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Abstract	The need for renewable alternative sources of liquid biofuels has lead to tremendous interest in the conversion of lignocellulosic biomass to fuel compounds via microbial routes. A key aspect of the research involves the engineering of robust and stable microbial host platforms that can produce these compounds at high titer. Impact on growth caused by inhibitory compounds in the deconstructed biomass and accumulation of toxic metabolic intermediates and final product are bottlenecks that severely limit product titers. This chapter reviews known sources of toxicity arising from various aspects of this process and discusses native and heterologous mechanisms of microbial stress response and defense that can be used to engineer better production hosts.

Control of Stress Tolerance in Bacterial Host Organisms for Bioproduction of Fuels

Aindrila Mukhopadhyay, Nathan J. Hillson, and Jay D. Keasling

Contents

Abstract The need for renewable alternative sources of liquid biofuels has lead to tremendous interest in the conversion of lignocellulosic biomass to fuel compounds via microbial routes. A key aspect of the research involves the engineering of robust and stable microbial host platforms that can produce these compounds at high titer. Impact on growth caused by inhibitory compounds in the deconstructed biomass and accumulation of toxic metabolic intermediates and final product are bottlenecks that severely limit product titers. This chapter reviews known sources of toxicity arising from various aspects of this process and discusses native and heterologous mechanisms of microbial stress response and defense that can be used to engine better production hosts.

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1 Introduction

Microorganisms have been engineered to produce an astonishingly large array of compounds ranging from high value pharmaceuticals, fragrances, and nutritional supplements to fine chemicals such as amino acids, solvents, and building blocks for paints, plastics, and polymers (Wackett 2008; Fortman et al. 2008; Klein-Marcuschamer et al. 2007). While the majority of research focuses on the development of optimal biosynthetic enzymes and pathways to convert selected carbon sources to target end products, the recent focus of microbial metabolic engineering on the production of bulk commodities, specifically biofuels and compounds otherwise derived from petrochemical sources (Burk 2010; Ryder 2009; Steen et al. 2010; Atsumi et al. 2008), has imposed the staggering additional challenge of maximizing production. Pursuing sustainable, ecologically friendly bio-routes remains important, but a key metric of success in the microbial production of biofuel compounds is reaching high production levels at minimal cost to compete with inexpensive petrochemical and synthetic methods. Several analyses have emphasized this overarching requirement of high biofuel production levels (Hill et al. 2006). For example, while typical production levels of n-butanol with clostridium strains are about 13 g/L, optimization to increase production to 19 g/L was required to make this process economically viable (Papoutsakis 2008). With ever-higher levels of production and the use of minimally processed biomass, other aspects of microbial cellular physiology become acutely significant (Zhang et al. 2009). Growth inhibitory factors from deconstructed lignocellulosic biomass, as well as the accumulation of toxic intermediates in the biosynthetic pathway and the final product itself, can limit production. Cellular engineering efforts must therefore shift to developing microbes that cope with growth inhibition, toxicity, and stress. Studies of microbial stress response toward these inhibitory factors are key to elucidating the mechanisms that may be utilized to generate a robust industrial host that can cope with all aspects of growth and production inhibition. Furthermore, microbial diversity, both in the form of the ever-growing repository of sequenced genomes as well as bio-prospecting new ecosystems, contains an immense potential to provide the mechanisms required to tolerate a range of inhibitory aspects presented by this biofuel production pipeline. This chapter describes commonly encountered inhibitors and toxic factors generated during the conversion of lignocellulosic material to biofuel, the corresponding mechanisms that can be brought to bear on stress mitigation, and the strategies to overcome current limitations in obtaining stable, engineered hosts for industrial use. While applicable to all microbial hosts used for large-scale production of compounds from deconstructed biomass, including *S. cerevisiae*, this chapter focuses on bacterial systems as the production host.

2 Sources of Microbial Stress in Deconstructed Biomass

Lignocellulosic biomass presents the most promising renewable source of feed for the production of liquid biofuels (Ragauskas et al. 2006). Plant biomass is largely comprised of cellulose, hemicellulose and lignin (Somerville et al. 2004). While it would be ideal to use all components of this available material, the present goal of biofuel programs is to maximize the use of sugar polymers, cellulose, and hemicellulose. Despite the focus on these sugar polymers, most downstream biological processes (saccharification and microbial conversion) cannot utilize this material directly. The main factors that contribute to the intractability of lignocellulosic material are the inaccessibility of cellulose in its crystalline form and the occlusion of hemicellulose and cellulose by lignin (Himmel et al. 2007; Simmons et al. 2008). Pretreatment of plant biomass is therefore necessary to simplify the lignocellulosic material prior to saccharification and microbial conversion. Methods for deconstructing plant biomass include dilute acid hydrolysis, ammonia fiber expansion, and most recently, the use of ionic liquids. All deconstruction methodologies generate by-products that are detrimental to microbial growth and/or impact the bioconversion of sugars to biofuels (Fig. 1).

Dilute acid pretreatment is the most widely utilized and best documented method for plant biomass deconstruction and is known to generate inhibitory by-products that fall into three main categories: (1) furan aldehydes (furfural and hydroxymethylfurfural (HMF)) formed via the degradation of xylose and glucose, respectively (Klinke et al. 2004; Palmqvist and Hahn-Hagerdal 2000a; Pienkos and Zhang 2009); (2) organic acids, namely acetic acid produced by the deacetylation of hemicellulose and lignin, formic and levulinic acids from furans and HMF, respectively, and gluconic acid (Himmel et al. 2007; Palmqvist and Hahn-Hagerdal 2000a); and (3) phenolic compounds and other aromatics from lignin breakdown (Palmqvist and Hahn-Hagerdal 2000b; Pienkos and Zhang 2009). Detailed studies have also identified a range of aromatic compounds, aldehydes, ketones, and other acids (Klinke et al. 2004; Ranatunga et al. 1997).

Ammonia fiber expansion (AFEX) is an alternate strategy to the dilute acid treatment. AFEX minimizes the formation of sugar degradation products and converts a greater portion of the cellulose to sugars (Wyman et al. 2009; Lau and Dale 2009) compared to the dilute acid procedure, though the latter may be more efficient for biomass with high woody content (Sun and Cheng 2002). Common inhibitors associated with AFEX are the phenolics derived from depolymerized lignin and their associated aromatic degradation products (Balan et al. 2009).

Ionic liquid-based pretreatment of cellulose, though suggested as early as 1934 (Swatloski et al. 2002), is a relatively new procedure for deconstructing lignocellulosic material (Li et al. 2009; Swatloski et al. 2002) and provides an alternative to dilute acid processing and AFEX (Liu et al. 2010; Li et al. 2009; Singh et al. 2009). As the most recent technology to be explored in this context, studies are still ongoing that will elucidate the composition of the deconstructed plant material derived from ionic liquid pretreatment. The pros and cons of water-immiscible

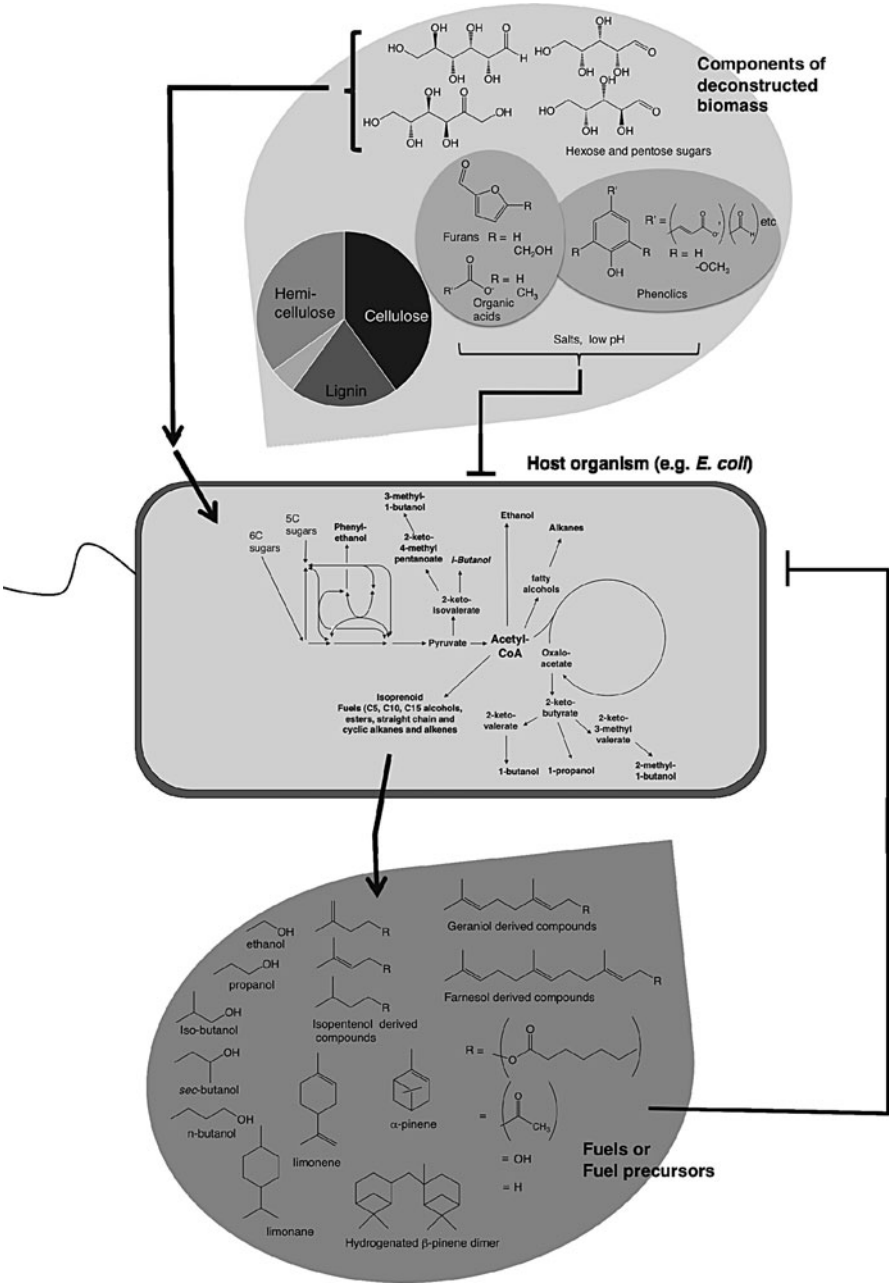

Fig. 1 Sources of common inhibitory compounds and toxic products. Sugar components of lignocellulosic plant biomass hydrolysates serve as carbon sources, while other components can have inhibitory impacts. A gram-negative bacterial host (e.g., *E. coli*) serves as the general host organism model with central metabolic routes leading to various classes of biofuels. Candidate biofuel compounds with known microbial toxicity are shown below. Note: the toxicities of *sec*-butanol, limonane, and the hydrogenated β-pinene dimer in *E. coli* have yet to be tested

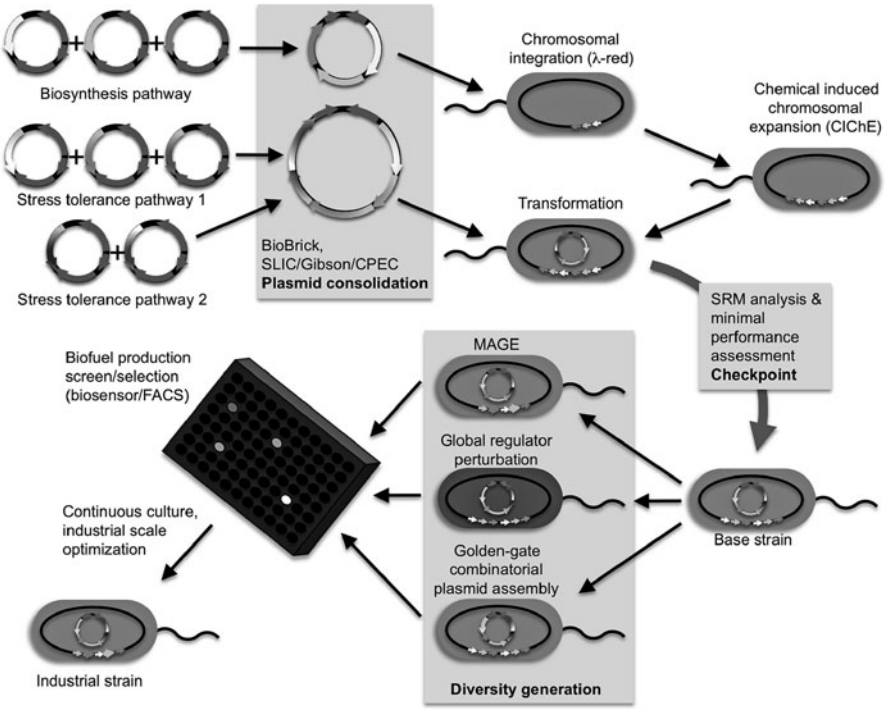

Fig. 2 Plasmid consolidation, ClChE, diversity generation, and strain selection process workflow. Biosynthesis and stress tolerance pathways are condensed into single plasmids using BioBrick (Anderson et al. 2010; Shetty et al. 2008), or SLIC/Gibson/CPEC (Gibson et al. 2009; Li and Elledge 2007; Quan and Tian 2009) methodologies. The biosynthetic pathway is then integrated into the chromosome, using the λ-red system, and subsequently expanded in the chromosome via ClChE (Tyo et al. 2009). The plasmid bearing the stress tolerance pathways is then transformed into the resulting strain. SRM analysis and performance assessments are conducted for each biosynthetic and stress tolerance pathway, ensuring that each pathway is at least minimally functional before proceeding to subsequent diversity generation with MAGE (Wang et al. 2009) (targeting the chromosomally expanded biosynthetic pathway and potentially other chromosomal loci), global regulator perturbation (Alper and Stephanopoulos 2007; Alper et al. 2006), and Golden-gate combinatorial plasmid assembly (Engler et al. 2008, 2009). Candidate strains are then screened or selected using a high-throughput assay such as a biofuel production biosensor (Dietrich and Keasling, unpublished data). Selected strains must then be optimized

ionic liquids have only recently begun to be explored (Park and Kazlauskas 2003; Li et al. 2009). Specifically, the impact of any residual levels of this reagent in the deconstructed soup on downstream processes, such as the saccharification steps or the microbial culture, requires to be studied.

Furan compounds (Fig. 1) have been thoroughly investigated for their impact on several bacterial hosts, such as *Zymomonas mobilis* (Ranatunga et al. 1997; Franden et al. 2009), several *E. coli* strains (Gutierrez et al. 2002, 2006; Zaldivar et al. 1999) including the ethanologenic *E. coli* LY180 (Miller et al. 2009a), and the solventogenic *Clostridium beijerinckii* (Ezeji et al. 2007). Very well studied in

S. cerevisiae (Horvath et al. 2001; Liu et al. 2005; Banerjee and Bhatnagar 1981; Gorsich et al. 2006), the toxicity is mitigated by dehydrogenases that reduce the aldehydes to their corresponding furan methanols (Petersson et al. 2006).

Lignin depolymerization yields a diverse array of phenolic alcohols, including coumaryl (no methoxy groups at the position *ortho-* to the OH- group), coniferyl (one methoxy group), and synapyl (two methoxy groups) (Fig. 1). Klinke et al. (2004) provide an extensive review of the toxicities of various alcohol, carbonyl and acid derivatives of these phenolic compounds, as well as their relative toxicities based on the number of methoxy groups. Ferulic acid and vanillin are among the best-studied phenolic compounds. Vanillin, in particular, has been used as an antimicrobial agent in the food industry (Fitzgerald et al. 2004; Gasson et al. 1998). The primary mechanism of phenolic toxicity universally appears to be the disruption of cell wall integrity.

Though not as toxic as the furan aldehydes or aromatic compounds, acetic acid is typically released in significant quantities and has been shown to impact not only growth but also target compound production. The latter has been documented for *Z. mobilis*, where the impact on ethanol production was greater than that explained by the impact on growth alone (Osman and Ingram 1985). Several studies have evaluated the effect of weak organic acids on bacterial physiology (Polen et al. 2003; Arnold et al. 2001). Acetic acid toxicity mainly arises from the membrane permeability of the undissociated acid. Upon entry into the cell, the acid dissociates and increases intracellular H^+ levels, decreasing the transmembrane proton gradient and disrupting the energy balance that is regulated by the proton motive force (Axe and Bailey 1995). Among other organic acids, accumulation of formic acid is another potential source of toxicity and is reported to elicit a very different general response from that of acetate accumulation (Kirkpatrick et al. 2001). Formic acid is reported to be more toxic than acetic acid (Pienkos and Zhang 2009) but typically accumulates at much lower levels during the pretreatment process. As such, most strain improvement efforts for small organic acids were focused on acetic acid (Dien et al. 2003; Pienkos and Zhang 2009; Warnecke and Gill 2005).

The most extensive studies examining the impact of a complete deconstructed soup on microbial host growth and production have focused on *S. cerevisiae* (Palmqvist and Hahn-Hagerdal 2000b) and implicate weak acids, phenolics and furans. Broad groups of inhibitory compounds and biomass hydrolysates have also been evaluated with ethanologenic *E. coli* (Klinke et al. 2004), *Z. mobilis* (Franden et al. 2009), and solventogenic clostridia (Mitchell et al. 2008). Similar toxic responses were identified in other bacterial hosts and methods to detoxify the deconstruction soup are often necessary. For example, overliming is a commonly used process for dilute acid pretreated material that has been shown to degrade many aromatic acids and ketones, (Klinke et al. 2004; Palmqvist and Hahn-Hagerdal 2000a) resulting in the production of gypsum and adding to the process cost (Galbe and Zacchi 2002). Cho et al. (2009) specifically targeted the peroxide-based removal of *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, vanillic acid, syringaldehyde, and vanillin, and demonstrated a clear improvement in butanol production using *C. berjenkii*. While such removal methods have the

potential to improve microbial conversion yields, they also add cost to the workflow. Therefore, it is worth examining microbial engineering of more resistant production strains so that residual amounts of these molecules do not impose any substantial impact on the microbial host.

3 Targets for Engineering Stress Tolerance from Biomass Inhibitory Compounds

The studies outlined in the previous sections provide a basis for cellular engineering for improved tolerance to the classes of inhibitory compounds discussed above. Dehydrogenases that convert furan aldehydes to less harmful alcohols have been documented in a wide variety of microbes, including *S. cerevisiae*, *P. putida*, and *E. coli*. However, despite their ability to metabolize HMF, these strains remain sensitive to the compound. The *E. coli* strain EMFR9, derived from the ethanologenic *E. coli* strain LY180, showed greater tolerance to furan aldehydes (Miller et al. 2009a). Analysis of this strain, under exposure to HMF, indicated that the genes (*yqhD* and *dkgA*) encoding two NADPH-dependent alcohol dehydrogenases that catalyze the conversion of furfural to furan methanol were repressed (Miller et al. 2009b). Although furan methanol is less toxic than furfural, the additional draw on NADPH impacted processes that use this cofactor, such as sulfur assimilation, and lead to a greater growth impact. Therefore, even though HMF detoxification pathways exist, the cofactors being utilized in the process should be kept in mind. In this regard, a recent study of an inhibitor tolerant *S. cerevisiae* strain found up-regulation in mechanisms that may offset the cofactor requirement for furfural and HMF reduction (Liu et al. 2009). Alternately, in situ detoxification strategies have also been explored that involve treating deconstructed biomass with strains that contain degradation pathways for aldehyde inhibitors prior to use with the fuel production host (Koopman et al. 2010; Wierckx et al. 2010). For discussions on molecular mechanisms of in situ detoxification of the aldehyde inhibitors in yeast, see Chap. 1.

In the case of phenolic compounds, several potential mechanisms exist that may alleviate or provide resistance to these inhibitory compounds. Efflux pumps that export inhibitory molecules provide a direct tolerance mechanism. Homologs of the aromatic acid efflux (Aae) pump system from *E. coli* (Van Dyk et al. 2004) and the toluene tolerance (Ttg) pumps in *P. putida* (Ramos et al. 2002) are potential candidates for the export of phenolic compounds. Modulation of the cell wall fatty acid composition has also been documented to provide benefit in coping with the disruptive action of phenolic compounds in *E. coli* (Keweloh et al. 1991). The metabolism or degradation of phenolic compounds to non-toxic metabolites is another potential route to mitigate phenolic stress. For example, phenol peroxidases (laccases) have been used to treat processed biomass (Jönsson et al. 1998), and laccases from heterologous sources, such as the *Bacillus*

licheniformis laccase *cotA*, have been functionally expressed in *E. coli* (Koschorreck et al. 2009). Other degradation mechanisms include decarboxylation, such as from ferulic acid to vinylguaiacol, a less toxic compound, which has been demonstrated in *Bacillus pumilus* (Lee et al. 1998). Interestingly, there are pathways to convert phenolic compounds to central metabolic intermediates, such as to acetyl-CoA via catechol, that are well documented in bacteria such as *Pseudomonas* (Feist and Hegeman 1969; Ng et al. 1994; Herrmann et al. 1995). The meta-pathway that converts phenols to catechol and finally to acetyl-CoA consists of seven steps. Though it may be an elaborate route to obtain resistance, it provides the additional benefit of converting the inhibitory material to a central metabolism intermediate that can be channeled into cellular growth and production. This could be developed to maximize the use of all components of the lignocellulosic biomass, rather than just the sugar polymers.

With respect to small organic acids, long-term adaptation of *E. coli* to acetate has been undertaken and involved changes in metabolism (Holms and Bennett 1971; Polen et al. 2003) and is impacted by the choice of sugars in the carbon source (Lasko et al. 2000). Tolerance to these compounds has been studied and engineered in several host microbes (Dien et al. 2003; Pienkos and Zhang 2009; Warnecke and Gill 2005). See Chap. 5 for mechanisms of cell defense and tolerance to organic acid in yeast.

4 Impact of Engineering a Pathway

Commercially viable titers for bio-products can range from several mg/L in the case of pharmaceuticals to hundreds of g/L for commodity chemicals such as biofuels and are the primary driving force behind most metabolic engineering efforts. Reaching these production levels requires a significant amount of pathway optimization. Strain development is an iterative process whereby pathway manipulation is followed by system-level studies to identify potential bottlenecks and reveal detrimental side effects (Mukhopadhyay et al. 2008). Once it has been successfully demonstrated that a product of interest can be produced in vivo, achieving economically viable production levels requires minimizing the generation of less desirable side products and maximizing carbon flux toward the target product. For example, improvements in bio-ethanol production in *E. coli* utilized many such steps, and this progress has been very well reviewed (Jarboe et al. 2007).

Most metabolic engineering efforts use a combination of native and heterologous genes. Examples include the production of the sesquiterpene amorphadiene (Newman et al. 2006; Martin et al. 2003), 1,3-propanediol (Saxena et al. 2009; Sauer et al. 2008; Biebl et al. 1999), 1,4-butanediol (Burgard and Van Dien 2007), iso-butanol (Connor et al. 2010; Cann and Liao 2008), and most recently, fatty acid ethyl ester production in *E. coli* (Steen et al. 2010). Understanding how the incorporation of an engineered exogenous pathway perturbs the host system is important for overcoming pathway bottlenecks. For example, codon optimization

of heterologous genes may be required to minimize stress caused by the depletion of the pool of available charged tRNA (Gustafsson et al. 2004; Welch et al. 2009). Additionally, the burden of expressing both native and non-native pathways can cause imbalances in the cellular redox state by altering the cofactor balance or levels of ATP, which can lead to overflow metabolism (Vemuri et al. 2006). Imbalances in enzymatic activity can also result in the accumulation of toxic or inhibitory pathway intermediates, which may drastically reduce cellular growth as well as production levels. A systematic evaluation in *E. coli* of intermediate buildup was conducted for an engineered isoprenoid pathway, converting the five carbon pyrophosphate intermediate to the final sequiterpene via the 10 and 15 carbon pyrophosphates, to examine the individual impact of each intermediate (Martin et al. 2003). The pyrophosphates were found to be highly detrimental to cellular growth in the order of C5 > C10 > C15, and a highly efficient final enzyme to convert the C15 farnesyl pyrophosphate to amorphadiene was required to relieve the system of stress. In another study, the accumulation of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) was found to be a bottleneck in the production of mevalonate via a heterologous pathway in *E. coli*. Downregulating the synthesis of HMG-CoA or the overexpression of *tHMG1,* encoding the enzyme downstream of HMG-CoA, alleviated the growth impact (Pfleger et al. 2006; Pitera et al. 2007).

Optimization of heterologous pathways is essential for maximizing production and minimizing the buildup of toxic intermediates. An elegant approach to alleviate HMG-CoA stress utilized scaffolding domains from metazoan signaling proteins to recruit the first three enzymes of the mevalonate pathway; all of which were tagged with the corresponding peptide ligands (Dueber et al. 2009). The scaffold co-localizes the enzymes thereby reducing the accumulation of toxic intermediates while increasing the effective concentrations of pathway intermediates in the vicinity of the enzymes. Varying the stoichiometry of the scaffold binding domains, effectively controlling enzyme ratios, resulted in 77-fold more mevalonate than the unscaffolded system.

In another instance, the host stress response was also a problem in the production of the *p*-hydroxy styrene precursor, *p*-hydroxy cinnamate, in *E. coli*. Systematic evaluation of the toxicity of *p*-hydroxy cinnamate led to discovery of the aromatic acid efflux genes (*aae*) (Van Dyk et al. 2004). Overexpression of the *aaeAB* genes using an inducible P_{trc} promoter resulted in a twofold increase in *p*-hydroxy cinnamate tolerance, while the toxicity from *p*-hydroxy styrene final product was alleviated using a biphasic reaction system (Sariaslani 2007; Van Dyk 2008). Recently optimization of *p*-hydroxy-styrene production using a solvent-resistant *P. putida* S12 strain also used an organic phase extraction system and improved production by twofold (Verhoef et al. 2009).

The accumulation of a particular intermediate does not necessarily indicate if it is due to excess levels of the upstream enzyme or the low levels of the downstream enzyme. Methods to compensate often express the limiting enzyme from a second plasmid and/or tune parameters such as the gene's promoter and ribosome binding site or the plasmid's origin of replication. However, arbitrary enzyme

overproduction can rob the cell of resources that could otherwise be devoted to generating the target compound. Tools that allow the quantitative interrogation of target enzymes and diagnostic methods that enable the evaluation of biosynthetic pathway expression provide key information for resolving pathway bottlenecks. High throughput mass spectrometric methods, such as selected reaction monitoring (SRM), are useful for diagnosing and optimizing protein production for biofuel production (Keasling 2008). Correlation of protein production levels with metabolite titers from different strains is integral to optimizing the productivity and stability of the engineered microbe (Dueber et al. 2009).

5 Accumulation of Toxic Products

The diversity of microbial biosynthetic pathways allows for a large number of biofuel candidates to be envisioned, but compounds must meet several criteria to serve as biofuel targets (Fig. 1). Several recent reviews comprehensively cover the range of microbially derived compounds that meet these criteria, ranging from small chain alcohols to alkanes and alkenes (for bio-gasoline) to longer chain hydrocarbons (for biodiesel) (Wackett 2008; Peralta-Yahya and Keasling 2010; Lee et al. 2008; Keasling and Chou 2008; Fortman et al. 2008; Chemier et al. 2009; Antoni et al. 2007) as well as cyclic hydrocarbons that may serve as bio-jet fuel components (Harvey et al. 2010; Ryder 2009).

Many of these compounds have solvent-like properties presenting a severe impact on cell growth and consequently limiting product titer. Even ethanol, the most well-established biofuel, is toxic at some level to the organisms used to produce it. Exposure to alcohols and solvents has been reported to impact bacterial growth via a variety of mechanisms including increased membrane fluidity, ion leakage, changes in fatty acid composition, difficulties in translation, and elongated cells (Baer et al. 1987; Ingram 1990; Sikkema et al. 1995; Tomas et al. 2004). In general, toxicity increases with solvent hydrophobicity, which is determined by the length of the carbon backbone. In general, the toxicity of the alcohol correlates well with the octanol–water partition coefficient, P_{ow}; at saturating concentrations, solvents with a log P_{ow} greater than 3.8 are not toxic to *E. coli*. The degree of toxicity of an alcohol varies across bacteria, with some bacteria being more affected by the length of the alkyl chain while others by saturation of the carbon backbone (fewer double bonds). The majority of toxicity studies propose the cell membrane as the most affected by organic solvents and as contributing significantly to stress adaptation. Short- and long-chain alcohols are known to cause stress by desiccation, and by intercalating into the hydrophobic cell wall fatty acids, respectively. Their similarities to other well-understood stresses, such as desiccation or hypersalinity, may suggest gene candidates for engineering fuel tolerant hosts. See Chap. 6 for more descriptions on microbial stress response to toxic compounds and organic solvent.

5.1 Ethanol

Microbial ethanol production from glucose and mixed sugars is now a well-established process. See Chap. 3 for molecular mechanisms of ethanol tolerance in yeast. A vast body of literature also focuses on bacterial ethanologenic hosts such as *E. coli* and *Z. mobilis* (Ingram et al. 1998; Jarboe et al. 2007; Lawford and Rousseau 2003; Lee 1997; Lin et al. 2005; Yomano et al. 1998; Zaldivar et al. 2001). The impact of various other parameters on ethanol production has also been investigated in *E. coli*. Examples include choice of sugar (Alterthum and Ingram 1989), acetic acid accumulation (Lawford and Rousseau 1992), inhibitors from lignocellulosic biomass (Zaldivar and Ingram 1999; Zaldivar et al. 1999, 2000), and loss of osmolytes (Underwood et al. 2004). *Z mobilis* is one of the best natural producers of ethanol and is naturally tolerant to greater amounts of ethanol than wild type *S. cerevisiae* or *E. coli* (Rogers et al. 1984), making it a focal point of many efforts for optimized ethanol production (Joachimsthal and Rogers 2000). *Z. mobilis*'s response to ethanol implicates heat shock response chaperones (Michel and Starka 1986; Barbosa et al. 1994), and early studies also found the lipid composition of *Z. mobilis* to be well suited for ethanol accumulation in having a large percentage of vaccenic acid in the acyl groups of its polar membrane phospholipids (Carey and Ingram 1983). However, even in *Z. mobilis*, the accumulation of ethanol eventually inhibits glucose uptake and conversion (Osman and Ingram 1985).

5.2 Butanol

Butanol stress has been extensively studied, for example, in solventogenic *Clostridia*, which is a native producer. In *C. acetobutylicum*, transcript analysis after exposure to 0.75% n-butanol (6 g/L) indicated that the primary response is the increase of transcripts encoding chaperones, proteases, and other heat shock-related protei[illegible]mas et al. 2004), and further, the overexpression of GroELS chaperones produced strains with greater n-butanol tolerance (Tomas et al. 2003). To date, the most optimized butanol production in *C. acetobutylicum* leads to a titer of about 13.6 g/L while that in *C. berjenkii* is 19 g/L (Papoutsakis 2008). Several other clostridial strains are being investigated due to their ability to produce higher levels of n-butanol, such as *C. pasteurianum*, which can produce as high as 17 g/L using glycerol as a carbon source (Biebl 2001). Despite the availability of such natural producers, it has been argued that metabolic engineering of more tractable industrial hosts such as *E. coli* may be a better strategy for bio-butanol production. The main impediment toward this goal is the low concentration at which butanol is toxic to *E. coli*. Recent studies have examined the effect of n-butanol and iso-butanol exposure on *E. coli*. In the case of iso-butanol, transcript analysis revealed several key mechanisms including the disruption of quinone function and the involvement

of global regulators such as ArcA (Brynildsen and Liao 2009). n-Butanol stress in *E. coli* DH1 has also been examined in a comprehensive functional genomics study and was found to elicit strong cell envelope and oxidative stresses as well as cause perturbations to several ArcA-regulated electron transport and respiratory mechanisms (Rutherford et al. 2010).

The distribution of response among several major regulons makes it difficult to engineer all modes of stress relief, suggesting evolution and adaptation as important strategies to obtain stress-tolerant strains. Such non-targeted approaches were used in *Pseudomonas* spp, where n-butanol tolerance was improved from 3% to 6% (Ruhl et al. 2009). Nevertheless, the identification of genes impacted by n-butanol exposure in the functional genomics studies enables a systematic approach in which the corresponding knockdowns or overexpressions can be implemented and evaluated for improvement in solvent resistance. This targeted strategy has the distinct advantage of a well-defined approach that may be translated to other hosts. For example, n-butanol exposure caused a disruption of redox balance, points to candidates such as the alcohol dehydrogenase YqhD and superoxide dismutases (Rutherford et al. 2010).

5.3 C5-10 Alcohols and Hydrocarbons

An important class of biofuels can be derived from isoprenoid biosynthetic pathways. Hemi-, mono-, and sesquiterpenes (C5, C10, and C15, respectively) have all been suggested as potential fuel candidates. Specific examples include isopentenol, isopentanol (Connor and Liao 2009), limonene, limonane (from limonene (Ryder 2009)), dimethyl octane (from geraniol (Martin et al. 2007)); farnesane (from farnesene (Ryder 2009)), hydrogenated pinene dimers (Harvey et al. 2010); and others (Peralta-Yahya and Keasling 2010). Terpenes have historically been studied as medicinal, flavoring, and fragrance compounds. Limonene, pinene, geraniol and citronellol, putative biofuel compound precursors, are associated with plant extracts and are used in a wide array of cosmetics, insect repellant, sanitizing agents, and solvent applications. Toxicity of these isoprenoid compounds has been evaluated in a variety of bacteria such as *E. coli, Samonella enterica,* and *Staphylococcus aureus* (Kim et al. 1995; Trombetta et al. 2005; Cristani et al. 2007). Specific modes of antibiotic resistance have also been evaluated for several of these compounds. The common household disinfectant, Pinesol, contains a mixture of cyclic monoterpenes, and its antimicrobial impact on *E. coli* has been studied via transcript analysis (Gill et al. 2002). Following up on these initial studies, it was shown that the derepression of the AcrAB-TolC pump genes provided a significantly higher resistance to Pine oil (Moken et al. 1997). Similarly, oxidation-based mechanisms have been found in *Pseudomonas aeruginosa* for the metabolism of geraniol and citronellol (Hoschle and Jendrossek 2005).

Very few studies have evaluated the impact of longer carbon chain compounds on bacterial cultures as the solubility of these compounds drop below measurable

levels. It is likely that long-chain compounds beyond a certain carbon length no longer intercalate into the cell wall and will impose no toxic effect on cell growth or production. Consistent with this, no growth defect was observed for an *E. coli* strain developed to produce fatty acid ethyl esters (FAEE) at almost gram per liter scales (Steen et al. 2010). It is noteworthy, however, that addition of an organic phase to FAEE production cultures improved production by 1.6-fold (from 427 to 674 g/L), which may be suggestive of product accumulation causing a push back on the biosynthetic pathway.

5.4 Targets for Engineering Stress Tolerance from Toxic End Products

Targeted and systems-level studies in bacterial systems for solvent stress point to several candidates that may be explored to generate fuel-tolerant hosts. Selection of ethanol-tolerant *E. coli* is a much-explored area as is the application of cell-wide stress response studies and mutagenesis approaches (Ingram 1990; Jarboe et al. 2007; Jeffries and Jin 2000; Alper et al. 2006; Gonzalez et al. 2003; Yomano et al. 1998). Tolerance mechanisms range from modulation of cell wall fluidity (Ingram and Vreeland 1980; Ingram et al. 1980), expression of chaperones (Barbosa et al. 1994), to the use of osmoprotective agents such as glycine betain (Gonzalez et al. 2003). The response to ethanol is more like salt or desiccation stress in that ethanol appears to have a water exclusion effect. Consequently, studies implicate the role of osmoprotectants in stress mitigation (Gonzalez et al. 2003; Underwood et al. 2004). *E. coli* shows a similar response in cell wall fatty acid composition in response to salt and ethanol stresses and pretreatment with salt resulted in greater resistance to ethanol (Ingram and Vreeland 1980); specifically, an increase in unsaturated fatty acids was found in response to ethanol stress (Ingram et al. 1980). The opposite trends were observed during exposure to longer, more hydrophobic solvents such as hexenol, where pre-exposure to salt had no impact (Ingram et al. 1980). An increase in trans unsaturated fatty acids in response to both ethanol and NaCl was also found in *P. putida* (Loffeld and Keweloh 1996). A previous study found similar trends in *P. putida* exposed to toluene and ethanol: an increase in saturated fatty acids in cells exposed to toluene, but the reverse in cells exposed to ethanol, leading the authors to suggest that the reduction in saturation in ethanol is a cause rather than a response (Heipieper and de Bont 1994). Modulation of cell wall fluidity appears to be a key response in several other microbes such as *Oenococcus oeni* (Grandvalet et al. 2008; Silveira et al. 2004) and *Z. mobilis* (Carey and Ingram 1983; Michel and Starka 1986). Genetic engineering of cell wall fatty acid distribution has been used in several bacteria with measured impact on stress tolerance or sensitivity. Unsaturated fatty acids in the membrane of the cyanobacterium *Synechocystis* spp. were increased by deleting the desaturase genes *desA* and *desD* (Sakamoto and Murata 2002; Allakhverdiev et al. 1999) and resulted in an increased salt tolerance.

Similarly, a knockout in the *cis/trans* isomerase *cti* in *P. putida* DOR-T1E (Junker and Ramos 1999) resulted in an increased sensitivity to toluene. Finally, the cyclopropyl fatty acid synthase (*cfa*) from *O. oeni* has been used to complement a corresponding knockout in *E. coli* to restore ethanol sensitivity (Grandvalet et al. 2008).

Butanol is more hydrophobic than ethanol and does not cause the same type of water exclusion stress. However, butanol is toxic to *E. coli* and other bacteria at much lower concentrations. Being membrane permeable, butanol causes stress at both cell envelope as well as intracellular levels. Therefore, addressing the impacted cellular components, as discovered via cell wide studies, might provide appropriate stress relief. Mechanisms that correct the disrupted redox state of the cell, such as superoxide dismutases or dehydrogenases (e.g., *yqhD*), may be effective. Though these mechanisms have never been directly explored for relieving butanol stress, they have been effective in dealing with redox stress (Kang et al. 2007; Perez et al. 2008), which is also observed during n-butanol exposure in *E. coli* (Rutherford et al. 2010). Deletion of *ydhD* specifically reduced iso-butanol production in *E. coli* (Atsumi et al. 2009), consistent with its importance in stress from these target compounds. Cues from other bacteria, such as *C. acetobutyliticum*, include the overexpression of GroELS chaperones (Tomas et al. 2003).

A limited number of studies have evaluated the impact of longer chain alcohols, alkanes, alkenes, cyclic hydrocarbons, and aromatic compounds on bacteria. With respect to terpenoid compounds, studies in *E. coli* point to export pumps as a key mechanism to reduce toxicity. Given its wide substrate range, the AcrAB-TolC system native to *E. coli* holds the potential of providing tolerance toward several terpene compounds (Gill et al. 2002; Moken et al. 1997). Homologous pumps exist in other more solvent-resistant bacteria and are worthy of examination for engineering host resistance. With respect to solvent tolerance, a large body of knowledge comes from studies in *Pseudomonas* spp (Ramos et al. 2002). The involvement of efflux pumps is documented in *P. putida* DOT-T1E (Ramos et al. 1998), *P. putida* S12 (Kieboom et al. 1998a,b), *P. putida* MTB6 (Huertas et al. 2000), *P. putida* GM73 (Kim et al. 1998), and *P. putida* F1 (Phoenix et al. 2003) and is possibly the primary mechanism of solvent tolerance in these bacteria. Of special note is the versatile, solvent-resistant pump (*srp*) from *P. putida* S12, which was shown to be induced in response to a variety of relevant compounds such as C5-C9 alkanes (moderate induction) and C5-C8 alcohols (strong induction), as well as aromatic solvents (Kieboom et al. 1998b). The other key resistance mechanism reported in several *P. putida* strains is the increase in *cis* to *trans* isomerization of cell wall fatty acids, which modulates cell wall fluidity. Solvent tolerant *E. coli* strains have been reported to demonstrate resistance to cyclohexane at concentrations typically lethal to the parent *E. coli* strain (Aono and Kobayashi 1997). Subsequent analysis of these solvent-resistant strains found a decrease in cell wall hydrophobicity and specifically reported changes in the lipopolysaccharide content. *P. putida* strains have also been documented to use vesicles to sequester and export toxic metabolites (Kobayashi et al. 2000); however, this is neither a widely observed mechanism nor would it be straightforward to engineer into a

heterologous host. Alternate responses include metabolism of the offending alkane (Roling et al. 2002; van Beilen et al. 2001; Spormann and Widdel 2000); however, with respect to improving product titers, product catabolism is not an ideal strategy to alleviate the stress.

6 Engineered Controls of Stress Tolerance Pathways

Typical laboratory systems use carefully selected combinations of inducible promoters, plasmid copy numbers, ribosomal binding sites, and terminators to expressed genes and pathways (Smolke 2009). Such systems are invaluable for demonstrating feasibility for a biosynthetic pathway or stress response function. Furthermore, in the case of high value commodities where the impact on cell growth due to stress or the cost of maintaining plasmid-borne systems is completely offset by the value of the target compound, no further engineering may be necessary. In this regard, maximizing the amount of target compound per culture cycle is necessary to reduce the reliance on scale up alone. Therefore, the longer the host can perform optimally under production conditions, the greater the yield from a given quantity of starting material and correspondingly the cost associated with deconstruction per cycle of production. In such large-scale settings, especially in a continuous process, it becomes a significant hindrance to (1) maintain a plasmid using a selection marker and (2) provide a constant concentration of the external inducer. The elimination one or both may result in significant cost benefit to the process.

A basic strategy to bypass the cost and effort associated with the addition of the inducers would entail the use of constitutive promoters that provide a constant level of gene expression. However, stress response mechanisms may not be the ideal systems for functional expression under conditions where stress is not present. Even with the most benign mechanisms, such as the expression of chaperones, constitutive expression burdens the cell with excessive protein production. In most cases, however, expression of the stress response mechanism comes at an even higher cost. For example, overexpression of efflux pumps can be toxic to the cell due to overloading of the protein translocation machinery used to target proteins to the membrane (Wagner et al. 2007), and careful tuning of pump expression is required to avoid growth inhibition (Wagner et al. 2008). A better strategy, therefore, is to have expression systems that are regulated using cellular cues rather than an externally added induc[illegible]here are at least two interesting approaches for an internally regulated system. One such system would be where sources of toxicity, specifically the inhibitory compounds or the accumulating harmful target compound, would be detected and used to trigger the expression of the appropriate mechanism. The other approach would use regulatory mechanisms that become active during the conditions imposed by the inhibitory compounds or the accumulating harmful target compound, and place the genes encoding the resistance mechanism under the control of these regulators.

To execute the former, sensory proteins that can sense the inhibitory compounds and effect downstream responses are required. Bacterial two-component systems, typically comprised of sensor histidine kinase (HK) and a response regulator (RR), are an ideal mechanism for such a strategy. In these systems, the histidine kinase functions to sense extra- and intracellular signals and triggers signal transduction via a phosphotransfer to the cognate RR that in its active phophorylated state regulates cellular response, often by gene induction (Stock et al. 2000; Galperin et al. 2001; Gao and Stock 2009). The biodiversity from sequenced organisms provides a variety of two-component systems (Mascher et al. 2006). These include systems that sense many relevant compounds discussed in this chapter or conditions associated with their presence. For example, phenolic and aromatic compounds serve as signals for the *Agrobacterium tumefaciens* VirA/VirG system (e.g., acetosyringone) (Lee et al. 1995) and the *P. putida* TodS/TodR (Toluene) (Lau et al. 1997) (Busch et al. 2007). Acidic pH is sensed by variety of sensors including the *E. coli* PhoQ (Bearson et al. 1997), the *A. tumefaciens* VirA (Gao and Lynn 2005), and the *Sinorhizobium* ActX (Tittabutr et al. 2006). Sensor kinases are also known for other pertinent signals or stress responses, such as hexose sugar sensing by the *E. coli* UhbP (Island and Kadner 1993; Wright and Kadner 2001; Wright et al. 2000), cell density or quorum sensing by the *E. coli* QseC (Sperandio et al. 2002), cell envelope stress by the *E. coli* CpxA, and redox stress by the *E. coli* ArcB (Iuchi et al. 1990; Malpica et al. 2004).

It should be pointed out that heterologous expression of a two-component system in *E. coli* may not be sufficient to accomplish signal sensing, transduction, and gene regulation, and the corresponding response regulator may also require native sigma factors etc. (Lohrke et al. 2001). However, this problem can be bypassed by using only the sensory domains of the appropriate two-component system as a fusion protein with native *E. coli* systems. Such fusion systems have been made successfully using the well-characterized EnvZ/OmpR two-component systems that natively control *E. coli's* response to changes in osmolarity (Cai and Inouye 2002; Kishii et al. 2007). Well-cited examples include fusion sensory HKs in which the periplasmic and transmembrane domains of chemoreceptor Tar (aspartate sensor) or Trg (ribose sensor) were fused with the catalytic core of EnvZ (Baumgartner et al. 1994; Utsumi et al. 1989). The resulting Taz1 and Trz1 proteins enabled response regulator activation to Asp or ribose rather than osmotic change. In another example, intracellular O_2 was sensed by a FixL-EnvZ fusion and used to induce *ompC-gfp* (Kumita et al. 2003). Factors to be taken into consideration in order to generate such chimeric sensor histidine kinases for microbial engineering have been described recently (Salis et al. 2009). The advantage of such an approach is that it will be highly specific to the inhibitor in question and will not be triggered by other conditions. The obvious drawback is that a suitable sensor may not be known (e.g., furfural). However, in such cases, signals that are less specific (e.g., change in pH) but that correlate with the presence of the toxic compound or growth stage can be used.

The second strategy uses cues pertinent to the stress response or a pertinent growth/metabolic condition to control the expression of stress mitigating

mechanisms. Systems biology studies provide a broad suite of differentially expressed genes for the conditions of interest. Done with the right controls, these data sets allow the identification of genes that change specifically in response to the stressor, in this case, the inhibitors and toxic final products. The regulatory system that controls the up- or downregulation of the specifically responding genes can then be used to control the expression of the selected stress response mechanism. Conditions that correlate with the stress or the growth mode corresponding to target compound production can be selected, and genes that are differentially modulated in response to these conditions provide potential targets for this approach. For example, candidates to drive key pathways would be those that correlate with sugar utilization (e.g., diauxic shift), cell density (e.g., via quorum sensing), and stationary phase promoters; all of which are an integral part of culturing conditions for target compound production.

In *E. coli*, the availability of gene libraries can also provide powerful strategies to identify ideal candidates for creating such control systems. An important resource in this regard is the library of fluorescent transcriptional reporters generated in *E. coli* K12, in which *gfp* (green fluorescent protein) has been placed under control of about 2,000 native *E. coli* promoters (Zaslaver et al. 2006). This library was used to assess promoter function for the glucose–lactose diauxic shift and could potentially be used to screen for relevant promiscuous or specific pumps for a wide variety of conditions pertinent to the biofuel production workflow.

7 Robust Engineering of Multiple Tolerance Mechanisms into the Same Host

As discussed above, it is possible to engineer several classes of stress tolerance mechanisms into various biofuel-producing hosts. Naturally, it is of more importance to approach a simultaneous incorporation of multiple tolerance characteristics and biofuel synthesis pathways into the same host. This approach assumes that each of the individual stress tolerance and biofuel synthesis pathways has (separately) been introduced into the host organism, assays have been developed to gauge the performance of each pathway, the function of each pathway has been verified in the host, and the associated expression control systems have been minimally optimized. With those assumptions satisfied, three major challenges arise when attempting to engineer multiple pathways into a single host: (1) optimizing the performance of a given biological pathway often adversely affects other pathways in the same cell, (2) simultaneous optimization of all the pathways is required, and (3) generating sufficient library diversity within the collective pathways (from which to screen or select) and maintaining pathway stability become more difficult with each additional pathway.

In stark contrast with the engineering ideal, biological pathways are generally far from orthogonal. All activities transpiring within the cell are coupled to a greater or

lesser extent with each other. Any given stress tolerance or biofuel synthesis pathway may affect the intracellular environment (available cellular resources, membrane structure, redox balance, pH, etc.) to the detriment of the other engineered pathways. For example, adding an AcrA/B-TolC efflux pump to relieve terpene/limonene toxicity would result in exporting tetracycline from the cell (Okusu et al. 1996), a side effect that would diminish the expression of a biosynthetic pathway placed under the control of the P_{tet} promoter. Thus, although a pathway may have previously been introduced into and optimized for the host organism, the performance of the pathway might greatly diminish after the introduction of the other pathways of interest.

Since the introduced pathways will likely perturb each other's performance, this naturally leads to the requirement to screen or select for the desired function of multiple pathways simultaneously. At the beginning of this process, it is important to identify any grossly under-performing pathway(s) (whether stress tolerance or biosynthetic) and only initiate a systems-wide combinatorial screen/selection once a minimal level of activity is achieved for every pathway. The effort at this stage should be modest, because each pathway has previously been demonstrated to be functional, but some serial re-optimization of pathways that perform extremely poorly in their new context may be required. The targeted SRM approach (Anderson and Hunter 2006) could prove invaluable at this point to determine a functional pathway's component ratios before placing it in a new context. If other stress tolerance and biosynthetic pathways dramatically perturb these component ratios, SRM analysis can be applied in an iterative fashion to improve the under-performing pathway(s).

When incorporating multiple pathways into a single host, there are potentially many different parameters to optimize for each gene (gene variant, promoter, RBS, copy number), and the aggregate parameters must be combinatorially assessed by each pathway's assay. However, generating a large and diverse combinatorial library to screen, or from which to select, for optimal systemic performance, is rather futile without comprehensive high-throughput assays. Some functional assays are higher throughput than others, and an immediate concern is that an assay whose throughput is acceptable for optimizing an individual pathway may not be feasible for use within the context of a combinatorial screen. Since tolerance pathways are generally assessed via growth rates under increasing titers of exogenously introduced stress, and are readily transferable to high-throughput screens or selections, the burden falls predominantly upon assaying the biosynthetic pathway. In addition, restraint should be applied against over-optimizing stress tolerance pathways to the detriment of biofuel production. It may be best to exclusively screen or select for biofuel production, because optimizing production will implicitly address any underlying stress tolerance limitations.

While a validated fluorescence-activated cell sorting (FACS)-based assay of biofuel production would be ideal, such as a recent finding of an intracellular n-butanol biosensor (Dietrich and Keasling, unpublished data), single cell assays may not be applicable in all situations, especially if the cellular export of the biofuel is limiting. A general caveat to screening or selecting for biofuel production in

batch mode at a small scale is that the results may not be particularly applicable to continuous culture at an industrial scale, and further strain and culture optimizations will likely be necessary (Burgard and Van Dien 2007). Finally, in addition to screening combinations of stress tolerance and biosynthetic pathways, it can also be very fruitful to perturb global regulators for improved performance (Alper and Stephanopoulos 2007; Alper et al. 2006).

Before any attempts have been made to incorporate multiple pathways into the same host organism, stress tolerance and biofuel synthesis pathways are generally introduced into the cell on one or more replicating plasmid vectors. Since the number of compatible origins of replication is limited, the pathways of interest, the genes for which may be distributed across multiple plasmids, must often be consolidated into one or a few vectors. The traditional approach of restriction enzyme/multiple cloning site plasmid construction impedes this process, as it becomes more difficult to find amenable restriction sites with each pathway added to a given plasmid, and increasingly likely to necessitate the introduction of silent point mutations to disrupt the undesirable recognition sites. Furthermore, the traditional implementation of this process will almost certainly vary for each new combination of stress tolerance and biofuel synthesis pathways (since new restriction sites will be selected and new point mutations must be introduced), and therefore, it will often be necessary to restart the process from scratch. An alternative approach is to employ a standardized assembly strategy, such as the BioBricks method (Shetty et al. 2008; Anderson et al. 2010), which easily allows for the concatenation of multiple pathways together, in any combination.

Even though each pathway has already been incorporated into the target host and minimally optimized, it will likely be necessary to do so again within the context of all of the other pathways. Consolidating multiple pathways into a single plasmid (increasing plasmid size) or transforming multiple plasmids into the same host can affect plasmid copy number. It will generally be required, then, to screen/select various different combinations as described above. It should be noted that combinatorial library creation is potentially at odds with the binary BioBrick assembly method, because the cumulative library size is limited by number of colonies pooled after each assembly step (only two sequences are assembled together at time). However, it is possible to generate combinatorial libraries using other methods (Li and Elledge 2007; Gibson et al. 2009; Quan and Tian 2009; Engler et al. 2008, 2009) that allow for concurrent multi-part assembly while maintaining BioBrick compatibility for downstream applications.

Even after consolidating all of the desired stress tolerance and biosynthetic pathways into a few plasmids and selecting/screening for optimal combinations thereof, there remain numerous drawbacks to plasmid systems. Plasmids are not often utilized in an industrial context, because enforcing antibiotic selection pressure is not cost-effective, introduces additional cellular stress, and potentially reduces biofuel production. In addition, some plasmids do not segregate in an ordered fashion (unlike the chromosome), and this segregational instability can result in plasmid loss and accelerate the spread of mutations through the plasmid population that curtails the biofuel pathway while allowing the plasmid to

propagate (Tyo et al. 2009). Note that this concern is generally only applicable to the biofuel synthesis pathway (which itself may be responsible for cell stress), since in the presence of the cell-stress, even in the absence of antibiotic selection pressure, the stability of stress tolerance pathways may not justify significant concern.

Chromosomal integration is an important route to stabilizing biosynthetic pathways. While replicating plasmids offers variable copy-numbers to chose from (e.g., pUC vs. pSC101 (Smolke 20[illegible]hromosomal integrations have historically been limited to a single copy. Chromosomal integrations with multiple copies have recently been demonstrated with the *c*hemical *i*nduced *ch*romosome *e*xpansion (CIChE) method (Tyo et al. 2009). Whereas it has been relatively facile to generate combinatorial plasmid libraries (with variable promoters, RBS, etc.) from which to screen or select, it has been more challenging to accomplish the analogous chromosomal modifications. To some extent, with the advent of *m*ultiplex *a*utomated *g*enomic *e*ngineering (MAGE) (Wang et al. 2009), it is becoming feasible to achieve combinatorial diversity within the chromosome itself.

Recombination is yet another means available to the host organism to disable biosynthetic pathways. Cellular recombination machinery can remove (from the chromosome or a plasmid) portions of a deleterious pathway that contains high-homology sequence repeats (e.g., a repeated promoter sequence). In addition, since the aforementioned CIChE methodology relies upon only one repeat flanki[illegible]e pathway to be integrated (Tyo et al. 2009), it is likely that the CIChE process will not result in chromosomal repeats of the entire pathway if the pathway internally contains repeated sequences. Perhaps the best defense against undesired pathway recombination is to avoid sequence repeats altogether, utilizing multiple gene operons and the minimal number of promoters and terminators, where possible. When multiple promoters and terminators with similar function are required, it is advisable to choose those with maximally divergent sequences. An additional means to mitigate pathway recombination instability is to delete *recA*, as performed at the completion of CIChE (and before initiating the MAGE process).

8 Conclusion and Perspectives

A biofuel-producing host must harbor not only the biosynthetic pathway, but also the carefully engineered tolerance mechanisms to enable stable growth and high production. Serious consideration of a bacterial host for the production of a bulk commodity must address issues of pretreatment inhibitors, metabolic engineering burden and toxicity from target compound. End product toxicity especially is a common problem in strain engineering for biotechnology applications and is possibly the most critical in biofuel production due the absolute requirement to maximize production titer. *E. coli* engineered to serve as an industrial host to produce bulk chemicals such as 1,4-butanediol and 1,3-propanediol, had substantial engineering devoted to improving tolerance was required to make production cost

effective (Burk 2010; Zeng and Biebl 2010). While the toxicity from some of current and advanced biofuel candidates are well understood, entire classes of fuels that can be produced microbially remain to be explored further. For example recent discoveries of novel pathways enabled the production of hydrocarbons longer than C15 in *E. coli* (Beller et al. 2010), and further developments will reveal if such targets impose toxic limits on the production titer and the strain engineering required to overcome these.

Eventually, the goals of a well-engineered microbial host system go beyond a typical laboratory inducer controlled, plasmid borne s s. Beyond just the deployment of key tolerance mechanisms, not only will the target genes be expressed using cues from the system, but they will also have more sophisticated positive and negative feedback controls like those found in native microbial systems. Such engineered systems would allow more optimal levels of stress response to be maintained in the face of fluctuating stress conditions and variable product formation, potentially resulting in a more robust producer (Dunlop et al. 2010). Strategies for developing resistant marker free strains with chromosomally encoded pathways and tolerance mechanisms are also essential both enerate a stable host platform and also for ease and safety of use in large industrial scales.

In this chapter, we sought to identify common sources of cellular growth and toxicities that might be encountered by a biofuel producer and discuss several targeted approaches that may help in the development of a better producer. However, other combinatorial and evolutionary strategies also exist to address similar problems and are a well-reviewed topic (Zhang et al. 2009). Recent studies promise new strategies that can be brought to bear on strain engineering such as the genome scale technologies developed for compiling and transplanting a complete mycoplasma genome into a heterologous host (Gibson et al. 2010). Alternate genome level approaches are the use of metagenomic fosmids to engineer tolerance to pretreatment inhibitors (Sommer et al. 2010). The importance of cellular engineering to optimize microbial physiology beyond pathway optimization is being recognized as an important aspect of strain development especially in the conversion of lignocellulosic biomass to biofuels.

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Author Queries

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Query Refs.	Details Required	Author's response
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