(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
08 May 2025 (08.05.2025)

(10) International Publication Number
WO 2025/096363 A2

(51) International Patent Classification:
C12N 9/04 (2006.01) *C12P 7/18* (2006.01)
C12N 15/52 (2006.01)

(21) International Application Number:
PCT/US2024/053309

(22) International Filing Date:
29 October 2024 (29.10.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/594,062 30 October 2023 (30.10.2023) US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2025/096363 A2

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

(57) Abstract: Disclosed herein are genetically engineered yeast cells capable of producing xylitol and comprise an exogenous polynucleotide sequence encoding a ZWF enzyme 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:15, 16, and 23. The genetically engineered yeast cell may additionally be engineered to overexpress a native RPE enzyme, express an exogenous XPDH enzyme, express an exogenous XKS enzyme, express an exogenous XDH enzyme, overexpress a native X5PP enzyme, and/or express an exogenous X5PP enzyme.

GENETICALLY MODIFIED YEAST AND FERMENTATION PROCESSES FOR THE PRODUCTION OF XYLITOL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/594,062, filed October 30, 2023, 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-1794-WO-PCT.xml" which is 377,000 bytes in size created on October 28, 2024 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.

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 glucose-6-phosphate dehydrogenase (ZWF) enzyme 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:15, 16, and 23. The ZWD enzyme may be at least 85% identical to SEQ ID NO:15 or 16. 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 form 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*. When the engineered cell is used in a fermentation process in the presence of dextrose, yield of polyols production is increased relative to yield of polyols in an equivalent fermentation process using an equivalent cell that does not express a ZWF enzyme 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:15, 16, and 23.

[0006] The yeast cell may be a *Moniliella pollinis* cell and additionally comprise a deletion or disruption of a gene encoding an erythrose reductase enzyme 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:60 and 62. The cell may have a deletion of at least one allele of the gene encoding the erythrose reductase enzyme. The cell may have a deletion of both alleles of the gene encoding the erythrose reductase enzyme. The gene encoding the erythrose reductase enzyme may be 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:61 and 63.

[0007] The cell may additionally comprise an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence 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:5, 95-101, and 103. The XPDH enzyme may have a sequence at least 85% identical to at least one of SEQ ID NOs: 5, 95-101, and 103 or to at least one of SEQ ID NOs:5, 87, 98, or 101. 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:5, 97, 98, 101.

[0008] The cell may additionally comprise an exogenous polynucleotide sequence encoding a xylulokinase (XKS) enzyme comprising a sequence 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:90 and 91. The cell may additionally comprise an exogenous polynucleotide sequence encoding a xylitol dehydrogenase (XDH) enzyme comprising a sequence 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:104-106.

[0009] The cell may additionally comprise a genetic modification resulting in overexpression of a native enzyme with ribulose-5-phosphate epimerase (RPE) activity. The yeast cell may be a yeast cell of the genus *Moniliella*. The yeast cell may be a *Moniliella pollinis* cell and the native RPE 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:46 and 94. The genetic modification resulting in overexpression of a native RPE enzyme comprises addition of an exogenous polynucleotide encoding the native RPE enzyme such that the genetically engineered cell comprises at least one additional copy of a sequence encoding the RPE enzyme.

[0010] The yeast may additionally comprise a genetic modification resulting in overexpression of a native enzyme with xylitol-5-phosphate phosphatase (X5PP) activity; and/or an exogenous polynucleotide sequence encoding an enzyme with xylitol-5-phosphate phosphatase (X5PP) activity. The cell may be a *Moniliella pollinis* cell and the genetic modification results in overexpression of a native X5PP enzyme with a sequence 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:74, 88, 92, and 93. The genetic modification may comprise addition of an exogenous polynucleotide sequence encoding the native X5PP enzyme such that the genetically engineered cell comprises at least one additional copy of a sequence encoding the native X5PP enzyme. The yeast cell may comprise an exogenous polynucleotide sequence encoding an enzyme with X5PP activity and a sequence 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:74-91. The yeast cell comprises an exogenous polynucleotide sequence encoding an enzyme with X5PP activity and a sequence 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: 74, 76, 77, 78, 79, 80, 84, and 86. The yeast cell comprises an exogenous polynucleotide sequence encoding an enzyme with X5PP activity and 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: 74, 77, 78, 80, and 86. X5PP activity in the genetically engineered yeast cell may be higher than X5PP activity in an equivalent cell lacking the genetic modification or exogenous polynucleotide sequence.

[0011] One or more of the exogenous polynucleotide sequences described above may be operably linked to a heterologous or artificial promoter. The heterologous or artificial promoter

may be selected from the group consisting of pyruvate kinase 1 promoter (PYK1p; SEQ ID NO:6), 6-phosphogluconate dehydrogenase promoter (6PGDp; SEQ ID NO:107), glyceraldehyde-3-phosphate dehydrogenase promoter (TDH3p; SEQ ID NO:108), translational elongation factor 1 promoter (TEFp; SEQ ID NO:109), phosphoglucomutase 1 promoter (PGM1p; SEQ ID NO:110), 3-phosphoglycerate kinase promoter (PGK1p; SEQ ID NO:111), enolase promoter (ENO1p ; SEQ ID NO:112), asparagine synthetase promoter (ASNSp; SEQ ID NO:113), 50S ribosomal protein L1 promoter (RPLAp; SEQ ID NO:114), and RPL16B (SEQ ID NO:115). The promoter may be a constitutive promoter. One or more of the exogenous polynucleotide sequences is integrated into the genome of the yeast cell at a locus selected from the ER1 locus, the ER3 locus, the PDC1 locus, the pyrF locus, the TRP3 locus, the gpdIIA locus, and the gpdIIB locus.

[0012] The disclosure also provides a method for producing xylitol using the engineered cells described herein, the method comprising contacting a substrate comprising dextrose with an engineered cell described herein, wherein fermentation of the substrate by the engineered cell 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 5-80, 10-75, 15-70, 20-60, 30-50, or 40-50 mmol O_2/(L • h) The xylitol may be produced at a rate of at least 0.2, 0.3, 0.5, 0.75, or at least 1.0 g L^{-1} h^{-1}. Xylitol production may be at least at least 20, 30, 50, 75, or 100 g/L when the fermentation is run at 35 °C for 96 hours. The concentration of dextrose may be at least 100 g/L. The xylitol yield of the fermentation is higher than yield in the same fermentation process run using an equivalent yeast lacking the exogenous polynucleotide sequence encoding the ZWF enzyme.

[0013] The disclosure also provides use of the engineered cells described herein to produce xylitol.

BRIEF DESCRIPTION OF THE FIGURES

[0014] 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.

[0015] The drawings illustrate generally, by way of example, but not by way of limitation, various aspects discussed herein.

[0016] FIG. 1 shows the native pentose phosphate pathway (dotted lines and arrows) and the native glycolysis pathways (solid lines and arrows) in *Moniliella pollinis*.

[0017] FIG. 2 shows erythritol, ribitol, and xylitol titers for the reactions outlined in Example 3.

[0018] FIG. 3 shows erythritol, ribitol, and xylitol titers for the reactions outlined in Example 4.

[0019] FIG. 4 shows erythritol, ribitol, and xylitol titers for the reactions outlined in Example 6.

[0020] FIG. 5 shows erythritol, ribitol, and xylitol titers for the reactions outlined in Example 7.

DETAILED DESCRIPTION

[0021] 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.

[0022] 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.

[0023] 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.

[0024] 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.

[0025] This disclosure relates to various recombinant cells engineered to produce xylitol. In general, the recombinant cells described herein are capable of producing xylitol and comprise an exogenous polynucleotide sequence encoding a ZWF enzyme. The recombinant yeast may additionally be characterized by overexpression of a ribulose 5-phosphatase epimerase (RPE) enzyme; inclusion of an exogenous polynucleotide sequence encoding a xylitol phosphate dehydrogenase (XPDH) enzyme; inclusion of an exogenous polynucleotide sequence encoding a xylulokinase (XKS) enzyme; inclusion of an exogenous polynucleotide sequence encoding a xylitol dehydrogenase (XDH) enzyme); and/or overexpression of a xylitol-5-phosphate phosphatase (X5PP) enzyme. The disclosure further provides fermentation methods for the production of xylitol from dextrose using the genetically engineered cells described herein.

[0026] 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, Kluveromyces 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 kudriavzevvi, Trichosporonoides* (e.g., *Trichosporonoides megachiliensis, Trychosporonoides oedocephalis, Trychosporonoides 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.

[0027] 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. ocedocephalis, M. nigrescens*), and *Pseudozyma* (e.g., *Pseudozyma tsukubaensis*), and *Trichosporonoides* (e.g., *Trichosporonoides megachiliensis, Trychosporonoides oedocephalis, Trychosporonoides 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 Ustilaginacceae 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).

[0028] 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+ ($NAD(P)^+$), and water to NADPH or NADH (NAD(P)H), 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.

[0029] 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).

[0030] 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).

[0031] 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 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:54, and a 5' portion, SEQ ID NO:55), G418 resistance gene (for example broken into a 3' portion, SEQ ID NO:9, and a 5' portion, SEQ ID NO:8), zeocin resistance gene (for example broken into a 3' portion, SEQ ID NO:47, and a 5' portion, SEQ ID NO:48), nourseothricin N-acetyl transferase (NAT) (for example broken into a 3' portion, SEQ ID NO:57, and a 5' portion, SEQ ID NO:56), and invertase gene (SUC2) (for example a 3' portion of SEQ ID NO:58 and a 5' portion of SEQ ID NO:59).

[0032] The recombinant cells described herein include one or more exogenous polynucleotide sequences encoding one or more polypeptides that, when expressed, improve the fermentation of glucose to xylitol by the recombinant cells.

[0033] The terms "glucose" and "dextrose" are used interchangeably herein and refer to D-glucose except where expressly indicated otherwise.

[0034] 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*.

[0035] 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.

[0036] 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

Amino Acid	Three-letter symbol	One-letter symbol
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

[0037] 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.

[0038] 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.

[0039] 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.

[0040] 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.

[0041] 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.

[0042] 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).

[0043] 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.

[0044] 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 erythrose reductase 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.

[0045] The recombinant cells described herein may include deletions or disruptions in one or more native genes. The phase “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. Deletion or

disruption of a native host cell gene can be coupled to the incorporation of one or more polynucleotide sequences (e.g., an exogenous or native polynucleotide sequence) into the host cell at the locus of the host cell gene to be deleted or disrupted. The polynucleotide sequence to be inserted may be designed to replace all or a portion of the host cell gene to be deleted or disrupted. The polynucleotide sequence may encode for a gene product of interest, for example, a polypeptide, an enzyme, and the like. The deletion or disruption can also be accomplished using a deletion construct that does not contain a polynucleotide sequence to be integrated. Other methods for gene disruption or deletion are known and described in the art.

[0046] The recombinant cells described herein have a deletion or disruption in one or more native genes encoding an enzyme involved in erythritol fermentation or consumption. Deletion or disruption of one or more of these biosynthetic pathway enzymes decreases the ability of the recombinant cell to produce erythritol and may, depending on the deletion or disruption, increasing carbon flux into the fermentation pathway for the production of xylitol.

[0047] The recombinant cells described herein may include a deletion or disruption of a native erythrose reductase (ER) gene. The native ER gene encodes an enzyme that has erythrose reductase activity. As used herein "erythrose reductase activity" and "ER activity" are used interchangeably and refer to enzymes that catalyze the reversible conversion of erythrose or erythrose-4-phosphate to erythritol or erythritol-4-phosphate using a nicotinamide adenine dinucleotide (phosphate) (hydrogen) (NAD(P)(H)) cofactor. In the art, enzymes that catalyze the reversible conversion of erythrose-4-phosphate to erythritol-4-phosphate using a nicotinamide adenine dinucleotide (phosphate) (hydrogen) (NAD(P)(H)) cofactor may also be described as erythrose or erythritol phosphate dehydrogenases. When the host cell contains multiple ER genes, it is preferred to delete or disrupt at least one of them. When the host cell contains multiple alleles of a given ER gene, it is preferred to delete or disrupt one allele or both alleles of the given ER gene.

[0048] As used herein, "NAD(P)H" refers to nicotinamide adenine dinucleotide (phosphate) hydrogen and is inclusive of both NADH and NADPH. As is understood in the art, inclusion of the phosphate (or "P" abbreviation) in parentheses indicates that the phosphate may be absent or present and the name and abbreviation are inclusive of both. Similarly, "NAD(H)" or "NADP(H)" refers to both the reduced and oxidized forms of the cofactor.

[0049] When the recombinant cell is a *Moniliella pollinis* cell, the recombinant cell may comprise a deletion or disruption of an ER gene encoding an amino acid 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 SEQ ID NO:60. When the recombinant cell is a *Moniliella pollinis* cell, the recombinant cell may comprise a deletion or disruption of an ER gene with a nucleotide 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 SEQ ID NO:61. See, for example, US Provisional Application No. 63/499,990, filed May 4, 2023, and US Provisional Application No. 63/499,989, filed May 4, 2023.

[0050] When the recombinant cell is a *Moniliella pollinis* cell, the recombinant cell may comprise a deletion or disruption of an ER gene encoding an amino acid 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 SEQ ID NO:62. When the recombinant cell is a *Moniliella pollinis cell*, the recombinant cell may comprise a deletion or disruption of an ER gene with a nucleotide 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 SEQ ID NO:63. See, for example, US Provisional Application No. 63/499,990, filed May 4, 2023, and US Provisional Application No. 63/499,989, filed May 4, 2023.

[0051] 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:3 and SEQ ID NO:10), the *ER3* locus (defined as the locus flanked by SEQ ID NO:49 and SEQ ID NO:50), the *PDC1* locus (defined as the locus flanked by SEQ ID NO:64 and SEQ ID NO:65), the *pyrF* locus (defined as the locus flanked by SEQ ID NO:66 and SEQ ID NO:67), the *TRP3* locus (defined as the locus flanked by SEQ ID NO:68 and SEQ ID NO:69), the *gpdIIA* locus (defined as the locus flanked by SEQ ID NO:70 and SEQ ID NO:71); and the *gpdIIB* locus (defined as the locus flanked by SEQ ID NO:72 and SEQ ID NO:73). 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.

[0052] 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.

[0053] The recombinant cells described herein are capable of producing xylitol and include an exogenous polynucleotide sequence encoding an exogenous enzyme with glucose-6-phosphate dehydrogenase (ZWF) activity. The enzyme may be any suitable enzyme with ZWF activity. As used herein, “ZWF” and “ZWF enzyme” are used interchangeably and refer to an enzyme with ZWF activity. Herein, “ZWF activity” and “glucose-6-phosphate dehydrogenase activity” are used interchangeably and refer to the ability to reversibly catalyze the conversion of glucose-6-phosphate and $NAD(P)^+$ to 6-phosphogluconolactone and NAD(P)H. Suitable enzymes with ZWF activity may include, but are not limited to, those classified under EC 1.1.1.388, EC 1.1.1.49, and EC 1.1.1.363. Polynucleotides encoding ZWF enzymes may be derived from any suitable source. For example, a polynucleotide encoding a ZWF enzyme may be derived from *Moniliella pollinis*, *Pseudomonas putida*, *Pantoea* sp. Ap-967, and the like. The ZWF enzyme may be a polypeptide with an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:15, 16, and 23. The ZWF enzyme may be a polypeptide with an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:15 and 16. The ZWF enzyme may be a polypeptide with an amino acid sequence at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:15, 16, and 23. The ZWF enzyme may be a polypeptide with an amino acid sequence at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:15 and 16.

[0054] The recombinant cell capable of producing xylitol may include an exogenous polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:23 which is derived from a *Moniliella pollinis* ZWF enzyme sequence. The exogenous polynucleotide sequence may encode an amino acid sequence 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%, identical to SEQ ID NO:23. The exogenous polynucleotide sequence may encode an amino acid sequence at least 90% identical to SEQ ID NO:23.

[0055] The recombinant cell capable of producing xylitol may include an exogenous polynucleotide sequence that is or may be derived from *Pseudomonas putida* encoding the amino acid sequence of SEQ ID NO:15. The exogenous polynucleotide sequence may encode an amino acid sequence 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%, identical to SEQ ID NO:15. The exogenous polynucleotide sequence may encode an amino acid sequence at least 90% identical to SEQ ID NO:15.

[0056] The recombinant cell capable of producing xylitol may include an exogenous polynucleotide sequence that is or may be derived from *Pantoea* sp. Ap-967 encoding the amino acid sequence of SEQ ID NO:16. The exogenous polynucleotide sequence may encode an amino acid sequence 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%, identical to SEQ ID NO:16. The exogenous polynucleotide sequence may encode an amino acid sequence at least 90% identical to SEQ ID NO:16.

[0057] The recombinant cells described herein may additionally include one or more enzymes in the xylitol biosynthetic pathway. For example, the recombinant cells may include one or more of (i) a genetic modification resulting in overexpression of a native X5PP enzyme, (ii) an exogenous polynucleotide sequence encoding an exogenous X5PP enzyme, (iii) a genetic modification resulting in overexpression of a native RPE enzyme, (iv) an exogenous polynucleotide sequence encoding an exogenous XPDH enzyme; (v) an exogenous polynucleotide sequence encoding an exogenous XKS enzyme; and (iv) an exogenous polynucleotide sequence encoding an exogenous XDH enzyme.

[0058] The final step in the xylitol pathway, from xylitol 5-phosphate to xylitol, requires a phosphatase enzyme. The *Saccharomyces cerevisiae* PYP1 (polyol phosphatase 1) gene encodes a sugar alcohol phosphatase that hydrolyzes sorbitol-6-phosphate, ribitol-5-phosphate, and (D)-glycerol-3-phosphase (Xu et al., “Discovery and functional characterization of a yeast sugar alcohol phosphatase,” ACS Chem. Biol., 13, 2018, 3011-3020). PYP1 is a member of the haloacid

dehalogenase (HAD)-like hydrolase superfamily (Kuznetsova, et a., "Functional diversity of haloacid dehalogenase superfamily phosphatases from *Saccharomyces cerevisiae*," J. Biol. Chem., 2015, 290, 18678-18698) and belongs to the enzyme class of sorbitol-6-phosphatases (Enzyme Commission (EC) 3.1.3.50). As xylitol 5-phosphate is a similar molecule to the known substrates of PYP1 it is demonstrated herein that one or more PYP-like enzymes or PYP orthologs have xylitol-5-phosphate phosphatase activity and can be used to increase xylitol production in the recombinant cells described herein. *E. coli* HxpA (hexitol phosphatase A) is a HAD-like enzyme belonging to EC 3.1.3.50 with a similar substrate profile to PYP1 (Kuznetsova et al., "Genome wide analysis of substrate specificities of the *Escherichia coli* haloacid dehalogenase-like phosphate family," 2006, J. Biol. Chem., 281, 36149-36161). Accordingly, it is also demonstrated herein that one or more HAD-like hydrolase enzymes or HAD-like hydrolase orthologs have xylitol-5-phosphate phosphatase activity and can be used to increase xylitol production in the recombinant cells describe here.

[0059] The recombinant cells described herein are capable of producing xylitol, include an exogenous polynucleotide sequence encoding a ZWF enzyme, and may be characterized by overexpression of a native enzyme with xylitol-5-phosphate phosphatase (X5PP) activity and/or include an exogenous polynucleotide sequence encoding a native or exogenous enzyme with xylitol-5-phosphate phosphatase (X5PP) activity. In general, the recombinant cell(s) including overexpression of an X5PP enzyme or expressing an exogenous X5PP enzyme produce more xylitol than an equivalent cell lacking the exogenous X5PP enzyme or lacking overexpression of the X5PP enzyme. The enzyme may be any suitable enzyme with X5PP activity. As used herein, "X5PP enzyme" and "X5PP" are interchangeable and refer to an enzyme with X5PP activity. Herein, "xylitol-5-phosphate phosphatase activity" and "X5PP activity" are used interchangeably and refer to the ability to catalyze the conversion of xylitol-5-phosphate to xylitol and phosphate. Suitable X5PP enzymes may include a divalent metal cation, for example, Mg^{2+}, Mn^{2+}, or Co^{2+}. Suitable enzymes with X5PP activity may include, but are not limited to, those classified under EC 3.1.3.50, for example, sugar alcohol phosphatases and HAD-like hydrolases. Polynucleotides encoding X5PP enzymes may be derived from any suitable source. For example, a polynucleotide encoding an X5PP enzyme may be derived from *Moniliella pollinis*, *Saccharomyces cerevisiae*, *Lachancea dasiensis*, *Tetrapisispora blattae*, *Saccharomyces pastorianus*, *Kazachstania Africana*, *Podospora comata*, *Geotrichum candidum*, *Ogattaea haglerorum*, *Debaryomyces fabryi*, *Monilinia fructicola*, *Nadsonia fulvescens* var. elongata DSM 6958, *Escherichia coli*,

Wickerhamomyces ciferrii, *Bacillus amyloliquefaciens*, and the like. The X5PP enzyme may be a polypeptide with an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, and 91. The X5PP enzyme may be a polypeptide with an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:74, 76, 77, 78, 79, 80, 84, and 86. The X5PP enzyme may be a polypeptide with an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or 100% identical to the amino acid sequence of at least one of SEQ ID NOs:74, 77, 78, 80, and 86. See for example, US Provisional Application No. 63/499,992, filed May 4, 2023, and incorporated herein by reference in its entirety.

[0060] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Moniliella pollinis* gene encoding the amino acid sequence of SEQ ID NO:74. The exogenous polynucleotide sequence may encode an amino acid sequence 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%, identical to SEQ ID NO:74.

[0061] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Saccharomyces cerevisiae* gene encoding the amino acid sequence of SEQ ID NO:75. The exogenous polynucleotide sequence may encode an amino acid sequence 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%, identical to SEQ ID NO:75.

[0062] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Lachancea dasiensis* gene encoding the amino acid sequence of SEQ ID NO:76. The exogenous polynucleotide sequence may encode an amino acid sequence 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%, identical to SEQ ID NO:76.

[0063] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Tetrapisispora blattae* gene encoding

the amino acid sequence of SEQ ID NO:77. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:77.

[0064] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Saccharomyces pastorianus* gene encoding the amino acid sequence of SEQ ID NO:78. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:78.

[0065] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Kazachstania africana* gene encoding the amino acid sequence of SEQ ID NO:79. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:79.

[0066] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Podospora comata* gene encoding the amino acid sequence of SEQ ID NO:80. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:80.

[0067] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Geotrichum candidum* gene encoding the amino acid sequence of SEQ ID NO:81. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:81.

[0068] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Ogattaea haglerorum* gene encoding the amino acid sequence of SEQ ID NO:82. The exogenous polynucleotide sequence may encode an

amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:82.

[0069] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Debaryomyces fabryi* gene encoding the amino acid sequence of SEQ ID NO:83. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:83.

[0070] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Monilinia fructicola* gene encoding the amino acid sequence of SEQ ID NO:84. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:84.

[0071] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Nadsonia fulvescens* var. elongata DSM 6958 gene encoding the amino acid sequence of SEQ ID NO:85. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:85.

[0072] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from an *Escherichia coli* gene encoding the amino acid sequence of SEQ ID NO:86. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:86.

[0073] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Wickerhamomyces ciferrii* gene encoding the amino acid sequence of SEQ ID NO:87. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:87.

[0074] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Moniliella pollinis* gene encoding the amino acid sequence of SEQ ID NO:88. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:88.

[0075] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Bacillus amyloliquefaciens* gene encoding the amino acid sequence of SEQ ID NO:89. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:89.

[0076] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Saccharomyces cerevisiae* DOG2 gene encoding the amino acid sequence of SEQ ID NO:90. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:90.

[0077] The recombinant cell is capable of producing xylitol, includes an exogenous polynucleotide sequence encoding a ZWF enzyme, and may comprise an exogenous polynucleotide sequence that is or may be derived from a *Saccharomyces cerevisiae* DOG1 gene encoding the amino acid sequence of SEQ ID NO:91. The exogenous polynucleotide sequence may encode an amino acid sequence at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99%, identical to SEQ ID NO:91.

[0078] The enzyme with X5PP activity may be native to the host cell. For example, when the host organism is *M. pollinis*, the X5PP enzyme may be an enzyme with a sequence at least 50%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% identical to at least one of SEQ ID NOs:74, 88, 92, and 93. The recombinant cell may comprise an exogenous polynucleotide encoding an X5PP enzyme with 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 at least one of SEQ ID NOs:92, 93, 200, or 221. The recombinant cell may include a genetic modification that increases expression of an X5PP enzyme 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:92, 93, 74, or 88. The genetic modification may include, but is not limited to, insertion of additional copies of a nucleic acid encoding the native X5PP enzyme into the cell (e.g., integration of additional copies of the X5PP encoding polynucleotide into it non-native locus in the cell), insertion of a constitutive promoter upstream of the coding region of the native X5PP enzyme encoding gene in the genome of the host cell, and/or modification of the existing promoter upstream of the coding region of the native X5PP enzyme encoding gene in the genome of the host cell. One of skill in the art will recognize that expression of a native X5PP enzyme encoding gene may be increased by a number of methods known in the art and will be able to select and apply such methods as appropriate.

[0079] As used herein, “overexpression” refers to an expression level of a polypeptide that is higher than the expression level of the same polypeptide in the absence of a genetic modification or exogenous polynucleotide encoding said polypeptide in an equivalent cell.

[0080] The recombinant cells described herein capable of producing xylitol and having an exogenous polynucleotide sequence encoding a ZWF enzyme may also be characterized by overexpression of a ribulose 5-phosphate epimerase (RPE enzyme). The recombinant cells described herein capable of producing xylitol and having an exogenous polynucleotide sequence encoding a ZWF enzyme may also include an exogenous polynucleotide sequence encoding an X5PP enzyme and/or overexpress a native X5PP enzyme, and/or may include an exogenous polynucleotide encoding a native or exogenous RPE enzyme or may have a genetic modification resulting in overexpression of a native RPE enzyme, as described herein. In general, the recombinant cell(s) including overexpression of the RPE enzyme produce more xylitol than an equivalent cell lacking the RPE enzyme or lacking overexpression of the RPE enzyme.

[0081] The recombinant cells described herein are capable of producing xylitol, have an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide encoding a native or exogenous RPE enzyme or may have a genetic modification resulting in overexpression of a native RPE enzyme. The RPE enzyme may be any suitable enzyme with ribulose 5-phosphate epimerase activity. As used herein, “ribulose 5-phosphate epimerase activity” and “RPE activity” are used interchangeably and refer to the ability to catalyze the conversion of ribulose-5-phosphate to xylulose-5-phosphate. The enzyme with RPE activity may be native to the host cell or the RPE enzyme may be an exogenous RPE enzyme. For example, when the host organism is *M. pollinis*, the RPE enzyme may be an enzyme with a sequence at least

70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, or at least 99% identical to at least one of SEQ ID NOs:46 and 94. The recombinant cell may comprise an exogenous polynucleotide encoding an RPE enzyme with 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 at least one of SEQ ID NOs:46 and 94. The recombinant cell may include a genetic modification that increases expression of an RPE enzyme 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:46 and 94. The genetic modification may include, but is not limited to, insertion of additional copies of a nucleic acid encoding the native RPE into the cell, insertion of a constitutive promoter upstream of the coding region of the native RPE gene in the genome of the host cell, and/or modification of the existing promoter upstream of the coding region of the native RPE gene in the genome of the host cell. One of skill in the art will recognize that expression of a native RPE gene may be increased by a number of methods known in the art and will be able to select and apply such methods as appropriate. See, for example, PCT Application No. PCT/US2023/066631 filed May 5, 2023.

[0082] The recombinant cells described herein are capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may 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. A recombinant cell described herein capable of producing xylitol has an exogenous polynucleotide sequence encoding a ZWF enzyme and may include an exogenous polynucleotide sequence encoding an X5PP enzyme and/or overexpress a native X5PP enzyme, may include an exogenous polynucleotide sequence encoding an XPDH enzyme, and/or may include an exogenous polynucleotide encoding a native or exogenous RPE enzyme or may have a genetic modification resulting in overexpression of a native RPE enzyme, as described herein.

[0083] 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*

soli, *Heyndrickxia sporothermodurans*, *Clostridium fungisolvens*, or *Neobacillus cucumis*. The XPDH gene may encode an amino acid 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:5 and 95-103. The XPDH gene may encode an amino acid 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:5, 97, 98, and101. See, for example, PCT Application No. PCT/US2023/066629, filed May 5, 2023, which is incorporated herein in its entirety.

[0084] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Clostridium difficile* gene encoding the amino acid of SEQ ID NO:95. The exogenous polynucleotide may encode an amino acid sequence with 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:95.

[0085] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Clostridium difficile* gene encoding the amino acid of SEQ ID NO:96. The exogenous polynucleotide may encode an amino acid sequence with 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:96.

[0086] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Lactobacillus rhamnosus* gene encoding the amino acid of SEQ ID NO:5. The exogenous polynucleotide may encode an amino acid sequence with 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:5.

[0087] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Bacillus halodurans* gene encoding the amino acid of SEQ ID NO:97. The exogenous polynucleotide may encode an amino acid sequence with 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:97.

[0088] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Alkalihalobacillus ligniniphilus* gene encoding the amino acid of SEQ ID NO:98. The exogenous polynucleotide may encode an amino acid sequence with 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:98.

[0089] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Jeotgalibacillus soli* gene encoding the amino acid of SEQ ID NO:99. The exogenous polynucleotide may encode an amino acid sequence with 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:99.

[0090] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Heyndrickxia sporothermodurans* gene encoding the amino acid of SEQ ID NO:100. The exogenous polynucleotide may encode an amino acid sequence with 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:100.

[0091] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Clostridium fungisolvens* gene encoding the amino acid of SEQ ID NO:101. The exogenous polynucleotide may encode an amino acid sequence with 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:101.

[0092] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Neobacillus cucumis* gene encoding the amino acid of SEQ ID NO:103. The exogenous polynucleotide may encode an amino acid sequence with 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:103.

[0093] The recombinant cells described herein are capable of producing xylitol, have an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous

polynucleotide sequence encoding a xylulokinase (XKS) enzyme. A recombinant cell described herein capable of producing xylitol and having an exogenous polynucleotide sequence encoding a ZWF enzyme may additionally include an exogenous polynucleotide sequence encoding an X5PP enzyme and/or overexpress a native X5PP enzyme, an exogenous polynucleotide sequence encoding an XKS enzyme, and/or include an exogenous polynucleotide encoding a native or exogenous RPE enzyme or may have a genetic modification resulting in overexpression of a native RPE enzyme, as described herein. The exogenous polynucleotide sequence may be an exogenous xylulose sugar phosphatase (XKS) gene.

[0094] A “xylulokinase gene” and an “XKS gene” are used interchangeably herein and refer to any gene or polynucleotide that encodes a polypeptide with xylulokinase activity. As used herein, “xylulokinase activity” refer to the ability to catalyze the conversion of xylulose-5-phosphate and ADP to xylulose and ATP. The XKS gene may be derived from any suitable source. For example, the XKS gene may be derived from *Saccharomyces cerevisiae*. The XKS gene may encode an amino acid 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:90 and 91. Additional description of recombinant cells capable of producing xylitol and including a polypeptide with xylulokinase activity is provided in PCT Application No. PCT/US2023/066627, filed May 5, 2023, which is incorporated herein by reference in its entirety.

[0095] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Saccharomyces cerevisiae* DOG1 sugar phosphatase gene encoding the amino acid of SEQ ID NO:91. The exogenous polynucleotide may encode an amino acid sequence with 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:91.

[0096] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Saccharomyces cerevisiae* DOG2 sugar phosphatase gene encoding the amino acid of SEQ ID NO:90. The exogenous polynucleotide may encode an amino acid sequence with 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:90.

[0097] The recombinant cells described herein are capable of producing xylitol, have an exogenous polynucleotide sequence encoding a ZWF enzyme, and an exogenous polynucleotide

sequence encoding a xylitol dehydrogenase (XDH) enzyme. A recombinant cell described herein capable of producing xylitol and having an exogenous polynucleotide sequence encoding a ZWF enzyme may additionally include an exogenous polynucleotide sequence encoding a xylitol dehydrogenase (XDH) enzyme, an exogenous polynucleotide encoding an XKS enzyme, an exogenous polynucleotide sequence encoding an X5PP enzyme and/or overexpress a native X5PP enzyme, an exogenous polynucleotide sequence encoding an XKS enzyme, and/or include an exogenous polynucleotide encoding a native or exogenous RPE enzyme or may have a genetic modification resulting in overexpression of a native RPE enzyme, as described herein. The exogenous polynucleotide sequence may be an exogenous XDH gene.

[0098] A “xylitol dehydrogenase gene” and an “XDH gene” are used interchangeably herein and refer to any gene or polynucleotide that encodes a polypeptide with xylitol dehydrogenase activity. As used herein, “xylitol dehydrogenase activity” refer to the ability to catalyze the conversion of xylulose and NADH or NADPH to xylitol and NAD^+ or $NADP^+$. The XDH gene may be derived from any suitable source. For example, the XDH gene may be derived from *Pichia stipitis*, *Rhodobacteraceae bacterium*, or *Bemisia argentofolii*. The XDH gene may encode an amino acid 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:104, 105, and 106. Additional description of recombinant cells capable of producing xylitol and including a polypeptide with xylulokinase activity and a polypeptide with xylitol dehydrogenase activity is provided in PCT Application No. PCT/US2023/066627, filed May 5, 2023, which is incorporated herein by reference in its entirety.

[0099] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a cofactor switched *Pichia stipitis* XDH gene encoding the amino acid of SEQ ID NO:104. The exogenous polynucleotide may encode an amino acid sequence with 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:104.

[0100] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Rhodobacteraceae bacterium* SDR family oxidoreductase gene encoding the amino acid of SEQ ID NO:105. The exogenous polynucleotide may encode an amino acid

sequence with 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:105.

[0101] The recombinant cell is capable of producing xylitol, has an exogenous polynucleotide sequence encoding a ZWF enzyme, and may include an exogenous polynucleotide that is, or may be derived from, a *Bemisia argentofolii* (Silverleaf Whitefly) ketose reductase (sorbitol dehydrogenase) gene encoding the amino acid of SEQ ID NO:106. The exogenous polynucleotide may encode an amino acid sequence with 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:106.

[0102] 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:6); 6-phosphogluconate dehydrogenase promoter (6PGDp; SEQ ID NO:107); glyceraldehyde-3-phosphate dehydrogenase promoter (TDH3p; SEQ ID NO:108); translational elongation factor 1 promoter (TEFp; SEQ ID NO:109); phosphoglucomutase 1 promoter (PGM1p; SEQ ID NO:110); 3-phosphoglycerate kinase promoter (PGK1p; SEQ ID NO:111); enolase promoter (ENO1p ; SEQ ID NO:112); asparagine synthetase promoter (ASNSp; SEQ ID NO:113); 50S ribosomal protein L1 promoter (RPLAp; SEQ ID NO:114); RPL16B (SEQ ID NO:115); glycerol-3-phosphate dehydrogenase 2a promoter (GPDIIap; SEQ ID NO:134); glycerol dehydrogenase NADP(H) 1 promoter (GDN1p; SEQ ID NO:135); erythritol-P dehydrogenase 1 promoter (EPDH1; SEQ ID NO:136), translation elongation factor 1A like gene 7 promoter (TEF7p; SEQ ID NO:137); heat shock protein 90 promoter (HSP90p; SEQ ID NO:138); translation elongation factor 1A like gene 4 promoter (TEF4p; SEQ ID NO:139); translation elongation factor 2A like gene 6 promoter (TEF6p; SEQ ID NO:140); transaldolase 1 promoter (TAL1p; SEQ ID NO:141); citrate synthase 1 promoter (CIT1p; SEQ ID NO:142); endoplasmic reticulum chaperone BiP (KAR2p; SEQ ID NO:143); heat shock protein 88 promoter (HSP88p; SEQ ID NO:144); heat shock protein 70 promoter (HPS70p; SEQ ID NO:145); tubulin alpha-1 chain promoter (TUB1p; SEQ ID NO:146); glyceraldehyde-3-phosphate dehydrogenase 2 promoter (TDH2p; SEQ ID NO:147); translation elongation factor 1A like gene 5 promoter (TEF5p; SEQ ID NO:148);

translation elongation factor 3 promoter (TEF3p; SEQ ID NO:149); thioredoxin peroxidase promoter (TPXp; SEQ ID NO:150); alginate lyase promoter (ALPp; SEQ ID NO:151); thiazole biosynthetic 4 promoter (THI4p; SEQ ID NO:152); phosphoglucomutase/phospomannomutase promoter (PMMp; SEQ ID NO:153); fructose-bisphosphate aldolase 1 promoter (FBA1p; SEQ ID NO:154); alcohol dehydrogenase 1 promoter (ADH1p; SEQ ID NO:155); and extracellular endoglucanase 1 promoter (GLX1p; SEQ ID NO:156).

[0103] 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:7); ASNS terminator (ASNSt; SEQ ID NO:116); ENO1 terminator (ENO1t; SEQ ID NO:117); hexokinase 1 terminator (HXK1t; SEQ ID NO:118); PGK1 terminator (PGK1t; SEQ ID NO:119); PGM1 terminator (PGM1t; SEQ ID NO:120); PYK1 terminator (PYK1t; SEQ ID NO:51); RPLA terminator (RPLAt: SEQ ID NO:121); transaldolase 1 terminator (TAL1t; SEQ ID NO:122); TDH3 terminator (TDH3t; SEQ ID NO:123); translation elongation factor 2 terminator (TEF2t; SEQ ID NO:53); triosephosphate isomerase 1 terminator (TPI1t; SEQ ID NO:124); MpTEF1 (SEQ ID NO:125); TEF7 terminator (TEF7t; SEQ ID NO:157); HSP90 terminator (HSP90t; SEQ ID NO:158); TEF4 terminator (TEF4t; SEQ ID NO:159); TEF6 terminator (TEF6t; SEQ ID NO:160); CIT1 terminator (CIT11t; SEQ ID NO:161): KAR2 terminator (KAR2t; SEQ ID NO:162); HPS88 terminator (HSP88t; SEQ ID NO:163); HSP70 terminator (HSP70t; SEQ ID NO:164); TUB1 terminator (TUB1t; SEQ ID NO:165); TKL1 terminator (TAL1t; SEQ ID NO:166); TDH2 terminator (TDH2t; SEQ ID NO:167); TEF5 terminator (TEF5t; SEQ ID NO:168); TGL2 terminator (TGL2t; SEQ ID NO:169); TEF3 terminator (TEF3t; SEQ ID NO:170); TPX terminator (TPXt; SEQ ID NO:171); ALP terminator (ALPt; SEQ ID NO:172); plasma membrane ATPase terminator (PMA1t; SEQ ID NO:173); THI4 terminator (THI4t; SEQ ID NO:174); glucose-6-phosphate isomerase 1 terminator (PGI1t; SEQ ID NO:175); PMM terminator (PMMt; SEQ ID NO:176); FBA1 terminator (FBA1t; SEQ ID NO:177); ADH1 terminator (ADH1t; SEQ ID NO:178); sodium/potassium transporting ATPase alpha chain terminator (ATP1t; SEQ ID NO:179); and GLX1 terminator (GLX1t; SEQ ID NO:180).

[0104] 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.

[0105] 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.

[0106] 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 cells described herein to produce 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 or a partially aerobic process.

[0107] 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.

[0108] 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.

[0109] 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 5-80, 10-75, 15-70, 20-60, 30-50, or 40-50 mmol O_2/(L • h). In some embodiments, the specific OUR can be in the range of 0.05 to 10, 0.1 to 9, 0.5 to 8, 1.0 to 7, 1.5 to 6, 2 to 5, or 2.5 to 4 mmol O_2/(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.

[0110] 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.

[0111] The initial dextrose concentration of the fermentation may be at least 100, 200, 250, 300, 350, or at least 400 g/L dextrose when run as a batch fermentation. The initial dextrose concentration may be between 100 to 500, 150 to 450, 400 to 200, or 250 to 350 g/L when run as a batch fermentation. When run in a fed batch fermentation process, the dextrose concentration may be at least 100, 200, 250, 300, 350, 400, or at least 450 g/L.

[0112] 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.

[0113] 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.

[0114] The xylitol production rate of the process may be at least 0.2, 0.3, 0.5, 0.75, or at least 1.0 g L^{-1} h^{-1}. The final xylitol titer of the process may be at least 5, 10, 20, 30, 50, 75, or 100 g/L.

The rate, titer and/or yield of xylitol production may be higher than in a fermentation process run with an equivalent yeast lacking the exogenous polynucleotide encoding a ZWF enzyme.

[0115] The xylitol yield of the process may be at least 25, at least 30, at least 35, at least 40, at least 50, at least 55 percent, at least 65 percent, or at least 70 percent. The xylitol yield of the process may be higher than the xylitol yield of the process run with an equivalent yeast cell lacking the exogenous polynucleotide sequence encoding a ZWF enzyme.

[0116] The total polyol yield of the process may be at least 25, at least 30, at least 35, at least 40, at least 50, at least 55 percent, at least 65 percent, or at least 70 percent. The total polyol yield of the process may be higher than the total polyol yield of the process run with an equivalent yeast cell lacking the exogenous polynucleotide sequence encoding a ZWF enzyme. Without being bound by any particular hypothesis, theory, or mode of action, it is believed that higher ZWF enzyme activity (e.g., through an increase in ZWF gene copy number) increases carbon flux into the pentose phosphate pathway and will lead to an increase in polyol yield in the process.

EXAMPLES

[0117] 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.

[0118] Throughout the Examples, strain numbering and sequence identification numbers are used consistently. For example, strain 1-2 in Example 2 is the same as strain 1-2 in Example 3, etc.

Example 1: Glucose-6-Phosphate Dehydrogenase Diversity

[0119] 330 glucose-6-phosphate (ZWF) enzyme candidate sequences were obtained from Uniprot based on EC 1.1.1.388 and analyzed using the Geneious protein aligner tool. These sequences showed relatively high sequence identity, 221 of the sequences are greater than 75% identical to one another. A total of 6 sequences were selected for evaluation based on their low sequence identity to one another.

[0120] Additionally, the enzymes sequence of the ZWF1 enzyme from *Arabidopsis thaliana* (Uniprot Q43727) was used as a basis for further sequence-based enzyme identification using

sequence homology against the National Center for Biotechnology Information (NCBI) database. The top 100 sequences from this search were aligned, demonstrating at least 80% sequence identity across the set. 5 ZWF enzyme candidate sequences from plants were chosen for further testing based on randomly varying the distance (% homology) away from the original sequence.

[0121] Four candidate ZWF encoding genes from Moniliella pollinis were also identified and selected for testing; RCSR20949, RCSR22401, RCSR24965, and RCSR02959. Three of these four, RCSR20949, RCSR22401, and RCSR24965, were mutated to contain an aspartic acid and isoleucine (DI) in place of alanine and arginine (AR) in the proposed co-factor binding site to potentially convert the enzyme to prefer NADH.

[0122] These 18 total ZWF enzyme candidate sequences were then cloned into *Moniliella pollinis* as described below to characterize *in vivo* enzyme activity.

Example 2: Genetically Modified Moniliella pollinis Strains

Strain 1-1

[0123] 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.

Strain 1-2

[0124] Strain 1-1 was transformed with SEQ ID NO:2 and SEQ ID NO:1 by first protoplasting the parent strain by adding an enzyme mixture containing 0.6M $MgSO_4$, 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 $MgSO_4$, 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) to approximately 200 µL protoplast mixture (10^8 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 ºC, 100 rpm, for 16 to 24 hours. Following the incubation, protoplasts were pelleted by centrifugation and resuspended in 1 mL PBS. The resuspended protoplasts were plated on PDA + 250 mg/L geneticin (G418) selection plates and incubated at 35 ºC for at least 2 days until transformants grew. 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 two copies of the *L. rhamnosus* XPDH sequence. A PCR verified isolate was designated strains 1-2.

[0125] SEQ ID NO:1 contains (i) 5' flanking DNA for targeted chromosomal integration into the ER1 locus (SEQ ID NO:3); (ii) a polynucleotide sequence, SEQ ID NO:4, encoding the XPDH homolog from *Lactobacillus rhamnosus* of SEQ ID NO:5, under the control of the PYK1 promoter of SEQ ID NO:6 and the PGD terminator of SEQ ID NO:7; and (iii) a 5' portion of the G418 selectable marker (SEQ ID NO:8).SEQ ID NO:2 contains (i) a 3' portion of the G418 selectable marker (SEQ ID NO:9); (ii) a polynucleotide sequence, SEQ ID NO:4, encoding the XPDH homolog from *Lactobacillus rhamnosus* of SEQ ID NO:5 , under the control of the PYK1 promoter of SEQ ID NO:6 and the PGD terminator of SEQ ID NO:7; and (iii) a 3' flanking DNA for targeted chromosomal integration into the ER1 locus (SEQ ID NO:10).

Strains 1-3 through 1-19

[0126] Table 2 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 transformation sequence. While the table lists the source of the encoded ZWF polypeptide sequences, the polynucleotide sequence encoding the polypeptide sequence was codon optimized prior to cloning and therefore does not match the gene sequence from the source organism. Gene sequences encoding the indicated polypeptide are part of the transformation fragment sequence.

[0127] For example, strain 1-2 was transformed with SEQ ID NO:28 and SEQ ID NO:45 using the transformation method described for strain 1-2, to create strains 1-3a-e. SEQ ID NO:28 contained (i) a 3' portion of a zeocin resistance marker (SEQ ID NO:47); (ii) a polynucleotide encoding the candidate ZWF enzyme of SEQ ID NO:11, under the control of a PYK1 promoter (SEQ ID NO:6) and a 6PGD terminator (SEQ ID NO:7); and (ii) a 3' flanking DNA for targeted chromosomal integration into the ER3 locus (SEQ ID NO:50). SEQ ID NO:45 contained (i) 5'

flanking DNA for targeted chromosomal integration into the ER3 locus (SEQ ID NO:49); (ii) a polynucleotide encoding the RPE enzyme of SEQ ID NO:46 under the control of a PYK1 promoter (SEQ ID NO:6) and a PYK1 terminator (SEQ ID NO:51); and (iii) a 5' portion of a zeocin resistance marker (SEQ ID NO:48).Transformed protoplasts were selected on PDA + zeocin selection plates and incubated at 35 °C for at least 2 days until transformants grow. Resulting transformants were streaked for single colony isolation on PDA + zeocin plates and single colonies were selected. Selected colonies were evaluated by colony PCR for integration of the ZWF and RPE encoding sequences. PCR verified sister isolates were designated strains 1-3a, 1-3b, 1-3c, 1-3d, and 1-3e.

[0128] The transformation fragments of SEQ ID NOs:29-44 included the same components as SEQ ID NO:28, except the sequence encoding the polypeptide of SEQ ID NO:11 was replaced with a polynucleotide encoding the polypeptide of SEQ ID NOs:12-27, respectively. In some instances, more than one PCR verified isolate, e.g., "sister" isolates, are indicated by letters following the strain number. For example, as described in the previous paragraph, strain 1-3 has 5 sister isolates, strains 1-3a, 1-3b, 1-3c, 1-3d, and 1-3e.

Table 2

Strain	Parent Strain	First Bipartite Transformation Fragment			Second Bipartite Transformation Fragment	
		Transformation SEQ ID NO:	Encoded Polypeptide SEQ ID NO:	Encoded Polypeptide Source	Transformation SEQ ID NO:	Encoded Polypeptide SEQ ID NO:
1-3a-e	1-2	28	11	*Arabidopsis thaliana*	45	46
1-4a-e	1-2	29	12	*Cephalotus follicularis*	45	46
1-5a-e	1-2	30	13	*Salix suchowensis*	45	46
1-6a-e	1-2	31	14	*Perilla frutescens*	45	46
1-7a-e	1-2	32	15	*Pseudomonas putida*	45	46
1-8a-e	1-2	33	16	*Pantoea sp. Ap-967*	45	46
1-9a-e	1-2	34	17	*Halorubrum coriense*	45	46
1-10a-e	1-2	35	18	*Haloferax larsenii*	45	46

1-11a-e	1-2	36	19	*Aeromonas caviae*	45	46
1-12a-e	1-2	37	20	*Natrialba hulunbeirensis*	45	46
1-13a-e	1-2	38	21	*M. pollinis* RCSR22401 mutant	45	46
1-14a-e	1-2	39	22	*M. pollinis* RCSR20949 mutant	45	46
1-15a-e	1-2	40	23	*M. pollinis* RCSR24965 mutant	45	46
1-16a-e	1-2	41	24	*M. pollinis* RCSR02929	45	46
1-17a-e	1-2	42	25	*M. pollinis* RCSR20949	45	46
1-18a-e	1-2	43	26	*M. pollinis* RCSR22401	45	46
1-19a-e	1-2	44	27	*M. pollinis* RCSR24965	45	46

Strain 1-20

[0129] Strain 1-2 was transformed with SEQ ID NO:45 and SEQ ID NO:52 using the transformation method outlined above for strain 1-2. SEQ ID NO:52 contained (i) a 3' portion of a zeocin resistance gene expression cassette (SEQ ID NO:47), an MpTEF2 terminator (SEQ ID NO:53), and a 3' ER3 flanking sequence (SEQ ID NO:50). Transformed protoplasts were selected on PDA + zeocin selection plates and incubated at 35 °C for at least 2 days until transformants grow. Resulting transformants were streaked for single colony isolation on PDA + zeocin plates and single colonies were selected. Selected colonies were evaluated by colony PCR for integration of the RPE encoding sequence. PCR verified sister isolates were designated strains 1-20a, 1-20b, 1-20c, 1-20d, and 1-20e.

Example 3: Shake Flask Fermentation Assay

[0130] Strains 1-2, 1-3a-e, 1-4a-e, 1-5a-e, 1-6a-e, 1-7a-e, 1-8a-e, 1-9a-e, 1-10a-e, 1-11a, 1-11c, 1-20a, and 1-20b were run in shake flasks to assess glucose consumption as well as ribitol, erythritol, glycerol, and ethanol production.

[0131] 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.

[0132] A 250 ml non-baffled flask containing 20mL production medium (Table 3) was inoculated with 0.4 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 48, 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. Results are shown in Table 4 and FIG. 2 and xylitol and polyol yield are reported in Table 5. Xylitol yield was calculated by dividing the concentration of xylitol (w/v) by the total glucose consumed at a given time point (w/v). For example, if the starting glucose was 100 g/L and at 48 hours 20g/L of xylitol was produced and 20 g/L glucose remained, the yield at the 48-hour time point would be the xylitol concentration at 48 hours (20g/L) divided by the total consumed glucose at 48 hours (100 g/L – 20 g/L), a resulting yield of 25%. Polyol was calculated using the same method, but in place of the xylitol concentration, the concentration of the total of xylitol, ribitol, and erythritol produced at a given time point was used.

[0133] Results show that several strains produced a higher polyol titer than control strains. For example, strains 1-3a, 1-3e, 1-6d, 1-7a, 1-7b, 1-7c, 1-7d, 1-8c, 1-8d, 1-8e, 1-9a, 1-9c, and 1-10b had a higher polyol titer and of those strains 1-7c, 1-7d, 1-8c, 1-8d, 1-8e, and 1-9a had higher xylitol titers. Additionally, strains 1-7a and 1-8b had a higher yield of xylitol while strains 1-3a, 1-3e, 1-7b, 1-7c, 1-7d, 1-8c, 1-8d, and 1-9a had a higher yield of total polyols.

[0134] While PCR verification indicated that the transformed polynucleotide sequence(s) was present in the indicated strains, further analysis indicated that in the majority of the strains, the RPE and ZWF encoding sequences were integrated into the strain, but not at the ER3 loci as planned. This may account for some of the sister strain variability in the results. However, the results still demonstrate that expression of the encoded ZWF helps increase yield of xylitol.

Table 3: Production Medium

Component	Concentration (units)
Glucose	300 (g/L)
KH2PO4	1.27 (g/L)
(NH4)2HPO4	0.13 (g/L)
(NH4)2SO4	1.80 (g/L)
Urea	2.85 (g/L)
Citric Acid	200.0 (mg/L)
MgSO4-7H2O	690.2 (mg/L)
FeSO4-7H2O	32.35 (mg/L)
ZnSO4-7H2O	4.40 (mg/L)
MnSO4-H2O	0.53 (mg/L)
CuSO4-5H2O	0.45 (mg/L)
CaCl2-2H2O	91.60 (mg/L)
Na2SO4	154.50 (mg/L)
Thiamine-HCl	10 (mg/L)
Choline-Cl	100 (mg/L)
Antifoam CF-32	1.00 (g/L)

Table 4.

Strain	Time Point (h)	Fermentation Broth Analyte (g/L)						Time Point (h)	Fermentation Broth Analyte (g/L)						Time Point (h)	Fermentation Broth Analyte (g/L)					
		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-2	48	164.00	3.22	9.13	17.23	18.67	16.49	72	63.32	10.53	24.35	42.55	21.73	20.49	96	36.59	14.15	29.59	55.84	22.62	13.25
1-20c	48	185.11	0.32	12.73	7.85	20.21	12.24	72	104.44	0.89	37.01	16.29	23.89	16.97	96	61.07	1.55	55.98	23.56	26.98	13.14
1-20d	48	183.58	0.29	14.34	7.17	21.88	13.03	72	95.75	0.81	41.79	16.57	25.89	19.12	96	80.16	1.11	53.03	19.13	26.48	12.65
1-3a	48	186.34	0.59	12.11	8.57	19.02	13.25	72	103.01	2.18	34.96	20.66	22.60	17.58	96	57.03	3.48	51.85	30.48	24.83	13.54
1-3b	48	195.16	0.23	7.61	7.16	23.77	12.89	72	115.38	0.88	24.60	14.68	28.52	19.99	96	87.09	1.47	35.80	20.19	30.36	14.76
1-3c	48	188.37	0.12	12.61	8.35	20.93	12.00	72	107.24	0.30	36.76	16.82	24.82	16.45	96	65.12	0.55	55.58	24.25	27.63	12.08
1-3d	48	208.97	0.39	9.67	7.91	17.31	8.48	72	132.12	1.22	28.33	16.41	23.16	13.56	96	86.90	2.06	45.55	24.57	26.59	11.57
1-3e	48	184.63	0.61	12.28	8.98	20.21	12.88	72	99.84	2.16	34.51	20.51	23.80	18.10	96	57.04	3.51	51.42	30.10	26.56	13.44
1-4a	48	193.54	0.26	11.53	7.23	20.06	11.92	72	107.41	0.81	35.89	15.72	24.49	18.69	96	68.21	1.41	53.98	22.88	27.49	13.49
1-4b	48	182.49	0.31	13.43	8.18	22.26	13.56	72	103.58	0.79	36.69	16.28	26.69	18.09	96	57.66	1.33	55.84	23.57	30.40	14.82
1-4c	48	210.28	0.27	5.43	7.32	20.51	9.66	72	129.53	1.27	21.08	16.84	24.68	16.95	96	91.61	2.46	32.15	26.61	27.45	12.28
1-4d	48	195.88	0.38	11.22	7.65	19.16	12.53	72	111.74	1.36	33.77	16.83	22.79	18.65	96	65.83	2.31	52.23	25.37	25.67	14.86
1-4e	48	193.73	0.27	12.07	7.41	20.20	11.27	72	108.80	0.76	35.64	15.46	24.40	17.06	96	61.23	1.31	55.37	22.64	27.44	14.07
1-5a	48	207.32	0.24	8.19	6.06	19.53	9.91	72	118.08	0.69	30.48	12.65	26.30	17.96	96	67.05	1.25	51.53	19.07	30.21	16.04
1-5b	48	192.71	0.26	12.27	7.26	18.94	12.15	72	112.42	0.73	35.42	14.95	22.30	17.43	96	64.49	1.25	55.40	21.87	24.59	15.59
1-5c	48	187.55	0.22	13.44	7.80	20.25	11.70	72	106.63	0.55	37.21	15.98	23.84	16.51	96	65.15	0.92	55.65	23.19	26.60	10.97
1-5d	48	209.45	0.22	4.32	7.76	23.99	9.65	72	126.04	1.05	19.19	15.64	30.78	18.56	96	78.37	2.13	32.63	26.06	33.78	15.82

1-5e	48	193.75	0.11	11.34	7.57	20.70	11.91	72	109.00	0.28	35.62	15.56	24.20	18.09	96	59.98	0.60	56.72	22.61	26.76	15.41
1-6a	48	191.79	0.18	8.80	7.52	24.10	11.89	72	113.21	0.50	27.76	14.83	28.50	17.48	96	82.85	0.89	40.40	20.30	31.43	12.08
1-6b	48	189.91	0.26	12.85	7.36	19.42	12.01	72	111.49	0.70	35.53	15.16	22.73	16.99	96	65.87	1.22	55.05	22.31	25.28	13.95
1-6c	48	195.87	0.25	11.67	7.30	19.58	10.91	72	112.31	0.72	34.23	15.22	23.76	16.65	96	65.19	1.21	52.98	22.50	26.26	13.33
1-6d	48	189.62	1.19	11.48	7.85	17.74	13.87	72	101.27	4.00	33.15	17.59	23.04	20.13	96	51.11	6.43	50.51	25.99	27.03	16.89
1-6e	48	202.41	0.56	10.18	8.08	16.87	10.02	72	122.37	2.05	29.69	18.01	20.15	15.64	96	73.32	3.55	48.11	27.84	22.40	14.06
1-7a	48	184.64	0.52	13.23	8.30	19.61	13.06	72	101.73	1.78	35.88	18.43	22.83	18.74	96	56.61	2.92	53.53	27.29	25.30	14.57
1-7b	48	211.65	0.22	13.41	6.83	19.01	6.09	72	135.51	0.55	40.27	12.09	24.08	11.60	96	83.27	0.93	67.78	17.75	27.33	10.29
1-7c	48	191.88	3.18	9.48	9.61	15.84	13.43	72	78.36	14.35	26.59	26.49	20.94	23.51	96	15.91	24.08	37.72	42.12	23.19	21.14
1-7d	48	200.30	1.41	9.80	10.31	15.63	11.84	72	95.79	6.67	28.60	28.72	18.53	20.85	96	42.37	10.59	44.71	45.29	19.92	16.09
1-7e	48	242.56	0.30	6.20	5.14	12.12	3.35	72	171.38	2.13	25.51	10.94	16.39	10.41	96	102.39	6.02	51.00	20.24	19.62	13.29
1-8a	48	241.20	0.30	8.50	5.07	11.63	1.75	72	175.20	1.12	27.58	9.42	16.32	8.77	96	114.00	2.59	53.59	16.00	19.44	11.60
1-8b	48	187.67	0.67	12.04	8.57	20.87	13.77	72	99.90	2.41	33.50	19.87	24.09	20.32	96	68.54	3.69	47.27	28.08	26.49	14.09
1-8c	48	201.17	1.39	11.36	9.82	16.19	11.30	72	96.01	6.10	31.71	26.20	19.69	18.61	96	43.03	9.73	47.36	42.42	22.06	11.87
1-8d	48	198.41	1.49	11.48	10.26	16.34	11.73	72	94.05	6.52	31.01	27.60	19.32	20.38	96	40.34	10.57	47.75	43.83	20.93	15.81
1-8e	48	188.50	1.92	9.40	12.32	16.14	13.77	72	83.26	7.89	26.62	32.75	18.72	22.66	96	43.33	12.02	36.29	47.50	19.74	16.80
1-9a	48	192.44	0.91	10.45	10.24	19.13	11.79	72	93.32	3.72	30.71	25.43	23.55	20.92	96	37.64	6.35	46.92	39.79	26.41	18.28
1-9b	48	189.90	0.27	12.76	7.35	18.94	12.67	72	112.46	0.71	34.71	14.85	21.62	17.41	96	69.09	1.21	53.59	21.38	23.69	14.82
1-9c	48	180.93	0.73	13.14	9.21	20.54	14.07	72	93.78	2.36	35.06	21.10	23.91	18.94	96	54.12	3.72	50.39	31.30	26.80	12.50
1-9d	48	203.75	0.25	10.39	7.33	21.38	10.13	72	116.09	0.71	31.64	14.30	24.74	18.59	96	92.37	1.12	44.14	17.62	26.74	12.17
1-9e	48	230.75	0.19	3.95	5.47	17.43	7.00	72	143.37	1.04	21.85	11.91	22.92	17.63	96	83.77	2.23	42.62	20.99	26.50	17.01
1-10a	48	189.43	0.31	11.83	7.36	19.89	12.03	72	119.84	0.74	31.90	14.36	23.70	16.32	96	70.95	1.28	50.10	20.99	26.34	15.63

1-10b	48	202.59	0.99	5.83	9.83	16.07	12.38	72	99.10	5.74	21.49	26.93	19.29	22.53	96	54.30	9.80	31.43	42.71	20.30	18.51
1-10c	48	197.90	0.13	5.80	6.86	27.84	11.08	72	121.83	0.39	19.98	11.79	34.99	16.33	96	94.49	0.72	30.13	16.91	38.90	10.84
1-10d	48	189.20	0.52	12.19	8.08	19.55	12.84	72	103.48	1.82	33.86	17.96	22.74	18.54	96	60.88	2.90	50.45	27.12	25.06	14.14
1-10e	48	192.37	0.24	11.05	7.15	21.87	11.74	72	106.43	0.68	33.71	14.92	26.01	18.61	96	53.44	1.28	54.51	22.69	28.64	16.97
1-11a	48	184.89	0.14	12.70	8.12	21.36	13.30	72	105.12	0.30	35.07	16.01	24.69	17.88	96	67.16	0.53	52.13	22.73	27.16	12.90
1-11c	48	179.64	0.38	13.55	7.50	23.21	13.56	72	94.84	0.90	36.68	14.71	28.20	19.42	96	55.02	1.54	53.75	20.17	31.49	14.90

PT-1794-WO-PCT

Table 5: Yield (%) at 96 Hours

Strain	Xylitol	Polyol
1-2	11.2	33.1
1-20c	23.3	27.0
1-20d	23.9	24.4
1-3a	21.2	28.6
1-3b	16.7	19.1
1-3c	23.5	26.8
1-3d	21.2	24.1
1-3e	21.0	28.4
1-4a	23.1	26.1
1-4b	22.9	26.9
1-4c	15.3	20.4
1-4d	22.1	26.6
1-4e	23.0	26.4
1-5a	22.0	23.9
1-5b	23.4	26.2
1-5c	23.5	26.6
1-5d	14.6	20.2
1-5e	23.5	26.6
1-6a	18.5	20.5
1-6b	23.3	26.2
1-6c	22.4	25.6
1-6d	20.2	27.7
1-6e	21.1	26.5
1-7a	21.8	27.9
1-7b	31.0	28.8
1-7c	13.2	34.6
1-7d	17.2	33.5
1-7e	25.6	25.7
1-8a	28.6	24.1
1-8b	20.3	26.4
1-8c	18.3	33.2
1-8d	18.3	34.0
1-8e	14.0	31.9
1-9a	17.8	31.0
1-9b	23.0	25.4
1-9c	20.4	28.5
1-9d	21.1	21.0
1-9e	19.6	22.0
1-10a	21.7	24.1
1-10b	12.7	27.9
1-10c	14.5	15.9
1-10d	20.9	26.8
1-10e	22.0	26.1
1-11a	22.2	25.1
1-11c	21.8	25.2

Example 4: Shake Flask Fermentation Assay

[0135] Strains 1-2, 1-20c, 1-20d, 1-11c, 1-11d, 1-11e, 1-12a-e, 1-13a-e, 1-14-a-e, 1-15a-e, 1-16a-d, 1-17a-e, 1-18a-e, and 1-19e-a were run in shake flasks to assess glucose consumption as well as ribitol, erythritol, glycerol, and ethanol production.

[0136] 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.

[0137] A 250 ml non-baffled flask containing 20mL production medium (Table 3) was inoculated with 0.4 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 48, 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. Results are shown in Table 6 and FIG. 3 and xylitol and polyol yield are reported in Table 7. Yield was calculated as outlined in Example 3.

[0138] Results show that several strains produced a higher polyol titer than control strains. For example, strains 1-11e, 1-12c, 1-12d, 1-13a, 1-13b, 1-13c, 1-13d, 1-13e, 1-14b, 1-14c, 1-14d, 1-14e, 1-15a, 1-15b, 1-15d, 1-15e, 1-16a, 1-16b, 1-16d, 1-18b, 1-18c, and 1-19c had a higher polyol titer Strains 1-13a, 1-13c, 1-14a, 1-14e, 1-16c, 1-17c, 1-19b, 1-19c, 1-19d, and 1-19e had higher xylitol titers than control strains1-20c and 1-20d. Additionally, strains 1-19b ad 1-19c had a higher yield of xylitol while strains 1-11c, 1-11e, 1-12b, 1-13b, 1-13c, 1-14b, 1-14c, 1-15a, 1-15b, 1-15d, 1-15e, 1-16a, 1-16b, 1-16d, 1-18c, and 1-19c had a higher yield of total polyols.

[0139] While PCR verification indicated that the transformed polynucleotide sequence(s) was present in the indicated strains, further analysis indicated that in the majority of the strains, the RPE and ZWF encoding sequences were integrated into the strain, but not at the ER3 loci as planned. This may account for some of the sister strain variability in the results. However, the results still demonstrate that expression of the encoded ZWF helps increase yield of xylitol.

Table 6

Strain	Time Point (h)	Fermentation Broth Analyte (g/L)						Time Point (h)	Fermentation Broth Analyte (g/L)						Time Point (h)	Fermentation Broth Analyte (g/L)					
		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
1-2	48	170.80	3.12	8.75	17.29	20.37	16.86	72	66.25	10.84	24.43	44.62	23.51	21.73	96	46.39	13.31	27.20	54.91	23.70	10.53
1-20c	48	189.11	0.38	12.99	7.88	22.06	12.72	72	108.42	0.91	37.90	16.34	26.14	17.38	96	63.39	1.35	53.60	23.66	27.67	11.18
1-20d	48	189.17	0.31	14.95	7.00	22.73	13.19	72	99.77	0.76	42.07	15.78	26.83	19.32	96	77.89	1.06	52.58	19.78	25.49	10.69
1-11c	48	194.16	0.42	12.62	7.51	18.54	13.53	72	113.75	1.32	36.10	16.94	21.80	18.30	96	67.49	1.98	52.20	24.72	22.95	13.51
1-11d	48	194.76	0.30	12.57	7.26	20.79	11.91	72	114.57	0.70	36.55	15.17	24.94	16.99	96	65.58	1.08	53.07	22.19	26.30	12.45
1-11e	48	197.40	0.61	11.38	8.81	21.51	11.53	72	107.63	2.17	34.37	20.35	26.15	16.37	96	60.20	3.39	48.93	30.21	28.01	10.22
1-12a	48	208.10	0.37	11.13	7.00	21.18	9.17	72	127.37	0.89	33.70	14.08	26.48	14.52	96	68.19	1.51	52.95	22.55	29.80	13.02
1-12b	48	191.67	0.48	12.77	7.88	18.58	14.31	72	110.26	1.48	35.57	18.42	22.32	18.34	96	67.57	2.23	49.68	26.96	23.78	11.47
1-12c	48	193.41	0.47	12.33	7.65	20.05	13.54	72	108.11	1.51	35.95	17.05	23.50	19.58	96	60.09	2.40	51.95	25.88	24.62	14.36
1-12d	48	197.31	0.59	10.25	8.08	19.80	13.53	72	107.74	2.45	32.32	19.60	23.52	19.72	96	57.31	3.73	47.36	30.79	24.52	14.44
1-12e	48	218.61	0.34	6.79	5.47	20.06	11.56	72	112.60	1.56	29.50	12.78	26.41	22.46	96	51.37	3.02	49.57	20.51	28.25	16.97
1-13a	48	197.02	0.37	12.95	6.89	21.16	12.46	72	112.44	1.07	39.05	14.58	25.78	18.52	96	58.38	1.75	57.88	21.46	26.94	14.31
1-13b	48	196.20	0.61	12.57	8.29	19.35	12.35	72	108.75	2.13	36.57	18.71	23.20	18.36	96	60.60	3.30	52.48	28.15	24.43	12.32
1-13c	48	194.44	0.46	13.02	7.81	20.88	13.11	72	109.35	1.40	37.49	16.89	24.68	18.70	96	60.70	2.25	55.33	25.31	26.21	13.94
1-13d	48	205.57	0.45	10.08	7.38	19.96	11.02	72	113.81	1.90	33.75	16.82	24.75	18.95	96	60.43	2.94	51.17	25.96	26.46	13.83
1-13e	48	210.84	0.46	10.97	7.24	19.24	9.95	72	118.24	1.77	35.34	16.28	24.17	18.07	96	65.64	2.76	53.35	25.08	25.67	12.72
1-14a	48	204.75	0.33	11.65	6.77	20.56	10.21	72	122.34	0.85	36.16	14.08	26.21	15.10	96	66.34	1.39	55.14	21.98	28.66	10.94

1-14b	48	194.00	0.62	12.96	8.41	20.36	12.74	72	109.10	2.17	36.53	19.64	24.48	17.76	96	59.24	3.36	52.32	29.56	25.73	12.15
1-14c	48	205.89	0.66	9.89	8.27	18.72	12.53	72	115.89	3.33	31.12	21.04	23.78	18.91	96	64.16	5.66	45.28	34.00	25.18	13.52
1-14d	48	203.78	0.94	10.13	7.56	19.38	11.79	72	111.71	3.99	31.97	17.92	24.54	19.44	96	55.68	6.66	47.95	28.04	26.12	15.16
1-14e	48	199.75	0.31	12.15	7.01	21.10	12.06	72	110.21	0.82	37.14	15.41	26.27	16.52	96	57.58	1.35	54.99	23.44	28.06	11.12
1-15a	48	209.09	0.51	11.47	7.09	20.22	10.39	72	123.18	2.09	35.84	16.62	25.16	17.28	96	72.79	3.33	53.22	26.18	26.62	10.88
1-15b	48	215.53	1.10	9.45	7.07	18.71	9.13	72	124.34	4.82	31.83	16.84	23.77	16.98	96	58.45	8.67	50.50	27.89	25.29	15.05
1-15c	48	195.47	0.71	12.67	9.00	18.93	13.22	72	106.46	2.26	34.84	20.27	22.85	18.02	96	74.81	3.10	43.91	28.20	23.94	11.13
1-15d	48	180.15	2.53	11.96	11.96	16.15	18.51	72	71.22	9.15	31.87	33.93	19.27	25.57	96	27.33	13.10	41.19	48.97	18.69	17.59
1-15e	48	195.38	0.64	12.69	8.33	19.64	12.35	72	106.63	2.09	36.33	19.64	23.74	16.76	96	56.13	3.08	52.86	30.55	25.96	8.37
1-16a	48	186.52	1.30	11.37	10.76	20.91	15.21	72	87.46	5.05	31.54	27.85	24.63	20.22	96	41.20	7.57	42.38	43.16	25.86	12.29
1-16b	48	209.86	1.05	5.25	10.38	18.41	12.00	72	98.28	7.33	21.15	30.70	23.27	23.19	96	41.40	12.10	30.23	49.85	23.44	17.21
1-16c	48	193.62	0.34	13.51	7.09	22.29	12.64	72	99.57	1.00	42.27	14.09	29.18	19.55	96	58.57	1.63	57.80	17.44	31.81	14.87
1-16d	48	209.55	0.95	5.70	9.99	18.79	12.43	72	100.37	6.80	21.87	29.50	23.42	23.38	96	38.39	11.74	32.05	50.03	23.33	18.19
1-17a	48	186.28	2.11	9.03	14.11	19.28	14.79	72	110.93	5.98	20.52	30.63	23.12	14.67	96	86.99	7.69	23.52	40.52	23.81	7.01
1-17b	48	200.82	1.04	9.22	9.66	19.45	13.91	72	103.64	5.14	27.03	26.71	23.27	21.07	96	74.61	7.01	32.98	36.23	23.54	14.00
1-17c	48	234.52	0.28	6.80	4.83	15.93	8.26	72	131.38	1.39	31.77	11.12	25.12	20.63	96	50.78	2.77	57.90	18.86	29.55	22.32
1-17d	48	218.49	0.27	8.63	6.33	18.00	9.92	72	134.53	0.80	31.55	13.23	22.69	16.43	96	78.32	1.45	51.15	21.38	24.27	13.87
1-17e	48	213.80	0.34	10.31	6.80	17.86	9.61	72	133.38	0.86	32.19	14.09	22.17	15.22	96	85.59	1.34	48.26	20.67	23.83	10.94
1-18a	48	215.21	0.31	9.87	6.61	17.74	10.33	72	130.30	0.92	31.74	14.21	22.27	15.80	96	78.22	1.51	48.82	22.37	23.76	11.19
1-18b	48	206.82	0.56	9.16	8.07	20.41	11.48	72	115.48	2.64	29.96	19.80	24.99	17.05	96	61.46	4.10	44.96	32.15	26.50	10.67
1-18c	48	199.84	0.69	10.95	8.33	19.89	12.86	72	110.63	2.81	32.02	20.06	23.61	18.81	96	60.24	4.32	46.97	31.97	24.85	12.77
1-18d	48	193.78	0.38	13.65	7.56	19.80	13.27	72	113.06	0.99	37.34	15.84	23.37	18.27	96	66.44	1.56	53.54	23.43	24.34	12.86

1-18e	48	183.64	1.58	11.21	12.13	20.02	17.11	72	88.66	5.31	29.10	27.14	22.72	24.80	96	79.33	6.06	31.65	29.09	23.14	16.17
1-19a	48	200.57	0.29	12.87	7.42	20.82	11.38	72	117.02	0.59	38.30	15.19	23.50	15.27	96	81.33	0.87	52.61	21.09	24.28	7.14
1-19b	48	192.81	0.36	13.74	7.17	20.94	13.85	72	115.17	0.87	39.85	14.61	25.58	17.53	96	61.88	1.37	57.66	21.54	26.43	12.97
1-19c	48	213.45	0.36	12.04	6.66	18.47	9.54	72	129.12	0.94	36.14	13.02	23.16	16.83	96	71.82	1.61	57.10	20.75	24.79	13.77
1-19d	48	191.37	0.18	13.15	7.55	22.22	13.19	72	108.86	0.35	37.81	15.08	26.07	18.93	96	59.32	0.60	55.76	22.49	27.21	13.77
1-19e	48	197.23	0.32	12.45	6.99	20.54	12.59	72	114.10	0.74	36.57	14.50	24.75	17.63	96	61.08	1.22	54.35	21.98	25.95	13.79

Table 7: Xylitol and Polyol Yield (%) at 96 Hours

Strain	Xylitol	Polyol
1-2	10.7	37.4
1-20c	22.5	33.0
1-20d	23.5	32.8
1-11c	22.3	33.7
1-11d	22.5	32.3
1-11e	20.3	34.2
1-12a	22.7	33.0
1-12b	21.2	33.7
1-12c	21.5	33.2
1-12d	19.4	33.5
1-12e	19.8	29.2
1-13a	23.8	33.3
1-13b	21.8	34.8
1-13c	23.0	34.4
1-13d	21.2	33.2

Strain	Xylitol	Polyol
1-13e	22.6	34.4
1-14a	23.4	33.4
1-14b	21.6	35.2
1-14c	19.1	35.8
1-14d	19.5	33.6
1-14e	22.5	32.7
1-15a	23.3	36.1
1-15b	20.8	35.8
1-15c	19.4	33.1
1-15d	15.0	37.6
1-15e	21.5	35.2
1-16a	16.3	35.7
1-16b	11.6	35.4
1-16c	23.8	31.6
1-16d	12.2	35.6

Strain	Xylitol	Polyol
1-17a	11.0	33.4
1-17b	14.5	33.6
1-17c	23.1	31.7
1-17d	22.9	33.1
1-17e	22.3	32.5
1-18a	21.8	32.5
1-18b	18.7	33.8
1-18c	19.5	34.5
1-18d	22.8	33.4
1-18e	14.2	30.0
1-19a	23.9	33.8
1-19b	24.0	33.6
1-19c	24.8	34.6
1-19d	23.0	32.5
1-19e	22.6	32.2

Example 5: Genetically Modified Moniliella pollinis Strains

Strain 2-1

[0140] Strain 1-2 was transformed with was transformed with SEQ ID NO:45 and SEQ ID NO:52 using the protocol outlined above. The transformation fragment of SEQ ID NO:45 contained, in order, a 5’ ER3 flanking sequence (SEQ ID NO:49), a MpPYK1 promoter (SEQ ID NO:6), a gene encoding the *M. pollinis* RPE2 polypeptide of SEQ ID NO:46, a MpPYK terminator (SEQ ID NO:51), and a 5’ portion of a zeocin resistance gene expression cassette (SEQ ID NO:48). The transformation fragment of SEQ ID NO:52 contained, in order, a 3’ portion of a zeocin resistance gene expression cassette (SEQ ID NO:47), an MpTEF2 terminator (SEQ ID NO:53), and a 3’ ER3 flanking sequence (SEQ ID NO:50). Resulting transformants were streaked for single colony isolation on PDA + zeocin

plates and single colonies were selected. Selected colonies were evaluated by colony PCR for integration of the *M. pollinis* RPE2 sequence. A PCR verified isolate was designated strains 2-1.

Strain 2-2

[0141] UV mutagenesis (using a Hoefer UV Crosslinker at an energy of 360 uJ/cm^3) and selection of strain 1-3 was used to generate a *Moniliella pollinis* strain with reduced foaming during shake flask fermentation. Strains with low-foaming phenotypes were selected based on visual evaluation of foaming in a shake flask fermentation compared to foaming on the parent 1-3 strain. The resulting low-foaming strain, containing two copies of an exogenous polynucleotide sequence encoding the XPDH of SEQ ID NO:5 integrated at the ER1 locus and one copy of a polynucleotide sequence encoding the RPE of SEQ ID NO:46 integrated at the ER3 locus, was designated 2-2.

Strain 2-3

[0142] Strain 2-2 was transformed with the Cre recombinase plasmid of SEQ ID NO:126 using the transformation method outlined above. The resulting transformants were evaluated by colony PCR for removal of the G418 and zeocin resistance selection marker. A PCR verified isolate was designated strain 2-3.

Strain 2-4

[0143] Strain 2-3 was grown non-selectively on YPD plates to allow for the loss of the plasmid of SEQ ID NO:126. Biomass was struck for single colonies and evaluated by PCR to confirm loss of the plasmid. A PCR verified isolate was designated strain 2-4.

Strain 2-5

[0144] Strain 2-4 was transformed with SEQ ID NO:127 and SEQ ID NO:45 using the transformation method outlined above. SEQ ID NO:127 contained, in order, a 3’ portion of a zeocin resistance gene expression cassette (SEQ ID NO:47), a MpPGK1 promoter (SEQ ID NO:111), a gene (SEQ ID NO:128) encoding the *M. pollinis* X5PP polypeptide of SEQ ID NO:74, a Mp6PGD terminator (SEQ ID NO:7), and a 3’ ER3 flanking sequence (SEQ ID NO:50). Resulting transformants were streaked for single colony isolation on PDA + zeocin plates and single colonies

were selected. Selected colonies were evaluated by colony PCR for integration of the indicated sequence. A PCR verified isolate was designated strain 2-5.

Strains 2-6 through 2-9

[0145] Strain 2-5 was transformed according to Table 8 with SEQ ID NO:129 and one of SEQ ID NOs:130-133 using the transformation method outlined above. SEQ ID NO:129 contained, in order, a 3' portion of the G418 selectable marker (SEQ ID NO:9), an MpTEF1 terminator (SEQ IDNO:125), and a 3' gpdIIB flanking sequence (SEQ ID NO:73) . Each of SEQ ID NOs:130-133 contained, in order, a 5' gpdIIB flanking sequence (SEQ ID NO:72), an MpPGK1 promoter (SEQ ID NO:111), a nucleotide sequence encoding one of SEQ ID NOs:15, 16, 23, or 24, an MpTDH3 terminator (SEQ ID NO:123), and a 5' portion of the G418 selectable marker (SEQ ID NO:8). Resulting transformants were streaked for single colony isolation on PDA + 250 mg/L 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 as outlined in Table 8.

[0146] For example, strain 2-5 was transformed with SEQ ID NO:129 and SEQ ID NO:130 using the transformation method outline above. Resulting transformants were streaked for single colony isolation on PDA + 250 mg/L geneticin (G418) plates and single colonies were selected. Selected colonies were evaluated by colony PCR for integration of the sequence encoding the polypeptide of SEQ ID NO:15. PCR verified isolates were designated strains 2-6a, 2-6b, 2-6c, 2-6d, and 2-6e.

Table 8.

		First Bipartite Transformation Fragment			Second Bipartite Transformation Fragment
Strain	Parent Strain	Transformation SEQ ID NO:	Encoded Polypeptide SEQ ID NO:	Encoded Polypeptide Source	Transformation SEQ ID NO:
2-6a-e	2-5	130	15	*P. putida*	129
2-7a-e	2-5	131	16	*Pantoea* sp. Ap-967	129

2-8a-e	2-5	132	23	*M. pollinis* RCSR24965 mutant	129
2-9a-e	2-5	133	24	*M. pollinis* RCSR02929	129

Example 6: Shake Flask Fermentation Assay

[0147] Strains 2-5, 2-6a-e, 2-7e-a, 2-8a, 2-8b, and 2-8e were run in shake flasks to assess glucose consumption as well as ribitol, erythritol, glycerol, and ethanol production.

[0148] 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.

[0149] A 250 ml non-baffled flask containing 20mL production medium (Table 3) was inoculated with 0.4 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 48, 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. Results are shown in Table 9 and FIG. 4 and xylitol and polyol yield are reported in Table 10. Yield was calculated as outlined in Example 3.

[0150] Results show that expression of the *Pantoea* sp. ZWF enzyme of SEQ ID NO:16 resulted in increased xylitol yield (strains 2-7a-e). Strains 2-7a, 2-7d, and 2-7e also showed an increase in polyol yield. Expression of the P. putida ZWF enzyme of SEQ ID NO: 15 resulted in increased xylitol and increased polyol yield (strains 2-6a, 2-6d, and 2-6e).

[0151] While PCR verification indicated that the transformed polynucleotide sequence(s) was present in the indicated strains, further analysis indicated that in the majority of the strains, the ZWF encoding sequence were integrated into the strain, but not at the gpdIIB loci as planned. Strains 2-6a, 2-6d, 2-7e, and 2-8e, and 2-9d included the encoding sequence correctly integrated at the gpdIIB loci. Further analysis was inconclusive on the integration loci of the encoding sequence in strain 2-7a. In

strains 2-6b, 2-6c, 2-6e, 2-7b, 2-7c, 2-7d, 2-8a, and 2-8b, the encoding sequence for the ZWF enzyme was not integrated at the gpdIIB loci. This may account for some of the sister strain variability in the results. However, the results still demonstrate that expression of the encoded *Pantoea* sp. ZWF helps increase yield of xylitol and polyols.

Table 9

Strain	Time Point (h)	Fermentation Broth Analyte (g/L)						Time Point (h)	Fermentation Broth Analyte (g/L)					
		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
2-5	24	291.90	0.07	2.01	1.34	5.39	0.34	48	169.25	0.13	28.94	8.46	20.44	18.44
2-5	24	290.42	0.06	2.05	1.27	5.24	0.45	48	174.76	0.13	28.24	8.13	19.57	18.58
2-6a	24	291.07	0.05	2.13	1.21	5.00	0.45	48	220.93	0.13	23.44	4.25	15.14	8.26
2-6b	24	296.09	0.05	1.69	0.63	3.20	0.30	48	154.39	0.14	36.05	9.24	13.93	24.85
2-6c	24	285.61	0.05	2.39	1.58	5.36	0.91	48	161.82	0.14	32.01	9.48	19.94	19.97
2-6d	24	297.84	0.05	1.56	0.61	2.98	0.20	48	223.28	0.14	21.08	4.36	16.13	7.62
2-6e	24	291.01	0.06	1.58	0.99	4.54	0.32	48	205.69	0.26	23.30	5.03	18.53	9.37
2-7a	24	298.59	0.06	1.14	0.28	1.71	0.15	48	263.05	0.12	11.34	2.79	12.17	1.81
2-7b	24	299.87	0.04	1.00	0.20	1.51	0.12	48	261.45	0.11	10.21	2.54	11.84	2.06
2-7c	24	293.05	0.06	2.25	0.35	1.82	1.11	48	216.34	0.09	23.15	1.44	7.27	13.60
2-7d	24	306.71	0.04	1.07	0.20	1.45	0.07	48	275.28	0.12	7.20	2.44	10.32	0.73
2-7e	24	299.68	0.04	1.36	0.40	2.21	0.16	48	256.46	0.11	12.39	2.84	12.43	2.24
2-8a	24	286.86	0.05	2.15	1.30	5.23	0.72	48	159.67	0.16	30.21	8.22	18.06	21.57
2-8a	24	287.96	0.05	2.03	1.23	5.27	0.54	48	172.77	0.15	28.79	8.33	20.75	17.82
2-8b	24	288.24	0.06	2.33	1.17	4.73	0.90	48	165.71	0.14	31.42	7.90	15.28	23.11
2-8b	24	289.81	0.05	2.03	1.24	4.93	0.34	48	181.92	0.14	25.98	7.68	20.56	16.27
2-8e	24	289.99	0.05	1.82	1.14	4.76	0.37	48	180.34	0.14	26.07	7.69	20.50	16.01
2-5	72	2.17	0.37	101.77	22.53	19.86	30.50	96	0.81	0.46	116.28	23.59	10.73	13.29
2-5	72	5.99	0.37	98.03	20.48	19.44	35.04	96	0.06	0.50	110.72	21.06	13.69	22.29

2-6a	72	81.47	0.34	84.75	13.67	17.50	20.15	96	0.05	0.63	140.08	19.51	8.73	18.38
2-6b	72	5.70	0.44	107.16	19.20	10.28	37.20	96	0.10	0.54	117.51	19.80	7.31	24.72
2-6c	72	5.12	0.41	96.42	22.18	20.35	33.87	96	1.55	0.46	101.81	22.62	20.35	22.89
2-6d	72	88.50	0.35	81.91	13.34	19.00	18.42	96	0.06	0.62	140.16	20.07	10.47	16.20
2-6e	72	66.65	0.74	86.91	14.49	21.64	19.87	96	0.05	1.18	134.26	19.90	12.66	15.61
2-7a	72	194.63	0.22	43.37	5.33	15.80	5.22	96	97.28	0.36	90.80	10.95	17.60	10.61
2-7b	72	170.45	0.22	46.58	5.72	16.69	10.82	96	74.87	0.38	92.60	10.97	18.77	16.34
2-7c	72	79.98	0.30	82.21	5.15	6.60	28.48	96	18.34	0.47	117.81	5.40	4.79	26.36
2-7d	72	218.56	0.20	34.43	4.44	14.98	3.10	96	138.97	0.32	76.45	7.50	17.36	6.53
2-7e	72	187.57	0.24	46.60	5.65	15.93	5.75	96	89.77	0.42	98.54	10.79	18.14	10.90
2-8a	72	16.28	0.42	92.28	18.47	19.37	33.26	96	5.46	0.50	102.04	18.96	19.72	22.73
2-8a	72	6.39	0.39	97.99	21.03	21.13	34.52	96	0.05	0.53	111.08	21.71	13.81	23.12
2-8b	72	9.25	0.42	99.76	17.53	13.76	38.88	96	0.13	0.54	111.00	18.17	11.68	26.41
2-8b	72	5.89	0.37	97.60	22.82	21.21	31.08	96	0.03	0.51	114.48	23.74	12.80	17.31
2-8e	72	8.45	0.37	96.70	22.34	21.46	32.08	96	0.05	0.49	112.22	22.65	14.33	20.19

Table 10 Yield (%) at 96 Hour

Strain	Xylitol	Polyol
2-5	39.0	47.1
2-5	37.0	44.3
2-6a	46.9	53.6
2-6b	39.3	46.1
2-6c	34.2	42.0
2-6d	46.9	53.8

Strain	Xylitol	Polyol
2-6e	44.9	52.0
2-7a	45.0	50.6
2-7b	41.3	46.4
2-7c	42.0	44.1
2-7d	47.8	52.7
2-7e	47.1	52.5

Strain	Xylitol	Polyol
2-8a	34.8	41.4
2-8a	37.2	44.6
2-8b	37.1	43.4
2-8b	38.3	46.4
2-8e	37.5	45.3

Example 7: Shake Flask Fermentation Assay

[0152] Strains 2-5 and 2-9a-e were run in shake flasks to assess glucose consumption as well as ribitol erythritol, glycerol, and ethanol production.

[0153] 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.

[0154] A 250 ml non-baffled flask containing 20mL production medium (Table 3) was inoculated with 0.4 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 48, 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. Results are shown in Table 11 and FIG. 5 and xylitol and polyol yield are reported in Table 12. Yield was calculated as outlined in Example 3.

[0155] Results show that in the 2x *L. rhamnosus* XPDH, 2x RPE2, *M. pollinis* X5PP of SEQ ID NO:74 background, overexpression of the *M. pollinis* RSCR02929 ZWF enzyme does not further increase the titer or yield of xylitol nor the titer or yield of total polyols in the fermentation.

[0156] While PCR verification indicated that the transformed polynucleotide sequence(s) was present in the indicated strains, further analysis indicated that in the majority of the strains, the ZWF encoding sequence were integrated into the strain, but not at the gpdIIB loci as planned. Strain 2-9d included the encoding sequence correctly integrated at the gpdIIB loci. In strains 2-9a, 2-9b, 2-9c, and 2-9e the encoding sequence for the ZWF enzyme was not integrated at the gpdIIB loci. This may account for some of the sister strain variability in the results.

Table 11

Strain	Time Point (h)	Fermentation Broth Analyte (g/L)						Time Point (h)	Fermentation Broth Analyte (g/L)					
		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol		Glucose	Ribitol	Xylitol	Erythritol	Glycerol	Ethanol
2-5	24	290.90	0.03	1.55	1.20	5.45	0.26	48	174.02	0.11	25.73	8.80	24.54	16.64
2-5	24	289.53	0.03	1.59	1.19	5.48	0.33	48	169.55	0.10	26.25	8.87	24.64	16.93
2-9a	24	290.29	0.03	1.54	1.15	5.33	0.35	48	182.01	0.10	22.45	7.46	24.91	14.73
2-9b	24	287.45	0.05	1.45	1.29	5.76	0.29	48	172.69	0.11	23.69	8.18	26.37	16.08
2-9c	24	291.06	0.02	1.49	1.21	5.31	0.42	48	177.86	0.09	22.23	7.77	24.57	16.48
2-9d	24	290.83	0.02	1.76	1.16	5.40	0.39	48	177.23	0.11	23.63	7.90	23.06	16.20
2-9e	24	293.21	0.02	1.56	1.14	5.45	0.18	48	178.87	0.08	23.22	7.86	25.65	15.30
2-5	72	5.96	0.27	94.31	24.95	24.37	30.44	96	0.04	0.42	110.77	26.17	14.88	17.13
2-5	72	5.64	0.31	94.69	24.05	24.40	30.57	96	0.04	0.42	109.31	25.71	15.33	18.51
2-9a	72	60.68	0.19	68.94	16.61	25.94	24.34	96	52.29	0.27	77.41	17.95	26.13	16.42
2-9b	72	40.97	0.26	77.82	19.79	27.39	25.26	96	23.09	0.34	91.09	21.74	26.79	16.71
2-9c	72	45.86	0.30	73.19	19.01	25.54	27.68	96	29.90	0.41	85.51	20.19	25.52	20.18
2-9d	72	35.03	0.22	79.15	19.07	23.66	30.38	96	20.27	0.35	90.14	20.13	23.98	24.25
2-9e	72	26.35	0.26	84.42	20.91	27.09	28.63	96	6.92	0.30	99.20	22.75	27.01	18.97

Table 12 Yield (%) at 96 hours

Strain	Xylitol	Polyol
2-5	36.2	44.9
2-5	35.8	44.3
2-9a	30.5	37.7
2-9b	32.2	40.0
2-9c	31.0	38.5
2-9d	31.6	38.7
2-9e	33.2	40.9

CLAIMS

1. A genetically engineered yeast cell capable of producing xylitol, the engineered yeast cell comprising:

an exogenous polynucleotide sequence encoding a glucose-6-phosphate dehydrogenase (ZWF) enzyme 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:15, 16, and 23, preferably at least 85% identical to SEQ ID NO:15 or 16.

2. The yeast cell of claim 1, wherein the yeast cell is an osmotolerant yeast cell.

3. The yeast cell of claim 1 or claim 2, wherein the yeast cell is a cell of the subphylum Ustilaginomycotina.

4. The yeast cell of any preceding claim, 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*, *Saccharomyces cerevisiae*, *Penicillium*, *Torula*, *Pichia*, *Candida*, *Candida magnoliae*, and *Aureobasidium*.

5. The yeast cell of any preceding claim, wherein the yeast cell comprises a deletion or disruption of a native gene encoding an erythrose reductase enzyme 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:60 and 62.

6. The yeast cell of any preceding claim, wherein the yeast cell is a *Moniliella pollinis* cell and the gene encoding the erythrose reductase enzyme is 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:61 and 63.

7. The yeast cell of any preceding claim, wherein the cell additionally comprises an exogenous polynucleotide sequence encoding a xylitol-phosphate dehydrogenase (XPDH) enzyme comprising a sequence 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:5, 95-101, and 103, preferably at least 85% identical to at least one of SEQ ID NOs: 5, 95-101, and 103, or most preferably at least 90% identical one of SEQ ID NOs:5, 97, 98, or 101.

8. The yeast cell of any preceding claim, wherein the cell additionally comprises an exogenous polynucleotide sequence encoding a xylulokinase (XKS) enzyme comprising a sequence 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:90 and 91.

9. The yeast cell of claim 8, wherein the cell additionally comprises an exogenous polynucleotide sequence encoding a xylitol dehydrogenase (XDH) enzyme comprising a sequence 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:104-106.

10. The yeast cell of any preceding claim, wherein the cell additionally comprises a genetic modification resulting in overexpression of a native enzyme with ribulose-5-phosphate epimerase (RPE) activity.

11. The yeast cell of claim 10, wherein the cell is a *Moniliella pollinis* cell and the native RPE enzyme comprises a sequence 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:46 and 94.

12. The yeast cell of claim 10 or 11, wherein the genetic modification resulting in overexpression of a native RPE enzyme comprises addition of an exogenous polynucleotide encoding the native RPE enzyme such that the genetically engineered cell comprises at least one additional copy of a sequence encoding the RPE enzyme.

13. The yeast cell of any preceding claim, wherein the yeast additionally comprises a genetic modification resulting in overexpression of a native enzyme with xylitol-5-phosphate phosphatase (X5PP) activity; and/or an exogenous polynucleotide sequence encoding an enzyme with xylitol-5-phosphate phosphatase (X5PP) activity.

14. The yeast cell of claim 13, wherein the cell is a *Moniliella pollinis* cell and the genetic modification results in overexpression of a native X5PP enzyme with 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:74, 88, 92, and 93.

15. The yeast cell of claim 13 or 14, wherein the genetic modification comprises addition of an exogenous polynucleotide sequence encoding the native X5PP enzyme such that the genetically engineered cell comprises at least one additional copy of a sequence encoding the native X5PP enzyme.

16. The yeast cell of any one of claims 13-15, wherein the yeast cell comprises an exogenous polynucleotide sequence encoding an enzyme with X5PP activity and 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:74-91, preferably at least one of SEQ ID NOs:74, 76, 77, 78, 79, 80, 84, and 86, most preferably at least one of SEQ ID NOs:74, 77, 78, 80, and 86.

17. The yeast cell of any preceding claim, wherein one or more of the exogenous polynucleotide sequence(s) is operably linked to a heterologous or artificial promoter.

18. The yeast cell of claim 17, wherein the heterologous or artificial promoter is selected from the group consisting of pyruvate kinase 1 promoter (PYK1p; SEQ ID NO:6), 6-phosphogluconate dehydrogenase promoter (6PGDp; SEQ ID NO:107), glyceraldehyde-3-phosphate dehydrogenase promoter (TDH3p; SEQ ID NO:108), translational elongation factor 1 promoter (TEFp; SEQ ID NO:109), phosphoglucomutase 1 promoter (PGM1p; SEQ ID NO:110), 3-phosphoglycerate kinase

promoter (PGK1p; SEQ ID NO:111), enolase promoter (ENO1p ; SEQ ID NO:112), asparagine synthetase promoter (ASNSp; SEQ ID NO:113), 50S ribosomal protein L1 promoter (RPLAp; SEQ ID NO:114), and RPL16B (SEQ ID NO:115).

19. The yeast cell of any preceding claim, wherein one or more of the exogenous polynucleotide sequence(s) is integrated into the genome of the yeast cell at a locus selected from the ER1 locus, the ER3 locus, the PDC1 locus, the pyrF locus, the TRP3 locus, the gpdIIA locus, and the gpdIIB locus.

20. A method for producing xylitol comprising

contacting a substrate comprising dextrose with the engineered yeast cell of any preceding claim, wherein fermentation of the substrate by the engineered cell produces xylitol.

21. The method of claim 20, 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 5-80, 10-75, 15-70, 20-60, 30-50, or 40-50 mmol O_2/(L • h).

22. The method of claim 20 or 21, wherein the xylitol is produced at a rate of at least 0.2, 0.3, 0.5, 0.75, or at least 1.0 g L^{-1} h^{-1}.

23. The method of any one of claims 20-22, wherein the xylitol titer is at least 20, 30, 50, 75, or 100 g/L when the fermentation is run at 35 °C for 96 hours.

24. The method of any one of claims 20-23, wherein the concentration of dextrose is at least 100 g/L.

25. The method of any one of claims 20-24, wherein xylitol yield of the fermentation is higher than yield in the same fermentation process run using an equivalent yeast lacking the exogenous polynucleotide sequence encoding the ZWF enzyme.

26. Use of the engineered yeast of any one of claims 1-19 to produce xylitol.

FIG. 1

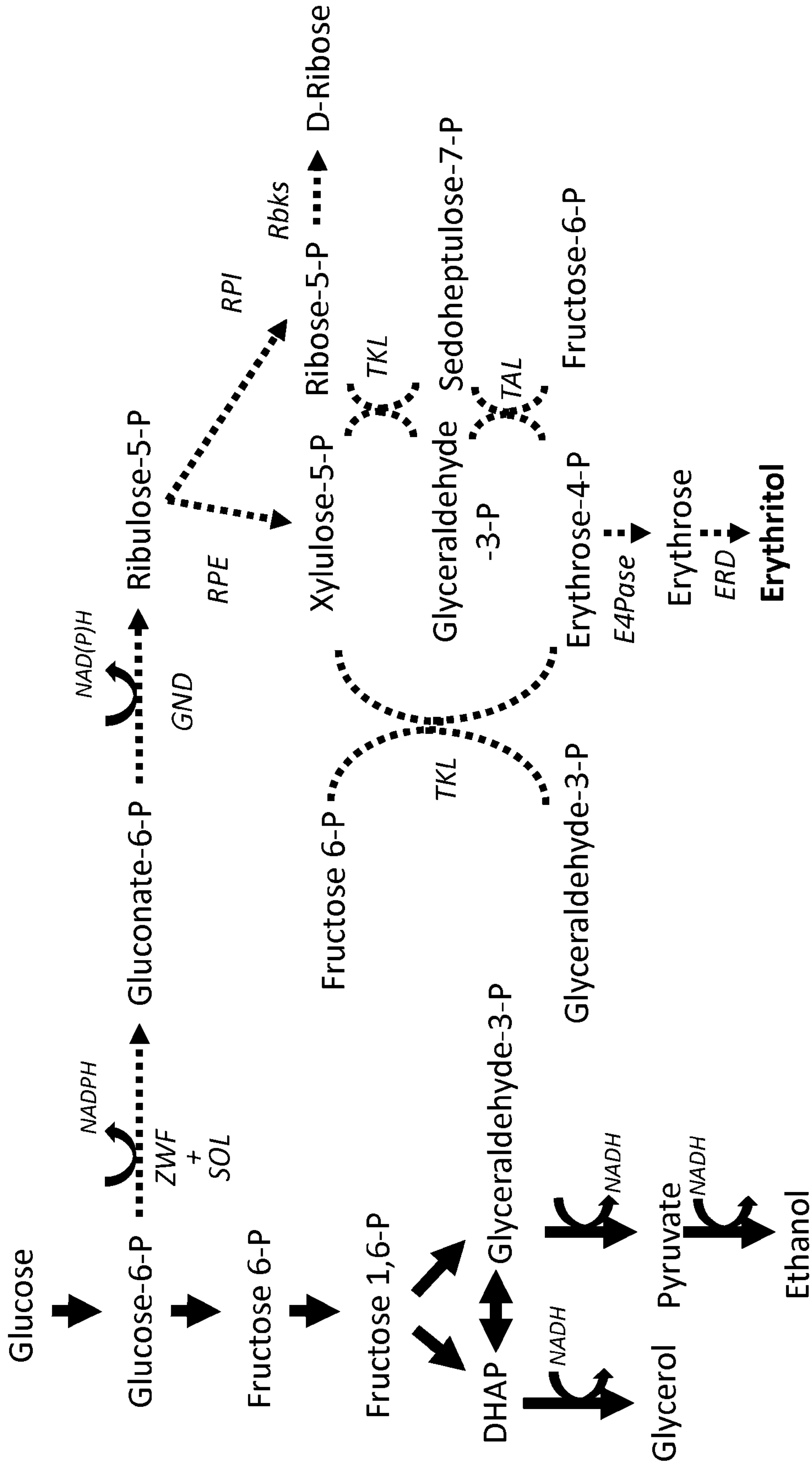

FIG. 2

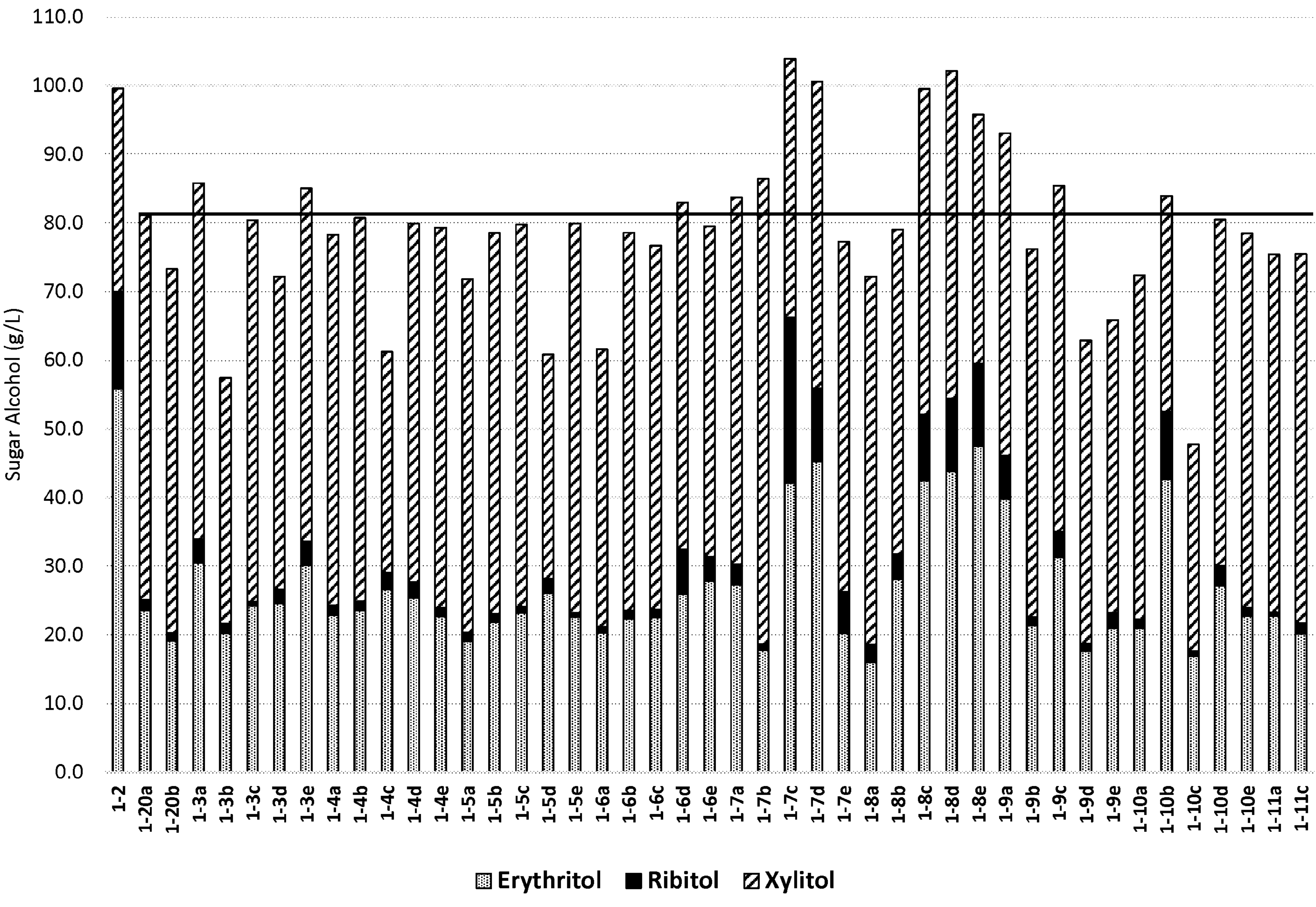

FIG. 3

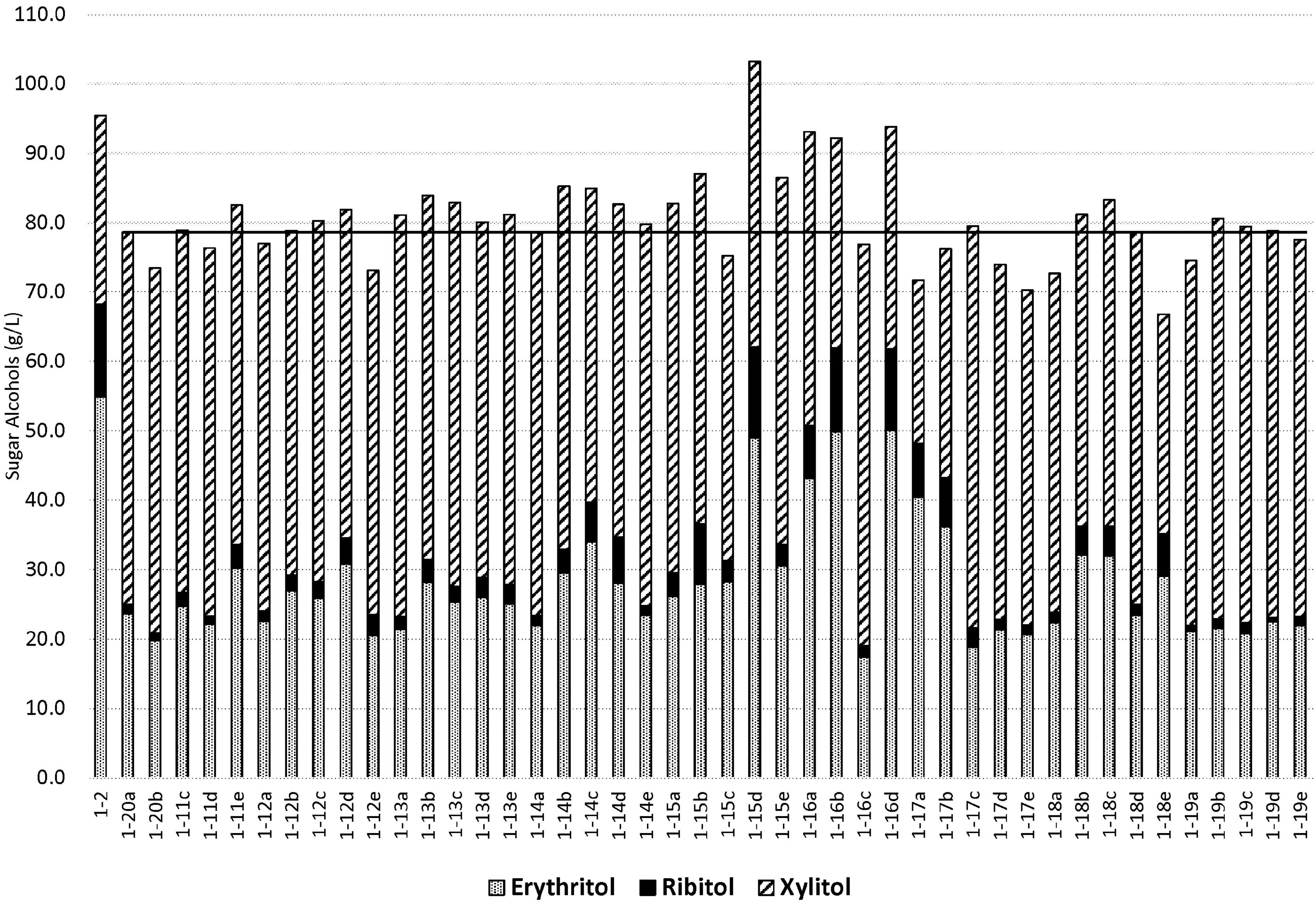

FIG. 4

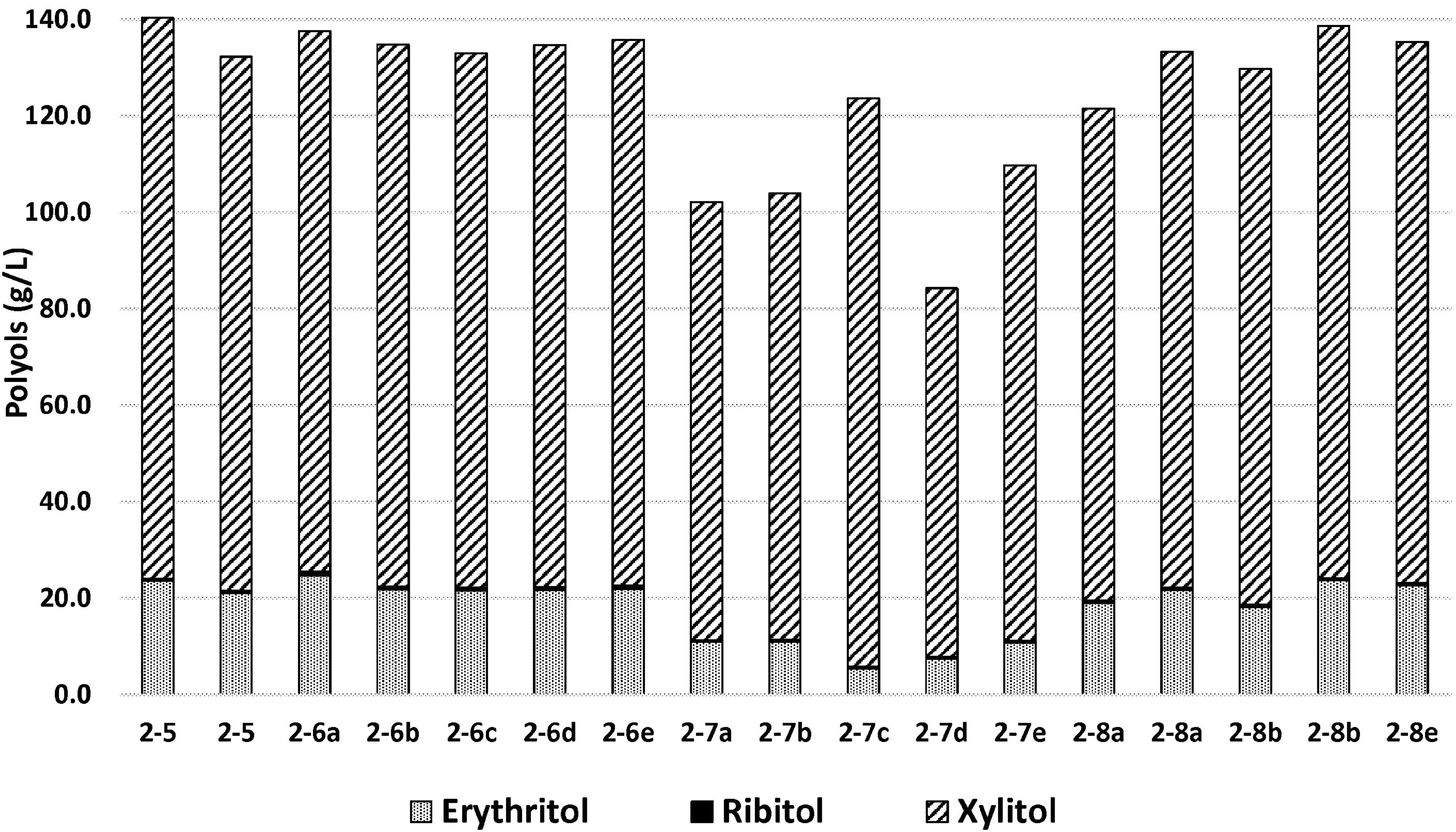

FIG. 5