RECOMBINANT YEAST HAVING ENHANCED GAMMA VALEROLACTONE TOLERANCE AND METHODS OF USE

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None
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References Cited
U.S. PATENT DOCUMENTS

OTHER PUBLICATIONS
Mukai et al., “PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in Saccharomyces cerevisiae”, Journal of Bioscience and Bioengineering, vol. 109, No. 6, pp. 564-569, 2010.*

* cited by examiner

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The present invention relates to materials and methods for the production of ethanol. More particularly, the present invention provides genetically modified strains of Saccharomyces cerevisiae having enhanced tolerance for gamma valerolactone (GVL) toxicity. Also provided are methods of using such genetically engineered yeast strains for improved GVL-mediated hydrolysis of lignocellulosic biomass for industrial-scale ethanol production.

22 Claims, 10 Drawing Sheets
FIGS. 2A-2C, CONTINUED

Late endosome to vacuole transport (p<0.01)

299 GVL sensitive mutants

Adj usted p-value

1E-27 1E-23 1E-19 1E-15 1E-11 1E-7 0.001

C

p<0.05

3.5 3.0 2.5 2.0 1.5 1.0

IC50 % GVVL

sec28

vps38

dil2A

Control
RECOMBINANT YEAST HAVING ENHANCED GAMMA VALEROLACTONE TOLERANCE AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 62/048,458, filed Sep. 10, 2014; which is incorporated herein by reference as if set forth in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under DE-FC02-07ER64494 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

FIELD OF THE INVENTION

Broadly, the present invention relates to materials and methods for the production of ethanol. In particular, the present invention relates to genetically modified yeast strains useful for glucose and xylose fermentation and, more specifically, to strains of Saccharomyces cerevisiae genetically engineered for enhanced tolerance for gamma valerolactone (GVL) toxicity and methods of using the same for improved GVL-mediated hydrolysis of lignocellulosic biomass for industrial-scale ethanol production.

BACKGROUND

Cellulosic biomass is a vast source of renewable energy and an abundant substrate for biofuel production. As an alternative to corn-based ethanol, bioethanol can be generated from lignocellulosic (LC) sugars derived from cellulosic biomass of renewable and sustainable plant feedstocks. Energy of cellulosic biomass is primarily stored as the recalcitrant polysaccharide cellulose, which is difficult to hydrolyze due to the highly crystalline structure, and in hemicellulose, which presents challenges because of its structural diversity and complexity. Many microbes cannot natively ferment pentose sugars (e.g., xylose) from complex lignocellulosic biomass, which is composed of cellulose, hemicellulose and lignin fractions. Even when engineered to express the minimal enzymes from native pentose sugar-metabolizing organisms, S. cerevisiae cannot ferment xylose from innocuous lab media at industrially-acceptable rates. Laluce et al., Applied Microbiol. Biotech. 166:1908 (2012); Almeida et al., Biotech. J. 6:286 (2011). Xylose is a prevalent sugar in both woody and herbaceous plants and a major component of hemicelluloses. Bioconversion of both xylose and glucose is required for the production of cellulosic biofuels. To further complicate matters, plant biomass must be chemically, mechanically, or thermally pretreated prior to enzymatic hydrolysis ex situ in order to produce fermentable glucose and xylose monomers. Such pretreatment processes generate a diverse array of degradation products derived from plant cell walls, such as hemicellulose and lignin-derived acetate and aromatic molecules, many of which inhibit cellular metabolism in S. cerevisiae and induce microbial stress during hydrolysate fermentation. Taylor et al., Biotechnology J. 7:1169 (2012); Liu, Applied Microbiol. Biotech. 90:809 (2011). At present, little is known about how such inhibitors impact xylose fermentation, particularly under strict industrially relevant, anaerobic conditions where ethanol production is maximized.

In view of the current state of the biofuel industry, particularly ethanol production based on glucose- and xylose-containing feedstocks, it can be appreciated that there remains a need for efficient and cost-effective processes for breaking down cellulose and hemicellulose into their constituent sugars.

SUMMARY OF THE INVENTION

The present invention is largely related to the inventors’ research efforts to better understand xylose utilization for microbial engineering. The invention relates generally to methods and compositions for digesting lignocellulosic material and more particularly to methods that involve exposing the material to genetically engineered Saccharomyces cerevisiae (S. cerevisiae) variants having enhanced tolerance for or resistance to gamma valerolactone (GVL)-mediated toxicity.

In a first aspect, provided herein is a recombinant yeast that has been genetically engineered to exhibit a reduced amount of functional PAD1 polypeptide. The recombinant yeast has increased tolerance to gamma valerolactone (GVL) toxicity relative to a wild-type yeast or another recombinant yeast not exhibiting a reduced amount of functional PAD1 polypeptide. The recombinant yeast can further exhibit a reduced amount of functional FDC1 polypeptide, wherein the recombinant yeast has increased tolerance to gamma valerolactone (GVL) toxicity relative to a wild-type yeast or another recombinant yeast not exhibiting reduced amounts of functional PAD1 and FDC1 polypeptides. In some cases, a recombinant yeast comprises a disabling mutation in a gene encoding PAD1 polypeptide. The recombinant yeast can further comprise a disabling mutation in a gene encoding FDC1 polypeptide. The gene encoding PAD1 polypeptide can be SEQ ID NO:8. The gene encoding FDC1 polypeptide can be SEQ ID NO:10.

In some cases, a recombinant yeast further exhibits reduced or undetectable amounts of functional ISU1, GRE3, and IRA2 polypeptides, wherein the recombinant yeast is capable of increased aerobic or anaerobic xylose fermentation relative to a wild-type yeast or another recombinant yeast not exhibiting reduced amounts of functional PAD1, ISU1, GRE3, and IRA2 polypeptides. The recombinant yeast can have disabling mutation at each of loci isu1, gre3, and ira2, whereby the mutations result in reduced amounts of functional ISU1, GRE3, and IRA2 polypeptides, respectively. The disabling mutation at the gre3 locus can comprise a substitution of a threonine for the alanine at amino acid residue position 46 of SEQ ID NO:4. The disabling mutation at the ira2 locus can comprise a substitution of a stop codon for the glutamate at amino acid residue position 2927 of SEQ ID NO:2. The disabling mutation at the isu1 locus can comprise a substitution of a tyrosine for the histidine at amino acid residue position 138 of SEQ ID NO:6. The recombinant yeast can produce ethanol at an increased rate relative to a wild-type yeast or another recombinant yeast not exhibiting reduced or undetectable amounts of functional ISU1, GRE3, and IRA2 polypeptides. The increased rate of ethanol production can occur under anaerobic conditions. The recombinant yeast can be of the genus Saccharomyces. The recombinant yeast can be of the species Saccharomyces cerevisiae. A portion of an extrachromosomal vector stably maintained in the recombinant yeast can comprise the disabling mutation. A nucleic acid sequence
comprising the disabling mutation can be integrated into a chromosome of the recombinant yeast.

In another aspect, a yeast inoculum is provided herein. The yeast inoculum can comprise a recombinant yeast as described herein and a culture medium.

In a further aspect of the invention, a method for fermenting cellulosic material into ethanol is provided. The method comprises contacting a GVL-treated hydrolysate to a recombinant yeast or a yeast inoculum provided herein for a period of time sufficient to allow fermentation of at least a portion of the cellulose material to ethanol, whereby the rate of fermentation of the cellulose material of the GVL-treated hydrolysate to ethanol is increased relative to the fermentation rate of a GVL-treated hydrolysate not contacted to the recombinant yeast or the yeast inoculum. The method can further comprise separating the ethanol from fermented cellulosic material. The GVL-treated hydrolysate can comprise xylose. The recombinant yeast can be Saccharomyces cerevisiae. The cellulosic material can comprise lignocellulosic biomass. In some cases, the lignocellulosic biomass comprises at least one material selected from the group consisting of agricultural residues, wood, municipal solid wastes, paper and pulp industry wastes, and herbaceous crops.

These and other features, objects, and advantages of the present invention will become better understood from the description that follows. In the description, reference is made to the accompanying drawings, which form a part hereof and in which there is shown by way of illustration, not limitation, embodiments of the invention. The description of preferred embodiments is not intended to limit the invention to cover all modifications, equivalents and alternatives. Reference should therefore be made to the claims recited herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be better understood and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIGS. 1A-1B show production of GVL hydrolysates and relative toxicity. Lignocellulosic biomass is heated with GVL to convert the cellulose and hemicellulose to sugar monomers, the result is a hydrolysate of sugars, amino acids, lignocellulosic derived fermentation inhibitors, and residual GVL that cannot be recovered (~1-3%) (A). The half-maximal inhibitory concentration (IC50) of GVL in rich media is 2.7% (B).

FIGS. 2A-2C show chemical genomic profiling of GVL. Chemical genomic profiling (A) revealed a significant enrichment for genes involved in late endosome to vacuole (p<0.01) among the top gene mutants sensitive to GVL (B). Single mutant validations of these individual mutants confirmed they were significantly more sensitive to GVL (C). (Mean±S.E., n=3).

FIGS. 3A-3C demonstrate that GVL rapidly compromises membrane integrity and is synergistic with ethanol. GVL compromises membrane integrity as determined by dye uptake after treatment, and this effect is apparent with as little as 2.5% GVL. (A, B). GVL is significantly synergistic with ethanol (C). (Mean±S.E., n=3).

FIGS. 4A-4D present genes mediating GVL toxicity by deletion and overexpression mutant profiling. Among deletion mutants significantly resistant to GVL, we saw significant enrichment for genes in phenylpropanoid metabolism (p<0.001), driven by the mutants of the decarboxylases Pad1p and Fdc1p (A). Individual mutants in these genes were more tolerant of GVL (B). Overexpression profiling using MoBY-ORF transformed Y133 demonstrated that overexpression of PAD1 conferred significant GVL sensitivity (C). Increased expression of PAD1 significantly reduced GVL tolerance in single mutant cultures (D). (Mean±S.E., n=3).

FIGS. 5A-5D demonstrate that deletion of PAD1 and FDC1 confers tolerance of GVL in a xylose fermenting yeast. A two-step PCR approach was used to simultaneously delete PAD1 and FDC1 in Y133, which are adjacent on chromosome IV (A), and confirmed deletion by PCR (B). The Y133 pad1Δfde1Δ mutant had significantly greater (p<0.01) tolerance of GVL (C), and also had faster growth, sugar consumption, and ethanol production under anaerobic conditions in synthetic hydrolysate with 1% GVL (D).

FIGS. 6A-6D demonstrate that vinyl products of PAD1 and FDC1 are more toxic than the acid precursors, and are synergistic with GVL. The decarboxylase Pad1p converts phenolic acids to a vinyl form (A). The vinyl derivative of coumaric acid (4-vinylphenol) is significantly more toxic than the acid form (B, p<0.001). 4-vinylphenol is significantly synergistic with GVL (C). Deletion of PAD1 and FDC1 confers resistance to coumaric acid (D).

While the present invention is susceptible to various modifications and alternative forms, exemplary embodiments thereof are shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the description of exemplary embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DETAILED DESCRIPTION OF THE INVENTION

In General

Before the present materials and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, materials, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. As well, the terms “a” (or “an”), “one or more” and “at least one” can be used interchangeably herein. It is also to be noted that the terms “comprising,” “including,” and “having” can be used interchangeably.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications and patents specifically mentioned herein are incorporated by reference for all purposes including describing and disclosing the chemicals, cell lines, vectors, animals, instruments, statistical analysis and methodologies which
are reported in the publications which might be used in connection with the invention. All references cited in this specification are to be taken as indicative of the level of skill in the art. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.


The nucleotides which occur in the various nucleotide sequences appearing herein have their usual single-letter designations (A, G, T, C or U) used routinely in the art. In the present specification and claims, references to Greek letters may either be written out as alpha, beta, etc. or the corresponding Greek letter symbols (e.g., α, β, etc.) may sometimes be used.

As used herein, the term polynucleotide generally refers to any polynucleotide (polyribonucleotide or polypeptide ionucleotide), which may be unmodified RNA or DNA or modified RNA or DNA. Polynucleotides include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. As used herein, the term polynucleotide also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are “polynucleotides” as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as trytiated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. The term polynucleotide also embraces short polynucleotides often referred to as oligonucleotide(s).

The term “isolated nucleic acid” as used herein means a nucleic acid isolated from its natural environment or prepared using synthetic methods such as those known to one of ordinary skill in the art. Complete purification is not required in either case. The nucleic acids of the invention can be isolated and purified from normally associated materials in conventional ways such that in the purified preparation the nucleic acid is the predominant species in the preparation. At the very least, the degree of purification is such that the extraneous material in the preparation does not interfere with use of the nucleic acid of the invention in the manner disclosed herein. The nucleic acid is preferably at least about 85% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

Further, an isolated nucleic acid has a structure that is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. An isolated nucleic acid also includes, without limitation, (a) a nucleic acid having a sequence of a naturally occurring genomic or extrachromosomal nucleic acid molecule but which is not flanked by the coding sequences that flank the sequence in its natural position; (b) a nucleic acid incorporated into a vector or into a prokaryote or eukaryote genome such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene. Specifically excluded from this definition are nucleic acids present in mixtures of clones, e.g., as those occurring in a DNA library such as a cDNA or genomic DNA library. An isolated nucleic acid can be modified or unmodified DNA or RNA, whether fully or partially single-stranded or double-stranded or even triple-stranded. A nucleic acid can be chemically or enzymatically modified and can include so-called non-standard bases such as inosine, as described in a preceding definition.

Compositions of the invention

Efficient biochemical conversion and fermentation of renewable lignocellulosic feedstocks is essential for the production of biofuels and other bioproducts from plant materials. While S. cerevisiae excel at fermentation of glucose from corn and sugar cane, the fermentation of renewable lignocellulosic biomass presents a significant challenge. Xylose, which is a pentose sugar and a major component of hemimcellulose, can comprise almost 30% of total cell wall carbohydrate in grasses. Its conversion, along with glucose, into ethanol is critical for any economically-viable cellulosic biofuel process. Biomass pretreatments and enzymatic hydrolysis are viable but costly ways of depolymerizing cellulose and hemicellulose fractions of biomass to produce soluble carbohydrates. Large-scale depolymerization cellulose and hemicellulose factions of biomass is increasingly economically feasible as the cost of pretreatment reagents drops. Acid-catalyzed hydrolysis methods are generally less expensive than enzyme-catalyzed methods. Gamma-valerolactone (GVL) is an inexpensive solvent that can be derived from cellulose or hemicelluloses. GVL promotes thermocatalytic saccharification through complete solubilization of all lignocellulosic biomass components including lignin, which makes GVL-mediated hydrolysis of lignocellulosic biomass a potentially transformative technology for biofuel production. Luterbacher et al., Science 343:277-280 (2014); see also Bond et al., Integrated Catalytic Conversion of γ-Valerolactone to Liquid Alkenes for Transportation Fuels, Science 26: (2010). Standard methods
of GVL-mediated hydrolysis yields hydrolysates having high sugar levels (glucose and xylose) but also having residual levels of GVL that are toxic to fermentative microorganisms such as yeast. Current GVL-mediated hydrolysis methods yield hydrolysates comprising about 2.3% residual GVL. The present invention is based, at least in part, on the Inventors’ discovery of genetic modifications that increase a yeast strain’s tolerance for GVL toxicity and increase its growth rate in the presence of GVL.

Accordingly, one aspect of the present invention relates to eukaryotic host cells genetically engineered for improved tolerance to GVL toxicity. In particular, the present invention provides eukaryotic cells that have been genetically engineered to have enhanced GVL toxicity tolerance, enhanced anaerobic and/or aerobic xylose fermentation, and increased ethanol production relative to an unmodified cell or a recombinant cell not genetically engineered as described herein. Modified cells of the present invention are well-suited for producing a variety of fermentation products, including ethanol, in fermentation processes that use xylose or a combination of xylose and glucose as carbon sources. As used herein, a “host cell” is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence. A host cell that has been transformed or transfected may be more specifically referred to as a “recombinant host cell.” A preferred host cell is a host cell that is naturally capable of alcoholic fermentation, preferably, anaerobic alcoholic fermentation. Host cells may also exhibit a high tolerance to ethanol, low pH, organic acids, and/or elevated temperatures. Such characteristics or activities of the host cell may be naturally present in the host cell or may be introduced or modified by genetic modification. Preferred host cells for the present invention include yeast cells, particularly yeast cells of the genus Saccharomyces. Preferred yeast species as host cells include Saccharomyces cerevisiae, S. bulderi, S. barnettii, S. exigua, S. sovarum, S. diastaticus, K. lactis, K. marxianus, and K. fragilis, of which yeast cells of the genus Saccharomyces and yeast cells of the species Saccharo- myces cerevisiae (S. cerevisiae) are preferred. Yeasts of the genus Saccharomyces possess both a metabolic pathway and a fermentative pathway for respiration.

“Yeasts” are eukaryotic micro-organisms classified in the kingdom Fungi. Most reproduce asexually by budding, although some yeasts undergo sexual reproduction by meiosis. Yeasts are unicellular, although some species with yeast forms may become multi-cellular through the formation of a string of connected budding cells known as pseudohyphae, or false hyphae, as seen in most molds. Yeasts do not form a single taxonomic or phylogenetic grouping. The term “yeast” is often taken as a synonym for Saccharomyces cerevisiae, but the phylogenetic diversity of yeasts is illustrated by their assignment to two taxonomic classes of fungi, the ascomycetes and the basidiomycetes.

In exemplary embodiments, a genetically modified yeast of the present invention comprises one or more genetic modifications that reduce or disrupt expression of functional PAD1 (phenylacrylic acid deacetylase) polypeptide or functional FDC1 (fenolic acid deacetylase) polypeptide. PAD1 and FDC1 are phenylacrylic acid deacetylases that deacetylate aromatic phenylacrylic acids (e.g., fenolic acid, p-coumaric acid, cinnamic acid) in S. cerevisiae. See Clausen et al., Gene 142(1):107-12 (1994); Mukai et al., J. Bioscience & Bioengineering 109(6):564-569 (2010). Full-length PAD1 (NCBI Gene ID: 852150) polypeptide is 242 amino acids. Full-length FDC1 (NCBI Gene ID: 852152) polypeptide is 503 amino acids. By “delete or disrupt”, it is meant that the entire coding region of the gene is eliminated (deletion), or the gene or its promoter and/or terminator region is modified (such as by deletion, insertion, or mutation) such that the gene no longer produces a partially or fully non-functional polypeptide (i.e., lacking enzymatic activity), or produces an enzyme with severely reduced activity. The deletion or disruption can be accomplished by genetic engineering methods, forced evolution or mutagenesis, and/or selection or screening.

In some cases, a recombinant yeast of the present invention comprises a genetic modification that deletes or disrupts a Pad1 nucleic acid that encodes PAD1 polypeptide, whereby the genetically modified yeast produces a reduced level of functional PAD1 polypeptide. In other embodiments, a recombinant yeast of the present invention comprises a genetic modification that deletes or disrupts a Fdc1 nucleic acid that encodes FDC1 polypeptide, whereby the genetically modified yeast produces a reduced level of functional FDC1 polypeptide. In some cases, such genetically modified yeast produce no or substantially no functional PAD1 polypeptide. In other embodiments, a recombinant yeast of the present invention comprises a genetic modification that deletes or disrupts a Fdc1 nucleic acid that encodes FDC1 polypeptide, whereby the genetically modified yeast produces a reduced level of functional FDC1 polypeptide. In some cases, such genetically modified yeast produces no or substantially no functional PAD1 polypeptide. In some cases, it will be advantageous to genetically modify a host cell to comprise genetic modifications that cause reduced levels of both functional polypeptides, PAD1 and FDC1. Recombinant yeast comprising one or more of the genetic modifications described herein exhibit improved fermentation rates relative to unmodified yeast or yeast not comprising the genetic modifications described herein. Such recombinant yeast also exhibit increased tolerance to GVL toxicity and improved growth rates in hydrolysates comprising residual GVL following GVL-mediated hydrolysis.

It is contemplated that certain additional genetic modifications may be advantageous or necessary to produce other desirable characteristics and/or to enable the yeast cell to produce certain products at industrially-acceptable levels. For example, genetic modifications that reduce or eliminate functional PAD1 polypeptide or functional FDC1 polypeptide can be introduced into S. cerevisiae yeast of the GLBRCY133 (“Y133”) strain (a GLBRCY128 derivative). Yeast of the GLBRCY128 (“Y128”) strain were evolved for robust, anaerobic xylose metabolism under industrially relevant conditions and high yields of extracellular ethanol. Forced evolution of the Y128 yeast strain from a background strain designated NRRL YB-210/GLBRCY0 (Mortimer and Johnston, Genetics 113(1):35-43 (1986)), has been described elsewhere. See U.S. Application No. 61/978,585, filed Apr. 11, 2014. Yeast of the Y133 strain comprise the genotype of GLBRCY128, but with the loxp-KanMX-loxp marker excised by Cre as previously described (Parrerias et al., PLoS One 2014; 9(9):e107490).

Accordingly, in some cases, a recombinant yeast of the present invention comprises a genetic modification that deletes or disrupts a Pad1 nucleic acid that encodes PAD1 polypeptide and further comprises a disabling mutation at each of loci isul1, gre3, and ira2, whereby the mutations result in reduced amounts of functional ISU1, GRE3, and IRA2 polypeptides.

The degree of GVL’s toxicity to a microorganism such as yeast depends on the yeast’s growth conditions. Generally, yeast grown in a minimal medium are more sensitive to chemical stress, while yeast grown in a nutrient-rich medium are more tolerant of chemical stress. Recombinant yeast of the present invention tolerate higher levels of GVL relative to a wild type yeast or yeast not comprising a genetic modification described herein when grown in either a nutri-
ent-rich medium or minimal medium. In exemplary embodiments, a recombinant yeast of the present invention that comprises a genetic modification resulting in reduced levels of functional PAD1 polypeptide has significantly more GVL tolerance (P<0.05) than a yeast having the same genetic background but having normal levels of functional PAD1 polypeptide, even when grown under industrially relevant conditions in a minimal medium with high sugar loading (osmotically stressful). In general, toxicity is expressed as the “half maximal inhibitory concentration” or “IC₅₀,” the terms “half maximal inhibitory concentration” and “IC₅₀” are used interchangeably and, as used herein, refer to a concentration of the compound that is required to inhibit a given biological or biochemical function by half. In a standard yeast lab strain, the IC₅₀ is about 1.98% GVL, while a PAD1 deletion mutant in the standard lab strain background has an IC₅₀ of about 2.4% GVL and FDC1 deletion mutant in the standard lab strain background has an IC₅₀ of about 2.1% GVL. In other words, a yeast having a genetic modification (in a standard lab strain background) that eliminates functional PAD1 polypeptide can tolerate GVL toxicity wherein GVL comprises about 2.4% of the hydrolysate. Similarly, yeast having a genetic modification (in a standard lab strain background) that eliminates functional FDC1 polypeptide can tolerate GVL toxicity wherein GVL comprises about 2.1% of the hydrolysate.

The GVL IC₅₀ for a genetically modified yeast of the present invention, when grown anaerobically in a minimal medium, is in the range between about 1.15% GVL and about 1.28% GVL, as compared to an IC₅₀ of about 1.0% for unmodified yeast of the xylose-fermenting background strain (Y128). When grown in a nutrient-rich media, yeast of the Y128 xylose-fermenting strain have an IC₅₀ of about 2.2% GVL, whereas genetically modified yeast of the present invention have an IC₅₀ of about 2.4% GVL to about 2.5% GVL. When grown in a nutrient-rich media, yeast of the Y133 xylose-fermenting strain have an IC₅₀ of about 2.7% GVL, whereas yeast of the genetically modified strain (e.g., Y133 pΔ1Δ1α1Δ1α) of the present invention have an IC₅₀ of about 2.9% GVL.

In some cases, a suitable host yeast cell comprises at least one native gene (a “xylose isomerase gene”) that produces an active xylose isomerase enzyme that is capable of catalyzing the interconversion of D-xylose to D-xylulose. Xylose isomerase can also catalyze the interconversion of D-ribose to D-ribulose and D-glucose to D-fructose. The enzyme can be specific to the reduction of xylose or non-specific (i.e., capable of catalyzing the conversion of a range of pentose sugars). In some cases, a suitable host yeast cell is genetically engineered to contain an expression cassette containing Clostridium phytofermentans xylose isomerase (CpIXYLA), which can confer anaerobic xylose fermentation by S. cerevisiae with additional genetic modifications (see Brat et al., Applied Environmental Microbiol. 75:2304 (2009)), driven by the ScerTDH3 promoter. In exemplary embodiments, the expression cassette further comprises ScarTAL1, a Pentose Phosphate Pathway transaldolase enzyme that can improve xylose metabolism when overexpressed (see Ni et al., Applied Environmental Microbiol. 73:2061 (2007); Walfurdsson et al., Applied Environmental Microbiol. 61:4184 (1995), and SstpXYL3 driven by the ScarPYGK1 and ScarTEF2 promoters, respectively. For example, the host yeast cell can comprise a TAL1-XYL-A-XYL3 gene expression cassette.

Genetic modification of the host cell can be accomplished in one or more steps via the design and construction of appropriate vectors and transformation of the host cell with those vectors. Nucleic acid constructs useful in the invention may be prepared in conventional ways, by isolating the desired genes from an appropriate host, by synthesizing all or a portion of the genes, or combinations thereof. Similarly, the regulatory signals, the transcriptional and translational initiation and termination regions, may be isolated from a natural source, be synthesized, or combinations thereof. The various fragments may be subjected to endonuclease digestion (restriction), ligation, sequencing, in vitro mutagenesis, primer repair, or the like. The various manipulations are well known in the literature and will be employed to achieve specific purposes.

The various nucleic acids and/or fragments thereof may be combined, cloned, isolated and sequenced in accordance with conventional ways. After each manipulation, the DNA fragment or combination of fragments may be inserted into the cloning vector, the vector transformed into a cloning host, e.g., E. coli, the cloning host grown up, lysed, the plasmid isolated and the fragment analyzed by restriction analysis, sequencing, combinations thereof, or the like.

Targeted integration can be accomplished by designing a vector having regions that are homologous to the upstream (5'-) and downstream (3'-) flanks of the targeted gene. Either of both of these regions may include a portion of the coding region of the targeted gene. The gene cassette (including associated promoters and terminators if different from those of the targeted gene) and selection markers (with associated promoters and terminators as may be needed) can reside on a vector between the regions that are homologous to the upstream and downstream flanks of the targeted gene. Targeted cassette insertion can be verified by any appropriate method such as, for example, PCR. A host cell may be transformed according to conventional methods that are known to practitioners in the art. Electroporation and/or chemical (such as calcium chloride- or lithium acetate-based) transformation methods can be used. The DNA used in the transformations can either be cut with particular restriction enzymes or used as circular DNA. Methods for transforming yeast strains are described in WO 99/14335, WO 00/71738, WO 02/42471, WO 03/102201, WO 03/102152 and WO 03/049525; these methods are generally applicable for transforming host cells in accordance with this invention. Other methods for transforming eukaryotic host cells are well known in the art such as from standard handbooks, such as Sambrook and Russel (2001) “Molecular Cloning: A Laboratory Manual (3rd edition),” Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, or F. Ausubel et al., eds., “Current protocols in molecular biology,” Green Publishing and Wiley Interscience, New York (1987).

In another aspect, compositions of the present invention further include yeast inocula comprising recombinant yeast as provided herein. A yeast inoculum of the present invention comprises (a) a recombinant yeast as provided herein and (b) a culture medium. In exemplary embodiments, the recombinant yeast is S. cerevisiae and the culture medium is a liquid culture medium. Yeast inocula of the present invention include large-scale preparations of sufficient quantities of viable yeast cells for use in, for example, xylose fermentation and other industrial ethanol-producing methods. When contacted to a GVL-treated hydrolysate comprising some level of residual GVL, a yeast inoculum of the present invention exhibits improved xylose fermentation rates and increased growth rates relative to a yeast inoculum that does not comprises a recombinant yeast of the present invention.

Methods of the Invention

The methods provided by the present invention involve the discovery and incorporation of genetic modifications
into genes encoding certain polypeptides into a single host organism and the use of those organisms to convert xyllose to ethanol. In particular, the present invention provides a method of fermenting cellulose material comprising the 5-carbon sugar xyllose into ethanol, where the method comprises use of a recombinant yeast having enhanced tolerance of GVL relative to wild type yeast or a recombinant yeast not comprising the genetic modifications described herein.

In exemplary embodiments, recombinant yeast of the present invention are used to make ethanol by converting xyllose and other sugars under appropriate fermentation conditions. The sugars can come from a variety of sources including, but not limited to, cellulose material. The cellulose material can be lignocellulosic biomass. As used herein, the term “lignocellulosic biomass” refers to any materials comprising cellulose, hemicellulose, and lignin, wherein the carbohydrate polymers (cellulose and hemicelluloses) are tightly bound to the lignin. Generally, lignocellulosic material for making ethanol is feedstock such as corn stover, which consists of the stems, cobs, and leaves from the corn plants (i.e., the non-grain material). Corn stover is typically shredded by mechanical means and incorporated by tillage into topsoil for decomposition. In addition to lignocellulosic ethanol production from corn stover, other feedstocks such as sorghum, wheat, or another grain can be used. In some cases, lignocellulosic biomass comprises material selected from the group consisting of materials that comprise at least 75% cellulose, cellulose/hemicelluloses, xyllose, biomass, and chitin. In other cases, the lignocellulosic biomass comprises at least one material selected from the group consisting of agricultural residues, wood, municipal solid wastes, paper and pulp industry wastes, and herbaceous crops. As used herein, the term “biomass” refers to a renewable energy source, is biological material from living or recently living organisms. As an energy source, biomass can either be used directly, or converted into other energy products such as biofuel. Biomass includes plant or animal matter that can be converted into fibers or other industrial chemicals, including biofuels. Industrial biomass can be grown from numerous types of plants, including miscanthus, switchgrass, hemp, corn, poplar, willow, sorghum, sugarcane, bamboo, and a variety of tree species, ranging from eucalyptus to oil palm (palm oil). Thus, biomass can include wood biomass and non-wood biomass.

In some cases, methods of the present invention include a hydrolysis step. For example, when cellulose material is used in the methods disclosed herein, the material can be hydrolyzed to produce a hydrolysate comprising xyllose and glucose, which is subsequently contacted to one or more recombinant yeasts of the present invention. As used herein, the term “hydrolysate” refers to a fermentable sugar-containing product produced from cellulose material (e.g., biomass), typically through pretreatment and saccharification processes. In exemplary embodiments, cellulose material is pretreated using a solvent comprising gamma-valerolactone (GVL or y-valerolactone). Such a pretreatment may also comprise one or more physical or chemical treatments such as grinding, milling, cutting, base treatment such as with ammonia or NaOH, and acid treatment.

In some cases, GVL-mediated hydrolysis further comprises an enzymatic saccharification treatment. Enzymatic saccharification typically makes use of an enzyme composition or blend to break down cellulose and/or hemicellulose and to produce a GVL-treated hydrolysate containing 6-carbon sugars (e.g., glucose) and 5-carbon sugars (e.g., xyllose, arabinose). For review of saccharification enzymes, see Lynd et al., *Microbiol. Mol. Biol. Rev.* 66:506-577 (2002). Saccharification enzymes may be obtained commercially. In some cases, saccharification enzymes may be produced using recombinant microorganisms that have been engineered to express one or more saccharifying enzymes.

Following hydrolyzation, a GVL-treated hydrolysate is contacted with one or more of the genetically engineered yeasts disclosed herein (e.g., a yeast strain genetically modified to exhibit reduced amounts of functional PADI polypeptide and/or functional FDC1 polypeptide) under conditions suitable for fermentation. Fermentation conditions can comprise aerobic or anaerobic conditions. In exemplary embodiments, a method of the invention comprises contacting under anaerobic conditions a recombinant yeast as provided herein to a GVL-treated hydrolysate for a period of time sufficient to allow fermentation of at least a portion of the cellulose material into ethanol. In exemplary embodiments, a recombinant yeast used according to the methods provided herein is *Saccharomyces cerevisiae*. As used herein, “anaerobic fermentation” refers to a fermentation process run in the absence of oxygen, or in which substantially no oxygen is consumed. Preferably less than 5, 2.5, or 1 mmol/L h, more preferably 0 mmol/L h is consumed, (i.e., oxygen consumption is not detectable), and where organic molecules serve as both electron donor and electron acceptors. In the absence of oxygen, NADH produced in glycolysis and biomass formation cannot be oxidized by oxidative phosphorylation.

In another aspect, the present invention provides a method of fermenting cellulose material comprising the 5-carbon sugar xyllose into ethanol, where the method comprises use of a recombinant yeast having enhanced tolerance of GVL relative to a wild type yeast or a recombinant yeast not comprising the genetic modifications described herein. In particular, the present invention provides a method whereby the rate of fermentation of cellulose material in a GVL-treated hydrolysate to ethanol is increased relative to the fermentation rate of a GVL-treated hydrolysate not contacted to a recombinant yeast or yeast inoculum provided by the present invention. In such cases, the method comprises contacting a GVL-treated hydrolysate to a recombinant yeast having increased tolerance to GVL toxicity, whereby cellulose material of the contacted hydrolysate is fermented to produce ethanol at an enhanced rate relative to fermentation of a GVL-treated hydrolysate that has not been contacted to a recombinant yeast of the present invention.

In some cases, methods of the present invention further comprise an ethanol separation or extraction step. Following conversion of sugars into ethanol, the ethanol can be separated from a fermentation culture using, for example, a standard distillation method or by filtration using membranes or membrane systems known in the art. Methods of separating or extracting are not restricted to those disclosed herein.

Methods of the present invention can be conducted continuously, batch-wise, or some combination thereof. In some cases, a genetically engineered yeast disclosed herein can be used to produce ethanol from glycerol. Glycerol is a by-product of biodiesel production, which, using a recombinant yeast of the present invention, could be further converted to ethanol. In some cases, a method of converting glycerol to ethanol can comprise contacting glycerol to one or more of the genetically engineered yeasts disclosed herein (e.g., a yeast strain genetically modified to exhibit reduced amounts of functional PADI polypeptide and/or functional FDC1 polypeptide) under appropriate fermentation conditions. For example, a method of converting glycerol into ethanol can comprise contacting under anaerobic conditions...
a recombinant yeast as provided herein to glycerol for a period of time sufficient to allow fermentation of at least a portion of the glycerol into ethanol. In exemplary embodiments, a recombinant yeast used according to the methods provided herein is *Saccharomyces cerevisiae* (S. cerevisiae). In some cases, the glycerol is crude glycerol.

Following conversion of glycerol into ethanol, the ethanol can be separated from a fermentation culture using, for example, a standard distillation method or by filtration using membranes or membrane systems known in the art. Methods of separating or extracting are not restricted to those disclosed or exemplified herein.

Articles of Manufacture

In a further aspect, the present invention provides an article of manufacture containing any one or more of the recombinant yeasts disclosed herein. An article of manufacture can contain one of the microorganisms disclosed herein (e.g., one or more of the yeasts strains), or an article of manufacture can contain two or more of the microorganisms disclosed herein. Articles of manufacture disclosed herein also can include, for example, components necessary for growth of the particular microorganism(s).

While the present invention is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the description herein of specific embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

The present invention will be more fully understood upon consideration of the following non-limiting Examples. All papers and patents disclosed herein are hereby incorporated by reference as if set forth in their entirety.

EXAMPLES

**Genetic Engineering and Directed Evolution of a S. cerevisiae Strain Tolerant to GVL Toxicity**

Lignocellulosic biomass derived fuels and chemicals provide a suite sustainable bioproducts. Before biomass can be converted to fuel or compounds, it must be converted to fermentable sugars (pre-treatment and hydrolysis), and these sugars converted to fuels by microorganisms. Both pre-treatment and hydrolysis can imbue the resultant hydrolysates with toxicity arising from residual pre-treatment chemicals or biomass derived inhibitors [2, 3], which throttle fermentation rates at a substantial economic cost [4].

Fermentation inhibitors come in many forms, and the landscape of these inhibitors is constantly changing as new pre-treatment, hydrolysis, and feedstocks technologies are developed [2]. Enzymatic hydrolysis of biomass for fermentation releases small acids, phenolics, and furans that are a ubiquitous challenge to bioconversion [3, 5]. Chemical hydrolysis methods such as γ-valerolactone (GVL) and ionic liquids offer an enzyme free route to fermentable sugars, but come with their own challenges [6-8]. In addition to the small acid inhibitors, the chemicals used for hydrolysis can persist in residual amounts into the resultant hydrolysate, and these compounds are not biologically benign to fermentative microorganisms [7, 8]. Further, as these chemical catalyst are used in relatively large amounts during hydrolysis, they residual concentrations are often much higher than the small acid and phenolic inhibitors generated from the biomass.

GVL is a promising, new chemical hydrolysis technology to breakdown the cellulose polysaccharides to fermentable sugar monomers [6]. The advantage of GVL is that it is a recoverable and renewable chemical. One challenge of this method is the toxicity of residual GVL to fermentative microbes. GVL is mildly toxic to yeast, but this toxicity can be magnified when in combination with other inhibitors and the ethanol produced. As such, engineering GVL tolerant microbes is a means of overcoming toxicity, minimizing the costs of reagent recovery, and improving biofuels produced via ionic liquid hydrolysis.

We have used chemical genomics to discover the genome-wide response to toxicity. Using this information we have identified specific genes that mediate toxicity, and have engineered these specific mutations into an industrially viable, xylose-fermenting strain of *Saccharomyces cerevisiae*. This approach offer a rapid method of tailoring existing strains to specific chemical stressors found in industrial biocconversion.

GVL is the Major Inhibitor Found in GVL Hydrolysates: GVL produced hydrolysates (FIG. 1A) are still largely unstudied, as such, our first goal was to identify the major inhibitors of GVL hydrolysates. LC/MS of hydrolysates revealed that three inhibitory compounds were highly abundant in the GVL hydrolysates: GVL, levulinic acid, and hydroxymethylfurfural (HMF); other lignocellulosic derived inhibitors were present, but at orders of magnitude lower concentrations (Table 1). GVL hydrolysates have a high level of residual GVL (230 mM), and as such this is the most toxic major inhibitor in GVL hydrolysates, given its half maximal inhibitory concentration (IC₅₀) is 270 mM (FIG. 1B). Because of this, we focused on understanding GVL toxicity and developing GVL-tolerant yeast strains.

**TABLE 1**

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Chemical Genomic Predicts GVL Targets Cellular Membranes and Membrane Bound Processes:

To understand the mode of action of GVL toxicity we conducted chemical genomic analysis (FIG. 2A). This is a reverse genetics method that uses collections of defined gene mutants, and uses the response of these mutants in the presence of a chemical stress to gain functional insight into the chemical's mode of action and cellular target. We first challenged the yeast deletion collection with media containing 230 mM GVL and used barcode sequencing to identify the fitness response of the individual deletion mutants.

Among the top 10 sensitive deletion mutants, we found significant enrichment for genes involved in late endosome to vacuole transport (p<0.01, FIG. 2B), driven by deletion mutants of SEC28, VPS38, DID2. We validated mutants within this GO terms using single mutants culture, and found all had a lower IC₅₀ compared to the control strain (FIG.
Deletion mutants of these 3 genes have increased sensitivity to ethanol, heat, and membrane disrupting agents such as micanozole and nigerin. When we correlated the chemical genomic profile of GVL with the yeast genetic interaction network [10], we found significant enrichment for genes involved in golgi-vesicle mediated transport among the top 10 correlations (p < 0.001). RET2 was consistently predicted as the top correlation for the GVL chemical genomic profile. Ret2p is a subunit of the coatamer complex involved in retrograde transport between Golgi and ER and is also involved in golgi transport of vesicles [11]. RET2 mutants similarity show increased sensitivity to heat and membrane disrupting agents. We correlated the chemical genomic profile to GVL to existing chemical genomic datasets, and found its profile was significantly similar to profiles of nigerin (p < 0.01) and papainamide (p < 0.01), membrane destabilizing compounds. Taken together, these data suggest GVL could exert toxicity by damaging membrane integrity.

GVL Damages Membranes and is Synergistic with Ethanol:
To confirm if GVL treatment can rapidly affect cell integrity, we assessed cell permeability after GVL treatment. Using FACS analysis combined with a dye that is only taken up by cells with damaged membranes, we found a rapid and dose dependent effect of GVL on leakage (FIGS. 3A, 3B), similar to the effects of ethanol but with a greater magnitude (FIG. 3B). Given that both GVL and ethanol can damage cellular membranes, we also tested if these compounds are synergistic. We found a strong synergism between GVL and ethanol in both our lab strain and xylose fermenting strain (FIG. 3C). At a 1% GVL concentration and 4% ethanol concentration, we saw a significant synergistic interaction between GVL and ethanol (p < 0.01). This suggest that as ethanol titers increase during fermentation, the toxic effects of GVL and ethanol will magnify each other, which ultimately affects yields.

Deletion of the Decarboxylases Pad1p and Fdc1p Enhance GVL Tolerance:
Importantly for our goal, we also looked for gene deletions that increased resistance to GVL. Among the top GVL resistance mutants we found a significant enrichment for genes involved in phenylpropanoid metabolic process (p < 0.002, FIG. 4A), driven by deletion mutations of PAD1 and FDC1. Single mutant validations reveals deletion of these genes improved GVL tolerance (FIG. 4B). Pad1p is phenylacrylic acid decarboxylase with a reported role in aromatic acid catabolism and the ability to detoxify cinnamic acid [12, 13]. Like Pad1p, Fdc1p is thought to be a similar phenylacrylic acid decarboxylase involved in detoxifying ferulic acid [13].

Using chemical genomics guided biodesign, we identified 2 genes that are key in mediating GVL tolerance of the fermentative yeast S. cerevisiae. Deletion of the genes encoding acid decarboxylases Pad1 and Fdc1 conferred greater tolerance to GVL. These specific mutations were introduced into an industrially viable, xylose fermenting yeast (GLBRC-Y128) to create an ethanol producing yeast (GVL-R1) specifically tailored for GVL hydrolysates. Yeast of the GVL-R1 strain has the advantage of being able to grow and ferment both glucose and xylose faster than the GLBRC-Y128 strain in up to 2.5% residual GVL. Under our test conditions, GLBRC-Y128 required 72 hours to completely ferment all glucose, whereas engineered strain GVL-R1 achieved this in 48 hours. Importantly, engineered strain GVL-R1 was also able to ferment xylose, allowing for greater ethanol yields.

Overexpression Chemical Genomic Profiling Confirms Pad1p Mediates GVL Toxicity:
We wanted to expand our chemical genomic analysis to industrially an industrially relevant, xylose ferment yeast strain. Presently there are no available genome-wide deletion mutant collections in industrial yeast, so we took a complementary approach. The MoBY-ORF 2.0 plasmid collection is with barcoded versions of 95% of all S. cerevisiae genes each expressed on a µl plasmid [14]. This collection of plasmids can be pooled and transformed into any yeast to allow investigations of the effect of gene dose under stress conditions. We transformed a version of the xylose-fermenting yeast GLBRC-Y133 [15] en masse with the pooled plasmid collection and selected over 50K individual transformants (10x genome coverage). We grew this pooled transformant collection in the presence of 2.5% GVL over water control under anaerobic conditions in glucose, xylose containing media and assessed the effects of increased gene dose on growth in the presence of GVL. We found the Pad1p overexpression mutant was one of the top sensitive strains (p < 0.01, FIG. 4C). We confirmed with single mutant cultures that overexpression of PAD1 causes GVL sensitivity. The IC50 of Y133*pad1Δ was 2.2%, compared to 2.56% of vector control (FIG. 4D, p < 0.001).

Deletion of PAD1 and FDC1 in a Xylose Fermenting Strain Confers GVL Tolerance:
Chemical genomic profiling and validation of individual mutants confirmed that the decarboxylase Pad1p (and Fdc1p) were involved in GVL toxicity. We chose to engineer these deletions into a xylose fermenting yeast strain GLBRC Y133 (henceforth Y133). PAD1 and FDC1 are adjacent on chromosome IV, and as such we were able to delete both at the same time using transformation with PCR product of the antibiotic resistance marker KanMX flanked by homologous regions upstream of PAM and downstream of FDC1 (FIG. 4A). We confirmed deletion of both genes by PCR (FIG. 4B).

The IC50 concentration of GVL of the Y133 pad1Δfdc1Δ strain was significantly higher than the Y133 background (FIG. 5C, p < 0.01). Finally, we tested the performance of the Y133 pad1Δfdc1Δ strain under industrially relevant anaerobic conditions in a synthetic hydrolysate containing 1% GVL. The double KO strain grew, consumed sugars, and produced ethanol, whereas the Y133 background strain did not (FIG. 5D).

Vinyl Products of Pad1p Decarboxylation are Synergistic with GVL:
PAD1 and FDC1 are known to convert phenolic acids into a vinyl form, and are thought to “detoxify” ferulic and coumaric acids; however, we posit that the vinyl derivatives (see FIG. 6A) may be more toxic than the acid forms. We found that the IC50 values of the vinyl derivative of coumaric acid, 4-vinylphenol, was an order of magnitude lower than that of coumaric acid (FIG. 6B). Further, we found a statistically significant synergistic interaction between the vinyl derivative of coumaric acid, 4-vinylphenol, and GVL (FIG. 6C, p < 0.01). Chemical genomic profiling of 4-vinylphenol revealed that, similar to GVL, RET2 was the top genetic interaction network correlation, and this compound was similarly predicted to target the membrane bound processes such as vesicle mediated transport. Deletion of PAD1 and FDC1 may reduce production of membrane damaging vinyls, which are synergistic with GVL. Our Y133 pad1Δfdc1Δ has a statistically significantly greater tolerance of coumaric acid (FIG. 6D, p < 0.01), and we contend this is because less of the more toxic vinyl form is being generated.
Discussion
Through chemical genomic analysis we predicted and confirmed the chemical hydrolysis reagent gamma-valerolactone exerts toxicity by damaging cellular membranes, similar to ethanol and other membrane damaging drugs. Further, this compound is synergistic with ethanol. While this compound is less toxic than other fermentation inhibitors (e.g., furfural, HMF), the abundance of residual GVL in hydrolysates may ultimately limit ethanol production through a documented synergism with end-product fuels such as ethanol or isobutanol. The toxic effects of GVL can be alleviated by specific deletion of the cellular decarboxylases Padlp and Fdp1p, which convert phenolic acids into a more toxic vinyl form that is synergistic with GVL. While these specific decarboxylases have been studied for their role in "detoxifying" hydrolysates by converting phenolic acids, the vinyl products may ultimately have a greater effect on growth in phenolic rich hydrolysates.

The process technologies of lignocellulosic biofuel production are still evolving, and as a result the landscape of fermentation inhibitors is dynamic. Strain development is necessary to keep pace with these new chemical stressors. Industry relies on tried and true industrial yeast strains with favorable performance and may be hesitant to adopt new genetic backgrounds, no matter how well they are predicted to perform. We have used a functional genomics approach to identify points of rational engineering. As our discovery system is based on *Saccharomyces cerevisiae*, the primary bioethanol organism, these genes identified can be directly modified in other yeast strains to rapidly tailor proven strains to new purposes.

This approach can also be applied to other bioproducts. Through synthetic biology drugs, green chemicals, and next-generation fuels can be produced by yeast and other engineered microbes, and many of these end-products can be toxic to the producing biocatalyst. The genome-wide, functional view of their effects provided by chemical genomics could be useful in improving production. This system is not limited to yeast, genomic wide mutant and overexpression collections exist in a number of industrial relevant microbes, such as *E. coli* and *Zymomonas mobilis*, and as such, the same approach is translatable.

Methods
Compounds, Initial Screening, and IC₅₀ Determination: Compounds tested were purchased from Sigma. Cells of *S. cerevisiae* (MATα pdr1Δ:natMX pdr3A::KILU1A3 sqn2A::KILUEU2 can1Δ::STE2::Sp_his5 lyp1Δ his3Δ1 leu2Δ ura3Δ1 met15Δ0), referred to as control strain, were grown in 200 μl cultures at 30°C in YPD, with a drug or DMSO control. Plates were read on a TECAN M1000 over a 48 h growth period. The specific growth rate was calculated using GCAI analysis software (available at geat-pub.glbc.org on the world wide web) [16]. When presented, IC₅₀ values for growth inhibition were calculated from triplicate 8 point dose curves and SigmaPlot 12.0. When presented, error bars are Mean±Standard error of at least 3 replicates.

Chemical Genomic Analysis:
Chemical genomic analysis of poacie acid was performed as described as described previously [17, 18]. The tested yeast deletion collection had ~4000 strains using the genetic background described in Andrusiak (2012) [19]. The optimal inhibitory concentration of poacie acid for chemical genomic profiling (70-80% growth versus solvent control in YP-galactose media after 24 hours of growth) was determined using an 8 point dose curve. A concentration of 88 μg/ml inhibited growth within this range. 200 μl cultures of the pooled, deletion collection of *S. cerevisiae* deletion mutants were grown with 88 μg/ml poacie acid or a DMSO control in triplicate for 48 h at 30°C. Genomic DNA was extracted using the Epicentre MasterPure™ Yeast DNA purification kit. Mutant-specific molecular barcodes were amplified with specially designed multiplex primers [20]. The barcodes were sequenced using an Illumina MiSeq. 5 replicates of each condition (poacie acid vs DMSO) were sequenced. One DMSO control was lost due to poor sequencing reads. The barcode counts for each yeast deletion mutant in the presence of poacie acid were normalized against the DMSO control conditions to define sensitivity or resistance of individual strains. To determine a p-value for each top sensitive and resistant mutant, we used the EdgeR package [21, 22]. A Bonferroni-corrected hypergeometric distribution test was used to search for significant enrichment of GO terms among the top 10 sensitive and resistant deletion mutants [23]. To understand the pathways that were most affected by poacie acid we developed a protein complex/pathway score based on the summation of the z-scores for each complex/pathway (Pathway z-score). Correlation of the chemical genomic profile of poacie acid with the yeast genetic interaction network was done as described in Costanzo et al. (2010) [10].

MoBY-ORF Profiling:
MoBY-ORF profiling of GVL was conducted by first generating a pooled collection of the yeast GLBRC-Y135 containing the plasmid collection. The plasmid pool for transformation was generated as described previously (HO). For yeast transformation, the plasmids were extracted from 150 μl of *E. coli* culture MAXI Prep. Plasmid was used to transform GLBRC-Y133 via high efficiency LiAc transformation. Transformed yeast were plated to YPD+Geneticin (G418) agar plates and incubated until colonies appeared. A total of 50,000 colonies were washed from the plates using 1×PBS, mixed 1:1 with 50% glycerol, and stored until use. For MoBY-ORF profiling, 25 μl of media containing YPD+2.5% GVL+G418 was allowed to degas overnight in an anaerobic chamber, and then inoculated with 100 μl of the transformed yeast pool (n=3). Cells were grown in culture for 48 hours. Genomic DNA was extracted from 1 ml from each culture using modified mini-prep with with zymolase and glass beads. Gene specific barcodes were amplified, processed, sequenced, and analyzed as described above.

Growth and Sugar Conversion Experiments:
6 25-ml anaerobic flasks were prepared with Synthetic hydrolysate (SynH) (6% glucose/3% xylose)+1% GVL, pH 5.0. Flasks were inoculated with rinsed 133 or 133 pad1ΔΔc1Δ cells to bring the initial OD to approximately 0.1. The tubes were grown for 72 hours with agitation anaerobically at 30°C. 1 ml samples were taken every 24 hours. Initial and daily samples were measured for OD and submitted for HPLC analysis to quantify sugar consumption and ethanol production.

Cell Leakage Assays:
A FungiLight™ cell viability assay (Invitrogen L34952) was used to determine if poacie acid caused membrane damage we used using a Guava Flow Cytometer (Millipore, USA). The population of stained cells (damaged integrity) vs non-stained cells can be determined by flow cytometry. Caspofungin (50 ng/ml) was included as a positive control. MMS and DMSO were included as a non-cell wall targeting and solvent control respectively. To test the effects of the compounds on both active and arrested cells, log-phase cultures were washed with 1×PBS and resuspended to an OD of 0.5 in either YPD media or YP (no carbon source) in the presence of the drugs (n=3) for 4 hours at 30°C. The cells were then stained and immediately read by flow cytometry. A one-way ANOVA and Tukey’s test was used to calculate the difference between drug treatments among cells with arrested growth.
Synergy Screening:
To test for synergy, a 6x6 dose matrix was initially used to identify potentially synergistic dose combinations, these points were then confirmed in triplicate. 200 μl cultures were grown with combinations of with propionic acid (125 μg/ml), caspofungin (12.5 ng/ml) and fluconazole (3.8 μg/ml) and the relevant single agent and solvent controls their OD measured after 24 h. Synergy was determined by comparing actual optical density in the presence of compound combinations to an expected value calculated using the multiplicative hypothesis. This assumes that, in the absence of an interaction, each compound would decrease the OD of the cell culture by the same fraction in the presence of the other compound as it does when applied alone, i.e., E=A*B*BC, where E is the expected OD, A is OD when compound A is applied alone, B is OD when compound B is applied alone, and C is OD of the control culture (DMSO). In the presence of synergy, the actual OD value is lower than the expected OD. A paired t-test was used to confirm statistical significance of this difference in 3 replicates of the experiment.

Determination of Farnesyl and Diterulates by RP-HPLC- HR/AM-MS in Hydrolysates:
ACSH samples were diluted 1:10 and 20 μl samples were analyzed by reverse phase (C18) HPLC—high resolution/accurate mass spectrometry. Peak areas of peaks matching in retention time and accurate mass+/-10 ppm of authentic reference standards were used to calculate concentrations by comparison to an external standard curve.

GVL Synergy Experiments:
The synergistic interaction between GVL and ethanol was initially discovered using a 6-point dose matrix of the two compounds. We identified the points of the greatest synergy and perform triplicate growth curves of GLBR-128 using a TECAN M1000 microplate reader. To confirm the GVL-R1 strain was less sensitive to the synergism between GVL and ethanol, used a 6-point dose matrix as described above with both GLBR-128 or GVL-R1 in a TECAN microplate reader for 48 hours at 30°C. Data from the most synergistic combination is presented (2% GVL-5% ethanol).

REFERENCES
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<223> OTHER INFORMATION: GRE3 coding sequence

<400> SEQUENCE: 3

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gtaagggaga atgggaatat tttaaaagat gttttcactgt ggtaacctta ataacggct 180
gaaatggcct ctagttgcgt taggggtgctg gaaatctggc aaaaaattct tgtggaatc 240
aatattgaa gctatatcaat taggctacgg tttatcagat ggtgcctgag actacgcaa 300
cgaagaaggg gttgggtgaag gttacagaga acgcaattcc ccagtgctttc tttctagaa 360
ggtatatgtt gttcttcaac aagttgagaa caatttcctaa catcttgatc atgttaaatc 420
agccttaag aagaccttta gcggataggg acttgatttt ttgaacactg attataatca 480
ccttccacct cgcctcaaat atggtcattt tgaaagaaaa tacccctcaag gatcttatac 540
ggagcgcagat gcagcagaga aagttcatac caaccgagca cctgtaccaaa tcatagatac 600
gtaccgggct gttgaagatg agcttcagtt aggtcttgtt gttttcctaa 660
ccttccagggga agcggatttc aagattttatt acgtgtgtgt aagatcaagc cggagctcctt 720
goaaaaaggg caacacottt atttgcaacctg aagacacactg tttgtatttt gttaaatca 780
cgtatcacc aagcttttttt atctctccct gtggcttcaaa ccctttactt cagaggtcct 840
acagttgcgca aaacaccgaa caaccctttt ggagaatgat gtaaccaagaa agttcctcaca 900
aaaacatccag cgcaggataac cttcaccagtt atgggtgatt gggtcgaact agagagcatt 960
tggtgctatt ccaaaaaatctt ccaagagagg aagggctctg ggcacactgaa aaatcggaaaa 1020
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atttaatgtt ccaaggaacct gttttgctgg aatttctccccc acttttgtgat gatcagcaca 1140
gtaaaactca cccttcagaca gcgtatagaa aaaaattctcct cttccagactg ctgtatagtt 1200
gtataattt ttcacagctg ttctgttagg ccacagagac cttttaaataa aatgttagtg 1260
tatactttttt ctggcttg 1278

<210> SEQ ID NO 4
<211> LENGTH: 127
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 4

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Gly Leu Gly Cys Trp Lys Ile Asp Lys Lys Val Cys Ala Asn Glu Ile 20 25 30
Tyr Glu Ala Ile Lys Leu Gly Tyr Arg Leu Phe Arg Gly Ala Cys Asp 35 40 45
Tyr Gly Asn Glu Lys Glu Val Gly Gly Ile Arg Lys Ala Ile Ser 50 55 60
Glu Gly Leu Val Ser Arg Lys Asp Ile Phe Val Val Ser Lys Leu Trp 65 70 75 80
Asn Asn Phe His His Pro Asp His Val Lys Leu Ala Leu Lys Lys Thr 85 90 95
Leu Ser Asp Met Gly Leu Asp Tyr Leu Asp Leu Tyr Tyr Ile His Phe
100 105 110
Pro Ile Ala Phe Lys Tyr Val Pro Phe Glu Glu Tyr Pro Pro Gly
115 120 125
Phe Tyr Thr Gly Ala Asp Glu Lys Gly His Ile Thr Glu Ala
130 135 140
His Val Pro Ile Ile Asp Thr Tyr Arg Ala Leu Glu Glu Cys Val Asp
145 150 155 160
Glu Gly Leu Ile Lys Ser Ile Gly Val Ser Asp Phe Gln Gly Ser Leu
165 170 175
Ile Gln Asp Leu Leu Arg Gly Cys Arg Ile Lys Pro Val Ala Leu Gln
180 185 190
Ile Glu His His Pro Tyr Leu Thr Glu His Leu Val Glu Phe Cys
195 200 205
Lys Leu His Asp Ile Gln Val Val Ala Tyr Ser Ser Phe Gly Pro Gln
210 215 220
Ser Phe Ile Glu Met Asp Leu Gln Leu Ala Lys Thr Thr Pro Thr Leu
225 230 235 240
Phe Glu Asn Asp Val Ile Lys Lys Val Ser Gln Asn His Pro Gly Ser
245 250 255
Thr Thr Ser Gln Val Leu Leu Arg Trp Ala Thr Gln Arg Gly Ile Ala
260 265 270
Val Ile Pro Lys Ser Ser Lys Glu Arg Leu Gly Am Leu Glu
275 280 285
Ile Glu Lys Lys Phe Thr Leu Thr Glu Glu Leu Lys Asp Ile Ser
290 295 300
Ala Leu Asn Ala Asn Ile Arg Phe Asn Asp Pro Thr Thr Thr Leu Asp
305 310 315 320
Gly Lys Phe Pro Thr Phe Ala
325

<210> SEQ ID NO 5
<211> LENGTH: 648
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: ISU1 coding sequence
<400> SEQUENCE: 5

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acacatatg aacctgatgc ttcctgttat aaccgagatt gcagggcctg ctcctgatgc 120
catcagacct gttgatgccg tgggggattt ggagagctcc agcatcaacga aagggctatta 180
tcgaaacaag tctataaggcccc tccaaagaaac gtcgcttcat tagataaaaa 240
attgtggcc gtcgagctc gtcagatggt gcagctccgg gtagaatttg 300
gcagctgata ggttaagagtct actagctctt actagcttagt gttcggcgggg 360
atgtggctgt gccggctgg ccctttccata tagaacagtaa tttgggagacag cagatgccct 420
ggagatgtcag gtaaaaaata agaactgtga aattctgtaga gagggtgtag ctgcagctgt 480
caggtttgat gttcggattg tagaagac gtcgatagc gtagttattg agaactacaat 540
atctaaaga acacactca cagcttatc gtaatgaaga aagatgtaaccc gagacacagac 600
acagatcata cccctcacta atcaacattg ttgacactaatt ttttg 648
<210> SEQ ID NO 6
<211> LENGTH: 165
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 6
Met Leu Pro Val Ile Thr Arg Phe Ala Arg Pro Ala Leu Met Ala Ile
1      5      10     15
Arg Pro Val Asn Ala Met Gly Val Leu Arg Ala Ser Ser Ile Thr Lys
20     25     30
Arg Leu Tyr His Pro Lys Val Ile Glu His Tyr Thr His Pro Arg Ann
35     40     45
Val Gly Ser Leu Asp Lys Lys Leu Pro Ann Val Gly Thr Gly Leu Val
50     55     60
Gly Ala Pro Ala Cys Gly Asp Val Met Arg Leu Gln Ile lys Val Ann
65     70     75     80
Asp Ser Thr Gly Val Ile Glu Asp Val Lys Phe Lys Thr Gly Cys
85     90     95
Gly Ser Ala Ile Ala Ser Ser Ser Tyr Met Thr Glu Leu Val Glu Gly
100    105    110
Met Thr Leu Asp Ala Ala Lys Ile Lys Ann Thr Glu Ile Ala Lys
115    120    125
Glu Leu Ser Leu Pro Pro Val Lys Leu His Cys Ser Met Leu Ala Glu
130    135    140
Asp Ala Ile Lys Ala Ala Ile Asp Tyr Lys Ser Lys Arg Ann Thr
145    150    155    160
Pro Thr Met Leu Ser
165

<210> SEQ ID NO 7
<211> LENGTH: 729
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 7
atgcctctat ttccagaaag aactaatata gcctttttca aaacaacag gcctttttgt 60
aattttccct tgctagagag aaccattaca actctaccat ctctctctac acataaactg 120
tccaccaagc taaccaggcc atcactctcg cctcacaagcc caagagaat tggctggcga 180
attacgggt gcacgggtgt ttgacttggga atcagacctt tacaagtgct aaaaaggttg 240
agcggagaaa ccaccattgt gatttttaaa tggggtgcag caaacaagaa atagaaaca 300
gatggggaac gcctagacgt gcgcgctcgg gcaacacaga catacttgtg tcggtgtgt 360
tttgaagatca ttttcttcag atcattttgg tagttttttg gcgcgtttcag 420
agtaatacaat tagctgtttc aagaatttctttacttgttgc atgttggttc 480
gatgtttccga taagacaagc tctttaatgt cttcagttta cttttttata cccttttct 540
tccacatctc tggagacac ggtgtaataa aaaaaatt cctccttcg 600
gatcagtcgt ttttacaagc acacacagcc cttcattgcct tttagaacaa aaggttgtggc 660
agatcttcsag acgctttttgt gcccacagtc gacacttttt cttgctggcga aggatcag 720
agacgatctg 729
<400> SEQUENCE: 8

Met Leu Leu Phe Pro Arg Arg Thr Asn Ile Ala Phe Phe Lys Thr Thr
1    5      10     15

Gly Ile Phe Ala Asn Phe Pro Leu Gln Gly Arg Thr Ile Thr Thr Ser
20   25     30

Pro Ser Phe Leu Thr His Lys Leu Ser Lys Glu Val Thr Arg Ala Ser
35   40     45

Thr Ser Pro Pro Pro Arg Pro Arg Phe Val Ala Lys Thr Gly Ala
50   55     60

Thr Gly Val Ala Leu Gly Ile Arg Leu Gln Val Leu Lys Glu Leu
65   70     75     80

Ser Val Glu Thr His Leu Val Ile Ser Lys Trp Gly Ala Ala Thr Met
85   90     95

Lys Tyr Glu Thr Asp Trp Glu Pro His Asp Val Ala Leu Ala Thr
100  105    110

Lys Thr Tyr Ser Val Arg Asp Val Ser Ala Cys Ile Ser Ser Gly Ser
115  120    125

Phe Glu His Asp Gly Met Ile Val Val Pro Cys Ser Met Lys Ser Leu
130  135    140

Ala Ala Ile Arg Ile Gly Phe Thr Glu Asp Leu Ile Thr Arg Ala Ala
145  150    155     160

Asp Val Ser Ile Lys Glu Asn Arg Lys Leu Leu Leu Val Thr Arg Glu
165  170    175

Thr Pro Leu Ser Ser Ile His Leu Glu Asn Met Leu Ser Leu Cys Arg
180  185    190

Ala Gly Val Ile Ile Phe Pro Pro Val Pro Ala Phe Tyr Thr Arg Pro
195  200    205

Lys Ser Leu His Asp Leu Glu Gln Ser Val Gly Arg Ile Leu Asp
210  215    220

Cys Phe Gly Ile His Ala Asp Thr Phe Pro Arg Trp Glu Gly Ile Lys
225  230    235     240

Ser Lys

<210> SEQ ID NO 9
<211> LENGTH: 1512
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 9

atgaggaagc taatccagc tttagaattt agagacttta tccaggtctt aaaaagttgaa
1    5      10     15

agtgactaa tcagaattac cgaagagatt gatccaanat tcgaagtagg tgcatttagg
20   25     30     35

agagaggctg atgaatccca cttaccagcc ccggtttattta aaaaaatccaa agggtcctcg
40   45     50     55

aagagtcttt tcagcatttt aggtggccaa gcggggtttg gaagagagga gaaggagat
70   75     80     85

catgtaggaa ttgcccaatt tctggggtgct gacccaaaaaa caacctacaa ggaatcata
100  105    110    115

gattatcttc tcggagttga ggaaagggaa cctcctcccc ccacctcgt ttctgtgcta
135  140    145    150

tctgcaccttt gtaaaaaaata ctaacctttt gcacaaaaaaa tatactcata aagctgtgcca
170  175    180    185

acaccatctc tatactgttc agacgggtgc aagtctctac aaccgtaagg aatgtgattg
200  205    210    215

ccttcaaacct cagataaaaa atttgcaaat tgcacaattg ttcaggttat ggttgtagat
235  240    245    250

gacaagcata tcagctgctc gtaaatcaca ccacaaccttt ctagcaaat tggctgactct
265  270    275    280

tggtcacgaa ttggaaaaag aatgcaaat cctttggtct cgagtttttt gcctccccca
295  300    305    310
```
gcagctattt tagttagttc catgccaatt cctgaaaaggt tttctgaaatc gattatggtt  
ggcgcacact ctggtgtagct ggttcagata gtaaaaatgtg aagccaaacg aaaaaatcgtt  
cctgcaaca ggtgtagatg atttgagagg actttaggtct tcaagatctt acacttggaa  
ggcacatgtt ttagatgtt tcttaaggccc aagttgatcct tggctcattg  
tacgttcaag actgtatagc ttagagac gctgtatattc taccctgttta aaaccccggt  
ccttgcattc ctagacacg traccctgttt ccctcctaatg ggtccaggt  
ccttgccatta tcttaaaaaggt gatttgaaaa gggctggcaag catgaaacac aacgccgtaa  
gaatttgctag taagagttag tcatatattac tttagaccaaa aaattggttt tatagttcatt  
gaataattt ttggggcaga tgcattgacg atatattact tcaaaaagact cactcgggcc  
tacgttaacaa gacattacac ccggaggttt ttgattagtt cactccccctttt  
cctttggtct cccttggttt gcgcataacc aagattgaa ccataaaggg tggaaagttgc  
gttactaaatt gccatattag acaagcaaat gacgcagcttg ttagctaatc aacctgttat  
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ggatataaat aat  

<210> SEQ ID NO 10
<211> LENGTH: 503
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 10

Met Arg Lys Leu Aaa Pro Ala Leu Glu Phe Arg Asp Phe Ile Gln Val
1   5  10  15

Leu Lys Asp Glu Asp Asp Leu Ile Glu Ile Thr Glu Glu Ile Asp Pro
20  25  30

Aaa Leu Glu Val Gly Ala Met Arg Lys Ala Tyr Glu Ser His Leu
35  40  45

Pro Ala Pro Leu Phe Lys Aaa Leu Leu Gly Ala Ser Lys Asp Leu Phe
50  55  60

Ser Ile Leu Gly Cys Pro Ala Gly Leu Arg Ser Lys Gly Lys Gly Asp
65  70  75  80

His Gly Arg Ile Ala His His Leu Gly Leu Asp Pro Lys Thr Thr Ile
85  90  95

Lys Glu Ile Ile Asp Tyr Leu Leu Glu Cys Lys Gly Lys Glu Pro Leu
100 105 110

Pro Pro Ile Thr Val Pro Val Ser Ser Ala Pro Cys Lys Thr His Ile
115 120 125

Leu Ser Glu Gly Lys Ile His Leu Gln Ser Leu Pro Thr Pro Tyr Leu
130 135 140

His Val Ser Asp Gly Lys Tyr Gly Lys Gly Thr Tyr Gly Met Trp Ile
145 150 155 160

Leu Gln Thr Pro Asp Lys Lys Trp Thr Asn Trp Ser Ile Ala Arg Gly
165 170 175

Met Val Val Asp Asp Lys His Ile Thr Gly Leu Val Ile Lys Pro Gln
180 185 190

His Ile Arg Gln Ile Ala Asp Ser Trp Ala Ala Ala Gly Lys Ala Asn
195 200 205

Glu Ile Pro Phe Ala Leu Cys Phe Gly Val Pro Pro Ala Ala Ile Leu
210 215 220
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We claim:

1. A recombinant yeast that has been genetically engineered to: ferment xylose and exhibit a reduced amount of functional PAD1 polypeptide, wherein the recombinant yeast has increased tolerance to gamma valerolactone (GVL) toxicity relative to a wild-type yeast or another recombinant yeast having the same genetic background but not exhibiting a reduced amount of functional PAD1 polypeptide.

2. The recombinant yeast of claim 1, further exhibiting a reduced amount of functional FDC1 polypeptide, wherein the recombinant yeast has increased tolerance to GVL toxicity relative to a wild-type yeast or another recombinant yeast having the same genetic background but not exhibiting reduced amounts of functional PAD1 and FDC1 polypeptides.

3. The recombinant yeast of claim 1, wherein the recombinant yeast comprises a disabling mutation in a gene encoding PAD1 polypeptide.

4. The recombinant yeast of claim 2, wherein the recombinant yeast further comprises a disabling mutation in a gene encoding FDC1 polypeptide.

5. The recombinant yeast of claim 3, wherein the PAD1 polypeptide is SEQ ID NO:8.

6. The recombinant yeast of claim 4, wherein the FDC1 polypeptide is SEQ ID NO:10.

7. The recombinant yeast of claim 1, further exhibiting reduced or undetectable amounts of functional ISU1, GRE3, and IRA2 polypeptides, wherein the recombinant yeast is capable of increased aerobic or anaerobic xylose fermentation relative to a wild-type yeast or another recombinant yeast not exhibiting reduced amounts of functional PAD1, ISU1, GRE3, and IRA2 polypeptides.

8. The recombinant yeast of claim 1, further comprising a disabling mutation at each of loci isu1, gre3, and ira2, whereby the mutations result in reduced amounts of functional ISU1, GRE3, and IRA2 polypeptides, respectively.

9. The recombinant yeast of claim 8, wherein the disabling mutation at the gre3 locus comprises a substitution of
a threonine for the alanine at amino acid residue position 46 of SEQ ID NO:4; wherein the disabling mutation at the ira2 locus comprises a substitution of a stop codon for the glutamate at amino acid residue at position 2927 of SEQ ID NO:2; and wherein the disabling mutation at the isu1 locus comprises a substitution of a tyrosine for the histidine at amino acid residue position 138 of SEQ ID NO:6.

10. The recombinant yeast of claim 8, wherein the recombinant yeast produces ethanol at an increased rate relative to a wild-type yeast or another recombinant yeast not exhibiting reduced or undetectable amounts of functional ISU1, GRE3, and IRA2 polypeptides.

11. The recombinant yeast of claim 10, wherein the increased rate of ethanol production occurs under anaerobic conditions.

12. The recombinant yeast of claim 1, wherein the recombinant yeast is of the genus Saccharomyces.

13. The recombinant yeast of claim 12, wherein the recombinant yeast is of the species Saccharomyces cerevisiae.

14. The recombinant yeast of claim 3, wherein a portion of an extrachromosomal vector stably maintained in the recombinant yeast comprises the disabling mutation.

15. The recombinant yeast of claim 3, wherein a nucleic acid sequence comprising the disabling mutation is integrated into a chromosome of the recombinant yeast.

16. A yeast inoculum, comprising: (a) a recombinant yeast of claim 1; and (b) a culture medium.

17. A method for fermenting cellulosic material into ethanol, comprising contacting a GVL-treated hydrolysate to the recombinant yeast of claim 1 or the yeast inoculum of claim 16 for a period of time sufficient to allow fermentation of at least a portion of the cellulosic material to ethanol, whereby the rate of fermentation of cellulosic material of the GVL-treated hydrolysate to ethanol is increased relative to the fermentation rate of a GVL-treated hydrolysate not contacted to the recombinant yeast or the yeast inoculum.

18. The method of claim 17, further comprising separating the ethanol from fermented cellulosic material.

19. The method of claim 17, wherein the GVL-treated hydrolysate comprises xylose.

20. The method of claim 17, wherein the recombinant yeast is Saccharomyces cerevisiae.

21. The method of claim 17, wherein the cellulosic material comprises lignocellulosic biomass.

22. The method of claim 21, wherein the lignocellulosic biomass comprises at least one material selected from the group consisting of agricultural residues, wood, municipal solid wastes, paper and pulp industry wastes, and herbaceous crops.

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