Title: METHOD FOR THE PRODUCTION OF GLUTACONATE

Abstract: The present invention relates to a novel method for the biocatalytic production of unsaturated dicarboxylic acids by cultivating a recombinant microorganism co-expressing a glutaconate CoA-transferase and a 2-hydroxygulutaryl-CoA dehydratase system. The present invention also relates to corresponding recombinant hosts, recombinant vectors, expression cassettes and nucleic acids suitable for preparing such hosts as well as a method of preparing polyamide or polyester copolymers making use of said dicarboxylic acids as obtained by said biocatalytic production method.
**Method for the production of glutaconate**

The present invention relates to a novel method for the biocatalytic production of unsaturated dicarboxylic acids by cultivating a recombinant microorganism co-expressing a glutaconate CoA-transferase and a 2-hydroxyglutaryl-CoA dehydratase system. The present invention also relates to corresponding recombinant hosts, recombinant vectors, expression cassettes and nucleic acids suitable for preparing such hosts as well as a method of preparing polymers, as for example polyamide or polyester copolymers, making use of said dicarboxylic acids as obtained by said biocatalytic production method.

**Background of the invention**

Glutaconic acid is an α,β-unsaturated C5-dicarboxylic acid (2-pentenedioic acid) that accumulates in individuals with glutaric acidemia type I (Hoffmann GF, Zschocke J (1999) Glutaric aciduria type I: from clinical, biochemical and molecular diversity to successful therapy. J Inherit Metab Dis 22:381-391). Glutaconic acid together with a diamine can polymerize to a polyamide related to Nylon®. The ideal material for biotechnological production of glutaconic acid would be glutamic acid, which can be produced by sugar fermentation. The chemical deamination of α-amino acids to α,β-unsaturated acids is very difficult. On the contrary, the strictly anaerobic bacteria *Acidaminococcus fermentans* and *Clostridium symbiosum* can easily deaminate glutamate via α-ketoglutarate, (R)-2-hydroxyglutarate, (R)-2-hydroxyglutaryl-CoA, and glutaconyl-CoA to (E)-glutaconate (Buckel W (2001b) Unusual enzymes involved in five pathways of glutamate fermentation. Appl Microbiol Biotechnol 57:263-273). *A. fermentans* and *C. symbiosum* are not suitable for the production of glutaconic acid because they decarboxylate glutaconyl-CoA to crotonyl-CoA. Genetic manipulation of these organisms has not been established yet. Thus, the genes coding for glutaconyl-CoA decarboxylase cannot be attenuated to a low level, whereas a complete deletion would deprive these organisms of the ability to produce ATP. Furthermore, the ultimate aim is the production of glutaconate not from glutamate but from glucose, on which *A. fermentans* and *C. symbiosum* are not able to grow.
The object of the present invention is therefore to provide a suitable method for the fermentative, biocatalytic production of glutaconic acid and related dicarboxylic acids or corresponding salts thereof.

**Description of the figures:**

Figure 1 depicts the pathway for glutaconate production. The enzymes of the final steps are numbered with 1, 2 and 3. 1: 2-hydroxyglutarate dehydrogenase; 2: glutaconate CoA-transferase; 3: 2-hydroxyglutaryl-CoA dehydratase.

Figure 2 depicts schematically the construction of recombinant plasmid pACYCDOuet-1 (Fig. 2A) in which the coding sequences for 2-hydroxyglutarate dehydrogenase (hgdH) and glutaconate CoA-transferase (gctAB), (subunits A and B) are inserted (Fig. 2B).

Figure 3 depicts schematically the construction of recombinant plasmids pASK-IBA3plus (Fig. 3A) in which the coding sequences for 2-hydroxyglutaryl-CoA dehydratase (subunits hgdA and hgdB) and its activator (hgdC) are inserted (Fig. 3B).

**Summary of the invention**

The above-mentioned problem was solved by the present invention teaching a biocatalytic method for the production of an unsaturated dicarboxylic acid compound, like glutaconate or a structurally similar glutaconate compound (of formula I) which method comprises converting a corresponding 2-hydroxy-substituted dicarboxylic acid in a recombinant microorganism co-expressing a glutaconate CoA-transferase and a 2-hydroxyglutaryl-CoA dehydratase system, so that said unsaturated dicarboxylic acid is formed.

For example, in order to convert *Escherichia coli* to a glutaconate producer, the present inventors expressed six genes, encoding 2-hydroxyglutarate dehydrogenase (HgdA, 1 in Fig. 1), glutaconate CoA-transferase (GcdAB, 2), 2-hydroxyglutaryl-CoA dehydratase (HgdAB, 3) and its activator (HgdC, 3). The new pathway can divert at α-ketoglutarate derived from glucose via the Embden-Meyerhof pathway and the citrate cycle.

**Detailed description of the invention**
1. Preferred embodiments

In a first embodiment the present invention provides a biocatalytic method for the production of an unsaturated dicarboxylic acid compound of the general formula I

\[ \text{HOOC-CH=CH-X-COOH} \] (I)

in particular the E-form of such a compound; wherein X represents a linear or branched, optionally unsaturated, optionally substituted hydrocarbon group, preferably having 1, 2, 3 or 4 carbon atoms;

which method comprises

- converting a 2-hydroxy-substituted dicarboxylic acid III compound

\[ \text{HOOC-C(OH)H-CH}_2\text{-X-COOH} \] (III)

wherein X is as defined above;

in a recombinant microorganism co-expressing a glutaconate CoA-transferase (E.C. 2.8.3.12) and a 2-hydroxyglutaryl-CoA dehydratase system, under conditions allowing formation of the desired product, and particularly or optionally in the presence of a coenzyme A (CoA) source, like acyl-CoA, like \( \text{C}_2\text{-C}_6\)-acyl-CoA, and in particular acetyl-coenzyme A, which CoA source may be of any origin, like endogenic to said microorganism, i.e. produced by said microorganism, or exogenic, i.e. added to said microorganism or production medium, so that said compound of formula I is formed, and/or optionally in the presence of any other exogenic or endogenic factors required for or improving formation of a compound of formula I;

- and optionally isolating said compound of formula I in the form of a substantially pure stereoisomer, as for example the Z-form or in particular the E-form, or as a mixture of stereoisomers, each either in the form of its salts or as free acid.

In compounds of formula I said group X preferably may be selected from \( \text{(CH}_2\text{)}_n\), with n being an integer from 1 to 4, \( \text{CH}=\text{CH}, \text{CH}_2\text{-C}(=\text{O})\), or \( \text{CH}=\text{C(OH)}\). In particular X is selected from \( \text{CH}_2\text{, C}_3\text{H}_4\), \( \text{CH}=\text{CH} \) and \( \text{CH}=\text{C(OH)}\).

According to the present invention said 2-hydroxy-substituted dicarboxylic acid III is preferably formed by said recombinant microorganism in a 2-hydroxyglutarate
dehydrogenase (E.C. 1.1.1.-) catalyzed conversion of a 2-oxo-dicarboxylic acid compound of formula II

\[ \text{HOOC-C(O)=CH}_2\text{X-COOH} \] (II)

wherein X is defined above. In particular, said 2-hydroxyglutarate dehydrogenase may be co-expressed by said recombinant microorganism as well.

In said method of the invention said oxo-dicarboxylic acid compound of formula II is either added to or fermentatively produced by said recombinant microorganism.

In particular, the method of the invention comprises the cultivation of at least one recombinant microorganism, which microorganism is derived from a parent microorganism having the ability to produce said 2-oxo-dicarboxylic acid compound of formula II as intermediary product of a metabolic pathway, and additionally having the ability to express heterologously at least one of the above mentioned enzymes and proteins. For example, said microorganism is a glutamate and/or glucose metabolizing aerobic or anaerobic recombinant bacterium, and said compound of formula II is 2-oxoglutarate formed by the biodegradation of glutamate (for example by the action of glutamate dehydrogenase) and/or glucose (for example via the Embden-Meyerhof pathway and the Krebs or citric acid cycle). If appropriate one or more, as for example 2, 3, 4 or 5, individual enzymes involved in said glutamate or glucose metabolism may be deregulated in order to further assist, or improve the formation of the intended product and/or to reduce or avoid the formation of undesired side products or secondary products (formed by the metabolism of the intended product) which otherwise would lessen or decrease the actual amount or concentration of the intended product as formed by the microorganism.

In particular, said glutamate and/or glucose metabolizing recombinant bacterium is selected from bacteria of the genus *Escherichia*, as for example *E. coli*, like the strain *E. coli* BL21-CodonPlus® (DE3)-RIL strain (Stratgene). The CodonPlus plasmid responsible for chloramphenicol resistance may also be removed.

In a particular embodiment of the method of the invention said 2-hydroxyglutaryl-CoA dehydratase system comprises a 2-hydroxyglutaryl-CoA dehydratase (E.C. 4.2.1.) and, if required for inducing and/or maintaining the intended dehydratase activity, optionally an activator protein for said enzyme. Said activator protein may be required for establishing dehydratase activity.
According to the present invention said enzymes and proteins (glutaconate CoA-transferase, 2-hydroxyglutaryl-CoA dehydratase, activator, 2-hydroxyglutarate dehydrogenase) are of prokaryotic or eukaryotic origin. In particular they may originate from different microbial genera or strains. For example it is not an absolute requirement that dehydratase and the activator for establishing dehydratase activity are derived from the same microbial genus or strain as long as the activator cooperates with (activates the) dehydratase enzyme as used for the bioconversion reaction.

In a particular embodiment, said enzymes and activator originate from the same or different anaerobic bacterium, which bacterium being able to convert glutamate into glutaconate. For example, said anaerobic bacterium is selected from bacteria of the genus Acidaminococcus, Clostridium, Fusobacterium or Peptostreptococcus, in particular Acidaminococcus fermentans, Clostridium symbiosum, Clostridium sporosphaeroides, Fusobacterium nucleatum including all subspecies, or Peptostreptococcus asaccharolyticus.

In particular, said 2-hydroxyglutarate dehydrogenase (HgdH) comprises at least one amino acid sequence of SEQ ID NO: 16 (FN0487, annotated as D-lactate dehydrogenase - Fusobacterium nucleatum subsp. nucleatum ATCC 25586; GenelID: 991766) or SEQ ID NO:2 (1XDW_A (A. fermentans); or a sequence having at least 50% identity to at least one of said sequences, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity, and still retaining the intended enzyme activity (or function), i.e. being applicable as HgdH enzyme; for example said enzyme may be a homodimer, as for example the A. fermentans enzyme; for example the A. fermentans enzyme shows 61% sequence identity to the enzyme from F. nucleatum.

In particular, said glutaconate CoA transferase (GctAB), which may be a heterooctamer (α₄β₄)

(a) comprises at least one alpha (A) and at least one beta (B) subunit, wherein said alpha subunit comprising an amino acid sequence according to SEQ ID NO: 4 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said beta subunit comprising an amino acid sequence according to SEQ ID NO: 6 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme activity, i.e. being applicable as GctAB enzyme; or
(b) comprises at least one alpha and at least one beta subunit, wherein said alpha subunit comprising an amino acid sequence according to SEQ ID NO: 22 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said beta subunit comprising an amino acid sequence according to SEQ ID NO: 24 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme activity, i.e. being applicable as GctAB enzyme; or

(c) comprises at least one alpha and at least one beta subunit, wherein said alpha subunit comprising an amino acid sequence according to SEQ ID NO: 18 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said beta subunit comprising an amino acid sequence according to SEQ ID NO: 20 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme activity, i.e. being applicable as GctAB enzyme.

In particular, said 2-hydroxyglutaryl-CoA dehydratase, which may be a heterodimer (AB) or trimer (ABD) with one [4Fe-4S] cluster in each A and each B subunit, and

(a) comprises at least one alpha (A) and at least one beta (B) subunit, wherein said alpha subunit comprising an amino acid sequence according to SEQ ID NO: 26 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said beta subunit comprising an amino acid sequence according to SEQ ID NO: 28 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme activity, i.e. being applicable as dehydratase enzyme; or

(b) comprises at least one alpha (A), at least one beta (B) subunit and at least one delta (D) subunit, wherein said alpha subunit comprising an amino acid sequence according to SEQ ID NO: 30 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said beta subunit comprising an amino acid sequence according to SEQ ID NO: 32 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said gamma
subunit comprising an amino acid sequence according to SEQ ID NO: 34 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme activity, i.e. being applicable as dehydratase enzyme; or

(c) comprises at least one alpha and at least one beta subunit, wherein said alpha subunit comprising an amino acid sequence according to SEQ ID NO: 8 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and wherein said beta subunit comprising an amino acid sequence according to SEQ ID NO: 10 or a sequence having at least 50% identity thereto, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme activity, i.e. being applicable as dehydratase enzyme.

In particular, said the activator protein comprises at least one amino acid sequence of SEQ ID NO: 12, 36 or 38 or a sequence having at least 50% identity to at least one of said sequences as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended activity, i.e. being applicable as dehydratase activator; an may be a homodimer with one [4Fe-4S] cluster between the two subunits.

In a further particular embodiment, said 2-hydroxyglutaryl-CoA dehydratase is from C. symbiosum (SEQ ID NO: 8 and/or 10); while said 2-hydroxyglutarate dehydrogenase (SEQ ID NO:2); said glutaconate CoA transferase (SEQ ID NO:4 and/or 6) and said activator protein (SEQ ID NO:12) are from A. fermentans; or independently of each other being derived therefrom, and having a sequence of at least 50% identity to the parent sequence, as for example at least 60, 70, 80, 85, 90, 92, 95, 96, 97, 98 or 99 % sequence identity; and still retaining the intended enzyme or activator activity. Said proteins may be present in the form of the active quaternary structure or may be present in the form of active fragments or subunits thereof.

Moreover, said proteins and enzymes may each be encoded by a nucleic acid sequence, which is adapted to the codon usage of said microorganism having the ability to produce said 2-oxo-dicarboxylic acid of formula I.

In addition, said proteins and enzymes may be encoded by nucleic acid sequences contained in one single or more expression vectors in may be encoded as single or multiple copies.
Additionally, at least one of said co-expressed proteins is heterologous to said recombinant microorganism.

The present invention also relates to:
- an expression cassette, comprising a combination of at least two different nucleic acid sequences encoding an enzyme or protein as defined above (i.e. the required set (or a subset of) of sequences encoding the required set (or a subset) of enzymes/proteins necessary for producing compounds of formula I via compounds of formula III), which sequences are operatively linked to at least one regulatory nucleic acid sequence;
- a recombinant vector, comprising at least one of said expression cassettes;
- a recombinant prokaryotic or eukaryotic host, transformed with at least one such vector.

In particular such recombinant hosts have the ability to produce a 2-oxo-dicarboxylic acid compound of formula (II), which is converted to a compound of formula (I) upon expression of said expression products as encoded by said at least one vector. For example said host may be a recombinant strain of a bacterium of the genus Escherichia.

In another embodiment the present invention relates to a method of preparing a polyamide or polyester, which method comprises
a) preparing a mono-unsaturated dicarboxylic acid compounds of the general formula (I) as defined above a by method as described herein;
b) isolating said compound optionally followed by hydrogenation in order to remove the C=C-double bond; and
c) polymerizing said compound as obtained according to step b), with at least one suitable polyvalent polymerizable amine monomer or polyvalent polymerizable hydroxyl compound.

In particular, said polyamine is a di-amine, a tri-amine or a mixture thereof and said polyvalent hydroxyl compound is a diol or triol or a mixture thereof. For example said polymerization reaction may be performed in the presence of a suitable catalyst, as for example acid or base catalyst.

In another embodiment the present invention relates to a method of preparing a polymer, which method comprises
preparing a mono-unsaturated dicarboxylic acid compounds of the general formula (I) as defined above by method as defined above; isolating said compound; and polymerizing said compound as obtained according to step b), with at least one suitable unsaturated polymerizable monomer.

For example, said polymerization may be performed in the presence of a suitable radical initiator. Suitable comonomers are those which may be applied in a radical-initiated polymerization, as for example monomers containing at least one polymerizable C=C-bond, like vinyl, acryl and methacryl.

In another embodiment of the method described herein at least one gene, as for example 1, 2, 3 or 4 genes, of a biosynthetic pathway in said recombinant microorganism directly or indirectly affecting the formation and/or decomposition of at least one compound of formula (I), (II) or (III), may deregulated (up or down regulated) in order to further optimize the method of the invention.

2. Explanation of particular terms

Unless otherwise stated the expressions "glutaconate" and "glutaconic acid" or the expressions "glutaconate compound" and "glutaconic acid compound" or the expressions "unsaturated dicarboxylic acid" or "unsaturated dicarboxylate compound" are considered to be synonymous. The glutaconate or dicarboxylic acid compound (of formula I) as obtained according to the present invention may be in the form of the free acid, in the form of a partial or complete salt of said acid or in the form of mixtures of the acid and its salt.

A dicarboxylic acid "salt" comprises for example metal salts, as for example zinc glutaconate, mono- or di-alkalimetal salts of said acid, like mono-sodium di-sodium, mono-potassium and di-potassium salts as well as alkaline earth metal salts as for example the calcium or magnesium salts.

The term "biocatalytic method" refers to any method performed in the presence of catalytic activity of an enzyme as defined herein, i.e. in the presence of isolated pure or crude enzyme or entire microbial cells containing or expression such enzyme activity.

The term "stereospecific" means that one of several stereoisomers or enantiomers is formed by the enzyme in high enantiomeric excess or purity, of at least 90%ee, preferably at least 95 %ee, in particular at least 98 %ee, or at least 99 %ee. the ee% value is calculated according to the following formula
\[ ee\% = \frac{X_A - X_B}{X_A + X_B} \times 100 , \]
wherein \( X_A \) and \( X_B \) refer to the molar fraction of enantiomer A or B, respectively.

Examples of "stereoisomers" are E-and Z- isomers or R- and S enantiomers.

"Deregulation" has to be understood in its broadest sense, and comprises an increase or decrease of complete switch off of an enzyme (target enzyme) activity by different means well known to those in the art. Suitable methods comprise for example an increase or decrease of the copy number of gene and/or enzyme molecules in an organism, or the modification of another feature of the enzyme affecting the its enzymatic activity, which then results in the desired effect on a metabolic pathway at issue, or any pathway or enzymatic reaction coupled thereto. Suitable genetic manipulation can also include, but is not limited to, altering or modifying regulatory sequences or sites associated with expression of a particular gene (e.g., by removing strong promoters, inducible promoters or multiple promoters), modifying the chromosomal location of a particular gene, altering nucleic acid sequences adjacent to a particular gene such as a ribosome binding site or transcription terminator, decreasing the copy number of a particular gene, modifying proteins (e.g., regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of a particular gene and/or translation of a particular gene product, or any other conventional means of deregulating expression of a particular gene routine in the art (including but not limited to use of antisense nucleic acid molecules), or other methods to knock-out or block expression of the target protein.

A preferred way of an "amplification" is an "up"- mutation which increases the gene activity e.g. by gene amplification using strong expression signals and/or point mutations which enhance the enzymatic activity.

A preferred way of an "attenuation" is a "down"- mutation which decreases the gene activity e.g. by gene deletion or disruption, using weak expression signals and/or point mutations which destroy or decrease the enzymatic activity.

The term "heterologous" or "exogenous" refers to sequences as described herein, which are introduced into or produced (transcribed or translated) by a genetically manipulated microorganism as defined herein and which microorganism prior to said manipulation did not contain or did not produce said sequence. In particular said microorganism prior to said manipulation may not contain or express said heterologous enzyme activity, or may contain or express an endogenous enzyme of comparable activity or specificity, which is encoded by a different coding sequence.
or by an enzyme of different amino acid sequence, and said endogenous enzyme may convert the same substrate or substrates as said exogenous enzyme.

A "parent" microorganism of the present invention is any microorganism having the ability to produce a compound of formula (II) as intermediary product.

An "intermediary product" is understood as a product, which is transiently or continuously formed during a chemical or biochemical process, in a not necessarily analytically directly detectable concentration. Said "intermediary product" may be removed from said biochemical process by a second, chemical or biochemical reaction.

A "recombinant host" may be any prokaryotic or eukaryotic cell, which contains either a cloning vector or expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell. For examples of suitable hosts, see Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

The term "recombinant microorganism" includes a microorganism (e.g., bacteria, yeast cell, fungal cell, etc.) which has been genetically altered, modified or engineered (e.g., genetically engineered) such that it exhibits an altered, modified or different genotype and/or phenotype (e.g., when the genetic modification affects coding nucleic acid sequences of the microorganism) as compared to the naturally-occurring microorganism or "parent" microorganism which it was derived from.

As used herein, a "substantially pure" protein or enzyme means that the desired purified protein is essentially free from contaminating cellular components, as evidenced by a single band following polyacrylamide-sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). The term "substantially pure" is further meant to describe a molecule, which is homogeneous by one or more purity or homogeneity characteristics used by those of skill in the art. For example, a substantially pure enzyme or protein will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: molecular mass, chromatographic migration, amino acid composition, amino acid sequence, blocked or unblocked N-terminus, HPLC elution profile, biological activity, and other such parameters. The term, however, is not meant to exclude artificial or synthetic mixtures of said enzyme or protein with other compounds. In addition, the term is not meant to exclude fusion proteins optionally isolated from a recombinant host.
3. Further embodiments of the invention

3.1 Proteins according to the invention

The present invention is not limited to the specifically mentioned enzymes/proteins, but also extends to functional equivalents thereof. "Functional equivalents" or "analogs" or "functional mutations" of the concretely disclosed enzymes are, within the scope of the present invention, various polypeptides thereof, which moreover possess the desired biological function or activity, e.g. enzyme activity.

For example, "functional equivalents" means enzymes, which, in a test used for enzymatic activity, display at least a 1 to 10\%, or at least 20\%, or at least 50\%, or at least 75\%, or at least 90\% higher or lower activity of an enzyme, as defined herein.

"Functional equivalents", according to the invention, also means in particular mutants, which, in at least one sequence position of the amino acid sequences stated above, have an amino acid that is different from that concretely stated, but nevertheless possess one of the aforementioned biological activities. "Functional equivalents" thus comprise the mutants obtainable by one or more amino acid additions, substitutions, deletions and/or inversions, where the stated changes can occur in any sequence position, provided they lead to a mutant with the profile of properties according to the invention. Functional equivalence is in particular also provided if the reactivity patterns coincide qualitatively between the mutant and the unchanged polypeptide, i.e. if for example the same substrates are converted at a different rate. Examples of suitable amino acid substitutions are shown in the following table:
<table>
<thead>
<tr>
<th>Original residue</th>
<th>Examples of substitution</th>
</tr>
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<tbody>
<tr>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>Arg</td>
<td>Lys</td>
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<tr>
<td>Asn</td>
<td>Gln; His</td>
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<tr>
<td>Asp</td>
<td>Glu</td>
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<td>Cys</td>
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<td>Gly</td>
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<td>His</td>
<td>Asn; Gln</td>
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<tr>
<td>Nε</td>
<td>Leu; Val</td>
</tr>
<tr>
<td>Leu</td>
<td>Lie; Val</td>
</tr>
<tr>
<td>Lys</td>
<td>Arg; Gln ; Glu</td>
</tr>
<tr>
<td>Met</td>
<td>Leu; Nε</td>
</tr>
<tr>
<td>Phe</td>
<td>Met ; Leu ; Tyr</td>
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<tr>
<td>Ser</td>
<td>Thr</td>
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<td>Thr</td>
<td>Ser</td>
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<td>Trp</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyr</td>
<td>Trp ; Phe</td>
</tr>
<tr>
<td>Val</td>
<td>Lie; Leu</td>
</tr>
</tbody>
</table>

"Functional equivalents" in the above sense are also "precursors" of the polypeptides described, as well as "functional derivatives" and "salts" of the polypeptides.

"Precursors" are in that case natural or synthetic precursors of the polypeptides with or without the desired biological activity.

The expression "salts" means salts of carboxyl groups as well as salts of acid addition of amino groups of the protein molecules according to the invention. Salts of carboxyl groups can be produced in a known way and comprise inorganic salts, for example sodium, calcium, ammonium, iron and zinc salts, and salts with organic bases, for example amines, such as triethanolamine, arginine, lysine, piperidine and the like. Salts of acid addition, for example salts with inorganic acids, such as hydrochloric acid or sulfuric acid and salts with organic acids, such as acetic acid and oxalic acid, are also covered by the invention.

"Functional derivatives" of polypeptides according to the invention can also be produced on functional amino acid side groups or at their N-terminal or C-terminal end using known techniques. Such derivatives comprise for example aliphatic esters of carboxylic acid groups, amides of carboxylic acid groups, obtainable by reaction with ammonia or with a primary or secondary amine; N-acyl derivatives of free amino...
groups, produced by reaction with acyl groups; or O-acyl derivatives of free hydroxy
groups, produced by reaction with acyl groups.

"Functional equivalents" naturally also comprise polypeptides that can be
obtained from other organisms, as well as naturally occurring variants. For example,
areas of homologous sequence regions can be established by sequence comparison,
and equivalent enzymes can be determined on the basis of the concrete parameters of
the invention.

"Functional equivalents" also comprise fragments, preferably individual domains
or sequence motifs, of the polypeptides according to the invention, which for example
display the desired biological function.

"Functional equivalents" are, moreover, fusion proteins, which have one of the
polypeptide sequences stated above or functional equivalents derived there from and
at least one further, functionally different, heterologous sequence in functional N-
terminal or C-terminal association (i.e. without substantial mutual functional impairment
of the fusion protein parts). Non-limiting examples of these heterologous sequences
are e.g. signal peptides, histidine anchors or enzymes.

"Functional equivalents" that are also included according to the invention are
homologues of the concretely disclosed proteins. These possess percent identity
values as stated above. Said values refer to the identity with the concretely disclosed
amino acid sequences, and may be calculated according to the algorithm of Pearson

The % identity values may also be calculated from BLAST alignments, algorithm
blastp (protein-protein BLAST) or by applying the Clustal setting as given below.
A percentage identity of a homologous polypeptide according to the invention
means in particular the percentage identity of the amino acid residues relative to the
total length of one of the amino acid sequences concretely described herein.

In the case of a possible protein glycosylation, "functional equivalents"
according to the invention comprise proteins of the type designated above in
deglycosylated or glycosylated form as well as modified forms that can be obtained by
altering the glycosylation pattern.

Such functional equivalents or homologues of the proteins or polypeptides
according to the invention can be produced by mutagenesis, e.g. by point mutation,
lengthening or shortening of the protein.
Such functional equivalents or homologues of the proteins according to the invention can be identified by screening combinatorial databases of mutants, for example shortening mutants. For example, a variegated database of protein variants can be produced by combinatorial mutagenesis at the nucleic acid level, e.g. by enzymatic ligation of a mixture of synthetic oligonucleotides. There are a great many methods that can be used for the production of databases of potential homologues from a degenerated oligonucleotide sequence. Chemical synthesis of a degenerated gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic gene can then be ligated in a suitable expression vector. The use of a degenerated genome makes it possible to supply all sequences in a mixture, which code for the desired set of potential protein sequences. Methods of synthesis of degenerated oligonucleotides are known to a person skilled in the art (e.g. Narang, S.A. (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acids Res. 11:477).

In the prior art, several techniques are known for the screening of gene products of combinatorial databases, which were produced by point mutations or shortening, and for the screening of cDNA libraries for gene products with a selected property. These techniques can be adapted for the rapid screening of the gene banks that were produced by combinatorial mutagenesis of homologues according to the invention. The techniques most frequently used for the screening of large gene banks, which are based on a high-throughput analysis, comprise cloning of the gene bank in expression vectors that can be replicated, transformation of the suitable cells with the resultant vector database and expression of the combinatorial genes in conditions in which detection of the desired activity facilitates isolation of the vector that codes for the gene whose product was detected. Recursive Ensemble Mutagenesis (REM), a technique that increases the frequency of functional mutants in the databases, can be used in combination with the screening tests, in order to identify homologues (Arkin and Yourvan (1992) PNAS 89:781 1-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331).

### 3.2 Coding nucleic acid sequences

The invention also relates to nucleic acid sequences that code for enzymes/proteins as defined herein.
The present invention also relates to nucleic acids with a certain degree of "identity" to the sequences specifically disclosed herein. "Identity" between two nucleic acids means identity of the nucleotides, in each case over the entire length of the nucleic acid.

For example the identity may be calculated by means of the Vector NTI Suite 7.1 program of the company Informax (USA) employing the Clustal Method (Higgins DG, Sharp PM. Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl. Biosci. 1989 Apr; 5(2):151-1) with the following settings:

Multiple alignment parameters:

- Gap opening penalty: 10
- Gap extension penalty: 10
- Gap separation penalty range: 8
- Gap separation penalty: off
- % identity for alignment delay: 40
- Residue specific gaps: off
- Hydrophilic residue gap: off
- Transition weighing: 0

Pairwise alignment parameter:

- FAST algorithm: on
- K-tuplesize: 1
- Gap penalty: 3
- Window size: 5
- Number of best diagonals: 5


- DNA Gap Open Penalty: 15.0
- DNA Gap Extension Penalty: 6.66
- DNA Matrix: Identity
All the nucleic acid sequences mentioned herein (single-stranded and double-stranded DNA and RNA sequences, for example cDNA and mRNA) can be produced in a known way by chemical synthesis from the nucleotide building blocks, e.g. by fragment condensation of individual overlapping, complementary nucleic acid building blocks of the double helix. Chemical synthesis of oligonucleotides can, for example, be performed in a known way, by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press, New York, pages 896-897). The accumulation of synthetic oligonucleotides and filling of gaps by means of the Klenow fragment of DNA polymerase and ligation reactions as well as general cloning techniques are described in Sambrook et al. (1989), see below.

The invention also relates to nucleic acid sequences (single-stranded and double-stranded DNA and RNA sequences, e.g. cDNA and mRNA), coding for one of the above polypeptides and their functional equivalents, which can be obtained for example using artificial nucleotide analogs.

The invention relates both to isolated nucleic acid molecules, which code for polypeptides or proteins according to the invention or biologically active segments thereof, and to nucleic acid fragments, which can be used for example as hybridization probes or primers for identifying or amplifying coding nucleic acids according to the invention.

The nucleic acid molecules according to the invention can in addition contain non-translated sequences from the 3’ and/or 5’ end of the coding genetic region.

The invention further relates to the nucleic acid molecules that are complementary to the concretely described nucleotide sequences or a segment thereof.

The nucleotide sequences according to the invention make possible the production of probes and primers that can be used for the identification and/or cloning of homologous sequences in other cellular types and organisms. Such probes or primers generally comprise a nucleotide sequence region which hybridizes under
"stringent" conditions (see below) on at least about 12, preferably at least about 25, for example about 40, 50 or 75 successive nucleotides of a sense strand of a nucleic acid sequence according to the invention or of a corresponding antisense strand.

An "isolated" nucleic acid molecule is separated from other nucleic acid molecules that are present in the natural source of the nucleic acid and can moreover be substantially free from other cellular material or culture medium, if it is being produced by recombinant techniques, or can be free from chemical precursors or other chemicals, if it is being synthesized chemically.

A nucleic acid molecule according to the invention can be isolated by means of standard techniques of molecular biology and the sequence information supplied according to the invention. For example, cDNA can be isolated from a suitable cDNA library, using one of the concretely disclosed complete sequences or a segment thereof as hybridization probe and standard hybridization techniques (as described for example in Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). In addition, a nucleic acid molecule comprising one of the disclosed sequences or a segment thereof, can be isolated by the polymerase chain reaction, using the oligonucleotide primers that were constructed on the basis of this sequence. The nucleic acid amplified in this way can be cloned in a suitable vector and can be characterized by DNA sequencing. The oligonucleotides according to the invention can also be produced by standard methods of synthesis, e.g. using an automatic DNA synthesizer.

Nucleic acid sequences according to the invention or derivatives thereof, homologues or parts of these sequences, can for example be isolated by usual hybridization techniques or the PCR technique from other bacteria, e.g. via genomic or cDNA libraries. These DNA sequences hybridize in standard conditions with the sequences according to the invention.

"Hybridize" means the ability of a polynucleotide or oligonucleotide to bind to an almost complementary sequence in standard conditions, whereas nonspecific binding does not occur between non-complementary partners in these conditions. For this, the sequences can be 90-100% complementary. The property of complementary sequences of being able to bind specifically to one another is utilized for example in Northern Blotting or Southern Blotting or in primer binding in PCR or RT-PCR.
Short oligonucleotides of the conserved regions are used advantageously for hybridization. However, it is also possible to use longer fragments of the nucleic acids according to the invention or the complete sequences for the hybridization. These standard conditions vary depending on the nucleic acid used (oligonucleotide, longer fragment or complete sequence) or depending on which type of nucleic acid - DNA or RNA - is used for hybridization. For example, the melting temperatures for DNA:DNA hybrids are approx. 10°C lower than those of DNA:RNA hybrids of the same length.

For example, depending on the particular nucleic acid, standard conditions mean temperatures between 42 and 58°C in an aqueous buffer solution with a concentration between 0.1 to 5 x SSC (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.2) or additionally in the presence of 50% formamide, for example 42°C in 5 x SSC, 50% formamide. Advantageously, the hybridization conditions for DNA:DNA hybrids are 0.1 x SSC and temperatures between about 20°C to 45°C, preferably between about 30°C to 45°C. For DNA:RNA hybrids the hybridization conditions are advantageously 0.1 x SSC and temperatures between about 30°C to 55°C, preferably between about 45°C to 55°C. These stated temperatures for hybridization are examples of calculated melting temperature values for a nucleic acid with a length of approx. 100 nucleotides and a G + C content of 50% in the absence of formamide. The experimental conditions for DNA hybridization are described in relevant genetics textbooks, for example Sambrook et al., 1989, and can be calculated using formulae that are known by a person skilled in the art, for example depending on the length of the nucleic acids, the type of hybrids or the G + C content. A person skilled in the art can obtain further information on hybridization from the following textbooks: Ausubel et al. (eds), 1985, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Hames and Higgins (eds), 1985, Nucleic Acids Hybridization: A Practical Approach, IRL Press at Oxford University Press, Oxford; Brown (ed), 1991, Essential Molecular Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.


"Stringent" hybridization conditions mean in particular: Incubation at 42°C overnight in a solution consisting of 50% formamide, 5 x SSC (750 mM NaCl, 75 mM
tri-sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt Solution, 10% dextran sulfate and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing of the filters with 0.1 x SSC at 65°C.

The invention also relates to derivatives of the concretely disclosed or derivable nucleic acid sequences.

Thus, further nucleic acid sequences according to the invention can be derived from the sequences specifically disclosed herein and can differ from it by addition, substitution, insertion or deletion of individual or several nucleotides, and furthermore code for polypeptides with the desired profile of properties.

The invention also encompasses nucleic acid sequences that comprise so-called silent mutations or have been altered, in comparison with a concretely stated sequence, according to the codon usage of a special original or host organism, as well as naturally occurring variants, e.g. splicing variants or allelic variants, thereof.

It also relates to sequences that can be obtained by conservative nucleotide substitutions (i.e. the amino acid in question is replaced by an amino acid of the same charge, size, polarity and/or solubility).

The invention also relates to the molecules derived from the concretely disclosed nucleic acids by sequence polymorphisms. These genetic polymorphisms can exist between individuals within a population owing to natural variation. These natural variations usually produce a variance of 1 to 5% in the nucleotide sequence of a gene.

Derivatives of nucleic acid sequences according to the invention mean for example allelic variants, having at least 60% homology at the level of the derived amino acid, preferably at least 80% homology, quite especially preferably at least 90% homology over the entire sequence range (regarding homology at the amino acid level, reference should be made to the details given above for the polypeptides). Advantageously, the homologies can be higher over partial regions of the sequences.

Furthermore, derivatives are also to be understood to be homologues of the nucleic acid sequences according to the invention, for example animal, plant, fungal or bacterial homologues, shortened sequences, single-stranded DNA or RNA of the coding and noncoding DNA sequence. For example, homologues have, at the DNA level, a homology of at least 40%, preferably of at least 60%, especially preferably of at least 70%, quite especially preferably of at least 80% over the entire DNA region given in a sequence specifically disclosed herein.
Moreover, derivatives are to be understood to be, for example, fusions with promoters. The promoters that are added to the stated nucleotide sequences can be modified by at least one nucleotide exchange, at least one insertion, inversion and/or deletion, though without impairing the functionality or efficacy of the promoters.

Moreover, the efficacy of the promoters can be increased by altering their sequence or can be exchanged completely with more effective promoters even of organisms of a different genus.

3.3 Preparation of functional mutants

The skilled reader is also aware of methods of generating functional mutants.

Depending on the technique applied, a skilled reader may generate arbitrary or directed mutations in genes or non-coding nucleic acid regions (which, for example, may be of importance for regulating gene expression) and, afterwards, may generate suitable gene libraries. The molecular biological method required therefore all well known in the art, and, for example, described by Sambrook and Russell, Molecular Cloning. 3. Edition, Cold Spring Harbor Laboratory Press 2001.

Methods of modifying genes and consequently of modifying the encoded proteins are well known to the skilled reader, as for example

- site-specific mutagenesis wherein single or multiple nucleotides of a gene are specifically replaced (Trower MK (Ed.) 1996; In vitro mutagenesis protocols. Humana Press, New Jersey),


- error-prone polymerase chain reaction (PCR), wherein nucleotide sequences are mutated via the action of an incorrectly functioning DNA-polymerase (Eckert KA, Kunkel TA (1990) Nucleic Acids Res 18:3739);

- SeSaM method (Sequence Saturation Method), wherein preferred substitutions are avoided by the polymerase (Schenk et al., Biospektrum, Vol. 3, 2006, 277-279)

- Passaging of genes in mutator-strains, showing an increased occurrence of mutations of nucleotide sequences, for example in view of a defective DNA-repair mechanism (Greener A, Callahan M, Jerpseth B (1996) An efficient random mutagenesis technique
using an E.coli mutator strain. In: Trower MK (Hrsg.) In vitro mutagenesis protocols. Humana Press, New Jersey), or

- DNA-Shuffling, wherein a pool of closely related genes is formed and digested and wherein the fragments are used as templates for a PCR reaction, and wherein full-length mosaic genes are formed (Stemmer WPC (1994) Nature 370:389; Stemmer WPC (1994) Proc Natl Acad Sci USA 91:10747).

By applying the so-called directed evolution technique (see for example Reetz MT und Jaeger K-E (1999), Topics Curr Chem 200:31; Zhao H, Moore JC, Volkov AA, Arnold FH (1999), Methods for optimizing industrial enzymes by directed evolution, In: Demain AL, Davies JE (Hrsg.) Manual of industrial microbiology and biotechnology. American Society for Microbiology) a skilled reader will be able to specifically prepare functional mutants in large scale. In a first step libraries of a specific protein are generated, for example, by applying any one of the above mentioned methods. Afterwards said libraries are expressed, for example by applying bacteria or phage display systems.

Those genes expressing functional mutants showing the desired feature profile may be selected and subjected to further mutation. The steps of mutation and selection or screening may be repeated iteratively until one of the obtained mutants shows the desired feature profile.

By the iterative approach a limited number of mutations, as for example 1 to 5 mutations, may be performed and their influence on the enzyme feature at issue may be evaluated and further improved mutants may be selected stepwise. Said selected mutant may then be subjected to a further mutation in substantially the same way. The number of single mutants to be evaluated may be reduced significantly in this way.

The teaching of the present invention provide important information as regards structure and sequence of the enzyme/ protein at issue, based on which it should be possible to generate further enzymes/proteins with the desired modified feature profile. In particular, so-called hot spots, i.e. sequence regions may be defined, which potentially may be suited for further mutation in order to modify or generate a desired feature of the enzyme/protein.

3.4 Constructs according to the invention

The invention also relates to expression constructs, containing, under the genetic control of regulatory nucleic acid sequences, a nucleic acid sequence coding
for a polypeptide or fusion protein according to the invention; as well as vectors comprising at least one of these expression constructs.

"Expression unit" means, according to the invention, a nucleic acid with expression activity, which comprises a promoter as defined herein and, after functional association with a nucleic acid that is to be expressed or a gene, regulates the expression, i.e. the transcription and the translation of this nucleic acid or of this gene. In this context, therefore, it is also called a "regulatory nucleic acid sequence". In addition to the promoter, other regulatory elements may be present, e.g. enhancers.

"Expression cassette" or "expression construct" means, according to the invention, an expression unit, which is functionally associated with the nucleic acid that is to be expressed or the gene that is to be expressed. In contrast to an expression unit, an expression cassette thus comprises not only nucleic acid sequences which regulate transcription and translation, but also the nucleic acid sequences which should be expressed as protein as a result of the transcription and translation.

The terms "expression" or "overexpression" describe, in the context of the invention, the production or increase of intracellular activity of one or more enzymes in a microorganism, which are encoded by the corresponding DNA. For this, it is possible for example to insert a gene in an organism, replace an existing gene by another gene, increase the number of copies of the gene or genes, use a strong promoter or use a gene that codes for a corresponding enzyme with a high activity, and optionally these measures can be combined.

Preferably such constructs according to the invention comprise a promoter 5'-upstream from the respective coding sequence, and a terminator sequence 3'-downstream, and optionally further usual regulatory elements, in each case functionally associated with the coding sequence.

A "promoter", a "nucleic acid with promoter activity" or a "promoter sequence" mean, according to the invention, a nucleic acid which, functionally associated with a nucleic acid that is to be transcribed, regulates the transcription of this nucleic acid.

"Functional" or "operative" association means, in this context, for example the sequential arrangement of one of the nucleic acids with promoter activity and of a nucleic acid sequence that is to be transcribed and optionally further regulatory elements, for example nucleic acid sequences that enable the transcription of nucleic acids, and for example a terminator, in such a way that each of the regulatory elements can fulfill its function in the transcription of the nucleic acid sequence. This does not
necessarily require a direct association in the chemical sense. Genetic control sequences, such as enhancer sequences, can also exert their function on the target sequence from more remote positions or even from other DNA molecules. Arrangements are preferred in which the nucleic acid sequence that is to be transcribed is positioned behind (i.e. at the 3’ end) the promoter sequence, so that the two sequences are bound covalently to one another. The distance between the promoter sequence and the nucleic acid sequence that is to be expressed transgenically can be less than 200 bp (base pairs), or less than 100 bp or less than 50 bp.

Apart from promoters and terminators, examples of other regulatory elements that may be mentioned are targeting sequences, enhancers, polyadenylation signals, selectable markers, amplification signals, replication origins and the like. Suitable regulatory sequences are described for example in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

Nucleic acid constructs according to the invention comprise in particular sequences selected from those, specifically mentioned herein or derivatives and homologues thereof, as well as the nucleic acid sequences that can be derived from amino acid sequences specifically mentioned herein which are advantageously associated operatively or functionally with one or more regulating signal for controlling, e.g. increasing, gene expression.

In addition to these regulatory sequences, the natural regulation of these sequences can still be present in front of the actual structural genes and optionally can have been altered genetically, so that natural regulation is switched off and the expression of the genes has been increased. The nucleic acid construct can also be of a simpler design, i.e. without any additional regulatory signals being inserted in front of the coding sequence and without removing the natural promoter with its regulation. Instead, the natural regulatory sequence is silenced so that regulation no longer takes place and gene expression is increased.

A preferred nucleic acid construct advantageously also contains one or more of the aforementioned enhancer sequences, functionally associated with the promoter, which permit increased expression of the nucleic acid sequence. Additional advantageous sequences, such as other regulatory elements or terminators, can also be inserted at the 3’ end of the DNA sequences. One or more copies of the nucleic acids according to the invention can be contained in the construct. The construct can
also contain other markers, such as antibiotic resistances or auxotrophy-
complementing genes, optionally for selection on the construct.

Examples of suitable regulatory sequences are contained in promoters such as
\(\text{cos}^-\), \(\text{tac}^-\), \(\text{trp}^-\), \(\text{tet}^-\), \(\text{trp-tet}^-\), \(\text{lpp}^-\), \(\text{lac}^-\), \(\text{lacI}^{\text{a}}\) \(\text{T7}^-\), \(\text{T5}^-\), \(\text{T3}^-\), \(\text{gal}^-\), \(\text{trc}^-\), \(\text{ara}^-\), \(\text{rhaP}\)
\(\text{(rhaP}_{\text{BAD}}\text{)}\)SP6-, \(\text{lambda-P}_{\text{R}}^-\) or in the \(\text{lambda-P}_{\text{L}}\) promoter, which find application
advantageously in Gram-negative bacteria. Other advantageous regulatory sequences
are contained for example in the Gram-positive promoters ace, amy and SPO2, in the
yeast or fungal promoters ADC1, MFalpha, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH.
Artificial promoters can also be used for regulation.

For expression, the nucleic acid construct is inserted in a host organism
advantageously in a vector, for example a plasmid or a phage, which permits optimum
expression of the genes in the host. In addition to plasmids and phages, vectors are
also to be understood as meaning all other vectors known to a person skilled in the art,
e.g. viruses, such as SV40, CMV, baculovirus and adenovirus, transposons, IS
elements, plasmids, cosmids, and linear or circular DNA. These vectors can be
replicated autonomously in the host organism or can be replicated chromosomally.
These vectors represent a further embodiment of the invention.

Suitable plasmids are, for example in \(\text{E. coli}\), pLG338, pACYC184, pBR322,
pUC18, pUC19, pKC30, pRep4, pH51, pKK223-3, pDHE19.2, pH52, pPLc236,
pMBL24, pLG200, pUR290, pN-IN\(^{113}\)-B1, \(\lambda\)gt1 1 or pBd C1; in nocard ioform
actinomycetes pJAM2; in \(\text{Streptomyces}\) plJ101, plJ364, plJ702 or plJ361; in bacillus
pUB1 10, pC194 or pBD214; in \(\text{Corynebacterium}\) pSA77 or pAJ667; in fungi pALS1,
pL2 or pBB1 16; in yeasts 2alphaM, pAG-1, YEp6, YEp13 or pEMBLYe23 or in plants
pLGV23, pGHIac\(^+\), pBIN19, pAK2004 or pDH51. The aforementioned plasmids
represent a small selection of the possible plasmids. Other plasmids are well known to
a person skilled in the art and will be found for example in the book Cloning Vectors
ISBN 0 444 904018). Further suitable plasmid are also mentioned in the experimental
part.

In a further embodiment of the vector, the vector containing the nucleic acid
construct according to the invention or the nucleic acid according to the invention can
be inserted advantageously in the form of a linear DNA in the microorganisms and
integrated into the genome of the host organism through heterologous or homologous
recombination. This linear DNA can comprise a linearized vector such as plasmid or just the nucleic acid construct or the nucleic acid according to the invention.

For optimum expression of heterologous genes in organisms, it is advantageous to alter the nucleic acid sequences in accordance with the specific codon usage employed in the organism. The codon usage can easily be determined on the basis of computer evaluations of other, known genes of the organism in question.


The recombinant nucleic acid construct or gene construct is inserted advantageously in a host-specific vector for expression in a suitable host organism, to permit optimum expression of the genes in the host. Vectors are well known to a person skilled in the art and will be found for example in "Cloning Vectors" (Pouwels P.H. et al., Publ. Elsevier, Amsterdam-New York-Oxford, 1985).

3.5 Hosts that can be used according to the invention

Depending on the context, the term "microorganism" means the starting microorganism (wild-type) or a genetically modified microorganism according to the invention, or both.

The term "wild-type" means, according to the invention, the corresponding starting microorganism, and need not necessarily correspond to a naturally occurring organism.

By means of the vectors according to the invention, recombinant microorganisms can be produced, which have been transformed for example with at least one vector according to the invention and can be used for the fermentative production according to the invention.

Advantageously, the recombinant constructs according to the invention, described above, are inserted in a suitable host system and expressed. Preferably,
common cloning and transfection methods that are familiar to a person skilled in the art are used, for example co-precipitation, protoplast fusion, electroporation, retroviral transfection and the like, in order to secure expression of the stated nucleic acids in the respective expression system. Suitable systems are described for example in Current Protocols in Molecular Biology, F. Ausubel et al., Publ. Wiley Interscience, New York 1997, or Sambrook et al. Molecular Cloning: A Laboratory Manual. 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

The parent microorganisms are typically those which have the ability to produce glutaconate, in particular (E)-glutaconate, from glucose and or glutamate.

Preferably they are bacteria, in particular of the orders Clostridials and Fusobacterales. In particular, the species A. fermentans (DSM 20731), C. symbiosium (DSM 934), C. sporosphaeroides (DSM 1294), P. assacharolyticus (ATCC 14963) and F. nucleatum subsp. nucleatum (DSM 15643) have to be mentioned.

ATCC designates American type strain culture collection, and DSM designates Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

The host organism or host organisms according to the invention preferably contain at least one of the nucleic acid sequences, nucleic acid constructs or vectors described in this invention, which code for an enzyme activity according to the above definition.

3.6 Fermentative production of glutaconate products of the invention

The invention relates to methods for the fermentative production of glutaconate and related compounds of formula (I).

The recombinant microorganisms as used according to the invention can be cultivated continuously or discontinuously in the batch process or in the fed batch or repeated fed batch process. A review of known methods of cultivation will be found in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und perifere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium that is to be used must satisfy the requirements of the particular strains in an appropriate manner. Descriptions of culture media for various microorganisms are given in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D. C., USA, 1981).
These media that can be used according to the invention generally comprise one or more sources of carbon, sources of nitrogen, inorganic salts, vitamins and/or trace elements.

Preferred sources of carbon are sugars, such as mono-, di- or polysaccharides. Very good sources of carbon are for example glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or cellulose. Sugars can also be added to the media via complex compounds, such as molasses, or other by-products from sugar refining. It may also be advantageous to add mixtures of various sources of carbon. Other possible sources of carbon are oils and fats such as soybean oil, sunflower oil, peanut oil and coconut oil, fatty acids such as palmitic acid, stearic acid or linoleic acid, alcohols such as glycerol, methanol or ethanol and organic acids such as acetic acid or lactic acid.

Sources of nitrogen are usually organic or inorganic nitrogen compounds or materials containing these compounds. Examples of sources of nitrogen include ammonia gas or ammonium salts, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex sources of nitrogen, such as corn-steep liquor, soybean flour, soybean protein, yeast extract, meat extract and others. The sources of nitrogen can be used separately or as a mixture.

Inorganic salt compounds that may be present in the media comprise the chloride, phosphate or sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron.

Inorganic sulfur-containing compounds, for example sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, but also organic sulfur compounds, such as mercaptans and thiols, can be used as sources of sulfur.

Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts can be used as sources of phosphorus.

Chelating agents can be added to the medium, in order to keep the metal ions in solution. Especially suitable chelating agents comprise dihydroxyphenols, such as catechol or protocatechuic acid, or organic acids, such as citric acid.

The fermentation media used according to the invention may also contain other growth factors, such as vitamins or growth promoters, which include for example biotin, riboflavin, thiamine, folic acid, nicotinic acid, pantothenate and pyridoxine. Growth
factors and salts often come from complex components of the media, such as yeast extract, molasses, corn-steep liquor and the like. In addition, suitable precursors can be added to the culture medium. The precise composition of the compounds in the medium is strongly dependent on the particular experiment and must be decided individually for each specific case. Information on media optimization can be found in the textbook "Applied Microbiol. Physiology, A Practical Approach" (Publ. P.M. Rhodes, P.F. Stanbury, IRL Press (1997) p. 53-73, ISBN 0 19 963577 3). Growing media can also be obtained from commercial suppliers, such as Standard 1 (Merck) or BHI (Brain heart infusion, DIFCO) etc.

All components of the medium are sterilized, either by heating (20 min at 2.0 bar and 121 °C) or by sterile filtration. The components can be sterilized either together, or if necessary separately. All the components of the medium can be present at the start of growing, or optionally can be added continuously or by batch feed.

The temperature of the culture is normally between 15°C and 45°C, preferably 25°C to 40°C and can be kept constant or can be varied during the experiment. The pH value of the medium should be in the range from 5 to 8.5, preferably around 7.0. The pH value for growing can be controlled during growing by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water or acid compounds such as phosphoric acid or sulfuric acid. Antifoaming agents, e.g. fatty acid polyglycol esters, can be used for controlling foaming. To maintain the stability of plasmids, suitable substances with selective action, e.g. antibiotics, can be added to the medium. Oxygen or oxygen-containing gas mixtures, e.g. the ambient air, are fed into the culture in order to maintain aerobic conditions. The temperature of the culture is normally from 20°C to 45°C. Culture is continued until a maximum of the desired product has formed. This is normally achieved within 10 hours to 160 hours.

The cells can be disrupted optionally by high-frequency ultrasound, by high pressure, e.g. in a French pressure cell, by osmolysis, by the action of detergents, lytic enzymes or organic solvents, by means of homogenizers or by a combination of several of the methods listed.

3.7 Product isolation

The methodology of the present invention can further include a step of recovering glutaconate or related compounds. The term "recovering" includes extracting, harvesting, isolating or purifying the compound from culture media. Recovering the
compound can be performed according to any conventional isolation or purification methodology known in the art including, but not limited to, treatment with a conventional resin (e.g., anion or cation exchange resin, non-ionic adsorption resin, etc.), treatment with a conventional adsorbent (e.g., activated charcoal, silicic acid, silica gel, cellulose, alumina, etc.), alteration of pH, solvent extraction (e.g., with a conventional solvent such as an alcohol, ethyl acetate, hexane and the like), distillation, dialysis, filtration, concentration, crystallization, recrystallization, pH adjustment, lyophilization and the like. For example glutaconate can be recovered from culture media by first removing the microorganisms. The remaining broth is then passed through or over a cation exchange resin to remove unwanted cations and then through or over an anion exchange resin to remove unwanted inorganic anions and organic acids.

3.8 Polymers

In another aspect, the present invention provides a process for the production of polyesters or polyamides (e.g. nylon ® or related polymers) comprising a step as mentioned above for the production of glutaconate compounds. The glutaconate compound is reacted in a known manner with di-, tri- or polyamines to get polyamides or with di-, tri- or polyols to obtain polyesters. For example, the glutaconate-type compound is reacted with polyamine or polyol containing 4 to 10 carbons.

In another aspect, the present invention provides method of preparing a polymer, in particular copolymers, which method comprises preparing a mono-unsaturated dicarboxylic acid compounds of the general formula (I) as defined above a by method as described therein, isolating said compound; and polymerizing said compound with at least one suitable unsaturated polymerizable monomer, preferably in the presence of a polymerization initiator, like for example sodiumperoxide disulfate (NAPS) as radical initiator.

As non-limiting examples of suitable co-monomers for performing the above polymerization reactions there may be mentioned:

polyols such as ethylene glycol, propylene glycol, glycerol, polyglycerols having 2 to 8 glycerol units, erythritol, pentaerythritol, and sorbitol.
polyamines, such as diamines, triamines and tetraamines, like ethylene diamine, propylene diamine, butylene diamine, neopentyl diamine, hexamethylene diamine, octamethylene diamine, diethylene triamine, triethylene tetramine, tetraethylene pentamine, dipropylene triamine, tripropylene tetramine, dihexamethylene triamine, aminopropylethlenediamine and bisaminopropylethlenediamine. Suitable polyamines are also polyalkylenepolyamines. The higher polyamines can be present in a mixture with diamines. Useful diamines include for example 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 1,8-diaminoctane.

alkenes, in particular C\textsubscript{2}-C\textsubscript{12}-alkenes which are monounsaturated linear or branched hydrocarbons having from 2 to 12 carbon atoms, as for example ethylene, 1- or 2-propylene, 1-, 2- and 3-butylene, 2-methyl-propylene, 1-, 2-, 3- and 4-pentenylene, 1-, 2-, 3-, 4- and 5-hexylene, 1-, 2-, 3-, 4-, 5- and 6-heptylene, or 1-, 2-, 3-, 4-, 5-, 6- and 7-octylene, 1-decene, 1-dodecene; and also their constitutional isomers.

mono-unsaturated C\textsubscript{3}-C\textsubscript{8}-carboxylic acids, like acrylic acid or (d-C \textsubscript{7}-alkyl) acrylic acids, vinylacetic acid, crotonic acid, fumaric acid, maleic acid, itaconic acid esters of monoethylenically unsaturated C\textsubscript{3}-C\textsubscript{8} monocarboxylic acids with C\textsubscript{1}-C\textsubscript{2}0 alkanols, C\textsubscript{5}-C\textsubscript{8} cycloalkanol, phenyl-C\textsubscript{1}-C\textsubscript{4} alkanols or phenoxy-C\textsubscript{1}-C\textsubscript{4} alkanols, examples being esters of acrylic acid with C\textsubscript{1}-C\textsubscript{2}0 alkanols, such as methyl acrylate, ethyl acrylate, n-butyl acrylate, 2-butyl acrylate, isobutyl acrylate, tert-butyl acrylate, 2-ethylhexyl acrylate, decyl acrylate, lauryl acrylate and stearyl acrylate, esters of acrylic acid with C\textsubscript{5}-C\textsubscript{10} cycloalkanols such as cyclohexyl acrylate, esters of acrylic acid with phenyl-C\textsubscript{1}-C\textsubscript{4} alkanols such as benzyl acrylate, 2-phenylethyl acrylate and 1-phenylethyl acrylate, esters of acrylic acid with phenoxy-C\textsubscript{1}-C\textsubscript{4} alkanols such as 2-phenoxyethyl acrylate, esters of methacrylic acid with CrC\textsubscript{20} alkanols, preferably d-C\textsubscript{10} alkanols, such as methyl methacrylate, ethyl methacrylate, n-butyl methacrylate, 2-butyl methacrylate, isobutyl methacrylate, tert-butyl methacrylate, 2-ethylhexyl methacrylate, decyl methacrylate, lauryl methacrylate and stearyl methacrylate, esters of methacrylic acid with C\textsubscript{5}-C\textsubscript{10} cycloalkanols, such as cyclohexyl methacrylate, esters of methacrylic acid with phenyl-C\textsubscript{1}-C\textsubscript{4} alkanols, such as benzyl methacrylate, 2-phenylethyl methacrylate and 1-phenylethyl methacrylate, and esters of methacrylic acid with phenoxy-C\textsubscript{1}-C\textsubscript{4} alkanols, such as 2-phenoxyethyl methacrylate; diesters of monoethylenically unsaturated C\textsubscript{4}-C\textsubscript{8} dicarboxylic acids with CrC\textsubscript{20} alkanols, such as diesters of maleic acid or of fumaric acid with CrC\textsubscript{20} alkanols,
examples being dimethyl maleate, diethyl maleate, di-n-butyl maleate, dimethyl fumarate, diethyl fumarate, and di-n-butyl fumarate;

C1-C20 alkylamides and di-Ci-C20 alkylamides of monoethylenically unsaturated C3-C8 monocarboxylic acids, especially the C1-C20 alkylamides and di-Ci-C20 alkylamides of acrylic acid and of methacrylic acid, for example,

amides of monoethylenically unsaturated carboxylic acids, such as acrylamide or methacrylamide,

anhydrides of monoethylenically unsaturated monocarboxylic and dicarboxylic acids having 3 to 8 C atoms, such as acrylic acid anhydride, methacrylic acid anhydride, maleic anhydride or itaconic anhydride,

hydroxyethyl-C2-C4 alkyl esters of monoethylenically unsaturated monocarboxylic or dicarboxylic acids having 3 to 8 C atoms, such as 2-hydroxyethyl acrylate, 2-hydroxyethyl propionate, 3-hydroxypropyl acrylate, 2-hydroxybutyl acrylate, 4-hydroxybutyl acrylate, 2-hydroxyethyl methacrylate, 2-hydroxypropyl methacrylate, 3-hydroxypropyl methacrylate, 2-hydroxybutyl methacrylate, 4-hydroxybutyl methacrylate,

monoethylenically unsaturated sulfonic acids and their salts, examples being vinylsulfonic acid, allylsulfonic acid, methallylsulfonic acid, styrenesulfonic acid, 2-acrylamido-2-methylpropanesulfonic acid, 2-methacrylamido-2-methylpropanesulfonic acid, 2-acrylamidoethanesulfonic acid, 2-methacrylamidoethanesulfonic acid, 2-acryloyloxyethanesulfonic acid, 2-methacryloyloxyethanesulfonic acid, 3-acryloyloxypropanesulfonic acid and 2-methacryloyloxypropanesulfonic acid,

monoethylenically unsaturated nitriles having 3 to 5 C atoms, such as acrylonitrile and methacrylonitrile,

N-vinyl heterocycles such as N-vinylpyrrolidone, N-vinylcaprolactam, N-vinylimidazole, and

monoethylenically unsaturated compounds having at least one Polyc2-C4 alkylene oxide group, examples being vinyl ethers and allyl ethers of poly-C2-C4 alkylene glycols or C1-C30 alkyl-poly-C2-C4 alkylene glycols, esters of monoethylenically unsaturated monocarboxylic and dicarboxylic acids having 3 to 8 C atoms with poly-C2-C4...
C₄ alkylene glycols or C₁-C₁₀ alkyl-poly-C₂-C₄ alkylene glycols, amides of monoethylenically unsaturated monocarboxylic and dicarboxylic acids having 3 to 8 C atoms with poly-C₂-C₄ alkylene glycol amines or C₁-C₇,0 alkyl-poly-C₂-C₄ alkylene glycol amines

ethylenically unsaturated compounds having at least one having a basic nitrogen or quaternized nitrogen atom such as diallyldimethyl ammonium chloride, N-methyl-N-vinylimidazolium salts such as the chloride, sulfate or methosulfate, N-(2-(dimethylamino)ethyl) acrylamide, 2-(N,N-dimethylamino)ethyl acrylate, 2-(N,N-dimethylamino)ethyl methacrylate, 2-(N,N-dimethylamino)ethylacrylamide, 3-(N,N-dimethylamino)propylacrylamide, 3-(N,N-dimethylamino)propylmethacrylamide, 2-(N,N-dimethylamino)ethylmethacrylamide, 2-(N,N,N-trimethylammonio)ethyl methacrylate chloride, 2-(N,N,N-trimethylammonio)ethylacrylamide chloride, 3-(N,N,N-trimethylammonio)propylacrylamide chloride, 3-(N,N,N-trimethylammonio)propylmethacrylamide chloride, and the corresponding sulfates and methyl sulfates.

vinyl aromatic monomers, such as styrene, α-methylstyrene, vinyltoluene, tert-butylstyrene, vinylpyridines;

vinyl and allyl esters of aliphatic carboxylic acids having 1 to 20 C atoms, examples being vinyl acetate, vinyl propionate, vinyl butyrate, vinyl hexanoate, vinyl laurate, and vinyl stearate;

conjugated diolefins such as butadiene and isoprene, and

halovinyl compounds such as chloroethene (vinyl chloride), 1,1-dichloroethene (vinylidene chloride), fluoroethene, 1,1-difluoroethene, and tetrafluoroethene.

Unless otherwise stated alkyl mar refer to C₁-C₇ alkyl and may be methyl, ethyl, propyl, isopropyl, n-butyl, 2-butyl, sec-butyl, tert-butyl, n-pentyl, 1-methylbutyl, 2-methylbutyl, 3-methylbutyl, 1,2-dimethylpropyl, 1,1-dimethylpropyl, 2,2-dimethylpropyl, 1-ethylpropyl, n-hexyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 4-methylpentyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 2,3-dimethylbutyl, 1,1-dimethylbutyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,1,2-trimethylpropyl,
1,2,2-trimethylpropyl, 1-ethylbutyl, 2-ethylbutyl, 1-ethyl-2-methylpropyl, n-heptyl, 2-heptyl, 3-heptyl, 2-ethylpentyl, 1-propylbutyl etc.

The following examples only serve to illustrate the invention. The numerous possible variations that are obvious to a person skilled in the art also fall within the scope of the invention.

**Experimental Part**

Unless otherwise stated the following experiments have been performed by applying standard equipment, methods, chemicals, and biochemcials as used in genetic engineering, fermentative production of chemical compounds by cultivation of microorganisms and in the analysis and isolation of products. See also Sambrook et al., and Chmiel et al as cited herein above.

**Materials and methods**

a) **Materials**

All chemicals and biochemcials were from Roche (Mannheim, Germany), Sigma (Deisenhofen, Germany), and AppliChem. The enzymes for DNA manipulation, DNA size markers, protein molecular mass markers and the molecular mass standard for SDS/PAGE were from Fermentas GmbH (St. Leon-Rot, Germany). *E. coli* strain BL21 was from Stratagene. Sequencing primers were purchased from MWG-Biotech AG (Ebersberg, Germany). Coenzyme A is from MP Biomedicals. CoA-esters of glutaric and acetic acids were prepared from the corresponding anhydrides (Simon, E.J & Shemin, D. (1953) *J. Am. Chem. Soc.* 75, 2530).

b) **Organisms and growth**

*E. coli* DH5α, used for cloning, and *E. coli* BL21, used for expression were grown under anaerobic conditions at room temperature on Standard I medium (1.5% peptone, 0.3% yeast extract, 100 mM NaCl, 5 mM glucose; Merck, Darmstadt), supplemented with 50 mM MOPS, 3 mM cysteine hydrochloride, 10 mM Na-glutamate, riboflavin and FeCl₂, at various concentrations.

c) **Enzyme activity assay**

2-Hydroxyglutarate dehydrogenase activity was measured at ambient temperature in cuvettes of 0.5 ml total volume containing 0.1 M Tris/HCl pH 8.0, 0.2
mM NADH and 2-hydroxyglutarate dehydrogenase at room temperature. After addition of 1 mM \(\alpha\)-ketoglutarate, the absorbance decrease of NADH was monitored at 340 nm \(\varepsilon = 6.3\, \text{mM}^{-1}\text{cm}^{-1}\) (Bresser J (1997) (R)-2-Hydroxyglutarat-Dehydrogenase aus Acidaminococcus fermentans. PhD Thesis, Philipps-Universita t Marburg, Germany; Martins BM, Macedo-Ribeiro S, Bresser J, Buckel W, Messerschmidt A (2005) Structural basis for stereo-specific catalysis in NAD\(^+\)-dependent (R)-2-hydroxyglutarate dehydrogenase from Acidaminococcus fermentans. Febs J 272:269-281).

**Glutaconate CoA-transferase** activity assay was performed aerobically at room temperature. The increase of absorbance was followed at 412 nm, \(\Delta\varepsilon = 14\, \text{mM}^{-1}\text{cm}^{-1}\).

Reagents used in assay are 0.1 M potassium phosphate pH 7.0, 0.2 M sodium acetate, 1 mM oxaloacetate, 1 mM 5,5'-dithiobis(2-nitrobenzoate) (DTNB), 20 \(\mu\)g citrate synthase, 0.1 mM glutaryl-CoA, total volume 0.5 ml (Buckel W, Dorn U, Semmler R (1981) Glutaconate CoA-transferase from Acidaminococcus fermentans. Eur J Biochem 118:315-321; Jacob U, Mack M, Clausen T, Huber R, Buckel W, Messerschmidt A (1997) Glutaconate CoA-transferase from Acidaminococcus fermentans: the crystal structure reveals homology with other CoA-transferases. Structure 5:415-426).

**2-Hydroxyglutaryl-CoA dehydratase** activity was measured under anoxic conditions at ambient temperature in cuvettes of 0.5 ml total volume containing 50 mM Mops/KOH pH 7.0, 10 mM dithiothreitol, 5 mM MgCl\(_2\), 0.1 mM dithionite, 0.4 mM ATP, and 2-hydroxyglutarate dehydrogenase with activator. After 10 minutes of incubation the reaction was started with 2 mM acetyl-CoA and 2 mM (R)-2-hydroxyglutarate. The increase of absorbance due to the formation of glutaryl-CoA was followed at 290 nm \(\varepsilon = 2.2\, \text{mM}^{-1}\text{cm}^{-1}\) (Kim J, Darley DJ, Buckel W, Pierik AJ (2008) An allylic ketyl radical intermediate in clostridial amino-acid fermentation. Nature 452:239-242).

**Glutaconate** was enzymatically determined with 2 mM acetyl-CoA, 50 mM potassium phosphate pH7.0, 0.25 mM NADPH, glutaconate and using a catalytic amount of enzymes glutaconate-CoA transferase, glutaconyl-CoA decarboxylase [3,5,6] and crotonyl-CoA carboxylase/reductase [9]. The formation of NADP\(^+\) was measured spectrophotometrically at 340 nm.

Glutaconate was also determined by HPLC at room temperature with UV detection at 210 nm using a C18 reverse-phase column in 20 mM sulfuric acid.

d) Other biochemical methods
Protein concentrations were determined using the Bio-Rad microassay with bovine serum albumin as standard. SDS-PAGE was done in a Mini Protein apparatus (Bio-Rad, Heidelberg, Germany). Proteins were stained with Coomassie brilliant blue (Serva, Heidelberg, Germany).

**Example 1: Cloning of genes**

**Cloning methods**

Routine manipulation of DNA, PCR and construction of recombinant plasmids were performed as described in (Sambrook J & Russell DW (2001) Molecular Cloning: a Laboratory Manual, 3rd edn. Cold Spring. Harbor Laboratory Press, Cold Spring Harbor, NY). PfuUltra High-Fidelity DNA Polymerase (Stratagene, USA) was used for the PCR amplification of gctAB using the following primers containing the Ndel: 5'-ATGGT A CATATG TGAGT AAAG TAATGACGT TAAAAGACGCAATCG-3' (SEQ ID NO:39) and XhoI: δ'-ATGGTA CTCGAG TTATTTTGCTTCC GTGGGGA CCTGG-3' (SEQ ID NO:40) restriction sites.

The genes hgdH (2-hydroxyglutarate dehydrogenase) and gctAB (glutaconate CoA-transferase) from A. fermentans were subcloned into pACYCDuet-1 vector (Novagen) (SEQ ID NO:13) from pET-Duet-1 and pJF1 18HE, respectively (Fig. 2) (Fürste JP, Pansegrau W, Frank R, Blocker H, Scholz P, Bagdasarian M, Lanka E (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector).

For cloning of hgdAB (2-hydroxyglutaryl-CoA dehydratase) from C. symbiosum and its activator hgdC from A. fermentans the pASK-IBA3plus vector (IBA GmbH, Gottingen, Germany) (SEQ ID NO:14) was used (Fig. 3).

**Ligation and transformation conditions for hgdH in pACYCDuet-1 vector**

The gene hgdH in pASKIBA7+ (laboratory collection) was subcloned into pETDuet (same procedures as described below) and then transferred to pACYCDuet-1, which can express up to 8 genes (10 kb). Before ligation, pETDuet_hgdH and pACYCDuet-1 vector were separately treated with restriction enzymes BamHI and EcoNI (Fermentas) for 1 h at 37°C (Table 1).
Table 1. Digestion with BamHI and EcoNI

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (dH₂O)</td>
<td>7.0</td>
</tr>
<tr>
<td>10x Buffer Tango</td>
<td>6.0</td>
</tr>
<tr>
<td>BamHI</td>
<td>1.0</td>
</tr>
<tr>
<td>EcoNI</td>
<td>1.0</td>
</tr>
<tr>
<td>pETDuet_hgdH/pACYCDuet-1 vector</td>
<td>15.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>30.0</td>
</tr>
</tbody>
</table>

Table 2. Ligation reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (dH₂O)</td>
<td>14.0</td>
</tr>
<tr>
<td>10 x Ligation buffer</td>
<td>3.0</td>
</tr>
<tr>
<td>T4 DNA ligase (Fermentas)</td>
<td>1.0</td>
</tr>
<tr>
<td>hgdH (Table 1)</td>
<td>10.0</td>
</tr>
<tr>
<td>pACYCDuet-1 vector (Novagen)</td>
<td>2.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The ligation reaction (Table 2) was performed at 23°C. After one hour T4 DNA ligase (Fermentas) was inactivated by incubation at 65°C for 10 minutes. The salts from the ligation mixture were removed by drop dialysis, using a 0.025 μm Millipore membrane, at ambient temperature for 30 minutes. Ligation mixture was added to 50μl competent E.coli DH₅α cells and transferred to an Electroporation Cuvette (Molecular BioProducts). A pulse was given to the cuvette using the following settings: 25 μF, 1.8 kV and 200 Ohm. The cuvette was washed with 300 μl Standard I medium and transferred to a sterile 1.5 ml Eppendorf tube. The transformation mixture was incubated for 1 h at 37°C and plated on a LB agar plate containing chloramphenicol (50 μg·ml⁻¹). The agar plate was incubated overnight at 37°C. Ten white colonies were picked and separately incubated in Standard medium overnight at 37°C: DNA was extracted using the Fermentas kit and digested with BamHI and EcoNI. One out of three correct fragments was extracted from the gel and ligated with gctAB.

**Ligation and transformation conditions for gctAB in pACYCDuet-1 vector, containing hgdH gene**

The gctAB genes were treated with two restriction enzymes Ndel and Xhol, which recognition sites were introduced by PCR reaction (Tables 3 and 4). Also the pACYCDuet-1 vector containing the hgdH gene, pACYCDuet-1_hgdH, was treated with the same restriction enzymes (Table 5). The ligation reaction and transformation were performed under conditions, explained in Table 6.
Table 3. PCR reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (dH₂O)</td>
<td>24.0</td>
</tr>
<tr>
<td>10x <em>PfuUltra</em> HF reaction buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>5.0</td>
</tr>
<tr>
<td>primer 1 (5 µM)</td>
<td>5.0</td>
</tr>
<tr>
<td>primer 2 (5 µM)</td>
<td>5.0</td>
</tr>
<tr>
<td>DNA template (8ng)</td>
<td>5.0</td>
</tr>
<tr>
<td><em>PfuUltra</em> HF DNA polymerase (2.5 U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Table 4. PCR reaction conditions

<table>
<thead>
<tr>
<th>Segment</th>
<th>Number of cycles</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>98°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>29</td>
<td>98°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>29</td>
<td>68°C</td>
<td>20 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>29</td>
<td>72°C</td>
<td>50 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

Table 5. Protocol for digestion

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (dH₂O)</td>
<td>6.0</td>
</tr>
<tr>
<td>10x Buffer R</td>
<td>2.0</td>
</tr>
<tr>
<td>DNA (PCR product)/ pACYCDuet-1_hgdH</td>
<td>10.0</td>
</tr>
<tr>
<td>NdeI</td>
<td>1.0</td>
</tr>
<tr>
<td>XhoI</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 6. Ligation reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (dH₂O)</td>
<td>10.0</td>
</tr>
<tr>
<td>10x Ligation buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>T4 DNA ligase (Fermentas)</td>
<td>1.0</td>
</tr>
<tr>
<td>gctAB</td>
<td>6.0</td>
</tr>
<tr>
<td>pACYCDuet-1_hgdH</td>
<td>1.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Ligation and transformation conditions for *hgdAB* and its activator gene *hgdC* in pASK-IBA 3 vector

The activator *hgdC* was introduced in pASK-IBA 3 vector, containing *hgdAB* genes. Both DNA were separately treated with restriction enzymes Eco47lll and MsiI (Fermentas) for 1 h at 37°C (Table 7). After digestion the enzyme was inactivated by incubation at 65°C for 20 minutes. The salts were removed by drop dialysis as above, at ambient temperature for 30 minutes. To the salt-free reaction, containing pASK-IBA 3_hgdAB, was added 1µl (1 U/µl) of Shrimp Alkaline Phosphatase (Fermentas), 2 µl of 10x Reaction buffer and incubated for 1 h at 37°C. The reaction was stopped by
heating at 65°C for 15 minutes. Ligation reaction (Table 8) and transformation were performed under the conditions explained above. Carbenicillin (100 µg-ml⁻¹) replaced chloramphenicol as antibiotic.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (dH₂O)</td>
<td>1.0</td>
</tr>
<tr>
<td>10x Buffer O</td>
<td>2.0</td>
</tr>
<tr>
<td>DNA (hgdC)/ pASK-IBA 3_hgdAB</td>
<td>15.0</td>
</tr>
<tr>
<td>Eco47III</td>
<td>1.0</td>
</tr>
<tr>
<td>Msl</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 8. Ligation reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Ligation buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>T4 DNA ligase (Fermentas)</td>
<td>1.0</td>
</tr>
<tr>
<td>hgdC</td>
<td>16.0</td>
</tr>
<tr>
<td>pASK-IBA3_hgdAB</td>
<td>1.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.0</td>
</tr>
</tbody>
</table>

**Gene expression**

The genes were transformed into modified *E. coli* BL21-CodonPlus(DE3). An overnight anaerobic preculture (100 ml) of a fresh single colony was used to inoculate 1 litre Standard I medium, described above, containing antibiotics (carbenicillin, 100 µg-ml⁻¹; chloramphenicol, 50 µg-ml⁻¹) and grown at the same conditions. When the culture reached OD₅₇₈ = 0.2, gene expression was induced with isopropyl-1-thio-β-D-galactoside, IPTG (240 mg-liter⁻¹) and anhydrotetracycline, AHT (200 µg-liter⁻¹). Cells were harvested 3 hours after induction (at OD₅₇₈ = 0.573), washed and suspended in 20 ml of buffer (50 mM MOPS, 5 mM MgCl₂ and 2 mM DTT) under anoxic conditions. The induced *E. coli* cells were lysed by French press and cell debris was removed by ultracentrifugation at 100 000 g at 4°C for 1 hour.

**Example 2: Fermentative production of glutaconate**

The first step was the construction of a plasmid, which comprises the six genes of 2-hydroxyglutarate dehydrogenase (*hgdH*), glutaconate CoA-transferase (*gctAB*), and the activator of 2-hydroxyglutaryl-CoA dehydratase (*hgdC*) from *A. fermentans* as well as 2-hydroxyglutaryl-CoA dehydratase (*hgdAB*) from *C. symbiosum* (see example 1).
Determination of enzyme activities

In Table 9 the enzymatic activities are shown, which were measured in the recombinant *E. coli* strain.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity, U</th>
<th>Specific activity, U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hydroxyglutarate dehydrogenase</td>
<td>730</td>
<td>250</td>
</tr>
<tr>
<td>Glutaconate CoA-transferase</td>
<td>0.71</td>
<td>0.25</td>
</tr>
<tr>
<td>2-Hydroxyglutaryl-CoA dehydratase + activator</td>
<td>0.23</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Determination of glutaconate

After growth, the glutaconate concentration in the medium was 0.30 ± 0.05 mM; when glutamate was omitted, the concentration decreased to 0.1 mM (Table 10). Hence, glutaconate indeed was produced indicating that the enzymes work also in vivo. However, the precursor of glutaconate is glutamate, either added as such or from peptone, rather than glucose as initially anticipated.

<table>
<thead>
<tr>
<th>Glutaconate, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>without glutamate</td>
</tr>
<tr>
<td>10 mM glutamate</td>
</tr>
</tbody>
</table>

Addition of riboflavin and iron(II) chloride in the Standard I medium, described above, increased the concentration of glutaconate (Table 11,12).

<table>
<thead>
<tr>
<th>Glutaconate, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM FeCl₂, without riboflavin</td>
</tr>
<tr>
<td>2 mM FeCl₂ + 0.2 mM riboflavin</td>
</tr>
<tr>
<td>2 mM FeCl₂ + 0.4 mM riboflavin</td>
</tr>
</tbody>
</table>
Table 12. Glutaconate in the cell free extract

<table>
<thead>
<tr>
<th>Glutaconate, mM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM FeCl₂, without riboflavin</td>
<td>0.19</td>
</tr>
<tr>
<td>2 mM FeCl₂ + 0.2 mM riboflavin</td>
<td>0.23</td>
</tr>
<tr>
<td>2 mM FeCl₂ + 0.4 mM riboflavin</td>
<td>0.21</td>
</tr>
</tbody>
</table>

The cell-free extract was prepared from 590 mg wet packed cells (approx. 118 mg dried cells) in 20 ml 50 mM MOPS pH 7.4. Assuming a volume of 2.5 ml/g dried cells (Brock M, Bucket W (2004) On the mechanism of action of the antifungal agent propionate. Eur J Biochem 271:3227-41) the internal concentration of glutaconate rises from 0.23 mM to 16 mM.

The original strain *E. coli* BL21 was grown and analysed under the same conditions as the recombinant strain and glutaconate was not detected.

**Discussion**

The data indicate that the recombinant *E. coli* strain indeed produced glutaconate. The substrate, however, is most likely glutamate rather than glucose, because glutamate enhanced the production glutaconate threefold. In the absence of the amino acid, the glutamate present in the peptone most likely is the precursor of glutaconate (Table 10). The use of glucose as precursor requires an electron acceptor, which cannot be oxygen because of the extreme sensitivity of the activator towards this agent (Buckel W, Golding BT (2006) Radical enzymes in anaerobes. Annu Rev Microbiol 60:27-49; Kim J, Darley DJ, Buckel W, Pierik AJ (2008) An allylic ketyl radical intermediate in clostridial amino-acid fermentation. Nature 452:239-242). The iron requirement (Table 11) stems from the iron sulfur clusters in the dehydratase (HgdAB) and its activator (HgdC). The slight improvement with riboflavin is probably due to riboflavin-5'-phosphate (FMN) as prosthetic group of the dehydratase (Hans M, Buckel W, Bill E (2000) The iron-sulfur clusters in 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans*. Biochemical and spectroscopic investigations. Eur J Biochem 267:7082-93). The data further indicate that the concentration of glutaconate inside the cells (16 mM) is about 12-times higher than outside (1.4 mM). Therefore, the export of glutaconate, probably mediated by the succinate transporter (Janausch IG, Zientz E, Tran QH, Kroger A, Unden G.J2002) C4-dicarboxylate carriers...
and sensors in bacteria. Biochim Biophys Acta. 1553:39-56), appears to limit the production of the dicarboxylic acid.

Example 3: Evaluation of alternative substrates for the enzymes of the glutaconate production pathway

Non-limiting examples of further substrates are given below:

a) 2-Hydroxyglutaryl-CoA dehydratase from Clostridium symbiosum and activator from Acidaminococcus fermentans

Table 13

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>(K_m) ((\mu)M)</th>
<th>(k_{cat}) (s(^{-1}))</th>
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<tr>
<td>(R)-2-Hydroxyglutaryl-CoA</td>
<td>(E)-Glutaconyl-CoA</td>
<td>52</td>
<td>83</td>
</tr>
<tr>
<td>(R,S)-2-Hydroxyadipoyl-CoA</td>
<td>(E)-Hex-2-enedioyl-CoA</td>
<td>100</td>
<td>17.4</td>
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<tr>
<td>(E,E)-Muconyl-CoA</td>
<td>(R)-2-Hydroxyhex-4-enedioyl-CoA</td>
<td>570</td>
<td>1.9</td>
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<tr>
<td>(E,E)-2-Hydroxymuconyl-CoA</td>
<td>(R)-2-Hydroxy-5-oxoadipoyl-CoA</td>
<td>1100</td>
<td>0.9</td>
</tr>
<tr>
<td>Butynediol-CoA</td>
<td>Oxaloacetate + CoA</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>(Acetylenedicarboxyl-CoA)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Scheme 1. Reactions catalysed by 2-hydroxyglutaryl-CoA dehydratase. It was assumed that the enzyme also exhibits also 2f?-specificity with substrates other than (R)-2-hydroxyglutaryl-CoA. (Anuthaman Parthasarathy and Wolfgang Buckel, unpublished)

b) (/?)-2-Hydroxyglutarate Dehydrogenase from A. fermentans
Scheme 2: Alternative substrates of (/?)-2-hydroxyglutarate dehydrogenase (NAD<sup>+</sup>). Most likely, both 2-oxoacids are reduced to the 2/?-enantiomers. $K_m$ and $k_{cat}$ values were not determined yet. The enzyme is specific for NAD7NADH. No activity was observed with NADPH/2-oxoglutarate or NADH/oxaloacetate.

c) Glutaconate CoA-transferase from A. fermentans


(R)-2-Hydroxyglutaryl-CoA, (E)-glutaconyl-CoA, muconyl-CoA, butynediyl-CoA (acetylenedicarboxyl-CoA), 4-oxo-hex-2-eneoyl-CoA, 4-nitro-but-2-enoyl-CoA and (RS)-2-hydroxyadipoyl-CoA were prepared by this enzyme using acetyl-CoA and an excess of the neutralised acid. Since the R-emantiomers react faster than S, the product should be mainly (R)-2-hydroxyadipoyl-CoA (Anutthaman Parthasarathy and Wolfgang Buckel, unpublished).

Conclusion:

As all three enzymes of the glutaconate production pathway can also use the C-6 homologues, the production of hex-2-enedioic acid from 2-oxoadipic or 2-aminoadipic acid is also feasible.

The following examples illustrate applicability of glutaconate (a compound of formula I) as obtainable by a biocatalytic method of the present invention for the preparation of organic polymers.
Example 4: Preparation of glutaconic acid - acrylic acid copolymers

Copolymers from glutaconic acid and acrylic acid with molar ratios from 1:1 to 1:3 (glutaconic acid:acrylic acid) were synthesized via radical polymerization methods in water solution using sodium peroxide disulfate (NAPS) as radical initiator.

a) Glutaconic acid - co - acrylic acid (1:1 mol)

In a 500 ml reactor flask were placed glutaconic acid (10.00 g) and distilled water (30.00 g). The mixture was stirred at 98°C under a nitrogen atmosphere for 15 minutes. A solution of NAPS (0.23 g) in distilled water (15.31 g) was added drop wise to the reactor over a period of 5 hours. 5 Minutes after the beginning of the addition of the initiator, a solution of acrylic acid (5.54 g) in distilled water (25.00 g) was also added drop wise to the reactor over a period of 4 hours. The temperature of the reaction was kept at 98°C. At the end of the initiator addition, the reaction was left at 98°C for other 2 hours and then cooled to room temperature.

A light yellow polymer solution was obtained with a solid content of 18.71 g. K-Value = 11.3 g (1wt% in deionized water).

b) Glutaconic acid - co - acrylic acid (1:2 mol)

In a 500 ml reactor flask were placed glutaconic acid (10.00 g) and distilled water (30.00 g). The mixture was stirred at 98°C under a nitrogen atmosphere for 15 minutes. A solution of NAPS (0.32 g) in distilled water (20.76 g) was added drop wise to the reactor over a period of 6 hours. 5 Minutes after the beginning of the addition of the initiator, a solution of acrylic acid (11.08 g) in distilled water (33.23 g) was also added drop wise to the reactor, over a period of 5 hours. The temperature of the reaction was kept at 98°C. At the end of the initiator addition, the reaction was left at 98°C for other 2 hours and then cooled to room temperature.

A light yellow polymer solution was obtained with a solid content of 20.59 g. K-Value = 14.7 g (1wt% in deionized water).
c) **Glutaconic acid - co - acrylic acid (1:3 mol)**

In a 500 ml reactor flask were placed glutaconic acid (10.00 g) and distilled water (30.00 g). The mixture was stirred at 98°C under a nitrogen atmosphere for 15 minutes.

A solution of NAPS (0.40 g) in distilled water (26.00 g) was added drop wise to the reactor over a period of 6 hours. 5 Minutes after the beginning of the addition of the initiator, a solution of acrylic acid (16.62 g) in distilled water (49.86 g) was also added drop wise to the reactor, over a period of 5 hours. The temperature of the reaction was kept at 98°C. At the end of the initiator addition, the reaction was left at 98°C for other 2 hours and then cooled to room temperature.

A light yellow polymer solution was obtained with a solid content of 20.90 g. K-Value = 17.20 g (1wt% in deionized water).

\[ M_n = 13.800 \text{ Da}; \quad M_w = 55.000 \text{ Da} \]

**K-Value determination:**

The K-values of the copolymer water solutions were determined in a deionized water solution at \( pH=7, \ T=25°C \) and with a polymer concentration of 1wt%, according to the procedure described by H. Fikenscher, in "Cellulose-Chemie" (1932), Band 13, 48-64, 71-74.

The documents as cited herein are all incorporated by reference.
### Table 14: List of SEQ ID NOs

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<th>Designation</th>
<th>Organism</th>
<th>Type</th>
<th>SEQ ID NO:</th>
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</tbody>
</table>

**Additional Information:**

- **HgdC**: *F. nucleatum* (NS 37)
- **Nde Primer**: Primer (39)
- **Xho Primer**: Primer (40)
Claims:

1. A biocatalytic method for the production of an unsaturated dicarboxylic acid compound of the general formula I

   \[ \text{HOOC-CH}=\text{CH-}X\text{-COOH} \]  
   \( (I) \)

   wherein
   
   \( X \) represents a linear or branched, optionally unsaturated, optionally substituted hydrocarbon group

   which method comprises;

   converting a 2-hydroxy-substituted dicarboxylic acid compound of the general formula III

   \[ \text{HOOC-C(OH)H-CH}_{2}\text{-}X\text{-COOH} \]  
   \( (III) \)

   wherein
   
   \( X \) is as defined above;

   in a recombinant microorganism co-expressing the genes encoding a glutaconate CoA-transferase (E.C. 2.8.3.12) and a 2-hydroxyglutaryl-CoA dehydratase system, optionally in the presence of a coenzyme A source so that said compound of formula I is formed;

   and optionally isolating said compound of formula I in the form of a substantially pure stereoisomer or as a mixture of stereoisomers.

2. The method of claim 1, wherein \( X \) is selected from \((\text{CH}_2)_n\), with \( n \) being an integer from 1 to 4, \( \text{CH}=\text{CH}, \text{CH}_2\text{-C(=O)}, \text{or CH}=\text{C(OH)} \)

3. The method of claim 1 or 2, wherein said 2-hydroxy-substituted dicarboxylic acid III is formed by said recombinant microorganism by a 2-hydroxyglutarate
dehydrogenase (E.C. 1.1.1.1-) catalyzed conversion of a 2-oxo-dicarboxylic acid compound of formula II

\[ \text{HOOC-C(=O)-CH}_2\text{-X-COOH} \] (II)

wherein

X is defined above.

4. The method of claim 3, wherein the gene of said 2-hydroxyglutarate dehydrogenase is co-expressed by said recombinant microorganism.

5. The method of one of the preceding claims, wherein said oxo-dicarboxylic acid compound of formula II is either added to or fermentatively produced by said recombinant microorganism.

6. The method of claim 5, wherein said microorganism is a glutamate and/or glucose metabolizing aerobic or anaerobic recombinant bacterium, and wherein said compound of formula II is 2-oxoglutarate.

7. The method of claim 6, wherein said glutamate and/or glucose metabolizing recombinant bacterium is selected from the genus *Escherichia*.

8. The method of one of the preceding claims, wherein said 2-hydroxyglutaryl-CoA dehydratase system comprises a 2-hydroxyglutaryl-CoA dehydratase (E.C. 4.2.1.-) and optionally an activator protein for said enzyme.

9. The method of one of the preceding claims, wherein said enzymes and activator protein are of prokaryotic or eukaryotic origin.

10. The method of claim 9, wherein said enzymes and activator originate from the same or different anaerobic bacterium having the ability to convert glutamate into glutaconate.
11. The method of claim 10, wherein said anaerobic bacterium is selected from bacteria of the genus *Acidaminococcus, Clostridium, Fusobacterium, Peptostreptococcus*

12. The method of claim 11, wherein said anaerobic bacterium is *Acidaminococcus fermentans, Clostridium symbiosum, Clostridium sporosphaeroides, Fusobacterium nucleatum* including all subspecies, or *Peptostreptococcus asaccharolyticus*

13. The method of one of the preceding claims, wherein said
   a) 2-hydroxyglutarate dehydrogenase (HgdH) comprises an amino acid sequence of; SEQ ID NO: 2 or 16; or sequences having at least 50% identity thereto;
   b) glutaconate CoA transferase (GctAB) comprises an amino acid sequence of SEQ ID NO: 4 and/or 6; SEQ ID NO: 18 and/or 20; or SEQ ID NO: 22 and/or 24; or sequences having at least 50% identity thereto;
   c) 2-hydroxyglutaryl-CoA dehydratase comprises an amino acid sequence of SEQ ID NO: 8 and/or 10; SEQ ID NO: 26 and/or 28; or SEQ ID NO: 30, 32 and/or 34; or sequences having at least 50% identity thereto;
   d) and the activator protein (HdgC) of c) comprises an amino acid sequence of SEQ ID NO: 12, 36 or 38; or a sequence having at least 50% identity thereto.

14. The method of claim 13, wherein said 2-hydroxyglutaryl-CoA dehydratase is from *C. symbiosum* while said 2-hydroxyglutarate dehydrogenase; said glutaconate CoA transferase and said activator protein are from *A. fermentans*.

15. The method as claimed in any of the preceding claims, wherein said proteins are each encoded by a nucleic acid sequence, which is adapted to the codon usage of said microorganism having the ability to produce said 2-oxo-dicarboxylic acid of formula II.
16. The method as claimed in any of the preceding claims, wherein said proteins are encoded by nucleic acid sequences contained in one or more expression vectors.

17. The method as claimed in any of the preceding claims, wherein at least one of said co-expressed proteins is heterologous to said recombinant microorganism.

18. The method as claimed in any of the preceding claims, wherein a compound of formula I is produced, wherein X is selected from CH₂, C₂H₄, CH=CH; CH=C(OH).

19. An expression cassette, comprising a combination of at least two different nucleic acid sequences each encoding an enzyme or protein as defined in one of the claims 1 to 17, which sequences are operatively linked to at least one regulatory nucleic acid sequence.

20. A recombinant vector, comprising at least one expression cassette as claimed in claim 19.

21. A recombinant prokaryotic or eukaryotic host, transformed with at least one vector as claimed in claim 20.

22. The recombinant host of claim 21 having the ability to produce a 2-oxo-dicarboxylic acid compound of formula (II), which is converted to a compound of formula (I) upon expression of said expression products as encoded by said at least one vector.

23. The host of claim 22, which is a recombinant strain of a bacterium of the genus Escherichia.

24. A method of preparing a polyamide, which method comprises

   a) preparing a mono-unsaturated dicarboxylic acid compounds of the general formula (I) as defined above by method of anyone of claims 1 to 18;

   b) isolating said compound optionally followed by hydrogenation in order to remove the C=C-double bond; and
c) polymerizing said compound as obtained according to step b), with at least one suitable polyvalent amine monomer

25. The method of claim 24, wherein the polyamine is a di-amine, a tri-amine or a mixture thereof.

26. A method of preparing a polyester, which method comprises
   a) preparing a mono-unsaturated dicarboxylic acid compounds of the general formula (I) as defined above a by method of anyone of claims 1 to 18;
   b) isolating said compound optionally followed by hydrogenation in order to remove the C=C-double bond; and
   c) polymerizing said compound as obtained according to step b), with at least one suitable polyvalent alcohol.

27. A method of preparing a polymer, which method comprises
   a) preparing a mono-unsaturated dicarboxylic acid compounds of the general formula (I) as defined above a by method of anyone of claims 1 to 18;
   b) isolating said compound; and
   c) polymerizing said compound as obtained according to step b), with at least one suitable unsaturated polymerizable monomer.
Fig. 1

- Glutamate
- Glucose
- 2-Oxoglutarate
- NADH
- OH
- (R)-2-Hydroxyglutarate
- (E)-Glutaconate
- (E)-Glutaryl-CoA
- (R)-2-Hydroxyglutaryl-CoA
- CoASH
- COO