Review

Microbial-based motor fuels: science and technology

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Summary

The production of biofuels via microbial biotechnology is a very active field of research. A range of fuel molecule types are currently under consideration: alcohols, ethers, esters, isoprenes, alkenes and alkanes. At the present, the major alcohol biofuel is ethanol. The ethanol fermentation is an old technology. Ongoing efforts aim to increase yield and energy efficiency of ethanol production from biomass. *n*-Butanol, another microbial fermentation product, is potentially superior to ethanol as a fuel but suffers from low yield and unwanted side-products currently. In general, biodiesel fuels consist of fatty acid methyl esters in which the carbon derives from plants, not microbes. A new biodiesel product, called microdiesel, can be generated in engineered bacterial cells that condense ethanol with fatty acids. Perhaps the best fuel type to generate from biomass would be biohydrocarbons. Microbes are known to produce hydrocarbons such as isoprenes, long-chain alkenes and alkanes. The biochemical mechanisms of microbial hydrocarbon biosynthesis are currently under study. Hydrocarbons and minimally oxygenated molecules may also be produced by hybrid chemical and biological processes. A broad interest in novel fuel molecules is also driving the development of new bioinformatics tools to facilitate biofuels research.

Introduction

Society in the early 21st century appears to be undergoing an unprecedented transition with respect to the fundamental source of its materials and energy. Petroleum, the fuel that has been driving modern society for one century, is showing signs of scarcity (Grant, 2005).

Demand for petroleum is increasing, but discoveries of fresh deposits are dwindling. The complex hydrocarbon mixtures found in crude oil deposits were formed under conditions of low heat and pressure over millions of years (Berner, 2003). Conventional petroleum is essentially non-renewable. Intertwined with this practical impediment, there is an apparent moral dilemma arising from petroleum usage. It has become widely accepted that the combustion of long-sequestered petroleum carbon is strongly contributing to the observed increase in atmospheric carbon dioxide, with concomitant global warming effects. The convergence of market pressure and concern for the environment is driving a headlong rush to new fuels that are largely bio-based. But are biofuels really new?

History and needs

For millennia, human societies depended on biological materials for energy and materials. Plant material was combusted for heat, used for building materials and clothing; animal power was harnessed for transportation. A little more than a century ago, society underwent another transition: from horse to automobile, from whale oil to crude oil. The 1890s was similar to today in that many fuel sources were being tested, production was in flux, and the industry was not yet integrated and consolidated. A similar situation exists today with the transitioning to a renewable energy society.

Not all of the technology is new; for example, humans have been purposely making ethanol and fatty acid derivatives for millenia. But current molecular biological tools are opening new doors for old technology, and making radical new technologies seem possible. The biology of today is vastly different from that of decades ago and most of the new technology is microbially based. So it is not surprising that microbial metabolic activities are coming into focus for solutions to our fuel needs for the 21st century.
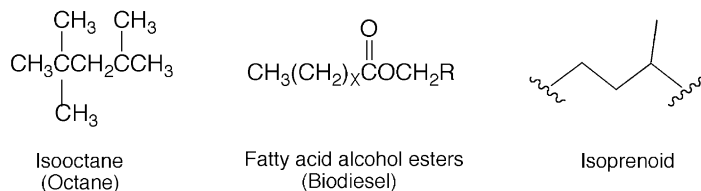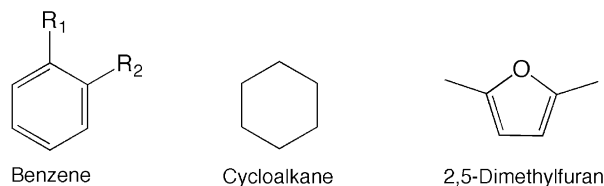
One of the important questions as we enter this stage of transition is, 'What constitutes a good motor fuel?' The answer is partly engine dependent but there are certain rules of thumb that span across different engine types. It is generally considered that a desirable fuel should be: (i) a liquid, (ii) highly combustible but not explosive, (iii)

0-1 Carbon

H_2 CH_4 CH_3OH CH_3NO_2

Hydrogen   Methane   Methanol   Nitromethane

2-5 Carbon

H_3COCH_3 CH_3CH_2OH $H_3CCH_2CH_3$ $CH_3(CH_2)_2CH_2OH$ $CH_3\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{C}}\!-\!OCH_3$

Dimethyl ether   Ethanol   Propane   *n*-Butanol   Methyl-*t*-butyl ester
(MTBE)

> 5 Carbon

$CH_3\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{C}}CH_2\overset{\displaystyle CH_3}{C}CH_3$ $CH_3(CH_2)_X\overset{\displaystyle O}{\overset{\|}{C}}OCH_2R$

Isooctane   Fatty acid alcohol esters   Isoprenoid
(Octane)   (Biodiesel)

Cyclic

Benzene   Cycloalkane   2,5-Dimethylfuran

Fig. 1. Gallery of fuel molecules in current, or proposed, use in spark ignition, diesel or construction vehicles.

something with a high energy to mass ratio, (iv) stable on long-term storage, (v) transportable by pipeline and (vi) inexpensive. A range of molecules meet most of those criteria (Fig. 1); it is expected that current microbiological research will lead to additional ones. Of those shown in Fig. 1, hydrogen, methane and propane are gaseous at 20°C, but they are important fuel molecules. Hydrogen suffers from storage problems making it unable to support long-distance travel currently. Methane, in the form of natural gas, is used principally for heating and electricity generation. Propane is used with construction vehicles, particularly forklifts (AmeriGas, America's Propane Company, 2007), but is not widely used for general transportation. Of the moderate-sized liquid fuels (C_2–C_5), about 20 billion pounds of methyl-*t*-butyl ether (MTBE) has been used annually but its use is declining due to water contamination issues (Suffet, 2007). Currently, the alcohol fuels, ethanol and *n*-butanol, are being developed as alternative biomass-derived oxygenated fuels. Of the two alcohols, ethanol is most widely used globally, with Brazil and the USA accounting for approximately 90% of ethanol production (Goldemberg, 2007).

The motor fuel of choice for spark ignition engines has consisted of, among other components, liquid, branched-chain alkanes exemplified by 2,2,4-trimethylpentane, or isooctane, which is ranked as 100 on the 'octane scale' for measuring suitability of molecules for fuel purposes. For diesel engines, a higher chain length of alkanes is desirable, and hexadecane is the comparable gold standard molecule. The use of longer-carbon-length hydrocarbons is due to the compression ignition in diesel engines; a spark plug is used to ignite a fuel–air mixture in spark ignition, or gasoline, engines. Both conventional gasoline and diesel hydrocarbons currently derive from crude oil, although recent microbial research is investigating a bio-based replacement as discussed in subsequent sections on alkanes and alkenes. Petroleum-based fuels are complex mixtures, consisting of benzene ring compounds, simple and alkylated cycloalkanes, alkanes and some heterocycles (Fig. 1).

Biodiesel, which serves as a replacement for hydrocarbon fuels in diesel engines, is generally comprised of fatty acid alcohol esters. The fatty acid precursors derive largely from animal and plant fatty acids (typically C_8–C_{22}) that are part of fats such as triacylglycerides, an esterified polyalcohol. Triacylglycerides themselves are not good fuels, but switching the alcohol moiety of a glycerol triester to make individual methanol or ethanol monoesters generates molecules that are excellent as a fuel for diesel engines. The chemical process used to switch the alcohol component is known as transesterification (Huber *et al.*, 2006). The upsurge in biodiesel production has resulted in a market glut of glycerol, a by-product of the transesterification process. However, new and creative uses for glycerol as

Table 1. Representative microorganisms for which genome sequences have been completed or are in progress that are important in the context of biofuels research.

Microorganism	Significance for biofuels
Anaebaena variabilis	Cyanobacterium producing hydrogen
Cladicellulosiruptor saccharolyticus	Degrades various polysaccharides; produces hydrogen
Clostridium acetobutylicum	Major organism producing *n*-butanol
Clostridium phytofermentans	Ferments pectin, cellulose, xylan producing ethanol and hydrogen gas
Clostridium thermocellum	Thermophilic ethanol producer
Methanosaeta thermophila	Common methanogen; converts acetate to methane
Micrococcus luteus	Produces long-chain alkenes
Pichia stipitis	Ethanol producing yeast fermenting xylose
Rhodospeudomonas palustris	Phototroph producing hydrogen gas
Saccharomyces cerevisiae	Major production organism for ethanol currently
Saccharophagus degradans	Degrades many biopolymers
Thermoanaerobacter pseudoethanolicus	Thermophilic ethanol producer
Vibrio furnissii M1	Reported to produce high levels of *n*-alkanes
Zymomonas mobilis	Ethanol fermentation with high ethanol tolerance

a fermentation feedstock are emerging as a result of the artificially low price created by increased biodiesel production.

Creative approaches are also emerging to take biomass and use chemical catalysts to dehydrate and reduce sugars for generating molecules that are sufficiently energy-dense to be used as fuels. An interesting recent example of this, described by Dumesic and co-workers (Roman-Leshkov *et al.*, 2007), is a process to generate 2,5-dimethylfuran, a molecule that is expected to have good combustion properties in vehicular engines. Such developments are indicative of the current perception in the biofuels arena. As we shift from petroleum, it has become unclear what the fuel molecule 'winners' will be. Which will have the requisite properties as fuels coupled with low production costs, and will the process of converting biomass be chemical, biological, or some mixture of both (Schmidt and Dauenhauer, 2007)? It is generally accepted that ethanol alone is not going to provide a long-term solution to meet society's energy needs (Hill *et al.*, 2006). It suffers from a somewhat low energy density, inability to be transported through pipelines and fairly high cost for extraction from fermentation broths. This is opening the door to developing many other molecules as replacements for ethanol and thus, discovering new fuel molecules to be produced via microbial biotechnology.

The desire for very different molecular structural class (hydrocarbons, alcohols, ethers, esters) is driving the idea that the field of synthetic biology will impact biofuels research. Synthetic biology is based on the premise that constructing biological systems from a 'parts lists' will lead to a greater understanding and utility of biology. A major tool of synthetic biology is genomics. Presently, the complete genome sequences for over 2000 microorganisms are in the public domain (GOLD, 2007). A significant number of the sequenced microbial strains have some significance in biofuels research due to the prominent role

of the United States Department of Energy in choosing and funding the public sequencing of non-pathogenic microbes. A representative list is shown in Table 1.

With respect to biofuels, ethanol, butanol and alkyl esters are biologically common. In contrast, many fuel molecules in current usage (Fig. 1) are not composed of biologically common functional groups. However, using synthetic biology, these or similar molecules could conceivably be constructed by exploiting known enzyme catalysis in new ways. This could be used to make new biomolecules, or make known molecules in much larger quantities than their natural abundance. This premise underlies a number of the emerging start-up biofuels companies that seek to create the next generation of bio-based fuel. The price of the product they would sell is much lower than a pharmaceutical product but the market is huge, and thus, potentially very lucrative.

Alcohols

Ethanol

Ethanol production is based on an old technology, if one considers the production and consumption of alcoholic beverages by human societies. A 6000-year-old Sumerian tablet depicts people drinking alcoholic beverages. Much more recently, investigations into the alcoholic fermentation were instrumental in establishing the early fields of microbiology and biochemistry. In 1839, Leibig ridiculed the cell theory and the proposition that grape juice fermentation was a biological process (Liebig, 1839). About 20 years later, Pasteur firmly associated the presence of yeast cells with 'good' fermentations that made ethanol (Pasteur, 1857; 1860). Some 6000 years after initiating the ethanol fermentation, humans had some understanding of the cellular basis underlying the process. In 1897, Buchner prepared a cell-free extract from *Saccharomyces cerevisiae* that transformed glucose to ethanol (Buchner, 1897). This was important for establishing a new way of

biochemical investigation and it also showed conceptually that metabolism could occur in the absence of a living cell. This paved the way for the measurement of cell-free enzyme activities and the general principles of enzyme kinetics (Michaelis and Menten, 1913). In fact, the field of enzymology was born out of studies on the ethanol fermentation.

It is somewhat astounding that ethanol produced for automobile engines in the current era is made using the same microorganism (*Saccharomyces*) and in similar titre (less than 15% of the aqueous fermentation broth) as has been done for centuries. The overall biochemical process used is also the same. Thus, 1 mole of glucose is converted to 2 moles of ethanol and 2 moles of carbon dioxide, with a net lost of one-third of the carbon atoms in generating the fuel. Bacterial alternatives to *Saccharomyces* yeast are being studied, principally *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca* (Ingram *et al.*, 1987; Dien *et al.*, 2003); however, these other organisms are not widely used commercially. The limitation in the titre of ethanol is due to its toxic effects on microorganisms at concentrations above 5%. Thus, ethanol must be obtained via distillation and drying, which are energy-intensive and somewhat costly steps. As a result, bioethanol production from corn starch is marginally energy yielding when one considers all the steps in the process (Hill *et al.*, 2006). Ethanol production from sugarcane, as practiced in Brazil and other tropical areas, has a better net energy yield.

Some major features of ethanol fermentation are currently the subject of intensive research. Specifically, the use of hemicellulosic material will require the fermentation of both hexose (glucose, mannose and galactose) and pentose (xylose and arabinose) sugars. Currently, there are no naturally occurring ethanol fermenting strains that handle all of the necessary sugars. This problem could be solved by appropriate metabolic engineering (Dien *et al.*, 2003). A novel approach to this problem would be to conduct a homoacetogenic fermentation, using strains that handle hexoses and pentoses simultaneously (Eggeman and Verser, 2006). In this approach, the resultant acetic acid is esterified with ethanol to make ethyl acetate, which is relatively water insoluble and thus readily recoverable. Hydrogenation of 1 mole of ethyl acetate, using well-known industrial chemical methods, produces 2 moles of ethanol. An advantage of this approach is the overall stochiometry; 1 mole of hexose sugar yields 3 moles of ethanol. This contrasts with the traditional ethanol fermentation in which two of the hexose carbon atoms are lost as carbon dioxide.

Another critical issue in making the ethanol fermentation cheap and energy efficient is for integration of the different elements in transforming biomass to ethanol. As currently envisioned, the overall process involves four steps: the production of saccharolytic enzymes, the hydrolysis of biomass carbohydrate polymers, the fermentation of hexoses and the fermentation of pentoses (Lynd *et al.*, 2005). It is hoped that genome sequencing will contribute to developing biological agents for a consolidated bioprocessing of cellulosic biomass. Specifically, organisms that are thermophilic, saccharolytic and ethanologenic may be used directly, or after genetic engineering, to develop a one-pot fermentation process for taking biomass to ethanol (Table 1).

Butanol

Butanol is another microbial fermentation product, studied principally with the anaerobe *Clostridium acetobutylicum*. *Clostridium acetobutylicum* was cultivated on an industrial scale nearly a century ago, although the desired compound in that process was the fermentation co-product acetone. Acetone was in great demand in 1915 because of its utility in the manufacture of cordite, an explosive used by countries warring against Germany. Germany had been the major manufacturer of acetone prior to World War I but acetone export was cut off with the war. Chaim Weizmann led the British effort to produce acetone using *C. acetobutylicum*, a process that met the war needs of England (Dixon, 1997). The large-scale fermentation process was shared with England's allies. By the end of the war, 22 fermentors of 30 000 gallon capacity were operating continuously in Canada (Kluyver, 1957). After World War I, however, the petroleum industry expanded and supplanted the bio-acetone process.

With the re-emergence of interest in producing fuels from biomass, there is again a focus on the acetone-butanol-ethanol fermentation of *C. acetobutylicum*. In the present day, *n*-butanol is the most desired product. *Clostridium acetobutylicum* is also of interest because it is saccharolytic, thus it could potentially hydrolyse starch directly, removing a need for pre-treatment. The fermentation pathway from glucose to ethanol is similar to that occurring in *S. cerevisiae*, proceeding through pyruvate and acetaldehyde (Fig. 2). Acetone is produced from 2 moles of acetyl-CoA, via acetoacetyl-CoA, thus there is a 50% loss of glucose carbon in generating one equivalent of acetone. A better carbon balance is obtained with *n*-butanol, which derives from reduction of acetoacetyl-CoA via butyryl-CoA, thus capturing four of the carbon atoms of glucose in the end-product. Recent fibrous bed reactor technology with *C. acetobutylicum*, developed by Huang and colleagues (2004), makes *n*-butanol as the major component in the fermentation broth with a productivity of 4.6 g l^{-1} h^{-1} and a yield of 0.42 g g^{-1}. These developments are beginning to make biobutanol an attractive fuel alternative to bioethanol.
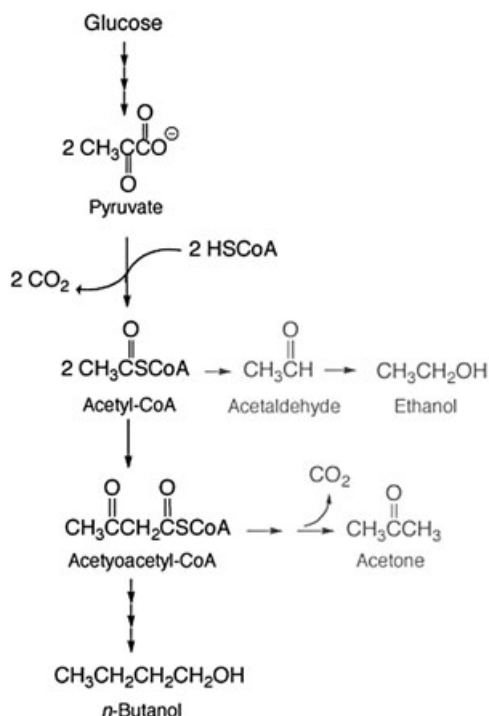
Fig. 2. Butanol synthesis by the fermentation pathway of *Clostridium acetobutylicum*.

As a motor fuel, *n*-butanol is superior to ethanol in several properties (Huber *et al.*, 2006). *n*-Butanol has a nearly 50% higher energy density than ethanol and is almost comparable to gasoline in this regard. *n*-Butanol carries less water than ethanol and could be transported through pipelines, unlike ethanol. Although the performance properties of butanol in car engines is still controversial, it clearly could be used in fuel blends, if not used directly as a motor fuel. British Petroleum and DuPont have recently teamed up in a major effort to further develop biobutanol for use as a motor fuel (Biobutanol, 2007).

Methanol

While methanol is not always considered as a serious contender in the alcohol fuel race, it does have history on its side. Methanol was used in blended motor fuels in the USA in the 1970s. Indy racing cars burn 100% methanol because methanol can run at extremely high compression ratios and thus generate maximal engine power. George Olah, hydrocarbon chemist and Nobel Laureate, has promoted a methanol-based economy, with methanol use as a fuel and chemical feedstock (Olah and Molnar, 1995). Currently, methanol is produced largely from natural gas, methane principally, by chemical catalytic processes that are mulistep. First, carbon monoxide and hydrogen are produced, and then those are reformed to generate

methanol. There has been some examination of the microbial counterpart to this chemistry that generates methanol from methane in one step with 100% yield. The enzyme catalysing this reaction, methane monooxygenase, is a multicomponent enzyme system biosynthesized by methanotrophic bacteria, those that grow on methane as a carbon and energy source (Colby *et al.*, 1979). The monooxygenase component is a μ-oxo-bridged di-iron protein that has been studied in structural and mechanistic detail (Lipscomb, 1994; Sazinsky and Lippard, 2005). One major issue in biotechnological applications is the relative instability of the enzyme to oxidative damage *in vitro*. *In vivo*, methanotrophs oxidize methanol completely to carbon dioxide as part of their overall energy metabolism. Recombinant methane monooxygenase systems suffer from low activity in comparison with that observed in native hosts (Wood, 2002).

Alcohol esters of fatty acids (biodiesel)

The current production strategy for biodiesel does not involve microbial biotechnology. As mentioned previously in the section on fuel molecules, the fatty acid carbon atoms in biodiesel come from animal fat waste or from plant oils. Lipids, principally triacylglycerides, are transesterified in a chemical process to produce fatty acid esters that constitute biodiesel (Fig. 3A). However, recent developments suggest that microbial biotechnology could yield a breakthrough in biodiesel production. This is because there are some current limitations in producing biodiesel. Primary among them is the limitation of oil-producing crops; there is only so much production capacity for rapeseed in Europe and soybean in the USA. Also, the methanol used for transesterification largely derives from natural gas. Thus, the resultant biodiesel is only partly derived from renewable sources. It would be more desirable to make fatty acid esters directly from cheaper and more widely available sugars such as glucose using bacteria as the catalysts for the entire transformation (Fig. 3B). This would dovetail with extensive work on bio-based cellulose processing to sugars and make for a potential one pot process from biomass to biodiesel. An important step to accomplishing this goal was recently taken.

While microorganisms do not typically make fatty acid methyl or ethyl esters, some microorganisms make copious amounts of storage lipids in the form of triacylglycerides and wax esters (Holdsworth and Ratledge, 1991; Kalscheuer et al., 2007). One bacterium, *Acinetobacter baylyi* strain ADP1, makes esters via an enzyme that catalyses acyltransfers to make wax esters and triacylglycerols and has very broad specificity with respect to the fatty acids and alcohols it binds (Kalscheuer and Steinbüchel, 2003). The observation that it would use ethanol as the acyl group acceptor, albeit at a lower rate
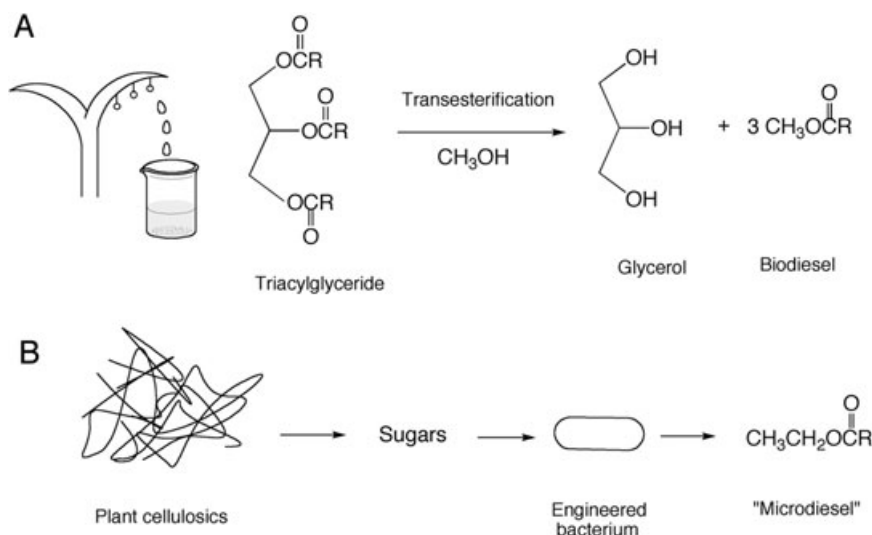
Fig. 3. Processes for making biodiesel (A) chemically and (B) biologically.

than longer-chain alcohols, provided the idea that a biodiesel-producing bacterium could be engineered (Kalscheuer *et al.*, 2006). Thus, the engineered *E. coli* strain contained the *Acinetobacter* wax ester synthase gene and ethanol-production genes from *Z. mobilis*. The recombinant strain, when fed exogenous fatty acids, produced fatty acid ethyl esters, up to 26% of the bacterial dry mass under ideal conditions (Fig. 3B). This was an excellent demonstration of feasibility. The product was called 'microdiesel' as this was a microbially produced fuel, and it was different from the majority of biodiesel by consisting of ethyl esters. In the chemical process, methanol has been used more often as that is cheaper than ethanol. To make microdiesel economically, the fatty acids would need to be biosynthesized *de novo* from sugars by the *E. coli* strain. That would require further genetic engineering. Another obstacle to a cheap bioprocess is the intracellular accumulation of the fatty acid esters. Harvesting the desired fuel product would require cell rupture and separation, a potentially expensive process step. It is unclear if a bioprocess can be created in which highly hydrophobic molecules, like fatty acid ethyl esters, can be engineered for biological excretion outside the cell.

Ethers

Low-molecular-weight ethers have suitable properties as fuel molecules in terms of stability, water insolubility and combustibility. The major ethers currently in use for fuel applications are MTBE and dimethyl ether. MTBE has been used as a gasoline additive. It serves to reduce carbon dioxide emissions and raise the octane number of fuels. Its use has been declining in the USA because it is reasonably soluble in water and imparts an unpleasant

taste and odour when present in drinking water at very low levels (Suffet, 2007). Dimethyl ether is more recently being used as a diesel fuel, for example, powering buses in Sweden (Wu *et al.*, 2006). Both MTBE and dimethyl ether are synthesized by chemical catalytic processes.

While biofuel ethers are not currently in general use, ether biosynthetic reactions, relevant to fuel type molecule, are becoming more fully understood. One example is the biosynthesis of ether lipids by methanogenic bacteria (Koga and Morii, 2007). In this case the alkenyl chains are attached to a glycerol moiety via an ether linkage. The enzymatic condensation of an activated hydrocarbon alcohol to another alcohol might be co-opted to make molecules that resemble biodiesel, but have superior properties.

Hydrocarbons

Hydrocarbons of the appropriate size are ideal fuels, meeting all the previously indicated fuel criteria: being (i) a liquid, (ii) highly combustible but not explosive, (iii) something with a high energy to mass ratio, (iv) stable on long-term storage, (v) transportable by pipeline and (vi) inexpensive. The last property, being inexpensive, has been predicated on readily available petroleum resources. As society is increasingly confronted with scarce petroleum reserves, there is widespread interest in developing a bio-based fuel that consists partly or wholly of hydrocarbons. It is widely believed that petroleum hydrocarbons derive from more highly oxygenated biological molecules deposited underground millions of years ago (Peters *et al.*, 2005). An alternative non-biogenic origin of petroleum has been proposed by Thomas Gold

and others (Gold, 1985). As proposed for the biogenic origins of petroleum, long-duration conditions of low heat and moderate to high pressure have given rise to less oxygenated molecules, including alkyl, cycloalkyl and aromatic hydrocarbons (Blumer, 1976). Thus, oil companies have often searched for biomarkers, bio-derived indicator molecules, as indices of oil deposit origin, size and composition (Moldowan and Seifert, 1979). Over the last 50 years, there have been numerous, scattered reports of direct hydrocarbon biosynthesis by living things, animals, plants and microbes (Kolattukudy, 1976; Ladygina *et al.*, 2006). The mechanisms underlying hydrocarbon biosynthesis have not been in the forefront of biochemistry, but these past reports are now being re-examined with great interest. There is ample opportunity to delve more deeply into the biochemical mechanisms of hydrocarbon-biosynthesis and apply the more recently acquired tools of synthetic biology.

Isoprenoid compounds

Microorganisms make many hydrocarbon molecules; some are found within the large class of molecules known as isoprenoid compounds, of which there are over 50 000 currently known (Walsh, 2007; Withers and Keasling, 2007). The simplest example is isoprene itself, 2-methylbuta-1,3-diene (Kuzma *et al.*, 1995). The physiological function of bacterial unsubstituted isoprene formation is unknown but it is proposed to be enzymatic (Fall and Copley, 2000). Recently, the genes underlying isoprene production by *Bacillus* were identified (Julsing *et al.*, 2007). The much larger class of isoprenoid compounds derive from the five-carbon precursors isopentyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP), the two isomeric building blocks for this class of molecules. Thus, the compounds typically consist of carbon atoms in multiples of five. There can be one condensation reaction to generate a C_{10} molecules or as many as 21 cycles to generate a C_{110} molecule (Walsh, 2007). While most known products arise from head-to-tail condensation reactions, other variations of IPP and DMAPP coupling have now been identified and this contributes to the enormous diversity of more than 50 000 molecules observed in natural systems.

Isoprenoid compounds are commonly known as terpenes and carotenoids. The term terpene arises from the high abundance of these compounds in turpentine; a carotenoid pigment is the major orange pigment in carrots. Isoprenoid compounds are typically important in nature as chemical signals; they comprise pigments or odorants that might attract or deter another organism (Harborne, 1988). For human applications, isoprenoid compounds comprise many important food additives, fragrances and medicinal compounds. For these reasons,

and the emerging potential for biofuel applications, there is burgeoning interest in genetically engineering microbial host strains to make large quantities of isoprenoid compounds. The host organisms used are generally those that have been most well studied with respect to molecular genetics: *E. coli*, *S. cerevisiae* and *Arabidopsis thaliana*.

Simple isoprene compounds have good potential for use as fuels if they can be produced on a large scale and cheaply. The company Amyris is developing a process to generate the anti-malarial drug artemisinin (Amyris, 2007). Artemisinin is a C_{15} isoprenoid compound that is effective in inhibiting the malarial parasite *Plasmodium falciparum* (Cumming *et al.*, 1997). Amyris' goal is the production of isoprenoid compounds cheaply and abundantly. They recognize that a collateral benefit may be the production of novel biofuel molecules. Before petroleum was in widespread use, the leading fuel in the USA was known as camphene, a mixture of ethyl alcohol and turpentine (Kovarik, 2007). In this context, the use of isoprenoid compounds as a fuel has precedence in an earlier age.

The simplest alkane – methane

It has long been appreciated that microbes generate methane and that natural gas, principally methane, can be used as a fuel (Conrad, 1996). Methane has been used largely in home heating and cooking and for electrical generation at municipal power plants. Commercially, methane is obtained largely from extraction of natural gas fields which are often associated with petroleum deposits. Biosynthetic methane is used in some local applications, meeting energy needs for farms and anaerobic digestor facilities. However, there are significant impediments for generating methane to use as a major biofuel. Methane is generated by strictly anaerobic bacteria, methanogens, that grow relatively slowly on biomass as part of complex anaerobic ecosystems. There are generally numerous end-products of these anaerobic fermentations that include volatile alkanoic acids in addition to methane. Moreover, the methane in the gas phase is mixed with nitrogen and carbon dioxide making it costly to purify. With present technology, biologically generated methane is probably most applicable to commerce on a small scale.

Longer-chain alkanes

In general, alkanes power most vehicles today, although the chain length is significantly longer than methane (C_1). The 'gold standard' fuel for spark combustion engines is isooctane (Fig. 1) and for diesel engines it is hexadecane. These derive almost totally from petroleum at present. Petroleum is believed to derive from biological molecules that have been reformed and become more reduced over

millions of years by diagenic processes (Blumer, 1976). Generally, it is not considered that living things make petroleum-like alkanes. However, there have been reports over many years demonstrating alkane biosynthesis by animals, plants and microbes (Kolattukudy, 1976). In most cases, the amount detected has been very low and the biochemical mechanisms have remained obscure. One of the more well-documented mechanisms for generating alkanes biologically has been studied in plants and occurs via decarbonylation of fatty acid aldehydes (Schneider-Belhaddad and Kolattukudy, 2000). In another example, a decarbonylase activity was purified from a microsomal membrane fractions of the alga *Botryococcus braunii* (Dennis and Kolattukudy, 1992). The purified enzyme was reported to transform octadecanal to carbon monoxide and the corresponding alkane heptadecane. It was suggested that the enzyme responsible contains a cobalt-containing porphyrin cofactor. *Botryococcus* is known to produce and accumulate a range of hydrocarbons and hydrophobic ether lipids (Metzger and Largeau, 2005).

Most well-documented studies of microbial alkane production have been conducted with marine eukaryotic algae. In one survey, Youngblood and Blumer (1973) reported that *n*-pentadecane was the major alkane in the brown algae tested, while *n*-heptadecane was found to predominate in red algae. Another species of green algae was reported to contain a C_{17}-cyclopropylalkane. *Dunalliella salina* has been reported to produce 6-methyl hexadecane and 4-methyl octadecane (Tornabene, 1980). Similar internally methyl branched alkanes have been reported in cyanobacteria (Han *et al.*, 1968; Han and Calvin 1969; Fehler and Light, 1970; Gelpi *et al.*, 1970)

Terrestrial microorganisms also produce alkanes other than methane (Jankowski and Zobell, 1948; Davis, 1968; Jones, 1969; Naccarato *et al.*, 1974). The reported alkanes are generally normal chain with a range of C_{16}–C_{30}. However, the following caveat is found in a review by T.G. Tornabene, who studied microbial hydrocarbon biosynthesis for several decades, 'Small amounts of nonisoprenoid hydrocarbons can be found in extracts from most bacterial cells. However, with appropriate precautions to eliminate extrinsic sources of hydrocarbons from the cultivation, extraction and analytical procedures, it is generally found that hydrocarbon biosynthesis is restricted to a relatively small number of bacteria' (Tornabene, 1980).

Most recently, a bacterium, *Vibrio furnissii* M1, was reported to make substantial levels of intermediate to long-chain alkanes (C_{16}–C_{28}) when grown on sugars or organic acids (Park *et al.*, 2001; 2005). *Vibrio furnissii* was obtained from activated sludge at a sewage disposal plant located in Japan and observed, in the laboratory, to produce an extensive floating layer on top of liquid cultures. The extracted polar and non-polar lipids were reported to consist of 48% alkanes (Park *et al.*, 2001).

Further research demonstrated the range of alkanes made by *V. furnissii* M1. A later paper reported that cell-free membrane fractions catalysed the reduction of hexadecanoic acid to hexadecane (Park, 2005). In addition, a patent was filed in Japan describing alkane biosynthesis by *V. furnissii* M1 and other *V. furnissii* strains obtained from the American Type Culture Collection (ATCC) and other sources (Miyamato, 2001).

The reports were widely noted by biofuels researchers for several reasons. Alkanes are a superior fuel and, being water insoluble, would be much cheaper to extract than ethanol. The reports by Park and co-workers indicated that a significant amount of the alkanes were outside the cells, and thus could potentially be recovered without expensive separation procedures. It was also notable that *V. furnissii* M1 produced alkanes when grown on renewable carbon sources such as sugars and polysaccharides, for example, starch, chitin and xylan. Moreover, the titre of alkanes was significant, accounting for as much as 30% of the carbon consumed. It was also very significant that all of the carbon atoms of the alkanes were conserved from their respective fatty acid precursors (Park, 2005), unlike decarbonylation in which a carbon atom is lost.

In light of these findings, Wackett and colleagues (2007) conducted research with *V. furnissii* M1 with the hope of furthering research on bacterial alkane production. Initially, the strain was confirmed to be *V. furnissii* based on 16S rRNA sequence and phenotypic characteristics. The organism showed many properties consistent with the reports of Park *et al.* with respect to growth properties of the bacterium. In subsequent work (Wackett *et al.*, 2007), the complete genome of *V. furnissii* M1 was sequenced at a coverage of 21-fold. The genome annotation effort failed to identify genes for enzymes likely to be involved in alkane biosynthesis; however, not all such genes are likely known. Genome annotation also failed to provide evidence for any known alkane oxidation genes. A series of experiments were conducted to screen for alkanes, either associated with the cells or free in the medium. All such experiments were negative. Other *V. furnissii* strains were tested, including one ATCC strain reported in the Japanese patent to make alkanes (Miyamato, 2001). This also proved negative. Low levels of alkanes were observed in extracts in preliminary experiments but these were shown to derive from glassware, stopcock grease and solvent contamination. In light of these observations, the high-level production of alkanes by *Vibrio* species remains to be verified.

Alkenes

Bacteria are also reported to produce alkenyl, waxy hydrocarbons, a class of molecules that might prove inter-
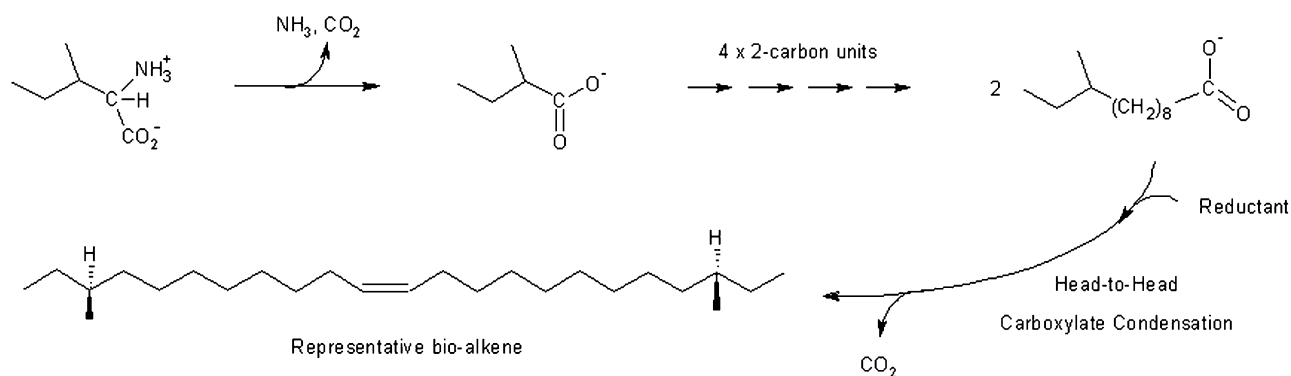
Fig. 4. Proposed pathway for alkene biosynthesis by *Micrococcus* sp.

esting in the context of fuel or specialty chemical applications. The most notable reports are with bacteria of the genus *Micrococcus* and related genera such as *Kocuria*. Micrococci are commensal residents of human skin from which various strains have been isolated in pure culture. *Micrococcus* species are high % G+C, Gram-positive cocci. At this stage, the physiological function of alkenes in *Micrococcus* species is unknown but alkenes have been documented to occur in many different members of the genus (Tornabene, 1980).

There is information on the structure of the alkenes but no detailed information on the mechanism of their biosynthesis. The biosynthetic pathway does not produce a single alkene, but rather a family of alkene products. The alkenes generally range from C_{21} to C_{29} (Fig. 4). The double bond is at or near the middle carbon(s) of the hydrocarbon chain. The alkenyl chains are subterminally methylated. In total, these observations provide clues as to plausible biosynthetic mechanisms. The alkenes are proposed to derive from branched chain fatty acids which are common bacterial lipids. The mechanism by which fatty acid chains may condense to make alkenes is more obscure. There have been reports of fatty acid head-to-head condensation reactions based on radiolabelling studies (Kolattukudy, 1976). *In vitro* studies conducted with crude protein extracts from *Micrococcus luteus* showed that label is lost when fatty acid precursors contain ^{14}C in carbon 1, but not carbon 16, consistent with the loss of a carboxy group during the condensation reaction (Albro and Dittmer, 1969). Additional studies are warranted to elucidate further mechanistic details and potentially engineer hydrocarbon production by *Micrococcus* strains.

Other fuels – hydrogen

Hydrogen is both ideal and problematic as a fuel choice. With hydrogen, issues of carbon dioxide or partially combusted atmospheric pollutants, carbon monoxide, for example, are skirted. Hydrogen reacts with oxygen to release considerable heat and generates water as an end-product. Hydrogen is gaseous even at very low temperatures such that storage density is an issue, especially in any potential vehicular fuel application. Creative efforts to overcome this problem focus on using porous solid materials that can serve as reservoir for hydrogen that can be released as a fuel stream (Rosi *et al.*, 2003). Despite these developments, there are still strong critics of using hydrogen, especially as a motor fuel (Zubrin, 2007).

How might hydrogen be generated in a future society that developed ways to use this energy source? Of course, hydrogen can be generated readily by electrolysis but this uses a high-grade energy source, electricity, to generate a lower-grade one, hydrogen, with only a 50% energy conversion efficiency. There are several practical scenarios being considered. For example, electricity could be generated at a remote site from a free resource, like wind, and hydrogen can be generated to store that energy chemically. Hydrogen can be made from biomass, both chemically and biologically. Salge and colleagues (2006) recently described the controlled combustion of vegetable oils and other biomass over a catalyst surface to generate hydrogen, carbon monoxide and small organic molecules, such as alkenes. Alternatively, biological systems are known to generate hydrogen. Biohydrogen derives from fermentation processes, but this is typically in low yield because of thermodynamic considerations (Nath and Das, 2004). Perhaps a more attractive possibility is the potential for 'bioeletrolysis' of water driven by photosynthesis. It is known that cyanobacteria and eukaryotic algae generate hydrogen gas. There is consideration of processes that might harvest hydrogen continuously from sunlight-driven biochemical reactions (Surzycki *et al.*, 2007).

Biohydrogen derives from nitrogenase (Einsle *et al.*, 2002), an accidental hydrogen generator, and hydrogenase, an enzyme class evolved to generate or consume

hydrogen (Shima and Thauer, 2007). Nitrogenase evolved to reduce atmospheric nitrogen to ammonia, an impressive six-electron reduction. The iron-molybdenum cofactor is a remarkable catalyst but its selectivity is imperfect; every 14 turnovers, approximately, nitrogenase will reduce protons to make hydrogen gas. This catalytic imperfection is being explored by some investigators for its potential to produce hydrogen biologically (Larimer *et al.*, 2004). Naturally, there are also investigations into using nature's designated hydrogen-generating catalyst, hydrogenase. Hydrogenases typically contain iron-sulfur clusters, some contain nickel and one is devoid of metal or other cofactors. To reduce protons, the redox centres must be low potential and electrons derive from low-potential donors, such as ferredoxins. A major issue with hydrogenase utilization and engineering is their sensitivity to molecular oxygen; in some cases this is due to destruction of the iron-sulfur clusters. Hydrogenase oxygen sensitivity is particularly an issue for photosynthetically powered hydrogen evolution. In this context, hydrogenases are being engineered for oxygen insensitivity (McTavish, 1998; Ghirardi *et al.*, 2005). Another bioengineering challenge is to link photosynthesis directly to hydrogen production rather than carbon fixation, the major biological outcome of photoenergy capture.

Biomass conversion

Regardless of the process or fuel, the source of biomass carbon and hydrogen is an important component of the biofuel equation. Generally, land plants are used to capture solar energy, make carbon molecules and give up the molecules in a transformable state. Ideally, the plants should grow quickly and not require large inputs of chemicals or labour. The most transformable molecules currently are glucose, fructose and starches and thus, commonly used plants are sugarcane, sugar beets and corn. There is currently intensive research to use cellulosic carbon efficiently (Lynd *et al.*, 2005). Cellulose is the most common biopolymer on earth and its efficient utilization would open the door to a much greater variety of biomass resources. One approach is to add cellulase enzymes after mechanically grinding biomass as an initial step of a bioprocess. However, commercial cellulase preparations are currently expensive and only moderately effective. The structure of plant cellulosics is complex, interwoven with lignin polymers and hemicellulose. Most strategies envision using cellulose and hemicellulose sugars following depolymerization, and burning lignin to provide energy for the processing plant. The strong heterogeneous lignin polymer is problematic however, as it blocks access to the usable cellulosic polymers. One emerging approach is to genetically engineer plants to be defective in lignin biosynthesis to make sugars more available and increase fuel yields per unit biomass (Chen and Dixon, 2007).

The use of hemicellulose will require fermentation of xylose and other five-carbon sugars. A more limited number of microorganisms grow on xylose, compared with glucose, and ferment the sugar to a usable fuel-like ethanol. Very recently, the genome sequence of a lignocellulose-converting, xylose-fermenting yeast, *Pichia stipitis*, has been obtained (Jeffries *et al.*, 2007). A concept is emerging that organisms must be used in a comprehensive way to minimize unit operations in a bio-processing plant. There might be benefits in efficiency as well. For example, *Clostridium thermocellum* is an anaerobic, thermophilic bacterium that breaks down cellulose and ferments the resultant sugars to ethanol (Lu *et al.*, 2006). There is an apparent synergistic effect of having the cellulase enzyme complex, the bacterium and the biomass substrate as a tripartite combination to enhance the rate of cellulose hydrolysis. Clearly, more research is needed to better understand these synergistic effects to best employ them for biofuel production. There are excellent reviews on the major issues in biomass conversion (Himmel *et al.*, 2007).

Chemical processing versus microbial biotechnology

Biologists, chemists and engineers alike all believe that bio-based carbohydrates will provide the carbon for the fuels of the future. There is much more uncertainty however about the conversion mechanism(s) and the ultimate fuel(s) that derive from those mechanisms. Along with widespread ongoing research pertaining to microbial biochemistry, there is a parallel line of research investigating chemical catalytic conversion of biomass to fuel molecules (Huber *et al.*, 2006; Schmidt and Dauenhauer, 2007). The overall guiding principles are the same; the molecules generated must be stable, energetic and economically produced. The challenge for chemical processing is to remove most of the oxygen atoms in starting materials such as sugars and ultimately produce molecules optimized for use as a fuel. The traditional chemical catalytic approach has been to break biomolecules down into single-carbon compounds like carbon monoxide and then build up larger molecules from those intermediates. There is a substantial entropic loss of energy in this process leading to inefficiency. Despite this problem, there is an attractiveness to chemical processes because they could, at least in theory, operate quickly and be amenable to large-scale processing.

A very recent study combined some of the best features of biological and chemical processing to generate a novel

biofuel molecule with reasonable efficiency (Roman-Leshkov *et al.*, 2007). In this process, biomass can be treated enzymatically to release glucose that can be isomerized enzymatically to fructose. Using chemical catalysts, fructose deoxygenation is accomplished in two steps. First, there is a dehydration, eliminating three molecules of water to produce 5-hydroxymethylfurfural. The second step removes two more oxygen atoms by hydrogenolysis to produce 2,5-dimethylfuran, the structure of which is shown in Fig. 1. 2,5-Dimethylfuran (DMF) has a research octane number of 119 and a high energy density of 30 kJ cm^{-3}. Moreover, DMF can be hydrogenated using a ruthenium catalyst to generate 2,5-diemthyltetrahydrofuran (DTHF), which has a higher energy content and may prove to be more stable to long-term storage. More research would be needed prior to use of DMF or DTHF as motor fuels, both with respect to engine compatibility and potential toxicological effects on humans and ecosystems.

An alternative approach is the production of synthesis gas (syngas), carbon monoxide and hydrogen, from biomass. Syngas can be converted via Fischer–Tropsch chemistry to form chemical feedstocks and fuels such as linear alkanes (Demirbas, 2007). Syngas can also be converted biologically using a range of organisms (Henstra *et al.*, 2007). There are a growing number of microbes known to oxidize carbon monoxide. Among the most interesting microbes for applications are the carboxydotrophic hydrogenogens that produce carbon dioxide and hydrogen from carbon monoxide. There are also a large class of bacteria containing the acetyl-CoA synthesis pathway involving carbon monoxide (CO) dehydrogenase. CO dehydrogenase condenses carbon monoxide, a methyl group fragment, and coenzyme A; it also catalyses the reversible oxidation of carbon monoxide to carbon dioxide. Bacteria containing CO dehydrogenase produce acetate, ethanol, butyrate and butanol. Metabolic engineering could be used to develop strains generating high titres of single products.

Overall, this research exemplifies the creativity that will be required to use biomass efficiently, using the best of what biology and chemistry have to offer, and generating new molecules for use in vehicular fuel applications.

Biofuels database, internet resources and knowledge integration

Biofuels research is exploding with new researchers bringing widely divergent expertise to the field. Each researcher will generally publish research findings in the scientific journals of their respective disciplines: chemical engineering, chemistry, biochemistry, genomics, plant biology, microbiology. There is a need to bring this divergent research knowledge together in one place. For example, biomass is often plant material; yield and molecular content is very important for feasibility as a fuel feedstock. Additionally, plant material must be processed to fermentation precursors, requiring chemical, biochemical and engineering knowledge. Manipulation of fermentation organisms will require a knowledge of genomics and molecular biology. Researchers working on biological aspects will need to appreciate the chemical and physical properties necessary for an ideal gasoline replacement. The introduction of new biofuels will also be influenced by economic, environmental and social factors.

To help meet these divergent information needs, the University of Minnesota Biofuels Database (UM-BFD) is a freely available web database developed to integrate research across fields and provide easy access to web resources pertaining to biofuels (Fig. 5). The database is highly curated. Links are provided to information that is frequently updated by other databases, for example, gene and protein sequence data. For these and other data as appropriate, the UM-BFD will link to the University of Minnesota Biocatalysis/Biodegradation Database (Bornscheuer, 2001; Leslie, 2005; Ellis *et al.*, 2006). This will also serve to link fuel biosynthesis with biodegradation, allowing users to investigate the impacts of fuel molecules should they spill, as they invariably will, into environmental compartments.

A major issue that the UM-BFD will address is nomenclature pertaining to biofuel molecules. Improper nomenclature impedes the development of clear thinking and new advances (DeTar, 2007). For example, current biodiesel largely consists of methyl esters of fatty acids. This derives from the component alcohol and fatty acids that are most available today but other esters might prove to be superior fuels when tested in engines. For example, the alcohol could be ethanol or butanol. Or the ester bond could be reversed if acetic acid were esterified to a long-chain alcohol. To our knowledge, many of these new combinations could be generated via metabolic engineering, but have never been tested as motor fuels. Depicting these different molecules, in a chemically correct nomenclature, will further people's understanding of what is possible to generate and test as motor fuels.

Synthetic biology in biofuels research – building hydrocarbons

Nature has evolved biochemical pathways and reactions to generate hydrocarbons that could be excellent fuels. The natural evolutionary process is based on selective pressure. Hydrocarbons are used biologically to impart water resistance to duck feathers, to coat fly wings and to dissolve insect toxins (Eisner *et al.*, 2005). The type and amount of hydrocarbon made by each organism arose via changes in the genetic material under the guiding force

Fig. 5. Homepage of the Biofuels Database (http://www.biofuelsdatabase.org).

of selective pressure. Thus, the hydrocarbon(s) produced aided in the survival of the organism. In contrast, the ideal biosynthetic route to a biofuel might consist of a somewhat different hydrocarbon end-product and production in much greater amounts. The tools of synthetic biology could come into play, using nature's building blocks, to engineer superior hydrocarbon fuels in high titre.

The first step in constructing a synthetic pathway is for a human to plan the pathway. There are many different combinatorial pathways that one could envision, using different types of reactions and genes. Envisioning the pathways would require an extensive knowledge of metabolic biochemistry, knowledge that for almost everyone is incomplete at best. Moreover, there might be combinations of reactions that could generate hydrocarbons in theory that have never been used in combination in naturally occurring organisms. Such combinations may not be obvious even to an experienced metabolic biochemist. This planning process could be enhanced by the use of computational tools that search and combine metabolic reaction types to generate novel metabolic pathways.

An *in silico* metabolism prediction tool is now freely available on the world wide web; it is known as the

Pathway Prediction System (PPS) (Hou *et al.*, 2003). The PPS was devised to predict the metabolic breakdown, or biodegradation, of chemicals by microorganisms; its major use is for predicting the fate of chemicals in the environment. Each predicted metabolic reaction is derived from a biotransformation rule. Each biotransformation rule represents a particular metabolic reaction type, for example, the oxidation of an alcohol to an aldehyde. There are a finite number of general metabolic reaction types. The current PPS rule set consists of slightly more than 200 rules. Theoretically, a rule set of several hundred rules could depict any known metabolic transformation and thus represent millions of plausible metabolic pathways, some of which exist in nature and some do not. The current PPS serves as a tool to 'envision' biodegradation pathways that might occur for any given chemical substance that the user enters into the PPS.

It is plausible to use the same metabolic rule base, chemical structure recognition software and output software to predict biosynthetic pathways leading to molecules of interest, including fuel hydrocarbons. The main difference is that the software would be directed to bio-

synthesize molecules, rather than to degrade them. Accomplishing this imposes some new computational challenges. However, there has been a similar logic already developed for computer-aided chemical synthesis, a process named by its developers as 'retro-synthesis' (Corey and Wipke, 1969). In retro-synthesis, a desired end-product is broken apart into fragments that might serve as building blocks. The software provides the synthetic chemist with ideas as to what fragments and reagents can be used to build up the larger molecule, and thus make it as efficiently as possible. In a similar manner, the metabolic logic of the PPS and the chemical construction logic of retro-synthesis could be combined to design molecules for biofuels and other applications.

Acknowledgements

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