

## Enzymes, *In Vivo* Biocatalysis, and Metabolic Engineering for Enabling a Circular Economy and Sustainability

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**ABSTRACT:** Since the industrial revolution, the rapid growth and development of global industries have depended largely upon the utilization of coal-derived chemicals, and more recently, the utilization of petroleum-based chemicals. These developments have followed a linear economy model (produce, consume, and dispose). As the world is facing a serious threat from the climate change crisis, a more sustainable solution for manufacturing, i.e., circular economy in which waste from the same or different industries can be used as feedstocks or resources for production offers an attractive industrial/business model. In nature, biological systems, i.e., microorganisms routinely use their enzymes and metabolic pathways to convert organic and inorganic wastes to synthesize biochemicals and energy required for their growth. Therefore, an understanding of how selected enzymes convert biobased feedstocks into special (bio)chemicals serves as an important basis from which to build on for applications in biocatalysis, metabolic engineering, and synthetic biology to enable biobased processes that are greener and cleaner for the environment. This review article highlights the current state of knowledge regarding the enzymatic reactions used in converting biobased wastes (lignocellulosic biomass, sugar, phenolic acid, triglyceride, fatty acid, and glycerol) and greenhouse gases ( $\text{CO}_2$  and  $\text{CH}_4$ ) into value-added products and discusses the current progress made in their metabolic engineering. The commercial aspects and life cycle assessment of products from enzymatic and metabolic engineering are also discussed. Continued development in the field of metabolic engineering would offer diversified solutions which are sustainable and renewable for manufacturing valuable chemicals.



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## 1. INTRODUCTION

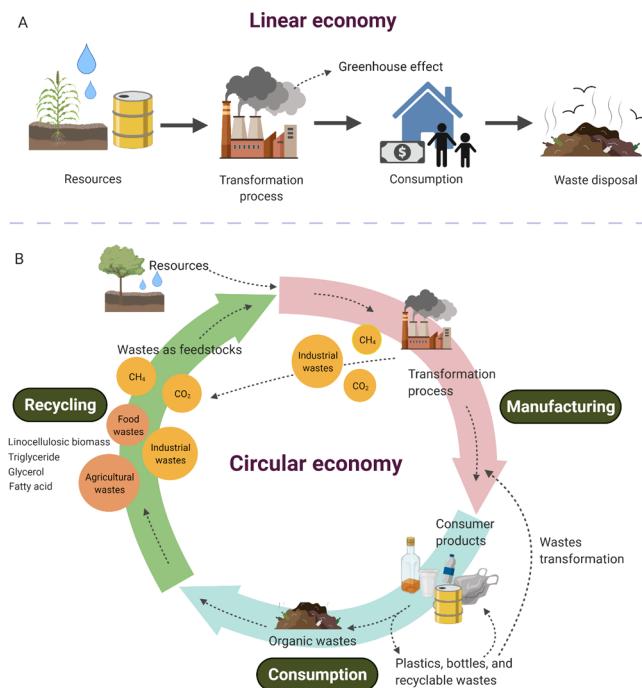
### 1.1. Circular versus Linear Economy Models

Unlimited demand for resources by human consumption behavior has resulted in global warming, climate change, and sustainability issues.<sup>1,2</sup> Unsustainable and nonrenewable resources such as fossil fuels are being depleted, while the demand for energy and material usage is still on the rise. Moreover, the majority of manufacturing industries, especially chemical industries, also generate toxic wastes which further contribute to environmental problems.<sup>3</sup> Use of high energy in production processes generally yields high amounts of greenhouse gases (GHGs) such as CO<sub>2</sub>, which directly causes global warming and leads to climate changes. These have affected the quality of life of all organisms on the Earth. Global warming has affected weather and climate patterns, which in turn, disturb agricultural industries and food supply chains worldwide. Climate change is also linked to new disease outbreaks because high temperatures can increase pathogen reproduction and extrinsic incubation rates.<sup>4,5</sup> For example, the extrinsic incubation period of Zika virus drops from 24.2 days at 21 °C to 5.1 days at 30 °C.<sup>6</sup> Recently, *Candida auris*, a new drug-resistant fungus with virulence factors including host evasion capabilities, and the ability to form biofilms and produce tissue-damaging hydrolytic enzymes,<sup>7</sup> has been proposed to be the first clear example of a pathogen which has emerged due to human-induced global warming.<sup>8</sup> The analysis showed that, in the past, *C. auris* resided mostly in the

human inner ear, in which the temperature was cooler than the rest of the human body, and only small numbers of strains could be grown at 37 °C. The higher current ambient temperature around the globe has resulted in the emergence of *C. auris* strains on three different continents that are more tolerant to higher temperatures and grow well at 37 °C each with distinct genetic differences.<sup>8,9</sup> It has been proposed that the thermotolerant *C. auris* may be one of the next superbugs which may even be more dangerous than COVID-19.<sup>10</sup>

High CO<sub>2</sub> emission has started to rise at an alarming rate since the industrial revolution 50 years ago. This CO<sub>2</sub> increase has resulted in a global temperature rise of 1.2 °C compared to the 1950s.<sup>11</sup> In order to maintain livable conditions for habitable areas on the planet, the global warming process must be slowed so that the overall temperature rise can be kept below 1.5 °C. Otherwise, the world may pass the tipping point of no return in which the temperature rising process will be accelerated because the “permafrost” (ice which never melts) will be thawed, releasing an enormous amount of GHGs.<sup>12</sup> The United Nations has recommended all government and private sectors to adopt rules of Sustainable Development Goal (SDG-17) into their operation policy.<sup>13,14</sup> These 17 goals address global challenges, of which three of them mainly concern responsible consumption and production, climate action (to stop global warming), and life on land and below water.<sup>14</sup> The lockdown activities during the COVID-19 crisis when transportation, energy demands, and consumption behavior decreased, resulted in a reduction in daily global emission of CO<sub>2</sub> by 17% in April 2020 compared to the same period in 2019.<sup>15</sup> Therefore, it is possible to control GHGs emission providing that more sustainable and responsible methods of consumption and production are implemented.<sup>13,14</sup>

Minimization of waste or elimination of waste production from man-derived processes or activities can be key to offering sustainable and renewable methods to supply chemicals, materials, and energy while maintaining (instead of stopping) current high standards of living, including the areas of transportation, health care, and consumption.<sup>16</sup> For traditional production processes which are part of the so-called “linear-economy”, resources are transformed into the desired products, consumed by consumers and subsequently disposed of as waste (Figure 1A). A linear economy has been the main economic model for most sectors because the interest of businesses is always on high profit and maintaining a fast speed of production with little concern regarding the environmental damage which may be incurred. The linear economy model has recently been challenged by a new economy model, termed the “circular economy” mode, in which waste generated after production, utilization, or consumption can be used as starting materials or feedstocks for another loop of production (Figure 1B).<sup>17,18</sup> A closed loop flow of materials via the circular economy model decreases demand on new resource consumption and also at the same time helps with waste management.<sup>19</sup> A powerful example of circular economy implementation is the use of recyclable materials such as paper, glass, plastics and metals for another loop of production. Poly(ethylene terephthalate) or PET can be broken down into ethylene glycol and terephthalic acid which can be used for production of another plastic form.<sup>20</sup> Therefore, effective waste sorting and recycling processes (instead of use and disposal via the linear economy) is important for regenerating waste back to new products, rather than disposing of wastes which



**Figure 1.** Comparison of linear and circular economy models. While the linear economy (A) disposes of waste after using the resources for production and consumption, the circular economy model (B) uses waste from other processes as starting resources to produce desired products.

eventually leaks into oceans, rivers, and freshwater resources and results in macro- and microplastics deposits which cause dramatic effects on marine animals.<sup>21,22</sup> Besides recyclable materials, the majority of municipal solid waste (MSW) worldwide is organic waste (~53%) such as food, agricultural, and agroindustry wastes which are low value and have not been used much as feedstocks for the production of valuable chemicals and materials.<sup>23–26</sup> It is estimated that 2.6 million tonnes of these organic wastes are generated per day.<sup>26</sup> Organic wastes have not been used much for production of valuable chemicals, materials and energy. It is mostly buried in landfills or burned without retrieving much benefit; this process is responsible for 15% of methane<sup>26</sup> and 5% of GHGs<sup>27</sup> generated worldwide. Effective and scalable technologies for conversion of these wastes into value-added materials will be very important for attaining a circular economy as part of global sustainable production processes.<sup>28,29</sup> In addition, improper treatment of organic waste from agriculture and households through burning also causes

air pollution problems such as high level of particulate matter (PM) 2.5 in many countries throughout the world.<sup>30–32</sup>

With our current wealth of knowledge on enzyme catalysis and the significant technological advances made in enzyme and metabolic engineering and synthetic biology, it is possible to construct new biocatalytic cascades and new metabolic pathways for production of chemicals and materials from biowastes. This approach will make production processes more sustainable with greener chemistry, and result in less GHGs and toxic wastes generation. We believe that a key factor toward reaching this goal would be the use of organic wastes which are available in large scale, such as biomass, food wastes and byproducts from food industries as feedstocks or starting materials for biobased transformation which employ reconstituted enzymes or engineered cells that can produce valuable products from low-value waste requiring disposal. As biobased transformation processes can be carried out under ambient temperatures and in aqueous and under mild conditions, their use in the conversion of organic waste serves multipurpose goals such as enabling less resource consumption and less GHGs and toxic wastes emission, and will allow industries and societies to employ a circular economy model as part of their new normal in business operation.

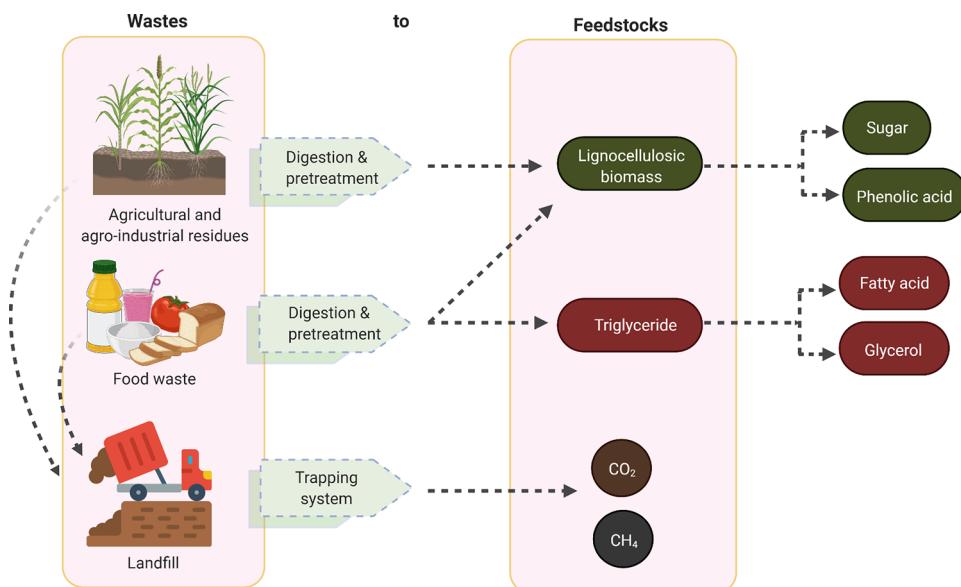
Therefore, this review aims to discuss the state-of-the-art knowledge in enzyme, *in vivo* biocatalysis, and metabolic engineering that make it possible to convert biomolecules such as sugars, phenolic acids, fatty acids, and glycerol, which can all be derived from major world organic wastes (lignocellulose, triglyceride, and food waste) into valuable products. As CO<sub>2</sub> and CH<sub>4</sub> are common final products from anaerobic digestion of organic waste under oxygen-limited conditions in landfills, we also include enzymatic and cellular reactions which can convert these two GHGs into valuable products. We do not include reactions or pathways converting food materials in this review as it would create competing food resource competition. In the last part, we discuss examples of biobased transformation processes which are currently conducted on a large scale in industrial processes and their positive impact on environments. We also share our perspectives on how we can employ biobased processes to convert food wastes into valuable products. Overall, the approach of using biobased transformation can serve as a powerful tool to loop problematic and low-value waste back to the production cycle and use them as starting resources for sustainable production via the circular economy.

## 1.2. Biocatalysis and Synthetic Biology to Increase the Value of Organic Wastes

Biocatalysis is defined as the use of enzymes in purified form, cell lysates, or whole cells to convert substrate(s) into

**Table 1. Major Advantages and Disadvantages of Using Enzymatic, Native Microbes, and Metabolic Engineering Approaches in Biotransformation**

	enzymatic approach	native microbe approach	metabolic engineering approach
major advantages	high specific productivity <sup>33</sup> high purity <sup>33</sup> of product compared to whole-cell catalysis <sup>33</sup>	containing specific and effective pathways <sup>39</sup> using cocultivation to improve yields of desired products <sup>40</sup> no need to purify biocatalysts <sup>33</sup> low yield compared to metabolic engineering approaches <sup>43</sup> generally low productivity when using cocultivation <sup>44</sup>	unlimited pathway designs to adjust metabolic flux <sup>41</sup> yield can be adjusted by genetic manipulation no need to purify biocatalysts <sup>33</sup> requiring extensive pathway optimization in different organisms <sup>45</sup> requiring recovery processes for some intracellular products <sup>46</sup>
major disadvantages	requiring biocatalyst purification and cofactor/ cosubstrate regeneration <sup>33</sup> high cost of enzyme preparation and operation process <sup>42</sup>		



**Figure 2.** Relationship of organic wastes and their breakdown into smaller molecules. Breakdown of lignocellulosic biomass can result in sugars and phenolic acids. Food wastes contain a high content of lignocellulose, starch, and triglyceride which can break down into fatty acids and glycerol, sugar, lignocellulose, and GHGs. Digestion of organic waste under limited oxygen conditions (anaerobic digestion) such as in landfills typically results in high amounts of CO<sub>2</sub> and CH<sub>4</sub>.

product(s).<sup>33</sup> The technology has been used for many decades in several fields including food, agriculture, medicine, and energy.<sup>34</sup> Different types of biocatalysts have different advantages (Table 1). Whole-cell (*in vivo*) biocatalysts which do not require costly enzyme purification processes have been used by industries to perform large-scale bioconversion.<sup>35</sup> Currently, metabolic engineering and synthetic biology have been used to improve yields or titers of products by adjusting the regulation of metabolic pathways.<sup>36</sup> In many examples, metabolic engineering and synthetic biology allow economical synthesis of products in a one-pot process or in engineered host(s).<sup>37</sup> Several factors including genetic stability (host selection or genome integration), expression-related stability (fine-tuning of gene expression or codon usage), and substrate/product toxicity (*in situ* product removal or strain selection) need to be adjusted in order to maximize the productivity of *in vivo* biocatalysis.<sup>38</sup> The advantages and disadvantages of using an enzymatic approach and *in vivo* biocatalysis (including native microbes and metabolically engineered cells) to carry out bioconversion processes are compared in Table 1.

### 1.3. Biobased Feedstocks Derived from Organic and Digested Wastes

While physical recycling or up-cycling processes of recyclable materials are well-known, the biological approaches for converting organic wastes into valuable materials and chemicals are not well established. The best well-known methods for converting organic waste into bioenergy and biomaterials are using yeast fermentation for production of ethanol and anaerobic digestion to produce biogas (CH<sub>4</sub>) and biofertilizers. While both technologies can use homogeneous organic waste from agricultural industries as feedstocks quite well, the use of heterogeneous waste such as food waste is more challenging. The key success of anaerobic digestion to convert heterogeneous organic waste into biogas requires an effective waste segregation program which has been implemented in many European countries such as Germany,

Sweden, and Finland.<sup>47</sup> In order to employ biocatalysis and synthetic biology to increase the value of organic waste by conversion into high-value products, one needs to understand reaction scopes and capacities which are suitable for each type of organic waste.

In this review, we group reactions of enzymatic and metabolically engineered cells according to the types of renewable low value feedstocks or organic wastes that they can convert. Management of these wastes for disposal consumes time and financial resources due to the massive amounts of waste produced by countries around the world. An overview of the types of organic wastes and the biomolecules derived from their breakdown and degradation is depicted in Figure 2. For agricultural waste, lignocellulosic biomass is the dominant waste, from which its breakdown can result in various types of sugars and phenolic acids (derived from lignin depolymerization). Various types of enzymatic reactions can convert these biomolecules into biochemicals, food additives, and pharmaceutical ingredients (sections 2.1 and 2.2). Food waste is another type of organic waste which is rich in triglycerides, starch, and lignocellulose. The breakdown of triglycerides can result in production of fatty acids and glycerol, which can be used by various enzymatic reactions and engineered cells to produce biofuels and biochemicals (sections 3.1–3.3). As anaerobic digestion of food waste in landfills generally results in CO<sub>2</sub> and CH<sub>4</sub>, enzymes and synthetic cells capable of converting these GHGs into usable chemicals are especially needed. The utilization of CO<sub>2</sub> and CH<sub>4</sub> is especially important for the environment because it can be instrumental for solutions against global warming problems in the future (sections 4.1 and 4.2). Table 2 summarizes the sources and properties of organic wastes discussed in this review as starting materials.

In the following sections, each type of feedstock from organic and digested wastes based on categories enlisted in Table 2 and Figure 2 are discussed in detail regarding their composition.

**Table 2. Overview of Organic Wastes, Breakdown Products, and High-Value Products Derived from Enzymatic and Cellular Bioconversion**

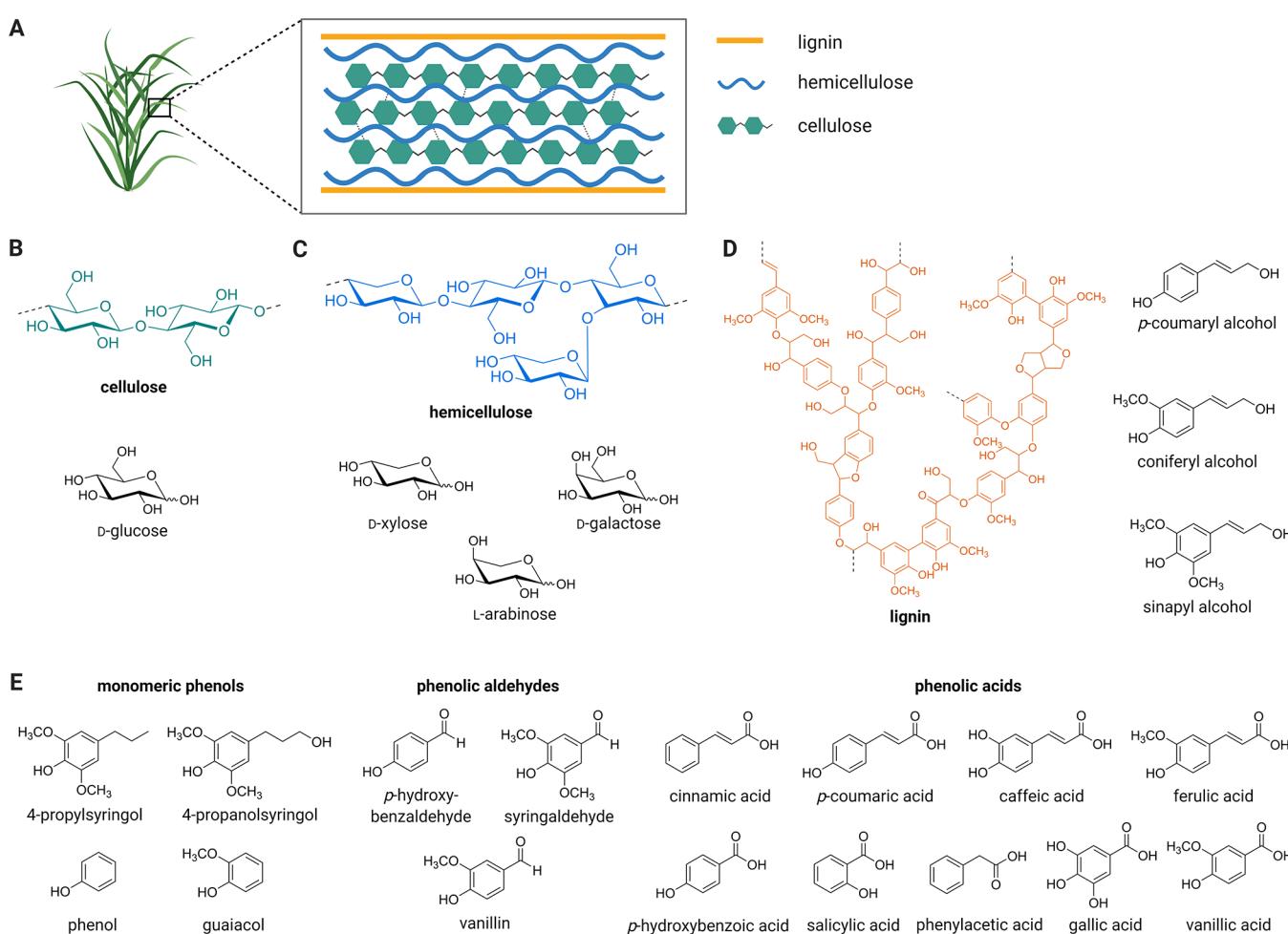
wastes (feedstocks)	sources	products	product applications
lignocellulosic biomass	agricultural residues (stover, straw, bagasse), agro-industrial residues (paper industry), and fruit industry (wine and coffee)	rare sugars, functionalized sugars, and phenolic compounds	food additives, fragrance, nutraceuticals, pharmaceuticals, and material components
sugar			
-phenolic acid	animal fats, vegetable oils, used cooking oils, food wastes, and sewage scum	biodiesel, alkane, and alkene	biofuels and monomers for polymer materials
triglyceride	food wastes and agro-industrial wastes e.g., animal fat, cooking oils, and palm oil mill effluent (POME)	hydrocarbons (alkanes and alkenes), fatty alcohols, fatty acid esters, and fatty acid methyl ketones	biofuels, chemicals, and monomers for polymer materials
fatty acid	food wastes, biodiesel wastes, and bioethanol wastes	short-chain alcohols, short-chain acids, and hydrogen gas	
glycerol			
carbon dioxide	anaerobic fermentation in landfills, industrial heating processes, and petroleum industries	short-chain acids, alcohols, aldehydes, ketones, and isoprene derivatives	chemicals, monomers for polymer materials, biofuels, preservatives, and pharmaceuticals
methane	anaerobic fermentation in landfills, food wastes, animal digestive tracts, farms, agricultural industries, and agro-industries	methanol, polyhydroxybutyrate, and lactic acid	biofuels and polymers

**1.3.1. Lignocellulosic Biomass.** Biotransformation of lignocellulosic biomass particularly from agricultural and forestry residues is an active area of research in both academic and industrial sectors because these starting materials are abundant, low cost, readily available, and renewable.<sup>48</sup> Lignocellulosic biomass waste has great potential to be used as platform chemicals for further conversion into value-added compounds. Lignocellulose is typically composed of carbohydrate and aromatic polymers, in which the carbohydrate polymers are typically composed of five and six carbon sugar monomers.<sup>49,50</sup> Structurally, lignocellulosic biomass is arranged into three types of biopolymers: cellulose, hemicellulose, and lignin (Figure 3A). The majority of lignocellulose content is cellulose (40–45%). The other major components are hemicellulose, which usually makes up 30–35%, while lignin components make up 20–30%.<sup>51–55</sup> Generally, feedstock from biomass can be depolymerized through pretreatment and subsequent hydrolysis. These conversion processes break down complex structures into their respective monomeric compounds. Cellulose and hemicellulose are built from sugars, whereas lignin is composed of polyphenols connected through a complex network of monomeric phenyl propanoic units.<sup>51</sup> Cleavage of cellulose results in glucose as the main monomer (Figure 3B),<sup>56</sup> whereas hemicellulose lysates are composed of other types of 5- and 6-carbon monosaccharides such as xylose, arabinose, and galactose (Figure 3C).<sup>57</sup>

In general, monomeric sugars such as glucose and xylose have long been recognized as potential sustainable sources to be applied in bioconversion processes due to their abundance and inexpensive cost.<sup>58</sup> They possess functional moieties which are beneficial for direct transformation into value-added compounds with various applications. Furthermore, these sugars are primary carbon sources which can be used for facilitating cell growth as a part of microbial cell factories.

Lignin is a phenolic polymer which is composed of three major phenylpropanol units, namely *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 3D).<sup>59,60</sup> Depolymerization of lignin using either chemical, physico-chemical, or biological processes or combined processes can liberate substantial amounts of phenolic monomers including monomeric phenols, phenolic aldehydes, and phenolic acids (Figure 3E).<sup>61–64</sup> The phenolic monomers are generally obtained as mixed compounds, in which their specific structures depend upon the depolymerization method used and the plant sources.<sup>62</sup> A method enabling reductive catalytic fractionation (RCF), known as the lignin-first approach, has recently been developed as a one-pot process for extraction and *in situ* catalytic conversion of the lignin released from lignocellulose into aromatic monomers.<sup>62,65</sup> The method enables extraction and catalytic processing of lignin to obtain high yields of aromatic monomers without modifying their structures.

Phenolic compounds derived from lignin are regarded as important precursors for food, pharmaceutical, cosmetic, and chemical industries.<sup>61,66</sup> Production of phenolic compounds has been increased due to their superior antioxidant activity, as well as their antimicrobial, dietary, and health benefits.<sup>66</sup> Several phenolic compounds such as vanillin,<sup>67</sup> terephthalic acid,<sup>68</sup> and resveratrol,<sup>69</sup> have been commercialized on industrial scales. Interestingly, many reports have shown that sugars as well as phenolic acids can be obtained as byproducts from agro-industrial wastes, particularly accumulated in agricultural residues and waste streams. Monosaccharides can



**Figure 3.** Compositions of lignocellulosic biomass. (A) Three major polymer compositions. (B) Cellulose is mostly composed of glucose. (C) Hemicellulose is composed of 5- and 6-carbon monosaccharides. (D) Lignin is mainly built from three phenylpropane alcohol units. (E) Phenolic compounds are typically derived from treatment processes of lignin.

be obtained from the hydrolysis of sugar cane bagasse and paper mill sludge.<sup>70,71</sup> Phenolic acids can be directly obtained as byproducts from fruit industries such as wine and coffee production.<sup>72,73</sup> As agro-industrial waste has been regarded as a sustainable feedstock for biorefinery,<sup>74</sup> valorization of this waste type can potentially make significant contributions toward developing a circular and sustainable bioeconomy. However, plant wastes usually contain mixtures of individual compounds which can limit their valorization in terms of selective transformation.<sup>75</sup> Many developments in enzymatic catalysis, metabolic engineering, and synthetic biology can help with these aspects to specifically convert these lignocellulosic-derived starting materials for the production of commodity or high-value chemicals.

**1.3.2. Triglyceride.** Triglycerides or triacylglycerols are generally found in food products such as animal fats and vegetable oils or in food wastes such as animal fat, cooking oils, plant oils, milk, and dairy products.<sup>76,77</sup> It is estimated that globally at least one-third of all food production processes results in waste without use or consumption (from production, processing, retailing, and consumption), to an amount equivalent to 1.3 billion tonnes of food waste per year.<sup>78</sup> In European countries, ~53% of household waste is organic waste and the costs of food waste management are around 98 billion euro per year.<sup>79</sup>

As the amount of cooking oil waste produced annually is extremely large, its disposal poses a concern to several countries.<sup>80</sup> Waste oils should not be disposed of to the ocean, rivers, or fresh-water reservoirs or deposited in the soil because it has harmful effects on animal health and ecosystems. A few decades ago, these wastes were used as animal feeds.<sup>80</sup> However, such operation has been legally terminated in many countries. The EU prohibited mixing of cooking oil waste into animal feed in 2002 because carcinogens generated during frying and heating processes can be deposited in animal meat and further transmitted through the food chain to pose toxicity risks to humans.<sup>81</sup> Thus, triglycerides are one of the most interesting feedstocks to be converted into valuable compounds, as successfully using it can significantly reduce the burden of its disposal costs for society.

**1.3.3. Fatty Acid.** Fatty acids and fatty acid derivatives can be directly derived from the hydrolysis of triglycerides. As mentioned in the previous section (1.3.2), food wastes are the main source of triglycerides. Therefore, fatty acids and fatty acid derivatives can also be derived from household food wastes<sup>82–84</sup> and wastes from food industries.<sup>85</sup> In addition to food wastes, industrial wastes such as palm oil mill effluent (POME) from palm oil industries also consist of a high content of fatty acids with carbon backbones ranging from C8 to C20.<sup>85</sup> Oil palm plantations and production facilities are

abundant in Southeast Asian countries such as Malaysia, Indonesia, and Thailand.<sup>86</sup> Therefore, using fatty acids and fatty acid derivatives from food wastes or agro-industrial wastes to produce valuable chemicals can decrease waste usually deposited in landfills and reduce emission of GHGs in general. Normally, oil waste alone such as waste cooking oil is estimated to be disposed of in water and land (as biomass waste) in enormous amounts. Just in Jakarta, 1.8 million tonnes of oil waste were disposed into the soil and gutters each week.<sup>87</sup> This number provides economy of scale if their derivatives (fatty acid and glycerol) can be used for production of valuable compounds. With their hydrophobic nature, fatty acids can serve as precursors in many enzymatic reactions to generate fuels or chemicals which traditionally can only be derived from petroleum.<sup>88</sup> Moreover, fatty acids are crucial substances by themselves in pharmaceutical, food, and nutrition industries.<sup>89</sup>

**1.3.4. Glycerol.** Similar to fatty acids, glycerol can be obtained from the hydrolysis of triglycerides derived from food and oil wastes. In addition, glycerol is also the main byproduct of biodiesel and bioethanol industries. Biodiesel and bioethanol are important green and renewable fuels which have been used in transportation worldwide to decrease dependency on fossil fuels.<sup>90</sup> Biodiesel is formed by transesterification between triacylglycerol and alcohol (typically methanol) resulting in glycerol as a major byproduct. Normally, 100 kg of biodiesel can generate around 10 kg of crude glycerol byproduct.<sup>90</sup> When biodiesel production and utilization increase, the amount of excess glycerol produced also increases, resulting in a huge amount of waste. The glycerol composition in biodiesel waste is around 70–90%. Although it cannot be used as a substance of cosmetics, food, and pharmaceuticals industries which require 99.5–99.7% purity,<sup>91</sup> it can serve as a feedstock for other industries. Glycerol can also be derived from the production of bioethanol; the remaining distillates after ethanol separation generally contain mixtures of various chemicals, including 2% glycerol.<sup>92</sup> Apart from biodiesel and bioethanol, glycerol is also a byproduct from several industries that use fats or oils as starting materials such as polymer, pharmaceutical and biochemical industries.<sup>93–101</sup> As the glycerol waste purification process is expensive, using crude glycerol as a feedstock for other products is more economically feasible.

Although chemical processes to transform crude glycerol into value-added products have been well developed and currently applied in industrial processes, these processes require high temperatures and pressure and the use of specialized metal catalysts.<sup>102,103</sup> The use of biological approaches offers better solutions in terms of environmental aspects because of less toxic waste discharge.<sup>104</sup> Several enzymatic reactions can specifically convert glycerol into high-value products. Glycerol is especially attractive as a carbon source for fermentation to produce valuable biochemicals with the same number of carbon atoms (C3 compounds), and its polyol nature gives it a higher reducing equivalent than common sugars such as glucose or xylose. Therefore, the use of glycerol byproducts from one industry as a starting material for a second industry is a clear example of a circular economy approach to create a greener process.<sup>101</sup>

**1.3.5. Carbon Dioxide.** Carbon dioxide ( $\text{CO}_2$ ) is one of the most common end products produced from various industrial processes. Generally, any industrial activity involved with carbonaceous fuels combustion (especially natural gas,

crude oil, coal, and petroleum) generates high amounts of  $\text{CO}_2$ . Aerobic digestion or yeast fermentation to produce bioethanol is another source of  $\text{CO}_2$  generation.<sup>105,106</sup> Since the industrial revolution,  $\text{CO}_2$  is one of the major GHGs produced by human activities ( $\text{CO}_2$  in the atmosphere was increased ~30% since then).<sup>107</sup> Global  $\text{CO}_2$  emission increased from 22 gigatonnes per year in 1990 to 36.2 gigatonnes per year in 2026 and may increase to 60 gigatonnes per year by 2050 if the same trend continues.<sup>108–110</sup> This can cause the global surface temperature to rise by around 3.7–4.8 °C by 2050 compared to the preindustrial period which can result in catastrophic changes to the Earth.<sup>111</sup> Therefore, the Intergovernmental Panel on Climate Change (IPCC) has urged that the global temperature must be limited to be less than 1.5 °C and net  $\text{CO}_2$  emission from human activities around 45% must be decreased by 2030 compared to those for 2010.<sup>111</sup> In order to reduce the amount of  $\text{CO}_2$  emission, the Kyoto Conference on Climate Change (COP) and Paris Climate Agreement of the United Nations Framework Convention on Climate Change or UNFCCC urged all countries to adopt a  $\text{CO}_2$  reduction policy.<sup>112</sup> Moreover, carbon taxation policy has been implemented to reduce  $\text{CO}_2$  produced from industries. The tax of released  $\text{CO}_2$  is around \$40–80 per tonne  $\text{CO}_2$ .<sup>113</sup> For example, the pulp and paper industry generates around 116 million tonnes  $\text{CO}_2$  per year which is a large amount of carbon tax to spend.<sup>114</sup> Carbon tax implementation is expected to force industries to convert  $\text{CO}_2$  into valuable chemicals and materials which is key to reducing  $\text{CO}_2$  emission into the atmosphere.

Various physical technologies such as chemical catalysis, electrochemistry, and photochemistry can convert  $\text{CO}_2$  to C1 compounds, e.g., formic acid and methanol. However, the thermodynamics of  $\text{CO}_2$  reduction technologies is challenging and generally energy demanding because  $\text{CO}_2$  is extremely stable.<sup>115,116</sup> In 2019 alone,  $\text{CO}_2$  in the atmosphere was 411.77 ppm.<sup>117</sup> This amount is challenging because a large amount of energy is required for practical entrapment of the gas. In contrast, biological approaches to utilize  $\text{CO}_2$  are more attractive because some organisms in nature (e.g., plants, algae, cyanobacteria, and bacteria) have enzymes and pathways to fix  $\text{CO}_2$ , incorporating it into other carbon skeletons that are eventually used to produce lignocellulosic biomass and other biochemicals through biosynthesis.<sup>118,119</sup> However, photosynthetic efficiency is not very high, 47% of light can be absorbed by plant pigments, and 11.8% of energy can be stored in the form of nicotinamide adenine dinucleotide phosphate (NADPH). A large amount of energy is used for other processes in photorespiration. The overall photosynthetic efficiency is calculated to be only 1% of the photosynthetic efficiency which is equivalent to removal of approximately 2.7 kg/m<sup>2</sup>  $\text{CO}_2$ .<sup>120</sup> In order to increase the efficiency of  $\text{CO}_2$  conversion and to convert it into the desired products, several artificial enzymatic pathways are available for fixing  $\text{CO}_2$ . We are entering into an era in which one can custom design cascade reactions or metabolically engineered cells to convert  $\text{CO}_2$  from industrial waste into  $\text{CO}_2$ -derived products.

**1.3.6. Methane.** Methane ( $\text{CH}_4$ ) is produced in natural ecosystems, such as wetlands, which account for ~40% of global methane production.<sup>121–123</sup> Animal and human activities are accounted for the rest of total methane production.  $\text{CH}_4$  generated by humans is mostly related to agricultural and household activities such as anaerobic digestion of sewage sludge, animal manure, agro-industrial

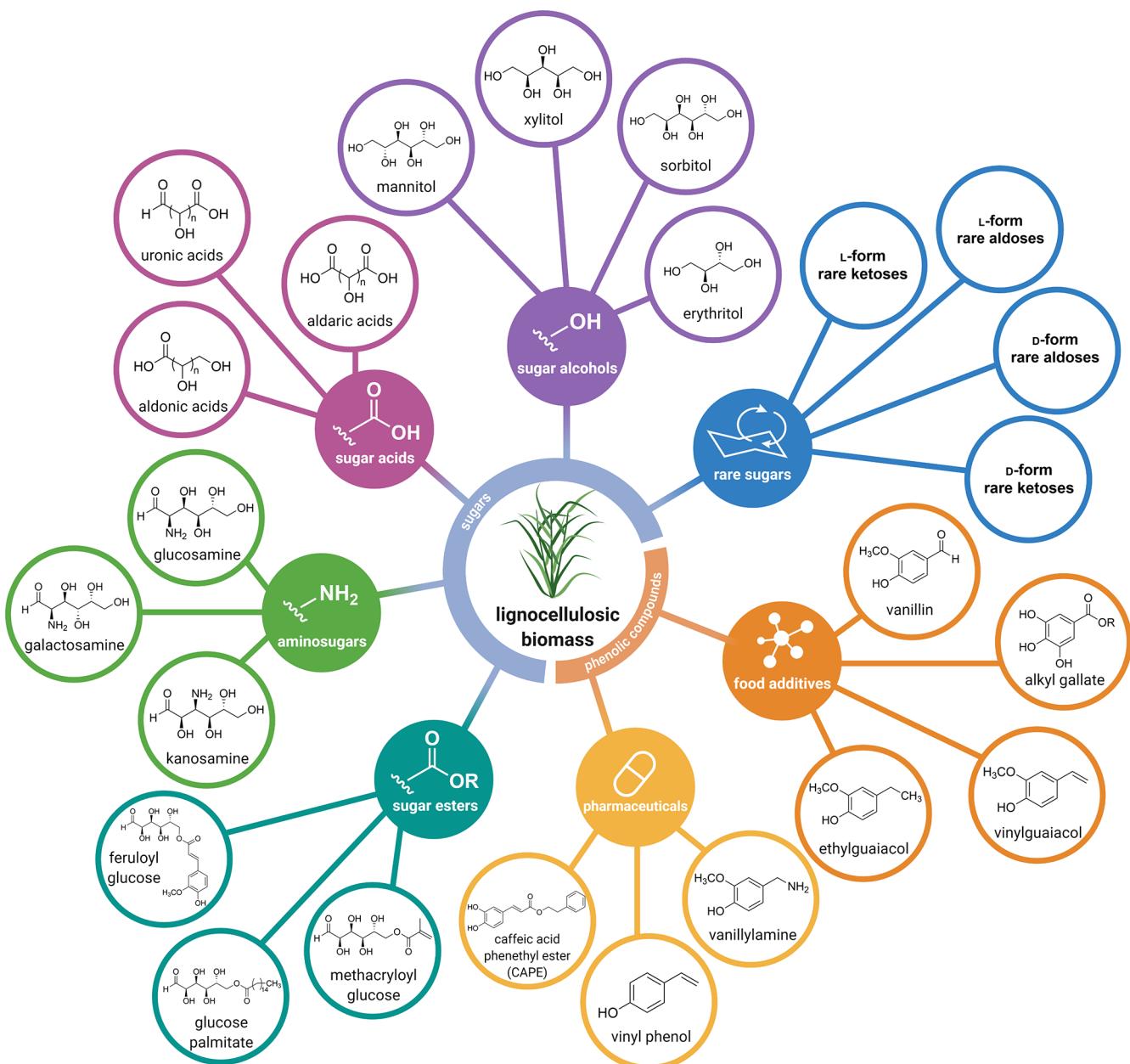


Figure 4. Overview of products from the bioconversion of sugars and phenolic compounds derived from lignocellulose.

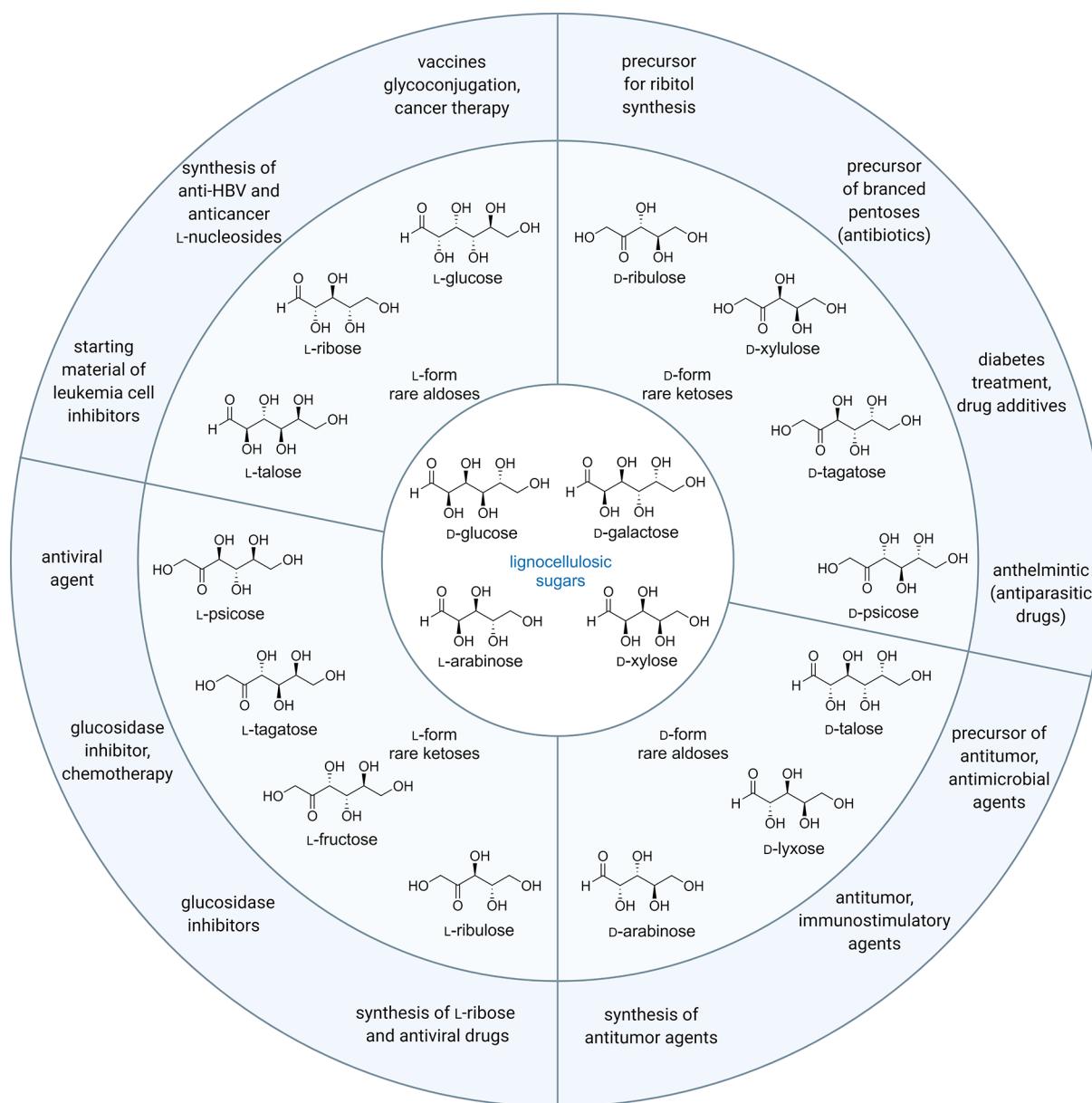
sludge, and composting of organic waste in landfills. Global emissions of  $\text{CH}_4$  reached nearly 600 million tonnes in 2017, and its emission has increased continually since then.<sup>124</sup> Even though the total amount of  $\text{CH}_4$  in GHGs is estimated to be much less than  $\text{CO}_2$ ,  $\text{CH}_4$  is 34-times more potent GHGs than  $\text{CO}_2$  and is responsible for approximately 20% of the overall effects caused by GHGs since 1750.<sup>125</sup> In addition to its climate impacts, methane can also affect air quality because it can contribute to the formation of ground-level ozone (tropospheric) which is a dangerous air pollutant.<sup>126</sup>

Anaerobic digestion of biomass and agricultural waste produces methane as part of a mixture of gases called biogas. Biogas is typically composed of  $\text{CH}_4$ ,  $\text{CO}_2$ , and  $\text{H}_2$  and other minor gases.<sup>127</sup> It can be directly used for heating as an energy source in which the final product is  $\text{CO}_2$ . Methane produced by bioprocesses can be considered as a renewable and sustainable alternative energy to substitute for fossil fuels.

For example, the use of methane produced from agricultural waste to generate fuels is one of the well-known examples of a circular economy model which couples waste management to value creation. Current technological advances with enzymes and engineered cells also facilitate the use of methane directly as a starting carbon precursor for the production of other valuable chemicals.

## 2. PRODUCTION OF LIGNOCELLULOSIC BIOMASS-DERIVED PRODUCTS

This section provides an overview of a collection of efficient and selective biotransformation of primary components derived from lignocellulose including sugars and phenolic compounds to generate valuable chemicals which are of interests for various applications. An overview of mono-saccharide and phenolic compound bioconversion is summarized in Figure 4.



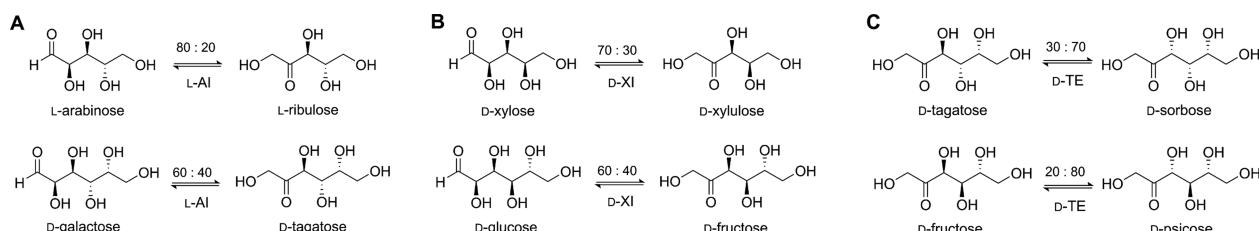
**Figure 5.** Various rare sugars can be produced from lignocellulosic monosaccharides and their applications.

## 2.1. Sugar-Derived Products

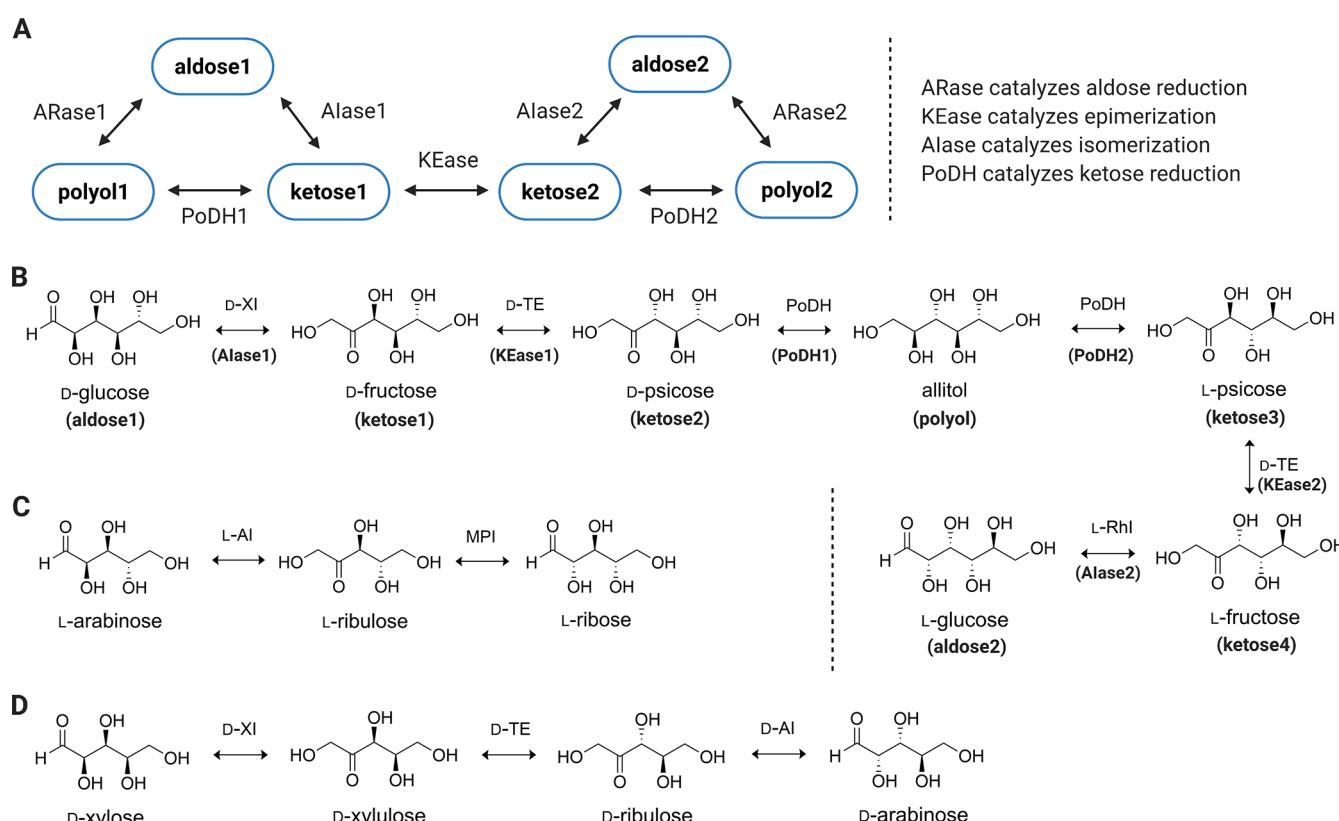
Sugars are versatile substrates for numerous chemical transformations. They can be metabolized by fermentative microbes such as yeast to produce ethanol. However, a more economically attractive conversion would be to transform commonly available monomeric sugars (glucose, xylose, galactose, arabinose, and mannose) into rare sugars or sugar derivatives which are parts of drug components. Typically, lignocellulosic hydrolysates consist of glucose and xylose as the major monosaccharide components in which glucose makes up approximately 30–50% and xylose approximately 20% of feedstock dry weight.<sup>128–130</sup> Galactose, arabinose, and mannose can be obtained in much lower quantities (1–10% of dry weight). Sugar derivatives have various biological activities because they contain a variety of stereospecific structures which can interact with different target receptors or enzymes and trigger biological reaction cascades.<sup>131</sup> Their high solubility in aqueous media also makes synthesis of sugars by classical chemical synthesis not very convenient because the

compounds cannot be solubilized well in organic solvent.<sup>132</sup> Moreover, high contents of hydroxy groups of sugars do not allow regio-specific chemical synthesis to be achieved easily and require blocking/deblocking protocols.<sup>133</sup> Therefore, sugar biotransformation via enzymatic reactions is attractive because enzymes naturally catalyze the desired reactions in a highly selective manner and can be conducted in a hydrophilic environment where sugars are more soluble.

**2.1.1. Rare Sugars.** Isomers, epimers, and derivatives of common natural monosaccharides are regarded as rare sugars. Rare sugars have diverse applications as food additives, nutraceuticals, and active pharmaceutical ingredients.<sup>134</sup> Although rare sugars can also be found as monosaccharides in nature, they are only presented in scarce amounts. Their functions are mostly involved in the pentose phosphate pathway and carbohydrate metabolism.<sup>135,136</sup> Biosynthesis pathways of rare sugars can be found in many living organisms, and rare sugar-producing enzymes have been explored and studied for more than half century. For example, in bacteria,



**Figure 6.** Interconversion of different reactions catalyzed by isomerases. (A) L-Arabinose isomerase (L-AI). (B) D-Xylose isomerase (D-XI). (C) D-Tagatose 3-epimerase (D-TE). Ratio of substrate and product at equilibrium under ambient conditions is displayed for each reaction.



**Figure 7.** Multienzymatic cascade reactions employing isomerases and oxidoreductases for the production of rare sugars. (A) An overall interconversion between aldoses, ketoses and polyols proposed by the Izumoring strategy. The figure was adapted with permission from Zhang et al.<sup>134</sup> Copyright © (2017) Elsevier. Enzymes used in this strategy include ketose 3-epimerase (KEase), aldose isomerase (Alase), polyol dehydrogenase (PoDH), and aldose reductase (ARase). (B) Enzymatic cascades for producing rare L-glucose from D-glucose based on the Izumoring strategy. (C) Isomerase-based synthesis of L-ribose from L-arabinose. (D) Isomerase-based synthesis of D-arabinose from D-xylose. Abbreviations: L-AI: L-arabinose isomerase; MPI: mannose-6-phosphate isomerase. D-XI: D-xylose isomerase; D-TE: D-tagatose 3-epimerase; D-AI: D-arabinose isomerase.

isomerization of D-galactose into D-tagatose is mediated by L-arabinose isomerase (L-AI),<sup>137</sup> while isomerization of D-xylose into D-xylulose is catalyzed by D-xylose isomerase (D-XI).<sup>138</sup> The conversion of D-tagatose into D-sorbose is catalyzed by D-ketohexose 3-epimerase,<sup>139</sup> while epimerization of D-ribulose 5-phosphate into D-xylulose 5-phosphate can be catalyzed by D-ribulose 5-phosphate 3-epimerase (D-RPE).<sup>140</sup> In yeasts and some mammalian cells, interconversion between D-ribose 5-phosphate and D-ribulose 5-phosphate is catalyzed by pentose phosphate isomerase.<sup>135</sup> In this section, we discuss the production of rare sugars from four types of primary lignocellulosic sugars including D-glucose, D-xylose, L-arabinose, and D-galactose based on enzymatic and whole-cell catalysis (Figure 5).

### 2.1.1. Enzymatic Reactions for the Synthesis of Rare Sugars. Using Isomerase

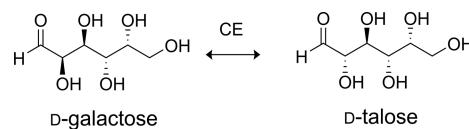
A variety of rare sugar producing enzymes in natural biosynthetic pathways have been discovered. Most of these enzymes have broad substrate utilization and can use more than one type of sugars as substrates. For example, L-AI can catalyze isomerization of D-galactose into D-tagatose as well as conversion of L-arabinose into L-ribulose (Figure 6A).<sup>141,142</sup> D-XI catalyzes isomerization between D-xylose and D-xylulose and interconversion between D-glucose and D-fructose (Figure 6B).<sup>143,144</sup> D-Tagatose 3-epimerase (D-TE) catalyzes epimerization between D-tagatose and D-sorbose and between D-fructose and D-psicose (Figure 6C).<sup>145,146</sup> However, employment of only isomerases alone to produce rare sugars often faces problems of low product yield because the reaction is reversible, preventing achievement of total conversion. At equilibrium, high amounts of substrate are present, and the total conversion cannot be obtained.

Several reaction prototypes have been explored in the development of multistep cascade reactions to convert abundant monosaccharides derived from lignocellulosic feedstocks into rare sugars. One of the promising approaches is the Izumoring strategy, which was established by Prof. Ken Izumori's group in 2002.<sup>147</sup> This strategy consists of four types of enzymes including ketose 3-epimerase (KEase), aldose isomerase (AIase), polyol dehydrogenase (PoDH), and aldose reductase (ARase), which are used to interconvert between different aldoses and ketoses (Figure 7A).<sup>148</sup> KEase catalyzes ketose C-3 epimerization between epimeric ketoses, whereas AIase catalyzes aldose-ketose isomerization. Oxidation/reduction of aldoses/ketoses to yield corresponding hexitols is catalyzed by PoDH, while ARase catalyzes the interconversion between D- and L-sugars. The combination of these enzymes allows the catalysis of interconversion between aldonketoses and their corresponding rare sugar forms. By using this method, all tetroses, pentoses, and hexoses could be interconverted to the desired L- or D-sugars. For example, Prof. Izumori designed an approach for producing L-glucose using six steps of biotransformation starting from abundant D-glucose (Figure 7B). The first step is isomerization of D-glucose to D-fructose by D-XI followed by epimerization to yield D-psicose by D-TE. The third step is the reduction of D-psicose to yield an enantiomeric mixture of D- and L-allitol by PoDH. In the next step, a more specific PoDH can be used to oxidize only L-allitol to form L-psicose, which can be further epimerized to form L-fructose by D-TE. The final step is isomerization of L-fructose to L-glucose by L-rhamnose isomerase (L-RhI). In summary, the key steps for the biotransformation are epimerization by D-TE to interconvert two ketohexoses directly and specific reduction by PoDH to generate desired hexitols.

Several rare monosaccharides and polyols have been produced based on the Izumoring strategy. L-Ribose, a potential starting material for pharmaceutical compounds, can be synthesized from abundant L-arabinose in a one-step two-enzyme process in which L-ribulose is first produced from L-arabinose by L-AI and subsequently converted to L-ribose by mannose-6-phosphate isomerase (MPI) (Figure 7C).<sup>149</sup> The process can give a conversion yield of 23.6% and a productivity of 39 g/L·h. In another example, three enzymes including D-XI, D-TE and D-AI can be employed for preparation of a rare sugar, D-arabinose, from inexpensive D-xylose (Figure 7D).<sup>150</sup> The cascade reaction starts from isomerization of D-xylose to produce D-xylulose by D-XI. Epimerization between D-xylulose and D-ribulose can then be catalyzed by D-TE, and the resulting D-ribulose can be converted to D-arabinose by D-AI simultaneously in the same reaction mixture. The D-xylose conversion can reach equilibrium within 12 h to obtain D-arabinose with 40% yield. As D-arabinose occurrence in nature is very rare and its production by chemical synthesis generally requires many steps,<sup>151</sup> the development of D-arabinose production from D-xylose is thus valuable for future employment of enzyme technology in production of valuable compounds.

Alternatively, novel isomerases have been searched and employed to explore new isomerization reactions. L-Rhamnose isomerase, which isomerizes L-rhamnose to L-rhamnulose, has been found to catalyze previously unknown bioconversion between common and rare sugars in both D- and L-forms.<sup>152</sup> The enzyme displays its ability to use various rare sugars such as L-mannose, L-lyxose and D-ribose, and is thus useful for using together with other isomerases. Recently, cellobiose 2-

epimerase (CE) was found to be capable of synthesizing D-talose from D-galactose in a single step (Figure 8).<sup>153</sup> However,



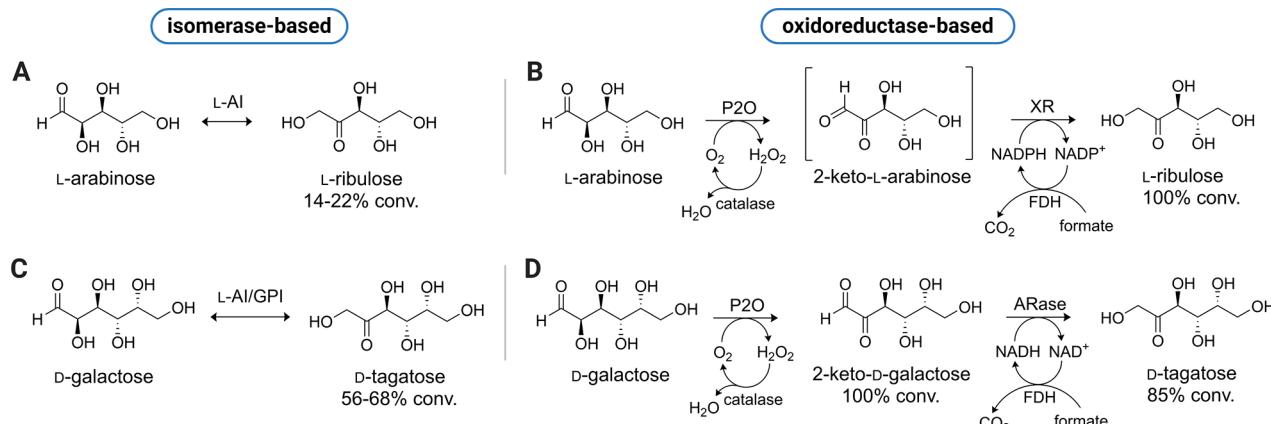
**Figure 8.** Single step interconversion of D-galactose to D-talose catalyzed by cellobiose 2-epimerase (CE).

the specific activity of this conversion is quite low and less than 1% with its natural substrate, cellobiose. Therefore, further optimization via enzyme engineering is necessary for improving these reactions for real industrial applications.

Current advancements of enzyme engineering achieved via directed evolution have been used to find new pathways for synthesizing rare sugars with increased efficiency. Yeom et al. created variants of phosphosugar isomerases including ribose-5-phosphate isomerase (RPI), glucose-6-phosphate isomerase (GPI) and MPI using site-directed mutagenesis for altering their activities toward rare monosaccharides that are rarely found in nature.<sup>154</sup> The phosphate binding residue of each isomerase was mutated based on analysis by homology modeling and docking of monosaccharides onto the three-dimensional structure models of each isomerase. Three variants including RPI-R133D, MPI-R192N, and GPI-T85Q showed activities toward D-ribose isomerase, L-ribose isomerase (L-RI), and L-talose isomerase about 2-, 3-, and 5-fold higher than those of wild-type enzymes, respectively. It was found that these mutated isomerases can no longer use their natural substrate phosphosugars, demonstrating that activity of sugar isomerases can be altered and tailored toward new substrates. In addition to expanding new activities, enzyme engineering has been carried out to increase thermostability,<sup>155</sup> improve the pH tolerance range toward acidic conditions,<sup>156</sup> or increase the reactivity toward non-native substrates.<sup>157</sup> However, only moderate results could be achieved thus far. Therefore, further identification of new isomerases or highly stable enzymes from thermophilic microorganisms is still required.

Isomerization reactions, in particular, require the enzymes to be thermotolerant because the thermodynamic equilibrium toward product formation is generally low at ambient temperature, and can be increased by raising the reaction temperature.<sup>158</sup> Examples of applications can be found in several cases such as enzymatic conversion of D-galactose to D-tagatose with an immobilized thermostable L-AI in a packed-bed reactor.<sup>159</sup> The immobilized L-AI exhibited a broader pH working range and can be used at higher temperature, ~15 °C compared to free enzyme. Recently, the L-AI immobilized as cross-linked enzyme aggregates (CLEAs) using magnetic particles for cross-linking exhibited a 51-fold increase in stability under acidic conditions (pH 5.6) and high temperature (60 °C), as compared to the soluble enzyme.<sup>160</sup> The magnetic cross-link increased the operational stability of the isomerase because around 70% of its initial activity could be retained after 9 cycles. The use of magnetic CLEAs allows better reusability and makes the application convenient because the enzyme can be efficiently removed from the product. Thus, enzyme immobilization technology should be useful for future implementation of isomerases in real applications.

### Using Sugar Oxidase/Reductase



**Figure 9.** Comparison of isomerase- and oxidoreductase-based approaches for rare sugars production. L-Ribulose production from L-arabinose using isomerization (A) or oxidation–reduction (B). D-Tagatose production from D-galactose using isomerization (C) or oxidation–reduction (D). Abbreviations; L-AI: L-arabinose isomerase; P2O: pyranose 2-oxidase; XR: xylose reductase; GPI: glucose-6-phosphate isomerase; ARase: aldose reductase; FDH: formate dehydrogenase.

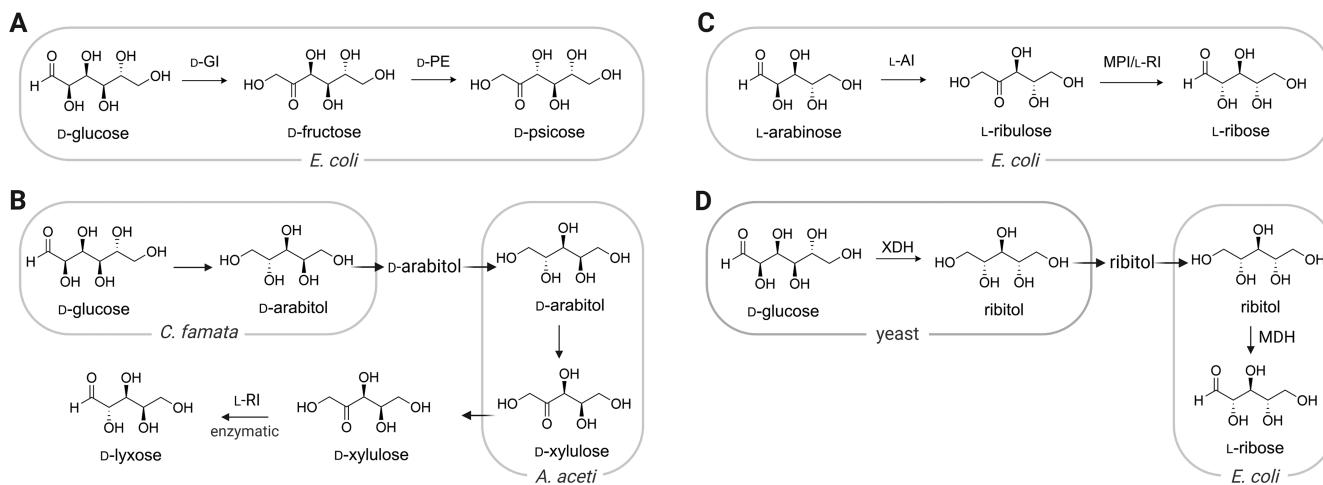
The overall sugar isomerization can also be achieved successfully using another approach of combining site-specific oxidation and reduction instead of isomerization. This approach can help overcome inefficiencies of the isomerase when the thermodynamic favorability does not support the product formation direction. For instance, although production of high-value L-ribulose can be produced by a single step isomerization by L-AI,<sup>161</sup> only a low yield could be obtained by this method because of low equilibrium conversion. The *in vitro* production of L-ribulose from L-arabinose by L-AI gives a product yield of around 14–19%,<sup>141,149</sup> and can be enhanced up to 22% by immobilization methods at high temperature (Figure 9A).<sup>162</sup> To overcome the problem, a new biocatalytic route to convert L-arabinose to L-ribulose without using direct isomerization was developed using oxidation/reduction in which the thermodynamics of the reaction is more favorable. A reaction of pyranose 2-oxidase (P2O) has been employed in the combined oxidation/reduction one-pot synthesis to generate L-ribulose from L-arabinose by first oxidizing L-arabinose at the C-2 position followed by reduction at the C-1 position by xylose reductase (XR; Figure 9B).<sup>163</sup> In order to achieve higher substrate specificity of P2O toward L-arabinose, the enzyme was rationally engineered to obtain the variant which has 40-fold higher activity toward L-arabinose than the wild-type enzyme. With this approach, the enzymatic cascade can catalyze 100% conversion of L-arabinose to L-ribulose with a production yield of 0.3 g/L in less than 7 h.

Synthesis of D-tagatose from inexpensive D-galactose is another example of using oxidation–reduction instead of isomerization. For the same isomerization using L-AI<sup>164,165</sup> or GPI<sup>166</sup> (Figure 9C), the conversion is not complete and the yield can only be increased at high temperature. A two-step chemoenzymatic process using P2O was reported to oxidize D-galactose at the C-2 position followed by catalytic hydrogenation using Pd to obtain D-tagatose.<sup>167</sup> The first step yields the oxidized sugar with around 60% efficiency; however, the final yield after reduction is only 30%. The low yield is the result of nonspecific reduction which generates other side products. Further development was carried out to replace the second chemical reduction step of 2-keto-D-galactose by the reaction of ARase (Figure 9D).<sup>168</sup> The first step of oxidation was found to be complete, whereas the final yield after the enzymatic reduction was 85%, corresponding to a productivity

of 0.99 g/L h. However, significant amounts of byproducts were detected (presumably due to degradation of the 2-keto intermediate) because the overall reaction was quite slow, as the catalytic rates of P2O toward D-galactose are much slower than other lignocellulosic sugars such as D-glucose and D-xylose.<sup>169</sup> Later on, a catalytically related enzyme, pyranose dehydrogenase (PDH) was employed for the galactose oxidation step.<sup>170</sup> Unlike P2O, PDH utilizes benzoquinone as an electron acceptor instead of molecular oxygen ( $O_2$ ). Kinetic studies of PDH indicate that the enzyme has a catalytic efficiency ( $k_{cat}/K_m$ ) toward D-galactose of  $46.2 \text{ mM}^{-1} \text{ s}^{-1}$  which is ~140 folds higher than that of P2O. In addition to D-galactose, PDH can efficiently use other lignocellulosic sugars such as D-xylose and L-arabinose as substrates. A broad range of substrates preference and high catalytic efficiency of PDH makes the enzyme attractive for use in the synthesis of rare sugars from renewable sources.

As illustrated above, the total conversion can be obtained using an enzymatic oxidation–reduction approach, which is much greener and causes fewer environmental hazards than the chemical process.<sup>167</sup> Although sugar isomerases have a wide substrate scope and can use a variety of monosaccharides and phosphosugars as substrates, their applications are limited because of an unfavorable thermodynamic equilibrium. The incomplete conversion of sugars can cause expensive and complicated downstream processes because sugar substrates and products have similar physical properties, making them difficult to separate.<sup>134</sup> Consequently, the oxidoreductases could be an excellent choice for achieving high yields and facile product purification processes. However, the utilization of oxidoreductase requires redox partners, and the expensive costs of cosubstrates cause problems in large scale preparation applications.<sup>171</sup> These problems can be overcome by using an efficient cosubstrate regenerating system such as the use of formate dehydrogenase to constantly supply NAD(P)H for oxidation–reduction in the synthesis of rare sugars (Figure 9B,D).<sup>172</sup>

**2.1.1.2. Whole-Cell Biocatalysis for Production of Rare Sugars.** Despite the ability of reconstituted enzymatic systems to combine reactions of several purified enzymes in one-pot to achieve synthesis of rare sugars, the cost of enzyme production and purification make this approach not economically viable in some cases. In order to achieve industrial-scale application,



**Figure 10.** Production of rare sugars using microorganisms. (A) Production of D-psicose from D-glucose. (B) Three-step process for production of D-lyxose from D-glucose through two fermentation batches and an enzymatic conversion. (C) Production of L-ribose from L-arabinose. (D) Production of L-ribose from D-glucose by two-step fermentation processes. Abbreviations; D-GI: D-glucose isomerase; D-PE: D-psicose 3-epimerase; L-RI: L-ribose isomerase; L-AI: L-arabinose isomerase; MPI: mannose-6-phosphate isomerase; XDH: xylitol dehydrogenase; MDH: mannitol dehydrogenase.

whole-cell biocatalysis approaches have been explored in order to use free or immobilized cells to synthesize rare sugars. With developments in metabolic engineering and synthetic biology, bacteria such as *Escherichia coli*, or eukaryotes such as yeast, can serve as platforms for rare sugar production. For general products used as food and drugs, *Saccharomyces cerevisiae* may be preferred over *E. coli* because the presence of endotoxin in *E. coli* can hamper its application in the food industry.<sup>173</sup>

Based on the Izumoring strategy and isomerase-based method, several biotransformations of rare sugars have been explored using whole-cell biocatalysts. D-Psicose (also known as D-allulose), a noncaloric sweetener used in patients with diabetes, can be produced from D-glucose by engineered *E. coli* cells.<sup>174</sup> The whole-cell biotransformation was conducted by coexpressing heterologous D-glucose isomerase (D-GI) and D-psicose 3-epimerase (D-PE) for conversion of D-glucose to D-psicose. D-Glucose can be converted to D-fructose by D-GI before D-fructose is epimerized to form D-psicose by the reaction of D-PE (Figure 10A). The final product was obtained with 17.8% yield and production of 89.1 g/L. At equilibrium, the product solution was found as a mixture consisting of leftover D-glucose and D-fructose, an intermediate resulting from the reaction of D-GI. D-Glucose can also be used for microbial production of D-lyxose, which is a precursor for the synthesis of antitumor and immunostimulatory agents through a three-step process comprised of two fermentation batches and enzymatic conversion.<sup>175</sup> The process could be carried out by the first conversion of D-glucose to D-arabitol by *Candida famata*, followed by dehydrogenation of D-arabitol to form D-xylulose by *Acetobacter aceti*, followed by isomerization to D-lyxose by the reaction of L-RI (Figure 10B). The overall yield of D-lyxose was approximately 35%. The bioproduction of L-ribose, a potent antiviral agent and a starting material of L-nucleoside-based pharmaceuticals,<sup>154</sup> has been investigated using *E. coli* cells. Production of L-ribose from cheap and abundant L-arabinose can be accomplished by co-overexpression of L-arabinose isomerase (L-AI) and mannose-6-phosphate isomerase (MPI)<sup>176</sup> or L-ribose isomerase (L-RI)<sup>161</sup> to convert L-ribulose to L-ribose (Figure 10C). In another approach, a synthetic platform for direct conversion of ribitol

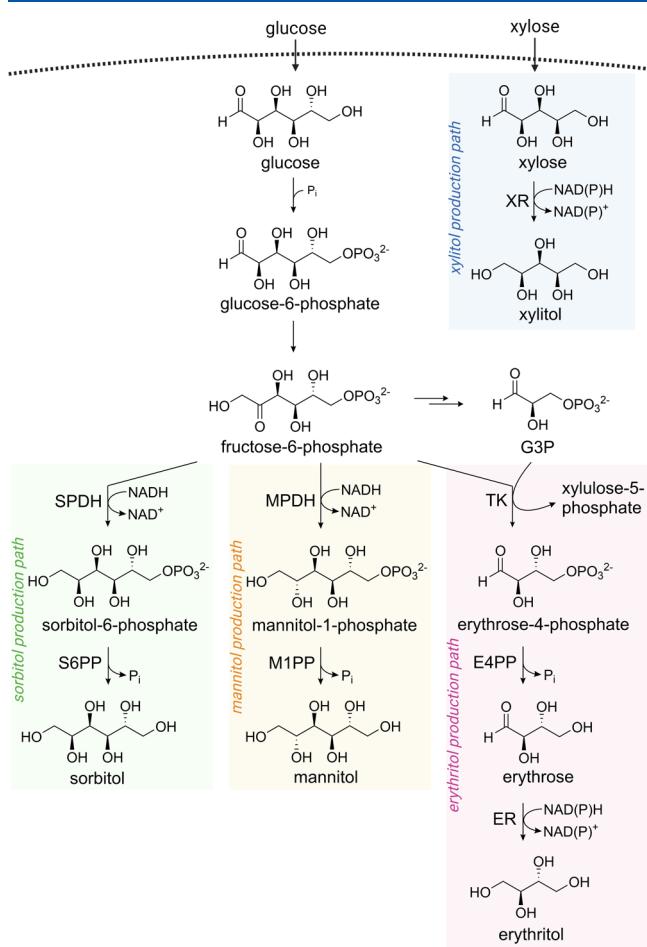
to L-ribose could be accomplished using *E. coli* overexpressing mannitol dehydrogenase (MDH).<sup>177</sup> Since ribitol is expensive and thus not practical for use as a starting material for industry, yeast cells overexpressing xylitol dehydrogenase (XDH) which can convert D-glucose into ribitol and excreted into the fermentation medium is used together with *E. coli* overexpressing MDH (Figure 10D).

Entrapment or immobilization of enzymes as whole-cell biocatalysts has also been shown to circumvent some thermodynamic limitations while simultaneously enhancing enzyme stability. For instance, expression of secretory L-AI in sporulating cells results in entrapment of the enzyme in the periplasmic space of the outermost layer of the spore wall.<sup>179</sup> The entrapped enzyme was found to have increased resistance toward environmental stresses, tolerating a broad range of pH conditions ranging from 3 to 10 and functioning well at high temperatures of up to 95 °C. Recently, L-AI was overexpressed in the cytoplasm of Gram-positive bacteria to be used as whole-cell biocatalysts for catalyzing the isomerization of D-galactose to D-tagatose.<sup>180</sup> The whole-cell encapsulating L-AI was shown to protect the enzyme from thermal deactivation and achieved a conversion yield of 85% within 48 h in a batch process conducted at 50 °C. Although these conditions gave nearly complete conversion, the remaining amount of substrate still poses a challenge in product purification. Because tagatose and galactose have similar physical properties such as their structures and solubility in aqueous media, the downstream process requires selective extraction or isolation for recovery of tagatose from the crude mixture.<sup>181</sup>

Although the production of rare sugars using cell factories is an economically attractive synthetic route for applications in various industrial sectors, the utilization of cells can be complicated because most lignocellulosic monosaccharides substrates can also be used for cell growth and metabolic functions via native pathways in host cells. D-Glucose can be utilized by the glycolysis pathway for cellular metabolism, drawing away from its full use as a substrate for rare sugar production. When xylose or arabinose is used as a substrate, its uptake into cells is often inhibited by the presence of glucose in the growth media due to the low preference of cells for these

substrates.<sup>182</sup> In addition to direct consumption of substrates in metabolic pathways, intermediates for rare sugar production such as L-ribulose and D-xylulose can also be assimilated into several metabolic ways.<sup>183</sup> In order to enable the whole-cell biocatalysts to be compatible with rare sugar production on a commercial scale, more improvement using advanced multidisciplinary approaches such as metabolic engineering, synthetic biology, and enzyme evolution is required for increasing catalytic properties such as activity, stability, and substrate-product preference of microbial cell factories.

**2.1.2. Sugar Alcohols.** Sugar alcohols such as xylitol, mannitol, sorbitol, and erythritol are mainly reduced forms of monosaccharides which have been extensively used as low-calorie sweeteners in food and pharmaceutical industries.<sup>184</sup> Sugar alcohols can also serve as starting precursors for transformation into commodity chemicals.<sup>33</sup> In particular, xylitol and sorbitol have potential applications as building blocks for producing various value-added compounds such as lactic acid<sup>185</sup> and vitamin C,<sup>186</sup> respectively. This section summarizes four examples of sugar alcohols synthesis from monosaccharides in hemicellulosic hydrolysates by wild-type and engineered whole-cell biocatalysts (Figure 11).



**Figure 11.** Enzymatic pathways for conversion of glucose and xylose into various sugar alcohols. Abbreviations; XR: xylose reductase; G3P: glyceraldehyde-3-phosphate; SPDH: sorbitol-6-phosphate dehydrogenase; S6PP: sorbitol-6-phosphatase; MPDH: mannitol-1-phosphate dehydrogenase; M1PP: mannitol-1-phosphatase; TK: transketolase; E4PP: erythrose-4-phosphatase; ER: erythrose reductase.

**2.1.2.1. Xylitol.** Xylitol is ranked as one of the most valuable building block chemicals which can be produced by renewable biomass.<sup>185</sup> Xylitol is widely used as an alternative sweetener because it has similar sweetness to sucrose but with much fewer calories. In addition, it can be used in pharmaceutical and nutraceutical products to give sweetness without causing tooth decay because it has activity against dental plaque formation.<sup>187</sup> Metabolic engineering and microbial fermentation processes have been extensively explored to construct economically viable microbial platforms for production of xylitol. The content below focuses on the current progress in metabolic pathway engineering for xylitol production from major sugars found in lignocellulosic biomass and agricultural waste such as glucose and xylose (Table 3).

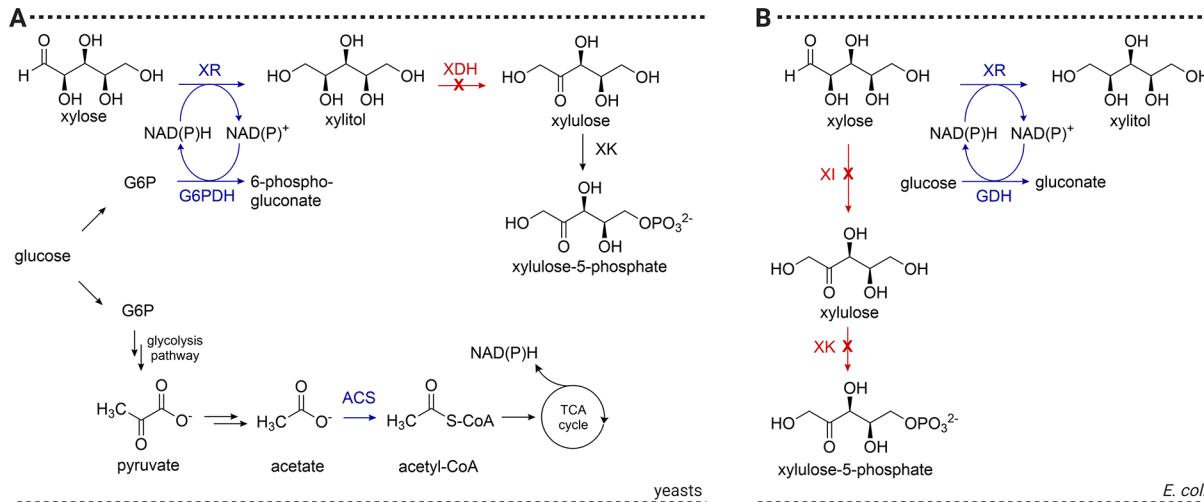
Xylitol can be naturally produced from xylose by fermentation of xylose-utilizing yeasts such as *Pichia stipitis*<sup>193</sup> and *Candida* species.<sup>194</sup> Reduction of xylose to xylitol is catalyzed by xylose reductase (XR) in the first step of xylose metabolism (Figure 11). As a native producer, *Candida* sp. is among the major species investigated for its xylitol production capabilities. The problem of xylitol production in yeast is that xylitol can be used in the pentose phosphate metabolic pathway via xylitol oxidation to xylulose by xylitol dehydrogenase (XDH) and then subsequently phosphorylated to form xylulose-5-phosphate by xylulose kinase (XK; Figure 12A). This subsequent metabolic pathway is regarded as a weak point for xylitol fermentation in yeast. Therefore, an XDH-deficient mutant ( $\Delta XYL2$ ) of *Candida tropicalis* was constructed to generate a system which can accumulate xylitol in the culture.<sup>188</sup> The engineered strain can produce xylitol with a yield of 0.97 g/g glucose and a productivity of 3.23 g/L h. However, *Candida* sp. cell growth is significantly repressed in a medium that contains glucose as a carbon source.<sup>195</sup> Moreover, utilization of *Candida* sp. in the food industry is problematic because of their pathogenic properties.<sup>196</sup> For production of products used as food and drugs, *S. cerevisiae* is a practical choice for use as a cell factory. A recombinant *S. cerevisiae* which can produce xylitol has been established by incorporation of XR. Since the reaction of XR requires utilization of NAD(P)H, simultaneous production of the reducing cosubstrate can be done by co-overexpressing glucose-6-phosphate dehydrogenase (G6PD) and acetyl-CoA synthetase (ACS) in the recombinant *S. cerevisiae*.<sup>189</sup> G6PD and ACS increase cellular NAD(P)H by oxidation of glucose-6-phosphate (G6P) and production of acetyl-CoA for feeding the tricarboxylic acid (TCA) cycle, respectively (Figure 12A). The engineered strain resulted in a full conversion of xylose to xylitol with a productivity of 4.27 g/L h using continuous xylose feeding.

The attempts to use bacteria as alternative hosts strain were later developed. Lactic acid bacteria (LAB) are classified as generally recognized as safe status (GRAS). However, they are mostly incapable of using xylose and thus require incorporation of the xylose assimilating system. For instance, *Lactococcus lactis* widely used in dairy and food processes<sup>197</sup> was engineered to overexpress a xylose transporter (XT) and XR from yeasts.<sup>191</sup> The engineered strain could produce xylitol from xylose with a productivity of 2.72 g/L h within 20 h. *E. coli* was also investigated as a host for xylitol production due to its rapid growth, ease of handling, and the abundance of genetic tools. Their native pathways can also assimilate both hexose and pentose sugars. In xylose metabolism of *E. coli*, xylose is first isomerized to xylulose by XI and then reduced to xylulose-5-phosphate by XK. Thus, *E. coli* was primarily

Table 3. Xylitol Production by Engineered Microorganisms

substrates	microorganisms	genetic manipulations <sup>a</sup>	cell types	yield (g <sub>product</sub> /g <sub>substrate</sub> )	productivity (g <sub>product</sub> /L h)	ref
glucose, xylose	<i>C. tropicalis</i>	$\Delta XYL2$	free cells	0.97	3.23	188
xylose	<i>S. cerevisiae</i>	$XYL1^+$ , $ACS1^+$ , $ZWF1^+$	free cells	1.00	4.27	189
glucose, xylose	<i>S. cerevisiae</i>	$XYL1^+$	immobilized	0.32	5.80	190
xylose	<i>L. lactis</i>	$XYL1^+$ , $xylT^+$	free cells	1.00	2.72	191
glucose, xylose	<i>E. coli</i>	$XR^+$ , $\Delta xylA$ , $\Delta xylB$	free cells	1.00	1.40	185
xylose	<i>E. coli</i>	$XR^+$ , $GDH^+$	free cells	1.00	6.37	192
xylose	<i>E. coli</i>	$XR^+$ , $GDH^+$	immobilized	1.00	9.10	192

<sup>a</sup>Marks denote for heterologous expression (+) or deletion (Δ) of genes. Manipulated genes; *XYL2*: xylitol dehydrogenase; *XYL1/XR*: xylose reductase; *ACS1*: acetyl-CoA synthetase; *ZWF1*: glucose-6-phosphate dehydrogenase; *xylT*: xylose transporter; *xylA*: xylose isomerase; *xylB*: xylulose kinase; and *GDH*: glucose dehydrogenase.



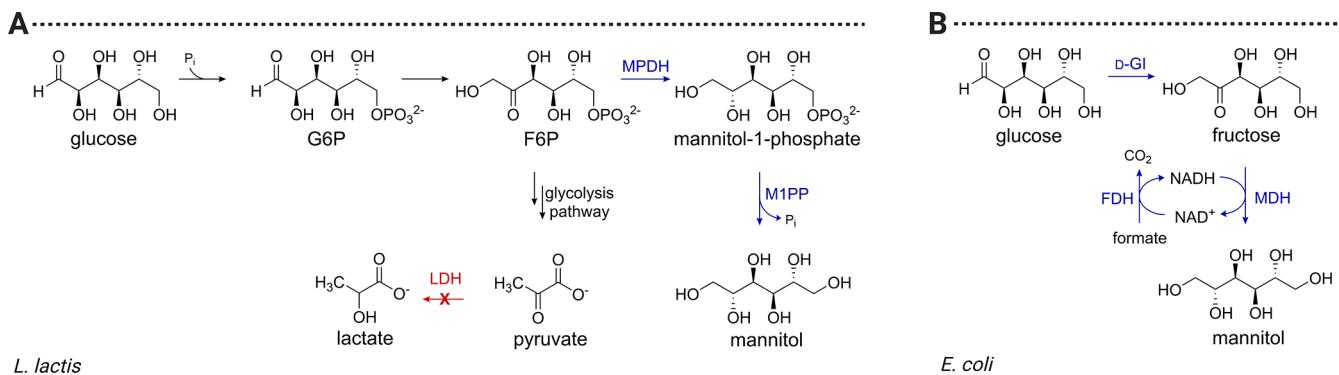
**Figure 12.** Engineering of metabolic pathways for xylitol production in different microorganisms. (A) *S. cerevisiae*. (B) *E. coli*. Pathway manipulations are highlighted as heterologous expression (blue) or deletion (crossed-red). Abbreviations: G6PD: glucose-6-phosphate dehydrogenase; XR: xylose reductase; XDH: xylitol dehydrogenase; XK: xylulose kinase; ACS: acetyl-CoA synthetase; GDH: glucose dehydrogenase; XI: xylose isomerase.

engineered by overexpressing XR for xylitol production and blocking xylose metabolism by disrupting the expression of XI and XK (Figure 12B).<sup>185</sup> A putative pathway for xylitol phosphorylation was also blocked by disrupting the phosphoenolpyruvate-dependent fructose phosphotransferase system (ptsF), allowing further increase in the xylose consumption rate of 0.6 g/L h. The overall productivity of 1.40 g/L h after 107-h batch fermentation could be achieved. To further enhance production of xylitol from xylose, the coexpression of XR and glucose dehydrogenase (GDH) which can generate NADPH from glucose was carried out (Figure 12B), resulting in a whole-cell biocatalyst which can synthesize xylitol from xylose with high productivity of 6.37 g/L h within only 12-h of incubation.<sup>192</sup>

To reuse microbial cells in a fed-batch process or prolonged fermentation during continuous production, immobilization of cells through several approaches was investigated.<sup>198</sup> *S. cerevisiae* expressing XR was entrapped in a packed-bed bioreactor for production of xylitol from xylose.<sup>190</sup> The highest productivity of 5.88 g/L h was achieved. In stability tests, the immobilized cells retained approximately 50% of the original activity after 15 days of continuous fermentation. Recently, another immobilization approach was applied to *E. coli* cells harboring XR and GDH by cross-linking cells with diatomite as solid-phase carriers through glutaraldehyde links.<sup>192</sup> Stability of the immobilized cells was evaluated by reusing them for 10 batches. After recycling for 3 batches, the cells retained 100%

of the original activity and retained 76.4% of their activity after 10 batches of recycling. The immobilized cells were additionally investigated for the conversion of xylose from mother liquor (XML) to xylitol, as this would be the approach of using manufacturing waste to produce a more valuable product. The results showed that XML at a concentration of 50 g/L could be converted to xylitol with 100% yield with a productivity of 9.1 g/L h. It can thus be seen that recent developments in metabolic and process engineering have allowed xylitol bioproduction by fermentation to be both facile and economically viable. Currently, xylitol bioproduction is still an active area of research with various investigations to explore the use of engineered cells to convert crude lysates from biomass treatments or even waste residues from industry to produce xylitol in a single fermentation process.

**2.1.2.2. Mannitol.** Mannitol is an important ingredient for food and pharmaceutical industries. In food applications, it serves as a low-calorie sweetener and can be used to increase the shelf life of food products.<sup>199</sup> Mannitol can also be used as a filler in pharmaceuticals to add volume and stabilize products.<sup>200</sup> In natural metabolic pathways, mannitol can be produced from glucose in LAB<sup>201</sup> and fungi<sup>202</sup> or from fructose by yeasts.<sup>203</sup> Therefore, whole-cell biocatalysis is efficient means for mannitol production by biotransformation. In the glucose metabolism, fructose-6-phosphate is reduced to mannitol-1-phosphate by mannitol-1-phosphate dehydrogen-



**Figure 13.** Metabolic engineering approaches for increasing mannitol production in microorganisms. (A) Enhancement of the mannitol production pathway in *L. lactis*. (B) Enhancement of glucose conversion into mannitol in *E. coli*. Pathway manipulations are highlighted as heterologous expression (blue) or deletion (crossed-red). Abbreviations; MPDH: mannitol-1-phosphate dehydrogenase; M1PP: mannitol-1-phosphatase; LDH: lactate dehydrogenase; d-GI: glucose isomerase; MDH: mannitol-1-dehydrogenase; FDH: formate dehydrogenase.

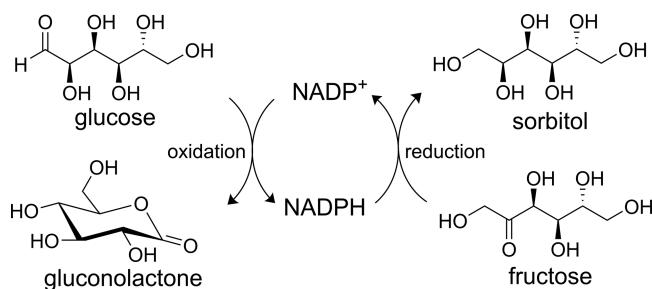
ase (MPDH) and subsequently dephosphorylated to yield mannitol by mannitol-1-phosphatase (M1PP; Figure 11).

Metabolic engineering approaches for mannitol production mostly focus on improvement of product yield in microbes which can naturally produce mannitol from glucose and to incorporate heterologous genes into hosts which cannot produce mannitol naturally. LAB have been extensively explored as host strains for mannitol production due to their safety and prevalent use in the food industry. The engineering approach was based on reducing lactic acid production paths and facilitating efficient conversion of fructose-6-phosphate (F6P) into mannitol. Lactate dehydrogenase (LDH)-deficient *L. lactis* was constructed and the engineered strain also co-overexpressed two enzymes, mannitol-1-phosphate dehydrogenase (MPDH) and mannitol-1-phosphatase (M1PP; Figure 13A).<sup>204</sup> The engineered strain achieved a maximum mannitol production yield from glucose of 39 mol/mol. Another approach to develop whole-cell biotransformation for the production of mannitol from glucose in *E. coli* was employment of isomerase and dehydrogenase as key catalysts.<sup>205</sup> d-GI catalyzes conversion of glucose into fructose, while mannitol dehydrogenase (MDH) catalyzes reduction of fructose to mannitol (Figure 13B). To facilitate fructose reduction to mannitol, FDH was incorporated to enhance NADH regeneration inside the cell. Results from whole-cell biocatalysis showed production of mannitol from glucose at a yield of 0.42 g/g. Another approach was developed using a mutant strain of the yeast *C. magnoliae* exhibiting higher glucose uptake than the wild-type strain.<sup>203</sup> Using two-stage fermentation in which the first stage was aerobic growth using glucose as a carbon source and the second stage was an anaerobic conversion of fructose to mannitol, mannitol production yield reached 81% conversion and a productivity of 4 g/L h. This two-stage fermentation process comprised of a growth phase and production phase allows optimization of mannitol production in yeast and it is the best system to date regarding the use of yeast for production of mannitol.

**2.1.2.3. Sorbitol.** Sorbitol has been widely used in the food and chemical industries as a precursor for L-ascorbic acid or vitamin C synthesis since 1933.<sup>206</sup> It can be converted to vitamin C by fermentation using the bacterium *Gluconobacter oxidans*, which has also been recognized as the first instance of artificial synthesis of vitamin C. Besides its applications in food, it has been used for synthesis of polymer building blocks such as isosorbide and propylene glycol.<sup>207,208</sup> In natural pathways,

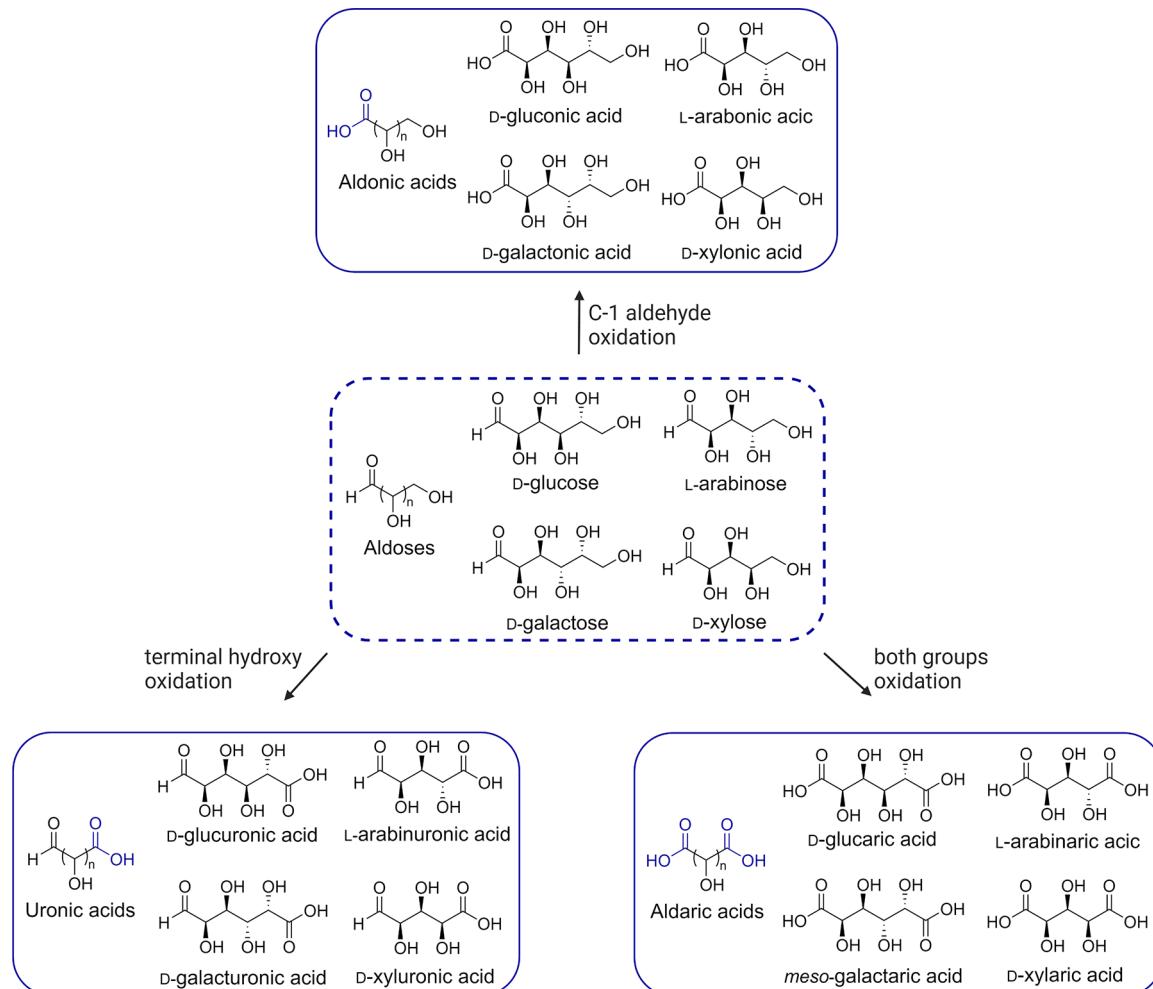
two enzymes, sorbitol-6-phosphate dehydrogenase (SPDH) and sorbitol-6-phosphatase (S6PP), are involved in sorbitol biosynthesis from fructose-6-phosphate derived from the glycolysis pathway (Figure 11).

Although the production of sorbitol (approximately 700 000 tonnes per year) can be achieved by reduction of glucose by H<sub>2</sub> using nickel catalysts,<sup>33</sup> the development of biobased approaches has received considerable interest due to its greater sustainability. Most of the studies investigated the use of *Zymomonas mobilis*, which is capable of coproducing sorbitol and D-gluconic acid from sucrose or a mixture of glucose and fructose in the production of sorbitol.<sup>186</sup> The sorbitol biosynthesis in this strain is catalyzed by NADP-bound glucose-fructose oxidoreductase (GFOR; Figure 14). The



**Figure 14.** Enzymatic coproduction of sorbitol and gluconolactone catalyzed by glucose-fructose oxidoreductase (GFOR).

enzyme catalyzes a ping-pong mechanism reaction where glucose is first converted to gluconolactone and nicotinamide adenine dinucleotide phosphate (NADPH) and then fructose is reduced by NADPH to form sorbitol.<sup>209</sup> Thus, the strain always coproduces gluconolactone (gluconic acid) and sorbitol. The highest sorbitol yield obtained to date was from *Z. mobilis* strain ATCC 29191 produced in a batch system.<sup>210</sup> The microbe is capable of conversion of glucose and fructose into an equimolar ratio of gluconic acid and sorbitol with a yield of 91% for both products. The final concentration of produced sorbitol was 300 g/L. For LAB, the LDH-deficient *Lactobacillus plantarum* overexpressing SPDH was constructed to improve the pathway of sorbitol production.<sup>211</sup> The engineered strain could produce sorbitol with 66% yield from glucose conversion. However, mannitol was found to be coproduced with sorbitol in considerable amount (around 15–



**Figure 15.** Sugar acids derived from lignocellulosic aldose sugars.

20% of total polyols) due to the presence of mannitol catabolic enzymes (MPDH and M1PP, Figure 13A) in *L. plantarum*. The coproduction of sorbitol with gluconolactone and other polyols are the main hurdles which make biotechnological production of sorbitol infeasible for commercialization.

**2.1.2.4. Erythritol.** Unlike other sugar alcohols, erythritol is a four-carbon structure which can be produced from glucose by yeasts, LAB, and other fungal species via the pentose phosphate pathway through erythrose-4-phosphate and erythrose as intermediates (Figure 11). Erythritol can be naturally produced by osmophilic yeasts including *Candida magnoliae*<sup>212</sup> and *Yarrowia lipolytica*<sup>212</sup> and LAB such as *Leuconostoc oenos*.<sup>213</sup>

Erythritol bioproduction from lignocellulosic sugars relies mostly on the isolation of microorganisms that are capable of producing erythritol at a high yield.<sup>194</sup> *Trichosporonoides megachiliensis* and *Pseudozyma tsukubaensis* have been employed to produce erythritol commercially because of their high yield and productivity. *T. megachiliensis* mutant obtained from UV irradiation was investigated for its potential for production of erythritol from glucose.<sup>214</sup> Analysis of enzyme specific activities in crude lysates during culturing suggested that activities of enzymes in the pentose phosphate pathway, particularly erythrose reductase (ER) and transketolase (TK) were higher than those of the TCA cycle. Industrial production of erythritol from glucose by this strain was explored in a

100 000-L fermentation tank, and the results showed that erythritol production reached 0.47 g/g of glucose used with a productivity of 2 g/L h. For erythritol production by *P. tsukubaensis* strain,<sup>215</sup> production in laboratory (7 L), pilot (300 L), and plant (50 000 L) scales were explored. In the plant scale, an erythritol yield of around 60% conversion from glucose with a productivity of 2.84 g/L h could be obtained.

**2.1.3. Sugar Acids.** Sugar acids along with alcohols are commonly found as common products from natural fermentative processes. Sugar acids are generally defined as single or double oxidation products of aldose sugars commonly derived from lignocellulosic monosaccharides. They can be classified into three types according to their acid functional groups: aldonic, aldaric, and uronic acids (Figure 15).<sup>216,217</sup> Aldonic acids are obtained by oxidation of an aldehyde group at the C-1 position to form a carboxylic acid, whereas uronic acids are derivatives from oxidation at the terminal hydroxyl group to form acid. Oxidation of both terminal groups results in diacid compounds of the aldaric acid class. These sugar acids are valuable materials used in food, cosmetic and pharmaceutical industries. Moreover, their biocompatibility and possession of active functional groups make them compatible with applications in biofuels and biopolymers. For instance, D-gluconic acid- and D-xylonic acid-based monomers are promising building blocks for ring-opening polymerization reactions and preparation of biopolymers such as polyamides

Table 4. Production of Sugar Acids by Whole-Cell Biocatalysts and Enzymatic Cascade Reactions

acid types	sugar acids	substrates	biocatalysts	key enzymes <sup>a</sup> /genetic manipulations <sup>b</sup>	yield (g <sub>product</sub> /g <sub>substrate</sub> )	productivity (g <sub>product</sub> /L h)	ref
aldonic	D-gluconic acid	D-glucose	<i>A. niger</i>	GOx	1.05	21.09	220
	D-gluconic acid	D-glucose	<i>A. niger</i>	GOx	0.98	31.05	221
	D-gluconic acid	D-glucose	<i>G. oxidans</i>	GDH	0.95	4.07	222
	D-xylonic acid	D-xylose	<i>S. cerevisiae</i>	xylB <sup>+</sup> , xylC <sup>+</sup>	0.88	0.44	223
	D-xylonic acid	D-xylose	<i>E. coli</i>	xylB <sup>+</sup> , xylC <sup>+</sup> , ΔyjhG, ΔyagF, ΔxylA	1.09	1.80	224
	D-xylonic acid	D-xylose	enzymatic cascade	XYD, ADH	0.95	15.66	225
	L-arabonic acid	L-arabinose	<i>S. cerevisiae</i>	araDH <sup>+</sup> , GAL2 <sup>+</sup>	0.86	0.25	226
	L-arabonic acid	L-arabinose	<i>E. coli</i>	araDH <sup>+</sup> , AraA	0.99	1.22	227
	D-galactonic acid	D-galactose	<i>E. coli</i>	gld <sup>+</sup>	0.88	0.24	227
	uronic	D-glucuronic acid	D-glucose	<i>E. coli</i>	ino1 <sup>+</sup> , suhB <sup>+</sup> , miox <sup>+</sup>	0.03	228
aldaric	D-glucaric acid	D-glucose	<i>S. cerevisiae</i>	ino1 <sup>+</sup> , miox <sup>+</sup> , udh <sup>+</sup>	0.20	0.03	229
	D-glucaric acid	D-glucose	<i>E. coli</i>	ino1 <sup>+</sup> , suhB <sup>+</sup> , miox <sup>+</sup> , udh <sup>+</sup> , Δzwf, Δpgi, ΔuxaC, ΔgudD	0.46	230	

<sup>a</sup>Enzymes are GOx: glucose oxidase; GDH: glucose dehydrogenase; XYD: xylose dehydrogenase; ADH: alcohol dehydrogenase. <sup>b</sup>Marks denote heterologous expression (+) or deletion (Δ) of genes encoding key enzymes. Manipulated genes are xylB: xylose dehydrogenase; xylC: xylonolactone lactonase; yjhG/yagF: xylonate dehydratase; xylA: xylose isomerase; aradh: arabinose dehydrogenase; GAL2: galactose permease; araA: arabinose isomerase; gld: galactose dehydrogenase; ino1: myo-inositol-1-phosphate synthase; suhB: inositol 1-monophosphatase; miox: myo-inositol oxygenase; udh: uronate dehydrogenase; zwf: glucose-6-phosphate dehydrogenase; pgi: phosphoglucomutase; uxuC: uronate isomerase; gudD: glucarate dehydratase.

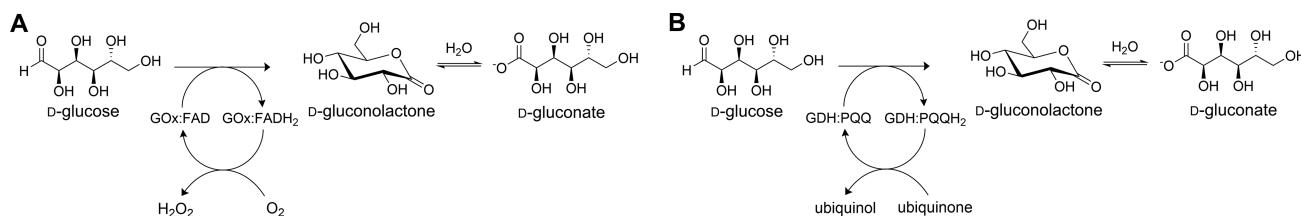


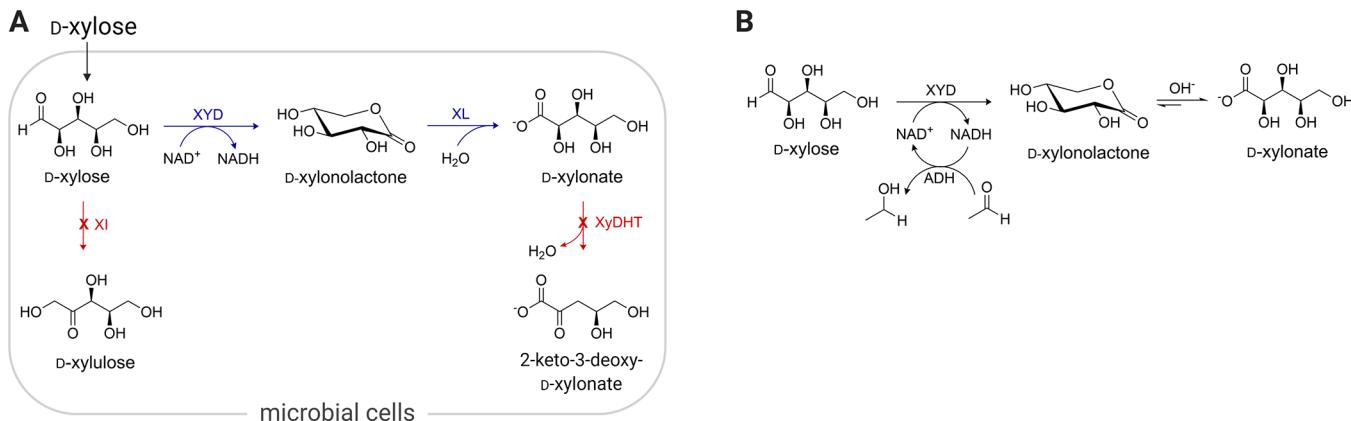
Figure 16. Conversion of D-glucose to D-gluconate by glucose oxidizing enzymes. (A) Glucose oxidase (GOx). (B) PQQ-dependent glucose dehydrogenase (GDH:PQQ).

and polyesters.<sup>218,219</sup> In this section, we review the current knowledge of biotechnological production of major sugar acids using metabolically engineered microorganisms or multiple-enzyme cascade reactions (Table 4).

**2.1.3.1. D-Gluconic Acid.** Gluconic acid/gluconate is one of the most well-known sugar acids that have been produced in industrial scale for use in various applications such as in food additives, and as monomers for biodegradable polymers and cement industry.<sup>231</sup> Glucose can be oxidized by oxidoreductases to generate gluconolactone, which is spontaneously hydrolyzed by water or enzymatically catalyzed by gluconolactonase to yield gluconic acid.<sup>232</sup> Glucose oxidizing enzymes can be divided into two groups, glucose oxidase (GOx) and glucose dehydrogenase (GDH), depending on their electron acceptors. GOx is a flavin adenine dinucleotide (FAD)-harboring enzyme that catalyzes glucose oxidation to form gluconolactone by utilizing molecular oxygen as an electron acceptor with simultaneous production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; Figure 16A).<sup>232,233</sup> On the other hand, GDH uses other electron acceptors (not oxygen) for oxidation of glucose. GDHs are commonly classified into three subgroups depending on the types of electron mediators including nicotinamide adenine dinucleotide (phosphate) (NAD(P)<sup>+</sup>), pyrroloquinoline quinone (PQQ) or FAD. Unlike NAD(P)<sup>+</sup> which binds loosely to the enzyme and acts as a substrate to receive electrons from glucose, PQQ and FAD bind tightly to the enzymes and serve as cofactors by mediating electron transfer from glucose to various natural and artificial electron acceptors. For instance, PQQ-dependent GDH found in *Gluconobacter*

sp. catalyzes glucose oxidation and the corresponding reduction of ubiquinone to ubiquinol (Figure 16B).<sup>234</sup>

Currently, the annual production of gluconic acid is estimated to be around 100 000 tonnes and it is mainly produced by biotechnological processes.<sup>235</sup> The compound is commercially produced by microbes possessing GOx or GDH such as *Aspergillus niger*<sup>236</sup> or *Gluconobacter oxidans*.<sup>237</sup> *Aspergillus* species is the most well-known species for high level expression of GOx and it still remains as the most commonly used microorganism for gluconic acid production.<sup>236</sup> GOx in *A. niger* are expressed in two forms; one is predominantly located in the cell wall and the other one is released to extracellular fluid.<sup>231</sup> Their presence in two locations increases gluconic acid production because gluconolactone can be hydrolyzed into gluconic acid by spontaneous hydrolysis with extracellular water. Most industrial processes for gluconic acid production involving *A. niger* use batch processes because continuous processes would accumulate formation of mycelial filaments during fungal growth which can inhibit GOx activity. For instance, the highest gluconic acid production rate in batch could be obtained by performing fermentation with high initial glucose content and controlling optimal mycelial morphology during submerged fermentation.<sup>220</sup> The fermentation media containing large amount of glucose (330 g/L) could be used to perform bioconversion for 14.5 h, resulting in an overall yield of 1.05 g/g and gluconic acid productivity rate at 21.09 g/L h. Recently, an improved continuous fermentation by improvement of mass transfer and dispersing mycelia of fungi was established.<sup>221</sup> The highest



**Figure 17.** Biotransformation of D-xylose to D-xylonate. (A) Metabolic engineering to construct whole-cell biocatalysts for production of D-xylonate. Pathways are highlighted as heterologous expression (blue) and deletion (crossed-red) for increasing metabolic flux. (B) Enzymatic cascades for production of D-xylonate. Abbreviations; XYD: xylose dehydrogenase; XL: xylonolactonase; XI: xylose isomerase; XyDHT: xylonate dehydratase; ADH: alcohol dehydrogenase.

production rate of 31.05 g/L h was achieved with a yield of 0.98 g sodium gluconate/glucose. However, several disadvantages of using *A. niger* were observed including the microbe high sensitivity toward lignocellulosic hydrolysates such as weak acids and phenolic compounds. These compounds can act as inhibitors and *A. niger* tends to form large fungus pellets which subsequently limit the oxygen transfer to cells.<sup>238,239</sup> *G. oxidans* has been attractive for production of gluconic acid because it can oxidize monosaccharides to form acids and ketones rapidly.<sup>240</sup> Unlike GOx expressed in *A. niger*, two systems of GDHs are overexpressed in *G. oxidans*; one is a soluble NADP<sup>+</sup>-dependent GDH in cytoplasm and the second one is PQQ-dependent GDH which is localized in the cytoplasmic membrane.<sup>241</sup> Overexpression of the membrane-bound GDH in *G. oxidans* was found to increase the gluconate production yield by 70% compared to the wild-type strain activity.<sup>242</sup> Recently, batch fermentation of *G. oxidans* with agricultural waste was successfully achieved.<sup>222</sup> The *G. oxidans* strain DSM 2003 was utilized to produce gluconic acid from potato waste hydrolysates with an overall conversion yield of 0.95 g product/g glucose and productivity of 4.07 g/L h.

There have been considerable interests in overexpressing glucose oxidizing enzymes in other bacterial strains widely used in industrial biotechnology such as *E. coli* and *S. cerevisiae*. Overexpression of GOx or GDH in *E. coli* were investigated,<sup>243,244</sup> but the results showed that more than 60% of the recombinant proteins were inactive and localized in inclusion bodies. They need to be refolded into soluble forms *in vitro* by dialysis in urea and FAD-containing buffer. Similar production of recombinant GOx in yeast such as *S. cerevisiae* often results in a decrease in enzymatic activity due to hyperglycosylation.<sup>245</sup> Consequently, current approaches for gluconic acid production by microbial fermentation are mainly achieved by using either native *Aspergillus* or *Gluconobacter* species.

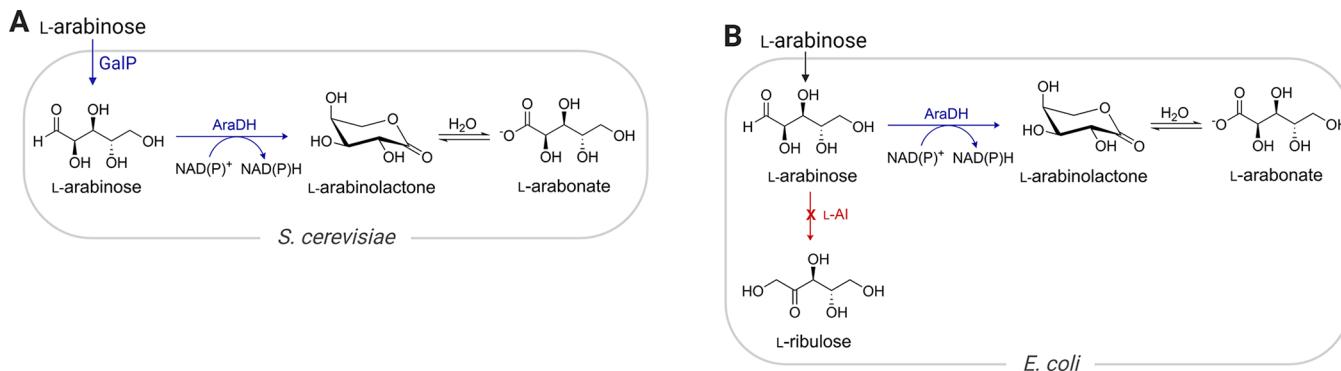
**2.1.3.2. D-Xylic Acid.** Xylic acid/xylonate is a versatile platform chemical with various applications. It can be used as a complexing agent for concrete<sup>246</sup> and as a precursor for polymers<sup>247</sup> such as copolyamides, polyesters, and hydrogels. Naturally, high extracellular concentrations of xylonate have been reported for various bacteria, particularly *G. oxidans* and *P. putida*.<sup>247</sup> However, these species require complex growth medium and conditions such as low pH and precise control of

cell growth in order to maintain continuing bioconversion in batch.

The attempt to engineer xylonic acid production in yeast such as *S. cerevisiae* could be achieved by incorporation of xylose dehydrogenase (XYD) and xylonolactonase (XL).<sup>223</sup> After xylose is transported into yeast, it can be oxidized to form xylonolactone by XYD and followed by hydrolysis to form xylonic acid by XL (Figure 17A). The engineered *S. cerevisiae* can convert xylose to xylonate with a conversion yield of 0.88 g/g and a productivity rate at 0.44 g/L h. A similar approach of incorporating the xylonate biosynthetic pathway with disruption of xylose and xylonate metabolism pathways including inactivation of XI and xylonate dehydratase (XyDHT) to prevent isomerization of xylose to xylulose and xylonate dehydration was carried out in *E. coli* (Figure 17A).<sup>224</sup> The results showed that 108.2 g/L of D-xylonate could be produced with a yield of 1.09 g xylonate/g xylose and a specific productivity of 1.80 g/L h.

In addition to whole-cell biocatalysis, the use of isolated enzymes to construct an enzymatic cascade of XYD and alcohol dehydrogenase (ADH) under alkaline conditions was established (Figure 17B).<sup>225</sup> XYD catalyzes conversion of xylose to xylonolactone whereas ADH catalyzes reduction of aldehyde to alcohol with simultaneous regeneration of NADH to NAD<sup>+</sup> to be used in the XYD reaction. The reactions were maintained at pH 8.0 which pushed the equilibrium toward xylonate formation because the produced lactone can undergo spontaneous hydrolysis at alkaline pH. Product of 95% yield was obtained from xylose with a production rate of 15.66 g/L h. Purified xylonate could be obtained after enzyme filtration and evaporation to remove alcohol byproducts and buffer. This biocatalytic cascade for producing xylonate based on the use of xylose dehydrogenase and an NAD<sup>+</sup> regeneration in alkaline pH could be used for high yield production and easy purification of xylonate. The use of chemoenzymatic reactions thus could be feasible for scaling up.

**2.1.3.3. L-Arabinic Acid.** L-Arabinic acid/arabonate is a 4-epimer of D-xylonate and it has similar applications for polymer synthesis as those of D-xylonate described above. Moreover, it was reported that it can be used as a drug for treating calcium oxalate urolithiasis.<sup>226</sup> Investigation of oxidation products from L-arabinose utilization receives much less attention than those



**Figure 18.** Metabolic engineering for production of L-arabonate from L-arabinose. (A) *S. cerevisiae*. (B) *E. coli*. Pathways are highlighted as heterologous expression (blue) or deletion (crossed-red) for increasing metabolic flux. Abbreviations; GalP: galactose permease; AraDH: arabinose dehydrogenases; L-AI: L-arabinose isomerase.

of D-glucose and D-xylose due to its low content in waste compared to those monosaccharides.

Metabolic engineering of *S. cerevisiae* was investigated to construct a whole-cell biocatalyst to produce arabonate from arabinose.<sup>226</sup> Oxidation of L-arabinose to form L-arabonic acid is catalyzed by arabinose dehydrogenases (AraDH) that uses NAD(P)<sup>+</sup> as a cosubstrate or electron acceptor.<sup>226</sup> Well characterized AraDHs include the enzymes found in various root-associated nitrogen-fixing bacteria such as *Rhizobium* species and *Azospirillum brasiliense*.<sup>248,249</sup> As *S. cerevisiae* can consume L-arabinose very slowly, recombinant engineering for expression of arabinose assimilating pathways is required. Galactose permease (GalP) and AraDH from the bacterium *Rhizobium leguminosarum* were overexpressed to enable constant uptake and oxidation of L-arabinose to form L-arabonate (Figure 18A).<sup>226</sup> The engineered yeast strain resulted in the production of 18 g/L arabonate in culture, at a rate of 0.25 g/L h with an 86% yield from arabinose.

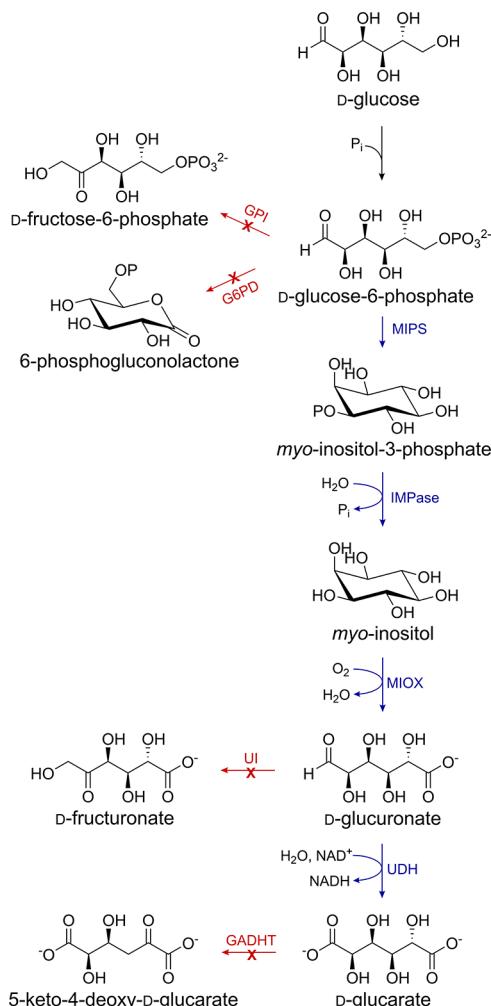
Later on, metabolic engineering of *E. coli* to construct an L-arabonate producing strain by heterologous expression of AraDH was performed. Unlike the yeast pathway mentioned above, *E. coli* can take up pentose sugars into cells before metabolizing them via the pentose phosphate pathway.<sup>250</sup> Thus, AraDH from *Azospirillum brasiliense* was overexpressed in *E. coli* to enable direct transformation of consumed arabinose into arabonate (Figure 18B).<sup>227</sup> In addition, a gene encoding for L-AI was deleted to reduce a competitive pathway of arabinose utilization. The resulting strain was shown to produce 43.9 g/L arabonate with a productivity of 1.22 g/L h and a 99% yield from arabinose. In addition to the oxidation of L-arabinose, AraDH has promiscuous activity toward galactose. The enzyme can catalyze galactose oxidation to form galactonate at a rate comparable to the rate of arabinose oxidation. Therefore, improving the purity of the sugar feedstock can be the key to decreasing the formation of undesirable products. Currently, production of L-arabonate via biobased approaches still requires further improvements before the reaction can meet industrial requirements.

**2.1.3.4. D-Glucaric Acid.** Glucaric acid (glucarate) and its derivatives are widely used as food additives, dietary supplements, drugs, detergents, corrosion inhibitors, and biodegradable materials.<sup>251</sup> Although it can be synthesized by chemical oxidation of D-glucose by nitric acid, the reaction is inefficient and generates nitrogen oxide (NO<sub>x</sub>), a GHG, as a byproduct.<sup>252</sup> Therefore, biological production of glucaric acid using whole-cell catalysts has received attention. S.

*cerevisiae* can be used for production of glucarate by coexpression of four key enzymes including *myo*-inositol-1-phosphate synthase (MIPS), inositol-1-monophosphatase (IMPAse), *myo*-inositol oxygenase (MIOX), and uronic acid/uronate dehydrogenase (UDH) to produce glucaric acid from extracellular glucose (Figure 19).<sup>229</sup> By fed-batch fermentation in a 5-L bioreactor, the bioconversion of glucose to glucaric acid could be achieved with a yield of 0.20 g/g and production rate at 0.03 g/L h. Recently, a biosynthetic pathway for glucaric acid production in *E. coli* from raw glucose was established by overexpression of the same four key enzymes as in *S. cerevisiae*.<sup>230</sup> The flux of the glucaric acid biosynthetic pathway could be enhanced by blocking the conversion of glucose into G6P by disrupting G6PD and GPI expression. Moreover, genes expressing uronic acid isomerase (UI) and glucaric acid/glucarate dehydratase (GADHT) were deleted to increase the glucarate production titer. Subsequently, the culture medium was optimized to obtain a maximum yield of 5.35 g/L culture with 46% conversion from glucose. The yield of D-glucarate production is quite low compared to production of other sugar acids (Table 4). Therefore, further development of D-glucarate production pathways to redirect flux into the target pathways is needed in order to develop technologies which meet industrial requirements.

**2.1.3.5. Other Sugar Acids.** Other sugar acids which can be achieved via the biotransformation of other monosaccharides include D-galactonic acid and D-glucuronic acid. D-Galactonic acid (galactonate) is an oxidized product of D-galactose which can be used as a polyester precursor, pharmaceutical intermediate, and cosmetic raw material.<sup>253</sup> Galactonic acid production in *E. coli* was established by overexpression of galactose dehydrogenase (GalDH).<sup>227</sup> Batch fermentation of the engineered *E. coli* gave galactonic acid with an 88% yield from galactose conversion with an average productivity of 0.24 g/L h in a 5-L bioreactor (Table 4). D-Glucuronic acid can be obtained from the glucaric biosynthesis pathway (Figure 19). Its derivative could be used to synthesize paclitaxel glucuronyl prodrugs.<sup>254</sup> By coexpression of two enzymes involved in glucuronic production (MIPS and MIOX) in *E. coli*, the engineered strain was found to be able to produce glucuronic acid from D-glucose, although at a low concentration (Table 4).<sup>228</sup>

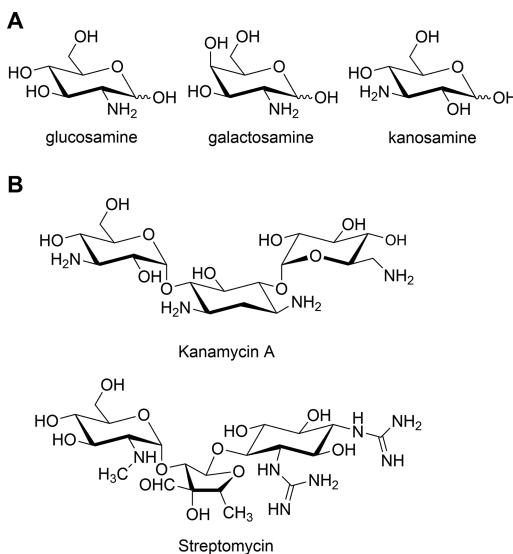
Production of these acids has only been shown on a small scale and their yields are quite low. The technology has not yet been commercialized.<sup>227,255</sup> In addition, the main challenge of sugar acid production from nonglucose sugars has been their



**Figure 19.** Metabolic engineering for D-glucarate production from D-glucose.  $P_i$  denotes a phosphate ( $\text{PO}_4^{3-}$ ) group. Pathways are highlighted for heterologous expression (blue) or deletion (crossed-red). Abbreviations; GPI: glucose-6-phosphate isomerase; G6PD: glucose-6-phosphate dehydrogenase; MIPS: myo-inositol-1-phosphate synthase; IMPase: inositol-1-monophosphatase; MIOX: myo-inositol oxygenase; UDH: uronate dehydrogenase; UI: uronic acid isomerase; GADHT: glucarate dehydratase.

low content in lignocellulose hydrolysates compared to glucose and xylose. Nevertheless, developments in this field are important for the sustainable production of specialty chemicals and biomass utilization.

**2.1.4. Aminosugars.** Aminosugars are important constituents in many natural biomacromolecules such as chitin, glycoproteins, lipopolysaccharides, and mucopolysaccharides.<sup>256</sup> Most common aminosugars in nature are found as derivatives of hexoses, such as 2-amino-2-deoxy-D-glucosamine (glucosamine), 2-amino-2-deoxy-D-galactosamine (galactosamine), and 3-amino-3-deoxy-D-glucose (kanosamine; Figure 20A).<sup>257</sup> Glucosamine (GlcN) is an essential building block for the biosynthesis of chitin, which is the second most abundant polysaccharide after cellulose, making up the exoskeletons of crustaceans, mollusks, and insects and the fibrillar polymers found in fungal cell walls.<sup>258</sup> Incorporation of an amino group into monosaccharide structures significantly changes their physicochemical and biological activities. For instance, naturally produced aminosugars can serve as precursors in the synthesis of several antibiotics. In *Streptomyces* species, the



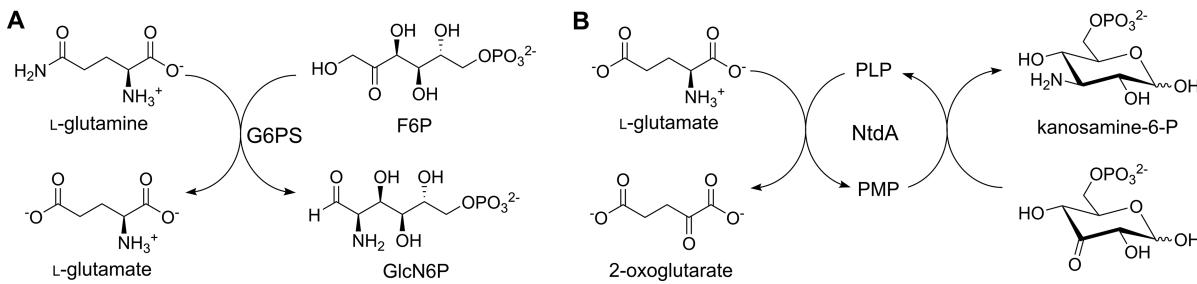
**Figure 20.** Aminosugars found in nature. (A) Common naturally produced aminosugars. (B) Incorporation of amino sugars into antibiotics.

antibiotic kanamycin A is synthesized from kanosamine,<sup>259</sup> while streptomycin is assembled from a methylated derivative of glucosamine (Figure 20B).<sup>260</sup>

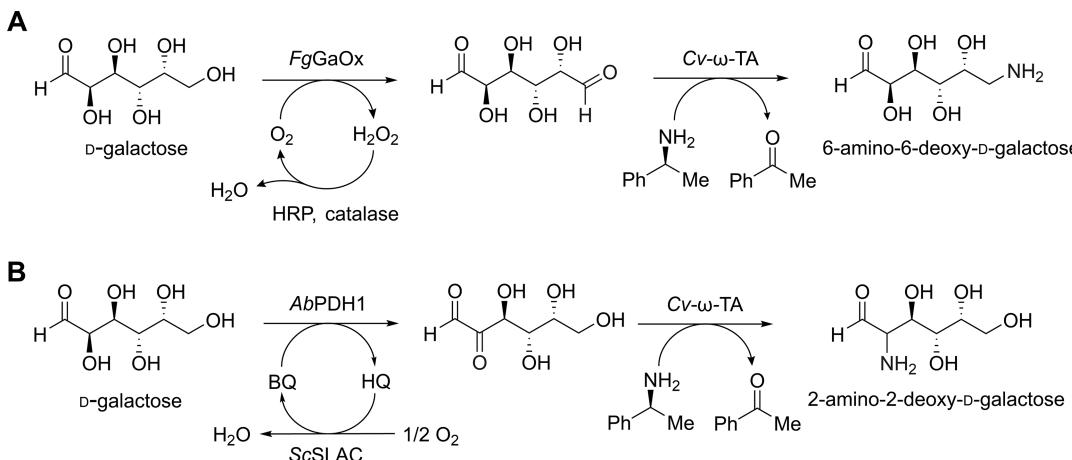
In addition to their biological activities, aminosugars also have applications as specialty chemicals. These sugars can be used for the synthesis of glycopolypeptides which have useful therapeutic and biotechnological applications such as drug delivery materials.<sup>261</sup> Recently, aminosugars, especially D-glucosamine can be applied as aminosugar-based catalysts catalyze organic transformations such as aldol reactions, Michael addition, epoxidation, fluorination, and imine reduction.<sup>262</sup> For example, the chirality and rigidity of D-glucosamine help control the stereoselectivity of the reaction.

**2.1.4.1. Enzymatic Reactions for Aminosugar Synthesis.** In biological systems, aminosugars are synthesized by enzymes in a stereospecific manner. The amination is catalyzed by a pyridoxal phosphate (PLP)-dependent aminotransferase or a cofactor-independent amidotransferase.<sup>256</sup> An amino group is usually transferred from L-glutamine or L-glutamate (amine donor) to a keto group of sugar phosphates or sugar nucleotides. An example of cofactor-independent amidotransferring enzymes is an L-glutamine: D-fructose-6-phosphate amidotransferase, commonly known as glucosamine-6-phosphate synthase (G6PS), which catalyzes a transamination from L-glutamine to D-fructose-6-phosphate (F6P), forming D-glucosamine-6-phosphate (GlcN6P) and L-glutamate (Figure 21A). For PLP-dependent aminotransferases, a well-known example is in the 3,3'-neotrehalosadiamine (NTD) biosynthetic pathway in *Bacillus subtilis*.<sup>263</sup> The PLP-dependent 3-oxo-glucose-6-phosphate:glutamate aminotransferase (NtdA) catalyzes transamination from L-glutamate to 3-oxo-glucose-6-phosphate, yielding kanosamine-6-phosphate and 2-oxoglutarate as products (Figure 21B).

These enzyme-catalyzed aminations are regioselective, making them attractive for biocatalysis. Recently, the enzymatic cascade to generate regio-specific functionalized monosaccharides using a combination of oxidation and transamination to establish a two-step process for synthesis of amino carbohydrates was reported.<sup>264</sup> The first step is oxidation of galactose using galactose oxidase from *Fusarium*



**Figure 21.** Biological synthesis of aminosugars catalyzed by enzymes. (A) G6PS: cofactor-independent amidotransferase; (B) NtdA: PLP-dependent aminotransferase; PLP: pyridoxal-5'-phosphate; PMP: pyridoxamine-5'-phosphate.



**Figure 22.** Enzymatic cascades for regiospecific amination of galactose. (A) C-6 oxidation of galactose catalyzed by *FgGaOx* and subsequent amination. (B) C-2 oxidation of galactose catalyzed by *AbPDH1* and subsequent amination. Abbreviations: *FgGaOx*: galactose oxidase from *F. graminearum*; *Cv-w-TA*: transaminase from *C. violaceum*; HRP: horseradish peroxidase; *AbPDH1*: pyranose dehydrogenase from *A. bisporus*; *ScSLAC*: small laccase from *S. coelicolor*; BQ: benzoquinone; HQ: hydroquinone.

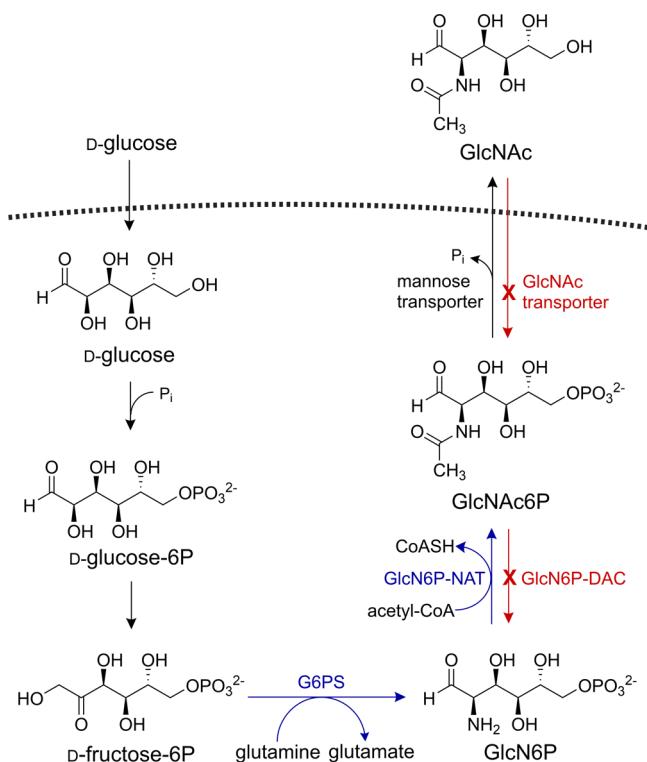
*graminearum* (*FgGaOx*) or pyranose dehydrogenase from *Agaricus bisporus* (*AbPDH1*) to form a ketohexose intermediate while the second step is catalyzed by  $\omega$ -transaminase from *Chromobacterium violaceum* (*Cv-w-TA*) to yield aminosugar. For the GaOx-catalyzed reaction, D-galactose is oxidized at the C-6 position, yielding an aldehyde intermediate (Figure 22A) while the reaction catalyzed by PDH1 oxidizes at the C-2 position to result in a ketone intermediate (Figure 22B). Transamination reaction can be used to incorporate an amino group at different positions of sugars. The highest yield of aminosugar production from galactose of around 67% could be achieved using the pathway illustrated in Figure 22A. These examples illustrate a full biocatalytic cascade including oxidoreductases and transaminases allowing the amination of lignocellulosic sugars and implementation of multiple reactions in one pot.

**2.1.4.2. Metabolic Engineering for Production of Aminosugars.** Although production of common aminosugars such as glucosamine (GlcN) can be achieved by production of chitinolytic enzymes in *E. coli* for breaking down chitins,<sup>265</sup> lignocellulosic biomass also serves as attractive starting material for production of N-acetylglucosamine (GlcNAc) and other specialty amino sugars using metabolically engineered cells.<sup>266</sup> Commonly used hosts in bioindustries such as *E. coli*, *Bacillus subtilis* have been engineered for production of aminosugars such as GlcN from glucose.<sup>267</sup> GlcN is rapidly degraded in *E. coli* and its degradation products can inhibit cell growth.<sup>265</sup> Thus, its acetylated derivative, GlcNAc is more preferred as a final product from microbial fermentation than GlcN. Two

genes encoding glucosamine-6-phosphate synthase (G6PS) and glucosamine-6-phosphate N-acetyltransferase (GlcN6P-NAT) were introduced into *E. coli* for increasing the production of GlcNAc from glucose, while two genes encoding for GlcN6P deacetylase (GlcN6P-DAC) and GlcNAc transporter were deleted to prevent cellular utilization of GlcNAc and its reuptake into cells, respectively (Figure 23).<sup>268</sup> Consequently, the GlcNAc production titer could reach 110 g/L after fermentation for 72 h with a 67% conversion yield from glucose. The acetylated glucosamine is more stable than glucosamine at neutral pH and does not create any inhibitory effects on cellular activities. At the final stage, the GlcNAc product is hydrolyzed under acidic conditions to yield glucosamine as a final product. This study highlighted a strategic combination of metabolic and process engineering for production of valuable chemicals.

#### 2.1.5. Sugar Esters and Glucose-Based Monomers.

Ester derivatives of sugars have received increased attention, as they have properties which make them usable as specialty polymers and materials. The incorporation of sugar-derived units into traditional polymers such as polyamides, polyesters, and polyurethanes can generate novel biodegradable and biocompatible polymers and materials. These sugar esters and sugar-based monomers can be synthesized by esterification or transesterification of mono- or disaccharides with various acyl donors including natural fatty acids, phenolic acids, and synthetic monomers.<sup>269,270</sup> Common sugars and functionalized sugars such as aldonic acids, aldaric acids, and aminosugars can be used as substrates to synthesize sugar esters with new

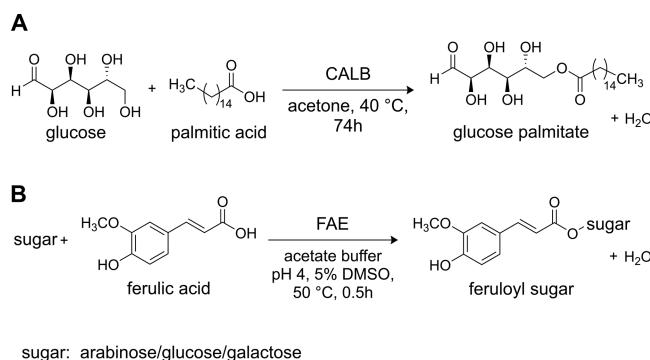


**Figure 23.** Metabolic engineering for production of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc). Pathways were modified by gene expression (blue) and gene deletion (crossed-red). Abbreviations; G6PS: glucosamine-6-phosphate synthase; GlcNAc6P-NAT: glucosamine-6-phosphate *N*-acetyltransferase; GlcNAc6P-DAC: GlcN-6P deacetylase.

properties.<sup>218</sup> Interestingly, some of these ester derivatives have shown their therapeutic potentials such as antitumor, plant growth inhibition and antibiotic activities, and can also serve as enhancing additives for cosmetics and pharmaceuticals.<sup>269</sup>

**2.1.5.1. Esterification.** Lipase is a well-known and effective catalyst used for enantioselective esterification reactions.<sup>271</sup> It can be employed for ester synthesis of glucose palmitate from glucose and palm fatty acid distillates. As both substrates are abundant and direct feedstocks derived from agricultural processes, the synthesis of glucose palmitate is a good example of biocatalysis in an application which can contribute to the global bioeconomy. The commercially available immobilized Lipase B from *Candida antarctica* (CALB) was used as a biocatalyst for catalyzing esterification of glucose and palmitic acid at 40 °C in acetone (Figure 24A).<sup>272</sup> After 74 h, the reaction resulted in formation of 6-O-palmitoyl- $\alpha$ -D-glucopyranose (glucose palmitate) with a 76% yield conversion of glucose. This ester and its derivatives are nonionic surfactants which can be employed as ingredients in foods, detergents, and cosmetics.

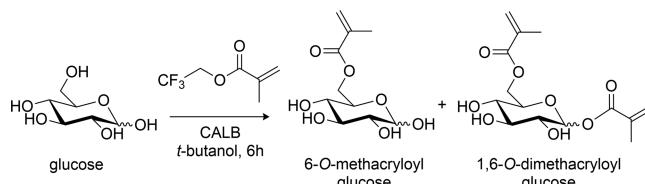
Recently, feruloyl esterases or ferulic acid esterases (FAEs) have been demonstrated to be another promising set of enzymes for esterification and transesterification reactions for the production of sugar esters. FAEs can catalyze direct esterification of natural phenolic acids including various hydroxycinnamic acids such as ferulic, *p*-coumaric, caffeic, and sinapinic acids with sugars in organic–water mixtures.<sup>273</sup> For example, sugar-feruloyl esters such as feruloylated monosaccharides can be catalyzed by FAE from *A. niger*.<sup>274</sup>



**Figure 24.** Esterification of monosaccharides. (A) Using CALB for the synthesis of glucose palmitate. (B) Using FAE for esterification of sugars with ferulic acid. Abbreviations; CALB: Lipase B from *Candida antarctica*; FAE: feruloyl esterase.

The enzyme can use various monomeric sugars including arabinose, glucose, and galactose to condense with ferulic acid in acetate buffer containing 5% dimethyl sulfoxide (DMSO) at pH 4.0 and 50 °C (Figure 24B). Among the tested sugars evaluated by comparing product peak areas in HPLC, glucose was the best substrate for generating feruloylated esters. The produced feruloyl esters display various important biological functions such as antitumor, antimicrobial, antiviral and anti-inflammatory activities.<sup>275</sup>

**2.1.5.2. Transesterification.** Sugars can also be modified to form ester derivatives using transesterification catalyzed by lipase. Recently, an enzymatic-catalyzed carbohydrate (meth)-acrylation of glucose could be achieved using CALB and 2,2,2-trifluoroethyl methacrylate (TFMA) as a methacrylic donor to covalently link to glucose in *t*-butanol, resulting in a mixture of mono- and dimethacrylated products at a ratio of 87:13 (mol %), respectively (Figure 25).<sup>276</sup> The byproduct of trans-

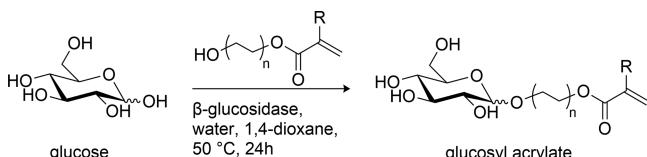


**Figure 25.** Methacrylation of glucose catalyzed by CALB.

esterification using TFMA was 2,2,2-trifluoroethanol which can be easily removed from the reaction mixture. The total yield of bioconversion from glucose to methacrylated products was only 34%. The low yield was probably due to the poor solubility of sugars in organic solvents. Therefore, this synthetic method may be more compatible with alkylated derivatives such as methyl glycosides. The produced methacrylated sugar monomers can be used to produce polymers with hydrophilic compatibility without backbone degradation.<sup>277</sup> These characteristics are useful for creating polymers with biodegradability, biocompatibility and also specific biological interactions.

**2.1.5.3. Glycosidation.** As the synthesis of glucosyl-acrylate monomers is not effective due to the low compatibility of these sugars and their products with organic solvents, a recent approach was established using  $\beta$ -glucosidase as an alternative enzyme to synthesize glucoside-acrylate in an aqueous solution.<sup>278</sup> The reaction components were investigated to

enhance the solubility of glucose and maintain glucosidase activity. The optimized conditions were obtained by controlling the ratio of acrylate and water. Additionally, the presence of water-miscible cosolvents is essential for reaction homogeneity, which can be enhanced by adding 1,4-dioxane. The optimum condition obtained used a water/acrylate/1,4-dioxane ratio of 13:80:7% (v/v), and the reaction was performed at 50 °C to obtain glycosidation of glucose with various types of acrylate monomers using  $\beta$ -glucosidase (Figure 26). The highest yield under these conditions was



**Figure 26.** Glycosidation of acrylates catalyzed by  $\beta$ -glucosidase. R = H,  $\text{CH}_3$ ; n = 1, 2.

50% (w/v, based on glucose amount) after 24 h. The glucosyl-acrylate monomers were successfully copolymerized with various vinyl monomers in DMF and water. The produced polymers can be used for drug carrier systems or as nonionic polymeric surfactants.

**2.1.6. Critical Evaluation of Production of Sugar-Derived Compounds.** Based on the discussion above regarding the synthesis of five types of sugar derivatives from lignocellulosic sugars, their bioproduction can be achieved via enzymatic and microbial conversion. While employment of enzymatic reactions is advantageous for selective transformation and facile downstream processing, the cost of production is still very high and (to the best of our knowledge) not yet feasible for commercial scale production. On the other hand, production via microbial fermentation, has been successfully employed for production of special sugars on an industrial scale despite requiring methods for circumventing competitive pathways of native sugar metabolism in the host cells. With more knowledge of enzyme catalysis and cellular metabolism, these two approaches can be merged via metabolic engineering in which the engineered cells can be optimized in order to increase the product titer in the future. With increasing efforts toward development of a circular economy,<sup>278,279</sup> sugars in the hydrolysates obtained from agricultural and agro-industrial residues will continue to receive increasing attention for their use as resources for starting reagents.

Production of sugar derivatives such as sugar alcohols and sugar acids has been carried out in various industries. Manufacturing of gluconic acid has been commercially produced by fermentation using *A. niger* to oxidize glucose directly.<sup>236</sup> Because production of gluconic acid from glucose using chemical methods generates nonspecific oxidation, the microbial conversion is thus dominant in this industrial sector.<sup>280</sup> Sugar acids such as gluconic acid have been produced commercially with its current production estimated as 80–100 000 tonnes annually.<sup>281</sup> For xylitol, a sugar alcohol in which its market size has continuously increased due to expansion in health and wellness sectors,<sup>282</sup> its current manufacturing is mainly via chemical catalytic hydrogenation of xylose isolated from hemicellulosic hydrolysates.<sup>283</sup> As the cost of purification and crystallization processes of xylose from hemicellulose hydrolysates is high,<sup>284</sup> biotechnological pro-

cesses which do not require xylose purification have received increased attention. The largest scale of xylitol production from agricultural residues by means of biotransformation has been successfully conducted at a pilot scale level by Wang and co-workers using coculture of yeast and bacteria to convert the xylose in waste xylose mother liquor (WXML) to xylitol in a 30 000 L bioreactor.<sup>285</sup> The process achieved 75% yield (xylitol/xylose), demonstrating its feasibility in industrial applications. For microbial production of other sugar derivatives from lignocellulosic biomass, their processes suffer from toxicity of feedstock hydrolysates and expensive product separation from the fermentation broth.<sup>283,286</sup> In our opinion, more development in lignocellulosic waste valorization and improved efficiency of biotransformation processes will allow real implementation of microbial bioproduction of special sugars to support sustainability and address environmental issues.

Although from the perspective of value addition, rare sugar synthesis can be considered to provide the largest economic gain from lignocellulosic materials, their production scale is mostly limited to lab demonstrations. Major obstacles for industrial implementation include the expensive cost of using purified enzymes and the additional downstream processes. However, rare sugar production via enzymatic routes holds its own promise because most of these products are high in price and require low market volume. Rare sugars are important components of nucleoside analogues, which are potent antiviral and anticancer drugs produced by pharmaceutical companies.<sup>287</sup> Rare sugars, especially L-enantiomers are highly important for pharmaceuticals because they have better metabolic stability and more favorable toxicological profiles.<sup>171,287</sup> In recent years, various examples have demonstrated high efficiencies of bioconversion, which would reduce the costs of downstream processing. For example, L-ribulose can be derived from abundantly cheap L-arabinose (10 000-fold increase in commercial value) via one-pot enzymatic cascades with 100% conversion,<sup>163</sup> while D-tagatose (\$191/g) can be produced from D-galactose (\$0.15/g) with excellent yield at (85% conversion).<sup>180</sup> D-Galactose can also be converted to D-talose, enabling production with an approximately 8300-fold increase in commercial value for the product relative to the starting material.<sup>153</sup> With more developments emerging in enzyme catalysis and engineering to increase enzyme robustness and efficiency, biocatalysts may be reused to reduce cost. Further improvement of process engineering especially via flow systems allow the unused substrates to be reused to further reduce the process costs, promoting development in this area to achieve economic feasibility required by industrial sectors.

## 2.2. Phenolic Acid-Derived Products

Lignin is a heterogeneous biopolymer consisting of aromatic compounds. Currently, the most valuable components of lignocellulosic biomass feedstock are carbohydrate fractions including cellulose and hemicellulose (previous sections) which make up the major part of the feedstock, while lignin is only a minor part (about 15–30%) and is thus considered as a byproduct of the industry.<sup>288</sup> The main building block units of lignin are *p*-hydroxyphenyl, guaiacyl, and syringyl phenolic structures. Therefore, phenolic acids (PAs) are among the major compounds derived from lignin fractionation and depolymerization.<sup>289–292</sup> Several fractionation methods with different degrees of delignification, such as ammonia-based

**Table 5.** Bioconversion of Phenolic Acids<sup>a</sup>

substrates	products	(bio)catalysts	applications	ref
gallic acid	alkyl gallate	-immobilized lipase from <i>Staphylococcus xylosus</i> -thermophilic lipase from <i>Bacillus licheniformis</i> SCD11501	food additive	297 298
ferulic acid	vanillin	whole-cell <i>E. coli</i> harboring phenolic acid decarboxylase (PAD) and aromatic dioxygenase (ArDO)	food additive	299
creosol	vanillin	vanillyl-alcohol oxidase (VAO)	food additive	300
ferulic acid	4-vinylguaiacol	-whole-cell <i>E. coli</i> harboring ferulic acid decarboxylase from <i>Bacillus pumilus</i> -immobilized cells of <i>Bacillus licheniformis</i> DLF-17056 - <i>E. coli</i> overexpressing PAD from <i>B. licheniformis</i> (BiPAD) on cell surface -immobilized PAD from <i>Bacillus atrophaeus</i> (BaPAD) on zeolite	food additive and monomer for polymer industries	301 302 303 304
	4-ethylguaiacol	-Pd/C reduction -vinylphenol reductase	food additive and flavoring agents	305 306
<i>p</i> -coumaric acid	4-vinylphenol	- <i>E. coli</i> overexpressing PAD from <i>B. licheniformis</i> (BiPAD) on cell surface	monomer for polymer industries	303
caffeic acid	CAPE	lipase CALB	pharmaceutical additives	307,308
methyl caffete	CAPE	whole-cell <i>Aspergillus niger</i> EXF 4321 harboring lipase	pharmaceutical additives	309
vanillin	vanillylamine	lysate or whole-cell CV2025 $\omega$ -transaminase ( $\omega$ -TA) from <i>Chromobacterium violaceum</i> DSM3019	precursor for synthesis of pharmaceutical additives	310
<i>p</i> -coumaric acid	3,4,5-THCA	<i>p</i> -hydroxyphenylacetate (HPA) 3-hydroxylase (C <sub>2</sub> )	pharmaceutical and cosmetic additives	311,312

<sup>a</sup>Abbreviations; 3,4,5-THCA: 3,4,5-trihydroxycinnamic acid; CAPE: Caffeic acid phenethyl ester.

fractionation, reductive catalytic fractionation, and form-aldehyde-assisted fractionation are available to remove lignin from lignocellulosic biomass.<sup>290</sup> For conversion of lignin to PAs, several depolymerization methods including reductive, oxidative, and acid/base-catalyzed depolymerization can be employed.<sup>290</sup> It is important to note that these reported methods normally involve the use of organic solvents and noble metal or alkaline metal catalysts. Recently, a milder and more sustainable, noncatalytic oxidative depolymerization reaction of lignin using molecular oxygen with perfluorodecalin has been reported.<sup>293</sup> Up to about 10-fold of PAs monomer could be achieved in a short time by this method compared to conventional methods under similar conditions.<sup>293</sup>

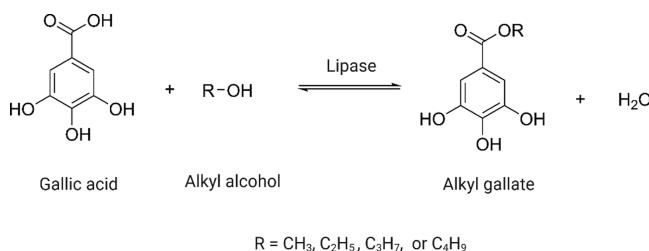
Therefore, PAs are abundant and renewable feedstocks which can provide unique natural properties for further construction of valuable chemicals. Moreover, a high amount of PAs cannot be excreted to freshwater resources without proper treatment because it would create toxicity to animals.<sup>294–296</sup> Therefore, efforts to convert these low-value PAs into high-value products are an active research field. In recent decades, attention has been paid to upgrade low-value lignin to more valuable compounds. Most of the investigations have focused on using enzymatic reactions either in cell-free or whole-cell form. Our review in this section focuses on biotransformation using enzymes to convert abundant and low-value PAs into high-value compounds, i.e., food additives, active pharmaceutical ingredients, monomers for materials, and specialty chemicals (Table 5). More comprehensive reviews of bioconversion of phenolic acids to valuable chemicals can be found in other recently published reviews.<sup>59,61,290,291</sup>

**2.2.1. Food Additives.** *2.2.1.1. Alkyl Gallates.* Gallic acid or 3,4,5-trihydroxybenzoic acid is an important PA because of its biological potential, including antifungal, antimicrobial, and anticancer activities.<sup>313–315</sup> Gallic acid can be extracted from food wastes or waste from agri-food industries. Agri-food industries have been one of the major contributors to the generation of organic waste and a source of environmental pollution.<sup>316,317</sup> Various types of food and drink processing industries are also important sources of PAs such as gallic

acid.<sup>318–321</sup> For example, 132 mg of gallic acid equivalents (GAE) can be extracted per gram of saffron processing waste dry mass.<sup>322</sup> A GAE of 17.75 mg/g can be extracted from spent ground coffee,<sup>323</sup> while 29.56 mg of GAE can be extracted from 100 g of potato peel.<sup>324</sup> The most abundant source of gallic acid—based on currently available data is tea.<sup>325</sup>

Applications of most PAs such as gallic acid are limited by their solubility and stability.<sup>326</sup> Esterification of PAs is a general means to make the compounds soluble in hydrophobic or lipophilic media.<sup>326</sup> Esterification of PAs can also be employed to broaden the scope of applications and reactivities.<sup>326</sup> For gallic acid, various types of alkyl gallates including propyl gallate, octyl gallate, and dodecyl gallate are widely used as food additives especially in food containing lipid to prevent lipid oxidation.<sup>327</sup> This application leverages upon the antioxidative activity of gallic acid and solubility of the ester compound in lipid environments.<sup>328–330</sup> Recently, epigallocatechin-3-gallate has been found to stimulate the growth of beneficial bacteria and inhibit the growth of pathogenic bacteria in human intestine, which may boost human health due to increased antioxidant, antibacterial, and antiviral activities.<sup>331,332</sup> Although chemical synthesis of phenolic esters can be carried out using acidic or basic catalysis, the use of harsh and hazardous chemicals is generally not preferred by food and cosmetic industries. Therefore, enzymatic synthesis of phenolic esters offers a much more elegant way for the preparation of alkyl gallate.

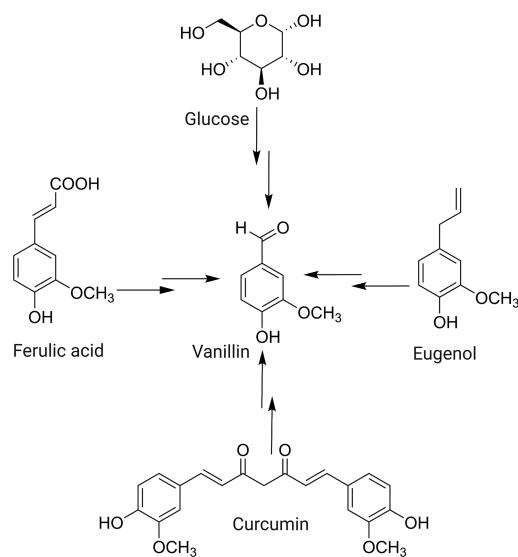
Esterification of gallic acid and propanol can be catalyzed by the immobilized *Staphylococcus xylosus* lipase to synthesize propyl gallate in a solvent-free system.<sup>297</sup> About a 90% conversion could be obtained after 8 h of incubation using a 160 molar ratio of propanol/gallic acid with 400 U of the immobilized lipase at 52 °C.<sup>297</sup> Various alkyl gallates including methyl gallate, ethyl gallate, propyl gallate, and butyl gallate could also be synthesized using a thermophilic lipase from *Bacillus licheniformis* SCD11501 (Figure 27).<sup>298</sup> After 10-h incubation at 55 °C, about 58%, 67%, 72%, and 64% yields of methyl gallate, ethyl gallate, propyl gallate, and butyl gallate could be obtained, respectively.<sup>298</sup>



**Figure 27.** Esterification reaction of gallic acid and alkyl alcohol (methanol, ethanol, propanol, or butanol) catalyzed by lipase to produce alkyl gallate.

**2.2.1.2. Vanillin.** Vanilla is an important fragrance for the food and perfume industries, which has been extensively used worldwide. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the key component in vanilla. Natural vanillin is extracted from orchid vanilla pods in which the extraction process is costly.<sup>333</sup> About 500 kg of vanilla pod is needed for producing around 1 kg of vanillin. Due to its high demand, natural vanillin is very expensive and the cost varies between \$1200–\$4000/kg, while chemically synthesized vanillin only costs \$15/kg.<sup>334</sup> As the annual global market demand of vanillin is high (about 15 000 000 kg in 2010) and the worldwide supply of vanilla pods is limited (only 2 000 000 kg/year) because vanilla pods grow slowly, biobased production of vanillin using microbial fermentation offers an alternative way to supply natural and biobased vanillin for food and fragrance industries.

Vanillin can be biologically synthesized from various substrates including glucose, eugenol, curcumin, and ferulic acid (Figure 28). Ferulic acid is one of the key components in

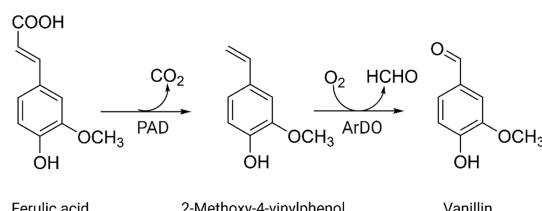


**Figure 28.** Different routes of vanillin synthesis from biobased chemicals.

lignin of lignocellulose. It can be found in agricultural byproducts including oat hulls, corn bran, corn fibers, sugar beet pulp, and rice.<sup>335–338</sup> These feedstocks offer great potential of using a circular economy model to turn low-value biobased waste into valuable biochemicals such as vanillin. For example, the world production of maize in 2017 was 6527 million tonnes,<sup>339</sup> of which its production in Thailand alone was 29 million tonnes.<sup>339</sup> In many developing countries, especially in Asia, corn stover or husk is often

processed through burning which is the cheapest way to process biomass but it causes PM2.5 air pollution problems in the region.<sup>340</sup> If this biomass can be used as a source of ferulic acid for the synthesis of vanillin, the problem may be alleviated because the economic value would support proper biomass processing and valorization rather than burning.

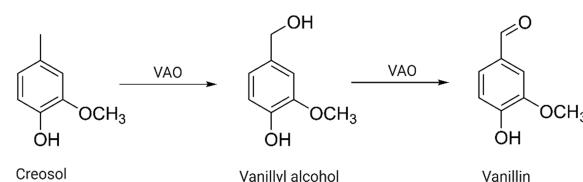
Several biobased technologies for the synthesis of vanillin including enzyme and whole-cell conversion via metabolic engineering are available. One of the most efficient biosynthetic methods recently reported is the use of *E. coli* harboring phenolic acid decarboxylase (PAD) and aromatic dioxygenase (ArDO) in which a 90% yield of vanillin from ferulic acid can be obtained after 2-h incubation at 50 °C and pH 9.5 in a chloroform/water system (Figure 29).<sup>299</sup> The



**Figure 29.** Synthesis of vanillin using phenolic acid decarboxylase (PAD) and aromatic dioxygenase (ArDO) in the metabolically engineered *E. coli*.

bioconversion in a 5-L bioreactor gave 13.3 g/L of vanillin within 18 h, and the process can be carried out without extra addition of cofactors.<sup>299</sup> The use of the chloroform/water system can simultaneously separate the product out from a substrate mixture. Therefore, production and purification can be done in one-pot.<sup>299</sup> However, chloroform is generally not acceptable as a solvent for food industries. In our opinion, further development in process engineering to carry out the reaction in simple alcohols such as ethanol would make the process attractive for food industries.

Besides the synthesis of vanillin from ferulic acid, vanillin can also be synthesized from vanillylamine or creosol.<sup>341</sup> However, vanillylamine is not a widely available substrate, while creosol from creosote could be obtained from oak<sup>342</sup> and coal tar.<sup>343</sup> The synthesis of vanillin from creosol could be carried out at ambient conditions using vanillyl-alcohol oxidase (VAO) (Figure 30).<sup>300</sup> However, the wild-type VAO has poor



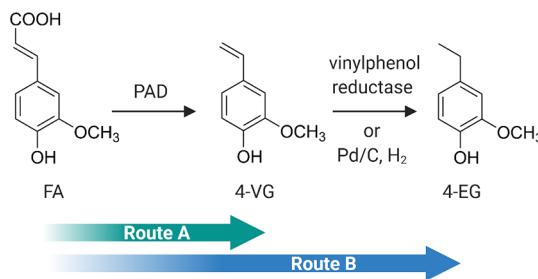
**Figure 30.** Synthesis of vanillin from creosol using vanillyl-alcohol oxidase (VAO). Two-step conversion of creosol to vanillin. Creosol is converted to vanillyl alcohol before being converted to vanillin.

activity at high substrate concentrations.<sup>300</sup> Protein engineering using random mutagenesis was employed to improve VAO activity toward creosol. Four single-site mutation variants including I238T, F454Y, E502G, and T505S showed up to a 40-fold increase in catalytic efficiency ( $k_{cat}/K_m$ ) compared to the wild-type enzyme.<sup>344</sup> Although the VAO catalytic efficiency was improved, the overall efficiency was still low. The best variant, T505S, has a  $k_{cat}/K_m$  value of about 10  $\mu\text{M}^{-1} \text{s}^{-1}$ . In

our opinion, extensive protein engineering is required to further develop VAO for this biocatalytic reaction.

**2.2.1.3. Vinylphenols.** 4-Vinylguaiacol (4-VG) or 2-methoxy-4-vinylphenol is a vinylphenol that is widely used in the food and polymer industries as flavoring agent in several beverages<sup>345</sup> and as a monomer for biodegradable polymers.<sup>346</sup> 4-VG can be produced from ferulic acid (FA) derived from lignin as discussed above in the production of vanillin. The price of 4-VG is about 30–40 times higher than the price of FA.<sup>347,348</sup> Although chemical synthesis of 4-VG from FA can be achieved using base-catalyzed decarboxylation of FA via microwave heating,<sup>349</sup> biobased methods for synthesis of 4-VG are under demand because it can be more economically and environmentally viable.

Several microorganisms have been shown to be capable of converting ferulic acid to 4-VG or other high value-added compounds. Recently, *Enterobacter* spp. including *Enterobacter soli* and *Enterobacter aerogenes* have been used to convert ferulic acid to 4-VG with 100% and 84% yields, equivalent to ~5.15 mM of product (1000 ppm) at pH 5.0, respectively.<sup>345</sup> Since 4-VG is insoluble in water, a two-phase bioreactor was employed in the biocatalysis of *E. coli* harboring ferulic acid decarboxylase from *Bacillus pumilus*. The system could produce 13.8 g of 4-VG from 25 g of ferulic acid in a two-phase bioreactor using octane as cosolvent at 310 °C, pH 6.8 (Figure 31 (route



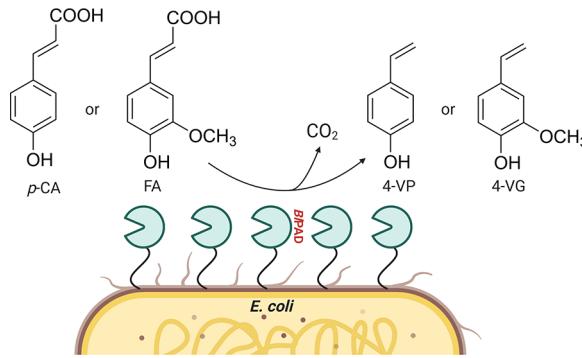
**Figure 31.** Synthesis of 4-vinylguaiacol (4-VG) and 4-ethylguaiacol (4-EG) from ferulic acid (FA). Abbreviations; PAD: phenolic acid decarboxylase; Pd/C: palladium on charcoal.

A)).<sup>301</sup> Due to the high cost of refined ferulic acid, crude ferulic acid from extracted rice bran was also tested as a substrate for the synthesis of 4-VG by alginate-immobilized cells of *B. licheniformis* DLF-17056 to reduce the overall process cost. 4-VG (0.76 g/L) could be produced from 1.0 g/L ferulic acid within 24 h of biotransformation at 40 °C pH 7.0.<sup>302</sup> Moreover, the immobilized *B. licheniformis* DLF-17056 can be reused up to 8 times with more than 60% of the initial activity retained. This process system is efficient and gives good economic feasibility.

In addition, 4-VG can be further used for the synthesis of 4-ethylguaiacol (4-EG), which is an additive in various foods, including soy sauce, Scotch whiskey, and Arabica coffee beans.<sup>303</sup> The gram-scale synthesis of 4-EG can be carried out via palladium on charcoal (Pd/C) reduction (Figure 31 (route B)).<sup>305</sup> Although this method produces a good yield of product, the high energy input and complexity of the method hinder its usage in real applications. To make the synthesis of 4-EG greener, enzymatic reduction instead of charcoal (Pd/C) reduction has been explored. The charcoal (Pd/C) reduction step can be replaced by the reaction of vinylphenol reductase to convert 4-VG to 4-EG.<sup>306,350–352</sup> However, the current usage of vinylphenol reductase is still limited at low substrate

concentrations. Future development using enzyme and process engineering are required for improving the synthesis of vinylphenols by vinylphenol reductase.

The synthesis of 4-VG or 4-vinylphenol (4-VP) from FA or *p*-coumaric acid (*p*-CA) using CA has been carried out using *E. coli* overexpressing phenolic acid decarboxylase (*BIPAD*) from *B. licheniformis* on the cell surface by fusing *BIPAD* on the outer membrane of cell and exposing *BIPAD* to the outer cellular environment (Figure 32).<sup>303</sup> 4-VG or 4-VP with 94.9%



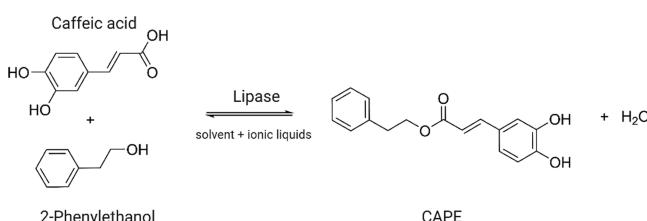
**Figure 32.** Synthesis of 4-vinylguaiacol (4-VG) or 4-vinylphenol (4-VP) from ferulic acid (FA) or *p*-coumaric acid (*p*-CA) using *E. coli* harboring phenolic acid decarboxylase from *B. licheniformis* (*BIPAD*) on the cell surface.

or 99.8% yield could be obtained from the reaction of intracellular *BIPAD* when 300 mM of FA or *p*-CA was solubilized in the toluene/ $\text{Na}_2\text{HPO}_4$ -citric acid buffer system.<sup>303</sup> Using the cell harboring cell surface displayed *BIPAD*, 4-VP could be obtained with 72.6% or 80.4% yield under the same conditions (Figure 32).<sup>303</sup> While only 11% of the activity of intracellular *BIPAD* remained after reusing the cells for 3 cycles, the cell surface displaying *BIPAD* could be reused up to 7 cycles with 63% of the activity maintained.<sup>303</sup> However, the yield of 4-VP catalyzed by extracellular *BIPAD* is slightly lower than intracellular *BIPAD* although the extracellular *BIPAD* is more stable and reusable. Recently, immobilized phenolic acid decarboxylase from *Bacillus atrophaeus* (*BaPAD*) via a peptide linker on zeolite could remarkably produce 4-VG about 295 g/L from FA after a 13-h reaction under ambient conditions at 30 °C, pH 6.5.<sup>304</sup> Moreover, this biocatalyst is recyclable because 73% of its original activity remained after 10 cycles. This method should be useful for large-scale bioconversion of PAs and other related compounds.

**2.2.2. Pharmaceutical Additives.** **2.2.2.1. Caffeic Acid Phenethyl Ester (CAPE).** Caffeic acid (3,4-dihydroxycinnamic) is one of the hydroxycinnamate metabolites widely distributed in plant tissues.<sup>353</sup> Caffeic acid has high antioxidant activity and is widely used in medicines and cosmetics.<sup>353</sup> Therefore, the demand for caffeic acid in the bioindustry is increasing. Caffeic acid can be derived from various types of plants such as potatoes. Potatoes are one of the most cultivated crops globally, with an annual production of around 325 million tonnes.<sup>354</sup> It was predicted to reach production of 9 billion tonnes by 2050.<sup>354</sup> Potato peel, which is the major waste from potato processing can be a feedstock for caffeic acid production because 332.6 mg of caffeic acid can be extracted from 100 g of dry weight of potato peel.<sup>355</sup>

Although caffeic acid has a wide range of biological activities, its application is limited due to its low solubility and stability in various solvent systems.<sup>308</sup> Therefore, modification of caffeic acid derivatives is important for improving their solubility and still maintaining excellent biological activities. One example of a caffeic acid derivative, caffeic acid phenethyl ester (CAPE), is well-known for its broad range of activities including anticancer, antiviral, and antioxidant activities.<sup>356–360</sup> CAPE is an active ingredient in a wide range of cosmetic and pharmaceutical products and can be naturally found in honeybee propolis. However, extraction of CAPE from natural sources is insufficient for its high demand because the process is costly and time-consuming and results in a low yield of product and low purity.<sup>361</sup>

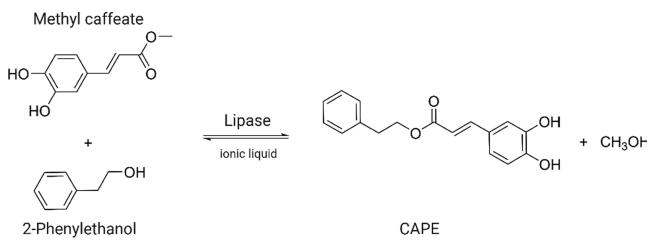
Although chemical synthesis of CAPE can be carried out via Wittig reaction<sup>362,363</sup> and malonic acid monoester methods,<sup>364</sup> chemically synthesized CAPE is not well accepted as pharmaceutical additives. The development of biocatalysis to synthesize CAPE is necessary. In recent years, attempts to synthesize CAPE from caffeic acid and 2-phenylethanol in solvent and ionic liquids systems have been carried out (Figure 33).<sup>307,308</sup> Most of the methods use the lipase CALB to



**Figure 33.** Esterification of caffeic acid and 2-phenylethanol catalyzed by lipase. Abbreviation; CAPE: caffeic acid phenethyl ester.

produce CAPE.<sup>365–367</sup> CALB can catalyze esterification of caffeic acid and 2-phenylethanol in isooctane at 70 °C to yield nearly 100% conversion within 48 h.<sup>308</sup> Applying ultrasonic power can accelerate the reaction of CALB to give about 93% conversion of caffeic acid within 9.6 h.<sup>307</sup> Despite the good yield of CAPE using these protocols, the procedure is not ready for industrial scale-up because the process is slow and energy consuming. Moreover, the water content of the reaction must be minimized to maintain an excellent yield. Direct esterification of caffeic acid is also difficult because caffeic acid can inhibit lipase activity.<sup>368,369</sup>

The most promising method to produce CAPE is transesterification of methyl caffeate by 2-phenylethanol in ionic liquid using CALB as a biocatalyst (Figure 34).<sup>370</sup> Methyl caffeate can be prepared from caffeic acid and methanol using cation exchange resin at 60 °C for 4 h.<sup>371</sup> The trans-

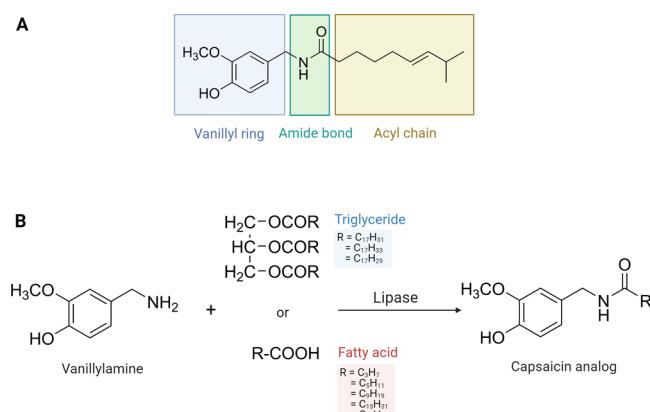


**Figure 34.** Transesterification of methyl caffeate and 2-phenylethanol using lipase or the whole-cell containing lipase as a biocatalyst. Abbreviation; CAPE: caffeic acid phenethyl ester.

esterification results in about 93% yield of CAPE after 2.5 h using CALB in a packed bed microreactor at 60 °C.<sup>370</sup> Moreover, the microreactor can be reused for 20 cycles without reduction of the lipase activity.<sup>370</sup>

Recently, a whole cell-based catalyst was employed for CAPE synthesis. Whole-cell biocatalysis can reduce the cost of enzyme preparation. The halotolerant fungus *A. niger* EXF 4321 harboring lipase was used to synthesize CAPE in 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide medium. At a molar ratio of caffeic acid/2-phenylethanol of 1:20, about an 84% yield of CAPE could be obtained after 12 h at 30 °C.<sup>309</sup> This method is promising for a large scale synthesis because the high yield of CAPE can be achieved at a much lower temperature (30 °C) than other reported methods (60–80 °C).<sup>309</sup> However, this method is still time-consuming and needs further process optimization for scaling up.<sup>368,369</sup>

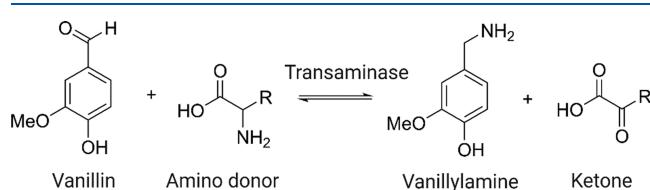
**2.2.2.2. Vanillylamine.** Capsaicin is an active ingredient in chili (Figure 35A) which has a wide range of biological



**Figure 35.** Synthesis of capsaicin analogues. (A) Capsaicin structure. (B) Synthesis of capsaicin analogs via amidation of vanillylamine and free fatty acids or triglycerides catalyzed by lipases.

activities including antioxidative,<sup>372</sup> anti-inflammatory,<sup>373</sup> antiobesity,<sup>374</sup> antimicrobial,<sup>375,376</sup> and anticancer activities.<sup>377–380</sup> Due to its potent activities, capsaicin is attractive for pharmaceutical industries. Although capsaicin can be directly extracted from chili, the pungency of capsaicin limits its use and diminishes its intrinsic value. Therefore, several attempts have been made to chemically or biologically synthesize nonpungent capsaicin analogs to retain its active value and broaden its utilization (Figure 35B).<sup>381–387</sup>

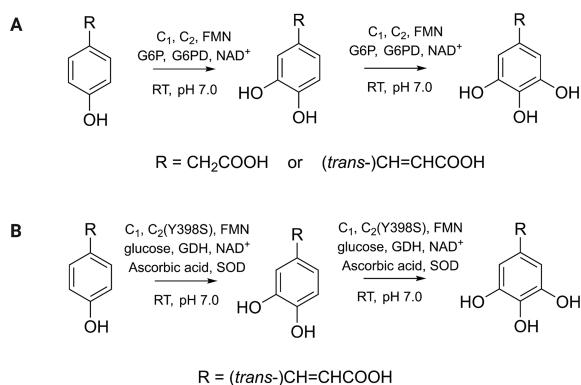
Vanillylamine is a precursor for capsaicin synthesis via amidation catalyzed by lipases (Figure 35B). Vanillylamine can be synthesized via transamination of vanillin and an amino donor (Figure 36). An enzyme that can catalyze vanillin transamination is CV2025  $\omega$ -transaminase ( $\omega$ -TA) from *Chromobacterium violaceum* DSM30191.<sup>310</sup> In this reaction, (*S*)- $\alpha$ -methylbenzylamine was used as an amino donor.<sup>310</sup>



**Figure 36.** Biosynthesis of vanillylamine by transaminase.

Total conversion could be achieved within 25 min when the reaction was carried out with excess amino donor to vanillin (<10 mM) in about 4:1 ratio.<sup>310</sup> Both  $\omega$ -TA lysate and whole-cell forms can be used for the synthesis of vanillylamine.<sup>310</sup> Near total conversion was obtained within 60 min when using the cell lysate, while an 88% (w/w) conversion was obtained when using the whole-cell biocatalyst.<sup>310</sup> Both forms of the biocatalyst were stable over 24 h.<sup>310</sup> Since  $\omega$ -TA is sensitive to substrate inhibition, a fed-batch process to keep a low concentration of vanillin was carried out to produce vanillylamine up to 5.5 g/L.<sup>310</sup>

**2.2.3. Other Specialty Phenolic Acid-Derived Products.** Trihydroxyaromatic acids have potential in therapeutics because they display various properties including antioxidant, anticancer, and anti-inflammatory properties.<sup>388–391</sup> Chemical synthesis of trihydroxyaromatic acids often involves usage of toxic reagents and high temperatures.<sup>392,393</sup> Therefore, an enzymatic reaction for the synthesis of trihydroxyaromatic acids was developed. The oxygenase component ( $C_2$ ) of *p*-hydroxyphenylacetate (HPA) 3-hydroxylase (HPAH) from *Acinetobacter baumannii* which catalyzes the hydroxylation of phenolic compounds under mild conditions was used coupled with associated systems including the  $C_1$  flavin reductase and G6PD to continuously supply reduced flavin, a cosubstrate for the  $C_2$  reaction. Although the catalytic activity of wild-type  $C_2$  for converting the native substrate (4-HPA) to 2-(3,4,5-trihydroxyphenyl)acetic acid (3,4,5-THPA) is reasonable (Figure 37A) with a total turnover number (TTN) of 100 at



**Figure 37.** Synthesis of trihydroxyaromatic acids by *p*-hydroxyphenylacetate (HPA) 3-hydroxylase ( $C_2$ ). (A) Synthesis of 2-(3,4,5-trihydroxyphenyl)acetic acid (3,4,5-THPA) and 3,4,5-trihydroxycinnamic acid (3,4,5-THCA) by  $C_2$ . (B) Synthesis of 3,4,5-THCA from *p*-coumaric acid and caffeic acid extracted from POME by Y398S variant. Abbreviations:  $C_1$ : flavin reductase;  $C_2$ : the oxygenase component of *p*-hydroxyphenylacetate (HPA) 3-hydroxylase (HPAH); FMN: flavin mononucleotide; G6P: glucose-6-phosphate; G6PD: glucose-6-phosphate dehydrogenase; NAD<sup>+</sup>: nicotinamide adenine dinucleotide; GDH: glucose dehydrogenase; SOD: superoxide dismutase.

100 min, and the enzyme is quite stable.<sup>311</sup>  $C_2$  is sluggish in using non-native substrates such as *p*-CA<sup>311</sup> and 4-amino-phenylacetate (4-APA).<sup>394</sup> Therefore, residues around the substrate binding site were engineered to obtain variants which can catalyze the hydroxylation of 4-APA and *p*-CA. The Y398S variant can convert *p*-CA to 3,4,5-trihydroxycinnamic acid (3,4,5-THCA) reasonably well (Figure 37A)<sup>311</sup> and can also be used in a semi-large scale reaction to produce 3,4,5-THCA from *p*-CA and caffeic acid extracted from POME waste

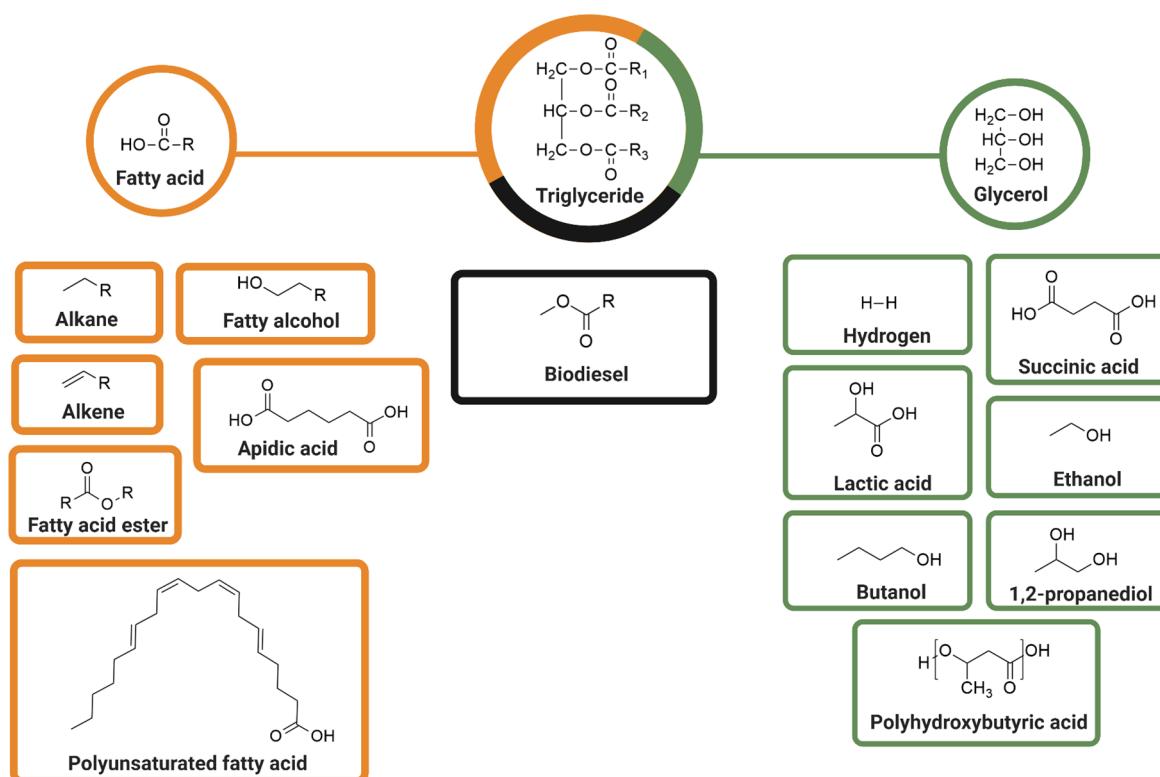
(Figure 37B).<sup>312</sup> The reaction condition of Y398S was further optimized by adding superoxide dismutase (SOD) and ascorbic acid to stabilize the hydroxylated products. Moreover, the NADH regeneration system was later changed from the G6P/G6PD system to glucose/GDH which is more economical. This established protocol should be useful for future development of scale-up processes. For the S146A variant, the enzyme showed better activity toward 4-APA as compared to the wild-type,<sup>394</sup> implying that residues around the binding site of  $C_2$  can be changed to accommodate various types of substrates.

The biotransformation process mentioned above is potentially useful as a sustainable waste refinery process to support a circular economy. However, as the two-component flavin-dependent monooxygenase such as  $C_2$  requires a cognate partner, a  $C_1$  flavin reductase, to supply reduced flavin, both enzymes need to be robust enough for the prolonged bioconversion process. Therefore,  $C_1$  was engineered using computational approaches to obtain two variants, A166L and A58P, which have greater thermostability than the wild-type enzyme.<sup>395</sup> Moreover, these two variants, especially A166L exhibits solvent-tolerant activity toward several solvents including dimethyl sulfoxide (DMSO), methanol, and ethanol. Further optimization of the  $C_2$  system with the engineered  $C_1$ , or by replacing  $C_1$  with a more thermostable flavin reductase (e.g., PheA2 from *Bacillus thermoglucosidasi* A7<sup>396</sup> or Th-Fre from *Streptomyces violaceusniger* strain SPC6<sup>397</sup>) should be useful for further improvement of hydroxylation of PA derivatives to synthesize valuable chemicals in the future.

In addition to its use as starting precursors for hydroxylation, various PAs have just recently been shown to serve as substrates for a side activity of carboxylic acid reductase (CAR) from *Mycobacterium marinum*.<sup>398</sup> CAR could be used to synthesize methyl cinnamate, methyl ferulate, methyl 4-hydroxyphenylacetate, and cinoxate (a commercial sunscreen ester formed from methoxycinnamic acid and 2-ethoxyethanol). Although yields of these compounds are rather low at this point, the esterification catalyzed by CAR can be conducted in aqueous environments, making the system attractive for future applications in metabolic engineering. In addition to CAR, acyltransferase from *Mycobacterium smegmatis* (*MsAcT*) was recently found to catalyze transesterification of benzyl alcohol and vinyl acetate, generating many products similar to the CAR system mentioned above.<sup>399</sup>

**2.2.4. Critical Evaluation of Production of Phenolic Acid-Derived Compounds.** Discussion of the previous sections have shown the significant developments in enzyme-based production of valuable compounds from phenolic acids during the past few decades. Various bioconversions of PAs are robust and feasible under mild and environmentally friendly conditions. Both native and engineered biocatalysts with promising catalytic efficiency and excellent stability were employed in purified, lysate, or immobilized forms as well as in whole-cell harboring or displaying targeted enzymes on the cell surface for PAs bioconversion. Products from PA derivatives such as alkyl gallates, vanillin, CAPE, vinylphenols, vanillylamine, and trihydroxyaromatic acids exhibit outstanding biological activities which are useful for applications in the food, pharmaceutical, cosmetic, and polymer industries.

Several examples of bioconversions gave high conversion percentages in short reaction times with excellent biocatalyst reusability. Yield of CAPE production of 93% can be achieved within 2.5 h and the biocatalyst can be reused for up to 20



**Figure 38.** Overall products which can be derived from triglycerides, fatty acids, and glycerol by enzymatic reactions and metabolic engineering.

**Table 6. Triglyceride, Fatty Acid, and Glycerol-Derived Products and Their Applications**

feedstocks	products	applications	ref
triglyceride	biodiesel	biofuels for transportation	400
fatty acid	alkane	biofuels for transportation, domestic heating and cooking in household activities	88
fatty acid	alkene	biofuels and starting materials for production of lubricants, plastics, detergents, surfactants, and other chemicals	401–404
fatty acid	fatty alcohol	key ingredients in daily products such as detergents, shampoos, body washes, dishwashing liquid, fabric softener, and other cleaning reagents	405
fatty acid	adipic acid	intermediates for production of nylon, resins, adhesives, and lubricants	406
fatty acid	polyunsaturated fatty acid such as arachidonic acid	lubricants, cosmetics, surface coatings, and printing inks	407
fatty acid	fatty acid ester	consumer products, components in drugs, and adhesives	408,409
glycerol	hydrogen	fuel cells for energy source	95
glycerol	succinic acid	consumer products, components in drugs, and adhesives	96
glycerol	lactic acid	preservatives, acidulant, textile, pharmaceutical, and chemicals	97
glycerol	ethanol	biofuel and chemicals	98
glycerol	butanol	biofuel and chemicals	99
glycerol	1,2-propanediol	fibers in polymer industry, e.g., polytrimethylene terephthalate	100
glycerol	PHB	biodegradable plastics	410

cycles.<sup>370</sup> Process engineering is also important for improving efficiency of bioconversion of PAs. Efficiency of CAPE synthesis can be further improved using a packed bed microreactor<sup>370</sup> while the production of vanillin can be improved using a two-phase bioreactor.<sup>299</sup> These processes provide a beneficial impact on product purification cost because PA products could be simultaneously separated into the nonaqueous phase after being produced from reactions in an aqueous environment. Most importantly, it allows scale-up of the bioconversion process. An example of the remarkable achievement of PAs bioconversion is the synthesis of 4-VG, in which about 300 g/L of the product could be obtained.<sup>304</sup> These accomplishments are useful guidelines for further development of PA bioconversion.

In our opinions, development in this topic lacks behind research related to usage of real feedstocks such as lignocellulosic biomass or waste. Most of the published work has used purified PA substrates because utilization of biomass waste for PA bioconversion is usually hampered by the presence of other compounds which can be inhibitors of the employed enzymes. Furthermore, variable PA concentrations make it difficult to control the production yield. However, usage of real waste can be found for trihydroxyaromatic acids synthesis from POME<sup>312</sup> and 4-VG synthesis from crude FA.<sup>302</sup> More development toward the use of biomass or biowastes possibly by practical and sustainable pretreatment processes would allow the process to achieve a fully sustainable circular economy model.

### 3. PRODUCTION OF TRIGLYCERIDE-, FATTY ACID-, AND GLYCEROL-DERIVED PRODUCTS

Triglyceride is a major component in oil and fat and its hydrolysis yields fatty acid and glycerol. Therefore, triglyceride, fatty acid, and glycerol are cheap and abundant feedstocks which can be used for synthesizing fuels, specialty chemicals, and materials. Triglyceride and its hydrolysis products can be derived from household and industrial wastes. An overview of valuable compounds that can currently be produced from triglyceride, fatty acid, and glycerol by enzymatic reactions and metabolic engineering pathways are shown in Figure 38 and Table 6.

Table 6 summarizes compounds which can be synthesized from triglyceride, fatty acid, and glycerol. However, not all of these compounds can be produced on a large scale. We therefore have selected to discuss in detail only the reactions and pathways which have the possibility for scale up and such that their use may contribute to lessening our dependence on fossil fuels. For example, reactions to convert fatty acids and fatty acid derivatives into alkanes, alkenes, and fatty alcohols are discussed because these technologies can contribute to the future production of biofuels for transportation and starting materials for various industries. For glycerol, the bioconversion leading to the production of hydrogen, succinic acid, 1,2-propanediol, and 1,3-propanediol are discussed.

#### 3.1. Triglyceride-Derived Products

The content in this section focuses on the enzymatic reactions that can convert triglycerides into products such as biodiesel. Biodiesel properties and combustion conditions can affect the performance of vehicles and dictate their impact on the environment. In addition to CO<sub>2</sub>, ignition of biodiesel can emit CO, CO<sub>2</sub>, sulfur oxides (SO<sub>x</sub>), and nitrogen oxides (NO<sub>x</sub>).<sup>411</sup> It was found that the nature of the fatty acid components in triglycerides can affect the quality of combustion because the chain length of the fatty acids determines the boiling point.<sup>412</sup> Increasing long-chain fatty acid content in biodiesel can reduce NO<sub>x</sub> emissions.<sup>411</sup> Therefore, cooking oil waste, which contains long-chain fatty acids, has been of great interest for biofuel production.

**3.1.1. Biodiesel.** Fatty acid alkyl ester or biodiesel is one of the most important biofuels and is regarded as a clean energy alternative to petroleum-derived diesel. However, advantages and disadvantages of biodiesel utilization are still debatable. Biodiesel contains low sulfur and aromatic contents which results in lower levels of PM2.5 pollutants than diesel.<sup>413</sup> Biodiesel also has high combustion efficiency and high cetane number.<sup>414</sup> In terms of safety, it has a high flash point (~150 °C) which is higher than that of diesel (~65 °C), making it safer for transportation.<sup>415</sup> Normally, biodiesel is used in a pure form or mixed with petroleum-based diesel at a certain ratio to reduce production costs. Generally, the partial supplementation of diesel such as in B5 (5% biodiesel and 95% diesel), B10 (10% biodiesel and 90% diesel) and B20 (20% biodiesel and 80% diesel) diesel is widely used in the EU, U.S.A. and Southeast Asia.<sup>416</sup> However, biodiesel also has drawbacks such as it causes more filter clogging in an engine than diesel due to its high moisture content.<sup>417</sup> The increased demand for feedstocks (oils and fats) can lead to deforestation for palm cultivation which in turn affects CO<sub>2</sub> balance in rainforest areas. As mentioned in sections 1.3.2 and 1.3.3, a large amount of cooking oils from food wastes (around 1 million tonnes per year in the EU, 1.5 million tonnes per year in the US and 2–3

million tonnes per year in China<sup>418</sup>) needs to be disposed of. Conversion of cooking oil waste into biodiesel (total demand worldwide of 16.2 million tonnes per year) can help decrease resource consumption and CO<sub>2</sub> emission.

Biodiesel can be produced from triglyceride and alcohol using chemical catalysts under alkaline or acid conditions or using enzymes. Generally, the process requires variation of several factors such as alcohol molar ratio, free fatty acid composition, temperature, reaction time and catalyst type.<sup>419</sup> The majority of commercial processes producing biodiesel use alkaline compounds, such as sodium hydroxide or potassium hydroxide as catalysts due to their inexpensive cost.<sup>420,421</sup> However, the alkaline process can form soap as a byproduct which needs to be removed by further purification steps.<sup>422</sup> For acid catalysts, although no soap is formed in the reaction, the condition requires high temperature and high substrate molar ratios. Compared to a methanol/vegetable oil ratio of 3:1 required for alkaline conditions, the acid catalyst requires a methanol/vegetable oil ratio of 30:1 due to its slow rate and low yield.<sup>422</sup> Therefore, enzymatic reactions offer attractive routes for biodiesel production because the product can be produced under mild conditions and the overall process does not generate toxic waste for environment.<sup>422,423</sup> Many enzymes have been reported for their abilities to catalyze biodiesel production. The section below summarizes the key enzymatic reactions that can catalyze efficient transesterification.

##### 3.1.1.1. Key Enzymatic Reactions for Biodiesel Production.

Lipases are enzymes which can catalyze transesterification of triglyceride and alcohol (methanol or ethanol) to produce fatty acid alkyl ester or biodiesel (Figure 39). This enzymatic

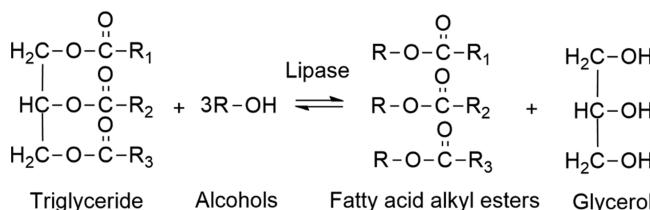


Figure 39. Synthesis of biodiesel or fatty acid alkyl esters from triglyceride and alcohols by lipase.

transesterification consists of two steps which are hydrolysis of an ester bond and esterification for alcohol joining.<sup>424</sup> Lipases are present in many organisms, e.g., plants (papaya latex and castor seed lipases), animals (pig and human pancreatic lipases), bacteria, fungi, and yeasts.<sup>425</sup> Lipases have a wide substrate utilization range. Many highly efficient lipases are commercially available such as CALB (immobilized lipase originally from *Candida antarctica*), Lipozyme RM IM (immobilized lipase originally from *Rhizomucor miehei*), and Lipozyme TL IM (immobilized lipase originally from *Thermomyces lanuginosus*). Lipases are generally stable in diverse media such as aqueous, organic solvents, ionic liquids, supercritical fluids, and deep eutectic solvents, making the enzymes suitable for a wider range of applications in industries.<sup>426</sup> Among them, CALB which is immobilized on acrylic resin is known for its high stability and activity.<sup>427</sup> The immobilization scaffold facilitates recovery and reuse of biocatalysts which also reduces the cost and often also increases enzyme thermostability at high temperature.<sup>428–432</sup>

Many reports have demonstrated the use of CALB as a biocatalyst in biodiesel production. Although most lipases

catalyze hydrolysis at a specific position of glycerol, such as Lipozyme IM which has regio-specificity at the 1- and 3-positions of glycerol, CALB is a nonspecific lipase which can catalyze ester bond cleavage and join at any positions.<sup>425</sup> However, lipase can be inactivated by a high concentration of methanol. This problem can be overcome by adding methanol stepwise in the transesterification using CALB, resulting in a process which gained 90% yield of fatty acid methyl ester (FAME) produced from waste oil.<sup>433</sup> The enzyme used in the process was stable for 100 days and could be reused for 50 times without activity loss. Although the process was shown to be inhibited by glycerol (byproduct from the reaction) possibly by limiting the mass transfer of the process, a continuous removal of glycerol can be employed to alleviate the problem and control the production of biodiesels.<sup>434,435</sup> Therefore, the transesterification of soybean oils by ethanol to produce biodiesel can be achieved using CALB to yield 100% ethyl esters at 25 °C.<sup>436,437</sup>

**3.1.1.2. Microbial Transformation for Biodiesel Production.** The cost of lipase for biodiesel production is \$0.14 per kg ester product, while the cost of using NaOH is \$0.006, which is significantly lower than the cost of using enzymes. Thus, lipase immobilization was introduced to allow for enzyme reuse and, thus, cost reduction.<sup>438</sup> It was reported that the preparation of CALB on macroporous polypropylene (Accurel MP1000) would cost around \$0.06 per lipase activity unit in which 1 enzyme molecule can convert 1 μmol substrate (olein fatty acid) per minute. This immobilized CALB can catalyze 80% conversion yield and can be reused for 5 cycles (10 h per cycles). This is equivalent to a cost of around \$0.09 per 1 kg product which is cheaper than the cost of using free lipase.<sup>439</sup> Although a very high yield for biodiesel production using immobilized lipase as a biocatalyst can be achieved, many steps are required to prepare these immobilized enzymes including extraction, purification and immobilization.<sup>440,441</sup>

The approach of using lipases in whole-cell biocatalysts was explored as an alternative method to reduce operational cost for preparing biocatalysts. Lipases from bacteria, fungi and yeasts can be used for production of biodiesel. For bacteria, lipase from *Serratia marcescens* YXJ-1002 can be used to convert industrial waste grease to biodiesel. Lipase from *S. marcescens* YXJ-1002 was expressed in *E. coli* and used for converting waste grease collected from grease traps in Singapore's sewage to FAME as a product with 97% yield. Moreover, the whole-cell biocatalyst could be reused for 4 cycles and still gave a 74% yield.<sup>442</sup> For fungal cells, *Rhizopus oryzae* IFO4697 overexpressing lipase with specificity for the 1,3-position can be immobilized on biomass support particles (BSPs). Methanolysis using stepwise addition of methanol and BSP-immobilized cells gave the methyl ester product of 90% in 15% water content medium.<sup>443</sup> For yeast, the lipase originally from *R. oryzae* could be overexpressed on the cell surface of *S. cerevisiae* ATCC 60715 and the cells could be employed to produce methyl esters from triglyceride and methanol with 78.3% yield in 72 h.<sup>444</sup> As the yield of whole-cell bioconversion is not much different from the immobilized enzymes, this whole-cell method can serve as an alternative approach for biodiesel production with lower cost.

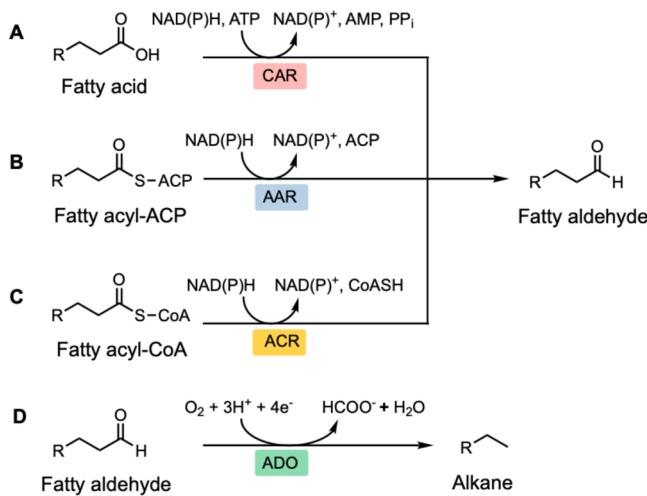
### 3.2. Fatty Acid-Derived Products

The content in this section focuses on enzymatic and metabolically engineered cells for production of hydrocarbons and fatty alcohol as value-added products from fatty acids.

Alkanes and alkenes are hydrocarbon organic materials mainly found in fossil fuels.<sup>445</sup> They are major components in natural gas and petroleum, and are used as fuels for combustion in engines, as monomers for polymer production, and as solvents and plastics. Fatty alcohols are mainly used for production of detergents and surfactants, and also as components in cosmetics, foods, and industrial solvents.<sup>405</sup> As fossil fuels are not renewable and not sustainable, enzymatic and metabolically engineered systems capable of producing hydrocarbons and fatty alcohols from agricultural wastes and agro-industries are attractive technology.

**3.2.1. Hydrocarbons.** **3.2.1.1. Alkanes.** Alkanes can be divided into three groups depending on their chain lengths, ranging from short-chain, medium-chain, and long-chain alkanes. C2 to C4 alkanes which are flammable hydrocarbon gases are classified as short-chain alkanes; they are the main components of liquefied petroleum gas (LPG), which contains mostly propane ( $C_3H_8$ ) and butane ( $C_4H_{10}$ ) liquid under high pressure. They are used as fuels to support transportation as well as for domestic heating and cooking in household activities.<sup>446,447</sup> Propane is also used as a refrigerant in air conditioning and in refrigerating industries because it has good heat transfer properties, and does not create damage to the ozone layer.<sup>448</sup> C5 to C17 alkanes which can be used as gasoline and diesel fuels in combustion engines for transportation are classified as medium-chain alkanes.<sup>449</sup> The third type of alkanes having their chain lengths longer than C18 and used as wax oil such as lubricants is classified as long-chain alkanes.<sup>450</sup> We summarize below the currently available metabolic engineering processes capable of turning fatty acids to alkanes.

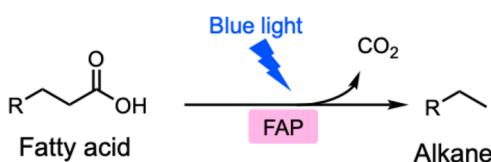
**3.2.1.2. Key Enzymatic Reactions for Alkane Production from Fatty Acids or Fatty Acid Derivatives.** Fatty acids or fatty acid derivatives can be converted to alkanes via two schemes. The first scheme involves initial reduction of fatty acid to generate fatty aldehyde which is then decarbonylated or deformylated to form alkane, while the second scheme is direct decarboxylation of fatty acid to form alkane. The first scheme employs enzymes capable of using fatty acids or fatty acid derivatives as substrates to generate aldehydes. These enzymes include carboxylic acid reductase (CAR), acyl-[acyl carrier protein (ACP)] reductase (AAR), and acyl-CoA reductase (ACR) which can reduce fatty acids or fatty acid derivatives to form aldehydes.<sup>88</sup> CAR is an ATP- and NADPH-dependent enzyme which catalyzes reduction of carboxylic acids to their corresponding aldehydes (Figure 40A).<sup>451–456</sup> Aldehyde is a key intermediate for production of alkane and other value-added compounds. CAR shows high catalytic efficiency with medium-chain fatty acids (C8–C12).<sup>452,457,458</sup> Crystal structures of CAR show that CAR contains three domains: an adenylation domain, a thiolation domain, and a reductase domain. Structural analysis indicates that the motions of these domains are necessary for catalysis. Crystal structures of *Mycobacterium marinum* CAR indicate two conformations associated with active and inactive reductase activity.<sup>88,453</sup> AAR from *Nostoc punctiforme* is an NAD(P)H-dependent enzyme catalyzing the reduction of a long-chain fatty acyl-ACP to form aldehyde, ACP, and NAD(P)<sup>+</sup> in cyanobacteria (Figure 40B).<sup>459,460</sup> ACR which is encoded by the *acr* gene from *Acinetobacter calcoaceticus* catalyzes NAD(P)H-dependent reduction to generate fatty aldehyde from fatty acyl-CoA (C14–C22). This enzyme shows the highest activity with a C16 substrate (Figure 40C).<sup>461,462</sup> The following reaction



**Figure 40.** Reductions of (A) fatty acid, (B) fatty acyl-ACP, and (C) fatty acyl-CoA to form fatty aldehyde using CAR, AAR, and ACR, respectively. (D) The reduction (oxidative deformylation) of fatty aldehyde to alkane and formate using ADO. Abbreviations; CAR: carboxylic acid reductase; AAR: acyl-[acyl carrier protein] reductase; ACR: acyl-CoA reductase; ADO: aldehyde deformylating oxygenase.

under the first scheme utilizes a nonheme iron-dependent enzyme, aldehyde deformylating oxygenase (ADO), found in cyanobacteria to catalyze decarbonylation or cleavage of a terminal aldehyde group to generate alkanes and formate as byproduct (Figure 40D).<sup>463–467</sup>

The second scheme of alkane generation uses fatty acid photodecarboxylase (FAP) which is a light-dependent flavoenzyme from microalgae *Chlorella variabilis*. This enzyme offers a short-cut pathway for alkane biosynthesis by catalyzing decarboxylation of fatty acids to yield alkanes and  $\text{CO}_2$  (Figure 41).<sup>468–471</sup> Its reaction mechanism involves photoexcitation of



**Figure 41.** Reactions of alkane production. Decarboxylation of fatty acid to alkane and  $\text{CO}_2$  using FAP. Abbreviations; FAP: fatty acid photodecarboxylase.

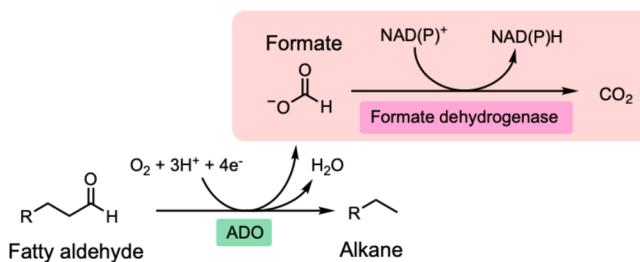
the oxidized FAD cofactor by blue light illumination to form a singlet state FAD. The reaction is proposed to involve electron transfer from amino acids surrounding the FAD to generate a radical pair of amino acid, fatty acid and flavin semiquinone. The radical intermediate rearranges to eliminate  $\text{CO}_2$  and produce alkane.<sup>472,473</sup> FAP can use a broad range of fatty acids as substrates.<sup>474</sup>

**3.2.1.3. Metabolic Engineering for Alkane Production from Fatty Acids or Fatty Acid Derivatives.** Two types of metabolic engineering schemes, two-step versus one-step according to the type of enzymatic reactions involved (see above section), can be used for production of alkanes *in vivo* (bioalkane). For the two-step alkane biosynthesis in *E. coli*, heterologous expression of enzymes of both steps such as AAR and ADO from cyanobacteria *N. punctiforme* PCC73102 convert 3% of a glucose carbon source to yield 300 mg/L of hydrocarbon containing 40% of C17-alkene and C15 odd-

chain alkanes as the major products. It also showed that 80% of the hydrocarbon produced was secreted outside the cells (Figure 43, purple and green).<sup>459</sup> Variants of AAR from cyanobacteria *Synechococcus elongatus* PCC 7942 with mutation of four residues improved its activity and solubility. Under limited nitrogen supply, AAR and ADO from *Synechococcus elongatus* (SeAAR and SeADO, respectively) could be heterologously expressed in the engineered *Cupriavidus necator* in which the genes for polyhydroxyalkanoates (PHAs) synthesis were deleted. The cells produced 670 mg/L of total hydrocarbon consisting of 286 mg/L of long-chain alkanes.<sup>475</sup> For the fungal *Aspergillus carbonarius* ITEM 5010 overexpressing fatty acyl-CoA reductase (FAR) and ADO from *S. elongatus* PCC 7942, the system showed only 2.7 and 10.2 mg/L of pentadecane and heptadecane production, respectively.<sup>476</sup>

In addition to the AAR-ADO system, the *E. coli* system overexpressing ADO and CAR from *Mycobacterium marinum* (*MmCAR*) together with auxiliary enzymes could produce a long-chain alkane with a yield of 2 mg/L. The auxiliary enzymes were thioesterase (TE) and maturase from *B. subtilis* (Sfp) which Sfp catalyzes the transfer of a phosphopantetheinyl group to CAR to fully activate the fatty acid reduction activity of CAR.<sup>477</sup> Biosynthesis of short chain alkanes, such as propane could be achieved using the engineered ADO from *Prochlorococcus marinus* MIT9313 (ADO<sub>A134F</sub>) which has a different substrate scope from the wild-type enzyme. The activity and protein solubility of the variant are also better than the wild-type enzyme.<sup>478</sup> Alkane production by ADO<sub>A134F</sub> was further improved by replacing acetoacetyl-CoA synthase with TE and deleting two endogenous aldehyde reductases to prevent competitive pathways. Incorporation of ferredoxin (Fd) and ferredoxin oxidoreductase (FNR) to regenerate more of reduced Fd for ADO increased propane production to up to 3.4 mg/L, or an equivalent to 0.06% of propane yield in *E. coli*.<sup>479</sup> Interestingly, a higher propane production yield of 32 mg/L in *E. coli* could be obtained when overexpressing catalase (KatE) to remove  $\text{H}_2\text{O}_2$ , which is aside product resulting from nonspecific electron transfer to oxygen. This was a 107-fold increase compared to the condition containing the two aldehyde reductases.<sup>480</sup> The same system can also be used to synthesize medium-chain alkanes in *S. cerevisiae*.<sup>457</sup> However, the last step of this pathway generates formate as a byproduct, which upon accumulation leads to a lower pH that is harmful to the cells.<sup>480</sup>

Another two-step alkane bioproduction using cascade reactions of ACR1 and ADO to convert fatty acyl-CoA to alkanes has been explored (Figure 43, purple and green). Although ACR1 from *Acinetobacter baylyi* ADP1 was mostly used in the pathway to synthesize fatty alcohols and wax esters from fatty acid derivatives,<sup>462,481–483</sup> recent work has shown that combined expression of *Acinetobacter baumannii* ACR1 and *P. marinus* MIT9313 ADO in *E. coli* can be used for efficient biosynthesis of alkanes.<sup>484</sup> This work also added expression of FDH from *Xanthobacter* sp. 91 in *E. coli* to prevent formate accumulation and pH drop in the cells. The addition of FDH also gives an extra advantage to the system because it recycles electrons from formate to generate more NAD(P)H which can eventually generate extra reduced ferredoxin, resulting in increased alkane production yield to nearly 50%, the best yield to date for the alkane generation by ADO.<sup>484</sup> The reaction of FDH is shown in Figure 42.

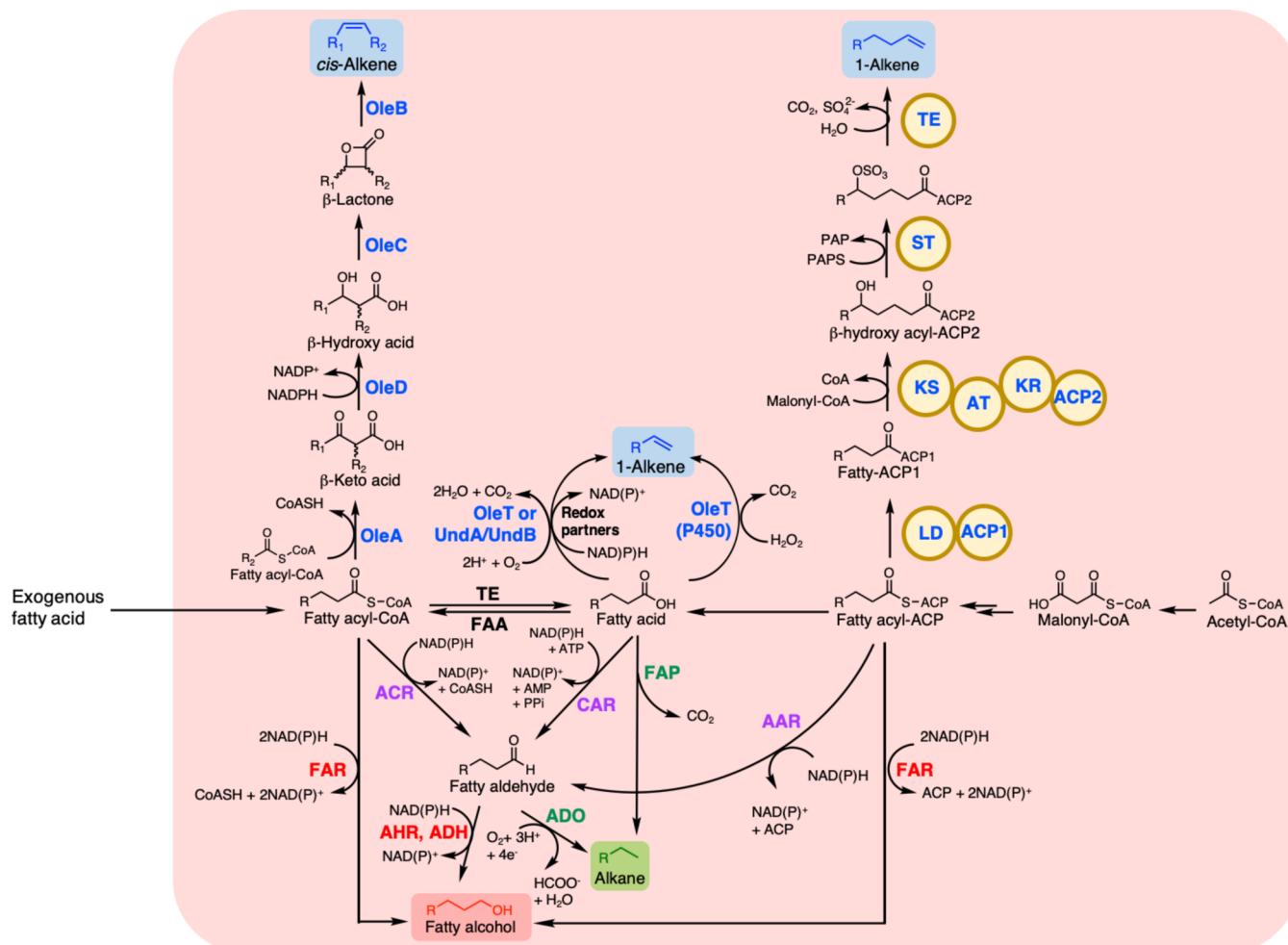


**Figure 42.** Formate eliminated from the reaction of aldehyde deformylating oxygenase can be recycled back to be a reductant for alkane production using the reaction of formate dehydrogenase. Abbreviations; ADO: aldehyde deformylating oxygenase.

An emerging and promising system for production of alkanes is the use of a one-step reaction FAP from chloroplast of *C. variabilis* NC64A FAP (*CvFAP*) and *Chlamydomonas reinhardtii* (*CrFAP*) to catalyze decarboxylation of long-chain fatty acids to form alkanes (Figure 43, green). The reaction of FAP requires strong blue light.<sup>468,469</sup> When the enzyme was

co-overexpressed with a medium-chain fatty acid thioesterase in *E. coli*, the system could continuously produce hydrocarbons.<sup>485</sup> Expression of *CvFAP* in *Yarrowia lipolytica* yeast could be used to generate diesel-like and odd-chain alkanes of C17 and C15 carbon lengths.<sup>486</sup> Recently, the production of short-chain alkanes such as propane, butane and isobutane has been successfully demonstrated by incorporating the engineered *CvFAP*<sub>G462I</sub> in *E. coli* (43.8 mg/L of propane) and *Halomonas* (157.1 mg/L of propane). The engineered *Halomonas* can be grown in nonsterile seawater, reducing the cost of the system for larger scale production. This technology shows promising economic feasibility and may be used for future development of cooking-gas production.<sup>445–447</sup>

**3.2.1.4. Alkenes.** Alkenes are unsaturated hydrocarbons containing at least one double bond. They are commonly used as fuels and starting materials for the production of lubricants, plastics, detergents, surfactants and other chemicals.<sup>401–404</sup> Alkenes are generally produced from fossil fuels such as natural gas and crude oil. Recent developments in metabolic



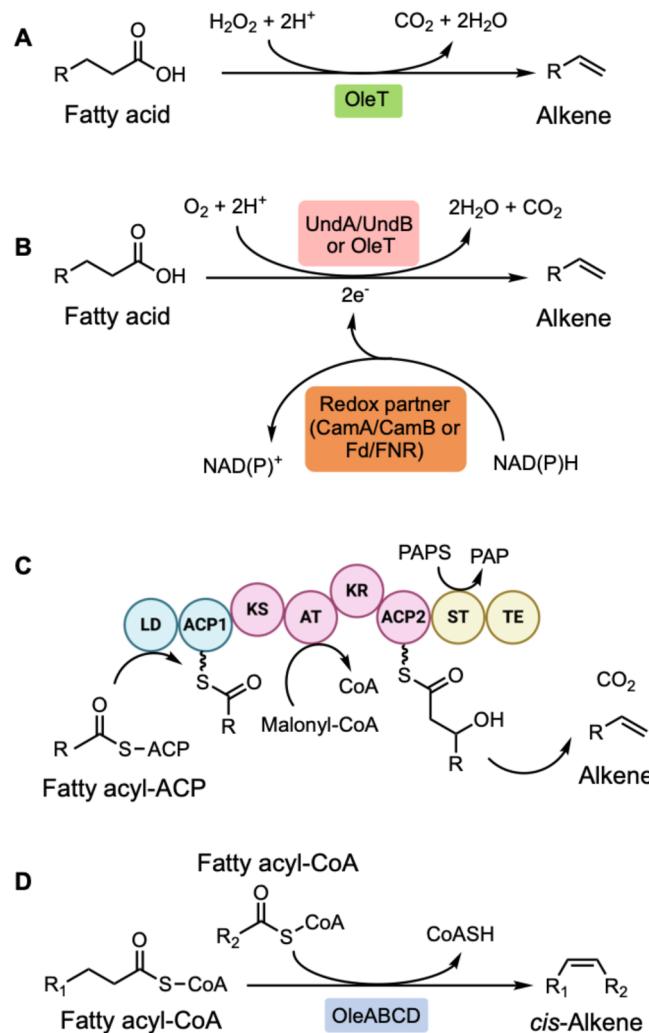
**Figure 43.** Engineered metabolic pathways which can be used for production of alkane, alkene, and fatty alcohol from fatty acids and fatty acid derivatives. Abbreviations; CAR: carboxylic acid reductase; AAR: acyl-[acyl carrier protein] reductase; ACR: acyl-CoA reductase; ADO: aldehyde deformylating oxygenase; FAP: fatty acid photodecarboxylase; FAR: fatty acyl-CoA reductase; AHR: aldehyde reductase; ADH: alcohol dehydrogenase; FAAS: fatty acyl-CoA synthase; OleT, UndA and UndB: fatty acid decarboxylases for alkene synthesis; LD: loading domain; ACP1: acyl carrier protein-1; ACP2: acyl carrier protein-2; KS: ketosynthase; AT: acyltransferase; KR: ketoreductase; ST: sulfotransferase; TE: thioesterase; OleA: thiolase; OleB:  $\alpha/\beta$ -hydrolase; OleC:  $\beta$ -lactone synthetase; OleD: NAD(P)H-dependent reductase; PAP: adenosine 3',5'-diphosphate; PAPS: 3'-phosphoadenosine 5'-phosphosulfate.

engineering offer alternative bioprocesses for alkene production.

**3.2.1.5. Key Enzymatic Reactions for Alkene Production from Fatty Acids.** Various enzymes that can catalyze decarboxylation of fatty acid can be used to produce alkenes from fatty acids or fatty acid derivatives. Depending on the end products of alkenes biosynthesis, the systems can be divided into two groups to produce terminal and internal alkenes. The terminal alkenes can be synthesized via two types of reactions consisting of a one-step reaction and cascade reactions encoded by a gene cluster. Enzymes catalyzing one-step decarboxylation of fatty acids to produce terminal alkenes include cytochrome P450 (CYP) fatty acid decarboxylases (OleT) and fatty acid decarboxylases for alkene synthesis (UndA/UndB). For the cascade decarboxylation, two pathways have been reported to produce terminal and internal alkenes. Olefin synthase (Ols) is a single enzyme with multiple domains which can synthesize a terminal alkene, while the internal alkene or *cis*-alkene production can be synthesized by a three- or four-enzyme cluster of OleABCD.<sup>88</sup>

For one-step decarboxylation of fatty acids, OleT which is a member of the CYP152 family containing a heme-iron cofactor ligated with an axial cysteine thiolate catalyzes the decarboxylation of medium- and long-chain fatty acids or fatty acid derivatives (C8–C20) to yield 1-alkenes in a one-step reaction (Figure 44A).<sup>487</sup> The reaction of OleT requires electrons from redox protein partners such as putidaredoxin/NADH-putidaredoxin reductase (CamA/CamB)<sup>488</sup> or ferredoxin/ferredoxin reductase (Fd/FNR; Figure 44B).<sup>489</sup> Alternatively, H<sub>2</sub>O<sub>2</sub> can be used as a H<sub>2</sub>O<sub>2</sub> shunt to provide electrons to the system. OleTs from various species including *Methylobacterium populi* (OleT<sub>MP</sub>),<sup>490</sup> *Jeotgalicoccus* sp. (OleT<sub>JE</sub>),<sup>491</sup> *Jeotgalicoccus halophilus* (OleT<sub>JH</sub>), *Salinicoccus qindaonensis* (OleT<sub>SQ</sub>), and *Straphylococcus aureus* (OleT<sub>SA</sub>) have been identified and demonstrated to catalyze the synthesis of alkenes.<sup>492</sup> However, OleT can also perform side reactions of  $\alpha$ - and  $\beta$ -fatty acid hydroxylation.<sup>490–494</sup>

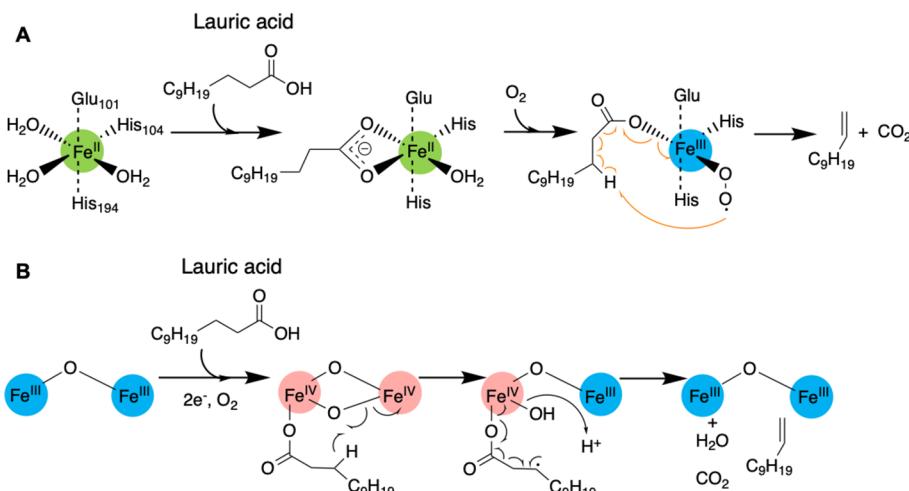
OleT enzymes in various forms from several species have been explored for their ability to produce alkenes from fatty acids. For example, CYP-Sm46 $\Delta$ 29 is a fatty acid decarboxylase in the CYP152 family from *Staphylococcus massiliensis* S46 CYP which has its 29 amino acids at the N-terminal removed to improve protein solubility. However, not all members of the CYP152 family catalyze decarboxylation, such as CYP-Aa162 from *Alicyclobacillus acidocaldarius* which mainly catalyzes fatty acid hydroxylation.<sup>494</sup> Catalytic activity, substrate specificity and the functions of active site residues of several OleT enzymes were investigated by structural analysis, substrate screening and mutagenesis.<sup>489,491,492,496</sup> One limitation of alkene production by OleT is the use of H<sub>2</sub>O<sub>2</sub> as a cosubstrate because high concentrations of H<sub>2</sub>O<sub>2</sub> can cause toxicity to the OleT system. Recently, several H<sub>2</sub>O<sub>2</sub>-generating systems have been used to supply H<sub>2</sub>O<sub>2</sub> for the OleT reaction. Alditol oxidase from *Streptomyces coelicolor* which catalyzes glycerol oxidation to form H<sub>2</sub>O<sub>2</sub> was fused with OleT<sub>JE</sub> to generate a chimeric enzyme that showed increased alkene production and prolonged activity of OleT<sub>JE</sub> compared to the wild-type system.<sup>497</sup> In another example, glucose oxidase (GOx) which uses glucose as a substrate to generate H<sub>2</sub>O<sub>2</sub> could be used with OleT<sub>SA</sub> to give a high yield of alkene production of up to 99% from using stearic acid (C18) and eicosanoic acid (C20) as substrates.<sup>498</sup> The bioconversion of OleT<sub>JE</sub> using NAD(P)H and redox partners including ferredoxin from *S. elongatus* PCC



**Figure 44.** Reactions of alkene productions from fatty acids and fatty acid derivatives using (A) OleT, (B) OleT or UndA/UndB with redox partner, (C) olefin synthase with protein modules, and (D) OleABCD cluster. Panel C is adapted with permission from Mendez-Perez et al.<sup>495</sup> Copyright © (2011) American Society for Microbiology. Abbreviations: OleT, UndA and UndB: fatty acid decarboxylase for alkene synthesis; CamA: NAD(P)H-dependent putidaredoxin reductase; CamB: putidaredoxin; Fd: ferredoxin; FNR: ferredoxin reductase; LD: loading domain; ACP1: acyl carrier protein-1; ACP2: acyl carrier protein-2; KS: ketosynthase; AT: acyltransferase; KR: ketoreductase; ST: sulfotransferase; TE: thioesterase; OleA: thiolase; OleB:  $\alpha/\beta$ -hydrolase; OleC:  $\beta$ -lactone synthetase; OleD: NAD(P)H-dependent reductase; PAP: adenosine 3',5'-diphosphate; PAPS: 3'-phosphoadenosine 5'-phosphosulfate.

7942 (SeFd) and ferredoxin reductase from *Corynebacterium glutamicum* ATCC 13032 (CgFNR) also gave a higher yield of conversion (94.4%) compared to the system using free H<sub>2</sub>O<sub>2</sub> (49.5%) when myristic acid was used as a substrate.<sup>489</sup> In addition, decarboxylation of short-chain fatty acids (C4–C9) could be achieved using cascade reactions containing the purified OleT<sub>JE</sub>, the redox partner (CamA and CamB) and FDH to generate NAD(P)H from formate. The system generated 0.93 g/L of alkane titers (C3–C8; Figure 44B).<sup>488</sup>

The terminal alkenes can also be synthesized from aliphatic medium-chain fatty acids using the enzymes UndA and UndB to catalyze the one-step decarboxylation reaction. UndA isolated from *Pseudomonas* is a nonheme iron(II)- and O<sub>2</sub>-



**Figure 45.** Reaction mechanisms of fatty acid decarboxylation by UndA (A)<sup>499</sup> and UndB (B).<sup>500</sup> Panel A is adapted with permission from Rui et al.<sup>499</sup> Copyright © (2014) The National Academy of Sciences. Abbreviations; UndA and UndB: nonheme iron-dependent fatty acid decarboxylases for alkene synthesis.

dependent oxidase/decarboxylase. It catalyzes the decarboxylation of fatty acids such as lauric acid using  $O_2$  to generate 1-undecene, carbon dioxide and water via  $\beta$ -hydrogen abstraction of fatty acids (Figure 44B and 45A). Analysis of the X-ray crystal structure of UndA showed that bulky amino acid side chains such as F239, L240, and Y41 limit the size of the fatty acid chain length (C10–C14) capable of binding to the enzyme active site.<sup>499</sup> Investigation of 1-undecene production in bacteria showed that 1–100 ng/mL of 1-undecane could be produced only in *Pseudomonas* sp., but not in *Shewanella* or *E. coli*.<sup>499</sup> Another enzyme that can catalyze fatty acid decarboxylation is UndB from *Pseudomonas fluorescens* PF-5 which is a membrane-bound desaturase-like decarboxylase which can form a double bond by removing two hydrogen atoms from fatty acids (Figure 45B). It desaturates medium-chain fatty acids to result in 1-alkenes. The range of fatty acid substrates (C6–C18) for UndB is wider than that of UndA.<sup>500</sup>

For the cascade decarboxylation to generate terminal alkenes, the biosynthesis of medium-chain alkenes from fatty acid derivatives can be achieved by the reaction of olefin synthase (Ols), which is a large multidomain enzyme isolated from the cyanobacterium *Synechococcus* strain PCC 7002. Ols is comprised of three protein modules. Each module has its C-terminal region fused with the N-terminal region of a nearby module. The first module is a substrate activation module consisting of a loading domain (LD) which binds and consumes ATP and an ACP domain called ACP1 which generates fatty acyl-ACP1. The second module is an elongation module which has ketosynthase (KS) and acyltransferase (AT) activities, and it contains a motif to bind malonyl-CoA, ketoreductase (KR), and acyl carrier protein 2 (ACP2). This module catalyzes incorporation of two carbons from malonyl-CoA to elongate a fatty acyl substrate and generate fatty acyl-ACP2. KR then binds to this complex to reduce a  $\beta$ -keto group of fatty acyl-ACP2 to result in a  $\beta$ -hydroxyl moiety of fatty acyl-ACP2. The last module is a terminal alkene formation module which consists of sulfotransferase (ST) and C-terminal thioesterase (TE). ST performs sulfation at the  $\beta$ -hydroxyl group using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfate group donor. TE consequently catalyzes dehydration and decarboxylation to generate a terminal alkene

at the final step (Figure 43, blue, and Figure 44C).<sup>88,495</sup> During the catalytic reaction, a dynamic loop of ST needs to adopt the conformation that is closest to the  $\beta$ -hydroxy acyl-ACP-linked substrate. The disordered dynamic movement of the ST flap region limits the substrate selectivity of the Ols system because ST is highly selective. For example, it cannot react with substrate analogues containing a methoxy group at the C5-position of  $\beta$ -hydroxyacyl-ACP-linked substrate.<sup>501</sup>

For bioproduction of an internal alkene, a cluster containing three- or four genes involved in olefin biosynthesis defined as *oleABCD* was identified in *Xanthomonas campestris*,<sup>502</sup> *Stenotrophomonas maltophilia*,<sup>503</sup> and *Shewanella oneidensis*.<sup>504</sup> Enzymes encoded by the cluster, OleABCD, catalyze the conversion of long-chain fatty acyl-CoA or fatty acid derivatives to produce internal *cis*-alkenes.<sup>502–504</sup> OleA acts as a thiolase catalyzing head-to-head Claisen condensation of two long-chain fatty acyl-CoA molecules without decarboxylation. The roles of active site residues H285, E117, and C143 of OleA have been studied using site-directed mutagenesis and X-ray crystal structures. All of these residues are important for the first step of olefin biosynthesis.<sup>505–509</sup> OleD is an NAD(P)H-dependent reductase and a member of the short-chain dehydrogenase superfamily which catalyzes reduction of  $\beta$ -keto acid to generate  $\beta$ -hydroxy acid.<sup>503</sup> OleC is a  $\beta$ -lactone synthetase found in *X. campestris*, *S. maltophilia*, *Micrococcus luteus*, *Lysobacter dokdonensis*, and *Arenimonas malthae* which catalyzes  $\beta$ -lactone synthesis from  $\beta$ -hydroxy acid.<sup>502</sup> OleB is an  $\alpha/\beta$ -hydrolase catalyzing  $\beta$ -lactone decarboxylation to form the internal *cis*-alkene at the last step of long-chain alkene biosynthesis (Figure 43, blue route).<sup>508</sup>

**3.2.1.6. Metabolic Engineering for Alkene Production from Fatty Acids.** Bacteria and yeast overexpressing enzymes catalyzing alkene production and their redox partners have been explored for the production of terminal alkenes. To enhance alkene production by OleT<sub>JE</sub> from fatty acids in yeast, *S. cerevisiae* was engineered to remove the *faa1* gene encoding for fatty acyl-CoA synthethase to prevent utilization of fatty acid substrates by other pathways. This system generated a 6.2-fold increase of total alkene production as compared to the native strain. Additionally, the gene *ctt1* encoding for the cytoplasmic catalase T, the gene *cta1* encoding the peroxisomal catalase A and the *ccp1* gene encoding the mitochondrial

cytochrome c peroxidase were deleted from the genome to reduce the systems capable of destroying H<sub>2</sub>O<sub>2</sub>. This modification resulted in a 7.4-fold increase in total alkene production compared to the host cell expressing OleT<sub>JE</sub>. Despite the fact that OleT<sub>JE</sub> is a heme-iron dependent enzyme, integration of the gene *hem3* encoding porphobilinogen deaminase for heme synthesis in the genome did not enhance the total alkene production. However, the *S. cerevisiae* with the *hem3* gene integrated into its genome, supplemented with heme and H<sub>2</sub>O<sub>2</sub> generated a 3.6-fold increase in alkene production compared to growth without any supplements. The *S. cerevisiae* overexpressing the codon-optimized OleT<sub>JE</sub> with the early mentioned modifications and a strong constitutive promoter grown in rich media in a bioreactor for 144 h could produce 3.7 mg/L of intracellular alkenes (C11–C19) with 1-heptadecene as the main product. This production was 67.4-fold higher than that of the native *S. cerevisiae* overexpressing OleT<sub>JE</sub>.<sup>510</sup>

To enhance alkene production in *E. coli*, OleT from *Macrococcus caseolyticus* (OleT<sub>MC</sub>) and TE were co-overexpressed in *E. coli* BL21 (DE3). The system could produce 17.8 mg/L of C11–C15 odd-chain terminal alkenes. Because electron transfer to OleT<sub>MC</sub> was thought to be the rate-limiting step in the overall decarboxylation process, two-component redox partners, including CamA and CamB were added to increase the alkene yield further to 49.64 mg/L, which is about 2.8-fold more than that of the condition without the extra redox partners (Figure 43, blue).<sup>511</sup> Later on, OleT<sub>JE</sub> was fused with a flavin/Fe–S reductase from *Rhodococcus* sp. NCIMB 9784 (*RhFRED*) which can catalyze electron transfer from NADPH to oxidized flavin, then to the Fe–S cluster of *RhFRED*, and finally to the heme to generate the reactive ferryl-oxo cation radical for the OleT reaction. The fusion enzyme of OleT<sub>JE</sub> and *RhFRED* was expressed using a plasmid system in *E. coli*, resulting in an increase in the total amount of terminal intracellular and extracellular alkenes produced, giving a yield of 97.6 mg/L.<sup>493,512</sup> In the latest studies, deletion of endogenous catalases (*katE* and *katG*) in *E. coli* overexpressing OleT<sub>JE</sub> resulted in improvement of decarboxylation yield (98.2% from conversion of lauric acid) and a ratio of decarboxylation over hydroxylation of 5.8 to 1.<sup>513</sup>

For terminal alkene production by UndA and UndB, which also catalyze one-step decarboxylation, *E. coli* BL21 overexpressing UndA using the plasmid system resulted in production of 5.5 µg/mL 1-undecene from lauric acid. This amount is about 5.5-fold higher than the amount of 1-undecene obtained from the *Pseudomonas* original system (Figure 43, blue).<sup>499</sup> *E. coli* overexpressing UndB and *ucfatB2* encoding for TE could produce 22 mg/mL of 1-undecene without feeding lauric acid as a substrate. A similar *E. coli* system overexpressing an UndB homologue, the *Pmen\_4370* gene from *Pseudomonas mendocina* ymp, resulted in 1-undecene production of 55 mg/L.<sup>500</sup> For the production of terminal alkenes in other bacterial species, TE from *E. coli* and UndA were overexpressed in *A. baylyi* ADP1, resulting in the engineered *A. baylyi* ADP1 which can generate 72 µg/L of 1-undecene in high salt medium.<sup>514</sup> The cyanobacterial *Synechocystis* sp. PCC 6803 overexpressing codon optimized UndB and TE could give a product yield of 19 mg/g cell dry weight.<sup>515</sup> Recently, two copies of UndB from *P. mendocina* and TE from *E. coli* were incorporated into the *Pseudomonas aeruginosa* chromosome together with deletion of *fadE* and

*rhlAB* genes encoding for acyl-CoA dehydrogenase and rhamnosyltransferase chain A and B, respectively, to reduce the competitive pathway. This system could generate 778.4 and 1102.6 mg/L of 1-alkenes, the highest yield to date for alkene production with UndB, when using palm oil and lauric acid as substrates, respectively.<sup>516</sup>

For metabolic engineering of the *ols* gene cluster, a heterologous promoter which controls expression of the native *ols* gene cluster was incorporated into the cyanobacterial host, *Synechococcus* sp. PCC 7002, to increase the production of 1-alkene C19:2 and C19:1 to 4.2 µg/mL/OD of culture, which is higher than that of the nonengineered system by approximately 2.4-fold (Figure 43, blue).<sup>495</sup> Replacing the *ols* pathway with the genes of *undA* and *fap* encoding fatty acid photo-decarboxylase from *Chlorella variabilis* NC64A into the genome resulted in products with carbon chain lengths longer than that of the wild-type *Synechococcus* sp. PCC 7002.<sup>517</sup> Unfortunately, the *ols* gene cluster could not be expressed in *E. coli* DH10B due to difficulty in protein translation of a big gene module.<sup>518</sup>

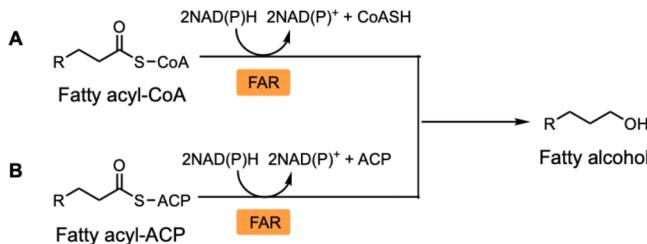
For internal alkene production by the OleABCD cluster isolated from the Gram-positive bacterium *M. luteus* ATCC 4698, cell lysate from *E. coli* with heterologous expression of this gene cluster showed production of long-chain alkenes C27:3 and C29:3.<sup>519</sup> Native biosynthesis of very long-chain fatty acids by head-to-head condensation has been reported in several microorganisms such as *Chloroflexus aurantiacus* J-10-fl, *Kocuria rhizophila* DC2201, *Brevibacterium fuscum* ATCC 15993, *X. campestris*, *S. oneidensis* MR-1, *Shewanella putrefaciens* CN-32, *Shewanella baltica* OS185, *Shewanella frigidimarina* NCIMB 400, *Shewanella amazonensis* SB2B, *Shewanella denitrificans* OS217, *Colwellia psychrerythraea* 34H, *Geobacter bemandjiensis* Bem, *Opitutaceae bacterium* TAV2, and *Planctomyces maris* DSM 8797 (Figure 43, blue). These organisms contain proteins similar to those in the OleABD cluster, such as thiolase, α/β-hydrolase, AMP-dependent ligase/synthase, and short-chain dehydrogenase. *X. campestris* produced a wide range of very long-chain olefins from C24 to C31.<sup>504,519</sup> However, there have been no reports about a preferred carbon atom range of substrates for olefin synthesis.

**3.2.2. Fatty Alcohols.** Fatty alcohols are important commodity chemicals. They were produced at an amount of 3.04 million tonnes in 2015, in which 0.84 million tonnes were from petroleum-based production and 2.20 million tonnes were from biobased production. The value of fatty alcohols in the global market was estimated to be 6.9 billion US dollars in 2017,<sup>405</sup> and the market demand will continue to increase because fatty alcohols are key ingredients in lotions, soaps, liquid detergents and skin care products. Currently, the COVID-19 pandemic situation requires more use of cleansing reagents such as detergents, shampoos, body washes, dish-washing liquids, and fabric softeners which contain fatty alcohols and fatty alcohol derivatives as ingredients. Fatty alcohols are also used for production of fragrance additives found in other cleansing products.<sup>405</sup> Therefore, metabolic engineering for fatty alcohol biosynthesis offers an attractive choice for a sustainable economy because the production process does not create environmental toxic waste.<sup>405</sup>

**3.2.2.1. Key Enzymatic Reactions for Fatty Alcohol Production from Fatty Acids.** Fatty acids and their derivatives such as fatty acyl-CoA and fatty acyl-ACP can be used for the biosynthesis of fatty alcohols via enzymatic reactions.

Enzymatic pathways to synthesize fatty alcohols consist of two major pathways containing one- and two-step reductions.

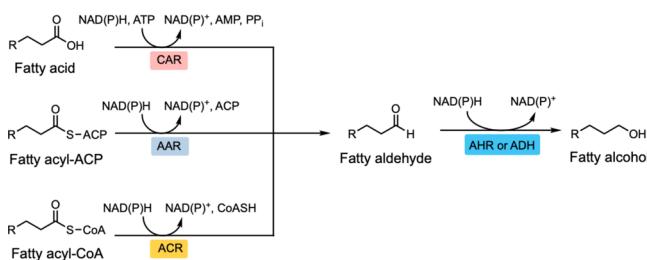
For the one-step reduction, reductions of fatty acyl-CoA and fatty acyl-ACP are catalyzed by FAR, an NADPH-dependent enzyme that can transfer four electrons from two molecules of NADPH to synthesize primary fatty alcohols (Figure 46).



**Figure 46.** One-step reduction of fatty acyl-CoA and fatty acyl-ACP using FAR to generate fatty alcohol. Abbreviations; FAR: fatty acid reductase for fatty alcohol synthesis; ACP: acyl carrier protein.

FARs are present in plants,<sup>520–522</sup> bacteria,<sup>523,524</sup> insects,<sup>525</sup> birds,<sup>526</sup> and mammals.<sup>525</sup> FAR isolated from *Marinobacter aquaeolei* VT8 (MaFAR) catalyzes reduction of a wide range of fatty acyl-CoA or fatty acyl-ACP from C8:0 to C20:4 to result in fatty alcohols. The reaction proceeds directly to formation of a fatty alcohol without generating a free fatty aldehyde intermediate. Results from steady-state kinetics indicate that the FAR reaction with palmitoyl-CoA as a substrate gives the highest rate of production.<sup>523,527</sup> In plants, AtFAR6 isolated from *Arabidopsis thaliana* was found to be localized in the chloroplasts. When it was heterologously expressed in *Nicotiana tabacum* and *Nicotiana benthamiana* leaves, the enzymes showed activities for converting C16 fatty acyl-ACP and -CoA to C16-fatty alcohol.<sup>520</sup>

For the two-step reduction process, fatty acid, fatty acyl-ACP or fatty acyl-CoA is first reduced to form fatty aldehyde by different enzymes i.e. CAR,<sup>477</sup> AAR,<sup>528–531</sup> and ACR,<sup>532,533</sup> respectively, similar to the first step reaction in alkane production (Figure 40). Subsequently, the second step of fatty aldehyde reduction is catalyzed by aldehyde reductase (AHR) or alcohol dehydrogenase (ADH) to yield corresponding fatty alcohols (Figure 47).<sup>528,529</sup> Members of the AHRs family include NADPH-dependent aldehyde reductases AdhE and YqhD, which are encoded by the genes *adhE* and *yqhD*, respectively. These enzymes are available in native *E. coli* and they prefer to produce alcohols with side chains longer than



**Figure 47.** Two-step reduction. The first-step is the reduction of fatty acid, fatty acyl-ACP or fatty acyl-CoA using ACR, CAR and AAR, respectively. The second-step is the generation of fatty aldehyde using AHR or ADH to generate fatty alcohol. Abbreviations; CAR: carboxylic acid reductase; AAR: acyl-[acyl carrier protein] reductase; ACR; acyl-CoA reductase; AHR: aldehyde reductase; ADH: alcohol dehydrogenase.

C3-fatty alcohols. Examples of ADHs include ethanol-active dehydrogenase and acetaldehyde-active reductase encoded by the genes *adhP* and *yigB* from endogenous *E. coli*, respectively. Additionally, Zn-dependent alcohol dehydrogenase, Slr1192, isolated from *Synechocystis sp.* PCC 6803 can also be used in conjunction with the acid reductase to produce fatty alcohols as final products.<sup>405,534</sup>

**3.2.2.2. Metabolic Engineering for Fatty Alcohol Production from Fatty Acids.** Metabolic engineering to synthesize fatty alcohols has been widely investigated in yeast strains because their growth is robust and the process is readily amenable to scale-up for industrial production.<sup>535</sup> FARs from various strains were explored for their use in fatty alcohol production because they can directly generate fatty alcohols from fatty acyl-CoAs and fatty acyl-ACPs as previously mentioned (Figure 43, red). The genes encoding FAR from *Tyto alba*, ATP-dependent citrate lyase (ACL) from *Y. lipolytica* and endogenous acetyl-CoA carboxylase (Acc1) genes from *S. cerevisiae* were overexpressed in *S. cerevisiae* and the system can generate fatty alcohols at a yield of around 300 mg/L. While fatty alcohol production of up to 655 mg/L could be obtained from a batch culture with high cell density, higher production of 11.1 g/L of fatty alcohols could be obtained from a fed-batch culture.<sup>536</sup> For *L. starkeyi*, which is an oleaginous yeast tolerant to lipid accumulation, the engineered *L. starkeyi* overexpressing FAR could accumulate high amounts of intracellular lipid as high as 65% of the dried cell weight.<sup>537</sup> Overexpression of MaFAR in *L. starkeyi* and *Y. lipolytica* produced 770 mg/L and 167 mg/L of fatty alcohols in flask culture, respectively. Up-regulation of carbohydrate metabolism can also enhance production of fatty acids and fatty acid derivatives. When used in conjunction with overexpression of MaFAR, the system resulted in high fatty alcohol production of up to 5.75 g/L of fatty alcohols from 50 mL culture in shake flask containing 91 g/L of glucose.<sup>405,538</sup> Moreover, scaling-up of fatty alcohol production using a 2-L culture in bioreactor was performed with *Y. lipolytica* strain L36DGA1 overexpressing MaFAR and diacylglycerol acyl-transferase 1 (DGA1) with mutation at the Mga2 regulator to force lipogenesis.<sup>539</sup> The engineered *Y. lipolytica* strain L36DGA1 can produce 5.8 g/L of total fatty alcohols using 80 g/L of glucose as a substrate. The system can also use  $\alpha$ -linolenic acid to generate linolenyl alcohol.<sup>540</sup>

The process of fatty alcohol production can be enhanced by addition of solvent for *in situ* extraction. When the culture was overlaid with 10% v/v of dodecane, up to 99% of fatty alcohols could be recovered. Dodecane is thought to reduce the toxicity of fatty alcohol accumulation inside the cells.<sup>541</sup> Moreover, addition of a biodegradable surfactant, such as 0.1% v/v tergitol together with 20% v/v dodecane in a 2-L bioreactor overexpressing MaFAR from *Rhodoporfidium toruloides* resulted in a 4.3-fold increase in fatty alcohol production to reach 352.6 mg/L compared to the titer of 81.8 mg/L obtained from the condition without tergitol. A 2-L culture in a bioreactor of *Rhodoporfidium toruloides* produced fatty alcohols at a yield of 1.6 g/L from 50 g/L of glucose with addition of 0.1% and 0.075% tergitol and dodecane, respectively. The higher amount of fatty alcohol produced in the presence of tergitol may be caused by the easier transfer of fatty alcohols via binding to surfactant which facilitates membrane penetration and migration to dodecane.<sup>542</sup>

For metabolic engineering in *E. coli*, genes associated with competing pathways of intermediate utilization including *edd*

encoding phosphogluconate dehydrogenase from Eutner-Doudoroff pathway, *ppsA* encoding phosphoenolpyruvate synthase from gluconeogenesis pathway, *lahA*, *poxB*, *pta*, *pflB* encoding D-lactate dehydrogenase, pyruvate oxidase, phosphate acetyltransferase, and pyruvate formate lyase (PFL) from the fermentative pathway, respectively, *aceA* encoding isocitrate lyase from the glyoxylate pathway and *plsX* encoding phosphate acyltransferase were deleted together with over-expression of AAR and AHR with upregulation of *zwf* encoding G6PD. This system resulted in fatty alcohol production of 12.5 g/L from glucose in 3-L culture media after 60 h. This amount is equivalent to 22.16% of the theoretical yield,<sup>543</sup> and it is the highest yield of fatty alcohol production by metabolic engineering to date.<sup>405</sup>

### 3.3. Glycerol-Derived Products

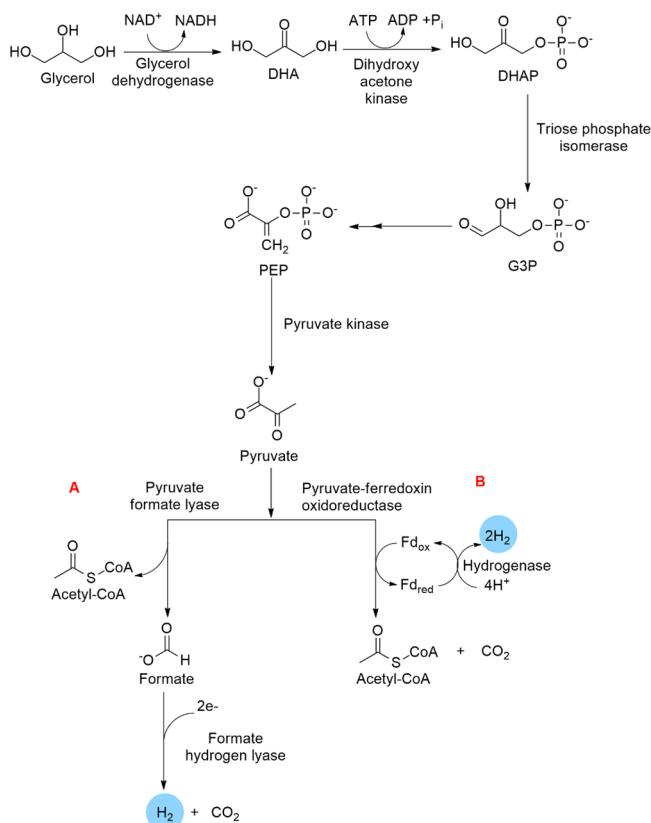
Although glycerol has a wide range of direct applications, it also has great potential to serve as a starting precursor for production of other valuable compounds. In this section, key enzymatic and metabolic engineering processes which can convert glycerol into products important for industries are discussed. As glycerol is a C3 compound, it can be assimilated through intermediates involved with main metabolic pathways. For example, glycerol can be converted to pyruvate, which is an intermediate required for cell growth and energy production. Pyruvate can be converted to C4 compounds such as oxaloacetate (OAA) or malate in the TCA cycle and to many different products.<sup>544</sup> Glycerol can be used for production of hydrogen, succinic acid, 1,2-PDO and 1,3-PDO which are commodity chemicals, by microbial fermentation.<sup>90</sup>

**3.3.1. Hydrogen.** Hydrogen, together with solar, wind, biomass, and hydropower offer alternative solutions for clean energy carriers without harmful effects on the environment. Hydrogen is an ideal energy carrier because it provides low density energy source and high heating value compared to solar, wind and geothermal energy.<sup>95,545</sup> In addition, hydrogen serves as an important reactant in various catalytic reactions in industries such as ammonia synthesis and hydrogenation processes for food and pharmaceuticals.<sup>95</sup> The global hydrogen production total is estimated to be over 65 million tonnes/year.<sup>546</sup> Due to rapid developments in hydrogen production technology, the cost of hydrogen production is expected to be competitive with oil and natural gas in the future.<sup>547</sup> However, current industrial hydrogen production still relies mostly on the steam reforming process which is not green and requires very high temperatures (>700 °C), generating a large amount of GHGs emission.<sup>546,548</sup> Alternatively, biological systems offer a greener solution to production of hydrogen gas.

**3.3.1.1. Key Enzymatic Reactions for Hydrogen Production.** Several microorganisms such as cyanobacteria<sup>549</sup> and bacteria<sup>550</sup> can produce molecular hydrogen from various substrates. A key enzyme for hydrogen synthesis is hydrogenase which converts  $2\text{H}^+$  and  $2\text{e}^-$  to form  $\text{H}_2$ . Hydrogenase also catalyzes a reversible heterolytic cleavage of  $\text{H}_2$ . Hydrogenases can be classified into three types depending on their metal in the active site [NiFe]-, [FeFe]-, and [Fe]-hydrogenase. In *E. coli*, the hydrogen-producing reaction is catalyzed by the formate hydrogen lyase (Fhl) complex which is membrane-bound. This complex is composed of formate dehydrogenase (FDH) which catalyzes formate oxidation to form  $\text{CO}_2$  and  $2\text{H}^+$  and transfers two electrons to [NiFe]-hydrogenase, which catalyzes the formation of  $\text{H}_2$  product

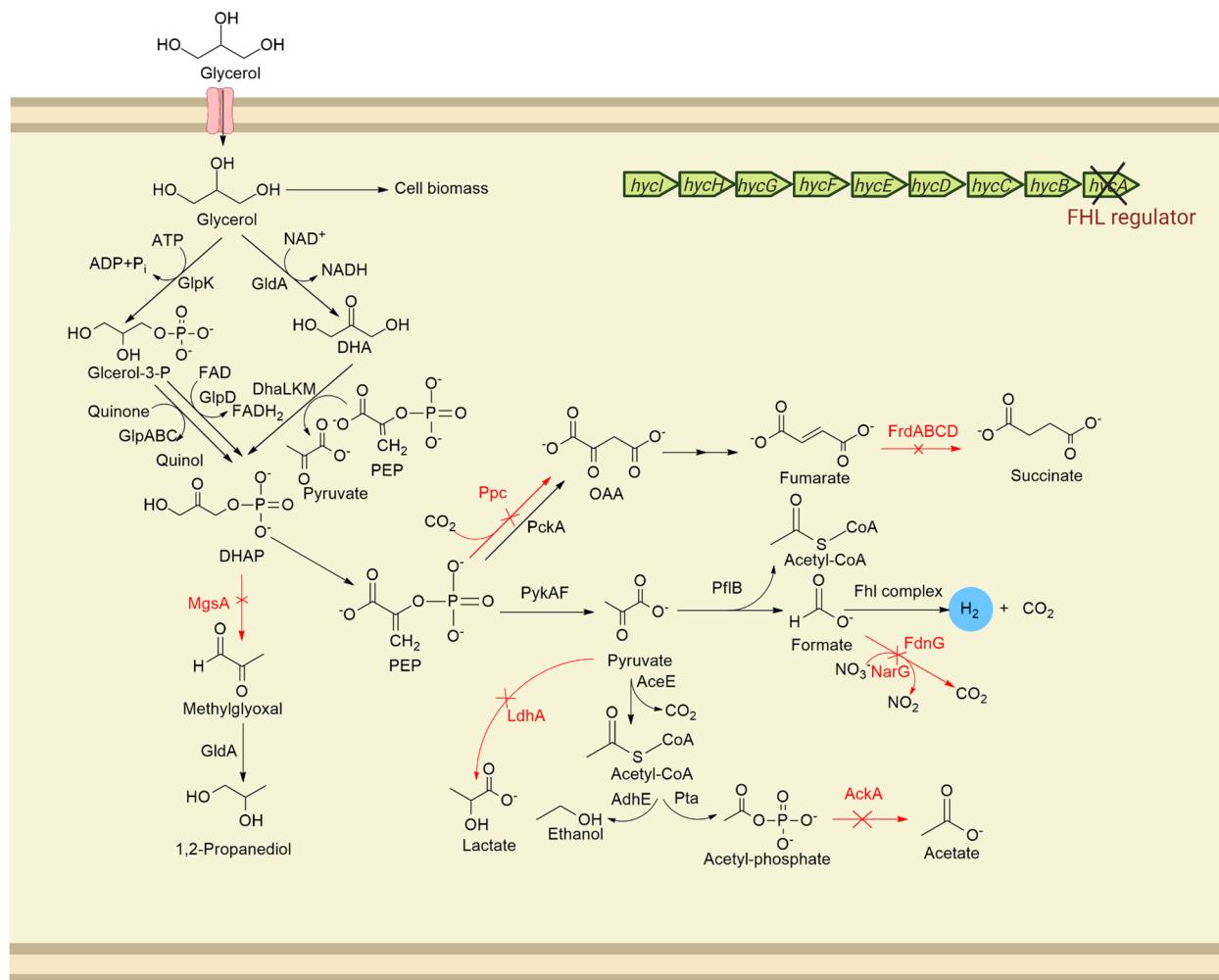
from  $2\text{H}^+$  and  $2\text{e}^-$  transferred from formate.<sup>551</sup> As glycerol can be used as a starting precursor for formate production, hydrogen production from glycerol is thus possible.

A pathway to generate formate or hydrogen from glycerol starts from the reaction of glycerol dehydrogenase which converts glycerol to dihydroxyacetone (DHA). DHA is then converted into dihydroxyacetonephosphate (DHAP) which can enter the glycolytic pathway to be transformed to pyruvate. The key junction where pyruvate can be converted into formate is at the reaction of PFL, which is responsible for the conversion of pyruvate and Coenzyme A (CoA) into formate and acetyl-CoA under anaerobic conditions (Figure 48).<sup>552</sup>



**Figure 48.** Metabolic pathways for  $\text{H}_2$  synthesis from glycerol feedstock in *E. coli* (A) and *Clostridium* spp. (B).

PFL is found in obligatory or facultative anaerobes such as Firmicutes and Enterobacteriaceae. The pathway of metabolizing glycerol into hydrogen can also be found in other facultative anaerobes such as *E. coli* and *Klebsiella pneumoniae*; strict anaerobes such as *Clostridium acetobutylicum*, *Clostridium pasteurianum*, *Clostridium paraputrificum*, and *Clostridium thermocellum*; and aerobes such as cyanobacteria and algae.<sup>553–557</sup> Another important enzyme to generate reducing equivalents for hydrogenase is the enzyme pyruvate: ferredoxin oxidoreductase which catalyzes oxidative decarboxylation of pyruvate to form acetyl-CoA,  $\text{CO}_2$  and reduced ferredoxin.<sup>558</sup> The resulting reduced ferredoxin provides electrons in the hydrogenase reaction to produce  $\text{H}_2$  (Figure 48).<sup>559</sup> Several native organisms can produce high yields of hydrogen. For example, the photosynthetic bacterium *Rhodopseudomonas palustris* can produce nearly 90% of the theoretical yield (7 mol of  $\text{H}_2$  from 8 mol of theoretical yield) at a rate of 34 mL  $\text{H}_2$ /g dry cell weight·h.<sup>560</sup>

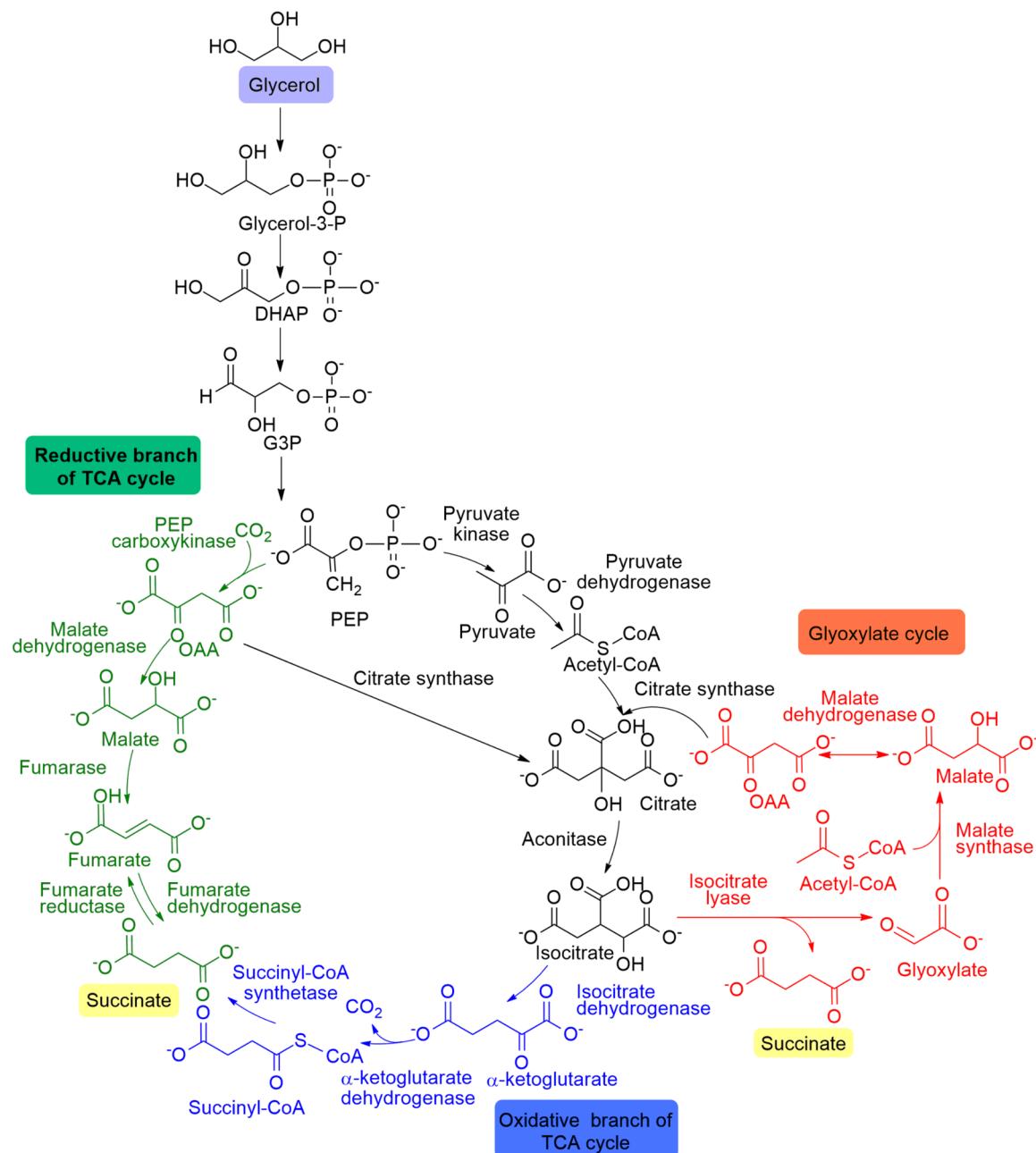


**Figure 49.** Metabolic engineering pathways for  $\text{H}_2$  production from glycerol in *E. coli*. Genetic manipulations carried out to increase the yield of hydrogen production are indicated by a cross symbol. Pathway was manipulated by deleting genes expressing fumarate reductase (*FrdC*),  $\text{D}-\text{lactate dehydrogenase}$  (*LdhA*),  $\alpha$ -subunit of formate dehydrogenase N (*FdnG*), phosphoenolpyruvate carboxylase (*Ppc*),  $\alpha$ -subunit of nitrate reductase A (*NarG*), methylglyoxal synthase (*MgsA*), and *FhlA* transcriptional regulator (*HycA*). Abbreviations; DHA: dihydroxyacetone; DHAP: dihydroxyacetonephosphate; PEP: phosphoenolpyruvate; OAA: oxaloacetate; Fhl: formate hydrogen lyase; GldA: glycerol dehydrogenase.

**3.3.1.2. Microbial Transformation for Hydrogen Production by Metabolic Engineering.** Although glycerol can be converted to hydrogen by the native pathways of several organisms, the use of these organisms may be limited at the industrial level because some of them are pathogenic, require strictly anaerobic fermentation and supplementation with rich nutrients, and suffer from a lack of genetic tools.<sup>561</sup> A common host, *E. coli*, which can produce many products for industries has been engineered to increase the yield of hydrogen production. *E. coli* grown in glycerol under anaerobic conditions gives a lower yield of hydrogen production than when grown on glucose.<sup>562</sup>  $\text{CO}_2$  can enhance glycerol utilization under low pH, whereas high  $\text{H}_2$  partial pressure decreases  $\text{H}_2$  production and cell growth. Therefore, a low partial pressure fermentation has been used to enhance the yield of  $\text{H}_2$  production.<sup>563–565</sup> However, the product titer and yield are still low. Thus, metabolic engineering approach which deletes genes related to pathways diverting metabolites from hydrogen production was carried out. Tran and Maeda used a low partial pressure bioconversion together with deletion of genes of fumarate reductase (*frdABCD*), lactate dehydrogenase (*ldhA*), formate dehydrogenase (*fndG*), phosphoenolpyr-

uvate carboxylase (*ppc*), nitrate reductase (*narG*), methylglyoxal synthase (*mgsA*), and a regulator of the transcriptional regulator *FhlA* (*hycA*) to prevent utilization of intermediates required for  $\text{H}_2$  production (Figure 49).<sup>566</sup> The engineered *E. coli* gave a hydrogen yield of 0.67 mol  $\text{H}_2$ /mol glycerol, which is 4.5-fold higher than that of the wild-type cell. After 48 h of fermentation, the maximum theoretical yield of 1 mol  $\text{H}_2$  could be obtained from 1 mol glycerol, which is 5.5-fold higher than the hydrogen productivity of the wild-type strain.<sup>566</sup>

**3.3.2. Succinic Acid.** Succinic acid (SA) is a dicarboxylic acid that can be classified as a platform chemical because of its various applications.<sup>567</sup> SA has been used as an ingredient in surfactants or detergents, and as an ion chelator (to prevent corrosion of metals), as well as a food additive (for pH adjustment and as an antimicrobial agent) and an additive in pharmaceuticals such as antibiotics.<sup>568</sup> Moreover, because its structure contains two functional carboxylic groups, it can serve as a precursor for synthesizing monomers for material products such as 1,4-butanediol, butyrolactone, tetrahydrofuran, adipic acid, etc.<sup>569–571</sup> The size of SA in the global market is expected to reach  $\sim \$1.1$  billion by 2022.<sup>567</sup>



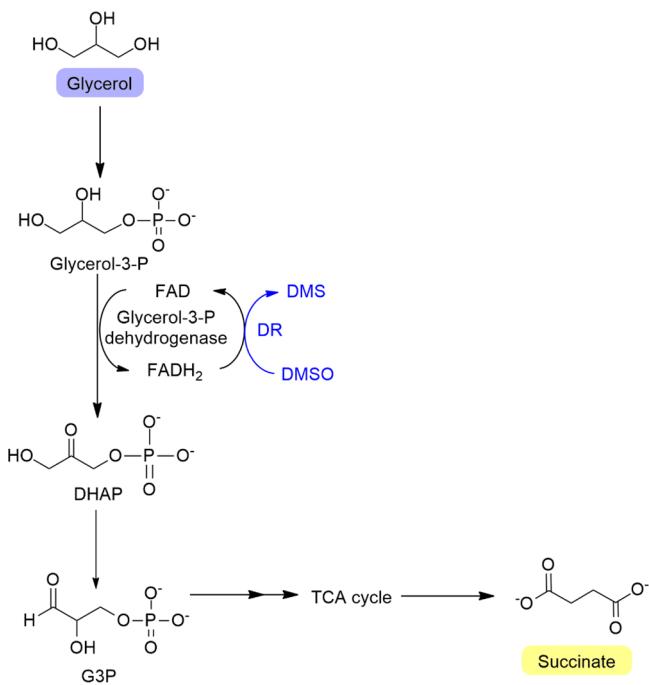
**Figure 50.** Succinate production from glycerol carbon source in bacteria using the reductive branch of the TCA cycle (as shown in green), the oxidative branch of the TCA cycle (as shown in blue), or (and) the glyoxylate cycle (as shown in red).

**3.3.2.1. Key Enzymatic Reactions for Production of Succinic Acid.** SA is found in plants, animals, and microbes because it is a key metabolite of the tricarboxylic acid (TCA) cycle.<sup>567</sup> Many studies reported the ability of microorganisms such as *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, and *Basfia succiniciproducens* DD1 to produce SA from glycerol as a carbon source.<sup>572–579</sup> Three metabolic routes are possible for producing SA from glycerol (and glucose) including the reductive branch of the TCA cycle, the oxidative branch of the TCA cycle, and the glyoxylate cycle (Figure 49). For the reductive branch (green route, Figure 50), SA serves as a final electron sink under anaerobic conditions. Key enzymes in this route include PEP carboxykinase, malate dehydrogenase, fumarase, and fumarate reductase. The committed step is at

the reaction of PEP carboxykinase which is regulated by the level of CO<sub>2</sub>. Under high CO<sub>2</sub> levels and high PEP carboxykinase activity, SA production, and cell growth increase.<sup>580</sup> For the oxidative branch of the TCA cycle, acetyl-CoA is converted to citrate, isocitrate, α-ketoglutarate, and further to succinate. In another route which is part of the glyoxylate shunt, isocitrate is converted to succinate. This pathway generates 1 mol succinate from 2 mol acetyl-CoA.

Native microbes such as *B. succiniproducens* DD1 can be used to produce a high yield of SA from glycerol, 1.02 g SA/g glycerol with a production rate of 0.094 g/L h. This productivity is relatively low compared to that achievable with the use of glucose as substrate, which can produce 106 g/L SA with a production rate of 1.36 g/L h.<sup>579</sup> The use of glycerol as a substrate is limited by redox imbalance during cell

growth because only one reducing equivalent is produced from one glycerol by the glycolysis pathway. After glycerol is converted to glycerol-3-phosphate, this glycerol-3-phosphate can be further converted to DHAP by glycerol 3-phosphate dehydrogenase which uses FAD and generates FADH<sub>2</sub>. Addition of an extra electron acceptor to regenerate FAD was reported to promote the glycerol conversion to DHAP and improve SA synthesis. By using dimethyl sulfoxide (DMSO) as an electron acceptor to regenerate the reducing equivalent (Figure 51), SA production reached 49.62 g/L with a product yield of 0.87 g SA/g glycerol and a production rate of 2.31 g/L h.<sup>579</sup>



**Figure 51.** Reducing equivalent regenerating system using DMSO as an electron acceptor. Abbreviations; DMS: dimethyl sulfide; DMSO: dimethyl sulfoxide; DR: DMSO reductase; FAD: flavin adenine dinucleotide; FADH<sub>2</sub>: reduced FAD.

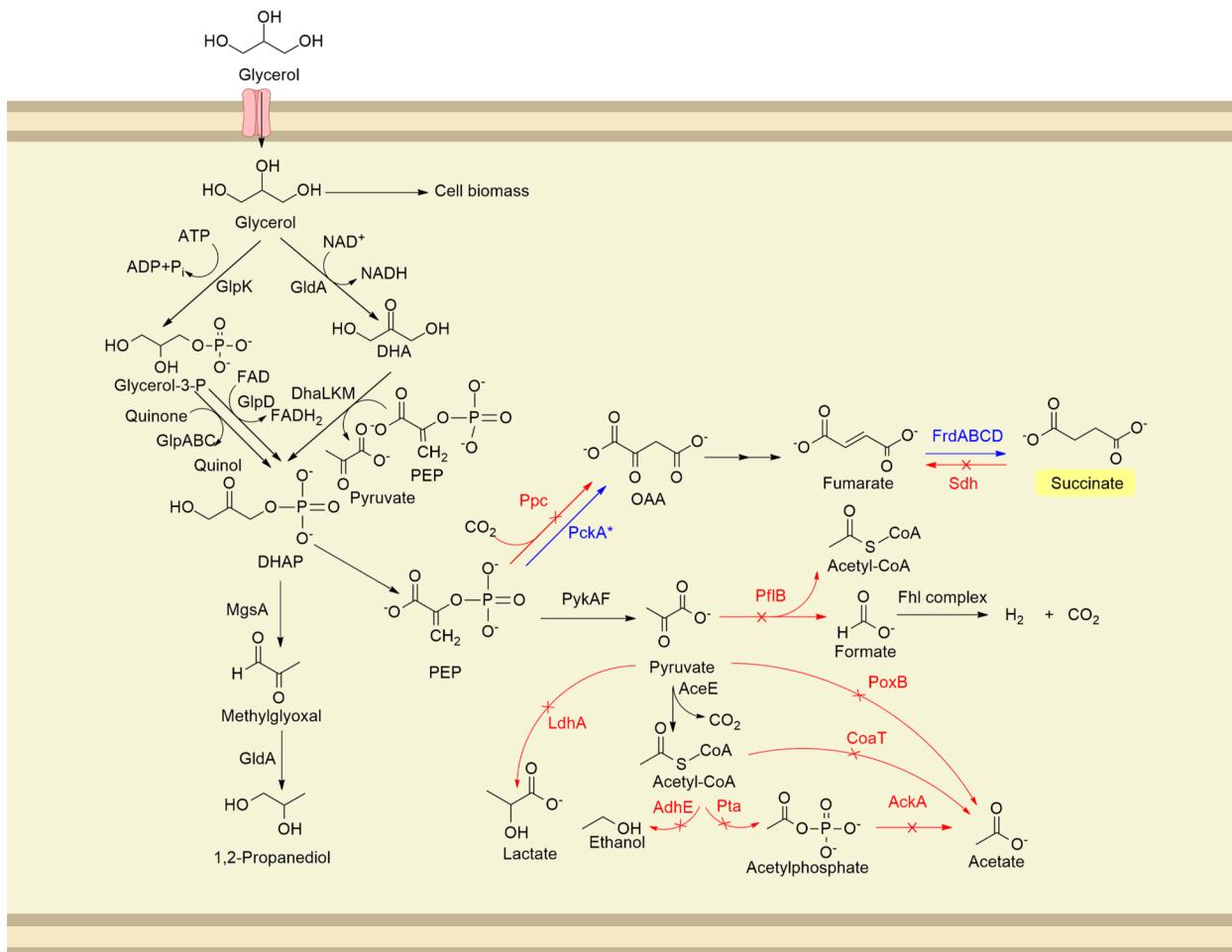
**3.3.2.2. Microbial Transformation for Succinate Production by Metabolic Engineering.** Several microbes do not have all of the pathways and enzymes for succinate production. For example, *A. succinogenes* lacks the glyoxylate shunt, and it lacks citrate synthase and isocitrate dehydrogenase in the TCA cycle. Therefore, this organism cannot produce SA via the glyoxylate cycle and the oxidative path of the TCA cycle, thus leading to a small yield of SA production.<sup>567</sup> Various hosts such as *E. coli*, *Corynebacterium glutamicum*, and *Y. lipolytica* have been engineered to increase their yield of SA production from glycerol. Production of SA can be increased by elimination of enzymes diverting glycerol flux to metabolites of other pathways. In *E. coli*, the gene encoding pyruvate formate lyase (*pflB*) was deleted to eliminate production of formate and ethanol and also to reduce acetate production. However, the engineered cells displayed a slow growth rate because of a lack of sufficient energy or a lack of a sufficient electron source. To solve the problem, addition of energy through formation of ATP was done by increasing the level of phosphoenolpyruvate (PEP) carboxykinase expression by promoter mutation (*pck\**). A high level of PEP carboxykinase can also increase the level of

OAA, which can also push the flux toward succinate production. With increased PEP carboxykinase expression and deletion of phosphoenolpyruvate-dependent phosphotransferase and pyruvate formate lyase genes (*pck<sup>+</sup>*, *ΔldhA*, and *ΔpflB*, and ) in this *E. coli*, the major routes for glycerol utilization under anaerobic conditions to produce ethanol, lactate and acetate were decreased and the production of SA was enhanced, yielding 0.8 mol SA/mol glycerol with a titer of 9.4 g/L.<sup>581,582</sup> In another report, deletion of the competing pathways for production of lactate, ethanol, and acetate (*ΔadhE*, *Δpta*, *ΔpoxB*, *ΔldhA*, and *Δppc*) was carried out in conjunction with expression of *L. lactis* pyruvate carboxylase to drive more of SA production from pyruvate. The insufficiency of ATP was solved by maintaining the condition to be microaerobic so that ATP can be synthesized by oxidative phosphorylation during glycerol utilization. The engineered cells resulted in a titer for succinate production of 14 g/L.<sup>583</sup> For *C. glutamicum*, although the organism has pathways to produce SA, it cannot use glycerol as a carbon source because it lacks a glycerol transporter. Incorporation of the *glpFKD* operon (encoding for a glycerol facilitator, glycerol kinase and glycerol-3-phosphate dehydrogenase) from *E. coli* into *C. glutamicum* resulted in an engineered *C. glutamicum* which can use glycerol as both carbon and energy source.<sup>584</sup> With further deletion of the genes *pqo* (pyruvate:menaquinone oxidoreductase), *ackA* (acetate kinase), *pta* (phosphotransacetylase), and *CoAT* (acetyl-CoA:CoA transferase) to prevent acetate production and the incorporation of *glpFKD* operon in *C. glutamicum*, the engineered cells could produce 9.3 g/L SA (0.21 mol S/mol glycerol) under aerobic conditions (an aerobic process generally gives a maximal yield of 0.5 mol SA/mol glycerol).<sup>585</sup>

For larger scale production, the engineered *E. coli* (*ΔldhA*, *ΔpflB*, and *pck<sup>+</sup>*) was used to produce SA by two-stage fermentation from purified crude glycerol. First, the cells were grown under the aerobic stage, and the expression of PCK was induced. The cells were then harvested and transferred into an anaerobic fermentation process to achieve 0.97 mol SA/mol glycerol.<sup>586</sup> In *Y. lipolytica*, deletion of the *sdh* gene coding for succinate dehydrogenase complex which can turn SA to fumarate in the TCA cycle gave 198.2 g/L SA using fed-batch fermentation (Figure 52).<sup>587</sup>

**3.3.3. 1,2-Propanediol and 1,3-Propanediol.** 1,2-Propanediol (1,2-PDO) or propylene glycol is an important chemical that can be used as a monomer to synthesize polyester resin, paints, antifreeze agents, food additives, flavoring agents, liquid detergents and pharmaceuticals.<sup>588,589</sup> 1,2-PDO is a commodity chemical with a big market size of about 1.4 million tonnes annually with a 4% annual growth.<sup>588</sup> Similar to 1,2-PDO, 1,3-propanediol (1,3-PDO) is also widely used in various applications as an ingredient in ink, polyester fibers, polyurethanes and pharmaceuticals.<sup>590,591</sup> Glycerol can serve as a precursor for synthesis of 1,2-PDO and 1,3-PDO by chemical catalysts. Although a high yield can be obtained for these compounds, the process needs high pressure of more than 2 000 psi and high temperature of more than 200 °C.<sup>588</sup> To reduce the production cost and use mild and environmental friendly conditions, cell factories designed via metabolic engineering approaches offers a route that enables industrial production of 1,2-PDO and 1,3-PDO from glycerol.

**3.3.3.1. Key Enzymatic Reactions for 1,2-Propanediol and 1,3-Propanediol Production.** Some native microorganisms can produce 1,2-PDO from sugar<sup>592–594</sup> while some can produce



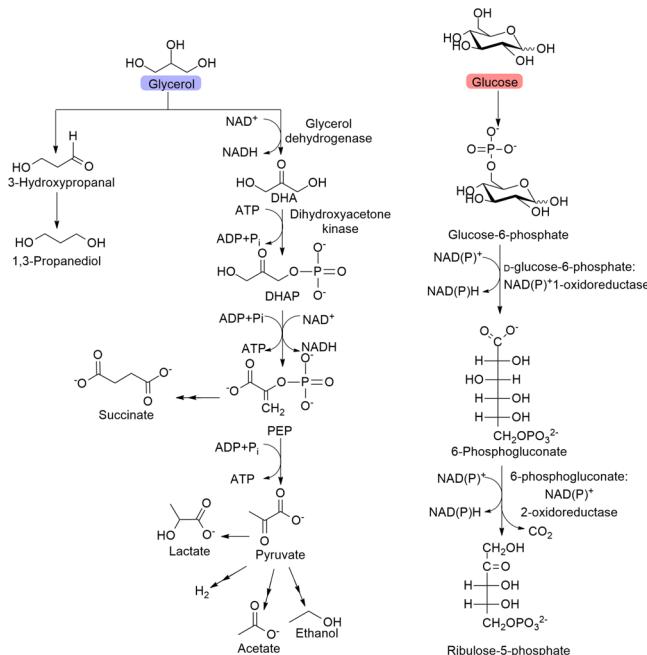
**Figure 52.** Metabolic engineering pathways for succinate synthesis. Abbreviations; DHA: dihydroxyacetone; DHAP: dihydroxyacetonephosphate; PEP: phosphoenolpyruvate; OAA: oxaloacetate; GldA: glycerol dehydrogenase; GlpK: glycerol kinase; GlpD: glycerol-3-phosphate dehydrogenase; MgSA: methylglyoxal synthase; DhaKLM: dihydroxyacetone kinase; Ppc: phosphoenolpyruvate carboxylase; PckA: phosphoenolpyruvate carboxykinase; A: lactate dehydrogenase A; PykAF: pyruvate kinases A and F; AceEF: pyruvate dehydrogenase complex; LpdA: dihydrolipoyle dehydrogenase; AdhE: aldehyde-alcohol dehydrogenase; Pta: phosphate acetyltransferase; PflB: pyruvate formate lyase; PoxB: pyruvate dehydrogenase; CoaT: acetyl-CoA transferase; AckA: acetate kinase; FrdABCD: fumarate reductase subunit A,B,C and D; Sdh: succinate dehydrogenase; Fhl complex: formate hydrogenlyase complex.

1,3-PDO using glycerol as a starting material. These include *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus diolivorans*, *C. pasteurianum*, *C. butyricum*, *K. pneumoniae*, and *Citrobacter freundii*.<sup>595–601</sup> For *L. brevis* and *L. buchneri*, their growth rate on glycerol culture medium is very low. This problem can be solved by using a mixture of glucose and glycerol which provides better growth for microbes. Analysis of products from a mixed medium of glycerol and glucose indicates that glucose is converted to ethanol, lactate, and CO<sub>2</sub>, whereas glycerol is converted to 3-hydroxypropanal and 1,3-PDO.<sup>595</sup> In most of these microbes, glycerol is converted into 3-hydroxypropanal in the first reaction step using a coenzyme B12-dependent glycerol dehydratase. The key enzyme of this pathway, 1,3-PDO oxidoreductase, uses NADH as a reductant, and then converts 3-hydroxypropanal to 1,3-PDO. It was reported that high concentrations of reducing equivalents in the form of NADH can enhance 1,3-PDO formation from glycerol.<sup>602</sup> In general, reducing equivalents can be obtained from the pathway converting glycerol into pyruvate or from other routes that contains NADH-generating dehydrogenases (Figure 53). Using cofeeding of 0.1 mol glucose/mol glycerol, 73.7 g/L of 1,3-PDO could be obtained from the culture of *L.*

*diolivorans*. The titer could be improved to 84.5 g/L by adding vitamin B12, which is a coenzyme of glycerol dehydratase into the culture medium.<sup>601</sup>

**3.3.3.2. Microbial Transformation for 1,2-Propanediol and 1,3-Propanediol Production by Metabolic Engineering.** The native *E. coli* cannot produce 1,2-PDO and 1,3-PDO from glycerol directly. For the pathway leading to production of 1,2-PDO, there is a requirement for the formation of methylglyoxal, which is a toxic metabolite leading to cell growth inhibition.<sup>603</sup> Normally, *E. coli* has a glyoxylase which can convert methylglyoxal into S-lactoylglutathione and lactate. Therefore, the end product of methylglyoxal in native *E. coli* is lactate.<sup>604</sup> For 1,3-PDO synthesis, *E. coli* lacks the activity of an NADH-dependent 1,3-PDO oxidoreductase encoded by *dhaB* gene to convert 3-hydroxypropanal to produce 1,3-PDO because the *dha* regulon is absent.<sup>605</sup> Therefore, a synthetic biology approach has been applied to engineered *E. coli* to produce high amounts of 1,2-PDO and 1,3-PDO to levels applicable for industries.<sup>606,607</sup>

For 1,2-PDO production in the engineered *E. coli*, the genes of *mgsA* (methylglyoxal synthase), *gldA* (glycerol dehydrogenase), and *yqhD* (aldehyde oxidoreductase) were overexpressed,



**Figure 53.** Native metabolic pathways in microbes capable of producing 1,3-PDO using glycerol or a mixture of glycerol and glucose to enhance 1,3-PDO production.

whereas the genes of *ldhA*, *frdA*, *adhE*, and *ackA-pta* were deleted to remove the peripheral pathways which lead to lactate, succinate, ethanol, and acetate production (Figure 54). The gene encoding for *E. coli* PEP-dependent dihydroxyacetone kinase (DHAK) was also replaced by the gene encoding *Citrobacter freundii* ATP-dependent DHAK, resulting in the engineered cell which can produce up to 5.6 g/L of 1,2-PDO.<sup>608</sup> In yeast, the genes of *mgsA*, *gldA*, and *yqhD* were incorporated into *S. cerevisiae*, resulting in the system that can produce 1,2-PDO in high amounts.<sup>609–611</sup> To improve glycerol uptake, addition of a glycerol facilitator of uptake showed significant improvement of *S. cerevisiae* growth on the glycerol synthetic medium.<sup>612</sup> As several enzymes in this pathway require the use of reducing equivalents, the genes encoding for glycerol dehydrogenase from *Ogataea parapolymorpha* (*Opgdh*) and dihydroxyacetone kinase (DAK1) from *S. cerevisiae* were overexpressed, and the endogenous gene of glycerol kinase (GUT1) was deleted to increase NADH availability for the production of 1,2-PDO.<sup>609</sup> In addition, to increase DHAP which is a precursor of methylglyoxal, the activity of endogenous triosephosphate isomerase (TPI1) which also uses DHAP as the substrate was downregulated by substituting the native promoter by a weak promoter, resulting in production of 4.3 g/L of 1,2-PDO.<sup>609</sup>

For production of 1,3-PDO, glycerol can be converted to 3-hydroxypropanal by glycerol dehydratase encoded by *gdht* or *dhaB*. Because *E. coli* lacks this *dhaB* gene, the overexpression of glycerol dehydratase encoded by *dhaB* from *C. freundii* in *E. coli* thus solved this problem and 3-hydroxypropanal can be produced from glycerol. In addition, alcohol dehydrogenase encoded by *yqhD* in *E. coli* identified as an isozyme of 1,3-PDO dehydrogenase was also overexpressed, resulting in the increase of 1,3-PDO production from 41.1 g/L to 62 g/L with a production ratio of 0.81 mol 1,3-PDO/mol glycerol.<sup>613</sup> In another report, the genes of *C. butyricum* *dhaB1* (vitamin B12-independent glycerol dehydratase), *dhaB2* (activating protein

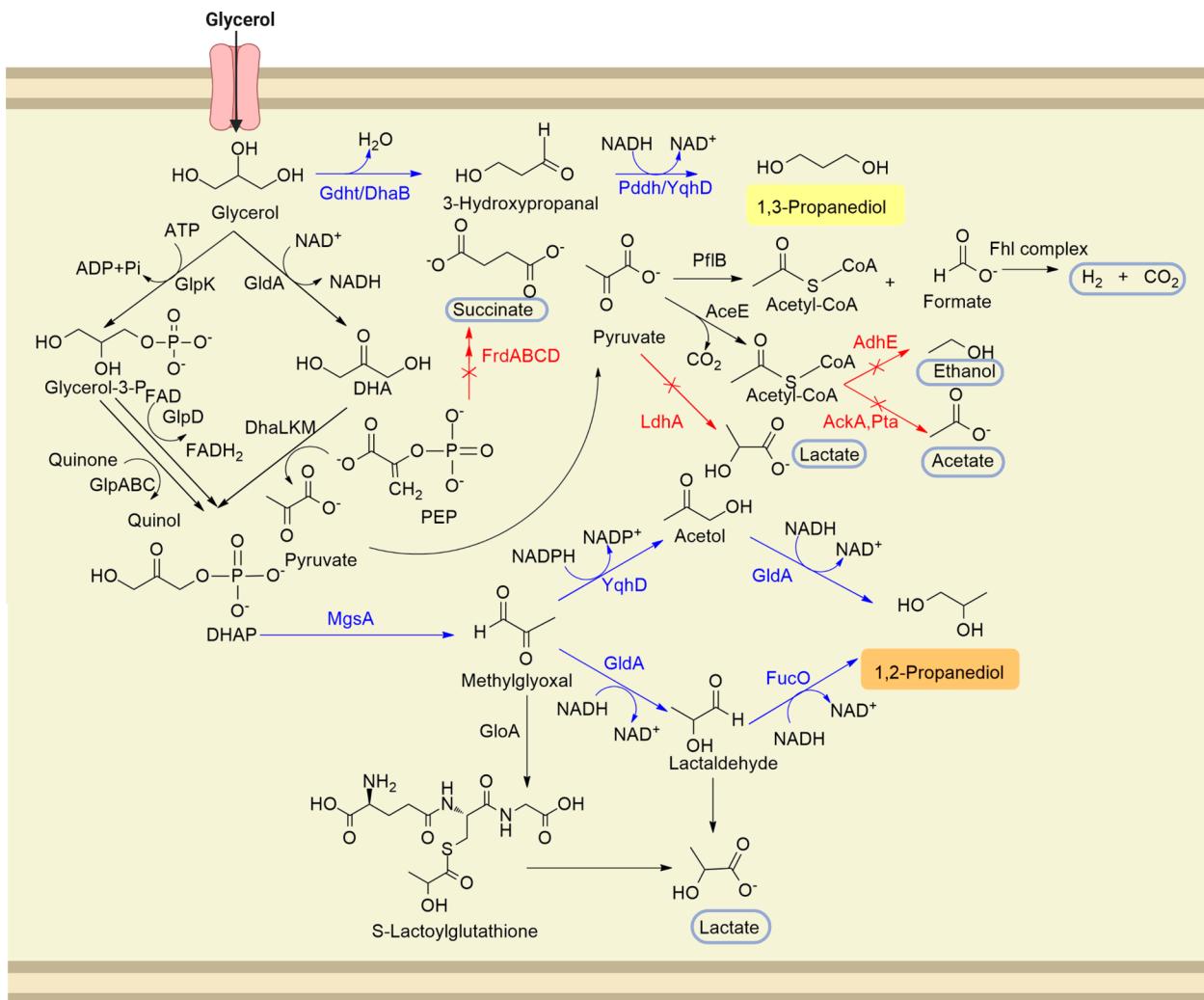
of glycerol dehydratase and alcohol dehydrogenase, *yqhD*) were overexpressed in *E. coli*. They also used pBV220 which is a temperature-sensitive vector that drives the gene from the  $\lambda$  phage PLPR promoter which can be controlled under temperature-sensitive gene induction. Using two-stage fermentation in which the first stage is cell biomass production and the second stage is the glycerol conversion, 1,3-PDO could be produced at the second stage with a titer of 104.4 g/L and a rate of 2.61 g/L h.<sup>614</sup>

### 3.4. Critical Evaluation of Production of Compounds from Triglycerides, Fatty Acids, and Glycerol

Discussion in the previous section highlighted examples demonstrating the production of various valuable and commodity chemicals from triglycerides, fatty acids, and glycerol via enzymes and metabolic engineering. These feedstocks can be obtained from used cooking oil, which is a problematic type of waste that requires disposal. Thus, the bioconversion of these feedstocks to produce biodiesel, one of the most common biofuels, exemplifies the advantage of using a circular economy for waste management and sustainable production. However, due to the rapid development of alternative energy technology, global demand for biodiesel will face challenges, while opportunities for application of the bioconversion of triglycerides, fatty acids and glycerol for clean production of H<sub>2</sub> or synthesis of monomers for specialty materials will continue to grow.

Triglycerides and fatty acids can be converted to common biofuels such as biodiesel and bioalkanes. While enzymatic and microbial production of biodiesel gives high product yield and can be produced at a large scale, the technology to produce bioalkane is only at the lab scale and largescale production has not yet been demonstrated. Recently, production of short-chain alkanes has been demonstrated using the reaction of fatty acid photodecarboxylase (FAP) in *Halomonas* sp.,<sup>615,616</sup> which is much more efficient than the ADO system.<sup>447,484</sup> The engineered *Halomonas* sp. could be grown under nonsterile and alkaline conditions with high salt to prevent growth of other microbes. The alkane production process can continue for 65 days. In future plans, seawater will be used in large scale production to reduce the process cost.<sup>445–447</sup> This technology shows high potential in production of propane/butane for heating, transportation and cooking systems, but will require light energy activation which may be difficult for large scale production. In our opinions, the most challenging issue for production of biofuels in the future is the decrease of product demand. Rapid development of alternative energy technology and the trend toward use of electric vehicle (EV) will decrease the use of internal combustion engine (ICE) cars worldwide.<sup>617</sup> Norway and Netherlands aim to ban all gasoline and diesel cars by 2025, and Germany before 2030.<sup>617</sup> We think that production of bioalkanes via metabolic engineering should be developed to provide material-related, not energy-supplied products in order to maximize the technology value.

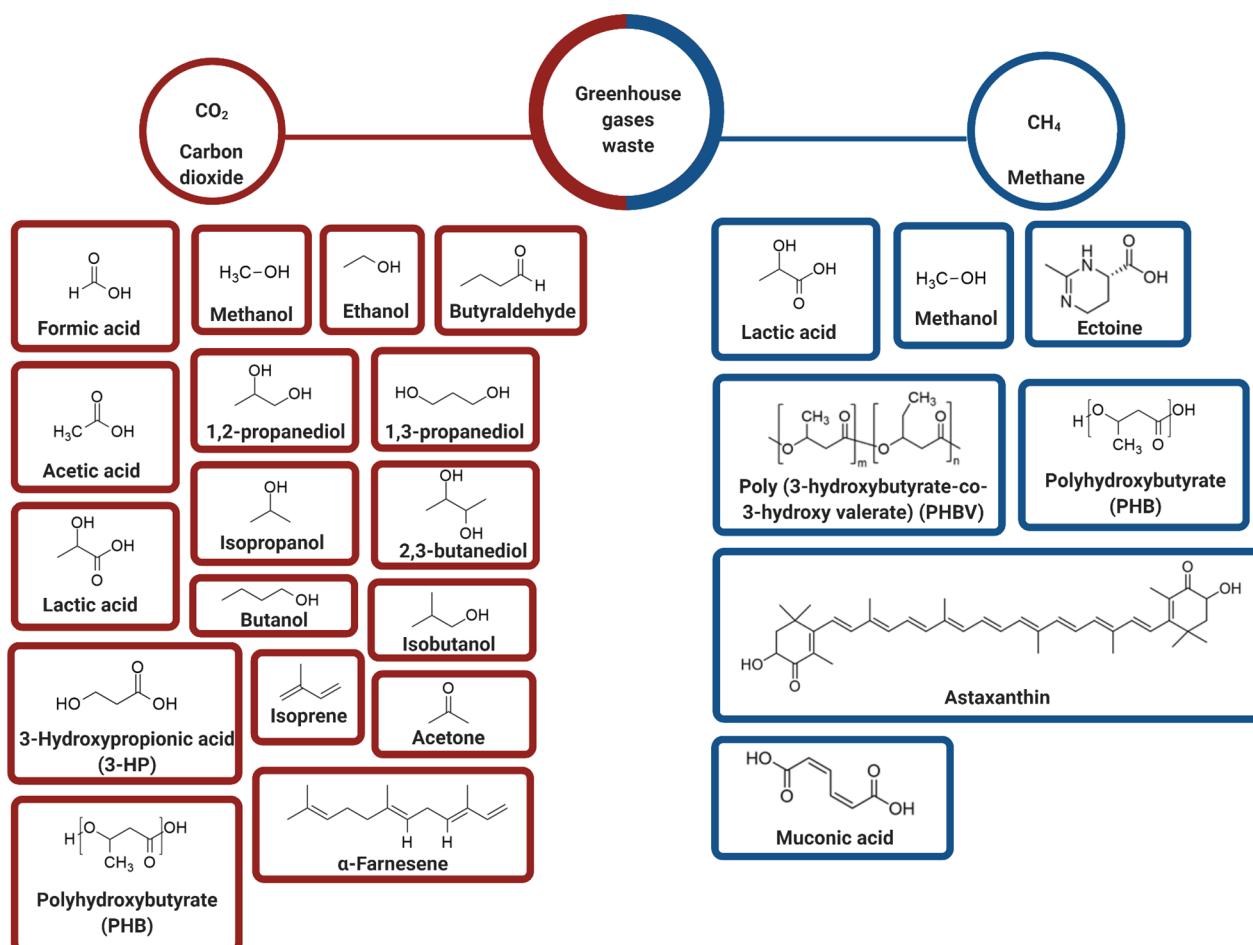
Valuable hydrocarbons such as alkenes and fatty alcohols can be produced from fatty acids. Terminal alkenes are used as starting materials for lubricants.<sup>401–404,404</sup> Their global market was more than \$12.5 billion in 2016 and expected to be \$19 billion in 2024.<sup>618</sup> In our opinions, one-step decarboxylation using a one enzyme system (OleT<sup>510</sup> or UndA<sup>499</sup>/UndB<sup>500</sup>) rather than the protein modules of the OleABCD cluster should be more practical for biocatalyst development because it is easier to manipulate their enzyme and metabolic



**Figure 54.** Engineered pathway for propanediol synthesis using glycerol metabolisms in *E. coli*. Abbreviations; AckA: acetate kinase; AdhE: alcohol dehydrogenase; DhaKLM: dihydroxyacetone kinase; FrdABCD: fumarate reductase; FucO: 1,2-propanediol:NAD<sup>+</sup> 1-oxidoreductase; Gdh/DhaB: glycerol dehydratase; Pddh: 1,3-PDO dehydrogenase; Pta: phosphotransacetylase; GlpK: glycerol kinase; GldA: glycerol dehydrogenase; GlpABC: quinone-dependent glycerol-3-phosphate dehydrogenase; GlpD: glycerol-3-phosphate dehydrogenase; MgsA: methylglyoxal synthase; YqhD: alcohol dehydrogenase; 3-HPA: 3-hydroxypropionaldehyde.

engineering processes. As these enzymes need redox partners which require usage of NAD(P)H inside cells, optimization of redox systems inside hosts would also be required. Each host strain has different redox properties and competing pathways which may need to be modified to enhance the alkene production.<sup>405</sup> For fatty alcohols which are key ingredients in daily products such as cleaning reagents,<sup>405</sup> their global market value was \$6.9 billion in 2016 and expected to increase to over \$10 billion in 2023 due to the COVID-19 pandemic which increased demand for hygienic cleaning reagents.<sup>405</sup> Several metabolic engineering systems give good yield of fatty alcohol production such as the use of FAR expressed in yeast *L. starkeyi* because this strain can tolerate high lipid accumulation.<sup>537</sup> In our opinions, engineered cells capable of producing fatty alcohols from lignocellulosic sugar or high water content oil waste should be useful for attaining a circular economy. Unlike chemical catalysts which require water removal,<sup>619,620</sup> these cells works well in aqueous conditions and can use feedstocks or waste directly to produce fatty alcohols.

Production of succinic acid, 1,2-PDO, 1,3-PDO, and hydrogen from crude glycerol from food wastes or biodiesel wastes provide an attractive circular economy model for glycerol waste utilization. In general, the purity of glycerol from these wastes is 70–90%, preventing its direct use in cosmetics, foods, or pharmaceuticals components. Metabolically engineered cells that can convert glycerol waste to commodity chemicals such H<sub>2</sub>, SA, 1,2-PDO, and 1,3-PDO provide useful paths for increasing the feedstock value. In this case, the use of engineered cells rather than enzymes should be more practical because of the high viscosity of glycerol, which can directly impact enzyme activities while whole-cell biocatalysts would be able to control glycerol levels by localizing the reaction to a cellular compartment. Among all products, we think that H<sub>2</sub> production from glycerol is very interesting due to the likely high demand of H<sub>2</sub> in the future. In addition to industrial processes (i.e., NH<sub>3</sub> synthesis and hydrogenation in food industry), H<sub>2</sub> can be used in electric cars and other fuel cell equipment. Although the engineered *E. coli* harboring the pathway for H<sub>2</sub> production obtained from multiple gene deletion can result in high H<sub>2</sub> production yield (close to



**Figure 55.** Overall products possibly produced from major GHGs, carbon dioxide and methane, via enzymatic reactions and metabolically engineered microbes.

**Table 7. CO<sub>2</sub> and CH<sub>4</sub>-Derived Products and Their Applications**

feedstocks	products	applications	ref
CO <sub>2</sub>	formic acid	chemicals, preservatives, and antibacterial agent	623
CO <sub>2</sub>	acetic acid	composition of vinegar and used as chemicals	624,625
CO <sub>2</sub> , CH <sub>4</sub>	lactic acid	preservatives, acidulant, and flavoring chemicals.	97
CO <sub>2</sub>	3-hydroxypropionic acid	precursor for polymers, metal lubricants, and antistatic agents	626
CO <sub>2</sub> , CH <sub>4</sub>	polyhydroxybutyrate	biocompatible and biodegradable polymer	410
CH <sub>4</sub>	poly(3-hydroxy butyrate- <i>co</i> -3-hydroxy valerate)	biocompatible and biodegradable polymer	627
CH <sub>4</sub>	muconic acid	bioplastics, food additives, agrochemicals, and pharmaceuticals	628
CO <sub>2</sub> , CH <sub>4</sub>	methanol	solvent and chemicals	629
CO <sub>2</sub>	ethanol	biofuel and solvent	98
CO <sub>2</sub>	1,2-propanediol and 1,3-propanediol	precursor for making fibers and polymers, e.g., polytrimethylene terephthalate	100
CO <sub>2</sub>	isopropanol	solvent and chemicals	630
CO <sub>2</sub>	2,3-butanediol	precursor for synthesis of commodity chemicals such as methyl ethyl ketone, gamma-butyrolactone, and 1,3-butadiene	631
CO <sub>2</sub>	butanol	biofuel	99
CO <sub>2</sub>	isobutanol	biofuel and solvent	632
CO <sub>2</sub>	isoprene	precursor for rubber material	633,634
CO <sub>2</sub>	farnesene	lubricants, cosmetics, fragrances, and biofuels	635
CO <sub>2</sub>	acetone	solvent	636
CO <sub>2</sub>	butyraldehyde	precursor for synthesis of alcohols, carboxylic acids, amines, and esters	637
CH <sub>4</sub>	ectoine	moisturizers and osmo-protectant	638
CH <sub>4</sub>	astaxanthin	food additive and anti-inflammatory substance	639

100%),<sup>566</sup> these cells are often weak and showed slow growth rates. Their applications in continuous or large-scale processes

have not yet been demonstrated. We think that an engineering approach to preserve cell robustness should help in the

application of H<sub>2</sub> production from glycerol waste. As glycerol waste obtained from biodiesel production often has a basic pH and contains methanol, host systems should be robust and able to tolerate these conditions.

#### 4. PRODUCTION OF CARBON DIOXIDE- AND METHANE-DERIVED PRODUCTS

As CO<sub>2</sub> and CH<sub>4</sub> are two major GHGs produced by human activities, development of enzymatic or whole-cell catalysts to convert these two GHGs into valuable products is the important hallmark and challenging issues to enable sustainable circular economy. Figure 5S summarizes possible CO<sub>2</sub> and CH<sub>4</sub>-derived products which can be produced based on currently feasible technology. CO<sub>2</sub> is a common byproduct from the metabolism of aerobic microbes; it can be seen that various enzymatic reactions and metabolic engineering systems discussed in the previous sections (3.2 and 3.3) generate CO<sub>2</sub>. In anaerobic digestion systems such as in landfills and waste refinery of agro-industries, CH<sub>4</sub> is produced as a major end product.<sup>621</sup> Several routes of biological reactions can convert these gaseous carbon wastes into chemicals and precursors used in polymers, biofuels, and pharmaceutical industries as summarized in Table 7. However, not all reactions are currently promising for large scale product development. Therefore, only reactions which are potentially possible for future scale up and creating high impact for industries were selected for in-depth discussion. For example, enzymatic reactions and engineered microorganisms which can convert CO<sub>2</sub> and CH<sub>4</sub> to polyhydroxybutyrate (PHB), which is a biocompatible and biodegradable polymer that can be used for fabrication of biomaterials for surgery, tissue engineering, and pharmacology applications,<sup>622</sup> and to C1 products (formic acid and methanol), acetic acid, and lactic acid, are discussed.

##### 4.1. Carbon Dioxide-Derived Products

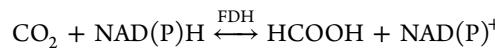
Well-known CO<sub>2</sub> fixation pathways can be classified into six pathways comprised of (1) the Calvin cycle which relates to the pentose phosphate pathway, (2) the Wood–Ljungdahl pathway which directly fixes CO<sub>2</sub> by C<sub>1</sub> carriers, (3) the dicarboxylate/4-hydroxybutyrate (DC/HB) cycle which fixes CO<sub>2</sub> and bicarbonate by pyruvate synthase and PEP carboxylase, respectively, (4) the 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle, which converts one molecule of bicarbonate into malonyl-CoA and another molecule of bicarbonate into methylmalonyl-CoA by acetyl-CoA/propionyl-CoA carboxylase, (5) the reductive TCA cycle which fixes two molecules of CO<sub>2</sub> by reversing the oxidative TCA cycle, and (6) the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle, which uses an *in vitro* reaction network employing 17 enzymes.<sup>640</sup>

In this section, we choose to discuss only selected enzymatic reactions and metabolic routes for production of value-added products from CO<sub>2</sub> because not all pathways referred to above show clear product routes. Metabolic paths of CO<sub>2</sub> utilization in these pathways are different. For example, metabolic pathways for production of formic acid, methanol, and acetic acid are different from those to produce lactic acid, 3-HP, and PHB. The latter compound group requires the biosynthetic route to go through a common precursor molecule such as acetyl-CoA. CO<sub>2</sub> first needs to be entrapped and converted to glyceraldehyde-3-phosphate (G3P), PEP, pyruvate, and then acetyl-CoA. Because acetyl-CoA is an intermediate for several pathways, the metabolic flux can be directed to the desired

pathway by deleting genes and inhibit enzymes of other competing pathways to finally achieve the desired products with high yield and titer.

**4.1.1. Formic Acid and Methanol.** Formic acid and methanol have versatile applications. Formic acid is widely used as a chemical component in preservatives and antibacterial agents.<sup>623</sup> For methanol, it is a common solvent in chemical laboratories and industries. Although methanol exposure (ingestion, dermal absorption, and inhalation) is hazardous because it can be converted into formaldehyde and formic acid in human body,<sup>641</sup> the overall demand of methanol is increasing because of its wide applications. Methanol can be used as the energy source for direct methanol fuel cells (DMFC) or used in transportation fuels (such as gasoline methanol blends or as ingredient in biodiesels).<sup>642</sup> Moreover, it can be directly used as transportation fuel due to its high volumetric energy density.<sup>643</sup> Formic acid and methanol can be synthesized from CO<sub>2</sub> by proton-coupled double-electron reduction using metal and chemical catalysts.<sup>644–647</sup> Methanol and formic acid can be produced through thermal conversion of natural gas and coal, which have long been used in manufacturing processes for centuries.<sup>648</sup> However, the process requires high energy input and releases a large amount of CO<sub>2</sub>.<sup>649</sup> Due to its low utilization of energy and sustainability of the process, the use of biocatalysts to synthesize formic acid and methanol from CO<sub>2</sub> has received increasing attention.

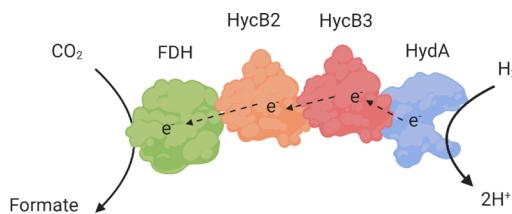
**4.1.1.1. Key Enzymatic Reactions for Production of Formic Acid from CO<sub>2</sub>.** Formate dehydrogenase (FDH) catalyzes a reversible electron transfer reaction from formate to NAD(P)<sup>+</sup> to form CO<sub>2</sub> and NAD(P)H. Therefore, the reverse direction of this reaction can be used to synthesize formic acid from CO<sub>2</sub> under the appropriate conditions. The reversibility of the reaction is controlled by thermodynamic factors i.e. substrate concentrations. Generally, CO<sub>2</sub> reduction is an unfavorable endergonic reaction. Normally, CO<sub>2</sub> is a stable molecule because its molecular structure is linear and nonpolar. The reduction reaction of CO<sub>2</sub> to form formic acid has a reduction potential value of  $-0.61\text{ V}$  which is very low and requires a strong reductant.<sup>650</sup> Therefore, most of the FDHs favor catalysis of formate oxidation using NAD(P)<sup>+</sup> to generate CO<sub>2</sub> and NAD(P)H.<sup>651</sup> For oxidoreductases that can catalyze CO<sub>2</sub> reduction, NADH or NADPH are used as reductants to transfer electrons and H<sup>+</sup> to CO<sub>2</sub>. Various types of enzymes have been reported to be capable of catalyzing the CO<sub>2</sub> reduction to form formate.



FDHs that can catalyze the reduction of carbon dioxide to form formate can be classified into three types: metal-independent/NAD(P)<sup>+</sup>-dependent FDHs, metal-containing/NAD(P)<sup>+</sup>-dependent FDHs and metal-containing/NAD(P)<sup>+</sup>-independent FDHs.<sup>652</sup> For metal-independent/NAD(P)<sup>+</sup>-dependent FDHs, FDH from the yeast *Candida boidinii* (CbFDH) has been studied for its ability to reduce CO<sub>2</sub>. Nevertheless, its CO<sub>2</sub>-reducing activity is very low ( $k_{\text{cat}}$  for CO<sub>2</sub> is  $0.015\text{ s}^{-1}$ ).<sup>176</sup> For metal-dependent/NAD(P)<sup>+</sup>-dependent FDHs which contains tungsten or molybdenum in the active sites, they generally have higher rates of CO<sub>2</sub> reduction than other types of enzymes, and can be found in autotrophic microbes such as *Syntrophobacter fumaroxidans*. Two formate dehydrogenases (FDH1 and FDH2) from *S. fumaroxidans* have a  $k_{\text{cat}}$  of  $2.5 \times 10^3$  and  $0.2 \times 10^3\text{ s}^{-1}$ , respectively.<sup>653</sup>

However, these enzymes are  $O_2$  sensitive, making them difficult for industrial applications. In 2017, FDH from *C. necator* (also known as *Ralstonia eutropha*) (FdsABG), which is an  $O_2$ -tolerant molybdenum-containing/NAD $^{+}$ -dependent FDH, was shown to catalyze a high rate of  $CO_2$  reduction with good oxygen tolerance.<sup>654</sup> Although the  $k_{cat}$  for the reduction of  $CO_2$  reduction is 14-fold lower than the  $k_{cat}$  for the oxidation of formate, FdsABG can catalyze  $CO_2$  reduction by NADH and can be coupled with the reaction of glucose dehydrogenase which can continuously generate NADH; the system has a  $k_{cat}$  of 200 s $^{-1}$ .<sup>179</sup>

For the third group of enzymes, metal-containing/NAD(P) $^{+}$ -independent FDHs, generally contain tungsten, molybdenum, or iron as cofactors in their active sites.<sup>179</sup> Their reactions are limited by their slow rate of  $CO_2$  reduction and their high sensitivity to oxygen.<sup>654</sup> Instead of using NAD(P)H as a reductant, they can use other reductants such as hydrogen as a substrate for their carbon dioxide reductase activities. An example of this enzyme type is hydrogen-dependent carbon dioxide reductase (HDCR) from *Acetobacterium woodii* which has a large protein complex composed of 4 subunits, formate dehydrogenase, Fe–Fe hydrogenase, and two small electron transfer subunits (Figure 56).<sup>655</sup> The purified HDCR can



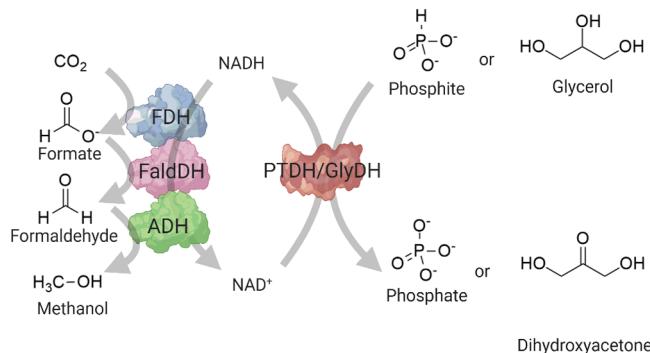
**Figure 56.** Hydrogen-dependent carbon dioxide reductase (HDCR) protein complex which consists of formate dehydrogenase (FDH), Fe–Fe hydrogenase (HydA), and two small electron transfer subunits (HycB2 and HycB3).

catalyze the  $CO_2$  reduction with a turnover frequency (TOF) of about 101 600 h $^{-1}$  at 30 °C which is still much faster than the rate of chemical catalysts (TOF about 70 h $^{-1}$ ) but slower than the metal-dependent/NAD(P) $^{+}$ -dependent FDHs mentioned above.<sup>655</sup> However, a limitation for the use of HDCR is on its requirement to be under anaerobic conditions because its Fe–Fe hydrogenase active site is sensitive to oxygen, making the scale up process challenging. Another enzyme, the Fhl complex in *E. coli* was also reported for its ability to convert  $CO_2$  into formate, similar to HDCR.<sup>656</sup> Fhl consists of FDH and hydrogenase components and it catalyzes the consecutive reduction of protons to generate molecular hydrogen and reduction of  $CO_2$  by hydrogen to form formic acid.<sup>551</sup>

**4.1.1.2. Microbial Transformation for Formic Acid Production by Metabolic Engineering.** As the reduction of  $CO_2$  to form formate has a lower redox potential value than their reductants ( $E^{\circ}$  of  $CO_2$ /formate is  $-420$  mV whereas  $E^{\circ}$  of  $H^+/H_2$  is  $-410$  mV and  $E^{\circ}$  of NAD(P) $^{+}$ /NAD(P)H is  $-320$  mV),<sup>657</sup> the concentrations of reductant per  $CO_2$  need to be maintained at a high ratio in order to drive the reaction forward. It can be seen that, for whole-cell biocatalysis, the reaction of HDCR or Fhl are more promising than the reaction of metal-independent/NAD(P) $^{+}$ -dependent because  $H^+/H_2$  has a lower  $E^{\circ}$  value than NAD(P) $^{+}$ /NAD(P)H. A metabolic engineering approach has been employed to maintain a high concentration of hydrogen inside the cell by deleting the genes

encoding hydrogenase 1 and hydrogenase 2 which catalyze  $H_2$  oxidation, and also the genes encoding PFL activating enzyme and FDH ( $\Delta hyaB$ ,  $\Delta hybC$ ,  $\Delta pflA$ , and  $\Delta fdhE$ ). The engineered *E. coli* could be used to convert  $CO_2$  from a mixture of  $CO_2$ : $H_2$  with a ratio of 56:44 (137.62 mmol/L  $CO_2$  in solution) into formic acid with a 100% yield.<sup>656</sup>

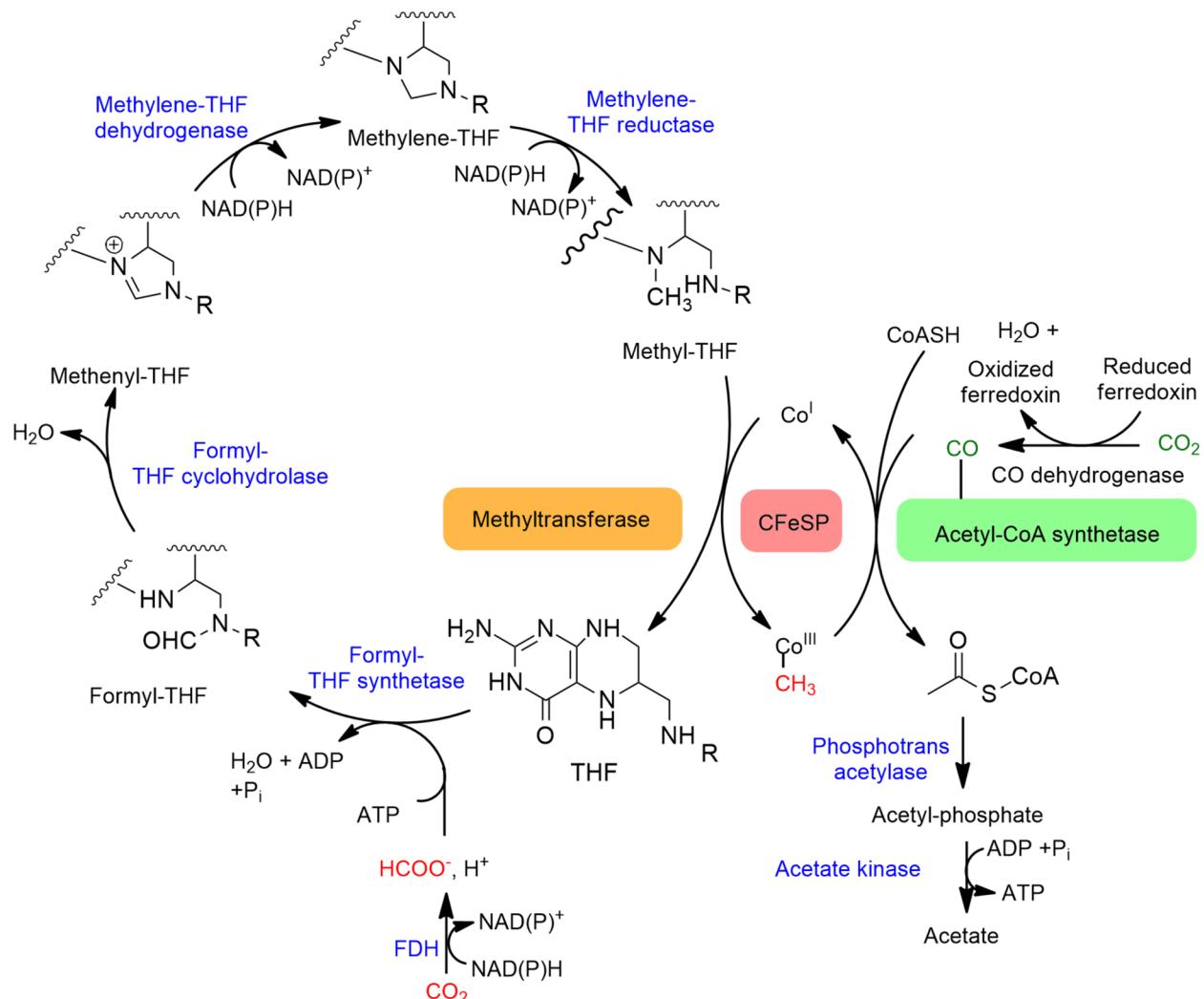
**4.1.1.3. Key Enzymatic Reactions for Methanol Production.** Several types of multienzymatic cascade reactions can be used to convert  $CO_2$  into methanol.<sup>658</sup> Direct and well-known cascade reactions for methanol synthesis make use of three enzymes, formate dehydrogenase (FDH) which catalyzes conversion of  $CO_2$  to formic acid, formaldehyde dehydrogenase (FaldDH) which converts formic acid to formaldehyde, and alcohol dehydrogenase (ADH) which reduces formaldehyde to form methanol (Figure 57).<sup>659–663</sup> These three



**Figure 57.** Multienzymatic cascade reactions for methanol synthesis from  $CO_2$ . This system is comprised of formate dehydrogenase (FDH), formaldehyde dehydrogenase (FaldDH), alcohol dehydrogenase (ADH), and NADH-regenerating systems such as phosphite dehydrogenase (PTDH) or glycerol dehydrogenase (GlyDH).

enzymes need reduced NADH as a reductant.<sup>664</sup> By feeding a high concentration of NADH into a solution containing these three enzymes with  $CO_2$  bubbling, a yield of methanol synthesis of 91.2% could be achieved (1 mol of methanol requires 3 mol of NADH). However, in order to maintain a thermodynamic driving force for the forward direction, this reaction requires high NADH concentrations (up to 100 mM) and produces a very low concentration of methanol (less than 1 mM).<sup>664</sup> As NADH is rather expensive, phosphite dehydrogenase (PTDH), formate dehydrogenase (FDH), or glycerol dehydrogenase (GlyDH) has been applied in the cascade reactions to regenerate NADH.<sup>664,665</sup> Because PTDH gives a higher activity in NADH regeneration than GlyDH,<sup>665</sup> the PTDH reaction can be used in conjunction with 1-ethyl-3-methylimidazolium acetate, an ionic liquid organic cosolvent which also helps increase the solubility of  $CO_2$  in the aqueous phase, resulting in production of 7.86 mM methanol.<sup>664</sup> For metabolically engineered cells, there is no report of any engineered microbes which can produce methanol from  $CO_2$  *in vivo*.

**4.1.2. Acetic Acid.** Acetic acid or acetate is widely used as a precursor for the production of chemicals. For example, acetate is a key component for production of polyvinyl acetate, which is a thermoplastic polymer, and acetate is also commonly used in the food industry in products such as vinegar.<sup>666,667</sup> The acetic acid market size is large, with production amounts reaching greater than 10 trillion kg annually.<sup>668</sup> It can be produced from natural pathways in aerobic acetic acid bacteria such as *Acetobacter* and



**Figure 58.** Acetic acid biosynthesis from the Wood–Ljungdahl pathway by formate dehydrogenase (FDH), formyl-THF synthetase, formyl-THF cyclohydrolase, methylene-THF dehydrogenase, methylene-THF reductase, methyltransferase, corrinoid iron–sulfur protein (CFeSP), acetyl-CoA synthase, phosphotransacetylase, and acetate kinase.

*Gluconobacter* by oxidative fermentation of molasses.<sup>669</sup> Apart from aerobic acetic acid bacteria, another group of bacteria, acetogens, can also produce acetic acid from synthesis gas or Syngas under anaerobic conditions.<sup>670</sup> Acetogens are anaerobic bacteria that can use the Wood–Ljungdahl pathway to conserve their energy and synthesize acetate from  $\text{CO}_2$  as shown below.



The Wood–Ljungdahl pathway is usually found in many ancient organisms including acetogens and methanogens such as *Moorella thermoacetica*,<sup>671</sup> *C. ljungdahlii*,<sup>672</sup> *C. carboxidivorans*,<sup>673</sup> *Eubacterium limosum*,<sup>674</sup> *Methanosarcina barkeri*,<sup>675</sup> *Methanobacterium thermoautotrophicum*,<sup>676</sup> and *Methanobacterium wolfei*.<sup>677</sup> Using the patterns of  $^{12}\text{C}/^{13}\text{C}$  isotopic labeling, the Wood–Ljungdahl pathway in these microbes was investigated. The results also imply that they might be original autotrophs which could use inorganic compounds such as  $\text{CO}$ ,  $\text{CO}_2$ , and  $\text{H}_2$  to synthesize and maintain their cellular ATP level 1 billion years ago before  $\text{O}_2$  was present on the Earth. Therefore, most of enzymes in this metabolic pathway are sensitive to oxygen.<sup>671</sup>

#### 4.1.2.1. Key Enzymatic Reactions for Acetic Acid Production from $\text{CO}_2$

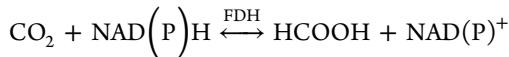
In the Wood–Ljungdahl pathway, the first key step is the conversion of  $\text{CO}_2$  to form methyl-tetrahydrofolate (THF) by formate dehydrogenase. In the next step, formyl-THF synthetase converts THF and formate to formyl-THF using ATP as a cosubstrate. The following two reactions are catalyzed by formyl-THF cyclohydrolase and methylene-THF dehydrogenase to produce methylene-THF from formyl-THF. In *M. thermoacetica*, the cyclohydrolase and dehydrogenase are part of the same bifunctional enzyme, while they are separate monofunctional enzymes in *C. formicoaceticum* and *A. woodii*. Methylene-THF reductase then catalyzes the conversion of methylene-THF to methyl-THF. Methyltransferase then transfers the methyl group of methyl-THF to a cobalt center of the corrinoid iron–sulfur protein (CFeSP) which is a methyl group carrier protein commonly found in anaerobic  $\text{CO}_2$  fixation microbes. In another branch of  $\text{CO}_2$  fixation, another enzyme CO dehydrogenase generates another intermediate, CO from  $\text{CO}_2$ . In the next step, CO is transferred to the active site of acetyl-CoA synthase as a first substrate, followed by transfer of a methyl group from the methylated CFeSP. Acetyl-CoA synthesis is achieved by conversion of two molecules of  $\text{CO}_2$  to methyl and a carbonyl

group, and then binding of CoA to trigger thiolysis of the acetyl-metal bond. Finally, acetate is liberated by the reactions of phosphotransacetylase and acetate kinase (Figure 58).<sup>671</sup>

**4.1.2.2. Microbial Transformation for Acetic Acid Production by Metabolic Engineering.** For several decades, *A. woodii* has been used in industry to produce acetate from CO<sub>2</sub> and H<sub>2</sub> by its Wood–Ljungdahl pathway.<sup>678</sup> Although the microbe can produce acetate under autotrophic conditions, acetate yield obtained from the system is still low.<sup>669</sup> To increase the carbon flux of the Wood–Ljungdahl pathway, THF-dependent enzymes (formyl-THF synthetase, formyl-THF cyclohydrolase, methylene-THF dehydrogenase, and methylene-THF reductase), phosphotransacetylase were overexpressed. In addition, acetate kinase was also overexpressed to increase the ATP level. The genetically modified *A. woodii* could produce 51 g/lacetate under pH-controlled conditions in a stirred-tank reactor with continuous sparging of H<sub>2</sub> and CO<sub>2</sub>.<sup>669</sup>

**4.1.3. Lactic Acid.** Two isomeric forms of lactic acid, D- and L-lactic acid, are widely used as monomers for synthesis of polylactide or poly(lactic acid), a well-known biodegradable polymer used in textiles, drug delivery systems, packaging materials and agricultural films. As the properties of poly(lactic acid) depend on the mixing ratio of D (−) and L (+) lactic acid, production of a pure form of both isomers is necessary for polymer industries.<sup>679</sup> Additionally, D- and L-lactic acid are also used in other important applications such as in food industries.<sup>680</sup> In 2015, the global lactic acid market size was 330 000 tonnes,<sup>681</sup> and it is expected to reach 1 000 000 tonnes/year by 2020 because of increasing demand in bioplastic utilization.<sup>682</sup> With this high demand in production, a sustainable way to produce lactic acid is highly needed. Although lactic acid can be produced via fermentation by microorganisms from crop-derived sugars,<sup>683–687</sup> the cost of biomass pretreatment to obtain sugar materials is still the bottleneck of the whole process. Biomass needs to be saccharified by physicochemical and enzymatic methods using α-amylase or glucoamylase.<sup>688</sup> A more sustainable method to produce lactic acid would be to convert CO<sub>2</sub> which is a major and abundant GHGs to synthesize lactic acid.<sup>689–691</sup> By native pathways, cyanobacteria can produce lactic acid by photosynthesis via the Calvin cycle using only CO<sub>2</sub> as a carbon source and light as an energy source.<sup>692</sup>

**4.1.3.1. Key Enzymatic Reactions for Lactic Acid Production from CO<sub>2</sub>.** Cyanobacteria is a photosynthetic organism that uses CO<sub>2</sub> in the Calvin cycle to produce ATP and G3P, which is a C3 intermediate. G3P can enter the central metabolic pathway to produce pyruvate.<sup>692</sup> Lactate dehydrogenase (LDH) can further convert pyruvate into lactate using NADH as a reductant as shown below. However,

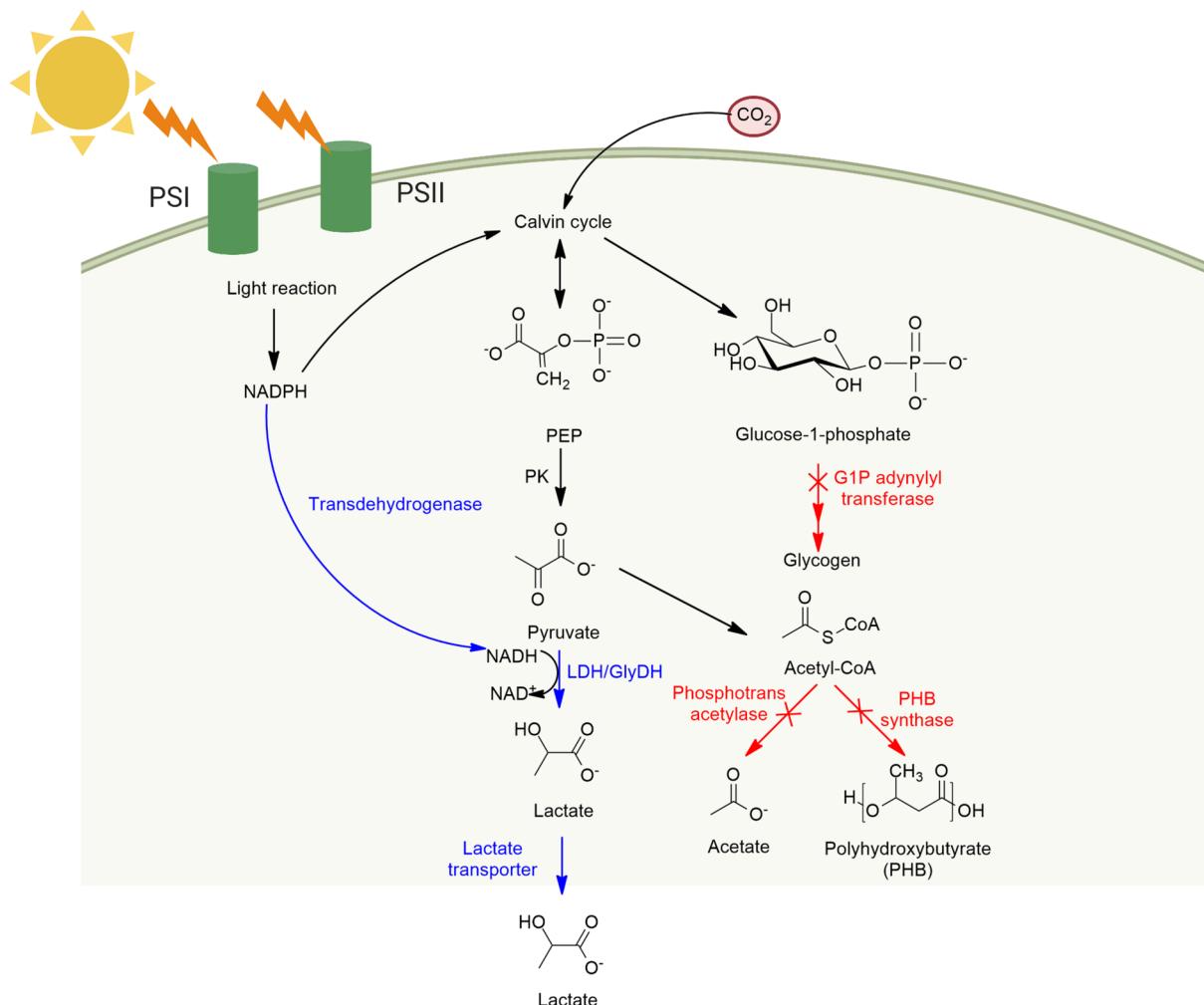


the energy or reductant gained from the photosynthetic process cannot drive the synthesis of lactic acid directly because photosynthesis produces only NADPH, while LDH requires NADH. This challenge makes the LDH reaction the rate-limiting step in lactic acid production by photosynthetic organisms.<sup>692–694</sup> Based on this reason, LDH was engineered to broaden its substrate utilization to be able to use both NADH and NADPH. The triple variant of NADH-dependent LDH (D-LDH) from *Lactobacillus delbrueckii* 11842 (D176S,

I177R, and F178T) has a  $k_{\text{cat}}/K_m$  using NADPH 184-fold (from  $1.82 \times 10^5$  to  $3.36 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) and  $k_{\text{cat}}/K_m$  using NADH ( $5.57 \times 10^6$  to  $8.46 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ) greater than the wild-type enzyme (1.51-fold).<sup>695</sup> Therefore, future approaches for enzyme engineering should be useful for removing the limitations of lactic acid production by photosynthetic microbes.

**4.1.3.2. Microbial Transformation for Lactic Acid Production from CO<sub>2</sub> by Metabolic Engineering.** Besides the limitation of LDH as described in the previous section, the use of cyanobacteria for producing lactic acid is also limited by the fact that it lacks many transporters to transport organic compounds such as sugars, amino acids and lactic acid out of the cell.<sup>696</sup> Therefore, the genes of lactate dehydrogenase (*ldhA*) and lactate transporter-encoding genes (*lldP*) from *E. coli* were introduced into *S. elongatus* PCC7942 to increase the ability of the cyanobacteria to excrete lactic acid into the extracellular media.<sup>696</sup> The gene of NADPH/NADH transhydrogenase from *E. coli* was also expressed in *Synechococcus* in order to convert NADPH produced from photosynthesis to NADH, which can be used by LDH. The engineered *Synechococcus* could produce lactate with a rate of about 54 mg/L/day/OD.<sup>696</sup> The genes encoding for the engineered glycerol dehydrogenase (GlyDH) and soluble transhydrogenase from *P. aeruginosa* were incorporated into *Synechocystis* sp. PCC 6803 to synthesize the optically pure D-lactic acid from CO<sub>2</sub>.<sup>679</sup> The engineered microbe could produce about 1.14 g/L of lactate under photoautotrophic conditions.<sup>679</sup> In 2014, *Synechocystis* sp. PCC6803 was further engineered by increasing the expression level of LDH, coexpressing pyruvate kinase to enhance a pyruvate flux and knocking down phosphoenolpyruvate carboxylase to decrease fluxes of other competing pathways. The engineered microbe was further improved by incorporating the LDH variant which has high affinity to NADPH. This results in a bioprocess which can produce 0.83 mg/L L-lactic acid.<sup>691</sup> Further investigation incorporated the LDH variant in which its substrate preference was switched from NADH to NADPH and the lactic acid transporter, resulted in the engineered *S. elongatus* PCC7942 which could produce D-lactate product at an amount of 1.31 g/L from CO<sub>2</sub>-enriched air bubbling (Figure 59).<sup>697</sup>

**4.1.4. 3-Hydroxypropionic Acid.** 3-Hydroxypropionic acid or 3-hydroxypropanoate (3-HP) is ranked as one of the top three important chemicals which can be derived from biomass by the US Department of Energy (DOE).<sup>626</sup> As 3-HP consists of carboxyl and hydroxyl groups, the compound is versatile for further synthesis or bioconversion process to produce specialty chemicals and materials. For example, it can be used to produce acrylic acid, methyl acrylate, malonic acid, ethyl 3-HP, 1,3-PDO, propiolactone, and acrylonitrile which are common cross-linking agents for polymers.<sup>626</sup> Moreover, 3-HP can be used as a starting material for the synthesis of propiolactone and polyesters by cyclization and polymerization reactions, respectively.<sup>698</sup> Another important property of 3-HP is its biocompatibility. 3-HP can be used to produce poly(3-hydroxypropionic acid), a biodegradable polymer which has been used in drug industries as drug-delivery materials.<sup>699</sup> Although the market size of 3-HP is expected to be as high as 3.6 million tonnes/year,<sup>255</sup> 3-HP production in large industrial scale has not yet been widely operated because the process cost is high and not green for the environment.<sup>700</sup> Therefore, production of 3-HP from biobased feedstock and using biological approaches has been an active area of research.



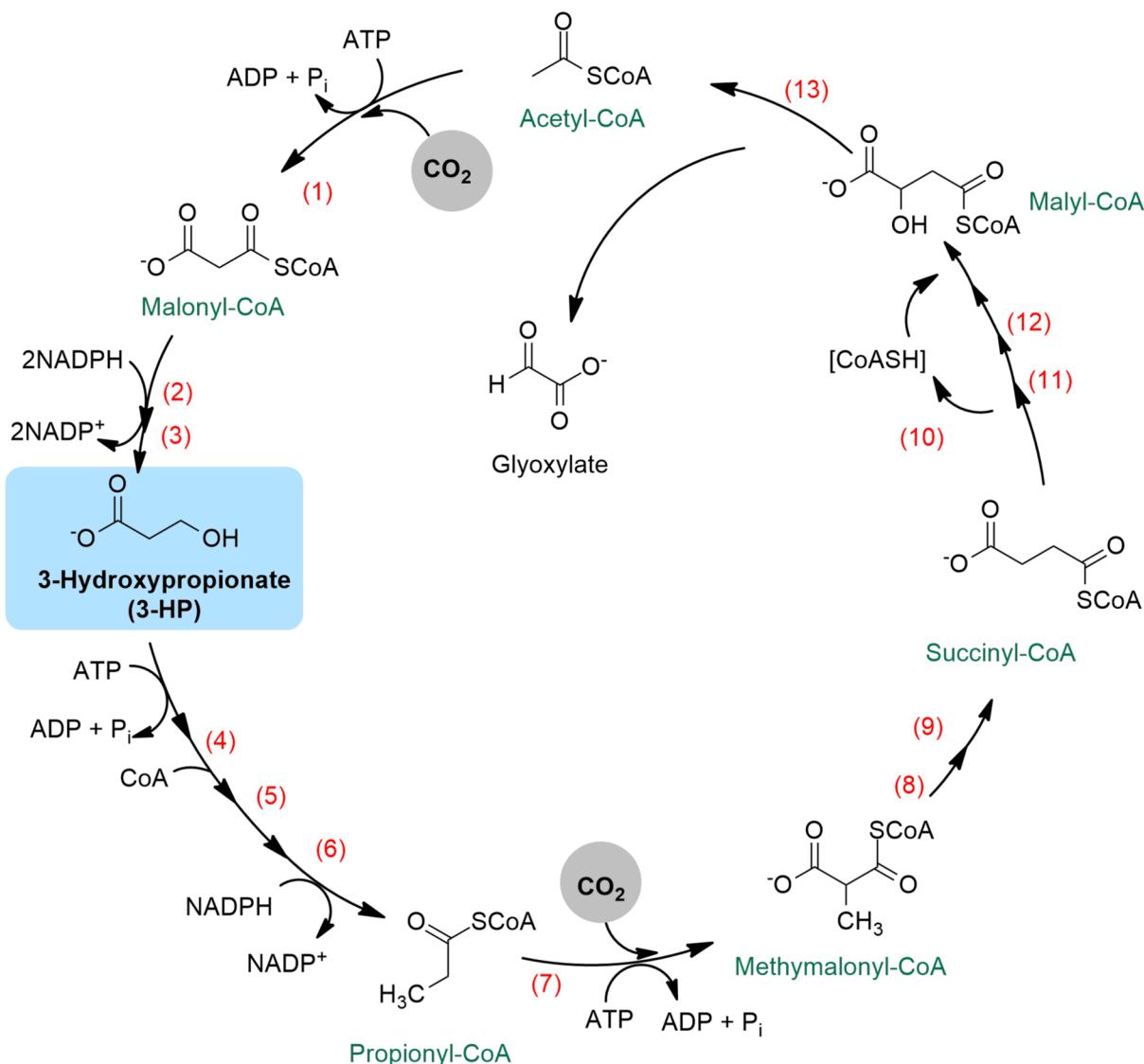
**Figure 59.** Metabolic engineering of lactic acid production pathways in cyanobacteria which use  $\text{CO}_2$  as a carbon source and light as an energy source. To reduce competing pathways, the lactate-utilizing pathway was deleted as shown in red (G1P adenylyltransferase; glucose-1-phosphate adenylyl transferase, PHB synthase, polyhydroxybutyrate synthase and phosphotransacetylase) and lactate producing enzymes with improved activities were incorporated as shown in blue (LDH: lactate dehydrogenase; GlyDH: glycerol dehydrogenase).

Several native microbes are known to be natural 3-HP producers such as *Alcaligenes faecalis* and *Rhodococcus erythropolis*, producing 3-HP from acrylic acid,<sup>264</sup> and *S. kluuyveri* producing 3-HP from ammonia and carbon dioxide derived from degradation products of a pyrimidine base via uracil catabolisms.<sup>626,701</sup> However, these technologies still are not economically feasible because the price of starting materials mentioned is quite high.<sup>698</sup> Therefore, the approach of using  $\text{CO}_2$  to synthesize 3-HP directly has been explored (Figure 60). A thermophilic photosynthetic bacterium *Chloroflexus aurantiacus* can also synthesize 3-HP during phototrophic growth through the 3-hydroxypropionate cycle.<sup>626</sup> With the capability to directly fix  $\text{CO}_2$  and convert it into 3-HP product, this technology is more attractive in terms of cost and environmental impact.

**4.1.4.1. Key Enzymatic Reactions for 3-Hydroxypropionic Acid Production.** Key enzymatic steps to synthesize 3-hydroxypropionate in the 3-HP pathway occur via the reactions to condense two molecules of  $\text{CO}_2$  (or bicarbonate) to form one molecule of glyoxylate. As shown in Figure 60,<sup>702</sup> the reaction can occur through carboxylation of acetyl-CoA to form malonyl-CoA by ATP-dependent biotin-containing acetyl-CoA carboxylase or Acc which requires covalent biotin

attachment (1). Malonyl-CoA reductase or Mcr (2) then converts malonyl-CoA into malonate semialdehyde (2) and malonate semialdehyde reductase or Msr catalyzes further reduction to form 3-hydroxypropionate by using two NADPH molecules (3).<sup>703</sup> However, availability of the key precursor, malonyl-CoA, is generally limited for 3-HP synthesis because the compound is important for cellular synthesis of other important compounds such as ketones and fatty acids.<sup>704</sup> Another challenge of 3-HP synthesis is the rate-limiting step of this pathway, previously identified as the reaction of Mcr.<sup>705</sup> Effects of Mcr and Msr on 3-HP production were investigated using *in vitro* assays and measurement of steady-state kinetics parameters of Mcr and Msr. The  $K_m$  and specific activity of Mcr for malonyl-CoA were  $100 \mu\text{M}$  and  $4.6 \mu\text{M}/\text{min}/\text{mg}$ ,<sup>706</sup> respectively, whereas the  $K_m$  and specific activity of Msr for malonic semialdehyde were  $70 \pm 10 \mu\text{M}$  and  $200 \mu\text{M}/\text{min}/\text{mg}$ , respectively.<sup>707</sup> Therefore, several research groups have focused on increasing the Mcr activity and availability of malonyl-CoA for 3-HP synthesis.

**4.1.4.2. Microbial Transformation for 3-Hydroxypropionic Acid Production by Metabolic Engineering.** Cyanobacteria are known prokaryotes capable of carrying out plant-like oxygenic photosynthesis. They are important in assimilation of

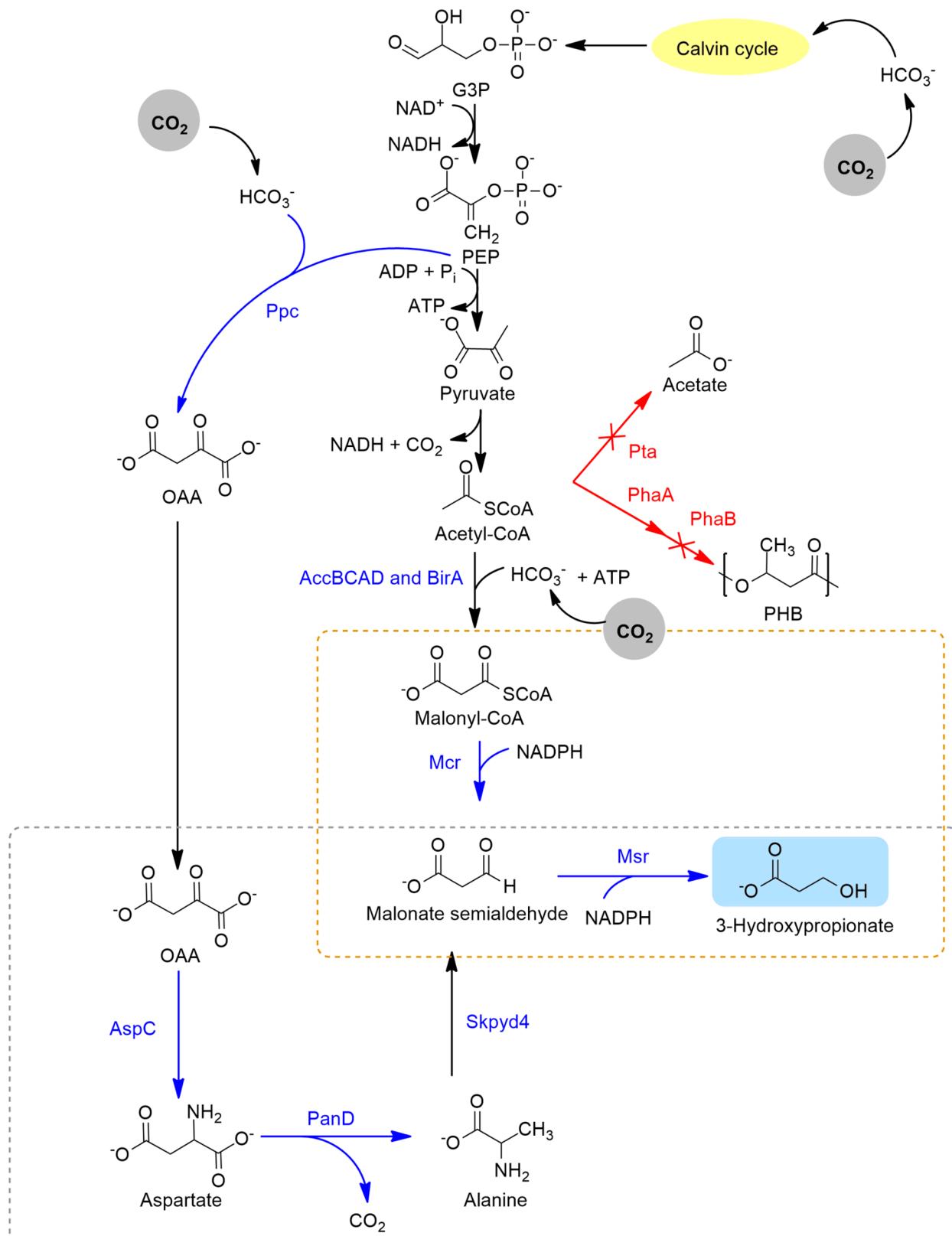


**Figure 60.** Pathway for production of 3-hydroxypropionate from  $\text{CO}_2$ . The pathway for  $\text{CO}_2$  fixation is comprised of (1) acetyl-CoA carboxylase; (2) malonyl-CoA reductase ( $\text{NADPH}$ ); (3) malonate semialdehyde reductase ( $\text{NADP}^+$ ); (4) 3-hydroxypropionyl-CoA synthetase; (5) 3-hydroxypropionyl-CoA dehydratase; (6) acryloyl-CoA reductase ( $\text{NADPH}$ ); (7) propionyl-CoA carboxylase; (8) methylmalonyl-CoA epimerase; (9) methylmalonyl-CoA mutase; (10) succinyl-CoA: L-malate-CoA transferase; (11) succinate dehydrogenase; (12) fumarate hydratase; and (13) L-malyl-CoA lyase. The figure was adapted with permission from Herter et al.<sup>702</sup> Copyright © (2001) American Society for Microbiology.

$\text{CO}_2$  and recycling of  $\text{O}_2$ .<sup>708</sup> *Synechocystis* sp. has been successfully engineered to use  $\text{CO}_2$  and produce several products.<sup>709</sup> For 3-HP synthesis, 3-HP is synthesized through a malonate semialdehyde (Msa) intermediate (generally called the malonyl-CoA dependent pathway), which is reduced to form 3-HP.<sup>710</sup> To improve the titer of 3-HP formation, malonyl-CoA dependent and  $\beta$ -alanine dependent pathways were incorporated into *S. elongatus* PCC 7942 to increase formation of precursors for 3-HP. For the malonyl-CoA dependent pathway, the genes of malonyl-CoA reductase (*mcr*) from *Sulfolobus tokodaii* was and malonate semialdehyde dehydrogenase (*msr*) from *Metallosphaera sedula* were integrated into the *S. elongatus* PCC 7942 genome. For expressing enzymes in the  $\beta$ -alanine dependent pathway (aspartate transaminase or AspC from *E. coli*, aspartate decarboxylase or PanD from *E. coli* and  $\beta$ -alanine transaminase from *S. kluyveri*), their genes were integrated into the *S. elongatus* PCC 7942 genome to increase the carbon flux for 3-

HP synthesis from the  $\beta$ -alanine pathway (Figure 61). These engineering efforts yielded a final titer of 665 mg/L 3-HP.<sup>710</sup> In 2016, *Synechocystis* sp. PCC 6803 was used as a host for 3-HP synthesis. Expression of *mcr* was controlled by a different promoter, and expression of Acc and biotinilase were also increased. The level of NADPH, which is a reductant substrate for *Mcr*, was increased by overexpression of NAD(P) transhydrogenase to convert NADPH to NADH. These efforts resulted in a cell that can generate 837.2 mg/L of 3-HP.<sup>711</sup>

**4.1.5. Polyhydroxybutyrate.** Polyhydroxybutyrate (PHB) is a biopolymer that can be produced by sugar and lipid fermentation of bacteria. PHB can be accumulated in bacterial cells and used as a conserved carbon and energy source.<sup>712</sup> As mechanical properties of PHB are comparable to polypropylene, it can be used to replace synthetic polymer.<sup>713</sup> PHB is classified as a biodegradable thermoplastic with a high melting temperature, a high degree of crystallinity and low permeability to  $\text{O}_2$ ,  $\text{H}_2\text{O}$  and  $\text{CO}_2$ .<sup>714</sup> PHB has wide



**Figure 61.** Enhancing 3-HP biosynthesis from  $\text{CO}_2$  in cyanobacteria *via* metabolic engineering. The malonyl-CoA dependent pathway is shown in the yellow box, while the  $\beta$ -alanine dependent pathway is shown in the gray box. Abbreviations: G3P: glyceraldehyde-3-phosphate; PEP: phosphoenolpyruvate; Ppc: phosphoenolpyruvate carboxylase; AspC: aspartate aminotransferase; PanD: PLP-independent aspartate decarboxylase; Adc: PLP-dependent aspartate decarboxylase; Skpyd4:  $\beta$ -alanine aminotransferase; Mcr: malonyl-CoA reductase; Msr: malonate semialdehyde reductase; AccBCAD: acetyl-CoA carboxylase subunit B, C, A and D; BirA: biotinilase; PntA: NAD(P) transhydrogenase subunit A; PntB: NAD(P) transhydrogenase subunit B; PhaB: PHA-specific acetoacetyl-CoA reductase; Pta: phosphate acetyltransferase.

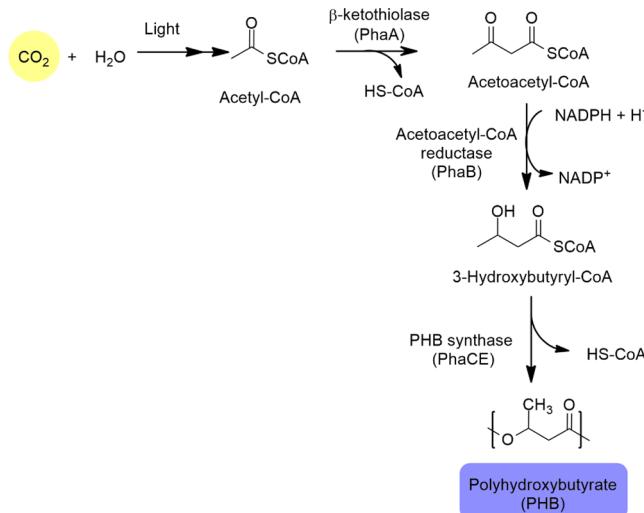
applications, especially as high value plastics used in food packaging,<sup>410</sup> medical implantation, and as material in controlled release systems for drug release in the human body because it is biocompatible, is slowly degraded, and does not cause inflammatory effects.<sup>715</sup>

**4.1.5.1. Key Enzymatic Reactions for Polyhydroxybutyrate Production from CO<sub>2</sub>.** Several species of cyanobacteria including *Gloeocapsa* sp., *Spirulina platensis*, *Aphanothecace* sp., *Oscillatoria limosa*, *Anabaena cylindrica*, *Synechococcus* sp. and *Synechocystis* sp. can produce PHB from CO<sub>2</sub>.<sup>716–720</sup> CO<sub>2</sub> is assimilated through acetyl-CoA via the Calvin cycle. Two molecules of acetyl-CoA are then condensed via Claisen condensation to form acetoacetyl-CoA by  $\beta$ -ketothiolase (PhaA). Acetoacetyl-CoA is then reduced by acetoacetyl-CoA reductase (PhaB) using NADPH as a reductant to form 3-hydroxybutyryl-CoA. Finally, PHB synthase (comprising PhaC and PhaE) catalyzes liberation of CoA to form the PHB polymeric product (Figure 62).<sup>721–723</sup> Besides cyanobac-

containing only PhaA and PhaB (26% product in dry cell weight).<sup>725</sup> To increase the productivity and efficiency of using CO<sub>2</sub> as a carbon source, random mutagenesis of *Synechocystis* sp. PCC 6714 by UV mutagenesis treatment was carried out, resulting in a mutant with 2.5-fold higher PHB productivity and higher CO<sub>2</sub> consumption rate than the wild-type strain (37  $\pm$  4% PHB accumulated per dry cell weight). Results from quantitative real-time PCR showed that the genes *cmpA* (bicarbonate-binding protein CmpA), *cmpB* (bicarbonate transport system permease protein), *cmpC* (bicarbonate transport ATP-binding protein), and *sbtA* (bicarbonate transporter) which are involved in bicarbonate transportation in the mutant, were upregulated, and there were also higher expression levels for *rbcL* (Rubisco), which is involved in CO<sub>2</sub> uptake. However, the expression level of *phaA*, *phaB*, *phaC*, and *phaE* genes were unchanged. These results suggested that CO<sub>2</sub> transport and uptake are important for improving PHB synthesis.<sup>721</sup>

Instead of obtaining PHB accumulated inside cells as insoluble granules, obtaining the product in monomeric forms, especially in stereospecific compounds such as (*S*)- and (*R*)-3-hydroxybutyrate are more helpful for downstream processing and applications. The monomeric PHB can also be secreted into the extracellular environment, decreasing the chances of damaging the cell and also reducing the cost for downstream processing.<sup>658</sup> Because (*S*)- and (*R*)-3-hydroxybutyrate can be naturally produced and secreted from *Synechocystis* cells,<sup>726</sup> this species was selected as a host for further engineering. To synthesize (*S*)-3-hydroxybutyrate, several genes encoding relevant enzymes were integrated into the *Synechocystis* sp. PCC 6803 genome. These include genes encoding thiolase (*thil*) from *C. acetobutylicum* which can produce acetoacetyl-CoA from acetyl-CoA, acetoacetyl-CoA reductase (*hbd*) from *C. acetobutylicum* which has a specific activity to produce (*S*)-3-hydroxybutyryl-CoA from acetoacetyl-CoA, thioesterase (*tesB*) from *E. coli* to liberate CoA and generate (*S*)-3-hydroxybutyrate. For production of (*R*)-3-hydroxybutyrate, the genes encoding *R. eutropha* thiolase (*phaA*), *R. eutropha* acetoacetyl-CoA reductase (*phaB*), and *E. coli* thioesterase (*tesB*) were integrated into the *Synechocystis* sp. PCC 6803 genome. The key step for (*R*)-3-hydroxybutyrate production is *R*-specific PhaB which can convert acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA. TesB is a general hydrolase which can cleave CoA from both (*S*)- and (*R*)-hydroxybutyryl-CoA. To avoid formation of PHB granules, the gene encoding PHB polymerase (PhaEC) in cyanobacteria was deleted (Figure 63). The current technology can produce (*R*)-3-hydroxybutyrate by deletion of *phaEC*, integration of *phaA*, *phaB*, and *tesB* in *Synechocystis* sp. PCC 6803, resulting in production of 533.4 mg/L after continuous cultivation for 21 days.<sup>726,727</sup>

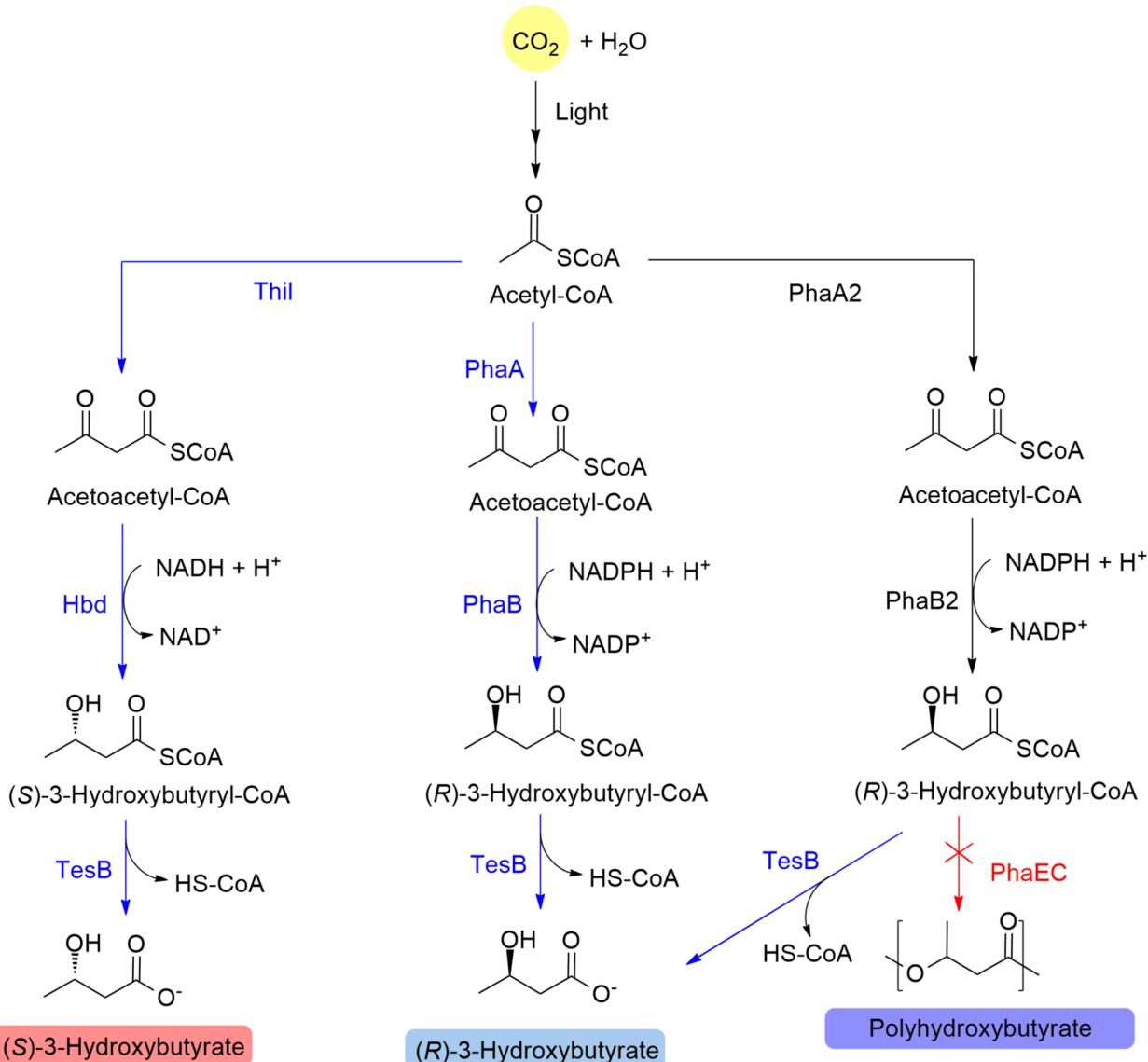
In addition to enzymatic reactions and metabolically engineered pathways discussed above which focus on production of commodity products from CO<sub>2</sub>, other new catalytic routes, particularly the CETCH cycle have also shown a promising approach to overcoming the limitations of the natural CO<sub>2</sub> fixation pathways. In the CETCH cycle, the CO<sub>2</sub> fixation is catalyzed by the engineered carboxylase (enoyl-CoA carboxylases/reductases (ECRs)) which is oxygen-insensitive and much more effective than RuBisCO. In the latest version of the CETCH cycle, 17 enzymes from 9 different organisms which were optimized by enzyme engineering and metabolic proofreading can catalyze CO<sub>2</sub> fixation with a specificity of 5



**Figure 62.** Polyhydroxybutyrate (PHB) synthesis pathway using CO<sub>2</sub> as a starting compound.

teria, *R. eutropha* can also use H<sub>2</sub>/CO<sub>2</sub> for growth to synthesize PHB.<sup>724</sup> Under limiting nutrients, *R. eutropha* can store a large amount of PHB in granules at amounts as high as 61 g/L.

**4.1.5.2. Microbial Transformation for Polyhydroxybutyrate Production by Metabolic Engineering.** As the genetics of cyanobacteria is easier to engineer than that of *R. eutropha*, most of the metabolic engineering work to improve PHB production from CO<sub>2</sub> was mostly done with *Synechocystis*. The native *Synechocystis* sp. PCC 6803 genome also contains the sequence of *phaA*, *phaB*, and *phaEC* encoding for  $\beta$ -ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHB synthase (PhaEC) that are important for producing PHB as mentioned in the previous section. Expression of these genes in *Synechocystis* sp. PCC 6803 was done by gene upregulation using nitrogen-deprived medium (BG11 medium without NaNO<sub>3</sub>) containing 0.4% (w/v) acetate. Under nitrogen depletion, the amino acid synthesis pathway is blocked, especially glutamate synthesis from  $\alpha$ -ketoglutarate, leading to accumulation of cellular NADPH, which is needed for the PhaB reaction. With upregulation of *phaA*, *phaB*, and *phaEC* expression, a 1.5-fold increase of PHB production compared to the wild-type strain was found. However, the maximum PHB production (2.6-fold increase) was observed in the cells



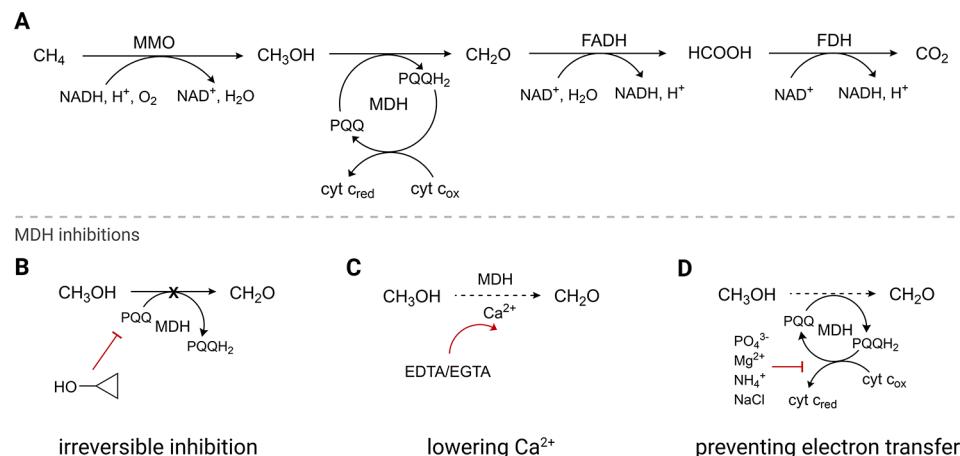
**Figure 63.** Metabolic engineered pathways for production of (S)- and (R)-3-hydroxybutyrate in *Synechocystis* sp. PCC 6803. Abbreviations; Thil: thiolase from *C. acetobutylicum*; Hbd: acetoacetyl-CoA reductase from *C. acetobutylicum*; TesB: thioesterase from *E. coli* K12; PhaA: thiolase from *R. eutropha* H16; PhaB: acetoacetyl-CoA reductase from *R. eutropha* H16; PhaA2: thiolase in *Synechocystis* sp. PCC 6803; PhaB2: acetoacetyl-CoA reductase in *Synechocystis* sp. PCC 6803; PhaEC: PHB polymerase in *Synechocystis* sp. PCC 6803. The figure was adapted with permission from Wang et al.<sup>727</sup> Copyright © (2013) Elsevier.

nmol/min/mg<sub>protein</sub> which is higher than the  $\text{CO}_2$  fraction in the Calvin cycle (1 to 3 nmol/min/mg<sub>protein</sub>).<sup>728</sup> It should be interesting to see further development of the CETCH cycle to produce biochemicals or biomaterials from  $\text{CO}_2$  in the future.

#### 4.2. Methane-Derived Products

Methane-utilizing bacteria or methanotrophs are microorganisms capable of carbon sequestration and conversion of GHGs to valuable compounds including fuels and chemicals.<sup>729</sup> The conversion of gaseous waste streams from industry or from anaerobic digestion-derived biogas obtained from sludge or landfills by methanotrophs to valuable chemicals provides a sustainable means for reducing the negative environmental impact of GHGs. Therefore, gaining an understanding of methanotrophic metabolisms is important for guiding their applications as biocatalysts in this respect and for fighting against the climate change crisis.

Several products such as liquid fuels, bioplastics and commodity chemicals can be obtained from biotransformation of methane by native or genetically engineered methanotrophs (Figure 55). Production of methanol from  $\text{CH}_4$  is one of the most investigated systems, as it can be produced by the native metabolic pathways. Production of PHBs and lactic acids has received more attention and has become increasingly attractive due to the value of PHBs as described in the previous section. Methanotrophs can also convert methane to ectoine<sup>730</sup> and astaxanthin.<sup>731</sup> However, the yield was still quite low, ranging from 1 to 100 mg/g biomass. A recent report showed that methane can be used to produce muconic acid, a monomer for production of high-value commodity chemicals such as terephthalic acid and adipic acid although the yield is still low.<sup>732</sup> Therefore, we discuss herein the recent advances in metabolic engineering and synthetic biology for production of methanol, PHB, and lactic acid from methane, as their



**Figure 64.** Methane metabolism in methanotrophs and different mechanisms of MDH inhibition for increasing methanol yield. (A) Methane oxidation pathway in methanotrophs. (B) Irreversible inhibition. (C) Inhibition via lowering the concentration of free  $\text{Ca}^{2+}$  ions. (D) Inhibition via preventing electron transfer. Abbreviations; MMO: methane monooxygenase; MDH: methanol dehydrogenase; FADH: formaldehyde dehydrogenase; FDH: formate dehydrogenase.

production yields are more promising and it should be possible to reach production levels to meet the demands of real applications in the near future.

**4.2.1. Methanol.** As previously described in section 4.1.1, methanol is an important commodity chemical with rising global demand. Besides  $\text{CO}_2$ , methane, which is another GHG, can also be used as a carbon source for methanol bioproduction by microorganisms. Compared to  $\text{CO}_2$ , use of  $\text{CH}_4$  is more feasible for implementation in a large scale bioproduction of methanol because methane oxidation can be combined with waste treatment in one system. In nature, two groups of bacteria have been found to be able to oxidize  $\text{CH}_4$  to methanol under ambient conditions.<sup>733</sup> One group consists of aerobic methanotrophic bacteria or methanotrophs which utilize  $\text{CH}_4$  as their carbon source. Another group includes ammonia-oxidizing bacteria (AOB), which can partially oxidize  $\text{CH}_4$  to methanol when using ammonia as an energy source. Among these two bacterial groups, methane metabolism in methanotrophs is better understood and the microbes have been extensively investigated for potential biomethanol production from methane. This section will focus on the recent bioconversion of methane from natural and engineered strains, particularly in methanotrophs.

**4.2.1.1. Methanotrophic Bacteria (Methanotrophs).** Methane-utilizing microbes or methanotrophs can utilize methane as a sole carbon source and generate carbon dioxide as the final oxidized product through its oxygen-dependent methane metabolic pathway (Figure 64A).<sup>734,735</sup> The pathway starts with oxidation of methane to methanol by metalloenzyme methane monooxygenase (MMO). Methanol is then converted to formaldehyde by PQQ-dependent methanol dehydrogenase (MDH). Then, formaldehyde dehydrogenase (FADH) converts formaldehyde into formate. Finally, formate is oxidized to carbon dioxide by formate dehydrogenase (FDH). The most well-known and extensively studied methanotroph is *Methylotinus trichosporium* OB3b, which expresses two forms of MMO responsible for initial conversion of methane to methanol: the membrane bound particulate MMO (pMMO) and the soluble cytoplasmic MMO (sMMO).<sup>736,737</sup> Moreover, its growth rate and level of MMO expression can be regulated by addition of low-cost metals such as copper in small amounts.<sup>738</sup>

Current approaches to obtaining methanol as the final product include inhibiting further oxidation of methanol to formaldehyde. MDH is thus the primary target required for inhibition according to the methane oxidation pathway (Figure 64A). Several inhibitors have been found to directly affect the activity of this enzyme, including cyclopropanol, chelating agents, and salts.<sup>739</sup> Among these inhibitors, cyclopropanol has the highest inhibitory effect. Its mechanism of inhibition occurs via irreversible adduct formation with PQQ, a cofactor present in the MDH active site (Figure 64B).<sup>740</sup> The optimum concentration of cyclopropanol required for MDH inhibition in cells was found to be 67 nM per  $3.46 \times 10^{-2}$  mg dry cell/mL. This cell density could yield methanol as 152 mmol/g dry cell or equivalent to 60.5% consumption of methane.<sup>736</sup> Using higher cyclopropanol concentrations will reduce the activity of MMO, resulting in a decrease in methanol accumulation. However, because cyclopropanol is unstable under aerobic conditions, its use in methanotroph aerobic fermentation requires good control of oxygen levels.

Chelating agents such as ethylenediaminetetraacetic acid/ethylene glycol tetraacetic acid (EDTA/EGTA) were investigated for MDH inhibition activities both *in vitro* and *in vivo*.<sup>741</sup> Although EDTA was found to have no effect on the activity of purified MDH, it showed complete inhibition of MDH in the whole-cell biocatalysis. *In vivo*, the EDTA inhibits the activity of MDH by lowering the amount of  $\text{Ca}^{2+}$  ions available (Figure 64C). It was postulated that  $\text{Ca}^{2+}$  is required for maintaining the correct conformation of the active site of MDH.<sup>742</sup> Moreover, the chelating agents can also specifically inhibit MDH by binding to the lysine or arginine residues located close to the cytochrome-binding domain on a subunit of MDH, preventing further electron transfer to an electron acceptor cytochrome.

Other inhibitors including  $\text{CO}_2$ ,  $\text{Mg}^{2+}$ , phosphate,  $\text{NH}_4\text{Cl}$ , and  $\text{NaCl}$  have been shown to inhibit oxidation of methanol to form formaldehyde (Figure 64D).<sup>743</sup>  $\text{CO}_2$  was explored for its application as an inhibitor because it can be directly obtained from biogas. However,  $\text{CO}_2$  showed only small inhibitory effects even at a high concentration (40% v/v).<sup>744</sup> Other inhibitors including cations and anions such as  $\text{NH}_4^+$ ,  $\text{Mg}^{2+}$ ,  $\text{PO}_4^{3-}$ , and  $\text{NaCl}$  have been explored because they may disrupt the cytochrome binding to MDH, which may lead to inhibition

of the enzyme activity.<sup>745,746</sup> Studies have shown that when using high concentrations of MgCl<sub>2</sub>,<sup>747</sup> phosphate,<sup>748</sup> or NaCl,<sup>749</sup> methanol oxidation is indeed inhibited. This inhibition can be relieved by increasing the cytochrome concentration. For example, *Methylosinus trichosporium* OB3b can be grown to reach a high cell density in a reactor containing high phosphate contents and minimum Mg<sup>2+</sup> concentration.<sup>748</sup> By using a cell density of 17 g dry cell/L with 400 mM phosphate and 10 mM MgCl<sub>2</sub>, a maximum yield of methanol of 1.1 g/L could be achieved. Mg<sup>2+</sup>, phosphate, and NaCl have advantages over other types of inhibitors, in that they are effective, stable, and economical. A summary of MDH inhibitors employed for methanol production is shown in Table 8. Results indicate that some of the systems show promising potential for further development of large-scale methanol production from methane.

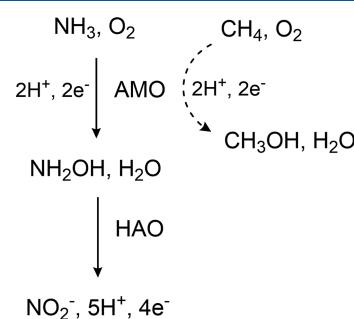
**Table 8. Methanol Production by Different Methanotrophic Strains and the Use of MDH Inhibitors in Fermentation**

strains	MDH inhibitors	methanol produced	ref
<i>Methylosinus trichosporium</i> OB3b	phosphate, cyclopropanol	0.17 g/L	736
<i>Methylosinus trichosporium</i> OB3b	NaCl	0.12 g/L	737
<i>Methylosinus trichosporium</i> IMV 3011	CO <sub>2</sub> , Mg <sup>2+</sup>	1.32 μg/L	744
<i>Methylocystis bryophila</i>	phosphate, Mg <sup>2+</sup>	0.07 g/L	747
<i>Methylosinus trichosporium</i> OB3b	phosphate, Mg <sup>2+</sup>	1.12 g/L	748
consortium of mixed species	NaCl	0.04 g/L	749
<i>Methylosinus trichosporium</i> OB3b	phosphate, NaCl, EDTA	0.44 g/L	750

Methanotrophs have also been explored for their use in applications involving conversion of biogases obtained from anaerobic digestion of municipal wastewater for methanol production.<sup>751</sup> As anaerobic digestion can convert organic waste to methane by methanogens, the use of methanogens and methanotrophs in tandem can serve as an effective way to convert organic waste into valuable liquid fuel. A recent study has shown that municipal wastewater could be collected and used for alternative culture media for methanol production. The wastewater was collected from waste treatment plants and then further used for biogas production.<sup>751</sup> The final anaerobic sludge digester biogas of the wastewater treatment plant consisting mainly of CH<sub>4</sub> (70%) and CO<sub>2</sub> (29%) was collected and supplemented with nitrate mineral salts and NaCl (MDH inhibitor). The maximum methanol production level obtained was 0.20 g/L by culturing mixed methanotrophs in the medium. Even the yield was low; the technology still shows positive outcome in converting unwanted waste into a valuable fuel. In another example, food waste was integrated with several post-transformations including bioproduction of ethanol, methanol, and energy.<sup>752</sup> Unprocessed waste was first fermented to generate ethanol. Ethanol was removed by distillation, resulting in solid residue, which could be processed through anaerobic digestion to generate biogas. Methanol in biogas was converted into methanol by a methanotroph, *Methyloferula stellata*. The maximum yield of methanol obtained was 0.10 g/L. In addition, the liquid residual waste was utilized to generate electricity through a microbial fuel cell. The low yield of methanol may be due to inhibitory effects of

CO<sub>2</sub> and H<sub>2</sub>S on methanotroph growth because these gases are normally generated along with methane during anaerobic digestion.<sup>753</sup> Nevertheless, this integrated process for food waste valorization demonstrated that it is possible to design a comprehensive biotransformation system which can convert low value waste into useful bioenergy and value-added chemicals.

**4.2.1.2. Ammonia-Oxidizing Bacteria (AOB).** Apart from methanotrophs that can utilize CH<sub>4</sub> as a carbon source, AOB can also oxidize methane to methanol using a homologous enzyme of MMO, an ammonia monooxygenase (AMO).<sup>739,743</sup> This microbe oxidizes ammonia (NH<sub>3</sub>) to generate nitrite (NO<sub>2</sub><sup>-</sup>) using CO<sub>2</sub> as a carbon source for cell viability. The oxidation of CH<sub>4</sub> to CH<sub>3</sub>OH can partially occur via nonspecific action of AMO under conditions where a reducing equivalent such as hydroxylamine (NH<sub>2</sub>OH) is high (Figure 65) because extra electrons from oxidation of hydroxylamine by hydroxylamine oxidoreductase (HAO) are available.

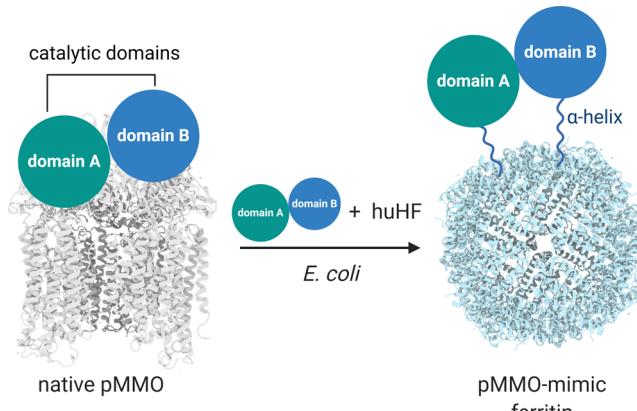


**Figure 65.** Metabolic pathways of oxidation of ammonia to nitrite and co-oxidation of CH<sub>4</sub> to methanol by AOB. Plain arrows indicate a regular pathway, while a dashed arrow indicates a side pathway, depending on availability of an electron donor. Abbreviations: AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase.

It was illustrated that methanol production by AOB can be achieved by fermentation of mixed gases in a fed-batch reactor.<sup>754</sup> A mixed culture of AOB and nitrite-oxidizing bacteria (NOB) for nitrifying performance enrichment was employed in a reactor containing a mixture of CH<sub>4</sub>, NH<sub>3</sub>, and O<sub>2</sub>. The maximum methanol produced was 0.82 mg CH<sub>3</sub>OH/mg biomass after 2 h of bioconversion. Recently, the study demonstrated that a high rate and yield of methanol production by AOB can be achieved by continuous feeding of electron donors for supplying AMO activity in AOB cultivation.<sup>755</sup> A nitrifying enrichment culture was employed for the oxidation of CH<sub>4</sub> with continuous addition of NH<sub>2</sub>OH or NH<sub>3</sub> (electron sources). The maximum rate of methane production was obtained by continuous feeding of NH<sub>2</sub>OH within a hydraulic retention time of 7.5 h. Methanol of 41 mg/L equivalent to a yield of 1.61 mg CH<sub>3</sub>OH/mg AOB was produced. AOB has an advantage over methanotrophs in that methanol is not further oxidized. However, the overall yield is still quite low because the pathway only depends on AMO side activity.

**4.2.1.3. Escherichia coli (E. coli).** Although both methane-utilizing organisms have been studied and demonstrated as promising technology for industrial biomanufacturing, it has never been implemented on a large scale for production purposes. This is mainly due to the slow growth of these bacteria and the lack of genetic tools for modifying organisms.<sup>756</sup> Moreover, MMOs cannot be used for *in vitro*

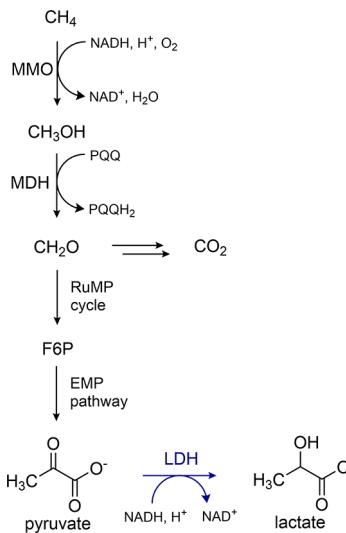
direct conversion of methane to methanol because the enzyme cannot be overexpressed in active forms.<sup>757</sup> To overcome these challenges, production of a MMO mimic and expression in fast-growing bacterial strains has been developed. Recently, Kim et al. reported a development of an MMO-mimetic enzyme by using a ferritin particle as a scaffold.<sup>758</sup> A human heavy-chain ferritin (huHF) particle was used as a scaffold because it has a globular cage shape consisting of 24 identical subunits which can help preserve MMOs native interdomain interactions. The genes encoding for two catalytic domains of the membrane-bound particulate form of MMO (pMMO) identified from *Methylococcus capsulatus*, along with a huHF gene were constructed and overexpressed in *E. coli*. The catalytic domain A and domain B of pMMO were reassembled on the ferritin scaffold through  $\alpha$ -helix conjugation sites (Figure 66). In addition, an excess amount of copper was



**Figure 66.** Molecular reconstruction of pMMO mimic in *E. coli*. Abbreviation; huHF: human heavy-chain ferritin.

added to stabilize the catalytic sites of the pMMOs. Notably, the expressed pMMOs was close to 90% soluble, accounting for more than 35% of the total expressed proteins in the engineered *E. coli*. The MMO-mimic enzymes were evaluated for their catalytic efficiency in methane oxidation at 45 °C. The maximal yield of 1350 mol methanol/mol MMO mimic was obtained within 12 h. This technology is promising and can overcome problems in the slow speed of the bacterial culture and eliminate obstacles associated with the native system for converting methane to methanol efficiently.

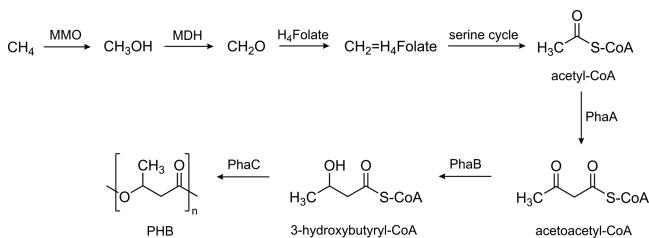
**4.2.2. Lactic Acid.** *Methylomicrobium buryatense* has been identified as a promising methanotroph which can be used for industrial-scale bioproduction of lactic acid or lactate (previously discussed in section 4.1.3) due to its robustness under harsh conditions and the availability of genetic tools.<sup>756</sup> In methane metabolism pathways, pyruvate is produced via assimilation of formaldehyde into the ribulose monophosphate (RuMP) cycle and the EMP pathway.<sup>759</sup> Using this route, pyruvate can be directly converted into lactate using NADH-dependent lactate dehydrogenase (LDH; Figure 67). It was demonstrated that overexpression of LDH in *M. buryatense* resulted in the engineered strain that initially produced 0.06 g/L lactate.<sup>760</sup> Further process optimization in a 5-L bioreactor with continued stirring, resulted in an increased yield of 0.8 g/L. However, further improvement of the production yield is difficult because lactate concentrations above 0.5 g/L inhibit the growth of the microbe.



**Figure 67.** Metabolic engineering of methanotrophs for lactate production from methane. The blue arrow indicates incorporation of LDH to produce lactate. Abbreviations; MMO: methane monooxygenase; MDH: methanol dehydrogenase; LDH: lactate dehydrogenase.

Since lactate accumulation can be toxic to methanotrophs and thus limit efficient production of lactate, a lactate-tolerant mutant of methanotroph was developed by adaptive laboratory evolution.<sup>758</sup> A methanotroph strain *Methyloimonas* sp. DH-1 isolated from brewery sludge was selected as a microbe model due to its fast growth and the recent availability of annotated genome sequences. The evolved strain exhibits growth in the presence of 8.0 g/L lactate. A D-specific lactate dehydrogenase was then heterologously introduced to produce D-lactate as a sole enantiomeric product. The maximum lactate production yield was further enhanced by optimization of the medium, resulting in a production titer of 1.19 g/L and a yield of 0.245 g/g CH<sub>4</sub>. However, the highest titer of lactate production from methane is still significantly lower than the yield obtained from sugar-utilizing microbes. Currently, lactate produced from glucose by engineered microbes can reach titers approximately 100-fold greater than native methanotrophs.<sup>761</sup>

**4.2.3. Polyhydroxyalkanoates.** **4.2.3.1. Polyhydroxybutyrate (PHB).** As PHB can be synthesized by several bacteria including methanotrophs,<sup>762</sup> it has been of great interest to use methanotrophs to produce PHB from methane.<sup>763,764</sup> Production of PHB is found to be limited to type II methanotrophs due to its employment of the serine pathway for formaldehyde assimilation, whereas other types (type I and type X) utilize the ribulose monophosphate pathway for formaldehyde assimilation.<sup>733</sup> In type II methanotrophs such as *Methylosinus* and *Methylocystis*, PHB is produced from methane by employing the serine pathway for formaldehyde assimilation into acetyl-CoA before further transformation into PHB via the PHA synthesis pathway (Figure 68).<sup>765,766</sup> The methanotrophs assimilate formaldehyde produced from the methanol oxidation pathway into tetrahydrofolate, resulting in methylenetetrahydrofolate (CH<sub>2</sub>=H<sub>4</sub>Folate) which is an intermediate in the serine pathway and linked to synthesis of acetyl-CoA.<sup>763</sup> Under nutrient-deficient conditions, the produced acetyl-CoA is then subsequently metabolized to PHB through PHA synthesis pathway. It was demonstrated that cultivation of *Methylocystis* sp. in batch cultures under nutrient limitation (ammonium, phosphorus or magnesium) resulted in



**Figure 68.** PHB biosynthesis pathway in type II methanotrophs. Abbreviations; MMO: methane monooxygenase; MDH: methanol dehydrogenase;  $\text{H}_4\text{Folate}$ : tetrahydrofolate;  $\text{CH}_2=\text{H}_4\text{Folate}$ : methyl- $\text{H}_4\text{Folate}$ ; PhaA, PhaB and PhaC are enzymes in PHB biosynthesis pathway previously described in section 4.1.5.

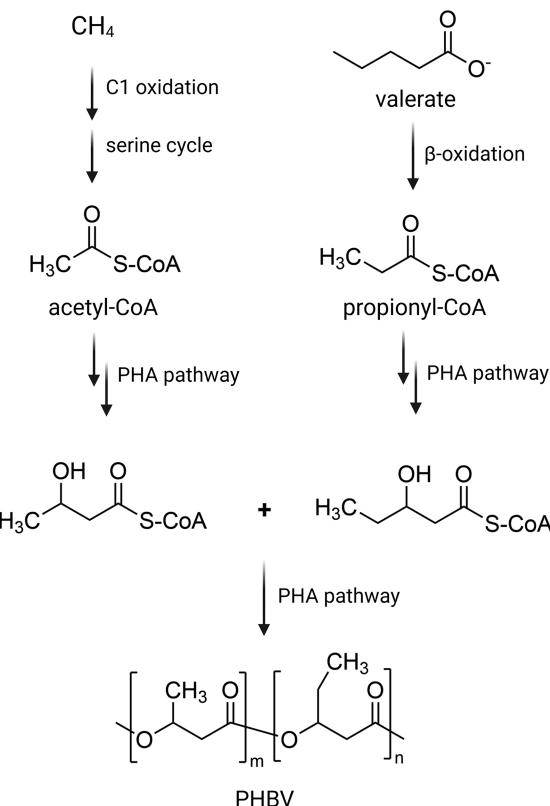
a maximum conversion yield of 51% PHB produced per input biomass by weight, equivalent to production of 0.55 g PHB/g  $\text{CH}_4$ .<sup>767</sup>

Mixed and pure cultures were compared for their ability to accumulate PHB.<sup>764</sup> Results suggest that coculture of methanotrophs are superior in several aspects: (1) removing accumulated toxic byproducts (such as methanol and formaldehyde) from the medium, (2) increasing nutrient supply, and (3) allowing the system to operate under nonsterile processes. For instance, a process using a mixed culture of methanotrophs with a dominant strain *Methylocystis* sp. GB 25 in bioreactors under nonsterile conditions gave PHB up to 50% yield based on methane consumption.<sup>768</sup> The ability of this process to be carried out under nonsterile conditions is particularly important because it reduces the production cost significantly, making the technology economically attractive. However, optimization of culture growth and selection of strains remain the challenging issues.

Compared to the previously discussed systems for production of PHAs from  $\text{CO}_2$  (section 4.1.5), methane has greater potential for PHB biosynthesis. This is largely due to thermodynamic aspects, as oxidation of methane is energetically favored. Using  $\text{CO}_2$  as a feedstock to generate PHB requires a large amount of reductant to transfer electrons to the system.<sup>764</sup> Moreover, PHA bioproduction from methane requires less energy when compared to use of other feedstocks. It is estimated that the total energy required for PHB production from waste biogas is 37.4 MJ/kg PHB, while PHB production from corn-derived sugar requires 41.9 MJ/kg PHB.<sup>769</sup> The advantages of combining waste management and sustainable means of PHB production make the production of PHB from methane a good example of using biobased processes to contribute to a circular economy.

**4.2.3.2. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV).** Methanotrophs can produce a copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). PHBV is generally produced by mixing PHB and 3-hydroxyvalerate. PHBV has superior physical properties than pure PHB because it has a higher melting temperature ( $T_m$ ) and glass transition temperature ( $T_g$ ), as well as greater crystallinity and flexibility than PHB.<sup>770,771</sup> It was shown that pure methanotrophic cultures fed with methane and valerate can produce PHBV.<sup>772</sup> The added valerate is presumably transformed into propionyl-CoA during beta oxidation.<sup>773,774</sup> At the PHA production step, the propionyl-CoA is condensed with acetyl-CoA which subsequently gets incorporated and becomes valerate units in the resulting PHBV copolymer (Figure 69).

The PHBV production was investigated by culturing a pure culture of *Methylocystis* sp. WRRC1 in a reactor fed with



**Figure 69.** Plausible pathway of PHBV biosynthesis by cultivation of methanotrophs fed with methane and valerate.

methane and valerate.<sup>775</sup> The total yield of hydroxybutyrate-hydroxyvalerate (HB-HV) copolymers obtained was 78% of the cell dry weight with more than 50% HV content. The ratio of HB to HV monomer depends on the concentration of valeric acid, in which higher levels of valerate can produce more HV content. When using valerate as 0.34% (w/v) in media, a maximum of 60% HV content could be achieved. However, the yield could not be increased beyond this level, because high valerate concentrations inhibit cell growth.

#### 4.3. Critical Evaluation of Production of Compounds from Carbon Dioxide and Methane

The previous sections 4.1 and 4.2 have shown that various natural and engineered microbes can be used to convert  $\text{CO}_2$  and  $\text{CH}_4$  to various products. However, it is still difficult at this state to use purified enzymes to perform such tasks as many reactions involved are oxygen-sensitive and some enzymes are membrane-bound. Among all products mentioned in section 4.1 and 4.2, lactate is among the most interesting products produced from both  $\text{CO}_2$  and  $\text{CH}_4$  by different pathways. It can be produced with a high titer (1.31 g/L lactate from  $\text{CO}_2$  in the engineered *S. elongatus* and 1.19 g/L lactate from  $\text{CH}_4$  in *Methylomonas*). This should allow the use of  $\text{CO}_2$  and  $\text{CH}_4$  feedstocks for scaled-up processes. As assimilation and usage of these two gases to produce valuable compounds is a global trend and there is an urgent demand to reduce GHGs, we can expect to see more exciting developments in metabolic engineering approaches for  $\text{CO}_2$  conversion. Cyanobacteria and algae are generally prime hosts for this engineering effort because their native cells can assimilate  $\text{CO}_2$  as a carbon source. The case of methane bioconversion is more challenging and can be accomplished through the use of cocultivation or

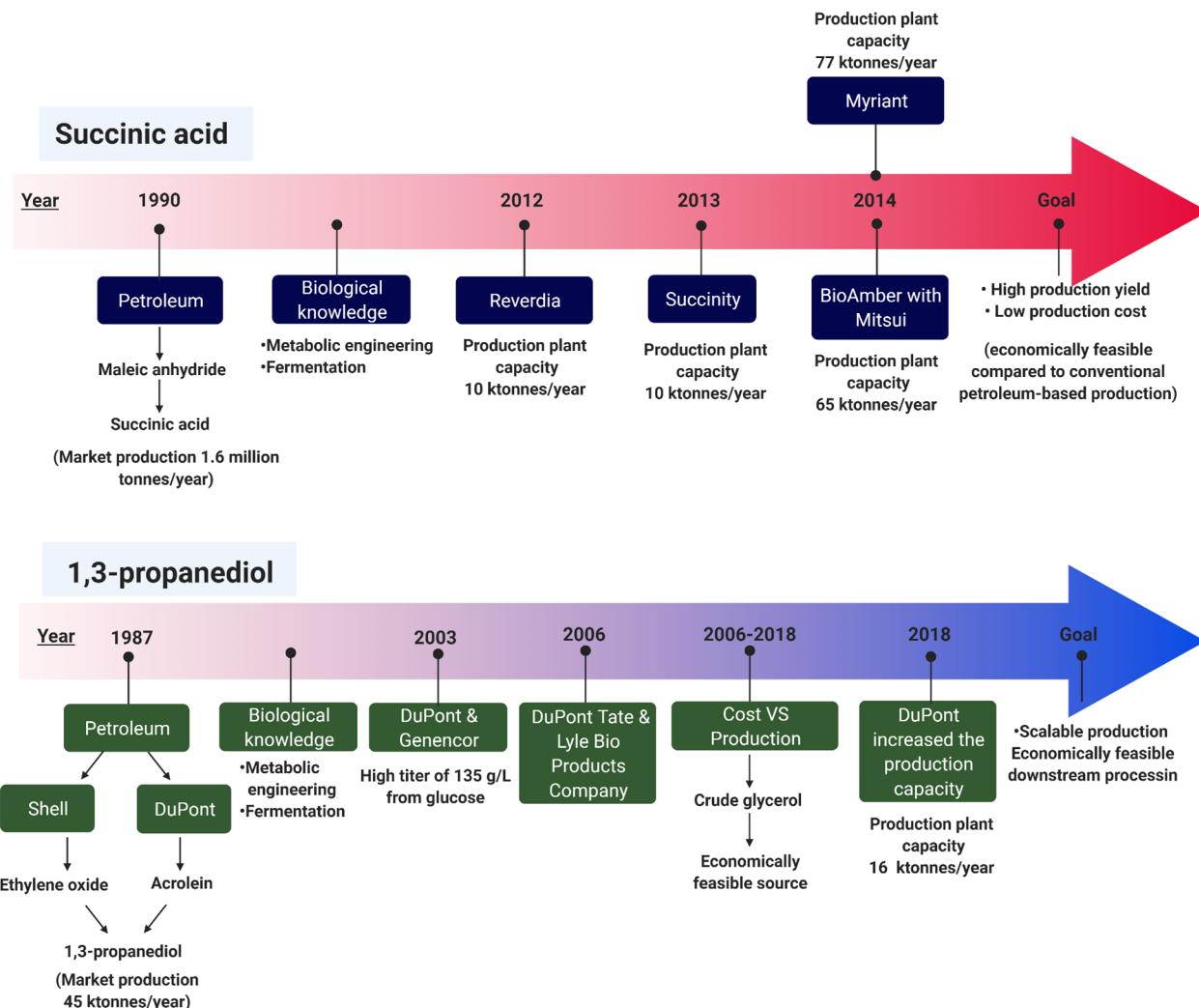


Figure 70. Milestones of development of commercial products from biobased feedstocks and production.<sup>781,786,788</sup>

addition of additives to promote or inhibit particular pathways. Engineering of methanotrophs is not widely investigated as their genetic tools and knowledge related to their biochemistry is not well-known.<sup>776</sup>

$\text{CO}_2$  and  $\text{CH}_4$  bioconversion are still limited by the need for high investment cost because of the special materials, equipment, operational processes and product purification steps required for the process. For example,  $\text{CO}_2$  conversion by engineered cyanobacteria requires a photoreactor and  $\text{CH}_4$  bioconversion requires an anaerobic reactor for fermentation, leading to high expense of equipment and facility setup. Another challenging issue is at the step of engineering process because  $\text{CO}_2$  and  $\text{CH}_4$  are gases which require high energy input to collect. Although these gases in the atmosphere are difficult to deal with, it should be possible to use  $\text{CO}_2$  and  $\text{CH}_4$  generated from industrial process as feedstocks for the process. In general, energy content of products should be higher than the energy input of the engineering process.<sup>777</sup> In addition, several factors for bioconversion conditions including conversion efficiency, cell viability and tolerance (high temperature tolerance, pH tolerance and ability for recyclability) should all be considered to prolong the fermentation process. With more emerging knowledge of enzyme engineering, enzyme activity or tolerance can be improved. The CETCH cycle exemplifies de novo reconstitution of synthetic  $\text{CO}_2$

fixation pathway derived from knowledge of *in vitro* and *in vivo* biocatalysis to surpass the capabilities of the natural pathway. With more improvement, such a concept may achieve large scale  $\text{CO}_2$  bioconversion in the near future.

## 5. COMMERCIAL ASPECTS FOR USING ENZYMATIC AND METABOLIC ENGINEERING IN INDUSTRIES

Enzymatic and metabolic engineering have served as important tools to improve product yields in industrial biotechnology. These tools have been developed in conjunction with process engineering in order to ensure that implementation of the overall process can be justified for large-scale commercial production, both technically and financially. There have been a number of examples in which enzymatic and metabolic engineering can be used in industrial processes. The two early examples are production of SA and 1,3-PDO. SA is a common building block for commodity chemicals. Although a biological route for synthesizing SA has emerged as an alternative method to the petrochemical one, the yield from the natural route in the TCA cycle is low.<sup>778</sup> Therefore, multiple gene mutations and gene deletions were performed to inhibit any byproduct formation. Successful metabolic engineering was reported with production of high yields of SA.<sup>779,780</sup> This had prompted a number of companies, including Bioamber, Myriant (later become PTT MCC

BioChem), Reverdia and Succinity to begin their scale-up to bring biobased production of SA to 10 000–77 000 tonnes/year.<sup>781</sup> However, this was only possible with advanced process engineering. For instance, in Bioamber's technology, a reactive distillation or an esterification unit was integrated to recycle CO<sub>2</sub> and NH<sub>3</sub> back to the fermenter as a feedstock and a neutralizing agent, respectively.<sup>782</sup> Further downstream processing steps including distillation and chemical derivation to products, alkaline-based precipitation, electrodialysis, and reactive extractions have also been demonstrated as options for SA recovery.<sup>783,784</sup> Unfortunately, although the biobased process for SA production is technically feasible, most of the large-scale operations have recently become idle or less active since oil prices crashed.<sup>785</sup>

Another industrial application of metabolic engineering is the production of 1,3-PDO, which has a growing demand as it is used in polyester and polyurethane synthesis and in several organic syntheses. Conventional production involves high temperature and pressure. There were two conventional routes for synthesis by chemical methods. The first route was known as the "Shell" method, which employed ethylene oxide as the feedstock. This process involves hydroformylation and hydrogenation to obtain 1,3-PDO. In another route, the Degussa-DuPont route, the starting material was acrolein, which needs to be further hydrogenated to form 1,3-PDO.<sup>786</sup> Alternatively, a biobased process has been proposed with glycerol or glucose as feedstocks.<sup>787</sup> Metabolic engineering has been implemented to enable the feasibility of the production.<sup>788,614</sup> Among all discoveries, the most significant development is the engineering of *E. coli* strain by DuPont and Genencor that allowed the efficient uptake of glucose, producing a high titer of 135 g/L in 2003.<sup>787,789–792</sup> In 2006, DuPont Tate & Lyle Bio Products began their production of 1,3-PDO using their proprietary bioprocess in Tennessee U.S.A., and later sold products under the brand names Susterra and Zemea. The production costs of the bioprocess, either from glucose or glycerol, are estimated to be comparable with the chemical routes at a high throughput (65 000 tonnes/year).<sup>793,794</sup> In fact, 50% of the costs comes from the downstream steps.<sup>794</sup> Downstream processing of the 1,3-PDO broth is typically done through biomass separation via ultrafiltration, desalination via electrodialysis, water removal via evaporation and final purification via distillation.<sup>794</sup> Other techniques such as reactive extraction,<sup>795</sup> chromatography,<sup>796,797</sup> and alcohol-based precipitation<sup>798</sup> have been developed, but their large-scale operations have not been demonstrated.

Apart from these early applications of SA and 1,3-PDO, production by metabolically engineered microbes has been growing toward more diverse products. In 2016, 1,4-butanediol was announced to be produced biologically after BASF and Novamont acquired IP and licensed the technology from Genomatica.<sup>799</sup> Newlight Technologies engineered cells over-expressing methane monooxygenase for synthesizing PHA from using methane as a carbon source.<sup>800</sup> The company sold the thermoplastic under the brand name AirCarbon, and targeted expansion to a capacity to 43 000 tonnes/year over the next two decades.<sup>801,802</sup>

These industrial examples have proven that the economic feasibility of bioprocess methods can be significantly improved as advances in metabolic engineering allow achievement of higher yields as well the ability to use cheaper starting materials. This leads to growing interests in replacing chemical approaches with bioprocesses (Figure 70). Not only do

bioprocesses eliminate the use of toxic catalysts and chemicals, but they have clearly been demonstrated to reduce energy usage and GHGs emissions. For instance, the life cycle assessment (LCA) on Myriant's biobased production of SA has shown that their technology can reduce the environmental burden on climate change and use of nonrenewable energy by factors of 3.85 and 10.44, respectively, relative to those of the petrochemical-based approach.<sup>803,804</sup> Similarly, the biobased production of 1,3-PDO was estimated to create a climate change potential of 5.5 kg CO<sub>2</sub>/kg 1,3-PDO, which is lower than the ethylene oxide-based approach that has a climate change potential of 7.0 kg CO<sub>2</sub>/kg 1,3-PDO.<sup>805</sup> It is noteworthy that the downstream steps of bioprocesses are responsible for a major portion of the energy usage. Further improvement should focus on process and heat integration to minimize the demand for nonrenewable resources.<sup>806</sup> Nevertheless, these studies clearly demonstrate that the bioprocess is a promising approach for more sustainable and cleaner production.

## 6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

While it is clear that a wide range of enzymatic and metabolic pathways to convert biowaste feedstocks to valuable compounds are available, real implementation of these systems in large scale commercial production is still limited. This is largely due to low yields and expensive downstream costs associated with biobased processes preventing the technology from meeting economic requirements. Adoption of new technology by private sectors also requires new investment by industries and also changes to existing workforce practices, meaning training of new jobs and technical skills for workers are required. Therefore, value addition by biocatalysts and engineered cells needs to be large so that the process is economically feasible and sustainable. However, manufacturing in the current era can no longer solely focus on profit margin but also needs to take environmental cost and impact into account. In order to make technology practical for real industries, we think that researchers must further develop technology in four aspects: (a) constructing more robust and efficient biocatalytic and metabolic pathways to produce high-value compounds from biobased waste, (b) improving efficiency of bioprocesses to be more practical and economical, (c) collaborating with target user groups so that new research findings can be adopted into real practice, and (d) counting environmental damage to be part of manufacturing cost and responsibility.

As major weak points of biobased processes are mostly on cost (biocatalyst preparation and up-scaling cost) and product yield, it is important to develop technology to overcome these issues. More diversified and economically attractive cascade reactions would promote more research of using the biobased processes in large scale manufacturing. The higher value products, the better chance is for enzymatic and metabolic engineering processes to be adopted for large scale production because industries can afford to spend on cost of manufacturing. One of the key improvements generally required to achieve this goal is to obtain efficient biocatalysts to use the desired (which often are not native) substrates effectively which have good thermostability and solvent tolerance. This issue can be addressed through enzyme engineering i.e. directed evolution, rational or semirational designs.<sup>807–809</sup> Frances Arnold, Nobel laureate in Chemistry

(2018), has performed pioneering work in using directed evolution to create enzymes capable of catalyzing formidable reactions. For example, they evolved cytochrome P450 from *Labrenzia aggregate* to catalyze the first enantioselective anti-Markovnikov oxidation of substituted styrenes.<sup>808,810</sup> Another example, P2O (section 5) which uses D-glucose as a native substrate can be rationally engineered to use L-arabinose, resulting in an enzyme variant which can be used together with xylose reductase, formate dehydrogenase and catalase to construct the cascade reaction that can convert L-arabinose (\$0.1/g) into L-ribulose (\$995/g) with 100% yield.<sup>163</sup> Engineering of enzymes to be more tolerant to high temperatures and organic solvents can also reduce the cost of biocatalysts because expensive biocatalysts can be reused and have a long life span. This can be done through target engineering of residues with high B-factor values<sup>811</sup> or using computer assisted methodology via several softwares such as FireProt,<sup>812</sup> FRESCO,<sup>813</sup> or disulfide by designed.<sup>814</sup> For example, the thermostability of flavin reductase<sup>395</sup> and dehalogenating monooxygenase<sup>815</sup> can be increased by rational engineering, allowing their reactions to be used in converting low-value toxic waste into high-value compounds such as D-luciferin.<sup>816</sup> Enzyme engineering also allows incorporation of genes encoding for enzymes with improved properties into cells so that new and efficient metabolic pathways can be directly obtained.

Development of process engineering to be integrated and efficient for material and energy utilization is also important for reducing the cost of manufacturing. As around 50% of the total cost of bioprocesses is often associated with downstream processing, hybrid separation techniques such as reactive extraction, or reactive distillation, should be explored to improve efficiency. Bioprocesses are currently batch-wise and time-consuming. Effective process design to allow the systems to be continuous can also help reduce the cost of the protocols involved. New technologies such as flow chemistry and enabling separation devices can also be helpful to make the production system more efficient.<sup>817–822</sup> Besides development of product separation and purification processes, a major cost of bioprocesses is on biocatalyst production, either in purified enzyme or whole-cell forms. Large scale production and storage of active biocatalysts can also be costly. To overcome this challenge, synthetic biology approaches can be used to up regulate production of enzymes of interest, participating in the metabolically engineered pathways so that manufacturing cost per amount of biocatalyst obtained can be decreased. High cell density can also improve the product titer and yield. Another approach to lessen manufacturing cost is to recycle enzymes or engineered cells through immobilization. Besides reusing enzymes or cells in the process, immobilization also allows easy product recovery and protects biocatalysts against denaturants or inactivation.<sup>823</sup>

Research in these fields aims to make an impact on invention and development of greener and cleaner technology which can be implemented in real applications. This requires demonstration of technology feasibility on a large scale and not stopping development at only the proof-of-concept (or publication) level. In addition to technical merit, the development should also focus on “pain points” or real issues to be solved and aim for the goal of technoeconomic feasibility. Without technoeconomic feasibility, implementation of discoveries in enzymes and metabolic engineering technology to make cleaner manufacturing processes is improbable. How-

ever, a large-scale demonstration requires considerable support which may be difficult in some countries. For midincome countries such as Thailand, a funding scheme through triple helix (government, academia, and industry) model or quadruple helix which has additional partners from local communities is helpful in supporting this type of research. Participation from private sectors helps facilitate technology adoption, while the government can invest in basic knowledge and facilities. For example, our research group receives funding from the Thai government and three private companies to build capacity in demonstrating the use of enzymes and whole-cell biocatalysts to convert household organic or food wastes into energy and chemicals. Our research aims to develop technologies to promote waste segregation under a program called C-ROS (Cash Return from ZeroWaste and Segregation of Trash).<sup>824</sup> We develop technologies to convert organic waste, which is of the least value, and problematic wastes because their decomposition obstructs recycling of recyclable materials and decomposition of food waste also account for 2.05 gigatonne per year of GHGs generation globally.<sup>825</sup> Using anaerobic digestion coupled with metabolic and process engineering technology, we aim to create several reaction prototypes that can convert food waste into useful energy and (bio)chemicals. Currently, we have succeeded in transferring some of our technology to local communities in Nan and Rayong provinces. Users have used our technology to convert their household or community food waste into biogas and biofertilizer. In the next phase of development, we will work with local industries to use enzymes and metabolic engineered cells to convert organic waste from bioindustries to (bio)-chemicals.

In the next decade to come, the situation of the climate change crisis will be even more imposing and policies to control GHGs emissions will be more seriously addressed. We will experience a lot of new rules to act upon this issue. In 2020, the EU set new rules valid up to 2030, which directly affected five sectors including (1) energy and industry, (2) transport, buildings and agriculture, (3) land use and forestry, (4) energy efficiency, and (5) renewable energy.<sup>826</sup> GHGs from sectors (1) and (2) need to be reduced by 43% and 30%, respectively (compared to 2005). For sector (3), this is the first time that land use (such as deforestation or draining of wetlands) needs to be responsible for GHGs emission. For sector (4), energy consumption needs to be decreased by 30% compared to business-as-usual projections. For sector (5), use of renewable energy needs to be increased, with the EU setting a target of a 32% increase over this period.<sup>826</sup> Furthermore, they aim to remove the same or greater amounts of CO<sub>2</sub> from the atmosphere as that released between 2021 to 2030. The EU also aims for a systemic transition to a low-carbon circular economy such as the increase of circular materials, biobased materials and bioenergy.<sup>827</sup> In Asia, China's Third National Assessment Report on Climate Change reported that the temperature in China increased up to 1.5 °C (since 1909), which is higher than the global temperature average.<sup>828</sup> Moreover, 20% of GHGs emission was released from China. Therefore, China has implemented a policy to adopt a climate response including mitigation (help with decarbonization), adaptation (change at the core of planning and infrastructure) and support of sustainable economic development globally. In the U.S., the country aims to reduce CO<sub>2</sub> emissions to 50% by 2050 (compared to 2005). With these new policies and actions, more new research findings or reaction prototypes of

enzymes and metabolically engineered cells are needed so that more prototype candidates can be further developed along with the pipeline to become techno-economic capable technology that can contribute to sustainable development for a circular economy.

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### Notes

The authors declare no competing financial interest.

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Thanyaporn Wongnate is a faculty member at the school of Biomolecular Science and Engineering (BSE), Vidyasirimedhi Institute of Science and Technology (VISTEC). She has recently been awarded the 2019 L'Oréal-UNESCO For Women in Science National Fellowship and selected by Generation T Asia as one of the people who have made a positive contribution to the Asian sustainability sector. Her research work is in the fields of bioenergy and biorefinery. She believes that bioenergy research can contribute significantly to global energy demand in the future and biomass can be converted to produce valuable products.

Pimchai Chaiyen is professor and Dean of the School of Biomolecular Science and Engineering (BSE), Vidyasirimedhi Institute of Science and Technology (VISTEC), Thailand. She is one of the most accomplished researchers in Thailand and has received numerous awards, including L'Oreal-UNESCO Woman in Science Crystal Award for the most accomplished woman scientist in Thailand (2017), Outstanding Scientist of Thailand (2015), and Outstanding Researcher Award (2012). She was named in the Asian Scientists 100 list by Asian Scientist Magazine and has recently joined ACS Catalysis in an Associate Editor role. Her research interests are in the broad areas of enzyme catalysis, enzyme engineering, biocatalysis, metabolic engineering, synthetic biology, and technology development in the areas of green, bio, and circular economy.

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