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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 October 2009 (01.10.2009)

PCT

(10) International Publication Number
WO 2009/120806 A2

(51) International Patent Classification:
C12N 1/21 (2006.01) *C12P 7/06* (2006.01)
C12N 15/53 (2006.01) *C12N 9/02* (2006.01)
C12P 7/16 (2006.01) *C12R 1/145* (2006.01)

(21) International Application Number:
PCT/US2009/038300

(22) International Filing Date:
25 March 2009 (25.03.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/039,313 25 March 2008 (25.03.2008) US
61/154,505 23 February 2009 (23.02.2009) US

(71) Applicant *(for all designated States except US)*: **COBALT TECHNOLOGIES, INC.** [US/US]; 500 Clyde Ave., Suite 500, Mountain View, CA 94043 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CONTAG, Pamela, Reilly** [US/US]; 6110 Bollinger Rd., San Jose, CA 95129 (US). **BURNS-GUYDISH, Stacy, M.** [US/US]; 620 El Patio Drive, Cambpell, CA 95008 (US). **MEERMAN, Hendrikus, J.** [US/US]; 111 Bean Creek Road, Unit 114, Scotts Valley, CA 95066 (US).

(74) Agents: **GOETZ, David, H.** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

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

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

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2009/120806 A2

(54) **Title**: EXPRESSION OF OXIDOREDUCTASES TO INCREASE VOLUMETRIC PRODUCTIVITY

(57) **Abstract**: Compositions and methods are provided for increased volumetric productivity of microorganisms by expression of oxidoreductases. Such methods are useful in increasing the volumetric production of alcohols by microorganisms such as Clostridium.

EXPRESSION OF OXIDOREDUCTASES TO INCREASE VOLUMETRIC PRODUCTIVITY

CROSS REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application Nos. 61/039,313, filed March 25, 2008, and 61/154,505, filed February 23, 2009, which applications are herein incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Obligate and facultative anaerobic microorganisms are used in a number of industrial and pharmaceutical applications to produce products of interest. For example, there is increasing interest in using microorganisms such as E. coli, yeast and *Clostridium* for production of biofuels. It would be useful to increase the volumetric productivity of such products in these microorganisms.

SUMMARY OF THE INVENTION

[0003] The present invention provides a method comprising transforming an anaerobic microorganism such as a *Clostridium* such as *Clostridium acetobutylicum* with a recombinant nucleic acid such as a heterologous nucleic acid comprising a nucleotide sequence encoding an oxidoreductase. The method further comprises culturing the recombinant anaerobic microorganism to produce a fermentation product under microaerobic fermentation conditions. In one aspect of the method, the anaerobic microorganism is an obligate anaerobe. In another aspect of the method, the anaerobic microorganism is an obligate anaerobe and the recombinant anaerobic recombinant microorganism is an anaerobe that is resistant to a microaerobic environment. In one aspect of the method, the oxidoreductase is an oxygenase. In another aspect of the method, the oxidoreductase is luciferase. In one aspect of the method, the nucleotide sequence includes a constituitive or an inducible promoter. In another aspect of the method, the expression of the recombinant heterologous oxidoreductase such as luciferase is independent of the expression levels or activity of the biosynthetic pathway or the genes in the biosynthetic pathway that produce the fermentation product. In one aspect of the method, the nucleotide sequence has an A/T content of at least about 50%, 55%, 60%, 65%, 70%, 75% or more. In another aspect of the method, the fermentation product is a solvent such as an alcohol such as butanol, isobutanol, ethanol, methanol, propanol, or isopropanol; or a ketone such as acetone, or butanone; or an organic acid such as acetate, butyrate, isobutyrate, isopropionate, propionate, lactate, citrate, or aminovalerate. In one aspect, the microaerobic conditions include but are not limited to sparging with a gas of at least 0.1% to at least 5% or more O_2 (e.g. 0.1%, 0.5%, 0.6%, 0.7%, 0.8%, 1.0%, 1.2%, 1.5%, 1.75%, 2%, 3%, 4%, 5% or more O_2). In another aspect, the microaerobic conditions include but are not limited to culture conditions with at least about 0.05ppm dissolved O_2 or more (e.g. 0.05, 0.075, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6ppm or more). In one aspect, the method provides for improved volumetric productivity, improved product titer, improved oxygen tolerance, or improved resistance to phenolic growth inhibitors in the recombinant anaerobic microorganism as compared to the parent strain. In one aspect, the method provides for at least a 10% increase in volumetric productivity of butanol. In another aspect, the method provides for at least a 10% increase in the product titer of

butanol. In one aspect, the method provides for a 10% increase in oxygen or phenolic inhibitor tolerance. In some cases, the increase is relative to the same strain cultured at a different O_2 concentration. In other cases, the increase is relative to a parent strain or a control strain cultured at the same or a different O_2 concentration. In some cases, the phenolic inhibitors include furfural, hydroxymethyl furfural, vanillin, *p*-coumarate, ferulic acid, 4-hydroxybenzoate, vanillic acid, and syringaldehyde. In one aspect, the method provides for continuous culture of recombinant anaerobic microorganisms expressing oxidoreductase such as luciferase. In another aspect, the method provides for an increase in butanol production or titer relative to acetone or ethanol production or titer.

[0004] In one embodiment, the present invention provides a method for making fuel comprising culturing a recombinant anaerobic bacteria or microorganism in a bioreactor under microaerobic conditions, and collecting a fuel product from the bioreactor such as ethanol, isopropanol, methanol, or butanol. In one aspect of the method, the recombinant anaerobic bacteria is a gram-positive bacteria. In another aspect, the recombinant anaerobic bacteria is resistant to a microaerobic fermentation or culture condition. In another aspect, the bacteria is from the genus *Clostridium*. In another aspect, the recombinant bacteria comprises a heterologous nucleic acid sequence encoding an oxidoreductase such as a luciferase. In one aspect, the microaerobic conditions produce fuel at a rate that is at least 10% greater than a recombinant anaerobic bacteria that does not comprise a heterologous nucleotide sequence encoding an oxidoreductase. In another aspect, the conditions comprise sparging gas comprising at least 0.1% O_2. In another aspect, the conditions comprise at least about 0.5% dissolved oxygen or at least about 0.1ppm to at least about 0.6ppm or more.

[0005] In one embodiment the present invention provides a bioreactor comprising a fermentation product-producing recombinant anaerobic microorganism, oxygen, and a fermentation product. In one aspect, the oxygen concentration is at least about 0.1ppm. In another aspect, the fermentation product-producing recombinant anaerobic microorganism comprises a heterologous nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase such as luciferase. In some cases, the fermentation product is an alcohol, a ketone, or an organic acid. In one aspect, the bacteria is from the genus *Clostridium*.

[0006] In one embodiment the present invention provides a bioreactor comprising a transformed gram-positive bacteria, and culture conditions that permit the bacteria to produce butanol at a rate of greater than 0.1g/L/h. In one aspect, the culture conditions permit the bacteria to produce a fermentation product at a rate that is 10% greater than the non-transformed gram-positive bacteria. In another aspect, the gram-positive bacteria are from the genus *Clostridium*. In one aspect, the transformed Gram-positive bacteria comprise a heterologous recombinant nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase. In another aspect, the bioreactor further comprises oxygen, wherein the oxygen is at a dissolved oxygen content of at least 0.1ppm.

[0007] In one embodiment the present invention provides a method for converting an obligate anaerobic microorganism to an anaerobic microorganism resistant to a microaerobic environment comprising transforming said organism with a polynucleotide sequence encoding luciferase, and growing said transformed anaerobic microorganism in a microaerobic environment or greater amounts of oxygen.

[0008] In one embodiment the present invention provides a strict anaerobe of a genus selected from the group consisting of *Clostridium*, *Fusobacterium*, *Peptostreptococcus*, *Bacteriodes*, *Butyrivibrio*, *Leptptrichia*, *Selenomonas*, *Succinimonas*, *Succinivibrio*, *Eubacterium*, *Lachnospira*, *Aracnia*, *Propionibacterium*, *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Treponema*, *Borrelia*, and *Campylobacter*, wherein said strict anaerobe is transformed with a heterologous nucleotide sequence encoding an oxidoreductase.

[0009] In one embodiment, the present invention provides a method for making a fuel (e.g. butanol, methanol, isopropanol, ethanol, etc.) comprising transforming a parent strain of bacteria (e.g. a gram-positive bacteria such as a *Clostridium*) with a recombinant heterologous nucleic acid comprising a nucleotide sequence encoding for an oxidoreductase, culturing the transformed bacteria under conditions suitable for biosynthesis of the fuel, and collecting the fuel. In some cases, the method provides 10% more fuel than culturing the parent strain. In some cases, the method provides for not monitoring the level of expression, or the activity of the oxidoreductase. In some cases, the method provides that the oxidoreductase is not a luciferase (e.g. *luc* or *lux*).

[0010] In one embodiment, the present invention provides a recombinant nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase operatively linked to a transcriptional regulatory sequence that is functional in anaerobes. Expression of the oxidoreductase in an anaerobe increases the volumetric productivity of an alcohol in the anaerobe. The increase can be at least 10%. In some aspects, the oxidoreductase is luciferase. The alcohol is a useful product such as butanol. The transcriptional regulatory sequence can comprise a constitutive promoter. The nucleotide sequence of the oxidoreductase can have an A/T content of 62-75%. In another aspect of the present invention, a recombinant anaerobic microorganism comprising the recombinant nucleic acid molecule is provided. The microorganism can be an obligate or facultative anaerobe, such as *Clostridium*. In another aspect of the invention, a parent strain is an anaerobic microorganism, and the transformed recombinant microorganism expressing an oxidoreductase is resistant to a microaerobic environment. The microorganism can be cultured and fermented.

[0011] In yet another embodiment of the present invention, a method of increasing the volumetric productivity of an alcohol in an anaerobic microorganism is provided. The method comprises fermenting an alcohol-producing anaerobic recombinant microorganism comprising a recombinant nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase operatively linked to a transcriptional regulatory sequence that is functional in the microorganism. Expression of the nucleotide sequence can be independent of the production of a product in the fermentative or synthetic pathway.

INCORPORATION BY REFERENCE

[0012] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0014] **FIG. 1** depicts a number of biochemical pathways in *Clostridium acetobutylicum* that are active during the acidogenic or solventogenic phases. Enzymes that catalyze specific reactions are identified by letters as follows: (A) glyceraldehyde 3-phosphate dehydrogenase; (B) pyruvate-ferredoxin oxidoreductase; (C) NADH-ferredoxin oxidoreductase; (D) NADPH-ferredoxin oxidoreductase; (E) NADH rubredoxin oxidoreductase; (F) hydrogenase; (G) phosphotransacetylase (phosphate acetyltransferase), (*pta*, CAC1742); (H) acetate kinase (*askA*, CAC1743); (I) acetyl-CoA acetyltransferase (thiolase), (*thil*, CAP0078, and CAC2873)); (J) 3-hydroxybutyryl-CoA dehydrogenase; (K) crotonase (3-hydroxybutyryl-CoA dehydratase, beta-hydroxybutyryl-CoA dehydrogenase), (*bhd*, CAC2708); (L) butyryl-CoA dehydrogenase (*bcd*, CAC2711); (M) phosphotransbutyrylase (phosphate butyltransferase) (*ptb*, CAC3076); (N) butyrate kinase, (*buk*, CAC3075, and CAC1660); (O) acetaldehyde dehydrogenase (possibly *adhe1*, CAP0162 and *adhe*, CAP0035); (P) ethanol dehydrogenase (*adhe1*, CAP0162; *bdhB*, CAC3298; and *bdhA*, CAC3299); (Q) butyraldehyde dehydrogenase, (*adhe1*, CAP0162 and *adhe*, CAP0035); (R) butanol dehydrogenase (*adhe1*, CAP0162; *adhe*, CAP0035; *adh*, CAP0059; *bdhB*, CAC3298; *bdhA*, CAC3299; and CAC3392); (S) butyrate-acetoacetate CoA-transferase (acetoacetyl-CoA:acetate/butyrate:CoA transferase), (*ctfa*, CAP0163(A) and *ctfb*, CAP0164(B); (T) acetoacetate decarboxylase (*adc*, CAP0165); (U) pyruvate decarboxylase (*pdc*, CAP0025). Select enzymes are further detailed in Table 1. Others can be found in readily available reference materials, such as on The Institute for Genomic Research's website (www.tigr.org).

[0015] **FIG. 2** illustrates an expression construct comprising lux CDABE, with ribosome binding sites (asterisks) inserted upstream of each gene.

[0016] **FIG. 3** is a graph of butanol productivity by strains Co-0115 and Co-5878 under various O_2 sparging regimes as indicated on the X-axis. Co-0115 refers to a strain of *Clostridium acetobutylicum* derived from a parent strain Co-0124 by transformation of an expression vector lacking in a functional heterologous oxidoreductase operably linked to a promoter, and Co-5878 refers to a strain derived from Co-0124 that expresses a heterologous oxidoreductase.

[0017] **FIG. 4** is a graph of maximum butanol production, and the ratio of butanol to acetone production in a fermentation run under various indicated O_2 sparging regimes.

DETAILED DESCRIPTION OF THE INVENTION

[0018] The present invention provides compositions and methods for increasing the volumetric productivity i.e. the amount of a fermentation product made per unit volume per unit time, e.g. [g/L/h] of commercially valuable products from microorganisms. The methods can be used to increase the volumetric productivity production of any product of a synthetic or fermentative pathway, for example, for the production of solvents useful as fuels. The methods are achieved by providing the microorganisms with a recombinant nucleic acid comprising a nucleotide sequence encoding an oxidoreductase operatively linked to a transcriptional regulatory sequence. Expression of an oxidoreductase in anaerobic microorganisms, such as luciferase, increases the rate of formation of fermentation products made by such microorganisms, i.e. the volumetric productivity. It also increases the production of products i.e. titer (g/L), made by such microorganisms, for example, it increases the rate of formation of butanol by *Clostridium* or the amount of production of butanol by *Clostridium*, or both. Additionally, the methods of the present invention alter the ratio of production of different commercially valuable products form microorganisms. For example, the methods increase ratio of butanol production by *Clostridium* over acetone production.

Microorganisms

[0019] The present invention provides microorganisms with increased volumetric productivity of commercially valuable products such as alcohols. The microorganisms have a recombinant nucleic acid with a nucleotide sequence encoding an oxidoreductase operatively linked to a transcriptional regulatory sequence. The microorganisms of the present invention are generally anaerobic. They can be obligate or facultative anaerobes, or they can be anaerobes that are resistant to a microaerobic environment or microaerobic fermentation conditions, additionally they can be eukaryotic or prokaryotic.

[0020] The microorganism can be yeast. Examples include, but are not limited to, *Saccharomyces cerevisiae, S. bayanus, S. carlsbergensis, S. Monacensis, S. Pastorianus, S. uvarum, Kluyveromyces* including *K. marxianus*, and *Aspergillus* and *Candida* species. . The microorganisms can also be bacteria. The bacteria can be Gram-negative or Gram-positive. Non-limiting examples of Gram-positive bacteria include: Staphylococcus, Streptococcus, certain Bacillus, Anthrax, Mycobacterium, etc. The bacteria can be low-GC bacteria.

[0021] The bacteria can be an anaerobe such as *Clostridium.* For example, selected from a species including, but not limited to *C. acetobutylicum* (e.g., p262). With *C. acetobutylicum*, wild types strains contemplated for use with this invention include ATCC 43084 and ATCC 824 from the American Tissue Culture Collection (ATCC) and DSM 792 and DSM 1731 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. High butanol producing mutants of C. *acetobutylicum* contemplated for use with this invention include strains such as ATCC 55025, and ATCC 39058 from ATCC. Another high producing strain contemplated for use with this invention is B643. (Contag, P. R., et al, Cloning of a lactate dehydrogenase gene from *Clostridium acetobutylicum* B643 and expression in Escherichia coli. Appl. Environ. Microbiol. 56:3760-3765, 1990.)

[0022] Other examples of *Clostridium* include *C. beijerinckii* (e.g., ATCC 25752, ATCC51743), *C. puniceum* (e. g. ATCC43978), or *C. saccharobutylicum* (e.g., ATCC BAA-117). The *Clostridium* can also be selected from

C. acidisoli, C. aciditolerans, C. acidurici, C. aerotolerans, C. akagii, C. aldenense, C. algidicarnis, C. algidixylanolyticum, C. alkalicellulosi, C. aminovalericum, C. amygdalinum, C. arcticum, C. argentinense, C. aurantibutyricum, C. baratii, C. botulinum, C. bowmanii, C. butyricum, C. cadaveris, C. caminithermale, C. carboxidivorans, C. carnis, C. celatum, C. celerecrescens, C. cellulolyticum, C. cellulosi, C. chartatabidum, C. clostridioforme, C. coccoides, C. cochlearium, C. cocleatum, C. colinum, C. difficile, C. diolis, C. disporicum, C. drakei, C. durum, C. estertheticum, C. fallax, C. felsineum, C. fervidum, C. fimetarium, C. formicaceticum, C. ghonii, C. glycolicum, C. glycyrrhizinilyticum, C. haemolyticum, C. halophilum, C. tetani, C. perfringens, C. phytofermentans, C. piliforme, C. polysaccharolyticum, C. populeti, C. propionicum, C. proteoclasticum, C. proteolyticum, C. psychrophilum, C. puri, C. putrefaciens, C. putrificum, C. quercicolum, C. quinii, C. ramosum, C. roseum, C. saccharolyticum, C. saccharoperbutylacetonicum, C. sardiniense, C. stercorarium subsp. Thermolacticum, C. sticklandii, C. paradoxum, C. paraperfringens, C. paraputrificum, C. pascui, C. pasteurianum, C. novyi, C. septicum, C. histolyticum, C. hydroxybenzoicum, C. hylemonae, C. innocuum, C. kluyveri, C. lactatifermentans, C. lacusfryxellense, C. laramiense, C. lentocellum, C. lentoputrescens, C. ljungdahlii, C. methoxybenzovorans, C. methylpentosum, C. nitrophenolicum, C. novyi, C. oceanicum, C. oroticum, C. oxalicum, C. tertium, C. tetani, C. tetanomorphum, C. thermaceticum,C. thermautotrophicum, C. thermoalcaliphilum, C. thermobutyricum, C. thermocellum, C. thermocopriae, C. thermohydrosulfuricum, C. thermolacticum, C. thermopalmarium, C. thermopapyrolyticum, C. thermosaccharolyticum, C. thermosulfurigenes, C. tyrobutyricum, C. uliginosum, C. ultunense, C. villosum, C. viride, C. xylanolyticum, C. xylanovorans, C. bifermentans, and *C. sporogenes.*

[0023] Other bacteria that may be used include *Corynebacteria*, such as *C. diphtheriae*, *Pneumococci*, such as *Diplococcus pneumoniae, Streptococci*, such as *S. pyogenes* and *S. salivarus*, *Staphylococci*, such as *S. aureus* and *S. albus*, *Myoviridae, Siphoviridae,* Aerobic Spore-forming Bacilli, Bacilli, such as *B. anthracis, B. subtilis, B. megaterium, B. cereus, Butyrivibrio fibrisolvens,* Anaerobic Spore-forming Bacilli, Mycobacteria, such as *M. tuberculosis hominis, M. bovis, M. avium , M. paratuberculosis, Actinomycetes (fungus-like bacteria), such as, A. israelii, A. bovis, A. naeslundii, Nocardia asteroides, Nocardia brasiliensis, the Spirochetes, Treponema pallidium, Treponema pertenue, Treponema carateum, Borrelia recurrentis, Leptospira icterohemorrhagiae, Leptospira canicola, Spirillum minus, Streptobacillus moniliformis, Trypanosomas, Mycoplasmas, Mycoplasma pneumoniae, Listeria monocytogenes, Erysipelothrix rhusiopathiae, Streptobacillus monilformis, Donvania granulomatis, Bartonella bacilliformis, Rickettsiae, Rickettsia prowazekii, Rickettsia mooseri, Rickettsia rickettsiae,* and *Rickettsia conori.* Other bacteria used can include *Escherichia coli, Zymomonas mobilis, Erwinia chrysanthemi,* and *Klebsiella planticola.*

[0024] The microorganism can be grown in cultures and fermented to generate alcohols or solvents, such as butanol. In one aspect of the invention the microorganisms are grown in a culture. In some embodiments, the microorganisms are grown in suspension cultures. In some embodiments, the suspension cultures are batch cultures. In other embodiments the suspension cultures are fed-batch cultures. In still other embodiments the suspension cultures are continuous cultures. In some embodiments, the microorganisms are grown on solid supports. In some embodiments, the microorganisms are immobilized on solid supports.

[0025] Conventional bioreactors and methods for culturing microorganisms to produce target products are known and are contemplated for use with the present invention methods and compositions. For example, fermentors for use in the batch fermentation of *C. acetobutylicum* are well known in the art. (Beesch, S. C. Acetone-butanol fermentation of sugars. Eng. Proc. Dev. 44:1677-1682, 1952; Beesch, S. C. Acetone-butanol fermentation of starches. Appl. Microbiol. 1:85-96, 1953; Killeffer, D. H. Butanol and acetone from corn. A description of the fermentation process. Ind. Eng. Chem. 19:46-50, 1927; MuCutchan W. N., and Hickey, R. J. The butanol-acetone fermentations. Ind. Ferment. 1:347-388, 1954.) Typically, the fermentors to be used have capacities of 50,000 to 200,000 gallons and are frequently without mechanical agitation systems. Mixing of the fermentor contents can be achieved through the sparging of sterile gas, e.g. carbon dioxide or N_2, which may also serve to prevent contamination of the culture through the maintenance of positive pressure within the fermentor. Other techniques of mixing culture contents include the use of agitators or the recirculation of fermentation broth, particularly broth returned to the fermentor after the removal of a fermentation product. In some embodiments, the contents of the fermentor are not mixed, but may rely on the production and movement of evolved gases to mix contents.

[0026] In some of the embodiments of the methods of the present invention, the microorganism may be grown in cultures and fermented in a fermentor or other bioreactor with oxygen sparging such as, for example, sparging of oxygen in a substantially inert carrier gas such as, for example, N_2. In some cases, concentrations of oxygen in the sparging gas may be at least about 0.1%, 0.2%, 0.3%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.75%, or about 3%, 5%, 10% or about 15% to provide a microaerobic environment or fermentation conditions In other cases, concentrations of oxygen in the sparging gas are substantially 0% to provide an anaerobic environment. In some cases, the fermentor may be operated to control for maximum or minimum dissolved oxygen content by manipulating, for example, gas or air sparging rates, pH, carbon source feed rates, or nitrogen source feed rates. Suitable dissolved oxygen concentrations expressed as a percentage of the maximum possible dissolved oxygen content in an aqueous medium that are contemplated by the methods of the present invention for culturing microorganisms include but are not limited to about 0.1%, 0.2%, 0.3%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.75%, or about 3%, 5%, 10%, or 15% to provide a microaerobic environment or fermentation conditions. In other cases, an anaerobic environment may be provided by maintaining a dissolved oxygen concentration of substantially 0%. In some cases, dissolved oxygen may be expressed in terms of parts per million (ppm), in which case suitable dissolved oxygen concentrations contemplated by the methods of the present invention for culturing microorganisms include but are not limited to about 0.05 ppm, 0.1ppm, 0.15ppm, 0.2ppm, 0.3ppm, 0.35ppm, 0.4ppm, 0.45 ppm, 0.5ppm, 0.55ppm, 0.6ppm, 0.7ppm, 0.75ppm, 0.8ppm, 0.9ppm, 1ppm, 2ppm, 3ppm, or 5ppm, or 10ppm, 15ppm, 20ppm, or about 30ppm to provide a microaerobic environment or fermentation conditions. In other cases, an anaerobic environment may be provided by culturing microorganisms under conditions of substantially 0ppm dissolved oxygen.

[0027] In some of the embodiments of the methods of the present invention, the recombinant anaerobic microorganism expressing an oxidoreductase such as luciferase may be resistant to incidental, accidental, or inadvertent exposure to oxygen. For example, the culture container may inadvertently allow entrance of outside air, or an inappropriate sparging gas may be utilized during culture. In some cases, the oxidoreductase may allow the

survival and/or growth of the recombinant anaerobic microorganism despite culturing in an environment that is not anaerobic (i.e. oxygen is present). In some cases, the presence of an inadvertent, incidental, or accidental amount of oxygen in the culture of a recombinant microorganism of the present invention may result in little or no discernable detrimental effect on volumetric productivity or product titer.

[0028] Fed-batch fermentation processes may also be used with *C. acetobutylicum* fermentations. Fermentors for the continuous fermentation of *C. acetobutylicum* are also known in the art. (U.S. Pat. No. 4,424,275, and U.S. Pat. No. 4,568,643.) Since a high density, steady state culture can be maintained through continuous culturing, with the attendant removal of solvent containing fermentation broth, smaller capacity fermentors can be used. The fermentation processes can also utilize immobilized cells as disclosed in WO 81/01012. Immobilization creates cell-free fermentation broth simplifying product recovery and may increase the cell density thereby increasing the production rate of solvents. In some of the embodiments of the methods of the present invention, the microorganism may be grown in continuous cultures and fermented continuously in a fermentor or other bioreactor with oxygen sparging such as, for example, sparging of oxygen in a substantially inert carrier gas such as, for example, N_2. In some cases, concentrations of oxygen in the sparging gas may be at least about 0.1%, 0.2%, 0.3%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.75%, or about 3%, 5%, 10%, or 15% to provide a microaerobic environment or fermentation conditions. In other cases, concentrations of oxygen in the sparging gas are substantially 0% to provide an anaerobic environment. In some cases, the fermentor may be operated to control for maximum or minimum dissolved oxygen content by manipulating, for example, gas or air sparging rates, pH, carbon source feed rates, or nitrogen source feed rates. Suitable dissolved oxygen concentrations expressed as a percentage of the maximum possible dissolved oxygen content in an aqueous medium that are contemplated by the methods of the present invention for continuous culturing of microorganisms include but are not limited to about 0.1%, 0.2%, 0.3%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.75%, or about 3%, 5%, 10%, or 15% to provide a microaerobic environment or fermentation conditions. In other cases, an anaerobic environment may be provided by maintaining a dissolved oxygen concentration of substantially 0%. In some cases, dissolved oxygen may be expressed in terms of parts per million (ppm), in which case suitable dissolved oxygen concentrations contemplated by the methods of the present invention for continuous culturing of microorganisms include but are not limited to about 0.05 ppm, 0.1ppm, 0.15ppm, 0.2ppm, 0.3ppm, 0.35ppm, 0.4ppm, 0.45 ppm, 0.5ppm, 0.55ppm, 0.6ppm, 0.7ppm, 0.75ppm, 0.8ppm, 0.9ppm, 1ppm, 2ppm, 3ppm, 5ppm, 10ppm, 15ppm 20ppm, or about 30ppm to provide a microaerobic environment or fermentation conditions. In other cases, an anaerobic environment may be provided by culturing microorganisms under conditions of substantially 0ppm dissolved oxygen..

[0029] The cultures of the present invention can be monitored and optimized (e.g., to optimize growth conditions, optimize expression of a desired gene of interest, optimize production of a compound), for example, monitored by light emitting reporters so that necessary modifications can be made to culture conditions (e.g., addition of nutrients, change of temperature/pH, etc). The microorganisms may also produce a light emitting reporter that signals the status of target production. The use of light-emitting reporters may be applicable for the monitoring of all types of fermentative, synthetic pathways, expression of particular genes in a host cell, or the presence of a compound in the

environment (e.g., mercury, metals, organic pollutants). See, for example, USSN 11/853,681, filed September 11, 2007.

[0030] The microorganisms may be "wild type" wherein they natively produce the desired target, or they may have undergone mutagenesis and positive selection to overproduce the desired target. Alternatively, the cells can be previously engineered to express enzymes required for the desired fermentative or synthetic pathway. This can be in the form of overexpressing the native enzymes required for the fermentative or synthetic pathways or the expression of heterologous enzymes required for a fermentative or synthetic pathway. Additionally, signal enzymes can be introduced simultaneously into the host cells with either native or heterologous fermentative or synthetic pathway enzymes. With simultaneous introduction, the signal enzymes can be on the same operon as the introduced fermentative or synthetic pathway enzymes or the signal enzymes can be located on different operons. Furthermore, the host can also be genetically modified so that expression of a necessary enzyme for a competing fermentative or synthetic pathway is down regulated or negated, thereby forcing substrate down the fermentative or synthetic pathway of interest. In some cases, the host can be genetically modified so that expression of a protein such as an enzyme such as, for example, an oxidoreductase, such as luciferase, may provide for increased tolerance of one or more compounds, such as O_2, present in the fermentation medium. In some embodiments, the microorganisms express at least one additional heterologous gene besides an oxidoreductase. In some embodiments, the at least one additional heterologous gene expresses a desired protein. In other embodiments, the additional heterologous gene expresses an enzyme involved in a fermentative, synthetic, metabolic, or respiratory pathway of interest. In some embodiments, the at least one additional heterologous gene increases the productivity of a desired product that is already produced in the microorganism. For example, with *C. acetobutylicum* the additional heterologous gene can be an alcohol dehydrogenase or an aldehyde-alcohol dehydrogenase, the expression of which increases the production of butanol. In some embodiments RNA molecules can be expressed that interfere with, block or activate the expression or translation of other proteins.

[0031] Genetic modifications as described above may provide a desired benefit in producing commercially useful products in the microorganisms, but decrease the rate of production, i.e. the productivity, of said desired product. Different culture conditions or shifting of the microorganism's biochemical pathways from one phase to another may also affect the productivity of a microorganism. The present invention of expressing an oxidoreductase can increase the productivity of the organism by increasing amount of product made per unit volume per unit time, i.e. the volumetric productivity, or by increasing the amount of product made, i.e. the product titer..

Biochemical Pathways

[0032] The present invention contemplates increasing the volumetric productivity of products in the fermentative or synthetic biochemical pathways of microorganisms. A biochemical pathway is a sequence of enzymatic or other reactions by which one biological compound is converted to another. A biochemical pathway product, or target product, is a compound produced by an organism or an *in vitro* system wherein the product is the desired compound to be produced from the pathway. The target product can be a pathway "end product." A pathway end product is a compound produced by an organism or an *in vitro* system wherein no further conversion of

the compound is possible because there is no enzyme available that converts the compound to another compound. For example, no further enzymatic conversion is possible in a microorganism because, there is no gene in the genome that encodes such an enzyme. Examples of end products in some *Clostridia* such as *C. acetobutylicum,* include the solvents: acetone, butanol and ethanol. A target product can also be a biochemical pathway intermediate wherein further conversion of the compound is possible. For example, in some *Clostridia,* such as *C. acetobutylicum,* pathway intermediates include "acid intermediates." The acid intermediates, acetate and butyrate, accumulate in the culture media when *Clostridia* is in the acidogenic culture phase. Later in the solventogenic phase, these acid intermediates are re-assimilated and used to synthesize solvents. Another acid intermediate, lactate, accumulates in the culture media when *Clostridia* is cultured under conditions of iron limitation and high pH. Other *Clostridia* do not produce appreciable quantities of solvents, but instead produce acids as the fermentative end product. Additional fermentation products anticipated by the present invention include butanone, isobutanol, propanol, isopropanol, methanol, isobutyrate, isopropionate, propionate, citrate, and aminovalerate.

[0033] The biochemical pathways of the microorganisms described herein may change, for example, the flux through various biochemical pathways can change, shifting the rate of production of various targets. For example, in a batch culture of *C. acetobutylicum*, the initial production of acids as described above, such as acetate and butyrate, decreases the pH of the culture, however, once the concentration of undissociated butyrate reaches approximately 9 mM, a shift occurs wherein the *C. acetobutylicum* re-assimilates the secreted acids and switches to the production of solvents such as butanol and acetone. Butanol has a toxic effect upon the cells and its accumulation may inhibit the expression of the enzymes that produce it. To increase the production of butanol by a microorganism, butanol can be removed from the fermentation broth or water or culture media can be added to the fermentor to dilute the accumulated butanol below the inhibitory threshold. The volumetric productivity of the microorganism can also be increased, for example, by expressing an oxidoreductase such as luciferase in the microorganism, such that more butanol is produced per unit time, thereby reaching the maximal concentration of butanol in a shorter period of time. Alternatively, the product titer of the microorganism can be increased for example, by expressing an oxidoreductase such as luciferase in the microorganism, such that a higher maximal butanol concentration is achieved. In some cases, the expression of an oxidoreductase such as luciferase in the microorganism in combination with culturing in a microaerobic environment provides for enhanced volumetric productivity, enhanced growth, enhanced product titer, or a combination thereof.

[0034] The volumetric productivity of products in the fermentative pathways of microorganisms can also be increased. A fermentative pathway is a metabolic pathway that proceeds anaerobically, wherein an organic molecule functions as the terminal electron acceptor rather than oxygen, as happens with oxidative phosphorylation under aerobic conditions. Glycolysis is an example of a wide-spread fermentative pathway in bacteria (*C. acetobylicium* and *E. coli*) and yeast. During glycolysis, cells convert simple sugars, such as glucose, into pyruvate with a net production of ATP and NADH. At least 95% of the pyruvate is consumed in short pathways which regenerate NAD^+, an obligate requirement for continued glycolysis and ATP production. The waste or end products of these NAD^+ regeneration systems are referred to as fermentation products. Depending upon the organism and culturing conditions, pyruvate is ultimately converted into end products such as organic acids (formate, acetate,

lactate, pyruvate, butyrate, succinic, dicarboxylic acids, adipic acid, and amino acids), and neutral solvents (ethanol, butanol, acetone, 1,3-propanediol, 2,3-propanediol, acetaldehyde, butyraldehyde, 2,3-butanediol).

[0035] The Comprehensive Microbial Resource (CMR) of TIGR lists nine types of fermentation pathways in its atlas based on the fermentative end product: homolactic acid (lactic acid); heterolactic acid (lactic acid), ethanolic, propionic acid, mixed (formic and acetic acid), butanediol, butyric acid, amino acid, and methanogenesis. The method of this invention can be used in any of the fermentative pathways described above. The fermentative pathways described in this invention can be naturally occurring or engineered.

[0036] Solvents are a class of end products produced by microbes that have commercial value. These include, for example, alcohols (ethanol, butanol, propanol, isopropanol, 1,3-propanediol, 2,3-propanediol, 2,3-butanediol, glycerol) , ketones (acetone) and aldehydes (acetaldehyde, butyraldehyde). **FIG. 1** illustrates the production of the solvents acetone, butanol and ethanol in a number of biochemical pathways that are active during the acidogenic or solventogenic phases in *Clostridium acetobutylicum* (see also **Table 1** for select enzymes involved).

[0037] Enzymes can be overexpressed in *C. acetobutylicum* for the production of butanol, including enzymes such as butyraldehyde dehydrogenase and butanol dehydrogenase. Enzymes of competing fermentative or biochemical pathways can be down regulated or deleted in *C. acetobutylicum* including enzymes such as pyruvate decarboxylase, lactate dehydrogenase and acetate kinase. By overexpressing and downregulating or deleting enzymes or genes in *C. acetobutylicum*, the volumetric productivity and/or the product titer provided by fermentation of the microorganism may be affected. For example, expression of a plasmid expressing an enzyme to be overexpressed in *C. acetobutylicum* to increase the production of a desired solvent may decrease the overall volumetric productivity of solvents as compared to a wild type strain. Expression of an oxidoreductase, such as luciferase, can increase the volumetric productivity of solvents to wild type levels, or levels greater than wild type levels. Similarly, the expression of an oxidoreductase can increase the volumetric productivity or product titer of solvents and other fermentation products of recombinant microorganisms engineered to produce or overproduce these products.

Table 1. Select *C. acetobutylicum* Enzymes Involved in Acidogenesis or Solventogenesis

Letter	Gene ID	Name	Definition
G	CAC1742	*pta*	Phosphotransacetylase [another source called it Phosphate acetyltransferase]
H	CAC1743	*askA*	Acetate kinase
I	CAC2873		Acetyl-CoA acetyltransferase
I	CAP0078	*thil*	Acetyl coenzyme A acetyltransferase [thiolase]

J	CAC2708	*hbd* Also listed as *Hdb*	Beta-hydroxybutyryl-CoA dehydrogenase [Also listed as 3-hydroxybutyryl-CoA dehydrogenase]
K	CAC2712	*crt*	Crotonase [3-hydroxybutyryl-CoA dehydratase]
L	CAC2711	*bcd*	Butyryl-CoA dehydrogenase
M	CAC3076	*ptb*	Phosphate butyryltransferase
N	CAC1660		Butyrate kinase
N	CAC3075	*buk*	Butyrate kinase, BUK
O	CAP0162	*adhe1*	Alcohol dehydrogenase / acetaldehyde dehydrogenase [aldehyde dehydrogenase (NAD+)]
O	CAP0035	*adhe*	Aldehyde-alcohol dehydrogenase [ADHE1]
P	CAP0162	*adhe1*	Alcohol dehydrogenase / acetaldehyde dehydrogenase [aldehyde dehydrogenase (NAD+)]
P	CAP0036		Uncharacterized, ortholog of YgaT gene of B. subtillis
P	CAC3298	*bdhB*	NADH-dependent butanol dehydrogenase B [BDH II]
P	CAC3299	*bdhA*	NADH-dependent butanol dehydrogenase A [BDH I]
P	CAP0059	*adh*	Alcohol dehydrogenase
Q	CAP0162	*adhe1*	Alcohol dehydrogenase / acetaldehyde dehydrogenase [aldehyde dehydrogenase (NAD+)]
Q	CAP0035	*adhe*	Aldehyde-alcohol dehydrogenase [ADHE1]
R	CAP0059	*adh*	Alcohol dehydrogenase
R	CAC3298	*bdhB*	NADH-dependent butanol dehydrogenase B [BDH II]

R	CAC3299	*bdhA*	NADH-dependent butanol dehydrogenase A [BDH I]
R	CAC3392		NADH-dependent butanol dehydrogenase
R	CAP0162	*adhe1*	Alcohol dehydrogenase / acetaldehyde dehydrogenase [aldehyde dehydrogenase (NAD+)]
R	CAP0035	*adhe*	Aldehyde-alcohol dehydrogenase [ADHE1]
S	CAP0163(A)	*ctfa*	Butyrate-acetoacetate CoA-transferase subunit A
S	CAP0164(B)	*ctfb*	Butyrate-acetoacetate CoA-transferase subunit B
T	CAP0165	*adc*	Acetoacetate decarboxylase
U	CAP0025	*pdc*	Pyruvate decarboxylase

[0038] The solvents *C. acetobutylicum* typically co-produces include acetone, butanol and ethanol (ABE) in a ratio roughly 3:6:1 by weight. Hydrogen and carbon dioxide are also produced during fermentation by *C. acetobutylicum*. Different species of butanol-producing *Clostridia* are known and they are differentiated mainly by the type and ratio of the solvents they produce. *C. beijerinckii* (synonym *C. butylicum*) produces solvents in approximately the same ratio as *C. acetobutylicum* and in some strains of *C. beijerinckii* isopropanol is produced in place of acetone. (George, H. A., et al. Acetone, isopropanol, and butanol production by *Clostridium beijernickii* (syn. *Clostridium butylicum*) and *Clostridium Aurantibutyricum*. Appl. Environ. Microbiol. 45:1160-1163, 1983.) *C. saccharobutylicum* is the proposed name for a *Clostridium* species identified through genetic and physiologic traits from saccharolytic industrial strains. (Keis, S., et al. Emended descriptions of *Clostridium acetobutylicum*, and *Clostridium beijerinckii* and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov. Intl. J. System. Evol. Microbio. 51:2095-2103, 2001.) *C. aurantibutyricum* produces both acetone and isopropanol in addition to butanol. (George, H. A., supra.) *C. tetanomorphum* produces almost equimolar amounts of butanol and ethanol, but not other solvents. (Gottwald, M., et al. Formation of n-butanol from D-glucose by strains of "*Clostridium tetanomorphum*" group. Appl. Environ. Microbio. 48:573-576, 1984.)

[0039] Solvent production in batch cultures of *C. acetobutylicum* proceeds through two phases. In the first, termed the acidogenic phase, that occurs during the exponential growth phase, *C. acetobutylicum* produces hydrogen, carbon dioxide, acetate and butyrate. The accumulation of acids in the culture media lowers the pH. The transition to the second or solventogenic phase occurs when the undissociated concentration of butyric acid in the culture reaches approximately 9 mM. (Hüsemann, M. H. W., and E. T. Papoutsakis. Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioeng.32:843-852,

1988.) This phase begins when *C. acetobutylicium* reaches early stationary phase. (Davies, R. and Stephenson M. Studies on the acetone-butyl alcohol fermentation. I. Nutritional and other factors involved in the preparation of active suspensions of *Clostridium acetobutylicum*. Biochem. J. 35:1320-1331, 1941.) Here, acetone, butanol and ethanol are synthesized concomitantly from the re-assimilated acids and the continued consumption of carbohydrates, raising the culture's pH. Hydrogen and carbon dioxide production continues.

[0040] When *C. acetobutylicum* is grown in batch culture different proportions of acids and solvents may be produced. The dilution rate, medium composition, and temperature may vary. (see for example, U.S. Pat. No. 5,063,156.) The addition of acetate or propionate does not typically affect the initiation of solventogenesis, but will generally increase the total concentration of solvents produced. (Hüsemann, M. H. W., and E. T. Papoutsakis. Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioeng.32:843-852, 1988.)

[0041] Solvent yields can also be changed by sparging the culture with CO gas. This causes a reversal of the butyrate production pathway with the resultant uptake of butyrate that is then unavailable as a subsequent substrate for acetone production. (Hartmanis, M. G. N., et al.Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 20:66-71, 1984.)

[0042] Changing the fermentation temperature can also affect butanol and solvent yield. In batch fermentation experiments conducted with three different solvent-producing strains, solvent yields remained fairly constant at around 31% at 30° C and 33° C, but decreased to 23-25% at 37° C. (McCutchan, W. N., and Hickey, R. J. The butanol-acetone fermentations. Ind. Ferment. 1:347-388, 1954.) Similar results were obtained in a more recent study with *C. acetobutylicum* NCIB 852 in which solvent yields were found to decrease from 29% at 25° C to 24% at 40° C, although the fermentation time decreased as the temperature was increased. (McNeil, B., and Kristiansen, B., Effect of temperature upon growth rate and solvent production in batch cultures of *Clostridium acetobutylicum*. Biotech Lett. 7:499-502, 1985.) The decrease in solvent yield appeared to reflect a decrease in acetone production, while the yield of butanol was unaffected.

[0043] In continuous culture, *C. acetobutylicum* can be maintained in three different stable metabolic states. Acidogenic, when grown at neutral pH on glucose, solventogenic when grown at low pH on glucose and alcohologenic when grown at neutral pH under conditions of high NAD(P)H availability. (Girbal, L. et al. Regulation of metabolic shifts in *Clostridium acetobutylicum* ATCC824, FEMS Microbiol. Rev. 17:287-297, 1995.) An acidogenic culture will switch to the solventogenic phase with a lowering of pH, a lowering of acetate and/or butyrate concentration, or with growth limiting quantities of phosphate or sulfate, but plentiful nitrogen and carbon sources. (Bahl, H. Andersch, W, and Gottschalk G. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur. J. Appl. Microbiol. Biotechnol. 15:201-205, 1982; Bahl, H., and Gottschalk G., Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture, p. 215-223. In Wang D. I. C. and Scott. C. D. (ed.), Biotechnology and bioengineering Symposium no. 14, Sixth Symposium on Biotechnology for Fuels and Chemicals, John Wiley & Sons, Inc., New York, 1984.) The physiologic signals for solventogenesis induce the biosynthesis of all terminal enzymes that catalyze solvent

production with a concomitantly decrease in acidogenic enzymatic activity. (Andersch, W., Hubert, B., and Gottschalk, G. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. Eur. J. Appl. Microbiol. Biotechnol. 18:327-332, 1983. Rogers, P. Genetics and biochemistry of Clostridium relevant to development of fermentation processes. Adv. Appl. Microbiol. 31:1-60, 1986.)

[0044] The biosynthesis of solvent production can be affected by mutagenesis. *C. acetobutylicum* is amenable to conventional mutational methodologies such as the use of alkylating agents like ethylmethylsulfonate (EMS), N-methyl N'-nitro N-nitrosoguanidine (NG), ICR 191, nitrous acid, nitroquinoline-N-oxide, and triethylene melamine, and selection by growth on increasing concentrations of butanol, resistance to allyl alcohol, or for cellulase, xylanase or amylase activity. Through such strategies regulatory mutants have been identified, along with mutants with increased solvent production, greater tolerance for higher solvent concentrations, decreased production of acids, and greater amolytic activity. (U.S. Pat. No. 4,757,010; Rogers, P., and Palosaari, N. *Clostridium acetobutylicum* mutants that produce butyraldehyde and altered quantities of solvents. Appl. Env. Microbio. 53:2761-2766, 1987.)

[0045] Studies exploring the overexpression of homologous genes and the expression of heterologous genes in low G+C gram-positive organisms such as *C. acetobutylicum* have lagged those of higher G+C organisms like *E. coli*, because low G+C gram-positive organisms are genetically distinct based on codon usage, amino acid usage and base content. (*C. acetobutylicum* has 29% GC content compared to *E. coli* with 50% GC content.) The design of new vectors and the sequencing and use of appropriate regulatory sequences and the study and use of low G+C gram-positive organisms is proceeding apace. (Gram-positive/negative shuttle vectors, U.S. Pat. No. 6,737,245; transposons, U.S. Pat. No. 7,056,728; bacteriophages, Reid S. J. et al. Transformation of *Clostridium acetobutylicum* Protoplasts with Bacteriophage DNA. Appl Environ Microbiol. 1983 Jan;45(1):305-307.) As a result, *C. acetobutylicum* is an attractive host organism for the methods of this invention. For example, the expression of oxidoreductases can easily be expressed in *C. acetobutylicum* to increase the volumetric productivity of solvents such as butanol.

[0046] For butanol and butyrate production, glucose is first converted by way of glycolysis to pyruvate. The enzyme, glyceraldehyde-3-phosphate dehydrogenase catalyzes the last enzymatic step, the conversion of glyceraldehyde-3-phosphate to pyruvate. (**Step A, FIG. 1**) Next, pyruvate is converted to acetyl-CoA with the concomitant loss of a molecule of carbon dioxide by the enzyme pyruvate-ferredoxin oxidoreductase. (**Step B, FIG. 1**.) Two acetyl CoA molecules are then condensed to acetoacetyl-CoA by acetyl-CoA acetyltransferases (*thil*, (thiolase), CAP0078; and CAC2873) with the production of one free CoA group. (**Step I, FIG. 1**) Acetoacetyl-CoA is converted to 3-hydroxybutyrl-CoA (β-hydroxybutyrl-CoA) by 3-hydroxybutyrl-CoA dehydrogenase (*hbd*, CAC2708) a process that requires the oxidation of NADH to NAD^+. (**Step J, FIG. 1**) 3-hydroxybutyrl-CoA is then converted to crotonyl-CoA by crotonase (*crt,* CAC2712) with the concomitant loss of a molecule of water. (**Step K, FIG. 1**) Crotonyl-CoA is converted to butyryl-CoA by butyryl-CoA dehydrogenase (*bcd*, CAC2711) with the concomitant oxidation of NADH to NAD^+. (**Step L, FIG. 1**)

[0047] When *C. acetobutylicum* is in the solventogenic phase, butyraldehyde dehydrogenase catalyzes the reaction to produce butyraldehyde that is subsequently converted to butanol. Butyryl-CoA is reduced to butyraldehyde by butyraldehyde dehydrogenase (*adhe*, CAP0035, and *adhe1*, CAP0162) and NADH. (**Step Q, FIG. 1**) Butyraldehyde is then reduced to butanol by dehydrogenases (*adhe*, CAP0035, *adhe1*, CAP0162, *adh*, CAP0059, *bdhA*, CAC3299, *bdhB*, CAC3298, and CAC3392) and NADPH. (**Step R, FIG. 1**).

[0048] During acidogenesis, butyryl-CoA is phosphorylated by phosphotransbutyrylase (*ptb*, CAC3076, **Step M, FIG. 1**) to make butyrylphosphate, which is then converted to butyrate by butyrate kinase (CAC1660 and *buk*, CAC3075, **Step N, FIG. 1**) with the production of one molecule of ATP. Butyrate production can be a signal to initiate solventogenesis and be recycled back to butyryl-CoA by the *ctfa/ctfb* complex (acetoacetyl-CoA:acetate/butyrate:CoA transferase). (**Step S, FIG. 1**). During acidogenesis, the expression of the genes coding for the enzymes that are responsible for the catalyzing the final steps of butyrate production, butyrate kinase (*buk*, **Step N, FIG. 1**) (as well as acetate kinase (*ack,* **Step H, FIG. 1**), for the production of acetate) is high. (Durre, P. et al. Transcriptional regulation of solventogenesis in *Clostridium acetobutylicum*. J. Mol. Microbiol. Biotechnol. 4:295-300, 2002.)

[0049] The methods of this invention may also be used to increase the volumetric productivity of ethanol. For ethanol production, glucose is first converted by way of glycolysis to pyruvate. The enzyme, glyceraldehyde-3-phosphate dehydrogenase catalyzes the last enzymatic step, the conversion of glyceraldehyde-3-phosphate to pyruvate. (Step **A in Fig. 1**) From here, pyruvate can flow through two separate ethanologenic pathways as *Clostridia* is one of the few genera of bacteria that possess pyruvate decarboxylase. In one pathway, pyruvate is converted to acetyl-CoA with the concomitant loss of a molecule of carbon dioxide by the enzyme pyruvate-ferredoxin oxidoreductase. (**Step B, FIG. 1**) Acetyl-CoA is then converted to acetylaldehyde by acetaldehyde dehydrogenase (**Step O, FIG. 1**) and NADH. Finally, acetylaldehyde is reduced to ethanol by dehydrogenase (*bdhB*, CAC3298; *bdhA*, CAC3299; and possibly *adhe1*, CAP0162, and CAP0035, **Step P, FIG. 1**) and NADH. In the other pathway, pyruvate is decarboxylated by pyruvate decarboxylase (**Step U, FIG. 1**) to form acetylaldehyde that is then reduced to ethanol by dehydrogenases (*bdhB*, CAC3298; *bdhA*, CAC3299; and possibly *adhe1*, CAP0162, and CAP0035, **Step P, FIG. 1**) and NADH.

[0050] The methods of this invention can also be used to increase the volumetric productivity of acetone. For the production of acetone, glucose is first converted by way of glycolysis to pyruvate. The enzyme, glyceraldehyde-3-phosphate dehydrogenase catalyzes the last enzymatic step, the conversion of glyceraldehyde-3-phosphate to pyruvate. (**Step A, FIG. 1**) Next, pyruvate is converted to acetyl-CoA with the concomitant loss of a molecule of carbon dioxide by the enzyme pyruvate-ferredoxin oxidoreductase. (**Step B, FIG. 1**) Two acetyl CoA molecules are then condensed to acetoacetyl-CoA by acetyl-CoA acetyltransferases (*thil*, (thiolase), CAP0078, and CAC2873) with the production of one free CoA group. (**Step I, FIG. 1**) Acetoacetyl-CoA is converted to acetoacetate by acetoacetyl-CoA: acetate/butyrate CoA transferase. (**Step S, Fig. 1**) Acetoacetate is converted to acetone by acetoacetate decarboxylase with the production of one molecule of carbon dioxide. (**Step T, FIG. 1**)

[0051] Several competing pathways can draw intermediates away from the aforementioned pathways. For example, lactate dehydrogenase can reduce pyruvate into lactate using lactate dehydrogenase. (**Step U, FIG. 1.**), drawing intermediates away from producing the solvents ethanol, acetone, and butanol, as well as the acids acetate and butyrate. This, however, is minimal except under conditions of iron limitation and high pH. Alternatively, pyruvate decarboxylase can convert pyruvate into acetaldehyde, drawing pyruvate off to produce ethanol. (**Step U, FIG. 1**) Acetyl-CoA can also be drawn off to make acetate or ethanol. (**Steps G and H; Steps 0 and P, FIG. 1**) Furthermore, acetoacetyl-CoA can be converted to acetone by way of acetoacetyl-CoA:acetate/butyrate-CoA transferase and acetoacetate decarboxylase. (**Steps S and T, FIG. 1.**)

[0052] The methods of the present invention can also be used to increase the volumetric productivity of ethanologenic organisms like *Zymomonas mobilis* and *Saccharomyces cerevisiae*, as well as *E. coli.* Both *Z. mobilis* and *S. cerevisiae* ferment one molecule of glucose into two molecules of ethanol and two molecules of CO_2. Two enzymatic steps are typically required. First pyruvate decarboxylase cleaves pyruvate into acetaldehyde and carbon dioxide. Alcohol dehydrogenase then regenerates NAD+ by transferring hydrogen equivalents from NADH to acetaldehyde, thereby producing ethanol. *Z. mobilis* is a bacterium commonly found in plant saps and honey, and relies on the Entner-Doudoroff pathway as a fermentative path. This shorter pathway yields one ATP per glucose molecule. *Z. mobilis* possesses two alcohol dehydrogenase isozymes that catalyze the reduction of acetaldehyde to ethanol during fermentation, accompanied by the oxidation of NADH to NAD+. The production of ethanol by *S. cerevisiae* is well known and results in the net production of two molecules of ATP for every molecule of glucose. Both *Z. mobilis and S. cerevisiae* have served as the source of heterologous genes for the production of ethanol in other microorganisms, such as *E. coli.*

[0053] *E. coli* does not naturally possess the enzyme pyruvate decarboxylase and therefore when it is grown anaerobically, minimal ethanol is produced along with mixed acids (or example, fermentative growth on 25 mM glucose typically yields 6.5 mM ethanol, 8.2 mM acetate, 6.5 mM lactate, 0.5 mM succinate, and about 1 mM formate leaving 10.4 mM residual glucose) Brau & Sahm (1986a) Arch. Microbiol. 144:296-301, (1986b) Arch. Microbiol. 146:105-110. When the genes encoding alcohol dehydrogenase II (*adhB*) and pyruvate decarboxylase (*pdc*) cloned from *Z. mobilis* are introduced and expressed into in *E. coli*, the initial concentration of 25 mM glucose was completely converted yielding up to 41.5 mM ethanol while almost forming no acids, and has been shown by other researchers. (Conway et al. (1987a) J. Bacteriol. 169:2591-2597; Neale et al. (1987) Nucleic Acids Res. 15:1752-1761; Ingram and Conway [1988] Appl. Environ. Microbiol. 54:397-404; Ingram et al. (1987) Appl. Environ. Microbiol. 53:2420-2425.) The extent of ethanol production under anaerobic and aerobic conditions was directly related to the level of expression of the *Z. mobilis* ethanologenic gene. Therefore, using appropriate transcription regulatory nucleotide sequences, a construct can be designed with a nucleotide sequence that encodes an oxidoreductase, the construct can additionally include ethanologenic heterologous sequences, to improve the volumetric productivity of ethanol production.

[0054] This technique is not limited to *E. coli*, since subsequent studies demonstrated the general applicability of this approach by the use of two other enteric bacteria, *Erwinia chrysanthemi* and *Klebsiella planticola*, to increase

ethanol yields and productivity from hexoses, pentoses, and sugar mixtures. (Tolan and Finn. Appl. Environ. Microbiol. 53:2033-2038, 2039-2044,1987; Beall et al., 1993; Ingram and Conway, 1988; Wood and Ingram, 1992.)

[0055] The present invention also provides methods and compositions for increasing the volumetric productivity of commercially valuable products produced by synthetic pathways. Synthetic pathways can include natural, pre-existing pathways that generate secondary metabolites, also known as natural products, such as aliphatic, aromatic, and heteroaromatic organic acids, alkaloids, terpenoids, polyketides, , phenols, iridoids , steroids, saponins, peptides, ethereal oils, resins and balsams. Additionally, a synthetic pathway can include pathways introduced either whole or in part, into an organism through genetic engineering, cell fusion, conjugation, or other means. For example, the introduction of an ethanologenic pathway in *E. coli* through the use of plasmids encoding the heterologous proteins from *Z. mobilis* for alcohol dehydrogenase II and pyruvate decarboxylase. Or the engineering of a terpenoid pathway in *E. coli*, through the expression of a synthetic amorpha-4,11-diene synthase gene derived from the plant *Artemisia annua* coupled with the mevalonate isoprenoid pathway from *S. cerevisiae.* (Martin, V. J. J. et al. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nature Biotech. 21:796-802, 2003; US Pat. Application Publication 2004/0005678 A1.)

[0056] The volumetric productivity of products in the fermentative or synthetic biochemical pathways are increased by expressing an oxidoreductase.

Oxidoreductases

[0057] The volumetric productivity in microorganisms is increased by expressing an oxidoreductase in the microorganism. Volumetric productivity of biochemical and fermentative products such as solvents can be increased in microorganisms such as *C. acetobutylicum*, wherein the microorganism comprises a recombinant nucleic acid with a nucleotide sequence encoding an oxidoreductase operatively linked to a transcriptional regulatory sequence.

[0058] Oxidoreductases are enzymes that catalyzes the transfer of electrons from one molecule, the reductant or electron donor, to another, the oxidant or electron acceptor. Oxidoreductases include oxygenases, which oxidizes a substrate by transferring oxygen atoms to a substrate. Oxygenases include monooxygenases, which transfer one oxygen atom from O_2 to a substrate, and reduce the other oxygen atom to water, and dioxygenases, which transfer both atoms of molecular oxygen onto a substrate. Oxygenases include cytochrome P450 oxidases and luciferase.

[0059] Luciferases are oxygenases that act on a substrate which, through an enzyme catalyzed reaction in the presence of molecular oxygen and ATP, transform the substrate into an excited state. Luciferase is a low molecular weight oxidoreductase which catalyzes the dehydrogenation of luciferin or other substrate in the presence of oxygen, ATP and magnesium ions. Luciferases, as well as aequorin-like molecules, require a source of energy, such as ATP, NAD(P)H, and a substrate to oxidize, such as luciferin (a long chain fatty aldehyde) or coelentrizine and oxygen. During this process, about 96% of the energy released appears as visible light. Despite the loss of energy, volumetric productivity of a microorganism may be increased because, without being bound by theory, microorganisms benefit from the dehydrogenation of a substrate. Dehydrogenation of a fatty acid aldehyde in the

present of oxygen and $FMNH_2$ can lower the intracellular oxygen concentration, which can protect the oxygen-sensitive enzymes of the microorganism. The oxidoreductase may also offer resistance to oxidative stress or provide a redox sink by regenerating NAD^+, thereby increasing the productivity of the microorganism.

[0060] The recombinant nucleic acids of the present invention can comprise nucleotide sequences encoding a luciferase in the luciferase family (de Wet, J. R, et al., Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7:725-737, 1987) and the aequorin family (Prasher, et al. Cloning and expression of the cDNA coding for Aequorin, a bioluminescent calcium-binding protein. Biochem Biophys Res Commun 126: 1259-1268,1985). Members of the luciferase family have been identified in a variety of prokaryotic and eukaryotic organisms. Prokaryotic and eukaryotic luciferases, as well as variants possessing varied or altered optical properties, such as luciferases that produce different colors of light, can be used in the present invention (e.g., Kajiyama, N., and Nakano, E., (1991) Protein Engineering 4(6):691-693). During this process, about 96% of the energy released appears as visible light. "Lux" refers to prokaryotic genes associated with luciferase and photon emission, whereas "Luc" refers to eukaryotic genes associated with luciferase and photon emission. Luciferase is a well known real time reporter protein and can be expressed in most Gram negative aerobic bacteria and some Gram positive aerobes.

[0061] Prokaryotic luciferase is typically encoded by two subunits (luxAB) of a five gene complex that is termed the lux operon (luxCDABE). The remaining three genes comprise the luxCDE subunits and code for the fatty acid reductase responsible for the biosynthesis of the aldehyde substrate used by luciferase for the luminescent reaction. For example, bacterial luciferase ("lux") is typically made up of two subunits (α and β) encoded by two different genes (luxA and luxB) on the lux operon. Three other genes on the operon (lux C, lux D and luxE) encode the enzymes required for biosynthesis of the aldehyde substrate. Bacterial lux is present in certain bioluminescent Gram-negative bacteria (e.g., *Photorhabdus luminescens*) and is ordered CDABE.

[0062] Eukaryotic luciferase ("luc") is typically encoded by a single gene (de Wet, J. R., et al., Proc. Natl. Acad. Sci. U.S.A. 82:7870-7873, 1985; de Wet, J. R, et al., Mol. Cell. Biol. 7:725-737, 1987). An exemplary eukaryotic organism containing a luciferase system is the North American firefly *Photinus pyralis*. Firefly luciferase has been extensively studied, and is widely used in ATP assays. cDNAs encoding luciferases (lucOR) from *Pyrophorus plagiophthalamus*, another species of click beetle, have been cloned and expressed. (Wood, et al. Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors. Science 244:700-702, 1989.) This beetle is unusual in that different members of the species emit bioluminescence of different colors. Four classes of clones, having 95-99% homology with each other, were isolated. They emit light at 546 nm (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm (orange).

[0063] The recombinant nucleic acid of the present invention can comprise a nucleotide sequence encoding the lux operon obtained from the soil bacterium *Photorhabdus luminescens*, formerly *Xenorhabdus luninescens* (Frackman, et al., Cloning, organization, and expression of the bioluminescence genes of *Xenorhabdus luninescens.* J. Bacteriol. 172"5767-5773,1990), confers on transformed *E coli* optimal bioluminescence at 37° C. (Xi, et al. Cloning and nucleotide sequences of lux genes and characterization of Luciferase of *Xenorhabdus luninescens* from a human wound. J. Bacter. 173:1399-1405, 1991.) The sequence is available from GenBank under the accession

number M90092. In contrast to luciferase from *P. luminescens*, other luciferases isolated from luminescent prokaryotic and eukaryotic organisms have optimal bioluminescence at lower temperatures. (Campbell, A. K. Chemiluminescence, Principles and Applications in Biology and Medicine. Ellis Horwood, Chichester, UK. 1988 .)

[0064] Lux genes which can be utilized in the compositions and methods of the invention, can also be obtained from organisms including but not limited to *Photobacterium phosphoreum, Vibrio salmonicida, Photobacterium leiognathi, Vibrio harvey, Photobacterium leiognathi, Vibrio fischeri,* and *Photinus pyralis.* A variety of other luciferase encoding genes have been identified including, but not limited to, the following: Sherf, B. A., and Wood, K. V., U.S. Pat. No. 5,670,356; Kazami, J., et al., U.S. Pat. No. 5,604,123; Zenno, S., et al, U.S. Pat. No. 5,618,722; Wood, K. V., U.S. Pat. No. 5,650,289; Wood, K. V, U.S. Pat. No. 5,641,641; Kajiyama N., and Nakano, E., U.S. Pat. No. 5,229,285; Cormier, M. J., and Lorenz, W. W., U.S. Pat. No. 5,292,658; ; Cormier, M. J., and Lorenz, W. W., U.S. Pat. No. 5,418,155; de Wet, J. R., et al, Molec. Cell. Biol. 7:725-737, 1987; Tatsumi, H. N., et al, Biochim. Biophys. Acta 1131:161-165, 1992; and Wood, K. V., et al, Science 244:700-702, 1989, all herein incorporated by reference. Such luciferase encoding genes may be modified by the methods described herein to produce polypeptide sequences and/or expression cassettes useful, for example, in Gram-positive microorganisms.

[0065] The nucleic sequences encoding oxidoreductases such as luciferase can be modified from the naturally occurring sequences to facilitate its expression in a particular microorganism. For example, to express *P. luminescens* lux in *C. acetobutylicum*, the nucleotide sequence of the wild type lux operon (luxCDABE) was reengineered to have an AT content of 69%. This was accomplished by taking advantage of the degeneracy of the genetic code so that codons that include C or G at degenerate positions could be replaced with codons that encode the same amino acid, but have an A or T in the degenerate positions. The sequences of the individual genes of the *C. acetobutylicum* optimized lux operon, along with their corresponding amino acid sequences are given in SEQ ID NO: 1-10. One can similarly modify other oxidoreductases so that they are optimized for expression in *C. acetobutylicum* and other organisms with high AT content in the range of 60-80%.

Nucleic Acid Molecules

[0066] The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pa.: Mack Publishing Company, 1990); Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D. M. Weir and C. C. Blackwell, eds., 1986, Blackwell Scientific Publications); Ausubel, F. M., et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Media, Pa. (1995.); Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.) (2001).)

[0067] According to the present invention, microorganisms have a recombinant nucleic acid with a nucleotide sequence encoding an oxidoreductase operatively linked to a transcriptional regulatory sequence, e.g., promoters, which increases the volumetric productivity of the microorganism. A given a transcriptional regulatory sequence

that is operably linked to a coding sequence (e.g., a gene encoding an oxidoreductase) is capable of effecting the expression of the coding sequence when the proper enzymes are present. The promoter or other control elements does not need to be contiguous with the coding sequence, but still function to direct the expression thereof. For example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence. The recombinant nucleic acid can be inserted into an appropriate vector that is then used to transform the intended host.

[0068] In one aspect of the invention, sequences encoding oxidoreductases are optimized for expression in a host cell. Thus one aspect of the invention is directed to altered sequences or codon usage manipulation for expression of the altered sequence in Gram positive bacteria. Sequences can be codon optimized to comprise high A/T content for expression in low-GC bacteria. Such low-GC bacteria can be obligate or strict anaerobe Gram positive bacteria. For example, sequences encoding oxidoreductases can be optimized for expression in Gram positive anaerobes of high A/T content. Oxidoreductases (in particular the Lux proteins of luciferase) that are genetically modified to have nucleotide sequences that are A/T rich, are useful, among other things, for expression and activity in cells that have a preference for A/T rich genes, e.g., *Clostridium*. To make such genes one can engineer the codons to replace G or C with A or T, in particular at positions that do not change the amino acid encoded at that codon by taking advantage of the degeneracy of the genetic code so as to replace codons that include C or G at degenerate positions with A or T. In certain cases, such as *lux*, a reporter construct can be part of a larger operon containing several cistrons and the entire coding sequence of the operon can be engineered to have A/T rich content. These engineered genes then can be operatively linked with expression control sequences, such as promoters and/or ribosome binding sites that are compatible with the intended host organism.

[0069] In various embodiments, nucleic acid sequences encoding oxidoreductases, such as lux genes, are altered to comprise A/T content of from about 62% to about 75%, about 62% to about 65%, 62% to 70%, 65% to 75% or 70% to 75% of the total sequence based on codon degeneracy. Thus in various embodiments, A/T content is about 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74% or 75%.

[0070] A lux operon sequence can be optimized for expression in Gram positive anaerobes of high A/T (i.e., low-GC) content, with a specified ribosome binding site and an altered substrate binding site. For example, a lux operon can be modified as to A/T content (for illustrative purposes, A/T optimization = "X"), ribosome binding sites, for example, particular to the desired host (for illustrative purposes, ribosome binding site modification = "Y"), LuxA catalytic site for substrate binding (for illustrative purposes, substrate binding site modification = "Z"). Thus, a nucleic acid construct of the invention can have single modifications of X, Y, or Z, or various combinations such as X, XY, XYZ or XZ. Any of the preceding variations can be modified by a promoter or expression control sequence obtained from the particular host in which the nucleic acid construct (X, XY, XYZ or XZ) is integrated or provided via an episomal vector. As such, luciferases expressed in bacteria (e.g., Gram positive anaerobe) can have the various combinations and be used to increase the volumetric productivity of the bacteria. For example, *Clostridial* codon usage for a low-G/C bacteria (e.g., GC content is less than about 40%) can be optimized for the enhanced expression of luciferase, wherein the lux genes are optimized and ribosome binding sites provided upstream of each lux gene to allow expression in *Clostridium.*

[0071] In one aspect of the invention, the expression of a sequence encoding a recombinant oxidoreductase provides oxygen tolerance. For example, where such sequences are expressed in a microorganism (e.g., in an obligate anaerobe or anaerobic bacterium), such microorganisms are able to grow in low oxygen partial pressures or oxygen environments, because of expression of said sequence. Low oxygen partial pressures include microaerobic environments. In another aspect of the invention, the sequence encoding an oxidoreductase is optimized for expression in a Gram positive bacterium. In some embodiments, the sequence encoding an oxidoreductase is optimized so that it contains an AT content of at least 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, or 75% to provide oxygen tolerance. In some embodiments, the sequence encoding an oxidoreductase is optimized so that the AT is between about 62% to about 75%. For example, where such optimized sequences are expressed in a microorganism (e.g., in an obligate anaerobe or anaerobic bacterium), such microorganisms are able to grow in a low oxygen or oxygen environment, because of said optimization. Therefore, the optimization provides a protein that functions as an oxygenase. For example, the entire *Photorhabdus luminescens* lux operon (SEQ ID NO: 11) has been reengineered to introduce A/T content necessary to express in high A/T Gram positive bacterium (SEQ ID NO: 12).

[0072] In some cases, expression of one or more recombinant oxidoreductases in a microorganism, allows an anaerobe such as a strict anaerobe, an obligate anaerobe or a facultative anaerobe to survive or grow in the presence of oxygen, whereas the unmodified, parent strain would be unable to survive or grow in the presence of oxygen, or would exhibit a reduced level of growth, product titer, or volumetric productivity. In other cases, expression of one or more recombinant oxidoreductases in a microorganism, allows an anaerobe such as a strict anaerobe, an obligate anaerobe or a facultative anaerobe to survive for a longer period of time than the parent strain would be able to survive in the presence of oxygen. For example, expression of a recombinant oxidoreductase may allow for survival for seconds, minutes (e.g. about 1, 2, 3, 5, 10, 15, 20 30, 35, 40, 45, 50, or 60), hours (e.g. about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 20, 22, or 24), days (e.g. about 1, 2, 3, 4, 5, 6, or 7), or weeks (e.g. about 1, 2, 3, 4 or more) longer than the parent strain in the presence of oxygen. In some cases, the parent strain is an anaerobe, and the recombinant strain expressing one or more recombinant heterologous oxidoreductases such as luciferase is resistant to a microaerobic environment (i.e. grows in the presence of low concentrations of oxygen). In some embodiments, expression of one or more optimized recombinant oxidoreductases allows the microorganism to be cultured in batch, fed-batch, or continuous cultures under conditions, including the presence of oxygen that the unmodified, parent microorganism is unable to grow or produce a desired product.

[0073] In another aspect of the invention, the expression of an optimized or wild-type recombinant heterologous sequence encoding an oxidoreductase provides tolerance to inhibitors of organismal growth, such as phenolic compounds including para-coumarate (*p*-coumarate), ferulic acid, 4-hydroxybenzoate, vanillic acid, ferulic acid, and aromatic aldehydes including but not limited to furfural, vanillin, syringaldehyde, or hydroxymethyl furfural. In some cases, the oxidoreductase provides a detoxifying function by detoxifying the phenolic inhibitors of growth. In some cases, the oxidoreductase provides for volumetric productivity of a fermentation product that exhibits little or no detrimental affect by the presence of phenolic growth inhibitors as compared to fermentation in the absence or substantial absence of phenolic growth inhibitors. For example, the recombinant microorganism may show little or no reduction in volumetric productivity, or growth in the presence of about 0.05g/L phenolic inhibitor,

0.1g/L, 0.2g/L, 0.3g/L, 0.4g/L, 0.5g/L, 0.75g/L, 1 g/L, 2g/L, 3g/L, 5g/L, 7.5g/L, 10g/L, 15g/L, or about 20g/L or more phenolic inhibitor. In some cases, the recombinant microorganism may exhibit greater growth or volumetric productivity in the presence of about 0.05g/L phenolic inhibitor, 0.1g/L, 0.2g/L, 0.3g/L, 0.4g/L, 0.5g/L, 0.75g/L, 1 g/L, 2g/L, 3g/L, 5g/L, 7.5g/L, 10g/L, 15g/L, or about 20g/L or more phenolic inhibitor than a parent strain, or a strain that does not express a heterologous oxidoreductase such as luciferase.

Transcription Regulatory Sequences

[0074] The nucleotide sequence encoding an oxidoreductase is operatively linked to a transcriptional regulatory sequence. The transcription regulatory nucleotide sequences are typically selected based on compatibility with the intended host. For example, for expression of an oxidoreductase in *C. acetobutylicum*, the transcription regulatory nucleotide sequences include those from genes including but not limited to those listed in **Table 2**. In various embodiments, promoters are selected from genes including but not limited to butanol dehydrogenase, butyraldehyde dehydrogenase, ethanol dehydrogenase, acid aldehyde dehydrogenase, acetoacetate decarboxylase, butyrate kinase, phosphobutyryltransferase, phosphotransacetylase, acetate kinase, acyl CoA transferase, lactate dehydrogenase and butyl CoA.

[0075] The promoter sequence can be an inducible or constitutive promoter. Depending on the host cell, there are hundreds of constitutive and inducible promoters which are known and that can be engineered with the optimized reporters of the invention. Examples of constitutive promoters include the *int* promoter of bacteriophage λ, the *bla* promoter of the β-lactamase gene sequence of pBR322, *hydA* or *thlA* in *Clostridium, S. coelicolor hrdB*, or *whiE*, the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, Staphylococcal constitutive promoter P_{blaZ}, and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage (P_L and P_R), the *trp, reca, lacZ, AraC* and *gal* promoters of *E. coli*, the α-amylase (Ulmanen Ett at., J. Bacteriol. 162:176-182, 1985) and the sigma-28-specific promoters of *B. subtilis* (Gilman et al., Gene sequence 32:11-20(1984)), the promoters of the bacteriophages of *Bacillus* (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982)), Streptomyces promoters (Ward et at., Mol. Gen. Genet. 203:468-478, 1986), Staphylococcal cadmium-inducible P_{cad}-cadC promoters and the like. Exemplary prokaryotic promoters are reviewed by Glick (J. Ind. Microtiot. 1:277-282, 1987); Cenatiempo (Biochimie 68:505-516, 1986); and Gottesman (Ann. Rev. Genet. 18:415-442, 1984). Further examples of inducible promoters, such as in *Clostridium* species, include *recA* or *recN* gene promoters can be utilized which are part of the SOS repair system in *Clostridium*, or T5, CP25, P32, P59, P1P2 and PL promoters which can be linked to at least one operator selected from the group consisting of xylO, tetO, trpO, malO and λc1O. (See US Patent Application 20030027286).

[0076] In some embodiments, a promoter which is constitutively active under certain culture conditions, may be inactive in other conditions. For example, the promoter of the *hydA* gene from *Clostridium acetobutylicum*, expression is known to be regulated by the environmental pH. Therefore, in some embodiments, depending on the desired host cell, a pH-regulated promoter can be utilized with the expression constructs of the invention (e.g., **FIG. 2** with *hydA* promoter driving expression of the optimized lux genes in response to variations in environmental pH).

Other pH regulatable promoters are known, such as P170 functioning in lactic acid bacteria, as disclosed in US Patent Application No. 20020137140.

Table 2. Sources for Transcription Regulatory Nucleotide Sequences for Select Genes of *C. acetobutylicum*

Gene ID	Direction	Annotation	IR Length	Description	Reference
CAC1742	+	pta	264	Phosphotransacetylase	Boynton. Appl. Environ. Microbiol. 1996
CAC1743	+	askA	11	Acetate kinase	Boynton. Appl. Environ. Microbiol.1996
CAC2708	-	hbd	104	Beta-hydroxybutyryl-CoA dehydrogenase, NAD-dependent	Boynton. J. Bacteriol. 1996
CAC2711	-	bcd	13	Butyryl-CoA dehydrogenase	Boynton. J. Bacteriol. 199
CAC2712	-	crt	175	Crotonase (3-hydroxybutyryl-COA dehydratase)	Boynton. J. Bacteriol. 199
CAC2873	-		326	Acetyl-CoA acetyltransferase	Stim-Herndon. Gene. 1995
CAC3075	-	buk	27	Butyrate kinase, BUK	Walter. Gene. 1993
CAC3076	-	ptb	108	Phosphate butyryltransferase	Walter. Gene. 1993
CAC3298	-	bdhB	276	NADH-dependent butanol dehydrogenase B (BDH II)	Walter. J. Bacteriol. 1992
CAC3299	-	bdhA	147	NADH-dependent butanol dehydrogenase A (BDH I)	Walter. J. Bacteriol. 1992
CAP0035	-	adhe	476	Aldehyde-alcohol dehydrogenase ADHE1	Fontaine. J. Bacteriol. 2002
CAP0078	-	thil	105	Acetyl coenzyme A	Winzer. J. Mol. Microbiol.

				acetyltransferase (thiolase)	Biotechnol., 2000
CAP0162	+	adhe1	666	Aldehyde dehydrogenase (NAD+)	Nair. J. Bacteriol. 1994 Fischer. J. Bacteriol. 175:6959-6969, 1993
CAP0163	+	ctfa	63	Butyrate-acetoacetate COA-transferase subunit A	Nair. J. Bacteriol. 1994 Fischer. J. Bacteriol. 175:6959-6969, 1993
CAP0164	+	ctfb	4	Butyrate-acetoacetate COA-transferase subunit B	Nair. J. Bacteriol. 1994 Fischer. J. Bacteriol. 175:6959-6969, 1993
CAP0165	-	adc	232	Acetoacetate decarboxylase	Gerischer. J. Bacteriol. 172, 1990 Gerischer. J. Bacteriol. 174:426-433, 1992

a) Gene ID: Systematic gene code from TIGR; b) Direction: Coding strand; c) Annotation: Gene symbol according to TIGR; d) IR length: Length of the upstream Intergenic Region; e) Description: Description of gene function.

[0077] In general, to express the desired gene/nucleotide sequence efficiently, various promoters may be used; e.g., the original promoter of the gene, promoters of antibiotic resistance genes such as for instance kanamycin resistant gene of Tn5, ampicillin resistant gene of pBR322, and promoters of lambda phage and any promoters which may be functional in the host cell. For expression, other regulatory elements, such as for instance a Shine-Dalgarno (SD) sequence (e.g., AGGAGG and so on including natural and synthetic sequences operable in the host cell) and a transcriptional terminator (inverted repeat structure including any natural and synthetic sequence) which are operable in the host cell (into which the coding sequence will be introduced to provide a recombinant cell of this invention) may be used with the above described promoters.

[0078] Moreover, methods of identifying bacterial promoters can be practiced in selecting a promoter to be utilized in expression constructs of the present invention. Such methods are known, such as disclosed in US Patent Application No. 20060029958, US Patent No. 6617156. Through the analysis of the transcription regulatory nucleotide sequences, the appropriate primers can be designed so that the transcription regulatory nucleotide

sequence of interest can be cloned from genomic DNA by use of the technique of polymerase chain reaction (PCR). The transcription regulatory sequences for genes from any desired host can be identified through the use of computational methods utilizing the sequenced genome of the host (e.g., genome of *C. acetobutylicum* ATCC 824 to obtain promoters therefrom). See, Paredes, C. J. et al. Transcriptional organization of the Clostridium acetobutylicum genome, Nuc. Acids Res. 32:1973-1981. Furthermore, sequences for many pathways are known and available through internet based services such as TIGR or the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), the transcription regulatory nucleotide sequences can be identified through standard molecular biology techniques such as cDNA primer extension using primers derived from the gene sequences of interest coupled with reverse transcription.

[0079] The transcription regulatory nucleotide sequences to increase the volumetric productivity of solvents such as butanol may be those active during the solventogenesis stage. For example, the transcription regulatory nucleotide sequence of the *sol* operon, found on the pSOL1 megaplasmid of *C. acetobutylicum* ATCC 824 can be used. The *sol* operon controls the transcription of three genes, *adhE*, CAP0035 (aldehyde-alcohol dehydrogenase), *ctfA*, CAP0163(A), and *ctfB*, CAP0164(B) (butyrate-acetoacetate CoA-transferase subunits A and B) the expression of which increases approximately 10-fold with the initiation of solventogenesis. (Feustel, L., et al. Characterization and development of two reporter gene systems for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 70:798-803,2004.) The gene product of *adhE*, butyraldehyde/butanol dehydrogenase, is typically active only during the onset of solventogenesis. Also on the pSOL1 megaplasmid is *adc*, CAP0165, (acetoacetate decarboxylase, **Step T, FIG. 1**) the transcription of which also increases approximately 10-fold with the onset of solventogenesis. (Feustel, L., et al. supra.) It is transcribed from its own promoter in the opposite direction of that of the *sol* operon. Transcription of acetoacetate decarboxylase occurs at the onset of the solventogenic phase and the activity of the enzyme was found to be stable throughout the solventogenic phase. (Gerischer, U., and Durre, P. mRNA analysis of the adc gene region of *Clostridium acetobutylicum* during the shift to solventogenesis. J. Bact. 174:426-433, 1992) Therefore the use of the transcription regulatory nucleotide sequence for *adc* or *adhE*, or that of pSOL1 may be used in conjunction with a nucleotide sequence encoding an oxidoreductase to increase the volumetric productivity of solvents.

[0080] During the later portion of solvent production another aldehyde-alcohol dehydrogenase, *bdhB,* found on its own monocistronic operon, takes over. (Petersen, D. J., et al. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 173:1831-1834, 1991; Sauer, U., and P. Dürre. Differential induction of genes related to solvent formation during the shift from acidogenesis to solventogenesis in continuous culture of *Clostridium acetobutylicum.* FEMS Microbiol. Lett. 125:115-120,1995) The transcription regulatory nucleotide sequence of the *bdhB* operon, may be operatively linked to an oxidoreductase to increase the volumetric productivity of butanol since the aldehyde-alcohol dehydrogenase encoded for by *bdhB* is believed to be responsible for high butanol production. (Feustel, L., et al., supra.)

[0081] Other transcription regulatory nucleotide sequences of interest include CAC3392 (NADH-dependent butanol dehydrogenase) and *adh*, CAP0059 (alcohol dehydrogenase), since these genes encode for enzymes used in the last step of butanol production, the reduction of butyraldehyde to butanol. Additionally, the transcription

regulatory nucleotide sequence for *adhe1* (CAP0162, alcohol dehydrogenase/acetaldehyde dehydrogenase) could be used since butyraldehyde is one enzymatic step away from butanol.

[0082] Any of the nucleic acid sequences, constructs and the like disclosed herein can be comprised in an expression cassette. For example, a desired transcription regulatory nucleotide sequence can be operably linked to a gene encoding an oxidoreductase along with the appropriate translational regulatory elements (e.g., Gram-positive Shine-Dalgarno sequences), short, random nucleotide sequences, and selectable markers, to form what is termed an expression cassette. The methodologies utilized in making the individual components of an expression cassette and in assembling the components are well known in the art of molecular biology (see, for example, Ausubel, F. M., et al., or Sambrook, et al.) in view of the teachings of the specification. Examples of expression cassettes useful in the present invention include the gusA reporter cassette (Girbal, L., et al. supra) and the lacZ reporter cassette (Tummala, S. B. et al. Development and characterization of a gene expression reporter system for Clostridium acetobutylicum ATCC 824, Appl. Envir. Mircobiol. 65:3793-3799, 1999).

[0083] In various embodiments, the present invention also includes expression cassettes that allow for expression of eukaryotic luciferase. In one embodiment, the luc expression cassette includes a polynucleotide encoding the luc gene product operably linked to a constitutively expressed promoter. In one embodiment, the promoter is obtained from Gram-positive bacteria. In a further embodiment, the promoter is obtained from low-GC Gram positive bacteria. In yet further embodiments, the promoter are obtained from obligate or strict anaerobe Gram positive bacteria. In various such embodiments, an expression cassette can then be introduced into a suitable vector backbone, for example as a shuttle vector. In one embodiment, the shuttle vector includes a selectable marker and two origins of replication, one for replication in Gram-negative organisms, and the other for replication in Gram-positive organisms. Appropriate promoters can be identified by any method known in the art in view of the teachings of the present specification.

[0084] In one embodiment an expression cassette comprises a bacterial lux operon with the genes arranged in either the native orientation, luxCDABE (**FIG. 2**), or in a rearranged orientation, such as luxABCDE (U.S. Pat. No. 6,737,245). One embodiment of the present invention uses a luciferase expression cassette wherein the lux operon from *P. luminescens* is operationally linked to the appropriate transcription regulatory nucleotide sequence for an enzyme in a fermentative pathway of *C. cetobutylicum* in a manner analogous to U.S. Pat. No. 6,737,245. Any expression cassettes described herein optionally contain a site for insertion of known or unknown sequence. For example, an insertion site can typically be located 5' to the luxB gene (i.e., between luxA and luxB). An expression construct can comprise the sequences of SEQ ID NO: 1 and SEQ ID NO: 3. In other embodiments, the expression construct comprises the sequences of SEQ ID NO: 1, 3, 5, 7 and 9. Sequences illustrated with a stop codon can be substituted with any equivalent stop codon (e.g., UAG ("amber"), UAA ("ochre"), and UGA ("opal" or "umber"). Furthermore, any stop codon can be used where a sequence encoding a protein is listed without a stop codon.

[0085] Expression cassettes can comprise a polynucleotide encoding luxA, and luxB gene products, wherein (a) transcription of the polynucleotide results in a polycistronic RNA encoding both gene products, and (b) polynucleotide sequences comprising Gram-positive ribosome-binding site sequences are located adjacent the 5' end

of the luxA coding sequences and adjacent the 5' end of the luxB coding sequences (e.g., SEQ ID NO: 12) is contemplated by the present invention. The expression cassette can further comprise an insertion site 5' to at least one of either the luxA or luxB coding sequences. The insertion site may, for example, further comprise a multiple-insertion site. The multiple-insertion site can be located 5' to the luxA coding sequences or 5' to the luxB coding sequences. The nucleotide sequence can further encode luxC, luxD and luxE gene products. The arrangement of the coding sequences for the lux gene products may be, for example, in the following relative order 5'-luxA-luxB-luxC-luxD-luxE-3' or CDABE (**FIG. 2**), CDEAB, CABDE, or ABCDE.

[0086] In yet another aspect, the invention includes an expression cassette comprising a polynucleotide encoding luxA, luxB, and luc gene products, wherein (a) transcription of the polynucleotide results in a polycistronic RNA encoding all three gene products, and (b) polynucleotide sequences comprising Gram-positive bacterial Shine-Dalgarno sequences are located adjacent the 5' end of the lux coding sequences, and adjacent the 5' end of the lux coding sequences. In one embodiment, the polynucleotide further encodes luxC, luxD and luxE gene products (e.g., **FIG. 2**). In another embodiment, Gram-positive bacterial Shine-Dalgarno sequences are located 5' to all of the lux coding sequences or 5' to luxA and/or luxC only. In yet other embodiments, transcription of the polynucleotide is mediated by a promoter contained in an enhancer sequence, such as Sp1, Sp5, Sp6, Sp9, Sp16 or Sp17. Enhancer sequences can be Sp16, or Sa1-Sa6, such as Sa2 or Sa4.

[0087] The expression cassette may further include a multiple-insertion site located adjacent the 5' end of the lux coding sequences (**FIG. 2**). In various embodiments, the coding sequences for luxA and luxB are from *Photorhabdus luminescens*, and are optimized for expression in a low DNA G+C content host. In one embodiment, LuxA and LuxB are encoded by SEQ ID NO: 2 and SEQ ID NO: 4, respectively. In other embodiments a lux operon is modified to include mutation of the catalytic site of luxA to enhance the enzymatic activity of the luciferase at less partial pressure of oxygen. In various embodiments of the invention lux genes (e.g., lux ABCDE) are provided in an expression construct, and are provided via an episomal vector or integrated into the host genome. The order for the various lux genes can be CABDE, ABCDE, CDABE or CDEAB. In one embodiment, the lux genes are provided in a construct as illustrated in **FIG. 2**, arranged in a CDABE fashion, where ribosome binding sites functional in desired bacteria are operatively linked to each gene (asterisks in **FIG. 2**). In one embodiment, the lux expression cassette is optimized to match the codon usage of the bacterial species. In the case of *Clostridium*, the codon usage is optimized to 60-70% A/T content (or low G/C content) and a gram-positive ribosome binding site (5'-AGGAGG-3') is added 8-10 base pairs upstream of the start codon of each gene. Restriction enzyme sites are included for the rearrangement of genes. The expression cassettes may also comprise Gram-positive bacterial Shine-Dalgarno sequences, for example, 5' to all of the lux coding sequences. Transcription of the nucleotide can be mediated by a promoter contained in an expression enhancer sequence, such as Sa1-Sa6, e.g., Sa2 or Sa4. In another group of embodiments, transcription of the polynucleotide is mediated by a promoter contained in an enhancer sequence can be Sp1, Sp5, Sp6, Sp9, Sp16 and Sp17, such as Sp16, or those disclosed in **Table 2**. In one embodiment, the coding sequences for luxA and luxB are obtained from *Photorhabdus luminescens* and comprise SEQ ID NO: 2 and SEQ ID NO: 4, respectively. In another embodiment, there are several copies of the luxCDABE operon genes.

[0088] Expression cassettes can be inserted into "shuttle vectors", plasmids that can replicate in two or more hosts. A shuttle vector to be used with gram negative and gram positive organisms requires the shuttle vector to contain an origin of replication from each class. Examples of shuttle vectors include the pAUL-A vector (Chakraborty, et al. (1992) J. Bacteriol. 174:568 574), pMK4 and pSUM series (U.S. Pat. No. 6,737,245), and pIMP1 (Mermelstein, L. D., et al. Bio/Technology 10:190-195, 1992). Other vectors are well known to those skilled in the art and are readily available from catalogs.

[0089] Numerous methods for the introduction of nucleic acid constructs of the invention into cells or protoplasts of cells are known to those of skill in the art and include, but are not limited to, the following: lipid-mediated transfer (e.g., using liposomes, including neutral and cationic lipids), direct injection (e.g., microinjection), conjugation, cell fusion, microprojectile bombardment (e.g., biolistic methods, such as DNA particle bombardment), co-precipitation (e.g., with calcium phosphate, or lithium acetate), DEAE-dextran- or polyethylene glycol-mediated transfer, viral vector-mediated transfer and electroporation.

[0090] Electroporation is one method for transforming *C. acetobutylicum*. In one variation of this method, electrocompetent *C. acetobutylicum* cells prepared from mid-logarithmic growth phase are used. Following electroporation, cells are incubated at 37° C in an appropriate broth, like 2 x YT broth while under a nitrogen atmosphere. Following a recovery period, the cells are transferred to an anaerobic glovebox, and serial dilutions are then plated on nutrient plates like 2 x YT agar plates that are supplemented with the requisite antibiotic concentration.

[0091] Transformed *C. acetobutylicum* can be selected based on a selective marker of, for example, colonies of microorganisms that containing nucleic acid constructs derived from the complete luxCDABE operon, can be identified by manual visual inspection in a darkened room or by the use of an image detection system such as one that incorporates a charge coupled device (CCD) camera, screening clones with a luminometer or through standard molecular biology techniques. Since oxygen is required for the bioluminescence reaction, plates may need to be exposed to low concentrations of oxygen in order to detect positive colonies. The expression cassettes derived from luc and luxAB require the addition of an exogenous substrate in order to produce light. In one embodiment of the present invention, the substrate is aldehyde. When administered to cells, aldehyde may be applied in the atmosphere surrounding the culture media as a vapor or directly to the culture media. In another embodiment, the selectable marker may comprise nucleic acid sequences encoding for a reporter protein, such as, for example, green fluorescent protein (GFP), DS-Red (red fluorescent protein), acetohydroxyacid synthase (AHAS), beta glucoronidase (GUS), secreted alkaline phosphatase (SEAP), beta-galactosidase, chloramphenicol acetyltransferase (CAT), horseradish peroxidase (HRP), luciferase, nopaline synthase (NOS), octopine synthase (OCS), or derivatives thereof, or any number of other reporter proteins known to one skilled in the art.

[0092] Instead of transforming an organism with a plasmid, an oxidoreductase can be integrated into a chromosome of the host. Use of chromosomal integration of the reporter construct offers several advantages over plasmid-based constructions, including greater stability, and the elimination of the use of antibiotics to maintain

selective pressure on the organisms to retain the plasmids. In general, chromosomal integration is accomplished by the use of a DNA fragment containing the desired gene upstream from an antibiotic resistance gene such as the chloramphenicol gene and a fragment of homologous DNA from the target organism. This DNA fragment can be ligated to form circles without replicons and used for transformation. For example, the pfl gene can be targeted in the case of E. coli, and short, random Sau3A fragments can be ligated in Klebsiella to promote homologous recombination. In this way, ethanologenic genes have been integrated chromosomally in E. coli. (Ohta et al. Appl. Environ. Microbiol. 57: 893-900, 1991.) Another method for chromosomal integration uses a transposable element such as a transposon that provides for the introduction of an engineered cassette.

[0093] The copy number of the oxidoreductase can be controlled by the concentration of the antibiotic used in the selection process. For example, when a low concentration of antibiotics is used for selection, clones with single copy integrations are found, albeit at very low frequency. While this may be disadvantageous for many genes, a low copy number for luciferase may be ideal given the high sensitivity of the detectors employed in light measurement. Higher level expression can be achieved in a single step by selection on plates containing much higher concentrations of antibiotic.

Volumetric Productivity

[0094] The present invention provides methods and compositions for increasing the volumetric productivity of microorganisms. The volumetric productivity for a batch fermentation is calculated by dividing the product concentration (titer, for example, the amount of product in a given volume) by the time required to reach this value. For example, in batch fermentation, the volumetric productivity is calculated by dividing the peak product concentration (fermentation titer) by the time required to reach this value. For example, a microorganism produces 200g of butanol per liter in 2 hours. The volumetric productivity would be 100g/(L*h). In a continuous fermentation at steady state the volumetric productivity is calculated by multiplying the titer by the dilution rate. For example, at a dilution rate of 2.0 (h^{-1}) and a steady state (constant) titer of 50 g/L, the volumetric productivity is 100 g/(L*h).

[0095] The product can be the total amount of solvents, for example the amount of acetone, butanol and/or ethanol produced by *Clostridium* in a given time frame, or the amount of a particular solvent or alcohol, such as the amount of butanol produced in a given time frame. The volumetric productivity may be increased by expressing oxidoreductases in a microorganism.

[0096] The increase in volumetric productivity may be determined by comparing the amount of product produced by a microorganism modified to express an oxidoreductase, to the amount produced by an unmodified microorganism. The unmodified microorganism may be a wild type strain, without introduction of any recombinant molecules into the organism, or a microorganism with a control recombinant nucleic acid introduced, such as a control plasmid. For example, a strain of *Clostridium* may be transformed by a plasmid comprising a nucleotide sequence encoding luciferase, and the amount of butanol produced is compared to a wild type strain of *Clostridium* without any recombinant nucleic acid introduced. In other embodiments, the amount of butanol produced by a

strain of *Clostridium* transformed by a plasmid comprising a nucleotide sequence encoding luciferase is compared to a strain transformed with a control plasmid not encoding luciferase (see Example 4).

[0097] Alternatively, the increase in volumetric productivity can be determined by comparing the amount of product produced by a microorganism expressing an oxidoreductase from a recombinant nucleic acid to the amount produced by a genetically modified microorganism not expressing oxidoreductase. For example, a strain of *Clostridium* may comprise recombinant nucleic acid molecules expressing luciferase and an enzyme in the solventogenesis pathway such as butanol dehydrogenase. The amount of butanol produced is compared to a strain of *Clostridium* comprising recombinant nucleic acid molecules expressing butanol dehydrogenase. The oxidoreductase may be expressed from the same or different expression cassettes as another protein or enzyme, for example, butanol dehydrogenase. For example, *Clostridium* may be transformed with two different plasmids, one with lux genes, and the other with a nucleotide sequence encoding butanol dehydrogenase. Alternatively, a single plasmid containing nucleotide sequences that comprise the lux genes as well as a nucleotide sequence encoding butanol dehydrogenase. The production of a solvent such as butanol, from either of the aforementioned strains can be compared to a *Clostridium* expressing the butanol dehydrogenase from a recombinant nucleic acid alone. Variations may be easily contemplated by one of ordinary skill in the arts. For example, more than one other nucleotide sequence encoding other genes can be expressed in a single or multiple recombinant nucleic acid molecules, along with an oxidoreductase in the same or different recombinant nucleic acid molecule, in a microorganism and compared to a microorganism expressing the same recombinant nucleic acid molecules but without oxidoreductase expression.

[0098] The addition of nucleotide sequence encoding an oxidoreductase can provide an increase of at least 10% in volumetric productivity. In some embodiments, at least 15% increase in volumetric productivity is achieved. In yet other embodiments, at least 20%, 25%, 35%, 50%, 60%, 75%, 80%, 95%, 100%, 125%, 150%, or 200% increase in volumetric productivity results. For example, the addition of lux genes to a recombinant nucleic acid can result in a greater than 100% increase in volumetric productivity, measured by butanol alone or by total solvents (acetone, butanol, and ethanol) in *Clostridium* when compared to *Clostridium* comprising a plasmid not expressing the lux genes. In one embodiment, of the invention, the expression of a nucleotide sequence encoding an oxidoreductase in a microorganism provides an increase in volumetric productivity or product titer of at least about 10% (e.g. 15%, 20%, 25%, 30%, 40%, 50%, 60%, 75%, 80%, 100% or more) as compared to a control strain not expressing the oxidoreductase, or as compared to a parent strain. In some cases, the comparison is between two or more strains cultured under the same conditions (e.g. anaerobically or microaerobically). In other cases, the comparison is between two or more strains cultured under different conditions (e.g. anaerobically or microaerobically).

[0099] In one aspect of the invention, the expression of a sequence encoding an oxidoreductase provides oxygen tolerance or resistance to a microaerobic environment. For example, where such sequences are expressed in a recombinant microorganism (e.g., in an obligate anaerobe or anaerobic bacterium), such microorganisms are able to grow in a microaerobic, low oxygen or oxygen environment. Therefore, the recombinant microorganism may exhibit an increase in volumetric productivity in the presence of oxygen due to an increase in tolerance to oxygen or

resistance to a microaerobic environment. For example a recombinant microorganism expressing a heterologous oxidoreductase may be grown with oxygen sparging at concentrations of oxygen in the sparging gas of at least about 0.1%, 0.2%, 0.3%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.75%, or about 3%, 5%, 10%, or abou 15%; whereas the parent strain may be unable to grow at one or more of these concentrations. Alternatively, a recombinant microorganism expressing a heterologous oxidoreductase may be grown in a medium containing about 0.05 ppm, 0.1ppm, 0.15ppm, 0.2ppm, 0.3ppm, 0.35ppm, 0.4ppm, 0.45 ppm, 0.5ppm, 0.55ppm, 0.6ppm, 0.7ppm, 0.75ppm, 0.8ppm, 0.9ppm, 1ppm, 2ppm, 3ppm, 5ppm, 10ppm, 15ppm, 20ppm, or about 30ppm dissolved oxygen; whereas the parent strain may be unable to grow at one or more of these concentrations. In some cases, growth in the presence of oxygen provides increased volumetric productivity, and expression of one or more recombinant heterologous oxidoreductases provides the ability of a microorganism to grow in the presence of the oxygen. In such cases, oxygen and oxidoreductase expression may both be required for increased volumetric productivity. The methods of the present invention may further be useful for transforming an obligate anaerobic microorganism (e.g. microorganisms from the following genera: *Clostridium*, *Fusobacterium*, *Peptostreptococcus*, *Bacteriodes*, *Butyrivibrio*, *Leptptrichia*, *Selenomonas*, *Succinimonas*, *Succinivibrio*, *Eubacterium*, *Lachnospira*, *Aracnia*, *Propionibacterium*, *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Treponema*, *Borrelia*, and *Campylobacter*) into an anaerobe that is resistant to a microaerobic environment.

[00100] In another aspect of the invention, the expression of an optimized recombinant heterologous sequence encoding an oxidoreductase provides tolerance to inhibitors of volumetric productivity, such as phenolic inhibitors including aromatic aldehydes including but not limited to furfural, vanillin, or hydroxymethyl furfural. In some cases, the heterologous oxidoreductase may provide for detoxification of the inhibitors (see e.g. Cho, D.H., Lee, Y.J., Um, Y., Sang, B., Kim, H.Y. Appl. Microbiol. Biotechnol. Published Online March 20, 2009).

[00101] The amount of product produced by a microorganism can be assessed from cells grown in batch mode using feeding strategies such as open-loop (non-feedback) or closed-loop (feedback). (U.S. Pat. No. 6,955,892.) The open-loop feeding strategies are typically pre-determined feed profiles for carbon/nutrient addition. Commonly used feed schedules include constant or increasing feed rates (constant, stepwise or exponential) in order to keep up with the increasing cell densities. The closed-loop feeding strategies, on the other hand, typically rely on measurements that indicate the metabolic state of the culture. The two most commonly measured online variables for fermentation are dissolved oxygen (DO) concentration and pH. With DO monitoring, a rising DO signifies a reduction of oxygen consumption that in turn is based on nutrient limitation or depletion. When the DO rises above a threshold value or the rate of change is above a threshold value, the process controller will increase the nutrient feed rate. Conversely, when the DO drops below the desired set point or the rate of change is above a threshold value, the process control will reduce the nutrient feed rate to reflect metabolic demand. Similarly, changes in culture pH or the rate of change of a culture pH can be used alone or in combination with DO measurements to adjust the rate at which nutrient feed is added to the fermentor. The alternative to the batch-fed process is the continuous process, wherein typically, fermentation broth is simultaneously removed from the fermentor and fresh nutrients and/or water is added to maintain fermentor volume and desired cell density.

[00102] The solvents produced by batch fermentation, can be isolated and used to measure the productivity. Numerous means are available for the isolation of solvents from fermentation broth including continuous extraction with solvents (U.S. Pat. No. 4,424,275 and U.S. Pat. No. 4,568, 643), the use of fluorocarbons (U.S. Pat. No. 4,777,135), the use of absorbent material (U.S. Pat. No. 4,520,104), the use of a pervaporation membrane (U.S. Pat. No. 5,755,967), and the use of a stripping gas (U.S. Pat. Appl. No. 10/945,551).

[00103] One embodiment of this invention uses a vapor compression distillation system. (U.S Pat. Nos. 4,671,856, 4,769,113, 4,869,067, 4,902,197, 4,919,592, 4,978,429, 5,597,453, and 5,968,321.) For batch fermentations of *C. acetobutylicum* the harvesting of solvents contained in the spent fermentation media first requires that the broth be centrifuged to remove cells and particulate matter. The clarified broth is then sent to the distillation system wherein the clarified broth enters a heat exchanger and is preheated by heat transfer from outgoing distilled product and waste fluid. In some embodiments, non-clarified broth may be used, such as commonly done with beer columns in ethanol production facilities. The preheated broth is degassed and fed to a plate-type evaporator/condenser which has counter-flow evaporating and condensing chambers formed alternately between stacked metal plates which are separated by gaskets. The media enters the evaporating chambers where it boils. Heated vapor leaving the evaporating chambers passes through a mesh that removes mist, and is then pressurized by a low pressure compressor. The pressurized vapor is delivered to the condenser chambers, where it condenses as the distilled product, giving up heat to broth in the boiling chambers, and is then discharged from the system. Unvaporized broth containing dissolved solids is likewise collected and discharged from the system.

[00104] In some embodiments, with continuous cultures of *C. acetobutylicum,* the fermentation broth drawn is off the fermentor and centrifuged to concentrate cells and particulate matter. Alternately, the fermentation broth can be filtered, for example, by tangential flow filtration. The concentrated cells and matter can be added back to the fermentor if desired to increase cell density or for further fermentation of partially fermented substrate. Alternately, the clarified or filtered fermentation broth can be added back to the fermentor if it contains soluble fermentable substrate. When it is desired to harvest solvents from the media numerous strategies are available including storage of the clarified or filtered fermentation broth until a reasonable quantity is present to initiate a distillation run or the the continuous feeding of clarified or filtered fermentation broth to the vapor distillation system. In some embodiments, the fermentation broth can be processed without centrifugation or filtration.

[00105] Fermentation broth composed of certain butanol containing solvent mixtures may undergo spontaneous phase separate based on specific gravity. The use of a float level indicator can be used to assist in separating the butanol containing solvent layer from the remaining aqueous fraction.

Products

[00106] The products produced by the microorganism of the present invention may be commercially valuable. The products may be used, for example, as biofuel (e.g. alcohols, fatty acids or alkanes), flavoring or odorants for food and perfumes (e.g. esters and terpenes), or for pharmacological use (e.g. antibodies, enzymes), such as for the treatment or prevention of medical conditions.

[00107] The microorganism can produce products such as organic compounds with increased volumetric productivity. For example, the microorganism can produce hydrocarbons, alcohols, esters, ethers, ketones, and their derivatives. Hydrocarbons such as alkanes, alkenes, alkynes and their derivatives can be produced, including compounds such as terpenes, terpenoids, alkaloids, can be produced in the microorganisms. The compounds may be used as biofuels. Compounds that may be produced include methane, ethane, propane, butane, pentane, hexane, heptane, octane and others, as well as their derivatives such as their respective alcohols, esters or ethers. Alcohols include methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol and others. Fatty acids and esters, including C_8 to C_{34} fatty acids, also can be products of microbial biosynthesis. Ketones such as acetone, acetophenone, methyl ethyl ketone, acetoacetate, beta-hydroxbutyrate and others can also be produced. They can be used to make plastics, fibers, drugs, paint, and other commercially valuable goods.

[00108] Proteins that may be produced include recombinant proteins, or endogenous proteins. Recombinant proteins may be enzymes, antibodies, peptides, ligands, receptors, or any other cellular signaling molecule. The proteins may be useful as a pharmaceutical agent. They may also be useful in diagnostics or in research applications.

[00109] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

EXAMPLES

Example 1: Making Lux Expression Vectors

[00110] In various embodiments, a luciferase expression cassettes is inserted into a vector backbone, e.g., a shuttle vector, such as pMK4 (Sullivan, M., et al., (1984) Gene 29:21-26), pDL289 (Buckley, N., et al., (1995) J. Bacteriol 177:5028-5034) and the pSUM series (Ainsa, J. A., et al., (1996) Gene 176:23-26). Typically, the shuttle vectors include the following: (1) a Gram-positive origin of replication; (2) a Gram-negative origin of replication (3) polylinkers; and (4) a polynucleotide encoding a selectable marker (e.g., ampicillin, chloramphenicol).

[00111] The expression cassettes can be constructed utilizing methodologies known in the art of molecular biology (see, for example, Ausubel, F. M., et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., Media, Pa. (1995), or Sambrook, et al.) in view of the teachings of the specification. Typically, expression cassettes are assembled from polynucleotides encoding lux or luc genes by operably linking these polynucleotides to

suitable transcriptional (e.g., a promoter) and translational regulatory elements (e.g., Gram-positive Shine-Dalgarno sequences). Short, random nucleotide sequences, selectable markers, and the like can also be introduced into the expression cassettes at suitable positions.

[00112] For example, a vector backbone can comprise luxABCDE (in different order as desired) (e.g., SEQ ID NO: 12), with a promoter insertion site upstream of SEQ ID NO: 12. Furthermore, the backbone comprises selectable markers (e.g., Amp, CAT), one or more origin of replication and multiple cloning sites (MCSs). Therefore, various polynucleotide sequences can be mobilized into and out of the vector as desired, by obtaining polynucleotide sequences comprising a restriction site present in the MCSs.

[00113] One method of obtaining polynucleotides, suitable regulatory sequences and short, random nucleotide sequences is PCR. General procedures for PCR as taught in MacPherson et al., PCR: A Practical Approach, (IRL Press at Oxford University Press, (1991)). PCR conditions for each application reaction may be empirically determined. A number of parameters influence the success of a reaction. Among these parameters are annealing temperature and time, extension time, Mg2+ and ATP concentration, pH, and the relative concentration of primers, templates and deoxyribonucleotides. Exemplary primers are described below in Example 1. After amplification, the resulting fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination.

[00114] Another method for obtaining polynucleotides, for example, short, random nucleotide sequences, is by enzymatic digestion. As described below in the Examples, short DNA sequences generated by digestion of DNA from a suitable bacterium with, e.g., a blunt-cutting four-nucleotide recognition restriction enzyme such as AluI, HaeIII and Sau3AI, were ligated with the modified lux cassette.

[00115] Polynucleotides are inserted into vector genomes using methods known in the art. For example, insert and vector DNA can be contacted, under suitable conditions, with a restriction enzyme to create complementary or blunt ends on each molecule that can pair with each other and be joined with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of a polynucleotide. These synthetic linkers can contain nucleic acid sequences that correspond to a particular restriction site in the vector DNA. Other means are known and available in the art as well.

Example 2: Optimization of Lux Operon

[00116] Optimize codon to ~60-70% A/T for a light-emitting protein to be expressed in a low DNA G + C content bacteria (e.g., *Clostridium*).

[00117] Furthermore, a gram positive Shine-Dalgarno sequence (AGGAGG) 5-10 nucleotides upstream of start codon is inserted for each gene. In addition, a promoter is selected for expression in the host cell , e.g., thlA promoter (thiolase gene) (SEQ ID NO: 13), or bdhB promoter (SEQ. ID: 14).

[00118] Engineer restriction sites before promoter, after promoter/before lux genes, after lux genes:

[00119] Engineer restriction sites for luxAB

[00120] Restriction Enzyme Design: various restriction enzyme recognition sequences are known and can be used in the nucleic acid constructs of the invention. Exemplary sequences used include: SphI, BamHI - thl promoter - BamHI, PspOMI, XhoI - luxCD - PstI, NotI - luxAB - NheI - luxE - NsiI, SalI, XbaI, SphI

[00121] Thus optimization of a low A/T sequence to a high A/T sequence, the desired sequence (e.g., luxABCDE) are designed *in silico*, substituting codons enriched in AT residues and known to more efficiently expressed in low-GC bacteria. Furthermore, Shine-Dalgarno sequences (e.g., selected from a gene also highly expressed in the target host cell) are added upstream to each gene to facilitate efficient initiation of translation. Synthetic DNAs of 80 to 120 nt can be designed based on the top strand of each gene. Each oligonucleotide can be analyzed using a ssDNA folding program. Sequences that exhibit significant intramolecular base pairing can be modified to reduce or eliminate base pairing (e.g., by making a corresponding shift in a neighboring oligonucleotide or making codon changes consistent with translation in the target host cell. For example, modifications to preclude intramolecular base pairing can be made at the 5' or 3' ends in a 15 to 25 stretch at the ends. Subsequently, bridging oligonucleotides complementary to the ends of the top strand oligonucleotide are designed to produce a set of oligonucleotides encoding the top strand of the lux genes. Sequences for recombination and/or restriction endonuclease recognition can be inserted using PCR primers. (e.g., EXAMPLE 3).

EXAMPLE 3: Assembly and Amplification

[00122] Each lux gene is assembled in reactions containing the top strand and corresponding bridging oligonucleotides (both at 25 mM) in Pfu reaction buffer and 5 mM dNTPs. Amplification of each gene follows : an initial denaturation step was carried out at 948C followed by 55 cycles of denaturation (948C for 1 min), annealing (458C for 40 s) and extension (728C for 1 min). Following this, 15 mM of the appropriate amplifications primers were used to amplify each lux gene in reactions primed with 5 ml of assembly reaction—conditions for PCR were identical except that 25 cycles of amplification were used instead of 55 and a terminal extension incubation of 4 min, at 728C was added. All reactions were carried out using a robo-cycler 96 (Stratagene) with zero ramp time between temperatures. Fragments are visualized by agarose gel electrophoresis, excised and purified using QIAEX II gel extraction.

[00123] Amplified fragments can be cloned into the vector of choice using compatible restriction sites. Alternatively, optimized sequences can be submitted to commercial companies for preparation (e.g., Codon Devices, PerkinElmer, BioSynthesis, Inc.).

EXAMPLE 4: Increased Volumetric Productivity of Butanol

[00124] The genes required for bioluminescence, luxCDABE, from the soil bacterium, *Photorhabdus luminescence*, were optimized for expression in *Clostridium* by increasing the AT codon usage and adding gram-positive ribosome binding sites. The bdhB promoter was used (SEQ ID NO: 14). The *Clostridium* Co-0124 strain was transformed with the optimized luciferase genes contained on the plasmid pJIR418, creating the strain Co-5878.

A control strain, Co-0115, was generated by transforming Co-0124 with pJIR418 plasmid without the luciferase genes. The strains were compared in 15L batch fermentors with P2 medium containing 4% glucose with erythromycin added at 50ug/mL to maintain selection for each plasmid. Samples were taken from the fermentations periodically to monitor OD_{600} and for analysis of solvent product accumulation by HPLC. A MAX300-LO online mass spectrophotometer (Extra CMS, Pittsburg, PA) measured the concentration of H_2, CO_2, O_2, butanol, acetone, and ethanol in the fermentative off gas from each fermentor.

[00125] Performance was determined by calculating the productivity and yield and comparing each strain. In batch fermentation, volumetric productivity is calculated by dividing the peak product concentration (titer) by the time required to reach this value. The performance of Co-5878 was compared to Co-0115. The amount of butanol, and total solvents (acetone, butanol, and ethanol) formed was measured by a Waters HPLC (Milford, MA) equipped with a refractive index detector. The Co-5878 strain had a butanol volumetric productivity of 0.27g/(liter*hour) compared to 0.12g/(L*h) for the Co-0115 strain. The total solvent productivity was 0.41 versus 0.18g/(L*h) for Co-5878 versus Co-0115. As a result the addition of the lux genes resulted in a greater than 100% increase in volumetric productivity, measured by both butanol alone and total solvents. The parent strain, Co-0124, demonstrated butanol and solvent volumetric productivity of 0.28 and 0.43g/(L*h).

[00126] Yield, the conversion of input glucose substrate to solvent product, was comparable for all strains, 0.21, 0.24 and 0.21 g butanol/glucose, and 0.32, 0.34, and 0.34 g total solvents (acetone, butanol, and ethanol)/g glucose for Co-5878, Co-0115, and Co-0124, respectively. The slightly higher yield of Co-0115 may have been artificially inflated since the charged glucose was not completely consumed despite the much longer fermentation time.

SEQUENCE LISTING

SEQ ID NO: 1 Optimized luxA Nucleotide Sequence

ATGAAATTTGGATTATTTTTTCTTAATTTTATAAATAGTACAACTATTCAAGAACAGTCAATAGCAAGA
ATGCAGGAGATTACAGAGTATGTTGATAAGCTAAATTTTGAGCAGATTCTTGTATGTGAAAATCATTTT
TCAGATAATGGTGTTGTAGGTGCTCCTTTAACTGTTAGTGGTTTTTTATTAGGACTTACAGAAAAAATT
AAGATAGGTTCATTAAATCATGTAATTACTACACATCATCCAGTTAGAATAGCAGAAGAGGCTTGCCT
TTTAGATCAACTTTCTGAAGGAAGATTTATATTAGGTTTTAGTGATTGTGAAAGAAAAGATGAGATGC
ACTTTTTTAATAGACCTGAACAATATCAACAACAACTTTTTGAAGAGTGCTATGATATTATAAATGACG
CATTAACTACAGGATATTGTAATCCAAATGGAGATTTTTATAATTTTCCTAAAATTTCAGTAAATCCAC
ATGCTTATACTCAGAATGGTCCTAGAAAGTATGTTACAGCAACTTCTTGTCATGTAGTTGAATGGGCAG
CTAAGAAGGGTATACCATTAATTTTTAAATGGGATGATAGTAATGAAGTAAAACATGAGTATGCTAAG
AGATATCAAGCAATAGCTGGTGAATATGGAGTTGATCTTGCAGAAATTGATCATCAATTAATGATATT

AGTTAATTATTCAGAGGATTCTGAAAAAGCTAAGGAAGAGACAAGAGCATTTATAAGTGATTATATTT
TAGCTATGCACCCTAATGAAAATTTTGAAAAAAAACTTGAGGAAATAATAACTGAAAATTCAGTTGGT
GATTATATGGAGTGCACAACTGCTGCAAAACTTGCAATGGAAAAATGTGGAGCTAAAGGTATTCTTTT
ATCTTTTGAAAGTATGTCAGATTTTACACATCAGATTAATGCAATAGATATAGTAAATGATAATATTAA
GAAATATCATATGTAA

SEQ ID NO: 2 Optimized LuxA Amino Acid Sequence

MKFGNFLLTYQPPQFSQTEVMKRLVKLGRISEECGFDTVWLLEHHFTEFGLLGNPYVAAAYLLGATKKLN
VGTAAIVLPTAHPVRQLEEVNLLDQMSKGRFRFGICRGLYNKDFRVFGTDMNNSRALMECWYKLIRNGM
TEGYMEADNEHIKFHKVKVLPTAYSQGGAPIYVVAESASTTEWAAQHGLPMILSWIINTNEKKAQIELYNE
VAQEYGHDIHNIDHCLSYITSVDHDSMKAKEICRNFLGHWYDSYVNATTIFDDSDKTKGYDFNKGQWRDF
VLKGHKNTNRRVDYSYEINPVGTPQECIDIIQTDIDATGISNICCGFEANGTVDEIISSMKLFQSDVMPFLKEK
QRSLLY

SEQ ID NO: 3 Optimized luxB Nucleotide Sequence

ATGAAATTTGGATTATTTTTTCTTAATTTTATAAATAGTACAACTATTCAAGAACAGTCAATAGCAAGA
ATGCAGGAGATTACAGAGTATGTTGATAAGCTAAATTTTGAGCAGATTCTTGTATGTGAAAATCATTTT
TCAGATAATGGTGTTGTAGGTGCTCCTTTAACTGTTAGTGGTTTTTTATTAGGACTTACAGAAAAAATT
AAGATAGGTTCATTAAATCATGTAATTACTACACATCATCCAGTTAGAATAGCAGAAGAGGCTTGCCT
TTTAGATCAACTTTCTGAAGGAAGATTTATATTAGGTTTTAGTGATTGTGAAAGAAAAGATGAGATGC
ACTTTTTTAATAGACCTGAACAATATCAACAACAACTTTTTGAAGAGTGCTATGATATTATAAATGACG
CATTAACTACAGGATATTGTAATCCAAATGGAGATTTTTATAATTTTCCTAAAATTTCAGTAAATCCAC
ATGCTTATACTCAGAATGGTCCTAGAAAGTATGTTACAGCAACTTCTTGTCATGTAGTTGAATGGGCAG
CTAAGAAGGGTATACCATTAATTTTTAAATGGGATGATAGTAATGAAGTAAAACATGAGTATGCTAAG
AGATATCAAGCAATAGCTGGTGAATATGGAGTTGATCTTGCAGAAATTGATCATCAATTAATGATATT
AGTTAATTATTCAGAGGATTCTGAAAAAGCTAAGGAAGAGACAAGAGCATTTATAAGTGATTATATTT
TAGCTATGCACCCTAATGAAAATTTTGAAAAAAAACTTGAGGAAATAATAACTGAAAATTCAGTTGGT
GATTATATGGAGTGCACAACTGCTGCAAAACTTGCAATGGAAAAATGTGGAGCTAAAGGTATTCTTTT
ATCTTTTGAAAGTATGTCAGATTTTACACATCAGATTAATGCAATAGATATAGTAAATGATAATATTAA
GAAATATCATATGTAA

SEQ ID NO: 4 Optimized Lux B Amino Acid Sequence

MKFGLFFLNFINSTTIQEQSIARMQEITEYVDKLNFEQILVCENHFSDNGVVGAPLTVSGFLLGLTEKIKIGSL
NHVITTHHPVRIAEEACLLDQLSEGRFILGFSDCERKDEMHFFNRPEQYQQQLFEECYDIINDALTTGYCNP
NGDFYNFPKISVNPHAYTQNGPRKYVTATSCHVVEWAAKKGIPLIFKWDDSNEVKHEYAKRYQAIAGEYG
VDLAEIDHQLMILVNYSEDSEKAKEETRAFISDYILAMHPNENFEKKLEEIITENSVGDYMECTTAAKLAME
KCGAKGILLSFESMSDFTHQINAIDIVNDNIKKYHM

SEQ ID NO: 5 Optimized luxC Nucleotide Sequence

ATGAATAAAAAGATATCATTTATTATAAATGGAAGAGTTGAAATATTTCCTGAGTCAGATGATTTAGT
ACAATCTATAAATTTTGGTGATAATTCTGTTCATCTTCCAGTACTTAATGATTCACAGGTTAAGAATAT
TATAGATTATAATGAGAATAATGAGCTTCAGCTTCATAATATTATAAATTTTCTTTATACAGTAGGACA
GAGATGGAAGAATGAGGAGTATAGCAGAAGAAGAACTTATATAAGAGATCTTAAGAGATATATGGGT
TATAGTGAGGAAATGGCAAAATTAGAAGCTAATTGGATTTCAATGATATTATGTTCTAAGGGAGGTTT
ATATGATTTAGTTAAAAATGAATTAGGAAGTAGACATATTATGGATGAATGGTTACCTCAAGATGAAT
CATATATAAGAGCATTTCCAAAAGGTAAAAGTGTACATCTTTTAACAGGAAATGTTCCTTTAAGTGGA
GTACTTTCAATTTTAAGAGCTATACTTACTAAAAATCAGTGCATTATAAAGACATCTAGTACTGATCCA
TTTACAGCAAATGCTTTAGCACTTAGTTTTATAGATGTTGATCCTCATCATCCAGTAACTAGATCTTTA
AGTGTTGTATATTGGCAACATCAAGGTGATATTTCACTTGCTAAAGAAATAATGCAACATGCAGATGT
TGTAGTTGCTTGGGGAGGTGAAGATGCAATTAATTGGGCTGTAAAGCACGCACCTCCAGATATAGATG
TTATGAAATTTGGACCTAAAAAGTCTTTTTGTATTATAGATAATCCAGTAGATTTAGTTAGTGCTGCAA
CAGGTGCTGCACATGATGTATGCTTTTATGATCAGCAGGCTTGTTTTTCAACTCAAAATATATATTATA
TGGGATCACATTATGAAGAATTTAAACTTGCATTAATTGAAAAACTTAATTTATATGCTCATATACTTC
CAAATACAAAGAAAGATTTTGATGAAAAGGCAGCTTATAGTTTAGTTCAGAAAGAATGTTTATTTGCA
GGACTTAAAGTAGAAGTTGATGTACATCAAAGATGGATGGTTATTGAATCAAATGCTGGTGTAGAATT
AAATCAGCCACTTGGAAGATGCGTTTATTTACATCATGTAGATAATATAGAGCAAATTTTACCTTATGT
TAGAAAGAATAAAACTCAAACAATATCTGTATTTCCATGGGAAGCAGCTTTAAAGTATAGAGATCTTT
TAGCACTTAAAGGTGCTGAAAGAATTGTTGAGGCAGGAATGAATAATATATTTAGAGTAGGTGGTGCT
CATGATGGAATGAGGCCTTTACAGAGACTTGTTACTTATATAAGTCATGAAAGACCAAGTCATTATAC
AGCAAAAGATGTAGCTGTAGAGATTGAGCAAACTAGATTTTTAGAAGAAGATAAGTTTTTAGTATTTG
TTCCTTAA

SEQ ID NO: 6 LuxC Amino Acid Sequence

MNKKISFIINGRVEIFPESDDLVQSINFGDNSVHLPVLNDSQVKNIIDYNENNELQLHNIINFLYTVGQRWKN
EEYSRRRTYIRDLKRYMGYSEEMAKLEANWISMILCSKGGLYDLVKNELGSRHIMDEWLPQDESYIRAFPK
GKSVHLLTGNVPLSGVLSILRAILTKNQCIIKTSSTDPFTANALALSFIDVDPHHPVTRSLSVVYWQHQGDIS
LAKEIMQHADVVVAWGGEDAINWAVKHAPPDIDVMKFGPKKSFCIIDNPVDLVSAATGAAHDVCFYDQQ
ACFSTQNIYYMGSHYEEFKLALIEKLNLYAHILPNTKKDFDEKAAYSLVQKECLFAGLKVEVDVHQRWMV
IESNAGVELNQPLGRCVYLHHVDNIEQILPYVRKNKTQTISVFPWEAALKYRDLLALKGAERIVEAGMNNI
FRVGGAHDGMRPLQRLVTYISHERPSHYTAKDVAVEIEQTRFLEEDKFLVFVP

SEQ ID NO: 7 Optimized luxD Nucleotide Sequence

ATGGAAAATAAAAGTAGATATAAGACAATAGATCATGTTATTTGTGTAGAGGAGAATAGAAAGATAC
ATGTTTGGGAAACTTTACCTAAAGAAAATTCACCAAAAAGAAAAAATACACTTATTATAGCATCTGGA
TTTGCTAGAAGAATGGATCATTTTGCTGGTTTAGCTGAATATTTATCTCAAAATGGATTTCATGTAATT
AGATATGATTCATTACATCATGTTGGTTTAAGTTCAGGAACTATAGATGAATTTACAATGTCAATTGGT
AAGCAGAGTTTACTTGCAGTAGTTGATTGGTTAAATACTAGAAAAATAAATAATCTTGGAATGTTAGC
TAGTTCATTATCTGCAAGAATAGCTTATGCAAGTCTTTCAGAGATTAATGTATCTTTTCTTATAACAGC
TGTTGGTGTAGTTAATTTAAGATATACTTTAGAAAGAGCACTTGGATTTGATTATCTTAGCCTTCCTATT
GATGAATTACCAGATAATCTTGATTTTGAGGGACATAAGTTAGGTGCTGAAGTATTTGCAAGAGATTG
CTTTGATTCAGGATGGGAAGATCTTACATCTACTATAAATAGTATGATGCACTTAGATATTCCTTTTAT
AGCTTTTACAGCAAATAATGATGATTGGGTTAAACAAGATGAGGTAATTACTCTTCTTTCTAGTATAAG
AAGTCATCAGTGTAAAATATATTCACTTTTAGGTTCTAGTCATGATCTTGGAGAAAATTTAGTTGTATT
AAGAAATTTTTATCAATCAGTTACAAAGGCTGCAATTGCTATGGATAATGGTTGCCTTGATATAGATGT
AGATATTATAGAACCATCTTTTGAGCATTTAACTATTGCAGCTGTTAATGAAAGAAGAATGAAAATAG
AAATAGAGAATCAAGTAATTAGTTTAAGTTAA

SEQ ID NO: 8 Optimized Lux D Amino Acid Sequence

MENKSRYKTIDHVICVEENRKIHVWETLPKENSPKRKNTLIIASGFARRMDHFAGLAEYLSQNGFHVIRYDS
LHHVGLSSGTIDEFTMSIGKQSLLAVVDWLNTRKINNLGMLASSLSARIAYASLSEINVSFLITAVGVVNLR
YTLERALGFDYLSLPIDELPDNLDFEGHKLGAEVFARDCFDSGWEDLTSTINSMMHLDIPFIAFTANNDDW
VKQDEVITLLSSIRSHQCKIYSLLGSSHDLGENLVVLRNFYQSVTKAAIAMDNGCLDIDVDIIEPSFEHLTIAA
VNERRMKIEIENQVISLS

SEQ ID NO: 9 Optimized luxE Nucleotide Sequence

ATGACATCTTATGTTGATAAACAAGAAATAACTGCAAGTTCAGAGATTGATGATTTAATATTTAGTTCA
GATCCTCTTGTATGGTCTTATGATGAACAGGAAAAGATTAGAAAAAAGTTAGTTCTTGATGCTTTTAGA
CATCATTATAAACATTGTCAAGAGTATAGACATTATTGCCAGGCACATAAAGTAGATGATAATATAAC
AGAAATTGATGATATACCAGTTTTTCCTACTTCAGTATTTAAGTTTACAAGATTACTTACTTCAAATGA
AAATGAGATTGAATCATGGTTTACAAGTTCAGGAACTAATGGTTTAAAATCTCAAGTTCCAAGAGATA
GACTTAGTATAGAAAGACTTTTAGGATCAGTATCTTATGGTATGAAGTATATAGGAAGTTGGTTTGATC
ATCAAATGGAGTTAGTTAATCTTGGTCCTGATAGATTTAATGCTCATAATATTTGGTTTAAATATGTAA
TGTCACTTGTAGAACTTTTATATCCAACAAGTTTTACTGTAACAGAAGAGCATATAGATTTTGTTCAGA
CTTTAAATAGTCTTGAAAGAATTAAACATCAAGGAAAGGATATATGTTTAATTGGTTCACCTTATTTTA
TATATCTTTTATGCAGATATATGAAAGATAAGAATATTTCTTTTAGTGGAGATAAATCACTTTATATAA
TAACTGGAGGTGGATGGAAATCTTATGAAAAGGAGAGTTTAAAAAGAAATGATTTTAATCATCTTTTA
TTTGATACTTTTAATCTTTCAAATATTAATCAAATAAGAGATATTTTTAATCAGGTAGAATTAAATACA
TGTTTTTTTGAGGATGAAATGCAAAGAAAACATGTTCCACCTTGGGTATATGCAAGGGCTCTTGATCCA
GAAACTTTAAAGCCTGTTCCAGATGGTATGCCTGGACTTATGTCTTATATGGATGCTTCAAGTACTAGT
TATCCAGCTTTTATAGTAACTGATGATATTGGTATAATAAGTAGAGAATATGGACAATATCCTGGAGTT
TTAGTTGAGATTTTAAGAAGAGTTAATACAAGAAAACAGAAGGGTTGTGCACTTTCATTAACTGAGGC
TTTTGGATCTTGA

SEQ ID NO: 10 Optimized LuxE Amino Acid Sequence

MTSYVDKQEITASSEIDDLIFSSDPLVWSYDEQEKIRKKLVLDAFRHHYKHCQEYRHYCQAHKVDDNITEI
DDIPVFPTSVFKFTRLLTSNENEIESWFTSSGTNGLKSQVPRDRLSIERLLGSVSYGMKYIGSWFDHQMELV
NLGPDRFNAHNIWFKYVMSLVELLYPTSFTVTEEHIDFVQTLNSLERIKHQGKDICLIGSPYFIYLLCRYMKD
KNISFSGDKSLYIITGGGWKSYEKESLKRNDFNHLLFDTFNLSNINQIRDIFNQVELNTCFFEDEMQRKHVPP
WVYARALDPETLKPVPDGMPGLMSYMDASSTSYPAFIVTDDIGIISREYGQYPGVLVEILRRVNTRKQKGC
ALSLTEAFGS (**SEQ ID NO: 10)**

SEQ ID NO: 11 Sequence for LuxCDABE from Accession Number M90092

gaattctcag actcaaatag aacaggattc taaagactta agagcagctg tagatcgtgattttagtacg atagagccaa cattgagaaa ttatggggca
acggaagcac aacttgaagacgccagagcc aaaatacaca agcttaacca agaacagagg ttatacaaat gacagttaatacagaggcac taataaacag
cctaggcaag tcctaccaag aaatttttga tgaagggctaattccttata ggaataagcc aagtggttct cctggggtgc ctaatatttg tattgacatg
gtgaaagagg ggatttttt gtcgtttgaa cggaatagta aaatattaaa cgaaattact ttaagattgc ttagagacga taaagctttg tttatatttc caaatgaatt

gccatcaccg ttgaagcatt ctatggatag gggatgggtt agagaaaatt taggtgatct gattaaatca ataccaccga gacaaatttt aaaaaggcag tttggttgga aagatctata tcgttttacg

gatgaaatca gtatgcagat ttcttatgat ttacgtgaac aggttaattc agtgactttc ttgcttacat cagacgtgag ttggtaattt aatatatata cccttcatcc ttcaagttgc

tgctttgttg gctgctttct ctcaccccag tcacatagtt atctatgctc ctggggattc gttcacttgc cgccgcgctg caacttgaaa tctattgggt atatgctatt ggtaattatg gaaaattgcc tgatttatat ataacttaac ttgtaaacca gataataatt tacatgaata ttatcacgta taaaaaaatt gcgattcttt taatttgaaa tagttcaatt taattgaaac tttttattaa caaatcttgt tgatgtgaaa attttcgttt gctattttaa cagatattgttaaacggaga aggcagcatg ttgatgattc actcagccag actgacagtt ttaagcggaa aattgcagag tatgatcgca ttctgataaa ggttacaggt cactcgcaac cagaatttca tctttgtata ttttgttttg ttatttacgt tgcagcaaga caaaaatага agaaacaaatatttatacaa cccgtttgca agagggttaa acagcaattt aagttgaaat tgccctatta aatggatggc aaatatgaac aaaaaaattt cattcattat taacggtcga gttgaaatat ttcctgaaag tgatgattta gtgcaatcca ttaattttgg tgataatagt gttcatttgc cagtattgaa tgattctcaa gtaaaaaaca ttattgatta taatgaaaat aatgaattgc aattgcataa cattatcaac tttctctata cggtagggca acgatggaaa aatgaagaat attcaagacg caggacatat attcgtgatc taaaaagata tatgggatat tcagaagaaa tggctaagct agaggccaac tggatatcta tgattttgtg ctctaaaggt ggcctttatg atcttgtaaa aaatgaactt ggttctcgcc atattatgga tgaatggcta cctcaggatg aaagttatat tagagctttt ccgaaaggaa aatccgtaca tctgttgacg ggtaatgtgc

cattatctgg tgtgctgtct atattgcgtg caattttaac aaagaatcaa tgcattataa aaacctcatc aactgatcct tttaccgcta atgcattagc gctaagtttt atcgatgtgg

accctcatca tccggtaacg cgttctttgt cagtcgtata ttggcaacat caaggcgata tatcactcgc aaaagagatt atgcaacatg cggatgtcgt tgttgcttgg ggaggggaag atgcgattaa ttgggctgta aagcatgcac cacccgatat tgacgtgatg aagtttggtc ctaaaaagag tttttgtatt attgataacc ctgttgattt agtatccgca gctacagggg cggctcatga tgtttgtttt tacgatcagc aagcttgttt ttccacccaa aatatatatt acatgggaag tcattatgaa gagtttaagc tagcgttgat agaaaaattg aacttatatg cgcatatatt accaaacacc aaaaaagatt ttgatgaaaa ggcggcctat tccttagttc aaaaagaatg tttatttgct ggattaaaag tagaggttga tgttcatcag cgctggatgg ttattgagtc aaatgcgggt gtagaactaa atcaaccact tggcagatgt gtgtatcttc atcacgtcga taatattgag caaatattgc cttatgtgcg aaaaaataaa acgcaaacca tatctgtttt tccttgggag gccgcgctta agtatcgaga cttattagca ttaaaaggtg cagaaaggat tgtagaagca ggaatgaata atatatttcg ggttggtggt gctcatgatg gaatgagacc tttacaacga ttggtgacat atatttccca tgaaagacca tcccactata ctgctaaaga tgttgcggtc gaaatagaac agactcgatt cctggaagaa gataagttcc tggtatttgt cccataatag gtaaaagaat atggaaaata aatccagata taaaaccatc gaccatgtta tttgtgttga agaaaataga aaaattcatg tctgggagac gctgccaaaa gaaaatagtc caaagagaaa aaataccctt attattgcgt cgggttttgc ccgcaggatg gatcattttg ccggtctggc agagtatttg tcgcagaatg gatttcatgt gatccgctatgattctcttc accacgttgg attgagttca gggacaattg atgaatttac aatgtccata ggaaaacaga gtttattagc agtggttgat tggttaaata cacgaaaaat aaataacctcggtatgctgg cttcaagctt atctgcgcgg atagcttatg caagtctatc tgaaattaat gtctcgtttt taattaccgc agtcggtgtg gttaacttaa gatatactct cgaaagagct ttaggatttg attatctcag cttacctatt gatgaattgc cagataattt agattttgaaggtcataaat tgggtgctga ggtttttgcg agagattgct ttgattctgg ctgggaagat ttaacttcta caattaatag tatgatgcat cttgatatac cgtttattgc ttttactgca aataatgacg attgggtaaa gcaagatgaa gttattacat tactatcaag catccgtagt catcaatgta agatatattc tttactagga agctcacatg atttgggtga gaacttagtg gtcctgcgca atttttatca atcggttacg aaagccgcta tcgcgatgga taatggttgt ctggatattg atgtcgatat tattgagccg tcattcgaac atttaaccat tgcggcagtc aatgaacgcc gaatgaaaat tgagattgaa aatcaagtga tttcgctgtc ttaaaaccta taccaataga tttcgagttg cagcgcggcg gcaagtgaac gcattcccag gagcatagat aactctgtga ctggggtgcg tgaaagcagc caacaaagca gcaacttgaa ggatgaaggg tatattggga tagatagtta actctatcac tcaaatagaa atataaggac tctctatgaa atttggaaac tttttgctta cataccaacc cccccaattt tctcaaacag aggtaatgaa acggttggtt

aaattaggtc gcatctctga ggaatgcggt tttgataccg tatggttact tgagcatcat ttcacggagt ttggtttgct tggtaaccct tatgtggctg ctgcttattt acttggcgca accaagaaat tgaatgtagg gactgcggct attgttctcc ccaccgctca tccagttcgc cagcttgaag aggtgaattt gttggatcaa atgtcaaaag gacgatttcg atttggtatt tgtcgggggc tttacaataa agattttcgc gtatttggca cagatatgaa

taacagtcgt gccttaatgg agtgttggta taagttgata cgaaatggaa tgactgaggg atatatggaa gctgacaacg aacatattaa gttccataag gtaaaagtgc tgccgacggc atatagtcaa ggtggtgcac ctatttatgt cgttgctgaa tccgcttcca cgactgaatg ggccgctcaa catggtttac cgatgatttt aagttggatt ataaatacta acgaaaagaa agcacaaatt gagctttata acgaggtcgc tcaagaatat ggacacgata ttcataatat cgaccattgc ttatcatata taacatcggt agaccatgac tcaatgaaag cgaaagaaat ttgccggaat tttctggggc attggtatga ttcctatgtt aatgccacaa ccatttttga tgattcagac aaaacaaagg gctatgattt caataaagga caatggcgcg actttgtctt aaaaggacat aaaaatacta atcgtcgcgt tgattacagt tacgaaatca atccggtggg

aaccccgcag gaatgtattg atataattca aacagacatt gacgccacag gaatatcaaa tatttgttgt gggtttgaag ctaatggaac agtagatgaa attatctctt ccatgaagct cttccagtct gatgtaatgc cgtttcttaa agaaaaacaa cgttcgctat tatattagct aaggaaaatg aaatgaaatt tggcttgttc ttccttaact ttatcaattc aacaactatt caagagcaaa gtatagctcg catgcaggaa ataacagaat atgtcgacaa attgaatttt gagcagattt tggtgtgtga aaatcatttt tcagataatg gtgttgtcgg cgctcctttg actgtttctg gtttttttact tggcctaaca gaaaaaatta aaattggttc attgaatcat gtcattacaa ctcatcatcc tgtccgcata gcggaagaag cgtgcttatt ggatcagtta agcgaaggaa gatttatttt aggatttagt gattgcgaga gaaaggatga aatgcatttt ttcaatcgcc ctgaacaata ccagcagcaa ttatttgaag aatgctatga cattattaac gatgctttaa caacaggcta ttgtaatcca aatggcgatt tttataattt ccccaaaata tccgtgaatc cccatgctta tacgcaaaac gggcctcgga aatatgtaac agcaacaagt tgtcatgttg ttgagtgggc tgctaaaaaa ggcattcctc taatctttaa gtgggatgat tccaatgaag ttaaacatga atatgcgaaa agatatcaag ccatagcagg tgaatatggt gttgacctgg cagagataga tcatcagtta atgatattgg ttaactatag tgaagacagt gagaaagcta aagaggaaac gcgtgcattt ataagtgatt atattcttgc aatgcaccct aatgaaaatt tcgaaaagaa acttgaagaa ataatcacag aaaactccgt cggagattat atggaatgta caactgcggc taaattggca atggagaaat gtggtgcaaa aggtatatta ttgtcctttg aatcaatgag tgattttaca catcaaataa acgcaattga tattgtcaat gataatatta aaaagtatca catgtaatat accctatgga tttcaaggtg catcgcgacg gcaagggagc gaatccccgg gagcatatac ccaatagatt tcaagttgca gtgcggcggc aagtgaacgc atccccagga gcatagataa ctatgtgact ggggtaagtg aacgcagcca

acaaagcagc agcttgaaag atgaagggta tagataacga tgtgaccggg gtgcgtgaac gcagccaaca aagaggcaac ttgaaagata acgggtataa aagggtatag cagtcactct gccatatcct ttaatattag ctgccgaggt aaaacaggta tgacttcata tgttgataaa caagaaatca cagcaagttc agaaattgat gatttgattt tttcgagtga tccattagtc tggtcttacg acgaacagga aaagattaga aaaaaacttg tgcttgatgc gtttcgtcat cactataaac attgtcaaga ataccgtcac tactgtcagg cacataaagt agatgacaat attacggaaa ttgatgatat acctgtattc ccaacatcag tgtttaagtt tactcgctta ttaacttcta atgagaacga aattgaaagt tggtttacca gtagtggcac aaatggctta aaaagtcagg taccacgtga cagactaagt attgagaggc tcttaggctc tgtaagttat

ggtatgaaat atattggtag ttggttcgat catcaaatgg aattggtcaa cctgggacca gatagattta atgctcataa tatttggttt aaatatgtta tgagcttggt agagttatta

tatcctacgt cattcaccgt aacagaagaa cacatagatt tcgttcagac attaaatagt cttgagcgaa taaaacatca agggaaagat atttgtctta ttggttcgcc atactttatt tatttgctct gccgttatat gaaagataaa aatatctcat tttctggaga taaaagtctt tatattataa cgggggggagg ctggaaaagt tacgaaaaag aatctttgaa gcgtaatgat ttcaatcatc ttttattcga cactttcaac ctcagtaata ttaaccagat ccgtgatata tttaatcaag ttgaactcaa cacttgtttc tttgaggatg aaatgcaacg taaacatgtt ccgccgtggg tatatgcgcg agcacttgat cctgaaacat tgaaaccggt acctgatggg atgcctggtt tgatgagtta tatggatgca tcatcaacga gttatccggc atttattgtt accgatgata tcggaataat tagcagagaa tatggtcaat atcctggtgt attggttgaa attttacgtc gcgttaatac gaggaaacaa aaaggttgtg ctttaagctt aactgaagca tttggtagtt gatagtttct

ttggaaagag gagcagtcaa aggctcattt gttcaatgct tttgcgaaac gttttgtcga actctaggcg aaggttctcg actttccccg catcaggggt atatacaagt aaaaaagctc agggggtaaa cctgagcttg ggatgttgat ttttaagtat

gagatacatg ggcggattta aataacggag tcagtttgga aatatcaacg gtcttttctg ctttatcgag gctataagtt tcttgcagtt ttaaccacaa ccgcggagag ctgccaagta cttgtgacag ttttattgcc atctctggcg tgactgctgc tttacacgat actaaacgtt gaaccgtaga gggagcaaca ttcaatgccc gcgctaagtt cacgaattc

SEQ ID NO: 12 Optimized Lux the CDABE genes are separated by gram-positive ribosome binding sites

aattcgaattctcagactcaaatagaacaggattctaaagacttaagagcagctgtagatcgtgattttagtacgatagagccaacattgagaaattatggggcaacggaag cacaacttgaagacgccagagccaaaatacacaagcttaaccaagaacagaggttatacaaatgacagttaatacagaggcactaataaacagcctaggcaagtcctac caagaaatttttgatgaagggctaattccttataggaataagccaagtggttctcctggggtgcctaatatttgtattgacatggtgaaagaggggatttttttgtcgtttgaacg gaatagtaaaatattaaacgaaattactttaagattgcttagagacgataaagctttgtttatatttccaaatgaattgccatcaccgttgaagcattctatggataggggatggg ttagagaaaatttaggtgatctgattaaatcaataccaccgagacaaatttttaaaaaggcagtttggttggaaagatctatatcgttttacggatgaaatcagtatgcagatttct tatgatttacgtgaacaggttaattcagtgactttcttgcttacatcagacgtgagttggtaatttaatatatataccccttcatccttcaagttgctgctttgttggctgctttctctcac cccagtcacatagttatctatgctcctggggattcgttcacttgccgccgcgctgcaacttgaaatctattgggtatatgctattggtaattatggaaaattgcctgatttatatat aacttaacttgtaaaccagataataatttacatgaatattatcacgtataaaaaaaattgcgattcttttaatttgaaatagttcaatttaattgaaacttttattaacaaatcttgttgat gtgaaaattttcgtttgctattttaacagatattgttaaacggagaaggcagcatgttgatgattcactcagccagactgacagttttaagcggaaaattgcagagtatgatcgc attctgataaaggttacaggtcactcgcaaccagaatttcatctttgtatattttgttttgttatttacgttgcagcaagacaaaaatagaagaaacaaatatttatacaacccgttt gcaagagggttaaacagcaatttaagttgaaattgccctattaaatggagcatgcggatcctcgacttttaacaaaatatattgataaaaataataggatccgggcccctcg agaggaggatggcaaatatgaataaaagatatcatttattataaatggaagagttgaaatatttcctgagtcagatgatttagtacaatctataaattttggtgataattctgttc atcttccagtacttaatgattcacaggttaagaatattatagattataatgagaataatgagcttcagcttcataatattataaattttctttatacagtaggacagagatggaaga atgaggagtatagcagaagaagaacttatataagagatcttaagagatatatgggttatagtgaggaaatggcaaaattagaagctaattggatttcaatgatattatgttcta agggaggtttatatgatttagttaaaaatgaattaggaagtagacatattatggatgaatggttacctcaagatgaatcatatataagagcatttccaaaaggtaaaagtgtac atcttttaacaggaaatgttcctttaagtggagtactttcaattttaagagctatacttactaaaaatcagtgcattataaagacatctagtactgatccatttacagcaaatgcttta gcacttagttttatagatgttgatcctcatcatccagtaactagatctttaagtgttgtatattggcaacatcaaggtgatatttcacttgctaaagaaataatgcaacatgcagat gttgtagttgcttggggaggtgaagatgcaattaattgggctgtaaagcacgcacctccagatatagatgttatgaaatttggacctaaaaagtctttttgtattatagataatcc agtagatttagttagtgctgcaacaggtgctgcacatgatgtatgcttttatgatcagcaggcttgtttttcaactcaaaatatatattatatgggatcacattatgaagaatttaaa cttgcattaattgaaaaacttaatttatatgctcatatacttccaaatacaaagaaagattttgatgaaaaggcagcttatagtttagttcagaaagaatgtttatttgcaggactta aagtagaagttgatgtacatcaaagatggatggttattgaatcaaatgctggtgtagaattaaatcagccacttggaagatgcgtttatttacatcatgtagataatatagagca aattttaccttatgttagaaagaataaaactcaaacaatatctgtatttccatgggaagcagctttaaagtatagagatcttttagcacttaaaggtgctgaaagaattgttgagg caggaatgaataatatatttagagtaggtggtgctcatgatggaatgaggcctttacagagacttgttacttatataagtcatgaaagaccaagtcattatacagcaaaagatg tagctgtagagattgagcaaactagatttttagaagaagataagtttttagtatttgttccttaataggaggtaaaagaatatggaaaataaaagtagatataagacaatagatc atgttatttgtgtagaggagaatagaaagatacatgtttgggaaacttacctaaagaaaattcaccaaaaagaaaaaatacacttattatagcatctggatttgctagaagaat ggatcattttgctggtttagctgaatatttatctcaaaatggatttcatgtaattagatatgattcattacatcatgttggtttaagttcaggaactatagatgaatttacaatgtcaatt ggtaagcagagtttacttgcagtagttgattggttaaatactagaaaaataaataatcttggaatgttagctagttcattatctgcaagaatagcttatgcaagtctttcagagatt aatgtatcttttcttataacagctgttggtgtagttaatttaagatatactttagaaagagcacttggatttgattatcttagccttcctattgatgaattaccagataatcttgattttg agggacataagttaggtgctgaagtatttgcaagagattgctttgattcaggatgggaagatcttacatctactataaatagtatgatgcacttagatattccttttatagctttta

cagcaaataatgatgattgggttaaacaagatgaggtaattactcttctttctagtataagaagtcatcagtgtaaaatatattcacttttaggttctagtcatgatcttggagaaa
atttagttgtattaagaaatttttatcaatcagttacaaaggctgcaattgctatggataatggttgccttgatatagatgtagatattatagaaccatcttttgagcatttaactattg
cagctgttaatgaaagaagaatgaaaatagaaatagagaatcaagtaattagtttaagttaaaacctataccaatagatttcgagttgcagcgcggcggcaagtgaacgca
ttcccaggagcatagataactctgtgactggggtgcgtgaaagcagccaacaaagcagcaacttgaaggatgaagggtatattgggatagatagttaactctatcactcaa
atagaaatatactgcaggcggccgcaggaggactctctatgaaatttggaaatttttacttacatatcaacctccacagtttagtcaaactgaagttatgaagagattagtaa
aacttggtagaatatcagaggaatgtggatttgatacagtttggttacttgaacatcattttactgagtttggtcttttaggaaatccttatgtagcagctgcatatttacttggtgct
acaaagaaattaaatgtaggtacagcagctattgttttacctacagcacatcctgttagacagttagaagaagtaaatcttttagatcaaatgtctaaaggtagatttagatttgg
aatatgcagaggattatataataaggattttagagttttggtactgatatgaataatagtagggctcttatggagtgttggtataaattaattagaaatggaatgacagaaggtt
atatggaagcagataatgagcatataaagtttcataaagtaaaagtacttccaactgcttattcacagggaggtgcacctatttatgtagttgctgaatctgcaagtacaactg
aatgggctgcacagcatggattaccaatgatactttcatggattataaatacaaatgagaagaaagctcaaatagaattatataatgaagtagcacaagagtatggacatga
tattcataatatagatcattgcctttcttatattactagtgttgatcatgattcaatgaaagctaaagaaatatgtagaaatttttaggtcattggtatgattcttatgtaaatgcaaca
actatttttgatgatagtgataaaacaaagggatatgattttaataaaggtcagtggagagattttgttcttaaaggacataagaatactaatagaagagtagattattcatatga
aataaatcctgttggaactccacaagagtgtattgatataatacaaactgatattgatgctacaggaatatctaatatttgctgtggatttgaagcaaatggtactgtagatgaa
ataattagtagtatgaagttatttcagtctgatgttatgccttttcttaaggagaaacaaagaagtttactttattagctaaggaggaaaatgaaatgaaatttggattatttttttctta
attttataaatagtacaactattcaagaacagtcaatagcaagaatgcaggagattacagagtatgttgataagctaaatttgagcagattcttgtatgtgaaaatcatttttca
gataatggtgttgtaggtgctcctttaactgttagtggttttttattaggacttacagaaaaaattaagataggttcattaaatcatgtaattactacacatcatccagttagaatag
cagaagaggcttgccttttagatcaactttctgaaggaagatttatattaggttttagtgattgtgaaagaaaagatgagatgcacttttttaatagacctgaacaatatcaacaa
caacttttgaagagtgctatgatattataaatgacgcattaactacaggatattgtaatccaaatggagatttttataattttcctaaaatttcagtaaatccacatgcttatactca
gaatggtcctagaaagtatgttacagcaacttcttgtcatgtagttgaatgggcagctaagaagggtataccattaatttttaaatgggatgatagtaatgaagtaaaacatga
gtatgctaagagatatcaagcaatagctggtgaatatggagttgatcttgcagaaattgatcatcaattaatgatattagttaattattcagaggattctgaaaaagctaaggaa
gagacaagagcatttataagtgattatattttagctatgcaccctaatgaaaattttgaaaaaaaacttgaggaaataataactgaaaattcagttggtgattatatggagtgca
caactgctgcaaaacttgcaatggaaaaatgtggagctaaaggtattcttttatcttttgaaagtatgtcagattttacacatcagattaatgcaatagatatagtaaatgataata
ttaagaaatatcatatgtaatataccctatggatttcaaggtgcatcgcgacggcaagggagcgaatccccgggagcatatacccaatagatttcaagttgcagtgcggcg
gcaagtgaacgcatccccaggagcatagataactatgtgactggggtaagtgaacgcagccaacaaagcagcagcttgaaagatgaagggtatagataacgatgtgac
cggggtgcgtgaacgcagccaacaaagaggcaacttgaaagataacgggtataaaagggtatagcagtcactctgccatatccttaatattagctgccggctagcagg
aggtaaaacaggtatgacatcttatgttgataaacaagaaataactgcaagttcagagattgatgatttaatatttagttcagatcctcttgtatggtcttatgatgaacaggaaa
agattagaaaaaagttagttcttgatgcttttagacatcattataaacattgtcaagagtatagacattattgccaggcacataaagtagatgataatataacagaaattgatgat
ataccagtttttcctacttcagtatttaagtttacaagattacttacttcaaatgaaaatgagattgaatcatggtttacaagttcaggaactaatggtttaaaatctcaagttccaa
gagatagacttagtatagaaagacttttaggatcagtatcttatggtatgaagtatataggaagttggtttgatcatcaaatggagttagttaatcttggtcctgatagatttaatg
ctcataatatttggtttaaatatgtaatgtcacttgtagaacttttatatccaacaagttttactgtaacagaagagcatatagattttgttcagactttaaatagtcttgaaagaatta
aacatcaaggaaaggatatatgtttaattggttcaccttattttatatatcttttatgcagatatatgaaagataagaatatttcttttagtggagataaatcactttatataataactg
gaggtggatggaaatcttatgaaaaggagagtttaaaaagaaatgattttaatcatcttttatttgatacttttaatctttcaaatattaatcaaataagagatatttttaatcaggta
gaattaaatacatgttttttgaggatgaaatgcaaagaaaacatgttccaccttgggtatatgcaagggctcttgatccagaaactttaaagcctgttccagatggtatgcctg
gacttatgtcttatatggatgcttcaagtactagttatccagcttttatagtaactgatgatattggtataataagtagagaatatggacaatatcctggagttttagttgagatttta
agaagagttaatacaagaaaacagaagggttgtgcactttcattaactgaggcttttggatcttgaatgcatgtcgactctagagcatgctagtttctttggaaagaggagca
gtcaaaggctcatttgttcaatgcttttgcgaaacgttttgtcgaactctaggcgaaggttctcgactttccccgcatcaggggtatatacaagtaaaaaagctcaggggta
aacctgagcttgggatgttgatttttaagtatgagatacatgggcggatttaaataacggagtcagtttggaaatatcaacggtcttttctgctttatcgaggctataagtttcttg
cagttttaaccacaaccgcggagagctgccaagtacttgtgacagttttattgccatctctggcgtgactgctgctttacacgatactaaacgttgaaccgtagagggagca
acattcaatgcccgcgctaagttcacgaattc

SEQ ID NO: 13 Sequence for thiolase A promoter

TAGAATGAAGTTTCTTATGCACAAGTATTTTTTATTACATTAATATAGTTAAAATATAAACTTATGTATTTATGCTAA
AACATGATTTTAAGGGGGTTAGCATATGCATAAGTTTAATTTTTTTGTTAAAAAATATTAAACTTTGTGTTTTTTTTA
ACAAAATATATTGATAAAAATAATAATAGTGGGTATAATTAAGTTGTTAGAGAAAACGTATAAATTAGGGATAAACT
ATGGAACTTATGAAATAGATTGAAATGGTTTATCTGTTACCCCGTATCAAAATTT

SEQ ID NO: 14 Sequence for bdhB promoter

TAGAAACTGTAGAGGTATTTTTATAATTTAAAAGATGTTAAAGAGTGAGGAGTAATTTTGTTCTAACGCCTCACTCT
TTTCATTTTATGATTAAATGTATGCTGATTTACGCTAACTTAAATCCTAAATAATAACCTAATGTTAATATTTTGTAA
CAAATGGATAAAAGCGTAAAAATATTATTGTAATAATTTTAAGTAGGTTTAAAATATATATAATGTAGAAGCATTCC
TACATTATATTATTTAAATAATAATCTAAACAGGAGGGGTTAAA

EXAMPLE 4: Increased Volumetric Productivity in the Presence of Oxygen

[00127] Volumetric Productivity of strains Co-0115 and Co-5878 are compared in 15L batch fermentors with P2 medium containing 4% glucose with erythromycin added at 50ug/mL to maintain selection for each plasmid under 3 different sparging regimes: sparging with N_2 gas comprising 0% O_2, sparging with N_2 gas comprising 0.5% O_2, sparging with N_2 gas comprising 1% O_2, and sparging with N_2 gas comprising 2% O_2. Samples are taken from the fermentations periodically to monitor OD_{600} and for analysis of solvent product accumulation by HPLC. A MAX300-LO online mass spectrophotometer (Extra CMS, Pittsburg, PA) measures the concentration of H_2, CO_2, O_2, butanol, acetone, and ethanol in the fermentative off gas from each fermentor.

[00128] Performance is determined by calculating the productivity and yield and comparing each strain. In batch fermentation, volumetric productivity is calculated by dividing the peak product concentration (titer) by the time required to reach this value. The performance of Co-5878 is compared to Co-0115. The amount of butanol, and total solvents (acetone, butanol, and ethanol) formed is measured by a Waters HPLC (Milford, MA) equipped with a refractive index detector. The Co-5878 strain exhibits a tolerance to oxygen concentrations in the sparging gas of at least about 2%, corresponding to about 0.1 to about 0.5 ppm dissolved O_2.

[00129] As shown in FIG. 3, the lux expressing strain Co-5878 exhibits a 16% greater volumetric productivity when fermented in a bioreactor with sparging gas comprising 1% oxygen relative to fermentation of the same strain with no added oxygen. As also shown in FIG. 3, the lux expressing strain Co-5878 exhibits a 146% greater volumetric productivity when fermented in a bioreactor with sparging gas comprising 1% oxygen relative to fermentation of the control strain Co-0115 without oxygen.

[00130] As shown in FIG. 4, the lux expressing strain Co-5878 exhibits a 25% greater selectivity in production of butanol over production of acetone when fermented in a bioreactor with sparging gas comprising 1% oxygen

relative to fermentation of the same strain with no added oxygen. As also shown in FIG. 4, the lux expressing strain Co-5878 exhibits a 15% greater maximum butanol concentration in the fermentation medium when fermented in a bioreactor with sparging gas comprising 1% oxygen relative to fermentation of the same strain with no added oxygen.

CLAIMS

WHAT IS CLAIMED IS:

1. A method comprising:

(a) transforming an anaerobic microorganism with a heterologous recombinant nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase to produce a recombinant anaerobic microorganism; and

(b) culturing said recombinant anaerobic microorganism under microaerobic fermentative conditions to produce a fermentation product.

2. The method of claim 1 wherein the anaerobic microorganism is an obligate anaerobe.

3. The method of claim 1 wherein the anaerobic microorganism is an obligate anaerobe, and the anaerobic recombinant microorganism is an anaerobe resistant to a microaerobic environment.

4. The method of claim 1 wherein the anaerobic microorganism is from the genus *Clostridium*.

5. The method of claim 1 wherein said oxidoreductase is an oxygenase.

6. The method of claim 1 wherein said oxidoreductase is luciferase.

7. The method of claim 1 wherein said transcriptional regulatory sequence comprises a constitutive promoter.

8. The method of claim 1 wherein expression of said nucleotide sequence is independent of expression of a gene or set of genes in a biosynthetic pathway for production of the fermentation product.

9. The method of claim 1 wherein said nucleotide sequence has an A/T content of at least 50%.

10. The method of claim 1, wherein said fermentation product is a solvent.

11. The method of claim 1, wherein said fermentation product is an organic acid.

12. The method of claim 10, wherein said solvent is an alcohol.

13. The method of claim 10, wherein said solvent is a ketone.

14. The method of claim 11, wherein said organic acid is a carboxylic acid.

15. The method of claim 11, wherein said organic acid is selected from the group consisting of acetate, butyrate, isobutyrate, isopropionate, propionate, lactate, citrate, and aminovalerate.

16. The method of claim 12, wherein said alcohol is selected from the group consisting of butanol, isobutanol, ethanol, methanol, propanol, and isopropanol.

17. The method of claim 13, wherein said ketone is selected from the group consisting of acetone, and butanone.

18. The method of claim 1, wherein the conditions comprise sparging with gas comprising at least 0.1% O_2.

19. The method of claim 1, wherein the microaerobic fermentative conditions comprise at least about 0.5% dissolved oxygen.

20. The method of claim 1, wherein the microaerobic fermentative conditions comprise a dissolved oxygen content of at least about 0.1ppm.

21. The method of claim 1 wherein volumetric productivity, product titer, oxygen-tolerance, or phenolic growth inhibitor tolerance is increased in the recombinant anaerobic microorganism as compared to the anaerobic microorganism.

22. The method of claim 21, wherein the volumetric productivity of butanol is increased by at least 10% as compared to the anaerobic microorganism.

23. The method of claim 21, wherein the titer of butanol is increased by at least 10% as compared to the anaerobic microorganism.

24. The method of claim 21, wherein the oxygen-tolerance is increased by at least 10% as compared to the anaerobic microorganism.

25. The method of claim 21, wherein the tolerance of phenolic growth inhibitors is increased by at least 10% as compared to the anaerobic microorganism.

26. The method of claim 21, wherein volumetric productivity of butanol is increased by at least 10% relative to culturing the recombinant anaerobic microorganism anaerobic ally.

27. The method of claim 21, wherein volumetric productivity of butanol is increased by at least 50% relative to culturing a control anaerobic microorganism anaerobic ally.

28. The method of claim 21, wherein fermentation product titer is increased by at least 10% relative to culturing the recombinant anaerobic microorganism anaerobic ally.

29. The method of claim 21, wherein the culture conditions comprise at least about 0.1g/L phenolic growth inhibitor.

30. The method of claim 21, wherein the phenolic growth inhibitor is selected from the group consisting of furfural, hydroxymethyl furfural, vanillin, *p*-coumarate, ferulic acid, 4-hydroxybenzoate, vanillic acid, and syringaldehyde.

31. The method of claim 1, wherein the method further comprises continuous culture.

32. The method of claim 1, wherein said method alters production of two fermentation products, wherein the ratio of the two fermentation products is at least about 10% higher or lower in the recombinant anaerobic microorganism compared to the anaerobic microorganism.

33. The method of claim 32, wherein the two fermentation products are solvents.

34. The method of claim 33, wherein the solvents are alcohols.

35. The method of claim 34, wherein the two alcohols are selected from the group consisting of butanol, isobutanol, ethanol, methanol, propanol, and isopropanol.

36. The method of claim 33, wherein the solvent are an alcohol and a ketone.

37. The method of claim 36, wherein said alcohol is butanol, isobutanol, ethanol, methanol, propanol, or isopropanol, and said ketone is acetone, or butanone.

38. The method of claim 33, wherein the two solvents are organic acids.

39. The method of claim 38, wherein the organic acids are selected from the group consisting of acetate, butyrate, isobutyrate, isopropionate, propionate, lactate, citrate, and aminovalerate.

40. A method for making fuel comprising:

(a) culturing a recombinant anaerobic bacteria in a bioreactor under microaerobic conditions; and

(b) collecting a fuel product from said bioreactor.

41. The method of claim 40, wherein said recombinant anaerobic bacteria is a gram-positive bacteria.

42. The method of claim 40, wherein said recombinant anaerobic bacteria is resistant to a microaerobic environment.

43. The method of claim 40, wherein said recombinant anaerobic bacteria is from the genus *Clostridium*.

44. The method of claim 40, wherein said recombinant anaerobic bacteria comprises a heterologous nucleotide sequence encoding an oxidoreductase.

45. The method of claim 44, wherein said oxidoreductase is a luciferase.

46. The method of claim 44, wherein said oxidoreductase is heterologous.

47. The method of claim 40, wherein said fuel is selected from the group consisting of ethanol, butanol, isopropanol, and methanol.

48. The method of claim 44, wherein the culturing a recombinant anaerobic bacteria in a bioreactor under microaerobic conditions produces said fuel at a rate of greater than 0.15 g/L/h.

49. The method of claim 44, wherein the culturing a recombinant anaerobic bacteria in a bioreactor under microaerobic conditions produces said fuel at a rate that is at least 10% greater than a recombinant anaerobic bacteria that does not comprise a heterologous nucleotide sequence encoding an oxidoreductase.

50. The method of claim 40, wherein the conditions comprise sparging with gas comprising at least 0.1% O_2.

51. The method of claim 40, wherein the microaerobic conditions comprise at least about 0.5% dissolved oxygen.

52. The method of claim 40, wherein the microaerobic conditions comprise a dissolved oxygen content of at least about 0.1ppm.

53. A bioreactor comprising a fermentation product-producing recombinant anaerobic microorganism, oxygen, and a fermentation product.

54. The bioreactor of claim 53, wherein said oxygen comprises dissolved oxygen present at concentration of at least about 0.1ppm.

55. The bioreactor of claim 53, wherein said fermentation product-producing recombinant anaerobic microorganism comprises a heterologous recombinant nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase.

56. The bioreactor of claim 53, wherein said fermentation product is a solvent.

57. The bioreactor of claim 56, wherein said solvent is an alcohol.

58. The bioreactor of claim 57, wherein said alcohol is selected from the group consisting of butanol, isobutanol, ethanol, methanol, propanol, and isopropanol.

59. The bioreactor of claim 56, wherein said solvent is a ketone.

60. The bioreactor of claim 59, wherein said ketone is selected from the group consisting of acetone and butanone.

61. The bioreactor of claim 53, wherein said fermentation product is an organic acid.

62. The bioreactor of claim 61, wherein said organic acid is selected from the group consisting of acetate, butyrate, isobutyrate, isopropionate, propionate, lactate, citrate, and aminovalerate.

63. The bioreactor of claim 53, wherein the recombinant anaerobic microorganism is an obligate anaerobe.

64. The bioreactor of claim 53, wherein the microorganism is from the genus *Clostridium*.

65. The bioreactor of claim 53, wherein said oxidoreductase is an oxygenase.

66. The bioreactor of claim 53, wherein said oxidoreductase is luciferase.

67. A bioreactor comprising:

(a) transformed Gram-positive bacteria; and

(b) culture conditions that permit said bacteria to produce butanol at a rate of greater than 0.1 g/L/hr.

68. The bioreactor of claim 67, wherein the culture conditions permit said bacteria to produce a fermentation product at a rate 10 % greater than the non-transformed gram-positive bacteria.

69. The bioreactor of claim 67, wherein the bacteria is from the genus *Clostridium*.

70. The bioreactor of claim 67, wherein said transformed Gram-positive bacteria comprises a heterologous recombinant nucleic acid molecule comprising a nucleotide sequence encoding an oxidoreductase.

71. The bioreactor of claim 67, further comprising oxygen, wherein the oxygen is at a dissolved oxygen content of at least 0.1ppm.

72. A method for converting an obligate anaerobic microorganism to an anaerobic microorganism resistant to a microaerobic environment comprising:

(a) transforming said organism with a polynucleotide sequence encoding luciferase; and

(b) growing said transformed anaerobic microorganism in a microaerobic environment or greater amounts of oxygen.

73. A strict anaerobe of a genus selected from the group consisting of *Clostridium*, *Fusobacterium*, *Peptostreptococcus*, *Bacteriodes*, *Butyrivibrio*, *Leptptrichia*, *Selenomonas*, *Succinimonas*, *Succinivibrio*, *Eubacterium*, *Lachnospira*, *Aracnia*, *Propionibacterium*, *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Treponema*, *Borrelia*, and *Campylobacter*, wherein said strict anaerobe is transformed with a heterologous nucleotide sequence encoding an oxidoreductase.

74. A method for making fuel comprising:

(a) transforming a bacteria with a recombinant heterologous nucleic acid comprising a nucleotide sequence encoding for an oxidoreductase;

(b) culturing the transformed bacteria under conditions suitable for biosynthesis of a fuel; and

(c) collecting the fuel.

75. The method of claim 74, wherein the step of culturing does not include monitoring of the level of oxidoreductase expression or activity.

76. The method of claim 74, wherein the oxidoreductase is not a luciferase.

77. The method of claim 74, wherein the oxidoreductase is not selected from the group consisting of *lux* and *luc*.

78. The method of claim 74, wherein the step of collecting provides collecting at least 10% more fuel than is provided by culturing bacteria that are not transformed with a heterologous oxidoreductase.

79. The method of claim 74, wherein the fuel is butanol, methanol, ethanol, or isopropanol.

80. The method of claim 79, wherein the fuel is butanol.

FIGURE 1

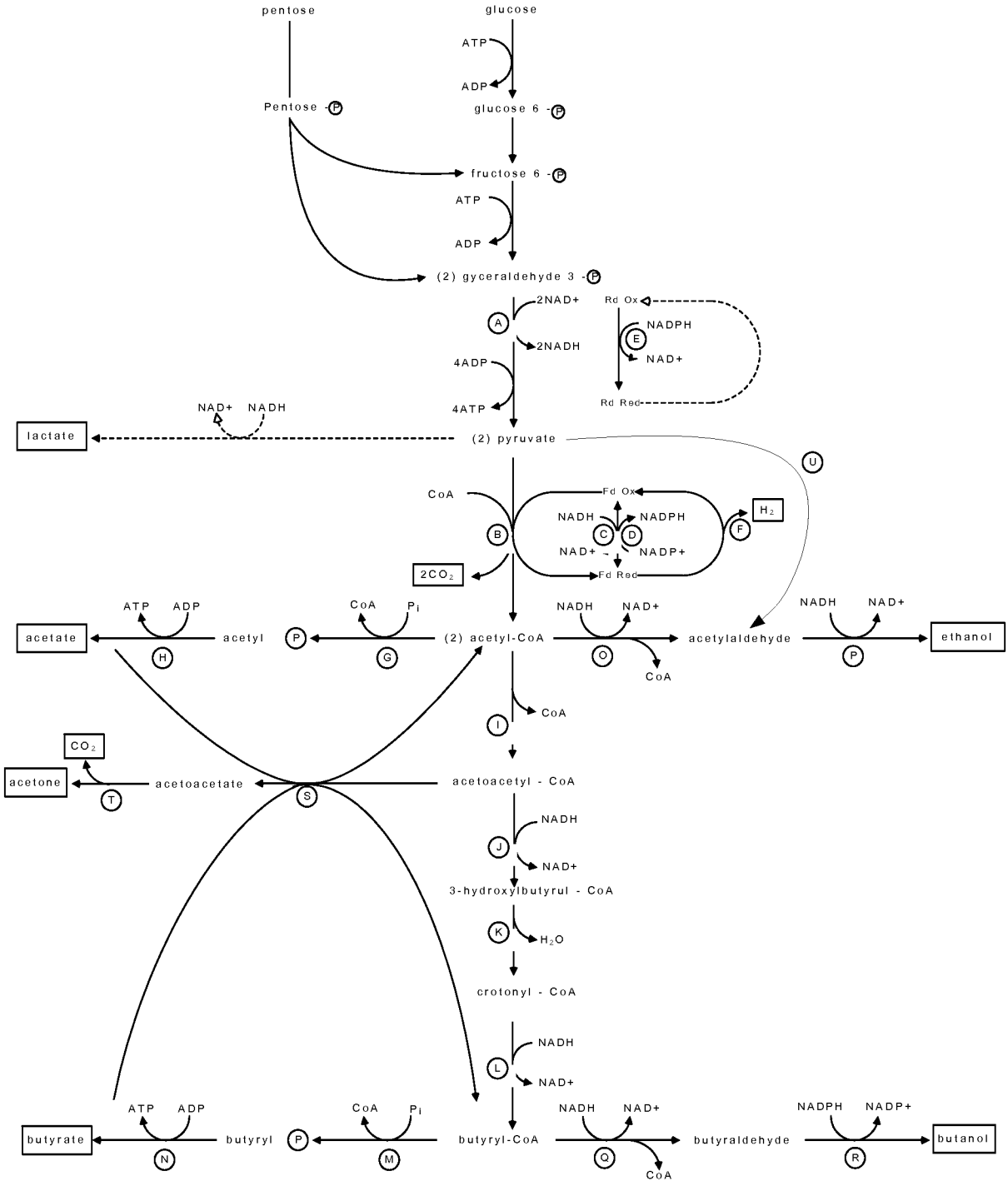

pentose
glucose
ATP
ADP
Pentose -P
glucose 6 -P
fructose 6 -P
ATP
ADP
(2) gyceraldehyde 3 -P
2NAD+
2NADH
A
Rd Ox
NADPH
E
NAD+
Rd Red
4ADP
4ATP
NAD+ NADH
lactate
(2) pyruvate
U
CoA
Fd Ox
NADH
NADPH
H2
B
C
D
F
NAD+
NADP+
Fd Red
2CO2
ATP ADP
CoA Pi
NADH NAD+
NADH NAD+
acetate
acetyl
P
(2) acetyl-CoA
acetylaldehyde
ethanol
H
G
O
P
CoA
I
CO2
acetone
acetoacetate
acetoacetyl - CoA
T
S
NADH
J
NAD+
3-hydroxylbutyrul - CoA
K
H2O
crotonyl - CoA
NADH
L
NAD+
ATP ADP
CoA Pi
NADH NAD+
NADPH NADP+
butyrate
butyryl
P
butyryl-CoA
butyraldehyde
butanol
N
M
Q
R
CoA

FIGURE 2

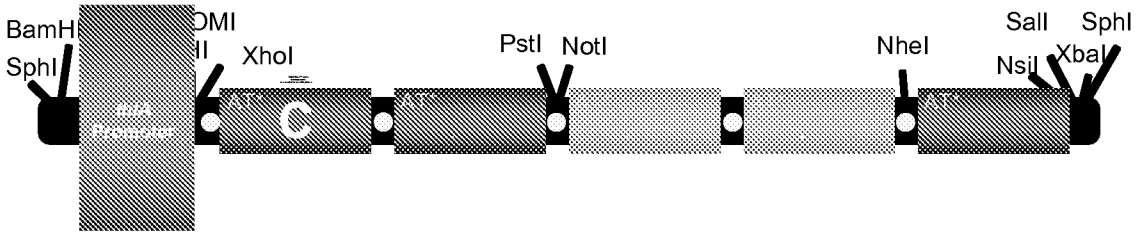


FIGURE 3

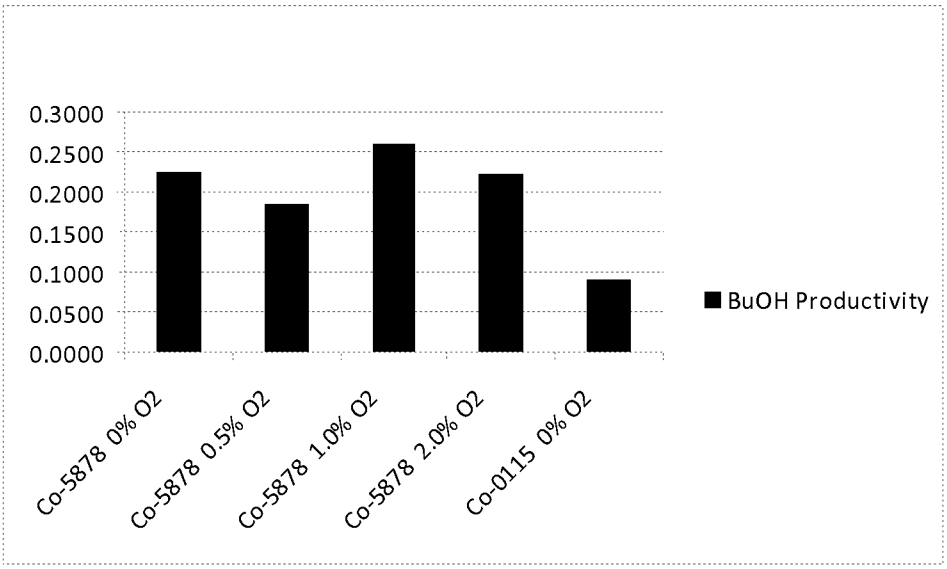
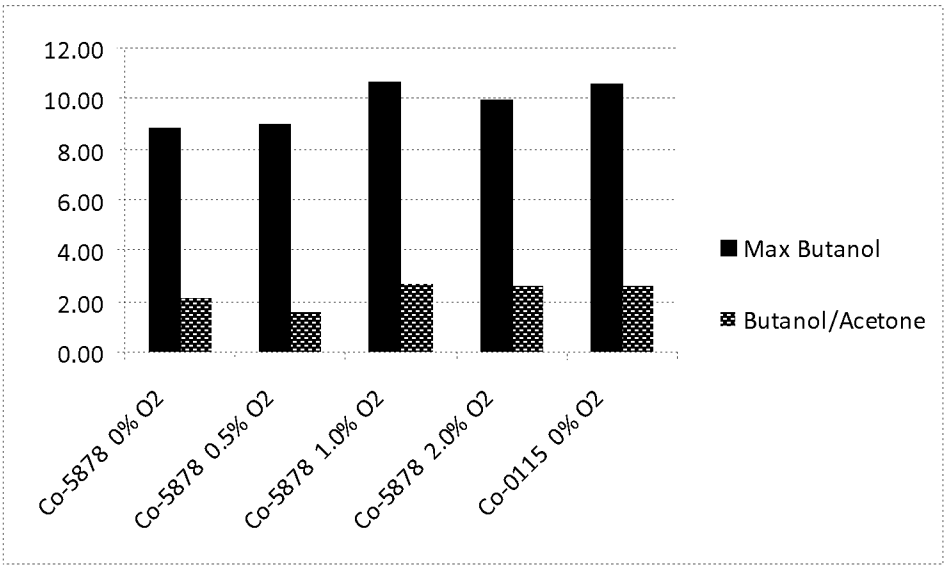

0.3000
0.2500
0.2000
0.1500
0.1000
0.0500
0.0000
Co-5878 0% O2
Co-5878 0.5% O2
Co-5878 1.0% O2
Co-5878 2.0% O2
Co-0115 0% O2
BuOH Productivity

FIGURE 4


12.00
10.00
8.00
6.00
4.00
2.00
0.00
Co-5878 0% O2
Co-5878 0.5% O2
Co-5878 1.0% O2
Co-5878 2.0% O2
Co-0115 0% O2
Max Butanol
Butanol/Acetone