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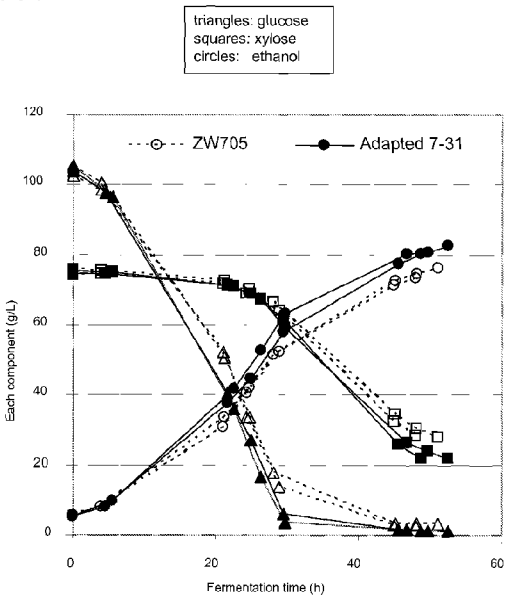
(54) Title: XYLOSE UTILIZING ZYMOMONAS MOBILIS WITH IMPROVED ETHANOL PRODUCTION IN BIOMASS HYDROLYSATE MEDIUM

(57) Abstract: Xylose-utilizing, ethanol producing strains of *Zymomonas mobilis* with improved performance in medium comprising biomass hydrolysate were isolated using an adaptation process. Independently isolated strains were found to have independent mutations in the same coding region. Mutation in this coding may be engineered to confer the improved phenotype.

Figure 1



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— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*

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TITLE

XYLOSE UTILIZING *ZYMOMONAS MOBILIS* WITH IMPROVED ETHANOL PRODUCTION IN BIOMASS HYDROLYSATE MEDIUM

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with United States Government support under Award No. DE-FC36-07GO17056 awarded by the Department of Energy. The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to the fields of microbiology and fermentation. More specifically, mutant *Zymomonas* strains with improved growth and ethanol production in biomass hydrolysate medium were isolated and characterized.

BACKGROUND OF THE INVENTION

Production of ethanol by microorganisms provides an alternative energy source to fossil fuels and is therefore an important area of current research. It is desirable that microorganisms producing ethanol, as well as other useful products, be capable of growing and producing ethanol in a medium that does not impact the human food supply, such as avoiding use of sugars produced from corn grain. As a result of developments in cellulosic biomass processing, glucose, xylose, and other sugars may be released in high concentrations in a biomass hydrolysate for use in fermentation. As such, conversion of biomass to ethanol poses great possibility for improving environmental impacts by using renewable non-food resources to provide an alternative to fossil fuels.

Zymomonas mobilis and other bacterial ethanologens which do not naturally utilize xylose have been genetically engineered for xylose utilization to improve growth and ethanol production by using more of the sugars in biomass hydrolysate. However, growth and ethanol production in biomass-hydrolysate containing medium is typically not optimal due to the presence of acetate and other compounds that are inhibitory to microorganisms. Disclosed in commonly owned and co-pending United States Patent Publication US20110014670A1 is a method for producing an improved xylose-utilizing *Zymomonas* strain that is more tolerant to acetate and ethanol in the medium, as well as strains isolated by the method.

The toxic effect of single compounds likely to be found in the hydrolysates of pretreated biomass is described in Delegenes et al. ((1996) Enzymes and Microbial Technology 19:220-224). Adaptation of xylose-fermenting *Zymomonas mobilis* to conditioned dilute acid yellow poplar hemicellulose hydrolysate is described in Lawford et al. ((1999), Applied Biochemistry and Biotechnology 77:191-204).

There remains a need for isolated xylose-utilizing *Zymomonas* ethanologen strains with improved ethanol production during fermentation in biomass hydrolysate medium, and methods for genetic engineering to produce improved strains.

SUMMARY OF THE INVENTION

The invention provides recombinant xylose-utilizing *Zymomonas* strains with improved growth and ethanol production in biomass hydrolysate medium. In addition, the invention provides methods of making improved *Zymomonas* strains for use in hydrolysate medium and methods of making ethanol using said strains.

Accordingly, the invention provides a recombinant, xylose-utilizing, ethanol-producing microorganism of the genus *Zymomonas*, having at least one genetic modification in the zmo1432 open reading frame.

In one aspect the invention provides a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, and polynucleotides encoding the same.

In another aspect the invention provides a method for the production of a recombinant *Zymonomas* ethanologen comprising:

a) providing a xylose-utilizing, ethanol-producing microorganism of the genus *Zymomonas*;

b) providing a polynucleotide encoding a protein having the amino acid sequence selected from the group consisting of: SEQ ID NO:3 and SEQ ID NO:4; and

c) introducing the polynucleotide of b) into the microorganism of a); wherein the endogenous zmo1432 coding region is disrupted.

In an alternate aspect the invention provides a method for the production of a recombinant *Zymonomas* ethanologen comprising:

a) providing a xylose-utilizing, ethanol-producing microorganism of the genus *Zymomonas* comprising a zmo1432 open reading frame encoding a polypeptide having the amino acid sequence as set forth in SEQ ID NO:

2; and

b) introducing a mutation in the zmo1432 open reading frame of a) such that expression of the mutated open reading frame expresses a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:4.

In another aspect the invention provides a method for the production of ethanol comprising:

a) providing the recombinant *Zymomonas* of the invention;

b) providing a biomass hydrolysate medium comprising xylose; and

c) growing the *Zymomonas* of a) in the biomass hydrolysate medium of b) wherein ethanol is produced.

BRIEF DESCRIPTION OF THE FIGURES, BIOLOGICAL DEPOSITS AND SEQUENCE DESCRIPTIONS

Applicants have made the following biological deposits under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure:

INFORMATION ON DEPOSITED STRAINS

Depositor Identification Reference	International Depository Designation	Date of Deposit
Zymomonas ZW658	ATCC No PTA-7858	Sept. 12, 2006

Figure 1 shows a graph of fermentation of corn cob hydrolysate over time, comparing glucose and xylose utilization and ethanol production for Zymomonas strains and Adapted 7-31.

Figure 2 shows a graph of fermentation of corn cob hydrolysate over time, comparing glucose and xylose utilization and ethanol production for Zymomonas strains ZW705, Adapted 7-31, and Adapted 5-6.

Figure 3 shows an alignment of the protein encoded by zmo1432 and the Bcemn03_1426 protein of *Burkholderia cenocepacia*.

Figure 4 shows an alignment of the protein encoded by zmo1432 and the *E. coli* AaeB protein.

The following sequences conform with 37 C.F.R. §§ 1.821-1.825

("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (2009) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. § 1.822.

SEQ ID NO:1 is the nucleotide sequence of the coding region designated zmo1432 in the published *Zymomonas mobilis* genomic sequence (NCBI Reference: NC_006526.2).

SEQ ID NO:2 is the amino acid sequence encoded by the coding region designated zmo1432 in the published *Zymomonas mobilis* genomic sequence (NCBI Reference: NC_006526.2).

SEQ ID NO:3 is the amino acid sequence of SEQ ID NO:2 but with the amino acid at position No. 366 changed to arginine.

SEQ ID NO:4 is the amino acid sequence of SEQ ID NO:2 but with the amino acid at position No. 117 changed to phenylalanine.

SEQ ID NO:5 is the amino acid sequence of the *Burkholderia cenocepacia* protein encoded by fusC (Accession P24128).

SEQ ID NO:6 is the amino acid sequence of the *Klebsiella oxytoca* fusaric acid detoxification protein (Accession: Q48403)

SEQ ID NO:7 is the amino acid sequence of the *Burkholderia cenocepacia* protein encoded by Bcenmc03_1426. SEQ ID NO:8 is the amino acid sequence of the *E. coli* protein AaeB.

SEQ ID NO:9 is the amino acid sequence of the immature Xyn3 which incorporates a predicted signal sequence corresponding to positions 1 to 16.

SEQ ID NO:10 is the amino acid sequence of the immature Fv3A which incorporates a predicted signal sequence corresponding to positions 1 to 23.

SEQ ID NO: 11 is the amino acid sequence of the immature Fv43D, which incorporates a predicted signal sequence corresponding to positions 1 to 20.

SEQ ID NO:12 is the amino acid sequence of the immature Fv51A which incorporates a predicted signal sequence corresponding to

positions 1 to 19.

DETAILED DESCRIPTION

The present invention describes adaptation of xylose-utilizing *Zymomonas* cells in biomass hydrolysate medium, to improve growth and ethanol production. Characterization of mutations in adapted strains identified mutations characteristic for the improvement, which may be engineered in non-adapted xylose-utilizing *Zymomonas* strains. These strains are used to make ethanol more efficiently, to produce ethanol as a fossil fuel replacement.

The following abbreviations and definitions will be used for the interpretation of the specification and the claims.

As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains" or "containing," or any other variation thereof, are intended to cover a non-exclusive inclusion. For example, a composition, a mixture, process, method, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

Also, the indefinite articles "a" and "an" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances (i.e. occurrences) of the element or component. Therefore "a" or "an" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

As used herein "xylose-utilizing *Zymomonas* cell(s)" refers to a cell or cells of a strain that are genetically engineered to express enzymes conferring the ability to use xylose as a carbohydrate source for fermentation.

The term "adapted strain" refers to a microorganism that has been selected for growth under particular conditions in order to improve it's ability to grow and produce a product in those conditions.

As used herein "corresponding non-adapted strain" refers to the

original xylose-utilizing *Zymomonas* strain that is a strain from which improved strains are produced using the biomass hydrolysate adaptation process disclosed herein.

As used herein "feeding growth medium" refers to the medium that is added into the continuous culture vessel.

The term "lignocellulosic biomass" or "biomass" refers to any lignocellulosic material and includes materials comprising cellulose, hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Biomass may also comprise additional components, such as protein and/or lipid. Biomass may be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example, biomass could comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Lignocellulosic biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat straw, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum plant material, soybean plant material, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, and flowers.

As used herein "biomass hydrolysate" and "cellulosic hydrolysate" refer to a product produced from biomass, which is cellulosic material, typically through pretreatment and saccharification processes. Fermentable sugars are present in the hydrolysate, as well as other products.

As used herein "biomass hydrolysate medium" refers to medium which contains at least about 50% hydrolysate prepared from cellulosic and/or lignocellulosic biomass. Hydrolysate is prepared by saccharification of biomass, typically preceeded by pretreatment. In addition to the hydrolysate, the medium may include defined components for biocatalyst growth and production.

"Xyn3" is a GH10 family xylanase from *Trichoderma reesei*. Xyn3 (SEQ ID NO:9) was shown to have endoxylanase activity indirectly by observation of its abaility to catalyze increased xylose monomer production in the presence of xylobiosidase when the enzymes act on

pretreated biomass or on isolated hemicellulose. .

'Fv3A" is a GH3 family enzyme from *Fusarium verticillioides*. Fv3A (SEQ ID NO:10) was shown to have β-xylosidase activity, for example, in an enzymatic assay using *p*-nitrophenyl-β-xylopyranoside, xylobiose, mixed linear xylo-oligomers, branched arabinoxylan oligomers from hemicellulose, or dilute ammonia pretreated corncob as substrates..

"Fv43D" is a GH43 family enzyme from *Fusarium verticillioides*. Fv43D (SEQ ID NO:11) was shown to have β-xylosidase activity in, for example, an enzymatic assay using *p*-nitophenyl-β-xylopyranoside, xylobiose, and/or mixed, linear xylo-oligomers as substrates.

"Fv51A" is a GH51 family enzyme from *Fusarium verticillioides*. Fv51A (SEQ ID NO:12) was shown to have L-α-arabinofuranosidase activity in, for example, an enzymatic assay using 4-nitrophenyl-α-L-arabinofuranoside as a substrate. .

"Gene" refers to a nucleic acid fragment that expresses a specific protein or functional RNA molecule, which may optionally include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" or "wild type gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes.

The term "genetic construct" refers to a nucleic acid fragment that encodes for expression of one or more specific proteins or functional RNA molecules. In the gene construct the gene may be native, chimeric, or foreign in nature. Typically a genetic construct will comprise a "coding sequence". A "coding sequence" refers to a DNA sequence that encodes a specific amino acid sequence.

"Promoter" or "Initiation control regions" refers to a DNA sequence

capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters".

The term "expression", as used herein, refers to the transcription and stable accumulation of coding (mRNA) or functional RNA derived from a gene. Expression may also refer to translation of mRNA into a polypeptide. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

The term "transformation" as used herein, refers to the transfer of a nucleic acid fragment into a host organism, resulting in genetically stable inheritance. The transferred nucleic acid may be in the form of a plasmid maintained in the host cell, or some transferred nucleic acid may be integrated into the genome of the host cell. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid" and "vector" as used herein, refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence

(i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "selectable marker" means an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, i.e., resistance to an antibiotic, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid of interest.

The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: 1.) Computational Molecular Biology (Lesk, A. M., Ed.) Oxford University: NY (1988); 2.) Biocomputing: Informatics and Genome Projects (Smith, D. W., Ed.) Academic: NY (1993); 3.) Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., Eds.) Humania: NJ (1994); 4.) Sequence Analysis in Molecular Biology (von Heinje, G., Ed.) Academic (1987); and 5.) Sequence Analysis Primer (Gribskov, M. and Devereux, J., Eds.) Stockton: NY (1991).

Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences is performed using the "Clustal method of alignment" which encompasses several varieties of the algorithm including the "Clustal V method of alignment" corresponding to the alignment method labeled Clustal V (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.,* 8:189-191 (1992)) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are

KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program. Additionally the "Clustal W method of alignment" is available and corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.* 8:189-191(1992)) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). Default parameters for multiple alignment (GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB). After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

Producing *Zymomonas* Strains With Improved Fermentation In Hydrolysate Medium

The invention provides a recombinant, xylose-utilizing, ethanol-producing microorganism of the genus *Zymomonas*, having at least one genetic modification in the zmo1432 open reading frame. The effect of this mutation is to express a polypeptide that improves the behavior of the strain in a hydrolysate medium, increasing the strain's tolerance to various growth inhibitors in the hydrolysate and increasing the yield of ethanol. The improvements in fermentation behavior have been linked to mutations in the zmo 1432 region of the Zymomonas genome, (NCBI Reference: NC_006526.2), defined herein as SEQ ID NO:1, encoding the polypetptide of SEQ ID NO:2.

Accordingly it is put forth here that improved fermentation in hydrolysate medium may be conferred to *Zymomonas* strains that are able to utilize xylose as a carbon source and that produce ethanol by introducing at least one genetic modification in an open reading frame (ORF) that encodes a protein having at least about 95% amino acid identity to SEQ ID NO:2, prior to modification. The protein may have at least about 95%, 96%, 96%, 98%, 99%, or 100% identity to SEQ ID NO:2. Any genetic modification in said protein may be made which increases ethanol production by the strain harboring said mutant protein in the presence of hydrolysate medium. Increase in ethanol production is determined by comparison to production by a *Zymomonas* strain lacking the genetic

modification, under the same conditions of fermentation. A strain with a genetic modification in said ORF may be readily assayed by one of skill in the art to assess increased ethanol production in the presence of biomass hydrolysate by methods such as described in Example 3 herein. Said improved strain has higher tolerance to biomass hydrolysate, and inhibitors present in biomass hydrolysate, where tolerance refers to the ability of the strain to grow and produce ethanol similarly in media with a specified level of hydrolysate (level of tolerance) as compared to in media with less or no hydrolysate. Higher tolerance is determined by comparison with a *Zymomonas* strain lacking the genetic modification.

In one embodiment the genetic modification results in an alteration at position 366 of the amino acid sequence of SEQ ID NO:2 that substitutes arginine for threonine. In another embodiment the genetic modification results in an alteration at position 117 of the amino acid sequence of SEQ ID NO:2 that substitutes phenylalanine for serine. Any change may be made in the nucleotide sequence which results in the change of codon 366 to encode arginine, or results in the change of codon 117 to encode phenylalanine. Codons encoding arginine are CGT, CGC, CGA, CGG, AGA, and AGG. Codons encoding phenylalnine are TTT and TTC.

In one embodiment the genetic modification is in a coding region that has at least 95% sequence identity to the ORF zmo1432 as named in the published *Zymomonas mobilis* genomic sequence (NCBI Reference: NC_006526.2) which is the complement of nucleotides 1446603 to 1448633 having SEQ ID NO:1. The ORF of SEQ ID NO:1 may be called alternative names, but in any case it may be identified as zmo1432 by comparison to the sequence of SEQ ID NO:1. There may be some variation in the sequence of coding regions identified as zmo1432 among *Zymomonas* species or strains. Thus a coding region identified as zmo1432 may have at least about 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:1 and the coding region with the genetic modification may have at least about 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:1 prior to genetic modification. This coding region, prior to modification, is the target for genetic modification.

At least one of the described genetic modifications may be created in a *Zymomonas* strain that is able to utilize xylose as a carbon source and that produces ethanol by any method known to one of skill in the art. Such methods include by adaptation as described below and in Examples 1 and 2 herein, by chemical mutagenesis and screening, and by genetic engineering.

Genetic engineering to introduce a genetic modification in the target coding region may be by methods including using double-crossover homologous recombination to replace the endogenous target coding region with the same coding region harboring a mutation that is described above. In homologous recombination, DNA sequences flanking the target integration site are placed bounding a spectinomycin-resistance gene or other selectable marker, and the replacement mutant sequence leading to insertion of the selectable marker and the replacement mutant sequence into the target genomic site. The selectable marker is outside of the coding region so that in the product, the coding region is expressed. In addition, the selectable marker may be bounded by site-specific recombination sites, so that after expression of the corresponding site-specific recombinase, the resistance gene is excised from the genome. Particularly suitable for integration of a replacement mutant sequence is transposition using EPICENTRE®'s EZ::Tn in vitro transposition system, which is used in Examples 1 and 6 of United States Patent Publication US-2009-0246846-A1.

Alternatively, expression of the target endogenous coding region may be disrupted in a cell by a manipulation such as insertion, mutation, or deletion and a gene expressing the coding region harboring a mutation, as described above, may be introduced into the cell. An example of double-crossover, homologous recombination in *Zymomonas* used to inactivate a coding region is described in United States Patent U.S. 7,741,119. The introduced gene may be the endogenous gene (with mutant coding region) including its native promoter, or the introduced gene may be a chimeric gene comprising an operably linked promoter and a coding region with at least about 95%, 96%, 97%, 98%, or 99% identity to the disrupted coding region. Typically a 3' termination region is included in a chimeric gene. Promoters that may be used in *Zymomonas* include *Zm*P*gap* and the promoter of the *Zymomonas* enolase gene.

The introduced gene may be maintained on a plasmid, or integrated into the genome using, for example, homologous recombination, site-directed integration, or random integration. A gene to be introduced is typically constructed in or transferred to a vector for further manipulations. Vectors are well known in the art. Particularly useful for expression in *Zymomonas* are vectors that can replicate in both *E. coli* and *Zymomonas*, such as pZB188 which is described in United States Patent U.S. 5,514,583. Vectors may include plasmids for autonomous replication in a cell, and plasmids for carrying constructs to be integrated into bacterial genomes. Plasmids for DNA integration

may include transposons, regions of nucleic acid sequence homologous to the target bacterial genome, or other sequences supporting integration. An additional type of vector may be a transposome produced using, for example, a system that is commercially available from EPICENTRE®. It is well known how to choose an appropriate vector for the desired target host and the desired function.

Host Strain

Any strain of *Zymomonas* that is able to utilize xylose as a carbon source and that produces ethanol may be a starting strain for the present invention. Such strains are used for adaption in hydrolysate medium, for chemical mutagenesis and screening, or are genetically engineered to produce the improved strains disclosed herein. Strains of *Zymomonas,* such as *Z. mobilis,* that have been engineered to express a xylose to ethanol fermentation pathway are particularly useful. Endogenous genes may provide part of the metabolic pathway, or may be altered by any known genetic manipulation technique to provide a protein with enzyme activity useful for xylose metabolism. For example, the endogenous transketolase may complement other introduced enzyme activities in creating a xylose utilization pathway. Typically four genes may be introduced into a *Zymomonas* strain, such as *Z mobilis,* for expression of four enzymes involved in xylose metabolism as described in United States Patent, U.S. 5,514,583, which is herein incorporated by reference. These include genes encoding xylose isomerase, which catalyzes the conversion of xylose to xylulose and xylulokinase, which phosphorylates xylulose to form xylulose 5-phosphate. In addition, transketolase and transaldolase, two enzymes of the pentose phosphate pathway, convert xylulose 5-phosphate to intermediates that couple pentose metabolism to the glycolytic Entner-Douderoff pathway permitting the metabolism of xylose to ethanol. DNA sequences encoding these enzymes may be obtained from any of numerous microorganisms that are able to metabolize xylose, such as enteric bacteria, and some yeasts and fungi. Sources for the coding regions include *Xanthomonas, Klebsiella, Escherichia, Rhodobacter, Flavobacterium, Acetobacter, Gluconobacter, Rhizobium, Agrobacterium, Salmonella, Pseudomonads*, and *Zymomonas*. Particularly useful are the coding regions of *E. coli.*

The encoding DNA sequences are operably linked to promoters that are expressed in *Z. mobilis* cells such as the promoters of *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase (GAP promoter), and *Z. mobilis* enolase (ENO promoter). The coding regions may individually be expressed from promoters, or

two or more coding regions may be joined in an operon with expression from the same promoter. The resulting chimeric genes may be introduced into *Zymomonas* and maintained on a plasmid, or integrated into the genome using, for example, homologous recombination, site-directed integration, or random integration. Xylose-utilizing strains that are of particular use include ZM4(pZB5) (described in United States Patents, U,S 5,514,583, U.S. 6,566,107, and U.S. 5,571,2133, and incorporated by reference herein), 8b (United States Patent Application U.S. 2003/0162271; Mohagheghi et al., (2004) Biotechnol. Lett. 25; 321-325), as well as ZW658 (ATCC PTA-7858), ZW800, ZW801-4, ZW801-5, and ZW801-6 (described in commonly owned and co-pending United States Patent Application Publication U.S. 2008-0286870 A1, which is herein incorporated by reference).

Zymomonas strains that are additionally engineered to utilize other sugars that are not natural substrates, may also be used in the present process. An example is a strain of *Z. mobilis* engineered for arabinose utilization as described in United States Patent U.S. 5,843,760, which is herein incorporated by reference.

Adaptation

For adaptation, a xylose-utilizing strain of *Zymomonas* (a starting strain as described above) is continuously grown in a medium containing biomass hydrolysate. Biomass hydrolysate is produced by saccharification of biomass. Typically the biomass is pretreated prior to saccharification. Biomass may be treated by any method known by one skilled in the art to produce fermentable sugars in a hydrolysate. Typically the biomass is pretreated using physical and/or chemical treatments, and saccharified enzymatically. Physical and chemical treatments may include grinding, milling, cutting, base treatment such as with ammonia or NaOH, and acid treatment. Particularly useful is a low ammonia pretreatment where biomass is contacted with an aqueous solution comprising ammonia to form a biomass-aqueous ammonia mixture where the ammonia concentration is sufficient to maintain an alkaline pH of the biomass-aqueous ammonia mixture but is less than about 12 wt.% relative to dry weight of biomass, and where dry weight of biomass is at least about 15 wt% solids relative to the weight of the biomass-aqueous ammonia mixture, as disclosed in co-pending and commonly owned United States Patent Application Publication US-20070031918-A1, which is herein incorporated by reference.

Enzymatic saccharification typically makes use of an enzyme composition or blend to break down cellulose and/or hemicellulose and to produce a hydrolysate containing sugars such as, for example, glucose, xylose, and arabinose. Saccharification enzymes are reviewed in Lynd, L. R., *et al.* (Microbiol. Mol. Biol. Rev., 66:506-577, 2002). At least one enzyme is used, and typically a saccharification enzyme blend is used that includes one or more glycosidases. Glycosidases hydrolyze the ether linkages of di-, oligo-, and polysaccharides and are found in the enzyme classification EC 3.2.1.x (Enzyme Nomenclature 1992, Academic Press, San Diego, CA with Supplement 1 (1993), Supplement 2 (1994), Supplement 3 (1995, Supplement 4 (1997) and Supplement 5 [in Eur. J. Biochem., 223:1-5, 1994; Eur. J. Biochem., 232:1-6, 1995; Eur. J. Biochem., 237:1-5, 1996; Eur. J. Biochem., 250:1-6, 1997; and Eur. J. Biochem., 264:610-650 1999, respectively]) of the general group "hydrolases" (EC 3.). Glycosidases useful in the present method can be categorized by the biomass components they hydrolyze. Glycosidases useful for the present method include cellulose-hydrolyzing glycosidases (for example, cellulases, endoglucanases, exoglucanases, cellobiohydrolases, β-glucosidases), hemicellulose-hydrolyzing glycosidases (for example, xylanases, endoxylanases, exoxylanases, β-xylosidases, arabino-xylanases, mannases, galactases, pectinases, glucuronidases), and starch-hydrolyzing glycosidases (for example, amylases, α-amylases, β-amylases, glucoamylases, α-glucosidases, isoamylases). In addition, it may be useful to add other activities to the saccharification enzyme consortium such as peptidases (EC 3.4.x.y), lipases (EC 3.1.1.x and 3.1.4.x), ligninases (EC 1.11.1.x), or feruloyl esterases (EC 3.1.1.73) to promote the release of polysaccharides from other components of the biomass. It is known in the art that microorganisms that produce polysaccharide-hydrolyzing enzymes often exhibit an activity, such as a capacity to degrade cellulose, which is catalyzed by several enzymes or a group of enzymes having different substrate specificities. Thus, a "cellulase" from a microorganism may comprise a group of enzymes, one or more or all of which may contribute to the cellulose-degrading activity. Commercial or non-commercial enzyme preparations, such as cellulase, may comprise numerous enzymes depending on the purification scheme utilized to obtain the enzyme.

Saccharification enzymes may be obtained commercially. Such enzymes include, for example, Spezyme® CP cellulase, Multifect® xylanase, Accelerase® 1500, and Accellerase® DUET (Danisco U.S. Inc., Genencor International,

Rochester, NY). In addition, saccharification enzymes may be unpurified and provided as a cell extract or a whole cell preparation. The enzymes may be produced using recombinant microorganisms that have been engineered to express one or more saccharifying enzymes.

Additional enzymes for saccharification include, for example, glycosyl hydrolases such as members of families GH3, GH39, GH43, GH55, GH10, and GH11. GHs are a group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a noncarbohydrate moiety. Families of GHs have been classified based on sequence similarity and the classification is available in the Carbohydrate-Active enzyme (CAZy) database (Cantarel et al. (2009) Nucleic Acids Res. 37 (Database issue):D233-238). Certain of these enzymes are able to act on various substrates and have demonstrated effecacy as saccharification enzymes. Glycoside hydrolase family 3 ("GH3") enzymes have a number of known activities, including, for example, β-glucosidase (EC:3.2.1.21); β-xylosidase (EC:3.2.1.37); N-acetyl β-glucosaminidase (EC:3.2.1.52); glucan β-1,3-glucosidase (EC:3.2.1.58); cellodextrinase (EC:3.2.1.74); exo-1,3-1,4-glucanase (EC:3.2.1); and/or β-galactosidase (EC 3.2.1.23) activities. Glycoside hydrolase family 39 ("GH39") enzymes also have a number of known activities, including, for example, α-L-iduronidase (EC:3.2.1.76) and/or β-xylosidase (EC:3.2.1.37) activities. Glycoside hydrolase family 43 ("GH43") enzymes have a number of known activities including, for example, L-α-arabinofuranosidase (EC 3.2.1.55); β-xylosidase (EC 3.2.1.37); endoarabinanase (EC 3.2.1.99); and/or galactan 1,3-β-galactosidase (EC 3.2.1.145) activities. Glycoside hydrolase family 51 ("GH51") enzymes are known to have, for example, L-α-arabinofuranosidase (EC 3.2.1.55) and/or endoglucanase (EC 3.2.1.4) activities. Glycoside hydrolase family 10 ("GH10") have beendescribed in detail in Schmidt *et al.*, 1999, Biochemistry 38:2403-2412 and Lo Leggio *et al.*, 2001, FEBS Lett 509: 303-308) and the Glycoside hydrolase family 11 ("GH11") have been described in Hakouvainen *et al.*, 1996, Biochemistry 35:9617-24.

In the present adaptation process, xylose-utilizing *Zymomonas* is continuously grown in the presence of increasing proportions of biomass hydrolysate in the growth medium as described in Examples 1 and 2 herein. At periodic intervals samples are taken and assayed for performance in hydrolysate medium, including for xylose and glucose utilization, and for ethanol production.

Multiple rounds of adaptation in a medium containing increasing proportions of hydrolysates, as well as other stress components such as acetate and ethanol, may be used to produce strains with improved performance, such as those described above.

Fermentation Of Improved Xylose-Utilizing Strain

An engineered xylose-utilizing and ethanol producing *Zymomonas* strain with at least one genetic modification described herein may be used in fermentation to produce ethanol. The *Z. mobilis* strain is brought in contact with medium that contains biomass hydrolysate that includes sugars comprising glucose and xylose. At least a portion of the sugars are derived from pretreated and saccharified cellulosic or lignocellulosic biomass. Additional sugars and/or other media components may be included in the medium.

When the sugars concentration is high such that growth is inhibited, the medium includes sorbitol, mannitol, or a mixture thereof as disclosed in commonly owned United States Patent U.S. 7,629,156. Galactitol or ribitol may replace or be combined with sorbitol or mannitol. The *Z. mobilis* grows in the medium where fermentation occurs and ethanol is produced. The fermentation is run without supplemented air, oxygen, or other gases (which may include conditions such as anaerobic, microaerobic, or microaerophilic fermentation), for at least about 24 hours, and may be run for 30 or more hours. The timing to reach maximal ethanol production is variable, depending on the fermentation conditions. The fermentations may be run at temperatures that are between about 30 ° C and about 37 ° C, at a pH of about 4.5 to about 7.5.

The present *Z. mobilis* may be grown in medium containing mixed sugars including xylose in laboratory scale fermenters, and in scaled up fermentation where commercial quantities of ethanol are produced. Where commercial production of ethanol is desired, a variety of culture methodologies may be applied. For example, large-scale production from the present *Z. mobilis* strains may be produced by both batch and continuous culture methodologies. A classical batch culturing method is a closed system where the composition of the medium is set at the beginning of the culture and not subjected to artificial alterations during the culturing process. Thus, at the beginning of the culturing process the medium is inoculated with the desired organism and growth or metabolic activity is permitted to occur adding nothing to the system. Typically, however, a "batch" culture is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen

concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the culture is terminated. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase are often responsible for the bulk of production of ethanol.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch culture processes are also suitable for growth of the present *Z. mobilis* strains and comprise a typical batch system with the exception that the substrate is added in increments as the culture progresses. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch culturing methods are common and well known in the art and examples may be found in Biotechnology: A Textbook of Industrial Microbiology, Crueger, Crueger, and Brock, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36, 227, (1992), herein incorporated by reference.

Commercial production of ethanol may also be accomplished with a continuous culture. Continuous cultures are open systems where culture medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous cultures generally maintain the cells at a constant high liquid phase density where cells are primarily in log phase growth. Alternatively, continuous culture may be practiced with immobilized cells where carbon and nutrients are continuously added, and valuable products, by-products or waste products are continuously removed from the cell mass. Cell immobilization may be performed using a wide range of solid supports composed of natural and/or synthetic materials as is known to one skilled in the art.

Particularly suitable for ethanol production is a fermentation regime as follows. The desired *Z. mobilis* strain of the present invention is grown in shake flasks in semi-complex medium at about 30 °C to about 37 °C with shaking at about 150 rpm in orbital shakers and then transferred to a 10 L seed fermentor containing similar medium. The seed culture is grown in the seed fermentor anaerobically until OD_{600} is between 3 and 10, when it is transferred to the production fermentor where the fermentation parameters are optimized for

ethanol production. Typical inoculum volumes transferred from the seed tank to the production tank range from about 2% to about 20% v/v. Fermentation medium for the present strains contains biomass hydrolysate in at least about 50% and may be supplemented with other nutrients, as known to one of skill in the art..A final concentration of about 5 mM sorbitol or mannitol is present in the medium.

The fermentation is controlled at pH 5.0 - 6.0 using caustic solution (such as ammonium hydroxide, potassium hydroxide, or sodium hydroxide) and either sulfuric or phosphoric acid. The temperature of the fermentor is controlled at 30 °C - 35 °C. In order to minimize foaming, antifoam agents (any class- silicone based, organic based etc) are added to the vessel as needed. An antibiotic, for which there is an antibiotic resistant marker in the strain or to which the strain is resistant, such as kanamycin, may be used optionally to minimize contamination.

Any set of conditions described above, and additionally variations in these conditions that are well known in the art, are suitable conditions for production of ethanol by a xylose-utilizing recombinant *Zymomonas* strain.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is disclosed herein that xylose-utilizing *Zymomonas* strains with improved utilization of glucose and xylose, and production of ethanol, in the presence of biomass hydrolysate can be obtained by a process of adaptation and screening in biomass hydrolysate medium. Increase in glucose and xylose utilization, and ethanol production, is measured by comparison to glucose and xylose utilization by the xylose-utilizing corresponding non-adapted strain. The corresponding non-adapted strain is the strain used as the starting strain for the hydrolysate adaptation process.

Zymomonas mobilis strains were isolated from hydrolysate adaptation experiments as described in Examples 1and 2 herein. Two isolated strains were named Adapted 5-6 (or AR2 5-6) and Adapted 7-31 (or AR3 7-31), and were further characterized. When grown in corn cob hydrolysate as described in Example 3 herein, these strains utilized glucose and xylose more rapidly than the corresponding non-adapted strain ZW705. At 21 hours of a fermentation run, about 20% more glucose had been utilized. In addition, at 21 hours about 22% more ethanol was produced, while at 52 hours about 21% more xylose had been used and 5% more ethanol was produced. In general, the exact percent increases in glucose utilization, xylose utilization, and ethanol production in a

strain with new genetic changes of the Adapted 5-6 or Adapted 7-31 strains, that are described below, will depend on many factors. These factors include the conditions of the fermentation as well as the genetic characteristics of the strain that are in addition to the new genetic changes disclosed herein.

To identify any changes in the genomes of the Adapted 5-6 and Adapted 7-31 strains, the genomes were sequenced. By comparison of these genomic sequences to genomic sequences of the corresponding starting strain, ZW705 (described in United States Patent Publication US20110014670A1), wild type strain ZW1 (ATCC 31821), and the published *Zymomonas mobilis* sequence of strain ATCC 31821 (Seo et. al, Nat. Biotech. 23:63-8 2005; NCBI Reference: NC_006526.2), it was determined that both strains had a single, new mutation in the same coding region. This coding region is identified as the zmo1432 open reading frame (ORF) in the published *Zymomonas mobilis* genomic sequence (NCBI Reference: NC_006526.2) and is the complement of nucleotides 1446603 to 1448633 having SEQ ID NO:1.

Adapted 5-6 has a mutation at position No. 1097 in SEQ ID NO:1 that is a change from C to G. This results in the change of codon 366 from ACA encoding threonine to AGA encoding arginine in the zmo1432 encoded protein (SEQ ID NO:2) resulting in the protein of SEQ ID NO:3 where arginine is substituted for threonine No. 366. Adapted 7-31 has a mutation at position No. 350 in SEQ ID NO:1 that is a change from C to T. This results in the change of codon 117 from TCT encoding serine to TTT encoding phenylalanine in the zmo1432 encoded protein (SEQ ID NO:2)) resulting in the protein of SEQ ID NO:4 where phenylalanine is substituted for serine No. 117.

The hypothetical coding region zmo1432 (or ORF) is annotated in the *Zymomonas mobilis* complete genome sequence (NCBI Reference: NC_006526.2) as encoding a "fusaric acid resistance protein". It is annotated as having a fusaric acid resistance protein conserved region which is protein motif: PFAM:PFO4632 (Wellcome Trust Sanger Institute, Genome Research Limited, Hinxton, England). This motif is found in proteins associated with fusaric acid resistance from *Burkholderia cepacia* (Swiss-Prot::P24128 (SEQ ID NO:5); Utsumi et al. (1991) Agric. Biol. Chem. 55:1913-1918; the organism was renamed from *Pseudomonas cepacia*) and *Klebsiella oxytoca* (Swiss-Prot::Q48403 (SEQ ID NO:6); Toyoda et al. (1991) J Phytopathol. 133:165-277), which are likely to be membrane transporter proteins.

A region named Bcenm03_1426 of the *Burkholderia cepacia* chromosome 1 complete sequence is annotated as encoding a putative fusaric acid resistance transporter protein (Accession YP_001764723; Copeland et al. submitted 2/27/2008). This protein (SEQ ID NO:7) has similarity to the zmo1432 encoded protein as described in Example 4 herein. In addition, the *E. coli* protein AaeB (SEQ ID NO:8), which is a component of an aromatic carboxylic acid efflux pump (VanDyk et al J. Bact. 186:7196-7204 (2004)), has similarity to the zmo1432 encoded protein, as described in Example 4 herein. Thus a transporter protein of *Zymomonas* may be the target of mutations that improve glucose and xylose utilization, and ethanol production in xylose-utilizing *Zymomonas* strains that are grown in hydrolysate medium.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

The meaning of abbreviations is as follows: "kb" means kilobase(s), "bp" means base pairs, "nt" means nucleotide(s), "hr" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "L" means liter(s), "ml" means milliliter(s), "μL" or "μl " means microliter(s), "μg" means microgram(s), "ng" means nanogram(s), "g" means gram(s), "mM" means millimolar, "μM" means micromolar, "nm" means nanometer(s), "μmol" means micromole(s), "pmol" means picomole(s), "OD600" is optical density at 600 nm.

GENERAL METHODS

Turbidostat

Adaptation was in a turbidostate (United States Patent U.S. 6,686,194; Heurisko USA, Inc. Newark, DE), which is a continuous flow culture device where the concentration of cells in the culture was kept constant by controlling the flow of medium into the culture, such that the turbidity of the culture was kept within specified narrow limits. Two media were available to the growing culture in the continuous culture device, a resting medium (Medium A) and a challenge medium (Medium B). A

culture was grown on a resting medium in a growth chamber to a turbidity set point and then was diluted at a dilution rate set to maintain that cell density. Dilution was performed by adding media at a defined volume once every 10 minutes. When the turbidostat entered a media challenge mode, the choice of adding challenge medium or a resting medium was made based on the rate of return to the set point after the previous media addition. The steady state concentration of medium in the growth chamber was a mix of Medium A and Medium B, with the proportions of the two media dependent upon the rate of draw from each medium that allowed maintenance of the set cell density at the set dilution rate. A sample of cells representative of the population in the growth chamber was recovered from the outflow of the turbidostat (in a trap chamber) at weekly intervals. The cell sample was grown once in MRM3G6 medium and saved as a glycerol stock at -80 ^{0}C.

Enzymes

Spezyme® CP-100, Multifect® CX12L, and Accellerase® 1500 were from Danisco U.S. Inc., Genencor (Rochester, NY).

Novozyme 188 was from Novozymes (2880 Bagsvaerd, Denmark).

Additional enzymes used in the saccharification process(es) herein were the glycosyl hydrolases (GH) Xyn3, Fv3A, Fv51A and Fv43D. Xyn3 (SEQ ID NO:9) is a GH10 family xylanase from *Trichoderma reesei*, Fv3A (SEQ ID NO:10) is a GH3 family enzyme from *Fusarium verticillioides*, Fv43D (SEQ ID NO:11) is a GH43 family enzyme from *Fusarium verticillioides*, and Fv51A (SEQ ID NO:12) is a GH51 family of enzyme from *Fusarium verticillioides*.

Media.

Corn cob hydrolysate used in the adaptation was prepared first by dilute ammonia pretreatment of ground corn cob. A horizontal Littleford Day 130L reactor vessel containing a jacket for passing steam around the body of the vessel and one of the sides (Littleford Day, Inc., Florence, KY) was loaded with milled cob. Vacuum was applied to the vessel to reach 0.1 atm prior to introduction of a 29 wt% ammonium hydroxide solution and water near the top of the vessel to give a 6 wt% NH_3 relative to dry weight biomass. Steam was introduced near the top of the vessel to raise the internal vessel temperature to 145 °C. This temperature was held for 20 mins. At the end of pretreatment, the reactor was depressurized to reach atmospheric pressure. Vacuum (approximately to less than 1 atm) was subsequently applied to lower the

temperature to less than 60 °C.

The pretreated cob was then treated with and enzyme consortium allowing for enzymatic hydrolysis of the cellulose and hemicellulose polymers using an enzyme mix containing: Spezyme CP-100 at 34 mg protein per g of glucan in the ptretreated cob; Multifect CX12L at 12.5 mg protein per g of xylan in the pretreated cob; Novozymes 188 at 6.6 mg protein per g glucan in the pretreated cob. The hydrolysis reaction was carried out at 25% (weight/volume) pretreated cob dry matter, pH 5.3 and 47 °C. The reaction was stirred continuously and ran for 72 hrs.

Solids were removed from the hydrolysate using an initial, continuous centrifugation to partially clarify the mixture. The partially clarified mix was again centrifuged at 18,000 xG for 20 mins then passed first through a 0.45 micron filter followed by a 0.22 micron filter to produce clarified, filter sterilized hydrolysate.

Each of the glucose, xylose and acetate concentrations was determined by HPLC analysis. The clarified hydrolysate had a 68 g/L glucose concentration, 46 g/L xylose concentration, and 5 g/L acetate concentration. The clarified hydrolysate was supplemented with 6.2 g/L ammonium acetate to increase the total ammonium acetate concentration such that the concentration falls within the range of 11 to 12 g/L. Where noted, 0.5% yeast extract (Difco Yeast Extract, Becton, Dickinson and Co., Sparks, MD) was added to provide additional nutrients. This medium was labeled HYAc/YE. The pH of the HYAc/YE medium was adjusted to 5.8 and the medium was filter sterilized.

A hydrolysate medium for 1 L fermentation test was prepared using the method described above, except that the enzyme composition used in the hydrolysis was changed to an enzyme blend comprising Accellerase® 1500, Xyn3, Fv3A, Fv51A, and Fv43D, which was added to the hydrolysis reaction at 21.3 mg protein/g glucan + xylan. The hydrolysate was used without clarification in the 1 L scale fermentations.

Additional Media

MRM3 contains per liter: yeast extract (10 g), KH_2PO_4 (2 g) and $MgSO_4.7H_2O$ (1g)

MRM3G6.5X4.5NH_4Ac12.3 is MRM3 containing 65 g/L glucose, 45 g/L xylose, 12.3 g/L ammonium acetate

G5 or MRM3G5 is MRM3 containing 50g/L glucose

G10 or MRM3G10 is MRM3 containing 100g/L glucose

MRM3G2 is MRM3 containing 20 g/L glucose

MRM3X2 is MRM3 containing 20 g/l xylose

halfYEMaxSM:10 g/L Difo yeast extract, 2 g/L KH_2PO_4, 5 g/L $MgSO_4.7H_2O$, 10 mM sorbitol, 150 g/L glucose

For plating studies noted in Figure 2, MRM3G2 (20g/L glucose) and MRM3X2 (20g/L xylose) were supplemented with 1.5% agar, heated to dissolve the agar, cooled to 45 °C and poured into petri dishes.

Preparation of Frozen Stock Cultures

Frozen stock cultures were prepared for strain ZW705, weekly turbidostat sample population cultures, and isolated mutant clones. Additional frozen stocks were prepared by growing the original frozen stock on G5 or G10 medium and using the biomass generated to prepare new frozen stocks.

Inoculation of Seed, Batch Adaptation and Turbidostat Cultures

Frozen stock was used to inoculate overnight G5 or G10 seed cultures. The seed cultures were used to inoculate batch adaptation cultures by centrifuging the seed culture medium and diluting the cell pellet in fresh adaptation medium. For turbidostat inoculation, the entire seed pellet was re-suspended in 10-15 ml of turbidostat resting medium and used to inoculate the turbidostat reactor or 5 ml of overnight seed was used as a 10% inoculum.

Culture OD600 Measurements

To measure culture OD, samples were diluted in 100 g/L xylose (diluent and blank). The diluted culture was allowed to sit for 15 minutes before taking the OD measurement. All OD measurements were at 600 nm.

HPLC Analysis

HPLC analyses were performed with a Waters Alliance HPLC system. The column used was a Transgenomic ION-300 column (#ICE-99-9850, Transgenomic, Inc) with a BioRad Micro-Guard Cartridge Cation-H (#125-0129, Bio-Rad, Hercules, CA). The column was run at 75 °C and 0.4 mL/min flow rate using 0.01 N H_2SO_4 as solvent. The concentrations of starting sugars and products were determined with a refractive index detector using external standard calibration curves.

Strain ZW705 description

Zymomonas mobilis strainZW705 was produced from strain ZW804-1. ZW801-4 is a recombinant xylose-utilizing strain of *Z. mobilis* that was described in commonly owned United States Patent U.S. 7,741,119, which is incorporated herein by reference. Strain ZW801-4 was derived from strain ZW800, which was derived from strain ZW658, all

as described in United States Patent U.S. 7,741,119. ZW658 was constructed by integrating two operons, P_{gap}*xylAB* and P_{gap}*taltkt*, containing four xylose-utilizing genes encoding xylose isomerase, xylulokinase, transaldolase and transketolase, into the genome of ZW1 (ATCC 31821) via sequential transposition events, and followed by adaptation on selective media containing xylose (U.S 7,629,156). ZW658 was deposited as ATCC PTA-7858. In ZW658, the gene encoding glucose-fructose oxidoreductase was insertionally-inactivated using host-mediated, double-crossover, homologous recombination and spectinomycin resistance as a selectable marker to create ZW800 (U.S. 7,741,119). The spectinomycin resistance marker, which was bounded by loxP sites, was removed by site specific recombination using Cre recombinase to create ZW801-4.

Cultures of *Z. mobilis* strain ZW801-4 were adapted for growth under stress conditions of medium containing ammonium acetate to produce ZW705 as described in commonly owned United States Patent Publication US20110014670A1 , which is incorporated herein by reference. A continuous culture of ZW801-4 was run in 250 ml stirred, pH and temperature controlled fermentors (Sixfors; Bottmingen, Switzerland). The basal medium for fermentation was 5 g/L yeast extract, 15 mM ammonium phosphate, 1 g/L magnesium sulfate, 10 mM sorbitol, 50 g/L xylose and 50 g/L glucose. Adaptation to growth in the presence of high concentrations of acetate and ammonia was effected by gradually increasing the concentration of ammonium acetate added to the above continuous culture media while maintaining an established growth rate as measured by the specific dilution rate over a period of 97 days. Ammonium acetate was increased to a concentration of 160 mM. Further increases in ammonium ion concentration were achieved by addition of ammonium phosphate to a final total ammonium ion concentration of 210 mM by the end of 139 days of continuous culture. Strain ZW705 was isolated from the adapted population by plating to single colonies and amplification of one chosen colony.

Example 1

Adaptation To Corn Cob Hydrolysate Using An Automated, Cell Density Controlled Continuous Culture Apparatus

The turbidostat described in General Methods was used to adapt *Zymomonas mobilis* cultures to growth in corn cob hydrolysate medium. A culture of strain ZW705 was grown in the turbidostat described in General Methods to an arbitrary turbidity set point that dictated that the culture use all of the glucose and approximately half of the xylose present in the incoming media to meet the set point cell density at the set dilution rate. Using resting medium that was 50% HYAc/YE and 50% MRM3G6.5X4.5NH_4Ac12.3. The turbidostat was run as described in General Methods using as challenge medium HYAc/YE. Cell samples were taken weekly for 6 weeks from the trap chamber.

After 6 weeks of continuous culture, samples from the weekly saved cell stocks were revived in MRM3G6 and grown to about 1 OD600 in 10 ml static cultures at 33 °C. These were used to inoculate 12 ml cultures of HYAc/YE medium to approximately 0.4 OD600 nm, and the cultures were grown at 30 °C. Samples were taken at different times as in Table 1, and assayed for OD600, sugar consumption, and ethanol production. The results are shown in Table 1.

Table 1 Analysis of weekly trubidostat samples grown in 12 ml HYAc/YE fermentations

culture	Sampling time	OD 600 nm	remaining glucose (g/L)	remaining xylose (g/L)	ethanol (g/L)
ZW705	0	0.400	69.8	46.0	0.0
	24	0.760	52.8	45.4	8.4
	48	2.140	5.4	30.7	38.2
	72	2.300	2.3	16.0	45.1
week 1	0	0.417	69.8	46.0	0.0
	24	0.78	48.8	45.6	9.7
	48	1.430	4.8	34.2	35.6
	72	1.890	2.4	25.7	37.7
week 2	0	0.351	69.8	46.0	0.0
	24	0.810	53.2	46.1	7.7
	48	1.670	7.6	36.1	33.5
	72	1.710	2.7	26.6	39.3
week 3	0	0.395	69.8	46.0	0.0
	24	1.090	42.9	44.3	13.4
	48	1.920	2.9	27.3	40.2
	72	2.410	1.9	19.7	42.1
week 4	0	0.386	69.8	46.0	0.0
	24	1.070	42.2	44.2	13.7
	48	1.510	3.3	30.7	38.4
	72	2.010	2.1	25.2	39.4
week 5	0	0.328	69.8	46.0	0.0
	24	0.920	52.7	45.5	7.7

	48	1.350	8.5	36.2	34.1
	72	1.650	2.9	25.8	40.4
week 6	0	0.402	69.8	46.0	0.0
	24	0.600	55.5	45.9	6.9
	48	1.360	13.9	39.8	29.1
	72	1.530	4.1	31.6	37.5

All cultures retained the ability to grow on xylose but the rate of xylose use seemed to have decreased after the 3rd week of adaptation. Single colonies were isolated from the culture of cells sampled at the end of week 3. Colonies were isolated by growing the retained glycerol stock in MRM3G5, then plating on MRM3X2 plates. Single colonies were replica patched onto MRM3X2 and MRM3G2 plates. Large, dense patches on both carbon sources were chosen as strains and maintained as frozen glycerol stocks. Selected strains were grown in 12 ml test cultures as described for the frozen stock population test described above. Results for six strains and ZW705 are shown in Table 2.

Table 2 Analysis of isolated pure cultures from week 3 of the turbidosat adaptation of ZW705 grown in 12 ml HYAc/YE fermentations

Strain	Sampling time	OD 600 nm	remaining glucose (g/L)	remaining xylose (g/L)	ethanol (g/L)
ZW705	0	0.383	74.9	44.4	0.0
	24	0.450	51.1	43.4	13.4
	48	1.350	5.7	35.1	31.4
	72	2.770	1.9	17.1	49.0
12-18X-1-10	0	0.333	74.9	44.4	0.0
	24	0.760	47.7	44.4	14.6
	48	2.530	3.1	28.3	36.7
	72	2.800	1.7	12.1	50.4
12-18X-2-36	0	0.400	74.9	44.4	0.0
	24	0.900	46.9	44.3	15.1
	48	2.220	2.8	24.2	38.9
	72	3.430	1.8	9.1	53.3
12-18X-5-34	0	3.820	74.9	44.4	0.0
	24	0.530	52.7	44.9	13.1
	48	2.430	4.3	30.3	35.7
	72	3.050	1.9	13.0	51.1
12-18X-6-9	0	0.401	74.9	44.4	0.0
	24	0.530	51.2	44.4	13.1
	48	2.590	3.2	28.7	36.6
	72	3.050	1.8	11.4	52.5
12-18X-7-43	0	0.351	74.9	44.4	0.0
	24	0.600	49.6	43.9	14.3
	48	2.200	3.1	26.9	38.3
	72	3.330	1.7	9.9	53.8

12-18X-8-19	0	0.409	74.9	44.4	0.0
	24	0.749	45.2	44.4	16.0
	48	2.230	3.4	27.3	34.8
	72	2.910	1.7	11.7	52.1

All of the selected strains produced more ethanol in the 12 ml test than did the strain that entered adaptation (ZW705). The strains used slightly more xylose than the parent strain. Strain 12-18X-2-36 was chosen for an additional round of adaptation.

Example 2

Adaptation To Corn Cob Hydrolysate With Added Ethanol Using An Automated, Cell Density Controlled Continuous Culture Apparatus

A culture of strain 12-18X-2-36 (described in Example 1) was grown in the turbidostat as described above for strain ZW705 and in General Methods, except that the resting medium was HYAc/YE and the challenge medium was HYAc/YE + 9 weight% ethanol. The turbidostat was run for 4 weeks with weekly sampling of the chamber outflow. Frozen cell stocks were made from the outflow samples. Frozen cell stocks were revived in MRM3G6 for testing in 12 ml hydrolysate fermentations under the same conditions as described for the first turbidosat run, but with starting density of about 0.5 OD600. One fermentation was in the same HYAc/YE medium, and another fermentation was in HYAc/YE to which ethanol was added at 30 g per L of medium . Results for both fermentations are shown in Table 3.

Table 3 Analysis of weekly trubidostat samples grown in 12 ml HYAc/YE or HYAc/YE + 30g/L ethanol fermentations

		HYAc/YE with no added ethanol				HYAc /YE with 30 g/L ethanol			
culture	samp-ling time	OD 600 nm	remain-ing glucose (g/L)	remain-ing xylose (g/L)	ethanol (g/L)	OD 600 nm	remain-ing glucose (g/L)	remain-ing xylose (g/L)	ethanol (g/L)
ZW705	0	0.530	75.5	47.7	0.0	0.530	73.3	48.3	26.2
	24	0.890	50.1	48.0	13.7	0.470	63.6	46.8	27.3
	48	2.200	3.9	33.6	43.6	0.760	51.1	45.7	32.7
	72	2.500	1.8	19.7	50.3	1.060	39.0	45.7	39.6
week 1	0	0.513	75.5	47.7	0.0	0.515	73.3	48.3	26.2
	24	1.360	20,1	44.2	30.2	1.370	45.2	46.9	36.3
	48	2.200	2.1	27.3	47.7	1.810	14.7	42.5	50.1
	72	2.520	1.7	18.5	50.6	1.920	3.8	40.1	57.7
week 2	0	0.504	75.5	47.7	0.0	0.504	73.3	48.3	26.2
	24	1.140	32.4	47.1	23.2	0.950	51.6	46.8	34.1
	48	1.910	2.4	27.8	47.7	1.500	19.4	42.8	50.4
	72	2.290	1.7	18.5	51.4	1.780	5.7	38.0	60.5
week 3	0	0.527	75.5	47.7	0.0	0.527	73.3	48.3	26.2
	24	0.640	53.5	48.7	12.3	0.570	62.1	48.2	28.5
	48	1.420	7.3	37.7	39.5	0.600	51.9	46.1	33.2
	72	2.010	2.0	25.5	47.3	1.060	35.3	44.8	38.7
week 4	0	0.518	75.5	47.7	0.0	0.518	73.3	48.3	26.2
	24	0.730	25.5	45.2	27.8	0.480	64.3	47.7	25.5
	48	1.760	2.3	23.7	49.3	0.560	52.8	46.8	32.9
	72	1.890	1.8	15.8	52.6	1.080	38.4	45.2	40.4

All cultures were similar to the ZW705 control in the test fermentations with no ethanol added to the HYAc/YE medium. In fermentations with 30 g/L ethanol added to the HYAc/YE medium, the cultures taken after weeks 1 and 2 were much better at utilizing glucose and producing ethanol than ZW705 and the week 3 and 4 samples. The culture stored after week 2 had the highest ending ethanol titer in the test that began with added ethanol and was chosen for isolation of single cell derived strains. The strain isolation procedure described for the screen that derived strain 12-18X-2-36 was run twice and the results of both rounds of screening are shown in Tables 4 and 5. Screens were started in HYAc/YE medium with no added ethanol, or with 40 g/L added ethanol.

Table 4 Analysis of isolated pure cultures from week 2 of the turbidosat adaptation of 12-18X-2-36 grown in 12 ml HYAc/YE or HYAc/YE + ethanol fermentations (round 1)

		HYAc/YE with no added ethanol				HYAc/YE with 40 g/L ethanol			
culture	Sampling time	OD 600 nm	remain-ing glucose (g/L)	Remain-ing xylose (g/L)	ethanol (g/L)	OD 600 nm	Remain-ing glucose (g/L)	Remain-ing xylose (g/L)	ethanol (g/L)
ZW705	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.8	56.5	50.2	9.0	0.42	69.8	50.0	24.5
	48.0	1.8	12.6	41.6	28.2	0.75	62.6	49.6	27.5
	72.0	1.9	3.8	24.0	46.7	0.60	52.7	48.7	36.5
1-32	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.8	55.3	49.6	12.0	0.41	65.3	48.0	35.2
	48.0	1.9	7.8	38.5	39.1	0.68	57.3	47.6	38.2
	72.0	2.4	2.6	21.2	51.6	0.72	50.8	47.5	41.0
1-44	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.8	53.7	49.9	12.6	0.44	66.7	48.2	34.7
	48.0	1.9	6.5	37.5	40.1	0.75	60.8	48.5	35.5
	72.0	2.2	1.8	21.7	50.1	0.65	55.8	48.1	37.4
2-24	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.7	59.4	49.7	9.8	0.41	67.6	48.6	32.0
	48.0	1.9	4.8	34.9	42.8	0.69	61.2	48.6	34.2
	72.0	2.3	2.5	18.6	51.3	0.64	55.9	48.6	36.9
2-47	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.9	52.1	49.5	13.5	0.49	66.3	48.7	30.8
	48.0	1.8	9.6	40.3	37.0	0.90	58.6	48.8	34.4
	72.0	1.8	2.7	27.6	46.3	0.61	51.8	48.4	38.2
3-8	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.8	56.9	49.8	10.9	0.44	66.6	48.0	33.8
	48.0	2.0	50.0	34.3	43.0	0.76	58.3	47.9	37.2
	72.0	2.4	2.6	19.6	50.7	0.85	50.6	47.7	40.8
3-45	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.6	60.4	950.0	9.2	0.44	67.6	48.4	34.3
	48.0	1.3	14.1	43.4	33.6	0.55	61.4	48.1	36.7
	72.0	1.8	2.6	19.6	45.1	0.52	57.2	47.9	38.0
4-17	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.6	58.8	49.6	10.0	0.44	66.7	48.3	32.8
	48.0	1.4	5.7	38.0	40.4	0.60	59.7	48.4	34.8
	72.0	1.9	2.4	29.6	49.6	0.54	55.2	48.6	35.5
4-27	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.8	49.8	49.4	14.6	0.52	63.6	48.4	33.7
	48.0	1.6	4.3	34.1	44.0	0.74	53.8	48.2	37.9
	72.0	2.1	2.6	23.7	48.1	0.68	47.5	48.1	40.3
5-6	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.6	53.9	79.7	12.6	0.46	59.7	48.7	35.5
	48.0	1.8	5.8	36.9	41.9	0.90	47.7	48.8	40.3
	72.0	1.9	2.7	23.8	48.7	0.59	**41.5**	48.6	**42.9**
5-35	0.0	0.5	77.7	50.9	0.0	0.50	75.1	49.2	28.1
	24.0	0.8	52.7	49.7	13.1	0.42	65.8	48.2	33.3
	48.0	1.7	5.7	36.8	41.6	0.74	58.4	48.0	36.3
	72.0	1.9	2.4	22.7	49.6	0.80	53.6	48.1	38.1

When starting without added ethanol most strains used all of the glucose in the hydrolysate medium but did not use all of the xylose, so that total ethanol production was dependent on the extent of xylose utilization. Several strains used more xylose and produced more ethanol than did the non-adapted parent strain ZW705. When 40 g/L ethanol was added to the starting medium, growth and sugar utilization was much less in all strains. In this test xylose use was very low or xylose was not consumed at all in most cases. Three strains used significantly more glucose and produced more ethanol than ZW705. The best of those was the strain Adapted 5-6, shown in Table 4.

Table 5 Analysis of isolated pure cultures from week 2 of the turbidosat adaptation of 12-18X-2-36 grown in 12 ml HYAc/YE or HYAc/YE + ethanol fermentations (round 2)

		HYAc/YE with no added ethanol				HYAc/YE with 40 g/L ethanol			
culture	Sampling time	OD 600 nm	remain-ing glucose (g/L)	remain-ing xylose (g/L)	ethanol (g/L)	OD 600 nm	remain-ingg glucose (g/L)	remain-ing xylose (g/L)	ethanol (g/L)
ZW705	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	0.9	40.7	48.6	19.0	0.60	56.2	49.0	34.0
	48.0	2.5	2.9	26.5	48.3	1.14	28.4	46.9	48.8
	72.0	2.9	2.2	17.9	52.7	1.49	17.5	44.7	55.3
6-4	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	0.9	19.0	46.0	30.6	0.79	54.4	49.0	34.9
	48.0	2.4	2.9	28.4	47.2	1.75	18.9	44.2	55.2
	72.0	2.6	2.1	18.4	51.8	1.98	9.2	39.8	61.7
6-43	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.3	28.0	47.7	25.0	0.52	58.6	48.3	36.5
	48.0	2.7	2.7	20.9	50.5	0.65	44.9	47.6	44.0
	72.0	2.8	2.2	14.6	52.9	1.12	37.9	47.3	46.8
7-13	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.6	17.5	46.6	29.7	0.51	57.0	48.2	38.0
	48.0	32.7	2.6	18.2	50.6	0.99	35.7	46.3	49.4
	72.0	3.3	2.2	11.8	53.3	1.99	25.9	44.9	53.8
7-31	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.4	23.1	46.9	27.7	0.77	39.5	48.0	39.5
	48.0	2.5	2.7	19.2	52.3	1.35	22.3	45.2	56.7
	72.0	3.0	2.1	12.8	53.7	2.09	12.7	43.6	56.5
8-2	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.5	22.1	47.5	20.7	0.59	54.8	48.3	37.8
	48.0	3.3	2.8	20.3	50.3	1.24	30.4	46.3	51.7
	72.0	3.4	2.3	14.8	51.8	1.14	20.4	44.8	55.0
8-22	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.2	28.7	47.6	24.6	0.56	58.1	48.6	35.5
	48.0	2.3	2.8	19.2	51.5	0.99	33.6	46.4	49.2
	72.0	2.8	2.2	11.4	55.2	1.14	21.7	44.1	54.9

9-5	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.3	32.0	49.2	23.0	0.67	57.9	48.2	37.0
	48.0	2.5	2.9	23.0	48.8	1.07	32.0	46.2	51.8
	72.0	2.7	2.3	14.7	52.4	1.68	22.2	44.3	55.6
9-21	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.4	28.4	48.0	24.9	0.51	58.7	48.3	35.9
	48.0	2.6	2.7	21.1	50.4	0.88	36.4	47.0	48.4
	72.0	2.7	2.3	13.4	53.5	1.06	26.1	45.3	52.2
10-17	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	0.9	42.1	49.4	18.4	0.32	62.8	48.4	33.9
	48.0	2.4	3.0	24.7	49.0	0.43	51.7	47.8	41.2
	72.0	2.8	2.3	14.0	52.7	0.55	45.6	47.7	41.0
10-32	0.0	0.5	77.8	51.5	0.0	0.50	74.9	49.1	30.6
	24.0	1.1	37.6	48.9	20.1	0.50	59.3	48.4	34.1
	48.0	2.3	2.8	23.4	47.3	1.05	35.2	46.9	46.6
	72.0	2.4	2.3	15.7	51.0	1.34	22.6	45.0	52.3

Results were similar in the second strain isolation experiment. Several strains used more xylose than ZW705 in the no added ethanol test, and used more glucose in the added ethanol test. Many used more glucose in the first 24 hours of growth and achieved higher cell mass as measured by OD600 nm in the first 24 hours also. Of these, the strain Adapted 7-31 was chosen for further testing.

Example 3

Performance Testing Of Hydrolysate Adapted Strains

Seed Fermentation

Seed fermentation was performed in a 1 L fermenter (Sartorious Stedim BIOSTAT). Sterilized, empty fermenters were filled with filter-sterilized halfYEMaxSM. Seed fermentations were performed at 33 °C and pH 5.5, using unfiltered 4 N NH_4OH as the base to control to pH 5.5. Seed fermentations were inoculated with sufficient volume of frozen stock cells that were grown for about 7.5 hr to OD600 of about 2.5, in halfYEMaxSM. Cells were diluted into the 1L fermenter to give a starting OD600 nm of about 0.025. In general, seed fermentations were harvested when ~120 g/L glucose had been consumed and/or OD600 of about 10 had been reached. The seed fermentations were periodically sampled to monitor growth, and harvested at about 18.5 hr.

Hydrolysate Fermentation

Hydrolysate fermentation was performed in a 1 L fermentor (Sartorious Stedim BIOSTAT). Sterilized, empty fermentors were filled with 450 ml of corn cob hydrolysate prepared as described in General Methods. Hydrolysate

fermentations were performed at pH 5.8 using unfiltered 4 N NaOH as the base to adjust pH. Hydrolysate fermentation began at 33 °C. Hydrolysate fermentations were inoculated with 10 volume% (50 ml) of seed from the seed fermentation (see above) to generate an initial OD of about 1.0. The hydrolysate fermentations were periodically sampled to monitor reaction progress. The samples were assayed for glucose, xylose and ethanol as described in General Methods.

Two hydrolysate fermentations were run one week apart, each with the control strain ZW705 and the selected strain from adaptation, Adapted 7-31 (Example 2). The time course of the fermentations is shown in Figure 1. Adapted 7-31 used glucose faster than ZW705 and used more total xylose to achieve a higher ethanol titer during the course of the fermentation.

Hydrolysate fermentations were run and assayed as described above to compare the control strain ZW705, strain Adapted 7-31, and strain Adapted 5-6 (see Example 2). Results are shown in Figure 2. Strain Adapted 5-6 performance was equivalent to that of strain Adapted 7-31. Both of these adapted strains achieved a higher final ethanol titer by using glucose more rapidly at the beginning of the fermentation and by using more xylose by the end of the fermentation.

Example 4

Comparative DNA Sequencing Of Adapted Strains

The whole genome sequence of wild type *Zymomonas mobilis* (ZM4) has been described (Jeong-Sun Seo et. al, Nature Biotechnology. 23, 2005). A wild type starting strain (ZW1; ATCC 31821), an intermediate strain (ZW658; ATCC PTA-7858) and ZW705, were sequenced using high throughput 454 technology (Shendure and Ji, Nature Biotech. 26:1135 (2008)) and compared to the published wild type sequence. Strains Adapted 5-6 and Adapted 7-31 were sequenced using Illumina technology (Shendure and Ji, Nature Biotech 26:1135 (2008)). From the wild type sequence, the sequence of ZW658 and the separately determined insertion sites of the intentional sequence changes coming from insertion of the two xylose utilization operons and the knockout of the GFOR gene (described in General Methods), a consensus sequence was made to which sequences from adapted strains could be compared. The description of sequencing and genome assembly follows.

Sequencing

Strains were sequenced using the sequencing technologies Illumina/Solexa and Roche-454. These massively parallel sequencing methods give very high throughput of short reads. In the case of Illumina, it gives more than 200 million reads that are 100 bp long. For Roche-454 outputs 1 million reads are 500 bp long. Both methods allow sequencing from both ends of genomic DNA fragments to produce paired-end reads

Hundreds of millions of short reads from Adatped 5-6 and Adapted 7-31 strains were aligned to a reference genome sequence prepared from the wild type, ZW658, and ZW705 sequences. The resulting alignment was analyzed to collect information on coverage and variations. Since many reads can align to a specific region, the alignment is a pile up of reads against the reference and the depth coverage of a given position is the number of reads that cover that position. If at a position the consensus base is different from the reference base, the position is said to have a Single Nucleotide Polymorphism (SNP) variation.

In the comparison of the Adapted 5-6 and Adapted 7-31 sequences to the starting strain for adaptation ZW705 sequence, a single base change, or SNP, was identified for each adapted strain. The SNP for Adapted 5-6 was at position 1453116 which is in the open reading frame of a gene designated zmo1330. The SNP for Adapted 7-31 was in the same open reading frame, but the change was at a different position (1453863). The change in Adapted 5-6 is in position 1097 of the coding region (SEQ ID NO:1) and is a change from C to G at that position. This mutation results in a codon change for amino acid 366 from ACA to AGA, resulting in a change in amino acid 366 from threonine to arginine in the encoded protein. The change in Adapted 7-31 is in position 350 of the coding region (SEQ ID NO:1) and is a change from C to T at that position. This mutation results in a codon change for amino acid 117 from TCT to TTT resulting in a change in amino acid 117 from serine to phenylalanine.

The protein encoded by zmo1330 was used in a BLAST of the transporter classification data base (Saier Lab Bioinformatics Group; Saier et al. (2009), Nucl. Acids Res., 37: D274-8) which identified the protein as belonging to the aaeB family. This family of proteins is characterized by having a hydrophobic N terminus that predicts 5 to 6 membrane crosses, a fairly long and less hydrophobic middle section, and then a similar 5 to 6 membrane crosses in the C- terminus. This result suggested that the zmo1330 encoded protein is a membrane transport protein.

In the published *Z. mobilis* genome sequence (Seo et al., *ibid*; NCBI

Reference: NC_006526.2), zmo1330 corresponds to zmo1432 which is annotated as encoding a "fusaric acid resistance protein". The set of proteins required for resistance to fusaric acid in *Pseudomonas cepacia* was identified by Utsumi et al. (Agric. Biol. Chem. 55:1919-1918 (1991)). Among the set of open reading frames (ORFs) in what appeared to be one operon conferring fusaric acid resistance is one designated fusB by Utsumi et al. (*ibid*) The sequence of the protein encoded by fusB is identical to Bcenm03_1426 from *Burkholderia cenocepacia* (SEQ ID NO:7), which was aligned with the protein encoded by zmo1432. The two protein sequences align with 3 small gaps and are 22% identical, but 63% similar (Clustal W alignment) as shown in Figure 3. The sequence of Bcenm03_1426 has about the same identity and similarity to the sequence of AaeB (SEQ ID NO:8), which is the larger of two proteins encoded in an operon in *E. coli* that were shown to be required for tolerance to p-aminobenzoic acid (pABA) (VanDyk et al J. Bact. 186:7196-7204 (2004)). Zmo1432 is 17% identical and 55% similar to *E. coli* aaeB. Alignment of proteins encoded by zmo1432 and to aaeB is shown in Figure 4.

CLAIMS

What is claimed is:

1. A recombinant, xylose-utilizing, ethanol-producing microorganism of the genus *Zymomonas*, having at least one genetic modification in the zmo1432 open reading frame.

2. The recombinant *Zymomonas* of Claim 1 wherein the zmo1432 open reading frame having at least one genetic modification encodes a polypeptide having an amino acid sequence with at least about 95% identity to the amino acid sequence as set forth in SEQ ID NO:2, wherein the presence of the mutant protein increases ethanol production by the *Zymomonas* during fermentation in biomass hydrolysate medium as compared to production by a *Zymomonas* lacking the genetic modification under the same conditions.

3. The recombinant *Zymomonas* of Claim 2 wherein the genetic modification is a mutation in the zmo1432 coding region that results in an amino acid substitution selected from the group consisting of : 1) a substitution at position 366 in SEQ ID NO: 2 of arginine for threonine; and 2) a substitution at position 117 in SEQ ID NO: 2 of phenylalanine for serine.

4. The recombinant *Zymomonas* of Claim1 having a higher tolerance to biomass hydrolysate as compared with a *Zymomonas* lacking the at least one genetic modification in the zmo1432 open reading frame.

5. A polynucleotide encoding a protein having the amino acid sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO: 4.

6. A polypeptide having the amino acid sequence selected from the group consisting of in SEQ ID NO: 3 and SEQ ID NO:4.

7. A method for the production of a recombinant *Zymonomas* ethanologen comprising:

a) providing a xylose-utilizing, ethanol-producing microorganism of the genus *Zymomonas* comprising a zmo1432 open reading frame encoding a polypeptide having the amino acid sequence as set forth in SEQ ID NO: 2; and

b) introducing a mutation in the zmo1432 open reading frame of a) such that expression of the mutated open reading frame expresses a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:4.

8. A method of the production of ethanol comprising:
a) providing the recombinant *Zymomonas* of claim 1;
b) providing a biomass hydrolysate medium comprising xylose; and
c) growing the *Zymomonas* of a) in the biomass hydrolysate medium of b) wherein ethanol is produced.

Figure 1

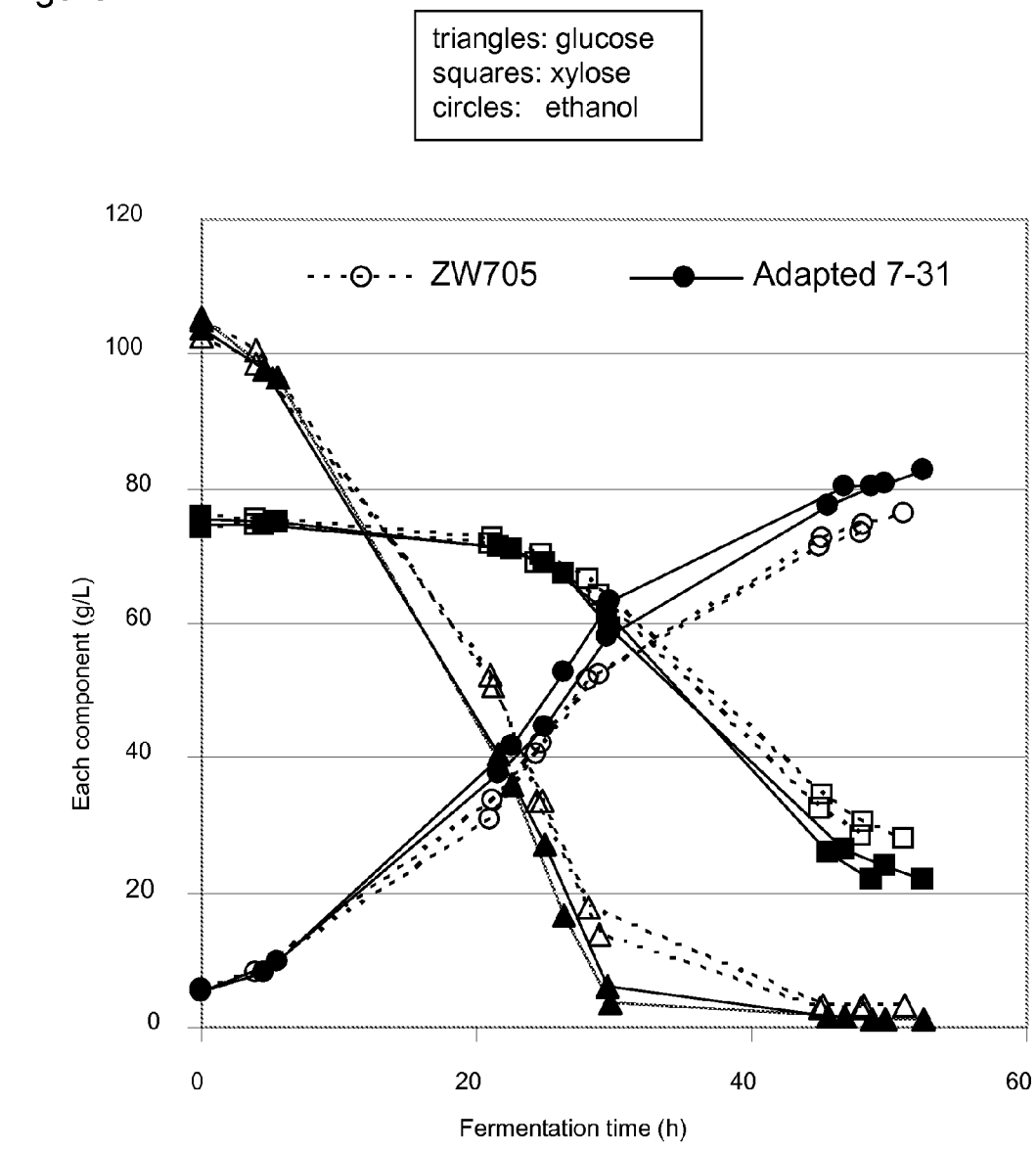
triangles: glucose
squares: xylose
circles: ethanol
ZW705
Adapted 7-31
120
100
80
60
40
20
0
Each component (g/L)
0
20
40
60
Fermentation time (h)

Figure 2

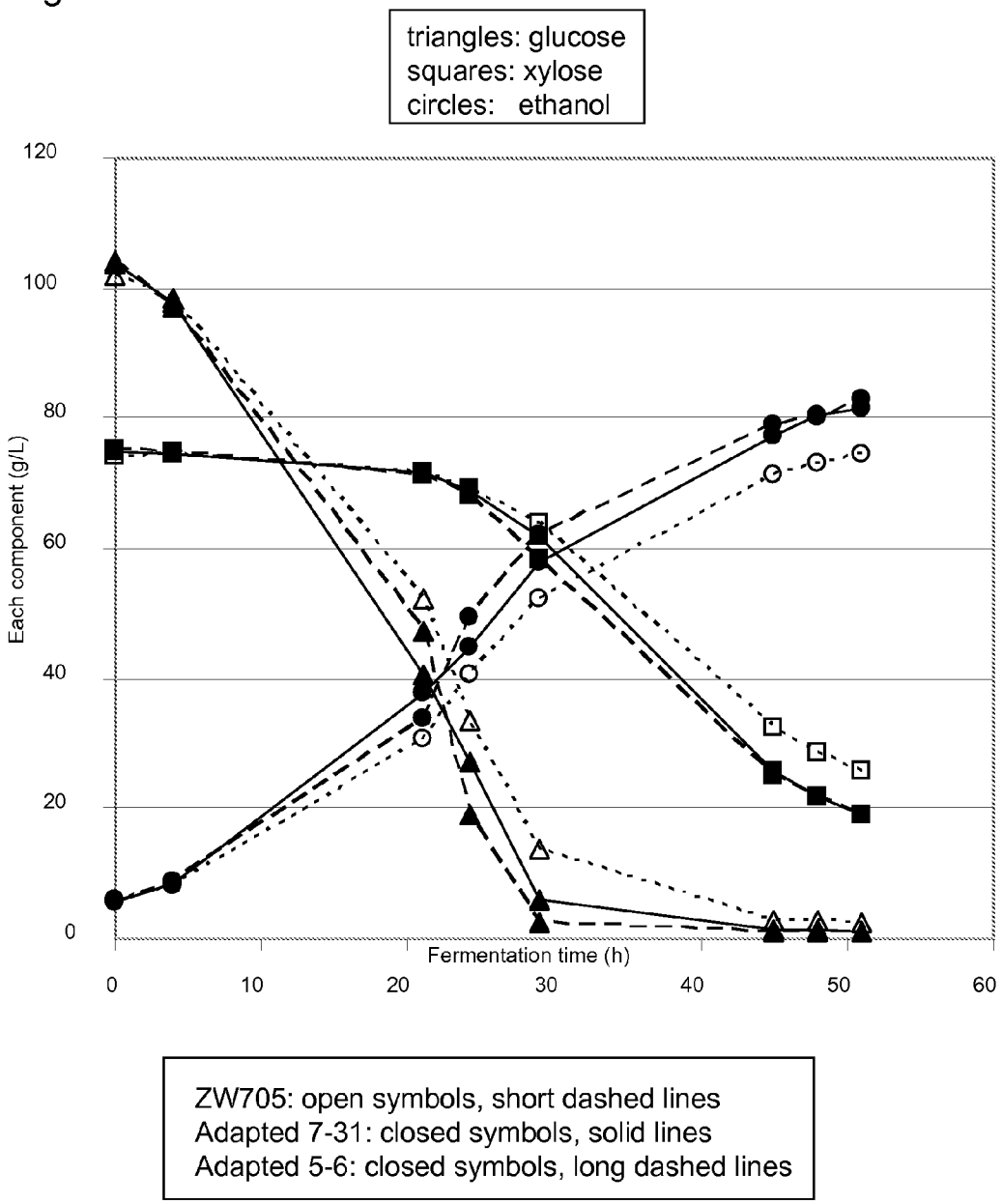
triangles: glucose
squares: xylose
circles: ethanol
120
100
80
60
40
20
0
Each component (g/L)
0
10
20
30
40
50
60
Fermentation time (h)
ZW705: open symbols, short dashed lines
Adapted 7-31: closed symbols, solid lines
Adapted 5-6: closed symbols, long dashed lines

Figure 3

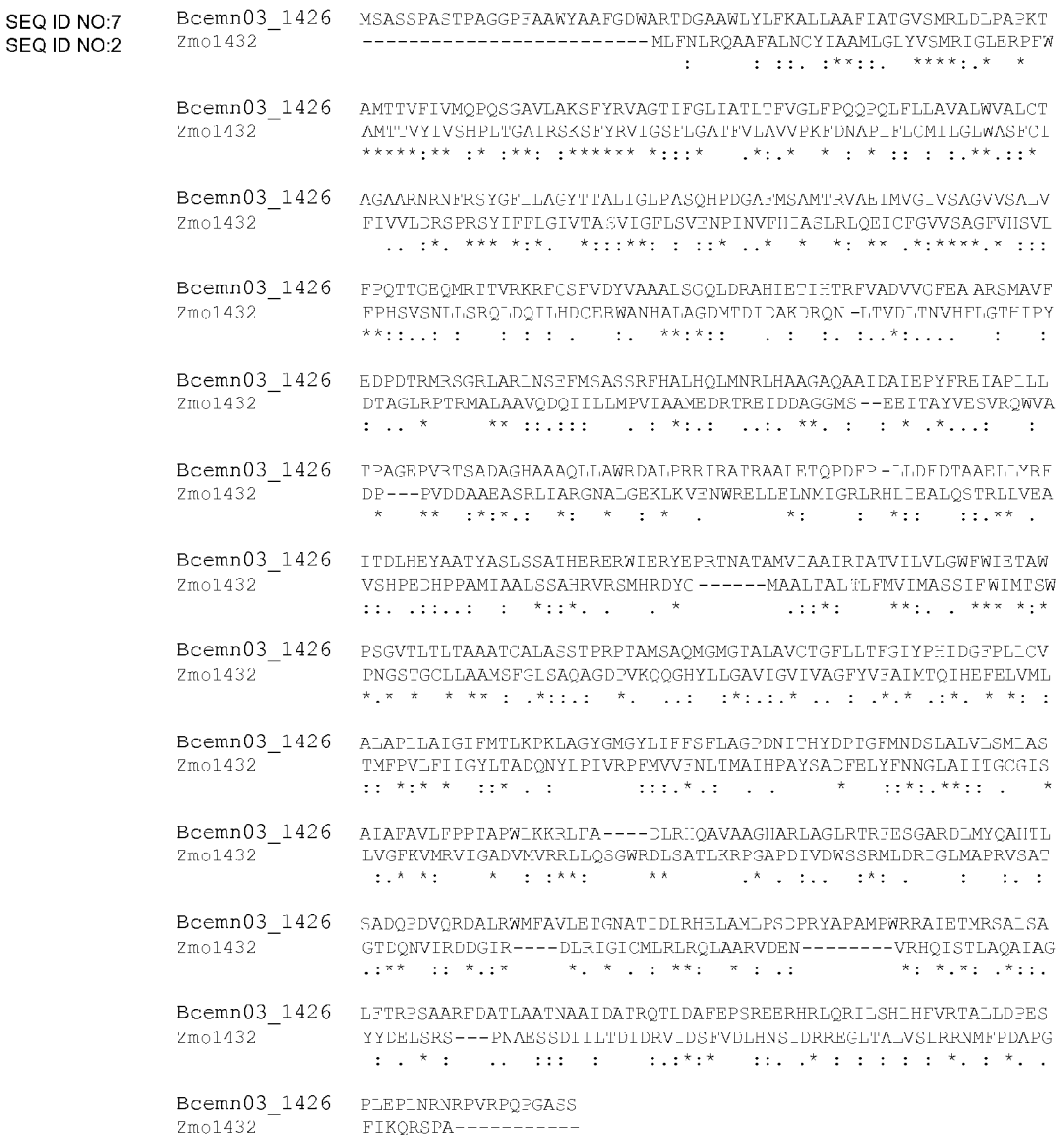

Figure 4


SEQ ID NO:2
SEQ ID NO:8
zmo1432
EcaaeB
---MLFNLRQAAFALNCYIAAMLGLYVSMRIGLERPFWAMTTVYIVSH---------PLT
MGIFSIANQHIRFAVKLATAIVLALFVGFHFQLETPRWAVLTAAIVAAGPAFAAGGEPYS
GAIRSKSFYRVIGSFLGATFVLAVVPKFDNAPLFLCMILGLWASFCIFIVVLDRSPRSYI
GAIRYRGFLRIIGTFIGCIAGLVIIIAMIRAPLLMILVCCIWAGFCTWISSLVRIENSYA
FFLGIVTASVIGFLSVENPINVFHIASLRLQEICFGVVSAGFVHSVLFPHSVSNLLSRQL
WGLAGYTALIIVITIQPEPLLTPQFAVERCSEIVIGIVCAIMADLLFSPRSIKQEVDREL
DQILHDCERWANHALAGDMTDIDAKDRQNLTVDLTNVHFLGTHIPYDTAGLRPTRMALAA
ESLLVAQYQLMQLCIKHGDGEVVDKAWGDLVRRTTALQGMRSNLNMESSRWARANRRLKA
VQDQIILLMPVIAAMEDRTREIDDAGGMSEEITAYVESVRQWVADPPVDDAAEASRLIAR
IN--TLSLTLITQSCETYLIQNTRPELITDTFREFFD--------TPVETAQDVHKQLKR
GNALGEKLKVENWRELLELNMIGRLRHLIEALQSTRLLVEAVSHPEDHPPAMIAALSSAH
------LRRVIAWTGERETPVT--IYSWVAAATRYQLLKRGVISNTKINATEEEILQGEP
RVRSMHRDYGMAALTALTLFMVIMASSIFWIMTSWPNGSTGCLLAAMSFGLSAQAGDPVK
EVKVESAERHHAMVNFWRTTLSCILGTLFWLWTGWTSGSGAMVMIAVVTSLAMRLPNPRM
QQGHYLLGAVIGVIVAGFYVFAIMTQIHEFELVMLTMFPVLFIIGYLTADQNYLPIVRPF
VAIDFIYGTLAALPLGLLYFLVIIPNTQQSMLLLCISLAVLGFFLGIEVQKRRLGSMGAL
MVVFNLTMAIHPAYSADFELYFNNGLAIITGCGISLVGFKVMRVIGADVMVRRLLQSGWR
ASTINIIVLDNP-MTFHFSQFLDSALGQIVGCVLAFTVILLVRDKSRDRTGRVLLNQFVS
DLSATLKRPGAPDIVDWSSRMLDRIGLMAPRVSATGTDQNVIRDDGIRDLRIGICMLRLR
AAVSAMTTNVARRKENHLPALYQQLFLLMNKFPG-----------DLPKFRLALTMIIAH
QLAARVDENVRHQISTLAQAIAGYYDELSRSPNAESSDIILTDIDRVIDSFVDLHNSIDR
QRLRDAPIPVNEDLSAFHRQMRRTADHVISARSDDKRRRYFGQLLEELEIYQEKLRIWQA
REGLTALVSLRRNMFPDAPGFIKQRSPA
PPQVTEPVHRLAGMLHKYQHALTDS---