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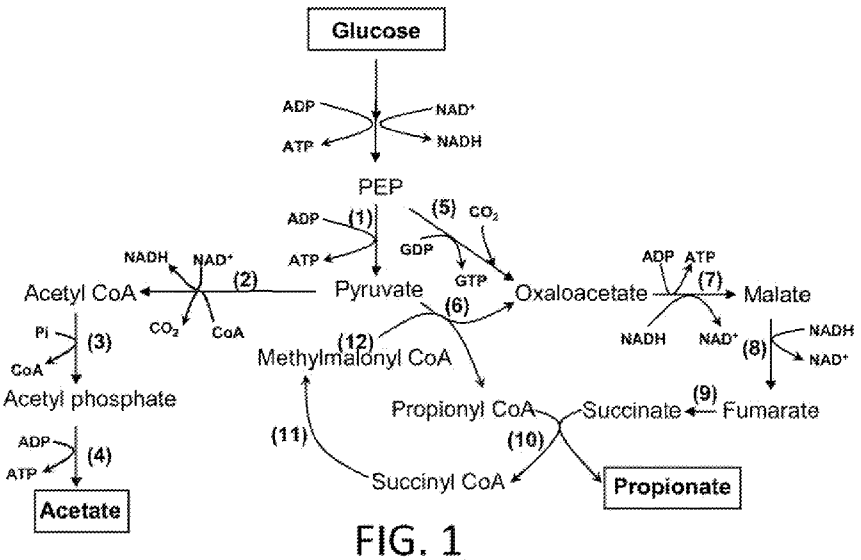

FIG. 1

(57) **Abstract**: Metabolically engineered organisms capable of producing propionic acid. Host organisms are transfected with recombinant vectors containing a CoA transferase gene. Mutants exhibit increased CoA transferase activity, increased propionic acid yield and productivity, and increased tolerance to propionic acid and acidic pHs. Methods for producing propionic acid through fermentation of mutants overexpressing a CoA transferase gene are also included.

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SYSTEMS AND METHODS FOR PROPIONIC ACID PRODUCTION

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CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority from US Provisional Patent Application Number 61/411,804, filed November 9, 2010, which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] Metabolically engineered microorganisms and methods of producing propionic acid, particularly metabolically engineered microorganisms having genes for the overexpression of CoA transferase and methods of using metabolically engineered microorganisms to produce propionic acid from sugars and other substrates in fermentation.

BACKGROUND OF THE INVENTION

[0003] Propionic acid is a carboxylic acid. Colorless and soluble in water, propionic acid has many industrial uses. It is used as a food preservative in both human and animal food, and is also used in pharmaceuticals, plastics, perfumes, artificial flavorings and herbicides. While the majority of propionic acid is produced in a petrochemical process, market demand and price have been increasing steadily, spurring interest in producing the acid by other means. Propionic acid is the major fermentation product produced by propionibacteria, which are Gram-positive, nonspore-forming, rod-shaped, facultative anaerobes. Two species of wild-type propionibacteria, *P. acidipropionici* and *P. freudenreichii ss. shermanii*, are commonly used in Swiss cheese production and have been extensively studied for their potential in commercial propionic acid production. A wide range of carbon sources, including glucose, lactose, sucrose, xylose, lactate, pyruvate, glycerol, and sorbitol, can be consumed by propionibacteria such as *P. acidipropionici* and *P. freudenreichii ss. shermanii* for cell growth and product synthesis.

[0004] However, historically product yields and production rates during fermentation of propionibacteria are not high. As in most organic acid fermentations, propionic acid fermentation is strongly inhibited by acidic pH and the fermentation product itself, propionic acid. The specific growth rate of propionibacteria drops by more than 50% when there is 1% (w/v) propionic acid present in the growth medium. The optimal pH range for cell growth is between 6 and 7, and at a pH of less than 4.5 there is practically no growth. Furthermore, because of the nature of the heterofermentative metabolism of propionibacteria, propionic acid is scarcely ever formed as a sole fermentation product. Instead, the production of propionic acid is usually accompanied by acetate, succinate, and carbon dioxide. Acetate and succinate also have an inhibitory effect on cell growth during fermentation. Additional efforts must be taken in order to remove these growth-inhibiting byproducts during fermentation which has historically made the fermentation of propionibacteria a less than desirable means of producing propionic acid.

SUMMARY OF THE INVENTIVE CONCEPT

[0005] Provided herein are metabolically-engineered microorganisms that include recombinant biochemical pathways useful for producing propionic acid from various substrates. Also provided herein are methods of producing propionic acid using the microorganisms described herein.

[0006] The invention features recombinant microorganisms capable of producing propionic acid that are constructed by transferring into a host organism a gene for propionyl-CoA:succinate CoA transferase (CoA transferase). The host organism may be capable of producing propionic acid and be engineered to overexpress CoA transferase, resulting in increased propionic acid production as well as a greater resistance to propionic acid and metabolic byproducts resulting for propionic acid fermentation.

[0007] In certain embodiments the host organism is *P. acidipropionici* and the CoA transferase gene comes from *P. acidipropionici*. In certain embodiments the host organism is *P. freudenreichii* subsp. *shermanii* and the CoA transferase gene comes from *P. freudenreichii* subsp. *shermanii*.

[0008] In certain embodiments the recombinant microorganism is capable of producing propionic acid from glucose. In other embodiments the recombinant microorganism is capable of producing propionic acid from other monosaccharides, oligosaccharides, and polysaccharides. In other embodiments the recombinant microorganism is capable of producing propionic acid from other carbon sources, including glycerol, lactate and pyruvate.

[0009] In certain embodiments the recombinant microorganisms may be Pa-01CoA, Pa-04CoA, Pfs-01CoA, or Pfs-04CoA.

[0010] In certain embodiments, the invention features vectors that enable a host organism to overexpress CoA transferase and produce increased amounts of propionic acid. The vectors may be plasmids that contain genes for propionyl-CoA:succinate CoA transferase. Depending on the embodiment, the vector may be pGEM-CoA, pET-CoA, pKH01COA, or pKH04COA. The vectors may be transferred to *P. acidipropionici* or *P. freudenreichii* subsp. *shermanii* to overexpress CoA transferase to increase propionic acid production.

[0011] In certain embodiments, the invention features methods for producing propionic acid. The methods include obtaining microbial hosts having a metabolic pathway capable of producing propionic acid, transfecting the microbial host with a vector containing a gene for CoA transferase, fermenting the transfected microbial host in a medium comprising a substrate, and recovering propionic acid. In certain embodiments the microbial host is *P. acidipropionici* or *P. freudenreichii* subsp. *shermanii*. In certain embodiments the gene for CoA transferase is obtained from *P. acidipropionici* or *P. freudenreichii* subsp. *shermanii*. In certain embodiments the transfected microbial host may be fermented with a substrate selected from the group consisting of glucose, lactose, sucrose, xylose, fructose, and maltose. In certain embodiments the transfected microbial host may be fermented with a substrate comprised of a different type of carbon source, such as glycerol, lactate or pyruvate.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] **Figure 1** depicts an exemplary metabolic pathway for propionic acid synthesis in Propionibacteria;

[0013] **Figure 2** shows the nucleotide and deduced amino acid sequences of the CoA transferase gene from *P. acidipropionici*;

[0014] **Figure 3** shows the alignment of *P. acidipropionici CoA* transferase with analogous CoA transferases from *Propionibacterium acnes*, *Saccharopolyspora erythraea*, *Corynebacterium efficiens*, *Azoarcus sp.* and *Pseudoalteromonas atlantica* T6c;

[0015] **Figure 4** shows a diagram of the construction of expression plasmid pET-CoA;

[0016] **Figure 5** shows a SDS-PAGE analysis of cloned CoA transferase gene expression in *E. coli* BL21(DE3);

[0017] **Figure 6** is a chart showing the expression of CoA transferase in *E. coli* BL21(DE3) wild-type and transformants harboring pET-CoA;

[0018] **Figure 7** is a diagram of the construction of overexpression plasmids pKH04CoA and pKH01CoA;

[0019] **Figure 8** is a chart showing the expression of CoA transferase in wild-type *P. acidipropionici* and mutants Pa-01CoA and Pa-04CoA;

[0020] **Figure 9** shows the kinetics of propionic acid fermentation of glucose in serum bottles by *P. acidipropionici* wild-type;

[0021] **Figure 10** shows the kinetics of propionic acid fermentation of glucose in serum bottles by mutant Pa-01CoA;

[0022] **Figure 11** shows the kinetics of propionic acid fermentation of glucose in serum bottles by mutant Pa-04CoA;

[0023] **Figure 12** shows the kinetics of propionic acid fermentation of glucose in batch fermentation by *P. acidipropionici* wild-type in a 5-L stirred-tank fermentor;

[0024] **Figure 13** shows the kinetics of propionic acid fermentation of glucose in batch fermentation by mutant Pa-01CoA in a 5-L stirred-tank fermentor;

[0025] **Figure 14** shows the kinetics of propionic acid fermentation of glucose in batch fermentation by mutant Pa-04CoAin a 5-L stirred-tank fermentor;

[0026] **Figure 15** shows the effects of pH on the growth rate of *P. acidipropionici* wild-type;

[0027] **Figure 16** shows the effects of pH on the growth rate of mutant Pa-01CoA;

[0028] **Figure 17** shows the effects of pH on the growth rate of mutant Pa-04CoA;

[0029] **Figure 18** shows the effects of propionic acid concentration on the growth of *P. acidipropionici* and mutants Pa-01CoA and Pa-04CoA;

[0030] **Figure 19** is a graph comparing the enzyme activities of CoA transferase in wild type *P. shermanii* and the Pfs-01CoA and Pfs-04CoA mutants;

[0031] **Figure 20** shows the results of SDS-PAGE of protein extracts from wild type, and Pfs-01CoA and Pfs-04CoA mutants;

[0032] **Figure 21** shows a fermentation profiles for wild-type *P. shermanii*;

[0033] **Figure 22** shows a fermentation profile for mutant Pfs-01CoA-5;

[0034] **Figure 23** shows a fermentation profile for mutant Pfs-01CoA-9;

[0035] **Figure 24** shows a fermentation profile for mutant Pfs-01CoA-9 without hygromycin B;

[0036] **Figure 25** shows the relative yields of wild-type *P. shermanii* and mutants in co-substrate fermentation;

[0037] **Figure 26** shows the relative productivity of the wild-type *P. shermanii* and mutants in co-substrate fermentation;

[0038] **Figure 27** shows the ratios of propionic acid to acetic acid from wild-type *P. shermanii* and mutants in co-substrate fermentation;

[0039] **Figure 28** shows the ratios of propionic acid to succinic acid from wild-type *P. shermanii* and mutants in co-substrate fermentation; and

[0040] **Figure 29** shows a comparison of carbon consumption rates by wild-type *P. shermanii* and mutants in co-substrate fermentation.

SEQUENCE DESCRIPTIONS

Nucleotide/Amino Acid	Description	SEQ. ID Nos.
Propionyl-CoA: succinate coenzyme A transferase gene from *P. acidipropionici*	GenBank Accession No. FJ641063	SEQ. ID No. 1

ATCC 4875		
Amplification of CoA transferase gene from ATCC 4875 - Forward Primer	5'-AGGAC*CC*ATG*G*CAGATCGGATTGCCAAC-3′	SEQ. ID No. 2
Amplification of CoA transferase gene from ATCC 4875 - Reverse Primer	5′-GGTCCAT*ATG*CCTGGGACGCAGAAC-3′	SEQ. ID No. 3
Amplification of CoA transferase gene from NC_014215 - Forward Primer	5'-AGGAGAAATTCCATGAACGAACGCATCTCC-3'	SEQ. ID No. 4
Amplification of CoA transferase gene from NC_014215 - Reverse Primer	5'-TGAGAGTGCACCATAATCACCCGGAAAACCATTG-3'	SEQ. ID No. 5
Propionibacterium acnes CoA transferase gene	GenBank Accession No. Q6A647	SEQ. ID No. 6
Saccharopolyspora erythraea CoA transferase gene (strain NRRL 2338)	GenBank Accession No. A4FLX0	SEQ. ID No. 7
Corynebacterium efficiens CoA transferase gene	GenBank Accession No. Q8FMP3	SEQ. ID No. 8
Azoarcus sp. (strain BH72) CoA transferase gene	GenBank Accession No. A1KAR7	SEQ. ID No. 9
Pseudoalteromonas atlantica T6c CoA transferase gene	GenBank Accession No. Q15P97	SEQ. ID No. 10
P. freudenreichii subsp. *Shermanii* CoA transferase gene	Gene from *P. freudenreichii* subsp. *shermanii* (GenBank Accession No. NC_014215)	SEQ. ID No. 11

DETAILED DESCRIPTION

[0041] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety for all purposes. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0042] Any publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0043] The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way. In addition, the materials, methods, and examples are illustrative only and not intending to be limiting. The use of the singular includes the plural unless specifically stated otherwise. Also, the use of “comprise,” “comprises,” “comprising,” “contain,” “contains,” “containing,” “include,” “includes,” and “including” are not intended to be limiting. It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention. The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[0044] All numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical

parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

[0045] As utilized in accordance with the embodiments provided herein, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0046] The term "plasmid" refers to a circular nucleic acid vector. Generally, plasmids contain an origin of replication that allows many copies of the plasmid to be produced in a bacterial (or sometimes eukaryotic) cell without integration of the plasmid into the host cell DNA.

[0047] The term "construct" as used herein refers to a recombinant nucleotide sequence, generally a recombinant nucleic acid molecule, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences. In general, "construct" is used herein to refer to a recombinant nucleic acid molecule.

[0048] The term "host cell" refers to a cell that is to be transformed using the methods and compositions of the invention. In general, host cell as used herein means a microorganism cell into which a nucleic acid of interest is to be transformed.

[0049] The term "transformation" refers to a permanent or transient genetic change, preferably a permanent genetic change, induced in a cell following incorporation of non-host nucleic acid sequences. Transformation (or transduction, or transfection), can be achieved by any one of a number of means including electroporation, conjugation, microinjection, biolistics (or particle bombardment-mediated delivery), or *agrobacterium* mediated transformation.

[0050] The term "vector" generally refers to a polynucleotide that can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes, that are able to replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can

be an organism which comprises one or more of the above polynucleotide constructs such as an *agrobacterium*.

[0051] The term "promoter" refers to a minimal nucleic acid sequence sufficient to direct transcription of a nucleic acid sequence to which it is operably linked. The term "promoter" is also meant to encompass those promoter elements sufficient for promoter-dependent gene expression controllable for cell-type specific expression or inducible by external signals or agents; such elements may be located in the 5′ or 3′ regions of the naturally-occurring gene.

[0052] The term "native" or "wild-type" as used with a protein, enzyme, polynucleotide, gene, or cell, means a protein, enzyme, polynucleotide, gene, or cell that occurs in nature.

[0053] Accordingly, metabolically "engineered" or "modified" organisms are produced via the introduction of genetic material into a host or parental microorganism of choice thereby modifying or altering the cellular physiology and biochemistry of the microorganism. Through the introduction of genetic material the parental microorganism acquires new properties, e.g., the ability to produce a new, or greater quantities of, an intracellular metabolite. In an illustrative embodiment, the introduction of genetic material into a parental microorganism acquires new properties, e.g., the ability to produce a new, or greater quantities of, an intracellular metabolite. In an illustrative embodiment, the introduction of genetic material into a parental microorganism results in a new or modified ability to produce propionic acid. The genetic material introduced into the parental microorganism contains gene(s), or parts of genes, coding for one or more of the enzymes involved in a biosynthetic pathway for the production of propionic acid and may also include additional elements for the expression and/or regulation of expression of these genes, e.g. promoter sequences.

[0054] An engineered or modified microorganism can also include in the alternative or in addition to the introduction of a genetic material into a host or parental microorganism, the disruption, deletion, or knocking out of a gene or polynucleotide to alter the cellular physiology and biochemistry of the microorganism. Through the reduction, disruption or knocking out of a gene or polynucleotide the microorganism acquires new or improved properties (e.g., the ability to produce new or greater

quantities of an intracellular metabolite, improve the flux of a metabolite down a desired pathway, and/or reduce the production of undesirable by-products).

[0055] Microorganisms provided herein are modified to produce metabolites in quantities not available in the parental organism. A "metabolite" refers to any substance produced by metabolism or a substance necessary for taking part in a particular metabolic process. A metabolite can be an organic compound that is a starting material (e.g., glucose), an intermediate (e.g., succinate) in, or an end product (e.g., propionic acid) of metabolism.

[0056] Referring to **Figure 1**, the metabolic pathway for propionic acid synthesis in Propionibacteria is shown, where the following numbers identify the following: (1) Pyruvate kinase; (2) Pyruvate dehydrogenase or Pyruvate synthase; (3) Phosphate acetyl transferase; (4) Acetate kinase; (5) Phosphoenolpyruvate carboxykinase or Phosphoenolpyruvate carboxylase; (6) Methylmalonyl-CoA carboxytransferase; (7) Malate dehydrogenase; (8) Fumarate hydratase; (9) Succinate dehydrogenase; (10) Propionyl-CoA:succinate CoA transferase; (11) Methylmalonyl-CoA mutase; and (12) Methylmalonyl-CoA epimerase. Propionyl-CoA:succinate CoA transferase, or Co-A transferase, is the enzyme that catalyzes the transfer of CoA from propionyl-CoA to succinic acid and results in the production of propionic acid.

[0057] The DNA sequence of the gene encoding propionyl-CoA:succinate CoA transferase (CoA transferase), EC#2.8.3.- (1512 bp) in *P. acidipropionici* ATCC 4875 is identified and has been given GenBank accession No. FJ641063. The CoA transferase gene is obtained by PCR amplification and then overexpressed in *E. coli* BL21(DE3) through transformation with expression plasmid pET-CoA. The *E. coli* transformants exhibit increased CoA transferase activity compared to the wild-type *E. coli*. The gene sequence and upstream/downstream nucleotide sequences are analyzed. An alignment of the CoA transferase gene identified in *P. acidipropionici* and several reported CoA transferases from *Propionibacterium acnes*, *Saccharopolyspora erythraea*, *Corynebacterium efficiens*, *Azoarcus sp.*, and *Pseudoalteromonas atlantica* identifies several highly conserved amino acid fragments.

[0058] The CoA transferase gene is also overexpressed in *P. acidipropionici* ATCC 4875 to create mutants capable of enhanced propionic acid production. First,

pGEM-CoA vector is created using the CoA transferase gene fragment from *P. acidipropionici*. Then the CoA transferase gene is released from the pGEM-CoA vector and is ligated with pKHEM01 and pKHEM04 to construct the overexpression plasmids pKH01COA and pKH04COA, respectively. These plasmids are transferred into *P. acidipropionici* to create mutants capable of comparatively higher propionic acid production as well as higher resistance to propionic acid inhibition and better tolerance to acidic pHs.

[0059] Metabolic engineering of *P. freudenreichii*subsp. s*hermanii* is also performed to create mutants capable of increased propionic acid production, as well as greater inhibition to propionic acid and higher tolerance to acidic pHs. The mutants are engineered by first ligating the CoA transferase gene from *P. shermanii* into pKHEM01 and pKHEM04 plasmids to construct plasmids pKH01COA and pKH04COA, respectively.

[0060] *P. shermanii* cells are then transformed with pKH01COA and pKH04COA to create mutant strains overexpressing the CoA transferase gene. The mutant strains exhibit increased CoA transferase enzyme activity in comparison to the wild-type *P. shermanii* strain. During fermentation, the mutants exhibit higher propionic acid yield and productivity than the wild-type, as well as increased propionic acid/acetic acid and propionic/succinic acid ratios in comparison with the wild-type.

[0061] The *P. acidipropionici* and *P. shermanii* mutants are able to produce propionic acid during fermentation using any carbohydrate or carbon source as a substrate. Carbohydrates used as substrate may include lactose, sucrose, glucose, fructose, maltose, xylose, and any other monosaccharides, disaccharides, oligosaccharides, or polysaccharides. Other carbon sources that may be used as substrates include glycerol, lactate and pyruvate.

[0062] EXAMPLES

[0063] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those skilled in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the

art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

[0064] Bacterial strains and growth conditions

[0065] Bacterial strains and plasmids used in the examples are listed in Table 1.

[0066] Table 1: Bacterial strains and plasmids used in the examples

Strains or plasmids	Description	Reference or source
Strains		
P. acidipropionici	Wild type	ATCC 4875
P. freudenreichii subsp. *shermanii*	Wild type	DSM 4902
E. coli BL21(DE3)	Cm^r	Novagen
E. coli DH5α		Invitrogen
Plasmids		
pETDuet-1	Ap^r; ColE1 replicon; T7 promoter	Merck
pGEM-T	Ap^r; for cloning of PCR products	Promega
pGEM-CoA	1.6 kb PCR product in pGEM-T	The examples
pET-CoA	1.5 kb NcoI/NdeI fragment from pGEM-CoA in pETDuet-1	The examples
pKHEM01	Shuttle vector pPK705 with *hemA* gene, P138 promoter, hyg^r	Murooka et al., 2001
pKHEM04	Shuttle vector pPK705 with *hemA* gene, P4 promoter, hyg^r	Murooka et al., 2001
pKH01COA	pKHEM01 with CoA transferase, P138 promoter	The examples
pKH04COA	pKHEM04 with CoA transferase, P4 promoter	The examples

Cm^r: chloramphenical resistance; Ap^r: ampicillin resistance; ColE1: *E. coli*replicon; hyg^r: hygromycin B resistance; *hemA*: 5-aminolevulinic acid synthase

[0067] Example 1: Metabolic Engineering of *Propionibacterium acidipropionici*

[0068] Bacterial strains and culture media

[0069] *P. acidipropionici* was cultured anaerobically at 32℃ in a medium containing 1% (w/v) yeast extract, 0.5% trypticase, 0.025% K_2HPO_4, 0.005% $MnSO_4$, and an appropriate amount of carbon source. All *E. coli* strains were grown at 37℃ aerobically in 250 ml flasks or anaerobically in 150 ml serum bottles containing Luria broth (LB) with 100 μg/ml of ampicillin when necessary. Both recombinant and wild-type strains were stored at -85℃ in 15% (v/v) glycerol.

[0070] DNA isolation and manipulation

[0071] Chromosome DNA of *P. acidipropionici*, used as PCR template, was isolated using the QIAGEN genomic DNA kit (Qiagen, Valencia, CA). Plasmid isolation from *E. coli* was performed using QIAprep®MiniPrep plasmid purification kit (Qiagen). DNA fragments for subcloning were extracted from gel by QIAquick gel extraction kit. Restriction endonucleases and T4 DNA ligase were purchased from Invitrogen and used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

[0072] PCR amplification and construction of plasmids

[0073] PCR primers were designed using the software Primer 3 based on the DNA sequence of the CoA transferase gene obtained from 454 sequencing of *P. acidipropionici* ATCC 4875. The CoA transferase gene has a gene length of 1512 bp, a protein length of 503 amino acids, and a molecular weight of 55kDa. The nucleotide sequence of the CoA transferase gene has been submitted to the GenBank database under accession no. FJ641063 (SEQ. ID No. 1). The complete nucleotide and deduced amino acid sequences of the CoA transferase gene, which begins with a methionine codon and ends with a TGA stop codon, are presented in **Figure 2**. In Fig. 2, the bold letters represent ribosome binding sites, open arrows represent inverted repeats, double-underlined letters represent the -35 and -10 regions, and single-underlined letters indicate termination sequences.

[0074] The G + C content in the open reading frame (ORF) of the CoA transferase gene is 64%, which is in the G + C content range of 53-67% previously

reported for the genus propionibacteria. The DNA alignment was determined by using an EMBOSS program (http://www.ebi.ac.uk/Tools/emboss/align) and shows significant homology (Identity: 82.9%) between *P. acidipropionici* CoA transferase and the succinyl-CoA or butyryl-CoA:coenzyme A transferase from *P. acnes*(GenBank accession no. Q6A647).

[0075] The distances between the CoA transferase gene and the upstream or downstream adjacent gene ORFs are 161 bp and 186 bp, respectively, indicating that these three neighboring genes may not constitute an operon. In addition, the annotated up- and down-stream genes were putative aldose 1-epimerase subfamily and nucleoside 5'-monophosphate phosphohydrolase, respectively, which may not be related to CoA transferase or CoA hydrolase. These results suggest that CoA transferase in *P. acidipropionici* consists of one polypeptide, which is similar to the propionate CoA transferase from *Clostridium propionicum*. Compared to *P. acnes*, the upstream vicinity genes of *P. acidipropionici* CoA transferase gene have the same gene arrangement order (rRNA small subunit methyltransferase → phosphoserine phosphatase → aldose 1-epimerase family protein → CoA transferase) and high similarities (>62%) in their protein amino acid sequences. However, there is no similarity observed in the downstream vicinity of the gene.

[0076] As shown in Fig. 2, the putative ribosomal binding site (Shine-Dalgarno sequence, AGGAGG) is located 4 nucleotides upstream of the start codon AUG. Two consensus sequences, CGCACA and CCGTTG, were found at the upstream of ORF corresponding to putative -35 and -10 regions, respectively, by comparison of the consensus promoter sequences (-35, C-A/G-N-A/C-A; -10, A/G-T-G/C-T-T-G) of *Propionibacterium freudenreichii*. Two inverted repeats (11 bp and 12 bp) were found in the promoter region. No consensus sequences of ACGCGCA or 5'-TG-3' were observed, corresponding to putative -16 regions, in the promoter element. Several inverted repeats (AGCTCGGTTCTGAGCT; GGCCCCGACCACCAGGCCGGGGCC) were found in the downstream of the CoA transferase gene, 9 bp and 32 bp distances from the TGA stop codon.

[0077] Amino acid sequence comparison

[0078] The deduced amino acid sequence of *P. acidipropionici* CoA transferase indicates that this protein has a calculated molecular mass of 55.2 kDa and an isoelectric point (pI) of 5.62. The predicted amino acid sequence was searched through the protein database using the BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast). Significant homology is observed between the query sequence of *P. acidipropionici* and the Coenzyme A transferase/hydrolase of several prokaryotic species. Except for the highest identity (83%) to the putative succinyl-CoA or butyryl-CoA:coenzyme A transferase from *P. acnes*, the range of identity to other prokaryotic CoA transferases was approximately 49-61%.

[0079] Referring to **Figure 3**,the alignment of *P. acidipropionici* CoA transferase with analogous CoA transferasesfrom *Propionibacterium acnes* (GenBank accession no. Q6A647),*Saccharopolysporaerythraea* (strain NRRL 2338)(GenBank accession no. A4FLX0),*Corynebacteriumefficiens (GenBank*accession no. Q8FMP3),*Azoarcus sp.* (strain BH72)(GenBank accession no. A1KAR7) and *Pseudoalteromonas atlantica* T6c (GenBank accession no. Q15P97). This alignment was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2). From the results, several highly conserved regions were located. In Fig. 3, identical residues among four of the six proteins are shown in black. A "*" identifies that residues in that particular column are identical in all sequences in the alignment; ":" identifies where conserved substitutions have been observed, and "." means that semi-conserved substitutions are observed.

[0080] A glycine cluster (D-G-D-Q-I-*G*-F-**G**-**G**-**F**-T-G-S-**G**-Y-**P**) (Fig.3, region "1") was observed in the N-terminal amino acid sequence of putative propionyl-CoA: succinate CoA transferase of *P. acidipropionici*. This cluster is significantly identical to the consensus sequence (Prosite PS01273: [DN]-[GN]-x(2)-[LIVMFA](3)-**G**-**G**-**F**-x(3)- **G**-x-**P**) reported by Wierenga et al. One more glycine was found in the *P. acidipropionici* glycine cluster (italic letter). Another conserved glycine-rich region (**G**-I-**G**-**G**-S-**G**) was found in the amino acid sequence (Fig. 3, region "2", which was highly similar to the reported conserved motif (**G**-x-**G**-x-x-**G**) for nucleotide binding.

[0081] Referring to **Figure 4**, the overall construction of the expression plasmid pET-CoA containing the CoA transferase gene from *P. acidipropionici* is shown. The

primers used in the PCR amplification of the CoA transferase gene are shown in Table 2.

[0082] Table 2: Primers used in PCR amplification of CoA transferase gene.

Primer	DNA Sequence
Amplification of CoA transferase gene from ATCC 4875 - Forward Primer (SEQ. ID No. 2).	5'-AGGAC*CC*ATG*G*CAGATCGGATTGCCAAC-3'
Amplification of CoA transferase gene from ATCC 4875 - Reverse Primer (SEQ. ID No. 3).	5'-GGTC*CAT**ATG***CCTGGGACGCAGAAC-3'

[0083] Three nucleotides A, G, and T were substituted by C, C and G (represented in italics) respectively to generate an internal *Nco*I restriction site (underlined) in the forward primer (5'-AGGAC*CC*ATG*G*CAGATCGGATTGCCAAC-3'). In the reverse primer (5'-GGTC*CAT**ATG***CCTGGGACGCAGAAC-3'), two nucleotides, C and G, were substituted by A and T respectively (represented in italics) to generate an internal *Nde*I restriction site (underlined). The reaction system (50 μl) contained 5 μl of 10×PCR buffer, 1 μl of 10 mMdNTP, 1.5 μl of 50 mM $MgCl_2$, 1.5 μl of 10 μM forward and reverse primers, 2 μl of genomic DNA, 2.5 μl of PCR enhancer, and 1 μl of Platimum®*Pfx*DNA polymerase (Invitrogen, Carlsbad, CA). The reaction parameters were set as the following: one cycle at 95°C for 10 min followed by 10 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 30 s, and extension at 72°C for 1 min and then 20 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 30 s, and extension at 72°C for 1 min. Subsequently, 1 μl of *Taq*DNA polymerase was added in the reaction system and incubated for another 30 min, 4°C hold. The PCR products with an expected size of ~1.5 kb were purified via 0.7% (w/v) agarose gel electrophoresis. The gel extracted PCR fragment was ligated into pGEM-T vector (Promega, Madison, WI) and verified by restriction enzyme analysis and DNA sequencing.

[0084] DNA sequencing and analysis

[0085] DNA fragment sequencing of plasmids was determined by the dideoxy chain termination method performed at the Plant-Microbe Genomics Facility (PMGF), the Ohio State University. The whole genome of *P. acidipropionici* ATCC 4875 was sequenced into 45 contigs by using 454 sequencing technology and annotated at J. Craig Venter Institute (Maryland).

[0086] Cloning CoA transferase gene in *E. coli*

[0087] As illustrated in Fig. 4, the PCR product containing the open reading frame of CoA transferase was inserted into pGEM-T vector to construct plasmid pGEM-CoA. Then, the CoA transferase gene was cut using *Nco*I-*Not*I digestion and ligated into the same sites of pETDuet-1 (Merck, Darmstadt, Germany), downstream of *T7* promoter, to form plasmid pET-CoA. The recombinant plasmid, pET-CoA, was transformed into *E. coli* BL21(DE3). The recombinant mutants were selected by ampicillin resistance.

[0088] Gene expression

[0089] For protein overexpression experiments, 100 μg/ml of ampicillin and 30 μg/ml of chloroamphenicol were supplemented in LB medium when *E. coli* BL21(DE3) harboring pET-CoA was cultivated under aerobic or anaerobic conditions. 0.5 ml of overnight culture was inoculated in 4.5 ml of fresh LB medium and incubated at 37℃ until its OD_{600} reached 0.6. Then, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the medium and cultured at 16℃ for another 2 hours. The induced cells were harvested by centrifugation at 6,000×g for 5 min at 4℃ (Eppendorf Centrifuge 5415R, Eppendorf, Hamburg, Germany).

[0090] Cell extracts preparation and enzyme assays

[0091] About 50 ml of cells in the exponential phase were harvested by centrifuging at 6,000×g for 10 min and washed three times, and then resuspended in 3 mL of ice cold Tris/HCl buffer (25 mM, pH 7.4). The cell suspension was then ultrasonicated using a sonic dismembrator (Fisher Scientific, Model 100). Samples were kept in an ice bath during cell disruption. Sonication was conducted for 5 seconds followed by 25 seconds of resting to prevent overheating, total 20 cycles, and then centrifuged at 16,000×g, 4℃ for 1 h to remove cell debris. Cell extracts were kept cold on ice before they were used in the enzyme activity assay. The protein content of the

extracts was determined in triplicate by Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard protein.

[0092] The activity of CoA transferase was assayed based on the method of Schulman and Wood. The reaction was conducted at 25°C, followed by measuring the absorbance at 340 nm, which increased linearly with time for 3-5 minutes. A cuvette lacking CoA transferase was used as a blank control. One standard unit of CoA transferase is defined as the amount of enzyme causing an absorbency change of 1.0 per minute, and the specific activity is defined as units per milligram of protein.

[0093] SDS-PAGE

[0094] Protein samples or cell pellets were mixed with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) loading buffer and put in boiling water for 10 min. After 10 min of centrifugation at 16,000×g, 4°C, the supernatant was used for SDS-PAGE (Mini-PROTEAN® 3 Cell, Bio-Rad) according to the protocol of Bio-Rad. The gels were stained with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO).

[0095] Overexpression of the CoA transferase gene in *E. coli*

[0096] To overexpress the CoA transferase of *P. acidipropionici* in *E. coli* BL21(DE3), the PCR reaction was carried out using the genomic DNA of *P. acidipropionici* as a template. Once the construction of pET-CoA was complete, the sequence of the CoA transferase gene fragment from PCR amplification was confirmed by the dideoxy chain termination method. The recombinant *E. coli* BL21(DE3) cells harboring pET-CoA were cultured at 16°C under both aerobic and anaerobic conditions with the presence of 0.5 mM of IPTG. Protein samples were prepared and the supernatants of protein solution were analyzed with SDS-PAGE.

[0097] Referring to **Figure 5**, SDS-PAGE analysis of cloned CoA transferase gene expression in *E. coli* BL21(DE3) is shown. Lanes 1-4 are cells grown aerobically, and lanes 5-7 are cells grown anaerobically. Lane 1:BL21 without IPTG induction; lanes 2, 5: BL21 with IPTG induction; lanes 3,6: BL21 harboring pET-CoA without IPTG induction; lanes 4, 7: LB21 harboring pET-CoA with IPTG induction. Arrows indicate the overexpressed CoA transferase, and MW is molecular weight markers (kDa). As can be seen in Figure 5, CoA transferase gene was overexpressed under both aerobic and

anaerobic growth conditions after IPTG induction (lanes 4 and 7). The distinct bands in Lanes 4 and 7 have the same molecular mass of approximately 55 kDa as that of *P. acidipropionici* CoA transferase.

[0098] Crude cell lysate of *E. coli* BL21 (DE3) harboring pET-CoA was assayed for CoA transferase activity. The propionyl-CoA: succinate CoA transferase activity was determined by measuring the formation rate of acetyl-CoA, which equals to the rate of formation of NADH in the presence of malate dehydrogenase and citrate synthase. The enzyme assay was performed with cells cultured under anaerobic conditions. To reduce the background activity of the host, rifampin was added to selectively inhibit the host RNA polymerase. Referring to **Figure 6**, a chart showing the expression of CoA transferase in *E. coli* BL21(DE3) wild-type and transformant harboring pET-CoA (transformant with and without rifampin). As shown in Figure 6, propionyl-CoA: succinate CoA transferase activity was detected in the recombinant carrying the plasmid pET-CoA under these conditions, whereas negligible activity was detected in the wild-type host. Furthermore, CoA transferase activity was further increased in the presence of rifampin.

[0099] Construction of CoA transferase overexpression plasmids pKH01COA and pKH04COA

[00100] Referring to **Figure 7**, construction of the overexpression plasmids pKH04CoA and pKH01CoA are shown. PCR amplification methods described above were used to create a PCR fragment of the CoA transferase gene that was inserted into pGEM-T vector and verified by restriction enzyme analysis and DNA sequencing. The CoA gene fragment was then released from pGEM-T vector by digestion with *NcoI* and *NdeI*, and then it was ligated by T4 DNA ligase with NcoI/NdeI digested pKHEM01 or pKHEM04. The overexpression plasmids pKH01COA and pKH04COA were thus constructed from pKHEM01 and pKHEM04, respectively.

[00101] Electroporation and selection of transformants (mutants)

[00102] The next step was to transfer the overexpression plasmids pKH01COA and pKH04COA into *P. acidipropionici*. An appropriate volume of overnight culture of *P. acidipropionici* was inoculated into 50 ml of fresh NLB medium to reach OD_{600} = ~ 0.05. The cells were cultured at 32℃ until OD_{600} = ~ 0.8. Then cells were harvested by

centrifugation and washed with 0.5 volume of 1 mM HEPES buffer (pH 7.0). The cell pellet was resuspended in 0.1 volume of pre-chilled 10% glycerol and incubated on ice for 30 min. After centrifugation, cells were resuspended in 0.02 volume of pre-chilled 10% glycerol. 100 µl aliquot was mixed with 1 µg plasmid and transferred into a pre-chilled 0.2-cm electroporation cuvette. Electroporation was performed by using the following conditions: 12.5 kV/cm, 25 µF, 100 Ω. After electroporation, cells were diluted with 900 µl NLB medium and incubated at 32℃ for 8 h. An appropriate volume of culture was then plated on NLB agar containing 250 µg/ml hygromycin B. Plates were incubated in anaerobic chamber at 32℃ for 7-10 days until colonies formed. Two colonies, designated as Pa-01CoA and Pa-04CoA, were picked and tested for their ability to produce propionic acid.

[00103] Gene expression in Pa-01CoA and Pa-04CoA mutants

[00104] An enzyme assay of wild-type (WT) *P. acidipropionici* and mutants Pa-01CoA and Pa-04CoA was performed. The results are illustrated in **Figure 8**. The specific enzyme activity of CoA transferase of *P. acidipropionici* wild type strain was 174.9 U/mg protein, whereas the CoA transferase activity of the Pa-01CoA mutant was higher at 182.9 U/mg. The CoA transferase activity of the Pa-04CoA mutant was even higher at 214.1 U/mg. This confirms that CoA transferase was overexpressed and functional in the *P. acidipropionici* mutants Pa-01CoA and Pa-04CoA.

[00105] Batch fermentations of glucose by *P. acidipropionici* wild type and mutants overexpressing CoA transferase were studied in serum bottles and a 5-liter fermentor. Referring to **Figures 9**, **10**, and **11**, the kinetics of propionic acid fermentation of glucose in serum bottles by *P. acidipropionici* wild-type (WT) (Fig. 9) and the mutants overexpressing CoA transferase, Pa-01CoA (Fig. 10) and Pa-04CoA (Fig. 11) is shown. The results are summarized in Table 3 below.

[00106] Table 3: Batch fermentation kinetics of wild-type and mutants in serum bottles without pH control.

	Serum bottle		
	WT	Pa-01CoA	Pa-04CoA
Product yield (g/g)			
Propionic acid	0.33	0.36	0.39

Acetic acid	0.056	0.052	0.059
Productivity (g/L·h)			
Propionic acid	0.08	0.074	0.072
Acetic Acid	0.004	0.006	0.007
Specific growth rate (h^{-1})	0.17	0.14	0.13
P/A	6.2	6.5	6.3
Final propionic acid (g/L)	4.6	6.0	6.2
Final $O.D._{600}$	8.9	9.3	9.8
Final pH	4.2	4.0	4.0

[00107] As shown in Figs. 9 - 11 and Table 3 above, the mutants overexpressing CoA transferase produced more propionic acid than the wild-type *P. acidipropionici* did.

[00108] Referring to **Figures 12**, **13**, and **14**, kinetics of propionic acid fermentation of glucose in batch fermentation by *P. acidipropionici* wild-type (WT) (Fig. 12) and the mutants overexpressing CoA transferase, Pa-01CoA (Fig 13) and Pa-04CoA (Fig. 14) in 5-L stirred-tank fermentors is shown. The results are summarized in Table 4 below. Pa-04CoA-1 and Pa-04CoA-2 represent two batches of fermentation using the same Pa-04CoA mutant.

[00109] Table 4: Batch fermentation kinetics of wild-type and mutants in stirred-tank fermentors.

	Bioreactor			
	WT	Pa-01CoA	Pa-04CoA-1	Pa-04CoA-2
Product yield (g/g)				
Propionic acid	0.29	0.36	0.43	0.34
Acetic acid	0.061	0.054	0.074	0.065
Productivity (g/L·h)				
Propionic acid	0.34	0.34	0.49	0.38
Acetic Acid	0.068	0.058	0.078	0.075
Specific growth rate (h^{-1})	0.16	0.17	0.19	0.17
P/A	2.6	3.4	3.0	2.9
Final propionic acid (g/L)	15.8	17.5	20.5	16.3
Final $O.D._{600}$	14.7	13.2	14.7	14.1

[00110] As shown in Figs. 12 - 14 and Table 4 above, the mutants overexpressing CoA transferase produced more propionic acid than the wild-type *P. acidipropionici* did. The increased propionic acid production was attributed to the higher resistance to propionic acid inhibition and better tolerance to acidic pHs. Referring to **Figures 15**, **16**, **and 17**, the effects of pH on the growth rate of *P. acidipropionici* wild-type (Fig. 15) and Pa-01CoA (Fig. 16) and Pa-04CoA (Fig. 17) mutants are shown. As shown, both mutants were able to maintain higher cell densities than the wild-type strain at acidities ranging from pH 4.0 to pH 6.5. Referring to **Figure 18**, Panel A, the relative specific growth rates of *P. acidipropionici* wild-type and Pa-01CoA and Pa-04CoA mutants are shown at different propionic acid concentrations. As shown in Fig. 18, the mutants exhibited higher growth rates than the wild-type *P. acidipropionici* as the acid concentration was increased. Panel B of Fig. 18 is a chart showing how the inhibition constant, K_i, and the maximum specific growth rate, μ_{max}, are calculated. In the chart of Panel B, the slope of each line is $1/ \mu_{max}K_i$ and the intersection on the Y axis is $1/ \mu_{max}$. P is the concentration of propionate. The relationship between μ_{max}, K_i, P, and μ is set by Equation 1:

[00111] Equation 1: Specific Growth Rate

$$\mu = \frac{\mu_{max} K_i}{K_i + P} \quad \text{or} \quad \frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{1}{\mu_{max} K_i} P$$

[00112] The values of μ_{max} (h^{-1}) and K_i (g/L) for the strains are as follows: WT: μ_{max}, .103, K_i, 18.3; Pa-01CoA: μ_{max}, .122, K_i, 24.5; Pa-04CoA: μ_{max}, .134, K_i, 21.7.

[00113] Example 2: Metabolic Engineering of *Propionibacterium freudenreichii*

[00114] Bacterial strains and culture media

[00115] *P. freudenreichii* subsp. *Shermanii* (*P. shermanii*) DSM 4902 was cultured anaerobically at 32°C in the NLB medium containing 1% yeast extract, 1% trypticase and 1% sodium lactate. All *E. coli* strains were grown at 37°C aerobically in 250 ml flasks or anaerobically in 150 ml serum bottles containing Luria broth (LB) with 100 µg/ml of ampicillin when necessary. Both recombinant and wild-type strains were stored at -85°C in 15% (v/v) glycerol.

[00116] DNA isolation and manipulation

[00117] Chromosome DNA of *P. shermanii*, used as PCR template, was isolated using the Promega Wizard® Genomic DNA Purification Kit. Plasmid isolation from *E. coli* and propionibacteria was performed using QiagenQIAprep®MiniPrep plasmid purification kit. Vectors were purified by QiagenQIAquick® gel extraction kit and PCR fragments for subcloning were purified usingQiagenQIAquick® PCR purification kit. Restriction endonucleases were purchased from New England Biolabs and used according to the manufacturer's instructions. Ligation of PCR product with vectors was performed using Clontech In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA).

[00118] Construction of overexpression plasmids

[00119] PCR primers used for the construction of overexpression plasmids are shown in Table 5 below.

[00120] Table 5: Primers used in PCR amplification of CoA transferase gene.

Primer	DNA Sequence
Amplification of CoA transferase gene from NC_014215 - Forward Primer (SEQ. ID No. 4)	5'-AGGAGAAATTCCATGAACGAACGCATCTCC-3'
Amplification of CoA transferase gene from NC_014215 - Reverse Primer (SEQ. ID No. 5)	5'- TGAGAGTGCACCATAATCACCCGGAAAACCATTG-3'

[00121] PCR primers were designed based on the DNA sequence of propionyl-CoA: succinate CoA transferase (CoA transferase) gene of *P. shermanii*, Accession NO. NC_014215 (SEQ. ID No. 11). The reaction system (50 µl) contained 25 µl of 2×PCR buffer, 2.5 µl of 10 µM forward and reverse primers, 0.5 µl of genomic DNA and 19.5 µl ddH_2O. The reaction parameters were set as: initial denaturation at 98℃ for 2 min followed by 35 cycles of denaturation at 98℃ for 10 s, annealing at 59℃ for 30 s, and extension at 72℃ for 50 s and the final extension at 72℃ for 10 min. The PCR product was purified usinga PCR purification kit. pKHEM01 and pKHEM04 plasmids were digested with *NcoI* and *NdeI*. Enzyme digest products were gel extracted. Purified CoA transferase gene was ligated with *NcoI* and *NdeI* digested linear pKHEM01 and pKHEM04 plasmids using In-Fusion® HD Cloning Kit to construct pKH01COA and pKH04COA.

[00122] Electroporation of propionibacteria

[00123] *P. shermanii* was precultivated in NLB media overnight at 32℃. Electrocompetent cells were prepared from this preculture by diluting 1:50 (50 ml) in NLB media and further cultivating for 16 hours at 32℃ (OD_{600}~0.8). The culture was placed on ice for 20 min and then centrifuged at 5000×g for 4 min (4℃). The cells were washed twice by 50 ml ice cold sterile ddH_2O and 25 ml ice cold sterile 10% glycerol, respectively. The cells were resuspended in 0.5 ml ice cold sterile 10% glycerol and dispensed in 70 µl aliquots. Electroporation of propionibacteria was performed by using a Bio-Rad Gene Pulser apparatus. 70 µl competent cells were mixed with 2 µg overexpression plasmids and incubated in 0.1 cm electroporation cuvette on ice for 10 min. The electroporation parameters were set as 200 Ω resistance and 25 µF capacitance at 25 kV/cm and an electric pulse was delivered. 1.5 ml NLB media was immediately mixed with electroporated cells and incubated in an anaerobic tube at 32℃ for 4 hours. Cell suspension was centrifuged and plated on NLB agar plate supplemented with 250 µg/ml hygromycin B. The plates were incubated in an anaerobic jar at 32℃ for 5-7 days and transformants could be detected.

[00124] Cell extracts preparation and enzyme assay

[00125] About 50 ml of cells in the exponential phase were harvested by centrifuging at 6,000×g for 10 min and washed three times, and then resuspended in 3 mL of ice cold Tris/HCl buffer (25 mM, pH 7.4). The cell suspension was mixed with 0.1 mm silica beads in 2 ml centrifuge tube. Cells were disrupted by using a bead beater. After 10 cycles, cells were centrifuged at 16,000×g, 4℃ for 1 h to remove cell debris. Cell extracts were kept cold on ice before they were used in the enzyme activity assay. The protein content of the extracts was determined in triplicate by Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard protein. The activity of CoA transferase was assayed based on the method of Schulman and Wood. The assay mixture (250 µl) contained 0.1 ml of Mixture 1 (1.0 M, pH 8.0 Tris/HCl buffer, 1 ml; 0.4 M sodium malate, 0.01 ml; 0.01 M NAD, 1.0 ml; water to 4 ml), 0.01 ml of 1.5 M sodium acetate, 0.01 ml of Mixture 2 (944 unit/mg of malic dehydrogenase, 14 µl; 355 unit/mg of citrate synthase, 11 µl; 0.1M, pH 6.8 phosphate buffer, 975 µl), 0.15 µmole (in 0.01 ml) of succinyl-CoA, 0.05 ml of cell extract, and water to 0.25 ml. The reaction was

conducted at 25℃, followed by measuring the absorbance at 340 nm, which increased linearly with time for 3-5 minutes. A cuvette lacking CoA transferase was used as a blank control. One standard unit of CoA transferase is defined as the amount of enzyme causing one micromole NADH increase per minute, and the specific activity is defined as units per milligram of protein.

[00126] SDS-PAGE

[00127] Protein samples were mixed with 2×Laemmli loading buffer (Bio-Rad) and put in boiling water for 10 min. After 10 min centrifugation at 16,000×g, 4℃, the supernatant was used for SDS-PAGE (Mini-PROTEAN® 3 Cell, Bio-Rad) according to the protocol of Bio-Rad. The gels were stained with Coomassie brilliant blue.

[00128] Fermentation kinetics

[00129] *P. shermanii* wild type strain and mutants were cultivated in 50 ml fermentation media containing 1% yeast extract, 0.5% trypticase, 0.025% K2HPO4, 0.005% MnSO4, 10 g/l glucose and 20 g/l glycerol. The fermentation was performed in serum bottles with sealed caps. Nitrogen gas was used to purge the media to establish anaerobic atmosphere and 1 g $CaCO_3$ was added in each bottle to control the pH. Seed media was prepared by cultivating cells in NLB media to late exponential phase and 2 ml seed media was inoculated into serum bottle to initiate fermentation. Samples were withdrawn from bottles regularly and analyzed with HPLC (Shimadzu).

[00130] Gene expression in Pfs-01CoA and Pfs-04CoA mutants

[00131] Referring to **Figure 19**, the enzyme activity of wild-type (WT) *P. shermanii* and the Pfs-01CoA and Pfs-04CoA mutants is shown. The Pfs-01CoA mutant has been filed with the ATCC prior to the filing of this application. It was received by the ATCC in Manassas, Virginia on November 3, 2011 and has been granted ATCC No. _____. The specific enzyme activity of CoA transferase of *P. shermanii* wild type strain was 69.8 U/mg protein, whereas the CoA transferase activity of mutant strain harboring pKH01COA (Pfs-01CoA mutant) was much higher at 133.4 U/mg. The CoA transferase activity of mutant strain harboring pKH04COA (Pfs-04CoA mutant) was also higher at 94.7 U/mg. Compared with the wild type strain, the specific enzyme activities for two overexpression mutants increased 91% and 36%, respectively. This confirms that CoA transferase was overexpressed and functional in *P. shermanii* mutants.

[00132] Referring to **Figure 20**, the results of SDS-PAGE of protein extracts from wild type, and Pfs-01CoA and Pfs-04CoA mutants are shown. The protein band with a molecular mass of 56 kDa, as indicated by the arrow, was higher for both mutants, indicating that the CoA transferase was overexpressed. The *in vivo* overexpression of CoA transferase was hence verified.

[00133] Two Pfs-01CoA mutants were selected and named Pfs-01CoA-5 and Pfs-01CoA-9. These two mutants and *P. shermanii* wild type strain were studied for their fermentation kinetics in serum bottles. For both mutants, hygromycin B (250 µg/ml) was supplemented in the fermentation media. To test the plasmid stability and the effect of antibiotics selection pressure, the fermentation with Pfs-01CoA-9 was also performed without hygromycin B in the medium. The fermentation profiles for the wild-type and mutants under various conditions are shown in **Figures 21** through **24** (Fig. 21, wild-type; Fig. 22, Pfs-01CoA-5 mutant; Fig. 23: Pfs-01CoA-9 mutant; and Fig. 24: Pfs-01CoA-9 without hygromycin B)**.** The propionic acid yield, productivity, and substrate consumption rates in these fermentations were calculated and are listed in Table 6 below.

[00134] **Table 6:** Comparison of fermentation performance between the wild type (WT) and mutants overexpressing CoA transferase (Pfs-01C0A-5 and Pfs-01C0A-9 with and without hygromycin B in the medium).

	WT	Pfs-01CoA-5	Pfs-01CoA-9	Pfs-01CoA-9 w/o
Propionic acid yield (g/g)	0.49	0.54	0.53	0.51
Propionate productivity (g/L·h)	0.077	0.101	0.098	0.075
Propionate/Acetate ratio (w/w)	3.5	5.0	4.8	4.1
Propionate/Succinate ratio (w/w)	8.4	14.9	15.9	9.4
Glucose consumption rate (g/L·h)	0.113	0.083	0.085	0.093
Glycerol consumption rate (g/L·h)	0.062	0.104	0.102	0.073

Total carbon consumption rate (g/L·h)	0.175	0.187	0.187	0.166

[00135] Both mutant strains showed significantly higher propionic acid yield and productivity than the wild type. **Figures 25** and **26** show the relative yields and productivity of the wild type and mutants during fermentation, respectively. Compared with wild type, the propionic acid yield increased ~10% and the productivity increased ~30% for both mutants (with hygromycin B in the medium). The increased propionic acid production can be attributed to the increased CoA transferase activity in these mutants, which allowed more carbon fluxes through the propionic acid biosynthesis pathway than the acetate pathway. Referring to **Figures 27** and **28**, the ratios of propionic acid to acetic acid and propionic acid to succinic acid are shown, respectively, for the different strains. As shown, the propionic acid/acetic acid ratio in the mutants increased 43% to ~5.0 from 3.5 for the wild type. The propionic acid/succinic acid ratio for the mutant also increased 80% to ~15 from 8.4 for the wild type. The significantly higher propionic acid/succinic acid ratio can be attributed to more and faster CoA groups being transferred from propionyl-CoA to succinate. The higher concentration ratio of propionic acid to byproducts is important as it would ease the downstream separation process for propionic acid purification from the fermentation broth.

[00136] For all strains, glucose and glycerol were consumed simultaneously in the co-substrate fermentation. However, the mutants showed different consumption pattern. Referring to **Figure 29**, a comparison of carbon consumption rates by wild-type and the mutant strains during co-substrate fermentation is shown. As shown in both Table 6 and **Figure 29**, the wild-type strain had higher glucose consumption rate than glycerol, whereas both mutant strains had higher glycerol consumption rate than glucose. CoA transferase catalyzes propionic acid formation and its overexpression diverted more carbon flow toward NADH consuming propionic acid synthesis pathway. As a result, glycerol, providing more reducing power than glucose, became a preferential substrate for mutants. For the Pfs-01CoA-9 mutant cultivated without hygromycin B, it also showed better fermentation performance than the wild type.

[00137] Other Embodiments

[00138] The foregoing description and Examples detail certain specific embodiments of the invention and describe the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear, the invention can be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

WHAT IS CLAIMED IS:

1. A method of metabolically engineering a microorganism capable of propionic acid production, comprising the steps of:

   obtaining a microbial host capable of propionic acid production;

   transferring a gene that encodes propionyl-CoA:succinate CoA transferase into the microbial host.

2. The method of Claim 1, wherein the gene that encodes propionyl-CoA:succinate CoAtransferase is a DNA comprising the base sequence of SEQ. ID No. 1 or a DNA which hybridizes to a DNA comprising the base sequence of SEQ. ID No. 1 or a complimentary base sequence of SEQ. ID No. 1 under stringent conditions and which encodes a polypeptide having propionyl-CoA:succinate CoA transferase activity.

3. The method of Claim 1, wherein the gene that encodes propionyl-CoA:succinate CoAtransferase is a DNA comprising the base sequence of SEQ. ID No. 11 or a DNA which hybridizes to a DNA comprising the base sequence of SEQ. ID No. 11 or a complimentary base sequence of SEQ. ID No. 11 under stringent conditions and which encodes a polypeptide having propionyl-CoA:succinate CoA transferase activity.

4. The method of Claim 1, wherein the microbial host is selected from the group consisting of *P. acidipropionici* and *P. freudenreichii* subsp. *shermanii*.

5. The method of Claim 1, wherein the transferring step is performed by transfecting the microbial host with pKH04COA.

6. The method of Claim 1, wherein the transferring step is performed by transfecting the microbial host with pKH01COA.

7. A method for producing propionic acid, comprising the steps of:

obtaining a microbial host having a metabolic pathway capable of producing propionic acid;

transfecting the microbial host with a vector containing a gene for expressing propionyl-CoA:succinate CoA tranferase;

fermenting the transfected microbial host in a medium comprising a substrate;

recovering propionic acid.

8. The method of claim 7, wherein the substrate is selected from the group consisting of: glucose, lactose, sucrose, xylose, fructose, and maltose.

9. The method of claim 7, wherein the substrate is selected from the group consisting of: glycerol, lactate and pyruvate.

10. The method of claim 7, wherein the microbial host is selected from the group consisting of *P. acidipropionici* and *P. freudenreichii* subsp. *shermanii*.

11. The method of claim 7, wherein the gene for expressing propionyl-CoA:succinate CoA transferase is obtained from *P. acidipropionici*.

12. The method of claim 7, wherein the gene for expressing propionyl-CoA:succinate CoA transferase is obtained from *P. freudenreichii* subsp. *shermanii*.

13. A metabolically engineered microorganism capable of producing propionic acid, said microorganism capable of overexpressing CoA transferase.

14. The metabolically engineered microorganism of Claim 13, wherein the microorganism is transformed with a CoA transferase gene from *P. acidipropionici*.

15. The metabolically engineered microorganism of Claim 13, wherein the microorganism is transformed with a CoA transferase gene from *P. freudenreichii* subsp. *shermanii*.

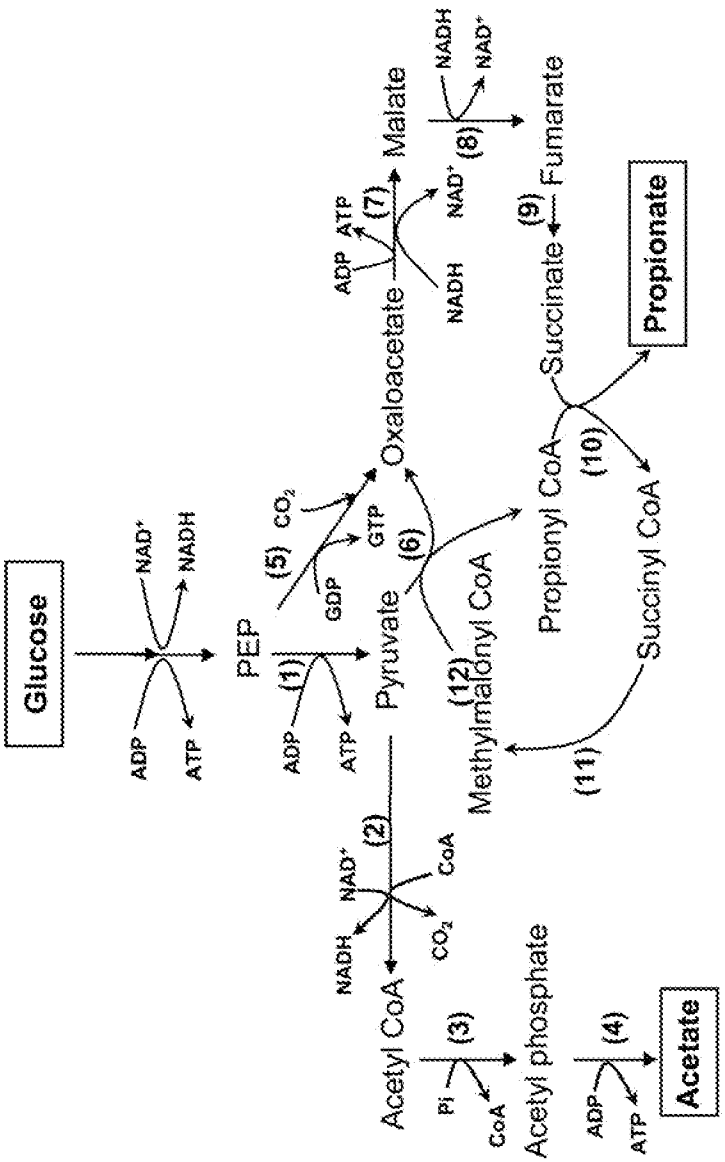

Glucose
NAD+
NADH
ADP
ATP
PEP
(1)
ADP
ATP
Pyruvate
(2)
NAD+
CoA
NADH
CO2
Acetyl CoA
(3)
Pi
CoA
Acetyl phosphate
(4)
ADP
ATP
Acetate
(5)
CO2
GDP
GTP
Oxaloacetate
(6)
Methylmalonyl CoA
(7)
ADP
ATP
NADH
Malate
(8)
NADH
NAD+
NAD+
Fumarate
(9)
Succinate
Propionyl CoA
(10)
Propionate
(11)
Succinyl CoA
(12)

FIG. 1

FIG. 2

FIG. 3

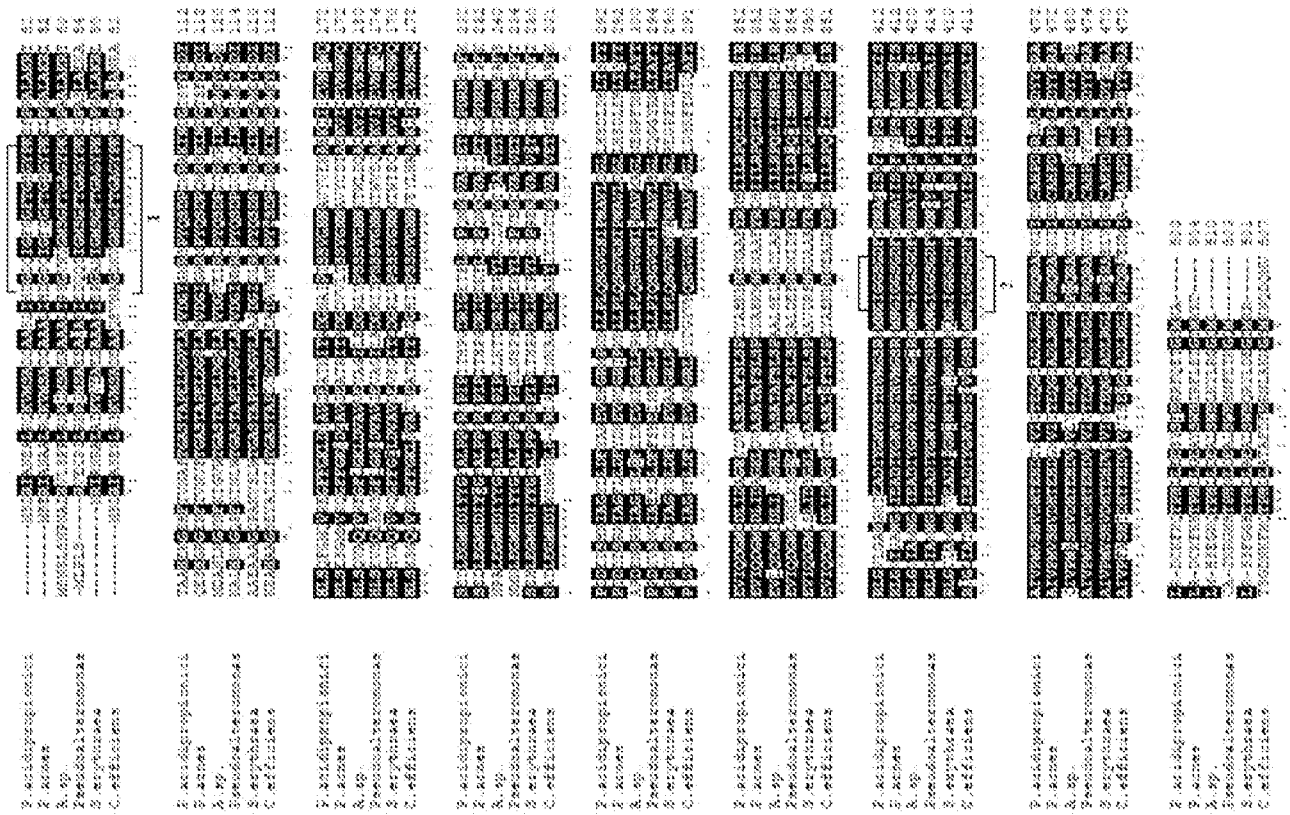
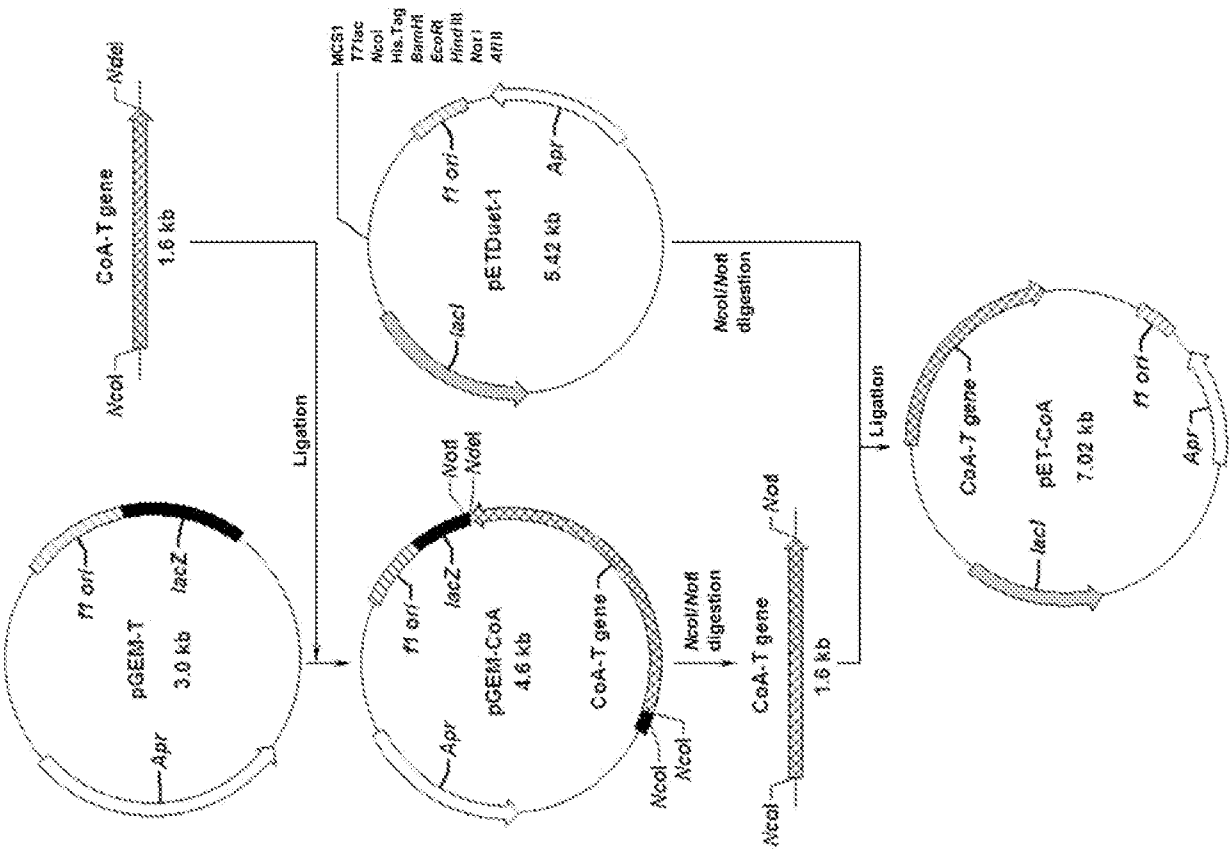


FIG. 4


CoA-T gene
NdeI
NcoI
1.6 kb
Ligation
MCS1
T7lac
NcoI
His.Tag
BamHI
EcoRI
HindIII
NotI
XhoI
f1 ori
lacI
pETDuet-1
5.42 kb
Apr
NcoI/NotI digestion
f1 ori
lacZ
pGEM-T
3.0 kb
Apr
NcoI
NdeI
f1 ori
lacZ
pGEM-CoA
4.6 kb
Apr
CoA-T gene
NcoI
NcoI
NcoI/NotI digestion
CoA-T gene
NotI
1.6 kb
NcoI
Ligation
CoA-T gene
pET-CoA
7.02 kb
lacI
f1 ori
Apr

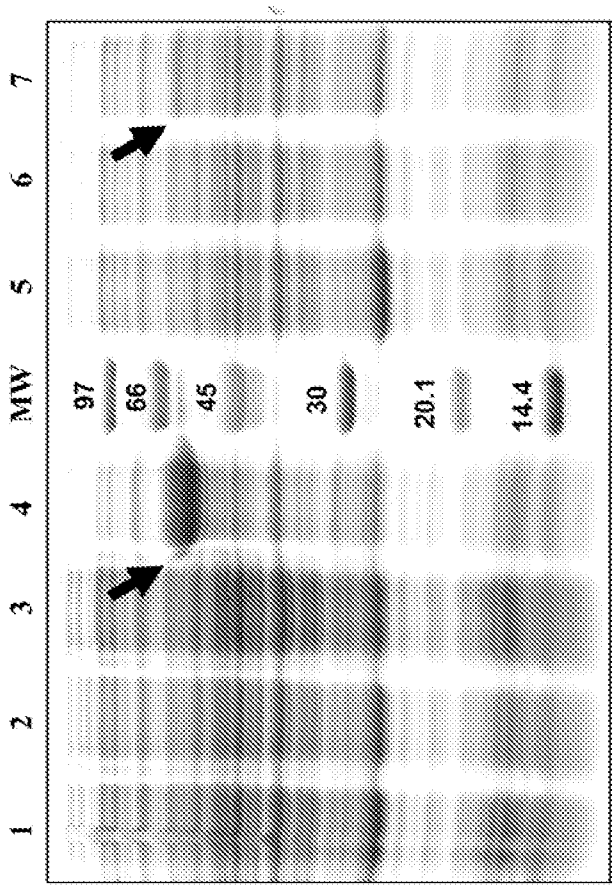


FIG. 5

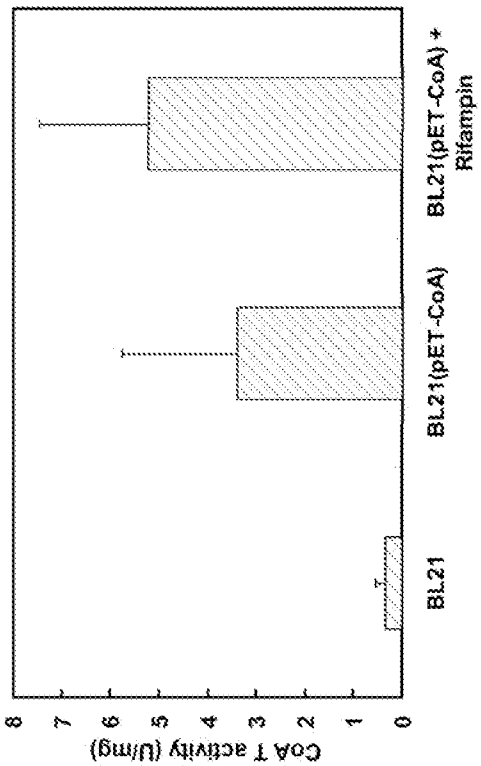

CoA T activity (U/mg)
0
1
2
3
4
5
6
7
8
BL21
BL21(pET-CoA)
BL21(pET-CoA) + Rifampin

FIG. 6

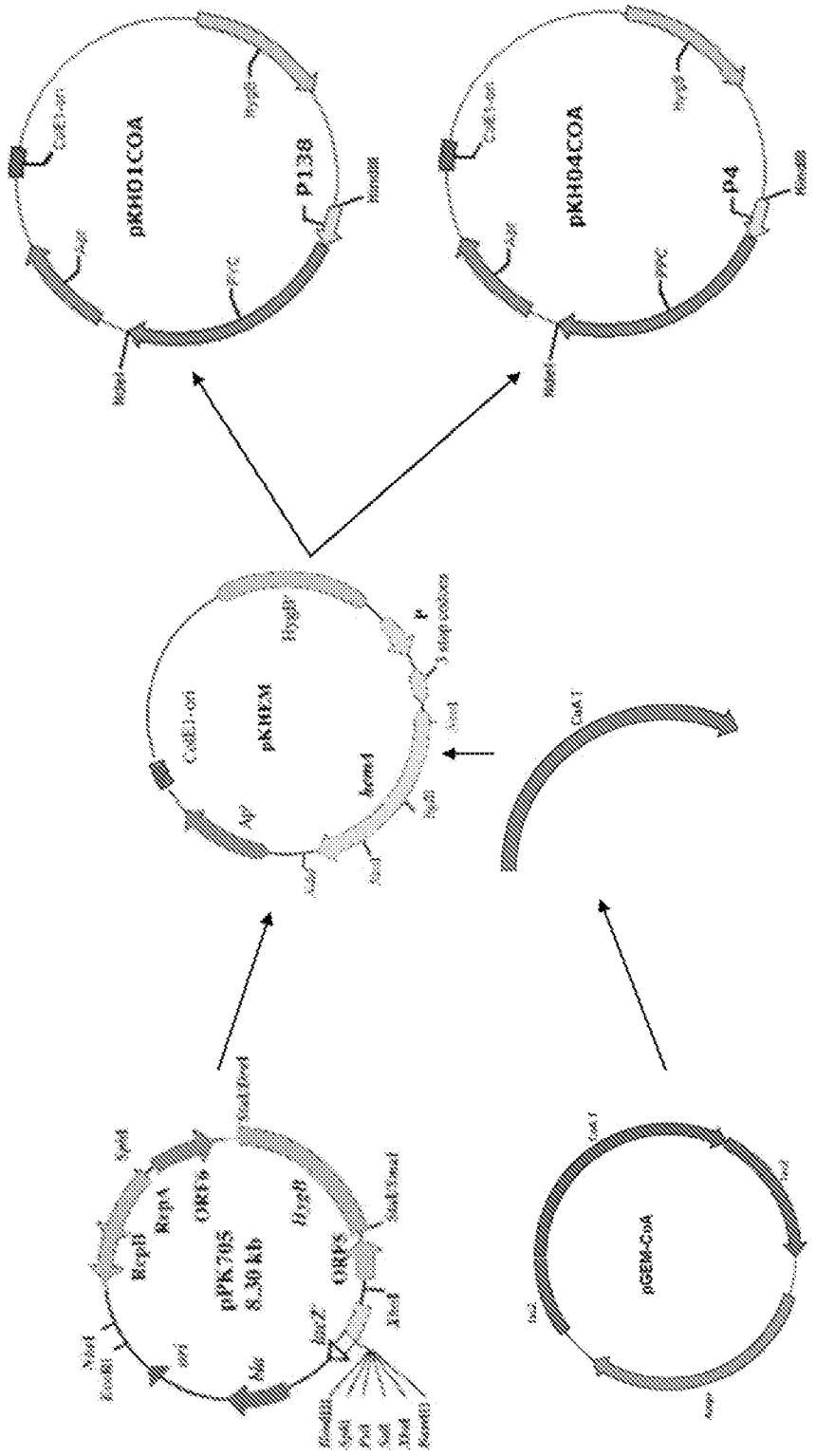


FIG. 7

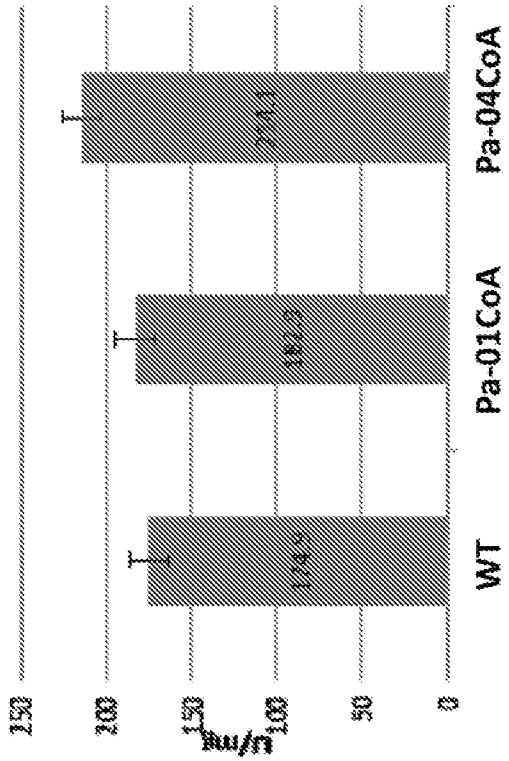


FIG. 8

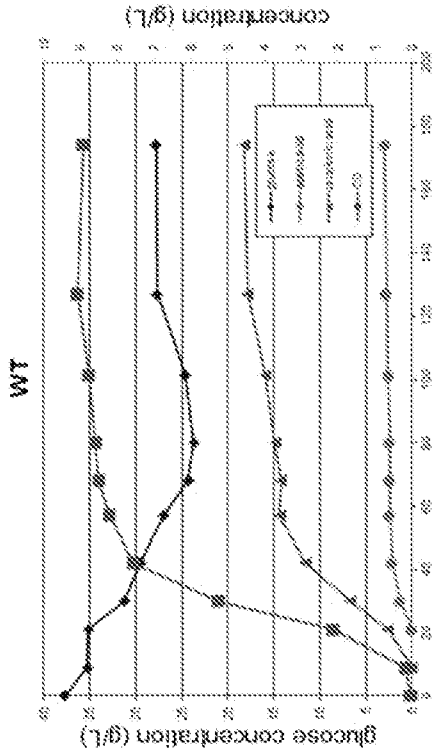


FIG. 9

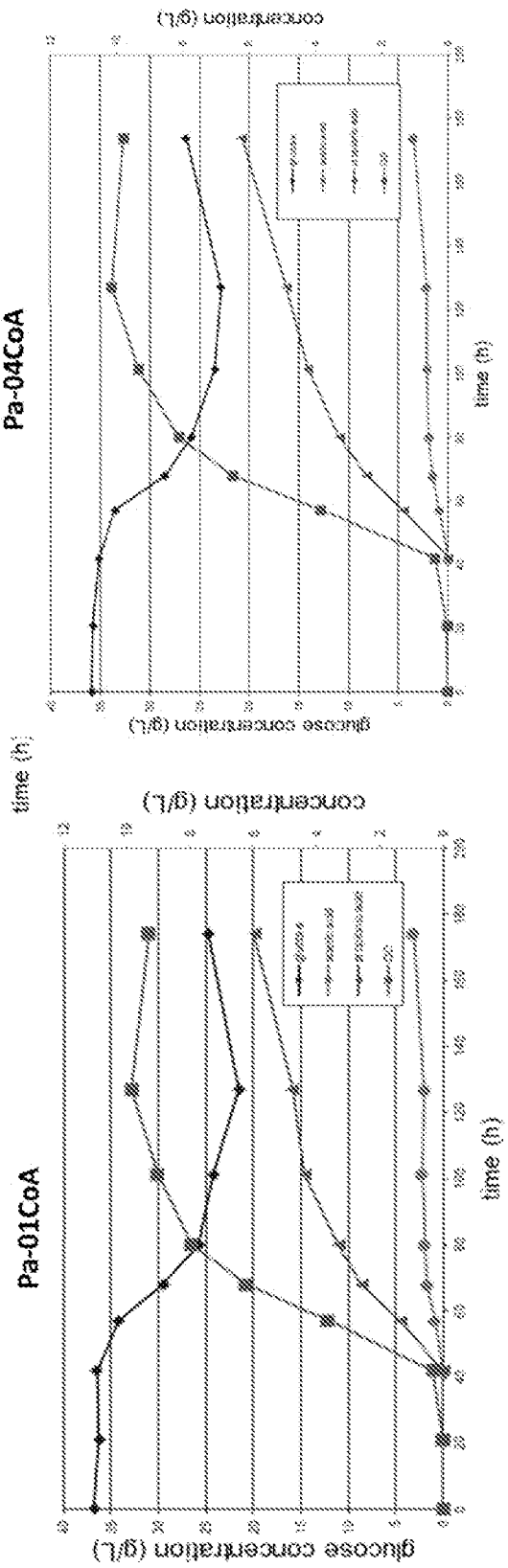


FIG. 10

FIG. 11

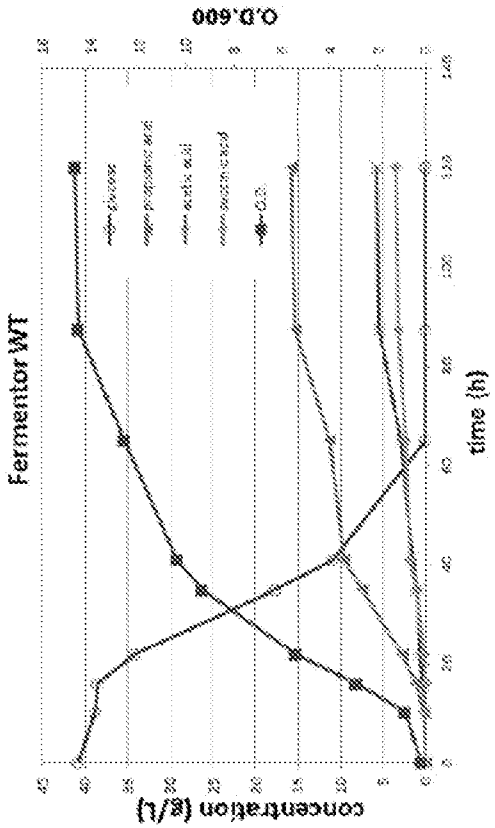


FIG. 12

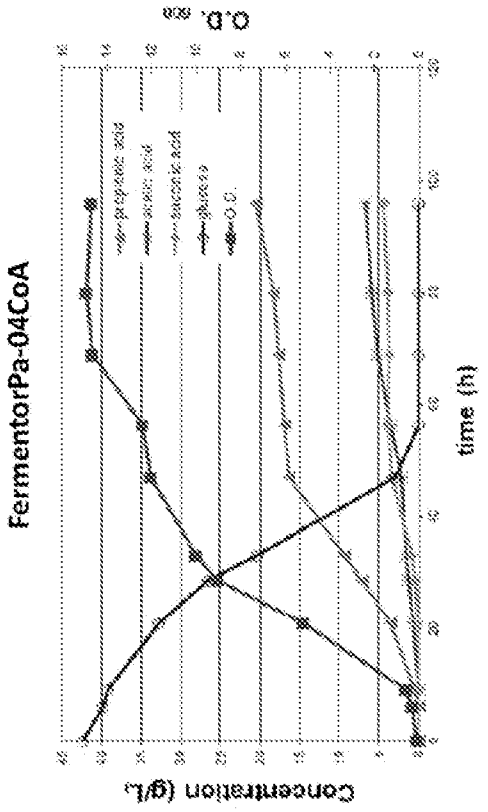


FIG. 14

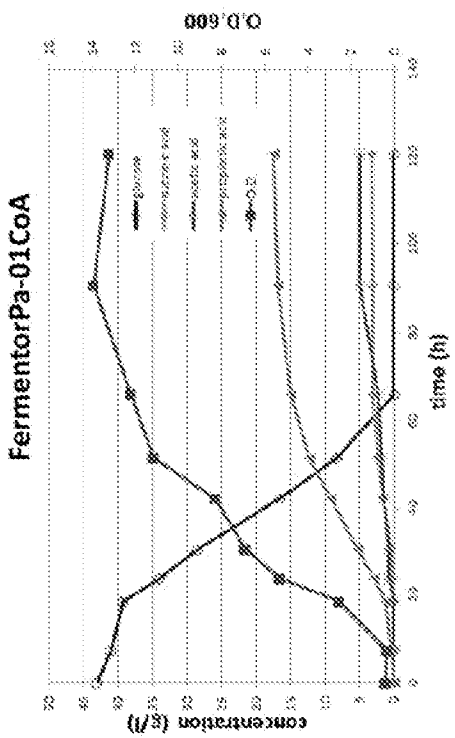


FIG. 13

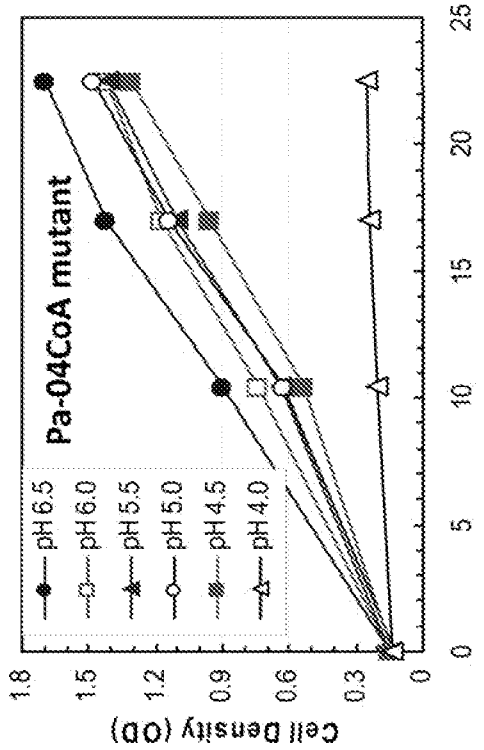


FIG. 17

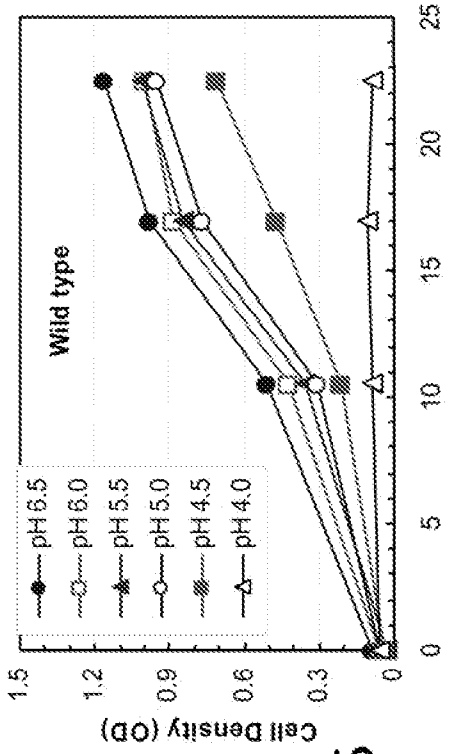


FIG. 15

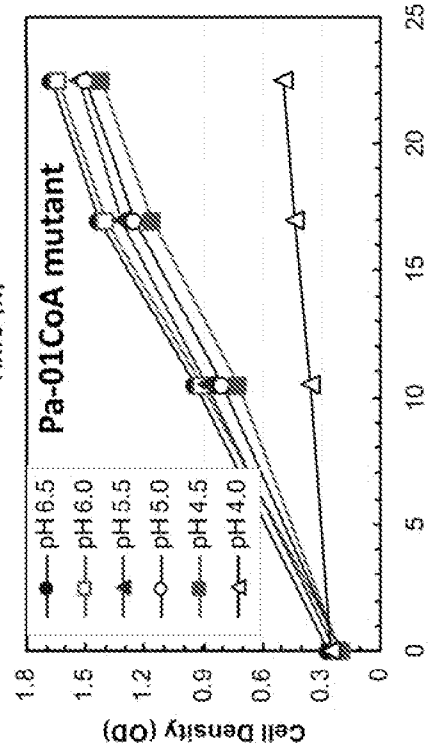


FIG. 16

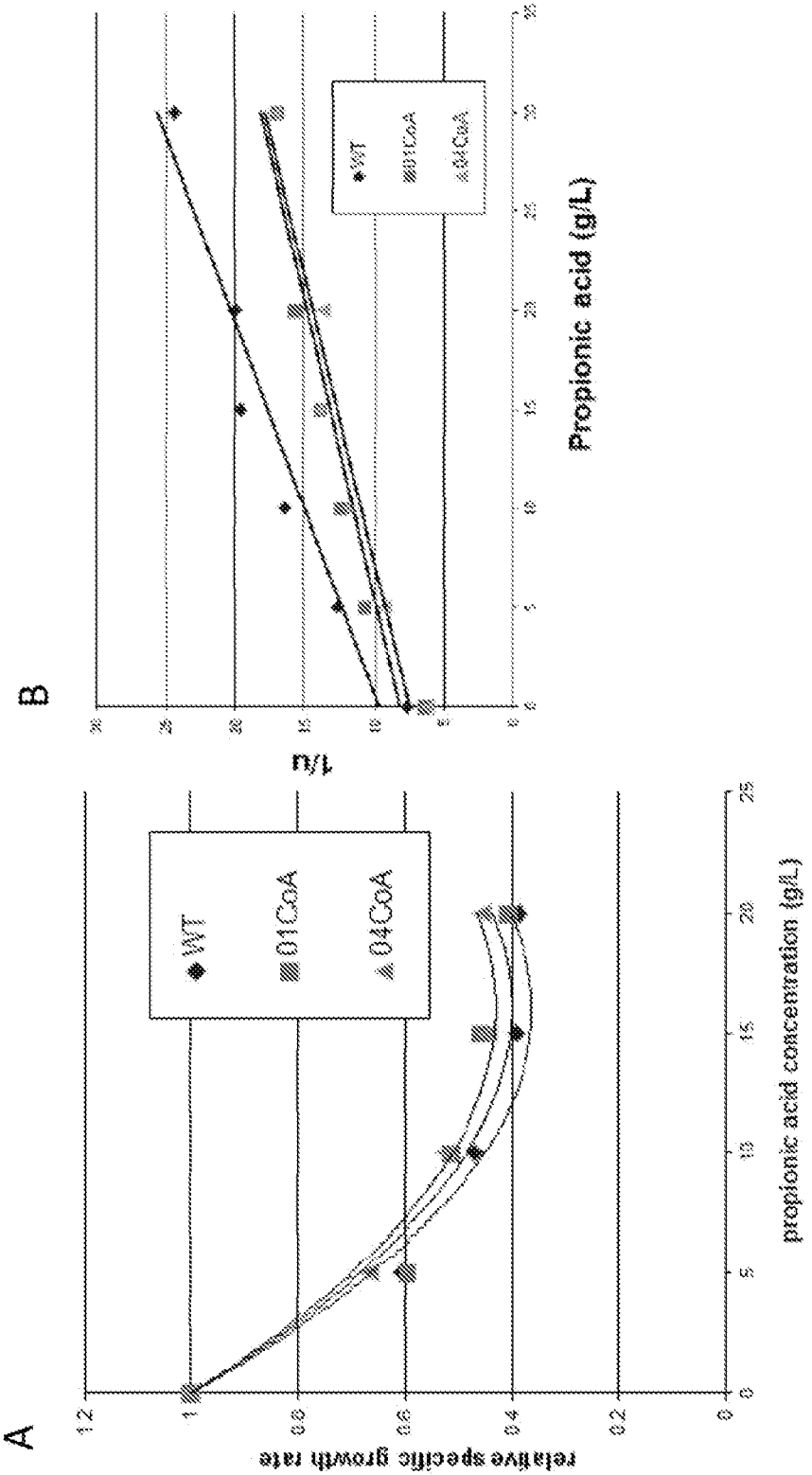

A
relative specific growth rate
propionic acid concentration (g/L)
WT
01CoA
04CoA
B
1/u
Propionic acid (g/L)
WT
01CoA
04CoA

FIG. 18

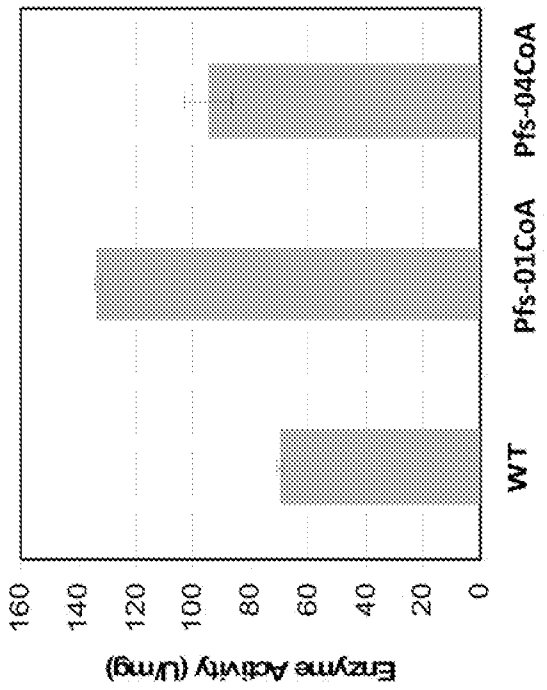

Enzyme Activity (U/mg)
160
140
120
100
80
60
40
20
0
WT
Pfs-01CoA
Pfs-04CoA

FIG. 19

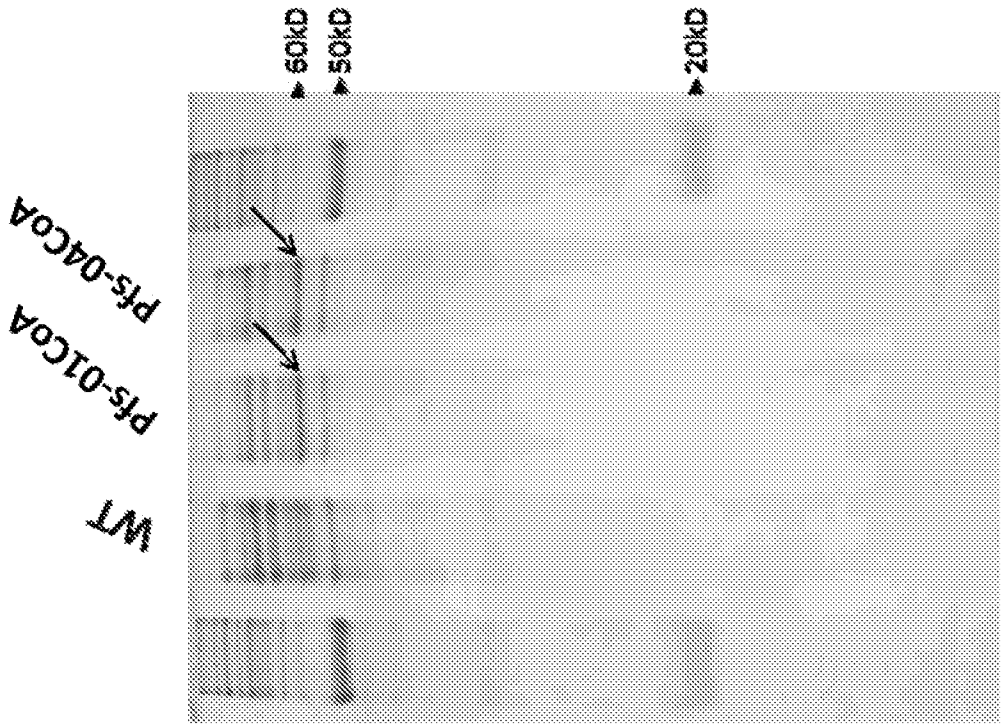


FIG. 20

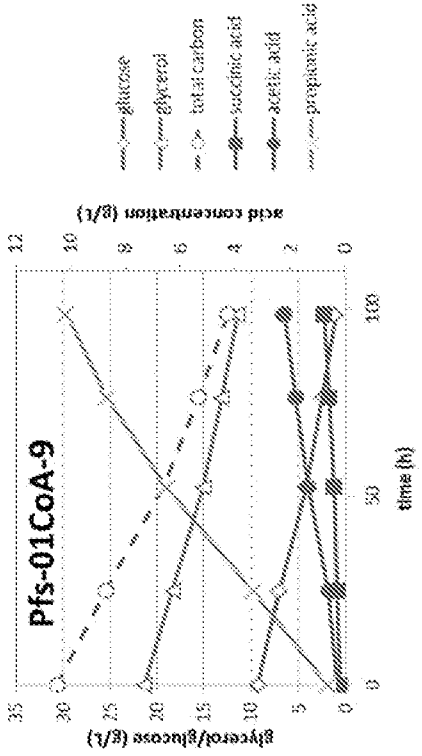


FIG. 23

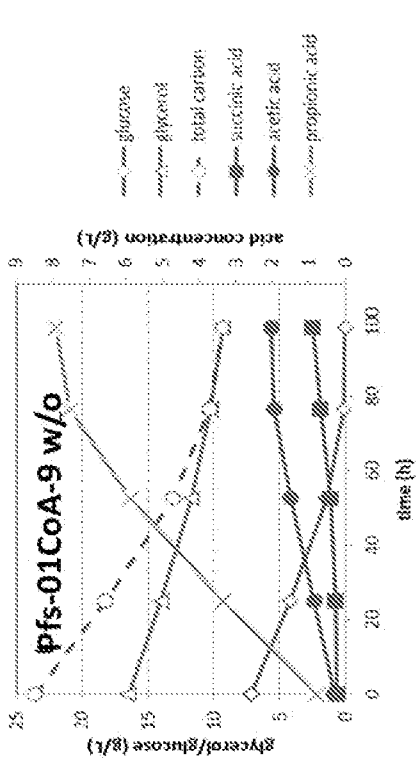


FIG. 24

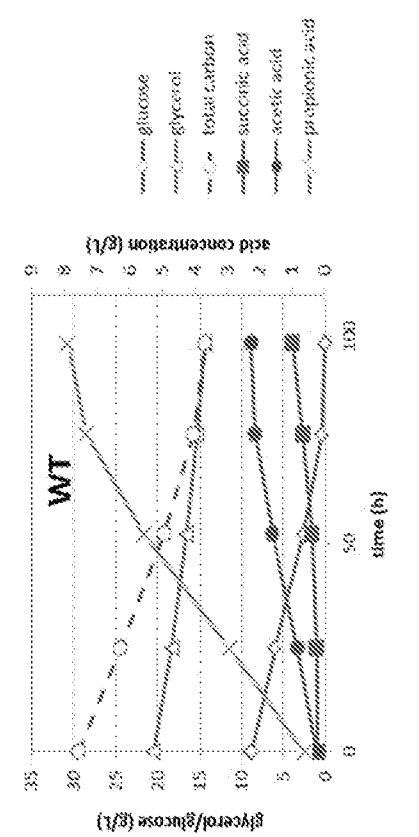


FIG. 21

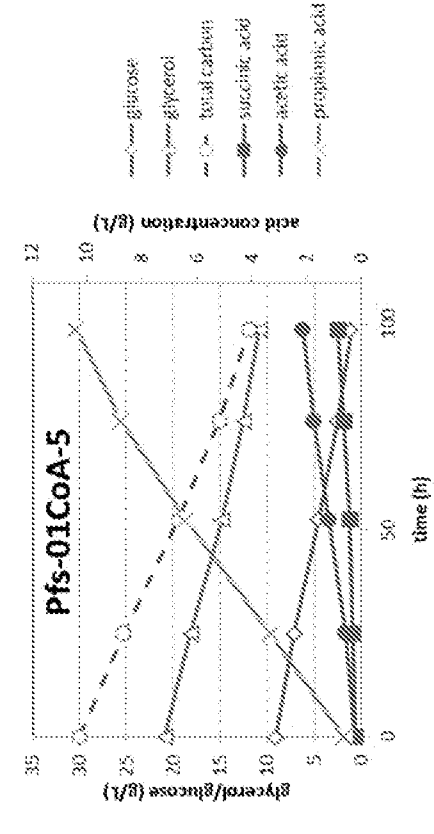


FIG. 22

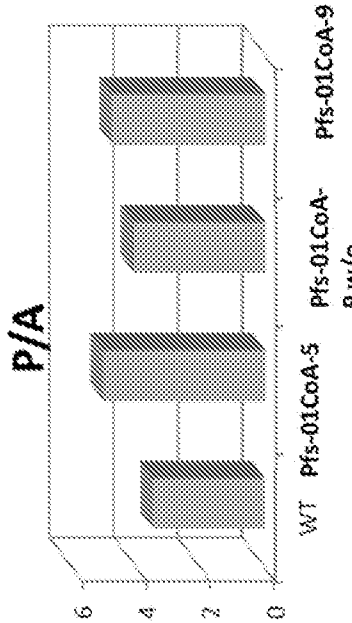


FIG. 27

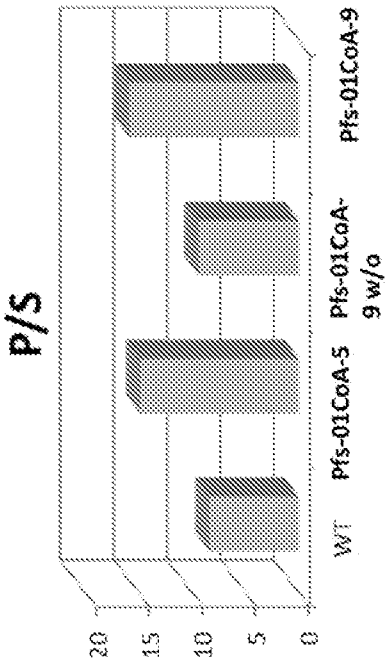


FIG. 28

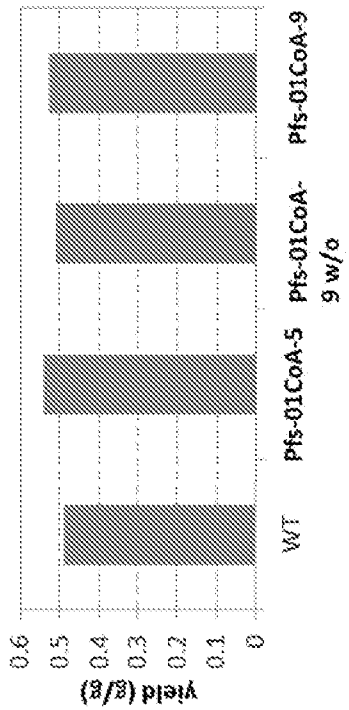


FIG. 25

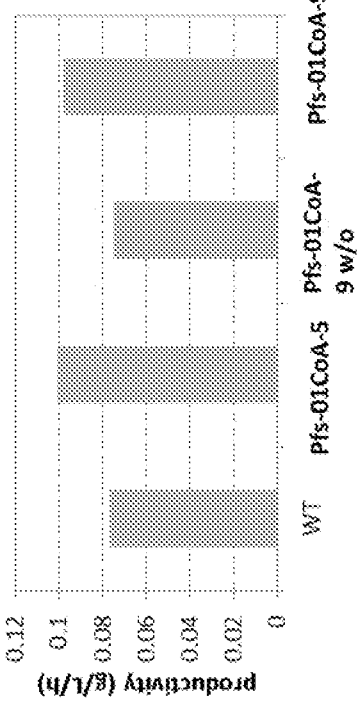


FIG. 26

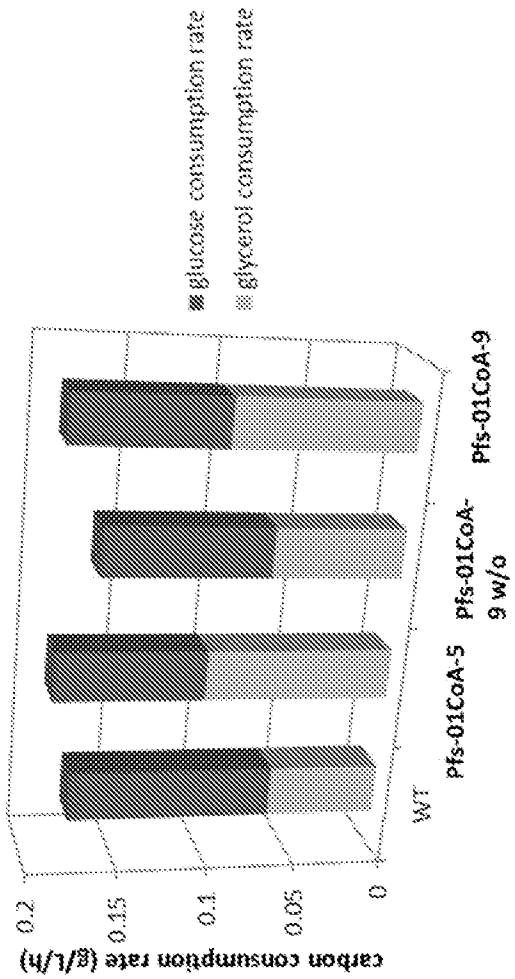

glucose consumption rate
glycerol consumption rate
carbon consumption rate (g/L/h)
0.2
0.15
0.1
0.05
0
WT
Pfs-01CoA-5
Pfs-01CoA-9 w/o
Pfs-01CoA-9

FIG. 29