US010006057B2

(12) **United States Patent**
Piotrowski et al.

(10) **Patent No.: US 10,006,057 B2**
(45) **Date of Patent: Jun. 26, 2018**

(54) **RECOMBINANT YEAST HAVING ENHANCED GAMMA VALEROLACTONE TOLERANCE AND METHODS OF USE**

(71) Applicants: **Wisconsin Alumni Research Foundation**, Madison, WI (US); **Regents of the University of Minnesota**, Minneapolis, MN (US)

(72) Inventors: **Jeff S. Piotrowski**, Madison, WI (US); **Trey K. Sato**, Madison, WI (US); **Chad L. Myers**, Arden Hills, MN (US)

(73) Assignee: **Wisconsin Alumni Research Foundation**, Madison, WI (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days. days.

(21) Appl. No.: **14/848,440**

(22) Filed: **Sep. 9, 2015**

(65) **Prior Publication Data**

US 2016/0068869 A1 Mar. 10, 2016

Related U.S. Application Data

(60) Provisional application No. 62/048,458, filed on Sep. 10, 2014.

(51) **Int. Cl.**

C12P 7/10	(2006.01)
C12N 1/14	(2006.01)
C12N 15/81	(2006.01)

(52) **U.S. Cl.**

CPC ***C12P 7/10*** (2013.01); ***C12N 1/14*** (2013.01); ***C12N 15/81*** (2013.01); *Y02E 50/16* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2007/0141690	A1*	6/2007	Karhumaa	C12N 9/0006 435/161
2016/0040153	A1*	2/2016	Froehlich	C12N 9/92 435/161

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

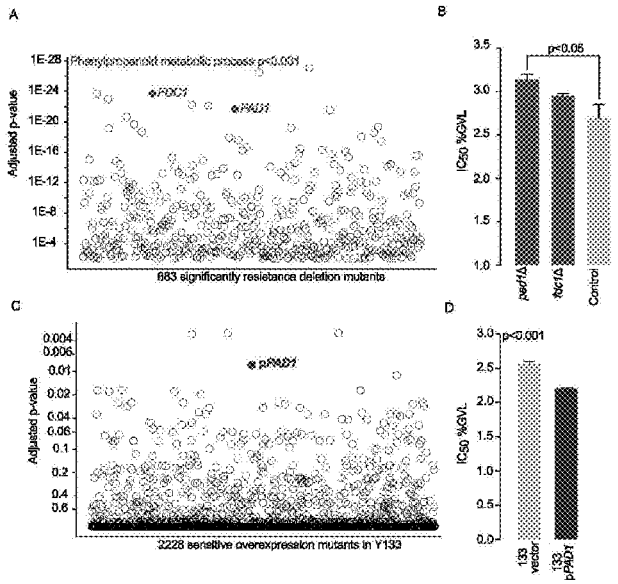
Primary Examiner — Richard C Ekstrom

(74) *Attorney, Agent, or Firm* — Boyle Fredrickson S.C.

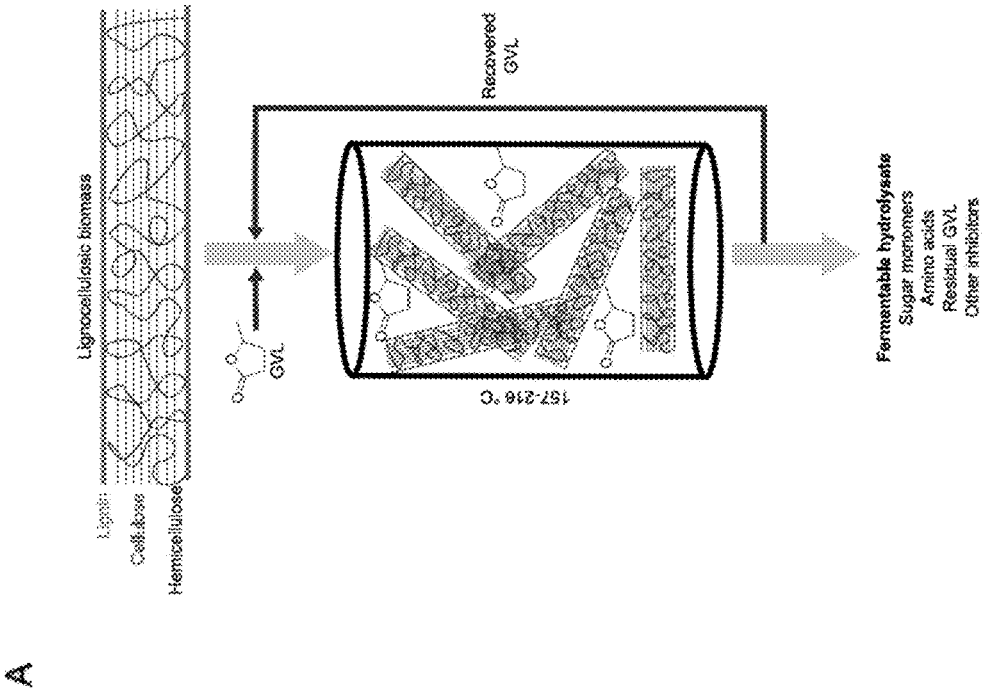
(57) **ABSTRACT**

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.

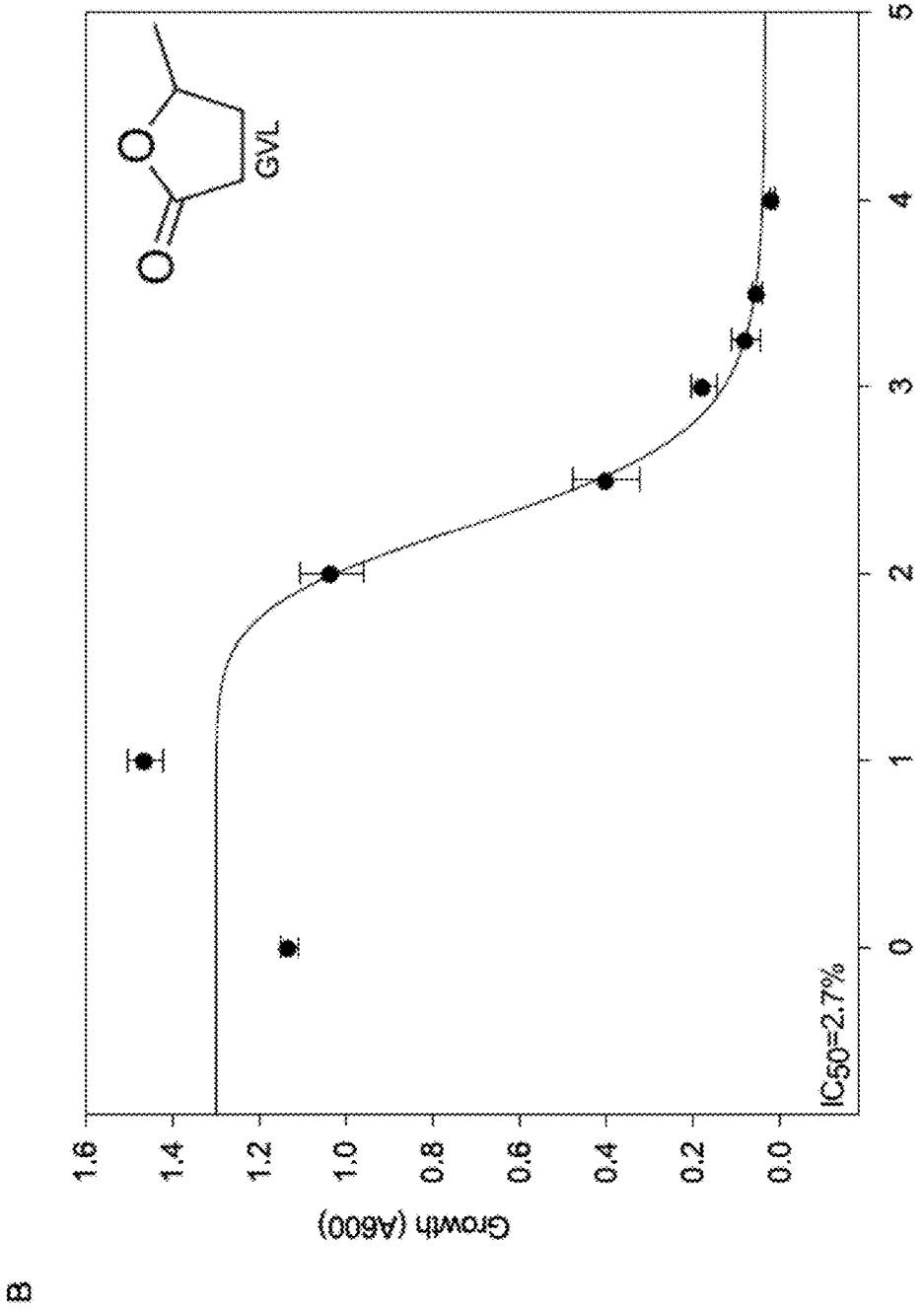
22 Claims, 10 Drawing Sheets



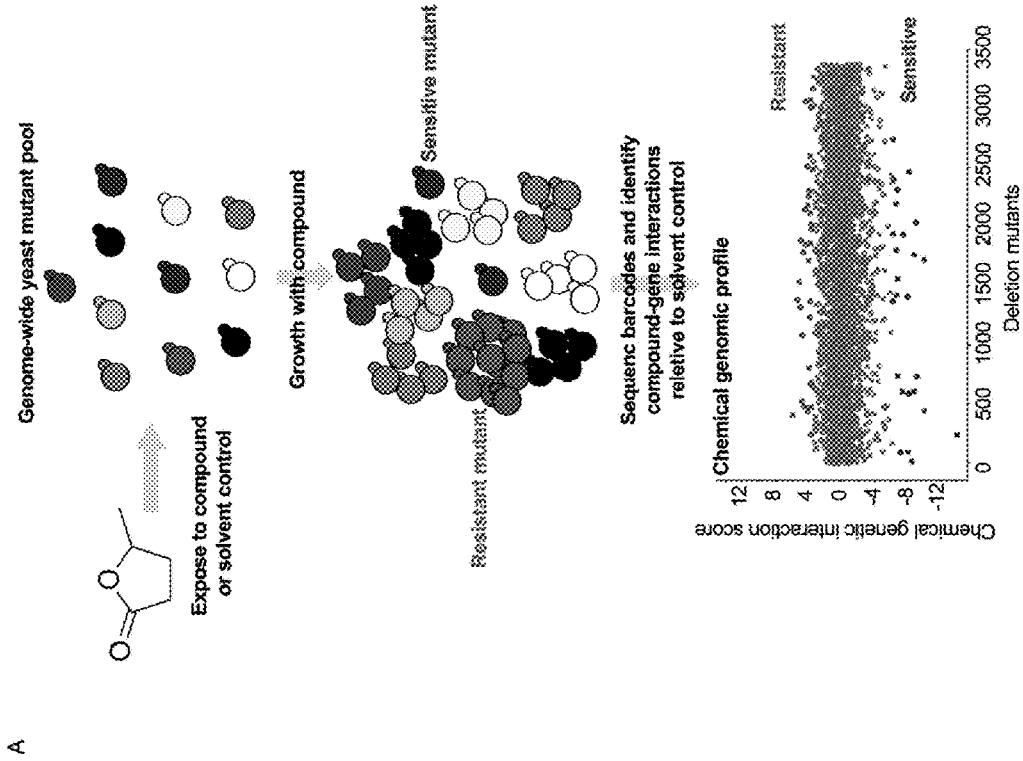
FIGS. 1A-1B


A
Lignocellulosic biomass
Lignin
Cellulose
Hemicellulose
GVL
157-216 °C
Recovered GVL
Fermentable hydrolysate
Sugar monomers
Amino acids
Residual GVL
Other inhibitors

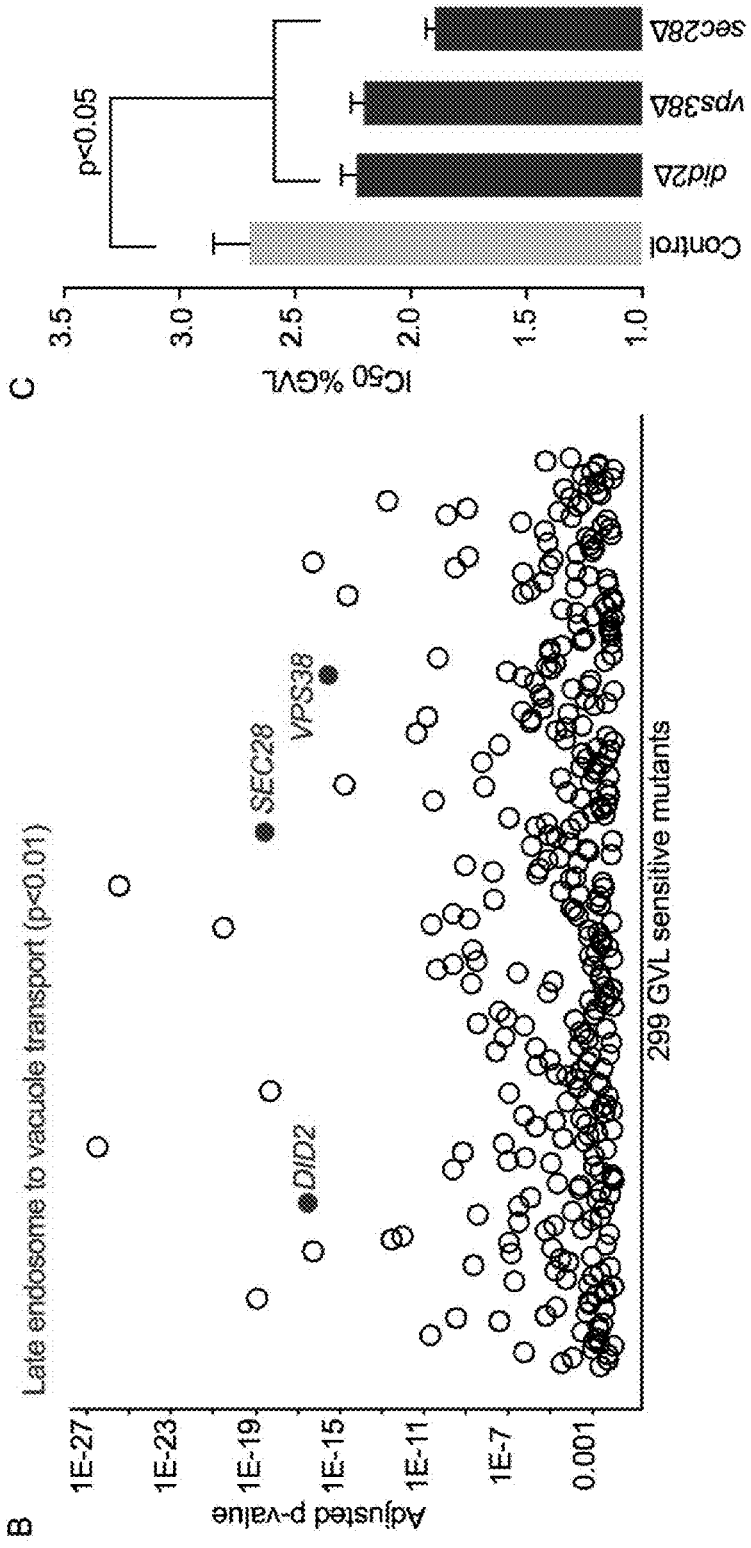
FIGS. 1A-1B, CONTINUED



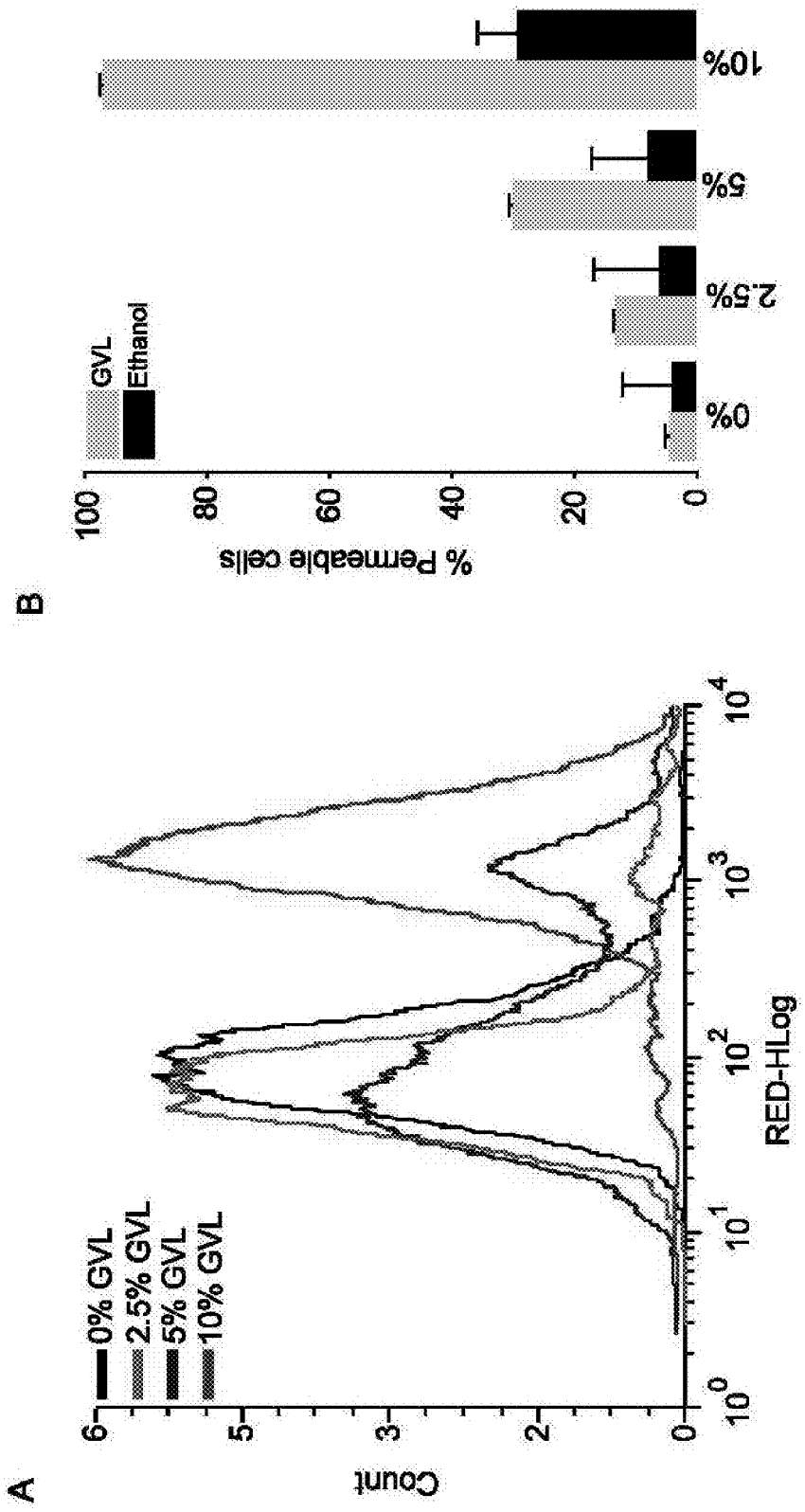
FIGS. 2A-2C


A
Genome-wide yeast mutant pool
Expose to compound or solvent control
Growth with compound
Sensitive mutant
Resistant mutant
Sequenc barcodes and identify compound-gene interactions relative to solvent control
Chemical genomic profile
Resistant
Sensitive
Chemical genetic interaction score
12
8
4
0
-4
-8
-12
0
500
1000
1500
2000
2500
3000
3500
Deletion mutants

FIGS. 2A-2C, CONTINUED

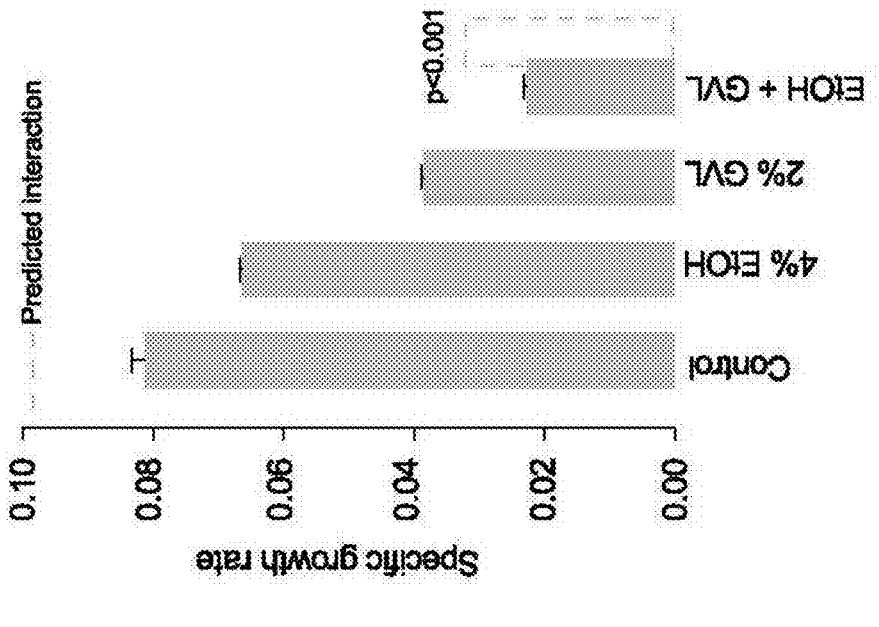


FIGS. 3A-3C

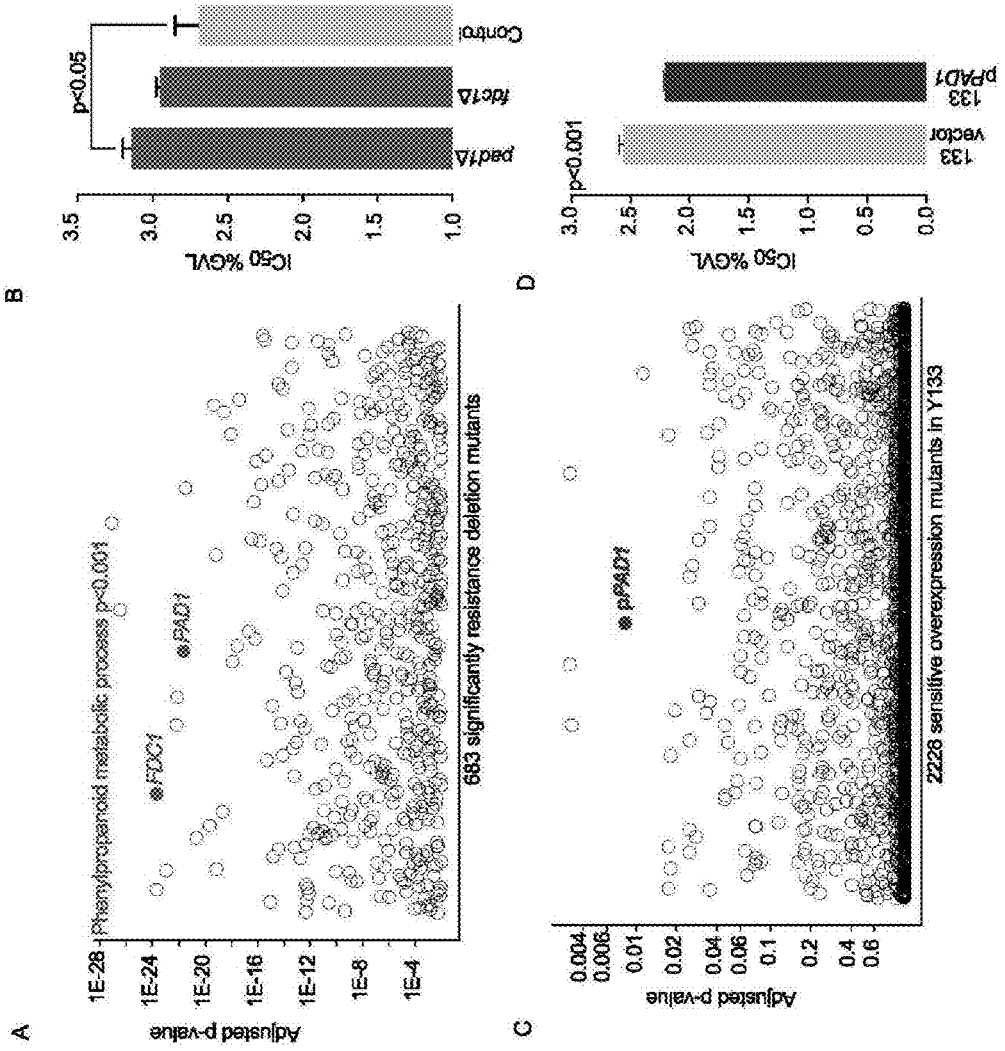

A
Count
RED-HLog
0% GVL
2.5% GVL
5% GVL
10% GVL
B
% Permeable cells
GVL
Ethanol
0%
2.5%
5%
10%

FIGS. 3A-3C, CONTINUED

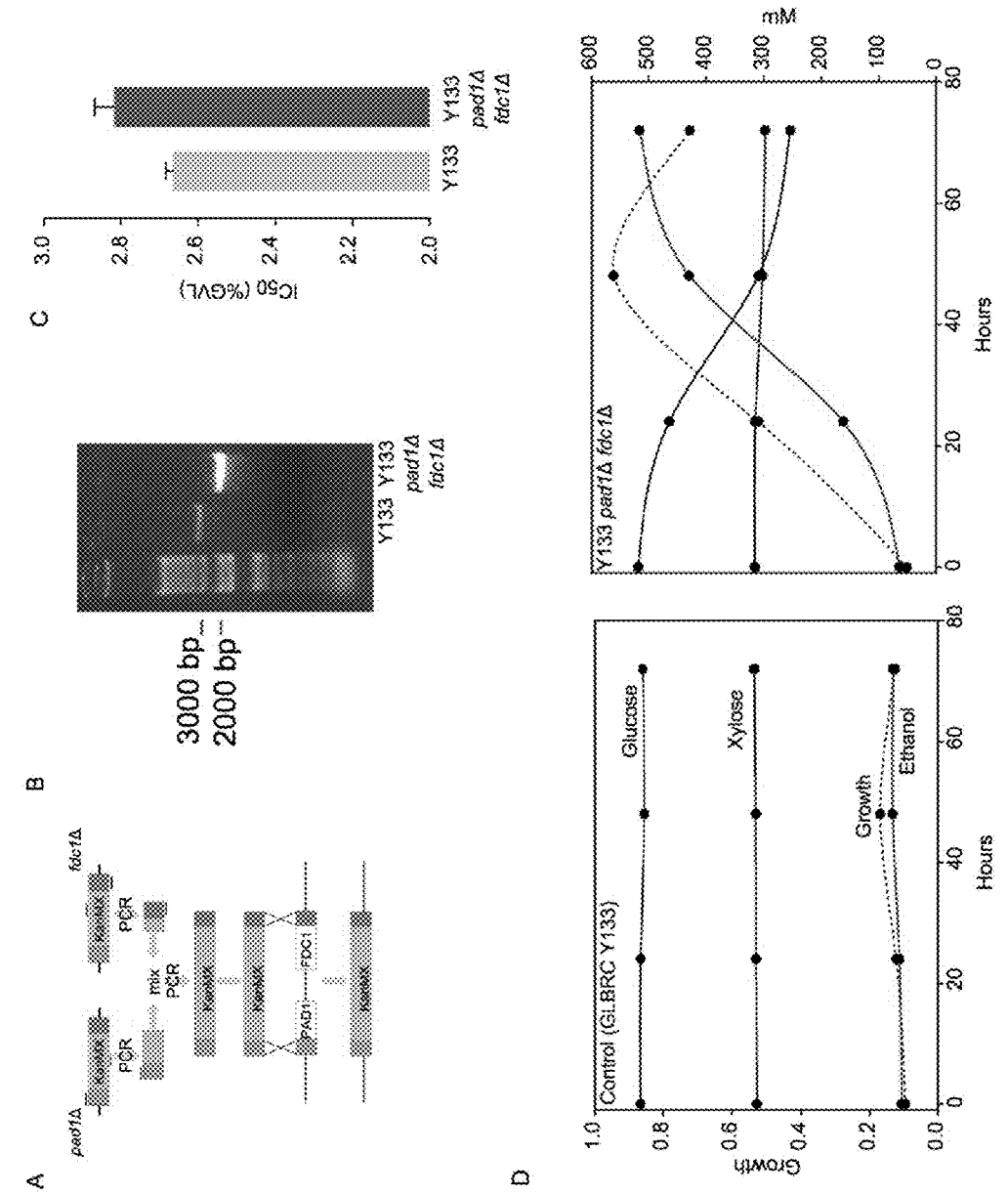
C



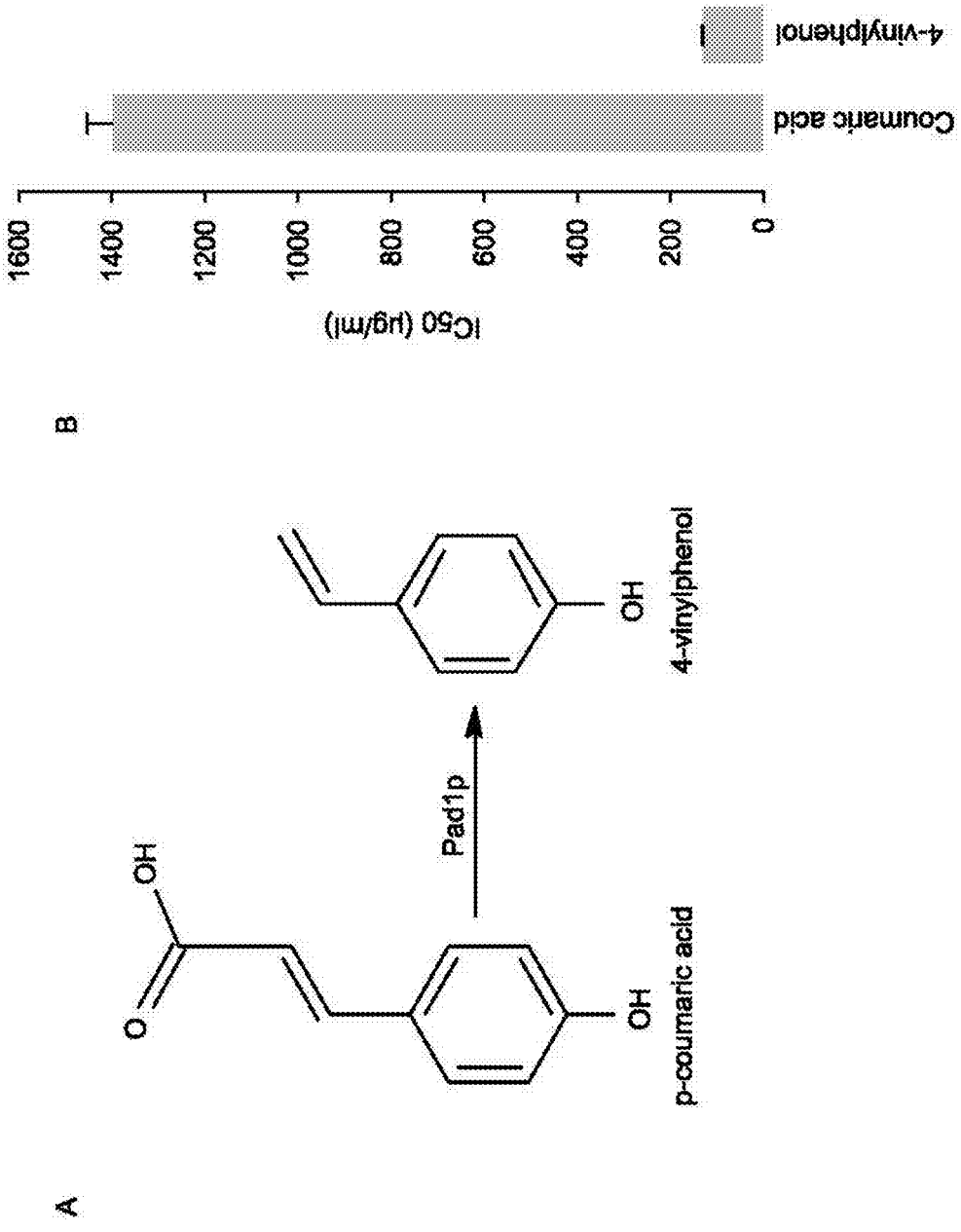
FIGS. 4A-4D


A
Adjusted p-value
1E-28
1E-24
1E-20
1E-16
1E-12
1E-8
1E-4
Phenylpropanoid metabolic process p<0.001
FDC1
PAD1
683 significantly resistance deletion mutants
B
IC50 %GVL
3.5
3.0
2.5
2.0
1.5
1.0
p<0.05
pad1Δ
fdc1Δ
Control
C
Adjusted p-value
0.004
0.006
0.01
0.02
0.04
0.06
0.1
0.2
0.4
0.6
pPAD1
2228 sensitive overexpression mutants in Y133
D
IC50 %GVL
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p<0.001
133 vector
133 pPAD1

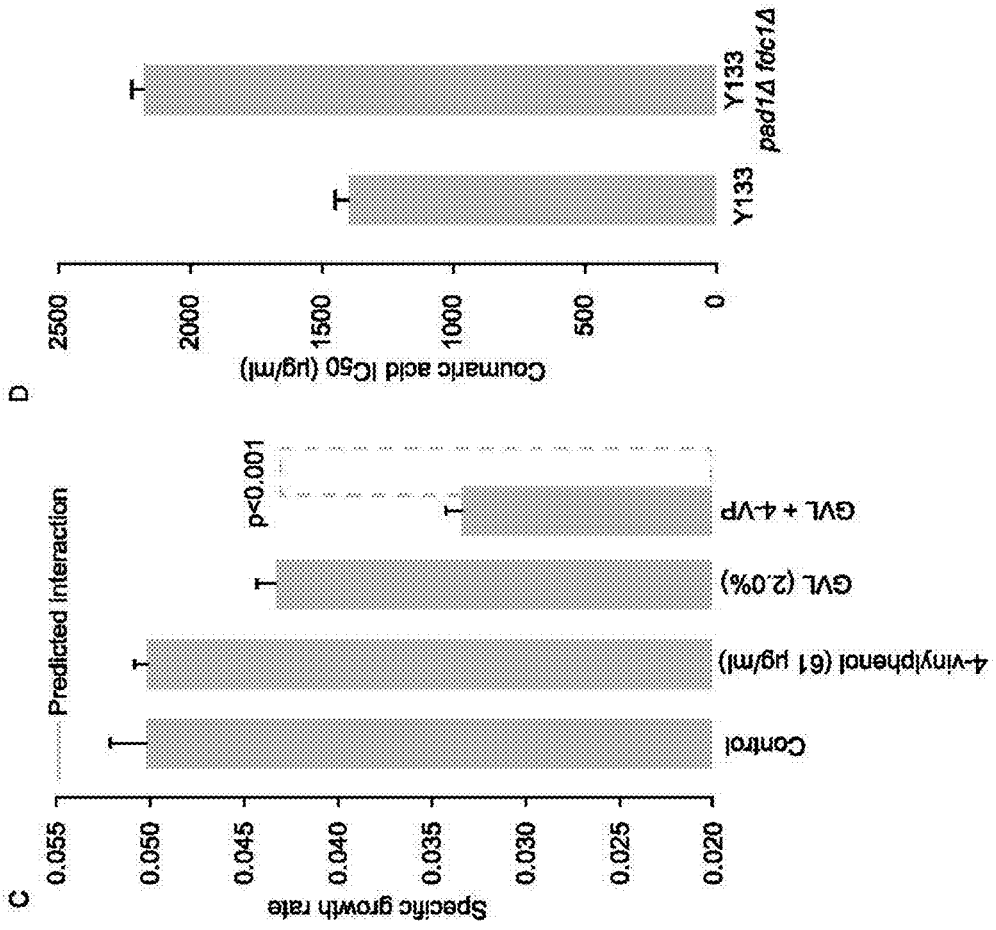
FIGS. 5A-5D


A
pad1Δ
fdc1Δ
PCR
mix
PCR
PAD1
FDC1
B
3000 bp
2000 bp
Y133
Y133 pad1Δ fdc1Δ
C
IC50 (%GVL)
3.0
2.8
2.6
2.4
2.2
2.0
Y133
Y133 pad1Δ fdc1Δ
D
Control (GLBRC Y133)
Glucose
Xylose
Growth
Ethanol
Growth
Hours
Y133 pad1Δ fdc1Δ
mM
Hours

FIGS. 6A-6D


A
p-coumaric acid
Pad1p
4-vinylphenol
OH
O
OH
OH
B
IC50 (µg/ml)
0
200
400
600
800
1000
1200
1400
1600
Coumaric acid
4-vinylphenol

FIGS. 6A-6D, CONTINUED



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 because of 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 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 at 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 hydrosylate 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 cellulosic material to ethanol, whereby the rate of fermentation of cellulosic material of the GVL-treated hydrosylate to ethanol is increased relative to the fermentation rate of a GVL-treated hydrosylate 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. **1**A-**1**B 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 (IC_{50}) of GVL in rich media is 2.7% (B).

FIGS. **2**A-**2**C 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. **3**A-**3**C 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 effects is apparent with as little as 2.5% GVL (A, B). GVL is significantly synergistic with ethanol (C). (Mean±S.E, n=3).

FIGS. **4**A-**4**D 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. **5**A-**5**D 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 pad1Δfdc1Δ 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. **6**A-**6**D 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 practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); and Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986).

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 polyribonucleotide or polydeoxyribonucleotide, 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 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 "polynucleotide(s)" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated 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 material 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 hemicellulose, 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 fractions 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 hydrosylation 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 hydrosylates 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. barnetti, S. exiguus, S. uvarum, S. diastaticus, K. lactis, K. marxianus*, and *K. fragilis*, of which yeast cells of the genus *Saccharomyces* and yeast cells of the species *Saccharomyces cerevisiae* (*S. cerevisiae*) are preferred. Yeasts of the genus *Saccharomyces* posses 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 decarboxylase) polypeptide or functional FDC1 (ferulic acid decarboxylase) polypeptide. PAD1 and FDC1 are phenylacrylic acid decarboxylases that decarboxylate aromatic phenylacrylic acids (e.g., ferulic 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 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 a 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 improve 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 hydrosylates 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 (Parreiras et al., *PLoS One.* 2014; 9(9):e107499).

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 isu1, 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 growth 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 "IC_{50}." The terms "half maximal inhibitory concentration" and "IC_{50}" 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 IC_{50} is about 1.98% GVL, while a PAD1 deletion mutant in the standard lab strain background has an IC_{50} of about 2.4% GVL and FDC1 deletion mutant in the standard lab strain background has an IC_{50} 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 hydrosylate. 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 hydrosylate.

The GVL IC_{50} 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 IC_{50} 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 IC_{50} of about 2.2% GVL, whereas genetically modified yeast of the present invention have an IC_{50} 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 IC_{50} of about 2.7% GVL, whereas yeast of the genetically modified strain (e.g., Y133 pad1Δfdc1Δ) of the present invention has an IC_{50} 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 (CphytoXylA), 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 ScerTAL1, a Pentose Phosphate Pathway transaldolase enzyme that can improve xylose metabolism when overexpressed (see Ni et al., *Applied Environmental Microbiol.* 73:2061 (2007); Walfridsson et al., *Applied Environmental Microbiol.* 61:4184 (1995)), and SstipXYL3 driven by the ScerPGK1 and ScerTEF2 promoters, respectively. For example, the host yeast cell can comprise a TAL1-XylA-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 target gene. Either of both of these regions may include a portion of the coding region of the target gene. The gene cassette (including associated promoters and terminators if different from those of the target 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 target 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 hydrosylate 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 xylose to ethanol. In particular, the present invention provides a method of fermenting cellulosic material comprising the 5-carbon sugar xylose 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 xylose and other sugars under appropriate fermentation conditions. The sugars can come from a variety of sources including, but not limited to, cellulosic material. The cellulosic 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, xylose, 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 hydrolyzation step. For example, when cellulosic material is used in the methods disclosed herein, the material can be hydrolyzed to produce a hydrolysate comprising xylose 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 cellulosic material (e.g., biomass), typically through pretreatment and saccharification processes. In exemplary embodiments, cellulosic material is pretreated using a solvent comprising gamma-valerolactone (GVL or γ-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., xylose, 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 hydrosylate 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 PAD1 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 hydrosylate for a period of time sufficient to allow fermentation of at least a portion of the cellulosic 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 cellulosic material comprising the 5-carbon sugar xylose 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 cellulosic material in a GVL-treated hydrosylate to ethanol is increased relative to the fermentation rate of a GVL-treated hydrosylate not contacted to a recombinant yeast or yeast inoculum provided by the present invention. In such cases, the method comprises contacting a GVL-treated hydrosylate to a recombinant yeast having increased tolerance to GVL toxicity, whereby cellulosic material of the contacted hydrosylate is fermented to produce ethanol at an enhanced rate relative to fermentation of a GVL-treated hydrosylate 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 PAD1 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 is provided. An article of manufacture can contain one of the microorganisms disclosed herein (e.g., one or more of the yeast 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 bioconversion.

GVL is the Major Inhibitor Found in GVL Hydrolysates:

GVL produced hydrolysates (FIG. **1**A) are still largely unstudied, as such, our first goal was to identify the major inhibitors of GVL hydrolyates. 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 (IC_{50}) is 270 mM (FIG. **1**B). Because of this, we focused on understanding GVL toxicity and developing GVL-tolerant yeast strains.

TABLE 1

Quantification of the 10 most abundant fermentation inhibitors found in GVL hydrolysates

Inhibitor	mM
GVL	100-230
Acetate	30.27
Formate	25.12
Levulinic acid	17.13
HMF	10.80
Acetaldehyde	1.98
Furfural	1.33
2-ketoglutaric acid	0.15
Furoic Acid	0.13
Coumaric acid	0.11

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. **2**A). 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. **2**B), 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 IC_{50} compared to the control strain (FIG.

2C). Deletion mutants of these 3 genes have increased sensitivity to ethanol, heat, and membrane disrupting agents such as miconazole and nigercin. 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 coatomer complex involved in retrograde transport between Golgi and ER 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 nigericin ($p<0.01$) and papuamide ($p<0.01$), 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. **3**A, **3**B), similar to the effects of ethanol but with a greater magnitude (FIG. **3**B). 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. **3**C). 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 yield.

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. **4**A), driven by deletion mutants of PAD1 and FDC1. Single mutant validations reveals deletion of these genes improved GVL tolerance (FIG. **4**B). 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 to an industrially viable, xylose fermenting yeast (GLBRCY-128) to create a 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 extend 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 2μ 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 (10× genome coverage). We grew this pooled transformant collection in the presence of 2.5% GVL or a 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. **4**C). We confirmed with single mutant cultures that overexpression of PAD1 causes GVL sensitivity. The IC_{50} of $Y133^{+}$ pPAD1 was 2.2%, compared to 2.56% of vector control (FIG. **4**D, $p<0.001$).

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. **4**A). We confirmed deletion of both genes by PCR (FIG. **4**B).

The IC_{50} concentration of GVL of the Y133 pad1Δfdc1Δ strain was significantly higher than the Y133 background (FIG. **5**C; $p<0.01$). 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. **5**D).

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. **6**A) may be more toxic than the acid forms. We found that the IC_{50} values of the vinyl derivative of coumaric acid, 4-vinylphenol, was an order of magnitude lower than that of coumaric acid (FIG. **6**B). Further, we found a statistically significant synergistic interaction between the vinyl derivative of coumaric acid, 4-vinylphenol, and GVL (FIG. **6**C, $p<0.01$). 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. **6**D, $p<0.01$), 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 my 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 Pad1p and Fdc1p, 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 IC_{50} Determination:

Compounds tested were purchased from Sigma. Cells of *S. cerevisiae* (MATα pdr1Δ::natMX pdr3Δ::KI.URA3 snq2Δ::KI.LEU2 can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 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 GCAT analysis software (available at gcat3-pub.glbrc.org on the world wide web) [16]. When presented, IC_{50} 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 poacic 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 poacic 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 poacic 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. 3 replicates of each condition (poacic 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 poacic 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 poacic 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 poacic acid with the yeast genetic interaction network to 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-Y133 containing the plasmid collection. The plasmid pool for transformation was generated as described previously (HO). For yeast transformation, the plasmids were extracted from 150 mL 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 mL of media containing YPD+2.5% GVL+G418 was allowed to degas overnight in an anerobic 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 zymolyase 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 pad1Δfdc1Δ 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 FungaLight™ cell viability assay (Invitrogen L34952) was used to determine if poacic 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 6×6 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 poacic 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/C, 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 Ferulate and Diferulates 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 GLBRY-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 GLBRCY-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

1. Ho et al., Combining functional genomics and chemical biology to identify targets of bioactive compounds. *Curr Opin Chem Biol* 2011, 15:66-78.
2. Piotrowski et al., Death by a thousand cuts: the challenges and diverse landscape of lignocellulosic hydrolysate inhibitors. *Front Microbiol* 2014, 5.
3. Palmqvist and Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour Technol* 2000, 74:25-33.
4. Keating et al., Aromatic inhibitors derived from ammonia-pretreated lignocellulose hinder bacterial ethanologenesis by activating regulatory circuits controlling inhibitor efflux and detoxification. *Microb Physiol Metab* 2014, 5:402.
5. Almeida et al., Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J Chem Technol Biotechnol* 2007, 82:340-349.
6. Luterbacher et al., Nonenzymatic Sugar Production from Biomass Using Biomass-Derived γ-Valerolactone. *Science* 2014, 343:277-280.
7. Ouellet et al., Impact of ionic liquid pretreated plant biomass on *Saccharomyces cerevisiae* growth and biofuel production. *Green Chem* 2011, 13:2743.
8. Docherty et al., Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. *Green Chem* 2005, 7:185-189.
9. McNew et al., Gos1p, a *Saccharomyces cerevisiae* SNARE protein involved in Golgi transport. *FEBS Lett* 1998, 435:89-95.
10. Costanzo et al., The genetic landscape of a cell. *Science* 2010, 327:425-431.
11. Cosson et al., Delta- and zeta-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. *EMBO J* 1996, 15:1792-1798.
12. Clausen et al., PAD1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. *Gene* 1994, 142:107-112.
13. Mukai et al., PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 2010, 109:564-569.
14. Magtanong et al., Dosage suppression genetic interaction networks enhance functional wiring diagrams of the cell. *Nat Biotechnol* 2011, 29:505-511.
15. Parreiras et al., Engineering and Two-Stage Evolution of a Lignocellulosic Hydrolysate-Tolerant *Saccharomyces cerevisiae* Strain for Anaerobic Fermentation of Xylose from AFEX Pretreated Corn Stover. *PLoS ONE* 2014, 9:e107499.
16. Sato et al., Harnessing genetic diversity in *Saccharomyces cerevisiae* for improved fermentation of xylose in hydrolysates of alkaline hydrogen peroxide pretreated biomass. *Appl Environ Microbiol* 2013:AEM.01885-13.
17. Fung S-Y et al., Unbiased screening of marine sponge extracts for anti-inflammatory agents combined with chemical genomics identifies girolline as an inhibitor of protein synthesis. *ACS Chem Biol* 2013.
18. Parsons et al., Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. *Cell* 2006, 126:611-625.
19. Andrusiak K: Adapting *S. cerevisiae* Chemical Genomics for Identifying the Modes of Action of Natural Compounds. Thesis; 2012.
20. Smith et al., Quantitative phenotyping via deep barcode sequencing. *Genome Res* 2009, 19:1836-1842.
21. Robinson et al., Design and analysis of bar-seq experiments. G3 *GenesGenomesGenetics* 2014, 4:11-18.
22. Robinson et al., edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinforma Oxf Engl* 2010, 26:139-140.
23. Boyle et al., GO::TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics* 2004, 20:3710-3715.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 10

<210> SEQ ID NO 1
<211> LENGTH: 12012
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

-continued

<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: IRA2 coding sequence

<400> SEQUENCE: 1

ataccgatac tttccatacg cacttgattt agaggccggc tccactatcg agattgaaaa 60

tcagtatgga gaagtgatct tcttgggcaa gtatggctct tctccaatga ttaacttaag 120

gccaccttca agattatctg cagaaagttt acaggcatcc caagagccat tttactcctt 180

tcaaatcgat acgttaccag aactggatga ctctagtatc atcagtacat ccatttcact 240

ctcttatgac ggtgacgaca atgaaaaagc cctgacttgg gaagaactct aggtcaaact 300

ctatttttat acagcaatga gtacctttc acatacacat aaatatatta aatataataa 360

atacaataaa tataaaataa ccctttcga aactctttac ttcctaaggc ctctcattac 420

ttatcaccta gcatcatgct cttcatacat gccatctact ttcaaacgat atacggctaa 480

ccagaaaagt acggacaatc agggacgagc agggacatga cgccttccgc acagatccca 540

gagaaaagca gggaaacaag aaaataagaa aacaagaaaa acagtagtta cccttgtaag 600

tgtcatccac aacagaacca atactcttac tcttccgctc attccccgca gaatgataga 660

actattctaa atccccttgc cttgcttggc cttgacttgg gttgggactt ggacctctag 720

aaccgatgtg ccttcaaaca tcttagcgag ataagagtct gccattaatg catcgagcca 780

atatcttgga ctagtacgag gttcacgtag cctttcttgc acggcaccag cgtcccaccc 840

caagttcttt gctatactga ccgccttaca cattttagtg tgattccaac ccaaagtagc 900

tcctcaaaaa gggtcacccg atcggtgaat caatccgcgg cgcagaaatg ttcgtagagc 960

tctcgtttct tctagaacgc tcccttgatg aaacgaaaat ttccatcaca ataaagctcg 1020

cacgcctttt tgaaaacccc aagctttgct gtgtcttctt gtgaaaagtt ttgcccaacg 1080

attatctatt ctacatataa ccggggagtt aagcacatcc tattgcccta cattctctcc 1140

gcttacttca ttgtctagag ctcctgggaa acaaaagacc gaaaaagcga aaacaaaatc 1200

agaacaaggc ttaagtactt tttcaccaat tgtagcaaac atttaaccac attttagcac 1260

actagcatat agcattgtcc tctgttattc gttttgcttt tctcctttag tgttactttt 1320

cccccaacgt tacaccattt tttgatatca actaaactgt atacattatc tttcttcagg 1380

gagaagcatg tcccagccca ctaagaataa gaagaaagaa cacgggaccg attccaagtc 1440

atcccgcatg actcggacgt tggttaatca tattcttttt gaaagaattc tcccgatcct 1500

tccggtggag tctaatctaa gtacctattc ggaagtggaa gagtattcct cattcatttc 1560

atgcagatct gtgctcatta acgttaccgt ttcccgagat gcaaatgcta tggtggaagg 1620

caccttggag ttgatagaat cgcttcttca agggcacgaa atcatttcag ataagggtag 1680

cagtgacgtt attgaatcaa tactgattat actaagattg ttaagtgatg cgctagagta 1740

taattggcaa aatcaagaaa gccttcatta caacgacatt tcgactcacg tagaacatga 1800

ccaagaacag aagtacagac caaagcttaa cagtattctg cccgactact cgtcgactca 1860

ttccaatggc aacaaacact ttttccacca gagcaaacct caggcactga taccggaact 1920

ggcatcgaaa ttgcttgaga gttgcgcgaa gttgaagttc aatacaagaa ctttgcaaat 1980

tttacaaaat atgatcagtc atgttcatgg aaacattcta acgactttga gttcctcgat 2040

tcttccccgc cacaaatcct atctgacaag gcacaaccat ccttctcatt gtaaaatgat 2100

tgactctact ctaggccata ttctccgatt tgtagcggct tccaatccgt ccgagtattt 2160

tgaatttatc agaaagagtg tgcaagtgcc cgtaacacag acacacacgc attcacactc 2220

-continued

ccattcacac tctttgccat cttccgttta taacagcata gtgccccact ttgatctttt 2280

cagcttcatc tatttaagca agcataattt taagaaatac ttggaactca tcaaaaactt 2340

atcggtgacg ttaaggaaaa cgatttatca ttgcctactt ttgcattaca gcgccaaagc 2400

aataatgttt tggataatgg ctaggcctgc ggaatattat gaactcttca acttattaaa 2460

agataataac aatgaacact cgaaatcctt aaacacgtta aaccatacac ttttcgagga 2520

gatccattcg acttttaatg tgaatagcat gataaccacc aatcaaaatg ctcatcaagg 2580

ctcatcttcc ccttcgtcct cctcgccatc gtcaccacct agctcatcat catcggataa 2640

caacaatcaa aacataatag caaaatcctt aagtcgtcag ctttctcacc accagtcata 2700

cattcaacag cagtctgaaa gaaaactaca ttcttcatgg actacaaact ctcaatcctc 2760

tacttcactg tcatcttcaa cgtctaattc aacaacaact gatttctcta ctcacactca 2820

accaggagaa tatgaccctt ccttaccaga tactcccacg atgtctaaca tcactattag 2880

tgcatcttca ttattatctc aaactccaac tccaacaaca caattgcaac agcggttgaa 2940

ctcagcagct gcagccgccg ccgcagctgc ttcaccatcg aattccaccc caactggata 3000

cacagcagag caacaaagtc gcgcttcata cgatgcacac aaaactggcc atactggtaa 3060

ggattatgac gaacattttt tgtctgtcac tcgtttggat aatgttttgg agttatacac 3120

gcactttgat gatactgagg tactaccaca cacatccgta ctgaagtttt taactacttt 3180

gacaatgttc gatattgacc tttttaatga attaaacgct acatcattca aatatattcc 3240

tgattgtact atgcatcgtc caaaagaaag aacaagttct ttcaataata ctgcacacga 3300

gacaggttcc gaaaagactt cgggtataaa acatattaca caaggcttaa agaaattaac 3360

ttctttacct tcctcaacca aaaaaactgt aaaatttgtg aagatgttgc taagaaattt 3420

aaatgggaat caagctgtat cagatgttgc cctcttagat acaatgaggg ccttactatc 3480

attctttaca atgacttctg cggtctttct cgtggataga aacttaccct cagtactttt 3540

tgccaagaga ctcatcccca taatggggac aaatttaagc gtcggtcaag actggaattc 3600

aaaaataaat aacagtttga tggtttgttt gaaaaaaaac tccaccacgt ttgttcaatt 3660

acaattaata ttcttctctt cagctattca attcgatcat gaattattac tggcacgtct 3720

gagcatcgat acaatggcca acaatttaaa catgcagaag ctatgccttt atactgaagg 3780

attcaggata ttcttcgaca taccaagtaa gaaggaattg cggaaggcaa ttgcggttaa 3840

aatttctaaa tttttcaaaa cattattctc cattatagca gatattcttt tacaagaatt 3900

tccgtatttt gatgagcaaa tcaccgacat agttgcttcc attcttgacg gtacaattat 3960

caatgagtat ggtacgaaga aacatttcaa ggggagctca ccctctttat gttcgacaac 4020

ccggtcaaga tcaggatcta catctcaaag ttcaatgaca ccagtttctc cgctgggact 4080

ggatactgat atatgtccaa tgaacaccct gtctttagtt ggttcaagta cttcaagaaa 4140

ttctgacaac gttaattcat taaacagttc accaaagaac ttgtcttctg atccatactt 4200

gtcacatctt gtggccccaa gagcgcgtca tgctttaggt gggccatcta gtattataag 4260

gaataaaata ccgactacat tgacttcacc tccaggaacg gaaaaatctt caccagtaca 4320

acgtccgcaa acggaaagca tcagtgccac accaatggcc ataacaaatt ctactccatt 4380

atcgtcggca gcattcggaa ttcgatcgcc tttgcagaaa ataagaacga ggcgttattc 4440

cgatgaaagt ttaggaaaat tcatgaaatc aacaaataat tacattcaag aacatttgat 4500

accaaaagat ttgaatgaag caactcttca agatgctaga agaataatga ttaatatttt 4560

cagtattttt aagagaccga atagttactt catcattcct cacaatataa actcgaattt 4620

-continued

acaatgggtt tcgcaggatt ttagaaatat tatgaaaccg attttcgtcg ccatcgtaag 4680

tccggatgta gatttacaga atactgctca atcattcatg gataccttat tatcgaatgt 4740

tattacttat ggtgaatcag atgagaatat cagtattgaa gggtatcatc ttctttgcag 4800

ttacactgta acattatttg caatgggcct tttcgatttg aaaattaata atgaaaagcg 4860

tcaaattctc ttggatataa ctgtcaagtt tatgaaggtt agatcacatt tagcagggat 4920

cgcggaggcc tcacaccaca tggaatacat aagtgattct gaaaaactca cctttccgct 4980

gattatgggg actgttggta gggccctatt tgtttcatta tactctagtc aacaaaaaat 5040

tgaaaagact ttaaagattg cttacacaga gtatctttct gcaatcaatt ttcatgagag 5100

gaatattgat gatgctgata aaacttgggt tcataatatt gagtttgtag aagcgatgtg 5160

tcatgacaac tacacaactt ctggttcaat tgctttccaa aggaggacaa gaaataatat 5220

tttacgattt gctactattc ctaacgctat cttacttgat tctatgagga tgatctataa 5280

gaagtggcat acttacacac acagtaaaag tttagaaaaa caagaacgga acgacttcag 5340

aaatttcgcg ggtattttag cctctttgtc gggtatccta ttcatcaata aaaagatatt 5400

gcaagaaatg tatccatacc tactcgacac cgtttcagaa ttgaaaaaaa atatagactc 5460

ttttatctca aaacaatgcc aatggttaaa ctatccggat ttattaacga gagaaaattc 5520

aagagatatt ctaagtgtag aactgcatcc tttgtctttt aacttacttt ttaataattt 5580

gaggctcaag ttaaaagaac ttgcttgttc agacttatca ataccagaaa atgaaagttc 5640

ctatgtttta ttagaacaaa taatcaaaat gctgcggaca atcctaggtc gtgatgatga 5700

caattatgta atgatgcttt tttccacaga gattgtagat cttattgatt tattgacaga 5760

tgaaataaaa aaaataccag cctattgtcc aaaatatctc aaggcaatta ttcaaatgac 5820

caaaatgttc agtgccttgc agcactcaga ggttaattta ggtgtcaaaa atcattttca 5880

cgttaaaaat aaatggttga ggcaaatcac tgattggttt caagtgagta ttgcgagaga 5940

gtacgatttc gaaaacttgt caaaacctct aaaagaaatg gatttggtaa aaagagacat 6000

ggatattcta tacatagata cggcaatcga agcttcaacc gctattgcgt acctcacgag 6060

acatactttc ttagagattc cacctgccgc gtcagatccc gaactatctc gatctaggtc 6120

tgtgatattt gggttttatt tcaacatctt aatgaaaggc cttgaaaaaa gtagtgatcg 6180

tgacaattac ccagtattct tgaggcacaa aatgagtgtc ctcaacgaca atgtaatact 6240

ttcattaaca aatctttcaa acaccaatgt tgatgcgagt ttgcagttca ccttaccgat 6300

gggctattcc ggaaatcgaa acattaggaa tgcatttttg gaggtcttca ttaatatcgt 6360

tacgaactat cggacataca cggctaaaac tgaccttgga aaattagagg cagcagacaa 6420

atttttgcga tatacgattg aacatcccca gctatcgtcc tttggagcag cggtttgtcc 6480

cgctagcgat attgatgctt atgctgctgg cttaataaat gcatttgaaa cgaggaatgc 6540

cacccacatt gtagtggcac agttgattaa aaatgaaatt gaaaaatctt ccagacctac 6600

ggatatcctt agaagaaata gctgtgctac gagatcatta tctatgctag ccaggtccaa 6660

gggtaacgaa tatttgattc gcactttgca accattacta aaaaaaatta tccagaacag 6720

agatttttt gaaattgaga aactaaaacc ggaagattca gatgctgaac gtcaaataga 6780

gctcttcgtt aaatacatga atgaattatt ggaatccata tccaactccg tatcttattt 6840

tccccctcct ttattttata tttgccaaaa catttataaa gttgcgtgtg aaaaatttcc 6900

ggatcacgca attatcgccg ctgggtcttt cgtgttttta cggtttttt gtcctgcttt 6960

agtcagccct gattctgaaa atatcataga tatttctcac ttgagcgaaa agcgtacctt 7020

-continued

catcagcttg gctaaagtta tccaaaatat tgccaatggc tcagaaaatt tctccagatg 7080

gccagctttg tgttcccaaa aggattttct taaggaatgt agcgatagaa ttttcagatt 7140

cctagctgaa ctttgtagaa cagatcgcac gatagacatc caagtgagaa cagacccaac 7200

gccaattgca tttgactatc aattccttca ttcctttgtt tacctttacg gtcttgaggt 7260

gagaaggaat gtgctaaatg aagcaaaaca tgatgatggt gacattgatg gtgacgattt 7320

ctataagacc acatttttac ttattgatga tgttcttggc caattaggcc aacctaaaat 7380

ggaattttcc aatgaaatac caatatacat aagagaacat atggacgact atccggaact 7440

gtatgagttc atgaataggc acgcgttcag aaacattgag acttcaacag cgtacagccc 7500

aagcgttcac gagtccacct caagtgaagg cattccaatt attacgttaa caatgtcaaa 7560

tttctcagac agacatgtgg acattgatac agttgcttac aagttcttgc aaatttatgc 7620

tcgaatctgg accaccaaac actgtttaat aatcgactgt acagaatttg acgagggagg 7680

gcttgatatg aggaaattta tttctttggt tatgggacta ttaccagaag ttgcacccaa 7740

aaattgtata ggctgttact actttaacgt aaacgagaca tttatggata attatggaaa 7800

atgtttggac aaagacaacg tatatgtttc ctcgaaaatt cctcattatt tcattaatag 7860

taactctgat gaaggactta tgaaatctgt gggtataact ggacaagggt tgaaggttct 7920

gcaagatatt cgtgtctctc tgcatgatat cacgctttat gacgaaaaaa gaaatagatt 7980

tacgccggta tcgttgaaaa taggcgatat ttactttcaa gtcttgcatg aaactcctag 8040

gcaatataaa ataagggaca tgggtacttt attcgacgta aaattcaatg atgtctacga 8100

aattagccga atatttgaag tacatgtttc gtcaataact ggagtggcag ctgaatttac 8160

agtaactttt caggacgaga gaaggttgat ttttagtagt ccgaaatacc ttgaaattgt 8220

gaagatgttc tattacgcac agatccggtt agaaagtgaa tatgaaatgg ataataattc 8280

gagtacctcc tccccaaatt caaacaacaa ggacaaacag cagaaagaga gaacaaaact 8340

attgtgccac ctactgttag tatctcttat tggtctgttt gatgagagta aaaaaatgaa 8400

aaacagttcg tataacctaa tagctgccac tgaggcgtca tttggtttga actttggctc 8460

ccattttcat cgctctcccg aggtgtacgt ccccgaagat actacaacat ttttaggtgt 8520

tattggaaag tctcttgcag agtctaatcc agaactcaca gcctatatgt ttatctatgt 8580

tttggaggca ttgaagaaca acgtaattcc tcacgtttac atccctcata ccatttgcgg 8640

tttgtcttat tggatcccta atttatacca acatgtgtat ttggctgatg atgaagaagg 8700

ccccgaaaac atatctcaca ttttccgaat tcttatcagg ctctctgtga gagagactga 8760

ctttaaagcc gtatacatgc aatatgtttg gttgctactt ttagatgatg gccgcttaac 8820

tgacattatc gttgatgaag ttattaatca tgcgttagaa agagactccg aaaaccgcga 8880

ttggaagaaa acaatatcgt tactgactgt cctacccact actgaggttg ctaataatat 8940

tattcaaaaa atattggcaa aaattagatc atttttaccg tcattgaagt tagaagctat 9000

gacccaaagt tggtctgaac taacaatatt agttaagata agcatccacg tttttttga 9060

aacttctttg ctggtacaga tgtacttacc agagatcctg tttatcgtat ccttattaat 9120

tgatgttggt ccaagggaac tcagatcatc actacaccag ctattaatga atgtatgcca 9180

ttccttggct attaactcag ctttaccaca agatcataga aataatctag atgaaataag 9240

tgatatattt gcacatcaaa aggtgaagtt tatgtttggg ttcagcgagg acaaaggacg 9300

aattttacag atttttagcg cttcttcttt tgcaagcaag tttaatattc tggatttctt 9360

catcaataat atattattgc tgatggaata ttcttcaacg tacgaagcaa acgtgtggaa 9420

-continued

gacaagatac aagaaatatg tcttggaatc tgtgtttaca agtaattctt ttctttcggc 9480

acgttcaatc atgattgttg gtataatggg taaatcttac ataactgaag ggttatgcaa 9540

ggctatgtta attgaaacca tgaaagttat cgccgaacca aagattactg acgagcatct 9600

tttcttagcc atatctcata tttttactta ttccaaaatt gttgaaggtt tggatcccaa 9660

ccttgactta atgaagcact tattttggtt ttcaacactc ttccttgaat cacgtcaccc 9720

gataattttt gagggtgccc ttctctttgt gtcaaactgt ataaggcgcc tatacatggc 9780

ccagtttgaa aatgaaagcg aaacatcatt gataagtact ttacttaagg ggagaaagtt 9840

tgctcatacc tttttaagca agatagagaa tcttagtggt attgtttgga atgaagataa 9900

ttttacacac attctgattt tcatcattaa taaaggacta tccaatcctt tcattaagag 9960

tacggctttt gatttcttga agatgatgtt tagaaactcc tactttgagc atcaaatcaa 10020

tcagaaatct gatcattatt tgtgctatat gttcctattg tattttgttt taaactgcaa 10080

tcaatttgag gaacttttag gtgacgttga ttttgaagga gaaatggtta acattgaaaa 10140

caagaacacc attcctaaaa ttttgttaga gtggttgagt tcggataacg aaaatgcaaa 10200

cattaccctc tatcaaggtg cgatactgtt caaatgttca gttacggatg aaccaagtag 10260

atttaggttt gcgttgatta ttaggcatct attgacaaag aaacccattt gtgcattgcg 10320

tttttacagt gttattcgta acgaaataag aaaaatatca gcatttgagc aaaattcgga 10380

ttgtgttcca cttgctttcg atattttaaa cttattagtg acgcattcag agtctaattc 10440

gttagaaaaa cttcacgaag aatccattga acgtctaacc aaaagaggtt tatcgattgt 10500

gacttcttct ggtatatttg cgaagaactc cgacatgatg ataccttag atgtaaaacc 10560

tgaagatatc tatgaacgta agagaataat gacaatgatt ttatcaagga tgtcatgttc 10620

tgcttagagg tgttacataa actaatgaaa gaaatatcaa tatctatctg taagcatgaa 10680

tgtacatatc tcatgttagg gttttcttat cgctaatttt tcgcaatttg ttacgtgggt 10740

tgcttttata cagctacaat ttttatatat tctatcgtgt aatgaatggc tcagtaaatt 10800

caagcgccac atagactaat gtacatacca atgcatttta attgtaagaa taaaaggggc 10860

cattcatcta ccgtcttagt tgaaagtgtt tctgtgaatt ttttcaaatt ccgtttttc 10920

ctttttatat aatagcatgg tggcacgagc atcttcgact gaagaatgct caccttcttg 10980

aatggaaatt tttaaaacct ccctggttaa tttctttaag ctgggtgttt tacccttagc 11040

atacaacttc ctgaatggga ggtgtcttga agtgtccctg agtagtgact ttgggtggga 11100

taacatcaat gcttcgagat catgctttaa agcgtgccct acaagaattc taccttccag 11160

aatatccgca gtctttttt gagcttcttt gaatgtaatg gcatttttca tatgctctgg 11220

tttaatacca ctaacccaag ttctccattc tacaactttc tctcttggct taacaaattc 11280

atcgaggacg acatgtccaa aataatttac gattgatatt ctagctaacg cagactcttt 11340

accctcggga ccaacgccta caaattcaca atccatggcg atgtatttcc caatttcttt 11400

actcttatta ctattaatac gagtatcttc cgaaataccc acttttactg gctctttaat 11460

agtagtacta gtatttgctt tatttgggtt gaattcaaac acttttccct cgagcttgtc 11520

tttctcatgc ttactaatct ccttgttcat gttatatacc atgtccataa ttttactgcc 11580

gtttttgcgc tttcgagggg catattgggt tgtactactg acatttactg ttttgctaac 11640

tttcttaacg tttctgattt tcctatttga ttgcttattc tttccattgg aggtaggatt 11700

gctttcggag gctaatagtg cctgccagtt tgaagagaga gccattaaaa ctgtacgcta 11760

tctactttat taaatctgta aacgtctatg aagcctctag aaccaggctt taaatggatt 11820

-continued

gtggttgcga tgaggttagt ttaacttttg aaatttttct ttttttagc cgaccttaca 11880

tatcagcccg cgtcaaaaaa tatacggtat aataattctc aatagataca ggctactgaa 11940

caggaaaact aaataaaaca gtgtttgtaa aacccccacc acaccataat aagacgataa 12000

tggacacggt ga 12012

<210> SEQ ID NO 2
<211> LENGTH: 3079
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 2

Met Ser Gln Pro Thr Lys Asn Lys Lys Lys Glu His Gly Thr Asp Ser
1 5 10 15

Lys Ser Ser Arg Met Thr Arg Thr Leu Val Asn His Ile Leu Phe Glu
20 25 30

Arg Ile Leu Pro Ile Leu Pro Val Glu Ser Asn Leu Ser Thr Tyr Ser
35 40 45

Glu Val Glu Glu Tyr Ser Ser Phe Ile Ser Cys Arg Ser Val Leu Ile
50 55 60

Asn Val Thr Val Ser Arg Asp Ala Asn Ala Met Val Glu Gly Thr Leu
65 70 75 80

Glu Leu Ile Glu Ser Leu Leu Gln Gly His Glu Ile Ile Ser Asp Lys
85 90 95

Gly Ser Ser Asp Val Ile Glu Ser Ile Leu Ile Ile Leu Arg Leu Leu
100 105 110

Ser Asp Ala Leu Glu Tyr Asn Trp Gln Asn Gln Glu Ser Leu His Tyr
115 120 125

Asn Asp Ile Ser Thr His Val Glu His Asp Gln Glu Gln Lys Tyr Arg
130 135 140

Pro Lys Leu Asn Ser Ile Leu Pro Asp Tyr Ser Ser Thr His Ser Asn
145 150 155 160

Gly Asn Lys His Phe Phe His Gln Ser Lys Pro Gln Ala Leu Ile Pro
165 170 175

Glu Leu Ala Ser Lys Leu Leu Glu Ser Cys Ala Lys Leu Lys Phe Asn
180 185 190

Thr Arg Thr Leu Gln Ile Leu Gln Asn Met Ile Ser His Val His Gly
195 200 205

Asn Ile Leu Thr Thr Leu Ser Ser Ser Ile Leu Pro Arg His Lys Ser
210 215 220

Tyr Leu Thr Arg His Asn His Pro Ser His Cys Lys Met Ile Asp Ser
225 230 235 240

Thr Leu Gly His Ile Leu Arg Phe Val Ala Ala Ser Asn Pro Ser Glu
245 250 255

Tyr Phe Glu Phe Ile Arg Lys Ser Val Gln Val Pro Val Thr Gln Thr
260 265 270

His Thr His Ser His Ser His Ser His Ser Leu Pro Ser Ser Val Tyr
275 280 285

Asn Ser Ile Val Pro His Phe Asp Leu Phe Ser Phe Ile Tyr Leu Ser
290 295 300

Lys His Asn Phe Lys Lys Tyr Leu Glu Leu Ile Lys Asn Leu Ser Val
305 310 315 320

Thr Leu Arg Lys Thr Ile Tyr His Cys Leu Leu Leu His Tyr Ser Ala
325 330 335

-continued

```
Lys Ala Ile Met Phe Trp Ile Met Ala Arg Pro Ala Glu Tyr Tyr Glu
            340                 345                 350

Leu Phe Asn Leu Leu Lys Asp Asn Asn Asn Glu His Ser Lys Ser Leu
        355                 360                 365

Asn Thr Leu Asn His Thr Leu Phe Glu Glu Ile His Ser Thr Phe Asn
    370                 375                 380

Val Asn Ser Met Ile Thr Thr Asn Gln Asn Ala His Gln Gly Ser Ser
385                 390                 395                 400

Ser Pro Ser Ser Ser Ser Pro Ser Ser Pro Pro Ser Ser Ser Ser Ser
                405                 410                 415

Asp Asn Asn Asn Gln Asn Ile Ile Ala Lys Ser Leu Ser Arg Gln Leu
            420                 425                 430

Ser His His Gln Ser Tyr Ile Gln Gln Gln Ser Glu Arg Lys Leu His
        435                 440                 445

Ser Ser Trp Thr Thr Asn Ser Gln Ser Ser Thr Ser Leu Ser Ser Ser
    450                 455                 460

Thr Ser Asn Ser Thr Thr Thr Asp Phe Ser Thr His Thr Gln Pro Gly
465                 470                 475                 480

Glu Tyr Asp Pro Ser Leu Pro Asp Thr Pro Thr Met Ser Asn Ile Thr
                485                 490                 495

Ile Ser Ala Ser Ser Leu Leu Ser Gln Thr Pro Thr Pro Thr Thr Gln
            500                 505                 510

Leu Gln Gln Arg Leu Asn Ser Ala Ala Ala Ala Ala Ala Ala Ala Ala
        515                 520                 525

Ser Pro Ser Asn Ser Thr Pro Thr Gly Tyr Thr Ala Glu Gln Gln Ser
    530                 535                 540

Arg Ala Ser Tyr Asp Ala His Lys Thr Gly His Thr Gly Lys Asp Tyr
545                 550                 555                 560

Asp Glu His Phe Leu Ser Val Thr Arg Leu Asp Asn Val Leu Glu Leu
                565                 570                 575

Tyr Thr His Phe Asp Asp Thr Glu Val Leu Pro His Thr Ser Val Leu
            580                 585                 590

Lys Phe Leu Thr Thr Leu Thr Met Phe Asp Ile Asp Leu Phe Asn Glu
        595                 600                 605

Leu Asn Ala Thr Ser Phe Lys Tyr Ile Pro Asp Cys Thr Met His Arg
    610                 615                 620

Pro Lys Glu Arg Thr Ser Ser Phe Asn Asn Thr Ala His Glu Thr Gly
625                 630                 635                 640

Ser Glu Lys Thr Ser Gly Ile Lys His Ile Thr Gln Gly Leu Lys Lys
                645                 650                 655

Leu Thr Ser Leu Pro Ser Ser Thr Lys Lys Thr Val Lys Phe Val Lys
            660                 665                 670

Met Leu Leu Arg Asn Leu Asn Gly Asn Gln Ala Val Ser Asp Val Ala
        675                 680                 685

Leu Leu Asp Thr Met Arg Ala Leu Leu Ser Phe Phe Thr Met Thr Ser
    690                 695                 700

Ala Val Phe Leu Val Asp Arg Asn Leu Pro Ser Val Leu Phe Ala Lys
705                 710                 715                 720

Arg Leu Ile Pro Ile Met Gly Thr Asn Leu Ser Val Gly Gln Asp Trp
                725                 730                 735

Asn Ser Lys Ile Asn Asn Ser Leu Met Val Cys Leu Lys Lys Asn Ser
            740                 745                 750
```

-continued

```
Thr Thr Phe Val Gln Leu Gln Leu Ile Phe Phe Ser Ser Ala Ile Gln
        755                 760                 765

Phe Asp His Glu Leu Leu Leu Ala Arg Leu Ser Ile Asp Thr Met Ala
    770                 775                 780

Asn Asn Leu Asn Met Gln Lys Leu Cys Leu Tyr Thr Glu Gly Phe Arg
785                 790                 795                 800

Ile Phe Phe Asp Ile Pro Ser Lys Lys Glu Leu Arg Lys Ala Ile Ala
                805                 810                 815

Val Lys Ile Ser Lys Phe Phe Lys Thr Leu Phe Ser Ile Ile Ala Asp
            820                 825                 830

Ile Leu Leu Gln Glu Phe Pro Tyr Phe Asp Glu Gln Ile Thr Asp Ile
        835                 840                 845

Val Ala Ser Ile Leu Asp Gly Thr Ile Ile Asn Glu Tyr Gly Thr Lys
    850                 855                 860

Lys His Phe Lys Gly Ser Ser Pro Ser Leu Cys Ser Thr Thr Arg Ser
865                 870                 875                 880

Arg Ser Gly Ser Thr Ser Gln Ser Ser Met Thr Pro Val Ser Pro Leu
                885                 890                 895

Gly Leu Asp Thr Asp Ile Cys Pro Met Asn Thr Leu Ser Leu Val Gly
            900                 905                 910

Ser Ser Thr Ser Arg Asn Ser Asp Asn Val Asn Ser Leu Asn Ser Ser
        915                 920                 925

Pro Lys Asn Leu Ser Ser Asp Pro Tyr Leu Ser His Leu Val Ala Pro
    930                 935                 940

Arg Ala Arg His Ala Leu Gly Gly Pro Ser Ser Ile Ile Arg Asn Lys
945                 950                 955                 960

Ile Pro Thr Thr Leu Thr Ser Pro Pro Gly Thr Glu Lys Ser Ser Pro
                965                 970                 975

Val Gln Arg Pro Gln Thr Glu Ser Ile Ser Ala Thr Pro Met Ala Ile
            980                 985                 990

Thr Asn Ser Thr Pro Leu Ser Ser  Ala Ala Phe Gly Ile  Arg Ser Pro
        995                 1000                1005

Leu Gln  Lys Ile Arg Thr Arg  Arg Tyr Ser Asp Glu  Ser Leu Gly
    1010                1015                1020

Lys Phe  Met Lys Ser Thr Asn  Asn Tyr Ile Gln Glu  His Leu Ile
    1025                1030                1035

Pro Lys  Asp Leu Asn Glu Ala  Thr Leu Gln Asp Ala  Arg Arg Ile
    1040                1045                1050

Met Ile  Asn Ile Phe Ser Ile  Phe Lys Arg Pro Asn  Ser Tyr Phe
    1055                1060                1065

Ile Ile  Pro His Asn Ile Asn  Ser Asn Leu Gln Trp  Val Ser Gln
    1070                1075                1080

Asp Phe  Arg Asn Ile Met Lys  Pro Ile Phe Val Ala  Ile Val Ser
    1085                1090                1095

Pro Asp  Val Asp Leu Gln Asn  Thr Ala Gln Ser Phe  Met Asp Thr
    1100                1105                1110

Leu Leu  Ser Asn Val Ile Thr  Tyr Gly Glu Ser Asp  Glu Asn Ile
    1115                1120                1125

Ser Ile  Glu Gly Tyr His Leu  Leu Cys Ser Tyr Thr  Val Thr Leu
    1130                1135                1140

Phe Ala  Met Gly Leu Phe Asp  Leu Lys Ile Asn Asn  Glu Lys Arg
    1145                1150                1155
```

-continued

```
Gln Ile  Leu Leu Asp Ile Thr  Val Lys Phe Met Lys  Val Arg Ser
    1160                 1165                 1170

His Leu  Ala Gly Ile Ala Glu  Ala Ser His His Met  Glu Tyr Ile
    1175                 1180                 1185

Ser Asp  Ser Glu Lys Leu Thr  Phe Pro Leu Ile Met  Gly Thr Val
    1190                 1195                 1200

Gly Arg  Ala Leu Phe Val Ser  Leu Tyr Ser Ser Gln  Gln Lys Ile
    1205                 1210                 1215

Glu Lys  Thr Leu Lys Ile Ala  Tyr Thr Glu Tyr Leu  Ser Ala Ile
    1220                 1225                 1230

Asn Phe  His Glu Arg Asn Ile  Asp Asp Ala Asp Lys  Thr Trp Val
    1235                 1240                 1245

His Asn  Ile Glu Phe Val Glu  Ala Met Cys His Asp  Asn Tyr Thr
    1250                 1255                 1260

Thr Ser  Gly Ser Ile Ala Phe  Gln Arg Arg Thr Arg  Asn Asn Ile
    1265                 1270                 1275

Leu Arg  Phe Ala Thr Ile Pro  Asn Ala Ile Leu Leu  Asp Ser Met
    1280                 1285                 1290

Arg Met  Ile Tyr Lys Lys Trp  His Thr Tyr Thr His  Ser Lys Ser
    1295                 1300                 1305

Leu Glu  Lys Gln Glu Arg Asn  Asp Phe Arg Asn Phe  Ala Gly Ile
    1310                 1315                 1320

Leu Ala  Ser Leu Ser Gly Ile  Leu Phe Ile Asn Lys  Lys Ile Leu
    1325                 1330                 1335

Gln Glu  Met Tyr Pro Tyr Leu  Leu Asp Thr Val Ser  Glu Leu Lys
    1340                 1345                 1350

Lys Asn  Ile Asp Ser Phe Ile  Ser Lys Gln Cys Gln  Trp Leu Asn
    1355                 1360                 1365

Tyr Pro  Asp Leu Leu Thr Arg  Glu Asn Ser Arg Asp  Ile Leu Ser
    1370                 1375                 1380

Val Glu  Leu His Pro Leu Ser  Phe Asn Leu Leu Phe  Asn Asn Leu
    1385                 1390                 1395

Arg Leu  Lys Leu Lys Glu Leu  Ala Cys Ser Asp Leu  Ser Ile Pro
    1400                 1405                 1410

Glu Asn  Glu Ser Ser Tyr Val  Leu Leu Glu Gln Ile  Ile Lys Met
    1415                 1420                 1425

Leu Arg  Thr Ile Leu Gly Arg  Asp Asp Asp Asn Tyr  Val Met Met
    1430                 1435                 1440

Leu Phe  Ser Thr Glu Ile Val  Asp Leu Ile Asp Leu  Leu Thr Asp
    1445                 1450                 1455

Glu Ile  Lys Lys Ile Pro Ala  Tyr Cys Pro Lys Tyr  Leu Lys Ala
    1460                 1465                 1470

Ile Ile  Gln Met Thr Lys Met  Phe Ser Ala Leu Gln  His Ser Glu
    1475                 1480                 1485

Val Asn  Leu Gly Val Lys Asn  His Phe His Val Lys  Asn Lys Trp
    1490                 1495                 1500

Leu Arg  Gln Ile Thr Asp Trp  Phe Gln Val Ser Ile  Ala Arg Glu
    1505                 1510                 1515

Tyr Asp  Phe Glu Asn Leu Ser  Lys Pro Leu Lys Glu  Met Asp Leu
    1520                 1525                 1530

Val Lys  Arg Asp Met Asp Ile  Leu Tyr Ile Asp Thr  Ala Ile Glu
    1535                 1540                 1545
```

-continued

```
Ala Ser  Thr Ala Ile Ala Tyr  Leu Thr Arg His Thr  Phe Leu Glu
    1550                1555                1560

Ile Pro  Pro Ala Ala Ser Asp  Pro Glu Leu Ser Arg  Ser Arg Ser
    1565                1570                1575

Val Ile  Phe Gly Phe Tyr Phe  Asn Ile Leu Met Lys  Gly Leu Glu
    1580                1585                1590

Lys Ser  Ser Asp Arg Asp Asn  Tyr Pro Val Phe Leu  Arg His Lys
    1595                1600                1605

Met Ser  Val Leu Asn Asp Asn  Val Ile Leu Ser Leu  Thr Asn Leu
    1610                1615                1620

Ser Asn  Thr Asn Val Asp Ala  Ser Leu Gln Phe Thr  Leu Pro Met
    1625                1630                1635

Gly Tyr  Ser Gly Asn Arg Asn  Ile Arg Asn Ala Phe  Leu Glu Val
    1640                1645                1650

Phe Ile  Asn Ile Val Thr Asn  Tyr Arg Thr Tyr Thr  Ala Lys Thr
    1655                1660                1665

Asp Leu  Gly Lys Leu Glu Ala  Ala Asp Lys Phe Leu  Arg Tyr Thr
    1670                1675                1680

Ile Glu  His Pro Gln Leu Ser  Ser Phe Gly Ala Ala  Val Cys Pro
    1685                1690                1695

Ala Ser  Asp Ile Asp Ala Tyr  Ala Ala Gly Leu Ile  Asn Ala Phe
    1700                1705                1710

Glu Thr  Arg Asn Ala Thr His  Ile Val Val Ala Gln  Leu Ile Lys
    1715                1720                1725

Asn Glu  Ile Glu Lys Ser Ser  Arg Pro Thr Asp Ile  Leu Arg Arg
    1730                1735                1740

Asn Ser  Cys Ala Thr Arg Ser  Leu Ser Met Leu Ala  Arg Ser Lys
    1745                1750                1755

Gly Asn  Glu Tyr Leu Ile Arg  Thr Leu Gln Pro Leu  Leu Lys Lys
    1760                1765                1770

Ile Ile  Gln Asn Arg Asp Phe  Phe Glu Ile Glu Lys  Leu Lys Pro
    1775                1780                1785

Glu Asp  Ser Asp Ala Glu Arg  Gln Ile Glu Leu Phe  Val Lys Tyr
    1790                1795                1800

Met Asn  Glu Leu Leu Glu Ser  Ile Ser Asn Ser Val  Ser Tyr Phe
    1805                1810                1815

Pro Pro  Pro Leu Phe Tyr Ile  Cys Gln Asn Ile Tyr  Lys Val Ala
    1820                1825                1830

Cys Glu  Lys Phe Pro Asp His  Ala Ile Ile Ala Ala  Gly Ser Phe
    1835                1840                1845

Val Phe  Leu Arg Phe Phe Cys  Pro Ala Leu Val Ser  Pro Asp Ser
    1850                1855                1860

Glu Asn  Ile Ile Asp Ile Ser  His Leu Ser Glu Lys  Arg Thr Phe
    1865                1870                1875

Ile Ser  Leu Ala Lys Val Ile  Gln Asn Ile Ala Asn  Gly Ser Glu
    1880                1885                1890

Asn Phe  Ser Arg Trp Pro Ala  Leu Cys Ser Gln Lys  Asp Phe Leu
    1895                1900                1905

Lys Glu  Cys Ser Asp Arg Ile  Phe Arg Phe Leu Ala  Glu Leu Cys
    1910                1915                1920

Arg Thr  Asp Arg Thr Ile Asp  Ile Gln Val Arg Thr  Asp Pro Thr
    1925                1930                1935
```

-continued

```
Pro Ile  Ala Phe Asp Tyr Gln  Phe Leu His Ser Phe  Val Tyr Leu
    1940                1945                1950

Tyr Gly  Leu Glu Val Arg Arg  Asn Val Leu Asn Glu  Ala Lys His
    1955                1960                1965

Asp Asp  Gly Asp Ile Asp Gly  Asp Asp Phe Tyr Lys  Thr Thr Phe
    1970                1975                1980

Leu Leu  Ile Asp Asp Val Leu  Gly Gln Leu Gly Gln  Pro Lys Met
    1985                1990                1995

Glu Phe  Ser Asn Glu Ile Pro  Ile Tyr Ile Arg Glu  His Met Asp
    2000                2005                2010

Asp Tyr  Pro Glu Leu Tyr Glu  Phe Met Asn Arg His  Ala Phe Arg
    2015                2020                2025

Asn Ile  Glu Thr Ser Thr Ala  Tyr Ser Pro Ser Val  His Glu Ser
    2030                2035                2040

Thr Ser  Ser Glu Gly Ile Pro  Ile Ile Thr Leu Thr  Met Ser Asn
    2045                2050                2055

Phe Ser  Asp Arg His Val Asp  Ile Asp Thr Val Ala  Tyr Lys Phe
    2060                2065                2070

Leu Gln  Ile Tyr Ala Arg Ile  Trp Thr Thr Lys His  Cys Leu Ile
    2075                2080                2085

Ile Asp  Cys Thr Glu Phe Asp  Glu Gly Gly Leu Asp  Met Arg Lys
    2090                2095                2100

Phe Ile  Ser Leu Val Met Gly  Leu Leu Pro Glu Val  Ala Pro Lys
    2105                2110                2115

Asn Cys  Ile Gly Cys Tyr Tyr  Phe Asn Val Asn Glu  Thr Phe Met
    2120                2125                2130

Asp Asn  Tyr Gly Lys Cys Leu  Asp Lys Asp Asn Val  Tyr Val Ser
    2135                2140                2145

Ser Lys  Ile Pro His Tyr Phe  Ile Asn Ser Asn Ser  Asp Glu Gly
    2150                2155                2160

Leu Met  Lys Ser Val Gly Ile  Thr Gly Gln Gly Leu  Lys Val Leu
    2165                2170                2175

Gln Asp  Ile Arg Val Ser Leu  His Asp Ile Thr Leu  Tyr Asp Glu
    2180                2185                2190

Lys Arg  Asn Arg Phe Thr Pro  Val Ser Leu Lys Ile  Gly Asp Ile
    2195                2200                2205

Tyr Phe  Gln Val Leu His Glu  Thr Pro Arg Gln Tyr  Lys Ile Arg
    2210                2215                2220

Asp Met  Gly Thr Leu Phe Asp  Val Lys Phe Asn Asp  Val Tyr Glu
    2225                2230                2235

Ile Ser  Arg Ile Phe Glu Val  His Val Ser Ser Ile  Thr Gly Val
    2240                2245                2250

Ala Ala  Glu Phe Thr Val Thr  Phe Gln Asp Glu Arg  Arg Leu Ile
    2255                2260                2265

Phe Ser  Ser Pro Lys Tyr Leu  Glu Ile Val Lys Met  Phe Tyr Tyr
    2270                2275                2280

Ala Gln  Ile Arg Leu Glu Ser  Glu Tyr Glu Met Asp  Asn Asn Ser
    2285                2290                2295

Ser Thr  Ser Ser Pro Asn Ser  Asn Asn Lys Asp Lys  Gln Gln Lys
    2300                2305                2310

Glu Arg  Thr Lys Leu Leu Cys  His Leu Leu Leu Val  Ser Leu Ile
    2315                2320                2325
```

-continued

```
Gly Leu  Phe Asp Glu Ser Lys  Lys Met Lys Asn Ser  Ser Tyr Asn
    2330                2335                2340

Leu Ile  Ala Ala Thr Glu Ala  Ser Phe Gly Leu Asn  Phe Gly Ser
    2345                2350                2355

His Phe  His Arg Ser Pro Glu  Val Tyr Val Pro Glu  Asp Thr Thr
    2360                2365                2370

Thr Phe  Leu Gly Val Ile Gly  Lys Ser Leu Ala Glu  Ser Asn Pro
    2375                2380                2385

Glu Leu  Thr Ala Tyr Met Phe  Ile Tyr Val Leu Glu  Ala Leu Lys
    2390                2395                2400

Asn Asn  Val Ile Pro His Val  Tyr Ile Pro His Thr  Ile Cys Gly
    2405                2410                2415

Leu Ser  Tyr Trp Ile Pro Asn  Leu Tyr Gln His Val  Tyr Leu Ala
    2420                2425                2430

Asp Asp  Glu Glu Gly Pro Glu  Asn Ile Ser His Ile  Phe Arg Ile
    2435                2440                2445

Leu Ile  Arg Leu Ser Val Arg  Glu Thr Asp Phe Lys  Ala Val Tyr
    2450                2455                2460

Met Gln  Tyr Val Trp Leu Leu  Leu Leu Asp Asp Gly  Arg Leu Thr
    2465                2470                2475

Asp Ile  Ile Val Asp Glu Val  Ile Asn His Ala Leu  Glu Arg Asp
    2480                2485                2490

Ser Glu  Asn Arg Asp Trp Lys  Lys Thr Ile Ser Leu  Leu Thr Val
    2495                2500                2505

Leu Pro  Thr Thr Glu Val Ala  Asn Asn Ile Ile Gln  Lys Ile Leu
    2510                2515                2520

Ala Lys  Ile Arg Ser Phe Leu  Pro Ser Leu Lys Leu  Glu Ala Met
    2525                2530                2535

Thr Gln  Ser Trp Ser Glu Leu  Thr Ile Leu Val Lys  Ile Ser Ile
    2540                2545                2550

His Val  Phe Phe Glu Thr Ser  Leu Leu Val Gln Met  Tyr Leu Pro
    2555                2560                2565

Glu Ile  Leu Phe Ile Val Ser  Leu Leu Ile Asp Val  Gly Pro Arg
    2570                2575                2580

Glu Leu  Arg Ser Ser Leu His  Gln Leu Leu Met Asn  Val Cys His
    2585                2590                2595

Ser Leu  Ala Ile Asn Ser Ala  Leu Pro Gln Asp His  Arg Asn Asn
    2600                2605                2610

Leu Asp  Glu Ile Ser Asp Ile  Phe Ala His Gln Lys  Val Lys Phe
    2615                2620                2625

Met Phe  Gly Phe Ser Glu Asp  Lys Gly Arg Ile Leu  Gln Ile Phe
    2630                2635                2640

Ser Ala  Ser Ser Phe Ala Ser  Lys Phe Asn Ile Leu  Asp Phe Phe
    2645                2650                2655

Ile Asn  Asn Ile Leu Leu Leu  Met Glu Tyr Ser Ser  Thr Tyr Glu
    2660                2665                2670

Ala Asn  Val Trp Lys Thr Arg  Tyr Lys Lys Tyr Val  Leu Glu Ser
    2675                2680                2685

Val Phe  Thr Ser Asn Ser Phe  Leu Ser Ala Arg Ser  Ile Met Ile
    2690                2695                2700

Val Gly  Ile Met Gly Lys Ser  Tyr Ile Thr Glu Gly  Leu Cys Lys
    2705                2710                2715
```

-continued

```
Ala Met  Leu Ile Glu Thr Met  Lys Val Ile Ala Glu  Pro Lys Ile
    2720                2725                2730

Thr Asp  Glu His Leu Phe Leu  Ala Ile Ser His Ile  Phe Thr Tyr
    2735                2740                2745

Ser Lys  Ile Val Glu Gly Leu  Asp Pro Asn Leu Asp  Leu Met Lys
    2750                2755                2760

His Leu  Phe Trp Phe Ser Thr  Leu Phe Leu Glu Ser  Arg His Pro
    2765                2770                2775

Ile Ile  Phe Glu Gly Ala Leu  Leu Phe Val Ser Asn  Cys Ile Arg
    2780                2785                2790

Arg Leu  Tyr Met Ala Gln Phe  Glu Asn Glu Ser Glu  Thr Ser Leu
    2795                2800                2805

Ile Ser  Thr Leu Leu Lys Gly  Arg Lys Phe Ala His  Thr Phe Leu
    2810                2815                2820

Ser Lys  Ile Glu Asn Leu Ser  Gly Ile Val Trp Asn  Glu Asp Asn
    2825                2830                2835

Phe Thr  His Ile Leu Ile Phe  Ile Ile Asn Lys Gly  Leu Ser Asn
    2840                2845                2850

Pro Phe  Ile Lys Ser Thr Ala  Phe Asp Phe Leu Lys  Met Met Phe
    2855                2860                2865

Arg Asn  Ser Tyr Phe Glu His  Gln Ile Asn Gln Lys  Ser Asp His
    2870                2875                2880

Tyr Leu  Cys Tyr Met Phe Leu  Leu Tyr Phe Val Leu  Asn Cys Asn
    2885                2890                2895

Gln Phe  Glu Glu Leu Leu Gly  Asp Val Asp Phe Glu  Gly Glu Met
    2900                2905                2910

Val Asn  Ile Glu Asn Lys Asn  Thr Ile Pro Lys Ile  Leu Leu Glu
    2915                2920                2925

Trp Leu  Ser Ser Asp Asn Glu  Asn Ala Asn Ile Thr  Leu Tyr Gln
    2930                2935                2940

Gly Ala  Ile Leu Phe Lys Cys  Ser Val Thr Asp Glu  Pro Ser Arg
    2945                2950                2955

Phe Arg  Phe Ala Leu Ile Ile  Arg His Leu Leu Thr  Lys Lys Pro
    2960                2965                2970

Ile Cys  Ala Leu Arg Phe Tyr  Ser Val Ile Arg Asn  Glu Ile Arg
    2975                2980                2985

Lys Ile  Ser Ala Phe Glu Gln  Asn Ser Asp Cys Val  Pro Leu Ala
    2990                2995                3000

Phe Asp  Ile Leu Asn Leu Leu  Val Thr His Ser Glu  Ser Asn Ser
    3005                3010                3015

Leu Glu  Lys Leu His Glu Glu  Ser Ile Glu Arg Leu  Thr Lys Arg
    3020                3025                3030

Gly Leu  Ser Ile Val Thr Ser  Ser Gly Ile Phe Ala  Lys Asn Ser
    3035                3040                3045

Asp Met  Met Ile Pro Leu Asp  Val Lys Pro Glu Asp  Ile Tyr Glu
    3050                3055                3060

Arg Lys  Arg Ile Met Thr Met  Ile Leu Ser Arg Met  Ser Cys Ser
    3065                3070                3075

Ala
```

<210> SEQ ID NO 3
<211> LENGTH: 1278
<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

-continued

<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: GRE3 coding sequence

<400> SEQUENCE: 3

cttctagggg gcctatcaag taaattactc ctggtacact gaagtatata agggatatag 60

aagcaaatag ttgtcagtgc aatccttcaa gacgattggg aaaatactgt aatataaatc 120

gtaaaggaaa attggaaatt ttttaaagat gtcttcactg gttactctta ataacggtct 180

gaaaatgccc ctagtcggct tagggtgctg gaaaattgac aaaaaagtct gtgcgaatca 240

aatttatgaa gctatcaaat taggctaccg tttattcgat ggtgcttgcg actacggcaa 300

cgaaaaggaa gttggtgaag gtatcaggaa agccatctcc gaaggtcttg tttctagaaa 360

ggatatattt gttgtttcaa agttatggaa caattttcac catcctgatc atgtaaaatt 420

agctttaaag aagaccttaa gcgatatggg acttgattat ttagacctgt attatattca 480

cttcccaatc gccttcaaat atgttccatt tgaagagaaa taccctccag gattctatac 540

gggcgcagat gacgagaaga aaggtcacat caccgaagca catgtaccaa tcatagatac 600

gtaccgggct ctggaagaat gtgttgatga aggcttgatt aagtctattg gtgtttccaa 660

ctttcaggga agcttgattc aagatttatt acgtggttgt agaatcaagc ccgtggcttt 720

gcaaattgaa caccatcctt atttgactca agaacaccta gttgagtttt gtaaattaca 780

cgatatccaa gtagttgctt actcctcctt cggtcctcaa tcattcattg agatggactt 840

acagttggca aaaaccacgc caactctgtt cgagaatgat gtaatcaaga aggtctcaca 900

aaaccatcca ggcagtacca cttcccaagt attgcttaga tgggcaactc agagaggcat 960

tgccgtcatt ccaaaatctt ccaagaagga aaggttactt ggcaacctag aaatcgaaaa 1020

aaagttcact ttaacggagc aagaattgaa ggatatttct gcactaaatg ccaacatcag 1080

atttaatgat ccatggacct ggttggatgg taaattcccc acttttgcct gatccagcca 1140

gtaaaatcca tactcaacga cgatatgaac aaatttccct cattccgatg ctgtatatgt 1200

gtataaattt ttacatgctc ttctgtttag acacagaaca gctttaaata aaatgttgga 1260

tatacttttt ctgcctgt 1278

<210> SEQ ID NO 4
<211> LENGTH: 327
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 4

Met Ser Ser Leu Val Thr Leu Asn Asn Gly Leu Lys Met Pro Leu Val
1 5 10 15

Gly Leu Gly Cys Trp Lys Ile Asp Lys Lys Val Cys Ala Asn Gln Ile
20 25 30

Tyr Glu Ala Ile Lys Leu Gly Tyr Arg Leu Phe Asp Gly Ala Cys Asp
35 40 45

Tyr Gly Asn Glu Lys Glu Val Gly Glu 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

-continued

```
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 Lys Tyr Pro Pro Gly
        115                 120                 125

Phe Tyr Thr Gly Ala Asp Asp Glu Lys 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 Asn 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 Gln 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 Lys Glu Arg Leu Leu Gly Asn Leu Glu
        275                 280                 285

Ile Glu Lys Lys Phe Thr Leu Thr Glu Gln Glu Leu Lys Asp Ile Ser
    290                 295                 300

Ala Leu Asn Ala Asn Ile Arg Phe Asn Asp Pro Trp Thr Trp 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

```
tcttaaatcc ataagaacat cccttcatat aacaattgaa taaggaaaac acaacacata     60

acacatattt aacctgatgc ttcctgttat aacgagattt gcaaggcctg ctctgatggc    120

catcagacct gtgaatgcca tgggggtttt gagagcgtcc agcataacga aaaggcttta    180

tcatcccaag gtcatagagc attatacaca tccaagaaac gtcggctcat tagataaaaa    240

attgcccaac gtcggcactg gtctagtggg tgcgccagcg tgcggtgatg tgatgaggtt    300

gcagatcaaa gtcaacgact ctactggcgt tattgaagat gtcaaattca aaacttttgg    360

atgtggctcc gccattgcct cctcttcata tatgactgaa ttggtacagg ggatgacctt    420

ggacgatgcg gcaaaaatta agaacactga aattgctaag gagttgagct tgcccccagt    480

caagttgcat tgctctatgt tagcggaaga tgcgatcaag gcagctatta aggactacaa    540

atctaagaga aacactccaa ccatgttatc gtaatgaata agaagataac cgggacaaga    600

acaagatcaa accctcacta atcaacaagt tggacttaat ttgtgcaa                 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 Asn
35 40 45

Val Gly Ser Leu Asp Lys Lys Leu Pro Asn Val Gly Thr Gly Leu Val
50 55 60

Gly Ala Pro Ala Cys Gly Asp Val Met Arg Leu Gln Ile Lys Val Asn
65 70 75 80

Asp Ser Thr Gly Val Ile Glu Asp Val Lys Phe Lys Thr Phe Gly Cys
85 90 95

Gly Ser Ala Ile Ala Ser Ser Ser Tyr Met Thr Glu Leu Val Gln Gly
100 105 110

Met Thr Leu Asp Asp Ala Ala Lys Ile Lys Asn 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 Lys Asp Tyr Lys Ser Lys Arg Asn 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

atgctcctat ttccaagaag aactaatata gccttttttca aaacaacagg cattttgct 60

aattttcctt tgctaggtag aaccattaca acttcaccat ctttccttac acataaactg 120

tcaaaggaag taaccagggc atcaacttcg cctccaagac caaagagaat tgttgtcgca 180

attactggtg cgactggtgt tgcactggga atcagacttc tacaagtgct aaaagagttg 240

agcgtagaaa cccatttggt gatttcaaaa tggggtgcag caacaatgaa atatgaaaca 300

gattgggaac cgcatgacgt ggcggccttg gcaaccaaga catactctgt tcgtgatgtt 360

tctgcatgca tttcgtccgg atctttccag catgatggta tgattgttgt gccctgttcc 420

atgaaatcac tagctgctat tagaatcggt tttacagagg atttaattac aagagctgcc 480

gatgtttcga ttaaagagaa tcgtaagtta ctactggtta ctcgggaaac ccctttatct 540

tccatccatc ttgaaaacat gttgtcttta tgcagggcag gtgttataat ttttcctccg 600

gtacctgcgt tttatacaag acccaagagc cttcatgacc tattagaaca aagtgttggc 660

aggatcctag actgctttgg catccacgct gacacttttc ctcgttggga aggaataaaa 720

agcaagtaa 729

<210> SEQ ID NO 8
<211> LENGTH: 242
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

-continued

<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 Leu 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 Arg Pro Lys Arg Ile Val Val Ala Ile Thr Gly Ala
50 55 60

Thr Gly Val Ala Leu Gly Ile Arg Leu 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 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 Gln 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 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 taaatccagc tttagaattt agagacttta tccaggtctt aaaagatgaa 60

gatgacttaa tcgaaattac cgaagagatt gatccaaatc tcgaagtagg tgcaattatg 120

aggaaggcct atgaatccca cttaccagcc ccgttattta aaaatctcaa aggtgcttcg 180

aaggatcttt tcagcatttt aggttgccca gccggtttga gaagtaagga gaaaggagat 240

catggtagaa ttgcccatca tctggggctc gacccaaaaa caactatcaa ggaaatcata 300

gattatttgc tggagtgtaa ggagaaggaa cctctccccc caatcactgt tcctgtgtca 360

tctgcacctt gtaaaacaca tatactttct gaagaaaaaa tacatctaca aagcctgcca 420

acaccatatc tacatgtttc agacggtggc aagtacttac aaacgtacgg aatgtggatt 480

cttcaaactc cagataaaaa atggactaat tggtcaattg ctagaggtat ggttgtagat 540

gacaagcata tcactggtct ggtaattaaa ccacaacata ttagacaaat tgctgactct 600

tgggcagcaa ttggaaaagc aaatgaaatt cctttcgcgt tatgttttgg cgttccccca 660

-continued

gcagctattt tagttagttc catgccaatt cctgaaggtg tttctgaatc ggattatgtt 720

ggcgcaatct tgggtgagtc ggttccagta gtaaaatgtg agaccaacga tttaatggtt 780

cctgcaacga gtgagatggt atttgagggt actttgtcct taacagatac acatctggaa 840

ggcccatttg gtgagatgca tggatatgtt ttcaaaagcc aaggtcatcc ttgtccattg 900

tacactgtca aggctatgag ttacagagac aatgctattc tacctgtttc gaaccccggt 960

ctttgtacgg atgagacaca taccttgatt ggttcactag tggctactga ggccaaggag 1020

ctggctattg aatctggctt gccaattctg gatgccttta tgccttatga ggctcaggct 1080

ctttggctta tcttaaaggt ggatttgaaa gggctgcaag cattgaagac aacgcctgaa 1140

gaattttgta agaaggtagg tgatatttac tttaggacaa aagttggttt tatagtccat 1200

gaaataattt tggtggcaga tgatatcgac atatttaact tcaaagaagt catctgggcc 1260

tacgttacaa gacatacacc tgttgcagat cagatggctt ttgatgatgt cacttctttt 1320

cctttggctc cctttgtttc gcagtcatcc agaagtaaga ctatgaaagg tggaaagtgc 1380

gttactaatt gcatatttag acagcaatat gagcgcagtt ttgactacat aacttgtaat 1440

tttgaaaagg gatatccaaa aggattagtt gacaaagtaa atgaaaattg gaaaaggtac 1500

ggatataaat aa 1512

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

<400> SEQUENCE: 10

Met Arg Lys Leu Asn 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

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

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

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

His Val Ser Asp Gly Gly Lys Tyr Leu Gln 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 Ile 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

-continued

```
Val Ser Ser Met Pro Ile Pro Glu Gly Val Ser Glu Ser Asp Tyr Val
225                 230                 235                 240

Gly Ala Ile Leu Gly Glu Ser Val Pro Val Val Lys Cys Glu Thr Asn
                245                 250                 255

Asp Leu Met Val Pro Ala Thr Ser Glu Met Val Phe Glu Gly Thr Leu
            260                 265                 270

Ser Leu Thr Asp Thr His Leu Glu Gly Pro Phe Gly Glu Met His Gly
        275                 280                 285

Tyr Val Phe Lys Ser Gln Gly His Pro Cys Pro Leu Tyr Thr Val Lys
    290                 295                 300

Ala Met Ser Tyr Arg Asp Asn Ala Ile Leu Pro Val Ser Asn Pro Gly
305                 310                 315                 320

Leu Cys Thr Asp Glu Thr His Thr Leu Ile Gly Ser Leu Val Ala Thr
                325                 330                 335

Glu Ala Lys Glu Leu Ala Ile Glu Ser Gly Leu Pro Ile Leu Asp Ala
            340                 345                 350

Phe Met Pro Tyr Glu Ala Gln Ala Leu Trp Leu Ile Leu Lys Val Asp
        355                 360                 365

Leu Lys Gly Leu Gln Ala Leu Lys Thr Thr Pro Glu Glu Phe Cys Lys
    370                 375                 380

Lys Val Gly Asp Ile Tyr Phe Arg Thr Lys Val Gly Phe Ile Val His
385                 390                 395                 400

Glu Ile Ile Leu Val Ala Asp Asp Ile Asp Ile Phe Asn Phe Lys Glu
                405                 410                 415

Val Ile Trp Ala Tyr Val Thr Arg His Thr Pro Val Ala Asp Gln Met
            420                 425                 430

Ala Phe Asp Asp Val Thr Ser Phe Pro Leu Ala Pro Phe Val Ser Gln
        435                 440                 445

Ser Ser Arg Ser Lys Thr Met Lys Gly Gly Lys Cys Val Thr Asn Cys
    450                 455                 460

Ile Phe Arg Gln Gln Tyr Glu Arg Ser Phe Asp Tyr Ile Thr Cys Asn
465                 470                 475                 480

Phe Glu Lys Gly Tyr Pro Lys Gly Leu Val Asp Lys Val Asn Glu Asn
                485                 490                 495

Trp Lys Arg Tyr Gly Tyr Lys
            500
```

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 hydrosylate 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 hydrosylate to ethanol is increased relative to the fermentation rate of a GVL-treated hydrosylate 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.

* * * * *