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MINI-REVIEW

Pentanol isomer synthesis in engineered microorganisms

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Abstract Pentanol isomers such as 2-methyl-1-butanol and 3-methyl-1-butanol are a useful class of chemicals with a potential application as biofuels. They are found as natural by-products of microbial fermentations from amino acid substrates. However, the production titer and yield of the natural processes are too low to be considered for practical applications. Through metabolic engineering, microbial strains for the production of these isomers have been developed, as well as that for 1-pentanol and pentenol. Although the current production levels are still too low for immediate industrial applications, the approach holds significant promise for major breakthroughs in production efficiency.

Keywords Pentanol · 2-Methyl-1-butanol · 3-Methyl-1-butanol · Biofuels

Introduction

Modern society relies heavily on energy, especially the energy required for the transportation of goods, services, and people. For the past century, most of our transportation energy has come from fuels derived from petroleum. However, petroleum is a non-renewable resource, and recent efforts have pushed for alternatives. A heavily produced alternative to gasoline is ethanol primarily because ethanol production is a long-established art. Yet as a fuel, ethanol does not compare well to gasoline because it has a much lower energy density (only 21 MJ/L versus about 32 MJ/L for gasoline) and a high hygroscopicity. Pentanol isomers, as well as other higher alcohols, have a low affinity for water and an energy density of about 28 MJ/L and fit well into the current transportation infrastructure. Beyond their potential use as fuel, pentanol isomers also have a multitude of other applications.

Several reviews about microbial production of biofuels in general have been written in the past 2 years (Antizar-Ladislao and Turrion-Gomez 2008; Atsumi and Liao 2008; Fortman et al. 2008; Connor and Liao 2009). These reviews address a wide range of topics but are limited in their discussion about the exciting new research on pentanol production. In this paper, we take a more in-depth look at the pentanols: how they are biologically produced, how that can be improved upon, and what applications they can serve.

Natural production of pentanols

Pentanols, or amyl alcohols, have long been known to the food industry as one of many flavor compounds produced during fermentation. Studies have shown pentanols, along with their related aldehydes, acids, and esters, to be the most abundant odor and flavor components of fermented beverages (Soccol et al. 2007). In particular, 3-methyl-1-butanol (isoamyl alcohol) is often the main fermentation flavor compound after ethanol (Lurton et al. 1995; Boulton et al. 1996). Initially, the focus was on how these compounds affected the taste of fermented food products, but eventually, more research was conducted to determine the mechanism for their production.

In the beginning of the twentieth century, Ehrlich (1907) first discovered the connection between certain amino acids and the presence of higher alcohols in beer. Ehrlich's work led to the discovery of the eponymous pathway and decades of research (Sentheshanuganathan 1960; Dickinson et al.

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1997, 2000; Hazelwood et al. 2008) about how these alcohols are produced in fermentations. The Ehrlich pathway is the shortest path to higher alcohols through the direct degradation of amino acids (Fig. 1). The amino acids first undergo transamination by an amino acid aminotransferase enzyme to form 2-ketoacids, the reverse direction of the biosynthetic reaction. The next step is a decarboxylation reaction by a 2-ketoacid decarboxylase (KDC), of which the most well known is pyruvate decarboxylase for ethanol production found in a diverse array of yeast and bacteria. Decarboxylation of a 2-ketoacid yields an aldehyde and CO_2, and the final reduction from aldehyde to alcohol via an alcohol dehydrogenase (ADH) is a standard fermentation reaction. For example, isoleucine added to a yeast fermentation can be converted to its 2-ketoacid, 2-keto-3-methylvalerate (KMV), which can then be decarboxylated to form 2-methyl-1-butyraldehyde, which is in turn reduced to 2-methyl-1-butanol (active amyl alcohol). Likewise, leucine is converted to 3-methyl-1-butanol, and several other amino acids can also use this pathway.

However, it is not necessary to add amino acids to a fermentation to produce these higher alcohols. The yeast and bacteria themselves synthesize all of the amino acids as well as their 2-ketoacid precursors. Yeast can produce higher alcohols from their native amino acid production pathways (Eden et al. 2001; Schoondermark-Stolk et al. 2005). In fact, the first push toward engineering the production of pentanols in microorganisms came about by exploiting this biosynthetic pathway. By using mutagenesis and selection based on the biosynthetic amino acid pathways, several groups have been able to increase higher alcohol formation in yeast (Kielland-Brandt et al. 1979; Fukuda et al. 1993; Suzzi et al. 1998). By using the toxicity of chemical analogues of the amino acids isoleucine and leucine as selection pressure, they were able to augment production of 2-methyl-1-butanol and 3-methyl-1-butanol, respectively, during fermentation. Because these studies were attempting to improve flavor rather than produce either alcohol in particular, the overall titers were still fairly low. This endogenous production and improvement in yeast can serve as a model from which to engineer the production of pentanols in other organisms.

Engineered production of 2-methyl-1-butanol in *Escherichia coli*

In *E. coli*, the KDC enzyme catalyzing the decarboxylation of higher 2-ketoacids is missing. Combined with an ADH, these two enzymes are all that is needed for conversion of 2-ketoacids in *E. coli* into higher alcohols. Atsumi et al. (2008b) have shown that the broad substrate range KDC from *Lactococcus lactis* encoded by the *kivd* gene is functional in *E. coli* and enables the conversion of many 2-ketoacids into alcohols, including KMV to 2-methyl-1-butanol and 2-ketoisocaproate (KIC) to 3-methyl-1-butanol. The synthesis of 2-methyl-1-butanol is dependent on the 2-ketoacid precursor of the amino acid isoleucine whose biosynthetic pathway is naturally occurring in bacteria such as *E. coli* (Fig. 2). Thus, the isoleucine pathway, up to the common precursor KMV, is the primary target for the engineering of 2-methyl-1-butanol production.

The first committed reaction in the biosynthesis of 2-methyl-1-butanol is the condensation of pyruvate and 2-ketobutyrate (2KB), catalyzed by the enzyme aceto-hydroxyacid synthase (AHAS). This enzyme also catalyzes the condensation of two pyruvate molecules in the first step of valine production, though most organisms have only one isozyme to carry out this reaction. In the enterobacteria, however, there are often three isozymes present, one of which is better suited for reacting with 2KB (Barak et al. 1987; Gollop et al. 1990) and thus better for the production of 2-methyl-1-butanol. Cann and Liao (2008) confirmed that overexpression of this isozyme, AHAS II, is in fact a good choice for 2-methyl-1-butanol production, especially AHAS II from *Salmonella typhimurium*.

For most microorganisms, the source of 2KB for the AHAS reaction is the amino acid threonine. Threonine is

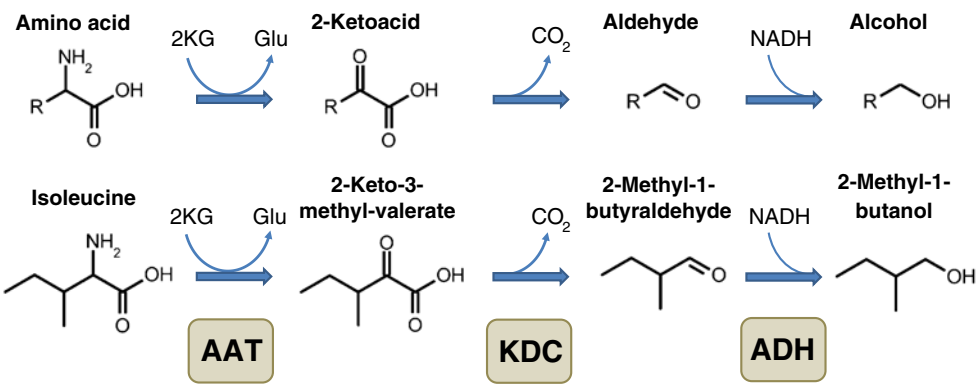

Fig. 1 Ehrlich degradation pathway from amino acid to alcohol. *AAT* amino acid amino-transferase, *KDC* 2-ketoacid decarboxylase, *ADH* alcohol dehydrogenase, *2KG* 2-ketoglutarate, *Glu* glutamate

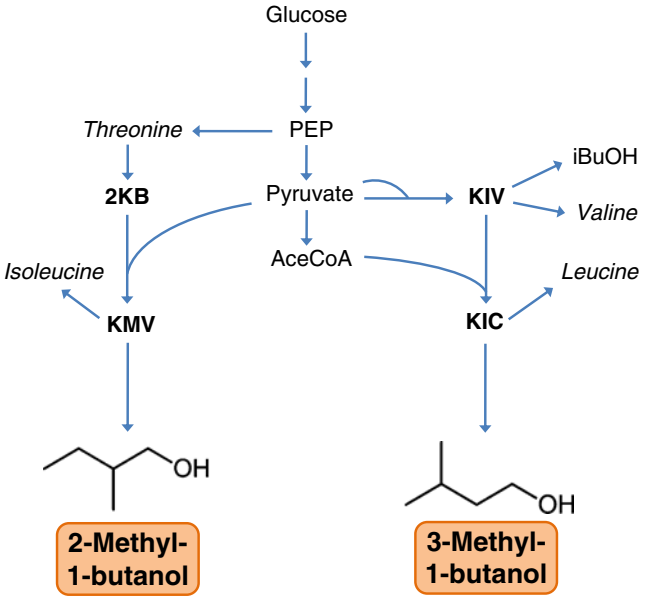

Fig. 2 Production of 2-methyl-1-butanol and 3-methyl-1-butanol from the amino acid biosynthetic pathways of isoleucine and leucine, respectively. *PEP* phosphoenol pyruvate, *2KB* 2-ketobutyrate, *KIV* 2-ketoisovalerate, *KMV* 2-keto-3-methylvalerate, *KIC* 2-ketoisocaproate, *iBuOH* isobutanol

converted to 2KB in one reaction carried out by the enzyme threonine deaminase. Several studies have given insights to increasing threonine production in *E. coli* and other organisms (Shimizu et al. 1995; Lee et al. 2007), and much of this knowledge is transferrable to 2-methyl-1-butanol biosynthesis. In the case of *Corynebacterium glutamicum*, it was shown that a lysine-producing strain can be converted into an isoleucine-producing strain by overexpression and removal of feedback inhibition of the threonine biosynthetic pathway and threonine deaminase (Morbach et al. 1996). Recent work has validated this approach for the production of higher alcohols in *E. coli*, including 2-methyl-1-butanol, where the efficacy of overexpressing a feedback-resistant threonine deaminase and threonine biosynthetic operon (*thrABC*) with artificial transcription regulation has been clearly demonstrated (Cann and Liao 2008; Shen and Liao 2008).

Further improvement in 2-methyl-1-butanol production was achieved through gene knockouts. Many knockout strains were cultured and tested for improved 2-methyl-1-butanol production, mostly genes in competing pathways and genes that lead to side product formation. However, only knockout strains leading to increased flux to threonine and 2KB (*metA* and *tdh*) were significantly improved (Cann and Liao 2008). The best strain tested achieved 1.25 g/L of 2-methyl-1-butanol with a yield of 0.17 g/g glucose. Additional work in strain design should lead to even better 2-methyl-1-butanol production strains.

Engineered production of 3-methyl-1-butanol in *E. coli*

The leucine biosynthetic pathway in *E. coli* produces the 2-ketoacid precursor of 3-methyl-1-butanol, KIC (Fig. 2). Analogous to 2-methyl-1-butanol production, the expression of *kivd* from *L. lactis* facilitates the conversion of KIC to 3-methyl-1-butanol. Leucine biosynthesis also builds from the valine pathway in *E. coli*, consuming the precursor of valine, 2-ketoisovalerate (KIV). Previous work in isobutanol production has demonstrated the ability to produce enough KIV to enable 22 g/L of isobutanol in *E. coli* cultures (Atsumi et al. 2008b). A key factor in this level of production was the expression of *alsS* from *Bacillus subtilis* coding for aceto-lactate synthase for very efficient condensation of two pyruvate molecules in the first committed step in KIV production. Another important aspect of high KIV production for isobutanol is a strain in which competing fermentation pathways are deleted, leading to an increased availability of both NADH and pyruvate. The strategies shown here to increase KIV are useful for further conversion of KIV to 3-methyl-1-butanol.

The leucine biosynthetic pathway combines KIV with acetyl coenzyme-A (acetyl-CoA) to produce KIC for 3-methyl-1-butanol production. However, simply overexpressing the native leucine operon from *E. coli* (*leuABCD*) does little to divert KIV from isobutanol production (Connor and Liao 2008). It was previously demonstrated that removal of feedback inhibition of the leucine operon is effective for increasing the production of leucine in microorganisms (Gusyatiner et al. 2002). Connor and Liao (2008) showed that in addition to the expression of a feedback-resistant leucine operon, a synthetic ribosomal binding site is important for the efficient expression of the operon. These additions led to a dramatic increase in 3-methyl-1-butanol production in *E. coli*.

Further improvement in the production of 3-methyl-1-butanol can come from the deletion of competing pathways. As mentioned previously, gene knockouts of fermentation pathways such as lactate (*ldhA*), ethanol (*adhE*), acetate (*pta*), and succinate (*frdBC*), as well as knockouts of *pflB* and *fnr* to increase efficiency in low oxygen conditions (Atsumi et al. 2008a), contributed to an increase in the pools of NADH, pyruvate, and acetyl-CoA for increased production of 3-methyl-1-butanol. Two other vital precursors, KIV and KIC, are also precursors for the transamination reaction to form the amino acids valine and leucine, respectively. Deletion of the two genes responsible for the transamination of most of the KIV and KIC, *ilvE* and *tyrB*, further increased 3-methyl-1-butanol production in *E. coli* to a final titer of nearly 1.3 g/L with a yield of 0.11 g/g glucose (Connor and Liao 2008).

Isopentenol production from the terpenoid pathway

An oxidized extension of the pentanol family, isopentenol can be found in trace amounts in the resin of certain tree species (Dewick 2002). Isopentenol is derived from the five-carbon building blocks of terpenoid synthesis, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP; Fig. 3). There are at least two biosynthetic pathways to IPP and DMAPP common in microorganisms, one dependent on the intermediate mevalonate, made from acetyl-CoA, and found in *Saccharomyces cerevisiae*, and another dependent on methylerythritol phosphate, from pyruvate and glyceraldehyde-3-phosphate, and found in *E. coli*. There have been many studies concerning the engineering of these pathways for the production of higher terpenoids (Farmer and Liao 2000; Martin et al. 2003), but very little work has been done to engineer the production of isopentenol.

Isopentenol was detected at very low levels in *E. coli* cultures as a by-product of the overexpression of most of the native methylerythritol phosphate pathway (Rohdich et al. 2002). More recently, Withers et al. (2007) actually discovered some enzymes responsible for the direct production of isopentenol from IPP and DMAPP from *B. subtilis*. They accomplished this by screening a genomic library for the enzymatic relief of toxic levels of farnesyl diphosphate, a product of IPP and DMAPP polymerization, in *E. coli* cultures. Expression of the *nudF* gene from *B. subtilis* allowed the production of about 110 mg/L of isopentenol.

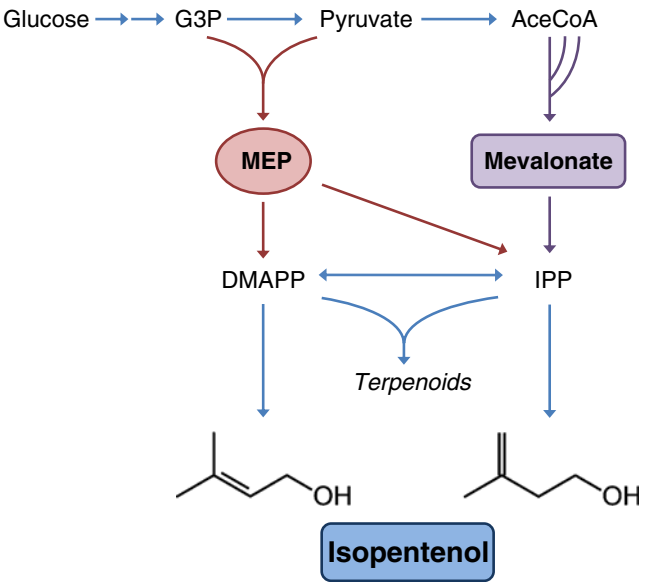

Fig. 3 Pentenol production via parallel pathways involving either the intermediate mevalonate or methylerythritol phosphate. *G3P* glyceraldehyde-3-phosphate, *AceCoA* acetyl coenzyme-A, *MEP* methylerythritol phosphate, *DMAPP* dimethylallyl diphosphate, *IPP* isopentenyl diphosphate

1-Pentanol production using novel pathway functions

Normal pentanol, the straight chain isomer, is a semi-natural biological compound. Its presence has been detected in trace amounts in yeast fermentation (Williams et al. 1981; Mauricio et al. 1997), but the biosynthetic pathway is not well understood. Recently, however, it has been shown how 1-pentanol can be produced in microorganisms by expanding the range of substrates for known biosynthetic pathways (Zhang et al. 2008).

It was previously established that the KDC coded by *kivd* from *L. lactis* has a wide substrate range for converting 2-ketoacids into aldehydes (de la Plaza et al. 2004; Atsumi et al. 2008b), and we have already seen how well this enzyme helps in the production of two other pentanols, 2-methyl-1-butanol and 3-methyl-1-butanol. However, the 2-ketoacid that would be converted to 1-pentanol, 2-ketocaproate, is not normally found in microorganisms. Interestingly, it turns out that the leucine biosynthetic pathway in *E. coli* (*leuABCD*) can also be engineered to accommodate a broad substrate range (Fig. 4).

Previous work has already shown that the leucine pathway, which normally converts KIV to KIC, can convert 2KB to 2-ketovalerate, the precursor in 1-butanol production (Atsumi et al. 2008b; Shen and Liao 2008). The natural substrate for the leucine pathway is very similar to 2-ketovalerate; however, overexpression in *E. coli* of wild-type *leuABCD* along with *kivd* produces only a small amount of 1-butanol, and no 1-pentanol is detected (Zhang et al. 2008). Protein engineering of the two promiscuous enzymes LeuA and Kivd proved to be instrumental in the production of 1-pentanol. Removal of leucine-induced feedback inhibition of LeuA first allowed 1-pentanol to be detected, and an enlargement of the Kivd substrate-binding pocket allowed a production level of 750 mg/L of 1-pentanol in *E. coli* cultures (Zhang et al. 2008). Considering that 1-pentanol was not the final goal of this protein engineering, it seems likely that more focused work could yield even greater production of 1-pentanol in the future.

Applications of pentanols

The current push for alternative fuels has brought about our recent discussion of pentanols and other higher alcohols as potential biofuels. As discussed, pentanols have good physical properties for use as liquid transportation fuels, and they are currently used as fuel additives in gasoline blends. This section will discuss the many other applications of pentanol isomers, both current and potential.

There are a couple of ways to diversify the type of fuel that pentanols can become. For example, current biodiesel production relies mostly on methanol to esterify fatty acid

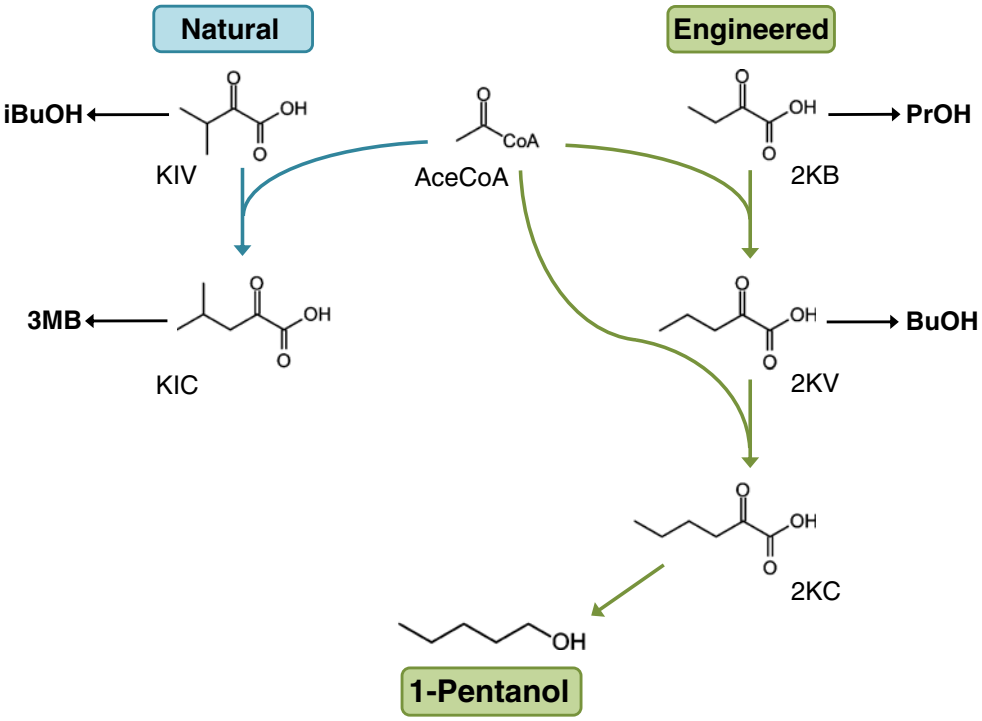

Fig. 4 Natural and engineered reactions catalyzed by the leucine biosynthetic operon, *leuABCD*. *2KV* 2-ketovalerate, *2KC* 2-ketocaproate, *PrOH* 1-propanol, *BuOH* 1-butanol, *3MB* 3-methyl-1-butanol

triglycerides into a diesel fuel (Gerpen 2005). However, longer chain alcohols, like pentanols, are readily used in the esterification reaction and can be preferable due to their greater fat solubility as compared to methanol (Salis et al. 2005). Another potential fuel application for pentanols and other higher alcohols could come from their polymerization to longer compounds suitable for diesel, jet fuel, or other fuels (Marchionna et al. 2001). Through a simple chemical activation of the hydroxyl group to a carbon–carbon double bond, these alcohols could be converted to a wide range of useful hydrocarbons.

Besides diesel esters, pentanols can form other esters for more applications. One of the primary flavor esters found in food fermentations is isoamyl acetate, which comes from the reaction of 3-methyl-1-butanol and acetyl-CoA. Recent work has demonstrated the viability of engineering *E. coli* for the production of isoamyl acetate from added 3-methyl-1-butanol by expressing genes from *S. cerevisiae* (Vadali et al. 2004). Other work has demonstrated the potential to combine ethanol production with simultaneous expression of esterification enzymes for in vivo biodiesel production (Kalscheuer et al. 2006). This suggests that the endogenous production of 3-methyl-1-butanol demonstrated in *E. coli* (Connor and Liao 2008) could be combined with genes for its esterification with acetyl-CoA for engineering of the direct production of isoamyl acetate in *E. coli*.

A product used in the mining industry, amyl xanthate, is another useful pentanol ester. Amyl xanthate can come from any of the primary pentanol isomers, e.g., 1-pentanol, 2-methyl-1-butanol (The Dow Chemical Company 2002c; Lappe and Hofmann 2005). Pentanol isomers are often used as solvents for chemical reaction, liquid extraction, and products such as paint and coatings (The Dow Chemical Company 2002a, b, c; Lappe and Hofmann 2005). They are especially useful as chemical intermediates or starting materials for a wide variety of products, including pharmaceuticals, antioxidants, cosmetics, herbicides, liquid crystals, dyes, flavorings, and catalysts (Gershanovskii et al. 1973; The Dow Chemical Company 2002a, b, c; Lappe and Hofmann 2005). Other applications for pentanols are additives for lubrication oil, corrosion inhibitors, and plasticizers and stabilizers for polymer plastics (The Dow Chemical Company 2002b, c; Lappe and Hofmann 2005).

Table 1 Summary of highest titers and yields for engineered pentanol production

Pentanol	Titer (g/L)	Yield (g/g)	Reference
2-Methyl-1-butanol	1.25	0.17	Cann and Liao 2008
	0.37	N/A[a]	Fukuda et al. 1993
	0.25	N/A[a]	Kielland-Brandt et al. 1979
3-Methyl-1-butanol	1.28	0.11	Connor and Liao 2008
	0.41	0.008	Watanabe et al. 1990
	0.28	0.002	Suzzi et al. 1998
1-Pentanol	0.75	0.038	Zhang et al. 2008
	0.08	N/A[a]	Mauricio et al. 1997
Pentenol	0.11	0.006	Withers et al. 2007

[a] Not able to be determined from available data

Conclusions

Pentanols are a useful class of compounds that have garnered increasing attention due to recent pressure to find alternatives to petroleum. While a lot of this attention is toward the fuel applications of petroleum, it should be remembered that petroleum-derived products permeate modern society. Biologically produced pentanols can be a part of the solution to moving beyond petroleum, and this review has summarized current methods of pentanol production (Table 1) and discussed many of the diverse ways that pentanols are useful.

As far as engineered production in biological systems, production of pentanols is just beginning. While long known as minor fermentation products, engineered production of pentanols has only taken place within the last 2 years. This means that much more research is required before biologically produced pentanols will be industrially relevant. The next major challenge likely to be faced in the microbial production of pentanols is the issue of toxicity. Pentanols are basically short-chain fatty alcohols and thus have a hydrophobic region of significant size. While the large hydrophobic region translates to a low solubility (about 2%) and thus easier separation from water, it also means that these compounds are more fat-soluble and thus tend to associate in the cellular membranes of microbes. There is already much research into the toxicity of hydrophobic compounds in *E. coli* and other organisms (Okolo et al. 1987; Aono et al. 1994; Sikkema et al. 1995), and some solutions have been studied (Roffler et al. 1987; Aono 1998; Izak et al. 2008). As biological production of pentanols is pushed further, the issue of its toxicity will become more prominent.

There are several directions for future research into biologically engineered pentanol production. Thus far, *E. coli* has been the organism of choice for this production, though many of the key enzymes utilized are derived from other important and useful organisms such as yeast strains and lactobacilli. These microbes along with others can potentially offer advantages to make them better hosts for pentanol production. Further desirable research might be to include cellulose degradation directly to pentanols or even incorporate CO_2 for pentanol production. The area of biologically engineered pentanol is certainly still a nascent field.

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