COMPOSITIONS AND METHODS FOR ROBUST DYNAMIC METABOLIC CONTROL

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 62/461,436, filed February 21, 2017, which application is incorporated herein by reference in its entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with Government support under Federal Grant Nos. HR0011-14-C-0075, 12043956, and 1445726 awarded by the DOD/DARPA, NAVY/ONR, and NSF, respectively. The Government has certain rights to this invention.

REFERENCE TO A SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been filed electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 21, 2018, is named 52240_702_601_SL.txt and is 81,697 bytes in size.

BACKGROUND OF THE INVENTION

[0004] Biotechnology-based fermentation processes have been successfully developed to produce everything from biologics and small molecule therapies to specialty, bulk and commodity chemicals, and even next generation biofuels. These processes have made rapid advancements in recent years due to technology developments in the fields of fermentation science and synthetic biology, as well as metabolic and enzyme engineering. Despite these substantial advances, most successful examples of rational and directed engineering approaches have also greatly relied on numerous and often lengthy cycles of trial and error. The present disclosure provides a strategy that simultaneously reduces the complexity of the problem (as well as the size of the relevant design space), while also minimizing metabolic responses to environmental conditions, increasing robustness and scalability of engineered strains.

SUMMARY OF THE INVENTION

[0005] The present disclosure provides, in part, a high-throughput engineering platform that enables the rapid development of microbial production strains.

[0006] In one aspect, the present disclosure provides a cell for generating a product, wherein the cell comprises: a heterologous polynucleotide for controlled reduction of expression of an enzyme of a metabolic pathway, wherein the controlled reduction of expression of the enzyme induces a stationary phase of the cell; and a heterologous production polynucleotide for mediating controlled increase in expression of a production enzyme for

generation of the product; wherein a rate of production of the product during the stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking the enzyme.

[0007] In some embodiments, the heterologous polynucleotide reduces flux through the metabolic pathway. In some embodiments, the enzyme is selected from the group consisting of enoyl-ACP/CoA reductase, glucose-6-phosphate dehydrogenase, lipoamide dehydrogenase, citrate synthase, soluble transhydrogenase, and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase. In some embodiments, the production enzyme is selected from the group consisting of NADPH-dependent alanine dehydrogenase, an alanine exporter, and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. In some embodiments, the change of an environmental condition comprises increasing or decreasing a concentration of a sugar in a culture medium contacting the cell. In some embodiments, the sugar is glucose. In some embodiments, the change of an environmental condition comprises increasing or decreasing oxygenation of a culture medium contacting the cell. In some embodiments, the product comprises 3-hydroxypropionic acid.

[0008] In some embodiments, the product comprises an amino acid. In some aspects, the amino acid comprises alanine. In some aspects, the cell is grown in a culture, and a rate of production of the alanine by the culture is at least 0.5 g/L/hour. In some aspects, the rate of production of the alanine is at least 1.0 g/L/hour. In some aspects, the rate of production of the alanine is at least 1.5 g/L/hour. In some aspects, the rate of production of the alanine is at least 1.6 g/L/hour. In some aspects, the culture produces at least 80 g/L of the alanine. In some aspects, the culture produces at least 100 g/L of the alanine. In some aspects, the culture produces at least 120 g/L of the alanine. In some aspects, the culture produces at least 140 g/L of the alanine. In some aspects, the production polynucleotide encodes an alanine exporter. In some aspects, the alanine exporter is alaE.

[0009] In some embodiments, the product comprises mevalonic acid. In some embodiments, the cell is grown in a culture, and a rate of production of the mevalonic acid by the culture is at least 0.5 g/L/hour. In some embodiments, the rate of production of the mevalonic acid is at least 1.0 g/L/hour. In some embodiments, the rate of production of the mevalonic acid is at least 1.2 g/L/hour. In some embodiments, the rate of production of the mevalonic acid is at least 1.25 g/L/hour. In some aspects, the cell is grown in a culture, and the culture produces at least 50 g/L of the mevalonic acid. In some embodiments, the culture produces at least 70 g/L of the mevalonic acid. In some embodiments, the culture produces at least 90 g/L of the mevalonic acid. In some embodiments, the culture produces at least 95 g/L of the mevalonic acid. In some embodiments, the heterologous

polynucleotide is selected from the group consisting of: a silencing polynucleotide for repressing transcription of a gene encoding the enzyme; and a degradation polynucleotide for mediating cellular degradation of the enzyme.

[0010] In some aspects, the heterologous polynucleotide comprises a silencing polynucleotide, and the silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter of a gene encoding the enzyme. In some aspects, the heterologous polynucleotide encodes a CRISPR enzyme, and the CRISPR enzyme specifically binds to the promoter sequence when bound to the gRNA. In some aspects, the CRISPR enzyme is catalytically inactive. In some aspects, the heterologous polynucleotide comprises a degradation polynucleotide, wherein the degradation polynucleotide comprises a sequence encoding a degradation tag, wherein the degradation tag mediates degradation of the enzyme. In some embodiments, expression of the heterologous polynucleotide is regulated by phosphate availability in the cell. In some embodiments, expression of the production polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the cell is an *E. coli* cell.

[0011] In another aspect, disclosed herein is a method comprising: culturing independently a plurality of strains of a cell, wherein each strain comprises (i) a heterologous polynucleotide for mediating controlled reduction of expression of an enzyme of a metabolic pathway, wherein the controlled reduction of expression of the enzyme induces a stationary phase of the cell; and (ii) a heterologous production polynucleotide for mediating controlled increase in expression of a production enzyme for generation of the product; wherein each strain of the plurality of strains differs from another strain in a sequence of at least one of the heterologous polynucleotide or the heterologous production polynucleotide; growing the plurality of strains to stationary phase; and selecting a strain of the plurality of strains based on a level of the product produced by the selected strain during the stationary phase.

[0012] In some embodiments, the method comprises determining the level of the product. In some embodiments, the method comprises growing the selected strain. In some embodiments, the selected strain is grown in a bioreactor. In some embodiments, a culture medium comprising the selected strain has a volume of at least 500ml. In some embodiments, the culture medium has a volume of at least 1L. In some embodiments, the heterologous polynucleotide is selected from the group consisting of: a silencing polynucleotide for repressing transcription of a gene encoding the enzyme; and a degradation polynucleotide for mediating cellular degradation of the enzyme. In some embodiments, a first and second strain of the plurality of strains comprises a silencing polynucleotide. In some

embodiments, the silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter sequence of a gene encoding the enzyme. In some embodiments, the gRNA sequence differs between the first and second strains. In some embodiments, the first and second strain of the plurality of strains comprise a degradation polynucleotide. In some embodiments, the degradation polynucleotide differs between the first and second strains. In some embodiments, the enzyme is selected from the group consisting of enoyl-ACP/CoA reductase, glucose-6-phosphate dehydrogenase, lipoamide dehydrogenase, citrate synthase, soluble transhydrogenase, and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase. In some embodiments, the production enzyme is selected from the group consisting of NADPH-dependent alanine dehydrogenase, an alanine exporter, and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. In some embodiments, the product is selected from the group consisting of mevalonic acid, 3-hydroxypropionic acid, and an amino acid.

[0013] In some embodiments, the product is an amino acid and the amino acid is alanine. In some embodiments, the cell of the selected strain a rate of production of the product during the stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking the heterologous polynucleotide. In some embodiments, the change of an environmental condition comprises a change in concentration of a sugar of a culture medium contacting the cell. In some embodiments, the change of an environmental condition comprises a change in oxygenation of a culture medium contacting the cell.

[0014] In another aspect, disclosed herein is a method of generating a cellular product comprising: culturing a heterologous cell in a culture medium, wherein the heterologous cell comprises: (i) a heterologous polynucleotide for mediating controlled reduction of expression of an enzyme of a metabolic pathway, wherein the controlled reduction of expression of the enzyme induces a stationary phase of the cell; and (ii) a heterologous production polynucleotide for mediating controlled increase in expression of a production enzyme for generation of the product; wherein a rate of production of the product during the stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking the enzyme.

[0015] In one embodiment, the method further comprises changing the environmental condition. In one embodiment, the environmental condition comprises a concentration of a sugar of the culture medium, and changing the environmental condition comprises increasing or decreasing the concentration. In some embodiments, the sugar is glucose. In some embodiments, the environmental condition comprises an oxygen concentration of the culture medium, and changing the environmental condition comprises increasing or

decreasing the oxygen concentration. In some embodiments, the culturing is performed in a bioreactor. In some embodiments, the culture medium has a volume of at least 500ml. In some embodiments, the culture medium has a volume of at least 1L. In some embodiments, the product comprises 3-hydroxypropionic acid. In some embodiments, the product comprises an amino acid. In some embodiments, the amino acid comprises alanine. In some embodiments, the rate of production of the alanine is at least 0.5 g/L/hour. In some embodiments, the rate of production of the alanine is at least 1.0 g/L/hour. In some embodiments, the rate of production of the alanine is at least 1.5 g/L/hour. In some embodiments, the rate of production of the alanine is at least 1.6 g/L/hour. In some embodiments, the production polynucleotide encodes an alanine exporter. In some embodiments, the alanine exporter is alaE.

[0016] In some embodiments, the product comprises mevalonic acid. In some embodiments, the rate of production of the mevalonic acid is at least 0.5 g/L/hour. In some embodiments, the rate of production of the mevalonic acid is at least 1.0 g/L/hour. In some embodiments, the rate of production of the mevalonic acid is at least 1.2 g/L/hour. In some embodiments, the rate of production of the mevalonic acid is at least 1.25 g/L/hour. In some embodiments, the heterologous polynucleotide is selected from the group consisting of: a silencing polynucleotide for repressing transcription of a gene encoding the enzyme; and a degradation polynucleotide for mediating cellular degradation of the enzyme. In some embodiments, the heterologous polynucleotide comprises a silencing polynucleotide, and the silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter sequence of a gene encoding the enzyme. In some embodiments, the heterologous polynucleotide encodes a CRISPR enzyme, wherein the CRISPR enzyme specifically binds to the promoter sequence when bound to the gRNA. In some embodiments, the CRISPR enzyme is catalytically inactive. In some embodiments, the heterologous polynucleotide comprises a degradation polynucleotide, wherein the degradation polynucleotide comprises a sequence encoding a degradation tag, wherein the degradation tag mediates degradation of the enzyme. In some embodiments, the expression of the heterologous polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the expression of the production polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the cell is an *E. coli* cell.

[0017] In another aspect, disclosed herein is a cell for production of alanine, wherein the cell comprises: (i) a heterologous polynucleotide for controlled reduction of expression of an enzyme of a metabolic pathway, wherein the enzyme is selected from the group consisting of enoyl-ACP/CoA reductase, glucose-6-phosphate dehydrogenase, lipoamide

dehydrogenase (lpd), citrate synthase (gltA), soluble transhydrogenase, and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase; and (ii) an alanine exporter, wherein the alanine exporter is expressed at increased levels as compared to a wildtype cell.

[0018] In some embodiments, the alanine exporter is encoded by an alaE gene. In some embodiments, the controlled reduction of expression of the enzyme induces a stationary phase of the cell. In some embodiments, the cell further comprises a heterologous production polynucleotide for controlled increase in expression of a production enzyme for generation of the alanine. In some embodiments, the production enzyme is selected from the group consisting of NADPH-dependent alanine dehydrogenase and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. In some embodiments, the heterologous polynucleotide is selected from the group consisting of: a silencing polynucleotide for mediating transcriptional repression of a gene encoding the enzyme; and a degradation polynucleotide for mediating cellular degradation of the enzyme. In some embodiments, the heterologous polynucleotide comprises a silencing polynucleotide, and the silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter sequence of a gene encoding the enzyme. In some embodiments, the polynucleotide further encodes a CRISPR enzyme, wherein the CRISPR enzyme specifically binds to the promoter sequence when bound to the gRNA. In some embodiments, the CRISPR enzyme is catalytically inactive. In some embodiments, the heterologous polynucleotide comprises a degradation polynucleotide, wherein the degradation polynucleotide comprises a sequence encoding a degradation tag, wherein the degradation tag mediates degradation of the enzyme. In some embodiments, the polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the production polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the cell is an *E. coli* cell.

[0019] In some embodiments, a culture comprises the cell. In some embodiments, a rate of production of the alanine by the culture is at least 0.5g/L/hour. In some embodiments, a rate of production of the alanine by the culture is at least 1.0g/L/hour. In some embodiments, a rate of production of the alanine by the culture is at least 1.5g/L/hour. In some embodiments, a rate of production of the alanine by the culture is at least 1.6g/L/hour. In some embodiments, the culture produces at least 100 g/L of the alanine. In some embodiments, the culture produces at least 120 g/L of the alanine. In some embodiments, the culture produces at least 140 g/L of the alanine.

[0020] In some aspects, disclosed herein is a method of production of alanine comprising growing in a culture medium a cell comprising (i) a heterologous polynucleotide for controlled reduction of expression of a enzyme of a metabolic pathway, wherein the

enzyme is selected from the group consisting of enoyl-ACP/CoA reductase, glucose-6-phosphate dehydrogenase, lipoamide dehydrogenase, citrate synthase, soluble transhydrogenase, and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase; and (ii) an alanine exporter, wherein the alanine exporter is expressed at increased levels as compared to a wildtype cell.

[0021] In some embodiments, the controlled reduction of expression of the enzyme induces a stationary phase of the cell. In some embodiments, the method further comprises decreasing an oxygenation level or a sugar concentration of the culture medium during the stationary phase, wherein a rate of production of the cellular product is reduced less in response to the decreasing as compared to a cell lacking the heterologous polynucleotide. In some embodiments, the sugar is glucose. In some embodiments, the alanine exporter is encoded by an alaE gene. In some embodiments, the cell further comprises a heterologous production polynucleotide for controlled increase in expression of a production enzyme for generation of the alanine. In some embodiments, the production enzyme is selected from the group consisting of: NADPH-dependent alanine dehydrogenase and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. In some embodiments, the heterologous polynucleotide is selected from the group consisting of: a silencing polynucleotide for mediating transcriptional repression of a gene encoding the enzyme; and a degradation polynucleotide for mediating cellular degradation of the enzyme. In some embodiments, the heterologous polynucleotide comprises a silencing polynucleotide, and the silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter sequence of a gene encoding the enzyme. In some embodiments, the heterologous polynucleotide encodes a CRISPR enzyme, wherein the CRISPR enzyme specifically binds to the promoter sequence when bound to the gRNA. In some embodiments, the CRISPR enzyme is catalytically inactive. In some embodiments, the heterologous polynucleotide comprises a degradation polynucleotide, wherein the degradation polynucleotide comprises a sequence encoding a degradation tag, wherein the degradation tag mediates degradation of the enzyme.

[0022] In some embodiments, the expression of the heterologous polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the production polynucleotide is regulated by phosphate availability in the cell. In some embodiments, the cell is an *E. coli* cell. In some embodiments, a rate of production of the alanine is at least 0.5g/L/hour. In some embodiments, a rate of production of the alanine is at least 1.0g/L/hour. In some embodiments, a rate of production of the alanine is at least 1.5g/L/hour. In some embodiments, a rate of production of the alanine is at least 1.6g/L/hour.

INCORPORATION BY REFERENCE

[0023] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The novel features of the invention are set forth with particularity in the appended 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 utilized, and the accompanying drawings of which:

[0025] Figure 1A depicts an overview of dynamic metabolic control in 2-stage fermentations.

[0026] Figure 1B depicts strain and bioprocess optimization.

[0027] Figures 2A-D depict an example of implementation of 2-stage Synthetic Metabolic Valves (SMVs) in *E. coli*.

[0028] Figures 3A-K depict an example of alanine production in *E. coli* utilizing 2-stage dynamic control.

[0029] Figures 4A-F depict example robustness comparison between 2-stage and growth associated approaches.

[0030] Figures 5A-J depict example comparisons of "Valve" and growth associated alanine production in micro-fermentations and 1L fermentation.

[0031] Figure 6A-H depict an example of mevalonate production in *E. coli* utilizing 2-stage dynamic control.

[0032] Figure 7 depicts an example of phosphate depletion promoter characterization.

[0033] Figure 8 depicts an example of insulated phosphate depletion promoter characterization.

[0034] Figure 9 depicts an example of insulated constitutive promoter characterization.

[0035] Figure 10 depicts an example of metabolic modeling results for optimal 3-HP flux in two stage fermentations.

[0036] Figure 11 depicts examples of chromosomal modifications.

[0037] Figure 12 depicts an example of average maximal growth rates of starting host strains in 1L FGM10 minimal medium fermentations, n=2.

[0038] Figure 13A-E depict examples of distribution of glucose utilized during the growth phase of starting host strains in 1L standard minimal medium fermentations.

[0039] Figure 14 depicts pCASCADE-control plasmid construction scheme.

[0040] Figures 15A-B depict pCASCADE construction scheme.

[0041] Figures 16A-C depict an overview of micro-fermentation process.

[0042] Figure 17 depicts micro-fermentation for L-alanine production using different insulated phosphate promoters in DLF_0025 strain.

[0043] Figure 18 depicts Heatmap for L-alanine production by gapN/gapA strains.

[0044] Figures 19A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0045] Figures 20A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0046] Figures 21A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0047] Figures 22A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0048] Figures 23A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0049] Figures 24A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0050] Figures 25A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0051] Figures 26A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0052] Figures 27A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0053] Figures 28A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0054] Figures 29A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0055] Figures 30A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0056] Figures 31A-D depict alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[0057] Figure 32 depicts alanine production in response to different OTR and glucose concentration in micro-fermentation for one strain evaluated for robustness.

[0058] Figures 33A-B depict growth profile for all valve and growth associated strains at 1L scale evaluated in this paper.

[0059] Figure 34 depicts specific Productivity (SP) comparison for strain with highest mevalonate titer from literature and mevalonate strain 1 evaluated in this work.

[0060] Figure 35 depicts alanine standard curve from MS measurement. Average and standard deviation for mass spec response from triplicate standard measurement were plotted.

[0061] Figures 36A-B depict glucose and ethanol standard curves from RI measurement.

[0062] Figure 37 depicts 3-Hydroxypropionic acid standard curve from TUV measurement.

[0063] Figures 38A-D depict TUV standard curves for L-alanine, D-alanine, mevalonic acid, and mevalonolactone.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0064] 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.

[0065] As used herein, "reduced enzymatic activity," "reducing enzymatic activity," and the like is meant to indicate that a microorganism cell's, or an isolated enzyme, exhibits a lower level of activity than that measured in a comparable cell of the same species or its native enzyme. That is, enzymatic conversion of the indicated substrate(s) to indicated product(s) under known standard conditions for that enzyme is at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 percent less than the enzymatic activity for the same biochemical conversion by a native (non-modified) enzyme under a standard specified condition. This term also can include elimination of that enzymatic activity. A cell having reduced enzymatic activity of an enzyme can be identified using any method known in the art. For example, enzyme activity assays can be used to identify cells having reduced enzyme activity. See, for example, *Enzyme Nomenclature*, Academic Press, Inc., New York 2007.

[0066] 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 foreign to (i.e., not naturally found in) a given host microorganism; (b) the sequence may be naturally found in a given host microorganism, but in an unnatural (e.g., greater than expected) amount; or (c) the sequence of nucleic acids comprises two or more subsequences that are not found in the same relationship to each other in nature. For example, regarding instance (c), a heterologous nucleic acid sequence that is recombinantly produced will

have two or more sequences from unrelated genes arranged to make a new functional nucleic acid, such as a nonnative promoter driving gene expression.

[0067] 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.

[0068] 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).

[0069] 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 reducing activities 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.

[0070] Bio-production or fermentation, as used herein, may be aerobic, microaerobic, or anaerobic.

[0071] When the genetic modification of a gene product, e.g., 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, e.g., the enzyme.

[0072] 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.

[0073] Species and other phylogenic identifications are according to the classification known to a person skilled in the art of microbiology.

[0074] Enzymes are listed here within, with reference to a Universal Protein Resource (Uniprot) identification number, which would be well known to one skilled in the art (Uniprot is maintained by and available through the UniProt Consortium).

[0075] 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.

[0076] 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.

Overview

[0077] Provided herein is a high-throughput metabolic engineering platform enabling the rapid optimization of microbial production strains. The platform, which bridges a gap between current *in vivo* and *in vitro* bio-production approaches, relies on dynamic minimization of the active metabolic network. Dynamic metabolic network minimization can be accomplished using combinations of CRISPR interference and controlled proteolysis to reduce the activity of multiple enzymes in essential central metabolism. Minimization can be implemented in the context of standardized 2-stage bio-processes. This approach not only can result in a design space with greatly reduced complexity, but also in increased metabolic fluxes and production rates as well as in strains which are robust to environmental conditions. Robustness can lead to predictable scalability from high-throughput small-scale screens, or "micro-fermentations", to fully instrumented bioreactors. Predictive high-throughput approaches may be critical for metabolic engineering programs to truly take advantage of the rapidly increasing throughput and

decreasing costs of synthetic biology. The examples provided herein have not only demonstrated proof of principle for this approach in the common industrial microbe: *E. coli*, and has validated this approach with the rapid optimization of *E. coli* strains producing two important industrial chemicals: alanine and mevalonic acid, at commercially meaningful rates, titers (147 g/L and 97 g/L, respectively), and yields.

[0078] Also provided herein are systems and methods to rapidly optimize a microorganism for chemical productions in a high-throughput fashion.

[0079] Also provided herein are microorganisms that can be used with the disclosed platform and/or methods for chemical productions.

Synthetic metabolic valves (SMVs)

[0080] The current disclosure describes the construction of synthetic metabolic valves (SMVs) 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.

[0081] The development of platform microbial strains that utilize SMVs can decouple growth from product formation. These strains enable the dynamic control of metabolic pathways, including those that when altered have negative effects on microorganism growth. Dynamic control over metabolism is accomplished via a combination of methodologies including but not limited to transcriptional silencing and controlled enzyme proteolysis. These microbial strains are utilized in a multi-stage bioprocess encompassing as least two stages, the first stage in which microorganisms are grown and metabolism can be optimized for microbial growth and at least one other stage in which growth can be slowed or stopped, and dynamic changes can be made to metabolism to improve production of desired product, such as a chemical or fuel. The transition of growing cultures between stages and the manipulation of metabolic fluxes can be controlled by artificial chemical inducers or preferably by controlling the level of key limiting nutrients. In addition, genetic modifications may be made to provide metabolic pathways for the biosynthesis of one or more chemical or fuel products. Also, genetic modifications may be made to enable the utilization of a variety of carbon feedstocks including but not limited sugars such as glucose, sucrose, xylose, arabinose, mannose, and lactose, oils, carbon dioxide, carbon monoxide, methane, methanol and formaldehyde.

[0082] This approach 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 enables these synthetic valves: 1) transcriptional gene silencing or repression

technologies in combination with 2) inducible 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.

[0083] In various cases, one SMV can refer to the manipulation of one gene (or its protein product). The manipulation can be controlled silencing of the gene and/or controlled degradation of its protein product. In certain cases, combination of SMVs can lead to improved production in yields, rate and/or robustness, which includes manipulation of two genes (or their protein products). In some cases, an engineered microorganism comprises at least one SMV. In some cases, an engineered microorganism comprises more than one SMV. In some cases, an engineered microorganism comprises two, three, four, five, six, seven, eight, nine, or ten, or more SMVs.

Method and Systems for Bio-production

[0084] Provided herein are methods or systems for robust large scale production of molecules from biologics and small molecule therapeutics to specialty, bulk and commodity chemicals, and biofuels. The methods or systems provided herein comprise using engineered microorganism which comprises a limited set of metabolic enzymes. In some embodiments, the engineered microorganism comprises at least one metabolic enzyme that has reduced level or activity. In some embodiments, the engineered microorganism comprises two, three, four, five, six, seven, eight, nine, or ten, or more metabolic enzymes that have reduced level or activity. The methods and systems provided herein can reduce metabolic responses to environmental conditions and can be easily transferred from small scale (e.g. mgs) production to large scale (e.g. kgs) production. The methods and systems provided herein can reduce the time and costs associated with transitioning from small scale (e.g. mgs) to large scale (e.g. kgs) production.

[0085] Within the scope of the current disclosure are genetically modified microorganism, wherein the microorganism is capable of producing a product derived from any key metabolic intermediate including but not limited to malonyl-CoA, pyruvate, oxaloacetate, erthyrose-4-phosphate, xylulose-5-phosphate, alpha-ketoglutarate and citrate 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.

[0086] In various embodiments, the invention includes a culture system comprising a carbon source in an aqueous medium and a genetically modified microorganism, 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.

Carbon Sources

[0087] 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).

Microorganisms

[0088] 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.

[0089] The examples describe specific modifications and evaluations to certain bacterial and fungal microorganisms. The scope of the invention is not meant to be limited to such species, but to be generally applicable to a wide range of suitable microorganisms.

[0090] Suitable host cells or host microorganisms for bio-production can be either prokaryotic or eukaryotic. Suitable host cells or host microorganisms can be bacteria such as Citrobacter, Enterobacter, Clostridium, Klebsiella, Aerobacter, Lactobacillus, Aspergillus, Saccharomyces, Schizosaccharomyces, Zygosaccharomyces, Pichia, Kluyveromyces, Candida, Hansenula, Debaryomyces, Mucor, Torulopsis, Methylobacter, Escherichia, Salmonella, Bacillus, Streptomyces, and Pseudomonas. In some embodiments, a host cell or an engineered cell is *E. coli*. In some embodiments, a host cell or an engineered cell is *S. cerevisiae*.

[0091] In certain aspects, provided herein is a microorganism genetically modified to comprise: a production pathway comprising at least one enzyme for the biosynthesis of a product, and a combination of multiple synthetic metabolic valves to controllably reduce or eliminate flux through multiple metabolic pathways. In some embodiments, each of the multiple synthetic metabolic valves comprises one or more genes for (i) controlled silencing of gene expression of at least one gene or (ii) the controlled proteolytic inactivation of at least one protein. In some embodiments, a rate of the biosynthesis of the product is increased in a productive stationary phase upon a depletion of a nutrient, wherein the depletion of the nutrient induces the multiple synthetic metabolic valves. In some cases, the controlled silencing of gene expression is accomplished by RNA interference, CRISPR interference or transcriptional repression. In some cases, the controlled proteolytic inactivation is accomplished by protein cleavage by a specific protease or targeted degradation by specific peptide tags. In some cases, the nutrient is phosphate, nitrogen, sulfur, magnesium, or a combination thereof.

[0092] In certain aspects, provided herein is a genetically modified microorganism comprising: a production pathway comprising at least one enzyme for the biosynthesis of a product from one of the following metabolites: pyruvate, acetolactate, acetyl-CoA, acetoacetyl-CoA or malonyl-CoA; and a combination of multiple synthetic metabolic valves, wherein each of the multiple synthetic metabolic valves comprises one of a fabI, gltA, lpd, zwf or udhA gene for (i) controlled silencing of gene expression of a corresponding one of said fabI, gltA, lpd, zwf or udhA genes or (ii) controlled proteolytic inactivation of a protein encoded by a corresponding one of said fabI, gltA, lpd, zwf or udhA genes. In some embodiments, a rate of the biosynthesis of the product is increased in a productive stationary phase upon a depletion of a nutrient, wherein the depletion of the nutrient induces the multiple synthetic metabolic valves. In some embodiments, the product is alanine or a derivative thereof. In some embodiments, the product is mevalonate or a derivative thereof. In some embodiments, the product is malonic acid or a derivative thereof. In some embodiments, the nutrient is phosphate, nitrogen, sulfur, magnesium, or a combination thereof.

[0093] In certain aspects, provided herein is a genetically modified microorganism comprising: a production pathway to produce alanine from pyruvate; and a combination of multiple synthetic metabolic valves, wherein each of the multiple synthetic metabolic valves comprises one of a fabI, gltA, lpd, zwf or udhA gene for (i) controlled silencing of gene expression of a corresponding one of said fabI, gltA, lpd, zwf or udhA genes or (ii) controlled proteolytic inactivation of a protein encoded by one of said fabI, gltA, lpd, zwf or udhA genes. In some embodiments, a rate of the biosynthesis of alanine is increased in a productive stationary phase upon a depletion of a nutrient, wherein the depletion of the nutrient induces the multiple

synthetic metabolic valves. In some embodiments, the nutrient is phosphate, nitrogen, sulfur, magnesium, or a combination thereof.

[0094] In some cases, a genetically modified microorganism is a heterologous cell. In some cases, provided herein is a heterologous cell for generating a product. In some cases, a heterologous cell comprises an engineered valve polynucleotide for mediating controlled reduction of expression of a valve enzyme acting in a metabolic pathway. In certain cases, a controlled reduction of expression of a valve enzyme reduces flux through a metabolic pathway, wherein the controlled reduction of expression of the valve enzyme induces a stationary phase of the heterologous cell. In some cases, a heterologous cell further comprises an engineered production polynucleotide for mediating controlled increase in expression of a production enzyme for generation of the product. In some situations, a heterologous cell comprises an engineered valve polynucleotide for mediating controlled reduction of expression of a valve enzyme acting in a metabolic pathway, wherein a rate of production of a product during a stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking the controlled reduction of expression of the valve enzyme.

[0095] In some cases, provided herein is a heterologous cell for generating a product, wherein said cell comprises: an engineered valve polynucleotide for mediating controlled reduction of expression of a valve enzyme acting in a metabolic pathway, wherein said controlled reduction of expression of said valve enzyme reduces flux through said metabolic pathway, wherein said controlled reduction of expression of said valve enzyme induces a stationary phase of said cell; and an engineered production polynucleotide for mediating controlled increase in expression of a production enzyme for generation of said product; wherein a rate of production of said product during said stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking said controlled reduction of expression of said valve enzyme.

[0096] In some cases, provided herein is a cell comprising a reduced expression or activity of a valve enzyme, wherein the valve enzyme comprises an enzyme selected from the group consisting of enoyl-ACP/CoA reductase (fabI), glucose-6-phosphate dehydrogenase (zwf), lipoamide dehydrogenase (lpd), citrate synthase (gltA), soluble transhydrogenase (udhA), NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (gapA), and a combination thereof.

[0097] In some cases, provided herein is a cell comprising a production enzyme, wherein the production enzyme comprises an enzyme selected from the group consisting of NADPH-dependent alanine dehydrogenase (ald), alanine exporter (alaE), NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (gapN), and a combination thereof.

Environmental Conditions

[0098] Environmental conditions can comprise medium and culture conditions. Environmental factors that may influence production can be temperature, pH, acidity, ethanol, sulfite, and availability of nutrients.

[0099] In addition to an appropriate carbon source, such as selected from one of the herein disclosed types, bio-production media may 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 the enzymatic pathway necessary for chemical product bio-production under the present disclosure. Another aspect of the invention regards media and culture conditions that comprise genetically modified microorganisms of the invention and optionally supplements.

[00100] 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.

[00101] 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.

[00102] Bio-productions may be performed under aerobic, microaerobic or anaerobic conditions with or without agitation.

[00103] In some cases, a change of an environmental condition comprises a change in sugar concentration of a culture medium contacting a cell. In some cases, a change in sugar concentration of a culture medium is an increase of sugar concentration. In some other cases, a change in sugar concentration is a decrease of sugar concentration. In some situations, an increase of sugar concentration is from 1% to 2%, from 2% to 3%, from 3% to 4%, from 4% to 5%, from 5% to 10%, from 10% to 15%, from 15% to 20%, from 20% to 30%, from 30% to 40%, from 40% to 50% , from 50% to 60%, from 60% to 70%, from 70% to 80%, from 80% to 90%, or from 90% to 100% more sugar compared with the original sugar concentration in the culture medium. In some situations, a decrease of sugar concentration is from 1% to 2%, from 2% to 3%, from 3% to 4%, from 4% to 5%, from 5% to 10%, from 10% to 15%, from 15% to 20%, from 20% to 30%, from 30% to 40%, from 40% to 50% , from 50% to 60%, from 60% to 70%, from 70% to 80%, from 80% to 90%, or from 90% to 100% less sugar compared with the original sugar concentration in the culture medium.

[00104] In some cases, a change of an environmental condition comprises a change in oxygenation of a culture medium contacting a cell. In some cases, a change in oxygenation of a culture medium is an increase of oxygenation. In some other cases, a change in oxygenation of a culture medium is a decrease of oxygenation. In some situations, an increase of oxygenation is

the addition of oxygen from 1% to 2%, from 2% to 3%, from 3% to 4%, from 4% to 5%, from 5% to 10%, from 10% to 15%, from 15% to 20%, from 20% to 30%, from 30% to 40%, from 40% to 50% , from 50% to 60%, from 60% to 70%, from 70% to 80%, from 80% to 90%, or from 90% to 100% more than the original amount of oxygen added in a culture medium. In some situations, a decrease of oxygenation is the addition of oxygen from 1% to 2%, from 2% to 3%, from 3% to 4%, from 4% to 5%, from 5% to 10%, from 10% to 15%, from 15% to 20%, from 20% to 30%, from 30% to 40%, from 40% to 50% , from 50% to 60%, from 60% to 70%, from 70% to 80%, from 80% to 90%, or from 90% to 100% less than the original amount of oxygen added in a culture medium.

Bio-production Reactors and Systems

[00105] Fermentation systems utilizing methods and/or compositions according to the invention are also within the scope of the invention.

[00106] 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. 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).

Genetic Modifications, Nucleotide Sequences, and Amino Acid Sequences

[00107] Embodiments of the present disclosure 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.

[00108] 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.

[00109] 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.

[00110] 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 disclosure. 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.

[00111] For various embodiments of the invention the genetic manipulations may be described to include various genetic manipulations, including those 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 and/or identified culture conditions and/or to provision of additional nucleic acid sequences such as to increase copy number and/or 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.

[00112] 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 (IdhA), phosphate acetyltransferase (pta), pyruvate oxidase (poxB), pyruvateformate lyase (pflB), methylglyoxal synthase (mgsA), acetate kinase (ackA), alcohol dehydrogenase (adhE), the clpXP protease specificity enhancing factor (sspB), the ATPdependent Lon protease (Ion), 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.

[00113] 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. In some embodiments, a microorganism may comprise two, three, four, five, six, seven, eight, nine, or ten, or more synthetic metabolic valves. 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: fabl, zwf gltA, ppc, udhA, Ipd, sucD, aceA, pfkA, Ion, rpoS, 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.

[00114] 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.

[00115] Accordingly, as described in various sections above, some compositions, methods and systems of the present disclosure 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.

[00116] 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. 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.

[00117] 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-OHbutyrate, 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. VI.A Gene Silencing

[00118] In particular 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.

[00119] In some cases, a valve polynucleotide comprises a polynucleotide selected from the group consisting of: a silencing polynucleotide for repressing transcription of a gene encoding said valve enzyme; a degradation polynucleotide for mediating cellular degradation of said valve enzyme; and a combination thereof.

[00120] In some cases, a valve polynucleotide comprises a silencing polynucleotide, and said silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter of a gene encoding said valve enzyme.

[00121] In some cases, a valve polynucleotide further encodes a CRISPR enzyme, wherein said CRISPR enzyme specifically binds to said promoter sequence when bound to said gRNA. In some cases, a CRISPR enzyme is catalytically inactive.

[00122] In some cases, a valve polynucleotide comprises a degradation polynucleotide, wherein said degradation polynucleotide comprises a sequence encoding a degradation tag, wherein said degradation tag mediates degradation of said valve enzyme. In some cases, the expression of a valve polynucleotide is regulated by phosphate availability in a cell. In some cases, the expression of a production polynucleotide is regulated by phosphate availability in a cell. In certain cases, the cell is an *E. coli* cell.

Controlled Proteolysis

[00123] In particular the current disclosure describes the use of controlled protein degradation or proteolysis to help enable 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 can be used. 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, an N-terminal sequence can be added to a protein of interest to enable 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.

[00124] 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. The invention describes the use of controlled protein degradation or proteolysis to help enable 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.

Synthetic Metabolic Valve Control

[00125] In particular the current disclosure 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 multistage fermentations. These include but are not limited to artificial chemical inducers including: tetracycline, anhydrotetracycline, lactose, IPTG (isopropyl-beta-D-l-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.

[00126] 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.

Products

[00127] In some embodiments, provided herein is a microorganism or a cell for producing a product. In some cases, the product comprises 3-hydroxypropionic acid. In some cases, the product comprises an amino acid. In some cases, the amino acid comprises alanine. In some cases, the alanine is L-alanine. In some cases, the alanine is D-alanine. In some cases, a rate of production of alanine is at least 0.1 g/L/hr, 0.2 g/L/hr, 0.3 g/L/hr, 0.4 g/L/hr, 0.5 g/L/hr, 0.6 g/L/hr, 0.7 g/L/hr, 0.8 g/L/hr, 0.9 g/L/hr, 1.0 g/L/hr, 1.1 g/L/hr, 1.2 g/L/hr, 1.3 g/L/hr, 1.4 g/L/hr, 1.5 g/L/hr, 1.6 g/L/hr, 1.7 g/L/hr, 1.8 g/L/hr, 1.9 g/L/hr, 2.0 g/L/hr, 2.5 g/L/hr, 3.0 g/L/hr, 3.5 g/L/hr, 4.0 g/L/hr, 4.5 g/L/hr, 5.0 g/L/hr, 5.5 g/L/hr, 6.0 g/L/hr, 7.0 g/L/hr, 8.0 g/L/hr, 9.0 g/L/hr, or at least 10 g/L/hr.

[00128] In some cases, the alanine titers after 24 hours can be from 0 to 0.5 g/L, 0.5 g/L to 1 g/L, 1 g/L to 1.5 g/L, 1.5 g/L to 2 g/L, 2 g/L to 2.5 g/L, 2.5 g/L to 3 g/L, 3 g/L to 3.5 g/L, 3.5 g/L to 4 g/L, 4 g/L to 4.5 g/L, 4.5 g/L to 5 g/L, or from 5 g/L to 10 g/L. The dynamic range of alanine production offered by SMVs can be up to a 4-fold increase compared to that offered by solely altering the expression level of the production pathway enzymes (by changing the promoter). In some cases, the dynamic range of alanine production offered by SMVs can be up to a 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold increase compared to that offered by solely altering the expression level of the production pathway enzymes.

[00129] In some cases, a production polynucleotide in the microorganism encodes an alanine exporter. In some cases, the alanine exporter is alaE.

[00130] In some cases, the product comprises mevalonic acid. In some cases, a rate of production of mevalonic acid is at least 0.1 g/L/hr, 0.2 g/L/hr, 0.3 g/L/hr, 0.4 g/L/hr, 0.5 g/L/hr, 0.6 g/L/hr, 0.7 g/L/hr, 0.8 g/L/hr, 0.9 g/L/hr, 1.0 g/L/hr, 1.1 g/L/hr, 1.2 g/L/hr, 1.3 g/L/hr, 1.4 g/L/hr, 1.5 g/L/hr, 1.6 g/L/hr, 1.7 g/L/hr, 1.8 g/L/hr, 1.9 g/L/hr, 2.0 g/L/hr, 2.5 g/L/hr, 3.0 g/L/hr, 3.5 g/L/hr, 4.0 g/L/hr, 4.5 g/L/hr, 5.0 g/L/hr, 5.5 g/L/hr, 6.0 g/L/hr, 7.0 g/L/hr, 8.0 g/L/hr, 9.0 g/L/hr, or at least 10 g/L/hr.

Methods

[00131] Provided herein are methods for producing a product in an engineered microorganism in a large scale. Also provided herein are methods for engineering microorganisms for large-scale production of a product in a high-throughput fashion.

[00132] In some cases, provided herein is a method, comprising: culturing a plurality of strains of a cell, wherein each strain of said plurality of strains comprises (i) an engineered valve polynucleotide for mediating controlled reduction of expression of a valve enzyme acting in a metabolic pathway, wherein said controlled reduction of expression of said valve enzyme reduces flux through said metabolic pathway; and (ii) an engineered production polynucleotide for mediating controlled increase in expression of a production enzyme for generation of said product; wherein each strain of said plurality of strains differs from another strain in a sequence of at least one of said engineered valve polynucleotide or said engineered production polynucleotide; measuring a level of said product generated by each of said plurality of strains; and selecting a strain based on said level of said product. In some embodiments, the method further comprises growing said selected strain in a bioreactor. In some embodiments, a culture medium comprising said selected strain has a volume of at least 100 ml, 200 ml, 300 ml, 400 ml, 500 ml, 600 ml, 700 ml, 800 ml, 900 ml, or at least 1000 ml. In some embodiments, a culture medium has a volume of at least 1L.

[00133] In some embodiments, a valve polynucleotide comprises a polynucleotide selected from the group consisting of: a silencing polynucleotide for repressing transcription of a gene encoding said valve enzyme; a degradation polynucleotide for mediating cellular degradation of said valve enzyme; and a combination thereof. In some embodiments, a first and a second strain of said plurality of strains comprise a silencing polynucleotide. In some embodiments, a silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter of a gene encoding said valve enzyme. In some embodiments, a gRNA sequence differs between said first and second strains. In some embodiments, a promoter recognized by said gRNA differs between said first and second strains. In some embodiments, a first strain comprises said silencing polynucleotide and said degradation polynucleotide, and a second strain comprises said silencing polynucleotide but does not comprise said degradation polynucleotide. In some embodiments, a level of product is greater in said second strain than said first strain. In some embodiments, a level of product is greater in said first strain than said second strain. In some embodiments, a valve enzyme comprises an enzyme selected from the group consisting of enoyl-ACP/CoA reductase (fabI), glucose-6-phosphate dehydrogenase (zwf), lipoamide dehydrogenase (lpd), citrate synthase (gltA), soluble transhydrogenase (udhA),

NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (gapA), and a combination thereof. In some embodiments, a production enzyme comprises an enzyme selected from the group consisting of NADPH-dependent alanine dehydrogenase (ald), alanine exporter (alaE), NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (gapN), and a combination thereof.

[00134] In some embodiments, a product is selected from the group consisting of mevalonic acid, 3-hydroxypropionic acid, an amino acid, and a combination thereof. In some embodiments, the amino acid is alanine. In some embodiments, the alanine is L-alanine. In some embodiments, the alanine is D-alanine.

[00135] In some embodiments, a rate of production of the product during said stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking said controlled reduction of expression of said valve enzyme.

[00136] In some embodiments, a change of an environmental condition comprises a change in a sugar concentration of a culture medium contacting said cell.

[00137] In some embodiments, a change of an environmental condition comprises a change in oxygenation of a culture medium contacting said cell.

[00138] In some cases, provided herein is a method of generating a cellular product comprising: culturing a heterologous cell in a culture medium, wherein said heterologous cell comprises: (i) an engineered valve polynucleotide for mediating controlled reduction of expression of a valve enzyme acting in a metabolic pathway, wherein said controlled reduction of expression of said valve enzyme reduces flux through said metabolic pathway, wherein said controlled reduction of expression of said valve enzyme induces a stationary phase of said cell; and (ii) an engineered production polynucleotide for mediating controlled increase in expression of a production enzyme for generation of said product; wherein a rate of production of said product during said stationary phase is reduced less in response to a change of an environmental condition as compared to a cell lacking said controlled reduction of expression of said valve enzyme. In some embodiments, the method further comprises changing said environmental condition. In some embodiments, the environmental condition comprises a sugar concentration of said culture medium, and changing said environmental condition comprises increasing or decreasing said sugar concentration. In some cases, said sugar is glucose, sucrose, lactose, maltose, xylose, mannitol, or a combination thereof. In some cases, said sugar is glucose. In some cases, the environmental condition comprises an oxygen concentration of said culture medium, and changing said environmental condition comprises increasing or decreasing said oxygen concentration. In some cases, said culturing is performed in a bioreactor.

[00139] In some cases, said culture medium has a volume of at least 100 ml, 200 ml, 300 ml, 400 ml, 500 ml, 600 ml, 700 ml, 800 ml, 900 ml, or at least 1000. In some cases, said culture medium has a volume of at least 1L. In some case, said product comprises 3-hydroxypropionic acid. In some cases, said product comprises an amino acid. In some cases, said amino acid comprises alanine.

[00140] In some cases, a rate of production of said alanine is at least 0.1 g/L/hr, 0.2 g/L/hr, 0.3 g/L/hr, 0.4 g/L/hr, 0.5 g/L/hr, 0.6 g/L/hr, 0.7 g/L/hr, 0.8 g/L/hr, 0.9 g/L/hr, 1.0 g/L/hr, 1.1 g/L/hr, 1.2 g/L/hr, 1.3 g/L/hr, 1.4 g/L/hr, 1.5 g/L/hr, 1.6 g/L/hr, 1.7 g/L/hr, 1.8 g/L/hr, 1.9 g/L/hr, 2.0 g/L/hr, 2.5 g/L/hr, 3.0 g/L/hr, 3.5 g/L/hr, 4.0 g/L/hr, 4.5 g/L/hr, 5.0 g/L/hr, 5.5 g/L/hr, 6.0 g/L/hr, 7.0 g/L/hr, 8.0 g/L/hr, 9.0 g/L/hr, or at least 10 g/L/hr. In some cases, said production polynucleotide encodes an alanine exporter. In some cases, said alanine exporter is alaE. In some cases, said culturing occurs for less than 20 hours, 30 hours, 40 hours, 50 hours, 60 hours, 70 hours, 80 hours, 90 hours, or less than 100 hours. In some cases, said culturing occurs for less than 10 hours, 15 hours, 20 hours, 25 hours, 30 hours, 35 hours, 40 hours, or less than 45 hours. In some cases, said culturing occurs for less than 30 hours.

[00141] In some cases, said product comprises mevalonic acid. In some cases, a rate of production of said mevalonic acid is at least 0.1 g/L/hr, 0.2 g/L/hr, 0.3 g/L/hr, 0.4 g/L/hr, 0.5 g/L/hr, 0.6 g/L/hr, 0.7 g/L/hr, 0.8 g/L/hr, 0.9 g/L/hr, 1.0 g/L/hr, 1.1 g/L/hr, 1.2 g/L/hr, 1.3 g/L/hr, 1.4 g/L/hr, 1.5 g/L/hr, 1.6 g/L/hr, 1.7 g/L/hr, 1.8 g/L/hr, 1.9 g/L/hr, 2.0 g/L/hr, 2.5 g/L/hr, 3.0 g/L/hr, 3.5 g/L/hr, 4.0 g/L/hr, 4.5 g/L/hr, 5.0 g/L/hr, 5.5 g/L/hr, 6.0 g/L/hr, 7.0 g/L/hr, 8.0 g/L/hr, 9.0 g/L/hr, or at least 10 g/L/hr. In some cases, said culturing occurs for less than 20 hours, 30 hours, 40 hours, 50 hours, 60 hours, 70 hours, 80 hours, 90 hours, or less than 100 hours. In some cases, said culturing occurs for less than 80 hours.

[00142] In some embodiments, a valve polynucleotide comprises a polynucleotide selected from the group consisting of: a silencing polynucleotide for repressing transcription of a gene encoding said valve enzyme; a degradation polynucleotide for mediating cellular degradation of said valve enzyme; and a combination thereof. In some cases, a valve polynucleotide comprises a silencing polynucleotide, and said silencing polynucleotide comprises a guide RNA (gRNA) comprising a gRNA sequence that recognizes a promoter of a gene encoding said valve enzyme. In some cases, a valve polynucleotide further encodes a CRISPR enzyme, wherein said CRISPR enzyme specifically binds to said promoter sequence when bound to said gRNA. In some cases, a CRISPR enzyme is catalytically inactive. In some case, a valve polynucleotide comprises a degradation polynucleotide, wherein said degradation polynucleotide comprises a sequence encoding a degradation tag, wherein said degradation tag mediates degradation of said valve enzyme. In some cases, an expression of said valve polynucleotide is regulated by phosphate. In

some cases, an expression of said production polynucleotide is regulated by phosphate. In some cases, said cell is an *E. coli* cell.

Optimization of Bio-production

[00143] Biotechnology based fermentation processes have been successfully developed to produce everything from biologics and small molecule therapeutics to specialty, bulk and commodity chemicals, and even next generation biofuels [1-3]. These processes have made rapid advancements in recent years due to numerous technology developments [4, 5]. It has never been easier to produce new molecules using synthetic biology. Despite these advances, a major challenge remains in taking molecules from proof of concept (POC) to commercially meaningful levels. Strain optimization, or overcoming the "mg" to "kg" hurdle has remained a key barrier to the successful commercialization of bio-processes. After the demonstration of POC, successful bio-process development routinely requires lengthy iterations of both microbial strain and fermentation optimization [6-8] (**Figure 1B**). These optimization efforts are often specific to the product or host strain of interest. The throughput of synthetic biology has outpaced that of metabolic engineering, partly due to a lack of broadly useful tools to perform meaningful and standardized optimization of engineered microbial strains in a high-throughput manner [9].

[00144] There are numerous challenges in strain optimization and moving past POC levels, not the least of which are the size and complexity of the potential design space. In contrast to simpler gene circuits, amenable to electrical circuit models[10-12], metabolic networks are highly interconnected. Each metabolite and/or enzyme can interact with endless others. This combinatorial complexity results in a huge potential design space, which is intractable to the kinds of systematic experimentation required for the development of standardized design principles (Supplemental Materials, Table 1). The challenges in addressing such a large design space have persisted despite the dramatic advances in, and decreased costs of, reading and writing DNA that have led to new high-throughput DNA assembly and microbial strain construction methods [13-16]. It is not surprising that new synthetic biology technologies involving strain engineering are often demonstrated with easily screened or selected phenotypes [13, 17-19]. Most of these are limited to a focus on optimizing a limited set of pathway specific enzymes.

[00145] One approach to overcome the complexity of this challenge is the use of *in vitro* systems for bio-production, which comprise a limited set of metabolic enzymes. However, these approaches have challenges in replicating key advantages of *in vivo* systems, including cofactor recycling and energy generation [20, 21]. Another approach to deal with this complexity is to develop faster screening methods for strain evaluation [22]. However, increased throughput alone can never evaluate the full complexity of the potential design space. In addition, results obtained

from high-throughput studies often do not translate, even in the same microbe, to a different environment [20, 23, 24]. Small scale screens do not readily translate to larger scale production processes, leading to iterations of process optimization on top of strain optimization (**Figure 1B**). This is because metabolism is highly regulated and can respond, sometimes dramatically, to changes in environmental conditions [25 20, 26-28]. A lack of environmental robustness is traditionally one factor making the scale up of fermentation based processes difficult. This issue has led to the development of specialized complex micro-reactor systems for scale down offering only modest improvements in throughput [20, 29-31].

[00146] There remains a significant need for broadly applicable, rapid and robust approaches to greatly reduce the time and costs transitioning from "mgs" to "kgs". Ideally, approaches should be amenable to multiple products and production hosts. Provided herein is the development of a generalizable, high-throughput strain optimization approach that enables the use of truly scalable, standardized fermentation processes. This approach, as outlined in **Figure 1B**, panel b, involves the dynamic minimization of the active metabolic network [32], which combines the benefits of a smaller design space common to *in vitro* approaches while maintaining the benefits of *in vivo* biosynthesis [20]. We can isolate and focus on the minimal metabolic networks required for production. Utilizing combinations of synthetic metabolic valves (SMVs) [32, 33] (**Figures 2A-D**) we can dynamically minimize the metabolic network and redirect metabolic flux in the context of a standardized 2-stage fermentation process [20].

[00147] This approach can reduce the complexity of the problem and the size of the relevant design space, greatly speeding up optimization. In various embodiments, it is demonstrated herein that dynamic metabolic network minimization can improve pathway fluxes beyond those achievable with production pathway modifications alone (**Figures 3A-K and 6A-H**). Simultaneously, we demonstrate that dynamic network minimization reduces metabolic responses to environmental conditions, which increases the robustness and scalability of engineered strains (**Figures 3A-K and 5A-J**).

Examples

2-Stage Synthetic Metabolic Valves in E. coli

[00148] We first developed improved synthetic metabolic valves (SMVs) in *E. coli* that are capable of the dynamic reduction of protein levels in a 2-stage process. These SMVs can be used to reduce levels of key metabolic enzymes (or reduce enzymatic activities of key metabolic enzymes) and rely on controlled proteolysis or CRISPR-based gene silencing or both proteolysis and silencing in combination (**Figures 2A-D**) [32-35]. Cell growth and dynamic metabolic control can be implemented using phosphate depletion as an environmental trigger. Phosphate can be an

ideal candidate as a trigger, as one of the costliest components of minimal media. In addition, stationary phases induced in *E. coli* by phosphate depletion have retained glycolytic uptake as well as increased protein expression [31, 36]. Numerous promoter systems responding to phosphate are well characterized in *E. coli* as well as other microbes including *S. cerevisiae* [37]. Phosphate responsive promoter variants were evaluated (Supplemental Materials, Section 1) and subsequently used for 2-stage control.

[00149] SMVs were implemented in *E. coli* using the native Type I-E Cascade CRISPR system for induced gene silencing [34, 38], while controlled proteolysis was induced by incorporating C-terminal degron tags on target proteins, both as previously demonstrated [63,33] (**Figure 2A**). These systems were introduced into a host strain initially engineered for minimal byproduct formation and high biomass yields and growth rates (*E. coli* strain DLF_0025, Supplemental Materials, Section 3) [24, 27, 28 39]. Using this approach, as Figures 2A-D demonstrate, protein levels can be controlled in 2-stage processes, as exemplified by turning "ON" GFPuv and "OFF" mCherry fluorescent proteins with phosphate depletion in minimal medium. The combination of gene silencing with proteolysis results in the largest rates of protein degradation (**Figures 2C-D**). The specific impact of gene silencing and proteolysis on decay rates will likely vary depending on the host, target gene/enzyme, and its specific natural turnover rates and expression levels [40, 41].

Metabolic Network Minimization Leads to Improved Fluxes

[00150] With the successful demonstration of dynamic control of protein levels in a 2-stage process, we turned to investigate the dynamic control of metabolic fluxes in *E. coli* through controlled reduction of key central metabolic enzymes alone and in combination. Reducing fluxes through thermodynamically favored "committed" reactions in the network is expected to lead to increases in network metabolite pools (Supplemental Materials Section 5), and as a result, changes in pathway fluxes. Enzymes in key committed steps in central metabolic pathways were identified and chosen as initial SMV targets and alanine was chosen as an initial test product (**Figures 3A-K**). A set of strains were constructed for alanine production (**Figure 3A**), comprising an NADPH-dependent alanine dehydrogenase (*ald**)[42]. Variants with multiple combinations of SMVs in central metabolic enzymes were made, with either modifications to induce proteolysis or gene silencing or both in combination. (Supplemental Materials, Section 3). Together the set of strains having SMVs evaluated in 2-stage processes are identified as "Valve" strains. A panel of alanine "Valve" strains (~500 strains in total) were evaluated for alanine production in standardized, 2-stage, 96-well plate based micro-fermentations (Supplemental Materials, Section 7). Alanine titers after 24 hours of production are given in **Figures 3B-C**. Briefly, alanine titers after 24 hours ranged from ~ 0 g/L to ~ 4.7 g/L, and as expected, varied significantly with respect to the number and combination of SMVs; most SMV

combinations lead to improved performance when compared to the control with no SMVs and the alanine pathway alone. In some cases, the alanine titers after 24 hours can be from 0 to 0.5 g/L, 0.5 g/L to 1 g/L, 1 g/L to 1.5 g/L, 1.5 g/L to 2 g/L, 2 g/L to 2.5 g/L, 2.5 g/L to 3 g/L, 3 g/L to 3.5 g/L, 3.5 g/L to 4 g/L, 4 g/L to 4.5 g/L, 4.5 g/L to 5 g/L, or from 5 g/L to 10 g/L. The dynamic range of alanine production offered by SMVs can be up to a 4-fold increase compared to that offered by solely altering the expression level of the production pathway enzymes (by changing the promoter) (Supplemental Materials, Section 7). In some cases, the dynamic range of alanine production offered by SMVs can be up to a 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, or 10-fold increase compared to that offered by solely altering the expression level of the production pathway enzymes. Importantly, the use of proteolysis or silencing alone and/or in combination had significant impacts on production, indicating that for each enzyme the fine tuning of activity using SMVs is critical. One of the best performing strains from the micro-fermentations was then evaluated in a minimal medium, 2-stage, 1L fermentation with 10 gdcw/L of biomass (**Figure 3F**), which resulted in 80 g/L 100% L-alanine after 48 hours of production with a yield of 0.8 g/g. Further engineering of this strain by overexpressing an alanine exporter (encoded by the *E. coli alaE* gene [43]) resulted in 147 g/L 100% L-alanine after 27 hours of production with a yield within error of theoretical yield ~1 g/g, (**Figure 3G**).

Micro-fermentation Robustness

[00151] A central hypothesis was that by restricting metabolism in the production stage, strain performance could not only be improved, but would be more robust to environmental (process) conditions. Simply put, carbon flow is restricted through a minimized metabolic network, which can no longer adapt via cellular responses to the environment. To test this hypothesis, strains were evaluated under different "micro-fermentation" process conditions. Glucose concentration and oxygen transfer rate (key process variables impacting strain performance in traditional fermentations [26]) were varied (**Figure 3D**, Supplemental Materials, Section 8), and alanine production measured. A robustness score (RS) was developed to quantify environmental robustness. Larger RS scores indicate more robust strains. Whereas relative standard deviation (RSD) is one metric for robustness, we wanted to incorporate a stricter measure of robustness which also incorporates the maximal deviation (Max Dev) a strain has under all process conditions (RS, Equation (1)).

$$\text{Equation (1):} RS = 100 - \frac{average(RSD) + \max(Dev)}{2} * 100$$

[00152] Robustness scores for a subset of 48 alanine "Valve" strains are given in **Figure 3E**. Results from these experiments studies are tabulated in Supplemental Materials, Section 8. A Chi^2 analysis using a cutoff of RS >0.6 for robustness was used to identify key SMVs which statistically contribute to process robustness. The proteolytic degradation of *fabI* was a primary contributor to robustness (Chi^2 =13.85, $P_{value} < 0.001$) and as a result, "Valve" strains with proteolytic degradation of *fabI* were used in further studies. In addition, the "Valve" strains with proteolytic degradation of *gltA* and/or the combination of the proteolytic degradation of *fabI* and *gltA* were found to also be significant contributers of robustness, albeit with a large P_{value}.

2-Stage "Valve" Strains Compared to Traditional Growth Associated Strains

[00153] To compare the 2-stage approach enabled by SMVs to more traditional growth associated processes, we constructed 5 strains, with constitutively expressed alanine dehydrogenase (*ald**), capable of the growth associated production of alanine. These growth associated strains varied in the strength of the promoter used to drive *ald** expression [44] (Supplemental Materials, Section 2), yet utilized the same common no-valve control host strain. **Figure 5** illustrates the results of a direct comparison of "Valve" strains in a 2-stage process compared to "Growth Associated (GA)" strains in a traditional fermentation at the microtiter (**Figures 5A-D**) and 1L (**Figures 5E-J**) scales. In micro-fermentations, 2-stage "Valve" strains outperformed GA strains with respect to titer and process robustness. The most robust GA strain from the micro-fermentation analysis (also with the highest production level) was compared to a robust "Valve" strain in 1L fermentations with varied process conditions. The "Valve" strains showed consistent performance in all process conditions evaluated (**Figure 5E**), consistent with results from micro-fermentations, where the GA strain had significant performance variability dependent on process. We hypothesized that the increased environmental robustness observed in both "micro-" and 1L scale fermentations for "Valve" strains would lead to predictable scale up, where strains with improved performance in high-throughput micro-fermentations would reliably have improved performance in controlled bioreactors. To evaluate the scalability of the system, "Valve" alanine strains with statistically differentiated performance in micro-fermentations (P-value < 0.001) were evaluated in standardized 2-stage 1L fermentations and compared to all GA strains. Statistically different performances observed in "micro-fermentations" have scaled predictably to 1L fermentations for 2-stage "Valve" strains. This contrasts with results obtained with GA strains where no correlation between micro-fermentation and 1L performance was observed (**Figures 5G-H**).

Product Flexibility

[00154] With the successful and predictable scale-up of alanine strains into 1L fully instrumented fermentations, we moved to validate the technology platform for an additional

product: mevalonic acid. To this end, additional dynamic production pathways were constructed for mevalonic acid biosynthesis (**Figure 6A**). A set of two-gene production pathway plasmids encoding three enzymatic functions was constructed for mevalonic acid production, consisting of the *E. faecalis mvaE* and *mvaS* genes encoding a bifunctional acetyl-CoA acetyltransferase, NADPH dependent HMG-CoA reductase, and HMG-CoA synthase respectively. A mutant *mvaS* gene, *mvaS*(A110G) with higher activity was used [45, 46]. Production plasmids were initially evaluated for mevalonate production in the control strain (**Figure 6B**). The best producing plasmid was then introduced into a variety of engineered "Valve" strains and evaluated in micro-fermentations (**Figure 6C**). A subset of statistically differentiated strains were then evaluated in 1L fermentations to assess scalability (**Figure 6D**), which, as in the case of alanine, was predictive. In some cases, a performing strain produced meaningful titers and yields, 97 g/L in 78 hrs of production with a yield of 0.46 g/g (84% of theoretical yield) (**Figure 6E**). Specific productivity for this mevalonate strain is over 4-fold higher than the best previously reported results [47] (Supplemental Materials, Section 9).

Discussion

[00155] Historically some of the most successful efforts to metabolically engineer the production of small molecules have leveraged the power of anaerobic metabolism to couple product formation with growth. This has allowed for the classical design and selection of industrial strains to produce many products including ethanol, succinic acid, lactate and isobutanol, which have leveraged the power of evolution and selection to reach optimal metabolic fluxes in engineered networks [48, 49]. While growth associated production is not strictly linked to anaerobic metabolism, growth association greatly limits the number and variety of different molecules that can be made using synthetic biology. A generic, robust and accessible non-growth associated platform would greatly simplify the optimization and scale up of a diverse number of products.

[00156] In contrast to most existing 2-stage processes, which have relied on natural metabolic responses to environmental triggers for production improvement, we have taken the next step in actively minimizing the essential metabolic network and redirecting metabolites to products of interest. Many of the targeted essential central metabolic pathways in this work have traditionally been off limits to engineering strategies, as deleting essential enzymes is incompatible with growth and growth associated production in traditional fermentation. The dynamically minimized metabolic network also results in enhanced robustness to environmental variables enabling the faithful translation of high-throughput small-scale studies to larger instrumented fermentations. A current paradigm in the field is to improve the throughput of relevant strain evaluations by developing small-scale, custom-designed micro-reactors for

enhanced process control. In contrast, our approach is a move in a new direction involving engineering microbial metabolism to be less sensitive to process changes, simplifying high-throughput experimentation.

[00157] Beyond robustness, we have demonstrated that combinatorial modifications to essential enzymes in minimal metabolic networks can lead to significant improvements in production, particularly when compared to altering production pathway expression levels alone. These large variations in performance are due to changes in a limited subset of key central metabolic nodes, likely resulting in altered metabolite levels. Compared to previous approaches to dynamically control enzyme levels, we demonstrate improved potential for fine tuning of protein levels with a combination of gene silencing and proteolysis [50]. As stationary phase cells cannot dilute existing proteins with cell division, this dual approach makes sense. The specific control of the level of any given enzyme will of course also depend on natural turnover mechanisms. At first glance, it may still be surprising that the combination of both gene silencing and proteolysis together does not always result in improved performance, i.e. "more is not always better". Future efforts may be needed to explain these results, which could either be due to a requirement of maintaining minimal fluxes in the larger network or a consequence of changes in the levels of key regulatory metabolites that are not part of the minimal network, yet influence network activity.

[00158] While the approach as demonstrated can address many issues common to most bio-production processes, many product specific challenges remain. The toxicity of a product or pathway metabolite may limit titers or production rates. A minimal network that may be optimal at a low titer, may not be optimal at elevated titers. In addition, the engineering of improved enzymes is often a challenge in many "mg" to "kg" projects.

[00159] Feasibility of adapting this approach to other microbial hosts is expected. Key requirements for new hosts include a rapid and robust growth phase, the ability to engineer dynamic control over protein levels, and a metabolically active stationary phase. Numerous microbes have well characterized nutrient triggers for productive stationary phase metabolism [36], for example nitrogen limitation in *Ralstonia* species, *Yarrowia* species and others [51 52]. Even when these requirements are not naturally met, they can be engineered into the host such as *S. cerevisiae* or other microbes, with each potential host presenting unique challenges and corresponding solutions.

[00160] Future efforts can be aimed at applying this platform for molecules with more complex production pathways. This approach can offer a tractable route for rapid optimization to metabolic engineers and synthetic biologists, who wish to move past POC levels and begin to tackle problems at more industrially relevant rates, titers and yields.

Methods

Reagents and Media

[00161] 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 Supplemental Materials, Section 7.

E. coli Strain Construction

[00162] Oligonucleotides and synthetic linear DNA (Gblocks™) used for strain construction and confirmation are all given in Supplemental Materials, Section 3, 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) [62]. Chromosomal modifications were made using standard recombineering methodologies [63] 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, strictly following the protocols of Li et al [64]. 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 gBlocks 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 [65], and moved to the appropriate background strain using standard methodologies. The kanamycin resistance cassette was cured using the pCP20 plasmid, leaving an frt scar [63, 65]. 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.

E. coli Plasmid Construction

[00163] Primers used for the design and construction of CASCADE guides arrays were listed in Supplemental Materials, Section 6. 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 [73] with an insulated low phosphate induced ugpB promoter [74]. 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.

[00164] 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.

[00165] Production pathways enzymes were expressed from high copy plasmids via low phosphate inducible promoters. Production pathway gene sequences were codon optimized using the Codon Optimization Tool from the IDT website, phosphorylated G-blocks™ were designed and purchased from IDT for each pathway. Plasmids were assembled using NEBuilder® HiFi DNA Assembly Master Mix following manufacturer's protocol (NEB, MA).

pSMART-HC-Kan (Lucigen, WI) was used as backbone for all pathway plasmids. All plasmid sequences were confirmed by DNA sequencing (Eton Bioscience, NC) and deposited with Addgene.

E. coli BioLector

[00166] Single colonies of each strain were inoculated into 5 mL LB with appropriate antibiotics and cultured at 37 °C, 220 rpm for 9 hours or until OD600 reached > 2. 500 µL of the culture was inoculated into 10 mL SM10 medium with appropriate antibiotics, and cultured in a square shake flask (CAT#: 25-212, Genesee Scientific, Inc. San Diego, CA) at 37 °C, 220 rpm for 16 hours. Cells were pelleted by centrifugation and the culture density was normalized to OD600 = 5 using FGM3 media. Growth and fluorescence measurements were obtained in a Biolector (m2p labs, Baesweiler, Germany) using a high mass transfer FlowerPlate (CAT#: MTP-48-B, m2p-labs, Germany). 40 µL of the OD normalized culture was inoculated into 760 µL of FGM3 medium with appropriate antibiotics. Biolector settings were as follows: RFP gain=100, GFP gain=20, Biomass gain=20, shaking speed=1300 rpm, temperature=37 °C, humidity=85%. Every strain was analyzed in triplicate.

E. coli Micro-Fermentations

[00167] 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 °C, 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 enable adequate culture oxygenation.

[00168] 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 °C, 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 micro-fermentations were performed for each strain.

[00169] For growth associated alanine micro-fermentations, glycerol stock preparation and 16 hour overnight culture in SM10++ proceeded as described above. After 16 hours of growth in SM10++ medium, 5 µL of overnight culture was inoculated into 150 µL FGM3 with 40 mM phosphate containing appropriate antibiotic. Plates were covered with sandwich covers (Model # CR1596 obtained from EnzyScreen, Haarlam, The Netherlands) and 96 well plate cultures were incubated at 37 °C, 400 rpm for 24 hours. After 24 hours of production, OD600 was recorded, all samples from each well were then pelleted by centrifugation and the supernatant collected for subsequent analytical measurement. Triplicate micro-fermentations were performed for each strain.

[00170] Micro-fermentation robustness evaluations were conducted as described in Supplemental Materials, Section 8.

1L Fermentation Seeds

[00171] Single colony from transformation plate was inoculated into 5 mL LB with appropriate antibiotics and cultured at 37 °C, 220 rpm for 16 hours. 500 µL of the LB culture was inoculated into 50 mL SM10 media with appropriate antibiotics in square shake flask (CAT#: 25-214, Genesee Scientific, Inc. San Diego, CA), the culture was incubated at 37 °C 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 °C.

1L Fermentations

[00172] 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.

[00173] For the standardized 2-stage process with ~ 10 gcdw/L biomass, tanks were filled with 800 mL of FGM10 medium, with enough phosphate to target a final *E. coli* biomass concentration ~ 10 gcdw /L. 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 °C and pH 6.8 using 5 M ammonium hydroxide and 1 M hydrochloric acid as titrants. 10 M ammonium hydroxide was used for **Figure 3G** 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.

[00174] In the case of growth associated fermentation processes, tanks were filled with 800 mL of FGM10 medium with 40 mM phosphate, which was in great excess and ensured phosphate depletion doesn't happen for growth associated fermentation processes. 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 °C and pH 6.8 using 5 M ammonium hydroxide and 1 M hydrochloric acid as titrants. 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. Feed rate and dissolved oxygen concentration was set to desired values in the beginning, and maintained throughout the fermentation process. Fermentation runs were continued for up to ~ 50 hours and samples automatically withdrawn every 3 hours. Samples were saved for subsequent analytical analysis.

Analytical Methods

[00175] Sample standard curves for all compounds quantified are shown in Supplemental Materials, Section 10.

[00176] Glucose and Ethanol Quantification: A UPLC-RI method was developed for the simultaneous quantification of glucose and ethanol 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 °C. 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, for ethanol was 1-20 g/L. Samples were diluted as needed to be within the accurate linear range. Dilution was performed using ultrapure water.

[00177] Alanine Quantification: A reverse phase UPLC-MS/MS method was developed for alanine. 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 70 °C. The following eluents were used: solvent A: H_2O, 0.2% formic acid and 0.05% ammonium (v/v); solvent B: MeOH, 0.2% formic acid and 0.05% ammonium (v/v). The gradient elution was as follows: 0–0.1 min isocratic 5% B, flow rate increased from 0.65 mL/min to 0.75 mL/min; 0.1–0.3 min, linear from 5% to 95% B at 0.75 mL/min; 0.3–0.9 min isocratic 95% B at 0.75 mL/min; and 0.9–1.2 min linear from 95% to 5% B at 0.75 mL/min; 1.2–1.3 min isocratic 5% B at 0.75 mL/min. Sample injection volume was 5 μL. UPLC method development was carried out using standard aqueous stock solutions of analyte. Separations were performed using an Acquity H-Class UPLC integrated with a Xevo™ TQD Mass spectrometer (Waters Corp., Milford, MA. USA). MS/MS parameters including MRM transitions were tuned for each analyte and are listed in Table 22. Alanine (2,3-13C2, 99%) was used as internal standard for alanine at a concentration of 5 mg/L. Peak integration and further analysis was performed using MassLynx v4.1 software. The linear range for alanine was 1-100 mg/L. Samples were diluted as needed to be within the accurate linear range. Dilution was performed using ultrapure water, and the final 10-fold dilution was performed using solvent A, with 5 mg/L of C13 alanine (2,3-13C2, 99%).

[00178] Mevalonic Acid Quantification: A reverse phase UPLC-TUV method was developed for the simultaneous quantification of mevalonic acid and mevalonolactone. 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 1 mL/min. Sample injection volume was 10 µL. Absorbance was monitored at 210 nm. UPLC method development was carried out using standard aqueous stock solutions of analytes. 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 mevalonic acid and mevalonolactone were 0.01-0.1 g/L. Samples were diluted as needed to be within the accurate linear range. Mevalonic acid diluted in 20 mM phosphoric acid would spontaneously convert to mevalonolactone [80], thus, quantification of both mevalonic acid and mevalonolactone was necessary for fermentation samples. Mevalonic acid and mevalonolactone standards were prepared fresh each time, and ran immediately on UPLC. Dilution was performed using ultrapure water, and the final 10-fold dilution was performed using 20 mM phosphoric acid.

[00179] Alanine Stereoisomer Quantification: A reverse phase UPLC-TUV method was developed for the simultaneous quantification and differentiation of L-/D-alanine. Chromatographic separation was performed using a Chirex 3126 (D)-penicillamine column (150 x 4.6 mm, 5 µm; Phenomenex Inc., Torrance, CA) at 50 ℃. 2 mM Copper Sulfate was used as the eluent. The isocratic elution was as follows: 0–10 min at 0.75 mL/min. Sample injection volume was 10 µL. Absorbance was monitored at 254 nm. UPLC method development was carried out using standard aqueous stock solutions of analytes. 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 L-/D-alanine was 0.1-1 g/L. Samples were diluted as needed to be within the accurate linear range. Dilution was performed using ultrapure water.

Supplemental Materials

[00180] Table 1: Combinatorial complexity of metabolic networks.

Combination #	**Entire *E. coli* Gene Network**	**Reduced Central Metabolism Network**
	Number of Experiments	
1	4500	~45 (Glycolysis, TCA, PPP and ETC genes only)
2	1.0×10^6	990
3	1.5×10^{10}	14,190
4	1.7×10^{13}	148,995
5	1.5×10^{16}	1.2×10^6

Section 1: Phosphate promoters

[00181] Phosphate promoter sequences were obtained from the EcoCyc database [81] for PhoB regulated promoters (https://ecocyc.org/, **Table** 2). We sought to evaluate not only the relative strength of promoters previously characterized to respond to phosphate depletion, but in addition the relative leakiness in phosphate rich conditions. To this aim we constructed a set of fluorescent reporter plasmids. We cloned the ultraviolet excitable GFPuv gene behind a set of 12 phosphate dependent promoters, in the pSMART-HC-Kan (Lucigen, WI) backbone. These reporter strains were evaluated in a 2-stage micro-fermentation protocol in an m2p-labs Biolector™. Results are illustrated in **Figure** 7. The *ugpB* gene promoter was often chosen for high level tightly controlled expression when expression cassettes were chromosomally integrated or for the inducible expression of guide arrays.

[00182] Insulators [82] were added to both 5' and 3' end of a subset of phosphate promoters (**Table** 3) to help with consistent performance in different sequence contexts. To reduce read-through transcription, a unique terminator was added to the 5' end of each insulated promoter. Terminator sequences were from http://parts.igem.org/Terminators/Catalog. Insulated phosphate promoters were similarly characterized using GFPuv expression in a m2p-labs Biolector™ (**Figure 8**).

[00183] Table 2: Phosphate inducible promoter sequences evaluated, the ribosomal binding site is underlined, and the start codon of the gene (GFPuv) is shown in green.

Promoter Name	Sequence	SEQ ID NO
ugpBp	TCTTTCTGACACCTTACTATCTTACAAATGTAACAAAAAAGTTATTTTTCTGTAATTCGA GCATGTCATGTTACCCCGCGAGCATAAAACGCGTGTGTAGGAGGATAATCTATG	1
yibDp	GTGCGTAATTGTGCTGATCTCTTATATAGCTGCTCTCATTATCTCTCTACCCTGAAGTGAC TCTCTCACCTGTAAAAATAATATCTCACAGGCTTAATAGTTTCTTAATACAAAGCCTGTA AAACGTCAGGATAACTTCTGTGTAGGAGGATAATCTATG	2
phoAp	CGATTACGTAAAGAAGTTATTGAAGCATCCTCGTCAGTAAAAAGTTAATCTTTTCAACA GCTGTCATAAAGTTGTCACGGCCGAGACTTATAGTCGCTTTGTTTTTATTTTTTAATGTAT TTGTAGTGTAGGAGGATAATCTATGGCTAGCAAAGGAGAAGAACTTTTCACATG	3
phoBp	GCCACGGAAATCAATAACCTGAAGATATGTGCGACGAGCTTTTCATAAATCTGTCATAA ATCTGACGCATAATGACGTCGCATTAATGATCGCAACCTATTTATTGTGTAGGAGGATA ATCTATGGCTAGCAAAGGAGAAGAACTTTTCACATG	4
amnp	AGACAGTCAACGCGCTTGATAGCCTGGCGAAGATCATCCGATCTTCGCCTTACACTTTTG TTTCACATTTCTGTGACATACTATCGGATGTGCGGTAATTGTATAGGAGGATAATCTATG	5
ydfHp	GCTATGCCGGACTGAATGTCCACCGTCAGTAATTTTTATACCCGGCGTAACTGCCGGGTT ATTGCTTGTCACAAAAAAGTGGTAGACTCATGCAGTTAACTCACTGTGTAGGAGGATAA TCTATG	6

mipAp	CATCCATAAATTTTGCATAATTAATGTAAAGACCAGGCTCGCCAGTAACGCTAAATTCA TTTGGCTGTAAGCGCGGTGTCATCCGCGTCAGGAAAATTAAACAGTTACTTTAAAAAAT GAAAACGTAAAAAGGTTGGGTTTCGATGTATTGACGGGTAAACTTTGTCGCCCGCTAAA CATTTGTTTGTGTAGGAGGATAATCTATG	7
phoHp	AATCCTGCTGAAAGCACACAGCTTTTTTCATCACTGTCATCACTCTGTCATCTTTCCAGT AGAAACTAATGTCACTGAAATGGTGTTTTATAGTTAAATATAAGTAAATATATTGTTGCA ATAAATGCGAGATCTGTTGTACTTATTAAGTAGCAGCGGAAGTTCGTGTAGGAGGATAA TCTAT	8
yhjCp	CTACAGAGATGACGTGTAGAAAATAGTTACCGATATAAATAGTTACAGCTAAACGCCTG AAATTACATGTCGAGGGCACTATTTAAAACAATTTTGAGGATTTCCTTATATTGGTGGTT AGTACGCATGCAATTAAAAATGAAATTCCGCGACCACAAGCCAAAATAACAAACGGCA AGGAGACAAAAATAAGCACAAATAGCCAACACGTCCTCTGTTCACTTTAAAGGGAATCG CTGAAAAATACGCTCTGTTTAAGGGGATTCACCTTTCTCAGAAAGCTATTCCGCCCTTTT CCTGCTGAGAAATCGCCACATTCGGCATGACAACATTGTGAAAGTGTAGGAGGATAATC TATG	9
phoUp	ACCGAACTGAAGCAGGATTACACCGTGGTGATCGTCACCCACAACATGCAGCAGGCTGC GCGTTGTTCCGACCACACGGCGTTTATGTACCTGGGCGAATTGATTGAGTTCAGCAACA CGGACGATCTGTTCACCAGTGTAGGAGGATAATCTATG	10
pstSp	AAGACTTTATCTCTCTGTCATAAAACTGTCATATTCCTTACATATAACTGTCACCTGTTTG TCCTATTTTGCTTCTCGTAGCCAACAAACAATGCTTTATGAGTGTAGGAGGATAATCTAT GGCTAGCAAAGGAGAAGAACTTTTCACATG	11
phoEp	AGCATGGCGTTTTGTTGCGCGGGATCAGCAAGCCTAGCGGCAGTTGTTTACGCTTTTATT ACAGATTTAATAAATTACCACATTTTAAGAATATTATTAATCTGTAATATATCTTTAACA ATCTCAGGTTAAAAACTTTCCTGTTTTCAACGGGACTCTCCCGCTGGTGTAGGAGGATAA TCTATG	12

[00184] Table 3: Insulated promoter sequences. Insulator sequences are italicized. -35 and -10 boxes are highlighted in bold and underlined.

Insulated Promoter	**Sequence**	**SEQ ID NO**
BBa_B0015_IN_yibDp	CCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTT TTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCT TCGGGTGGGCCTTTCTGCGTTTATA*CACAGCTAACACCACGTCGTCCCTATCTG CTGCCCTAGGTCTATGAGTGGTTGCTGGATAAC*GTGCGTAATTGTGCTGATCTC TTATATAGCTGCTCTCATTATCTCTCTACCCTGAA**GTGACT**CTCTCACCTGTA AAAATAATATCTCACAGGCT**TAATA**GTTTCTTAATACAAAGCCTGTAAAACG TCAGGATAACTTCT*ATATTCAGGGAGACCACAACGGTTTCCCTCTACAAATAATTT TGTTTAACTTT*	13
BBa_B1002_IN_phoBp	CGCAAAAAACCCCGCTTCGGCGGGGTTTTTTCGCACGTCTCCATCGCTTGCC CAAGTTGTGAAG*CACAGCTAACACCACGTCGTCCCTATCTGCTGCCCTAGGTCT ATGAGTGGTTGCTGGATAAC*GCCACGGAAATCAATAACCTGAAGATATGTGCG ACGAGCTT**TTCATA**AATCTGTCATAAATCTGACG**CATAAT**GACGTCGCATTA ATGATCGCAACCTATTTATT*ATATTCAGGGAGACCACAACGGTTTCCCTCTACAA ATAATTTTGTTTAACTTT*	14

BBa_B1004_IN_mipAp	CGCCGAAAACCCCGCTTCGGCGGGGTTTTGCCGCACGTCTCCATCGCTTGCC CAAGTTGTGAAG*CACAGCTAACACCACGTCGTCCCTATCTGCTGCCCTAGGTCT ATGAGTGGTTGCTGGATAAC*CATCCATAAATTTTGCATAATTAATGTAAAGAC CAGGCTCGCCAGTAACGCTAAATTCATTTGGCTGTAAGCGCGGTGTCATCCG CGTCAGGAAAATTAAACAGTTACTTTAAAAAATGAAAACGTAAA**AAGGTT**G GGTTTCGATGTATTGACGG**GTAAAC**TTTGTCGCCCGCTAAACATTTGTTT*ATA TTCAGGGAGACCACAACGGTTTCCCTCTACAAATAATTTTGTTTAACTTT*	15
BBa_B1006_IN_phoUp	AAAAAAAAACCCCGCCCCTGACAGGGCGGGGTTTTTTTTACGTCTCCATCGC TTGCCCAAGTTGTGAAG*CACAGCTAACACCACGTCGTCCCTATCTGCTGCCCTA GGTCTATGAGTGGTTGCTGGATAAC*ACCGAACTGAAGCAGGATTACACCGTGG TGATCGTCACCCACAACATGCAGCAGGCTGCGCGTTGTTCCGACCACA**CGG CGT**TTATGTACCTGGGCGAATT**GATTGA**GTTCAGCAACACGGACGATCTGTT CACCA*ATATTCAGGGAGACCACAACGGTTTCCCTCTACAAATAATTTTGTTTAACTT T*	16
BBa_B1010_IN_phoHp	CGCCGCAAACCCCGCCCCTGACAGGGCGGGGTTTCGCCGCACGTCTCCATCG CTTGCCCAAGTTGTGAAG*CACAGCTAACACCACGTCGTCCCTATCTGCTGCCCT AGGTCTATGAGTGGTTGCTGGATAAC*AATCCTGCTGAAAGCACACAGCTTTTTT CATCACTGTCATCACT**CTGTCA**TCTTTCCAGTAGAAAC**TAATGT**CACTGAAA TGGTGTTTTATAGTTAAATATAAGTAAATATATTGTTGCAATAAATGCGAGA TCTGTTGTACTTATTAAGTAGCAGCGGAAGTTC*ATATTCAGGGAGACCACAAC GGTTTCCCTCTACAAATAATTTTGTTTAACTTT*	17

Section 2: Constitutive Promoters

[00185] A set of constitutive insulated promoters of varying strength were used for constitutive expression and taken directly from Davis et al., including the proA, proB, proC, proD promoters [82] and HCEp promoter [83]. Insulator was added to 5' and 3' of HCEp promoter. Similar to insulated phosphate promoters, a unique terminator was added to the 5' end of constitutive promoters. These were used to drive constitutive pathway expression in growth associated production strains as well as to make strain modifications where constitutive heterologous gene expression was appropriate. These promoter sequences are given in Table 4 below and promoter characterized using GFPuv expression (**Figure 9**).

[00186] Table 4: Constitutive promoter sequences.

Promoter	Sequence	SEQ ID NO
BBa_B1004_proA	CGCCGAAAACCCCGCTTCGGCGGGGTTTTGCCGCACGTC TCCATCGCTTGCCCAAGTTGTGAAGCACAGCTAACACCA CGTCGTCCCTATCTGCTGCCCTAGGTCTATGAGTGGTTG CTGGATAACTTTACGGGCATGCATAAGGCTCGTAGGCTA TATTCAGGGAGACCACAACGGTTTCCCTCTACAAATAAT TTTGTTTAACTTT	18

BBa_B1006_proB	AAAAAAAAACCCCGCCCCTGACAGGGCGGGGTTTTTTTT ACGTCTCCATCGCTTGCCCAAGTTGTGAAGCACAGCTAA CACCACGTCGTCCCTATCTGCTGCCCTAGGTCTATGAGT GGTTGCTGGATAACTTTACGGGCATGCATAAGGCTCGTA ATATATATTCAGGGAGACCACAACGGTTTCCCTCTACAA ATAATTTTGTTTAACTTT	19
BBa_B1010_proC	CGCCGCAAACCCCGCCCCTGACAGGGCGGGGTTTCGCC GCACGTCTCCATCGCTTGCCCAAGTTGTGAAGCACAGCT AACACCACGTCGTCCCTATCTGCTGCCCTAGGTCTATGA GTGGTTGCTGGATAACTTTACGGGCATGCATAAGGCTCG TATGATATATTCAGGGAGACCACAACGGTTTCCCTCTAC AAATAATTTTGTTTAACTTT	20
BBa_B1002_proD	CGCAAAAAACCCCGCTTCGGCGGGGTTTTTTCGCACGTC TCCATCGCTTGCCCAAGTTGTGAAGCACAGCTAACACCA CGTCGTCCCTATCTGCTGCCCTAGGTCTATGAGTGGTTG CTGGATAACTTTACGGGCATGCATAAGGCTCGTATAATA TATTCAGGGAGACCACAACGGTTTCCCTCTACAAATAAT TTTGTTTAACTTT	21
BBa_B0015_IN_HCEp	CCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGAC TGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTC TCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTT TCTGCGTTTATACACAGCTAACACCACGTCGTCCCTATC TGCTGCCCTAGGTCTATGAGTGGTTGCTGGATAACCTCC TTCACAGATTCCCAATCTCTTGTTAAATAACGAAAAAGC ATCAATTAAAACCCATGTCTTTCTATATTCCAGCAATGT TTTATAGGGGACATATTGATGAAGATGGGTATCACCTTA GTGAATTGCTATAAGCTGCTCTTTTTTGTTCGTGATATAC TGATAAATTGAATTTTCACACTTCATATTCAGGGAGACC ACAACGGTTTCCCTCTACAAATAATTTTGTTTAACTTT	22

Section 3: Chromosomally Modified Host Strains

[00187] Figure 11 depicts each chromosomal modification. Strains utilized and/or constructed for this study are listed in Table 5. Tables 6 and 7 lists oligonucleotides and synthetic DNA sequences used for strain construction and/or confirmation. **Figure 12** and **Figure 13A-E** show growth rates and glucose distribution during growth for control strains in 1L fermentation.

[00188] Table 5: List of chromosomally modified strains.

Strain	Genotype	Source
BW25113 (wt)	F-, λ-, Δ(araD-araB)567, lacZ4787(del)(::rrnB-3) , rph-1, Δ(rhaD-rhaB)568, hsdR514	CGSC
JW3197-1	BW25113, sspB756(del)::kan	[53]
Bwapldf	BW25113, ΔackA-pta, ΔpoxB, ΔpflB, ΔldhA, ΔadhE	[39]

DLF_0001	BWapldf, ΔiclR, ΔarcA	this study
DLF_0002	BWapldf, ΔiclR, ΔarcA, ΔsspB::frt	this study
DLF_0025	DLF_0002, Δcas3::tm-ugpb-sspB-pro-casA(N2S)	this study
DLF_0028	DLF_0025, fabI-DAS+4-gentR	this study
DLF_0031	DLF_0025, lpd-DAS+4-gentR	this study
DLF_0038	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR	this study
DLF_0039	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR	this study
DLF_0040	DLF_0025, fabI-DAS+4-gentR, zwf-DAS+4-bsdR	this study
DLF_0041	DLF_0025, lpd-DAS+4-gentR, gltA-DAS+4-zeoR	this study
DLF_0042	DLF_0025, lpd-DAS+4-gentR, udhA-DAS+4-bsdR	this study
DLF_0043	DLF_0025, gltA-DAS+4-zeoR	this study
DLF_0044	DLF_0025, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	this study
DLF_0045	DLF_0025, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR	this study
DLF_0046	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	this study
DLF_0047	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4::zeoR, udhA-DAS+4-bsdR	this study
DLF_0048	DLF_0025, lpd-DAS+4-gentR, gltA-DAS+4-zeoR, zwf-DAS+4-bsdR	this study
DLF_0049	DLF_0025, lpd-DAS+4-gentR, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR	this study
DLF_0165	DLF_0025, lpd-DAS+4-gentR, zwf-DAS+4-bsdR	this study
DLF_0763	DLF_0025, udhA-DAS+4-bsdR	this study
DLF_01002	DLF_0025, zwf-DAS+4-bsdR	this study
DLF_01517	DLF_0012, Δcas3::pro-casA(N2S)	this study
DLF_01530	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, zeoR-proDp-gapN-zeoR	this study
DLF_01531	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, gltA-DAS+4-purR	this study
DLF_01532	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, gapA-DAS+4-zeoR-proDp-gapN	this study
DLF_01533	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, gapA-DAS+4-zeoR-proDp-gapN, gltA-DAS+4-purR	this study
DLF_01536	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, zeoR-proDp-gapN, gltA-DAS+4-purR	this study
DLF_01537	DLF_0025, fabI-DAS+4-gentR, udhA-DAS+4-bsdR, gapA-DAS+4-zeoR	this study

DLF_01538	DLF_0025, fabI-DAS+4-gentR, gltA-DAS+4-zeoR, udhA-DAS+4-bsdR, gapA-DAS+4-zeoR	this study

[00189] Table 6: Oligonucleotides utilized for strain construction.

Oligo	Sequence	SEQ ID NO
ilcR_tetA_F	TAACAATAAAAATGAAAATGATTTCCACGATACAGAAA AAAGAGACTGTCATCCTAATTTTTGTTGACACTCTATC	23
ilcR_sacB_R	TGCCACTCAGGTATGATGGGCAGAATATTGCCTCTGCCC GCCAGAAAAAGATCAAAGGGAAAACTGTCCATATGC	24
iclR_500up	CCGACAGGGATTCCATCTG	25
iclR_500dn	TATGACGACCATTTTGTCTACAGTTC	26
arcA_tetA_F	GGACTTTTGTACTTCCTGTTTCGATTTAGTTGGCAATTTA GGTAGCAAACTCCTAATTTTTGTTGACACTCTATC	27
arcA_sacB_R	ATAAAAACGGCGCTAAAAAGCGCCGTTTTTTTTGACGGT GGTAAAGCCGAATCAAAGGGAAAACTGTCCATATGC	28
arcA_500up	CCTGACTGTACTAACGGTTGAG	29
arcA_500dn	TGACTTTTATGGCGTTCTTTGTTTTTG	30
sspB_kan_F	CTGGTACACGCTGATGAACACC	31
sspB_kan_R	CTGGTCATTGCCATTTGTGCC	32
sspB_conf_F	GAATCAGAGCGTTCCGACCC	33
sspB_conf_R	GTACGCAGTTTGCCAACGTG	34
cas3_tetA_F	AATAGCCCGCTGATATCATCGATAATACTAAAAAAACAG GGAGGCTATTATCCTAATTTTTGTTGACACTCTATC	35
cas3_sacB_R	TACAGGGATCCAGTTATCAATAAGCAAATTCATTTGTTCT CCTTCATATGATCAAAGGGAAAACTGTCCATATGC	36
cas3_conf_F	CAAGACATGTGTATATCACTGTAATTC	37
cas3_500dn	GCGATTGCAGATTTATGATTTGG	38
fabI_conf_F	GCAAAATGCTGGCTCATTG	39
gapA_conf_F	GAACTGAATGGCAAACTGACTG	40
gapA_500dn	TGGGGATGATCGACCACA	41
gltA_conf_F	TATCATCCTGAAAGCGATGG	42
lpd_conf_F	ATCTCACCGTGTGATCGG	43
udhA_conf_F	CAAAAGAGATTCTGGGTATTCACT	44
zwf_conf_F	CTGCTGGAAACCATGCG	45
zwf_500dn	AGAGCATGTCGTTATAGGAGGTGAT	46
ampR_intR	AGTACTCAACCAAGTCATTCTG	47
bsdR_intR	GAGCATGGTGATCTTCTCAGT	48

gentR_intR	GCGATGAATGTCTTACTACGGA	49
purR_intR	GTCGCTGGGTAATCTGCAA	50
tetA_intR	ATCAACGCATATAGCGCTAGCAG	51
zeoR_intR	ACTGAAGCCCAGACGATC	52

[00190] Table 7: Synthetic DNA utilized for strain construction.

tetA-sacB Cassette	**SEQ ID NO**
TCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTA TCAGTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAGATCGCATTGGTA ATTACGTTACTCGATGCCATGGGGATTGGCCTTATCATGCCAGTCTTGCCAA CGTTATTACGTGAATTTATTGCTTCGGAAGATATCGCTAACCACTTTGGCGT ATTGCTTGCACTTTATGCGTTAATGCAGGTTATCTTTGCTCCTTGGCTTGGAA AAATGTCTGACCGATTTGGTCGGCGCCCAGTGCTGTTGTTGTCATTAATAGG CGCATCGCTGGATTACTTATTGCTGGCTTTTTCAAGTGCGCTTTGGATGCTGT ATTTAGGCCGTTTGCTTTCAGGGATCACAGGAGCTACTGGGGCTGTCGCGGC ATCGGTCATTGCCGATACCACCTCAGCTTCTCAACGCGTGAAGTGGTTCGGT TGGTTAGGGGCAAGTTTTGGGCTTGGTTTAATAGCGGGGCCTATTATTGGTG GTTTTGCAGGAGAGATTTCACCGCATAGTCCCTTTTTTATCGCTGCGTTGCTA AATATTGTCACTTTCCTTGTGGTTATGTTTTGGTTCCGTGAAACCAAAAATAC ACGTGATAATACAGATACCGAAGTAGGGGTTGAGACGCAATCGAATTCGGT ATACATCACTTTATTTAAAACGATGCCCATTTTGTTGATTATTTATTTTTCAG CGCAATTGATAGGCCAAATTCCCGCAACGGTGTGGGTGCTATTTACCGAAA ATCGTTTTGGATGGAATAGCATGATGGTTGGCTTTTCATTAGCGGGTCTTGG TCTTTTACACTCAGTATTCCAAGCCTTTGTGGCAGGAAGAATAGCCACTAAA TGGGGCGAAAAAACGGCAGTACTGCTCGGATTTATTGCAGATAGTAGTGCA TTTGCCTTTTTAGCGTTTATATCTGAAGGTTGGTTAGTTTTCCCTGTTTTAATT TTATTGGCTGGTGGTGGGATCGCTTTACCTGCATTACAGGGAGTGATGTCTA TCCAAACAAAGAGTCATCAGCAAGGTGCTTTACAGGGATTATTGGTGAGCC TTACCAATGCAACCGGTGTTATTGGCCCATTACTGTTTGCTGTTATTTATAAT CATTCACTACCAATTTGGGATGGCTGGATTTGGATTATTGGTTTAGCGTTTTA CTGTATTATTATCCTGCTATCGATGACCTTCATGTTAACCCCTCAAGCTCAGG GGAGTAAACAGGAGACAAGTGCTTAGTTATTTCGTCACCAAATGATGTTATT CCGCGAAATATAATGACCCTCTTGATAACCCAAGAGCATCACATATACCTGC CGTTCACTATTATTTAGTGAAATGAGATATTATGATATTTTCTGAATTGTGAT TAAAAAGGCAACTTTATGCCCATGCAACAGAAACTATAAAAAATACAGAGA	53

ATGAAAAGAAACAGATAGATTTTTTAGTTCTTTAGGCCCGTAGTCTGCAAAT CCTTTTATGATTTTCTATCAAACAAAAGAGGAAAATAGACCAGTTGCAATCC AAACGAGAGTCTAATAGAATGAGGTCGAAAAGTAAATCGCGCGGGTTTGTT ACTGATAAAGCAGGCAAGACCTAAAATGTGTAAAGGGCAAAGTGTATACTT TGGCGTCACCCCTTACATATTTTAGGTCTTTTTTTATTGTGCGTAACTAACTT GCCATCTTCAAACAGGAGGGCTGGAAGAAGCAGACCGCTAACACAGTACAT AAAAAAGGAGACATGAACGATGAACATCAAAAAGTTTGCAAAACAAGCAA CAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGGCGCAACTCAAGCGTT TGCGAAAGAAACGAACCAAAAGCCATATAAGGAAACATACGGCATTTCCCA TATTACACGCCATGATATGCTGCAAATCCCTGAACAGCAAAAAAATGAAAA ATATCAAGTTCCTGAGTTCGATTCGTCCACAATTAAAAATATCTCTTCTGCA AAAGGCCTGGACGTTTGGGACAGCTGGCCATTACAAAACGCTGACGGCACT GTCGCAAACTATCACGGCTACCACATCGTCTTTGCATTAGCCGGAGATCCTA AAAATGCGGATGACACATCGATTTACATGTTCTATCAAAAAGTCGGCGAAA CTTCTATTGACAGCTGGAAAAACGCTGGCCGCGTCTTTAAAGACAGCGACA AATTCGATGCAAATGATTCTATCCTAAAAGACCAAACACAAGAATGGTCAG GTTCAGCCACATTTACATCTGACGGAAAAATCCGTTTATTCTACACTGATTT CTCCGGTAAACATTACGGCAAACAAACACTGACAACTGCACAAGTTAACGT ATCAGCATCAGACAGCTCTTTGAACATCAACGGTGTAGAGGATTATAAATC AATCTTTGACGGTGACGGAAAAACGTATCAAAATGTACAGCAGTTCATCGA TGAAGGCAACTACAGCTCAGGCGACAACCATACGCTGAGAGATCCTCACTA CGTAGAAGATAAAGGCCACAAATACTTAGTATTTGAAGCAAACACTGGAAC TGAAGATGGCTACCAAGGCGAAGAATCTTTATTTAACAAAGCATACTATGG CAAAAGCACATCATTCTTCCGTCAAGAAAGTCAAAAACTTCTGCAAAGCGA TAAAAAACGCACGGCTGAGTTAGCAAACGGCGCTCTCGGTATGATTGAGCT AAACGATGATTACACACTGAAAAAAGTGATGAAACCGCTGATTGCATCTAA CACAGTAACAGATGAAATTGAACGCGCGAACGTCTTTAAAATGAACGGCAA ATGGTACCTGTTCACTGACTCCCGCGGATCAAAAATGACGATTGACGGCATT ACGTCTAACGATATTTACATGCTTGGTTATGTTTCTAATTCTTTAACTGGCCC ATACAAGCCGCTGAACAAAACTGGCCTTGTGTTAAAAATGGATCTTGATCCT AACGATGTAACCTTTACTTACTCACACTTCGCTGTACCTCAAGCGAAAGGAA ACAATGTCGTGATTACAAGCTATATGACAAACAGAGGATTCTACGCAGACA AACAATCAACGTTTGCGCCAAGCTTCCTGCTGAACATCAAAGGCAAGAAAA CATCTGTTGTCAAAGACAGCATCCTTGAACAAGGACAATTAACAGTTAACA AATAAAAACGCAAAAGAAAATGCCGATATTGACTACCGGAAGCAGTGTGAC CGTGTGCTTCTCAAATGCCTGATTCAGGCTGTCTATGTGTGACTGTTGAGCT GTAACAAGTTGTCTCAGGTGTTCAATTTCATGTTCTAGTTGCTTTGTTTTACT	

GGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTCATCTGTTACATTGTCGA TCTGTTCATGGTGAACAGCTTTAAATGCACCAAAAACTCGTAAAAGCTCTGA TGTATCTATCTTTTTTACACCGTTTTCATCTGTGCATATGGACAGTTTTCCCTT TGAT	
ΔiclR-cure	
AAATGATTTCCACGATACAGAAAAAAGAGACTGTCATGGGCAGAATATTGC CTCTGCCCGCCAGAAAAAG	54
ΔarcA-cure	
CTGTTTCGATTTAGTTGGCAATTTAGGTAGCAAACTCGGCTTTACCACCGTC AAAAAAAACGGCGCTTTT	55
Δcas3-pro-casA	
CAAGACATGTGTATATCACTGTAATTCGATATTTATGAGCAGCATCGAAAAA TAGCCCGCTGATATCATCGATAATACTAAAAAAACAGGGAGGCTATTACCA GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTA TCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCG GGTGGGCCTTTCTGCGTTTATATCTTTCTGACACCTTACTATCTTACAAATGT AACAAAAAAGTTATTTTTCTGTAATTCGAGCATGTCATGTTACCCCGCGAGC ATAAAACGCGTGTGTAGGAGGATAATCTTTGACGGCTAGCTCAGTCCTAGGT ACAGTGCTAGCCATATGAAGGAGAACAAATGAATTTGCTTATTGATAACTG GATCCCTGTACGCCCGCGAAACGGGGGGAAAGTCCAAATCATAAATCTGCA ATCGCTATAC	56
Δcas3::ugBp-sspB-pro-casA	
CAAGACATGTGTATATCACTGTAATTCGATATTTATGAGCAGCATCGAAAAA TAGCCCGCTGATATCATCGATAATACTAAAAAAACAGGGAGGCTATTACCA GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTA TCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCG GGTGGGCCTTTCTGCGTTTATATCTTTCTGACACCTTACTATCTTACAAATGT AACAAAAAAGTTATTTTTCTGTAATTCGAGCATGTCATGTTACCCCGCGAGC ATAAAACGCGTGTGTAGGAGGATAATCTATGGATTTGTCACAGCTAACACC ACGTCGTCCCTATCTGCTGCGTGCATTCTATGAGTGGTTGCTGGATAACCAG CTCACGCCGCACCTGGTGGTGGATGTGACGCTCCCTGGCGTGCAGGTTCCTA TGGAATATGCGCGTGACGGGCAAATCGTACTCAACATTGCGCCGCGTGCTGT CGGCAATCTGGAACTGGCGAATGATGAGGTGCGCTTTAACGCGCGCTTTGGT GGCATTCCGCGTCAGGTTTCTGTGCCGCTGGCTGCCGTGCTGGCTATCTACG CCCGTGAAAATGGCGCAGGCACGATGTTTGAGCCTGAAGCTGCCTACGATG AAGATACCAGCATCATGAATGATGAAGAGGCATCGGCAGACAACGAAACC GTTATGTCGGTTATTGATGGCGACAAGCCAGATCACGATGATGACACTCATC	57

CTGACGATGAACCTCCGCAGCCACCACGCGGTGGTCGACCGGCATTACGCG TTGTGAAGTAATTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCCATATG AAGGAGAACAAATGAATTTGCTTATTGATAACTGGATCCCTGTACGCCCGCG AAACGGGGGGAAAGTCCAAATCATAAATCTGCAATCGCTATAC	
fabI-DAS+4-gentR	
CTATTGAAGATGTGGGTAACTCTGCGGCATTCCTGTGCTCCGATCTCTCTGC CGGTATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTTCAGCATTGCTGCA ATGAACGAACTCGAACTGAAAGCGGCCAACGATGAAAACTATTCTGAAAAC TATGCGGATGCGTCTTAATAGGAAGTTCCTATTCTCTAGAAAGTATAGGAAC TTCCGAATCCATGTGGGAGTTTATTCTTGACACAGATATTTATGATATAATA ACTGAGTAAGCTTAACATAAGGAGGAAAAACATATGTTACGCAGCAGCAAC GATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGTGGCTCAAGT ATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCCATGC GGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTACTC CCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCGTAGTAAGACA TTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCGG CTTACGTTCTGCCCAAGTTTGAGCAGCCGCGTAGTGAGATCTATATCTATGA TCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTGCCACCGCGCTCAT CAATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTATGTGATCTACGTG CAAGCAGATTACGGTGACGATCCCGCAGTGGCTCTCTATACAAAGTTGGGC ATACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGTACCGCCACCTAA GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCGTTCTGTTGGTAAAGAT GGGCGGCGTTCTGCCGCCCGTTATCTCTGTTATACCTTTCTGATATTTGTTAT CGCCGATCCGTCTTTCTCCCCTTCCCGCCTTGCGTCAGG	58
gapA-DAS+4-zeoR-proDp-gapN	
TCTCCAAAGCGGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGT CTTGATTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCAACTTTAAAATT AAAGAGGTATATATTAATGACTAAGCAATATAAGAATTACGTAAATGGGGA GTGGAAGCTTTCGGAGAATGAAATTAAGATCTATGAACCAGCCAGTGGGGC GGAATTGGGGTCAGTCCCGGCAATGTCCACTGAAGAAGTTGACTATGTCTAC GCCTCGGCCAAAAAAGCGCAGCCAGCATGGCGCTCGCTTTCCTATATTGAGC GTGCGGCTTATTTGCACAAAGTCGCAGACATCCTGATGCGTGACAAGGAGA AAATTGGAGCGGTATTGTCCAAGGAAGTAGCGAAAGGCTACAAATCCGCAG TATCGGAGGTCGTCCGCACCGCCGAGATTATTAATTATGCGGCCGAAGAAG GGCTTCGCATGGAGGGTGAGGTCTTGGAGGGCGGCAGTTTTGAGGCGGCAT CCAAGAAAAAAATCGCTGTCGTCCGTCGCGAGCCGGTGGGACTTGTGCTTG CTATTAGTCCGTTCAATTACCCCGTGAATCTGGCCGGCTCCAAGATTGCCCC	59

TGCACTGATCGCGGGCAATGTAATCGCTTTTAAACCACCGACCCAAGGATCG ATTAGTGGACTTCTTTTAGCGGAGGCGTTTGCGGAGGCAGGTCTTCCAGCCG GCGTATTCAATACCATCACGGGGCGTGGAAGTGAAATCGGGGATTACATCG TGGAGCACCAGGCAGTAAATTTCATCAACTTCACGGGTTCCACGGGGATCG GGGAGCGTATCGGTAAGATGGCTGGGATGCGTCCGATCATGTTGGAACTTG GCGGCAAGGATAGTGCGATTGTGCTGGAAGACGCAGACTTGGAATTGACAG CTAAAAACATTATCGCTGGAGCCTTCGGGTATAGTGGTCAACGTTGCACGGC AGTTAAGCGCGTTCTTGTTATGGAAAGTGTCGCGGATGAATTGGTCGAGAA GATTCGCGAGAAAGTGTTAGCTCTTACGATTGGAAATCCAGAGGACGATGC TGACATCACTCCATTGATCGACACGAAATCCGCGGATTACGTCGAGGGGCT GATCAACGACGCGAACGATAAGGGAGCAGCGGCTTTGACCGAGATCAAACG CGAGGGGAACCTGATCTGCCCGATTCTTTTTGACAAAGTCACAACTGACATG CGCTTGGCATGGGAAGAACCCTTCGGCCCAGTCTTGCCTATTATCCGCGTTA CTAGCGTAGAGGAAGCAATTGAAATTTCCAATAAATCCGAATATGGGTTGC AAGCGAGTATCTTTACTAACGATTTTCCACGTGCCTTTGGTATTGCGGAACA GTTAGAAGTCGGGACAGTTCACATCAACAACAAGACGCAGCGCGGGACAGA TAACTTCCCCTTTTTGGGAGCAAAGAAGTCTGGGGCTGGAATCCAAGGGGT GAAATACTCCATCGAAGCCATGACGACGGTGAAGAGCGTTGTTTTTGACATC AAGTAAAACATAAGGAGGAAAAACAGATGGCGAAACTGACCTCGGCGGTT CCGGTTCTGACGGCACGTGATGTGGCGGGCGCGGTTGAATTTTGGACGGATC GTCTGGGCTTCAGTCGTGATTTTGTGGAAGATGACTTCGCAGGCGTGGTTCG CGATGACGTCACCCTGTTTATTTCCGCAGTTCAGGATCAAGTCGTGCCGGAC AACACGCTGGCTTGGGTGTGGGTTCGTGGCCTGGATGAACTGTATGCGGAAT GGAGCGAAGTTGTCTCTACCAATTTCCGTGACGCGAGCGGTCCGGCCATGAC GGAAATCGGCGAACAGCCGTGGGGTCGCGAATTTGCTCTGCGTGACCCGGC TGGCAACTGTGTCCATTTCGTGGCTGAAGAACAAGATTGAGTTGAGATGAC ACTGTGATCTAAAAAGAGCGACTTCGGTCGCTCTTTTTTTTACCTGA	
gapA- zeoR-proDp-gapN	
ACGAAACCGGTTACTCCAACAAAGTTCTGGACCTGATCGCTCACATCTCCAA ATGATTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCAACTTTAAAATTA AAGAGGTATATATTAATGACTAAGCAATATAAGAATTACGTAAATGGGGAG TGGAAGCTTTCGGAGAATGAAATTAAGATCTATGAACCAGCCAGTGGGGCG GAATTGGGGTCAGTCCCGGCAATGTCCACTGAAGAAGTTGACTATGTCTACG CCTCGGCCAAAAAAGCGCAGCCAGCATGGCGCTCGCTTTCCTATATTGAGCG TGCGGCTTATTTGCACAAAGTCGCAGACATCCTGATGCGTGACAAGGAGAA AATTGGAGCGGTATTGTCCAAGGAAGTAGCGAAAGGCTACAAATCCGCAGT ATCGGAGGTCGTCCGCACCGCCGAGATTATTAATTATGCGGCCGAAGAAGG	60

GCTTCGCATGGAGGGTGAGGTCTTGGAGGGCGGCAGTTTTGAGGCGGCATC CAAGAAAAAAATCGCTGTCGTCCGTCGCGAGCCGGTGGGACTTGTGCTTGCT ATTAGTCCGTTCAATTACCCCGTGAATCTGGCCGGCTCCAAGATTGCCCCTG CACTGATCGCGGGCAATGTAATCGCTTTTAAACCACCGACCCAAGGATCGAT TAGTGGACTTCTTTTAGCGGAGGCGTTTGCGGAGGCAGGTCTTCCAGCCGGC GTATTCAATACCATCACGGGGCGTGGAAGTGAAATCGGGGATTACATCGTG GAGCACCAGGCAGTAAATTTCATCAACTTCACGGGTTCCACGGGGATCGGG GAGCGTATCGGTAAGATGGCTGGGATGCGTCCGATCATGTTGGAACTTGGC GGCAAGGATAGTGCGATTGTGCTGGAAGACGCAGACTTGGAATTGACAGCT AAAAACATTATCGCTGGAGCCTTCGGGTATAGTGGTCAACGTTGCACGGCA GTTAAGCGCGTTCTTGTTATGGAAAGTGTCGCGGATGAATTGGTCGAGAAG ATTCGCGAGAAAGTGTTAGCTCTTACGATTGGAAATCCAGAGGACGATGCT GACATCACTCCATTGATCGACACGAAATCCGCGGATTACGTCGAGGGGCTG ATCAACGACGCGAACGATAAGGGAGCAGCGGCTTTGACCGAGATCAAACGC GAGGGGAACCTGATCTGCCCGATTCTTTTTGACAAAGTCACAACTGACATGC GCTTGGCATGGGAAGAACCCTTCGGCCCAGTCTTGCCTATTATCCGCGTTAC TAGCGTAGAGGAAGCAATTGAAATTTCCAATAAATCCGAATATGGGTTGCA AGCGAGTATCTTTACTAACGATTTTCCACGTGCCTTTGGTATTGCGGAACAG TTAGAAGTCGGGACAGTTCACATCAACAACAAGACGCAGCGCGGGACAGAT AACTTCCCCTTTTTGGGAGCAAAGAAGTCTGGGGCTGGAATCCAAGGGGTG AAATACTCCATCGAAGCCATGACGACGGTGAAGAGCGTTGTTTTTGACATCA AGTAAAACATAAGGAGGAAAAACAGATGGCGAAACTGACCTCGGCGGTTCC GGTTCTGACGGCACGTGATGTGGCGGGCGCGGTTGAATTTTGGACGGATCGT CTGGGCTTCAGTCGTGATTTTGTGGAAGATGACTTCGCAGGCGTGGTTCGCG ATGACGTCACCCTGTTTATTTCCGCAGTTCAGGATCAAGTCGTGCCGGACAA CACGCTGGCTTGGGTGTGGGTTCGTGGCCTGGATGAACTGTATGCGGAATGG AGCGAAGTTGTCTCTACCAATTTCCGTGACGCGAGCGGTCCGGCCATGACGG AAATCGGCGAACAGCCGTGGGGTCGCGAATTTGCTCTGCGTGACCCGGCTG GCAACTGTGTCCATTTCGTGGCTGAAGAACAAGATTGAGTTGAGATGACACT GTGATCTAAAAAGAGCGACTTCGGTCGCTCTTTTTTTTACCTGA	
gapA-DAS+4-zeoR	
TCTACCGATTTCAACGGCGAAGTTTGCACTTCCGTGTTCGATGCTAAAGCTG GTATCGCTCTGAACGACAACTTCGTGAAACTGGTATCCTGGTACGACAACGA AACCGGTTACTCCAACAAAGTTCTGGACCTGATCGCTCACATCTCCAAAGCG GCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCTTGATCCTGAC GGATGGCCTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACAT TCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAAT	61

ATTGAAAAAGGAAGAGTAATGGCGAAACTGACCTCGGCGGTTCCGGTTCTG ACGGCACGTGATGTGGCGGGCGCGGTTGAATTTTGGACGGATCGTCTGGGC TTCAGTCGTGATTTTGTGGAAGATGACTTCGCAGGCGTGGTTCGCGATGACG TCACCCTGTTTATTTCCGCAGTTCAGGATCAAGTCGTGCCGGACAACACGCT GGCTTGGGTGTGGGTTCGTGGCCTGGATGAACTGTATGCGGAATGGAGCGA AGTTGTCTCTACCAATTTCCGTGACGCGAGCGGTCCGGCCATGACGGAAATC GGCGAACAGCCGTGGGGTCGCGAATTTGCTCTGCGTGACCCGGCTGGCAAC TGTGTCCATTTCGTGGCTGAAGAACAAGATTGAGTTGAGATGACACTGTGAT CTAAAAAGAGCGACTTCGGTCGCTCTTTTTTTTACCTGATAAAATGAAGTTA AAGGACTGCGTCATGATTAAGAAAATTTTTGCCCTTCCGGTCATCGAACAAA TCTCCCCTGTCCTCTCCCGTCGTAAACTGGATGAACTGGACCTCATTGTGGTC GATCATCCCCAGGTAAAAGCCTCT	
gltA-DAS+4-ampR	
GTATTCCGTCTTCCATGTTCACCGTCATTTTCGCAATGGCACGTACCGTTGGC TGGATCGCCCACTGGAGCGAAATGCACAGTGACGGTATGAAGATTGCCCGT CCGCGTCAGCTGTATACAGGATATGAAAAACGCGACTTTAAAAGCGATATC AAGCGTGCGGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCT TAATAGTCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTTGTTTAT TTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATA AATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGT GTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCA GAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTG GGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCC CCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGC GGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACA CTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTT ACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGT GATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTT GGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACG ATGCCTACAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTA CTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAA GTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTG ATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGG GGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTC AGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCAC TGATTAAGCATTGGTAACTGTCAGACTAATGGTTGATTGCTAAGTTGTAAAT	62

ATTTTAACCCGCCGTTCATATGGCGGGTTGATTTTTATATGCCTAAACACAA AAAATTGTAAAAATAAAATCCATTAACAGACCTATATAGATATTTAAAAAG AATAGAACAGCTCAAATTATCAGCAACCCAATACTTTCAATTAAAAACTTCA TGGTAGTCGCATTTATAACCCTATGAAA	
gltA-DAS+4-purR	
ACCGTCATTTTCGCAATGGCACGTACCGTTGGCTGGATCGCCCACTGGAGCG AAATGCACAGTGACGGTATGAAGATTGCCCGTCCGCGTCAGCTGTATACAG GATATGAAAAACGCGACTTTAAAAGCGATATCAAGCGTGCGGCCAACGATG AAAACTATTCTGAAAACTATGCGGATGCGTCTTAATCCTGACGGATGGCCTT TTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGT ATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAA GGAAGAGTATGACTGAATACAAGCCCACGGTACGCTTGGCGACGCGCGACG ATGTTCCCCGCGCTGTTCGTACATTAGCTGCGGCCTTTGCAGATTACCCAGC GACGCGCCATACGGTCGATCCGGACCGCCATATCGAGCGTGTCACAGAATT GCAGGAACTTTTCTTAACTCGCGTGGGCCTTGACATCGGAAAGGTCTGGGTG GCTGACGATGGCGCTGCAGTGGCTGTTTGGACCACTCCGGAGAGTGTAGAG GCTGGTGCAGTGTTCGCCGAAATTGGTCCTCGTATGGCCGAATTAAGTGGAA GTCGTCTGGCAGCCCAACAACAAATGGAAGGGTTGCTTGCGCCCCACCGTC CGAAAGAACCCGCGTGGTTCCTTGCCACCGTTGGAGTAAGCCCAGATCACC AGGGGAAGGGTTTAGGATCTGCCGTAGTTTTACCAGGTGTGGAGGCAGCAG AACGTGCGGGAGTTCCGGCCTTCCTTGAGACGTCGGCGCCGCGCAATTTACC GTTTTACGAACGTCTTGGATTCACCGTTACGGCGGACGTGGAGGTGCCGGAG GGACCCCGTACTTGGTGTATGACTCGTAAACCGGGAGCCTGATAATGGTTGA TTGCTAAGTTGTAAATATTTTAACCCGCCGTTCATATGGCGGGTTGATTTTTA TATGCCTAAACACAAAAAATTGTAAAAATAAAATCCATTAACAGACCTATA TAGATATTTAAAAAGAATAGAACAGCTCAAATTATCAGCAACCCA	63
gltA-DAS+4-zeoR	
GTATTCCGTCTTCCATGTTCACCGTCATTTTCGCAATGGCACGTACCGTTGGC TGGATCGCCCACTGGAGCGAAATGCACAGTGACGGTATGAAGATTGCCCGT CCGCGTCAGCTGTATACAGGATATGAAAAACGCGACTTTAAAAGCGATATC AAGCGTGCGGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCT TAATAGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGA CTCACTATAGGAGGGCCATCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCT CACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGG GTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGAC GTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACC CTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCG	64

GAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAG ATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGC AACTGCGTGCACTTTGTGGCAGAGGAGCAGGACTGAGGATAAGTAATGGTT GATTGCTAAGTTGTAAATATTTTAACCCGCCGTTCATATGGCGGGTTGATTTT TATATGCCTAAACACAAAAAATTGTAAAAATAAAATCCATTAACAGACCTA TATAGATATTTAAAAAGAATAGAACAGCTCAAATTATCAGCAACCCAATAC TTTCAATTAAAAACTTCATGGTAGTCGCATTTATAACCCTATGAAA	
lpd-DAS+4-gentR	
GCGGCGAGCTGCTGGGTGAAATCGGCCTGGCAATCGAAATGGGTTGTGATG CTGAAGACATCGCACTGACCATCCACGCGCACCCGACTCTGCACGAGTCTGT GGGCCTGGCGGCAGAAGTGTTCGAAGGTAGCATTACCGACCTGCCGAACCC GAAAGCGAAGAAGAAGGCGGCCAACGATGAAAACTATTCTGAAAACTATG CGGATGCGTCTTAATAGCGAATCCATGTGGGAGTTTATTCTTGACACAGATA TTTATGATATAATAACTGAGTAAGCTTAACATAAGGAGGAAAAACATATGT TACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGT TAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCA AGTCAAATCCATGCGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGAC GTAGCCACCTACTCCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGC TCCGTAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTTGT TGGCGCTCTCGCGGCTTACGTTCTGCCCAAGTTTGAGCAGCCGCGTAGTGAG ATCTATATCTATGATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGCATTG CCACCGCGCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTTGGTGCTTA TGTGATCTACGTGCAAGCAGATTACGGTGACGATCCCGCAGTGGCTCTCTAT ACAAAGTTGGGCATACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGT ACCGCCACCTAATTTTTCGTTTGCCGGAACATCCGGCAATTAAAAAAGCGGC TAACCACGCCGCTTTTTTTACGTCTGCAATTTACCTTTCCAGTCTTCTTGCTC CACGTTCAGAGAGACGTTCGCATACTGCTGACCGTTGCTCGTTATTCAGCCT GACAGTATGGTTACTGTC	65
udhA-DAS+4-bsdR	
TCTGGGTATTCACTGCTTTGGCGAGCGCGCTGCCGAAATTATTCATATCGGT CAGGCGATTATGGAACAGAAAGGTGGCGGCAACACTATTGAGTACTTCGTC AACACCACCTTTAACTACCCGACGATGGCGGAAGCCTATCGGGTAGCTGCG TTAAACGGTTTAAACCGCCTGTTTGCGGCCAACGATGAAAACTATTCTGAAA ACTATGCGGATGCGTCTTAATAGTTGACAATTAATCATCGGCATAGTATATC GGCATAGTATAATACGACTCACTATAGGAGGGCCATCATGAAGACCTTCAA CATCTCTCAGCAGGATCTGGAGCTGGTGGAGGTCGCCACTGAGAAGATCAC CATGCTCTATGAGGACAACAAGCACCATGTCGGGGCGGCCATCAGGACCAA	66

GACTGGGGAGATCATCTCTGCTGTCCACATTGAGGCCTACATTGGCAGGGTC ACTGTCTGTGCTGAAGCCATTGCCATTGGGTCTGCTGTGAGCAACGGGCAGA AGGACTTTGACACCATTGTGGCTGTCAGGCACCCCTACTCTGATGAGGTGGA CAGATCCATCAGGGTGGTCAGCCCCTGTGGCATGTGCAGAGAGCTCATCTCT GACTATGCTCCTGACTGCTTTGTGCTCATTGAGATGAATGGCAAGCTGGTCA AAACCACCATTGAGGAACTCATCCCCCTCAAGTACACCAGGAACTAAAGTA AAACTTTATCGAAATGGCCATCCATTCTTGCGCGGATGGCCTCTGCCAGCTG CTCATAGCGGCTGCGCAGCGGTGAGCCAGGACGATAAACCAGGCCAATAGT GCGGCGTGGTTCCGGCTTAATGCACGG	
zwf-DAS+4-bsdR	
GAAGTGGAAGAAGCCTGGAAATGGGTAGACTCCATTACTGAGGCGTGGGCG ATGGACAATGATGCGCCGAAACCGTATCAGGCCGGAACCTGGGGACCCGTT GCCTCGGTGGCGATGATTACCCGTGATGGTCGTTCCTGGAATGAGTTTGAGG CGGCCAACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCTTAATAGTT GACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACTCACTAT AGGAGGGCCATCATGAAGACCTTCAACATCTCTCAGCAGGATCTGGAGCTG GTGGAGGTCGCCACTGAGAAGATCACCATGCTCTATGAGGACAACAAGCAC CATGTCGGGGCGGCCATCAGGACCAAGACTGGGGAGATCATCTCTGCTGTC CACATTGAGGCCTACATTGGCAGGGTCACTGTCTGTGCTGAAGCCATTGCCA TTGGGTCTGCTGTGAGCAACGGGCAGAAGGACTTTGACACCATTGTGGCTGT CAGGCACCCCTACTCTGATGAGGTGGACAGATCCATCAGGGTGGTCAGCCC CTGTGGCATGTGCAGAGAGCTCATCTCTGACTATGCTCCTGACTGCTTTGTG CTCATTGAGATGAATGGCAAGCTGGTCAAAACCACCATTGAGGAACTCATC CCCCTCAAGTACACCAGGAACTAAAGTAATATCTGCGCTTATCCTTTATGGT TATTTTACCGGTAACATGATCTTGCGCAGATTGTAGAACAATTTTTACACTTT CAGGCCTCGTGCGGATTCACCCACGAGGCTTTTTTTATTACACTGACTGAAA CGTTTTTGCCCTATGAGCTCCGGTTACAGGCGTTTCAGTCATAAATCCTCTGA ATGAAACGCGTTGTGAATC	67
dadX-DAS+4-purR	
GCGTGCGCACCATGACGGTGGGGACCGTCTCGATGGATATGCTAGCGGTCG ATTTAACGCCTTGCCCGCAGGCGGGTATTGGTACGCCGGTTGAGCTGTGGGG CAAGGAGATCAAAATTGATGATGTCGCCGCCGCTGCCGGAACGGTGGGCTA TGAGTTGATGTGCGCGCTGGCGCTACGCGTCCCGGTTGTGACGGTGGCGGCC AACGATGAAAACTATTCTGAAAACTATGCGGATGCGTCTTAATCCTGACGG ATGGCCTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTC AAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATAT TGAAAAAGGAAGAGTATGACTGAATACAAGCCCACGGTACGCTTGGCGACG	68

CGCGACGATGTTCCCCGCGCTGTTCGTACATTAGCTGCGGCCTTTGCAGATT ACCCAGCGACGCGCCATACGGTCGATCCGGACCGCCATATCGAGCGTGTCA CAGAATTGCAGGAACTTTTCTTAACTCGCGTGGGCCTTGACATCGGAAAGGT CTGGGTGGCTGACGATGGCGCTGCAGTGGCTGTTTGGACCACTCCGGAGAG TGTAGAGGCTGGTGCAGTGTTCGCCGAAATTGGTCCTCGTATGGCCGAATTA AGTGGAAGTCGTCTGGCAGCCCAACAACAAATGGAAGGGTTGCTTGCGCCC CACCGTCCGAAAGAACCCGCGTGGTTCCTTGCCACCGTTGGAGTAAGCCCA GATCACCAGGGGAAGGGTTTAGGATCTGCCGTAGTTTTACCAGGTGTGGAG GCAGCAGAACGTGCGGGAGTTCCGGCCTTCCTTGAGACGTCGGCGCCGCGC AATTTACCGTTTTACGAACGTCTTGGATTCACCGTTACGGCGGACGTGGAGG TGCCGGAGGGACCCCGTACTTGGTGTATGACTCGTAAACCGGGAGCCTGAT AACTTGTTGTAAGCCGGATCGGAGGCAACGTCTTCTGGGTGCAAAAAAATC ATCCATCCGGCTGGTCAGCAACTGTAGTTGTTAATGTGACAGAGCCATTGCC CATGATAGTGTCCATTAAAAGGATGGACACTATTTCCCCGGAACCTGAACTC ACCGCACAGGCGTTCTACATAAAACGCTTACGCTTCATTGTTGACTC	

Section 4: Dynamic Control over Protein Levels.

[00191] Plasmids expressing fluorescent proteins and silencing guides were transformed into the corresponding hosts strain listed in Table 8. Strains were evaluated in triplicate in an m2p-labs Biolector™, which simultaneously measures fluorescence including GFPuv and mCherry levels, as well as biomass levels.

[00192] Table 8: Strains used for Dynamic Control over protein levels

Microbe	Synthetic Metabolic Valves	Plasmid	Host Strain
E. coli	RFP-control	pCDF-mcherry1+pSMART-IN:yibDp-GFPuv	DLF_0002
	Proteolysis	pCDF-mcherry2+pSMART-IN:yibDp-GFPuv	DLF_0025
	Silencing	pCDF-mcherry1+pCASCADE-proD+pSMART-IN:yibDp-GFPuv	DLF_01517
	Proteolysis+Silencing	pCDF-mcherry2+pCASCADE-proD+pSMART-IN:yibDp-GFPuv	DLF_0025

[00193] OD600 readings were corrected using the formula below, where OD600 refers to an offline measurement, OD600* refers to Biolector biomass reading, *t0* indicates the start point, and *tf* indicates the final point.

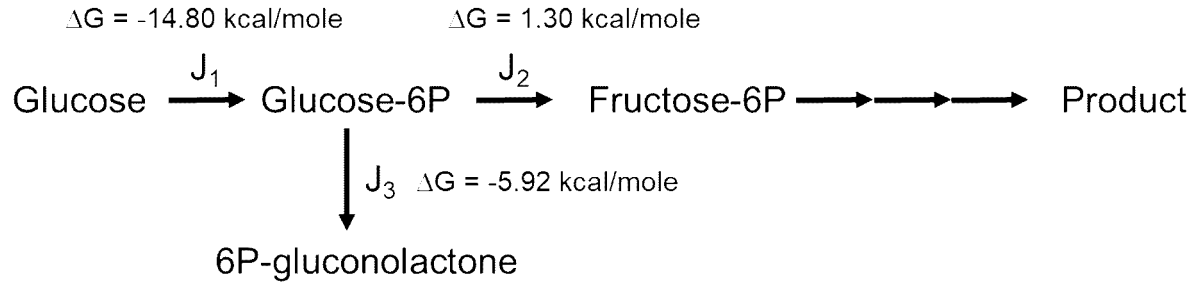
Equation S1: $OD600_t = (OD600_t^* - OD600_{t0}^*) * \frac{(OD600_{tf} - OD600_{t0})}{(OD600_{tf}^* - OD600_{t0}^*)} + 0.25$

Section 5: Metabolic Control

[00194] Near Equilibrium Reactions

[00195] The impact of Valves on metabolite pools for near equilibrium reactions is illustrated using the G6P node as an example. Abbreviations: Gluc, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PGl, 6-phosphate-gluconolactone.

[00196] ***G6P node without Valves***



Equation S2: $Steady\ State\ Mass\ balance\ J_1 = J_2 + J_3$

Equation S3: $Net\ Flux = J_i = e^{\frac{-dG}{RT}} - 1$

Equation S4: $e^{\frac{-dG1}{RT}} - 1 = e^{\frac{-dG2}{RT}} - 1 + e^{\frac{-dG3}{RT}} - 1$

Equation S5: $e^{\frac{-dG1}{RT}} = e^{\frac{-dG2}{RT}} + e^{\frac{-dG3}{RT}} - 1$

Equation S6: $Keq1 = Keq2 + Keq3 - 1$

Equation S7: $Keq1 + 1 = Keq2 + Keq3$

Equation S8: $\frac{[G6P]}{[Gluc]} + 1 = \frac{[F6P]}{[G6P]} + \frac{[6PGl]}{[G6P]}$

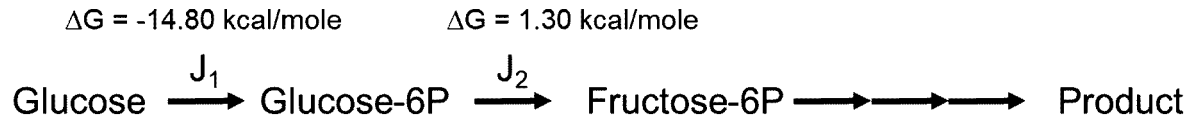
Equation S9: $\frac{[G6P]}{[Gluc]} + 1 = \frac{[F6P] + [6PGl]}{[G6P]}$

Equation S10: $\frac{[G6P]^2}{[Gluc]} + [\mathrm{G6P}] = [F6P] + [6PGl]$

Equation S11: $[F6P] = \frac{[G6P]^2}{[Gluc]} + [\mathrm{G6P}] - [\mathrm{6PGl}]$

[00197] ***G6P node with Valves***

When zwf valve is in effect, $J_3 \approx 0$.



Equation S12: $Steady\ State\ Mass\ balance\ J_1 = J_2$

Equation S13: $Net\ Flux = J_i = e^{\frac{-dG}{RT}} - 1$

Equation S14: $e^{\frac{-dG1}{RT}} - 1 = e^{\frac{-dG2}{RT}} - 1$

Equation S15: $Keq1 = Keq2$

Equation S16: $\frac{[G6P]}{[Gluc]} = \frac{[F6P]}{[G6P]}$

Equation S17: $[\mathrm{F6P}] = \frac{[G6P]^2}{[Gluc]}$

[00198] ***Impact of Valves***

Equation S11: $[F6P]network = \frac{[G6P]^2}{[Gluc]} + [G6P] - [6PGl]$

Equation S17: $[F6P]valve = \frac{[G6P]^2}{[Gluc]}$

Since close to equilibrium $[6PGl] > [G6P]$

$[F6P]valve >= [F6P]network$

The removal of thermodynamically favored reactions near equilibrium from the network will result in increased metabolite pools.

Section 6: Gene Silencing Arrays & Pathway Expression Constructs

[00199] The design and construction of CASCADE guides and guide arrays is illustrated below in **Figure 14** and **Figure 15A-B**. The pCASCADE-control plasmid was prepared by swapping the pTet promoter in pcrRNA.Tet [88] with an insulated low phosphate induced ugpB promoter [82]. Two promoters were responsible for regulating gltA gene, and sgRNA was designed for both promoters, resulting in guide gltA1 (G1) and gltA2 (G2).[89] Four promoters were responsible for regulating gapA gene, and sgRNA was designed for the first promoter, since during exponential phase of growth, gapA mRNAs were mainly initiated at the highly efficient gapA P1 promoter and remained high during stationary phase compared to the other three gapA promoters.[90] Multiple promoters upstream of lpd gene were involved in lpd regulation (https://ecocyc.org/gene?orgid=ECOLI&id=EG10543#tab=showAll), thus design of unique and effective sgRNA for lpd only was not possible. Promoter sequences for fabI, udhA and zwf were obtained from EcoCyc database (https://ecocyc.org/). 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. The pCASCADE-control vector was used as template. pCASCADE plasmids with arrays of two or more guides were prepared as illustrated in **Figure 15A-B**. The pCASCADE guide array plasmid was prepared by sequentially amplifying complementary halves of each smaller guide plasmid by PCR, followed by subsequent DNA assembly. Table 9 lists sgRNA guide sequences and primers

used to construct them. All pCASCADE silencing plasmids are listed in Table 10 below and are available at Addgene.

[00200] Table 9: List of sgRNA guide sequences and primers used to construct them. Spacers are italicized.

sgRNA/Primer Name	Sequence	SEQ ID NO	Template
fabI	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*	69	
fabI-FOR	GTTTATCTGTTCGTA*TCGAGTT CCCCGCGCCAGCGGGGATAAAC CG*AAAAAAAAACCCC	70	pCASCADE control
fabI-REV	GGTTATTATAATCAACGGTTTA TCCCCGCTGGCGCGGGGAACT CGAGGTGGTACCAGATC	71	
gapAP1	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*GTTTTTGTAATTTT ACAGGCAACCTTTTAT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*	72	
gapAP1-FOR	CAGGCAACCTTTTAT*TCGAGTT CCCCGCGCCAGCGGGGATAAAC CG*AAAAAAAAACCCC	73	pCASCADE control
gapAP1-REV	TAAAATTACAAAAACCGGTTT ATCCCCGCTGGCGCGGGGAAC TCGAGGTGGTACCAGATC	74	
gltA1	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*AAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*	75	
gltA1-FOR	GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*AAAAAAAAACCCC	76	pCASCADE control
gltA1-REV	ATTATATGCTTTTCGGTTTATC	77	

	CCCGCTGGCGCGGGGAACTCG AGGTGGTACCAGATCT		
gltA2	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TATTGACCAATTC ATTCGGGACAGTTATTAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*	78	
gltA2-FOR	GGGACAGTTATTAGT*TCGAGTT CCCCGCGCCAGCGGGGATAAAC CG*AAAAAAAAACCCC	79	pCASCADE control
gltA2-REV	GAATGAATTGGTCAATACGGT TTATCCCCGCTGGCGCGGGGA ACTCGAGGTGGTACCAGATCT	80	
proD	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*AGTGGTTGCTGGA TAACTTTACGGGCATGC*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*	81	
proD-FOR	AACTTTACGGGCATGC*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*AAAAAAAAACCCC	82	pCASCADE control
proD-REV	ATCCAGCAACCACTCGGTTTAT CCCCGCTGGCGCGGGGAACTC GAGGTGGTACCAGATCT	83	
udhA	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTACCATTCTGTT GCTTTTATGTATAAGAA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*	84	
udhA-FOR	TTTTATGTATAAGAA*TCGAGTT CCCCGCGCCAGCGGGGATAAAC CG*AAAAAAAAACCCC	85	pCASCADE control
udhA-REV	GCAACAGAATGGTAACGGTTT ATCCCCGCTGGCGCGGGGAAC TCGAGGTGGTACCAGATC	86	
zwf	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*CTCGTAAAAGCAG	87	

	TACAGTGCACCGTAAGA*TCGA GTTCCCCGCGCCAGCGGGGATA AACCG*		
zwf-FOR	CAGTGCACCGTAAGA*TCGAGTT CCCCGCGCCAGCGGGGATAAAC CG*AAAAAAAAACCCC	88	pCASCADE control
zwf-REV	TACTGCTTTTACGAGCGGTTTA TCCCCGCTGGCGCGGGGAACT CGAGGTGGTACCAGATC	89	
FG1	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*	90	
gltA1-FOR	GCGCCAGCGGGGATAAACCG*A AAAGCATATAAT*GCG	91	pCASCADE-gltA1
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	92	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	93	pCASCADE-fabI
fabI-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATACGAACAGAT AAACGGTTATTATAATC	94	
FG2	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*TATTGACCAATTCATTCG GGACAGTTATTAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*	95	
gltA2-FOR	GCGCCAGCGGGGATAAACCG*T ATTGACCAATTCATTC*	96	pCASCADE-gltA2
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC	97	

	ATCCGG		
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	98	pCASCADE-fabI
fabI-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATACGAACAGAT AAACGGTTATTATAATC	99	
FU	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*TTACCATTCTGTTGCTTT TATGTATAAGAA*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*	100	
udhA-FOR	GCGCCAGCGGGGATAAACCGT TACCATTCTGTTG	101	pCASCADE-udhA
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	102	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	103	pCASCADE-fabI
fabI-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATACGAACAGAT AAACGGTTATTATAATC	104	
FZ	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*CTCGTAAAAGCAGTACA GTGCACCGTAAGA*TCGAGTTCC CCGCGCCAGCGGGGATAAACCG*	105	
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	106	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	107	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	108	pCASCADE-fabI
fabI-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATACGAACAGAT	109	

	AAACGGTTATTATAATC		
G1G2	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*AAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCGT*ATTGACCAATTCATT CGGGACAGTTATTAGT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*	110	
gltA2-FOR	GCGCCAGCGGGGATAAACCG*T ATTGACCAATTCATTC*	111	pCASCADE-gltA2
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	112	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	113	pCASCADE-gltA1
gltA1-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTTCATAACT TTTAC	114	
G1U	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCGAAAAGCATATAAT*G CGTAAAAGTTATGAAGT*TCGA GTTCCCCGCGCCAGCGGGGATA AACCG*TTACCATTCTGTTGCTT TTATGTATAAGAA*TCGAGTTCC CCGCGCCAGCGGGGATAAACCG*	115	
udhA-FOR	GCGCCAGCGGGGATAAACCGT TACCATTCTGTTG	116	pCASCADE-udhA
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	117	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	118	pCASCADE-gltA1
gltA1-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTTCATAACT TTTAC	119	
G1Z	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*AAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG*	120	

	*AGTTCCCCGCGCCAGCGGGGAT AAACCG*CTCGTAAAAGCAGTA CAGTGCACCGTAAGA*TCGAGTT CCCCGCGCCAGCGGGGATAAAC CG*		
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	121	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	122	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	123	pCASCADE-gltA1
gltA1-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTTCATAACT TTTAC	124	
G2U	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCGTATTGACCAATTCA TTC*GGGACAGTTATTAGT*TCGA GTTCCCCGCGCCAGCGGGGATA AACCG*TTACCATTCTGTTGCTT TTATGTATAAGAA*TCGAGTTCC CCGCGCCAGCGGGGATAAACCG*	125	
udhA-FOR	GCGCCAGCGGGGATAAACCGT TACCATTCTGTTG	126	pCASCADE-udhA
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	127	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	128	pCASCADE-gltA2
gltA2-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTAATAACTG TC	129	
G2Z	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCGTATTGACCAATTCA TTC*GGGACAGTTATTAGT*TCGA GTTCCCCGCGCCAGCGGGGATA AACCG*CTCGTAAAAGCAGTAC AGTGCACCGTAAGA*TCGAGTTC CCCGCGCCAGCGGGGATAAACC*	130	

	G		
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	131	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	132	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	133	pCASCADE-gltA2
gltA2-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTAATAACTG TC	134	
UZ	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTACCATTCTGTT GCTTTTATGTATAAGAA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*CTCGTAAAAGCAGTACA GTGCACCGTAAGA*TCGAGTTCC CCGCGCCAGCGGGGATAAACCG*	135	
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	136	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	137	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	138	pCASCADE-udhA
udhA-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATTCTTATACAT AAAAGC	139	
FG1G2	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*TATTGACCAATTCATTCGGG ACAGTTATTAGT*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*	140	
gltA2-FOR	GCGCCAGCGGGGATAAACCG*T*	141	pCASCADE-gltA2

	ATTGACCAATTCATTC		
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	142	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	143	pCASCADE-FG1
gltA1-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTTCATAACT TTTAC	144	
G1G2A	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*GAAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCGT*ATTGACCAATTCATT CGGGACAGTTATTAGT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*GTTTTTGTAATTT TACAGG CAACCTTTTAT*TCGAGTTCCCC GCGCCAGCGGGGATAAACCG*	145	
gapAP1-FOR	GCGCCAGCGGGGATAAACCGG TTTTTGTAATTT TACAGGC	146	pCASCADE-gapAP1
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	147	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	148	pCASCADE-G1G2
gltA2-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTAATAACTG TC	149	
G1G2U	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*GAAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*TATTGACCAATTCATT CGGGACAGTTATTAGT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*TTACCATTCTGTTGCTTTT ATGTATAAGAA*TCGAGTTCCCC GCGCCAGCGGGGATAAACCG*	150	

udhA-FOR	GCGCCAGCGGGGATAAACCGT TACCATTCTGTTG	151	pCASCADE-udhA
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	152	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	153	pCASCADE-G1G2
gltA2-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTAATAACTG TC	154	
G1G2Z	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*GAAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*TATTGACCAATTCATT CGGGACAGTTATTAGT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*CTCGTAAAAGCAGTACAG TGCACCGTAAGA*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*	155	
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	156	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	157	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	158	pCASCADE-G1G2
gltA2-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTAATAACTG TC	159	
FG1G2A	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*TATTGACCAATTCATTCGGG ACAGTTATTAGT*TCGAGTTCCC*	160	

	*CGCGCCAGCGGGGATAAACCG*GTTTTTGTAATTT TACAGGCAACCTTTTAT*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*		
gapAP1-FOR	GCGCCAGCGGGGATAAACCGGTTTTTGTAATTT TACAGGC	161	pCASCADE-gapAP1
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	162	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	163	pCASCADE-FG1G2
gltA2-REV	CGGTTTATCCCCGCTGGCGCGGGGAACTCGAACTAATAACTGTC	164	
FG1G2U	*TCGAGTTCCCCGCGCCAGCGGGGATAAACCG*TTGATTATAATAACCGTTTATCTGTTCGTA*TCGAGTTCCCCGCGCCAGCGGGGATAAACCG*AAAAGCATATAATGCGTAAAAGTTATGAAGT*TCGAGTTCCCCGCGCCAGCGGGGATAAACCG*TATTGACCAATTCATTCGGGACAGTTATTAGT*TCGAGTTCCCCGCGCCAGCGGGGATAAACCG*TTACCATTCTGTTGCTTTTATGTATAAGAA*TCGAGTTCCCCGCGCCAGCGGGGATAAACCG*	165	
gltA2-FOR	GCGCCAGCGGGGATAAACCG*TATTGACCAATTCATTC*	166	pCASCADE-udhA
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTCATCCGG	167	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGCGGGCAAG	168	pCASCADE-FG1G2
gltA1-REV	CGGTTTATCCCCGCTGGCGCGGGGAACTCGAACTTCATAACTTTTAC	169	
FG1G2Z	*TCGAGTTCCCCGCGCCAGCGGGGATAAACCG*TTGATTATAATAA	170	

	CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*TATTGACCAATTCATTCGGG ACAGTTATTAGT*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*C TCGTAAAAGCAGTACAGTGCA CCGTAAGA*TCGAGTTCCCCGCG CCAGCGGGGATAAACCG*		
gltA2-FOR	GCGCCAGCGGGGATAAACCG*T ATTGACCAATTCATTC*	171	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	172	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	173	pCASCADE-FG1G2
gltA1-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGAACTTCATAACT TTTAC	174	
G1G2UA	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*AAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*TATTGACCAATTCATT CGGGACAGTTATTAGT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*TTACCATTCTGTTGCTTTT ATGTATAAGAA*TCGAGTTCCCC GCGCCAGCGGGGATAAACCG*GT TTTTGTAATTT TACAGGCAAC CTTTTAT*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*	175	
gapAP1-FOR	GCGCCAGCGGGGATAAACCGG TTTTTGTAATTT TACAGGC	176	pCASCADE-gapAP1
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	177	

pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	178	pCASCADE-G1G2U
udhA-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATTCTTATACAT AAAAGC	179	
G1G2UZ	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*AAAAGCATATAAT GCGTAAAAGTTATGAAGT*TCG AGTTCCCCGCGCCAGCGGGGAT AAACCG*TATTGACCAATTCATT CGGGACAGTTATTAGT*TCGAGT TCCCCGCGCCAGCGGGGATAAA CCG*TTACCATTCTGTTGCTTTT ATGTATAAGAA*TCGAGTTCCCC GCGCCAGCGGGGATAAACCG*CT CGTAAAAGCAGTACAGTGCAC CGTAAGA*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*	180	
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	181	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	182	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	183	pCASCADE-G1G2U
udhA-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATTCTTATACAT AAAAGC	184	
FG1G2UA	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC GTATTGACCAATTCATTCGGG ACAGTTATTAGT*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*T	185	

	TACCATTCTGTTGCTTTTATGT ATAAGAA*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*GTTTTT GTAATTT TACAGGCAACCTTT TAT*TCGAGTTCCCCGCGCCAGC GGGGATAAACCG*		
gapAP1-FOR	GCGCCAGCGGGGATAAACCGG TTTTTGTAATTT TACAGGC	186	pCASCADE-gapAP1
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	187	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	188	pCASCADE-FG1G2U
udhA-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATTCTTATACAT AAAAGC	189	
FG1G2UZ	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*TATTGACCAATTCATTCGGG ACAGTTATTAGT*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*T TACCATTCTGTTGCTTTTATGT ATAAGAA*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*CTCGTA AAAGCAGTACAGTGCACCGTA AGA*TCGAGTTCCCCGCGCCAGC GGGGATAAACCG*	190	
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	191	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	192	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	193	pCASCADE-FG1G2U

udhA-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATTCTTATACAT AAAAGC	194	
FG1G2UZA	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTGATTATAATAA CCGTTTATCTGTTCGTA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*AAAAGCATATAATGCGT AAAAGTTATGAAGT*TCGAGTTC CCCGCGCCAGCGGGGATAAACC G*TATTGACCAATTCATTCGGG ACAGTTATTAGT*TCGAGTTCCC CGCGCCAGCGGGGATAAACCG*T TACCATTCTGTTGCTTTTATGT ATAAGAA*TCGAGTTCCCCGCGC CAGCGGGGATAAACCG*CTCGTA AAAGCAGTACAGTGCACCGTA AGA*TCGAGTTCCCCGCGCCAGC GGGGATAAACCG*GTTTTTGTAA TTT TACAGGCAACCTTTTAT*TC GAGTTCCCCGCGCCAGCGGGGA TAAACCG*	195	
gapAP1-FOR	GCGCCAGCGGGGATAAACCGG TTTTTGTAATTT TACAGGC	196	pCASCADE-gapAP1
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	197	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	198	pCASCADE-FG1G2UZ
zwf-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATCTTACGGTGC ACTGTAC	199	
UZ	*TCGAGTTCCCCGCGCCAGCGGG GATAAACCG*TTACCATTCTGTT GCTTTTATGTATAAGAA*TCGAG TTCCCCGCGCCAGCGGGGATAA ACCG*CTCGTAAAAGCAGTACA GTGCACCGTAAGA*TCGAGTTCC*	200	

	CCGCGCCAGCGGGGATAAACCG		
zwf-FOR	GCGCCAGCGGGGATAAACCGC TCGTAAAAG	201	pCASCADE-zwf
pCASCADE-REV	CTTGCCCGCCTGATGAATGCTC ATCCGG	202	
pCASCADE-FOR	CCGGATGAGCATTCATCAGGC GGGCAAG	203	pCASCADE-udhA
udhA-REV	CGGTTTATCCCCGCTGGCGCG GGGAACTCGATTCTTATACAT AAAAGC	204	

[00201] Table 10: List of plasmids used in this study.

Plasmid Utilized in this Study		
Plasmid	**Purpose**	**Source**
pSIM5	Recombineering and Strain Construction	Court Lab[54]
pCP20	FRT kanamycin cassette curing	Court Lab[54]
pSMART-HC-Kan	Backbone Vector	Lucigen
pcrRNA.Tet	pCASCADE-control backbone	Beisel Lab[34]
Plasmid Constructed in this Study		
Plasmid	**Plasmid Name**	**Addgene ID**
pSMART-Ala2	pSMART-HCKan-IN:yibDp-ald*	71326
pSMART-Ala3	pSMART-HCKan-IN:phoBp-ald*	71327
pSMART-Ala4	pSMART-HCKan-IN:phoHp-ald*	71328
pSMART-Ala5	pSMART-HCKan-IN:mipAp-ald*	71329
pSMART-Ala11	pSMART-HCKan-proA-ald*	87172
pSMART-Ala12	pSMART-HCKan-proC-ald*	87173
pSMART-Ala13	pSMART-HCKan-proD-ald*	87174
pSMART-Ala14	pSMART-HCKan-proB-ald*	101079
pSMART-Ala15	pSMART-HCKan-HCEp-ald*	101080
pSMART-Mev2	pSMART-IN:yibDp1-mvaE-IN:phoBp2-mvaS(A110G)	66642
pSMART-Mev3	pSMART-IN:yibDp1-mvaE-IN:mipAp2-mvaS(A110G)	102761
pSMART-Mev4	pSMART-IN:yibDp1-mvaE-IN:phoHp2-mvaS(A110G)	102762
pSMART-Mev5	pSMART-IN:mipAp1-mvaE-IN:yibD2-mvaS(A110G)	102763
pSMART-3HP	pSMART-3HP-NADPH-rhtA	87143
pCDF-mcherry1	pCDF-proD-mcherry	87144

pCDF-mcherry2	pCDF-proD-mcherry-DAS4	87145
pSMART-GFPuv	pSMART-IN:yibDp-GFPuv	65822
pSMART-GFPuv2	pSMART-IN:phoBp-GFPuv	71517
pSMART-GFPuv3	pSMART-IN:phoUp-GFPuv	71518
pSMART-GFPuv4	pSMART-IN:phoHp-GFPuv	71519
pSMART-GFPuv5	pSMART-IN:mipAp-GFPuv	71520
pCASCADE-control	pCASCADE	65821
pCASCADE-proD	pCASCADE-proD	65820
pCASCADE-gapAP1	pCASCADE-gapAP1	87146
pCASCADE-fabI	pCASCADE-fabI	66635
pCASCADE-FG1	pCASCADE-fabI-gltA1	71340
pCASCADE-FG1G2	pCASCADE-fabI-gltA1-gltA2	71342
pCASCADE-FG1G2A	pCASCADE-fabI-gltA1-gltA2-gapA	87147
pCASCADE-FG1G2U	pCASCADE-fabI-gltA1-gltA2-udhA	66637
pCASCADE-FG1G2UA	pCASCADE-fabI-gltA1-gltA2-udhA-gapA	87154
pCASCADE-FG1G2UZ	pCASCADE-fabI-gltA1-gltA2-udhA-zwf	87148
pCASCADE-FG1G2UZA	pCASCADE-fabI-gltA1-gltA2-udhA-zwf-gapA	87149
pCASCADE-FG1G2Z	pCASCADE-fabI-gltA1-gltA2-zwf	66638
pCASCADE-FG2	pCASCADE-fabI-gltA2	71341
pCASCADE-FU	pCASCADE-fabI-udhA	66636
pCASCADE-FZ	pCASCADE-fabI-zwf	71335
pCASCADE-G1G2	pCASCADE-gltA1-gltA2	71348
pCASCADE-G1G2A	pCASCADE-gltA1-gltA2-gapA	87150
pCASCADE-G1G2U	pCASCADE-gltA1-gltA2-udhA	71343
pCASCADE-G1G2UA	pCASCADE-gltA1-gltA2-udhA-gapA	87151
pCASCADE-G1G2UZ	pCASCADE-gltA1-gltA2-udhA-zwf	87152
pCASCADE-G1G2Z	pCASCADE-gltA1-gltA2-zwf	71347
pCASCADE-G1U	pCASCADE-gltA1-udhA	71339
pCASCADE-G1Z	pCASCADE-gltA1-zwf	71337
pCASCADE-G2U	pCASCADE-gltA2-udhA	65819
pCASCADE-G2Z	pCASCADE-gltA2-zwf	71338
pCASCADE-gltA1	pCASCADE-gltA1	71334
pCASCADE-gltA2	pCASCADE-gltA2	65817
pCASCADE-udhA	pCASCADE-udhA	65818
pCASCADE-UZ	pCASCADE-udhA-zwf	87153

pCASCADE-zwf	pCASCADE-zwf	65825

Section 7: 2-Stage Micro-fermentations

[00202] *E. coli* Media Stock Solutions

- 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.
- 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 $ZnSO_4 \cdot 7H_2O$, 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.

[00203] Media Components

[00204] Prepare the final working medium by aseptically mixing stock solutions based on the following tables in the order written to minimize precipitation, then filter sterilize (with a 0.2 µm filter).

[00205] Table 11: 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
$ZnSO_4 \cdot 7H_2O$	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

[00206] Table 12: 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
$ZnSO_4 \cdot 7H_2O$	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

[00207] Micro-fermentations

[00208] An overview of the micro-fermentation protocol is illustrated in **Figure 16A-C**. Strains were evaluated for production in 96 well plate micro-fermentations, wherein cells were initially grown to mid-log phase, harvested, washed, resuspended and normalized in a phosphate free production medium to an OD600 = 1, for a 24 hour production stage. The success of the micro-fermentations required: (1) syncing strains up by harvesting all strains in exponential phase; (2) the use of low biomass levels, so that batch sugar could be kept low while enabling significant potential product accumulation; and (3) a method to supply adequate mixing and aeration, while minimizing evaporative losses. To address the final requirement, commercially available microplate sandwich covers and clamps from EnzyScreen™ was used, which greatly reduce evaporative losses while enabling high levels of mixing and aeration in standard 25 mm orbit shakers operating at 400 rpm [92-93]. Micro-fermentation results for alanine production with different insulated phosphate promoters are shown in **Figure 17**. Micro-fermentation results for strains evaluated with *gapA* and *gapN* gene alterations are given in **Figure 18**.

Section 8: Micro-fermentations robustness evaluation

[00209] During micro-fermentation oxygen robustness studies, production culture volume was varied to achieve desired oxygen transfer rate (OTR) values as previously reported (http://www.enzyscreen.com/oxygen_transfer_rates.htm) [92-93], and as listed below in Table 14. Batch glucose levels during the production stage were altered to assess robustness to glucose. Strains utilized in the robustness experiments at the micro-fermentation scale are listed in Table 15. Results from the micro-fermentation robustness studies are given in Figures 19A-D, Figures 20A-D, Figures 21A-D, Figures 22A-D, Figures 23A-D, Figures 24A-D, Figures 25A-D, Figures 26A-D, Figures 27A-D, Figures 28A-D, Figures 29A-D, Figures 30A-D, Figures 31A-D, and Figure 32.

[00210] Table 14: Culture conditions for different OTR values.

25mm orbit shaker		
Max OTR	**Shaking Speed**	**Fill Volume**
(mmol/L-hr)	**(rpm)**	**(µL)**
25	400	100
20	400	150
15	400	200

[00211] Table 15: List of strains used for micro-fermentation robustness evaluations and their RS scores.

Strain #	**Silencing**	**Proteolysis**	**Plasmid**	**RS**
1	gltA1	FU	pSMART-Ala2	89.6
2	gltA1	F	pSMART-Ala2	89.5
3	gltA1	GU	pSMART-Ala2	89.4
4	FG1G2	None	pSMART-Ala2	89.3
5	G1G2	GU	pSMART-Ala2	88.8
6	FG1G2	G	pSMART-Ala2	88.2
7	G1G2	F	pSMART-Ala2	83.4
8	gltA2	FGU	pSMART-Ala2	83.4
9	gltA1	FGU	pSMART-Ala2	83.1
10	G1G2	FGU	pSMART-Ala2	82.3
11	gltA2	U	pSMART-Ala2	82.2
12	gltA2	F	pSMART-Ala2	80.6
13	FG1G2	FG	pSMART-Ala2	80.5
14	None	G	pSMART-Ala2	79.9
15	gltA2	GU	pSMART-Ala2	77.9
16	fabI	FGU	pSMART-Ala2	75.7
17	None	FG	pSMART-Ala2	75.4
18	G1G2	FU	pSMART-Ala2	75.3
19	None	FGU	pSMART-Ala2	73.4
20	None	FU	pSMART-Ala2	73.3
21	gltA1	U	pSMART-Ala2	72.9
22	fabI	FG	pSMART-Ala2	69.1
23	FG1G2	FU	pSMART-Ala2	67.6
24	gltA2	FU	pSMART-Ala2	67.5

25	None	F	pSMART-Ala2	65.6
26	gltA2	FG	pSMART-Ala2	62.1
27	FG1G2	F	pSMART-Ala2	61.1
28	fabI	GU	pSMART-Ala2	59.9
29	fabI	F	pSMART-Ala2	59.6
30	gltA1	FG	pSMART-Ala2	58.1
31	gltA1	None	pSMART-Ala2	57.1
32	None	None	pSMART-Ala2	55.5
33	G1G2	None	pSMART-Ala2	54.1
34	fabI	U	pSMART-Ala2	53.9
35	gltA2	G	pSMART-Ala2	52.8
36	fabI	None	pSMART-Ala2	50.3
37	fabI	FU	pSMART-Ala2	48.4
38	gltA2	None	pSMART-Ala2	47.8
39	FG1G2	FGU	pSMART-Ala2	44.6
40	None	GU	pSMART-Ala2	42.9
41	None	U	pSMART-Ala2	39.3
42	fabI	G	pSMART-Ala2	39.2
43	gltA1	G	pSMART-Ala2	34.7
44	G1G2	FG	pSMART-Ala2	32.8
45	FG1G2	U	pSMART-Ala2	29.4
46	FG1G2	GU	pSMART-Ala2	24.3
47	G1G2	G	pSMART-Ala2	24.1
48	G1G2	U	pSMART-Ala2	-25.3
49	None	None	pSMART-Ala13	55.7
50	None	None	pSMART-Ala12	-31.5
51	None	None	pSMART-Ala15	-103.2
52	None	None	pSMART-Ala11	-114.1
53	None	None	pSMART-Ala14	-441.5

Section 9: Standardized 2-Stage Fermentations

[00212] A standardized phosphate limited 2-stage fermentation protocol was utilized for evaluation of all valve strains. This protocol yields highly reproducible growth stage results, with minimal strain to strain variability even with strains making different products. More significant variability was observed during the production stage as a result of differing feed rates and base utilization by different strains. **Figure 33A** gives the growth curves for all valve strains

with a 10 g·cdw/L biomass level in 1 L fermentations performed in this study. This consistency is contrasted to the more variable growth of growth associated production strains, given in **Figure 33B**.

[00213] Table 16: Strains used for mevalonic acid scalability.

Strain #	Silencing	Proteolysis	Plasmid
1	FG1G2	FU	pSMART-Mev2
2	G2Z	FGUA	pSMART-Mev2
3	FG1G2A	FUN	pSMART-Mev2
4	UZ	FGUA	pSMART-Mev2

Section 10: Analytical Methods

[00214] Table 17: UPLC-MS/MS parameters

Analyte	Retention Time (min)	ESI Mode	MRM Transition(s)	Cone Voltage	Collision Energy
Alanine	0.5	+	89.95→44.08	15	9
C13-Alanine	0.5	+	91.95→46.08	15	9

Detailed description of figures

[00215] Figure 1A: An Overview of Dynamic Metabolic Control in 2-Stage Fermentations. Metabolic engineering involves optimizing a metabolic pathway to a desired product to the existing metabolic network of a host, converting feedstocks to a desired product. Filled circles indicate metabolites and lines indicate enzymatic reactions. Traditional optimization in metabolic engineering, often involves three key steps (a) the deletion of competing non-essential metabolic pathways including those leading to undesired byproducts and the overexpression of enzymes in the pathway converting feedstock molecules to the product (indicated by thicker lines) and potentially (b) attenuating enzymes in essential metabolism (indicated by orange lines) to further increase production. This process is iterated to optimize the yield to the desired product (pie charts). By contrast, dynamic metabolic network minimization can be used to fully unlock the potential of commonly used 2-stage fermentation processes (c-d). In the first stage of these processes (c) biomass growth and yield are optimized, while in the second stage (d) product formation is optimized, which is well suited for a 2-stage process (e) in which biomass levels accumulate and consume a limiting nutrient (in this case inorganic phosphate), which

when depleted triggers entry into a productive stationary phase. Synthetic metabolic valves utilizing CRISPRi based gene silencing and/or controlled proteolysis can be used (f and g) to greatly reduce the pertinent metabolic network upon the transition to the production stage, (f) and array of silencing guides can be induced, processed by the CASCADE complex into individual guides and used to silencing target multiple genes of interest (GOI). (g) If C-tcrminal DAD+4 lags are added to enzymes of interest (EOI) through chromosomal modification, they can be inducibly degraded by the clpXP protease in the present of and inducible sspB chaperone. (h) Dynamic control over protein levels in *E. coli* using 2 stage dynamic control with inducible proteolysis and CRISPRi silencing. As cells grow phosphate is depleted, and cells "turn off mCherry and "turn on" GFPuv. Shaded areas represent one standard deviation from the mean, n=3. (i) Relative impact of proteolysis and gene silencing alone and in combination on mCherry degradation, with (j) decays rates.

[00216] Figure 1B: **Strain and Bioprocess Optimization**. (a) Conventional approaches for strain and process optimization in metabolic engineering often involves deletion of competing non-essential metabolic pathways and overexpression of pathway enzymes (Filled circles: metabolites; lines: enzymatic reactions. green indicated a production pathway). (a-i) Strain variants are evaluated at screening scale (microtiter plates, shake flasks, etc), (a-ii) the best strains are assessed in larger scale instrumented bioreactors. Numerous design-build-test cycles (a-vi-vii) are used to iteratively optimize both the production strain and process, including the often-critical optimization of environmental (process) variables (a-vii). (a-iii) The best performing strains and associated optimized process conditions are scaled to industrially relevant levels. (b) Rapid strain and bioprocess optimization using 2-stage dynamic metabolic control. The metabolic network in the cell is dynamically minimized to only the steps essential for product formation. This is accomplished in a standardized 2-stage bioprocess (c), where a biomass accumulating growth stage is followed by a production stage, with only a minimal metabolic network. The limitation of a macronutrient can be used to “switch” cellular metabolism from growth to production. The approach results in a smaller subset of potential strain variants for screening (b-i). Metabolic network minimization helps increase relevant metabolite levels (d) and thus production levels, it also enhances process robustness (e), and as a result process and strain scalability (f). The best producers identified from screening are predictably and rapidly scaled to (b-ii) larger instrumented bioreactors, and (b-iii) subsequently to industrially relevant levels. If needed, limited design-build-test cycles (b-iv) are incorporated to guide improvements. Product independent, standardized protocols are followed for strain evaluation at all scales, eliminating the need for intensive process optimization.

[00217] Figures 2A-D: **Implementation of 2-stage Synthetic Metabolic Valves (SMVs) in *E. coli***. **Figure 2A** depicts SMVs utilizing CRISPRi based gene silencing and/or controlled proteolysis were constructed. (Top) Silencing: An array of inducible silencing guide RNAs (i) can be used to silence expression of multiple genes of interest (GOI) when the native *E. coli* CRISPR/Cascade machinery is expressed, which can process guide arrays into individual guides (ii). (Bottom) Proteolysis: When C-terminal DAS+4 tags are added to enzymes of interest (EOI) (through chromosomal modification), they can be degraded by the clpXP protease (iv) upon the controlled induction of the *sspB* chaperone (iii). **Figure 2B** depicts dynamic control over protein levels in *E. coli* using inducible proteolysis and CRISPRi silencing. As cells grow phosphate is depleted, cells "turn OFF" mCherry and "turn ON" GFPuv. Shaded areas represent one standard deviation from the mean, r.f.u, relative fluorescence units. **Figure 2C** depicts relative impact of proteolysis and gene silencing alone and in combination on mCherry degradation, n.f.u. normalized fluorescence units (normalized to maximal fluorescence). **Figure 2D** depicts relative impact of proteolysis and gene silencing alone and in combination on observed mCherry fluorescence decays rates (per hour).

[00218] Figures 3A-K: Alanine Production in *E. coli* utilizing 2-stage Dynamic Control. **Figure 3A** depicts strain variant design. Primary pathways in central metabolism are shown including: Glycolysis, the Pentose Phosphate Pathway, the Citric Acid Cycle (TCA), Fatty Acid Biosynthesis, and the Soluble Transhydrogenase. Key valve candidate enzymes/genes that are "turned OFF" to reduce flux through central metabolism can include: glucose-6-phosphate dehydrogenase (*zwf-"Z"*), lipoamide dehydrogenase (*lpd-"L"*), citrate synthase (*gltA-"G"*), enoyl-ACP reductase (*fabI-"F"*), and the soluble transhydrogenase (*udhA-"U"*). Importantly, dynamic elimination of *fabI* has been previously demonstrated to increase intracellular malonyl-CoA pools as well as malonyl-CoA flux [55]. Enzymes that are dynamically "turned ON" can include the metabolic pathways to produce the products of interest, in this case alanine. Specific pathway enzymes include an NADPH-dependent alanine dehydrogenase (*ald**) and an alanine exporter (*alaE*). Additionally, as the alanine production pathway utilizes NADPH as a cofactor, the NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase encoded by the *gapN* gene [56] from *S. mutans* was turned on alone and in combination with turning off the native *gapA-"A"* gene (NADH dependent glyceraldehyde dehydrogenase). Abbreviation: 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, MSA – malonate semialdehyde, ACP – acyl carrier protein, Ru – ribulose, Xu –xylulose, E - erthryose, Ri – ribose, S - sedoheptulose. Strains were engineered

with SMVs for the dynamic control of all combinations of valve genes/enzymes, either through gene silencing alone, proteolysis alone, or the combination of both. These strains were evaluated for alanine production in standardized micro-fermentations. **Figure 3B** depicts rank order plot for average alanine titer (black) of all valve strains examined in 2-stage micro-fermentation, grey area represents standard deviation. Alanine production in the control strain was colored in red. **Figure 3C** depicts average alanine titer in 2-stage production in response to different proteolysis and silencing combinations, from 0 g/L (purple) to 5 g/L (red). **Figure 3D** depicts average alanine titer in response to different oxygen transfer rates (OTR) and glucose concentrations evaluated for a single "Valve" alanine strain (Silencing of gltA1 ("G1"), Proteolysis of fabI and udhA ("FU")). The results of this surface were used to calculate a strain-specific robustness score (RS) (refer to text), this strain has the highest RS score. **Figure 3E** depicts a heat map of the robustness score for a subset of 48 "Valve" strains evaluated across multiple process conditions. **Figure 3F** depicts scale up of one of the best producing strain from micro-fermentations (Silencing of fabI-gltA1-gltA2 ("FG1G2"), Proteolysis of fabI, gltA and udhA ("FGU")) to 1L bioreactors results in a titer of 80 g/L after 48 hrs of production, with a yield of 0.8 g/g. **Figure 3G** depicts overexpression of the *alaE* alanine exporter in this strain (Panel f) results in significantly improved production, reaching 147 g/L in 27 hrs of production, with a yield of ~1 g/g. (Refer to Supplemental Materials, Section 3 for additional details). **Figure 3H** depicts strains selected for robustness evaluation in micro-fermentations. **Figure 3I** depicts robustness and titer for the most robust "Valve" alanine strain (Silencing_gltAl, Proteolysis_FU). Bottom surface shows heat map for the alanine titer normalized to the median of all process conditions assessed, upper surface shows alanine tiler under all process conditions, the same color scale (alanine titer in g/L) was used for both panels. **Figure 3J** depicts RS3 scores for the selected strains. **Figure 3K** depicts process reproducibility heat map for all conditions evaluated, the same grayscale was used for Figure 3J and Figure 3K.

[00219] Figures 4A-F: Robustness Comparison Between 2-Stage and Growth Associated Approaches. Figure 4A depicts rank order of the RS3 scores for all alanine strains evaluated, red bars indicate valve alanine strains, and blue bars indicate growth associated (GA) alanine strains. **Figure 4B** depicts average RS3 score for "Valve" alanine strains with proteolysis "F" valve, and growth associated alanine strains. **Figure 4C** depicts max titer plot for a representative "Valve" alanine (Proteolysis_FGU, Silencing_gltA1), and growth associated alanine strains in micro-fermentation of all conditions evaluated. **Figure 4D** depicts process reproducibility for growth associated alanine strains under all conditions evaluated. **Figure 4E** depicts robustness and titer for a representative robust "Valve" alanine (Proteolysis_FGU, Silencing_gltA1). **Figure 4F** depicts robustness and titer for the GA2 strain. Bottom surface,

heat map for the alanine titer normalized to the median of all process conditions assessed, upper surface, alanine titer under all process conditions, the same color scale (alanine titer in g/L) was used for both panels.

[00220] Figures 5A-J: Comparisons of "Valve" and growth associated alanine production in micro-fermentations (**Figures 5A-D**) and 1L fermentation (**Figures 5E-J**). Average alanine titer (**Figure 5A**) and robustness score (**Figure 5B**) for all strains used for robustness analysis. Average alanine titer in response to different OTR and glucose concentrations for selected "Valve" (**Figure 5C**) and growth associated (**Figure 5D**) alanine strains. Strains marked by asterisk in (**Figure 5B**) were used for this analysis. These two strains were selected for 1L performance comparison. **Figure 5E** and **Figure 5F** depicts 1L performance metrics evaluated, including average specific productivity (SP, g/gdcw-h), average glucose uptake rate (GUR, g/gcdw-h), max titer (g/L), and max yield (g/g). **Figure 5G** and **Figure 5H** depicts µL to 1L scalability. 1L data was standardized to the maximal titer within 50 hours of production. Adequate feed was used for growth associated strains to avoid glucose depletion. **Figure 5I** and **Figure 5J** depicts 1L production profiles for all strains used in scalability plot **Figure 5G** and **Figure 5H** respectively, darker symbols represent growth curves, lighter symbols represent production curves, shape of symbols encode the same strains in **Figure 5G** or **Figure 5H**.

[00221] Figure 6A-E: Mevalonate Production in *E. coli* utilizing 2-stage Dynamic Control. **Figure 6A** depicts Metabolic Pathways and SMVs for mevalonate production. **Figure 6B** depicts mevalonate production using several production pathway plasmid variants with varied promoter combinations in the control strain. **Figure 6C** depicts micro-fermentation results for a subset of "Valve" strains producing mevalonate, using the best production pathway from Figure 6B, along with combinations of proteolytic and silencing SMVs. **Figure 6D** depicts µL to 1L scalability for a subset of mevalonate strains evaluated at the 1L scale. n=3 for µL data and n=1 for 1L data. The maximal titer within 50 hours of production time was used for the correlation. **Figure 6E** depicts production of the best mevalonate strain from Figure 6D (Silencing of fabI-gltA1-gltA2 ("FG1G2"), Proteolysis of fabI and udhA ("FU")) in 1L bioreactors. A titer of 97 g/L was observed in 78 hrs of production. Yields during the production stage reached 0.46 g/g (84% of theoretical yield). (Refer to Supplemental Materials, Section 9 for additional details). **Figure 6F** depicts micro-fermentation results for a subset of strains producing 3-HP. **Figure 6G** depicts µL to 1L scalability for a subset of 3-HP strains evaluated at the 1L scale (Supplemental Materials Tables S21 and S22). **Figure 6H** depicts production performance for the best 3-HP strains in the 1L systems, squares, 3-HP/mevalonic acid titer; circles, OD600. Yields during the production stage reached for the 0.46 g/g for mevalonic acid and 0.63 g/g for 3-HP in the highest producers.

[00222] Figure 7: Phosphate depletion promoter characterization. A set of GFP reporter vectors were constructed to assess the expression level of 12 previously identified phosphate regulated promoters. Strains were evaluated continuously for GFP expression in the Biolector™ using a standardized protocol wherein in minimal medium limited for phosphate is used. After Biomass levels reach a peak (not shown for clarity), GFP expression begins. Importantly the current set of promoters enables a large range of expression levels.

[00223] Figure 8: Insulated phosphate depletion promoter characterization. A set of GFP reporter vectors were constructed to assess the expression level of five insulated phosphate regulated promoters in FGM3 media. Strains were evaluated continuously for GFP expression in the Biolector™ using a standardized protocol wherein in minimal medium limited for phosphate is used. After Biomass levels reach a peak (not shown for clarity), GFP expression begins. Importantly the current set of promoters enables a large range of expression levels.

[00224] Figure 9: Insulated constitutive promoter characterization. A set of GFP reporter vectors were constructed to assess the expression level of five insulated constitutive promoters in FGM3 with 40 mM phosphate media. Shaded area represents standard deviations, n=3. Strains were evaluated continuously for GFP expression in the Biolector™. GFP expression was observed only for promoters proA, proB and proD.

[00225] Figure 10: Metabolic modeling results for optimal 3-HP flux in two stage fermentations. LEFT: Optimized fluxes during the growth stage where biomass production was used as the objective function. RIGIIT: Optimized fluxes during the 3-HP production stage where 3-HP production was used as the objective function (biomass production was set to 0). Fluxes are listed as relative ratios or moles of flux through a given reaction per 100 moles of glucose utilized.

[00226] Figure 11: Chromosomal modifications.

[00227] Figure 12: Average maximal growth rates of starting host strains in 1L FGM10 minimal medium fermentations, n=2.

[00228] Figure 13A-E: Distribution of glucose utilized during the growth phase of starting host strains in 1L standard minimal medium fermentations**.** Mid exponential and final growth period results are given for DLF_0025 as "production" begins in mid-late exponential phase. Results are averages of duplicate fermentations. Figure 13A, BW25113; Figure 13B, BWapldf; Figure 13C, DLF_0001; Figure 13D, DLF_0025 at mid-exponential; Figure 13E, DLF_0025 at end of growth phase. Unit was gram glucose.

[00229] Figure 14: pCASCADE-control plasmid construction scheme.

[00230] Figure 15A-B: pCASCADE construction scheme. Figure 15A, single sgRNA cloning; Figure 15B, double sgRNA.

[00231] Figure 16A-C: Micro-fermentation process overview. (A) An overview of the high throughput micro-fermentation protocol. Freezer stocks (alternatively colonies may be used) are used to inoculate into SM10++ in 96 well plates. Cultures are grown overnight for 16 hours, harvested by centrifugation, washed with no-phosphate medium and resuspended in no-phosphate medium at target biomass levels. (OD600nm = 1.0). EnzyScreen™ covers and clamps are used to reduce evaporation and enable high oxygen transfer rates. The protocol is implemented with a Tecan Evo liquid handler. (B) Representative overnight growth in a 96 well plates culture, distribution of OD600 for overnight culture was plotted. (C) Representative OD600 distribution after normalization using Tecan Evo liquid handler.

[00232] Figure 17: Micro-fermentation for L-alanine production using different insulated phosphate promoters in DLF_0025 strain.

[00233] Figure 18: Heatmap for L-alanine production by gapN/gapA strains.

[00234] Figures 19A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00235] Figures 20A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00236] Figures 21A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00237] Figures 22A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00238] Figures 23A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00239] Figures 24A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00240] Figures 25A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00241] Figures 26A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00242] Figures 27A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00243] Figures 28A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00244] Figures 29A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00245] Figures 30A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00246] Figures 31A-D: Alanine production in response to different OTR and glucose concentration in micro-fermentation for 4 strains evaluated for robustness.

[00247] Figure 32: Alanine production in response to different OTR and glucose concentration in micro-fermentation for one strain evaluated for robustness.

[00248] Figures 33A-B: Growth profile for all (**Figure 33A**) valve and (**Figure 33B**) growth associated strains at 1L scale evaluated in this paper**.** Growth curves were synced to account for any variations in lag time. Valve strains growth curves were synced to the same mid-exponential point. Growth associated strains growth curves were synced to the same take-off point.

[00249] Figure 34: Specific Productivity (SP) comparison for strain with highest mevalonate titer from literature and mevalonate strain 1 evaluated in this work.

[00250] Figure 35: Alanine standard curve from MS measurement. Average and standard deviation for mass spec response from triplicate standard measurement were plotted.

[00251] Figures 36A-B: Glucose (**Figure 36A**) and ethanol (**Figure 36B**) standard curves from RI measurement. Average and standard deviation for peak area from triplicate standard measurement were plotted.

[00252] Figure 37: 3-Hydroxypropionic acid standard curve from TUV measurement. Average and standard deviation for peak area from duplicate standard measurement were plotted.

[00253] Figures 38A-D: TUV standard curves for (**Figure 38A**) L-alanine, (**Figure 38B**) D-alanine, (**Figure 38C**) mevalonic acid, and (**Figure 38D**) mevalonolactone. Average and standard deviation for peak area from triplicate standard measurement were plotted.

References

1. Cameron, D. E.; Bashor, C. J.; Collins, J. J., A brief history of synthetic biology. *Nat Rev Microbiol* **2014,** *12* (5), 381-90.

2. Cheong, S.; Clomburg, J. M.; Gonzalez, R., Energy- and carbon-efficient synthesis of functionalized small molecules in bacteria using non-decarboxylative Claisen condensation reactions. *Nature biotechnology* **2016,** *34* (5), 556-61.

3. Choi, S. Y.; Park, S. J.; Kim, W. J.; Yang, J. E.; Lee, H.; Shin, J.; Lee, S. Y., One-step fermentative production of poly(lactate-co-glycolate) from carbohydrates in Escherichia coli. *Nature biotechnology* **2016,** *34* (4), 435-40.

4. Jarboe, L. R.; Zhang, X.; Wang, X.; Moore, J. C.; Shanmugam, K. T.; Ingram, L. O., Metabolic engineering for production of biorenewable fuels and chemicals: contributions of synthetic biology. *Journal of biomedicine & biotechnology* **2010**, 761042.

5. Lee, J. W.; Na, D.; Park, J. M.; Lee, J.; Choi, S.; Lee, S. Y., Systems metabolic engineering of microorganisms for natural and non-natural chemicals. *Nat Chem Biol* **2012,** *8* (6), 536-46.

6. Dellomonaco, C.; Clomburg, J. M.; Miller, E. N.; Gonzalez, R., Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals. *Nature* **2011,** *476* (7360), 355-9.

7. Kim, S.; Clomburg, J. M.; Gonzalez, R., Synthesis of medium-chain length (C6-C10) fuels and chemicals via beta-oxidation reversal in Escherichia coli. *J Ind Microbiol Biotechnol* **2015,** *42* (3), 465-75.

8. Meadows, A. L.; Hawkins, K. M.; Tsegaye, Y.; Antipov, E.; Kim, Y.; Raetz, L.; Dahl, R. H.; Tai, A.; Mahatdejkul-Meadows, T.; Xu, L.; Zhao, L.; Dasika, M. S.; Murarka, A.; Lenihan, J.; Eng, D.; Leng, J. S.; Liu, C. L.; Wenger, J. W.; Jiang, H.; Chao, L.; Westfall, P.; Lai, J.; Ganesan, S.; Jackson, P.; Mans, R.; Platt, D.; Reeves, C. D.; Saija, P. R.; Wichmann, G.; Holmes, V. F.; Benjamin, K.; Hill, P. W.; Gardner, T. S.; Tsong, A. E., Rewriting yeast central carbon metabolism for industrial isoprenoid production. *Nature* **2016,** *537* (7622), 694-697.

9. Yadav, V. G.; De Mey, M.; Lim, C. G.; Ajikumar, P. K.; Stephanopoulos, G., The future of metabolic engineering and synthetic biology: towards a systematic practice. *Metab Eng* **2012,** *14* (3), 233-41.

10. Brophy, J. A.; Voigt, C. A., Principles of genetic circuit design. *Nat Methods* **2014,** *11* (5), 508-20.

11. Koutinas, M.; Kiparissides, A.; Pistikopoulos, E. N.; Mantalaris, A., Bioprocess systems engineering: transferring traditional process engineering principles to industrial biotechnology. *Comput Struct Biotechnol J* **2012,** *3*, e201210022.

12. Rodrigo, G.; Jaramillo, A., AutoBioCAD: full biodesign automation of genetic circuits. *ACS Synth Biol* **2013,** *2* (5), 230-6.

13. Garst, A. D.; Bassalo, M. C.; Pines, G.; Lynch, S. A.; Halweg-Edwards, A. L.; Liu, R.; Liang, L.; Wang, Z.; Zeitoun, R.; Alexander, W. G.; Gill, R. T., Genome-wide mapping of mutations at single-nucleotide resolution for protein, metabolic and genome engineering. *Nat Biotech* **2017,** *35* (1), 48-55.

14. Church, G. M.; Elowitz, M. B.; Smolke, C. D.; Voigt, C. A.; Weiss, R., Realizing the potential of synthetic biology. *Nat Rev Mol Cell Biol* **2014,** *15* (4), 289-94.

15. Thomas, S.; Maynard, N. D.; Gill, J., DNA library construction using Gibson Assembly[reg]. *Nat Meth* **2015,** *12* (11).

16. Goodwin, S.; McPherson, J. D.; McCombie, W. R., Coming of age: ten years of next-generation sequencing technologies. *Nat Rev Genet* **2016,** *17* (6), 333-51.

17. Lynch, M. D.; Warnecke, T.; Gill, R. T., SCALEs: multiscale analysis of library enrichment. *Nat Methods* **2007,** *4* (1), 87-93.

18. Zeitoun, R. I.; Garst, A. D.; Degen, G. D.; Pines, G.; Mansell, T. J.; Glebes, T. Y.; Boyle, N. R.; Gill, R. T., Multiplexed tracking of combinatorial genomic mutations in engineered cell populations. *Nat Biotechnol* **2015,** *33* (6), 631-7.

19. Crook, N.; Abatemarco, J.; Sun, J.; Wagner, J. M.; Schmitz, A.; Alper, H. S., In vivo continuous evolution of genes and pathways in yeast. *Nat Commun* **2016,** *7*, 13051.

20. Burg, J. M., Reed, BJ., Ye, Z., Cooper, C.B., Moreb, E.A., and Lynch, M.D, Large-Scale Bioprocess Competitiveness: The Potential of Dynamic Metabolic Control in Two-Stage Fermentations. *Current Opinions in Chemical Engineering* **2016,** *(In Review).*

21. Zhang, Y. H., Production of biofuels and biochemicals by in vitro synthetic biosystems: Opportunities and challenges. *Biotechnol Adv* **2015,** *33* (7), 1467-83.

22. Dietrich, J. A.; McKee, A. E.; Keasling, J. D., High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu Rev Biochem* **2010,** *79*, 563-90.

23. Formenti, L. R.; Norregaard, A.; Bolic, A.; Hernandez, D. Q.; Hagemann, T.; Heins, A. L.; Larsson, H.; Mears, L.; Mauricio-Iglesias, M.; Kruhne, U.; Gernaey, K. V., Challenges in industrial fermentation technology research. *Biotechnol J* **2014,** *9* (6), 727-38.

24. Levanon, S. S.; San, K. Y.; Bennett, G. N., Effect of oxygen on the Escherichia coli ArcA and FNR regulation systems and metabolic responses. *Biotechnol Bioeng* **2005,** *89* (5), 556-64.

25. Logue, J. B.; Findlay, S. E.; Comte, J., Editorial: Microbial Responses to Environmental Changes. *Front Microbiol* **2015,** *6*, 1364.

26. Garcia-Ochoa, F.; Gomez, E., Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnol Adv* **2009,** *27* (2), 153-76.

27. Waegeman, H.; Beauprez, J.; Moens, H.; Maertens, J.; De Mey, M.; Foulquie-Moreno, M. R.; Heijnen, J. J.; Charlier, D.; Soetaert, W., Effect of iclR and arcA knockouts on biomass formation and metabolic fluxes in Escherichia coli K12 and its implications on understanding the metabolism of Escherichia coli BL21 (DE3). *BMC Microbiol* **2011,** *11*, 70.

28. Waegeman, H.; Maertens, J.; Beauprez, J.; De Mey, M.; Soetaert, W., Effect of iclR and arcA deletions on physiology and metabolic fluxes in Escherichia coli BL21 (DE3). *Biotechnol Lett* **2012,** *34* (2), 329-37.

29. Hemmerich, J.; Adelantado, N.; Barrigon, J. M.; Ponte, X.; Hormann, A.; Ferrer, P.; Kensy, F.; Valero, F., Comprehensive clone screening and evaluation of fed-batch strategies in a microbioreactor and lab scale stirred tank bioreactor system: application on Pichia pastoris producing Rhizopus oryzae lipase. *Microb Cell Fact* **2014,** *13* (1), 36.

30. Ramirez-Vargas, R.; Vital-Jacome, M.; Camacho-Perez, E.; Hubbard, L.; Thalasso, F., Characterization of oxygen transfer in a 24-well microbioreactor system and potential respirometric applications. *J Biotechnol* **2014,** *186*, 58-65.

31. Huber, R.; Roth, S.; Rahmen, N.; Buchs, J., Utilizing high-throughput experimentation to enhance specific productivity of an E.coli T7 expression system by phosphate limitation. *BMC biotechnology* **2011,** *11*, 22.

32. Lynch, M. D., Into new territory: improved microbial synthesis through engineering of the essential metabolic network. *Curr Opin Biotechnol* **2016,** *38*, 106-11.

33. McGinness, K. E.; Baker, T. A.; Sauer, R. T., Engineering controllable protein degradation. *Mol Cell* **2006,** *22* (5), 701-7.

34. Luo, M. L.; Mullis, A. S.; Leenay, R. T.; Beisel, C. L., Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression. *Nucleic acids research* **2015,** *43* (1), 674-81.

35. Qi, L. S.; Larson, M. H.; Gilbert, L. A.; Doudna, J. A.; Weissman, J. S.; Arkin, A. P.; Lim, W. A., Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **2013,** *152* (5), 1173-83.

36. Chubukov, V.; Sauer, U., Environmental dependence of stationary-phase metabolism in Bacillus subtilis and Escherichia coli. *Applied and environmental microbiology* **2014,** *80* (9), 2901-9.

37. Santos-Beneit, F., The Pho regulon: a huge regulatory network in bacteria. *Front Microbiol* **2015,** *6*, 402.

38. Brouns, S. J.; Jore, M. M.; Lundgren, M.; Westra, E. R.; Slijkhuis, R. J.; Snijders, A. P.; Dickman, M. J.; Makarova, K. S.; Koonin, E. V.; van der Oost, J., Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **2008,** *321* (5891), 960-4.

39. Jian, J.; Zhang, S. Q.; Shi, Z. Y.; Wang, W.; Chen, G. Q.; Wu, Q., Production of polyhydroxyalkanoates by Escherichia coli mutants with defected mixed acid fermentation pathways. *Appl Microbiol Biotechnol* **2010,** *87* (6), 2247-56.

40. Grunenfelder, B.; Rummel, G.; Vohradsky, J.; Roder, D.; Langen, H.; Jenal, U., Proteomic analysis of the bacterial cell cycle. *Proc Natl Acad Sci U S A* **2001,** *98* (8), 4681-6.

41. Hintsche, M.; Klumpp, S., Dilution and the theoretical description of growth-rate dependent gene expression. *J Biol Eng* **2013,** *7* (1), 22.

42. Lerchner, A.; Jarasch, A.; Skerra, A., Engineering of alanine dehydrogenase from Bacillus subtilis for novel cofactor specificity. *Biotechnol Appl Biochem* **2016,** *63* (5), 616-624.

43. Hori, H.; Yoneyama, H.; Tobe, R.; Ando, T.; Isogai, E.; Katsumata, R., Inducible L-alanine exporter encoded by the novel gene ygaW (alaE) in Escherichia coli. *Applied and environmental microbiology* **2011,** *77* (12), 4027-34.

44. Davis, J. H.; Rubin, A. J.; Sauer, R. T., Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic acids research* **2011,** *39* (3), 1131-41.

45. Hedl, M.; Sutherlin, A.; Wilding, E. I.; Mazzulla, M.; McDevitt, D.; Lane, P.; Burgner, J. W., 2nd; Lehnbeuter, K. R.; Stauffacher, C. V.; Gwynn, M. N.; Rodwell, V. W., Enterococcus faecalis acetoacetyl-coenzyme A thiolase/3-hydroxy-3-methylglutaryl-coenzyme A reductase, a dual-function protein of isopentenyl diphosphate biosynthesis. *J Bacteriol* **2002,** *184* (8), 2116-22.

46. Steussy, C. N.; Robison, A. D.; Tetrick, A. M.; Knight, J. T.; Rodwell, V. W.; Stauffacher, C. V.; Sutherlin, A. L., A structural limitation on enzyme activity: the case of HMG-CoA synthase. *Biochemistry* **2006,** *45* (48), 14407-14.

47. Xiong, M.; Schneiderman, D. K.; Bates, F. S.; Hillmyer, M. A.; Zhang, K., Scalable production of mechanically tunable block polymers from sugar. *Proc Natl Acad Sci U S A* **2014,** *111* (23), 8357-62.

48. Otterstedt, K.; Larsson, C.; Bill, R. M.; Stahlberg, A.; Boles, E.; Hohmann, S.; Gustafsson, L., Switching the mode of metabolism in the yeast Saccharomyces cerevisiae. *EMBO Rep* **2004,** *5* (5), 532-7.

49. Hubmann, G.; Guillouet, S.; Nevoigt, E., Gpd1 and Gpd2 fine-tuning for sustainable reduction of glycerol formation in Saccharomyces cerevisiae. *Applied and environmental microbiology* **2011,** *77* (17), 5857-67.

50. Lascaris, R.; Bussemaker, H. J.; Boorsma, A.; Piper, M.; van der Spek, H.; Grivell, L.; Blom, J., Hap4p overexpression in glucose-grown Saccharomyces cerevisiae induces cells to enter a novel metabolic state. *Genome Biol* **2003,** *4* (1), R3.

51. Mittal, N.; Babu, M. M.; Roy, N., The efficiency of mitochondrial electron transport chain is increased in the long-lived mrg19 Saccharomyces cerevisiae. *Aging Cell* **2009,** *8* (6), 643-53.

52. Thomas, M. R.; O'Shea, E. K., An intracellular phosphate buffer filters transient fluctuations in extracellular phosphate levels. *Proc Natl Acad Sci U S A* **2005,** *102* (27), 9565-70.

53. Gray, J. V.; Petsko, G. A.; Johnston, G. C.; Ringe, D.; Singer, R. A.; Werner-Washburne, M., "Sleeping beauty": quiescence in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev* **2004,** *68* (2), 187-206.

54. Grilly, C.; Stricker, J.; Pang, W. L.; Bennett, M. R.; Hasty, J., A synthetic gene network for tuning protein degradation in Saccharomyces cerevisiae. *Mol Syst Biol* **2007,** *3*, 127.

55. Orth, J. D.; Thiele, I.; Palsson, B. O., What is flux balance analysis? *Nat Biotechnol* **2010,** *28* (3), 245-8.

56. Yim, H.; Haselbeck, R.; Niu, W.; Pujol-Baxley, C.; Burgard, A.; Boldt, J.; Khandurina, J.; Trawick, J. D.; Osterhout, R. E.; Stephen, R.; Estadilla, J.; Teisan, S.; Schreyer, H. B.; Andrae, S.; Yang, T. H.; Lee, S. Y.; Burk, M. J.; Van Dien, S., Metabolic engineering of Escherichia coli for direct production of 1,4-butanediol. *Nat Chem Biol* **2011,** *7* (7), 445-52.

57. Gupta, A.; Reizman, I. M.; Reisch, C. R.; Prather, K. L., Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. *Nature biotechnology* **2017,** *35* (3), 273-279.

58. Wang, J.; Yu, H. Q., Biosynthesis of polyhydroxybutyrate (PHB) and extracellular polymeric substances (EPS) by Ralstonia eutropha ATCC 17699 in batch cultures. *Appl Microbiol Biotechnol* **2007,** *75* (4), 871-8.

59. Xu, P.; Qiao, K.; Ahn, W. S.; Stephanopoulos, G., Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proc Natl Acad Sci U S A* **2016,** *113* (39), 10848-53.

60. Lynch, M. D.; Warnecke, T.; Gill, R. T. Method for Producing 3-Hydroxypropionic Acid and Other Products. Sept 8, 2011.

61. Qiao, K.; Wasylenko, T. M.; Zhou, K.; Xu, P.; Stephanopoulos, G., Lipid production in Yarrowia lipolytica is maximized by engineering cytosolic redox metabolism. *Nat Biotechnol* **2017**.

62. Jian, J.; Zhang, S. Q.; Shi, Z. Y.; Wang, W.; Chen, G. Q.; Wu, Q., Production of polyhydroxyalkanoates by Escherichia coli mutants with defected mixed acid fermentation pathways. *Appl Microbiol Biotechnol* **2010,** *87* (6), 2247-56.

63. Sharan, S. K.; Thomason, L. C.; Kuznetsov, S. G.; Court, D. L., Recombineering: a homologous recombination-based method of genetic engineering. *Nature protocols* **2009,** *4* (2), 206-23.

64. Li, X. T.; Thomason, L. C.; Sawitzke, J. A.; Costantino, N.; Court, D. L., Positive and negative selection using the tetA-sacB cassette: recombineering and P1 transduction in Escherichia coli. *Nucleic acids research* **2013,** *41* (22), e204.

65. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H., Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology* **2006,** *2*, 2006 0008.

66. van Dijken, J. P.; Bauer, J.; Brambilla, L.; Duboc, P.; Francois, J. M.; Gancedo, C.; Giuseppin, M. L. F.; Heijnen, J. J.; Hoare, M.; Lange, H. C.; Madden, E. A.; Niederberger, P.; Nielsen, J.; Parrou, J. L.; Petit, T.; Porro, D.; Reuss, M.; van Riel, N.; Rizzi, M.; Steensma, H. Y.; Verrips, C. T.; Vindeløv, J.; Pronk, J. T., An interlaboratory comparison of physiological and genetic properties of four Saccharomyces cerevisiae strains. *Enzyme and Microbial Technology* **2000,** *26* (9-10), 706-714.

67. Otterstedt, K.; Larsson, C.; Bill, R. M.; Stahlberg, A.; Boles, E.; Hohmann, S.; Gustafsson, L., Switching the mode of metabolism in the yeast Saccharomyces cerevisiae. *EMBO Rep* **2004,** *5* (5), 532-7.

68. Wieczorke, R.; Krampe, S.; Weierstall, T.; Freidel, K.; Hollenberg, C. P.; Boles, E., Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses inSaccharomyces cerevisiae. *FEBS Letters* **1999,** *464* (3), 123-128.

69. Gietz, R. D.; Schiestl, R. H., High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature protocols* **2007,** *2* (1), 31-4.

70. Stovicek, V.; Borodina, I.; Forster, J., CRISPR–Cas system enables fast and simple genome editing of industrial Saccharomyces cerevisiae strains. *Metabolic Engineering Communications* **2015,** *2*, 13-22.

71. Labun, K.; Montague, T. G.; Gagnon, J. A.; Thyme, S. B.; Valen, E., CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic acids research* **2016,** *44* (W1), W272-6.

72. Hoffman, C. S.; Winston, F., A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformaion of Escherichia coli. *Gene* **1987,** *57* (2-3), 267-272.

73. Luo, M. L.; Mullis, A. S.; Leenay, R. T.; Beisel, C. L., Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression. *Nucleic acids research* **2015,** *43* (1), 674-81.

74. Davis, J. H.; Rubin, A. J.; Sauer, R. T., Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic acids research* **2011,** *39* (3), 1131-41.

75. Smith, J. D.; Suresh, S.; Schlecht, U.; Wu, M.; Wagih, O.; Peltz, G.; Davis, R. W.; Steinmetz, L. M.; Parts, L.; St Onge, R. P., Quantitative CRISPR interference screens in yeast

identify chemical-genetic interactions and new rules for guide RNA design. *Genome Biol* **2016,** *17*, 45.

76. Gilbert, L. A.; Larson, M. H.; Morsut, L.; Liu, Z.; Brar, G. A.; Torres, S. E.; Stern-Ginossar, N.; Brandman, O.; Whitehead, E. H.; Doudna, J. A.; Lim, W. A.; Weissman, J. S.; Qi, L. S., CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **2013,** *154* (2), 442-51.

77. Sikorski, R. S.; Hieter, P., A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **1989,** *122* (1), 19-27.

78. Duetz, W. A.; Ruedi, L.; Hermann, R.; O'Connor, K.; Buchs, J.; Witholt, B., Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Applied and environmental microbiology* **2000,** *66* (6), 2641-6.

79. Duetz, W. A.; Witholt, B., Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. *Biochem Eng J* **2001,** *7* (2), 113-115.

80. Lindemann, C. J.; Singh, M. M.; Ramjit, H. G.; Bell, C.; Ip, D. P., Determination of mevalonolactone in capsules by capillary gas-liquid chromatography. *J Pharm Biomed Anal* **1991,** *9* (4), 311-6.

81. Keseler, I. M.; Mackie, A.; Peralta-Gil, M.; Santos-Zavaleta, A.; Gama-Castro, S.; Bonavides-Martinez, C.; Fulcher, C.; Huerta, A. M.; Kothari, A.; Krummenacker, M.; Latendresse, M.; Muniz-Rascado, L.; Ong, Q.; Paley, S.; Schroder, I.; Shearer, A. G.; Subhraveti, P.; Travers, M.; Weerasinghe, D.; Weiss, V.; Collado-Vides, J.; Gunsalus, R. P.; Paulsen, I.; Karp, P. D., EcoCyc: fusing model organism databases with systems biology. *Nucleic acids research* **2013,** *41* (Database issue), D605-12.

82. Davis, J. H.; Rubin, A. J.; Sauer, R. T., Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic acids research* **2011,** *39* (3), 1131-41.

83. Poo, H.; Song, J. J.; Hong, S.-P.; Choi, Y.-H.; Yun, S. W.; Kim, J.-H.; Lee, S. C.; Lee, S.-G.; Sung, M. H., Novel high-level constitutive expression system, pHCE vector, for a convenient and cost-effective soluble production of human tumor necrosis factor-α. *Biotechnology Letters* **2002,** *24* (14), 1185-1189.

84. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H., Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2006,** *2*, 2006 0008.

85. Jian, J.; Zhang, S. Q.; Shi, Z. Y.; Wang, W.; Chen, G. Q.; Wu, Q., Production of polyhydroxyalkanoates by Escherichia coli mutants with defected mixed acid fermentation pathways. *Appl Microbiol Biotechnol* **2010,** *87* (6), 2247-56.

86. van Dijken, J. P.; Bauer, J.; Brambilla, L.; Duboc, P.; Francois, J. M.; Gancedo, C.; Giuseppin, M. L. F.; Heijnen, J. J.; Hoare, M.; Lange, H. C.; Madden, E. A.; Niederberger, P.; Nielsen, J.; Parrou, J. L.; Petit, T.; Porro, D.; Reuss, M.; van Riel, N.; Rizzi, M.; Steensma, H.

Y.; Verrips, C. T.; Vindeløv, J.; Pronk, J. T., An interlaboratory comparison of physiological and genetic properties of four Saccharomyces cerevisiae strains. *Enzyme and Microbial Technology* **2000,** *26* (9-10), 706-714.

87. Otterstedt, K.; Larsson, C.; Bill, R. M.; Stahlberg, A.; Boles, E.; Hohmann, S.; Gustafsson, L., Switching the mode of metabolism in the yeast Saccharomyces cerevisiae. *EMBO Rep* **2004,** *5* (5), 532-7.

88. Luo, M. L.; Mullis, A. S.; Leenay, R. T.; Beisel, C. L., Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression. *Nucleic acids research* **2015,** *43* (1), 674-81.

89. Wilde, R. J.; Guest, J. R., Transcript analysis of the citrate synthase and succinate dehydrogenase genes of Escherichia coli K12. *J Gen Microbiol* **1986,** *132* (12), 3239-51.

90. Charpentier, B.; Branlant, C., The Escherichia coli gapA gene is transcribed by the vegetative RNA polymerase holoenzyme E sigma 70 and by the heat shock RNA polymerase E sigma 32. *Journal of Bacteriology* **1994,** *176* (3), 830-839.

91. Li, X. T.; Thomason, L. C.; Sawitzke, J. A.; Costantino, N.; Court, D. L., Positive and negative selection using the tetA-sacB cassette: recombineering and P1 transduction in Escherichia coli. *Nucleic acids research* **2013,** *41* (22), e204.

92. Duetz, W. A.; Ruedi, L.; Hermann, R.; O'Connor, K.; Buchs, J.; Witholt, B., Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Applied and environmental microbiology* **2000,** *66* (6), 2641-6.

93. Duetz, W. A.; Witholt, B., Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. *Biochem Eng J* **2001,** *7* (2), 113-115.

[00254] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

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

providing a genetically modified microorganism for producing a product;

growing the provided genetically modified microorganism in a media in a growth phase, the genetically modified microorganism comprising:

i. a production pathway comprising at least one production enzyme for biosynthesis of the product; and

ii. one or more synthetic metabolic valves for reducing or eliminating flux through multiple metabolic pathways within the GMO when the synthetic metabolic valves are induced, the one or more synthetic metabolic valves comprising:

a) at least one silencing SMV that silences gene expression of a gene encoding an enzyme, or

b) at least one proteolytic SMV that controls proteolysis of an enzyme;

transitioning to a productive stationary phase wherein, compared to a microorganism lacking the production pathway or the one or more synthetic metabolic valves, the growth of the genetically modified microorganism is slowed or stopped and product production is enhanced, the productive stationary phase being entered by at least inducing the one or more synthetic metabolic valves; and

producing the product.

2. The multi-stage fermentation bioprocess of claim 1, wherein the product is selected from the group consisting of an amino acid, acetate, acetoin, acetone, acrylic, malate, fatty acid ethyl esters, 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, fused alcohols, and 1,2-propanediol, 1,3-propanediol, formate, fumaric acid, propionic acid, succinic acid, valeric acid, maleic acid and poly-hydroxybutyrate.

3. The multi-stage fermentation bioprocess of claim 1, wherein:

the enzyme that is silenced by the silencing metabolic valve and the enzyme subject to proteolysis by the proteolytic synthetic metabolic valve are each encoded by a gene selected from the group consisting of: fabI, gltA, ldp, zwf, and udhA; or

the silencing synthetic metabolic valve is characterized by CRISPR interference with gene expression and expression of a CASCADE plasmid comprising an array of guide RNA genes; or

the proteolytic synthetic metabolic valve is characterized by expression of the proteolyzed enzyme operably linked to a N-terminal or C-terminal tag and controlled proteolysis is selective for enzymes comprising the N-terminal or C-terminal tag upon induction of the synthetic metabolic valve.

4. The multi-stage fermentation bioprocess of claim 1, wherein the step of providing a genetically modified microorganism for producing a product comprises:

providing a plurality of distinct genetically modified microorganisms, each genetically modified microorganism therein comprising:

i. a production pathway comprising at least one production enzyme for biosynthesis of a product; and

ii. a combination of one or more synthetic metabolic valves for reducing or eliminating flux through multiple metabolic pathways within the genetically modified microorganism when the synthetic metabolic valve is induced, the synthetic metabolic valve comprising:

a) a silencing SMV that silences gene expression of a gene encoding an enzyme, or

b) a proteolytic SMV that controls proteolysis of an enzyme,

wherein each of the plurality of genetically modified microorganisms is distinct therein by one or more of the production enzyme, the enzyme to be silenced or the enzyme to be proteolyzed;

independently growing each of the plurality of genetically modified microorganisms in culture media in a growth phase;

transitioning to a productive stationary phase where growth of at least one of the plurality of genetically modified microorganisms is slowed or stopped and product production is enhanced, as compared to a microorganism lacking the production pathway or synthetic metabolic valve, the productive stationary phase being entered by at least inducing the synthetic metabolic valve;

producing the product;

measuring the level of product produced in the production phase for each of the plurality of genetically modified microorganisms after a period of time, and

selecting a genetically modified microorganism to be provided for a fermentation process based on the level of product produced.

5. The multi-stage fermentation bioprocess of claim 1, wherein:

the product is alanine;

the production enzyme or a promotor sequence of a gene operably linked to a gene encoding the production enzyme is heterologous to the species from which the genetically modified microorganism is derived; and

a heterologous enzyme of alanine production is one of: NADPH-dependent alanine dehydrogenase (ald), alanine exporter (alaE), glyceraldehyde-3-phosphate dehydrogenase (gapN) or a combination thereof.

6. The multi-stage fermentation bioprocess of claim 1, wherein:

the product is mevalonate or 3-HP;

the production enzyme or a promotor sequence operably linked to a gene encoding the production enzyme is heterologous to the species from which the genetically modified microorganism is derived; and

a heterologous enzyme of mevalonate or 3-HP production is one of: an NADPH dependent 3-hydroxypropionic dehydrogenase, an acetyl-CoA acetyltransferase, NADPH dependent HMG-CoA reductase, HMG-CoA synthase or a combination thereof.

7. A genetically modified microorganism strain comprising:

i. a production pathway comprising at least one production enzyme for biosynthesis of a product; and

ii. a combination of one or more synthetic metabolic valves capable of reducing or eliminating flux through multiple metabolic pathways within the genetically modified microorganism when the one more synthetic metabolic valves are induced, the one or more synthetic metabolic valves comprising:

a) at least one silencing synthetic metabolic valve that silences gene expression of a gene encoding an enzyme, or

b) at least one proteolysis synthetic metabolic valve that controls proteolysis of an enzyme; and

wherein growth of the genetically modified microorganism is slowed or stopped and product production is enhanced by inducing the synthetic metabolic valve, and

wherein at least one enzyme that is silenced by the silencing metabolic valve and at least one enzyme that is controlled by the proteolytic metabolic valve are each encoded by a gene selected from the group consisting of: fabI, gltA, ldp, zwf, and udhA.

8. The genetically modified microorganism strain of claim 7, wherein the product is alanine;

the enzyme of the production pathway is one of: NADPH-dependent alanine dehydrogenase (ald), alanine exporter (alaE), glyceraldehyde-3-phosphate dehydrogenase (gapN) or a combination thereof; and

the synthetic metabolic valve comprises a combination of silencing of gene expression of fabI, gltA1 or gltA2 and controlled proteolysis of fabI or udhA.

9. The genetically modified microorganism of claim 7, wherein the product is mevalonate or 3-HP;

the enzyme of the production pathway is one of: an NADPH dependent 3-hydroxypropionic dehydrogenase, an acetyl-CoA acetyltransferase, NADPH dependent HMG-CoA reductase, HMG-CoA synthase or a combination thereof; and

the synthetic metabolic valve comprises a combination of silencing of gene expression of fabI, gltA1 or gltA2 and controlled proteolysis of fabI or udhA.

10. A plurality of about 50 to about 500 genetically modified microorganism strains of claim 7, wherein each of the plurality of genetically modified microorganisms is distinct therein by one or more of the production enzyme, the enzyme to be silenced or the enzyme to be proteolyzed.