musculus* C57BL/6.<sup>115</sup> The WS/DGAT from bacterium *A. baylyi* ADP1 is an acyltransferase extensively used for microbial biodiesel production.<sup>33,108-111,116</sup> However, the reaction rate of the WS/DGAT

enzyme is low with ethanol as substrate,<sup>110,113</sup> since the WS/DGAT from *A. baylyi* ADP1 displayed a general preference for long-chain alcohols and a lower activity for short-chain alcohols. Luckily, WS/DGAT homologs are frequently found in many organism genomes.<sup>112</sup> All the acyltransferase from *A. baylyi* ADP1, *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *M. musculus* C57BL/6 and *Psychrobacter arcticus* 273-4 can responsible for the formation of biodiesel.<sup>39</sup> It is very likely that more effective candidates for biodiesel synthesis can be found among the acyltransferases from different organisms with various substrate and product chain-length preferences. Indeed, an acyltransferase from *M. hydrocarbonoclasticus* DSM 8798 without DGAT activity was found to be more suitable for producing biodiesel since it avoids direction of fatty acyl-CoAs toward TAG biosynthesis.<sup>117</sup> Additionally, directed evolution of acyltransferases may result in selection of enzymes with higher specificity toward ethanol. Furthermore, the substrate specificities of WSs vary with the different expression hosts,<sup>109,114</sup> indicating the importance of exploring suitable hosts for the biodiesel synthesis. The WS/DGAT from *A. baylyi* strain ADP1 is active in different microbial hosts, such as *E. coli*, *Pseudomonas citronellolis* and *S. cerevisiae*.<sup>109,118,119</sup> Active expression of the WS/DGAT homolog from *M. smegmatis* could also be achieved in *E. coli* and *Rhodococcus opacus*.<sup>118,120</sup>

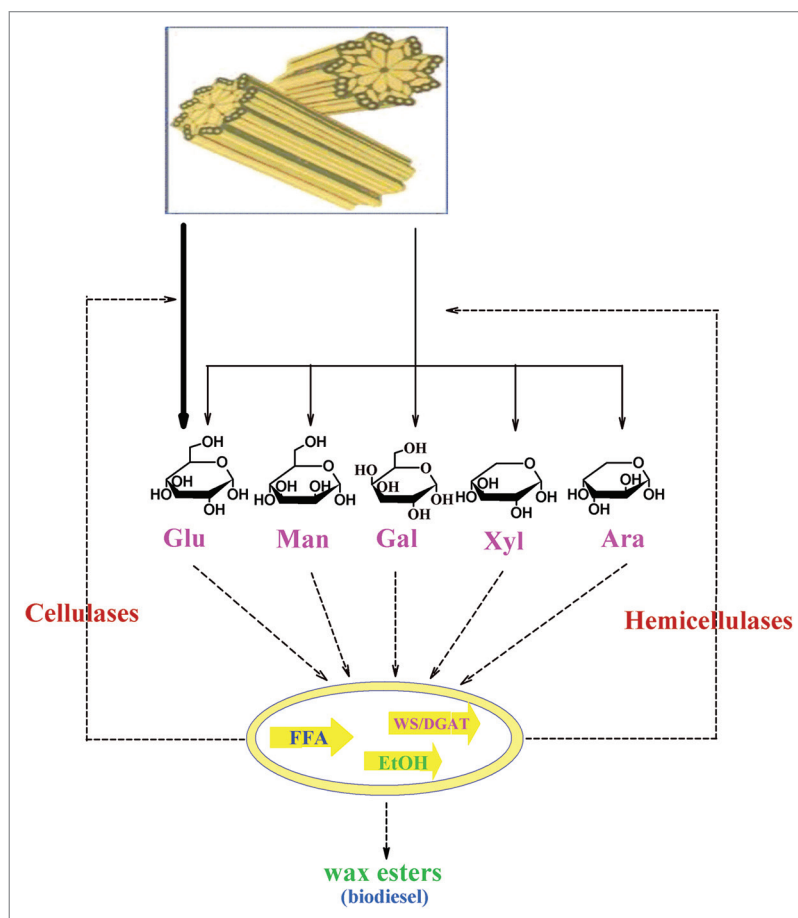
### Genetic Engineering in Substrates Metabolism

Fermentation economics depend on a variety of factors, including investment costs for the fermentation equipment, raw materials and maintenance plus costs for upstream and downstream processes as well as the process yield and throughput.<sup>121</sup> In particular, it may be highly desirable and perhaps necessary to incorporate the feature of naturally utilization of agro-industrial wastes into these cell factories to decrease the investment costs when developing recombinant biodiesel producers. Utilization of agro-industrial residues to make biofuel has become one of the hottest points in current researches.<sup>122-125</sup>

**Lignocellulosic biomass.** De novo biosynthesis of FAEEs from lignocellulosic biomass has been achieved by genetic manipulation in the study of Duan et al.<sup>32</sup> As inexpensive materials for biotechnology production, lignocellulosic plant biomass represents the largest source of renewable carbon and consists of 40–55% cellulose, 25–50% hemicellulose and 10–40% lignin. The social and economic benefits of producing biofuel from lignocellulosic biomass instead of crops are widely appreciated.<sup>126</sup> To do so, lignocellulosic plant biomass must be broken down and hydrolyzed into fermentable sugars.<sup>127</sup> Large quantities of glycoside hydrolase

(GH) enzymes are demand for the efficiently conversion of lignocellulose into fermentable sugars. Actually, the substantial capital and material expense occurred in the enzymes preparation has become the second highest contribution to raw material cost after the feedstock itself.<sup>127</sup> Combination of lignocellulose-utilization and product formation properties in one single microorganism, also called consolidated bioprocess (CBP), might be a potential breakthrough. Hydrolyze the cellulose and hemicelluloses in biomass and produce a valuable product currently accomplished in different reactors or different organisms, are combined in CBP.<sup>128</sup> The key point of CBP for biodiesel production is the engineering of a microorganism that can efficiently de-polymerize biomass polysaccharides to fermentable sugars and efficiently convert this mixed-sugar hydrolysate into FAEEs (Fig. 5).

Two strategies can be used to develop CBP organisms: (1) engineering naturally cellulolytic microorganisms to improve product-related properties; and (2) engineering non-cellulolytic organisms with high product yields to become cellulolytic.<sup>129</sup> Selection of suitable and efficient cellulolytic enzymes is a key factor in the process of engineering non-cellulolytic organisms with high product yields. Full enzymatic hydrolysis of crystalline cellulose usually requires synergistic action of three major types of enzymatic activity: (1) endoglucanases (EGs) (1,4- $\beta$ -D-glucan 4-glucanohydrolases; EC 3.2.1.4); (2) exoglucanases, including cellodextrinases (1,4- $\beta$ -D-glucan glucanohydrolases; EC 3.2.1.74), and cellobiohydrolases (CBHs) (1,4- $\beta$ -D-glucan cellobiohydrolases; EC 3.2.1.91); and (3)  $\beta$ -glucosidases (BGLs) ( $\beta$ -glucoside glucohydrolases; EC 3.2.1.21).<sup>130</sup> Endoglucanases are active on the amorphous regions of cellulose and yield cellobiose and cello-oligosaccharides as hydrolysis products, while cellobiohydrolases are active on the crystalline regions of cellulose and yield almost exclusively cellobiose as their main hydrolysis product. Furthermore,  $\beta$ -glucosidases convert cellobiose and some cellooligosaccharides to glucose and then taken up by organisms. Likewise, the synergistic action of a plethora of enzymes is required for the hydrolysis of hemicellulose, another fraction contained in the lignocellulosic biomass with high content. Over the past decade, a lot of efficient lignocellulose degrading enzymes have been exploited and functionally active expressed in non-cellulolytic or weak-cellulolytic microorganisms (Table 1). Directly into ethanol or other fuels from some model cellulosic and hemicellulosic substrates has been achieved in several non-cellulolytic microorganisms after the introduction of lignocellulose degrading enzyme genes. For example, introduction of cellulase genes enabled the yeast *S. cerevisiae*<sup>123</sup> and the bacterium *Klebsiella oxytoca*<sup>131</sup> directly convert phosphoric acid swollen cellulose (PASC) to ethanol without the addition of exogenous cellulase and hemicellulase. *E. coli* is a popular host for engineering



**Figure 5.** Graphic illustration of a lignocellulose conversion to biodiesel in a single microbe by consolidated bioprocess (CBP). The enzymatic hydrolysis of the cellulose and hemicellulose fractions to fermentable hexoses and pentoses requires the production of both cellulases and hemicellulases (dashed lines). The subsequent conversion of the hexoses and pentoses to biodiesel initiated by the modifying fatty acids pathways, introducing ethanol producing pathway and overexpressing acyltransferase. FFA, fatty acid synthesis; WS/DGAT, acyltransferase for fatty acid esters synthesis; EtOH, ethanol producing pathway.

in the biofuel production. Construction of recombinant *E. coli* with CBP capability is of great practical significance. However, the inferior capacity in *E. coli* for protein export has rendered it unable to extracellularly produce cellulase or hemicellulase in quantities required for industrial-scale lignocellulose hydrolysis. Various techniques, developed over decades of research, can be applied to generate secreted yields from *E. coli*,<sup>132</sup> although these concentrations are still too low for an industrial process. In 2010, Steen et al.<sup>33</sup> constructed an FAEEs-producing *E. coli* with extracellular hemicellulase activity to use the xylan in the growth medium. Then, Bokinsky et al. engineered *E. coli* extracellularly expressing cellulase, xylanase,  $\beta$ -glucosidase, and xylobiosidase enzymes under control of native *E. coli* promoters by the fusion with export related protein (e.g., protein OsmY). The engineered *E. coli* grew well and produced fuel substitutes using either the cellulose or hemicellulose components of ionic liquid-pretreated switchgrass or on both components when combined as a coculture.<sup>133</sup> Removal of soluble inhibitors in the hydrolysates



**Table 1.** Common lignocellulose degrading enzymes expressed in non-cellulolytic or weak-cellulolytic microorganisms

Enzyme or gene	Source-species	Receiver-species
<b>Cellulose degradation: endoglucanase (EG)</b>		
EGI	<i>Trichoderma reesei</i>	<i>Saccharomyces cerevisiae</i> , <sup>143</sup> <i>Yarrowia lipolytica</i> , <sup>144</sup> <i>Ashbya gossypii</i> <sup>145</sup>
EGII	<i>T. reesei</i>	<i>S. cerevisiae</i> , <sup>124</sup> <i>Y. lipolytica</i> , <sup>146</sup> <i>Pichia pastoris</i> , <sup>147</sup> <i>Kluyveromyces marxianus</i> <sup>148</sup>
EG(CeIE)	<i>Clostridium thermocellum</i>	<i>S. cerevisiae</i> , <sup>149</sup> <i>Escherichia coli</i> <sup>150</sup>
EG (CeIA)	<i>C. thermocellum</i>	<i>Bacillus subtilis</i> , <sup>151</sup> <i>Lactobacillus gasserii</i> , <sup>152</sup> <i>Lactobacillus johnsonii</i> <sup>152</sup>
EG (CeIA)	<i>Thermobifida fusca</i>	<i>S. cerevisiae</i> <sup>153</sup>
EGIII	<i>Trichoderma harzianum</i>	<i>P. pastoris</i> <sup>154</sup>
<b>Cellobiohydrolase (CBH)</b>		
CBHI	<i>T. reesei</i>	<i>S. cerevisiae</i> , <sup>153</sup> <i>P. pastoris</i> <sup>155</sup>
CBHII	<i>T. reesei</i>	<i>S. cerevisiae</i> , <sup>156</sup> <i>Y. lipolytica</i> , <sup>146</sup> <i>P. pastoris</i> , <sup>146</sup> <i>Aspergillus oryzae</i> <sup>156</sup>
CBHII	<i>Magnaporthe oryzae</i>	<i>A. oryzae</i> <sup>156</sup>
CBHI	<i>Talaromyces emersonii</i>	<i>S. cerevisiae</i> <sup>157</sup>
CBH (Cel48)	<i>Clostridium phytofermentans</i>	<i>E. coli</i> , <sup>158</sup> <i>B. subtilis</i> <sup>158</sup>
<b><math>\beta</math>-Glucosidase (BGL)</b>		
BGL1	<i>Saccharomycopsis fibuligera</i>	<i>S. cerevisiae</i> <sup>149</sup>
BGL1	<i>Aspergillus aculeatus</i>	<i>S. cerevisiae</i> , <sup>148</sup> <i>K. marxianus</i> , <sup>147</sup> <i>T. reesei</i> , <sup>159</sup> <i>Issatchenkia orientalis</i> <sup>160</sup>
BGL1	<i>A. oryzae</i>	<i>S. cerevisiae</i> <sup>125</sup>
BGL	<i>Penicillium brasilianum</i>	<i>A. oryzae</i> <sup>161</sup>
<b>Hemicellulose degradation:</b>		
$\beta$ -Xylanase(Xyn2)	<i>T. reesei</i>	<i>E. coli</i> , <sup>162</sup> <i>P. pastoris</i> , <sup>163</sup> <i>S. cerevisiae</i> <sup>164</sup>
$\beta$ -Xylanase (xynA)	<i>Penicillium citrinum</i>	<i>P. pastoris</i> <sup>165</sup>
$\beta$ -Xylosidase	<i>T. reesei</i>	<i>S. cerevisiae</i> <sup>166</sup>
$\beta$ -Mannanase	<i>A. aculeatus</i>	<i>Y. lipolytica</i> , <sup>167</sup> <i>S. cerevisiae</i> , <sup>168</sup> <i>Aspergillus niger</i> <sup>169</sup>
endo- $\beta$ -1,4-Glucanase	<i>A. aculeatus</i>	<i>A. oryzae</i> <sup>170</sup>

may reduce the enzyme loading required for the industrial process in the biomass hydrolysis.<sup>134,135</sup> Therefore, even though the yields of secreted protein (<0.1 mg enzymes/g solids) in the study of Bokinsky et al.<sup>133</sup> was low, the *E. coli* is capable of growing on the cellulose and hemicellulose fractions of the ionic liquid-pretreated plant biomass.

**Crude glycerol.** The upsurge in biodiesel production has resulted in a market surplus of glycerol, a byproduct of the chemical transesterification process.<sup>14</sup> Glycerol can be used as inexpensive and readily available raw material to produce fuels and chemicals in some industrial fermentation processes.<sup>136</sup> There have been intensive attempts to identify glycerol assimilation pathway in bacterial or yeast strains.<sup>137-140</sup> The possibility to recycle glycerol, which is the byproduct of FAEEs, for FAEEs production was demonstrated in the report of Yu et al.<sup>40</sup> and microbial biodiesel production from glycerol was successfully achieved in *S. cerevisiae*. The application of glycerol for biodiesel production was also demonstrated by Elbahloul and Steinbuchel<sup>34</sup> by the pilot-scale cultivation of *E. coli* harboring a plasmid with ethanol producing enzymes and acyltransferases. Besides, crude glycerol contains water, salts and other organic materials including residual methanol and free fatty acids and each component varies widely in contents.<sup>141</sup> Therefore, exploration of microbial strains tolerant to undesirable inhibitory components such as salts and organic solvents that present in

crude glycerol becomes a challenge in low-grade crude glycerol fermentation.

**Restaurant oil wastes.** Restaurant oil wastes, rich in free fatty acids, have been utilized to provide fatty acid sources for FAEEs-based biodiesel production in the report of Wang et al.<sup>31</sup> The exploration of strategies to utilize restaurant oil wastes for biodiesel production offers significant advantages, such as enhancing the economic viability of biodiesel, not competing with the food market and maintaining the health of the environment.<sup>142</sup> However, it should be noted that the FAEEs yielded from the restaurant oil wastes is still extremely lower than that from the standard medium containing sodium oleate, which indicates that much more further investigations are needed before its industrial application.

## Conclusion

Biodiesel is produced by transesterification of fatty acid sources (e.g., TAGs) with short-chain alcohols yielding monoalkyl esters of long-chain fatty acids such as FAMES and FAEEs. To realize this process, microorganisms must be developed to have the ability to produce fatty acids and short-chain alcohols available for transesterification and to possess acyltransferases with a higher activity for short-chain alcohols. Although all of these capabilities are involved by known microorganisms, de novo synthesis

of fatty acid ester based biodiesel (FAEEs) has not been found in nature microorganisms to date. Experimental evidence and fundamental principles of the related pathways support the feasibility of de novo biosynthesis of biodiesel by one single microorganism after reasonable modification of potential host organisms. Strains of *E. coli* are found to be the most popular host for this engineering. However, it is clear that biodiesel production in the current engineered strains is still far from industrial application. Developing microbes with both higher efficiency and capability of costless substrates is urgent in order to compete with the fossil fuel. It could be noted many pathways would influence the biodiesel synthesis. Overexpression of one or several specific enzyme genes may not cause dramatic facilitation. Systems metabolic engineering might be more suitable for the development of such microbial strains having potential

commercial applications. Furthermore, how robust the microorganism for a large-scale industrial process is another problem should be given adequate consideration.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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