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(54) Title: COMPOSITIONS AND METHODS FOR THE PRODUCTION OF PYRUVIC ACID AND RELATED PRODUCTS USING DYNAMIC METABOLIC CONTROL

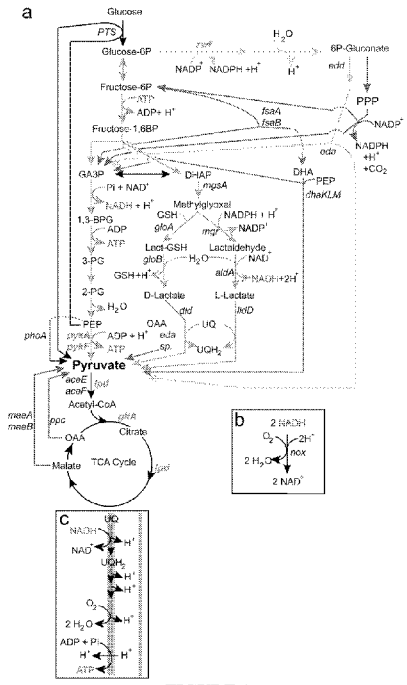

FIGURE 1

(57) Abstract: The present disclosure is related to genetically engineered microbial strains and related bioprocesses for the production of pyruvate and related products. Specifically, the use of dynamically controlled synthetic metabolic valves to reduce the activity of enzymes known to contribute to pyruvate synthesis, leads to increased pyruvate production in a two-stage process rather than a decrease in production.

[Continued on next page]

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COMPOSITIONS AND METHODS FOR THE PRODUCTION OF PYRUVIC ACID AND RELATED PRODUCTS USING DYNAMIC METABOLIC CONTROL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/687,874, filed June 21, 2018, 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 pyruvic acid or pyruvate and pyruvate derived products.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] This invention was made with government support under Federal Grant No. MCB-1445726 awarded by the National Science Foundation and Federal Contract No. HR0011-14-C-0075 awarded by the United States Department of Defense and Federal Grant No. ONR YIP 12043956 awarded by the United States Department of Defense. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0004] Petroleum is the primary feedstock, not only for the fuels we use but the majority of the chemicals we consume as well. The chemical industry is heavily reliant on this non-renewable resource. Replacement of petroleum with renewable feedstocks ensures longer-term viability and environmental sustainability. Novel fermentation based processes to make chemicals have been a contributing technology, enabling the change to renewable feedstocks (Werpy &Peterson, Top Value Added Chemicals from Biomass. Volume I - Results of Screening for Potential Candidates from Sugars and Synthesis Gas., Yixiang et al. "Green" Chemicals from Renewable Agricultural Biomass - A Mini Review. The Open Agriculture Journal, 2008). These fermentation processes have made rapid advancements in recent years due to technology developments in the fields of fermentation science, synthetic biology, as well as metabolic and enzyme engineering (Jarboe, L.R., et al., Metabolic engineering for production of biorenewable fuels and chemicals: contributions of synthetic biology. J Biomed Biotechnol, 2010 and Lee, J.W., et al., Systems metabolic engineering of microorganisms for natural and non-natural chemicals. Nat Chem Biol, 2012). Despite these substantial advances, most successful examples of rational directed engineering approaches have also greatly relied on numerous cycles of trial and error. The field of metabolic engineering has historically been limited in predicting the behavior of complex biological systems in-vivo, from simplified

models and basic in-vitro biochemical principles. Such rational approaches have required significant a priori knowledge of microbial physiology that in many cases is incomplete. This is particularly true for complex phenotypes that require an intricate balance between the activities of many seemingly unrelated gene products. In many cases, it has proven much more difficult than expected to integrate a possibly well characterized production pathway into a living host and balance the complex requirements of both biomass growth and production.

[0005] Pyruvic acid (pyruvate at neutral pH) is a three carbon oxo-monocarboxylic acid, also known as 2-oxopropanoic acid, 2-ketopropionic acid or acetylformic acid. Having both a keto and carboxylic groups, pyruvate is a potential precursor for many chemicals, pharmaceuticals, food additives, and polymers, useful in the synthesis of its esters such as ethyl pyruvate as well as L-DOPA, N-acetyl-D-neuraminic acid, and (R)-phenylacetylcarbinol. In addition, pyruvate is a central metabolite with significant potential as precursor to numerous additional commercial products that can be produced via subsequent biochemical conversions either *in vitro* or *in vivo*.

SUMMARY OF THE INVENTION

[0006] Provided herein are microbial strains for scalable biofermentation processes vie the use of synthetic metabolic valves (SMVs) that can 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.

[0007] Also provided are methods to construct microbial strains using controllable synthetic metabolic valves for the production of pyruvate or pyruvic acid as well as derivatives and further metabolic products of pyruvate or pyruvic acid. Synthetic metabolic valves are used to controllably reduce or eliminate flux through one more metabolic pathways known to produce pyruvic acid, resulting in strains unexpectedly producing high rates of pyruvic acid through alternative pathways. Flux is reduced or eliminated through one or more metabolic pathways, the enzymes of which may be essential for microbial growth in a given environment. The genetically modified microorganisms described herein use one or more synthetic metabolic valves alone or in combination thereby enabling dynamic control over metabolic pathways resulting in pyruvate production. Additional genetic modifications including gene deletions may be added to a microorganism to provide improved pyruvate production.

[0008] Provided herein are multi-stage bioprocesses that use the described genetically modified microorganism containing one or more synthetic metabolic valves that provide dynamic flux control. In certain embodiments, carbon feedstocks can include, but are not limited to the sugars: glucose, sucrose xylose, 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 pyruvate to additional chemical or fuel products.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] 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:

[0010] **Figure 1** depicts metabolic pathways involved in the production of pyruvate or pyruvic acid from glucose. The following abbreviations are found in the Figure: PTS –glucose phosphotransferase transport system, P – phosphate, BP- bisphosphate, OAA – oxaloacetate, DHAP- dihydroxyacetone phosphate, GA3P-glyceraldehyde- 3-phosphate, 1,3-BPG – 1,3 bisphosphoglycerate, 3-PG – 3-phosphoglycerate, 2-PG – 2- phosphoglycerate, PEP- phosphoenolpyruvate, PPP – pentose phosphate pathway, GSH – glutathione , Lact- lactaldehyde, UQ – oxidized ubiquinone, UQH2 – reduced ubiquinone, TCA- tricarboxylic acid.

[0011] **Figure 2** depicts the production of pyruvate at the L fermentation scale in *E. coli* strain DLF_01542. (Genotype: (F-, *λ-*, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*, *ΔldhA::frt*, *ΔpoxB::frt*, *ΔpflB::frt*, *ΔackA-pta::frt*, *ΔadhE::frt*, *ΔiclR*, *ΔarcA*, *ΔsspB*, *Δcas3::ugpBp-sspB-proB*, *lpd-DAS+4*, *gltA-DAS+4*, *zwf-DAS+4*, *pykA-DAS+4*, *pykF-DAS+4)* containing plasmids pCASCADE-gltA2-zf-pykA-pykF and pCDF-nox. Biomass and pyruvate titers are plotted as a function of time.

[0012] **Figure 3** depicts an example standard curve for the quantification of glucose.

[0013] **Figure 4** depicts an example standard curve for the quantification of pyruvate.

DETAILED DESCRIPTION OF THE INVENTION

[0014] The present invention is related to various production methods and/or genetically modified microorganisms that have utility for production of pyruvate and or pyruvic acid as well as to related chemical products, to methods of making such chemical products that use populations of these microorganisms in vessels, and to systems for chemical production that employ these microorganisms and methods.

Definitions

[0015] 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.

[0016] 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 an nonnative promoter driving gene expression.

[0017] 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.

[0018] 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.

[0019] 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.

[0020] Bio-production, Micro-fermentation (microfermentation) or Fermentation, as

used herein, may be aerobic, microaerobic, or anaerobic.

[0021] 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.

[0022] 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.

[0023] Species and other phylogenic identifications are according to the classification known to a person skilled in the art of microbiology.

[0024] 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.

[0025] 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.

[0026] 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, "OD_{600}" 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-thiogalactopyranoiside, "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

[0027] 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 glucose, sucrose, xylose, mannose, arabinose, oils, carbon dioxide, carbon monoxide, methane, methanol, formaldehyde and 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

[0028] 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.

[0029] 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 Common Methods Section.

III. Media and Culture Conditions

[0030] 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.

[0031] Another aspect of the invention regards media and culture conditions that comprise genetically modified microorganisms of the invention and optionally supplements.

[0032] 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

[0033] 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.

[0034] 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

[0035] 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.

[0036] 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.

[0037] 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.

[0038] 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.

[0039] 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.

[0040] 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.

[0041] 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.

[0042] 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.

[0043] 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.

[0044] 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.

[0045] 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.

[0046] More generally, and depending on the particular metabolic pathways of a microorganism selected for genetic modification, any subgroup of genetic modifications may be made to decrease cellular production of fermentation product(s) other than the desired fermentation product, selected from the group consisting of acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, isoprenoids, glycerol, ethylene glycol, ethylene, propylene, butylene, isobutylene, ethyl acetate, vinyl acetate, other acetates, 1,4-butanediol, 2,3-butanediol, butanol, isobutanol, sec-butanol, butyrate, isobutyrate, 2-OH-isobutryate, 3-OH-butyrate, ethanol, isopropanol, D-lactate, L-lactate, pyruvate, itaconate, levulinate, glucarate, glutarate, caprolactam, adipic acid, propanol, isopropanol, fusel alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate. Gene deletions may be made as disclosed generally herein, and other approaches may also be used to achieve a desired decreased cellular production of selected fermentation products other than the desired products.

[0047] VI. Synthetic Metabolic Valves

[0048] 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.

[0049] 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

[0050] 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 CRISRPR 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 CRISRPR 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 additional numerous transcriptional repressor systems are well known in the art and can be used to turn off gene expression.

VI.B Controlled Proteolysis

[0051] 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 additional 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.

[0052] 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

[0053] 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.

[0054] 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.

VII. Pyruvate and Product Producing Strains

[0055] Referring now to **Figure 1**: Enzymes/genes that are candidates to either "turned off" with metabolic valves or deleted as potentially overexpressed are shown: glucose-6-phosphate dehydrogenase (*zwf-"Z"*), lipoamide dehydrogenase (*lpd-"L"*), citrate synthase (*gltA-"G"*), pyruvate dehydrogenase complex subunit (*aceEF*, *lpd*-"L"), pyruvate kinase A (*pykA*-"A"), pyruvate kinase F (*pykF*-"F"), nox, methylglyoxal synthase (*mgsA*), multisubunit dihydroxyacetone kinase (*dhaKLM*), methylglyoxal reductase (*mgr*), aldehyde dehydrogenase (*aldA*), phosphoenolpyruvate carboxylase (*ppc*), phosphatase (*phoA*), malate dehydrogenase – NAD+ specific (*maeA*), malate dehydrogenase (*maeB*), glyoxylase I (*gloA*), glyoxylase II (*gloB*), phosphogluconate dehydratase *(edd*), multifunctional 2-keto-3-deoxygluconate 6-phosphate aldolase and 2-keto-4-hydroxyglutarate aldolase and oxaloacetate decarboxylase (*eda*), fructose-6-phosphate aldolase I (*fsaA*), fructose-6-phosphate aldolase II (*fsaB*), D-lactate dehydrogenase (*dld*), L-lactate dehydrogenase (*lld*).

[0056] In certain embodiments pyruvate production is greatly enhanced in strains engineered to reduce or eliminate flux through biochemical steps in pathways known to be responsible for pyruvate biosynthesis as illustrated in **Figure 1**. In various embodiments, this can either be accomplished by gene deletions, in the case of non essential genes, or through the use of dynamic metabolic valves, as described above when a gene is essential and/or important for growth. In certain embodiments valves alone and or in the combination in the following genes can lead to enhanced pyruvate production: glucose-6-phosphate dehydrogenase (*zwf-"Z"*), lipoamide dehydrogenase (*lpd-"L"*), citrate synthase (*gltA-"G"*), pyruvate dehydrogenase complex subunit (*aceEF*, *lpd*-"L"), pyruvate kinase A (*pykA*-"A"), pyruvate kinase F (*pykF*-"F"), methylglyoxal synthase (*mgsA*), multisubunit dihydroxyacetone kinase (*dhaKLM*), methylglyoxal reductase (*mgr*), aldehyde dehydrogenase (*aldA*), phosphoenolpyruvate

carboxylase (*ppc*), phosphatase (*phoA*), malate dehydrogenase – NAD+ specific (*maeA*), malate dehydrogenase (*maeB*), glyoxylase I (*gloA*), glyoxylase II (*gloB*), phosphogluconate dehydratase (*edd*), multifunctional 2-keto-3-deoxygluconate 6-phosphate aldolase and 2-keto-4-hydroxyglutarate aldolase and oxaloacetate decarboxylase (*eda*), fructose-6-phosphate aldolase I (*fsaA*), fructose-6-phosphate aldolase II (*fsaB*), D-lactate dehydrogenase (*dld*), L-lactate dehydrogenase (*lld*) or erythrose-4-phosphate dehydrogenase (*epd*). Pyruvate is then presumably synthesized through one or more uncharacterized routes or through rerouting flux through one or more alternative known pyruvate synthesis pathways. In additional embodiments, deletions of many of these potential alternative pathways alone and in combination does not eliminate pyruvate synthesis. In other embodiments, additionally overexpression an NADH oxidase enzyme capable of removing excess NADH formed as a byproduct of pyruvate synthesis can enhance pyruvate production. In still other embodiments strains engineered with one or more of the genetic modification discussed above can be grown in large scale fermentations enabling pyruvate production at high rates, titers and yields. Yet in still additional embodiments, biochemical pathways well known in the art capable of converting pyruvate into additional numerous chemicals can be incorporated into the above described strains to convert pyruvate into additional products.

[0057] The microbial strains are engineered for pyruvate product production. Pyruvate product refers to pyruvate, pyruvic acid, or any other form of pyruvate. However, the strains are also able to produce a variety of pyruvate derived products. The microorganisms may also comprise a pyruvate derived product production pathway or alternatively, the pyruvate product produced by the microorganism may be a source for pyruvate derived product production. In some cases, the pyruvate derived product is produced from pyruvate by an enzymatic pathway. The enzymatic pathway may comprise one or more than one enzymatic action. The pyruvate derived product production may also occur by chemical action on pyruvate.

[0058] Some exemplary pyruvate derived products include: an amino acid, alanine, valine, isoleucine, leucine, serine, cysteine, aspartate, acetylaldehyde, phosphoenolypyruvate, citrate, oxaloacetate, ethyl pyruvate, L-DOPA, N-acetyl-D-neuraminic acid, (R)-phenylacetylcarbinol, acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, acetylCoA, isoprenoids, glycerol, ethylene glycol, ethylene, propylene, butylene, isobutylene, ethyl acetate, vinyl acetate, 1,4-butanediol, 2,3-butanediol, butanol, isobutanol, sec-butanol, butyrate, isobutyrate, 2-OH-isobutryate, 3-OH-butyrate, ethanol, isopropanol, D-lactate, L-lactate, pyruvate, itaconate, levulinate, glucarate, glutarate, caprolactam, adipic acid, propanol, isopropanol, fusel alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate. Of course, the

microorgansims and methods described herein provide a source of pyruvate available for production of any product formed from pyruvate.

[0059] Within the scope of the invention are genetically modified microorganism, wherein the microorganism is capable of producing pyruvate or a pyruvate derived product 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.

[0060] Within the scope of the invention are genetically modified microorganism, wherein the microorganism is capable of producing a pyruvate product or a pyruvate derived product from a hexose sugar source at a yield greater than 0.5 g product /g hexose, greater than 0.6 g product /g hexose, greater than 0.7 g product /g hexose, greater than 0.8 g product /g hexose, greater than 0.9 g product /g hexose, greater than 0.95 g product /g hexose, or greater than 0.98 g product /g hexose.

[0061] 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.

Summary

[0062] Herein described is a genetically modified microorganism and biofermentation methods for producing a pyruvate product comprising use of a genetically modified microorganism having a combination of at least one of three types of modifications: a synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes; a synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes; and a chromosomal deletion of a gene encoding an enzyme of a pyruvate metabolism pathway.

[0063] A gene expression-silencing synthetic metabolic valve refers to a synthetic metabolic valve that is at least characterized by silencing gene expression of one or more genes

encoding one or more enzymes. An enzymatic degradation synthetic metabolic valve refers to a synthetic metabolic valve characterized by at least inducing enzymatic degradation of one or more enzymes. In cases where there are more than one enzymes controlled by the gene silencing valve and the enzymatic valve, the gene groups of each valve may be the exactly the same, have some gene in common and some genes that are different or the two valves may regulate groups of genes with no overlap.

[0064] The gene expression-silencing synthetic metabolic valve and the enzymatic degradation synthetic metabolic valve are activated under conditions that are suitable for causing a transition in a biofermentation process. Such as chemical induction or nutrient depletion.

[0065] The one or more enzymes of each synthetic metabolic valve are the same or different. That is, the enzyme selection for silencing and enzymatic degradation may be the same enzyme or two enzymes may be subject to control by the synthetic metabolic valves of the microorganism.

[0066] The genetically modified microorganism may have a single modification or combination of modifications. For example, multiple metabolic valves directed to multiple enzymes may be used. A combination of two of the three modifications may be used, for example, a gene deletion combined with enzymatic degradation of one or more enzymes. In some cases a single valve may control expression of more than one enzyme simultaneously.

[0067] The genetically modified microorganism characterized by an increased production of pyruvate in a biofermentation process as compared to pyruvate produced from biofermentation of a non-genetically modified microorganism.

[0068] The genetically modified microorganism may be characterized in that the one or more enzymes are selected from the group consisting of: *fabI, zwf, gltA, ppc, udhA, lpd, sucD, aceA, pfkA, lon, rpoS, pykA, pykF, tktA or tktB*. The one or more enzymes is selected from the group consisting of: citrate synthase (*gltA*), pyruvate dehydrogenase (*lpd*), and glucose-6-phosphate dehydrogenase (*zwf*). The one or more enzymes may be a pyruvate kinase. The one or more enzymes is a pyruvate kinase A (*pykA*) or pyruvate kinase F (*pykF*).

[0069] The chromosomal deletion of the genetically modified microorganism may be selected from the group consisting of: methylglyoxal synthase (*mgsA*), dihydroxyacetone kinase (*dhaL*), D-erythrose-4-phosphate dehydrogenase (*epd*), 2-keto-3-deoxygluconate 6-phosphate/2-keto-4-hydroxyglutarate aldolase (*eda*), and PTS multiphosphoryl transfer protein (*ptsA*).

[0070] In some cases, the genetically modified microorganism also expresses, or overexpresses a NADH oxidase.

[0071] Silencing of gene expression may occur via CRISPR interference and the genetically modified microorganism may also express 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.

[0072] In some cases, the genetically modified microorganism produces a pyruvate product titer of greater than 0.08 g/L at twenty four in a biofermentation process.

[0073] The genetically modified microorganism may also include a production pathway for producing a pyruvate derived product. That is, the genetically modified microorganism may express an enzyme for conversion of pyruvate to a derived product, such as an amino acid. This conversion of pyruvate to a pyruvate derived product may occur in one step or in multiple steps. The conversion may occur within the microorganism itself or within a reactor vessel containing the microorganism. The pyruvate derived product may be selected from the group consisting of: an amino acid, alanine, valine, isoleucine, leucine, serine, cysteine, aspartate, acetylaldehyde, phosphoenolypyruvate, citrate, oxaloacetate, ethyl pyruvate, L-DOPA, N-acetyl-D-neuraminic acid, (R)-phenylacetylcarbinol, acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, acetylCoA, isoprenoids, glycerol, ethylene glycol, ethylene, propylene, butylene, isobutylene, ethyl acetate, vinyl acetate, 1,4-butanediol, 2,3-butanediol, butanol, isobutanol, sec-butanol, butyrate, isobutyrate, 2-OH-isobutryate, 3-OH-butyrate, ethanol, isopropanol, D-lactate, L-lactate, pyruvate, itaconate, levulinate, glucarate, glutarate, caprolactam, adipic acid, propanol, isopropanol, fusel alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate.

[0074] Exemplary methods for producing a pyruvate product from a genetically modified microorganism may comprise (a) in a first stage, growing a genetically modified microorganism, the genetically modified microorganism comprising a combination of at least one of: i. a synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes; ii. a synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes; and iii. a chromosomal deletion, wherein the one or more enzymes of each synthetic metabolic valve are the same or different. A second stage of the method may include: (i) inducing the synthetic metabolic valve(s) to slow or stop the growth of the microorganism and to change metabolism within the microorganism; and (ii) producing a pyruvate product.

[0075] The method may further include a step of (c) centrifugation to separate the genetically modified microorganism and the pyruvate product. The method may include a further step of formation of a pyruvate salt from the pyruvate product or formation of a pyruvate ester from the pyruvate product. The method may include a further step of producing a pyruvate derived product by biochemical conversion of the pyruvate product to a derived product selected from the group consisting of: an amino acid, alanine, valine, isoleucine, leucine, serine, cysteine,

aspartate, acetylaldehyde, phosphoenolypyruvate, citrate, oxaloacetate, ethyl pyruvate, L-DOPA, N-acetyl-D-neuraminic acid, (R)-phenylacetylcarbinol, acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, acetylCoA, isoprenoids, glycerol, ethylene glycol, ethylene, propylene, butylene, isobutylene, ethyl acetate, vinyl acetate, 1,4-butanediol, 2,3-butanediol, butanol, isobutanol, sec-butanol, butyrate, isobutyrate, 2-OH-isobutryate, 3-OH-butyrate, ethanol, isopropanol, D-lactate, L-lactate, pyruvate, itaconate, levulinate, glucarate, glutarate, caprolactam, adipic acid, propanol, isopropanol, fusel alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate.

Disclosed Embodiments Are Non-Limiting

[0076] 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 **Figure 1**), 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.

[0077] 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.

[0078] 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).

[0079] 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.

EXAMPLES

[0080] The examples herein provide some examples, not meant to be limiting. All reagents, unless otherwise indicated, are obtained commercially. Species and other phylogenic identifications are according to the classification known to a person skilled in the art of microbiology, molecular biology and biochemistry.

[0081] Example 1: Production of Pyruvate in Microfermentations using *E. coli* engineered with metabolic valves.

[0082] This example describes the increased production of pyruvate in *E. coli* using metabolic valves of several genes alone and in combination including *pykA* (encoding pyruvate kinase A), *pykF* (encoding pyruvate kinase B), *gltA* (encoding citrate synthase), *zwf* (encoding glucose-6-phosphate dehydrogenase), *lpd* (encoding a subunit of the pyruvate dehydrogenase multi-enzyme complex. Briefly, *E. coli* strain DLF_0025 (F-, λ^-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*, *ΔldhA::frt*, *ΔpoxB::frt*, *ΔpflB::frt*, *ΔackA-pta::frt*, *ΔadhE::frt*, *ΔiclR*, *ΔarcA*, *ΔsspB*, *Δcas3::ugpBp-sspB-proB)* which is capable of dynamic metabolic control was further genetically modified to contain DAS+4 tags at the C-terminus of various enzymes as well as gene deletions. These strains were then transformed with pCASCADE plasmids expressing gRNA arrays capable of the silencing of several gene promoters, as well as optionally a plasmid allowing for the induction of NADH oxidase encoded by the nox gene which is under the control of the *ugpB* gene promoter and induced by phosphate depletion (Addgene Plasmid # 1010894). The strains and plasmids were constructed as described in the common methods section. Following strain construction, strains were evaluated in standard microfermentations in microtiter plates in triplicate. Cells were harvested by centrifugation and pyruvate quantified in the supernatant by UPLC (Refer to Common methods Section). Results are given in Table 1 below as a function of strain, both with measured titers and biomass normalized titers.

[0083] **Table 1:** Pyruvate Production in MicroFermentations by engineered strains of *E. coli.*

Strain #	Genes with Proteolysis Tags	Chromosomal Gene Deletions	Gene Silencing	NADH Oxidase Expression Vector	24 hour Pyruvate Titer (g/L)	24 Hour Normalized Pyruvate Titer (g/L-
1	gltA	None	pCASCADE-empty vector	None	6.926334	3.103196
2	gltA	None	pCASCADE-gltA1	None	2.435827	0.661909
3	gltA	None	pCASCADE-gltA1-gltA2	None	1.644148	7.339948
4	gltA	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	5.958417	3.252411
5	gltA	None	pCASCADE-gltA1-gltA2-zwf	None	2.087338	0.483628
6	gltA	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	2.117973	3.601994
7	gltA	None	pCASCADE-gltA1-pykA-pykF	None	7.562506	3.322718
8	gltA	None	pCASCADE-gltA1-zwf	None	2.744115	2.858453
9	gltA	None	pCASCADE-gltA1-zwf-pykA-pykF	None	1.982524	0.496624
10	gltA	None	pCASCADE-gltA2	None	1.839331	3.308149
11	gltA	None	pCASCADE-gltA2-pykA-pykF	None	3.377558	3.68729
12	gltA	None	pCASCADE-gltA2-zwf	None	1.862437	0.972044
13	gltA	None	pCASCADE-gltA2-zwf-pykA-pykF	None	2.173982	0.697683
14	gltA	None	pCASCADE-pykA-pykF	None	2.808667	0.794306
15	gltA	None	pCASCADE-zwf	None	7.906433	2.256402
16	gltA	None	pCASCADE-zwf-pykA-pykF	None	1.806812	0.460921
17	gltA, pykA, pykF	None	pCASCADE-empty vector	None	6.692441	4.61519
18	gltA, pykA, pykF	None	pCASCADE-gltA1	None	2.033619	1.570556
19	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2	None	1.03772	2.753742
20	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.081247	2.936575
21	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf	None	1.409172	1.184098
22	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	1.792465	2.4126
23	gltA, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	None	2.253633	2.611062
24	gltA, pykA, pykF	None	pCASCADE-gltA1-zwf	None	2.113928	2.554688
25	gltA, pykA, pykF	None	pCASCADE-gltA1-zwf-pykA-pykF	None	2.243429	3.99528
26	gltA, pykA, pykF	None	pCASCADE-gltA2	None	1.442783	2.466254
27	gltA, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	None	1.381587	2.406065
28	gltA, pykA, pykF	None	pCASCADE-gltA2-zwf	None	1.382044	1.542374
29	gltA, pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.183367	0.854238
30	gltA, pykA, pykF	None	pCASCADE-pykA-pykF	None	5.914064	5.735963
31	gltA, pykA, pykF	None	pCASCADE-zwf	None	0.668855	0.476328
32	gltA, pykA, pykF	None	pCASCADE-zwf-pykA-pykF	None	7.186233	5.833313
33	gltA, zwf	None	pCASCADE-empty vector	None	6.481895	4.151521
34	gltA, zwf	None	pCASCADE-gltA1	None	1.805899	1.140859
35	gltA, zwf	None	pCASCADE-gltA1-gltA2	None	1.363764	3.535358
36	gltA, zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.773825	3.693546
37	gltA, zwf	None	pCASCADE-gltA1-gltA2-zwf	None	2.907301	3.041142
38	gltA, zwf	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	6.768355	4.839656
39	gltA, zwf	None	pCASCADE-gltA1-pykA-pykF	None	2.627482	2.937604

40	gltA, zwf	None	pCASCADE-gltA1-zwf	None	2.214957	2.763343
41	gltA, zwf	None	pCASCADE-gltA1-zwf-pykA-pykF	None	3.132929	7.155092
42	gltA, zwf	None	pCASCADE-gltA2	None	1.69175	2.680298
43	gltA, zwf	None	pCASCADE-gltA2-pykA-pykF	None	1.828265	2.751795
44	gltA, zwf	None	pCASCADE-gltA2-zwf	None	1.451611	1.626382
45	gltA, zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.717691	2.611901
46	gltA, zwf	None	pCASCADE-pykA-pykF	None	5.561404	3.495474
47	gltA, zwf	None	pCASCADE-zwf	None	0.248783	0.121037
48	gltA, zwf	None	pCASCADE-zwf-pykA-pykF	None	5.219484	5.439796
49	gltA, zwf, pykA, pykF	None	pCASCADE-empty vector	None	5.772126	3.779944
50	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1	None	3.206812	2.3104
51	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2	None	1.63791	3.639719
52	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.511753	3.482661
53	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf	None	1.814503	2.026223
54	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None		0
55	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	None	3.211797	3.542762
56	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-zwf	None	2.880992	3.144158
57	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-zwf-pykA-pykF	None	4.107069	4.565642
58	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2	None	1.643419	2.548133
59	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	None	1.265883	1.936015
60	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-zwf	None	1.778775	2.016111
61	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.715338	2.722327
62	gltA, zwf, pykA, pykF	None	pCASCADE-zwf	None	8.480922	5.741642
63	gltA, zwf, pykA, pykF	None	pCASCADE-zwf-pykA-pykF	None	7.410325	14.25967
64	lpd	None	pCASCADE-empty vector	None	1.626551	0.412412
65	lpd	None	pCASCADE-gltA1	None	2.128559	4.257117
66	lpd	None	pCASCADE-gltA1-gltA2	None	2.070359	3.175397
67	lpd	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.054934	0.332158
68	lpd	None	pCASCADE-gltA1-gltA2-zwf	None	2.263284	2.707277
69	lpd	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	1.334917	0.975817
70	lpd	None	pCASCADE-gltA1-pykA-pykF	None	1.087234	0.269651
71	lpd	None	pCASCADE-gltA1-zwf	None	2.451736	0.832791
72	lpd	None	pCASCADE-gltA1-zwf-pykA-pykF	None	0.876629	1.274169
73	lpd	None	pCASCADE-gltA2	None	1.14362	0.746488
74	lpd	None	pCASCADE-gltA2-pykA-pykF	None	3.374363	0.833588
75	lpd	None	pCASCADE-gltA2-zwf	None	1.071412	3.151213
76	lpd	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.383387	1.801285
77	lpd	None	pCASCADE-pykA-pykF	None	1.348538	0.54028
78	lpd	None	pCASCADE-zwf	None	1.439127	0.922517
79	lpd	None	pCASCADE-zwf-pykA-pykF	None	1.669276	0.925319

80	lpd, gltA	None	pCASCADE-empty vector	None	5.590723	3.831887
81	lpd, gltA	None	pCASCADE-gltA1	None	6.355754	6.76144
82	lpd, gltA	None	pCASCADE-gltA1-gltA2	None	1.650923	1.727412
83	lpd, gltA	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	8.728136	3.565415
84	lpd, gltA	None	pCASCADE-gltA1-gltA2-zwf	None	2.35574	1.335454
85	lpd, gltA	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	2.344075	2.675885
86	lpd, gltA	None	pCASCADE-gltA1-pykA-pykF	None	7.569212	2.045733
87	lpd, gltA	None	pCASCADE-gltA1-zwf	None	6.968887	4.45581
88	lpd, gltA	None	pCASCADE-gltA1-zwf-pykA-pykF	None	2.368826	2.322378
89	lpd, gltA	None	pCASCADE-gltA2	None	2.416623	3.082428
90	lpd, gltA	None	pCASCADE-gltA2-pykA-pykF	None	7.801392	4.88809
91	lpd, gltA	None	pCASCADE-gltA2-zwf	None	2.344589	3.733422
92	lpd, gltA	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.924203	1.444597
93	lpd, gltA	None	pCASCADE-pykA-pykF	None	7.148925	1.946875
94	lpd, gltA	None	pCASCADE-zwf	None	7.49176	2.051413
95	lpd, gltA	None	pCASCADE-zwf-pykA-pykF	None	2.144874	0.624963
96	lpd, gltA, pykA, pykF	None	pCASCADE-empty vector	None		0
97	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1	None		0
98	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2	None		0
99	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	None		0
100	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf	None	1.887489	1.815906
101	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None		0
102	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	None		0
103	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-zwf	None		0
104	lpd, gltA, pykA, pykF	None	pCASCADE-gltA2	None		0
105	lpd, gltA, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	None		0
106	lpd, gltA, pykA, pykF	None	pCASCADE-gltA2-zwf	None	2.507271	3.663619
107	lpd, gltA, pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	None		0
108	lpd, gltA, pykA, pykF	None	pCASCADE-pykA-pykF	None		0
109	lpd, gltA, pykA, pykF	None	pCASCADE-zwf	None	0.412556	0.266492
110	lpd, gltA, pykA, pykF	None	pCASCADE-zwf-pykA-pykF	None		0
111	lpd, gltA, zwf	None	pCASCADE-empty vector	None	4.163291	4.756634
112	lpd, gltA, zwf	None	pCASCADE-gltA1	None	3.90461	2.297046
113	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2	None	1.815815	2.846417
114	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.351396	3.223518
115	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2-zwf	None	2.495417	1.969081
116	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	4.065545	4.626404
117	lpd, gltA, zwf	None	pCASCADE-gltA1-pykA-pykF	None	4.617462	4.703824
118	lpd, gltA, zwf	None	pCASCADE-gltA1-zwf	None	3.555241	4.097316
119	lpd, gltA, zwf	None	pCASCADE-gltA1-zwf-pykA-pykF	None	4.5642	3.105891

120	lpd, gltA, zwf	None	pCASCADE-gltA2	None	2.208222	2.828299
121	lpd, gltA, zwf	None	pCASCADE-gltA2-pykA-pykF	None	1.770202	2.686235
122	lpd, gltA, zwf	None	pCASCADE-gltA2-zwf	None	1.806209	2.062353
123	lpd, gltA, zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	None	2.224844	1.363429
124	lpd, gltA, zwf	None	pCASCADE-pykA-pykF	None	6.923406	4.839343
125	lpd, gltA, zwf	None	pCASCADE-zwf	None	0.190025	0.11843
126	lpd, gltA, zwf	None	pCASCADE-zwf-pykA-pykF	None	7.182654	4.351277
127	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-empty vector	None	4.242052	4.090736
128	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1	None		0
129	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2	None	3.324025	3.839208
130	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.319675	3.166208
131	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	4.420189	5.357545
132	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	None	1.782848	3.78251
133	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-zwf	None		0
134	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA2	None		0
135	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	None	2.478112	4.155466
136	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	None		0
137	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-pykA-pykF	None		0
138	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-zwf	None		0
139	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-zwf-pykA-pykF	None	3.693888	7.828024
140	lpd, zwf	None	pCASCADE-empty vector	None	2.195519	0.782995
141	lpd, zwf	None	pCASCADE-gltA1	None	1.87622	2.132068
142	lpd, zwf	None	pCASCADE-gltA1-gltA2	None	1.964431	2.698394
143	lpd, zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	1.418538	0.604147
144	lpd, zwf	None	pCASCADE-gltA1-gltA2-zwf	None	1.9114	1.137738
145	lpd, zwf	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	1.105357	0.714055
146	lpd, zwf	None	pCASCADE-gltA1-pykA-pykF	None	2.218935	0.645039
147	lpd, zwf	None	pCASCADE-gltA1-zwf	None	2.43108	0.724398
148	lpd, zwf	None	pCASCADE-gltA1-zwf-pykA-pykF	None	1.202002	0.805632
149	lpd, zwf	None	pCASCADE-gltA2	None	1.188285	0.83447
150	lpd, zwf	None	pCASCADE-gltA2-pykA-pykF	None	2.268933	0.57763
151	lpd, zwf	None	pCASCADE-gltA2-zwf	None	1.075495	0.759531
152	lpd, zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.609353	1.146263
153	lpd, zwf	None	pCASCADE-pykA-pykF	None	2.55484	0.716042
154	lpd, zwf	None	pCASCADE-zwf	None	2.692122	0.964227
155	lpd, zwf	None	pCASCADE-zwf-pykA-pykF	None	1.66086	0.542765
156	None	None	pCASCADE-empty vector	None		0
157	None	None	pCASCADE-gltA1	None		0
158	None	None	pCASCADE-gltA1-gltA2	None	1.935203	2.658246
159	None	None	pCASCADE-gltA1-gltA2-pykA-pykF	None		0
160	None	None	pCASCADE-gltA1-gltA2-zwf	None	1.220768	0.678204

161	None	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None		0
162	None	None	pCASCADE-gltA1-pykA-pykF	None		0
163	None	None	pCASCADE-gltA1-zwf	None		0
164	None	None	pCASCADE-gltA1-zwf-pykA-pykF	None		0
165	None	None	pCASCADE-gltA2	None		0
166	None	None	pCASCADE-gltA2-pykA-pykF	None		0
167	None	None	pCASCADE-gltA2-zwf	None		0
168	None	None	pCASCADE-gltA2-zwf-pykA-pykF	None		0
169	None	None	pCASCADE-pykA-pykF	None		0
170	None	None	pCASCADE-zwf	None		0
171	None	None	pCASCADE-zwf-pykA-pykF	None	1.531818	0.479893
172	pykA, pykF	None	pCASCADE-empty vector	None		0
173	pykA, pykF	None	pCASCADE-gltA1	None		0
174	pykA, pykF	None	pCASCADE-gltA1-gltA2	None	0.994998	2.381061
175	pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	None		0
176	pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf	None	0.743998	0.868244
177	pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None		0
178	pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	None		0
179	pykA, pykF	None	pCASCADE-gltA1-zwf	None		0
180	pykA, pykF	None	pCASCADE-gltA1-zwf-pykA-pykF	None		0
181	pykA, pykF	None	pCASCADE-gltA2	None		0
182	pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	None		0
183	pykA, pykF	None	pCASCADE-gltA2-zwf	None		0
184	pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	None		0
185	pykA, pykF	None	pCASCADE-pykA-pykF	None		0
186	pykA, pykF	None	pCASCADE-zwf	None		0
187	pykA, pykF	None	pCASCADE-zwf-pykA-pykF	None		0
188	zwf	None	pCASCADE-empty vector	None	0.48452	0.140359
189	zwf	None	pCASCADE-gltA1	None	1.677519	3.584443
190	zwf	None	pCASCADE-gltA1-gltA2	None	2.44634	0.798414
191	zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	None	0.733646	0.264663
192	zwf	None	pCASCADE-gltA1-gltA2-zwf	None	1.983733	2.637943
193	zwf	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	None	2.266191	0.565417
194	zwf	None	pCASCADE-gltA1-pykA-pykF	None	1.001772	0.30654
195	zwf	None	pCASCADE-gltA1-zwf	None	1.552254	0.437008
196	zwf	None	pCASCADE-gltA1-zwf-pykA-pykF	None	1.932433	3.378379
197	zwf	None	pCASCADE-gltA2	None	2.215242	0.575089
198	zwf	None	pCASCADE-gltA2-pykA-pykF	None	1.848844	0.461749
199	zwf	None	pCASCADE-gltA2-zwf	None	1.899225	2.472949
200	zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	None	1.37267	2.660213
201	zwf	None	pCASCADE-pykA-pykF	None	0.948317	0.487817
202	zwf	None	pCASCADE-zwf	None	0.774174	0.875763
203	zwf	None	pCASCADE-zwf-pykA-pykF	None	4.251237	4.251237
204	gltA	None	pCASCADE-empty vector	pCDF-nox	2.994474	3.771425
205	gltA	None	pCASCADE-gltA1	pCDF-nox	1.862628	2.593756

206	gltA	None	pCASCADE-gltA1-gltA2	pCDF-nox	1.007197	3.14877
207	gltA	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.169942	3.316068
208	gltA	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox	1.148563	1.754416
209	gltA	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	1.756779	5.076515
210	gltA	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	1.862411	3.223726
211	gltA	None	pCASCADE-gltA1-zwf	pCDF-nox	1.725361	3.059423
212	gltA	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox	1.678689	2.667593
213	gltA	None	pCASCADE-gltA2	pCDF-nox	1.683244	4.230319
214	gltA	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	1.269628	3.680294
215	gltA	None	pCASCADE-gltA2-zwf	pCDF-nox	1.685963	1.992629
216	gltA	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	1.5489	3.475285
217	gltA	None	pCASCADE-pykA-pykF	pCDF-nox	3.401353	4.661111
218	gltA	None	pCASCADE-zwf	pCDF-nox	3.478002	4.920911
219	gltA	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	3.925427	7.573365
220	gltA, pykA, pykF	None	pCASCADE-empty vector	pCDF-nox	5.787515	5.907134
221	gltA, pykA, pykF	None	pCASCADE-gltA1	pCDF-nox	3.815812	3.654957
222	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2	pCDF-nox	1.405854	3.85493
223	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.562872	3.857133
224	gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	1.077614	3.094636
225	gltA, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	2.690626	3.65202
226	gltA, pykA, pykF	None	pCASCADE-gltA1-zwf	pCDF-nox	2.114894	2.897114
227	gltA, pykA, pykF	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox	2.200602	2.049016
228	gltA, pykA, pykF	None	pCASCADE-gltA2	pCDF-nox	1.637188	3.391869
229	gltA, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	1.581651	3.579125
230	gltA, pykA, pykF	None	pCASCADE-gltA2-zwf	pCDF-nox	1.395467	1.661488
231	gltA, pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox		0
232	gltA, pykA, pykF	None	pCASCADE-pykA-pykF	pCDF-nox	4.943349	5.012369
233	gltA, pykA, pykF	None	pCASCADE-zwf	pCDF-nox	5.154877	5.303808
234	gltA, pykA, pykF	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	7.308858	7.275463
235	gltA, zwf	None	pCASCADE-empty vector	pCDF-nox	6.068373	5.302436
236	gltA, zwf	None	pCASCADE-gltA1	pCDF-nox	2.906389	4.428513
237	gltA, zwf	None	pCASCADE-gltA1-gltA2	pCDF-nox	1.891147	6.640964
238	gltA, zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.216316	2.779578
239	gltA, zwf	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	3.218554	4.248692
240	gltA, zwf	None	pCASCADE-gltA1-zwf	pCDF-nox	2.049991	2.791686
241	gltA, zwf	None	pCASCADE-gltA2	pCDF-nox		0
242	gltA, zwf	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	1.667479	3.308424
243	gltA, zwf	None	pCASCADE-gltA2-zwf	pCDF-nox	0.940073	1.907074
244	gltA, zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	1.309984	2.897104
245	gltA, zwf	None	pCASCADE-pykA-pykF	pCDF-nox	6.668118	313.7938
246	gltA, zwf	None	pCASCADE-zwf	pCDF-nox	0.169753	8.091173
247	gltA, zwf	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	6.250439	7.890972
248	gltA, zwf, pykA, pykF	None	pCASCADE-empty vector	pCDF-nox		0
249	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2	pCDF-nox	2.074567	4.761458

250	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.545728	3.583217
251	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	0.175741	7.421514
252	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	2.715614	3.702218
253	gltA, zwf, pykA, pykF	None	pCASCADE-gltA1-zwf	pCDF-nox	2.613912	3.338671
254	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2	pCDF-nox	1.712319	3.201853
255	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	1.849683	3.629106
256	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-zwf	pCDF-nox	1.154281	2.364942
257	gltA, zwf, pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	1.240534	2.982126
258	gltA, zwf, pykA, pykF	None	pCASCADE-pykA-pykF	pCDF-nox		0
259	gltA, zwf, pykA, pykF	None	pCASCADE-zwf	pCDF-nox	0.234393	0.186545
260	gltA, zwf, pykA, pykF	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	0.16027	0.256555
261	lpd	None	pCASCADE-empty vector	pCDF-nox	4.376713	3.361195
262	lpd	None	pCASCADE-gltA1	pCDF-nox	2.04663	2.057742
263	lpd	None	pCASCADE-gltA1-gltA2	pCDF-nox	2.184127	6.115776
264	lpd	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.803429	5.178993
265	lpd	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox	1.867143	2.835664
266	lpd	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	1.673461	5.99592
267	lpd	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	0.547288	27.50189
268	lpd	None	pCASCADE-gltA1-zwf	pCDF-nox	2.375918	3.533016
269	lpd	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox		0
270	lpd	None	pCASCADE-gltA2	pCDF-nox	2.528549	4.302375
271	lpd	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	1.380511	2.352209
272	lpd	None	pCASCADE-gltA2-zwf	pCDF-nox	1.425502	2.860501
273	lpd	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	0.228188	0.553209
274	lpd	None	pCASCADE-pykA-pykF	pCDF-nox	3.595425	4.370753
275	lpd	None	pCASCADE-zwf	pCDF-nox		0
276	lpd	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	3.542121	5.410545
277	lpd, gltA	None	pCASCADE-empty vector	pCDF-nox	5.179109	5.445217
278	lpd, gltA	None	pCASCADE-gltA1	pCDF-nox	2.894256	2.311374
279	lpd, gltA	None	pCASCADE-gltA1-gltA2	pCDF-nox	1.204914	3.415192
280	lpd, gltA	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.827513	4.462683
281	lpd, gltA	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	3.199911	3.929936
282	lpd, gltA	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	4.093769	6.196953
283	lpd, gltA	None	pCASCADE-gltA1-zwf	pCDF-nox	3.36976	5.852004
284	lpd, gltA	None	pCASCADE-gltA2	pCDF-nox	2.939899	6.572397
285	lpd, gltA	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	2.278146	4.946683
286	lpd, gltA	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	2.820681	5.358233
287	lpd, gltA	None	pCASCADE-zwf	pCDF-nox	3.233077	2.87451
288	lpd, gltA	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	6.800414	9.71446
289	lpd, gltA, pykA, pykF	None	pCASCADE-empty vector	pCDF-nox		0

290	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1	pCDF-nox	8.03826	8.340001
291	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.552227	4.725054
292	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox	2.185541	6.115112
293	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox		0
294	lpd, gltA, pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	4.280626	6.225369
295	lpd, gltA, pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	4.26763	8.841531
296	lpd, gltA, pykA, pykF	None	pCASCADE-gltA2-zwf	pCDF-nox	3.855886	8.57874
297	lpd, gltA, pykA, pykF	None	pCASCADE-pykA-pykF	pCDF-nox		0
298	lpd, gltA, pykA, pykF	None	pCASCADE-zwf	pCDF-nox	8.447011	7.519215
299	lpd, gltA, zwf	None	pCASCADE-empty vector	pCDF-nox	4.822733	4.659195
300	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2	pCDF-nox	2.158706	5.196068
301	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.604957	3.843196
302	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox	2.101259	2.026306
303	lpd, gltA, zwf	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	1.141694	4.03982
304	lpd, gltA, zwf	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	2.991522	3.936369
305	lpd, gltA, zwf	None	pCASCADE-gltA1-zwf	pCDF-nox	2.303922	3.215522
306	lpd, gltA, zwf	None	pCASCADE-gltA2	pCDF-nox	2.227893	4.514656
307	lpd, gltA, zwf	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	2.153473	3.78413
308	lpd, gltA, zwf	None	pCASCADE-gltA2-zwf	pCDF-nox	1.858938	3.720182
309	lpd, gltA, zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	2.062681	4.223776
310	lpd, gltA, zwf	None	pCASCADE-pykA-pykF	pCDF-nox		0
311	lpd, gltA, zwf	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	5.10091	7.184178
312	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA1	pCDF-nox		0
313	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-gltA2	pCDF-nox		0
314	lpd, gltA, zwf, pykA, pykF	None	pCASCADE-zwf	pCDF-nox		0
315	lpd, zwf	None	pCASCADE-empty vector	pCDF-nox	3.295953	2.717124
316	lpd, zwf	None	pCASCADE-gltA1	pCDF-nox	2.004334	1.737009
317	lpd, zwf	None	pCASCADE-gltA1-gltA2	pCDF-nox	2.14836	5.742128
318	lpd, zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.615304	4.175747
319	lpd, zwf	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox	1.646336	2.637682
320	lpd, zwf	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	1.959566	6.973794
321	lpd, zwf	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	3.382341	3.928615
322	lpd, zwf	None	pCASCADE-gltA1-zwf	pCDF-nox	3.418304	4.087022
323	lpd, zwf	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox	1.429885	3.883446
324	lpd, zwf	None	pCASCADE-gltA2	pCDF-nox	1.17526	2.278122
325	lpd, zwf	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	0.99175	1.730411
326	lpd, zwf	None	pCASCADE-gltA2-zwf	pCDF-nox	1.303131	2.203132
327	lpd, zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	1.476491	2.806216
328	lpd, zwf	None	pCASCADE-pykA-pykF	pCDF-nox	3.372472	3.379772
329	lpd, zwf	None	pCASCADE-zwf	pCDF-nox	3.362499	3.15467
330	lpd, zwf	None	pCASCADE-zwf-pykA-pykF	pCDF-nox	3.89799	6.046395

331	None	None	pCASCADE-empty vector	pCDF-nox		0
332	None	None	pCASCADE-gltA1	pCDF-nox		0
333	None	None	pCASCADE-gltA1-gltA2	pCDF-nox	1.550647	3.857811
334	None	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	1.626857	3.941507
335	None	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox	2.067611	2.753914
336	None	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox		0
337	None	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox		0
338	None	None	pCASCADE-gltA1-zwf	pCDF-nox		0
339	None	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox		0
340	None	None	pCASCADE-gltA2	pCDF-nox	0.47467	0.945332
341	None	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	0.505176	0.87895
342	None	None	pCASCADE-gltA2-zwf	pCDF-nox	0.690594	1.171828
343	None	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	0.326887	0.602724
344	None	None	pCASCADE-pykA-pykF	pCDF-nox		0
345	None	None	pCASCADE-zwf	pCDF-nox		0
346	None	None	pCASCADE-zwf-pykA-pykF	pCDF-nox		0
347	pykA, pykF	None	pCASCADE-empty vector	pCDF-nox		0
348	pykA, pykF	None	pCASCADE-gltA1	pCDF-nox		0
349	pykA, pykF	None	pCASCADE-gltA1-gltA2	pCDF-nox		0
350	pykA, pykF	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox		0
351	pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox		0
352	pykA, pykF	None	pCASCADE-gltA1-gltA2-zwf-pykA-pykF	pCDF-nox	2.045377	4.620023
353	pykA, pykF	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox		0
354	pykA, pykF	None	pCASCADE-gltA1-zwf	pCDF-nox		0
355	pykA, pykF	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox		0
356	pykA, pykF	None	pCASCADE-gltA2	pCDF-nox	1.118483	1.983301
357	pykA, pykF	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox		0
358	pykA, pykF	None	pCASCADE-gltA2-zwf	pCDF-nox		0
359	pykA, pykF	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox		0
360	pykA, pykF	None	pCASCADE-pykA-pykF	pCDF-nox		0
361	pykA, pykF	None	pCASCADE-zwf	pCDF-nox		0
362	pykA, pykF	None	pCASCADE-zwf-pykA-pykF	pCDF-nox		0
363	zwf	None	pCASCADE-empty vector	pCDF-nox		0
364	zwf	None	pCASCADE-gltA1	pCDF-nox		0
365	zwf	None	pCASCADE-gltA1-gltA2	pCDF-nox	1.776522	2.064739
366	zwf	None	pCASCADE-gltA1-gltA2-pykA-pykF	pCDF-nox	0.459614	0.565033
367	zwf	None	pCASCADE-gltA1-gltA2-zwf	pCDF-nox		0
368	zwf	None	pCASCADE-gltA1-pykA-pykF	pCDF-nox	0.607424	0.776648
369	zwf	None	pCASCADE-gltA1-zwf	pCDF-nox	0.344144	0.527415
370	zwf	None	pCASCADE-gltA1-zwf-pykA-pykF	pCDF-nox		0
371	zwf	None	pCASCADE-gltA2	pCDF-nox	0.50789	0.668066
372	zwf	None	pCASCADE-gltA2-pykA-pykF	pCDF-nox	0.513826	0.584531
373	zwf	None	pCASCADE-gltA2-zwf	pCDF-nox	1.142363	2.518881
374	zwf	None	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	0.586813	1.446314
375	zwf	None	pCASCADE-pykA-pykF	pCDF-nox		0

376	zwf	None	pCASCADE-zwf	pCDF-nox		0
377	zwf	None	pCASCADE-zwf-pykA-pykF	pCDF-nox		0
378	gltA, zwf, pykA, pykF	ΔdhaL	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.492333	3.415342
379	gltA, zwf, pykA, pykF	Δepd	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	4.262333	3.280439
380	gltA, zwf, pykA, pykF	ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.902	3.753735
381	gltA, zwf, pykA, pykF	ΔmgsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.657667	3.569623
382	gltA, zwf, pykA, pykF	Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.542	3.76073
383	gltA, zwf, pykA, pykF	ΔdhaL, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.966333	3.411112
384	gltA, zwf, pykA, pykF	ΔdhaL, ΔmgsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.551667	3.371653
385	gltA, zwf, pykA, pykF	Δepd, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.804667	3.356804
386	gltA, zwf, pykA, pykF	Δepd, ΔmgsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.742333	3.491614
387	gltA, zwf, pykA, pykF	Δepd, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.356333	3.354152
388	gltA, zwf, pykA, pykF	ΔmgsA, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.457	3.303145
389	gltA, zwf, pykA, pykF	ΔptsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	4.002	3.158236
390	gltA, zwf, pykA, pykF	ΔmgsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.788	3.836733
391	gltA, zwf, pykA, pykF	ΔdhaL, Δepd, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.559667	3.613042
392	gltA, zwf, pykA, pykF	ΔdhaL, Δepd, ΔmgsA_0%	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	4.545333	2.131413
393	gltA, zwf, pykA, pykF	ΔdhaL, Δepd, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.779	3.88175
394	gltA, zwf, pykA, pykF	ΔdhaL, ΔmgsA, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.673	3.374992
395	gltA, zwf, pykA, pykF	ΔdhaL, ΔptsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	4.133	3.408303
396	gltA, zwf, pykA, pykF	ΔdhaL, ΔmgsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.637	2.858076
397	gltA, zwf, pykA, pykF	Δepd, ΔmgsA, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.959667	3.664137
398	gltA, zwf, pykA, pykF	Δepd, ΔptsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	4.402333	3.479784
399	gltA, zwf, pykA, pykF	Δepd, ΔmgsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.770333	3.760646
400	gltA, zwf, pykA, pykF	ΔptsA, ΔmgsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.573667	3.245052
401	gltA, zwf, pykA, pykF	ΔdhaL, Δepd, ΔmgsA, ΔptsA	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.398667	3.787656
402	gltA, zwf, pykA, pykF	ΔdhaL, ΔmgsA, ΔptsA, Δeda	pCASCADE-gltA2-zwf-pykA-pykF	pCDF-nox	3.482333	2.642224

[0084] Example 2: Production of Pyruvate in Fermentations using engineered *E. coli* strain DLF_01542 containing plasmids pCASCADE-gltA2-zf-pykA-pykF and pCDF-nox.

[0085] *E. coli* strain DLF_01542 (Genotype: (F-, *λ*-, *Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *rph-1, Δ(rhaD-rhaB)568, hsdR514, ΔldhA::frt, ΔpoxB::frt, ΔpflB::frt, ΔackA-pta::frt, ΔadhE::frt, ΔiclR, ΔarcA, ΔsspB, Δcas3::ugpBp-sspB-proB, lpd-DAS+4, gltA-DAS+4, zwf-DAS+4, pykA-DAS+4, pykF-DAS+4)* containing plasmids pCASCADE-gltA2-zf-pykA-pykF and pCDF-nox, was used to produce pyruvate in fully instrumented lab scale fermentations (Refer to Common Methods Section for methods). Biomass levels and pyruvate were measured as a function of time. Pyruvate was quantified using UPLC (Refer to Common Methods Section). Results are given in **Figure 2**.

[0086] Example 3: Purification of Pyruvic acid and salts thereof from fermentation broth

[0087] There are several well known methods for the purification of pyruvic acid and or its salts from aqueous broth. First broth may be clarified (removal of cells) by either centrifugation or filtration, such as ultrafiltration. Following clarification additional purification may include ion exchange chromatography (US Patent No: 4918013), such as a weakly-basic anion-exchange resin, which adsorbs the pyruvate anion. After rinsing out contaminants, the pyruvate can be eluted with a strong mineral acid such as sulfuric or hydrochloric acid (Japanese patent H06345683A). Alternatively, pyruvate in its acid form can be extracted into ether, the ether removed by vacuum evaporation and subsequently crystalized by addition of a miscible liquid. (US Patent No: 3993543). Yet another alternative purification involves complex-formation extraction using Tri-n-octanylamine (TOA) as an extractant. (Ma, C. Q., Li, J. C., Qiu, J. H., Wang, M., & Xu, P. (2005). *Recovery of pyruvic acid from biotransformation solutions. Applied Microbiology and Biotechnology, 70(3), 308–314.* doi:10.1007/s00253-005-0072-0).

[0088] Example 4: Production of pyruvate salts

[0089] Salts of pyruvate can be produced from clarified broth by extraction into an organic ester solvent such as tributyl phosphate, and then removed by addition of base to produce the metal pyruvate salts, which can be back-extraction into aqueous solution. (Chinese patent CN1103331C). Specifically, the addition of sodium hydroxide would lead to production of sodium pyruvate, the addition of magnesium hydroxide would lead to production of magnesium pyruvate, the addition of potassium hydroxide would lead to production of potassium pyruvate, addition of calcium hydroxide would lead to production of calcium pyruvate, and the addition of alternative hydroxides would lead to production of additional salts

of pyruvate.

[0090] Example 5: Conversion of pyruvic acid to pyruvic acid esters

[0091] Pyruvic acid, once produced, may be converted to the corresponding ester by reaction with an alcohol. For example, the addition of methanol to a mixture of pyruvic acid in the presence of an acid catalyst will result in the production of methyl pyruvate. As another example, the addition of ethanol to a mixture of pyruvic acid in the presence of an acid catalyst will result in the production of ethyl pyruvate. For example, Japanese Patent JPH1180088A teaches a method for the production of ethyl pyruvate by adding ethanol to pyruvic acid and heating in the presence of an acid catalyst. Since water reduces the yield of the reaction, 1,2-dichloroethane is added to enhance water removal. One skilled in the art may conduct this reaction with any alcohol and suitable catalyst to produce pyruvic acid esters

[0092] Esters of pyruvate may include for example polyol-pyruvate esters, pyruvate thioesters, glycerol-pyruvate esters or dihydroxyacetone-pyruvate esters. In vitro, a Fisher or Fischer-Speier esterification may be performed to produce a pyruvate ester from pyruvate. Methods of preparing a pyruvate ester by oxidation with hydrogen peroxide in the presence of a Ti--Si catalyst have been described (U.S. Patent No. 8,877,959; and Lopalco et al "Mechanism of Decarboxylation of pyruvic acid in the presence of hydrogen peroxide" J. Pharm Sci 2016 Feb; 105(2): 705-713. Doi:10.1002/jps.24653). Preparation of other pyruvate thiolesters has also been described (U.S. Patent No. 5,968,727).

COMMON METHODS SECTION

[0093] All methods in this Section are provided for incorporation into the Examples where so referenced. The names and city addresses of major suppliers are provided herein.

Subsection I. Microorganism Species and Strains, Cultures, and Growth Media

[0094] Microbial species, that may be used as needed, are as follows: *Escherichia coli strain BW25113* is obtained from the Yale Genetic Stock Center (www.cgsc.biology.yale.edu) and is obtained as an actively growing culture. Serial dilutions of the actively growing *E. coli K12* culture are made into Luria Broth (RPI Corp, Mt. Prospect, IL, USA) and are allowed to grow for aerobically for 24 hours at 37°C at 250 rpm until saturated.. *Escherichia coli strain BWapldf* was a generous gift from George Chen from Tsinghua University in China. Serial dilutions of the actively growing *E. coli BWapldf* is culture are made into Luria Broth (RPI Corp, Mt. Prospect, IL, USA) and are allowed to grow for aerobically for 24 hours at 37°C at 250 rpm until saturated.

[0095] Unless otherwise stated, all materials and reagents were of the highest grade possible and purchased from Sigma (St. Louis, MO). C13 labeled Alanine (2,3-13C2, 99%) (Item # CLM-2734-PK) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury,

MA). Luria Broth was used for routine strain and plasmid propagation and construction. Working antibiotic concentrations were as follows: ampicillin (100 µg/mL), kanamycin (35 µg/mL), chloramphenicol (35 µg/mL), spectinomycin (100 µg/mL), zeocin (50 µg/mL), gentamicin (10 µg/mL), blasticidin (100 µg/mL), puromycin (150 µg/mL), tetracycline (5 µg/mL). Luria broth with low salt (Lennox formulation) was used to select for zeocin, blasticidin and puromycin resistant clones. In addition, for puromycin selection, phosphate buffer (pH=8.0) was added to LB Lennox to a final concentration of 50 mM. Media formulations including stock solutions are described in the Tables below.

[0096] Stock Solutions are prepared as follows.

- 10X concentrated Ammonium-Citrate 30 salts (1L), mix 30 g of $(NH_4)_2SO_4$ and 1.5 g citric acid in water with stirring, adjust pH to 7.5 with 10 M NaOH. Autoclave and store at room temperature (RT).
- 10X concentrated Ammonium-Citrate 90 salts (1L), mix 90 g of $(NH_4)_2SO_4$ and 2.5 g citric acid in water with stirring, adjust pH to 7.5 with 10 M NaOH. Autoclave and store at RT.
- 10X concentrated Ammonium-Citrate 90 salts (1L), mix 90 g of $(NH_4)_2SO_4$ and 2.5 g citric acid in water with stirring, adjust pH to 7.5 with 10 M NaOH. Autoclave and store at RT.
- 1 M Potassium 3-(N-morpholino) propanesulfonic Acid (MOPS), adjust to pH 7.4 with 50% KOH. Filter sterilize (0.2 µm) and store at RT.
- 0.5 M potassium phosphate buffer, pH 6.8, mix 248.5 mL of 1.0 M K_2HPO_4 and 251.5 mL of 1.0 M KH_2PO_4 and adjust to a final volume of 1000 mL with ultrapure water. Filter sterilize (0.2 µm) and store at RT.
- 2 M $MgSO_4$ and 10 mM $CaSO_4$ solutions. Filter sterilize (0.2 µm) and store at RT.
- 50 g/L solution of thiamine-HCl. Filter sterilize (0.2 µm) and store at 4°C.
- 500 g/L solution of glucose, dissolve by stirring with heat. Cool, filter sterilize (0.2 µm), and store at RT.
- 100 g/L yeast extract, autoclave, and store at RT.
- 100 g/L casamino acid, autoclave, and store at RT.
- 500X Trace Metal Stock: Prepare a solution of micronutrients in 1000 mL of water containing 10 mL of concentrated H_2SO_4. 0.6 g $CoSO_4 \cdot 7H_2O$, 5.0 g $CuSO_4 \cdot 5H_2O$, 0.6 g $ZnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g H_3BO_3, and 0.3 g $MnSO_4 \cdot H_2O$. Filter sterilize (0.2 µm) and store at RT in the dark.
- Prepare a fresh solution of 40 mM ferric sulfate heptahydrate in water, filter sterilize

(0.2 μm) before preparing media each time.

[0097] **Table CM1:** Seed Media, pH 6.8:

Ingredient	Unit	SM10	SM10++
$(NH_4)_2SO_4$	g/L	9	9
Citric Acid	g/L	0.25	0.25
Potassium Phosphate	mM	5	5
$CoSO_4 \cdot 7H_2O$	g/L	0.0048	0.0048
$CuSO_4 \cdot 5H_2O$	g/L	0.04	0.04
$ZnSO_4 \cdot H_2O$	g/L	0.0048	0.0048
$Na_2MoO_4 \cdot 2H_2O$	g/L	0.0016	0.0016
H_3BO_3	g/L	0.0008	0.0008
$MnSO_4 \cdot H_2O$	g/L	0.0024	0.0024
$FeSO_4 \cdot 7H_2O$	g/L	0.044	0.044
$MgSO_4$	mM	2.5	2.5
$CaSO_4$	mM	0.06	0.06
Glucose	g/L	45	45
MOPS	mM	200	200
Thiamine-HCl	g/L	0.01	0.01
Yeast Extract	g/L	1	2.5
Casamino Acids	g/L	0	2.5

[0098] **Table CM2:** Production/Wash Media, pH 6.8:

Ingredient	Unit	FGM3	FGM3 No Phosphate	FGM3 Wash	FGM3+40 mM phosphate	FGM10
$(NH_4)_2SO_4$	g/L	3	3	3	3	9
Citric Acid	g/L	0.15	0.15	0.15	0.15	0.25
Potassium Phosphate	mM	1.8	0	0	40	5
$CoSO_4 \cdot 7H_2O$	g/L	0.0024	0.0024	0	0.0024	0.0048
$CuSO_4 \cdot 5H_2O$	g/L	0.02	0.02	0.00	0.02	0.04
$ZnSO_4 \cdot H_2O$	g/L	0.0024	0.0024	0	0.0024	0.0048
$Na_2MoO_4 \cdot 2H_2O$	g/L	0.0008	0.0008	0	0.0008	0.0016
H_3BO_3	g/L	0.0004	0.0004	0	0.0004	0.0008
$MnSO_4 \cdot H_2O$	g/L	0.0012	0.0012	0	0.0012	0.0024
$FeSO_4 \cdot 7H_2O$	g/L	0.022	0.022	0	0.022	0.044
$MgSO_4$	mM	2	2	0	2	2.5
$CaSO_4$	mM	0.05	0.05	0	0.05	0.06
Glucose	g/L	45	25	0	45	25

MOPS	mM	200	200	0	200	0
Thiamine-HCl	g/L	0.01	0.01	0	0.01	0.01

[0099] **Table CM3:** Fermentation Media, pH 6.8:

Ingredient	FLNM10	FLNM10_S	FLNM10_P	Unit
NH4Cl	2	2	2.486	g/L
Citric Acid	1.7	1.7	1.7	g/L
(NH4)2HPO4	1	1	0.4	g/L
CoSO4·7H2O	0.002	0.002	0.002	g/L
CuSO4·5H2O	0.002	0.002	0.002	g/L
ZnSO4·7H2O	0.0137	0.0137	0.0137	g/L
Na2MoO4·2H2O	0.012	0.012	0.012	g/L
H3BO3	0.003	0.003	0.003	g/L
MnSO4·H2O	0.0038	0.0038	0.0038	g/L
FeSO4·7H2O	0.3	0.3	0.3	g/L
MgSO4 7H2O	1.4	1.4	1.4	g/L
CaCl2 2H2O	0.02	0.02	0.02	g/L
glucose	25	25	25	g/L
Thiamine-HCl	0.01	0.01	0.01	g/L
H2SO4		0.384		mL

Subsection II. Strain Construction

[00100] Oligonucleotides and synthetic linear DNA (G-blocks™) used for strain construction and confirmation are all given in Tables CM4 –CM8 below. and they were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Strain BW25113 was obtained from the Yale Genetic Stock Center (CGSC http://cgsc.biology.yale.edu/). Strain BWapldf was a kind gift from George Chen (Tsinghua University). Chromosomal modifications were made using standard recombineering methodologies either with direct antibiotic cassette integration in the case of C-terminal DAS+4 tags carrying antibiotic resistance cassettes, or through scarless tet-sacB selection and counterselection. The recombineering plasmid pSIM5 and the tet-sacB selection/counterselection marker cassette were kind gifts from Donald Court (NCI, https://redrecombineering.ncifcrf.gov/court-lab.html). Briefly, the tet-sacB selection/counterselection cassette was amplified using the appropriate oligos supplying ~50 bp flanking homology sequences using Econotaq (Lucigen Middleton, WI) according to manufacturer's instructions, with an initial 10 minutes denaturation at 94 °C, followed by 35 cycles of 94 °C, for 15 seconds, 52 °C for 15 seconds, and 72 °C for 5 minutes. Cassettes used for "curing" of the tet-sacB cassette or direct integration (when an antibiotic marker is present) were obtained as G-blocks™ from IDT. In the case of the *sspB* gene deletion, the open reading frame deletion replaced with a kanamycin resistance was amplified from the

Keio Collection strain, JW3197-1 (the keio Collection), and moved to the appropriate background strain using standard methodologies. The kanamycin resistance cassette was cured using the pCP20 plasmid, leaving an frt scar. Chromosomal modifications were confirmed by PCR amplification and sequencing (Eton Biosciences) using paired oligonucleotides, either flanking the entire region, or in the case of DAS+4 tag insertions an oligo 5' of the insertion and one internal to the resistance cassette. Oligos and DNA sequences for DAS+4 tagging and tet-secB based genome engineering are given in Tables CM4 and CM5.

[00101] Table CM4: Oligonucleotides used for strain construction.

Oligo	Sequence	SEQ ID NO
ilcR_tetA_F	TAACAATAAAAATGAAAATGATTTCCACGATACAGAAAAAAGAGACTG TCATCCTAATTTTTGTTGACACTCTATC	1
ilcR_sacB_R	TGCCACTCAGGTATGATGGGCAGAATATTGCCTCTGCCCGCCAGAAAAA GATCAAAGGGAAAACTGTCCATATGC	2
iclR_500up	CCGACAGGGATTCCATCTG	3
iclR_500dn	TATGACGACCATTTTGTCTACAGTTC	4
arcA_tetA_F	GGACTTTTGTACTTCCTGTTTCGATTTAGTTGGCAATTTAGGTAGCAAAC TCCTAATTTTTGTTGACACTCTATC	5
arcA_sacB_R	ATAAAAACGGCGCTAAAAAGCGCCGTTTTTTTTGACGGTGGTAAAGCCG AATCAAAGGGAAAACTGTCCATATGC	6
arcA_500up	CCTGACTGTACTAACGGTTGAG	7
arcA_500dn	TGACTTTTATGGCGTTCTTTGTTTTTG	8
sspB_kan_F	CTGGTACACGCTGATGAACACC	9
sspB_kan_R	CTGGTCATTGCCATTTGTGCC	10
sspB_conf_F	GAATCAGAGCGTTCCGACCC	11
sspB_conf_R	GTACGCAGTTTGCCAACGTG	12
cas3_tetA_F	AATAGCCCGCTGATATCATCGATAATACTAAAAAAACAGGGAGGCTAT TATCCTAATTTTTGTTGACACTCTATC	13
cas3_sacB_R	TACAGGGATCCAGTTATCAATAAGCAAATTCATTTGTTCTCCTTCATATG ATCAAAGGGAAAACTGTCCATATGC	14
cas3_conf_F	CAAGACATGTGTATATCACTGTAATTC	15
cas3_500dn	GCGATTGCAGATTTATGATTTGG	16
gltA_conf_F	TATCATCCTGAAAGCGATGG	17
lpd_conf_F	ATCTCACCGTGTGATCGG	18
udhA_conf_F	CAAAAGAGATTCTGGGTATTCACT	19
zwf_conf_F	CTGCTGGAAACCATGCG	20
zwf_500dn	AGAGCATGTCGTTATAGGAGGTGAT	21
ampR_intR	AGTACTCAACCAAGTCATTCTG	22
bsdR_intR	GAGCATGGTGATCTTCTCAGT	23
gentR_intR	GCGATGAATGTCTTACTACGGA	24
purR_intR	GTCGCTGGGTAATCTGCAA	25
tetA_intR	ATCAACGCATATAGCGCTAGCAG	26
zeoR_intR	ACTGAAGCCCAGACGATC	27
tetR_intR	ATCAACGCATATAGCGCTAGCAG	28
specR_intR	CACTGTGTGGCTTCAGGC	29
ampR_intR	AGTACTCAACCAAGTCATTCTG	30
purR_intR	GTCGCTGGGTAATCTGCAA	31

PykA-FOR1	CCTGACTGCTCTCTATCG	32
PykA-FOR2	CGAAGCGGTTAATCTGCTG	33
PykF-FOR1	CGGCTCATCAGTTGGTACTG	34
PykF-FOR2	GGGTAAAGAACTGGCTCTGC	35

[00102] **Table CM5:** Synthetic DNA used for strain construction.

tetA-sacB Cassette SEQ ID NO 36
TCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGAA
AAGTGAAATGAATAGTTCGACAAAGATCGCATTGGTAATTACGTTACTCGATGCCATGGGGATTG
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GTCTGACCGATTTGGTCGGCGCCCAGTGCTGTTGTTGTCATTAATAGGCGCATCGCTGGATTACTT
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GCTACTGGGGCTGTCGCGGCATCGGTCATTGCCGATACCACCTCAGCTTCTCAACGCGTGAAGTGG
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Δcas3::ugBp-sspB-pro-casA SEQ ID NO 39
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gltA-DAS+4-zeoR SEQ ID NO 42

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lpd-DAS+4-gentR SEQ ID NO 43

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zwf-DAS+4-bsdR SEQ ID NO 44

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CCATTGAGGAACTCATCCCCCTCAAGTACACCAGGAACTAAAGTAATATCTGCGCTTATCCTTTAT
GGTTATTTTACCGGTAACATGATCTTGCGCAGATTGTAGAACAATTTTTACACTTTCAGGCCTCGTG
CGGATTCACCCACGAGGCTTTTTTTATTACACTGACTGAAACGTTTTTGCCCTATGAGCTCCGGTTA
CAGGCGTTTCAGTCATAAATCCTCTGAATGAAACGCGTTGTGAATC

pykA-DAS+4:ampR SEQ ID NO 45

TGAACCTGACTGCTCTCTATCGTGGCGTTACGCCGGTGCACTTTGATAGCGCTAATGACGGCGTAG
CAGCTGCCAGCGAAGCGGTTAATCTGCTGCGCGATAAAGGTTACTTGATGTCTGGTGACCTGGTGA
TTGTCACCCAGGGCGACGTGATGAGTACCGTGGGTTCTACTAATACCACGCGTATTTTAACGGTAG
AGGCGGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCTTAATAGTCCTGACGGATG
GCCTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCA
TGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACAT
TTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCT
GGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCA
ACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAG
TTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATAC
ACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGA

CAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTG ACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCG CCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGC CTACAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGC AACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCG GCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCA CTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTAT GGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAG ACTAAGTACGTTGCCGGATGCGGCGAAAACGCCACATCCGGCCTACAGTTCAATGATAGTTCAAC AGATTTCGAATATTCTGAAGCAAACTTGAACTTATCATCAGGCGAAGGCCTCTCCTCGCGAGAGGC TTTTTTATTTGATGGGATAAAGATCTTTGCGCTTATACGGCTGGATTTCGCCCGGTTTGCGAGTTTT CAGCAAT
pykF-DAS+4:purr SEQ ID NO 46
AAACGGCTCATCAGTTGGTACTGAGCAAAGGCGTTGTGCCGCAGCTTGTTAAAGAGATCACTTCTA CTGATGATTTCTACCGTCTGGGTAAAGAACTGGCTCTGCAGAGCGGTCTGGCACACAAAGGTGAC GTTGTAGTTATGGTTTCTGGTGCACTGGTACCGAGCGGCACTACTAACACCGCATCTGTTCACGTC CTGGCGGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCTTAATCCTGACGGATGGC CTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATG AGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGACTGAATACAAGCC CACGGTACGCTTGGCGACGCGCGACGATGTTCCCCGCGCTGTTCGTACATTAGCTGCGGCCTTTGC AGATTACCCAGCGACGCGCCATACGGTCGATCCGGACCGCCATATCGAGCGTGTCACAGAATTGC AGGAACTTTTCTTAACTCGCGTGGGCCTTGACATCGGAAAGGTCTGGGTGGCTGACGATGGCGCTG CAGTGGCTGTTTGGACCACTCCGGAGAGTGTAGAGGCTGGTGCAGTGTTCGCCGAAATTGGTCCTC GTATGGCCGAATTAAGTGGAAGTCGTCTGGCAGCCCAACAACAAATGGAAGGGTTGCTTGCGCCC CACCGTCCGAAAGAACCCGCGTGGTTCCTTGCCACCGTTGGAGTAAGCCCAGATCACCAGGGGAA GGGTTTAGGATCTGCCGTAGTTTTACCAGGTGTGGAGGCAGCAGAACGTGCGGGAGTTCCGGCCT TCCTTGAGACGTCGGCGCCGCGCAATTTACCGTTTTACGAACGTCTTGGATTCACCGTTACGGCGG ACGTGGAGGTGCCGGAGGGACCCCGTACTTGGTGTATGACTCGTAAACCGGGAGCCTGATAATAT TGCTTTTGTGAATTAATTTGTATATCGAAGCGCCCTGATGGGCGCTTTTTTTATTTAATCGATAACC AGAAGCAATAAAAAATCAAATCGGATTTCACTATATAATCTCACTTTATCTAAGATGAATCCGATG GAAGCATCCTGTTTTCTCTCAATTTTTTTATCTAAAACCCAGCGTTCGATGCTTCTTTGAGCGAACG ATCAAAAATAAGTGCCTTCCCATCAAAAAAATATTCTCAACATAAAAAACTTTGTGTAATACTTGT AACGCTACATGGAGATTAACTCAATCTAGAGGGTATTAATAATGAAAGCTACTAAACTGG

[00103] In the case of deletions for the following genes: mgsA, ptsA, epd, dhaL, and eda, lambda red recombineering along with a CRISPR-cas based gene deletion methodology was used as recently described by Moreb et al, 2017 (doi: 10.1021/acssynbio.7b00174) . Briefly, gRNA expression plasmids were constructed to express gRNAs to target a given locus, donor DNA ordered to delete genes using recombineering, and primer s for locus confirmation by PCR and sequencing. These sequences are given in Table CM6, CM7 and CM8.

[00104] **Table CM6.** Primers to construct gRNA Cas9 cutting plasmids.

Name	Sequence	SEQ ID NO
AM512_gRNAmgsA	CTGTATGCAACAGGCACTACGTTTTAGAGCTAGAAATAGCAAG	47
AM225_sgRNA_F3	GTGCTCAGTATCTCTATCACTGA	48
AM514_gRNAptsA	ATGTGTTCTGATTTGCTGTGGTTTTAGAGCTAGAAATAGCAAG	49
AM225_sgRNA_F3	GTGCTCAGTATCTCTATCACTGA	50
AM536_gRNAepd	TAAATGGCTTCGGTCGCATCGTTTTAGAGCTAGAAATAGCAAG	51
AM225_sgRNA_F3	GTGCTCAGTATCTCTATCACTGA	52
AM537_gRNAdhaL	CTTTATCAGATGTTCCGCGAGTTTTAGAGCTAGAAATAGCAAG	53
AM225_sgRNA_F3	GTGCTCAGTATCTCTATCACTGA	54
AM550_gRNAeda	GGTGCTGAATCCACAGCAGCGTTTTAGAGCTAGAAATAGCAA G	55

AM225_sgRNA_F3	GTGCTCAGTATCTCTATCACTGA	56

[00105] **Table CM7.** Donor DNA sequences to delete genes.

Name	Sequence	SEQ ID NO:
AM519_DONmgsA	GCAGCATAAGTGCTTACAGTAATCTGTAGGAAAGTTAACTACGGATCCCCG ATTATCAGCGTTATCTCGCGGACCGTCTGAAGTAA	57
AM549_DONPtsA2	CCTTTTACAGTTCCAGTTCATGTTGCAGCAGGCTGGCGATAGCGTTTTGCGG CATGCTTCCGGTTTATCGCAAGTTATGAGGCGGATCGC	58
AM538_DONepd	ACGCATCATCTAACAATTTGATGACGGGAATTATGCAATTCGTGGAATTGTC GAACATTTAATCGACTGAAACGCTTCAGCTAGGATAAG	59
AM539_DONdhaL	GATTGACTATCGAACGTAATTTAATTGGCGCGTACTGCACCTCACGACTGGG AGAAGGTGTCGGTGAATTAGCCCGTCAGATGTTAATGA	60
AM551_DONeda	GCCTTCTACAGCTTCACGCGCCAGCTTAGTAATGCGGTCGTAATCGCCTGAT TACAAATTTGTCGTCTTAAAAAGTGATACAGGTTGCGC	61

[00106] **Table CM8.** Primers to confirm gene deletions.

Name	Sequence	SEQ ID NO
AM525_byemgsA_F	CTGACCCACAAACGCGAAAT	62
AM526_byemgsA_R	GGTGGCGAGAAAACCGTAAG	63
AM547_byeptsA_F2	CGCCCGTCATTAAATGCTGA	64
AM548_byeptsA_R2	GGCTAATAACCCTTGTGCGG	65
AM540_byeEpd_F	TTCGGCTGGACAAACATTCC	66
AM541_byeEpd_R	AACCTGTTGATCGTGCATGG	67
AM542_byeDhaL_F	CGTCTATAACCGCCTGACCA	68
AM543_byeDhaL_R	TTGTGGATCGTCAATTCCCG	69
AM552_byeEda_F	CTGGTAGACGAAGCGGAACT	70
AM553_byeEda_R	CCTCGATCGGGCATTTTGAC	71

Subsection III. Plasmid Construction

pCASCADE Plasmid Construction

[00107] Gene silencing guide arrays were expressed from a series of pCASCADE plasmids. The pCASCADE-control plasmid was prepared by swapping the pTet promoter in pcrRNA.Tet (Luo et al, 2015, NAR. doi: 10.1093/nar/gku971) with an insulated low phosphate induced ugpB gene promoter. Promoter sequences for all genes were obtained from EcoCyc database (https://ecocyc.org/). In order to design CASCADE guide array, CASCADE PAM sites near the -35 or -10 box of the promoter of interest were identified, 30 bp at the 3' end of PAM site was selected as the guide sequence and cloned into pCASCADE plasmid using Q5 site-directed mutagenesis (NEB, MA) following manufacturer's protocol, with the modification that 5% v/v DMSO was added to the Q5 PCR reaction. PCR cycles were as follows: amplification involved an initial denaturation step at 98 °C for 30 second followed by cycling at 98 °C for 10 second, 72 °C for 30 second, and 72 °C for 1.5 min (the extension rate was 30 second/kb) for 25

cycles, then a final extension for 2 min at 72 °C. 2 µL of PCR mixture was used for 10 µL KLD reaction, which proceeded under room temperature for 1 hour, after which, 1 µL KLD mixture was used for electroporation.

[00108] The pCASCADE guide array plasmids were prepared by sequentially amplifying complementary halves of each smaller guide plasmid by PCR, followed by subsequent DNA assembly. The pCASCADE-control vector was used as template. pCASCADE plasmids with arrays of two or more guides were prepared using Q5 High-Fidelity 2X Master Mix (NEB, MA). PCR cycles were as follows: amplification involved an initial denaturation step at 98 °C for 30 second followed by cycling at 98 °C for 10 second, 66 °C for 30 second, and 72 °C for 45 second (the extension rate was 30 second/kb) for 35 cycles, then a final extension for 2 min at 72 °C. PCR product was purified by gel-extraction, 20 µL ultrapure water was used to elute 50 µL PCR reaction purification. 1 µL of each eluted PCR product was used for 10 µL of Gibson Assembly (NEB, MA), which was completed by incubation at 50 °C for 15 min. 1 µL Gibson Assembly mix was used for electroporation. Sequence information for silencing guides is given in Table CM9 and CM10.

[00109] **Table CM9:** List of pCASCADE plasmids used in this study. Sequences available in Addgene where submitted.

Silencing Plasmid	Promoter(s) Silenced	Addgene #
pCASCADE-control	none	65821
pCASCADE-gltA1	gltAp1	71334
pCASCADE-gltA2	gltAp2	65817
pCASCADE-zwf	zwf	65825
pCASCADE-gltA1-gltA2	gltAp1, gltAp2	71348
pCASCADE-gltA1-zwf	gltAp1, zwf	71337
pCASCADE-gltA2-zwf	gltAp2, zwf	71338
pCASCADE-pykA-pykF	pykA, pykF	NA
pCASCADE-gltA1-pykA-pykF	gltAp1, pykA, pykF	NA
pCASCADE-gltA2-pykA-pykF	gltAp2, pykA, pykF	NA
pCASCADE-gltA1-gltA2-pykA-pykF	gltAp1, gltAp2, pykA, pykF	NA
pCASCADE-gltA1-gltA2-zwf-pykA-pykF	gltAp1, gltAp2,zwf,pykA, pykF	NA
pCASCADE-gltA1-zwf-pykA-pykF	gltAp1, zwf, pykA, pykF	NA
pCASCADE-gltA2-zwf-pykA-pykF	gltAp2, zwf, pykA, pykF	NA

[00110] **Table CM10:** List of sgRNA guide array sequences and primers used to construct them from given templates. Sequences for guide arrays containing guides with pykA or pykF gRNAs are given. Spacers are italicized.

sgRNA/Primer Name	Sequence	SEQ ID NO	Template
pykA	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*TGACGATCGCTAAAAACGACTGTCAC TGTC*TCGAGTTCCCCGCGCCAGCGGGGAT AAACCG*	72	

pykA-sgRNA-FOR	ACGACTGTCACTGTCTCGAGTTCCCCGCG CCAGCGGGGATAAACCGAAAAAAAAAC CCC	73	pCASCADE control
pykA-sgRNA-REV	TTTTAGCGATCGTCACGGTTTATCCCCGC TGGCGCGGGGAACTCGAGGTGGTACCAG ATC	74	
pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*CACCACCACTTTCGTAATACCGGATTC GCT*TCGAGTTCCCCGCGCCAGCGGGGATA AACCG*	75	
pykF-sgRNA-FOR	AATACCGGATTCGCTTCGAGTTCCCCGCG CCAGCGGGGATAAACCGAAAAAAAAAC CCC	76	pCASCADE control
pykF-sgRNA-REV	ACGAAAGTGGTGGTGCGGTTTATCCCCG CTGGCGCGGGGAACTCGAGGTGGTACCA GATC	77	
pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*TGACGATCGCTAAAAACGACTGTCAC TGTC*TCGAGTTCCCCGCGCCAGCGGGGAT AAACCG*CACCACCACTTTCGTAATACCGG ATTCGCT*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*	78	
pykF-FOR	AATACCGGATTCGCTTCGAGTTCCCCGCG CCAGCGGGGATAAACCGAAAAAAAAAC CCC	79	pCASCADE-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-pykA
pykA-REV	ACGAAAGTGGTGGTGCGGTTTATCCCCG CTGGCGCGGGGAACTCGAGGTGGTACCA GATC	82	
gltA1-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*AAAAGCATATAATGCGTAAAAGTTAT GAAGT*TCGAGTTCCCCGCGCCAGCGGGGA TAAACCG*TGACGATCGCTAAAAACGACTG TCACTGTC*TCGAGTTCCCCGCGCCAGCGG GGATAAACCG*CACCACCACTTTCGTAATA CCGGATTCGCT*TCGAGTTCCCCGCGCCAG CGGGGATAAACCG*	83	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	84	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-gltA1
gltA1-REV	CGGTTTATCCCCGCTGGCGCGGGGAACT CGAACTTCATAACTTTTAC	85	
gltA2-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*TATTGACCAATTCATTCGGGACAGTTA TTAGT*TCGAGTTCCCCGCGCCAGCGGGGA TAAACCG*TGACGATCGCTAAAAACGACTG TCACTGTC*TCGAGTTCCCCGCGCCAGCGG GGATAAACCG*CACCACCACTTTCGTAATA CCGGATTCGCT*TCGAGTTCCCCGCGCCAG CGGGGATAAACCG*	86	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	87	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-gltA2
gltA2-REV	CGGTTTATCCCCGCTGGCGCGGGGAACT CGAACTAATAACTGTC	88	

zwf-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*CTCGTAAAAGCAGTACAGTGCACCGT AAGA*TCGAGTTCCCCGCGCCAGCGGGGAT AAACCG*TGACGATCGCTAAAAACGACTG TCACTGTC*TCGAGTTCCCCGCGCCAGCGG GGATAAACCG*CACCACCACTTTCGTAATA CCGGATTCGCT*TCGAGTTCCCCGCGCCAG CGGGGATAAACCG*	89	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	90	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-zwf
zwf-REV	CGGTTTATCCCCGCTGGCGCGGGGAACT CGATCTTACGGTGCACTGTAC	91	
gltA1-gltA2-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*AAAAGCATATAATGCGTAAAAGTTAT GAAGT*TCGAGTTCCCCGCGCCAGCGGGGA TAAACCG*TATTGACCAATTCATTCGGGAC AGTTATTAGT*TCGAGTTCCCCGCGCCAGC GGGGATAAACCG*TGACGATCGCTAAAAA CGACTGTCACTGTC*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*CACCACCACTTTC GTAATACCGGATTCGCT*TCGAGTTCCCCG CGCCAGCGGGGATAAACCG*	92	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	93	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-gltA1-gltA2
gltA2-REV	CGGTTTATCCCCGCTGGCGCGGGGAACT CGAACTAATAACTGTC	94	
gltA1-zwf-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*AAAAGCATATAATGCGTAAAAGTTAT GAAGT*TCGAGTTCCCCGCGCCAGCGGGGA TAAACCG*CTCGTAAAAGCAGTACAGTGCA CCGTAAGA*TCGAGTTCCCCGCGCCAGCGG GGATAAACCG*TGACGATCGCTAAAAACG ACTGTCACTGTC*TCGAGTTCCCCGCGCCA GCGGGGATAAACCG*CACCACCACTTTCGT AATACCGGATTCGCT*TCGAGTTCCCCGCG CCAGCGGGGATAAACCG*	95	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	96	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-gltA1-zwf
zwf-REV	CGGTTTATCCCCGCTGGCGCGGGGAACT CGATCTTACGGTGCACTGTAC	97	
gltA2-zwf-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*TATTGACCAATTCATTCGGGACAGTTA TTAGT*TCGAGTTCCCCGCGCCAGCGGGGA TAAACCG*CTCGTAAAAGCAGTACAGTGCA CCGTAAGA*TCGAGTTCCCCGCGCCAGCGG GGATAAACCG*TGACGATCGCTAAAAACG ACTGTCACTGTC*TCGAGTTCCCCGCGCCA GCGGGGATAAACCG*CACCACCACTTTCGT AATACCGGATTCGCT*TCGAGTTCCCCGCG CCAGCGGGGATAAACCG*	98	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	99	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	

pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-gltA2-zwf
zwf-REV	CGGTTTATCCCCGCTGGCGCGGGGAACT CGATCTTACGGTGCACTGTAC	100	
gltA1-gltA2-zwf-pykA-pykF	*TCGAGTTCCCCGCGCCAGCGGGGATAAAC CG*AAAAGCATATAATGCGTAAAAGTTAT GAAGT*TCGAGTTCCCCGCGCCAGCGGGGA TAAACCG*TATTGACCAATTCATTCGGGAC AGTTATTAGT*TCGAGTTCCCCGCGCCAGC GGGGATAAACCG*CTCGTAAAAGCAGTAC AGTGCACCGTAAGA*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*TGACGATCGCTAA AAACGACTGTCACTGTC*TCGAGTTCCCCG CGCCAGCGGGGATAAACCG*CACCACCACT TTCGTAATACCGGATTCGCT*TCGAGTTCC CCGCGCCAGCGGGGATAAACCG*	101	
pykA-FOR	GCGCCAGCGGGGATAAACCGTGACGATC GCTAAAAAC	102	pCASCADE-pykA-pykF
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	80	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	81	pCASCADE-gltA1-gltA2-zwf
zwf-REV	CGGTTTATCCCCGCTGGCGCGGGGAA CTCGATCTTACGGTGCACTGTAC	103	

pCDF-nox Plasmid Construction

[00111] NADH oxidase from *Streptococcus mutans* (UniProt Q54453) was codon optimized using the Codon Optimization Tool from the IDT website, phosphorylated G-blocks™ were designed and purchased from IDT. pCDF-nox was assembled using NEBuilder® HiFi DNA Assembly Master Mix following manufacturer's protocol (NEB, MA) with the G-blocks™ and a PCR product of pCDF-1b containing only the origin and spectinomycin resistance marker. Plasmid sequence was confirmed by DNA sequencing (Eton Bioscience, NC).

Subsection IV. Microfermentations

[00112] Plasmids were transformed into host strains by electroporation using ECM 630 High Throughput Electroporation System (Harvard Apparatus, Inc. Holliston, MA) following manufacturer's protocol or using individual electroporation cuvettes. Glycerol stocks were prepared for each transformation plate by adding equal volume of sterile 20% glycerol, and 3 μL were used to inoculate overnight culture in 150 μL SM10++ medium with appropriate antibiotics. Plates were covered with sandwich covers (Model # CR1596 obtained from EnzyScreen, Haarlam, The Netherlands). These covers ensured minimal evaporative loss during incubation. Unless otherwise stated, 96 well plates were cultured at 37 □, 400 rpm for 16 hours, shaker orbit is 25 mm. This combination of orbit and minimal shaking speed is required to obtain needed mass transfer coefficient and provide adequate culture oxygenation.

[00113] After 16 hours of growth, cells were pelleted by centrifugation, excess media was removed and cells were resuspended in 150 μL of FGM3 Wash solution. Subsequently cells

were once again pelleted and again excess media was removed, pellet was resuspended in 50 µL FGM3 No Phosphate media containing appropriate antibiotics. 5 µL of the resuspended culture was added to 195 µL of water for OD600 measurement using standard flat bottom 96 well plate. OD600 for production was normalized to OD600 = 1, using FGM3 No Phosphate media containing appropriate antibiotics, in a total volume of 150 µL using standard 96 well plate. Plates were covered with sandwich covers (Model # CR1596 obtained from EnzyScreen, Haarlam, The Netherlands) and 96 well plate cultures were incubated at 37 □, 400 rpm for 24 hours. After 24 hours of production, all samples from each well were pelleted by centrifugation and the supernatant collected for subsequent analytical measurement. Triplicate microfermentations were performed for each strain.

Subsection V. Lab Scale Fermentations

[00114] *1L Fermentation Seeds:* Single colony from transformation plate was inoculated into 5 mL LB with appropriate antibiotics and cultured at 37 □, 220 rpm for 16 hours. 500 µL of the LB culture was inoculated into 50 mL FLNM10 media supplemented with 200mM MOPS buffer pH = 7.4, with appropriate antibiotics in square shake flask (CAT#: 25-214, Genesee Scientific, Inc. San Diego, CA), the culture was incubated at 37 □ with a shaking speed of 220 rpm for 24 hours, at which time OD600 is usually between 3 and 10, the culture was harvested by centrifugation at 4000 rpm for 15 min, supernatant was discarded and cell culture was normalized to OD600 = 10 using SM10 media. For 1 L fermentation seed, 6 mL of normalized OD600 = 10 culture was added to 1.5 mL of 50% glycerol in cryovials, and stored at -80 □.

[00115] *1L Fermentations*: An Infors-HT Multifors (Laurel, MD, USA) parallel bioreactor system was used to perform 1L fermentations, including three gas connection mass flow controllers configured for air, oxygen and nitrogen gases. Vessels used had a total volume of 1400 mL and a working volume of up to 1L. Online pH and pO2 monitoring and control were accomplished with Hamilton probes. Offgas analysis was accomplished with a multiplexed Blue-in-One BlueSens gas analyzer (BlueSens. Northbrook, IL, USA). Culture densities were continually monitored using Optek 225 mm OD probes, (Optek, Germantown, WI, USA). The system used was running IrisV6.0 command and control software and integrated with a Seg-flow automated sampling system (Flownamics, Rodeo, CA, USA), including FISP cell free sampling probes, a Segmod 4800 and FlowFraction 96 well plate fraction collector.

[00116] For the standardized 2-stage process with ~ 10 gcdw/L biomass, tanks were filled with 800 mL of FLNM10 medium (Table CM3). Antibiotics were added as appropriate. Frozen seed vials were thawed on ice and 7.5 mL of seed culture was used to inoculate the tanks. After inoculation, tanks were controlled at 37 □ and pH 6.8 using 5 M ammonium hydroxide and 1 M

hydrochloric acid as titrants. 10 M ammonium hydroxide was used as the base fermentation run. The following oxygen control scheme was used to maintain the desired dissolved oxygen set point. First gas flow rate was increased from a minimum of 0.3 L/min of air to 0.8 L/min of air, subsequently, if more aeration was needed, agitation was increased from a minimum of 300 rpm to a maximum of 1000 rpm. Finally, if more oxygen was required to achieve the set point, oxygen supplementation was included using the integrated mass flow controllers. Starting glucose concentration was 25 g/L. A constant concentrated sterile filtered glucose feed (500g/L) was added to the tanks at specified rate, i.e. 2 g/h, once agitation reached 800 rpm. In cases where feed rate or dissolved oxygen content needed to be varied for robustness study, changes were made after cells entered stationary phase. Fermentation runs were extended for up to ~ 50 hours after entry into stationary phase and samples automatically withdrawn every 3 hours. Samples were saved for subsequent analytical measurement.

Subsection VI: Determination of Strain Dry Weight

[00117] Culture samples (5 ml, n=3) were taken and washed 2X with deionized water via centrifugation and resuspension. After wash steps the OD of the samples were determined at 600 nm. Subsequently, samples were filtered over pre-weighed nitrocellulose filters (pore size, 0.45 µm). Filters were washed extensively with demineralized water and dried in a microwave oven for 2 min and weighed to determine correlation of OD600 and gDCW, which was 0.5.

Subsection VII: Analytical Methods

[00118] *Glucose Quantification:* A UPLC-RI method was developed for the quantification of glucose concentrations, using an Acquity H-Class UPLC integrated with a Waters 2414 Refractive Index (RI) detector (Waters Corp., Milford, MA. USA). Chromatographic separation was performed using a Bio-Rad Fast Acid Analysis HPLC Column (100 x 7.8 mm, 9 µm particle size; CAT#: #1250100, Bio-Rad Laboratories, Inc., Hercules, CA) at 65 □. 5 mM sulfuric acid was used as the eluent. The isocratic elution was as follows: 0–0.1 min, flow rate increased from 0.4 mL/min to 0.42 mL/min, 0.1–12 min flow rate at 0.48 mL/min. Sample injection volume was 10 µL. UPLC method development was carried out using standard aqueous stock solutions of analytes. Peak integration and further analysis was performed using MassLynx v4.1 software. The linear range used for glucose was 1-10 g/L. Samples were diluted as needed to be within the accurate linear range. Dilution was performed using ultrapure water. A sample standard curve is shown in **Figure 3**.

[00119] *Pyruvate Quantification:* A reverse phase UPLC-TUV method was developed for the quantification of pyruvic acid. Chromatographic separation was performed using a Restek Ultra AQ C18 column (150 mm × 2.1 i.d., 3 µm; CAT#: 9178362, Restek Corporation,

Bellefonte, PA) at 30 □. 20 mM phosphoric acid was used as the eluent. The isocratic elution was as follows: 0–3 min isocratic at 0.8 mL/min. Sample injection volume was 10 µL. Absorbance was monitored at 210 nm. UPLC method development was carried out using standard aqueous stock solution of analyte. Separations were performed using an Acquity H-Class UPLC (Waters Corp., Milford, MA. USA). Peak integration and further analysis was performed using MassLynx v4.1 software. The linear range for pyruvic acid was 0. 1-1 g/L. Samples were diluted as needed to be within the accurate linear range. Dilution was performed using ultrapure water. A sample standard curve is shown in **Figure 4**.

CLAIMS

1. A genetically modified microorganism for producing a pyruvate product comprising, compared to a corresponding non-genetically modified microorganism, one or more of:

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

ii. an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes; and

iii. a chromosomal deletion of a gene encoding an enzyme of a pyruvate metabolic pathway,

wherein the one or more enzymes are the same or different between the synthetic metabolic valves,

the genetically modified microorganism characterized by an increased production of pyruvate in a biofermentation process as compared to pyruvate produced from biofermentation of the non-genetically modified microorganism.

2. The genetically modified microorganism of claim 1, wherein the a 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.

3. The genetically modified microorganism of claim 1, wherein the one or more enzymes of the gene expression-silencing synthetic metabolic valve and of the enzymatic degradation synthetic metabolic valve are selected from the group consisting of: *fabI, zwf, gltA, ppc, udhA, lpd, sucD, aceA, pfkA, lon, rpoS, pykA, pykF, tktA or tktB*.

4. The genetically modified microorganism of claim 1, the one or more enzymes of the gene expression-silencing synthetic metabolic valve and of the enzymatic degradation synthetic metabolic valve are selected from the group consisting of: citrate synthase (*gltA*), pyruvate dehydrogenase (*lpd*), and glucose-6-phosphate dehydrogenase (*zwf*).

5. The genetically modified microorganism of claim 1, wherein one of the one or more enzymes of the a gene expression-silencing synthetic metabolic valve or of the enzymatic degradation synthetic metabolic valve is a pyruvate kinase.

6. The genetically modified microorganism of claim 1, wherein the one of the one or more enzymes of the gene expression-silencing synthetic metabolic valve or of the enzymatic degradation synthetic metabolic valve is a pyruvate kinase A (*pykA*) or pyruvate kinase F (*pykF*).

7. The genetically modified microorganism of claim 1, wherein the chromosomal deletion is selected from the group consisting of: methylglyoxal synthase (*mgsA*), dihydroxyacetone kinase (*dhaL*), D-erythrose-4-phosphate dehydrogenase (*epd*), 2-keto-3-deoxygluconate 6-phosphate/2-keto-4-hydroxyglutarate aldolase (*eda*), and PTS multiphosphoryl transfer protein (*ptsA*).

8. The genetically modified microorganism of claim 1, wherein the genetically modified microorganism further comprises overexpression of a NADH oxidase.

9. 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.

10. The genetically modified microorganism of claim 1, wherein the microorganism produces a pyruvate product titer of greater than 0.08 g/L at twenty four in a biofermentation process.

11. The genetically modified microorganism of claim 1, further comprising a production pathway for production of a pyruvate derived product selected from the group consisting of: an amino acid, alanine, valine, isoleucine, leucine, serine, cysteine, aspartate, acetylaldehyde, phosphoenolypyruvate, citrate, oxaloacetate, ethyl pyruvate, L-DOPA, N-acetyl-D-neuraminic acid, (R)-phenylacetylcarbinol, acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, acetylCoA, isoprenoids, glycerol, ethylene glycol, ethylene, propylene, butylene, isobutylene, ethyl acetate, vinyl acetate, 1,4-butanediol, 2,3-butanediol, butanol, isobutanol, sec-butanol, butyrate, isobutyrate, 2-OH-isobutryate, 3-OH-butyrate, ethanol, isopropanol, D-lactate, L-lactate, pyruvate, itaconate, levulinate, glucarate, glutarate, caprolactam, adipic acid, propanol,

isopropanol, fusel alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate.

12. A multi-stage fermentation bioprocess for producing a pyruvate product from a genetically modified microorganism, comprising:

(a) growing a genetically modified microorganism, the genetically modified microorganism comprising a combination of at least one of:

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

ii. an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes; and

iii. a chromosomal deletion,

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

(b)

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

(ii) producing a pyruvate product.

13. The multi-stage fermentation bioprocess of claim 12, further comprising:

a centrifugation to separate the genetically modified microorganism and the pyruvate product.

14. The multi-stage fermentation bioprocess of claim 12, further comprising formation of a pyruvate salt from the pyruvate product.

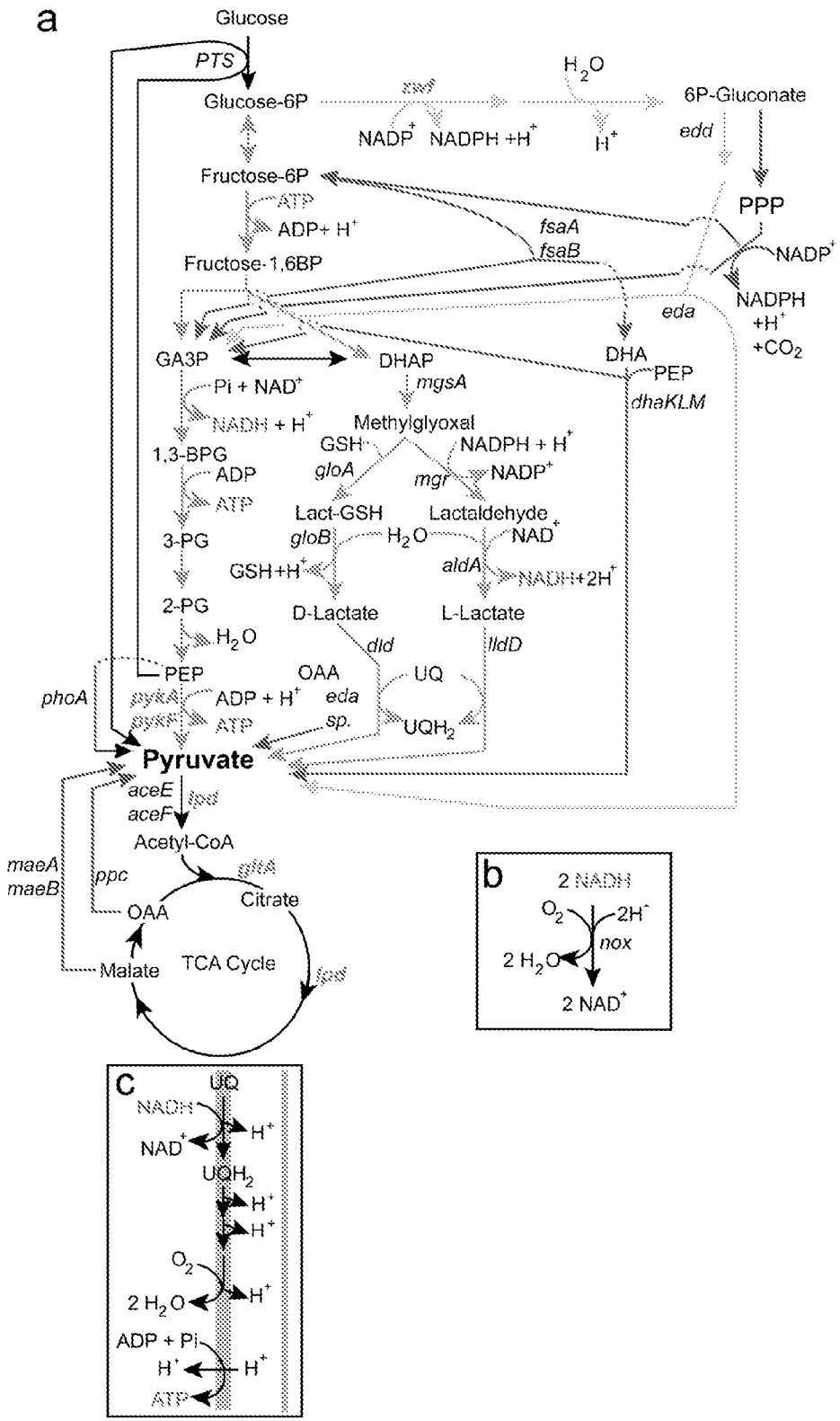
15. The multi-stage fermentation bioprocess of claim 12, further comprising formation of a pyruvate ester from the pyruvate product.

16. The multi-stage fermentation bioprocess of claim 12, further comprising producing a pyruvate derived product by biochemical conversion of the pyruvate product to a derived product selected from the group consisting of: an amino acid, alanine, valine, isoleucine, leucine, serine, cysteine, aspartate, acetylaldehyde, phosphoenolypyruvate, citrate, oxaloacetate,

ethyl pyruvate, L-DOPA, N-acetyl-D-neuraminic acid, (R)-phenylacetylcarbinol, acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, acetylCoA, isoprenoids, glycerol, ethylene glycol, ethylene, propylene, butylene, isobutylene, ethyl acetate, vinyl acetate, 1,4-butanediol, 2,3-butanediol, butanol, isobutanol, sec-butanol, butyrate, isobutyrate, 2-OH-isobutryate, 3-OH-butyrate, ethanol, isopropanol, D-lactate, L-lactate, pyruvate, itaconate, levulinate, glucarate, glutarate, caprolactam, adipic acid, propanol, isopropanol, fusel alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate.

17. The multi-stage fermentation bioprocess of claim 12 wherein the one or more enzymes is selected from the group consisting of: citrate synthase (*gltA*), pyruvate dehydrogenase (*lpd*), glucose-6-phosphate dehydrogenase (*zwf*), pyruvate kinase A (*pykA*) and pyruvate kinase F (*pykF*).

18. The multi-stage fermentation bioprocess of claim 12 wherein the genetically modified microorganism further comprises overexpression of a NADH oxidase.


a
Glucose
PTS
Glucose-6P
zwf
NADP+
NADPH +H+
H2O
H+
6P-Gluconate
edd
PPP
Fructose-6P
ATP
ADP+ H+
fsaA
fsaB
NADP+
Fructose-1,6BP
NADPH
+H+
+CO2
eda
GA3P
DHAP
DHA
PEP
dhaKLM
Pi + NAD+
mgsA
NADH + H+
Methylglyoxal
GSH
NADPH + H+
1,3-BPG
gloA
mgr
NADP+
ADP
ATP
Lact-GSH
Lactaldehyde
3-PG
gloB
H2O
NAD+
GSH+H+
aldA
NADH+2H+
2-PG
D-Lactate
L-Lactate
H2O
dld
lldD
PEP
OAA
UQ
phoA
pykA
pykF
ADP + H+
ATP
eda
sp.
UQH2
Pyruvate
aceE
aceF
lpd
Acetyl-CoA
maeA
maeB
ppc
gltA
OAA
Citrate
Malate
TCA Cycle
fpd
b
2 NADH
O2
2H+
nox
2 H2O
2 NAD+
c
UQ
NADH
H+
NAD+
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H+
H+
O2
2 H2O
H+
ADP + Pi
H+
H+
ATP

FIGURE 1

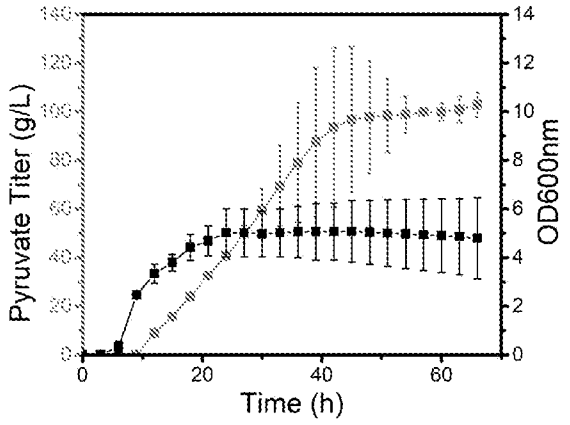

FIGURE 2

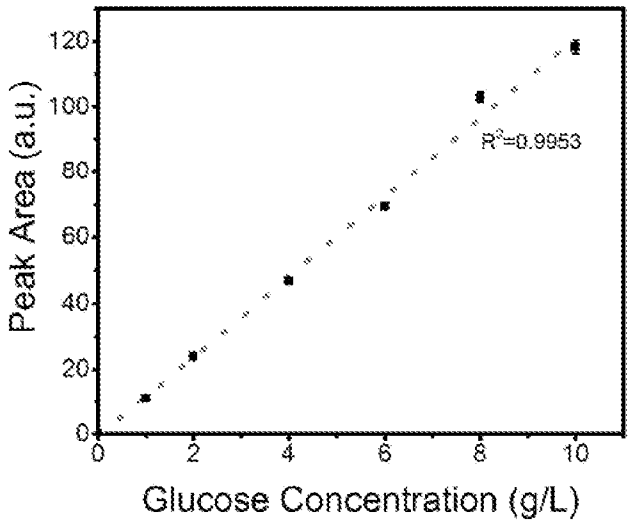

FIGURE 3

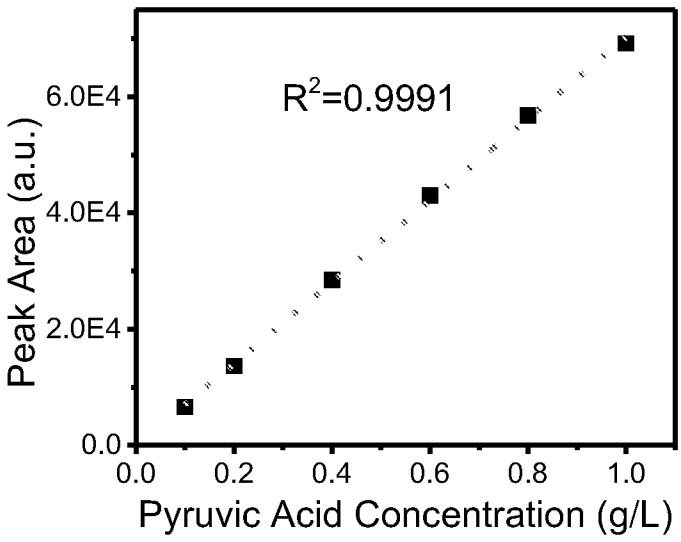

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/38415

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/09, C12N 15/11, C12N 15/52 (2019.01)
CPC - C12N 15/113, C12N 15/63, C12N 15/70, C12N 2310/14, C12N 2320/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/0121707 A1 (DUKE UNIVERSITY) 4 May 2017 (04.05.2017) para abstract, [0007], [0014], [0032], [0084-0085], [0092], [0099], [0115], [00127-00128]	1-11
Y	US 2010/0304450 A1 (EITEMAN et al.) 2 December 2010 (02.12.2010) Abstract, [0010], [0012], [0017], [0045], [0081], [0108]	1-11
Y	JP2015154768 A (UNIV KYUSHU) 27 August 2015 (27.08.2015) para [0010], [0030], [0058]	5-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 September 2019	12 NOV 2019

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/38415

Continuation of Box No. III Observations where unity of invention is lacking:

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Groups I requires an isolated genetically modified microorganism composition that is not required by group II.

Group II requires a multi-stage fermentation bioprocess producing a pyruvate product that is not required by group I.

Common Technical Features

The common technical feature shared by Groups I and II, is a genetically modified microorganism for producing a pyruvate product, comprising:
i. a gene expression-silencing synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes;
ii. an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes; and
iii. a chromosomal deletion of a gene encoding an enzyme of a pyruvate metabolic pathway,
wherein the one or more enzymes are the same or different between the synthetic metabolic valves.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated by US 2017/0121707 A1 to Duke University (hereinafter "Duke Univ."). Duke Univ. teaches a genetically modified microorganism for producing a pyruvate product (para [00127-00128] - "Example 13: Production of Alanine in E. coli, from Pyruvate in 96 Well Plates ... Several E. coli strains were constructed utilizing a combination of host strains"), comprising
i. a gene expression-silencing synthetic metabolic valve characterized by silencing gene expression of one or more genes encoding one or more enzymes (para [0094] "the invention describes the use of controlled gene silencing to help enable 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"),
ii. an enzymatic degradation synthetic metabolic valve characterized by inducing enzymatic degradation of one or more enzymes (para [0007] "This approach allows for simpler models of metabolic fluxes and physiological demands during a production phase ... 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 enables these synthetic valves ... with 2) inducible enzyme degradation"),
iii. a chromosomal deletion of a gene encoding an enzyme of a pyruvate metabolic pathway (para [0084] - "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) ...pyruvate oxidase (poxB), pyruvate-formate lyase (pflB), ... may be disrupted").

As the technical feature was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.