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Toxicological challenges to microbial bioethanol production and strategies for improved tolerance

Hannah Akinosho[1,2] · Thomas Rydzak[2,3] · Abhijeet Borole[3,4,5] · Arthur Ragauskas[1,2,4] · Dan Close[3]

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Abstract Bioethanol production output has increased steadily over the last two decades and is now beginning to become competitive with traditional liquid transportation fuels due to advances in engineering, the identification of new production host organisms, and the development of novel biodesign strategies. A significant portion of these efforts has been dedicated to mitigating the toxicological challenges encountered across the bioethanol production process. From the release of potentially cytotoxic or inhibitory compounds from input feedstocks, through the metabolic co-synthesis of ethanol and potentially detrimental byproducts, and to the potential cytotoxicity of ethanol itself, each stage of bioethanol production requires the application of genetic or engineering controls that ensure the host organisms remain healthy and productive to meet the necessary economies required for large scale production. In addition, as production levels continue to increase, there is an escalating focus on the detoxification of the resulting waste streams to minimize their environmental impact. This review will present the major toxicological challenges encountered throughout each stage of the bioethanol production process and the commonly employed strategies for reducing or eliminating potential toxic effects.

Keywords Toxicology · Biofuels · Bioethanol · Fermentation · Process engineering

✉ Dan Close
closedm@ornl.gov

[1] Renewable BioProducts Institute, Georgia Institute of Technology, Atlanta, GA, USA

[2] BioEnergy Science Center, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831, USA

[3] Biosciences Division, Oak Ridge National Laboratory, P.O. Box 2008, MS6342, Oak Ridge, TN 37831-6342, USA

[4] Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, TN, USA

[5] Bredesen Center for Interdisciplinary Research and Education, University of Tennessee, Knoxville, TN, USA

Introduction

The rise and importance of bio-based ethanol production approaches

The potential for ethanol to serve as a transportation fuel has been recognized for over a century. In 1826, Samuel Morey developed one of the earliest versions of the internal combustion engine, which he engineered to run using a combination of ethanol and turpentine. While his technological approach was innovative, the prevalent use of steam power overshadowed its adoption. Nicholas Otto renewed interest in ethanol-fueled combustion engines in 1860, however, the advent of the industrial age, which redistributed jobs from farming to manufacturing and increased taxes on alcohol, again caused the widespread use of ethanol to lose momentum. Several decades later, by the

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1890s, ethanol had nonetheless reestablished itself as a common fuel, and in 1896 Henry Ford developed the Quadricycle, the first known vehicle to operate on 100 % ethanol. However, soon after, the discovery of significant oil reserves in Texas led to widespread use of gasoline, which continues to this day. Nonetheless, despite gasoline's popularity, several countries including Brazil and France adopted 90 % gasoline and 10 % ethanol blends beginning in the early 1900s, and several gasoline/ethanol demand and price wars have ensued due to the great depression, World War II, and new innovations in transportation developed during this time period. More recently, ethanol demand again rose in response to the United States oil crisis of the 1970s but rapidly decreased as the country recovered during the 1990s. However, as a result of the political turmoil generated by these events, the United States government began to give farmers subsidies for ethanol production during this time, which has continued to shape the use of ethanol even today (Kirakosyan and Kaufman 2009).

Outside of the United States, governmental policies have greatly influenced the widespread adoption of ethanol as a transportation fuel in Brazil over the past 40 years. Beginning in 1975, the Brazilian government launched ProAlcool, a national program that strived to displace gasoline with bioethanol. Within 10 years of ProAlcool's launch, the bulk of cars in Brazil required ethanol blends containing 96 % bioethanol and 4 % water. By 1993 the Brazilian government had mandated that all remaining gasoline be blended to contain 20–25 % ethanol, and by 1999 the ProAlcool program began to be phased out as sustained bioethanol production required less government intervention and private companies penetrated the market (Balat and Balat 2009). Despite Brazil's heavy exploitation of ethanol, the United States remains as the world's largest ethanol producer (Chen and Khanna 2013), with governmental policies such as tax legislation, tax credits, and subsidies driving ethanol production and maintaining its competitiveness. The Energy Policy Act of 2005 established a renewable fuel standard (RFS) within the United States, which, as revised 2 years later by the Energy Policy Act of 2007, required that the amount of renewable fuels blended into transportation fuels increase by 27 billion liters between 2008–2022, with a minimum of 44 % of this ethanol derived from cellulosic sources (Balat and Balat 2009). By 2013 88.7 billion liters of ethanol were being produced annually in the United States, representing a significant increase from the 48.0 billion liters produced in 2007 (Scully and Orlygsson 2015). This increase may be due in part to the enactment of the volumetric ethanol excise tax credit (VEETC), which provided tax credits for blending ethanol with gasoline to offset the cost of producing ethanol domestically (Yacobucci et al. 2010). In addition to VEETC, the United States also established tariffs to curb the importation of sugarcane ethanol and encourage domestic ethanol production (Chen and Khanna 2013), however, both VEETC and the tariffs were allowed to expire in 2011.

Nonetheless, despite the application of these government policies supporting ethanol fuel use, factors such as cost and energy density per unit volume have continued to sustain the use of gasoline and diesel over ethanol fuel sources (EIA 2013). However, as technologies for ethanol production and utilization continue to improve, it is important to continually reevaluate the benefits of bioethanol-based fuels relative to their petroleum-derived counterparts (Table 1). Perhaps the important step towards realizing these benefits will be the reduction in ethanol production costs to the point where they are competitive with existing transportation fuels at the commercial level. To achieve this goal, the market will need to focus on either a thermochemical or bio-based production format in order to standardize production infrastructure and reach an appropriate economy of scale. In the thermochemical approach, steam is mixed with ethene, a hydrocarbon isolated from crude oil. This approach involves a phosphoric acid catalyst and requires high pressure (60 atm) and temperature (300 °C). While this approach is not only energy intensive and costly, it also still requires the use of fossil fuels as precursors. In contrast, the bio-based approach employs renewable materials, commonly in the form of agricultural wastes or plant biomass, to generate ethanol using the metabolic activity of microorganisms. These feedstocks contain complex carbohydrates, starches and/or sugars, which are substrates for microbial fermentation and conversion to ethanol. While the thermochemical production approach provides more rapid reactant conversions and ensures pure product formation, the bio-based approach provides a significant advantage in that it is fully independent from crude oil and holds the potential for lower production costs at a fully operational scale (Hirst 2002).

These bio-based strategies can employ any number of a diverse group of microorganisms that produce ethanol directly from sugars or hydrolyzed starches, or from pretreated, hydrolyzed biomass. Alternatively, more complex feedstocks such as cellulosic biomass can also be converted to ethanol using consolidated bioprocessing techniques, which utilize the performance of enzymatic hydrolysis and fermentation by a single microorganism (Lynd et al. 2002, 2005). While many microorganisms generate ethanol, the efficiency amongst them varies greatly and eliminates the practical industrial usage of many. Ideally, therefore, a viable candidate organism for ethanol production will generate more than 1 g ethanol/l/h, yield ethanol in excess of 90 % of its theoretical value for the provided feedstock

Table 1 Benefits of increased bioethanol fuel usage

Promotes energy independence by reducing crude oil imports
Allows domestic production from renewable resources
Lowers emissions when used as a gasoline additive or replacement
Promotes carbon dioxide offset when produced using bio-based sources
Stimulates agricultural employment in rural areas
Competes with gasoline and diesel to lower crude oil and refined fuel prices
Aids in meeting the world's increasing energy demands

input, tolerate ethanol concentrations greater than 40 g/l, be capable of growth at elevated temperatures and/or in acidic conditions, demonstrate resistance towards typical inhibitory compounds, and be economical to grow and maintain (Dien et al. 2003). Finally, the microbe should be able to utilize an extensive set of substrates, require only minimal input of simple nutrients to sustain growth, and generate ethanol as its major or exclusive product (Scully and Orlygsson 2015).

Commonly employed microbes for bio-based ethanol production

Saccharomyces cerevisiae, *Zymomonas mobilis*, *Escherichia coli*, and *Clostridium thermocellum* have emerged as common fermentation hosts for bio-based ethanol production (Table 2). *S. cerevisiae* is a yeast that has an extensive history in industrial fermentation and exhibits exceptional ethanol tolerance (Ginley and Cahen 2011). *Z. mobilis* is a bacterium that produces ethanol at concentrations 2.5-times greater than *S. cerevisiae* and can generate ethanol in excess of 90 % the theoretical yield (Weber et al. 2010). The classic bacterial production host *E.* coli is also used since it requires only simple nutritional inputs for growth and maintenance and has a lengthy history of use in recombinant technologies. Similarly, *C. thermocellum*, a thermophilic, Gram-positive bacterium, is employed due to its unique ability to naturally produce enzymes that hydrolyze a number of plant components and ferment cellulose-derived hydrolysis products.

Each of these microorganisms possesses unique advantages for ethanol production. However, none is naturally capable of expressing all of the idealistic ethanol production traits. For instance, yeasts such as *S. cerevisiae* can carry out fermentation at low pH, which minimizes contamination, and are traditionally separated from fermentation media easier than bacteria. Thermophilic microorganisms such as *C. thermocellum* can carry out fermentation at higher temperatures, which lowers viscosities, increases potential substrate loadings, and minimizes the need for cooling, but have limited genetic tractability and are not as well characterized as *S. cerevisiae* or *E. coli* (Scully and Orlygsson 2015; Weber et al. 2010). *E. coli*, unlike some ethanologenic microbes, can metabolize pentose sugars, but also experiences growth difficulties in the presence of glucose due to pentose catabolism repression. Furthermore, it does not tolerate ethanol as well as yeast, and is sensitive to environmental factors such as temperature and pH (Weber et al. 2010). These shortcomings do not immediately eliminate any of these microorganisms as bioethanol production hosts, but require the innovative application of toxicological mitigation strategies for improving their growth and ethanol synthesis characteristics if they are to usher in a new age of commercially viable bioethanol production.

Common culture strategies for bioethanol production and methods for mitigating their toxicological challenges

Batch culture

Batch cultures are closed systems where an inoculum is added to a vessel, and the cells then propagate over time. Throughout the batch process, neither the medium nor the culture broth are withdrawn, nor are additional volumes

Table 2 Representative microorganisms used for bioethanol production

Organism	Type	Gram + /−	Typical fermentation conditions
E. coli	Anaerobic, mesophilic bacterium	−	pH 6.0–8.0, 35 °C
Z. mobilis	Facultative anaerobic, mesophilic bacterium	−	pH 4.0–5.0, 30–40 °C
C. thermocellum	Anaerobic, thermophilic bacterium	+	pH 6.0–8.0, 60 °C
S. cerevisiae	Anaerobic, mesophilic yeast	N/A	pH 4.5–6.5, 25–35 °C

added to the vessel (with the exception of chemical additions for environmental regulatory processes such as pH control). As a result of this strategy, nutrient levels and cell populations will continually vary over the course of the fermentation. Cell growth in batch cultures can be divided into four phases: lag, exponential, stationary, and death. The lag phase describes the period between the introduction of the inoculum to the medium and the period when growth becomes apparent. The exponential phase describes the progression of microbial propagation, where nutrients are being exhausted and toxins are accumulating. The stationary phase describes the stage at which the rate of cell propagation equals the rate of cell death, while the death phase describes the period at which microbial population levels decline. Upon completion of fermentation, the vessel is drained, cleaned, sterilized, and filled with new medium in preparation for the next fermentation batch. Using the batch culture strategy, the primary toxicological limitations are the corresponding nutrient limitations and the accumulation of waste or cytotoxic metabolic products (Fig. 1a).

Modified (or fed) batch culture

Modified batch cultures are similar to batch cultures, with the exception that nutrients are either constantly or occasionally fed to the culture medium in the fermentation vessel. Under this design, a low initial substrate concentration is commonly employed, and the medium is generally added to less than half of the volume of the fermentation vessel. The inoculum is then added to the vessel and fermentation proceeds. As the substrate is consumed, additional substrate is slowly added back to the vessel, and the volume increases accordingly. Following the completion of fermentation, the vessel is drained and a small percentage of the inoculum is retained to inoculate the next batch (Ezeji et al. 2006). This strategy permits extended growth periods and greater cell densities and

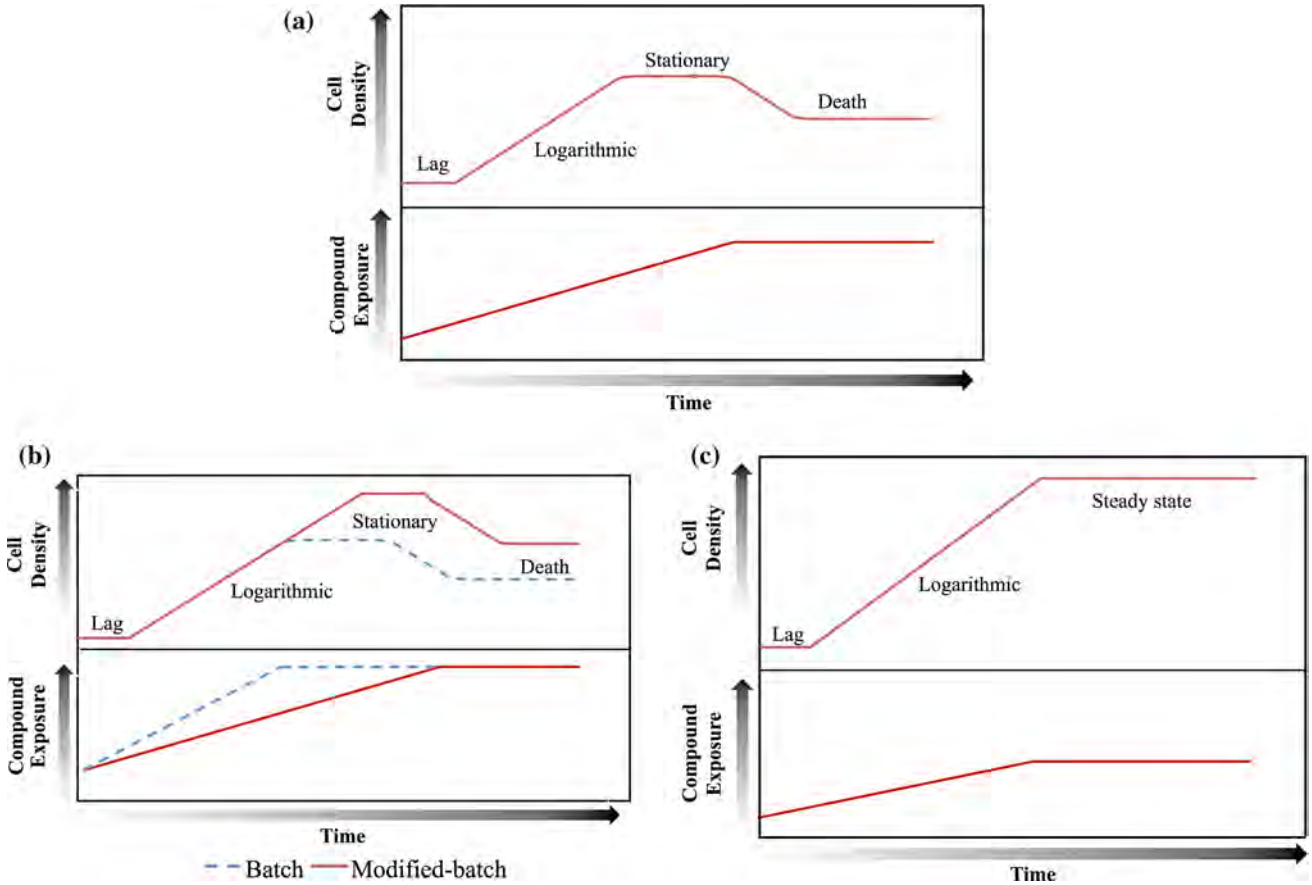

Fig. 1 Cytotoxic exposure profiles vary among the different culture strategies. **a** Using a batch fermentation strategy, potentially cytotoxic compounds can accumulate throughout the process due to the closed nature of the system. **b** The extended logarithmic phase of the modified-batch fermentation process temporarily reduces cytotoxic compound exposure comparison to batch fermentation through the dilutive effect of medium addition. **c** By employing an equal rate of medium addition and culture extraction, the continuous fermentation strategy can reduce exposure to cytotoxic byproducts or fermentation end points

encourages secondary metabolite production. However, because the culture medium is not removed, the toxicological challenges of waste and cytotoxic metabolic product formation remain, but are subject to dilution effects concurrent with medium addition (Fig. 1b).

Continuous culture

The use of a continuous culture strategy extends the exponential phase of microbial growth by maintaining steady growth rates (Fig. 1c). This growth method is commonly performed in a chemostat, which automatically withdraws spent culture medium and replaces it with fresh medium in a fixed-volume vessel. Under this design, densities, which are nutrition limited, dictate the feed rate of the medium to maintain constant growth rates. Perfusion culture continuously feeds and withdraws cell-free medium from the fermentation vessel to maintain the steady state. This circumvents the lag phase of growth, as well as the extensive cleaning and sterilization procedures observed in batch cultures, but incurs significant additional cost and increasingly complex equipment to perform.

Combined (or co-) culture

Co-culture, which can be performed under any of the above mentioned culture strategies, uses two microorganisms to ferment a substrate in a sterilized environment. The co-culture approach presents several unique benefits such as improved feedstock utilization efficiency and the simultaneous performance of metabolic reactions that cannot be achieved efficiently using a single organism, but also requires the careful selection of co-culture partners in order to ensure they are compatible under the same fermentation conditions and do not adversely affect one another due to their disparate metabolic processes and varying toxicological resistances.

Limitations on bioethanol production imposed by toxicological challenges

Restrictions imposed on feedstock inputs

Bio-based ethanol production will need to accommodate a variety of different feedstocks in order to achieve the economic targets required for competition with fossil fuels. However, the incorporation of varying feedstock classes poses toxicological challenges that must be dealt with in order to support downstream microbial fermentation. The lignocellulosic biomass feedstock class represents perhaps the largest untapped resource for bioethanol production, but its intricate structure has hindered its cost-effectiveness and marketability. Due to its high level of complexity, lignocellulosic biomass often requires a pretreatment step that employs chemical, physical, and/or biological means to facilitate the enzymatic hydrolysis of cellulose into sugars, often generating inhibitors that can lead to cytotoxicity (Jönsson et al. 2013). As a result, strategies must be developed to achieve the detoxification of pretreated lignocellulosic materials. These strategies can take the form of biological methods, such as the development of a recombinant *Z. mobilis* ZM4 (pHW20a-*fdh*) with the ability to break down the inhibitor formate (Dong et al. 2013) and the overexpression of oxidoreductases in *S. cerevisiae* to manage furfural toxicity (Heer et al. 2009), or chemical engineering methods such as overliming and neutralization of pretreatment hydrolysates (Mohagheghi et al. 2004, 2006).

The hemicellulose biomass feedstock class, while not directly inhibitory, also poses a challenge to obtaining optimal yields because it contains both hexose and pentose sugars as potential fermentation substrates. Therefore, when this class of feedstock is employed, it is important to use a production host capable of metabolizing the full suite of released substrates. *Z. mobilis*, for example, can ferment glucose, fructose, sucrose, raffinose, and sorbitol, and has also been engineered to utilize pentoses (Kuhad et al. 2011). *S. cerevisiae* ferments a broader range of pentoses, hexoses, disaccharides, oligosaccharides, dextrins, and starches. *C. thermocellum* is capable of fermenting glucose, cellobiose, and cellooligosaccharides, and *E. coli* can ferment a wide range of sugars (Rao et al. 2011). When feedstocks with high hemicellulose contents are employed, ethanologenic microorganisms that can ferment both pentoses and hexoses are often employed. This approach minimizes waste product generation and maximizes ethanol yield by using a greater proportion of the biomass sugars, but limits the number of potential hosts that can be used.

Restrictions imposed on product formation

Ethanol production by microorganisms is the result of a long chain of metabolic reactions. The root metabolic process leading to ethanol production is glycolysis, a series of enzyme-catalyzed reactions that convert glucose into pyruvate in order to generate the energy and reducing equivalents required for driving downstream cellular activity such as ethanol production. While glucose catabolism occurs via the Embden-Meyerhof-Parnas pathway for the majority of ethanol production hosts, in *Z. mobilis* it is achieved through the Entner-Doudoroff pathway instead (Joachimsthal et al. 1998). Regardless of the route taken to catabolize glucose to pyruvate, downstream fermentation pathways are then used to convert pyruvate to organic compounds such as ethanol. While, in this context, ethanol

is the product of interest, it is by no means the only product generated in this process. As a result, there are ample opportunities for deleterious byproducts to accumulate during the fermentation process. In some cases, these products are toxic and can result in detrimental effects on growth and metabolism. However, even when these byproducts are not cytotoxic, it is beneficial to minimize their formation in order to improve ethanol yields (Herrero et al. 1985b; Warnecke and Gill 2005), as has been demonstrated following metabolic engineering of flux in *C. thermocellum* (Argyros et al. 2011; Biswas et al. 2014, 2015; Rydzak et al. 2011). Unfortunately, caution must also be exercised to ensure that ethanol production does not reach concentrations that themselves have detrimental effects towards these microorganisms and their fermentative capabilities (Ma et al. 2013). These opposing challenges present a particularly interesting tightrope that must be walked in order to achieve economical bioethanol production, where, from an engineering standpoint, production should always be increased, but, from a biological standpoint, lower production levels support improved host health and synthesis efficiency.

Restrictions imposed on co-culture strategies

Co-culture, during which two microorganisms are concomitantly grown under aseptic conditions, is sometimes applied to enhance the fermentative abilities of a single microorganism or to augment substrate utilization. This approach is commonly encountered when hosts such as *C. thermocellum*, which can hydrolyze cellulose and hemicellulose to hexoses and pentoses, but can utilize only hexoses, are employed. Rather than forfeit the use of the released pentose sugars, *C. thermocellum* can be paired with *Thermoanaerobacterium saccharolyticum*, a non-hemicellulolytic anaerobic thermophile that metabolizes a broader range of substrates, to increase ethanol yield. Using this strategy, metabolically engineered *C. thermocellum* and *T. saccharolyticum* have yielded 38.1 g ethanol/l from 92.2 g/l of Avicel (Argyros et al. 2011). Similarly, Xu and Tschirner created a *C. thermocellum* ATCC 27405 and *Clostridium thermolacticum* ATCC 43739 co-culture that enhanced saccharification. While this co-culture did not eliminate side product formation, it did generate higher ethanol yields than the individual monocultures alone (Xu and Tschirner 2011). *Z. mobilis* has also been employed for co-culture with *Pichia stipitis,* and has been shown to efficiently ferment glucose and xylose to 96 % of their theoretical yield (Fu et al. 2009). However, the co-culture approach also presents a difficult balancing act with cytotoxicity. For example, one microorganism may generate ethanol as the primary byproduct, while the other microorganism carries out mixed acid fermentations. The resulting secondary metabolites may also be toxic to one organism despite not being toxic to the other. Other environmental factors, such as pH or temperature, may also affect the growth of one of the partners. For example, yeast will ferment most efficiently at a low pH, which may be inhibitory to some bacteria.

Common toxicity inputs and their mechanisms of action

Regardless of the production format employed, microbes tasked with bioethanol production face a variety of potentially cytotoxic compounds that are inherent to the ethanol production process. Some of these, such as the furans furan-2-carbaldehyde (furfural), 5-hydroxymethylfurfural (HMF), and various phenolic compounds, result from the treatment processes employed to breakdown biomass and provide the sugars needed for ethanol production. Others, such as acetate and ethanol itself, result as natural byproducts of the organism's metabolism under fermentation conditions. Some compounds, such as acids, remain almost ubiquitous, and can be derived from nearly all stages of the process in one form or another. While the timing and intensity of compound exposure can be controlled through the use of differential culture and substrate treatment methods (Taylor et al. 2012), these chemicals represent the most common, and problematic, cytotoxicological challenges encountered throughout the ethanol production process (Luo et al. 2002). A thorough understanding of their toxicological mechanisms of action is therefore warranted in order to understand how they manifest their negative impacts on ethanol producing organisms, as such an understanding is key to mitigating these effects through the application of proper genetic or engineering controls.

Cytotoxic byproducts of biomass substrate treatments

The only economically sustainable method for bioethanol production is one that relies on the liberation of sugar from renewable biomass feedstocks. However, this strategy is complicated in that pretreatment of these materials is often a necessary prerequisite to obtaining efficient saccharification (Klinke et al. 2004; Lin and Tanaka 2006). Along with the sugars released during this process, the treatment conditions employed also give rise to the release of furans, phenolics, and organic acids (Chandel et al. 2007a, 2010; Mussatto and Roberto 2004; Palmqvist and Hahn-Hägerdal 2000), all of which have been demonstrated to induce downstream cytotoxic effects (Chandel et al. 2007b, 2011; Palmqvist and Hahn-Hägerdal 2000). While the resulting

endpoints of exposure all similarly manifest in the form of reduced yields and titers, the cytotoxicological mechanisms that give rise to these detriments vary by compound, and therefore often require different strategies for mitigation.

Furan compounds

The major furan compounds released during biomass treatment are furfural and HMF. Furfural is derived from the hemicellulose component of lignocellulosic materials (Zaldivar et al. 1999). During the treatment process, furfural is formed via the dehydration of pentose monomers, resulting in the formation of an aldehyde moiety that is significantly more reactive (Nimlos et al. 2006; Watanabe et al. 2005). Via a similar mechanism, HMF is formed from the dehydration of glucose sourced from the cellulosic biomass component (Taylor et al. 2012). Both furfural and HMF have been demonstrated to occur at concentrations between 0 and 5 g/l during dilute acid pretreatment of lignocellulosic biomass (Larsson et al. 1999a, 1999b; Martinez et al. 2001; Ranatunga et al. 2000), however, despite their negative effects they remain a consistent consideration due to the prevalence of this treatment method at the industrial scale (Sierra et al. 2008; Wooley et al. 1999; Yat et al., 2008).

The primary route of cytotoxicity for the furan compounds results from their reactivity with intracellular components such as nucleic acids and enzymatic proteins (Mills et al. 2009). Furfural, in particular, has been demonstrated to lead directly to the mutation of DNA through the introduction of single stranded breaks at sequences containing short repeats of either thymine or adenine residues (Hadi and Rehman 1989; Rahman and Hadi 1991), as well as through the mediation of large genetic rearrangements in plasmids (Khan and Hadi 1993). However, despite the negative genetic impact of exposure, furans primarily impose growth and metabolic limitations rather than outright cellular death. In yeast, furans become inhibitory between 0.5 and 2.0 g/l (Rumbold et al. 2009), leading to prolonged lag phases as the cells attempt to detoxify or circumvent their inhibitory effects (Almeida et al. 2011; Ma and Liu 2010). A similar effect on growth is observed in prokaryotic species, where exposures up to six-fold above observed growth-inhibitory concentrations have not resulted in significant cellular destruction (Zaldivar et al. 1999). The primary mechanism through which the furans act, therefore, appears to be their disruption of metabolic enzymatic functionality. Studies in *E. coli* have demonstrated that furans can serve as substrates for alcohol dehydrogenase as well as aldehyde dehydrogenase and pyruvate dehydrogenase (Modig et al. 2002). In addition to these inhibitory roles, their reactivity can also result in the generation of reactive oxygen species, providing a secondary pathway through which DNA mutation and protein inactivation can occur (Allen et al. 2010; Yasokawa and Iwahashi 2010).

Phenolic compounds

Phenolic compounds such as vanillin, syringaldehyde, and ferulate, are released primarily from lignin during biomass processing (Sannigrahi and Ragauskas 2013) and have been shown to possesses any number of functional moieties, such as hydroxyl, carboxyl, or formyl groups, in addition to their characteristic phenolic base (Mills et al. 2009). Previous studies have suggested that, following biomass processing steps, organisms can be exposed to phenolic compounds at concentrations up to 3 g/l, but that the specific chemicals observed are dependent on both the biomass and treatment methods employed (Fenske et al. 1998; Larsson et al. 1999b; Martinez et al. 2000). However, despite the differences in exposure levels and functional groups presented, the primary cytotoxic mechanism of action for these compounds is the destabilization or disruption of the cellular membrane. As a result of this mechanism, the toxicity of phenolic compounds correlates with their octanol–water partition coefficient (K_{ow}) (Mills et al. 2009), which represent their relative hydrophobicity (the lower the K_{ow} value, the more polar the compound) (Zingaro et al. 2013). Those phenolics with larger K_{ow} values are therefore more favored for membrane integration, resulting in the disruption of membrane gradients and cellular transport (Fitzgerald et al. 2004) and the alteration of membrane lipid/protein ratios (Keweloh et al. 1990).

Residual or liberated acids

Ethanol producing microorganisms are exposed to acidic compounds, primarily weak organic acids, either through their residual presence following biomass treatment procedures, as liberated compounds during the biomass breakdown process, or as naturally produced metabolic byproducts generated by the organisms themselves. While steps are traditionally taken to limit the amount of acid that is carried over from biomass treatment procedures, it remains important to understand the cytotoxic mechanisms resulting from acid exposure in order to develop improved engineering controls and genetic modification strategies that enhance both fermentation workflow design and the organism's tolerance to any residual or liberated acidic compounds. These mechanisms can manifest through one of several different cytotoxicological routes. At a basic level, following introduction into the cell, any acid can dissociate into its anionic and proton components, resulting in disruption of transmembrane pH potentials. When this occurs, ATP production within the cell becomes limited as the gradient required for ATP synthesis is diminished

without a corresponding increase in ATP formation (Axe and Bailey 1995). Under this mechanism, the toxicity of a given acid is highly correlated with its membrane permeability, with more permeable acids having a greater disruptive effect on ATP generation and resulting in decreased growth. The resultant anionic component of these acids can also contribute to cytotoxicity directly through alteration of cell turgor pressure, which can result in deleterious growth phenotypes that negatively affect product yield and titer (Roe et al. 1998). In addition to these indirect effects, some acids, such as formic acid and propionic acid, have also been demonstrated to interfere with DNA, RNA, protein, and cell wall synthesis. By limiting synthesis of these components, acid exposure was found to induce a bacteriostatic growth phase for *E. coli*, with the negative growth effects maintained even following a recovery period in the absence of acid treatment when compared to non-acid-exposed controls (Cherrington et al. 1990). Similarly, in *S. cerevisiae*, exposure to acetic acid has been shown to effectively knock out pentose sugar consumption, leading to the over accumulation of pentose-phosphate pathway intermediates and inhibiting growth (Hasunuma et al. 2011).

Residual or liberated salts

The presence of ionic compounds, such as salts, within the growth medium is necessary to support microbial metabolism. However, unlike routine laboratory cultivation in defined medium, the use of biomass feedstocks can result in the presence of ionic salts at levels capable of inhibiting growth. This inhibition is realized due to an increase in osmotic pressure, which places stress upon the host as it attempts to maintain its membrane integrity and metabolic activity levels (Wadskog and Adler 2003). *S. cerevisiae* has been demonstrated to tolerate up to 1.5 M concentrations of sodium chloride (Maiorella et al. 1984), and has been observed to increase ethanol output in response to moderate increases in salt concentrations, likely due to an increase in ATP demand resulting from up regulated plasma membrane transport (Jönsson et al. 2013). Cellulolytic thermophillic bacteria, as well as *E. coli*, have routinely demonstrated product inhibition in the presence of organic acids and salts (Lynd et al. 2005), and controlling their presence within the growth medium can greatly increase product output (Ingram et al. 1998).

Potentially inhibitory metabolic products

In addition to the exogenously applied and biomass derived inhibitory compounds discussed above, self-produced metabolic products can also negatively impact ethanol-producing microorganisms under relevant fermentation conditions. The two primary deleterious metabolic products encountered during this process are the carboxylic acid acetate and the process target product, ethanol. As natural metabolic products, acetate and ethanol are normally not toxic to host cells under normal growth conditions, however, once ethanol production has been shifted beyond the scale of the host's natural requirements, the overproduction of these components necessary to meet artificially inflated industrial targets can result in accumulation concentrations at scales for which cells have not developed a means of efficient processing.

Acetate

Acetate is the primary carboxylic acid generated during lignocellulosic hydrolysis (Taylor et al. 2012), but is also crucial to normal cellular development during the routine synthesis of ATP, a process that is especially important in fermentive bacteria (Papoutsakis and Meyer 1985). Under the fermentation conditions employed for industrial scale ethanol production, this process can be inhibited or overwhelmed, and acetate can begin to accumulate at greater concentrations than can be efficiently processed by the cells. When this occurs, the physical properties of acetate will cause it to impart negative cellular effects similar to those manifested through exposure to any of the previously detailed acidic compounds. Furthermore, the cumulative effects of acetate liberation and endogenous production can also conspire to jointly inhibit product synthesis. As acetate is naturally produced within the cell, the host's metabolism will seek to reduce its intracellular presence in order to avoid the negative effects of transmembrane pH potential disruption, turgor pressure alteration, and macromolecule synthesis inhibition. However, as increasing concentrations of acetate are then introduced into the medium following liberation from biomass, the environmental pH will undergo a corresponding decrease, resulting in corresponding alterations to production strain metabolism. These effects have been well detailed through observation of solventogenic and non-solventogenic *Clostridia* species, which have been shown to induce solvent production or cease metabolic activity upon acetate-mediated pH changes, respectively (Nicolaou et al. 2010).

Ethanol

Ethanol represents an interesting production target due to the fact that it is inherently cytotoxic to all microorganisms at high concentrations. However, as the desired fermentation product, there is significant human-mediated evolutionary pressure to continuously increase the amount that can be tolerated in order to increase production efficiency and reduce the associated costs of production. These

seemingly opposed goals therefore require that only organisms with naturally high ethanol tolerances be employed for these procedures, or that ethanol tolerance be engineered into the production host via genetic means. Understanding the diverse mechanisms underlying ethanol's toxicity is therefore key to achieving the latter of these approaches.

Regardless of the host employed, ethanol's primary toxicological mechanism is imparted through detrimental increases in membrane fluidity, which leads to increased permeability, altered membrane gradients, disordering of membrane associated proteins, and changes in cellular osmolality. In addition to this general mechanism, ethanol has also been demonstrated to impart targeted effects in several key fermentation hosts. Various studies in *S. cerevisiae* have demonstrated that ethanol also alters vacuole morphology (Meaden et al. 1999), induces heat shock protein synthesis, which leads to corresponding reductions in RNA and protein synthesis activity (Hu et al. 2007), and leads directly to protein denaturation (Hallsworth et al. 1998). Similar studies in *E. coli* have served to further elucidate the potential underlying causes for these effects, demonstrating that protein synthesis inhibition may be due to ethanol-induced ribosomal stalling at non-start methionine codons, while existing proteins may be inactivated through solvent exposure of their hydrophobic regions following direct interaction, leading to miss-folding and resulting in abnormal function (Bull and Breese 1978; Haft et al., 2014). Beyond the classical *S. cerevisiae* and *E. coli* hosts, these same targeted protein interactions may also apply to the more recently employed *Clostridium*-based production hosts, which have shown similar membrane and proteomic effects when exposed to ethanol as well (Zhu et al. 2013).

Underlying cellular detoxification mechanisms

To mitigate the cytotoxic effects encountered during bioethanol fermentation, cells have developed several primary strategies for compound detoxification or avoidance, or for the maintenance of growth and metabolism under the suboptimal conditions encountered during compound exposure. While recent research has made it clear that individual organisms may respond with unique approaches (Dunlop 2011), several general responses have been identified including the enactment of general stress response pathways, modification of membrane component composition, direct chemical efflux, metabolic conversion, and the alteration of transcriptional and translational expression. By eliciting one or more of these general pathways, the production host is able to increase its tolerance to the cytotoxic compounds discussed above and allow for the attainment of higher production titers if ethanol synthesis can be maintained under the energetic burden of the applied response pathway (Nicolaou et al. 2010).

General stress response pathway activation

General stress response pathways represent a catchall method for dealing with adverse environmental conditions such as heat shock, cold shock, or solvent exposure. As such, the enactment of these pathways following toxicological challenge has been widely observed across the common bioethanol production hosts (Nicolaou et al. 2010). In yeast, ethanol-initiated activation of general stress response pathways has been observed to closely parallel heat shock-induced pathway expression (Piper 1995), although many organisms share common stress response strategies, so these results may well be applicable in prokaryotic species as well. The enactment of this response profile centers primarily on the employment of transcriptional regulators and chaperone proteins. Transcriptional regulators, such as RpoS in *E. coli* (Lombardo et al. 2004), or Hsf1 in *S. cerevisiae* (Ding et al. 2009) serve as a means for quickly up- or down-regulating large numbers of genes in order to enact changes that maximize protein stability, maintain key metabolic activities, enable DNA repair, and support physiological maintenance. Of these activities, the one that plays the most well characterized role during the bioethanol fermentation process is that of maximizing protein stability. This task is mediated through the expression of chaperone proteins. Under normal growth conditions, these proteins aid in the assembly of nascent peptides, however, during times of stress they are re-purposed to re-fold damaged proteins in order to prevent errant activity or the loss of crucial enzymatic functions. Several studies have demonstrated that the up-regulation of these chaperones, such as GroESL and Hsp33 in prokaryotes such as *E. coli* and *Clostridium* species (Lund 2009; Tomas et al. 2003) and Hsp30 in *S. cerevisiae* (Seymour and Piper, 1999), can significantly improve ethanol tolerance by securing protein function within the cells and permitting continued cellular metabolic activity.

Alterations to membrane composition

The cellular membrane represents the first line of defense against cytotoxicity. It must remain capable of providing an effective barrier to prevent the entry of cytotoxic compounds, but is constrained by an absolute requirement to maintain nutrient uptake and electrical gradient potentials at a rate capable of supporting cellular survival. The traditional production hosts *E. coli* and *S. cerevisiae* both display similar membrane modifications under ethanol exposure, with each increasing the incorporation of

unsaturated fatty acids to maintain the function of their membrane associated protein machinery (Beaven et al. 1982; Ingram 1976). Other production hosts, such as *Clostridium* species, take an opposing approach and increase the incorporation of saturated fatty acids, longer chain fatty acids, or plasmalogens, as this allows them to increase rigidity and combat the increased membrane fluidity imparted by ethanol exposure (Timmons et al. 2009; Weber and de Bont 1996). With continued exposure, these changes can even become fixed within the membrane, further demonstrating their utility for providing a selective growth advantage during ethanol exposure (Isken and de Bont 1998). Beyond these changes to membrane composition, there is also evidence suggesting that integral membrane protein expression patterns can be altered following ethanol exposure, with up-regulated expression of the TolC protein in the *E. coli* outer membrane, for example, having been shown to increase solvent tolerance (Aono et al. 1998). Similarly, omics-based approaches have demonstrated evidence indicating yeast may alter their cell wall composition in response to solvent exposure as well (Zhao and Bai 2009).

Direct chemical efflux

Perhaps the most basic route for preventing compound cytotoxicity is simply the removal of the chemical from the cell so that it cannot impart its negative effects. This approach is accomplished through the action of efflux pumps. These pumps are multi-protein complexes that consume energy, often in the form of ATP, to move their target compound against its diffusion gradient and out of the cell. These pumps have been best characterized for their role in preventing ethanol fermentation-associated cytotoxicity in yeasts, where the ATP binding cassette pump Pdr12p has been shown to remove acidic compounds from the cell in order to prevent the loss of proton motive force and to aid in the maintenance of physiological turgor pressure (Hazelwood et al. 2006, Holyoak et al. 1999). Similarly, but although not as well characterized, *E. coli* has been suggested to utilize the MdfA efflux pump to maintain its intracellular pH during fermentation (Krulwich et al. 2005), although at this time there has been no work to indicate that any known efflux pump can confer increased tolerance to ethanol itself (Ankarloo et al. 2010).

Metabolic conversion to less toxic species

Compounds that cannot be directly removed from the cell may also be detoxified via the host's metabolism. When this occurs, the toxic compound is processed into a non-toxic or less toxic form that can then either be further processed by additional metabolic reactions or excreted in its modified form. This strategy is a common approach for mitigating the toxicity of furan compounds, which are converted to their less toxic alcohol forms via various furfural reductases/alcohol dehydrogenases (Clarkson et al. 2014; Gutierrez et al. 2002; Gutierrez et al. 2006; Palmqvist and Hahn-Hägerdal 2000), and for phenolic compounds, including cinnamic, p-coumaric, and frolic acids, which can be similarly detoxified via phenylacrylic acid decarboxylase (Clausen et al. 1994; Goodey and Tubb 1982; Mukai et al. 2010). Furthermore, these detoxification enzymes appear to be widespread within solvent exposed organisms. Expression of a *Sphingomonas* sp 14DN61 genomic library in *E. coli* identified an aldehyde dehydrogenase (PhnN) capable of converting vanillin, another toxic aldehyde found in lignocellulosic hydrolysates, to its less toxic carboxylic acid, vanillate, and a homologous vanillin dehydrogenase (*ligV*) from *S. paucimobilis* SYK-6 has also been shown to be essential for *E. coli* growth on vanillin (Peng et al. 2005).

Alterations in gene expression dynamics

In addition to the transcriptional changes imparted through general stress response activation, there is mounting evidence indicating that ethanol production hosts are similarly capable of altering their gene expression dynamics in response to prolonged growth under ethanol fermentation conditions, such as those that would be encountered during strain development. This evidence stems from a series of investigations that compared ethanol-adapted strains with their non-adapted counterparts. A comparison of ethanol-producing *E. coli* has demonstrated that, in response to ethanol stress, over 5 % of all genes demonstrate significantly altered transcriptional levels (Gonzalez et al. 2003). As anticipated, many of these genes were implicated in general stress response regulation or membrane synthesis, however, several other gene categories such as glycine and betaine metabolism were identified as well, with the hypothesis that these components are likely involved in the mitigation of osmotic stress (Wood et al. 2001). Targeted engineering strategies have also been employed in *E. coli* to demonstrate the utility of non-stress response gene transcriptional alteration for improved ethanol tolerance. A recent approach that modified the global transcription factor cAMP receptor protein to increase tolerance revealed that, in addition to general stress response genes, the resulting ethanol-tolerant strains had also accumulated transcriptional changes in both central intermediary metabolism and iron transport as well (Chong et al. 2013).

Similar approaches have been taken in *S. cerevisiae*, where a global gene analysis following ethanol exposure revealed that 6.3 % of all genes transcriptional levels were altered as a result of the exposure, with an almost equal

number of up- and down-regulated transcripts (Alexandre et al. 2001). While just over half of all of the genes displaying transcriptional changes were involved in general stress responses, the remaining genes represented processes such as ionic homeostasis, trehalose synthesis, antioxidant defense, and energy metabolism, providing ideal targets for downstream targeted engineering approaches. Other production hosts, such as *Clostridium* species, also present significant alterations to gene expression. While the majority of work with these species has highlighted functional expression changes such as nutrient sensing and cellulosome synthesis (Akinosho et al. 2014), additional studies demonstrate that these expression dynamics can influence energy and redox metabolism as well (Linville et al. 2013; Wilson et al. 2013).

Methods for mitigating toxicity during bioethanol production

A number of comprehensive reviews have detailed tolerance and stress responses to solvents and lignocellulosic hydrolysates in *S. cerevisiae*, *E. coli*, *P. putida*, and *C. acetobutylicum* (Almeida et al. 2007; Mills et al. 2009; Nicolaou et al. 2010; Ramos et al. 2002; Sardessai and Bhosle 2002), however, there has been less emphasis placed on detailing the specific mechanisms employed to mitigate the toxicological challenges encountered by hemicellulolytic and thermophilic microorganisms. Given the recent advances that have been made in using these organisms as hosts for bioethanol production, it is important to consider the potential evolutionary and genetic engineering strategies that have been enacted to mitigate ethanol and lignocellulosic hydrolysate toxicity in representatives of these species such as *C. thermocellum* and other *Clostridia*, and to parallel these findings with those of more intensively studied organisms. Using this approach, valuable insights can be obtained towards understanding both the general and organism specific strategies for overcoming cytotoxicity during bioethanol production.

Methods for identifying genomic engineering targets

Tolerance to ethanol and other toxic byproducts resulting from lignocellulosic hydrolysis is a complex, multigenic, and pleitropic process. Due to this complexity, the most effective strategy for selecting stains that are tolerant to, or thrive in, the presence of high ethanol or other toxic chemical titers has been through strain evolution. However it is important to note that tolerance (and metabolism), versus growth, as well as ethanol production capabilities, may have additional and unforeseen implications on downstream bioprocesses that need to be considered as well. Furthermore, ambiguity remains in separating inherent stress responses in relation to the adaptive tolerance afforded though the evolutionary adaption process. In general, however, responses to chemicals that an organism encounters routinely (e.g., ethanol as a metabolic byproduct in *S. cerevisiae* or *Z. mobilis*) are considered to be evolutionarily based, and likely require a complex pleiotropic signal transduction system to accommodate the various changes responsible for their physiological adaptation (e.g., changes in membrane composition, stress responses, energy metabolism, protective metabolite metabolism). Nonetheless, there are several compelling examples that demonstrate how single mutations or genetic complementation can confer significant tolerance to toxins as well.

Identification of targets known to classically influence ethanol tolerance

Understanding the physiological responses and expression profile dynamics of organisms exposed to ethanol can elucidate potential targets for metabolic engineering approaches aimed at synthetically increasing ethanol tolerance. For example, using information from studies demonstrating that heat shock proteins are induced in response to solvent stress in *C. acetobutylicum* (Terracciano and Kashket 1986), the *groESL* operon was targeted for overexpression. This resulted in an 85 % decrease in growth inhibition in response to butanol, and 33 % higher butanol yields (Tomas et al. 2003). Subsequent studies on this strain demonstrated that *groESL* overexpression also increased the expression of other heat shock proteins as well (Tomas et al. 2004). In addition, recent inverse metabolic engineering approaches have also proven effective in increasing ethanol tolerance. Using this strategy, Hong et al. (Hong et al. 2010) employed endogenous genomic libraries to demonstrate that overexpression of four genes (*INO1*, *DOG1*, *HAL1*, or a truncated version of *MSN2*) was capable of increasing ethanol tolerance, specific growth rate, titers, and productivities. A similar approach was also employed to identify a xenobiotic responsive element that confers butanol resistance in *C. acetobutylicum* (Borden and Papoutsakis 2007).

Identification of targets using genome sequencing

Increasingly, whole genome sequencing is being employed to aid in the identification of novel engineering targets. When combined with enzyme analysis and complementation studies, this approach has proven to be a powerful method for identifying targets that can significantly enhance ethanol tolerance. In a recent study, an ethanol tolerant *C. thermocellum* strain was sequenced and

500 genetic differences were identified relative to the parent strain (Brown et al. 2011). While these mutations were randomly distributed among non-coding regions, mutations within coding regions were most prevalent in membrane protein encoding genes previously shown to be differentially expressed (Williams et al. 2007), genes in mutational hotspots encoding proteins involved in cellulose degradation, genes for hypothetical proteins adjacent to phage/transposase genes, and a particular hotspot consisting of a putative 10 gene operon (Cthe_0422-Cthe_0431) previously proposed to be involved in ethanol and H_2 production. Interestingly, a bifunctional acetylaldehyde/alcohol dehydrogenase (*AdhE*) within this hotspot was shown to contain mutations that resulted in a change of cofactor specificity from NADH to NADPH. Subsequent introduction of this mutant allele in place of the wild type gene in the wild type strain was shown to enhance ethanol tolerance to the same level as the ethanol tolerant strain, without affecting ethanol yields (Brown et al. 2011). This not only demonstrates how genomic sequencing can serve as a powerful tool for identifying metabolic engineering targets, but also highlights how a single gene can enhance ethanol tolerance by impacting redox and electron balancing to confer increased ethanol tolerance.

Identification of targets using integrated omics approaches

When applied in lieu of genomic approaches, which identify changes influencing how proteins function, the transcriptomic, proteomic, and metabolomic analysis of wild type strains challenged with ethanol can provide insight towards how the timing and coordination of gene expression and pathway activation can be employed to mitigate toxicity. However, when applied in tandem with genome re-sequencing of strains with increased ethanol tolerance, these approaches can also help to differentiate between inherent adaptation mechanisms and evolutionary-based mechanisms. This has been exemplified in recent work investigating how *C. thermocellum* strains can adapt to ethanol stress. Proteomic membrane profiling in an ethanol tolerant *C. thermocellum* determined that 73 % of all membrane proteins, including those involved in carbohydrate transport and energy metabolism, were detected at lower levels relative to wild type strains (Williams et al. 2007). Additional work by Yang et al. (2012) employed an integrated transcriptomic, metabolomic, and proteomic approach to assess the physiological and regulatory responses of wild type cells challenged with ethanol in continuous cultures. Their analysis revealed that observed reductions in growth following ethanol stress correlated with inhibition of glycolysis and pyruvate catabolism, and the buildup of cellobiose and glycolytic intermediates. These results corroborated previously published results showing that ethanol addition impeded glycolysis and led to intracellular accumulation of glucose-6-phosphate and fructose-6-phosphate (Herrero et al. 1985a), suggesting that decreased ATP availability due to ethanol-induced H^+ membrane permeability was the primary cause of growth inhibition. Furthermore, an observed differential expression of hydrogenases and an RNF-like NADH:ferredoxin oxidoreductase suggested that these enzymes may also play a role in rebalancing the cellular redox state in response to ethanol stress, providing several potential targets for downstream metabolic engineering efforts aimed at increasing ethanol tolerance.

Common methods for genetically engineering ethanol tolerance

Natural and directed strain evolution

The natural evolution strategy for selecting mutant strains with elevated ethanol tolerance involves exposing a microorganism to increasing concentrations of ethanol and repeatedly selecting for the fastest-growing (or surviving) strains, followed by the purification of single-colony lineages from each successive treatment. This approach may be performed either by using a serial transfer approach that selects for improved-growth phenotypes in the presence of ethanol through sequential batch culture inoculations (Herrero and Gomez 1980; Shao et al. 2011; Williams et al. 2007), by progressively increasing ethanol concentrations in continuous cultures (Joachimsthal et al. 1998), or through growth on gradient agar plates and subsequent selection of the largest colonies. The directed evolution approach expedites this process by treating seed cultures with mutagens such as *N*-methyl *N*-nitro *N*-nitrosoguanidine or ultraviolet light, via whole genome shuffling, or through the expression of deletion libraries (for a review of this approach, see Nicolaou et al. 2010). For both methods, genomic re-sequencing of the isolated tolerant mutants is employed following selection to provide insight towards the mechanisms that have evolved to improve tolerance (Brown et al. 2011; Linville et al. 2013).

Overexpression of endogenous or exogenous tolerance genes

Following the identification of genes endowing mutant strains with increased ethanol tolerance, customized strains can be specifically engineered that overexpress either native or exogenous versions of these genes in order to endow the strain with an improved tolerance phenotype without the incorporation of additional, potentially deleterious genetic alterations. The effectiveness of this strategy has been demonstrated on multiple occasions, such as

through overexpression of the native NADPH-dependent alcohol dehydrogenase ADH6p in *S. cerevisiae*, which results in increased HMF tolerance (Petersson et al. 2006). Similarly, increasing flux through the pentose phosphate pathway by overexpressing glucose-6-phosphate dehydrogenase has been shown to increase NADPH availability for furfural reductases/alcohol dehydrogenases and further improve furfural tolerance (Gutierrez et al. 2006). In some cases, the overexpression strategy can also be used to increase production rates as well, as has been demonstrated with *PAD1* overexpression, which improved ethanol production rates by 29 % on spruce dilute acid hydrolysate (Larsson et al. 2001), or heterologous expression of the *Trametes versicolor* laccase gene, which was used to convert phenolic compounds to radicals that, in turn, polymerized to form high-molecular mass products (Larsson et al. 2001).

Reduction or elimination of genes producing deleterious byproducts

Although less common, reductive genomic approaches that eliminate native genetic architecture can also be employed to increase tolerance and improve ethanol output. In these strategies, genes that are not required for survival, but have been demonstrated to produce potentially cytotoxic metabolic intermediates or byproducts are removed from the genome to prevent deleterious effects on cellular growth or metabolism. Similarly, genes that permit the shuttling of metabolic intermediates into pathways that compete with ethanol production can also be eliminated in order to disable these pathways and more effectively route flux towards ethanol production. An example of this reductive approach can be seen in the silencing of the *yqhD* and *dkgA* NADPH-dependent oxidoreductases of *E. coli*, which increases tolerance to HMF exposure (Miller et al. 2009).

Common engineering controls for mitigating toxicity

A number of non-biological engineering control methods can be employed to minimize toxicity during the bioethanol production process. These include enzymatic treatments, chemical treatments, liquid–liquid or liquid–solid extraction procedures, and heating and vaporization applications (Jönsson et al. 2013). In addition, supplementation of zinc in continuous yeast cultures has been demonstrated to increase trehalose tolerance and significantly improve ethanol tolerance (Zhao et al. 2009), demonstrating that medium manipulation may also be used to enhance ethanol tolerance. From a workflow development perspective, increased inoculum sizes, the optimization of fermenter pH, recirculation or immobilization of cells, and the use of lower substrate dilution rates have all also been demonstrated to result in increased ethanol productivity and can be employed as well, however, these consideration must be made such that they remain compatible with the chosen bioreactor design to ensure efficient production rates (Brethauer and Wyman 2010; Palmqvist and Haggett 1997).

Mitigating the ecological impacts of bioethanol production

Toxicological compounds in bioethanol production waste streams

Between 5 and 10 % of the biomass entering an ethanol biorefinery will end up in the waste stream. While this includes pretreatment byproducts, fermentation byproducts, cell mass generated during fermentation, and any unconverted biomass polymers and oligomers, the primary toxic compounds found in these wastewater streams are the same phenolic, furan aldehyde, and organic acid compounds that presented cytotoxicological challenges during the fermentation process. The presence of these compounds in the waste stream means that it cannot be directly recycled until they are effectively detoxified to the point where they do not pose an environmental risk. Of these compounds, acetate is particularly concerning as a bioethanol production wastewater component because, while it is not toxic at low concentrations, it can add to the biological oxygen demand (BOD) of wastewater streams. In general, the concentration of acetate in biorefinery wastewater resulting from conversion of corn stover to ethanol is in the range of 6–10 g/l (Humbir et al. 2011), however, these levels can result in toxicity due to their potential for oxygen deprivation within natural water bodies. Therefore, the removal of acetate is essential for managing waste stream toxicity. A number of technologies have been investigated for this process, including ion exchange and membrane-based technologies (Nilvebrant et al. 2001), polymeric adsorption (Weil et al. 2002), precipitation using chemicals such as $Ca(OH)_2$ (overliming) (Martinez et al. 2000), and solvent extraction (Palmqvist and Hahn-Hägerdal 2000). However, these approaches are only partially effective and add considerable costs to the fermentation process, while still leaving some of the inhibitors in the process streams. Any attempt to recycle and reuse process water using these processes is therefore significantly limited due to the potential for cytotoxic build-up.

The use of microbial fuel cells for mitigating bioethanol production ecotoxicology

Detoxification of bioethanol waste streams using compound degradation via aerobic digestion has served as the

traditional method for wastewater treatment. However, owing to its high energy consumption, it will likely be limited in future systems that strive to increase energy efficiency. In light of this consideration, microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) have emerged as potential alternatives for the treatment of wastewater streams because they can produce energy while simultaneously removing toxic compounds to allow for a net reduction in the energy input consumed during the treatment process (Borole et al. 2009; Borole 2011; Borole et al. 2013). Microbial fuel cell-based removal of acetate has previously been reported (Lee et al. 2003; Liu et al. 2005; Rabaey et al. 2005) and has demonstrated an ability to produce electricity using microbial consortia developed specifically for biorefinery wastewaters containing acetate and other typical bioethanol production byproducts (Borole et al. 2009, 2013). Specifically, the electrogenic conversion of furans and phenolic molecules has emerged as a key application of this technology, with studies by Borole et al. demonstrating the removal of acetate, furfural, HMF and phenolic model compounds such as vanillic acid, 4-hydroxybenzaldehyde and 4-hydroxyacetophenone (Borole et al. 2009).

In addition, when properly deployed, MECs can also serve to improve the overall energy efficiency of ethanol biorefineries by generating hydrogen, which can be utilized in the biorefinery for improving fuel yield or as a separate product. The potential volume of hydrogen that can be produced using biorefinery wastewaters is estimated to be as high as 7200 m^3/h from a typical biorefinery processing 2000 ton per day of biomass into ethanol via fermentation. Including other types of biorefineries, the range of hydrogen production is estimated to be 750 to 8900 m^3/h. Thus, the MFC/MEC technology is beneficial not only from the standpoint of toxicity reduction, but also for generating value from the wastes for the biorefinery (Borole and Mielenz 2011). There are, however, several important points that must be considered when applying MFCs for removal of the toxic compounds from bioethanol wastewater streams, such as the percent removal of fermentation inhibitors and the degree of mineralization incurred, the ability of the MFC to handle the concentration of the inhibitors presented, the performance of the MFC in the presence of mixed-substrate feeds (i.e., when multiple inhibitors are presented simultaneously), the performance stability of the MFC (including power output and the identification of parameters requiring control), and the total power generation realized through MFC implementation.

Toxicant removal capabilities of microbial fuel cells

The concentration of acetate and other cytotoxic compounds in bioethanol production wastewater, which can range from a few mM to 20 mM or more following product separation (Ade et al. 2002; Klinke et al. 2004), is an important factor affecting their conversion. The effects of furfural on electricity production using MFCs has been studied at concentrations ranging from 0.1 to 2 g/l, with no negative effects on current output observed at any surveyed level. Energy recovery from furfural and other typical wastewater components such as HMF, vanillic acid, 4-hydroxybenzaldehyde, and 4-hydroxyacetophenone was reported to range from 60 to 69 %. These values are promising, and indicate substantial conversion of the compounds in the MFC bioanode. Further studies demonstrated that the bioanode was capable of sustaining the same conversion rate above a particular threshold concentration of the individual compounds, with maximum power densities of 3490 mW/m^2 (336 W/m^3) for furfural, 2510 mW/m^2 (238 w/m^3) for HMF and 630 mW/m^2 (62 W/m^3) for 4-hydroxybenzaldehyde (Borole et al. 2009).

Simultaneous detoxification of bioethanol waste stream components

The ability of the MFC bioanode to handle mixed-substrate waste streams has also been reported using a corn stover-derived process stream, where it was demonstrated to remove of all the above-referenced model compounds, as well as many of the phenolic compounds commonly generated during bioethanol production (Borole et al. 2013). During these studies, the effect of compound concentration was also examined, which revealed excessive growth of biofilms at high concentrations leading to mass transfer limitations. This was potentially due to the presence of residual sugars in the stream, which led to rapid biofilm growth even in the presence of the toxic compounds. The ability of the resultant biofilms to form a protective exopolysaccharide layer preventing exposure to high concentrations of the toxic compounds may be a potential explanation for the observed effects, however, further work will be necessary to fully support this hypothesis.

Performance stability under toxicological challenge

Evaluating the stability of MFCs will be key to determining if they can serve as long term solutions to the ecotoxicological challenges presented by bioethanol production. In a recent study by Borole et al., MFC performance was evaluated under challenge from model waste stream compounds for a period of 10 months. With a ferricyanide cathode being used for the last 4 months of operation, a power density of 3700 mW/m^2 was reported when furfural was employed as the substrate (Borole and Mielenz 2011). This output and longevity is promising for the future use of

MFCs in detoxifying the waste streams resulting from bioethanol production, which will serve as a key factor in both their production economics and their adoption by the general public.

Future directions within the field

Significant strides have been made on all levels of the bioethanol production process, however, issues still remain that must be addressed before the process can be performed at industrial scales with minimal ecological impacts. At the small scale, efforts must be made to further engineer the employed microorganisms to produce ethanol with minimal side products so as to both maximize efficiency and minimize environmental risk. Furthermore, work must continue to seek out or develop hosts that can more efficiently access cellulosic materials and convert them into products with minimal up stream pretreatment requirements. This will serve not only to limit the costs of bioethanol production, but will also limit the use of acids and other hazardous chemicals that must then be processed as wastes, and will minimize the total power consumption of the process. At the large scale, work must continue to harness what is currently considered waste from the bioethanol production process, and leverage it for further generation of energy or to better facilitate detoxification. As this work continues, the bioethanol production process as a whole is steadily moving towards a point where efficiency and ecotoxicological impact minimization will intersect to allow the long-term commercial survival of this industry.

Conclusions

Despite the abundance of cyto- and ecotoxicological concerns that result from the bioethanol production process, significant achievements have been made in reducing or overcoming their impacts. The advent of new genetic and metabolic tools and the discovery of new organisms that can potentially serve as bioethanol production hosts has led to significant advancements in both production capacity and economy. Therefore, as this industry continues to move forward it is important to fully understand both the inherent toxicological challenges and the methods that have been employed to overcome them. By leveraging this knowledge and applying it in combination with the new biotechnological and engineering strategies that are shaping the future of the bioethanol production field, it may be possible to move forward in a way that maximizes efficiency while minimizing the greater environmental impact.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest and that they have performed these studies in accordance with acceptable ethical procedures

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