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Biomass Conversion Inhibitors and *In Situ* Detoxification

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12.1 Introduction

Overcoming the impact of inhibitory compounds derived from lignocellulosic biomass is one of the major challenges for a sustainable biomass-to-biofuels industry. Lignocellulosic substrates are plant materials including softwood (gymnosperms), hardwood (woody angiosperms), and annual plants such as crops of herbaceous angiosperms. The major useful components of lignocelluloses for biofuels conversion are the polysaccharides cellulose and hemicellulose, that can be further degraded into simple sugars and utilized by fermentative microorganisms. *Cellulose* is a high-molecular-weight polymer of hexoses, mainly glucose. *Hemicellulose* is a polymer of mainly pentoses including xylose and arabinose, and some hexoses such as glucose, mannose, galactose, and rhamnose. Unlike the easily available sugars that are utilized in starch-based fermentations, lignocellulose biomass must be treated specifically to depolymerize and release simple sugars for microbial utilization. In this respect, several pretreatment methods have been commonly applied, including dilute acid hydrolysis, steam explosion, acid-catalyzed steam explosion, wet oxidation, wet explosion, alkaline hydrolysis, ammonia fiber explosion, and enzymatic hydrolysis. [For a comprehensive review of pretreatment technologies, the reader should consult Chapters 10 and 11 of this book, in addition to other reviews on the subject by Duff and Murray (1996), Sun and Cheng (2002), Moisier *et al.* (2005), Wyman *et al.* (2005), and Jörgensen *et al.* (2007).] Research and development in this area is under way in order to
develop methods that limit the production of potential inhibitors produced during the
deconstruction of biomass. Each of the currently available methods has different advan­
tages and disadvantages. However, there is as yet no universally ideal pretreatment
procedure that takes into consideration energy requirements, cost-efficiency, and practical
feasibility. One commonly observed problem is the generation of inhibitory compounds of
various byproducts during the pretreatment, mainly by dehydration of sugars and degra­
dation of lignin fractions.

12.2 Inhibitory Compounds Derived from Biomass Pretreatment

For economic reasons, dilute acid hydrolysis is commonly used in biomass degradation for
hydrolysis of the hemicellulose fraction and increased fiber porosity to allow enzymatic
saccharification and fermentation of the cellulose fraction (Bothast and Saha, 1997;
Saha, 2003). However, a major limitation of this method is the generation of numerous
byproducts and compounds that inhibit microbial growth and fermentation. Hemicellulosic
fractions of biomass hydrolysate contain various organic acids (Figure 12.1). Pentoses and
some hexoses are released from hemicelluloses, from which 2-furaldehde (furfural) and
5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural; HMF) can be formed by
dehydration of these sugars. Further degradation of hexoses released from cellulose can
lead to more HMF formation under high temperature and acidic conditions (Dunlop, 1948;
Antal et al., 1990; Antal et al., 1991; Larsson et al., 1999b; Lewkowski, 2001). Furfural and
HMF are considered to be the representative inhibitors of yeast and bacterial growth and
fermentation (Chung and Lee, 1985; Olsson and Hahn-Hägerdal, 1996; Taherzadeh
et al., 2000; Ezeji et al., 2007). HMF and furfural can further break down to produce
levulinic acid, formic acid, and furoic acid. Acetic acid and other organic acids are also
released from hemicellulosic fractions. Many other aldehyde compounds and phenolic

![Figure 12.1 Schematic showing the degradation of lignocellulosic biomass into fractions of
cellulose, hemicellulose and lignin, from which inhibitory compounds are generated during the
biomass pretreatment process](image)

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Compounds are generated from lignin degradation (McMillan, 1994). In addition, metals and SO₂ inhibitors resulting from hydrolytic equipment and additives can also be harmful to microbial growth and metabolic activities. Although more than 100 compounds were detected as potential inhibitors from biomass hydrolysates (Luo et al., 2002), many have not been well studied. Moreover, the synergistic effects of inhibitory compounds in a mixture are clearly beyond a simple sum of the individual compound effects. Most yeasts, including industrial strains, are susceptible to the complexes associated with dilute acid hydrolysis pretreatment (Palmqvist et al., 1999; Taherzadeh et al., 2000; Martin et al., 2003; Liu et al., 2004). In order to facilitate fermentation processes, additional remediation treatments – including physical, chemical, or biochemical detoxification procedures – are often required to remove these inhibitory compounds. However, these additional steps add cost and complexity to the process and generate extra waste products (Martinez et al., 2000; Mussatto and Roberto, 2004).

Due to the heterogeneous nature of lignocellulosic biomass, the degradation of by-products produced during the fermentation can vary significantly. The variety and concentration of inhibitory compounds also depend upon the pretreatment conditions such as treatment materials, temperature, pH, pressure, and time duration. Numerous inhibitors have generally been recognized as weak acids, furan inhibitors, and phenolics. With increased knowledge and understanding of the mechanisms of inhibition and detoxification, it is understood that specific chemical functional groups are responsible for the inhibitory effect and toxicity to microbes. For example, furfural and HMF are furan derivatives and commonly called ‘furan inhibitors.’ Evidence has shown that the metabolic conversion products of furfural and HMF, furan methanol (FM) and furan-2,5-dimethanol (FDM), are also furan derivatives, but less toxic to fermentative microorganisms (Liu et al., 2004; Liu et al., 2008b) (see below for more detailed discussion on this subject). The inhibitor and toxic effects appear to be caused by the aldehyde functional group rather than the furan ring. Naming the inhibitors by functional group implies likely mechanisms of the inhibition, and potentially helps to facilitate the investigation and understanding of the detoxification of the inhibitors. Therefore, in this chapter we present a classification of inhibitors based on their chemical functional groups as aldehydes, ketones, phenols, and organic acids. Each inhibitor is presented with a chemical structure, identification, common name, and molecular weight (MW) (Figure 12.2). In general, it was observed that low-molecular-weight compounds show more toxic effects to microbes than do high-MW compounds (Clark and Mackie, 1984; Sierra-Alvarez and Lettinga, 1991). This property could perhaps be ascribed to an easier transport of the smaller molecules via a variety of mechanisms, including passive diffusion.

Aldehyde inhibitors are compounds with one or more functional aldehyde groups, regardless of the base structure of a furan ring, a benzene ring or a phenol-related structure. For example, this class of compounds includes inhibitors such as furfural and HMF, each containing a furan ring and an aldehyde functional group (Delgenes et al., 1996; Ranatunga et al., 1997a; Ranatunga et al., 1997b). Other aldehyde inhibitors include 4-hydroxybenzaldehyde (Ando et al., 1986; Baquinero et al., 1980; Buchert et al., 1990; Jönsson et al., 1998; Klinke et al., 2002), vanillin (Ando et al., 1986; Buchert et al., 1990; Clark and Mackie, 1984; Jönsson et al., 1998; Klinke et al., 2002; Larsson et al., 1999b; Tran and Chambers, 1985; Fenske et al., 1999), syringaldehyde (Buchert et al., 1990; Larsson et al., 1999; Tran and Chambers, 1985; Fenske et al., 1999), and other compounds having...
Figure 12.2 A classification of compounds inhibitory to fermentative microorganisms derived from biomass pretreatment based on chemical functional groups including aldehydes, ketones, organic acids and phenols. The molecular weights are shown, with common names in parentheses.
Figure 12.2 (Continued)
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a benzene ring or a phenol-based structure including isovanillin (Larsson et al., 2000), ortho-vanillin (Larsson et al., 2000), and coniferylaldehyde (Buchert et al., 1990; Clark and Mackie, 1984; Larsson et al., 1999; Tran and Chambers, 1986; Fenske et al., 1999). Cinnamaldehyde is another aldehyde inhibitor typically present in lignocellulosic biomass hydrolysates. Ketone inhibitors include 4-hydroxyacetophene and the closely related compounds acetovanillone and acetosyringone (Klinke et al., 2001; Klinke et al., 2003); these compounds all share a common ketone functional group.

Similarly, inhibitors sharing a common carboxylic acid functional group are now collectively classified as organic acid inhibitors. This class of compounds includes simple acids as well as furoic acid with a furan ring that was previously considered as being a furan inhibitor. Moreover, many previously recognized phenolic compounds are now grouped as members of the organic acid inhibitor class based on their functional structure. Inhibitory compounds of this class all contain a carboxyl functional group and include acetic acid, formic acid (Ranatunga et al., 1997a; Ranatunga et al., 1997b; Delgenes et al., 1996; Zaldivar and Ingram, 1999), levulinic acid (Zaldivar et al., 1999), caproic acid (Ranatunga et al., 1997; Zaldivar et al., 1999), furoic acid (Klinke et al., 2001; Klinke et al., 2003; Zaldivar and Ingram, 1999), 4-hydroxybenzoic acid (Ando et al., 1986; Baquinero et al., 1980; Fenske et al., 1999; Jönsson et al., 1998; Klinke et al., 2002; Larsson et al., 1999), 3-hydroxybenzoic acid (Jönsson et al., 1998), 2-hydroxybenzoic acid (Ando et al., 1986), 2,5-dihydroxybenzoic acid (Jönsson et al., 1998), protocatechic acid (Larsson et al., 1999a), vanillic acid (Ando et al., 1986; Tran and Chambers, 1985; Klinke et al., 2002), gallic acid, syringic acid (Ando et al., 1986; Baquinero et al., 1980; Buchert et al., 1990; Jönsson et al., 1998; Klinke et al., 2002; Tran and Chambers, 1985), 4-hydroxycinnamic acid (Ando et al., 1986; Barquinero et al., 1980; Fenske et al., 1999; Klinke et al., 2002), ferulic acid (Klinke et al., 2002; Larsson et al., 2000), homovanillic acid (Larsson et al., 1999), guaiacylglycolic acid (Buchert et al., 1990), and sinapic acid (Baquinero et al., 1980). These inhibitors are thought to be exert their inhibitory actions via their carboxyl functional groups.

The remaining phenol-based inhibitors are grouped together including phenol (Clark and Mackie, 1984; Klinke et al., 2002), benzene-1,2-diol (catechol) (Jönsson et al., 1998; Larsson et al., 1999a), benzene-1,4-diol (hydroquinone) (Larsson et al., 1999a), 4-ethylbenzene-1,2-diol (ethylcatechol), 2-methylphenol, 3-methylbenzene-1,2-diol (methylcatechol), 2-methoxyphenol (guaiacol), 2-methoxy-4-(prop-2-en-1-yl) phenol (eugenol), 2-methoxy-4-[(1E)-prop-1-en-1-yl] phenol (isoeugenol), 4-(hydroxymethyl)-2-methoxyphenol (vanillyl alcohol), 4-[(1E)-3-hydroxyprop-1-en-1-yl]-2-methoxyphenol (coniferyl alcohol), and 2,6-dimethoxybenzene-1,4-diol (2,6-dimethoxy-hydroquinone) (Clark and Mackie, 1984; Buchert et al., 1990; Jönsson et al., 1998; Klinke et al., 2002).

### 12.3 Inhibitory Effects

The effects of different inhibitors vary widely among different strains of yeast and bacteria (Beall et al., 1991; Klinke et al., 2003; Martin et al., 2003; Talebnia et al., 2005; Sakai et al., 2007; Ezeji et al., 2007). Notably, furfural and HMF inhibit cell growth and ethanol production rates at lower concentrations. Individual strains have been isolated that retain their ability to produce ethanol in the presence of 10 to 79 mM of either furfural or HMF,
including strains of the following species: *Saccharomyces cerevisiae*, *Pichia stipitis*, *Candida shehatae*, *Corynebacterium glutamicum*, *Zymomonas mobilis*, and *Escherichia coli* (Delgenes et al., 1996; Y.Y. Lee et al., 1999; Palmqvist et al., 1999; Ranatunga et al., 1997a; Ranatunga et al., 1997b; Sakai et al., 2007; Talebnia et al., 2005; Zaldivar and Ingram, 1999). Dose-dependent inhibition effects of furfural and HMF were particularly characterized for the yeast *S. cerevisiae* (Taherzadeh et al., 2000; Liu et al., 2004).

On a defined medium under controlled conditions, yeast demonstrate dose-dependent cell growth and metabolic conversion activities in response to varied doses of HMF and/or furfural (Liu et al., 2004). When added at a concentration of 30 mM to a yeast culture, the major effects of these inhibitors comprise an extended lag phase of both growth and metabolic activities (Figure 12.3A and B). Most notably, metabolic conversion activities in transformation of HMF to FDM, furfural to FM, and glucose to ethanol are significantly delayed in the presence of inhibitors as compared to a control culture. However, in the presence of a high concentration of a single inhibitor such as 120 mM HMF, yeasts are completely inhibited and no cell growth is observed even after 128 h incubation (Figure 12.3C and D). The cells appear to be completely repressed at this inhibitor concentration, and no biological activity or HMF transformation is observed. Most yeasts, including industrial strains, are susceptible to these inhibitors. Furthermore, synergistic repression is commonly observed when a combination of inhibitors or inhibitor complexes

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**Figure 12.3**  Cell growth (A and C) as measured by absorbance at OD<sub>600</sub>, biotransformation, and metabolic conversion activities (B and D) of *S. cerevisiae* NRRL Y-12632 in response to 5-(hydroxymethyl)-2-furaldehyde (HMF) challenges at 30 mM (A and B) and 120 mM (C and D). Figure legends of HPLC assay data (B and D) labeled as glucose (○), ethanol (●), HMF (▲), and furan dimethanol (△). Glucose and ethanol are given in g l<sup>-1</sup>; the remaining values are units of mM.
is added; this can perhaps be best exemplified by the observation that, in the presence of a combination of inhibitors, yeast cells can be killed even at low concentrations (Liu et al., 2004). These inhibitors are reported to reduce enzymatic biological activities, to break down DNA, and to inhibit protein and RNA synthesis (Sanchez and Bautista, 1988; Khan and Hadi, 1994; Modig et al., 2002). Cell walls and membranes of yeast cells grown under furfural and HMF-challenged conditions appear damaged when compared to those of controls grown in the absence of any inhibitor (S.W. Gorsich and Z.L. Liu, unpublished results). As a result, cell growth is delayed and ethanol productivity significantly reduced.

Other aldehyde inhibitors, including phenol aldehydes such as 4-hydroxybenzaldehyde, coniferyl aldehyde, syringaldehyde and vanillins, were observed to be inhibitory at less than 10 mM to most yeast and bacterial strains (Ando et al., 1986; Delgenes et al., 1996; Ezeji et al., 2007; Klinke et al., 2001; Klinke et al., 2003; Larsson et al., 2000; Lee et al., 1999; Palmqvist et al., 1999; Ranatunga et al., 1997; Ranatunga et al., 1997b; Sakai et al., 2007; Zaldivar et al., 1999). Moreover, aldehyde inhibitors derived from lignin degradation appear to be more inhibitory than those derived from sugar dehydration (Lee et al., 1999). The degree of inhibition on cell growth and ethanol production also varies depending on the strains tested. Phenolic compounds cause increased membrane fluidity and affect membrane permeability (Heipieper et al., 1994). Such alterations when combined may enhance synergistic inhibition.

Ketones appear to exert a greater inhibitory effect on bacteria such as *Thermoanaerobacter mathranii* than on yeasts, in terms of reduced growth and ethanol yield (Klinke et al., 2001; Klinke et al., 2003). Phenols such as cathecol, hydroquinone, and coniferyl alcohol almost completely inhibit *E. coli* (Zaldivar et al., 2000), but are relatively less toxic to yeast (Larsson et al., 2000). However, eugenol and isoeugenol are inhibitory to yeasts at low concentrations. The three main phenol structure building blocks in lignin are described as *p*-hydroxyphenyl, guaiacyl, and syringyl. These chemicals differ in their methoxy-groups ortho to the phenol group (Klinke et al., 2004). The general toxicity of a phenol compound to yeasts has been correlated to the degree of its methoxy substituents ortho to the phenol hydroxyl group. The order of inhibitory effects, ranked from strong to weak, is as follows: (1) hydroxyphenol, (2) guaiacyl, and (3) syringyl (Ando et al., 1986; Clark and Mackie, 1984; Delgenes et al., 1996; Klinke et al., 2003).

Organic acids, in general, are more toxic to isolates of bacteria than yeasts. However, some acids such as 4-hydroxycinnamic acid and ferulic acid can severely restrict ethanol productivity by yeast at low concentrations (Larsson et al., 2000). Other common organic acids such as 4-hydroxybenzoic acid and vanillic acid cause inhibition of growth and ethanol production at relatively low concentrations (Ando et al., 1986; Clark and Mackie, 1984; Klinke et al., 2001; 2003; Lee et al., 1999; Palmqvist et al., 1999; Ranatunga et al., 1997b; Zaldivar et al., 1999). While demonstrating mild inhibition to most yeasts and bacteria, syringic acid is extremely toxic to *Clostridium beijerinckii* even at very low concentrations (Klinke et al., 2003; Lee et al., 1999; Ezeji et al., 2007; Ranatunga et al., 1997b; Zaldivar et al., 1999). The toxicity of organic acids has been correlated with their degree of hydrophobicity, suggesting the involvement of a hydrophobic target such as the cell membrane (Zaldivar and Ingram, 1999; Zaldivar et al., 1999).

In general, aldehydes and phenols are more toxic than organic acids (Leonard and Hajny, 1945). Inorganic salts produced from the biomass process and heavy metal ions such
as iron, chromium, nickel, and copper that originate from the corrosion of hydrolysis equipment can also be inhibitory to microorganisms (Mussatto and Roberto, 2004). In reality, inhibitory compounds present in a hydrolysate have synergistic inhibitive effects over that of the sum of individual toxic effects. However, removal of only the major inhibitors often results in significantly improved microbial growth and fermentation (Bucher et al., 1990; Larsson et al., 1999; Tran and Chambers, 1986; Klinke et al., 2003).

12.4 Removal of Inhibitors

Since biomass conversion inhibitors can be problematic for various fermentative microorganisms, the removal of inhibitory compounds from hydrolysates is typically necessary to facilitate efficient microbial growth and fermentation (Mussatto and Roberto, 2004). However, the type of inhibitory compounds present in a hydrolysate depends upon the types of pretreatment and biomass materials utilized in the process. Inhibitory compounds also vary according to each specific strain of fermentative microorganism utilized. The most commonly employed inhibitor removal methods are physical, chemical, or biological in nature. Vacuum evaporation is a physical method that is used to reduce the amounts of volatile compounds present in different hydrolysates. Notably, the concentrations of furfural, vanillin, and acetic acid in test hydrolysates were reported to be significantly reduced from 29 to 100% following such evaporation treatment (Converti et al., 2000; Larsson et al., 1999a; Rodrigues et al., 2001). There again, the efficiency of inhibitor removal varies according to the source of the hydrolysate. Nonetheless, unsatisfactory performance resulting in increased inhibition was also observed using such treatment. This was mainly ascribed to that this method concentrates the non-volatile toxic compounds as well. For example, fermentative microorganisms have been observed to be inhibited by concentrated non-volatiles such as lignin derivatives and extractives (Parajo et al., 1997; Palmqvist et al., 1996; Silva and Roberto, 1999).

Several chemical methods have been applied to precipitate toxic compounds such as alkali treatment using Ca(OH)$_2$ or NaOH. By employing this overliming treatment, the pH of the hydrolysate can be increased to 9–10, and subsequently readjusted to an appropriate value using acid addition prior to microbial fermentation. This method in general reduces aldehyde and ketone inhibitors, including furfural and HMF, and improves microbial growth and fermentation performance (Martinez et al., 2001; Palmqvist and Hahn-Hagerdal, 2000a; Palmqvist and Hahn-Hagerdal, 2000b; Roberto et al., 1991). An obvious disadvantage of this method is that it generates a CaSO$_4$ precipitation product that must be removed. This additional removal step complicates the processing procedures, increases energy requirements, increases cost, and also generates additional waste.

On the other hand, the level of the toxic compounds can be reduced by applying activated charcoal for attaining improved microbial fermentation performance (Domínguez et al., 1996; Silva et al., 1998; Y.Y. Lee et al., 1999). Similarly, diatomaceous earth has been used to absorb undesirable compounds (Ribeiro et al., 2001). The application of both anion- and cation-exchange resins has been reported to result in better detoxification results and improved fermentability when compared with other methods (Gong et al., 1993; W.G. Lee et al., 1999; Larsson et al., 1999a; Nilvebrant et al., 2001). However, this approach
may not be practical due to its high cost. Often, a combination of different inhibitor removal methods is more efficient than any single method alone to remove a variety of inhibitory compounds, such as applying pH adjustments, activated charcoal adsorption, boiling, and evaporation (Alves et al., 1998; Converti et al., 1999; 2000).

Interestingly, enzymatic treatment using peroxides and laccase obtained from the ligninolytic fungus *Trametes versicolor* has been reported to improve ethanol productivity of a fermentation process based on a willow hemicellulosic hydrolysate (Jönsson et al., 1998). This approach removes phenolic monomers and phenolic acids and appears to involve oxidative reaction of low-molecular-weight phenolic compounds. The soft-rot fungus *Trichoderma reesei* has been reported to be able to degrade inhibitory compounds in a hydrolysate after steam pretreatment (Palmqvist et al., 1997).

The mix of inhibitory compounds present in hydrolysates varies based upon the source of the biomass. Therefore, inhibitor removal is a very selective process and it is difficult to identify a standard process which provides satisfactory results for all substrates. In addition, not all potentially inhibitory compounds have been identified to this date. It is possible that some undiscovered compounds have synergistic inhibitory effects even at low concentrations, as is the case for the aldehyde inhibitors furfural and HMF. Therefore, continuing efforts to identify and understand the profiles of inhibitory compounds present in various hydrolysates remains a critical area of research for enabling the development of improved detoxification methods. Considering the need of keeping low process costs of commodity products such as ethanol, the removal of inhibitors from hydrolysates using the above mentioned methods may not be an economically worthwhile approach given the costs associated with additional processing steps and the loss of fermentable sugars.

### 12.5 Inhibitor-Tolerant Strain Development

The economics of fermentation-based bioprocesses for biofuels production rely extensively on the performance of microbial biocatalysts in industrial applications. The development of yeast or bacterial strains that can withstand the presence of inhibitors is one of the keys for developing a sustainable lignocellulosic biomass-to-biofuels industry. However, many of the industrially interesting microorganisms obtained thus far are not robust enough to withstand the stress conditions associated with the biomass conversion process. Nonetheless, improved hydrolysate fermentation by adaptation of fermentative microorganisms to hydrolysates has been reported (Olsson and Hahn-Hagerbal, 1996; Parajo et al., 1998; Silva and Roberto, 2001; Sene et al., 2001). While dose-dependent inhibition of yeast by furfural and HMF has been observed and characterized (Taherzaadeh et al., 2000; Liu et al., 2004), inhibitor-tolerant strains of ethanologenic *S. cerevisiae* with enhanced ability to detoxify the inhibitor furfural or HMF have been developed through directed evolutionary engineering (Liu, 2006; Liu et al., 2005).

Recently, a further improved tolerant yeast strain designated NRRL Y-50049 was generated that withstands the synergistic inhibition caused by inhibitor complexes; noteworthy, this strain has been observed to complete an ethanol fermentation cycle in 48 h (Liu et al., 2008b). The parent strain, on the other hand, is unable to grow in the presence of the HMF and furfural complexes. In contrast, and as demonstrated by HPLC
measurements, strain Y-50049 grows well on such media and dramatically reduces furfural and HMF. The furfural was completely depleted at 15 h as measured by HPLC assays. At 32 h, the HMF became completely undetectable while the conversion product of HMF, FDM, reached its peak concentration. Glucose was completely consumed and a normal ethanol yield obtained at or prior to 48 h. The typical inhibitor conversion products furan methanol (FM) and FDM were detected at the end of the fermentation, along with ethanol. These results indicate that it is possible to in situ detoxify furfural and HMF when using the ethanologenic yeast S. cerevisiae.

Fed-batch and increased inoculum size are two important conventional methods that have been used to overcome the inhibitory effects of furfural and HMF, since no strains have been available to grow in the presence of these inhibitors (Chung and Lee, 1985; Sanchez and Bautista, 1988; Tessier et al., 1998; Nilsson et al., 2005; Petersson et al., 2006). On the other hand, the tolerant yeast strain Y-50049 does not require any acclimatization to the presence of inhibitors, but rather grows readily and completes the fermentation following typical kinetics. Recently, another inhibitor-tolerant yeast strain was obtained via an adaptation method, which was able to grow in a medium obtained by diluting to 50% a sugarcane bagasse hydrolysate containing inhibitors (Martin et al., 2007). The screening of microorganisms tolerant or able to utilize inhibitory compounds as a carbon source have been reported (Lopez et al., 2004; Nichols et al., 2005). However, most of these isolates are not capable of ethanologenic fermentation.

Selection under pressure is an evolutionary process of Nature. Adaptation methods have a long history of use in the yeast utilization industry. The basis of success of these simple methods depends upon the innate genetic potential of the yeast being engineered combined with appropriate selection procedures. The directed evolutionary engineering procedures under laboratory settings that have been previously described (Liu et al., 2005) provide an easy and practical approach that can be extended to a broad range of applications. Such methods significantly reduce the time that is necessary to obtain desirable strain characteristics through nature evolutionary adaptation of yeast cells. However, a specific enrichment of the genetic background of ethanologenic yeast may be needed that can be achieved by introducing exogenous genes. For example, efficient xylose-utilizing strains of S. cerevisiae were obtained through directed evolution after introduction of a single exogenous xylose isomerase gene (Kuyper et al., 2005). Empirical data suggest that, when using an evolutionary engineering method, different populations with varied phenotypes can be recovered from a single recombinant strain under selection pressure (Sonderegger and Sauer, 2003). Multiple types of mutation have been successfully induced as a result of the application of selection pressure on yeasts (Z.L. Liu et al., unpublished results). Persistent gene expression pattern shifts have been observed in the ethanologenic yeast under HMF challenge conditions, which suggests that genomic adaptations occur during the laboratory evolutionary selection (Liu, 2006; Liu and Slininger, 2006). This process primarily takes place during the lag phase of growth. As a result, the development of ethanologenic yeasts with desirable characteristics using directed evolutionary engineering appears to be a promising arena and can constitute a very useful alternative for improving microbial strain performance (Liu and Slininger, 2005). Obviously, the process can be iterated and such adapted strains could be efficiently used for further genetic manipulation. Additional studies in this area are expected to result in strains with significantly improved inhibitor tolerance.
12.6 Inhibitor Conversion Pathways

The furfural conversion pathway to FM by yeasts has been described (Morimoto and Murakami, 1967; Villa et al., 1992; Liu, 2006) (Figure 12.4). It is currently commonly accepted that furfural is first converted to FM and further reduced to furoic acid (Palmqvist et al., 1999; Sarvari et al., 2003; Taherzadeh et al., 1999; Nemirovskii and Kostenko, 1991). Furfural can also be cleaved to form formic acid (Palmqvist and Hahn-Hägerdal, 2000b).

Unlike the well-studied furfural conversion pathway, knowledge of the HMF pathway has remained limited because there is no readily available commercial source for any of the HMF degradation products. This limitation makes it virtually impossible to study HMF conversion mechanisms. Based on the furfural conversion route, the current hypothesis is that HMF is first converted into HMF alcohol (Nemirovskii et al., 1989). Recently, an HMF metabolic conversion product was isolated and identified as being furan-2,5-dimethanol (FDM), also termed as 2,5-bis-hydroxymethylfuran (Figure 12.4) (Liu et al., 2004; Liu, 2006). HMF has a maximum absorbance at 282 nm, and FDM at 222 nm. Following a vigorous investigative effort, FDM was further isolated from cell-free culture supernatants, purified, and characterized using mass and NMR spectra analysis (Liu et al., 2004).

An important clue to the symmetrical nature of the HMF degradation products was that the signals for the aldehyde proton and the asymmetric spectra of HMF were absent when the purified HMF-conversion product was analyzed using NMR. The NMR spectra thus obtained are consistent with that of a symmetrical molecule with a furan ring. The chemical structure of the metabolite has been identified as that of a compound with \( C_6H_8O_3 \) composition and a molecular weight of 128 Da. The identification of FDM is an important development as it provides a basis for subsequent studies on the mechanisms of HMF inhibitor detoxification. Furthermore, an FDM preparation procedure has recently been described that can be used to prepare the necessary FDM standards for conducting by HPLC-based metabolic profiling analyses of HMF degradation (Liu et al., 2008b).

As revealed by HPLC assays, at the end of the fermentation, FM and FDM – the chief conversion products of furfural and HMF – are accumulated at high levels in the fermentation medium by the tolerant strains. Furfural and HMF are furan derivatives that comprise a furan ring and an aldehyde functional group with a composition of \( C_5H_4O_2 \) and \( C_6H_6O_3 \), respectively. Their conversion products FM and FDM, respectively with a composition of \( C_5H_6O_2 \) and \( C_6H_5O_3 \), retain the furan rings and an alcohol group which replaced the aldehyde structure (Figure 12.4). Apparently, FM and FDM are less toxic to microbes, since the yeast does not appear to be inhibited for its growth and ethanol fermentation in the presence of these compounds. Therefore, the aldehyde functional group in furfural and HMF is toxic to yeast, but the furan ring or associated alcohol functional groups are not (or are less) inhibitory. The mechanisms of the detoxification of furfural and HMF by yeast cells are unlikely to be involved in either the utilization or the degradation of the furan compound, but rather a reduction of the aldehyde into alcohol. Consequently, the use of ‘furan derivative’ as a general term for inhibitors such as furfural and HMF should be avoided, as FM and FDM are also furan derivatives.

A major metabolite of the organic acid ferulic acid has been identified as vinyl guaiacol (2-methoxy-4-vinylphenol). On the other hand, 4-hydroxyccinnamic acid was found to be converted by yeast cells into styrene (vinylbenzene) (Larsson et al., 2001a). Moreover, under oxygen-limited conditions, dihydroferulic acid [3-(4-hydroxy-3-methoxyphenyl)
Figure 12.4 Conversion pathways of 2-furaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (HMF) into 2-furanmethanol (FM) and furan-2,5-dimethanol (FDM) coupled with NADH and/or NADPH and catalyzed by multiple enzymes possessing aldehyde reduction activities.
propanic acid] is produced from ferulic acid, whereas dihydrocinnamic acid (3-phenylpropanoic acid) is produced from cinnamic acid (Figure 12.5).

### 12.7 Molecular Mechanisms of In Situ Detoxification

The biotransformation of furfural and HMF by yeast can be primarily ascribed to the action of NADH- and NADPH-coupled enzymes (Palmqvist et al., 1999; Larroy et al., 2002; Nilsson et al., 2005; Petersson et al., 2006; Liu et al., 2008b). In the presence of furfural, the ATP level is low and cell replication is thus limited. Likewise, glycerol formation is reduced. Furfural has been characterized as an electron acceptor (Wahlbom and Hahn-Hägerdal, 2002). Consistently, a shortage of NADH is observed in yeast when cells are incubated in the presence of furfural. It appears that furfural reduction competes for NADH and interferes with cell glycolysis during the regeneration of NAD\(^+\). As a result, furfural can cause an accumulation of acetaldehyde that results in a delay of acetate and ethanol production. Similarly, xylitol excretion by *S. cerevisiae* is reduced during xylose fermentation when furfural is added to the medium (Wahlbom and Hahn-Hägerdal, 2002). It has been reported that reduced furfural tolerance was observed for selective deletion mutants of genes coding for significant enzymes involved with the pentose phosphate pathway (Gorsich et al., 2006).

Most *in vitro* enzyme assays for HMF and furfural reduction were reported using whole-cell protein extracts. Varied cofactor preferences were observed; for example, HMF reduction by yeast cells was reported to have a preference for the cofactor NADPH (Wahlbom and Hahn-Hägerdal, 2002), whereas in a later study a different strain of *S. cerevisiae* was found that exhibited a NADH preference rather than NADPH (Nilsson et al., 2005). Similar observations have been reported for a crude protein extract that requires cofactor NADH for furfural reduction (Gutieerez et al., 2002). In contrast, a partially purified furfural reductase from *E. coli* by the same group demonstrated NADPH-dependent activity on furfural (Gutieerez et al., 2006). It is worth noting that recent studies on the reduction of furfural and HMF showed that mutants overexpressing a gene coding for a furfural and/or HMF reduction function have distinct cofactor preferences. For example, alcohol dehydrogenase VII (*ADH7*), aldehyde dehydrogenase IV (*ALD4*), and aldose reductase III (*GRE3*) exhibit on both substrates furfural and HMF a clear NADH preference, while alcohol dehydrogenase VI (*ADH6*) showed NADPH preference (Table 12.1) (Liu et al., 2008b). In contrast to the cofactor preference shown by these individually expressed genes, the whole-cell protein extract from *S. cerevisiae* Y-50049 demonstrates that reduction activities of both furfural and HMF are coupled with either of these two cofactors. This observation can be ascribed to the more diverse enzymatic activities displayed by the whole-cell extract of Y-50049, which reflects the activity of a pool of the functional enzymes rather than that of a single gene product, and therefore, either NADH or NADPH can be used for the aldehyde inhibitor reduction reactions. Consequently, depending upon varied pathways and the composition of the functional aldehyde reductases involved, the main trend of cofactor preference may be strain-dependent.

With respect to the substrate furfural, *ADH6* appears to be less selective to either cofactor, although the specific activity of this enzyme is higher with NADPH than with NADH (Table 12.1) (Petersson et al., 2006; Liu et al., 2008b). Studies performed at different
Figure 12.5 Conversion pathways of (2E)-3-phenylprop-2-enoic acid (cinnamic acid) to ethenylbenzene (styrene), and (2E)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enoic acid (ferulic acid) to 3-ethenyl-2-methoxyphenol (vinylguaiacol) catalyzed by phenylacrylic acid decarboxylase (Pad1p) and possible other enzymes. Under oxygen-limited conditions, ferulic acid and cinnamic acid were converted into dihydroferulic acid (4-hydroxy-3-methoxyphenyl acetic acid hydrate) and dihydrocinnamic acid (phenylacetic acid), respectively. Adapted from Larsson et al. (2001a)
Table 12.1  Specific activities and relative activities of whole-cell extract for selective overexpressed genes of *Saccharomyces cerevisiae* and *Escherichia coli* for reduction of furfural and 5-hydroxymethylfurfural (HMF)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Specific activity (mU mg(^{-1}) protein)</th>
<th>Relative activity(^{*}) Std (mU mg(^{-1}) protein or %)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADH</td>
<td>NADPH</td>
<td>NADPH</td>
</tr>
<tr>
<td>ADH6</td>
<td>Furfural</td>
<td>190-210</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ADH6</td>
<td>Furfural</td>
<td>—</td>
<td>900-990</td>
<td>—</td>
</tr>
<tr>
<td>ADH6</td>
<td>HMF</td>
<td>6-8</td>
<td>—</td>
<td>—</td>
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<td>—</td>
<td>~1300</td>
<td>—</td>
</tr>
<tr>
<td>ADH6</td>
<td>Furfural</td>
<td>62.0</td>
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<td>—</td>
</tr>
<tr>
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<td>—</td>
<td>97.7</td>
<td>—</td>
</tr>
<tr>
<td>ADH6</td>
<td>HMF</td>
<td>—</td>
<td>78.7</td>
<td>—</td>
</tr>
<tr>
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<td>—</td>
<td>—</td>
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<td>HMF</td>
<td>157.4</td>
<td>—</td>
<td>—</td>
</tr>
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<td>Furfural</td>
<td>66.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ALD4</td>
<td>HMF</td>
<td>92.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GRE3</td>
<td>Furfural</td>
<td>114.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>—</td>
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</tr>
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</tr>
<tr>
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<td>Furfural</td>
<td>353.6</td>
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</tr>
<tr>
<td>Y63</td>
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<td>—</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
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<td>HMF</td>
<td>—</td>
<td>—</td>
<td>25</td>
</tr>
<tr>
<td>Y63</td>
<td>Acetaldehyde</td>
<td>—</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>Y63</td>
<td>Propanal</td>
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<td>—</td>
<td>105</td>
</tr>
<tr>
<td>Y63</td>
<td>Butanal</td>
<td>—</td>
<td>—</td>
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</tr>
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</tr>
<tr>
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<td>175</td>
</tr>
<tr>
<td>Y63</td>
<td>Heptanal</td>
<td>—</td>
<td>—</td>
<td>156</td>
</tr>
<tr>
<td>Y63</td>
<td>Octanal</td>
<td>—</td>
<td>—</td>
<td>133</td>
</tr>
<tr>
<td>Y63</td>
<td>Trans-2-Nonanal</td>
<td>—</td>
<td>—</td>
<td>37</td>
</tr>
<tr>
<td>Y63</td>
<td>Benzaldehyde</td>
<td>—</td>
<td>—</td>
<td>60</td>
</tr>
<tr>
<td>Y63</td>
<td>Cinnamaldehyde</td>
<td>—</td>
<td>—</td>
<td>46</td>
</tr>
<tr>
<td>Y63</td>
<td>Anisaldehyde</td>
<td>—</td>
<td>—</td>
<td>7</td>
</tr>
<tr>
<td>Y63</td>
<td>Phenylacetaldehyde</td>
<td>—</td>
<td>—</td>
<td>135</td>
</tr>
</tbody>
</table>

\(^{*}\)Relative activity measured as relative percentage to that of furfural reduction activity.
laboratories have supported the observation that ADH6 exhibits an NADPH cofactor preference. The activity of this enzyme is significantly increased when its gene is overexpressed in ethanologenic yeast (Petersson et al., 2006). On the other hand, alcohol dehydrogenase ADH7 shows significant reduction activities not only towards furfural and HMF when coupled with NADH, but also with other aldehydes such as cinnamaldehyde (Liu et al., 2008b). Such an important property makes this enzyme an excellent candidate for efficient detoxification of inhibitors present in industrial biomass hydrolysates. This clearly represents an interesting area of development. It is interesting that kinetics studies indicated reductive reactions of ADH6 and ADH7 with various aldehydes and alcohol substrates are 50- to 100-fold more efficient than the corresponding oxidation reactions (Larroy et al., 2002). Therefore, the enzymes ADH6 or ADH7 seem to act as aldehyde reductases and have similar substrate specificities toward various aldehydes (Larroy et al., 2002). In fact, yeast clones overexpressing ADH6 and ADH7 show significantly higher reduction capabilities towards HMF and furfural (Liu et al., 2008b). As compared to the wild type, overexpression of the ADH6 and ADH7 genes in ethanologenic yeast improves inhibitor tolerance as well as growth rates in the presence of furfural and HMF. These improved characteristics can be directly attributed to the enhanced aldehyde reductase activities.

ALD4 is a major mitochondrial aldehyde dehydrogenase that is required for growth on ethanol and the conversion of acetaldehyde to acetate via equally utilizing NADP⁺ or NAD⁺ as coenzymes (Tessier et al., 1998). This enzyme was also found to function as a reductase, converting respectively HMF and furfural to FDM and FM while utilizing NADH as a cofactor (Liu et al., 2008b). Despite aldehyde dehydrogenases having been known to play an important role in the acetaldehyde metabolism of yeasts (Aranda and del Olmo, 2003), their potential for carrying out the detoxification of furfural and HMF during conversion processes of biomass to ethanol (Liu et al., 2008b) has only recently been observed.

Likewise, GRE3 is an aldo-keto reductase that is involved primarily in the catabolism of xylose and arabinose (Träff et al., 2002). However, GRE3 was recently reported to have strong reduction activities to furfural and HMF (Liu et al., 2008b). An enhanced expression of GRE3 is also observed under different stress conditions, such as NaCl or H₂O₂ challenge, heat shock, and carbon starvation (Aguilera and Prieto, 2001). Furthermore, overexpression of the GRE3 has been shown to increase methylglyoxal tolerance in S. cerevisiae, and it has been reported to be an endogenous substrate of GRE3. Methylglyoxal is an aldehyde form of pyruvic acid; it is a byproduct of metabolism that cannot be utilized by yeast cells. In particular, it impairs energy production, contributes to the generation of free radicals, and kill cells of a wide variety of species (Kalapos, 1999). It is noteworthy that in numerous improved strains of S. cerevisiae, GRE3 is deleted to reduce undesirable xylitol production levels (Träff et al., 2001; Kuyper et al., 2005). Nevertheless, GRE3 also functions as an aldehyde reductase that also converts HMF and furfural to FDM and FM, respectively. As a result, the current practice of deleting the gene that code for GRE3 for strain improvement could thus potentially affect the comprehensive stress tolerance and detoxification ability of yeasts, despite a single gene deletion, is probably unlikely to result in a dramatically increased susceptibility to either furfural or HMF. A more comprehensive understanding of the role of GRE3 and its interactions among its corresponding functional enzyme group is needed in order to engineer strains with optimized detoxification and pentose utilization balance.

A furfural reductase was reported to be instrumental in the reductive detoxification of furfural to furan dimethanol by the ethanologenic bacterium E. coli strain LYO1 (Table 12.1) A partially purified protein of this enzyme demonstrated a strict NADPH
cofactor preference for furfural reduction activity (Gutierrez et al., 2006). Searching for similar activities in yeasts, the mRNA expression levels of a few recently identified genes of \textit{S. cerevisiae} were observed to be significantly induced under both furfural and HMF challenges. Notably, crude whole-cell extracts of different clones overexpressing one of these genes exhibit significant aldehyde reduction activities to furfural and other toxic substrates (Table 12.1).

In total, more than 300 genes have been identified as being differentially expressed under inhibitor stress conditions (Liu, 2006). To date, fewer than a dozen functional genes have been examined, but the detoxification of furfural and HMF by yeast apparently is performed by a complex metabolic network that is not limited to these genes. In addition to functional enzymes, a significant number of genes with enhanced expression in the presence of various inhibitors were also observed to share common transcriptional factors (Liu and Sinha, 2006). For example, members of the pleiotropic drug resistance (PDR) gene family may play a significant role in coping with stress in order to promote cell survival (Liu et al., 2006). Among the 12 regulatory interactions identified using discrete dynamic system modeling studies, the transcription factor Yap1p and Pdr3p are considered as being significant regulatory elements for HMF detoxification (Song and Liu, 2007). As shown in a recent study, none of the tested yeast mutants carrying a single gene deletion in either \textit{ADH6}, \textit{ADH7}, \textit{ALD4} or \textit{GRE3} demonstrated a detectable growth defect, nor any susceptibility to either furfural or HMF under the controlled conditions (Liu et al., 2008b). This clearly indicates that a single gene deletion in any of the above-mentioned genes does not significantly affect cell growth or tolerance to these inhibitors. Therefore, as previously mentioned, it is unlikely that a single gene could play a decisive role in furfural or HMF detoxification. Instead, the \textit{in situ} detoxification of furfural and HMF likely involves multiple genes, including functional genes and regulatory genes, as well as regulatory cascades occurring among these genes.

Phenylacrylic acid decarboxylase (Padlp) catalyzes a decarboxylation step, by which aromatic carboxylic acids are converted to the corresponding vinyl derivatives (Larsson et al., 2001b). This was demonstrated during conversions of cinnamic acid and ferulic acid into styrene and vinylguaiacol (Figure 12.5). However, several other enzyme activities are also likely involved in this conversion (Larsson et al., 2001b). Interestingly, the overexpression of laccase from \textit{Trametes versicolor} in \textit{S. cerevisiae} also improved yeast tolerance to phenolic inhibitors (Larsson et al., 2000).

Single-gene studies have significantly contributed to our knowledge of gene functions during the past 50 years, and this approach will continue to be important in the future. However, investigations and advances in genomic biology have revolutionized our understanding and changed our view of yeast processing events. It is now clear that significant gene interactions and genomic regulatory networks need to be considered for achieving further improvement of the attributes of industrial strains. Genomic-based technologies will allow greater flexibility and power to design and develop more desirable and robust biocatalysts for achieving, within the next decade, a cost-effective and highly productive lignocellulosic conversion to ethanol.

A prototype of furfural and HMF conversion pathways relevant or critical to glycolysis and ethanol production, and particularly aldehyde inhibitors detoxification pathways, has been proposed (Liu, 2006; Liu et al., 2008b). An illustrative diagram of ethanologenic yeast responses to inhibitor stress and corresponding detoxification pathways is presented in Figure 12.6. It must be emphasized that this diagram remains largely incomplete, however,
Figure 12.6  Schematic diagram showing 2-furaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (HMF) conversion pathways relevant to glycolysis, and potential interactions with other candidate genes in a yeast cell. Furfural is converted into 2-furanmethanol (FM) and HMF into furan-2,5-dimethanol (FDM) coupled with NADH and/or NADPH and catalyzed by multiple enzymes possessing aldehyde reduction activities. Cinnamic acid and ferulic acid are converted into styrene and vinylguaiacol, catalyzed by phenylacrylic acid decarboxylase (Padlp). A shaded upward arrow indicates upregulated or induced gene expression, while an open downward arrow indicates downregulated or repressed expression for members of the pleiotropic drug resistance (PDR) gene family and other candidate genes and thus further studies of microbial stress tolerance and in situ detoxification are needed to attain a sufficiently deep knowledge of these pathways to enable the construction of optimal cellular biocatalysts. The biotransformation catalyzed by multiple aldehyde reductases of furfural, HMF, vanillin, and other aldehyde inhibitors by yeast results in the formation of the corresponding alcohol coupled with the cofactors NADH or NADPH. On the other hand, cinnamic acid and ferulic acid are respectively converted into styrene and vinylguaiacol, likely via the action of Padlp and other enzymes. The presence of aldehyde inhibitors appears to cause a redox imbalance that interferes with glycolysis, cell growth, and biosynthesis. A shortage of the cofactor NADH has been observed in the presence of furfural (Wahlbom and Hahn-Hägerdal, 2002). As a result of this reducing equivalent imbalance, aldehyde inhibitors could cause acetaldehyde accumulation that in turn would delay acetate and ethanol production. Empirical evidence accumulated to this date tends to demonstrate that the regeneration of a sufficient NADH pool from NAD+ is an apparent requirement for achieving efficient cell glycolysis and the efficient reduction of furfural, HMF, and other aldehydes interferes. In the presence of these inhibitors, the ATP level is typically low, cell replication is limited, and glucose is not consumed until adequate furfural and/or HMF reduction levels are reached (Larsson et al., 1999a; Taherzadeh et al., 2000;
Wahlbom and Hahn-Hägerdal, 2002; Liu et al., 2004). Notably, the NADPH-coupled furfural and HMF reduction activities of the whole-cell extract contribute to a great extent to \textit{in situ} detoxification. Consequently, synergistic competition for NADPH during the reduction reaction adds additional stress on cells, thus impacting growth, since NADPH is involved in numerous biosynthesis pathways. As a result, many metabolic process may be significantly altered and delayed in the presence of these inhibitors. Members of the PDR gene family mainly code for membrane and transport-related proteins; the latter are important as they are anticipated to play a significant role for yeast inhibitor tolerance and the regulation of detoxification gene interactions.

12.8 Perspective

Based on their chemical functional groups, inhibitory compounds derived from lignocellulosic biomass pretreatment are classified into four groups, comprising: (i) aldehydes; (ii) ketones; (iii) phenols; and (iv) organic acids. The mix of inhibitors – and thus the effects of inhibition on fermentative microbes – is of course strain-specific, but varies largely depending upon the biomass sources used as a primary raw material. This implies one major difficulty, which is to design a detoxification process of lignocellulosic biomass that is as generic as possible in order to maximize flexibility in manufacturing operations. One major economic hurdle to creating a sustainable biomass-to-biofuels industry is that the removal of inhibitors by physical or chemical means is unlikely to be a cost-competitive practice. Consequently, one of the leading paths of development is to derive tolerant strains that are able to \textit{in situ} detoxify harmful aldehydes, phenols, and organic acids. Despite a promising start, to this date the ability to overcome inhibitor complexes in biomass hydrolysates remains a significant challenge. The development of tolerant strains by directed evolutionary adaptation under laboratory settings is expected to play a significant role when combined with the necessary enhancements of genetic background through recombinant engineering. A deeper understanding of inhibitor conversion pathways and mechanisms of \textit{in situ} detoxification will undoubtedly facilitate the development of tolerant strains. Furfural is reduced to furan methanol and can be further catabolized to furoic acid and formic acid. HMF is reduced into furandimethanol and further to formic acid and levulinic acid. Many genes have been identified that code for enzymes possessing aldehyde reductase activities, including \textit{ADH6, ADH7, ALD4}, and \textit{GRE3}, in addition to several uncharacterized genes. The known mechanisms of the \textit{in situ} detoxification of furfural, HMF, cinnamaldehyde, and other aldehyde inhibitors are NAD(P)H-dependent aldehyde reductions catalyzed by multiple reductases. It appears critical to maintain a redox balance by reprogrammed pathways during the detoxification and ethanol production phases of the biomass-to-ethanol process. Furthermore, members of the PDR gene family are involved in the adaptive response to inhibitor stress conditions. Among the eight candidate transcriptional factors identified involving the inhibitor tolerance, \textit{Pdr3} and \textit{Yap1} are significantly involved in positively regulating gene responses and interactions during the coping reaction to HMF stress. The organic acid ferulic acid is metabolized to vinyl guaiacol, while 4-hydroxycinnamic acid is decarboxylated to styrene. These reactions are catalyzed by phenylacrylic acid decarboxylase and most likely involve other enzymes. Under oxygen-limited conditions, dihydrocinnamic acid and dihydroferulic acid are also produced.
Studies using genomic approaches to understanding inhibitor stress tolerance will allow a better understanding of cell response and *in situ* detoxification by various fermentative microorganisms.

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