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(72) **Inventeurs/Inventors:**
SUN, JUN, US;
PHARKYA, PRITI, US;
BURGARD, ANTHONY P., US

(73) **Propriétaire/Owner:**
GENOMATICA, INC., US

(74) **Agent:** SMART & BIGGAR LLP

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(57) **Abrégé/Abstract:**
The invention provides a non-naturally occurring microbial organism having a microbial organism having at least one exogenous gene insertion and/or one or more gene disruptions that confer production of primary alcohols. A method for producing long chain alcohols includes culturing these non-naturally occurring microbial organisms.

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(71) Applicant *(for all designated States except US)*: **GENOMATICA, INC.** [US/US]; 10520 Wateridge Circle, San Diego, CA 92121 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SUN, Jun** [CN/US]; 8274 Bryn Glen Way, San Diego, CA 92129 (US). **PHARKYA, Priti** [IN/US]; 11125 Caminito Alvarez, San Diego, CA 92126 (US). **BURGARD, Anthony, P.** [US/US]; 237 Walker Crossing, Bellefonte, PA 16823 (US).

(74) Agents: **GAY, David, A.** et al.; McDERMOTT, WILL & EMERY LLP, 11682 El Camino Real, Suite 400, San Diego, CA 92130-2047 (US).

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(54) Title: PRIMARY ALCOHOL PRODUCING ORGANISMS

(57) Abstract: The invention provides a non-naturally occurring microbial organism having a microbial organism having at least one exogenous gene insertion and/or one or more gene disruptions that confer production of primary alcohols. A method for producing long chain alcohols includes culturing these non-naturally occurring microbial organisms.

PRIMARY ALCOHOL PRODUCING ORGANISMS

BACKGROUND

This disclosure relates generally to biosynthetic processes and, more specifically to organisms having primary alcohol biosynthetic capability.

Primary alcohols are a product class of compounds having a variety of industrial applications which include a variety of biofuels and specialty chemicals. Primary alcohols also can be used to make a large number of additional industrial products including polymers and surfactants. For example, higher primary alcohols (C_4-C_{20}) and their ethoxylates are used as surfactants in many consumer detergents, cleaning products and personal care products worldwide such as laundry powders and liquids, dishwashing liquid and hard surface cleaners. They are also used in the manufacture of a variety of industrial chemicals and in lubricating oil additives. Long-chain primary alcohols, such as octanol and hexanol, have useful organoleptic properties and have long been employed as fragrance and flavor materials. Smaller chain (C4-C8) higher primary alcohols (e.g., butanol) are used as chemical intermediates for production of derivatives such as acrylates used in paints, coatings, and adhesives applications.

Primary alcohols are currently produced from, for example, hydrogenation of fatty acids, hydroformylation of terminal olefins, partial oxidation of n-paraffins and the Al- catalyzed polymerization of ethylene. Unfortunately, it is not commercially viable to produce primary alcohols directly from the oxidation of petroleum-based linear hydrocarbons (n-paraffins). This impracticality is because the oxidation of n-paraffins produces primarily secondary alcohols, tertiary alcohols or ketones, or a mixture of these compounds, but does not produce high yields of primary alcohols. Additionally, currently known methods for producing primary alcohols suffer from the disadvantage that they are restricted to feedstock which is relatively expensive, notably ethylene, which is produced via the thermal cracking of petroleum. In addition, current methods require several steps, and several catalyst types.

LCA production by microorganisms involves fatty acid synthesis followed by acyl-reduction steps. The universal fatty acid biosynthesis pathway found in most cells has been investigated for production of LCAs and other fatty acid derivatives. There is currently a great deal of improvement that can be achieved to provide more efficient biosynthesis pathways for LCA production with significantly higher theoretical product and energy yields.

Thus, there exists a need for alternative means for effectively producing commercial quantities of primary alcohols. The present invention satisfies this need and provides related advantages as well.

SUMMARY

In some aspects, embodiments disclosed herein relate to a non-naturally occurring microbial organism having a microbial organism having a malonyl-CoA-independent fatty acid synthesis (FAS) pathway and an acyl-reduction pathway having at least one exogenous nucleic acid encoding a malonyl-CoA-independent FAS pathway enzyme expressed in sufficient amounts to produce a primary alcohol, the malonyl-CoA-independent FAS pathway having ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase, the acyl-reduction pathway having an acyl-CoA reductase and an alcohol dehydrogenase.

In other aspects, embodiments disclosed herein relate to a method for producing a primary alcohol. The method includes culturing a non-naturally occurring microbial organism have having a malonyl-CoA-independent fatty acid synthesis (FAS) pathway and an acyl-reduction pathway having at least one exogenous nucleic acid encoding a malonyl-CoA-independent FAS pathway enzyme expressed in sufficient amounts to produce a primary alcohol under substantially anaerobic conditions for a sufficient period of time to produce the primary alcohol, the malonyl-CoA-independent FAS pathway having ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase, the acyl-reduction pathway having an acyl-CoA reductase and an alcohol dehydrogenase.

In some aspects, embodiments disclosed herein relate to a non-naturally occurring microbial organism that includes one or more gene disruptions occurring in genes encoding enzymes that couple long-chain alcohol (LCA) production to growth of the non-naturally occurring microbial organism. In other embodiments, LCA production can be accomplished during non-growth phases using the same disruption strategies. The one or more gene disruptions reduce the activity of the enzyme, whereby the gene disruptions confer production of LCA onto the non-naturally occurring microbial organism.

In other aspects, embodiments disclosed herein relate to a method for producing LCA that includes culturing a non-naturally occurring microbial organism having one or more gene disruptions. The one or more gene disruptions occur in genes encoding an enzyme that confers LCA production in the organism.

In some aspects, embodiments disclosed herein relate to a non-naturally occurring eukaryotic organism, that includes one or more gene disruptions. The one or more gene disruptions occur in genes that encode

enzymes such as a cytosolic pyruvate decarboxylase, a mitochondrial pyruvate dehydrogenase, a cytosolic ethanol-specific alcohol dehydrogenase and a mitochondrial ethanol-specific alcohol dehydrogenase. These disruptions confer production of long chain alcohols in the cytosol of the organism.

In some aspects, embodiments disclosed herein relate to a non-naturally occurring eukaryotic organism that includes one or more gene disruptions. The one or more gene disruptions occur in genes encoding enzymes such as a cytosolic pyruvate decarboxylase, a cytosolic ethanol-specific alcohol dehydrogenase, and a mitochondrial ethanol-specific alcohol dehydrogenase. These disruptions confer production of long chain alcohols in the mitochondrion of said organism.

In other aspects, embodiments disclosed herein relate to a method for producing long chain alcohols, including culturing these non-naturally occurring eukaryotic organisms.

The claimed invention relates to a microbial organism comprising: (i) a first set of heterologous nucleic acids encoding a malonyl-CoA-independent fatty acid synthesis (FAS) pathway, said malonyl-CoA-independent FAS pathway comprising one or more malonyl-CoA-independent FAS pathway enzymes selected from ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase expressed from said first set of heterologous nucleic acids; and (ii) a second set of heterologous nucleic acids encoding an acyl-reduction pathway expressed in sufficient amounts to produce primary alcohol selected from the group consisting of hexanol, heptanol, octanol, nonanol, decanol, dodecanol, tetradecanol and hexadecanol; wherein said acyl-reduction pathway comprises one or more acyl-reduction pathway enzymes having the activity of one or both of an acyl-CoA reductase and an alcohol dehydrogenase expressed from said second set of heterologous nucleic acids; wherein said ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase converts acyl-CoA to β-ketoacyl-CoA, wherein said 3-hydroxyacyl-CoA dehydrogenase converts β-ketoacyl-CoA to β-hydroxyacyl-CoA, wherein said enoylCoA hydratase converts β-hydroxyacyl-CoA to trans-2-enoyl-CoA, wherein said enoyl-CoA reductase converts trans-2-enoyl-CoA to acyl-CoA; and wherein said acyl-CoA reductase converts acyl-CoA to an aldehyde and wherein said alcohol dehydrogenase converts an aldehyde to said primary alcohol.

Aspects of the disclosure also relate to a microbial organism comprising heterologous nucleic acids encoding a malonyl-CoA-independent fatty acid synthesis (FAS) pathway comprising at least one malonyl-CoA-independent FAS pathway enzyme expressed in sufficient amounts to produce a fatty acyl-CoA, wherein the fatty acid chain of said fatty acyl-CoA has 6-10, 12, 14 or 16 carbon atoms, said

malonyl-CoA-independent FAS pathway comprising ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase, wherein said 3-hydroxyacyl-CoA dehydrogenase converts β-ketoacyl-CoA to β-hydroxyacyl-CoA, wherein said enoyl CoA hydratase converts β-hydroxyacyl-CoA to trans-2-enoyl-CoA, wherein said enoyl-CoA reductase converts trans-2-enoyl-CoA to acyl-CoA. Also disclosed is a composition comprising such an organism and a substantially anaerobic culture medium. Also disclosed is a method for producing a fatty acyl-CoA comprising culturing such an organism, wherein the malonyl-CoA-independent fatty acid synthesis (FAS) pathway enzyme is expressed in sufficient amounts to produce said fatty acyl-CoA having a fatty acid chain of said fatty acyl-CoA has 6-10, 12, 14 or 16 carbon atoms under anaerobic conditions for a sufficient period of time to produce said fatty acyl-CoA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the malonyl-CoA-independent fatty acid synthesis and reduction (MI-LCA) pathway to produce LCAs.

Figure 2 shows the contrasted hypothetical production envelopes of an OptKnock-designed strain against a typical non-growth-coupled production strain. The potential evolutionary trajectories of the OptKnock strain lead to a high producing phenotype.

Figure 3 shows the growth-coupled LCA production characteristics of strain design I (alternating dotted and dashed) compared with those of wild-type *E. coli* (black). A glucose uptake rate of 10 mmol/gDW/hr is assumed.

Figure 4 shows the growth-coupled LCA production characteristics of strain designs II (alternating dotted and dashed), III-V (dashed), and VI-XI (dotted) compared with those of wild-type *E. coli* (black). A glucose uptake rate of 10 mmol/gDW/hr is assumed.

Figure 5 shows the growth-coupled LCA production characteristics of strain designs XII (alternating dotted and dashed) and XIII-XV (dashed) compared with those of wild-type *E. coli* (black). A glucose uptake rate of 10 mmol/gDW/hr is assumed.

Figure 6 shows the growth-coupled LCA production characteristics of strain designs XVI-XVIII (alternating dotted and dashed) and XIX-XXI (dashed) compared with those of wild-type *E. coli* (black). A glucose uptake rate of 10 mmol/gDW/hr is assumed.

Figure 7 shows the growth-coupled LCA production characteristics of Designs I (alternating dotted and dashed), V (dashed), and V_A (dotted) compared with those of wild-type *E. coli* (black). A glucose uptake rate of 10 mmol/gDW/hr is assumed. Point A refers to the dodecanol production rate at maximum growth of a strain engineered according to design V_A and point B refers to the minimal dodecanol production rate required for growth.

Figure 8 shows the growth-coupled LCA production characteristics of Designs I (alternating dotted and dashed, XII (long dashed), XII_A (short dashed), and XII_B (dotted) compared with those of wild-type *E. coli* (black). A glucose uptake rate of 10 mmol/gDW/hr is assumed. Point A refers to the dodecanol production rate at maximum growth of a strain engineered according to design XII_B and point B refers to the minimal dodecanol production rate required for growth.

Figure 9a shows the formation of dodecanol in the cytosol by relying on the AMP-forming acetyl CoA synthetase for the formation of acetyl CoA for dodecanol production. The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 9b shows the growth-coupled production envelopes for the production of dodecanol in *S. cerevisiae* in the scenario where acetyl CoA synthetase is used for acetyl CoA production in the cytosol. The black curve shows the production envelope for the wild-type network under aerobic conditions, and the dark gray curve shows the growth-coupled production characteristics for the mutant network. A glucose uptake rate of 10 mmol/gDCW.hr is assumed.

Figure 10a shows the formation of dodecanol in the cytosol by relying on the ADP-forming acetate CoA ligase for the formation of acetyl CoA for dodecanol production. The gray arrow represents the addition of a heterologous enzyme. The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 10b shows the growth-coupled production envelopes for the production of dodecanol in *S. cerevisiae* in the scenario where acetate CoA ligase is employed for acetyl-CoA production in the cytosol. The black curve shows the production envelope for the wild-type network under aerobic conditions. The light gray curve shows the increase in feasible space after acetate CoA ligase is added to the network and the dark gray curve shows the growth-coupled production characteristics for the mutant network in the presence of oxygen. A glucose uptake rate of 10 mmol/gDCW.hr is assumed.

Figure 11a shows the formation of dodecanol in the cytosol by relying on the acylating acetaldehyde dehydrogenase for the formation of acetyl CoA for dodecanol production. The gray arrow shows a heterologous enzyme. The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 11b shows the growth-coupled production envelopes for the anaerobic production of dodecanol in *S. cerevisiae*. The black curve shows the production capabilities for the wild-type network, the light gray dotted curve shows the production characteristics when acylating acetaldehyde dehydrogenase is added to the network and the dark gray curve shows the growth-coupling when alcohol dehydrogenase is deleted from the augmented network. Note the increase in the theoretical maximum when acylating acetaldehyde dehydrogenase is functional. A glucose uptake rate of 10 mmol/gDCW.hr is assumed.

Figure 12 shows the formation of dodecanol in the cytosol by relying on a cytosolic pyruvate dehydrogenase for acetyl CoA and NADH production. This can be accomplished by introducing a heterologous cytosolic enzyme (shown in gray) or by retargeting the native mitochondrial enzyme to the cytosol. The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 13 shows the formation of dodecanol in the cytosol by relying on a cytosolic pyruvate:NADP oxidoreductase for acetyl CoA and NADH production. This can be accomplished by introducing a heterologous enzyme in the cytosol (shown in gray). The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 14 shows the formation of dodecanol in the cytosol by the introduction of a heterologous pyruvate formate lyase (shown in gray) in the cytosol. The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 15a shows the formation of dodecanol in the mitochondrion by using the pyruvate dehydrogenase for the formation of acetyl-CoA. The dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 15b shows the growth-coupled production envelopes for the production of dodecanol in *S. cerevisiae* mitochondrion. The black curve shows the production capabilities for the wild-type network under anaerobic conditions and the dark gray curve shows the production characteristics in the absence of oxygen when pyruvate decarboxylase is deleted from the network. A glucose uptake rate of 10 mmol/gDCW.hr is assumed.

Figure 16 shows the formation of dodecanol in the mitochondrion by using the pyruvate:NADP oxidoreductase for formation of acetyl CoA. The gray arrow shows the heterologous enzyme and the dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 17 shows the formation of dodecanol in the mitochondrion by using the pyruvate formate lyase for formation of acetyl CoA. The gray arrow shows the heterologous enzyme and the dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 18 shows the formation of dodecanol in the mitochondrion by adding the mitochondrial acylating acetaldehyde dehydrogenase. The gray arrow shows the heterologous enzyme(s) and the dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 19a shows the formation of dodecanol in the mitochondrion by using the acetyl CoA synthetase for formation of acetyl CoA. The gray arrow shows the heterologous enzyme(s) and the dotted arrows depict the flow of the majority of the carbon flux in this production scenario.

Figure 19b shows the growth-coupled production envelopes for the production of dodecanol in *S. cerevisiae* mitochondrion when acetyl-CoA is formed through the mitochondrial acetyl-CoA synthetase. The black curve shows the production envelope for the wild-type network under aerobic conditions, the light dark gray curve shows the production characteristics when the deletions have been imposed upon the network. The growth coupling can be improved further (dark gray curve) when flux through the oxidative part of the pentose phosphate pathway is decreased. A glucose uptake rate of 10 mmol/gDCW.hr is assumed.

Figure 20 shows the formation of dodecanol in the mitochondrion by using the acetate CoA ligase for formation of acetyl CoA. The gray arrows show the heterologous enzyme(s) and the dotted arrows depict the flow of the majority of the carbon flux in this production scenario

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed, in part, to recombinant microorganisms capable of synthesizing the primary alcohols using a malonyl-CoA-independent fatty acid synthesis and reduction pathway. The modified microorganisms of the invention also are capable of secreting the resultant primary alcohol into the culture media or fermentation broth for further manipulation or isolation. Recombinant microorganisms of the invention can be engineered to produce commercial quantities of a variety of different primary alcohols having different chain lengths between 4 (C4) and 24 (C24) or more carbon atoms. Production of primary alcohols through the modified pathways of the invention is particularly useful because it results in higher product and ATP yields than through naturally occurring biosynthetic pathways such as the well-documented malonyl-CoA dependent fatty acid synthesis pathway. Using acetyl-CoA as a C2 extension unit instead of malonyl-acyl carrier protein (malonyl-ACP) saves one ATP molecule per unit flux of acetyl-CoA entering the elongation cycle. The elongation cycle results in acyl-CoA instead of acyl-ACP, and precludes the need of the ATP-consuming acyl-CoA synthase reactions for the production of octanol and other primary alcohols. The primary alcohol producing organisms of the invention can additionally allow the use of biosynthetic processes to convert low cost renewable feedstock for the manufacture of chemical products.

In one specific embodiment, the invention utilizes a heterologous malonyl-CoA-independent fatty acid synthesis pathway coupled with an acyl-CoA reduction pathway to form primary alcohol species. The coupling of these two pathways will convert a carbon or energy source into acetyl-CoA, which is used as both primer and extension unit in biosynthetic elongation cycle. The elongation cycle includes ketoacyl-CoA thiolase (or ketoacyl-CoA acyltransferase), 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase. Each cycle results in the formation of an acyl-CoA extended by one C2 unit compared to the acyl-CoA substrate entering the elongation cycle. Carbon chain-length of the primary alcohols can be controlled by chain-length specific enoyl-CoA reductase, ketoacyl-CoA thiolase and/or acyl-CoA reductase. Acyl-CoA products with desired chain-lengths are funneled into a reduction pathway and reduced through the combination of acyl-CoA reductase and alcohol dehydrogenase or the fatty alcohol forming acyl-CoA reductase to form desired primary alcohol. These

reduction steps serve as another mechanism for control of chain length, for example, through the use of chain-length specific acyl-CoA reductases.

As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes within a malonyl-CoA-independent fatty acid biosynthetic pathway and enzymes within an acyl-reduction pathway.

As used herein, the term "isolated" when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally occurring environments. Specific examples of isolated microbial organisms include partially pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

As used herein, the terms "microbial," "microbial organism" or "microorganism" is intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

As used herein, the term "primary alcohol" is intended to mean an alcohol which has the hydroxyl radical connected to a primary carbon. The term includes an alcohol that possesses the group $-CH_2OH$ which can be oxidized so as to form a corresponding aldehyde and acid having the same number of carbon atoms. Alcohols include any of a series of hydroxyl compounds, the simplest of which are derived from saturated hydrocarbons, have the general formula $C_nH_{2n+1}OH$, and include ethanol and methanol. Exemplary primary alcohols include butanol, hexanol, heptanol, octanol, nananol, decanol, dodecanol, tetradecanol, and hexadecanol.

As used herein, the term "CoA" or "coenzyme A" is intended to mean an organic cofactor or prosthetic group (nonprotein portion of an enzyme) whose presence is required for the activity of many enzymes (the apoenzyme) to form an active enzyme system. Coenzyme A functions, for example, in certain condensing enzymes, acts in acetyl or other acyl group transfer and in fatty acid synthesis and oxidation.

As used herein, the term "substantially anaerobic" when used in reference to a culture or growth condition is intended to mean that the amount of oxygen is less than about 10% of saturation for dissolved oxygen in liquid media. The term also is intended to include sealed chambers of liquid or solid medium maintained with an atmosphere of less than about 1% oxygen.

"Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acids that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

As used herein, the term "growth-coupled" when used in reference to the production of a biochemical is intended to mean that the biosynthesis of the referenced biochemical is a product produced during the growth phase of a microorganism. "Non-growth-coupled when used in reference to the production of a biochemical is intended to mean that the biosynthesis of the referenced biochemical is a product produced during a non-growth phase of a microorganism. Production of a biochemical product can be optionally obligatory to the growth of the organism.

As used herein, the term "metabolic modification" is intended to refer to a biochemical reaction that is altered from its naturally occurring state. Metabolic modifications can include, for example, elimination of a biochemical reaction activity by functional disruptions of one or more genes encoding an enzyme participating in the reaction. Sets of exemplary metabolic modifications are illustrated in Table 1. Individual reactions specified by such metabolic modifications and their corresponding gene complements are exemplified in Table 2 for *Escherichia coli*. Reactants and products utilized in these reactions are exemplified in Table 3.

As used herein, the term "gene disruption," or grammatical equivalents thereof, is intended to mean a genetic alteration that renders the encoded gene product inactive. The genetic alteration can be, for example, deletion of the entire gene, deletion of a regulatory sequence required for transcription or translation, deletion of a portion of the gene with results in a truncated gene product or by any of various mutation strategies that inactivate the encoded gene product. One particularly useful method of gene disruption is complete gene deletion because it reduces or eliminates the occurrence of genetic reversions in the non-naturally occurring microorganisms of the invention. The term "gene disruption" is also intended to mean a genetic alteration that lowers the activity of a given gene product relative to its activity in a wild-type organism. This attenuation of activity can be due to, for example, a deletion in a portion of the gene which results in a truncated gene product or any of various mutation strategies that render the encoded gene product less active than its natural form, replacement or mutation of the promoter sequence leading to lower or less efficient expression of the gene, culturing the organism under a condition where the gene is less highly expressed than under normal culture conditions, or introducing antisense RNA molecules that interact with complementary mRNA molecules of the gene and alter its expression.

As used herein, the term "stable" when used in reference to growth-coupled production of a biochemical product is intended to refer to microorganism that can be cultured for greater than five generations without loss of the coupling between growth and biochemical synthesis. Generally, stable growth-coupled biochemical production will be greater than 10 generations,

particularly stable growth-coupled biochemical production will be greater than about 25 generations, and more particularly, stable growth-coupled biochemical production will be greater than 50 generations, including indefinitely. Stable growth-coupled production of a biochemical can be achieved, for example, by disruption of a gene encoding an enzyme catalyzing each reaction within a set of metabolic modifications. The stability of growth-coupled production of a biochemical can be enhanced through multiple disruptions, significantly reducing the likelihood of multiple compensatory reversions occurring for each disrupted activity.

Those skilled in the art will understand that the metabolic modifications exemplified herein are described with reference to *Escherichia* coli and *Saccharomyces cerevisae* genes and their corresponding metabolic reactions. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *Escherichia coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous gene disruptions in the other species. Such disruptions can include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

As used herein, the term "confers production" refers not only to organisms that lack operational metabolic pathways for the production of LCAs, but also to organisms that may have some level of production of LCAs. Thus, an organism that already generates LCAs can benefit from improved production conferred onto the organism by the disruption of one or more genes.

As used herein, the term "eukaryotic organism" refers to any organism having a cell type having specialized organelles in the cytoplasm and a membrane-bound nucleus enclosing genetic material organized into chromosomes. The term is intended to encompass all eukaryotic organisms including eukaryotic microbial organisms such as yeast and fungi. The term also includes cell cultures of any eukaryotic species that can be cultured for the production of a biochemical where the eukaryotic species need not be a microbial organism. A "eukaryotic microbial organism," "microbial organism" or "microorganism" is intended to mean any eukaryotic organism that exists as a microscopic cell that is included within the domain of eukarya.

An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less that 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities. Members of the serine protease family of enzymes, including tissue plasminogen activator and elastase, are considered to have arisen by vertical descent from a common ancestor.

Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding products are considered to be orthologs. For the growth-coupled production of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species. A specific example is the separation of elastase proteolysis and plasminogen proteolysis, two types of serine protease activity, into distinct molecules as plasminogen activator and elastase. A second example is the separation of mycoplasma 5'-3' exonuclease and Drosophila DNA polymerase III activity. The DNA polymerase from the first species can be considered an ortholog to either or both of the exonuclease or the polymerase from the second species and vice versa.

In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved from a

common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene compared to a gene encoding the function sought to be substituted. Therefore, a nonorthologous gene includes, for example, a paralog or an unrelated gene.

Therefore, in identifying and constructing the non-naturally occurring microbial organisms of the invention having growth-coupled production of a biochemical, those skilled in the art will understand applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications should include identification and disruption of orthologs. To the extent that paralogs and/or nonorthologous gene displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can disrupt these evolutionally related genes to ensure that any functional redundancy in enzymatic activities do not short circuit the designed metabolic modifications.

Orthologs, paralogs and nonorthologous gene displacements can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is sufficiently high to indicate the proteins are related through evolution from a common ancestor. Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compared and determine a raw sequence similarity or identity, and also determine the presence or significance of gaps in the sequence which can be assigned a weight or

score. Such algorithms also are known in the art and are similarly applicable for determining nucleotide sequence similarity or identity. Parameters for sufficient similarly to determine relatedness are computed based on well known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 25% to 100% sequence identity. Proteins that are unrelated can have an identity which is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%). Sequences between 5% and 24% may or may not represent sufficient homology to conclude that the compared sequences are related. Additional statistical analysis to determine the significance of such matches given the size of the data set can be carried out to determine the relevance of these sequences.

Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan-05-1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sept-16-1998) and the following parameters: Match: 1; mismatch: -2; gap open: 5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off. Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

The non-naturally occurring microbial organisms of the invention can contain stable genetic alterations, which refer to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein are described with reference to *Euglena gracilis*, *E. coli* and *S. cerevisiae* genes and their corresponding metabolic reactions. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance

provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

In some embodiments, the invention provides a non-naturally occurring microbial organism having a malonyl-CoA-independent fatty acid synthesis (FAS) pathway and an acyl-reduction pathway having at least one exogenous nucleic acid encoding a malonyl-CoA-independent FAS pathway enzyme expressed in sufficient amounts to produce a primary alcohol, said malonyl-CoA-independent FAS pathway comprising ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase, said acyl-reduction pathway comprising an acyl-CoA reductase and an alcohol dehydrogenase.

Malonyl-CoA-independent fatty acid synthesis is a metabolic process used by photosynthetic flagellate such as *Euglena gracilis* (Inui et al., *Euro. J. Biochem.* 96:931-34 (1984). These single cell organisms exhibit both algae and protozoan characteristics and, depending on conditions, can utilize either light energy (photosynthesis) or chemical energy (eating) for biochemical processes. Under anaerobic conditions, *E. gracilis* converts paramylon, the reserve beta-1,2-glucan polysaccharide, into wax ester with concomitant generation of ATP, a phenomenon named wax ester fermentation (Inui et al., *supra*, 1982; Inui et al., Agricultural and Biological Chemistry 47:2669-2671 (1983)). Fatty acid synthesis through the malonyl-CoA-independent pathway results in a net gain of ATP, whereas other fatty acid synthesis systems can not support the net gain of ATP. ATP also can be produced under aerobic conditions (Inui et al., *Archives Biochemistry and Biophysics* 237:423-29 (1985)).

In the absence of oxygen, acetyl-CoA is generated from pyruvate via an oxygen-sensitive pyruvate:NADP+ oxidoreductase (Inui et al., *supra*, 1984; Inui et al., *supra*, 1985; Inui et al., *Archives of Biochemistry and Biophysics* 280:292-98 (1990); Inui et al., *Journal of Biological Chemistry* 262:9130-35 (1987)), and serves as the terminal electron acceptor of glucose oxidation via the malonyl-CoA-independent fatty acid synthesis to form wax ester (Inui et al., *supra*, (1985)). *E. gracilis* contains five different systems of fatty acid synthesis, including four fatty acid synthesis systems located in different compartments, and the mitochondrial malonyl-CoA-independent FAS system involved in anaerobic wax ester fermentation (Hoffmeister et al., *J. of Biological Chemistry* 280:4329-38 (2005)). The malonyl-CoA-independent FAS system has been shown to produce C8-C18 fatty acids. A fatty acid is reduced to alcohol, esterified with

another fatty acid, and deposited in the cytosol as waxes (Inui et al., *Febs Letters* 150:89-93 (1982); Inui et al., *European Journal of Biochemistry* 142:121-126 (1984)). The wax can constitute approximately 50% of the total lipid in dark grown cells (Rosenberg, A., *Biochemistry* 2:1148 (1963)). A particularly useful embodiment of the invention harness the malonyl-CoA-independent fatty acid synthesis (FAS) system under anaerobic conditions to produce large quantities of alcohols using the modified biosynthetic pathways described herein.

The malonyl-CoA-independent fatty acid synthesis pathway is similar to the reversal of fatty acid oxidation and is referred as the fatty acid synthesis in mitochondria or acyl-carrier protein (ACP)-independent fatty acid synthesis as it is known in the art. Compared to the malonyl-CoA-dependent fatty acid synthesis (a.k.a. ACP dependent fatty acid synthesis; Smith et al., *Progress in Lipid Research* 42:289-317 (2003); White et al., *Annual Review of Biochemistry* 74:791-831 (2005)), there are several differences. First, acetyl-CoA is used as the extension unit instead of malonyl-ACP. Utilization of acetyl-CoA as elongation substrate in the malonyl-CoA-independent pathway eliminates the need for acetyl-CoA carboxylase complex (ACC), which converts acetyl-CoA to malonyl-CoA, and thus conserves one ATP molecule per unit flux of acetyl-CoA entering the elongation cycle. Second, all of the intermediates in the elongation cycle are attached to CoA instead of ACP. The elongation cycle can include (i) ketoacyl-CoA acyltransferase (or ketoacyl-CoA thiolase, EC 2.3.1.16), (ii) 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35 and 1.1.1.211), (iii) enoyl-CoA hydratase (EC 4.2.1.17 and 4.2.1.74), and (iv) enoyl-CoA reductase (EC 1.3.1.44 and 1.3.1.38). Third, the product from the elongation cycle is acyl-CoA, which can be utilized directly by acyl-CoA reductase, followed by a dehydrogenase for conversion to alcohol, or by fatty acid forming acyl-CoA reductase (FAR), which converts acyl-CoA directly to alcohol. Therefore, thioesterase and acyl-CoA synthase are not required for the production of primary alcohols, as is the case with the malonyl-CoA-dependent pathways.

For example, the microorganisms of the invention utilize the malonyl-CoA-independent fatty acid synthesis pathway coupled with the reduction of the fatty acid to form primary alcohol as illustrated in Figure 1. The microorganism can additionally be modified to convert, for example, renewable feedstock to acetyl-CoA. In the bioengineered pathways of the invention, acetyl-CoA can be used as both a primer and an extension unit in the elongation cycle described above. At the end of each elongation cycle, an acyl-CoA is formed that is one C2 unit longer than the acyl-CoA entering the elongation cycle. Coupling the above synthesis pathway to a reduction pathway yields the primary alcohol products of the invention. Particularly useful is the coupling of acyl-CoA having a desired chain-length to a reduction pathway that uses the combination of

chain-length specific acyl-CoA reductase (EC 1.2.1.50) and alcohol dehydrogenase (1.1.1.1) or the fatty alcohol forming acyl-CoA reductase (FAR, EC 1.1.1) to form desired primary alcohol. Carbon chain-length of the primary alcohols can be controlled by chain-length specific enoyl-CoA reductase, ketoacyl-CoA thiolase and/or acyl-CoA reductase.

The microorganisms of the invention having the coupled biosynthetic pathways described above can produce primary alcohols at very high levels. For example, the maximum theoretical yield for octanol using the malonyl-CoA-independent fatty acid biosynthetic pathway and the associated energetics were calculated by adding the malonyl-CoA-independent fatty acid synthesis, acyl-CoA reductase and alcohol dehydrogenase reactions to a predictive *E. coli* metabolic stoichiometric network using the *in silico* metabolic modeling system known in the art as SimPheny™ (see, for example, U.S. Patent Application Serial No. 10/173,547, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003). The model assumes that the secretion of octanol does not require energy. Table 4 shows the maximum theoretical yield for octanol under both aerobic and anaerobic conditions. The malonyl-CoA-independent fatty acid biosynthetic pathway is much more energy-efficient than the malonyl-CoA-dependent fatty acid synthesis pathways, and allows for a maximum theoretical yield of 0.5 mole octanol/mole of glucose and maximum ATP yield of 2.125 mole/mole of glucose under both aerobic and anaerobic conditions.

Table 4: Comparison of the maximum theoretical yield of octanol using (1) the malonyl-CoA-independent fatty acid synthesis and acyl-reduction pathway and (2) the ACP-dependent fatty acid synthesis and pathway.

	Malonyl-CoA-independent fatty acid biosynthetic and reduction pathway		**Typical fatty acid biosynthetic and reduction pathway**	
	Anaerobic	**Aerobic**	**Anaerobic**	**Aerobic**
Octanol Yield (mole/mole glucose)	0.5	0.5	0.375	0.48
Max ATP Yield @ max octanol yield (mole/mole glucose)	2.125	2.125	0	0

A non-naturally occurring microbial organism of the invention employs combinations of metabolic reactions for biosynthetically producing a target primary alcohol or a target mixture of primary alcohols of the invention. The combination of metabolic reactions can be engineered in a variety of different alternatives to achieve exogenous expression of a malonyl-CoA-independent FAS pathway in sufficient amounts to produce a primary alcohol. The non-naturally occurring microbial organisms will express at least one exogenous nucleic acid

encoding a malonyl-CoA-independent FAS pathway enzyme. In certain embodiments, the non-naturally occurring microbial organisms of the invention will be engineered to exogenously express more than one, including all, nucleic acids encoding some or all of the enzymes for the complete pathway of malonyl-CoA independent FAS pathway enzymes. Some or all of the enzymes for acyl-reduction also can be exogenously expressed. Exogenous expression should be at levels sufficient to produce metabolically utilizable gene product and result in the production of a target primary alcohol or set of alcohols.

The biochemical reactions for formation of primary alcohols from a carbon or other energy source through a malonyl-CoA independent FAS pathway is shown in Figure 1. The malonyl-CoA independent FAS pathway produces acyl-CoA. Concomitant utilization of this intermediate product to produce target primary alcohols by an acyl-reduction pathway also is shown in Figure 1.

The invention is described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more nucleic acids or genes encoding an enzyme associated with or catalyzing the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction and that reference to any of these metabolic constitutes also references the gene or genes encoding the enzymes that catalyze the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene or encoding nucleic acid also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes as well as the reactants and products of the reaction.

Microbial organisms other than *Euglena gracilis* generally lack the capacity to synthesize acyl-CoA through a malonyl-CoA independent FAS pathway. Moreover, organisms having all of the requisite metabolic enzymatic capabilities are not known to produce acyl-CoA from the enzymes described and biochemical pathways exemplified herein. Rather, microorganisms having the enzymatic constituents of malonyl-CoA independent FAS pathway operate to degrade short, medium, and long chain fatty-acyl-CoA compounds to acetyl-CoA. *E. gracilis*, having a malonyl-CoA independent FAS pathway, utilizes this pathway to produce acylglycerols, trihydric sugar alcohols, phospholipids, wax esters and/or fatty acids. In contrast, the non-naturally occurring microbial organisms of the invention generate acyl-CoA as a product of the malonyl-CoA independent FAS pathway and funnel this product into an acyl-reduction pathway

via favorable thermodynamic characteristics. Product biosynthesis of using the non-naturally occurring organisms of the invention is not only particularly useful for the production of primary alcohols, it also allows for the further biosynthesis of compounds using acyl-CoA and/or primary alcohols as an intermediate reactant.

The non-naturally occurring primary alcohol-producing microbial organisms of the invention are generated by ensuring that a host microbial organism includes functional capabilities for the complete biochemical synthesis of a malonyl-CoA independent fatty acid biosynthetic pathway and for an acyl-reduction pathway of the invention. Ensuring complete functional capabilities for both pathways will confer primary alcohol biosynthesis capability onto the host microbial organism. The enzymes participating in a malonyl-CoA independent FAS pathway include ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase. The enzymes participating in an acyl-reduction pathway include an acyl-CoA reductase and an alcohol dehydrogenase or an enzyme having dual reductase and dehydrogenase activity.

The non-naturally occurring microbial organisms of the invention can be produced by introducing expressible nucleic acids encoding one or more of the enzymes participating in the malonyl-CoA independent FAS and/or acyl-reduction pathways. Depending on the host microbial organism chosen for biosynthesis, nucleic acids for some or all of these biosynthetic pathways can be expressed. For example, if a chosen host is deficient in all of the enzymes in the malonyl-CoA independent FAS pathway, then expressible nucleic acids for each of the four enzymes ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase are introduced into the host for subsequent exogenous expression. Alternatively, for example, if the chosen host is deficient less than all four of the above enzymes, then all that is needed is to express nucleic acids encoding the deficient enzymes. For example, if a host is deficient in 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase a functionally complete malonyl-CoA independent FAS pathway can be engineererd by introduction of nucleic acids encoding these two enzymes.

In like fashion, where endogenous host biosynthetic machinery is complete for an acyl-reduction pathway, then genetic modification is unnecessary. However, if host capabilities are deficient in either or both of the acyl-CoA reductase and/or alcohol dehydrogenase activities, then introduction of the deficient activity by expression of an exogenous encoding nucleic acid is needed. Accordingly, depending on the malonyl-CoA independent FAS and acyl-reduction pathway constituents of a selected host microbial organism, the non-naturally occurring

microbial organisms of the invention will include at least one exogenously expressed malonyl-CoA independent FAS pathway-encoding nucleic acid and up to all six malonyl-CoA independent FAS and acyl-reduction pathway encoding nucleic acids.

Given the teachings and guidance provided herein, those skilled in the art will understand that the number of encoding nucleic acids to introduce in an expressible form will parallel the malonyl-CoA independent FAS and acyl-reduction pathway deficiencies of the selected host microbial organism. Therefore, a non-naturally occurring microbial organism of the invention can have one, two, three, four, five or six encoding nucleic acids encoding the above enzymes constituting the malonyl-CoA independent FAS pathway, an acyl-reduction pathway or both the malonyl-CoA independent FAS and acyl-reduction biosynthetic pathways. In some embodiments, the non-naturally occurring microbial organisms also can include other genetic modifications that facilitate or optimize acyl-CoA and/or primary alcohol biosynthesis or that confer other useful functions onto the host microbial organism. One such other functionality can include, for example, augmentation of the synthesis of one or more of the malonyl-CoA independent FAS pathway precursors such as acetyl-CoA, β-ketoacyl-CoA, β-hydroxyacyl-CoA, *trans*-2-enoyl-CoA and/or fatty aldehyde.

In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that contains the enzymatic capability to synthesize acyl-CoA through a malonyl-CoA independent FAS pathway, or having the capability to catalyze one or more of the enzymatic steps within the malonyl-CoA independent FAS and/or acyl-reduction pathways. In these specific embodiments it can be useful to increase the synthesis or accumulation of a malonyl-CoA independent FAS pathway product or an acyl-reduction pathway product to, for example, efficiently drive malonyl-CoA independent FAS and/or acyl-reduction pathway reactions toward primary alcohol production. Increased synthesis or accumulation can be accomplished by, for example, overexpression of nucleic acids encoding one or more of the above-described malonyl-CoA independent FAS and/or acyl-reduction pathway enzymes. Over expression of the desired pathway enzyme or enzymes can occur, for example, through exogenous expression of the endogenous gene or genes, or through exogenous expression of a heterologous gene or genes. Therefore, naturally occurring organisms can readily be generated to be non-naturally primary alcohol producing microbial organisms of the invention through overexpression of one, two, three, four, five or all six nucleic acids encoding a malonyl-CoA independent FAS and/or a acyl-reduction pathway enzymes. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an

increase in activity of an enzyme in the malonyl-CoA independent FAS and/or acyl-reduction biosynthetic pathways.

In particularly useful embodiments, exogenous expression of the encoding nucleic acids is employed. Exogenous expression confers the ability to custom tailor the expression and/or regulatory elements to the host and application to achieve a desired expression level that is controlled by the user. However, endogenous expression also can be utilized in other embodiments such as by removing a negative regulatory effector or induction of the gene's promoter when linked to an inducible promoter or other regulatory element. For example, activation of *fadB*, an *E. coli* gene having malonyl-CoA independent FAS activity corresponding to 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities can be accomplished by genetically knocking out a negative regulator, *fadR*, and co-expressing a heterologous ketothiolase (*phaA* from *Ralstonia eutropha*; Sato et al., *Journal of Bioscience and Bioengineering* 103:38-44 (2007)). Thus, an endogenous gene having a naturally occurring inducible promoter can be up-regulated by providing the appropriate inducing agent, or the regulatory region of an endogenous gene can be engineered to incorporate an inducible regulatory element, thereby allowing the regulation of increased expression of an endogenous gene at a desired time. Similarly, an inducible promoter can be included as a regulatory element for an exogenous gene introduced into a non-naturally occurring microbial organism.

Additionally, for example, if an endogenous enzyme or enzymes operate in a reverse direction to the desired malonyl-CoA independent FAS pathway, genetic modifications can be made to attenuate or eliminate such activities. For example, within the malonyl-CoA independent FAS pathway, the ketothiolase, dehydrogenase, and enoyl-CoA hydratase steps are reversible whereas the enoyl-CoA reductase step is primarily oxidative under physiological conditions (Hoffmeister et al., *Journal of Biological Chemistry* 280:4329-4338 (2005); Campbell, J. W. and J. E. Cronan, Jr., *J Bacteriol.* 184:3759-3764 (2002)). To accomplish reduction of a 2-enoyl-CoA intermediate a genetic modification can be introduced to attenuate or eliminate the reverse oxidative reaction.

Sources of encoding nucleic acids for a malonyl-CoA independent FAS and/or acyl-reduction pathway enzyme can include, for example, any species where the encoded gene product is capable of catalyzing the referenced reaction. Such species include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including human. For example, the microbial organisms having primary alcohol biosynthetic production are exemplified herein with reference to an *E. coli* host. However, with the complete genome

sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite malonyl-CoA independent FAS and/or acyl-reduction biosynthetic activity for one or more genes in related or distant species, including for example, homologues, orthologs, paralogs and nonorthologous gene displacements of known genes, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling biosynthesis of primary alcohols of the invention described herein with reference to a particular organism such as *E. coli* can be readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

In some instances, such as when an alternative malonyl-CoA independent FAS constituent enzyme or pathway exists in an unrelated species, primary alcohol biosynthesis can be conferred onto the host species by, for example, exogenous expression of a paralog or paralogs from the unrelated species that catalyzes a similar, yet non-identical metabolic reaction to replace the referenced reaction. Because certain differences among metabolic networks exist between different organisms, those skilled in the art will understand that the actual genes usage between different organisms may differ. However, given the teachings and guidance provided herein, those skilled in the art also will understand that the teachings and methods of the invention can be applied to all microbial organisms using the cognate metabolic alterations to those exemplified herein to construct a microbial organism in a species of interest that will synthesize the primary alcohol products of the invention.

Encoding nucleic acids and species that can be used as sources for conferring malonyl-CoA independent FAS and/or acyl-reduction pathway capability onto a host microbial organism are exemplified further below. In one exemplary embodiment, the genes *fadA* and *fadB* encode a multienzyme complex that exhibits three constituent activities of the malonyl-CoA independent FAS pathway, namely, ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase activities (Nakahigashi, K. and H. Inokuchi, *Nucleic Acids Research* 18:4937 (1990); Yang et al., *Journal of Bacteriology* 173:7405-7406 (1991); Yang et al, *Journal of Biological Chemistry* 265:10424-10429 (1990); Yang et al., *Biochemistry* 30:6788-6795 (1990)). The *fadI* and *fadJ* genes encode similar activities which can substitute for the above malonyl-CoA independent FAS conferring genes *fadA* and *fadB*. These genes are naturally expressed

under anaerobic conditions (Campbell and Cronan, *supra*, (2002)). The nucleic acid sequences for each of the above *fad* genes are well known in the art and can be accessed in the public databases such as Genbank using the following accession numbers.

fadA	YP_026272.1	*Escherichia coli*
fadB	NP_418288.1	*Escherichia coli*
fadI	NP_416844.1	*Escherichia coli*
fadJ	NP_416843.1	*Escherichia coli*
fadR	NP_415705.1	*Escherichia coli*

Other exemplary genes for the ketothiolase step include *ato*B which can catalyze the reversible condensation of 2 acetyl-CoA molecules (Sato et al., *supra*, 2007), and its homolog *yqeF*. Non-*E. coli* genes that can be used include *phaA* from *R. eutropha* (Jenkins, L. S. and W. D. Nunn. *Journal of Bacteriology* 169:42-52 (1987)), and the two ketothiolases, *thiA* and *thiB*, from *Clostridium acetobutylicum* (Winzer et al., *Journal of Molecular Microbiology and Biotechnology* 2:531-541 (2000)). The sequences for these genes can be found at the following Genbank accession numbers:

atoB	NP_416728.1	*Escherichia coli*
yqeF	NP_417321.2	*Escherichia coli*
phaA	YP_725941	*Ralstonia eutropha*
thiA	NP_349476.1	*Clostridium acetobutylicum*
thiB	NP_149242.1	*Clostridium acetobutylicum*

An exemplary gene from *E. coli* which can be used for conferring 3-hydroxyacyl-CoA dehydrogenase transformation activity is *paaH* (Ismail et al., *European Journal of Biochemistry* 270:3047-3054 (2003)). Non-*E. coli* genes applicable for conferring this activity include AAO72312.1 from *E. gracilis* (Winkler et al., *Plant Physiology* 131:753-762 (2003)), *paaC* from *Pseudomonas putida* (Olivera et al., *PNAS USA* 95:6419-6424 (1998)), *paaC* from *Pseudomonas fluorescens* (Di Gennaro et al., *Archives of Microbiology* 188:117-125 (2007)), and *hbd* from *C. acetobutylicum* (Atsumi et al., *Metabolic Engineering* (2007) and Boynton et al., *Journal of Bacteriology* 178:3015-3024 (1996)). The sequences for each of these exemplary genes can be found at the following Genbank accession numbers:

paaH	NP_415913.1	*Escherichia coli*
	AA072312.1	*Euglena gracilis*
paaC	NP_745425.1	*Pseudomonas putida*
paaC	ABF82235.1	*Pseudomonas fluorescens*
hbd	NP_349314.1	*Clostridium acetobutylicum*

Exemplary genes encoding the enoyl-CoA hydratase step include, for example, *maoC* (Park and Lee, *Journal Bacteriology* 185:5391-5397 (2003)), *paaF* (Ismail et al., *European Journal of Biochemistry* 270:3047-3054 (2003); Park and Lee, *Appl. Biochem. Biotechnol.* 113-116:335-346 (2004) and Park and Yup, *Biotechnol. Bioeng.* 86:681-686 (2004)), and *paaG* (Ismail et al., *European Journal of Biochemistry* 270:3047-3054 (2003); Park and Lee, Appl. *Biochem. Biotechnol.* 113-116:335-346 (2004) and Park and Yup, *Biotechnol. Bioeng.* 86:681-686 (2004)). Other genes which can be used to produce the gene product catalyzing this step , for example, *paaA*, *paaB*, and *paaN* from *P. putida* (Olivera et al., *PNAS USA* 95:6419-6424 (1998)) and *P. fluorescens* (Di Gennaro et al., *Archives of Microbiology* 188:117-125 (2007)). The gene product of *crt* from *C. acetobutylicum* also can be used (Atsumi et al., *Metabolic Engineering* (2007) and Boynton et al., *Journal of Bacteriology* 178: 3015-3024 (1996. The sequences for each of these exemplary genes can be found at the following Genbank accession numbers:

maoC	NP_415905.1	*Escherichia coli*
paaF	NP_415911.1	*Escherichia coli*
paaG	NP_415912.1	*Escherichia coli*
paaA	NP_745427.1	*Pseudomonas putida*
paaA	ABF82233.1	*Pseudomonas fluorescens*
paaB	NP_745426.1	*Pseudomonas putida*
paaB	ABF82234.1	*Pseudomonas fluorescens*
paaN	NP_745413.1	*Pseudomonas putida*
paaN	ABF82246.1	*Pseudomonas fluorescens*
crt	NP_349318.1	*Clostridium acetobutylicum*

An exemplary gene which can be introduced into a non-naturally occurring microbial organism of the invention to confer enoyl-CoA reductase activity is the mitochondrial enoyl-CoA reductase from *E. gracilis* Hoffmeister et al., *supra* (2005)). A construct derived from this sequence following the removal of its mitochondrial targeting leader sequence has been cloned and expressed in *E. coli*. This approach for heterologous expression of membrane targeted polypeptides in a soluble form is well known to those skilled in the art of expressing eukaryotic genes, particularly those with leader sequences that may target the gene product to a specific intracellular compartment, in prokaryotic organisms. A close homolog of this gene, TDE0597, from the prokaryote *Treponema denticola* represents also can be employed to confer enoyl-CoA reductase activity (Tucci and Martin, *FEBS Letters* 581:1561-1566 (2007)). Butyryl-CoA dehydrogenase, encoded by *bcd* from *C. acetobutylicum*, is a further exemplary enzyme that can be used to confer enoyl-CoA reductase activity onto a host microbial organism of the invention (Atsumi et al., *Metabolic Engineering* (2007) and Boynton et al., *Journal of Bacteriology* 178: 3015-3024 (1996)). Alternatively, *E. coli* genes exhibiting this activity can be obtained using methods well known in the art (see, for example, Mizugaki et al., *Chemical & Pharmaceutical*

Bulletin 30:206-213 (1982) and Nishimaki et al., *Journal of Biochemistry* 95:1315-1321 (1984)). The sequences for each of the above exemplary genes can be found at the following Genbank accession numbers:

TER	Q5EU90.1	*Euglena gracilis*
TDE0597	NP_971211.1	*Treponema denticola*
bcd	NP_349317.1	*Clostridium acetobutylicum*

At least three mitochondrial enoyl-CoA reductase enzymes exist in *E. gracilis* that similarly are applicable for use in the invention. Each enoyl-CoA reductase enzyme exhibits a unique chain length preference (Inui et al., *European Journal of Biochemistry* 142:121-126 (1984)), which is particularly useful for dictating the chain length of the desired primary alcohol products of the invention. EST's ELL00002199, ELL00002335, and ELL00002648, which are all annotated as mitochondrial trans-2-enoyl-CoA reductases, can be used to isolate these additional enoyl-CoA reductase genes as described further below.

Those skilled in the art also can obtain nucleic acids encoding any or all of the malonyl-CoA independent FAS pathway or acyl-reduction pathway enzymes by cloning using known sequences from available sources. For example, any or all of the encoding nucleic acids for the malonyl-CoA independent FAS pathway can be readily obtained using methods well known in the art from *E. gracilis* as this pathway has been well characterized in this organism. *E. gracilis* encoding nucleic acids can be isolated from, for example, an *E. gracilis* cDNA library using probes of known sequence. The probes can be designed with whole or partial DNA sequences from the following EST sequences from the publically available sequence database TBestDB (http://tbestdb.bcm.umontreal.ca). The nucleic acids generated from this process can be inserted

into an appropriate expression vector and transformed into *E. coli* or other microorganisms to generate primary alcohol production organisms of the invention.

ketoacyl-CoA acyltransferase (or ketoacyl-CoA thiolase)
ELL00002550
ELL00002493
ELL00000789
3-hydroxyacyl-CoA dehydrogenase
ELL00000206
ELL00002419
ELL00006286
ELL00006656
enoyl-CoA hydratase
ELL00005926
ELL00001952
ELL00002235
ELL00006206
enoyl-CoA reductase
ELL00002199
ELL00002335
ELL00002648

Alternatively, the above EST sequences can be used to identify homologue polypeptides in GenBank through BLAST search. The resulting homologue polypeptides and their corresponding gene sequences provide additional encoding nucleic acids for transformation into *E. coli* or other microorganisms to generate the primary alcohol producing organisms of the invention. Listed below are exemplary homologue polypeptide and their gene accession numbers in GenBank which are applicable for use in the non-naturally occurring organisms of the invention.

ketoacyl-CoA acyltransferase (or ketoacyl-CoA thiolase)	
YP_001530041	*Desulfococcus oleovorans* Hxd3
ZP_02133627	*Desulfatibacillum alkenivorans* AK-01
ZP_01860900	*Bacillus sp.* SG-1
YP_001511817	*Alkaliphilus oremlandii* OhILAs
NP_781017	*Clostridium tetani* E88
YP_001646648	*Bacillus weihenstephanensis* KBAB4
YP_001322360	*Alkaliphilus metalliredigens* QYMF
YP_001397054	*Clostridium kluyveri* DSM 555
NP_070026	*Archaeoglobus fulgidus* DSM 4304
YP_001585327	*Burkholderia multivorans* ATCC 17616
3-hydroxyacyl-CoA dehydrogenase	
AA072312	*Euglena gracilis*
XP_001655993	*Aedes aegypti*
NP_001011073	*Xenopus tropicalis*
NP_001003515	*Danio rerio*
XP_973042	*Tribolium castaneum*

XP_001638329	*Nematostella vectensis*
CAG11476	*Tetraodon nigroviridis*
XP_787188	*Strongylocentrotus purpuratus*
XP_001749481	*Monosiga brevicollis* MX1
NP_509584	*Caenorhabditis elegans*
XP_572875	*Cryptococcus neoformans var*
enoyl-CoA hydratase	
XP_844077	*Trypanosoma brucei*
XP_802711	*Trypanosoma cruzi* strain CL Brener
XP_806421	*Trypanosoma cruzi* strain CL Brener.
YP_001669856	*Pseudomonas putida* GB-1
YP_641317	*Mycobacterium* sp. MCS
YP_959434	*Marinobacter aquaeolei* VT8
ABK24445	*Picea sitchensis*
XP_640315	*Dictyostelium discoideum*
YP_633978	*Myxococcus xanthus* DK 1622
YP_467905	*Rhizobium etli* CFN 42
YP_419997	*Magnetospirillum magneticum* AMB-1
YP_001172441	*Pseudomonas stutzeri* A1501
enoyl-CoA reductase.	
XP_642118	*Dictyostelium discoideum* AX4
XP_001639469	*Nematostella vectensis*
XP_001648220	*Aedes aegypti*
XP_974428	*Tribolium castaneum*
XP_535334	*Canis lupus familiaris* (dog)
NP_001016371	*Xenopus tropicalis*
XP_320682	*Anopheles gambiae* str. PEST
ZP_01645699	*Stenotrophomonas maltophilia*
XP_001679449	*Caenorhabditis briggsae* AF16
ZP_01443601	*Roseovarius* sp. HTCC2601
XP_395130	*Apis mellifera*
XP_001113746	*Macaca mulatta*
ZP_01485509	*Vibrio cholerae* V51
ZP_02012479	*Opitutaceae bacterium* TAV2
ZP_01163033	*Photobacterium* sp. SKA34
YP_267463	*Colwellia psychrerythraea* 34H
ZP_01114282	*Reinekea* sp. MED297
ZP_01732824	*Flavobacteria bacterium* BAL38

As described previously, after the malonyl-CoA independent elongation cycle, the resulting acyl-CoA can be reduced to produce a primary alcohol by either a single enzyme or pair of enzymes that exhibit acyl-CoA reductase and alcohol dehydrogenase activities. Exemplary genes that encode enzymes for catalyzing the reduction of an acyl-CoA to its corresponding aldehyde include the *Acinetobacter calcoaceticus acr1* encoding a fatty acyl-CoA reductase (Reiser and Somerville, *Journal of Bacteriology* 179:2969-2975 (1997)), the Acinetobacter sp. M-1 fatty acyl-CoA reductase (Ishige et al., *Appl. Environ. Microbiol.* 68:1192-1195 (2002)), and the *sucD*

gene from *Clostridium kluyveri* (Sohling and Gottschalk, *Journal Bacteriology* 178:871-880 (1996)).

acr1	YP_047869.1	*Acinetobacter calcoaceticus*
	AAC45217	*Acinetobacter baylyi*
	BAB85476.1	*Acinetobacter* sp. Strain M-1
sucD	P38947.1	*Clostridium kluyveri*

Exemplary genes encoding enzymes that catalyze the conversion of an aldehyde to alcohol (i.e., alcohol dehydrogenase or equivalently aldehyde reductase) include *alrA* encoding a medium-chain alcohol dehydrogenase for C2-C14 (Tani et al., *Appl. Environ. Microbiol.* 66:5231-5235 (2000)), ADH2 from *Saccharomyces cerevisiae* (Atsumi et al., *Nature* 451:86-89 (2008)), and *yqhD* from *E. coli* which has preference for molecules longer than C3_(Sulzenbacher et al., *Journal of Molecular Biology* 342:489-502 (2004)).

alrA	BAB12273.1	*Acinetobacter* sp. Strain M-1
ADH2	NP_014032.1	*Saccharymyces cerevisiae*
yqhD	NP_417484.1	*Escherichia coli*

Alternatively, the fatty acyl-CoA can be reduced in one step by a fatty alcohol forming acyl-CoA reductase or any other enzyme with dual acyl-CoA reductase and alcohol dehydrogenase activity. For example, the jojoba (*Simmondsia chinensis*) *FAR* encodes an alcohol-forming fatty acyl-CoA reductase and its overexpression in *E. coli* resulted in FAR activity and the accumulation of fatty alcohol (Metz et al., *Plant Physiology* 122:635-644 (2000)). The reductase with narrow substrate chain-length specificities will also function as additional control for product chain-length. Additional gene candidates include the *E. coli adhE* (Kessler et al., *FEBS Letters* 281:59-63 (2000)) and *C. acetobutylicum bdh* I and *bdh* II (Walter et al., *Journal of Bacteriology* 174:7149-7158 (1992)) which can reduce acetyl-CoA and butyryl-CoA to ethanol and butanol, respectively.

FAR	AAD38039.1	*Simmondsia chinensis*
adhE	NP_415757.1	*Escherichia coli*
bdh I	NP_349892.1	*Clostridium acetobutylicum*
bdh II	NP_349891.1	*Clostridium acetobutylicum*

In addition, the *E. gracilis* nucleic acid sequences encoding enzymes for the reduction step can be obtained and transformed into a host as described previously for the malonyl-CoA independent FAS pathway encoding nucleic acids. Isolated from an *E. gracilis* cDNA library using probes, designed with whole or partial DNA sequences from the following EST sequences from TBestDB (http://tbestdb.bcm.umontreal.ca) can be performed as described previously.

aldehyde dehydrogenase
ELL00002572
ELL00002581
ELL00000108

In addition to the above exemplary encoding nucleic acids, nucleic acids other than those within the malonyl-CoA independent FAS and/or acyl-reduction pathways of the invention also can be introduced into a host organism for further production of primary alcohols. For example, the *Ralstonia eutropha BktB* and *PhbB* genes catalyze the condensation of butyryl-CoA and acetyl-CoA to form β-keto-hexanoyl-CoA and the reduction of β-keto-hexanoyl-CoA to 3-hydroxy-hexanoyl-CoA (Fukui et al., *Biomacromolecules* 3:618-624 (2002)). To improve the production of primary alcohols, exogenous DNA sequences encoding for these specific enzymes can be expressed in the production host of interest. Furthermore, the above described enzymes can be subjected to directed evolution to generate improved versions of these enzymes with high activity and high substrate specificity. A similar approach also can be utilized with any or all other enzymatic steps in the primary alcohol producing pathways of the invention to, for example, improve enzymatic activity and/or specificity and/or to generate long chain alcohols of a predetermined chain length or lengths.

In addition, fatty acyl-CoA and fatty alcohols generated as described above can be applied to produce esters of various lengths. These esters can be formed between: 1) fatty acyl-CoA and short-chain alcohols such as methanol, ethanol, propanol, etc.; 2) fatty alcohols and short-chain acyl-CoA such as formyl-CoA, acetyl-CoA, and propionyl-CoA, etc.; 3) fatty acyl-CoA and fatty alcohols as shown in the following equations.

fatty acyl-CoA + short-chain alcohols → fatty esters + CoA
fatty alcohols + short-chain acyl-CoA → fatty esters + CoA
fatty acyl-CoA + fatty alcohols = wax → CoA

The fatty (or long-chain) alcohols can be synthesized intracellularly by the pathways described herein or can be added to the medium and taken up by the engineered microbe. Similarly, short-chain alcohols can be added to the medium or produced endogenously. Ethanol is an exemplary short chain alcohol that is naturally produced by many microorganisms including *Escherichia coli* and *Saccahyromyces cerevisiae*. Exemplary fatty esters include, but not limited to, fatty acid methyl esters (FAMEs), fatty acid ethyl esters (FAEEs), acetyl esters, and wax. Such molecules have broad applications including in food, personal care, coatings, surfactants, and biodiesel (Gerhard Knothe, Energy & Fuels 2008, 22, 1358–1364). Fatty esters, in this context, are differentiated from wax by the size of the hydrocarbon chain on each side of the ester bond.

Waxes have long chain hydrocarbons on each side of the ester bond, whereas fatty esters have one short chain and one long chain hydrocarbon on each side of the ester bond, respectively.

The reactions to produce these esters can be catalyzed by enzymes with acyl-CoA:alcohol transacylase activities. Exemplary enzymes for catalyzing the formation of fatty esters include the acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS, EC 2.3.1.75) and acetyl-CoA:alcohol O-acetyltransferase (EC 2.3.1.84). Exemplary genes coding for these enzymes include the *Acinetobacter* sp. ADP1 *atfA* encoding a bifunctional enzyme with both wax ester synthase (WS) and acyl-CoA: diacylglycerol acyltransferase (DGAT) activities (Kalscheuer et al. A*J Biol Chem* 2003, **278:** 8075-8082.); the *Simmondsia chinensis* gene AAD38041 encoding a WS required for the accumulation of waxes in jojoba seeds (Lardizabal et al. *Plant Physiology* 2000, **122:** 645-655.); the *Alcanivorax borkumensis atfA1* and *atfA2* encoding bifunctional WS/DGAT enzymes (Kalscheuer et al. *J Bacteriol* 2007, **189:** 918-928.); the *Fragaria x ananassa* AAT encoding an alcohol acetyltransferasae (Noichinda et al. *Food Sci Technol Res* 1999, **5:** 239-242.); the *Rosa hybrid cultivar*AAT1 encoding an alcohol acetyltransferase (Guterman et al. *Plant Mol Biol* 2006, **60:** 555-563.); and the *Saccharomyces cerevisiae* ATF1 and ATF2 encoding alcohol acetyltransferases (Mason et al. *Yeast* 2000, **16:** 1287-1298.).

atfA	Q8GGG1	*Acinetobacter* sp. ADP1
	AAD38041	*Simmondsia chinensis*
atfA1	YP_694462	*Alcanivorax borkumensis* SK2
atfA2	YP_693524	*Alcanivorax borkumensis* SK2
AAT	AAG13130	*Fragaria x ananassa*
AAT1	Q5I6B5	*Rosa hybrid cultivar*
ATF1	P40353	*Saccharomyces cerevisiae*
ATF2	P53296	*Saccharomyces cerevisiae*

Host microbial organisms can be selected from, and the non-naturally occurring microbial organisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *E. coli*, *Rhodococcus opacus*, *Ralstonia eutropha*, *Klebsiella oxytoca*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *E. gracilis*. Exemplary yeasts or fungi include species selected from *Saccharomyces*

cerevisiae, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger* and *Pichia pastoris*.

Methods for constructing and testing the expression levels of a non-naturally occurring primary alcohol-producing host can be performed, for example, by recombinant and detection methods well known in the art. Such methods can be found described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual, Third Ed.*, Cold Spring Harbor Laboratory, New York (2001); Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1999). For example, nucleic acids encoding enzymes in the malonyl-CoA independent FAS and/or acyl-reduction pathway can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. For exogenous expression in *E. coli* or other prokaryotic cells, for example, mitochondrial genes will encode an N-terminal targeting signals, which can be removed before transformation into host cells. For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of the targeting sequence, or alternatively, can be targeted to mitochondrion with the addition of mitochondrial targeting signal functional in the host organism. Furthermore, genes can be subjected for codon optimization with techniques well known in the art, to achieve optimal expression of the one or more malonyl-CoA independent FAS and/or acyl-reduction pathway gene products.

An expression vector or vectors can be constructed to harbor one or more malonyl-CoA independent FAS and/or acyl-reduction pathway encoding nucleic acids operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors and artificial chromosomes. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. When two or more exogenous nucleic acids encoding are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one

constitutive promoter. The transformation of exogenous DNA sequences involved in a metabolic or synthetic pathway will be confirmed using methods well known in the art.

Primary alcohol production can be detected and/or monitored using methods well known to those skilled in the art. For example, final product of primary alcohol and/or intermediates such as acyl-CoA and organic acids can be analyzed by HPLC, GC-MS and LC-MS. For example, primary alcohols can be separated by HPLC using a Spherisorb 5 ODS1 column and a mobile phase of 70% 10 mM phosphate buffer (pH=7) and 30% methanol, and detected using a UV detector at 215 nm (Hennessy et al. 2004, J. Forensic Sci. 46(6):1-9). The release or secretion of primary alcohol into the culture medium or fermentation broth also can be detected using these procedures. Activities of one or more enzymes in the malonyl-CoA independent FAS and/or acyl-reduction pathway also can be measured using methods well known in the art.

The non-naturally occurring microbial organisms of the invention are constructed using methods well known in the art as exemplified above to exogenously express at least one nucleic acid encoding a malonyl-CoA independent FAS pathway enzyme in sufficient amounts to produce primary alcohol. Following the teachings and guidance provided herein, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of greater than that which can be synthesized in naturally occurring organisms. Generally, the intracellular concentration of, for example, octanol is about 54μg/L and decanol is about 148 μg/L.

As described further below, one exemplary growth condition for achieving biosynthesis of primary alcohols includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N_2/CO_2 mixture or other suitable non-oxygen gas or gases.

The invention further provides a method for the production of primary alcohols. The method includes culturing a non-naturally occurring microbial organism have having a malonyl-CoA-independent fatty acid synthesis (FAS) pathway and an acyl-reduction pathway comprising at

least one exogenous nucleic acid encoding a malonyl-CoA-independent FAS pathway enzyme expressed in sufficient amounts to produce a primary alcohol under substantially anaerobic conditions for a sufficient period of time to produce said primary alcohol, said malonyl-CoA-independent FAS pathway comprising ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase, said acyl-reduction pathway comprising an acyl-CoA reductase and an alcohol dehydrogenase.

Any of the non-naturally occurring microbial organisms described previously can be cultured to produce the biosynthetic products of the invention. For example, the primary alcohol producers can be cultured for the biosynthetic production of its engineered target primary alcohol. The primary alcohol can be isolated or isolated and further utilized in a wide variety of products and procedures.

In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described below and are well known in the art. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art.

The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described further below in the Examples, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions. Exemplary growth procedures include, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. All of these processes are well known in the art.

Fermentation procedures are particularly useful for the biosynthetic production of commercial quantities of primary alcohols. Generally, and as with non-continuous culture procedures, the continuous and/or near-continuous production of primary alcohols will include culturing a non-naturally occurring primary alcohol producing organism of the invention in sufficient neutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can be include, for example, 1 day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous culture can include 1 week, 2, 3, 4 or 5 or more weeks and up to several months. Alternatively, organisms of the invention can be cultured for hours, if suitable for a

particular application. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods.

Fermentation procedures are well known in the art. Briefly, fermentation for the biosynthetic production of primary alcohol products of the invention can be utilized in, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. Examples of batch and continuous fermentation procedures well known in the art are exemplified further below in the Examples.

In a further embodiment, the primary alcohol producing microbial organisms of the invention utilize renewable feedstocks and carbon-containing gas as carbon sources for growth. Employing these alternative materials as a feedstock is particularly useful because they are beneficial from an environmental standpoint and lower production costs of bioprocess-derived products such as the primary alcohols of the invention.

Renewable feedstocks useful for growth of the primary alcohol producing organisms of the invention, including fermentation processes with the modified organisms of the invention, can include any regenerative raw material which can be used by the cell as a supply a carbon or other energy source. In general, renewable feedstock are derived from living organisms or their metabolic byproducts including material derived from biomass, often consisting of underutilized components like chaff. Agricultural products specifically grown for use as renewable feedstocks and useful in the methods of the invention include, for example, corn, soybeans and cotton; flaxseed and rapeseed; sugar cane and palm oil. Renewable feedstocks that can be used therefore include an array of carbohydrates, fats and proteins derived from agricultural and/or animal matter which can be harnessed by the primary alcohol producing organisms of the invention as a source for carbon.

Plant-derived biomass which is available as an energy source on a sustainable basis includes, for example, herbaceous and woody energy crops, agricultural food and feed crops, agricultural crop wastes and residues, wood wastes and residues, aquatic plants, and other waste materials including some municipal wastes (see, for example, the URL 1.eere.energy.gov/biomass/information_resources.html, which includes a database describing more than 150 exemplary kinds of biomass sources). Exemplary types of biomasses that can be used as feedstocks in the methods of the invention include cellulosic biomass, hemicellulosic biomass and lignin feedstocks or portions of feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates useful as carbon sources such as glucose, xylose, arabinose,

galactose, mannose, fructose and starch. Given the teachings and guidance provided herein, those skilled in the art will understand that renewable feedstocks and biomass other than those exemplified above also can be used for culturing the microbial organisms of the invention for the production of a wide variety of primary alcohols.

In addition to renewable feedstocks such as those exemplified above, the primary alcohol producing microbial organisms of the invention also can be modified for growth on syngas as its source of carbon. In this specific embodiment, one or more proteins or enzymes are expressed in the primary alcohol producing organisms to provide a metabolic pathway for utilization of syngas or other gaseous carbon source.

Synthesis gas, also known as syngas or producer gas, is the major product of gasification of coal and of carbonaceous materials such as biomass materials, including agricultural crops and residues. Syngas is a mixture primarily of H_2 and CO and can be obtained from the gasification of any organic feedstock, including but not limited to coal, coal oil, natural gas, biomass, and waste organic matter. Gasification is generally carried out under a high fuel to oxygen ratio. Although largely H_2 and CO, syngas can also include CO_2 and other gases in smaller quantities. Thus, synthesis gas provides a cost effective source of gaseous carbon such as CO and, additionally, CO_2.

The Wood-Ljungdahl pathway catalyzes the conversion of CO and H_2 to acetyl-CoA and other products such as acetate. Organisms capable of utilizing CO and syngas also generally have the capability of utilizing CO_2 and CO_2/H_2 mixtures through the same basic set of enzymes and transformations encompassed by the Wood-Ljungdahl pathway. H_2-dependent conversion of CO_2 to acetate by microorganisms was recognized long before it was revealed that CO also could be used by the same organisms and that the same pathways were involved. Many acetogens have been shown to grow in the presence of CO_2 and produce compounds such as acetate as long as hydrogen is present to supply the necessary reducing equivalents (see for example, Drake, Acetogenesis, pp. 3-60 Chapman and Hall, New York, (1994)). This can be summarized by the following equation:

$$2\ CO_2 + 4\ H_2 + n\ ADP + n\ Pi \rightarrow CH_3COOH + 2\ H_2O + n\ ATP$$

Hence, non-naturally occurring microorganisms possessing the Wood-Ljungdahl pathway can utilize CO_2 and H_2 mixtures as well for the production of acetyl-CoA and other desired products.

The Wood-Ljungdahl pathway is well known in the art and consists of 12 reactions which can be separated into two branches: (1) methyl branch and (2) carbonyl branch. The methyl branch converts syngas to methyl-tetrahydrofolate (methyl-THF) whereas the carbonyl branch converts methyl-THF to acetyl-CoA. The reactions in the methyl branch are catalyzed in order by the following enzymes: ferredoxin oxidoreductase, formate dehydrogenase, formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclodehydratase, methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase. The reactions in the carbonyl branch are catalyzed in order by the following enzymes: cobalamide corrinoid/iron-sulfur protein, methyltransferase, carbon monoxide dehydrogenase, acetyl-CoA synthase, acetyl-CoA synthase disulfide reductase and hydrogenase. Following the teachings and guidance provided above for introducing a sufficient number of encoding nucleic acids to complete the either or both the malonyl-CoA independent FAS and/or the acyl-reduction pathway, those skilled in the art will understand that the same engineering design also can be performed with respect to introducing at least the nucleic acids encoding the Wood-Ljungdahl enzymes absent in the host organism. Therefore, introduction of one or more encoding nucleic acids into the microbial organisms of the invention such that the modified organism contains the complete Wood-Ljungdahl pathway will confer syngas utilization ability.

The invention is also directed, in part, to the design and creation of cells and organisms having growth-coupled production of LCA. In one embodiment, the invention utilizes optimization-based approaches based on *in silico* stoichiometric model of *Escherichia coli* metabolism that identify metabolic designs for optimal production of LCA. A bilevel programming framework, OptKnock, is applied within an iterative algorithm to predict multiple sets of gene disruptions, that collectively result in the growth-coupled production of LCA. The results described herein indicate that combinations of strategically placed gene deletions or functional disruptions of genes significantly improve the LCA production capabilities of *Escherichia coli* and other cells or organisms. The strain design strategies are equally applicable if an organism other than *E. coli* is chosen as the production host, even if the organism naturally lacks the activity or exhibits low activity of a subset of the gene products marked for disruption. In those cases, disruptions must only be introduced to eliminate or lessen the enzymatic activities of the gene products that are naturally present in the chosen production host. Growth-coupled production of LCA for the *in silico* designs are confirmed by construction of strains having the designed metabolic genotype.

These metabolically engineered cells or organisms also can be subjected to adaptive evolution to further augment growth-coupled product production.

The invention is also directed, in part, to the design and creation of cells and organisms that produce long chain alcohols, LCAs based on *in silico* stoichiometric model of *Saccharomyces cerevisiae* metabolism. One skilled in the art will recognize the ability to also produce LCAs by non-growth-coupled production by providing a non-producing growth phase, followed by a non-growth production phase, for example. The results described herein indicate that combinations of gene deletions or functional disruptions of genes significantly improve the LCA production capabilities of *Saccharomyces cerevisaie* and other cells of eukaryotic organisms and eukaryotic microbial organisms. The strain design pathways are equally applicable if a eukaryotic microbial organism other than *S. cerevisiae* is chosen as the production host, even if the organism naturally lacks the activity or exhibits low activity of a subset of the gene products marked for disruption. In the latter case, disruptions can be introduced to eliminate or lessen the enzymatic activities of the gene products that are naturally present in the chosen production host. In some embodiments, growth-coupled production of LCA for the *in silico* determined metabolic pathways is confirmed by construction of strains having the designed metabolic genotype. These metabolically engineered cells or organisms can also be subjected to adaptive evolution to further augment growth-coupled product production. In some embodiments, the engineered cells or organisms can also incorporate additional copies of beneficial genes to increase flux through a particular metabolic pathway. Alternatively, exogenous gene insertions from another organism can be used to install functionality that is not present in the host organism.

In some embodiments, the designed LCA production pathway utilizes a malonyl-CoA-independent fatty acid synthesis pathway coupled with reduction of the fatty acid to form primary alcohol as shown in Figure 1. The malonyl-CoA independent LCA production pathway (MI-LCA pathway) comprises the malonyl-CoA-independent fatty acid synthesis steps and the acyl-CoA reduction steps. An engineered microorganism possessing the MI-LCA pathway will convert low cost renewable feedstocks, such as glucose and sucrose, to acetyl-CoA through glycolysis. Acetyl-CoA then is used as both primer and extension units in an elongation cycle that involves the ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and enoyl-CoA reductase. At the end of each elongation cycle, an acyl-CoA is formed that is one C_2 unit longer than the acyl-CoA entering the elongation cycle. The acyl-CoA with a desired chain-length is then reduced through the combination of acyl-CoA reductase and alcohol dehydrogenase or the fatty alcohol forming acyl-CoA reductase to form the desired

primary alcohol. The carbon chain-length of the LCA can be controlled by chain-length specific enoyl-CoA reductase, ketoacyl-CoA thiolase, and/or acyl-CoA reductase.

The MI-LCA pathway has the advantage of better product and ATP yields than that through the typical energy-intensive fatty acid synthesis pathways for LCA production. For example, the maximum theoretical yield for dodecanol (C_{12}) using the MI-LCA pathway is 0.333 mol per mol of glucose consumed under both aerobic and anaerobic conditions:

$$3C_6H_{12}O_6 \rightarrow C_{12}H_{26}O + 6CO_2 + 5H_2O$$

Additionally, the energy and redox characteristics of the MI-LCA pathway make it suited for the creation of strains that couple LCA production to growth using OptKnock algorithms (Burgard, A.P., P. Pharkya, and C.D. Maranas, *Optknock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization.* Biotechnol Bioeng, 2003. **84**(6): p. 647-57; Pharkya, P., A.P. Burgard, and C.D. Maranas, *Exploring the overproduction of amino acids using the bilevel optimization framework OptKnock.* Biotechnol Bioeng, 2003. **84**(7): p. 887-99; Pharkya, P., A.P. Burgard, and C.D. Maranas, *OptStrain: a computational framework for redesign of microbial production systems.* Genome Res, 2004. **14**(11): p. 2367-76.). The resulting growth-coupled production strains will be inherently stable, self-optimizing, and suited for batch, fed-batch, and continuous process designs.

In some embodiments, the invention is directed to an integrated computational and engineering platform for developing metabolically altered microorganism strains having enhanced LCA producing characteristics. Strains identified via the computational component of the platform are put into actual production by genetically engineering the predicted metabolic alterations which lead to the enhanced production of LCA. Production of the desired product is coupled to optimal growth of the microorganism to optimize yields of this product during fermentation. In yet another embodiment, strains exhibiting growth-coupled production of LCA are further subjected to adaptive evolution to further augment product biosynthesis. The levels of growth-coupled product production following adaptive evolution also can be predicted by the computational component of the system where, in this specific embodiment, the elevated product levels are realized only following evolution.

In some embodiments, the invention provides a non-naturally occurring microbial organism, that includes one or more gene disruptions. The disruptions occur in genes encoding an enzyme that couples LCA production to growth of the organism when the gene disruption reduces the activity

of the enzyme, such that the gene disruptions confer stable growth-coupled production of LCA onto the non-naturally occurring organism.

In particular embodiments, the invention provides a non-naturally occurring eukaryotic organism, that includes one or more gene disruptions. The one or more gene disruptions occur in genes that encode enzymes that include, for example a cytosolic pyruvate decarboxylase, a mitochondrial pyruvate dehydrogenase, a cytosolic ethanol-specific alcohol dehydrogenase or a mitochondrial ethanol-specific alcohol dehydrogenase. These gene disruptions confer production of long chain alcohols in the cytosol or mitochondrion (*vide infra*)of the organism.

Further, the present invention provides methods of producing such non-naturally microbial organisms having stable growth-coupled production of LCA. For LCA production, for example, the method includes: (a) identifying *in silico* a set of metabolic modifications requiring LCA production during cell growth, and (b) genetically modifying a microorganism to contain the set of metabolic modifications requiring LCA production.

One consideration for bioprocessing is whether to use a batch or continuous fermentation scheme. One difference between the two schemes that will influence the amount of product produced is the presence of a preparation, lag, and stationary phase for the batch scheme in addition to the exponential growth phase. In contrast, continuous processes are kept in a state of constant exponential growth and, if properly operated, can run for many months at a time. For growth-associated and mixed-growth-associated product formation, continuous processes provide much higher productivities (i.e., dilution rate times cell mass) due to the elimination of the preparation, lag, and stationary phases. For example, given the following reasonable assumptions:

Monod kinetics (i.e., $\mu = \mu_m \cdot S/(K_s+S)$)
μ_m=1.0 hr^{-1}
final cell concentration/initial cell concentration = 20
$t_{prep} + t_{lag} + t_{stat}$ = 5 hr
feed concentration of limiting nutrient >> *Ks*

increased productivity from a continuous process has been estimated at 8-fold, Shuler et al, *Prentice Hall, Inc.*: Upper Saddle River, NJ., 245-247.

Despite advantages in productivity, many more batch processes are in operation than continuous processes for a number of reasons. First, for non-growth associated product formation (e.g., penicillin), the productivity of a batch system may significantly exceed that of a continuous process because the latter would have to operate at very low dilution rates. Next, production

strains generally have undergone modifications to their genetic material to improve their biochemical or protein production capabilities. These specialized strains are likely to grow less rapidly than their parental complements whereas continuous processes such as those employing chemostats (fermenters operated in continuous mode) impose large selection pressures for the fastest growing cells. Cells containing recombinant DNA or carrying point mutations leading to the desired overproduction phenotype are susceptible to back-mutation into the original less productive parental strain. It also is possible for strains having single gene deletions to develop compensatory mutations that will tend to restore the wild-type growth phenotype. The faster growing cells usually out-compete their more productive counterparts for limiting nutrients, drastically reducing productivity. Batch processes, on the other hand, limit the number of generations available by not reusing cells at the end of each cycle, thus decreasing the probability of the production strain reverting back to its wild-type phenotype. Finally, continuous processes are more difficult to operate long-term due to potential engineering obstacles such as equipment failure and foreign organism contamination. The consequences of such failures also are much more considerable for a continuous process than with a batch culture.

For small-volume production of specialty chemicals and/or proteins, the productivity increases of continuous processes rarely outweigh the risks associated with strain stability and reliability. However, for the production of large-volume, growth-associated products such as LCA, the increases in productivity for a continuous process can result in significant economic gains when compared to a batch process. Although the engineering obstacles associated with continuous bioprocess operation would always be present, the strain stability concerns can be overcome through metabolic engineering strategies that reroute metabolic pathways to reduce or avoid negative selective pressures and favor production of the target product during the exponential growth phase.

One computational method for identifying and designing metabolic alterations favoring growth-coupled production of a product is the OptKnock computational framework, Burgard et al., *Biotechnol Bioeng*, 84: 647-57 (2003). OptKnock is a metabolic modeling and simulation program that suggests gene disruption strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become a byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered

strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production.

The concept of growth-coupled biochemical production can be visualized in the context of the biochemical production envelopes of a typical metabolic network calculated using an *in silico* model. These limits are obtained by fixing the uptake rate(s) of the limiting substrate(s) to their experimentally measured value(s) and calculating the maximum and minimum rates of biochemical production at each attainable level of growth. Although exceptions exist, typically the production of a desired biochemical is in direct competition with biomass formation for intracellular resources. Thus, enhanced rates of biochemical production will necessarily result in sub-maximal growth rates. The disruptions suggested by OptKnock are designed to restrict the allowable solution boundaries forcing a change in metabolic behavior from the wild-type strain as depicted in Figure 2. Although the actual solution boundaries for a given strain will expand or contract as the substrate uptake rate(s) increase or decrease, each experimental point should lie within its calculated solution boundary. Plots such as these enable one to visualize how close strains are to their performance limits or, in other words, how much room is available for improvement. The OptKnock framework has already been able to identify promising gene disruption strategies for biochemical overproduction, (Burgard, A.P., P. Pharkya, and C.D. Maranas, *Biotechnol Bioeng*, 84(6):647-657 (2003); Pharkya, P., A.P. Burgard, and C.D. Maranas, *Biotechnol Bioeng*, 84(7):887-899 (2003)) and establishes a systematic framework that will naturally encompass future improvements in metabolic and regulatory modeling frameworks.

Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or disruptions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear

programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. Patent Application Serial No. 10/043,440, filed January 10, 2002, and in International Patent No. PCT/US02/00660, filed January 10, 2002.

Another computational method for identifying and designing metabolic alterations favoring growth-coupled production of a product is metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. Patent Application Serial No. 10/173,547, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003.

SimPheny® is a computational system that can be used to produce a network model *in silico* and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components. Analysis methods such as convex analysis, linear programming and the calculation of extreme pathways as described, for example, in Schilling et al., *J. Theor. Biol.* 203:229-248 (2000); Schilling et al., *Biotech. Bioeng.* 71:286-306 (2000) and Schilling et al., *Biotech. Prog.* 15:288-295 (1999), can be used to determine such phenotypic capabilities.

As described above, one constraints-based method used in the computational programs applicable to the invention is flux balance analysis. Flux balance analysis is based on flux balancing in a steady state condition and can be performed as described in, for example, Varma and Palsson, *Biotech. Bioeng.* 12:994-998 (1994). Flux balance approaches have been applied to reaction networks to simulate or predict systemic properties of, for example, adipocyte metabolism as described in Fell and Small, *J. Biochem.* 138:781-786 (1986), acetate secretion from *E. coli* under ATP maximization conditions as described in Majewski and Domach, *Biotech. Bioeng.* 35:732-738 (1990) or ethanol secretion by yeast as described in Vanrolleghem et al., *Biotech. Prog.* 12:434-448 (1996). Additionally, this approach can be used to predict or simulate the growth of *S. cerevisiae* on a variety of single-carbon sources as well as the metabolism of *H. influenzae* as described in Edwards and Palsson, *Proc. Natl. Acad. Sci.*

97:5528-5533 (2000), Edwards and Palsson, *J. Bio. Chem.* 274:17410-17416 (1999) and Edwards et al., *Nature Biotech.* 19:125-130 (2001).

Once the solution space has been defined, it can be analyzed to determine possible solutions under various conditions. This computational approach is consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement growth-coupled production of a biochemical product. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For simplicity in illustrating the invention, the methods and strains will be described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The ability of a cell or organism to couple growth to the production of a biochemical product can be illustrated in the context of the biochemical production limits of a typical metabolic network calculated using an *in silico* model. These limits are obtained by fixing the uptake rate(s) of the limiting substrate(s) to their experimentally measured value(s) and calculating the maximum and minimum rates of biochemical production at each attainable level of growth. As shown in Figure 2, the production of a desired biochemical generally is in direct competition with biomass formation for intracellular resources. Under these circumstances, enhanced rates of biochemical production will necessarily result in sub-maximal growth rates. The disruptions suggested by the above metabolic modeling and simulation programs such as OptKnock are designed to restrict the allowable solution boundaries forcing a change in metabolic behavior from the wild-type strain as depicted in Figure 2. Although the actual solution boundaries for a given strain will expand or contract as the substrate uptake rate(s) increase or decrease, each experimental point will lie within its calculated solution boundary. Plots such as these enable accurate predictions

of how close the designed strains are to their performance limits which also indicates how much room is available for improvement.

The OptKnock mathematical framework is exemplified herein for pinpointing gene disruptions leading to growth-coupled biochemical production as illustrated in Figure 2. The procedure builds upon constraint-based metabolic modeling which narrows the range of possible phenotypes that a cellular system can display through the successive imposition of governing physico-chemical constraints, Price et al., *Nat Rev Microbiol*, 2: 886-97 (2004). As described above, constraint-based models and simulations are well known in the art and generally invoke the optimization of a particular cellular objective, subject to network stoichiometry, to suggest a likely flux distribution.

Briefly, the maximization of a cellular objective quantified as an aggregate reaction flux for a steady state metabolic network comprising a set N = {1,..., *N*} of metabolites and a set M = {1,..., *M*}of metabolic reactions is expressed mathematically as follows:

$$\text{maximize} \quad v_{cellular\ objective}$$

$$\text{subject to} \quad \sum_{j=1}^{M} S_{ij} v_j = 0, \qquad \forall\, i \in \mathrm{N}$$

$$v_{substrate} = v_{substrate\_uptake}\ mmol/gDW{\cdot}hr \qquad \forall\, i \in \{\text{limiting substrate(s)}\}$$

$$v_{atp} \geq v_{atp\_main}\ mmol/gDW{\cdot}hr$$

$$v_j \geq 0, \qquad \forall\, j \in \{\text{irrev. reactions}\}$$

where S_{ij} is the stoichiometric coefficient of metabolite i in reaction j, v_j is the flux of reaction j, $v_{substrate\_uptake}$ represents the assumed or measured uptake rate(s) of the limiting substrate(s), and v_{atp_main} is the non-growth associated ATP maintenance requirement. The vector v includes both internal and external fluxes. In this study, the cellular objective is often assumed to be a drain of biosynthetic precursors in the ratios required for biomass formation, Neidhardt, F.C. et al., 2nd ed. 1996, Washington, D.C.: ASM Press. 2 v. (xx, 2822, lxxvi). The fluxes are generally reported per 1 *gDW·hr* (gram of dry weight times hour) such that biomass formation is expressed as *g biomass produced/gDW·hr* or *1/hr*.

The modeling of gene deletions, and thus reaction elimination, first employs the incorporation of binary variables into the constraint-based approach framework, Burgard et al., *Biotechnol Bioeng*, 74: 364-375 (2001), Burgard et al., *Biotechnol Prog*, 17: 791-797 (2001). These binary variables,

$$y_j = \begin{cases} 1, \text{ if reaction flux } v_j \text{ is active} \\ 0, \text{ if reaction flux } v_j \text{ is not active} \end{cases}, \forall\, j \in \mathsf{M}$$

assume a value of 1 if reaction j is active and a value of 0 if it is inactive. The following constraint,

$$v_j^{\min} \cdot y_j \le v_j \le v_j^{\max} \cdot y_j, \quad \forall\, j \in \mathsf{M}$$

ensures that reaction flux v_j is set to zero only if variable y_j is equal to zero. Alternatively, when y_j is equal to one, v_j is free to assume any value between a lower v_j^{min} and an upper v_j^{max} bound. Here, v_j^{min} and v_j^{max} are identified by minimizing and maximizing, respectively, every reaction flux subject to the network constraints described above, Mahadevan et al., *Metab Eng*, 5: 264-76 (2003).

Optimal gene/reaction disruptions are identified by solving a bilevel optimization problem that chooses the set of active reactions ($y_j = 1$) such that an optimal growth solution for the resulting network overproduces the chemical of interest. Schematically, this bilevel optimization problem is illustrated in Figure 2. Mathematically, this bilevel optimization problem is expressed as the following bilevel mixed-integer optimization problem:

$$\underset{y_j}{\text{maximize}} \quad v_{chemical} \qquad \textbf{(OptKnock)}$$

$$\left(\begin{array}{llll} \underset{v_j}{\text{subject to}} & \underset{}{\text{maximize}} & v_{biomass} & \\ & \underset{}{\text{subject to}} & \sum_{j=1}^{M} S_{ij} v_j = 0, & \forall\, i \in \mathsf{N} \\ & & v_{substrate} = v_{substrate\_uptake} & \forall\, i \in \{\text{limiting substrate(s)}\} \\ & & v_{atp} \geq v_{atp\_main} & \\ v_{biomass} \geq v_{biomass}^{target} & & & \end{array} \right)$$

$$v_j^{\min} \cdot y_j \leq v_j \leq v_j^{\max} \cdot y_j, \qquad \forall\, j \in \mathsf{M}$$

$$\sum_{j \in \mathsf{M}^{\text{forward}}} (1 - y_j) = K$$

$$y_j \in \{0,1\}, \qquad \forall\, j \in \mathsf{M}$$

where $v_{chemical}$ is the production of the desired target product, for example LCA or other biochemical product, and K is the number of allowable knockouts. Note that setting K equal to zero returns the maximum biomass solution of the complete network, while setting K equal to one identifies the single gene/reaction knockout ($y_j = 0$) such that the resulting network involves the maximum overproduction given its maximum biomass yield. The final constraint ensures that the resulting network meets a minimum biomass yield. Burgard et al., *Biotechnol Bioeng*, 84: 647-57 (2003), provide a more detailed description of the model formulation and solution procedure. Problems containing hundreds of binary variables can be solved in the order of minutes to hours using CPLEX 8.0, *GAMS: The Solver Manuals*. 2003: GAMS Development Corporation, accessed via the GAMS, Brooke et al., *GAMS Development Corporation* (1998), modeling environment on an IBM RS6000-270 workstation. The OptKnock framework has already been able to identify promising gene disruption strategies for biochemical overproduction, Burgard et al., *Biotechnol Bioeng*, 84: 647-57 (2003), Pharkya et al., *Biotechnol*

Bioeng, 84: 887-899 (2003), and establishes a systematic framework that will naturally encompass future improvements in metabolic and regulatory modeling frameworks.

Any solution of the above described bilevel OptKnock problem will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in LCA as a product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve growth-coupled LCA production are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. As described previously, one particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or *cis* binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the product coupling are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the growth-coupled production of LCA, or other biochemical products, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions: $y_1 + y_2 + y_3 \geq 1$. The integer cut method is well known in the art and can be found described in, for example, reference, Burgard et al., *Biotechnol Prog*, 17: 791-797 (2001). As

with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny.

Constraints of the above form preclude identification of larger reaction sets that include previously identified sets. For example, employing the integer cut optimization method above in a further iteration would preclude identifying a quadruple reaction set that specified reactions 1, 2, and 3 for disruption since these reactions had been previously identified. To ensure identification of all possible reaction sets leading to growth-coupled production of a product, a modification of the integer cut method was employed.

Briefly, the modified integer cut procedure begins with iteration 'zero' which calculates the maximum production of the desired biochemical at optimal growth for a wild-type network. This calculation corresponds to an OptKnock solution with K equaling 0. Next, single disruptions are considered and the two parameter sets, $objstore_{iter}$ and $ystore_{iter,j}$, are introduced to store the objective function ($v_{chemical}$) and reaction on-off information (y_j), respectively, at each iteration, *iter*. The following constraints are then successively added to the OptKnock formulation at each iteration.

$$v_{chemical} \geq objstore_{iter} + \varepsilon - M \cdot \sum_{j \in ystore_{iter,j}=0} y_j$$

In the above equation, ε and M are a small and a large numbers, respectively. In general, ε can be set at about 0.01 and M can be set at about 1000. However, numbers smaller and/or larger then these numbers also can be used. M ensures that the constraint can be binding only for previously identified disruption strategies, while ε ensures that adding disruptions to a previously identified strategy must lead to an increase of at least ε in biochemical production at optimal growth. The approach moves onto double disruptions whenever a single disruption strategy fails to improve upon the wild-type strain. Triple disruptions are then considered when no double disruption strategy improves upon the wild-type strain, and so on. The end result is a ranked list, represented as desired biochemical production at optimal growth, of distinct disruption strategies that differ from each other by at least one disruption. This optimization procedure as well as the identification of a wide variety of reaction sets that, when disrupted, lead to the growth-coupled production of a biochemical product are exemplified in detail further below. Given the teachings and guidance provided herein, those skilled in the art will understand that the methods and

metabolic engineering designs exemplified herein are applicable to the coupling of cell or microorganism growth to any biochemical product.

Employing the methods exemplified above, the methods of the invention enable the construction of cells and organisms that couple the production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. In this regard, metabolic alterations have been identified that obligatorily couple the production of LCA to organism growth. Microbial organism strains constructed with the identified metabolic alterations produce elevated levels of LCA during the exponential growth phase. These strains can be beneficially used for the commercial production of LCA in continuous fermentation process without being subjected to the negative selective pressures described previously.

Therefore, the methods of the invention provide a set of metabolic modifications that are identified by an *in silico* method selected from OptKnock. The set of metabolic modifications can include functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion. For LCA production metabolic modifications can be selected from the set of metabolic modifications listed in Table 1.

Also provided is a method of producing a non-naturally occurring microbial organism having stable growth-coupled production of LCA. The method includes: (a) identifying *in silico* a set of metabolic modifications requiring LCA production during exponential growth; (b) genetically modifying an organism to contain the set of metabolic modifications requiring product production, and culturing the genetically modified organism. Culturing can include adaptively evolving the genetically modified organism under conditions requiring product production. The methods of the invention are applicable to bacterium, yeast and fungus as well as a variety of other cells and microorganism. Exemplary bacteria include species selected from *E. coli*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. Exemplary eukaryotic organisms include species selected from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger*, *Rhizopus arrhizus*, *Rhizopus oryzae*, and *Pichia pastoris*.

A microbial organism produced by the methods of the invention is further provided. Additionally, the invention provides a non-naturally occurring microbial organism comprising one or more gene disruptions encoding an enzyme associated with growth-coupled production of LCA and exhibiting stable growth-coupled production of these products. The non-naturally occurring microbial organism of the invention includes one or more gene disruptions occurring in genes encoding an enzyme obligatorily coupling LCA production to growth of the microbial organism when the gene disruption reduces an activity of the enzyme, whereby the one or more gene disruptions confers stable growth-coupled production of LCA onto the non-naturally occurring microbial organism.

The non-naturally occurring microbial organism can have one or more gene disruptions included in a metabolic modification listed in Table 1. The one or more gene disruptions can be a deletion. The non-naturally occurring microbial organism of the invention can be selected from a group of microbial organism having a metabolic modification listed in Tables 1. Non-naturally occurring microbial organisms of the invention include bacteria, yeast, fungus, or any of a variety of other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *E. coli*, *A. succiniciproducens*, *A. succinogenes*, *M. succiniciproducens*, *R. etli*, *Bacillus subtilis*, *C. glutamicum*, *G. oxydans*, *Z. mobilis*, *L. lactis*, *L. plantarum*, *S. coelicolor*, *C. acetobutylicum*, *P. fluorescens*, and *P. putida*. Exemplary eukaryotic organisms include species selected from *S. cerevisiae*, *S. pombe*, *K. lactis*, *K. marxianus*, *A. terreus*, *A. niger*, *R. arrhizus*, *R. oryzae*, and *P. pastoris*.

The microbial organisms having growth-coupled LCA production are exemplified herein with reference to an *Escherichia coli* genetic background. However, with the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of an alternate species homolog for one or more genes, including for example, orthologs, paralogs and nonorthologous gene displacements, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling growth-coupled production of LCA described herein with reference to a particular organism such as *Escherichia coli* can be readily applied to other microorganisms. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

As described previously, homologues can include othologs and/or nonorthologous gene displacements. In some instances, such as when a substitute metabolic pathway exists in the species of interest, functional disruption can be accomplished by, for example, deletion of a paralog that catalyzes a similar, yet non-identical metabolic reaction which replaces the referenced reaction. Because certain differences among metabolic networks between different organisms, those skilled in the art will understand that the actual genes disrupted between different organisms may differ. However, the given the teachings and guidance provided herein, those skilled in the art also will understand that the methods of the invention can be applied to all microorganisms to identify the cognate metabolic alterations between organisms and to construct an organism in a species of interest that will enhance the coupling of LCA biosynthesis to growth.

The invention will be described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more genes associated with the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction and that reference to any of these metabolic constitutes also references the gene or genes encoding the enzymes that catalyze the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes as well as the reactants and products of the reaction. As described previously and further below, exemplary reactions, reaction nomenclature, reactants, products, cofactors and genes encoding enzymes catalyzing a reaction involved in the growth-coupled production of LCA are set forth in Tables 2 and 3.

The invention provides non naturally occurring microbial organisms having growth-coupled production of LCA. Product production is obligatorily linked to the exponential growth phase of the microorganism by genetically altering the metabolic pathways of the cell. The genetic alterations make the desired product a product during the growth phase. Sets of metabolic alterations or transformations that result in elevated levels of LCA biosynthesis are exemplified in Table 1, respectively. Each alteration within a set corresponds to the requisite metabolic reaction that should be functionally disrupted. Functional disruption of all reactions within each set results in the production of LCA by the engineered strain during the growth phase. The corresponding reactions to the referenced alterations and the gene or genes that potentially

encode them in *Escherichia coli*, are set forth in Table 2. The various metabolites, their abbreviations and location are set forth in Table 3.

For example, for each strain exemplified in Table 1, the metabolic alterations that can be generated for growth coupled LCA production are shown in each row. These alterations include the functional disruption of from one to six or more reactions. In particular, 995 strains are exemplified in Table 1 that have non-naturally occurring metabolic genotypes. Each of these non-naturally occurring alterations result in an enhanced level of LCA production during the exponential growth phase of the microbial organism compared to a wild-type strain, under appropriate culture conditions. Appropriate conditions include, for example, those exemplified further below in the Example I such as particular carbon sources or reactant availabilities and/or adaptive evolution.

Given the teachings and guidance provided herein, those skilled in the art will understand that to disrupt an enzymatic reaction it is necessary to disrupt the catalytic activity of the one or more enzymes involved in the reaction. Disruption can occur by a variety of means including, for example, deletion of an encoding gene or incorporation of a genetic alteration in one or more of the encoding gene sequences. The encoding genes targeted for disruption can be one, some, or all of the genes encoding enzymes involved in the catalytic activity. For example, where a single enzyme is involved in a targeted catalytic activity disruption can occur by a genetic alteration that reduces or destroys the catalytic activity of the encoded gene product. Similarly, where the single enzyme is multimeric, including heteromeric, disruption can occur by a genetic alteration that reduces or destroys the function of one or all subunits of the encoded gene products. Destruction of activity can be accomplished by loss of the binding activity of one or more subunits in order to form an active complex, by destruction of the catalytic subunit of the multimeric complex or by both. Other functions of multimeric protein association and activity also can be targeted in order to disrupt a metabolic reaction of the invention. Such other functions are well known to those skilled in the art. Further, some or all of the functions of a single polypeptide or multimeric complex can be disrupted according to the invention in order to reduce or abolish the catalytic activity of one or more enzymes involved in a reaction or metabolic modification of the invention. Similarly, some or all of enzymes involved in a reaction or metabolic modification of the invention can be disrupted so long as the targeted reaction is reduced or destroyed.

Given the teachings and guidance provided herein, those skilled in the art also will understand that an enzymatic reaction can be disrupted by reducing or eliminating reactions encoded by a common gene and/or by one or more orthologs of that gene exhibiting similar or substantially the same activity. Reduction of both the common gene and all orthologs can lead to complete abolishment of any catalytic activity of a targeted reaction. However, disruption of either the common gene or one or more orthologs can lead to a reduction in the catalytic activity of the targeted reaction sufficient to promote coupling of growth to product biosynthesis. Exemplified herein are both the common genes encoding catalytic activities for a variety of metabolic modifications as well as their orthologs. Those skilled in the art will understand that disruption of some or all of the genes encoding a enzyme of a targeted metabolic reaction can be practiced in the methods of the invention and incorporated into the non-naturally occurring microbial organisms of the invention in order to achieve the growth-coupled product production.

Herein below are described the designs identified for increasing LCA production in *Escherichia coli*. The OptKnock algorithm identified designs based on a stoichiometric model of *Escherichia coli* metabolism. Assumptions include (i) a glucose uptake rate of 10 mmol/gdw/hr; (ii) anaerobic or microaerobic conditions; and (iii) a minimum non-growth associated maintenance requirement of 3 mmol/gdw/hr. Dodecanol, a C_{12} molecule, was chosen as an exemplary long chain alcohol whose production can be coupled to growth following the teachings of this invention. Although glucose was assumed to be the growth substrate, it is understood that the strategies are applicable to any substrate including glucose, sucrose, xylose, arabinose, or glycerol. The complete set of growth-coupled LCA productions designs are listed in Table 1. The enzyme names, their abbreviations, and the corresponding reaction stoichiometries are listed in Table 2. Finally, metabolites names corresponding to the abbreviations in the reaction equations are listed in Table 3. Although the designs were identified using a metabolic model of *E. coli* metabolism, and the gene names listed in Table 2 are specific to *E. coli*, the method of choosing the metabolic engineering strategies and also the designs themselves are applicable to any LCA-producing organism. Thus the designs are essentially lists of enzymatic transformations whose activity must be either eliminated, attenuated, or initially absent from a microorganism to enable the growth coupled production of long chain alcohols.

One criterion for prioritizing the final selection of designs was the growth-coupled yield of dodecanol. To examine this, production cones were constructed for each strategy by first maximizing and, subsequently minimizing the dodecanol yields at different rates of biomass formation (as described in the previous section). If the rightmost boundary of all possible

phenotypes of the mutant network is a single point, it implies that there is a unique optimum yield of the product at the maximum biomass formation rate possible in the network. In other cases, the rightmost boundary of the feasible phenotypes is a vertical line, indicating that at the point of maximum biomass the network can make any amount of the dodecanol in the calculated range, including the lowest amount at the bottommost point of the vertical line. Such designs were given a lower priority. A short list of the highest priority OptKnock designs is provided here in Table I which represents a subset of the designs of Table 1.

TABLE I

Design	Enzyme activity	Abbreviation	Other notes
I	Acetaldehyde-CoA dehydrogenase	ADHEr	
	D-lactate dehydrogenase	LDH_D	
II	Acetaldehyde-CoA dehydrogenase	ADHEr	Design I + PFL
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
III	Acetaldehyde-CoA dehydrogenase	ADHEr	Design II + FRD2
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Fumarate reductase	FRD2	
IV	Acetaldehyde-CoA dehydrogenase	ADHEr	Design II + FUM
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Fumarase	FUM	
V	Acetaldehyde-CoA dehydrogenase	ADHEr	Design II + MDH
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Malate dehydrogenase	MDH	
VI	Acetaldehyde-CoA dehydrogenase	ADHEr	Design III + GLUDy
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Fumarate reductase	FRD2	
	Glutamate dehydrogenase	GLUDy	
VII	Acetaldehyde-CoA dehydrogenase	ADHEr	Design IV + GLUDy
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Fumarase	FUM	
	Glutamate dehydrogenase	GLUDy	
VIII	Acetaldehyde-CoA dehydrogenase	ADHEr	Design V + GLUDy
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Malate dehydrogenase	MDH	
	Glutamate dehydrogenase	GLUDy	
IX	Acetaldehyde-CoA dehydrogenase	ADHEr	Design III + THD2
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Fumarate reductase	FRD2	
	NAD(P) transhydrogenase	THD2	
X	Acetaldehyde-CoA dehydrogenase	ADHEr	Design IV + THD2
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Fumarase	FUM	
	NAD(P) transhydrogenase	THD2	
XI	Acetaldehyde-CoA dehydrogenase	ADHEr	Design V + THD2
	D-lactate dehydrogenase	LDH_D	
	Pyruvate formate lyase	PFLi	
	Malate dehydrogenase	MDH	
	NAD(P) transhydrogenase	THD2	
XII	Acetaldehyde-CoA dehydrogenase	ADHEr	Design I + PTAr and/or ACKr
	D-lactate dehydrogenase	LDH_D	
	Phosphotransacetylase and/or Acetate kinase	PTAr and/or ACKr	
XIII	Acetaldehyde-CoA dehydrogenase	ADHEr	Design XII + FRD2
	D-lactate dehydrogenase	LDH_D	
	Phosphotransacetylase and/or Acetate kinase	PTAr and/or ACKr	
	Fumarate reductase	FRD2	
XIV	Acetaldehyde-CoA dehydrogenase	ADHEr	Design XII + FUM
	D-lactate dehydrogenase	LDH_D	
	Phosphotransacetylase and/or Acetate kinase	PTAr and/or ACKr	
	Fumarase	FUM	
XV	Acetaldehyde-CoA dehydrogenase	ADHEr	Design XII + MDH
	D-lactate dehydrogenase	LDH_D	
	Phosphotransacetylase and/or Acetate kinase	PTAr and/or ACKr	
	Malate dehydrogenase	MDH	
XVI	Acetaldehyde-CoA dehydrogenase	ADHEr	Design I + FRD
	D-lactate dehydrogenase	LDH_D	
	Fumarate reductase	FRD2	
XVII	Acetaldehyde-CoA dehydrogenase	ADHEr	Design I + FUM
	D-lactate dehydrogenase	LDH_D	
	Fumarase	FUM	
XVIII	Acetaldehyde-CoA dehydrogenase	ADHEr	Design I + MDH
	D-lactate dehydrogenase	LDH_D	
	Malate dehydrogenase	MDH	
XIX	Acetaldehyde-CoA dehydrogenase	ADHEr	Design XVI + ATPS4r
	D-lactate dehydrogenase	LDH_D	
	Fumarate reductase	FRD2	
	ATP synthase	ATPS4r	
XX	Acetaldehyde-CoA dehydrogenase	ADHEr	Design XVII + ATPS4r
	D-lactate dehydrogenase	LDH_D	
	Fumarase	FUM	
	ATP synthase	ATPS4r	
XXI	Acetaldehyde-CoA dehydrogenase	ADHEr	Design XVIII + ATPS4r
	D-lactate dehydrogenase	LDH_D	
	Fumarate reductase	MDH	
	ATP synthase	ATPS4r	

All growth coupled designs in this document build upon Design I which calls for the joint disruption of acetylaldehyde-CoA dehydrogenase (ADHEr) and lactate dehydrogenase (LDH_D) activities to reduce the formation of ethanol and lactate, respectively. A dodecanol yield of 0.14 mol/mol glucose is predicted to be attained upon achieving a maximum growth rate of 0.20 1/hr (Design I, Figure 3). Design II specifies the removal, attenuation, or absence of ADHEr, LDH_D, and pyruvate formate lyase (PFLi) and is predicted to result in a dodecanol yield of 0.28 mol/mol glucose at maximum growth as shown in Figure 4. A tighter coupling of LCA production to growth is attained by the further disruption of fumarate reductase (FRD2), fumarase (FUM), or malate dehydrogenase (MDH) activity as indicated by the solution boundary of Designs III-V in Figure 4. An even tighter coupling of production to growth is attained by the further disruption of glutamate dehydrogenase (GLUDy) or NADP transhydrogenase (THD2) activity as shown in solution boundary of Designs VI – XI in Figure 4. Designs VI – XI actually require a non-insignificant yield of LCA, specifically, 0.05 mol dodecanol/mol glucose, to enable a minimal amount of cell growth.

Design XII calls for the disruption of phosphotransacetylase (PTAr) and/or acetate kinase (ACKr) activity in addition to ADHEr and LDH_D to prevent or lessen the production of acetate, ethanol, and lactate, respectively. A dodecanol yield of 0.28 mol/mol is required to attain a maximum growth rate of 0.16 1/hr assuming a glucose uptake rate of 10 mmol/gDW/hr as shown in Figure 5. A tighter coupling of LCA production to growth is attained by the further disruption of FRD2, FUM, or MDH as indicated by the solution boundary of Designs XIII - XV. Designs XVI – XVIII specify that the disruption of FRD2, FUM, or MDH activity in addition to ADHEr and LDH_D results in a tighter coupling of dodecanol production to cell growth as compared to Design I as shown in Figure 6. Further disrupting ATP synthase activity in designs XIX – XXI is predicted to result in a dodecanol yield of 0.30 mol/mol at a maximum growth rate of 0.13 1/hr as shown in Figure 6. The disruption of this activity forces the organism to rely on the MI-LCA pathway for energy generation. Accordingly, a minimum dodecanol yield of 0.05 mol/mol is required for any growth to be attained assuming that the organism lacks the activities listed in Designs XIX – XXI.

It is understood that the disruption of certain activities in addition to those listed by Designs I – XXI can lead to even higher production yields as illustrated in the following examples. Design V_A involves disruption of Acetaldehyde-CoA dehydrogenase (ADHEr), lactate dehydrogenase (LDH_D), malate dehydrogenase (MDH), pyruvate formate lyase (PFLi), L-aspartase (ASPT), pyruvate kinase (PYK), glucose 6-phosphate dehydrogenase (G6PDHy), and dihydroxyacetone phosphotransferase (DHAPT). Upon addition of the MI-LCA pathway, an engineered strain

containing disruptions in these activities is predicted to have a growth-coupled dodecanol yield of 0.327 mol/mol glucose at the maximum growth rate of 0.02 1/hr (Figure 7, point A). This corresponds to 98% of the maximum theoretical yield of 0.333 mol dodecanol/mol glucose. The maximum growth rate of such a strain is predicted to be approximately 10% of the wide type strain while a minimum dodecanol yield of 0.09 mol/mol is required for growth (Figure 7, point B). A recombinant strain containing reduced activity ofthese functionalities can be constructed in a single step or in subsequent steps by, for example, disrupting 2-3 activities each step. For example, one can engineer *E. coli* for growth coupled LCA production by first removing genes encoding ADHEr and LDH_D activities resulting in Design I. Design V is then constructed by further deleting genes responsible for MDH and PFLi activities. Design V_A is then constructed by deleting genes encoding ASPT, PYK, G6PDHy, and DHAPT activities. Finally, note that several activities (i.e., 6-phosphogluconolactonase (PGL), phosphogluconate dehydratase (PGDHY), or 2-dehydro-3-deoxy-phosphogluconate aldolase (EDA)) can replace G6PDHy for disruption and yield the same characteristics as Design V_A.

Design XII_A involves disruption of Acetaldehyde-CoA dehydrogenase (ADHEr), lactate dehydrogenase (LDH_D), acetate kinase (ACKr) and/or phosphotransacetylase (PTAr), glutamate dehydrogenase (NADP) (GLUDy), phosphogluconate dehydrogenase (PGDH), and glucose-6-phosphate isomerase (PGI). Design XII_B involves disruption of Acetaldehyde-CoA dehydrogenase (ADHEr), lactate dehydrogenase (LDH_D), acetate kinase (ACKr) and/or phosphotransacetylase (PTAr), glutamate dehydrogenase (NADP) (GLUDy), phosphogluconate dehydrogenase (PGDH), glucose-6-phosphate isomerase (PGI), and D-glucose transport via PEP:Pyr PTS (GLCpts). Upon addition of the MI-LCA pathway, an engineered strain lacking the activities specified by Design XII_B is predicted to have a growth-coupled dodecanol yield of 0.322 mol/mol glucose at the maximum growth rate of 0.04 1/hr (Figure 8, point A). This corresponds to 97% of the maximum theoretical yield of 0.333 mol dodecanol/mol glucose. The maximum growth rate of such a strain is predicted to be approximately 20% of the wild type strain while a minimum dodecanol yield of 0.05 mol/mol is required for growth (Figure 8, point B). A recombinant strain containing reduced activity of these functionalities can be constructed in a single step or in subsequent steps by, for example, removing additional activities each step. For example, one can engineer *E. coli* for growth coupled LCA production by first removing genes encoding ADHEr and LDH_D activities resulting in Design I. Design XII is then constructed by further deleting genes encoding PTAr and/or ACKr activities. Design XII_A is then constructed by deleting the genes responsible for GLUDy, PGDH, and PGI activities. Finally, Design XII_B is constructed by further deleting a gene essential for GLCpts activity.

Accordingly, the invention also provides a non-naturally occurring microbial organism having a set of metabolic modifications coupling LCA production to growth of the organism, the set of metabolic modifications includes disruption of one or more genes selected from the set of genes encoding proteins that include an acetylaldehyde-CoA dehydrogenase and a lactate dehydrogenase.

The present invention also provides a strain lacking the activities listed for Design I above that further lack at least one of the following activities: pyruvate formate lyase (PFLi), phosphotransacetylase (PTAr), acetate kinase (ACKr), fumarate reductase (FRD2), fumarase (FUM), or malate dehydrogenase (MDH) as exemplified by Designs II, XII, XVI, XVII, and XVIII.

In further embodiments, the invention provides a strain lacking the activities listed for Design II above and further lacks at least one of the following activities: fumarate reductase (FRD2), fumarase (FUM), or malate dehydrogenase (MDH) as exemplified by Designs III, IV, and V.

In still further embodiments, the invention provides strains lacking the activities listed for Designs III, IV, or V, above and further lack glutamate dehydrogenase (GLUDy) activity as exemplified by Designs VI, VII, and VIII.

The invention also provides strains lacking the activities listed for designs III, IV, or V, above and further lack NAD(P) transhydrogenase (THD2) activity as exemplified by Designs IX, X, and XI.

In yet further embodiments, the invention provides a strain lacking the activities listed for Design XII above and further lack at least one of the following activities: fumarate reductase (FRD2), fumarase (FUM), or malate dehydrogenase (MDH) as exemplified by Designs XIII, XIV, and XV.

Finally, the invention provides strains lacking the activities listed for designs XVI, XVII, and XVIII, above and further lack ATP synthase (ATPS4r) activity as exemplified by Designs XIX, XX, and XXI.

Herein below are described the pathways identified for increasing LCA production in *S. cerevisiae*. The OptKnock algorithm, described herein further below, identified designs based on a stoichiometric model of *Saccharomyces cerevisaie*metabolism. Assumptions include (i) a glucose uptake rate of 10 mmol/gdw/hr; (ii) anaerobic or microaerobic conditions; and (iii) a minimum non-growth associated maintenance requirement of 3 mmol/gdw/hr. Dodecanol, a C_{12}

molecule, was chosen as an exemplary long chain alcohol whose production can be coupled to growth following the teachings of this invention. Although glucose was assumed to be the growth substrate, it is understood that the methods are applicable to any substrate including glucose, sucrose, xylose, arabinose, or glycerol. Although the designs were identified using a metabolic model of *S. cerevisiae* metabolism the method of choosing the metabolic engineering pathways and also the designs themselves are applicable to any LCA-producing eukaryotic organism. Thus, the designs are essentially lists of enzymatic transformations whose activity must be either eliminated, attenuated, or initially absent from a microorganism to enable the production of long chain alcohols.

One criterion for prioritizing the final selection of pathways was the yield of dodecanol. To examine this, production cones were constructed for each set of pathways by first maximizing and, subsequently minimizing the dodecanol yields at different rates of biomass formation. If the rightmost boundary of all possible phenotypes of the mutant network is a single point, it implies that there is a unique optimum yield of the product at the maximum biomass formation rate possible in the network. In other cases, the rightmost boundary of the feasible phenotypes is a vertical line, indicating that at the point of maximum biomass the network can make any amount of the dodecanol in the calculated range, including the lowest amount at the bottommost point of the vertical line. Such designs were given a lower priority.

The organisms of the present invention can be cultured in a substantially anaerobic culture medium or a microaerobic culture medium as detailed herein below further. Such organisms have one or more gene disruptions which may include complete deletion in some embodiments, or disruption by removal or changes in functional portions encoded by fragments of the entire gene.

In some embodiments, the present invention provides non-naturally occurring eukaryotic microbial organisms that produce LCAs in the cytosol. Note that cytosol herein refers to any compartment outside the mitochondrion. In such embodiments, one or more gene disruptions in the eukaryotic organism encoding an enzyme include, for example, a cytosolic pyruvate decarboxylase, a mitochondrial pyruvate dehydrogenase, a cytosolic ethanol-specific alcohol dehydrogenase and a mitochondrial ethanol-specific alcohol dehydrogenase. Exemplary genes endocing these enzymes include, for example, YLR044C, YLR134W, YGR087C, PDC3, YNL071W, YER178W, YBR221C, YGR193C, YFL018C, YBR145W, YGL256W, YOL086C, YMR303, YMR083W, YPL088W, YAL061W, YMR318C, YCR105W, and YDL168W.

Other gene disruptions encoding an enzyme include, for example, a cytosolic malate dehydrogenase, a glycerol-3-phospate dehydrogenase shuttle, an external NADH dehydrogenase, and an internal mitochondrial NADH dehydrogenase can also be effected. Exemplary genes of the later include, for example, YOL126C, YDL022W, YOL059W, YIL155C, YMR145C, YDL085W, and YML120C.

These organisms can also include an exogenous nucleic acid encoding an enzyme in the cytosol including, for example, an acetyl-CoA synthetase (AMP-forming), an ADP-dependent acetate-CoA ligase, an acylating acetaldehyde dehydrogenase, a pyruvate dehydrogenase, a pyruvate:NADP oxidoreductase, and a pyruvate formate lyase, or their corresponding gene regulatory regions. An exogenous nucleic acid encoding a cytosolic transhydrogenase or its gene regulatory region can also be incorporated. In some embodiments these gene products may be natively expressed in the cytosol, while in other embodiments, they may be overexpressed by, for example, adding copies of the gene from the same source or from other organisms, or by introducing or changing gene regulatory regions. Such gene regulatory regions include, for example, alternate promoters, inducible promoters, variant promoters or enhancers to enhance gene expression. Functional disruption of negative regulatory elements such as repressors and/or silencers also can be employed to enhance gene expression. Similar modifications can be made to translation regulatory regions to enhance polypeptide synthesis and include, for example, substitution of a ribosome binding site with an optimal or consensus sequence and/or removing secondary structures.

These organisms maximize the availability of acetyl CoA, ATP and reducing equivalents (NADH) for dodecanol production. Acetyl CoA is the primary carbon precursor for the production of LCA via the proposed MI-LCA route. All the reactions enabling the formation of dodecanol via the malonyl-CoA independent pathway are operational in the cytosol. Specifically, ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and enoyl-CoA reductase function in the appropriate direction to form acyl CoA which is then reduced to fatty aldehyde and dodecanol via acyl CoA reductase and alcohol dehydrogenase.

Introduction of the MI-LCA pathway in the cytosol prevented any flux through the native pyruvate dehydrogenase *in silico*. Under anaerobic conditions and in conditions where glucose concentrations are high in the medium, the capacity of this mitochondrial enzyme is very limited and there is no significant flux through it. However, in some embodiments, this enzyme can be deleted or attenuated to increase LCA production.

In one embodiment, LCA production in the cytosol uses the AMP-forming acetyl-CoA synthetase. Dodecanol production in the cytosol relies on the native cell machinery to provide the precursors needed in LCA production. A majority of the pyruvate flux generated by glycolysis is channeled into the formation of acetyl CoA via the pyruvate dehydrogenase bypass comprised of the pyruvate decarboxylase, the acetaldehyde dehydrogenase and the AMP-forming acetyl-CoA synthetase (Figure 9a). This bypass is reported to have significant flux through it even under aerobic conditions at high concentrations of glucose (Pronk et al., *Yeast* 12:1607-1633 (1996)).

The last step of the bypass that converts acetate into acetyl-CoA is catalyzed by acetyl-CoA synthetase, encoded by the *ACS1* and *ACS2* genes. Since *ACS2* is constitutively expressed on glucose and is present in cytosol among other compartments, in some embodiments the non-naturally occurring eukarotyic organism is engineered to overexpress *ACS2*. In other embodiments the *ACS2* gene is replaced with a mutant *ACS* from *Salmonellas enterica* (Genbank id NP_807785.1) that is not subject to post-translational modification and has higher activity in *S. cerevisiae* as compared to *ACS1* or *ACS2* (Shiba et al., *Metab Eng.* 9:160-168 (2007)).

The AMP-generating acetyl CoA synthetase uses two ATP equivalents for the conversion of each molecule of acetate into acetyl CoA (CoA+ acetate + ATP → acetyl-CoA + PPi + AMP). Under anaerobic conditions, when energy is available only through substrate-level phosphorylation, the production of dodecanol via the AMP-forming acetyl CoA synthetase is not energetically favorable. Therefore, a small amount of oxygen is made available to the cell to fulfill its energetic requirements, simultaneously increasing the conversion of acetate into acetyl CoA.

The production of dodecanol can be improved by disruption of ethanol-specific alcohol dehydrogenases to prevent acetyl-CoA and NADH from being used for ethanol production. Additionally, the production of LCA benefits from preventing NADH from being used in the respiratory electron-transport chain. Thus, disruptions in the internal mitochondrial NADH dehydrogenase, the glycerol-3-phosphate dehydrogenase shuttle (consisting of cytosolic NADH-linked glycerol-3-phosphate dehydrogenase and a membrane-bound glycerol-3-phosphate:ubiquinone oxidoreductase) (Bakker et al., *FEMS Microbiol.* Rev. 25:15-37 (2001)) and the external NADH dehydrogenase are introduced in some embodiments. Further, cytosolic malate dehydrogenase that can potentially draw NADH away from dodecanol production is also disrupted. A growth-coupled production envelope after imposing these disruptions is shown in

dark gray in Figure 9b and compared with the dodecanol production characteristics under aerobic conditions.

In some embodiments, the non-naturally occurring eukaryotic organism incorporates an exogenous gene encoding an ADP-forming acetate CoA ligase. In this embodiment, the AMP-forming acetyl CoA synthetase in the cytosol is replaced by the ADP-forming acetate CoA ligase (CoA+ acetate + ATP → acetyl-CoA + Pi + ADP) (Figure 10a). Exogenous genes to introduce acetate CoA ligase include, for example, *acdA* and *acdB* from *Pyrococcus furiosus* (Glasemacher et al., *Eur. J. Biochem.* 244:561-567 (1997)) (Mai and Adams, *J. Bacteriol.* 178: 5897-5903 (1996)). The introduction of this enzyme that uses one equivalent of ATP for formation of each molecule of acetyl CoA (as opposed to 2 ATP equivalents) allows the production of dodecanol to be energetically neutral. In this embodiment, a small amount of oxygen or other electron acceptor respiration is used to generate energy to support growth. Such small amounts of oxygen are referred to as microaerobic conditions, as described further below. In some embodiments, the ethanol-specific alcohol dehydrogenases is disrupted to prevent ethanol formation. In embodiments incorporating CoA ligase, one or more of the following knockouts can be introduced for LCA production: cytosolic malate dehydrogenase, glycerol-3-phospate dehydrogenase shuttle, the external NADH dehydrogenase, and the internal mitochondrial NADH dehydrogenase. The growth-coupled production after imposition of these disruptions is shown in Figure 10b in dark gray. The black curve shows the production envelope for the wild-type strain under aerobic conditions and the light gray curve shows the envelope when the network is augmented with acetate-CoA ligase. Note the increase in the maximum theoretical yield of dodecanol after introduction of this enzyme.

In some embodiments, the non-naturally occurring eukaryotic organism incorporates an exogenous gene encoding an acylating acetaldehyde dehydrogenase. Improvement in the energetics of the dodecanol process can be accomplished by using the acylating acetaldehyde dehydrogenase (acetaldehyde + CoA + NAD → acetyl-CoA + NADH) for the conversion of acetaldehyde into acetyl CoA (Figure 11a). The benefits of using this enzyme are that (i) no energy is expended for production of acetyl CoA, and (ii) one molecule of NADH is formed for every molecule of acetyl CoA formed. Thus, the reducing equivalents needed for the production of acetyl CoA can also be generated. The introduction of this enzyme allows production of LCA under anaerobic conditions.

Acylating acetaldehyde dehydrogenase has been reported in several bacteria, including *Acetobacterium woodii* (Mai and Adams, *J. Bacteriol.* 178:5897-5903 (1996)), *Clostridium kluyveri* (Seedorf et al., *Proc. Natl. Acad. Sci. U. S. A* 105:2128-2133 (2008); Smith and Kaplan, Arch. Biochem. Biophys. 203:663-675(1980)), *Clostridium beijerinckii* (Yan et al., *Appl. Environ. Microbiol* 56:2591-2599 (1990)), and in species of *Pseudomonas* such as strain CF600 (Lei et al., *Biochemistry* 47:6870-6882 (2008); Manjasetty et al., *Acta Crystallogr. D. Biol. Crystallogr.* 57:582-585 (2001)). The Genbank ids of genes are shown in Table 5 below.

Table 5

Ald	YP_001394464.1	*Clostridium kluyveri*
dmpF	CAA43226.1	*Pseudomonas sp. CF600*
bphG	BAA03892.1	*Pseudomonas sp*
mhpD	NP_414884.1	*Escherichia coli* K12 MG1655

In some embodiments each of the strains above can be supplemented with additional disruptions. Alternatively, some other enzymes not known to possess significant activity under the growth conditions can become active due to adaptive evolution or random mutagenesis and can also be disrupted.

The anaerobic growth-coupled production of dodecanol (or any LCA) can be accomplished by disrupting ethanol-specific alcohol dehydrogenase activity. The introduction of an acylating acetaldehyde dehydrogenase, with its favorable energetics, prevents or reduces carbon flux through the native acetaldehyde dehydrogenase and the acetyl-CoA synthetase. The production envelope is shown in Figure 11b. The wild-type *S. cerevisiae* (black) network can form only small amounts of dodecanol as an byproduct of growth under anaerobic conditions. When the network is augmented with acylating dehydrogenase, there is an increase in the theoretical maximum yield in the network, but no growth-coupling is observed (dotted light gray curve). However, disruption of ethanol-specific alcohol dehydrogenase from the augmented network shows that dodecanol production is coupled to growth at the maximum feasible biomass in the network (dark gray curve).

In some embodiments, the non-naturally occurring eukaryotic organism uses a cytosolic pyruvate dehydrogenase for dodecanol production. Cytosolic pyruvate dehydrogenase for generating the precursors for the MI-LCA pathway are shown in Figure 12. In such embodiments, (i) pyruvate

is directly converted into acetyl CoA in the cytosol without the expenditure of energy, and (ii) more reducing equivalents are available to the cell.

In some embodiments, the non-naturally occurring eukaryotic organism is engineered to retarget the native mitochondrial pyruvate dehydrogenase to the cytosol. In other embodiments, a heterologous cytosolic enzyme is introduced into the organism. The retargeting of an enzyme to a different compartment can be accomplished by changing the targeting sequence of the protein (van Loon and Young, *EMBO J.* 5:161-165 (1986)). Disruption of the native pyruvate decarboxylase enables a majority of the carbon flux to be introduced into the cytosol for processing by cytosolic pyruvate dehydrogenase. This also allows the production of dodecanol under anaerobic conditions. The growth-coupled production envelope is similar to that depicted in Figure 11b. Note that pyruvate decarboxylase is disrupted instead of alcohol dehydrogenase to achieve growth-coupling in the network.

In some embodiments, the non-naturally occurring eukaryotic organism uses a cytosolic pyruvate:NADP oxidoreductase. Pyruvate: NADP oxidoreductase allows for the production of acetyl CoA and reducing equivalents in the cytosol as shown in Figure 13. The addition of this enzyme allows for the production of acetyl CoA without expending energy that would otherwise have been required by acetyl CoA synthetase. The enzyme has been purified from the mitochondrion of *Euglena gracilis* and is oxygen-sensitive (Inui et al., *Journal of Biochemistry* 96:931-934 (1984); Inui et al., *Archives of Biochemistry and Biophysics* 237:423-429 (1985); Inui et al., *Archives of Biochemistry and Biophysics* 274:434-442 (1989); Inui et al., *Archives of Biochemistry and Biophysics* 280:292-298 (1990)). It is used for generating acetyl CoA from pyruvate, simultaneously producing NADPH. The corresponding gene is *pno* and its Genbank id is: CAC37628.1. It can be targeted to the cytosol by removing the mitochondrial targeting sequence. In some embodiments, a transhydrogenase is also added. This enzyme can be introduced as an exogenous gene from an organism such as *E. coli* to convert the generated NADPH into NADH (Nissen et al., *Yeast* 18:19-32 (2001)).

With its low ATP requirements, the pathway is energetically favorable even under anaerobic conditions. To prevent or reduce the utilization of NADH and pyruvate for ethanol production, pyruvate decarboxylase activity can be disrupted. This leads to a growth-coupled production of dodecanol similar to that shown in Figure 11b.

In some embodiments, a non-naturally occurring eukaryotic organism uses a pyruvate formate lyase. In such embodiments, a heterologous cytosolic pyruvate formate lyase (*pfl*) is used to generate both acetyl CoA and NADH as shown in Figure 14. This enzyme is active typically

under anaerobic conditions in organisms such as *E. coli*. The lack of energy requirement for conversion of pyruvate into acetyl CoA makes the production of dodecanol feasible under anaerobic conditions.

The conversion of pyruvate into acetyl CoA is accompanied by the production of formate. This is metabolized by the native formate dehydrogenase, leading to additional generation of reducing equivalents in stoichiometric quantities. In some embodiments that use this strain design, one or more of the three pyruvate decarboxylases, *PDC1*, *PDC5 and PDC6*, can be disrupted. The Genbank ids of exemplary genes encoding pyruvate formate lyase are shown in Table 6 below.

Table 6

pflB	NP_415423.1	*Escherichia coli*
pfl	YP_001588758.	*Lactococcus lactis*
pfl	YP_001394497.1	*Clostridium kluyveri*

The disruption of pyruvate decarboxylase along with the introduction of a heterologous pyruvate formate lyase in the network leads to a growth-coupled production of dodecanol. The production curve is similar to what is shown in Figure 11b.

While the non-naturally occurring eukaryotic organisms described above produce LCAs in the cytosol, it is also possible to produce LCAs in the mitochondrion. Exemplary designs for the distribution of the carbon flux towards dodecanol production are detailed herein below. Organisms that produce LCAs in the mitochondrion include one or more disruptions in genes that encode enzymes such as a cytosolic pyruvate decarboxylase, a cytosolic ethanol-specific alcohol dehydrogenase, and amitochondrial ethanol-specific alcohol dehydrogenase. Exemplary genese encoding these enzymes include, for example, YLR044C, YLR134W, YGR087C, PDC3, YBR145W, YGL256W, YOL086C, YMR303, YMR083W, YPL088W, YAL061W, YMR318C, YCR105W, and YDL168W.

Other genes disruptions include those encoding an enzyme suchas a cytosolic malate dehydrogenase, glycerol-3-phospate dehydrogenase shuttle, catalyzed by, the external NADH dehydrogenase, and internal NADH dehydrogenase. Exemplary genes of the latter include, for example, YOL126C, YDL022W, YOL059W, YIL155C, YMR145C, YDL085W, and YML120C.

Organisms that produce LCAs in the mitochondrion can also include an exogenous nucleic acid encoding an enzyme such as a pyruvate dehydrogenase, a pyruvate: NADP oxidoreductase, a pyruvate formate lyase, an acylating acetaldehyde dehydrogenase, an acetate CoA ligase, and an

AMP-forming acetyl CoA synthetase or their corresponding gene regulatory regions as described above. Additionally, such organisms benefit from enhanced NADH transporting shuttle systems for transport of NADH from the cytosol into the mitochondrion. Other exogenous nucleic acids encoding an enzyme that can be inserted in such organisms include a transhydrogenase, formate dehydrogenase, a pyruvate decarboxylase, and a pyruvate oxidase, all in the mitochondrion, or their corresponding gene regulatory regions.

In one embodiment a mitochondrial pyruvate dehydrogenase is used in the non-naturally occurring eukaryotic organism. This can be the native pyruvate dehydrogenase which produces both acetyl CoA and NADH as shown in Figure 15a. Since, there is no energy requirement for the conversion of pyruvate to acetyl CoA via this route; the production of dodecanol, for example, is energetically favorable even under anaerobic conditions.

The mitochondrial pyruvate dehydrogenase is known to be active in both aerobic and anaerobic conditions in *S. cerevisiae* (Pronk et al., *Yeast* 12:1607-1633 (1996)). In some embodiments the enzyme is overexpressed in its native or a heterologous form. The native enzyme can be overexpressed by using a stronger promoter. Additionally, mutations can be introduced aimed at increasing its activity under anaerobic conditions (Kim et al., *J. Bacteriol.* 190:3851-3858 (2008)). Reducing equivalents generated in the cytosol are made available in the mitochondrion for dodecanol production by using the redox shuttles present in *S. cerevisiae*. Note that these shuttles transport NADH into the mitochondrion for energy generation under respiratory conditions (Overkamp et al., *J. Bacteriol.* 182:2823-2830 (2000)). For growth-coupled production, pyruvate decarboxylase activity is disrupted to allow for pyruvate flux to be directed towards pyruvate dehydrogenase and to inhibit ethanol formation. The production curve for the mutant network is shown in Figure 15b.

In some embodiments, a non-naturally occurring eukaryotic organism uses a heterologous pyruvate:NADP-oxidoreductase. The production of dodecanol in the mitochondrion can be achieved by introduction of the pyruvate:NADP oxidoreductase in the mitochondrion as shown in Figure 16. This enzyme is purified from *E. gracilis*. Since the enzyme is naturally present in mitochondrion and is active under anaerobic conditions, it is possible to get high activity of the enzyme under anaerobic conditions. The introduction of this enzyme provides the precursor acetyl CoA for dodecanol production and also reducing equivalents. The NADPH generated by the enzyme is converted into NADH by a transhydrogenase, which can be introduced into the mitochondrion. For additional reducing equivalents, the redox shuttles need to transport NADH from the cytosol to the mitochondrion. The growth-coupled production of LCA using this

enzyme can be obtained by disruption of pyruvate decarboxylase. The production curve of the mutant strain is very similar to the one shown in Figure 15b.

In some embodiments, a non-naturally occurring eukaryotic organism uses a heterologous pyruvate formate lyase. The production of dodecanol using a pyruvate formate lyase in mitochondrion is shown in Figure 17. These genes have been outlined herein above. In such embodiments, the native formate dehydrogenase is retargeted to the mitochondrion to allow for further metabolizing formate and generating more reducing equivalents. This strain can be adopted to carry sufficient flux to sustain high yield and productivity of LCA production in the mitochondrion in the absence of oxygen.

Anaerobic growth conditions are feasible for the production of dodecanol using this strain design. Redox shuttles can be overexpressed to transport NADH generated in the cytosol to the mitochondrion. Production in this scenario is possible by disrupting the cytosolic pyruvate decarboxylase activity. The production characteristics of the mutant strain are similar to that shown in Figure 15b.

In some embodiments, a non-naturally occurring eukaryotic organism uses a heterologous acetaldehyde dehydrogenase (acylating). In such embodiments, an acylating acetaldehyde dehydrogenase is introduced into the mitochondrion to provide both acetyl-CoA and NADH for LCA production as shown in Figure 18. A pyruvate decarboxylase isozyme is retargeted to the mitochondrion to convert pyruvate into acetaldehyde in some embodiments. The expression of these two activities in the mitochondrion is equivalent to the activity of pyruvate dehydrogenase. The growth-coupled production curve is the same as that shown in Figure 15b. The growth-coupled production strain has the native mitochondrial acetaldehyde dehydrogenase (Pronk et al., *Yeast* 12:1607-1633 (1996)) and the cytosolic pyruvate decarboxylase disrupted in some embodiments. In other embodiments, the mitochondrial ethanol-specific alcohol dehydrogenase is also disrupted to prevent the conversion of acetaldehyde into ethanol.

In some embodiments, a non-naturally occurring eukaryotic organism uses a mitochondrial acetyl CoA synthetase (AMP-forming). As discussed above, the expression of this enzyme requires oxygen for favorable energetics. ACS1, an isozyme of acetyl CoA synthetase is expressed in *S. cerevisiae* in the mitochondrion under aerobic conditions but is repressed by glucose. This enzyme can be mutated to eliminate the repression or a heterologous enzyme that is expressed under the conditions of interest can be introduced. Additionally, pyruvate decarboxylase also can be expressed in the mitochondrion to form acetate. *S. cerevisiae*, for example, already possesses a mitochondrial acetaldehyde dehydrogenase (Pronk et al., *Yeast*

12:1607-1633 (1996)). Alternatively, enzymes such as pyruvate oxidase can be heterologously expressed to convert pyruvate into acetate. One such enzyme candidate is pyruvate oxidase from *E. coli* (Genbank id: NP_451392.1). This enzyme is naturally expressed in the presence of oxygen.

The production of LCA using this strain design benefits from one or more of the following disrupted enzymes: cytosolic malate dehydrogenase, the glycerol-3-phospate dehydrogenase shuttle, the external NADH dehydrogenase, and the internal mitochondrial NADH dehydrogenase. The glycerol-3-phosphate shuttle is comprised of the cytosolic glycerol-3-phosphate dehydrogenase and the membrane-bound glycerol-3-phosphate:ubiquionone oxidoreductase, with the latter also functioning as the mitochondrial glycerol-3-phosphate dehydrogenase. In some embodiments, the mitochondrial ethanol-specific alcohol dehydrogenase is also disrupted to prevent or reduce the conversion of acetaldehyde into ethanol. The production curve for the wild type strain with a mitochondrial pyruvate decarboxylase added to the network is shown in black in Figure 19b. This curve is shown for aerobic conditions. The production characteristics when the aforementioned disruptions are imposed on the network are shown in light gray. The downregulation of the oxidative part of the pentose phosphate pathway, especially the committing step, glucose-6-phosphate dehydrogenase, further improves the LCA production characteristics of the network.

In some embodiments, a non-naturally occurring eukaryotic organism uses a mitochondrial acetate CoA ligase (ADP-forming). Mitochondrial LCA production can also be accomplished using an acetate-CoA ligase to convert acetate into acetyl-CoA as shown in Figure 20. As described above, the use of this enzyme is energetically favorable and LCA production is energetically neutral unless oxygen is supplied. The mitochondrial expression of pyruvate decarboxylase is used in such embodiments. LCA production is obtained by imposing disruptions in cytosolic malate dehydrogenase, the glycerol-3-phospate dehydrogenase shuttle, the external NADH dehydrogenase, and the internal NADH dehydrogenase. The down-regulation of the oxidative part of the pentose phosphate pathway further improves the growth-coupled production characteristics to yield a production curve similar to the one shown in Figure 19b. In some embodiments, the mitochondrial ethanol-specific alcohol dehydrogenase is also disrupted to prevent or reduce the conversion of acetaldehyde into ethanol.

The design strategies described herein are useful not only for enhancing growth-coupled production, but they are also well-suited for enhancing non-growth coupled production because they link the production of long chain alcohols to energy generation and/or redox balance.

Exemplary non-growth coupled production methods include implementing an aerobic growth phase followed by an anaerobic production phase. For example, Vemuri *et al. J. Ind. Microbiol. Biotechnol.* (6):325-332, (2002) describe a dual-phase process for the production of succinate in *E. Coli*. Okino *et al. Appl. Microbiol. Biotechnol.* Sep 6. (2008) [Currently available in online edition]. describe a similar non-growth couple production process in a strain of *Corynebacterium glutamicum* strain.

Another such method involves withholding an essential nutrient from a propogated cell culture, thereby limiting growth, but not precluding production as described in Durner *et al. Appl. Environ. Microbiol.* (8):3408-3414(2000). Yet another strategy aimed at decoupling growth from production involves replacing the growth substrate with another compound that is more slowly metabolizable as described in Altamirano *et al. Biotechnol. Bioeng.* 76:351-360 (2001). Growth decoupled-product formation can also be brought about by specific genetic modifications as described in Blombach *et al. Appl. Microbiol. Biotechnol.* 79:471-9 (2008).

Some microbial organisms capable of LCA production are exemplified herein with reference to an *Saccharomyces cerevisaie* genetic background. However, with the complete genome sequence available now for more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of an alternate species homolog for one or more genes, including for example, orthologs, paralogs and nonorthologous gene displacements, and the interchange of genetic alterations between eukaryotic organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling production of LCA described herein with reference to a particular organism such as *Saccharomyces cerevisaie* can be readily applied to other microorganisms. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

The methods of the invention are applicable to various eukarotic organisms such as yeast and fungus. The yeast can include *S. cerevisiae* and *Rhizopus arrhizus*, for example. Exemplary eukaryotic species include those selected from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Candida albicans*, *Candida boidinii* and *Pichia pastoris*. Additionally, select cells from larger eukaryotic organisms are also applicable to methods of the present invention.

Genes can be inserted into *S. cerevisiae*, using several methods; some of these are plasmid-based whereas others allow for the incorporation of the gene in the chromosome. The latter approach employs an integrative promoter based expression vector, for example, the pGAPZ or the pGAPZα vector based on the GAP promoter. The expression vector constitutes the GAP promoter, the HIS4 wild-type allele for integration and the 3' AOX transcription termination region of *P. pastoris* in addition to a KanMX cassette, flanked by loxP sites enabling removal and recycling of the resistance marker. The vectors are commercially available from Invitrogen. The details of which are elaborated in the Example below.

The engineered strains are characterized by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. Cultures are grown overnight and used as inoculum for a fresh batch culture for which measurements are taken during exponential growth. The growth rate is determined by measuring optical density using a spectrophotometer (A600). Concentrations of glucose, alcohols, and other organic acid byproducts in the culture supernatant will be determined by analytical methods including HPLC using an HPX-87H column (BioRad), or GC-MS, and used to calculate uptake and secretion rates. All experiments are performed with triplicate cultures.

The invention also provides a method for producing long chain alcohols by culturing the non-naturally occurring eukaryotic organism described herein above. The one or more gene disruptions occur in genes encoding an enzyme to coupling long chain alcohol production to growth of the organism when the gene disruption reduces an activity of the enzyme. The one or more gene disruptions confers stable growth-coupled production of long chain alcohols onto the organism. In alternate embodiments the gene disruptions can enhance LCA production in a non-growth dependent manner.

Each of the strains presented herein may be supplemented with additional disruptions if it is determined that the predicted strain designs do not sufficiently couple the formation of LCAs with biomass formation. However, the list of gene disruption sets provided here serves as an excellent starting point for the construction of high-yielding growth-coupled LCA production strains.

Each of the proposed strains can be supplemented with additional disruptions if it is determined that the predicted strain designs do not sufficiently couple the formation of the product with biomass formation. Alternatively, some other enzymes not known to possess significant activity under the growth conditions can become active due to adaptive evolution or random mutagenesis

and can also be disrupted. However, the list of gene disruption sets provided here serves as a starting point for construction of high-yielding growth-coupled LCA production strains.

The non-naturally occurring microbial organisms of the invention can be employed in the growth-coupled production of LCA. Essentially any quantity, including commercial quantities, can be synthesized using the growth-coupled LCA producers of the invention. Because the organisms of the invention obligatorily couple LCA to continuous growth or near-continuous growth processes are particularly useful for biosynthetic production of LCA. Such continuous and/or near continuous growth processes are described above and exemplified below in the Example I. Continuous and/or near-continuous microorganism growth processes also are well known in the art. Briefly, continuous and/or near-continuous growth processes involve maintaining the microorganism in an exponential growth or logarithmic phase. Procedures include using apparatuses such as the Evolugator™ evolution machine (Evolugate LLC, Gainesville, FL), fermentors and the like. Additionally, shake flask fermentation and grown under microaerobic conditions also can be employed. Given the teachings and guidance provided herein those skilled in the art will understand that the growth-coupled LCA producing microorganisms can be employed in a variety of different settings under a variety of different conditions using a variety of different processes and/or apparatuses well known in the art.

Generally, the continuous and/or near-continuous production of LCA will include culturing a non-naturally occurring growth-coupled LCA producing organism of the invention in sufficient nutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can be grown, for example, for a day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous cultures can include time durations of 1 week, 2, 3, 4 or 5 or more weeks and up to several months. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods. In particular embodiments, culturing is conducted in a substantially anaerobic culture medium.

LCA can be harvested or isolated at any time point during the continuous and/or near-continuous culture period exemplified above. As exemplified below, the longer the microorganisms are maintained in a continuous and/or near-continuous growth phase, the proportionally greater amount of LCA can be produced.

Therefore, the invention provides a method for producing LCA that includes culturing a non-naturally occurring microbial organism that includes one or more gene disruptions. The disruptions can occur in genes encoding an enzyme to coupling LCA production to growth of the

microorganism when the gene disruption reduces an activity of the enzyme, such that the disruptions confer stable growth-coupled production of LCA onto the non-naturally microbial organism.

In some embodiments, the gene disruption can include a complete gene deletion. In some embodiments other means to disrupt a gene include, for example, frameshifting by omission or addition of oligonucleotides or by mutations that render the gene inoperable. One skilled in the art will recognize the advantages of gene deletions, however, because of the stability it may confer to the non-naturally occurring organism from reverting to its wild-type. In particular, the gene disruptions are selected from the gene set that includes genes detailed herein above.

The metabolic engineering strategies listed in this disclosure assume that the organism can produce long chain alcohols via the malonyl-CoA independent pathway. The construction of a recombinant host organism capable of producing long chain alcohols via the malonyl-CoA independent pathway involves engineering a non-naturally occurring microbial organism having a malonyl-CoA-independent fatty acid synthesis (FAS) pathway and an acyl-reduction pathway having at least one exogenous nucleic acid encoding a malonyl-CoA-independent FAS pathway enzyme expressed in sufficient amounts to produce a primary alcohol. Such a malonyl-CoA-independent FAS pathway includes a ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase. The acyl-reduction pathway includes an acyl-CoA reductase and an alcohol dehydrogenase.

In order to validate the computational predictions presented herein, the strains must be constructed, evolved, and tested. *Escherichia coli* K-12 MG1655 housing the MI-LCA pathway will serve as the strain into which the disruptions will be introduced. The disruptions will be constructed by incorporating in-frame deletions using homologous recombination via the λ Red recombinase system of Datsenko and Wanner (Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.). The approach involves replacing a chromosomal sequence (i.e., the gene targeted for removal) with a selectable antibiotic resistance gene, which itself is later removed. Knockouts are integrated one by one into the recipient strain. No antibiotic resistance markers remain after each deletion allowing accumulation of multiple mutations in each target strain. The deletion technology completely removes the gene targeted for removal so as to substantially reduce the possibility of the constructed mutants reverting back to the wild-type.

As intermediate strains are being constructed, strain performance will be quantified by performing shake flask fermentations. Anaerobic conditions will be obtained by sealing the flasks with a rubber septum and then sparging the medium with nitrogen. For strains where growth is not observed under strict anaerobic conditions, microaerobic conditions can be applied by covering the flask with foil and poking a small hole for limited aeration. All experiments are performed using M9 minimal medium supplemented with glucose unless otherwise stated. Pre-cultures are grown overnight and used as inoculum for a fresh batch culture for which measurements are taken during exponential growth. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time. LCAs, ethanol, and organic acids are analyzed by GC-MS or HPLC using routine procedures. Triplicate cultures are grown for each strain.

The performance of select strains is tested in anaerobic, pH-controlled batch fermentations. This enables reliable quantification of the growth, glucose uptake, and formation rates of all products, as well as ensuring that the accumulation of acidic fermentation products will not limit cell growth. In addition, it allows accurate determination of LCA volumetric productivity and yield, two important parameters in benchmarking strain performance. Fermentations are carried out in 1-L bioreactors with 600 mL working volume, equipped with temperature and pH control. The reactor is continuously sparged with N_2 at approximately 0.5 L/min to ensure that DO levels remain below detection levels. The culture medium is the same as described above, except that the glucose concentration is increased in accordance with the higher cell density achievable in a fermentation vessel.

Chemostat experiments will be conducted to obtain a direct measure of how the switch in fermentation mode from batch to continuous affects LCA yield and volumetric productivity. The bioreactors described above using batch mode are operated in chemostat mode through continuous supply of medium and removal of spent culture. The inlet flow rate is set to maintain a constant dilution rate of 80% of the maximum growth rate observed for each strain in batch, and the outlet flow is controlled to maintain level. Glucose is the limiting nutrient in the medium, and set to achieve the desired optical density in the vessel.

The recombinant strains are initially expected to exhibit suboptimal growth rates until their metabolic networks have adjusted to their missing functionalities. To enable this adjustment, the strains are adaptively evolved. By subjecting the strains to adaptive evolution, cellular growth rate becomes the primary selection pressure and the mutant cells are compelled to reallocate their metabolic fluxes in order to enhance their rates of growth. This reprogramming of metabolism

has been recently demonstrated for several *E. coli* mutants that had been adaptively evolved on various substrates to reach the growth rates predicted *a priori* by an *in silico* model (Fong, S.S. and B.O. Palsson, *Metabolic gene-deletion strains of Escherichia coli evolve to computationally predicted growth phenotypes.* Nat Genet, 2004. **36**(10): p. 1056-8.). The OptKnock-generated strains are adaptively evolved in triplicate (running in parallel) due to differences in the evolutionary patterns witnessed previously in *E. coli* (Fong, S.S. and B.O. Palsson, *Metabolic gene-deletion strains of Escherichia coli evolve to computationally predicted growth phenotypes.* Nat Genet, 2004. **36**(10): p. 1056-8; Fong, S.S., J.Y. Marciniak, and B.O. Palsson, *Description and interpretation of adaptive evolution of Escherichia coli K-12 MG1655 by using a genome-scale in silico metabolic model.* J Bacteriol, 2003. **185**(21): p. 6400-8; Ibarra, R.U., J.S. Edwards, and B.O. Palsson, *Escherichia coli K-12 undergoes adaptive evolution to achieve in silico predicted optimal growth.* Nature, 2002. **420**(6912): p. 186-189.) that could potentially result in one strain having superior production qualities over the others. Evolutions are run for a period of 2-6 weeks, depending upon the rate of growth improvement attained. In general, evolutions are stopped once a stable phenotype is obtained. The growth-coupled biochemical production concept behind the OptKnock approach results in the generation of genetically stable overproducers.

The engineered strains can be characterized by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. Cultures are grown overnight and used as inoculum for a fresh batch culture for which measurements are taken during exponential growth. The growth rate can be determined by measuring optical density using a spectrophotometer (A600). Concentrations of glucose and other organic acid byproducts in the culture supernatant are determined by HPLC using an HPX-87H column (BioRad), and used to calculate uptake and secretion rates. All experiments are performed with triplicate cultures.

Following the adaptive evolution process, the new strains are characterized again by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. These results will be compared to the OptKnock predictions by plotting actual growth and production yields along side the production envelopes in the above figures. The most successful OptKnock design/evolution combinations are chosen to pursue further, and are characterized in lab-scale batch and continuous fermentations. The growth-coupled biochemical production concept behind the OptKnock approach should also result in the generation of genetically stable overproducers. Thus, the cultures are maintained in continuous mode for one month to evaluate long-term stability. Periodic samples are taken to ensure that yield and productivity are maintained throughout the experiment.

As previously mentioned, one computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)). The framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become a byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or disruptions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed January 10, 2002, in International Patent No. PCT/US02/00660, filed January 10, 2002, and U.S. patent application serial No. 11/891,602, filed August 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003. SimPheny® is a computational system that can be used to produce a network model *in silico* and to simulate the flux of mass, energy or charge through

the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as a product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., *Biotechnol. Prog.* 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an *in silico* method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene disruption combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction disruptions requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)).

An *in silico* stoichiometric model of *Escherichia coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Patent No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene disruptions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of disruptions. To enumerate all meaningful solutions, that is, all sets of disruptions leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

Adaptive evolution is a powerful experimental technique that can be used to increase growth rates of mutant or engineered microbial strains, or of wild-type strains growing under unnatural environmental conditions. It is especially useful for strains designed via the OptKnock formalism, which results in growth-coupled product formation. Therefore, evolution toward optimal growing strains will indirectly optimize production as well. Unique strains of *E. coli* K-12 MG1655 were created through gene knockouts and adaptive evolution. (Fong, S. S. and B. O. Palsson, *Nat.Genet.* 36:1056-1058 (2004).) In this work, all adaptive evolutionary cultures were maintained in prolonged exponential growth by serial passage of batch cultures into fresh medium before the stationary phase was reached, thus rendering growth rate as the primary selection pressure. The genes that were selected for this knockout study were *ackA, frdA, pckA, ppc, tpiA,* and *zwf.* Knockout strains were constructed and evolved on minimal medium supplemented with different carbon substrates (four for each knockout strain). Evolution cultures were carried out in duplicate or triplicate, giving a total of 50 evolution knockout strains.

The evolution cultures were maintained in exponential growth until a stable growth rate was reached. The computational predictions were accurate (i.e., within 10%) at predicting the post-evolution growth rate of the knockout strains in 38 out of the 50 cases examined. Furthermore, a combination of OptKnock design with adaptive evolution has led to improved lactic acid production strains. (Fong, S. S., A. P. Burgard, C. D. Herring, E. M. Knight, F. R. Blattner, C. D. Maranas, and B. O. Palsson, *Biotechnol Bioeng* 91:643-648 (2005).) The guidance of these teachings relevant to *E. coli* can be applied to other organisms.

There are a number of developed technologies for carrying out adaptive evolution. Exemplary methods are provided herein below. In some embodiments, optimization of a non-naturally occurring organism of the present invention includes subject the use of any of the these adaptive evolution techniques.

Serial culture involves repetitive transfer of a small volume of grown culture to a much larger vessel containing fresh growth medium. When the cultured organisms have grown to saturation in the new vessel, the process is repeated. This method has been used to achieve the longest demonstrations of sustained culture in the literature, (Lenski, R. E. and M. Travisano, *Proc Natl Acad Sci U S.A.* 91:6808-6814 (1994).) in experiments which clearly demonstrated consistent improvement in reproductive rate over period of years. In the experiments performed in the Palsson lab described above, transfer is usually performed during exponential phase, so each day the transfer volume is precisely calculated to maintain exponential growth through the next 24 hour period. This process is usually done manually, with considerable labor investment, and is subject to contamination through exposure to the outside environment. Furthermore, since such small volumes are transferred each time, the evolution is inefficient and many beneficial mutations are lost. On the positive side, serial dilution is inexpensive and easy to parallelize.

In continuous culture the growth of cells in a chemostat represents an extreme case of dilution in which a very high fraction of the cell population remains. As a culture grows and becomes saturated, a small proportion of the grown culture is replaced with fresh media, allowing the culture to continually grow at close to its maximum population size. Chemostats have been used to demonstrate short periods of rapid improvement in reproductive rate. (Dykhuizen, D. E., *Methods Enzymol.* 613-631 (1993).) The potential power of these devices was recognized, but traditional chemostats were unable to sustain long periods of selection for increased reproduction rate, due to the unintended selection of dilution-resistant (static) variants. These variants are able to resist dilution by adhering to the surface of the chemostat, and by doing so, outcompete less sticky individuals including those that have higher reproductive rates, thus obviating the intended

purpose of the device. (Chao, L. and G. Ramsdell *J.Gen.Microbiol* 20:132-138 (1985).) One possible way to overcome this drawback is the implementation of a device with two growth chambers, which periodically undergo transient phases of sterilization, as described in the patent by the Pasteur Institute (Marliere and Mutzel, US Patent 6,686,194, filed 1999).

Evolugator™ is a continuous culture device developed by Evolugate, LLC (Gainesville, FL) exhibits significant time and effort savings over traditional evolution techniques. (de Crecy, E., Metzgar, D., Allen, C., Penicaud, M., Lyons, B., Hansen, C.J., de Crecy-Lagard, V. *Appl. Microbiol. Biotechnol.* 77:489-496 (2007).) The cells are maintained in prolonged exponential growth by the serial passage of batch cultures into fresh medium before the stationary phase is attained. By automating optical density measurement and liquid handling, the Evolugator can perform serial transfer at high rates using large culture volumes, thus approaching the efficiency of a chemostat in evolution of cell fitness. For example, a mutant of *Acinetobacter* sp ADP1 deficient in a component of the translation apparatus, and having severely hampered growth, was evolved in 200 generations to 80% of the wild-type growth rate. However, in contrast to the chemostat which maintains cells in a single vessel, the machine operates by moving from one "reactor" to the next in subdivided regions of a spool of tubing, thus eliminating any selection for wall-growth. The transfer volume is adjustable, and normally set to about 50%.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Primary Alcohol Biosynthesis

This Example describes the generation of a microbial organism capable of producing primary alcohols using a malonyl-CoA independent FAS metabolic pathway and acyl-reduction metabolic pathways.

Escherichia coli is used as a target organism to engineer a malonyl-CoA-independent FAS and acyl-reduction pathway as shown in Figure 1.. *E. coli* provides a good host for generating a non-naturally occurring microorganism capable of producing primary alcohol, such as octanol. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, effectively under anaerobic conditions.

To generate an *E. coli* strain engineered to produce primary alcohol, nucleic acids encoding the enzymes utilized in the malonyl-CoA-independent FAS and acyl-reduction pathway as described previously, are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, *supra*, 2001; Ausubel *supra*, 1999; Roberts et al., *supra*, 1989). In particular, the *fadI/fadJ* genes (NP_416844.1 and NP_416843.1), encoding the multienzyme complex with ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase activities under anaerobic conditions, and the TDE0597 (NP_971211.1), encoding enoyl-CoA reductase, are cloned into the pZE13 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. The *acr1* gene (YP_047869.1), encoding acyl-CoA reductase, and the *alrA* gene (BAB12273.1), encoding alcohol dehydrogenase, are cloned into the pZA33 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. The two sets of plasmids are transformed into *E. coli* strain MG1655 to express the proteins and enzymes required for the malonyl-CoA-independent FAS and acyl-reduction pathway.

The resulting genetically engineered organism is cultured in glucose containing medium following procedures well known in the art (see, for example, Sambrook et al., *supra*, 2001). The expression of malonyl-CoA-independent FAS and acyl-reduction pathway genes is corroborated using methods well known in the art for determining polypeptide expression or enzymatic activity, including for example, Northern blots, PCR amplification of mRNA, immunoblotting. Enzymatic activities of the expressed enzymes are confirmed using assays specific for the individually activities (see, for example, Tucci, *supra*, 2007; Hoffmeister et al., 2005; Inui et al., *supra*, 1984; Winkler, 2003; Tani, 2000; Reiser, 1997; Ishige, 2000). The ability of the engineered *E. coli* strain to produce primary alcohol, such as octanol is confirmed using HPLC, gas chromatography-mass spectrometry (GCMS) or liquid chromatography-mass spectrometry (LCMS).

Microbial strains engineered to have a functional malonyl-CoA-independent FAS and acyl-reduction pathway is further augmented by optimization for efficient utilization of the pathway. Briefly, the engineered strain is assessed to determine whether any of the exogenous genes are expressed at a rate limiting level. Expression is increased for any enzymes expressed at low levels that can limit the flux through the pathway by, for example, introduction of additional gene copy numbers.

To generate better producers, metabolic modeling is utilized to optimize growth conditions. Modeling is also used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US

2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of primary alcohols. One modeling method is the bilevel optimization approach, OptKnock (Burgard et al., *Biotechnol. Bioengineer.* 84:647-657 (2003)), which is applied to select gene knockouts that collectively result in better production of primary alcohols. Adaptive evolution also can be used to generate better producers of, for example, the acetyl-CoA intermediate or the primary alcohol product. Adaptive evolution is performed to improve both growth and production characteristics (Fong and Palsson, *Nat. Genet.* 36:1056-1058 (2004); Alper et al., *Science* 314:1565-1568 (2006)). Based on the results, subsequent rounds of modeling, genetic engineering and adaptive evolution can be applied to the primary alcohol producer to further increase their production.

For large-scale production of primary alcohols, the above malonyl-CoA independent FAS pathway-containing organism is cultured in a fermenter using a medium known in the art to support growth of the organism under anaerobic conditions. Fermentations are performed in either a batch, fed-batch or continuous manner. Anaerobic conditions are maintained by first sparging the medium with nitrogen and then sealing culture vessel (e.g., flasks can be sealed with a septum and crimp-cap). Microaerobic conditions also can be utilized by providing a small hole for limited aeration. The pH of the medium is maintained at a pH of 7 by addition of an acid, such as H2SO4. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time. Byproducts such as undesirable alcohols, organic acids, and residual glucose can be quantified by HPLC (Shimadzu) with an HPX-087 column (BioRad), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids, Lin et al., *Biotechnol. Bioeng.*, 775-779 (2005).

Isolation of the product primary alcohol is performed based their insolubility in water. In particular, a two-phase fermentation process is used for separation of these product alcohols where they can either form a separate phase or be readily extracted in an organic phase from the fermentation broth. Residual cells and any other insoluble impurities are removed by filtration, allowing a continuous or semi-continuous fermentation process.

EXAMPLE II

Microorganisms Having Growth-coupled Production of LCA

This Example describes the construction *in silico* designed strains for the growth-coupled production of LCA.

E. coli K-12 MG1655 serves as the wild-type strain into which the disruptions are introduced. The disruptions are constructed by incorporating in-frame deletions using homologous recombination via the λ Red recombinase system of Datsenko and Wanner. (Datsenko, K.A. and B.L. Wanner, *Proc Natl Acad Sci U S A.*, 97(12):6640-5 (2000).) The approach involves replacing a chromosomal sequence (i.e., the gene targeted for removal) with a selectable antibiotic resistance gene, which itself is later removed. Knockouts are integrated one by one into the recipient strain. No antibiotic resistance markers will remain after each deletion allowing accumulation of multiple mutations in each target strain. The deletion technology completely removes the gene targeted for removal so as to substantially reduce the possibility of the constructed mutants reverting back to the wild-type.

As described further below, one exemplary growth condition for achieving biosynthesis of LCA includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organism of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N_2/CO_2 mixture or other suitable non-oxygen gas or gases.

The engineered strains are characterized by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. Cultures are grown overnight and used as inoculum for a fresh batch culture for which measurements are taken during exponential growth. The growth rate is determined by measuring optical density using a spectrophotometer (A600). Concentrations of glucose, LCA, and other organic acid byproducts in the culture supernatant are determined by HPLC using an HPX-87H column (BioRad), and are used to calculate uptake and secretion rates. All experiments are performed with triplicate cultures.

The recombinant strains can exhibit suboptimal growth rates until their metabolic networks have adjusted to their missing functionalities. To enable this adjustment, the strains are adaptively evolved. By subjecting the strains to adaptive evolution, cellular growth rate becomes the primary selection pressure and the mutant cells are compelled to reallocate their metabolic fluxes in order to enhance their rates of growth. This reprogramming of metabolism has been recently

demonstrated for several *E. coli* mutants that had been adaptively evolved on various substrates to reach the growth rates predicted *a priori* by an *in silico* model. (Fong, S.S. and B.O. Palsson, *Nat Genet*, 36(10):1056-8 (2004).) These teachings can be applied to *Escherichia coli.*

Should the OptKnock predictions prove successful; the growth improvements brought about by adaptive evolution will be accompanied by enhanced rates of LCA production. The OptKnock-generated strains are adaptively evolved in triplicate (running in parallel) due to differences in the evolutionary patterns witnessed previously in *E. coli* (Fong, S.S. and B.O. Palsson, *Nat Genet*, 36(10):1056-8 (2004); Fong, S.S., J.Y. Marciniak, and B.O. Palsson, *J Bacteriol*, 185(21):6400-8 (2003); Ibarra, R.U., J.S. Edwards, and B.O. Palsson, *Nature*, 420(6912):186-189 (2002)) that could potentially result in one strain having superior production qualities over the others. Evolutions are run for a period of 2-6 weeks, depending upon the rate of growth improvement attained. In general, evolutions are stopped once a stable phenotype is obtained.

The adaptive evolution procedure involves maintaining the cells in prolonged exponential growth by the serial passage of batch cultures into fresh medium before the stationary phase is attained. Briefly, one procedure allows cells to reach mid-exponential growth (A_{600}=0.5) before being diluted and passed to fresh medium (i.e., M9 minimal media with 2 g/L carbon source). This process is repeated, allowing for about 500 generations for each culture. Culture samples are taken, frozen with liquid nitrogen, and the optical culture density recorded for each day throughout the course of the evolutions. The evolutions are performed in triplicate due to differences in the evolutionary patterns witnessed previously Donnelly et al., *Appl Biochem Biotechnol* 70-72: 187-98 (1998); Vemuri et al., *Appl Environ Microbiol* 68:1715-27 (2002), that could potentially result in one strain having superior production qualities over the others. The adaptive evolution step can take up to about two months or more. The adaptive evolution step also can be less than two months depending on the strain design, for example.

Another process can evolve cells using automation technology and is commercially available by Evolugate, LLC (Gainesville, FL) under a service contract. The procedure employs the Evolugator™ evolution machine which results in significant time and effort savings over non-automated evolution techniques. Cells are maintained in prolonged exponential growth by the serial passage of batch cultures into fresh medium before the stationary phase is attained. By automating optical density measurement and liquid handling, the Evolugator can perform serial transfer at high rates using large culture volumes, thus approaching the efficiency of a chemostat for evolution of cell fitness. For example, a mutant of *Acinetobacter* sp ADP1 deficient in a component of the translation apparatus, and having severely hampered growth, was evolved in

200 generations to 80% of the wild-type growth rate. However, in contrast to the chemostat which maintains cells in a single vessel, the machine operates by moving from one "reactor" to the next in subdivided regions of a spool of tubing, thus eliminating any selection for wall-growth. The transfer volume is adjustable, and normally set to about 50%.

In contrast to a chemostat, which maintains cells in a single vessel, the machine operates by moving from one "reactor" to the next in subdivided regions of a spool of tubing, thus eliminating any selection for wall-growth. Culture samples are taken, frozen with liquid nitrogen, and the optical culture density recorded each day throughout the course of the evolutions. The Evolugator is used for each strain until a stable growth rate is achieved. Growth rate improvements of nearly 50% have been observed in two weeks using this device. The above-described strains are adaptively evolved in triplicate (running in parallel). At ten day intervals, culture samples are taken from the Evolugator, purified on agar plates, and cultured in triplicate as discussed above to assess strain physiology. Evolugator™ is a continuous culture device that exhibits significant time and effort savings over traditional evolution techniques. (de Crecy et al., *Appl. Microbiol. Biotechnol.* 77:489-496 (2007)).

Following the adaptive evolution process, the new strains are again characterized by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. These results are compared to the OptKnock predictions by plotting actual growth and production yields along side the production envelopes. The most successful OptKnock design/evolution combinations are chosen to pursue further, and is characterized in lab-scale batch and continuous fermentations. The growth-coupled biochemical production concept behind the OptKnock approach should also result in the generation of genetically stable overproducers. Thus, the cultures can be maintained in continuous mode for one month to evaluate long-term stability. Periodic samples will be taken to ensure that yield and productivity are maintained throughout the experiment.

EXAMPLE III

Microorganisms Having Growth-coupled Production of LCA

This Example describes the construction *in silico* designed strains for the growth-coupled production of LCA.

Gene deletions are introduced into *S. cerevisiae* by homologous recombination of the gene interrupted by the KanMX cassette, flanked by loxP sites enabling removal and recycling of the resistance marker (e.g. URA3) (Wach, A., et al., *PCR-based gene targeting in Saccharomyces*

cerevisiae, in *Yeast Gene Analysis*, M.F. Tuite, Editor. 1998, Academic Press: San Diego). Starting with a loxP-kanMX-loxP sequence on a plasmid, an artificial construct with this sequence flanked by fragments of the gene of interest are created by PCR using primers containing both 45-50 bp target sequence followed by a region homologous to the above cassette. This linear DNA is transformed into wild-type *S. cerevisiae*, and recombinants are selected by geneticin resistance (Wach, A., *et al. supra*]. Colonies are purified and tested for correct double crossover by PCR. To remove the KanMX marker, a plasmid containing the Cre recombinase and bleomycin resistance are introduced, promoting recombination between the loxP sites (Gueldener, U., *et al.*, *Nucleic Acids Res.* e23 (2002))]. Finally, the resulting strain is cured of the Cre plasmid by successive culturing on media without any antibiotic present. The final strain will have a markerless gene deletion, and thus the same method can be used to introduce multiple deletions in the same strain.

The strains are constructed, evolved, and tested by methods disclosed herein. Genes can be inserted into *S. cerevisiae*, for example, using several methods. These methods can be plasmid-based whereas others allow for the incorporation of the gene in the chromosome. The latter approach employs an integrative promoter based expression vector, for example, the pGAPZ or the pGAPZα vector based on the GAP promoter. The expression vector constitutes the GAP promoter, the HIS4 wild-type allele for integration and the 3' AOX transcription termination region of *P. pastoris* in addition to a KanMX cassette, flanked by loxP sites enabling removal and recycling of the resistance marker. Both of these vectors are commercially available from Invitrogen (Carlsbad, CA).

The method entails the synthesis and amplification of the gene of interest with suitable primers, followed by the digestion of the gene at a unique restriction site, such as that created by the *EcoRI/XhoI* enzymes (Vellanki *et al.*, Biotechnol. Lett. 29:313-318 (2007)). The gene is inserted at the *EcoRI* and *XhoI* sites in the expression vector, downstream of the GAP promoter. The gene insertion is verified by PCR and/or DNA sequence analysis. The recombinant plasmid is then linearized with *NarI* for histidine integration, purified and integrated into the chromosomal DNA of *S. cerevisiae* using an appropriate transformation method. The cells are plated on the YPD medium with the appropriate selection marker (e.g., kanamycin) and incubated for 2-3 days. The transformants will then be analyzed for the requisite gene insert by colony PCR.

To remove the antibiotic marker, a plasmid containing the Cre recombinase is introduced, promoting recombination between the loxP sites (Gueldener *et al.*, *supra*). Finally, the resulting strain is cured of the Cre plasmid by successive culturing on media without any antibiotic

present. The final strain will have a markerless gene deletion, and thus the same method can be used to introduce multiple insertions in the same strain.

The engineered strains are characterized by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. Cultures are grown overnight and used as inoculum for a fresh batch culture for which measurements are taken during exponential growth. The growth rate is determined by measuring optical density using a spectrophotometer (A600). Concentrations of glucose, alcohols, and other organic acid byproducts in the culture supernatant are determined by analytical methods including HPLC using an HPX-87H column (BioRad), or GC-MS, and used to calculate uptake and secretion rates. All experiments are performed with triplicate cultures.

The knockout strains are initially anticipated to exhibit suboptimal growth rates until their metabolic networks have adjusted to their missing functionalities. To enable this adjustment, the strains will be adaptively evolved. By subjecting the strains to adaptive evolution, cellular growth rate becomes the primary selection pressure and the mutant cells will be compelled to reallocate their metabolic fluxes in order to enhance their rates of growth. This reprogramming of metabolism has been recently demonstrated for several *E. coli* mutants that had been adaptively evolved on various substrates to reach the growth rates predicted *a priori* by an *in silico* model. The growth improvements brought about by adaptive evolution can be accompanied by enhanced rates of LCA production. The OptKnock-generated strains can be adaptively evolved in triplicate (running in parallel) due to differences in the evolutionary patterns witnessed previously in *E. coli* that could potentially result in one strain having superior production qualities over the others. Evolutions can be run for a period of 2-6 weeks, or longer depending upon the rate of growth improvement attained. In general, evolutions can be stopped once a stable phenotype is obtained.

The adaptive evolution procedure involves maintaining the cells in prolonged exponential growth by the serial passage of batch cultures into fresh medium before the stationary phase is attained. Briefly, one procedure allows cells to reach mid-exponential growth (A_{600}=0.5) before being diluted and passed to fresh medium (i.e., M9 minimal media with 2 g/L carbon source). This process is repeated, allowing for about 500 generations for each culture. Culture samples are taken, frozen with liquid nitrogen, and the optical culture density recorded for each day throughout the course of the evolutions. The evolutions are performed in triplicate due to differences in the evolutionary patterns witnessed previously Donnelly et al., *Appl Biochem Biotechnol.* 70-72: 187-98 (1998); Vemuri et al., *Appl Environ Microbiol.* 68:1715-27 (2002),

that could potentially result in one strain having superior production qualities over the others. The adaptive evolution step can take up to about two months or more. The adaptive evolution step also can be less than two months depending on the strain design, for example.

Another process can evolve cells using automation technology and is commercially available by Evolugate, LLC (Gainesville, FL) under a service contract. The procedure employs the Evolugator™ evolution machine which results in significant time and effort savings over non-automated evolution techniques. Cells are maintained in prolonged exponential growth by the serial passage of batch cultures into fresh medium before the stationary phase is attained. By automating optical density measurement and liquid handling, the Evolugator can perform serial transfer at high rates using large culture volumes, thus approaching the efficiency of a chemostat for evolution of cell fitness[25]. In contrast to a chemostat, which maintains cells in a single vessel, the machine operates by moving from one "reactor" to the next in subdivided regions of a spool of tubing, thus eliminating any selection for wall-growth. Culture samples are taken, frozen with liquid nitrogen, and the optical culture density recorded each day throughout the course of the evolutions. The Evolugator is used for each strain until a stable growth rate is achieved. Growth rate improvements of nearly 50% have been observed in two weeks using this device. The above-described strains are adaptively evolved in triplicate (running in parallel). At ten day intervals, culture samples are taken from the Evolugator, purified on agar plates, and cultured in triplicate as discussed above to assess strain physiology.

Following the adaptive evolution process, the new strains are again characterized by measuring the growth rate, the substrate uptake rate, and the product/byproduct secretion rate. These results are compared to the OptKnock predictions by plotting actual growth and production yields along side the production envelopes. The most successful OptKnock design/evolution combinations are chosen to pursue further, and is characterized in lab-scale batch and continuous fermentations. The growth-coupled biochemical production concept behind the OptKnock approach should also result in the generation of genetically stable overproducers. Thus, the cultures can be maintained in continuous mode for one month to evaluate long-term stability. Periodic samples will be taken to ensure that yield and productivity are maintained throughout the experiment.

Described herein above, is the application of the OptKnock methodology for generating useful gene disruption targets. Multiple disruption strategies were enumerated for establishing the coupling between LCA production and *Escherichia coli* growth. This methodology is applicable

to a wide variety of cells and microorganisms other than *Escherichia coli* and also can utilize metabolic modeling and simulation systems other than OptKnock.

The combined computational and engineering platform described herein is generally applicable to any stoichiometric model organism and the teachings and guidance provided herein will allow those skilled in the art to design and implement sets of genetic manipulations for metabolic alterations that lead to the growth-coupled production of any biochemical product.

The present disclosure provides gene disruption strategies for growth-coupled production of LCA in *Escherichia coli* under anaerobic conditions. The suggested strategies can increase product yields significantly over the reported yields for each of these products. A comprehensive list of the strategies is listed in Table 1 for LCA production. The associated genes and stoichiometries for each of the reaction disruption are catalogued in Table 2. Table 3 lists metabolite abbreviations and their corresponding names along with their location.

Table 1: The list of all disruption strategies identified by OptKnock that are most likely to provide growth-coupled LCA produciton.

	1	ADHEr	LDH_D	ASPT	MDH	PFLi	PGDHY	PYK	DHAPT
5	2	ADHEr	LDH_D	ASPT	MDH	PFLi	PGL	PYK	DHAPT
	3	ADHEr	LDH_D	ASPT	G6PDHy	MDH	PFLi	PYK	DHAPT
	4	ADHEr	LDH_D	ASPT	EDA	MDH	PFLi	PYK	DHAPT
	5	ADHEr	LDH_D	GLCpts	GLUDy	PGDH	PGI	PTAr	
	6	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGDH	PGI	
10	7	ADHEr	LDH_D	GLCpts	GLUDy	PGI	PTAr	TAL	
	8	ADHEr	LDH_D	GLCpts	GLUDy	PGI	PTAr	TKT1	
	9	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGI	TKT1	
	10	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGI	TAL	
	11	ADHEr	LDH_D	FBA	GLCpts	GLUDy	PTAr		
15	12	ADHEr	LDH_D	GLCpts	GLUDy	PTAr	TPI		
	13	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	TPI		
	14	ADHEr	LDH_D	GLCpts	GLUDy	PFK	PTAr		
	15	ADHEr	LDH_D	ACKr	FBA	GLCpts	GLUDy		
	16	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PFK		
20	17	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGI	RPE	
	18	ADHEr	LDH_D	GLCpts	GLUDy	PGI	PTAr	RPE	
	19	ADHEr	LDH_D	GLCpts	GLUDy	PGI	PTAr	TKT2	
	20	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGI	TKT2	
	21	ADHEr	LDH_D	ACKr	GLCpts	PGDH	PGI		
25	22	ADHEr	LDH_D	GLCpts	PGDH	PGI	PTAr		
	23	ADHEr	LDH_D	ACKr	GLCpts	PGI	TKT1		
	24	ADHEr	LDH_D	ACKr	GLCpts	PGI	TAL		
	25	ADHEr	LDH_D	GLCpts	PGI	PTAr	TAL		
	26	ADHEr	LDH_D	GLCpts	PGI	PTAr	TKT1		
30	27	ADHEr	LDH_D	ACKr	GLCpts	PFK			
	28	ADHEr	LDH_D	ACKr	GLCpts	TPI			
	29	ADHEr	LDH_D	ACKr	FBA	GLCpts			
	30	ADHEr	LDH_D	FBA	GLCpts	PTAr			
	31	ADHEr	LDH_D	GLCpts	PFK	PTAr			
35	32	ADHEr	LDH_D	GLCpts	PTAr	TPI			
	33	ADHEr	LDH_D	ACKr	GLCpts	PGI	RPE		
	34	ADHEr	LDH_D	GLCpts	PGI	PTAr	RPE		
	35	ADHEr	LDH_D	FRD2	GLCpts	GLUDy	PFLi	PGI	
	36	ADHEr	LDH_D	ACKr	GLCpts	PGI	TKT2		
40	37	ADHEr	LDH_D	GLCpts	PGI	PTAr	TKT2		
	38	ADHEr	LDH_D	FRD2	GLCpts	GLUDy	PFLi	TPI	
	39	ADHEr	LDH_D	FBA	FRD2	GLCpts	GLUDy	PFLi	
	40	ADHEr	LDH_D	FRD2	GLCpts	GLUDy	PFK	PFLi	
	41	ADHEr	LDH_D	ASPT	ATPS4r	FUM	NADH6	PGI	
45	42	ADHEr	LDH_D	ASPT	ATPS4r	MDH	NADH6	PGI	
	43	ADHEr	LDH_D	ASPT	ATPS4r	MDH	NADH6	PFK	
	44	ADHEr	LDH_D	ASPT	ATPS4r	FBA	FUM	NADH6	
	45	ADHEr	LDH_D	ASPT	ATPS4r	FUM	NADH6	TPI	
	46	ADHEr	LDH_D	ASPT	ATPS4r	MDH	NADH6	TPI	
50	47	ADHEr	LDH_D	ASPT	ATPS4r	FBA	MDH	NADH6	
	48	ADHEr	LDH_D	ASPT	ATPS4r	FUM	NADH6	PFK	
	49	ADHEr	LDH_D	FUM	GLCpts	GLUDy	PFLi	PGI	

	50	ADHEr	LDH_D	GLCpts	GLUDy	MDH	PFLi	PGI
	51	ADHEr	LDH_D	GLCpts	PFLi	PGI	SUCD4	
	52	ADHEr	LDH_D	GLCpts	NADH6	PFLi	PGI	
	53	ADHEr	LDH_D	FRD2	GLCpts	PFLi	PGI	
5	54	ADHEr	LDH_D	ACKr	GLUDy	HEX1	PGDH	PGI
	55	ADHEr	LDH_D	GLUDy	HEX1	PGDH	PGI	PTAr
	56	ADHEr	LDH_D	FUM	GLCpts	GLUDy	PFK	PFLi
	57	ADHEr	LDH_D	FBA	FUM	GLCpts	GLUDy	PFLi
	58	ADHEr	LDH_D	GLCpts	GLUDy	MDH	PFK	PFLi
10	59	ADHEr	LDH_D	FUM	GLCpts	GLUDy	PFLi	TPI
	60	ADHEr	LDH_D	FBA	GLCpts	GLUDy	MDH	PFLi
	61	ADHEr	LDH_D	GLCpts	GLUDy	MDH	PFLi	TPI
	62	ADHEr	LDH_D	GLCpts	NADH6	PFLi	TPI	
	63	ADHEr	LDH_D	FRD2	GLCpts	PFLi	TPI	
15	64	ADHEr	LDH_D	FBA	FRD2	GLCpts	PFLi	
	65	ADHEr	LDH_D	FBA	GLCpts	NADH6	PFLi	
	66	ADHEr	LDH_D	FBA	GLCpts	PFLi	SUCD4	
	67	ADHEr	LDH_D	FRD2	GLCpts	PFK	PFLi	
	68	ADHEr	LDH_D	GLCpts	PFLi	SUCD4	TPI	
20	69	ADHEr	LDH_D	GLCpts	PFK	PFLi	SUCD4	
	70	ADHEr	LDH_D	GLCpts	NADH6	PFK	PFLi	
	71	ADHEr	LDH_D	ASPT	GLCpts	MDH	PFLi	PGI
	72	ADHEr	LDH_D	ASPT	FUM	GLCpts	PFLi	PGI
	73	ADHEr	LDH_D	ASPT	ATPS4r	MDH	PGI	PPS
25	74	ADHEr	LDH_D	ASPT	ATPS4r	FUM	PGI	PPS
	75	ADHEr	LDH_D	GLUDy	HEX1	PGI	PTAr	TAL
	76	ADHEr	LDH_D	ACKr	GLUDy	HEX1	PGI	TAL
	77	ADHEr	LDH_D	ACKr	GLUDy	HEX1	PGI	TKT1
	78	ADHEr	LDH_D	GLUDy	HEX1	PGI	PTAr	TKT1
30	79	ADHEr	LDH_D	ACKr	GLUDy	HEX1	TPI	
	80	ADHEr	LDH_D	ACKr	GLUDy	HEX1	PFK	
	81	ADHEr	LDH_D	GLUDy	HEX1	PTAr	TPI	
	82	ADHEr	LDH_D	GLUDy	HEX1	PFK	PTAr	
	83	ADHEr	LDH_D	ACKr	FBA	GLUDy	HEX1	
35	84	ADHEr	LDH_D	FBA	GLUDy	HEX1	PTAr	
	85	ADHEr	LDH_D	ASPT	GLCpts	MDH	PFLi	TPI
	86	ADHEr	LDH_D	ASPT	FBA	GLCpts	MDH	PFLi
	87	ADHEr	LDH_D	ASPT	GLCpts	MDH	PFK	PFLi
	88	ADHEr	LDH_D	ASPT	FUM	GLCpts	PFK	PFLi
40	89	ADHEr	LDH_D	ASPT	FUM	GLCpts	PFLi	TPI
	90	ADHEr	LDH_D	ASPT	FBA	FUM	GLCpts	PFLi
	91	ADHEr	LDH_D	GLUDy	HEX1	PGI	PTAr	RPE
	92	ADHEr	LDH_D	ACKr	GLUDy	HEX1	PGI	RPE
	93	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	PGI
45	94	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	PGI
	95	ADHEr	LDH_D	ASPT	ATPS4r	FBA	MDH	PPS
	96	ADHEr	LDH_D	ASPT	ATPS4r	FUM	PFK	PPS
	97	ADHEr	LDH_D	ASPT	ATPS4r	MDH	PFK	PPS
	98	ADHEr	LDH_D	ASPT	ATPS4r	MDH	PPS	TPI
50	99	ADHEr	LDH_D	ASPT	ATPS4r	FUM	PPS	TPI
	100	ADHEr	LDH_D	ASPT	ATPS4r	FBA	FUM	PPS
	101	ADHEr	LDH_D	ACKr	GLUDy	HEX1	PGI	TKT2

	102	ADHEr	LDH_D	GLUDy	HEX1	PGI	PTAr	TKT2
	103	ADHEr	LDH_D	ASPT	ATPS4r	FBA	FUM	GLUDy
	104	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	PFK
	105	ADHEr	LDH_D	ASPT	ATPS4r	FBA	GLUDy	MDH
5	106	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	TPI
	107	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	PFK
	108	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	TPI
	109	ADHEr	LDH_D	ACKr	GLUDy	PGDH	PGI	
	110	ADHEr	LDH_D	GLUDy	PGDH	PGI	PTAr	
10	111	ADHEr	LDH_D	ACKr	GLUDy	PGI	TAL	
	112	ADHEr	LDH_D	GLUDy	PGI	PTAr	TKT1	
	113	ADHEr	LDH_D	ACKr	GLUDy	PGI	TKT1	
	114	ADHEr	LDH_D	GLUDy	PGI	PTAr	TAL	
	115	ADHEr	LDH_D	ACKr	GLUDy	TPI		
15	116	ADHEr	LDH_D	GLUDy	PFK	PTAr		
	117	ADHEr	LDH_D	FBA	GLUDy	PTAr		
	118	ADHEr	LDH_D	ACKr	FBA	GLUDy		
	119	ADHEr	LDH_D	ACKr	GLUDy	PFK		
	120	ADHEr	LDH_D	GLUDy	PTAr	TPI		
20	121	ADHEr	LDH_D	ACKr	GLUDy	PGI	RPE	
	122	ADHEr	LDH_D	GLUDy	PGI	PTAr	RPE	
	123	ADHEr	LDH_D	GLUDy	PGI	PTAr	TKT2	
	124	ADHEr	LDH_D	ACKr	GLUDy	PGI	TKT2	
	125	ADHEr	LDH_D	HEX1	PGDH	PGI	PTAr	
25	126	ADHEr	LDH_D	ACKr	HEX1	PGDH	PGI	
	127	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FUM	PGI
	128	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	MDH	PGI
	129	ADHEr	LDH_D	HEX1	PGI	PTAr	TAL	
	130	ADHEr	LDH_D	HEX1	PGI	PTAr	TKT1	
30	131	ADHEr	LDH_D	ACKr	HEX1	PGI	TKT1	
	132	ADHEr	LDH_D	ACKr	HEX1	PGI	TAL	
	133	ADHEr	LDH_D	GLUDy	HEX1	PFLi	PGI	SUCD4
	134	ADHEr	LDH_D	FRD2	GLUDy	HEX1	PFLi	PGI
	135	ADHEr	LDH_D	GLUDy	HEX1	NADH6	PFLi	PGI
35	136	ADHEr	LDH_D	ACKr	FBA	HEX1		
	137	ADHEr	LDH_D	FBA	HEX1	PTAr		
	138	ADHEr	LDH_D	HEX1	PFK	PTAr		
	139	ADHEr	LDH_D	ACKr	HEX1	PFK		
	140	ADHEr	LDH_D	ACKr	HEX1	TPI		
40	141	ADHEr	LDH_D	HEX1	PTAr	TPI		
	142	ADHEr	LDH_D	HEX1	PGI	PTAr	RPE	
	143	ADHEr	LDH_D	ACKr	HEX1	PGI	RPE	
	144	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FBA	FUM
	145	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FBA	MDH
45	146	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	MDH	TPI
	147	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FUM	PFK
	148	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FUM	TPI
	149	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	MDH	PFK
	150	ADHEr	LDH_D	FBA	GLUDy	HEX1	NADH6	PFLi
50	151	ADHEr	LDH_D	GLUDy	HEX1	NADH6	PFK	PFLi
	152	ADHEr	LDH_D	FBA	GLUDy	HEX1	PFLi	SUCD4
	153	ADHEr	LDH_D	FRD2	GLUDy	HEX1	PFK	PFLi

	154	ADHEr	LDH_D	GLUDy	HEX1	PFK	PFLi	SUCD4
	155	ADHEr	LDH_D	GLUDy	HEX1	NADH6	PFLi	TPI
	156	ADHEr	LDH_D	FBA	FRD2	GLUDy	HEX1	PFLi
	157	ADHEr	LDH_D	GLUDy	HEX1	PFLi	SUCD4	TPI
5	158	ADHEr	LDH_D	FRD2	GLUDy	HEX1	PFLi	TPI
	159	ADHEr	LDH_D	GLUDy	HEX1	MDH	PFLi	PGI
	160	ADHEr	LDH_D	FUM	GLUDy	HEX1	PFLi	PGI
	161	ADHEr	LDH_D	HEX1	PGI	PTAr	TKT2	
	162	ADHEr	LDH_D	ACKr	HEX1	PGI	TKT2	
10	163	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	MDH	PFK
	164	ADHEr	LDH_D	ATPS4r	FBA	GLUDy	HEX1	MDH
	165	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	MDH	TPI
	166	ADHEr	LDH_D	ATPS4r	FBA	FUM	GLUDy	HEX1
	167	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	HEX1	PFK
15	168	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	HEX1	TPI
	169	ADHEr	LDH_D	FBA	FUM	GLUDy	HEX1	PFLi
	170	ADHEr	LDH_D	FUM	GLUDy	HEX1	PFLi	TPI
	171	ADHEr	LDH_D	GLUDy	HEX1	MDH	PFLi	TPI
	172	ADHEr	LDH_D	GLUDy	HEX1	MDH	PFK	PFLi
20	173	ADHEr	LDH_D	FBA	GLUDy	HEX1	MDH	PFLi
	174	ADHEr	LDH_D	FUM	GLUDy	HEX1	PFK	PFLi
	175	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	HEX1	PGI
	176	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	MDH	PGI
	177	ADHEr	LDH_D	ASPT	ATPS4r	MDH	PGI	
25	178	ADHEr	LDH_D	ASPT	ATPS4r	FUM	PGI	
	179	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	NADH6	PGI
	180	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	NADH6	PGI
	181	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PGDH	PGI
	182	ADHEr	LDH_D	PGDH	PGI	PTAr		
30	183	ADHEr	LDH_D	ACKr	PGDH	PGI		
	184	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PFLi	PGI
	185	ADHEr	LDH_D	ASPT	ATPS4r	MDH	TPI	
	186	ADHEr	LDH_D	ASPT	ATPS4r	FUM	TPI	
	187	ADHEr	LDH_D	ASPT	ATPS4r	MDH	PFK	
35	188	ADHEr	LDH_D	ASPT	ATPS4r	FBA	FUM	
	189	ADHEr	LDH_D	ASPT	ATPS4r	FBA	MDH	
	190	ADHEr	LDH_D	ASPT	ATPS4r	FUM	PFK	
	191	ADHEr	LDH_D	ACKr	PGI	TKT1		
	192	ADHEr	LDH_D	PGI	PTAr	TAL		
40	193	ADHEr	LDH_D	PGI	PTAr	TKT1		
	194	ADHEr	LDH_D	ACKr	PGI	TAL		
	195	ADHEr	LDH_D	ATPS4r	FBA	GLUDy	MDH	NADH6
	196	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	NADH6	PFK
	197	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	NADH6	TPI
45	198	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	NADH6	TPI
	199	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	NADH6	PFK
	200	ADHEr	LDH_D	ATPS4r	FBA	FUM	GLUDy	NADH6
	201	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PGI	TAL
	202	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PGI	TKT1
50	203	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PFK	
	204	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	TPI	
	205	ADHEr	LDH_D	ATPS4r	FBA	GLUDy	HEX1	

	206	ADHEr	LDH_D	GLUDy	PTAr	PYK	SUCD4	
	207	ADHEr	LDH_D	ACKr	GLUDy	PYK	SUCD4	
	208	ADHEr	LDH_D	FRD2	GLUDy	PTAr	PYK	
	209	ADHEr	LDH_D	ACKr	FRD2	GLUDy	PYK	
5	210	ADHEr	LDH_D	FDH2	GLUDy	NADH6	PTAr	PYK
	211	ADHEr	LDH_D	ACKr	FDH2	GLUDy	NADH6	PYK
	212	ADHEr	LDH_D	PFK	PTAr			
	213	ADHEr	LDH_D	ACKr	TPI			
	214	ADHEr	LDH_D	ACKr	FBA			
10	215	ADHEr	LDH_D	PTAr	TPI			
	216	ADHEr	LDH_D	FBA	PTAr			
	217	ADHEr	LDH_D	ACKr	PFK			
	218	ADHEr	LDH_D	FRD2	GLUDy	PFLi	PGI	
	219	ADHEr	LDH_D	GLUDy	PFLi	PGI	PRO1z	SUCD4
15	220	ADHEr	LDH_D	ACKr	PGI	RPE		
	221	ADHEr	LDH_D	PGI	PTAr	RPE		
	222	ADHEr	LDH_D	ACKr	PGI	TKT2		
	223	ADHEr	LDH_D	PGI	PTAr	TKT2		
	224	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PGI	RPE
20	225	ADHEr	LDH_D	FRD2	GLUDy	PFLi	TPI	
	226	ADHEr	LDH_D	FRD2	GLUDy	PFK	PFLi	
	227	ADHEr	LDH_D	FBA	FRD2	GLUDy	PFLi	
	228	ADHEr	LDH_D	GLUDy	PFK	PFLi	PRO1z	SUCD4
	229	ADHEr	LDH_D	GLUDy	PFLi	PRO1z	SUCD4	TPI
25	230	ADHEr	LDH_D	FBA	GLUDy	PFLi	PRO1z	SUCD4
	231	ADHEr	LDH_D	GLUDy	MDH	PFLi	PGI	SUCD4
	232	ADHEr	LDH_D	FUM	GLUDy	NADH6	PFLi	PGI
	233	ADHEr	LDH_D	GLUDy	MDH	NADH6	PFLi	PGI
	234	ADHEr	LDH_D	FUM	GLUDy	PFLi	PGI	SUCD4
30	235	ADHEr	LDH_D	ASPT	GLUDy	MDH	PFLi	PGI
	236	ADHEr	LDH_D	ASPT	FUM	GLUDy	PFLi	PGI
	237	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PGI	TKT2
	238	ADHEr	LDH_D	FUM	GLUDy	PFK	PFLi	SUCD4
	239	ADHEr	LDH_D	GLUDy	MDH	NADH6	PFK	PFLi
35	240	ADHEr	LDH_D	FUM	GLUDy	PFLi	SUCD4	TPI
	241	ADHEr	LDH_D	FUM	GLUDy	NADH6	PFK	PFLi
	242	ADHEr	LDH_D	FBA	FUM	GLUDy	PFLi	SUCD4
	243	ADHEr	LDH_D	GLUDy	MDH	PFLi	SUCD4	TPI
	244	ADHEr	LDH_D	GLUDy	MDH	PFK	PFLi	SUCD4
40	245	ADHEr	LDH_D	FBA	FUM	GLUDy	NADH6	PFLi
	246	ADHEr	LDH_D	FBA	GLUDy	MDH	PFLi	SUCD4
	247	ADHEr	LDH_D	FBA	GLUDy	MDH	NADH6	PFLi
	248	ADHEr	LDH_D	GLUDy	MDH	NADH6	PFLi	TPI
	249	ADHEr	LDH_D	FUM	GLUDy	NADH6	PFLi	TPI
45	250	ADHEr	LDH_D	ASPT	ATPS4r	FUM	NADH6	PYK
	251	ADHEr	LDH_D	ASPT	ATPS4r	MDH	NADH6	PYK
	252	ADHEr	LDH_D	GLCpts	GLUDy	PFLi	PGI	PTAr
	253	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PFLi	PGI
	254	ADHEr	LDH_D	ASPT	FBA	GLUDy	MDH	PFLi
50	255	ADHEr	LDH_D	ASPT	GLUDy	MDH	PFK	PFLi
	256	ADHEr	LDH_D	ASPT	FBA	FUM	GLUDy	PFLi
	257	ADHEr	LDH_D	ASPT	GLUDy	MDH	PFLi	TPI

	258	ADHEr	LDH_D	ASPT	FUM	GLUDy	PFLi	TPI
	259	ADHEr	LDH_D	ASPT	FUM	GLUDy	PFK	PFLi
	260	ADHEr	LDH_D	ME2	PGL	PTAr	PYK	SUCD4
	261	ADHEr	LDH_D	FRD2	G6PDHy	ME2	PTAr	PYK
5	262	ADHEr	LDH_D	ACKr	ME2	PGL	PYK	SUCD4
	263	ADHEr	LDH_D	ACKr	FRD2	ME2	PGL	PYK
	264	ADHEr	LDH_D	FRD2	ME2	PGL	PTAr	PYK
	265	ADHEr	LDH_D	G6PDHy	ME2	PTAr	PYK	SUCD4
	266	ADHEr	LDH_D	ACKr	FRD2	G6PDHy	ME2	PYK
10	267	ADHEr	LDH_D	ACKr	G6PDHy	ME2	PYK	SUCD4
	268	ADHEr	LDH_D	G6PDHy	MDH	PTAr	PYK	SUCD4
	269	ADHEr	LDH_D	ACKr	G6PDHy	MDH	NADH6	PYK
	270	ADHEr	LDH_D	FRD2	G6PDHy	MDH	PTAr	PYK
	271	ADHEr	LDH_D	FRD2	MDH	PGL	PTAr	PYK
15	272	ADHEr	LDH_D	ACKr	G6PDHy	MDH	PYK	SUCD4
	273	ADHEr	LDH_D	ACKr	MDH	PGL	PYK	SUCD4
	274	ADHEr	LDH_D	MDH	NADH6	PGL	PTAr	PYK
	275	ADHEr	LDH_D	ACKr	MDH	NADH6	PGL	PYK
	276	ADHEr	LDH_D	ACKr	FRD2	G6PDHy	MDH	PYK
20	277	ADHEr	LDH_D	MDH	PGL	PTAr	PYK	SUCD4
	278	ADHEr	LDH_D	ACKr	FRD2	MDH	PGL	PYK
	279	ADHEr	LDH_D	G6PDHy	MDH	NADH6	PTAr	PYK
	280	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	PGI	
	281	ADHEr	LDH_D	FUM	GLUDy	PTAr	PYK	
25	282	ADHEr	LDH_D	ACKr	GLUDy	MDH	PYK	
	283	ADHEr	LDH_D	ACKr	FUM	GLUDy	PYK	
	284	ADHEr	LDH_D	GLUDy	MDH	PTAr	PYK	
	285	ADHEr	LDH_D	ATPS4r	HEX1	PGDH	PGI	
	286	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	TPI	
30	287	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	PFK	
	288	ADHEr	LDH_D	ATPS4r	FBA	GLUDy	NADH6	
	289	ADHEr	LDH_D	HEX1	PFLi	PGI		
	290	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	PYK
	291	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	PYK
35	292	ADHEr	LDH_D	ATPS4r	HEX1	PGI	TKT1	
	293	ADHEr	LDH_D	ATPS4r	HEX1	PGI	TAL	
	294	ADHEr	LDH_D	ATPS4r	HEX1	PFK		
	295	ADHEr	LDH_D	ATPS4r	FBA	HEX1		
	296	ADHEr	LDH_D	ATPS4r	HEX1	TPI		
40	297	ADHEr	LDH_D	HEX1	PFLi	TPI		
	298	ADHEr	LDH_D	HEX1	PFK	PFLi		
	299	ADHEr	LDH_D	FBA	HEX1	PFLi		
	300	ADHEr	LDH_D	ATPS4r	HEX1	PGI	RPE	
	301	ADHEr	LDH_D	ACKr	GLUDy	NADH6	PGI	PYK
45	302	ADHEr	LDH_D	GLUDy	NADH6	PGI	PTAr	PYK
	303	ADHEr	LDH_D	ATPS4r	HEX1	PGI	TKT2	
	304	ADHEr	LDH_D	ACKr	FRD2	PYK		
	305	ADHEr	LDH_D	ACKr	PYK	SUCD4		
	306	ADHEr	LDH_D	FRD2	PTAr	PYK		
50	307	ADHEr	LDH_D	PTAr	PYK	SUCD4		
	308	ADHEr	LDH_D	ACKr	FDH2	NADH6	PYK	
	309	ADHEr	LDH_D	FDH2	NADH6	PTAr	PYK	

	310	ADHEr	LDH_D	ATPS4r	NADH6	PGI		
	311	ADHEr	LDH_D	ACKr	GLCpts	PFLi	PGI	
	312	ADHEr	LDH_D	GLCpts	PFLi	PGI	PTAr	
	313	ADHEr	LDH_D	FRD2	GLUDy	PFLi	PYK	
5	314	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	PGDH	PGI
	315	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	PGDH	PGI
	316	ADHEr	LDH_D	FUM	GLUDy	PFLi	PGI	
	317	ADHEr	LDH_D	GLUDy	MDH	PFLi	PGI	
	318	ADHEr	LDH_D	ATPS4r	FBA	NADH6		
10	319	ADHEr	LDH_D	ATPS4r	NADH6	PFK		
	320	ADHEr	LDH_D	ATPS4r	NADH6	TPI		
	321	ADHEr	LDH_D	ATPS4r	FBA	FUM	GLUDy	
	322	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	PFK	
	323	ADHEr	LDH_D	ATPS4r	FBA	GLUDy	MDH	
15	324	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	TPI	
	325	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	TPI	
	326	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	PFK	
	327	ADHEr	LDH_D	FRD2	G6PDHy	ME2	PFLi	PYK
	328	ADHEr	LDH_D	FRD2	ME2	PFLi	PGL	PYK
20	329	ADHEr	LDH_D	EDA	FRD2	ME2	PFLi	PYK
	330	ADHEr	LDH_D	FRD2	ME2	PFLi	PGDHY	PYK
	331	ADHEr	LDH_D	GLUDy	MDH	PFK	PFLi	
	332	ADHEr	LDH_D	FBA	GLUDy	MDH	PFLi	
	333	ADHEr	LDH_D	GLUDy	MDH	PFLi	TPI	
25	334	ADHEr	LDH_D	FBA	FUM	GLUDy	PFLi	
	335	ADHEr	LDH_D	FUM	GLUDy	PFLi	TPI	
	336	ADHEr	LDH_D	FUM	GLUDy	PFK	PFLi	
	337	ADHEr	LDH_D	PFLi	PGI	SUCD4		
	338	ADHEr	LDH_D	FRD2	PFLi	PGI		
30	339	ADHEr	LDH_D	NADH6	PFLi	PGI		
	340	ADHEr	LDH_D	FRD2	MDH	PFLi	PGL	PYK
	341	ADHEr	LDH_D	FRD2	G6PDHy	MDH	PFLi	PYK
	342	ADHEr	LDH_D	FRD2	MDH	PFLi	PGDHY	PYK
	343	ADHEr	LDH_D	EDA	FRD2	MDH	PFLi	PYK
35	344	ADHEr	LDH_D	ACKr	ASPT	MDH	PYK	
	345	ADHEr	LDH_D	ASPT	MDH	PTAr	PYK	
	346	ADHEr	LDH_D	ACKr	ASPT	FUM	PYK	
	347	ADHEr	LDH_D	ASPT	FUM	PTAr	PYK	
	348	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	PGI	
40	349	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	PGI	
	350	ADHEr	LDH_D	FBA	PFLi	SUCD4		
	351	ADHEr	LDH_D	FRD2	PFK	PFLi		
	352	ADHEr	LDH_D	PFLi	SUCD4	TPI		
	353	ADHEr	LDH_D	FBA	FRD2	PFLi		
45	354	ADHEr	LDH_D	PFK	PFLi	SUCD4		
	355	ADHEr	LDH_D	FRD2	PFLi	TPI		
	356	ADHEr	LDH_D	NADH6	PFLi	TPI		
	357	ADHEr	LDH_D	FBA	NADH6	PFLi		
	358	ADHEr	LDH_D	NADH6	PFK	PFLi		
50	359	ADHEr	LDH_D	ASPT	MDH	PFLi	PGI	
	360	ADHEr	LDH_D	ASPT	FUM	PFLi	PGI	
	361	ADHEr	LDH_D	ASPT	GLUDy	MDH	PFLi	PYK

362	ADHEr	LDH_D	ASPT	FUM	GLUDy	PFLi	PYK	
363	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FUM	PYK	
364	ADHEr	LDH_D	ASPT	MDH	PFLi	TPI		
365	ADHEr	LDH_D	ASPT	FUM	PFLi	TPI		
366	ADHEr	LDH_D	ASPT	FBA	MDH	PFLi		
367	ADHEr	LDH_D	ASPT	FBA	FUM	PFLi		
368	ADHEr	LDH_D	ASPT	MDH	PFK	PFLi		
369	ADHEr	LDH_D	ASPT	FUM	PFK	PFLi		
370	ADHEr	LDH_D	ACKr	NADH6	PGI	PYK		
371	ADHEr	LDH_D	NADH6	PGI	PTAr	PYK		
372	ADHEr	LDH_D	ASPT	ATPS4r	FUM	PYK		
373	ADHEr	LDH_D	ASPT	ATPS4r	MALS	MDH	PYK	
374	ADHEr	LDH_D	ASPT	ATPS4r	ICL	MDH	PYK	
375	ADHEr	LDH_D	GLUDy	PFLi	PGDH	PGI		
376	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	PGI		
377	ADHEr	LDH_D	FBA	GLUDy	PFLi			
378	ADHEr	LDH_D	GLUDy	PFLi	TPI			
379	ADHEr	LDH_D	GLUDy	PFK	PFLi			
380	ADHEr	LDH_D	GLUDy	PFLi	PGI	TAL		
381	ADHEr	LDH_D	GLUDy	PFLi	PGI	TKT1		
382	ADHEr	LDH_D	GLUDy	PFLi	PRO1z	PYK	SUCD4	
383	ADHEr	LDH_D	GLUDy	MDH	NADH6	PFLi	PYK	
384	ADHEr	LDH_D	GLUDy	MDH	PFLi	PYK	SUCD4	
385	ADHEr	LDH_D	FUM	GLUDy	PFLi	PYK	SUCD4	
386	ADHEr	LDH_D	FUM	GLUDy	NADH6	PFLi	PYK	
387	ADHEr	LDH_D	GLUDy	PFLi	PGI			
388	ADHEr	LDH_D	EDA	MDH	PFLi	PYK	SUCD4	
389	ADHEr	LDH_D	MDH	PFLi	PGDHY	PYK	SUCD4	
390	ADHEr	LDH_D	MDH	PFLi	PGL	PYK	SUCD4	
391	ADHEr	LDH_D	G6PDHy	MDH	PFLi	PYK	SUCD4	
392	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	NADH6	PYK	
393	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	NADH6	PYK	
394	ADHEr	LDH_D	ACKr	AKGD	ATPS4r	GLUDy	PYK	
395	ADHEr	LDH_D	AKGD	ATPS4r	GLUDy	PTAr	PYK	
396	ADHEr	LDH_D	FRD2	PFLi	PYK			
397	ADHEr	LDH_D	ALAR	PFLi	PRO1z	PYK	SUCD4	
398	ADHEr	LDH_D	DAAD	PFLi	PRO1z	PYK	SUCD4	
399	ADHEr	LDH_D	PFLi	PGDH	PGI			
400	ADHEr	LDH_D	ATPS4r	PFLi	PGI			
401	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	PFLi	PYK	
402	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	PFLi	PYK	
403	ADHEr	LDH_D	PFLi	TPI				
404	ADHEr	LDH_D	FBA	PFLi				
405	ADHEr	LDH_D	PFK	PFLi				
406	ADHEr	LDH_D	ASPT	FUM	PFLi	PYK		
407	ADHEr	LDH_D	ASPT	MDH	PFLi	PYK		
408	ADHEr	LDH_D	PFLi	PGI	TKT1			
409	ADHEr	LDH_D	PFLi	PGI	TAL			
410	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	NADH6	
411	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	NADH6	
412	ADHEr	LDH_D	G6PDHy	ME2	PFLi	PYK	SUCD4	
413	ADHEr	LDH_D	EDA	ME2	PFLi	PYK	SUCD4	

	414	ADHEr	LDH_D	ME2	PFLi	PGDHY	PYK	SUCD4
	415	ADHEr	LDH_D	ME2	PFLi	PGL	PYK	SUCD4
	416	ADHEr	LDH_D	MDH	NADH6	PFLi	PGDHY	PYK
	417	ADHEr	LDH_D	G6PDHy	MDH	NADH6	PFLi	PYK
5	418	ADHEr	LDH_D	EDA	MDH	NADH6	PFLi	PYK
	419	ADHEr	LDH_D	MDH	NADH6	PFLi	PGL	PYK
	420	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	MDH	NADH6
	421	ADHEr	LDH_D	ASPT	ATPS4r	CBMK2	FUM	NADH6
	422	ADHEr	LDH_D	CBMK2	PFLi	PGI	RPE	
10	423	ADHEr	LDH_D	ASNS2	GLU5K	PFLi	PGI	RPE
	424	ADHEr	LDH_D	ASNS2	G5SD	PFLi	PGI	RPE
	425	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	PTAr
	426	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	PTAr
	427	ADHEr	LDH_D	PFLi	PGI			
15	428	ADHEr	LDH_D	ASPT	ATPS4r	FUM	GLUDy	
	429	ADHEr	LDH_D	ASPT	ATPS4r	GLUDy	MDH	
	430	ADHEr	LDH_D	ACKr	AKGD	ATPS4r	PYK	
	431	ADHEr	LDH_D	AKGD	ATPS4r	PTAr	PYK	
	432	ADHEr	LDH_D	ASPT	ATPS4r	MDH	NADH6	
20	433	ADHEr	LDH_D	ASPT	ATPS4r	FUM	NADH6	
	434	ADHEr	LDH_D	G6PDHy	GLCpts	GLUDy	PTAr	
	435	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGL	
	436	ADHEr	LDH_D	GLCpts	GLUDy	PGDH	PTAr	
	437	ADHEr	LDH_D	GLCpts	GLUDy	PGL	PTAr	
25	438	ADHEr	LDH_D	ACKr	G6PDHy	GLCpts	GLUDy	
	439	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	PGDH	
	440	ADHEr	LDH_D	GLCpts	GLUDy	PTAr	TKT1	
	441	ADHEr	LDH_D	GLCpts	GLUDy	PTAr	TAL	
	442	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	TKT1	
30	443	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	TAL	
	444	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	RPE	
	445	ADHEr	LDH_D	GLCpts	GLUDy	PTAr	RPE	
	446	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	TKT2	
	447	ADHEr	LDH_D	GLCpts	GLUDy	PTAr	TKT2	
35	448	ADHEr	LDH_D	GLCpts	PGDH	PTAr	THD2	
	449	ADHEr	LDH_D	G6PDHy	GLCpts	PTAr	THD2	
	450	ADHEr	LDH_D	ACKr	G6PDHy	GLCpts	THD2	
	451	ADHEr	LDH_D	ACKr	GLCpts	PGL	THD2	
	452	ADHEr	LDH_D	ACKr	GLCpts	PGDH	THD2	
40	453	ADHEr	LDH_D	GLCpts	PGL	PTAr	THD2	
	454	ADHEr	LDH_D	ACKr	GLCpts	THD2	TKT1	
	455	ADHEr	LDH_D	ACKr	GLCpts	TAL	THD2	
	456	ADHEr	LDH_D	GLCpts	PTAr	TAL	THD2	
	457	ADHEr	LDH_D	GLCpts	PTAr	THD2	TKT1	
45	458	ADHEr	LDH_D	ASPT	ATPS4r	MDH		
	459	ADHEr	LDH_D	ASPT	ATPS4r	FUM		
	460	ADHEr	LDH_D	GLCpts	PTAr	RPE	THD2	
	461	ADHEr	LDH_D	ACKr	GLCpts	RPE	THD2	
	462	ADHEr	LDH_D	ACKr	ATPS4r	PYK	SUCOAS	
50	463	ADHEr	LDH_D	ATPS4r	PTAr	PYK	SUCOAS	
	464	ADHEr	LDH_D	FRD2	GLCpts	GLUDy	PFLi	
	465	ADHEr	LDH_D	GLCpts	PTAr	THD2	TKT2	

	466	ADHEr	LDH_D	ACKr	GLCpts	THD2	TKT2
	467	ADHEr	LDH_D	FRD2	GLCpts	PFLi	THD2
	468	ADHEr	LDH_D	ACKr	GLUDy	PGDH	THD2
	469	ADHEr	LDH_D	GLUDy	PGL	PTAr	THD2
5	470	ADHEr	LDH_D	G6PDHy	GLUDy	PTAr	THD2
	471	ADHEr	LDH_D	GLUDy	PGDH	PTAr	THD2
	472	ADHEr	LDH_D	ACKr	GLUDy	PGL	THD2
	473	ADHEr	LDH_D	ACKr	G6PDHy	GLUDy	THD2
	474	ADHEr	LDH_D	FRD2	GLUDy	PFLi	THD2
10	475	ADHEr	LDH_D	GLUDy	PTAr	THD2	TKT1
	476	ADHEr	LDH_D	GLUDy	PTAr	TAL	THD2
	477	ADHEr	LDH_D	ACKr	GLUDy	TAL	THD2
	478	ADHEr	LDH_D	ACKr	GLUDy	THD2	TKT1
	479	ADHEr	LDH_D	ACKr	GLCpts	PGDH	
15	480	ADHEr	LDH_D	ACKr	GLCpts	PGL	
	481	ADHEr	LDH_D	GLCpts	PGDH	PTAr	
	482	ADHEr	LDH_D	GLCpts	PGL	PTAr	
	483	ADHEr	LDH_D	ACKr	G6PDHy	GLCpts	
	484	ADHEr	LDH_D	G6PDHy	GLCpts	PTAr	
20	485	ADHEr	LDH_D	GLUDy	PTAr	RPE	THD2
	486	ADHEr	LDH_D	ACKr	GLUDy	RPE	THD2
	487	ADHEr	LDH_D	GLCpts	GLUDy	PTAr	
	488	ADHEr	LDH_D	ACKr	GLCpts	GLUDy	
	489	ADHEr	LDH_D	GLCpts	PTAr	TKT1	
25	490	ADHEr	LDH_D	GLCpts	PTAr	TAL	
	491	ADHEr	LDH_D	ACKr	GLCpts	TAL	
	492	ADHEr	LDH_D	ACKr	GLCpts	TKT1	
	493	ADHEr	LDH_D	NADH6	PFLi	PTAr	PYK
	494	ADHEr	LDH_D	ACKr	NADH6	PFLi	PYK
30	495	ADHEr	LDH_D	ACKr	GLUDy	THD2	TKT2
	496	ADHEr	LDH_D	GLUDy	PTAr	THD2	TKT2
	497	ADHEr	LDH_D	ACKr	GLCpts	RPE	
	498	ADHEr	LDH_D	GLCpts	PTAr	RPE	
	499	ADHEr	LDH_D	ACKr	GLCpts	TKT2	
35	500	ADHEr	LDH_D	GLCpts	PTAr	TKT2	
	501	ADHEr	LDH_D	ACKr	GLUDy	PGDH	
	502	ADHEr	LDH_D	GLUDy	PGL	PTAr	
	503	ADHEr	LDH_D	ACKr	GLUDy	PGL	
	504	ADHEr	LDH_D	ACKr	G6PDHy	GLUDy	
40	505	ADHEr	LDH_D	GLUDy	PGDH	PTAr	
	506	ADHEr	LDH_D	G6PDHy	GLUDy	PTAr	
	507	ADHEr	LDH_D	GLUDy	PTAr	TKT1	
	508	ADHEr	LDH_D	ACKr	GLUDy	TKT1	
	509	ADHEr	LDH_D	ACKr	GLUDy	TAL	
45	510	ADHEr	LDH_D	GLUDy	PTAr	TAL	
	511	ADHEr	LDH_D	GLUDy	PTAr	RPE	
	512	ADHEr	LDH_D	ACKr	GLUDy	RPE	
	513	ADHEr	LDH_D	GLUDy	PTAr	TKT2	
	514	ADHEr	LDH_D	ACKr	GLUDy	TKT2	
50	515	ADHEr	LDH_D	PGDH	PTAr	THD2	
	516	ADHEr	LDH_D	ACKr	PGDH	THD2	
	517	ADHEr	LDH_D	G6PDHy	PTAr	THD2	

518	ADHEr	LDH_D	PGL	PTAr	THD2	
519	ADHEr	LDH_D	ACKr	PGL	THD2	
520	ADHEr	LDH_D	ACKr	G6PDHy	THD2	
521	ADHEr	LDH_D	PTAr	TAL	THD2	
522	ADHEr	LDH_D	ACKr	THD2	TKT1	
523	ADHEr	LDH_D	ACKr	TAL	THD2	
524	ADHEr	LDH_D	PTAr	THD2	TKT1	
525	ADHEr	LDH_D	PTAr	RPE	THD2	
526	ADHEr	LDH_D	ACKr	RPE	THD2	
527	ADHEr	LDH_D	FRD2	GLUDy	PFLi	
528	ADHEr	LDH_D	GLUDy	PFLi	PRO1z	SUCD4
529	ADHEr	LDH_D	FRD2	GLCpts	PFLi	
530	ADHEr	LDH_D	PTAr	THD2	TKT2	
531	ADHEr	LDH_D	ACKr	THD2	TKT2	
532	ADHEr	LDH_D	ACKr	GLCpts		
533	ADHEr	LDH_D	GLCpts	PTAr		
534	ADHEr	LDH_D	FRD2	PFLi	THD2	
535	ADHEr	LDH_D	ATPS4r	FUM	GLUDy	
536	ADHEr	LDH_D	ATPS4r	GLUDy	MDH	
537	ADHEr	LDH_D	FUM	GLCpts	PFLi	SUCD4
538	ADHEr	LDH_D	GLCpts	MDH	PFLi	SUCD4
539	ADHEr	LDH_D	FUM	GLUDy	PFLi	SUCD4
540	ADHEr	LDH_D	GLUDy	MDH	PFLi	SUCD4
541	ADHEr	LDH_D	GLUDy	MDH	NADH6	PFLi
542	ADHEr	LDH_D	FUM	GLUDy	NADH6	PFLi
543	ADHEr	LDH_D	MDH	PFLi	SUCD4	THD2
544	ADHEr	LDH_D	FUM	PFLi	SUCD4	THD2
545	ADHEr	LDH_D	ASPT	FUM	GLCpts	PFLi
546	ADHEr	LDH_D	ASPT	GLCpts	MDH	PFLi
547	ADHEr	LDH_D	ASPT	FUM	GLUDy	PFLi
548	ADHEr	LDH_D	ASPT	GLUDy	MDH	PFLi
549	ADHEr	LDH_D	GLCpts	PFLi	SUCD4	THD2
550	ADHEr	LDH_D	PGDH	PTAr		
551	ADHEr	LDH_D	PGL	PTAr		
552	ADHEr	LDH_D	ACKr	PGL		
553	ADHEr	LDH_D	G6PDHy	PTAr		
554	ADHEr	LDH_D	ACKr	G6PDHy		
555	ADHEr	LDH_D	ACKr	PGDH		
556	ADHEr	LDH_D	ASPT	FUM	PFLi	THD2
557	ADHEr	LDH_D	ASPT	MDH	PFLi	THD2
558	ADHEr	LDH_D	ACKr	GLUDy		
559	ADHEr	LDH_D	GLUDy	PTAr		
560	ADHEr	LDH_D	PTAr	TAL		
561	ADHEr	LDH_D	ACKr	TAL		
562	ADHEr	LDH_D	ACKr	TKT1		
563	ADHEr	LDH_D	PTAr	TKT1		
564	ADHEr	LDH_D	ACKr	RPE		
565	ADHEr	LDH_D	PTAr	RPE		
566	ADHEr	LDH_D	GLCpts	GLUDy	PFLi	SUCD4
567	ADHEr	LDH_D	FUM	GLCpts	GLUDy	PFLi
568	ADHEr	LDH_D	GLCpts	GLUDy	MDH	PFLi
569	ADHEr	LDH_D	ACKr	TKT2		

	570	ADHEr	LDH_D	PTAr	TKT2		
	571	ADHEr	LDH_D	GLUDy	PFLi	SUCD4	THD2
	572	ADHEr	LDH_D	FUM	GLUDy	PFLi	THD2
	573	ADHEr	LDH_D	GLUDy	MDH	PFLi	THD2
5	574	ADHEr	LDH_D	GLCpts	GLUDy	NADH6	PFLi
	575	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	PFLi
	576	ADHEr	LDH_D	GLCpts	MDH	PFLi	THD2
	577	ADHEr	LDH_D	FUM	GLCpts	PFLi	THD2
	578	ADHEr	LDH_D	ACKr	CBMK2	FRD2	PFLi
10	579	ADHEr	LDH_D	CBMK2	FRD2	PFLi	PTAr
	580	ADHEr	LDH_D	MDH	PTAr	SUCD4	
	581	ADHEr	LDH_D	FRD2	MDH	PTAr	
	582	ADHEr	LDH_D	ACKr	MDH	SUCD4	
	583	ADHEr	LDH_D	ACKr	FRD2	MDH	
15	584	ADHEr	LDH_D	FDH2	MDH	NADH6	PTAr
	585	ADHEr	LDH_D	ACKr	FDH2	MDH	NADH6
	586	ADHEr	LDH_D	GLCpts	NADH6	PFLi	THD2
	587	ADHEr	LDH_D	GLCpts	PFLi	SUCD4	
	588	ADHEr	LDH_D	GLCpts	NADH12	NADH6	PFLi
20	589	ADHEr	LDH_D	ATPS4r	FUM	PGL	
	590	ADHEr	LDH_D	ATPS4r	MDH	PGDH	
	591	ADHEr	LDH_D	ATPS4r	FUM	PGDH	
	592	ADHEr	LDH_D	ATPS4r	FUM	G6PDHy	
	593	ADHEr	LDH_D	GLCpts	MDH	NADH6	PFLi
25	594	ADHEr	LDH_D	FUM	GLCpts	NADH6	PFLi
	595	ADHEr	LDH_D	FRD2	PFLi		
	596	ADHEr	LDH_D	ALAR	PFLi	PRO1z	SUCD4
	597	ADHEr	LDH_D	DAAD	PFLi	PRO1z	SUCD4
	598	ADHEr	LDH_D	ACKr			
30	599	ADHEr	LDH_D	PTAr			
	600	ADHEr	LDH_D	FUM	PFLi	SUCD4	
	601	ADHEr	LDH_D	MDH	PFLi	SUCD4	
	602	ADHEr	LDH_D	FUM	NADH12	NADH6	PFLi
	603	ADHEr	LDH_D	MDH	NADH12	NADH6	PFLi
35	604	ADHEr	LDH_D	ATPS4r	MDH	TKT1	
	605	ADHEr	LDH_D	ATPS4r	FUM	TKT1	
	606	ADHEr	LDH_D	ATPS4r	MDH	TAL	
	607	ADHEr	LDH_D	ATPS4r	FUM	TAL	
	608	ADHEr	LDH_D	ATPS4r	NADH6	PFLi	PYK
40	609	ADHEr	LDH_D	ASPT	FUM	PFLi	
	610	ADHEr	LDH_D	ASPT	MDH	PFLi	
	611	ADHEr	LDH_D	ATPS4r	MDH	RPE	
	612	ADHEr	LDH_D	ATPS4r	FUM	RPE	
	613	ADHEr	LDH_D	PFLi	SUCD4	THD2	
45	614	ADHEr	LDH_D	NADH12	NADH6	PFLi	THD2
	615	ADHEr	LDH_D	FUM	NADH6	PFLi	THD2
	616	ADHEr	LDH_D	MDH	NADH6	PFLi	THD2
	617	ADHEr	LDH_D	ATPS4r	MDH	TKT2	
	618	ADHEr	LDH_D	ATPS4r	FUM	TKT2	
50	619	ADHEr	LDH_D	GLCpts	NADH6	PFLi	
	620	ADHEr	LDH_D	GLUDy	NADH6	PFLi	THD2
	621	ADHEr	LDH_D	GLUDy	PFLi	SUCD4	

	622	ADHEr	LDH_D	GLUDy	NADH12	NADH6	PFLi
	623	ADHEr	LDH_D	FUM	GLUDy	PFLi	
	624	ADHEr	LDH_D	GLUDy	MDH	PFLi	
	625	ADHEr	LDH_D	ATPS4r	FUM	NADH6	
5	626	ADHEr	LDH_D	ATPS4r	MDH	NADH6	
	627	ADHEr	LDH_D	ATPS4r	G6PDHy	GLUDy	NADH6
	628	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	PGDH
	629	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	PGL
	630	ADHEr	LDH_D	ATPS4r	MDH	PFLi	THD2
10	631	ADHEr	LDH_D	ATPS4r	FUM	PFLi	THD2
	632	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	TKT1
	633	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	TAL
	634	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	THD2
	635	ADHEr	LDH_D	GLCpts	MDH	PFLi	
15	636	ADHEr	LDH_D	FUM	GLCpts	PFLi	
	637	ADHEr	LDH_D	GLUDy	NADH6	PFLi	
	638	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	RPE
	639	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	TKT2
	640	ADHEr	LDH_D	FUM	PFLi	THD2	
20	641	ADHEr	LDH_D	MDH	PFLi	THD2	
	642	ADHEr	LDH_D	NADH6	PFLi	THD2	
	643	ADHEr	LDH_D	PFLi	SUCD4		
	644	ADHEr	LDH_D	NADH12	NADH6	PFLi	
	645	ADHEr	LDH_D	ATPS4r	NADH6	PFLi	
25	646	ADHEr	LDH_D	FUM	NADH6	PFLi	
	647	ADHEr	LDH_D	MDH	NADH6	PFLi	
	648	ADHEr	LDH_D	ATPS4r	NADH6	PGL	
	649	ADHEr	LDH_D	ATPS4r	NADH6	PGDH	
	650	ADHEr	LDH_D	ATPS4r	G6PDHy	NADH6	
30	651	ADHEr	LDH_D	ATPS4r	NADH6	TAL	
	652	ADHEr	LDH_D	ATPS4r	NADH6	TKT1	
	653	ADHEr	LDH_D	CBMK2	GLU5K	NADH6	PFLi
	654	ADHEr	LDH_D	CBMK2	G5SD	NADH6	PFLi
	655	ADHEr	LDH_D	ASNS2	CBMK2	NADH6	PFLi
35	656	ADHEr	LDH_D	ATPS4r	PFLi	THD2	
	657	ADHEr	LDH_D	NADH6	PFLi		
	658	ADHEr	LDH_D	ATPS4r	NADH6	RPE	
	659	ADHEr	LDH_D	ATPS4r	NADH6	TKT2	
	660	ADHEr	LDH_D	CBMK2	FUM	G5SD	PFLi
40	661	ADHEr	LDH_D	CBMK2	GLU5K	MDH	PFLi
	662	ADHEr	LDH_D	CBMK2	FUM	GLU5K	PFLi
	663	ADHEr	LDH_D	CBMK2	G5SD	MDH	PFLi
	664	ADHEr	LDH_D	ASNS2	CBMK2	FUM	PFLi
	665	ADHEr	LDH_D	ASNS2	CBMK2	MDH	PFLi
45	666	ADHEr	LDH_D	MDH	PFLi		
	667	ADHEr	LDH_D	FUM	PFLi		
	668	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	RPE
	669	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	TAL
	670	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	TKT1
50	671	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	TKT2
	672	ADHEr	LDH_D	ATPS4r	GLUDy	PFLi	
	673	ADHEr	LDH_D	ATPS4r	GLUDy	NADH6	

674	ADHEr	LDH_D	ATPS4r	PFLi	RPE		
675	ADHEr	LDH_D	ATPS4r	PFLi	TAL		
676	ADHEr	LDH_D	ATPS4r	PFLi	TKT1		
677	ADHEr	LDH_D	ATPS4r	PFLi	TKT2		
678	ADHEr	LDH_D	ATPS4r	CBMK2	PFLi		
679	ADHEr	LDH_D	ATPS4r	PFLi			
680	ADHEr	LDH_D	ASPT	MDH	PGDHY	PYK	
681	ADHEr	LDH_D	ASPT	EDA	MDH	PYK	
682	ADHEr	LDH_D	ATPS4r	CBMK2	NADH6		
683	ADHEr	LDH_D	ATPS4r	NADH6			
684	ADHEr	LDH_D	ATPS4r	HEX1	PGI	PPS	
685	ADHEr	LDH_D	G6PDHy	ME2	THD2		
686	ADHEr	LDH_D	ME2	PGL	THD2		
687	ADHEr	LDH_D	ME2	PGDH	PGDHY	THD2	
688	ADHEr	LDH_D	EDA	ME2	PGDH	THD2	
689	ADHEr	LDH_D	EDA	ME2	TAL	THD2	
690	ADHEr	LDH_D	ME2	PGDHY	TAL	THD2	
691	ADHEr	LDH_D	ME2	PGDHY	THD2	TKT1	
692	ADHEr	LDH_D	EDA	ME2	THD2	TKT1	
693	ADHEr	LDH_D	ME2	PGDHY	RPE	THD2	
694	ADHEr	LDH_D	EDA	ME2	RPE	THD2	
695	ADHEr	LDH_D	MDH	PGL	THD2		
696	ADHEr	LDH_D	G6PDHy	MDH	THD2		
697	ADHEr	LDH_D	EDA	MDH	PGDH	THD2	
698	ADHEr	LDH_D	MDH	PGDH	PGDHY	THD2	
699	ADHEr	LDH_D	ME2	PGDHY	THD2	TKT2	
700	ADHEr	LDH_D	EDA	ME2	THD2	TKT2	
701	ADHEr	LDH_D	MDH	PGDHY	THD2	TKT1	
702	ADHEr	LDH_D	EDA	MDH	THD2	TKT1	
703	ADHEr	LDH_D	MDH	PGDHY	TAL	THD2	
704	ADHEr	LDH_D	EDA	MDH	TAL	THD2	
705	ADHEr	LDH_D	ATPS4r	GLUDy	HEX1	PGI	
706	ADHEr	LDH_D	MDH	PGDHY	RPE	THD2	
707	ADHEr	LDH_D	EDA	MDH	RPE	THD2	
708	ADHEr	LDH_D	MDH	PGDHY	THD2	TKT2	
709	ADHEr	LDH_D	EDA	MDH	THD2	TKT2	
710	ADHEr	LDH_D	ATPS4r	HEX1	PGI		
711	ADHEr	LDH_D	FRD2	HEX1	MDH	PGI	
712	ADHEr	LDH_D	HEX1	MDH	PGI	SUCD4	
713	ADHEr	LDH_D	HEX1	PGI	SUCOAS		
714	ADHEr	LDH_D	HEX1	MDH	NADH6	PGI	
715	ADHEr	LDH_D	FUM	HEX1	NADH6	PGI	
716	ADHEr	LDH_D	FRD2	FUM	HEX1	PGI	
717	ADHEr	LDH_D	HEX1	PGI			
718	ADHEr	LDH_D	SUCOAS	THD2			
719	ADHEr	LDH_D	THD2				
720	ADHEr	LDH_D	GLCpts	SUCOAS	TKT2	TPI	
721	ADHEr	LDH_D	GLCpts	PFK	SUCOAS	TKT2	
722	ADHEr	LDH_D	FBA	GLCpts	SUCOAS	TKT2	
723	ADHEr	LDH_D	GLCpts	GLUDy	TKT2	TPI	
724	ADHEr	LDH_D	FBA	GLCpts	GLUDy	TKT2	
725	ADHEr	LDH_D	GLCpts	GLUDy	PFK	TKT2	

	726	ADHEr	LDH_D	GLCpts	PGI	SUCOAS	
	727	ADHEr	LDH_D	GLCpts	GLUDy	PGI	
	728	ADHEr	LDH_D	GLCpts	PFK	RPE	SUCOAS
	729	ADHEr	LDH_D	GLCpts	RPE	SUCOAS	TPI
5	730	ADHEr	LDH_D	FBA	GLCpts	RPE	SUCOAS
	731	ADHEr	LDH_D	GLCpts	GLUDy	RPE	TPI
	732	ADHEr	LDH_D	FBA	GLCpts	GLUDy	RPE
	733	ADHEr	LDH_D	GLCpts	GLUDy	PFK	RPE
	734	ADHEr	LDH_D	FBA	GLUDy	SUCOAS	TKT2
10	735	ADHEr	LDH_D	GLUDy	PFK	SUCOAS	TKT2
	736	ADHEr	LDH_D	GLUDy	SUCOAS	TKT2	TPI
	737	ADHEr	LDH_D	GLCpts	GLUDy	PFK	SUCOAS
	738	ADHEr	LDH_D	GLCpts	GLUDy	SUCOAS	TPI
	739	ADHEr	LDH_D	FBA	GLCpts	GLUDy	SUCOAS
15	740	ADHEr	LDH_D	GLCpts	PFK	TKT2	
	741	ADHEr	LDH_D	FBA	GLCpts	TKT2	
	742	ADHEr	LDH_D	GLCpts	TKT2	TPI	
	743	ADHEr	LDH_D	GLUDy	PGI	SUCOAS	
	744	ADHEr	LDH_D	PGDHY	PGI		
20	745	ADHEr	LDH_D	EDA	PGI		
	746	ADHEr	LDH_D	GLCpts	PGI		
	747	ADHEr	LDH_D	GLUDy	PFK	RPE	SUCOAS
	748	ADHEr	LDH_D	GLUDy	RPE	SUCOAS	TPI
	749	ADHEr	LDH_D	FBA	GLUDy	RPE	SUCOAS
25	750	ADHEr	LDH_D	GLCpts	RPE	TPI	
	751	ADHEr	LDH_D	GLCpts	PFK	RPE	
	752	ADHEr	LDH_D	FBA	GLCpts	RPE	
	753	ADHEr	LDH_D	PFK	SUCOAS	TKT2	
	754	ADHEr	LDH_D	FBA	SUCOAS	TKT2	
30	755	ADHEr	LDH_D	SUCOAS	TKT2	TPI	
	756	ADHEr	LDH_D	GLCpts	SUCOAS	TPI	
	757	ADHEr	LDH_D	GLCpts	PFK	SUCOAS	
	758	ADHEr	LDH_D	FBA	GLCpts	SUCOAS	
	759	ADHEr	LDH_D	FBA	GLCpts	GLUDy	
35	760	ADHEr	LDH_D	GLCpts	GLUDy	TPI	
	761	ADHEr	LDH_D	GLCpts	GLUDy	PFK	
	762	ADHEr	LDH_D	GLUDy	PFK	TKT2	
	763	ADHEr	LDH_D	FBA	GLUDy	TKT2	
	764	ADHEr	LDH_D	GLUDy	TKT2	TPI	
40	765	ADHEr	LDH_D	PGI	SUCOAS		
	766	ADHEr	LDH_D	GLUDy	PGI		
	767	ADHEr	LDH_D	ASPT	G6PDHy	MDH	PYK
	768	ADHEr	LDH_D	ASPT	MDH	PGL	PYK
	769	ADHEr	LDH_D	FBA	RPE	SUCOAS	
45	770	ADHEr	LDH_D	PFK	RPE	SUCOAS	
	771	ADHEr	LDH_D	RPE	SUCOAS	TPI	
	772	ADHEr	LDH_D	HEX1	PFK	SUCOAS	TKT1
	773	ADHEr	LDH_D	FBA	HEX1	SUCOAS	TAL
	774	ADHEr	LDH_D	HEX1	PFK	SUCOAS	TAL
50	775	ADHEr	LDH_D	HEX1	SUCOAS	TKT1	TPI
	776	ADHEr	LDH_D	FBA	HEX1	SUCOAS	TKT1
	777	ADHEr	LDH_D	HEX1	SUCOAS	TAL	TPI

	778	ADHEr	LDH_D	GLUDy	RPE	TPI	
	779	ADHEr	LDH_D	FBA	GLUDy	RPE	
	780	ADHEr	LDH_D	GLUDy	PFK	RPE	
	781	ADHEr	LDH_D	GLUDy	HEX1	TKT1	TPI
5	782	ADHEr	LDH_D	GLUDy	HEX1	PFK	TKT1
	783	ADHEr	LDH_D	FBA	GLUDy	HEX1	TKT1
	784	ADHEr	LDH_D	GLUDy	HEX1	TAL	TPI
	785	ADHEr	LDH_D	FBA	GLUDy	HEX1	TAL
	786	ADHEr	LDH_D	GLUDy	HEX1	PFK	TAL
10	787	ADHEr	LDH_D	GLUDy	SUCOAS	TPI	
	788	ADHEr	LDH_D	GLUDy	PFK	SUCOAS	
	789	ADHEr	LDH_D	FBA	GLUDy	SUCOAS	
	790	ADHEr	LDH_D	FRD2	PYK	SUCOAS	TKT2
	791	ADHEr	LDH_D	PYK	SUCD4	SUCOAS	TKT2
15	792	ADHEr	LDH_D	GLCpts	TPI		
	793	ADHEr	LDH_D	GLCpts	PFK		
	794	ADHEr	LDH_D	FBA	GLCpts		
	795	ADHEr	LDH_D	FRD2	GLUDy	PYK	TKT2
	796	ADHEr	LDH_D	GLUDy	PYK	SUCD4	TKT2
20	797	ADHEr	LDH_D	PFK	TKT2		
	798	ADHEr	LDH_D	FBA	TKT2		
	799	ADHEr	LDH_D	TKT2	TPI		
	800	ADHEr	LDH_D	CBMK2	SUCOAS	TAL	TPI
	801	ADHEr	LDH_D	CBMK2	FBA	SUCOAS	TAL
25	802	ADHEr	LDH_D	CBMK2	FBA	SUCOAS	TKT1
	803	ADHEr	LDH_D	CBMK2	PFK	SUCOAS	TAL
	804	ADHEr	LDH_D	CBMK2	PFK	SUCOAS	TKT1
	805	ADHEr	LDH_D	CBMK2	SUCOAS	TKT1	TPI
	806	ADHEr	LDH_D	CBMK2	FBA	HEX1	SUCOAS
30	807	ADHEr	LDH_D	CBMK2	HEX1	SUCOAS	TPI
	808	ADHEr	LDH_D	CBMK2	HEX1	PFK	SUCOAS
	809	ADHEr	LDH_D	PGI			
	810	ADHEr	LDH_D	HEX1	PFK	TAL	
	811	ADHEr	LDH_D	HEX1	TAL	TPI	
35	812	ADHEr	LDH_D	FBA	HEX1	TAL	
	813	ADHEr	LDH_D	HEX1	PFK	TKT1	
	814	ADHEr	LDH_D	HEX1	TKT1	TPI	
	815	ADHEr	LDH_D	FBA	HEX1	TKT1	
	816	ADHEr	LDH_D	PYK	RPE	SUCD4	SUCOAS
40	817	ADHEr	LDH_D	FRD2	PYK	RPE	SUCOAS
	818	ADHEr	LDH_D	FRD2	GLUDy	PYK	RPE
	819	ADHEr	LDH_D	GLUDy	PYK	RPE	SUCD4
	820	ADHEr	LDH_D	RPE	TPI		
	821	ADHEr	LDH_D	PFK	RPE		
45	822	ADHEr	LDH_D	FBA	RPE		
	823	ADHEr	LDH_D	SUCOAS	TPI		
	824	ADHEr	LDH_D	PFK	SUCOAS		
	825	ADHEr	LDH_D	FBA	SUCOAS		
	826	ADHEr	LDH_D	GLUDy	TPI		
50	827	ADHEr	LDH_D	FBA	GLUDy		
	828	ADHEr	LDH_D	GLUDy	PFK		
	829	ADHEr	LDH_D	FRD2	GLUDy	PYK	SUCOAS

830	ADHEr	LDH_D	GLUDy	PYK	SUCD4	SUCOAS	
831	ADHEr	LDH_D	HEX1	MDH	PFK	SUCD4	
832	ADHEr	LDH_D	HEX1	MDH	SUCD4	TPI	
833	ADHEr	LDH_D	FBA	HEX1	MDH	SUCD4	
834	ADHEr	LDH_D	FRD2	HEX1	MDH	TPI	
835	ADHEr	LDH_D	FBA	FRD2	HEX1	MDH	
836	ADHEr	LDH_D	FRD2	HEX1	MDH	PFK	
837	ADHEr	LDH_D	FRD2	MDH	TKT1	TPI	
838	ADHEr	LDH_D	FRD2	MDH	TAL	TPI	
839	ADHEr	LDH_D	MDH	PFK	SUCD4	TKT1	
840	ADHEr	LDH_D	MDH	PFK	SUCD4	TAL	
841	ADHEr	LDH_D	FBA	MDH	SUCD4	TKT1	
842	ADHEr	LDH_D	FBA	MDH	SUCD4	TAL	
843	ADHEr	LDH_D	MDH	SUCD4	TAL	TPI	
844	ADHEr	LDH_D	FRD2	MDH	PFK	TKT1	
845	ADHEr	LDH_D	FRD2	MDH	PFK	TAL	
846	ADHEr	LDH_D	FBA	FRD2	MDH	TAL	
847	ADHEr	LDH_D	MDH	SUCD4	TKT1	TPI	
848	ADHEr	LDH_D	FBA	FRD2	MDH	TKT1	
849	ADHEr	LDH_D	PYK	SUCD4	TKT2		
850	ADHEr	LDH_D	FRD2	PYK	TKT2		
851	ADHEr	LDH_D	FDH2	NADH6	PYK	TKT2	
852	ADHEr	LDH_D	CBMK2	PFK	TAL		
853	ADHEr	LDH_D	CBMK2	TAL	TPI		
854	ADHEr	LDH_D	CBMK2	FBA	TKT1		
855	ADHEr	LDH_D	CBMK2	TKT1	TPI		
856	ADHEr	LDH_D	CBMK2	FBA	TAL		
857	ADHEr	LDH_D	CBMK2	PFK	TKT1		
858	ADHEr	LDH_D	CBMK2	HEX1	PFK		
859	ADHEr	LDH_D	CBMK2	HEX1	TPI		
860	ADHEr	LDH_D	CBMK2	FBA	HEX1		
861	ADHEr	LDH_D	GLU5K	TAL	TPI		
862	ADHEr	LDH_D	G5SD	TAL	TPI		
863	ADHEr	LDH_D	FBA	GLU5K	TKT1		
864	ADHEr	LDH_D	G5SD	TKT1	TPI		
865	ADHEr	LDH_D	G5SD	PFK	TKT1		
866	ADHEr	LDH_D	GLU5K	PFK	TAL		
867	ADHEr	LDH_D	FBA	G5SD	TAL		
868	ADHEr	LDH_D	FBA	G5SD	TKT1		
869	ADHEr	LDH_D	G5SD	PFK	TAL		
870	ADHEr	LDH_D	GLU5K	TKT1	TPI		
871	ADHEr	LDH_D	GLU5K	PFK	TKT1		
872	ADHEr	LDH_D	FBA	GLU5K	TAL		
873	ADHEr	LDH_D	GLU5K	HEX1	TPI		
874	ADHEr	LDH_D	GLU5K	HEX1	PFK		
875	ADHEr	LDH_D	G5SD	HEX1	PFK		
876	ADHEr	LDH_D	FBA	G5SD	HEX1		
877	ADHEr	LDH_D	FBA	GLU5K	HEX1		
878	ADHEr	LDH_D	G5SD	HEX1	TPI		
879	ADHEr	LDH_D	ASNS2	PFK	TKT1		
880	ADHEr	LDH_D	ASNS2	TKT1	TPI		
881	ADHEr	LDH_D	ASNS2	PFK	TAL		

882	ADHEr	LDH_D	ASNS2	FBA	TKT1		
883	ADHEr	LDH_D	ASNS2	FBA	TAL		
884	ADHEr	LDH_D	ASNS2	TAL	TPI		
885	ADHEr	LDH_D	ASNS2	HEX1	PFK		
886	ADHEr	LDH_D	ASNS2	FBA	HEX1		
887	ADHEr	LDH_D	ASNS2	HEX1	TPI		
888	ADHEr	LDH_D	PYK	SUCD4	SUCOAS	TKT1	
889	ADHEr	LDH_D	FRD2	PYK	SUCOAS	TAL	
890	ADHEr	LDH_D	PYK	SUCD4	SUCOAS	TAL	
891	ADHEr	LDH_D	FRD2	PYK	SUCOAS	TKT1	
892	ADHEr	LDH_D	PYK	RPE	SUCD4		
893	ADHEr	LDH_D	FRD2	PYK	RPE		
894	ADHEr	LDH_D	FDH2	NADH6	PYK	RPE	
895	ADHEr	LDH_D	GLUDy	MDH	PYK	TKT2	
896	ADHEr	LDH_D	FUM	GLUDy	PYK	TKT2	
897	ADHEr	LDH_D	GLCpts	GLUDy	SUCOAS	TKT2	
898	ADHEr	LDH_D	GLUDy	PYK	SUCD4		
899	ADHEr	LDH_D	FRD2	GLUDy	PYK		
900	ADHEr	LDH_D	FDH2	GLUDy	NADH6	PYK	
901	ADHEr	LDH_D	FBA				
902	ADHEr	LDH_D	TPI				
903	ADHEr	LDH_D	PFK				
904	ADHEr	LDH_D	PYK	SUCD4	SUCOAS		
905	ADHEr	LDH_D	FRD2	PYK	SUCOAS		
906	ADHEr	LDH_D	FDH2	NADH6	PYK	SUCOAS	
907	ADHEr	LDH_D	FRD2	ME2	PGDHY	PYK	
908	ADHEr	LDH_D	EDA	FRD2	ME2	PYK	
909	ADHEr	LDH_D	FRD2	ME2	PGL	PYK	
910	ADHEr	LDH_D	EDA	ME2	PYK	SUCD4	
911	ADHEr	LDH_D	ME2	PGDHY	PYK	SUCD4	
912	ADHEr	LDH_D	ME2	PGL	PYK	SUCD4	
913	ADHEr	LDH_D	FRD2	G6PDHy	ME2	PYK	
914	ADHEr	LDH_D	G6PDHy	ME2	PYK	SUCD4	
915	ADHEr	LDH_D	MDH	NADH6	PGDHY	PYK	
916	ADHEr	LDH_D	MDH	PGL	PYK	SUCD4	
917	ADHEr	LDH_D	FRD2	MDH	PGL	PYK	
918	ADHEr	LDH_D	FRD2	MDH	PGDHY	PYK	
919	ADHEr	LDH_D	G6PDHy	MDH	PYK	SUCD4	
920	ADHEr	LDH_D	MDH	NADH6	PGL	PYK	
921	ADHEr	LDH_D	EDA	FRD2	MDH	PYK	
922	ADHEr	LDH_D	EDA	MDH	PYK	SUCD4	
923	ADHEr	LDH_D	MDH	PGDHY	PYK	SUCD4	
924	ADHEr	LDH_D	EDA	MDH	NADH6	PYK	
925	ADHEr	LDH_D	FRD2	G6PDHy	MDH	PYK	
926	ADHEr	LDH_D	G6PDHy	MDH	NADH6	PYK	
927	ADHEr	LDH_D	GLUDy	MDH	PYK	RPE	
928	ADHEr	LDH_D	FUM	GLUDy	PYK	RPE	
929	ADHEr	LDH_D	FRD2	PYK	TAL		
930	ADHEr	LDH_D	PYK	SUCD4	TKT1		
931	ADHEr	LDH_D	PYK	SUCD4	TAL		
932	ADHEr	LDH_D	FRD2	PYK	TKT1		
933	ADHEr	LDH_D	FDH2	NADH6	PYK	TAL	

	934	ADHEr	LDH_D	FDH2	NADH6	PYK	TKT1
	935	ADHEr	LDH_D	GLCpts	GLUDy	RPE	SUCOAS
	936	ADHEr	LDH_D	GLUDy	MDH	PYK	SUCOAS
	937	ADHEr	LDH_D	FUM	GLUDy	PYK	SUCOAS
5	938	ADHEr	LDH_D	FUM	GLUDy	NADH6	PYK
	939	ADHEr	LDH_D	GLUDy	MDH	NADH6	PYK
	940	ADHEr	LDH_D	GLCpts	SUCOAS	TKT2	
	941	ADHEr	LDH_D	GLUDy	SUCOAS	TKT2	
	942	ADHEr	LDH_D	ASPT	MDH	PYK	TKT2
10	943	ADHEr	LDH_D	ASPT	FUM	PYK	TKT2
	944	ADHEr	LDH_D	FRD2	PYK		
	945	ADHEr	LDH_D	PYK	SUCD4		
	946	ADHEr	LDH_D	FDH2	NADH6	PYK	
	947	ADHEr	LDH_D	GLCpts	GLUDy	TKT2	
15	948	ADHEr	LDH_D	GLCpts	GLUDy	SUCOAS	TAL
	949	ADHEr	LDH_D	GLCpts	GLUDy	SUCOAS	TKT1
	950	ADHEr	LDH_D	FUM	GLUDy	PYK	
	951	ADHEr	LDH_D	GLUDy	MDH	PYK	
	952	ADHEr	LDH_D	GLCpts	RPE	SUCOAS	
20	953	ADHEr	LDH_D	ASPT	FUM	PYK	RPE
	954	ADHEr	LDH_D	ASPT	MDH	PYK	RPE
	955	ADHEr	LDH_D	GLUDy	RPE	SUCOAS	
	956	ADHEr	LDH_D	GLCpts	GLUDy	RPE	
	957	ADHEr	LDH_D	ASPT	FUM	PYK	SUCOAS
25	958	ADHEr	LDH_D	ASPT	MDH	PYK	SUCOAS
	959	ADHEr	LDH_D	GLCpts	GLUDy	SUCOAS	
	960	ADHEr	LDH_D	ASPT	FUM	NADH6	PYK
	961	ADHEr	LDH_D	ASPT	MDH	NADH6	PYK
	962	ADHEr	LDH_D	SUCOAS	TKT2		
30	963	ADHEr	LDH_D	GLCpts	TKT2		
	964	ADHEr	LDH_D	ASPT	MDH	PYK	TKT1
	965	ADHEr	LDH_D	ASPT	FUM	PYK	TAL
	966	ADHEr	LDH_D	ASPT	MDH	PYK	TAL
	967	ADHEr	LDH_D	ASPT	FUM	PYK	TKT1
35	968	ADHEr	LDH_D	GLCpts	SUCOAS	TAL	
	969	ADHEr	LDH_D	GLCpts	SUCOAS	TKT1	
	970	ADHEr	LDH_D	GLUDy	TKT2		
	971	ADHEr	LDH_D	GLCpts	GLUDy	TKT1	
	972	ADHEr	LDH_D	GLCpts	GLUDy	TAL	
40	973	ADHEr	LDH_D	GLUDy	SUCOAS	TKT1	
	974	ADHEr	LDH_D	GLUDy	SUCOAS	TAL	
	975	ADHEr	LDH_D	ASPT	MDH	PYK	
	976	ADHEr	LDH_D	ASPT	FUM	PYK	
	977	ADHEr	LDH_D	RPE	SUCOAS		
45	978	ADHEr	LDH_D	GLCpts	RPE		
	979	ADHEr	LDH_D	GLCpts	SUCOAS		
	980	ADHEr	LDH_D	GLUDy	RPE		
	981	ADHEr	LDH_D	GLCpts	GLUDy		
	982	ADHEr	LDH_D	GLUDy	SUCOAS		
50	983	ADHEr	LDH_D	TKT2			
	984	ADHEr	LDH_D	GLCpts	TAL		
	985	ADHEr	LDH_D	GLCpts	TKT1		

986	ADHEr	LDH_D	SUCOAS	TAL
987	ADHEr	LDH_D	SUCOAS	TKT1
988	ADHEr	LDH_D	GLUDy	TKT1
989	ADHEr	LDH_D	GLUDy	TAL
990	ADHEr	LDH_D	RPE	
991	ADHEr	LDH_D	GLCpts	
992	ADHEr	LDH_D	SUCOAS	
993	ADHEr	LDH_D	GLUDy	
994	ADHEr	LDH_D	TAL	
995	ADHEr	LDH_D	TKT1	

Table 2: A list of all the reaction stoichiometries and the associated genes known to be associated with the reactions identified for disruption in the strategies listed in Tables 1.

Reaction Abbreviation	Reaction Name	Reaction Stoichiometry	Assigned Genes
ACKr	acetate kinase	[c] : ac + atp <==> actp + adp	b2296, b3115
ADHEr	acetaldehyde-CoA dehydrogenase	[c] : accoa + (2) h + (2) nadh <==> coa + etoh + (2) nad	b1241
AKGD	2-oxoglutarate dehydrogenase	[c] : akg + coa + nad --> co2 + nadh + succoa	b0727, b0726, b0116
ALAR	alanine racemase	[c] : ala-L <==> ala-D	b4053
ASNS2	asparagine synthetase	[c] : asp-L + atp + nh4 --> amp + asn-L + h + ppi	b3744
ASPT	L-aspartase	[c] : asp-L --> fum + nh4	b4139
ATPS4r	ATP synthase (four protons for one ATP)	adp[c] + (4) h[e] + pi[c] <==> atp[c] + (3) h[c] + h2o[c]	b3738+b3736+b3737, b3739, b3734+b3732+b3735+b3733+ b3731
CBMK2	Carbamate kinase	[c] : atp + co2 + nh4 --> adp + cbp + (2) h	b0323, b0521, b2874
DAAD	D-Amino acid dehydrogenase	[c] : ala-D + fad + h2o --> fadh2 + nh4 + pyr	b1189
EDA	2-dehydro-3-deoxy-phosphogluconate aldolase	[c] : 2ddg6p --> g3p + pyr	b1850
FBA	fructose-bisphosphate aldolase	[c] : fdp <==> dhap + g3p	b1773, b2097, b2925
FDH2	formate dehydrogenase (quinone-8: 2 protons)	for[c] + (3) h[c] + ubq8[c] --> co2[c] + (2) h[e] + ubq8h2[c]	b3893+b3894+b3892, b1476+b1475+b1474, b4079
FRD2	fumarate reductase	[c] : fum + mql8 --> mqn8 + succ	b4153+b4152+b4151+b4154
FUM	fumarase	[c] : fum + h2o <==> mal-L	b1612, b4122, b1611
G5SD	glutamate-5-semialdehyde dehydrogenase	[c] : glu5p + h + nadph --> glu5sa + nadp + pi	b0243
G6PDHy	glucose 6-phosphate dehydrogenase	[c] : g6p + nadp <==> 6pgl + h + nadph	b1852
GLCpts	D-glucose transport via PEP:Pyr PTS	glc-D[e] + pep[c] --> g6p[c] + pyr[c]	b1817, b1818, b2417, b1621, b2416, b1819, b1101, b2415
GLU5K	glutamate 5-kinase	[c] : atp + glu-L --> adp + glu5p	b0242
GLUDy	glutamate dehydrogenase (NADP)	[c] : glu-L + h2o + nadp <==> akg + h + nadph + nh4	b1761
HEX1	hexokinase (D-glucose:ATP)	[c] : atp + glc-D --> adp + g6p + h	b2388
ICL	Isocitrate lyase	[c] : icit --> glx + succ	b4015

LDH_D	D-lactate dehydrogenase	[c] : lac-D + nad <==> h + nadh + pyr	b2133, b1380
MALS	malate synthase	[c] : accoa + glx + h2o --> coa + h + mal-L	b4014, b2976
MDH	malate dehydrogenase	[c] : mal-L + nad <==> h + nadh + oaa	b3236
ME2	malic enzyme (NADP)	[c] : mal-L + nadp --> co2 + nadph + pyr	b2463
NADH12	NADH dehydrogenase (ubiquinone-8)	[c] : h + nadh + ubq8 --> nad + ubq8h2	b1109
NADH6	NADH dehydrogenase (ubiquinone-8 & 3.5 protons)	(4.5) h[c] + nadh[c] + ubq8[c] --> (3.5) h[e] + nad[c] + ubq8h2[c]	b2288+b2277+b2285+b2278+ b2276+b2286+b2287+b2279+ b2280+b2284+b2283+b2282+ b2281
PFK	phosphofructokinase	[c] : atp + f6p --> adp + fdp + h	b3916, b1723
PFLi	pyruvate formate lyase	[c] : coa + pyr --> accoa + for	b3114, b3951+b3952, b0902+b2579+b0903
PGDH	phosphogluconate dehydrogenase	[c] : 6pgc + nadp --> co2 + nadph + ru5p-D	b2029
PGDHY	phosphogluconate dehydratase	[c] : 6pgc --> 2ddg6p + h2o	b1851
PGI	glucose-6-phosphate isomerase	[c] : g6p <==> f6p	b4025
PGL	6-phosphogluconolactonase	[c] : 6pgl + h2o --> 6pgc + h	b0767
PPS	phosphoenolpyruvate synthase	[c] : atp + h2o + pyr --> amp + (2) h + pep + pi	b1702
PRO1z	proline oxidase	[c] : fad + pro-L --> 1pyr5c + fadh2 + h	b1014
PTAr	phosphotransacetylase	[c] : accoa + pi <==> actp + coa	b2297
PYK	pyruvate kinase	[c] : adp + h + pep --> atp + pyr	b1854, b1676
RPE	ribulose 5-phosphate 3-epimerase	[c] : ru5p-D <==> xu5p-D	b4301, b3386
SUCD4	succinate dehyrdogenase	[c] : fadh2 + ubq8 <==> fad + ubq8h2	b0723+b0721+b0724+b0722
SUCOAS	succinyl-CoA synthetase (ADP-forming)	[c] : atp + coa + succ <==> adp + pi + succoa	b0729+b0728
TAL	transaldolase	[c] : g3p + s7p <==> e4p + f6p	b2464, b0008
THD2	NAD(P) transhydrogenase	(2) h[e] + nadh[c] + nadp[c] --> (2) h[c] + nad[c] + nadph[c]	b1602+b1603
TKT1	transketolase	[c] : r5p + xu5p-D <==> g3p + s7p	b2935, b2465
TKT2	transketolase	[c] : e4p + xu5p-D <==> f6p + g3p	b2935, b2465
TPI	triose-phosphate isomerase	[c] : dhap <==> g3p	b3919

Table 3: List of the metabolite abbreviations, the corresponding names and locations of all the metabolites that participate in the reactions listed in Table 2.

Metabolite Abbreviation	Compartment	Metabolite Name
1pyr5c	Cytosol	1-Pyrroline-5-carboxylate
2ddg6p	Cytosol	2-Dehydro-3-deoxy-D-gluconate 6-phosphate
6pgc	Cytosol	6-Phospho-D-gluconate
6pgl	Cytosol	6-phospho-D-glucono-1,5-lactone
ac	Cytosol	Acetate
accoa	Cytosol	Acetyl-CoA
actp	Cytosol	Acetyl phosphate
adp	Cytosol	ADP
akg	Cytosol	2-Oxoglutarate
ala-D	Cytosol	D-Alanine
ala-L	Cytosol	L-Alanine
amp	Cytosol	AMP
asn-L	Cytosol	L-Asparagine
asp-L	Cytosol	L-Aspartate
atp	Cytosol	ATP
cbp	Cytosol	Carbamoyl phosphate
cit	Cytosol	Citrate
co2	Cytosol	CO2
coa	Cytosol	Coenzyme A
ctp	Cytosol	CTP
dha	Cytosol	Dihydroxyacetone
dhap	Cytosol	Dihydroxyacetone phosphate
e4p	Cytosol	D-Erythrose 4-phosphate
etoh	Cytosol	Ethanol
f6p	Cytosol	D-Fructose 6-phosphate
fad	Cytosol	FAD
fadh2	Cytosol	FADH2
fdp	Cytosol	D-Fructose 1,6-bisphosphate
for	Cytosol	Formate
fum	Cytosol	Fumarate
g3p	Cytosol	Glyceraldehyde 3-phosphate
g6p	Cytosol	D-Glucose 6-phosphate
glc-D	Cytosol	D-Glucose
glc-D[e]	Extra-organism	D-Glucose
glu5p	Cytosol	L-Glutamate 5-phosphate
glu5sa	Cytosol	L-Glutamate 5-semialdehyde
glu-L	Cytosol	L-Glutamate
glx	Cytosol	Glyoxylate
h	Cytosol	H+
h[e]	Extra-organism	H+
h2	Cytosol	H2
h2o	Cytosol	H2O
icit	Cytosol	Isocitrate
k	Cytosol	K+
lac-D	Cytosol	D-Lactate
mal-L	Cytosol	L-Malate
mql8	Cytosol	Menaquinol 8
mqn8	Cytosol	Menaquinone 8
nad	Cytosol	Nicotinamide adenine dinucleotide
nadh	Cytosol	Nicotinamide adenine dinucleotide - reduced

nadp	Cytosol	Nicotinamide adenine dinucleotide phosphate
nadph	Cytosol	Nicotinamide adenine dinucleotide phosphate - reduced
nh4	Cytosol	Ammonium
o2	Cytosol	O2
oaa	Cytosol	Oxaloacetate
pep	Cytosol	Phosphoenolpyruvate
pi	Cytosol	Phosphate
ppi	Cytosol	Diphosphate
pro-L	Cytosol	L-Proline
pyr	Cytosol	Pyruvate
r5p	Cytosol	alpha-D-Ribose 5-phosphate
ru5p-D	Cytosol	D-Ribulose 5-phosphate
s7p	Cytosol	Sedoheptulose 7-phosphate
succ	Cytosol	Succinate
succoa	Cytosol	Succinyl-CoA
ubq8	Cytosol	Ubiquinone-8
ubq8h2	Cytosol	Ubiquinol-8
xu5p-D	Cytosol	D-Xylulose 5-phosphate

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific examples and studies detailed above are only illustrative of the invention. It should be understood that various modifications can be made without departing from the scope of the invention.

WHAT IS CLAIMED IS:

1. A microbial organism comprising:

(i) a first set of heterologous nucleic acids encoding a malonyl-CoA-independent fatty acid synthesis (FAS) pathway, said malonyl-CoA-independent FAS pathway comprising one or more malonyl-CoA-independent FAS pathway enzymes selected from ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase expressed from said first set of heterologous nucleic acids; and

(ii) a second set of heterologous nucleic acids encoding an acyl-reduction pathway expressed in sufficient amounts to produce primary alcohol selected from the group consisting of hexanol, heptanol, octanol, nonanol, decanol, dodecanol, tetradecanol and hexadecanol;

wherein said acyl-reduction pathway comprises one or more acyl-reduction pathway enzymes having the activity of one or both of an acyl-CoA reductase and an alcohol dehydrogenase expressed from said second set of heterologous nucleic acids;

wherein said ketoacyl-CoA acyltransferase or ketoacyl-CoA thiolase converts acyl-CoA to β-ketoacyl-CoA, wherein said 3-hydroxyacyl-CoA dehydrogenase converts β-ketoacyl-CoA to β-hydroxyacyl-CoA, wherein said enoylCoA hydratase converts β-hydroxyacyl-CoA to trans-2-enoyl-CoA, wherein said enoyl-CoA reductase converts trans-2-enoyl-CoA to acyl-CoA; and

wherein said acyl-CoA reductase converts acyl-CoA to an aldehyde and wherein said alcohol dehydrogenase converts an aldehyde to said primary alcohol.

2. The microbial organism of claim 1, wherein said microbial organism comprises at least two heterologous nucleic acids each encoding one of said malonyl-CoA-independent FAS pathway enzymes.

3. The microbial organism of claim 1, wherein said microbial organism comprises at least three heterologous nucleic acids each encoding one of said malonyl-CoA-independent FAS pathway enzymes.

4. The microbial organism of claim 1, 2 or 3, wherein said first set of heterologous nucleic acids encoding said malonyl-CoA-independent FAS pathway comprise nucleic acids encoding ketoacyl-CoA acyltransferase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase.

5. The microbial organism of claim 1, 2 or 3, wherein said first set of heterologous nucleic acids encoding said malonyl-CoA-independent FAS pathway comprise nucleic acids encoding ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase.

6. The microbial organism of claim 1, wherein said microbial organism comprises at least four heterologous nucleic acids each encoding a malonyl-CoA-independent FAS pathway enzyme.

7. The microbial organism of claim 6, wherein said four heterologous nucleic acids encode ketoacyl-CoA acyltransferase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase.

8. The microbial organism of claim 6, wherein said four heterologous nucleic acids encode ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase.

9. The microbial organism of any one of claims 1 to 8, wherein said second set of heterologous nucleic acids encoding said acyl-reduction pathway encode a heterologous acyl-CoA reductase.

10. The microbial organism of any one of claims 1 to 9, wherein said second set of heterologous nucleic acids encoding said acyl-reduction pathway encode a heterologous alcohol dehydrogenase.

11. The microbial organism of any one of claims 1 to 10, wherein said one or more acyl-reduction pathway enzymes are expressed from two heterologous nucleic acids.

12. The microbial organism of claim 11, wherein one heterologous nucleic acid encodes the acyl-CoA reductase and a second heterologous nucleic acid encodes the alcohol dehydrogenase.

13. The microbial organism of any one of claims 1 to10, wherein said second set of heterologous nucleic acids encode an enzyme having acyl-CoA reductase activity and alcohol dehydrogenase activity.

14. The microbial organism of claim 13, wherein said enzyme having acyl-CoA reductase and alcohol dehydrogenase activity is a fatty alcohol forming acyl-CoA reductase (FAR).

15. The microbial organism of any one of claims 1 to 10, wherein said second set of heterologous nucleic acids encoding said acyl-reduction pathway encode (i) the acyl-CoA reductase and the alcohol dehydrogenase, and (ii) an enzyme having acyl-CoA reductase and alcohol dehydrogenase activity.

16. The microbial organism of claim 15, wherein said enzyme having acyl-CoA reductase and alcohol dehydrogenase activity is a fatty alcohol forming acyl-CoA reductase (FAR).

17. The microbial organism of any one of claims 1 to 16, wherein said primary alcohol is produced in amounts as a measurement of mmol/gDW/hr of at least 10% greater levels compared to a microbial organism lacking said first set of heterologous nucleic acids encoding the malonyl-CoA-independent FAS pathway and the second set of heterologous nucleic acids encoding the acyl-reduction pathway.

18. A composition comprising the microbial organism as defined in any one of claims 1 to 17, and a substantially anaerobic culture medium.

19. A method for producing a primary alcohol, comprising culturing the microbial organism as defined in any one of claims 1 to 17, wherein the heterologous nucleic acids encoding said malonyl-CoA-independent fatty acid synthesis (FAS) pathway and the heterologous nucleic acids encoding said acyl-reduction pathway are expressed in sufficient amounts and under substantially anaerobic conditions for a sufficient period of time to produce said primary alcohol.

20. The method of claim 19, wherein said primary alcohol is produced in amounts as a measurement of mmol/gDW/hr of at least 10% greater levels compared to a microbial

organism lacking said first set of heterologous nucleic acid encoding the malonyl-CoA-independent FAS pathway.

21. The method of claim 19 or 20, further comprising isolating said primary alcohol.

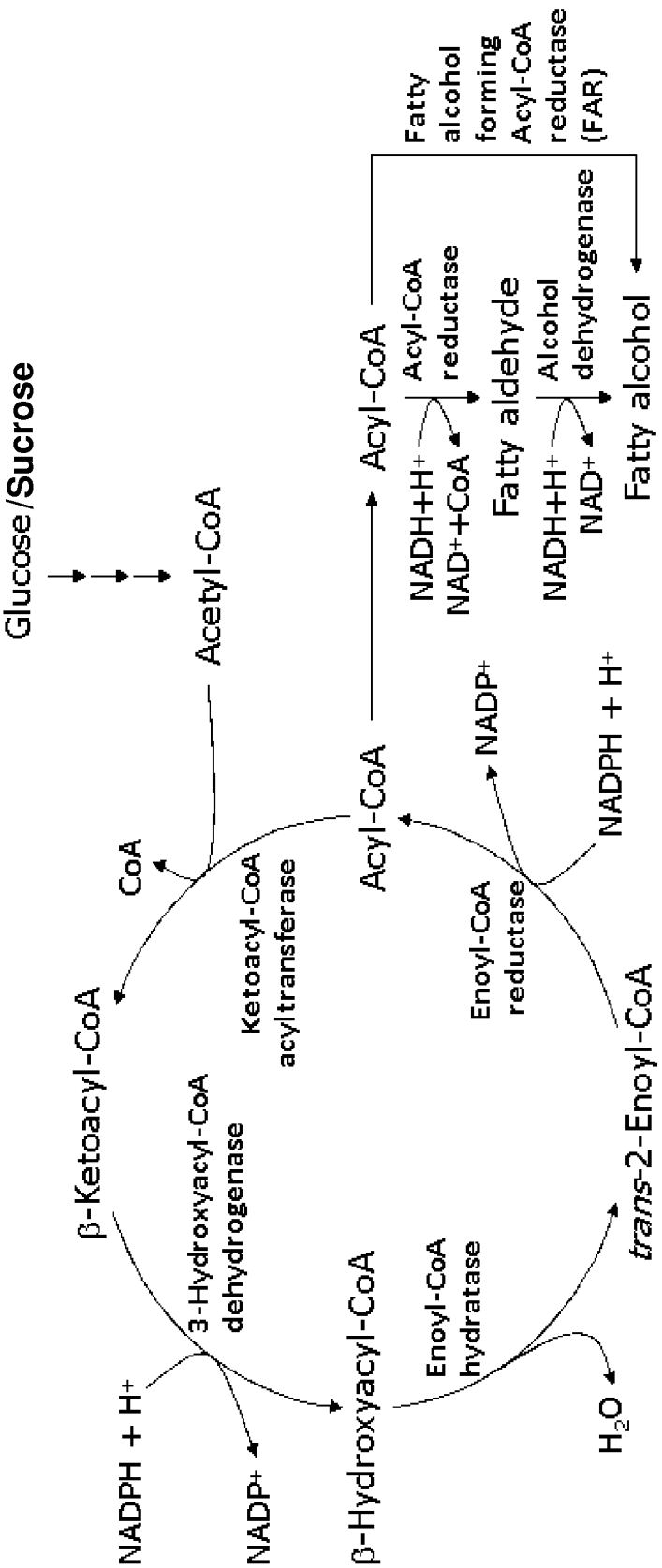
Glucose/Sucrose
Acetyl-CoA
CoA
β-Ketoacyl-CoA
Ketoacyl-CoA acyltransferase
3-Hydroxyacyl-CoA dehydrogenase
NADPH + H+
NADP+
β-Hydroxyacyl-CoA
Enoyl-CoA hydratase
H2O
trans-2-Enoyl-CoA
Enoyl-CoA reductase
NADP+
NADPH + H+
Acyl-CoA
Acyl-CoA
Acyl-CoA reductase
NADH+H+
NAD+ +CoA
Fatty aldehyde
Alcohol dehydrogenase
NADH+H+
NAD+
Fatty alcohol
Fatty alcohol forming Acyl-CoA reductase (FAR)

Figure 1

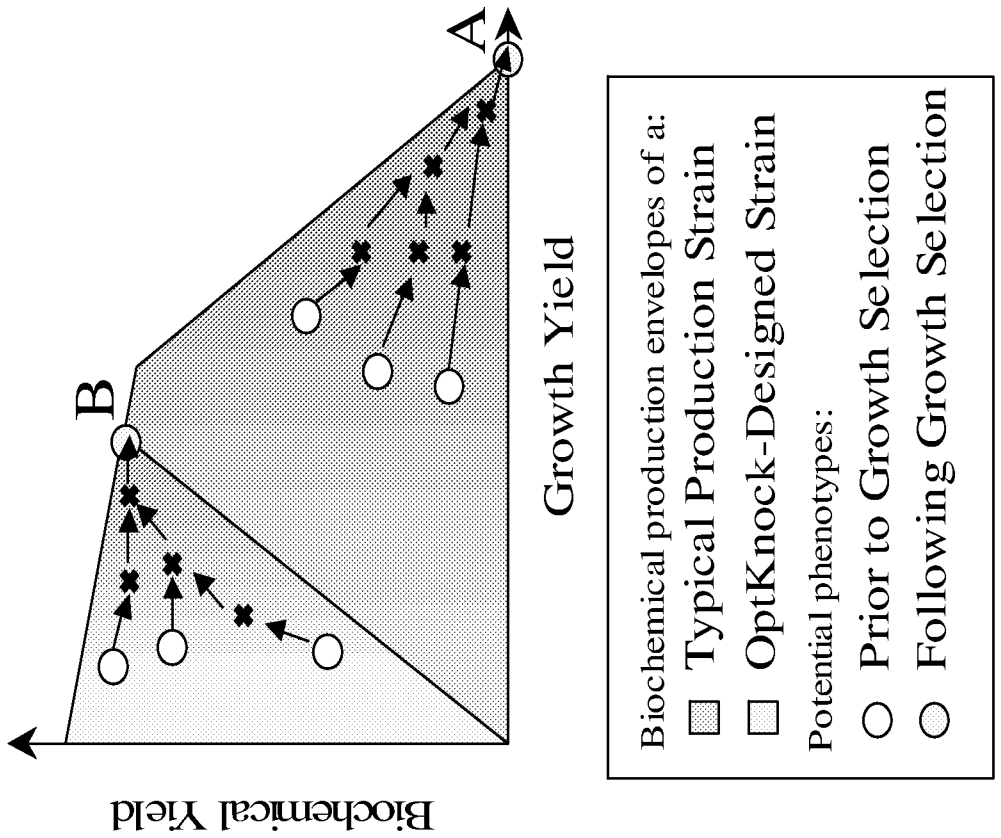
A
B
Growth Yield
Biochemical Yield
Biochemical production envelopes of a:
Typical Production Strain
OptKnock-Designed Strain
Potential phenotypes:
Prior to Growth Selection
Following Growth Selection

Figure 2

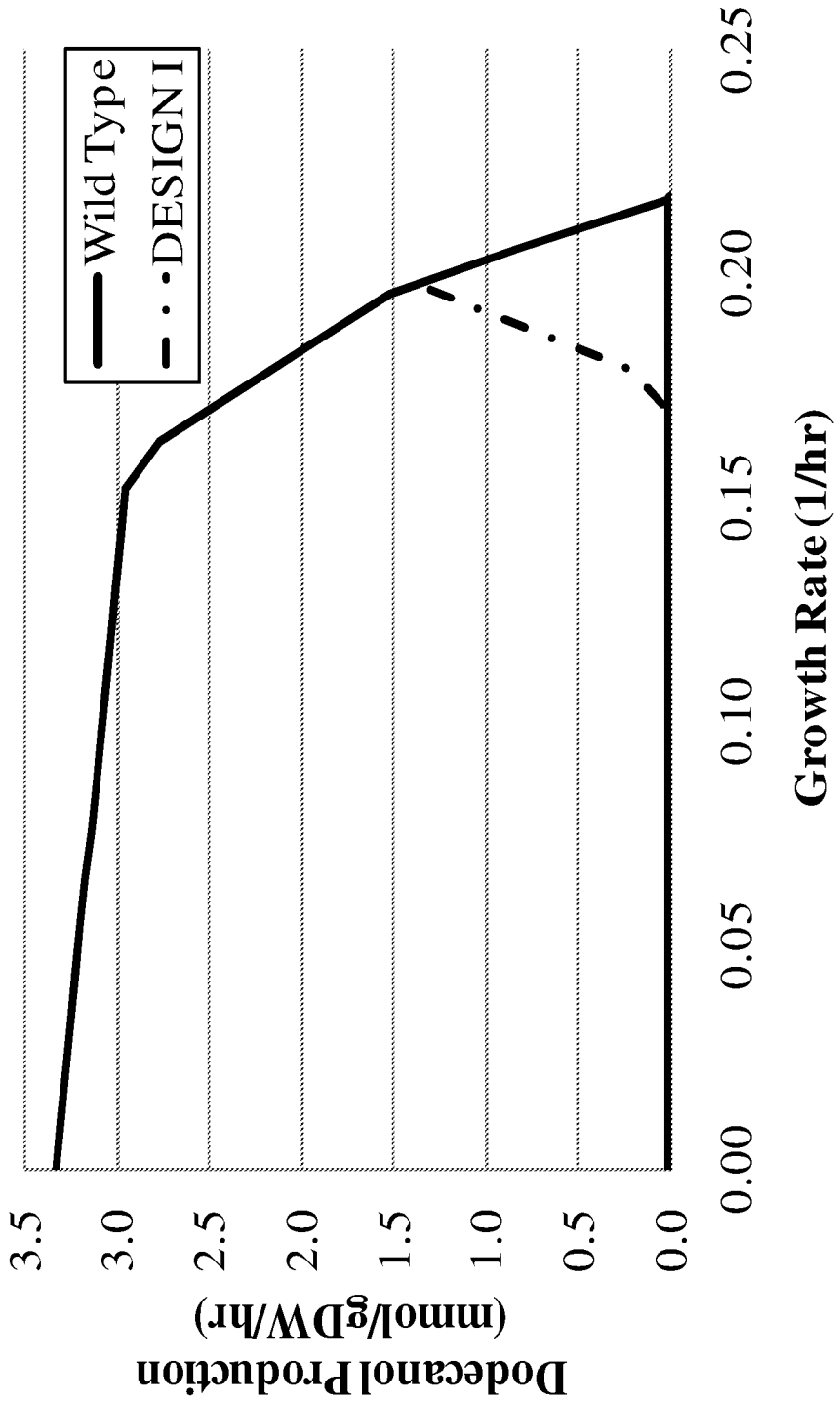
Wild Type
DESIGN I
Dodecanol Production (mmol/gDW/hr)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.05
0.10
0.15
0.20
0.25
Growth Rate (1/hr)

Figure 3

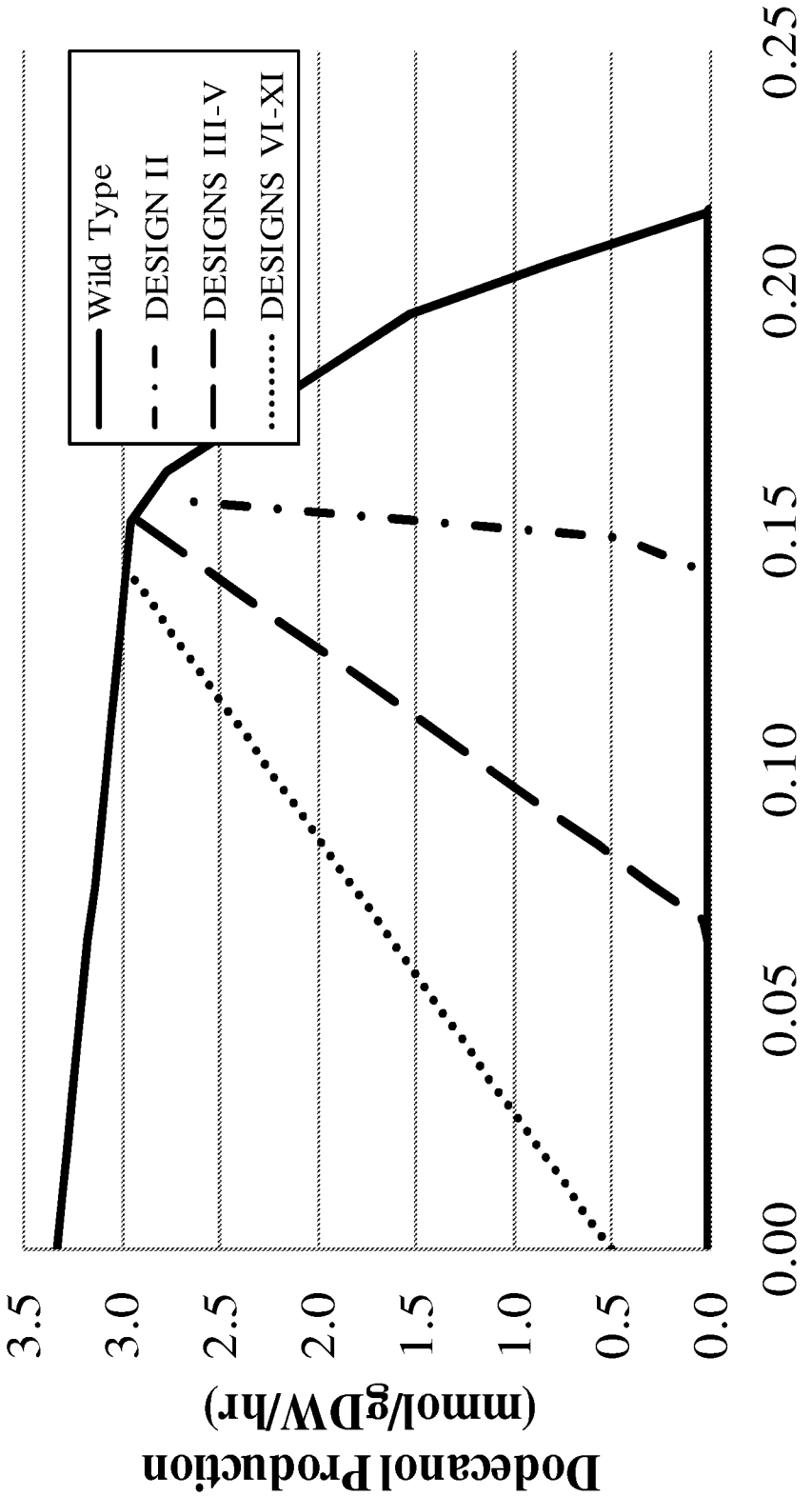
Wild Type
DESIGN II
DESIGNS III-V
DESIGNS VI-XI
Dodecanol Production (mmol/gDW/hr)
Growth Rate (1/hr)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.00
0.05
0.10
0.15
0.20
0.25

Figure 4

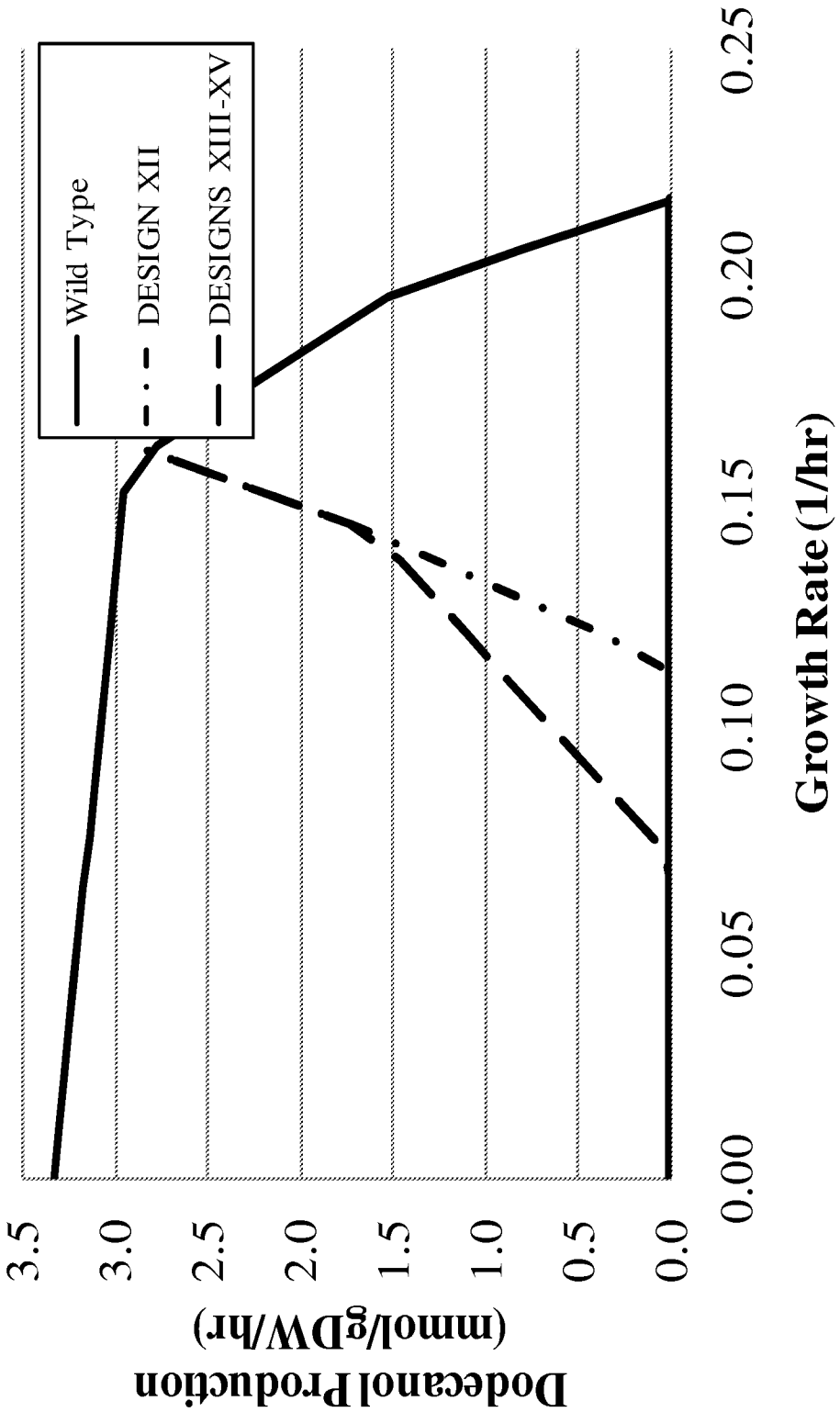
Wild Type
DESIGN XII
DESIGNS XIII-XV
Dodecanol Production (mmol/gDW/hr)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.05
0.10
0.15
0.20
0.25
Growth Rate (1/hr)

Figure 5

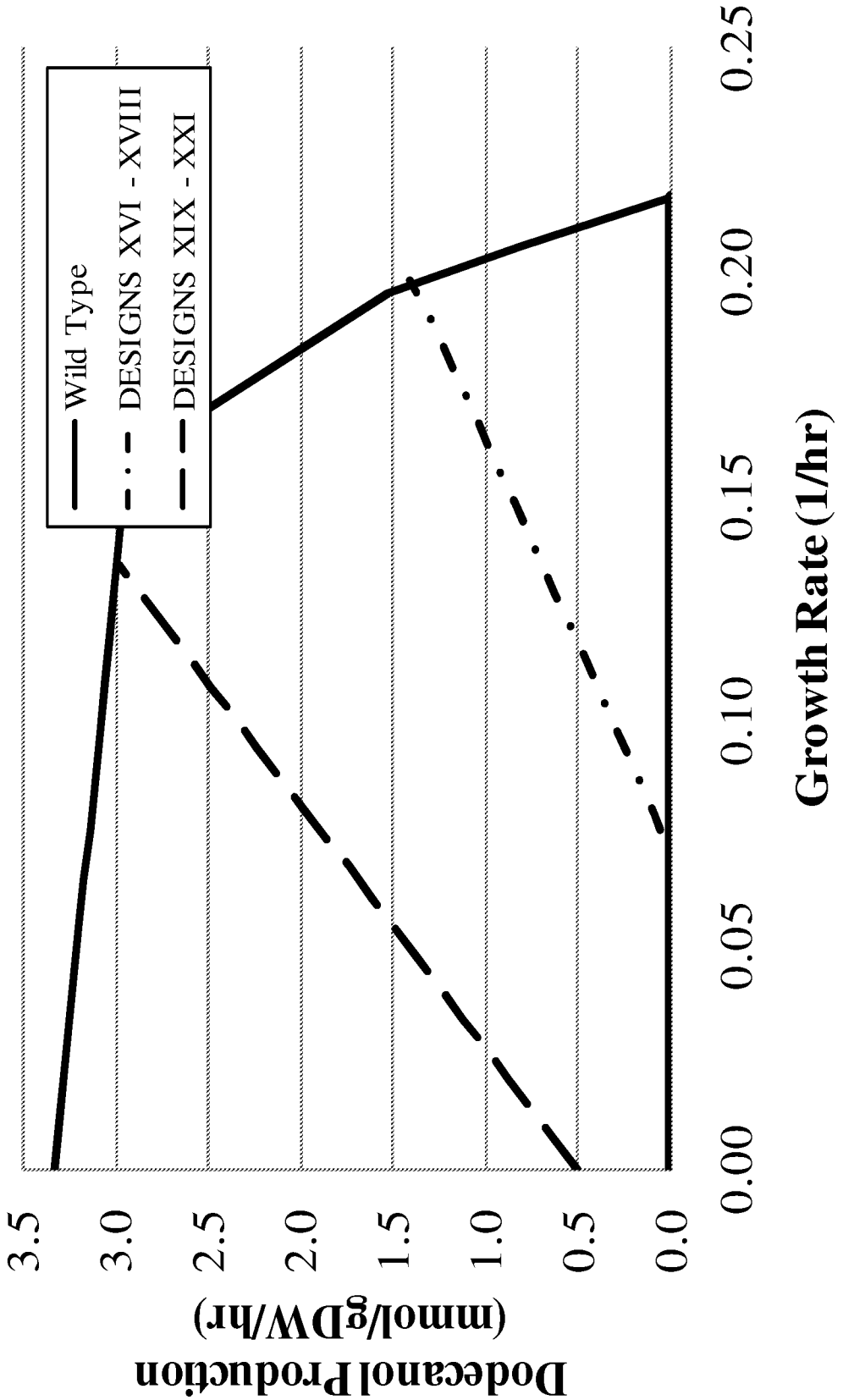
Wild Type
DESIGNS XVI - XVIII
DESIGNS XIX - XXI
Dodecanol Production (mmol/gDW/hr)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Growth Rate (1/hr)
0.00
0.05
0.10
0.15
0.20
0.25

Figure 6

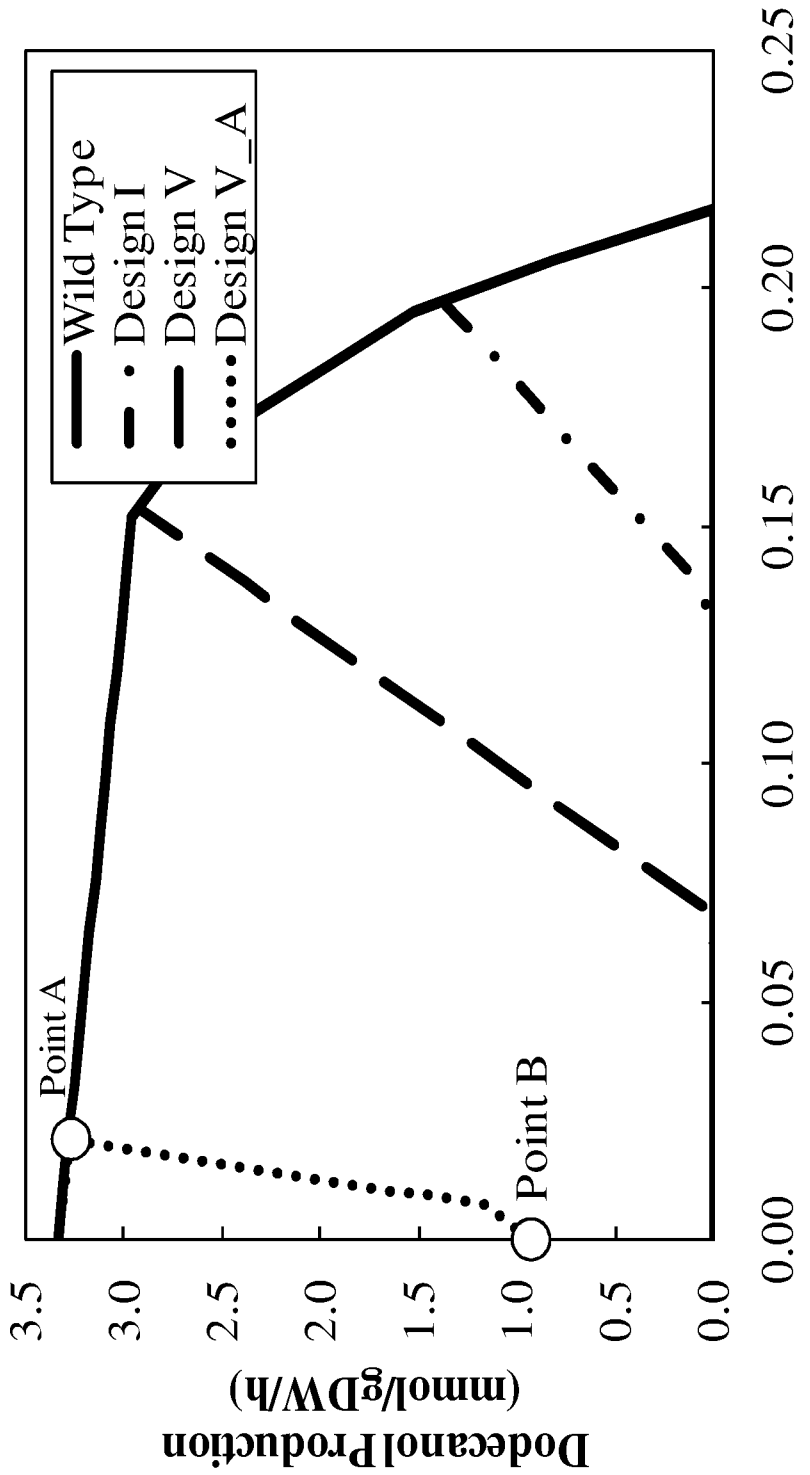
Wild Type
Design I
Design V
Design V_A
Point A
Point B
Dodecanol Production (mmol/gDW/h)
Growth Rate (1/hr)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.00
0.05
0.10
0.15
0.20
0.25

Figure 7

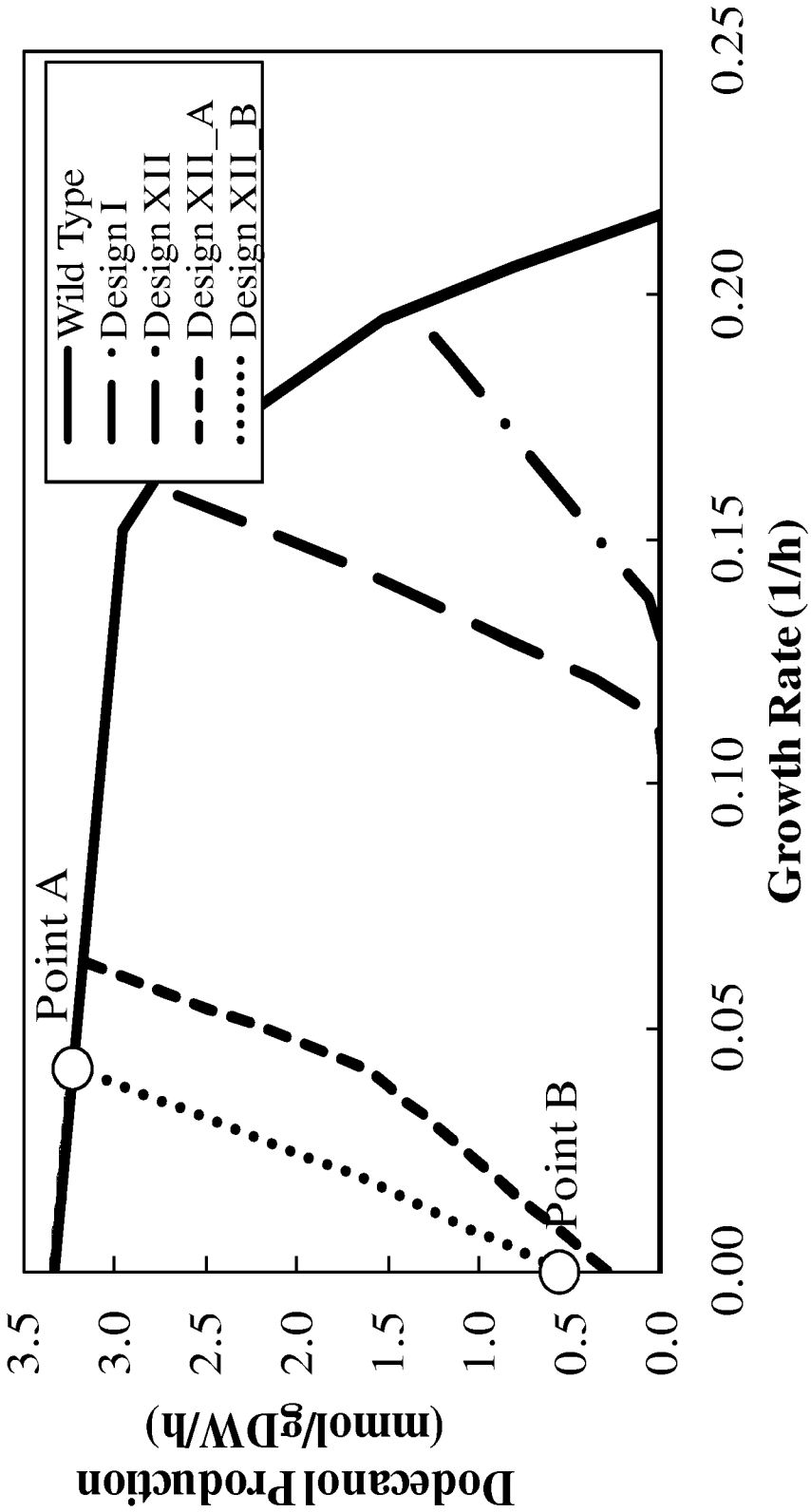
Wild Type
Design I
Design XII
Design XII_A
Design XII_B
Point A
Point B
Dodecanol Production
(mmol/gDW/h)
Growth Rate (1/h)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.00
0.05
0.10
0.15
0.20
0.25

Figure 8

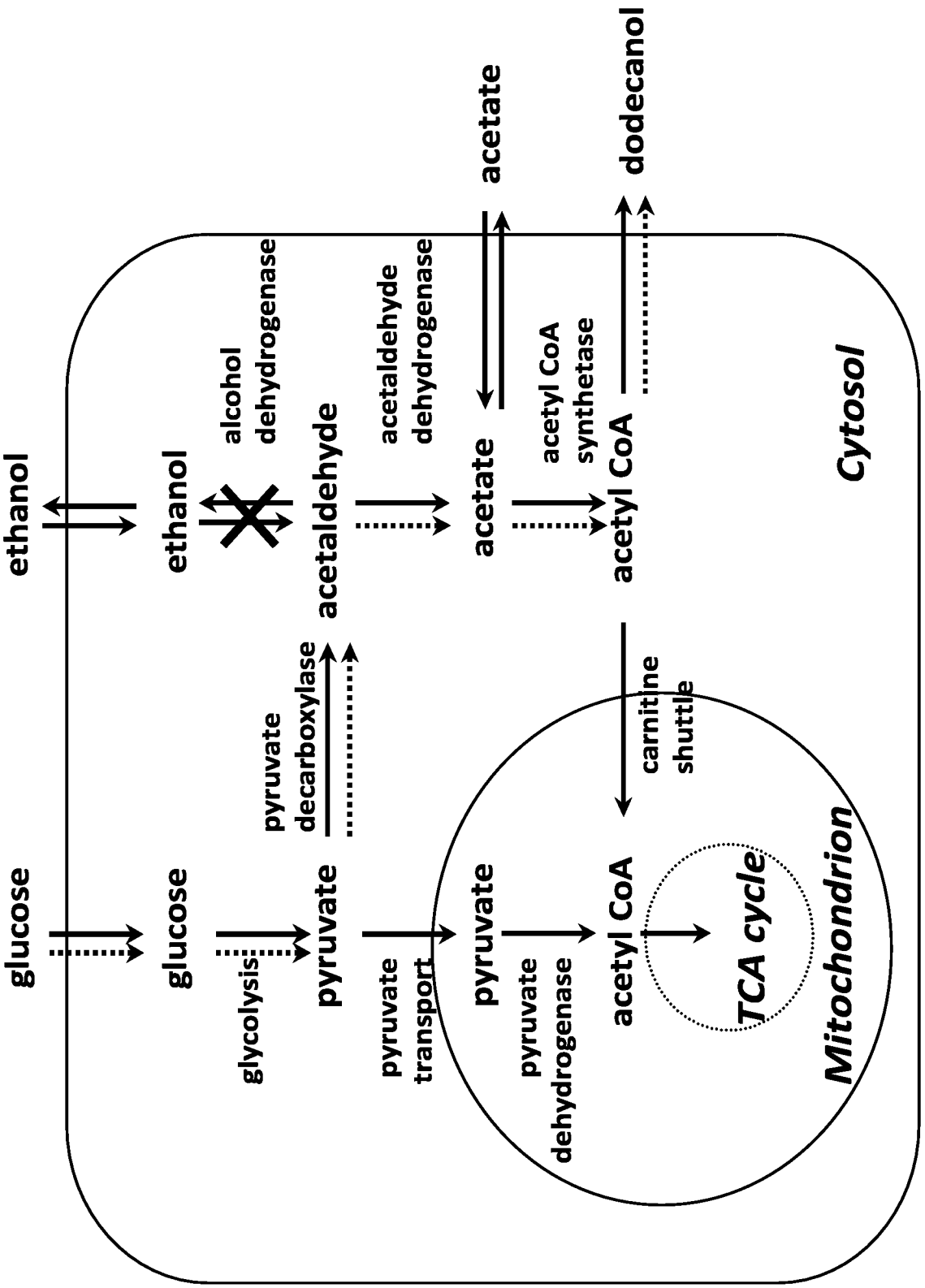
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetate
acetyl CoA synthetase
acetyl CoA
dodecanol
Cytosol
glucose
glucose
glycolysis
pyruvate
pyruvate decarboxylase
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
carnitine shuttle
TCA cycle
Mitochondrion

Figure 9a

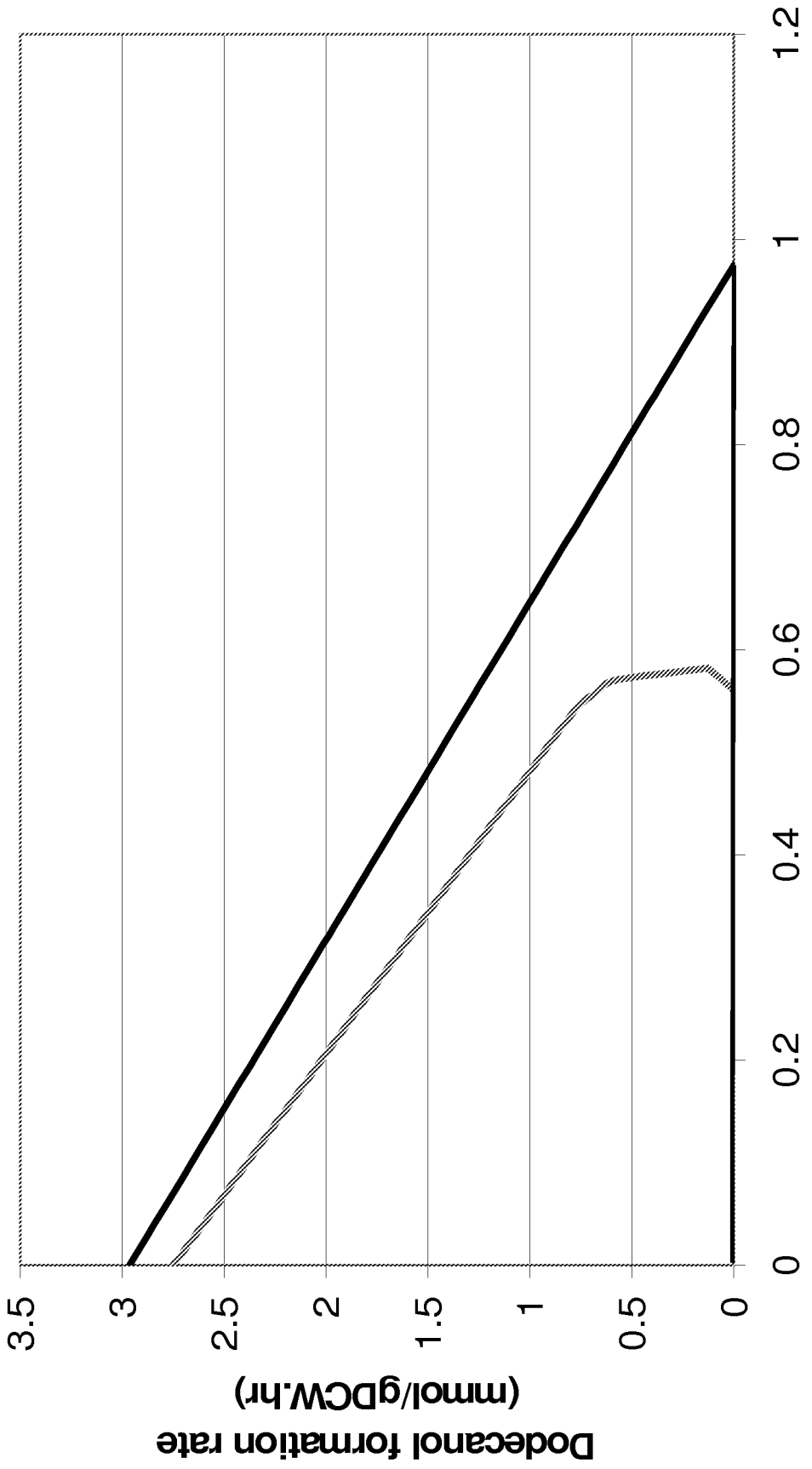
Dodecanol formation rate
(mmol/gDCW.hr)
3.5
3
2.5
2
1.5
1
0.5
0
Biomass formation rate (1/hr)
0
0.2
0.4
0.6
0.8
1
1.2

Figure 9b

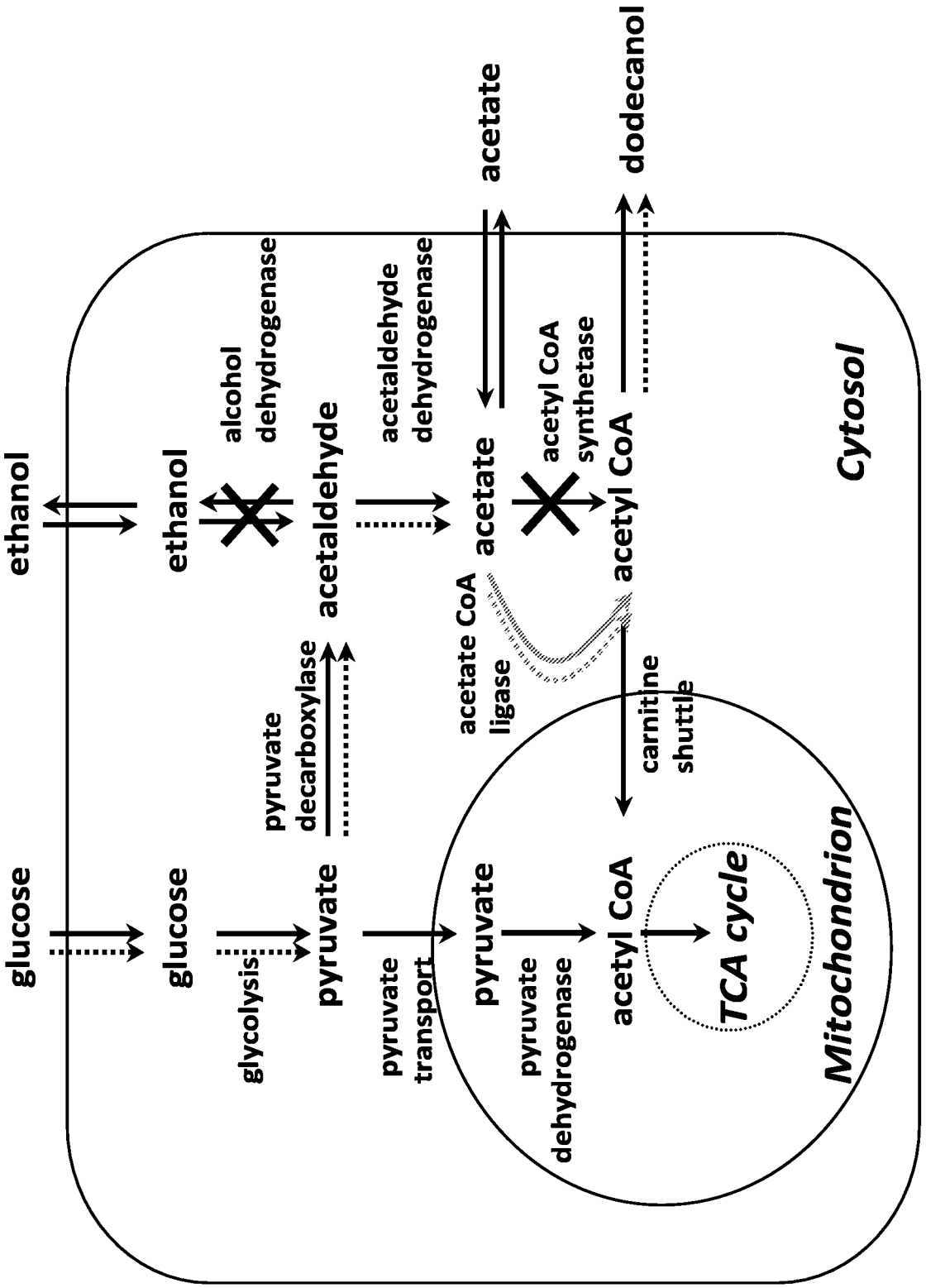
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
carnitine shuttle
acetyl CoA ligase
pyruvate decarboxylase
acetaldehyde
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde dehydrogenase
acetate
acetate
acetyl CoA synthetase
acetyl CoA
dodecanol
Cytosol

Figure 10a

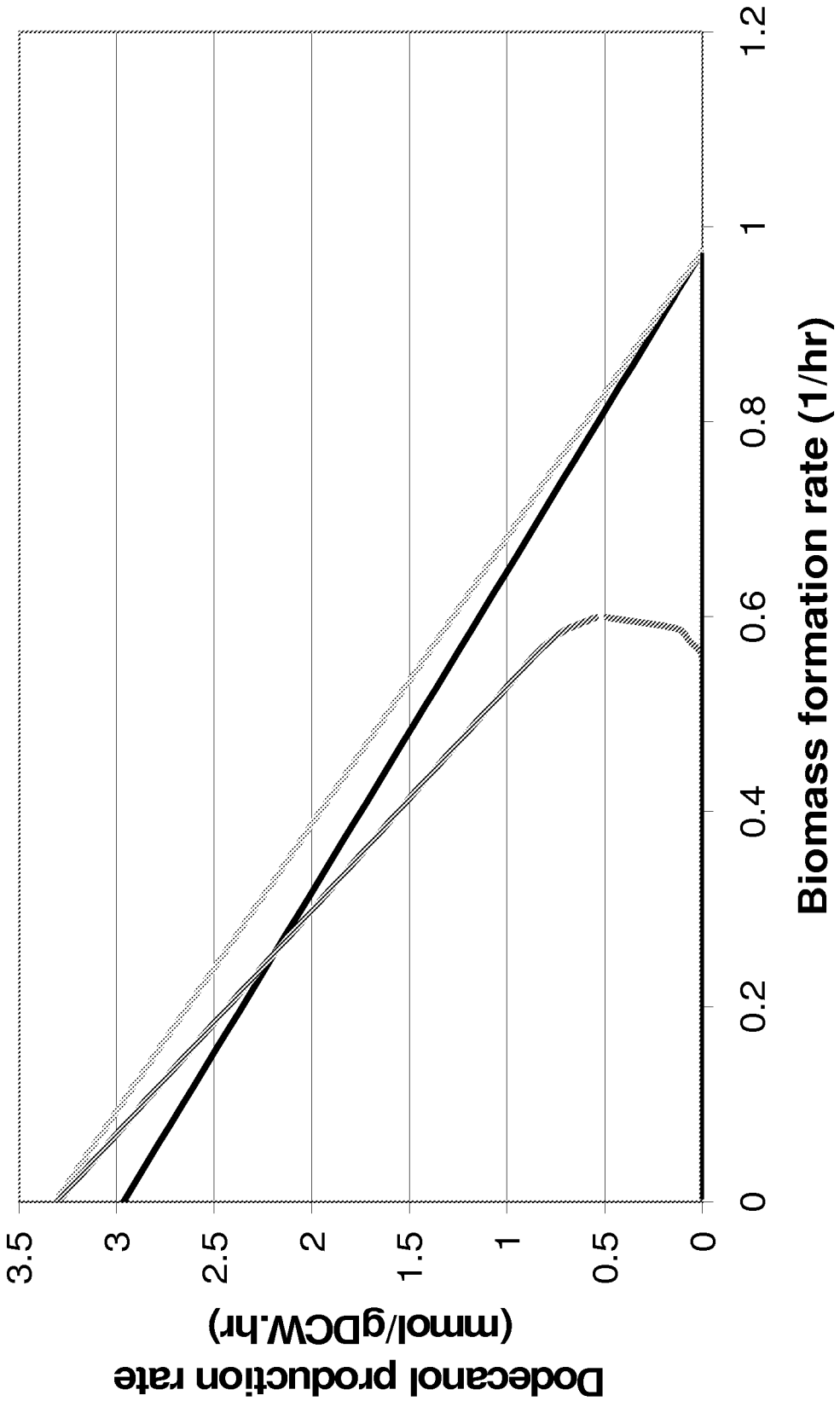
Dodecanol production rate
(mmol/gDCW.hr)
3.5
3
2.5
2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
1.2
Biomass formation rate (1/hr)

Figure 10b

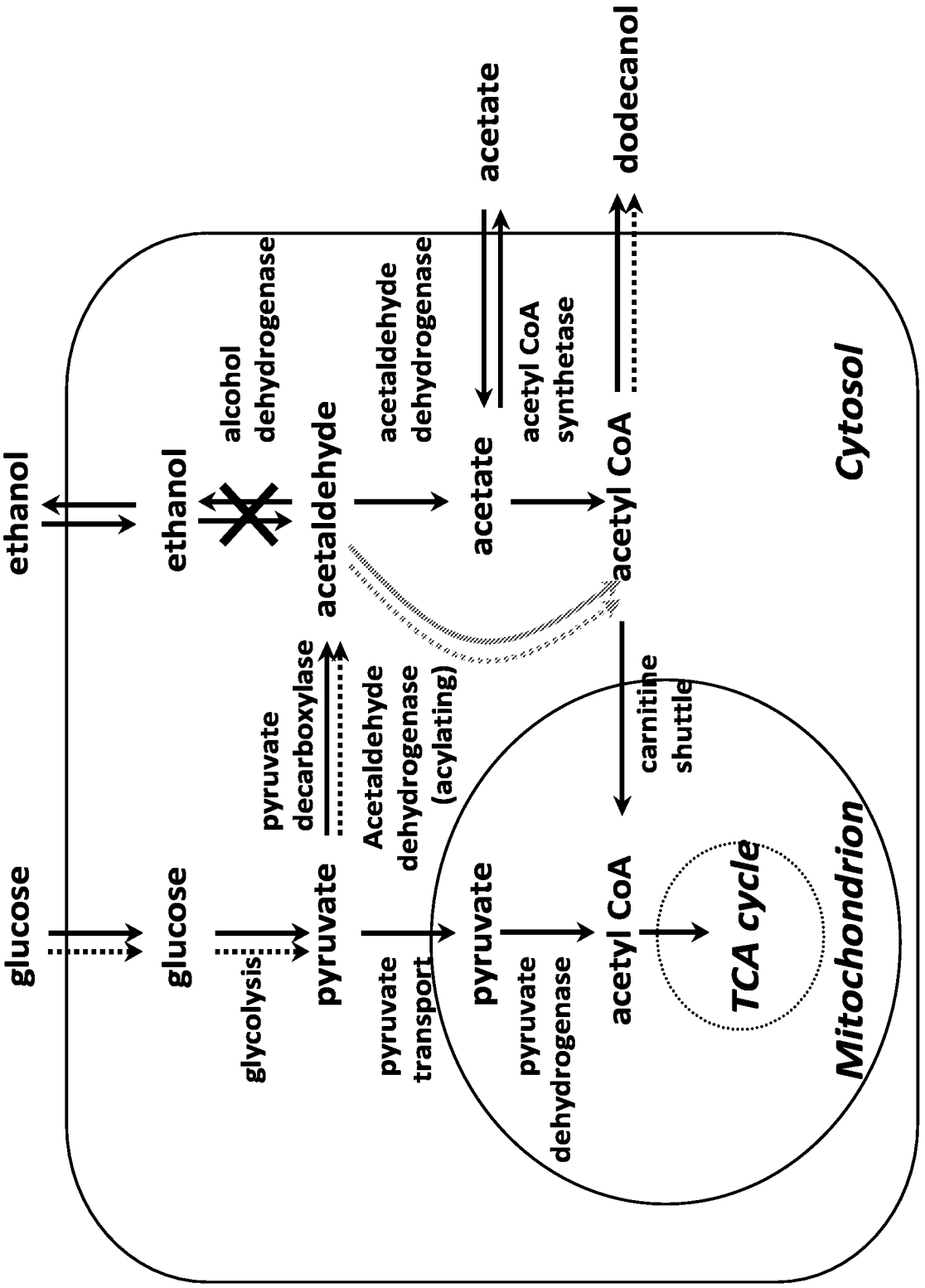
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
carnitine shuttle
Acetaldehyde dehydrogenase (acylating)
pyruvate decarboxylase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
dodecanol
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetate
Cytosol

Figure 11a

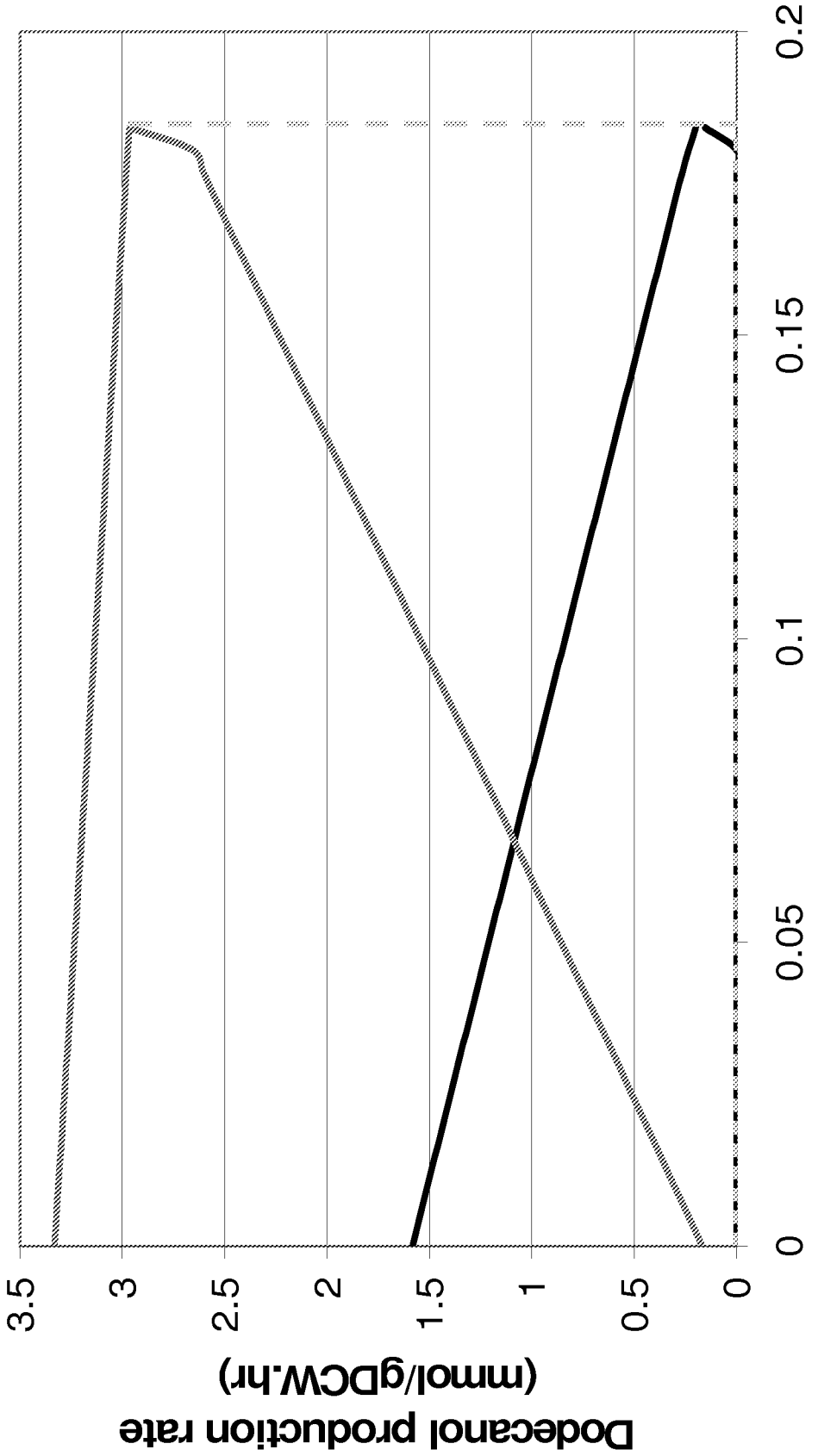
Dodecanol production rate
(mmol/gDCW.hr)
3.5
3
2.5
2
1.5
1
0.5
0
Biomass formation rate (1/hr)
0
0.05
0.1
0.15
0.2

Figure 11b

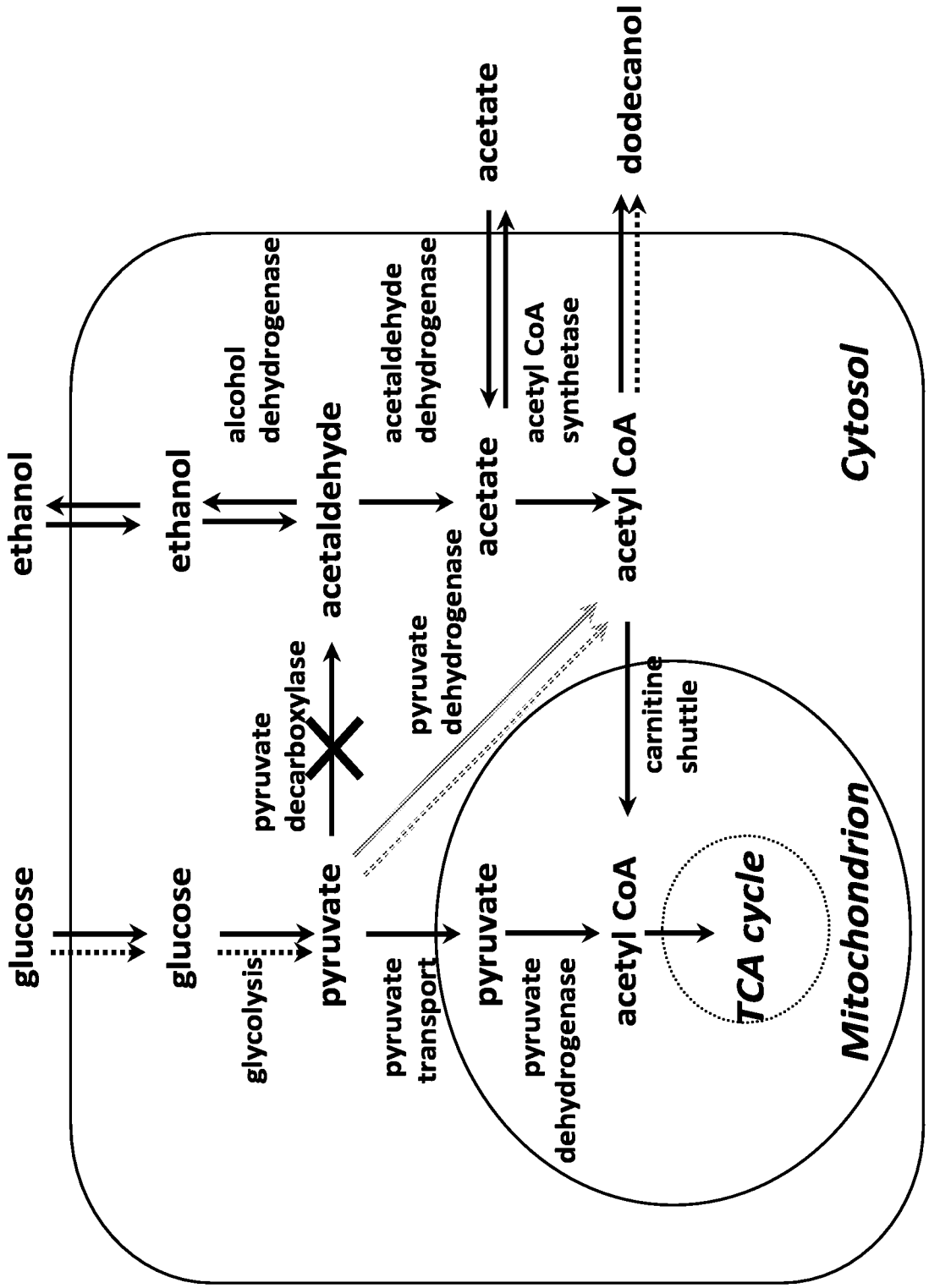
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
carnitine shuttle
pyruvate decarboxylase
pyruvate dehydrogenase
acetaldehyde
alcohol dehydrogenase
ethanol
ethanol
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetate
acetyl CoA
dodecanol
Cytosol

Figure 12

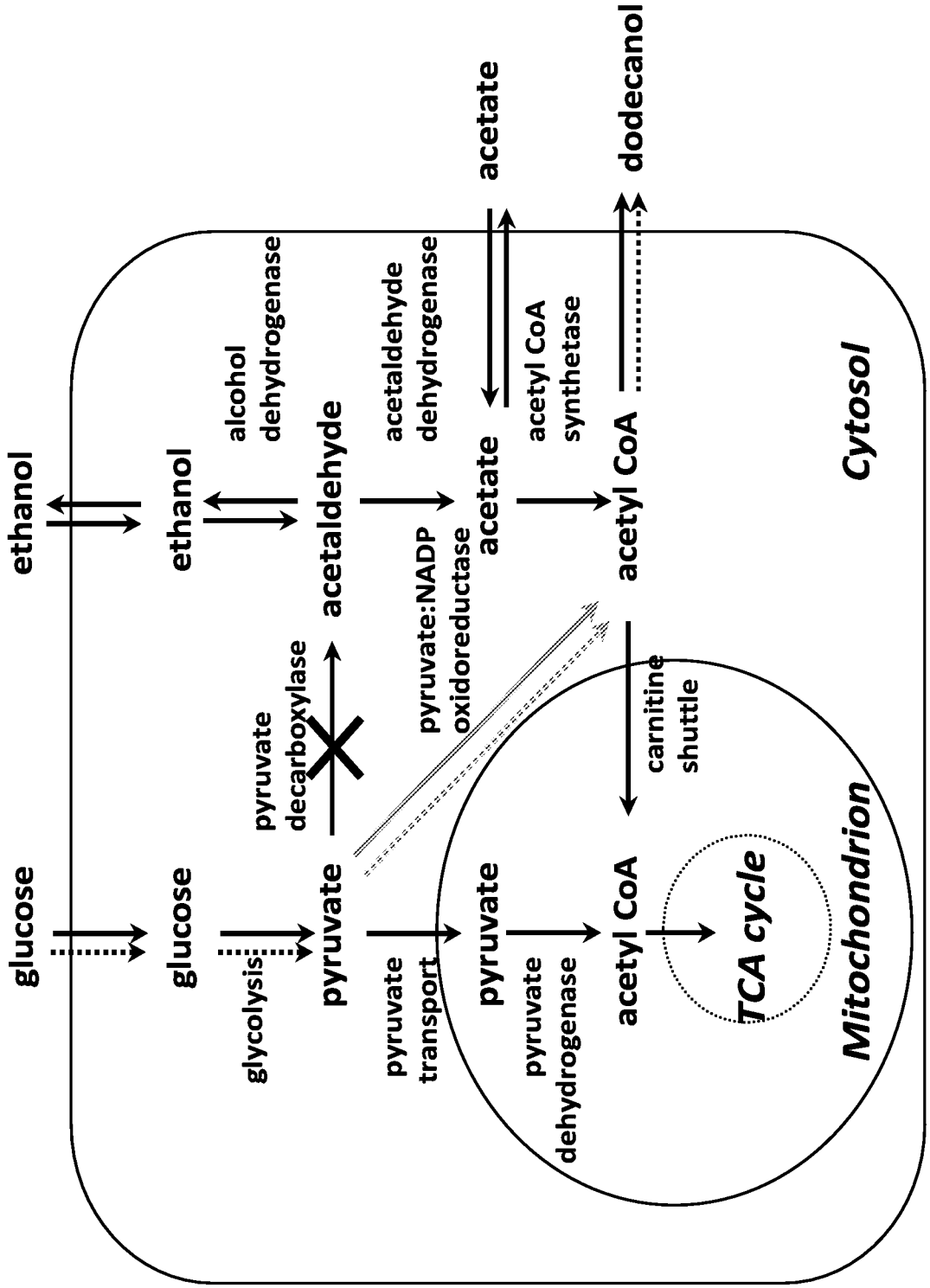
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
carnitine shuttle
pyruvate decarboxylase
pyruvate:NADP oxidoreductase
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetate
acetyl CoA
dodecanol
Cytosol

Figure 13

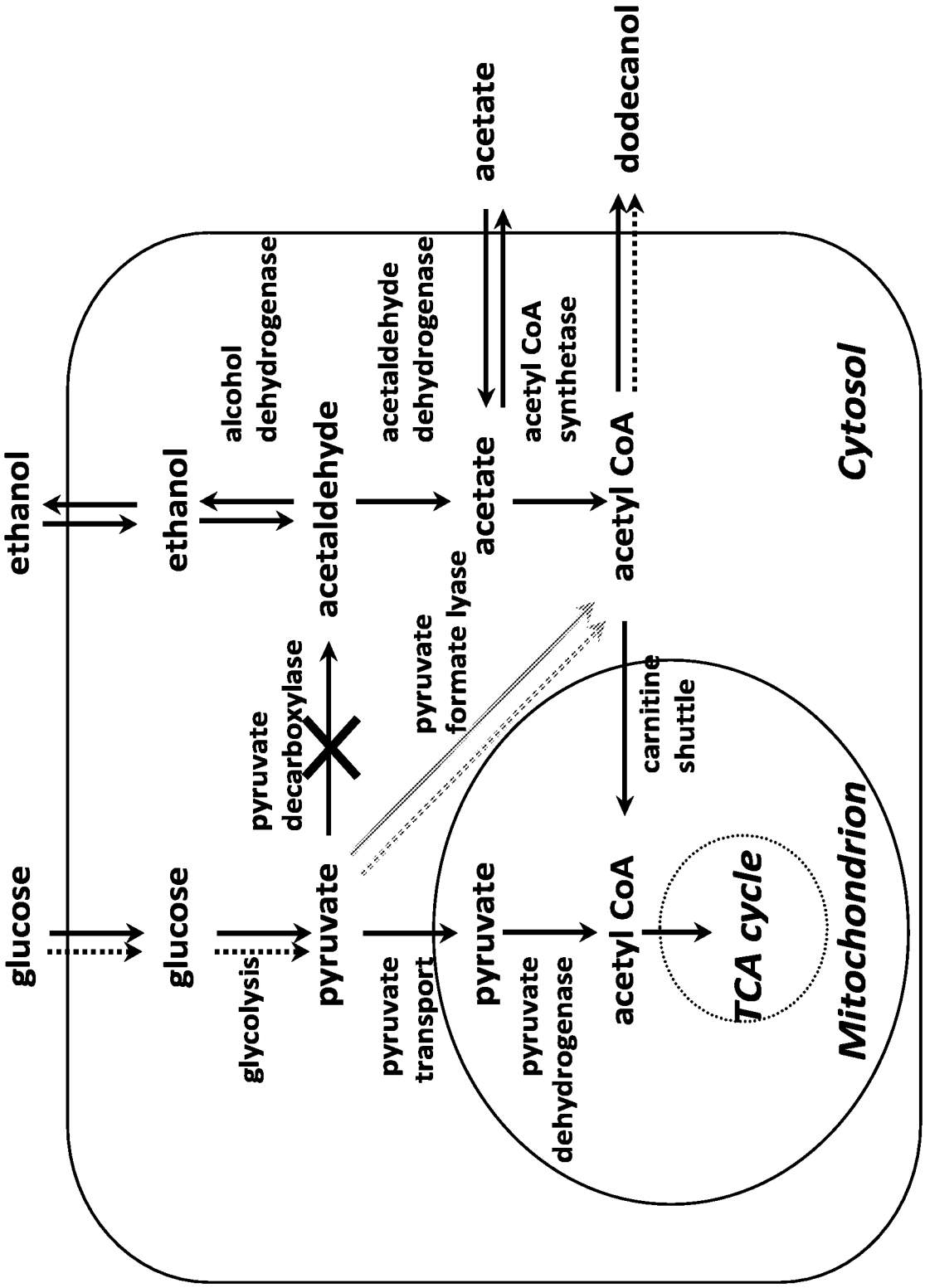
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
carnitine shuttle
pyruvate decarboxylase
pyruvate formate lyase
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
acetate
dodecanol
Cytosol

Figure 14

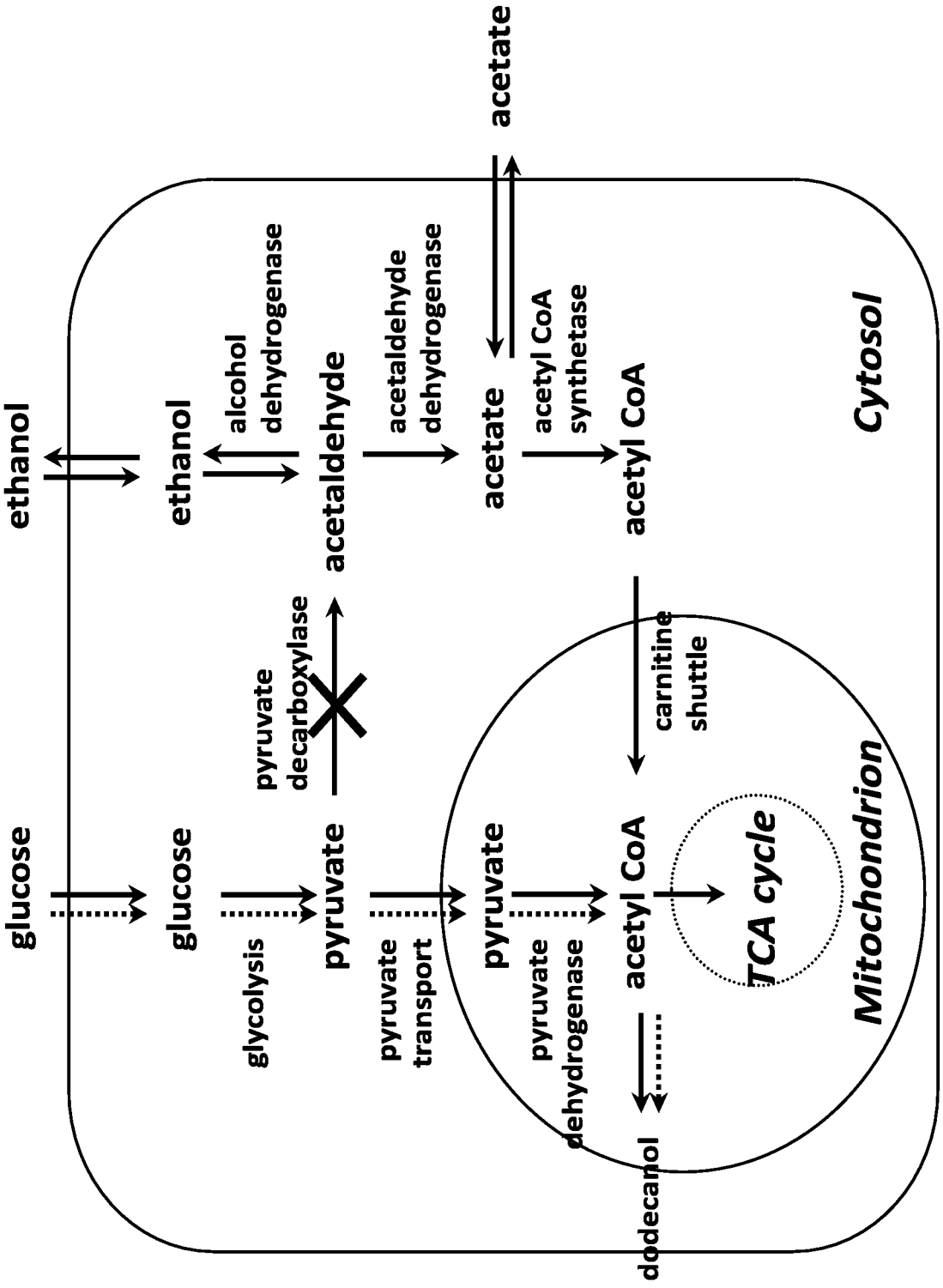
acetate
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
Cytosol
pyruvate decarboxylase
carnitine shuttle
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
dodecanol

Figure 15a

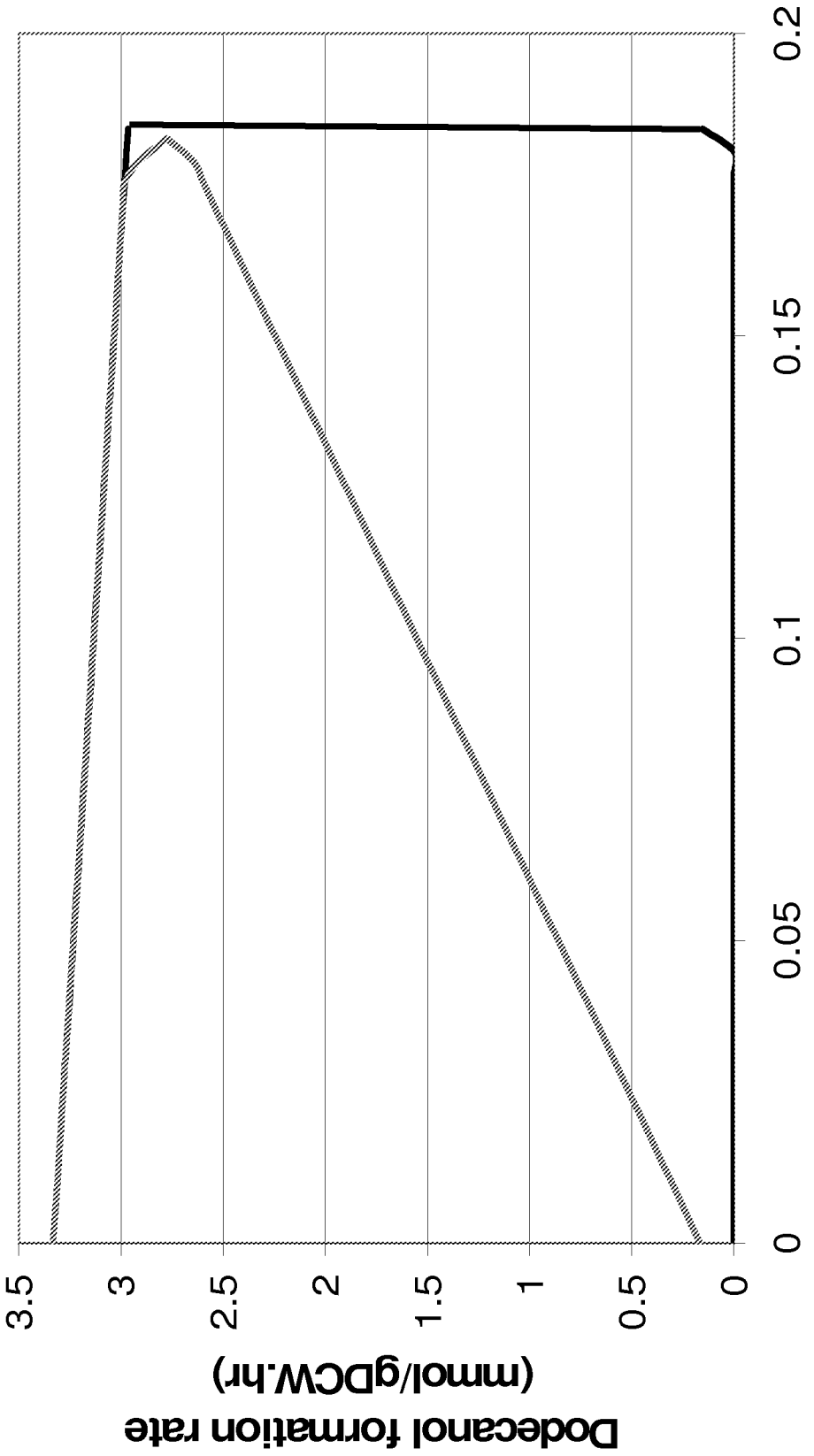
Dodecanol formation rate
(mmol/gDCW.hr)
3.5
3
2.5
2
1.5
1
0.5
0
Biomass formation rate (1/hr)
0
0.05
0.1
0.15
0.2

Figure15b

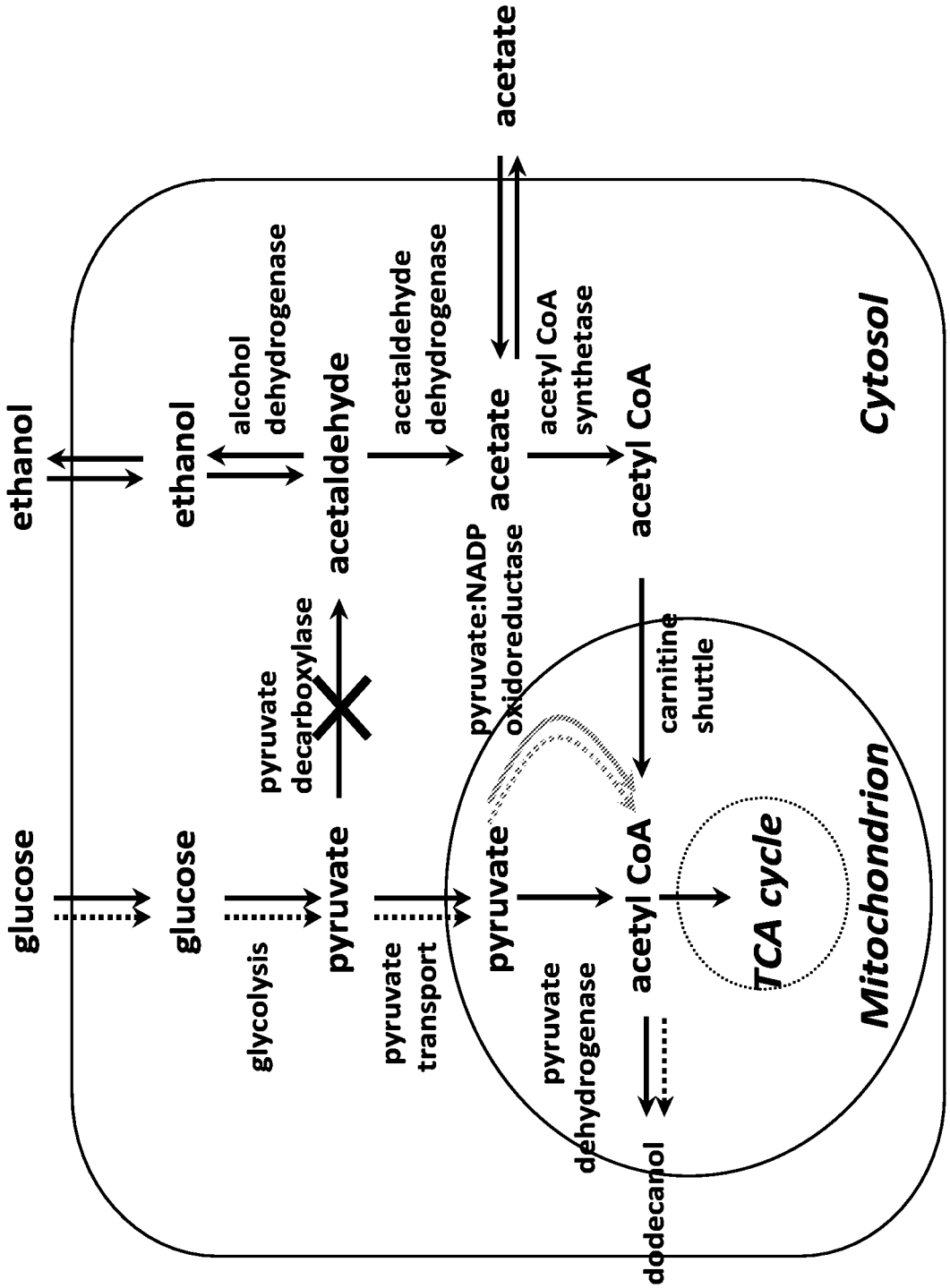
acetate
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
Cytosol
pyruvate decarboxylase
pyruvate:NADP oxidoreductase
carnitine shuttle
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
acetyl CoA
TCA cycle
Mitochondrion
pyruvate dehydrogenase
dodecanol

Figure 16

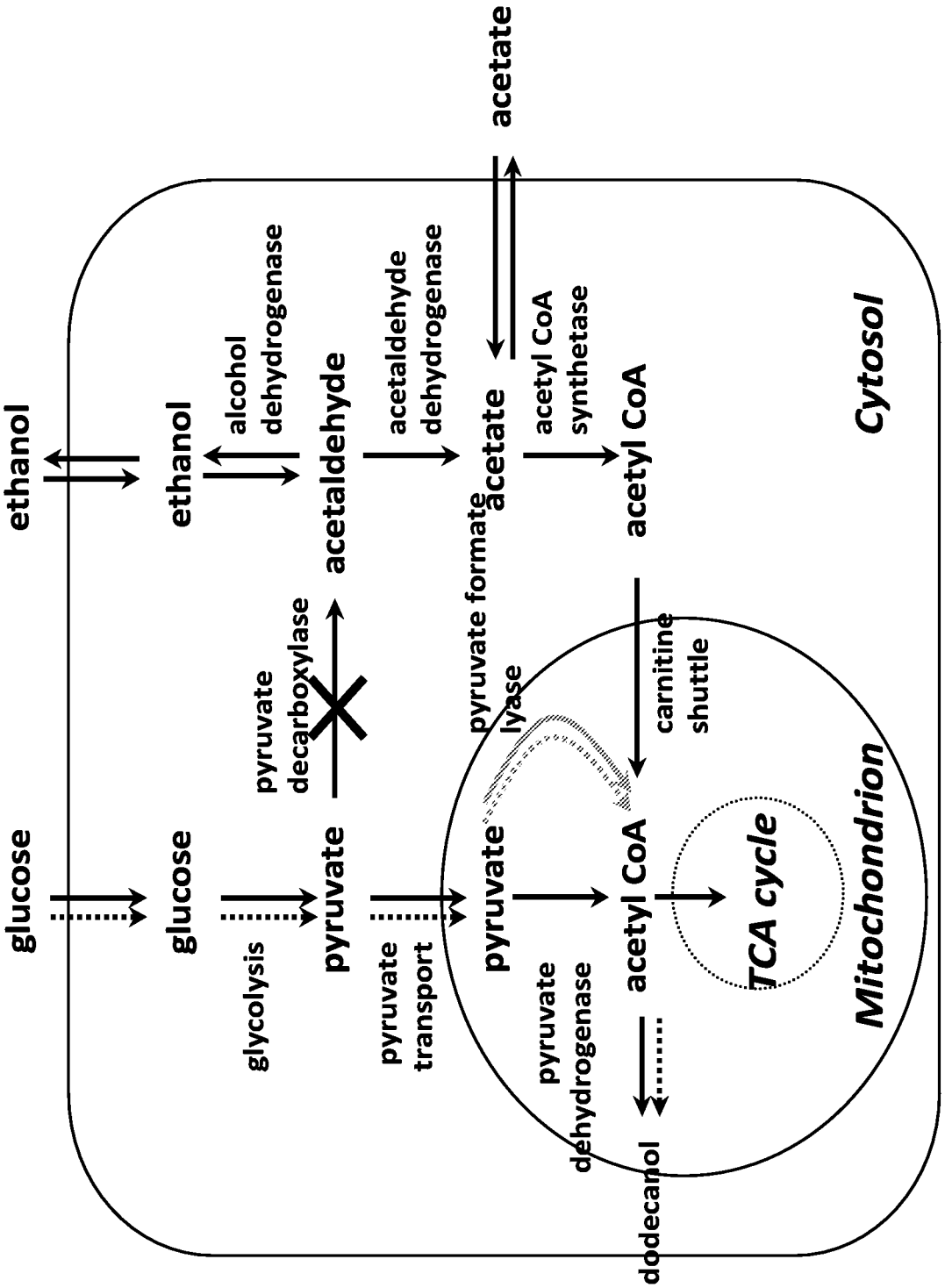
acetate
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
Cytosol
pyruvate formate lyase
pyruvate decarboxylase
carnitine shuttle
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
acetyl CoA
TCA cycle
Mitochondrion
pyruvate dehydrogenase
dodecanol

Figure 17

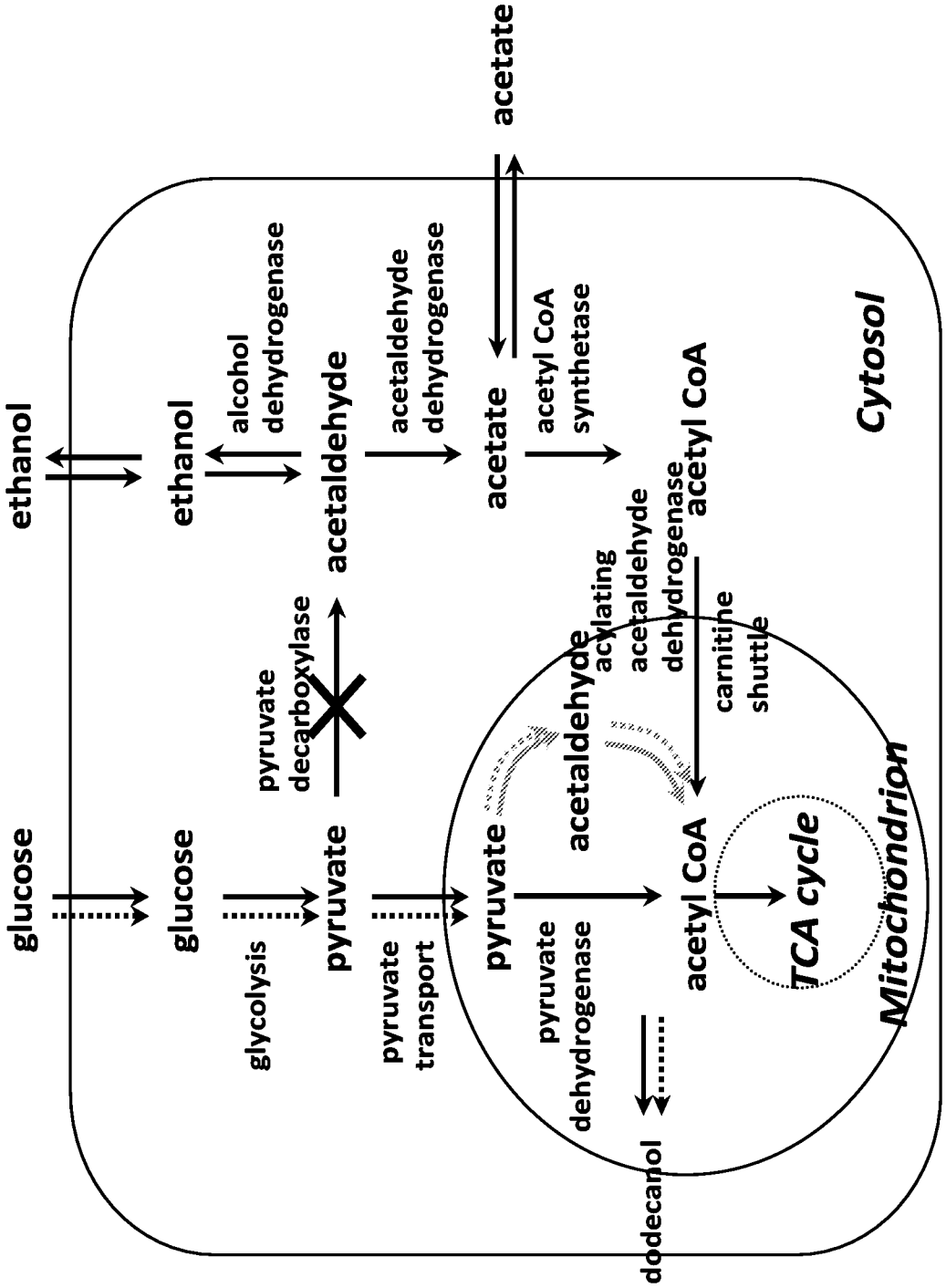
acetate
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
Cytosol
pyruvate decarboxylase
acetaldehyde
acylating acetaldehyde dehydrogenase
carnitine shuttle
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
acetaldehyde
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
dodecanol

Figure 18

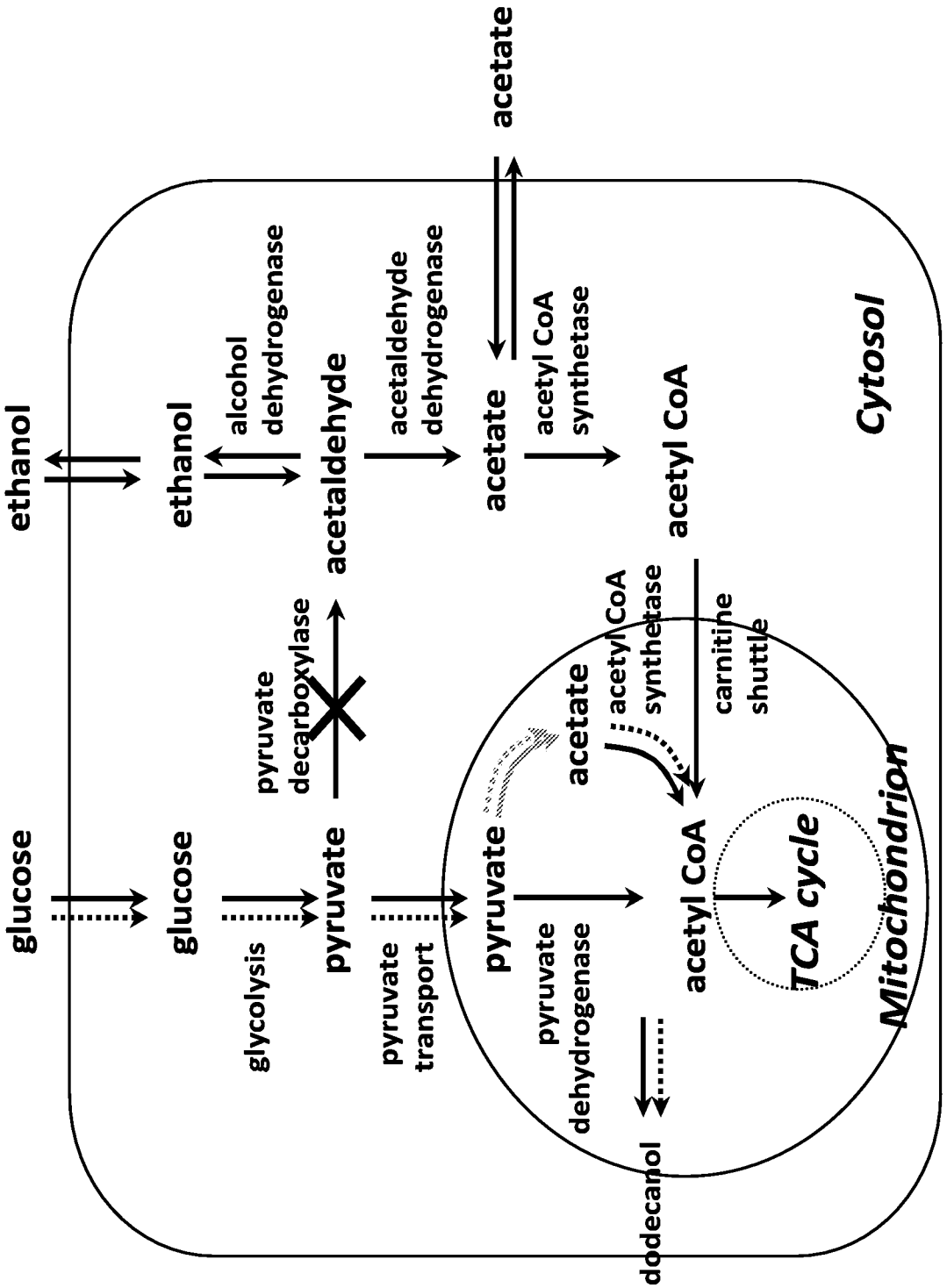
acetate
ethanol
ethanol
alcohol dehydrogenase
acetaldehyde
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetyl CoA
Cytosol
pyruvate decarboxylase
acetate
acetyl CoA synthetase
carnitine shuttle
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
dodecanol

Figure 19a

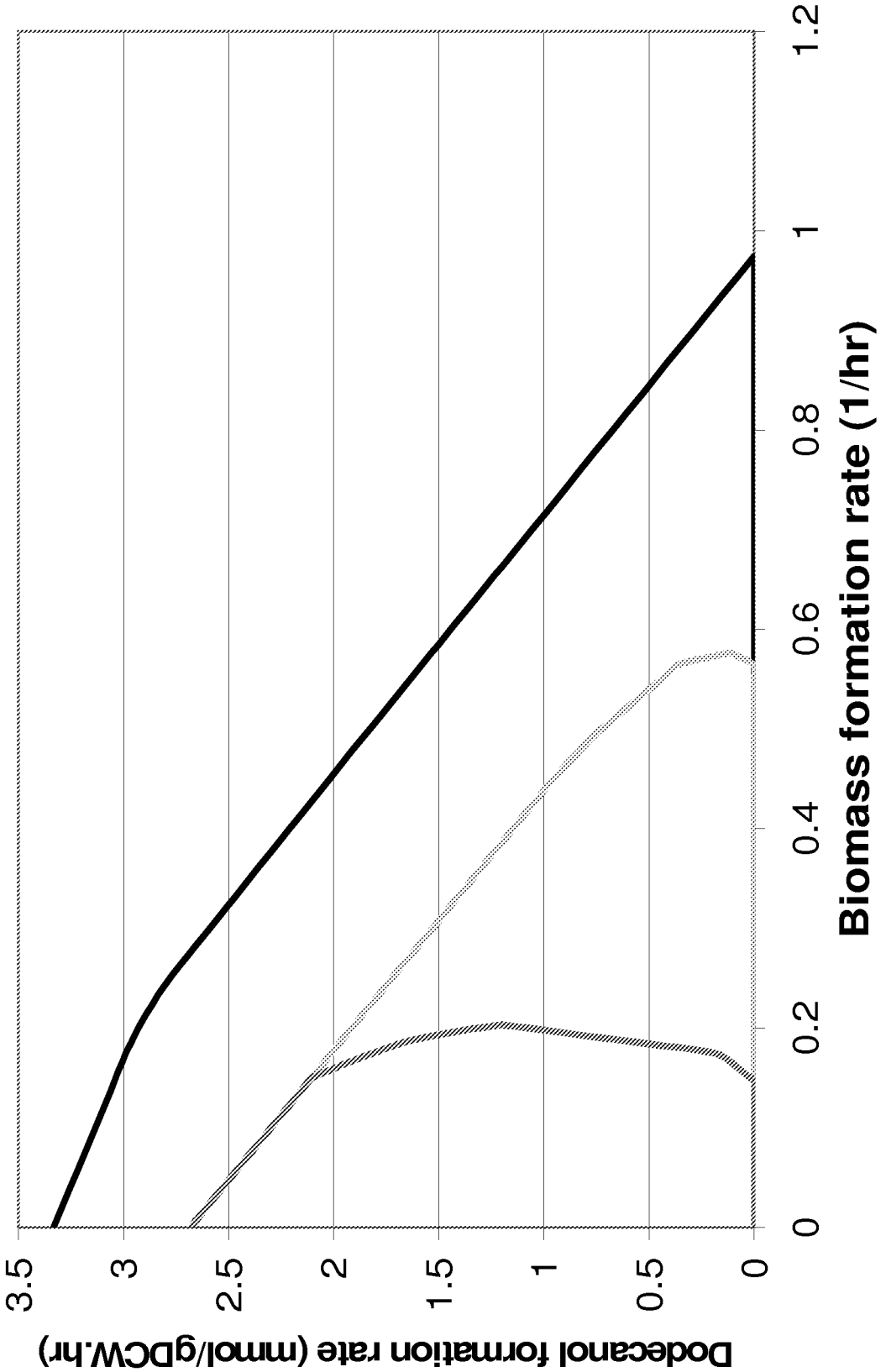
Dodecanol formation rate (mmol/gDCW.hr)
3.5
3
2.5
2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
1.2
Biomass formation rate (1/hr)

Figure 19b

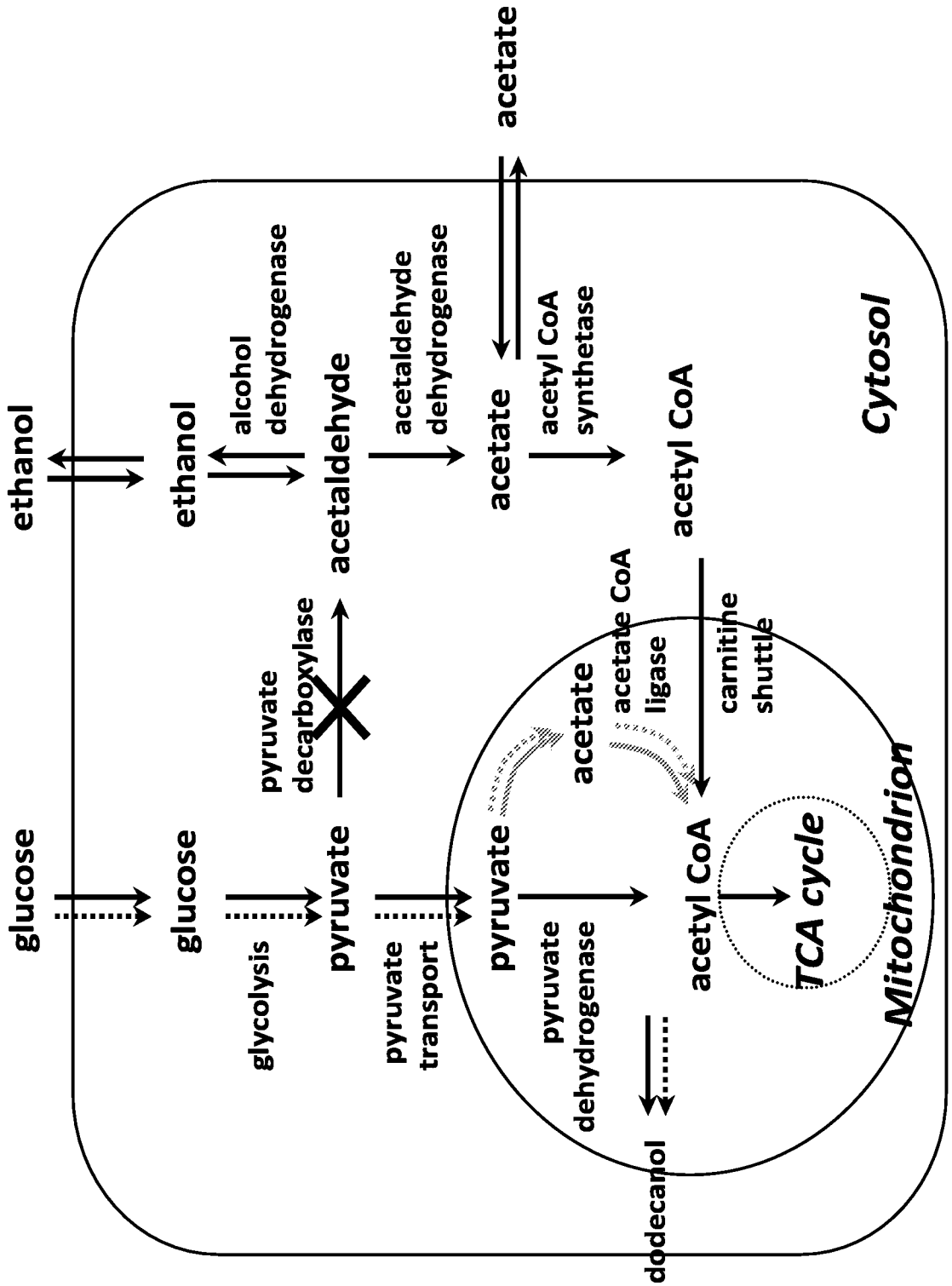
glucose
glucose
glycolysis
pyruvate
pyruvate transport
pyruvate
pyruvate dehydrogenase
acetyl CoA
TCA cycle
Mitochondrion
dodecanol
pyruvate decarboxylase
acetaldehyde
alcohol dehydrogenase
ethanol
ethanol
acetaldehyde dehydrogenase
acetate
acetyl CoA synthetase
acetate
acetate CoA ligase
acetyl CoA
carnitine shuttle
Cytosol

Figure 20