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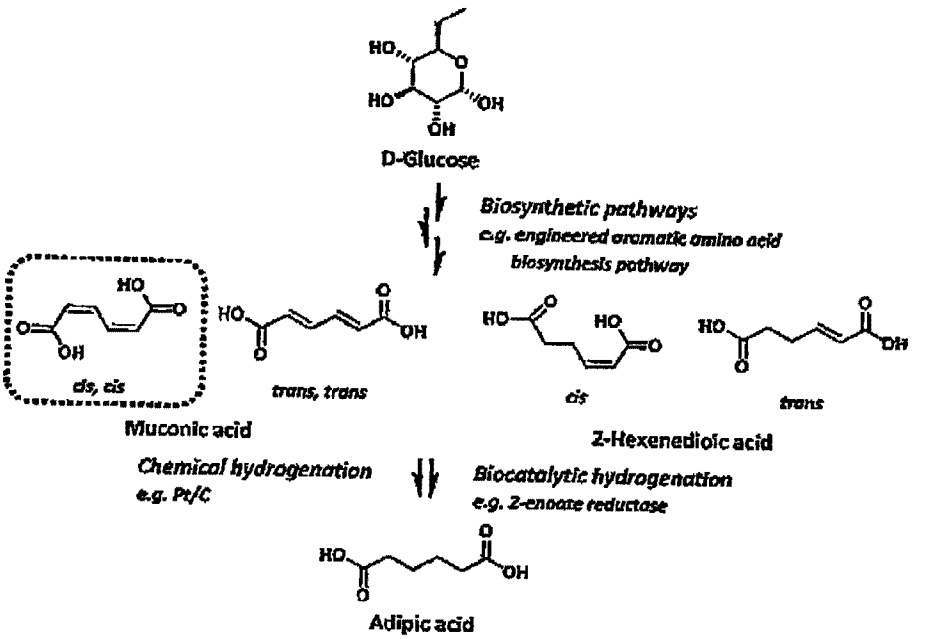

(57) Abrégé/Abstract:
A method for biosynthesis of adipic acid from six-carbon dicarboxylic acids having α, β-enoate reductase activity by treatment with an enzyme is provided. Also provided are genetically engineered microorganisms for use in the biosynthetic processes.

ABSTRACT

A method for biosynthesis of adipic acid from six-carbon dicarboxylic acids having α, β-enoate reductase activity by treatment with an enzyme is provided. Also provided are genetically engineered microorganisms for use in the biosynthetic processes.

MICROORGANISMS AND METHODS FOR BIOSYNTHESIS OF ADIPIC ACID

TECHNICAL FIELD

This disclosure relates to 2-enoate reductases and to synthesis of adipic acid.

BACKGROUND OF THE ART

Adipic acid is the most important commercial aliphatic dicarboxylic acid used for the synthesis of Nylon-6,6 polyamide (~$ 6 billion global market). 2.6 Million metric tonnes per year of adipic acid is produced from petroleum-derived benzene but known chemical processes can produce toxic by-products such as nitrous oxide (N_2O).

There is a need for sustainable production of commodity chemicals, including adipic acid, from renewable biomass instead of petroleum due to growing concerns over climate change, energy security, and human health.

BRIEF SUMMARY

In one aspect, there is provided a host microorganism in which exogenous nucleic acids are introduced, wherein the exogenous nucleic acids encode an enzyme having α,β-enoate reductase activity. In one embodiment, the enzyme comprises an amino acid sequence of SEQ ID NO: 1 or active fragment or homologue thereof. In one embodiment, the enzyme comprises amino acids (363) to (382) of SEQ ID NO: 1.

In another aspect, the enzyme comprises an amino acid sequence of SEQ ID NO: 2 or active fragment or homologue thereof. In another aspect, the enzyme comprises amino acids (363) to (382) of SEQ ID NO: 2.

In one embodiment, the enzyme having α,β-enoate reductase activity is derived from a microorganism of the genus Bacillus, Clostridium, or Moorella. In one embodiment, the microorganism is *Bacillus coagulans*.

In one embodiment, the host microorganism is *Escherichia coli*.

In another aspect, there is provided a process for producing adipic acid comprising culturing a host microorganism as described herein under conditions and for a sufficient period of time to produce adipic acid.

In another aspect, there is provided a process for producing adipic acid comprising enzymatically converting a 6-carbon unsaturated dicarboxylic acid to adipic acid using a 2-enoate reductase.

In one embodiment of the process,the enzyme comprises the amino acid sequence of SEQ ID NO:1 or 2 or is a fragment or homologue of an enzyme comprising the amino acid sequence of SEQ ID NO: 1 or 2. In one embodiment, the enzyme comprises the amino acid sequence of SEQ ID NO: 1 or a fragment or homologue thereof.

In one embodiment, the unsaturated 6-carbon dicarboxylic acid is muconic acid or 2-hexenedioic acid.

In one embodiment, the process comprises culturing a host microorganism under conditions and for a sufficient period of time to produce adipic acid.

In one embodiment, the culture medium is substantially aerobic.

In one embodiment, the 6-carbon unsaturated dicarboxylic acid is produced from biosynthetic methods.

In another aspect, there is provided a composition comprising a host microorganism and muconic acid and/or 2-hexenedioic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows chemocatalytic and biocatalytic hydrogenation of unsaturated six-carbon dicarboxylic acids into adipic acid using 2- enoate reductases (EREDs).

Figure 2 shows activity profiles of 25 Old Yellow Enzymes (OYEs; EC 1.6.99.1). The cut-off value for enzyme activity of OYE is 0.05 U/mg protein. 1 mM of substrates, 0.25 mM of NADPH, and 2.5 µg of proteins were used in the reaction mixture (potassium phosphate 100 mM and pH 7.0). A mixture of cis- and trans- isomers of 2-hexenedioic acid was used as a substrate.

Figure 3 shows SDS-PAGE analysis of the ERED expression in *E. coli* and protein purification. a) EREDCA, EREDCK, and EREDBC. b) EREDMT, EREDCL, and EREDCT. LC-MS analysis revealed that soluble proteins at 75 kDa in the E lane of EREDCT are hypothetical protein ECD_02181 (74.2 kDA, GenBank: ACT44004.1), heat

shock protein 90 (71.4 kDa, ACT42323.1), and molecular chaperone DnaK (69.1 kDa, ACT41916.1) (data not shown). M: marker, E: purified ERED, S: supernatant (soluble fraction), P: pellet (insoluble fraction).

Figure 4 shows time course of the anaerobic biotransformation of 2-hexenedioic acid into adipic acid by the *E. coli* cells expressing the recombinant EREDs. A mixture of *cis*- and *trans*- isomers of 2-hexenedioic acid was used as a substrate, and the results are means from at least two independent determinations. 20 mM 2-hexenedioic acid was used for the biotransformation.

Figure 5 shows complete biotransformation of 2-hexenedioic acid into adipic acid by the *E. coli* cells expressing the recombinant a) EREDCL and b) EREDMT. 20 mM 2-hexenedioic acid was used for the biotransformation under anaerobic conditions.

Figure 6 shows 24 h anaerobic biotransformation of *cis,cis*-muconic acid to adipic acid by the *E. coli* cells expressing the recombinant a) EREDBC, b) EREDCA, c) EREDMT, d) EREDCK and e) EREDCL. The saturated *cis,cis*-muconic acid solution (0.7 mM) was used for the biotransformation. Substrates and products were detected by LC-MS. 1: *cis,cis*-muconic acid, 2: *trans,trans*-muconic acid, 3: 2-hexenedioic acid, 4: adipic acid, 5: 3-hexenedioic acid-like compound with m/z 143.0319.

Figure 7 shows UV-visible absorption spectra of purified EREDBC and EREDCA. Purified protein was kept on ice in the presence of air (oxidation, Air lines) or 3mM NADH (reduction, NADH lines) under argon. Subsequent addition of 6 mM *trans*-cinnamic acid to the reduced protein resulted in partial re-oxidation (absorbance increase at 450 nm). The spectrophotometric cuvettes show brown-colored preparations of purified EREDBC and EREDCA used in these experiments.

Figure 8 shows *in vitro* biochemical characterization of purified EREDBC. Reductase activity of EREDBC as a function of a) 2-hexenedioic acid, b) 2-cyclohexen-1-one, c) 3-methyl-2-hexenone, and d) *trans*-cinnamic acid concentration.

Figure 9 shows *in vitro* biochemical characterization of purified EREDCA. Reductase activity of EREDCA as a function of a) *trans*-cinnamic acid concentration or b) 2-hexenedioic acid concentration in the presence of NADH.

Figure 10 shows *in vitro* biochemical characterization of purified EREDBC and EREDCA. Reductase activity as a function of 2-hexenedioic acid dissolved in a reaction mixture containing isopropanol in the presence of NADH.

Figure 11 shows *in vitro* oxygen tolerance (residual activity) of purified a) EREDBC and b) EREDCA in the presence of 21% of oxygen.

Figure 12 shows multiple sequence alignment of EREDs studied. Multiple sequence alignment was performed and visualized by Cluster Omega and ESPript 3.0, respectively.

Figure 13 shows whole-cell biotransformation of 2-hexenedioic acid (15 mM) into adipic acid by the *E. coli* cells expressing recombinant EREDBC (black) and EREDCA (white) under aerobic conditions. The cells were grown and the proteins were expressed anaerobically.

DETAILED DESCRIPTION

In one aspect, the present disclosure provides novel enzymes capable of reducing C=C bonds of unsaturated dicarboxylic acids and microorganism for producing the same.

As used herein "enzyme" includes proteins produced by a cell capable of catalyzing biochemical reactions. Further, unless context dictates otherwise, as used herein "enzyme" includes protein fragments that retain the relevant catalytic activity, and may include artificial enzymes synthesized to retain the relevant catalytic activity.

In one aspect, the present disclosure provides processes for producing cells and organisms for the biochemical synthesis of adipic acid from unsaturated six-carbon dicarboxylic acids using biocatalysts having hydrogenation activity toward α,β-enoate. In one embodiment, adipic acid can be produced from muconic acid or 2-hexenedioc acid using 2-enoate reductases as illustrated in Figure 1.

In another aspect, the present disclosure provides processes for the biochemical synthesis of adipic acid. In one embodiment, this process is performed in a substantially aerobic environment.

There have been tremendous efforts to develop sustainable production of commodity chemicals from renewable biomass instead of petroleum due to growing concerns over climate change, energy security, and human health.

Bio-based adipic acid has been produced from glucose via sequential chemical reactions i.e., oxidation of glucose into glucaric acid and hydrodeoxygenation of glucaric acid into adipic acid, which can be only catalyzed by chemical catalysts (US20100317823 A1). Bio-based production of adipic acid has been also attempted using metabolic engineering approaches. Industrial microorganisms such as *E. coli* or *Saccharomyces cerevisiae* with engineered aromatic amino acid biosynthesis pathway can produce *cis,cis*-muconic acid from glucose, which is then hydrogenated into adipic acid using chemical catalysts (see e.g. Niu, W., Draths, K.M. & Frost, J.W. Benzene-free synthesis of adipic acid. Biotechnology Progress 18, 201-211 (2002).) However, a biosynthetic pathway to produce adipic acid from 2-hexenedioic acid or muconic acid has not been experimentally demonstrated.

The present disclosure provides for the biochemical synthesis of adipic acid from unsaturated six-carbon dicarboxylic acids using biocatalysts having hydrogenation activity toward α,β-enoate. In one embodiment, adipic acid is produced from muconic acid or 2-hexenedioc acid using 2-enoate reductases (ERED; EC 1.3.1.31). However, there are several known pathways to produce muconic acid and 2-hexenedioic acid starting from common renewable feedstocks, and in one embodiment, one or more of such pathways in suitably introduced into a host microorganism.

The expression "derived from" in relation to an enzyme or (poly)peptide denotes that the enzyme or poly(peptide) was isolated from a (micro)organism or that it includes all or a biologically active part of the amino acid sequence of an enzyme or (poly)peptide isolated or characterized from such a (micro)organism.

In certain embodiments, EREDs as provided herein are derived from organisms of the genus *Bacillus*, *Clostridium* or *Moorella*. In one embodiment, the ERED is derived from the species *Clostridium acetobutylicum*. In one embodiment, the ERED is derived from the genus *Bacillus*. In one embodiment, the ERED is derived from the species *Bacillus coagulans*.

In one embodiment, the ERED comprises or consists of the amino acid of SEQ ID NO: 1 or an active fragment or a homologue thereof.

In another embodiment, the ERED comprises or consists of the amino acid of SEQ ID NO: 2 or an active fragment or a homologue thereof.

In one aspect, the enzyme is at least 80%, 85%, 90%, 95% or 100% identical to the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

In various aspects, a homologue of each enzyme refers to a protein which has an identity of at least 25%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% with the amino acid sequence of SEQ ID NO corresponding to the enzyme. Algorithms for determining sequence identity are publicly available and include e.g. BLAST available through the National Center for Biotechnology Information (NCBI). One skilled in the art can determine if the sequences are similar to a degree that indicates homology and thus similar or identical function.

A person skilled in the art can obtain a polynucleotide encoding a homologue of each enzyme by appropriately introducing substitution, deletion, insertion, and/or addition to the DNA of the enzyme which is composed of a nucleotide sequence disclosed herein, using methods such as site-specific mutagenesis (Nucleic Acid Res. 10, pp. 6487 (1982), Methods in Enzymol. 100 pp. 448 (1983), Molecular Cloning 2nd Edt., Cold Spring Harbor Laboratory Press (1989), PCR A Practical Approach IRL Press pp. 200 (1991)). The polynucleotide encoding a homologue of each enzyme can be introduced and expressed in a host to obtain the homologue.

Enzymes as described herein may be produced by a non-naturally occurring microorganism.

As used herein, the term "microorganism" is intended to mean any organism that exists as a microscopic cell and encompasses 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.

As used herein, the term "non-naturally occurring" when used in reference to a microorganism refers to a microorganism that 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.

The term "endogenous" refers to a referenced molecule or activity that originates in a host microorganism. 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 microorganism.

As used herein the term "exogenous" refers to molecules or activity that is introduced into a host microorganism. 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. In reference to expression of an encoding nucleic acid the term refers to introduction of the encoding nucleic acid in an expressible form into the microorganism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into a reference host organism. The source can be, for example, an encoding nucleic acid that expresses the activity following introduction into the host microorganism.

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 can use either or both a heterologous or homologous encoding nucleic acid.

As used herein, the term "isolated" when used in reference to a microorganism refers to an organism that is substantially free of at least one component as the referenced microorganism is found in nature. The term includes a microorganism that is removed from some or all components as it is found in its natural environment and includes substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

As used herein, the term "operably linked" refers to a linkage between one or more expression control sequences and the coding region in a polynucleotide to be expressed

in such a way that expression is achieved under conditions compatible with the expression control sequence.

In a preferred embodiment, the microorganism used in a process as described herein is a microorganism which is genetically modified so as to contain a nucleic acid molecule encoding a 2-enoate reductase as described herein.

A nucleic acid molecule encoding a 2-enoate reductase can be used alone or as part of a vector.

In one embodiment, the enzyme comprises an amino acid sequence of SEQ ID NO: 1 or active fragment or homologue thereof. In one embodiment, the enzyme comprises an amino acid sequence that is at least 80%, 85%, 90%, 95% or 100% identical to the amino acid sequence of SEQ ID NO. 1. In one aspect, the enzyme comprises amino acids (363) to (382) of SEQ ID NO: 1. In one embodiment, the enzyme comprises an amino acid sequence of SEQ ID NO: 2 or active fragment or homologue thereof. In one embodiment, the enzyme comprises an amino acid sequence that is at least 80%, 85%, 90%, 95% or 100% identical to the amino acid sequence of SEQ ID NO. 2. In another aspect, the enzyme comprises amino acids (363) to (382) of SEQ ID NO: 2.

In one embodiment, the enzyme comprises an 4Fe-4S cluster:

$CX_1SCX_2EGCMGRX_3QX_4YSX_5LX_6C$

wherein

X_1 is any amino acid, preferably a hydrophobic amino acid, preferably leucine or isoleucine, preferably leucine;

X_2 is any amino acid, preferably a polar amino acid, preferably histidine or glutamine, preferably histidine;

X_3 is any amino acid, preferably a hydrophobic amino acid preferably isoleucine or valine, preferably isoleucine;

X_4 is any amino acid, preferably glutamic acid or histidine, preferably glutamic acid;

X_5 is any amino acid, preferably a polar amino acid, preferably serine or methionine, preferably serine;

X_6 is any amino acid, preferably glycine or asparagine, preferably glycine.

In one embodiment, the enzyme comprises or substantially comprises (> 80%, > 85%, > 90%, > 95% or > 99%) of the consensus sequence as between SEQ ID NO: 1 and SEQ ID NO: 2.

In one embodiment, the enzyme is substantially aerostable, which in one embodiment comprises an enzyme that retains at least 20, at least 25 or at least 30% of its activity when stored in air for 3 days.

The nucleic acid molecules can further include expression control sequences operably linked to the polynucleotide comprised in the nucleic acid molecule. These expression control sequences may be suited to ensure transcription and synthesis of a translatable RNA in bacteria or fungi. Expression refers to the transcription of the heterologous DNA sequence, preferably into a translatable mRNA. Regulatory elements ensuring expression in bacteria, yeasts or fungi, cyanobacteria and algae are well known to those skilled in the art and encompass promoters, enhancers, termination signals, targeting signals and the like. Promoters for use in connection with the nucleic acid molecule may be homologous or heterologous with regard to its origin and/or with regard to the gene to be expressed. Suitable promoters are for instance promoters which lend themselves to constitutive expression. However, promoters which are only activated at a point in time determined by external influences can also be used. Artificial and/or chemically inducible promoters may be used.

The polynucleotide introduced into a microorganism is expressed so as to lead to the production of a polypeptide having the 2-enoate reductase activity described above.

An overview of different expression systems is for instance contained in Bitter et al. (Methods in Enzymology 153 (1987), 516-544) and in Sawers et al. (Applied Microbiology and Biotechnology 46 (1996), 1-9), Billman-Jacobe (Current Opinion in Biotechnology 7 (1996), 500-4), Hockney (Trends in Biotechnology 12 (1994), 456-463), Griffiths et al., (Methods in Molecular Biology 75 (1997), 427-440). An overview of yeast expression systems is for instance given by Hensing et al. (Antonie van Leuwenhoek 67 (1995), 261-279), Bussineau et al. (Developments in Biological Standardization 83 (1994), 13-19), Gellissen et al. (Antonie van Leuwenhoek 62 (1992), 79-93, Fleer (Current Opinion in Biotechnology 3 (1992), 486-496), Vedvick (Current Opinion in Biotechnology 2 (1991), 742-745) and Buckholz (Bio/Technology 9 (1991), 1067-1072).

Expression vectors have been widely described in the literature. Generally, they contain not only a selection marker gene and a replication-origin ensuring replication in the host selected, but also a bacterial or viral promoter, and in most cases a termination signal for transcription. Between the promoter and the termination signal there is in general at least one restriction site or a polylinker which enables the insertion of a coding DNA sequence. The DNA sequence naturally controlling the transcription of the corresponding gene can be used as the promoter sequence, if it is active in the selected host organism. However, this sequence can also be exchanged for other promoter sequences. It is possible to use promoters ensuring constitutive expression of the gene and inducible promoters which permit a deliberate control of the expression of the gene. Bacterial and viral promoter sequences possessing these properties are described in detail in the literature. Regulatory sequences for the expression in microorganisms, including *E. coli* and *S. cerevisiae*, are described in the literature known to those of skill in the art. Promoters permitting a particularly high expression of a downstream sequence are for instance the T7 promoter (Studier et al., Methods in Enzymology 185 (1990), 60-89), lacUV5, trp, trp-lacUV5 (DeBoer et al., in Rodriguez and Chamberlin (Eds), Promoters, Structure and Function; Praeger, New York, (1982), 462-481; DeBoer et al., Proc. Natl. Acad. Sci. USA (1983), 21-25), lp1, rac (Boros et al., Gene 42 (1986), 97-100).

Inducible promoters which may provide higher polypeptide yields than constitutive promoters can be used. Suitably, in one embodiment, a two-stage process is used: the host cells are first cultured under optimum conditions up to a relatively high cell density; and transcription is then induced.

Termination signals for transcription are also described in the literature.

In addition, it is possible to insert different mutations into the polynucleotides by methods well known in molecular biology (see for instance Sambrook and Russell (2001), *Molecular Cloning: A Laboratory Manual*, CSH Press, Cold Spring Harbor, N.Y., USA), enabling the synthesis of polypeptides having modified biological properties. In one embodiment, point mutations are introduced at positions at which a modification of the amino acid sequence increases the catalytic activity or stability of the enzyme.

Alternatively, the substrate preference of enzymes can also be changed using directed evolution. Enhancement in specific activity can be accomplished by using random

mutagenesis over the whole length of the protein (Sheryl B. Rubin-Pitel et al. (2007), "Chapter 3: Directed Evolution Tools in Bioproduct and Bioprocess Development" in *Bioprocessing for Value-Added Products from Renewable Resources*, S.-T. Yang, Ed., Elsevier, Amsterdam, The Netherlands.)Protein solubility can be increased by site-directed mutagenesis to make hydrophobic to hydrophilic mutations on the protein surface (Saul R. Trevino et al. Journal of Molecular Biology 366 (2007), 449-460).

Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

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.

Host microorganisms can be selected from, and the non-naturally occurring microorganisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms applicable to fermentation processes.

Host microorganisms can be selected from, and the non-naturally occurring microorganisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms may be used as a host organism. Suitable host microorganisms can be selected for example from Bacteria phylum like Proteobacteria, Firmicutes, Actinocacteria, Thermotogae, Bacteroides, Cyanobacteria. Archaea phyla Euryarchaeota, Fungi phylum Ascomycota and Zygomycota or Eukariota phylum like Chlorophyta, Dinoflagellata, Bacillariophyta, Eustigmatophyceae, Haptophyta and Heterokontophyta.

Suitable bacterial and archaeal species include:

Escherichia coli, Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, Bacillus megaterium, Bacillus brevis, Bacillus pumilus, Corynebacterium glutamicum, Zymomonas mobilis, Clostridium acetobutylicum, Clostridium butylicum, Clostridium kluyveri, Clostridium autoethanogenum, Moorella thermoacetica, Clostridium aceticum, Clostridium beijerinckii, Clostridium ljungdahlii, Clostridium saccharoperbutylacetonicum, Clostridium perfringens, Clostridium difficile, Clostridium botulinum, Clostridium tyrobutyricum, Clostridium tetanomorphum, Clostridium tetani, Clostridium propionicum,

Clostridium aminobutyricum, Clostridium subterminale, Clostridium sticklandii, Ralstonia eutropha, Mycobacterium bovis, Mycobacterium tuberculosis, Porphyromonas gingivalis, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas carboxidovorans (Oligotropha carboxidovorans), Pseudomonas stutzeri, Klebsiella pneumonia, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Gluconobacter oxydans, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Citrobacter freundii, Citrobacter amalonaticus, Acinetobacter calcoaceticus, Acinetobacter baylyi, Thermotoga maritima, Halobacterium salinarum, Serratia marcescens, Rhodospirillum rubrum, Ideonella sp., Rhodobacter capsulatus, Methylococcus capsulatus, Methylosinus trichosporium, Methylobacterium extorquens, Methylocystis GB25, Methylotrophus capsulatus, Methylomonas sp. 16a, *Pyrococcus furiosus.*

Suitable yeasts or fungi include:

Saccharomyces cerevisiae, Schizosaccharomyces pombe, Saccharomycopsis crataegensis, Kluyveromyces lactis, Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger, Pichia stipitis, Pichia pastoris, Rhizopus arrhizus, Rhizopus oryzae, Yarrowia lipolytica, Issatchenkia orientalis, Issatchenkia occidentalis, Candida lambica, Candida sorboxylosa, Candida zemplinina, Candida geochares, Pichia membranifaciens, Zygosaccharomyces kombuchaensis, Candida sorbosivorans, Candida vanderwaltii, Candida sorbophila, Zygosaccharomyces bisporus, Zygosaccharomyces lentus, Saccharomyces bayanus, Saccharomyces bulderi, Debaryomyces castellii, Candida boidinii, Candida etchellsii, Pichia jadinii, Pichia anomala, Penicillium chrysogenum, Candida tropicalis.

Suitable cyanobacteria include: *Acaryochloris marina* MBIC11017, *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, *Agmenellum quadruplicatum, Chlorobium tepidum*TLS, *Cyanothece* sp. ATCC 51142, *Gloeobacter violaceus* PCC 7421, *Microcystis aeruginosa* NIES-843, *Nostoc punctiforme* ATCC 29133, *Prochlorococcus marinus* MED4, *Prochlorococcus marinus* MIT9313, *Prochlorococcus marinus*SS120, *Prochlorococcus marinus* str. AS9601, *Prochlorococcus marinus* str. MIT 9211, *Prochlorococcus marinus* str. MIT 9215, *Prochlorococcus marinus* str. MIT 9301, *Prochlorococcus marinus* str. MIT 9303, *Prochlorococcus marinus* str. MIT 9312,

Prochlorococcus marinus str. MIT 9515, *Prochlorococcus marinus* str. NATL1A, *Prochlorococcus marinus* str. NATL2A, *Rhodopseudomonas palustris* CGA009, *Synechococcus elongatus* PCC 6301, *Synechococcus elongatus* PCC 7942, *Synechococcus* sp. CC9311, *Synechococcus* sp. CC9605, *Synechococcus* sp. CC9902, *Synechococcus* sp. JA-2-3B\'a(2-13), *Synechococcus* sp. JA-3-3Ab, *Synechococcus* sp. PCC 7002, *Synechococcus* sp. RCC307, *Synechococcussp.* WH 7803, *Synechococcus* sp. WH8102, *Synechocystis* sp. PCC 6803, *Thermosynechococcus elongatus* BP-1, *Trichodesmium erythraeum* IMS101.

Suitable algae include: *Botryococcus braunii, Chlamydomonas reinhardii, Chlorella* sp., *Crypthecodinium cohnii, Cylindrotheca* sp., *Dunaliella primolecta, Isochrysis* sp., *Monallanthus salina, Nannochloris* sp., *Nannochloropsis* sp., *Neochloris oleoabundans, Nitzschia* sp., *Phaeodactylum tricornutum, Schizochytrium* sp., *Tetraselmis sueica.*

In one embodiment, the host microorganism is an aerobic organism that can express an enzyme having α,β-enoate reductase activity in an aerobic environment. Expression of such an enzyme in an aerobic or a microaerobic environment may allow improved growth rate leading to improved productivity. Furthermore, this enzyme can be used to make chemicals from compounds that require aerobic biosynthesis pathways.

E. coli is a particularly useful host organism since it is a well characterized microorganism suitable for genetic engineering.

Methods for constructing and testing the expression levels of a non-naturally occurring adipic acid-producing host can be performed by recombinant and detection methods, with techniques which are 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). Specific methods for constructing and testing the expression levels of an adipic acid producing *E. coli* host are provided in the Examples.

When reference is made to more than one exogenous nucleic acid being included in a microorganism, it is to be understood that this refers to the referenced encoding nucleic acids or biochemical activities and not the number of separate nucleic acids introduced

into the host organism. As will be understood by those of skill in the art, such exogenous nucleic acids may be introduced into the host organism on separate nucleic acid molecules, on polycistronic nucleic acid molecules, or a combination thereof. For example, where two or more exogenous nucleic acids encoding different enzymatic activities are introduced into a host organism, the two or more exogenous nucleic acids can be introduced as a single nucleic acid, for example, on a single plasmid, on separate plasmids, can be integrated into the host chromosome at a single site or multiple sites, and still be considered as two or more exogenous nucleic acids.

As will be apparent to persons of skill in the art, depending on the host microorganism selected, nucleic acids for the pathway enzyme(s) described can be introduced into the host organism. As will be apparent to persons of skill in the art, where a host microorganism expresses the pathway gene(s), the microorganism may be engineered such that the gene encoding the enzyme is overexpressed and/or genes encoding enzymes or proteins of competing pathways may be deleted.

In one embodiment, methods are carried out with live cells. In another embodiment, the process is carried out *in vitro* with lysed cells or with partially or completely purified enzyme or with permeabilized cells. In one embodiment, the method is carried out *in vitro* and the enzyme is immobilized. Means and methods for immobilizing enzymes on different supports are well-known to the person skilled in the art. Such in vitro process can be similar to the processes described in (1) Dudley, Q.M., Karim, A.S., and Jewett, M.C. 2014. Cell-Free Metabolic Engineering: Biomanufacturing beyond the cell. Biotechnology Journal. 10:69–82., 2) Zhang Y-HP*. 2014. Production of biofuels and biochemicals by in vitro synthetic biosystems: opportunities and challenges. Biotechnology Advances, Epub, DOI: 10.1016/j.biotechadv.2014.10.009)

In one embodiment, the method is carried out in culture, with the host organism, producing the enzyme. The growth medium can include, for example, any inorganic or organic carbon source which can supply a source of carbohydrates or other precursors that the host organisms can naturally use or is engineered to use. In one embodiment, the host organism is provided with a feedstock of sugars. Such sources include, for example, sugars such as glucose, xylose, galactose, mannose, fructose and starch. Glucose can be obtained from various carbohydrate-containing sources including conventional biorenewable sources such as corn (maize), wheat, potato, cassava and

rice as well as alternative sources such as energy crops, plant biomass, agricultural wastes, forestry residues, sugar processing residues and plant-derived household wastes.

Sources of carbohydrate include renewable feedstocks and biomass, e.g. cellulosic biomass, hemicellulosic biomass and lignin feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates that can yield through biosynthetic pathways, unsaturated six-carbon dicarboxylic acids, muconic acid and 2-hexendioic acid. In addition, the enzyme can be incorporated in host microbes capable of using other substrates such as methanol, syngas, glycerol, CO2 and producing muconic acid and 2-hexenedioic acid. Other renewable feedstocks and biomass will be known to persons of skill in the art.

Biorenewable feedstock sources that may be used in accordance with the present invention include any renewable organic matter that includes a source of carbohydrates. These include, for example, grasses, trees (hardwood and softwood), vegetation and crop residues. Other sources can include, for example, organic waste materials (e.g., spent paper, green waste, municipal waste, etc.). Suitable carbohydrates, including glucose, may be isolated from biorenewable materials using methods that are known in the art. See, for example, Centi and van Santen, *Catalysis for Renewables*, Wiley-VCH, Weinheim 2007; Kamm, Gruber and Kamm, *Biorefineries-Industrial Processes and Products*, Wiley-VCH, Weinheim 2006; Shang-Tian Yang, *Bioprocessing for Value-Added Products from Renewable Resources New Technologies and Applications*, Elsevier B. V. 2007; Furia, *Starch in the Food Industry, Chapter* 8, *CRC Handbook of Food Additives* 2nd *Edition* CRC Press, 1973. See also chapters devoted to Starch, Sugar and Syrups within *Kirk-Othmer Encyclopedia of Chemical Technology* 5th *Edition*, John Wiley and Sons 2001. Processes to convert starch to glucose are also well known in the art, see, for example, Schenck, "Glucose and Glucose containing Syrups" in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH 2009. Furthermore, methods to convert cellulose to glucose are known in the art, see, for example, Centi and van Santen, *Catalysis for Renewables*, Wiley-VCH, Weinheim 2007; Kamm, Gruber and Kamm, *Biorefineries-Industrial Processes and Products*, Wiley-VCH, Weinheim 2006; Shang-Tian Yang, *Bioprocessing for Value-Added Products from Renewable Resources New Technologies and Applications*, Elsevier B. V. 2007.

In one embodiment, the processes as provided may be carried out in a fermenter.

The engineered organism can be cultivated in a variety of reactors systems, and the process can be carried out in different modes of operations. The most commonly used bioreactor is a stirred tank bioreactor or aerated fermenter. The fermenter is equipped with sterile air supply, the mixing of bubble dispersion is achieved by mechanical agitation, and the temperature may be maintained using a jacket or coil that circulates steam or cooling water. For aerated vessels, high height/diameter ratio (>3) may be chosen to increase the contact time between the bubbles and liquid phase. Other variations of bioreactors are airlift bioreactor where mixing is achieved without mechanical agitation, and packed bed or fluidized bed bioreactors which are used when the biocatalyst is immobilized.

The fermentation can be carried out in three different modes: batch, fed-batch and continuous mode. A standard batch bioreactor is considered a "closed" system. In batch mode, all the media components are added to bioreactor while ensuring the sterility. Once the medium has been prepared, the bioreactor is inoculated with an appropriate inoculum and the fermentation is allowed to proceed until the end without any changes to the medium, i.e., without feeding of any additional components. Components such as acid and/or base can, however, be added to maintain the pH, and air/oxygen can be added to maintain the dissolved oxygen levels. In batch fermentation biomass and product concentration change over time until the fermentation is complete. The cells undergo classical lag-phase, exponential growth-phase, stationary phase growth, followed by death phase.

A variation of the batch mode is fed-batch mode where the nutrients including the carbon source is added to the fermenter as the process progresses.

In addition to batch or fed-batch mode, continuous mode of fermentation can also be used. A continuous system is considered to be "open" system in contrast to the batch mode. In continuous mode, defined production medium is added continuously to the bioreactor and equal amounts of bioreactor contents are removed at the same rate. Continuous operation can be carried out in a chemostat where the vessel contents, including the cells are removed, or in a bioreactor that uses perfusion culture, which allows recycling of the viable cells back to the bioreactor, allowing high cell densities to be achieved.

The commonly used fermenter designs and different operation modes are very well-established in the literature [Biochemical Engineering Fundamentals, 2nd Ed. J. E. Bailey and D. F. Ollis, McGraw Hill, New York, 1986; Development of Sustainable Bioprocesses: Modeling and Assessment, E. Heinzle, A. P. Biwer and C. L. Cooney, John Wiley & Sons, Ltd., 2006; Bioprocess Engineering: Basic Concepts, 2nd Ed., M. L. Shuler and F. Kargi, Prentice Hall, 2001].

Batch, fed-batch or continuous fermentation procedures may be employed.

The organisms can be grown in any suitable medium for growth such as Luria-Bertani broth, Terrific broth or yeast extract-peptone-dextrose (YPD) medium. For production, depending up on the choice of the host, synthetic minimal media such as M9 minimal medium, yeast synthetic minimal medium, yeast nitrogen base, BG-11, or variations thereof can be used. A suitable minimal medium may contain at least one carbon source, at least one nitrogen source, salts, cofactors, buffers, and other components required to grow and maintain the recombinant microorganism. The carbon source can be one or more of the carbon sources described previously, the nitrogen source can be an ammonium salt or nitrate salt including but not limited to $(NH_4)2SO_4$, NH_4Cl, $(NH_4)2HPO_4$, NH_4OH, KNO_3, $NaNO_3$. The medium may be supplemented with complex or organic nitrogen sources such as urea, yeast extract, casamino acids, peptone, tryptone, soy flour, corn steep liquor, or casein hydrolysate. Additionally, the minimal medium can be supplied with trace metals including but not limited to H_3BO_3, $MnCl_2$, $ZnSO_4$, Na_2MoO_4, $CuSO_4$, $Co(NO_3)_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_3$, KI. The minimal medium may be supplemented with vitamins and/or non-vitamin compounds including but not limited to biotin, pantothanate, folic acid, inositol, nicotinic acid, p-aminobenzoic acid, pyridoxine, riboflavin, thiamine, cyanocobalamin, citric acid, ethylenediamine tetraacetic acid (EDTA), ferric ammonium citrate. The medium can be supplied by carbon dioxide either by direct sparging or in the form of $NaHCO_3$, or Na_2CO_3.

In one embodiment, the processes are conducted under substantially aerobic conditions.

As used herein the term "substantially aerobic" when used in reference to a culture or growth condition means, in one embodiment, that the amount of oxygen is equal to or greater than about 10% of saturation for dissolved oxygen in liquid media. In one embodiment, the term includes sealed chambers of liquid or solid medium maintained with an atmosphere greater than about 1% oxygen.

In one embodiment, the processes as described herein may suitably be performed at a pH range of between about 4 to about 8.

While in one embodiment, the temperature at which the methods as described herein are performed is not particularly restricted, generally, the processes as described herein may be performed at temperatures of between about 20°C and about 60°C.

A person skilled in the art will be readily able to determine an effective amount of enzyme to be used per quantity of substrate and, in one embodiment, the effective amount is not particularly restricted. The present inventors have found that an enzyme ratio of 1 µg of enzyme per 200 µg of substrate effective, although higher (e.g. 1:100) and lower (e.g. 1:1000) ratios of enzyme to substrate may be effective and an appropriate ratio can be readily determined by a person of skill in the art in relation to the specific reaction conditions.

The amount of product in the medium can be determined using methods known in the art such as High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Liquid Chromatography-Mass Spectrometry (LC-MS), Gas Chromatography-Mass Spectrometry (GC-MS).

Methods of assaying for the production of adipic acid are known to those of skill in the art and further are exemplified below. For example, product, intermediate and byproduct formation 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). Individual enzymatic activities from the exogenous DNA sequences can also be assayed using methods well known in the art.

In some embodiments, processes as disclosed herein further include purifying the product of the processes products of the methods described herein. Such methods of purification are known to those of skill in the art and include e.g. by filtration, distillation or evaporation. Isolation of compound from the fermentation broth depends on the final purity of the compound required. The separation techniques may include: centrifugation, microfiltration, ultrafiltration, nano-filtration, evaporation, crystallization, distillation, and ion-exchange. Typical downstream processing operation would include a series of processes including separation of cells using centrifugation or microfiltration, removal of additional solids in the broth using ultrafiltration, removal of salts from the broth using

nanofiltration, ion-exchange, or evaporative crystallization, and finally purification of adipic acid using distillation.

Industrial Applicability

Adipic acid has various uses as will be known to those of skill in the art. The majority of adipic acid produced is used as a monomer for the production of nylon by a polycondensation reaction with hexamethylene diamine forming 6,6-nylon. Other major applications also involve polymers: it is a monomer for production of polyurethane and its esters are used as plasticizers, especially in polyvinyl chloride (PVC).

A number of documents are referenced herein, however, it should be appreciated that any patent, publication, or other disclosure material, in whole or in part, that is reference herein is done so only to the extent that the referenced material does not conflict with definitions, statements, or other disclosure material set forth in this disclosure. As such, and to the extent necessary, the disclosure as explicitly set forth herein supersedes any conflicting material referenced herein.

It will be understood that numerous modifications thereto will appear to those skilled in the art. Accordingly, the above description and accompanying drawings should be taken as illustrative of the invention and not in a limiting sense. It will further be understood that it is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth, and as follows in the scope of the appended claims.

The embodiments of the invention described above are intended to be exemplary only. The scope of the invention is therefore intended to be limited solely by the scope of the appended claims.

Examples

The two families of flavoenzymes, Old Yellow Enzymes (OYEs; EC 1.6.99.1) and 2-enoate reductases (EREDs; EC 1.3.1.31) have been studied for biocatalytic hydrogenation of alkenes and exhibited a broad substrate specificity toward α,β-unsaturated substrates bearing an electron-withdrawing group such as aldehyde,

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ketone, and carboxylic acid. The C=C reducing activity of OYEs and EREDs from different organisms was analyzed.

Example 1 - Cloning, expression, and purification of proteins

Genes encoding the selected OYE and ERED proteins were PCR-amplified from genomic DNAs and cloned into p15Tv-Lic plasmid via a ligation-independent method as described in D. Bonsor, S. F. Butz, J. Solomons, S. Grant, I. J. S. Fairlamb, M. J. Fogg, G. Grogan, Organic and Biomolecular Chemistry 2006, 4, 1252-1260

Recombinant ERED plasmids were transformed into the *E. coli* BL21 (DE3) *ΔiscR* strain for the overexpression of iron-sulfur containing proteins (per methods described in M. K. Akhtar, P. R. Jones, Applied Microbiology and Biotechnology 2008, 78, 853-862). *E. coli* transformants were cultured aerobically at 37°C in Terrific Broth (TB) medium (1L) supplemented with 100 µg/mL ampicillin until the optical density (OD 600nm) reached 0.6-0.8. At this point cultures were transferred to tightly-closed flasks with a magnetic stir bar and protein expression was induced with 0.4 mM IPTG after a 30 min-anaerobic pre-cultivation in the closed flasks. Cultures were also incubated with DMSO (50 mM) as the final electron acceptor for 15-19 h at room temperature on a magnetic stir plate. *E. coli* cells were harvested by centrifugation (9,000g) and the cell pellets were stored in liquid N_2 protein purification. For the whole-cell biotransformation cells were used immediately. Protein purification was performed in an anaerobic glove-box (Coy Laboratory Products, Grass Lake, MI, USA) under an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. All buffers were degassed and sparged with Ar before use. Cell pellets were resuspended in lysis buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 5 mM imidazole, 0.05% Tween-20, 1 mg/mL lysozyme, 3 U/mL benzonase, 0.5 mM EDTA, and 1 mM DTT) and incubated at room temperature for 30 min. Cell lysates were cleared by centrifugation and the supernatant was incubated with Ni-affinity resin (Qiagen, Valencia, CA, USA) at 4°C for 1h. The resin was then washed with 100 mL of washing buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 30 mM imidazole, 0.5 mM EDTA, and 1 mM DTT) and eluted with elution buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 250 mM imidazole, 0.5 mM EDTA, and 1 mM DTT). Purified EREDs were frozen and stored in liquid N_2. Protein concentration was determined using Bradford assay and protein purity was evaluated on 10% SDS-PAGE gels.

Recombinant OYE plasmids were transformed into the *E. coli* BL21-Gold (DE3) strain (Stratagene, La Jolla, CA, USA). *E. coli* transformants were cultured at 37°C in TB medium supplemented with 100 μg/mL of ampicillin until the OD at 600nm reached 0.6-0.8. Protein expression was induced with 0.4 mM IPTG and the *E. coli* cells were grown overnight at 16°C. The *E. coli* cells were harvested by centrifugation (9,000g) and the pellets were resuspended in lysis buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, and 5 mM imidazole) followed by sonication to break cells. Lysates were cleared by centrifugation and the supernatant was incubated with Ni-affinity resin at 4°C for 30 min. The resin was then washed with washing buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% Glycerol, and 30 mM imidazole) and eluted with elution buffer (400 mM NaCl, 50 mM HEPES, pH 7.5, 5% Glycerol, and 250 mM imidazole). Purified OYEs were frozen in liquid N_2 and stored at −80°C.

Example 2 – Comparative Example

All results in this study are means from at least two independent determinations. Control experiments were performed in parallel to correct substrate-independent oxidation of cofactors for OYEs. All buffers and reagents used in anaerobic reactions were rigorously sparged with Ar to remove traces of oxygen before use.

OYEs do not contain an iron-sulfur cluster, but they can catalyze hydrogenation of activated alkenes by hydride transfer from NAD(P)H to FMN in a similar way to EREDs. 25 putative OYE genes (Table 1) were cloned based on the Pfam domain of the FMN oxidoreductase family PF00724. Purified OYEs were screened for hydrogenation activity against acrolein, 2-cyclohexn-1-one, *cis,cis*-muconic acid, *trans,trans*-muconic acid, and 2-hexendioic acid. Substrate specificity was measured spectrophotometrically in 96-well plates by following incubation for 20 min at room temperature in the anaerobic chamber. Buffers and reagents were also degassed and sparged with Ar. Reaction mixtures contained potassium phosphate (50 mM and pH 7.0), NaCl (10 mM), NADPH (0.25 mM), various substrates (1 mM) and protein (2.5 μg) in a final volume of 200 μL. Enzyme reactions were monitored by following the decrease in absorbance at 340 nm (ε340 nm = 6,200/M•cm) as a measure of the conversion of the cofactor NADPH to NADP+.

Table 1. The OYE proteins tested for biocatalytic hydrogenation

Protein ID	SEQ ID NO	GenBank ID	Microorganism	Activity [U/mg protein]*	
				acrolein	2-cyclohexen-1-one
BH1481	7	BAB05200.1	*Bacillus halodurans* C-125	ND	0.13
BSU2381	8	CAB14314.1	*Bacillus subtilis* subsp. subtilis str. 168	ND	0.21
CV3501	9	AAQ61162.1	*Chromobacterium violaceum* ATCC 12472	ND	0.07
EC5625	10	AAC74722.1	*Escherichia coli* K12	ND	0.18
LMO2471	11	CAD00549.1	*Listeria monocytogenes* EGD-e	ND	0.08
NE2398	12	CAD86422.1	*Nitrosomonas europaea* ATCC 19718	ND	0.19
PA2932	13	AAG06320.1	*Pseudomonas aeruginosa* PAO1	ND	0.13
PA4356	14	AAG07744.1	*Pseudomonas aeruginosa* PAO1	ND	0.15
PP0911	15	AAN66545.1	*Pseudomonas putida* KT2440	ND	0.15
PP1244	16	AAN66878.1	*Pseudomonas putida* KT2440	0.06	0.25
PP1466	17	AAN67100.1	*Pseudomonas putida* KT2440	ND	0.14
PS1143	18	AAO54700.1	*Pseudomonas syringae* pv. tomato str. DC3000	ND	0.18
PS2358	19	AAO55915.1	*Pseudomonas syringae* pv. tomato str. DC3000	ND	0.13
PS4251	20	AAO57808.1	*Pseudomonas syringae* pv. tomato str. DC3000	ND	0.09
PSPH1060	21	AAZ36642.1	*Pseudomonas syringae* pv. phaseolicola 1448A	ND	0.14
PSPH1370	22	AAZ33328.1	*Pseudomonas syringae* pv. phaseolicola 1448A	ND	0.18
PSPH4058	23	AAZ35385.1	*Pseudomonas syringae* phaseolicola 1448A	ND	0.22

RHA03305	24	ABG95009.1	*Rhodococcus* sp. RHA1	0.08	ND
RHA09668	25	ABG99960.1	*Rhodococcus* sp. RHA1	ND	ND
SA0956	26	BAB57118.1	*Staphylococcus aureus* subsp. aureus Mu50	ND	ND
SAV1511	27	BAC69220.1	*Streptomyces avermitilis* MA-4680	0.05	0.23
SM4273	28	AAK65532.1	*Sinorhizobium meliloti* 1021	ND	ND
SO4153	29	AAN57126.1	*Shewanella oneidensis* MR-1	ND	ND
XCC0307	30	AAM39626.1	*Xanthomonas campestris* pv. campestris str. ATCC 33913	ND	0.15
YST4373	31	AAA83386.1	*Saccharomyces cerevisiae*	0.17	0.23

* The cut-off value for enzyme activity of OYE is 0.05 U/mg protein. ND = not detected.

25 putative OYEs were overexpressed in *E. coli* and purified using Ni-NTA resin. OYE activity of purified proteins was tested with typical substrates of OYEs, i.e., acrolein and 2-cyclohexen-1-one, and 21 out of 25 proteins showed OYE activity (Table 1). However, no purified OYEs could hydrogenate 2-hexenedioic acid or muconic acid. Similarly, whole-cell biotransformations with three *E.coli* strains over-expressing OYEs: EC5625 (SEQ ID NO: 10) from *E. coli* (, YST4373 (SEQ ID NO: 31) from *S. cerevisiae*, and BSU2381 (SEQ ID NO: 8) from *Bacillus subtillis*, revealed no hydrogenation of 2-hexenedioic acid into adipic acid. This results are consistent with previous reports that OYEs cannot easily reduce α,β-unsaturated carboxylic acids without additional electron-withdrawing groups such as second acid- or -ester, a halogen or nitrile.

Example 3 - Enzyme screening: ERED family (EC 1.3.1.31)

All results in this study are means from at least two independent determinations. Control experiments were performed in parallel to correct substrate-independent oxidation of cofactors and EREDs. All buffers and reagents used in anaerobic reactions were rigorously sparged with Ar to remove traces of oxygen before use.

The C=C reducing activity of EREDs was examined. Enzyme activity of purified EREDs was measured spectrophotometrically in 96-well plates by following incubation for 3-5 min at 30°C in the anaerobic chamber as described in M. Bühler, H. Simon, Hoppe-Seyler's Zeitschrift fur Physiologische Chemie 1982, 363, 609-625. Buffers and reagents were degassed and sparged with Ar. Reaction mixture contained potassium phosphate (100 mM and pH 7.0), NADH (0.5

mM), indicated substrates, and proteins (0.1-50 µg) in a final volume of 200 µL. Enzyme reactions were monitored by following the decrease in absorbance at 340 nm (ϵ340 nm = 6,220/M•cm) due to oxidation of NADH to NAD+.

It is known that ERED from *C. tyrobutyricum* (EREDCT, SEQ ID NO: 5 CAA71086.1) exhibits a broad substrate specificity toward α,β-unsaturated carboxylates in vivo. (Buhler and Simon 1982) EREDCT shows 50 ~ 82 % sequence identity to EREDs (Figure 12) from *Bacillus coagulans* 36D1 (EREDBC, SEQ ID NO: 1), *C. acetobutylicum* (EREDCA, SEQ ID NO: 2), C. kluveri (EREDCK, SEQ ID NO: 3), *C. ljungdahlii* (EREDCL, SEQ ID NO: 4) and *Moorella thermoacetica* (EREDMT, SEQ ID NO: 6). Whole-cell anaerobic biotransformation of 2-hexenedioic acid (20mM) using these EREDs expressed in *E.coli* BL21 (DE3) *ΔiscR* (Figure 3) revealed production of adipic acid by all EREDs (except EREDCT) (Figure 4). EREDBC, EREDCA and EREDCK catalyzed a complete conversion of 2-hexenedioic acid to adipic acid within 3hr, while EREDMT and EREDCL showed complete hydrogenation of 2-hexenedioic acid after 6 and 48h, respectively (Figure 5) The inactivity of EREDCT can be attributed to its low expression in *E. coli* (Figure 3b), which can be improved using the established methods of codon optimization for recombinant protein expression in *E. coli*. Although the studied EREDCT is known to be an oxygen sensitive enzyme (Bühler, M. & Simon, H. On the kinetics and mechanism of enoate reductase. *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* **363**, 609-625 (1982)), for anaerobically grown EREDBC (SEQ ID NO: 1) and EREDCA (SEQ ID NO: 2) aerobic biotransformation of 2-hexenedioic acid (15 mM) was performed. All used buffers and solvents were prepared and stored under aerobic conditions. Under aerobic incubation conditions, both cultures catalyzed full conversion of 2-hexenedioic acid to adipic acid within 20 minutes (Figure 13). This result can be explained by high cell density used for biotransformation (average ~ 100 mg of dry cell biomass per ml) and *E.coli* high respiration rates in *E. coli* cells maintaining anaerobic conditions inside the cells.

Table 2. The ERED proteins tested for biocatalytic hydrogenation

Protein ID	GenBank ID	Microorganism
EREDBC (SEQ ID NO: 1)	AEO99944.1	*Bacillus coagulans* 36D1
EREDCA (SEQ ID NO: 2)	AEI32805.1	*Clostridium acetobutylicum* DSM 1731
EREDCK (SEQ ID NO: 3)	EDK32796.1	*Clostridium kluyveri* DSM 555

EREDCL (SEQ ID NO: 4)	ADK16394.1	*Clostridium ljungdahlii* DSM 13528
EREDCT (SEQ ID NO: 5)	CAA71086.1	*Clostridium tyrobutyricum*
EREDMT (SEQ ID NO: 6)	ABC20352.1	*Moorella thermoacetica* ATCC 39073

Whole-cell biotransformation of *cis,cis*- and *trans,trans*-isomers of muconic acid (0.7 mM) revealed that EREDBC, EREDCA, and EREDMT also hydrogenated these substrates to adipic acid. After a 24-hr incubation, no muconic acid or 2-hexenedioic acid were detected in the culture, and a 99% yield of adipic acid was obtained (Figures 6a, 6b, and 6c). EREDCK and EREDCL also exhibited a 99% yield of adipic acid from *trans,trans*-muconic acid, but a lower yield (< 35%) from the *cis,cis*-isomer (Figures 6d and 6e). Interestingly, with the latter substrate these EREDs produced three additional products identified as *trans,trans*-muconic acid, 2-hexenedioic acid, and 3-hexenedioic acid-like compound with m/z 143.0319 (Figures 6d and 6e). The formation of *trans,trans*-muconic acid from a *cis,cis*-isomer suggests the presence of *cis-trans* isomerase activity in EREDCK and EREDCL. The 3-hexenedioic acid-like compound appears to be an inefficient substrate for these EREDS. This is similar to the mutated 2,4-dienoyl-CoA reductases from *E. coli*, which catalyze hydrogenation of 2,4-dienoyl CoA into 3-enoyl CoA (Tu, X., Hubbard, P.A., Kim, J.J.P. & Schulz, H. Two distinct proton donors at the active site of Escherichia coli 2,4-dienoyl-CoA reductase are responsible for the formation of different products. *Biochemistry* **47**, 1167-1175 (2008).). Thus, the results indicate that the studied EREDs can catalyze the sequential hydrogenations of two C=C bonds of the six-carbon dicarboxylic acids, but appear to have different isomeric preferences.

Example 4 - Biochemical characterization of EREDCA and EREDBC

Anaerobic over-expression and affinity purification of six cloned EREDs produced significant amounts of soluble protein for EREDBC, EREDCA, and EREDCK (> 5 mg/L), whereas the other three EREDs showed lower expression (Figure 3). Purified EREDBC and EREDCA exhibited a brown colour in solution and an absorption spectrum with a shoulder at 380 nm and flavin-like maximum at 450 nm (Figure 7). Both the brown colour of purified EREDBC and EREDCA and the 380 nm shoulder in its absorption spectrum suggest the presence of a functional [4Fe-4S] cluster. Our spectral studies revealed that purified EREDBC and EREDCA can be completely

oxidized by oxygen (in the presence of air), reduced with an excess of NADH (3 mM), and then partially re-oxidized by substrate addition (i.e. 6 mM *trans*-cinnamic acid).

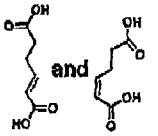
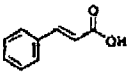
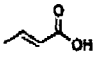
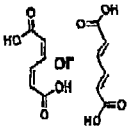
For *in vitro* biochemical characterization, enzyme activities of purified EREDs were measured with 2-hexenedioic acid, cis-cis-muconic acid, trans-trans muconic acid, *trans*-cinnamic acid, *trans*-2-methyl-2-butenoic acid, *trans*-2-butenoic acid, acrolein, 2-cyclohexen-1-one, and 3-methyl-2-cyclohexenone. Purified EREDBC and EREDCA showed NADH-dependent reductase activity, while the other purified EREDs were inactive with the tested substrates, probably , due to the loss of the iron-sulfur cluster, or flavin cofactors, or different substrate preference. [Feng, J. et al. Discovery and Characterization of BlsE, a Radical S-Adenosyl-L-methionine Decarboxylase Involved in the Blasticidin S Biosynthetic Pathway. *PLoS ONE* 8 (2013).]. In the EREDBC and EREDCA spectra shown in Figure 7, the peaks for the associated flavine and FeS cluster cofactors are clearly visible, which can help to differentiate between the active and inactive enzymes (while purifying).

Enzymatic assays under anaerobic conditions revealed that purified EREDCA has high hydrogenation activity against *trans*-cinnamic acid (3.5 U/mg protein) and low activity with *trans*-2-butenoic acid (0.008 U/mg protein). The kinetic constants (k_{cat} and K_m) for *trans*-cinnamic acid were 5.2 s^{-1} and 0.17 mM respectively, and its specific activity was comparable to that of cinnamate reductase from *C. sporogenes* (5 U/mg protein). EREDCA exhibited substrate inhibition by *trans*-cinnamic acid at substrate concentrations higher than 0.5 mM with an inhibition constant (K_i) of 1.2 mM (Figure 9a). Purified EREDCA was also active against 2-hexenedioic acid (up to 0.05U/mg protein), but showed no saturation by this substrate in the concentration range from 4 to 28 mM (Figure 9b).

Purified EREDBC exhibited significant reductase activity against 2-hexenedioic acid (0.09 U/mg protein), 2-cyclohexen-1-one (0.08 U/mg protein), 3-methyl-2-cyclohexenone (0.13 U/mg protein), and *trans*-cinnamic acid (0.39 U/mg protein) and detectable activity against *trans*-2-butenoic acid (crotonic acid, 0.036 U/mg protein) and acrolein (0.037 U/mg protein). EREDBC had a broad substrate specificity compared to EREDCA (**Table 3**). A biocompatible palladium catalyst was proposed for non-enzymatic hydrogenation of alkene metabolites in growth media including cinnamic acid variants and dicarboxylic acids[9]. EREDBC exhibited a comparable substrate spectrum to that of the palladium catalyst (Sirasani, G., Tong, L. & Balskus, E.P. A biocompatible alkene hydrogenation merges organic synthesis with microbial metabolism.

Angew. Chem. Int. Ed. (2014), suggesting EREDBC can be a good substitute for alkene hydrogenation in microbial production of biochemicals.

Table 3. *In vitro* C=C reductase activity of EREDBC against unsaturated carbonyl substrates.

Substrates		Activity [U/mg protein]
2-Hexenedioic acid[a]		
[29 mM]		0.09 ± 0.02
[35 mM]		2.3 ± 0.04
trans-Cinnamic acid [1.0 mM][b]		0.39 ± 0.001
3-Methyl-2-cyclohexenone [200 mM]		0.13 ± 0.03
2-Cyclohexen-1-one [200 mM]		0.08 ± 0.03
Acrolein [15 mM]		0.036 ± 0.005
trans-2-Butenoic acid [50 mM]		0.037 ± 0.010
Muconic acid [0.7 mM]		
cis,cis-isomer		ND[c]
trans,trans-isomer		ND[c]

[a] 2-Hexendioic acid was dissolved in aqueous buffer (29 mM) or in 0.25 mM isopropanol (35 mM).

[b] *trans*-Cinnamic acid titrated with 1N NaOH was used for enzyme assay.

[c] ND: not detected.

Kinetic studies of purified EREDBC were performed using 2-hexenedioic acid, 2-cyclohexen-1-one, 3-methyl-2-cyclohexenone, and *trans*-cinnamic acid as substrates (Figure 8). EREDBC showed no saturation by 2-hexenedioic acid, 2-cyclohexen-1-one, and 3-methyl-2-cyclohexenone, but saturation kinetics was observed for *trans*-cinnamic acid (Figure 8d). The kinetic constants (k_{cat} and K_m) for *trans*-cinnamic acid were 0.50 s^{-1} and 0.51 mM, respectively. EREDBC also exhibited moderate substrate inhibition by *trans*-cinnamic acid at substrate concentrations higher than 1.0 mM with the inhibition constant K_i 1.4 mM. In contrast to *trans*-cinnamic acid, EREDBC showed a sigmoid-like profile for 2-hexenedioic acid dissolved in aqueous buffer solution but no saturation was observed despite its significant *in vitro* activity (0.09 U/mg protein) (Figure 8a), which might be due to limited solubility of 2-hexenedioic acid in aqueous solutions.

To increase the dissolved substrate concentration, 2-hexenedioic acid was dissolved in a reaction mixture containing 0.25 mM (final concentration) isopropanol, and kinetic constants of EREDBC and EREDCA were determined. Both ERED enzymes showed sigmoidal kinetics in the concentration range from 2.5 to 35 mM (Figure 10). Compared to EREDCA, EREDBC exhibited a higher turnover rate (k_{cat}, 1.86 vs. 0.138 s^{-1}) and similar affinity (K_m, 18.9 vs. 20.47 mM) for 2-hexenedioic acid, resulting in a 14.6-fold higher catalytic efficiency of EREDBC compared to EREDCA (k_{cat}/K_m, 0.984 vs. 0.0674 $s^{-1}mM^{-1}$).

Example 5 – Oxygen Tolerance

It is known that EREDs are oxygen-sensitive enzymes, which are rapidly inactivated by oxygen due to the presence of an oxygen sensitive [4Fe-4S] iron-sulfur cluster coordinated by four cysteine residues in strictly conserved motif C-2X-C-3X-C-11X-C (Figure 12) [Gall, M. et al. Enzymatic conversion of flavonoids using bacterial chalcone isomerase and enoate reductase. Angewandte Chemie International Edition 53, 1439-1442 (2014).]. However, in our experiments purified EREDBC exhibited significant resistance to inactivation by oxygen, whereas EREDCA was inactivated only after two days of incubation under air (Figure 11).

Purified EREDs were placed in 5 mL glass tubes tightly capped with air-tight rubber stoppers. Anaerobic tubes were filled with anaerobic gas mixture (80% N_2, 10% H_2, and 10% CO_2) and aerobic tubes were filled with atmospheric air. The tubes were kept on ice for a week and a small aliquots of EREDs (5ul) were withdrawn by a syringe every day to measure residual

activity in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM NADH, indicated substrates (0.25 mM 2-hexenedioic acid for EREDBC and 0.75 mM cinnamic acid for EREDCA, and protein (1 µg) in a final volume of 200 µL.

After three days of storage in the absence of oxygen at 4°C, both EREDBC and EREDCA retained 35 and 19% of initial activity, respectively. In the presence of air (21% oxygen), EREDBC showed significant oxygen tolerance (i.e. no oxygen inactivation after retaining 33% of initial activity after three days of storage) (Figure 11a). In contrast, EREDCA exhibited 30% residual activity after one day of storage and then, was completely inactivated by oxygen after two additional days of storage (Figure 11b). The oxygen tolerance of EREDBC and EREDCA may be associated with restricted access of oxygen to its [4Fe-4S]. [Jervis, A.J. et al. The O_2 sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion. Proceedings of the National Academy of Sciences of the United States of America 106, 4659-4664 (2009).].

In some aspects, described herein is one or more of the following items:

1. An enzyme having 2-enoate reductase activity for use in enzymatically converting a 6-carbon unsaturated dicarboxylic acid to adipic acid, the enzyme comprising an amino acid sequence that is at least 85% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**, or a fragment thereof having 2-enoate reductase activity.
2. The enzyme for use of item 1, wherein the enzyme comprises an amino acid sequence that is at least 90% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**.
3. The enzyme for use of item 1, wherein the enzyme comprises an amino acid sequence that is at least 95% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**.
4. The enzyme for use of item 1, wherein the enzyme comprises an amino acid sequence that is at least 99% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**.
5. The enzyme for use of any one of items 1 to 4, wherein the enzyme comprises the 4Fe-4S cluster defined by amino acid residues 363 to 382 of **SEQ ID NO: 1 or 2**.
6. The enzyme for use of any one of items 1 to 5, wherein the enzyme is from a microorganism of the genus *Bacillus*, or *Clostridium*.
7. The enzyme for use of item 6, wherein the enzyme is from *Bacillus coagulans* or *Clostridium acetobutylicum*.
8. An enzyme having 2-enoate reductase activity for use in enzymatically converting a 6-carbon unsaturated dicarboxylic acid to adipic acid, wherein the enzyme is a variant of a *Bacillus coagulans* 2-enoate reductase (EREDBC) or a variant of a *Clostridium acetobutylicum* 2-enoate reductase (EREDCA), wherein the EREDBC comprises an amino acid sequence at least 95% identical to the amino acid sequence of **SEQ ID NO: 1** and the EREDCA comprises an amino acid sequence at least 95% identical to the amino acid sequence of **SEQ ID NO: 2**.
9. The enzyme for use of any one of items 1 to 8, wherein the unsaturated 6-carbon dicarboxylic acid is muconic acid.
10. The enzyme for use of any one of items 1 to 8, wherein the unsaturated 6-carbon dicarboxylic acid is 2 hexenedioic acid.
11. A microorganism for use in the production of adipic acid, as a product or an intermediate, the microorganism expressing the enzyme having 2-enoate reductase activity as defined in any one of items 1 to 8.
12. The microorganism for use of item 11, wherein the microorganism is bacteria.
13. The microorganism for use of item 11, wherein the microorganism is *Escherichia coli*.

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14. A process for producing adipic acid, as a product or an intermediate, the process comprising providing the enzyme as defined in any one of items 1 to 8; and contacting the enzyme with a 6-carbon unsaturated dicarboxylic acid substrate under conditions and for a sufficient period of time to enable enzymatic conversion of the 6-carbon unsaturated dicarboxylic acid substrate to adipic acid.
15. A process for producing adipic acid, as a product or an intermediate, the process comprising providing the microorganism as defined in any one of items 11 to 13; and culturing the microorganism in the presence of a 6-carbon unsaturated dicarboxylic acid substrate under conditions and for a sufficient period of time to enable the enzymatic conversion of the 6-carbon unsaturated dicarboxylic acid substrate to adipic acid.
16. The process of item 15, wherein the microorganism is cultured under conditions and for a sufficient period of time to produce adipic acid in substantially aerobic culture medium.
17. The process of any one of items 14 to 16, wherein the unsaturated 6-carbon dicarboxylic acid is muconic acid.
18. The process of any one of items 14 or 16, wherein the unsaturated 6-carbon dicarboxylic acid is hexenedioic acid.
19. A composition comprising: the enzyme as defined in any one of items 1 to 8 or the microorganism as defined in any one of items 11 to 13; and a substrate for conversion to adipic acid, the substrate comprising muconic acid and/or 2-hexenedioic acid.

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CLAIMS

1. An enzyme having 2-enoate reductase activity for use in enzymatically converting a 6-carbon unsaturated dicarboxylic acid to adipic acid, the enzyme comprising an amino acid sequence that is at least 85% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**, or a fragment thereof having 2-enoate reductase activity.

2. The enzyme for use of claim 1, wherein the enzyme comprises an amino acid sequence that is at least 90% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**.

3. The enzyme for use of claim 1, wherein the enzyme comprises an amino acid sequence that is at least 95% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**.

4. The enzyme for use of claim 1, wherein the enzyme comprises an amino acid sequence that is at least 99% identical overall to the amino acid sequence of **SEQ ID NO: 1 or 2**.

5. The enzyme for use of any one of claims 1 to 4, wherein the enzyme comprises the 4Fe-4S cluster defined by amino acid residues 363 to 382 of **SEQ ID NO: 1 or 2**.

6. The enzyme for use of any one of claims 1 to 5, wherein the enzyme is from a microorganism of the genus *Bacillus*, or *Clostridium*.

7. The enzyme for use of claim 6, wherein the enzyme is from *Bacillus coagulans* or *Clostridium acetobutylicum*.

8. An enzyme having 2-enoate reductase activity for use in enzymatically converting a 6-carbon unsaturated dicarboxylic acid to adipic acid, wherein the enzyme is a variant of a *Bacillus coagulans* 2-enoate reductase (EREDBC) or a variant of a *Clostridium acetobutylicum* 2-enoate reductase (EREDCA), wherein the EREDBC comprises an amino acid sequence at least 95% identical to the amino acid sequence of **SEQ ID NO: 1** and the EREDCA comprises an amino acid sequence at least 95% identical to the amino acid sequence of **SEQ ID NO: 2**.

9. The enzyme for use of any one of claims 1 to 8, wherein the unsaturated 6-carbon dicarboxylic acid is muconic acid.

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10. The enzyme for use of any one of claims 1 to 8, wherein the unsaturated 6-carbon dicarboxylic acid is 2 hexenedioic acid.

11. A microorganism for use in the production of adipic acid, as a product or an intermediate, the microorganism expressing the enzyme having 2-enoate reductase activity as defined in any one of claims 1 to 8.

12. The microorganism for use of claim 11, wherein the microorganism is bacteria.

13. The microorganism for use of claim 11, wherein the microorganism is *Escherichia coli*.

14. A process for producing adipic acid, as a product or an intermediate, the process comprising providing the enzyme as defined in any one of claims 1 to 8; and contacting the enzyme with a 6-carbon unsaturated dicarboxylic acid substrate under conditions and for a sufficient period of time to enable enzymatic conversion of the 6-carbon unsaturated dicarboxylic acid substrate to adipic acid.

15. A process for producing adipic acid, as a product or an intermediate, the process comprising providing the microorganism as defined in any one of claims 11 to 13; and culturing the microorganism in the presence of a 6-carbon unsaturated dicarboxylic acid substrate under conditions and for a sufficient period of time to enable the enzymatic conversion of the 6-carbon unsaturated dicarboxylic acid substrate to adipic acid.

16. The process of claim 15, wherein the microorganism is cultured under conditions and for a sufficient period of time to produce adipic acid in substantially aerobic culture medium.

17. The process of any one of claims 14 to 16, wherein the unsaturated 6-carbon dicarboxylic acid is muconic acid.

18. The process of any one of claims 14 or 16, wherein the unsaturated 6-carbon dicarboxylic acid is hexenedioic acid.

19. A composition comprising: the enzyme as defined in any one of claims 1 to 8 or the microorganism as defined in any one of claims 11 to 13; and a substrate for conversion to adipic acid, the substrate comprising muconic acid and/or 2-hexenedioic acid.

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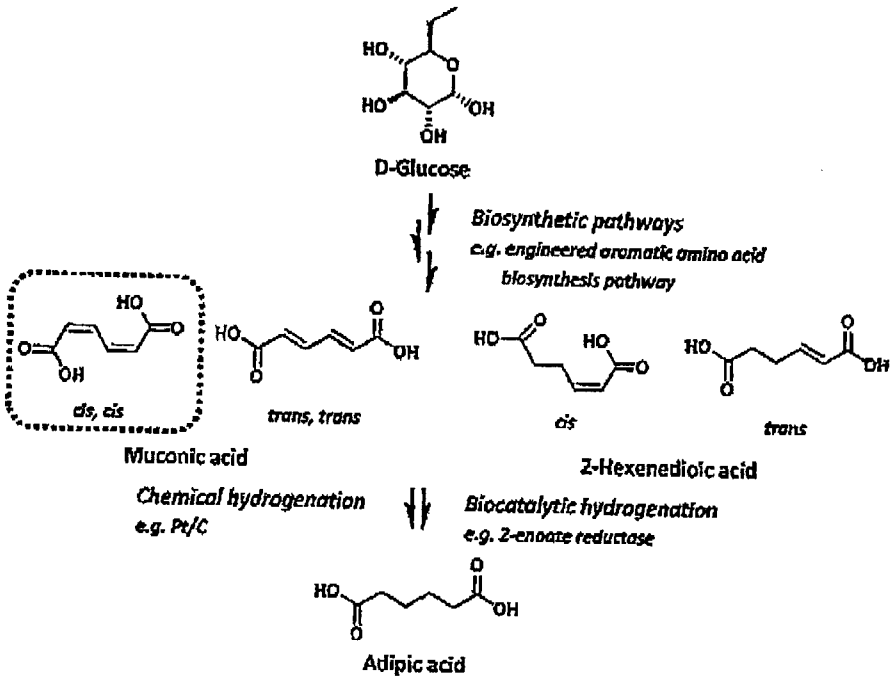

D-Glucose
Biosynthetic pathways
e.g. engineered aromatic amino acid biosynthesis pathway
cis, cis
trans, trans
Muconic acid
cis
trans
2-Hexenedioic acid
Chemical hydrogenation
e.g. Pt/C
Biocatalytic hydrogenation
e.g. 2-enoate reductase
Adipic acid

FIG. 1

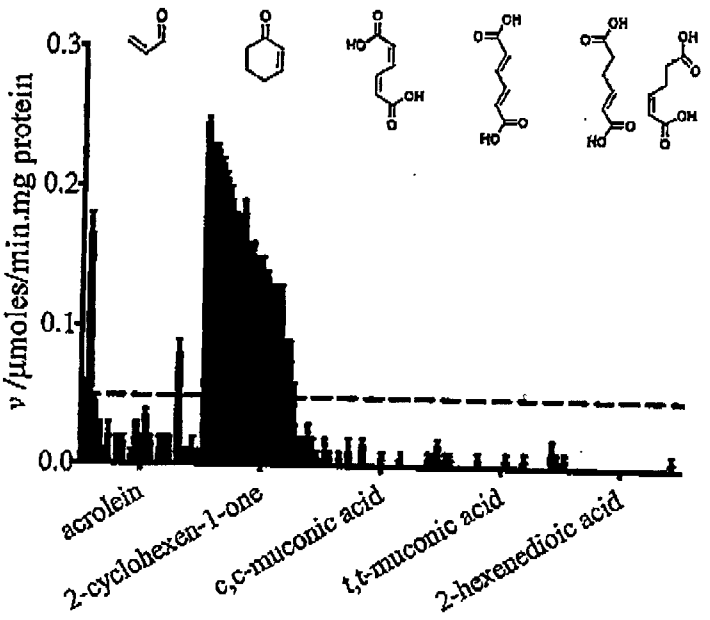

0.3
0.2
0.1
0.0
v /µmoles/min.mg protein
acrolein
2-cyclohexen-1-one
c,c-muconic acid
t,t-muconic acid
2-hexenedioic acid

FIG. 2

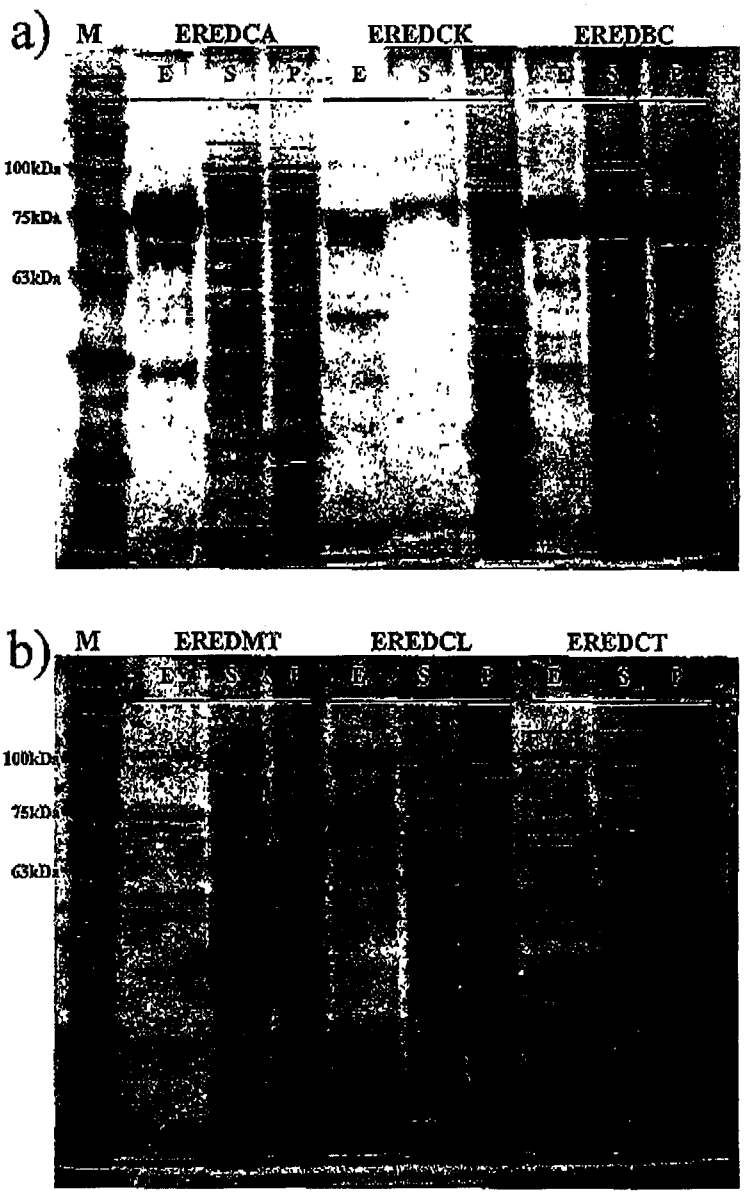

a)
M
EREDCA
EREDCK
EREDBC
E
S
P
100kDa
75kDa
63kDa
b)
M
EREDMT
EREDCL
EREDCT
E
S
P
100kDa
75kDa
63kDa

FIG. 3

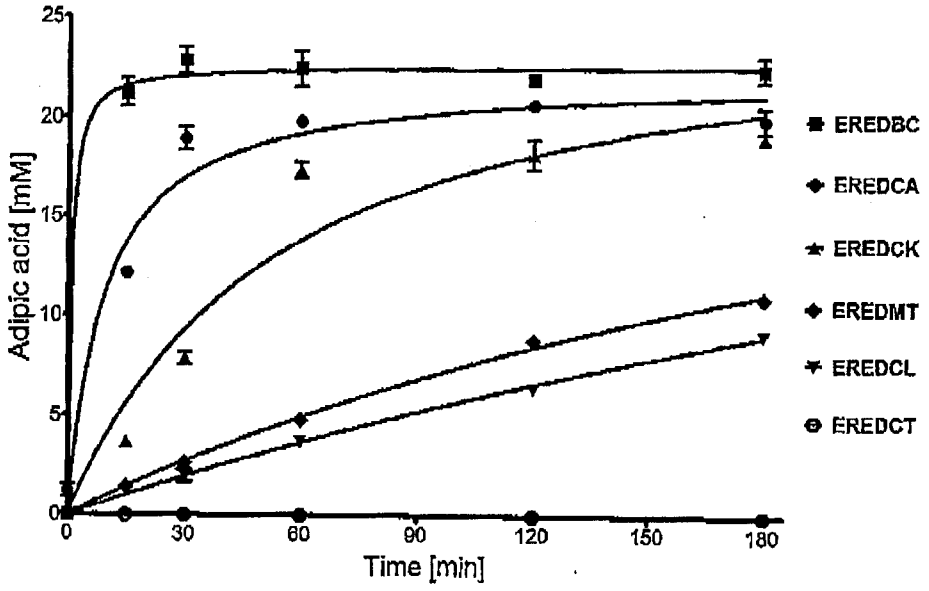

Adipic acid [mM]
25
20
15
10
5
0
0
30
60
90
120
150
180
Time [min]
EREDBC
EREDCA
EREDCK
EREDMT
EREDCL
EREDCT

FIG. 4

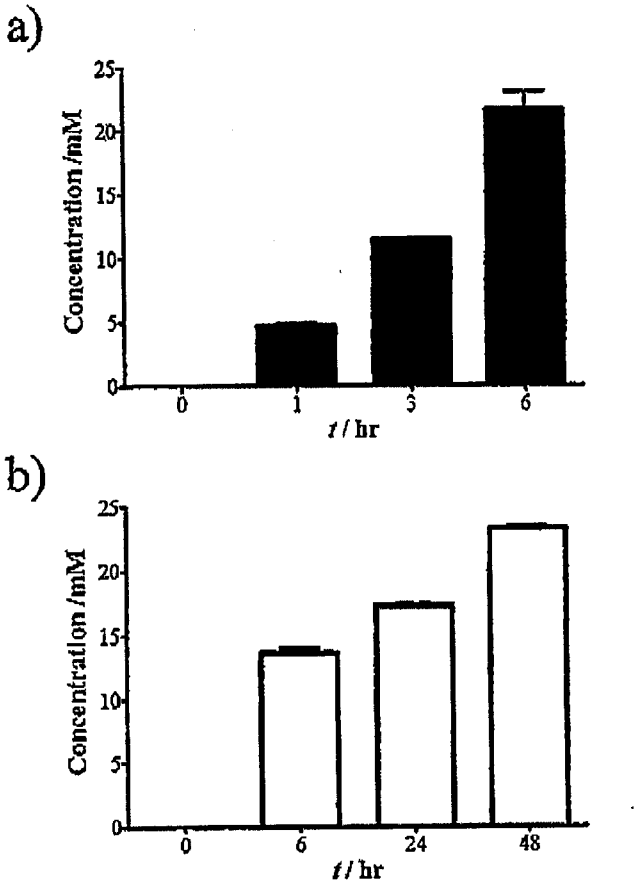

a)
Concentration /mM
25
20
15
10
5
0
0
1
3
6
t / hr
b)
Concentration /mM
25
20
15
10
5
0
0
6
24
48
t / hr

FIG. 5

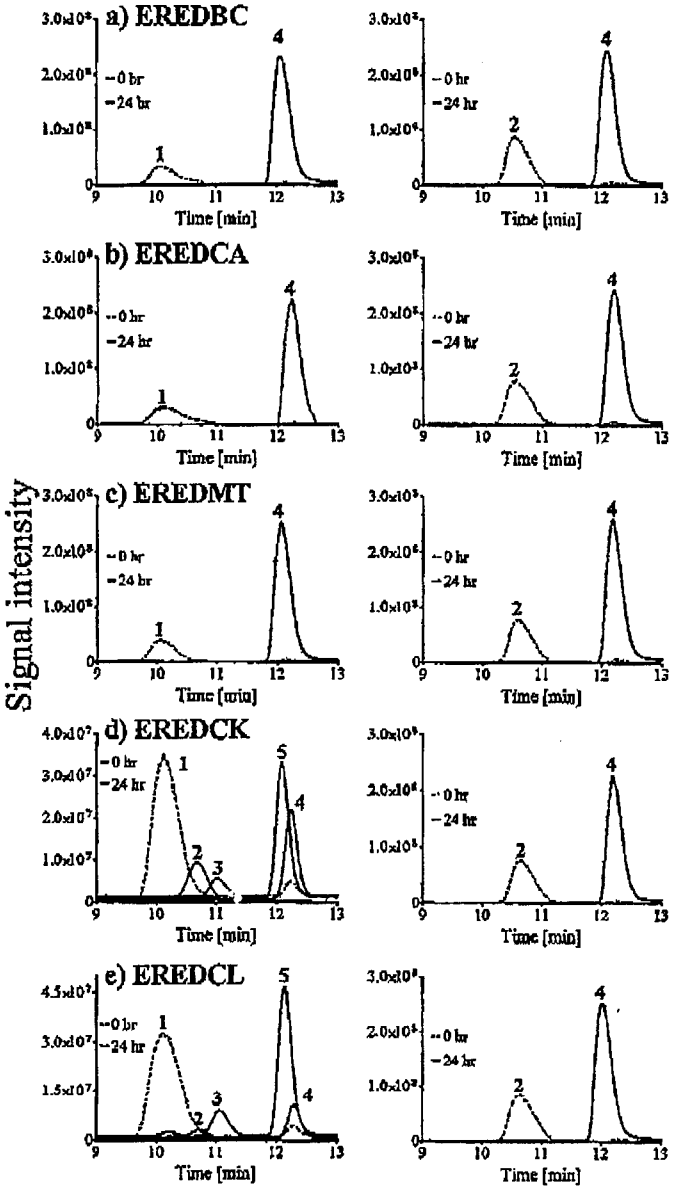

a) EREDBC
b) EREDCA
c) EREDMT
d) EREDCK
e) EREDCL
Signal intensity
Time [min]
0 hr
24 hr

FIG. 6

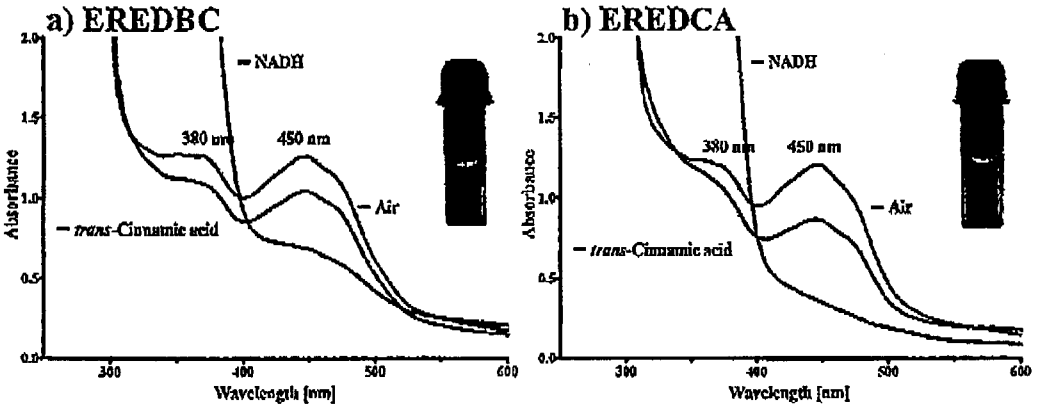

a) EREDBC
NADH
380 nm
450 nm
Air
trans-Cinnamic acid
Absorbance
Wavelength [nm]
b) EREDCA
NADH
380 nm
450 nm
Air
trans-Cinnamic acid
Absorbance
Wavelength [nm]

FIG. 7

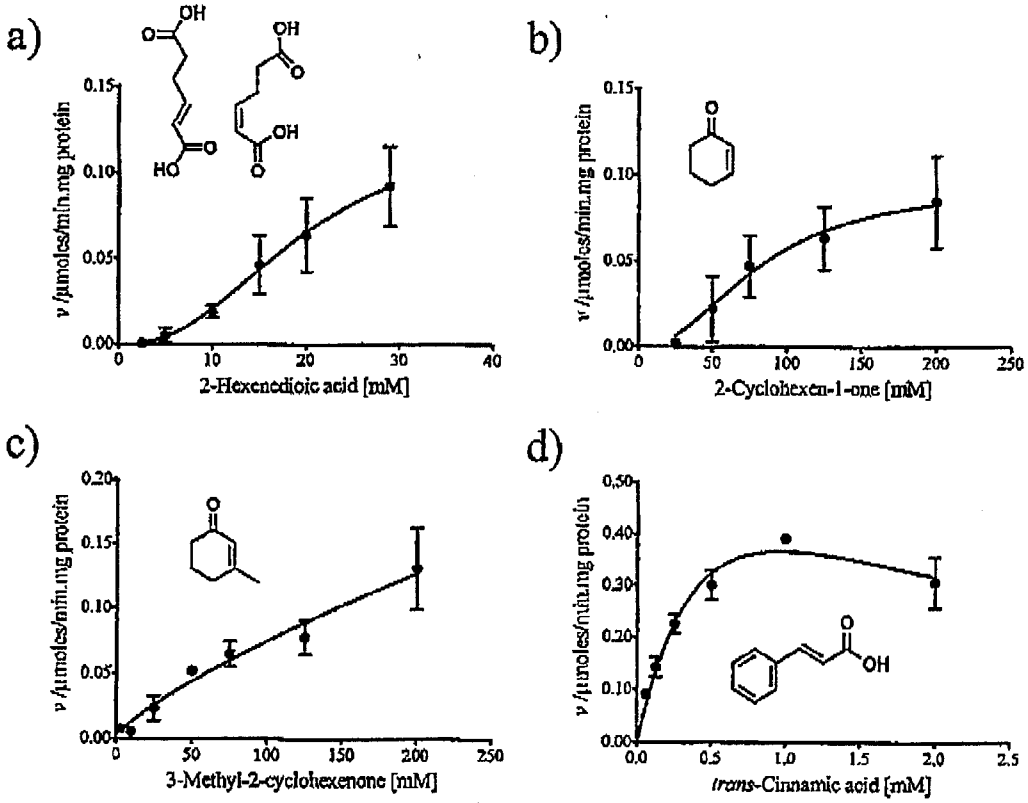

a)
OH
O
OH
O
HO
O
OH
O
O
v /µmoles/min.mg protein
0.15
0.10
0.05
0.00
0
10
20
30
40
2-Hexenedioic acid [mM]
b)
O
v /µmoles/min.mg protein
0.15
0.10
0.05
0.00
0
50
100
150
200
250
2-Cyclohexen-1-one [mM]
c)
O
v /µmoles/min.mg protein
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
250
3-Methyl-2-cyclohexenone [mM]
d)
O
OH
v /µmoles/min.mg protein
0,50
0.40
0.30
0.20
0.10
0.00
0.0
0.5
1.0
1.5
2.0
2.5
trans-Cinnamic acid [mM]

FIG. 8

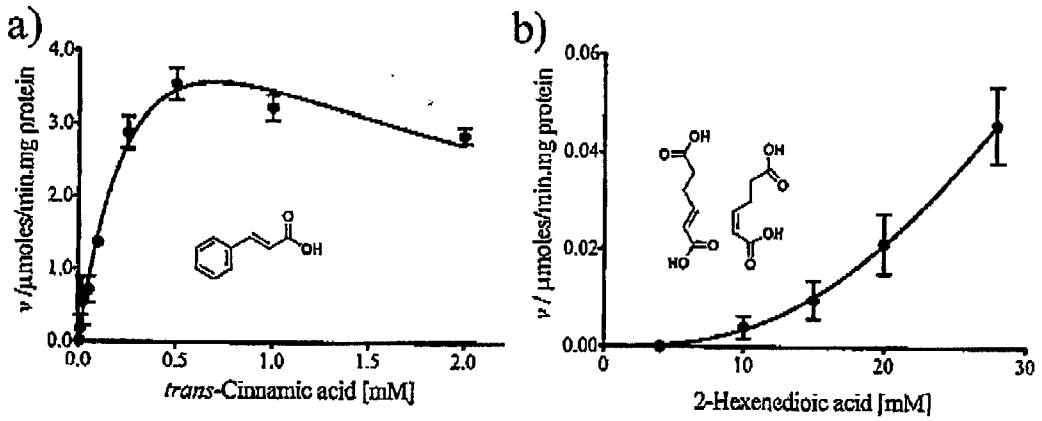

a)
v /µmoles/min.mg protein
4.0
3.0
2.0
1.0
0.0
0.0
0.5
1.0
1.5
2.0
trans-Cinnamic acid [mM]
OH
b)
v / µmoles/min.mg protein
0.06
0.04
0.02
0.00
0
10
20
30
2-Hexenedioic acid [mM]
OH
HO

FIG. 9

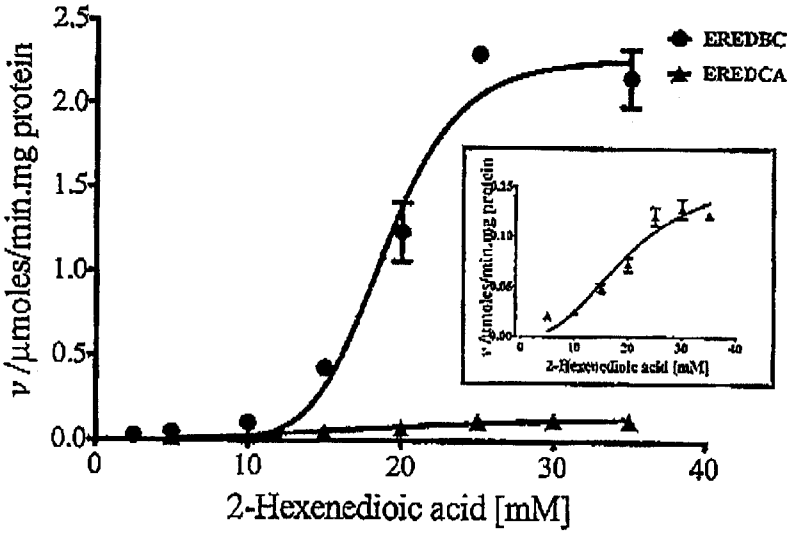

2.5
2.0
1.5
1.0
0.5
0.0
v /µmoles/min.mg protein
0
10
20
30
40
2-Hexenedioic acid [mM]
EREDBC
EREDCA

FIG. 10

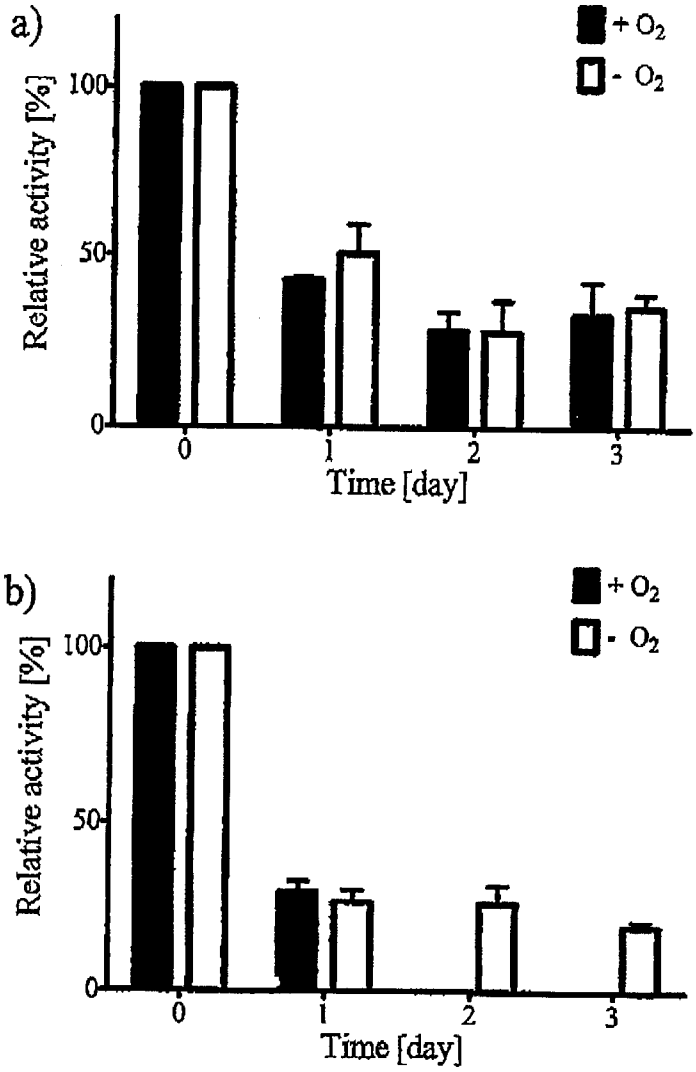

a)
+ O2
- O2
Relative activity [%]
100
50
0
0
1
2
3
Time [day]
b)
+ O2
- O2
Relative activity [%]
100
50
0
0
1
2
3
Time [day]

FIG. 11

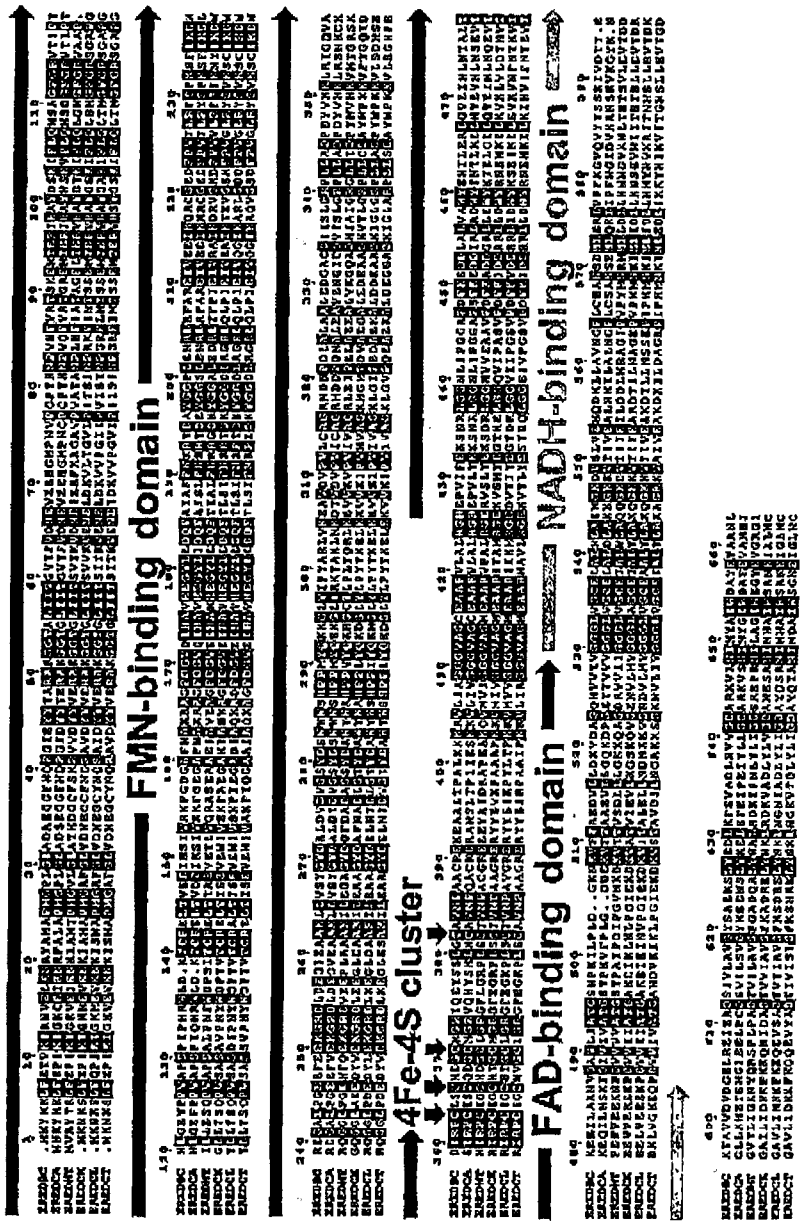

FMN-binding domain
4Fe-4S cluster
FAD-binding domain
NADH-binding domain

FIG. 12

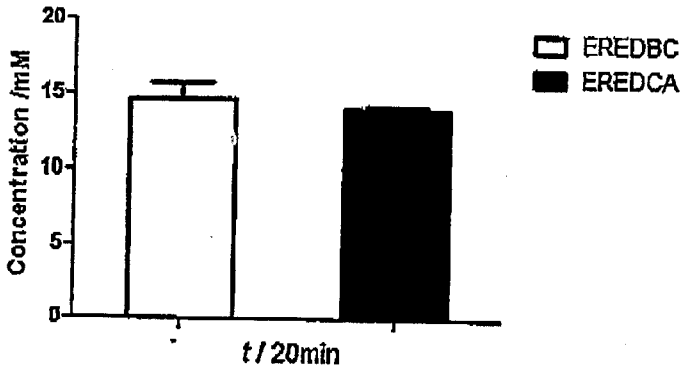
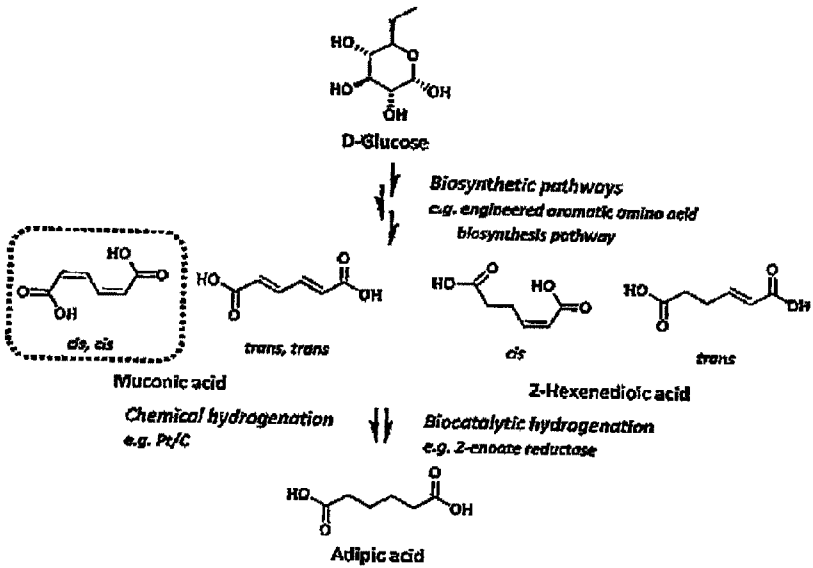


FIG. 13


D-Glucose
Biosynthetic pathways
e.g. engineered aromatic amino acid biosynthesis pathway
cis, cis
Muconic acid
trans, trans
cis
trans
2-Hexenedioic acid
Chemical hydrogenation
e.g. Pt/C
Biocatalytic hydrogenation
e.g. 2-enoate reductase
Adipic acid