



(51) International Patent Classification:

Not classified

(21) International Application Number:

PCT/US2021/025487

(22) International Filing Date:

02 April 2021 (02.04.2021)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/004,740 03 April 2020 (03.04.2020) US

(71) Applicant: DUKE UNIVERSITY [US/US]; 2812 Erwin Rd., STE 306, Durham, North Carolina 27709 (US).

(72) Inventors: LYNCH, Michael David; 402 Englewood Avenue, Durham, North Carolina 27701 (US). LI, Shuai; 2812 Erwin Road, Suite 306, Durham, North Carolina 27705 (US).

(74) Agent: SHOEMAKER, Debra et al.; 41 South High Street, Suite 2600, Columbus, Ohio 43215 (US).

(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, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD,

ME, MG, MK, MN, 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, GH, GM, KE, LR, LS, MW, MZ, NA, RW, 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, 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))

(54) Title: METHODS AND COMPOSITIONS FOR THE PRODUCTION OF XYLITOL FROM XYLOSE UTILIZING DYNAMIC METABOLIC CONTROL

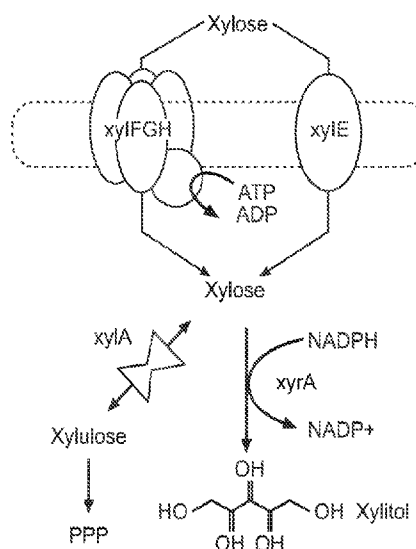


FIG. 1

(57) **Abstract:** The present disclosure is related to genetically engineered microbial strains and related bioprocesses for the production of xylitol. Specifically, the use of dynamically controlled synthetic metabolic valves to reduce the activity of certain enzymes, leads to increased xylitol production in a two-stage process.



METHODS AND COMPOSITIONS FOR THE PRODUCTION OF XYLITOL FROM XYLOSE UTILIZING DYNAMIC METABOLIC CONTROL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Numbers 63/004,740 filed April 3, 2020, and 63/056,085 filed July 24, 2020, both of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] This invention relates to metabolically engineered microorganisms, such as bacterial strains, and bioprocesses utilizing such strains. These strains provide dynamic control of metabolic pathways resulting in the production of xylitol from xylose.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been filed electronically in ASCII format as 49196-46_ST25 created March 29, 2021 that is 17051 bytes in size and is hereby incorporated by reference in its entirety.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0004] This invention was made with government support under Federal Grant No. EE0007563 awarded by the Department of Energy; Federal Contract No. HR0011-14-C-0075 awarded by the United States Department of Defense; Federal Grant No. ONR YIP 12043956 awarded by the United States Department of Defense; DARPA# HR0011-14-C-0075; ONR YIP #N00014-16-1-2558; DOE EERE grant #EE0007563; N00014-16-1-2558 awarded by NAVY/ONR, and NIH Biotechnology Training Grant (T32GM008555). The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0005] Xylitol is an industrial sugar alcohol primarily used as a sweetener, having a similar sweetness but fewer calories than sucrose. Annual production of Xylitol is ~125,000 tons and is produced via the reduction of xylose. Xylose is the second most abundant natural sugar (after glucose), therefore it is an attractive feedstock. Many studies have demonstrated the use of xylose as a feedstock for the biosynthesis of numerous products ranging from biofuels (ethanol) to chemicals, including lactic acid, succinic acid, xylonate, 1,2,4-butanetriol, and xylitol.

[0006] The industrial production of xylitol relies on traditional chemistry, and the process has remained relatively unchanged for decades. This conversion requires expensive catalysts and requires relatively pure xylose as a feedstock. Efforts have been made to identify more

economical ways to produce xylitol from lower cost, cellulosic sugar streams, including the development of biosynthetic processes. Biosynthetic production has the potential to decrease costs, utilize lower quality feedstocks, avoid the use of organic solvents, eliminate the need for expensive reduction catalysts. However, most previous biosynthetic studies producing xylitol from xylose rely on a bioconversion requiring an additional sugar (usually glucose) as an electron donor. Oxidation of glucose (producing the byproduct gluconic acid) generates NAD(P)H which is then used for xylose reduction. While these processes offer high xylitol titers and a good yield when just considering xylose, the requirement for glucose at equimolar levels to xylose is a significant inefficiency.

[0007] Perhaps the simplest conversion is xylose to xylitol, which requires only a single enzyme, a xylose reductase. Biosynthetic production of xylitol, over chemical conversion, has the potential to decrease costs, while avoiding the use of organic solvents, eliminating the need for expensive reduction catalysts, and improving product purity.

SUMMARY OF THE INVENTION

[0008] We rationally designed genetically modified microorganism strains to optimize xylitol production from xylose utilizing two stage dynamic metabolic control. As illustrated in FIG 1, this design included overexpression of xylose reductase and the dynamic reduction in xylose isomerase (*xylA*) activity to reduce xylose metabolism which competes with xylitol production. Toward this goal we constructed strains and plasmids to enable the dynamic induction of *xylA*, and dynamic reduction in XylA activity upon phosphate depletion, or other causative event, either through gene silencing, proteolysis of XylA or a combination of both functions. Provided herein are microbial strains for scalable biofermentation processes the use synthetic metabolic valves (SMVs) to decouple growth from product formation. The described strains provide dynamic control of metabolic pathways, including pathways that, when altered, have negative effects on microorganism growth under certain inducible conditions.

[0009] We also fully describe improved NADPH flux coincident with xylitol biosynthesis in engineered *E. coli*. Xylitol is produced from xylose via an NADPH dependent reductase. We utilize two-stage dynamic metabolic control to compare two approaches to optimize xylitol biosynthesis, a stoichiometric approach, wherein competitive fluxes are decreased, and a regulatory approach wherein the levels of key regulatory metabolites are reduced. The stoichiometric and regulatory approaches lead to a 16 fold and 100 fold improvement in xylitol production, respectively. Strains with reduced levels of enoyl-ACP reductase and glucose-6-phosphate dehydrogenase, led to altered metabolite pools resulting in the activation of the membrane bound transhydrogenase and a new NADPH generation pathway, namely pyruvate

ferredoxin oxidoreductase coupled with NADPH dependent ferredoxin reductase, leading to increased NADPH fluxes, despite a reduction in NADPH pools. These strains produced titers of 200 g/L of xylitol from xylose at 86% of theoretical yield in instrumented bioreactors. Dynamic control over enoyl-ACP reductase and glucose-6-phosphate dehydrogenase will broadly enable improved NADPH dependent bioconversions.

[0010] Also provided herein are multi-stage bioprocesses for xylitol production that use the described genetically modified microorganism containing one or more synthetic metabolic valves that provide dynamic flux control and result in improved xylitol production. In certain embodiments, carbon feedstocks can include xylose, or a combination of xylose and glucose, arabinose, mannose, lactose, or alternatively carbon dioxide, carbon monoxide, methane, methanol, formaldehyde, or oils. Additional genetic modifications may be added to a microorganism to provide further conversion of xylitol to additional chemical or fuel products.

[0011] Other methods, features and/or advantages is, or will become, apparent upon examination of the following Figures and detailed description. It is intended that all such additional methods, features, and advantages be included within this description and be protected by the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The novel features of the invention are set forth with particularity in the claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are used, and the accompanying drawings of which:

[0013] **FIG 1** depicts the design of metabolic valves for the bioproduction of xylitol. The biosynthesis process of xylitol in *E. coli* by xylose reductase (XyrA) with NADPH as cofactor (bold arrow). The main competitive pathway for the consumption of xylose is to xylose by xylose isomerase (XylA, valve).

[0014] **FIG 2A-C** depicts Xylose Reductase Expression and Enzyme Kinetics. FIG2A, Expression of XyrA in BL21 using media combination of SM10++(for growth) and SM10-No phos(for expression). After the expression, the postproduction cells were lysed by freeze-thawing cycle. Next, the xyrA protein was extracted by N-N Resin because of the His-tag on XyrA which was design into plasmid sequence. FIG2B, Activity of xyrA with NADPH as co-factor. Reaction velocity is plotted as function of xylose concentration. In these assays, NADPH was held at a constant initial level of 50 μ M. FIG 2C, Kinetic Parameters for XyrA from this project and from other research sources as comparison.

[0015] **FIG 3** depicts the xylitol titer/OD (g/L-OD) were measured under different xylA silencing and xylA proteolysis combinations. The specific productivity of different strains was

significantly different with the control strain DLF-0025-EV. While all three valve combinations made statistically significant amount more than the DLF25-EV control, xylA silencing or proteolysis alone were better than the combination.

[0016] FIG 4 depicts xylitol Production in *E. coli* utilizing 2-stage Dynamic Control. Strain metabolic network design. The main metabolic pathways include: Fatty Acid Biosynthesis, the Citric Acid Cycle (TCA), NADPH supply, the Pentose Phosphate Pathway Transhydrogenase and Glycolysis. The valves which may be ‘switched off’ in the metabolic system include xylose isomerase (xylA-X), the soluble transhydrogenase (udhA-U) enoyl-ACP reductase (fabI-F), citrate synthase (gltA-G) and glucose-6-phosphate dehydrogenase (zwf-Z). These valves are all highlighted by red valves. Xylose reductase (xylA) may be dynamically ‘switched on’ for xylitol production with NADPH as cofactor.

[0017] FIG 5A-B depicts (5A) Rank order plot for average xylitol titer of all valve strains examined in 2-stage micro fermentation, as well as with standard deviation. Xylitol production in the control strain was colored in red. A post hoc Dunnett test shows combinations that differ from the DLF025-Empty vector control significantly at $p < 0.05$, which are indicated as darkened (instead of gray bar, meaning non-significant) in the sorted titer per unit OD plot. (5B) Heatmap of xylitol titer in 2-stage production in response to different proteolysis and silencing combinations, from 0 g/L (white) to 12 g/L (darker). The x-axis stands for different proteolysis valves while the y-axis represents the different pCASCADE silencing. The DLF_25 empty valve control is in the red circle. The gray dots indicate combinations that are not assayed or have no proper cell growth for all replicates. According to the heatmap result, for the combinations which the titer/OD > 3 , 6 replications were performed to avoid the false positive results.

[0018] FIG 6 depicts p-value map of micro-fermentation results of FIG 5.

[0019] FIG 7A-B depicts plots of instrumented fermentation of (7A) an exemplary production strain Z-FZ (Silencing of zwf (“Z”), proteolysis of fabI and zwf (“FZ”)) and (7B) the control strain (DLF-0025-EV) to 1L bioreactors. The Blue lines indicate the OD₆₀₀ values and orange lines represents the xylitol titer at various time points. The Z-FZ combination resulted in a titer of 104 $\pm$ 11.31g/L after 160 hours of production, while the control strain (DLF_0025-EV) only produced -3 g/L at the same production time. We replicated the Z-FZ tank fermentation using the same seed and fermentation conditions, the results here are the average of these two replicated tanks and standard deviation was noted in the plot sample points.

[0020] FIG 8 depicts a conceptual model of two-stage NADPH production in our engineered system. Glucose-6-phosphate dehydrogenase (encoded by the zwf gene) is normally responsible for the biosynthesis of a majority of NADPH. This irreversible reaction drives an NADPH set point, in which the SoxRS oxidative stress response is OFF (gray area). Dynamic reduction in

Zwf levels reduces NADPH pools activating the SoxRS response, which in turn activates expression of Pyruvate ferredoxin oxidoreductase (Pfo, encoded by the ydbK gene) and NADPH dependent ferredoxin reductase (Fpr). Together Pfo and Fpr (operating in reverse) constitute a new pathway to generate NADPH as well as allow for continued pyruvate oxidation and generation of acetyl-CoA for entry into the tricarboxylic acid cycle (TCA cycle). NADPH flux is further enhanced by reducing fatty acid biosynthesis whose products inhibit the membrane bound transhydrogenase (encoded by the pntAB genes). Activated PntAB uses the proton motive force to convert NADH from the TCA cycle to NADPH. NADPH can be used for bioconversions such as for xylitol production.

[0021] FIG 9A-B depict enzyme levels of 9A) XylA and 9B) UdhA in response to inducible proteolysis and/or gene silencing in a phosphate depleted stationary phase. ev -empty vector, x- xylA promoter, u- udhA promoter.

[0022] FIG 10 A-C: Specific xylitol production in strains engineered for dynamic control over levels of 10A) xylose isomerase (XylA), 10B) soluble transhydrogenase (UdhA) and 10C) the combined control over xylose isomerase soluble transhydrogenase. ev -empty vector, x- xylA promoter, u- udhA promoter. All results were obtained from microfermentations.

[0023] FIG 11: XyrA expression and purification from BL21(DE3). Left: A time course of expression post phosphate depletion, whole cell lysates demonstrate expression of XyrA. Densitometry indicates an expression level of ~ 20%. Middle: Purification of XyrA (which contains an N-terminal 6 X histidine tag) via IMAC. Right Kinetic analysis of purified XyrA. Initial velocity ($\mu\text{M/s}$) is plotted as a function of substrate (xylose) concentration.

[0024] FIG 12 A-D: 12A) An overview of xylitol production and the location of metabolic valves in central metabolism. Xylitol is produced from xylose by a xylose reductase (xyrA). Valves comprise inducible proteolysis and/or silencing of 5 enzymes: citrate synthase (gltA), xylose isomerase (xylA), glucose-6-phosphate dehydrogenase (zwf), enoyl-ACP reductase (fabI) and soluble transhydrogenase (udhA). The membrane bound transhydrogenase (pntAB) is also shown. 12B) Specific xylitol production (g/L-OD600nm) in microfermentations as a function of silencing and or proteolysis. 12C) P-values for the data in 12B, comparing each strain to the no-valve control using a Welch's t-test. 12D) a rank order plot of the data from panel. Bars indicate a p-value < 0.05. Abbreviations: xylE : xylose permease, xylFGH : xylose ABC transporter, PPP: pentose phosphate pathway, PDH: pyruvate dehydrogenase multienzyme complex, TCA: tricarboxylic acid, G6P: glucose-6-phosphate, 6-PGL: 6-phosphogluconolactone, 6PG: 6-phosphogluconate, GA3P: glyceraldehyde-3-phosphate, PEP: phosphoenolpyruvate, OAA: oxaloacetic acid, X5P: xylulose-5-phosphate, Fd: ferredoxin. Silencing: ev: empty vector, g2: gltAp2 promoter, z: zwf promoter, x: xylA promoter, u: udhA promoter. Proteolysis: F: fabI-

DAS+4, G: gltA-DAS+4, Z: zwf-DAS+4, U: udhA-DAS+4, X: xylA-DAS+4. All results were obtained from microfermentations.

[0025] FIG 13 A-D: Agarose gel electrophoretic analysis of gRNA array stability. Colony PCR was used to amplify and size gRNA arrays from 8 clones after transformation into host strains engineered for dynamic metabolic control. “Guide” indicates PCR products are taken from sequence confirmed gRNA arrays with 0, 1, or 2 gRNAs respectively. 13A) Strain DLF_Z0025, white labels: pCASCADE-ev, yellow labels: pCASCADE-g2, 13B) Strain DLF_Z0025, white labels: pCASCADE-z, yellow labels: pCASCADE-fg2, 13C) White labels: Strain DLF_Z0044, pCASCADE-fg2, yellow labels: DLF_Z0025, pCASCADE-fg2, 13D) White labels: Strain DLF_Z0046, pCASCADE-g2, gray labels: DLF_Z1002 pCASCADE-fg2.

[0026] FIG 14: Dynamic Control over FabI (enoyl-ACP reductase) levels due to inducible proteolysis with a DAS+4 degron tag. The chromosomal fabI gene was tagged with a C-terminal sfGFP. Protein levels were measured by ELISA, 24 hour post induction by phosphate depletion in microfermentations.

[0027] FIG 15 A-D: Identification of pathways responsible for NADPH and xylitol production in the “FZ” valve strain 15A) the impact of deletions of ydbK and fpr on specific xylitol production, 15B) the impact of pntAB overexpression on xylitol production. (15C-D) “FZ” valve strains further modified for dynamic control over 15C) GltA levels and 15D) UdhA levels. ev - empty vector, z- zwf promoter, g2- gltAp2 promoter, u- udhA promoter. All results were obtained from microfermentations.

[0028] FIG 16 A-B: Stoichiometric flux models of 16A) cellular growth and 16B) stationary phase xylitol production in “FZ” valve strains. Pathway flux is relative to xylose uptake rates. During growth the majority of flux is through the pentose phosphate pathway (PPP), pyruvate dehydrogenase multienzyme complex (PDH) with minimal flux through the pentose membrane bound transhydrogenase. Upon dynamic control, a 4-fold increase in membrane bound transhydrogenase flux is accompanied by increased flux through Pfo (ydbK) and FPr. Abbreviations: G6P: glucose-6-phosphate, 6-PGL: 6-phosphogluconolactone, 6PG: 6-phosphogluconate, GA3P: glyceraldehyde-3-phosphate, OAA: oxaloacetic acid.

[0029] FIG 17 A-B Modeled NADPH producing reactions and pathways for xylitol production in different production strains. 17A) Specific reactions fluxes during xylitol production. 17B) Pathway percentage fluxes for xylitol production.

[0030] FIG 18 A-C: Xylitol production in minimal media fed batch fermentations in instrumented bioreactors by 18A) the control strain expressing xylose reductase (DLF_Z0025, pCASCADE-ev, pHCKan-xyrA), 18B) the “FZ” valve strain (DLF_Z0025-fabI-DAS+4-zwf-DAS+4, pCASCADE-z, pHCKan-xyrA), 18C) the “FZ” valve strain also overexpressing the

membrane bound transhydrogenase pntAB (DLF_Z0025-fabI-DAS+4-zwf-DAS+4, pCASCADE-z, pHCKan-xyrA, pCDF-pntAB). Biomass (black) and xylitol (blue) are given as a function of time. For FIG 18B and 18C, x's and triangles represent the measured values of two duplicate runs.

[0031] FIG 19 depicts stationary phase NADPH pools in engineered strain. Pools were measured 24 hours post phosphate depletion.

DETAILED DESCRIPTION OF THE INVENTION

[0032] The present invention is related to various genetically modified microorganisms that have utility for production of xylitol or a related chemical products to methods of making such chemical products using these microorganisms.

Definitions

[0033] As used in the specification and the claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to an “expression vector” includes a single expression vector as well as a plurality of expression vectors, either the same (e.g., the same operon) or different; reference to “microorganism” includes a single microorganism as well as a plurality of microorganisms; and the like.

[0034] The term “heterologous DNA,” “heterologous nucleic acid sequence,” and the like as used herein refers to a nucleic acid sequence wherein at least one of the following is true: (a) the sequence of nucleic acids is foreign to (i.e., not naturally found in) a given host microorganism; (b) the sequence may be naturally found in a given host microorganism, but in an unnatural (e.g., greater than expected) amount; or (c) the sequence of nucleic acids comprises two or more subsequences that are not found in the same relationship to each other in nature. For example, regarding instance (c), a heterologous nucleic acid sequence that is recombinantly produced will have two or more sequences from unrelated genes arranged to make a new functional nucleic acid, such as a nonnative promoter driving gene expression. The term “heterologous” is intended to include the term “exogenous” as the latter term is generally used in the art. With reference to the host microorganism's genome prior to the introduction of a heterologous nucleic acid sequence, the nucleic acid sequence that codes for the enzyme is heterologous (whether or not the heterologous nucleic acid sequence is introduced into that genome). As used herein, chromosomal, and native and endogenous refer to genetic material of the host microorganism.

[0035] The term “synthetic metabolic valve,” and the like as used herein refers to either the use of controlled proteolysis, gene silencing or the combination of both proteolysis and gene silencing to alter metabolic fluxes.

[0036] As used herein, the term “gene disruption,” or grammatical equivalents thereof (and

including “to disrupt enzymatic function,” “disruption of enzymatic function,” and the like), is intended to mean a genetic modification to a microorganism that renders the encoded gene product as having a reduced polypeptide activity compared with polypeptide activity in or from a microorganism cell not so modified. The genetic modification can be, for example, deletion of the entire gene, deletion or other modification of a regulatory sequence required for transcription or translation, deletion of a portion of the gene which results in a truncated gene product (e.g., enzyme) or by any of various mutation strategies that reduces activity (including to no detectable activity level) the encoded gene product. A disruption may broadly include a deletion of all or part of the nucleic acid sequence encoding the enzyme, and also includes, but is not limited to other types of genetic modifications, e.g., introduction of stop codons, frame shift mutations, introduction or removal of portions of the gene, and introduction of a degradation signal, those genetic modifications affecting mRNA transcription levels and/or stability, and altering the promoter or repressor upstream of the gene encoding the enzyme.

[0037] Bio-production, Micro-fermentation (microfermentation) or Fermentation, as used herein, may be aerobic, microaerobic, or anaerobic.

[0038] When the genetic modification of a gene product, i.e., an enzyme, is referred to herein, including the claims, it is understood that the genetic modification is of a nucleic acid sequence, such as or including the gene, that normally encodes the stated gene product, i.e., the enzyme.

[0039] As used herein, the term “metabolic flux” and the like refers to changes in metabolism that lead to changes in product and/or byproduct formation, including production rates, production titers and production yields from a given substrate.

[0040] Species and other phylogenic identifications are according to the classification known to a person skilled in the art of microbiology.

[0041] Enzymes are listed here within, with reference to a UniProt identification number, which would be well known to one skilled in the art. The UniProt database can be accessed at <http://www.UniProt.org/>. When the genetic modification of a gene product, i.e., an enzyme, is referred to herein, including the claims, it is understood that the genetic modification is of a nucleic acid sequence, such as or including the gene, that normally encodes the stated gene product, i.e., the enzyme.

[0042] Where methods and steps described herein indicate certain events occurring in certain order, those of ordinary skill in the art will recognize that the ordering of certain steps may be modified and that such modifications are in accordance with the variations of the invention. Additionally, certain steps may be performed concurrently in a parallel process when possible, as well as performed sequentially.

[0043] The meaning of abbreviations is as follows: “C” means Celsius or degrees Celsius, as is

clear from its usage, DCW means dry cell weight, “s” means second(s), “min” means minute(s), “h,” “hr,” or “hrs” means hour(s), “psi” means pounds per square inch, “nm” means nanometers, “d” means day(s), “μL” or “uL” or “ul” means microliter(s), “mL” means milliliter(s), “L” means liter(s), “mm” means millimeter(s), “nm” means nanometers, “mM” means millimolar, “μM” or “uM” means micromolar, “M” means molar, “mmol” means millimole(s), “μmol” or “uMol” means micromole(s), “g” means gram(s), “μg” or “ug” means microgram(s) and “ng” means nanogram(s), “PCR” means polymerase chain reaction, “OD” means optical density, “OD₆₀₀” means the optical density measured at a photon wavelength of 600 nm, “kDa” means kilodaltons, “g” means the gravitation constant, “bp” means base pair(s), “kbp” means kilobase pair(s), “% w/v” means weight/volume percent, “% v/v” means volume/volume percent, “IPTG” means isopropyl-μ-D-thiogalactopyranoside, “aTc” means anhydrotetracycline, “RBS” means ribosome binding site, “rpm” means revolutions per minute, “HPLC” means high performance liquid chromatography, and “GC” means gas chromatography.

I. Carbon Sources

[0044] Bio-production media, which is used in the present invention with recombinant microorganisms must contain suitable carbon sources or substrates for both growth and production stages. Suitable substrates may include but are not limited to xylose or a combination of xylose and glucose, sucrose, xylose, mannose, arabinose, oils, carbon dioxide, carbon monoxide, methane, methanol, formaldehyde, or glycerol. It is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention as a carbon source(s).

II. Microorganisms

[0045] Features as described and claimed herein may be provided in a microorganism selected from the listing herein, or another suitable microorganism, that also comprises one or more natural, introduced, or enhanced product bio-production pathways. Thus, in some embodiments the microorganism(s) comprise an endogenous product production pathway (which may, in some such embodiments, be enhanced), whereas in other embodiments the microorganism does not comprise an endogenous product production pathway.

[0046] More particularly, based on the various criteria described herein, suitable microbial hosts for the bio-production of a chemical product generally may include, but are not limited to the organisms described in the Methods Section.

[0047] The host microorganism or the source microorganism for any gene or protein described here may be selected from the following list of microorganisms: *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*,

Debaryomyces, Mucor, Torulopsis, Methylobacter, Escherichia, Salmonella, Bacillus, Streptomyces, and Pseudomonas. In some aspects the host microorganism is an E.coli microorganism.

III. Media and Culture Conditions

[0048] In addition to an appropriate carbon source, such as selected from one of the herein-disclosed types, bio-production media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of chemical product bio-production under the present invention.

[0049] Another aspect of the invention regards media and culture conditions that comprise genetically modified microorganisms of the invention and optionally supplements.

[0050] Typically cells are grown at a temperature in the range of about 25° C to about 40° C in an appropriate medium, as well as up to 70° C for thermophilic microorganisms. Suitable growth media are well characterized and known in the art. Suitable pH ranges for the bio-production are between pH 2.0 to pH 10.0, where pH 6.0 to pH 8.0 is a typical pH range for the initial condition. However, the actual culture conditions for a particular embodiment are not meant to be limited by these pH ranges. Bio-productions may be performed under aerobic, microaerobic or anaerobic conditions with or without agitation.

IV. Bio-production Reactors and Systems

[0051] Fermentation systems utilizing methods and/or compositions according to the invention are also within the scope of the invention. Any of the recombinant microorganisms as described and/or referred to herein may be introduced into an industrial bio-production system where the microorganisms convert a carbon source into a product in a commercially viable operation. The bio-production system includes the introduction of such a recombinant microorganism into a bioreactor vessel, with a carbon source substrate and bio-production media suitable for growing the recombinant microorganism, and maintaining the bio-production system within a suitable temperature range (and dissolved oxygen concentration range if the reaction is aerobic or microaerobic) for a suitable time to obtain a desired conversion of a portion of the substrate molecules to a selected chemical product. Bio-productions may be performed under aerobic, microaerobic, or anaerobic conditions, with or without agitation. Industrial bio-production systems and their operation are well-known to those skilled in the arts of chemical engineering and bioprocess engineering.

[0052] The amount of a product produced in a bio-production media generally can be determined using a number of methods known in the art, for example, high performance liquid chromatography (HPLC), gas chromatography (GC), or GC/Mass Spectroscopy (MS).

V. Genetic Modifications, Nucleotide Sequences, and Amino Acid Sequences

[0053] Embodiments of the present invention may result from introduction of an expression vector into a host microorganism, wherein the expression vector contains a nucleic acid sequence coding for an enzyme that is, or is not, normally found in a host microorganism.

[0054] The ability to genetically modify a host cell is essential for the production of any genetically modified (recombinant) microorganism. The mode of gene transfer technology may be by electroporation, conjugation, transduction, or natural transformation. A broad range of host conjugative plasmids and drug resistance markers are available. The cloning vectors are tailored to the host organisms based on the nature of antibiotic resistance markers that can function in that host. Also, as disclosed herein, a genetically modified (recombinant) microorganism may comprise modifications other than via plasmid introduction, including modifications to its genomic DNA.

[0055] More generally, nucleic acid constructs can be prepared comprising an isolated polynucleotide encoding a polypeptide having enzyme activity operably linked to one or more (several) control sequences that direct the expression of the coding sequence in a microorganism, such as *E. coli*, under conditions compatible with the control sequences. The isolated polynucleotide may be manipulated to provide for expression of the polypeptide. Manipulation of the polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well established in the art.

[0056] The control sequence may be an appropriate promoter sequence, a nucleotide sequence that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter sequence may contain transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any nucleotide sequence that shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell. The techniques for modifying and utilizing recombinant DNA promoter sequences are well established in the art.

[0057] For various embodiments of the invention the genetic manipulations may include a manipulation directed to change regulation of, and therefore ultimate activity of, an enzyme or enzymatic activity of an enzyme identified in any of the respective pathways. Such genetic modifications may be directed to transcriptional, translational, and post-translational modifications that result in a change of enzyme activity and/or selectivity under selected culture conditions. Genetic manipulation of nucleic acid sequences may increase copy number and/or comprise use of mutants of an enzyme related to product production. Specific methodologies and approaches to achieve such genetic modification are well known to one skilled in the art.

[0058] In various embodiments, to function more efficiently, a microorganism may comprise one or more gene deletions. For example, in *E. coli*, the genes encoding the lactate dehydrogenase (*ldhA*), phosphate acetyltransferase (*pta*), pyruvate oxidase (*poxB*), pyruvate-formate lyase (*pflB*), methylglyoxal synthase (*mgsA*), acetate kinase (*ackA*), alcohol dehydrogenase (*adhE*), the *clpXP* protease specificity enhancing factor (*sspB*), the ATP-dependent Lon protease (*lon*), the outer membrane protease (*ompT*), the *arcA* transcriptional dual regulator (*arcA*), and the *iclR* transcriptional regulator (*iclR*) may be disrupted, including deleted. Such gene disruptions, including deletions, are not meant to be limiting, and may be implemented in various combinations in various embodiments. Gene deletions may be accomplished by numerous strategies well known in the art, as are methods to incorporate foreign DNA into a host chromosome.

[0059] In various embodiments, to function more efficiently, a microorganism may comprise one or more synthetic metabolic valves, composed of enzymes targeted for controlled proteolysis, expression silencing or a combination of both controlled proteolysis and expression silencing. For example, one enzyme encoded by one gene or a combination of numerous enzymes encoded by numerous genes in *E. coli* may be designed as synthetic metabolic valves to alter metabolism and improve product formation. Representative genes in *E. coli* may include but are not limited to the following: *fabI*, *zwf*, *gltA*, *ppc*, *udhA*, *lpd*, *sucD*, *aceA*, *pfkA*, *lon*, *rpoS*, *pykA*, *pykF*, *tktA* or *tktB*. It is appreciated that it is well known to one skilled in the art how to identify homologues of these genes and or other genes in additional microbial species.

[0060] For all nucleic acid and amino acid sequences provided herein, it is appreciated that conservatively modified variants of these sequences are included and are within the scope of the invention in its various embodiments. Functionally equivalent nucleic acid and amino acid sequences (functional variants), which may include conservatively modified variants as well as more extensively varied sequences, which are well within the skill of the person of ordinary skill in the art, and microorganisms comprising these, also are within the scope of various embodiments of the invention, as are methods and systems comprising such sequences and/or microorganisms.

[0061] Accordingly, as described in various sections above, some compositions, methods and systems of the present invention comprise providing a genetically modified microorganism that comprises both a production pathway to make a desired product from a central intermediate in combination with synthetic metabolic valves to redistribute flux.

[0062] Aspects of the invention also regard provision of multiple genetic modifications to improve microorganism overall effectiveness in converting a selected carbon source into a selected product. Particular combinations are shown, such as in the Examples, to increase

specific productivity, volumetric productivity, titer and yield substantially over more basic combinations of genetic modifications.

[0063] In addition to the above-described genetic modifications, in various embodiments genetic modifications, including synthetic metabolic valves also are provided to increase the pool and availability of the cofactor NADPH and/or NADH which may be consumed in the production of a product.

[0064] VI. Synthetic Metabolic Valves

[0065] Use of synthetic metabolic valves allows for simpler models of metabolic fluxes and physiological demands during a production phase, turning a growing cell into a stationary phase biocatalyst. These synthetic metabolic valves can be used to turn off essential genes and redirect carbon, electrons, and energy flux to product formation in a multi-stage fermentation process. One or more of the following provides the described synthetic valves: 1) transcriptional gene silencing or repression technologies in combination with 2) inducible and selective enzyme degradation and 3) nutrient limitation to induce a stationary or non-dividing cellular state. SMVs are generalizable to any pathway and microbial host. These synthetic metabolic valves allow for novel rapid metabolic engineering strategies useful for the production of renewable chemicals and fuels and any product that can be produced via whole cell catalysis.

[0066] In particular, the invention describes the construction of synthetic metabolic valves comprising one or more or a combination of the following: controlled gene silencing and controlled proteolysis. It is appreciated that one well skilled in the art is aware of several methodologies for gene silencing and controlled proteolysis.

VI.A Gene Silencing

[0067] In particular, the invention describes the use of controlled gene silencing to provide the control over metabolic fluxes in controlled multi-stage fermentation processes. There are several methodologies known in the art for controlled gene silencing, including but not limited to mRNA silencing or RNA interference, silencing via transcriptional repressors and CRISPR interference. Methodologies and mechanisms for RNA interference are taught by Agrawal et al. “RNA Interference: Biology, Mechanism, and Applications” Microbiology and Molecular Biology Reviews, December 2003; 67(4) p657-685. DOI: 10.1128/MMBR.67.657-685.2003. Methodologies and mechanisms for CRISPR interference are taught by Qi et al. “Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression” Cell February 2013; 152(5) p1173-1183. DOI: 10.1016/j.cell.2013.02.022. In addition, methodologies, and mechanisms for CRISPR interference using the native *E. coli* CASCADE system are taught by Luo et al. “Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression” NAR. October 2014; DOI: 10.1093. In addition numerous

transcriptional repressor systems are well known in the art and can be used to turn off gene expression.

VI.B Controlled Proteolysis

[0068] In particular, the invention describes the use of controlled protein degradation or proteolysis to provide the control over metabolic fluxes in controlled multi-stage fermentation processes. There are several methodologies known in the art for controlled protein degradation, including but not limited to targeted protein cleavage by a specific protease and controlled targeting of proteins for degradation by specific peptide tags. Systems for the use of the *E. coli* clpXP protease for controlled protein degradation are taught by McGinness et al, “Engineering controllable protein degradation”, Mol Cell. June 2006; 22(5) p701-707. This methodology relies upon adding a specific C-terminal peptide tag such as a DAS4 (or DAS+4) tag. Proteins with this tag are not degraded by the clpXP protease until the specificity enhancing chaperone sspB is expressed. sspB induces degradation of DAS4 tagged proteins by the clpXP protease. In addition, numerous site specific protease systems are well known in the art. Proteins can be engineered to contain a specific target site of a given protease and then cleaved after the controlled expression of the protease. In some embodiments, the cleavage can be expected lead to protein inactivation or degradation. For example Schmidt et al (“ClpS is the recognition component for Escherichia coli substrates of the N-end rule degradation pathway” Molecular Microbiology March 2009. 72(2), 506–517. doi:10.1111), teaches that an N-terminal sequence can be added to a protein of interest in providing clpS dependent clpAP degradation. In addition, this sequence can further be masked by an additional N-terminal sequence, which can be controllable cleaved such as by a ULP hydrolase. This allows for controlled N-rule degradation dependent on hydrolase expression. It is therefore possible to tag proteins for controlled proteolysis either at the N-terminus or C-terminus. The preference of using an N-terminal vs. C-terminal tag will largely depend on whether either tag affects protein function prior to the controlled onset of degradation.

[0069] The invention describes the use of controlled protein degradation or proteolysis to provide the control over metabolic fluxes in controlled multi-stage fermentation processes, in *E. coli*. There are several methodologies known in the art for controlled protein degradation in other microbial hosts, including a wide range of gram-negative as well as gram-positive bacteria, yeast and even archaea. In particular, systems for controlled proteolysis can be transferred from a native microbial host and used in a non-native host. For example Grilly et al, “A synthetic gene network for tuning protein degradation in *Saccharomyces cerevisiae*” Molecular Systems Biology 3, Article 127. doi:10.1038, teaches the expression and use of the *E. coli* clpXP protease in the yeast *Saccharomyces cerevisiae*. Such approaches can be used to transfer the

methodology for synthetic metabolic valves to any genetically tractable host.

VI.C Synthetic Metabolic Valve Control

[0070] In particular the invention describes the use of synthetic metabolic valves to control metabolic fluxes in multi-stage fermentation processes. There are numerous methodologies known in the art to induce expression that can be used at the transition between stages in multi-stage fermentations. These include but are not limited to artificial chemical inducers including: tetracycline, anhydrotetracycline, lactose, IPTG (isopropyl-beta-D-1-thiogalactopyranoside), arabinose, raffinose, tryptophan and numerous others. Systems linking the use of these well-known inducers to the control of gene expression silencing and/or controlled proteolysis can be integrated into genetically modified microbial systems to control the transition between growth and production phases in multi-stage fermentation processes.

[0071] In addition, it may be desirable to control the transition between growth and production in multi-stage fermentations by the depletion of one or more limiting nutrients that are consumed during growth. Limiting nutrients can include but are not limited to: phosphate, nitrogen, sulfur, and magnesium. Natural gene expression systems that respond to these nutrient limitations can be used to operably link the control of gene expression silencing and/or controlled proteolysis to the transition between growth and production phases in multi-stage fermentation processes.

[0072] Within the scope of the invention are genetically modified microorganism, wherein the microorganism is capable of producing xylitol at a specific rate selected from the rates of greater than 0.05 g/gDCW-hr, 0.08g/gDCW-hr, greater than 0.1g/gDCW-hr, greater than 0.13g/gDCW-hr, greater than 0.15g/gDCW-hr, greater than 0.175g/gDCW-hr, greater than 0.2g/gDCW-hr, greater than 0.25g/gDCW-hr, greater than 0.3g/gDCW-hr, greater than 0.35g/gDCW-hr, greater than 0.4g/gDCW-hr, greater than 0.45g/gDCW-hr, or greater than 0.5g/gDCW-hr.

[0073] Within the scope of the invention are genetically modified microorganism, wherein the microorganism is capable of producing xylitol from xylose or another sugar source at a yield greater than 0.5 g product /g xylose, greater than 0.6 g product /g xylose,, greater than 0.7 g product /g xylose,, greater than 0.8 g product /g xylose,, greater than 0.9 g product /g xylose,, greater than 0.95 g product /g xylose,, or greater than 0.98 g product /g xylose.

[0074] In various embodiments, the invention includes a culture system comprising a carbon source in an aqueous medium and a genetically modified microorganism according to any one of claims herein, wherein said genetically modified organism is present in an amount selected from greater than 0.05 gDCW/L, 0.1 gDCW/L, greater than 1 gDCW/L, greater than 5 gDCW/L, greater than 10 gDCW/L, greater than 15 gDCW/L or greater than 20 gDCW/L, such as when the volume of the aqueous medium is selected from greater than 5 mL, greater than 100 mL, greater than 0.5L, greater than 1L, greater than 2 L, greater than 10 L, greater than 250 L, greater

than 1000L, greater than 10,000L, greater than 50,000 L, greater than 100,000 L or greater than 200,000 L, and such as when the volume of the aqueous medium is greater than 250 L and contained within a steel vessel.

Overview of Invention Aspects

[0075] In one aspect, a genetically modified microorganism for producing xylitol comprising is provided. The genetically modified microorganism characterized by inducible modification of expression of xylose reductase (xylA) and an inducible synthetic metabolic valve. The synthetic metabolic valve characterized by a gene expression-silencing synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes; or an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes, or a combination thereof.

[0076] In one aspect the xylose reductase of the genetically modified microorganism is an NADPH dependent xylose reductase or the xylose reductase may be the *xylA* gene of *A. niger*.

[0077] In one aspect, the genetically modified microorganism produces xylitol from a xylose feedstock. Of course the genetically modified microorganism may use a feedstock comprising xylose and a second sugar blending in any ratio.

[0078] In one aspect the gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve of the genetically modified microorganism may be directed to control of the gene encoding xylose isomerase or the xylose isomerase enzyme; or the gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme.

[0079] In one aspect the gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve of the genetically modified microorganism may be directed to control more than one gene, for example a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and a gene encoding xylose isomerase or the xylose isomerase enzyme.

[0080] In yet another aspect, In one aspect the gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve of the genetically modified microorganism may be directed to control more than one gene, for example a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and a gene encoding enoyl-ACP reductase (*fabI*) or the enoyl-ACP reductase (*fabI*) enzyme.

[0081] In yet another aspect, In one aspect the gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve of the genetically modified microorganism may be directed to control silencing of a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) and

enzyme degradation of glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and enoyl-ACP reductase (*fabI*) enzyme.

[0082] In another aspect, expression of xylose reductase, gene expression-silencing synthetic metabolic valve, and the enzymatic degradation synthetic metabolic valve are induced under conditions of a transition phrase of a multi-stage biofermentation process. The induction may occur via nutrient depletion or via phosphate depletion.

[0083] In one aspect, the genetically modified microorganism may further comprise a chromosomal deletion.

[0084] In one aspect, the silencing of gene expression comprises CRISPR interference and the genetically modified microorganism also expresses a CASCADE guide array, the array comprising two or more genes encoding small guide RNAs each specific for targeting a different gene for simultaneous silencing of multiple genes.

[0085] In one aspect, the genetically modified microorganism produces a xylitol product titer of greater than 0.08 g/L at twenty four in a biofermentation process.

[0086] In one aspect, the invention provides for a multi-stage fermentation bioprocess for producing xylitol from a genetically modified microorganism, including the steps of (a) providing a genetically modified microorganism. The genetically modified microorganism characterized by a modification of expression of xylose reductase and a synthetic metabolic valve comprising: a gene expression-silencing synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes; or an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes, or a combination thereof. The one or more enzymes of each synthetic metabolic valve are the same or different. The method further includes the steps of growing the genetically modified microorganism in a media with a xylose feedstock and transitioning from a growth phase to a xylitol. The transition step includes inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism; and inducing expression of xylose reductase, thereby producing xylitol.

[0087] In some aspects, the multi-stage fermentation bioprocess may use a genetically modified microorganism characterized by the gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve of the genetically modified microorganism are directed to control of at least two genes, including a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and a gene encoding enoyl-ACP reductase (*fabI*) or the enoyl-ACP reductase (*fabI*) enzyme.

[0088] In some aspects, the multi-stage fermentation bioprocess will produce a xylitol product titer of greater than 0.08 g/L at twenty four in a biofermentation process.

[0089] In some aspects, the transition phase of the multi-stage fermentation bioprocess occurs via phosphate depletion of the growth media. In some aspects, the genetically modified microorganism of the multi-stage fermentation bioprocess is further characterized by a chromosomal deletion.

[0090] In one aspect the genetically modified microorganism for producing xylitol, the microorganism comprises: inducible reduction of xylose isomerase; inducible reduction of glucose-6-phosphate dehydrogenase activity so that the microorganism produces xylitol from the feedstock xylose upon induction. In another aspect the microorganism is an E.coli microorganism. In one aspect, the induction of the microorganism occurs by via nutrient depletion. In one aspect, the induction of the microorganism occurs via phosphate depletion.

[0091] In one aspect, the invention provides a multi-stage fermentation bioprocess for producing xylitol from a genetically modified microorganism including inducible reduction of xylose isomerase and inducible reduction of glucose-6-phosphate dehydrogenase activity. The bioprocess includes the steps of (a) providing a genetically modified microorganism, (b) growing the genetically modified microorganism in a media with a xylose feedstock; (c) transitioning from a growth phase to a xylitol producing stage by inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism; and inducing expression of xylose isomerase, thereby (d) producing xylitol.

[0092] In one aspect the genetically modified microorganism for producing xylitol, the microorganism comprises: inducible reduction of xylose reductase; inducible reduction of glucose-6-phosphate dehydrogenase activity; inducible reduction of enoyl-ACP reductase; wherein the strain produces xylitol from the feedstock xylose upon induction. In one aspect, the microorganism is an E.coli microorganism. In some aspect, induction of the microorganism occurs by via nutrient depletion or phosphate depletion.

[0093] In one aspect, the invention provides a multi-stage fermentation bioprocess for producing xylitol from a genetically modified microorganism including inducible reduction of xylose reductase; inducible reduction of glucose-6-phosphate dehydrogenase activity; inducible reduction of enoyl-ACP reductase. The bioprocess includes the steps of (a) providing a genetically modified microorganism; (b) growing the genetically modified microorganism in a media with a xylose feedstock; (c) transitioning from a growth phase to a xylitol producing stage by inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism; and inducing expression of xylose reductase, thereby (d) producing xylitol.

[0094] In one aspect the genetically modified microorganism for producing xylitol, the microorganism comprises: activity of a membrane bound transhydrogenase activity is increased; activity of a pyruvate ferredoxin oxidoreductase is increased; activity of a NADPH dependent

ferredoxin reductase is increased; and wherein the microorganism produces at least one chemical product whose biosynthesis requires NADPH.

Disclosed Embodiments Are Non-Limiting

[0095] While various embodiments of the present invention have been shown and described herein, it is emphasized that such embodiments are provided by way of example only. Numerous variations, changes and substitutions may be made without departing from the invention herein in its various embodiments. Specifically, and for whatever reason, for any grouping of compounds, nucleic acid sequences, polypeptides including specific proteins including functional enzymes, metabolic pathway enzymes or intermediates, elements, or other compositions, or concentrations stated or otherwise presented herein in a list, table, or other grouping (such as metabolic pathway enzymes shown in a **FIGs 1 and 4**), unless clearly stated otherwise, it is intended that each such grouping provides the basis for and serves to identify various subset embodiments, the subset embodiments in their broadest scope comprising every subset of such grouping by exclusion of one or more members (or subsets) of the respective stated grouping. Moreover, when any range is described herein, unless clearly stated otherwise, that range includes all values therein and all sub-ranges therein.

[0096] Also, and more generally, in accordance with disclosures, discussions, examples and embodiments herein, there may be employed conventional molecular biology, cellular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook and Russell, "Molecular Cloning: A Laboratory Manual," Third Edition 2001 (volumes 1 - 3), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Animal Cell Culture, R. I. Freshney, ed., 1986. These published resources are incorporated by reference herein.

[0097] The following published resources are incorporated by reference herein for description useful in conjunction with the invention described herein, for example, methods of industrial bio-production of chemical product(s) from sugar sources, and also industrial systems that may be used to achieve such conversion (Biochemical Engineering Fundamentals, 2nd Ed. J. E. Bailey and D. F. Ollis, McGraw Hill, New York, 1986, e.g. Chapter 9, pages 533-657 for biological reactor design; Unit Operations of Chemical Engineering, 5th Ed., W. L. McCabe et al., McGraw Hill, New York 1993, e.g., for process and separation technologies analyses; Equilibrium Staged Separations, P. C. Wankat, Prentice Hall, Englewood Cliffs, NJ USA, 1988, e.g., for separation technologies teachings).

[0098] All publications, patents, and patent applications mentioned in this specification are entirely incorporated by reference herein, including U.S. Provisional Application No.s 62/010,574, filed June 11, 2014, and 62/461,436, filed February 21, 2017, and

PCT/US2015/035306 filed June 11, 2015 and PCT/US2018/019040, filed February 21, 2018.

EXAMPLES

[0099] The examples herein provide some examples, not meant to be limiting. All reagents, unless otherwise indicated, are obtained commercially. Species and other phylogenetic identifications are according to the classification known to a person skilled in the art of microbiology, molecular biology, and biochemistry.

[00100] Common Methods

[00101] Reagents and Media

[00102] All reagents and chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted. MOPS (3-(N-morpholino)propanesulfonic acid) was obtained from BioBasic, Inc. (Amherst, NY). Crystalline xylose was obtained from Profood International (Naperville, IL). All media: SM10++, SM10 No Phosphate, and FGM25 were prepared as previously reported (Menacho-Melgar, R. *et al.* Scalable, two-stage, autoinduction of recombinant protein expression in *E. coli* utilizing phosphate depletion. *Biotechnol. Bioeng.* **26**, 44 (2020)) except that xylose was substituted for glucose (1 gram xylose for 1 gram glucose) in all media formulations. LB, Lennox formulation, was used for routine strain propagation. Working antibiotic concentrations were as follows: kanamycin: 35 µg/mL, chloramphenicol: 35 µg/mL, gentamicin: 10 µg/mL, 10 zeocin: 100 µg/mL, blasticidin: 100 µg/mL, spectinomycin: 25 µg/mL, tetracycline: 5 µg/mL.

[00103] Strains & Plasmids Construction

[00104] Refer to Supplemental Table S1 for a list of strains and plasmids used in this study. Sequences of synthetic DNA used in this study are given in Supplemental Table S2. Chromosomal modifications were constructed using standard recombineering methodologies (Liochev, S. I. *et al.* *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1328–1331 (1994)). The recombineering plasmid pSIM5 was a kind gift from Donald Court (NCI, <https://redrecombineering.ncifcrf.gov/court-lab.html>). 53,54 C-terminal DAS+4 tags were added by direct integration and selected through integration of antibiotic resistance cassettes 3' of the gene as previously described.²⁴ All strains were confirmed by PCR, agarose gel electrophoresis and confirmed by sequencing. Refer to Table S3 for oligos used for strain confirmation and sequencing.

[00105] The *xyrA* gene from *Aspergillus niger* was codon optimized for expression in *E. coli* and the plasmid, pHCKan-*xyrA* (Addgene #58613), enabling the low phosphate induction of xylose reductase, was constructed by TWIST Biosciences (San Francisco, CA). pCDF-pntAB (Addgene # 158609) was constructed using PCR and Gibson Assembly from pCDF-ev 30 to drive expression of the pntAB operon from the low phosphate inducible *ugpBp* promoter

(Moreb, E. A. *et al.* Media Robustness and scalability of phosphate regulated promoters useful for two-stage autoinduction in *E. coli*. *ACS Synthetic Biology* (2020)

doi:10.1021/acssynbio.0c00182). pCASCADE guide RNA array plasmids were prepared by the combination of PCR and Gibson assembly as previously described. Refer to Table S4 for oligos used for pCASCADE plasmid construction.

[00106] Table 1: Plasmids used in these Examples:

Plasmids	Promoter	Ori	Res	Addgene Number	Source
pSMART-HC-Kan	None	colE1	Kan	NA	Lucigen
pHC-Kan-yibDp-xyrA	yibDp	colE1	Kan	TBD	Previous Lab Work
pCASCADE-EV	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA1	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA2	ugpBp	p15A	Cm	65817	Previous Lab Work
pCASCADE-zwf	ugpBp	p15A	Cm	65825	Previous Lab Work
pCACADE-udhA	ugpBp	p15A	Cm	65818	Previous Lab Work
pCASCADE-xylA	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA1-zwf	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA2-zwf	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA1-udhA	ugpBp	p15A	Cm	TBD	Previous Lab Work

pCACADE-gltA2-udhA	ugpBp	p15A	Cm	65819	Previous Lab Work
pCASCADE-gltA1-gltA2	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA1-gltA2-zwf	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-gltA1-gltA2-udhA	ugpBp	p15A	Cm	TBD	Previous Lab Work
pCASCADE-zwf-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-udhA-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA1-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA2-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA1-zwf-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA2-zwf-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA1-udhA-xylA	ugpBp	p15A	Cm	TBD	This study
pCACADE-gltA2-udhA-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA1-gltA2-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA1-gltA2-zwf-xylA	ugpBp	p15A	Cm	TBD	This study
pCASCADE-gltA1-gltA2-udhA-xylA	ugpBp	p15A	Cm	TBD	This study

[00107] Table S1: Additional plasmids used in the Examples:

Plasmid	Insert	promoter	Ori	Res	Addgene	Source
pSMART-HC-Kan	None	None	colE1	Kan	NA	Lucigen
pCDF-ev	None	None	cloDF13	Sm	89596	Previous Lab Work https://paperpile.com/c/15x0oz/U3k3Y
pHCKan-xyrA	xyrA	yibDp ²	colE1	Kan	158610	This study
pCDF-pntAB	pntAB	ugpBp ²	cloDF13	Sm	158609	This study
pCASCADE-ev	none	ugpBp ²	p15	Cm	65821	Previous Lab Work
pCASCADE-g2	gltAp2 gRNA	ugpBp ²	p15	Cm	65817	Previous Lab Work
pCASCADE-f	fabIp gRNA	ugpBp ²	p15	Cm	66635	This study
pCASCADE-z	zwfp gRNA	ugpBp ²	p15	Cm	65825	Previous Lab Work
pCASCADE-u	udhAp gRNA	ugpBp ²	p15	Cm	65818	This study

pCASCADE-x	xylAp gRNA	ugpBp ²	p15	Cm	158611	This study
pCASCADE-g2z	gltAp2 , zwfp gRNA array	ugpBp ²	p15	Cm	71338	Previous Lab Work
pCASCADE-g2u	gltAp2 udhAp gRNA array	ugpBp ²	p15	Cm	65819	This study
pCASCADE-g2x	gltAp2, xylAp gRNA array	ugpBp ²	p15	Cm	158613	This study
pCASCADE-zx	zwfp xylAp gRNA array	ugpBp ²	p15	Cm	158614	This study
pCASCADE-ux	udhAp , xylAp gRNA array	ugpBp ²	p15	Cm	158612	This study
pCASCADE-fg2	fabIp, gltAp2 gRNA array	ugpBp ²	p15	Cm	71341	This study
pCASCADE-fz	fabIp, zwf gRNA array	ugpBp ²	p15	Cm	71335	This study

[00108] Table 2: Host strains used in these Examples:

Strain	Proteolytic Abbreviation	Genotype	Source
DLF_0025	Control/None	F-, λ-, Δ(araD-araB)567, lacZ4787(del)(::rrnB-3) , rph-1, Δ(rhaD-rhaB)568, hsdR514, ΔackA-pta, ΔpoxB, ΔpflB, ΔldhA, ΔadhE, ΔsspB, ΔiclR, ΔarcA, Δcas3::tm-ugpb-sspB-pro [casA*]	Previous Lab Work
DLF_0025-X	X	DLF_0025, xylA-DAS+4-ampR	This Study
DLF_0025-F	F	DLF_0025, fabI-DAS+4-gentR	Previous Lab Work

DLF_0025-G	G	DLF_0025, gltA-DAS+4-zeoR	Previous Lab Work
DLF_0025-Z	Z	F_0025, zwf-DAS+4-bsdR	Previous Lab Work
DLF_0025-U	U	DLF_0025, udhA-DAS+4-bsdR	Previous Lab Work
DLF_0025-GU	GU	DLF_0025, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR	Previous Lab Work
DLF_0025-GZ	GZ	DLF_0025, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	Previous Lab Work
DLF_0025-FG	FG	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR	Previous Lab Work
DLF_0025-FZ	FZ	DLF_0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR	Previous Lab Work
DLF_0025-FU	FU	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR	Previous Lab Work
DLF_0025-FGU	FGU	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR	Previous Lab Work
DLF_0025-FGZ	FGZ	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	Previous Lab Work
DLF_0025-FX	FX	DLF_0025, fabI-DAS+4-gentR, xylA-DAS+4-ampR	This Study
DLF_0025-FGX	FGX	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, xylA-DAS+4-ampR	This Study
DLF_0025-FZX	FZX	DLF_0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR, xylA-DAS+4-ampR	This Study
DLF_0025-FUX	FUX	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, xylA-DAS+4-ampR	This Study

DLF_0025-FGUX	FGUX	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR, xylA-DAS+4-ampR	This Study
DLF_0025-FGZX	FGZX	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR, xylA-DAS+4-ampR	This Study
DLF_0025-UX	UX	DLF_0025, udhA-DAS+4-bsdR, xylA-DAS+4-ampR	This Study
DLF_0025-ZX	ZX	DLF_0025, zwf-DAS+4-bsdR, xylA-DAS+4-ampR	This Study

[00109] Table S2: Additional Strains used in the Examples:

Strain	Genotype	Source
DLF_Z0025	F-, λ^- , $\Delta(\text{araD-araB})567$, $\text{lacZ}4787(\text{del})(::\text{rrnB-3})$, rph-1 , $\Delta(\text{rhaD-rhaB})568$, $\text{hsdR}514$, $\Delta\text{ackA-pta}$, ΔpoxB , ΔpflB , ΔldhA , ΔadhE , ΔiclR , ΔarcA , ΔsspB , $\Delta\text{cas3}::\text{tm-ugpb-sspB-pro-casA}$.	Previous Lab Work
DLF_Z0043	DLF_Z0025, gltA-DAS+4-zeoR	Previous Lab Work
DLF_Z1002	DLF_Z0025, zwf-DAS+4-bsdR	Previous Lab Work
DLF_Z0044	DLF_Z0025, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	Previous Lab Work
DLF_Z0028	DLF_Z0025, fabI-DAS+4-gentR	This study
DLF_Z0028 G	DLF_Z0025, fabI-sfGFP-gentR	This study
DLF_Z0028 GD	DLF_Z0025, fabI-sfGFP-DAS+4-gentR	This study
DLF_Z0763	DLF_Z0025, udhA-DAS+4-bsdR	This study
DLF_Z0039	fDLF_Z0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR	This study

DLF_Z0040	DLF_Z0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR	This study
DLFZ_0045	DLF_Z0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR	This study
DLFZ_0046	DLF_Z0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	This study
DLFZ_0047	DLF_Z0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR	This study
SL_0001	DLF_Z0025, xylA-DAS+4-ampR	This study
SL_0002	DLF_Z0025, fabI-DAS+4-gentR, xylA -DAS+4-ampR	This study
SL_0003	DLF_Z0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, xylA -DAS+4-ampR	This study
SL_0004	DLF_Z0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR, xylA -DAS+4-ampR	This study
SL_0005	DLF_Z0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, xylA -DAS+4-ampR	This study
SL_0006	DLF_Z0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR, xylA -DAS+4-ampR	This study
SL_0007	DLF_Z0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR, xylA -DAS+4-ampR	This study
SL_0008	DLF_Z0025, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR	This study
SL_0009	DLF_Z0025, zwf-DAS+4, -bsdR xylA -DAS+4-ampR	This study
SL_0010	DLF_Z0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR, $\Delta ydbK$	This study
SL_0011	DLF_Z0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR, Δfpr	This study
Ori- origin of replication, Res - resistance marker, Sm - spectinomycin, Cm- chloramphenicol, Kan - kanamycin, Amp - ampicillin		

[00110] Chromosomal modifications were constructed using standard recombineering methodologies. A C-terminal DAS+4 tag on the *xylA* gene was added by direct integration and selected through integration of antibiotic resistance cassettes 3' of the gene. All strains were confirmed by PCR, agarose gel electrophoresis and confirmed by sequencing.

[00111] Table 3: Sequences of synthetic DNA:

xylA-DAS4-ampR
GATACGATGGCACTGGCGCTGAAAATTGCAGCGCGCATGATTGAAGATGGCGAG CTGGATAAACGCATCGCGCAGCGTTATTCCGGCTGGAATAGCGAATTGGGCCAG CAAATCCTGAAAGGCCAAATGTCACTGGCAGATTTAGCCAAATATGCTCAGGAA CATCATTTGTCTCCGGTGCATCAGAGTGGTCGCCAGGAACAACCTGGAAAATCTGG TAAACCATTATCTGTTCGACAAAGCGGCCAACGATGAAAATCTGAAAACTA TGCGGATGCGTCTTAATGATAAGGACCGTGTTGACAATTAATCATCGGCATAGTA TATCGGCATAGTATAATACGACAAGGTGAGGAACTAAACCATGAGTATTCAACA TTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCA CCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGT GGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTACGCCCC GAAGAACGTTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTAT TATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTGCGCGCATACACTATTCTCA GAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTCACGGATGGCAT GACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACTGCGGC CAACTTACTTCTGGCAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGAC AACATGGGGGATCATGTAACCTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAA GCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACG TTGCGCAAACCTATTAACCTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAA TAGACTGGATGGAGGCGGATAAAGTTGCAGGATCACTTCTGCGCTCGGCCCTCCC GGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGT ATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGCATCGTAGTTATCTACA CGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG GTGCCTCACTGATTAAGCATTGGTAGTAAGTAGGGATAACAGGGTAATCGGCTA ACTGTGCAGTCCGTTGGCCCGGTTATCGGTAGCGATACCGGGCATTTTTTTAAGG AACGATCGATATGTATATCGGGATAGATCTTGGCACCTCGGGCGTAAAAGTTATT TTGCTCAACGAGCAGGGTGAGGTGGTTGCTGCGCAAACGGAAAAGCTGACCGTT TCGCGCCCGCATCCACTCTGGTCGGAACAAGACCCGGAACAGTGGTGGCAGGCA

ACTGATCGCGCAA (SEQ ID NO: 1)
fabI-DAS+4-gentR
CTATTGAAGATGTGGGTA ACTCTGCGGCATTCCTGTGCTCCGATCTCTCTGCCGGT ATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTTCAGCATTGCTGCAATGAACG AACTCGAACTGAAAGCGGCCAACGATGAAA ACTATTCTGAAA ACTATGCGGATG CGTCTTAATAGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCGAATCCATG TGGGAGTTTATTCTTGACACAGATATTTATGATATAATAA ACTGAGTAAGCTTAAC ATAAGGAGGAAAAACATATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCA GTCGCCCTAAAACAAAGTTAGGTGGCTCAAGTATGGGCATCATTTCGCACATGTAG GCTCGGCCCTGACCAAGTCAAATCCATGCGGGCTGCTCTTGATCTTTTCGGTCGT GAGTTCGGAGACGTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTCG GGA ACTTGCTCCGTAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGC GGTTGTTGGCGCTCTCGCGGCTTACGTTCTGCCCAAGTTTGAGCAGCCGCGTAGT GAGATCTATATCTATGATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTG CCACCGCGCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTATGT GATCTACGTGCAAGCAGATTACGGTGACGATCCCGCAGTGGCTCTCTATACAAAG TTGGGCATACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGTACCGCCACCT AAGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCGTTCTGTTGGTAAAGATG GGCGGCGTTCTGCCGCCCGTTATCTCTGTTATACCTTTCTGATATTTGTTATCGCC GATCCGTCTTTCTCCCCTTCCCGCCTTGCGTCAGG (SEQ ID NO: 2)
gltA-DAS+4-zeoR
GTATTCCGTCTTCCATGTTACCGTCATTTTCGCAATGGCACGTACCGTTGGCTGG ATCGCCC ACTGGAGCGAAATGCACAGTGACGGTATGAAGATTGCCCGTCCGCGT CAGCTGTATACAGGATATGAAAAACGCGACTTTAAAAGCGATATCAAGCGTGCG GCCAACGATGAAA ACTATTCTGAAA ACTATGCGGATGCGTCTTAATAGTTGACAA TTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTATAGGAGGGC CATCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCC GGAGCGGTTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAG GACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTTCATCAGCGCGGTCC AGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGG ACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCT CCGGGCCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCC TGCGCGACCCGGCCGGCAACTGCGTGCACTTTGTGGCAGAGGAGCAGGACTGAG GATAAGTAATGGTTGATTGCTAAGTTGTAAATATTTTAACCCGCCGTTTCATATGG

CGGGTTGATTTTTATATGCCTAAACACAAAAAATTGTAAAAATAAAATCCATTAA CAGACCTATATAGATATTTAAAAAGAATAGAACAGCTCAAATTATCAGCAACCC AATACTTTCAATTAAAAACTTCATGGTAGTCGCATTTATAACCCTATGAAA(SEQ ID NO: 3)
udhA-DAS+4-bsdR
TCTGGGTATTCACTGCTTTGGCGAGCGCGCTGCCGAAATTATTCATATCGGTCAG GCGATTATGGAACAGAAAGGTGGCGGCAACACTATTGAGTACTTCGTCAACACC ACCTTTAACTACCCGACGATGGCGGAAGCCTATCGGGTAGCTGCGTTAAACGGTT TAAACCGCCTGTTTGC GGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGC GTCTTAATAGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACG ACTCACTATAGGAGGGCCATCATGAAGACCTTCAACATCTCTCAGCAGGATCTGG AGCTGGTGGAGGTCGCCACTGAGAAGATCACCATGCTCTATGAGGACAACAAGC ACCATGTCGGGGCGGCCATCAGGACCAAGACTGGGGAGATCATCTCTGCTGTCC ACATTGAGGCCTACATTGGCAGGGTCACTGTCTGTGCTGAAGCCATTGCCATTGG GTCTGCTGTGAGCAACGGGCAGAAGGACTTTGACACCATTGTGGCTGTCAGGCAC CCCTACTCTGATGAGGTGGACAGATCCATCAGGGTGGTCAGCCCCTGTGGCATGT GCAGAGAGCTCATCTCTGACTATGCTCCTGACTGCTTTGTGCTCATTGAGATGAA TGGCAAGCTGGTCAAAACCACCATTTGAGGAACTCATCCCCCTCAAGTACACCAG GAACTAAAGTAAAACTTTATCGAAATGGCCATCCATTCTTGCGCGGATGGCCTCT GCCAGCTGCTCATAGCGGCTGCGCAGCGGTGAGCCAGGACGATAAACCAGGCCA ATAGTGCGGCGTGGTTCCGGCTTAATGCACGG(SEQ ID NO: 4)
zwf-DAS+4-bsdR

GAAGTGGAAGAAGCCTGGAAATGGGTAGACTCCATTACTGAGGCGTGGGCGATG
 GACAATGATGCGCCGAAACCGTATCAGGCCGGAACCTGGGGACCCGTTGCCTCG
 GTGGCGATGATTACCCGTGATGGTTCGTTCTGGAATGAGTTTGAGGCGGCCAACG
 ATGAAAACCTATTCTGAAAACCTATGCGGATGCGTCTTAATAGTTGACAATTAATCA
 TCGGCATAGTATATCGGCATAGTATAAATACGACTCACTATAGGAGGGCCATCATG
 AAGACCTTCAACATCTCTCAGCAGGATCTGGAGCTGGTGGAGGTGCGCCACTGAG
 AAGATCACCATGCTCTATGAGGACAACAAGCACCATGTCGGGGCGGCCATCAGG
 ACCAAGACTGGGGAGATCATCTCTGCTGTCCACATTGAGGCCTACATTGGCAGGG
 TCACTGTCTGTGCTGAAGCCATTGCCATTGGGTCTGCTGTGAGCAACGGGCAGAA
 GGACTTTGACACCATTGTGGCTGTCAGGCACCCCTACTCTGATGAGGTGGACAGA
 TCCATCAGGGTGGTCAGCCCCTGTGGCATGTGCAGAGAGCTCATCTCTGACTATG
 CTCCTGACTGCTTTGTGCTCATTGAGATGAATGGCAAGCTGGTCAAACACCACCAT
 TGAGGAACTCATCCCCCTCAAGTACACCAGGAACTAAAGTAATATCTGCGCTTAT
 CCTTTATGGTTATTTTACCGGTAACATGATCTTGCGCAGATTGTAGAACAATTTT
 ACACCTTTCAGGCCTCGTGCGGATTACCCACGAGGCTTTTTTTATTACACTGACTG
 AAACGTTTTTGCCTATGAGCTCCGGTTACAGGCGTTTCAGTCATAAATCCTCTGA
 ATGAAACGCGTTGTGAATC(SEQ ID NO: 5)

[00112] Table S3: Additional synthetic DNA:

xylA-DAS4-ampR

GATACGATGGCACTGGCGCTGAAAATTGCAGCGCGCATGATTGAAGATGGCGAGC
 TGGATAAACGCATCGCGCAGCGTTATTCCGGCTGGAATAGCGAATTGGGCCAGCA
 AATCCTGAAAGGCCAAATGTCCTGAGGATTTAGCCAAATATGCTCAGGAACAT
 CATTTGTCTCCGGTGCATCAGAGTGGTCGCCAGGAACAACCTGGAAAATCTGGTAA
 ACCATTATCTGTTTCGACAAAGCGGCCAACGATGAAAACCTATTCTGAAAACCTATGC
 GGATGCGTCTTAATGATAAGGACCGTGTTGACAATTAATCATCGGCATAGTATATC
 GGCATAGTATAAATACGACAAGGTGAGGAACTAAACCATGAGTATTCAACATTTCC
 GTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGTCTACCCAGA
 AACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTAC
 ATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTACGCCCCGAAGAAC
 GTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGT
 ATTGACGCCGGGCAAGAGCAACTCGGTGCGGCATACACTATTCTCAGAATGACT
 TGGTTGAGTACTCACCAGTCACAGAAAAGCATCTCACGGATGGCATGACAGTAAG

AGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTC
 TGGCAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGA
 TCATGTAACCTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAAC
 GACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTAT
 TAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAG
 GCGGATAAAAGTTGCAGGATCACTTCTGCGCTCGGCCCTCCCGGCTGGCTGGTTTAT
 TGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTG
 GGGCCAGATGGTAAGCCCTCCCGCATCGTAGTTATCTACACGACGGGGAGTCAGG
 CAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAA
 GCATTGGTAGTAAGTAGGGATAACAGGGTAATCGGCTAACTGTGCAGTCCGTTGG
 CCCGGTTATCGGTAGCGATAACCGGGCATTTTTTTTAAGGAACGATCGATATGTATAT
 CGGGATAGATCTTGGCACCTCGGGCGTAAAAGTTATTTTGCTCAACGAGCAGGGT
 GAGGTGGTTGCTGCGCAAACGGAAAAGCTGACCGTTTCGCGCCCGCATCCACTCT
 GGTCCGAACAAGACCCGGAACAGTGGTGGCAGGCAACTGATCGCGCAA (SEQ ID
 NO: 1)

fabI-DAS+4-gentR

CTATTGAAGATGTGGGTAACCTCTGCGGCATTCTGTGCTCCGATCTCTCTGCCGGT
 ATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTTCAGCATTGCTGCAATGAACGA
 ACTCGAACTGAAAGCGGCCAACGATGAAAACCTATTCTGAAAACCTATGCGGATGCG
 TCTTAATAGGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCGAATCCATGTGG
 GAGTTTATTCTTGACACAGATATTTATGATATAATAACTGAGTAAGCTTAACATAA
 GGAGGAAAAACATATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGC
 CCTAAAACAAAGTTAGGTGGCTCAAGTATGGGCATCATTGCGACATGTAGGCTCG
 GCCCTGACCAAGTCAAATCCATGCGGGCTGCTCTTGATCTTTTCGGTTCGTGAGTTC
 GGAGACGTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTCGGGAACT
 TGCTCCGTAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTT
 GCGCTCTCGCGGCTTACGTTCTGCCCAAGTTTGAGCAGCCGCGTAGTGAGATCTA
 TATCTATGATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTGCCACCGC
 GCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTATGTGATCTACG
 TGCAAGCAGATTACGGTGACGATCCCGCAGTGGCTCTCTATACAAAGTTGGGCAT
 ACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGTACCGCCACCTAAGAAGTT
 CCTATTCTCTAGAAAGTATAGGAACTTCCGTTCTGTTGGTAAAGATGGGCGGCGTT
 CTGCCGCGCGTTATCTCTGTTATACCTTTCTGATATTTGTTATCGCCGATCCGTCTTT

CTCCCCTTCCCGCCTTGCGTCAGG(SEQ ID NO: 2)

fabI-sfGFP-gentR

AAAGACTTCCGCAAAATGCTGGCTCATTGCGAAGCCGTTACCCCGATTGCGCGTAC
CGTTACTATTGAAGATGTGGGTAACTCTGCGGCATTCTGTGCTCCGATCTCTCTG
CCGGTATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTTCAGCATTGCTGCAATG
AACGAACTCGAACTGAAAGGGGGTTTCAGGCGGGTTCGGGTGGCgtgagcaagggcgaggagc
gtttaccgggggtggtgcccattcgtgagctggacggcgacgtaaacggccacaagttcagcgtgcgcggcgagggcgagggc
gatgccaccaacggcaagctgacctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccacctcgtgaccacctga
cctacggcgtgagctgcttcagccgtaccccgaccacatgaagcgccacgacttctcaagtccgccatgccgaaggctacgtcca
ggagcgcaccatcagcttcaaggacgacggcacctacaagaccgcgcggaggtgaagttcgagggcgacacctggtgaaccgc
atcgagctgaagggcatcgaacttcaaggaggacggcaacatcctggggcacaagctggagtacaacttcaacagccacaacgtctata
tcaccgccgacaagcagaagaacggcatcaaggccaacttcaagatccgccacaacgtggaggacggcagcgtgcagctcgccga
ccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccacaaccactacctgagcaccagtcctgctgagcaa
agaccccaacgagaagcgcgatcacatggtcctgctggagtctgtagccgcgggatcactcacggcatggacgagctgtacaa
gTAATGACGAATCCATGTGGGAGTTTATTCTTGACACAGATATTTATGATATAATA
ACTGAGTAAGCTTAACATAAGGAGGAAAAACATATGTTGCGTAGCTCTAACGATG
TGACGCAACAAGGTTTCGCGTCCAAAGACAAAATTGGGAGGCAGTAGCATGGGGAT
CATTCGCACTTGTCGCCTGGGGCCAGACCAGGTGAAGTCAATGCGTGCGGCTCTG
GACTTATTTCGGGCGCGAATTTGGAGATGTAGCCACTTACTCACAGCACCAACCGG
ACAGTGATTACTTGGGGAATTTACTTCGCAGTAAACTTTTATCGCTTTGGCCGCT
TTCGACCAGGAGGCTGTAGTAGGTGCGTTGGCAGCCTATGTTCTTCCTAAATTGGA
GCAACCGCGTAGCGAAATTTACATCTATGATCTTGCAGTCTCCGGCGAACATCGCC
GTCAGGGGATCGCCACAGCTTTAATCAACCTTTTGAAGCATGAGGCTAATGCACTT
GGAGCGTACGTGATTTATGTGCAGGCTGACTACGGTGATGATCCTGCAGTCGCTCT
GTACACCAAACCTGGGTATCCGCGAGGAGGTCATGCACTTTGATATTGACCCGTCG
ACGGCTACCTAAGTTCTGTTGGTAAAGATGGGCGGC GTTCTGCCGCCCGTTATCTC
TGTTATACCTTTCTGATATTTGTTATCGCCGATCCGTCTTTCTCCCCTTCCCGCCTTG
CGTCAGG(SEQ ID NO: 21)

fabI-sfGFP-DAS+4- gentR

AAAGACTTCCGCAAAATGCTGGCTCATTGCGAAGCCGTTACCCCGATTGCGCGTAC
CGTTACTATTGAAGATGTGGGTAACTCTGCGGCATTCTGTGCTCCGATCTCTCTG

CCGGTATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTTCAGCATTGCTGCAATG
AACGAACTCGAACTGAAAGGGGGTTCAGGCGGGTCGGGTGGCgtgagcaagggcgaggagc
tgttcaccgggggtggtgcccacctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgcgcggcgagggcgagggc
gatgccaccaacggcaagctgacctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctga
cctacggcgtgcagtgttcagccgtaccccgaccacatgaagcgccacgacttctcaagtccgcatgcccgaaggctacgtcca
ggagcgcaccatcagcttcaaggacgacggcacctacaagaccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgc
atcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaacttcaacagccacaacgtctata
tcaccgccgacaagcagaagaacggcatcaaggccaacttcaagatccgccacaacgtggaggacggcagcgtgcagctcgccga
ccactaccgcagaaacacccccatggcgacggccccgtgctgctgcccacaaccactacgtgagcaccagtcgctgctgagcaa
agaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgcccgggatcactcacggcatggacgagctgtacaa
gGGTGGGGGTGGGAGCGGCGGGTGGCTCCGCGGCCAACGATGAAAACCTATTCT
GAAAACCTATGCGGATGCGTCTTAATGACGAATCCATGTGGGAGTTTATTCTTGACA
CAGATATTTATGATATAATAACTGAGTAAGCTTAACATAAGGAGGAAAAACATAT
GTTGCGTAGCTCTAACGATGTGACGCAACAAGGTTTCGCGTCCAAAGACAAAATTG
GGAGGCAGTAGCATGGGGATCATTTCGCACTTGTCGCCTGGGGCCAGACCAGGTGA
AGTCAATGCGTGCGGCTCTGGACTTATTTCGGGCGCGAATTTGGAGATGTAGCCACT
TACTCACAGCACCAACCGGACAGTGATTACTTGGGGAATTTACTTCGCAGTAAAA
CTTTTATCGCTTTGGCCGCTTTTCGACCAGGAGGCTGTAGTAGGTGCGTTGGCAGCC
TATGTTCTTCCTAAATTCGAGCAACCGCGTAGCGAAATTTACATCTATGATCTTGC
AGTCTCCGGCGAACATCGCCGTCAGGGGATCGCCACAGCTTTAATCAACCTTTTGA
AGCATGAGGCTAATGCACTTGGAGCGTACGTGATTTATGTGCAGGCTGACTACGG
TGATGATCCTGCAGTCGCTCTGTACACCAAACTGGGTATCCGCGAGGAGGTCATGC
ACTTTGATATTGACCCGTCGACGGCTACCTAAGTTCTGTTGGTAAAGATGGGCGGC
GTTCTGCCGCCCGTTATCTCTGTTATACCTTTCTGATATTTGTTATCGCCGATCCGT
CTTTCTCCCCTTCCCGCCTTGCGTCAGG(SEQ ID NO: 22)

udhA-DAS+4-bsdR

TCTGGGTATTCACTGCTTTGGCGAGCGCGCTGCCGAAATTATTCATATCGGTCAGG
CGATTATGGAACAGAAAGGTGGCGGCAACACTATTGAGTACTTCGTCAACACCAC
CTTTAACTACCCGACGATGGCGGAAGCCTATCGGGTAGCTGCGTTAAACGGTTTAA
ACCGCCTGTTTGGGCCAACGATGAAAACCTATTCTGAAAACCTATGCGGATGCGTCT
TAATAGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCA
CTATAGGAGGGCCATCATGAAGACCTTCAACATCTCTCAGCAGGATCTGGAGCTG
GTGGAGGTCGCCACTGAGAAGATCACCATGCTCTATGAGGACAACAAGCACCATG

TCGGGGCGGCCATCAGGACCAAGACTGGGGAGATCATCTCTGCTGTCCACATTGA
 GGCCTACATTGGCAGGGTCACTGTCTGTGCTGAAGCCATTGCCATTGGGTCTGCTG
 TGAGCAACGGGCAGAAGGACTTTGACACCATTGTGGCTGTCAGGCACCCCTACTC
 TGATGAGGTGGACAGATCCATCAGGGTGGTCAGCCCCTGTGGCATGTGCAGAGAG
 CTCATCTCTGACTATGCTCCTGACTGCTTTGTGCTCATTGAGATGAATGGCAAGCT
 GGTCAAAACCACCATTTAGAGGAATCATCCCCCTCAAGTACACCAGGAACTAAAGT
 AAAACTTTATCGAAATGGCCATCCATTCTTGCGCGGATGGCCTCTGCCAGCTGCTC
 ATAGCGGCTGCGCAGCGGTGAGCCAGGACGATAAACCAGGCCAATAGTGCGGCG
 TGGTTCCGGCTTAATGCACGG (SEQ ID NO: 4)

[00113] The *xyrA* gene from *Aspergillus niger* was codon optimized for *E. coli* and the plasmid, pHCKan-INS: yibDp-6xhis-xyrA, enabling the low phosphate induction of xylose reductase, was constructed by TWIST Biosciences. pCASCADE guide RNA array plasmids were prepared by the combination of PCR and Gibson assembly as previously described.

[00114] Primers used for assembly are given in Table 4:

Plasmids/p rimers name	sequences
gltA1	TCGAGTTCCTCCGCGCCAGCGGGGATAAACCGAAAAGCATATAATG CGTAAAAGTTATGAAGTTCGAGTTCCTCCGCGCCAGCGGGGATAAA CCG (SEQ ID NO: 6)
gltA1-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG (SEQ ID NO: 7)
gltA1-REV	CGGTTTATCCCCGCTGGCGCGGGGAAGTTCGAACTTCATAACTTTTA C (SEQ ID NO: 8)
gltA2	TATTGACCAATTCATTCGGGACAGTTATTAGTTCGAGTTCCTCCGCG CCAGCGGGGATAAACCG (SEQ ID NO: 9)
gltA2-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG (SEQ ID NO: 10)
gltA2-REV	CGGTTTATCCCCGCTGGCGCGGGGAAGTTCGAACTAATAACTGTC (SEQ ID NO: 11)
udhA	TTACCATTCTGTTGCTTTTATGTATAAGAATCGAGTTCCTCCGCGCCA GCGGGGATAAACCG (SEQ ID NO: 12)

udhA-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG (SEQ ID NO: 13)
udhA-REV	CGGTTTATCCCCGCTGGCGCGGGGAACCTCGATTCTTATACATAAAAGC (SEQ ID NO: 14)
zwf	CTCGTAAAAGCAGTACAGTGCACCGTAAGATCGAGTTCCCCGCGCCAGCGGGGATAAACCG (SEQ ID NO: 15)
zwf-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG (SEQ ID NO: 16)
zwf-REV	CGGTTTATCCCCGCTGGCGCGGGGAACCTCGATCTTACGGTGCACTGTAC (SEQ ID NO: 17)
xylA	GGAGTGCCCAATATTACGACATCATCCATCTCGAGTTCCCCGCGCCAGCGGGGATAAACCG (SEQ ID NO: 18)
xylA-FOR	CCAGCGGGGATAAACCGGGAGTGCCCAATATTAC (SEQ ID NO: 19)
xylA-REV	CTTGCCCGCCTGATGAATGCTCATCCGG (SEQ ID NO: 20)

[00115] Table S4: Additional oligonucleotides:

Plasmids/primers name	Sequences
fabI_int_F	GCAAAATGCTGGCTCATTG(SEQ ID NO: 23)
gentR_intR	GCGATGAATGTCTTACTACGGA(SEQ ID NO: 24)
gltA_int_F	TATCATCCTGAAAGCGATGG(SEQ ID NO: 25)
zeo_intR	ACTGAAGCCCAGACGATC(SEQ ID NO: 26)
zwf_intF	CTGCTGGAAACCATGCG(SEQ ID NO: 27)
udhA_intF	CAAAAGAGATTCTGGGTATTCACT(SEQ ID NO: 28)
bsdR_intR	GAGCATGGTGATCTTCTCAGT(SEQ ID NO: 29)
xylA_intF	AGATGGCGAGCTGGATA(SEQ ID NO: 30)
ampR_intR	AGTACTCAACCAAGTCATTCTG(SEQ ID NO: 31)

Plasmids/primers name	Sequences
gltA2	TATTGACCAATTCATTTCGGGACAGTTATTAGTTCGAGTTCCCCGCGC CAGCGGGGATAAACCG(SEQ ID NO: 6)
gltA2-FOR	CCGGATGAGCATTTCATCAGGCGGGCAAG(SEQ ID NO: 7)
gltA2-REV	CGGTTTATCCCCGCTGGCGCGGGGAAGTTCGAACTAATAACTGTC(SEQ ID NO: 8)
udhA	TTACCATTCTGTTGCTTTTATGTATAAGAATCGAGTTCCCCGCGCCA GCGGGGATAAACCG(SEQ ID NO: 12)
udhA-FOR	CCGGATGAGCATTTCATCAGGCGGGCAAG(SEQ ID NO: 13)
udhA-REV	CGGTTTATCCCCGCTGGCGCGGGGAAGTTCGATTCTTATACATAAA GC(SEQ ID NO: 14)
xylA	GGAGTGCCCAATATTACGACATCATCCATCTCGAGTTCCCCGCGCCA GCGGGGATAAACCG(SEQ ID NO: 18)
xylA-FOR	CCAGCGGGGATAAACCGGAGTGCCCAATATTAC(SEQ ID NO: 19)
xylA-REV	CTTGCCCGCCTGATGAATGCTCATCCGG(SEQ ID NO: 20)

[00116] Micro and 1L Fermentations

[00117] Micro-fermentations and 1L fermentations in instrumented bioreactors were performed as previously reported, except that xylose was substituted for glucose (1gram xylose for 1gram glucose) in all media formulations. Guide array stability was confirmed after transformation of pCASCADE vector by PCR prior to evaluation in 96 well plate micro-fermentations.

[00118] Xylose and Xylitol Quantification

In micro-fermentations, xylose and xylitol were quantified by commercial bioassays from Megazyme (Wicklow, Ireland, Catalog #K-XYLOSE and K-SORB), according to the manufacturer's instructions. All the results were tested by measuring the absorbance at 492nm. For the quantification of tank fermentations, an HPLC method coupled with a refractive index detector was used to measure both xylose as well as xylitol. At 55 °C, a Rezex ROA-Organic Acid H+

(8%) Analysis HPLC Column (CAT#: #00H-0138-K0, Phenomenex, Inc., Torrance, CA, 300 x 7.8 mm;) was employed for the compound's separation. According to reference, we chose sulfuric acid as the isocratic eluent solvents, and the flow rate was set at 0.5mL/min. Waters Acquity H-Class UPLC integrated with a Waters 2414 Refractive Index (RI) detector (Waters Corp., Milford, MA, USA) was used for the chromatographic detection. Injection volume of sample and standard was set as 10 μ L. Samples were diluted in 20 times using filtered ultrapure water to make all the sample points appear within the standards linear range. The standard variation range was between 0.01 to 20 g/L. MassLynx v4.1 software was used for all the peak integration and concentration analysis.

[00119] Fermentations.

[00120] Minimal media microfermentations were performed as previously reported (Moreb, E. A. *et al.* Media Robustness and scalability of phosphate regulated promoters useful for two-stage autoinduction in *E. coli*. *ACS Synthetic Biology* (2020) doi:10.1021/acssynbio.0c00182 and Menacho-Melgar, R. *et al.* Scalable, two-stage, autoinduction of recombinant protein expression in *E. coli* utilizing phosphate depletion. *Biotechnol. Bioeng.* **26**, 44 (2020)) except that xylose was substituted for glucose (1 gram xylose for 1 gram glucose) in all media formulations. Guide array stability was confirmed after transformation of pCASCADE plasmids by PCR prior to evaluation according to Li et al.²⁴ Fed batch fermentations were performed as previously reported, again with xylose instead of glucose (Menacho-Melgar, R. *et al.* Scalable, two-stage, autoinduction of recombinant protein expression in *E. coli* utilizing phosphate depletion. *Biotechnol. Bioeng.* **26**, 44 (2020)). Xylose feeding was as modified as follows. The starting batch glucose concentration was 25 g/L. Concentrated sterile filtered xylose feed (500 g/L) was added to the tanks at an initial rate of 10 g/h when cells entered mid-exponential growth. This rate was then increased exponentially, doubling every 1.083 hours (65 min) until 40 g total glucose had been added, after which the feed was maintained at 1.75g/hr. The feed was reduced to 0.875 g/hr due to xylose accumulation at 85 hrs post inoculation, and stopped at 120hrs post inoculation.

[00121] (XyxA) Xylose reductase Purification and Activity assays

[00122] *E. coli* BL21(DE3) (New England Biolabs, Ipswich, MA) with plasmid pHCKan-xyrA (bearing a 6x his tag) was cultured overnight in Luria Broth (Lenox formulation). The overnight culture was used to inoculate SM10++ media (with xylose as a carbon source instead of glucose) with appropriate antibiotics. Cells were cultured at 37°C for 16 hours, then cells were centrifuged, and the pellet was washed with SM10 No phosphate media. Next, the washed pellet was resuspended and cultured in SM10 No Phosphate media again with the appropriate antibiotics. After the expression, the postproduction cells were lysed by a freeze-thaw cycle.

XyrA protein was purified using Ni-NTA Resin (G-Biosciences, Cat # 786-939) according to manufacturer's instructions. Kinetics assays for XyrA were performed in a reaction buffer composed of 50 mM sodium phosphate (pH 7.6, 5mM MgCl₂) with NADPH as cofactor (Suzuki, T. *et al.* Expression of xyrA gene encoding for D-Xylose reductase of *Candida tropicalis* and production of xylitol in *Escherichia coli*. *J. Biosci. Bioeng.* **87**, 280–284 (1999)). In these assays, NADPH was held at a constant initial level of 50 μ M. Results of the assay were measured through monitoring the absorbance of NADPH at 340nm for 1.5 hours (15s per read) using a SpectraMax Plus 384 microplate reader (Molecular Devices). Reaction velocity is plotted as a function of xylose concentration. Using the Eadie-hofstee equation, we got the parameters: $V_{max}=22.6 \pm 1.01$ U, $k_{cat}=13.56 \pm 3.05$ s⁻¹ and $K_m: 35.12 \pm 3.05$ mM.

[00123] (XylA) Xylose isomerase quantification

[00124] Xylose isomerase activities from cell extracts were quantified with a D-xylose reductase coupled enzyme assay, similar to methods previously described, and following a decrease in absorbance of NADPH at 340nm (Guamán, L. P. *et al.* xylA and xylB overexpression as a successful strategy for improving xylose utilization and poly-3-hydroxybutyrate production in *Burkholderia sacchari*. *J. Ind. Microbiol. Biotechnol.* **45**, 165–173 (2018) and Lee, S.-M., Jellison, T. & Alper, H. S. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **78**, 5708–5716 (2012)). Cultures were grown in shake flasks in SM10++ media and harvested in mid exponential phase, washed and resuspended in SM 10 No phosphate media. After 16 hours of phosphate depletion, cells were pelleted by 10 minutes of centrifugation (4122 RCF, 4 degrees C) and lysed with BugBuster protein extraction reagent (Millipore Sigma, Catalog #70584) according to the manufacturer's protocol. Cell debris was removed by two rounds of centrifugation, 20 minutes (4122 RCF, 4 degrees C) followed by a 20 minute hard spin (14000 RCF, 4 degrees C). The lysate was filtered with Amicon 30MWCO filters (Millipore Sigma, Catalog #UFC8030) according to the manufacturer's protocol and washed three times to exchange the buffer with the reaction buffer (45mM sodium phosphate, 10mM MgCl₂, pH 7.6) and remove metabolites. Samples were assayed in triplicate in a 96 well plate with 100uL of the filtered cell extract per well containing 31.25mM xyulose, 0.5mM NADPH, and 1ug/mL of purified D-xylose reductase (see above). The absorbance at 340nm was measured every 15seconds for 1.5hours and the slope of the linear region was used to quantify XylA activity. Total protein concentration of each sample was determined with a standard Bradford assay. Kinetic parameters were as follows: $k_{cat}: 13.56 \pm 3.05$ s⁻¹, $K_m: 35.12 \pm 3.05$ mM.

[00125] (UdhA) Soluble transhydrogenase quantification

[00126] The activity of the soluble transhydrogenase was quantified by method previously reported (Chou, H.-H., Marx, C. J. & Sauer, U. Transhydrogenase promotes the robustness and evolvability of *E. coli* deficient in NADPH production. *PLoS Genet.* **11**, e1005007 (2015) and Sauer, U., Canonaco, F., Heri, S., Perrenoud, A. & Fischer, E. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. *J. Biol. Chem.* **279**, 6613–6619 (2004)). The process of UdhA expression and cell lysis was carried out using the same method as the XyrA expression mentioned above. The lysates were centrifuged for 15 minutes (4200 RPM, 4°C) to remove large debris. A second hard spin was performed for 30 minutes (14000 RPM, 4°C) to remove remaining debris and further separate the membrane fraction from the soluble transhydrogenase. Lysates were diluted 1:5 with the assay reaction buffer (50mM Tris-HCl, 2mM MgCl₂, pH 7.6) and transferred to an Amicon Ultra centrifugal filter (10kDa MWCO). The samples were centrifuged for 30 minutes (4200 RPM, 4°C) and this step was repeated 3 times to remove metabolites and exchange the lysis buffer for the assay buffer. After filtration the protein concentrations of the samples were quantified with a standard Bradford assay.

[00127] Then soluble transhydrogenase activity was assayed at room temperature. Assays were performed in black 96 well plates by mixing equal volumes of lysate and reaction buffer for a final volume of 100uL per well and a final concentration of 0.5mM NADPH and 1mM 3-acetylpyridine adenine dinucleotide (APAD⁺). Changes in absorbance at 400nm and 310nm due to the reduction of APAD⁺ and the oxidation of NADPH, respectively, were monitored simultaneously by Spectramax Plus 384 microplate reader at 30 second intervals for 30 minutes. A standard curve was used to calculate the molar absorptivity of NADPH ($3.04 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The molar absorptivity was used to convert the measured slope of the linear region to the change in concentration per minute. The specific activity (Units per mg of total protein) was determined by dividing the change in concentration per minute by the protein concentration.

[00128] FabI Quantification

[00129] Quantification of FabI via a C-terminal GFP tags was performed using a GFP quantification kit from AbCam (Cambridge, UK, Cat # ab171581) according to manufacturer's instructions.

[00130] Xylose and Xylitol Quantification

[00131] In micro-fermentations, xylose and xylitol were quantified by commercial bioassays from Megazyme (Wicklow, Ireland, Cat # K-XYLOSE and K-SORB), according to the manufacturer's instructions. An HPLC method coupled with a refractive index detector was used to quantify both xylose as well as xylitol from instrumented fermentations. Briefly, a Rezex ROA-Organic Acid H⁺ (8%) Analysis HPLC Column (Cat #: #00H-0138-K0, Phenomenex, Inc.,

Torrance, CA, 300 x 7.8 mm;) was employed for the separation of xylose and xylitol. 5 mM Sulfuric acid as the isocratic mobile phase at a flow rate of 0.5mL/min, at 55 °C,. A Waters Acquity H-Class UPLC integrated with a Waters 2414 Refractive Index (RI) detector (Waters Corp., Milford, MA. USA) was used for detection. The injection volume of samples and standards was 10 µL. Samples were diluted 20 fold in water in order to be in the linear range (0.01 to 20 g/L). MassLynx v4.1 software was used for all the peak integration and analyses.

[00132] NADPH Pool Quantification

[00133] NADPH pools were measured using an NADPH Assay Kit (AbCam, Cambridge, UK, Cat # ab186031) according to manufacturer's instructions. Cultures and phosphate depletion were performed as described above for XyrA expression (except there was no xyrA plasmid in the cell). Cells were lysed using the lysis buffer in the assay kit.

[00134] Metabolic Modeling

[00135] *In silico* analyses were performed implementing Constraint-based (COBRA) models for *E. coli*, developed employing the COBRApy Python package with a previously reported reconstruction as a starting point. This curated *E. coli* K-12 MG1655 reconstruction includes 2,719 metabolic reactions and 1,192 unique metabolites. This model was adapted as follows. First, missing reactions and metabolites for xylitol production and export were added as shown in Table S5:

[00136] **Table S5.** Xylitol Specific reactions added to the metabolic model

Name	Reaction	Identifier
Xylose reductase	$h_c + nadph_c + xyl_D_c \rightleftharpoons nadp_c + xylt_c$	XYLR
Xylitol exchange	$xylt_e \rightleftharpoons$	EX_xylt_e
Xylitol transport via passive diffusion	$xylt_e \rightleftharpoons xylt_c$	XYLTt
Flavodoxin reductase (NADPH)	$2.0 flxso_c + nadph_c \rightleftharpoons 2.0 flxr_c + h_c + nadp_c$	FLDR2

[00137] All reactions, metabolites stoichiometry and identifiers were extracted from the BiGG Models database. The resulting model was validated for mass balances and metabolite compartment formulas with COBRApy validation methods. Once properly balanced, a growth

model was created and analyzed. Specific evaluated conditions and biomass fluxes are shown in Table S6.

[00138] Table S6. Metabolic model validation with different carbon sources and oxygen levels. (All flux values are expressed in nmol/gDW*hr).

Only Carbon Source	C.S Flux	Oxygen Flux	Biomass Flux obtained from model	Previously reported Biomass Flux*
Glucose	-8.8	-30 (Aerobic)	0.771914	0.70± 0.01
Glucose	-8.8	0 (Anaerobic)	0.253285	0.33 ± 0.02
Xylose	-9.5	-30 (Aerobic)	0.645282	0.50 ± 0.02
Xylose	-9.5	0 (Anaerobic)	0.115445	0.13 ± 0.02

[00139] *Numbers from (Prasad, S., Singh, A. & Joshi, H. C. Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Resour. Conserv. Recycl.* **50**, 1–39 (2007)).

[00140] Next, experimental data obtained from the xylitol micro-fermentations was used to constrain the model. Specific constraints included: i) setting the ratio for pyruvate consumption through Pyruvate Dehydrogenase (PDH) and Pyruvate-flavodoxin Oxidoreductase (*ybdk*), with 10% and 90% of total flux respectively and ii) setting Ferredoxin/flavodoxin reductase to a reversible reaction and iii) using xylose as a sole carbon source with an input flux of 10 mmol/gCDW*hr under minimal media conditions. Finally a set of specific xylitol production strains were constructed and evaluated *in silico* using Flux Balance Analysis (FBA) to obtain xylitol yields, analyze cofactor and/or metabolites of interest as well as production and consumption fluxes. Specific cases that were analyzed included reduction or increased activity of: Zwf, FabI, GltA, XylA, PntAB and UdhA as shown in Table S7. For each case/condition the following data was obtained: Xylitol yield, NADPH producing and consuming g reaction fluxes and escher maps of central metabolism for flux distribution visualization. Finally, major changes in fluxes between the most relevant strains were analyzed.

[00141] Table S7. Strains modeled

Individual Valves		Combinations of Valves	
Strain	Valves Silencing	Strain	Valves Silencing

WT	--	Z-F	zwf & fabI
Z	zwf	Z-G	zwf & gltA
F	fabI	Z-X	zwf & xylA
G	gltA	Z-pAB	zwf & pntAB
X	xylA	Z-F-G	zwf, fabI & gltA
pAB	pntAB	Z-G-U	zwf, gltA & udhA
U	udhA	Z-F-pAB	zwf, fabI & pntAB

[00142] Example 1: Characterization of XyrA Xylose reductase

[00143] Referring now to FIG 2(A), Expression of XyrA in BL21 using media combination of SM10++(for growth) and SM10-No phos(for expression). After the expression, the postproduction cells were lysed by freeze-thawing cycle. Next, the xyrA protein was extracted by N-N Resin because of the His-tag on XyrA which was design into plasmid sequence. In FIG2(B), Activity of xyrA with NADPH as co-factor. Reaction velocity is plotted as function of xylose concentration. In these assays, NADPH was held at a constant initial level of 50 uM. FIG 2(C) Kinetic Parameters for XyrA from this project and from other research sources as comparison. Kinetics for XyrA were measured using 50 mM sodium phosphate, pH 7.6 (containing 5mM MgCl₂).²⁶ 50 uM NADPH. Results of the assay were measured through monitoring the absorbance of NADPH at 340nm. Using Eadie-Hofstee equation, the parameters V_{max}=22.6U, k_{cat}=13.5s⁻¹ and k_m=35.12mM were established thus confirming protein enzyme activity that could be used in the tank fermentation process. Knowing the V_{max}, the minimal expression level needed to hit a desired specific production rate can then be established.

Example 2: Design of metabolic valves for bioproduction of xylitol

[00144] Rationally designed strains to optimize xylitol production from xylose utilizing two stage dynamic metabolic control, in a phosphate depleted stationary phase were developed. As illustrated in FIG 1, this design included overexpression of xylose reductase and the dynamic reduction in xylose isomerase (*xyIA*) activity to reduce xylose metabolism which competes with xylitol production. Toward this goal we constructed strains and plasmids to enable the dynamic induction of *xyrA*, and dynamic reduction in XylA activity upon phosphate depletion, either through gene silencing, proteolysis of XylA or the combination. Refer to Tables 1 and 2 for plasmids and strains used. These strains were evaluated in 96 well plate micro-fermentations as reported by Moreb et al and results are given in FIG 3.

Example 3: Xylitol production utilizing 2-stage dynamic control

[00145] Since dynamic control over XylA (“X”) activity only led to modest improvements in xylitol production, FIG 3, we evaluated the potential impact of a larger set of valves on xylitol production. We constructed a set of strains with valves in key metabolic pathways, FIG 4. These valves included: citrate synthase (GltA-“G”), glucose-6-phosphate dehydrogenase (Zwf-“Z”), enoyl-ACP reductase (FabI-“F”) and soluble transhydrogenase (udhA-“U”) which control flux through the tricarboxylic acid cycle, pentose phosphate pathway, fatty acid biosynthesis and NADPH supply respectively. Strains were constructed with combinations of X, U, G, Z and F valves and evaluated for xylitol production. As described above, dynamic metabolic control was accomplished by adding C-terminal DAS+4 degron tags to the *xylA*, *udhA*, *zwf*, *gltA* and *fabI* genes as well as the overexpression of guide RNAs enabling silencing of their transcription. Refer to the Methods section for detailed chromosomal modification and plasmid construction.

[00146] The panel consisted of ~370 valve combinations of X, U, G, Z and F that were evaluated for xylitol production in two stage 96 well plate micro-fermentations in at least triplicate. Results of these experiments are given in FIG 5A and 5B. Xylitol titers ranged from ~0g/L-OD(600nm) to ~9.35g/L-OD(600nm). Approximately ~80% of the silencing and proteolysis combinations performed better than the control strain, which only produced 0.106 g/L-OD. Significant differences in specific xylitol production (xylitol (g/L) per unit OD_{600nm}) between valve strains and the control strain were determined by one-way ANOVA ($F(414,851)=7.598$, $p<0.0001$).

[00147] P-values were used to generate a p-value heatmap (FIG 6), where only combinations with a p value less than 0.05 are highlighted. Combinations not assayed or with less than 2 successful replicates (lack of success is due to lack of cell growth) are indicated by a gray dot since they are not qualified for statistical analysis. While the incorporation of X valves generally led to increase xylitol production, to surprisingly the two highest xylitol producers had neither X or U valves (which should increase NADPH levels) but rather combinations of F, G and Z valves. The highest producer had a combination of F and Z valves, which the xylitol specific productivity could reach 9.35g/L-OD_{600nm}. The performance of this genetic combination was also synergistic above either F or Z valves alone. This was surprising since these two enzymes have no direct or predictable impact of xylitol biosynthesis as can be seen in FIG 4.

[00148] Example 4: Xylitol Production in Instrumented Bioreactors

[00149] Based on the results from the micro-fermentations (FIGs 5 and 6), we chose the “Z-FZ” valve strain (silencing of *zwf* “Z” and proteolysis of *fabI* and *zwf* “FZ”) which has titer of 9.35 g/L-OD₆₀₀ as well as the control for evaluation in instrumented bioreactors. Fermentations

were performed according to Menacho-Melgar et al, where phosphate is limiting in the media leading to phosphate depletion and xylitol production in stationary phase as illustrated above in FIG 5. Results of these fermentations are given in FIG 6 below. The Z-FZ strain (FIG 6A) enabled xylitol production up to 104 $\pm$ 11.31 g/L with 160 hours, while *xylA* expression in our control strain DLF_0025-EV (FIG 6B) led to only ~3g/L of xylitol within the same time.

[00150] Example 5: Improvement of NADPH flux and xylitol biosynthesis

[00151] Most previous studies producing xylitol from xylose rely on a bioconversion requiring an additional sugar (usually glucose) as an electron donor (Albuquerque, T. L. de, da Silva, I. J., de Macedo, G. R. & Rocha, M. V. P. Biotechnological production of xylitol from lignocellulosic wastes: A review. *Process Biochem.* **49**, 1779–1789 (2014).; Cirino, P. C., Chin, J. W. & Ingram, L. O. Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. *Biotechnol. Bioeng.* **95**, 1167–1176 (2006); and Su, B., Wu, M., Zhang, Z., Lin, J. & Yang, L. Efficient production of xylitol from hemicellulosic hydrolysate using engineered *Escherichia coli*. *Metab. Eng.* **31**, 112–122 (2015)). Oxidation of glucose (producing the byproduct gluconic acid) generates NADPH which is then used for xylose reduction (Jin, L.-Q., Xu, W., Yang, B., Liu, Z.-Q. & Zheng, Y.-G. Efficient Biosynthesis of Xylitol from Xylose by Coexpression of Xylose Reductase and Glucose Dehydrogenase in *Escherichia coli*. *Appl. Biochem. Biotechnol.* **187**, 1143–1157 (2019). While these processes offer high xylitol titers and a good yield when considering xylose, the requirement for glucose at equimolar levels to xylose is a significant inefficiency. More broadly, improving NADPH availability or flux, useful in the synthesis of numerous metabolites as well as cell based bioconversions, has been a long standing challenge in metabolic engineering.

[00152] We applied two-stage dynamic metabolic control (DMC) to improve NADPH flux and xylitol production using xylose as a sole feedstock (Burg, J. M., Cooper, C. B., Ye, Z., Reed, B. R. & Moreb, E. A. Large-scale bioprocess competitiveness: the potential of dynamic metabolic control in two-stage fermentations. *Current opinion in* (2016)). Dynamic control over metabolism has become a popular approach in metabolic engineering, and has been used for the production of various products from 3-hydroxypropionic acid to myo-inositol and many others. We have recently reported an extension of dynamic metabolic control to two-stage bioprocesses, where products are made in a metabolically productive phosphate depleted stationary phase. The implementation of this approach relies on combined use of controlled proteolysis and gene silencing, using degron tags and CRISPR interference respectively. Importantly, in these initial studies we demonstrated that improved metabolic fluxes resulting from dynamic metabolic control, can be a consequence of reducing levels of central metabolites which are feedback regulators of other key metabolic pathways. Specifically, we have recently shown that

decreasing glucose-6-phosphate dehydrogenase levels activates the SoxRS regulon increasing expression and activity of pyruvate ferredoxin/ferredoxin oxidoreductase (Pfo). Pfo leads to improved acetyl-CoA production in stationary phase. (Refer to FIG 11(A)). In this work we report the evaluation of combinations of synthetic metabolic valves on xylitol production from xylose. Firstly, increased Pfo activity not only leads to improved acetyl-CoA flux but also NADPH production. NADPH is produced from reduced flavodoxin/ferredoxin via the action of NADPH dependent flavodoxin/ferredoxin reductase (Fpr). We also identify a key regulatory mechanism controlling NADPH fluxes, namely the inhibition of the membrane bound transhydrogenase (PntAB) by fatty acid metabolites. By dynamically disrupting fatty acid biosynthesis, we alleviate inhibition of PntAB. This mechanism is synergistic with activating Pfo and greatly increases NADPH flux and xylitol production. We compare this “regulatory” approach with a more intuitive stoichiometric strategy where the levels of key enzymes competing with xylitol production are dynamically reduced. Importantly, improved NADPH fluxes are, in part, a consequence of reduced NADPH pools. Reduced NADPH pools drive changes in expression and activity that result in increased NADPH fluxes, presumably a regulatory mechanism which has evolved to restore set point NADPH levels. These results are a reminder that pools and flux are not equivalent and not necessarily correlated.

[00153] Stoichiometric Strategy

[00154] We initially rationally designed strains to optimize xylitol production from xylose utilizing two stage dynamic metabolic control, reliant on decreasing levels of key competitive pathways. As illustrated in FIG 1, this design included dynamic reduction in xylose isomerase (*xylA*) and soluble transhydrogenase (*udhA*) activities. These modifications were designed to reduce xylose metabolism which competes with xylitol production and increases NADPH supply. NADPH can be consumed by the soluble transhydrogenase. Toward this goal we constructed strains and plasmids to enable the dynamic reduction in XylA and UdhA levels upon phosphate depletion. Refer to Supplemental Table S1 for strains and plasmids used in this study. As described above and previously reported dynamic reduction in activity was accomplished by adding C-terminal DAS+4 degron tags to the chromosomal *xylA* and *udhA* genes as well as the overexpression of guide RNAs aimed at silencing their expression. The impact of these modifications on enzyme levels in two stage cultures is given in FIG 9A-B. In the case of XylA, proteolysis led to ~ 60% reduction in activity. To our surprise the silencing gRNA actually led to an increased XylA activity level. The mechanism behind this is currently unknown and requires further study. The combination of silencing and proteolysis resulted in no further reduction in activity when compared to proteolysis alone. In the case of UdhA, proteolysis resulted in a ~30%

reduction in activity, whereas silencing had no detectable impact on activity levels with or without proteolysis.

[00155] The combination of proteolysis and silencing for XylA or “X valves” and proteolysis alone in the case of UdhA, a “U Valve”, are evaluated for xylitol production. Specifically strains were engineered with these metabolic valves as well as for overexpression of a xylose reductase (*xyrA* from *A. niger*) and evaluated in two-stage minimal media microfermentations as reported by Moreb et al. Results are given in FIG 10A-C. Additionally, a confirmatory analysis of XyrA kinetics was performed, and results are given in Supplemental FIG 11. The combination of modifications resulted in a 16 fold increase in xylitol production compared to the control.

[00156] Regulatory Strategy

[00157] To investigate the impact of a regulatory strategy, we next sought to evaluate the potential impact of a larger set of valves on xylitol production as illustrated in FIG 12A. We constructed a set of strains with valves in citrate synthase (GltA), glucose-6-phosphate dehydrogenase (Zwf) and enoyl-ACP reductase (FabI) which control flux through the tricarboxylic acid cycle, pentose phosphate pathway and fatty acid biosynthesis, respectively. We have previously reported the construction of metabolic valves in GltA (“G Valves”), and Zwf (“Z Valves”) which comprised either proteolytic degradation (DAS+4 tags), gene silencing (either the *zwf* promoter or *gltAp2* promoter) or both. In the case of FabI, we constructed new strains and plasmids to evaluate two-stage dynamic control on FabI levels. Toward this goal, as similarly reported by Li et al., we appended a superfolder GFP to the C-terminus of the *fabI* allele to enable quantification of protein levels by an ELISA. Unfortunately and unexpectedly, when plasmids silencing *fabI* expression were evaluated, guide RNA protospacer loss was observed (FIG 13A-D) and as a result we could not reliably obtain results where *fabI* is silenced. FabI proteolysis led to a ~75 % reduction in FabI levels (FIG 14), and as a result proteolytic degradation alone will be referred to as an “F Valve”. Strains were constructed with combinations of “X”, “U”, “G”, “Z” and “F” valves and evaluated for xylitol production, again in minimal media microfermentations. Results are given in FIG 12A-D. To our surprise the highest xylitol producer had neither “X” or “U” valves but rather a combination of “F” and “Z” valves. Xylitol production in the “FZ” valve strain was synergistic above either “F” or “Z” valves alone (FIG 12B). This was surprising in that these two enzymes have no direct or predictable impact of xylitol biosynthesis as can be seen in FIG 12A. We have recently reported that the “Z” valve results in increased acetyl-CoA fluxes by leading to the activation of Pfo (encoded by the *ydbK* gene). With increased flux through Pfo we hypothesized that NADPH could be generated from reduced ferredoxin/ferredoxin through ferredoxin reductase (Fpr).

Using deletions in *ydbK* and *fpr*, as shown in FIG 15, we confirmed that this pathway, and specifically Fpr is indeed in part responsible for the elevated xylitol production and NADPH flux observed in our “FZ” valve strain. This is, to our knowledge, the first confirmation that Fpr is reversible *in vivo*. This reverse flux through Fpr may be dependent on low NADPH pools (as discussed below). The synergistic impact of the “F” valves was somewhat unanticipated. However, elevated NADPH fluxes due to dynamic control over FabI (enoyl-ACP reductase) can be attributed to reduced levels of fatty acid metabolites, specifically acyl-CoAs (and potentially their precursors acyl-ACPs). Fatty acyl-CoAs are competitive inhibitors of the membrane bound transhydrogenase encoded by the *pntAB* genes (FIG 12A). Palmitoyl-CoA, specifically, has a reported K_i of 1-5 μ M. Control over FabI levels and/or activity has been previously shown to reduce acyl-ACP pools and as a result alleviate feedback inhibition of acetyl-CoA carboxylase and malonyl-CoA synthesis. To our knowledge this is the first study demonstrating the importance of these metabolites in controlling NADPH fluxes. While previous reports demonstrate the inhibition of PntAB by acyl-CoAs (which in minimal media are derived from fatty acid biosynthesis) there remains a possibility that acyl-ACPs also act as inhibitors, although future work is needed to confirm this hypothesis.

[00158] We next evaluated several additional modifications on top of the “FZ” valves, with a potential to impact xylitol production. (FIG 15B-D). Specifically we evaluated the addition of “G” and “U” valves as well as overexpression of *pntAB*. Plasmid based overexpression of the *pntAB* genes (using a low phosphate inducible promoter led to a significant improvement in xylitol production (FIG 15B). In contrast, the addition of either the “G” or “U” valve to the “FZ” combination did not increase xylitol synthesis but rather led to a significant decrease in xylitol production (FIG 15C-D). This suggests that citrate synthase (GltA) activity, and flux through the TCA cycle, is required for optimal NADPH flux.

[00159] Using results from these experiments, we were able to estimate boundary conditions for several intracellular fluxes. For example from FIG 15B, we can estimate that flux through the Pfo/Fpr pathway accounts for at most ~ 55% of the NADPH/xylitol production. As a result we are able to build stoichiometric metabolic models, as illustrated in FIG 16A-B, comparing an optimal growth phase and xylitol production phase. Importantly, this model confirms that indeed TCA flux is critical for xylitol production (FIG 17A-B) and that a 4-fold increase in PntAB activity, in addition to flux through the Pfo/Fpr pathways is needed to explain increases in NADPH flux and xylitol production. The model predicts an overall maximal xylitol yield in this metabolic state of ~0.864g/g of xylose, in line with yields measured in fed batch fermentations as discussed below.

[00160] Production in Instrumented Bioreactors

[00161] Next, we compared xylitol production in instrumented bioreactors using the “FZ” valve strain with and without *pntAB* overexpression with a control strain. Minimal media fed batch fermentations were performed as described by Menacho-Melgar et al., wherein the media has enough batch phosphate to support target biomass levels ($\sim 25\text{gCDW/L}$) prior to phosphate depletion and induction of xylitol biosynthesis in stationary phase. Results of these studies are given in FIG 18. While *xylA* expression in our control strain (DLF_Z0025) led to only a few grams per liter of xylitol (FIG 18A), the incorporation of “FZ” valves led to titers over 100g/L in 160 hours of production (FIG 18B). The additional overexpression of *pntAB* (FIG 18C) resulted in maximal titers over 200g/L (185-204g/L) in 170 hrs. In these duplicate fermentations the average overall xylitol yield was 0.873 ± 0.026 g/g xylose, and the average production yield (in stationary phase) was 0.935 ± 0.011 g/g xylose.

[00162] *Improved NADPH Flux is not Correlated with NADPH pools.*

[00163] Lastly, we measured the levels of NADPH in a set of our engineered strains. Results are given in FIG 19A. Interestingly, there was no correlation between specific xylitol production and NADPH pools. In this case, the three strains having the highest NADPH pools were the control strain and the strains with dynamic control over enoyl-ACP levels (“F” valve) or soluble transhydrogenase (“U” valve) levels. The addition of the “Z” valve (reduced levels of glucose-6-phosphate dehydrogenase) led to a decrease in NADPH pools but an increase in NADPH flux. Deletions of either *ydbK* and or *fpr*, also led to decreases in NADPH levels, and while overexpression of *pntAB* increased xylitol production rates and fluxes it did not improve NADPH pools in the “FZ” background.

[00164] The use of 2-stage dynamic control generated an usual metabolic state leading to enhanced NADPH fluxes and xylitol production. To our knowledge this is the highest titer and yield of xylitol produced to date in engineered *E. coli*, particularly with xylose as a sole carbon source. Additionally, the productive stationary phase generated with these modifications can be extended to at least 170 hours. While the focus of this work has been on xylitol production, the identification of “F” and “Z” valves impacting NADPH flux has applicability to other NADPH dependent processes including more complicated pathways, and may represent a facile method for routine NADPH dependent bioconversions. The impact of FabI activity and fatty acid metabolite pools, on transhydrogenase activity, is consistent with previous biochemical studies, and has likely evolved to balance NADPH supply with fatty acid synthesis demand. Unfortunately, this feedback regulatory mechanism has been lost in the past several decades of metabolic engineering studies in *E. coli*, yet represents a powerful approach to improving NADPH fluxes. The unpredictable combination of “F” and “Z” valves is at odds with standard thinking regarding NADPH flux, where Zwf is often considered one of the primary sources of

NADPH in the cell and reducing Zwf activity would not be high on a list of changes to make in order to increase NADPH supply.

[00165] In order to explain the lack of correlation between NADPH pools and our results, we developed a conceptual model as illustrated in FIG 1. The “Z” valve leads to a decrease in NADPH pools which activate the SoxRS regulon, which is sensitive to oxidant and NADPH levels. SoxRS activation leads to increased expression of Pfo, which is required to maintain a high rate of pyruvate oxidation, generating NADPH via Fpr. Uniquely, this study identifies a previously unreported pathway for NADPH production utilizing Pfo and Fpr and supports that Fpr catalyzes a reversible reaction *in vivo*. Pfo expression is required, not only for pyruvate oxidation and sugar consumption but also NADH generation via the TCA cycle. Increased TCA flux produces excess NADH which is needed as a substrate for PntAB for maximal NADPH flux. Disruption of the TCA cycle (“G” Valve, FIG 15B) eliminates NADH production and acetyl-CoA consumption, greatly reducing NADPH flux. Increased NADPH levels due to the “F” valve make sense in light of the results discussed and are attributable to increased activity of the membrane bound transhydrogenase, PntAB. Reduced soluble transhydrogenase (UdhA, FIG 15D) levels leads to increased NADPH pools (FIG 19) which presumably reduce SoxRS activation and Pfo expression. Simply put, the metabolic network responds to decreased NADPH and acyl-CoA pools by increasing sugar consumption and NADPH flux to compensate. If “set” point NADPH pools are regained or if continued sugar catabolism stops, continued NADPH flux is halted.

[00166] Lastly, the metabolic state leading to enhanced NADPH flux and xylitol production would be hard to identify and/or engineer in a growth coupled process as it relies on the manipulation of feedback inhibition due to central metabolites. These central metabolic regulatory circuits have evolved to balance fluxes to both optimize growth and enable adaptive responses to environmental and physiological perturbations. Dynamic metabolic control, and in particular two-stage dynamic metabolic control, is uniquely suited to manipulate central metabolite levels without impacting cell growth or survival. This approach can lead to the discovery as well as the manipulation of central regulatory mechanisms, which in turn have a high potential to enhance metabolic fluxes and drive future metabolic engineering strategies.

CLAIMS

1. A genetically modified microorganism for producing xylitol from xylose comprising:
inducible modification of expression of xylose reductase and
an inducible synthetic metabolic valve comprising:
a gene expression-silencing synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes; or
an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes, or
a combination thereof.
2. The genetically modified microorganism of claim 1, wherein the xylose reductase is an NADPH dependent xylose reductase.
3. The genetically modified microorganism of claim 1, wherein the xylose reductase is the *xyrA* gene of *A. niger*.
4. The genetically modified microorganism of claim 1, wherein the genetically modified microorganism produces xylitol from a xylose feedstock.
5. The genetically modified microorganism of claim 1, wherein of gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve are directed to control of the gene encoding xylose isomerase or the xylose isomerase enzyme.
6. The genetically modified microorganism of claim 1, wherein of gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve are directed to control of the gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme.
7. The genetically modified microorganism of claim 1, wherein of gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve are directed to control of at least two genes, including
a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and

a gene encoding xylose isomerase or the xylose isomerase enzyme.

8. The genetically modified microorganism of claim 1, wherein of gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve are directed to control of at least two genes, including

a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and

a gene encoding enoyl-ACP reductase (*fabI*) or the enoyl-ACP reductase (*fabI*) enzyme.

9. The genetically modified microorganism of claim 1, wherein of gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve consist of

silencing of a gene encoding glucose-6-phosphate dehydrogenase (*zwf*)

and enzyme degradation of glucose-6-phosphate dehydrogenase (*zwf*) enzyme; and

enoyl-ACP reductase (*fabI*) enzyme.

10. The genetically modified microorganism of claim 1, wherein expression of xylose reductase, gene expression-silencing synthetic metabolic valve, and the enzymatic degradation synthetic metabolic valve are induced under conditions of a transition phase of a multi-stage biofermentation process.

11. The genetically modified microorganism of claim 1, wherein the induction occurs via nutrient depletion.

12. The genetically modified microorganism of claim 1, wherein the induction occurs via phosphate depletion.

13. The genetically modified microorganism of claim 1, further comprising a chromosomal deletion.

14. The genetically modified microorganism of claim 1, wherein the silencing of gene expression comprises CRISPR interference and the genetically modified microorganism also expresses a CASCADE guide array, the array comprising two or more genes encoding small guide RNAs each specific for targeting a different gene for simultaneous silencing of multiple genes.

15. The genetically modified microorganism of claim 1, wherein the microorganism produces a xylitol product titer of greater than 20 g/L at about twenty four hours in a biofermentation process.

16. A multi-stage fermentation bioprocess for producing xylitol from a genetically modified microorganism, comprising:

(a) providing a genetically modified microorganism comprising:

a modification of expression of xylose reductase and

a synthetic metabolic valve comprising:

a gene expression-silencing synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes; or

an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes, or

a combination thereof.

wherein the one or more enzymes of each synthetic metabolic valve are the same or different; and

(b) growing the genetically modified microorganism in a media with a xylose feedstock;

(c) transitioning from a growth phase to a xylitol producing stage by

inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism; and

inducing expression of xylose reductase, thereby

(d) producing xylitol.

17. The multi-stage fermentation bioprocess of claim 16, wherein the gene-silencing synthetic metabolic valve or the enzyme degradation synthetic metabolic valve of the genetically modified microorganism are directed to control of at least two genes, selected from the group consisting of:

a gene encoding glucose-6-phosphate dehydrogenase (*zwf*) or the glucose-6-phosphate dehydrogenase (*zwf*) enzyme;

a gene encoding enoyl-ACP reductase (*fabI*) or the enoyl-ACP reductase (*fabI*) enzyme; and

a gene encoding xylose isomerase or the xylose isomerase enzyme.

18. The multi-stage fermentation bioprocess of claim 16, wherein the bioprocess

produces a xylitol product titer of greater than 20 g/L at about twenty four hours in a biofermentation process.

19. The multi-stage fermentation bioprocess of claim 16, wherein the inducing transition phrase occurs via phosphate depletion of the growth media.

20. The multi-stage fermentation bioprocess of claim 16, the genetically modified microorganism further comprising a chromosomal deletion.

21. A genetically modified microorganism for producing xylitol, the microorganism comprising:

inducible reduction of xylose isomerase;
inducible reduction of glucose-6-phosphate dehydrogenase activity;
wherein the strain produces xylitol from the feedstock xylose upon induction.

22. The genetically modified microorganism of claim 21, wherein the microorganism is an *E.coli* microorganism.

23. The genetically modified microorganism of claim 21, wherein induction occurs by via nutrient depletion.

24. The genetically modified microorganism of claim 21, wherein induction occurs via phosphate depletion.

25. A multi-stage fermentation bioprocess for producing xylitol from the genetically modified microorganism of claim 21, comprising:

- (a) providing a genetically modified microorganism
- (b) growing the genetically modified microorganism in a media with a xylose feedstock;
- (c) transitioning from a growth phase to a xylitol producing stage by
 - inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism; and
 - inducing expression of xylose isomerase, thereby
- (d) producing xylitol.

26. A genetically modified microorganism for producing xylitol, the microorganism comprising:

inducible reduction of xylose reductase;
inducible reduction of glucose-6-phosphate dehydrogenase activity;
inducible reduction of enoyl-ACP reductase;
wherein the strain produces xylitol from the feedstock xylose upon induction.

27. The genetically modified microorganism of claim 26, wherein the microorganism is an *E.coli* microorganism.

28. The genetically modified microorganism of claim 26, wherein induction occurs by via nutrient depletion.

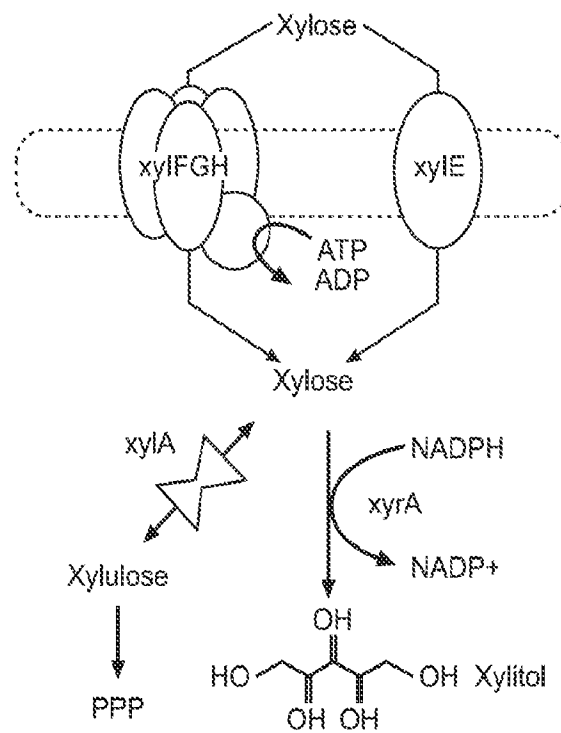
29. The genetically modified microorganism of claim 26, wherein induction occurs via phosphate depletion.

30. A multi-stage fermentation bioprocess for producing xylitol from the genetically modified microorganism of claim 26, comprising:

- (a) providing a genetically modified microorganism
- (b) growing the genetically modified microorganism in a media with a xylose feedstock;
- (c) transitioning from a growth phase to a xylitol producing stage by
 - inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism; and
 - inducing expression of xylose reductase, thereby
- (d) producing xylitol.

31. A genetically modified microorganism, in which:
activity of a membrane bound transhydrogenase activity is increased;
activity of a pyruvate ferredoxin oxidoreductase is increased;
activity of a NADPH dependent ferredoxin reductase is increased; and
wherein the microorganism produces at least one chemical product whose biosynthesis requires NADPH.

1/19

**FIG. 1**

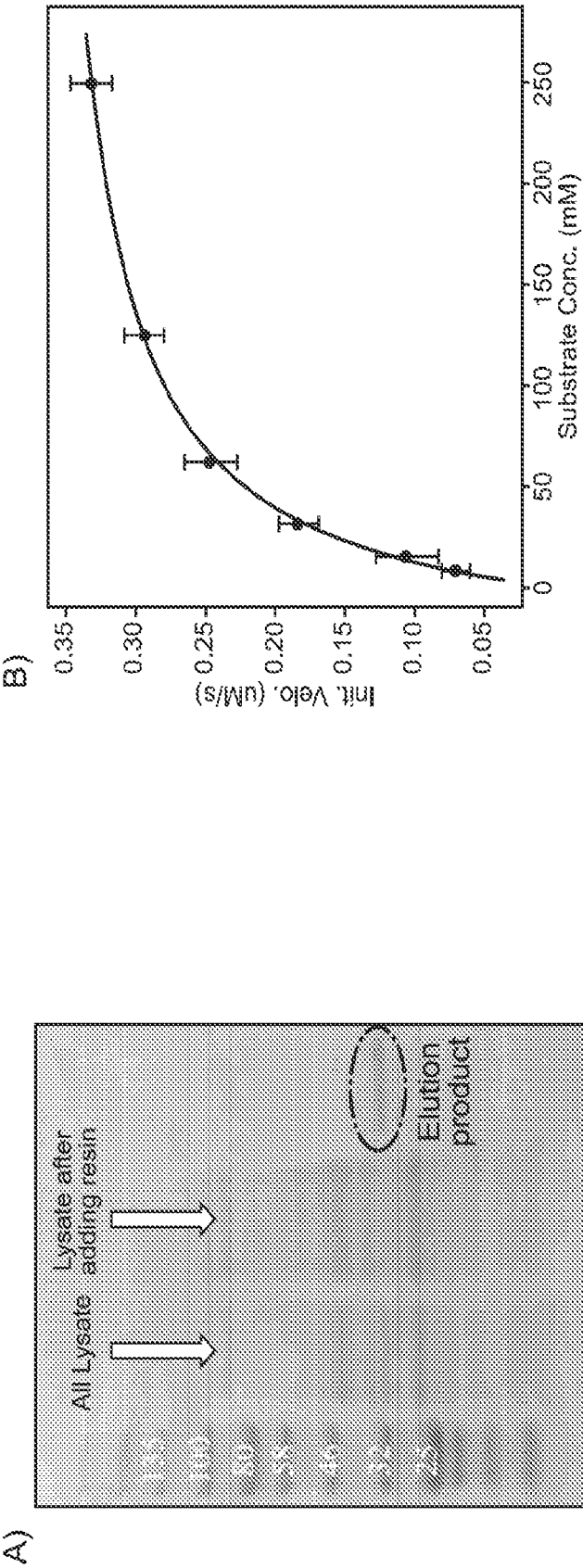


FIG. 2B

FIG. 2A

C)

	Vmax(U)	Kcat(s ⁻¹)	Km(uM)	Source
1	75		39	1
2		13	96	2
3	23.2		42	3
4	22.6	13.5	35	This Study

FIG. 2C

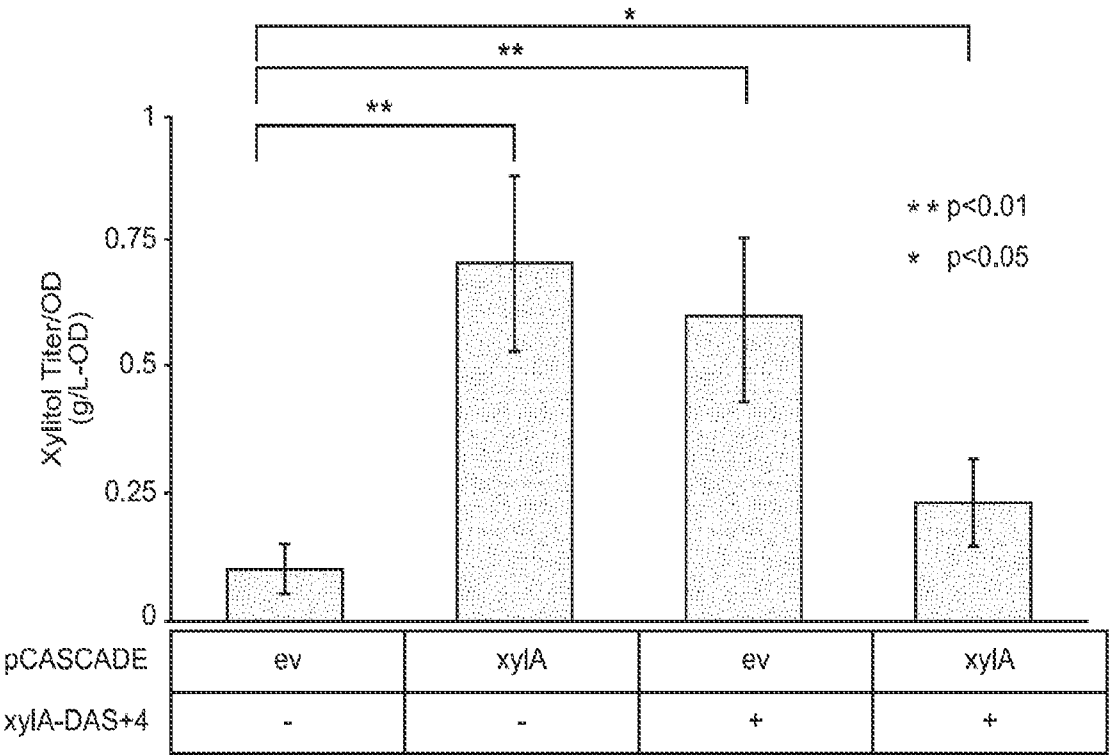


FIG. 3

4/19

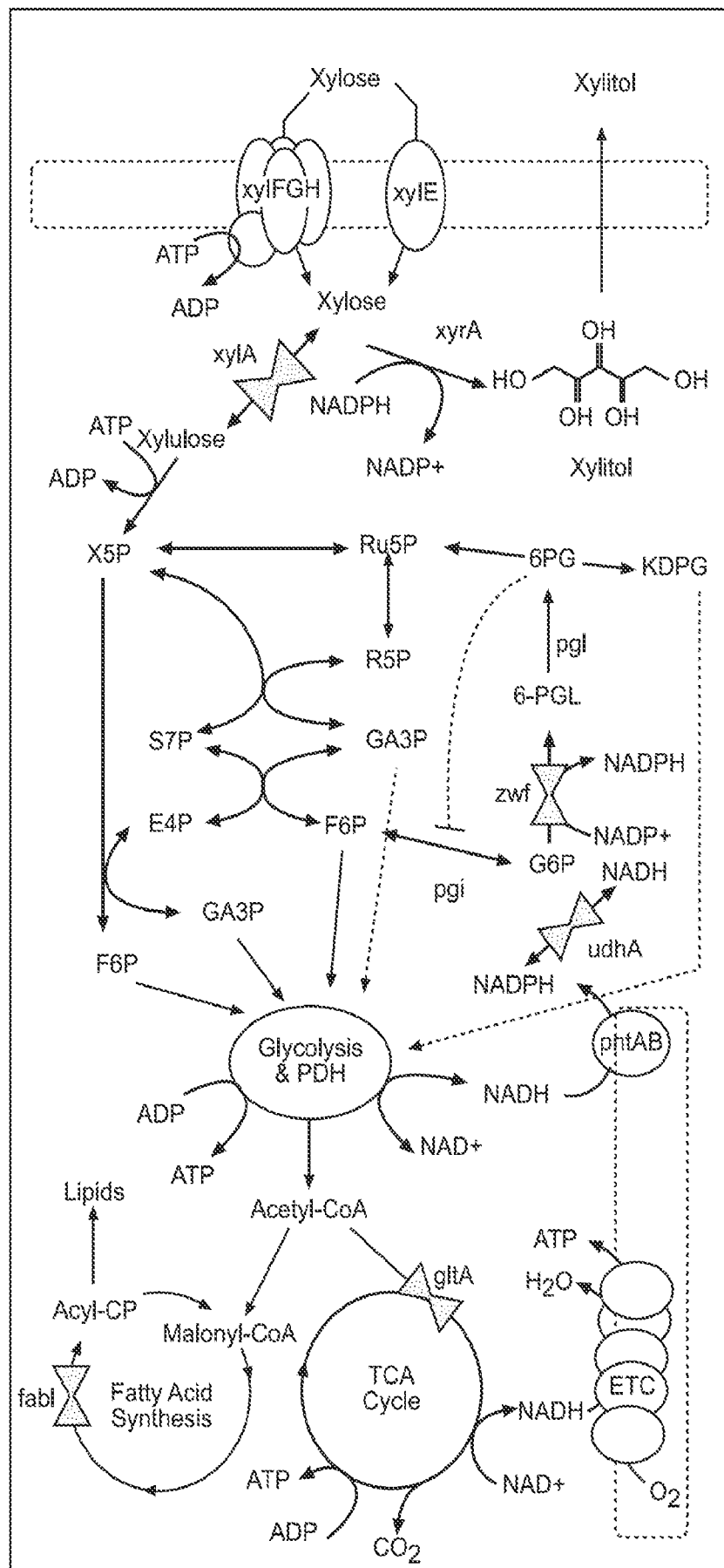
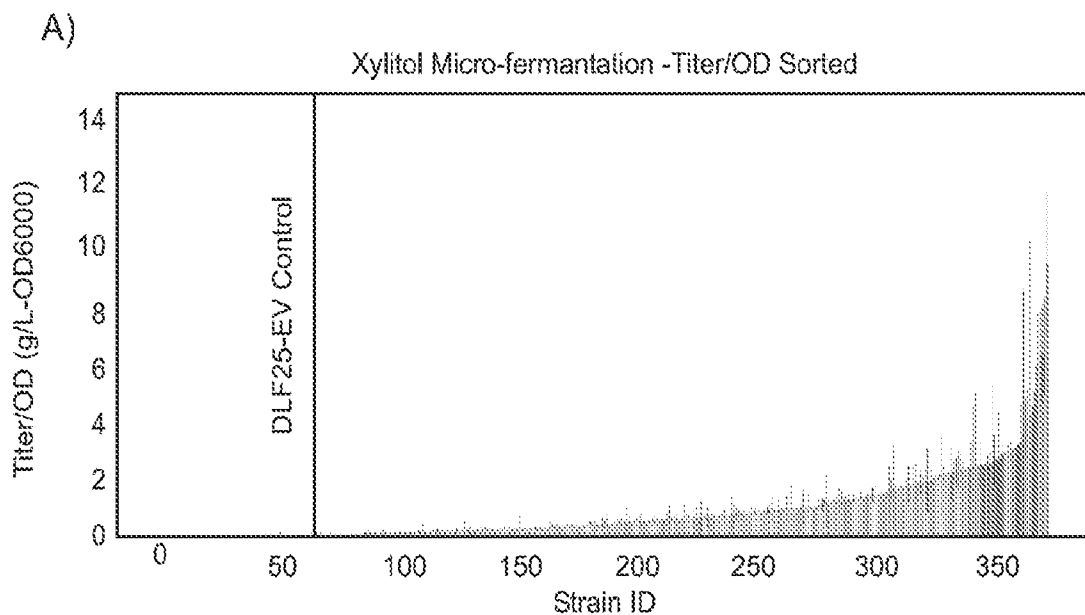
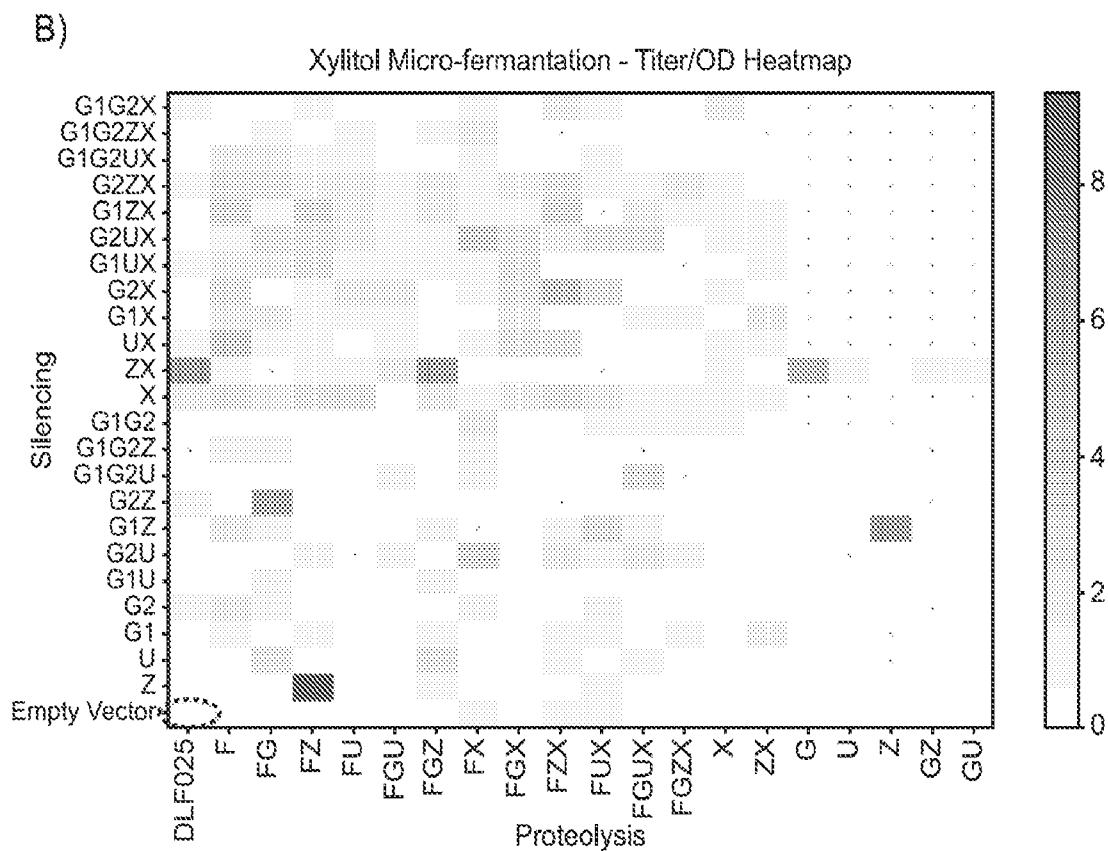


FIG. 4

SUBSTITUTE SHEET (RULE 26)

5/19

**FIG. 5A****FIG. 5B**

6/19

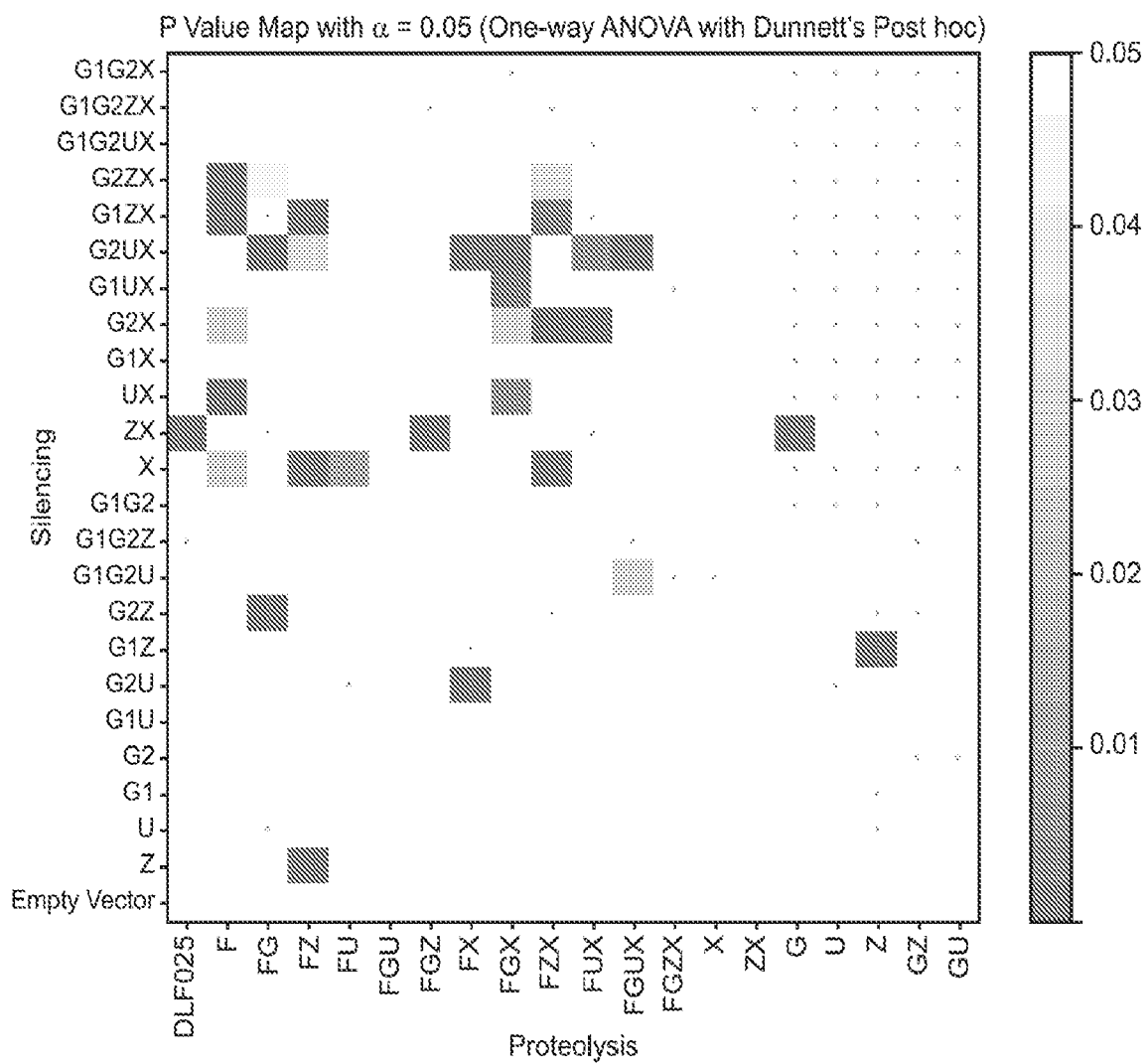


FIG. 6

7/19

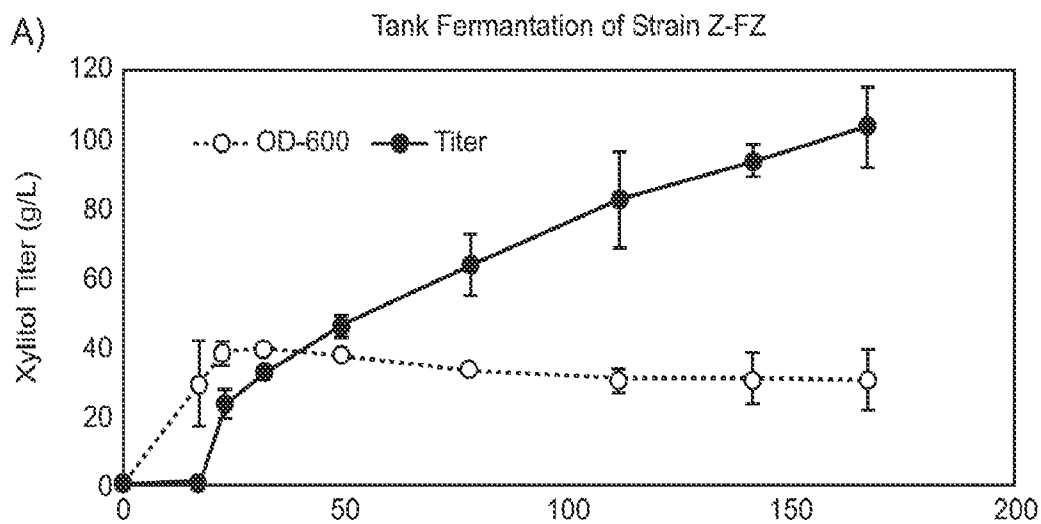


FIG. 7A

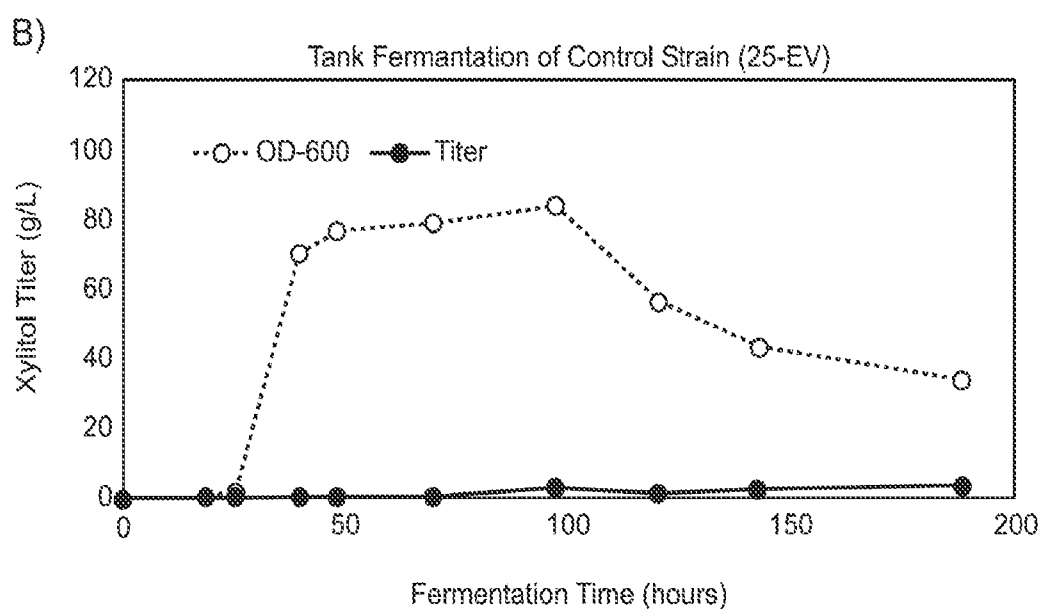


FIG. 7B

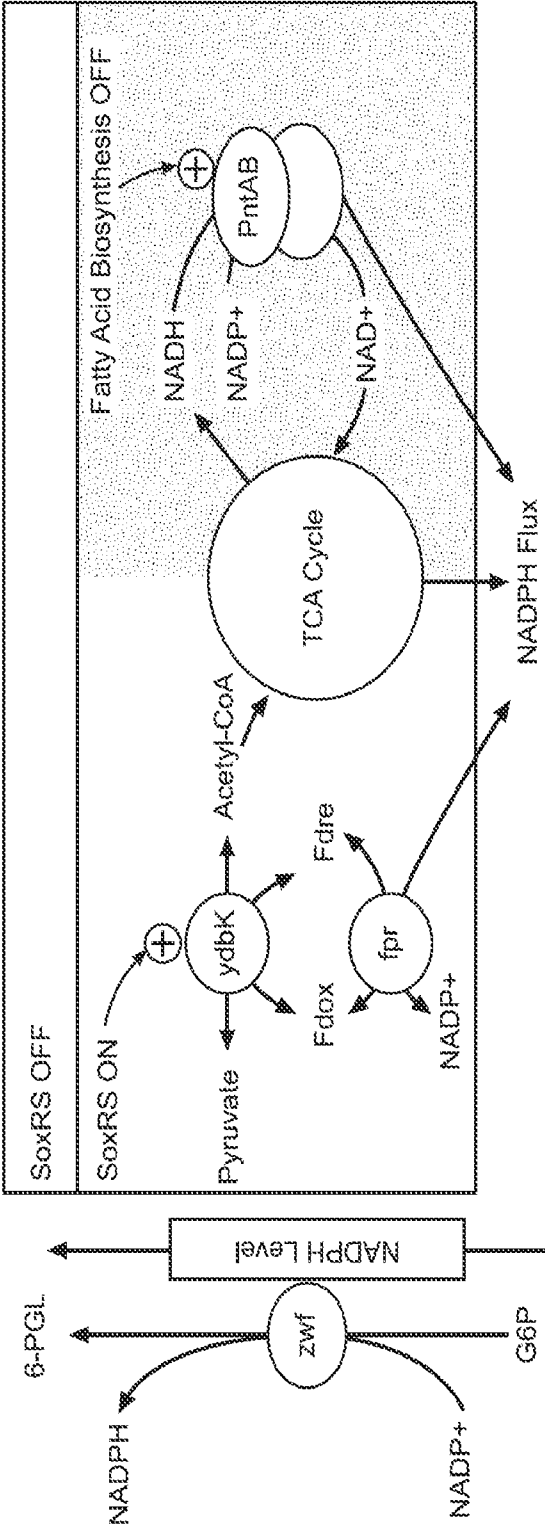


FIG. 8

9/19

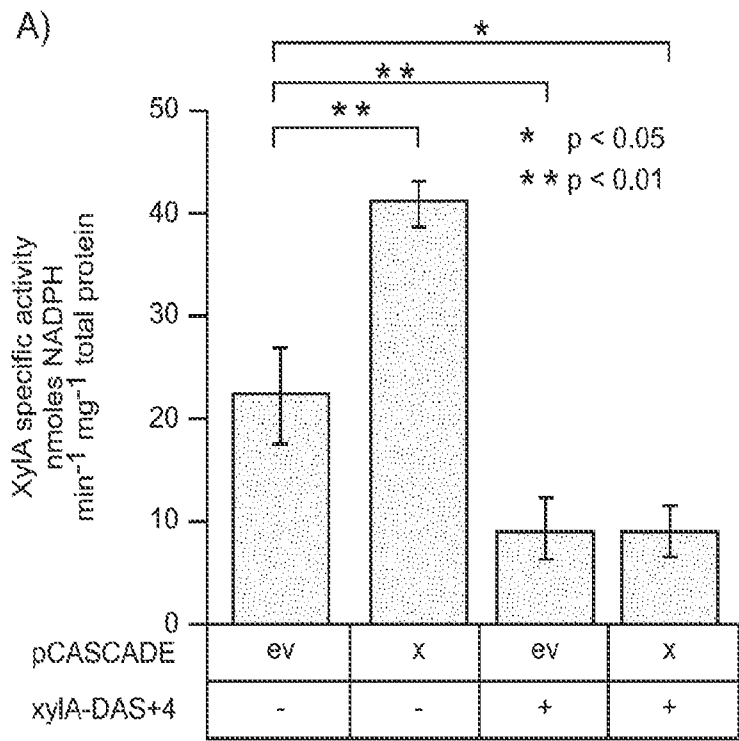


FIG. 9A

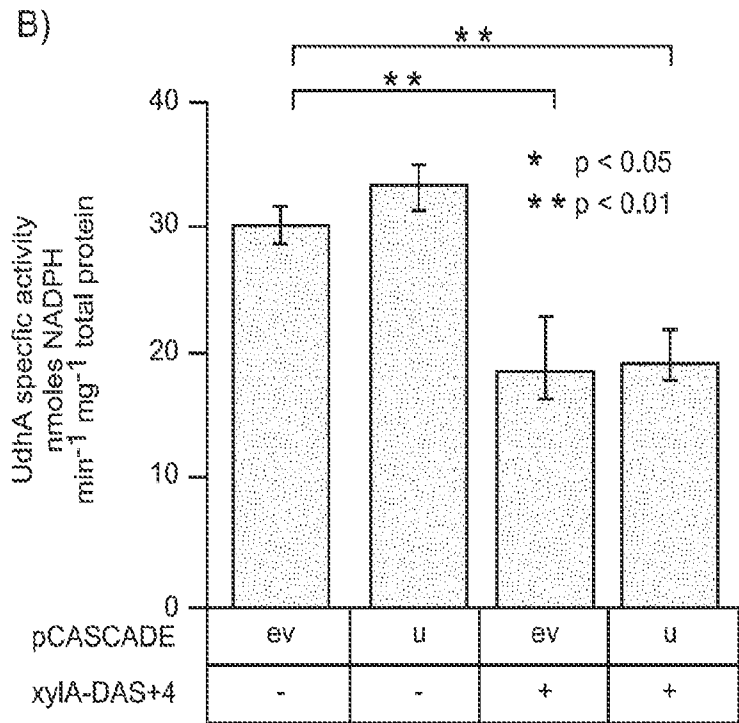


FIG. 9B

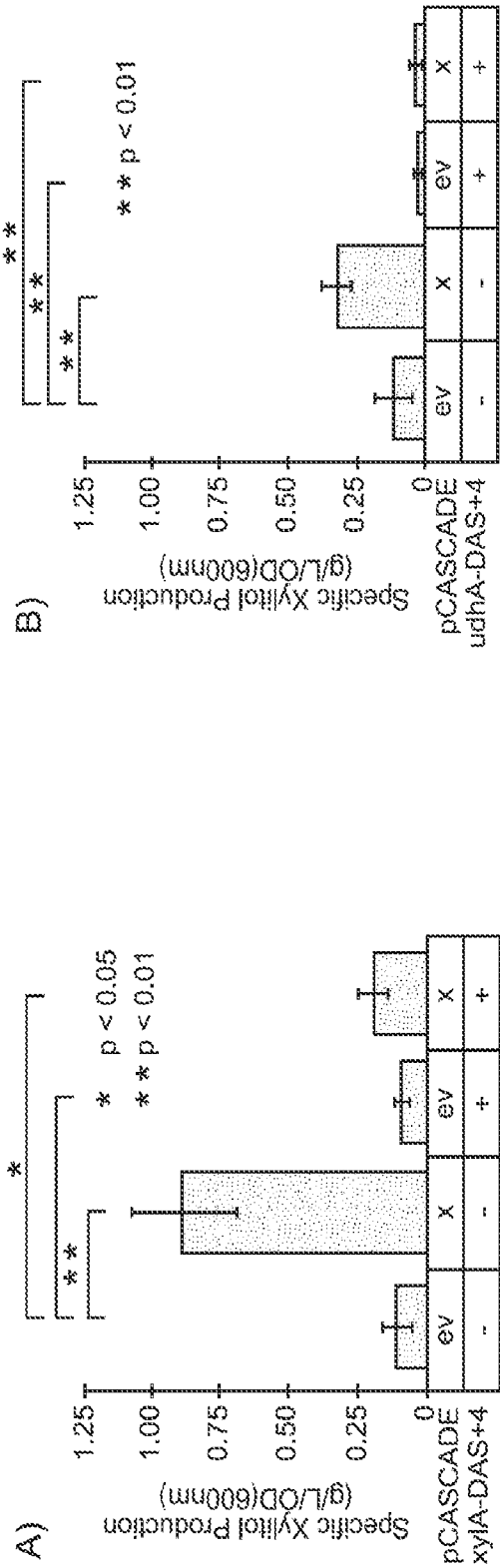


FIG. 10A

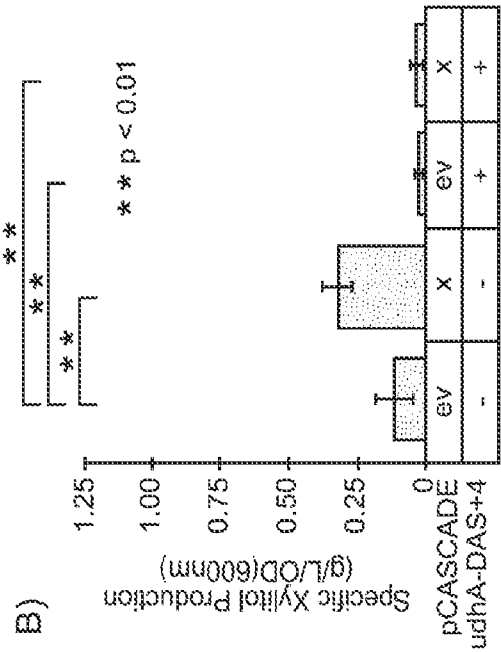


FIG. 10B

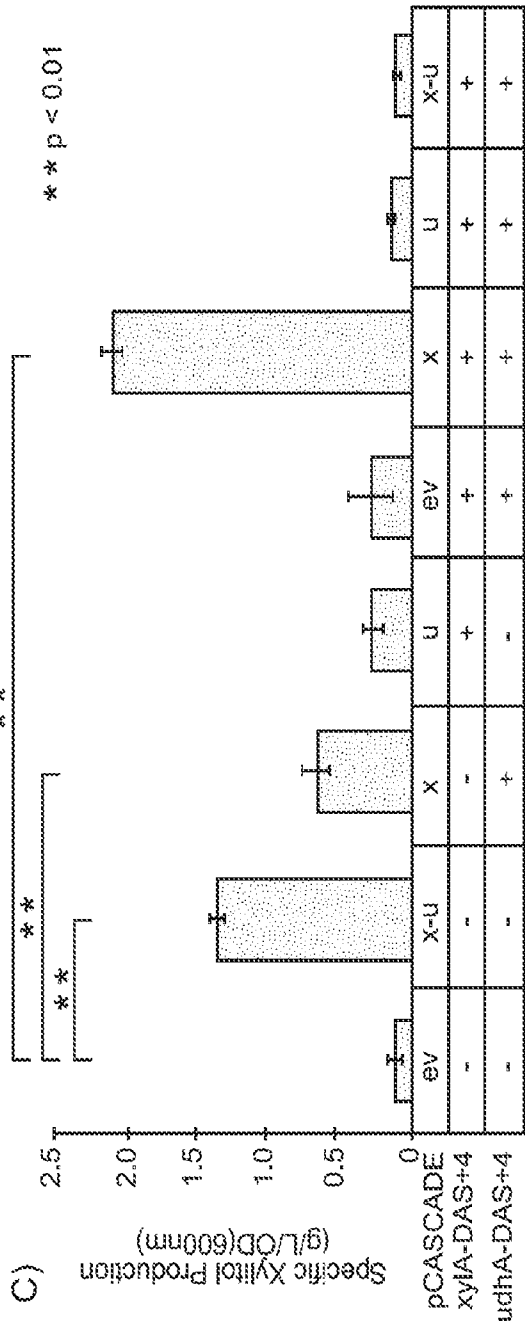


FIG. 10C

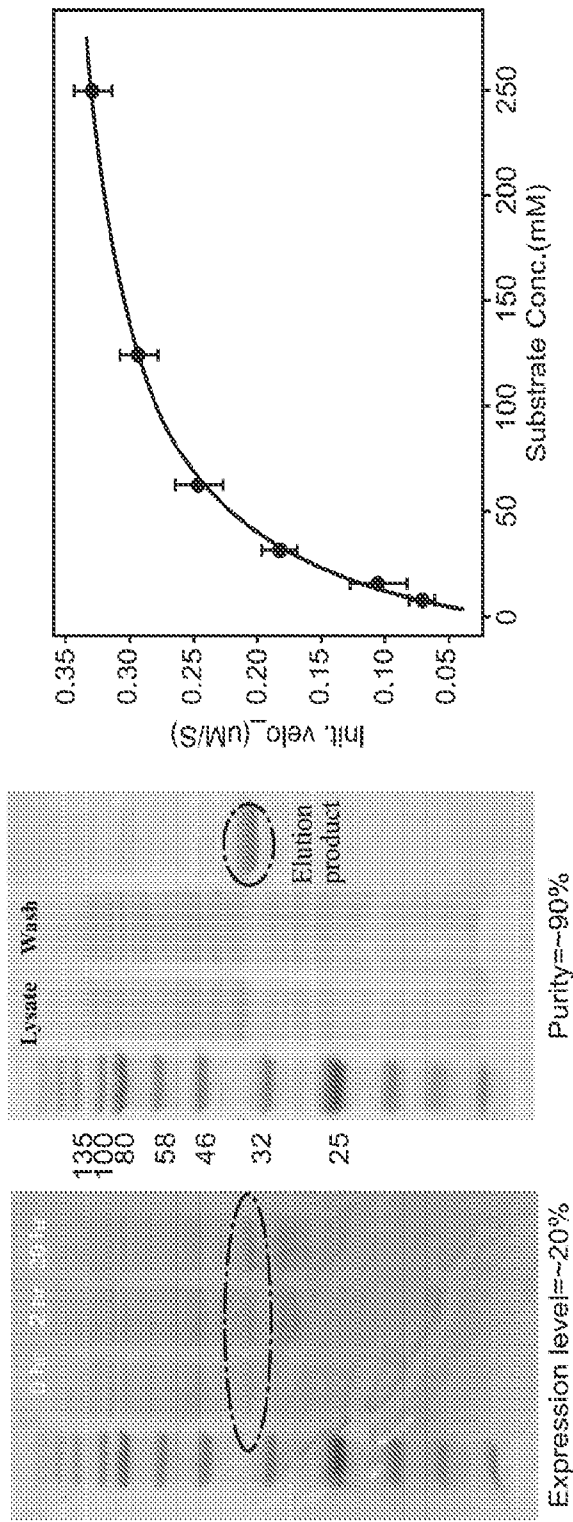
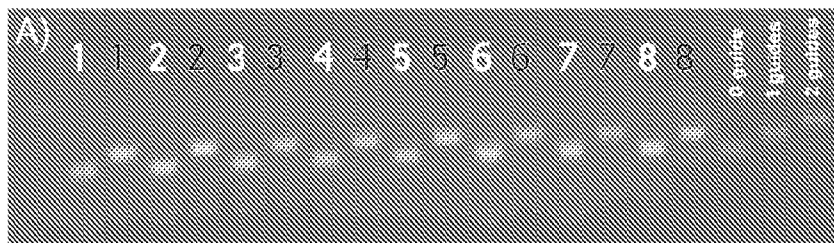
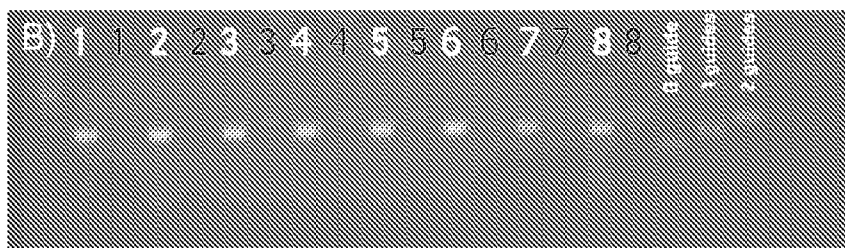
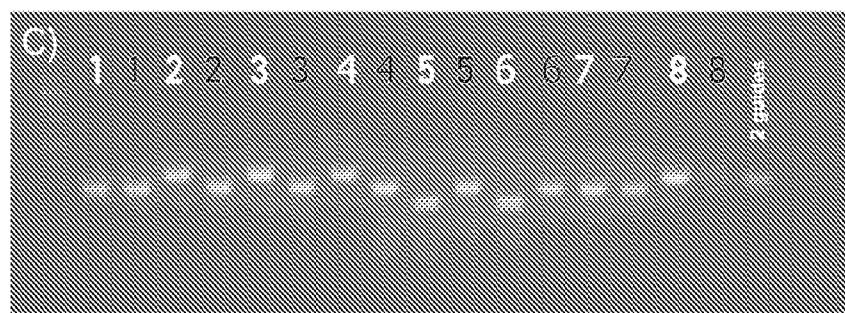
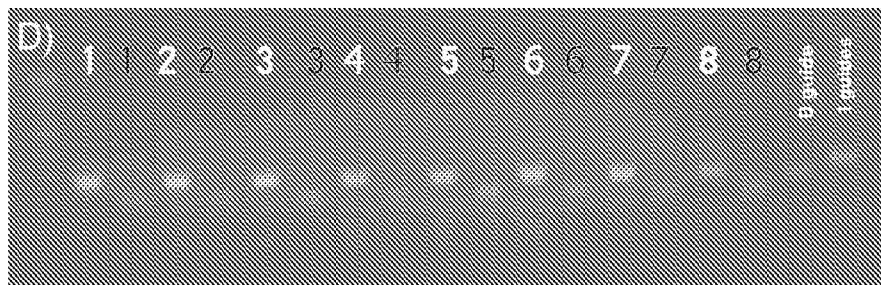
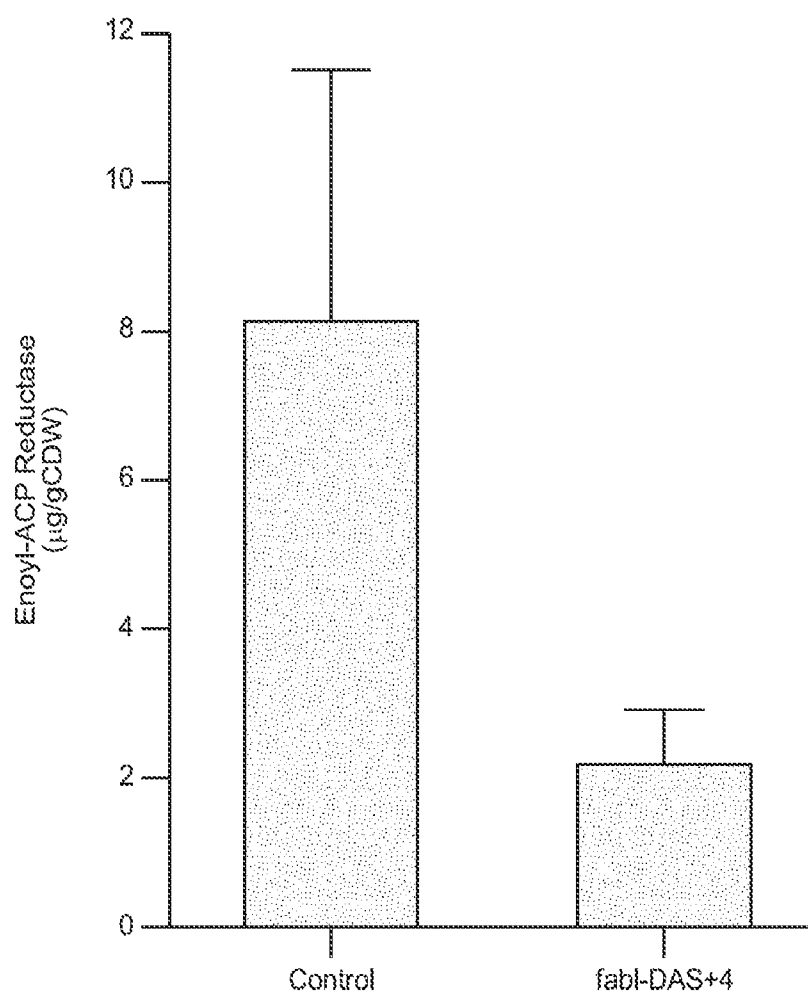


FIG. 11

**FIG. 13A****FIG. 13B****FIG. 13C****FIG. 13D**

14/19

**FIG. 14**

15/19

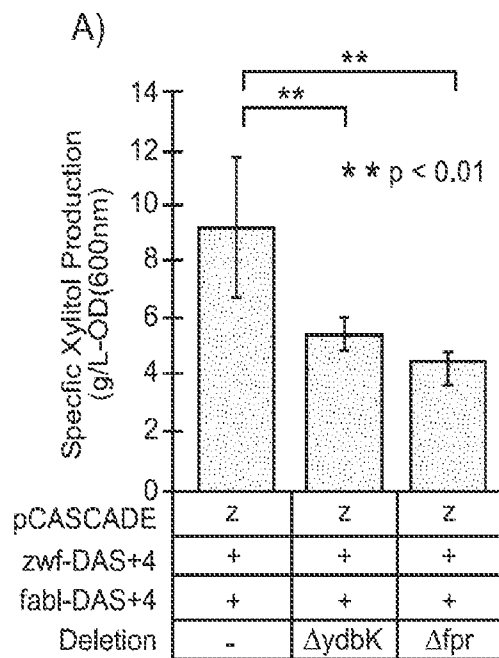


FIG. 15A

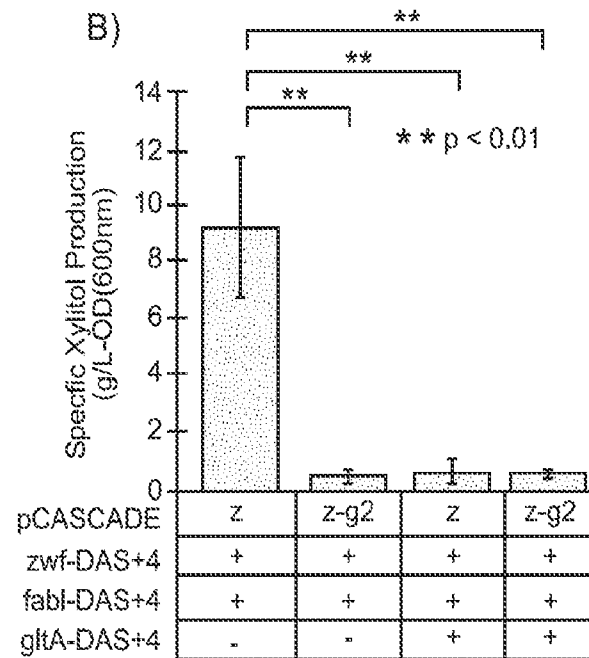


FIG. 15B

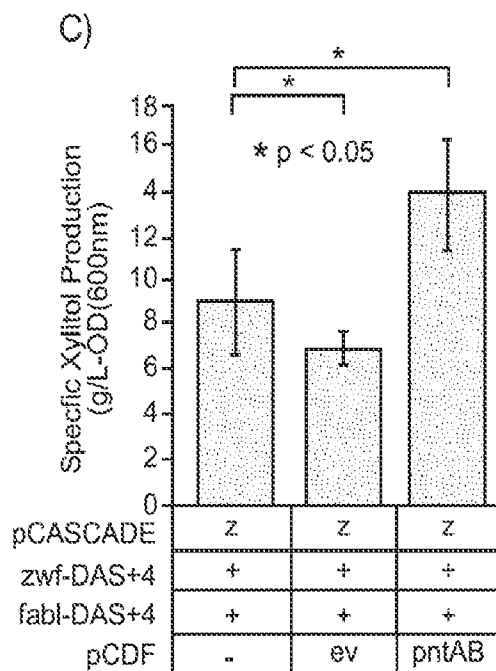


FIG. 15C

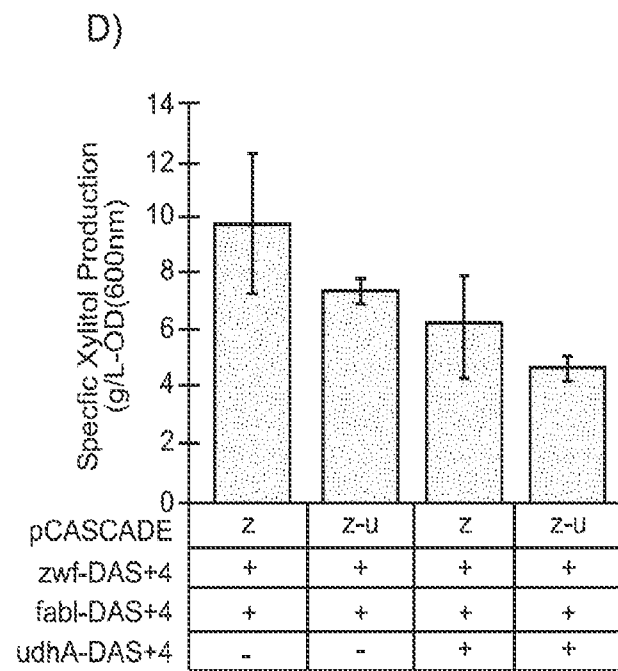


FIG. 15D

17/19

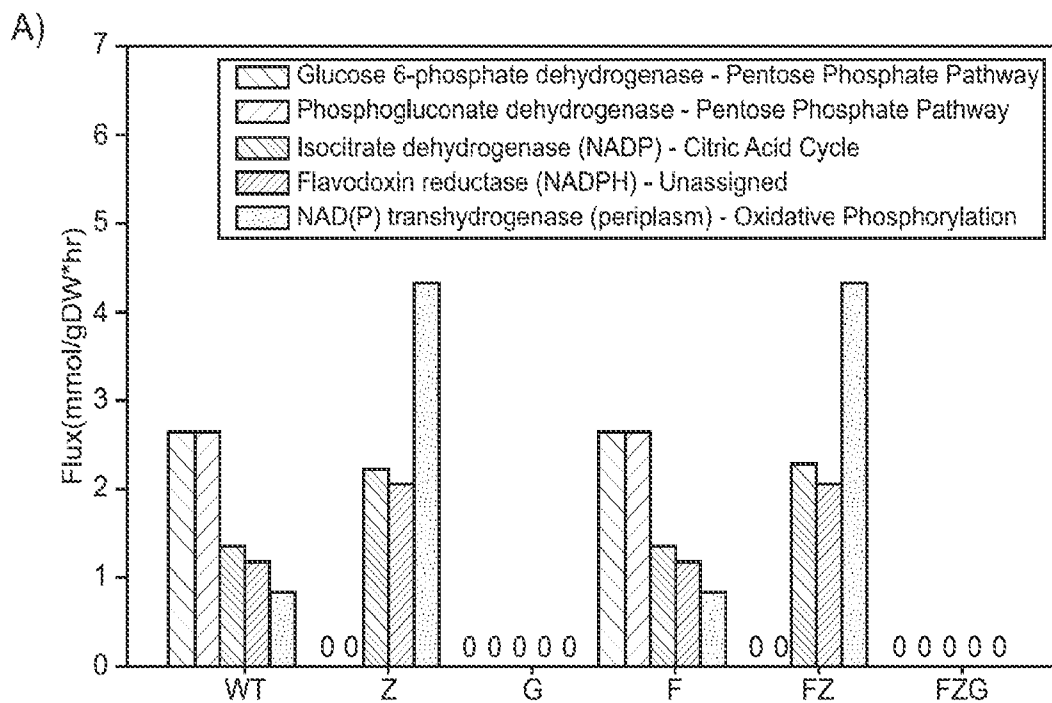


FIG. 17A

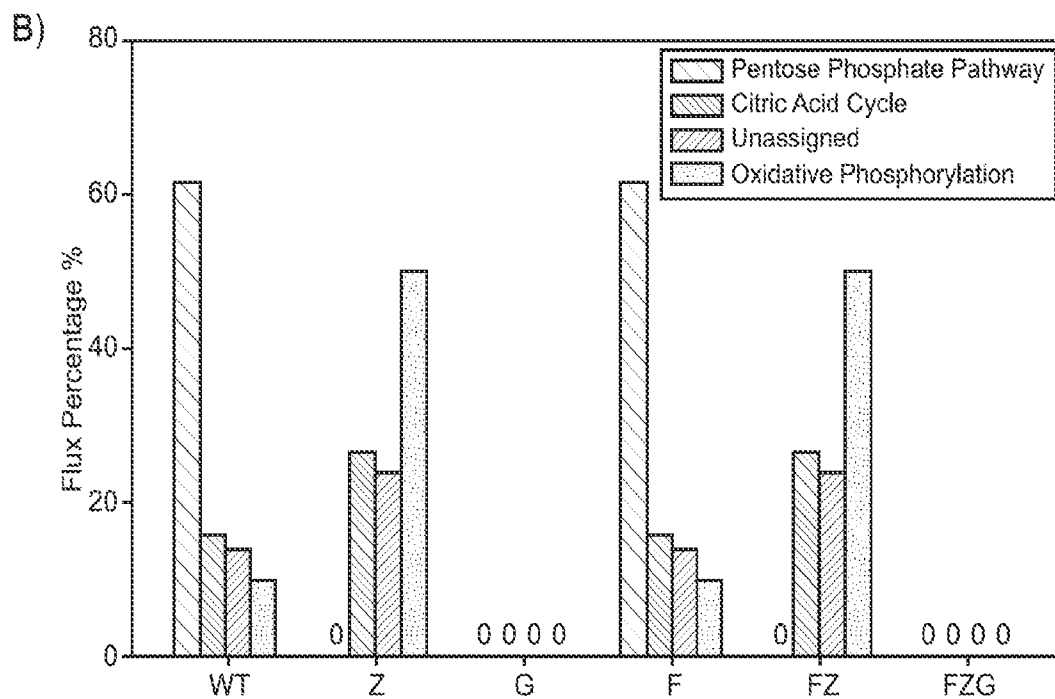
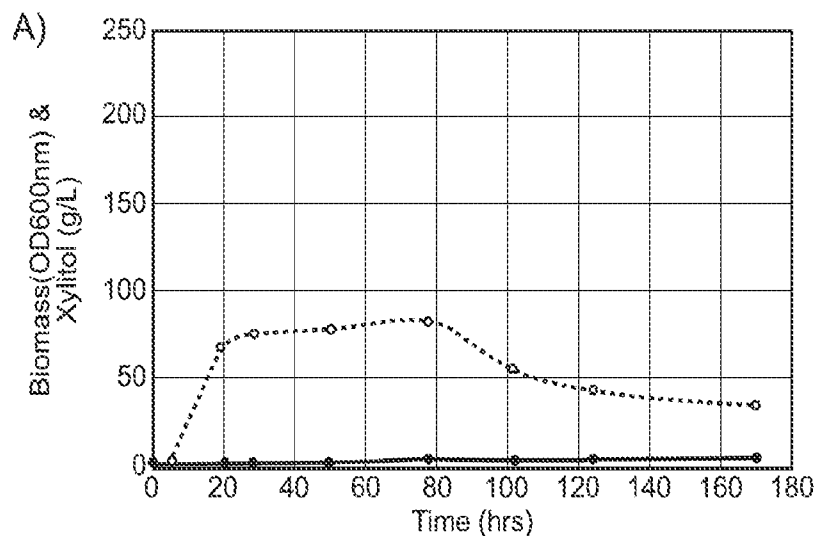
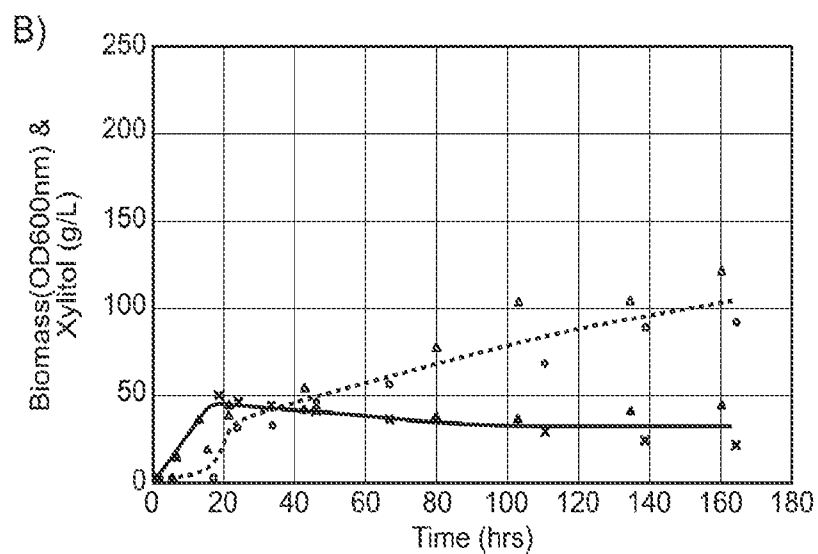
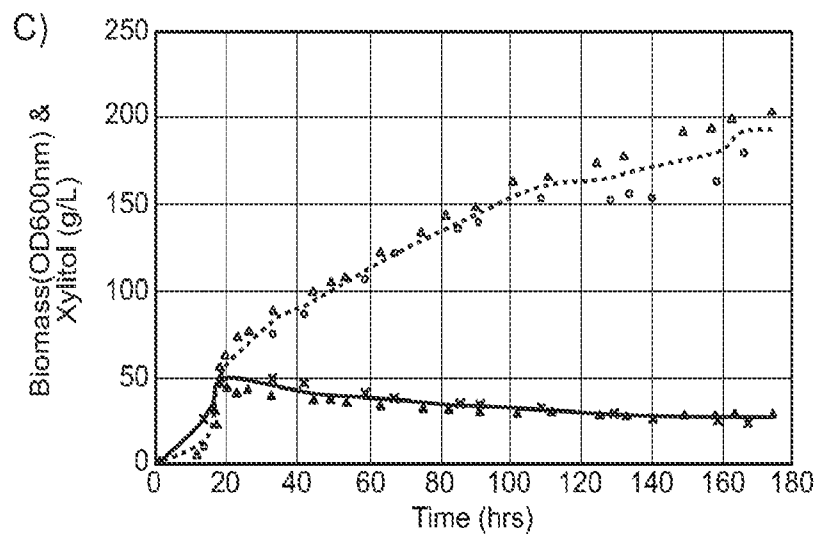
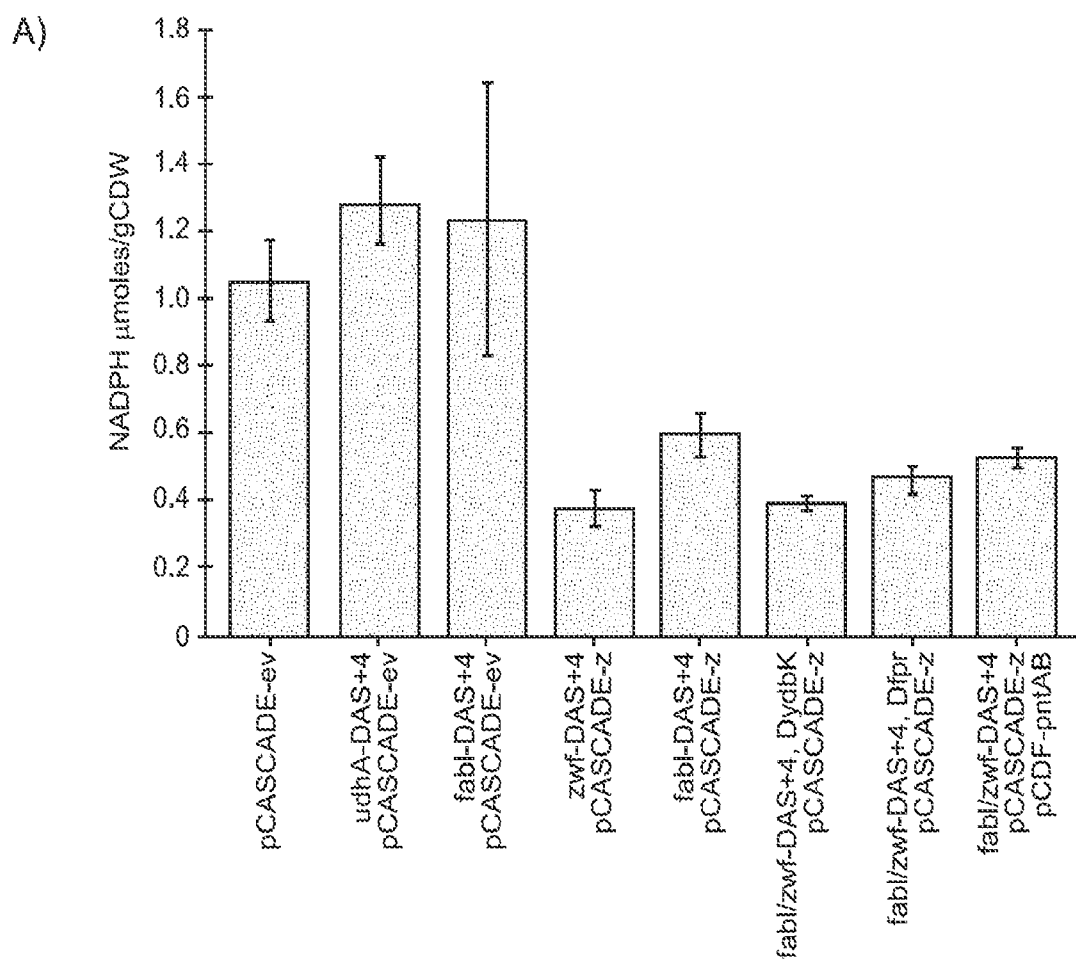


FIG. 17B

18/19

**FIG. 18A****FIG. 18B****FIG. 18C**

19/19

**FIG. 19**