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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 November 2019 (07.11.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/213033 A1

(51) International Patent Classification:
C12N 15/52 (2006.01) *C12P 7/42* (2006.01)
C12N 9/88 (2006.01) *C12P 7/18* (2006.01)
C12N 9/00 (2006.01) *C12P 7/48* (2006.01)
C12N 9/10 (2006.01) *C12P 7/44* (2006.01)
C12P 7/56 (2006.01) *C12P 13/14* (2006.01)
C12P 7/06 (2006.01) *C12P 13/00* (2006.01)
C12P 7/28 (2006.01) *C12P 13/08* (2006.01)
C12P 7/54 (2006.01) *C12P 1/04* (2006.01)
C12P 7/46 (2006.01)

(21) International Application Number:
PCT/US2019/029827

(22) International Filing Date:
30 April 2019 (30.04.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/665,777 02 May 2018 (02.05.2018) US

(71) Applicant *(for US only)*: **INVISTA NORTH AMERICA S.A.R.L.** [LU/US]; One Little Falls Centre, 2711 Centerville Road, Wilmington, DE 19808 (US).

(71) Applicant *(for all designated States except US)*: **INVISTA TEXTILES (U.K.) LIMITED** [GB/GB]; One St. Peter's Square, Manchester M2 3DE (GB).

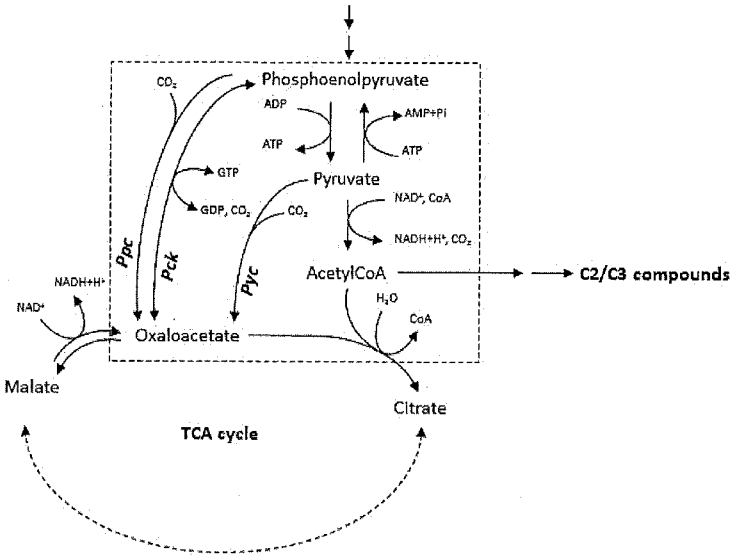
(72) Inventors: **FOSTER, Alexander, Brett**; Wilton Centre, Redcar TS10 4RF (GB). **FRITSCH, Emilie, Sophie**; Wilton Centre, Redcar TS10 4RF (GB).

(74) Agent: **TYRRELL, Kathleen, A.** et al.; Licata & Tyrrell P.C., 66 E. Main Street, Marlton, NJ 08053 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

(54) Title: MATERIALS AND METHODS FOR MAXIMIZING BIOSYNTHESIS THROUGH ALTERATION OF PYRUVATE-ACETYL-COA-TCA BALANCE IN SPECIES OF THE GENERA RALSTONIA AND CUPRIAVIDUS AND ORGANISMS RELATED THERETO

FIG. 1



(57) Abstract: Methods of redirecting carbon flux and increasing C2/C3 or a C4/5/6 carbon chain length carbon-based chemical product yield in an organism, nonnaturally occurring organisms with redirected carbon flux and increased C2/C3 or C4/5/6 carbon chain length carbon-based chemical product yield and methods for using these organisms in production of C2/C3 or C4/5/6 carbon chain length carbon-based chemical products are provided.

[Continued on next page]

WO 2019/213033 A1

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

MATERIALS AND METHODS FOR MAXIMIZING BIOSYNTHESIS THROUGH ALTERATION OF PYRUVATE-ACETYL-COA-TCA BALANCE IN SPECIES OF THE GENERA RALSTONIA AND CUPRIAVIDUS AND ORGANISMS RELATED THERETO

This patent application claims the benefit of priority from U.S. Provisional Patent Application Serial No. 62/665,777 filed May 2, 2018, teachings of which are herein incorporated by reference in their entirety.

FIELD

The present invention relates to methods of redirecting carbon flux and increasing C2/C3 or C4/5/6 carbon chain length carbon-based chemical product yield in an organism, nonnaturally occurring organisms with redirected carbon flux and increased C2/C3 or C4/5/6 carbon chain length carbon-based chemical product yield and methods for using these organisms in production of C2/C3 or C4/5/6 carbon chain length carbon-based chemical products.

BACKGROUND

The PEP-pyruvate-oxaloacetate node, also described as the anaplerotic node, has been described as a switch point for carbon distribution within the central metabolism (Sauer & Eikmanns FEMS Microbiology Reviews 2005 29(4):765-794). It involves a set of interconnected reactions, which can mediate the conversion of C3 and C4 compounds, notably via differential levels of acetyl-CoA and oxaloacetate (FIG. 1).

In silico analysis of the *C. necator* H16 genome has revealed the presence of a gene encoding a GTP-dependent phosphoenolpyruvate carboxykinase Pck (EC 4.1.1.32), H16_A3711, a gene encoding a phosphoenolpyruvate carboxylase Ppc (EC 4.1.1.31), H16_A2921, and a gene encoding a pyruvate

carboxylase Pyc (EC 6.4.1.1), H16_A1251 (Bruland et al. Journal of Applied Microbiology 2010 109:79-90). Some studies suggest that in *C. necator*, *ppc* and *pyc* are not expressed (Bruland et al. Journal of Applied Microbiology 2010 109:79-90; Schwartz et al. Proteomics 2009 9(22):5132-5142) while others have disclosed their expression (Alagesan et al. Metabolomics 2018 14:9).

Both Ppc and Pyc generate oxaloacetate from phosphoenolpyruvate or pyruvate, respectively. Pck catalyzes the reversible carboxylation of phosphoenolpyruvate to oxaloacetate (Schobert & Bowien J Bacteriol. 1984 159(1):167-172; Brämer & Steinbüchel FEMS Microbiol Lett. 2002 2;212(2):159-64). However, in *E. coli*, where there is a phosphoenolpyruvate carboxylase activity, it has been described that, due to Ppc and Pck's kinetic properties, Pck functions as the decarboxylating enzyme (Kim et al. Applied and Environmental Microbiology 2004 70(2):1238-1241).

Several studies have shown that deregulating enzymes involved in the anaplerotic node allowed redirection of the carbon flux into the TCA cycle for production of C4/C5/C6 compounds or towards the production of C2/C3 compounds (e.g. Segura & Espin Appl Microbiol Biotechnol. 2004 65(4):414-8; Kim et al. Applied and Environmental Microbiology 2004 70(2):1238-1241; Meng et al. Microbial Cell Factories 2016 15:141).

Inactivation of pyruvate carboxylase within *A. vinelandii* UW136 has been shown to increase the specific production of poly(3-hydroxybutyrate)(PHB) three-fold (Segura & Espin Appl Microbiol Biotechnol. 2004 65(4):414-8). This anaplerotic enzyme catalyzes the ATP dependent carboxylation of pyruvate to generate oxaloacetate that replenishes the TCA cycle. By diminishing flux of acetyl-CoA into the TCA cycle and slowing

down the TCA cycle due to low concentrations of oxaloacetate, acetyl-CoA instead becomes more available and is diverted toward PHB synthesis.

It has also been shown that the overexpression of *ppc* encoding a phosphoenolpyruvate carboxylase diverts flux in the TCA cycle towards the production of C4 compounds such as oxaloacetate and malate (Park et al. Bioprocess Biosyst Eng. 2013 36(1):127-31) and reduces acetate production in *E. coli* (Papagianni, M. Microbial Cell Factories 2012 11:50). This reduction of acetate production was also observed for *E. coli* strains overexpressing *pyc*, encoding a pyruvate carboxylase, suggesting that the pool of acetyl-CoA is redirected towards the TCA cycle (March et al. Applied and Environmental Microbiology 2002 68(11): 5620-5624; Vemuri et al. Biotechnology and Bioengineering 2005 90(1):64-76).

Additionally, in an *E. coli* strain deleted for *ppc*, heterologous expression of *pck* from *Actinobacillus succinogenes* was shown to replace the phosphoenolpyruvate carboxylase activity, and resulted in increased succinate production (Kim et al. Applied and Environmental Microbiology 2004 70(2):1238-1241; Papagianni, M. Microbial Cell Factories 2012 11:50).

Replacement of traditional chemical production processes relying on, for example fossil fuels and/or potentially toxic chemicals, with environmentally friendly and sustainable solutions is being considered, including work to identify suitable building blocks for use in the manufacturing of chemicals. Organisms and methods for their production and use are needed.

SUMMARY

Methods for increasing product yield of organisms and organisms capable of increased product yield are provided.

An aspect of the present invention related to methods of redirecting carbon flux in an organism. The methods comprises modulating one or more polypeptides, or functional fragments thereof, having an activity of a phosphoenolpyruvate carboxykinase and/or a phosphoenolpyruvate carboxylase and/or a pyruvate carboxylase and/or a citrate lyase or citrate lyase subunit in an organism.

In one nonlimiting embodiment, phosphoenolpyruvate carboxykinase classified under EC 4.1.1.32, EC 4.1.1.38, or EC 4.1.1.49 is modulated.

In one nonlimiting embodiment, the phosphoenolpyruvate carboxykinase modulated comprises SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof.

In one nonlimiting embodiment, phosphoenolpyruvate carboxylase classified under EC 4.1.1.31 is modulated.

In one nonlimiting embodiment, the phosphoenolpyruvate carboxylase modulated comprises SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%

sequence identity to an amino acid sequence set forth in SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof.

In one nonlimiting embodiment, pyruvate carboxylase classified under EC 6.4.1.1 is modulated.

In one nonlimiting embodiment, the pyruvate carboxylase modulated comprises SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof.

In one nonlimiting embodiment, a citrate lyase subunit classified under EC 4.1.3.34 and EC 2.8.3.10 is modulated.

In one nonlimiting embodiment the citrate lyase subunit modulated comprises SEQ ID NO: 42, 44, 46, 48, 50 and/or 52 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 42, 44, 46, 48, 50 and/or 52 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 41, 43, 45,

47, 49 and/or 51 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 41, 43, 45, 47, 49 and/or 51 or a functional fragment thereof.

In one nonlimiting embodiment, modulating an activity level of one or more polypeptides comprises overexpressing an endogenous or exogenous nucleic acid sequence in the organism.

In one nonlimiting embodiment, modulating an activity level of one or more polypeptides comprises downregulating, deleting or mutating an endogenous or exogenous nucleic acid sequence in the organism.

In one nonlimiting embodiment, carbon flux is redirected toward products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto.

Another aspect of the present invention relates to methods for increasing carbon-based chemical product yield in an organism. The methods comprise modulating one or more polypeptides, or functional fragments thereof, having an activity of a phosphoenolpyruvate carboxykinase and/or a phosphoenolpyruvate carboxylase and/or a pyruvate carboxylase and/or a citrate lyase or citrate lyase subunit in an organism. In the methods yield of a C2/C3 or a C4/5/6 carbon chain length product is increase.

Another aspect of the present invention relates to nonnaturally occurring organisms capable of redirecting carbon flux toward products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto. In these nonnaturally occurring organisms an activity level of one or more polypeptides, or functional fragments thereof, having an activity of a phosphoenolpyruvate carboxykinase and/or a

phosphoenolpyruvate carboxylase and/or a pyruvate carboxylase and/or a citrate lyase or citrate lyase subunit is modulated in the organism.

Another aspect of the present invention relates to a method for producing a carbon-based chemical product in an organism with a nonnaturally occurring organism of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the pathways through which flux may be altered by modulating the activity of an enzyme involved in the PEP-pyruvate-oxaloacetate node in an organism.

DETAILED DESCRIPTION

Provided by this disclosure and teachings are methods and materials for maximizing the production of products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto through modulation of enzymes involved in the PEP-pyruvate-oxaloacetate node. The inventors have found that it is possible to differentiate flux going to C4/C5/C6 compounds via the TCA cycle from compounds derived from acetyl-CoA for C2/C3 products. More specifically, in the methods and organism of the present invention one or more polypeptides having an activity of a phosphoenolpyruvate carboxykinase (Pck) and/or a phosphoenolpyruvate carboxylase (Ppc) and/or a pyruvate carboxylase (Pyc) and/or a citrate lyase or citrate lyase subunit (Cit) is modulated to redirect carbon flux.

By "modulated" or "modulate" or "modulating' for purposes of the present invention, it is meant to include overexpressing, downregulating, deleting, mutating or

replacing an endogenous or exogenous nucleic acid sequence or polypeptide in an organism.

In certain aspects, the organism is modulated by altering, engineering, or introducing one or more nucleic acid sequences within the organism. The altering of modifying of the nucleic acid sequences can be, for example and without limitation, via genetic engineering, by adaptive mutation, or by selective isolation of naturally occurring mutant strains.

In some nonlimiting embodiments, one or more enzymes or nucleic acids of the organism are modified via non-direct or rational enzyme design approaches with aims of improving activity, improving specificity, reducing feedback inhibition, reducing repression, improving enzyme solubility, changing stereo-specificity, or changing co-factor specificity. In some embodiments, the enzymes in the pathways outlined herein can be gene dosed (i.e., overexpressed by having a plurality of copies of the gene in the host organism), into the resulting genetically modified organism via episomal or chromosomal integration approaches. In some nonlimiting embodiments, genome-scale system biology techniques such as Flux Balance Analysis can be utilized to devise genome scale attenuation or knockout strategies for directing carbon flux. Attenuation strategies include, but are not limited to, the use of transposons, homologous recombination (double cross-over approach), mutagenesis, enzyme inhibitors, and RNA interference (RNAi). In some embodiments, fluxomic, metabolomic and transcriptomal data can be utilized to inform or support genome-scale system biology techniques, thereby devising genome-scale attenuation or knockout strategies in directing carbon flux. In some embodiments, the tolerance of the host microorganism to high concentrations of the

extracellular product can be improved through continuous cultivation in a selective environment.

The modified nucleic acid sequences of the organism can include, for example, one or more enzymes, one or more promoters, one or more transcription factors, or combinations thereof. The modifications can be to nucleic acids encoding polypeptides functioning as a transhydrogenase, reductase, dehydrogenase, or hydrogenase enzyme or functional fragments thereof. The modifications can be to nucleic acids not directly involved in encoding polypeptides functioning as a transhydrogenase, reductase, dehydrogenase, or hydrogenase enzyme or functional fragments thereof, but indirectly affecting the polypeptides through the interconnected metabolic network and metabolic control strategy of the organism. The modification of the nucleic acid sequences can include one or more deletions, one or more substitutions, one or more insertions, or combinations thereof.

Enzymes with substitutions will generally have not more than 50 (e.g., not more than 1, not more than 2, not more than 3, not more than 4, not more than 5, not more than 6, not more than 7, not more than 8, not more than 9, not more than 10, not more than 12, not more than 15, not more than 20, not more than 25, not more than 30, not more than 35, not more than 40, or not more than 50) amino acid substitutions (e.g., conservative or non-conservative substitutions). This applies to any of the enzymes described herein and functional fragments thereof. A conservative substitution is a substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and

glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The nonpolar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic, or acidic groups by another member of the same group can be deemed a conservative substitution. In contrast, a non-conservative substitution is a substitution of one amino acid for another with dissimilar characteristics. Deletion variants can, for example, lack 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid segments (of two or more amino acids) or non-contiguous single amino acids.

In one nonlimiting embodiment, modification of the organism is carried out by allele exchange. In this embodiment, genome edits are made in a *Cupriavidus* or *Ralstonia* organism with perturbed PHB synthesis or an organism with properties similar thereto by allele exchange (also referred to as allelic exchange). In one non-limiting embodiment, the organism is a ΔphaCAB H16 *C. necator* strain generated using allele exchange.

The term 'allele' is often used interchangeably with the term 'gene' more generally, and refers to a defined genomic locus. In allele exchange, a specific run of DNA sequence (i.e., the native allele) in a genome of an organism is literally exchanged for a recombinant, mutant, or synthetic

run of DNA sequence (i.e., the recombinant allele). Depending on the nature of the recombinant allele, this allele exchange can result in a gene deletion, a gene substitution, or a gene insertion.

In one nonlimiting embodiment, recombinant/synthetic alleles can be constructed via gene synthesis and/or standard molecular biology techniques. These alleles are then cloned into a plasmid vector for transfer into the organism and execution of the allele exchange procedure.

In some nonlimiting embodiments, the organism is modified to include one or more exogenous nucleic acid sequences.

The term "exogenous" as used herein with reference to a nucleic acid (or a protein) and an organism refers to a nucleic acid that does not occur in (and cannot be obtained from) a cell of that particular type as it is found in nature or a protein encoded by such a nucleic acid. Thus, a non-naturally-occurring nucleic acid is considered to be exogenous to a host once in the host. It is important to note that non-naturally-occurring nucleic acids can contain nucleic acid subsequences or fragments of nucleic acid sequences that are found in nature provided the nucleic acid as a whole does not exist in nature. For example, a nucleic acid molecule containing a genomic DNA sequence within an expression vector is non-naturally-occurring nucleic acid, and thus is exogenous to a host cell once introduced into the host, since that nucleic acid molecule as a whole (genomic DNA plus vector DNA) does not exist in nature. Thus, any vector, autonomously replicating plasmid, or virus (e.g., retrovirus, adenovirus, or herpes virus) that as a whole does not exist in nature is considered to be non-naturally-occurring nucleic acid. It follows that genomic DNA fragments produced by PCR or

restriction endonuclease treatment as well as cDNAs are considered to be non-naturally-occurring nucleic acid since they exist as separate molecules not found in nature. It also follows that any nucleic acid containing a promoter sequence and polypeptide-encoding sequence (e.g., cDNA or genomic DNA) in an arrangement not found in nature is non-naturally-occurring nucleic acid. A nucleic acid that is naturally-occurring can be exogenous to a particular host microorganism. For example, an entire chromosome isolated from a cell of yeast x is an exogenous nucleic acid with respect to a cell of yeast y once that chromosome is introduced into a cell of yeast y.

In contrast, the term "endogenous" as used herein with reference to a nucleic acid (e.g., a gene) (or a protein) and a host refers to a nucleic acid (or protein) that does occur in (and can be obtained from) that particular host as it is found in nature. Moreover, a cell "endogenously expressing" a nucleic acid (or protein) expresses that nucleic acid (or protein) as does a host of the same particular type as it is found in nature. Moreover, a host "endogenously producing" or that "endogenously produces" a nucleic acid, protein, or other compound produces that nucleic acid, protein, or compound as does a host of the same particular type as it is found in nature.

In certain aspects, the organism is modulated to include one or more functional fragments of enzymes, other polypeptides, or nucleic acids. The phrase "functional fragment" as used herein refers to a peptide fragment of a polypeptide or a nucleic acid sequence fragment encoding a peptide fragment of a polypeptide that has at least 25%, e.g., at least 30%, at least 40%, at least 50%, at least 60%, at

least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or at least 100% of the activity of the corresponding mature, full-length, polypeptide. The functional fragment can generally, but not always, be comprised of a continuous region of the polypeptide, wherein the region has functional activity.

For purposes of the present invention, by "redirecting carbon flux" it is meant that the modulated organisms and methods of the present invention are capable of producing increased levels of products having either a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto as compared to the same organism without modulation.

For purposes of the present invention, by "derivatives and compounds related thereto" it is meant to encompass compounds derived from the same substrates and/or enzymatic reactions as compounds having a C2/C3 or a C4/C5/C6 chain length, byproducts of these enzymatic reactions and compounds with similar chemical structure including, but not limited to, structural analogs wherein one or more substituents of compounds having a C2/C3 or a C4/C5/C6 chain length are replaced with alternative substituents. Nonlimiting examples of C2/C3 chain length compounds include lactic acid, ethanol, acetone, acetic acid, malonic acid, 3-hydroxypropanoic acid and 1,3-propanediol and derivatives and compounds related thereto. Nonlimiting examples of C4/C5/C6 chain length compounds comprise one or more of citric acid, maleic acid, succinic acid, glutaric acid, glutamic acid, pentamethylene diamine, 1,4-diaminobutane, fumaric acid, itaconic acid, lysine and adipic acid and derivatives and compounds related thereto. In some nonlimiting embodiments, the organism has been modified to

exhibit an increased synthesis of the extracellular product relative to that of the corresponding wild type organism.

Additional descriptions of the synthesis of similar carbon-based chemical products with *Ralstonia*, *Cupriavidus*, or an organism similar thereto can be found in U.S. Patent Nos. 10,196,657; 9,920,339; 9,862,973; and 9,580,733, the disclosures of which are incorporated by reference herein in their entirety for all purposes.

For compounds of the present invention containing carboxylic acid groups such as organic monoacids, hydroxyacids, aminoacids and dicarboxylic acids, these compounds may be formed or converted to their ionic salt form when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like. Acceptable inorganic bases include aluminum hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate and/or bicarbonate, sodium hydroxide, ammonia and the like. The salt can be isolated as is from the system as the salt or converted to the free acid by reducing the pH to below the lowest pKa through addition of acid or treatment with an acidic ion exchange resin.

For compounds of the present invention containing amine groups such as but not limited to organic amines, aminoacids and diamine, these compounds may be formed or converted to their ionic salt form by addition of an acidic proton to the amine to form the ammonium salt, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with

organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 2-naphthalenesulfonic acid, 4-methylbicyclo-[2.2.2]oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis-(3-hydroxy-2-ene-1-carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid or muconic acid. The salt can be isolated as is from the system as a salt or converted to the free amine by raising the pH to above the lowest pKa through addition of base or treatment with a basic ion exchange resin. Acceptable inorganic bases are known in the art and include aluminum hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate and/or bicarbonate, ammonia, sodium hydroxide, and the like.

For compounds of the present invention containing both amine groups and carboxylic acid groups such as but not limited to aminoacids, these compounds may be formed or converted to their ionic salt form by either 1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric

acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl)benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 2-naphthalenesulfonic acid, 4-methylbicyclo-[2.2.2]oct-2-ene-1-carboxylic acid, glucoheptonic acid, 4,4'-methylenebis-(3-hydroxy-2-ene-1-carboxylic acid), 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid. Acceptable inorganic bases include aluminum hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate and/or bicarbonate, sodium hydroxide, ammonia and the like or 2) when an acidic proton present in the parent compound either is replaced by a metal ion, e.g., an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base. Acceptable organic bases are known in the art and include ethanolamine, diethanolamine, triethanolamine, tromethamine, N-methylglucamine, and the like. Acceptable inorganic bases are known in the art and include aluminum hydroxide, calcium hydroxide, potassium hydroxide, sodium carbonate, sodium hydroxide, and the like. The salt can be isolated as is from the system or converted to the free acid by reducing the pH to below the lowest pKa through addition of acid or treatment with an acidic ion exchange resin.

For the generation of C4, C5 and C6 containing compounds in accordance with the methods and organism of the present invention, in one nonlimiting embodiment, this modulation can take the form of enhanced activity or expression of a Pck in an organism in which Ppc has been deleted. In another nonlimiting embodiment, the organism may be modulated by

altering activity or expression or replacing the endogenous or exogenous Pyc and/or endogenous or exogenous Ppc. In one nonlimiting embodiment, the organism may be modulated by increasing activity or expression of Pyc and/or Ppc.

Nonlimiting examples of C4, C5 and C6 containing compounds include citric acid, maleic acid, succinic acid, glutaric acid, glutamic acid, pentamethylene diamine, 1,4-diaminobutane, fumaric acid, itaconic acid, lysine and adipic acid and derivatives and compounds related thereto.

For the generation of C2 and C3 containing compounds, fatty acids and PHBs, isoprenoid and branched chain amino acids in accordance with the methods and organisms of the present invention, this modulation can take the form of decreased activity or expression of Pyc and/or endogenous Ppc.

Nonlimiting examples of C2/C3 chain length compounds include lactic acid, ethanol, acetone, acetic acid, malonic acid, 3-hydroxypropanoic acid and 1,3-propanediol and derivatives and compounds related thereto. In another embodiment, modulation may comprise mutation of isocitrate dehydrogenase in the organism as described, for example, by Park and Lee (Journal of Fermentation and Bioengineering 1996 81(3):197-205).

Additional nonlimiting examples of modulations to the organism to generate C4, C5 and C6 containing compounds or C2 and C3 containing compounds are set forth in the Examples.

Nonnaturally occurring organisms produced and used in accordance with the present invention are selected from a species of *Cupriavidus* or *Ralstonia* with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto.

For purposes of the present invention, by "diminishing" or "diminished" polyhydroxybutyrate synthesis, it is meant that the organism is altered to synthesize less polyhydroxybutyrate as compared to an unaltered wild-type organism of the same species. Organisms used in this disclosure can exhibit at least 20%, 25%, 30%, 40%, 50% or even greater decreased polyhydroxybutyrate synthesis as compared to an unperturbed wild-type organism of the same species.

Nonlimiting examples of species of *Cupriavidus* or *Ralstonia* useful in accordance with this disclosure include *Cupriavidus necator*, *Cupriavidus metallidurans*, *Cupriavidus taiwanensis*, *Cupriavidus pinatubonensis*, *Cupriavidus basilensis* and *Ralstonia pickettii*.

C. necator (also referred to as *Hydrogenomonas eutrophus*, *Alcaligenes eutropha*, *Ralstonia eutropha*, and *Wautersia eutropha*) is a Gram-negative, flagellated soil bacterium of the Betaproteobacteria class. This hydrogen-oxidizing bacterium is capable of growing at the interface of anaerobic and aerobic environments and easily adapts between heterotrophic and autotrophic lifestyles. Sources of energy for the bacterium include both organic compounds and hydrogen. Additional properties of *C. necator* include microaerophilicity, copper resistance (Makar, N.S. & Casida, L.E. Int. J. of Systematic Bacteriology 1987 37(4): 323-326), bacterial predation (Byrd et al. Can J Microbiol 1985 31:1157-1163; Sillman, C. E. & Casida, L. E. Can J Microbiol 1986 32:760-762; Zeph, L.E. & Casida, L.E. Applied and Environmental Microbiology 1986 52(4):819-823) and polyhydroxybutyrate (PHB) synthesis. In addition, the cells have been reported to be capable of either aerobic or nitrate

dependent anaerobic growth. A nonlimiting example of a *C. necator* organism useful in the present invention is a *C. necator* of the H16 strain. In one nonlimiting embodiment, a *C. necator* host of the H16 strain with at least a portion of the *phaC1AB1* gene locus knocked out (*ΔphaCAB*) is used. In one nonlimiting embodiment, the organism is further modified to eliminate *phaCAB*, involved in PHBs production and/or H16-*A0006-9* encoding endonucleases thereby improving transformation efficiency as described in U.S. Patent Application Serial No. 15/717,216, teachings of which are incorporated herein by reference. However, other means of eliminating PHB synthesis are included within the scope of the invention.

By "an organism with properties similar thereto" it is meant an organism having one or more of the above-mentioned properties of *C. necator*.

In one nonlimiting embodiment for the processes of the present invention, one or more polypeptides having an activity of a Pck and/or a Ppc and/or a Pyc and/or a Cit in the organism is modulated.

In one nonlimiting embodiment, the Pck is classified under EC 4.1.1.32, EC 4.1.1.38, or EC 4.1.1.49. In one nonlimiting embodiment, the Pck comprises SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof. In one nonlimiting embodiment, the Pck is encoded by a nucleic acid sequence comprising SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a

functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof.

In one nonlimiting embodiment, the Ppc is classified under EC 4.1.1.31. In one nonlimiting embodiment, the Ppc comprises SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof. In one nonlimiting embodiment, the Ppc is encoded by a nucleic acid sequence comprising SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof.

In one nonlimiting embodiment, the Pyc is classified under EC 6.4.1.1. In one nonlimiting embodiment, the Pyc comprises SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment

thereof. In one nonlimiting embodiment, the Pyc is encoded by a nucleic acid sequence comprising SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof.

Cit is composed of multiple subunits such as, but not limited to, CitF (citrate CoA-transferase) and CitE (citryl-CoA lyase) being classified under EC 2.8.3.10 or EC 4.1.3.34, respectively. In one nonlimiting embodiment, the CitE comprises SEQ ID NO: 42, 44, 46, 48 or 50 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NO: 42, 44, 46, 48 or 50 or a functional fragment thereof. In one nonlimiting embodiment, the CitE is encoded by a nucleic acid sequence comprising SEQ ID NO: 41, 43, 45,47 or 49 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 41, 43, 45, 47 or 49 or a functional fragment thereof. In one nonlimiting embodiment, the CitF comprises SEQ ID NO: 52 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or

99.5% sequence identity to an amino acid sequence set forth in SEQ ID NO: 52 or a functional fragment thereof. In one nonlimiting embodiment, the CitF is encoded by a nucleic acid sequence comprising SEQ ID NO: 51 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 51 or a functional fragment thereof.

In one nonlimiting embodiment for processes of the present invention, isocitrate dehydrogenase is modulated. In one nonlimiting embodiment, the isocitrate dehydrogenase comprises SEQ ID NO: 54, 56 or 58 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to an amino acid sequence set forth in SEQ ID NO: 54, 56 or 58 or a functional fragment thereof. In one nonlimiting embodiment, the isocitrate dehydrogenase is encoded by a nucleic acid sequence comprising SEQ ID NO: 53, 55 or 57 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 99.5% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 53, 55 or 57 or a functional fragment thereof.

The percent identity (and homology) between two amino acid sequences as disclosed herein can be determined as follows. First, the amino acid sequences are aligned using the BLAST 2 Sequences (B12seq) program from the stand-alone version of

BLAST containing BLASTP version 2.0.14. This stand-alone version of BLAST can be obtained from the U.S. government's National Center for Biotechnology Information web site (www with the extension ncbi.nlm.nih.gov). Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two amino acid sequences using the BLASTP algorithm. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\B12seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology (identity), then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology (identity), then the designated output file will not present aligned sequences. Similar procedures can be followed for nucleic acid sequences except that blastn is used.

Once aligned, the number of matches is determined by counting the number of positions where an identical amino acid residue is presented in both sequences. The percent identity (and homology) is determined by dividing the number of matches by the length of the full-length polypeptide amino acid sequence followed by multiplying the resulting value by 100. It is noted that the percent identity (homology) value is rounded to the nearest tenth. For example, 90.11, 90.12,

90.13, and 90.14 is rounded down to 90.1, while 90.15, 90.16, 90.17, 90.18, and 90.19 is rounded up to 90.2. It also is noted that the length value will always be an integer.

It will be appreciated that a number of nucleic acids can encode a polypeptide having a particular amino acid sequence. The degeneracy of the genetic code is well known to the art; i.e., for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. For example, codons in the coding sequence for a given enzyme can be modified such that optimal expression in a particular species (e.g., bacteria or fungus) is obtained, using appropriate codon bias tables for that species.

In one nonlimiting embodiment, the organism is further modified to eliminate *phaCAB*, involved in PHBs production and/or *A0006-9* encoding endonucleases thereby improving transformation efficiency as described in U.S. Patent Application Serial No 15/717,216, teachings of which are incorporated herein by reference.

In the process of the present invention, the modulated organism is then subjected to conditions wherein products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto are produced.

In the process described herein, a fermentation strategy can be used that entails anaerobic, micro-aerobic or aerobic cultivation. A fermentation strategy can entail nutrient limitation such as nitrogen, phosphate or oxygen limitation.

Under conditions of nutrient limitation a phenomenon known as overflow metabolism (also known as energy spilling, uncoupling or spillage) occurs in many bacteria (Russell, J.B. J Mol Microbiol Biotechnol. 2007 13(1-3):1-11). In growth conditions in which there is a relative excess of carbon

source and other nutrients (e.g. phosphorous, nitrogen and/or oxygen) are limiting cell growth, overflow metabolism results in the use of this excess energy (or carbon), not for biomass formation but for the excretion of metabolites, typically organic acids. In *R. eutropha* a modified form of overflow metabolism occurs in which excess carbon is sunk intracellularly into the storage carbohydrate polyhydroxybutyrate (PHB). In strains of *R. eutropha* which are deficient in PHB synthesis this overflow metabolism can result in the production of extracellular overflow metabolites. The range of metabolites that have been detected in PHB deficient *R. eutropha* strains include acetate, acetone, butanoate, cis-aconitate, citrate, ethanol, fumarate, 3-hydroxybutanoate, propan-2-ol, malate, methanol, 2-methyl-propanoate, 2-methyl-butanoate, 3-methyl-butanoate, 2-oxoglutarate, meso-2,3-butanediol, acetoin, DL-2,3-butanediol, 2-methylpropan-1-ol, propan-1-ol, lactate 2-oxo-3-methylbutanoate, 2-oxo-3-methylpentanoate, propanoate, succinate, formic acid and pyruvate. The range and quantity of overflow metabolites produced in a particular fermentation can depend upon the limitation applied (e.g. nitrogen, phosphate, oxygen), the extent of the limitation, the carbon source provided and fermentation conditions such as, but not limited to, pH, source of phosphates or ammonia. See for example, Schlegel and Vollbrecht Microbiology 1980 117:475-481; Vollbrecht et al. European Journal of Applied Microbiology and Biotechnology 1978 6(2):145-155; Vollbrecht and Schlegel European Journal of Applied Microbiology and Biotechnology 1978 6(2):157-166; and Vollbrecht et al. European Journal of Applied Microbiology and Biotechnology 1979 7(3):267-276.

Applying a suitable nutrient limitation in defined fermentation conditions can thus result in an increase in the flux through a particular metabolic node. The application of this knowledge to *R. eutropha* strains genetically modified to produce desired chemical products via the same metabolic node can result in increased production of the desired product.

For example, in glycolysis conditions, TCA cycle is favored. This can be switched by modifying the carbon flux towards the increase of the acetyl-coA pool. Under gluconeogenic conditions, the formation of pyruvate and PEP is favored, and this can be switched by modifying the carbon flux towards TCA cycle. A higher flux through the TCA cycle has also been described in mixotrophic conditions of glycerol and CO_2 (Alagesan et al. Metabolomics 2018 14:9).

A cell retention strategy using a ceramic hollow fiber membrane can be employed to achieve and maintain a high cell density during fermentation.

Feedstocks for fermentation may be gases such as carbon dioxide or hydrogen; sugars such as glucose, xylose or fructose; sugar acids such as gluconate; fatty acids or fats/oils , carboxylic acids such as propionic acid, lactic acid, and formic acid; amino acids, aromatics such as phenol and benzoic acid and/or alcohols such as glycerol.

The feedstocks may be carbon sources derived from by-product or waste streams such as brewing, dairy, plant oil, ethanol, corn, soy, fish, or sugar industries or any other food or agricultural waste such as used cooking oil.

The biological feedstock can be, or can derive from, monosaccharides, disaccharides, lignocellulose, hemicellulose, cellulose, paper-pulp waste, black liquor, lignin, levulinic acid and formic acid, triglycerides, glycerol, fatty acids,

agricultural waste, thin stillage, condensed distillers' solubles or waste streams from the food processing or dairy industries municipal waste such as fruit peel/pulp or whey. The non-biological feedstock can be, or can derive from, natural gas, syngas, CO_2/H_2, CO, H_2, O_2, methanol, ethanol, waste streams from processes to produce monomers for the Nylon-66 and Nylon-6 industries such as but not limited to non-volatile residues (NVRs) and caustic wash waste streams from the cyclohexane oxidation process used to manufacture adipic acid or caprolactam or waste stream from other chemical industry processes such as, but not limited to a carbon black industry or a hydrogen-refining industry, or petrochemical industry, a nonlimiting example being a PTA-waste stream.

In one nonlimiting embodiment, at least one of the enzymatic conversions of the production method comprises gas fermentation within the modulated *Ralstonia or Cupriavidus* organism or other organism with properties similar thereto. In this embodiment, the gas fermentation may comprise at least one of natural gas, syngas, CO, H_2, O_2, CO_2/H_2, methanol, ethanol, non-volatile residue, caustic wash from cyclohexane oxidation processes, or waste stream from a chemical industry such as, but not limited to a carbon black industry or a hydrogen-refining industry, or petrochemical industry. In one nonlimiting embodiment, the gas fermentation comprises CO_2/H_2.

The methods of the present invention may further comprise recovering produced products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto. Once produced, any method can be used to isolate these products or derivatives or compounds related thereto. The isolation of at least one product can involve any one or more downstream processes generally known to be suitable for

the at least partial separation and/or isolation of material from a reaction or bioprocess. The collection can, for example, involve centrifugations, cell disruptions, concentrations, precipitations, extractions, filtrations, crystallizations, distillations, chemical conversions, or combinations thereof. One or more biosynthetic products can be collected from the liquid or solid phase of the culture, or from the gas phase present in the headspace of a bioreactor or the off-gas.

The present invention also provides nonnaturally occurring organisms capable of redirecting carbon flux toward and increasing yield of carbon-based chemical products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto. The nonnaturally occurring organisms are selected from a species of *Cupriavidus* or *Ralstonia* with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto. Nonlimiting examples of species of *Cupriavidus* or *Ralstonia* useful in accordance with this disclosure include *Cupriavidus necator*, *Cupriavidus metallidurans*, *Cupriavidus taiwanensis*, *Cupriavidus pinatubonensis*, *Cupriavidus basilensis* and *Ralstonia pickettii*.

In one nonlimiting embodiment, the present invention relates to a substantially pure culture of the nonnaturally occurring organism capable of redirecting carbon flux toward and increasing yield of carbon-based chemical products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto.

As used herein, a "substantially pure culture" of an altered organism is a culture of that microorganism in which less than about 40% (i.e., less than about 35%; 30%; 25%; 20%;

15%; 10%; 5%; 2%; 1%; 0.5%; 0.25%; 0.1%; 0.01%; 0.001%; 0.0001%; or even less) of the total number of viable cells in the culture are viable cells other than the altered microorganism, e.g., bacterial, fungal (including yeast), mycoplasmal, or protozoan cells. The term "about" in this context means that the relevant percentage can be 15% of the specified percentage above or below the specified percentage. Thus, for example, about 20% can be 17% to 23%. Such a culture of nonnaturally occurring microorganisms includes the cells and a growth, storage, or transport medium. Media can be liquid, semi-solid (e.g., gelatinous media), or frozen. The culture includes the cells growing in the liquid or in/on the semi-solid medium or being stored or transported in a storage or transport medium, including a frozen storage or transport medium. The cultures are in a culture vessel or storage vessel or substrate (e.g., a culture dish, flask, or tube or a storage vial or tube).

In one nonlimiting embodiment, one or more polypeptides having an activity of a Pck and/or a Ppc and/or a Pyc and/or a Cit in the organism is modulated.

In one nonlimiting embodiment for processes of the present invention, isocitrate dehydrogenase is modulated.

In one nonlimiting embodiment, the organism is further modified to eliminate *phaCAB*, involved in PHBs production and/or *A0006-9* encoding endonucleases thereby improving transformation efficiency, as described in U.S. Patent Application Serial No 15/717,216, teachings of which are incorporated herein by reference. However, other means of eliminating PHB synthesis are included within the scope of the invention.

In addition, the present invention provides bio-derived, bio-based, or fermentation-derived products produced using the methods and/or altered organisms disclosed herein. In one nonlimiting embodiment, a bio-derived, bio-based or fermentation derived product is produced in accordance with the exemplary central metabolism depicted in FIG. 1. Examples of such products include, but are not limited to, compositions comprising at least one bio-derived, bio-based, or fermentation-derived compound or any combination thereof, as well as molded substances, formulations and semi-solid or non-semi-solid streams comprising one or more of the bio-derived, bio-based, or fermentation-derived compounds or compositions, combinations or products thereof.

While the invention has been described in detail, in some instances making reference to a specific aspect thereof, it is apparent to one of skill in the art that various changes and modifications can be made thereto without departing from its spirit and scope. The following section provides further illustration of the methods and materials of the present invention. These Examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

Two strains were used for the generation of RNAseq data: *C. necator* H16 Δ*phaCAB* and Δ*phaCAB*Δ*pimACD*. The strains were grown in triplicates at 30°C on *Cupriavidus* defined medium with 1% fructose. Samples were collected for RNA extraction at OD600 between 0.2 and 0.5. The RNAs were extracted and processed to generate cDNA libraries, which were then sequenced on Illumina MiSeq with chemistry v2.

Table 1 summarizes the RNA sequencing results for the genes ppc, pyc and pck. The expression units correspond to the relative expression unit, which is the expression normalized to the total number of mapped reads for each sample with an average of 3 biological replicates. DF and DDF refer to the strains *ΔphaCAB* and *ΔphaCABΔpimACD* respectively grown with fructose as sole carbon source.

Table 1. RNA sequencing results of the genes *ppc*, *pyc* and *pck*

	DF AVERAGE	DDF AVERAGE	Protein Name	Gene	Description
YP_727365	71.84	70.35	Ppc	H16_A2921	Phosphoenolpyruvate carboxylase
YP_725759	11.66	5.52	Pyc	H16_A1251	Pyruvate carboxylase
YP_728135	155.24	152.24	Pck	H16_A3711	Phosphoenolpyruvate carboxykinase

As shown by the RNAseq experiment, three genes ppc, pyc and pck were expressed in *C. necator* when grown on fructose and can thus be modulated.

Example 2: Deletion/Downregulation of *pyc* and/or *ppc*

The deletions of one or both *pyc* and/or *ppc* genes block the replenishment of the oxaloacetate. This is expected to lead to a decrease of the pool of available oxaloacetate, thus a slowdown of the TCA cycle and a higher availability of the acetyl-coA pool for synthesis of PHB (C2/C3 route) as described by Segura and Espin (Appl Microbiol Biotechnol. 2004 65(4):414-8).

Example 3: Overexpression of *pyc* and/or *ppc*

The overexpression of one or both *pyc* and/or *ppc* genes, either endogenous or exogenous, increases the anaplerotic flux to oxaloacetate and thus replenishes the oxaloacetate pool. The pool of acetyl-CoA will preferentially be fed into the TCA cycle. The overexpression will thus direct the carbon flux towards the production of C4/C5/C6 compounds. In addition to the overexpression of the endogenous *ppc* gene, the gene encoding the PEP carboxylase A from *Methanothermobacter thermoautotrophicus* can also be overexpressed as the enzyme activity is not influenced by the levels of acetyl-CoA and the enzyme is less sensitive to levels of aspartate (Sauer & Eikmanns FEMS Microbiology Reviews 2005 29(4):765-794). This effect of overexpression(s) may be even further accentuated in conjunction with the deletion of *pck* as it has been described in *E. coli* that Pck's kinetic properties favor the oxolacetate decarboxylation rather than the phosphoenolpyruvate carboxylation (Kim et al. Applied and Environmental Microbiology 2004 70(2):1238-41).

Example 4: Overexpression of *pck* in mutants in which *ppc* is deleted or downregulated

In *R. eutropha*, Pck catalyzes the reversible carboxylation of phosphoenolpyruvate to oxaloacetate (Schobert & Bowien J Bacteriol. 1984 159(1):167-172). Results in *E. coli* suggest however that Pck's kinetic properties favor the production of PEP from oxaloacetate rather than the reverse reaction. Nevertheless, it has also been described that this equilibrium can be shifted towards the production of oxaloacetate when *pck* is overexpressed in a *ppc* mutant (Kim et al. Applied and Environmental Microbiology. 2004 70(2):1238-1241; Meng et al.

Microbial Cell Factories 201615:141; Papagianni, M. Microbial Cell Factories 2012 11:50). For instance, the heterologous expression of *A. succinogenes* Pck in *E. coli* has been shown to increase the flux to succinate production (Kim et al. Applied and Environmental Microbiology. 2004 70(2):1238-1241; Meng et al. Microbial Cell Factories 201615:141).

In *R. eutropha*, the endogenous or an exogenous *pck* gene can be expressed in a Δ*ppc* mutant. The exogenous Pck can replace the defective phosphoenolpyruvate carboxylase activity and thus diverts the flux in the TCA cycle towards the production of C4, C5 and C6 compounds.

Example 5: Overexpression of Pck and combination with Ppc

Tan et al. have demonstrated in *E. coli* that modulating the expression of Pck and Ppc independently, had a positive impact on succinate production, indicating that the higher flux towards the production of C4 compounds was achieved (Appl. Environ. Microbiol. 2013 79(16): 4838-4844). In addition, they showed that combining activation of Pck and Ppc resulted in higher titers than the independent activations.

In *R. eutropha*, modulating the expression of Pck using different promoters, RBS or regulators or by protein engineering, independently or in combination with the modulation of Ppc's expression could result in higher flux for the production of C4/C5/C6 compounds.

Example 6: Overexpression of a citrate lyase

It has been shown in the *C. necator* PHB$^-$4 mutant that an excess of pyruvate is produced, which can thus be used to generate acetyl-CoA. This acetyl-coA can enter the TCA cycle and can react with oxaloacetate to generate citrate. A citrate

lyase activity can catalyze the conversion of citrate to acetate and oxaloacetate, which corresponds to an anaplerotic reaction to produce oxaloacetate that circumvents the full TCA cycle.

The citrate lyase is composed of several subunits, including CitF, the subunit alpha (EC 2.8.3.10), which converts acetyl-coA and citrate to acetate and citryl-CoA, and CitE, the subunit beta (EC 4.1.3.34), which catalyzes the conversion of citryl-CoA to acetyl-CoA and oxaloacetate.

In the PHB$^-$4 mutant, it has been found that CitE4, the subunit Beta of a citrate lyase, is more highly expressed (Raberg et al. PLoS ONE 2014 9(5):e95907).

In *C. necator* H16, four genes have been annotated as encoding a citryl-CoA lyase activity: *citE1* (H16_A2635, YP_727085), *citE2* (H16_B0353, YP_728518), *citE3* (H16_B0680, YP_728842) and *citE4* (H16_B2113, YP_841625). No orthologs of CitF have been found in *C. necator* H16, however in *C. necator* N-1 strain, both CitE and CitF are present, encoded by citF (CNE_BB1p09780) and citE (CNE_BB1p01450).

Heterologous overexpression of one or more of the citE genes together with citF in *R. eutropha*, in which PHB production is down-regulated or blocked, is expected to lead to higher levels of oxaloacetate, which could then be converted to C4 compounds such as malate or succinate.

In a strain in which PHB production has been blocked or downregulated, this strategy can also be combined with the overexpression of *pyc* and/or *ppc* (as high levels of pyruvate are expected in the mutant) or the overexpression of *pck* in mutants in which the phosphoenolpyruvate carboxylase activity (Ppc) has also been blocked.

Example 7: Down-regulation/Deletion of Isocitrate dehydrogenase activity

The partial blockage of the TCA cycle due to down-regulation/deletion of isocitrate dehydrogenase activity encoded by the genes *icd1, icd2* and *icd3* in *C. necator* increases the carbon flow to the PHB biosynthesis pathway rather than the TCA cycle. This has been observed in *C. necator* for an isocitrate dehydrogenase leaky mutant (Park & Lee Journal of Fermentation and Bioengineering 1996 81(3):197-205). This modification can be performed independently or in conjunction with the deletion/downregulation of *pyc* and/or *ppc* (see Example 2) to increase the flux towards the production of C2/C3 compounds.

WHAT IS CLAIMED IS:

1. A method of redirecting carbon flux in an organism, said method comprising modulating activity of one or more polypeptides, or functional fragments thereof, having an activity of a phosphoenolpyruvate carboxykinase and/or a phosphoenolpyruvate carboxylase and/or a pyruvate carboxylase and/or a citrate lyase or citrate lyase subunit in an organism selected from a species of *Cupriavidus* or *Ralstonia* with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto.

2. The method of claim 1 wherein the phosphoenolpyruvate carboxykinase is classified under EC 4.1.1.32, EC 4.1.1.38, or EC 4.1.1.49; the phosphoenolpyruvate carboxylase is classified under EC 4.1.1.31; the pyruvate carboxylase is classified under EC 6.4.1.1; and/or the citrate lyase subunit is classified under EC 4.1.3.34 and EC 2.8.3.10.

3. The method of claim 1 or 2 wherein the phosphoenolpyruvate carboxykinase comprises SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof.

4. The method of any of claims 1, 2 or 3 wherein the phosphoenolpyruvate carboxylase comprises SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof.

5. The method of any of the preceding claims wherein the pyruvate carboxylase comprises SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof.

6. The method of any of the preceding claims wherein the citrate lyase subunit comprises SEQ ID NO: 42, 44, 46, 48, 50 and/or 52 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set

forth in SEQ ID NO: 42, 44, 46, 48, 50 and/or 52 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 41, 43, 45, 47, 49 and/or 51 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 41, 43, 45, 47, 49 and/or 51 or a functional fragment thereof.

7. The method of any of the preceding claims wherein modulating the activity of one or more polypeptides comprises overexpressing or mutating an endogenous or exogenous nucleic acid sequence in the organism.

8. The method of any of claims 1 to 6 wherein modulating the activity of one or more polypeptides comprises downregulating, deleting or mutating an endogenous or exogenous nucleic acid sequence in the organism.

9. The method of any of the preceding claims wherein carbon flux is redirected toward products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto.

10. The method of claim 9, wherein said products having a C2/C3 chain length comprise one or more of lactic acid, ethanol, acetone, acetic acid, malonic acid, 3-hydroxypropanoic acid or 1,3-propanediol.

11. The method of claim 9, wherein said products having a C4/C5/C6 chain length comprise one or more of citric acid, maleic acid, succinic acid, glutaric acid, glutamic acid,

pentamethylene diamine, 1,4-diaminobutane, fumaric acid, itaconic acid, lysine or adipic acid.

12. A method for increasing carbon-based chemical product yield in an organism, wherein said product has a C2/C3 or a C4/5/6 carbon chain length, said method comprising modulating activity of one or more polypeptides, or functional fragments thereof, having an activity of a phosphoenolpyruvate carboxykinase and/or a phosphoenolpyruvate carboxylase and/or a pyruvate carboxylase and/or a citrate lyase or citrate lyase subunit in an organism selected from a species of *Cupriavidus* or *Ralstonia* with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, thereby increasing carbon-based chemical product yield in the organism as compared to an organism without said modulated polypeptides.

13. A nonnaturally occurring organism capable of redirecting carbon flux toward products having a C2/C3 or a C4/C5/C6 chain length, derivatives thereof and/or compounds related thereto, said organism selected from a species of *Cupriavidus* or *Ralstonia* with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto and having one or more polypeptides, or functional fragments thereof, having an activity of a phosphoenolpyruvate carboxykinase and/or a phosphoenolpyruvate carboxylase and/or a pyruvate carboxylase and/or a citrate lyase or citrate lyase subunit modulated in the organism.

14. The nonnaturally occurring organism of claim 13, wherein said products having a C2/C3 chain length comprise one

or more of lactic acid, ethanol, acetone, acetic acid, malonic acid, 3-hydroxypropanoic acid or 1,3-propanediol.

15. The nonnaturally occurring organism of claim 13, wherein said products having a C4/C5/C6 chain length comprise one or more of citric acid, maleic acid, succinic acid, glutaric acid, glutamic acid, pentamethylene diamine, 1,4-diaminobutane, fumaric acid, itaconic acid, lysine or adipic acid.

16. The nonnaturally occurring organism of claim 13 wherein the phosphoenolpyruvate carboxykinase is classified under EC 4.1.1.32, EC 4.1.1.38, or EC 4.1.1.49; the phosphoenolpyruvate carboxylase is classified under EC 4.1.1.31; the pyruvate carboxylase is classified under EC 6.4.1.1; and/or the citrate lyase subunit is classified under EC 4.1.3.34 and EC 2.8.3.10.

17. The nonnaturally occurring organism of claim 13 wherein

the phosphoenolpyruvate carboxykinase comprises SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 2, 8, 10, 12, 14, 16 or 18 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 1, 7, 9, 11, 13, 15 or 17 or a functional fragment thereof;

the phosphoenolpyruvate carboxylase comprises SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 4, 30, 32, 34, 36, 38 or 40 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 3, 29, 31, 33, 35, 37 or 39 or a functional fragment thereof;

the pyruvate carboxylase comprises SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 6, 20, 22, 24, 26 or 28 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 5, 19, 21, 23, 25 or 27 or a functional fragment thereof; and/or

the citrate lyase subunit comprises SEQ ID NO: 42, 44, 46, 48, 50 and/or 52 or a functional fragment thereof or is a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to an amino acid sequence set forth in SEQ ID NO: 42, 44, 46, 48, 50 and/or 52 or a functional fragment thereof or is encoded by a nucleic acid sequence comprising SEQ ID NO: 41, 43, 45, 47, 49 and/or 51 or

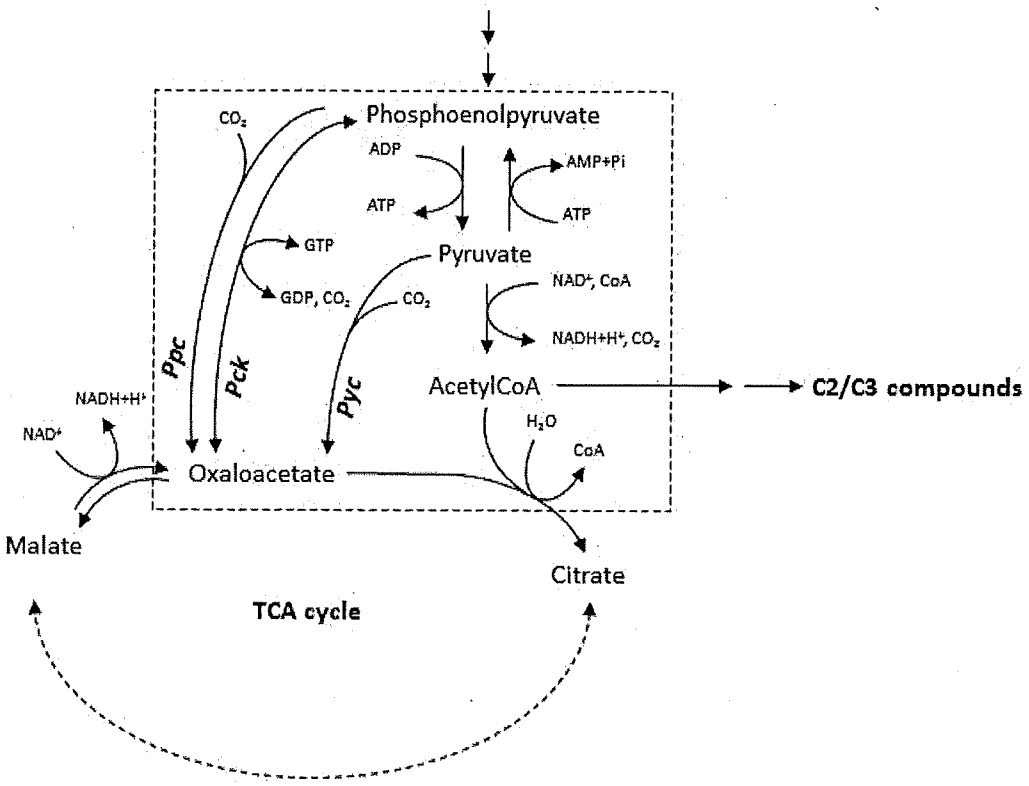
a functional fragment thereof or a nucleic acid sequence encoding a polypeptide with similar enzymatic activities exhibiting at least 50% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 41, 43, 45, 47, 49 and/or 51 or a functional fragment thereof.

18. The nonnaturally occurring organism of any of claims 13, 14, 15, 16 or 17 wherein modulating the activity of one or more polypeptides comprises overexpressing or mutating an endogenous or exogenous nucleic acid sequence in the organism.

19. The nonnaturally occurring organism of any of claims 13, 14, 15, 16 or 17 wherein modulating the activity of one or more polypeptides comprises downregulating, deleting or mutating an endogenous or exogenous nucleic acid sequence in the organism.

20. A method for producing a carbon-based chemical product in an organism, said method comprising fermenting the nonnaturally occurring organism of any of claims 13, 14, 15, 16, 17, 18 or 19 with a carbon source.

FIG. 1


Phosphoenolpyruvate
ADP
ATP
AMP+Pi
ATP
GTP
Pyruvate
GDP, CO2
CO2
NAD+, CoA
NADH+H+, CO2
Ppc
Pck
Pyc
AcetylCoA
C2/C3 compounds
NADH+H+
NAD+
H2O
CoA
Oxaloacetate
Malate
Citrate
TCA cycle

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/029827

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/52 C12N9/88 C12N9/00 C12N9/10 C12P7/56
C12P7/06 C12P7/28 C12P7/54 C12P7/46 C12P7/42
C12P7/18 C12P7/48 C12P7/44 C12P13/14 C12P13/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2018/005770 A2 (INVISTA NORTH AMERICA S Á R L [US]) 4 January 2018 (2018-01-04) the whole document examples 2-4 table 11 figures 13-16 claims 40,41,42,45,52 -----	1-3,7-20
Y	RABERG M. ET AL: "A Closer Look on the Polyhydroxybutyrate- (PHB-) Negative Phenotype of Ralstonia eutropha PHB-4", PLOS ONE, vol. 9, no. 5, 2 May 2014 (2014-05-02), page e95907, XP055605139, DOI: 10.1371/journal.pone.0095907 cited in the application the whole document table 2 ----- -/--	1-3,7-20

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2019	23/09/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Kamp, Mart

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 8 809 027 B1 (LYNCH MICHAEL D [US] ET AL) 19 August 2014 (2014-08-19) the whole document example 12 figures 3,4 claims 1,7,16,17	1-3,7-20
Y	ALAGESAN S. ET AL: "C-assisted metabolic flux analysis to investigate heterotrophic and mixotrophic metabolism in Cupriavidus necator H16", METABOLOMICS, vol. 14, no. 9, 4 December 2017 (2017-12-04), pages 1-10, XP055605143, cited in the application the whole document	1-3,7-20
Y	BRULAND N. ET AL: "Unravelling the C3/C4 carbon metabolism in Ralstonia eutropha H16", JOURNAL OF APPLIED MICROBIOLOGY., vol. 109, 1 December 2009 (2009-12-01), pages 79-90, XP055605137, GB ISSN: 1364-5072, DOI: 10.1111/j.1365-2672.2009.04631.x cited in the application the whole document	1-3,7-20
Y	SCHWARTZ E. ET AL: "A proteomic view of the facultatively chemolithoautotrophic lifestyle of Ralstonia eutropha H16", PROTEOMICS, vol. 9, no. 22, 1 November 2009 (2009-11-01), pages 5132-5142, XP055605095, DE ISSN: 1615-9853, DOI: 10.1002/pmic.200900333 cited in the application the whole document	1-3,7-20
A	LU J. ET AL: "Studies on the production of branched-chain alcohols in engineered Ralstonia eutropha", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 96, no. 1, 4 August 2012 (2012-08-04), pages 283-297, XP035107817, ISSN: 1432-0614, DOI: 10.1007/S00253-012-4320-9 the whole document	1-3,7-20
A	WO 2015/117019 A1 (EASEL BIOTECHNOLOGIES LLC [US]) 6 August 2015 (2015-08-06) the whole document	1-3,7-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/029827

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

3(completely); 1, 2, 7-20(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 3(completely); 1, 2, 7-20(partially)

A method of redirecting carbon flux in an organism, said method comprising modulating activity of one or more polypeptides, or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a phosphoenolpyruvate carboxykinase. A method for increasing carbon-based chemical product yield in an organism, wherein said product has a C2/C3 or a C4/5/6 carbon chain length, said method comprising modulating activity of one or more polypeptides or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, thereby increasing carbon-based chemical product yield in the organism as compared to an organism without said modulated polypeptides, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a phosphoenolpyruvate carboxykinase. A corresponding nonnaturally occurring organism. A method for producing a carbon-based chemical product in an organism, said method comprising fermenting said nonnaturally occurring organism with a carbon source.

---

2. claims: 4(completely); 1, 2, 7-20(partially)

A method of redirecting carbon flux in an organism, said method comprising modulating activity of one or more polypeptides, or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a phosphoenolpyruvate carboxylase. A method for increasing carbon-based chemical product yield in an organism, wherein said product has a C2/C3 or a C4/5/6 carbon chain length, said method comprising modulating activity of one or more polypeptides or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, thereby increasing carbon-based chemical product yield in the organism as compared to an organism without said modulated polypeptides, wherein the one or more polypeptides or functional fragments thereof are polypeptides or

functional fragments thereof having an activity of a phosphoenolpyruvate carboxylase. A corresponding nonnaturally occurring organism. A method for producing a carbon-based chemical product in an organism, said method comprising fermenting said nonnaturally occurring organism with a carbon source.

---

3. claims: 5(completely); 1, 2, 7-20(partially)

A method of redirecting carbon flux in an organism, said method comprising modulating activity of one or more polypeptides, or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a pyruvate carboxylase. A method for increasing carbon-based chemical product yield in an organism, wherein said product has a C2/C3 or a C4/5/6 carbon chain length, said method comprising modulating activity of one or more polypeptides or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, thereby increasing carbon-based chemical product yield in the organism as compared to an organism without said modulated polypeptides, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a pyruvate carboxylase. A corresponding nonnaturally occurring organism. A method for producing a carbon-based chemical product in an organism, said method comprising fermenting said nonnaturally occurring organism with a carbon source.

---

4. claims: 6(completely); 1, 2, 7-20(partially)

A method of redirecting carbon flux in an organism, said method comprising modulating activity of one or more polypeptides, or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a citrate lyase or citrate lyase subunit. A method for increasing carbon-based chemical product yield in an organism, wherein said product has a C2/C3 or a C4/5/6 carbon chain length, said method comprising modulating activity of one or more polypeptides or functional fragments thereof, in an organism selected from a species of Cupriavidus or Ralstonia with diminished polyhydroxybutyrate synthesis or an organism with properties similar thereto,

thereby increasing carbon-based chemical product yield in the organism as compared to an organism without said modulated polypeptides, wherein the one or more polypeptides or functional fragments thereof are polypeptides or functional fragments thereof having an activity of a citrate lyase or citrate lyase subunit. A corresponding nonnaturally occurring organism. A method for producing a carbon-based chemical product in an organism, said method comprising fermenting said nonnaturally occurring organism with a carbon source.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/029827

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018005770 A2	04-01-2018	US 2018002704 A1	04-01-2018
		WO 2018005770 A2	04-01-2018
US 8809027 B1	19-08-2014	US 8809027 B1	19-08-2014
		US 2015072384 A1	12-03-2015
WO 2015117019 A1	06-08-2015	EP 3099784 A1	07-12-2016
		US 2016348086 A1	01-12-2016
		US 2018216096 A1	02-08-2018
		WO 2015117019 A1	06-08-2015