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Selection and optimization of microbial hosts for biofuels production

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ABSTRACT

Currently, the predominant microbially produced biofuel is starch- or sugar-derived ethanol. However, ethanol is not an ideal fuel molecule, and lignocellulosic feedstocks are considerably more abundant than both starch and sugar. Thus, many improvements in both the feedstock and the fuel have been proposed. In this paper, we examine the prospects for bioproduction of four second-generation biofuels (*n*-butanol, 2-butanol, terpenoids, or higher lipids) from four feedstocks (sugars and starches, lignocellulosics, syngas, and atmospheric carbon dioxide). The principal obstacle to commercial production of these fuels is that microbial catalysts of robust yields, productivities, and titers have yet to be developed. Suitable microbial hosts for biofuel production must tolerate process stresses such as end-product toxicity and tolerance to fermentation inhibitors in order to achieve high yields and titers. We tested seven fast-growing host organisms for tolerance to production stresses, and discuss several metabolic engineering strategies for the improvement of biofuels production.

1. Metabolic engineering challenges in biofuels production

The creation of liquid transportation fuels from renewable biomass has been a long-standing research goal (Cysewski and Wilke, 1976). Biomass is renewable, and is abundant in places where other liquid fuels, chiefly petroleum and its byproducts, are not readily available (Perlach et al., 2005).

Biomass resources are widely distributed, but often with densities of 0.4 kg/L or lower. The challenge of fuels production from biomass is to liquefy and increase the bulk density of the resource, all while preserving its energy content. The final fuel product should (i) have a high energy density on a mass as well as a volume basis, (ii) be produced at yields near the stoichiometric maximum from a given biomass feed, and (iii) enjoy compatibility with existing fuel distribution infrastructure. No single fuel currently satisfies all of these criteria (Table 1).

Ethanol as made from corn or sugar cane has an acceptable energy density and can be produced from a variety of biomasses at excellent yields. However, ethanol largely fails the requirement for compatibility with existing fuel infrastructure. Gasoline, the predominant liquid fuel in the US, enjoys over 95,000 miles of dedicated pipeline for its distribution (http://www.pipeline101.com/overview/products-pl.html), over 160,000 filling stations (Yergin et al., 2006), usually with underground gasoline storage tanks, and over 240,000,000 heavy- and light-duty passenger vehicles already in use (http://www.pipeline101.com/overview/products-pl.html). Together, this infrastructure is worth hundreds of billions of dollars. Ethanol can cause materials corrosion and draw water into the fuel mixture, properties which render it unsuitable for use in the existing fuel distribution infrastructure. Increased use of ethanol in large quantities may thus necessitate large infrastructure investments.

Other proposed fuels, in particular, biodiesel, butanol, and various terpenoid compounds may be compatible with existing fuels infrastructure, but efficient, high-yielding processes for their production are not yet commercially feasible. The principal barrier to the production of these advanced biofuels is the development of robust, high-yielding microbes and processes for their production.

In this paper, we survey the feedstocks that may be useful for biofuels production, and similarly survey the molecules that have been proposed as second-generation liquid biofuels, and consider how various feedstocks and products influence the choice and optimization of suitable host organisms for biofuels production. Although substantial opportunities for metabolic engineering of higher plants exist (Ragauskas et al., 2006), our focus is limited to examining microbial conversions of biomass to liquid fuels.

2. Biofuels feedstocks and products

Metabolic pathways for the synthesis of most proposed biofuels proceed through common metabolic intermediates such as acetyl-CoA or pyruvate. The pathways can thus be effectively divided into "feed" pathways (Fig. 1a), which convert biomass to

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Table 1
Energy densities, maximum yields, and compatibility with existing fuel distribution infrastructure for various types of biomass and liquid fuels[a]

	Molar energy density (kJ/mol)	Formula mass	Volumetric energy density (MJ/L)	Mass energy density (MJ/kg)	Bulk density (kg/L)	Maximum stoichiometric yield from glucose (kg/kg)	Infrastructure compatibility?
Petrofuels							
Gasoline		100–105	34.7	42.3	0.72–0.78	–	Yes
No. 2 diesel		~200	38.3	45.3	0.80–0.89	–	Yes
Example biofuels feedstocks							
Hybrid poplar wood	–	–	6–7.1	19.38 (dry basis)	0.310–0.370	–	No
Glucose	2803	180.155	24.3	15.6	1.562	–	No
Syngas ($CO+H_2$)	569	30.026		18.95	–	–	No
Candidate biofuels							
Ethanol	1367	46.07	23.6	29.9	0.792	0.511	No
n-Butanol	2676	74.121	29.2	36.1	0.81	0.411	Probable
Vegetable oil (canola)	35390	~887	36.2–36.7	39.8–40.0	0.910–0.917	~0.353	No
Biodiesel (methyl-esterified virgin canola)	12080	~298	35.7	40.6	~0.88	–	Partial and increasing
α-Pinene (representative terpenoid)	6205	136.234	38.9	45.5	0.8539	0.324	Unknown

[a] Data compiled from Balatinecz and Kretschmann (2001), Brown (2003), Davis and Diegel (2007), Henstra et al. (2007), Lide (2006–2007), Shahidi (2005), Thomas (2000), Transportation Energy Data Book: Edition 26, (2007).

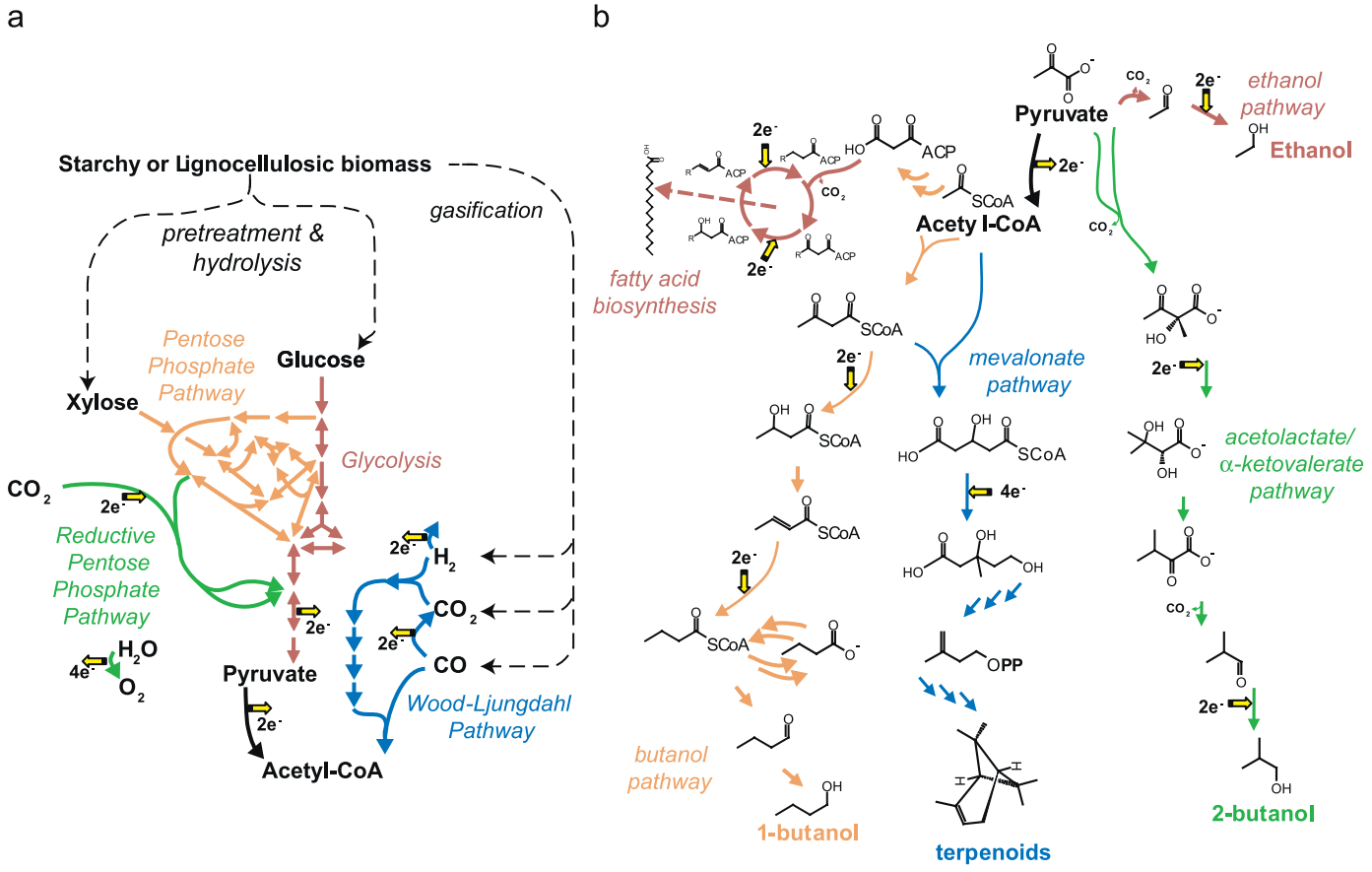

Fig. 1. Pathways for the metabolic conversion of biomass into biofuels can be loosely divided into (a) feed pathways, which convert carbohydrate biomass into the central metabolic intermediates pyruvate and acetyl-CoA; and (b) product pathways, which convert these central intermediates into fuels. Reducing equivalents, shown with yellow arrows, are generated in (a) and consumed in (b). For reasons of clarity and familiarity, molecular structures are not drawn in (a).

the common metabolic intermediates, and "production" pathways, which convert the intermediate to the chosen fuel (Fig. 1b). Generally speaking, the feed pathways create reducing equivalents which are required by production pathways for the synthesis of the biofuel. Consumption of reducing equivalents by other pathways, for example aerobic respiration, is thus undesirable.

2.1. Feedstocks

2.1.1. Starches and simple sugars

Ethanol, today's predominant biofuel, is presently manufactured using feedstocks such as cane-derived sucrose and corn-derived starch. In Brazil, sugar cane juice and sugar cane molasses are used as sources of sucrose. Using *Saccharomyces cerevisiae* and closely related yeast strains as the host, industrial ethanol yields on sucrose are up to 93% of the stoichiometric maximum. Both continuous and batch production is used, with residence times in the fermentors being 6–10 h (da Silva et al., 2005). Production is more than 15 billion gallons (57 billion liters) per year.

In the US, the predominant feedstock is maize. Studies have reported industrial yields in the range of 2.65–2.71 gal per bushel (0.388–0.397 L/kg) of maize, which amounts to approximately 93.6–95.8% of the stoichiometric maximum (Johnson, 2006; McAloon et al., 2000). Presently, industrially attained ethanol titers post-fermentation are around 9% by weight (McAloon et al., 2000), although this figure may increase with improvements in strain ethanol tolerance (see Section 3.2).

The process for production of ethanol from these sources is highly efficient, with feedstock cost generally amounting to 60–80% of the cost of production, although this figure is subject to the usual vagaries of the agricultural commodities markets. The metabolic pathway for starch or sucrose conversion into fuel begins with enzymatic hydrolysis, which yields glucose. The glucose is converted to pyruvate via the familiar pathway of glycolysis (Fig. 1a). Because processes for starch- or sugar-derived ethanol are so efficient, they are a convenient benchmark by which to evaluate other proposed biofuels processes.

2.1.2. Cellulosics

Cellulosic biomass enjoys a much more massive resource base than what is available from maize or sugarcane (Perlach et al., 2005). It is expected to play a dominant role in biofuels production in the near future.

However, cellulosic biomass is more difficult to convert into fermentable sugars than is corn or sugar cane, because (i) five-carbon sugars, mainly xylose, account for 10–25% of the total carbohydrates; (ii) of the presence of lignin, a highly recalcitrant network polymer of aromatic alcohols that accounts for 17–25% of common cellulosic biomasses (van Maris et al., 2006); and (iii) cellulose is much more resistant to hydrolysis than starches and simple oligosaccharides. The first obstacle can be overcome through the selection and/or engineering of microbes capable of the anaerobic fermentation of xylose and other five-carbon sugars to ethanol (see below). These five-carbon sugars are metabolized via the pentose phosphate pathway (Fig. 1a). Enzymatic degradation of lignin is too slow to be practical industrially, but lignin can be productively burned for power production, or gasified for thermochemical conversion.

The third obstacle is the most important. Cellulosic biomass recalcitrance necessitates chemical "pretreatment" of cellulosic biomass to a partially hydrolyzed product that cellulase enzymes can more easily digest. A number of pretreatment variations have been proposed, but many generate fermentation inhibitors (see Section 3.2).

Two main metabolic engineering approaches have long been studied for provision of cellulase and sugar fermentation to ethanol. The first is aimed at genetically modifying *S. cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, or other organisms so that pathways for utilization of five-carbon sugars and for ethanol synthesis exist in the same organism (Ho et al., 1998; Ingram et al., 1999; Mohagheghi et al., 1998). Recently, promising results with *S. cerevisiae* have been reviewed (van Maris et al., 2006). The advantages of *Saccharomyces* are high ethanol tolerance, and the ability to ferment at low pH and forgo medium sterilization. The disadvantage is that *Saccharomyces* (like *E. coli* and *Z. mobilis*) cannot utilize cellulose or derived oligosaccharides directly. This necessitates that a biorefinery install its own dedicated on-site cellulase production system, usually involving the aerobic cultivation of organisms such as *Trichoderma reseei* (Zhang et al., 2006).

The diversion of feedstock to cellulase production, and the expenses incurred by aerobic cell growth (e.g. electricity and capital for air compression and culture agitation) add to the cost of fuel production. A 2000 study estimated that cellulase production accounted for about ~$0.073/kg ($0.22/gal) of ethanol, or about 14%, of the production cost (McAloon et al., 2000) in a *Zymomonas*-based process, in rough agreement with past estimates (Lynd, 1996). The assumed biomass cost in these studies was $35–$42 per dry ton.

Another approach, called consolidated bioprocessing (CBP) and formerly referred to as direct microbial conversion (DMC), uses highly cellulolytic organisms like the thermophilic *Clostridium thermocellum* either exclusively or in co-culture with other thermophilic, higher-producing sugar fermenters (Lynd et al., 2002; Ng et al., 1981). These organisms permit cellulase production, cellulose hydrolysis, and fermentation to occur anaerobically in the same process vessel. Advantages are (i) that the thermophilic nature of these organisms may also obviate requirements for medium sterilization by allowing operation at 60 °C, (ii) most clostridia can efficiently use xylose without recombinant modification, and (iii) the cellulase production and cellulose digestion can occur the same vessel. Disadvantages of CBP are (i) the lower solvent resistance of clostridia (Demain et al., 2005), (ii) comparative difficulty in genetic modification of clostridia, even despite recent developments (Tyurin et al., 2004), and (iii) increased energetic demands for cellulase production in anaerobic environments (Lynd et al., 2002).

Thus, the effect of consolidated processing on effective cellulose conversion costs is unclear. Anaerobic, single-vessel cellulase production may appear to eliminate the tradeoff between resources to dedicated cellulase production and ultimate biomass-to-fuel conversion, but, "an analogous tradeoff exists for single organisms that both produce cellulase and ferment the resulting hydrolysis products" (Lynd, 1996). CBP may have the potential to lower cellulose conversion costs, but to be realized, the development of strains and processes which dramatically increase rates of anaerobic cellulose conversion must be developed (Lynd et al., 2002).

2.1.3. Syngas

Syngas, sometimes called producer gas, is a mixture of carbon monoxide, hydrogen, and carbon dioxide. It can be prepared easily from biomass (or fossil fuels) by treatments at high temperature in the absence of oxygen (Sutton et al., 2001). Syngas can serve as the sole carbon and energy source for a variety of microorganisms, including ethanol and butanol producers. Carbon monoxide is oxidized to carbon dioxide by water via carbon monoxide dehydrogenase (CODHs). This reaction provides reducing power for anabolic CO metabolism via the Wood–Ljungdahl pathway (Ragsdale, 2004), where CO is converted to acetyl-CoA via acetyl-CoA synthetase (ACS) enzyme (Fig. 1a).

Hydrogen can be metabolized by many of the same organisms which metabolize carbon monoxide. In some cases, however, hydrogen metabolism may be inhibited by carbon monoxide itself, or by other trace syngas components such as nitric oxide (Ahmed et al., 2006; Ragsdale, 2004). Increasing the tolerance of microbes to syngas-associated stresses is an intriguing possibility, but approaches are just beginning to be studied.

Nonetheless, a variety of clostridia such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, and *Clostridium carboxidivorans* can simultaneously utilize both of these substrates and overproduce ethanol, butanol, and/or acetate (Rajagopalan et al., 2002; Sipma et al., 2006). For example, Lewis and his co-workers (Datar et al., 2004; Rajagopalan et al., 2002) have reported ethanol yields on CO of 45% the theoretical, with productivities in the neighborhood of 1 g/L/day and titers of up to 3 g/L.

Relative to synthesis of fuels from syngas via heterogeneous catalysis (Phillips et al., 2007), fermentation-based processes may (i) be operated at lower pressures, (ii) enjoy superior specificity for the desired products, and (iii) are likely to be more tolerant of sulfur impurities in syngas than traditional heterogeneous catalysts.

2.1.4. Light and carbon dioxide

Photosynthetic organisms tap the energy in light to generate reducing equivalents from water, thus allowing fixation and use of carbon dioxide as a growth substrate. The pathway for incorporation of CO_2 is through the reductive pentose phosphate pathway, also known as the Calvin cycle. The enzyme Rubisco catalyzes the conversion of CO_2 and ribulose-5-phosphate to two trioses, which can subsequently be converted to pyruvate (Fig. 1a).

Biomass from microbial photosynthesis is being explored for biofuels production. Compared to higher plants, microbial photosynthesizers can be genetically modified more easily, and also enjoy faster life cycles and rapid growth.

Maximum attainable cell densities and productivities are important determinants of the cost of algal biomass. Generally, cell densities in outdoor algae ponds reach no more than 0.5–1.0 g/L of dry cell weight, even when supplying highly enriched sources of carbon dioxide (such as the flue gas of a coal or natural gas-fired power plant). This necessitates mobilization of considerable land and water resources for algal culture. Cost estimates made in the 1980s by a Department of Energy program for algal biomass grown in large-scale ponds was \$0.19–\$0.41/kg of biomass (unadjusted for inflation) (Sheehan et al., 1998).

An alternative is to use photobioreactors for growth. Algal photobioreactors can obtain cell densities near 20 g/L (Lee, 2001), but estimates of the cost of algal biomass grown in closed bioreactor systems have been up to \$12–\$32/kg dry weight (Grima et al., 2003; Lee, 2001), indicating that these systems are likely far more costly than open ponds. Various elaborate modulations of the intensity and the spatial and temporal distribution of the incident light flux have been proposed (Gordon and Polle, 2007; Ono and Cuello, 2004) as methods for increasing productivities.

A US Department of Energy review of its extensive research program on algal biomass production in the 1970s and 1980s details the isolation of several promising algal strains, as well as methods for genetic transformation of algae, and improvement of lipid yields (Sheehan et al., 1998). Representative productivities in outdoor pond systems were 15–20 $g/m^2/d$. Lipid contents of ~40% (w/w) and specific growth rates of ~2 d^{-1} were representative. More recently, robust genetic methods have been reported for *Chlamydomonas reinhardtii*, *Chlorella* subspecies, and other algae (Coll, 2006; Stevens and Purton, 1997).

Algal biomass may become an attractive option for fuels production if metabolic engineering can boost productivities, photosynthetic efficiencies, or lipid content dramatically over historical levels, or if carbon taxes or emissions caps necessitate CO_2 capture from flue gas emissions from fossil fuel power plants.

2.2. Products

2.2.1. Ethanol

Ethanol is produced from pyruvate in two steps: pyruvate decarboxylase converts pyruvate to acetaldehyde, and alcohol dehydrogenase reduces the acetaldehyde to ethanol (Fig. 1b). This pathway is commonly exploited in *Saccharomyces* yeast hosts, or in the Gram-negative gamma-proteobacteria *Z. mobilis* or recombinant *E. coli*.

The maximum attainable ethanol concentration depends on the host and medium composition, but is usually between 4 and 16 wt%, depending on the host. Processes for the distillation of ethanol from dilute fermentation broth were known even in ancient Egypt (Stichlmair, 2000), but distillative separation of ethanol from the fermentation broth requires considerable energy input. To provide a basis for comparison with other fuels without specifying separation process specifics, we estimated the reversible work of separation for ethanol and several other fuels from an aqueous mixture as a function of concentration (Fig. 2; full description of calculation methods are in the supplemental materials available online). For reference, energy input required to separate ~9 wt% ethanol from water is about 5 MJ/kg for corn ethanol in the US (Johnson, 2006). (This implies that distillation operates at about 5% of the thermodynamically maximal efficiency.)

Distillation represents a higher percentage of the cost of production for corn ethanol than for cellulosic ethanol, because feedstock pretreatment and cellulase production still dominate projected costs for cellulosic ethanol (see Section 2.1.2 and references therein).

2.2.2. Butanol

n-Butanol is a fermentation product of *Clostridium acetobutylicum* and *Clostridium bjerinkci* (Ezeji et al., 2007b). It is produced from acetyl-CoA through the dimerization of two acetyl-CoAs into acetoacetyl-CoA. Subsequent enzymes catalyze the four-electron reduction and dehydration of acetoacetyl-CoA to butyric acid. The butyrate can then be re-metabolized by some clostridial species via a further four-electron reduction to *n*-butanol. In clostridia, acetone (not shown in Fig. 1b) is often produced concomitantly with butanol by decarboxylation of acetoacetyl-CoA.

The total pathway from glucose to butanol is, in principle, redox balanced. However, in some organisms, including *C. acetobutylicum* and *E. coli*, hydrogen is produced via formate-hydrogen lyase (Liu et al., 2006) and represents a competing outlet for reducing equivalents. In principle, hydrogenase activity or alternate formate dehydrogenases (Sanchez et al., 2005) could recover these reducing equivalents. The clostridial pathway has been functionally expressed in recombinant *E. coli* hosts, although

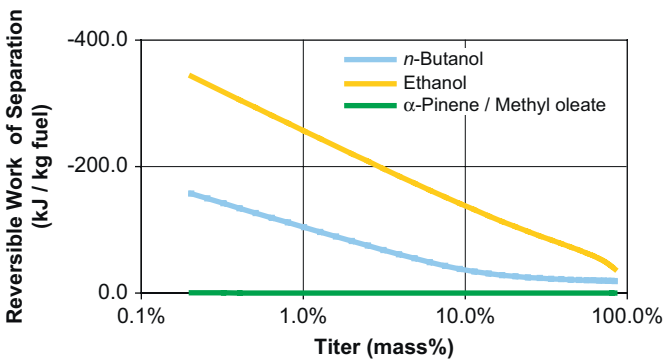

Fig. 2. The reversible work of separation of for ethanol, butanol, alpha-pinene, or methyl oleate (biodiesel) from aqueous solution as a function of titer.

the observed productivity fell short of what was observed for clostridia (Atsumi et al., 2008).

An alternative approach to butanol production that starts from pyruvate instead of acetyl-CoA has been proposed in the patent literature (Donaldson et al., 2007). Decarboxylative dimerization of pyruvate to acetolactate is followed by reduction to α-ketovalerate, decarboxylation, and further reduction to butanol. The enzymes composing this pathway are not usually found in the same organism. Notably, this pathway results in production of the 2-butanol isomer. Relative to 1-butanol, the 2-butanol isomer may be preferred as a fuel.

Butanol has a considerably lower reversible work of separation from water than ethanol, which implies that its separation from fermentation broth will be considerably more energy efficient (Fig. 2). However, it is far more toxic to most microorganisms. In clostridia, 13 g/L has been reported as a usual *n*-butanol titer (Ezeji et al., 2004). Butanol yields on glucose of 0.76 mol/mol and productivities of 0.76 g/L/h have been reported for clostridial cultures sparged with hydrogen (Ezeji et al., 2004).

2.2.3. Higher lipids

Acetyl-CoA is the ultimate source of carbon for fatty acid biosynthesis. In many organisms, long-chain fatty acids biosynthesized through the ATP-requiring carboxylation to malonyl-CoA, followed by cycles of decarboxylative addition of malonyl-CoA to acyl units and *β*-reduction (Fig. 1b). Long-chain fatty acids, as methyl esters (Zhang et al., 2003) or decarboxylated derivatives (Maki-Arvela et al., 2007) are attractive as renewable substitutes for diesel fuel.

Oils from higher plants are also synthesized in this way. Easily extracted oils from palm trees and soybeans can be converted to biodiesel and are widely used for biofuels production. The obstacle that has stopped wider adoption of this technology is the insufficient availability of low-cost feedstocks (Hill et al., 2006). Nonetheless, about 0.225 billion gallons (0.805 billion liters) were produced in the United States in 2006. Like corn ethanol, government subsidies for biodiesel production are currently required to sustain commercial interest in these technologies.

Lipid production by algae has the potential to get around current biodiesel feedstock limitations (Chisti, 2007; Hankamer et al., 2007). Improving lipid production by algae has been an important metabolic engineering goal for many years, and remains so today (see Section 2.1.4). More recently, interest has developed in the generation of biofuels via lipogenic (or oleaginous) yeasts and other microbes (Voelker and Davies, 1994). This technology would allow microbial conversion of cellulosic materials to lipids.

Lipids do not mix with water, and as such, the reversible work of separation from aqueous solution is near zero. One drawback may be that lipids are often accumulated intracellularly, which could require extraction of the lipids from crude cell pastes (Grima et al., 2003). Separation of the lipids from undesirable biomass components, perhaps by solvent extraction or other means, will require its own energy inputs. An alternative metabolic engineering strategy is to engineer secretion of the lipid products.

Many details of fatty acid secretion even in model organisms like *E. coli* and *S. cerevisiae* are poorly understood. These areas may be attractive targets for metabolic engineering.

2.2.4. Terpenoids

Isoprenoids are a broad class of metabolites synthesized from isoprenyl pyrophosphate (IPPP), the pyrophosphate ester of 3-methylbut-3-en-1-ol, or its isomer dimethylallyl pyrophosphate (DMAP), the pyrophosphate ester of 3-methylbut-2-en-1-ol. These molecules are synthesized either from glyceraldehyde-3-phosphate and pyruvate via the methylerythritol pathway (omitted from Fig. 1b), or from acetyl-CoA via the mevalonate pathway (Fig. 1b).

IPPP and DMAP can be dimerized or polymerized to an astonishing array of olefinic hydrocarbons or their alcohol derivatives. For fuel applications, water-insoluble liquid products such as the hemiterpenoid alcohol 3-methylbut-2-en-1-ol (prenol) (Withers et al., 2007), or perhaps monoterpene and sesquiterpene hydrocarbons such as limonene, pinene, or cadinene, may be preferred targets.

Strains and processes capable of converting sugars to terpenoids at yields similar to the ethanol process have not yet been reported in the scientific literature. For example, a representative titer for the sesquiterpene hydrocarbon amorphadiene has been reported as about 0.5 g/L (Ro et al., 2006).

Terpenoid production has usually been examined only under aerobic culture conditions, but for large-scale, high-yield production, anaerobic processes are desired. Obviation of the oxygen requirement for terpenoid overproduction is one key metabolic engineering objective for biofuels applications of this pathway.

2.3. Requirements for yield, titer, and productivity

Ultimately, any host organism for biofuels production must perform at high yield, because of the significant cost for feedstock. Years of research has revealed the enzymological and genetic details of metabolic pathways for ethanol and butanol fermentation, and allowed their functional pathway expression in convenient host organisms (Atsumi et al., 2008; Ingram et al., 1999). Carbon monoxide dehydrogenase has been functionally expressed in recombinant *E. coli* (Loke et al., 2000), suggesting that research to develop recombinant syngas fermenting organisms may also be attractive.

Equally important, comprehensive stoichiometric models of metabolism permit the design of efficient genetic backgrounds for fuel production (Alper et al., 2005b; Fong et al., 2003; Ibarra et al., 2002). These models can also be used to design genetic backgrounds in which biomass yield is positively correlated with the desired product yield, allowing simple evolutionary strategies to be used to optimize the yield of the desired product. We conjecture, therefore, that of yield, productivity, and titer, the yield of a given pathway for fuel production may be the most amenable to traditional metabolic engineering strategies for strain optimization.

However, a similarly diverse and effective array of techniques is not available to optimize productivities, or, in particular, titers. Mechanisms for dealing with the stresses associated with biofuels production, such as osmotic stress, membrane disruption, and the presence of various toxins appear to be host-specific and often poorly understood (Cakar et al., 2005; Taherzadeh et al., 1997a). The selection of a fitting host organism is thus essential for the development of biofuels production processes.

3. Biofuels hosts

3.1. Desirable properties

Finding or constructing an optimal host for biofuel production is the most obvious requisite of any metabolic engineering effort, but is far from trivial. In this context, the ideal host would degrade lignocellulosic components, ferment the resulting sugars (both hexoses and pentoses) at high rates and with high yields,

and tolerate high titers of the end-product at high temperatures and extreme pH (to avoid cooling and sterilization costs). The fact that biodiversity is remarkable, especially for microorganisms, may suggest that an ideal host for biofuel production already exists, so that the real challenge is finding it. Since there is no clear path for solving this needle-in-a-haystack problem, most researchers have opted to tackle the design problem of combining desirable characteristics into an engineered host using recombinant DNA technology. Any such effort usually entails the alteration of phenotypes dictated by multiple genes. The systematic implementation of this approach requires substantial knowledge of the host to be modified, which has favored the use of "laboratory strains" that are research-friendly, but not necessarily robust enough for industrial applications.

The characteristics that have made laboratory strains like *E. coli*, *S. cerevisiae*, and *Bacillus subtilis* so popular are also to a large extent those that will be needed for the development of industrially relevant production strains. The first requisite is genetic competence, the ability of a strain to accept foreign DNA in a controllable fashion. High transformation efficiencies are desirable, especially for the successful use of combinatorial libraries for screening genes or gene variants that confer a particular phenotype of interest (see discussion in following section). Even though transformation protocols have been used for a long time, they tend to be host-specific and based on empirical observations rather than on fundamental principles. Furthermore, their success seems to depend on a variety of factors such as the activity of host restriction and DNA-modification systems (Alegre et al., 2004; Matsushima et al., 1989), genetic background (Umemoto et al., 1996), origin of replication and marker of the vector (Aukrust et al., 1995), to name a few. A second trait that makes laboratory strains attractive is the availability of well-characterized metabolic engineering "modules" that allow manipulation of the genotype in different ways. Examples include promoters of various strengths (Alper et al., 2005a), termination sequences, repressor-inducer systems, plasmids (with known copy number, replication mechanism, compatibility with other plasmids, etc.), chromosome integration cassettes for building knockouts or stable replication of genes, etc. A third feature is the availability of "omics" platforms and algorithms for genome-wide characterization of cellular responses to different manipulations and environments (microarrays, metabolic network models, etc.). A fourth, and most understated feature of all, is the great amount of accumulated knowledge on the physiology of laboratory strains provided by generations of researchers.

The need for host strains that are "domesticable" and predictable arises also from overall process considerations. Understanding the relationship between growth phase and product formation, as well as the kinetics of both is essential for process design. For example, solventogenesis in *C. acetobutylicum* is a stationary-phase phenotype controlled by global transcriptional regulators that respond to different cues (Ravagnani et al., 2000; Wilkinson et al., 1995), knowledge of which should be considered in the implementation of butanol fermentations. Another important aspect associated with any large-scale fermentation is the cost of maintaining sterile conditions and the possibility of contamination. The use of antibiotics is undesirable not only for economic reasons, but also for the development of resistance. Running fermentations at low pH has been an effective measure in this regard, but requires acid-tolerant hosts. High temperature has also been used when the biotransformation is carried by a thermophilic microorganism (Sakai and Ezaki, 2006). However, the microorganism that produces the compound of interest most efficiently (i.e. with the highest productivity, titer, yield from cellulose, etc.), is rarely also highly tolerant to acid, heat, or similar restrictive environments. Because environmental tolerance is generally a phenotype dictated by the expression of many genes, those traits are hard to engineer or introduce into a desired host. For this reason, strains that are easy to manipulate and are well characterized are good starting points for the development of production platforms.

3.2. Strain improvement

Improving environmental tolerance has been an active area of study because maximum productivity and titer are key determinants for the profitable fermentative production of commodity chemicals. Creative reactor or process design strategies have been successfully implemented to ameliorate product toxicity problems (Ezeji et al., 2007b), but such approaches are mostly complemented by strain engineering. In general, genetic changes for strain improvement may be of two types: rational (or directed) and random (or combinatorial). The first is straightforward when a genetic sequence that is likely to impact a trait of interest is known. The second is preferred when this knowledge is lacking, and relies in our ability to construct genetically diverse populations and screen them.

Random approaches have gained special attention, because detailed physiological mechanisms of tolerance remain widely uncharacterized. Traditionally, these efforts have been focused on long-term adaptation or serial rounds of mutagenesis and selection, usually referred to as "classical strain improvement" (CSI). The mutagenesis step is accomplished with chemical or physical mutagens (e.g. nitrosoguanidine and UV, respectively), producing libraries of variants that can be then screened for improvement in tolerance. A clear limitation of this approach is the non-transferability and intractability of the resulting genotypic changes. More recently, a variety of random-search (i.e. library-based) methods for strain improvement have emerged that are based on phenotypic alteration using tractable elements. These include artificial transcription factors (Park et al., 2003), global transcription machinery engineering (Alper et al., 2006; Alper and Stephanopoulos, 2007), libraries of siRNAs (Berns et al., 2004), randomized ribozymes (Miyagishi et al., 2005), knockout and overexpression libraries (Jin and Stephanopoulos, 2007), and others.

Although obvious, the fact that any genetic manipulation results in changes to an existing, functional genome is sometimes ignored when pursuing a strain improvement effort. This fact implies that the type of changes resulting in improvement depend on the genetic background of the host strain. As such, there is a limit for improvement defined by the maximum number of changes that can be implemented experimentally. Let us take the engineering of thermotolerance as an example: even if chaperones and proteases are overexpressed, thermophilic versions of key enzymes are introduced, membrane properties are modified, etc., the makeup of an organism that makes it tolerant to heat is a property of the system in its entirety, and resists simplification. This does not imply that tolerance cannot be improved with respect to that of the wild-type, but that the practical relevance of the limit for improvement depends on the phenotype we choose to engineer. Therefore, the prospects of finding an ideal strain for biofuel production reside on a balance between selecting the right host as a starting point and choosing which properties to change.

This fact is underscored by the data in Fig. 3, which shows that the tolerance of commonly used mesophilic, facultatively anaerobic biofuels production hosts to two stresses. *B. subtilis* beats the other six hosts by a large margin in the case of tolerance to 1-butanol, but is very sensitive to the fermentation inhibitors in dilute-acid pretreated (Schell et al., 2003), quicklime-neutralized corn stover hydrolysate (CSH). (Details of the CSH material are

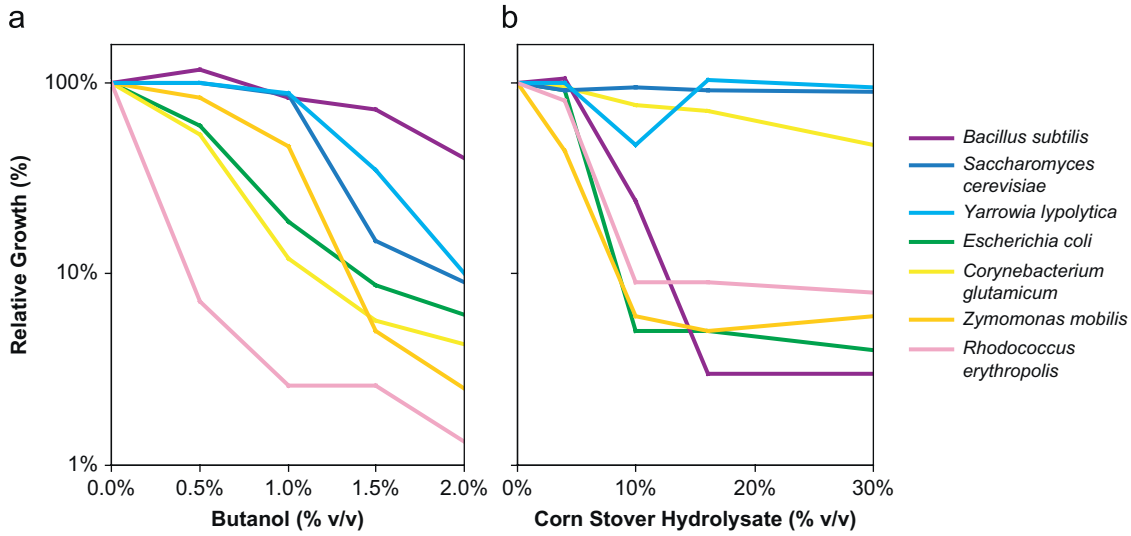

Fig. 3. The tolerance of seven common mesophilic, facultatively anaerobic host organisms to the stresses of (a) butanol tolerance, or (b) tolerance to neutralized corn stover hydrolysate. See supplemental material for full experimental details.

available in the supplemental materials.) Conversely, *Corynebacterium glutamicum* is comparatively much more tolerant to the CSH than to butanol. The yeasts *S. cerevisiae* and *Yarrowia lypolytica* are reasonably tolerant to both of these stresses. Data like that in Fig. 3 are useful for qualitatively comparing the different hosts. But, without tailoring temperatures, growth media, and, in the case of CSH, pretreatment and neutralization conditions to each particular host, it is difficult to extrapolate such data to process conditions.

3.3. Engineering environmental tolerance to common process stresses

Environmental stresses come in many disparate forms, only some of which have been reasonably well-studied. Heat tolerance has received significant attention, as running fermentations at higher temperatures curbs cooling costs and reduces the risk of contamination. Heat increases the fluidity of the membrane (Balogh et al., 2005; Mansilla et al., 2004; Shigapova et al., 2005), which in turn interferes with energy transduction and pH maintenance (Sikkema et al., 1995). In addition, it causes protein denaturation and aggregation, directly affecting most cellular functions (Riezman, 2004). Thermotolerance has been enhanced both by rational and random schemes. Overexpression of heat shock protein hsp22.4 (from *Chaetomium globosum*) in *S. cerevisiae* increased cell viability after a 4-h shift to 51C (Liu et al., 2007). This approach has been extended to other organisms, from bacteria (Fiocco et al., 2007) to very complex multicellular systems (Feder et al., 1996; Welte et al., 1993). Thermotolerance has also been improved by preconditioning (Shigapova et al., 2005), overproduction of metabolites such as trehalose (Purvis et al., 2005), and supplementation with protectants such as betaine and choline (Holtmann and Bremer, 2004). These three and other compounds act similarly by altering the osmolarity of the cytoplasm, changing the water–protein interactions, stabilizing them and preventing aggregation (Cayley and Record, 2003; Conlin and Nelson, 2007; Ignatova and Gierasch, 2006).

Combinatorial approaches have also been successful. For example, subjecting a mutagenized *Saccharomyces* population to serial freeze–thaw cycles followed by selection has delivered mutants with increased tolerance to multiple stresses, including heat (Cakar et al., 2005). Screening of artificial transcription factor libraries has been used to engineer thermotolerance in *S. cerevisiae* (Park et al., 2003) and to determine a gene responsible for this phenotypic alteration in *E. coli* (Park et al., 2005).

Osmotolerance has also been studied as a target for strain improvement, as high substrate, product or salt concentrations that increase osmotic pressure are commonly encountered in industrial fermentations (Thatipamala et al., 1992; Varela et al., 2004). Some of the same strategies outlined for enhancing thermotolerance can be or have been applied. For example, trehalose and betaine have been reported to increase osmotolerance in *E. coli* (Miller and Ingram, 2007), and the protective role of trehalose in yeast has been reported (Hounsa et al., 1998). Transcriptional engineering has delivered yeast strains improved for growth in high salt (Park et al., 2003) and glucose (Alper et al., 2006). Similarly, this method has improved the tolerance of *E. coli* to a variety of stresses, as well as improved metabolite overproduction (Alper and Stephanopoulos, 2007).

3.4. Biofuels-specific stresses

The problem of environmental tolerance is not only a challenge for fermentation technology in general, but is particularly relevant to the production of biofuels for two reasons. First, when the production strain cannot degrade lignocellulosic material directly, the substrate for fermentation may contain toxic compounds. This has been widely discussed for feedstocks derived from lignocellulosic hydrolysates, as pretreatment by acid hydrolysis produces a mixture of oligosaccharides, organic acids, phenolic derivatives, and furans (Sakai et al., 2007), all but the first of which are inhibitors of growth for many microorganisms. Second, the product of fermentation may itself be toxic, in which case the titer could be inherently limited. A classic example of this is ethanol production in *Saccharomyces* (Alper et al., 2006).

Lignocellulosic derivatives that have received most attention are furfural, hydroxymethyl furfural (HMF), acetic acid, and phenolic compounds. The amount and identity of the inhibitors after detoxification of hydrolysates depend on the method used (overliming, laccase treatment, charcoal, etc.) (Klinke et al., 2004). Although toxicity and detoxification issues have been mostly explored for ethanol, some studies for other fermentations such as butanol exist (Ezeji et al., 2007a). Because different compounds exert toxicity through different mechanisms and their effects

appear to be coupled (Ezeji et al., 2007a; Klinke et al., 2004; Taherzadeh et al., 1997a), solving the problem of environmental tolerance to the substrate cocktail by rational approaches and simple process modifications seems unlikely. However, partial successes from this front have been reported. Overexpression of ADH6 in *S. cerevisiae*, an 5-HMF-reducing enzyme, has enhanced conversion of the inhibitor which could be probably used in detoxification, but no increase in ethanol productivity was reported (Petersson et al., 2006). A similar effort with ZWF1, encoding a glucose-6-phosphate dehydrogenase, resulted in higher furfural tolerance (Gorsich et al., 2006). Simultaneous overexpression of the genes would probably be synergistic, as the reduction of the inhibitor by ADH6 is NADPH-dependent, and ZWF1 is hypothesized to help this step by committing its substrate to the pentose-phosphate pathway, which produces the reduced cofactor. Overexpression of the enzyme phenylacrylic acid decarboxylase (from PAD1) resulted in *Saccharomyces* strains improved in ethanol productivity in the presence of ferulic and cinnamic acids (Larsson et al., 2001).

Manipulation of the fermentation pH has been used for alleviating tolerance to acetic acid, as toxicity is mainly effected by the protonated species (Lawford and Rousseau, 1998; Taherzadeh et al., 1997b). However, pH control is undesirable because of the additional cost associated with it, and because low pH reduces the risk of contamination as discussed above. Transferring a gene of acid-resistant *Oenococcus oeni* that responds to different stresses resulted in an *E. coli* strain with improved low pH tolerance (Morel et al., 2001). These or similarly constructed hosts may be better suited for fermentations at low pH.

Improvements from random approaches have also been reported. Genome shuffling of ethanologenic *Candida krusei* has delivered acetic acid-resistant mutants that perform better than the parent in ethanol fermentations in the presence of the inhibitor (Wei et al., 2008). The usefulness of CSI methods for improving tolerance of yeasts to lignocellulosic hydrolysate components has also been reported (Liu et al., 2005; Sonderegger et al., 2004). Similarly, studies describe that *Pichia stipitis* long-term adapted to increasing concentrations of hardwood hydrolasate partially neutralized or alkalinized with lime had higher ethanol productivity and titer (Nigam, 2001a, b).

Product inhibition has also been addressed, mainly for yeast ethanol fermentations. Most fuels have solvent-like properties, and are thought to impact the cell physiology similarly. In general, solvents and other lipophilic hydrocarbons partition into the membrane and disrupt its fluidity, which in turn results in ion leakage (Graca da Silveira et al., 2002; Ingram and Vreeland, 1980). Similar to the case of heat, this increase in membrane fluidity causes dissipation of the transmembrane pH and leads to an energy shortage, which ultimately results in cessation of growth. The harmful effect of membrane fluidity is therefore accentuated at high temperature and also in the presence of feedstock-derived inhibitors (Demain et al., 2005; Klinke et al., 2004), which results in an especially challenging problem.

Many ethanol-tolerant yeast strains have been independently isolated in the context of alcoholic beverage production, through hundreds of years of artificial trait selection. These are usually poorly characterized, but application of modern techniques has helped in the elucidation of possible ethanol-tolerance mechanisms in these strains (Shobayashi et al., 2007; Watanabe et al., 2007). Following a comparison of sake-brewing and laboratory strains using microarrays, overexpression of tryptophan-biosynthesis genes (TRP1-5) resulted in enhanced ethanol tolerance (Hirasawa et al., 2007). The mechanism behind this phenotype seems to be similar to that of strains with higher levels of trehalose (Kim et al., 1996), and for the same reasons outlined for thermotolerance.

The complexity of fuel/solvent tolerant phenotypes has invited the use of random approaches. A mutant of the TATA-binding protein, coded by SPT15, increased yeast tolerance to high-glucose, high-ethanol conditions through the alteration in expression of hundreds of genes (Alper et al., 2006). The improved phenotype could not be recovered from localized changes in gene expression, which is in tune with the complex nature of the stresses and underlines the need for *global* cellular engineering for strain improvement. A similar effort in *E. coli* delivered a mutant sigma factor with improved ethanol tolerance (Alper and Stephanopoulos, 2007). Overexpression of two genes in *C. acetobutylicum* that were found by screening a genomic DNA library resulted in increased butanol tolerance (Borden and Papoutsakis, 2007). Other localized genetic manipulations have been explored, like null mutations ura7 and gal6 that were selected by challenging a knockout library of *Saccharomyces* in ethanol (Yazawa et al., 2007). The mutant strains grew and consumed glucose faster in the presence of 8% ethanol compared to the wild-type, through changes in membrane lipid composition and other mechanisms that were not fully understood. CSI programs have also delivered ethanol-tolerant *E. coli* (Yomano et al., 1998) and butanol-tolerant *C. beijerinckii* mutants (Annous and Blaschek, 1991; Qureshi and Blaschek, 2001).

Even with the substantial research interest that this problem has attracted, environmental tolerance for biofuel fermentations continues to be a challenge. A main reason for *Saccharomyces* fermentations of ethanol being traditionally preferred is that this microorganism has been bred to precisely this end for hundreds of generations. Artificial evolution has already solved many of the limitations, but it has not had time to solve others (like tolerance to lignocellulosic hydrolysates) that are of practical significance today. Rational genetic manipulations based on "omics" data will likely be part of the answer, but random methods will also play a central role. Given that the genotypic space is practically infinitely large, better and more efficient ways of searching the phenotypic space are needed. Such research will contribute to the improvement of industrial strains rapidly enough to face the challenges of liquid fuel shortages.

4. Conclusions

Today's predominant microbially produced biofuel is starch-derived ethanol. However, further expansion of production capacity will require use of lignocellulosic feedstocks. The two best-developed technologies for conversion of lignocellulosics to fuel are (i) pretreatment and enzymatic hydrolysis to fermentable sugars, or (ii) gasification to syngas. Microbial photosynthetic processes may also render carbon dioxide as an attractive biomass feedstock.

An additional and orthogonal problem with starch-derived ethanol is the chemical properties of ethanol itself. Other fuel molecules, such as butanol, or liquid hydrocarbons of lipid or terpenoid origin, have higher energy densities, mix less readily with water, can be easier to separate from fermentation broth, and are compatible with the existing fuel distribution infrastructure. A primary barrier to the introduction of these technologies is insufficient microbe performance. That is, metabolic engineering research to improve the yield, titer, and productivity of microbial strains will be essential to the development of later-generation biofuels. We conjecture that yield may be the easiest of the trifecta to engineer, because the experimental tools for pathway manipulation, and the metabolic models required for evolutionary strain optimization are already in place. However, a similar toolset for improvement in productivity and titer is not available; in many cases, regulatory pathways governing are incompletely

understood. Further elucidation of these pathways is a much-needed avenue for future research.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.06.009.

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