OXYGEN-REGULATED MICROORGANISMS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/715,702, filed September 8, 2005, and International Application PCT/2006/032525 filed August 18, 2006, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to novel strains of microorganisms and fermentation processes involving these microorganisms. More specifically, the present invention relates to genetically modified strains of microorganisms and the use thereof for the production of commercial products, such as recombinant proteins, nucleic acids, amino acids and specialty chemicals. The present invention also relates to methods of preparing strains of microorganisms for such uses.

BACKGROUND

[0003] Microorganisms, such as bacteria are used extensively in industrial processes to manufacture biopharmaceuticals, vaccine components, plasmid DNAs, vaccine DNAs and many specialty chemicals, including amino acids. Bacteria used in industrial processes are typically grown in liquid medium supplemented with glucose as a source of carbon. Large amounts of glucose are required in industrial processes to grow bacteria to the high densities desired for maximizing volumetric productivity, for producing specialty chemicals and for maintenance of the bacteria. Bacteria take up and assimilate metabolites at a high rate. The flux of metabolites can be so high that it overwhelms one or more of the biochemical reactions in the central carbon pathways of the bacteria as the concentration of certain metabolites rise. The bacteria dispose of the high concentrations of metabolites by utilizing one or more "overflow" pathways.

[0004] *E. coli* tend to secrete acetate when oxygen becomes scarce during high cell density fermentations. The root cause is the need for the cell to dispose of electrons to an acceptor other than O_2. The proximate cause is intracellular accumulation of acetyl-CoA, a product of glycolysis, which in the presence of O_2 is normally burned by the TCA cycle. However, when O_2 is low, the TCA cycle cannot metabolize acetyl-CoA efficiently, so it builds up in the cell. Excess acetyl-CoA is converted to acetate which is excreted along with other

organic acids to remove electrons from the cell. This provides a viable but slow metabolic solution for *E. coli* in the wild, but in industrial fermentors as cell density is driven to higher levels, excess acetate can accumulate to toxic levels.

[0005] This problem has traditionally been addressed by fed batch fermentation wherein the carbon source is metered into the culture so the cell growth is limited by carbon starvation to levels commensurate with the available O_2. Despite metering the carbon source, oxygen utilization eventually becomes limiting at high cell densities. In this situation the fermentor environment also becomes heterogeneous due to the lack of perfect mixing, and the O_2 level varies from place to place in pockets. Although acetate excreted in a low O_2 region can be taken back up by cells that are carried to regions with more O_2, this may be metabolically inefficient and, eventually, as more cells accumulate, the acetate level inexorably climbs even with fed batch technology. As the organic acid levels rise beyond the buffering capacity of the media, pH is maintained by titration with mineral bases such as NaOH which not only require additional equipment and attention from plant personnel, but can also increase the complexity of downstream product purification as well as the salt burden in the fermentor waste stream. A fermentation regimen that increases specific product formation by minimizing over flow metabolism would represent a significant improvement to the art.

SUMMARY OF THE INVENTION

[0006] In various embodiments, the present invention provides a microorganism with metabolic flux from a carbon source regulated by oxygen levels. The microorganism may be a prokaryote, such as *E. coli*, *Shigella*, *Salmonella, Corynebacter, Lactococcus* or *Streptomycetes*. The microorganism may also be a eukaryote, such as a yeast. The yeast may be *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Pichia* species. The carbon source may be glucose, fructose, galactose, mannose, sucrose, maltose, N-acetylglucosamine, β-glucosides, mannitol, cellobiose, sorbose, glucitol or galactitol.

[0007] The microorganism may comprise an oxygen-regulated promoter operatively linked to a gene encoding a protein of a phosphoenol pyruvate-dependent phosphotransferase system. The microorganism may comprise an oxygen-regulated promoter operatively linked to a gene encoding a protein of the *E. coli* O157.H7 sucrose metabolism module. The gene operatively linked to the oxygen-regulated promoter may encode a fusion protein. The fusion protein may comprise ubiquitin.

[0008] These an other embodiments of the present invention are discussed in further detail herein below,

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Figure 1 shows the *E. coli* O157:H7 sucrose metabolism module.

[0010] Figure 2 shows the wild type K12 *sodA* promoter.

[0011] Figure 3 shows the rearranged 0157:H7 sucrose metabolism module controlled by the *sodA* promoter.

[0012] Figure 4 shows PCR amplification products, a) *cscA*, b) *cscKB*, and c) *cscA* and *cscKB* recombinant product.

[0013] Figure 5 shows (a) MG1655 cells grown on M9 medium-containing 1% sucrose as the sole carbon source with no colony formation, and (b) MG1655 *sodA* pKD46 containing the *cscAKB* operon wherein recombinants were able to grow on M9 medium plus 1% sucrose.

[0014] Figure 6 shows an alternate embodiment of the invention wherein only *cscA* is controlled by oxygen by the *sodA* promoter and a separate operon ($Plac_p$) controls *cscKB*.

[0015] Figure 7 shows PCR amplification products, a) *cscKB*, and b) $Plac_{op}$-*cscKB*-terminator fragment

[0016] Figure 8 shows an alternate embodiment of the invention wherein only *cscA* is controlled by oxygen by the *sodA* promoter and a separate operon ($cscK_p$) controls *cscKB*.

[0017] Figure 9 shows PCR amplification products, a) *cscProK-B,* and b) *cscProK-B* fragment cut as a *Hind*III-*Xba*I fragment.

[0018] Figure 10 shows the growth curve of MG1655 *sodA*::*csc* on minimal MOPS media with 1% sucrose. The boxed area of the curve indicates the time that the culture was grown under anaerobic conditions.

[0019] Figure 11 shows the growth curve of MG1655 *sodA*::*csc* on minimal MOPS media with 0.4% sucrose as the sole carbon and energy source (gray diamonds) and MOPS media with 0.2% glucose and 0.4% sucrose (black squares). The boxed area of the curve indicates the time that the culture was grown under anaerobic conditions.

[0020] Figure 12 shows the viable cell counts (Panel A) and media pH (Panel B) for MG1655 *sodA*::*csc* grown on minimal MOPS media with 0.4% sucrose as the sole carbon and energy source (gray diamonds) and MOPS media with 0.2% glucose and 0.4% sucrose (black squares). The boxed area of the plots indicates the time that the culture was grown under anaerobic conditions.

[0021] Figure 13 illustrates the replacement of native PTS promoters with the *sodA* promoter.

[0022] Figure 14 illustrates the strategy for recombinant construction of PTS regulated by the *sodA* promoter and the oligonucleotides that may be used for PCR amplification.

DETAILED DESCRIPTION OF THE INVENTION

[0023] While the present invention is capable of being embodied in various forms, the description below of several embodiments is made with the understanding that the present disclosure is to be considered as an exemplification of the invention, and is not intended to limit the invention to the specific embodiments illustrated. Headings are provided for convenience only and are not to be construed to limit the invention in any way. Embodiments illustrated under any heading may be combined with embodiments illustrated under any other heading.

[0024] The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word "about." In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. As used herein, the terms "about" and "approximately" when referring to a numerical value shall have their plain and ordinary meanings to one skilled in the pertinent art at issue. Also, the disclosure of ranges is intended as a continuous range including every value between the minimum and maximum values recited as well as any ranges that can be formed thereby. This includes ranges that can be formed that do or do not include a finite upper and/or lower boundary. Accordingly, the skilled person will appreciate that many such ratios, ranges, and ranges of ratios can be unambiguously derived from the data and numbers presented herein and all represent embodiments of the present invention.

[0025] In one embodiment, the present invention relates to a microorganism with metabolic flux regulated by oxygen levels. When the microorganism is in an environment with below-threshold levels of oxygen, metabolic flux is reduced. By reducing metabolic flux at low levels of oxygen, overflow metabolism to undesirable metabolites produced in reduced oxygen environments may be reduced. When oxygen levels are increased above a threshold level, metabolic flux may be increased. Within a culture, there may be microenvironments with sufficient oxygen and other microenvironments with insufficient oxygen for efficient product synthesis. The microorganism may be able to self-regulate metabolic flux under varying oxygen levels and thus only consume substrate under conditions that allow efficient product formation.

[0026] By regulating the rate of metabolism as a function of oxygen levels, there may be a reduction in the production of acetate and other undesirable over flow metabolites. By

allowing the microorganism to self-regulate metabolism, a product of interest may be produced with greater efficiency and less cost.

1. Microorganism

[0027] The microorganism may be derived from any parent microorganism. Representative parent microorganisms are available from the American Type Culture Collection. Other representative microorganisms are described in S.Y. Lee, "*High Density Culture of Escherichia coli*," Tibtech 14:98-103 (1996). The parent organism may be a eukaryote, such as yeast. Representative examples of yeast include, but are not limited to, *S. cerevisiae* *S. pombe* and *Pichia* species. The parent organism may also be a prokaryote, such as bacteria and may be a chemoheterotrophic organotroph. Representative examples of bacteria include, but are not limited to, *E. coli*, *Shigella*, *Salmonella*, *Corynebacter*, *Lactococcus* and *Streptomycetes*.

[0028] The microorganism may also be a reduced genome microorganism, such as a reduced genome bacterium. Much of the genetic information contained within the genome of a microorganism may be deleted without detrimentally affecting production of the end metabolite. Moreover, a microorganism with a reduced genome may be advantageous in the production of many products of interest. For example, a reduced genome may lead to the production of fewer native products or lower levels thereof, which may lead to less complex purification of a product of interest. In addition, a microorganism with a reduced genome may be less metabolically demanding and thus may produce a product of interest more efficiently. Reduced genome bacteria are discussed in U.S. Patent No. 6,989,265, which is incorporated herein by reference. A reduced genome bacteria with limited production of natural periplasmic proteins may be beneficial for expressing recombinant proteins in the periplasm. Examples of suitable reduced genome bacteria strains include, but are not limited to, MDS12, MDS13. MDS39, MDS40, MDS41 MDS42, MDS43, MDS44, MDS45, MDS46, MDS47, MDS48, MDS49, MDS50, MDS51, MDS52, MDS53, MDS54, MDS55, MDS56, MDS57, MDS58, MDS59 and MDS60. The reduced genome microorganism may also be derived *de novo* from genes and operons from one or more microorganisms.

2. Oxygen-Regulated Metabolic Flux

[0029] Oxygen levels may regulate the metabolic flux of a carbon source in a microorganism. The metabolism of a carbon source may be regulated at the importation of the carbon source. For example, the expression of a protein involved in the importation of the carbon source may be placed under the control of an oxygen-regulated promoter.

[0030] The metabolism of a carbon source may also be regulated at the first intracellular metabolic step utilizing the carbon source or any metabolic step thereafter, or a combination thereof. For example, the expression of a protein involved in glycolysis or in conversion of the carbon source into a substrate for glycolysis may be placed under the control of an oxygen-regulated promoter.

a. Carbon Source

[0031] The carbon source may be any carbon source capable of supporting growth and/or metabolizing of the microorganism, either natively or by introducing heterologous genes or operons encoding enzymes capable of metabolizing the carbon source. The carbon source may be a monosaccharide, such as glucose, fructose, galactose, or mannose. The carbon source may also be a disaccharide, such as sucrose. Other carbon sources include, but are not limited to, maltose, N-acetylglucosamine, β-glucosides, mannitol, cellobiose, sorbose, glucitol and galactitol.

[0032] Bacteria may take up a carbon source via the phosphpoenolpyruvate:carbohydrate phosphotransferase (PTS) system. The PTS system may allow rapid and efficient uptake of high-quality exogenous carbon sources and may play a key role in the catabolic regulation of metabolism in many bacteria. Expression of one or more components of the PTS system may be under control of an oxygen-regulated promoter, which may reduce the production of over flow products. An alternative carbon source uptake pathway, regulated by the availability of oxygen may allow more efficient partitioning of substrate into product formation throughout the course of a fermentation.

[0033] The PTS system comprises a phospho-relay system in which the EI protein (encoded by *ptsH*) transfers the phosphate group of phosphoenolpyruvate to the Hpr protein (encoded by *ptsI*), which in turn phosphorylates a carbohydrate specific EIIA subunit (in *E. coli* the glucose specific EIIA protein is encoded by the *crr* gene), which can then interact with a membrane associated protein or subunit of the carbohydrate transport complex to internalize and phosphorylate the cognate sugar. The overall reaction converts PEP to pyruvate while transporting an exogenous sugar to an internal sugar-phosphate.

[0034] In *E. coli*, the genes encoding the common PTS elements, *ptsH* and *ptsI* are associated on the chromosome with the *crr* gene. All three genes are transcribed from a set of promoters found upstream of *ptsH*. In addition, an additional promoter or promoter pair within *ptsI* produces a transcript that includes only *ptsI*. Expression of some of the promoters upstream of *ptsH* increases under anaerobic conditions. Increased expression of these genes can result

in an increased level of sugar uptake and activation via any of the PTS systems for which a cognate sugar is present. Increased transcription of these genes may therefore play a role in the production of unwanted over-flow metabolites, since as oxygen becomes limiting as an electron acceptor for the TCA cycle, increasing expression of the PTS genes simultaneously increases the capacity for sugar uptake. Replacing the native *ptsH*-associated promoters with an oxygen-regulated promoter may regulate PTS sugar uptake in an oxygen dependent manner, thus ensuring that consumption of the carbon source is coupled to the presence of oxygen. By controlling expression of the common elements of one or more PTS systems in the cell, the availability and/or utilization of multiple carbon sources may be controlled. As discussed above, uptake and activation of carbon sources can exceed the capacity of the microorganism to efficiently metabolize them, resulting in production of unwanted over flow metabolites. In addition, the production of compounds directly derived from PEP, such as aromatics derived from the shikimic acid pathway, can be limited by consumption of PEP for PTS mediated sugar transport.

1) Sucrose

[0035] Sucrose may also be used as a carbon source for the microorganism. Sucrose is a disaccharide of glucose and fructose. In order to be metabolized, sucrose may be transported into the cell and then split into the monosaccharide units. Both glucose and fructose may then enter glycolysis as glucose-6-phosphate and fructose-6-phosphate.

[0036] A number of microorganisms, including most strains of *E. coli*, are unable to grow on sucrose. However, some strains have a gene system that allows the uptake and metabolism of sucrose as the sole carbon source. Such gene systems, or portions thereof, may be added to a microorganism to allow the use of sucrose as a carbon source. The genes may be added to the chromosome of the microorganism, or may be added to the microorganism on a plasmid. One or more of the genes added to the microorganism may be placed under the control of an oxygen-regulated promoter. It is also contemplated that gene systems allowing uptake and metabolism of other carbon sources may also be added to the microorganism.

[0037] U.S. Patent Nos. 6,365,723 and 6,855,814, which are incorporated herein by reference, describe a representative example of compact gene system for uptake and metabolism of sucrose that permits the use of sucrose as the sole carbon source. The *E. coli* O157:H7 sucrose metabolism module is shown in Figure 1. The *cscB* gene product transports sucrose in to the cell and *cscA* codes for sucrose hydrolase (invertase), which splits the disaccharide sucrose into fructose and glucose. Gene *cscK*, codes for fructokinase the first enzyme of glycolytic metabolism of fructose. The *cscK* gene shows high similarity to

other fructokinases, and may be redundant to the *fruK* gene of *E. coli* K-12; however, *E. coli* K-12 cells may lack invertase and sucrose hydrolase activities. The *cscR* gene product is a repressor that controls expression of the genes of the O157:H7 operon in relation to sucrose availability by binding to the promoters, typical of the *lacI-galR* family of repressor proteins.

b. Oxygen-Regulated Promoter

[0038] Metabolic flux from the carbon source may be regulated by using oxygen sensitive promoters operative linked to a gene encoding one or more of the proteins involved in metabolism of the carbon source. Representative examples of promoters that are activated in the presence of oxygen are promoters within the *cyo* operon and superoxide dismutase (*sodA*), which encodes an enzyme that protects the cell against oxidative damage. The wild type K12 *sodA* chromosomal region is shown in Figure 2.

[0039] By using a positive oxygen sensing promoter, sufficiently high oxygen levels may induce expression of proteins that allow metabolism of the carbon source while minimizing production of over flow metabolites or other by-products. The response time of the promoter may be increased by placing cytoplasmic metabolic proteins under the control of the oxygen sensitive promoter, while non-cytoplasmic proteins (e.g., transmembrane proteins) may be constitutively expressed or placed under control of different promoter. The speed at which metabolism is reduced under diminishing oxygen conditions may be increased by increasing the turnover rate of the metabolic proteins. For example, a protein involved in the metabolism of the carbon source may be expressed as fusion protein with ubiquitin. The invention also encompasses nucleic acid constructs comprising an oxygen regulated promoter operatively linked to a heterologous gene not normally under control of an oxygen sensitive promoter.

c. Growth Rates

[0040] The microorganism may have an improved growth rate when grown in the presence of an abundance of a carbon source. When grown in liquid medium comprising from 0% to the maximum tolerated % concentration of a carbon source the microorganism may have a growth rate including, but not limited to, greater than about 25% to about 400% as compared to the parent of the microorganism. The microorganism may be grown in medium comprising a carbon source at a w/v of at least about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.5, 2%, 2.5, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5%, 8%, 8.5%, 9%, 9.5% or 10%. At such conditions, the microorganism may have a growth rate

greater than about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, 150%, 155%, 160%, 165%, 170%, 175%, 180%, 185%, 190%, 195%, 200%, 205%, 210%, 215%, 220%, 225%, 230%, 235%, 240%, 245%, 250%, 255%, 260%, 265%, 270%, 275%, 280%, 285%, 290%, 295%, 300%, 305%, 310%, 315%, 320%, 325%, 330%, 335%, 340%, 345%, 350%, 355%, 360%, 365%, 370%, 375%, 380%, 385%, 390%, 395%, or 400% as compared to a parental microorganism.

d. Metabolic Flux

[0041] The microorganism may have improved metabolic flux when grown in the presence of an abundance of a carbon source. When grown in liquid medium comprising from 0% to the maximum tolerated % concentration of a carbon source, the microorganism may have a metabolic flux including, but not limited to, from about 5% to about 90% of the carbon source being directed to the desired products. A strain may have a metabolic flux of about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90%.

[0042] In general, flux of metabolites through each reaction step in any given pathway depends on the relative rates of the forward reaction and reverse reactions. The flux may refer to the rate of change in concentration of an analyte as a function of time and sample size. The metabolic flux through any single metabolic conversion may be determined using methodologies described in U.S. Patent Nos. 6,764,817 and 6,849,396, the contents of which are incorporated herein by reference. Further guidance on flux and methods for its determination is provided, for example, by Newsholme, E. A. et al., *Biochem. Soc. Symp.* 43:183-205 (1978); Newsholme, E. A., et al., *Biochem. Soc. Symp.* 41:61-110 (1976); and Newsholme, E. A., and Sart., C., *Regulation in Metabolism*, Chaps. 1 and 3, Wiley-Interscience Press (1973), the contents of which are incorporated herein by reference.

e. Production

[0043] The microorganisms of the present invention may be able to produce end metabolites or other products of interest at higher rates. When grown in liquid medium comprising from 0% to the maximum tolerated % concentration of a carbon source, the microorganism may produce end metabolites or other products of interest from about 0.001 g/L to about 100 g/L of the carbon source being directed to the desired products. A strain may have a metabolic

flux of about 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80%.

f. Rate of Production

[0044] In view of the ability to control the flux of metabolism, the microorganism may produce a variety of amounts of the end metabolite, at a variety of rates, and at variable rates of efficiency of carbon source utilization. The strains of the present invention may produce the end metabolite at least to levels including, but not limited to, about 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L, 80 g/L, 90 g/L, and 100 g/L.

[0045] The microorganism may produce the end metabolite at a rate including, but not limited to, at least about 0.50 g/L/hr, 0.75 g/L/hr, 1.00 g/L/hr, 1.25 g/L/hr, 1.50 g/L/hr, 1.75 g/L/hr, 2.00 g/L/hr, 2.25 g/L/hr, 2.50 g/L/hr, 2.75 g/L/hr, 3.00 g/L/hr, 3.25 g/L/hr, 3.50 g/L/hr, 3.75 g/L/hr, 4.00 g/L/hr, 4.25 g/L/hr, 4.50 g/L/hr, 4.75 g/L/hr, and 5.00 g/L/hr.

[0046] The microorganism may produce the end metabolite at a rate of efficiency of carbon source utilization including, but not limited to, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, and 75%.

3. Product of Interest

[0047] The microorganism may be used to produce a product of interest at high levels of efficiency in media with an abundance of a carbon source. The product of interest produced by the microorganism may be one or more of any products including, but not limited to, chemicals, amino acids, vitamins, cofactors, nucleic acids, such as DNA, fatty acids, growth factors, proteins and intermediates thereof. The product of interest may be a product that is naturally produced by the microorganism. The product of interest may also be a non-natural product that is produced as a result of heterologous genes being added to the microorganism.

[0048] The product of interest may be intracellularly located in the microorganism. The product of interest may also be secreted into the periplasm of the microorganism. The periplasm may be beneficial for protein production, because: (i) recombinant human protein may be produced with the correct amino terminus, whereas those produced in the cytoplasm may begin with an additional methionine not present in the natural protein; (ii) many proteins may fold correctly in the periplasmic space (iii) the correct disulfide bonds may be formed in the periplasm; (iv) the periplasmic space may contain much less and far fewer proteins than the cytoplasm, simplifying purification (v) there may be fewer proteases than in the cytoplasm, which may reduce protein digestion and loss; and (vi) expressed proteins may be readily released with other periplasmic proteins by specifically disrupting the outer

membrane, substantially free of the more abundant cytoplasmic proteins. The product of interest may also be excreted by the cell into the media.

4. Fermentation

[0049] The microorganism may be used to produce desired products in batch fermentations, where the entire required amount of the carbon source may be added at the beginning of the fermentation. Not withstanding the ability of the modified cells to tailor carbon source consumption to the availability of oxygen, the strains may also be used to produce desired products in fed batch fermentations. The feeding rate of the carbon source may be any amount up to that which produces the maximum tolerated concentration but is preferably the minimum amount required to maintain maximal growth rate. The strains may also be used to produce desired products in continuous or "chemostat" modes of fermentation, which may allow maintenance of a higher dilution rate.

[0050] The microorganism may be used for fermentation processes in a synthetic or natural medium containing at least one carbon source and at least one nitrogen source that may be utilized by the strain by virtue of its possessing the necessary metabolic pathway(s) and, as appropriate, inorganic salts, growth factors and the like.

[0051] Illustrative examples of suitable nitrogen sources include, but are not limited to: ammonia, including ammonia gas and aqueous ammonia; ammonium salts of inorganic or organic acids, such as ammonium chloride, ammonium phosphate, ammonium sulfate and ammonium acetate; and other nitrogen-containing substances, including amino acids, meat extract, peptone, molasses, corn steep liquor, casein hydrolysate, soybean cake hydrolysate and yeast extract.

[0052] Some amino acids present individually in minimal salts media may not be utilized well by bacteria as carbon sources. Each species of bacterium differs in their ability to utilize each natural amino acid. Amino acids that are not utilized individually may be utilized well in the presence of other amino acids, for instance serine may be used as a carbon source only if glycine, leucine, isoleucine and valine are present. In rich media, such as synthetic amino acid mixes, several amino acids may be utilized preferentially and consumed before other amino acids. Serine, proline, glycine, aspartate, threonine, glutamate and alanine may be completely removed from a mix of the 16 amino acids present in casamino acids, a popular media constituent, while the others are utilized more slowly and incompletely. Similar results are obtained in tryptone broth. If it is desirable to re-utilize amino acids accumulated as end metabolites, then the amino acid may be preferably serine, proline, glycine, aspartate,

threonine, glutamate or alanine, and the medium may contain the additional amino acids needed to stimulate its use as a carbon source. The medium may also contain a protein hydrolysate including, but not limited to, tryptones, casamino acids and soy hydrolysates.

[0053] The present invention has multiple aspects, illustrated by the following non-limiting examples.

EXAMPLE 1

Oxygen-Regulated Sucrose Uptake and Metabolism

[0054] A sucrose catabolizing (Suc^+) strain of *E.coli* K-12 was produced by replacing the native *sodA* gene with the sucrose metabolism module of *E. coli* O157:H7 as shown in Figure 3. The genes were rearranged from their original configuration within the module by inverting the *cscB* gene and placing it in line with *cscK*. The *cscR* gene was omitted because the promoter-operator region was replaced by the promoter and regulatory elements of the *sodA* gene. It should be noted that the strain could also have been produced without *cscK*, since the activity of the gene product is redundant to that of the *fruK* gene of *E. coli* K-12. The strain could also have been produced by introducing the sucrose metabolism module on a plasmid or into the genome under control of a separate promoter, such as *sodA*.

[0055] Primers 1 (P1) and 2 (P2) were designed to amplify both *cscK* and *cscB* as a single 2.2 Kb piece (Figure 4b). The sequence for P1 was: 5'-CCCACGGAGTGGCTGTGCTGC AACATGGAGCACTCTGGCTACTGGGTTAAGTCAGATGAATTTAAGGGAA-3' (SEQ ID NO: 1), which has a 50-bp overlap with the 3' end of the *cscA* gene and 20-bp of the 5' end of the *cscK* gene including the ribosome binding site (RBS) region of the *cscK* gene. The sequence for P2 is: 5'-CAAAACCACATCAATTGAAACGCTGTTTTATTTTTATCGGAT CATTGTTTCTATATTGCTGAAGGTACAG-3' (SEQ ID NO: 2), which has 50-bp of the 3' end sequence after the *sodA* terminator region and 20-bp of the 3' end of the *cscB* gene. Primers 3 and 4 were designed to amplify *cscA* (1.4 kb) (Figure 4a) from O157:H7 genomic DNA. The sequence for P4 is: 5'-CTGCTTACGCGGCATTAACAATCGGCCGCCCGACA ATACTGGAGATGAATATGACGCAATCTCGATTGCA-3' (SEQ ID NO: 4), which has 50-bp of the *sodA* promoter sequence including the RBS. The sequence for P3 is: 5'-TTAACCCAGTAGCCAGAGTG-3' (SEQ ID NO: 3), which matches the 3' end sequence of the *cscA* gene.

[0056] In the first round of PCR to amplify *cscA* or *cscKB*, a 50 µl PCR reaction was carried out in a tube containing 100 ng O157:H7 genomic DNA, 50 pmol of each primer, 0.2 mM

dNTPs and 2.5 U of *Pfu* polymerase. For the *cscA* amplification, the reaction went through 1 cycle of 1 min at 95°C, 25 cycles of 1 min of denaturing at 95°C; 15 sec of annealing at 55°C; and 4 min extension at 72°C, then a final cycle of 10 min extension at 72°C. For the *cscKB* amplification, the reaction went through the same cycling as above except the annealing temperature was 48°C.

[0057] In the second round of recombinant PCR reactions, an equal molar amount of *cscA* and *cscKB* products were mixed as templates in a 50 µl reaction containing 50 pmol of each primer, 0.2 mM dNTPs and 2.5 U of *Pfu* polymerase. The reaction went through 1 cycle of 5 min at 95°C, 5 cycles of 30 sec of denaturing at 95°C; 30 sec of annealing at 65°C; and 7.5 min extension at 72°C, then 25 cycles of 1 min of denaturing at 95°C; 7.5 min extension at 72°C and a final cycle of 10 min extension at 72°C. The resultant product was the expected size of 3.7 kb (Figure 4c).

[0058] The recombinant PCR products contained 50-bp of *sodA* 5' sequence, *cscA*, *cscK*, *cscB*, and 50 bp of the 3' end sequence after the *sodA* terminator. Thus, the *cscAKB* operon from O157:H7 was placed under control of the *sodA* promoter of MG1655, to produce MG1655 *sodA:csc*. The PCR products were purified and used for electroporation of the *sodA*::Tn*5* mutant of MG1655, containing plasmid pKD46. MG1655 cells cannot grow on M9 medium containing 1% sucrose as the sole carbon source, as shown in Figure 5a. The transformed cells containing the *cscAKB* operon, however, were able to grow on the same medium after 2 days at 30°C in the presence of ampicillin (100 µg/ml final concentration) as shown in Figure 5B.

EXAMPLE 2

Oxygen-Regulated Sucrose Metabolism

[0059] Invertase may be the bottleneck through which sucrose metabolism must flow. As a cytoplasmic enzyme, invertase can turn over more quickly than a membrane protein. This is important in achieving a rapid shutdown of function. In fact, the rate of its degradation can be speeded up if necessary by ubiquitin tags and other such modifications. By contrast, levels of *cscB* are is unlikely to be quickly regulated since the symporter is a membrane protein. As a result, we produced the Suc^+ strain shown in Figure 6. The construct provides a separate operon for independently controlling the sucrose symporter *cscB* using a *lac* promoter. As before, the fructokinase gene (*cscK*) may be superfluous.

[0060] Primers 4 and 5 were designed to amplify *cscA* (1.4 kb) from O157:H7 genomic DNA. P4 was the same primer described in Example 1. The sequence for P5 is: 5'-CAAAACCACATCAATTGAAACGCTGTTTTATTTTTATCGGATCATTGTTTTTAA CCCAGTAGCCAGAGTG-3' (SEQ ID NO: 5). Thus, the *cscA* gene is flanked by 50-bp of 5' and 3' sequences of *sodA* ($sodA_p$-*cscA*-$sodA_t$). Primers K5 and B3 were designed to amplify both *cscK* and *cscB* in one piece (2.2 Kb) (Figure 7a). The sequence for K5 is: 5'-GTAAGTCAGATGAATTTAAGGGAA-3' (SEQ ID NO: 6), which includes the RBS region of the *cscK* gene. The sequence for B3 is: 5'-CTATATTGCTGAAGGTACAGGCG T-3' (SEQ ID NO: 7), which has 20-bp of the 3' end of the *cscB* gene.

[0061] PCR was carried out to amplify *cscA* or *cscKB*. A 50-ml PCR reaction was carried out in a tube containing 100 ng O157 genomic DNA, 50 pmol of each primer, 0.2 mM dNTPs and 2.5 U of *Pfu* polymerase. The reaction went through 1 cycle of 1 min at 95°C, 25 cycles of 1 min of denaturing at 95°C; 15 sec of annealing at 55°C; and 4 min extension at 72° C, then a final cycle of 10 min extension at 72°C.

[0062] Four microliters of the *cscKB* product was cloned into a pCR-Blunt vector (Invitrogen) at the *Eco*RI site to create pFD1. Thus, *cscKB* product is placed under the control of an inducible *Plac* promoter. The 2.7-kb *Plac-cscKB*-terminator fragment was excised from the plasmid by *Pvu*II digestion and gel-purified (Figure 7b). This fragment was sub-cloned into the *Sma*I site of pJW23, which contains an *att* site. The new plasmid, named pFD6, was then digested with *Not*I, and the larger fragment recovered after gel purification. This fragment, containing the *Plac-cscKB*-terminator was re-ligated to generate pFD7. Plasmids pFD7 and pJW289 were co-transformed into a *sodA*::Tn*5* mutant of MG1655 or MDS42 (without pKD46) to enable integration. The pJW289 plasmid was cured later as described previously. The pKD46 plasmid was introduced back to the cured host for recombination.

[0063] The PCR product of $sodA_p$-*cscA*-$sodA_t$ was then used to transform the new host to enable recombination. The transformed cells were plated on M9 medium containing 1% sucrose as a sole carbon source. Transformants able to grow on the medium have a Suc+ phenotype.

EXAMPLE 3

Control of Sucrose Metabolism by an Oxygen-Sensitive Promoter and Sucrose Uptake by a Sucrose Repressor for Sucrose Positive Bacteria

[0064] The third example preserves the sucrose repressor CscR to control the level of sucrose in the cell in a normal fashion for sucrose positive strains such as O157:H7. This scheme is tantamount to transplanting the *cscA* gene from the normal position in the sucrose operon of O157:H7 into the *sodA* operon. This method of control may be the most effective in regulating acetate because the pool of sucrose will be fixed and the only controlling function is the level of invertase, the bottleneck enzyme. The *cscR* gene is eliminated from this scheme, which results in constitutive transport of sucrose and fructokinase activities. As before, the *sodA* gene may either be replaced by *cscA* or combined in series with *cscA* as shown in Figure 8.

[0065] Primers 4 and 5 were designed to amplify *cscA* (1.4 kb) from O157 genomic DNA. P4 and P5 were described in Example 1. Thus, the *cscA* gene is flanked by 50-bp of 5' and 3' sequences of *sodA*. Primers ProK5 and B3 were designed to amplify both *cscK* and *cscB* in one piece (2.2 Kb). The sequence for ProK5 is: 5'-AAGAGGTTTATCACTAACATTTTG TG-3' (SEQ ID NO: 8), which includes the entire promoter region of the *cscK* gene. The sequence for B3 was the same as described before.

[0066] PCR was carried out to amplify *cscProK-B* (2.3 kb) (Figure 9a). A 50-µl PCR reaction was carried out in a tube containing 100 ng O157 genomic DNA, 50 pmol of each primer, 0.2 mM dNTPs and 2.5 U of *Pfu* polymerase. The reaction went through 1 cycle of 1 min at 95°C, 25 cycles of 1 min of denaturing at 95°C; 15 sec of annealing at 55°C; and 4 min extension at 72° C, then a final cycle of 30 min extension at 72°C.

[0067] Four microliters of the *cscProK-B* product was cloned into a pCR-Blunt vector (Invitrogen) to create pFD8. The *cscProK-B* fragment was cut out as a *Hind*III-*Xba*I fragment (2.7 kb) (Figure 9b) and sub-cloned into pJW23, which contains an *att* site. The new plasmid pFD14 was then cut with *Not*I, and the larger fragment recovered after gel purification. This fragment, containing the *cscProK-B*-terminator was re-ligated to produce pFD15. Plasmids pFD15 and pJW289 were co-transformed into a *sodA*::Tn*5* mutant of MG1655 or MDS42 (without pKD46) to enable integration. Both plasmids were later cured as described previously. Plasmid pKD46 was then introduced back into the cured host.

[0068] The PCR products of *cscA* were then used to transform the above new hosts to enable recombination. The transformed cells were plated on M9 medium containing 1% sucrose as a sole carbon source. Transformants able to grow on the medium have a Suc+ phenotype.

EXAMPLE 4

Oxygen-Regulated Growth of Sucrose Metabolism

[0069] Aerobic dependent growth of the MG1655 *sodA*::*csc* strain of Example 1 was examined using sucrose as a sole carbon and energy source and monitoring growth throughout an aerobic-anaerobic-aerobic cycle. The cycle was initiated by switching the fermentor from air to a 95:5 mixture of Nitrogen and carbon dioxide, and then back to air after a suitable interval in 500 ml air-lift fermentations.

[0070] Figure 10 represents a typical growth curve of the strain on minimal MOPS media with 1% sucrose as the sole carbon and energy source. The lack of growth under anaerobic conditions indicates the cells were no longer able to catabolize sucrose. In contrast, growth of MG1655 *sodA*::*csc* on glucose plus sucrose is relatively unchanged by changes in head space gas, since glucose transport and glycolysis are not solely affected by the aerobic or anaerobic status of the culture.

[0071] Figure 11 depicts the results of two similar air lift fermentations, one carried out in minimal MOPS media containing 0.4% sucrose as the sole carbon and energy source and one carried out in the same media supplemented with 0.2% glucose, in addition to 0.4% sucrose as a carbon and energy source. The strain did not grow on sucrose under anaerobic conditions, but continued to catabolize glucose whether oxygen was present or not. In addition to the growth plots shown in Figure s 10 and 11 are confirmed by the observation that the overall viable cell counts did not change throughout the anaerobic phase in the sucrose only culture, while they continued to increase when exogenous glucose was present (shown in Figure 12A). This indicates that there is no biochemical restriction on glycolysis and that glucose produced from sucrose (by the action of the *csc* gene products) could support anaerobic growth if it occurred. Lack of growth on sucrose under anaerobic conditions is therefore due to lack of transcription of the *csc* genes from the oxygen-dependent *sodA* promoter.

[0072] In addition, the pH profiles (Figure 12B) demonstrate that growth on sucrose generates very little acid, whereas the culture containing both sucrose and glucose produces a large amount of acid. This also supports the notion that little or no sucrose catabolism takes

place during the anaerobic phase of the fermentation since anaerobic assimilation of glucose is known to generate large amounts of acetate and formate. The inability of the culture growing on sucrose to produce any acid during the course of the anaerobic shift indicates that little or no glucose is available within the cell in the absence of oxygen.

EXAMPLE 5

Control of PTS Carbohydrate System by an Oxygen-Sensitive Promoter

[0073] Replacement of the native PTS promoters with the *sodA* promoter is illustrated in Figures 13 and 14. The desired construct is produced in three steps. The first step involves the production of two separate PCR products sharing a unique sequence homologous to their chromosomal targets at one end, as well as a common sequence to allow fusion of the two PCR products at the opposite ends. The first of the initial PCR products includes 45 bases of sequence homologous to the 3' end of the *cysK* gene, the complete chloramphenicol acetyl-transferase gene (CAT) plus it's promoter as well as a non-coding linker sequence to provide homology to the second PCR product (described below). The CAT gene is amplified from plasmid pACYC184 using a primer designated as Oligo 1 with the sequence 5'- gcattgtttg ccgatctcttcactgagaaagaattgcaacagtaaTTACGCCCCGCCCTGCC ACTCATCG-3' (SEQ ID NO: 9), where the lower case sequence is homologous to the 3' end of *cysK* and the upper case sequence is homologous to the 3'-end of the CAT gene. The second oligo in this PCR reaction is designated Oligo 3 with the sequence 5'- acaaacctgaatttt aagtccagtacctaCGACGCACTTTGCGCCGAATAAATACCTG-3' (SEQ ID NO: 10), where the lower case sequence is the non-coding sequence homologous with the second PCR product and the upper case sequence is homologous to the 5'-end of the CAT promoter region. A 50-µl PCR reaction is carried out in a tube containing 1 ng pACYC184 DNA, 50 pmol of each primer, 0.2 mM dNTPs and 2.5 U of *Pfu* polymerase. The reaction went through 1 cycle of 1 min at 95°C, 30 cycles of 1 min of denaturing at 95°C; 15 sec of annealing at 55°C; and 2 min extension at 72° C, then a final cycle of 10 min extension at 72°C.

[0074] The second of the initial PCR products includes a short non-coding sequence to provide homology to the first PCR product, the intact oxygen-dependent *sodA* promoter and 45 bases of sequence homologous to the 5' end of the *ptsH* gene. The *sodA* promoter region is amplified from *E. coli* chromosomal DNA using a primer designated as Oligo 2 with the sequence 5'-tgttttggacttaaaattcaggtcatggatAATGCGTCGACTCCTGCAAAACCATACCC

T-3' (SEQ ID NO: 11), where the lower case sequence is the non-coding sequence homologous with the first PCR product and the upper case sequence is homologous to the 5'-side of the *sodA* promoter region of the *E. coli* chromosome. The second oligo of this PCR reaction is designated Oligo 4 with the sequence 5'-gggtgtgcagaccgttcggagcggtaatggtaacttcttg ctggaaCATATTCATCTCCAGTATTGTCGGG-3' (SEQ ID NO: 12), the lower case sequence is homologous to the 5'-end of *ptsH* and the upper case sequence is homologous to the 3'-side of the *sodA* promoter. This oligo effectively replaces the start codon of *sodA* with the start codon of *ptsH*. A 50-µl PCR reaction is carried out in a tube containing 100 ng *E. coli*MG1655 DNA, 50 pmol of each primer, 0.2 mM dNTPs and 2.5 U of Turbo *Pfu*. The reaction went through 1 cycle of 2 min at 95°C, 30 cycles of 1 min of denaturing at 95°C; 15 sec of annealing at 55°C; and 1 min extension at 72° C, then a final cycle of 5 min extension at 72°C.

[0075] The PCR products of each reaction are recovered from agarose gels and purified on Qiagen purification columns prior to a second round of PCR. In this reaction the two first round PCR products are denatured and allowed to re-anneal prior to extension. A 50-µl PCR reaction was carried out in a tube containing 5 ng of each purified, first round PCR product, 0.2 mM dNTPs and 2.5 U of *Pfu* polymerase. The reaction goes through 1 cycle of 2 min at 95°C, 10 cycles of 1 min of denaturing at 95°C; 15 sec of annealing at 55°C; and 1 min extension at 72° C. After the first 5 cycles 50 pmoles of Oligo 1 and Oligo 4 are added to the reactions and the reactions are continued for an additional 25 cycles, followed by a final cycle of 5 min extension at 72°C. The second round PCR product is purified using a Qiagen column and transformed into MG1655 or MDS42 containing pKD46 to enable integration. Stable integrants are isolated as chloramphenicol resistant colonies. The pKD46 plasmid is cured as described previously.

EXAMPLE 6

Oxygen-Regulated Growth of Sugar Metabolism

[0076] Transcriptional regulation of the PTS carbohydrate transport system from oxygen regulated promoters reduces the role the PTS system plays in sugar consumption under anaerobic conditions. Although glucose or other carbohydrates can still be taken up via non-PTS systems rates of assimilation are greatly reduced since the PTS system represents the highest velocity sugar uptake strategy in *E. coli*. An additional benefit of bringing expression of the *ptsH* and *ptsI* genes, which play a common role in all PTS systems in *E. coli*, under the

control of an oxygen regulated promoter allows a single point of regulation to control consumptionof all PTS sugars. By direct analogy with the sucrose model described in earlier examples, growth of *E. coli* strains on other sugars can be constrained to more closely match the availability of oxygen as a terminal electron acceptor thereby minimizing production of over flow metabolites by the engineered cell for a broad range of PTS sugars.

CLAIMS

1. A microorganism with metabolic flux from a carbon source regulated by oxygen levels.

2. The microorganism of claim 1 wherein the microorganism is a prokaryote.

3. The microorganism of claim 2 wherein the prokaryote is selected from the group consisting of *E. coli*, *Shigella*, *Salmonella*, *Corynebacter*, *Lactococcus* and *Streptomycetes*.

4. The microorganism of claim 1 wherein the microorganism is a eukaryote.

5. The microorganism of claim 4 wherein the eukaryote is a yeast.

6. The microorganism of claim 5 wherein the yeast is selected from the group consisting of *S. cerevisiae* and *S. pombe*.

7. The microorganism of claim 1 wherein the carbon source is selected from the group consisting of glucose, fructose, galactose, mannose, sucrose, maltose, N-acetylglucosamine, β-glucosides, mannitol, cellobiose, sorbose, glucitol and galactitol.

8. The microorganism of claim 1 wherein the microorganism comprises an oxygen-regulated promoter operatively linked to a gene encoding a protein of a phosphoenol pyruvate-dependent phosphotransferase system.

9. The microorganism of claim 1 wherein the microorganism comprises an oxygen-regulated promoter operatively linked to a gene encoding an *E. coli* O157.H7 sucrose metabolism module.

10. The microorganism of claim 8 wherein the gene encodes a fusion protein comprising the protein of the phosphoenol pyruvate-dependent phosphotransferase system and ubiquitin.

11. The microorganism of claim 9 wherein the gene encodes a fusion protein comprising the protein of the *E. coli* O157.H7 sucrose metabolism module and ubiquitin.

12. A method for regulating metabolic flux in a microorganism comprising culturing the microorganism of claim 1 under suitable nutrient conditions and oxygen concentrations to produce a desired metabolic flux.

13. A method for reducing the production of overflow metabolites in microorganism the method comprising culturing the microorganism of claim 1 under nutrient conditions and under oxygen concentrations below, above, or at a threshold level of oxygen thereby altering the flow of carbon through one or more metabolic pathway that produces said overflow metabolites.

A microorganism comprising an oxygen sensitive promoter said promoters capable of regulating metabolic flux of a carbon source through one or more metabolic pathways of said microorganism and wherein said oxygen sensitive promoter is operatively linked to a heterologous gene not naturally under the transcriptional control of said oxygen sensitive promoter.