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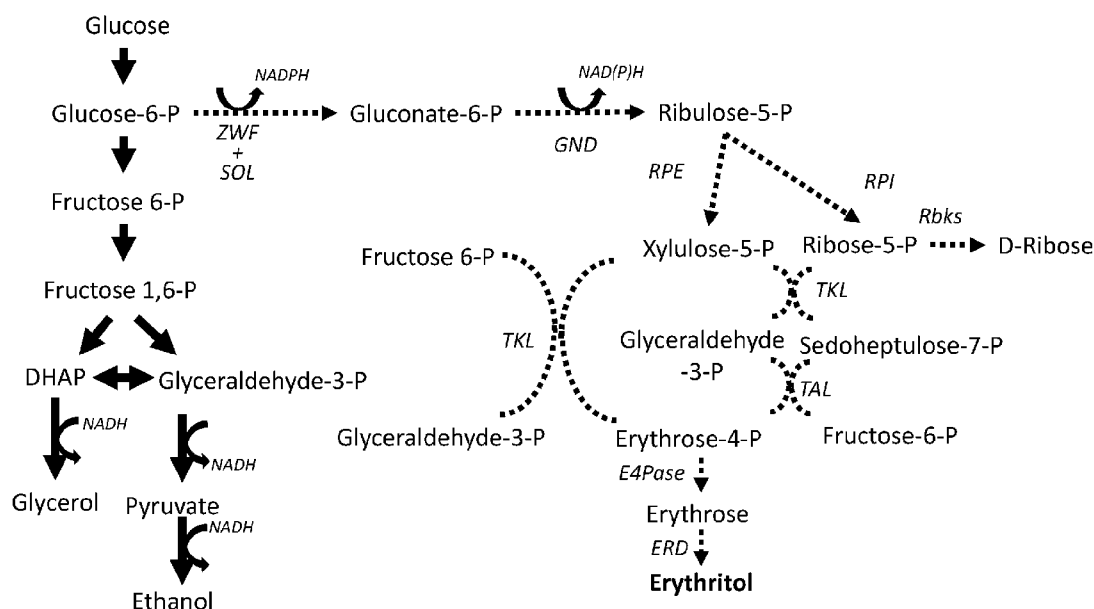
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(54) Title: GENETICALLY MODIFIED YEAST AND FERMENTATION PROCESSES FOR THE PRODUCTION OF XYLITOL

FIG. 1



(57) Abstract: Disclosed herein are genetically engineered yeast cells capable of producing xylitol. The engineered yeast cell may comprise an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ ID NOs: 12-15, 28-31, or 33.

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GENETICALLY MODIFIED YEAST AND FERMENTATION PROCESSES FOR THE PRODUCTION OF XYLITOL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Provisional Application No. 63/364,375, filed May 9, 2022, which is incorporated by reference herein in its entirety.

REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA PATENT CENTER

[0002] The content of the Sequence Listing XML file of the sequence listing named “PT-1349-WO-PCT.xml” which is 448,927 bytes in size created on May 4, 2023 and electronically submitted via Patent Center herewith the application is incorporated by reference in its entirety.

BACKGROUND

[0003] Xylitol is a low-calorie sweetener used as a food additive and sugar substitute. Commonly used in drug, dietary supplement, confectionary, and toothpaste compositions, xylitol has also been associated with anticariogenic properties when used in chewing gums. Traditional methods of xylitol production, including chemically catalyzed hydrogenation of xylose hydrolyzed from biomass extracted xylan, are both monetarily and environmentally costly. These methods require high temperatures and pressures, large amounts of water, and metal catalysts that must be mined. In contrast, fermentation processes have been used commercially at large scale to produce other organic molecules, such as ethanol, citric acid, lactic acid, and the like, and may offer a cost effective and sustainable alternative to traditional xylitol processing methods.

[0004] Accordingly, provided herein are genetically modified yeast and fermentation methods for the production of xylitol while reducing production of erythritol.

SUMMARY

[0005] The present disclosure provides a genetically engineered yeast cell capable of producing xylitol, the engineered yeast cell comprising an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme. The XPDH enzyme may comprise a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ ID NOs: 12-15, 28-

31, and 33. The yeast cell may be an osmotolerant yeast cell. The yeast cell may be a cell of the subphylum Ustilaginomycotina. The yeast cell may be selected from the group consisting of *Trichosporonoides megachiliensis*, *Trychosporonoides oedocephalis*, *Trychosporonoides nigrescens*, *Pseudozyma tsukubaensis*, *Trigonopsis variabilis*, *Moniliella*, *Ustilaginomycetes*, *Trichosporon*, *Yarrowia lipolytica*, *Penicillium*, *Torula*, *Pichia*, *Candida*, *Candida magnoliae*, and *Aureobasidium*. The yeast cell may be a yeast cell of the genus.

[0006] The disclosure also provides a genetically engineered *Moniliella* cell capable of producing xylitol, the engineered *Moniliella* cell comprising an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33.

[0007] The XPDH enzyme may have a sequence at least 85% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33 or to at least one of SEQ ID NOs: 14, 15, 28, or 31. The XPDH enzyme may have a sequence at least 90% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33 or to at least one of SEQ ID NOs: 14, 15, 28, or 31.

[0008] The engineered cell described herein may be a *Moniliella pollinis* cell. The yeast cell may be capable of producing xylitol at a titer of at least 20, 30, 50, 75, or 100 g/L when used in a fermentation process in the presence of dextrose at 35 ° for 96 hours. Erythritol production by the yeast cell may be reduced relative to erythritol production in an equivalent yeast cell lacking the exogenous polynucleotide sequence. The exogenous polynucleotide sequence may be integrated into the genome of the yeast cell at a loci selected from the ER1 locus, the ER3 locus, the PDC1 locus, the pyrF locus, the TRP3 locus, the gpdIIA locus, and the gpdIIB locus. The exogenous polynucleotide sequence may be operably linked to a heterologous or artificial promoter. The promoter may be a constitutive promoter. The promoter may be selected from the group consisting of pyruvate kinase 1 promoter (PYK1p; SEQ ID NO:86), 6-phosphogluconate dehydrogenase promoter (6PGDp; SEQ ID NO:130), glyceraldehyde-3-phosphate dehydrogenase promoter (TDH3p; SEQ ID NO:132), translational elongation factor 1 promoter (TEFp; SEQ ID NO:133), modified TEFp (SEQ ID NO:131), phosphoglucomutase 1 promoter (PGM1p; SEQ ID NO:134), 3-phosphoglycerate kinase promoter (PGK1p; SEQ ID NO:135), enolase promoter (ENO1p; SEQ

ID NO:136), asparagine synthetase promoter (ASNSp; SEQ ID NO:137), 50S ribosomal protein L1 promoter (RPLAp; SEQ ID NO:138), and RPL16B (SEQ ID NO:139).

[0009] The disclosure provides a method for producing xylitol, the method comprising contacting a substrate comprising dextrose with an engineered yeast cell described herein, wherein fermentation of the substrate by the engineered yeast produces xylitol. The disclosure also provides a method for producing xylitol, the method comprising contacting a substrate comprising dextrose with an engineered yeast cell comprising an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33, wherein fermentation of the substrate by the engineered yeast produces xylitol. The fermentation temperature may be at or between 25 °C to 45 °C, 30 °C to 40 °C, or 32 °C to 37 °C. The volumetric oxygen uptake rate (OUR) may be between 0.5 to 40, 1 to 35, 2 to 30, 3 to 25, 4 to 20, or 5 to 15 mmol O₂/(L • h). Xylitol may be produced at a rate of at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, or at least 1.0 g L⁻¹ h⁻¹. Xylitol production may be at least at least 10, 20, 30, 40, 50, 75, or 100 g/L when the fermentation is run at 35 °C for 96 hours. Erythritol production may be reduced relative to an equivalent fermentation run with an equivalent yeast cell lacking the exogenous polynucleotide sequence. Erythritol production may be less than 50, 40, 30, or less than 20 g/L when the fermentation is run at 35 °C for 96 hours. Glycerol production may be reduced relative to an equivalent fermentation run with an equivalent yeast cell lacking the exogenous polynucleotide sequence. Ethanol production may be reduced relative to an equivalent fermentation run with an equivalent yeast cell lacking the exogenous polynucleotide sequence.

BRIEF DESCRIPTION OF THE FIGURES

[0010] This patent or application contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and the payment of the necessary fee.

[0011] The drawings illustrate generally, by way of example, but not by way of limitation, various aspects discussed herein.

[0012] FIG. 1 shows the native pentose phosphate pathway (dotted lines and arrows) and the native glycolysis pathways (solid lines and arrows) in *Moniliella pollinis*.

[0013] FIG. 2 shows diversity in the galactitol-1-phosphate-5-dehydrogenase (G1PDH) / xylitol-phosphate dehydrogenase (XPDH) sequence space.

[0014] FIG. 3 shows the structural characteristics of the NAD or NADP binding pocket located +23 amino acids from the characteristic GXGXXG motif (SEQ ID NO:133) of XPDH enzymes.

[0015] FIG. 4 shows diversity in the ribulose-5-phosphate reductase sequence space.

[0016] FIG. 5 shows *in vitro* activity of TarJ' and XPDH enzymes as outlined in Example 3.

[0017] FIG. 6 shows erythritol, ribitol, and xylitol metabolite concentrations (g/L) at 96 hours of shake flask fermentations of strains 1-1, 1-13a-f, and 1-15a-f as outlined in Example 5. Data labels report the concentration (g/L) of xylitol.

[0018] FIG. 7 shows erythritol, ribitol, and xylitol metabolite concentrations (g/L) at 96 hours of shake flask fermentations of strains 1-1, 1-35a-d, 1-37a-d, 1-38a-f, and 1-39a-f as outlined in Example 5. Data labels report the concentration (g/L) of xylitol.

[0019] FIG. 8 shows erythritol, ribitol, and xylitol metabolite concentrations (g/L) at 96 hours of shake flask fermentations of strains 1-13c, 1-29a-e, 1-33a-e, and 1-34a-e as outlined in Example 6. Data labels report the concentration (g/L) of xylitol.

[0020] FIG. 9 shows erythritol, ribitol, arabitol, and xylitol metabolite concentrations (g/L) at 96 hours of shake flask fermentations of strains 1-13c, 1-12a-e, 1-14a-e, and 1-16a-e as outlined in Example 8. Data labels report the concentration (g/L) of xylitol (strains 1-13c, 1-14a-e, and 1-16a-e) or arabitol (strains 1-12a-e).

[0021] FIG. 10 shows erythritol, ribitol, and xylitol metabolite concentrations (g/L) at 96 hours of shake flask fermentations of strains 1-13c, 1-36a-e, and 1-40a-e as outlined in Example 9. Data labels report the concentration (g/L) of xylitol.

[0022] FIG. 11 shows erythritol, ribitol, and xylitol metabolite concentrations (g/L) at 96 hours of shake flask fermentations of strains 1-30a-e, 1-31a-e, 1-32a-e, and 1-13c as outlined in Example 10. Data labels report the concentration (g/L) of xylitol.

DETAILED DESCRIPTION

[0023] Reference will now be made in detail to certain aspects of the disclosed subject matter, examples of which are illustrated in part in the accompanying drawings. While the disclosed subject matter will be described in conjunction with the enumerated claims, it will be understood

that the exemplified subject matter is not intended to limit the claims to the disclosed subject matter.

[0024] In this document, the terms “a,” “an,” or “the” are used to include one or more than one unless the context clearly dictates otherwise. The term “or” is used to refer to a nonexclusive “or” unless otherwise indicated. All publications, patents, and patent documents referred to in this document are incorporated by reference herein in their entirety, as though individually incorporated by reference. In the event of inconsistent usages between this document and those documents so incorporated by reference, the usage in the incorporated reference should be considered supplementary to that of this document; for irreconcilable inconsistencies, the usage in this document controls.

[0025] Values expressed in a range format should be interpreted in a flexible manner to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range were explicitly recited. For example, a range of “about 0.1% to about 5%” or “about 0.1% to 5%” should be interpreted to include not just about 0.1% to about 5%, but also the individual values (e.g., 1%, 2%, 3%, and 4%) and the sub-ranges (e.g., 0.1% to 0.5%, 1.1% to 2.2%, 3.3% to 4.4%) within the indicated range. The statement “about X to Y” has the same meaning as “about X to about Y,” unless indicated otherwise. Likewise, the statement “about X, Y, or about Z” has the same meaning as “about X, about Y, or about Z,” unless indicated otherwise.

[0026] Unless expressly stated, ppm (parts per million), percentage, and ratios are on a by weight basis. Percentage on a by weight basis is also referred to as wt% or % (wt) below.

[0027] This disclosure relates to various recombinant cells engineered to produce xylitol. In general, the recombinant cells described herein have an active pentose phosphate pathway and are characterized by expression of an exogenous xylitol-phosphate dehydrogenase (XPDH) enzyme. The disclosure further provides fermentation methods for the production of xylitol from dextrose using the genetically engineered cells described herein.

[0028] In general, recombinant cells described herein are yeast cells. As used herein, “yeast” refers to eukaryotic single celled microorganisms classified as members of the fungus kingdom. Yeast are unicellular organisms which evolved from multicellular ancestors with some species retaining multicellular characteristics such as forming strings of connected budding cells known as pseudo hyphae or false hyphae. Yeast cells may also be referred to in the art as yeast-like cells,

and as used herein “yeast cell” encompasses both yeast and yeast-like cells. Suitable yeast and yeast-like host cells for modification may include, but are not limited to, *Saccharomyces cerevisiae*, *Komagataella* sp., *Kluyveromyces* (e.g., *Kluyveromyces lactis*, *Kluyveromyces marxianus*), *Yarrowia lipolytica*, *Issatchenkia orientalis*, *Pichia galeiformis*, *Pichia* sp. YB-4149 (NRRL designation), *Pichia pastoris*, *Candida* (e.g., *Candida magnoliae*, *Candida ethanolica*), *Pichia deserticola*, *Pichia membranifadens*, *Pichia fermentans*, *Aspergillus*, *Trichoderma*, *Myceliphthora thermophila*, *Moniliella* (e.g., *Moniliella pollinis*), *Pfaffia*, *Yamadazyma*, *Hansenula*, *Pichia kudriavzevii*, *Trichosporonoides* (e.g., *Trichosporonoides megachiliensis*, *Trichosporonoides oedocephalis*, *Trichosporonoides nigrescens*), *Pseudozyma tsukubaensis*, *Trigonopsis variabilis*, *Penicillium*, and *Torula*. An ordinarily skilled artisan would understand the requirements for selection of a suitable yeast cell, and recombinant yeast cells of the present disclosure are not limited to those expressly recited herein. Methods for genetic engineering of yeast cells are known and described in the art and a skilled artisan would understand the methods necessary to transform and engineer a suitable yeast cell.

[0029] A suitable yeast cell may be a cell of the phylum *Basidiomycota* and the subphylum *Ustilaginomycotina*. Suitable yeast of the subphylum *Ustilaginomycotina* include, but are not limited to, *Ustilago* (e.g., *U. cynodontis*, *U. maydis*, *U. sphaerogena*, *U. cordal*, *U. scitaminea*, *U. coicis*, *U. syntherismae*, *U. esculenta*, *U. neglecta*, *U. crus-galli*, *Ustilago avenae*), *Sporisorium* (e.g., *Sporisorium exsertum*), *Moniliella* (e.g., *M. pollinis*, *M. tomentosa*, *M. acetoabutans*, *M. fonsecae*, *M. madida*, *M. megachiliensis*, *M. oedocephalis*, *M. nigrescens*), and *Pseudozyma* (e.g., *Pseudozyma tsukubaensis*), and *Trichosporonoides* (e.g., *Trichosporonoides megachiliensis*, *Trichosporonoides oedocephalis*, *Trichosporonoides nigrescens*). Yeast of the subphylum *Ustilaginomycotina* have been known and described in the art as potential production organisms for valuable chemicals such as itaconate, malate, succinate, mannitol, and erythritol and other valuable biotechnological applications. See, for example, Geiser et al. (Prospecting the biodiversity of the fungal family Ustilaginaceae for the production of value-added chemicals,” *Fungal Biol Biotechnol*, 2014, 1:2), Feldbrugge et al., (“The biotechnological use and potential of plant pathogenic smut fungi,” *Appl Microbiol Biotechnol*, 2013, 97(8):3253-65), Guevarra et al., (“Accumulation of itaconic, 2-hydroxyparaconic, itatartaric, and malic acids by strains of the genus *Ustilago*, *Agric. Biol. Chem.*, 1990, 54(9), 2353-2358), and Moon et al., (“Biotechnological production of erythritol and its applications,” *Appl Microbiol Biotechnol*, 2010, 86:1017-1025).

[0030] A suitable yeast cell will have an active pentose phosphate pathway that produces ribulose-5-phosphate. As used herein “active pentose phosphate pathway” refers to expression of one or more functional enzymes which, together, convert glucose-6-phosphate, NADP⁺ or NAD⁺, and water to NADPH or NADH, CO₂, and ribulose-5-phosphate. Continuing in a non-oxidative phase, the pathway may also produce other pentose (i.e., 5-carbon) sugars. For example, the pentose phosphate pathway may produce ribulose-5-phosphate, ribose-5-phosphate, xylulose-5-phosphate, fructose 6-phosphate, combinations thereof, and the like, depending on the enzymatic activities present. The active pentose phosphate pathway may be native to the yeast cell or it may be introduced into the yeast cell by genetic engineering.

[0031] The yeast cell may be an osmotolerant yeast cell. As used herein, “osmotolerant” refers to a yeast capable of growth and reproduction under conditions of high osmolarity, such as at least 10% (w/v), at least 20% (w/v), at least 30% (w/v), at least 40% (w/v), at least 50% (w/v), or at least 60% (w/v) glucose and/or at least 6% (w/v), at least 10% (w/v), at least 12% (w/v), at least 13% (w/v), at least 15% (w/v) sodium chloride. Species and strains of osmotolerant yeast are known and described in the art, including many species of yeast used in industrial fermentation processes. Likewise, methods for assaying yeast osmotolerance are known and described in the art. See, for example, Tiwari, S., et al., (“Nectar yeast community of tropical flowering plants and assessment of their osmotolerance and xylitol-producing potential,” *Current Microbiology*, 2022, 79:28).

[0032] The recombinant yeast cell may be a recombinant *Moniliella* cell, for example, a *Moniliella pollinis* cell. FIG. 1 shows the predicted native pentose phosphate and glycolysis pathways in *Moniliella pollinis*. *Moniliella* has previously been used in the fermentation production of erythritol and methods for genetically modifying and fermenting *Moniliella* are known and described in the art. See, for example, Li et al. (“Methods for genetic transformation of filamentous fungi,” 2017, *Microb Cell Fact*, 16:168).

[0033] Various plasmids and methods for transformation of *Moniliella* are also described in the Examples below. For example, *Moniliella* may be transformed using a bipartite polynucleotide sequence(s) in which, following recombination, the exogenous polynucleotide of interest is integrated at the specified locus and the selection marker is expressible within the cell. Suitable selection markers are known and used in the art. The selectable marker may include, but is not limited to, amdS (for example broken into a 3' portion, SEQ ID NO:167, and a 5' portion, SEQ

ID NO:174), G418 resistance gene (for example broken into a 3' portion, SEQ ID NO:172, and a 5' portion, SEQ ID NO:175), zeocin resistance gene (for example broken into a 3' portion, SEQ ID NO:168, and a 5' portion, SEQ ID NO:169), nourseothricin N-acetyl transferase (NAT) (for example broken into a 3' portion, SEQ ID NO:171, and a 5' portion, SEQ ID NO:170), and invertase gene (SUC2) (for example a 3' portion of SEQ ID NO:173 and a 5' portion of SEQ ID NO:176).

[0034] The recombinant cells described herein include one or more exogenous polynucleotide sequences encoding one or more exogenous polypeptides that, when expressed improve the fermentation of glucose to ribitol by the recombinant cells.

[0035] The terms “glucose” and “dextrose” are used interchangeably herein and refer to D-glucose except where expressly indicated otherwise.

[0036] As used herein, “exogenous” refers to genetic material or an expression product thereof that originates from outside of the host organism. For example, the exogenous genetic material or expression product thereof can be a modified form of genetic material native to the host organism, it can be derived from another organism, it can be a modified form of a component derived from another organism, or it can be a synthetically derived component. For example, a *K. lactis* invertase gene is exogenous when introduced into *S. cerevisiae*.

[0037] As used herein, “native” refers to genetic material or an expression product thereof that is found, apart from individual-to-individual mutations which do not affect function or expression, within the genome of wild-type cells of the host cell. For the purposes of this application, the *Moniliella pollinis* cell “*Moniliella tomentosa* var *pollinis* TCV364” described in US 6,440,712, which is incorporated herein by reference in its entirety, and deposited under the Budapest Treaty at BCCM/MUCL (Belgian Coordinated Collections of Micro-organisms/Mycothèque de l'Université Catholique de Louvain by Eridania Béghin Say, Vilvoorde R&D Centre, Havenstraat 84, B-1800 Vilvoorde) on March 28, 1997 under number MUCL40385, is considered the wild-type *Moniliella pollinis* cell.

[0038] As used herein, the terms “polypeptide” and “peptide” are used interchangeably and refer to the collective primary, secondary, tertiary, and quaternary amino acid sequences and structure necessary to give the recited macromolecule its function and properties. As used herein, “enzyme” or “biosynthetic pathway enzyme” refer to a protein that catalyzes a chemical reaction. The recitation of any particular enzyme, either independently or as part of a biosynthetic pathway

is understood to include the co-factors, co-enzymes, and metals necessary for the enzyme to properly function. A summary of the amino acids and their three and one letter symbols as understood in the art is presented in Table 1. The amino acid name, three letter symbol, and one letter symbol are used interchangeably herein.

Table 1: Amino Acid three and one letter symbols

Amino Acid	Three letter symbol	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[0039] Variants or sequences having substantial identity or homology with the polypeptides described herein can be utilized in the practice of the disclosed recombinant cells, compositions, and methods. Such sequences can be referred to as variants or modified sequences. That is, a polypeptide sequence can be modified yet still retain the ability to exhibit the desired activity.

Generally, the variant or modified sequence may include greater than about 45%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity with the wild-type, naturally occurring polypeptide sequence, or with a variant polypeptide as described herein.

[0040] As used herein, the phrases “% sequence identity,” “% identity,” and “percent identity,” are used interchangeably and refer to the percentage of residue matches between at least two amino acid sequences or at least two nucleic acid sequences aligned using a standardized algorithm. Methods of amino acid and nucleic acid sequence alignment are well-known. Sequence alignment and generation of sequence identity include global alignments and local alignments which are carried out using computational approaches. An alignment can be performed using BLAST (National Center for Biological Information (NCBI) Basic Local Alignment Search Tool) version 2.2.31 software with default parameters. Amino acid % sequence identity between amino acid sequences can be determined using standard protein BLAST with the following default parameters: Max target sequences: 100; Short queries: Automatically adjust parameters for short input sequences; Expect threshold: 10; Word size: 6; Max matches in a query range: 0; Matrix: BLOSUM62; Gap Costs: (Existence: 11, Extension: 1); Compositional adjustments: Conditional compositional score matrix adjustment; Filter: none selected; Mask: none selected. Nucleic acid % sequence identity between nucleic acid sequences can be determined using standard nucleotide BLAST with the following default parameters: Max target sequences: 100; Short queries: Automatically adjust parameters for short input sequences; Expect threshold: 10; Word size: 28; Max matches in a query range: 0; Match/Mismatch Scores: 1, -2; Gap costs: Linear; Filter: Low complexity regions; Mask: Mask for lookup table only. A sequence having an identity score of XX% (for example, 80%) with regard to a reference sequence using the NCBI BLAST version 2.2.31 algorithm with default parameters is considered to be at least XX% identical or, equivalently, have XX% sequence identity to the reference sequence.

[0041] Polypeptide or polynucleotide sequence identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in

the tables, figures or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

[0042] The polypeptides disclosed herein may include “variant” polypeptides, “mutants,” and “derivatives thereof.” As used herein the term “wild-type” is a term of the art understood by skilled persons and means the typical form of a polypeptide as it occurs in nature as distinguished from variant or mutant forms. As used herein, a “variant,” “mutant,” or “derivative” refers to a polypeptide molecule having an amino acid sequence that differs from a reference protein or polypeptide molecule. A variant or mutant may have one or more insertions, deletions, or substitutions of an amino acid residue relative to a reference molecule.

[0043] The amino acid sequences of the polypeptide variants, mutants, derivatives, or fragments as contemplated herein may include conservative amino acid substitutions relative to a reference amino acid sequence. For example, a variant, mutant, derivative, or fragment polypeptide may include conservative amino acid substitutions relative to a reference molecule. “Conservative amino acid substitutions” are those substitutions that are a substitution of an amino acid for a different amino acid where the substitution is predicted to interfere least with the properties of the reference polypeptide. In other words, conservative amino acid substitutions substantially conserve the structure and the function of the reference polypeptide. Conservative amino acid substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge and/or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain.

[0044] As used herein, terms “polynucleotide,” “polynucleotide sequence,” and “nucleic acid sequence,” and “nucleic acid,” are used interchangeably and refer to a sequence of nucleotides or any fragment thereof. These phrases also refer to DNA or RNA of natural or synthetic origin, which may be single-stranded or double-stranded and may represent the sense or the antisense strand. The DNA polynucleotides may be a cDNA (e.g., coding DNA) or a genomic DNA sequence (e.g., including both introns and exons).

[0045] A polynucleotide is said to encode a polypeptide if, in its native state or when manipulated by methods known to those skilled in the art, it can be transcribed and/or translated to produce the polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

[0046] Those of skill in the art understand the degeneracy of the genetic code and that a variety of polynucleotides can encode the same polypeptide. In some aspects, the polynucleotides (e.g., polynucleotides encoding an XPDH polypeptide) may be codon-optimized for expression in a particular cell including, without limitation, a plant cell, bacterial cell, fungal cell, or animal cell. While polypeptides encoded by polynucleotide sequences found in various species are disclosed herein any polynucleotide sequences may be used which encodes a desired form of the polypeptides described herein. Thus, non-naturally occurring sequences may be used. These may be desirable, for example, to enhance expression in heterologous expression systems of polypeptides or proteins. Computer programs for generating degenerate coding sequences are available and can be used for this purpose. Pencil, paper, the genetic code, and a human hand can also be used to generate degenerate coding sequences.

[0047] The recombinant cells described herein may include deletions or disruptions in one or more native genes. The phrase “deletion or disruption” refers to the status of a native gene in the recombinant cell that has either a completely eliminated coding region (deletion) or a modification of the gene, its promoter, or its terminator (such as by a deletion, insertion, or mutation) so that the gene no longer produces an active expression product, produces severely reduced quantities of the expression product (e.g., at least a 75% reduction or at least a 90% reduction) or produces an expression product with severely reduced activity (e.g., at least 75% reduced or at least 90% reduced). The deletion or disruption can be achieved by genetic engineering methods, forced evolution, mutagenesis, RNA interference (RNAi), and/or selection and screening. The native gene to be deleted or disrupted may be replaced with an exogenous nucleic acid of interest for the expression of an exogenous gene product (e.g., polypeptide, enzyme, and the like).

[0048] The recombinant cells described herein may include one or more genetic modifications in which an exogenous nucleic acid is integrated into the genome of the host cell. One of skill in the art know how to select suitable loci in a yeast genome for integration of the exogenous nucleic acid. Suitable integration loci may include, but are not limited to, the *PDC1*, *GPD1*, *CYB2A*, *CYB2B*, *g4240*, *YMR226*, *MDHB*, *ATO2*, *Adh9091*, *Adh1202*, *ADE2*, *ADH2556*, *GAL6*, *MDH1*, *SCW11*, *ER1*, *ER3*, *pyrF*, *TRP3*, *gpdIIA*, and *gpdIIB* loci. For example, in a *M. pollinis* host cells, suitable interaction loci may include, but are not limited to, the *ER1* locus (defined as the locus flanked by SEQ ID NO:85 and SEQ ID NO:162), the *ER3* locus (defined as the locus flanked by SEQ ID NO:155 and SEQ ID NO:165), the *PDC1* locus (defined as the locus flanked

by SEQ ID NO:152 and SEQ ID NO:164), the *pyrF* locus (defined as the locus flanked by SEQ ID NO:153 and SEQ ID NO:163), the *TRP3* locus (defined as the locus flanked by SEQ ID NO:156 and SEQ ID NO:159), the *gpdIIA* locus (defined as the locus flanked by SEQ ID NO:157 and SEQ ID NO:161); and the *gpdIIB* locus (defined as the locus flanked by SEQ ID NO:158 and SEQ ID NO:166). The exogenous nucleic acid may also be integrated in an intergenic region or other location in the host cell genome not specifically specified herein. Other suitable integration loci may be determined by one of skill in the art. Furthermore, one of skill in the art would recognize how to use sequences to design primers to verify correct gene integration at the chosen locus.

[0049] The recombinant cell may have one or more copies of a given exogenous nucleic acid sequence integrated in a host chromosome(s) and replicated together with the chromosome(s) into which it has been integrated. For example, the yeast cell may be transformed with nucleic acid construct including a polynucleotide sequence encoding for a polypeptide described herein and the polynucleotide sequence encoding for the polypeptide may be integrated in one or more copies in a host chromosome(s). The recombinant cell may include multiple copies (two or more) of a given polynucleotide sequence encoding a polypeptide described herein. The recombinant cell may have one, two, three, four, five, six, seven, eight, nine, ten, or more copies of a polynucleotide sequence encoding a polypeptide described herein integrated into the genome. The multiple copies of said polynucleotide sequence may all be incorporated at a single locus or may be incorporated at multiple loci.

[0050] The recombinant cells described herein are capable of producing xylitol and include an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme. The exogenous polynucleotide sequence may be an exogenous xylitol-phosphate dehydrogenase (XPDH) gene. Following conversion of xylulose 5-phosphate to xylitol 5-phosphate, it is believed a native phosphatase enzyme removes the phosphate to produce xylitol.

[0051] A “xylitol-phosphate dehydrogenase gene” and an “XPDH gene” are used interchangeably herein and refer to any gene or polynucleotide that encodes a polypeptide with xylitol-phosphate dehydrogenase activity. As used herein, “xylitol-phosphate dehydrogenase activity” refer to the ability to catalyze the conversion of xylulose-5-phosphate and NADPH or NADH to xylitol 5-phosphate and NADP⁺ or NAD⁺. The XPDH gene may be derived from any suitable source. For example, the XPDH gene may be derived from *Clostridium difficile*, *Lactobacillus rhamnosus*, *Bacillus halodurans*, *Alkalihalobacillus ligniniphilus*, *Jeotgalibacillus*

*sol*i, *Heyndrickxia sporothermodurans*, *Clostridium fungisolvans*, or *Neobacillus cucumis*. The XPDH gene may encode an amino acid at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to the amino acid sequence of at least one of SEQ ID NOs:12-15, 28-32, or 33. The XPDH gene may encode an amino acid at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to the amino acid sequence of at least one of SEQ ID NOs:14, 15, 28, or 31.

[0052] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Clostridium difficile* gene encoding the amino acid of SEQ ID NO:12. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:12.

[0053] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Clostridium difficile* gene encoding the amino acid of SEQ ID NO:13. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:13.

[0054] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Lactobacillus rhamnosus* gene encoding the amino acid of SEQ ID NO:14. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:14.

[0055] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Bacillus halodurans* gene encoding the amino acid of SEQ ID NO:15. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:15.

[0056] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Alkalihalobacillus ligniniphilus* gene encoding the amino acid of SEQ ID NO:28. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least

60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:28.

[0057] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Jeotgalibacillus soli* gene encoding the amino acid of SEQ ID NO:29. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:29.

[0058] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Heyndrickxia sporothermodurans* gene encoding the amino acid of SEQ ID NO:30. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:30.

[0059] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Clostridium fungisolvans* gene encoding the amino acid of SEQ ID NO:31. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:31.

[0060] The recombinant cell may comprise an exogenous polynucleotide that is or may be derived from a *Neobacillus cucumis* gene encoding the amino acid of SEQ ID NO:33. The exogenous polynucleotide may encode an amino acid sequence with at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:33.

[0061] The exogenous polynucleotides in the recombinant cells described herein may be under the control of a promoter. For example, the exogenous nucleic acid may be operably linked to a heterologous or artificial promoter. Suitable promoters are known and described in the art. Promoters may include, but are not limited to, pyruvate decarboxylase promoter (PDC), translation elongation factor 2 promoter (TEF2), SED1, alcohol dehydrogenase 1A promoter (ADH1), hexokinase 2 promoter (HXK2), FLO5 promoter, pyruvate kinase 1 promoter (PYK1p; SEQ ID NO:86); 6-phosphogluconate dehydrogenase promoter (6PGDp; SEQ ID NO:130); glyceraldehyde-3-phosphate dehydrogenase promoter (TDH3p; SEQ ID NO:132); translational elongation factor 1 promoter (TEFp; SEQ ID NO:133); modified TEFp (SEQ ID NO:131);

phosphoglucomutase 1 promoter (PGM1p; SEQ ID NO:134); 3-phosphoglycerate kinase promoter (PGK1p; SEQ ID NO:135); enolase promoter (ENO1p ; SEQ ID NO:136); asparagine synthetase promoter (ASNSp; SEQ ID NO:137); 50S ribosomal protein L1 promoter (RPLAp; SEQ ID NO:138); and RPL16B (SEQ ID NO:139).

[0062] The exogenous nucleic acids in the recombinant cells described herein may be under the control of a terminator. For example, the exogenous nucleic acid may be operably linked to a heterologous or artificial terminator. Suitable terminators are known and described in the art. Terminators may include, but are not limited to, GAL10 terminator, PDC terminator, transaldolase terminator (TAL) 6PGD terminator (6PGDt; SEQ ID NO:140); ASNS terminator (ASNSt; SEQ ID NO:141); ENO1 terminator (ENO1t; SEQ ID NO:142); hexokinase 1 terminator (HXK1t; SEQ ID NO:143); PGK1 terminator (PGK1t; SEQ ID NO:144); PGM1 terminator (PGM1t; SEQ ID NO:145); PYK1 terminator (PYK1t; SEQ ID NO:146); RPLA terminator (RPLAt; SEQ ID NO:147); transaldolase 1 terminator (TAL1t; SEQ ID NO:148); TDH3 terminator (TDH3t; SEQ ID NO:149); translation elongation factor 2 terminator (TEF2t; SEQ ID NO:150); and triosephosphate isomerase 1 terminator (TPI1t; SEQ ID NO:151).

[0063] A promoter or terminator is “operably linked” to a given polynucleotide (e.g., a gene) if its position in the genome or expression cassette relative to said polynucleotide is such that the promoter or terminator, as the case may be, performs its transcriptional control function.

[0064] The polypeptides described herein may be provided as part of a construct. As used herein, the term “construct” refers to recombinant polynucleotides including, without limitation, DNA and RNA, which may be single-stranded or double-stranded and may represent the sense or the antisense strand. Recombinant polynucleotides are polynucleotides formed by laboratory methods that include polynucleotide sequences derived from at least two different natural sources or they may be synthetic. Constructs thus may include new modifications to endogenous genes introduced by, for example, genome editing technologies. Constructs may also include recombinant polynucleotides created using, for example, recombinant DNA methodologies. The construct may be a vector including a promoter operably linked to the polynucleotide encoding a polypeptide as described herein. As used herein, the term “vector” refers to a polynucleotide capable of transporting another polynucleotide to which it has been linked. The vector may be a plasmid, which refers to a circular double-stranded DNA loop into which additional DNA segments may be integrated.

[0065] The disclosure also provides fermentation methods for the production of xylitol using the recombinant cells described herein. The fermentation methods include the step of fermenting a substrate using the genetically engineered yeasts described herein to product xylitol. The fermentation method can include additional steps, as would be understood by a person skilled in the art. Non-limiting examples of additional process steps include maintaining the temperature of the fermentation broth within a predetermined range, adjusting the pH during fermentation, and isolating the xylitol from the fermentation broth. The fermentation process may be a fully aerobic process.

[0066] The fermentation method can be run using a suitable fermentation substrate. The substrate of the fermentation method can include glucose, sucrose, galactose, mannose, molasses, xylose, fructose, hydrolysates of starch, lignocellulosic hydrolysates, or a combination thereof. One skilled in the art will recognize what fermentation substrate is suitable for a given fermentation organism and system.

[0067] The fermentation process can be run under various conditions. The fermentation temperature, i.e., the temperature of the fermentation broth during processing, may be ambient temperature. Alternatively, or additionally, the fermentation temperature may be maintained within a predetermined range. For example, the fermentation temperature can be maintained in the range of 25 °C to 45 °C, 30 °C to 40 °C, or 32 °C to 37 °C, preferably about 35 °C. However, a skilled artisan will recognize that the fermentation temperature is not limited to any specific range or temperature recited herein and may be modified as appropriate.

[0068] The fermentation process can be run within certain oxygen uptake rate (OUR) ranges. The volumetric OUR of the fermentation process can be in the range of 0.5 to 40, 1 to 35, 2 to 30, 3 to 25, 4 to 20, or 5 to 15 mmol O₂/(L • h). In some embodiments, the specific OUR can be in the range of 0.05 to 10, 0.1 to 8, 0.15 to 5, 0.2 to 1, or 0.3 to 0.75 mmol O₂/(g cell dry weight • h). However, the volumetric or specific OURs of the fermentation process are not limited to any specific rates or ranges recited herein.

[0069] The fermentation process can be run at various cell concentrations. In some embodiments, the cell dry weight at the end of fermentation can be 5 to 40, 8 to 30, or 10 to 20 g cell dry weight/L. Further, the pitch density or pitching rate of the fermentation process can vary.

In some embodiments, the pitch density can be 0.05 to 11, 0.1 to 10, or 0.25 to 8 g cell dry weight/L.

[0070] The initial dextrose concentration of the fermentation may be at least 100, 200, 250, 300, 350, or at least 400 g/L dextrose. The initial dextrose concentration may be between 100 to 400, 150 to 350, or 250 to 325 g/L.

[0071] The fermentation process can be associated with various characteristics, such as, but not limited to, fermentation production rate, pathway fermentation yield, final titer, and peak fermentation rate. These characteristics can be affected by the selection of the yeast and/or genetic modification of the yeast used in the fermentation process. These characteristics can be affected by adjusting the fermentation process conditions. These characteristics can be adjusted via a combination of yeast selection or modification and the selection of fermentation process conditions.

[0072] The xylitol production rate of the process may be at least at least 0.2, 0.3, 0.5, 0.75, or at least 1.0 g L⁻¹ h⁻¹. The xylitol mass yield of the process may be at least 55 percent, at least 65 percent, at least 70 percent, at least 75 percent, at least 80 percent, or at least 85 percent. The final xylitol titer of the process may be at least 20, 30, 50, 75, or 100 g/L.

[0073] The fermentation process can be run as a dextrose-fed batch. Further, the fermentation process can be a batch process, continuous process, or semi-continuous process, as would be understood by a person skilled in the art.

EXAMPLES

[0074] The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1 – Xylitol-Phosphate Dehydrogenase Diversity

[0075] Roughly three thousand galactitol-1-phosphate-5-dehydrogenase (G1PDH)/xylitol-phosphate dehydrogenase (XPDH) enzyme sequences were obtained from Uniprot and analyzed.

FIG. 2 illustrates the natural sequence diversity for this set of sequences. This set is diverse, with ~25% of the enzymes having no homologue more than 75% identical. As these enzymes tend to prefer NAD to NADP as a cofactor, the cofactor binding preferences of the homologs were assessed in a manner similar to that described by Duax et al., (“Rational proteomics I. Fingerprinting identification and cofactor specificity in the short-chain oxidoreductase (SCOR) enzyme family,” *Proteins*, 2003, 53(4):931-943). Cofactor binding pockets were identified by proximity to the Rossman fold (+23 to +30 amino acids from the GXGXXG motif (SEQ ID NO:129)) and scored on the basis of total charge in an 8-residue window. The top 8 candidates that were predicted to use NADP were selected for further characterization, along with 4 candidates predicted to use NAD, and 3 controls.

[0076] Upon further review of the structural characteristics of the predicted binding pocket for factors that may influence cofactor preference, an important aspartate residue was identified. See FIG. 3. The polypeptide of SEQ ID NO:34 and substitutions thereof were used to construct a structural homology model to predict cofactor binding pocket confirmations FIG. 3 shows the C-terminal end of the penultimate β -strand on the outside of the Rossman Fold domain. Without wishing to be bound by any particular theory, it is predicted that enzymes in which the first residue in this region (residue 198 relative to SEQ ID NO:34) is an aspartate and the second residue (residue 199 relative to SEQ ID NO:34) is a large hydrophobic amino acid (for example, isoleucine) will prefer an NAD cofactor due to the hydrogen bonding of the aspartate to the hydroxyl groups of the NAD ribose. However, enzymes in which that first residue (residue 198 relative to SEQ ID NO:34) is an alanine, glycine, or serine and the second residue (residue 199 relative to SEQ ID NO:34) is lysine or arginine will prefer an NADP cofactor as the positive charge on the lysine or arginine residue will interact with the negative charge of the phosphate of the NADP and the smaller residue in the first position allows space in the binding pocket for said phosphate. Based on this analysis, 12 additional enzymes were selected for their predicted preference for NADP. Finally, 6 additional enzymes with sequence similarity to active XPDH enzymes were selected for testing.

Example 2 – TarJ’ Diversity

[0077] Roughly eight hundred ribulose 5-phosphate reductase sequences were obtained from Uniprot and analyzed. FIG. 3 illustrates the natural sequence diversity for this set of sequences.

Overall, the diversity in this set is low, as only 10% of the enzymes have no sequence similarity more than 75% identical. As these enzymes tend to prefer NADP to NAD as a cofactor, no scoring was performed, and the sequences were simply aligned in Geneious (ClustalW, default settings). Eight enzymes were selected for further analysis based on sequence similarity.

Example 3 – In vitro Enzyme Assays

[0078] Polynucleotides encoding suspected XPDH homologs (Table 2) or TarJ' homologs (Table 3) were cloned into a vector containing a T7 promoter and terminator for cell-free protein expression (New England Biolabs, PURExpress® In Vitro Protein Synthesis). Cell-free synthesized proteins were analyzed for activity on four substrates (ribulose 5-phosphate, xylulose 5-phosphate, ribulose, and xylulose) with either NADP or NAD cofactors. Seven enzymes (XPDH of SEQ ID NOs:12 and 34, TarJ' of SEQ ID NOs:36, 37, 38, 40, and 42), were able to catalyze the reduction of either ribulose 5-phosphate or xylulose 5-phosphate (FIG. 5) but not the reduction of xylulose or ribulose (data not shown).

Table 2: XPDH Homologs

Source Organism	Gene Name/Accession Numbers	Polypeptide SEQ ID NO:
<i>Mariniphaga sediminis</i>	D1164_16045	1
<i>Candidatus Dormibacteraeota bacterium</i>	DLM67_26945	2
<i>Candidatus Falkowbacteria bacterium</i>	COT93_01110	3
<i>Acidobacteria bacterium</i>	DMG39_01315	4
<i>Spirochaetaceae bacterium</i>	DCP56_03760	5
<i>Candidatus Hydrogenedentes bacterium</i>	ENW01_11985	6
<i>Rhizobium sp.</i>	E6Q76_20070	7
<i>Citrobacter freundii</i>	C2U38_22630	8
<i>Escherichia coli</i>	gatD	9
<i>Escherichia coli</i> (strain K12)	rspB	10
<i>Lactobacillus salivarius</i> cp400	LSCP400_11731	11
<i>Clostridium difficile</i>	WP_004454628.1	12

<i>Clostridium difficile</i>	WP_011860836.1	13
<i>Lactobacillus rhamnosus</i>	AAT02414.1	14
<i>Bacillus halodurans</i>	WP_010896369.1	15
<i>Lachnospiraceae bacterium</i>	A0A3C1WRV0_9FIRM	16
<i>Faecalibacterium sp.</i> OF03-6AC	DXA66_03105	17
<i>Agathobacter ruminis</i>	CSX02_08830	18
<i>Alicyclobacillus acidoterrestris</i>	AAC03nite_18160	19
<i>Ruminococcus sp.</i> KGMB03662	FFK04_01895	20
<i>Clostridiaceae bacterium</i>	D3Z58_08610	21
<i>Thermoplasmata archaeon</i>	A0A497HL05	22
<i>Lachnospiraceae bacterium</i>	A0A3B9I8G1	23
<i>Prevotella marshii</i> DSM 16973 = JCM 13450	E0NTD1	24
<i>Blautia sp.</i> OM05-6	A0A417S5N4	25
<i>Candidatus Bathyarchaeota archaeon</i>	A0A7C4RGE8	26
<i>Lachnospiraceae bacterium</i>	A0A7U9REN4	27

Table 3: TarJ' Homologs

Source Organism	Gene Name/Accession Numbers	Polypeptide SEQ ID NO:
<i>Staphylococcus aureus</i>	AMV81374.1	34
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> 71193	ST398NM01_0269	35
<i>Eubacterium ventriosum</i>	DW918_	36
<i>Pradoshia sp.</i> D12	F7984_17520	37
<i>Lactobacillus plantarum</i> EGD-AQ4	N692_02285	38
uncultured <i>Ruminococcus sp.</i>	DPQ25_03780	39
<i>Kandleria vitulina</i>	SAMN04487759_1396	40
<i>Lactiplantibacillus plantarum</i>	C7M34_02724 C7M35_00347 C7M36_02765 C7M47_01038	41

	FET70_01115 LPJSA22_01709 Nizo2802_1951	
<i>Lachnospiraceae bacterium</i>	IMSAGC019_01831	42

Example 4 – Genetically Modified *Moniliella pollinis* Strains

[0079] Strain 1-1 is the *Moniliella pollinis* host strain “*Moniliella tomentosa* var *pollinis* TCV364” described in US 6,440,712, which is incorporated herein by reference in its entirety, and deposited under the Budapest Treaty at BCCM/MUCL (Belgian Coordinated Collections of Micro-organisms/Mycothèque de l'Université Catholique de Louvain by Eridania Béghin Say, Vilvoorde R&D Centre, Havenstraat 84, B-1800 Vilvoorde) on March 28, 1997 under number MUCL40385. Table 4 below lists various *Moniliella pollinis* strains, including information on the parent strain, the sequence with which the parent strain was transformed, and characterizations of the expression cassette(s) contained on the transformed sequence. Each “XPDH/TarJ’ Homolog Expression Cassette” contained, in order, a 5’ ER1 flanking sequence (SEQ ID NO:85), a MpPYK1 promoter (SEQ ID NO:86), a gene encoding the indicated XPDH or TarJ’ homolog (one of SEQ ID NOs:87-128), a Mp6PGD terminator (SEQ ID NO:140), and a 5’ portion of a G418 resistance gene expression cassette (SEQ ID NO:175). Each “Selectable Marker Cassette” contained, in order, a 3’ portion of a G418 resistance gene expression cassette (SEQ ID NO:172), an MpTEF2 terminator (SEQ ID NO:150), and a 3’ ER1 flanking sequence (SEQ ID NO:160). Upon bipartite transformation with both the XPDH/TarJ’ Homolog Expression Cassette and the Selectable Marker Cassette, the two cassettes recombine for integration of both the nucleotide sequence encoding the XPDH or TarJ’ homolog and the G418 resistance marker at the ER1 locus.

[0080] The indicated *Moniliella pollinis* parent strain was transformed with the indicated sequence(s) by first protoplasting the parent strain by adding an enzyme mixture containing 0.6M MgSO₄, 7.5 g/L driselase, and 12.5 g/L *Trichoderma harzianum* lysing enzyme to a mycelial pellet of the parent strain. Protoplasts were then pelleted, washed with 0.6M MgSO₄, and resuspended in STC medium (0.6M sucrose, 50 mM CaCl₂, 10 mM Tris-HCl, pH 7.5). Bipartite transformations were prepared by adding 100 µg single stranded salmon sperm DNA and 1.5 to 5 µg each of the 5’ and 3’ DNA transformation fragments (3-10 µg total; see Table 4 for list of fragments) to approximately 200 µL protoplast mixture (10⁸ cells/mL). 1 mL 50% PEG in STC

medium was then added to the salmon sperm DNA, transformation DNA, and protoplast mixture and the resulting combination was incubated for 15 minutes at room temperature. Following incubation, recovery broth (0.4M sucrose, 1 g/L yeast extract, 1 g/L malt extract, 10 g/L glucose, pH 4.5) was added to the mixture and incubated at 27 °C, 100 rpm, for 16 to 24 hours. Following the incubation, protoplasts were pelleted by centrifugation and resuspended in 1 mL PBS.

[0081] The resuspended protoplasts were plated on PDA + 250 mg/L geneticin (G418) selection plates and incubated at 30-35 °C for at least 2-4 days until transformants grow. Resulting transformants were evaluated by colony PCR for integration of the indicated sequence. A PCR verified isolate was then designated as the indicated strain number. In some instances, more than one PCR verified isolate, e.g., “sister” isolates, are indicated by letters following the strain number. For example, strain 1-2 has 5 sister isolates, strains 1-2a, 1-2b, 1-2c, 1-2d, and 1-2e.

[0082] For example, Strain 1-1 was transformed with SEQ ID NO:43 and SEQ ID NO:44. SEQ ID NO:43 contains (i) 3' flanking DNA for targeted chromosomal integration into the ER1 locus (SEQ ID NO:162), and (ii) a 3' portion of the G418 resistance gene selectable marker (SEQ ID NO:172). SEQ ID NO:44 contains (i) an expression cassette for the XPDH homolog from *M. sediminis*, SEQ ID NO:87 encoding the amino acid sequence of SEQ ID NO:1, under the control of the PYK1 promoter of SEQ ID NO:86 and the PGD terminator of SEQ ID NO:140; (ii) 5' flanking DNA for targeted chromosomal integration into the ER1 locus (SEQ ID NO:85); and (iii) a 5' portion of the G418 resistance gene selectable marker (SEQ ID NO:175). Transformants were selected on PDA + 250 mg/L geneticin (G418) selection plates and incubated at 30-35 °C for at least 2 days until transformants grow. Resulting transformants were streaked for single colony isolation on PDA + geneticin (G418) plates and single colonies were selected. Selected colonies were evaluated by colony PCR for integration of the indicated sequence. PCR verified isolates were designated strains 1-2a, 1-2b, 1-2c, 1-2d, and 1-2e.

Table 4

Strain	Parent Strain	XPDH/TarJ' Homolog Expression Cassette			Selectable Marker Cassette
		Transformation SEQ ID NO:	Nucleotide SEQ ID NO:	Encoded Polypeptide SEQ ID NO:	Transformation SEQ ID NO:
1-2a-e	1-1	44	87	1	43
1-3a-e	1-1	45	88	2	43
1-4a-e	1-1	46	89	3	43
1-5a-e	1-1	47	90	4	43
1-6a-e	1-1	48	91	5	43
1-7a-e	1-1	49	92	6	43
1-8a-d	1-1	50	93	7	43
1-9a-e	1-1	51	94	8	43
1-10a-e	1-1	52	95	9	43
1-11a-e	1-1	53	96	10	43
1-12a-e	1-1	54	97	11	43
1-13a-f	1-1	55	98	12	43
1-14a-e	1-1	56	99	13	43
1-15a-f	1-1	57	100	14	43
1-16a-e	1-1	58	101	15	43
1-17a-e	1-1	59	102	16	43
1-18a-e	1-1	60	103	17	43
1-19a-e	1-1	61	104	18	43
1-20a-e	1-1	62	105	19	43
1-21a-e	1-1	63	106	20	43
1-22a-e	1-1	64	107	21	43
1-23a-e	1-1	65	108	22	43
1-24a-e	1-1	66	109	23	43
1-25a-e	1-1	67	110	24	43

1-26a-e	1-1	68	111	25	43
1-27a-e	1-1	69	112	26	43
1-28a-e	1-1	70	113	27	43
1-29a-e	1-1	71	114	28 (<i>Alkalihalobacillus ligniniphilus</i> XPDH WP_017729640.1)	43
1-30a-e	1-1	72	115	29 (<i>Jeotgalibacillus soli</i> XPDH WP_041086875.1)	43
1-31a-e	1-1	73	116	30 (<i>Heyndrickxia sporothermodurans</i> XPDH WP_066235909.1)	43
1-32a-e	1-1	74	117	31 (<i>Clostridium fungisolvans</i> XPDH WP_183276691.1)	43
1-33a-e	1-1	75	118	32 (<i>Alkalihalobacillus nanhaiisediminis</i> XPDH WP144449381.1)	43
1-34a-e	1-1	76	119	33 (<i>Neobacillus cucumis</i> XHPD WP_198313751.1)	43
1-35a-d	1-1	77	120	34	43
1-36a-e	1-1	78	121	35	43
1-37a-d	1-1	79	122	36	43
1-38a-f	1-1	80	123	37	43
1-39a-f	1-1	81	124	38	43
1-40a-e	1-1	82	125	39	43
1-41a-e	1-1	83	127	41	43
1-42a-f	1-1	84	128	42	43

Example 5 – Shake Flask Fermentation Assay

[0083] Strains 1-1, 1-35a-d, 1-37a-d, 1-38a-f, 1-39a-f, 1-42a-f, 1-13a-f, and 1-15a-f (outlined in Table 4 above), were run in shake flasks to assess glucose consumption as well as ribitol, xylitol, glycerol, and ethanol production.

[0084] Strains were streaked out for biomass growth on YPD plates (bacteriological peptone 20g/L, yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L) and incubated at 30 °C for 48-72 hours. Cells from the incubated YPD plates were scraped into 40 mL rich medium (170 g/L glucose, 10 g/L yeast extract) in a 250 mL non-baffled flask. Cells were incubated at 30 °C and 250 rpm until the optical density (OD600) reached 15-20 to form the seed culture. Optical density is measured at a wavelength of 600 nm with a 1 cm path length cuvette using a model Genesys20 spectrophotometer (Thermo Scientific). The seed culture reached an OD600 between 15-20 in about 32-50 hours.

[0085] A 250 ml non-baffled flask containing production medium (Table 5) was inoculated with 0.8 mL of the seed culture to form the production culture. The production culture was incubated at 35 °C and 250 rpm. Samples were taken from the production culture after 72 and 96 hours of incubation. Samples were analyzed for glucose, ribitol, xylitol, erythritol, glycerol, and ethanol by high performance liquid chromatography with refractive index detector. Fermentation results are reported in Table 6 and FIGS. 6 and 7.

Table 5: Production Medium

Component	Concentration (units)
Glucose	300 (g/L)
KH ₂ PO ₄	1.27 (g/L)
(NH ₄) ₂ HPO ₄	0.13 (g/L)
(NH ₄) ₂ SO ₄	1.80 (g/L)
Urea	2.85 (g/L)
Citric Acid	200.0 (mg/L)
MgSO ₄ ·7H ₂ O	690.2 (mg/L)
FeSO ₄ ·7H ₂ O	32.35 (mg/L)

ZnSO ₄ ·7H ₂ O	4.40 (mg/L)
MnSO ₄ ·H ₂ O	0.53 (mg/L)
CuSO ₄ ·5H ₂ O	0.45 (mg/L)
CaCl ₂ ·2H ₂ O	91.60 (mg/L)
Na ₂ SO ₄	154.50 (mg/L)
Thiamine-HCl	10 (mg/L)
Choline-Cl	100 (mg/L)
Antifoam CF-32	1.00 (g/L)

[0086] While PCR verification indicated that the transformed polynucleotide sequence was present in the indicated strains, further analysis indicated that in some strains, the sequence was not correctly integrated at the ER1 locus. Further analysis indicated that strains 1-35a, 1-37a-d, 1-38a-c, 1-39d-f, 1-42a-b, 1-42d, 1-13a-b, 1-13d-e, 1-15b-c, and 1-15e-f include the transformed polynucleotide sequence, but it is not integrated at the ER1 locus.

Table 6: 96-hour Shake Flask Results

Strain	Fermentation Broth Analyte (g/L)					
	Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-1	54.2270	0.5672	0.1156	56.9108	14.4101	45.2717
1-35a	19.0690	77.8018	5.5078	14.1262	11.6922	45.6143
1-35b	22.4343	69.5363	2.6051	18.4159	6.7667	51.1619
1-35c	31.4030	66.5826	2.4193	17.3705	7.5255	49.2545
1-35d	28.2827	77.4169	5.2674	13.9265	7.1954	49.1733
1-37a	42.3156	58.8415	1.1308	17.6218	8.1394	44.6216
1-37b	92.0525	30.1794	0.5249	29.1345	17.4800	28.4251
1-37c	77.9520	25.8442	0.3365	37.6779	28.5369	33.4608
1-37d	62.2726	0.9251	0.2062	50.0038	18.7245	45.7655
1-38a	19.5123	89.5643	4.5079	13.9258	6.0135	46.2436
1-38b	15.4952	97.2570	7.8962	13.6124	6.7302	35.1221
1-38c	9.1530	100.4624	8.8382	14.4767	6.4634	41.8789

1-38d	3.5262	93.3559	5.1135	14.0524	7.8424	46.9099
1-38e	5.8345	91.9564	4.7706	13.7265	7.3560	48.8018
1-38f	5.6278	90.6644	4.9442	13.6917	7.7348	47.5447
1-39a	18.6244	72.8649	2.8000	19.1466	8.1946	40.2287
1-39b	2.3933	100.5639	10.0874	14.2583	6.9797	44.4668
1-39c	31.7163	67.5513	2.4932	17.8548	7.1724	47.5427
1-39d	3.3290	101.3044	9.6496	13.9511	6.9558	46.4375
1-39e	38.9049	66.5485	2.4033	16.8345	7.1407	48.7270
1-39f	0.8569	91.5653	11.1004	24.3341	6.1712	37.6166
1-42a	53.2303	1.3617	0.1586	62.1601	16.1305	44.7835
1-42b	70.3640	0.5390	0.1319	52.0362	13.1695	44.2371
1-42c	54.3271	0.6740	0.1317	61.0264	17.1397	44.9797
1-42d	55.3052	0.7063	0.1484	63.2909	12.3513	43.1762
1-42e	61.0003	0.5738	0.1502	56.8237	13.3096	39.6208
1-42f	64.0496	0.5451	0.1352	55.2579	13.6874	42.7263
1-13a	58.2322	3.0713	39.0606	24.5223	9.9512	43.5850
1-13b	64.5326	2.8987	38.2994	23.4455	9.2364	42.1528
1-13c	62.8811	2.8168	38.3638	23.0771	9.2614	43.8806
1-13d	47.8236	3.3178	40.0543	25.7124	9.9847	40.8596
1-13e	59.3667	4.8671	28.8414	30.2839	9.5968	42.2960
1-13f	66.1336	2.8202	38.0642	22.4811	8.8765	42.3954
1-15a	58.6182	8.6323	38.8703	21.1152	9.3006	43.9871
1-15b	78.7837	1.2617	12.2069	34.2608	11.5806	43.9409
1-15c	55.9848	1.1212	10.2043	47.6605	12.0438	44.2130
1-15d	54.0847	8.9671	38.7047	21.4173	9.3022	44.1645
1-15e	60.7735	1.9279	16.0149	43.7036	11.1713	46.0607
1-15f	102.6556	8.2515	30.3843	19.3150	13.0177	32.7172

Example 6 – Shake Flask Fermentation Assay

[0087] Strains 1-13c, 1-29a-e, 1-33a-e, and 1-34a-e (outlined in Table 4 above), were run in shake flasks to assess glucose consumption as well as ribitol, xylitol, glycerol, and ethanol production.

[0088] Strains were streaked out for biomass growth on YPD plates (bacteriological peptone 20g/L, yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L) and incubated at 30 °C for 48-72 hours. Cells from the incubated YPD plates were scraped into 40 mL rich medium (170 g/L glucose, 10 g/L yeast extract) in a 250 mL non-baffled flask. Cells were incubated at 30 °C and 250 rpm until the optical density (OD600) reached 15-20 to form the seed culture. Optical density is measured at a wavelength of 600 nm with a 1 cm path length cuvette using a model Genesys20 spectrophotometer (Thermo Scientific). The seed culture reached an OD600 between 15-20 in about 32-50 hours.

[0089] A 250 ml non-baffled flask containing production medium (Table 5) was inoculated with 0.8 mL of the seed culture to form the production culture. The production culture was incubated at 35 °C and 250 rpm. Samples were taken from the production culture after 96 hours of incubation. Samples were analyzed for glucose, ribitol, xylitol, erythritol, glycerol, and ethanol by high performance liquid chromatography with refractive index detector. Fermentation results are reported in Table 7 and FIG. 8.

[0090] As seen in FIG. 8, while sister strains 1-34c and 1-34d produced 15.8 and 18.6 g/L xylitol, respectively, strains 1-34a, 1-34b, and 1-34e did not produce significantly more xylitol than wild-type (strain 1-1, FIG. 6). While strains 1-34a, 1-34b, and 1-34e were initially PCR verified, it was later determined that the integrated polynucleotide, which should encode the *N. cucumis* XPDH homolog, contained a frameshift mutation and no functional XPDH was expressed. Therefore, while the results appear varied, they are in fact consistent given that strains 1-34a, 1-34b, and 1-34e did not contain a polynucleotide that encoded a functional XPDH.

Table 7: 96-hour Shake Flask Results

Strain	Fermentation Broth Analyte (g/L)					
	Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-13c	104.7609	2.4041	25.3431	20.3872	8.3483	28.8597
1-29a	107.59	2.7375	23.9236	21.2592	8.327	29.7606
1-29b	117.0722	2.8833	21.624	21.9028	7.5941	30.1983

1-29c	92.8027	3.0122	25.0203	22.3197	8.607	34.1865
1-29d	113.7418	2.654	22.973	21.1195	7.992	30.2967
1-29e	106.1983	2.7505	24.6828	21.1105	8.3573	30.0138
1-33a	102.5508	0.4847	0.2175	48.9982	9.7203	31.4812
1-33b	101.4479	0.507	0.0751	50.0129	9.8179	31.3012
1-33c	231.4215	0.2896	0.0353	6.9913	16.2002	6.2899
1-33d	107.2532	0.5626	0.0542	48.3365	10.36	31.982
1-33e	89.76	0.5258	0.0847	54.4229	10.6124	27.6685
1-34a	97.4677	0.487	0.0849	49.4197	10.4031	31.8369
1-34b	99.6607	0.4724	0.0682	49.2765	9.7575	31.2798
1-34c	125.3303	1.7402	15.7865	23.7361	8.5658	28.3016
1-34d	109.6517	2.4549	18.5615	26.2014	8.1791	30.7102
1-34e	92.4856	0.434	0.22	47.7303	9.2374	32.5853

Example 7 – Shake Flask Fermentation Assay

[0091] Strains 1-13c, 1-17a-e, 1-18a-e, 19a-e, 1-21a-e, 1-22a-e, 1-23a-e, 1-24a-e, 1-25a-e, and 1-27a-d (outlined in Table 4 above), were run in shake flasks to assess glucose consumption as well as ribitol, xylitol, glycerol, and ethanol production.

[0092] Strains were streaked out for biomass growth on YPD plates (bacteriological peptone 20g/L, yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L) and incubated at 30 °C for 48-72 hours. Cells from the incubated YPD plates were scraped into 40 mL rich medium (170 g/L glucose, 10 g/L yeast extract) in a 250 mL non-baffled flask. Cells were incubated at 30 °C and 250 rpm until the optical density (OD₆₀₀) reached 15-20 to form the seed culture. Optical density is measured at a wavelength of 600 nm with a 1 cm path length cuvette using a model Genesys20 spectrophotometer (Thermo Scientific). The seed culture reached an OD₆₀₀ between 15-20 in about 32-50 hours.

[0093] A 250 ml non-baffled flask containing production medium (Table 5) was inoculated with 0.8 mL of the seed culture to form the production culture. The production culture was incubated at 35 °C and 250 rpm. Samples were taken from the production culture after 96 hours of incubation. Samples were analyzed for glucose, ribitol, xylitol, erythritol, glycerol, and ethanol

by high performance liquid chromatography with refractive index detector. Fermentation results are reported in Table 8.

Table 8: 96-hour Shake Flask Results

Strain	Fermentation Broth Analyte (g/L)					
	Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-13a	98.6422	2.6387	24.7712	25.2307	13.8005	28.8903
1-17a	74.915	0.5991	0.2543	60.8569	13.0048	33.3547
1-17b	79.2438	0.577	0.0725	58.9302	12.8216	35.3409
1-17c	151.0953	0.4237	0.0975	24.7655	21.0532	20.2202
1-17d	90.0013	1.4266	0.0645	55.808	18.8406	27.8706
1-17e	84.2381	0.7091	0.0428	59.7786	15.6082	29.9387
1-18a	187.0194	0.3163	0.1069	21.6446	15.8366	10.9522
1-18b	83.2939	0.6768	0.0524	63.3983	14.7387	31.6461
1-18c	88.21	0.6902	0.0625	59.74	14.7915	31.5115
1-18d	76.4267	0.8183	0.0692	57.1505	16.2282	34.0999
1-18e	89.0621	0.7336	0.0841	42.5495	26.7038	25.7627
1-19a	94.518	0.6166	0.0801	54.4468	18.8522	28.9871
1-19b	83.559	0.6632	0.0659	60.2352	14.9726	31.2065
1-19c	93.3168	0.7412	0.0646	60.6521	14.3042	29.9464
1-19d	165.946	0.4311	0.1236	24.1998	15.9581	19.0694
1-19e	78.3357	0.675	0.0637	61.3653	15.847	29.361
1-21a	77.8724	0.882	0.0789	65.2592	16.0186	31.302
1-21b	85.2124	0.6777	0.0619	56.1455	15.4518	33.3606
1-21c	85.008	1.0769	0.0802	58.6289	14.8934	32.8785
1-21d	83.4408	1.9855	0.0373	56.0976	14.2115	34.3856
1-21e	60.5121	2.039	0.0418	63.3641	15.5567	31.3166
1-22a	96.2241	0.8385	0.0644	62.4003	13.1106	29.8987
1-22b	90.5783	0.5506	0.0722	54.306	13.9584	34.1293
1-22c	85.3285	0.6442	0.0595	60.2244	15.0443	31.2101

1-22d	87.0015	0.6859	0.0824	59.9578	14.9889	31.3812
1-22e	78.5983	0.5432	0.0718	58.3192	14.9655	30.6604
1-23a	84.0794	0.4999	0.109	52.3855	13.872	35.266
1-23b	84.7042	0.5799	0.0696	58.9566	15.0869	33.9501
1-23c	87.9261	0.7983	0.0538	55.5429	17.3164	30.1523
1-23d	87.2081	0.6745	0.0623	59.4328	14.8259	31.3395
1-23e	82.9585	1.0568	0.0795	62.242	18.8816	23.5949
1-24a	86.7546	0.534	0.0725	53.0638	13.0757	34.4192
1-24b	104.4802	0.583	0.0821	48.7903	14.9732	29.8021
1-24c	104.5435	0.563	0.0872	48.1204	14.5086	31.0036
1-24d	169.2148	0.425	0.1078	25.2327	14.0784	19.6567
1-24e	78.1939	0.5172	0.082	55.4618	15.2743	36.423
1-25a	85.5913	0.8201	0.0711	57.2974	18.9442	29.1298
1-25b	83.3265	0.6891	0.0801	60.4083	15.034	31.4877
1-25c	139.0213	0.6194	0.1079	28.2606	22.9621	22.0197
1-25d	137.1956	0.6387	0.1254	28.3686	22.7144	22.2391
1-25e	78.0893	0.6945	0.0792	61.3866	15.5101	31.7923
1-27a	54.0049	0.8002	0.0653	73.3789	15.1766	36.3078
1-27b	88.2258	0.5067	0.0543	53.4474	13.5761	34.0372
1-27c	114.4683	0.7334	0.0729	43.7274	17.9455	26.3899
1-27d	76.682	0.5437	0.0552	58.8882	12.8684	38.8414

Example 8 – Shake Flask Fermentation Assay

[0094] Strains 1-13c, 1-3a-e, 1-10a-e, 1-11a-e, 1-12a-e, 1-14a-e, 1-16a-e, 1-28a-e, and 1-2a-e (outlined in Table 4 above), were run in shake flasks to assess glucose consumption as well as ribitol, xylitol, glycerol, and ethanol production.

[0095] Strains were streaked out for biomass growth on YPD plates (bacteriological peptone 20g/L, yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L) and incubated at 30 °C for 48-72 hours. Cells from the incubated YPD plates were scraped into 40 mL rich medium (170 g/L glucose, 10 g/L yeast extract) in a 250 mL non-baffled flask. Cells were incubated at 30 °C and 250 rpm until the optical density (OD600) reached 15-20 to form the seed culture. Optical density

is measured at a wavelength of 600 nm with a 1 cm path length cuvette using a model Genesys20 spectrophotometer (Thermo Scientific). The seed culture reached an OD₆₀₀ between 15-20 in about 32-50 hours.

[0096] A 250 ml non-baffled flask containing production medium (Table 5) was inoculated with 0.8 mL of the seed culture to form the production culture. The production culture was incubated at 35 °C and 250 rpm. Samples were taken from the production culture after 72 and 96 hours of incubation. Samples were analyzed for glucose, ribitol, xylitol, erythritol, glycerol, and ethanol by high performance liquid chromatography with refractive index detector. Fermentation results are reported in Table 9 and FIG. 9.

[0097] While PCR verification indicated that the transformed polynucleotide sequence was present in the indicated strains, further analysis indicated that in some strains, the sequence was not correctly integrated at the ER1 locus. Further analysis indicated that strain 1-16b-e includes the transformed polynucleotide sequence, but it is not at the ER1 locus. Further analysis was inconclusive on the integration location in strains 1-2c and 1-2d.

Table 9: 96-hour Shake Flask Results

Strain	Fermentation Broth Analyte (g/L)						
	Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol	Arabitol
1-13c	52.365	3.1859	40.2435	26.0209	11.1145	37.6398	
1-3a	58.7394	0.6129	0.3235	59.7362	15.12	40.891	
1-3b	40.836	0.4677	0.1051	49.0415	15.6583	51.2491	
1-3c	55.8652	0.5887	0.1253	59.2426	14.1511	42.761	
1-3d	47.4122	0.5038	0.1279	50.8589	16.8177	41.1038	
1-3e	45.8606	0.5127	0.1267	50.1914	16.3743	48.2382	
1-10a	58.5369	0.5891	0.1201	57.9712	15.3885	40.4663	
1-10b	58.9318	0.6075	0.1492	57.9368	14.8489	41.9797	
1-10c	67.992	0.4588	0.142	49.0939	14.307	40.394	
1-10d	46.0003	0.6906	0.0995	62.4692	19.849	32.9163	
1-10e	55.0568	0.655	0.136	61.0313	15.9185	38.2694	
1-11a	51.9376	0.429	0.0526	60.4417	14.8425	40.9479	

1-11b	45.6503	0.554	0.1326	61.3534	18.1726	44.3344	
1-11c	5.6815	1.5471	0.0801	86.3607	27.719	41.9823	
1-11d	40.3772	0.5489	0.1304	61.8324	18.171	41.9493	
1-11e	53.0558	0.6124	0.115	60.6504	15.489	39.7766	
1-12a	30.456	n.a.	n.a.	41.1173	9.6187	44.7618	46.2065
1-12b	28.2127	n.a.	n.a.	41.7725	9.3555	45.1461	47.4416
1-12c	45.2228	n.a.	n.a.	41.1422	9.0341	39.2179	42.4228
1-12d	30.6334	n.a.	n.a.	36.381	10.0025	39.6166	44.7009
1-12e	81.9896	n.a.	n.a.	34.348	12.8173	30.9024	30.2901
1-14a	36.1578	60.3639	19.8883	14.677	6.9789	39.9443	
1-14b	29.7316	53.6709	14.9948	11.3645	12.08	43.9787	
1-14c	27.9273	59.5839	17.4099	13.471	7.8201	45.0742	
1-14d	13.2601	64.6881	20.7494	15.3596	7.6729	41.5184	
1-14e	24.8568	64.1917	20.7387	15.176	7.2937	39.5752	
1-16a	53.8198	4.4367	36.058	32.5055	10.8652	39.1211	
1-16b	52.0735	4.9579	38.9526	23.8416	10.1467	41.0993	
1-16c	55.1727	1.6863	24.425	36.6549	11.7111	41.8186	
1-16d	45.5429	5.6345	22.6578	36.7881	11.2977	41.0565	
1-16e	41.996	2.3924	19.2866	43.8223	17.3556	40.5198	
1-28a	57.3853	0.6449	0.2041	58.1115	15.5452	41.2408	
1-28b	58.7477	0.5753	0.1016	56.9917	14.4135	41.1243	
1-28c	59.1807	0.5957	0.1202	57.6647	14.5907	42.3829	
1-28d	32.0943	0.6578	0.1353	62.5783	18.1666	40.946	
1-28e	56.5966	0.5859	0.1193	57.5414	14.0459	41.5865	
1-2a	50.0384	0.7556	0.1162	61.3047	15.3111	41.1167	
1-2b	34.3286	0.79	0.1848	58.4062	19.6802	48.8752	
1-2c	43.167	0.7455	0.0948	70.8234	11.4465	42.3114	
1-2d	41.842	0.6423	0.1082	63.7359	15.0666	39.6665	
1-2e	47.5807	0.6122	0.1415	60.543	13.8641	43.2135	

Example 9 – Shake Flask Fermentation Assay

[0098] Strains 1-13c, 1-8a-d, 1-26a-e, 1-36a-e, 1-41a-e, 1-40a-e, and 1-20a-e (outlined in Table 4 above), were run in shake flasks to assess glucose consumption as well as ribitol, xylitol, glycerol, and ethanol production.

[0099] Strains were streaked out for biomass growth on YPD plates (bacteriological peptone 20g/L, yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L) and incubated at 30 °C for 48-72 hours. Cells from the incubated YPD plates were scraped into 40 mL rich medium (170 g/L glucose, 10 g/L yeast extract) in a 250 mL non-baffled flask. Cells were incubated at 30 °C and 250 rpm until the optical density (OD600) reached 15-20 to form the seed culture. Optical density is measured at a wavelength of 600 nm with a 1 cm path length cuvette using a model Genesys20 spectrophotometer (Thermo Scientific). The seed culture reached an OD600 between 15-20 in about 32-50 hours.

[0100] A 250 ml non-baffled flask containing production medium (Table 5) was inoculated with 0.8 mL of the seed culture to form the production culture. The production culture was incubated at 35 °C and 250 rpm. Samples were taken from the production culture after 96 hours of incubation. Samples were analyzed for glucose, ribitol, xylitol, erythritol, glycerol, and ethanol by high performance liquid chromatography with refractive index detector. Fermentation results are reported in Table 10 and FIG. 10.

[0101] While PCR verification indicated that the transformed polynucleotide sequence was present in the indicated strains, further analysis indicated that in some strains, the sequence was not correctly integrated at the ER1 locus. Further analysis indicated that strains 1-8c, 1-8d and 1-41c include the transformed polynucleotide sequence, but it is not integrated at the ER1 locus. Further analysis was inconclusive on integration locus in strains 1-36a, 1-41b, 1-41e, and 1-20a-e.

Table 10: 96-hour Shake Flask Results

Strain	Fermentation Broth Analyte (g/L)					
	Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-13c	43.7126	3.1176	38.8613	24.5197	9.666	42.859
1-8a	46.3518	0.537	0.3381	57.0411	14.4597	46.2915
1-8b	47.5195	0.5334	0.1431	56.9461	13.7069	47.0977

1-8c	48.0894	0.5594	0.1063	55.9984	13.6091	47.8657
1-8d	37.4186	0.6121	0.1105	56.5403	13.8863	51.6109
1-26a	44.7559	0.6392	0.1211	58.7843	13.0302	45.1907
1-26b	26.8789	0.7454	0.1365	65.6702	14.7237	48.8557
1-26c	42.4506	0.7303	0.1018	65.269	12.7456	45.1127
1-26d	54.3955	0.5664	0.0934	50.543	16.7051	47.0098
1-26e	36.6369	0.7275	0.1162	70.0149	10.7288	48.273
1-36a	43.9038	23.4607	0.4003	37.8737	12.3842	43.023
1-36b	46.2296	17.4734	0.2509	28.3498	17.1992	51.7303
1-36c	33.5473	22.7172	0.384	32.9889	10.9137	53.8241
1-36d	30.3483	21.9108	0.3459	32.6397	12.4463	55.6903
1-36e	34.2625	22.4675	0.4111	33.3163	10.3248	53.7075
1-41a	50.84	0.6102	0.1238	53.2111	12.3563	47.0405
1-41b	27.0994	0.9407	0.0998	67.672	11.9654	54.0057
1-41c	54.6053	0.5387	0.1112	52.0424	14.0354	47.3747
1-41d	58.9086	0.6276	0.1701	51.36	21.0093	44.2126
1-41e	48.8262	0.5743	0.1143	57.4473	13.8819	45.8719
1-40a	38.7776	39.7726	0.2401	21.5304	12.246	44.721
1-40b	44.7092	49.927	0.3738	22.8583	10.4058	44.314
1-40c	43.6888	31.47	0.2197	29.7888	10.3401	50.5487
1-40d	43.0144	32.1802	0.1935	28.5121	13.9717	49.4035
1-40e	38.1811	32.5265	0.2004	31.8564	10.647	51.5381
1-20a	43.2545	0.7591	0.0973	60.0985	13.8425	44.7316
1-20b	44.7893	0.7386	0.1007	69.3394	13.0988	46.5606
1-20c	29.207	0.673	0.1105	63.3536	16.4597	49.4451
1-20d	39.0349	0.612	0.1224	57.5853	14.8878	49.7504
1-20e	48.061	0.7296	0.1017	61.7944	10.9017	47.5667

Example 10 – Shake Flask Fermentation Assay

[0102] Strains 1-13c, 1-30a-e, 1-31a-e, 1-32a-e, 1-4a-e, 1-5a-e, 1-6a-e, 1-7a-e, and 1-9a-e (outlined in Table 4 above), were run in shake flasks to assess glucose consumption as well as ribitol, xylitol, glycerol, and ethanol production.

[0103] Strains were streaked out for biomass growth on YPD plates (bacteriological peptone 20g/L, yeast extract 10 g/L, glucose 20 g/L, and agar 15 g/L) and incubated at 30 °C for 48-72 hours. Cells from the incubated YPD plates were scraped into 40 mL rich medium (170 g/L glucose, 10 g/L yeast extract) in a 250 mL non-baffled flask. Cells were incubated at 30 °C and 250 rpm until the optical density (OD600) reached 15-20 to form the seed culture. Optical density is measured at a wavelength of 600 nm with a 1 cm path length cuvette using a model Genesys20 spectrophotometer (Thermo Scientific). The seed culture reached an OD600 between 15-20 in about 32-50 hours.

[0104] A 250 ml non-baffled flask containing production medium (Table 5) was inoculated with 0.8 mL of the seed culture to form the production culture. The production culture was incubated at 35 °C and 250 rpm. Samples were taken from the production culture after 72 and 96 hours of incubation. Samples were analyzed for glucose, ribitol, xylitol, erythritol, glycerol, and ethanol by high performance liquid chromatography with refractive index detector. Fermentation results are reported in Table 11 and FIG. 11.

[0105] While PCR verification indicated that the transformed polynucleotide sequence was present in the indicated strains, further analysis indicated that in some strains, the sequence was not correctly integrated at the ER1 locus. Further analysis indicated that strains 1-30c and 1-30d include the transformed polynucleotide sequence, but it is not integrated at the ER1 locus. Further analysis was inconclusive on the integration locus in strain 1-6c.

Table 11: 96-hour Shake Flask Results

Strain	Fermentation Broth Analyte (g/L)					
	Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-13c	65.2534	2.6255	37.1061	24.3306	10.3659	40.8187
1-13c	63.7146	2.5391	36.7742	22.9396	9.9483	41.7289
1-30a	64.53	2.2035	28.9854	29.1288	11.3525	39.7976
1-30b	58.571	3.5146	29.9395	25.1729	12.5878	42.2656
1-30c	61.7808	1.8694	26.2541	26.3438	15.1573	43.1151

1-30d	57.4209	1.9473	26.054	27.0466	13.4411	44.8634
1-30e	74.7224	2.7504	28.386	28.8879	14.2582	37.7887
1-31a	65.2966	1.7919	23.8031	38.9002	11.2516	37.7702
1-31b	58.0672	1.528	25.7531	34.4798	12.434	40.5374
1-31c	60.9593	1.4068	24.8131	31.8421	11.6425	41.8413
1-31d	60.5991	1.4246	25.033	31.5056	11.5117	42.3126
1-31e	65.0496	1.7865	25.7844	33.8874	12.8272	40.8807
1-32a	67.1283	2.236	38.4541	23.5687	11.1949	38.707
1-32b	70.2815	2.1497	38.3141	22.7553	10.5468	38.8618
1-32c	68.0248	2.2063	38.5515	22.7602	10.3843	38.5577
1-32d	66.3494	2.1502	38.3931	21.9976	11.0603	40.7174
1-32e	63.8648	2.5133	38.8442	23.9287	10.8515	39.9071
1-4a	70.2783	0.4834	0.3673	46.1316	14.32	43.4293
1-4b	72.2855	0.7763	0.1831	57.21	19.3193	35.128
1-4c	56.1849	0.5101	0.1291	47.8537	15.7451	47.9659
1-4d	77.2722	0.5838	0.1901	50.3863	14.1389	39.9826
1-4e	70.9407	0.4671	0.1525	46.2662	15.3398	44.2176
1-5a	56.7359	0.6505	0.1281	69.7951	13.2652	40.3786
1-5b	66.4853	0.4758	0.1138	47.5548	17.2347	44.8984
1-5c	56.2946	0.4561	0.1072	46.0599	15.9891	48.8712
1-5d	63.8624	0.5188	0.1223	55.0207	15.5579	41.5538
1-5e	53.5706	0.4438	0.0978	46.4767	15.7478	49.9892
1-6a	53.5487	0.4466	0.0857	46.0621	14.3516	50.2479
1-6b	56.3098	0.5689	0.0987	59.4119	14.2449	44.3129
1-6c	62.2098	0.4975	0.1159	50.7104	13.912	46.5117
1-6d	88.3493	0.392	0.0963	46.7061	15.7906	39.0682
1-6e	66.3903	0.513	0.1236	54.7569	15.6941	42.6935
1-7a	84.3102	0.7204	0.0924	52.2635	19.827	33.7597
1-7b	69.0519	0.4897	0.1187	51.9057	14.6164	43.9185
1-7c	67.7409	0.5193	0.1426	53.5386	14.7419	42.8362

1-7d	61.1123	0.5279	0.1161	54.4933	14.502	43.378
1-7e	58.0693	0.4778	0.1356	50.1206	18.9325	46.1013
1-9a	52.6189	0.5986	0.1156	66.6905	13.1314	43.3405
1-9b	59.3034	0.464	0.1102	58.2455	14.7562	43.3825
1-9c	53.516	0.4302	0.1246	51.9753	15.0032	48.9462
1-9d	53.9501	0.4221	0.0876	46.6648	15.398	49.7159
1-9e	54.575	0.4136	0.0957	46.5254	15.3139	49.828

CLAIMS

What is claimed is:

1. A genetically engineered yeast cell capable of producing xylitol, the engineered yeast cell comprising:
an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme.
2. The yeast cell of claim 1, wherein the XPDH enzyme comprises a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33.
3. The yeast cell of claim 1 or claim 2, wherein the yeast cell is an osmotolerant yeast cell.
4. The yeast cell of any one of claims 1-3, wherein the yeast cell is a cell of the subphylum Ustilaginomycotina.
5. The yeast cell of any one of claims 1-4, wherein the yeast cell is selected from the group consisting of *Trichosporonoides megachiliensis*, *Trychosporonoides oedocephalis*, *Trychosporonoides nigrescens*, *Pseudozyma tsukubaensis*, *Trigonopsis variabilis*, *Moniliella*, *Ustilaginomycetes*, *Trichosporon*, *Yarrowia lipolytica*, *Penicillium*, *Torula*, *Pichia*, *Candida*, *Candida magnoliae*, and *Aureobasidium*
6. The yeast cell of any one of claims 1-5, wherein the yeast cell is a yeast cell of the *Moniliella* genus.

7. A genetically engineered *Moniliella* cell capable of producing xylitol, the engineered *Moniliella* cell comprising:
 - an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33.
8. The yeast cell of any one of claims 1-7, wherein the cell is a *Moniliella pollinis* cell.
9. The yeast cell of any one of claims 1-8, wherein the yeast cell is capable of producing xylitol at a titer of at least 20, 30, 50, 75, or 100 g/L when used in a fermentation process in the presence of dextrose at 35 ° for 96 hours.
10. The yeast cell of any one of claims 1-9, wherein erythritol production by the yeast cell is reduced relative to erythritol production in an equivalent yeast cell lacking the exogenous polynucleotide sequence.
11. The yeast cell of any one of claims 1-10, wherein the exogenous polynucleotide sequence is integrated into the genome of the yeast cell at a loci selected from the ER1 locus, the ER3 locus, the PDC1 locus, the pyrF locus, the TRP3 locus, the gpdIIA locus, and the gpdIIB locus.
12. The yeast cell of any one of claims 1-11, wherein the exogenous polynucleotide sequence is operably linked to a heterologous or artificial promoter.
13. The yeast cell of claim 12, wherein the promoter is a constitutive promoter.

14. The yeast cell of claim 12 or claim 13, wherein the constitutive heterologous or artificial promoter is selected from the group consisting of pyruvate kinase 1 promoter (PYK1p; SEQ ID NO:86), 6-phosphogluconate dehydrogenase promoter (6PGDp; SEQ ID NO:130), glyceraldehyde-3-phosphate dehydrogenase promoter (TDH3p; SEQ ID NO:132), translational elongation factor 1 promoter (TEFp; SEQ ID NO:133), modified TEFp (SEQ ID NO:131), phosphoglucomutase 1 promoter (PGM1p; SEQ ID NO:134), 3-phosphoglycerate kinase promoter (PGK1p; SEQ ID NO:135), enolase promoter (ENO1p ; SEQ ID NO:136), asparagine synthetase promoter (ASNSp; SEQ ID NO:137), 50S ribosomal protein L1 promoter (RPLAp; SEQ ID NO:138), and RPL16B (SEQ ID NO:139).

15. The yeast cell of any one of claims 1-14, wherein the XPDH enzyme has a sequence at least 85% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33 or to at least one of SEQ ID NOs:14, 15, 28, or 31.

16. The yeast cell of any one of claims 1-15, wherein the XPDH enzyme has a sequence at least 90% identical to at least one of SEQ ID NOs: 12-15, 28-31, and 33 or to at least one of SEQ ID NOs:14, 15, 28, or 31.

17. A method for producing xylitol, the method comprising:
contacting a substrate comprising dextrose with the engineered yeast cell of any one of claims 1-16, wherein fermentation of the substrate by the engineered yeast produces xylitol.

18. A method for producing xylitol, the method comprising:
contacting a substrate comprising dextrose with an engineered yeast cell comprising an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to at least one of SEQ

ID NOs: 12-15, 28-31, and 33, wherein fermentation of the substrate by the engineered yeast produces xylitol.

19. The method of claim 18, wherein the engineered yeast cell is a *Moniliella pollinis* cell.
20. The method of any one of claims 17-19, wherein the fermentation temperature is at or between 25 °C to 45 °C, 30 °C to 40 °C, or 32 °C to 37 °C and the volumetric oxygen uptake rate (OUR) is between 0.5 to 40, 1 to 35, 2 to 30, 3 to 25, 4 to 20, or 5 to 15 mmol O₂/(L • h).
21. The method of any one of claims 17-20, wherein xylitol is produced at a rate of at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, or at least 1.0 g L⁻¹ h⁻¹.
22. The method of any one of claims 17-21, wherein xylitol production is at least at least 10, 20, 30, 40, 50, 75, or 100 g/L when the fermentation is run at 35 °C for 96 hours.
23. The method of any one of claims 17-22, wherein erythritol production is reduced relative to an equivalent fermentation run with an equivalent yeast cell lacking the exogenous polynucleotide sequence.
24. The method of any one of claims 17-23, wherein erythritol production is less than 50, 40, 30, or less than 20 g/L when the fermentation is run at 35 °C for 96 hours.
25. The method of any one of claims 17-24, wherein glycerol production is reduced relative to an equivalent fermentation run with an equivalent yeast cell lacking the exogenous polynucleotide sequence.

26. The method of any one of claims 17-25, wherein ethanol production is reduced relative to an equivalent fermentation run with an equivalent yeast cell lacking the exogenous polynucleotide sequence.

FIG. 1

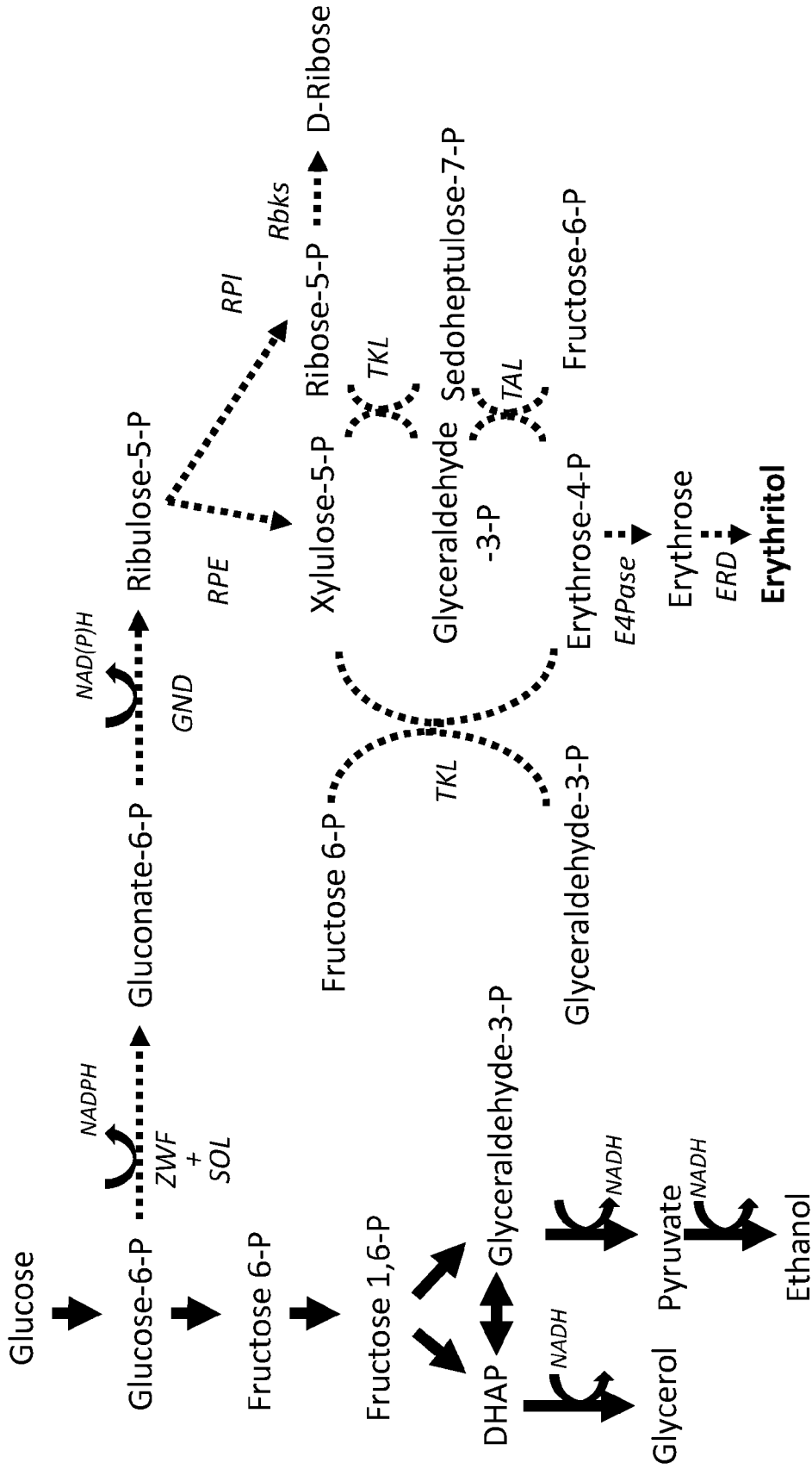


FIG. 2

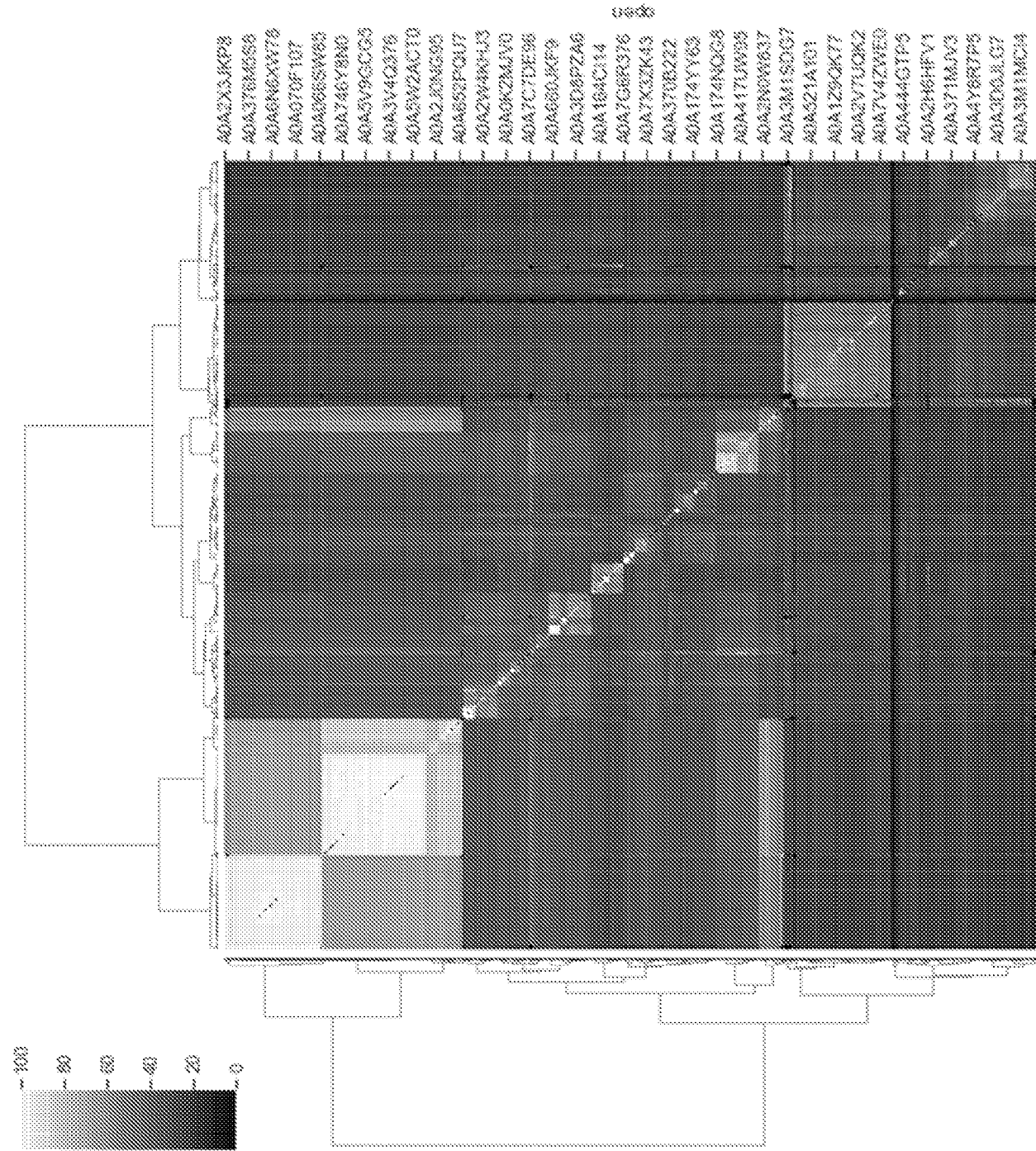


FIG. 3

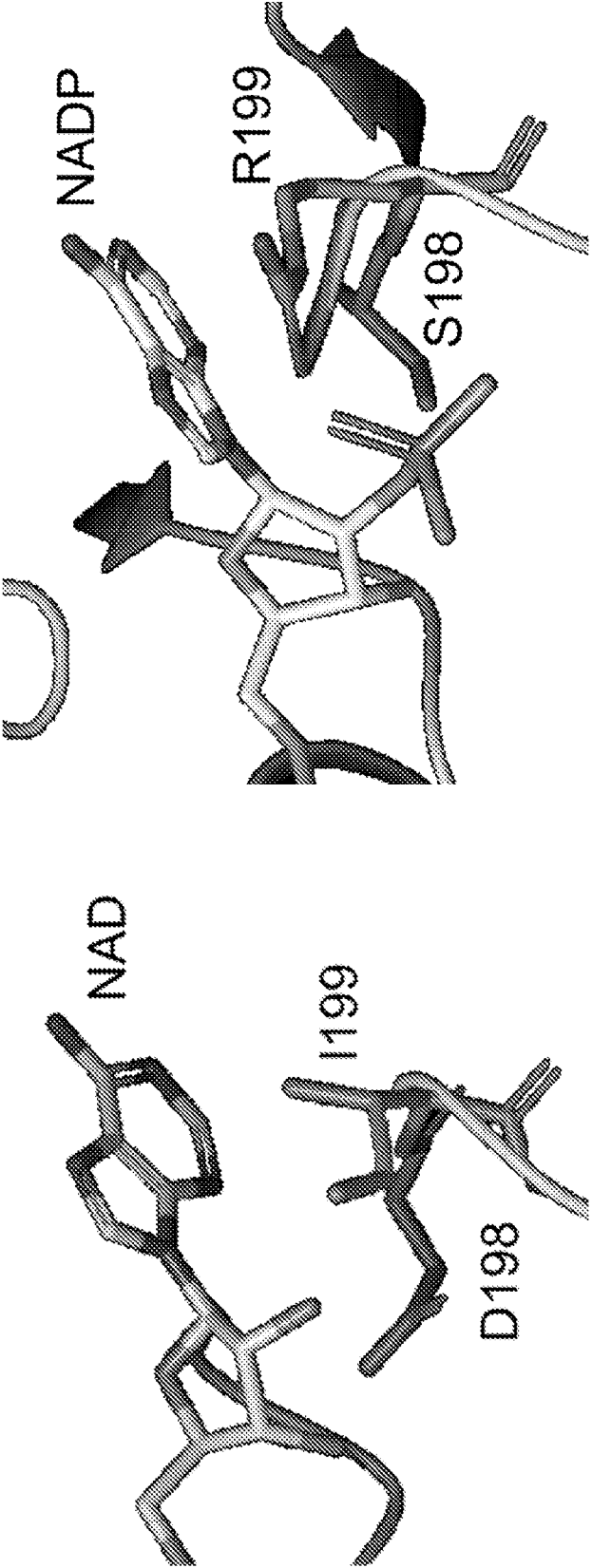


FIG. 4

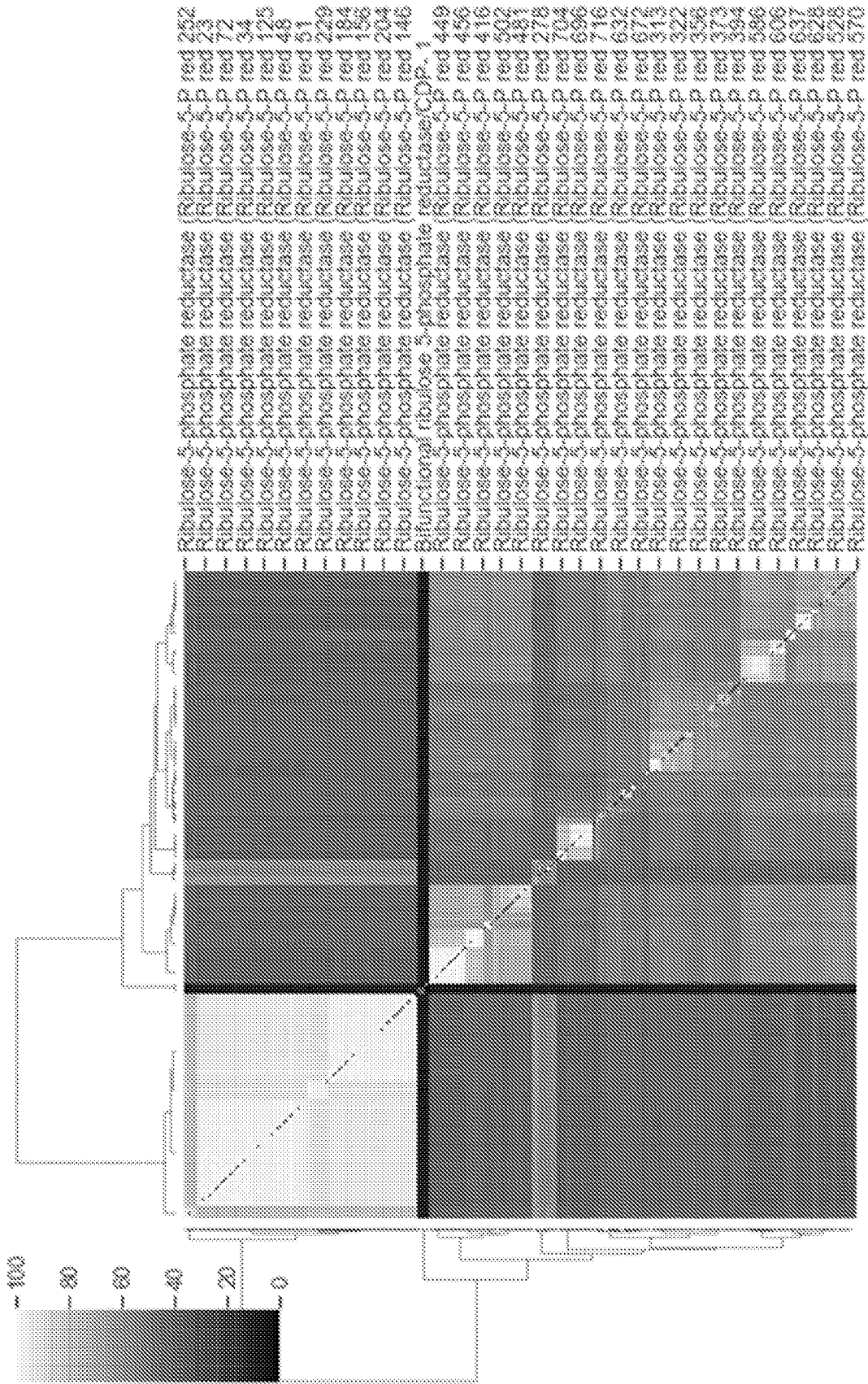


FIG. 5

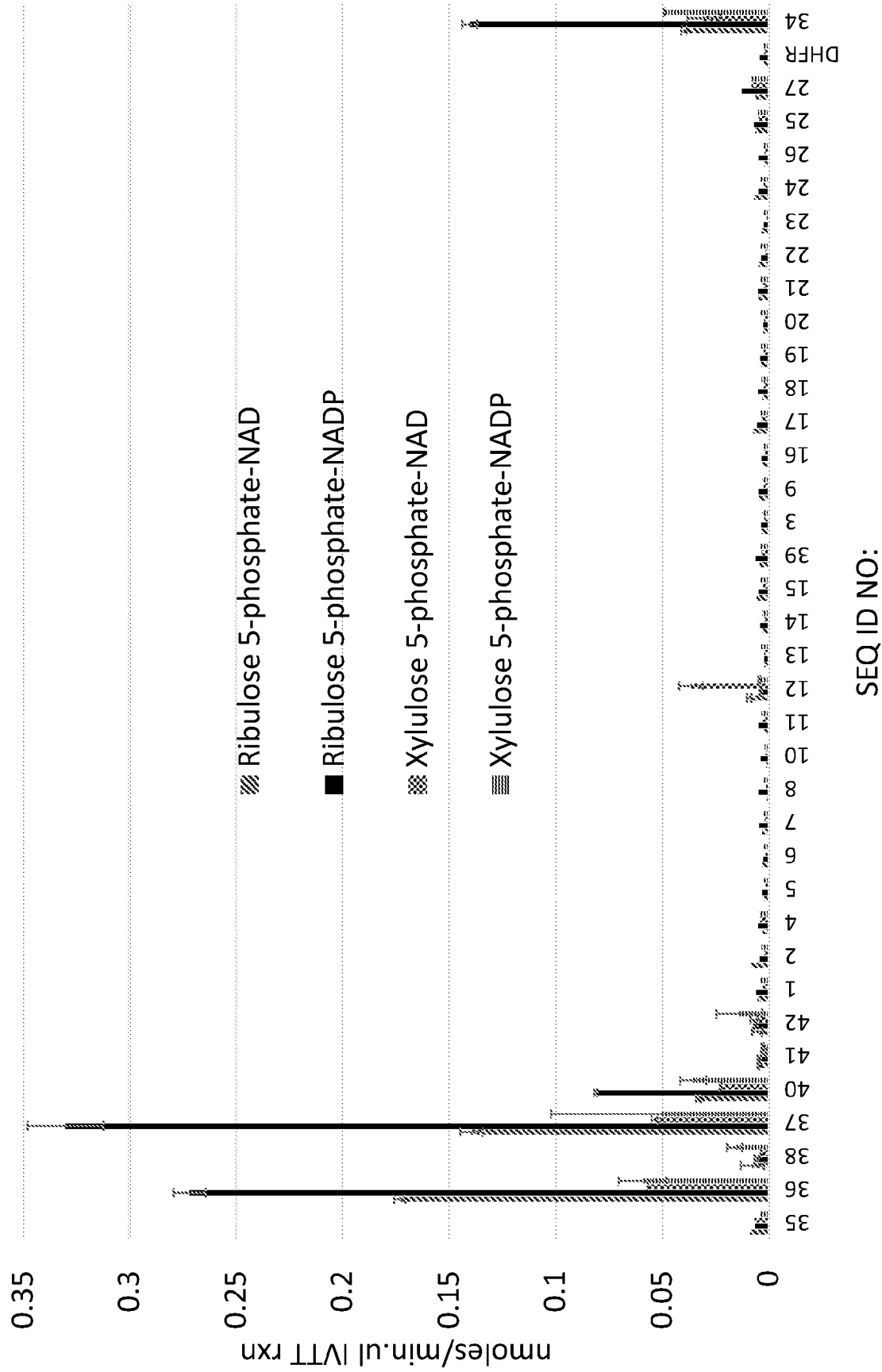


FIG. 6

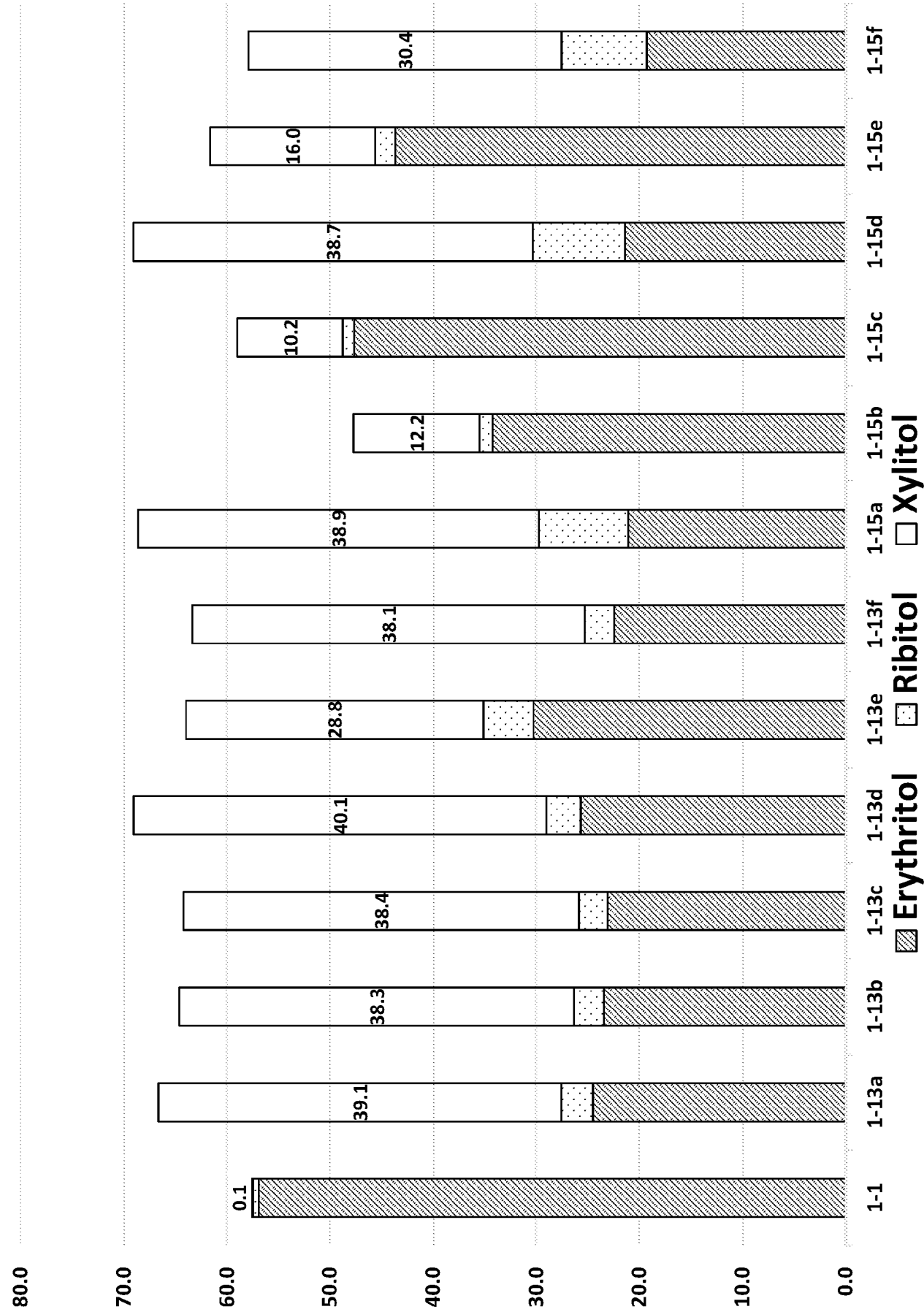


FIG. 7

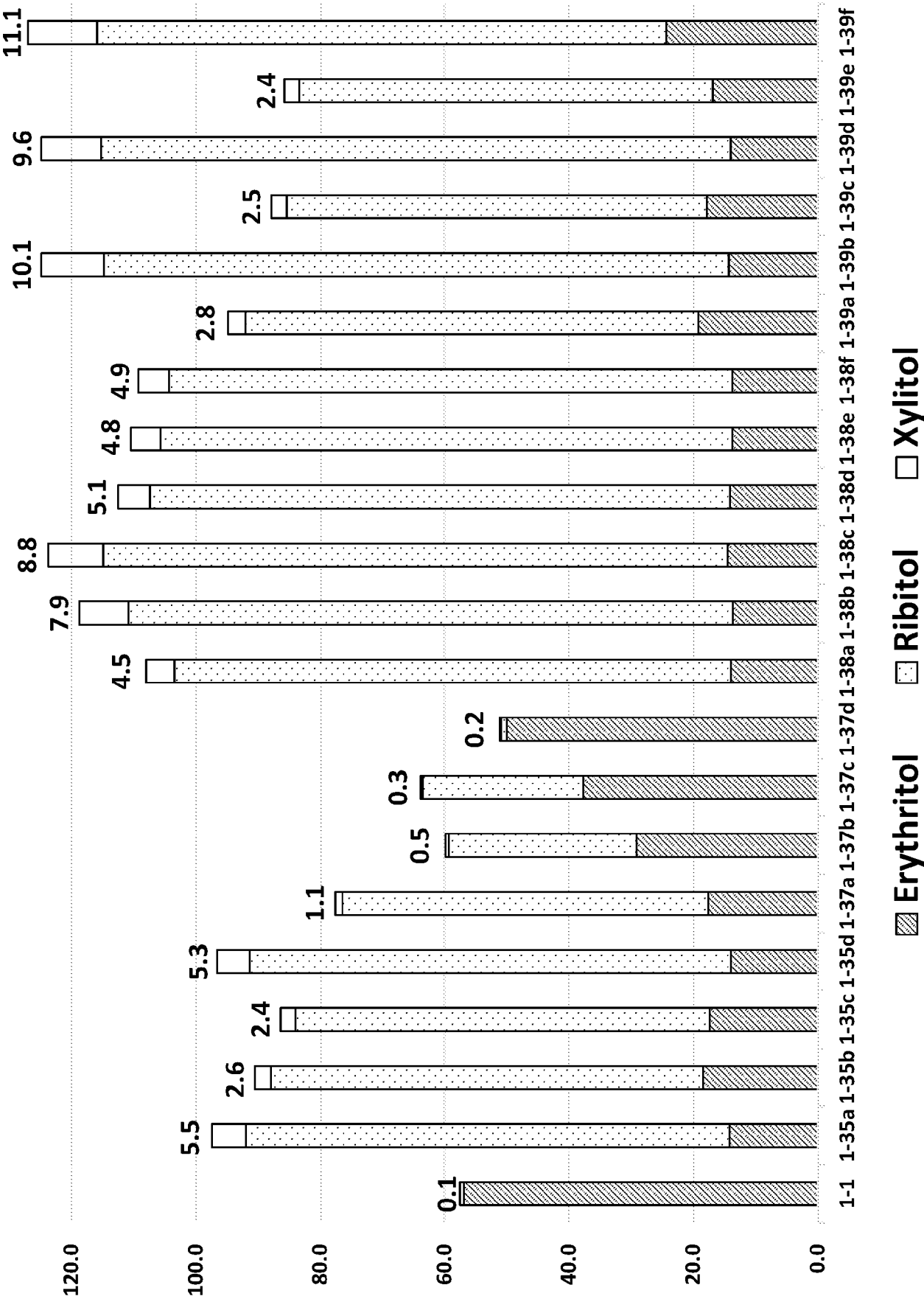


FIG. 8

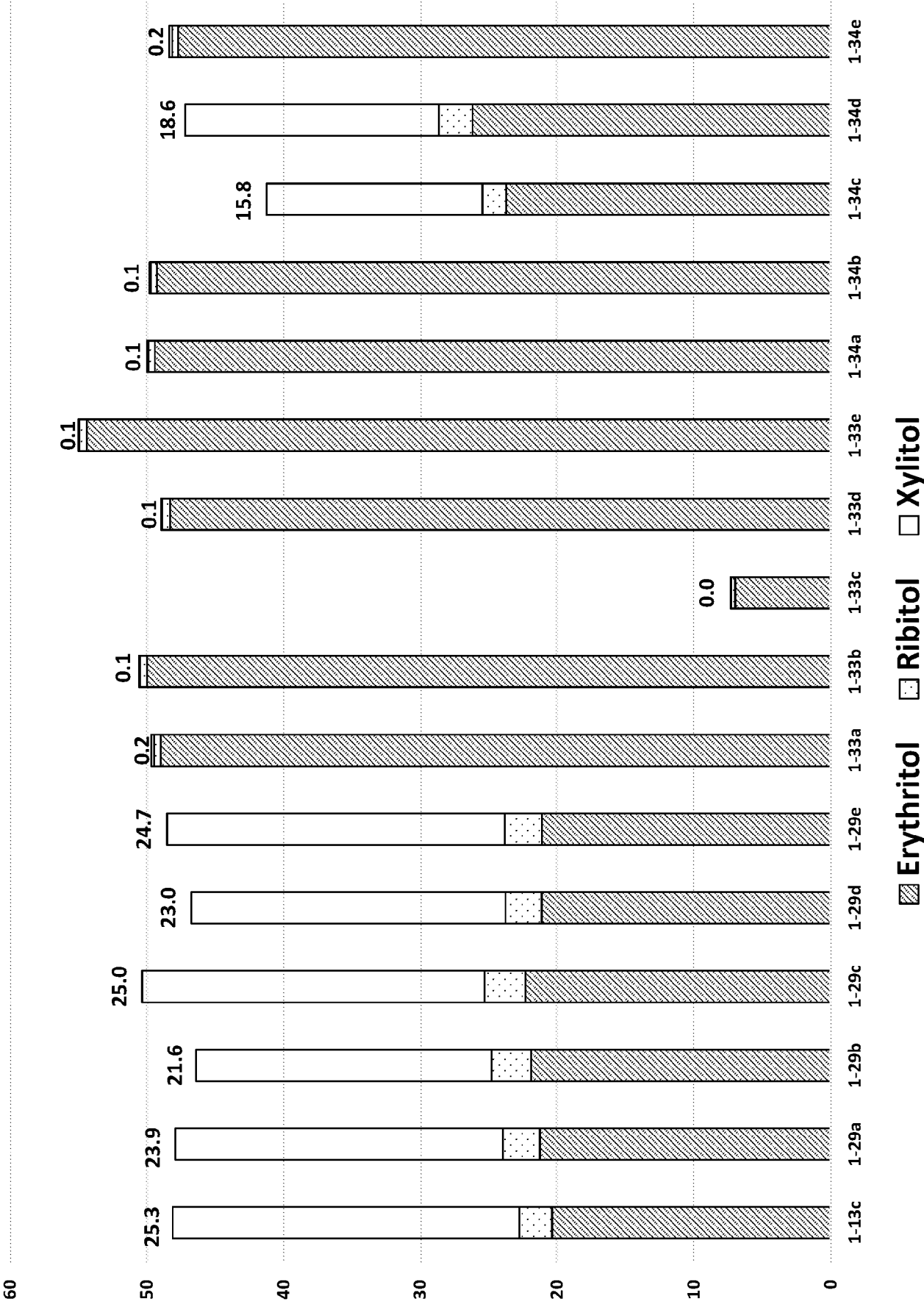


FIG. 9

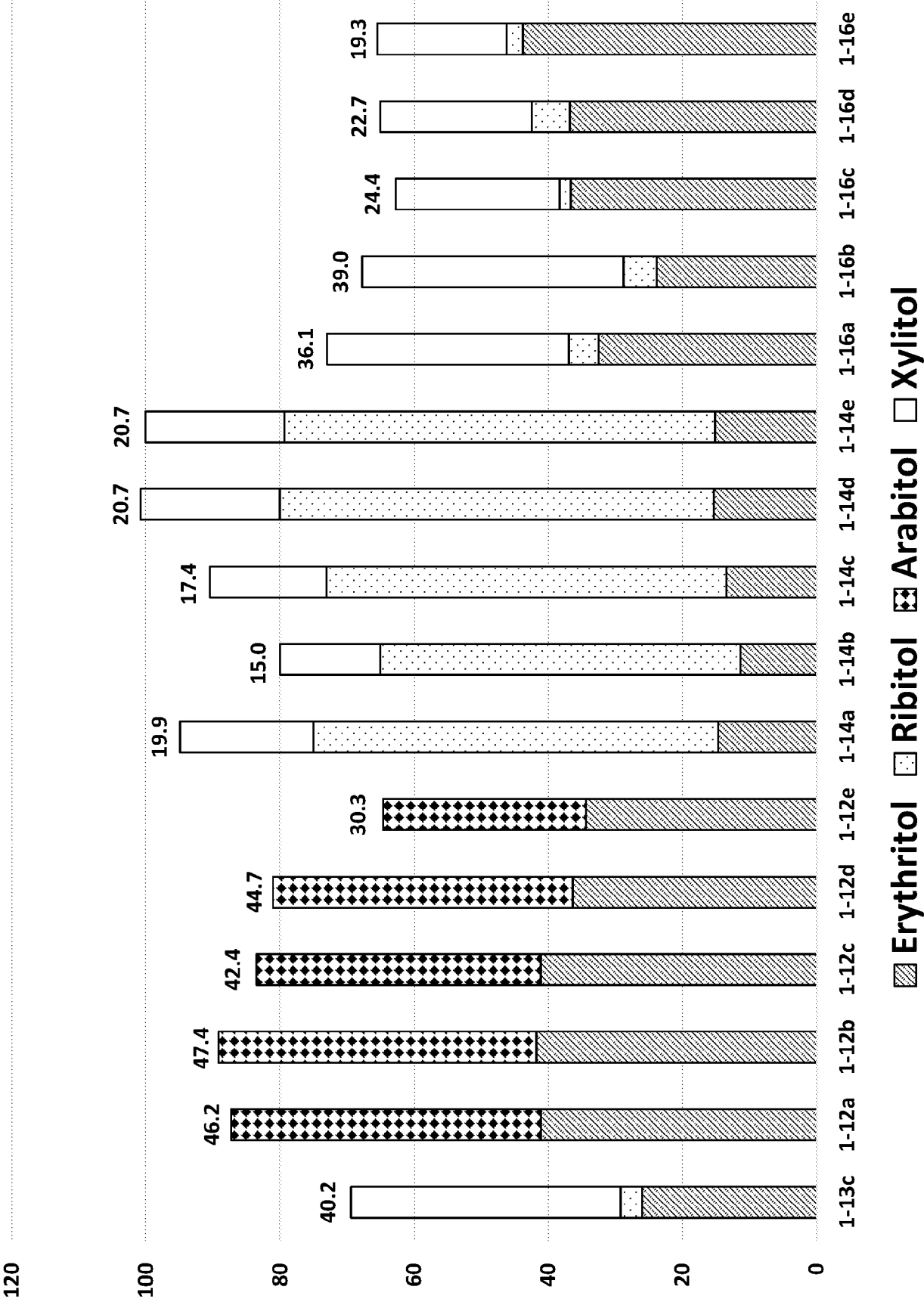


FIG. 10

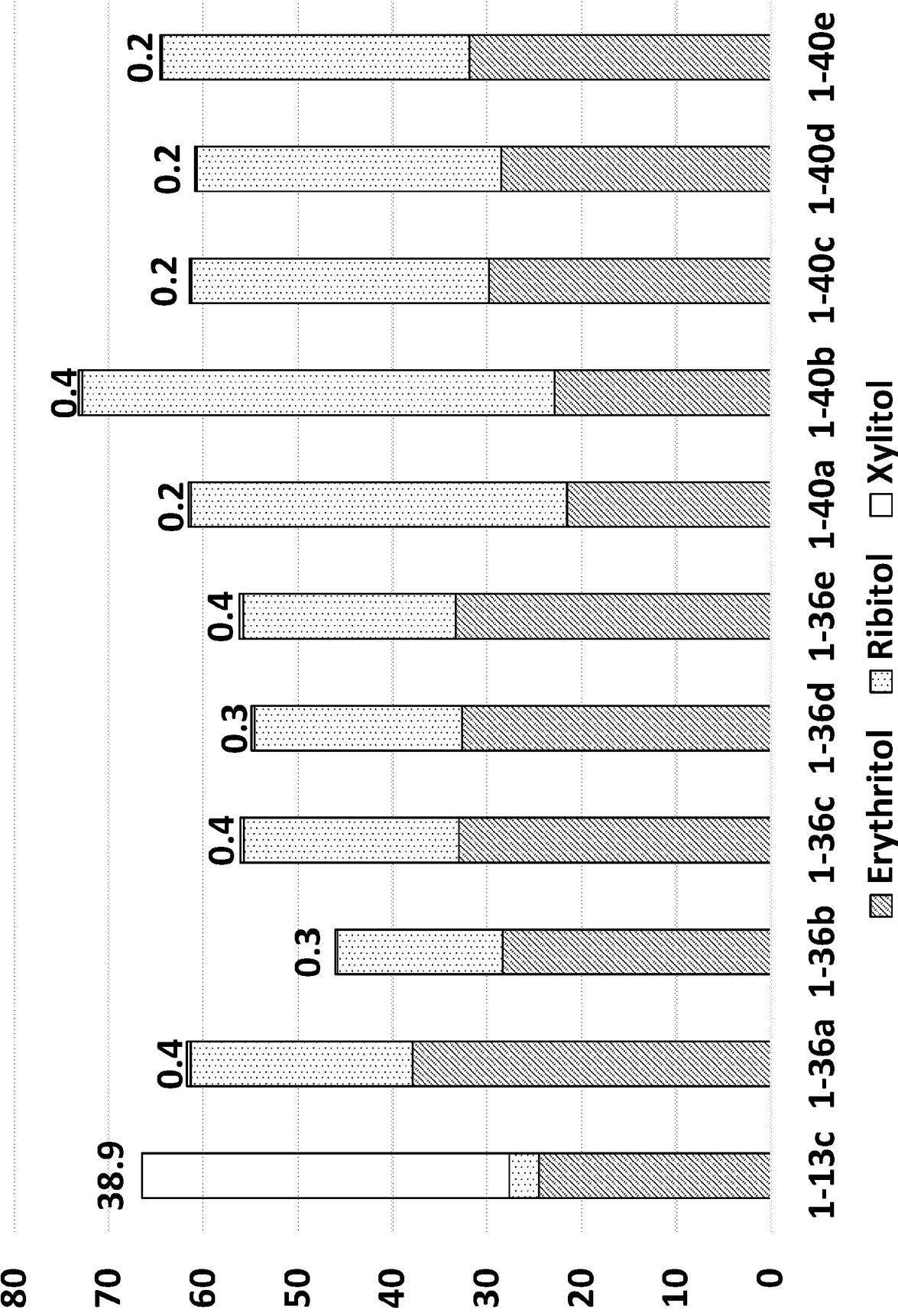


FIG. 11

