HU000035611T2

(19) **HU** (11) Lajstromszám: **E 035 611** (13) **T2**

MAGYARORSZÁG
Szellemi Tulajdon Nemzeti Hivatala

EURÓPAI SZABADALOM
SZÖVEGÉNEK FORDÍTÁSA

(21) Magyar ügyszám: **E 09 763021**
(22) A bejelentés napja: **2009. 03. 27.**

(96) Az európai bejelentés bejelentési száma:
EP 20090763021
(97) Az európai bejelentés közzétételi adatai:
EP 2265709 A2 **2009. 12. 17.**
(97) Az európai szabadalom megadásának meghirdetési adatai:
EP 2265709 B1 **2017. 11. 08.**

(51) Int. Cl.: ***C12N 1/21*** *(2006.01)*
C12P 13/04 *(2006.01)*
C12P 7/44 *(2006.01)*

(86) A nemzetközi (PCT) bejelentési szám:
PCT/US 09/038663
(87) A nemzetközi közzétételi szám:
WO 09151728

(30) Elsőbbségi adatok:
40059 P **2008. 03. 27.** **US**

(72) Feltaláló(k):
BURGARD, Anthony, P., Bellefonte PA 16823 (US)
PHARKYA, Priti, San Diego CA 92126 (US)
OSTERHOUT, Robin, E., San Diego CA 92107 (US)

(73) Jogosult(ak):
Genomatica, Inc., San Diego, CA 92121 (US)

(74) Képviselő:
SBGK Szabadalmi Ügyvivői Iroda, Budapest

(54) **Adipinsav és más vegyületek termelésére alkalmas mikroorganizmusok**

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 265 709 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention
of the grant of the patent:
08.11.2017 Bulletin 2017/45

(21) Application number: **09763021.4**

(22) Date of filing: **27.03.2009**

(51) Int Cl.:
C12N 1/21 $^{(2006.01)}$
C12P 13/04 $^{(2006.01)}$
C12P 7/44 $^{(2006.01)}$

(86) International application number:
PCT/US2009/038663

(87) International publication number:
WO 2009/151728 (17.12.2009 Gazette 2009/51)

(54) **MICROORGANISMS FOR THE PRODUCTION OF ADIPIC ACID AND OTHER COMPOUNDS**

MIKROORGANISMEN ZUR HERSTELLUNG VON ADIPINSÄURE UND ANDEREN VERBINDUNGEN

MICROORGANISMES POUR LA PRODUCTION D'ACIDE ADIPIQUE ET AUTRES COMPOSÉS

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK TR

(30) Priority: **27.03.2008 US 40059 P**

(43) Date of publication of application:
29.12.2010 Bulletin 2010/52

(73) Proprietor: **Genomatica, Inc.**
San Diego, CA 92121 (US)

(72) Inventors:
- **BURGARD, Anthony, P.**
Bellefonte
PA 16823 (US)
- **PHARKYA, Priti**
San Diego
CA 92126 (US)
- **OSTERHOUT, Robin, E.**
San Diego
CA 92107 (US)

(74) Representative: **Sharples, Andrew John**
EIP
Fairfax House
15 Fulwood Place
London WC1V 6HU (GB)

(56) References cited:
WO-A2-2005/068643
US-A- 5 616 496
US-A1- 2003 113 886
US-B1- 6 214 592
US-B1- 6 280 986

- **O. N. PONAMOREVA ET AL: "Transformation of low-molecular linear caprolactam oligomers by the caprolactam-degrading bacterium Pseudomonas putida BS394(pBS268)", MICROBIOLOGY, vol. 79, no. 3, 1 June 2010 (2010-06-01), pages 321-326, XP55032166, ISSN: 0026-2617, DOI: 10.1134/S0026261710030070**
- **BORONIN A M ET AL: "Plasmids specifying epsilon-caprolactam degradation in Pseudomonas strains", FEMS MICROBIOLOGY LETTERS, NO LONGER PUBLISHED BY ELSEVIER, vol. 22, no. 3, 1 May 1984 (1984-05-01), pages 167-170, XP023918547, ISSN: 0378-1097 [retrieved on 1984-05-01]**
- **GESHER ET AL.: 'Genes Coding for a New Pathway of Aerobic Benzoate Metabolism in Azoarcus evansii' J. BACTERIOL. vol. 184, no. 22, November 2002, pages 6301 - 6315, XP008133722**
- **KINOSHITA ET AL.: 'Purification and Characterization of 6-Aminohexanoic-Acid-Oligomer Hydrolase of Flavobacterium sp.' KI72, EUR. J. BIOCHEM. vol. 116, 1981, pages 547 - 551, XP008133724**
- **FUKUMURA: 'Hydrolysis of Cyclic and Linear Oligomers of 6-Aminocaproic Acid by a Bacterial Cell Extract' J. BIOCHEM. vol. 59, no. 6, 1966, pages 531 - 536, XP008133726**

Printed by Jouve, 75001 PARIS (FR)

Description

BACKGROUND OF THE INVENTION

[0001] The present invention relates generally to biosynthetic processes, and more specifically to organisms having 6-aminocaproic acid and caprolactam biosynthetic capability.

[0002] Adipic acid, a dicarboxylic acid, with molecular weight of 146.14, is a compound of commercial significance. Its major use is to produce nylon 6,6, a linear polyamide made by condensing adipic acid with hexamethylene diamine that is primarily employed for manufacturing different kinds of fibers. Other uses of adipic acid include its use in plasticizers, unsaturated polyesters, and polyester polyols. Additional uses include for production of polyurethane, lubricant components, and as a food ingredient as a flavorant and gelling aid.

[0003] Historically, adipic acid was prepared from various fats using oxidation. The current commercial processes for adipic acid synthesis rely on the oxidation of KA oil, a mixture of cyclohexanone, the ketone or K component, and cyclohexanol, the alcohol or A component, or of pure cyclohexanol using an excess of strong nitric acid. There are several variations of this theme which differ in the routes for production of KA or cyclohexanol. For example, phenol is an alternative raw material in KA oil production, and the process for the synthesis of adipic acid from phenol has been described. The other versions of this process tend to use oxidizing agents other than nitric acid, such as hydrogen peroxide, air or oxygen.

[0004] Caprolactam is an organic compound which is a lactam of 6-aminohexanoic acid (ε-aminohexanoic acid, aminocaproic acid). It can alternatively be considered cyclic amide of caproic acid. The primary industrial use of caprolactam is as a monomer in the production of nylon-6. Most of the caprolactam is synthesised from cyclohexanone via an oximation process using hydroxylammonium sulfate followed by catalytic rearrangement using the Beckmann rearrangement process step.

[0005] Thus, there exists a need for alternative methods for effectively producing commercial quantities of compounds such as adipic acid and carpolactam. The present invention satisfies this need and provides related advantages as well.

[0006] WO2005/068643 discloses biochemical synthesis of 6-aminocaproic acid from 6-aminohex-2-enoic acid compound or from 6-amino-2-hydroxyhexanoic acid by treatment with an enzyme having α,β-enoate reductase activity towards molecules containing an α,β-enoate group and a primary amino group.

SUMMARY OF INVENTION

[0007] The invention provides a non-naturally occurring microbial organism having a 6-aminocaproic acid pathway. The microbial organism contains at least two exogenous nucleic acids each encoding an enzyme in the 6-aminocaproic acid pathway and at least one pathway for producing adipyl-CoA. The invention additionally provides a method for producing 6-aminocaproic acid or caprolactam. The method can include culturing a 6-aminocaproic acid or caprolactam producing microbial organism, where the microbial organism expresses at least two exogenous nucleic acids encoding a 6-aminocaproic acid pathway enzyme in a sufficient amount to produce the respective product, under conditions and for a sufficient period of time to produce 6-aminocaproic acid or caprolactam.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008]

Figure 1 shows an exemplary pathway for adipate degradation in the peroxisome of *Penicillium chrysogenum.*

Figure 2 shows an exemplary pathway for adipate formation via a reverse degradation pathway. Several options are provided for the final conversion of adipyl-CoA to adipate.

Figure 3 shows an exemplary pathway for adipate formation via the 3-oxoadipate pathway.

Figure 4 show the similar enzyme chemistries of the last three steps of the 3-oxoadipate pathway for adipate synthesis and the reductive TCA cycle.

Figure 5 shows an exemplary pathway for synthesis of adipic acid from glucose via cis,cis-muconic acid. Biosynthetic intermediates (abbreviations): D-erythrose 4-phosphate (E4P), phosphoenolpyruvic acid (PEP), 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP), 3-dehydroquinic acid (DHQ), 3-dehydroshikimic acid (DHS), protocatechuic acid (PCA). Enzymes (encoding genes) or reaction conditions: (a) DAHP synthase (*aro*FFBR), (b) 3- dehydroquinate synthase (*aroB*), (c) 3-dehydroquinate dehydratase (*aroD*), (d) DHS dehydratase (*aroZ*), (e) protocatechuate decarboxylase (*aroY*), (f) catechol 1,2-dioxygenase (*catA*), (g) 10% Pt/C, H_2, 3400 kPa, 25 °C. Figure taken from Niu et al., Biotechnol. Prog. 18:201-211 (2002)).

Figure 6 shows an exemplary pathway for adipate synthesis via alpha-ketoadipate using alpha-ketoglutarate as a starting point.

Figure 7 shows an exemplary pathway for synthesis of adipate using lysine as a starting point.

Figure 8 shows an exemplary caprolactam synthesis pathway using adipyl-CoA as a starting point.

Figure 9 shows exemplary adipate synthesis pathways using alpha-ketoadipate as a starting point.

DETAILED DESCRIPTION OF THE INVENTION

[0009] The present invention is directed to the design and production of cells and organisms having biosynthetic production capabilities for 6-aminocaproic acid or caprolactam. The results described herein indicate that metabolic pathways can be designed and recombinantly engineered to achieve the biosynthesis of 6-aminocaproic acid or caprolactam in *Escherichia coli* and other cells or organisms. Biosynthetic production of 6-aminocaproic acid and caprolactam can be confirmed by construction of strains having the designed metabolic genotype. These metabolically engineered cells or organisms also can be subjected to adaptive evolution to further augment 6-aminocaproic acid or caprolactam biosynthesis, including under conditions approaching theoretical maximum growth.

[0010] As disclosed herein, a number of metabolic pathways for the production of adipate, 6-aminocaproate, and caprolactam are described. Two routes, the reverse adipate degradation pathway and the 3-oxoadipate pathway, were found to be beneficial with respect to (i) the adipate yields (92% molar yield on glucose), (ii) the lack of oxygen requirement for adipate synthesis, (iii) the associated energetics, and (iv) the theoretical capability to produce adipate as the sole fermentation product. Metabolic pathways for adipate production that pass through α-ketoadipate or lysine are also described but are lower yielding and require aeration for maximum production. A pathway for producing either or both of 6-aminocaproate and caprolactam from adipyl-CoA, a precursor in the reverse degradation pathway, is also disclosed herein.

[0011] As disclosed herein, a number of exemplary pathways for biosynthesis of adipate are described. One exemplary pathway involves adipate synthesis via a route that relies on the reversibility of adipate degradation as described in organisms such as *P. chrysogenum* (see Examples I and II). A second exemplary pathway entails the formation of 3-oxoadipate followed by its reduction, dehydration and again reduction to form adipate (see Examples III and IV). The adipate yield using either of these two pathways is 0.92 moles per mole glucose consumed. The uptake of oxygen is not required for attaining these theoretical maximum yields, and the energetics under anaerobic conditions are favorable for growth and product secretion. A method for producing adipate from glucose-derived cis,cis-muconic acid was described previously (Frost et al., United States Patent No. 5,487,987, issued January 30, 1996)(see Example V). Advantages of the methods disclosed herein over this previously described method are discussed. Metabolic pathways for adipate production that pass through α-ketoadipate (Example VI) or lysine (Example VII) precursors are lower yielding and require aeration for maximum production. A pathway for producing either or both of 6-aminocaproate and caprolactam from adipyl-CoA, a precursor in the reverse degradation pathway, is described (see Example VIII and IX). Additional pathways for producing adipate are described in Examples X and XI. Exemplary genes and enzymes required for constructing microbes with these capabilities are described as well as methods for cloning and transformation, monitoring product formation, and using the engineered microorganisms for production.

[0012] As disclosed herein, six different pathways for adipic acid synthesis using glucose/sucrose as a carbon substrate are described. For all maximum yield calculations, the missing reactions in a given pathway were added to the *E. coli* stoichiometric network in SimPheny that is similar to the one described previously (Reed et al., Genome Biol. 4:R54 (2003)). Adipate is a charged molecule under physiological conditions and was assumed to require energy in the form of a proton-based symport system to be secreted out of the network. Such a transport system is thermodynamically feasible if the fermentations are carried out at neutral or near-neutral pH. Low pH adipic acid formation would require an ATP-dependant export mechanism, for example, the ABC system as opposed to proton symport. The reactions in the pathways and methods of implementation of these pathways are described in Examples I-XI.

[0013] As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes within an adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway.

[0014] A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, non-naturally occurring microorganisms can have genetic modifications to nucleic acids encoding metabolic polypeptides or, functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

[0015] As used herein, the term "isolated" when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally occurring environments. Specific examples of isolated microbial organisms include partially pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

[0016] As used herein, the terms "microbial," "microbial organism" or "microorganism" is intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

[0017] As used herein, the term "CoA" or "coenzyme A" is intended to mean an organic cofactor or prosthetic group (nonprotein portion of an enzyme) whose presence is required for the activity of many enzymes (the apoenzyme) to form an active enzyme system. Coenzyme A functions in certain condensing enzymes, acts in acetyl or other acyl group transfer and in fatty acid synthesis and oxidation, pyruvate oxidation and in other acetylation.

[0018] As used herein, "adipate," having the chemical formula $-OOC-(CH_2)_4-COO-$ (see Figure 2) (IUPAC name hexanedioate), is the ionized form of adipic acid (IUPAC name hexanedioic acid), and it is understood that adipate and adipic acid can be used interchangeably throughout to refer to the compound in any of its neutral or ionized forms, including any salt forms thereof. It is understood by those skilled understand that the specific form will depend on the pH.

[0019] As used herein, "6-aminocaproate," having the chemical formula $-OOC-(CH_2)_5-NH_2$ (see Figure 8), is the ionized form of 6-aminocaproic acid (IUPAC name 6-aminohexanoic acid), and it is understood that 6-aminocaproate and 6-aminocaproic acid can be used interchangeably throughout to refer to the compound in any of its neutral or ionized forms, including any salt forms thereof. It is understood by those skilled understand that the specific form will depend on the pH.

[0020] As used herein, "caprolactam" (IUPAC name azepan-2-one) is a lactam of 6-aminohexanoic acid (see Figure 8).

[0021] As used herein, the term "substantially anaerobic" when used in reference to a culture or growth condition is intended to mean that the amount of oxygen is less than about 10% of saturation for dissolved oxygen in liquid media. The term also is intended to include sealed chambers of liquid or solid medium maintained with an atmosphere of less than about 1% oxygen.

[0022] "Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

[0023] The non-naturally occurring microbal organisms of the invention can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

[0024] Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein, are described with reference to a suitable host organism such as *E. coli* and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

[0025] An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less that 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities. Members of the serine protease family of enzymes, including tissue plasminogen activator and elastase, are considered to have arisen by vertical descent from a common ancestor.

[0026] Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding products are considered to be orthologs. For the production of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be introduced or disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species. A specific example is the separation of elastase proteolysis and plasminogen proteolysis, two types of serine protease activity, into distinct molecules as plasminogen activator and elastase. A second example is the separation of mycoplasma 5'-3' exonuclease and *Drosophila* DNA polymerase III activity. The DNA polymerase from the first species can be considered an ortholog to either or both of the exonuclease or the polymerase from the second species and vice versa.

[0027] In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved from a common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

[0028] A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene product compared to a gene encoding the function sought to be substituted. Therefore, a nonorthologous gene includes, for example, a paralog or an unrelated gene.

[0029] Therefore, in identifying and constructing the non-naturally occurring microbial organisms of the invention having adipate, 6-aminocaproic acid or caprolactam biosynthetic capability, those skilled in the art will understand with applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications can include identification and inclusion or inactivation of orthologs. To the extent that paralogs and/or nonorthologous gene displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can utilize these evolutionally related genes.

[0030] Orthologs, paralogs and nonorthologous gene displacements can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is sufficiently high to indicate the proteins are related through evolution from a common ancestor. Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compare and determine a raw sequence similarity or identity, and also determine the presence or significance of gaps in the sequence which can be assigned a weight or score. Such algorithms also are known in the art and are similarly applicable for determining nucleotide sequence similarity or identity. Parameters for sufficient similarity to determine relatedness are computed based on well known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 25% to 100% sequence identity. Proteins that are unrelated can have an identity which is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%). Sequences between 5% and 24% may or may not represent sufficient homology to conclude that the compared sequences are related. Additional statistical analysis to determine the significance of such matches given the size of the data set can be carried out to determine the relevance of these sequences.

[0031] Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan-05-1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sept-16-1998) and the following parameters: Match: 1; mismatch: -2; gap open: 5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off. Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

[0032] The invention provides non-naturally occurring microbial organisms capable of producing 6-aminocaproic acid or caprolactam. Also disclosed, for example is an adipate pathway which can be a reverse adipate degradation pathway (see Examples I and II). Disclosed in one example is a non-naturally occurring microbial organism having an adipate pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, the adipate pathway comprising succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and adipyl-CoA synthetase or phosphotransadipylase/adipate kinase or adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase. In addition, an adipate pathway can be through a 3-oxoadipate pathway (see Examples III and IV). Another disclosure provides a non-naturally occurring microbial organism having an adipate pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, the adipate pathway comprising succinyl-CoA:acetyl-CoA acyl transferase, 3-oxoadipyl-CoA transferase, 3-oxoadipate reductase, 3-hydroxyadipate dehydratase, and 2-enoate reductase.

[0033] In an embodiment, the invention provides a non-naturally occurring microbial organism having a 6-aminocaproic acid pathway comprising at least two exogenous nucleic acids each encoding a 6-aminocaproic acid pathway enzyme expressed in a sufficient amount to produce 6-aminocaproic acid, the 6-aminocaproic acid pathway comprising CoA-dependent aldehyde dehydrogenase and transaminase (see Examples VIII and IX). Alternatively, 6-aminocaproate dehydrogenase can be used to convert adipate semialdehyde to form 6-aminocaproate (see Figure 8). In a further embodiment, the invention provides a non-naturally occurring microbial organism having a caprolactam pathway comprising at least one exogenous nucleic acid encoding a caprolactam pathway enzyme expressed in a sufficient amount to produce caprolactam, the caprolactam pathway comprising CoA-dependent aldehyde dehydrogenase, transaminase or 6-aminocaproate dehydrogenase, and amidohydrolase (see Examples VIII and IX).

[0034] As disclosed herein, a 6-aminocaproic acid or caprolactam producing microbial organism of the invention can produce 6-aminocaproic acid and/or caprolactam from an adipyl-CoA precursor (see Figure 8 and Examples VIII and IX). Therefore, it is understood that a 6-aminocaproic acid or caprolactam producing microbial organism can further include a pathway to produce adipyl-CoA. For example an adipyl-CoA pathway can include the enzymes of Figure 2 that utilize succinyl-CoA and acetyl-CoA as precursors through the production of adipyl-CoA, that is, lacking an enzyme for the final step of converting adipyl-CoA to adipate. Thus, one exemplary adipyl-CoA pathway can include succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase and 5-carboxy-2-pentenoyl-CoA reductase.

[0035] In addition, as shown in Figure 1, an adipate degradation pathway includes the step of converting adipate to adipyl-CoA by an adipate CoA ligase. Therefore, an adipyl-CoA pathway can be an adipate pathway that further includes an enzyme activity that converts adipate to adipyl-CoA, including, for example, adipate-CoA ligase activity as in the first step of Figure 1 or any of the enzymes in the final step of Figure 2 carried out in the reverse direction, for example, any of adipyl-CoA synthetase (also referred to as adipate Co-A ligase), phosphotransadipylase/adipate kinase, adipyl-CoA;acetyl-CoA transferase or adipyl-CoA hydrolase. An enzyme having adipate to adipyl-CoA activity can be an endogenous activity or can be provided as an exogenous nucleic acid encoding the enzyme, as disclosed herein. Thus, it is understood that any adipate pathway can be utilized with an adipate to adipyl-CoA enzymatic activity to generate an adipyl-CoA pathway. Such a pathway can be included in a 6-aminocaproic acid or caprolactam producing microbial organism to provide an adipyl-CoA precursor for 6-aminocaproic acid and/or caprolactam production.

[0036] An additional exemplary adipate pathway utilizes alpha-ketoadipate as a precursor (see Figure 6 and Example VI). Yet another disclosure provides a non-naturally occurring microbial organism having an adipate pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, the adipate pathway comprising homocitrate synthase, homoaconitase, homoisocitrate dehydrogenase, 2-ketoadipate reductase, alpha-hydroxyadipate dehydratase and oxidoreductase. A further adipate pathway utilizes a lysine dedgradation pathway (see Figure 7 and Example VII). Another disclosure provides a non-naturally occurring microbial organism having an adipate pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, the adipate pathway comprising carbon nitrogen lyase, oxidoreductase, transaminase and oxidoreductase.

[0037] Yet another disclosed adipate pathway utilizes alpha-ketoadipate as a precursor (see Figure 9 and Examples X and XI). Thus, disclosed herein is a non-naturally occurring microbial organism having an adipate pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, the adipate pathway comprising alpha-ketoadipyl-CoA synthetase, phosphotransketoadipylase/alpha-ketoadipate kinase or alpha-ketoadipyl-CoA:acetyl-CoA transferase; 2-hydroxyadipyl-CoA dehydrogenase; 2-hydroxyadipyl-CoA dehydratase; 5-carboxy-2-pentenoyl-CoA reductase; and adipyl-CoA synthetase, phosphotransadipylase/adipate kinase, adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase. Still another disclosure provides a non-naturally occurring microbial organism having an adipate pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, the adipate pathway comprising 2-hydroxyadipate dehydrogenase; 2-hydroxyadipyl-CoA synthetase, phosphotranshydroxyadipylase/2-hydroxyadipate kinase or 2-hydroxyadipyl-CoA:acetyl-CoA transferase; 2-hydroxyadipyl-CoA dehydratase; 5-carboxy-2-pentenoyl-CoA reductase; and adipyl-CoA synthetase, phosphotransadipylase/adipate kinase, adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase.

[0038] In an additional embodiment, the invention provides a non-naturally occurring microbial organism having an adipate, 6-aminocaproic acid or caprolactam pathway, wherein the non-naturally occurring microbial organism comprises at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product, as disclosed herein. Thus, the invention provides a non-naturally occurring microbial organism containing at least one exogenous nucleic acid encoding a polypeptide, where the polypeptide is an enzyme or protein that converts the substrates and products of an adipate, 6-aminocaproic acid or caprolactam pathway, such as that shown in Figures 2, 3, 8 and 9.

[0039] Disclosed is a non-naturally occurring microbial organism having an adipate pathway, wherein the microbial organism contains at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product selected from succinyl-CoA and acetyl-CoA to 3-oxoadipyl-CoA; 3-oxoadipyl-CoA to 3-hydroxyadipyl-CoA; 3-hydroxyadipyl-CoA to 5-carboxy-2-pentenoyl-CoA; 5-carboxy-2-pentenoyl-CoA to adipyl-CoA; adipyl-CoA to adipate (see Figure 2). Also disclosed is a non-naturally occurring microbial organism having an adipate pathway, wherein the microbial organism contains at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product selected from succinyl-CoA and acetyl-CoA to 3-oxoadipyl-CoA; 3-oxoadipyl-CoA to 3-oxoadipate; 3-oxoadipate to 3-hydroxyadipate; 3-hydroxyadipate to hexa-2-enedioate; hexa-2-enedioate to adipate (see Figure 3).

[0040] In an additional embodiment, the invention provides a non-naturally occurring microbial organism having a 6-aminocaproic acid pathway, wherein the microbial organism contains at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product selected from adipyl-CoA to adipate semialdehyde; and adipate semialdehyde to 6-aminocaproate (see Figure 8). In still another embodiment, the invention provides a non-naturally occurring microbial organism having a caprolactam pathway, wherein the microbial organism contains at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product selected from adipyl-CoA to adipate semialdehyde; adipate semialdehyde to 6-aminocaproate; and 6-aminocaproate to caprolactam.

[0041] Still another disclosure provides a non-naturally occurring microbial organism having an adipate pathway, wherein the microbial organism contains at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product selected from alpha-ketoadipate to alpha-ketoadipyl-CoA; alpha-ketoadipyl-CoA to 2-hydroxyadipyl-CoA; 2-hydroxyadipyl-CoA to 5-carboxy-2-pentenoyl-CoA; 5-carboxy-2-pentenoyl-CoA to adipyl-CoA; and adipyl-CoA to adipate (see Figure 9). Additionally disclosed is a non-naturally occurring microbial organism having an adipate pathway, wherein the microbial organism contains at least one exogenous nucleic acid encoding a polypeptide that converts a substrate to a product selected from alpha-ketoadipate to 2-hydroxyadipate; 2-hydroxyadipate to 2-hydroxyadipyl-CoA; 2-hydroxyadipyl-CoA to 5-carboxy-2-pentenoyl-CoA; 5-carboxy-2-pentenoyl-CoA to adipyl-CoA; and adipyl-CoA to adipate (Figure 9).

[0042] The invention is described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more nucleic acids or genes encoding an enzyme associated with or catalyzing the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction, and reference to any of these metabolic constituents also references the gene or genes encoding the enzymes that catalyze the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene or encoding nucleic acid also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes as well as the reactants and products of the reaction.

[0043] The non-naturally occurring microbial organisms of the invention can be produced by introducing expressible nucleic acids encoding one or more of the enzymes participating in one or more adipate, 6-aminocaproic acid or caprolactam biosynthetic pathways. Depending on the host microbial organism chosen for biosynthesis, nucleic acids for some or all of a particular adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway can be expressed. For example, if a chosen host is deficient in one or more enzymes for a desired biosynthetic pathway, then expressible nucleic acids for the deficient enzyme(s) are introduced into the host for subsequent exogenous expression. Alternatively, if the chosen host exhibits endogenous expression of some pathway genes, but is deficient in others, then an encoding nucleic acid is needed for the deficient enzyme(s) to achieve adipate, 6-aminocaproic acid or caprolactam biosynthesis. Thus, a non-naturally occurring microbial organism of the invention can be produced by introducing exogenous enzyme activities to obtain a desired biosynthetic pathway or a desired biosynthetic pathway can be obtained by introducing one or more exogenous enzyme activities that, together with one or more endogenous enzymes, produces a desired product such as adipate, 6-aminocaproic acid or caprolactam.

[0044] Depending on the adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway constituents of a selected host microbial organism, the non-naturally occurring microbial organisms of the invention will include at least one exogenously expressed adipate, 6-aminocaproic acid or caprolactam pathway-encoding nucleic acid and up to all encoding nucleic acids for one or more adipate, 6-aminocaproic acid or caprolactam biosynthetic pathways. For example, adipate, 6-aminocaproic acid or caprolactam biosynthesis can be established in a host deficient in a pathway enzyme through exogenous expression of the corresponding encoding nucleic acid. In a host deficient in all enzymes of a adipate, 6-aminocaproic acid or caprolactam pathway, exogenous expression of all enzyme in the pathway can be included, although it is understood that all enzymes of a pathway can be expressed even if the host contains at least one of the pathway enzymes.

[0045] For example, exogenous expression of all enzymes in a pathway for production of adipate can be included in a host organism, such as succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and adipyl-CoA synthetase or phosphotransadipylase/adipate kinase or adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase. In particular, a host organism can contain the adipate pathway enzymes succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and adipyl-CoA synthetase. Alternatively, a host organism can contain the adipate pathway enzymes succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and phosphotransadipylase/adipate kinase. In addition, a host organism can contain the adipate pathway enzymes succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and adipyl-CoA:acetyl-CoA transferase. Further, a host organism can contain the adipate pathway enzymes succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and adipyl-CoA hydrolase.

[0046] In the case of a 6-aminocaproic acid producing microbial organism, exogenous expression of all enzymes in a pathway for production of 6-aminocaproic acid can be included in a host organism, such as CoA-dependent aldehyde dehydrogenase and transaminase or CoA-dependent aldehyde dehdrogenase and 6-aminocaproate dehydrogenase. For a caprolactam producing microbial organism, exogenous expression of all enzymes in a pathway for production of caprolactam can be included in a host organism, such as CoA-dependent aldehyde dehydrogenase, transaminase or 6-aminocaproate dehydrogenase, and amidohydrolase.

[0047] Given the teachings and guidance provided herein, those skilled in the art will understand that the number of encoding nucleic acids to introduce in an expressible form will, at least, parallel the adipate, 6-aminocaproic acid or caprolactam pathway deficiencies of the selected host microbial organism. Therefore, a non-naturally occurring microbial organism of the invention can have one, two, three, four, or five, up to all nucleic acids encoding the above enzymes constituting a adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway. In some embodiments, the non-naturally occurring microbial organisms also can include other genetic modifications that facilitate or optimize adipate, 6-aminocaproic acid or caprolactam biosynthesis or that confer other useful functions onto the host microbial organism. One such other functionality can include, for example, augmentation of the synthesis of one or more of the adipate, 6-aminocaproic acid or caprolactam pathway precursors such as succinyl-CoA and/or acetyl-CoA in the case of adipate synthesis, or adipyl-CoA in the case of 6-aminocaproic acid or caprolactam synthesis, including the adipate pathway enzymes disclosed herein.

[0048] In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that contains the enzymatic capability to synthesize adipate, 6-aminocaproic acid or caprolactam. In this specific embodiment it can be useful to increase the synthesis or accumulation of an adipate, 6-aminocaproic acid or caprolactam pathway product to, for example, drive adipate, 6-aminocaproic acid or caprolactam pathway reactions toward adipate, 6-aminocaproic acid or caprolactam production. Increased synthesis or accumulation can be accomplished by, for example, overexpression of nucleic acids encoding one or more of the above-described adipate, 6-aminocaproic acid or caprolactam pathway enzymes. Over expression of the adipate, 6-aminocaproic acid or caprolactam pathway enzyme or enzymes can occur, for example, through exogenous expression of the endogenous gene or genes, or through exogenous expression of the heterologous gene or genes. Therefore, naturally occurring organisms can be readily generated to be non-naturally occurring microbial organisms of the invention, for example, producing adipate, 6-aminocaproic acid or caprolactam, through overexpression of one, two, three, four, five, that is, up to all nucleic acids encoding adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway enzymes. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an increase in activity of an enzyme in the adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway.

[0049] In particularly useful embodiments, exogenous expression of the encoding nucleic acids is employed. Exogenous expression confers the ability to custom tailor the expression and/or regulatory elements to the host and application to achieve a desired expression level that is controlled by the user. However, endogenous expression also can be utilized in other embodiments such as by removing a negative regulatory effector or induction of the gene's promoter when linked to an inducible promoter or other regulatory element. Thus, an endogenous gene having a naturally occurring inducible promoter can be up-regulated by providing the appropriate inducing agent, or the regulatory region of an endogenous gene can be engineered to incorporate an inducible regulatory element, thereby allowing the regulation of increased expression of an endogenous gene at a desired time. Similarly, an inducible promoter can be included as a regulatory element for an exogenous gene introduced into a non-naturally occurring microbial organism.

[0050] It is understood that, in methods of the invention, any of the one or more exogenous nucleic acids can be introduced into a microbial organism to produce a non-naturally occurring microbial organism of the invention. The nucleic acids can be introduced so as to confer, for example, an adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway onto the microbial organism. Alternatively, encoding nucleic acids can be introduced to produce an intermediate microbial organism having the biosynthetic capability to catalyze some of the required reactions to confer adipate, 6-aminocaproic acid or caprolactam biosynthetic capability. For example, a non-naturally occurring microbial organism having an adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes. In the case of adipate production, the at least two exogenous nucleic acids can encode the enzymes such as the combination of succinyl-CoA:acetyl-CoA acyl transferase and 3-hydroxyacyl-CoA dehydrogenase, or succinyl-CoA:acetyl-CoA acyl transferase and 3-hydroxyadipyl-CoA dehydratase, or 3-hydroxyadipyl-CoA and 5-carboxy-2-pentenoyl-CoA reductase, or 3-hydroxyacyl-CoA and adipyl-CoA synthetase, and the like. In the case of caprolactam production, the at least two exogenous nucleic acids can encode the enzymes such as the combination of CoA-dependent aldehyde dehydrogenase and transaminase, or CoA-dependent aldehyde dehydrogenase and amidohydrolase, or transaminase and amidohydrolase.

[0051] Thus, it is understood that any combination of two or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention.

[0052] Similarly, it is understood that any combination of three or more enzymes of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention, for example, in the case of adipate production, the combination of enzymes succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyadipyl-CoA dehydratase; or succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase and 5-carboxy-2-pentenoyl-CoA reductase; or succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase and adipyl-CoA synthetase; or 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase and adipyl-CoA:acetyl-CoA transferase, and so forth, as desired, so long as the combination of enzymes of the desired biosynthetic pathway results in production of the corresponding desired product. Similarly, any combination of four or more enzymes of a biosynthetic pathway as disclosed herein can be included in a non-naturally occurring microbial organism of the invention, as desired, so long as the combination of enzymes of the desired biosynthetic pathway results in production of the corresponding desired product.

[0053] In addition to the biosynthesis of adipate, 6-aminocaproic acid or caprolactam as described herein, the non-naturally occurring microbial organisms and methods of the invention also can be utilized in various combinations with each other and with other microbial organisms and methods well known in the art to achieve product biosynthesis by other routes. For example, one alternative to produce adipate, 6-aminocaproic acid or caprolactam other than use of the adipate, 6-aminocaproic acid or caprolactam producers is through addition of another microbial organism capable of converting an adipate, 6-aminocaproic acid or caprolactam pathway intermediate to adipate, 6-aminocaproic acid or caprolactam. One such procedure includes, for example, the fermentation of a microbial organism that produces an adipate, 6-aminocaproic acid or caprolactam pathway intermediate. The adipate, 6-aminocaproic acid or caprolactam pathway intermediate can then be used as a substrate for a second microbial organism that converts the adipate, 6-aminocaproic acid or caprolactam pathway intermediate to adipate, 6-aminocaproic acid or caprolactam. The adipate, 6-aminocaproic acid or caprolactam pathway intermediate can be added directly to another culture of the second organism or the original culture of the adipate, 6-aminocaproic acid or caprolactam pathway intermediate producers can be depleted of these microbial organisms by, for example, cell separation, and then subsequent addition of the second organism to the fermentation broth can be utilized to produce the final product without intermediate purification steps.

[0054] In other embodiments, the non-naturally occurring microbial organisms and methods of the invention can be assembled in a wide variety of subpathways to achieve biosynthesis of 6-aminocaproic acid or caprolactam. In these embodiments, biosynthetic pathways for a desired product of the invention can be segregated into different microbial organisms, and the different microbial organisms can be co-cultured to produce the final product. In such a biosynthetic scheme, the product of one microbial organism is the substrate for a second microbial organism until the final product is synthesized. For example, the biosynthesis of 6-aminocaproic acid or caprolactam can be accomplished by constructing a microbial organism that contains biosynthetic pathways for conversion of one pathway intermediate to another pathway intermediate or the product. Alternatively, 6-aminocaproic acid or caprolactam also can be biosynthetically produced from microbial organisms through co-culture or co-fermentation using two organisms in the same vessel, where the first microbial organism produces an adipate, 6-aminocaproic acid or caprolactam intermediate and the second microbial organism converts the intermediate to 6-aminocaproic acid or caprolactam.

[0055] Given the teachings and guidance provided herein, those skilled in the art will understand that a wide variety of combinations and permutations exist for the non-naturally occurring microbial organisms and methods of the invention together with other microbial organisms, with the co-culture of other non-naturally occurring microbial organisms having subpathways and with combinations of other chemical and/or biochemical procedures well known in the art to produce adipate, 6-aminocaproic acid or caprolactam.

[0056] Sources of encoding nucleic acids for an adipate, 6-aminocaproic acid or caprolactam pathway enzyme can include, for example, any species where the encoded gene product is capable of catalyzing the referenced reaction. Such species include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including human. Exemplary species for such sources include, for example, *Escherichia coli, Pseudomonas kuackmussii, Pseudomonas putida, Pseudomonas fluorescens, Klebsiella pneumoniae, Serratia proteamaculans, Streptomyces* sp. 2065, *Pseudomonas aeruginosa, Ralstonia eutropha, Clostridium acetobutylicum, Euglena gracilis, Treponema denticola, Clostridium kluyveri, Homo sapiens, Rattus norvegicus, Acinetobacter* sp. ADP1, *Streptomyces coelicolor, Eubacterium barkeri, Peptostreptococcus asaccharolyticus, Clostridium botulinum, Clostridium tyrobutyricum, Clostridium thermoaceticum (Moorella thermoaceticum), Acinetobacter calcoaceticus, Mus musculus, Sus scrofa, Flavobacterium sp, Arthrobacter aurescens, Penicillium chrysogenum, Aspergillus niger, Aspergillus nidulans, Bacillus subtilis, Saccharomyces cerevisiae, Zymomonas mobilis, Mannheimia succiniciproducens, Clostridium ljungdahlii, Clostridium carboxydivorans, Geobacillus stearothermophilus, Agrobacterium tumefaciens, Achromobacter denitrificans, Arabidopsis thaliana, Haemophilus influenzae, Acidaminococcus fermentans, Clostridium sp. M62/1, Fusobacterium nucleatum,* as well as other exemplary species disclosed herein or available as source organisms for corresponding genes (see Examples). However, with the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite adipate, 6-aminocaproic acid or caprolactam biosynthetic activity for one or more genes in related or distant species, including for example, homologues, orthologs, paralogs and nonorthologous gene displacements of known genes, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling biosynthesis of adipate, 6-aminocaproic acid or caprolactam described herein with reference to a particular organism such as *E. coli* can be readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

[0057] In some instances, such as when an alternative adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway exists in an unrelated species, adipate, 6-aminocaproic acid or caprolactam biosynthesis can be conferred onto the host species by, for example, exogenous expression of a paralog or paralogs from the unrelated species that catalyzes a similar, yet non-identical metabolic reaction to replace the referenced reaction. Because certain differences among metabolic networks exist between different organisms, those skilled in the art will understand that the actual gene usage between different organisms may differ. However, given the teachings and guidance provided herein, those skilled in the art also will understand that the teachings and methods of the invention can be applied to all microbial organisms using the cognate metabolic alterations to those exemplified herein to construct a microbial organism in a species of interest that will synthesize 6-aminocaproic acid or caprolactam.

[0058] Host microbial organisms can be selected from, and the non-naturally occurring microbial organisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *Escherichia coli, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Bacillus subtilis, Corynebacterium glutamicum, Gluconobacter oxydans, Zymomonas mobilis, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Clostridium acetobutylicum, Pseudomonas fluoresceus,* and *Pseudomonas putida.* Exemplary yeasts or fungi include species selected from *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis,* *Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger* and *Pichia pastoris.* For example, *E. coli* is a particularly useful host organisms since it is a well characterized microbial organism suitable for genetic engineering. Other particularly useful host organisms include yeast such as *Saccharomyces cerevisiae.*

[0059] Methods for constructing and testing the expression levels of a non-naturally occurring 6-aminocaproic acid- or caprolactam-producing host can be performed, for example, by recombinant and detection methods well known in the art. Such methods can be found described in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Ed., Cold Spring Harbor Laboratory, New York (2001); and Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1999).

[0060] Exogenous nucleic acid sequences involved in a pathway for production of 6-aminocaproic acid or caprolactam can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. For exogenous expression in *E. coli* or other prokaryotic cells, some nucleic acid sequences in the genes or cDNAs of eukaryotic nucleic acids can encode targeting signals such as an N-terminal mitochondrial or other targeting signal, which can be removed before transformation into prokaryotic host cells, if desired. For example, removal of a mitochondrial leader sequence led to increased expression in *E. coli* (Hoffmeister et al., J. Biol. Chem. 280:4329-4338 (2005). For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of leader sequence, or can be targeted to mitochondrion or other organelles, or targeted for secretion, by the addition of a suitable targeting sequence such as a mitochondrial targeting or secretion signal suitable for the host cells. Thus, it is understood that appropriate modifications to a nucleic acid sequence to remove or include a targeting sequence can be incorporated into an exogenous nucleic acid sequence to impart desirable properties. Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

[0061] An expression vector or vectors can be constructed to include one or more adipate, 6-aminocaproic acid or caprolactam biosynthetic pathway encoding nucleic acids as exemplified herein operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and selection sequences or markers operable for stable integration into a host chromosome. Additionally, the expression vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The transformation of exogenous nucleic acid sequences involved in a metabolic or synthetic pathway can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in a sufficient amount to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

[0062] The invention additionally provides methods for producing a desired product such as 6-aminocaproic acid or caprolactam. Disclosed herein is a method for producing adipate, comprising culturing a non-naturally occurring microbial organism having an adipate pathway, the pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, under conditions and for a sufficient period of time to produce adipate, the adipate pathway comprising succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, 5-carboxy-2-pentenoyl-CoA reductase, and adipyl-CoA synthetase or phosphotransadipylase/adipate kinase or adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase. Another disclosure provides a method for producing adipate, comprising culturing a non-naturally occurring microbial organism having an adipate pathway, the pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, under conditions and for a sufficient period of time to produce adipate, the adipate pathway comprising succinyl-CoA:acetyl-CoA acyl transferase, 3-oxoadipyl-CoA transferase, 3-oxoadipate reductase, 3-hydroxyadipate dehydratase, and 2-enoate reductase.

[0063] In yet another embodiment, the invention provides a method for producing 6-aminocaproic acid, comprising culturing a non-naturally occurring microbial organism having a 6-aminocaproic acid pathway, the pathway comprising at least one exogenous nucleic acid encoding a 6-aminocaproic acid pathway enzyme expressed in a sufficient amount to produce 6-aminocaproic acid, under conditions and for a sufficient period of time to produce 6-aminocaproic acid, the 6-aminocaproic acid pathway comprising CoA-dependent aldehyde dehydrogenase and transaminase or 6-aminocaproate dehydrogenase. In a further embodiment, the invention provides a method for producing caprolactam, comprising culturing a non-naturally occurring microbial organism having a caprolactam pathway, the pathway comprising at least one exogenous nucleic acid encoding a caprolactam pathway enzyme expressed in a sufficient amount to produce caprolactam, under conditions and for a sufficient period of time to produce caprolactam, the caprolactam pathway comprising CoA-dependent aldehyde dehydrogenase, transaminase or 6-aminocaproate dehydrogenase, and amidohydrolase.

[0064] Also disclosed herein is a method for producing adipate, comprising culturing a non-naturally occurring microbial organism having an adipate pathway, the pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, under conditions and for a sufficient period of time to produce adipate, the adipate pathway comprising alpha-ketoadipyl-CoA synthetase, phosphotransketoadipylase/alpha-ketoadipate kinase or alpha-ketoadipyl-CoA:acetyl-CoA transferase; 2-hydroxyadipyl-CoA dehydrogenase; 2-hydroxyadipyl-CoA dehydratase; 5-carboxy-2-pentenoyl-CoA reductase; and adipyl-CoA synthetase, phosphotransadipylase/adipate kinase, adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase.

[0065] In still another disclosure there is provided a method for producing adipate, comprising culturing a non-naturally occurring microbial organism having an adipate pathway, the pathway comprising at least one exogenous nucleic acid encoding an adipate pathway enzyme expressed in a sufficient amount to produce adipate, under conditions and for a sufficient period of time to produce adipate, the adipate pathway comprising 2-hydroxyadipate dehydrogenase; 2-hydroxyadipyl-CoA synthetase, phosphotranshydroxyadipylase/2-hydroxyadipate kinase or 2-hydroxyadipyl-CoA:acetyl-CoA transferase; 2-hydroxyadipyl-CoA dehydratase; 5-carboxy-2-pentenoyl-CoA reductase; and adipyl-CoA synthetase, phosphotransadipylase/adipate kinase, adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase.

[0066] Suitable purification and/or assays to test for the production of 6-aminocaproic acid or caprolactam can be performed using well known methods. Suitable replicates such as triplicate cultures can be grown for each engineered strain to be tested. For example, product and byproduct formation in the engineered production host can be monitored. The final product and intermediates, and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography-Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) using routine procedures well known in the art. The release of product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual glucose can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 90:775-779 (2005)), or other suitable assay and detection methods well known in the art. The individual enzyme activities from the exogenous DNA sequences can also be assayed using methods well known in the art.

[0067] The 6-aminocaproic acid or caprolactam can be separated from other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, extraction procedures as well as methods that include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art.

[0068] Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the 6-aminocaproic acid or caprolactam producers can be cultured for the biosynthetic production of adipate, 6-aminocaproic acid or caprolactam.

[0069] For the production of 6-aminocaproic acid or caprolactam, the recombinant strains are cultured in a medium with carbon source and other essential nutrients. It is highly desirable to maintain anaerobic conditions in the fermenter to reduce the cost of the overall process. Such conditions can be obtained, for example, by first sparging the medium with nitrogen and then sealing the flasks with a septum and crimp-cap. For strains where growth is not observed anaerobically, microaerobic conditions can be applied by perforating the septum with a small hole for limited aeration. Exemplary anaerobic conditions have been described previously and are well-known in the art. Exemplary aerobic and anaerobic conditions are described, for example, in United State Patent application serial No. 11/891,602, filed August 10, 2007. Fermentations can be performed in a batch, fed-batch or continuous manner, as disclosed herein.

[0070] If desired, the pH of the medium can be maintained at a desired pH, in particular neutral pH, such as a pH of around 7 by addition of a base, such as NaOH or other bases, or acid, as needed to maintain the culture medium at a desirable pH. The growth rate can be determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time.

[0071] The growth medium can be, for example, any carbohydrate source which can supply a source of carbon to the non-naturally occurring microorganism. Such sources include, for example, sugars such as glucose, xylose, arabinose, galactose, mannose, fructose and starch. Other sources of carbohydrate include, for example, renewable feedstocks and biomass. Exemplary types of biomasses that can be used as feedstocks in the methods of the invention include cellulosic biomass, hemicellulosic biomass and lignin feedstocks or portions of feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates useful as carbon sources such as glucose, xylose, arabinose, galactose, mannose, fructose and starch. Given the teachings and guidance provided herein, those skilled in the art will understand that renewable feedstocks and biomass other than those exemplified above also can be used for culturing the microbial organisms of the invention for the production of adipate, 6-aminocaproic acid or caprolactam.

[0072] In addition to renewable feedstocks such as those exemplified above, the adipate, 6-aminocaproic acid or caprolactam microbial organisms of the invention also can be modified for growth on syngas as its source of carbon. In this specific embodiment, one or more proteins or enzymes are expressed in the adipate, 6-aminocaproic acid or caprolactam producing organisms to provide a metabolic pathway for utilization of syngas or other gaseous carbon source.

[0073] Synthesis gas, also known as syngas or producer gas, is the major product of gasification of coal and of carbonaceous materials such as biomass materials, including agricultural crops and residues. Syngas is a mixture primarily of H_2 and CO and can be obtained from the gasification of any organic feedstock, including but not limited to coal, coal oil, natural gas, biomass, and waste organic matter. Gasification is generally carried out under a high fuel to oxygen ratio. Although largely H_2 and CO, syngas can also include CO_2 and other gases in smaller quantities. Thus, synthesis gas provides a cost effective source of gaseous carbon such as CO and, additionally, CO_2.

[0074] The Wood-Ljungdahl pathway catalyzes the conversion of CO and H_2 to acetyl-CoA and other products such as acetate. Organisms capable of utilizing CO and syngas also generally have the capability of utilizing CO_2 and CO_2/H_2 mixtures through the same basic set of enzymes and transformations encompassed by the Wood-Ljungdahl pathway. H_2-dependent conversion of CO_2 to acetate by microorganisms was recognized long before it was revealed that CO also could be used by the same organisms and that the same pathways were involved. Many acetogens have been shown to grow in the presence of CO_2 and produce compounds such as acetate as long as hydrogen is present to supply the necessary reducing equivalents (see for example, Drake, Acetogenesis, pp. 3-60 Chapman and Hall, New York, (1994)). This can be summarized by the following equation:

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

[0075] Hence, non-naturally occurring microorganisms possessing the Wood-Ljungdahl pathway can utilize CO_2 and H_2 mixtures as well for the production of acetyl-CoA and other desired products.

[0076] The Wood-Ljungdahl pathway is well known in the art and consists of 12 reactions which can be separated into two branches: (1) methyl branch and (2) carbonyl branch. The methyl branch converts syngas to methyl-tetrahydrofolate (methyl-THF) whereas the carbonyl branch converts methyl-THF to acetyl-CoA. The reactions in the methyl branch are catalyzed in order by the following enzymes: ferredoxin oxidoreductase, formate dehydrogenase, formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclodehydratase, methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate reductase. The reactions in the carbonyl branch are catalyzed in order by the following enzymes: cobalamide corrinoid/iron-sulfur protein, methyltransferase, carbon monoxide dehydrogenase, acetyl-CoA synthase, acetyl-CoA synthase disulfide reductase and hydrogenase. Following the teachings and guidance provided herein for introducing a sufficient number of encoding nucleic acids to generate an adipate, 6-aminocaproic acid or caprolactam pathway, those skilled in the art will understand that the same engineering design also can be performed with respect to introducing at least the nucleic acids encoding the Wood-Ljungdahl enzymes absent in the host organism. Therefore, introduction of one or more encoding nucleic acids into the microbial organisms of the invention such that the modified organism contains the complete Wood-Ljungdahl pathway will confer syngas utilization ability.

[0077] Given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that secretes the biosynthesized compounds of the invention when grown on a carbon source such as a carbohydrate. Such compounds include, for example, 6-aminocaproic acid or caprolactam and any of the intermediate metabolites in the adipate, 6-aminocaproic acid or caprolactam pathway. All that is required is to engineer in one or more of the required enzyme activities to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the adipate, 6-aminocaproic acid or caprolactam biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that produces and/or secretes 6-aminocaproic acid or caprolactam when grown on a carbohydrate and produces and/or secretes any of the intermediate metabolites shown in the adipate, 6-aminocaproic acid or caprolactam pathway when grown on a carbohydrate. For example, the adipate producing microbial organisms can initiate synthesis from an intermediate, for example, 3-oxoadipyl-CoA, 3-hydroxyadipyl-CoA, 5-carboxy-2-pentenoyl-CoA, or adipyl-CoA (see Figure 2), as desired. In addition, an adipate producing microbial organism can initiate synthesis from an intermediate, for example, 3-oxoadipyl-CoA, 3-oxoadipate, 3-hydroxyadipate, or hexa-2-enedioate (see Figure 3). The 6-aminocaproic acid producing microbial organism of the invention can initiate synthesis from an intermediate, for example, adipate semialdehyde (see Figure 8). The caprolactam producing microbial organism of the invention can initiate synthesis from an intermediate, for example, adipate semialdehyde or 6-aminocaproic acid (see Figure 8), as desired.

[0078] The non-naturally occurring microbial organisms of the invention are constructed using methods well known in the art as exemplified herein to exogenously express at least one nucleic acid encoding an adipate, 6-aminocaproic acid or caprolactam pathway enzyme in sufficient amounts to produce 6-aminocaproic acid or caprolactam. It is understood that the microbial organisms of the invention are cultured under conditions sufficient to produce 6-aminocaproic acid or caprolactam. Following the teachings and guidance provided herein, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of 6-aminocaproic acid or caprolactam resulting in intracellular concentrations between about 0.1-200 mM or more. Generally, the intracellular concentration of adipate, 6-aminocaproic acid or caprolactam is between about 3-150 mM, particularly between about 5-125 mM and more particularly between about 8-100 mM, including about 10 mM, 20 mM, 50 mM, 80 mM, or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention.

[0079] In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described herein and are described, for example, in U.S. patent application serial No. 11/891,602, filed August 10, 2007. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions, the adipate, 6-aminocaproic acid or caprolactam producers can synthesize 6-aminocaproic acid or caprolactam at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified herein. It is understood that, even though the above description refers to intracellular concentrations, adipate, 6-aminocaproic acid or caprolactam producing microbial organisms can produce adipate, 6-aminocaproic acid or caprolactam intracellularly and/or secrete the product into the culture medium.

[0080] The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described herein, particularly useful yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

[0081] As described herein, one exemplary growth condition for achieving biosynthesis of 6-aminocaproic acid or caprolactam includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N_2/CO_2 mixture or other suitable non-oxygen gas or gases.

[0082] The culture conditions described herein can be scaled up and grown continuously for manufacturing of 6-aminocaproic acid or caprolactam. Exemplary growth procedures include, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. All of these processes are well known in the art. Fermentation procedures are particularly useful for the biosynthetic production of commercial quantities of 6-aminocaproic acid or caprolactam. Generally, and as with non-continuous culture procedures, the continuous and/or near-continuous production of adipate, 6-aminocaproic acid or caprolactam will include culturing a non-naturally occurring adipate, 6-aminocaproic acid or caprolactam producing organism of the invention in sufficient nutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can be include, for example, 1 day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous culture can include 1 week, 2, 3, 4 or 5 or more weeks and up to several months. Alternatively, organisms of the invention can be cultured for hours, if suitable for a particular application. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods. It is further understood that the time of culturing the microbial organism of the invention is for a sufficient period of time to produce a sufficient amount of product for a desired purpose.

[0083] Fermentation procedures are well known in the art. Briefly, fermentation for the biosynthetic production of 6-aminocaproic acid or caprolactam can be utilized in, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. Examples of batch and continuous fermentation procedures are well known in the art.

[0084] In addition to the above fermentation procedures using the adipate, 6-aminocaproic acid or caprolactam producers of the invention for continuous production of substantial quantities of adipate, 6-aminocaproic acid or caprolactam, the adipate, 6-aminocaproic acid or caprolactam producers also can be, for example, simultaneously subjected to chemical synthesis procedures to convert the product to other compounds or the product can be separated from the fermentation culture and sequentially subjected to chemical conversion to convert the product to other compounds, if desired. As described herein, an intermediate in the adipate pathway utilizing 3-oxoadipate, hexa-2-enedioate, can be converted to adipate, for example, by chemical hydrogenation over a platinum catalyst (see Example III).

[0085] To generate better producers, metabolic modeling can be utilized to optimize growth conditions. Modeling can also be used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of 6-aminocaproic acid or caprolactam.

[0086] One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework, Burgard et al., Biotechnol. Bioeng. 84:647-657 (2003). OptKnock is a metabolic modeling and simulation program that suggests gene deletion strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

[0087] Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed January 10, 2002, in International Patent No. PCT/US02/00660, filed January 10, 2002, and U.S. patent application serial No. 11/891,602, filed August 10,2007.

[0088] Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003. SimPheny® is a computational system that can be used to produce a network model *in silico* and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

[0089] These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

[0090] Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

[0091] The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

[0092] Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

[0093] To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., Biotechnol. Prog. 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

[0094] The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an *in silico* method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

[0095] As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., Biotechnol. Bioeng. 84:647-657 (2003)).

[0096] An *in silico* stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and in U.S. Patent No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

EXAMPLE I

Reverse Adipate Degradation Pathway

[0097] This example describes an exemplary adipate synthesis pathway via a reverse adipate degradation pathway.

[0098] Organisms such as *Penicillium chrysogenum* have the ability to naturally degrade adipate (Thykaer et al., Melab. Eng. 4:151-158. (2002)). The mechanism is similar to the oxidation of fatty acids (see Figure 1). The first step in adipate degradation is an ATP-dependent reaction that activates adipate with CoA. The second reaction is catalyzed by a dehydrogenase that forms 5-carboxy-2-pentenoyl-CoA from adipyl-CoA. During peroxisomal adipate degradation, the dehydrogenase enzyme contains FAD, which accepts the electrons and then transfers them directly to oxygen. A catalase enzyme dissipates the H_2O_2 formed by the reduction of oxygen. In mitochondrial fatty acid oxidation, the FAD from the dehydrogenase transfers electrons directly to the electron transport chain. A multi-functional fatty acid oxidation protein in eukaryotes such as *S. cerevisiae* and *P. chrysogenum* carries out the following hydratase and dehydrogenase steps. The final step is an acyl transferase that splits 3-oxoadipyl CoA into acetyl-CoA and succinyl-CoA.

[0099] A highly efficient pathway for the production of adipate is achieved through genetically altering a microorganism such that similar enzymatic reactions are employed for adipate synthesis from succinyl-CoA and acetyl-CoA (see Figure 2). Successful implementation of this entails expressing the appropriate genes, tailoring their expression, and altering culture conditions so that high acetyl-CoA, succinyl-CoA, and/or redox (for example, NADH/NAD+) ratios will drive the metabolic flux through this pathway in the direction of adipate synthesis rather than degradation. Strong parallels to butyrate formation in *Clostridia* (Kanehisa and Goto, Nucl. Acids Res. 28:27-30 (2000)) support that each step in the adipate synthesis pathway is thermodynamically feasible with reaction directionality governed by the concentrations of the participating metabolites. The final step, which forms adipate from adipyl-CoA, can take place either via a synthetase, phosphotransadipylase/kinase, transferase, or hydrolase mechanism.

[0100] The maximum theoretical yields of adipate using this pathway were calculated both in the presence and absence of an external electron acceptor such as oxygen. These calculations show that the pathway can efficiently transform glucose into adipate and CO_2 under anaerobic conditions with a 92% molar yield (Table I). The production of adipate using this pathway does not require the uptake of oxygen as NAD+ can be regenerated in the two hydrogenase steps that form 3-hydroxyadipyl-CoA and adipyl-CoA (see Figure 2). Further, the pathway is favorable energetically as up to 1.55 moles of ATP are formed per mole of glucose consumed at the maximum theoretical yield of adipate assuming either a synthetase, phosphotransadipylase/kinase, or transferase mechanism for the final conversion step. The ATP yield can be further improved to 2.47 moles of ATP produced per mole of glucose if phosphoenolpyruvate carboxykinase (PPCK) is assumed to function in the ATP-generating direction towards oxaloacetate formation. Maximum ATP yield calculations were then performed assuming that the adipyl-CoA to adipate transformation is a hydrolysis step. This reduces the maximum ATP yields at maximum adipate production to 0.85 and 1.77 mole ATP per mole glucose consumed if PPCK is assumed irreversible and reversible, respectively. Nevertheless, these ATP yields are sufficient for cell growth, maintenance, and production.

Table 1: The maximum theoretical yields of adipate and the associated ATP yields per mole of glucose using the reverse degradation pathway assuming the final step in the pathway is a synthetase, phosphotransadipylase/kinase, or transferase.

	Aerobic	Anaerobic
Adipate Yield	0.92	0.92
Max ATP yield @ max adipate yield	1.55	1.55
Max ATP yield @ max adipate yield PPCK assumed	2.47	2.47

[0101] Successfully engineering this pathway involves identifying an appropriate set of enzymes with sufficient activity and specificity. This entails identifying an appropriate set of enzymes, cloning their corresponding genes into a production host, optimizing fermentation conditions, and assaying for product formation following fermentation. To engineer a production host for the production of adipate, one or more exogenous DNA sequence(s) are expressed in a suitable host microorganism. In addition, the microorganisms can have endogenous gene(s) functionally deleted. These modifications allow the production of adipate using renewable feedstock.

[0102] Below is described a number of biochemically characterized candidate genes that encode enzymes that catalyze each step of the reverse adipate degradation pathway in a production host. Although described using *E. coli* as a host organism to engineer the pathway, essentially any suitable host organism can be used. Specifically listed are genes that are native to *E. coli* as well as genes in other organisms that can be applied to catalyze the appropriate transformations when properly cloned and expressed.

[0103] Referring to Figure 2, step 1 involves succinyl CoA:acetyl CoA acyl transferase (β-ketothiolase). The first step in the pathway combines acetyl-CoA and succinyl-CoA to form 3-oxoadipyl-CoA. The gene products encoded by *pcaF* in *Pseudomonas* strain B13 (Kaschabek et al., J. Bacteriol. 184:207-215 (2002)), *phaD* in *Pseudomonas putida* U (Olivera et al., Proc. Natl. Acad. Sci. USA 95:6419-6424 (1998)), *paaE* in *Pseudomonas fluorescens* ST (Di Gennaro et al., Arch. Microbiol. 188:117-125 (2007)), and *paaJ* from *E. coli* (Nogales et al., Microbiol. 153:357-365 (2007)) catalyze the conversion of 3-oxoadipyl-CoA into succinyl-CoA and acetyl-CoA during the degradation of aromatic compounds such as phenylacetate or styrene. Since β-ketothiolase enzymes catalyze reversible transformations, these enzymes can be employed for the first step in adipate synthesis shown in Figure 2. For example, the ketothiolase *phaA* from *R. eutropha* combines two molecules of acetyl-CoA to form acetoacetyl-CoA (Sato et al., J. Biosci. Bioengineer. 103:38-44 (2007)). Similarly, a β-keto thiolase (*bktB*) has been reported to catalyze the condensation of acetyl-CoA and propionyl-CoA to form β-ketovaleryl-CoA (Slater et al., J. Bacteriol. 180: 1979-1987 (1998)) in *R. eutropha.* The protein sequences for the above-mentioned gene products are well known in the art and can be accessed in the public databases such as GenBank using the following accession numbers.

Gene name	GenBank Accession #	Organism
paaJ	NP_415915.1	*Escherichia coli*
pcaF	AAL02407	*Pseudomonas knackmussii* (B13)
phaD	AAC24332.1	*Pseudomonas putida*
paaE	ABF82237.1	*Pseudomonas fluorescens*

[0104] These exemplary sequences can be used to identify homologue proteins in GenBank or other databases through sequence similarity searches (for example, BLASTp). The resulting homologue proteins and their corresponding gene sequences provide additional exogenous DNA sequences for transformation into *E. coli* or other suitable host microorganisms to generate production hosts.

[0105] For example, orthologs of *paaJ* from *Escherichia coli* K12 can be found using the following GenBank accession numbers:

YP_001335140.1	*Klebsiella pneumoniae*
YP_001479310.1	*Serratia proteamaculans*
AAC24332.1	*Pseudomonas putida*

[0106] Example orthologs of *pcaF* from *Pseudomonas knachmussii* can be found using the following GenBank accession numbers:

AAD22035.1	*Streptomyces* sp. 2065
AAN67000.1	*Pseudomonas putida*
ABJ15177.1	*Pseudomonas aeruginosa*

[0107] Additional native candidate genes for the ketothiolase step include *atoB,* which can catalyze the reversible condensation of 2 acetyl-CoA molecules (Sato et al., J. Biosci. Bioengineer. 103:38-44 (2007)), and its homolog *yqeF*. Non-native gene candidates include *phaA* (Sato et al., *supra,* 2007) and *bktB* (Slater et al., J. Bacteriol. 180:1979-1987 (1998)) from *R. eutropha*, and the two ketothiolases, *thiA* and *thiB*, from *Clostridium acetobutylicum* (Winzer et al., J. Mol. Microbiol. Biotechnol. 2:531-541 (2000)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

atoB	NP_416728.1	*Escherichia coli*
yqeF	NP_417321.2	*Escherichia coli*
phaA	YP_725941	*Ralstonia eutropha*
bktB	AAC38322.1	*Ralstonia eutropha*
thiA	NP_349476.1	*Clostridium acetobutylicum*
thiB	NP_149242.1	*Clostridium acetobutylicum*

[0108] Referring to Figure 2, step 2 involves 3-hydroxyacyl-CoA dehydrogenase. The second step in the pathway involves the reduction of 3-oxoadipyl-CoA to 3-hydroxyadipyl-CoA. The gene products encoded by *phaC* in *Pseudomonas putida* U (Olivera et al., Proc. Natl. Acad. Sci. USA 95:6419-6424 (1998)) and *paaC* in *Pseudomonas fluorescens* ST (Di Gennaro et al., Arch. Microbiol. 188:117-125 (2007)) catalyze the reverse reaction, that is, the oxidation of 3-hydroxyadipyl-CoA to form 3-oxoadipyl-CoA, during the catabolism of phenylacetate or styrene. The reactions catalyzed by such dehydrogenases are reversible and accordingly these genes represent candidates to carry out the second step of adipate synthesis as shown in Figure 2. A similar transformation is also carried out by the gene product of *hbd* in *Clostridium acetobutylicum* (Atsumi et al., Metab. Eng. (epub Sep. 14, 2007); Boynton et al., J. Bacteriol. 178:3015-3024 (1996)). This enzyme converts acetoacetyl-CoA to 3-hydroxybutyryl-CoA. Lastly, given the proximity in *E. coli* of *paaH* to other genes in the phenylacetate degradation operon (Nogales et al.,. Microbiol. 153:357-365 (2007)) and the fact that *paaH* mutants cannot grow on phenylacetate (Ismail et al., Eur. J. Biochem. 270:3047-3054 (2003)), it is expected that the *E. coli paaH* gene encodes a 3-hydroxyacyl-CoA dehydrogenase. The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

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

[0109] Referring to Figure 2, step 3 involves 3-hydroxyadipyl-CoA dehydratase. The gene product of *crt* from *C. acetobutylicum* catalyzes the dehydration of 3-hydroxybutyryl-CoA to crotonyl-CoA (see Figure 2) (Atsumi et al., *supra,* 2007; Boynton et al., J. Bacteriol. 178:3015-3024 (1996)). Homologs of this gene are strong candidates for carrying out the third step in the adipate synthesis pathway exemplified in Figure 2. In addition, genes known to catalyze the hydroxylation of double bonds in enoyl-CoA compounds represent additional candidates given the reversibility of such enzymatic transformations. For example, the enoyl-CoA hydratascs, *phaA* and *phaB*, of *P. putida* are believed to carry out the hydroxylation of double bonds during phenylacetate catabolism (Olivera et al., Proc. Natl. Acad. Sci. USA 95:6419-6424 (1998)) and thus represent additional candidates for incorporation into *E. coli.* The deletion of these genes precludes phenylacetate degradation in *P. putida.* The *paaA* and *paaB* from *P. fluorescens* catalyze analogous transformations (Olivera et al., *supra*, 1998). Lastly, a number of *Escherichia coli* genes have been shown to demonstrate enoyl-CoA hydratase functionality including *maoC* (Park and Lee, J. Bacteriol. 185:5391-5397 (2003)), *paaF* (Ismail et al., Eur. J. Biochem. 270:3047-3054 (2003); Park and Lee, Biotechnol. Bioeng. 86:681-686 (2004); Park and Lee, Appl. Biochem. Biotechnol. 113-116:335-346 (2004)), and *paaG* (Ismail et al., *supra,* 2003; Park and Lee, *supra*, 2004; Park and Lee, *supra*, 2004). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

maoC	NP_415905.1	*Escherichia coli*
paaF	NP_415911.1	*Escherichia coli*
paaG	NP_415912.1	*Escherichia coli*
crt	NP_349318.1	*Clostridium acetobutylicum*
paaA	NP_743427.1	*Pseudomonas putida*
paaB	NP_745426.1	*Pseudomonas putida*
phaA	ABF82233.1	*Pseudomonas fluorescens*
phaB	ABF82234.1	*Pseudomonas fluorescens*

[0110] Alternatively, β-oxidation genes are candidates for the first three steps in adipate synthesis. Candidate genes for the proposed adipate synthesis pathway also include the native fatty acid oxidation genes of *E. coli* and their homologs in other organisms. The *E. coli* genes *fadA* and *fadB* encode a multienzyme complex that exhibits ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase activities (Yang et al., Biochem. 30:6788-6795 (1991); Yang et al., J. Biol. Chem. 265:10424-10429 (1990); Yang et al., J. Biol. Chem. 266:16255 (1991); Nakahigashi and Inokuchi, Nucl. Acids Res. 18: 4937 (1990)). These activities are mechanistically similar to the first three transformations shown in Figure 2. The *fadI* and *fadJ* genes encode similar functions and are naturally expressed only anaerobically (Campbell et al., Mol. Microbiol. 47:793-805 (2003)). These gene products naturally operate to degrade short, medium, and long chain fatty-acyl-CoA compounds to acetyl-CoA, rather than to convert succinyl-CoA and acetyl-CoA into 5-carboxy-2-pentenoyl-CoA as proposed in Figure 2. However, it is well known that the ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase enzymes catalyze reversible transformations. Furthermore, directed evolution and related approaches can be applied to tailor the substrate specificities of the native β-oxidation machinery of *E. coli.* Thus these enzymes or homologues thereof can be applied for adipate production. If the native genes operate to degrade adipate or its precursors *in vivo*, the appropriate genetic modifications are made to attenuate or eliminate these functions. However, it may not be necessary since a method for producing poly[(R)-3-hydroxybutyrate] in *E. coli* that involves activating *fadB*, by knocking out a negative regulator, *fadR*, and co-expressing a non-native ketothiolase, *phaA* from *Ralstonia eutropha*, has been described (Sato et al., J. Biosci. Bioeng. 103:38-44 (2007)). This work clearly demonstrated that a β-oxidation, enzyme, in particular the gene product of *fadB* which encodes both 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities, can function as part of a pathway to produce longer chain molecules from acetyl-CoA precursors. The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

fadA	YP_026272.1	*Escherichia coli*
fadE	NP_418288.1	*Escherichia coli*

(continued)

fadI	NP_416844.1	*Escherichia coli*
fadJ	NP_416843.1	*Escherichia coli*
fadR	NP_415705.1	*Escherichia coli*

[0111] Referring to Figure 2, step 4 involves 5-carboxy-2-pentenoyl-CoA reductase. Whereas the ketothiolase, dehydrogenase, and enoyl-CoA hydratase steps are generally reversible, the enoyl-CoA reductase step is almost always oxidative and irreversible under physiological conditions (Hoffmeister et al., J. Biol. Chem. 280:4329-4338 (2005)). *FadE* catalyzes this likely irreversible transformation in *E. coli* (Campbell and Cronan, J. Bacteriol. 184:3759-3764 (2002)). The pathway requires an enzyme that can reduce a 2-enoyl-CoA intermediate, not one such as *FadE* that will only oxidize an acyl-CoA to a 2-enoyl-CoA compound. Furthermore, although it has been suggested that *E. coli* naturally possesses enzymes for enoyl-CoA reduction (Mizugaki et al., J. Biochem. 92:1649-1654 (1982); Nishimaki et al., J. Biochem. 95:1315-1321 (1984)), no *E. coli* gene possessing this function has been biochemically characterized.

[0112] One candidate gene for the enoyl-CoA reductase step is the gene product of *bcd* from C. *acetobutylicum* (Atsumi et al., *supra*, 2007; Boynton et al., J. Bacteriol. 178:3015-3024 (1996)), which naturally catalyzes the reduction of crotonyl-CoA to butyryl-CoA, a reaction similar in mechanism to the desired reduction of 5-carboxy-2-pentcnoyl-CoA to adipyl-CoA in the adipate synthesis pathway. Activity of this enzyme can be enhanced by expressing *bcd* in conjunction with expression of the *C. acetobutylicum etfAB* genes, which encode an electron transfer flavoprotein. An additional candidate for the enoyl-CoA reductase step is the mitochondrial enoyl-CoA reductase from *E. gracilis* (Hoffmeister et al., J. Biol. Chem. 280:4329-4338 (2005)). A construct derived from this sequence following the removal of its mitochondrial targeting leader sequence was cloned in *E. coli,* resulting in an active enzyme (Hoffmeister et al., *supra,* 2005). This approach is well known to those skilled in the art of expressing eukarytotic genes, particularly those with leader sequences that may target the gene product to a specific intracellular compartment, in prokaryotic organisms. A close homolog of this gene, TDE0597, from the prokaryote *Treponema denticola* represents a third enoyl-CoA reductase which has been cloned and expressed in *E. coli* (Tucci and Martin, FEBS Lett. 581:1561-1566 (2007)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

bcd	NP_349317.1	*Clostridium acetobutylicum*
etfA	NP_349315.1	*Clostridium acetobutylicum*
etfB	NP_349316.1	*Clostridium acetobutylicum*
TER	Q5EU90.1	*Euglena gracilis*
TDE0597	NP_971211.1	*Treponema denticola*

[0113] Referring to Figure 2, step 5 involves adipyl-CoA synthetase (also referred to as adipate-CoA ligase), phosphotransadipylase/adipate kinase, adipyl-CoA:acetyl-CoA transferase, or adipyl-CoA hydrolase. From an energetic standpoint, it is desirable for the final step in the adipate synthesis pathway to be catalyzed by an enzyme or enzyme pair that can conserve the ATP equivalent stored in the thioester bond of adipyl-CoA. The product of the *sucC* and *sucD* genes of *E. coli*, or homologs thereof, can potentially catalyze the final transformation shown in Figure 2 should they exhibit activity on adipyl-CoA. The *sucCD* genes naturally form a succinyl-CoA synthetase complex that catalyzes the formation of succinyl-CoA from succinate with the concaminant consumption of one ATP, a reaction which is reversible *in vivo* (Buck et al., Biochem. 24:6245-6252 (1985)). Given the structural similarity between succinate and adipate, that is, both are straight chain dicarboxylic acids, it is reasonable to expect some activity of the *sucCD* enzyme on adipyl-CoA. An enzyme exhibiting adipyl-CoA ligase activity can equivalently carry out the ATP-generating production of adipate from adipyl-CoA, here using AMP and PPi as cofactors, when operating in the opposite physiological direction as depicted in Figure 1. Exemplary CoA-ligases include the rat dicarboxylate-CoA ligase for which the sequence is yet uncharacterized (Vamecq et al., Biochem. J. 230:683-693 (1985)), either of the two characterized phenylacetate-CoA ligases from *P. chrysogenum* (Lamas-Maceiras et al., Biochem. J. 395, 147-155 (2005); Wang et al., Biochem. Biophy. Res. Commun. 360:453-458 (2007)), the phenylacetate-CoA ligase from *Pseudomonas putida* (Martinez-Blanco et al., J. Biol. Chem. 265:7084-7090 (1990)), and the 6-carboxyhexanoate-CoA ligase from *Bacilis subtilis* (Bower et al., J. Bacteriol. 178:4122-4130 (1996)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

sucC	NP_415256.1	*Escherichia coli*
sucD	AAC73823.1	*Escherichia coli*

[0114] Another option, using phosphotransadipylase/adipate kinase, is catalyzed by the gene products of *buk1*, *buk2,* and *ptb* from *C. acetobutylicum* (Walter et al., Gene 134:107-111 (1993); Huang et al., J. Mol. Microbiol. Biotechnol. 2:33-38 (2000)), or homologs thereof. The *ptb* gene encodes an enzyme that can convert butyryl-CoA into butyryl-phosphate, which is then converted to butyrate via either of the *buk* gene products with the concomitant generation of ATP. The analogous set of transformations, that is, conversion of adipyl-CoA to adipyl-phosphate followed by conversion of adipyl-phosphate to adipate, can be carried out by the *buk1*, *buk2,* and *ptb* gene products. The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

ptb	NP_349676	*Clostridium acetobutylicum*
buk1	NP_349675	*Clostridium acetobutylicum*
buk2	Q97II1	*Clostridium acetobutylicum*

[0115] Alternatively, an acetyltransferase capable of transferring the CoA group from adipyl-CoA to acetate can be applied. Similar transformations are catalyzed by the gene products of *cat1*, *cat2*, and *cat3* of *Clostridium kluyveri* which have been shown to exhibit succinyl-CoA, 4-hydroxybutyryl-CoA, and butyryl-CoA acetyltransferase activity, respectively (Sohling and Gottschalk, J. Bacteriol. 178:871-880 (1996); Seedorf et al., Proc. Natl. Acad. Sci. USA 105:2128-2133 (2008)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

cat1	P38946.1	*Clostridium kluyveri*
cat2	P38942.2	*Clostridium kluyveri*
cat3	EDK35586.1	*Clostridium kluyveri*

[0116] Finally, though not as desirable from an energetic standpoint, the conversion of adipyl-CoA to adipate can also be carried out by an acyl-CoA hydrolase or equivalently a thioesterase. The top *E. coli* gene candidate is *tesB* (Naggert et al., J. Biol. Chem. 266:11044-11050 (1991)), which shows high similarity to the human *acot8*, which is a dicarboxylic acid acetyltransferase with activity on adipyl-CoA (Westin et al., J. Biol. Chem. 280:38125-38132 (2005)). This activity has also been characterized in the rat liver (Deana, Biochem. Int. 26:767-773 (1992)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

tesB	NP_414986	*Escherichia coli*
acot8	CAA15502	*Homo sapiens*
acot8	NP_570112	*Rattus norvegicus*

[0117] Other native candidate genes include *tesA* (Bonner and Bloch, J. Biol. Chem. 247:3123-3133 (1972)), *ybgC* (Kuznetsova et al., FEMS Microbiol. Rev. 29:263-279 (2005); Zhuang et al., FEBS Lett. 516:161-163 (2002)), *paaI* (Song et al., J. Biol. Chem. 281:11028-11038 (2006)), and *ybdB* (Leduc et al., J. Bacteriol. 189:7112-7126 (2007)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

tesA	NP_415027	*Escherichia coli*
ybgC	NP_415264	*Escherichia coli*
paaI	NP_415914	*Escherichia coli*
ybdB	NP_415129	*Escherichia coli*

[0118] The above description provides an exemplary adipate synthesis pathway by way of a reverse adipate degradation pathway.

EXAMPLE II

Preparation of an Adipate Producing Microbial Organism Having A Reverse Degradation Pathway

[0119] This example describes the generation of a microbial organism capable of producing adipate using the reverse degradation pathway.

[0120] *Escherichia coli* is used as a target organism to engineer a reverse adipate degradation pathway as shown in Figure 2. *E. coli* provides a good host for generating a non-naturally occurring microorganism capable of producing adipate. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic acid, formic acid, lactic acid, and succinic acid, effectively under anaerobic or microaerobic conditions.

[0121] To generate an *E. coli* strain engineered to produce adipate, nucleic acids encoding the enzymes utilized in the reverse degradation pathway are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, *supra*, 2001; Ausubel *supra*, 1999). In particular, the *paaJ* (NP_415915.1), *paaH* (NP_415913.1), and *maoC* (NP_415905.1) genes encoding the succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyadipyl-CoA dehydratase activities, respectively, are cloned into the pZE13 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. In addition, the *bcd* (NP_349317.1), *etfAB* (349315.1 and 349316.1), and *sucCD* (NP_415256.1 and AAC73823.1) genes encoding 5-carboxy-2-pentenoyl-CoA reductase and adipyl-CoA synthetase activities, respectively, are cloned into the pZA33 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. The two sets of plasmids are transformed into *E. coli* strain MG1655 to express the proteins and enzymes required for adipate synthesis via the reverse degradation pathway.

[0122] The resulting genetically engineered organism is cultured in glucose-containing medium following procedures well known in the art (see, for example, Sambrook et al., *supra*, 2001). The expression of reverse degradation pathway genes is corroborated using methods well known in the art for determining polypeptide expression or enzymatic activity, including for example, Northern blots, PCR amplification of mRNA, immunoblotting, and the like. Enzymatic activities of the expressed enzymes are confirmed using assays specific for the individual activities. The ability of the engineered *E. coli* strain to produce adipate is confirmed using HPLC, gas chromatography-mass spectrometry (GCMS) and/or liquid chromatography-mass spectrometry (LCMS).

[0123] Microbial strains engineered to have a functional adipate synthesis pathway are further augmented by optimization for efficient utilization of the pathway. Briefly, the engineered strain is assessed to determine whether any of the exogenous genes are expressed at a rate limiting level. Expression is increased for any enzymes expressed at low levels that can limit the flux through the pathway by, for example, introduction of additional gene copy numbers.

[0124] To generate better producers, metabolic modeling is utilized to optimize growth conditions. Modeling is also used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of adipate. One modeling method is the bilevel optimization approach, OptKnock (Burgard et al., Biotechnol. Bioengineer. 84:647-657 (2003)), which is applied to select gene knockouts that collectively result in better production of adipate. Adaptive evolution also can be used to generate better producers of, for example, the acetyl-CoA and succinyl-CoA intermediates or the adipate product. Adaptive evolution is performed to improve both growth and production characteristics (Fong and Palsson, Nat. Genet. 36:1056-1058 (2004); Alper et al., Science 314:1565-1568 (2006)). Based on the results, subsequent rounds of modeling, genetic engineering and adaptive evolution can be applied to the adipate producer to further increase production.

[0125] For large-scale production of adipate, the above reverse degradation pathway-containing organism is cultured in a fermenter using a medium known in the art to support growth of the organism under anaerobic conditions. Fermentations are performed in either a batch, fed-batch or continuous manner. Anaerobic conditions are maintained by first sparging the medium with nitrogen and then sealing the culture vessel, for example, flasks can be sealed with a septum and crimp-cap. Microaerobic conditions also can be utilized by providing a small hole in the septum for limited aeration. The pH of the medium is maintained at a pH of around 7 by addition of an acid, such as H_2SO_4. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm) and the glucose uptake rate by monitoring carbon source depletion over time. Byproducts such as undesirable alcohols, organic acids, and residual glucose can be quantified by HPLC (Shimadzu, Columbia MD), for example, using an Aminex® series of HPLC columns (for example, HPX-87 series) (BioRad, Hercules CA), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 775-779 (2005)).

[0126] This example describes the preparation of an adipate producing microbial organism using a reverse degradation pathway.

EXAMPLE III

Adipate Synthesis Through 3-Oxoadipate

[0127] This example describes an exemplary adipate synthesis pathway through 3-oxoadipate.

[0128] An additional pathway from that described in Examples I and II that uses acetyl-CoA and succinyl-CoA as precursors for adipate formation and passes through the metabolic intermediate, 3-oxoadipate, is shown in Figure 3. The initial two transformations in this pathway are the two terminal steps of the degradation pathway for aromatic and choloroaromatic compounds operating in the reverse direction (Kaschabek et al., J. Bacteriol. 184:207-215 (2002); Nogales et al., Microbiol. 153:357-365 (2007); Ismail et al., Eur. J. Biochem. 270:3047-3054 (2003)). Specifically, the first step forms 3-oxoadipyl CoA by the condensation of succinyl- and acetyl-CoA. The second step forms 3-oxoadipate and is reported to be reversible in *Pseudomonas* sp. Strain B 13 (Kaschabek et al., J. Bacteriol. 184:207-215 (2002)).

[0129] The subsequent steps involve reduction of 3-oxoadipate to 3-hydroxyadipate (conversion of a keto group to hydroxyl group), dehydration of 3-hydroxyadipate to yield hexa-2-enedioate, and reduction of hexa-2-enedioate to form adipate. These steps of the pathway are analogous to the conversion of oxaloacetate into succinate via the reductive TCA cycle (see Figure 4). This supports the steps in the pathway being thermodynamically favorable subject to the presence of appropriate metabolite concentrations. The final reduction step can be carried out either biochemically or by employing a chemical catalyst to convert hexa-2-enedioate into adipate. Chemical hydrogenation can be performed using Pt catalyst on activated carbon as has been described in (Niu et al., Biotechnol. Prog. 18:201-211 (2002)).

[0130] The maximum theoretical yield of adipate using this pathway is 0.92 mole per mole glucose consumed, and oxygen is not required for attaining these yields (see Table 2). The associated energetics are identical to those of the reverse adipate pathway. Theoretically, ATP formation of up to 1.55 moles is observed per mole of glucose utilized through this pathway. The ATP yield improves to approximately 2.47 moles if phosphoenolpyruvate kinase (PPCK) is assumed to operate in the direction of ATP generation. Interestingly, the product yield can be increased further to 1 mole adipate per mole of glucose consumed if chemical hydrogenation is used for the last step and a 100% efficiency of catalysis is assumed. In this scenario, up to 1.95 moles of ATP are formed theoretically without assuming the reverse functionality of PPCK.

[0131] Table 2: The maximum theoretical yields of adipate and the associated ATP yields per mole of glucose using the 3-oxoadipate pathway.

	Final step enzymatic		Final step chemical hydrogenation	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Adipate Yield	0.92	0.92	1.00	1.00
Max ATP yield @ max adipate yield	1.55	1.55	1.95	1.95

[0132] Successfully engineering this pathway involves identifying an appropriate set of enzymes with sufficient activity and specificity. This entails identifying an appropriate set of enzymes, cloning their corresponding genes into a production host, optimizing fermentation conditions, and assaying for product formation following fermentation. To engineer a production host for the production of adipate, one or more exogenous DNA sequence(s) can be expressed in a host microorganism. In addition, the host microorganism can have endogenous gene(s) functionally deleted. These modifications allow the production of adipate using renewable feedstock.

[0133] Described below are a number of biochemically characterized candidate genes capable of encoding enzymes that catalyze each step of the 3-oxoadipate pathway for adipate synthesis. Although this method is described for *E. coli,* one skilled in the art can apply these teachings to any other suitable host organism. Specifically, listed below are genes that are native to *E. coli* as well as genes in other organisms that can be applied to catalyze the appropriate transformations when properly cloned and expressed.

[0134] Referring to Figure 3, step 1 involves succinyl CoA:acetyl CoA acyl transferase (β-ketothiolase). The first step in the pathway combines acetyl-CoA and succinyl-CoA to form 3-oxoadipyl-CoA. The gene products encoded by *pcaF* in *Pseudomonas* strain B 13 (Kaschabek et al., J. Bacteriol. 184:207-215 (2002)), *phaD* in *Pseudomonas putida* U (Olivera et al., Proc. Natl. Acad Sci. USA 95:6419-6424 (1998)), *paaE* in *Pseudomonas fluorescens* ST (Di Gennaro et al., Arch. Microbiol. 188:117-125 (2007)), and *paaJ* from *E. coli* (Nogales et al., Microbiol. 153:357-365 (2007)) catalyze the conversion of 3-oxoadipyl-CoA into succinyl-CoA and acetyl-CoA during the degradation of aromatic compounds such as phenylacetate or styrene. Since β-ketothiolase enzymes catalyze reversible transformations, these enzymes can be employed for the first step in adipate synthesis shown in Figure 3. For example, the ketothiolase *phaA* from *R. eutropha* combines two molecules of acetyl-CoA to form acetoacetyl-CoA (Sato et al., J. Biosci. Bioengineer. 103:38-44 (2007)). Similarly, a β-keto thiolase (*bktB*) has been reported to catalyze the condensation of acetyl-CoA and propionyl-CoA to form β-ketovaleryl-CoA (Slater et al., J. Bacteriol. 180:1979-1987 (1998)) in *R. eutropha.* The protein sequences for the above-mentioned gene products are well known in the art and can be accessed in the public databases such as GenBank using the following accession numbers.

Gene name	GenBank Accession #	Organism
paaJ	NP_415915.1	*Escherichia coli*
pcaF	AAL02407	*Pseudomonas knackmussii* (B13)
phaD	AAC24332.1	*Pseudomonas putida*
paaE	ABF82237.1	*Pseudomonas fluorescens*

[0135] These sequences can be used to identify homologue proteins in GenBank or other databases through sequence similarity searches, for example, BLASTp. The resulting homologue proteins and their corresponding gene sequences provide additional exogenous DNA sequences for transformation into *E. coli* or other microorganisms to generate production hosts.

[0136] For example, orthologs of *paaJ* from *Escherichia coli* K12 can be found using the following GenBank accession numbers:

YP_001335140.1	*Klebsiella pneumoniae*
YP_001479310.1	*Serratia proteamaculans*
AAC24332.1	*Pseudomonas putida*

[0137] Example orthologs of *pcaF* from *Pseudomonas knackmussii* can be found using the following GenBank accession numbers:

AAD22035.1	*Streptomyces* sp. 2065
AAN67000.1	*Pseudomonas putida*
ABJ15177.1	*Pseudomonas aeruginosa*

[0138] Additional native candidate genes for the ketothiolase step include *atoB* which can catalyze the reversible condensation of 2 acetyl-CoA molecules (Sato et al., J. Biosci. Bioengineer. 103:38-44 (2007)), and its homolog *yqeF.* Non-native gene candidates include *phaA* (Sato et al., *supra,* 2007) and *bktB* (Slater et al., J. Bacteriol. 180:1979-1987 (1998)) from *R. eutropha,* and the two ketothiolases, *thiA* and *thiB,* from *Clostridium acetobutylicum* (Winzer et al., J. Mol. Microbiol. Biotechnol. 2:531-541 (2000)). The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

atoB	NP_416728.1	*Escherichia coli*
yqeF	NP_417321.2	*Escherichia coli*
phaA	YP_725941	*Ralstonia eutropha*
bldB	AAC38322.1	*Ralstonia eutropha*
thiA	NP_349476.1	*Clostridium acetobutylicum*
thiB	NP_149242.1	*Clostridium acetobutylicum*

[0139] It is less desirable to use the thiolase-encoding genes *fadA* and *fadB,* genes in fatty acid degradation pathway in *E. coli,* in this exemplary pathway. These genes form a complex that encodes for multiple activities, most of which are not desired in this pathway.

[0140] Referring to Figure 3, step 2 involves 3-oxoadipyl-CoA transferase. In this step, 3-oxoadipate is formed by the transfer of the CoA group from 3-oxoadipyl-CoA to succinate. This activity is reported in a two-unit enzyme encoded by pcal and pcaJ in *Pseudomonas* (Kaschabek et al., J. Bacteriol. 184:207-215 (2002)). This enzyme catalyzes a reversible transformation. The protein sequences of exemplary gene products for subunit A of this complex can be found using the following GenBank accession numbers:

pcaI	AAN69545.1	*Pseudomonas putida*
pcaI	YP_046368.1	*Acinetobacter* sp. ADP1
pcaI	NP_630776.1	*Streptomyces coelicolor*

[0141] The protein sequences of exemplary gene products for subunit B of this complex can be found using the following GenBank accession numbers:

pcaJ	NP_746082.1	*Pseudomonas putida*
pcaJ	NP_630775.1	*Streptomyces coelicolor*
pcaJ	AAC37147.1	*Acinetobacter* sp, ADP1

[0142] Referring to Figure 3, step 3 involves 3-oxoadipate reductase. *E. coli* has several candidate alcohol dehydrogenases; two that have analogous functions are malate dehydrogenase (*mdh*) and lactate dehydrogenase (*ldhA*). While it has not been shown that these two enzymes have broad substrate specificities in *E. coli,* lactate dehydrogenase from *Ralstonia eutropha* has been shown to demonstrate high activities on substrates of various chain lengths such as lactate, 2-oxobutyrate, 2-oxopentanoate and 2-oxoglutarate (Steinbuchel and Schlegel, Eur. J. Biochem. 130:329-334 (1983)). An additional non-native enzyme candidate for this step is the mitochondrial 3-hydroxybutyrate dehydrogenase (*bdh*) from the human heart which has been cloned and characterized (Marks et al., J. Biol. Chem. 267:15459-15463 (1992)). This enzyme is particularly interesting in that it is a dehydrogenase that operates on a 3-hydroxyacid. Given that dehydrogenases are typically reversible, it is expected that this gene product, or a homlog thereof, will be capable of reducing a 3-oxoacid, for example, 3-oxoadipate, to the corresponding 3-hydroxyacid, for example, 3-hydroxyadipate. The protein sequences for each of these exemplary gene products can be found using the following GenBank accession numbers:

mdh	AAC76268.1	*Escherichia coli*
ldhA	NP_415898.1	*Escherichia coli*
ldh	YP_725182.1	*Ralstonia eutropha*
bdh	AAA58352.1	*Homo sapiens*

[0143] Referring to Figure 3, step 4 involves 3-hydroxyadipate dehydratase. In this reaction, 3-hydroxyadipate is dehydrated to hexa-2-enedioate. Although no direct evidence for this enzymatic transformation has been identified, most dehydratases catalyze the α, β-elimination of water. This involves activation of the α-hydrogen by an electron-withdrawing carbonyl, carboxylate, or CoA-thiol ester group and removal of the hydroxyl group from the β-position (Martins et al., Proc. Natl. Acad. Sci. USA 101:15645-15649 (2004); Buckel and Golding,. FEMS Microbiol. Rev. 22:523-541 (1998)). The protein sequences for exemplary gene products can be found using the following GenBank accession numbers:

acnA	P25516.3	*Escherichia coli*
fumB	P14407.2	*Escherichia coli*
ilvD	AAA24013.1	*Escherichia coli*

[0144] Other good candidates for carrying out this function are the serine dehydratases. These enzymes catalyze a very similar transformation in the removal of ammonia from serine as required in this dehydration step. The protein sequence for exemplary gene product can be found using the following GenBank accession number:

dsdA	P00926	*Escherichia coli*

[0145] Non-native gene candidates for this transformation have been identified as well. For example, the multi-subunit L-serine dehydratase from *Peptostreptococcus asaccharolyticus* was shown to complement an *E. coli* strain deficient in L-serine dehydratase activity (Hofmeister et al., J. Bacteriol. 179:4937-4941 (1997)). Further, a putative 2-(hydroxymethyl)glutarate dehydratase, encoded by the gene *hmd* in *Eubacterium barkeri* shows similarity to both α- and β-subunits of [4Fe-4S]-containing bacterial serine dehydratases (Alhapel et al., Proc. Natl. Acad. Sci. USA 103:12341-12346 (2006)). The protein sequence for exemplary gene product can be found using the following GenBank accession number:

hmd	ABC88407.1	*Eubacterium barkeri*

[0146] Referring to Figure 3, step 5 involves 2-enoate reductase. The final step in the 3-oxoadipate pathway is reduction of the double bond in hexa-3-enedioate to form adipate. Biochemically, this transformation can be catalyzed by 2-enoate reductase (EC 1.3.1.31) known to catalyze the NADH-dependent reduction of a wide variety of α, β-unsaturated carboxylic acids and aldehydes (Rohdich et al., J. Biol. Chem. 276:5779-5787 (2001)). This enzyme is encoded by *enr* in several species of *Clostridia* (Giesel and Simon, Arch. Microbiol. 135:51-57 (1983)) including *C. tyrobutyricum* and *C. thermoaceticum* (now called *Moorella thermoaceticum*) (Rohdich, et al., J. Biol. Chem. 276:5779-5787 (2001)). In the recently published genome sequence of C. *kluyveri,* 9 coding sequences for enoate reductases have been reported, out of which one has been characterized (Seedorf et al., Proc. Natl. Acad. Sci. USA 105:2128-2133 (2008)). The *enr* genes from both *C. tyrobutyricum* and *C. thermoaceticum* have been cloned and sequenced and show 59% identity to each other. The former gene is also found to have approximately 75% similarity to the characterized gene in *C. kluyveri* (Giesel and Simon, Arch. Microbiol. 135:51-57 (1983)). It has been reported based on these sequence results that *enr* is very similar to the dienoyl CoA reductase in *E. coli* (*fadH*) (Rohdich et al., J. Biol. Chem. 276:5779-5787 (2001)). Several gene candidates thus exist for catalyzing this last step in the 3-oxoadipate pathway and have been listed below. The *C. thermoaceticum enr* gene has also been expressed in an enzymatically active form in *E. coli* (Rohdich et al., *supra,* 2001). The protein sequences for exemplary gene products can be found using the following GenBank accession numbers:

fadH	NP_417552.1	*Escherichia coli*
enr	ACA54153.1	*Clostridium botulinum* A3 str
enr	CAA71086.1	*Clostridium tyrobutyricum*
enr	CAA76083.1	*Clostridium kluyveri*

[0147] The above description provides an exemplary adipate synthesis pathway by way of an 3-oxoadipate pathway.

EXAMPLE IV

Preparation of an Adipate Producing Microbial Organism Having A 3-Oxoadipate Pathway

[0148] This example describes the generation of a microbial organism capable of producing adipate using the 3-oxoadipate pathway.

[0149] *Escherichia coli* is used as a target organism to engineer the 3-oxoadipate pathway as shown in Figure 3. *E. coli* provides a good host for generating a non-naturally occurring microorganism capable of producing adipate. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic acid, formic acid, lactic acid, and succinic acid, effectively under anaerobic or microaerobic conditions.

[0150] To generate an *E. coli* strain engineered to produce adipate, nucleic acids encoding the enzymes utilized in the 3-oxoadipate pathway are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, *supra,* 2001; Ausubel *supra,* 1999). In particular, the *paaJ* (NP_415915.1), *pcaIJ* (AAN69545.1 and NP_746082.1), and *bdh* (AAA58352.1) genes encoding the succinyl-CoA:acetyl-CoA acyl transferase, 3-oxoadipyl-CoA transferase, and 3-oxoadipate reductase activities, respectively, are cloned into the pZE13 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. In addition, the *acnA* (P25516.3) and *enr* (ACA54153.1) genes encoding 3-hydroxyadipate dehydratase and 2-enoate reductase activities, respectively, are cloned into the pZA33 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. The two sets of plasmids are transformed into *E. coli* strain MG1655 to express the proteins and enzymes required for adipate synthesis via the 3-oxoadipate pathway.

[0151] The resulting genetically engineered organism is cultured in glucose containing medium following procedures well known in the art (see, for example, Sambrook et al., *supra,* 2001). The expression of the 3-oxoadipate pathway genes for adipate synthesis is corroborated using methods well known in the art for determining polypeptide expression or enzymatic activity, including for example, Northern blots, PCR amplification of mRNA, immunoblotting, and the like. Enzymatic activities of the expressed enzymes are confirmed using assays specific for the individual activities. The ability of the engineered *E. coli* strain to produce adipate is confirmed using HPLC, gas chromatography-mass spectrometry (GCMS) and/or liquid chromatography-mass spectrometry (LCMS).

[0152] Microbial strains engineered to have a functional adipate synthesis pathway are further augmented by optimization for efficient utilization of the pathway. Briefly, the engineered strain is assessed to determine whether any of the exogenous genes are expressed at a rate limiting level. Expression is increased for any enzymes expressed at low levels that can limit the flux through the pathway by, for example, introduction of additional gene copy numbers.

[0153] To generate better producers, metabolic modeling is utilized to optimize growth conditions. Modeling is also used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of adipate. One modeling method is the bilevel optimization approach, OptKnock (Burgard et al., Biotechnol. Bioengineer. 84:647-657 (2003)), which is applied to select gene knockouts that collectively result in better production of adipate. Adaptive evolution also can be used to generate better producers of, for example, the acetyl-CoA and succinyl-CoA intermediates or the adipate product. Adaptive evolution is performed to improve both growth and production characteristics (Fong and Palsson, Nat. Genet. 36:1056-1058 (2004); Alper et al., Science 314:1565-1568 (2006)). Based on the results, subsequent rounds of modeling, genetic engineering and adaptive evolution can be applied to the adipate producer to further increase production.

[0154] For large-scale production of adipate, the 3-oxoadipate pathway-containing organism is cultured in a fermenter using a medium known in the art to support growth of the organism under anaerobic conditions. Fermentations are performed in either a batch, fed-batch or continuous manner. Anaerobic conditions are maintained by first sparging the medium with nitrogen and then sealing the culture vessel, for example, flasks can be sealed with a septum and crimp-cap. Microaerobic conditions also can be utilized by providing a small hole in the septum for limited aeration. The pH of the medium is maintained at around a pH of 7 by addition of an acid, such as H_2SO_4. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm) and the glucose uptake rate by monitoring carbon source depletion over time. Byproducts such as undesirable alcohols, organic acids, and residual glucose can be quantified by HPLC (Shimadzu), for example, using an Aminex® series of HPLC columns (for example, HPX-87 series) (BioRad), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 775-779 (2005)).

[0155] This example describes the preparation of an adipate-producing microbial organism containing a 3-oxidoadipate pathway.

EXAMPLE V

Adipate Synthesis via cis,cis-Muconic Acid

[0156] This example describes an adipate synthesis pathway previously described (see Niu et al., Biotechnol. Prog. 18(2): p. 201-11. 2002; Frost et al., United States Patent No. 5,487,987, issued January 30, 1996).

[0157] Adipate synthesis via a combined biological and chemical conversion process has been previously described. (Niu et al., Biotechnol. Prog. 18:201-211 (2002)) and is shown in Figure 5. This method is further described in United States Patent No. 5,487,987. Adipate synthesis through this route entails introduction of three heterologous genes into *E. coli* that can convert dehydroshikimate into cis,cis-muconic acid (Niu et al., *supra,* 2002). A final chemical hydrogenation step leads to the formation of adipic acid. In this step, the pretreated fermentation broth that contained 150 mM cis,cis-muconate was mixed with 10% platinum (Pt) on activated carbon. The hydrogenation reaction was carried out at 3400 KPa of hydrogen pressure for two and a half hour at 250°C with stirring. The calculated adipate yields are shown in Table 3 assuming either an enzymatic or chemical catalysis step is utilized to convert cis,cis-muconate into adipate. Under aerobic conditions, an 85% molar yield of adipate can be obtained if a chemical reaction is employed for hydrogenation and a 75% molar yield is obtained if an NADH-based hydrogenase is used.

Table 3: The maximum theoretical yields of adipate per mole of glucose using the using the cis,cis-muconic acid pathway.

	Final step enzymatic		Final step chemical hydrogenation	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Adipate Yield	0.75	0.00	0.85	0.00

[0158] Although this is an exemplary method, there are disadvantages of this method compared to others, such as those described in Examples I-IV. For example, the first limitation of this method is the lower theoretical yields compared to the reverse adipate degradation and 3-oxoadipate pathways. The second limitation is that the ATP yields of this pathway are negligible. A third limitation of this pathway is that it involves a dioxygenase, necessitating a supply of oxygen to the bioreactor and precluding the option of anaerobic fermentation.

[0159] The above description provides an exemplary adipate synthesis pathway by way of a cis,cis-muconic acid pathway

EXAMPLE VI

Adipate Synthesis via Alpha-Ketoadipate

[0160] This example describes an exemplary adipate synthesis pathway via an alpha-ketoadipate pathway.

[0161] Alpha-keto adipate is a known intermediate in lysine biosynthesis in *S. cerevisiae,* and this information was used to identify an additional pathway for adipic acid biosynthesis (see Figure 6). Conversion of alpha-ketoglutarate to alpha-ketoadipate is catalyzed by homocitrate synthase, homoaconitase, and homoisocitrate dehydrogenase as indicated by dashed arrows in Figure 6. Conversion of alpha-ketoadipate into alpha-hydroxyadipate can be catalyzed by 2-ketoadipate reductase, an enzyme reported to be found in rat and in human placenta (Suda et al., Arch. Biochem. Biophys. 176:610-620 (1976); Suda et al., Biochem. Biophys. Res. Commun. 77:586-591 (1977). Subsequent steps involve a dehydratase for the conversion of alpha-hydroxyadipate into hcxa-2-enedioate followed by its reduction to adipic acid. This last step can be catalyzed either by an enzyme or can take place through a chemical reaction as described in Example II. Genes encoding the enzymes for the alpha-ketoadipate pathway are identified as described in Examples I-IV.

[0162] The adipate yields associated with this pathway are shown in Table 4. Because of the loss of two CO_2 molecules during the conversion of acetyl-CoA to adipate, only 67% of the glucose can be converted into adipate. This is reflected in the molar yields for this pathway under aerobic conditions. The yields are further reduced in the absence of oxygen uptake. Also since the maximum ATP yields under anaerobic conditions are negligible, the engineered organism will have to utilize additional substrate to form energy for cell growth and maintenance under such conditions.

Table 4: The maximum theoretical yields of adipate and the associated ATP yields per mole of glucose using the using the alpha-ketoadipate pathway.

	Final step enzymatic		Final step chemical hydrogenation	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Adipate Yield	0.67	0.45	0.67	0.40
Max ATP yield @ max adipate yield	6.17	0.00	7.50	0.00

[0163] The above description provides an exemplary adipate synthesis pathway by way of an alpha-ketoadipate pathway.

EXAMPLE VII

Adipate Synthesis via Lysine Degradation

[0164] This example describes an exemplary adipate synthesis pathway via a lysine degradation pathway.

[0165] Two additional pathways for adipate synthesis rely on lysine degradation to form adipate. One pathway starts from alpha-ketoglutarate to form lysine (pathway non-native to *E. coli* and found in *S. cerevisiae*), and the other uses aspartate as a starting point for lysine biosynthesis (pathway native to *E. coli*). Figure 7 shows adipate formation from lysine. The maximum theoretical yields for adipate, both in the presence and absence of oxygen, using the *E. coli* stoichiometric model are shown in Tables 5 and 6, with alpha-ketoglutarate and aspartate as the respective starting points for lysine. The maximum ATP yields accompanying these theoretical yields were also calculated and are shown in the same tables. These yields are lower in comparison to the other pathways described in Examples I-IV. Genes encoding the enzymes for the alpha-ketoadipate pathway are identified as described in Examples I-IV.

Table 5: The maximum theoretical yield of adipate and the accompanying ATP yield per mole of glucose assuming the lysine biosynthesis pathway with alpha-ketoglutarate as a starting point.

	Aerobic	Anaerobic
Adipate Yield	0.40	0.20
Max ATP yield @ max adipate yield	5.60	0.00

Table 6: The maximum theoretical yield of adipate and the accompanying ATP yield per mole of glucose assuming the lysine biosynthesis pathway with aspartate as a starting point.

	Aerobic	Anaerobic
Adipate Yield	0.50	0.34
Max ATP yield @ max adipate yield	0.50	0.04

[0166] The above description provides an exemplary adipate synthesis pathway by way of a lysine degradation pathway.

EXAMPLE VIII

Production of Caprolactam and 6-Aminocaproic Acid via Adipyl-CoA

[0167] This example describes an exemplary caprolactam and/or 6-aminocaproic acid synthesis pathway via an adipyl-CoA pathway.

[0168] An exemplary pathway for forming caprolactam and/or 6-aminocaproic acid using adipyl-CoA as the precursor is shown in Figure 8. The pathway involves a CoA-dependant aldehyde dehydrogenase that can reduce adipyl-CoA to adipate semialdehyde and a transaminase or 6-aminocaproate dehydrogenase that can transform this molecule into 6-aminocaproic acid. The terminal step that converts 6-aminocaproate into caprolactam can be accomplished either via an amidohydrolase or via chemical conversion (Guit and Buijs, U.S. Patent No. 6,353,100, issued March 7, 2002; Wolters et al., U.S. Patent No. 5,700,934, issued December 23, 1997; Agterberg et al., U.S. Patent No. 6,660,857, issued December 9, 2003). The maximum theoretical yield of caprolactam was calculated to be 0.8 mole per mole glucose consumed (see Table 7) assuming that the reverse adipate degradation pathway was complemented with the reaction scheme shown in Figure 8. The pathway is favorable energetically as up to 0.78 moles of ATP are formed per mole of glucose consumed at the maximum theoretical yield of caprolactam. The ATP yield can be further improved to 1.63 moles of ATP produced per mole of glucose if phosphoenolpyruvate carboxykinase (PPCK) is assumed to function in the ATP-generating direction towards oxaloacetate formation.

[0169] The final amidohydrolase step is energetically and redox neutral, and thus the product and ATP molar yields associated with 6-aminocaproic acid production are equivalent to those associated with caprolactam production. Thus one can alternatively envision a microorganism and associated fermentation process that forms 6-aminocaproic acid instead of caprolactam followed by an additional unit operation to dehydrate/cyclize 6-aminocaproic acid to caprolactam.

Table 7: The maximum theoretical yield of caprolactam and the accompanying ATP yield per mole of glucose assuming that the reverse fatty acid degradation pathway is complemented with the reaction scheme from Figure 8.

	Aerobic	Anaerobic
Caprolactam Yield	0.80	0.80
Max ATP yield @ max Caprolactam yield	0.78	0.78
Max ATP yield @ max Caprolactam yield PPCK assumed	1.63	1.63

[0170] Successfully engineering this pathway involves identifying an appropriate set of enzymes with sufficient activity and specificity. This entails identifying an appropriate set of enzymes, cloning their corresponding genes into a production host, optimizing fermentation conditions, and assaying for product formation following fermentation. To engineer a production host for the production of 6-aminocaproic acid or caprolactam, one or more exogenous DNA sequence(s) can be expressed in a host microorganism. In addition, the microorganism can have endogenous gene(s) functionally deleted. These modifications will allow the production of 6-aminocaproate or caprolactam using renewable feedstock.

[0171] Below is described a number of biochemically characterized candidate genes capable of encoding enzymes that catalyze each step of the caprolactam formation pathway described in Figure 8. Although described for *E. coli,* one skilled in the art can apply these teachings to any other suitable host organism. Specifically, the genes listed arc native to *E. coli* or are genes in other organisms that can be applied to catalyze the appropriate transformations when properly cloned and expressed.

[0172] Referring to Figure 8, step 1 involves CoA-dependant aldehyde dehydrogenase. Exemplary genes that encode enzymes for catalyzing the reduction of an acyl-coA to its corresponding aldehyde include the *Acinetobacter calcoaceticus acr1* encoding a fatty acyl-CoA reductase (Reiser and Somerville,. J. Bacteriol. 179:2969-2975 (1997)), the *Acinetobacter* sp. M-1 fatty acyl-CoA reductase (Ishige et al., Appl. Environ. Microbiol.. 68:1192-1195 (2002)) and the *sucD* gene from *Clostridium kluyveri* (Sohling and Gottschalk, J. Bacteriol. 178:871-880 (1996)), which can convert succinyl-CoA to succinate semialdehyde.

Gene name	GenBank Accession #	Organism
acr1	YP_047869.1	*Acinetobacter calcoaceticus*
	BAB85476.1	*Acinetobacter* sp. Strain M-1
sucD	P38947.1	*Clostridium kluyveri*

[0173] Referring to Figure 8, step 2 involves transaminase. The second step in the pathway is conversion of the 6-aldehyde to an amine. This transformation can likely be accomplished by gamma-aminobutyrate transaminase (GABA transaminase), a native enzyme encoded by *gabT* that transfers an amino group from glutamate to the terminal aldehyde of succinyl semialdehyde (Bartsch et al., J. Bacteriol. 172:7035-7042 (1990)). GABA transaminases in *Mus musculus, Pseudomonas fluorescens,* and *Sus scrofa* have been shown to react with 6-aminocaproic acid (Cooper, Methods Enzymol. 113:80-82 (1985); Scott and Jakoby, J. Biol. Chem. 234:932-936 (1959)). The protein sequences for exemplary gene products can be found using the following GenBank accession numbers:

gabT	NP_417148.1	*Escherichia coli*
abat	NP_766549.2	*Mus musculus*
gabT	YP_257332.1	*Pseudomonas fluorescens*
abat	NP_999428.1	*Sus scrofa*

[0174] Referring to Figure 8, step 2 can alternatively involve 6-aminocaproate dehydrogenase which comprises the reductive amination of adipate semialdehyde to form 6-aminocaproate. This transformation can be accomplished by lysine-6-dehydrogenase, which naturally converts L-lysine to 2-aminoadipate-6-semialdehyde. Exemplary enzymes can be found in *Geobacillus stearothermophilus* (Heydari et al., Appl. Environ. Microbiol. 70(2):937-942 (2004)), *Agrobacterium tumefaciens* (Hashimoto et al., J. Biochem. (Tokyo), 106(1):76-80 (1989); Misono et al., J. Biochem. (Tokyo), 105(6):1002-1008 (1989)), and *Achromobacter denitrificans* (Ruldeekulthamrong et al., BMB Reports 790-795 (2008)).

lysDH	BAB39707	*Geobacillus stearothermophilus*
lysDH	NP_353966	*Agrobacterium tumefaciens*
lysDH	AAZ94428	*Achromobacter denitrificans*

[0175] Referring to Figure 8, step 3 involves amidohydrolase. The final step of caprolactam synthesis is cyclization of 6-aminocaproic acid. This transformation has not been characterized enzymatically but it is very similar to the cyclization of lysine by D-lysine lactamase (EC 3.52.11) from *Cryptococcus laurentii* (Fukumura et al., FEBS Lett. 89:298-300 (1978)). However, the protein and nucleotide sequences of this enzyme are not currently known and, so far, lysine lactamase activity has not been demonstrated in other organisms.

[0176] Plasmids contained in several strains of *Pseudomonas* sp. isolated from soil have been shown to confer ability to grow on caprolactam as a sole carbon source (Boronin et al., FEMS Microbiol. Lett. 22:167-170 (1984)); however, associated gene or protein sequences have not been associated with this function to date.

[0177] The most closely related candidate enzyme with available sequence information is 6-aminohexanoate-cyclic dimer hydrolase, which has been characterized in *Pseudomonas* sp. and *Flavobacferium* sp. The *nylB* gene product from *Pseudomonas* sp NK87 was cloned and expressed in *E. coli* (Kanagawa et al., J. Gen. Microbiol. 139:787-795 (1993)). The substrate specificity of the enzyme was tested in *Flavobacterium* sp K 172 and was shown to react with higher-order oligomers of 6-aminohexanoate but not caprolactam (Kinoshita et al., Eur. J. Biochem. 116:547-551 (1981)). The reversibility and ability of 6-aminohexanoate dimer hydrolases in other organisms to react with the desired substrate in the direction of interest can be further tested. The protein sequences for exemplary gene products can be found using the following GenBank accession numbers:

nylB	AAA24929.1	*Pseudomonas* sp NK87
nylB	P13397	*Flavobacterium* sp K172
nylB	YP_949627.1	*Arthrobacter aurescens* TC1

[0178] The above description provides an exemplary pathway to produce caprolactam and/or 6-aminocaproic acid by way of an adipyl-CoA pathway.

EXAMPLE IX

Preparation of a 6-Aminocaproate or Caprolactam Producing Microbial Organism Having a 3-Oxoadipate Pathway

[0179] This example describes the generation of a microbial organism capable of producing adipate using the reverse degradation pathway and converting the intracellular adipate to 6-aminocaproate and/or caprolactam.

[0180] *Escherichia coli* is used as a target organism to engineer the necessary genes for adipate, 6-aminocaproate, and/or caprolactam synthesis (see Figure 2 and Figure 8). *E. coli* provides a good host for generating a non-naturally occurring microorganism capable of producing adipate, 6-aminocaproate, and/or caprolactam. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic acid, formic acid, lactic acid, and succinic acid, effectively under anaerobic or microaerobic conditions.

[0181] To generate an *E. coli* strain engineered to produce 6-aminocaproate and/or caprolactam, nucleic acids encoding the enzymes utilized in the reverse adipate degradation pathway and 6-aminocaproate or caprolactam synthesis pathways are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, *supra,* 2001; Ausubel, *supra,* 1999). In particular, the *paaJ* (NP_415915.1), *paaH* (NP_415913.1), and *maoC* (NP_415905.1) genes encoding the succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyadipyl-CoA dehydratase activities, respectively, are cloned into the pZE13 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. In addition, the *bcd* (NP_349317.1), *etfAB* (349315.1 and 349316.1), and *sucCD* (NP_415256.1 and AAC73823.1) genes encoding 5-carboxy-2-pentenoyl-CoA reductase and adipyl-CoA synthetase activities, respectively, are cloned into the pZA33 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. Lastly, the *acr1* (YP_047869.1), *gabT* (NP_417148.1), and *nylB* (AAA24929.1) genes encoding CoA-dependent aldehyde dehydrogenase, transaminase, and amidohydrolase activities arc cloned into a third compatible plasmid, pZS23, under the PA1/lacO promoter. pZS23 is obtained by replacing the ampicillin resistance module of the pZS 13 vector (Expressys, Ruelzheim, Germany) with a kanamycin resistance module by well-known molecular biology techniques. The three sets of plasmids are transformed into *E. coli* strain MG1655 to express the proteins and enzymes required for 6-aminocaproate and/or caprolactam synthesis.

[0182] The resulting genetically engineered organism is cultured in glucose containing medium following procedures well known in the art (see, for example, Sambrook et al., *supra,* 2001). The expression of the 6-aminocaproate and caprolactam synthesis genes is corroborated using methods well known in the art for determining polypeptide expression or enzymatic activity, including for example, Northern blots, PCR amplification of mRNA, immunoblotting, and the like. Enzymatic activities of the expressed enzymes are confirmed using assays specific for the individual activities. The ability of the engineered *E. coli* strain to produce 6-aminocaproate and/or caprolactam is confirmed using HPLC, gas chromatography-mass spectrometry (GCMS) and/or liquid chromatography-mass spectrometry (LCMS).

[0183] Microbial strains engineered to have a functional pathway for the synthesis of 6-aminocaproate and/or caprolactam are further augmented by optimization for efficient utilization of the pathway. Briefly, the engineered strain is assessed to determine whether any of the exogenous genes are expressed at a rate limiting level. Expression is increased for any enzymes expressed at low levels that can limit the flux through the pathway by, for example, introduction of additional gene copy numbers.

[0184] To generate better producers, metabolic modeling is utilized to optimize growth conditions. Modeling is also used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of 6-aminocaproate and/or caprolactam. One modeling method is the bilevel optimization approach, OptKnock (Burgard et al., Biotechnol. Bioengineer. 84:647-657 (2003)), which is applied to select gene knockouts that collectively result in better production of 6-aminocaproate and/or caprolactam. Adaptive evolution also can be used to generate better producers of, for example, the acetyl-CoA and succinyl-CoA intermediates of the products. Adaptive evolution is performed to improve both growth and production characteristics (Fong and Palsson, Nat. Genet. 36:1056-1058 (2004); Alper ct al., Science 314:1565-1568 (2006)). Based on the results, subsequent rounds of modeling, genetic engineering and adaptive evolution can be applied to the 6-aminocaproate and/or caprolactam producer to further increase production.

[0185] For large-scale production of 6-aminocaproate and/or caprolactam, the above organism is cultured in a fermenter using a medium known in the art to support growth of the organism under anaerobic conditions. Fermentations are performed in either a batch, fed-batch or continuous manner. Anaerobic conditions are maintained by first sparging the medium with nitrogen and then sealing the culture vessel, for example, flasks can be sealed with a septum and crimp-cap. Microaerobic conditions also can be utilized by providing a small hole in the septum for limited aeration. The pH of the medium is maintained at around a pH of 7 by addition of an acid, such as H_2SO_4. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm) and the glucose uptake rate by monitoring carbon source depletion over time. Byproducts such as undesirable alcohols, organic acids, and residual glucose can be quantified by HPLC (Shimadzu), for example, using an Aminex® series of HPLC columns (for example, HPX-87 series) (BioRad), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 775-779 (2005)).

EXAMPLE X

Adipate Synthesis via 2-Hydroxyadipyl-CoA

[0186] This example describes two exemplary adipate synthesis pathways proceeding from alpha-ketoadipate and passing through a 2-hydroxyadipyl-CoA intermediate.

[0187] As described in example VI, alpha-ketoadipate is a known intermediate in lysine biosynthesis that can be formed from alpha-ketoglutarate via homocitrate synthase, homoaconitase, and homoisocitrate dehydrogenase. Alpha-ketoadipate can be converted to 2-hydroxyadipyl-CoA by the two routes depicted in Figure 9. 2-hydroxyadipyl-CoA can be subsequently dehydrated and reduced to adipyl-CoA which can then be converted to adipate as shown in Figure 9. The maximum yield of adipate from glucose via these pathways is 0.67 mol/mol.

[0188] Conversion of alpha-ketoadipate into 2-hydroxyadipate can be catalyzed by 2-ketoadipate reductase, an enzyme reported to be found in rat and in human placenta (Suda et al., Arch. Biochem. Biophys. 176:610-620 (1976); Suda et al., Biochem. Biophys. Res. Commun. 77:586-591 (1977). Alternatively, enzymes capable of reducing alpha-ketoglutarate to 2-hydroxyglutarate may also show activity on alpha-ketoadipate, which is only one carbon atom longer. One such enzyme possessing alpha-ketoglutarate reductase activity is *serA* of *Escherichia coli* (Zhao and Winkler, J. Bacteriol. 178(1):232-9 (1996)). Additional exemplary enzymes can be found in *Arabidopsis thaliana* (Ho, et al., J. Biol. Chem. 274(I):397-402 (1999)) and *Haemophilus influenzae.*

serA	NP_417388.1	*Escherichia coli*
PGDH	NP_564034	*Arabidopsis thaliana*
serA	P43885	*Haemophilus influenzae*

[0189] Referring to Figure 9, 2-hydroxyadipate can likely be converted to 2-hydroxyadipyl-CoA by the synthetases, transferases, phosphotransadipylases and kinases described in example I. Alternatively, enzymes with 2-hydroxyglutarate CoA-transferase or glutaconate CoA-transferase activity are likely suitable to transfer a CoA moiety to 2-hydroxyadipate. One example of such an enzyme is encoded by the *gctA* and *gctB* genes *ofAcidaminococcus fermentans* (Buckel, et al., Eur. J. Biochem. 118(2):315-321 (1981); Mack, et al., Eur. J. Biochem. 226(1):41-51 (1994)). Similarly, synthetase, transferase, or phosphotransadipylase and kinase activities would be required to convert alpha-ketoadipate into alpha-ketoadipyl-CoA, as depicted in Figure 9. Conversion of alpha-ketoadipyl-CoA to 2-hydroxyadipyl-CoA can be carried out by an alpha-hydroxyacyl-CoA dehydrogenase enzyme. A similar activity was reported in propionate-adapted *E. coli* cells whose extracts catalyzed the oxidation of lactyl-CoA to form pyruvyl-CoA (Megraw et al., J. Bacteriol. 90(4): 984-988 (1965)). Additional hydroxyacyl-CoA dehydrogenases were described in example I.

gctA	Q59111	*Acidaminococcus fermentans*
gctB	Q59112	*Acidaminococcus fermentans*

[0190] The dehydration of 2-hydroxyadipyl-CoA to form 5-carboxy-2-pentenoyl-CoA can be carried out by a 2-hydroxyacyl-CoA dehydratase. A 2-hydroxyglutaryl-CoA dehydratase system has been characterized in *Acidaminococcus fermentans* and requires both the *hgdA* and *hgdB* subunits and the activator protein, *hgdC,* for optimal activity (Dutscho et al., Eur. J. Biochem. 181(3):741-746 (1989); Locher et al. J. Mol. Biol. 307(1):297-308; Muller and Buckel, Eur. J. Biochem. 230(2):698-704 (2001); Schweiger et al. Eur. J. Biochem. 169(2):441-448 (1987)). This enzyme system is similar in mechanism to the lactoyl-CoA dehydratase from *Clostridium propionicum* (Hofmeister and Buckel, Eur. J. Biochem. 206(2):547-552 (1992); Kuchta and Abeles, J. Biol. Chem. 260(24):13181-13189 (1985)). Homologs to *hgdA, hgdB,* and *hgdC* exist in several organisms.

hgdA	P11569	*Acidaminococcus fermentans*
hgdB	P11570	*Acidaminococcus fermentans*
hgdC	P11568	*Acidaminococcus fermentans*
hgdA	ZP_03731126.1	*Clostridium sp. M62/1*
hgdB	ZP_03731125.1	*Clostridium sp. M62/1*
hgdC	ZP_03731127.1	*Clostridium sp. M62/1*
hgdA	NP_603114.1	*Fusobacterium nucleatum ATCC 25586*
hgdB	NP_603115.1	*Fusobacterium nucleatum ATCC 25586*
hgdC	NP_603113.1	*Fusobacterium nucleatum ATCC 25586*

[0191] Conversion of 5-carboxy-2-pentenoyl-CoA to adipate is carried out by the enzymes described in Example I.

[0192] The above description provides an exemplary adipate synthesis pathway by way of a 2-hydroxyadipyl-CoA pathway.

EXAMPLE XI

Preparation of an Adipate Producing Microbial Organism Having a 2-Hydroxyadipyl-CoA Pathway

[0193] This example describes the generation of a microbial organism capable of producing adipate using a 2-hydroxyadipyl-CoA pathway.

[0194] *Escherichia coli* is used as a target organism to engineer the necessary genes for adipate synthesis (see Figure 9). *E. coli* provides a good host for generating a non-naturally occurring microorganism capable of producing adipate. *E. coli* is amenable to genetic manipulation and is known to be capable of producing various products, like ethanol, acetic acid, formic acid, lactic acid, and succinic acid, effectively under anaerobic or microaerobic conditions.

[0195] To generate an *E. coli* strain engineered to produce adipate, nucleic acids encoding the enzymes utilized in a 2-hydroxyadipyl-CoA to adipate pathway are expressed in *E. coli* using well known molecular biology techniques (see, for example, Sambrook, *supra,* 2001; Ausubel, *supra,* 1999). In particular, the *serA* (NP_417388.1), *gctA (*Q59111*)*, and *gctB (*Q59112*)* genes encoding the 2-hydroxyadipate dehydrogenase and 2-hydroxyadipyl-CoA:acetyl-CoA transferase activities, respectively, are cloned into the pZE13 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. In addition, the *hgdA* (P11569), *hgdB (*P11570*)*, and *hgdC* (P11568) genes encoding 2-hydroxyadipyl-CoA dehydratase activity, respectively, are cloned into the pZA33 vector (Expressys, Ruelzheim, Germany) under the PA1/lacO promoter. Further, the *bcd* (NP_349317.1), *etfAB* (349315.1 and 349316.1), and *sucCD* (NP_415256.1 and AAC73823.1) genes encoding 5-carboxy-2-pentenoyl-CoA reductase and adipyl-CoA synthetase activities are cloned into a third compatible plasmid, pZS23, under the PA1/lacO promoter. pZS23 is obtained by replacing the ampicillin resistance module of the pZS13 vector (Expressys, Ruelzheim, Germany) with a kanamycin resistance module by well-known molecular biology techniques. The three sets of plasmids are transformed into *E. coli* strain MG1655 to express the proteins and enzymes required for adipate synthesis.

[0196] The resulting genetically engineered organism is cultured in glucose containing medium following procedures well known in the art (see, for example, Sambrook et al., *supra,* 2001). The expression of the 2-hydroxyadipyl-CoA pathway genes for adipate synthesis is corroborated using methods well known in the art for determining polypeptide expression or enzymatic activity, including for example, Northern blots, PCR amplification of mRNA, immunoblotting, and the like. Enzymatic activities of the expressed enzymes are confirmed using assays specific for the individual activities. The ability of the engineered *E. coli* strain to produce adipate is confirmed using HPLC, gas chromatography-mass spectrometry (GCMS) and/or liquid chromatography-mass spectrometry (LCMS).

[0197] Microbial strains engineered to have a functional adipate synthesis pathway are further augmented by optimization for efficient utilization of the pathway. Briefly, the engineered strain is assessed to determine whether any of the exogenous genes are expressed at a rate limiting level. Expression is increased for any enzymes expressed at low levels that can limit the flux through the pathway by, for example, introduction of additional gene copy numbers.

[0198] To generate better producers, metabolic modeling is utilized to optimize growth conditions. Modeling is also used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of adipate. One modeling method is the bilevel optimization approach, OptKnock (Burgard et al., Biotechnol. Bioengineer. 84:647-657 (2003)), which is applied to select gene knockouts that collectively result in better production of adipate. Adaptive evolution also can be used to generate better producers of, for example, the alpha-ketoadipate intermediate or the adipate product. Adaptive evolution is performed to improve both growth and production characteristics (Fong and Palsson, Nat. Genet. 36:1056-1058 (2004); Alper et al., Science 314:1565-1568 (2006)). Based on the results, subsequent rounds of modeling, genetic engineering and adaptive evolution can be applied to the adipate producer to further increase production.

[0199] For large-scale production of adipate, the 2-hydroxyadipyl-CoA pathway-containing organism is cultured in a fermenter using a medium known in the art to support growth of the organism under anaerobic conditions. Fermentations are performed in either a batch, fed-batch or continuous manner. Anaerobic conditions are maintained by first sparging the medium with nitrogen and then sealing the culture vessel, for example, flasks can be sealed with a septum and crimp-cap. Microaerobic conditions also can be utilized by providing a small hole in the septum for limited aeration. The pH of the medium is maintained at around a pH of 7 by addition of an acid, such as H_2SO_4. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm) and the glucose uptake rate by monitoring carbon source depletion over time. Byproducts such as undesirable alcohols, organic acids, and residual glucose can be quantified by HPLC (Shimadzu), for example, using an Aminex® series of HPLC columns (for example, HPX-87 series) (BioRad), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., Biotechnol. Bioeng. 775-779 (2005)).

[0200] This example describes the preparation of an adipate-producing microbial organism containing a 2-hydroxyadipyl-CoA pathway.

Claims

1. A non-naturally occurring microbial organism, comprising a microbial organism having a pathway for converting adipyl-CoA to 6 aminocaproic acid (6-aminocaproic acid pathway) and at least one pathway for producing adipyl-CoA (adipyl-CoA pathway), said 6-aminocaproic acid pathway comprising two exogenous nucleic acids each encoding a 6-aminocaproic acid pathway enzyme expressed in a sufficient amount to produce 6-aminocaproic acid, said 6-aminocaproic acid pathway comprising CoA-dependent aldehyde dehydrogenase, and transaminase or 6-aminocaproate dehydrogenase,
wherein said at least one adipyl-CoA pathway comprises at least one of a pathway for converting adipate to adipyl CoA and a pathway for converting succinyl-CoA plus acetyl-CoA to adipyl-CoA,
wherein said pathway for converting adipate to adipyl CoA, comprises adipyl-CoA synthetase or phosphotransadipylase/adipate kinase; and
wherein said pathway for converting succinyl-CoA plus acetyl-CoA to adipyl-CoA comprises succinyl-CoA:acetyl-CoA acyl transferase, 3-oxoadipyl-CoA transferase, 3-oxoadipate reductase, 3-hydroxyadipate dehydratase, 2-enoate reductase, and adipyl-CoA synthetase, phosphotransadipylase/adipate kinase, adipyl-CoA:acetyl-CoA transferase or adipyl-CoA hydrolase.

2. The non-naturally occurring microbial organism of claim 1 wherein said two exogenous nucleic acids encode CoA-dependent aldehyde dehydrogenase and transaminase.

3. The non-naturally occurring microbial organism of claim 1 wherein said two exogenous nucleic acids encode CoA-dependent aldehyde dehydrogenase and 6-aminocaproate dehydrogenase.

4. The non-naturally occurring microbial organism of any one of claims 1-3, wherein said adipyl-CoA pathway comprises a pathway for converting adipate to adipyl CoA comprising adipyl-CoA synthetase.

5. The non-naturally occurring microbial organism of any one of claims 1-3, wherein said adipyl-CoA pathway comprises a pathway for converting adipate to adipyl CoA comprising phosphotransadipylase/adipate kinase.

6. The non-naturally occurring microbial organism of any one of claim 1-5, wherein said adipyl-CoA pathway comprises a pathway for converting succinyl-CoA plus acetyl-CoA to adipyl-CoA and said pathway for converting succinyl-CoA plus acetyl-CoA to adipyl-CoA comprises succinyl-CoA:acetyl-CoA acyl transferase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyadipyl-CoA dehydratase, and 5-carboxy-2-pentenoyl-CoA reductase.

7. The non-naturally occurring microbial organism of any one of claims 1-5, wherein said adipyl-CoA pathway comprises a pathway for converting adipate to adipyl CoA comprising adipyl-CoA synthetase and further comprises a pathway for converting succinyl-CoA plus acetyl-CoA to adipate (adipate pathway) comprising succinyl-CoA:acetyl-CoA acyl transferase, 3-oxoadipyl-CoA transferase, 3-oxoadipate reductase, 3-hydroxyadipate dehydratase, and 2-enoate reductase.

8. The non-naturally occurring microbial organism of claim 7, wherein said adipate pathway comprises one, two, three, four or five exogenous nucleic acids each encoding an adipate pathway enzyme.

9. The non-naturally occurring microbial organism of claim 7, wherein said adipyl-CoA pathway further comprises adipyl-CoA:acetyl-CoA transferase.

10. The non-naturally occurring microbial organism of claim 7, wherein said adipyl-CoA pathway further comprises adipyl-CoA hydrolase.

11. The non-naturally occurring microbial organism of any of claims 1-3, wherein said adipyl-CoA pathway comprises a pathway for converting succinyl-CoA plus acetyl-CoA to adipyl-CoA comprising one, two, three, four or five exogenous nucleic acids each encoding an adipyl-CoA pathway enzyme.

12. The non-naturally occurring microbial organism of any of claims 1-11, wherein at least one of said exogenous nucleic acids is a heterologous nucleic acid.

13. The non-naturally occurring microbial organism of any of claims 1-12, wherein the non-naturally occurring microbial organism is a species of bacteria, yeast or fungus.

14. The non-naturally occurring microbial organism of claim 13, wherein said bacteria is selected from the group consisting of *Escherichia coli, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Bacillus subtilis, Corynebacterium glutamicum, Gluconobacter oxydans, Zymomonas mobilis, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Clostridium acetobutylicum, Pseudomonas fluorescens,* and *Pseudomonoas putida.*

15. The non-naturally occurring microbial organism of claim 13, wherein said yeast or fungus is selected from the group consisting of *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger,* and *Pichia pastoris.*

16. The non-naturally occurring microbial organism of any one of claims 1-15, wherein said microbial organism further comprises a caprolactam pathway comprising amidohydrolase.

17. A substantially anaerobic culture comprising the non-naturally occurring microbial organism of any of claims 1-16.

18. A method for producing 6-aminocaproic acid, comprising culturing the non-naturally occurring microbial organism of any of claims 1-15, under conditions and for a sufficient period of time to produce 6-aminocaproic acid.

19. The method of claim 18, wherein said method further comprises separating the 6-aminocaproic acid from other components in the culture.

20. The method of claim 19, wherein the separating comprises extraction, continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration.

21. A method for producing caprolactam, comprising culturing the non-naturally occurring microbial organism of claim 16 under conditions and for a sufficient period of time to produce caprolactam.

22. The method of claim 21, wherein said method further comprises separating the caprolactam from other components in the culture.

23. The method of claim 22, wherein the separating comprises extraction, continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration.

Patentansprüche

1. Ein nicht-natürlich vorkommender mikrobieller Organismus, umfassend einen mikrobiellen Organismus mit einem Pfad zur Umwandlung von Adipyl-CoA zu 6-Aminocapronsäure (6-Aminocapronsäure-Pfad) und mindestens einen Pfad zur Herstellung von Adipyl-CoA (Adipyl-CoA-Pfad), wobei der 6-Aminocapronsäure-Pfad zwei exogene Nukleinsäuren umfasst, die jeweils für ein Enzym des 6-Aminocapronsäure-Pfad kodieren, das in einer ausreichenden Menge exprimiert wird, um 6-Aminocapronsäure zu erzeugen,
wobei der 6-Aminocapronsäure-Pfad CoA-abhängige Aldehyddehydrogenase und Transaminase oder 6-Aminocaproat-Dehydrogenase umfasst,
wobei der mindestens eine Adipyl-CoA-Pfad mindestens einen Pfad zum Umwandeln von Adipat zu Adipyl-CoA und einen Pfad zur Umwandlung von Succinyl-CoA plus Acetyl-CoA zu Adipyl-CoA umfasst,
wobei der Pfad zur Umwandlung von Adipat zu Adipyl-CoA, Adipyl-CoA-Synthetase oder Phosphotransadipylase/Adipatkinase umfasst; und
wobei der Pfad zur Umwandlung von Succinyl-CoA plus Acetyl-CoA zu Adipyl-CoA umfasst Succinyl-CoA:Acetyl-CoA-Acyltransferase, 3-Oxoadipyl-CoA-Transferase, 3-Oxoadipat-Reduktase, 3-Hydroxyadipat-Dehydratase, 2-Enoat-Reduktase und Adipyl-CoA-Synthetase, Phosphotransadipylase/Adipatkinase, Adipyl-CoA:Acetyl-CoA-Transferase oder Adipyl-CoA-Hydrolase.

2. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 1, wobei die beiden exogenen Nukleinsäuren für CoA-abhängige Aldehyd-Dehydrogenase und Transaminase kodieren.

3. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 1, wobei die beiden exogenen Nukleinsäuren für CoA-abhängige Aldehyd-Dehydrogenase und 6-Aminocaproat-Dehydrogenase kodieren.

4. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-3, wobei der Adipyl-CoA-Pfad einen Pfad zur Umwandlung von Adipat zu Adipyl-CoA umfasst, der Adipyl-CoA-Synthetase umfasst.

5. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1 bis 3, wobei der Adipyl-CoA-Pfad einen Pfad zur Umwandlung von Adipat zu Adipyl-CoA umfasst, der Phosphotransadipylase/Adipatkinase umfasst.

6. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-5, wobei der Adipyl-CoA-Pfad einen Pfad zur Umwandlung von Succinyl-CoA plus Acetyl-CoA zu Adipyl-CoA umfasst und wobei der Pfad zur Umwandlung von Succinyl-CoA plus Acetyl-CoA zu Adipyl-CoA Succinyl-CoA:Acetyl-CoA-Acyltransferase, 3-Hydroxyacyl-CoA-Dehydrogenase, 3-Hydroxyadipyl-CoA-Dehydratase und 5-Carboxy-2-pentenoyl-CoA-Reduktase umfasst.

7. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-5, wobei der Adipyl-CoA-Pfad einen Pfad zur Umwandlung von Adipat zu Adipyl-CoA umfasst, umfassend Adipyl-CoA-Synthetase und der ferner einen Pfad zur Umwandlung von Succinyl-CoA plus Acetyl-CoA zu Adipat (Adipad-Pfad) umfasst, umfassend Succinyl-CoA:Acetyl-CoA-Acyltransferase, 3-Oxoadipyl-CoA-Transferase, 3-Oxoadipat-Reduktase, 3-Hydroxyadipat-Dehydratase und 2-Enoat-Reduktase.

8. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 7, wobei der Adipat-Pfad eine, zwei, drei, vier oder fünf exogene Nukleinsäuren umfasst, die jeweils für ein Enzym des Adipat-Pfad kodieren.

9. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 7, wobei der Adipyl-CoA-Pfad ferner Adipyl-CoA:Acetyl-CoA-Transferase umfasst.

10. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 7, wobei der Adipyl-CoA-Pfad ferner Adipyl-CoA-Hydrolase umfasst.

11. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-3, wobei der Adipyl-CoA-Pfad einen Pfad zur Umwandlung von Succinyl-CoA plus Acetyl-CoA zu Adipyl-CoA umfasst, umfassend ein, zwei, drei, vier oder fünf exogene Nukleinsäuren, die jeweils für ein Enzym des Adipyl-CoA-Pfad kodieren.

12. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-11, wobei mindestens eine der exogenen Nukleinsäuren eine heterologe Nukleinsäure ist.

13. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-12, wobei der nicht-natürlich vorkommende mikrobielle Organismus eine Spezies von Bakterien, Hefen oder Pilzen ist.

14. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 13, wobei die Bakterien ausgewählt sind aus der Gruppe bestehend aus *Escherichia coli, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Bacillus subtilis, Corynebacterium glutamicum, Gluconobacter oxydans, Zymomonas mobilis, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Clostridium acetobutylicum, Pseudomonas fluorescens* und *Pseudomonoas putida.*

15. Der nicht-natürlich vorkommende mikrobielle Organismus nach Anspruch 13, wobei die Hefe oder der Pilz ausgewählt ist aus der Gruppe bestehend aus *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger* und *Pichia pastoris.*

16. Der nicht-natürlich vorkommende mikrobielle Organismus nach einem der Ansprüche 1-15, wobei der mikrobielle Organismus ferner einen Caprolactam-Pfad umfasst, der Amidohydrolase umfasst.

17. Eine im Wesentlichen anaerobe Kultur, umfassend den nicht-natürlich vorkommenden mikrobiellen Organismus nach einem der Ansprüche 1-16.

18. Ein Verfahren zur Herstellung von 6-Aminocapronsäure, umfassend das Kultivieren des nicht-natürlich vorkommen-

den mikrobiellen Organismus nach einem der Ansprüche 1-15, unter Bedingungen und für einen ausreichenden Zeitraum, um 6-Aminocapronsäure herzustellen.

19. Das Verfahren nach Anspruch 18, wobei das Verfahren ferner die Abtrennung der 6-Aminocapronsäure von anderen Komponenten in der Kultur umfasst.

20. Das Verfahren nach Anspruch 19, wobei die Abtrennung Extraktion, kontinuierliche Flüssig-Flüssig-Extraktion, Pervaporation, Membranfiltration, Membrantrennung, Umkehrosmose, Elektrodialyse, Destillation, Kristallisation, Zentrifugation, Extraktionsfiltration, Ionenaustauschchromatographie, Größenausschlusschromatographie, Adsorptionschromatographie und Ultrafiltration umfasst.

21. Das Verfahren zur Herstellung von Caprolactam, umfassend das Kultivieren des nicht-natürlich vorkommenden mikrobiellen Organismus nach Anspruch 16 unter Bedingungen und für einen ausreichenden Zeitraum, um Caprolactam herzustellen.

22. Das Verfahren nach Anspruch 21, wobei das Verfahren ferner das Abtrennen des Caprolactams von anderen Komponenten in der Kultur umfasst.

23. Das Verfahren nach Anspruch 22, wobei die Abtrennung Extraktion, kontinuierliche Flüssig-Flüssig-Extraktion, Pervaporation, Membranfiltration, Membrantrennung, Umkehrosmose, Elektrodialyse, Destillation, Kristallisation, Zentrifugation, Extraktionsfiltration, Ionenaustauschchromatographie, Größenausschlusschromatographie, Adsorptionschromatographie und Ultrafiltration umfasst.

Revendications

1. Organisme microbien non naturel, comprenant un organisme microbien possédant une voie pour la conversion de l'adipyl-CoA en acide 6-aminocaproïque (voie acide 6-amino-caproïque) et au moins une voie pour la production d'adipyl-CoA (voie adipyl-CoA), ladite voie acide 6-amino-caproïque comprenant deux acides nucléiques exogènes codant chacun pour une enzyme de la voie acide 6-aminocaproïque exprimée en une quantité suffisante pour produire ledit acide 6-amino-caproïque,
ladite voie acide 6-aminocaproïque comprenant une aldéhyde déshydrogénase CoA-dépendante, et une transaminase, ou une 6-aminocaproate déshydrogénase,
dans lequel ladite au moins une voie adipyl-CoA comprend au moins une voie pour la conversion de l'adipate en adipyl-CoA et/ou une voie pour la conversion d'une succinyl-CoA plus acétyl-CoA en adipyl-CoA,
dans lequel ladite voie pour la conversion de l'adipate en adipyl-CoA comprend une adipyl-CoA synthétase ou une phosphotransadipylase/adipate kinase ; et
dans lequel ladite voie pour la conversion de la succinyl-CoA plus acétyl-CoA en adipyl-CoA comprend une succinyl-CoA:acétyl-CoA acyltransférase, une 3-oxoadipyl-CoA transférase, une 3-oxoadipate réductase, une 3-hydroxyadipate déshydratase, une 2-énoate réductase, et une adipyl-CoA synthétase, une phosphotransadipylase/adipate kinase, une adipyl-CoA:acétyl-CoA transférase ou une adipyl-CoA hydrolase.

2. Organisme microbien non naturel selon la revendication 1, dans lequel lesdits deux acides nucléiques exogènes codent pour une aldéhyde déshydrogénase CoA-dépendante et une transaminase.

3. Organisme microbien non naturel selon la revendication 1, dans lequel lesdits deux acides nucléiques exogènes codent pour une aldéhyde déshydrogénase CoA-dépendante et une 6-aminocaproate déshydrogénase.

4. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 3, dans lequel ladite voie adipyl-CoA comprend une voie pour la conversion de l'adipate en adipyl-CoA comprenant une adipyl-CoA synthétase.

5. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 3, dans lequel ladite voie adipyl-CoA comprend une voie pour la conversion de l'adipate en adipyl-CoA comprenant une phosphotrans-adipylase/adipate kinase.

6. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 5, dans lequel ladite voie adipyl-CoA comprend une voie pour la conversion d'une succinyl-CoA plus acétyl-CoA en adipyl-CoA et ladite voie pour la conversion de la succinyl-CoA plus acétyl-CoA en adipyl-CoA comprend une succinyl-CoA:acétyl-CoA acyltransférase, une 3-hydroxyacyl-CoA déshydrogénase, une 3-hydroxyadipyl-CoA déshydratase, et une 5-carboxy-2-penténoyl-CoA réductase.

7. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 5, dans lequel ladite voie adipyl-CoA comprend une voie pour la conversion de l'adipate en adipyl-CoA comprenant une adipyl-CoA synthétase et comprend en outre une voie pour la conversion de la succinyl-CoA plus acétyl-CoA en adipate (voie adipate) comprenant une succinyl-CoA:acétyl-CoA acyltransférase, une 3-oxoadipyl-CoA transférase, une 3-oxoadipate réductase, une 3-hydroxyadipate déshydratase, et une 2-énoate réductase.

8. Organisme microbien non naturel selon la revendication 7, dans lequel ladite voie adipate comprend un, deux, trois, quatre ou cinq acides nucléiques exogènes codant chacun pour une enzyme de la voie adipate.

9. Organisme microbien non naturel selon la revendication 7, dans lequel ladite voie adipyl-CoA comprend en outre une adipyl-CoA:acétyl-CoA transférase.

10. Organisme microbien non naturel selon la revendication 7, dans lequel ladite voie adipyl-CoA comprend en outre une adipyl-CoA hydrolase.

11. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 3, dans lequel ladite voie adipyl-CoA comprend une voie pour la conversion de la succinyl-CoA plus acétyl-CoA en adipyl-CoA comprenant un, deux, trois, quatre ou cinq acides nucléiques exogènes codant chacun pour une enzyme de la voie adipyl-CoA.

12. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 11, dans lequel au moins un desdits acides nucléiques exogènes est un acide nucléique hétérologue.

13. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 12, dans lequel l'organisme microbien non naturel est une espèce bactérienne, une levure ou un champignon.

14. Organisme microbien non naturel selon la revendication 13, dans lequel ladite bactérie est choisie dans le groupe constitué par *Escherichia coli, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Bacillus subtilis, Corynebacterium glutamicum, Gluconobacter oxydans, Zymomonas mobilis, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Clostridium acetobutylicum, Pseudomonas fluorescens,* et *Pseudomonas putida.*

15. Organisme microbien non naturel selon la revendication 13, dans lequel ladite levure ou ledit champignon est choisi(e) dans le groupe constitué par *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger,* et *Pichia pastoris.*

16. Organisme microbien non naturel selon l'une quelconque des revendications 1 à 15, dans lequel ledit organisme microbien comprend en outre une voie caprolactame comprenant une amidohydrolase.

17. Culture essentiellement anaérobie comprenant l'organisme microbien non naturel selon l'une quelconque des revendications 1 à 16.

18. Procédé de production d'acide 6-aminocaproïque, comprenant la culture de l'organisme microbien non naturel selon l'une quelconque des revendications 1 à 15, dans certaines conditions et pendant un laps de temps suffisant pour produire l'acide 6-aminocaproïque.

19. Procédé selon la revendication 18, dans lequel ledit procédé comprend en outre la séparation de l'acide 6-aminocaproïque des autres composants dans la culture.

20. Procédé selon la revendication 19, dans lequel la séparation comprend l'extraction, l'extraction liquide-liquide continue, la pervaporation, la filtration sur membrane, la séparation sur membrane, l'osmose inverse, l'électrodialyse, la distillation, la cristallisation, la centrifugation, la filtration extractive, la chromatographie d'échange d'ions, la chromatographie d'exclusion de taille, la chromatographie d'adsorption, et l'ultrafiltration.

21. Procédé de production de caprolactame, comprenant la culture de l'organisme microbien non naturel selon la revendication 16 dans certaines conditions et pendant un laps de temps suffisant pour produire le caprolactame.

22. Procédé selon la revendication 21, dans lequel ledit procédé comprend en outre la séparation du caprolactame des autres composants dans la culture.

23. Procédé selon la revendication 22, dans lequel la séparation comprend l'extraction, l'extraction liquide-liquide continue, la pervaporation, la filtration sur membrane, la séparation sur membrane, l'osmose inverse, l'électrodialyse, la distillation, la cristallisation, la centrifugation, la filtration extractive, la chromatographie d'échange d'ions, la chromatographie d'exclusion de taille, la chromatographie d'adsorption, et l'ultrafiltration.

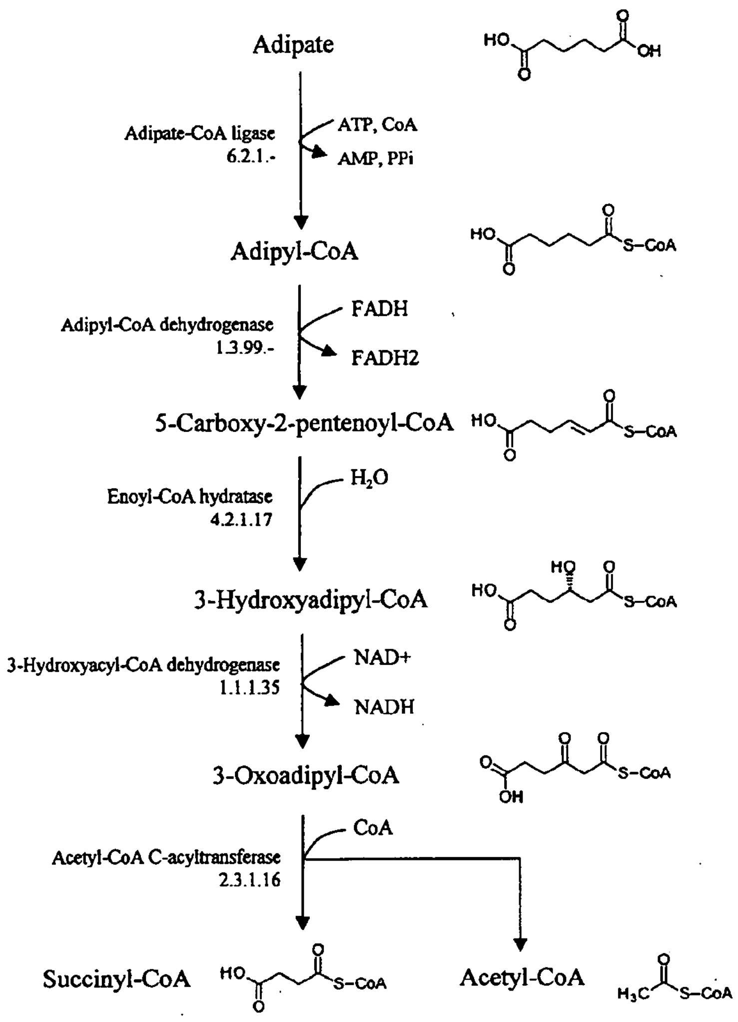
Adipate
Adipate-CoA ligase
6.2.1.-
ATP, CoA
AMP, PPi
Adipyl-CoA
Adipyl-CoA dehydrogenase
1.3.99.-
FADH
FADH2
5-Carboxy-2-pentenoyl-CoA
Enoyl-CoA hydratase
4.2.1.17
H_2O
3-Hydroxyadipyl-CoA
3-Hydroxyacyl-CoA dehydrogenase
1.1.1.35
NAD+
NADH
3-Oxoadipyl-CoA
Acetyl-CoA C-acyltransferase
2.3.1.16
CoA
Succinyl-CoA
Acetyl-CoA
HO
OH
O
S–CoA
H_3C

FIGURE 1

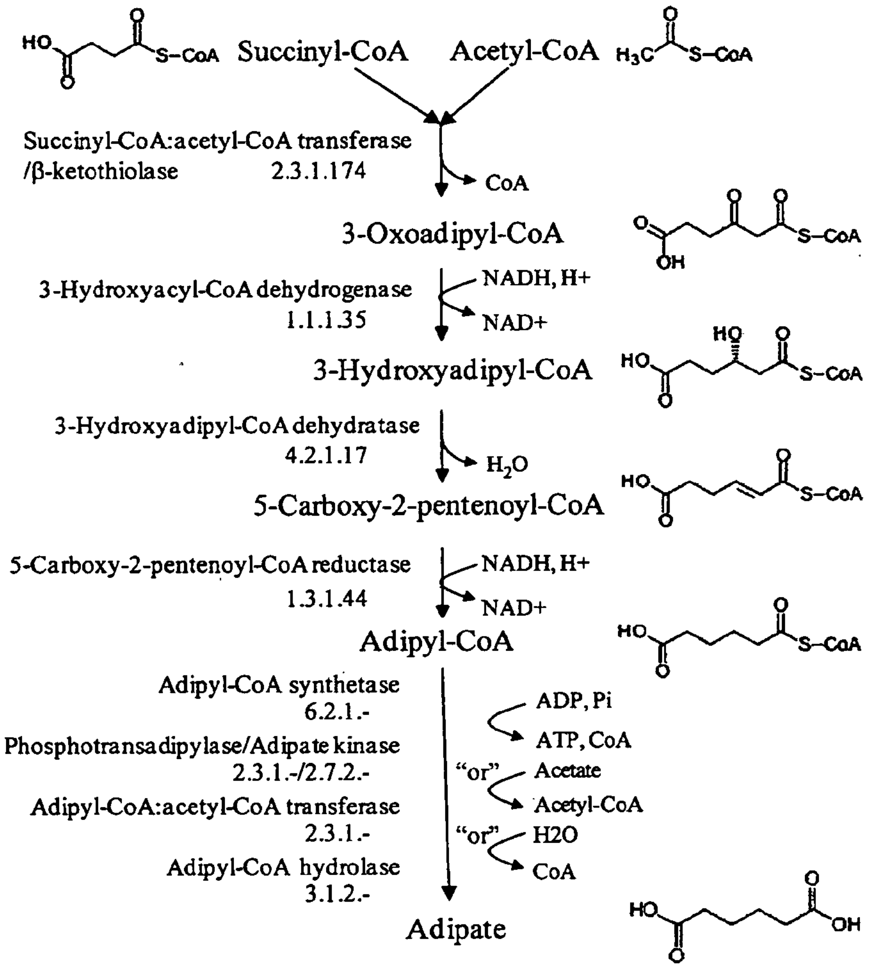
Succinyl-CoA
Acetyl-CoA
S-CoA
HO
O
H3C
Succinyl-CoA:acetyl-CoA transferase
/β-ketothiolase 2.3.1.174
CoA
3-Oxoadipyl-CoA
OH
3-Hydroxyacyl-CoA dehydrogenase
1.1.1.35
NADH, H+
NAD+
3-Hydroxyadipyl-CoA
3-Hydroxyadipyl-CoA dehydratase
4.2.1.17
H2O
5-Carboxy-2-pentenoyl-CoA
5-Carboxy-2-pentenoyl-CoA reductase
1.3.1.44
NADH, H+
NAD+
Adipyl-CoA
Adipyl-CoA synthetase
6.2.1.-
Phosphotransadipylase/Adipate kinase
2.3.1.-/2.7.2.-
Adipyl-CoA:acetyl-CoA transferase
2.3.1.-
Adipyl-CoA hydrolase
3.1.2.-
ADP, Pi
ATP, CoA
"or"
Acetate
Acetyl-CoA
"or"
H2O
CoA
Adipate

FIGURE 2

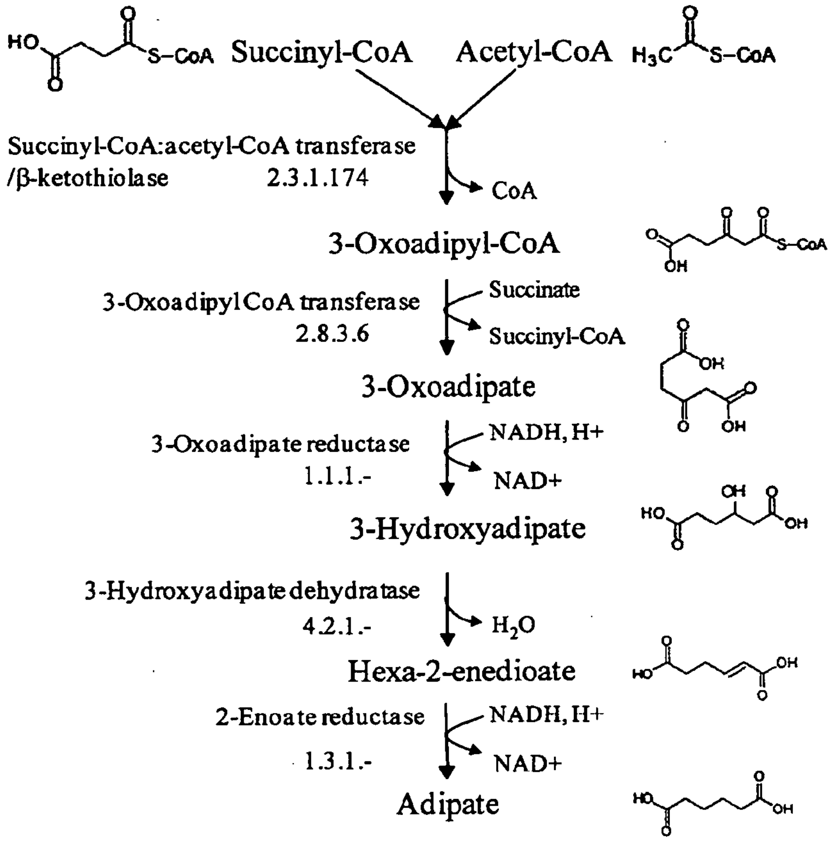
Succinyl-CoA
Acetyl-CoA
HO
S–CoA
H3C
S–CoA
Succinyl-CoA:acetyl-CoA transferase
/β-ketothiolase
2.3.1.174
CoA
3-Oxoadipyl-CoA
OH
3-OxoadipylCoA transferase
2.8.3.6
Succinate
Succinyl-CoA
3-Oxoadipate
OH
3-Oxoadipate reductase
1.1.1.-
NADH, H+
NAD+
3-Hydroxyadipate
HO
OH
3-Hydroxyadipate dehydratase
4.2.1.-
H2O
Hexa-2-enedioate
2-Enoate reductase
1.3.1.-
NADH, H+
NAD+
Adipate

FIGURE 3

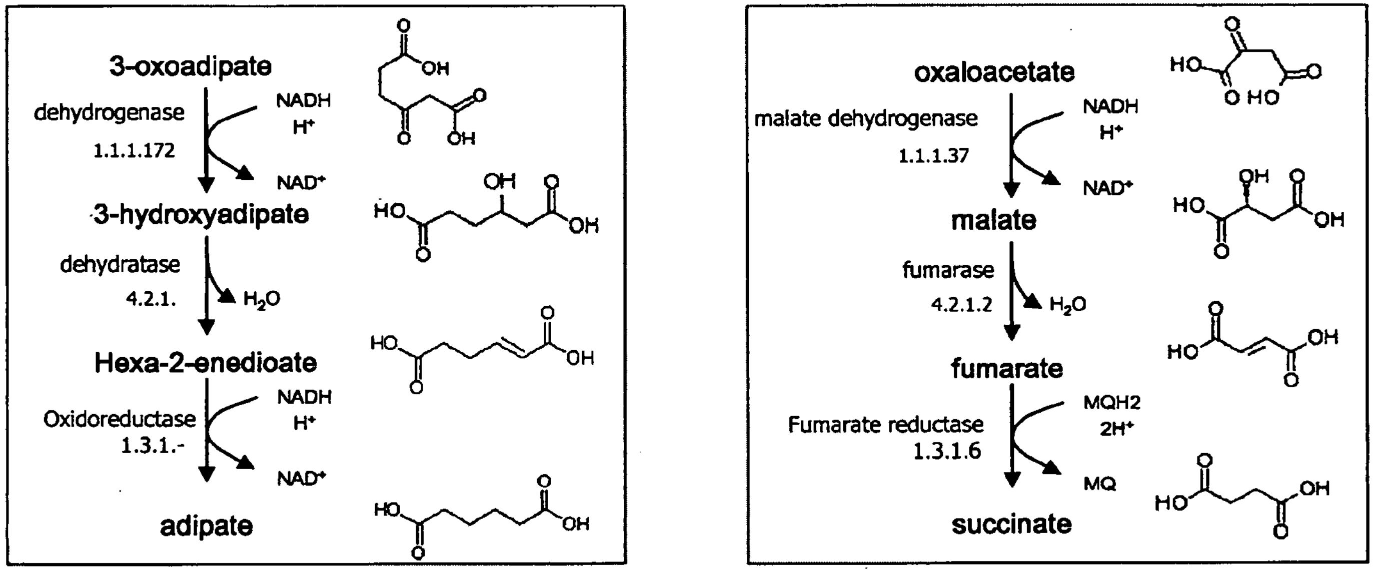
3-oxoadipate
dehydrogenase
1.1.1.172
NADH
H+
NAD+
3-hydroxyadipate
dehydratase
4.2.1.
H2O
Hexa-2-enedioate
Oxidoreductase
1.3.1.-
NADH
H+
NAD+
adipate
oxaloacetate
malate dehydrogenase
1.1.1.37
NADH
H+
NAD+
malate
fumarase
4.2.1.2
H2O
fumarate
Fumarate reductase
1.3.1.6
MQH2
2H+
MQ
succinate
HO
OH
O

FIGURE 4

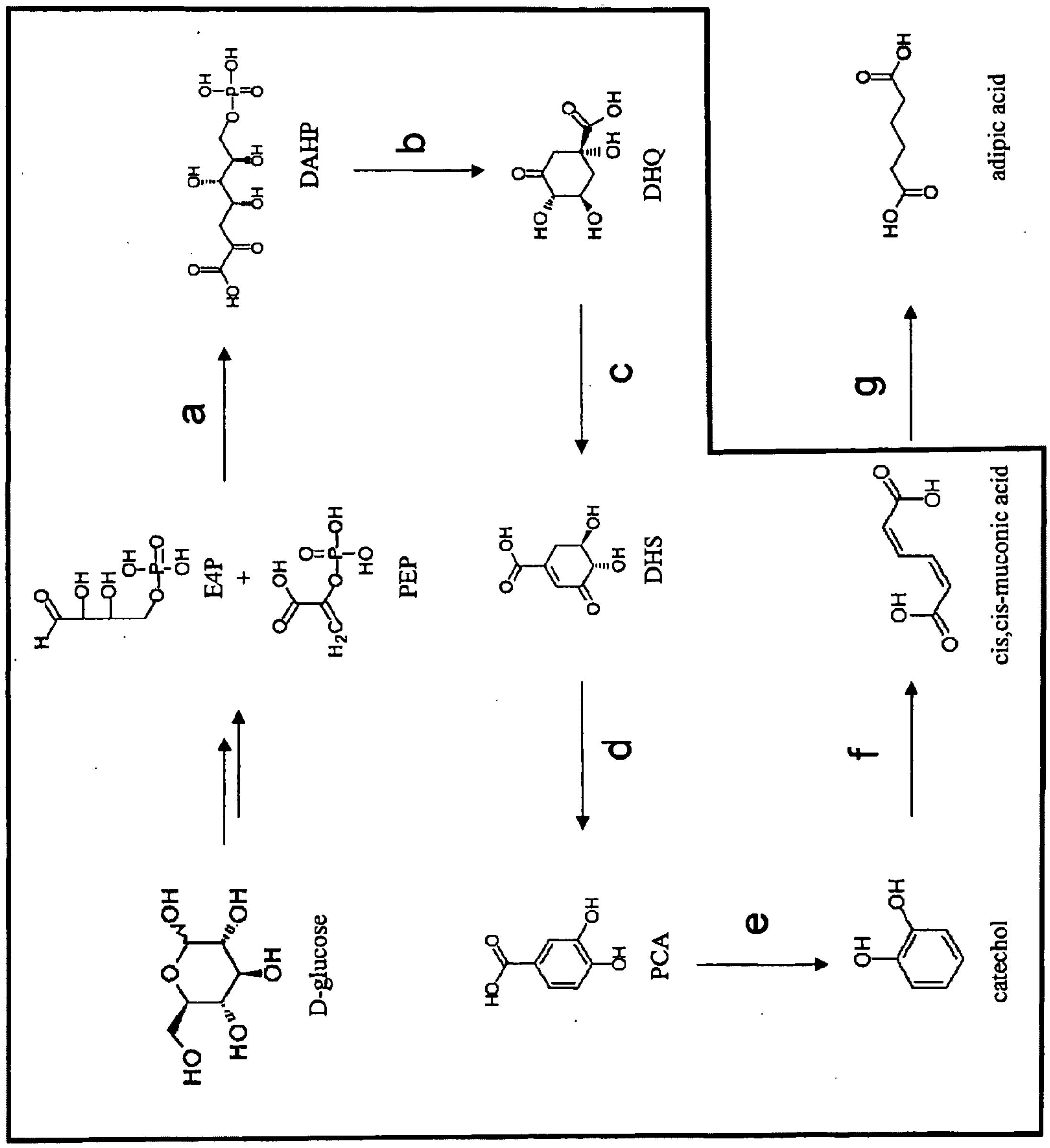
D-glucose
E4P
+
PEP
a
DAHP
b
DHQ
c
DHS
d
PCA
e
catechol
f
cis,cis-muconic acid
g
adipic acid

FIGURE 5

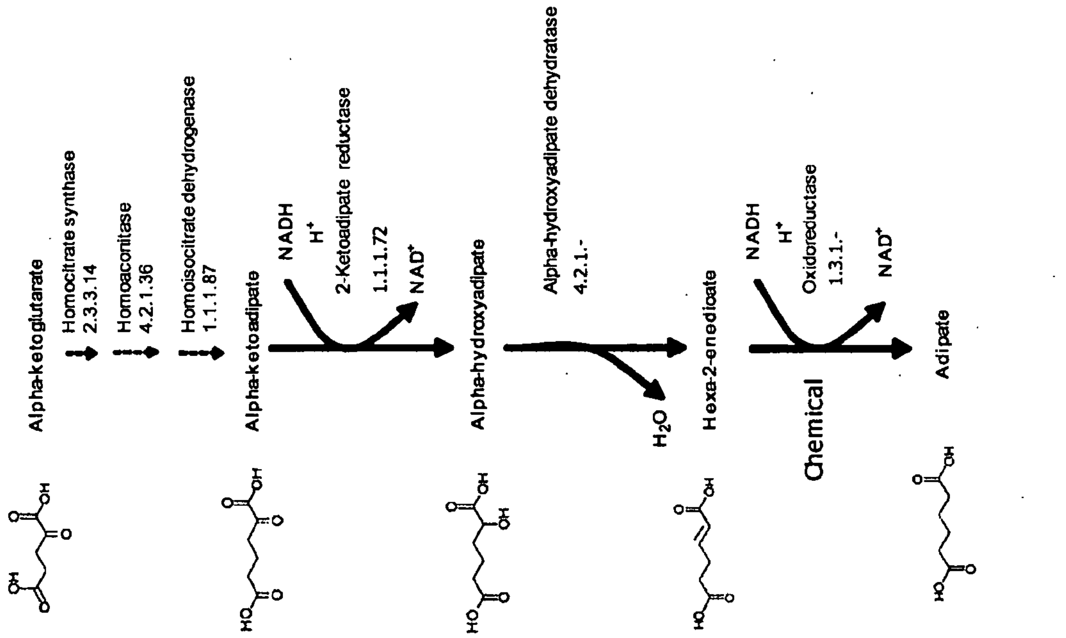
Alpha-ketoglutarate
Homocitrate synthase
2.3.3.14
Homoaconitase
4.2.1.36
Homoisocitrate dehydrogenase
1.1.1.87
Alpha-ketoadipate
NADH
H^+
2-Ketoadipate reductase
1.1.1.72
NAD^+
Alpha-hydroxyadipate
Alpha-hydroxyadipate dehydratase
4.2.1.-
H_2O
Hexa-2-enedioate
NADH
H^+
Oxidoreductase
1.3.1.-
NAD^+
Chemical
Adipate
OH
O
HO

FIGURE 6

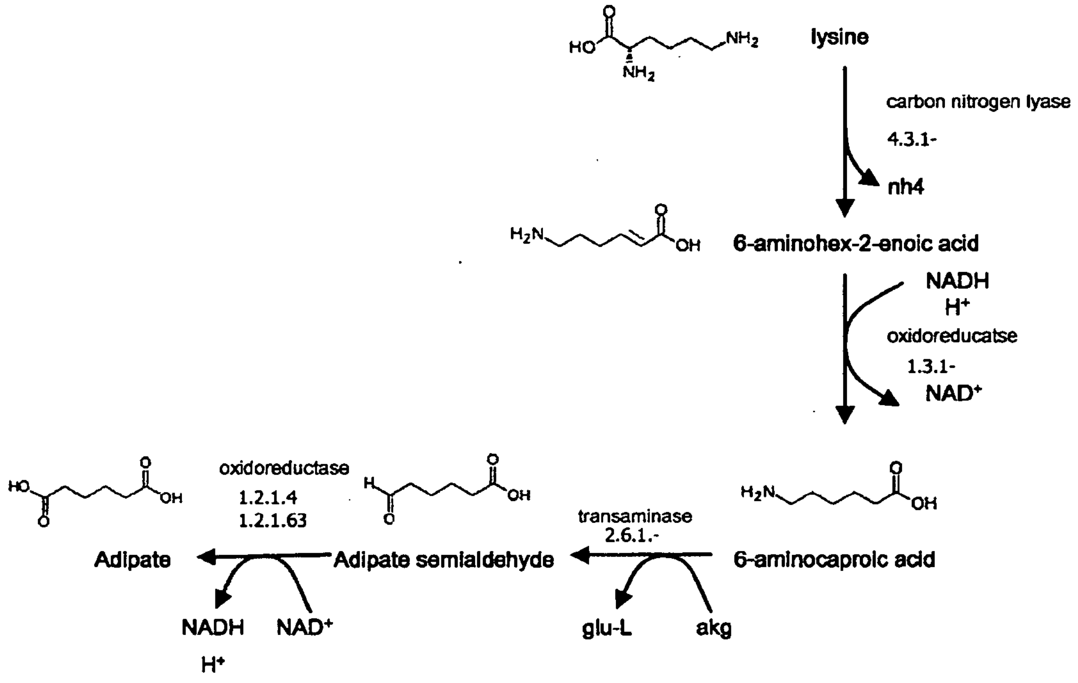
lysine
carbon nitrogen lyase
4.3.1-
nh4
6-aminohex-2-enoic acid
NADH
H+
oxidoreducatse
1.3.1-
NAD+
6-aminocaproic acid
transaminase
2.6.1.-
akg
glu-L
Adipate semialdehyde
oxidoreductase
1.2.1.4
1.2.1.63
NAD+
NADH
H+
Adipate

FIGURE 7

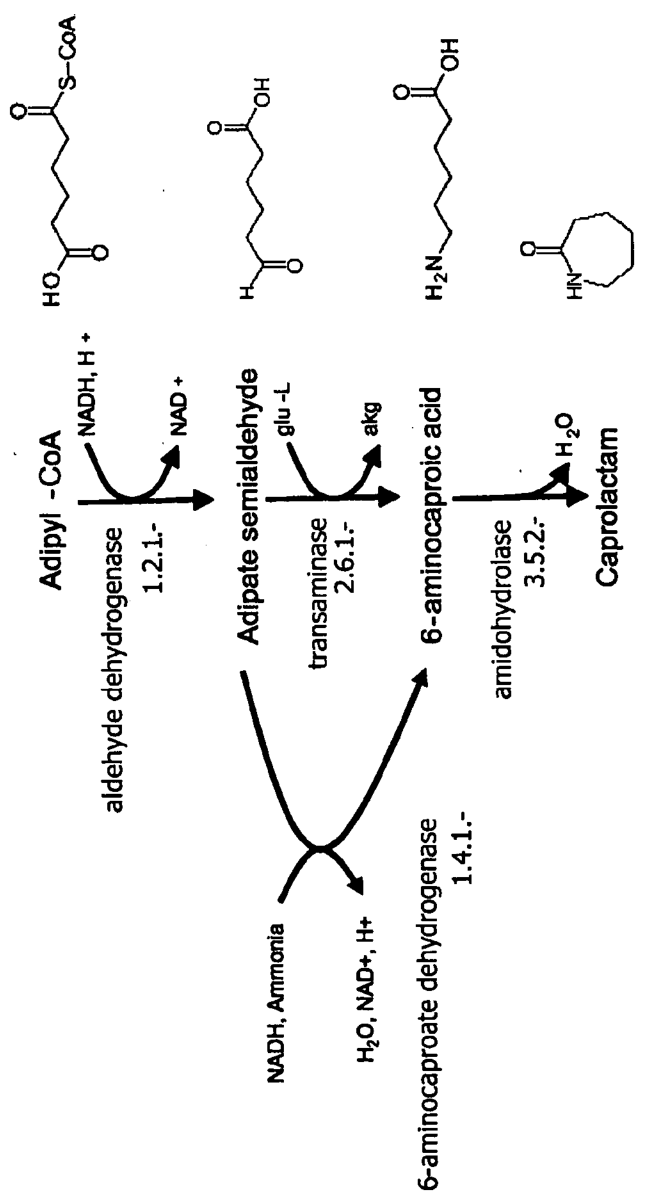
Adipyl -CoA
NADH, H +
NAD +
aldehyde dehydrogenase
1.2.1.-
Adipate semialdehyde
glu -L
akg
transaminase
2.6.1.-
6-aminocaproic acid
H2O
amidohydrolase
3.5.2.-
Caprolactam
NADH, Ammonia
H2O, NAD+, H+
6-aminocaproate dehydrogenase
1.4.1.-
HO
O
S-CoA
H
OH
H2N
HN

FIGURE 8

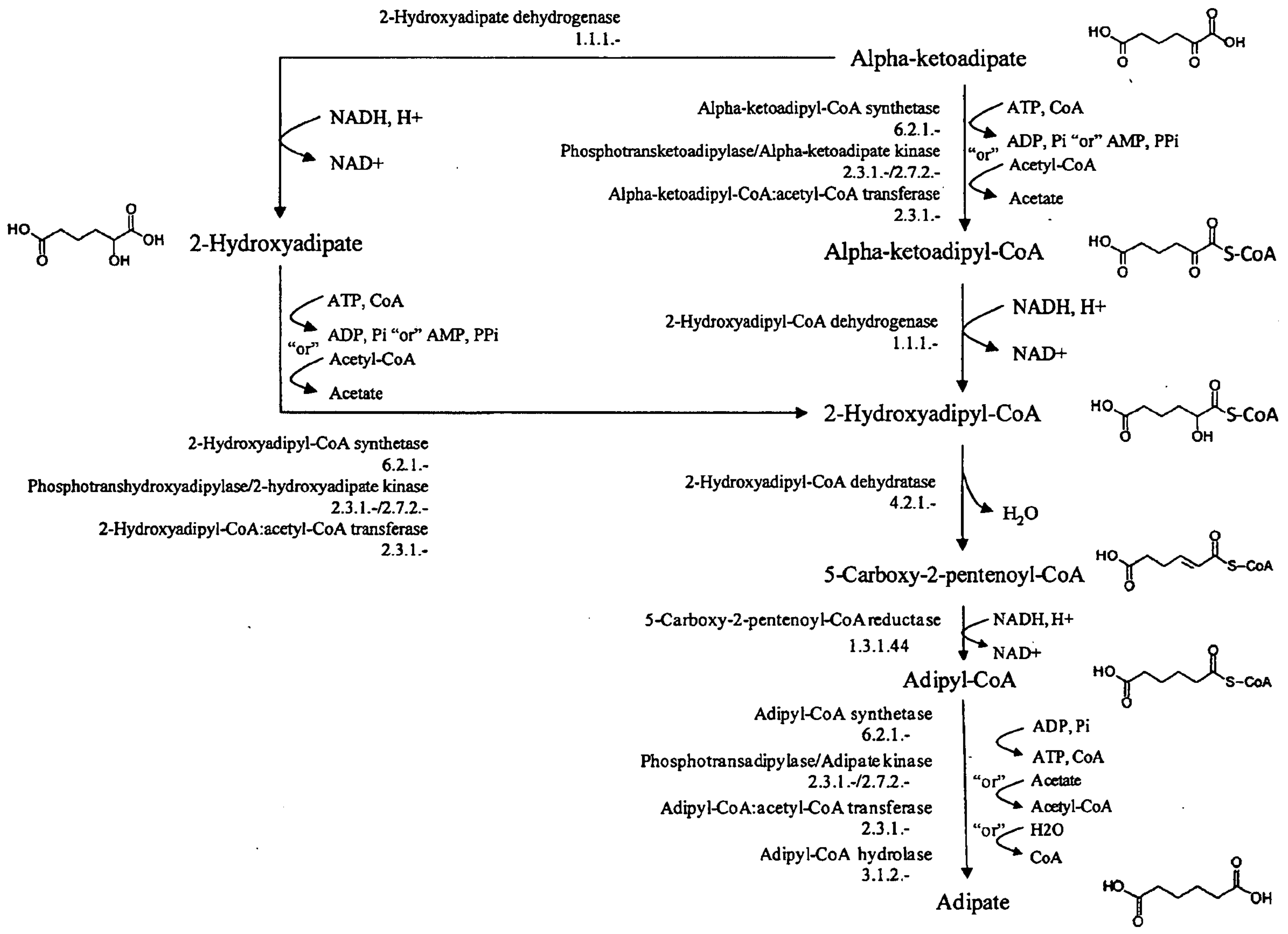
2-Hydroxyadipate dehydrogenase
1.1.1.-
Alpha-ketoadipate
NADH, H+
NAD+
2-Hydroxyadipate
ATP, CoA
ADP, Pi "or" AMP, PPi
"or"
Acetyl-CoA
Acetate
2-Hydroxyadipyl-CoA synthetase
6.2.1.-
Phosphotranshydroxyadipylase/2-hydroxyadipate kinase
2.3.1.-/2.7.2.-
2-Hydroxyadipyl-CoA:acetyl-CoA transferase
2.3.1.-
Alpha-ketoadipyl-CoA synthetase
6.2.1.-
Phosphotransketoadipylase/Alpha-ketoadipate kinase
2.3.1.-/2.7.2.-
Alpha-ketoadipyl-CoA:acetyl-CoA transferase
2.3.1.-
ATP, CoA
ADP, Pi "or" AMP, PPi
"or"
Acetyl-CoA
Acetate
Alpha-ketoadipyl-CoA
2-Hydroxyadipyl-CoA dehydrogenase
1.1.1.-
NADH, H+
NAD+
2-Hydroxyadipyl-CoA
2-Hydroxyadipyl-CoA dehydratase
4.2.1.-
H_2O
5-Carboxy-2-pentenoyl-CoA
5-Carboxy-2-pentenoyl-CoA reductase
1.3.1.44
NADH, H+
NAD+
Adipyl-CoA
Adipyl-CoA synthetase
6.2.1.-
Phosphotransadipylase/Adipate kinase
2.3.1.-/2.7.2.-
Adipyl-CoA:acetyl-CoA transferase
2.3.1.-
Adipyl-CoA hydrolase
3.1.2.-
ADP, Pi
ATP, CoA
"or"
Acetate
Acetyl-CoA
"or"
H2O
CoA
Adipate
HO
OH
O
S-CoA

FIGURE 9

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

- WO 2005068643 A **[0006]**
- US 5487987 A **[0011] [0156] [0157]**
- US 11891602 B **[0069]**
- US 89160207 A **[0079] [0087]**
- US 20020012939 A **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 20030224363 A **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 20040029149 A **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 20040072723 A **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 20030059792 A **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 20020168654 A **[0085] [0087] [0096] [0124] [0153] [0184] [0198]**
- US 20040009466 A **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 7127379 B **[0085] [0096] [0124] [0153] [0184] [0198]**
- US 0200660 W **[0087]**
- US 20030233218 A **[0088]**
- US 0318838 W **[0088]**
- US 6353100 B **[0168]**
- US 5700934 A, Wolters **[0168]**
- US 6660857 B, Agterberg **[0168]**

Non-patent literature cited in the description

- **NIU et al.** *Biotechnol. Prog.,* 2002, vol. 18, 201-211 **[0008] [0129] [0157]**
- **REED et al.** *Genome Biol.,* 2003, vol. 4, R54 **[0012]**
- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, 2001 **[0059]**
- **AUSUBEL et al.** Current Protocols in Molecular Biology. John Wiley and Sons, 1999 **[0059]**
- **HOFFMEISTER et al.** *J. Biol. Chem.,* 2005, vol. 280, 4329-4338 **[0060] [0111] [0112]**
- **LIN et al.** *Biotechnol. Bioeng.,* 2005, vol. 90, 775-779 **[0066]**
- **DRAKE.** Acetogenesis. Chapman and Hall, 1994, 3-60 **[0074]**
- **BURGARD et al.** *Biotechnol. Bioeng.,* 2003, vol. 84, 647-657 **[0086] [0095]**
- **BURGARD et al.** *Biotechnol. Prog.,* 2001, vol. 17, 791-797 **[0093]**
- **THYKAER et al.** *Melab. Eng.,* 2002, vol. 4, 151-158 **[0098]**
- **KANEHISA ; GOTO.** *Nucl. Acids Res.,* 2000, vol. 28, 27-30 **[0099]**
- **KASCHABEK et al.** *J. Bacteriol.,* 2002, vol. 184, 207-215 **[0103] [0128] [0134] [0140]**
- **OLIVERA et al.** *Proc. Natl. Acad. Sci. USA,* 1998, vol. 95, 6419-6424 **[0103] [0108] [0109]**
- **DI GENNARO et al.** *Arch. Microbiol.,* 2007, vol. 188, 117-125 **[0103] [0108] [0134]**
- **NOGALES et al.** *Microbiol.,* 2007, vol. 153, 357-365 **[0103] [0108] [0128] [0134]**
- **SATO et al.** *J. Biosci. Bioengineer.,* 2007, vol. 103, 38-44 **[0103] [0107] [0134] [0138]**
- **SLATER et al.** *J. Bacteriol.,* 1998, vol. 180, 1979-1987 **[0103] [0107] [0134] [0138]**
- **WINZER et al.** *J. Mol. Microbiol. Biotechnol.,* 2000, vol. 2, 531-541 **[0107] [0138]**
- **ATSUMI et al.** *Metab. Eng.* **[0108]**
- **BOYNTON et al.** *J. Bacteriol.,* 1996, vol. 178, 3015-3024 **[0108] [0109] [0112]**
- **ISMAIL et al.** *Eur. J. Biochem.,* 2003, vol. 270, 3047-3054 **[0108] [0109] [0128]**
- **PARK ; LEE.** *J. Bacteriol.,* 2003, vol. 185, 5391-5397 **[0109]**
- **PARK ; LEE.** *Biotechnol. Bioeng.,* 2004, vol. 86, 681-686 **[0109]**
- **PARK ; LEE.** *Appl. Biochem. Biotechnol.,* 2004, vol. 113-116, 335-346 **[0109]**
- **YANG et al.** *Biochem.,* 1991, vol. 30, 6788-6795 **[0110]**
- **YANG et al.** *J. Biol. Chem.,* 1990, vol. 265, 10424-10429 **[0110]**
- **YANG et al.** *J. Biol. Chem.,* 1991, vol. 266, 16255 **[0110]**
- **NAKAHIGASHI ; INOKUCHI.** *Nucl. Acids Res.,* 1990, vol. 18, 4937 **[0110]**
- **CAMPBELL et al.** *Mol. Microbiol.,* 2003, vol. 47, 793-805 **[0110]**
- **SATO et al.** *J. Biosci. Bioeng.,* 2007, vol. 103, 38-44 **[0110]**
- **CAMPBELL ; CRONAN.** *J. Bacteriol.,* 2002, vol. 184, 3759-3764 **[0111]**
- **MIZUGAKI et al.** *J. Biochem.,* 1982, vol. 92, 1649-1654 **[0111]**

- **NISHIMAKI et al.** *J. Biochem.,* 1984, vol. 95, 1315-1321 **[0111]**
- **TUCCI ; MARTIN.** *FEBS Lett.,* 2007, vol. 581, 1561-1566 **[0112]**
- **BUCK et al.** *Biochem.,* 1985, vol. 24, 6245-6252 **[0113]**
- **VAMECQ et al.** *Biochem. J.,* 1985, vol. 230, 683-693 **[0113]**
- **LAMAS-MACEIRAS et al.** *Biochem. J.,* 2005, vol. 395, 147-155 **[0113]**
- **WANG et al.** *Biochem. Biophy. Res. Commun.,* 2007, vol. 360, 453-458 **[0113]**
- **MARTINEZ-BLANCO et al.** *J. Biol. Chem.,* 1990, vol. 265, 7084-7090 **[0113]**
- **BOWER et al.** *J. Bacteriol.,* 1996, vol. 178, 4122-4130 **[0113]**
- **WALTER et al.** *Gene,* 1993, vol. 134, 107-111 **[0114]**
- **HUANG et al.** *J. Mol. Microbiol. Biotechnol.,* 2000, vol. 2, 33-38 **[0114]**
- **SOHLING ; GOTTSCHALK.** *J. Bacteriol.,* 1996, vol. 178, 871-880 **[0115] [0172]**
- **SEEDORF et al.** *Proc. Natl. Acad. Sci. USA,* 2008, vol. 105, 2128-2133 **[0115] [0146]**
- **NAGGERT et al.** *J. Biol. Chem,* 1991, vol. 266, 11044-11050 **[0116]**
- **WESTIN et al.** *J. Biol. Chem.,* 2005, vol. 280, 38125-38132 **[0116]**
- **DEANA.** *Biochem. Int.,* 1992, vol. 26, 767-773 **[0116]**
- **BONNER ; BLOCH.** *J. Biol. Chem.,* 1972, vol. 247, 3123-3133 **[0117]**
- **KUZNETSOVA et al.** *FEMS Microbiol. Rev.,* 2005, vol. 29, 263-279 **[0117]**
- **ZHUANG et al.** *FEBS Lett.,* 2002, vol. 516, 161-163 **[0117]**
- **SONG et al.** *J. Biol. Chem,* 2006, vol. 281, 11028-11038 **[0117]**
- **LEDUC et al.** *J. Bacteriol.,* 2007, vol. 189, 7112-7126 **[0117]**
- **BURGARD et al.** *Biotechnol. Bioengineer.,* 2003, vol. 84, 647-657 **[0124] [0153] [0184] [0198]**
- **FONG ; PALSSON.** *Nat. Genet.,* 2004, vol. 36, 1056-1058 **[0124] [0153] [0184] [0198]**
- **ALPER et al.** *Science,* 2006, vol. 314, 1565-1568 **[0124] [0153] [0198]**
- **LIN et al.** *Biotechnol. Bioeng.,* 2005, 775-779 **[0125] [0154] [0185] [0199]**
- **OLIVERA et al.** *Proc. Natl. Acad Sci. USA,* 1998, vol. 95, 6419-6424 **[0134]**
- **STEINBUCHEL ; SCHLEGEL.** *Eur. J. Biochem.,* 1983, vol. 130, 329-334 **[0142]**
- **MARKS et al.** *J. Biol. Chem.,* 1992, vol. 267, 15459-15463 **[0142]**
- **MARTINS et al.** *Proc. Natl. Acad. Sci. USA,* 2004, vol. 101, 15645-15649 **[0143]**
- **BUCKEL ; GOLDING.** *FEMS Microbiol. Rev.,* 1998, vol. 22, 523-541 **[0143]**
- **HOFMEISTER et al.** *J. Bacteriol.,* 1997, vol. 179, 4937-4941 **[0145]**
- **ALHAPEL et al.** *Proc. Natl. Acad. Sci. USA,* 2006, vol. 103, 12341-12346 **[0145]**
- **ROHDICH et al.** *J. Biol. Chem.,* 2001, vol. 276, 5779-5787 **[0146]**
- **GIESEL ; SIMON.** *Arch. Microbiol.,* 1983, vol. 135, 51-57 **[0146]**
- **NIU et al.** *Biotechnol. Prog.,* 2002, vol. 18 (2), 201-11 **[0156]**
- **SUDA et al.** *Arch. Biochem. Biophys.,* 1976, vol. 176, 610-620 **[0161] [0188]**
- **SUDA et al.** *Biochem. Biophys. Res. Commun.,* 1977, vol. 77, 586-591 **[0161] [0188]**
- **REISER ; SOMERVILLE.** *J. Bacteriol.,* 1997, vol. 179, 2969-2975 **[0172]**
- **ISHIGE et al.** *Appl. Environ. Microbiol..,* 2002, vol. 68, 1192-1195 **[0172]**
- **BARTSCH et al.** *J. Bacteriol.,* 1990, vol. 172, 7035-7042 **[0173]**
- **COOPER.** *Methods Enzymol.,* 1985, vol. 113, 80-82 **[0173]**
- **SCOTT ; JAKOBY.** *J. Biol. Chem.,* 1959, vol. 234, 932-936 **[0173]**
- **HEYDARI et al.** *Appl. Environ. Microbiol.,* 2004, vol. 70 (2), 937-942 **[0174]**
- **HASHIMOTO et al.** *J. Biochem.,* 1989, vol. 106 (1), 76-80 **[0174]**
- **MISONO et al.** *J. Biochem.,* 1989, vol. 105 (6), 1002-1008 **[0174]**
- **RULDEEKULTHAMRONG et al.** *BMB Reports,* 2008, 790-795 **[0174]**
- **FUKUMURA et al.** *FEBS Lett.,* 1978, vol. 89, 298-300 **[0175]**
- **BORONIN et al.** *FEMS Microbiol. Lett.,* 1984, vol. 22, 167-170 **[0176]**
- **KANAGAWA et al.** *J. Gen. Microbiol.,* 1993, vol. 139, 787-795 **[0177]**
- **KINOSHITA et al.** *Eur. J. Biochem.,* 1981, vol. 116, 547-551 **[0177]**
- **ALPER.** *Science,* 2006, vol. 314, 1565-1568 **[0184]**
- **ZHAO ; WINKLER.** *J. Bacteriol.,* 1996, vol. 178 (1), 232-9 **[0188]**
- **HO et al.** *J. Biol. Chem.,* 1999, vol. 274 (I), 397-402 **[0188]**
- **BUCKEL et al.** *Eur. J. Biochem.,* 1981, vol. 118 (2), 315-321 **[0189]**
- **MACK et al.** *Eur. J. Biochem.,* 1994, vol. 226 (1), 41-51 **[0189]**
- **MEGRAW et al.** *J. Bacteriol.,* 1965, vol. 90 (4), 984-988 **[0189]**
- **DUTSCHO et al.** *Eur. J. Biochem.,* 1989, vol. 181 (3), 741-746 **[0190]**
- **LOCHER et al.** *J. Mol. Biol.,* vol. 307 (1), 297-308 **[0190]**
- **MULLER ; BUCKEL.** *Eur. J. Biochem.,* 2001, vol. 230 (2), 698-704 **[0190]**
- **SCHWEIGER et al.** *Eur. J. Biochem.,* 1987, vol. 169 (2), 441-448 **[0190]**

- **HOFMEISTER ; BUCKEL.** *Eur. J. Biochem.,* 1992, vol. 206 (2), 547-552 **[0190]**
- **KUCHTA ; ABELES.** *J. Biol. Chem.,* 1985, vol. 260 (24), 13181-13189 **[0190]**

Szabadalmi igénypontok

1. Természetben elő nem forduló mikroorganizmus, amely az adipil-koenzim-A-t 6-amino-kapronsavvá átalakító bioszintézis útvonallal ("6-amino-kapronsav útvonal") és legalább egy adipil-koenzim-A-t termelő bioszintézis útvonallal ("adipil-koenzim-A útvonal") rendelkező mikroorganizmust foglal magában, ahol az említett 6-amino-kapronsav útvonalban két olyan nukleinsav vesz részt, amelyek mindegyike 6-amino-kapronsav termeléséhez megfelelő mennyiségben expresszálódó, 6-amino-kapronsav útvonalhoz tartozó enzimet kódol,

ahol az említett 6-amino-kapronsav útvonal lépéseiben koenzim-A-függő aldehid-dehidrogenáz, és transzamináz vagy 6-amino-kaproát-dehidrogenáz enzimek vesznek részt,

ahol az említett legalább egy adipil-koenzim-A útvonal, legalább egy, az adipátot adipil-koenzim-A-vá átalakító bioszintézis útvonalat, valamint szukcinil-koenzim-A-t és acetil-koenzim-A-t adipil-koenzim-A-vá átalakító bioszintézis útvonalat tartalmaz,

ahol az említett adipátot adipil-koenzim-A-vá átalakító bioszintézis útvonal lépéseiben adipil-koenzim-A szintetáz vagy foszfotranszadipiláz/adipát kináz enzimek vesznek részt, és

ahol a szukcinil-koenzim-A-t és acetil-koenzim-A-t adipil-koenzim-A-vá átalakító bioszintézis útvonal lépéseiben szukcinil-koenzim-A:acetil-koenzim-A acil-transzferáz, 3-oxoadipil-koenzim-A transzferáz, 3-oxoadipát-reduktáz, 3-hidroxiadipát-dehidratáz, 2-enoát-reduktáz, és adipil-koenzim-A szintetáz, foszfotranszadipiláz/adipát-kináz, adipil-koenzim-A:acetil-koenzim-A transzferáz vagy adipil-koenzim-A hidroláz enzimek vesznek részt.

2. Az 1. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett két exogén nukleinsav koenzim-A-függő aldehid-dehidrogenáz és transzamináz enzimeket kódol.

3. Az 1. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett két exogén nukleinsav koenzim-A-függő aldehid-dehidrogenáz és 6-aminokaproát-dehidrogenáz enzimeket kódol.

4. Az 1-3. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal az adipil-koenzim-A szintetáz enzimet tartalmazó, az adipátot adipil-koenzim-A-vá átalakító bioszintézis útvonalat tartalmaz.

5. Az 1-3. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal a foszfotranszadipiláz/adipát-kináz enzimet magában foglaló, az adipátot adipil-koenzim-A-vá átalakító bioszintézis útvonalat tartalmaz.

6. Az 1-5. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal a szukcinil-koenzim-A-t és acetil-koenzim-A-t adipil-koenzim-A-vá átalakító bioszintézis útvonalat tartalmaz, és ahol a szukcinil-koenzim-A-t és acetil-koenzim-A-t adipil-koenzim-A-vá átalakító bioszintézis útvonal lépéseiben szukcinil-koenzim-A:acetil-koenzim-A acil transzferáz, 3-hidroxiacilkoenzim-A dehidrogenáz, 3-hidroxiadipil-koenzim-A-dehidratáz és 5-karboxi-2-pentenoil-koenzim-A reduktáz enzimek vesznek részt.

7. Az 1-5. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal az adipil-koenzim-A szintetáz enzimet magában foglaló adipátot adipil-koenzim-A-vá átalakító bioszintézis útvonalat, továbbá a szukcinil-koenzim-A:acetil-koenzim-A acil transzferáz, 3-oxoadipil-koenzim-A transzferáz, 3-oxoadipát reduktáz, 3-hidroxadipát dehidratáz és 2-enoát reduktáz enzimeket magában foglaló, a szukcinil-koenzim-A-t és acetil-koenzim-A-t adipáttá ("adipát útvonal") átalakító bioszintézis útvonalat tartalmaz.

8. A 7. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipát útvonal, egy, kettő, három, négy vagy öt exogén nukleinsavat tartalmaz, amelyek mindegyike egy adipát útvonalhoz tartozó enzimet kódol.

9. A 7. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal lépéseiben az adipil-koenzim-A:acetil-koenzim-A transzferáz enzim is részt vesz.

10. A 7. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal lépéseiben adipil-koenzim-A hidroláz enzim is részt vesz.

11. Az 1-3. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett adipil-koenzim-A útvonal a szukcinil-koenzim-A-t és acetil-koenzim-A-t adipil-koenzim-A-vá átalakító bioszintézis útvonalat tartalmaz, amely egy, kettő, három, négy vagy öt olyan exogén nuleinsavat tartalmaz, amelyek mindegyike egy adipil-koenzim-A útvonal enzimet kódol.

12. Az 1-11. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett exogén nukleinsavak legalább egyike egy heterológ nukleinsav.

13. Az 1-12. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol a természetben elő nem forduló mikroorganizmus egy baktérium-, élesztő- vagy gombafaj.

14. A 13. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett baktériumot az *Escherichia coli*, *Klebsiella oxytoca*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, és *Pseudomonas putida* közül választjuk.

15. A 13. igénypont szerinti természetben elő nem forduló mikroorganizmus, ahol az említett élesztő vagy gombafajokat a *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger*, és *Pichia pastoris* közül választjuk.

16. Az 1-15. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmus, ahol az említett mikroorganizmus amidohidroláz enzimet tartalmazó kaprolaktám bioszintézis útvonalat is tartalmaz.

17. Lényegében anaerób sejttenyészet, amely 1-16. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmust tartalmaz.

18. Eljárás 6-aminokapronsav előállítására, azzal jellemezve, hogy az 1-15. igénypontok bármelyike szerinti természetben elő nem forduló mikroorganizmust a 6-aminokapronsav termeléséhez szükséges körülmények között és elegendő ideig növesztünk.

19. A 18. igénypont szerinti eljárás, azzal jellemezve, hogy a 6-aminokapronsavat el is választjuk a sejttenyészet egyéb komponenseitől.

20. A 19. igénypont szerinti eljárás, azzal jellemezve, hogy az elválasztás folyamata lehet extrakció, folytonos folyadék-folyadék extrakció, átpárologtatás, membránszűrés, fordított ozmózis, elektrodialízis, desztilláció, kristályosítás, centrifugálás, extraktív szűrés, ioncserélő kromatográfia, méretkizárásos kromatográfia, adszorpciós kromatográfia, vagy ultraszűrés.

21. Eljárás kaprolaktám előállítására, azzal jellemezve, hogy a 16. igénypont szerinti természetben elő nem forduló mikroorganizmust a kaprolaktám termeléséhez megfelelő körülmények között elegendő ideig növesztünk.

22. A 21. igénypont szerinti eljárás, azzal jellemezve, hogy a 6-aminokapronsavat el is választjuk a sejttenyészet egyéb komponenseitől.

23. A 22. igénypont szerinti eljárás, azzal jellemezve, hogy az elválasztás folyamata lehet extrakció, folytonos folyadék-folyadék extrakció, átpárologtatás, membránszűrés, fordított ozmózis, elektrodialízis, desztilláció, kristályosítás, centrifugálás, extraktív szűrés, ioncserélő kromatográfia, méretkizárásos kromatográfia, adszorpciós kromatográfia, vagy ultraszűrés.