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Advanced biofuels: Perspectives and possibilities

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1 Introduction

The economic engine that drives human welfare is dependent on fossil fuels. Among the various fossil fuel sources, coal, crude oil, and natural gas are the dominant sources of energy contributing to about 80% of the total world energy consumption in 2019 (IEA, 2020). The transportation industry that requires energy-dense liquid fuels consumes >70% of the crude oil extracted from the ground. Proven world reserves of crude oil ($\sim$1.65 trillion barrels in 2016) are expected to be about 50-times the current annual rate of consumption of this resource (about 35 billion barrels in 2016) assuming no new reserves are identified. It should be noted that this estimated oil reserve represents the total economically recoverable and not technically attainable resource. As the economically recoverable oil reserves dwindle, the cost of liquid transportation fuels is expected to rise due to an increase from the current average worldwide breakeven price of oil at about US $47 in 2018. Besides the putative negative impact of this potential increase in energy cost on the world economy, the use of fossil fuels contributes to adverse climate change due to the accumulation of atmospheric CO_2, the product of energy extraction from fossil fuels. It is imperative that renewable liquid fuels with no net CO_2 emission are developed to replace petroleum at a cost that is competitive with petroleum-derived transportation fuels.

Today's fossil fuels are transformed biomass produced millions of years ago and this temporal separation of production and consumption is the cause of current environmental pollution leading to significant global climate change. By short-circuiting this process, an environmentally sustainable process can be developed for the production of liquid fuels with no net CO_2 evolution (Fig. 1). Among the various potential fuel molecules, ethanol is an attractive alternative to petroleum-derived liquid fuels owing to the long history of the fermentation
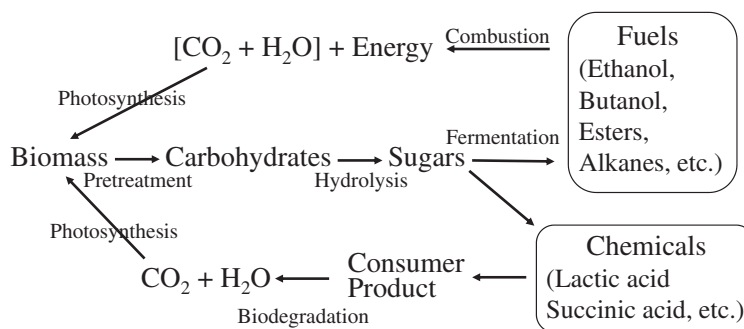
FIG. 1   A generalized scheme for conversion of lignocellulosic biomass to fuels and chemicals. Pretreatment of biomass by acid and heat generates free sugars, such as xylose and arabinose, while making cellulose accessible to enzyme hydrolysis. Other pretreatment processes using base or ionic liquids releases mostly carbohydrates. Only few examples of fuels and chemicals are listed. Combustion of the fuel molecules releases energy for transportation and the CO_2 released is reassembled into sugars, carbohydrates and biomass using the solar energy captured through photosynthesis. Sugars are also fermented to chemicals that are converted to products such as plastics. Upon end of life, these are converted to CO_2, for recapture by photosynthesis.

industry producing ethanol from grains and fruit juices (McGovern et al., 2004). The main polysaccharide in cereal grains is starch, an α-1,4 or α-1,6-linked homopolymer, that yields glucose upon enzyme hydrolysis. This glucose and the disaccharide sucrose from sugarcane and sugar beets are fermented effectively by *Saccharomyces cerevisiae* to ethanol accounting for more than 95% of the current world production of about 29 billion gallons (110 billion liters) in 2019 (https://afdc.energy.gov/data/10331). Volumetric productivity of ethanol as high as $5 g/(L.h)$ has been reported for yeast in simple batch fermentations indicating the effectiveness of this microbial biocatalyst in ethanol production (Rajoka et al., 2005).

The need for feeding an increasing world population negates the use of grains and sugars for the production of ethanol and other fuel molecules leaving nonedible lignocellulosic biomass as the preferred feedstock (Zilberman et al., 2013). As presented in Fig. 1, the carbohydrates in the recalcitrant biomass need to be hydrolyzed to sugars before fermentation by various native and/or engineered microorganisms to the desired biofuel or other chemicals that are currently derived from petroleum. This chapter will focus on critical factors that need to be addressed as an economically viable cellulosic biomass-based biofuel and biochemical industry is developed. It should be noted that Governments use mandates and subsidies to support biofuel industries. However, for long-term survival, these biorefineries need to develop technologies that can support effective competition in the marketplace without Governmental intervention. Specific discussions on pretreatment of biomass, enzyme hydrolysis of carbohydrates to sugars and fermentation are covered in other chapters in this book and the readers are referred to these chapters (Chapters 8, 9, and 10).

2 Cellulosic biomass as a feedstock for microbial fermentation to fuels and chemicals

The production cost of any biofuel, produced from grains and sugars or from cellulosic biomass, needs to match that of gasoline (petrol) for economic viability, now and in the near

future, due to the dominance of petroleum-derived liquid fuels in the world economy. The cost of production of gasoline varies with the price of crude oil and in October 2020 it averaged at about US$1.66/gal (about $0.44/L) in California, USA that uses a special blend (at a higher cost of production), at a world crude oil price of about $40.00 per barrel. Cost of distribution, taxes, and other margins account for the rest of the retail price. It should be noted that in August 2021, the cost of gasoline production in California, USA was $2.96 per gal (about $0.61/L) due to the higher price of crude oil (West Texas Intermediate) at $67.73 per barrel (https://ww2.energy.ca.gov/almanac/transportation_data/gasoline/margins/index_cms.php). In comparison, the cost of ethanol production from corn in 2019 in a commercial plant in Iowa, USA was calculated at about $1.70/gal ($0.45/L) (Irwin, 2020) and due to the low energy content of ethanol compared to gasoline (~0.7), this price increases to $2.40/gal ($0.63/L) on an equal energy basis (GGE, gallon gasoline equivalent). In August 2021, production cost of ethanol increased to $2.31 per gal (about $0/61/L) (https://www.extension.iastate.edu/agdm/energy/html/d1-10.html) but was still lower than the cost of gasoline production at that time. Apparently, market price of corn ($3.85 in 2019 to $6.33 per bushel (56 pounds) in August 2021; https://www.macrotrends.net/2532/corn-prices-historical-chart-data) is a major contributing factor to the production cost of ethanol. However, based on energy content (GGE), the production cost of ethanol in August 2021 ($3.30/gal; $0.87/L) was still higher than that of gasoline. Co-products generated during the ethanol production process, such as DDGS (distiller's dried grains with solubles) and corn oil, help improve the economics of the corn ethanol biorefinery. In addition, government subsidies ($0.45 per gallon of ethanol blended with gasoline in the USA) make ethanol a viable fuel.

In contrast to grains, lignocellulosic biomass is a complex mixture of several polymers with a composition that varies by plant species. Cellulose, a β-1,4-linked glucose homopolymer, is the dominant polysaccharide accounting for about 30%–50% of the total mass. Hemicellulose, a heteropolymer of hexoses, pentoses, and their derivatives, is another carbohydrate in this biomass (19%–25% of total weight) (Williams et al., 2016). These two carbohydrates, lignin, and other minor components form the structural part of plants providing rigidity and resistance to multiple attacks. Since cellulose and hemicellulose in the raw biomass are not readily accessible to appropriate enzymes to produce fermentable sugars, various physicochemical treatments of biomass are employed to improve enzyme access (Blanch et al., 2011; Kim et al., 2011; Tao et al., 2011).

Due to the complexity of sugar extraction from such biomass, the cost of production of cellulosic ethanol is expected to be higher than that of corn ethanol (Tao et al., 2011). Various analyses project a production cost of ethanol using dilute acid and steam pretreatment process between US$1.97 and $4.16 per gallon ($0.50–1.10/L) in a commercial scale biorefinery (Cheng et al., 2019; Gubiczaa et al., 2016; Johnson, 2016; van Rijn et al., 2018). For cellulosic ethanol to compete with gasoline, current biomass pretreatment and enzyme hydrolysis processes need significant improvements to lower the production cost of sugars (Brown et al., 2020).

Among the various pretreatment methods used for fermentative production of biofuels, dilute acid at elevated temperatures can be universally applied to plant materials, with appropriate modifications to suit plant density, to partially hydrolyze hemicellulose to hexoses, pentoses, and oligosaccharides (Kim et al., 2011). The cellulose in the posttreatment solids fraction is also readily accessible to cellulases. With sugarcane bagasse, this process (pretreatment and enzyme hydrolysis) can yield as high as 85% of the total sugars in biomass

for fermentation (Geddes et al., 2013; Nieves et al., 2011). This process also led to a theoretical ethanol yield of 86% with wheat straw as feedstock (Saha et al., 2015). However, a disadvantage of the dilute acid process is the co-generation of microbial growth inhibitors that hamper both enzyme hydrolysis and fermentation (Franden et al., 2013; Geddes et al., 2015; Kumar et al., 2013; Martin et al., 2018; Miller et al., 2009). In an alternate process, lignin can be selectively solubilized by alkali and the solids containing cellulose and hemicellulose are separated for hydrolysis by a mixture of enzymes that hydrolyze both cellulose and hemicellulose (Jin et al., 2010). Depending on the source of biomass, ammonia treatment is also reported to generate microbial growth inhibitors (Ong et al., 2016). Although a multistep pretreatment process that generates cellulose without contaminating solids and inhibitors can improve the efficiency of enzyme hydrolysis and allow enzyme recycling to lower the cost of enzymes in the overall process, the increased fixed and operating costs of such a pretreatment process in the production cost of ethanol needs to be addressed. A promising biomass pretreatment process is based on the use of low-cost protic ionic liquids for dissolving lignin away with or without also hydrolyzing hemicellulose (ionoSolv) from biomass leaving cellulose for effective enzyme hydrolysis (Brandt-Talbot et al., 2017; Sun et al., 2017). The advantages of this ionoSolv process are, (1) lower pretreatment reactor cost due to the less corrosive characteristics of the ionic liquid compared to other pretreatments discussed above, (2) lower enzyme loading for cellulose hydrolysis with potential enzyme recycling, and (3) minimal toxicity to enzymes and microorganisms based on the chemistry of the protic ionic liquid used in the process. However, recycling of ionic liquid and water is critical in this process. The estimated minimum selling price of ethanol with the low-cost protic ionic liquid pretreatment process at US$3.5/gal (Sun et al., 2017) indicates that this process is yet to meet the challenge of economically competitive biofuel from biomass. It would suffice to state, the pretreatment process including enzyme hydrolysis developed for generating sugars from lignocellulosic biomass for fermentation to fuels needs to be simple, minimal, and efficient (both fixed and operating costs) (Geddes et al., 2011; Gubiczaa et al., 2016).

The cost of enzymes is the second or third highest variable cost component of a cellulosic ethanol biorefinery after feedstock and chemicals and this can be 20%–25% of the total production cost of ethanol (Cheng et al., 2019; Gubiczaa et al., 2016; van Rijn et al., 2018). This is more than 10-times the cost of enzymes used in a corn to ethanol process (McAloon et al., 2000). It is unrealistic to accept that the cost of cellulases can be lowered to that of starch hydrolyzing enzymes per unit of ethanol produced, due to the differences in the complexity of the substrates and the variations in the specific activities of the two enzyme systems (Lynd et al., 2002). However, cellulases are another target for cost reduction in cellulosic ethanol production (Klein-Marcuschamer et al., 2012).

To further lower the cost of ethanol, all the released hexoses and pentoses need to be rapidly fermented to completion by a new class of microbial biocatalysts since *S. cerevisiae* and *Zymomonas mobilis* lack the ability to ferment pentoses to ethanol (Scalcinati et al., 2012; Yang et al., 2016). Although diverting part of the sugar stream, especially the pentoses, for the production of high-value co-products is attractive from an economic standpoint, it should be noted that the worldwide demand for liquid fuels far outstrips the demand for chemical feedstocks. In 2019, an average of 41% of petroleum was consumed in the USA as gasoline while less than 2% of petroleum was used for the production of petrochemical feedstocks for the manufacture of various chemicals, synthetic rubber and plastics (https://www.eia.gov/
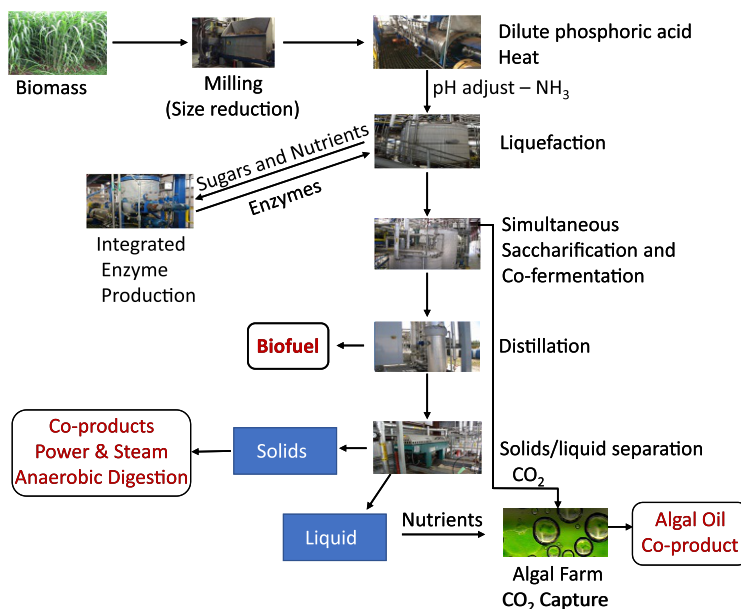
FIG. 2   An idealized biofinery.

). This shows the importance of R&D for maximizing the yield of liquid fuel from biomass while attempting to lower production cost.

Based on the discussion above, idealized integrated biomass to ethanol process can be visualized (Fig. 2) (Geddes et al., 2011; Gubiczaa et al., 2016). In this process, biomass after size reduction is treated with dilute phosphoric acid at high temperature and the slurry is liquified with enzymes to lower viscosity to decrease the capital cost associated with pumping and mixing. The liquified slurry at the highest solids loading is simultaneously saccharified and all the released sugars are fermented to ethanol (SScF) in a single vessel, preferably in a continuous mode. After removing ethanol by distillation or other means, liquid fraction of the stillage is separated and used as a nutrient-rich material to support an algal farm that captures the CO_2 released during fermentation. Algal oil is a secondary product of the biorefinery for conversion to biodiesel. Glycerol generated during this process can be fermented to ethanol or other desired chemicals by appropriate microbial biocatalysts. The solids from the stillage, rich in lignin, is a valuable feedstock for the production of other products (Abdelaziz et al., 2016; Beckham et al., 2016) as well as a source of power and steam, either directly or after anaerobic digestion (Khan and Ahring, 2019). Enzymes needed for the hydrolysis of carbohydrates in plant biomass are produced and consumed at site in this integrated biorefinery. This design achieves the following.

1. Simplified and integrated process lowers the capital cost since this is the highest contributor to cellulosic ethanol production cost (Brown et al., 2020).
2. Use of a less corrosive phosphoric acid (or low-cost protic ionic liquids) further lowers capital cost by eliminating the need for expensive alloys associated with the use of

corrosive acids like sulfuric acid. After pretreatment and pH adjustment with NH_3, the phosphate and ammonium salts are used as nutrients for various microorganisms, thus eliminating the cost of removal and disposal of the spent acid (Gubiczaa et al., 2016).

3. On-site production of enzymes lowers capital cost and eliminates the cost of concentration and transportation of enzymes (Johnson, 2016).
4. Liquefaction followed by SScF of all the sugars in one vessel at a high rate by an effective microorganism minimizes capital cost and also retention time (Geddes et al., 2010).
5. Additional co-products derived from algal oil and lignin improve the economics of the biorefinery while also lowering waste treatment cost, including CO_2 mitigation.

What are the challenges in achieving this idealized biorefinery for cost-competitive biofuel production?

1. Maximize conversion of biomass to sugars without generating growth-inhibitory side-products.
   (a) Maximize biomass pretreatment and enzyme hydrolysis to a hexose and pentose yield of $\geq 85\%$.
   (b) Lower the amount of enzyme needed for hydrolysis of carbohydrates by increasing catalytic efficiency of fungal enzymes and/or production of enzymes with high specific activity by the ethanol-producing microbial biocatalyst (consolidated bioprocessing) (Davison et al., 2020; Lopes et al., 2021; Lynd et al., 2005).
   (c) Lower the cost of fungal enzymes by increasing the rate and titer of enzyme production by the fungi beyond current levels (Agrawal et al., 2017; Fonseca et al., 2020; Ogunyewo et al., 2020).
2. Fermentation of sugars to ethanol.
   (a) Co-fermentation of both hexoses and pentoses at the same rate and product yield, either by appropriately engineered single or multiple microorganisms (Demeke et al., 2013; Xie et al., 2020).
   (b) Microbial biocatalysts are not inhibited by side-products generated during the pretreatment process (Martin et al., 2018).
   (c) Highest yield of ethanol (≥ 0.46 g ethanol/g sugar fermented) both on a total sugar and dry biomass basis.
3. Algal process for oil production
   (a) Cost-effective algal photobioreactors for continuous operation using solar energy for illumination (Anto et al., 2020). Although open ponds are inexpensive to construct and operate compared to photobioreactors, this culture system has the advantage of high algal productivity due to its ability to maintain axenic cultures in a controlled environment (Hannon et al., 2010). The higher energy demand of photobioreactors, in comparison to open ponds, is a challenge that can be met by appropriate integrated solar power stations.
   (b) Engineered algal strains for higher than current photosynthetic and CO_2 fixation efficiency (Gimpel et al., 2015).
   (c) Engineered algal strains convert a major fraction of photosynthate into neutral lipids without compromising growth and biomass production (Gimpel et al., 2015).
   Several strategies toward achieving these objectives in algal oil production are discussed in another chapter (Chapter 4) and are not detailed here.

4. Products from lignin

  (a) Extraction and purification of lignin for the production of chemical feedstocks and/or end products of significant commercial value from lignin (Wang et al., 2019b).
  (b) Chemical and microbial catalysts for cost-effective production of high-value products from lignin (Abdelaziz et al., 2016; Wang et al., 2019b).

Overcoming a part of these of challenges, especially at the level of sugar production and fermentation, can lower the production cost from the current estimates and close the gap to that of gasoline from petroleum (Shanmugam and Ingram, 2021; van Rijn et al., 2018).

In addition to ethanol, several other chemicals (fuels and chemical feedstocks) can be produced from sugars using microbial biocatalysts, although the rate, titer, and yield of these chemicals are not high enough for industrial deployment. As the microbial biocatalysts for production of these chemicals are being developed, it should be noted that the cost of production is expected to be the lowest in an anaerobic process compared to aerobic or O_2-limitation conditions. Besides the increase in capital cost associated with maintaining the needed O_2 concentration in large industrial-scale fermenters, microenvironments with differing O_2 levels within the large vessels have the potential to introduce unexpected side-products with an additional cost of product purification.

Ethanol and lactic acid are currently produced by anaerobic fermentation and butanol is another fuel molecule that was previously produced by fermentation. Improvements in microbial biocatalysts that can significantly lower the production cost of these chemicals are discussed in the following sections.

3 Cellulosic ethanol

Although several cellulosic ethanol biorefineries are being designed and constructed worldwide, only 5 plants are reported to be operational in 2019 and contribute less than 1% of the total ethanol produced (IEA, 2020; Padella et al., 2019). These biorefineries use the steam explosion of biomass as pretreatment with/without dilute acid followed by enzyme hydrolysis to generate fermentable sugars. Depending on the plant part and species, hemicellulose accounts for about 25%–44% of the total carbohydrates in lignocellulosic biomass (Robak and Balcerek, 2020) and the dilute acid and heat pretreatment hydrolyzes a fraction of the hemicellulose with pentoses as dominant monomeric sugars reaching a yield as high as 85%. Although glucose from cellulose can be readily fermented by *S. cerevisiae* or *Z. mobilis*, these microorganisms lack the metabolic potential to ferment pentoses in hemicellulose (Scalcinati et al., 2012; Yang et al., 2016). It should be noted that *S. cerevisiae* has the genetic capability of converting xylose to glyceraldehyde-3-phosphate, an intermediate in glycolysis, but fails to grow using xylose as C-source due to very low level of expression of these genes, especially xylitol dehydrogenase (Moyses et al., 2016; Toivari et al., 2004). Availability of a microbial biocatalyst that can ferment pentoses released from biomass to ethanol at a rate that is comparable to that of glucose fermentation by *S. cerevisiae*, an industrially preferred microbial biocatalyst, is a major challenge in the cellulosic ethanol biorefinery.

Two alternative approaches are used to construct microbial biocatalysts for fermentation of both hexoses and pentoses in the pretreated biomass slurries: endowing *S. cerevisiae* with pentose fermentation potential by introducing an active pathway and metabolic engineering of a
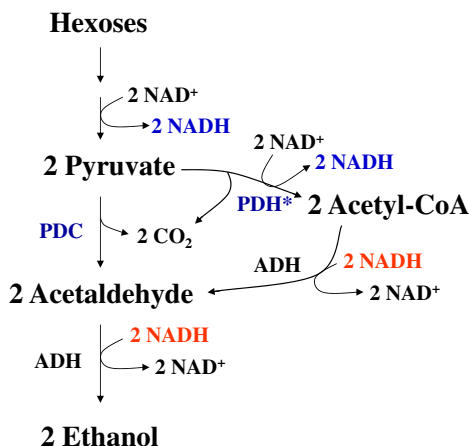
Hexoses

FIG. 3 Two alternative pathways for conversion of pyruvate to ethanol. PDC, Pyruvate decarboxylase; ADH, Alcohol dehydrogenase; PDH*, engineered pyruvate dehydrogenase.

microorganism with a native ability to ferment pentoses, such as *Escherichia coli*, for homo-ethanol production. A pioneering example of the second approach was introducing genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis* into *E. coli* (Ohta et al., 1991) (Fig. 3). In this example, the engineered *E. coli* converted close to 90% of hexoses and pentoses to ethanol while ethanol accounted for less than 10% of the sugar fermented in batch cultures of the wild-type *E. coli*. Further metabolic engineering removed the co-products resulting in ethanol as the only product. Ethanol production by an alternate pathway resulting from engineering native genes was also demonstrated in various pentose-fermenting bacteria (Jilani et al., 2017; Kim et al., 2007; Su et al., 2010; Zhou et al., 2008) (Fig. 3). However, the average productivities of ethanol that is ≤2.0 g ethanol/(L.h) by these engineered microbial biocatalysts including yeast is significantly lower than the rate of ethanol production from glucose by yeast of about 4.0 g/(L.h) (Alterthum and Ingram, 1989; Demeke et al., 2013; Gonzalez et al., 2002; Jilani et al., 2017; Ohta et al., 1991; Wang et al., 2019c; Xie et al., 2020). In addition, low ethanol tolerance of many of these bacterial biocatsalysts restricted ethanol titer to about 6% and limited the use of this group of engineered microbial biocatalysts by ethanol biorefineries.

In pentose fermenting microorganisms, sugar is transported into the cell by a specific transporter or by nonspecific sugar transporters. Pentose in the cytoplasm is phosphorylated, transformed in the pentose phosphate pathway (PPP) and the resulting metabolic intermediates enter the glycolytic pathway at the fructose-6-phosphate and glyceraldehyde-3-phosphate level for further conversion to the desired product. Since glucose and pentose fermentation pathways are common from fructose-6-phosphate to ethanol, the rate-limiting steps in pentose fermentation are in transport and conversion of pentoses to fructose-6-phosphate and glyceraldehyde-3-phosphate. Various strategies in the construction of a xylose-fermenting *S. cerevisiae* and improving the rate of fermentation are discussed in other chapters and are not discussed here. Toward this objective, a co-culture of two *S. cerevisiae* strains, each fermenting only one sugar (glucose or pentose), has the advantage of rapidly and simultaneously fermenting all the sugars released from biomass without the negative

effect of glucose on pentose fermentation (Eiteman et al., 2008; Hanly et al., 2012; Wang et al., 2019c). It is sufficient to say, that pentoses can account for as much as 25%–30% of biomass by weight and these readily available sugars need to be fermented to the primary product by the biorefinery to lower production cost of ethanol.

4 Butanol

Butanol, a 4-carbon alcohol, is more reduced than ethanol and as a result has a higher energy content than ethanol (ΔHc of -2.68 vs -1.41 MJ/mole, respectively) (Domalski, 1972). In addition to reaching about 94% of the energy content of gasoline, it is also less volatile, less hygroscopic, and less corrosive than ethanol and is considered a drop-in liquid fuel with gasoline. Several anaerobic bacteria do ferment sugars to butanol and this fermentation process was commercially exploited during the early- to mid-20th Century (Jones and Woods, 1986). However, current industrial production of butanol is based on petrochemical feedstocks since the minimum selling price of butanol produced by fermentation of biomass-derived sugars ($0.85/L; $3.22/gal) is higher than that of gasoline as seen with cellulosic ethanol as a fuel (Qureshi et al., 2020).

In contrast to ethanol, native butanol-producing bacteria, such as Clostridia, do not produce butanol as the sole fermentation product due to the make-up of the metabolic pathway that is not redox balanced (Fig. 4). Redox imbalance of the native pathway can be overcome by metabolic engineering of the organism leading to butanol as the sole organic product (Fig. 5) (Abdelaal et al., 2019; Atsumi et al., 2008; Kim et al., 2007; Shen et al., 2011; Tucci and Martin, 2007). In Clostridia, fermentative production of butanol from acetyl-CoA requires 5 NADHs while glycolysis and pyruvate-ferredoxin oxidoreductase (PFOR) combined only produce 2
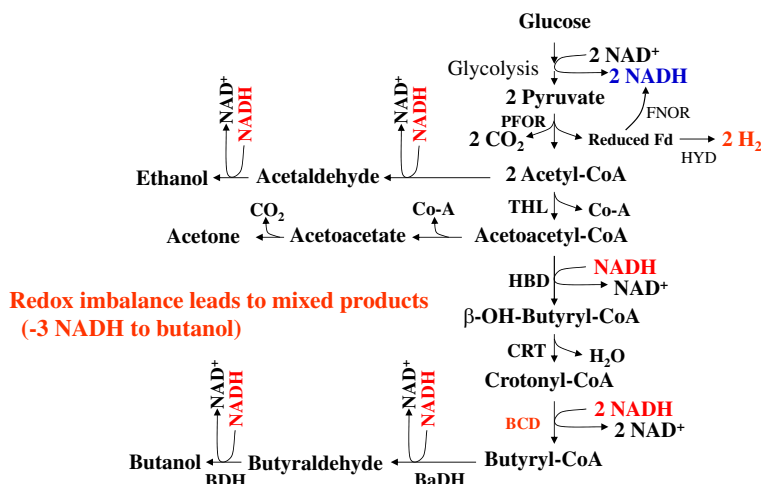

FIG. 4   Native butanol pathway is redox imbalanced. A combination of products are produced to maintain redox balance during growth and fermentation, as seen with *Clostridium acetobutylicum*. *BaDH*, butyraldehyde dehydrogenase; *BCD*, butyryl-CoA dehydrogenase complex; *BDH*, butanol dehydrogenase; *CRT*, crotonase; *FNOR*, ferredoxin-NADH oxidoreductase; *HBD*, hydroxybutyrate dehydrogenase; *HYD*, hydrogenase; *PFOR*, pyruvate-ferredoxin oxidoreductase; *THL*, thiolase.
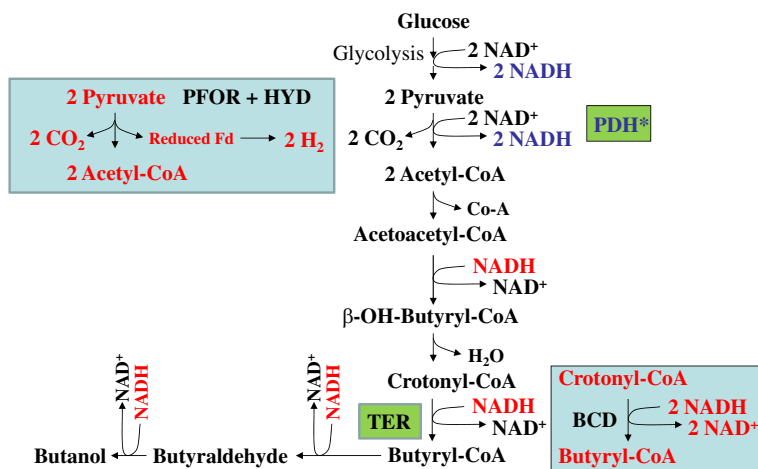
FIG. 5   An engineered fermentation pathway for production of butanol as the sole liquid fermentation product. In this pathway the native enzymes, pyruvate-ferredoxin oxidoreductase and butyryl-CoA dehydrogenase complex in the butanol pathway (reactions enclosed in a box) are replaced by an engineered pyruvate dehydrogenase (PDH*) and transenoyl-CoA reductase (TER), respectively. See Fig. 4 for other enzymes in the pathway.

NADHs during the conversion of glucose to 2 acetyl-CoAs (Fig. 4). Additional reductant available during the oxidative decarboxylation of pyruvate to acetyl-CoA is lost as H_2 with either reduced ferredoxin (Clostridia) or formate (enteric bacteria, such as *E. coli*) as intermediates. To capture this reductant, a formate dehydrogenase that generates NADH has been used in engineered *E. coli* (Fig. 6) (Shen et al., 2011). An alternate strategy was to capture the reductant directly as NADH during pyruvate decarboxylation using an engineered pyruvate dehydrogenase (PDH*), either by mutational alteration of the enzyme or by promoter exchange in *E. coli* (Fig. 5) (Abdelaal et al., 2019; Kim et al., 2008; Zhou et al., 2008).

The enzyme complex butyryl-CoA dehydrogenase (BCD) in native Clostridia utilizes 2 NADHs to reduce crotonyl-CoA to butyryl-CoA, a 2-electron/proton step. The reductant in the second NADH is released and lost as H_2 (Li et al., 2008). Replacing this enzyme with a trans-enoyl-CoA reductase (TER) from other microorganisms that utilize one NADH for this reduction eliminates this loss of reductant (Atsumi et al., 2008; Tucci and Martin, 2007). The engineered butanol fermentation pathway incorporates these two changes: PDH* and TER (Fig. 5) (Abdelaal et al., 2019).

Another challenge in butanol production, in contrast to ethanol production, is the low tolerance of microbial biocatalysts to this solvent that limits butanol titer to $\leq 20\,g/L$ during batch fermentations irrespective of the microbial biocatalyst (Qureshi et al., 2020; Wilbanks and Trinh, 2017). Several attempts to increase butanol tolerance of microbial biocatalysts have resulted in small increments in tolerance and final butanol titer. Process modifications enabling continuous product removal, such as gas stripping, vacuum fermentation, distillation in situ, etc. have the potential to minimize product toxicity and increase butanol titer but at an increase in capital and operating costs (Qureshi et al., 2020).

An alternative to process modifications to lower butanol toxicity is to alter the final product to butyrate (Fig. 6). Butyrate is comparatively less toxic than butanol to the microbial biocatalyst and the butyrate titer is reported to reach about $45\,g/L$ (Jang et al., 2014; Luo et al., 2018;
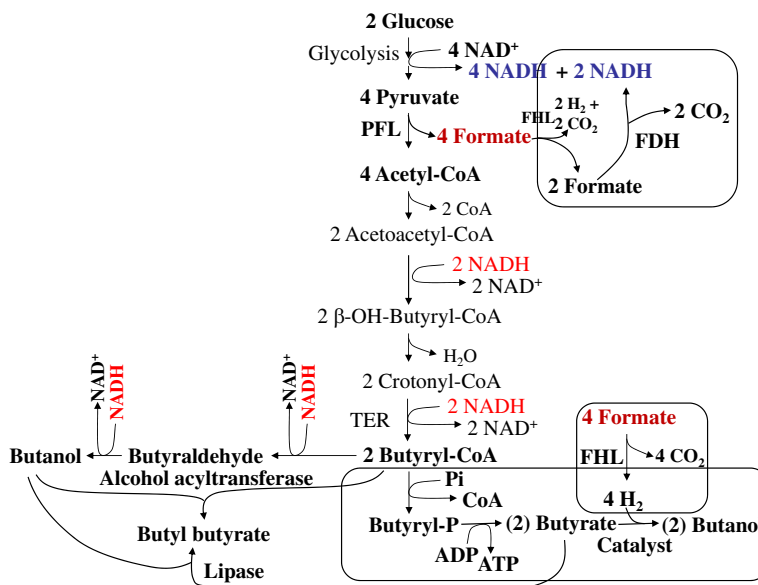
FIG. 6 Alternate pathways and products for production of butanol and butyl esters. The formate produced at the pyruvate formate lyase (PFL) reaction can serve as H_2 donor for chemical reduction of butyrate to butanol. NADH derived from formate is the needed additional reductant (NADH) for redox balanced production of butanol. One butanol and one butyrate can be combined to produce butyl butyrate in vivo using either enzyme or outside the cell using lipase. FDH, formate dehydrogenase; FHL, formate hydrogen lyase.

Wang et al., 2019a; Wang et al., 2015). Potential to increase this titer to over $100 \, g/L$ can be realized by engineering butyrate-tolerant bacteria that grow in the presence of 70–$80 \, g/L$ butyrate. Butyrate can be chemically reduced to butanol to very high concentration using the H_2 generated during the production of butyrate (Fig. 6) (Lee et al., 2014). This chemical reduction process overcomes the toxicity associated with butanol production by microbial biocatalysts while also eliminating ethanol as a co-product of butanol since the enzymes that convert butyryl-CoA to butanol also catalyzes the reduction of acetyl-CoA to ethanol.

Under appropriate conditions, butyrate can be combined with butanol as produced or separately to generate butyl butyrate, an eight-carbon ester as a drop-in biofuel for diesel (Noh et al., 2018; Sjoblom et al., 2017; Xin et al., 2016). Although butyl-butyrate production by microbial biocatalysts has been demonstrated, toxicity of this ester to the microorganism precludes the production of this ester at high concentration by fermentation (Wilbanks and Trinh, 2017).

5 Thermotolerant microbial biocatalyst for production of fuels

One of the major cost components of ethanol or other product production using biomass as feedstock is the fungal enzymes with a pH and temperature optima for the activity of about 5

and 50–55°C, respectively (Patel et al., 2005). In an idealized biorefinery, the microbial biocatalysts ferment the sugars as released by enzymes to product (SScF) to minimize the inhibitory effect of sugars on enzymes and to lower the capital cost (Lynd et al., 2002; Pemberton and Crawford, 1980). In cellulosic ethanol production process, the optimum temperature for yeast growth and fermentation activity is 30–35°C (Salvado et al., 2011). Because of the differences in temperature optimum for the yeast and cellulase, SScF is conducted at an intermediate temperature that lowers the specific activity of the enzymes and increases enzyme cost. A microbial biocatalyst that optimally grows and ferments sugars to ethanol at 50–55°C (*Bacillus coagulans, B. stearothermophilus*, etc.) can significantly lower enzyme loading and cost without compromising activity (Ou et al., 2009; San Martin et al., 1992; Su et al., 2010). Several attempts to evolve *S. cerevisiae* for growth at about 50°C are yet to produce a derivative appropriate for SScF. Yeast strains that can grow at 40°C have been described; however, at this temperature the fungal cellulase activity is only about 60% of the activity at the optimum temperature indicating the need for higher cellulase loading and enzyme cost compared to SScF at 50°C (Ogunmolu et al., 2017; Pandey et al., 2019; Patel et al., 2005). Metabolic engineering of thermotolerant microorganisms for production of ethanol at a rate and yield observed with *S. cerevisiae* is critically needed for an idealized biorefinery.

In addition to lowering enzyme cost in SScF at 50–55°C, cooling cost of fermentation tanks is also expected to be lower compared to fermentations at 37°C. Higher operating temperature can also lower energy cost associated with continuous product removal as suggested for butanol to minimize product toxicity and maintain high productivity. Additional advantage of fermentation at 50–55°C is lower risk of contamination of fermentation vessels by mesophilic microorganisms (Firmino et al., 2020) that can lower product yield.

6 Lactic acid

Besides ethanol, lactic acid is a fermentation product that is easy to produce at high titer, yield and productivity. Like ethanol, lactic acid fermentation by microorganisms also has a long history (Prajapati and Nair, 2017). Traditionally, lactic acid is used in food industry as a preservative and flavoring agent, and additionally has applications in cosmetics and pharmaceutical industries. Production of poly-lactic acid (PLA), a biodegradable and sustainable polymer, is a recent use of optically pure lactic acid (Auras et al., 2010). The market size of lactic acid in 2019 was about US$1.3 billion and PLA accounts for about 45% of this total. Demand for PLA is expected to increase by an annual rate of about 10% during the next 5 years as a bio-based alternative to petroleum-derived plastics (Ahuja and Mamtani, 2020).

Although lactic acid can be made from petroleum, fermentation is the preferred production method due to the enantiomeric purity of the product needed by the plastics industry. The calculated production cost of lactic acid from corn grain is US$ 844–1251 per ton depending on the microbial biocatalyst used and associated fermentation/product purification steps (Manandhar and Shah, 2020). The higher cost of PLA at about $0.85/lb. compared to PET at about $0.65/lb. in 2018 makes PLA less attractive (https://packaging360.in/insights/polylactic-acid—a-sustainable-bioplastics-packaging-option). This disadvantage in cost can be overcome by the sustainability and biodegradability of PLA. However, to
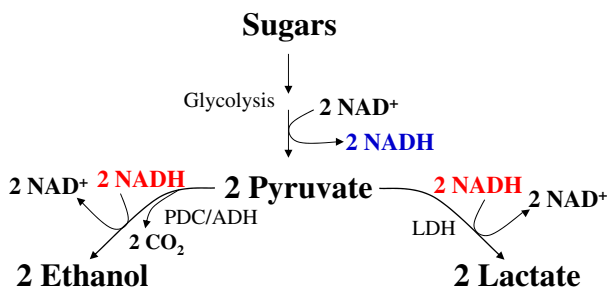
FIG. 7 Comparison of ethanol and lactate fermentation pathways. Pyruvate produced by glycolysis of sugars is either converted to ethanol or lactate as per the microbial biocatalyst to maintain redox balance during growth and fermentation. *ADH*, alcohol dehydrogenase; *LDH*, lactate dehydrogenase; *PDC*, pyruvate decarboxylase.

realize the shift from petroleum-based plastics to bio-based plastics, the production cost of lactic acid and other feedstocks needs to match that of petroleum-derived starting materials. Considering lactic acid and PLA as prime examples, the challenge is in minimizing product toxicity and simplifying product purification.

Various challenges in lactic acid production are listed below and some are already addressed successfully.

1. Fermentation of sugars to lactic acid is a simple process like that of ethanol (Fig. 7) but at a higher product yield of 1 g lactic acid per g sugar fermented (ethanol, 0.51 g/g). Considering the yield and ease of production of lactic acid, a significant part of production cost is associated with purification of lactic acid. Although ethanol can be readily removed by distillation from a postfermentation beer containing various solids and liquids, purification of lactic acid from such a complex mixture is difficult and expensive. Mineral salts medium with sugars as feedstock is preferred by the lactic acid industries to simplify purification.

2. Growth inhibition of lactic acid bacteria, such as *Lactobacillus*, by lactate lowers product titer (Goncalves et al., 1997) and requires a base for neutralization that needs to be removed and disposed. Extractive fermentation increases product titer, but the associated cost of this process is prohibitive at an industrial scale (Othman et al., 2017).

3. High titer of lactic acid is achieved by neutralization with $Ca(OH)_2$. The cost of disposal of gypsum generated during purification with the use of lime is an addition to the production cost. The use of yeast as a microbial biocatalyst that can tolerate a high concentration of lactic acid is an alternative to lactic acid bacteria (Manandhar and Shah, 2020).

4. Postfermentation purification of lactic acid is based on separation of lactic acid as calcium lactate, recovery of lactic acid from the calcium salt, esterification of the free acid form with methanol followed by distillation and hydrolysis (Filachione and Fisher, 1946; Manandhar and Shah, 2020). A microbial biocatalyst that can ferment sugars to lactic acid at a pH that is lower than the pK_a of lactic acid (3.86) that is also tolerant to a higher concentration of the product is preferred to lower production cost. Lactic acid-producing yeast strains appear to meet these requirements yielding a cost advantage over lactic acid bacteria as microbial biocatalysts (https://www.foodingredientsfirst.com/news/cargill-awarded-for-innovation-in-lactic-acid-production.html).

5. A thermotolerant microbial biocatalyst that grows and ferments sugars in mineral salts medium at a pH that is less than 4.0 at high rate and yield is an ideal microorganism for industrial production of optically pure lactic acid that in addition will also lower cooling cost of fermentation vessels as discussed above.

Although the fermentation conditions, yield and purification steps in the production of other organic acids may vary from that of lactic acid, product tolerance, thermotolerance, and mineral salts medium are process steps that are challenges shared with lactic acid production to achieve cost-effective industrial production of other bulk chemicals as chemical feedstocks.

7 Conclusion

As the world strives to wean away from fossil fuels as the dominant source of energy by envisioning alternate sustainable biofuels and biochemicals, numerous challenges stand in the way of achieving cost parity with petroleum-derived fuels and chemicals. An idealized biorefinery that has the potential to yield fuel ethanol that is cost-competitive with gasoline is presented and discussed. Minimal challenges associated with achieving this idealized lignocellulosic biomass biorefinery that uses nonfood carbohydrates as feedstock are identified and presented. Overcoming these obstacles to achieve a green economy is vital for the continued growth of the world economy without further deterioration of the environment.

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