Can Microbially Derived Advanced Biofuels Ever Compete with Conventional Bioethanol? A Critical Review

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Microbially derived alkanes and their derivatives are recognized as promising alternatives to petroleum-based fuels and chemicals. We review recent developments in their production, assess progress, and their potential against conventional bioethanol fermentation pathways. The success rate of genetic engineering efforts and their commercialization prospects are assessed, as well as challenges for producing fuels and chemicals from lignocellulosic biomass. Although significant progress has been made in the genetic engineering of microbes used in the production of long-chain hydrocarbons and their derivatives, titer and yield of these biomolecules are currently too low to compete with petroleum-derived products. As for microbially derived isoprenoids or fatty acids, the inherent complexity of micro-organism development will continue to present formidable challenges, making it highly unlikely of any short-term commercial take off. Nonetheless, first generation bioethanol (starch/sugar based) production is commercially established and therefore continued advancements in chemical synthesis should enable broad-scale use of bio-ethanol as a chemical feedstock for the production of advanced biofuels including butanol and other long-chain hydrocarbons.

Keywords: Biochemicals; Bioethanol; Biobutanol; Biofuels; Fermentation; Lignocellulosics

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INTRODUCTION

Climate change and energy security have placed enormous pressure on governments and industry to replace carbon-intensive petroleum with biomass-derived alternatives (Guo *et al.* 2013; Su *et al.* 2015). As a result of this global surge, bioethanol production and its use has increased exponentially in the last decade, reaching 25 billion gallons in 2013, which is more than three times higher than 2000 production levels (RFA 2015; Su *et al.* 2015). Although bioethanol currently dominates the biofuels market, there is increasing recognition that multiple biofuel options are needed to achieve broad-based substitution of petroleum.

Ethanol has several limitations as a "drop-in" replacement fuel (Lee *et al.* 2008; IEA 2011; Snow 2013). Ethanol can neither be a universal substitute to all types of fuels nor be a complete replacement of gasoline. Despite its high octane number, ethanol has a low energy density, which is 30% lower than gasoline (Atsumi *et al.* 2008). Ethanol is highly hygroscopic and corrosive, which not only limits its usage in existing engines, but makes it incompatible with the current petroleum storage and distribution infrastructure (Lee *et al.* 2008; Li *et al.* 2010b; IEA 2011). Therefore, in order to enable a sustainable transition from a hydrocarbon economy to a carbohydrate-based society, alternative biofuels must be compatible with the existing engine and fungible with respect to the supply chain infrastructure (Fortman *et al.* 2008).

Both natural and genetically engineered fermentation pathways in microorganisms can produce compounds that not only can substitute for gasoline, but can replace all petroleum-derived fuels (Steen *et al.* 2008; Yan and Liao 2009). These microbially-derived compounds can include long-chain alcohols, such as isopropanol, butanol, isobutanol, longer chain alcohols, isoprenoids, long-chain fatty acids, and their corresponding alcohols, esters, as well as alkanes (Peralta-Yahya and Keasling 2010; Steen *et al.* 2010; Carmann 2011). These biofuels are referred to as "advanced biofuels" to distinguish them from first-generation fuels, as these fuels are yet to be produced at commercial scale. Butanol and isopropanol are both naturally produced by the solventogenic *Clostridia* or can also be derived by the genetic engineering of natural *Clostridial* pathway or rerouting the amino acid biosynthesis pathways in both bacteria and yeasts (Papoutsakis 2008). Production of isoprenoids and long-chain fatty acids are achieved through the genetic engineering of the respective biosynthesis pathways. All such molecules have both higher energy content and hydrophobicity than ethanol, and most can be transported through existing pipelines (Dürre 2007). Many of these compounds, such as farnesane (isoprenoid based), have energy densities and other fuel properties equivalent to that of petroleum distillates and can be blended with the fossil-based counterparts in any proportions and used as "drop-in" fuels (Peralta-Yahya and Keasling 2010). The present article reviews different types of advanced biofuels that can be produced by microbial fermentation and assesses research progress against the developments in bioethanol production. The major liquid biofuels considered here are biochemically derived long-chain alcohols (> C3), long-chain fatty acids and their esters, and isoprenoid-based fuels. In addition to reviewing the progress made in the natural production pathways, the success rate is considered for the genetic engineering efforts on the *Clostridial* pathway and three other metabolic pathways: amino acid biosynthesis, fatty acid biosynthesis, and isoprenoid biosynthesis.

The feasibility of producing advanced biofuels from lignocellulosic substrates is a key focus. Much has been written on cellulosic biomass and its enormous potential for being a second generation feedstock (Perlack *et al.* 2005). Beyond its wide-scale availability, it is the only alternate carbon source to fossil fuels that does not compromise food security (Singhvi *et al.* 2014). Nonetheless, there are numerous challenges that must be addressed before advanced biofuels can be produced from lignocellulosic biomass. In addition to the presence of multiple sugars, lignocellulosic hydrolysate contains a spectrum of compounds, which are potentially toxic to the enzymes and/or microbes used in the bioconversion process (Azhar *et al.* 1981; Banerjee *et al.* 1981; Clark and Mackie 1984). These toxic compounds or inhibitors are both naturally present in the lignocellulosic substrates and also 'process derived,' typically from conversion processes such as steam pretreatment (Hahn-Hägerdal *et al.* 2007; Matsushika *et al.* 2009). However, the extent to which these different inhibitory compounds influence the rate and yield of the products is dependent on the nature of enzymes/microorganisms involved, the mode of fermentation, and the type of final products (Hahn-Hägerdal *et al.* 2007; Matsushika *et al.* 2009). The robustness of the genetically engineered strains to consume multiple sugars while being tolerant to a range of inhibitory compounds present in the lignocellulosic hydrolysates is assessed in this article. The additional and unique challenges related to producing each type of advanced fuels from cellulosic biomass are also analyzed. Finally, the status of commercialization of some of the advanced biofuels compared to bioethanol, potential research avenues, and the future perspectives for their commercial success are discussed.

ALTERNATIVE LIQUID BIOFUELS

Three different types of conventional liquid fuels used today include gasoline, middle distillates, and aviation fuels. All have different chemical compositions, primarily with respect to the type of hydrocarbons; consequently, they have different fuel properties (cetane and octane numbers, flash point, freezing point, *etc.*) and thus distinct applications (Table 1) (Ghosh *et al.* 2006; Fortman *et al.* 2008). Each category of petroleum fuels can be replaced by their biobased counterparts including short chain and long-chain alkanes, alcohols, esters, and fatty acids (Table 1). However, industrially relevant microorganisms and efficient bioprocesses are necessary to enable the commercial production of these compounds from biomass.

Table 1. Major Categories of Petroleum-based Conventional Fuels and their Potential Biomass-based Equivalents

Major Petroleum Derivatives	Major Composition	Application	Price per Gallon 2016 ($USD)	Potential Biofuel Alternatives
Gasoline	4-12 carbon compounds	Spark ignition engines	$2.00 to $2.90	Ethanol, butanol, isobutanol, 3-methyl 1-pentanol, hexanol
Middle distillates	9-23 carbon compounds	Compression ignition engines	$2.00 to $2.40	Biodiesel, long-chain alcohols, linier or cyclic isoprenoids
Aviation fuels	8-16 (kerosene type) & 5-15 (wide-cut) carbon compounds	Gas turbines	$4.10 to $5.30	Farnesane and other isoprenoids, fatty acid derived alkanes or alcohols

Isopropanol, Butanol, and Long-Chain Alcohols

Isopropanol and butanol

Various species of *Clostridium* naturally produce isopropanol and butanol from acetyl-CoA through the ABE/IBE (acetone/isopropanol, butanol, and ethanol) fermentation route (Fig. 1) (George and Chen 1983; Lin and Blaschek 1983; Chen and Hiu 1986; Ismaiel *et al.* 1993). Because this fermentation generally produces a mixture of solvents (Lee *et al.* 2008) (acetone/isopropanol, butanol and ethanol) and the solvent titer is very poor (< 2%) (Lee *et al.* 2008), several efforts have been made to genetically engineer *Clostridia* to selectively enhance the yield of isopropanol and butanol (Peralta-Yahya *et al.* 2010; Branduardi *et al.* 2014). However, with limited genetic tools available to engineer *Clostridia*, researchers soon realized that even with genetic improvements, *Clostridium*, a strict anaerobe, has several limitations affecting its potential use as an industrial host (Branduardi *et al.* 2014). Therefore, subsequent efforts were mainly limited to heterologously express the *Clostridial* pathway in industrially relevant microorganisms such as *E. coli* and *S. cerevisiae* (Branduardi *et al.* 2014).

Production of ABE/IBE (Acetone/Isopropanol-Butanol-Ethanol) Solvents by Natural Microbial Strains

Several bacterial genera including *Clostridium*, *Butylvibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Eusobacterium*, and *Megasphera* generate butanol (Ni and Sun 2009). Despite several of the inherent limitations, *Clostridium* strains are the most industrially relevant among natural butanol-producing strains (Ni and Sun 2009). Common solventogenic *Clostridium*

species are *Clostridium acetobutylicum*, *Clostridium saccharobutylicum*, *Clostridium beijerinckii*, and *Clostridium saccharoperbutyl-acetonicum* (George and Chen 1983; Lin and Blaschek 1983; Chen and Hiu 1986; Ismaiel *et al.* 1993). While most of *Clostridia* species such as *C. acetobutylicum* generate ABE solvents, some others, such as *C. beijerinckii*, produce IBE (George and Chen 1983; Chen and Hiu 1986). Some promising butanol-producing strains reported are *Clostridium acetobutylicum* ATCC 824, *C. saccharobutylicum* P262, *C. beijerinckii* P260, and *C. beijerinckii* BA 101 (Qureshi *et al.* 2006; Dürre 2007; Ni and Sun 2009; Green 2011).

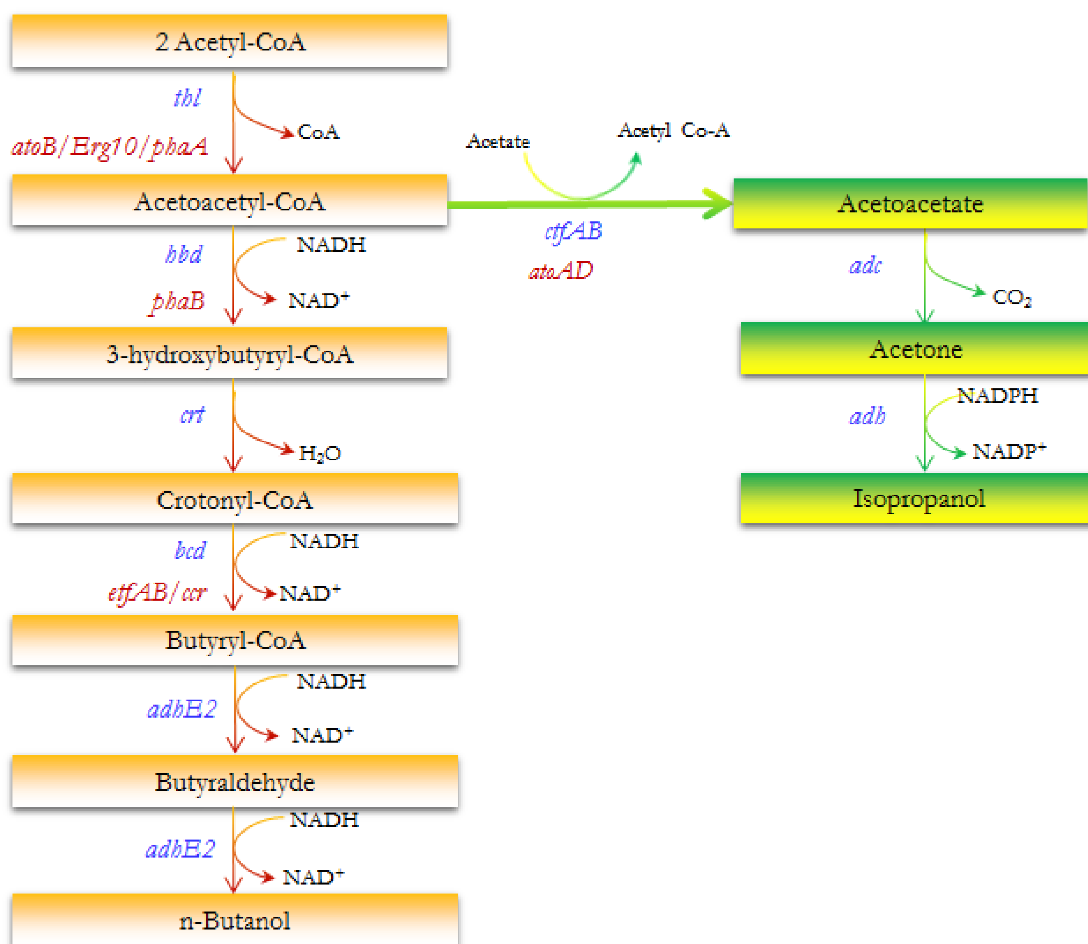

Fig. 1. Butanol and isopropanol biosynthesis pathway. Blue letters indicate *Clostridia* genes. Red letters refer to equivalent genes from microorganisms used in the genetic engineering studies.

In ABE/IBE fermentation, after glycolysis, pyruvate is converted to acetyl-CoA by the enzyme pyruvate ferredoxin oxidoreductases (Ezeji *et al.* 2007; Fischer *et al.* 2008; Zheng *et al.* 2009; Jang *et al.* 2012). Subsequently, two acetyl-CoA molecules undergo Claisen condensation to form acetoacetyl-CoA, which is reduced to 3-hydroxybutyryl-CoA, then dehydrated to form crotonyl-CoA and further reduced to butyryl-CoA, butyraldehyde, and finally butanol (Fig. 1) (Fischer *et al.* 2008; Nielsen *et al.* 2009; Zheng *et al.* 2009; Jang *et al.* 2012). Some of the acetyl-CoA is converted to acetaldehyde, which in turn is reduced by alcohol dehydrogenase to ethanol (Jones and Wood 1986). A smaller fraction of the acetoacetyl-CoA is converted to acetone, which in some species is further reduced to isopropanol by isopropanol dehydrogenase (Dürre

2007). In the IBE fermentation, depending on the strain and the cultivation conditions, residual acetone may also be an end-product (Ismaiel *et al.* 1993). During ABE fermentation, the acetone, butanol, and ethanol are typically generated in the ratio of 3:6:1 (Nimcevic and Gapes 2000).

The ABE/IBE fermentation involves a biphasic kinetic pattern comprising a first growth phase with the production of acetic acid and butyric acid (acidogenesis) followed by their re-assimilation into ABE/IBE solvents (solventogenesis) (Jones and Woods 1986; Girbal and Soucaille 1998) (Fig. 1). Production of organic acids in the acidogenic phase of the ABE/IBE fermentation lowers the pH outside the cell, and considerable amounts of undissociated acids can diffuse into the cytoplasm reducing the pH resulting in cell death (Huang *et al.* 1985; Jones and Wood 1986). Therefore, the cell is forced to re-assimilate the generated acids to alcohol to help maintain the external pH (Huang *et al.* 1985). This is the reason that subsequent production of acetic and butyric acids in the exponential growth, fermentation switches to the formation of neutral solvents shortly before entering the stationary phase (Dürre 2007). During the solventogenesis, microbial growth slows down and cells form granules and endospores (Paredes *et al.* 2005; Alsaker *et al.* 2004). In addition to ABE/IBE solvents, the process also produces carbon dioxide and hydrogen (Jones and Wood 1986).

One of the greatest challenges with using natural *Clostridia* strains for butanol production has been the low butanol titer (below 12 g/L) (Lee *et al.* 2008), which is more than ten times lower than the ethanol titer generally obtained in starch- or sugarcane-based ethanol fermentations (Amorim *et al.* 2011). Therefore, several genetic engineering studies were carried out to enhance the butanol titer, selectivity, and yield (Peralta-Yahya *et al.* 2010; Branduardi *et al.* 2014).

Genetic Engineering of *Clostridia* for Enhanced Butanol and Isopropanol Production

Several workers have investigated specific genetic manipulation, chemical mutagenesis, or their combination (Qureshi and Blaschek 1999; Gao *et al.* 2012; Branduardi *et al.* 2014). Successful examples of the specific genetic manipulations include the overexpression of acetone formation pathway by amplifying the *adc* (acetoacetate decarboxylase) and *ctfAB* (CoA transferase) genes, overexpression of *adhE* (aldehyde dehydrogenase), regulation of *soIR* (regulator of solvent production), reducing the activities of butyrate forming enzymes, and overexpression of heat shock proteins (Mermelstein *et al.* 1993; Nair *et al.* 1994; Harris *et al.* 2001; Lee *et al.* 2008; Papoutsakis, 2008). Controlling the regulator of solvent producing genes resulted in the best butanol concentration (17.6g/L) in *C. acetobutylicum* ATCC 824 (Nair *et al.* 1999; Harris *et al.* 2001; Thormann *et al.* 2002), which remains the most extensively studied and manipulated strain. Its genome sequence was published in 2001 (Nolling *et al.* 2001) followed by the genome sequence of *C. beijerinckii* NCIMB 8052 in 2007 (Ezeji *et al.* 2007).

The best genetically modified *Clostridia*—*C. beijerinckii* BA 101—produces a butanol concentration of 19.6 g/L (Qureshi and Blaschek 1999). This strain was developed by chemical mutagenesis with N-methyl N-9-nitro-N-nitrosoguanidine (Qureshi and Blaschek 1999; Gao *et al.* 2012). *C. beijerinckii* BA 101 was able to produce butanol and acetone yields two-fold greater than its parent strain (Qureshi and Blaschek 1999). The highest butanol ratio in the solvent mixture (0.7) was exhibited by *C. acetobutylicum* EA 2018, which was also developed using chemical mutagenesis (Hu *et al.* 2011). The acetone pathway was further knocked out in this strain increasing the butanol-to-total solvent ratio to 0.8 (Hu *et al.* 2011).

As isopropanol is commercially a more valuable chemical than acetone (Collas *et al.* 2012), genetic engineering efforts were also focused on the enhanced production of isopropanol (Lee *et al.* 2012). One of the earliest reported isopropanol concentrations from *Clostridium* was

1.8 g/L (Chen *et al.* 1986). Later, the *adh* gene from *C. beijerinckii* was cloned into the ABE-producing strain *C. acetobutylicum* ATCC 824. The resulting transformant excreted 6.1 g/L isopropanol and a minor amount of acetone (Lee *et al.* 2012). In *C. beijerinckii* NRRL B593, the reduction of acetone into isopropanol is catalyzed by a NADPH-dependent secondary-alcohol dehydrogenase (*s-adh*), which has been extensively characterized (Yan *et al.* 1988; Ismaiel *et al.* 1993; Korkhin *et al.* 1998; Goihberg *et al.* 2010). Although the *s-adh* was clearly distinct from *Clostridial* primary-alcohol dehydrogenases (Chen 1995) that reduce butyraldehyde into butanol, the *s-adh* showed activity on both primary and secondary alcohols, with a preference for secondary alcohols (Ismaiel *et al.* 1993). Kinetic studies confirmed that the physiological substrate was acetone.

Although *Clostridium* can naturally produce isopropanol and butanol, it is a strict anaerobe with a spore-forming life cycle characterized by a slow growth rate (Jones and Woods 1986; Zheng *et al.* 2009; Green 2011; Jang *et al.* 2012). In addition, limited genetic tools were available to engineer solvent production in this organism (Branduardi *et al.* 2014). These limitations have prompted researchers to attempt the heterologous expression of isopropanol and butanol pathways in *E. coli* and *S. cerevisiae* (Hanai *et al.* 2007; Atsumi *et al.* 2008a; Jojima *et al.* 2008; Inui *et al.* 2008; Steen *et al.* 2008; Nielsen *et al.* 2009; Peralta-Yahya *et al.* 2010; Branduardi *et al.* 2014).

Heterologous Expression of *Clostridial* Pathways in *E. coli* / *S. cerevisiae*

Metabolic engineering has been employed to create pathways for isopropanol production in *E. coli*. Introduction of four genes from *C. acetobutylicum* (*ctfA*, *ctfB*, *adc*, and thiolase (*thl*) into *E. coli* generated a strain capable of producing acetone, the precursor for isopropanol (Fig. 1) (Bermejo *et al.* 1998). Subsequent efforts on overexpressing the *C. beijerinckii adh* gene in combination with the above genes made it possible to overproduce isopropanol in *E. coli.* The engineered *E. coli* strains surpassed the best reported wild-type *Clostridial* strains, *C. beijerinckii* and *C. isopropylicum*, excreting approximately 4 g/L isopropanol (Groot and Luyben 1986; Matsumura *et al.* 1992). Later, use of *E. coli atoB* and *atoAD* instead of *thl* and *ctfAB* resulted in a concentration of 4.9 g/L (Hanai *et al.* 2007). Jojima and coworkers expressed *C. acetobutylicum* genes *ctfAB*, *ctfB*, and *thl* and *C. beijerinckii adh* genes from a dedicator promoter in a single vector instead of a polycistronic expression of the single operon and a two vector system (Jojima *et al.* 2008). This approach enabled them to obtain 13.6 g/L of isopropanol concentration, which was greater than what was reported with homologous expression (Jojima *et al.* 2008). A major advantage of the engineered *E. coli* strain compared to *S. cerevisiae* or *Clostridium* was the lack of important competing pathways for by-products such as ethanol or butanol (Hanai *et al.* 2007).

Heterologous expression of *Clostridial* butanol pathway in an industrial host has resulted in far lower butanol titer and yield than those obtained with *C. beijerinckii* BA 101. Atsumi *et al.* (2008a) employed a two operon system in *E. coli*. The first operon encoded *thl* and *adhE2*, and the second encoded the rest of the enzymes (*hbd*, *crt*, *bcd*/*etfAB*) (Atsumi *et al.* 2008a) (Fig. 1). Despite successful heterologous expression, the butanol concentration was only 13 mg/L, which was substantially lower than that obtained with natural *Clostridia* (Atsumi *et al.* 2008a). Subsequently, researchers have tried expressing several permutations and combinations of equivalent genes from different bacteria and yeasts including *E. coli*, *C. acetobutylicum*, *C. beijerinckii*, *Megasphaera elsdenii*, *Streptomyces coelicor*, and *S. cerevisiae* (Atsumi *et al.* 2008a; Inui *et al.* 2008). Efforts included deleting some of the host genes, which compete with the butanol production pathways primarily for acetyl-CoA and NADH (Hanai *et al.* 2007; Atsumi *et al.* 2008a; Inui *et al.* 2008). Among these, the most noted progress was made by Hanai

and coworkers (2007) in their work on *fnr* deletion to activate pyruvate dehydrogenase complex. They obtained a butanol concentration of 373 mg/L. Inui and coworkers (2008) expressed the butanol pathway in a single vector with the first promoter expressing *crt-bcd-/etfAB/hbd*, the second promoter expressing *thl*, and the third promoter expressing *adhE1/adhE2*. In this system, the use of *adhE2* instead of *adhE1* enhanced butanol production from 320 to 540 mg/L respectively, which was further enhanced to 1.2 g/L by extending the fermentation time to 60 hours. Nielsen *et al.* (2009) compared a polycistronic two-vector system with the individual expression of the genes from dedicated promoters in a four vector system (Nielsen *et al.* 2009). Individual gene expressions enabled a six-fold improvement in butanol concentration. This approach combined with the replacement of *thl* with *E. coli atoB*, incorporation of *S. cerevisiae fdh1* (formate dehydrogenase to enhance the NADH concentration) and overexpression of *gapA* to enhance the carbon flux through glycolytic pathway enhanced the butanol concentration up to 580 mg/L (Nielsen *et al.* 2009). Although the titers were higher than previous work, the values were considerably lower than the titer obtained with the natural *Clostridium* (Inui *et al.* 2008).

Despite many attempts to introduce the *Clostridial* pathway in *S. cerevisiae* (Fig. 1), the yields and productivities were poorer than engineered *E. coli*. Steen and coworkers (2008) have investigated expressing different combinations of acyl transferases (*Ralstonia eutropha phaA*, *E. coli atoB*, and *S. cerevisiae ERG10*), 3-hydro-xybutyryl-CoA dehydrogenase (*R. eutropha phaB*, *C. beijerinckii hbd*), crotonase (*C. beijerinckii*), butyryl-CoA dehydrogenase (*Streptomyces collinus ccr*), and alcohol dehydrogenase (Steen *et al.* 2008). The best optimized combination was *Erg10-hbd-crt-ccr-adhE2*, which resulted in 2.5 mg/L of butanol; 200 times lower than the best values obtained with engineered *E. coli* (Steen *et al.* 2008).

Bio-Alcohols via Rerouting of the Amino Acid Biosynthesis Pathway

In addition to the heterologous expression of *Clostridial* pathways in *E. coli* or *S. cerevisiae,* re-routing the amino acid biosynthesis pathway is another approach to produce a range of C4 or higher bioalcohols including n-butanol (Atsumi and Liao 2008a; Lamsen and Atsumi 2012; Bujis *et al.* 2013). In this approach, ketoacid intermediates generated during amino acid biosynthesis are rerouted via sequential decarboxylation to the aldehyde (by keto-acid decarboxylase) followed by the reduction of the aldehyde to alcohol (by alcohol dehydrogenase) (Lamsen and Atsumi 2012; Bujis *et al.* 2013) (Table 2). Interestingly, the same pathway—the Ehrlich pathway—naturally exists in yeasts for the production of "fusel oil", a by-product of fermentation (Fig. 2). There has been extensive research conducted to reconstitute the Erlich pathway in *E. coli* or *S. cerevisiae* for the production of different types of alcohols.

Table 2. Alcohols Produced by Re-routing the Amino Acid Biosynthesis Pathway in *E. coli / S. cerevisiae* and Ketoacid Intermediate Involved in their Production

Amino Acid Target	Ketoacid Intermediate	Potential Alcohol Products
Isoleucine	2-ketobutyrate	Isopropanol
	2-keto-3-methylvalerate	2-methyl-1-butanol
Valine	2-ketoisovalerate	Isobutanol
Leucine	2-keto-4-methylpentanoate	3-methyl-1-butanol
Phenylalanine	Phenylpyruvate	2-phenylethanol

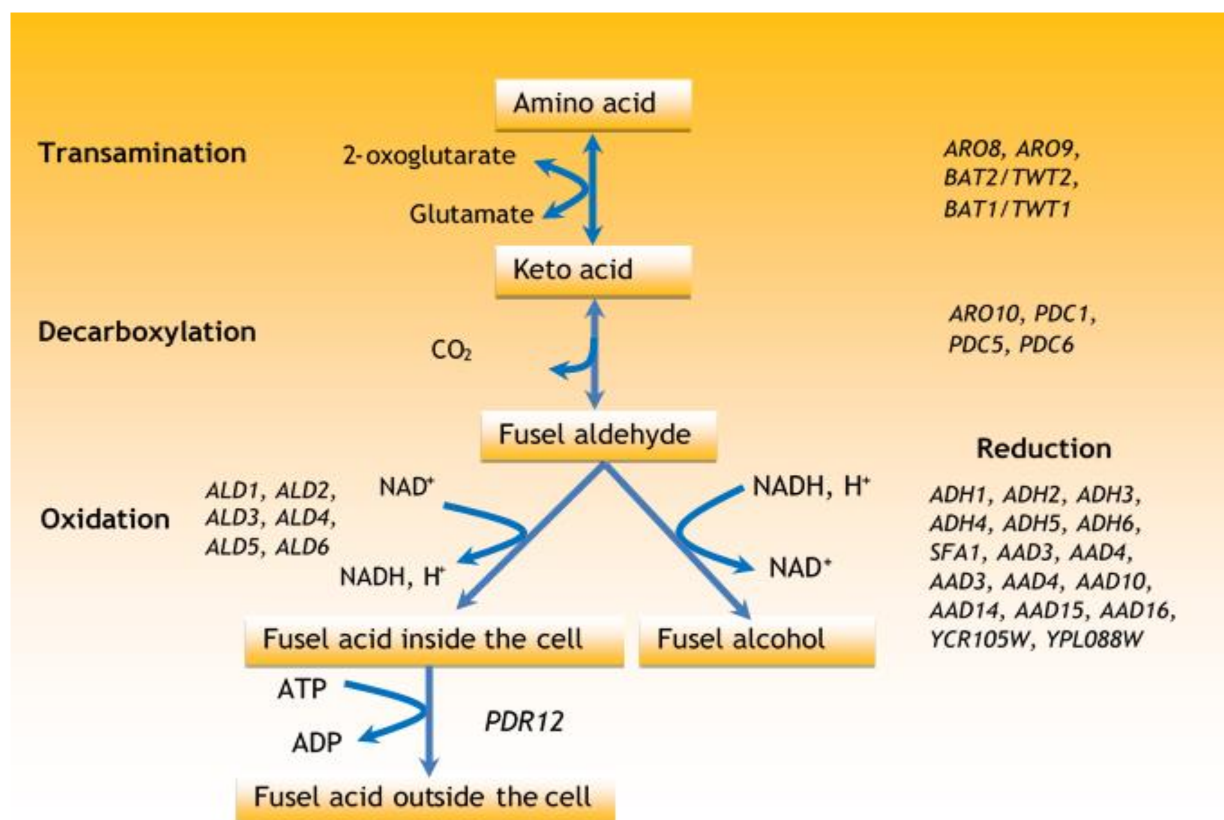

Fig. 2. Generic Erlich pathway for production of fusel oil (Hazelwood *et al.* 2008)

Lack of selectivity is one of the challenges when re-routing the amino acid biosynthesis pathway for the production of a given alcohol. Atsumi and coworkers (2008b) successfully introduced alpha-isoketovalerate decarboxylase from *Lactococcus lactis* (*kivd*) and alcohol dehydrogenase from *S. cerevisiae* (*adh2*) in *E. coli* with the objective of producing isobutanol. However, due to the broad substrate specificity of the decarboxylase enzymes, a range of keto-acids were converted to corresponding alcohols (Table 2), resulting in a mixture of six different alcohols (1-propanol, 2-methyl 1-butanol, isobutanol, 3-methyl 1-butanol, 2-phenyl ethanol and 1-butanol) with isobutanol produced in the highest concentration (389 mg/L) (Atsumi and Liao 2008b). Subsequent attempts have been made to enhance the selectiveness of isobutanol generated in the alcohol mix (Atsumi *et al.* 2008b).

The three main strategies that have been adapted to enhance the isobutanol concentration include: a) overexpression of native or heterologous expression of the enzymes of the ketoisovalerate pathway; b) overexpression of ketoacid decarboxylase; and c) overexpression of alcohol dehydrogenase. Selective increase in the concentration of the precursor acid isoketovalerate (by overexpressing of the *ilvHCD* genes in *E. coli*) together with deletion of the competing pathways that divert the isoketovalerate and pyruvate pools, resulted in an isobutanol concentration of 22 g/L, which surpassed the best reported n-butanol values (19.6 g/L) with *Clostridium* (Qureshi and Blaschek 1999; Atsumi *et al.* 2008b). Similar approaches were also investigated in *S. cerevisiae*, where the most effective combination was the heterologous expression of *Bacillus subtilis* acetolactase synthase (*AlsS*) to enhance the pool of 2-keto isovalerate and *L. lactis KivD* decarboxylase together with deletion of the ethanol formation

pathways (deletion of pyruvate decarboxylase genes) (Anthony *et al.* 2010; Festel *et al.* 2011; Urano *et al.* 2012; Lies *et al.* 2012). These attempts resulted in titers of isobutanol up to 12 g/L (Urano *et al.* 2012; Lies *et al.* 2012). Rerouting the isoketovalerate pathway later formed the basis of the industrial initiatives on isobutanol by Gevo and Butamax.

Similar to isobutanol, strategies have been developed to enhance the concentration of 1-butanol by re-routing the amino acid biosynthesis pathway. Some of the successful approaches include: deregulation of the threonine biosynthetic pathway; enhancing the concentration of the 2-ketovalerate by the overexpression of *LeuABCD*, which converts 2-ketobutyrate derived from the threonine biosynthetic pathway to 2-ketovalerate; and deletion of the interfering pathways that involve the production of 2-ketoisovalerate and pathways for threonine consumption. With all these strategies, the highest isopropanol/butanol concentration obtained was 2 g/L. Atsumi and Liao (2008c) have also constructed a more direct pathway for the production of 2-ketovalerate in *E. coli* by introducing the citramalate synthase pathway, which bypasses the threonine biosynthetic pathway in the production of 2-ketovalerate. This attempt resulted in a combined isopropanol/butanol production of 4 g/L (Atsumi and Liao 2008c).

Production of 3-methyl-1-butanol was accomplished by the conversion of 2-ketoisovalerate to 2-ketoisocaproate (2-keto-4-methylpentanoate), which was then decarboxylated and reduced (Connor *et al.* 2010). Enhancing the concentration of 2-ketoisovalerate (heterologous expression of *B. subtilis alsS* instead of *E. coli ilvlHCD* and enhancing their subsequent conversion to 2-ketoisocaproate by enhancing the activity of *leu ABCD*), the heterologous expression of alpha-isoketovalerate decarboxylase from *L. lactis kivd* and alcohol dehydrogenase from *S. cerevisiae adh2* were some of the genetic engineering tools employed. The best reported concentration has been 4.4 g/L of 3-methyl-1-butanol (Connor *et al.* 2010).

Similar to the production of 3-methyl-1-butanol, 2-methyl-1-butanol could also be produced from 2-keto-3-methylvalerate (Cann and Liao 2008). Acetohydroxyacid synthase (AHAS) is regarded as an important enzyme in this pathway because it catalyses the condensation of 2-ketobutyrate and pyruvate to form 2-aceto-2-hydroxy butyrate. Cann and Liao constructed an *E. coli* strain by overexpressing the native *thrABC* to enhance the pool of threonine and used threonine deaminase (*IlvA*) from *Corynebacterium glutamicum* to facilitate the transamination of threonine to 2-ketobutyrate and overexpressed *AHAS* gene from *Salmonella typhimurium* to catalyze the condensation of 2-ketobutyrate and pyruvate to form 2-aceto-2-hydroxy butyrate. Expressing the native *ilvC* and *ilvD* enabled the conversion of 2-aceto-2-hydroxy butyrate to 2-keto-3-methylvalerate, which is then decarboxylated by using *L. lactis kivd* and dehydrogenated using *S. cerevisiae adh2* to form 2-methyl-1-butanol. This optimization including the knocking out of competing pathways resulted in a production titer of 1.3 g/L.

Researchers were able to produce long chain (C5-C8) alcohols in *E. coli* by expanding the natural metabolic network by producing longer chain ketoacids and respective alcohols (Zhang *et al.* 2008). Because 2-ketoisovalerate can be naturally converted to 2-ketoisocaproate (leucine biosynthesis) by a three step chain elongation using *LeuABCD*, Zhang and coworkers (2008) explored the potential of *LeuA*, *LeuB*, *LeuC*, and *LeuD* to extend the elongation in the similar fashion for 2-keto-3-methylvalerate to 2-keto-4-methylhexanoate. Subsequently, *L. lactis* decarboxylase (*kivd*) and *S. cerevisiae* dehydrogenase (*adh6*) was used to generate corresponding six-carbon alcohols, 3-methyl-1-pentanol albeit a low concentration (794 mg/L) (Zhang *et al.* 2008; Lamsen and Atsumi 2012). Similar to 2-ketoisovaerate, 2-keto-valerate is elongated to 2-ketocaproate during isoleucine biosynthesis. The 2-ketocaproate was also shown to be further elongated by *LeuABCD* to 2-ketoheptanoate, which is subsequently decarboxylated and reduced to n-hexanol (Zhang *et al.* 2008; Lamsen and Atsumi 2012).

Similar to the heterologous expression of *Clostridium* pathway, re-routing amino acid biosynthesis pathways were relatively successful in *E. coli* compared to *S. cerevisiae* (Lamsen and Atsumi 2012; Buijs *et al.* 2013). One of the challenges with the yeasts has been the co-factor imbalances and the difficulty in dealing with a more complex system compared to bacteria (Buijs *et al.* 2013). Although researchers attempted to engineer for cofactor specificities of the involved oxidoreductases, the yields were substantially low (Buijs *et al.* 2013).

Despite a relatively higher success rate in the engineering of *Clostridia* pathway and amino acid biosynthesis pathway in *E. coli* as compared to *S. cerevisiae* (Rabinovitch-Deere *et al.* 2013), overcoming *E. coli* solvent toxicity presents a continued challenge (Table 3). It is apparent that *E. coli* can tolerate solvent concentrations in the vicinity of 1 to 2%. Increasing chain length increases the hydrophobicity of alcohol and its toxicity (Dunlop 2011). Increased hydrophobicity results in the disruption of the phospholipid component of the cell membrane (Ingram and Buttke 1984; Ingram 1986; Chin *et al.* 2013; Kolek *et al.* 2015). For example, increasing the butanol concentration from 0.5 to 1% can cause a 30% increase in the fluidity of lipid dispersal (Vollherbst-Schneck *et al.* 1984). The increase in the ratio of saturated to unsaturated fatty acids is a consequence of cell growth in the presence of long chain alcohols (Chin *et al.* 2013). Other consequences of solvent toxicity include inhibition of membrane bound ATPase activity, collapse of the membrane pH gradient, decreased intracellular ATP levels, and inhibition of the uptake of sugars and amino acids (Moreira *et al.* 1981; Ounine *et al.* 1985; Bowles and Ellefson 1985; Gottwald and Gottschalk 1985). Efforts to optimize the process strategies and decrease the butanol toxicity of *Clostridia* include the addition of saturated fatty acids, decreasing in temperature from 30 °C to 24 °C in the solventogenic phase, and developing mutants with increased butanol tolerance (Liu and Qureshi 2009; Dunlop 2011). Although *S. cerevisiae* may be a more robust industrial host that can tolerate higher product concentrations, a multi-fold increase in the product yield is necessary to achieve yields that are industrially relevant. Interestingly, isobutanol is comparatively less toxic than other alcohols, which largely enabled the researchers to obtain isobutanol titers surpassing the highest titer reported for n-butanol (Anthony *et al.* 2010; Festel *et al.* 2011; Lamsen and Atsumi 2012; Lies *et al.* 2012; Urano *et al.* 2012). Isobutanol is produced in wild type *S. cerevisiae via* valine metabolism. Therefore, if genetic engineering of *S. cerevisiae* is further optimized, isobutanol production seems promising (Hazelwood *et al.* 2008). However, it is worth highlighting that the amino acid biosynthesis pathway is tightly regulated in nature *via* feedback inhibition of the intermediates. Constructing a fast and deregulated pathway with high metabolic flux requires significant efforts on metabolic engineering and optimization (Buijs *et al.* 2013).

Table 3. Tolerance of Naturally Occurring, Genetically Engineered Microbial Strains to Ethanol, Butanol and Other Advanced Biofuel Molecules (Liu and Qureshi 2009; Dunlop 2011; Amyris 2013)

Culture	Maximum Tolerance (g/L)	Maximum Production (g/L)	Fermentation Substrates
Ethanol			
S. cerevisiae	180	180	Glucose
Z. mobilis ATCC31821	130	130	Glucose
L. buchneri	140	12	Glucose and xylose
L. heterohiochii	180	–	–
L. homohiochii	180	–	–
Butanol			
C. beijerinckii P260	19.5	19.5	Glucose, hemicellulosic sugars
C. beijerinckii BA101	19.6	19.6	Glucose, hemicellulosic sugars
C. acetobutylicum EA 2018	14.4	14.4	–
C. acetobutylicum solRH	17.6	17.6	–
Z. mobilis ATCC31821	10	–	–
E. coli W3110	10	–	–
L. delbrueckii	25	–	–
L. brevis	30	–	–
B. subtilis	13	0.024	–
P. putida	8	0.122	–
Other Advanced Biofuels (Genetically Engineered *E. coli / S. cerevisiae*)			
E. coli - Isobutanol	22	22	Glucose
E. coli - 2-Methyl-1-butanol	–	0.1	Glucose
E. coli - 3-Methyl-1-butanol	–	4.4	Glucose
E. coli - 1-Propanol	–	5.0	Glucose
E. coli - C6-C10 alcohol	–	0.42	Glucose
E. coli - Fatty acids	–	6.6	Glucose
E. coli - Fatty acid ethyl ester	–	0.67	–
E. coli - Farnesyl hexanoate	10	–	Glucose
E. coli - Geranyl acetate	5	–	Glucose
E. coli - Pinene	5	–	Glucose
E. coli - Limonene	0.25	–	Glucose
E. coli - Amorphadiene	–	37	Glucose
S. cerevisiae - Farnesene	–	14	Glucose and xylose

Fats, Lipids, and Esters

Long-chain fatty acids represent a key precursor for advanced fuels and chemicals because they can be transformed to a range of hydrocarbons and their derivatives. Fatty acids can be reduced to aldehydes and can then be subjected to decarbonylation, sequential reduction, or decarboxylation to produce equivalent alkanes as petroleum distillates (Fig. 3) (Lennen and Pfleger 2012). Fatty acid biosynthesis is one of the anabolic pathways capable of producing large hydrophobic fatty acid molecules and their derivatives (Fig. 4) Therefore, engineering fatty acid synthesis in industrial hosts such as *E. coli* and *S. cerevisiae* for the production of fatty acids is a potential route for the production of advanced biofuels and chemicals.

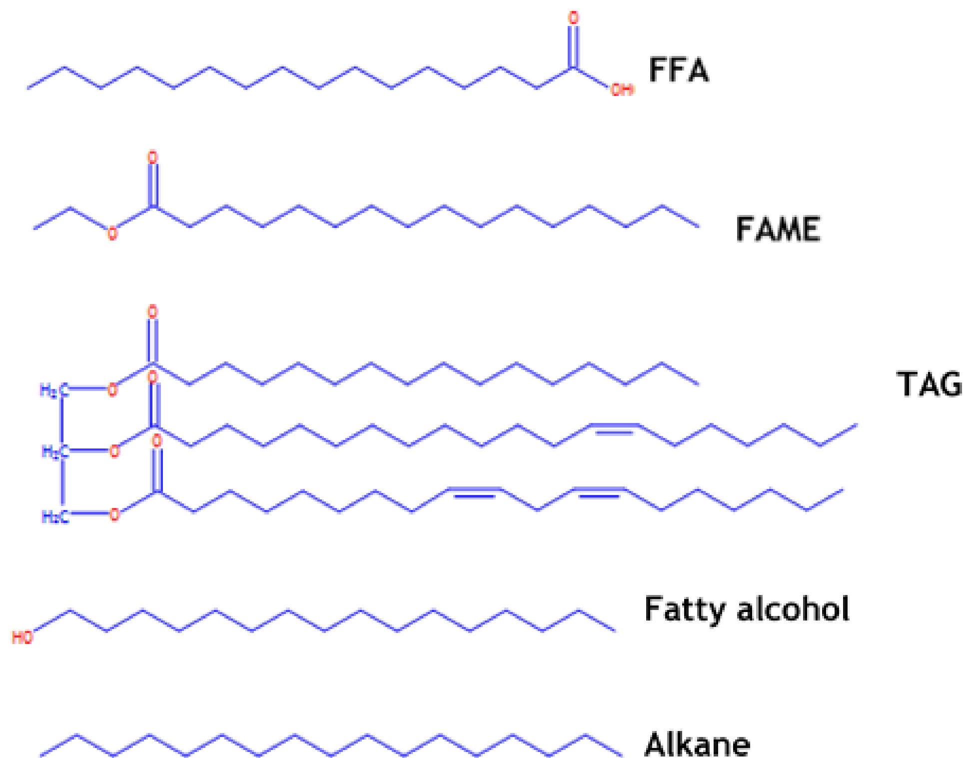

Fig. 3. Examples of target molecules obtained by fatty acid biosynthesis: free fatty acid (FFA), fatty acid methyl ester (FAME), triacylglyceride (TAG), fatty alcohol, and long-chain alkane

Microbial overproduction of fatty acids

Synthesis of fatty acid is an important part of lipogenesis in prokaryotes and eukaryotes. In the fatty acid biosynthesis pathway, fatty acids are generated from acetyl-CoA and malonyl-CoA precursors through action of fatty acid synthases (Steinbuechel 2014). Two carbon units are sequentially condensed or elongated onto growing fatty acyl chains. In the first step, acetyl-CoA carboxylase catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate. Subsequently, acetyl-CoA and malonyl-CoA converts to acetyl ACP and malonyl ACP by their respective transacylase enzymes. The next step is the condensation of aceyl-ACP and malonyl ACP to form acetoacetyl ACP (by the enzyme, ß-ketoacyl ACP synthase) followed by the reduction of acetoacetyl ACP (catalyzed by ß-keto acyl ACP reductase) and dehydration (by ß-hydroxy acyl-ACP dehydratase) and a second reduction (by enoyl-ACP reductase) to form butyryl ACP. This elongation cycle involving condensation, reduction, dehydration, and reduction is repeated (Fig. 4). After reaching the required chain length, the respective fatty acid-ACP is hydrolyzed by thioesterase to generate free fatty acid in eukaryotes, whereas in prokaryotes fatty acid-ACP is converted to glycerol-3-phosphate (Steinbuechel 2014).

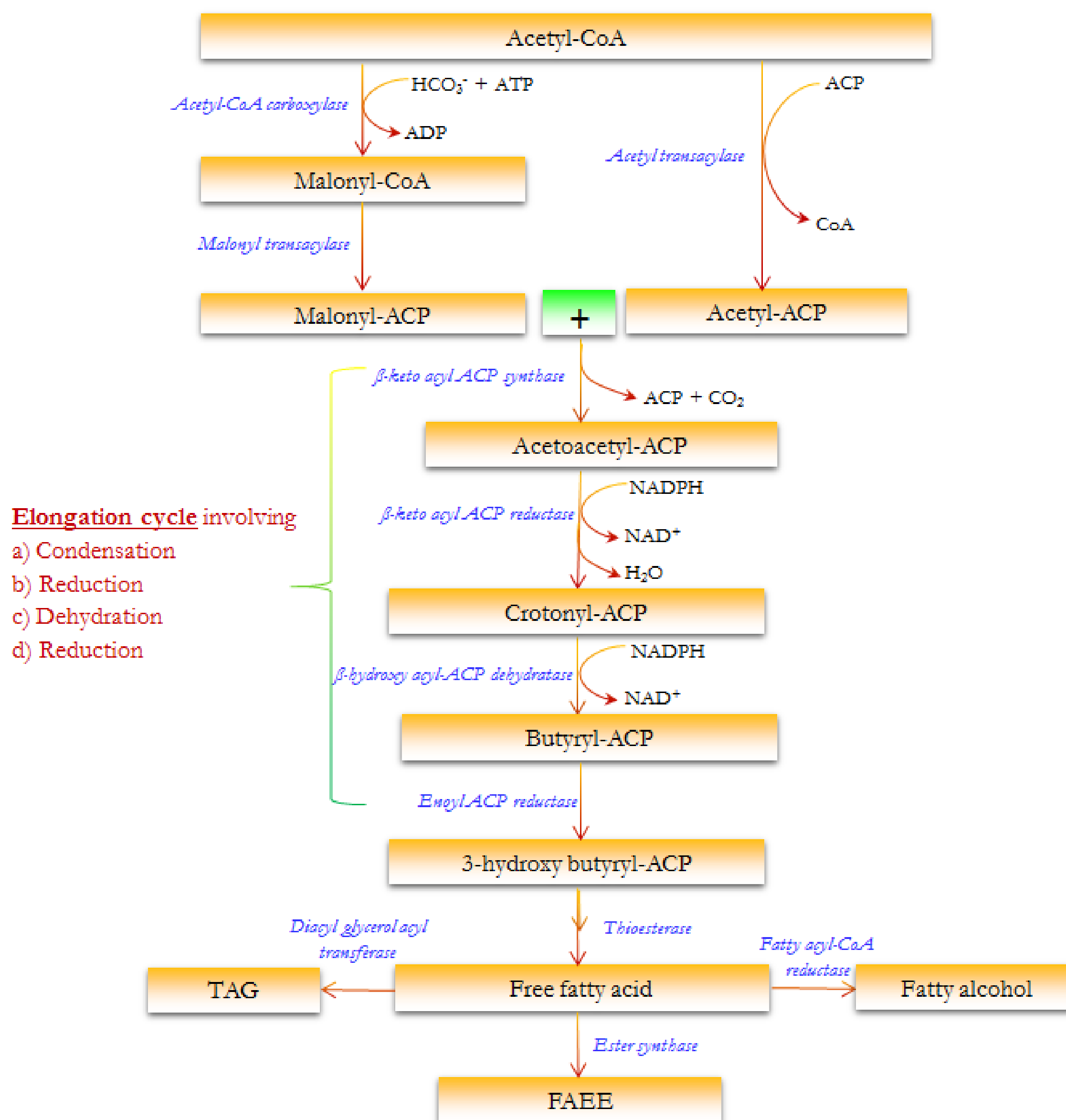

Fig. 4. Biosynthesis of fatty acid and its derivatives (Peralta-Yahya and Keasling 2010; Steinbuchel and JanBen 2014)

Thioesterases have been one of the key targets of metabolic engineering to achieve the desired carbon chain length (Davis *et al.* 2000; Lu *et al.* 2008). In eukaryotes, the four enzymes necessary for these four reaction steps in the elongation cycle are present in a multi-domain protein called type I fatty acid synthase, whereas in prokaryotes each step of the elongation cycle is performed by mono-functional enzyme called type II fatty acid synthase (Steinbuechel 2014). Two strategies have been adapted to engineer microorganisms for enhanced production of fatty acids in *E. coli* and *S. cerevisiae*; one is to minimize the breakdown of fatty acids by targeting the fatty acyl-CoA synthase genes, which are involved in the aerobic and anaerobic oxidation of fatty acid. The second strategy is to overexpress the genes involved in the fatty acid biosynthesis pathway, thus diverting maximum acetyl-CoA towards the production of fatty acids (Davis *et al.* 2000; Lu *et al.* 2008; Pfromm *et al.* 2010). Deletion of *E. coli fadD* and overexpression of the acetyl-CoA carboxylase (*acc*) and thioesterase enzymes were reported to result in a free fatty

acid titer of 2.5 g/L (Davis *et al.* 2000; Lu *et al.* 2008). Engineering the reversal of β-oxidation cycle to avoid the degradation of fatty acids has resulted in the best fatty acid titer, 6.6 g/L in engineered *E. coli* (Dellomonaco *et al.* 2010). However, it was found that majority of the fatty acids produced were reabsorbed by the cell to be used as a carbon and energy source (Lu *et al.* 2008).

Fatty acid has been esterified *in situ* in *E. coli* by introducing pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* and overexpressing the diacylglycerol acetyl transferase from *Acinetobacter baylyi*, resulting in 1.3 g/L of fatty acid ethyl ester (Kalscheuer *et al.* 2006). Shi and coworkers (2012) heterologously expressed five different wax ester synthases in *S. cerevisiae*, and the best results (6.3 mg/L) were obtained with the ester synthase from *Marinobacter hydrocarbonoclasticus*.

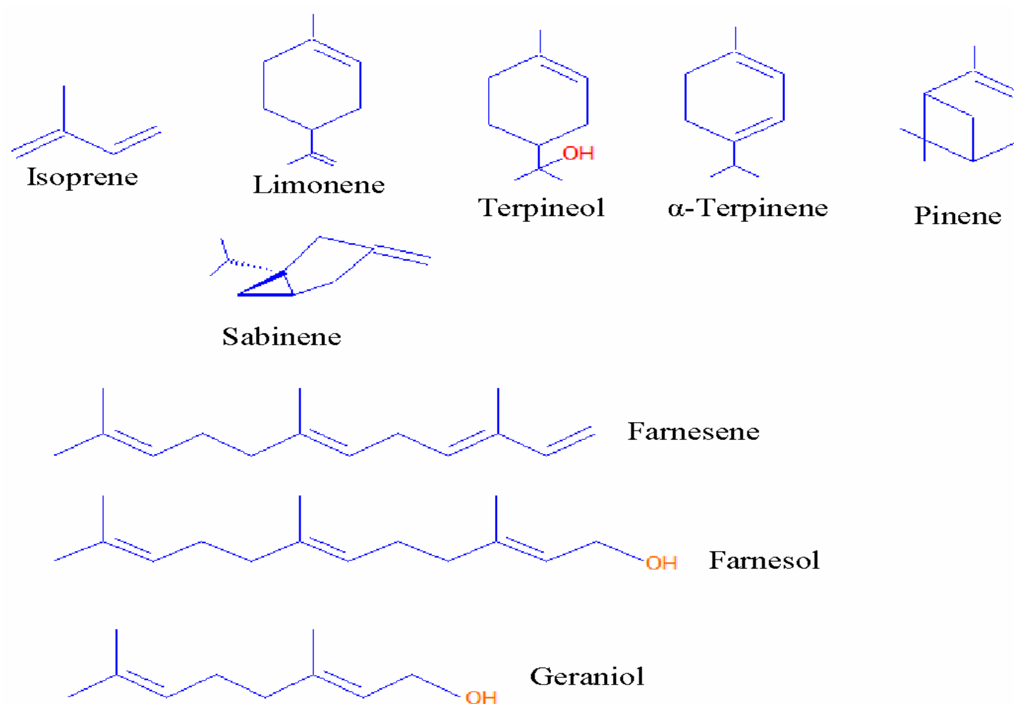

Fig. 5. Examples of isoprene derivatives (hemi-, mono-, and sesquiterpenoids) obtained *via* the isoprenoid biosynthesis pathway

Isoprenoids and Their Derivatives

Different types of isoprenoids

Terpenes belong to a large and diverse isoprenoid family with important medical and industrial applications (Chandran *et al.* 2011; Bujis *et al.* 2013; Schwab *et al.* 2013) (Fig. 5). As molecules in this category are diverse, it is uncertain as to what terpene would fit best as a gasoline, diesel, or jet fuel substitute (Chandran *et al.* 2011). While branched chain and short terpenes seem suitable as a gasoline substitute, longer cyclic or branched chain terpenes are appropriate as a diesel/jet fuel substitute (Fig. 5). Some monoterpenes currently explored for jet fuels include pinene, terpinene, and sabinene. A sesquiterpene, α-farnesane, which is reduced from α-farnesene, has also been proposed as a jet fuel precursor due to its low hygroscopicity and high energy density (Bujis *et al.* 2013).

Metabolic engineering for isoprenoid production

Terpenes are microbially produced by the metabolic engineering of the isoprenoid pathway (Chandran *et al.* 2011) (Fig. 6), which is a cellular metabolic pathway critical to the biosynthesis of many molecules including terpenoids and steroids (Wallaart *et al.* 2000). As the sesquiterpenes, including α-farnesene, are naturally produced in limited quantities (Wallaart *et al.* 2000), metabolic engineering is essential to overproduce these compounds (Peralta-Yahya *et al.* 2011; Westfall *et al.* 2012). The basic precursor monomers for isoprenoids are isoprenyl pyrophosphate (IPP) and its isomer dimethyl allyl pyrophosphate (DMAP) (Chandran *et al.* 2011).

These monomers are naturally produced from the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in *E. coli* (also known as deoxyxylulose pathway – DXP pathway) and the mevalonate (MVA) pathway in eukaryotes (Fig. 6). The monomers are condensed by IPP synthases to their corresponding monoterpenes, sesquiterpene, diterpenes, *etc.* (Peralta-Yahya *et al.* 2011) (Fig. 6). Resulting long-chain pyrophosphate molecules are further transformed into branched chain or cyclic alkenes by terpene synthases (Chandran *et al.* 2011) (Fig. 6). Finally, isoprenoid tailoring enzymes oxidize alkenes to alcohols or oxidize them to corresponding alcohols (Chandran *et al.* 2011).

Current metabolic engineering for isoprene biofuels is based on previous efforts to genetically engineer *E. coli* to produce two sesquiterpene derivatives: amorphadiene, a precursor for therapeutics/nutraceuticals, and artemisinic acid, a precursor for antimalarial drug (Peralta-Yahya and Keasling, 2010; Buijs *et al.* 2013). Since the production of IPPs and DMAPs are critical to the synthesis of isoprenoids, many researchers have attempted to optimize the deoxyxylulose pathway (DXP) (Farmer and Liao 2000; Alper *et al.* 2005; Alper *et al.* 2006; Yuan *et al.* 2006) and the heterologous expression of *S. cerevisiae* mevalonate pathway in *E. coli* (Martin *et al.* 2003) to enhance the production of these monomers (Fig. 6). Despite extensive efforts on optimizing DXP pathway, titers of amorphadiene and artemisinic acid were less than 500 mg/L (Martin *et al.* 2003). Much better results were obtained when the *S. cerevisiae* mevalonate pathway was expressed in *E. coli*, but it required extensive optimization efforts, as the native regulation of the pathway gets substantially altered resulting in an imbalance in gene expression and enzyme activity (Martin *et al.* 2003). Consequences faced were many including the depletion of the precursor compounds, accumulation of toxic intermediates, and changes in enzyme activity. For example, Pitera and coworkers (2007) found that enhanced concentrations of mevalonate led to the accumulation of the co-enzyme HMG-CoA. After extensive optimization efforts, the best titer was obtained with the combination of the heterologous expression of *Staphylococcus aureus mvaS*, *mvaA*, *and E. coli atoB* resulting in an amorphadiene concentration of 27 g/L. Over expression of the mevalonate pathway also enabled high titer amorphadiene production in *S. cerevisiae* (37 g/L) (Westfall *et al.* 2012).

In addition to amorphadiene and artemisinic acid, there were efforts to genetically engineer the isoprenoid pathway for biofuels. Yoshikuni and coworkers (2006) first introduced terpene synthase mutants into *E. coli* for the production of IPP and DMAP monomers. Eight different terpenes were obtained (α-farnesene, sabinene, γ-humulene, sibirene, longifolene, α-longpinene, α-ylange, and β-bisabolen), most of which have potential as biofuels.

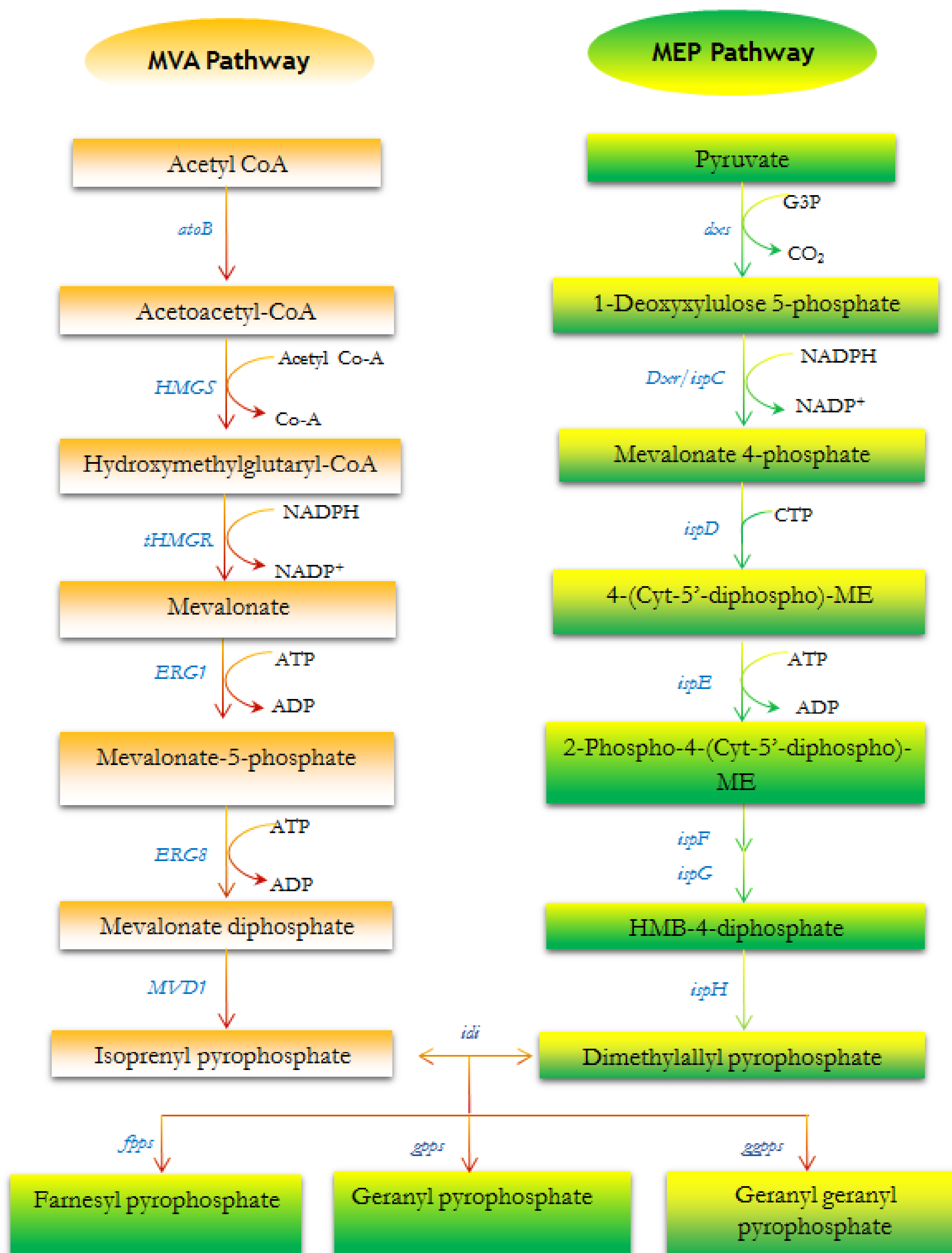

Fig. 6. The 2-C-methyl-d-erythritol-4-phosphate (MEP) and mevalonate (MVA) pathways for the biosynthesis of isoprenoids and their derivatives (Chandran *et al.* 2011)

A U.S. company, Amyris, has been working on developing microbes to overproduce farnesene, which can be further hydrogenated to farnesane. They used *S. cerevisiae* as the host and genetic engineering strategies focused on enhancing the acetyl-CoA pool, precursors for the

MVA pathway. Overexpression of the enzymes *ald6* and *Salmonella enterica acs1*, *adh2*, and repression of *ERG9*, which encodes squalene synthase, and over expression of *HMG1* were some of the strategies used to enhance farnesene yield (Shiba *et al.* 2007; Ohto *et al.* 2009; Asadollahi *et al.* 2008; Asadollahi *et al.* 2010; Chen *et al.* 2012). The titer of farnesane obtained was ~14 g/L, which still far lower than what is obtained in industrial ethanol fermentation (Amyris 2013).

TECHNOLOGICAL CHALLENGES FACING ADVANCED BIOFUEL PRODUCTION

Low Product Titer and Yield

Challenges with strain development

Robust microbial strains with high product yields, titers, and tolerance to products and other inhibitory compounds are prerequisites to the economic competitiveness of advanced biofuels (Connor and Liao 2008; Nicolaou *et al.* 2010; Dunlop 2011; Lamsen and Atsumi 2012; Bujis *et al.* 2013; Dellomonaco *et al.* 2010; Ezeji *et al.* 2010). However, the current titer and yield of advanced biofuel molecules is substantially lower than first-generation ethanol (Table 3). Despite extensive efforts on the screening of natural isolates together with genetic engineering, the highest butanol tolerance so far reported is 19.6 g/L (Qureshi and Blaschek 1999) with a total solvent production of 29 g/L (Qureshi and Blaschek 1999). Similarly, tolerance limits of most isoprenoids are < 40 g/L (see Table 3) and the highest titer obtained for amorphadiene at the lab scale (in engineered *S. cerevisiae*) is 37 g/L (Pitera *et al.* 2007). Achieving high titers with free fatty acids (maximum yield ~7 g/L) is even more challenging, with significant reduction in the colony-forming unit (CFU) occurring at concentrations as low as ~1 g/L. Economic viability requires a titer of at least 100 g/L (10%) with a productivity of 2 g/L/h and a theoretical yield of > 90% (Sheridan 2009). Although first-generation bioethanol production has reached these performance levels (Amorim *et al.* 2011), titer, productivity, and yields of advanced biofuel systems lag considerably, even when simple substrates such as glucose are used (Peralta-Yahya and Keasling 2010; Lamsen and Atsumi 2012; Bujis *et al.* 2013; Branduardi *et al.* 2014).

In spite of recent developments of a universal gene knock-out system for *Clostridia* and the expected advancements in metabolic engineering (Heap *et al.* 2007; Papoutsakis, 2008; Jang *et al.* 2012), *Clostridia* have several disadvantages in becoming an industrial host for the bulk production of fuel and chemicals (Bujis *et al.* 2013; Branduardi *et al.* 2014). *Clostridia* are strict anaerobes and have a slow growth rate with rapid degeneration (Jones and Wood 1986; Nimcevic and Gapes 2000). They are highly susceptible to bacterial contamination, which was one of the reasons for the closure of many ABE fermentation plants in the 19th century (Nimcevic and Gapes 2000). Therefore, due to the familiarity of the genetics, molecular biology, and fermentation characteristics of *S. cerevisiae* or *E. coli*, constructing the *Clostridia* pathway in these industrial hosts seems to be a relevant strategy (Lamsen and Atsumi 2012; Bujis *et al.* 2013; Branduardi *et al.* 2014). However, both *S. cerevisiae* and *E. coli* also exhibit limited growth in > 2% butanol (Table 3). Although Gram-positive *Lactobacillus* strains, *L. delbrueckii* and *L. brevis*, can tolerate up to 3% butanol (Table 3), they do not naturally produce butanol (Liu and Qureshi 2009). Heterologous expression of their genetic traits in *E. coli* or *S. cerevisiae* or introducing the *Clostridia* pathway in these *Lactobacillus* strains has not been explored to a large extent. Similarly, *Hypothermus butylicus* produces a considerable amount of butanol, and limited genetic engineering has been carried out on this organism (Zillig *et al.* 1990). Thus, these areas may present opportunities for future research.

Although genetically tractable, *E. coli* has several limitations as an industrial organism for the bulk production of biofuels. *E. coli* is highly sensitive to changes in pH, temperature, salt concentration, and product concentration. In addition, most current processes based on genetically engineered *E. coli* or *S. cerevisiae* require glucose as a substrate. Utilization of sucrose present in sugarcane feedstocks presents a formidable challenge to these systems. It should be noted that most *E. coli* cannot directly utilize sucrose and need to be engineered for sucrose utilization (Sabri *et al.* 2013). Furthermore, if lignocellulosic substrates are used, in addition to exhibiting product tolerance, microbial strains will need to be tolerant to a range of inhibitors while consuming C5 and C6 sugars present in feedstocks (Dien *et al.* 2003; Vickers *et al.* 2012; Ling *et al.* 2014). It is apparent that to be an industrially relevant biofuel producer, the strain will require substantial metabolic engineering and optimization (Yomano *et al.* 1998; Alper and Stephanopoulos 2007; Miller and Ingram 2007; Knoshaug and Zhang 2009).

No known microorganism has been isolated or engineered with all the necessary traits for the bulk production of advanced biofuels. The most relevant microorganism that has been extensively used in the food, beverage, and fuel ethanol industries is the yeast, *S. cerevisiae*. Yeasts have many promising features such as their shear stress tolerance, reasonably stable genetic composition, short generation times, intrinsically high sugar tolerances, minimal generation of undesired metabolic by-products (due to well-controlled glycolysis), and a unique extracellular growth-permitting pH range (Amorim *et al.* 2011; Kitagaki and Kitamoto 2013). These factors make them favorable for alcoholic fermentation. In addition, some yeasts are able to flocculate and therefore can be easily filtered out of culture broth, a useful feature for cell recycle fermentations and product recovery (Amorim *et al.* 2011). These qualities make *S. cerevisiae* a preferred organism for industrial processes (Bujis *et al.* 2013). However, substantial reengineering of cellular metabolism in *S. cerevisiae* is required to obtain desirable yield and titers to enable industrial production of advanced biofuels (Peralta-Yahya and Keasling 2010; Lamsen and Atsumi 2012; Bujis *et al.* 2013; Branduardi *et al.* 2014).

Viability of in situ product removal techniques

While distillation process is industrially established, it would be highly energy-intensive to recover advanced biofuels from a low titer fermentation broth using distillation alone. For every ton of butanol recovered from a 2% broth, 6 tons of steam are required (Abdehagh *et al.* 2013) (In this article, metric units are used throughout, and the term "tons" indicates metric tons). This corresponds to a distillation cost of $54 per ton of butanol assuming the use of a natural gas boiler for steam generation and a natural gas cost of $2.95 per 1000 ft^3 ($2.78 per GJ). Therefore, alternative techniques were developed that enable instantaneous product removal from the broth, which not only enriches the concentration of solvent prior to distillation, but allows continuous fermentation by maintaining a low enough titer to reduce the microbial toxicity. Some prominent strategies used for *in situ* product removal include gas stripping, solvent extraction, and pervaporation (Table 4) (Ezeji *et al.* 2003; Ezeji *et al.* 2010; Dürre 2007; Abdehagh *et al.* 2013).

Table 4. Typical *in situ* Product Removal Strategies for Enhancing Product Titer in ABE Fermentation (Ezeji *et al.* 2004; Dürre 2007; Inokuma *et al.* 2010; Baez *et al.* 2011; Abdehagh *et al.* 2013; Xue *et al.* 2014)

Technology	Reactor Type	Maximum Sugar Conc. (%)*	Max. Solvent Conc. (%)**	Major Process Considerations & Challenges
Gas stripping (N_2 or CO_2+H_2)	Batch	16	8	• Tightly controlling the concentration of butanol in the fermentation broth. • Substrate inhibition.
	Fed Batch	50	23	
	Continuous	116 (Butanol)	46 (Butanol) 14.3 (Isopropanol)*** 5.0 (Isobutanol)***	
Solvent Extraction	Fed Batch	34	14	• Toxicity of the extracting solvent and high partition coefficient with butanol. • Best results obtained with oleyl alcohol.
Pervaporation	Batch	15	5	• Fouling of the membrane. • Membrane chemistry can be affected by fermentation broth properties (lignocellulosics *vs.* starch *vs.* cane juice).
	Fed Batch	50	17	

** Substrate concentration*
*** Product concentration*
**** Not from the same fermentation broth. Reported in separate work aimed at recovering the respective product mentioned*

In gas stripping, nitrogen or fermentation gases are bubbled through the fermentation broth to strip away the products (de Vrije *et al.* 2013; Xue *et al.* 2013b; 2014a; 2014b; Ezeji *et al.* 2003). The stripped gas is then passed through a condenser to recover solvent vapors. After the solvents are condensed, inert gas is recycled back to the fermenter (Xue *et al.* 2014a). The efficiency of gas stripping depends on the flow rate, level of foam, and the presence of other components in fermentation broth (Xue *et al.* 2014). The highest butanol selectivity was obtained when gas stripping was performed at 67 °C (Abdehagh *et al.* 2013). Although batch, fed batch, and continuous fermentation can be coupled with *in situ* product removal, continuous fermentation had the highest beneficial effect. The highest recovered ABE concentration by this method was 420 to 460 g/L (Xue *et al.* 2013; Ezeji *et al.* 2013) and 143 g/L and 56 g/L for isopropanol and isobutanol, respectively (Table 4) (Inokuma *et al.* 2010). Gas stripping can be more effective when combined with adsorption, such that gas-phase molecules are adsorbed on a solid surface to recover products (Oudshoorn *et al.* 2009). Adsorption using hydrophobic columns is effective for butanol or higher hydrocarbon derivatives such as fatty acids or isoprenoids (Ezeji *et al.* 2004). Despite its low selectivity and poor removal efficiency, gas stripping does not incur the clogging or fouling associated with membrane separation (Xue *et al.* 2013b). Although for the reasons discussed, gas stripping is a promising alternative to distillation, it remains capital and energy intensive and therefore expensive (Xue *et al.* 2014b; Ezeji *et al.* 2010). Nonetheless, this integrated approach still reduces energy requirements 5 to 10 times over conventional distillation and costs less than directly distilling a 2% broth (Matsumura *et al.* 1988; Dürre 2007; Oudshoorn *et al.* 2009).

Efficiency of solvent extraction depends on the miscibility and affinity properties of the liquids (Matsumura *et al.* 1988). For butanol recovery, a non-polar solvent such as oleyl alcohol is mixed with the fermentation broth, and butanol selectively concentrates in the organic phase (Heipieper *et al.* 1994). An ideal solvent will have high partition coefficient for the products, be immiscible with the fermentation broth, be inexpensive and easily recoverable from the solvent, and be nontoxic to the microbial strain (Gyamerah and Glover 1996; Ezeji *et al.* 2004; Oudshoorn *et al.* 2009; Dellomonaca *et al.* 2010). Solvent toxicity can be minimized by using a membrane between the solvent phase and fermentation broth to restrict the direct contact of the solvent. In this technique, known as perstraction, products diffuse preferentially across the membrane and dissolve in the solvent; other components and fermentation intermediates (*e.g.*, acetic and butyric acids) are retained in the aqueous phase (Qureshi and Ezeji 2007). The main limitations with perstraction are membrane fouling and reduction in extraction efficiency (Ezeji *et al.* 2007).

Pervaporation combines membrane permeation and evaporation and has been recommended to enhance the recovery of ethanol and butanol from fermentation broths. The process uses a selective non-porous membrane. Target molecules diffuse through the membrane, leaving behind nutrients, sugar, and microbial cells. The permeate is removed in a vapor state from the other side of the membrane using a vacuum or sweeping gas such as N_2 (Matsumura *et al.* 1988; Ezeji *et al.* 2004; Vane 2008; Fouad and Feng 2008; Oudshoorn *et al.* 2009; Ezeji *et al.* 2010). Despite the advantages over distillation, the process has drawbacks such as fouling of the membrane (Vane 2008). However, the method is highly flexible. Higher efficiencies can be achieved by modifying the membrane chemistry by changing its charge or hydrophobicity (Nguyen 1999).

Although several product removal strategies have been developed to reduce the microbial toxicity in the fermentation broth, none have proven to be efficient in recovering absolute/near absolute concentrations of the targeted product. The best recovery reported for butanol was a titer of 46% (Table 4), which needs to be further distilled. Despite the development of numerous separation methods, very few have been proven on an industrial scale (Bujis *et al.* 2013). For the recovery of isobutanol, DuPont uses a combination of oleyl alcohol extraction with gas stripping, whereas Gevo uses a customized process involving flash distillation followed by phase separation (Bujis *et al.* 2013). The feasibility of these product removal strategies needs to be investigated on other advanced biofuel molecules such as fatty acids or isoprene derivatives.

Risk of Contamination

The characteristic low product titers, low cell densities, and prolonged incubation times associated with advanced biofuel fermentation indicate that they will be highly prone to contamination, especially in anaerobic environments. One of the challenges with ABE fermentation in the 19th century was the risk of contamination due to the low product titer (Jones and Wood 1986; Nimcevic and Gapes 2000). Notably, high gravity substrates and its resulting high bioethanol titer minimizes the risk of contamination in bioethanol fermentation (Puligundla *et al.* 2011). In contrast, an advanced biofuel system marked with low product tolerance makes it difficult to employ the high gravity approach (Dunlop 2011). Again, developing a microbial strain with increased substrate and product tolerance will be crucial to minimize the risk of contamination.

Additional Challenges Using Lignocellulosic Substrates

Carbon sourcing is the primary cost component of any biofuel system. Cheap sources of sugar are therefore a pre-requisite for the economic viability of any process (Singhvi *et al.* 2014).

Sustainable production of different biofuel equivalents on a large scale invariably requires abundantly available and inexpensive lignocellulosic feedstocks, which have the added benefit that they do not compromise food security (Singhvi *et al.* 2014). However, there are numerous technical challenges for obtaining commercially-relevant quantities of biomass and in achieving sufficient conversion efficiencies for lignocellulosic biomass to advanced biofuels (Galbe and Zacchi 2012; Huffer *et al.* 2012; Singhvi *et al.* 2014).

Inhibitor tolerance of genetically engineered microbial strains

When using lignocellulosic substrates, in addition to exhibiting product tolerance, the microbial strains used to produce biofuels should be tolerant to a range of inhibitors derived from the lignocellulosic substrates. Several inhibitors including organic acids, phenolics, and furan derivatives can both affect cell growth and interfere with metabolic pathways for the production of advanced biofuels (Ezeji *et al.* 2007; Nicolaou *et al.* 2010; Gao *et al.* 2012).

After ethanol, the largest body of work has been directed to butanol production from lignocellulosic feedstocks. Researchers have examined a range of biomass sources including corn stover, corn fiber, barley straw, and switch grass, as well as a variety of pretreatment conditions to assess their influence on n-butanol production (Parekh and Blaschek 1999; Parekh and Formanek 1999; Qureshi *et al.* 1999; Qureshi *et al.* 2006; Ezeji *et al.* 2007; Zhang *et al.* 2009; Qureshi *et al.* 2010; Lin *et al.* 2011; Mu *et al.* 2011; Gao *et al.* 2012; Guo *et al.* 2012, 2013). Sugar concentrations ranged from 31 to 58 g/L with solvent titers ranging from 2 to 26 g/L (Parekh and Blaschek 1999; Parekh and Formanek 1999; Qureshi *et al.* 1999, 2006; Ezeji *et al.* 2007; Zhang *et al.* 2009; Qureshi *et al.* 2010; Lin *et al.* 2011; Mu *et al.* 2011; Gao *et al.* 2012; Guo *et al.* 2012, 2013). The highest yield reported was 0.44 g/g (Qureshi *et al.* 2006, 2010), and the highest productivity was 5 g/L/h (Zhang *et al.* 2009). The lignocellulose-derived inhibitors consisted primarily of soluble phenolics that severely restrict the efficiency of solvent production (Guo *et al.* 2012).

For the production of advanced biofuels, fermentation can be carried out under aerobic/semi-aerobic conditions by re-routing the biosynthesis pathway, which could be advantageous to overcome some inhibitors (Amyris 2013). The robustness of genetically engineered strains for advanced biofuel production in surviving an inhibitor-rich lignocellulosic substrate is not yet known. However, Amyris, a company which produces farnesane using engineered *S. cerevisiae*, claims that their aerobic conditions helps the microbe effectively assimilate acetic acid, thus minimizing its inhibitory effect on fermentation (Amyris 2013); the influence of inhibitors such as phenolics and furan derivatives was not mentioned in their work. This is expected to present serious hurdles to commercialization, as the titer of terpenes produced from ionic liquid pretreated substrates was 12 times lower than the theoretical yield (Bokinsky *et al.* 2011). It appears that substantial metabolic engineering will be required to circumvent yield reduction from inhibition issues along with the ability to consume multiple sugars if reasonable production titers from lignocellulosic substrates are expected.

Previous work to increase the tolerance of micro-organisms to lignocellulosic inhibitors includes: rational engineering, evolutionary engineering, and inverse metabolic engineering (Nevoigt 2008; Jin *et al.* 2005). Some examples of successful rational engineering efforts include: engineering of the redox state by enhancing the intracellular glutathione by *GSH1* overexpression; co-expression of transaldolase and aldehyde dehydrogenase in the presence of furfural; over expression of aldehyde dehydrogenase to regenerate NAD^+ and relieve redox imbalance; over expression of RNA binding protein *lsm6* for improved tolerance to furfural acetic acid and sulfur compounds; and combining the NADH consuming acetic acid consumption pathway and NADH producing xylose utilization pathway (Fujitomi *et al.* 2012;

Gao and Xia 2012; Tanaka *et al.* 2012; Kim and Hahn 2013). Evolutionary engineering was carried out both by mutagenesis or adaptation to lignocellulose derived inhibitors. Examples include evolutionary engineering with 5HMF, which yielded a yeast mutant with high levels of *ari1* and a*dh7*, higher ethanol productivity, higher growth and higher alcohol dehydrogenase activity (Nevoigt 2008; Ling *et al.* 2014). To produce advanced biofuels from lignocellulosic biomass, genetic modifications used to achieve enhanced inhibitor tolerance will have to be synergistically combined with the genetic makeup required for the producing the advanced biofuels (Ling *et al.* 2014). Despite extensive research efforts, there has been limited success in developing a commercial microbial strain for producing ethanol that is both multiple-sugar consuming and inhibitor tolerant. As a result, substantial R&D would be required for metabolic engineering and optimization to develop a suitable microbial strain capable of producing advanced biofuels from lignocellulose (Ling *et al.* 2014).

High feedstock requirement

Even with a complete conversion, the typical requirement of monomeric sugars for producing most advanced biofuels would be higher than that of ethanol. As shown in Table 5, the maximum theoretical yield for isoprene is roughly 0.3 g/g sugar, whereas with ethanol it is 0.51 g/g.

This inherent limitation together with the low titer and low conversion efficiency of advanced biofuels places additional demands on feedstock requirements. For example, in ABE fermentation, low yield, together with the formation of acetone and ethanol as byproducts demands 6 to 7 tons of corn for every ton of butanol, whereas only 3 tons of corn is needed for the same amount of fuel ethanol production (Xue *et al.* 2013). Due to the presence of non-carbohydrate components and lower conversion efficiency, the amount of lignocellulosic substrate required would be up to 4 to 5 times higher than these values resulting in an increase in the feedstock cost. This, in turn, places additional demands on plant design as the capacity of the reactor systems will generally need to be larger, which escalates capital and operating costs.

Consumption of multiple sugars

In order to maximize the conversion efficiency of lignocellulosic biomass, both cellulose- and hemicellulose-derived sugars should be converted to products in high yield and titer. This means simultaneous or at least sequential consumption of multiple sugars (both C6 and C5 sugars) is a pre-requisite (Hahn-Hägerdal *et al.* 2007; Matsushika *et al.* 2009). Many of the biofuel producing microbial strains prefer glucose over other carbon sources due to carbon catabolite repression (Matsushika *et al.* 2009). Although considerable work has been done to genetically engineer yeast and bacteria to consume multiple sugars, most work was focused on the production of ethanol (Hahn-Hägerdal *et al.* 2007; Matsushika *et al.* 2009) and not on the production of advanced biofuels. Despite extensive efforts, construction of an efficient strain that can simultaneously metabolize C5 and C6 sugars while maintaining all of the required traits, (high sugar and ethanol tolerance, inhibitor tolerance *etc.*) for industrial scale ethanol production has been unsuccessful (Madhavan *et al.* 2012). Therefore, it is apparent that extensive research is required to convert both C6 and C5 sugars to advanced biofuels in reasonable titer and yield.

Table 5. Theoretical Yield of Microbially-Derived Fuel Precursors (Nakamura and Whited 2003; Atsumi *et al.* 2008; Huerta-Beristain *et al.* 2008; Whited *et al.* 2010; Wang *et al.* 2011; Huffer *et al.* 2012)

Product	Carbon Yield (%)	Mass Yield (g/g)	Redox Constrained
Alcohols			
1-butanol	65.3*	0.40	No
	66.7*	0.41	No
Propanol	49.5	0.33	No
1,3 propanediol	74.0	0.62	Yes
	75.0	0.63	Yes
3-Methyl-1-butanol	56.3	0.33	Yes
Isobutanol	66.7	0.41	Yes
Ethanol	63.9	0.49	No
	66.7	0.51	No
Isoprenoids			
Isoprene	69.9	0.32	Yes
	49.0	0.22	Yes
Farnesene	69.1	0.31	Yes
	46.3	0.21	Yes
Farnesol	54.6	0.27	Yes
	64.7	0.32	Yes
Geraniol	56.5	0.29	Yes
	66.2	0.34	Yes
Fatty Acid Derivatives			
Fatty alcohols	>60%*	0.34	Yes
Fatty acid ethyl ester	>85*	0.51	Yes

** Different numbers for the yield are due to the choice between aerobic and anaerobic conditions and redox constraints.*
*** Dependent on chain length*

Despite the high robustness and industrial relevance of *S. cerevisiae*, the strain naturally does not ferment pentose sugars (Palmqvist and Hahn-Hagerdal 2000). In addition to integrating xylose and arabinose utilization pathways (*e.g.*, expression of *XYL1*, *XYL2,* and *XYL3* genes), there have been several efforts focusing on the over expression of specific pentose transporters and the genes of the non-oxidative part of pentose phosphate pathways (Kuyper *et al.* 2005; Hahn-Hagerdal *et al.* 2007). One of the challenges in synergistically expressing xylitol dehydrogenase and xylose reductase was their co-enzyme specificities (NADPH *vs*. NADH). There have been several works to solve this redox imbalance including the heterologous expression of transhydrogenase enzymes (Kuyper *et al.* 2005). Later efforts in expressing xylose isomerase in *S. cerevisiae* partially resolved this problem, as the enzyme can directly convert xylose to xylulose, which can directly enter the pentose phosphate pathway (Brat *et al.* 2009; Matsushika *et al.* 2009). This approach was adapted in genetically engineered *S. cerevisiae* for the production of farnesane from a mixture of glucose and xylose (Amyris 2013).

Construction of a genetically engineered strain to consume multiple sugars has been shown to compromise product tolerance and yield as well as the ability to tolerate inhibitors (Bellissimi *et al.* 2009; Clomburg and Gonzalez 2010; Vickers *et al.* 2012). Wisselink and coworkers (2009) attempted to evolve the engineered *S. cerevisiae* on the pentose sugars without losing their performance on hexose sugars. However, researchers found that the strain was highly sensitive to inhibitors (Bellisimi *et al.* 2009). Although *E. coli* is well known for its wide

substrate utilization range (it can metabolize both C5 and C6 sugars), genetically engineered *E. coli*'s productivities and yields are not yet high enough for industrial use (Dien *et al.* 2003; Vickers *et al.* 2012). In addition to the recent work on advanced biofuel producing *E. coli*, research has been carried out for the construction of ethanologenic *E. coli*, which can consume multiple sugars from lignocellulose (Flores *et al.* 1996; Hernandez-Montalvo *et al.* 2001; Nichols *et al.* 2001; Yomano *et al.* 2009). Although researchers could successfully express pyruvate decarboxylase and alcohol dehydrogenase genes of *Z. mobilis*, the carbon catabolite expression still existed in *E. coli* in which glucose repressed the expression of sugar specific transporters and the key enzymes needed for the metabolism of alternative sugars (Dien *et al.* 2003). A more successful attempt was done by Yomino *et al.* (2009) in developing an *E. coli* ethanologenic strain by deleting the methyl glyoxal synthase gene which enabled the co-utilization of a 10% mixture of mannose, glucose, arabinose, xylose, and galactose (2% each). However, this sugar concentration is still considerably lower than what would be expected in an industrial process. The performance of this strain therefore needs to be assessed at high sugar and inhibitor concentrations.

Z. mobilis is not an ideal candidate for converting lignocellulosic sugars because it can only ferment glucose, fructose, and sucrose (Behera *et al.*2010). The strain lacks some of the glycolytic and pentose phosphate pathway enzymes necessary to ferment pentose sugars. Genetic engineering of *Z. mobilis* to express these enzymes had some successes (Zhang *et al.* 1995; Deanda *et al.* 1996), but the resulting strain had a much lower tolerance to inhibitors such as acetic acid (Lawford and Rousseau 2002; Mohagheghi *et al.* 2002). Through random mutagenesis and partial deletions, Ren and coworkers (2009) were able to evolve a glucose facilitator from *Z. mobilis* that transports xylose. However, the authors found that the intracellular xylose metabolism was inhibited by the presence of glucose (Ren *et al.* 2009).

Butanol-producing *Clostridial* strains are known for utilizing multiple sugars present in the lignocellulosic substrates including glucose, xylose, arabinose, and galactose (Ounine *et al.* 1983; Qureshi *et al.* 2007; Mu *et al.* 2011). When fermenting glucose, xylose, and galactose, the highest butanol titer (21.4 g/L) was obtained from glucose followed by arabinose (15.2 g/L), xylose (11.1 g/L), and galactose (10.1 g/L). Although *C. acetobutylicum* is recognized in utilizing both glucose and xylose, utilization of xylose is slower (Lin and Blaschek 1983; Ni and Sun 2009). The hyper-butanol producing strain *C. beijerinckii* BA101 can consume xylose together with glucose but exhibited poor performance in the presence of inhibitors (Ezeji *et al.* 2007a; b).

The Gram-negative bacterium *Klebsiella oxytoca* is well known for its ability to metabolize multiple sugars including some glucose oligomers: cellobiose and cellotriose (Wood and Ingram 1992). Genetic engineering of *K. oxytoca* to incorporate the *Z. mobilis* PET operon enabled the production of ethanol from a range of substrates (Wood and Ingram 1992; Wood *et al.* 1992). However, its ethanol tolerance was lower than that of even *E. coli*. *Scheffersomyces stipitis* (*Pichia stipitis*) is one of the naturally xylose fermenting strains, which can also ferment galactose, glucose, and cellobiose (Parekh *et al.* 1986). The redox balance between xylose reductase and xylitol dehydrogenase is less drastic in this yeast because the respective xylose reductase can use both NADH and NADPH (Verduyn *et al.* 1985). In addition, *P. stipitis* possesses a non-cytochrome electron transport chain that can resolve cofactor imbalances (Jeppsson *et al.* 1995). Despite these advantages, there are several limitations with *P. stipitis* compared to *S. cerevisiae* including lower sugar consumption rates and considerably lower ethanol and inhibitor tolerance (Bellido *et al.* 2011). Importantly, in glucose/xylose mixtures, glucose is still the preferred, while sugar and ethanol production is optimal only in micro-aerophilic conditions (Papini *et al.* 2012). Being Crabtree negative yeast, no ethanol production

was observed under aerobic conditions (Papini *et al.* 2012). All of these reasons explain why *P. stipitis* has not yet been considered for advanced biofuel production from lignocellulosic sugars despite its multiple substrate utilization potential.

Some thermotolerant yeast strains such as *Kluveromyces* and *Hansenula polymorpha* can ferment multiple sugars including pentoses (Lin *et al.* 2013; Dmytruk and Sibirny, 2013). *Kluveromyces* is highly Crabtree negative yeast, and its performance would heavily depend on the oxygen levels in the medium. Xylose fermentation is poor compared to that of glucose, which has a yield of up to 98% (Lin *et al.* 2013). Although *Hansenula polymorpha* was genetically modified to enhance the production of ethanol from multiple sugars, the ethanol production rate on xylose was found to be poor due to its lower uptake. No attempts have been reported to engineer these strains for the efficient production of advanced biofuels.

Most genetic engineering studies for re-routing the anabolic pathways for the production of advanced biofuels are carried out with pure glucose solutions. As a result, the ability of these strains to consume multiple sugars present in the lignocellulosic substrate and direct the metabolic flux to the desired product has yet to be engineered and optimized. The degree of carbon catabolite repression on the genetically engineered strains for the production of advanced biofuels is not yet known. With the limited success in developing *E. coli / S. cerevisiae* strains for the production of ethanol from the multiple sugars present in lignocellulosic substrates, construction of a microbe for lignocellulose-based advanced biofuel production would be extremely challenging.

Lack of a clean fractionation process and low-cost cellulase enzymes

The first step in the conversion of lignocellulose to advanced biofuel is the recovery of monomeric sugars in high yield and concentration. This generally requires a two-stage process involving both pretreatment and enzymatic hydrolysis to release sugars in hemicellulose and cellulose, while minimizing the formation of inhibitors (Bhutto *et al.* 2014). However, despite extensive efforts no such ideal fractionation process, which gives rise to high sugar yield in high concentration with a minimum amount of inhibitors, has yet to be developed. Compared to numerous pretreatment options, dilute acid/steam pretreatment is closest to commercialization (Galbe and Zacchi 2012). The cellulase enzyme loading used for hydrolysis of pretreated substrate is high and is a significant contributor to the overall production cost (Galbe and Zacchi 2012; Sorek *et al.*2014). Therefore, both pretreatment and enzymatic hydrolysis continue to be optimized to obtain overall high sugar yield and concentration from minimum enzyme and chemical/energy input (Hasunuma *et al.*2013). Above all, the presence of lignin presents a major hurdle that restricts the efficiency of pretreatment enzymatic hydrolysis processes. Incorporating a step for the valorization of lignin would potentially improve the economic viability of the overall process.

STATE OF COMMERCIALIZATION

Progress in the Commercialization of Bioethanol

Among different types of biofuel molecules that can be biochemically derived, the bioethanol industry, which has been commercialized for decades, is clearly dominant. Presently, bioethanol produced from sugar cane and starch feedstocks is the leading alternate fuel industry used throughout the world (Balat and Balat 2009; Balan *et al.* 2013). Although not completely optimized and economically competitive, many lignocellulose-based bioethanol plants started pre-commercial demonstration facilities (Balan *et al.* 2013). It should be noted that despite

significant investments, current large scale cellulosic ethanol facilities deliver less than six percent of their nameplate capacity (Rapier 2015). The major companies involved in the demonstration of lignocellulosic ethanol include: Abengoa Bioenergy, Chemtex/Beta Renewable, Poet-DSM, and DowDuPont (Balan *et al.* 2013). The success of these emerging production platforms will depend on a variety of factors including market conditions for liquid fuels, government and social support, and technical success factors.

Early Commercialization Efforts on ABE Fermentation and Lessons Learned

ABE fermentation was industrially established prior to the emergence of fossil based butanol. Beginning in the 1920s and through the 1950s, the ABE fermentation process ranked second to ethanol fermentation (Green 2011). Many ABE fermentation plants were built during the First and Second World Wars largely due to the demand for acetone used in the manufacture of cordite (Table 6) (Dürre 1998).

The bioacetone plant at King's Lynn, UK produced 440 kg of acetone per week using potato as the feedstock and *C. acetobutylicum* as a microbial strain, which was also called the Weizmann strain named after Chaim Weizmann, who first isolated this anaerobic bacterium (Gabriel 1928). The progress made by Weizmann's team further enabled the use of corn as a feedstock, which increased the production of acetone to 2000 tons a week. Subsequently, another plant was commissioned at Dorset and six additional distilleries were retrofitted for the production of acetone. Grain shortages prompted the use of horse chestnuts as a fermentation feedstock. However, the eventual shortage of chestnuts and the challenges with foaming made the British stop domestic production, which was transferred to Canada. In 1916, the Gooderham and Worts distillery in Toronto was retrofitted to adapt the Weizmann process to produce acetone, achieving an output of 3000 tons per week. As the United States joined the war in 1917, two plants were built in Terre Haute, Indiana located in the US corn belt. Some of the challenges faced in the bulk production of acetone were the poor titer, microbial contamination, and slow growth rate of *Clostridia,* which resulted in long incubation times. Bacterial contamination and consequent impact on the solvent production prompted the closure of many ABE fermentation plants in 1920s (Ross 1961). However, at the end of World War II, two-thirds of butanol and one-tenth of acetone production in the Unites States were from fermentation processes (Ross 1961; Ranjan and Moholkar 2012).

Initially, butanol was considered to be a waste by-product of ABE fermentation (Nimcevic and Gapes 2000). However, rapid expansion of the automobile industry created new markets for butanol as a solvent in quick-drying lacquers, leading to its re-birth in ABE fermentation prompting investment in new plants with greater capacity despite their inherently lower titer and yield (Table 6). Ultimately, fossil-based butanol production proved much cheaper. Together with the competition for molasses as cattle feed and the consequent rise in the feedstock prices, it outcompeted bio-butanol production. This resulted in a rapid decline of the butanol fermentation plants in 1950s and their total shut down in the 1960s in both United States and Britain (Jones and Wood 1986; Nimcevic and Gapes 2000).

Table 6. Examples of Bio-butanol Plants and Major Technical Challenges Faced (Jones and Wood 1986; Nimcevic and Gapes 2000; Green 2011)

Name and Location	Period	Substrate	Microbial Strain	Capacity (liters)	Highlights
CSC, Terre Haute	1920s	Corn	*Clostridium acetobutylicum*	–	Morphological and physiological changes in *Clostridia* is critical – Slow growth phase; Multiple fermenters; Continuous operation to reduce the residence time.
Dokshukino, USSR	1950s	Corn	*Clostridium acetobutylicum*	3500	Acidity in the plant. Danger of contamination.
	1950s	Corn, corn cobs, hemp waste, sunflower shells	*Clostridium acetobutylicum*	270,000	Continuous fermentation.
SunOpta, Soustons, France	1980s	Cereal straw, corn stover, sugar beet, Jerusalem artichoke	*Trichoderma reesei; Clostridium acetobutylicum*	1000	Ethanol production more economically favorable. Yield: 1 tons butanol from 7.7 tons of corn cobs; Economics depend on the market value of lignin.
EU Pilot Plant, Austria	1990s	Potato, rye, wheat and maize	*Clostridium beijerinckii*	–	Unhydrolyzed starchy mash was fermentable, but caused blockage. Partial liquefaction helps. Importance of product removal/recovery.

While plants were being shut down in the United States and Britain, butanol fermentation plants were still operating in China, South Africa and Russia until the early 1980s (Nimcevic and Gapes 2000; Chiao and Sun 2007). Availability of coal for energy requirements and less expensive molasses largely enabled their operation. However, the shortage of molasses in the early 1980s forced their closure as well (Nimcevic and Gapes 2000). Although butanol research has progressed considerably because its early development, there are pertinent lessons to be learned from early commercialization efforts, especially with regards to key challenges, namely: a) lack of a sustainable feedstock supply; b) the risk associated with purely "market driven" production; c) the limitation of strictly anaerobic *Clostridium* as an industrial microorganisms; d) the resulting low titer even with sugar cane/starch based feedstocks. It is also important to note that butanol was never used as a fuel in this early commercial era and that the applications were primarily in the production of high-value chemicals and materials (Green 2011).

Table 7. Recent Demonstration and Commercialization Activities of Bio-butanol and Isobutanol (Ni and Sun 2009; Balan *et al.* 2013; Bujis *et al.* 2013)

Company	Start-up Year	Feedstock	Microbe*	Annual Capacity	Technical Highlights
Cobalt Technologies Baton Rouge, LA	2013	Cellulosic feedstocks including wood chips	C	1 million gals	Steam pretreatment/ dilute acid pretreatment
Gevo St. Joseph, MO	2012	Corn	S	18 million gals	Retrofitting of starch-based ethanol plant
Butamax (BP-DuPont) Hull, England	2012	Wheat and corn	S	80 million gals	Retrofitting of starch-based ethanol plant
Ji-An Biochemical Co. Ltd., China	2007	—	CA	150,000 tons	—
Guiping Jinyuan Alcohol Industry Co. Ltd., China	2007	—	CA	100,000 tons	—
Cathay Industrial Biotech Co. Ltd., China	2008	—	CA	300,000 tons	—
Jinmaoyuan Biochemical Co. Ltd., China	2008	—	CA	60,000 tons	—
Lianyungang Lianhua Chemical Product Co.Ltd, China	2008	—	CA	—	—
Jiangsu Lianhai Biological Technology Co. Ltd., China	2008	—	CA	200,000 tons	—

***Key:** C = *Clostridium;* CA = *C. acetobutylicum;* S = *S. cerevisiae*

Current Commercialization Initiatives for n-Butanol and Isobutanol

Recent developments in butanol fermentation and an increasing surge for alternative fuel/chemicals have catalyzed increased interest in the fermentative butanol production. China is currently one of the leading countries spearheading the commercialization of the ABE fermentation process (Ni and Sun 2009). They have recently invested $200 million to install 200,000 tons of new annual production capacity with six large scale plants each producing approximately 30,000 t/a. These technologies use wild type *C. acetobutylicum* as the microbial strain. Fermentation is conducted in a semi-continuous mode with a residence time of 21 days. Each plant consists of a battery of approximately eight 300 to 400 m^3 fermentation reactors connected in series (Chiao and Sun 2007). Most of these plants use conventional distillation techniques to recover the solvents (Chiao and Sun 2007). In Brazil, an 8000 t/a bio-butanol plant was also constructed by HC Sucroquimica to produce butanol from sugarcane juice (Ni and Sun 2009).

Extensive research advancements in the last two decades to enhance butanol/isobutanol titer by the genetic engineering of *E. coli / S. cerevisiae* has resulted in recent demonstration and commercialization activities by the EU and U.S.-based companies (Table 7).

For example, Cobalt Technologies (now SGBio) targets production of n-butanol from cellulosic biomass using a proprietary pretreatment and fractionation process and a wild type *Clostridium* strain. The company entered into a joint venture with Solvay-Rhodia, the tenth largest global chemical company, to commercialize the technology in South America, primarily

using sugarcane bagasse as feedstock (Cobalt Technologies 2013). Gevo acquired a commercial scale ethanol plant to retrofit to produce isobutanol using engineered *E. coli*. DuPont (now DowDupont) also engineered several microbial strains for isobutanol and assigned the technology to Butamax™, which is a joint venture between BP and DuPont (Gevo 2014). The approach of both Butamax™ and Gevo used efficient heterologous enzymes (*e.g.*, *Bacillus subtilis AlsS*, *Lactococcus lactis Ilvd*) in *S. cerevisiae* and enhance the metabolic flux towards butanol by enhancing the pyruvate pool while minimizing by-product formation (Bujis *et al.* 2013).

Current commercialization efforts on bio-butanol and bio-isobutanol are targeted towards high value applications. Butanol and isobutanol are currently high value chemical commodities with global markets worth $6 billion and $560 million, respectively (Green 2011; Mascal 2012). Approximately 50% of the current butanol production is used for manufacturing acrylate esters, which are polymerized for applications in surface coatings, adhesives, elastomers, textiles, fibers, and plastics (Mascal 2012). Glycol ethers are important butanol derivatives used in industrial coatings and hard surface cleaners. Butyl acetate is used in paints, leather, ink, and coatings. Other applications are in the production of butyl amines, which have applications ranging from pharmaceuticals to pesticides to nylon manufacturing (Mascal 2012). Although not as large as n-butanol market, isobutanol is a valuable commodity with applications as a solvent in adhesives and surface coatings, dispersion and flotation agents, chemical synthesis intermediates, plastics, fibers, and bio-based jet fuel blend stocks (Green 2011).

Current Commercialization Efforts for Biochemically-derived Isoprenoids and Fatty Acid Esters

Microbial production of advanced biomolecules such as long-chain alcohols, fatty acids, and isoprenoids are mostly carried out at the lab scale. From the discussion in the earlier sections, it is apparent that substantial improvement in genetic engineering and process chemistry will be needed to enable their eventual commercialization. Because genetic engineering for the production of these molecules has been done mostly in two industrial hosts (namely, *E. coli* and *S. cerevisiae*), it may be important to analyze how such engineered microbes have been used in the bulk production of commodities. However, there are but few examples where research efforts with engineered *E. coli* resulted in industrial scale production of biobased bulk chemicals. One excellent example is the production of 1,3-propanediol, which is used in a variety of industrial applications including adhesives and coatings. Based on *E. coli* fermentation, the biochemical process for 1,3-propanediol has proven commercially viable and provides a product quality advantage compared to the petrochemical route (Vickers *et al.* 2012). While the scale of production of isoprenoids or fatty acids for fuel applications would be substantially higher than a “niche” commodity such as 1,3-propanediol, the feasibility of using engineered *E. coli* for such a large scale production is yet to be proven.

Amyris is working to commercialize the bulk production of sesquiterpenes (namely, farnesene under their trade name of Biofene®) using engineered *S. cerevisiae* (Balan *et al.* 2013; Bujis *et al.* 2013). These molecules are currently produced in their pilot-scale plants. Amyris’s farnesene strains seem to consume both hexose and pentose sugars. They have heterologously expressed *E. coli*’s xylose isomerase in their yeast strain, which enabled them to bypass the native route of xylose consumption in yeast (Amyris 2013). The company claims that they can produce farnesene from cellulosic feedstocks, which can then be chemically hydrogenated downstream to farnesane. As the microbial process is aerobic, the yeast cells can effectively assimilate the acetic acid, thus minimizing its inhibitory effects (Amyris 2013). However, the company has not discussed the influence of a range of other inhibitory compounds that are

produced during the pretreatment of lignocellulose. Despite these achievements, the maximum reported farnesane titer is 14 g/L (Amyris 2013), which indicates that the downstream processing needed to recover the product would be as expensive as in the case of butanol.

The biochemically-derived isoprenoids are also expected to have a number of high-value applications in the materials, specialty chemical, and pharmaceutical/nutraceutical markets due to their higher selectivity and purity (Adlington *et al.* 2015; Hernandez 2015). It is worth noting that biochemical pathways for isoprenoids are currently used for the production of artemisimic acid and amorphadiene, which have pharmaceutical applications. Likewise, oleochemicals are sold at much higher prices than fuels. Fatty alcohols, aldehydes/esters have large established markets ($3 billion) and used in the production of soaps, detergents, cosmetic additives, pheromones, and flavoring compounds (Rupilius and Ahamad 2006; Guzialowska *et al.* 2012). It is apparent that bulk production of isoprenoid precursors or fatty acid derivatives that are competitive with petroleum fuels will require extensive efforts on microbial development, process optimization, and engineering. Therefore, commercial processes that leverage microbial catalysts for the production of these molecules will likely target non-fuel applications.

SUMMARY AND FUTURE PROSPECTS

In this review, developments in the microbial production of advanced biofuels were examined and compared with progress in bioethanol fermentation. In addition to looking at the natural conversion pathways such as ABE fermentation, research achievements were analyzed in the genetic engineering of microbes for enhanced production of alcohols, isoprenoids, and fatty acids. Genetic engineering efforts were focused on either overexpressing the native production pathways to maximize the innate microbial capacity or heterologously expressing suitable genes in a genetically tractable and industrially relevant hosts such as *E. coli* and *S. cerevisiae*. Four metabolic pathways were primarily focused for the production of advanced biofuels: a) heterologous expression of ABE fermentation pathway; b) re-routing the amino acid biosynthesis pathways for the production of short and long-chain alcohols; c) the fatty acid biosynthesis pathway for the production of fatty acids and their corresponding alcohols and alkanes; and d) engineering of the isoprenoid pathway for the production of isoprene derivatives.

Industrial ABE/IBE fermentation for the production of butanol and isopropanol has a proven track record. Numerous commercial plants existed prior to 1980s. However, the bio-butanol industry met its demise once low-cost fossil-based butanol entered the market. Despite its ability to consume multiple sugars, *Clostridia*-based bio-butanol production proved to be uncompetitive with fossil-based butanol due to its extremely low butanol tolerance (< 12 g/L), susceptibility to microbial contamination, and the slow growth rate of spore-forming *Clostridia.* Regardless of whether it is biomass or fossil derived, butanol was never used as a fuel, but rather used in high value applications, mostly as an industrial solvent.

Lessons learned from the early commercialization efforts for bio-butanol contributed much to the development of antibiotics and the production of recombinant proteins. Later research efforts on genetic engineering of *Clostridia* to enhance butanol titer and yield resulted in improved butanol producing traits. The best organism for butanol production thus far is *C. beijerinckii* BA 101 developed by chemical mutagenesis. Using pure glucose as a feedstock, this strain has achieved the highest butanol titer of 19.6 g/L. In stark contrast, the highest ethanol titer obtained by *S. cerevisiae* from the industrial fermentation of starch and sugarcane-based feedstocks is 120-150 g/L, which is 6 to 8 times higher than the butanol titer. Therefore, to be an economically competitive biofuel will require substantially enhanced titers of bio-butanol.

Neither heterologous expression of the *Clostridial* pathway nor re-routing of the amino acid biosynthesis pathway have resulted in commercially-relevant yields of biobutanol. Re-routing the amino acid biosynthesis pathway was apparently more successful for isobutanol with a production titer of 22 g/L. Development of instantaneous product removal (*e.g.*, gas stripping) together with continuous fermentation enabled researchers to raise the butanol, isopropanol, and isobutanol concentrations to 460, 143 and 50 g/L respectively, which are acceptable titer values for further recovery by distillation. However, such *in situ* product removal techniques combined with distillation make the overall process highly capital intensive. Alternatively, bioethanol, as a commercially established bulk chemical, could be used as a feedstock in the chemical synthesis of long-chain alcohols and other value-added chemicals using one of the many established chemical conversion pathways.

Microbial bulk production of isoprenoids or fatty acids is at a very early stage of development and will require significant research, development, and demonstration activities to validate their potential. As such, these products will require a much longer time horizon for commercialization. Although long-chain alcohols, fatty acids, and isoprenoids were overproduced by the genetically engineered industrial hosts, *E. coli* and *S. cerevisiae*, their production has been limited to pure glucose, conducted mostly at the lab scale. The maximum titers obtained with isoprenoids (14 g/L farnesene) and fatty acid derivatives (6.6 g/L) are still too low to be commercially viable, indicating that further improvements and optimization in metabolic engineering will be necessary. Performance of these strains on sugarcane, starch-based, or lignocellulosic feedstocks are not yet fully assessed even at the lab scale, whereas bioethanol production from sugarcane and starch-based feedstocks has been commercial for years. In addition, many lignocellulosic ethanol plants have started operation at a near-commercial scale. A few studies conducted on the production of isoprene derivatives from lignocellulose indicated extremely low titers and yields in the vicinity of mg/L.

The ideal microbe, one that exhibits all of the required traits for the bulk production of advanced biofuels, does not exist. The combination of features required in an industrial biocatalyst is far from the optimal natural conditions at which these molecules are produced in a microbial cell. Achieving all of the desired traits will require the continued development of advanced genetically-engineered microbes. Although it is difficult to judge which microorganism would best suited for this purpose, our familiarity and experience in the use of yeasts for bulk industrial production of chemicals suggest that genetically engineered *S. cerevisiae* has the best chance to fulfill this function. *S. cerevisiae* has been industrially proven for bulk production in both the ethanol and food industries. While it can be argued that genetic engineering efforts with *E. coli* have been relatively more successful than *S. cerevisiae* (Rabinovitch-Deere *et al.* 2013), *E. coli* has yet to be used at a large scale. A successful example of smaller scale industrial applications of genetically engineered *E. coli* is the production of 1,3 propanediol and polyhydroxybutyrate (PHA), under development by Metabolix and ADM. High value “niche” industrial applications of genetically engineered *E. coli* include glycosylated pharmaceuticals, antibiotics, and recombinant protein production. However, the scale of production for advanced biofuels is expected to be orders of magnitude larger than these niche industrial applications and thus present a significant barrier to commercialization. In addition, *E. coli*’s tolerances to long-chain alcohols, fatty acids, and isoprenoids are much lower than *S. cerevisiae*, indicating that without enhancing the product tolerance of *E. coli*, the genetic modification efforts will not be effective. Either way, to be successfully applied in the production of advanced biofuels, the metabolic engineering in *E. coli / S. cerevisiae* must be substantially optimized.

Given the challenges for using lignocellulose, the production of advanced biofuels will require significant advances in metabolic and process engineering. Although some lignocellulosic ethanol/butanol plants are in the pre-commercial demonstration scale, these plants are not based on completely optimized processes. Continuous investments in R&D will be necessary to make advanced biofuels including lignocellulosic ethanol more competitive with fossil fuels. The upstream pretreatment and enzymatic hydrolysis steps need further research to enhance sugar yield and concentration with minimum formation of inhibitors. This will have to be done with minimum use of enzymes and chemicals. Although there have been several attempts to develop a single strain to carry out consolidated bioprocessing, the future of such an approach seems to be obscure as it requires the microbial cell to accommodate numerous and often competing functions without interfering with its basic physiological characteristics, growth rate, and tolerance. Therefore, it is highly likely that a commercial bioconversion scheme for lignocellulosic advanced biofuels will be based on a minimum of three process steps: pretreatment, enzymatic hydrolysis, and fermentation. To obtain reasonable performance in the fermentation step alone, microbial strains need to be constructed with substantial optimization to achieve fast and deregulated pathways for multiple sugar transport, good tolerance to inhibitors, high level of product tolerance, and high metabolic flux.

Due to the challenges discussed in this review and the relatively low cost of petroleum ($45 per barrel at the time of this writing), advanced biofuels are not attractive in the near to midterm. Therefore, these processes will first find commercial application in the production of chemical feedstocks, such as 1,3-propanediol or butanol. Among advanced biomolecules, butanol and isobutanol are closest to commercialization and will likely enter the chemical markets within the next few years as demonstration plants come on line. Global butanol capacity is 5 million tons with a market value over $6 billion. However, the justification for the commercial investment in biobutanol was made when the average price of industrial butanol was approximately $8/gal and pricing will need to recover before investments in additional capacity are made. Nonetheless, butanol remains an important bulk chemical with numerous applications in high-value industrial chemicals/materials such as acrylate esters, glycol ethers, butyl acetate, and butyl amines. While it may take a longer time for large scale production of microbe-derived isoprene and fatty acids to emerge, their first market applications will likely be high-value "niche" pharmaceuticals and/or nutraceutical applications.

Ethanol will continue to remain a major component of the global renewable energy mix due to its use as a fuel, oxygenate, and chemical feedstock. Moreover, its potential for growth is enormous, as bioethanol represents less than two percent of the global petroleum market, which is far below the blend wall. While starch and sugarcane will remain the primary feedstock supply for first generation bioethanol, further expansion of ethanol production will most likely rely on lignocellulosic biomass (second generation bioethanol) due to social opposition ("food *versus* fuel") and subsidies that require compliance with strict carbon dioxide reduction targets. Despite the significant research advancements required, the diversity of molecules that can be produced by microbial pathways and their suitability to replace the entire spectrum of fossil-derived molecules does provide great hope for the gradual transition of our hydrocarbon-based economy to one based on carbohydrates.

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