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(54) **MICROBIOLOGICAL PRODUCTION OF 3-HYDROXYISOBUTYRIC ACID**

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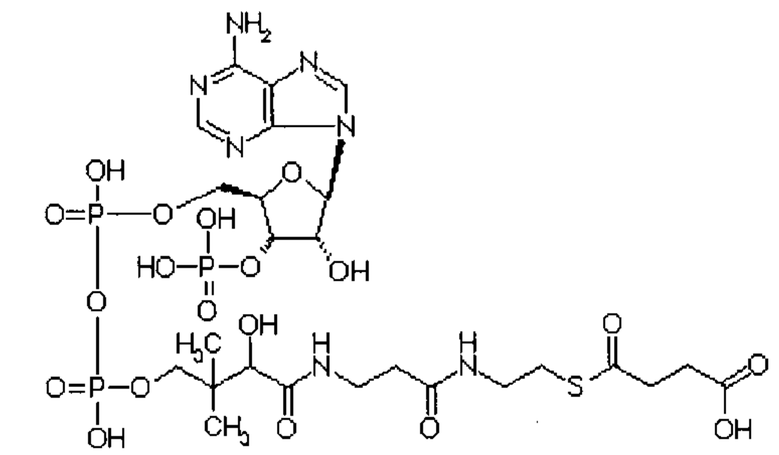
| ***C12N 15/74*** | (2006.01) |
| ***C07K 14/00*** | (2006.01) |

(52) **U.S. Cl.** ..... **435/135**; 435/252.32; 435/146; 536/23.1; 435/320.1; 530/350

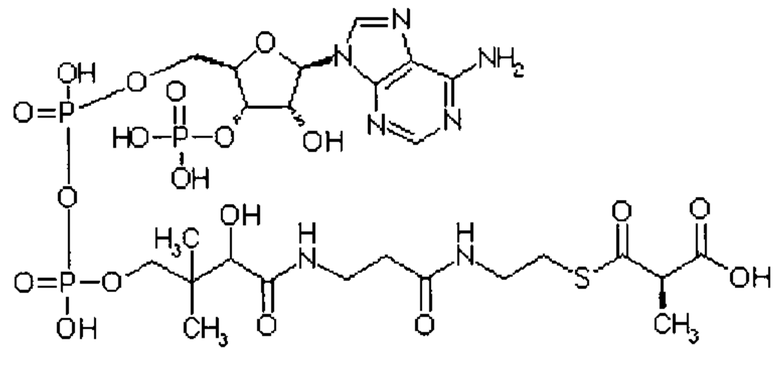
(57) **ABSTRACT**

The present invention relates to a cell which has been modified in comparison with its wild type in such a way that it is capable of forming more, by comparison with its wild, 3-hydroxyisobutyric acid or poly-hydroxyalkanoates based on 3-hydroxyisobutyric acid via methylmalonate-semialdehyde or 3-hydroxybutyryl-coenzyme A as precursors. The invention also relates to a method of generating a genetically modified cell, to the genetically modified cell obtainable by these methods, to a method of producing 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, to a method of producing methacrylic acid or methacrylic esters, and to a method of producing polymethacrylic acid or polymethacrylic esters. The present invention furthermore relates to an isolated DNA, to a vector, to the use of this vector for transforming a cell, to a transformed cell, and to a polypeptide.

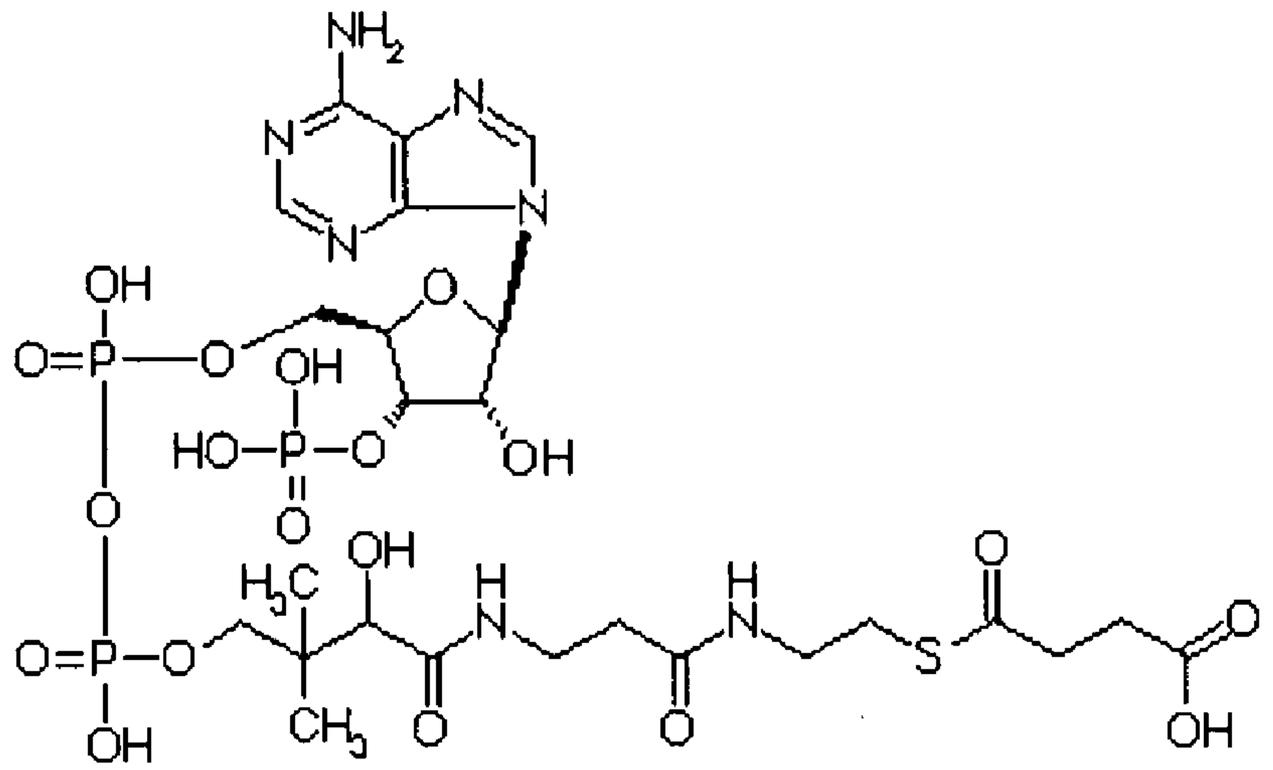


Succinyl-Coenzym A

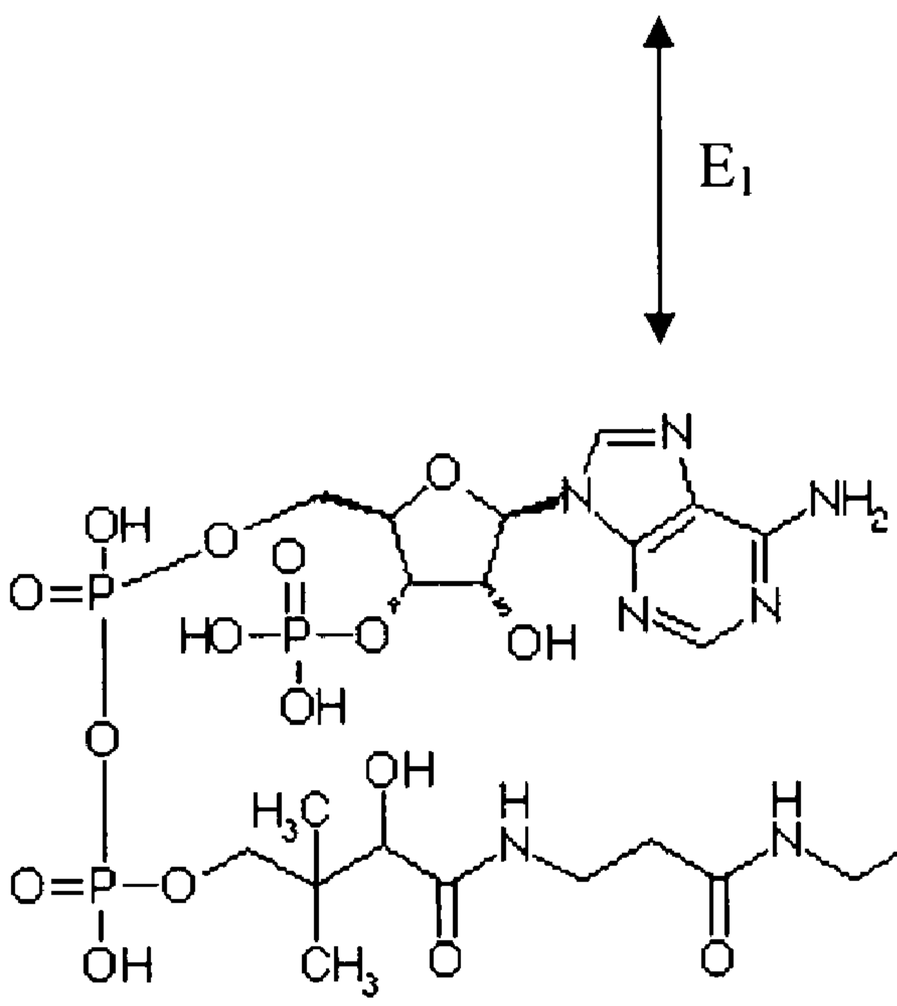
$E_1$



Methylmalonyl-Coenzym A

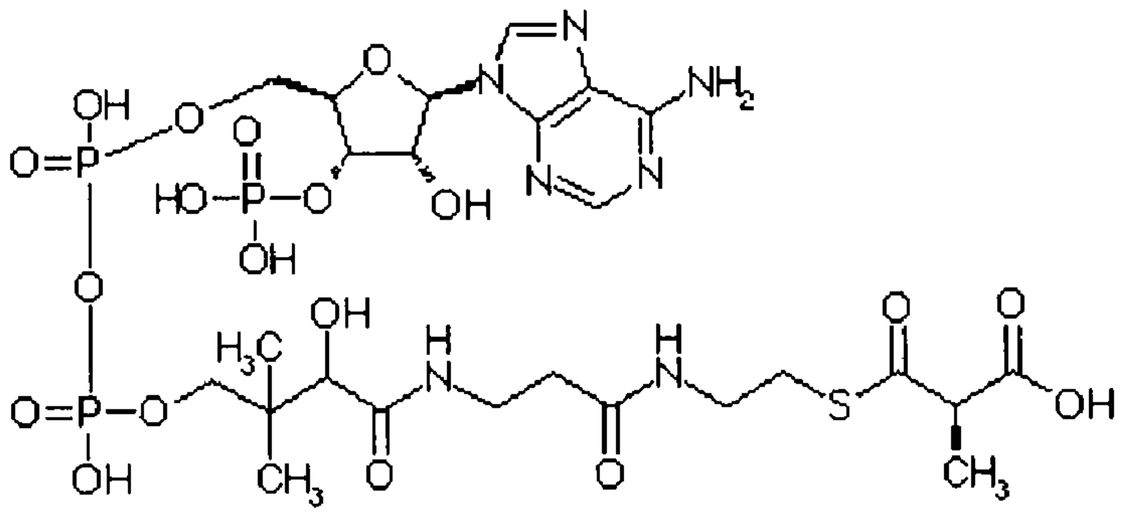


Succinyl-Coenzym A



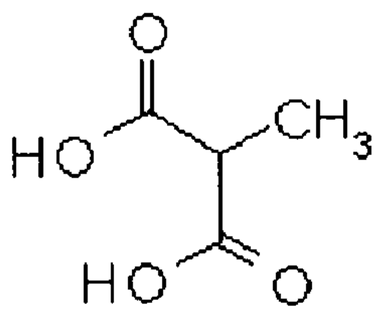
Methylmalonyl-Coenzym A

**Figur 1**



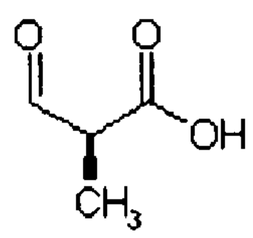
Methylmalonyl-Coenzym A

$E_2$



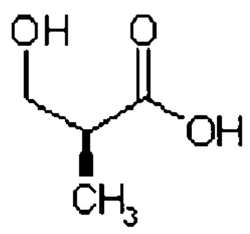
Methylmalonat

$E_3$



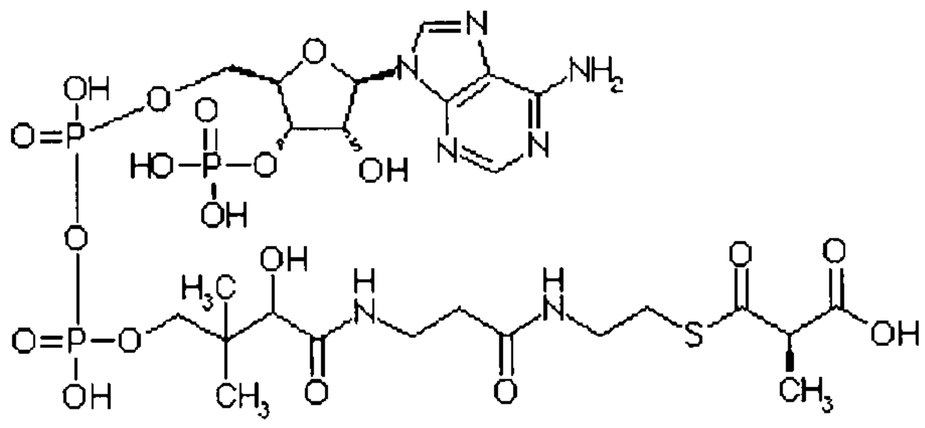
Methylmalonsäuresemialdehyd

$E_4$



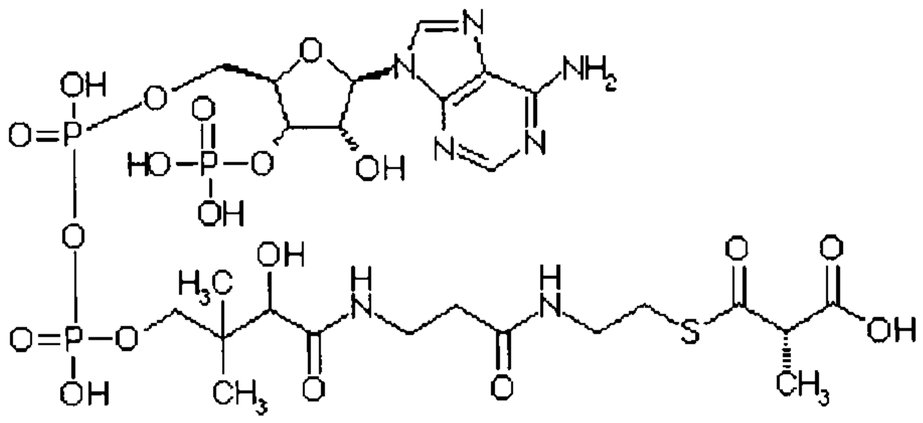
3-Hydroxyisobuttersäure

**Figur 2**



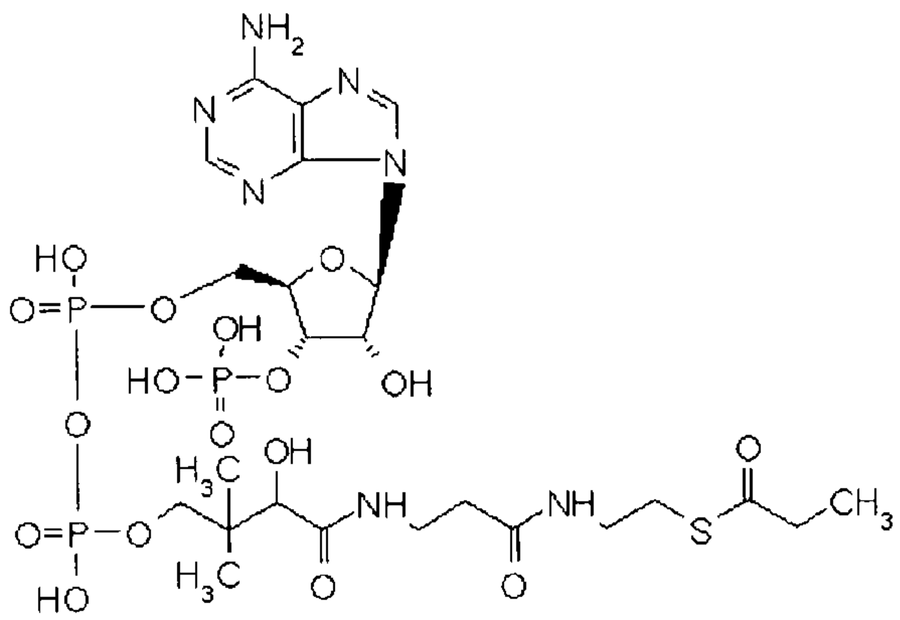
(R)-Methylmalonyl-Coenzym A

$E_6$



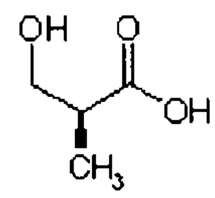
(S)-Methymalonyl-Coenzym A

$E_7$



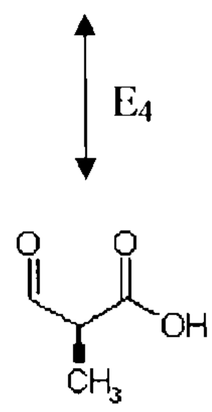
Propionyl-Coenzym A

$E_5$



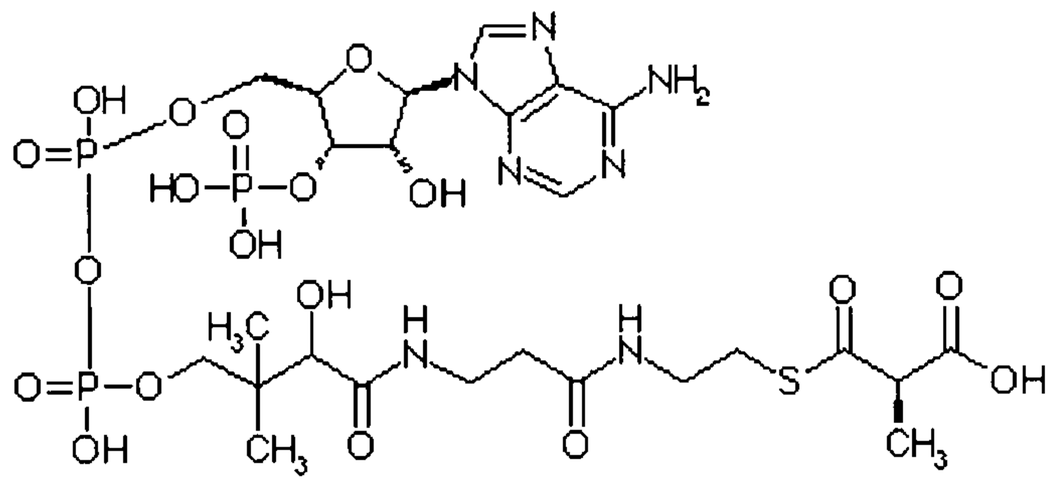
3-Hydroxyisobuttersäure

$E_4$

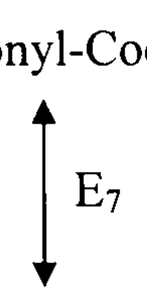


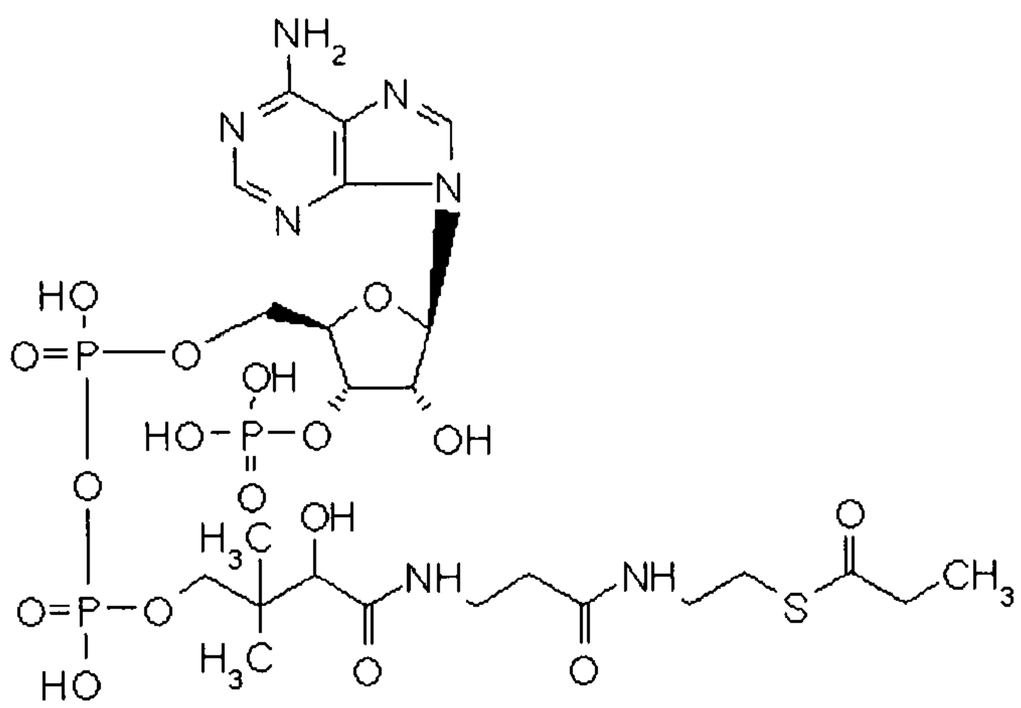
Methylmalonsäuresemialdehyd

**Figur 3**

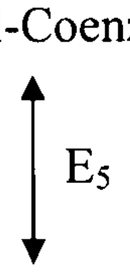


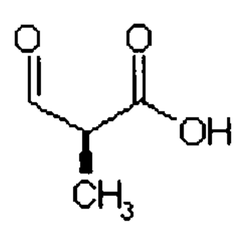
Methylmalonyl-Coenzym A



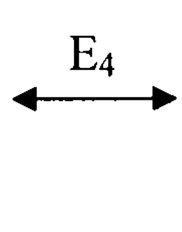



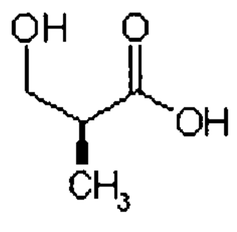
Propionyl-Coenzym A



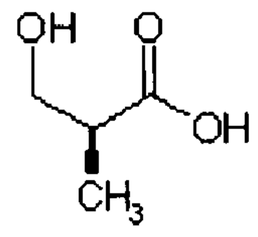
Methylmalonsäuresemialdehyd



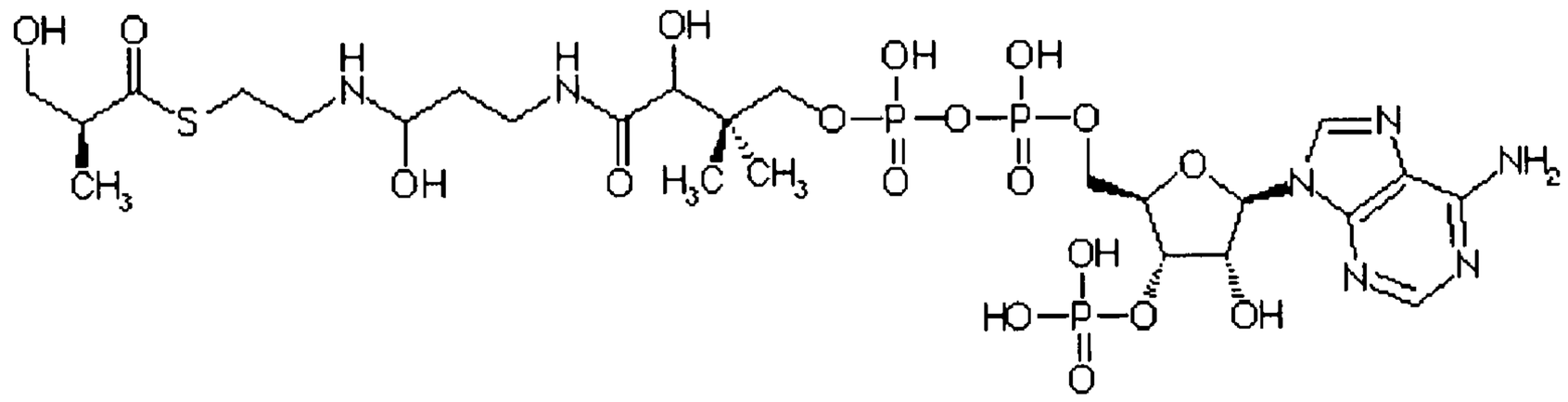
3-Hydroxyisobuttersäure

**Figur 4**



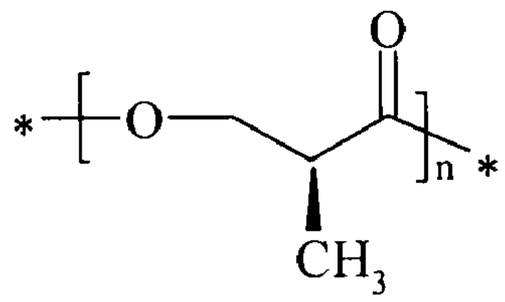
3-Hydroxyisobuttersäure

$E_8$



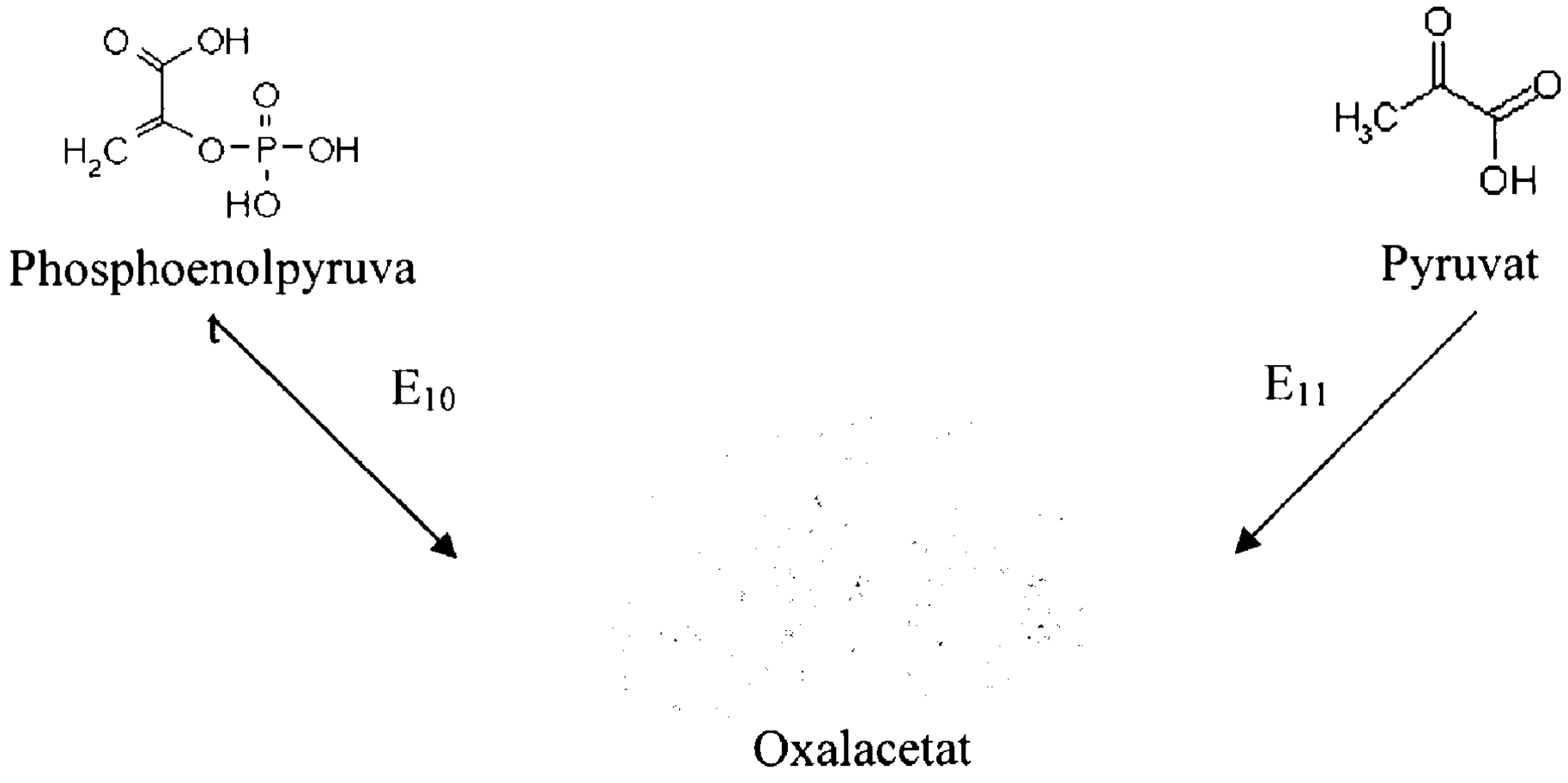
3-Hydroxyisobutyryl-Coenzym A

$E_9$

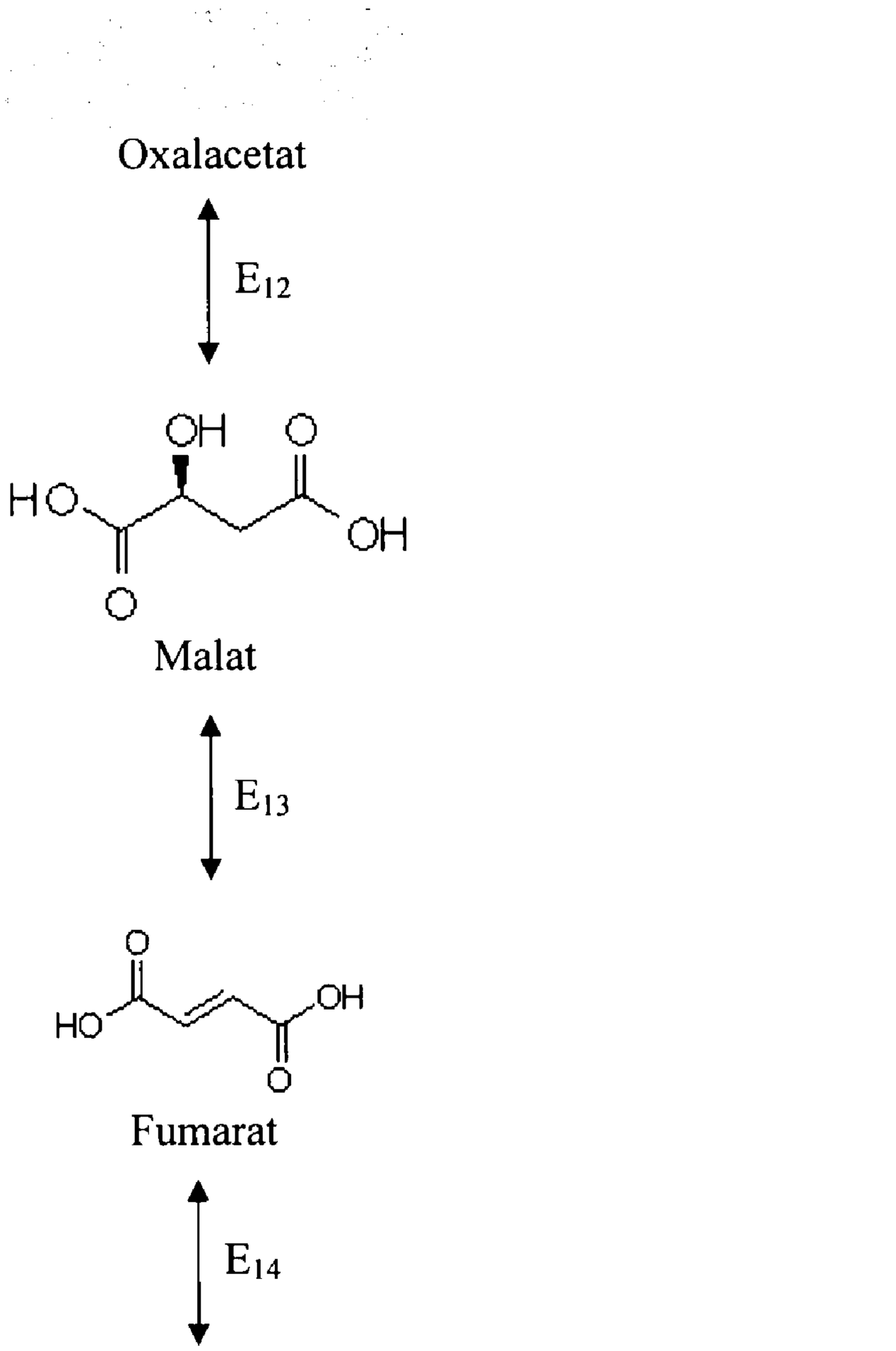


Polyhydroxyalkanoat

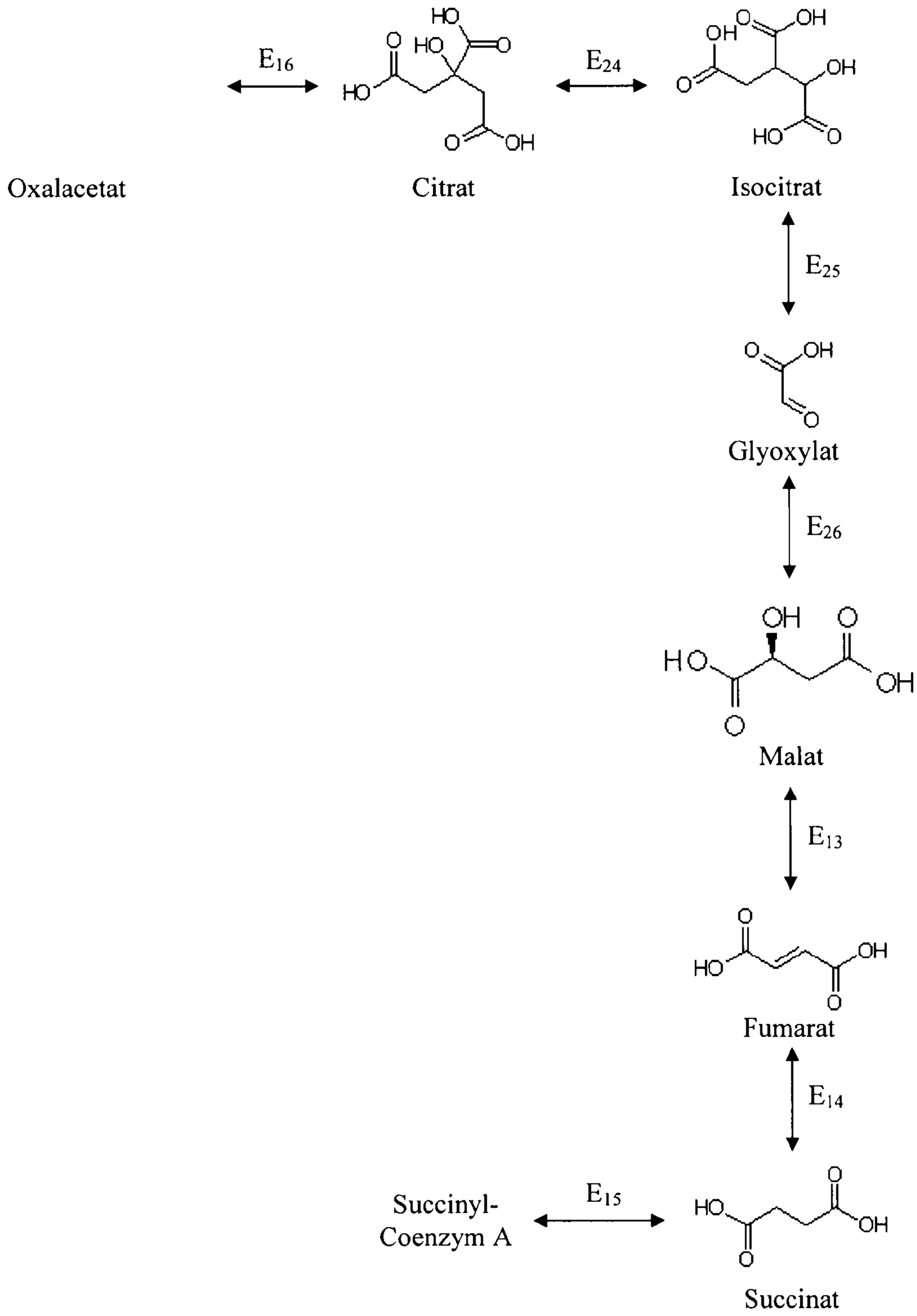
**Figur 5**



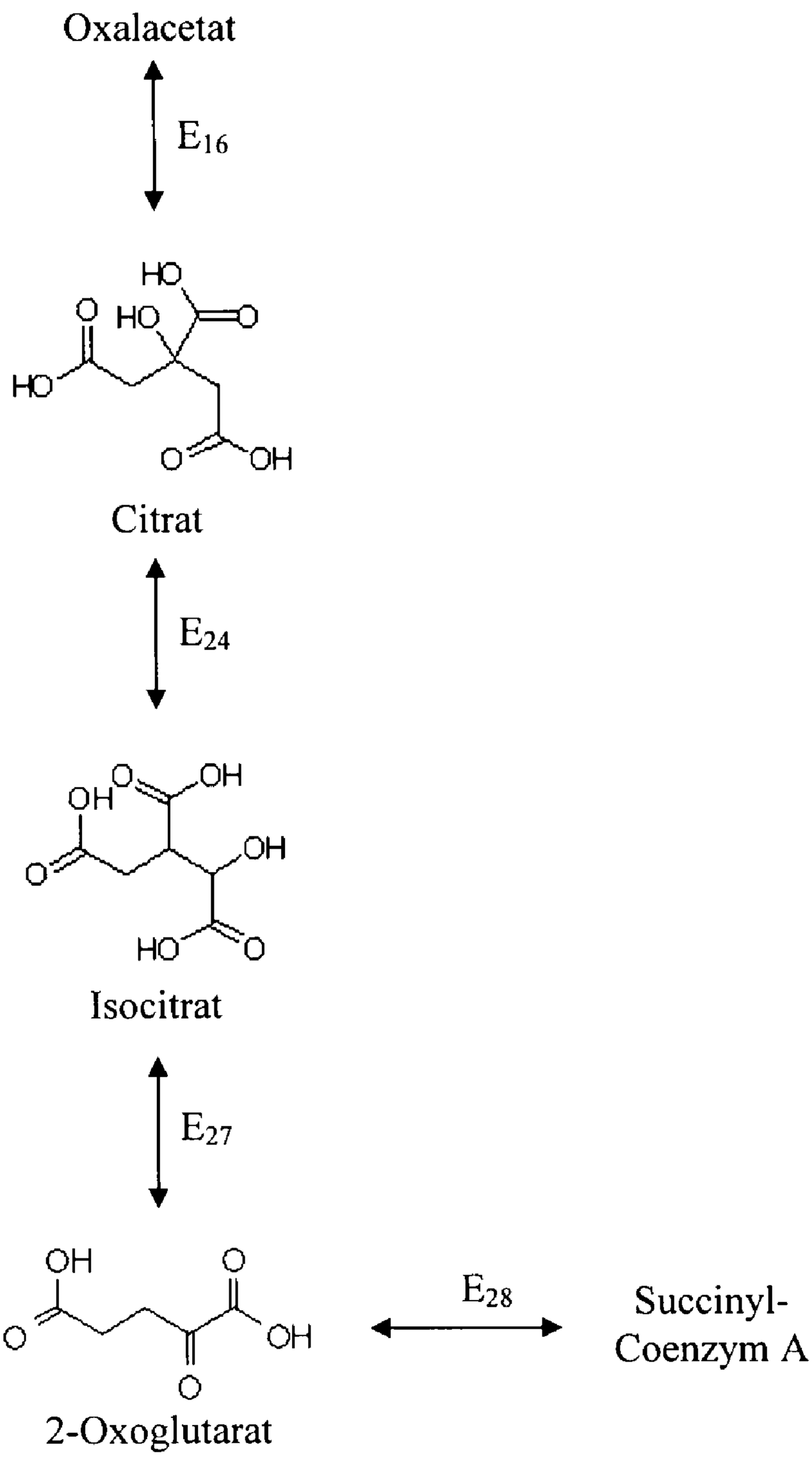

Figur 6


Oxalacetat
E12
OH
O
HO
OH
Malat
E13
O
HO
OH
O
Fumarat
E14
HO
O
OH
O
Succinat
E15
Succinyl-
Coenzym A

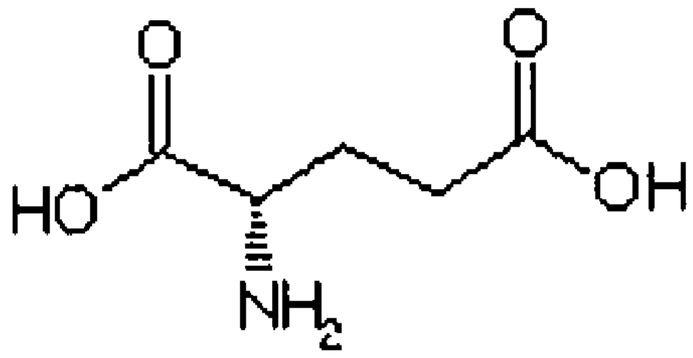
Figur 7


E16
E24
Oxalacetat
Citrat
Isocitrat
E25
Glyoxylat
E26
Malat
E13
Fumarat
E14
Succinyl-
Coenzym A
E15
Succinat

Figur 8

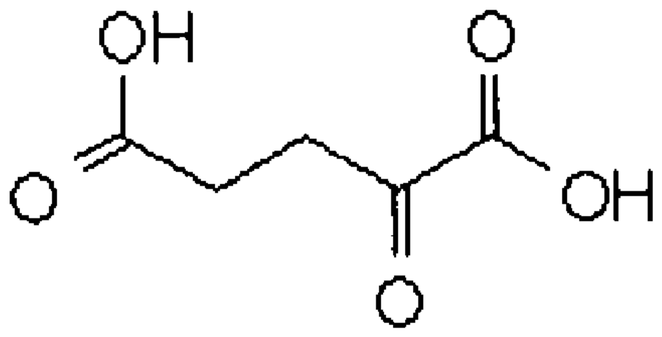

Oxalacetat
E16
Citrat
E24
Isocitrat
E27
2-Oxoglutarat
E28
Succinyl-
Coenzym A

Figur 9




L-Glutamat

$E_{46}$

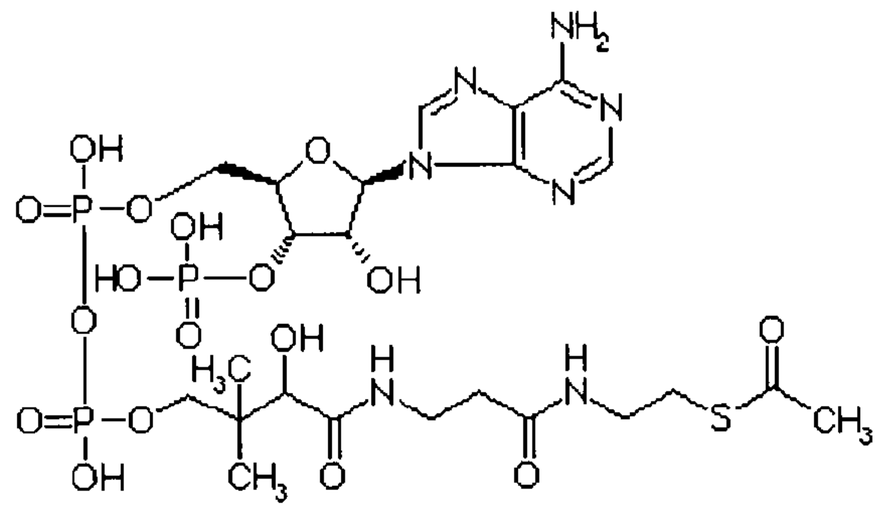



2-Oxoglutarat

$E_{28}$

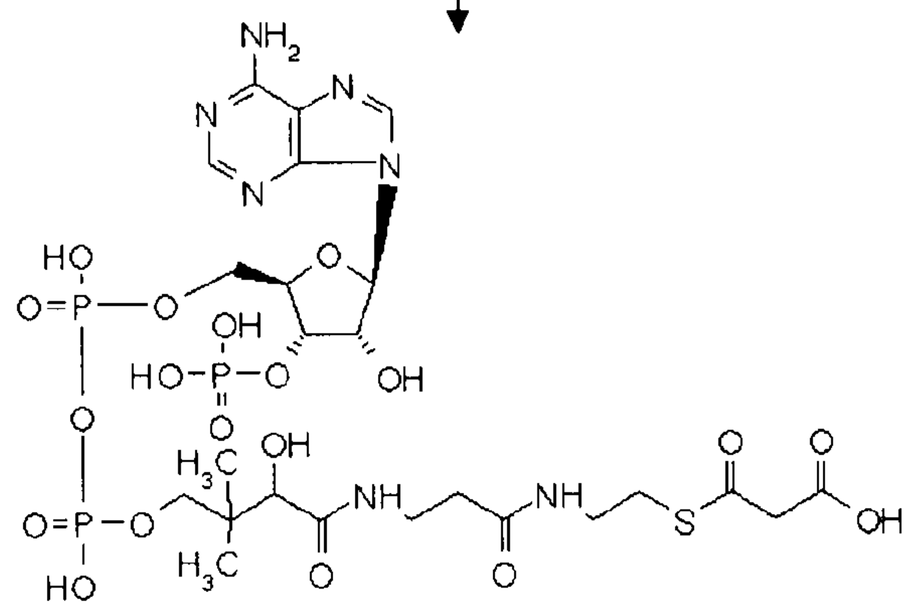
Succinyl-
Coenzym A

**Figur 10**



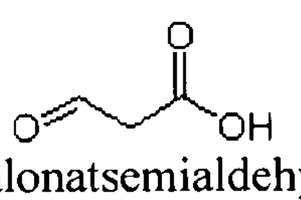
Acetyl-Coenzym A

$E_{47}$



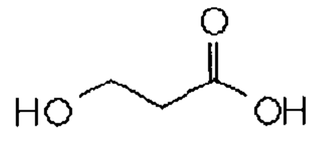
Malonyl-Coenzym A

$E_{48}$



Malonatsemialdehyd

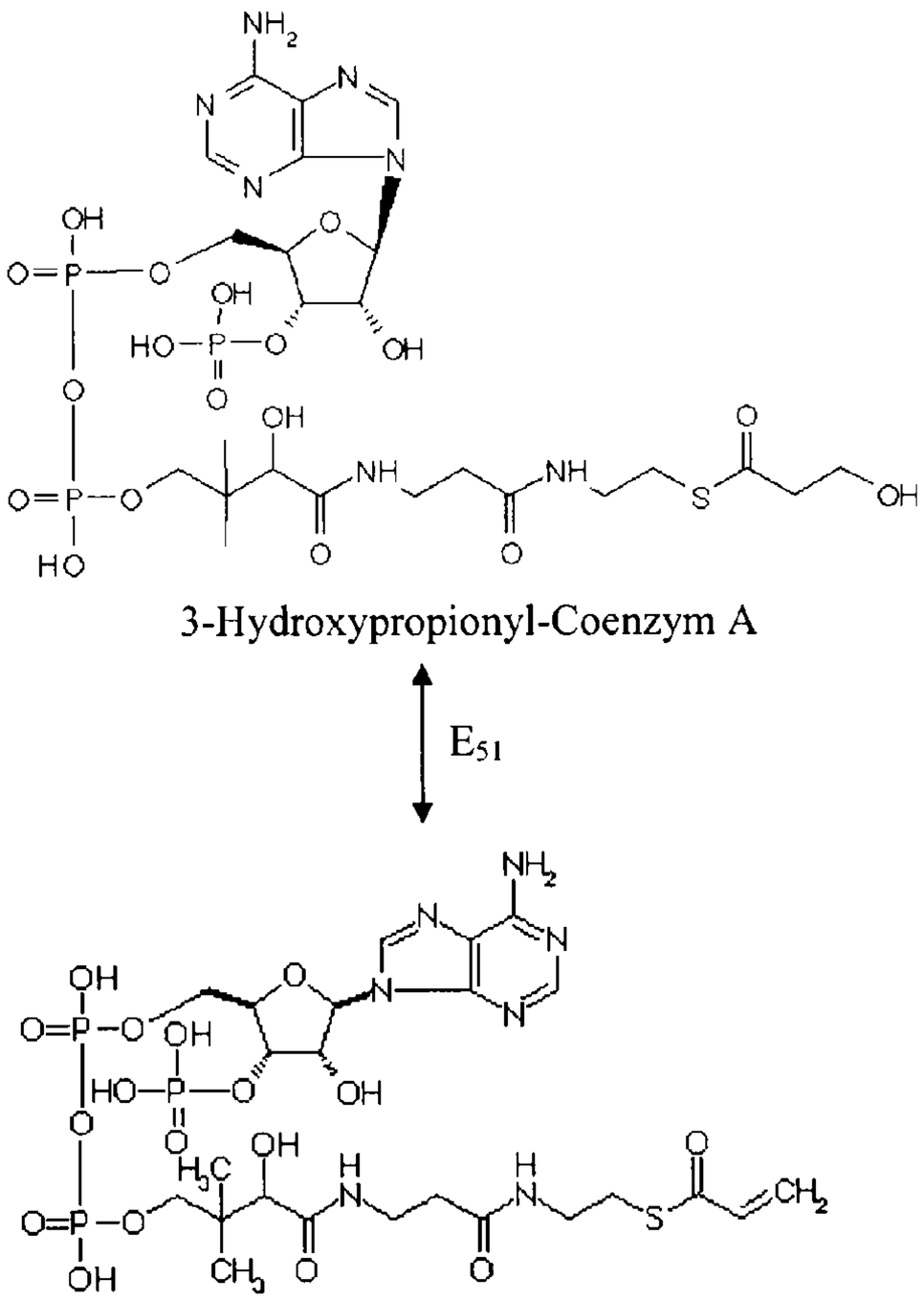
$E_{49}$



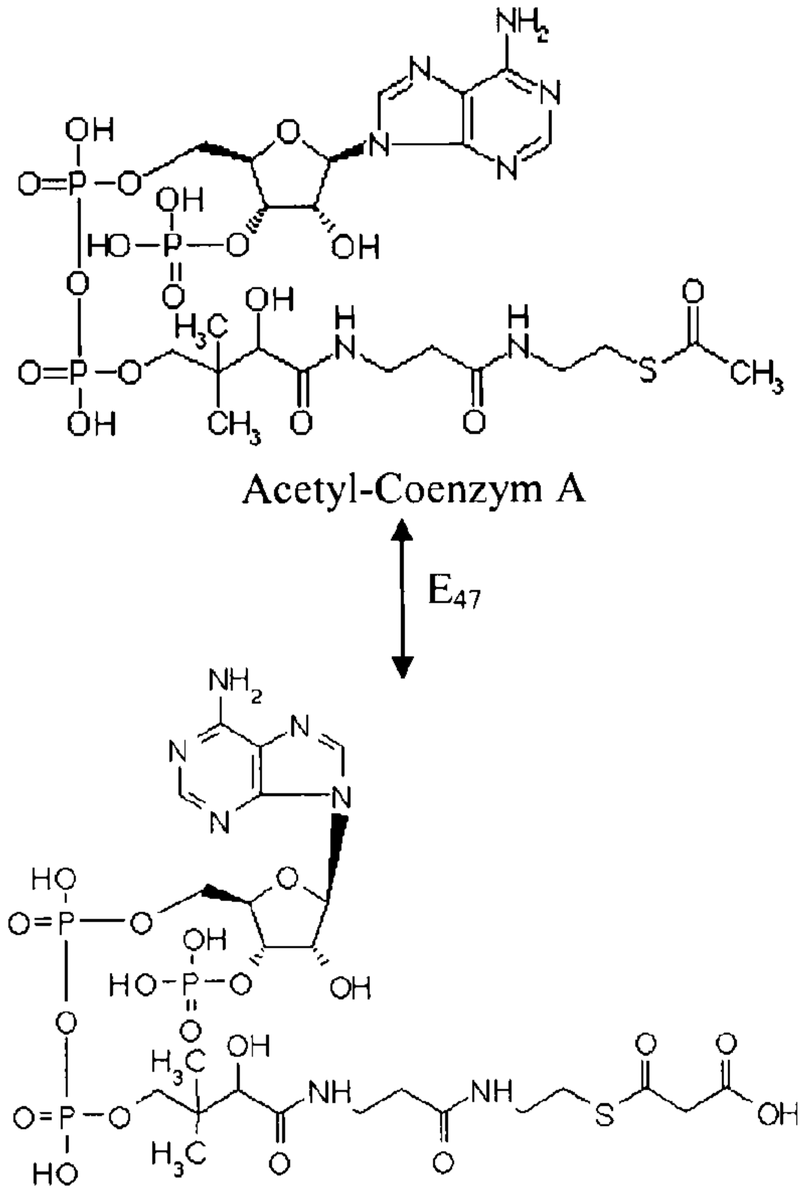
3-Hydroxypropionat

$E_{50}$

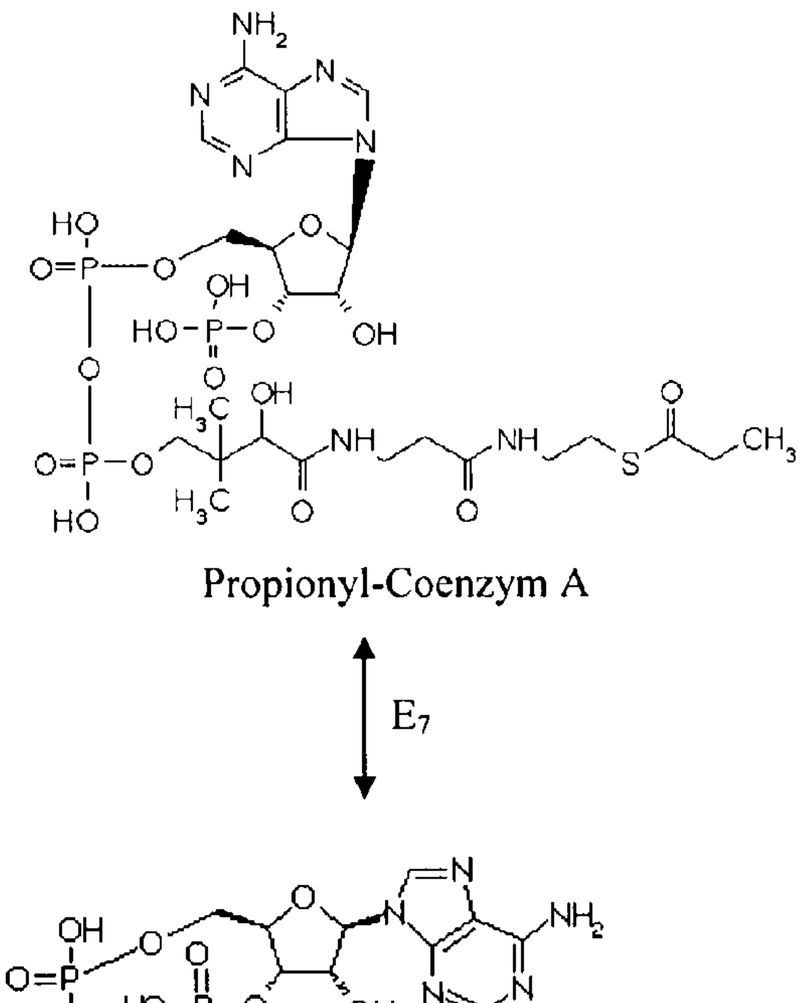
**Figur 11**


3-Hydroxypropionyl-Coenzym A
E51
Acryloyl-Coenzym A
E52
Propionyl-Coenzym A
3-Hydroxyisobuttersäure
E4
E5
Methylmalonsäuresemialdehyd

**Figur 12**

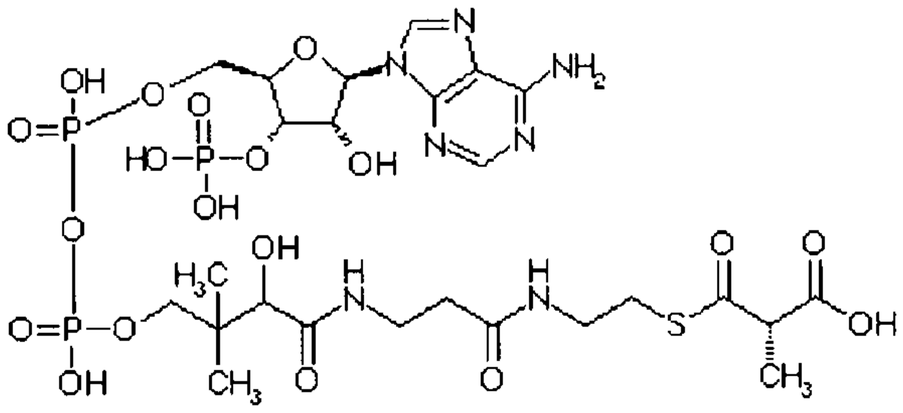

Acetyl-Coenzym A
E47
Malonyl-Coenzym A
E48
Malonatsemialdehyd
E49
3-Hydroxypropionat
E50
3-Hydroxypropionyl-Coenzym A
E51
Acryloyl-Coenzym A
E52

Figur 13



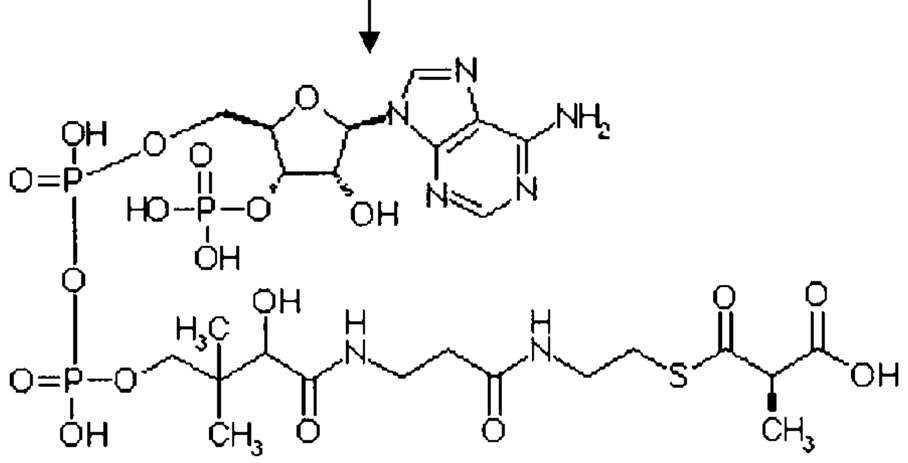
Propionyl-Coenzym A

$E_7$



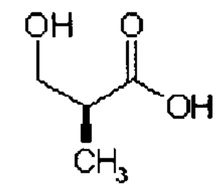
(S)-Methymalonyl-Coenzym A

$E_6$



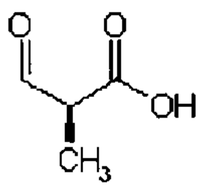
(R)-Methylmalonyl-Coenzym A

$E_2$



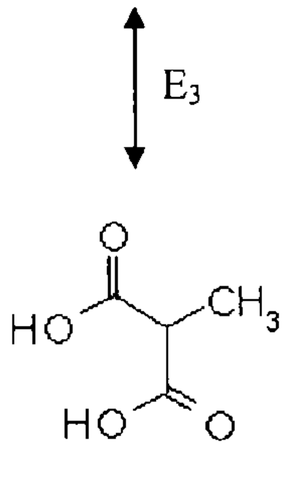
3-Hydroxyisobuttersäure

$E_4$



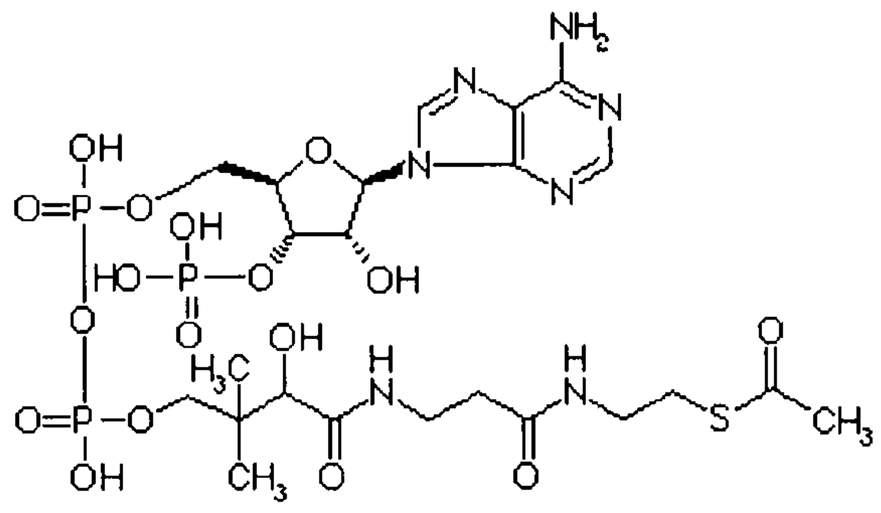
Methylmalonsäuresemialdehyd

$E_3$



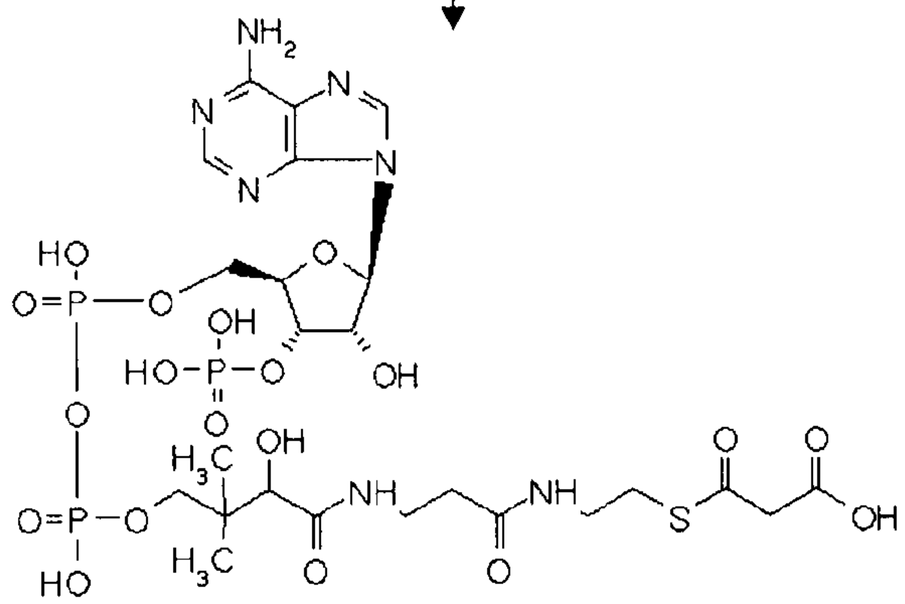
Methylmalonat

**Figur 14**



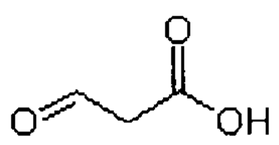
Acetyl-Coenzym A

$E_{47}$



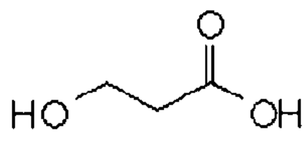
Malonyl-Coenzym A

$E_{48}$



Malonatsemialdehyd

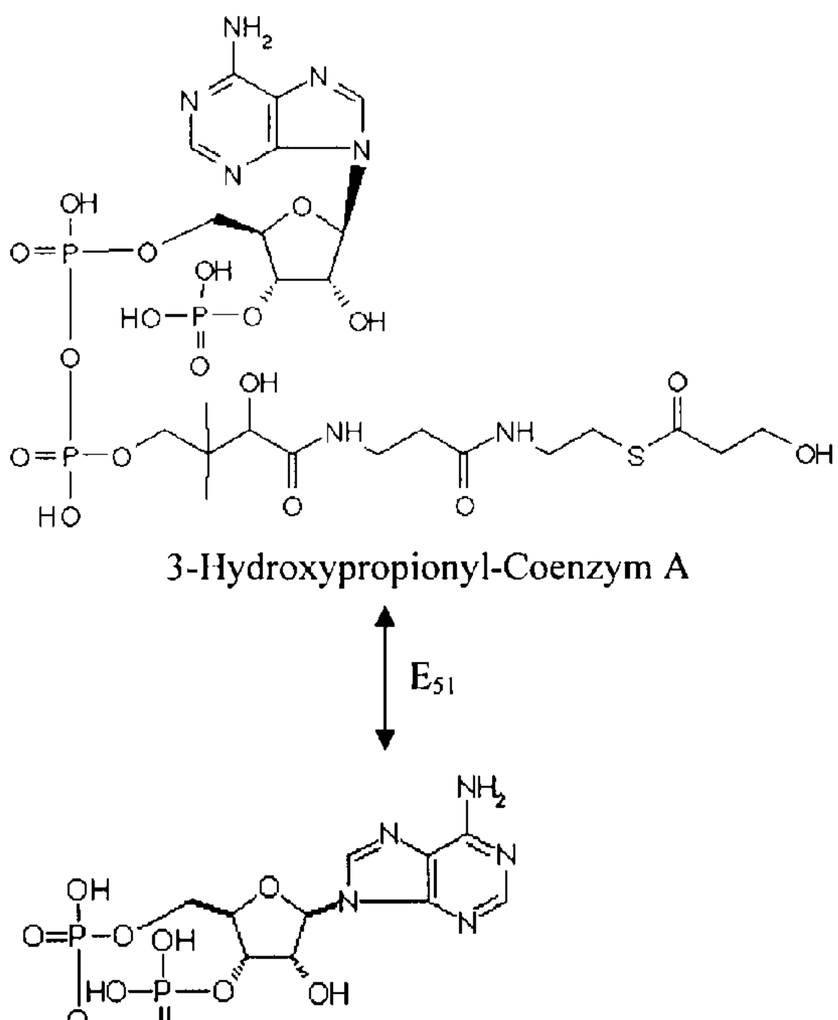
$E_{49}$



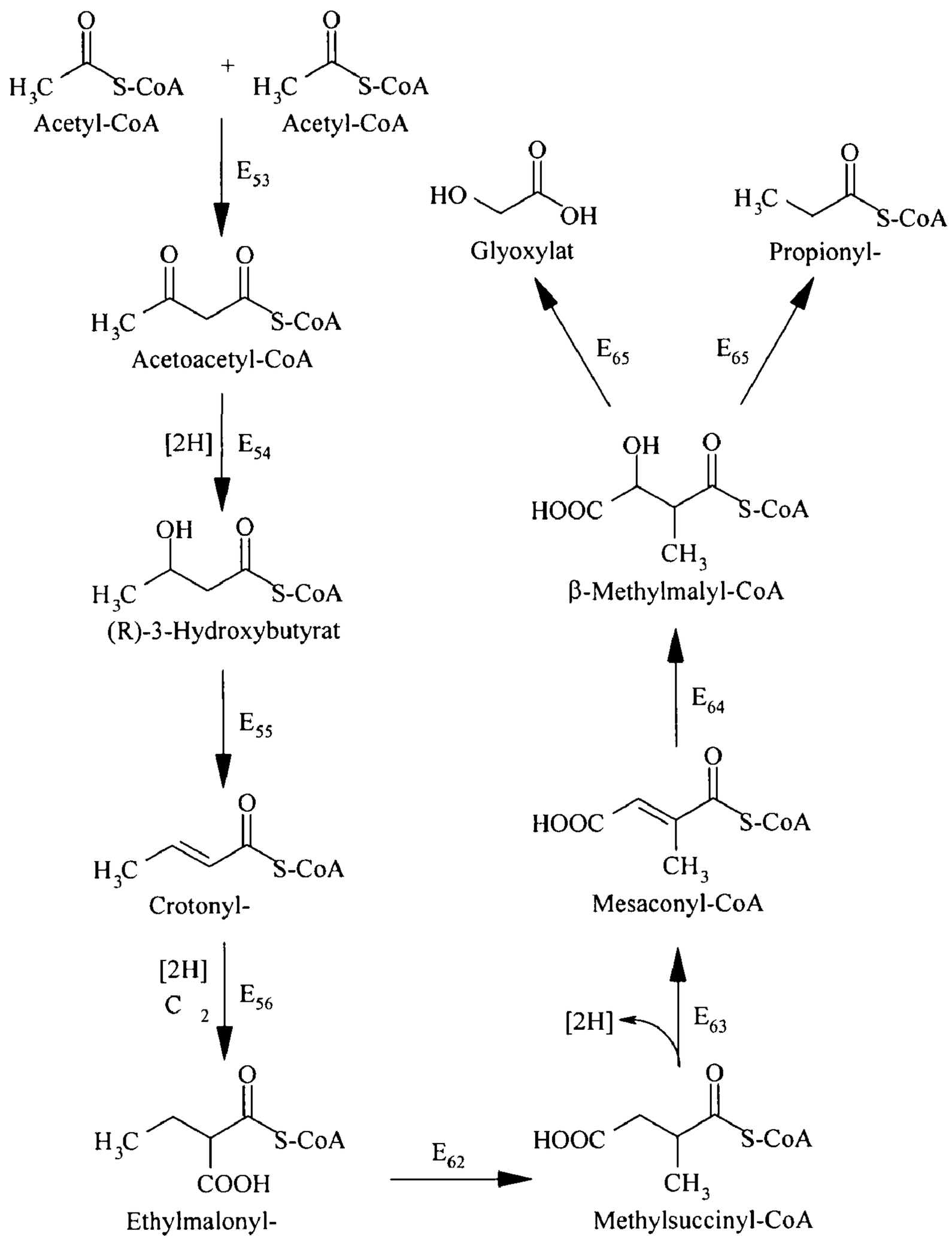
3-Hydroxypropionat

$E_{50}$

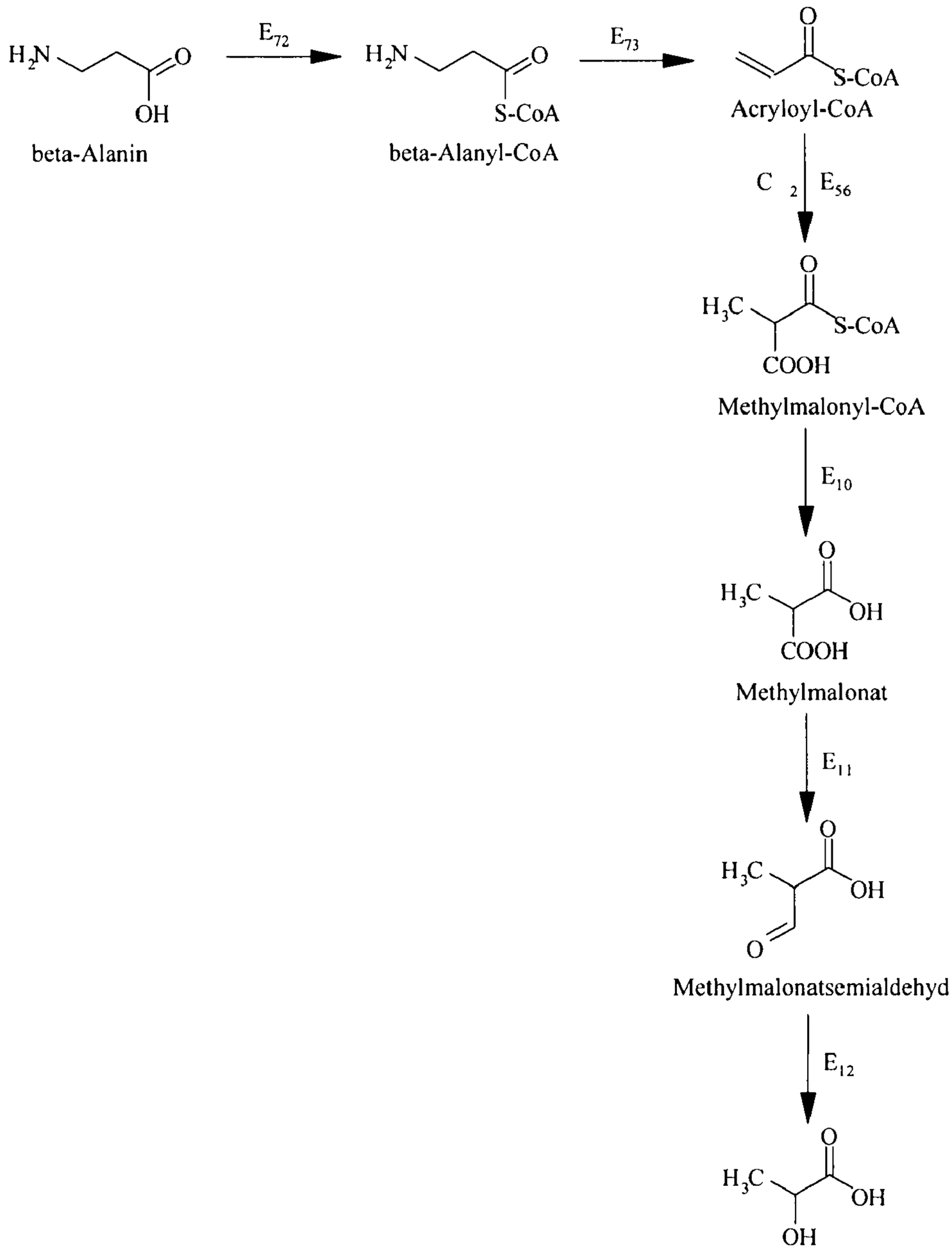
**Figur 15**


3-Hydroxypropionyl-Coenzym A
E51
Acryloyl-Coenzym A
E52
Propionyl-Coenzym A
E7
3-Hydroxyisobuttersäure
E4
Methylmalonsäuresemialdehyd
E3
Methylmalonat
E2
Methylmalonyl-Coenzym A

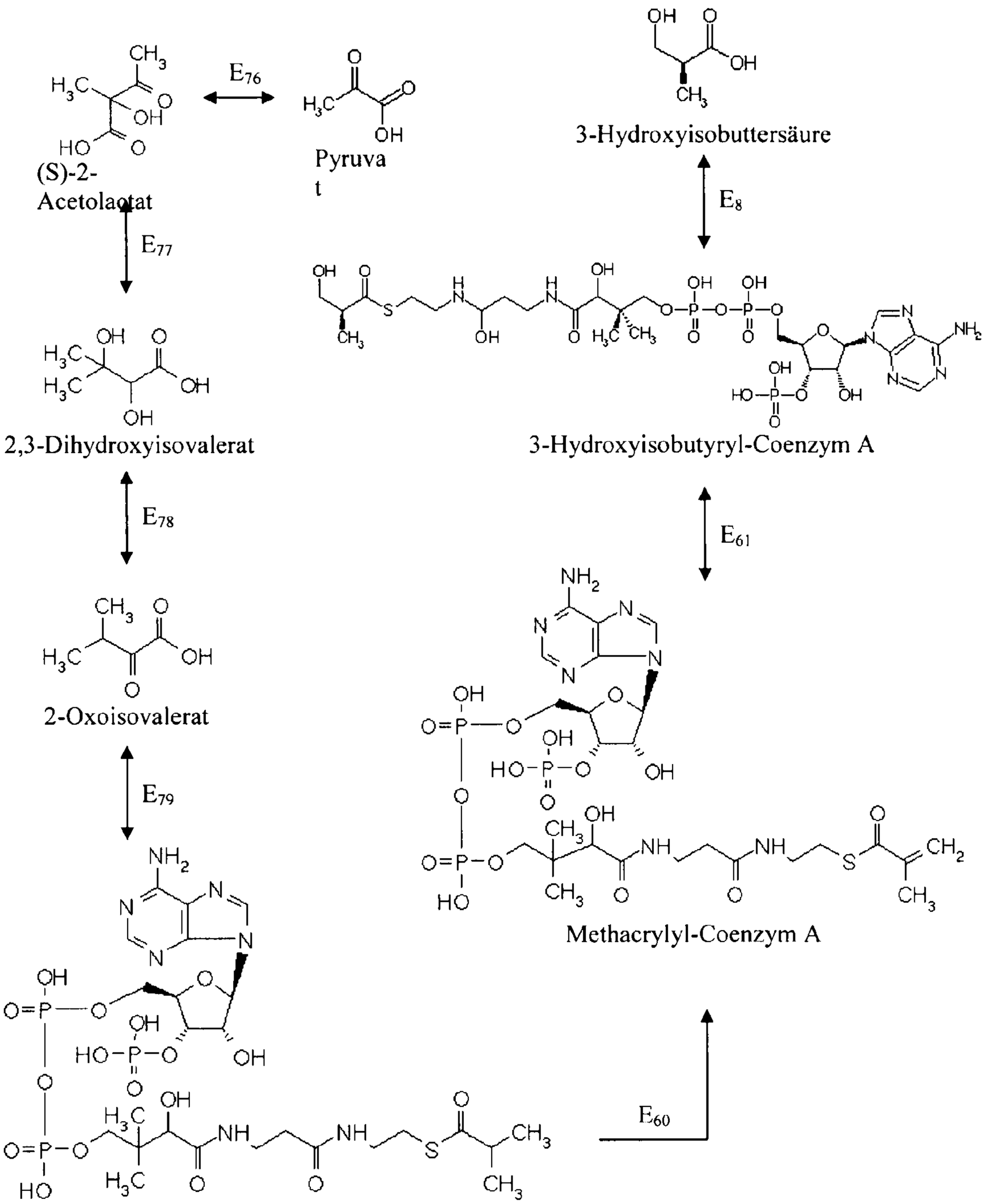
**Figur 16**


Acetyl-CoA
Acetyl-CoA
E53
Acetoacetyl-CoA
[2H]
E54
(R)-3-Hydroxybutyrat
E55
Crotonyl-
[2H]
C 2
E56
Ethylmalonyl-
E62
Methylsuccinyl-CoA
[2H]
E63
E64
Mesaconyl-CoA
E65
E65
β-Methylmalyl-CoA
Glyoxylat
Propionyl-

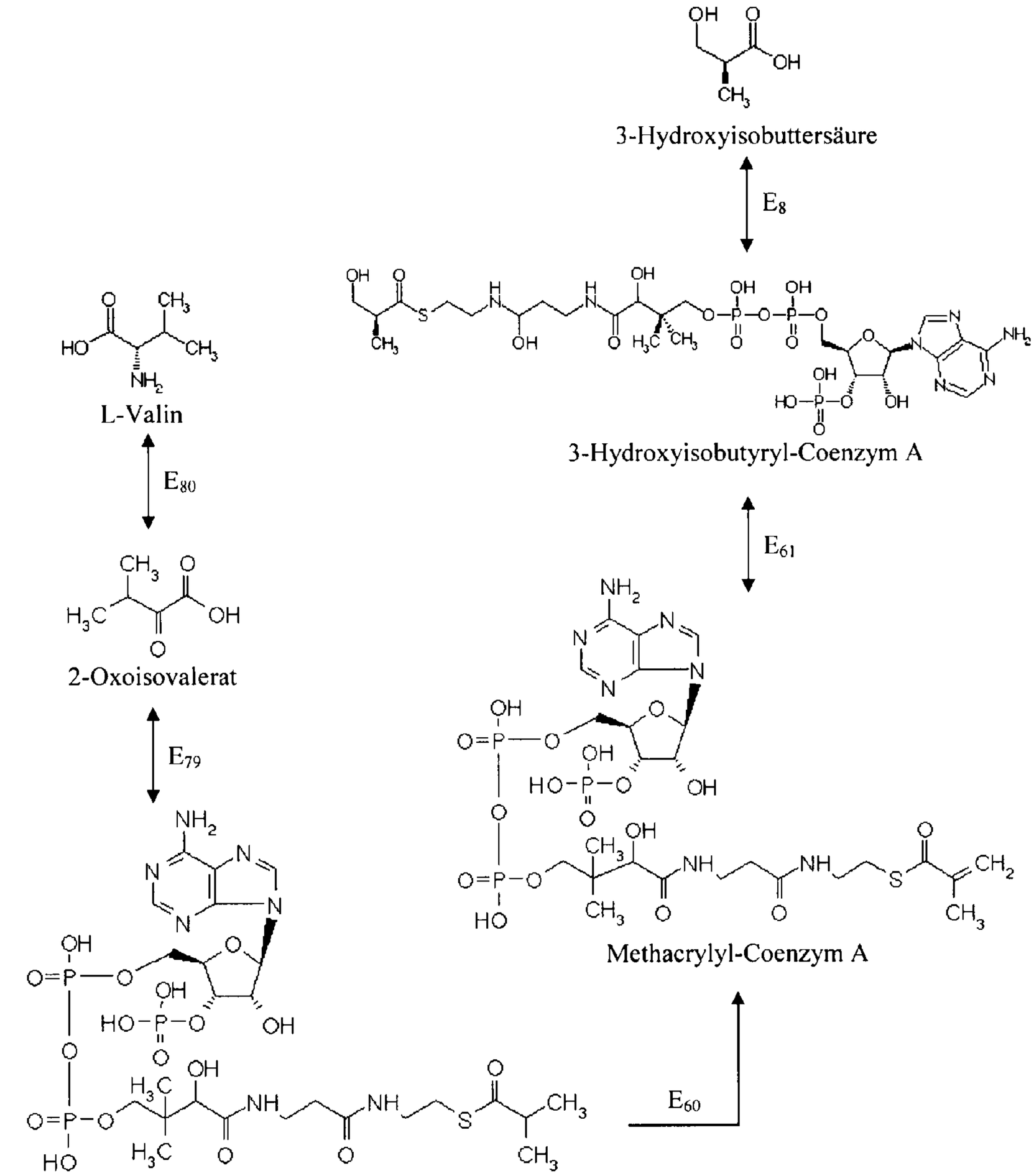
Figur 17


H2N
O
OH
beta-Alanin
E72
H2N
O
S-CoA
beta-Alanyl-CoA
E73
O
S-CoA
Acryloyl-CoA
C 2
E56
H3C
O
S-CoA
COOH
Methylmalonyl-CoA
E10
H3C
O
OH
COOH
Methylmalonat
E11
H3C
O
OH
O
Methylmalonatsemialdehyd
E12
H3C
O
OH
OH
3-Hydroxyisobuttersäure

**Figur 18**


(S)-2-Acetolactat
E76
Pyruvat
E77
2,3-Dihydroxyisovalerat
E78
2-Oxoisovalerat
E79
Isobutyryl-Coenzym A
E60
Methacrylyl-Coenzym A
E61
3-Hydroxyisobutyryl-Coenzym A
E8
3-Hydroxyisobuttersäure

**Figur 19**


3-Hydroxyisobuttersäure
E8
L-Valin
3-Hydroxyisobutyryl-Coenzym A
E80
E61
2-Oxoisovalerat
E79
Methacrylyl-Coenzym A
E60
Isobutyryl-Coenzym A

Figur 20

# MICROBIOLOGICAL PRODUCTION OF 3-HYDROXYISOBUTYRIC ACID

**[0001]** The present invention relates to cells which have been genetically modified in comparison with their wild type, to methods of generating a genetically modified cell, to the genetically modified cells obtainable by these methods, to a process for the preparation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, to a process for the preparation of methacrylic acid or methacrylic esters, and to a process for the preparation of polymethacrylic acid or polymethacrylic esters. The present invention furthermore relates to an isolated DNA, to a vector, to the use of this vector for the transformation of a cell, to a transformed cell, and to a polypeptide.

**[0002]** Methacrylic acid is an important intermediate which is employed for the preparation of polymers, in particular in the form of its alkyl esters. An example of a well-known methacrylic acid derivative is the methyl ester of methacrylic acid. The current global annual production of methyl methacrylate amounts to approximately 1.5 million tonnes. The polymethacrylic esters are raw materials in the plastics sector with a multiplicity of uses.

**[0003]** Methacrylic acid is usually produced commercially by means of the heterogeneous gas-phase oxidation of $C_4$-carbon compounds such as butylene, isobutylene, butane, isobutane, t-butyl alcohol or methacrolein by two-step catalysis on solid multi-metal oxide compositions as the catalyst. The resulting product gas mixture, which, besides methacrylic acid, also comprises a large number of secondary products, is subsequently either subjected to a total condensation reaction, generating aqueous methacrylic acid solution, or absorbed in a suitable solvent mixture. This is usually followed by further purification of the resulting liquid phases by means of distillation, crystallization, extraction, or a combination of these measures. Besides the catalytic gas-phase oxidation of $C_4$-carbon compounds, methacrylic acid can also be formed from isobutyric acid by catalytic oxidative dehydrogenation, as is described for example in EP-A-0 356 315. A further possibility for preparing methacrylic acid is what is known as the "ACH process", in which acetone cyanohydrin and sulfuric acid are reacted with the formation of methacrylamide as intermediate, which then reacts further with water to give methacrylic acid. The resulting methacrylic acid is subsequently purified by distillation. This process is described for example in EP-A-1 359 137.

**[0004]** The disadvantage of these conventional processes for the preparation of methacrylic acid is, inter alia, that during both the preparation of the methacrylic acid itself and during the subsequent steps, which involve purification by distillation, the process steps, which cause thermal stress, result, owing to the pronounced susceptibility of methacrylic acid to polymerization, in the formation of dimers or oligomers; this not only entails additional purification efforts, but also yield losses.

**[0005]** It was an object of the present invention to overcome the disadvantages of the prior art.

**[0006]** In particular, it was an object of the present invention to provide a process for the preparation of methacrylic acid which generates methacrylic acid with a minimum of steps which involve thermal stress.

**[0007]** Furthermore, it is intended that this process makes possible the preparation of methacrylic acid from renewable resources, in particular from carbohydrates and/or glycerol.

**[0008]** A contribution to achieving the abovementioned aims is provided by a cell which has been genetically modified in comparison with its wild type in such a way that it is capable of forming more 3-hydroxyisobutyric acid, or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, but preferably more 3-hydroxyisobutyric acid, in comparison with its wild type, this formation preferably taking place via methylmalonate semialdehyde or via 3-hydroxyisobutyryl-coenzyme A as precursor.

**[0009]** In the event that the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via methylmalonate semialdehyde as precursor, it is furthermore preferred that the formation takes place via succinyl-coenzyme A, propionyl-coenzyme A or acryloyl-coenzyme A, especially preferably via succinyl-coenzyme A, as further intermediate. In the event that the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via 3-hydroxyisobutyryl-coenzyme A as precursor, it is furthermore preferred that the formation takes place via isobutyryl-coenzyme A or via 3-hydroxybutyryl-coenzyme A, preferably via 3-hydroxybutyryl-coenzyme A, as further intermediate.

**[0010]** The term "precursor" as used in the present context defines a chemical compound which can be converted enzymatically into 3-hydroxyisobutyric acid in just one reaction step, while the term "intermediate" defines a chemical compound which cannot be converted enzymatically into 3-hydroxyisobutyric acid in just one reaction step.

**[0011]** The term "3-hydroxyisobutyric acid" as used in the present context always describes the corresponding $C_4$-carboxylic acid in the form in which it is present as a function of the pH, after having been formed by the microorganisms in question. As a consequence, the term always comprises the pure acid form (3-hydroxyisobutyric acid), the pure base form (3-hydroxyisobutyrate) and mixtures of protonated and deprotonated forms of the acid. Furthermore, the term "3-hydroxyisobutyric acid" comprises, in principle, both the (R) and the (S) stereoisomer, the (S) stereoisomer being especially preferred.

**[0012]** The wording "that it is capable of forming more 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid in comparison with its wild type" also applies in the event that the wild type of the genetically modified cell is not capable of forming any 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, but at least no detectable amounts of these compounds, and that detectable amounts of these components are only capable of being formed after the genetic modification.

**[0013]** A "wild type" of a cell preferably refers to a cell whose genome is present in a state as generated naturally as the result of evolution. The term is used both for the entire cell and for individual genes. As a consequence, the term "wild type" does not cover in particular those cells, or those genes, whose gene sequences have at least in part been modified by man by means of recombinant methods.

**[0014]** The 3-hydroxyisobutyric acid can subsequently give rise to methacrylic acid by subjecting it to a dehydration reaction under mild conditions. In the case of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, the vesicles present in the cells, which are filled with these polyhydroxyalkanoates, can be isolated and the polymers can subsequently be cleaved to give 3-hydroxyisobutyric acid, which can then be dehydrated to give methacrylic acid.

**[0015]** In this context, it is preferred according to the invention that the genetically modified cell has been genetically modified in such a way that it forms at least twice, especially preferably at least 10 times, more preferably at least 100 times, even more preferably at least 1000 times and most preferably at least 10 000 times more 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid than the wild type of the cell within a defined time interval, preferably within 2 hours, even more preferably within 8 hours and most preferably within 24 hours. The increase in the formation of product can be determined in this context for example by growing the cell according to the invention and the wild-type cell in each case separately, but under identical conditions (identical cell density, identical nutrient medium, identical culture conditions) for a particular time interval in a suitable nutrient medium and subsequently determining the amount of target product (3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid) in the nutrient medium.

**[0016]** The cells according to the invention may be prokaryotic or eukaryotic cells. They may take the form of mammalian cells (such as, for example, human cells), of plant cells or of microorganisms such as yeasts, fungi or bacteria, with microorganisms being especially preferred and bacteria and yeasts being most preferred.

**[0017]** Suitable bacteria, yeasts or fungi are in particular those bacteria, yeasts or fungi which have been deposited at the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ), Brunswick, Germany, as bacterial, yeast or fungal strains. Bacteria which are suitable according to the invention belong to the genera detailed under

http://www.dsmz.de/species/bacteria.htm,

yeasts which are suitable according to the invention belong to those genera which are detailed under

http://www.dsmz.de/species/yeasts.htm,

and fungi which are suitable according to the invention are those which are detailed under

http://www.dsmz.de/species/fungi.htm.

**[0018]** Cells which are especially preferred according to the invention are those of the genera *Corynebacterium, Brevibacterium, Bacillus, Acinetobacter, Lactobacillus, Lactococcus, Candida, Pichia, Kluveromyces, Saccharomyces, Escherichia, Zymomonas, Yarrowia, Methylobacterium, Ralstonia, Pseudomonas, Burkholderia* and *Clostridium*, with *Brevibacterium flavum, Brevibacterium lactofermentum, Escherichia coli, Saccharomyces cerevisiae, Kluveromyces lactis, Candida blankii, Candida rugosa, Corynebacterium glutamicum, Corynebacterium efficiens, Zymonomas mobilis, Yarrowia lipolytica, Methylobacterium extroquens, Ralstonia eutropha*, especially *Ralstonia eutropha* H16, *Rhodospirillum rubrum, Rhodobacter sphaeroides, Paracoccus versutus, Pseudomonas aeroginosa, Acinetobacter calcoaceticus* and *Pichia pastoris* being especially preferred.

**[0019]** In accordance with a first variant of the cell according to the invention, the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via methylmalonate semialdehyde as precursor.

**[0020]** In accordance with a first special embodiment of this first variant of the cell according to the invention, it is preferred that the formation of 3-hydroxyisobutyric acid or of the polyhydroxyalkanoate based on 3-hydroxyisobutyric acid preferentially takes place via succinyl-coenzyme A as intermediate, where the cell preferentially is capable of utilizing carbohydrates, glycerol or glutamate as the carbon source.

**[0021]** Here, it may be advantageous in the context of the first special embodiment of the first variant of the cell according to the invention that the cell according to the invention features an increased activity of an enzyme $E_1$, which catalyzes the conversion of succinyl-coenzyme A into methylmalonyl-coenzyme A, in comparison with its wild type (see FIG. **1**).

**[0022]** The term "increased activity of an enzyme" as used above in connection with the enzyme $E_1$ and in what follows in the context of the enzymes $E_2$ etc. is preferably to be understood as increased intracellular activity.

**[0023]** What now follows on increasing the enzymatic activity in cells applies both to increasing the activity of the enzyme $E_1$ and to all enzymes mentioned thereafter, whose activity can, if appropriate, be increased.

**[0024]** In principle, an increase in the enzymatic activity can be achieved by increasing the copy number of the gene sequence(s) which code for the enzyme, by using a strong promoter or by using a gene or allele which codes for a corresponding enzyme with an increased activity, and, if appropriate, combining these measures. Cells which have been genetically modified in accordance with the invention are generated for example by transformation, transduction, conjugation or a combination of these methods with a vector which comprises the desired gene, an allel of this gene or parts thereof, and a vector which makes possible the expression of the gene. The heterologous expression is achieved in particular by integration of the gene, or of the alleles, into the chromosome of the cell or into an extrachromosomally replicating vector.

**[0025]** An overview over the possibilities for increasing the enzymatic activity in cells with pyruvate carboxylase by way of example is found in DE-A-100 31 999, which is hereby incorporated by reference and whose disclosure content regarding the possibilities for increasing the enzymatic activity in cells forms part of the disclosure of the present invention.

**[0026]** The expression of the enzymes or genes mentioned hereinabove and in each case hereinbelow can be detected in the gel with the aid of 1- and 2-dimensional protein gel separation and subsequent visual identification of the protein concentration using suitable evaluation software. When the increase in an enzymatic activity is based exclusively on an increase in the expression of the gene in question, the quantification of the increase in the enzymatic activity can be determined in a simple manner by comparing the 1- or 2-dimensional protein separations between the wild type and the genetically modified cell. A conventional method of preparing the protein gels in *coryneform bacteria*, and of identifying the proteins, is the procedure described by Hermann et al. (*Electrophoresis,* 22: 1712.23 (2001)). The protein concentration can also be analyzed by Western blot hybridization using an antibody which is specific for the protein to be detected (Sambrook et al., *Molecular Cloning: a laboratory manual,* $2^{nd}$ Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA, 1989) followed by visual evaluation with suitable software for determining the concentration (Lohaus and Meyer (1989) *Biospektrum,* 5: 32-39; Lottspeich (1999), *Angewandte Chemie* 111: 2630-2647). The activity of DNA-binding proteins can be measured by means of DNA band shift assays (also referred to as gel retardation) (Wilson et al. (2001) *Journal of Bacteriology,* 183: 2151-2155). The

effect of DNA-binding proteins on the expression of other genes can be detected by various, extensively described methods of the reporter gene assay (Sambrook et al., *Molecular Cloning: a laboratory manual,* 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA, 1989). The intracellular enzymatic activities can be detected by various methods which have been described (Donahue et al. (2000) *Journal of Bacteriology* 182 (19): 5624-5627; Ray et al. (2000) *Journal of Bacteriology* 182 (8): 2277-2284; Freedberg et al. (1973) *Journal of Bacteriology* 115 (3): 816-823). In the event that no specific methods for determining the activity of a particular enzyme are detailed in what follows, the determination of the increase in the enzymatic activity, and also the determination of the reduction in an enzymatic activity, is preferably carried out by means of the methods described in Hermann et al., Electrophoresis, 22: 1712-23 (2001), Lohaus et al., Biospektrum 5 32-39 (1998), Lottspeich, *Angewandte Chemie* 111: 2630-2647 (1999) and Wilson et al. (2001) *Journal of Bacteriology,* 183: 2151-2155 (2001).

**[0027]** If increasing the enzymatic activity is brought about by mutating the endogenous gene, such mutations can be generated either undirected, using traditional methods such as for example by UV irradiation or by mutagenic chemicals, or directed by means of recombinant methods such as deletion(s), insertion(s) and/or nucleotide substitution(s). These mutations give rise to genetically modified cells. Especially preferred mutants of enzymes are in particular also those enzymes which are no longer capable of being feedback-inhibited, or which are at least less capable of being feedback-inhibited, in comparison with the wild-type enzyme.

**[0028]** If increasing the enzymatic activity is brought about by increasing the expression of an enzyme, then, for example, the copy number of the respective genes are increased, or the promoter and regulatory regions or the ribosomal binding site, which is located upstream of the structural gene, are mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same manner. By means of inducible promoters it is additionally possible to increase the expression at any desired point in time. Furthermore, the enzyme gene may also have assigned to it what are known as enhancer sequences as regulatory sequences; these also bring about an increased gene expression via an improved interaction between RNA polymerase and DNA. Measures for extending the life of the mRNA also improves expression. Furthermore, preventing the degradation of the enzyme protein also enhances the enzymatic activity. Here, the genes or gene constructs are either present in plasmids in different copy numbers, or else they are integrated and amplified in the chromosome. As an alternative, overexpression of the genes in question may also be achieved by modifying the media composition and the control of the culture.

**[0029]** Instructions for doing so can be found by the skilled worker in Martin et al. (*Bio/Technology* 5, 137-146 (1987)) in Guerrero et al. (*Gene* 138, 35-41 (1994)), Tsuchiya and Morinaga (*Bio/Technology* 6, 428-430 (1988)), in Eikmanns et al. (*Gene* 102, 93-98 (1991)), in EP-A-0 472 869, in U.S. Pat. No. 4,601,893, in Schwarzer and Pühler ((*Bio/Technology* 9, 84-87 (1991), in Reinscheid et al. (*Applied and Environmental Microbiology* 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in WO-A-96/15246, in Malumbres et al. (*Gene* 134, 15-24 (1993), in JP-A-10-229891, in Jensen and Hammer (*Biotechnology and Bioengineering* 58, 191-195 (1998)), inter alia, and in known textbooks of genetics and molecular biology. The above-described measures give rise to genetically modified cells, as do the mutations.

**[0030]** Plasmids, for example episomal plasmids, are employed for increasing the expression of the genes in question. Suitable plasmids are in particular those which are replicated in coryneform bacteria. A large number of known plasmid vectors such as, for example, pZ1 (Menkel et al., *Applied and Environmental Microbiology* 64: 549-554 (1989)), pEKEx1 (Eikmanns et al., *Gene* 107: 69-74 (1991)) or pHS2-1 (Sonnen et al., *Gene* 107: 69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors such as, for example, those based on pCG4 (U.S. Pat. No. 4,489,160) or pNG2 {Serwold-Davis et al., *FEMS Microbiology Letters* 66: 119-124 (1990)} or pAG1 (U.S. Pat. No. 5,158,891), may be employed in the same manner.

**[0031]** Others which are suitable are those plasmid vectors with the aid of which the method of amplifying genes by integration into the chromosome can be applied, as has been described for example by Reinscheid et al. (*Applied and Environmental Microbiology* 60: 126-132 (1994)) for duplicating or amplifying the hom-thrB operon. In this method, the entire gene is cloned into a plasmid vector which is capable of replication in a host (typically *Escherichia coli*), but not in *Corynebacterium glutamicum*. Suitable vectors are, for example, pSUP301 (Simon et al, *Bio/Technology* 1: 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., *Gene* 145: 69-73 (1994)), pGEM-T (Promega Corporation, Madison, Wis., USA), pCR2.1-TOPO (Shuman, *Journal of Biological Chemistry* 269: 32678-84 (1994)), pCR®Blunt (Invitrogen, Groningen, the Netherlands), pEM1 (Schrumpf et al., *Journal of Bacteriology* 173: 4510-4516)) or pBGS8 (Spratt et al., *Gene* 41: 337-342 (1986)). The plasmid vector, which contains the gene to be amplified, is subsequently transferred into the desired *Corynebacterium glutamicum* strain by means of conjugation or transformation. The conjugation method is described for example in Schäfer et al., *Applied and Environmental Microbiology* 60: 756-759 (1994). Transformation methods are described for example in Thierbach et al., *Applied Microbiology and Biotechnology* 29: 356-362 (1988), Dunican and Shivnan, *Bio/Technology* 7: 1067-1070 (1989) and Tauch et al., *FEMS Microbiology Letters* 123: 343-347 (1994). Following homologous recombination by means of a cross-over event, the resulting strain comprises at least two copies of the gene in question.

**[0032]** The wording "an activity of an enzyme $E_x$ which is increased in comparison with its wild type" used hereinabove and in what follows is preferably always understood as meaning an activity of the respective enzyme $E_x$ which is increased by a factor of at least 2, especially preferably of at least 10, more preferably of at least 100, even more preferably of at least 1000 and most preferably of at least 10 000. Furthermore, the cell according to the invention which features "an activity of an enzyme $E_x$ which is increased in comparison with its wild type", in particular also a cell whose wild type features no, or at least no detectable, activity of this enzyme $E_x$ and which only shows a detectable activity of this enzyme $E_x$ after increasing the enzymatic activity, for example by means of overexpression. In this context, the term "overexpression", or the wording "increase in the expression" used in what follows also comprises the case that a starting cell, for example a wild-type cell, features no, or at least no detectable,

expression and detectable expression of the enzyme $E_x$ is only induced by recombinant methods.

**[0033]** Accordingly, the wording "reduced activity of an enzyme $E_x$" used hereinbelow is understood as meaning an activity which is preferably reduced by a factor of at least 0.5, especially preferably of at least 0.1, more preferably of at least 0.01, even more preferably of at least 0.001 and most preferably of at least 0.0001. The reduction in the activity of a specific enzyme can be obtained for example by directed mutation, by the addition of competitive or non-competitive inhibitors or by other measures for reducing the expression of a specific enzyme which are known to the skilled worker.

**[0034]** In the case of the enzyme $E_1$, which catalyzes the conversion of succinyl-coenzyme A into methylmalonyl-coenzyme A, this preferably takes the form of a methylmalonyl-coenzyme A mutase (EC 5.4.99.2). This enzyme is preferably encoded by the gene selected from the group consisting of mut, mutA, mutB, sbm, sbmA, sbmB, sbm5, bhbA, mcmA, mcmA1, mcmA2, mcmB, mcm1, mcm2, mcm3, icmA, meaA1 and meaA2. The nucleotide sequence of these genes can be found for example in the "*Kyoto Encyclopedia of Genes and Genomes*" (KEGG database), the databases of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, Md., USA) or from the nucleotide sequence database of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany and Cambridge, UK).

**[0035]** In accordance with an especially preferred embodiment of the first variant of the cell according to the invention, the enzyme $E_1$ takes the form of the methylmalonyl-coenzyme A mutase from *Corynebacterium glutamicum* ATCC 13032, which is encoded by a gene with the DNA sequence as shown in SEQ ID No 01 and which has the amino acid as shown in SEQ ID No 02.

**[0036]** Furthermore, it is preferred in accordance with a first alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the preparation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, that the cell, if appropriate in addition to the increased activity of the enzyme $E_1$, features an activity of at least one of the following enzymes $E_2$ to $E_4$ which is increased in comparison with its wild type (see FIG. **2**):

**[0037]** of an enzyme $E_2$, which catalyzes the conversion of methylmalonyl-coenzyme A into methyl malonate;

**[0038]** of an enzyme $E_3$, which catalyzes the conversion of methyl malonate into methylmalonate semialdehyde;

**[0039]** of an enzyme $E_4$ which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyric acid.

**[0040]** In this context, cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_2$, $E_3$, $E_4$, $E_2E_3$, $E_2E_4$, $E_3E_4$, $E_2E_3E_4$, where $E_2E_3E_4$ is most preferred. Furthermore, it is possible that an enzyme is also capable of catalyzing at least two of the above-described reaction steps. Thus, for example, it is possible to employ an enzyme which features both the activity of enzyme $E_2$ and that of enzyme $E_3$ (and which therefore catalyzes the conversion of methylmalonyl-coenzyme A directly into methylmalonate semialdehyde) such as, for example, the malonyl coenzyme A reductase from *Sulfolobus tokodaii*, which is encoded by the DNA sequence with the SEQ ID No 03 and which has the amino acid sequence as shown in SEQ ID No 04, or else an enzyme which features all three enzymatic activities $E_2$, $E_3$ and $E_4$, such as the malonyl coenzyme A reductase from *Chloroflexus aurantiacus* (Hügler et al., Journal of Bacteriology 184, pages 2404-2410, 2002).

**[0041]** In this context, it is especially preferred that the enzyme

**[0042]** $E_2$ is a methylmalonyl-coenzyme A hydrolase (EC 3.1.2.17),

**[0043]** $E_3$ is an aldehyde dehydrogenase (EC 1.2.1.3) or an aldehyde oxidase (EC 1.2.3.1) and

**[0044]** $E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**[0045]** The enzyme $E_2$ is preferably encoded by the aox1 gene. The methylmalonyl-coenzyme A hydrolase from rat liver is described for example in Kovachy et al., "Recognition, isolation, and characterization of rat liver D-methylmalonyl coenzyme A hydrolase", *J. Biol. Chem.* 258 (1983), pages 11415-11421.

**[0046]** The enzyme $E_3$ is preferably encoded by genes selected from the group consisting of aldh2, aldh3a1, aldh3a2, aldh1b1, aldh9a1, aldh7a1, aldh1a4, aldh1a1, aldh1a2, mgc80785, mgc83352, mgc89020, dmel-CG31075, cg3752, cg9629, alh-9, alh-1, alh-2, f508.35, t7023.15, f15I1.19, tT17F15.130, ald1, ald2, ald4, ald5, ald6, ac1044Wp, adr417wp, msc7, tb06.5F5.780, aldH, puuC, putA, aldA, badH, alkH, pcD, rsp1591, rs01031, exaC, acoD, dhaL, pchA, aldB, dhaS, betB, ywdH, ycbD, aldX, aldY, aldA1, aldA2, aldC, pcd, cg10546, cg12668, cg12796, scg11A.05, sci30A.27c, sce9.27c, sck13.05c, sc5H4.03, thcA, gabD2, alkH, aldH, aldH1, aldY1, aldY2, aldY3, aldY4, aldY5, aldY6, aldY7 and aldhT.

**[0047]** Suitable genes for the enzyme $E_4$ are selected from the group consisting of hibadh, cg15093, cg15093, cg4747, mwL2.23, t13k14.90, f19b15.150, hibA, ygbJ, mmsB, mmsB, garR, tsar, mmsB-1, mmsB-2, yfjR, ykwC, ywjF, hibD, glxR, SCM1.40c, hibD, ehhahd, hadh2, hadhsc, hsd17B4, loc488110, had, mgC81885, hadh2-prov, cg3415, cg7113, ech-1, ech-8, ech-9, ard-1, yfcX, fadB, faoA, fadB2x, hbd-1, hbd-2, hbd-3, hbd-4, hbd-5, hbd-6, hbd-7, hbd-8, hbd-9, hbd-10, fadJ, rs04421, rs02946, rs05766, bbsD, bbsC, fadB1, fadB2, fadB5, hbdA, pimF, fabJ-1, fabJ, scbac19f3.11, sci35.13, scbac8d1.10c, sc5f2a.15, sc6a5.38, fadC2, fadC4, fadC5, fadC6, had and paaH. Further suitable 3-hydroxyisobutyrate dehydrogenases are described for example in Bannerjee et al. (1970), *J. Biol. Chem.*, 245, pages 1828 to 1835, Steele et al. (1992), *J. Biol. Chem.*, 267, pages 13585 to 13592, Harris et al. (1988), *J. Biol. Chem.*, 263, pages 327 to 331, Harris et al., *Biochim. Biophys. Acta*, 1645 (1), pages 89 to 95, Hawes et al. (2000), *Methods Enzymol.*, 324, pages 218 to 228, Harris et al., *J. Biol. Chem.*, 275 (49), pages 38780 to 38786, Rougraff et al. (1988), *J. Biol. Chem.*, 263(1), pages 327 to 331, Robinson et al., *J. Biol. Chem.*, 225, pages 511 to 521, Hawes et al. (1995), *Biochemistry*, 34, pages 4231 to 4237, Hasegawa J. (1981), *Agric. Biol. Chem.*, 45, pages 2805 to 2814, Hawes et al. (1996), *FEBS Lett.*, 389, pages 263 to 267, Hawes et al. (1996), *Enzymology and Molecular Biology of Carbonyl Metabolism*, Plenum Press, New York, pages 395 to 402, Adams et al. (1994), *Structure*, 2, pages 651 to 668, Zhang et et. (1999), *Biochemistry*, 38, pages 11231 to 11238, Mirny et al., (1999), *J. Mol. Biol.*, 291, pages 177 to 196 and Lokanath et al. (2005), *J Mol. Biol. The disclosure of these publications is hereby incorporated by reference and forms part of the disclosure of the present invention.*

**[0048]** The nucleotide sequences of the abovementioned genes and of further genes for the enzymes $E_2$ to $E_4$ can also be found in the KEGG database, the NCBI database or the EMBL database, inter alia.

**[0049]** In accordance with an especially preferred embodiment of this alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the preparation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, it is preferred that the malonyl coenzyme A reductase from *Sulfolobus tokodaii*, which is encoded by the DNA sequence with the SEQ ID No 03 and which has the amino acid sequence as shown in SEQ ID No 04, is employed for the conversion of methylmalonyl-coenzyme A into methylmalonate semialdehyde. In accordance with another especially preferred embodiment of this variant, the malonyl coenzyme A reductase from *Chloroflexus aurantiacus* (Hüler et al., Journal of Bacteriology 184, pages 2404-2410, 2002) is employed for the conversion of methylmalonyl-coenzyme A into 3-hydroxyisobutyric acid.

**[0050]** Furthermore, it is preferred in the context of this first alternative of the first special embodiment of the cell according to the invention that the cell features an activity of an enzyme $E_5$, which features the conversion of methylmalonate semialdehyde into propionyl-coenzyme A, which is reduced in comparison with its wild type, this enzyme preferably taking the form of a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27).

**[0051]** In accordance with a second alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the preparation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, it is preferred that the cell, if appropriate in addition to the increased activity of the enzyme $E_1$, features an activity of at least one of the following enzymes $E_4$ to $E_7$ which is increased in comparison with its wild type (see FIG. **3**):

**[0052]** of an enzyme $E_6$, which catalyzes the conversion of (R) methylmalonyl-coenzyme A into (S) methyl-malonyl-coenzyme A;

**[0053]** of an enzyme $E_7$, which catalyzes the conversion of (S) methylmalonyl-coenzyme A into propionyl-coenzyme A;

**[0054]** of an enzyme $E_5$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonate semialdehyde;

**[0055]** of an enzyme $E_4$, which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyric acid.

**[0056]** In this context, cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme

**[0057]** combinations is increased: $E_4$, $E_5$, $E_6$, $E_7$, $E_7$, $E_4E_5$, $E_4E_6$, $E_4E_7$, $E_5E_6$, $E_5E_7$, $E_6E_7$, $E_4E_5E_6$, $E_4E_5E_7$, $E_4E_6E_7$, $E_5E_6E_7$ and $E_4E_5E_6E_7$, with $E_4E_5E_6E_7$ being most preferred.

**[0058]** In this context, it is especially preferred that the enzyme

**[0059]** $E_6$ is a methylmalonyl-coenzyme A epimerase (EC 5.1.99.1)

**[0060]** $E_7$ is a methylmalonyl-coenzyme A decarboxylase (EC 4.1.1.41),

**[0061]** $E_5$ is a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27), and

**[0062]** $E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**[0063]** In this context, preferred enzymes $E_4$ are those which have already been mentioned above in the context of the first variant of the first preferred embodiment of the cell according to the invention.

**[0064]** The enzyme $E_6$ is preferably encoded by the mcee gene. A suitable methylmalonyl-coenzyme A decarboxylase (enzyme $E_7$) is described, for example, by Benning et al. in Biochemistry, Vol. 39 (2000), pages 4630-4639.

**[0065]** Suitable genes for the enzyme $E_5$ are preferably selected from the group consisting of aldh6a1, cg17896, t22c12.10, ald6, putA1, mmsA, mmsA-1, mmsA-2, mmsA-3, mmsA-4, msdA, iolA and iolAB.

**[0066]** Suitable genes for the enzyme $E_7$ are preferably selected from the group consisting of mmdA, bcc, oadB, oadB2, oadB3, SC1C2.16, SC1G7.10, pccB1, accA2, mmdB, mmdC and ppcB.

**[0067]** The nucleotide sequences of the abovementioned genes for the enzymes $E_5$, $E_6$ and $E_7$ may, inter alia, also be found in the KEGG database.

**[0068]** In accordance with a third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the preparation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, it is preferred that the cell, if appropriate in addition to the increased activity of the enzyme $E_1$, features an activity of at least one of the following enzymes $E_4$, $E_5$ and $E_7$ which is increased in comparison with its wild type (see FIG. **4**):

**[0069]** of an enzyme $E_7$, which catalyzes the conversion of methylmalonyl-coenzyme A into propionyl-coenzyme A;

**[0070]** of an enzyme $E_5$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonate-semialdehyde;

**[0071]** of an enzyme $E_4$, which catalyzes the conversion of methylmalonate-semialdehyde into 3-hydroxyisobutyric acid.

**[0072]** This pathway corresponds essentially to the second variant of the first preferred embodiment of the cell according to the invention, but, as opposed to the second variant, propionyl-CoA is prepared directly from methylmalonyl-coenzyme A. Preferred enzymes and genes for the enzymes $E_4$, $E_5$ and $E_7$ are those genes or enzymes which have already been mentioned above in connection with the second variant.

**[0073]** Furthermore, it may in accordance with the first special embodiment of the cell according to the invention (and also in accordance with all embodiments which are still to be described hereinbelow) also be preferred that the cell is capable of converting the formed 3-hydroxyisobutyric acid into a polyhydroxy-alkanoate. Such polyhydroxydalkanoates are deposited intracellularly by many microorganisms in the form of highly refractive granula. In this context, it is especially preferred that the cell according to the invention features an activity of at least one of, preferably of the two, the following enzymes $E_9$ and $E_{10}$ which is increased in comparison with its wild type (see FIG. **5**):

**[0074]** of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyric acid into 3-hydroxyisobutyryl-coenzyme A;

**[0075]** of an enzyme $E_9$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A to a polyhydroxyalkanoate based on 3-hydroxyisobutyric acid.

**[0076]** In this context, it is especially preferred that the enzyme

**[0077]** $E_8$ is a 3-hydroxyisobutyryl CoA hydrolase (EC 3.1.2.4) and

**[0078]** $E_9$ is a polyhydroxyalkanoate synthase.

**[0079]** As has already been explained above, the first preferred embodiment of the cell according to the invention generates 3-hydroxyisobutyric acid or the polyhydroxyalkanoates based on 3-hydroxyisobutyric acid from succinyl coenzyme A as intermediate and from methylmalonate semialdehyde as precursor. Here, it may make sense in principle to influence not only one or more of the abovementioned enzyme activities $E_1$ to $E_9$, but also those enzyme activities which lead to an increased formation of succinyl-coenzyme A in the cell.

**[0080]** In the event that, according to the first special embodiment of the first variant of the cell according to the invention, the formation of 3-hydroxyisobutyric acid or of the polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place from carbohydrates or glycerol via succinyl-coenzyme A as intermediate and methylmalonate semialdehyde as precursor, it is, according to a special embodiment of the above-described first, second or third alternative of the cell according to the invention, preferred that the cell features an activity of at least one of the, preferably of the two, following enzymes $E_{10}$ and $E_{11}$ which is increased in comparison with its wild type (see FIG. **6**):

**[0081]** of an enzyme $E_{10}$, which catalyzes the conversion of phosphoenolpyruvate into oxaloacetate;

**[0082]** of an enzyme $E_{11}$, which catalyzes the conversion of pyruvate into oxaloacetate.

**[0083]** In this context, it is especially preferred that the enzyme

**[0084]** $E_{10}$ is a phosphoenolpyruvate carboxylase (EC 4.1.1.31) and

**[0085]** $E_{11}$ is a pyruvate carboxylase (EC 6.4.1.1).

**[0086]** The enzyme $E_{10}$ is preferably encoded by the genes selected from the group consisting of f12 m16.21, f14n22.13, k15 m2.8, ppc, clpA, pepC, capP, cgl1585, pepC, pck ppc and pccA, where the ppc gene is especially preferred. Phosphoenolpyruvate carboxylases which are preferred according to the invention are also described in particular in U.S. Pat. No. 4,757,009, U.S. Pat. No. 4,980,285, U.S. Pat. No. 5,573,945, U.S. Pat. No. 6,872,553 and U.S. Pat. No. 6,599,732. As regards phosphoenolpyruvate carboxylases, the disclosure content of these publications is hereby incorporated by reference and forms part of the disclosure of the present invention.

**[0087]** The enzyme $E_{11}$ is preferably encoded by the genes selected from the group consisting of pc, pcx, cg1516, cg1516, pyc-1, pyc-2, aar162 Cp, pyr1, accC-2, pycA, pycA2, pca, cg10689, pyc, pycB, accC, oadA, acc and accC1, where the pyc gene is especially preferred. Pyruvate carboxylases which are preferred according to the invention are also described in particular in U.S. Pat. No. 6,455,284, U.S. Pat. No. 6,171,833, U.S. Pat. No. 6,884,606, U.S. Pat. No. 6,403,351, U.S. Pat. No. 6,852,516 and U.S. Pat. No. 6,861,246. A further pyruvate carboxylase which is especially preferred according to the invention is that mutant which is described in "*A novel methodology employing Corynebacterium glutamicum genome information to generate a new L-lysine-producing mutant*", Ohnishi J et al., Applied Microbiology and Biotechnology, Vol. 58 (2), pages 217-223 (2002).

**[0088]** The nucleotide sequences of suitable genes of the enzymes $E_{11}$ and $E_{12}$ can be found in the KEGG database, the NCBI database or the EMBL database.

**[0089]** Starting from the oxaloacetate intermediate stage, there are several possibilities for arriving at succinyl-coenzyme A, which can then be converted into 3-hydroxyisobutyric acid via methylmalonyl-coenzyme A by means of the three variants mentioned at the outset.

**[0090]** A first pathway leads via fumarate as intermediate. In this case it is preferred in accordance with a first special embodiment of the above-described first, second or third alternative of the cell according to the invention, where methylmalonate-semialdehyde is formed as precursor and succinyl-coenzyme A as intermediate, that the cell, if appropriate additionally to an increased activity of the enzyme $E_{10}$ or $E_{11}$, features an activity of at least one of the following enzymes $E_{12}$ to $E_{15}$ which is increased in comparison with its wild type (see FIG. **7**):

**[0091]** of an enzyme $E_{12}$, which catalyzes the conversion of oxaloacetate into malate;

**[0092]** of an enzyme $E_{13}$, which catalyzes the conversion of malate into fumarate;

**[0093]** of an enzyme $E_{14}$, which catalyzes the conversion of fumarate into succinate;

**[0094]** of an enzyme $E_{15}$, which catalyzes the conversion of succinate into succinyl-coenzyme A.

**[0095]** In this context, cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_{12}$, $E_{13}$, $E_{14}$, $E_{15}$, $E_{12}E_{13}$, $E_{12}E_{14}$, $E_{12}E_{15}$, $E_{13}E_{14}$, $E_{13}E_{15}$, $E_{14}E_{15}$, $E_{12}E_{13}E_{14}$, $E_{12}E_{13}E_{15}$, $E_{12}E_{14}E_{15}$, $E_{13}E_{14}E_{15}$, $E_{12}E_{13}E_{14}E_{15}$, with $E_{12}E_{13}E_{14}E_{15}$ being most preferred.

**[0096]** In this context, it is especially preferred that the enzyme

**[0097]** $E_{12}$ is a malate dehydrogenase (EC 1.1.1.37) or a malate quinone oxidoreductase (1.1.99.16),

**[0098]** $E_{13}$ is a fumarate hydratase (EC 4.2.1.2),

**[0099]** $E_{14}$ is a succinate dehydrogenase (EC 1.3.99.1 or EC 1.3.5.1) or a succinate quinone oxidoreductase (1.3.5.1), and

**[0100]** $E_{15}$ is a succinate coenzyme A ligase (EC 6.2.1.4 or EC 6.2.1.5).

**[0101]** The enzyme $E_{12}$ is preferably encoded by genes selected from the group consisting of mdh1, mdh2, mor1, cg10748, cg10749, cg5362, mdh-1, f46e10.10, f19p19.13, f12 m16.14, t30120.4, k15 m2.16, f1p2.70, f17i14.150, mnl12.18, mik19.17, mdh3, adl164 cp, adr152 cp, adr252wp, mdhA, mdhC, mdhB, ybiC, mdh, yiaK, ybiC, allD, citH, yjmC, citH, cg12380, ldh, sqdB, mqo, yojH, mqoA, mqoB, mqo1, mqo2, mqo3, mqo4 and cgl2001, where the mqo gene and the mdh gene are especially preferred.

**[0102]** The enzyme $E_{13}$ is preferably encoded by genes selected from the group consisting of fh, fh1, sc4094, sc4095, t30b22.19, k3k7.11, acr013/cp, fum1, fum2, fum3, fum4, fumH, fumA, fumB, fumC, fumC1, fumC2, fum, ttdA, ttdB, fumB-alpha, fumB-beta, citG, citB, fumX, fum-1 and fum-2, where the fum gene is especially preferred.

**[0103]** The enzyme $E_{14}$ is preferably encoded by genes selected from the group consisting of sdh1, sdh2, sdh3, sdh4, sdh5, sdh6, osm1, osm2, sdhA, sdhB, sdhC, sdhD, frdA, frdB, frdC, frdD, ifcA-1, ifcA-2, sdhB-1, sdhB-2, frdC2,

cgl0370, cgl0371, cgl0372, scm10.10c, scm10.11c, scm10.12c, sc5g8.25c, sc5g8.26c, scbac-31e11.02c, scbac31e11.02c, sc4b10.10c, sdhA2, sdhB2, sdhA1, sdhB1, qcrB2, sdhA3, sdhB3, frdB1 and frdB2, where the genes sdhA, sdhB and sdhC are especially preferred.

**[0104]** The enzyme $E_{15}$ is preferably encoded by genes selected from the group consisting of suclg1, suclg2, loc434885, cg10622, dmel-CG6255, f11a3.3, f8115.30, mkd15.11, lsc1, lsc2, ael211wp, afr134 cp, scsA, scsB, sucC and sucD.

**[0105]** Again, the nucleotide sequences of suitable genes of the enzymes $E_{12}$ to $E_{15}$, can also be found in the KEGG database, the NCBI database or the EMBL database.

**[0106]** In the event that the activity of one or more of the enzymes $E_{12}$ to $E_{15}$ is increased, it may also prove advantageous that the cell features an activity of one of the following enzymes $E_{16}$ to $E_{23}$ which is reduced in comparison with its wild type:

**[0107]** of an enzyme $E_{16}$, which catalyzes the conversion of oxaloacetate into citrate;

**[0108]** of an enzyme $E_{17}$, which catalyzes the conversion of malate into oxaloacetate;

**[0109]** of an enzyme $E_{18}$, which catalyzes the conversion of succinyl-coenzyme A into succinate,

**[0110]** of an enzyme $E_{19}$, which catalyzes the conversion of oxaloacetate into phosphoenolpyruvate,

**[0111]** of an enzyme $E_{20}$, which catalyzes the conversion of oxaloacetate into pyruvate,

**[0112]** of an enzyme $E_{21}$, which catalyzes the conversion of oxaloacetate into aspartate,

**[0113]** of an enzyme $E_{22}$, which catalyzes the conversion of malate into pyruvate,

**[0114]** of an enzyme $E_{23}$, which catalyzes the conversion of pyruvate into acetate.

**[0115]** Cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is reduced: $E_{16}$, $E_{17}$, $E_{18}$, $E_{19}$, $E_{20}$, $E_{21}$, and $E_{16}E_{17}E_{18}E_{19}E_{20}E_{21}E_{22}E_{23}$.

**[0116]** In this context, it is especially preferred that the enzyme

**[0117]** $E_{16}$ is a citrate synthase (EC 2.3.3.1 or EC 2.3.3.8),

**[0118]** $E_{17}$ is a malate oxidase (EC 1.1.3.3),

**[0119]** $E_{18}$ is a succinyl CoA hydrolase (EC 3.1.2.3),

**[0120]** $E_{19}$ is a phosphoenolpyruvate carboxykinase (EC 4.1.1.49 or 4.1.1.32),

**[0121]** $E_{20}$ is an oxaloacetate decarboxylase (EC 4.1.1.3),

**[0122]** $E_{21}$ is an aspartate transaminase (EC 2.6.1.1),

**[0123]** $E_{22}$ is a malate dehydrogenase (EC 1.1.1.38, EC 1.1.1.39 or EC 1.1.1.40),

**[0124]** $E_{23}$ is a pyruvate dehydrogenase (EC 1.2.1.51).

**[0125]** The enzyme $E_{16}$ is preferably encoded by genes selected from the group consisting of glt, cs, csl, cg3861, cts-1, f7f19.21, f4i1.16, t20n10.90, t20n10.100, t209.80, cit1, cit2, cit3, aar004 cp, agr002wp, cshA, gltA, citZ, cit, prpC, cisY, cis, mmgD, citA, gltA1, gltA2, gltA3, cgl0829, prpC1, scd10.20, citA1, citA2, citA3, acly, cg8322, f5e6.2, k7jJ8.14 and citE, where gltA is most preferred.

**[0126]** The enzyme $E_{19}$ is preferably encoded by genes selected from the group consisting of pckA, pck1, pck2, cg10924, cg17725, cg17725, pckG, ppcK, cgl2863, pck and 2sck36.02.

**[0127]** The enzyme $E_{20}$ is preferably encoded by genes selected from the group consisting of oadA, oadB, oadC, oadG, oag3, eda, dcoA, oadA1, oadA2, pycB and mmdB.

**[0128]** The enzyme $E_{21}$ is preferably encoded by genes selected from the group consisting of myn8.7, glt1, adr290wp, gltB, gltD, glt1, gls1, gltA, glt, glxD, gltD1, gltD2, gdh2, ag1040 Cp, gdhA1, gdhA, gdhA2, gluD, gluD1, gluD2, rocG, ypcA, gudB, t11i18.2, t2i1.150, mrg7.13, f19c24.7, gdh, gdh1, gdh2, gdh3, got1, got2, cg4233, cg8430, f23n19.17, f13j11.16, t26c19.9, f7f1.18, F10N7.200, t1611.170, f15n18.110, t20d1.70, aat1, aat2, ab1038wp, afr211 cp, agx1, bn a4, aatA, aatB, ybdL, aspC, yfbQ, aat, avtA1, avtA2, tyrB, avtA, avtB, argD1, argD2, aspB1, aspB2, aspB3, aspB, aspC1, aspC2, aspC3, aspC4, RS05143, aspAT, ywfG, yhdR, argD, mtnV, alaT, hisC, avtA1, avtA2, avtA3, cgl0240, cgl1103, cgl2599, cgl2844, 2sck36.07c, sc9e12.21, sc2h4.04c, tyrB, gtp, gtp1, gtp2, cg1640, f20d23.34, f26f24.16, f24j13.15, t10d10.20 and agr085wp, where aspC, aatA, gdh, gudB, gdhA, gltB and gltD are especially preferred.

**[0129]** The enzyme $E_{21}$ is preferably by genes selected from the group consisting of myn8.7, glt1, adr290wp, gltB, gltD, glt1, gls1, gltA, glt, glxD, gltD1, gltD2, gdh2, ag1040 Cp, gdhA1, gdhA, gdhA2, gluD, gluD1, gluD2, rocG, ypcA,

**[0130]** The enzyme $E_{22}$ is preferably encoded by genes selected from the group consisting of me, me1, me2, me3, mae, mae1, mae2, sfcA, sfcA1, maeA, maeB, tme, yqkJ, ywkA, yqkJ, malS, ytsJ, mleA, mleS, mez, sce59.10c, 2sc7g11.23, malS1, malS2, dme, maeB1, maeB2, mdh, mdh1, mdh2, dmel_cg10120, dmel_cg10120, dmel-cg5889, f19k16.27, f6f22.7, t22p22.60, f18a17.1, mod1, tme, mao, cg13007, malS and malE.

**[0131]** The enzyme $E_{23}$ is preferably encoded by genes selected from the group consisting of me, me1, me2, me3, mae, mae1, mae2, sfcA, sfcA1, maeA, maeB, tme, yqkJ, ywkA, yqkJ, malS, ytsJ, mleA, mleS, mez, sce59.10c, 2sc7g11.23, malS1, malS2, dme, maeB1, maeB2, mdh, mdh1, mdh2, dmel_cg10120, dmel_cg10120, dmel-cg5889, f19k16.27, f6f22.7, t22p22.60, f18a17.1, mod1, tme, mao, cgl3007, malS and malE.

**[0132]** Furthermore, it is preferred in accordance with the invention that, in the event where the increased provision of succinyl-coenzyme A in the cell takes place by means of the above-described pathway (oxaloacetate→malate→fumarate→succinyl-coenzyme A), the provision of reduction equivalents in the cell is also increased in a targeted manner.

**[0133]** One possibility of increasing the reduction equivalents consists in increasing the oxidative pentose phosphate pathway. In this context, it is especially preferred that the activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and/or of 6-phospho-gluconate dehydrogenase (EC 1.1.1.44), which is preferably encoded by the gnd gene, are increased while, if appropriate, simultaneously inhibiting glycolysis, for example by lowering the activity of glucose 6-phosphate isomerase, as described in WO-A-01/07626. In addition to, or instead of, the directed promotion of the pentose phosphate pathway, it may furthermore be preferred to provide reduction equivalents by supplying, to the cells, ethanol as the carbon source and by promoting, in the cells, the conversion of the ethanol into acetaldehyde by means of alcohol dehydrogenases (EC 1.1.1.1, EC 1.1.1.2, EC 1.1.1.71 or EC 1.1.99.8) and the further conversion of the acetaldehyde into acetyl coenzyme A by means of acetaldehyde dehydrogenases (EC 1.2.1.10). Again, suitable genes for alcohol dehydrogenases and acetaldehyde dehydrogenases, can be found in gene databases which are known to the skilled

worker, such as, for example, the KEGG database, the NCBI database or the EMBL database.

**[0134]** A second pathway from oxaloacetate to succinyl-coenzyme A leads via citrate as intermediate. In this case, it is preferred in accordance with a second special embodiment of the above-described first, second or third alternative of the cell according to the invention that the cell, if appropriate in addition to an increased activity of the enzyme $E_{10}$ or $E_{11}$, features an activity of at least one of the following enzymes $E_{13}$ to $E_{16}$ and $E_{24}$ to $E_{26}$ which is increased in comparison with its wild type (see FIG. **8**):

**[0135]** of an enzyme $E_{16}$, which catalyzes the conversion of oxaloacetate into citrate;

**[0136]** of an enzyme $E_{24}$, which catalyzes the conversion of citrate into isocitrate;

**[0137]** of an enzyme $E_{25}$, which catalyzes the conversion of isocitrate into glyoxalate and succinate;

**[0138]** of an enzyme $E_{26}$, which catalyzes the conversion of glyoxalate into malate;

**[0139]** of an enzyme $E_{13}$, which catalyzes the conversion of malate into fumarate;

**[0140]** of an enzyme $E_{14}$, which catalyzes the conversion of fumarate into succinate;

**[0141]** of an enzyme $E_{15}$, which catalyzes the conversion of succinate into succinyl-coenzyme A.

**[0142]** In this context, cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_{13}$, $E_{14}$, $E_{15}$, $E_{16}$, $E_{24}$, $E_{25}$, $E_{26}$, $E_{13}E_{14}$, $E_{13}E_{15}$, $E_{13}E_{16}$, $E_{13}E_{24}$, $E_{13}E_{25}$, $E_{13}E_{26}$, $E_{14}E_{15}$, $E_{14}E_{16}$, $E_{14}E_{24}$, $E_{14}E_{25}$, $E_{14}E_{26}$, $E_{15}E_{16}$, $E_{15}E_{24}$, $E_{15}E_{25}$, $E_{15}E_{26}$ and $E_{13}E_{14}E_{15}E_{16}E_{24}E_{25}E_{26}$, where $E_{13}E_{14}E_{15}E_{16}E_{24}E_{25}E_{26}$ is most preferred.

**[0143]** In this context, it is especially preferred that the enzyme

**[0144]** $E_{13}$ is a fumarate hydratase (EC 4.2.1.2),

**[0145]** $E_{14}$ is a succinate dehydrogenase (EC 1.3.99.1 or EC 1.3.5.1) or a succinate quinone oxidoreductase (1.3.5.1),

**[0146]** $E_{15}$ is a succinate coenzyme A ligase (EC 6.2.1.4 or EC 6.2.1.5),

**[0147]** $E_{16}$ is a citrate synthase (EC 2.3.3.1 or EC 2.3.3.8),

**[0148]** $E_{24}$ is an aconitate hydratase (EC 4.2.1.3),

**[0149]** $E_{25}$ is an isocitrate lyase (EC 4.1.3.1) and

**[0150]** $E_{26}$ is a malate synthase (EC 2.3.3.9).

**[0151]** Preferred genes for the enzymes $E_{13}$ to $E_{16}$ are those which have already been described above in connection with the first pathway from oxaloacetate to succinyl-coenzyme A.

**[0152]** The enzyme $E_{24}$ is preferably encoded by genes selected from the group consisting of aco1, aco2, ratireb, dmel-CG4706, dmel-CG4900, dmel-cg6342, cg9244, t3p4.5, f10 m23.310, f4b14.100, adl032Wp, afr629wp, acnA, acnB, acnC, acnD, rpfA, acnA1, acnA2, acnM, citB, leuC, cgl1540, sacA, can and aco, where acnA and acnB are especially preferred.

**[0153]** The enzyme $E_{25}$ is preferably encoded by genes selected from the group consisting of msd21.4, icl1, icl2, adl066 cp, agl057wp, aceA, icl, aceAa, aceAb, cgl0097 and cgl2331, where aceA is especially preferred. In accordance with a particular embodiment, genes which are preferred are those which code for an isocitrate lyase which is deregulated at the gene level or protein level.

**[0154]** The enzyme $E_{26}$ is preferably encoded by genes selected from the group consisting of med24.5, mlsS1, acr268 cp, masA, glcB, aceB, mls, glcB-1, glcB-2, cgl2329, masZ, aceB1, aceB2 and mas, where the aceB gene is especially preferred.

**[0155]** Again, the nucleotide sequences of suitable genes of the enzymes $E_{24}$ to $E_{26}$ can be found in the KEGG database, the NCBI database or the EMBL database.

**[0156]** When the provision of oxaloacetate from phosphoenol-pyruvate or from pyruvate is promoted by increasing the activity of the enzyme $E_{10}$ or $E_{11}$, the succinate which is formed, besides glyoxalate, upon cleavage of the isocitrate by the isocitrate lyase may also be utilized for the formation of succinyl-coenzyme A. Furthermore, it may be advantageous in this second pathway from the oxaloacetate to the succinate to reduce the activity of an enzyme $E_{27}$, which catalyzes the conversion of isocitrate into 2-oxoglutarate and which preferably takes the form of an isocitrate dehydrogenase (EC 1.1.1.41 or EC 1.1.1.42). Preferably, the isocitrate dehydrogenase takes the form of an enzyme which is encoded by a gene selected from the group consisting of idh1, idh2, cg7176, cg7176, cg7176, f20d21.16, f12p19.10, t15n1.80, idp1, idp2, idp3, aal022Wp, aer061 Cp, idhC, idhM, icdA, icd, idh, icd1, icd2, leuB, citC, citC, cgl0664, leuB2, idh3A, idg3B, idh3G, cg12233, dmel-CG5028, dmel-CG6439, f6p23.14, f23e12.180, f8d20.160, f12e4.20, adl223wp and afr137 cp, where icdA and citC are especially preferred.

**[0157]** A third pathway from the oxaloacetate to the succinyl-coenzyme A leads via 2-oxoglutarate as intermediate. In this case, it is preferred in accordance with a third special embodiment of the above-described first, second or third alternative of the cell according to the invention that the cell features an activity of at least one of the following enzymes $E_{16}$, $E_{24}$, $E_{27}$ and $E_{28}$ which is increased in comparison with its wild type, if appropriate in addition to an increased activity of the enzyme $E_{10}$ or $E_{11}$ (see FIG. **9**):

**[0158]** of an enzyme $E_{16}$, which catalyzes the conversion of oxaloacetate into citrate;

**[0159]** of an enzyme $E_{24}$, which catalyzes the conversion of citrate into isocitrate;

**[0160]** of an enzyme $E_{27}$, which catalyzes the conversion of isocitrate into 2-oxoglutarate;

**[0161]** of an enzyme $E_{28}$, which catalyzes the conversion of 2-oxoglutarate into succinyl-coenzyme A.

**[0162]** In this context, cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_{16}$, $E_{24}$, $E_{27}$, $E_{28}$, $E_{16}E_{24}$, $E_{16}E_{27}$, $E_{16}E_{28}$, $E_{24}E_{27}$, $E_{24}E_{28}$, $E_{27}E_{28}$, $E_{16}E_{24}E_{27}$, $E_{16}E_{24}E_{28}$, $E_{24}E_{27}E_{28}$ and $E_{16}E_{24}E_{27}E_{28}$, where $E_{16}E_{24}E_{27}E_{28}$ is most preferred.

**[0163]** In this context, it is especially preferred that the enzyme

**[0164]** $E_{16}$ is a citrate synthase (EC 2.3.3.1 or EC 2.3.3.8),

**[0165]** $E_{24}$ is an aconitate hydratase (EC 4.2.1.3),

**[0166]** $E_{27}$ is an isocitrate dehydrogenase (EC 1.1.1.41 or EC 1.1.1.42) and

**[0167]** $E_{28}$ is a 2-oxoglutarate synthase (EC 1.2.7.3).

**[0168]** Preferred genes for the enzymes $E_{16}$, $E_{24}$ and $E_{27}$ are those which have already been described above in connection with the first and second pathway from the oxaloacetate to the succinyl-coenzyme A.

**[0169]** The enzyme $E_{28}$ is preferably encoded by genes selected from the group consisting of korA, korB, kor D, korA1, korA2, korB1, korB2, oorA, oorB, oorC, oorD, oforA, oforB, porA, porB, porA1, porA2, porA3, porA4, porG, porG1, porG2, porB1, porB2, porB3, SCD20.12c,

SCD20.13c, SCAH10.34c, SCAH10.35c, korG, or A, orB, korG1 and korG2. Furthermore, $E_{28}$ may also take the form of a dehydrogenase complex consisting of a plurality of subunits which have different enzymatic activities. In particular, it may take the form of a dehydrogenase complex comprising an oxoglutarate dehydrogenase (EC 1.2.4.2), a dihydrolipoyl dehydrogenase (EC 1.8.1.4) and a dihydrolipoyllysine-residue succinyl transferase (EC 2.3.1.61). In this context, the oxoglutarate dehydrogenase (EC 1.2.4.2) is preferably encoded by genes selected from the group consisting of ogdh, ghdhl, loc239017, mgc68800, mgc80496, cg11661, t22e16.70, mpA24.10, kgd1, aer374 cp, sucA, odhA, kgdA and cgl1129, where sucA and odhA are especially preferred. The dihydrolipoyl dehydrogenase (EC 1.8.1.4) is preferably encoded by genes selected from the group consisting of dld, dld-prov, dldh, cg7430, t2j15.6, k14a17.6, at3g17240, mgd8.71pd1, afr512wp, dld1, lpd, tb03.26j7.650, tb04.3 m17.450, tb927.8.7380, tb08.10 k10.200, lpdA, lpdG, lpdV, lpd3, acoD, lpdA1, lpdA2, lpdA3, odhL, pdhD, pdhD1, pdhD2, pdhD3, pdhD42, lpdAchl1, lpdAch2, lpdAc, acoL, bfmbC, bkdD, cgl0366, cgl0688, scm1.17c, pdhL, sck13.11, lpdB2 and dld1, where lpd is especially preferred. In this context, the dihydrolipoyllysine-residue succinyl transferase (EC 2.3.1.61) is preferably encoded by genes selected from the group consisting of dlst, dlst-prov, mgc89125, dmel_CG5214, f10 m23.250, k13p22.8, kgd2agl200wp, kgd2, odhB, sucB, aceF, kgdB, sucB1, sucB2, pdhC, dlaT, kgd, sc5F7.20 and sc4B10.24c, where sucB and odhB are especially preferred.

**[0170]** The nucleotide sequences of suitable genes of the enzyme $E_{28}$ or of the abovementioned subunits of the enzyme $E_{28}$, can, again, be found in the KEGG database, the NCBI database or the EMBL database.

**[0171]** The above-described pathways from the oxaloacetate to the succinyl-coenzyme A depart from phosphoenolpyruvate or from pyruvate as substrate precursors. In this context, it may furthermore be preferred to genetically modify the cells in such a way that they are capable of providing especially large amounts of pyruvate or phosphoenolpyruvate starting from carbohydrates and/or from glycerol.

**[0172]** In the event that the cells are capable of utilizing glycerol as nutrient source, it is preferred that the cell according to the invention displays an activity of at least one, preferably all, of the following enzymes $E_{29}$ to $E_{42}$ which is increased in comparison with its wild type:

**[0173]** of an enzyme $E_{29}$, which facilitates the diffusion of glycerol into the cell,

**[0174]** of an enzyme $E_{30}$, which catalyzes the conversion of glycerol into glycerol 3-phosphate,

**[0175]** of an enzyme $E_{31}$, which catalyzes the conversion of glycerol 3-phosphate into dihydroxyacetone phosphate,

**[0176]** of an enzyme $E_{32}$, which catalyzes the transfer of sulfur to the sulfur acceptor thioredoxin 1,

**[0177]** of an enzyme $E_{33}$, which catalyzes the hydrolysis of phospholipids with formation of alcohols and glycerol,

**[0178]** of an enzyme $E_{34}$, which catalyzes the transport of glycerol 3-phosphate into the cell in exchange for phosphate;

**[0179]** of an enzyme $E_{35}$, which catalyzes the conversion of dihydroxyacetone phosphate into glyceraldehyde 3-phosphate,

**[0180]** of an enzyme $E_{36}$, which catalyzes the conversion of glyceraldehyde 3-phosphate into 1,3-biphosphoglycerate,

**[0181]** of an enzyme $E_{37}$, which catalyzes the conversion of 1,3-biphosphoglycerate into 3-phosphoglycerate,

**[0182]** of an enzyme $E_{38}$, which catalyzes the conversion of 3-phosphoglycerate into 2-phosphoglycerate,

**[0183]** of an enzyme $E_{39}$, which catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate,

**[0184]** of an enzyme $E_{40}$, which catalyzes the conversion of phosphoenolpyruvate into pyruvate,

**[0185]** of an enzyme $E_{41}$, which catalyzes the conversion of glycerol into dihydroxyacetone,

**[0186]** of an enzyme $E_{42}$, which catalyzes the conversion of dihydroxyacetone into dihydroxyacetone phosphate.

**[0187]** In this context, cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is reduced: $E_{29}$, $E_{30}$, $E_{31}$, $E_{32}$, $E_{33}$, $E_{34}$, $E_{35}$, $E_{36}$, $E_{37}$, $E_{38}$, $E_{39}$, $E_{40}$, $E_{41}$, $E_{42}$, $E_{29}E_{30}$, $E_{29}E_{31}$, $E_{29}E_{32}$, $E_{29}E_{33}$, $E_{29}E_{34}$, $E_{29}E_{35}$, $E_{29}E_{36}$, $E_{29}E_{37}$, $E_{29}E_{38}$, $E_{29}E_{39}$, $E_{29}E_{40}$, $E_{29}E_{41}$, $E_{29}E_{42}$, $E_{30}E_{31}$, $E_{30}E_{32}$, $E_{30}E_{33}$, $E_{30}E_{34}$, $E_{30}E_{35}$, $E_{30}E_{36}$, $E_{30}E_{37}$, $E_{30}E_{38}$, $E_{30}E_{39}$, $E_{30}E_{40}$, $E_{30}E_{41}$, $E_{30}E_{42}$, $E_{31}E_{32}$, $E_{31}E_{33}$, $E_{31}E_{34}$, $E_{31}E_{35}$, $E_{31}E_{36}$, $E_{31}E_{37}$, $E_{31}E_{38}$, $E_{31}E_{39}$, $E_{31}E_{40}$, $E_{31}E_{41}$, $E_{31}E_{42}$, $E_{32}E_{33}$, $E_{32}E_{34}$, $E_{32}E_{35}$, $E_{32}E_{36}$, $E_{32}E_{37}$, $E_{32}E_{38}$, $E_{32}E_{39}$, $E_{32}E_{40}$, $E_{32}E_{41}$, $E_{32}E_{42}$, $E_{33}E_{34}$, $E_{33}E_{35}$, $E_{33}E_{36}$, $E_{33}E_{37}$, $E_{33}E_{38}$, $E_{33}E_{39}$, $E_{33}E_{40}$, $E_{34}E_{41}$, $E_{33}E_{42}$, $E_{34}E_{35}$, $E_{34}E_{36}$, $E_{34}E_{37}$, $E_{34}E_{38}$, $E_{34}E_{39}$, $E_{34}E_{40}$, $E_{34}E_{41}$, $E_{34}E_{42}$, $E_{35}E_{36}$, $E_{35}E_{37}$, $E_{35}E_{38}$, $E_{35}E_{39}$, $E_{35}E_{40}$, $E_{35}E_{41}$, $E_{35}E_{42}$, $E_{36}E_{37}$, $E_{36}E_{38}$, $E_{36}E_{39}$, $E_{36}E_{40}$, $E_{36}E_{41}$, $E_{36}E_{42}$, $E_{37}E_{38}$, $E_{37}E_{39}$, $E_{37}E_{40}$, $E_{37}E_{41}$, $E_{37}E_{42}$, $E_{38}E_{39}$, $E_{39}E_{40}$, $E_{39}E_{41}$, $E_{39}E_{42}$, $E_{40}E_{41}$, $E_{40}E_{42}$, $E_{41}E_{42}$ and $E_{29}E_{30}E_{31}E_{32}E_{33}E_{34}E_{35}E_{36}E_{37}E_{38}E_{39}$-$E_{40}E_{41}E_{42}$.

**[0188]** In this context, it is especially preferred that the enzyme

**[0189]** $E_{29}$ is an aquaglyceroporin (glycerol facilitator) which is preferably encoded by the glpF gene,

**[0190]** $E_{30}$ is a glycerol kinase (EC 2.7.1.30) which is preferably encoded by the glpK gene,

**[0191]** $E_{31}$ is a glycerol 3-phosphate dehydrogenase (EC 1.1.99.5), preferably an FAD-dependent glycerol 3-phosphate dehydrogenase, where the glycerol 3-phosphate dehydrogenase is preferably encoded by the glpA gene, the glpB gene, the glpC gene or the glpD gene, especially preferably by the glpD gene,

**[0192]** $E_{32}$ is a sulfur transferase which is encoded by the glpE gene,

**[0193]** $E_{33}$ is a glycerol phosphodiesterase (EC 3.1.4.46) which is preferably encoded by the glpQ gene,

**[0194]** $E_{34}$ is a glycerol 3-phosphate permease which is preferably encoded by the glpT gene,

**[0195]** $E_{35}$ is a triose phosphate isomerase (EC 5.3.1.1),

**[0196]** $E_{36}$ is a glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12),

**[0197]** $E_{37}$ is a phosphoglycerate kinase (EC 2.7.2.3),

**[0198]** $E_{38}$ is a phosphoglycerate mutase (EC 5.4.2.1),

**[0199]** $E_{39}$ is an enolase (EC 4.2.1.11),

**[0200]** $E_{40}$ is a pyruvate kinase (EC 2.7.1.40),

**[0201]** $E_{41}$ is a glycerol dehydrogenase (EC 1.1.1.6) which is preferably encoded by the gldA gene, and

**[0202]** $E_{42}$ is a dihydroxyacetone kinase (EC 2.7.1.29) which is preferably encoded by the dhaK gene.

**[0203]** The gene sequences of the abovementioned enzymes can, again, be found in the gene databases which are

known to the skilled worker, in particular the KEGG database, the NCBI database or the EMBL database.

**[0204]** Furthermore, the gap gene, which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086), the tpi gene, which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086), and the pgk gene, which codes for 3-phospho-glycerate kinase (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086), are also known from other sources.

**[0205]** Using the known genes of the enzymes $E_{29}$ to $E_{42}$, it is possible to prepare genetically modified cells in which at least one, preferably at least two, more preferably at least three and most preferably all activities of the enzymes $E_{29}$ to $E_{42}$ has been increased by means of the techniques (mutation of the enzyme or increase in the expression of the enzyme) described at the outset in connection with the enzyme $E_1$. These cells are capable of being cultured in the presence of glycerol as the only carbon source (or else together with carbohydrates as further carbon source).

**[0206]** In addition to increasing one or more of the enzymatic activities $E_{29}$ to $E_{42}$, it may, in the event that the cell is capable of utilizing glycerol as carbon source, also be advantageous when the following genes are expressed, preferably heterologously expressed, in the cells according to the invention:

**[0207]** the glpG gene or the 3925 gene,

**[0208]** the glpX gene,

**[0209]** the dhaR gene, the ycgU gene or the b1201 gene,

**[0210]** the fsa gene, the mipB gene, the ybiZ gene or the B0825 gene,

**[0211]** the talC gene, the fsaB gene, the yijG gene or the b3946 gene.

**[0212]** Again, the nucleotide sequences of these genes can be found in the KEGG database, the NCBI database or the EMBL database.

**[0213]** In the event that the cells are capable of utilizing carbohydrates as nutrient source, it is preferred that the cell according to the invention features an activity of at least one, preferably of all, of the following enzymes $E_{43}$ to $E_{45}$ and $E_{36}$ to $E_{40}$ which is increased in comparison with its wild type:

**[0214]** of an enzyme $E_{43}$, which catalyzes the conversion of α-D-glucose 6-phosphate into β-D-fructose 6-phosphate,

**[0215]** of an enzyme $E_{44}$, which catalyzes the conversion of β-D-fructose 6-phosphate into β-D-fructose 1,6-biphosphate,

**[0216]** of an enzyme $E_{45}$, which catalyzes the conversion of β-D-fructose 1,6-biphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate,

**[0217]** of an enzyme $E_{36}$, which catalyzes the conversion of glyceraldehyde 3-phosphate into 1,3-biphosphoglycerate,

**[0218]** of an enzyme $E_{37}$, which catalyzes the conversion of 1,3-biphosphoglycerate into 3-phosphoglycerate,

**[0219]** of an enzyme $E_{38}$, which catalyzes the conversion of 3-phosphoglycerate into 2-phosphoglycerate,

**[0220]** of an enzyme $E_{39}$, which catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate, and

**[0221]** of an enzyme $E_{40}$, which catalyzes the conversion of phosphoenolpyruvate into pyruvate.

**[0222]** In this context genetically modified cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased:

**[0223]** $E_{36}$, $E_{37}$, $E_{38}$, $E_{39}$, $E_{40}$, $E_{43}$, $E_{44}$, $E_{45}$, $E_{36}E_{37}$, $E_{36}E_{38}$, $E_{36}E_{39}$, $E_{36}E_{40}$, $E_{36}E_{43}$, $E_{36}E_{44}$, $E_{36}E_{45}$, $E_{37}E_{38}$, $E_{37}E_{39}$, $E_{37}E_{40}$, $E_{37}E_{43}$, $E_{37}E_{44}$, $E_{37}E_{45}$, $E_{38}E_{39}$, $E_{38}E_{40}$, $E_{38}E_{43}$, $E_{38}E_{44}$, $E_{38}E_{45}$, $E_{39}E_{40}$, $E_{39}E_{43}$, $E_{39}E_{44}$, $E_{39}E_{45}$, $E_{40}E_{43}$, $E_{40}E_{44}$, $E_{40}E_{45}$, $E_{43}E_{44}$, $E_{43}E_{45}$, $E_{44}E_{45}$ and $E_{36}E_{37}E_{38}E_{39}$-$E_{40}E_{43}E_{44}E_{45}$.

**[0224]** In this context, it is especially preferred that the enzyme

**[0225]** $E_{43}$ is a glucose 6-phosphate isomerase (EC 5.3.1.9),

**[0226]** $E_{44}$ is a 6-phosphofructo kinase (EC 2.7.1.11),

**[0227]** $E_{45}$ is a fructose bisphosphate aldolase (EC 4.1.2.13),

**[0228]** $E_{36}$ is a glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12),

**[0229]** $E_{37}$ is a phosphoglycerate kinase (EC 2.7.2.3),

**[0230]** $E_{38}$ is a phosphoglycerate mutase (EC 5.4.2.1),

**[0231]** $E_{39}$ is an enolase (EC 4.2.1.11) and

**[0232]** $E_{40}$ is a pyruvate kinase (EC 2.7.1.40).

**[0233]** Again, the nucleotide sequences of these genes can be found be found in the KEGG database, the NCBI database or the EMBL database.

**[0234]** In the event that the cell is capable of utilizing carbohydrates as carbon source, it is furthermore preferred to promote not only the abovementioned enzymes $E_{43}$ to $E_{45}$ and $E_{36}$ to $E_{40}$, but also the uptake of glucose into the cells, for example by increasing the activity of enzymes of the phosphotransferase system, in particular those enzymes which are encoded by ptsI, ptsH and ptsM genes, or by enhancing glucokinase (EC 2.7.1.2), which is preferably encoded by the glk gene. In this context, reference is made in particular to U.S. Pat. No. 6,680,187, U.S. Pat. No. 6,818,432, U.S. Pat. No. 6,913,910 and U.S. Pat. No. 6,884,614, whose disclosure content with regard to the possibilities for overexpressing the ptsI, ptsH, ptsM and glk genes is hereby incorporated by reference and forms part of the disclosure of the present invention. In the event that carbohydrates act as carbon source, it may also be advantageous to promote the pentose phosphate pathway in a targeted manner, for example by increasing the activity of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and of 6-phosphogluconate dehydrogenase (EC 1.1.1.44), which is preferably encoded by the gnd gene, while, if appropriate, simultaneously inhibiting glycolysis, for example by weakening the activity of glucose 6-phosphate isomerase, as is described in WO-A-01/07626.

**[0235]** In the event that, according to the special embodiment of the cell according to the invention where methylmalonate semialdehyde is formed as precursor and succinyl-coenzyme A as intermediate, the cells form 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via oxaloacetate and pyruvate as intermediates, it may furthermore be preferred to reduce the activity of at least one, preferably of all, of the following enzymatic activities in the cell:

**[0236]** of an enzyme which catalyzes the conversion of oxaloacetate into phosphoenolpyruvate, such as, for example, phosphoenylpyruvate carboxykinase (EC 4.1.1.49) (see also DE-A-199 50 409),

**[0237]** of an enzyme which catalyzes the conversion of pyruvate into acetate such as, for example, pyruvate oxidase (EC 1.2.2.2) (see also DE-A-199 51 975),

**[0238]** of an enzyme which catalyzes the conversion of α-D-glucose 6-phosphate into β-D-fructose 6-phosphate (see also U.S. Ser. No. 09/396,478),

**[0239]** of an enzyme which catalyzes the conversion of pyruvate into lactate such as, for example, l-lactate dehydrogenase (EC 1.1.1.27) or lactate-malate transhydrogenase (EC 1.1.99.7),

**[0240]** of an enzyme which catalyzes the conversion of pyruvate into acetyl-coenzyme A such as, for example, pyruvate dehydrogenase (EC 1.2.1.51),

**[0241]** of an enzyme which catalyzes the conversion of pyruvate into acetyl phosphate such as, for example, pyruvate oxidase (EC 1.2.3.3),

**[0242]** of an enzyme which catalyzes the conversion of pyruvate into acetate, such as, for example, pyruvate dehydrogenase (EC 1.2.2.2),

**[0243]** of an enzyme which catalyzes the conversion of pyruvate into phosphoenolpyruvate such as, for example, phosphoenolpyruvate synthase (EC 2.7.9.2) or pyruvate, phosphate dikinase (EC 2.7.9.1),

**[0244]** of an enzyme which catalyzes the conversion of pyruvate into alanine such as, for example, alanine transaminase (2.6.1.2) or alanine-oxo-acid transaminase (EC 2.6.1.12), and/or

**[0245]** of an enzyme which converts pyruvate into acetolactate such as, for example, acetohydroxy acid synthase (EC 2.2.1.6).

**[0246]** Cells which are especially preferred in accordance with the invention and which are capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxybutyric acid from carbohydrates as carbon source via succinyl-coenzyme A as intermediate and in which one or more of the abovementioned enzymatic activities, in particular one of the enzymatic activities $E_1$ to $E_{45}$, more preferably the enzymatic activities $E_1$, $E_1E_2E_3E_4$, $E_1E_4E_5E_6E_7$ or and $E_1E_4E_5E_7$, can be increased are those microorganisms which have been described by Bennett et al., *Metab. Eng.* (2005), 7 (3), pages 229 to 239, Bennett et al., *Biotechnol. Bioeng.* (2005), 90 (6), pages 775 to 779, Bennett et al., *Biotechnol. Prog.* (2005), 21 (2), pages 358 to 365, Bennett et al. (2005), *Appl. Microbiol. Biotechnol.,* 67 (4), pages 515 to 523, Vemuri et al. (2002), *Applied and Environmental Microbiology* 68 (4), pages 1715 to 1727 and in U.S. Pat. No. 6,455,284.

**[0247]** If, according to the first special embodiment of the cell according to the invention, the formation of 3-hydroxyisobutyric acid or of the polyhydroxy-alkanoates based on 3-hydroxyisobutyric acid starting from L-glutamate as carbon source takes place via succinyl-coenzyme A as intermediate, it is, in a further special embodiment of the cell according to the invention, in which methylmalonat semialdehyde is formed as precursor and succinyl-coenzyme A as intermediate, furthermore preferred in accordance with the invention that it features an activity of at least one of the, preferably of the two, following enzymes $E_{28}$ and $E_{46}$ which is increased in comparison with its wild type (see FIG. **10**):

**[0248]** of an enzyme $E_{46}$, which catalyzes the conversion of L-glutamate into 2-oxoglutarate;

**[0249]** of an enzyme $E_{28}$, which catalyzes the conversion of 2-oxoglutarate into succinyl-coenzyme A.

**[0250]** In this context, it is especially preferred that the enzyme

**[0251]** $E_{46}$ is a glutamate synthase (EC 1.4.1.13 or EC 1.4.1.14), a glutamate dehydrogenase (EC 1.4.1.2, EC 1.4.1.3 or EC 1.4.1.4) or an aspartate transaminase (EC 2.6.1.1 or EC 2.6.1.2) and

**[0252]** $E_{28}$ is a 2-oxoglutarate synthase (EC 1.2.7.3).

**[0253]** Preferred as enzyme $E_{28}$ are those which have already been mentioned at the outset as preferred enzymes $E_{28}$.

**[0254]** The enzyme $E_{46}$ is preferably encoded by the genes selected from the group consisting of: myn8., glt1, adr290wp, gltB, gltD, yeiT, aegA, ygfT, gltD-1, gltD-2, glt1, glt2, gls1, gltA, glt, glxD, gltA, yerD, cgl0184, cgl0185, sc3c9.12, gdh1, gdh2, agl140 cp, gdhA, gdhA1, gdhA2, gluD, rocG, ypcA, gudB, gluD, gdhA, gdhA2, gdh, gdhA-1, gdhA2-2, gdhA-3, gluD1, gluD2, glud1-prov, glud1a, t11I18.2, t2I1.150, mrg7.13, got1, got2, caspat, got2-prov, xr406-prov, 406-prov, cg4233, cg4233, cg8430, cg8430, f23n19.17, f13j11.16, t26c19.9, f7f1.18, f10n7.200, t1611.170, f15n18.110, t20d1.70, aat, aat1, aat2, ab1038wp, afr211cp, agx1, bnA4, aatA, aatB, ybdL, aspC, yfbQ, ydcR, avtA2, aspC-1, aspC-2, aspC-3, aspC-4, aspB, aspB-1, aspB-2, aspB-3, aspB-4, argD1, argD2, aatAc, ywfG, mtnV, alaT, avtA1, avtA2, avtA3, cgl0240, cgl1103, cgl2599, cgl2844, dapC, 2sck36.07c, sc9e12.21, sc2h4.04c, aspB1, aspB2, aspB3, tyrB, gpt, gpt1, gpt2, mgc82097, cg1640, c32f10.8, f20d23.34, f26f24.16, f24j13.15, t10d10.20 or agrwp.

**[0255]** Again, the nucleotide sequences of these genes can be found be found in the KEGG database, the NCBI database or the EMBL database.

**[0256]** In accordance with a second special embodiment of the cell according to the invention, where the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via methylmalonate semialdehyde as precursor, it is preferred that the formation of 3-hydroxyisobutyric acid or of the polyhydroxyalkanoate based on 3-hydroxyisobutyric acid takes place via propionyl-coenzyme A as intermediate, where the cell is capable of preferentially utilizing carbohydrates, glycerol, methane or methanol as carbon source. In this context, a variety of pathways exist for arriving at 3-hydroxy-isobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, departing from propionyl-coenzyme A.

**[0257]** In accordance with a first alternative of this second special embodiment of the cell according to the invention, the formation of intermediate propionyl-coenzyme A takes place via acetyl-coenzyme A as further intermediate. In this context, it is especially preferred that the cell features an activity of at least one of the following enzymes $E_4$, $E_5$ and $E_{47}$ to $E_{52}$ which is increased in comparison with its wild type (see FIG. **11**):

**[0258]** of an enzyme $E_{47}$, which catalyzes the conversion of acetyl-coenzyme A into malonyl-coenzyme A;

**[0259]** of an enzyme $E_{48}$, which catalyzes the conversion of malonyl-coenzyme A into malonate semialdehyde;

**[0260]** of an enzyme $E_{49}$, which catalyzes the conversion of malonate semialdehyde into 3-hydroxypropionate;

**[0261]** of an enzyme $E_{50}$, which catalyzes the conversion of 3-hydroxypropionate into 3-hydroxypropionyl-coenzyme A;

**[0262]** of an enzyme $E_{51}$, which catalyzes the conversion of 3-hydroxypropionyl-coenzyme A into acryloyl-coenzyme A;

**[0263]** of an enzyme $E_{52}$, which catalyzes the conversion of acryloyl-coenzyme A into propionyl-coenzyme A;

**[0264]** of an enzyme $E_5$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonate semialdehyde;

**[0265]** of an enzyme $E_4$, which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxy-isobutyrate.

**[0266]** In this context, genetically modified cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_{47}$, $E_{48}$, $E_{49}$, $E_{50}$, $E_{51}$, $E_{52}$, $E_4$, $E_5$ and $E_{47}E_{48}E_{49}E_{50}E_{51}E_{52}E_4E_5$.

**[0267]** Furthermore, it is particularly preferred in this context that the enzyme

**[0268]** $E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35),

**[0269]** $E_5$ is a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27),

**[0270]** $E_{47}$ is a malonyl-coenzyme A decarboxylase (EC 4.1.1.9), a malonate-coenzyme A transferase (EC 2.8.3.3), a methylmalonyl-coenzyme A carboxy-transferase (EC 2.1.3.1) or an acetyl-coenzyme A carboxylase (EC 6.4.1.2),

**[0271]** $E_{48}$ is a malonate-semialdehyde dehydrogenase (EC 1.2.1.18),

**[0272]** $E_{49}$ is a 3-hydroxypropionate dehydrogenase (EC 1.1.1.59),

**[0273]** $E_{50}$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

**[0274]** $E_{51}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17) and

**[0275]** $E_{52}$ is an acyl-coenzyme A dehydrogenase (EC 1.3.99.3).

**[0276]** Preferred genes for the enzymes $E_4$ and $E_5$ are those which have already been described above in connection with the first special embodiment of the cell according to the invention.

**[0277]** The enzyme $E_{47}$ is preferably encoded by genes selected from the group consisting of mlycd, t19b17.4, tb08.2904.110, matA, acac, acaca, acacb, f5j5.21, f15c21.2, t8p21.5, acc1, aar071wp, accA, accB, accC, accD, accC1, accC2, mmdA, fabG, accD1, accD2, accD3, cgl0831, accBC, dtsR1, accDA, scc24.16c and cgl1327, where accA, accC and accD are most preferred.

**[0278]** The enzyme $E_{48}$ is preferably encoded by the iolD gene.

**[0279]** The enzyme $E_{51}$ is preferably encoded by genes selected from the group consisting of echs1, ehhadh, hadha, echs1-prov, cg4389, cg4389, cg6543, cg6984, cg8778, ech-1, ech-2, ech-3, ech-4, ech-5, ech-6, ech-7, FCAALL.314, fcaall.21, fox2, eci1, eci2, paaF, paaG, yfcX, fadB, faoA, rpfF, phaA, phaB, echA1, echA2, echA3, echA4, echA5, echA6, echA7, echA8, echA9, echA9, echA10, echA11, echA12, echA13, echA14, echA15, echA16, echA17, echA18, echA19, echA20, echA21, fad-1, fad-2, fad-3, dcaE, hcaA, fadJ, rsp0671, rsp0035, rsp0648, rsp0647, rs03234, rs03271, rs04421, rs04419, rs02820, rs02946, paaG1, paaG2, paaG3, ech, pksH, ydbS, eccH1, eccH2, pimF, fabJ1, fabJ2, caiD2, ysiB, yngF, yusL, fucA, cgl0919, scf41.23, scd10.16, sck13.22, scp8.07c, stbac16h6.14, sc5f2a.15, sc6a5.38, hbd-1, hbd-2, hdb-3, hdb-4, hdb-5, hdb-6, hdb-7, hdb-8, hdb-9, hdb-10, fad-1, fad-2, fad-3, fad-4, fad-5, paaF-1, paaF-2, paaF-3, paaF-4, paaF-5, paaF-6, paaF-7 and crt.

**[0280]** The enzyme $E_{52}$ is preferably encoded by genes selected from the group consisting of acadl, acadm, acad10, acad11, acadm-prov, acadl-prov, mgc81873, cg12262, cg4703, cg4860, f3e22.5, af1213wp, acdC, fadE13, acd-1, acd-2, acd-3, acd-4, acd-5, acd-6, acd-7, acd-8, acd-9, acd-10, acd-11, acd-12, acd, fadE1, fadE2, fadE3, fadE4, fadE5, fadE6, fadE7, fadE13, fadE14, fadE15, fadE16, fadE17, fadE18, fadE19, fadE20, fadE21, fadE22, fadE23, fadE26, fadE27, fadE30, fadE31, fadE33, fadE35, fadE38, fadE45, fadE, caiA, aidB, RSp0036, RS03588, mmgC, acdA-3, bcd, acdA, acdH1, acdH2, acdH3, aidB, acdI and acdH.

**[0281]** The nucleotide sequences of suitable genes for the enzymes $E_{47}$ to $E_{52}$, in particular also of the enzymes $E_{49}$ and $E_{50}$, can be found in the KEGG database, the NCBI database or the EMBL database.

**[0282]** According to a second alternative of this second special embodiment of the cell according to the invention, the formation of the intermediate propionyl-coenzyme A also takes place via acetyl-coenzyme A as further intermediate, where, according to this alternative, the propionyl-coenzyme A is not converted directly into the methylmalonate semialdehyde, but via methylmalonyl-coenzyme A. In this context, it is especially preferred that the cell features an activity of at least one of the following enzymes $E_2$ to $E_4$, $E_6$, $E_7$ and $E_{47}$ to $E_{52}$ which is increased in comparison with its wild type (see FIG. **12**):

**[0283]** of an enzyme $E_{47}$, which catalyzes the conversion of acetyl-coenzyme A into malonyl-coenzyme A;

**[0284]** of an enzyme $E_{48}$, which catalyzes the conversion of malonyl-coenzyme A into malonate semialdehyde;

**[0285]** of an enzyme $E_{49}$, which catalyzes the conversion of malonate semialdehyde into 3-hydroxypropionate;

**[0286]** of an enzyme $E_{50}$, which catalyzes the conversion of 3-hydroxypropionate into 3-hydroxypropionyl-coenzyme A;

**[0287]** of an enzyme $E_{51}$, which catalyzes the conversion of 3-hydroxypropionyl-coenzyme A into acryloyl-coenzyme A;

**[0288]** of an enzyme $E_{52}$, which catalyzes the conversion of acryloyl-coenzyme A into propionyl-coenzyme A;

**[0289]** of an enzyme $E_7$, which catalyzes the conversion of propionyl-coenzyme A into (S)-methylmalonyl-coenzyme A;

**[0290]** of an enzyme $E_6$, which catalyzes the conversion of (S)-methylmalonyl-coenzyme A into (R)-methylmalonyl-coenzyme A;

**[0291]** of an enzyme $E_2$, which catalyzes the conversion of (R)-methylmalonyl-coenzyme A into methyl malonate;

**[0292]** of an enzyme $E_3$, which catalyzes the conversion of methyl malonate into methylmalonate semialdehyde;

**[0293]** of an enzyme $E_4$, which catalyzes the conversion of methylmelonate-semialdehyde into 3-hydroxy-isobutyrate.

**[0294]** In this context, genetically modified cells which are especially preferred according to the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_2$, $E_3$, $E_4$, $E_6$, $E_7$, $E_{47}$, $E_{48}$, $E_{49}$, $E_{50}$, $E_{51}$, $E_{52}$ and $E_2E_3E_4E_6E_7E_{47}E_{48}E_{49}E_{50}E_{51}E_{52}$.

**[0295]** Preferred enzymes and genes of these enzymes are those genes and enzymes which have already been mentioned above in connection with the enzymes $E_2$ to $E_4$, $E_6$, $E_7$ and $E_{47}$ to $E_{52}$.

**[0296]** According to a third alternative of this first alternative of the second special embodiment of the cell according to the invention, the formation of the intermediate propionyl-coenzyme A also takes place via acetyl-coenzyme A as further intermediate, where, according to this alternative, the propionyl-coenzyme A is, again, not converted directly into methylmalonate-semialdehyde, but via (R)-methylmalonyl-coenzyme A (and not via (S)-methylmalonyl-coenzyme A). In this context, it is especially preferred that the cell features an activity of at least one of the following enzymes $E_2$ to $E_4$, $E_7$ and $E_{47}$ to $E_{52}$ which is increased in comparison with its wild type (see FIG. **13**):

**[0297]** of an enzyme $E_{47}$, which catalyzes the conversion of acetyl-coenzyme A into malonyl-coenzyme A;

**[0298]** of an enzyme $E_{48}$, which catalyzes the conversion of malonyl-coenzyme A into malonate semialdehyde;

**[0299]** of an enzyme $E_{49}$, which catalyzes the conversion of malonate semialdehyde into 3-hydroxypropionate;

**[0300]** of an enzyme $E_{50}$, which catalyzes the conversion of 3-hydroxypropionate into 3-hydroxypropionyl-coenzyme A;

**[0301]** of an enzyme $E_{51}$, which catalyzes the conversion of 3-hydroxypropionyl-coenzyme A into acryloyl-coenzyme A;

**[0302]** of an enzyme $E_{52}$, which catalyzes the conversion of acryloyl-coenzyme A into propionyl-coenzyme A;

**[0303]** of an enzyme $E_7$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonyl-coenzyme A;

**[0304]** of an enzyme $E_2$, which catalyzes the conversion of methylmalonyl-coenzyme A into methylmalonate;

**[0305]** of an enzyme $E_3$, which catalyzes the conversion of methyl malonate into methylmalonate-semialdehyde;

**[0306]** of an enzyme $E_4$, which catalyzes the conversion of methylmalonate-semialdehyde into 3-hydroxyisobutyrate.

**[0307]** In this context, genetically modified cells which are especially preferred according to the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_2$, $E_3$, $E_4$, $E_7$, $E_{47}$, $E_{48}$, $E_{49}$, $E_{50}$, $E_{51}$, $E_{52}$ and $E_2E_3E_4E_7E_{47}E_{48}E_{49}E_{50}E_{51}E_{52}$.

**[0308]** Preferred enzymes and genes of these enzymes are, again, those genes and enzymes which have already been mentioned above in connection with the enzymes $E_2$ to $E_4$, $E_7$ and $E_{47}$ to $E_{52}$.

**[0309]** According to a fourth alternative of the second special embodiment of the cell according to the invention, the formation of the intermediate propionyl-coenzyme A also takes place via acetyl-coenzyme A as further intermediate, where, according to this alternative, acetoacetyl-coenzyme A is formed as intermediate. In this context, it may be preferred that the cell features an activity of at least one of the following enzymes $E_8$ and $E_{53}$ to $E_{61}$ which is increased in comparison with its wild type:

**[0310]** of an enzyme $E_{53}$, which catalyzes the conversion of acetyl-coenzyme A into acetoacetyl-coenzyme A;

**[0311]** of an enzyme $E_{54}$, which catalyzes the conversion of acetoacetyl-coenzyme A into 3-hydroxybutanoyl-coenzyme A;

**[0312]** of an enzyme $E_{55}$, which catalyzes the conversion of 3-hydroxybutanoyl-coenzyme A into crotonyl-coenzyme A;

**[0313]** of an enzyme $E_{56}$, which catalyzes the conversion of crotonyl-coenzyme A into butyryl-coenzyme A;

**[0314]** of an enzyme $E_{57}$, which catalyzes the conversion of butyryl-coenzyme A into ethylmalonyl-coenzyme A;

**[0315]** of an enzyme $E_{58}$, which catalyzes the conversion of ethylmalonyl-coenzyme A into methylsuccinyl-coenzyme A;

**[0316]** of an enzyme $E_{59}$, which catalyzes the conversion of methylsuccinyl-coenzyme A isobutyryl-coenzyme A;

**[0317]** of an enzyme $E_{60}$, which catalyzes the conversion of isobutyryl-coenzyme A into methacrylyl-coenzyme A;

**[0318]** of an enzyme $E_{61}$, which catalyzes the conversion of methacrylyl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

**[0319]** of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**[0320]** In this context, genetically modified cells which are especially preferred according to the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_8$, $E_{53}$, $E_{54}$, $E_{55}$, $E_{56}$, $E_{57}$, $E_{58}$, $E_{59}$, $E_{60}$, $E_{61}$ and $E_8E_{53}E_{54}E_{55}E_{56}E_{57}E_{58}E_{59}E_{60}E_{61}$.

**[0321]** This metabolic pathway and the enzymes which play a role in this metabolic pathway are described, for example, in Korotkova et al., *Journal of Bacteriology* (2002), pages 1750 to 1758.

**[0322]** According to a fifth alternative of the second special embodiment of the cell according to the invention, the formation of the intermediate propionyl-coenzyme A takes place via, again, acetyl-coenzyme A as further intermediate, where, according to this alternative, acetoacetyl-coenzyme A is formed as further intermediate but where, in this case, ethylmalonyl-coenzyme A is formed directly from crotonyl-coenzyme A. In this context, it may be preferred that the cell features an activity of at least one of the following enzymes $E_8$ and $E_{53}$ to $E_{56}$ and $E_{62}$ to $E_{65}$ which is increased in comparison with its wild type (see FIG. **14**):

**[0323]** of an enzyme $E_{53}$, which catalyzes the conversion of two acetyl-coenzyme A units into acetoacetyl-coenzyme A;

**[0324]** of an enzyme $E_{54}$, which catalyzes the conversion of acetoacetyl-coenzyme A into 3-hydroxybutyryl-coenzyme A;

**[0325]** of an enzyme $E_{55}$, which catalyzes the conversion of 3-hydroxybutyryl-coenzyme A into crotonyl-coenzyme A;

**[0326]** of an enzyme $E_{56}$, which catalyzes the conversion of crotonyl-coenzyme A into ethylmalonyl-coenzyme A;

**[0327]** of an enzyme $E_{62}$, which catalyzes the conversion of ethylmalonyl-coenzyme A into methylsuccinyl-coenzyme A;

**[0328]** of an enzyme $E_{63}$, which catalyzes the conversion of methylsuccinyl-coenzyme A into mesaconyl-coenzyme A;

**[0329]** of an enzyme $E_{64}$, which catalyzes the conversion of mesaconyl-coenzyme A into β-methylmalyl-coenzyme A;

**[0330]** of an enzyme $E_{65}$, which catalyzes the conversion of β-methylmalyl-coenzyme A into glyoxylate and propionyl-coenzyme A.

**[0331]** Then, from propionyl-coenzyme A can in the above-described manner (increasing the activity of one or more of the enzymes $E_7$, $E_2$, $E_3$ and $E_4$, increasing the activity of one

or more of the enzymes $E_7$, $E_6$, $E_2$, $E_3$ and $E_4$, or increasing the activity of one of the, or of both, enzymes $E_4$ and $E_5$).

**[0332]** In this context, it is especially preferred that the enzyme

**[0333]** $E_{53}$ is a β-ketothiolase (EC 2.3.1.9),

**[0334]** $E_{54}$ is an acetoacetyl-coenzyme A reductase (an EC 1.1.1.36),

**[0335]** $E_{55}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17),

**[0336]** $E_{56}$ is a crotonyl-coenzyme A decarboxylase,

**[0337]** $E_{62}$ is an ethylmalonyl-coenzyme A mutase (EC 5.4.99.2),

**[0338]** $E_{63}$ is a methylsuccinyl-coenzyme A dehydrogenase,

**[0339]** $E_{64}$ is a mesaconyl-coenzyme A hydratase, and

**[0340]** $E_{65}$ is a β-methylmalyl/L-malyl-coenzyme A lyase.

**[0341]** The enzyme $E_{53}$ is preferably encoded by genes selected from the group consisting of acat1, acat2, loc484063, loc489-421, mgc69098, mgc81403, mgc81256, mgc83664, kat-1, erg10, ygeF, atoB, fadAx, phbA-1, phbA-2, atoB-2, pcaF, pcaF-2, phb-A, bktB, phaA, tioL, thlA, fadA, paaJ, phbAf, pimB, mmgA, yhfS, thl, vraB, th1, mvaC, thiL, paaJ, fadA3, fadA4, fadA5, fadA6, cgl12392, catF, sc8f4.03, thiL1, thiL2, acaB1, acaB2, acaB3, acaB4 or, where acat1, acat2, atoB and phbA and the corresponding gene from *Rhodobacter sphaeroides* are especially preferred.

**[0342]** The enzyme $E_{54}$ is preferably encoded by genes selected from the group consisting of phbB, fabG, phbN1, phbB2 or cgl12444, where phbB is especially preferred and the corresponding gene from *Rhodobacter sphaeroides* is especially preferred.

**[0343]** The enzyme $E_{55}$ is preferably encoded by genes selected from the group consisting of echS1, ehhadh, hadha, echs1-prov, Das Enzym $E_{55}$ wird vorzugsweise von Genen ausgewählt aus der Gruppe bestehend aus echS1, ehhadh, hadha, echs1-prov, cg4389, cg4389, cg6543, cg6984, cg8778, ech-1, ech-2, ech-3, ech-4, ech-5, ech-6, ech-7, FCAALL.314, fcaal1.21, fox2, eci1, eci2, paaF, paaG, yfcX, fadB, faoA, rpfF, phaA, phaB, echA1, echA2, echA3, echA4, echA5, echA6, echA7, echA8, echA9, echA9, echA10, echA11, echA12, echA13, echA14, echA15, echA16, echA17, echA18, echA19, echA20, echA21, fad-1, fad-2, fad-3, dcaE, hcaA, fadJ, rsp0671, rsp0035, rsp0648, rsp0647, rs03234, rs03271, rs04421, rs04419, rs02820, rs02946, paaG1, paaG2, paaG3, ech, pksH, ydbS, eccH1, ecCH2, pimF, fabJ1, fabJ2, caiD2, ysiB, yngF, yusL, fucA, cgl0919, scf41.23, scd10.16, sck13.22, scp8.07c, stbac16h6.14, sc5f2a.15, sc6a5.38, hbd-1, hbd-2, hdb-3, hdb-4, hdb-5, hdb-6, hdb-7, hdb-8, hdb-9, hdb-10, fad-1, fad-2, fad-3, fad-4, fad-5, paaF-1, paaF-2, paaF-3, paaF-4, paaF-5, paaF-6, paaF-7 and crt

where the corresponding gene from *Rhodobacter sphaeroides* is especially preferred.

**[0344]** The enzyme which is preferably employed as enzyme $E_{56}$ is an enzyme from *Rhodobacter sphaeroides* which is encoded by the DNA sequence with the SEQ ID No 05 and which has the amino acid sequence as shown in SEQ ID No 06.

**[0345]** Suitable genes for the enzyme $E_{62}$ are selected from the group consisting of mut, mutA, mutB, sbm, sbmA, sbmB, sbm5, bhbA, mcmA, mcmA1, mcmA2, mcmB, mcm1, mcm2, mcm3, icmA, meaA1 and meaA2, where, again, the corresponding gene from *Rhodobacter sphaeroides* is especially preferred.

**[0346]** Preferred genes for the enzymes $E_{63}$, $E_{64}$ and $E_{65}$ are, in particular, the genes for these enzymes from *Rhodobacter sphaeroides*.

**[0347]** Further examples of nucleotide sequences of the abovementioned genes can also be found in the KEGG database, the NCBI database or the EMBL database, inter alia.

**[0348]** As has already been explained above, the first alternative of the second preferred embodiment cell according to the invention generates 3-hydroxyisobutyric acid or the polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via propionyl-coenzyme A and acetyl-coenzyme A as intermediates. In this context, it may be meaningful, in principle, to influence not only one or more of the abovementioned enzymatic activities $E_2$ to $E_8$ and $E_{47}$ to $E_{65}$, but also those enzymatic activities which bring about an increase in the formation of acetyl-coenzyme A in the cell.

**[0349]** In the event that 3-hydroxyisobutyric acid is formed from carbohydrates or glycerol as carbon source, it may be preferred that the cell features an increased activity in an enzyme $E_{66}$, which catalyzes the conversion of pyruvate into acetyl-coenzyme A. This enzyme $E_{66}$ preferably takes the form of a pyruvate dehydrogenase (EC 1.2.1.51).

**[0350]** In the event that 3-hydroxyisobutyric acid is formed from $C_1$-carbon sources such as, for example, methane or methanol, it may be preferred that the cell features an activity of at least one of the enzymes $E_{67}$ to $E_{71}$ which is increased in comparison with its wild type:

**[0351]** of an enzyme $E_{67}$, which catalyzes the conversion of methane into methanol;

**[0352]** of an enzyme $E_{68}$, which catalyzes the conversion of methanol into formaldehyde;

**[0353]** of an enzyme $E_{69}$, which catalyzes the conversion of formaldehyde into 5,10-methylenetetrahydrofolate;

**[0354]** of an enzyme $E_{70}$, which catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate;

**[0355]** of an enzyme $E_{71}$, which catalyzes the conversion of 5-methyltetrahydrofolate into acetyl-coenzyme A.

**[0356]** In this context, it is especially preferred that the enzyme

**[0357]** $E_{67}$ is a methane monooxygenase (EC 1.14.13.25),

**[0358]** $E_{68}$ is a methanol dehydrogenase (EC 1.1.1.244),

**[0359]** $E_{69}$ is a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27),

**[0360]** $E_{70}$ is a methylenetetrahydrofolate reductase (EC 1.5.1.20),

**[0361]** $E_{71}$ is a carbon monoxide dehydrogenase (EC 1.2.99.2).

**[0362]** The nucleotide sequences of suitable genes for the enzymes $E_{63}$ to $E_{67}$ can be found in the KEGG database, the NCBI database or the EMBL database.

**[0363]** According to a third special embodiment of the cell according to the invention, where the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via methylmalonate-semialdehyde as precursor, it is preferred that the formation of 3-hydroxyisobutyric acid or of the polyhydroxyalkanoate based on 3-hydroxyisobutyric acid takes place via acryloyl-coenzyme A as intermediate, where the cell is capable of preferentially utilizing carbohydrates, glycerol or glutamate as carbon source.

**[0364]** In connection with the third special embodiment of the cell according to the invention, it is especially preferred when this cell features an activity of at least one of the fol-

lowing enzymes $E_{10}$ to $E_{12}$, $E_{56}$, $E_{72}$ and $E_{73}$ which is increased in comparison with its wild type (see FIG. **15**):

**[0365]** of an enzyme $E_{72}$, which catalyzes the conversion of beta-alanine to beta-alanyl-coenzyme A,

**[0366]** of an enzyme $E_{73}$, which catalyzes the conversion of beta-alanyl-coenzyme A into acrylyl-coenzyme A,

**[0367]** of an enzyme $E_{56}$, which catalyzes the conversion of acrylyl-coenzyme A into methylmalonyl-coenzyme A,

**[0368]** of an enzyme $E_{10}$, which catalyzes the conversion of methylmalonyl-coenzyme A into methyl malonate;

**[0369]** of an enzyme $E_{11}$, which catalyzes the conversion of methyl malonate into methylmalonate-semialdehyde;

**[0370]** of an enzyme $E_{12}$, which catalyzes the conversion of methylmalonate-semialdehyde into 3-hydroxyisobutyric acid.

**[0371]** In this context, cells which are especially preferred according to the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_{56}E_{10}$, $E_{56}E_{11}$, $E_{56}E_{12}$, $E_{56}E_{10}E_{11}$ and $E_{72}E_{73}E_{56}E_{10}E_{11}E_{12}$. In connection with the fourth special embodiment, too, of the cell according to the invention it may be advantageous to overexpress an enzyme which is capable of catalyzing at least two of the above-described reaction steps. Here too, it is possible for example to employ an enzyme which features both the activity of the enzyme $E_{10}$ and the activity of the enzyme $E_{11}$, such as, for example, the malonyl-coenzyme A reductase from *Sulfolobus tokodaii*, which is encoded by the DNA sequence with the SEQ ID No 03 and which features the amino acid sequence as shown in SEQ ID No 04. Furthermore, it is, in principle, also possible in the context of the fourth special embodiment of the cell according to the invention to employ a cell which is already capable of forming especially large amounts of acrylyl-coenzyme A.

**[0372]** In this context, it is especially preferred that the enzyme

**[0373]** $E_{72}$ is a coenzyme A transferase (EC 2.8.3.1) or a coenzyme A synthetase, preferably a coenzyme A transferase,

**[0374]** $E_{73}$ is a beta-alanyl-coenzyme A ammonia-lyase (EC 4.3.1.6),

**[0375]** $E_{56}$ is a crotonyl-coenzyme A decarboxylase

**[0376]** $E_{10}$ is a methylmalonyl-coenzyme A hydrolase (EC 3.1.2.17),

**[0377]** $E_{11}$ is an aldehyde dehydrogenase (EC 1.2.1.3) or an aldehyde oxidase (EC 1.2.3.1) and

**[0378]** $E_{12}$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**[0379]** Preferred enzymes $E_{72}$ with a CoA transferase activity are those from *Megasphaera elsdenii, Clostridium propionicum, Clostridium kluyveri* and also from *Escherichia coli*. Examples which may be mentioned at this point of a DNA sequence coding for a CoA transferase is the sequence from *Megasphaera elsdenii* referred to as SEQ ID No: 24 in WO-A-03/062173. Enzymes which are furthermore preferred are those variants of the CoA transferase which are described in WO-A-03/062173.

**[0380]** Suitable enzymes $E_{73}$ with a beta-alanyl-coenzyme A ammonia-lyase activity are, for example, those from *Clostridium propionicum*. DNA sequences which code for such an enzyme can be obtained for example from *Clostridium propionicum* as described in Example 10 in WO-A-03/062173. The DNA sequence which codes for the beta-alanyl-coenzyme A ammonia-lyase from *Clostridium propionicum* is specified in WO-A-03/062173 as SEQ ID No: 22.

**[0381]** An enzyme $E_{56}$ which is preferably employed is, again, the crotonyl-coenzyme A decarboxylase from *Rhodobacter sphaeroides*, which is encoded by the DNA sequence with the SEQ ID No 05 and which features the amino acid sequence as shown in SEQ ID No 06. This enzyme is not only capable of converting crotonyl-coenzyme A into ethylmalonyl-coenzyme A, but also of converting acrylyl-coenzyme A into methylmalonyl-coenzyme A.

**[0382]** Suitable genes for the enzymes $E_{10}$ to $E_{12}$ have already been mentioned in connection with the first variant of the cell according to the invention, where it is also preferred in connection with the second variant, the above-described gene from *Sulfolobus tokodaii* is especially preferred as gene for the enzyme $E_{11}$.

**[0383]** According to an especially preferred variant of the third special embodiment of the cell according to the invention, this cell features at least one activity of the enzyme $E_{10}$ and $E_{56}$ or of the enzymes $E_{10}$, $E_{11}$ and $E_{56}$ which is increased in comparison with its wild type, where the $E_{10}$ or the enzymes $E_{10}$ and $E_{11}$ is encoded by a DNA sequence as shown in SEQ ID No 03 and the enzyme $E_{56}$ is encoded by a DNA sequence as shown in SEQ ID No 05.

**[0384]** In this context, it is preferred when the increased activity of these two enzymes is achieved by overexpressing, in the cell, the polypeptides with SEQ ID No 04 and SEQ ID No 06 or else that amino acid sequences with at least 50%, preferably at least 55%, more preferably at least 60%, more preferably at least 65% and most preferably at least 70% identity with the amino acid sequence as shown in SEQ ID No 04 and SEQ ID No 06, respectively. In this context, these two DNA sequences may be integrated into the genome of the cell or else be present on a vector inside the cell.

**[0385]** In connection with the above-described third special embodiment of the cell according to the invention, it may furthermore be advantageous when the cell features not only an increase in the activity of the enzyme $E_{56}$ and/or of the activity of the enzyme $E_{10}$ or of the enzymes $E_{10}$ and $E_{11}$, but at least one, preferably both, of the following properties:

**[0386]** an activity of an enzyme $E_n$, which catalyzes the conversion of pyruvate into oxaloacetate or of an enzyme $E_{74}$, which catalyzes the conversion of phosphoenolpyruvate into oxaloacetate, but preferably of an enzyme $E_{11}$, which catalyzes the conversion of pyruvate into oxaloacetate, which is increased in comparison with its wild type, and

**[0387]** an increased activity of an enzyme $E_{75}$, which catalyzes the conversion of aspartate into beta-alanine.

**[0388]** The enzyme $E_{11}$ preferably takes the form of a carboxylase, especially preferably of a pyruvate carboxylase (EC number 6.4.1.1), which catalyzes the conversion of pyruvate into oxaloacetate. A pyruvate carboxylase which is especially preferred in this context is the mutant which is described in "*A novel methodology employing Corynebacterium glutamicum genome information to generate a new L-lysine-producing mutant*." Ohnishi J et al., Applied Microbiology and Biotechnology, Vol. 58 (2), pages 217-223 (2002). In this mutation, the amino acid proline at position 458 has been substituted by serine. The disclosure of this publication with regard to the possibilities of preparing pyruvate carboxylate mutants is hereby incorporated by referent and forms part of the disclosure of the present invention.

**[0389]** The enzyme $E_{75}$ preferably takes the form of a decarboxylase, especially preferably of a glutamate decarboxylate or of an aspartate decarboxylase, with a 1-aspartate 1-decarboxylase (EC number 4.1.1.11) which is encoded by the panD gene being most preferred. Aspartate decarboxylase catalyzes the conversion of aspartate into beta-alanine. Genes for aspartate decarboxylase (panD genes) from, inter alia, *Escherichia coli* (FEMS Microbiology Letters, 143, pages 247-252 (1996)), "*Photorhabdus luminescens* subsp. *Laumondii, Mycobacterium bovis* subsp. *Bovis*") and from a large number of other microorganisms have already been cloned and sequenced. DE-A-198 55 313 describes in particular the nucleotide sequence of the panD gene from *Corynebacterium glutamicum*. In principle, it is possible to use panD genes of any feasible origin, no matter whether from bacteria, yeasts or fungi. Furthermore, it is possible to employ all alleles of the panD gene, in particular also those which are the result of the degeneracy of the genetic code or of function-neutral sense mutations. An aspartate decarboxylase which is especially preferred according to the invention, besides the aspartate decarboxylase from *Corynebacterium glutamicum*, is the *Escherichia coli* mutant DV9 (Vallari and Rock, Journal of Bacteriology, 164, pages 136-142 (1985)). The disclosure of this publication with regard to the abovementioned mutant is hereby incorporated by reference and forms part of the disclosure of the present invention. The preparation of recombinant cells in which both the activity of the pyruvate carboxylase and the activity of the aspartate decarboxylase is increased is described in DE-A-10 2005 048 818.

**[0390]** According to a second variant of the cell according to the invention, the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxy-isobutyric acid takes place via 3-hydroxyisobutyryl-coenzyme A as precursor.

**[0391]** In the event that, in the cell according to the invention, the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via 3-hydroxyisobutyryl-coenzyme A as precursor, as specified in the second variant, it is preferred according to a first special embodiment that the formation of 3-hydroxyisobutyric acid or of the polyhydroxyalkanoate based on 3-hydroxyisobutyric acid takes place via isobutyryl-coenzyme A as intermediate, where the cell is capable of preferentially utilizing carbohydrates, glycerol or L-valine as carbon source.

**[0392]** In the event that carbohydrates or glycerol act as the carbon source, it is preferred, according to a first alternative of this first special embodiment of the second variant of the cell according to the invention that this cell features an activity of at least one of the following enzymes $E_{76}$ to $E_{79}$, $E_{60}$, $E_{61}$ and $E_8$ which is increased in comparison with its wild type (see FIG. **16**):

**[0393]** of an enzyme $E_{76}$, which catalyzes the conversion of pyruvate into 2-acetolactate;

**[0394]** of an enzyme $E_{77}$, which catalyzes the conversion of 2-acetolactate into 2,3-dihydroxyisovalerate;

**[0395]** of an enzyme $E_{78}$, which catalyzes the conversion of 2,3-dihydroxyisovalerate into 2-oxoisovalerate;

**[0396]** of an enzyme $E_{79}$, which catalyzes the conversion of 2-oxoisovalerate into isobutyryl-coenzyme A;

**[0397]** of an enzyme $E_{60}$, which catalyzes the conversion of isobutyryl-coenzyme A into methacrylyl-coenzyme A;

**[0398]** of an enzyme $E_{61}$, which catalyzes the conversion of methacrylyl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

**[0399]** of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**[0400]** In this context, genetically modified cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_8$, $E_{60}$, $E_{61}$, $E_{76}$, $E_{77}$, $E_{78}$, $E_{79}$ and $E_8E_{60}E_{61}E_{76}E_{77}E_{78}E_{79}$.

**[0401]** In this context, it is especially preferred that the enzyme

**[0402]** $E_8$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

**[0403]** $E_{76}$ is an acetolactate synthase (EC 2.2.1.6),

**[0404]** $E_{77}$ is a dihydroxyisovalerate dehydrogenase (EC 1.1.1.86),

**[0405]** $E_{78}$ is a 2,3-dihydroxyisovalerate dehydratase (EC 4.2.1.9),

**[0406]** $E_{79}$ is a 2-oxoisovalerate dehydrogenase (EC 1.2.1.25 or EC 1.2.4.4),

**[0407]** $E_{60}$ is an acyl-coenzyme A dehydrogenase (EC 1.3.99.3), a butyryl-coenzyme A dehydrogenase (EC 1.3.99.2) or a 2-methylacyl-coenzyme A dehydrogenase (EC 1.3.99.12), and

**[0408]** $E_{61}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17).

**[0409]** Preferred enzymes $E_8$, $E_{60}$ and $E_{61}$ are those which have already been described above.

**[0410]** The enzyme $E_{76}$ is preferably encoded by genes selected from the group consisting of ilvbl, t8p19.70, ilv1, ilv2, ilv6, aal021wp, ael305 cp, ilvI, ilvH, ilvN, ilvB, ilvM, ilvG, ilvN, budB, ilvN-1, ilvN-2, atrC, ilvX, iolD, budB, alsS, ilvK, ilvB1, ilvB2, ilvB3, ilvN1, ilvN2, cgl1271, cgl1272, iolD and scc57A.40c.

**[0411]** The enzyme $E_{77}$ is preferably encoded by genes selected from the group consisting of f14p22.200, ilv5, ac1198Wp, ilvC, ilvY, ilvC-1, ilvC-2, ilvC-3 and cgl1273, where the ilvC gene is most preferred.

**[0412]** The enzyme $E_{78}$ is preferably encoded by genes selected from the group consisting of f14o13.18, ilv3, acl117wp, ilvD, cgl1268, ilvD1 and ilvD2, where ilvD is most preferred.

**[0413]** In the event that L-valine acts as carbon source, it is preferred according to a second modification of the first special embodiment of the second alternative of the cell according to the invention, where the formation of 3-hydroxyisobutyric acid or of poly-hydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via 3-hydroxyisobutyryl-coenzyme A as precursor and isobutyryl-coenzyme A as intermediate, that this cell features an activity of at least one of the following enzymes $E_{79}$, $E_{80}$, $E_{60}$, $E_{61}$ and $E_8$ which is increased in comparison with its wild type (see FIG. **17**):

**[0414]** of an enzyme $E_{80}$, which catalyzes the conversion of L-valine into 2-oxoisovalerate;

**[0415]** of an enzyme $E_{79}$, which catalyzes the conversion of 2-oxoisovalerate into isobutyryl-coenzyme A;

**[0416]** of an enzyme $E_{60}$, which catalyzes the conversion of isobutyryl-coenzyme A into methacrylyl-coenzyme A;

**[0417]** of an enzyme $E_{61}$, which catalyzes the conversion of methacrylyl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

**[0418]** of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**[0419]** In this context, genetically modified cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_8$, $E_{60}$, $E_{61}$, $E_{79}$, $E_{80}$ and $E_8E_{60}E_{61}E_{79}E_{80}$.

**[0420]** In this context, it is especially preferred that the enzyme

**[0421]** $E_8$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

**[0422]** $E_{60}$ is an acyl-coenzyme A dehydrogenase (EC 1.3.99.3), a butyryl-coenzyme A dehydrogenase (EC 1.3.99.2) or a 2-methylacyl-coenzyme A dehydrogenase (EC 1.3.99.12),

**[0423]** $E_{61}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17),

**[0424]** $E_{79}$ is a 2-oxoisovalerate dehydrogenase (EC 1.2.1.25 or EC 1.2.4.4), and

**[0425]** $E_{80}$ is an amino acid transferase (EC 2.6.1.42).

**[0426]** Preferred enzymes $E_8$, $E_{60}$, $E_{61}$ and $E_{79}$ are those which have already been described above.

**[0427]** The enzyme $E_{80}$ is preferably encoded by genes selected from the group consisting of bcat1, bcat2, t27I1.8, t27i1.9, f2j10.5, f2j10.4, t12h1.16, mmb12.20, t9c5.3, mpa24.13, bat1, bat2, ad1384wp, eca39, bcaA, ilvE, ilvE1, ilvE2, ilvE3, ywaA, ybgE, bcaT and cgl2204, where ilvE is especially preferred.

**[0428]** The nucleotide sequences of suitable genes the enzyme $E_{80}$ can, again, be found in the KEGG database, the NCBI database or the EMBL database.

**[0429]** In connection with this second alternative of the first special embodiment of the second variant of the cell according to the invention, it may furthermore be advantageous to reduce the activity of an enzyme $E_4$ which catalyzes the conversion of methylmalonate-semialdehyde into 3-hydroxyisobutyric acid, where this enzyme $E_4$ preferably takes the form of a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or of a 3-hydroxy-acyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**[0430]** According to the second modification of the first special embodiment of the second variant of the cell according to the invention, where the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via 3-hydroxyisobutyryl-coenzyme A as precursor and isobutyryl-coenzyme A as intermediate and starting from L-valine as carbon source, it may furthermore be preferred to employ those cells which are already capable of forming large amounts of L-valine. In this context, suitable cells are in particular those which have been described by Blombach et al. in *Applied Environmental Microbiology, Vol.* 73 (7) (2007), pages 2079-2084.

**[0431]** In the event that $C_1$-compounds such as, for example, methane or methanol act as carbon source, it is preferred in a second special embodiment of the second variant of the cell according to the invention, where the formation of 3-hydroxyisobutyric acid or of poly-hydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via 3-hydroxyisobutyryl-coenzyme A as precursor, that the formation takes place via 3-hydroxyisobutyryl-coenzyme A as intermediate. In this context, it is preferred that the cell features an activity of at least one of the following enzymes $E_8$, $E_{53}$, $E_{54}$ and $E_{81}$ which is increased in comparison with its wild type:

**[0432]** of an enzyme $E_{53}$, which catalyzes the conversion of acetyl-coenzyme A into acetoacetyl-coenzyme A;

**[0433]** of an enzyme $E_{54}$, which catalyzes the conversion of acetoacetyl-coenzyme A into 3-hydroxybutyryl-coenzyme A;

**[0434]** of an enzyme $E_{81}$, which catalyzes the conversion of 3-hydroxybutyryl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

**[0435]** of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**[0436]** In this context, genetically modified cells which are especially preferred in accordance with the invention are those in which the activity of the following enzymes or enzyme combinations is increased: $E_9$, $E_{53}$, $E_{54}$, $E_{81}$ and $E_8E_{53}E_{54}E_{81}$.

**[0437]** In this context, it is especially preferred that the enzyme

**[0438]** $E_8$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

**[0439]** $E_{53}$ is a β-ketothiolase (EC 2.3.1.9),

**[0440]** $E_{54}$ is an acetoacetyl-coenzyme A reductase (an EC 1.1.1.36), and

**[0441]** $E_{81}$ is an isobutyryl-coenzyme mutase (EC 5.4.99.13).

**[0442]** Preferred enzymes $E_8$, $E_{53}$ and $E_{54}$ are those which have already been described hereinabove. A preferred enzyme $E_{81}$ is the isobutyryl-coenzyme mutase from β-proteo-bacterium strain L108 which is described in *Applied And Environmental Microbiology, Vol.* 72 (6), 2006, pages 4128-4135.

**[0443]** According to a special embodiment of the cell according to the invention, it is furthermore preferred that this cell features an expression of the glb0 gene which is increased in comparison with its wild type. Furthermore, it may under certain circumstances be preferred that the cell according to the invention features an activity of the citrate transport protein which is encoded by the dctA gene or the citP gene, which activity is reduced in comparison with its wild type.

**[0444]** A contribution to the solution of the problems mentioned at the outset is furthermore provided by a method of preparing a genetically modified cell which is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via methylmalonate-semialdehyde or isobutyryl-coenzyme A, as precursors, comprising the method step of increasing, in the cell, the activity of at least one of the above-described enzymes, preferably of one or more of the enzymes

**[0445]** $E_1$ to $E_4$,

**[0446]** $E_1$, $E_4$, $E_5$, $E_6$ and $E_7$,

**[0447]** $E_1$, $E_4$, $E_5$ and $E_7$,

**[0448]** $E_4$, $E_5$ and $E_{47}$ to $E_{52}$,

**[0449]** $E_2$ to $E_4$, $E_6$, $E_7$ and $E_{47}$ to $E_{52}$,

**[0450]** $E_2$ to $E_4$, $E_7$ and $E_{47}$ to $E_{52}$,

**[0451]** $E_8$ and $E_{53}$ to $E_{61}$,

**[0452]** $E_8$, $E_{60}$, $E_{61}$ and $E_{76}$ to $E_{79}$,

**[0453]** $E_8$, $E_{60}$, $E_{61}$, $E_{79}$ and $E_{80}$, or

**[0454]** $E_8$, $E_{53}$, $E_{54}$ and $E_{82}$

in the cell, where increasing the enzymatic activity is preferably carried out by the methods described at the outset.

**[0455]** Another contribution to the solution of the problems mentioned at the outset is provided by the cells obtainable by the above-described method.

**[0456]** Another contribution to the solution of the problems mentioned at the outset is provided by a method of producing 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on

3-hydroxyisobutyric acid, comprising the method step of bringing a cell according to the invention into contact with a nutrient medium comprising, as carbon source, carbohydrates, glycerol, carbon dioxide, methane, methanol, L-valine or L-glutamate under conditions under which 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid are formed from the carbon source, and, if appropriate, isolation of the 3-hydroxyisobutyric acid from the nutrient medium.

**[0457]** The genetically modified cells according to the invention can be into contact with the nutrient medium, and thus cultured, either continuously or batchwise in the batch method or in the fed-batch method or in the repeated-fed-batch method in order to produce 3-hydroxyisobutyrate or polyhydroxyalkanoates based on 3-hydroxyisobutyrate. A semicontinuous method as described in GB-A-1009370 is also feasible. An overview over known culture methods are described in the textbook by Chmiel ("*Bioprozesstechnik* 1. *Einführung in die Bioverfahrenstechnik*" [Bioprocess technology 1. introduction to bioprocess technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas ("*Bioreaktoren and periphere Einrichtungen*", [Bioreactors and peripheral equipment] Vieweg Verlag, Braunschweig/Wiesbaden, 1994).

**[0458]** The culture medium to be used must suitably meet the requirements of the strains in question. Descriptions of culture media for various microorganisms can be found in the textbook "*Manual of Methods for General Bacteriology*" of the American Society for Bacteriology (Washington D.C., USA, 1981).

**[0459]** Carbon sources which may be used are carbohydrates such as, for example, glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as, for example, soy oil, sunflower oil, peanut oil and coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and linolic acid, alcohols such as, for example, glycerol and methanol, hydrocarbons such as methane, amino acids such as L-glutamate or L-valine, or organic acids such as, for example, acetic acid. These substances may be used singularly or as a mixture. It is especially preferred to employ carbohydrates, in particular monosaccharides, oligosaccharides or polysaccharides, as described in U.S. Pat. No. 601, 494 and U.S. Pat. No. 6,136,576, or $C_5$-sugars, or glycerol.

**[0460]** Nitrogen sources which can be used are organic nitrogen-comprising compounds such as peptones, yeast extract, meat extract, malt extract, cornsteep liquor, soya mill and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used singularly or as a mixture.

**[0461]** Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-comprising salts can be used as sources of phosphorus. The culture medium must furthermore comprise salts of metals such as, for example, magnesium sulfate or iron sulfate, which are required for growth. Finally, essential growth factors such as amino acids and vitamins may be employed in addition to the abovementioned substances. Moreover, suitable precursors may be added to the culture medium. The abovementioned input materials may be added to the culture in the form of a single batch or else fed in a suitable manner during culturing.

**[0462]** The pH for the culture can be controlled by employing, in an appropriate manner, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia, or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids, it is possible to add to the medium suitable substances which have a selective effect, such as, for example, antibiotics. Aerobic conditions are maintained by introducing, into the culture, oxygen or oxygen-containing gas mixtures such as, for example, ambient air. The culture temperature is normally 20° C. to 45° C. and preferably 25° C. to 40° C. It may be preferred to employ, as cells, those cells which are described in U.S. Pat. No. 6,803, 218, in particular when using cells which are capable of converting glycerol as the substrate. In this case, the cells can be cultured at temperatures in the range of from 40 to 100° C.

**[0463]** The isolation of 3-hydroxyisobutyric acid from the nutrient solution is preferably carried out continuously, it being furthermore preferred in this context also to produce 3-hydroxyisobutyric acid by fermentation in a continuous manner, so that the entire process from the production of 3-hydroxyisobutyric acid up to its isolation from the fermentation liquor can be carried out continuously. For the continuous isolation of the production of 3-hydroxyisobutyric acid from the fermentation liquor, the former is continuously passed over a device for removing the microorganisms employed during fermentation, preferably through a filter with an exclusion level in the range of from 20 to 200 kDa, where a solid/liquid separation takes place. It is also feasible to employ a centrifuge, a suitable sedimentation device or a combination of these devices, it being especially preferred to first separate at least part of the microorganisms by sedimentation and subsequently to feed the fermentation liquor, which has been freed from part of the microorganisms, to ultrafiltration or to a centrifugation device.

**[0464]** After the microorganisms have been removed, the fermentation product, which is enriched with regard to its 3-hydroxyisobutyric acid fraction, is fed to a separation system, preferably a multistep separation system. This separation system provides a plurality of separation steps which are connected in series, from which steps in each case return lines lead away and back to the fermentation tank. Furthermore, exit pipes lead out of the respective separation steps. The individual separation steps may operate by the electrodialysis, the reverse osmosis, the ultrafiltration or the nanofiltration principle. As a rule, these are membrane separation devices in the individual separation steps. The selection of the individual separation steps is a function of the nature and the extent of the fermentation by-products and substrate residues.

**[0465]** Besides the 3-hydroxyisobutyric acid being separated off by means of electrodialysis, reverse osmosis, ultrafiltration or nanofiltration, in the course of which an aqueous 3-hydroxyisobutyric acid solution is obtained as the end product, the 3-hydroxyisobutyric acid can also be separated off by extractive methods from the fermentation solution which has been freed from microorganisms, in which case, finally, the pure 3-hydroxyisobutyric acid can be obtained. To separate the 3-hydroxyisobutryic acid by extraction, it is possible to add, to the fermentation solution, for example ammonium compounds or amines in order to form an ammonium salt of 3-hydroxyisobutyric acid. This ammonium salt can then be separated from the fermentation solution by adding an organic extractant and subsequently heating the resulting mixture, whereby the ammonium salt is concentrated in the organic phase. Then, the 3-hydroxyisobutyric acid can be

isolated from this phase for example by further extraction steps, giving the pure 3-hydroxyisobutyric acid. More details regarding the separation method can be found in WO-A-02/090312, whose disclosure regarding the separation of hydroxycarboxylic acids from fermentation solutions is hereby incorporated by reference and forms part of the disclosure of the present application.

**[0466]** Depending on the way in which the 3-hydroxyisobutyric acid is separated from the fermentation solution, either an aqueous solution of 3-hydroxyisobutyric acid comprising 2 to 90% by weight, preferably 7.5 to 50% by weight and especially preferably 10 to 25% by weight of 3-hydroxyisobutyric acid, or else pure 3-hydroxyisobutyric acid is obtained.

**[0467]** Furthermore, the 3-hydroxyisobutyric acid prepared by the method according to the invention can also be neutralized, either before, during or after the purification, for which purpose bases such as, for example, calcium hydroxide or sodium hydroxide can be employed.

**[0468]** A contribution to solving the problems mentioned at the outset is provided in particular also by a method of preparing methacrylic acid or methacrylic esters, comprising the method steps

**[0469]** IA) preparation of 3-hydroxyisobutyric acid by the method described above and, if appropriate, isolation and/or neutralization of the 3-hydroxyisobutyric acid of the 3-hydroxyisobutyric acid,

**[0470]** IB) dehydration of the 3-hydroxyisobutyric acid with formation of methacrylic acid and, if appropriate, esterification of the methacrylate or of the methacrylic acid.

**[0471]** According to method step IB), the 3-hydroxyisobutyric acid is dehydrated with formation of methacrylic acid, for which purpose it is possible either to employ the pure 3-hydroxyisobutyric acid isolated from the fermentation solution or else the aqueous solution of 3-hydroxyisobutryic acid, which has been isolated when working up the fermentation solution, it also being possible to concentrate the aqueous solution of 3-hydroxyisobutyric acid, if appropriate, before the dehydration step, for example by means of distillation, if appropriate in the presence of a suitable entrainer.

**[0472]** The dehydration reaction can, in principle, be carried out in liquid phase or in the gas phase. Furthermore, it is preferred in accordance with the invention that the dehydration reaction is carried out in the presence of a catalyst, with the nature of the catalyst employed depending on whether a gas-phase or a liquid-phase reaction is carried out.

**[0473]** Suitable dehydration catalysts are both acidic catalysts and alkaline catalysts. Acidic catalysts are preferred, in particular because they show less tendency to form oligomers. The dehydration catalyst may be employed both as a homogeneous and as a heterogeneous catalyst. If the dehydration catalyst is present in the form of a heterogeneous catalyst, it is preferred that the dehydration catalyst is in contact with a support x. Suitable supports x are all solids believed by the skilled worker to be suitable. In the present context, it is preferred that the solids have suitable pore volumes which are suitable for good binding and absorption of the dehydration catalyst. Furthermore, total pore volumes as specified by DIN 66133 in a range of from 0.01 to 3 ml/g are preferred, and total pore volumes in the range of from 0.1 to 1.5 ml/g are especially preferred. Moreover, it is preferred that the solids which are suitable as support x have a surface area in the range of from 0.001 to 1000 $m^2/g$, preferably in the range of from 0.005 to 450 $m^2/g$ and furthermore preferred in the range of from 0.01 to 300 $m^2/g$ as determined by BET test as specified in DIN 66131. A support which may be employed for the dehydration catalyst can firstly be bulk material with a mean particle diameter in the range of from 0.1 to 40 mm, preferably in the range of from 1 to 10 mm, and furthermore preferably in the range from 1.5 to 5 mm. The wall of the dehydration reactor may furthermore act as support. Furthermore, the support may be acidic or alkaline per se, or else an acidic or alkaline dehydration catalyst may be applied to an inert support. Application techniques which may be mentioned in particular are immersion or impregnation or else incorporation into a support matrix.

**[0474]** Suitable supports x, which may also feature dehydration catalyst properties, are, in particular, natural or synthetic silicates such as, in particular, mordenite, montmorillonite, acidic zeolites; supports which are coated with monobasic, dibasic or polybasic inorganic acids, in particular phosphoric acid, or with acidic salts of inorganic acids, such as substances of the oxide or silicate type, for example $Al_2O_3$, $TiO_2$; oxides and mixed oxides such as, for example, $\gamma$-$Al_2O_3$ and ZnO—$Al_2O_3$ mixed oxides of the heteropolyacids.

**[0475]** In accordance with an embodiment according to the invention, the support x consists at least in part of a compound of the oxide type. Such compounds of the oxide type should feature at least one of the elements selected from among Si, Ti, Zr, Al, P or a combination of at least two of these. Such supports may also act as dehydration catalyst themselves, owing to their acidic or alkaline properties. A preferred class of compounds, both as support by way of x and by way of dehydration catalyst comprise silicon/aluminum/phosphorus oxides. Preferred alkaline substances which act both as dehydration catalyst and also as support x comprise alkali, alkaline earth, lanthanum, lanthoids or a combination of at least two of these in the form of their oxides. Such acidic or alkaline dehydration catalysts are commercially available both from Degussa AG and from Südchemie AG. A further class are ion exchangers. Again, these may be present both in alkaline and in acidic form.

**[0476]** Suitable homogeneous dehydration catalysts are, in particular, inorganic acids, preferably phosphorus-containing acids and furthermore preferably phosphoric acid. These inorganic acids can be immobilized on the support x by immersion or impregnation.

**[0477]** The use of heterogeneous catalysts has proved particularly advantageous in particular in the case of gas phase dehydration. In the case of liquid-phase dehydration, however, both homogeneous and heterogeneous dehydration catalysts are employed.

**[0478]** Furthermore, it is preferred that the method according to the invention involves the use of a dehydration catalyst with an $H_0$ value in the range of from +1 to −10, preferably in the range of from +2 to −8.2 and furthermore preferably, in the case of liquid-phase dehydration, in the range of from +2 to −3 and in gas-phase dehydration in the range of from −3 to −8.2. The $H_0$ value corresponds to the acid function as defined by Hämmert and can be determined by what is known as amine titration and the use of indicators, or by the absorption of a gaseous base (see "*Studies in Surface Science and Catalytics*", vol. 51, 1989: "*New solid Acids and Bases, their catalytic Properties*", K. Tannabe et al).

**[0479]** According to a special embodiment of the method according to the invention, the acidic solid catalyst employed is a porous support structure which has been brought into

contact with an inorganic acid, preferably with phosphoric acid or with superacids such as, for example, sulfated or phosphated zirconium oxide and which is based preferably on at least 90% by weight, furthermore preferably at least 95% by weight and most preferably at least 99% by weight of a silicon oxide, preferably an $SiO_2$. The bringing into contact of the porous support structure with the inorganic acid is preferably carried out by impregnating the support structure with the acid, with the latter preferably being brought into contact with the former in an amount in a range of from 10 to 70% by weight, especially preferably in the range of from 20 to 60% by weight and more preferably in a range of from 30 to 50% by weight, based on the weight of the support structure, followed by drying. After drying, the support structure is heated in order to fix the inorganic acid, preferably at a temperature in a range of from 300 to 600° C., more preferably in a range of from 400 to 500° C.

**[0480]** According to a special embodiment of the method according to the invention, the dehydration reaction is carried out in the gas phase. Here, it is possible to employ conventional apparatuses as are known to the skilled worker in the field of gas phase reaction, for example tubular reactors. It is especially preferred to employ shell-and-tube heat exchangers and reactors which comprise thermoplates as heat exchangers.

**[0481]** According to an embodiment of the gas-phase dehydration reaction, pure 3-hydroxyisobutyric acid is introduced into a reactor comprising one of the abovementioned fixed-bed catalysts. According to another embodiment, the 3-hydroxyisobutyric acid is introduced into the reactor in the form of an aqueous solution comprising 2 to 80% by weight, especially preferably 5 to 50% by weight and more preferably 10 to 25% by weight of 3-hydroxyisobutyric acid, in each case based on the total weight of the aqueous solution. The pressure and temperature conditions inside the reactor are chosen such that the 3-hydroxyisobutyric acid, or the aqueous solution, is present in gaseous form when entering the reactor. The dehydration in the gas phase is preferably carried out in the temperature range of between 200 and 400° C., especially preferably between 250 and 350° C. The pressure inside the reactor during the gas-phase dehydration reaction is preferably in a range of from 0.1 to 50 bar, especially preferably in a range of from 0.2 to 10 bar and most preferably in a range of from 0.5 to 5 bar.

**[0482]** The amount of 3-hydroxyisobutyric acid introduced into the reactor in the gas-phase dehydration reaction is preferably in a range of from 10 to 100% by volume, especially preferably in a range of from 20 to 100% by volume and most preferably in a range of from 30 to 100% by volume.

**[0483]** According to another special embodiment of the method according to the invention, the dehydration reaction is performed in the liquid phase. The liquid-phase dehydration reaction can also be carried out in all apparatuses which are known to the skilled worker and in which a fluid can be heated to a desired reaction temperature, during which process a pressure can be applied to the apparatus which is sufficient for maintaining the reaction components in the liquid state under the desired temperature conditions.

**[0484]** According to a special embodiment of the method according to the invention, the liquid-phase dehydration method comprises a first method step, in which pure 3-hydroxyisobutyric acid or an aqueous solution comprising 5 to 100% by weight, especially preferably 20 to 100% by weight and most preferably 50 to 100% by weight of 3-hydroxyisobutyric acid, based on the total weight of the aqueous solution, is introduced into a reactor. The pressure and temperature conditions inside the reactor are chosen such that the 3-hydroxyisobutyric acid, or the aqueous solution, is present in liquid form when entering the reactor. According to a special embodiment of the method according to the invention in which the dehydration reaction is carried out in the liquid phase, the 3-hydroxyisobutyric acid, or the aqueous solution, is passed in such a way over a fixed catalyst bed inside the dehydration reactor that the liquid phase trickles over the surface of the catalyst particles. Such a procedure may be carried out for example in a trickle-bed reactor.

**[0485]** The dehydration in the liquid phase is preferably carried out in a temperature range of between 200 and 350° C., especially preferably between 250 and 300° C. The pressure inside the reactor in the case of liquid-phase dehydration is preferably in a range of from 1 to 50 bar, especially preferably in a range of from 2 to 25 bar and most preferably in a range of from 3 to 10 bar.

**[0486]** The catalysis of the dehydration reaction may be homogeneous or heterogeneous, both in the case of gas-phase dehydration and in the case of liquid-phase dehydration.

**[0487]** In the case of homogeneous catalysis, the catalyst, which in this case preferably takes the form of an inorganic acid such as, for example, phosphoric acid or sulfuric acid, is first brought into contact with the pure 3-hydroxyisobutyric acid or with the aqueous solution comprising the 3-hydroxyisobutyric acid. Thereafter, the resulting composition is introduced into the reactor and converted into methacrylic acid under the desired pressure and temperature conditions. It is also feasible to introduce the inorganic acid independently of the 3-hydroxyisobutyric acid or the aqueous solution into the reactor. In this case, the reactor features at least two feed lines, one for the 3-hydroxyisobutyric acid, or the aqueous solution comprising 3-hydroxyisobutyric acid, and one for the catalyst. If the dehydration reaction is carried out in liquid phase in a trickle-bed reactor, it is preferred to introduce the catalyst together with the 3-hydroxyisobutyric acid, or the aqueous solution comprising the 3-hydroxyisobutyric acid, at the top of the reactor.

**[0488]** In the case of heterogeneous catalysis, the catalyst is in the form of a solid substrate located in the reaction space, for example in the form of a fixed bed, in the form of catalyst-coated plates, preferably thermoplates, which are arranged inside the reactor, or else in the form of catalyst-coated reactor walls. Reactors which are possible are described for example in DE-A-198 48 208, DE-A-100 19 381 and EP-A-I 234 612. In the case of heterogeneous catalysis, preferred catalysts are support structures which have been brought into contact with inorganic acids, preferably impregnated porous support structures. The 3-hydroxyisobutyric acid, or the aqueous solution comprising the 3-hydroxyisobutyric acid, is then brought into contact with the surface of the solid catalyst material in the form of a vapor, or in liquid form.

**[0489]** According to an especially preferred embodiment of the method according to the invention, the dehydration of the 3-hydroxyisobutyric acid is carried out in liquid phase at a pressure in the range of from 200 to 500 mbar, at a temperature in a range of from 200 to 230° C. and in the presence of alkali metal ions as the catalyst.

**[0490]** The reaction mixture which is obtained after the dehydration reaction is either an aqueous methacrylic acid solution which does not contain any catalyst components (such a solution is obtained in the case of heterogeneously

catalyzed dehydration) or else an aqueous methacrylic acid solution which comprises catalysts (such a solution is obtained in the case of homogeneously catalyzed dehydration). Furthermore, the aqueous methacrylic acid solution may be in liquid form (if the dehydration reaction has been effected in the liquid phase) or in gaseous form (if the dehydration reaction has been carried out in the gas phase).

**[0491]** If appropriate, the resulting methacrylic acid solution can, according to a special embodiment of the method according to the invention, be esterified without further processing. In such a case, the methacrylic acid solution is brought into contact with suitable alcohols such as, for example, methanol, ethanol, 1-propanol, 2-propanol or 1-butanol and suitable esterification catalysts known to the skilled worker such as, for example, concentrated acids, with heating, and the methacrylic acid is so converted into the corresponding esters. However, it may be advantageous additionally to purify the methacrylic acid before esterification, it being possible to employ, in principle, any purification method which is known to the skilled worker and which is conventionally employed for the purification of contaminated (meth)acrylic acid obtained by catalytic gas-phase oxidation of propylene.

**[0492]** If the dehydration reaction has been carried out in the gas phase, it is preferred that the methacrylic acid is first condensed, generating an aqueous methacrylic acid solution. Here, any condensation method known to the skilled worker may be employed in principle, for example a fractional condensation as described in WO-A-2004/035514, WO-A-03/014172 or EP-A-EP 1 163 201 or by total condensation as described in EP-A-0 695 736. It is also feasible to add additional solvents, in particular water, during the condensation process in order to absorb the methacrylic acid as completely as possible.

**[0493]** The aqueous methacrylic acid solution obtained after condensation, or else the aqueous methacrylic acid solution obtained in the event of liquid-phase dehydration, can then be freed from water and other contaminants in further purification steps. Here, it is possible first to remove the water by azeotrope distillation in the presence of an entrainer as described, for example, in DE-A-198 53 064. It is also feasible to employ high-boiling organic solvents for absorbing the methacrylic acid, as is disclosed for example in EP-A-0 974 574. In addition to these distillation methods, membranes for dewatering may also be employed, as proposed for example in DE-A-44 01 405. Employing crystallization methods for purifying the aqueous methacrylic acid solution, which has been generated in the case of liquid-phase dehydration or which has been obtained by condensation, is furthermore feasible.

**[0494]** The methacrylic acid obtained after dehydration can be purified even further in further method steps. Thus, high-boiling contaminants which are still present can be removed by further distillation steps. However, it is especially preferred to further purify the methacrylic acid obtained by dehydration using crystallization methods as described for example in DE-A-101 49 353.

**[0495]** The resulting purified methacrylic acid can then be esterified, if appropriate.

**[0496]** A contribution to solving the problems mentioned at the outset is furthermore provided by a method of preparing methacrylic acid or methacrylic esters, comprising the method steps

**[0497]** IIA) preparation of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid by the method described above,

**[0498]** IB) cleavage of the polyhydroxyalkanoates based on 3-hydroxyisobutyric acid with formation of 3-hydroxyisobutyric acid and, if appropriate, neutralization of the 3-hydroxyisobutyric acid and/or isolation of the 3-hydroxyisobutyric acid,

**[0499]** IIC) dehydration of the 3-hydroxyisobutyric acid with formation of methacrylic acid and, if appropriate, esterification of the methacrylate or methacrylic acid.

**[0500]** A contribution to solving the problems mentioned at the outset is also provided by a method of preparing polymethacrylic acid or polymethacrylic esters, comprising the method steps

**[0501]** IIIA) preparation of methacrylic acid by the method described above,

**[0502]** IIIB) free-radical polymerization of the methacrylic acid,

it being possible, if appropriate, to esterify at least in part the carboxyl groups of the methacrylic acid before or after the free-radical polymerization reaction.

**[0503]** A contribution to solving the problem mentioned at the outset is furthermore provided by an isolated DNA, which is selected from the following sequences:

**[0504]** a) a sequence as shown in SEQ ID No 03,

**[0505]** b) an intron-free sequence which is derived from a sequence as specified in a) and which codes for the same protein or peptide as the sequence as shown in SEQ ID No 03,

**[0506]** c) a sequence which codes for a protein or peptide which comprises the amino acid sequence as shown in SEQ ID No 04,

**[0507]** d) a sequence with at least 80%, especially preferably at least 90%, more preferably at least 95% and most preferably 99% identity with a sequence as specified in one of groups a) to c), especially preferably as specified in group a), this sequence preferably coding for a protein or peptide which is capable of converting both S- or R-methylmalonyl-coenzyme A and malonyl-coenzyme A into the corresponding semialdehydes ((S)- or (R)-methylmalonate semialdehyde and malonate semialdehyde, respectively),

**[0508]** e) a sequence which hybridizes, or, taking into consideration the degeneration of the genetic code, would hybridize, with the counter strain of a sequence as specified in any of groups a) to d), especially preferably as specified in group a), this sequence preferably coding for a protein or peptide which is capable of converting both S- or R-methylmalonyl-coenzyme A and malonyl-coenzyme A into the corresponding semialdehydes ((S)- or (R)-methylmalonate semialdehyde and malonate semialdehyde, respectively),

**[0509]** f) a derivative of a sequence as specified in any of groups a) to e), especially preferably as specified in group a), this derivative preferably coding for a protein or peptide which is capable of converting both S- or R-methylmalonyl-coenzyme A and malonyl-coenzyme A into the corresponding semialdehydes ((S)- or (R)-methylmalonate semialdehyde and malonate semialdehyde, respectively), obtained by substitution, addition, inversion and/or deletion of at least one base, preferably of at least 2 bases, more preferably of at least 5 bases and most preferably at least 10

bases, but preferably of no more than 100 bases, especially preferably of no more than 50 bases and most preferably of no more than 25 bases, and

**[0510]** g) a sequence which is complementary to a sequence as specified in any of groups a) to f), especially preferably as specified in group a).

**[0511]** Surprisingly, it has been found that a DNA which has been isolated from bacteria of the strain *Sulfolobus tokodaii* (at the Deutsche Sammlung von Mikroorganismen [German collection of microorganisms], deposit number DSM 16993) and which has a DNA sequence as shown in SEQ ID No 03 codes for a polypeptide (SEQ ID No 04) which is capable even at temperatures of up to 75° C. of converting both S- or R-methylmalonyl-coenzyme A and malonyl-coenzyme A into the corresponding semialdehydes ((S)- or (R)-methylmalonate semialdehyde and malonate semialdehyde, respectively). Since (S)- or (R)-methylmalonate semialdehyde and malonate semialdehyde are natural metabolites which are formed for example during the degradation of valine, of leucin or of isoleucin, during the propanoate metabolism or during the pyruvate metabolism, and because the formed semialdehydes are capable of being reduced further in the course of the abovementioned metabolic pathways to give the corresponding 3-hydroxyalkanoates, the isolated DNA according to the invention can be utilized for generating recombinant bacteria which are capable of directly forming large amounts of 3-hydroxy-isobutyric acid (or 3-hydroxypropionic acid). If the cells are furthermore capable of polymerizing the formed 3-hydroxyalkanoates with formation of polyhydroxyalkanoates, this DNA would furthermore be suitable for generating recombinant bacteria capable of producing polyhydroxyalkanoates based on 3-hydroxy-isobuytric acid (or on 3-hydroxypropionic acid).

**[0512]** The "nucleotide identity" in relation to SEQ ID No 03, which is defined in alternative d), is determined with the aid of known methods here. In general, specialist computer programs with algorithms taking into consideration specific requirements are used.

**[0513]** Preferred methods of determining the identity first generate the maximum agreement between the sequences to be compared. Computer programs for determining the identity comprise the GCG program package, including but not limited thereto

**[0514]** GAP (Deveroy, J. et al., Nucleic Acid Research 12 (1984), page 387, Genetics Computer Group University of Wisconsin, Medicine (Wi)), and

**[0515]** BLASTP, BLASTN and FASTA (Altschul. S. et al., Journal of Molecular Biology 215 (1990), pages 403-410). The BLAST program may be obtained from the Center For Biotechnology Information (NCBI) and from other sources (BLAST Manual, Altschul S. et al., NCBI NLM NIH Bethesda Md. 22894; Altschul S. et al., above).

**[0516]** The Smith-Waterman algorithm, which is known, can also be used for determining the nucleotide identity.

**[0517]** Preferred parameters for the nucleotide alignment comprise the following:

**[0518]** Algorithmus Needleman and Wunsch, Journal of Molecular Biology 48 (1970), pages 443-453

**[0519]** alignment matrix

**[0520]** Matches=+10

**[0521]** Mismatches=0

**[0522]** Gap penalty=50

**[0523]** Gap length penalty=3

**[0524]** The GAP program is also suitable for use with the above parameters. The above parameters are the default parameters in the nucleotide sequence alignment.

**[0525]** An identity of 80% according to the above algorithm means 80% identity in the context of the present invention. The same applies to greater identities.

**[0526]** The feature "sequence which hybridizes, or, taking into consideration the degeneracy of the genetic code, would hybridize, with the counter strain of a sequence as specified in one of groups a) to d), especially preferably as specified in group a)," according to alternative e) indicates a sequence which hybridizes, or would hybridize taking into consideration the degeneracy of the genetic code, with the counter strand of a sequence as specified in one of groups a) to d), especially preferably as specified in group a), under preferably stringent conditions. For example, the hybridization reactions can be carried out at 68° C. in 2×SSC, or as described in the protocol of the dioxygenin labeling kit from Boehringer (Mannheim). Examples of preferred hybridization conditions are incubation overnight at 65° C. in 7% SDS, 1% BSA, 1 mM EDTA, 250 mM sodium phosphate buffer (pH 7.2), followed by washing at 65° C. with 2×SSC; 0.1% SDS.

**[0527]** The derivatives, of the isolated DNA according to the invention, which can be obtained according to alternative f) by substitution, addition, inversion and/or deletion of one or more bases of a sequence as specified in any of groups a) to e) include in particular those sequences which, in the protein which they encode, lead to conservative amino acid substitutions such as, for example, the substitution of glycine for alanine or of aspartate for glutamic acid. Such function-neutral mutations are referred to as sense mutations and do not lead to any principle modification of the activity of the polypeptide. It is furthermore known that modifications at the N and/or C terminus of a polypeptide do not have a considerable adverse effect on its function; indeed, they are even capable of stabilizing it, so that, as a consequence, the present invention also comprises DNA sequences where bases are added at the 3' terminus or at the 5' terminus of the sequence with the SEQ ID No 03. The skilled worker will find information on this subject in Ben Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6: 1321-1325 (1988)) inter alia, and in known textbooks of Genetics and Molecular Biology.

**[0528]** To isolate the DNA according to the invention, an NADPH-dependant malonyl-coenzyme A reductase was first isolated from a cell extract of *Metallosphaera sedula* and purified. The first 20 amino acids of the N terminus of the polypeptide of the resulting purified enzyme were sequenced. The gene for the malonyl-coenzyme A reductase was subsequently determined in the genome of *Sulfolobus tokodaii*, which has already been fully sequenced (Kawarabayasi et al., "*Complete genome sequence of an aerobic thermoacidophilic crenarchaeon, Sulfolobus tokodaii strain*7.", DNA Research 8:123-40), by identifying the derived protein sequence which is identical with the first 20 amino acids of the polypeptide isolated from *Metallosphaera sedula*. The DNA sequence according to the invention was then amplified by means of PCR, using suitable primers (see example 2).

**[0529]** A contribution to solving the problems mentioned at the outset is furthermore contributed by a vector, preferably an expression vector, comprising a DNA with a sequence as

specified in one of groups a) to f) as defined above. Suitable vectors are all vectors which are known to the skilled worker and which are traditionally employed for introducing DNA into a host cell. Preferred vectors are selected from the group consisting of plasmids, such as, for example, the *E. coli* plasmids pTrc99A, pBR345 and pBR322, viruses such as, for example, bacteriophages, adenoviruses, vaccinia viruses, baculoviruses, measles viruses and retroviruses, cosmids or YACs, with plasmids being most preferred as vectors.

**[0530]** According to a preferred embodiment of the vector according to the invention, the DNA with a sequence as specified in any of groups a) to f) is under the control of a promoter capable of being regulated, which promoter is suitable for expressing the polypeptide encoded by these DNA sequences in the cell of a microorganism, preferably in a bacterial cell, a yeast cell or a fungal cell, especially preferably in a bacterial cell, most preferably in an *E. Coli* cell. Examples of such promoters are the trp promoter or the tac promoter.

**[0531]** Besides a promoter, the vector according to the invention should preferably comprise a ribosomal binding site and terminator. Here, it is especially preferred that the DNA according to the invention is incorporated into an expression cassette of the vector comprising the promoter, the ribosomal binding site and terminator. Besides the above-mentioned structural elements, the vector may furthermore comprise selection genes known to the skilled worker.

**[0532]** A contribution to solving the problems mentioned at the outset is furthermore provided by the use of the above-described vector for transforming a cell and by the cell obtained by transformation of this vector. The cells which can be transformed with the vector according to the invention may be prokaryotes or eukaryotes. They may take the form of mammalian cells (such as, for example, cells from humans), of plant cells or of microorganisms such as yeasts, fungi or bacteria, with microorganisms being especially preferred and bacteria and yeasts being most preferred.

**[0533]** A contribution to solving the problems mentioned at the outset is also provided by a polypeptide which features the amino acid sequence with the SEQ ID No 04 or an amino acid sequence which is obtained when no more than 40 amino acids, preferably no more than 20 amino acids, even more preferably no more than 10 amino acids and most preferably no more than 5 amino acids in SEQ ID No 04 are deleted, inserted, substituted or else added to the C and/or N terminus of the amino acid sequence with the SEQ ID No 04. The polypeptide takes the form of an enzyme which is capable of catalyzing both the conversion of (S)- or (R)-methylmalonyl-coenzyme A into (S)- or (R)-methylmalonate semialdehyde and the conversion of malonyl-coenzyme A into malonate semialdehyde. Such a polypeptide can be obtained for example via the synthetic route, starting from the DNA sequence with the SEQ ID No 03, or by transformation of a suitable cell with a suitable vector comprising this nucleic acid sequence, expression, in the cell, of the protein encoded by this nucleic acid sequence, lysis of the cell, generating a cell extract, and subsequent purification of the enzyme by means of purification techniques known to the skilled worker, for example by means of HPLC or other chromatographic methods. Besides chromatographic purification of the polypeptide from cell extracts, one can also exploit the advantage that the polypeptide with the amino acid sequence SEQ ID No 04 is heat-resistant up to a temperature of at least 75° C. The cell extract can therefore be heated to a temperature of, for example, 75° C., which results in the coagulation, and thus precipitation, in the cell extract of those polypeptides which are not heat resistant. The polypeptide with the amino acid sequence SEQ ID No 04 is retained in the cell extract in nondenatured form.

**[0534]** The present invention will now be illustrated in greater detail with reference to nonlimiting figures and examples.

**[0535]** FIG. **1** shows the conversion of succinyl-coenzyme A into methylmalonyl-coenzyme A with catalysis by the enzyme $E_1$.

**[0536]** FIG. **2** shows the conversion of methylmalonyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_2$ to $E_4$ in accordance with the first alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0537]** FIG. **3** shows the conversion of (R)-methylmalonyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_4$, $E_6$ and $E_7$ in accordance with the second alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0538]** FIG. **4** shows the conversion of methylmalonyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_4$, $E_5$ and $E_7$ in accordance with the third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0539]** FIG. **5** shows the conversion of 3-hydroxyisobutyric acid into a polyhydroxyalkanoate with catalysis by the enzymes $E_5$ and $E_9$.

**[0540]** FIG. **6** shows the conversion of phosphoenolpyruvate or pyruvate into oxalacetate with catalysis by the enzymes $E_{10}$ or $E_{11}$ according to a special embodiment of the first, second or third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0541]** FIG. **7** shows the conversion of oxalacetate into succinyl-coenzyme A with catalysis by the enzymes $E_{12}$ to $E_{15}$ according to a first special embodiment of the first, second or third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0542]** FIG. **8** shows the conversion of oxalacetate into succinyl-coenzyme A with catalysis by the enzymes $E_{13}$ to $E_{16}$ and $E_{24}$ to $E_{26}$ according to a second special embodiment of the first, second or third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0543]** FIG. **9** shows the conversion of oxalacetate into succinyl-coenzyme A with catalysis by the enzymes $E_{16}$, $E_{24}$,

$E_{27}$ and $E_{28}$ according to a third special embodiment of the first, second or third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0544]** FIG. **10** shows the conversion of L-glutamate into succinyl-coenzyme A with catalysis by the enzymes $E_{46}$ and $E_{28}$ in accordance with a further special embodiment of the first, second or third alternative of the cell according to the invention, where succinyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0545]** FIG. **11** shows the conversion of acetyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_4$, $E_5$ and $E_{47}$ to $E_{52}$ in accordance with a first alternative of the second special embodiment of the cell according to the invention, where propionyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0546]** FIG. **12** shows the conversion of propionyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_2$ to $E_4$, $E_6$, $E_7$ and $E_{47}$ to $E_{52}$ in accordance with a second alternative of the second special embodiment of the cell according to the invention, where propionyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0547]** FIG. **13** shows the conversion of propionyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_2$ to $E_4$, $E_7$ and $E_{47}$ to $E_{52}$ in accordance with a third alternative of the second special embodiment of the cell according to the invention, where propionyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0548]** FIG. **14** shows the conversion of propionyl-coenzyme A into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_2$ to $E_4$, $E_7$ and $E_{47}$ to $E_{52}$ in accordance with a fourth, fifth alternative of the second special embodiment of the cell according to the invention, where propionyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0549]** FIG. **15** shows the conversion of β-alanine into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_{10}$ to $E_{12}$, $E_{56}$, $E_{72}$ and $E_{73}$ according to a third special embodiment of the cell according to the invention, where acrylyl-coenzyme A is formed as intermediate and methylmalonate semialdehyde as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0550]** FIG. **16** shows the conversion of pyruvate into 3-hydroxyisobuytric acid with catalysis by the enzymes $E_{76}$ to $E_{79}$, $E_{60}$, $E_{61}$ and $E_8$ according to a first alternative of the first special embodiment of the second variant of the cell according to the invention, where isobutyryl-coenzyme A is formed as intermediate and 3-hydroxyisobutyryl-coenzyme A as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

**[0551]** FIG. **17** shows the conversion of L-valine into 3-hydroxyisobutyric acid with catalysis by the enzymes $E_8$, $E_{60}$, $E_{61}$, $E_{79}$ and $E_{80}$ according to a second alternative of the first special embodiment of the second variant of the cell according to the invention, where isobutyryl-coenzyme A is formed as intermediate and 3-hydroxyisobutyryl-coenzyme A as precursor in the production of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid.

## EXAMPLES

### Example 1

**[0552]** The present invention is now illustrated in Example 1 with reference to a recombinant cell which is capable of producing 3-hydroxyisobutyric acid via 3-hydroxyisobutyryl-coenzyme A as precursor and isobutyryl-coenzyme A as intermediate, starting from L-valine as carbon source. To this end, the enzymes EC 2.6.1.42 and EC 1.2.4.4 (in each case from *Pseudomonas aeruginosa*) and a cluster comprising the three enzymes EC 1.3.99.12, EC 4.2.1.17 and EC 3.1.2.4 (from *Acinetobacter calcoaceticus*) were overexpressed in *E. coli* BL21 (DE3).

**[0553]** Here, the enzyme EC 1.2.4.4 is encoded by a gene with the DNA sequence as shown in SEQ ID No 07 and 08 (α and β subunit), while the enzyme EC 2.6.1.42 is encoded by a gene with the DNA sequence as shown in SEQ ID No 09. The enzyme EC 1.3.99.12 is encoded by a gene with the DNA sequence with the SEQ ID No 10, the enzyme EC 4.2.1.17 by a gene with the DNA sequence as shown in SEQ ID No 11, and the enzyme EC 3.1.2.4 by a gene with the DNA sequence as shown in SEQ ID No 12.

1. Organisms, Plasmids and Oligonucleotides

**[0554]** The following bacterial strains, vectors, genomic DNA and oligonucleotides were used for preparing this recombinant cell:

TABLE 1

Bacterial strains used

| Strain | Reference (manufacturer) |
|---|---|
| *E. coli* DH5 | NEB |
| *E. coli* BL21 (DE3) | Invitrogen |

TABLE 2

Vectors used

| Vector | Reference (manufacturer) |
|---|---|
| pCDFDuet-1 | Novagen |
| pET101/D-TOPO | Invitrogen |
| pCR2.1-TOPO | Invitrogen |

TABLE 3

Genomic DNA used

| Strain |
|---|
| *Pseudomonas aeruginosa* PAO1 |
| *Acinetobacter calcoaceticus* ADP1 |

TABLE 4

Oligonucleotides used

| Name | Sequence |
|---|---|
| Aca_VClus_fw | 5'-ATGCAATTTAATGAAGAACAGCTATTAATTC-3' (SEQ ID No. 13) |
| Aca_VClus_rev | 5'-CAGTCTGAAATGACTAACCTAATTGGC-3' (SEQ ID No. 14) |
| Pae_26142_fw | 5'-ACGGAATTCTGAAGGAGCTGGCAACTATG-3' (SEQ ID No. 15) |
| Pae_26142_rev | 5'-TTGTCGACTTACTTGACCAGGGTACGCC-3' (SEQ ID No. 16) |
| Pae_1244_fw | 5'-ACAGATCTGGAGGCCTGTCATGAGTGATTAC-3' (SEQ ID No. 17) |
| Pae_1244_rev | 5'-ATGGGTACCCATTCAGACCTCCATC-3' (SEQ ID No. 18) |

2. Amplification of the PCR Fragments 1.2.4.4 (2313 kb) and 2.6.1.42 (958 bp)

**[0555]** First, the fragments of 1.2.4.4 and 2.6.1.42 were amplified by means of PCR starting from the total DNA from *Pseudomonas aeruginosa*, using the primers as shown in SEQ ID No 15 to SEQ ID No 18, which are detailed in Table 4.

3. Digestion of the Vector pCDF-Duet-1 and of the PCR Fragment 2.6.1.42 (958 bp)

**[0556]** The vector pCDFDuet-1 (featuring a streptomycin-/spectinomycin resistance) is cleaved by means of EcoRI/SalI, as is the PCR fragment 2.6.1.42, and the restrictions thus obtained are ligated overnight with T4 ligase. This gives rise to the vector pCDFDuet::2.6.1.42.

4. Cloning of the PCR Fragments into the Vector pCR2.1-TOPO

**[0557]** The preparation of a cloning vector comprising the fragment 2.6.1.42 or the fragment 1.2.4.4, using the vector pCR2.1-TOPO, was performed as specified in the manufacturer's instructions. *E. coli* DH5α cells were transformed with the resulting cloning vectors pCR2.1-TOPO::1.2.4.4 and pCR2.1-TOPO::2.6.1.42. Since the pCR2.1-TOPO vectors feature a kanamycin resistance and an ampicillin resistance, the transformants were plated onto 2 AXI and KXI plates (20 and 40 μl). The plasmids of the resulting clones were isolated and digested:

| | |
|---|---|
| pCR2.1-TOPO::1.2.4.4 | BgIII + KpnI |
| fragment size 2313 bp | |
| pCR2.1-TOPO::2.6.1.42 | EcoRI + SalI |
| fragment size 958 bp | |

**[0558]** Each of the fragments was eluted from the gel and purified with the QIAquick kit from Qiagen (following instructions).

5. Preparation of the Vector pCDFDuet:2.6.1.42-1.2.4.4

**[0559]** The vector pCDFDuet::2.6.1.42 and the vector pCR2.1-TOPO::1.2.4.4 are digested with BgIII/KpnI.

**[0560]** This is followed by the ligation of pCDFDuet::2.6.1.42 (BgIII/KpnI) with pCR2.1-TOPO::1.2.4.4, giving rise to the vector pCDFDuet::2.6.1.42-1.2.4.4. Again, *E. coli* DH5α cells were transformed by means of this cloning vector. The plasmids were isolated. The plasmid pCDFDuet::2.6.1.42-1.2.4.4 features the DNA sequence as shown in SEQ ID No 19.

6. Cloning the Valine Cluster from *Acinetobacter calcoaceticus* ($V\text{-}Clus_{Aca}$)

**[0561]** Strain ATCC 33304 *Acinetobacter calcoaceticus* was cultured for the isolation of total DNA (HH agar or medium). Total DNA was isolated by means of the DNEasy kit from Qiagen (L1 and L2) and by a method comprising the method steps i) centrifugation of 1 ml of culture, ii) addition of 200 μl of $H_2O$ to the pellet, iii) heating for 10 min at 95° C., iv) centrifugation (10 min, 13 000 rpm), and v) removing the supernatant for a PCR.

**[0562]** To amplify the valine cluster from *A. calcoaceticus*, a PCR was carried out using the primers as shown in SEQ ID No 13 and SEQ ID No 14, which have been detailed in Table 4 (following the manufacturer's instructions using the polymerases Pfu and Taq, respectively).

**[0563]** The PCR products were purified and, following the instructions, ligated to the plasmid pET101/D-TOPO and transferred into *E. coli* DH5α. This gives rise to the plasmid pET101/D-TOPO::$V\text{-}Cluster_{Aca}$. Plasmid pET101/D-TOPO::$V\text{-}Cluster_{Aca}$ features the DNA sequence as shown in SEQ ID No 20.

7. Preparation of a Recombinant Cell which is Capable of Forming 3-Hydroxyisobutyric Acid from L-Valine

**[0564]** *E. coli* BL21 (DE3) was transformed with the plasmids pET101/D-TOPO::$V\text{-}Cluster_{Aca}$ and pCDF-Duet::2.6.1.42-1.2.4.4 (plated onto LB spec./amp medium). The resulting cells were capable of converting, in a nutrient medium comprising L-valine, the L-valine into 3-hydroxyisobutyric acid. In contrast, the wild type of the cells (*E. coli* BL21 (DE3)) was not capable of forming detectable amounts of 3-hydroxyisobutyric acid in such a nutrient medium.

Example 2

**[0565]** In this example, the DNA according to the invention is isolated and the gene is overexpressed in *E. coli*.

1. Culturing and Harvesting *Sulfolobus tokodaii*

**[0566]** *Sulfolobus tokodaii* was grown in a small culture volume (40-200 ml) at 75° C. and a pH of 3.0, with shaking (150 rpm). The growth was monitored photometrically via measuring the optical density at 578 nm ($OD_{578\ nm}$). A modified *Sulfolobus* medium was used (modified as described by Brock et al., *Archives of Microbiology* 84, pages 54-68, 1972; Suzuki et al., *Extremophiles,* 6, pages 39-44, 2002). The energy and carbohydrate source used were yeast extract, casamino acids and glucose. The medium consisted of the following components: basal medium, glucose stock solution, iron stock solution and trace element stock solution. At an $OD_{578\ nm}$ of 0.3-0.5 (exponential phase), the cells were harvested. The centrifugation was carried out in a Sorvall centrifuge (SS34 rotor) for 15 min at 9000 rpm. The cell pellet was employed directly for the DNA extraction.

**[0567]** Basal medium. $KH_2PO_4$ (0.28 g/l), $(NH_4)_2SO_4$ (1.3 g/l), $MgSO_4 \times 7\ H_2O$ (0.25 g/l), $CaCl_2 \times 6\ H_2O$

(0.07 g/l), yeast extract (1g/l) and casamino acids (1g/l). Before autoclaving, the pH was brought to 3.0 using $H_2SO_4$.

**[0568]** Glucose stock solution (100×). Glucose (100g/l).

**[0569]** The solution was filter-sterilized.

**[0570]** Iron stock solution (1000×). $FeCl_3 \times 6\ H_2O$ (20g/l). The solution was filter-sterilized.

**[0571]** Trace element stock solution (1000×). $MnCl_2 \times 4\ H_2O$ (1.8 g/l), $Na_2B_4O_7 \times 10\ H_2O$ (4.5 g/l), $ZnSO_4 \times 7H_2O$ (220 mg/l), $CuCl_2 \times 2\ H_2O$ (50 mg/l), $Na_2MoO_4 \times 2\ H_2O$ (30 mg/l), $VOSO_4 \times 5\ H_2O$ (30 mg/l), $CoCl_2 \times 6\ H_2O$ (8.4 mg/l). The individual components were dissolved in succession in distilled $H_2O$, the pH was brought to 3.0 using HCl, and the solution was filter-sterilized.

2. Isolation of Genomic DNA from *S. tokodaii*

**[0572]** Genomic DNA was isolated by the method of Murray and Thompson (*Nucleic Acid Research,* 8, pages 4321-4325, 1980). To this end, 10-50 mg (fresh weight) of freshly harvested cells are weighed into a 1.5 ml Eppendorf reaction vessel and resuspended in 570 ml of TE buffer (10 mM Tris/HCl (pH 8.0), 1 mM NaEDTA). 30 μl of a 10% (w/v) SDS solution (sodium dodecyl sulfate solution) and 3 μl of Proteinase K (20 μg/μl) were added and the mixture was incubated for 1 h at 52° C. Thereafter, 100 μl of 5 M NaCl solution and 80 μl of pre-warmed 10% (w/v) cetyltrimethylammonium bromide (CTAB) solution (10% (w/v) CTAB in 0.7 M NaCl) were added. After incubation for 10 min at 65° C., the complexes of CTAB, cell wall fragments and proteins were extracted with 780 μl of chloroform/iso-amyl alcohol (24:1 (v/v)) and spun down for 15 min at 14 000 rpm. The aqueous top phase was transferred into a fresh Eppendorf reaction vessel and the extraction was repeated. After the aqueous phase was free from pigments, it was covered with a layer of 400 μl of 100% isopropanol. By carefully mixing the two phases, the chromosomal DNA precipitated at the interface. Then, it was possible to fish out the DNA with a drawn-out Pasteur pipette and washed in 200 μl of 70% ethanol. After recentrifugation (5 min, 14 000 rpm), the supernatant was pipetted off and the DNA was dried for 2 h at room temperature and finally dissolved in 100 μl of TE buffer.

3. Amplification of the Malonyl-Coenzyme A Reductase Gene

**[0573]** The polymer chain reaction (PCR) (Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51, pages 263-273, 1986) was employed to amplify the malonyl-CoA reductase gene in a targeted fashion, from the genomic *Sulfolobus tokodaii* DNA obtained in Example 2. It was carried out in a thermocycler (Biometra, Göttingen).

**[0574]** A preparative PCR in which Pfu polymerase (Pfunds, Genaxxon) was used, was employed. The Pfu polymerase contains a 3'-5' exonuclease ("proofreading") function.

**[0575]** The following primers were used:

```
5'-ATTATCCCATGGGGAGAACATTAAAAGC-3'
("forward primer"; NcoI cleavage site is
underlined; (SEQ ID No 21)
and

5'-CGGGATCCTTACTTTTCAATATATCC-3'
("reverse primer"; BamHI cleavage site is
underlined; SEQ ID No 22)
```

**[0576]** The reaction mixture detailed in Table 1 hereinbelow was employed for the PCR reactions. The PCR was carried out as a hot start PCR, i.e. the reaction mixture was incubated for 2 min at 95° C. before adding the Pfu polymerase. This was followed by 30 cycles of in each case 1 minute at 95° C., 1 minute at 45° C. and 5 minutes at 72° C., followed by a last step of 30 seconds at 45° C., 15 minutes at 72° C. and, finally, a pause at 6° C.

TABLE 1

Standard reaction mixtures (50 μl) for proofreading PCR with Pfu polymerase

| Composition | μl/50 μl batch |
|---|---|
| 10 × Pfu PCR reaction buffer | 5 |
| dNTP mix (2 mM per nucleotide) | 5 |
| Forward primer (2 μM) | 12.5 |
| Reverse primer (2 μM) | 12.5 |
| Chromosomal DNA | 1 (10-50 ng) |
| Pfu polymerase (2.5 U/μl) | 2 |
| dd-$H_2O$ | 12 |

**[0577]** A gene fragment with a length of 1.1 kb was obtained.

4. Cloning the Malonyl-Coenzyme A Reductase Gene

**[0578]** To clone the malonyl-coenzyme A reductase gene from *Sulfolobus tokodaii*, the gene amplified in Example 3 was cloned unspecifically with the vector pCR T7/CT-Topo (Invitrogen, Karlsruhe), using the "pCR T7 Topo TA Expression Kit" (Invitrogen, Karlsruhe). This was done following the manufacturer's instructions.

**[0579]** To isolate the plasmid DNA, the plasmid DNA was prepared using the "QIAprep Spin Plasmid Miniprep Kit" from Qiagen (Hilden) following the manufacturer's instructions, starting from 5 ml overnight cultures of transformed *E. coli* TOP10F' cells.

5. Generation of an Expression Vector

**[0580]** To generate an expression vector comprising the malonyl-coenzyme A reductase gene, the isolated cloning vector obtained in Example 4 is subjected to restriction digestion with the restriction enzymes NCoI and BamHI. To this end, 25-27 μl of plasmid DNA (expression vector pTrc99A and pCR T7/CT-Topo vector, respectively, with the incorporated malonyl-coenzyme A reductase gene) are mixed thoroughly with 5 μl of a reaction buffer (10×) and 2-3 μl of restriction enzyme (10 U/μl; Fermentas, St. Leon-Rot). The reaction mixture was made up to 50 μl with distilled $H_2O$ and incu-

bated for 5 h at the temperature specified by the manufacturer. An ethanol precipitation was carried out before further use. To this end, the DNA was mixed with 3 volumes of 100% ethanol and 0.1 volumes of 3 M sodium acetate buffer (pH 5.3) and incubated for 2 h or overnight at −80° C. After a centrifugation step (20 min, 14 000 rpm, 4° C., Eppendorf table-top centrifuge), the supernatant is removed carefully, and the DNA was washed with 3 volumes of 70% (v/v) ethanol. After 10 min incubation at room temperature, the mixture was recentrifuged (10 min, 14 000 rpm, 4° C., Eppendorf table-top centrifuge) and the supernatant was discarded. The DNA was then dried for 1 hour at room temperature and subsequently taken up in the desired volume of $H_2O$ or TE buffer (10 mM Tris/HCl (pH 8.0), 1 mM NaEDTA).

**[0581]** Then, alkaline phosphatase is used for removing the 5'-phosphate groups of the linearized double-stranded vector. In this manner, the cloning efficiency is increased since religation of the vector is prevented. Calf intestinal alkaline phosphatase was used for dephosphorylating the digested vector.

**[0582]** The dephosphorylation was carried out in the same buffer as the restriction digestion. 50 μl of restriction mixture were mixed with 1.5 μl of CIAP (Calf Intestine Alkaline Phosphatase (1 U/μl; Fermentas, St. Leon-Rot) and the mixture was incubated for 30 min at 37° C. Before further use of the cleaved and dephosphorylated vector, an ethanol precipitation was carried out as described above.

**[0583]** T4 DNA ligase was used the ligation of the insert DNA with the expression vector, plasmid DNA and insert DNA being employed in a molar ratio of from 1:3-1:6.

**[0584]** Stock Solutions:

**[0585]** Ligation buffer (10×): 0.5 M Tris/HCl, pH 7.6

**[0586]** 100 mM $MgCl_2$

**[0587]** 0.5 mg/ml BSA

**[0588]** filter-sterilized, storage at room temperature

**[0589]** 5 mM ATP (adenosine triphosphate) Always make up freshly in sterile distilled $H_2O$

**[0590]** 50 mM DTE (dithioerythritol) Always make up freshly in ligation buffer

**[0591]** The ligation mixtures had a volume of 50 μl. Plasmid DNA (2-10 μl), insert DNA (2-20 μl), 5 μl of ligation buffer with DTE (50 mM) and the corresponding amount of sterile distilled $H_2O$ were pipetted together, vortexed, spun down briefly and subsequently incubated for 5 min at 45° C. The mixture was cooled on ice. 5 μl of 5 mM ATP and 1.5 μl of T4 DNA ligase (1 U/μl; Fermentas; St. Leon-Rot) were added, and everything was mixed. Ligation was performed overnight at 16° C.

**[0592]** The ligation mixture was employed directly for transforming chemically competent cells.

6. Transformation of *E. coli* Cells with the Expression Vector

**[0593]** A 5 ml overnight culture was grown starting from a single colony of *E. coli* Rosetta 2 cells. On the next morning, 50 ml of LB medium (Sambrook et al., "*Molecular Cloning: A Laboratory Manual*", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) were inoculated with 0.5-1.0 ml of this culture. After incubation for 1.5-2 h (37° C., shaking (180 rpm)), an $OD_{578}$ of 0.6 was reached. The cells were cooled on ice for 10 min and subsequently spun down for 5 min at 5000 rpm and 4° C. (GSA rotor, Sorvall centrifuge). The supernatant was discarded and the cell pellet was resuspended in 2.7 ml of cold 0.1 M $CaCl_2$ solution. After addition of 2.3 ml of sterile 50% (v/v) glycerol, the cell suspension was divided into portions (in each case 300 μl) in 1.5-ml Eppendorf reaction vessels. The competent cells were immediately frozen in liquid nitrogen and subsequently stored at −80° C.

**[0594]** To transform the cells, an aliquot of the chemically competent cells (300 μl) was defrosted on ice and treated with 25 μl of a ligation mixture. Everything was mixed carefully and incubated for 30 min on ice. After a heat shock (42° C., 1 min) the mixture was reincubated on ice for 5 min. Thereafter, 800 μl of LB medium (Sambrook et al., 1989) were added, and the cells were shaken for 1 h at 37° C. (Thermomixer, Eppendorf 5436). The mixture was concentrated and finally streaked onto LB medium. To this end, the mixture was spun down for 1 min at 10 000 rpm, 750 μm of the supernatant were discarded, and the cell pellet was resuspended. 50 μl, 100 μl and 200 μl of this concentrated mixture were streaked onto LB plates (Sambrook et al., 1989) supplemented with 100 μg/ml ampicillin and incubated overnight in the incubator at 37° C. The plates were washed with 1 ml LB medium. This cell suspension was used for subsequently inoculating 150 ml LB medium (supplemented with 100 μg/ml ampicillin) in 500 ml Erlenmeyer flasks with baffles. The cultures grew at 37° C. and 180 rpm. Overexpression was performed by inducing the promoter in pTrc99A by adding 0.5 M IPTG (isopropyl-β-D-thiogalactopyranoside) at an $OD_{578nm}$ of 0.6. The induced cultures were incubated for 3 h under the abovementioned conditions and subsequently harvested at an $OD_{578nm}$=2.7.

7. Detection of the Enzymatic Activity

**[0595]** The *E. coli* strain obtained in Example 6 was disrupted by means of a cell mill. The disrupted cells were heated for 15 min at 85° C. During this heat precipitation, nonheat resistant enzymes coagulate and are precipitated. Since the target protein is heat resistant, it is retained in the supernatant. To measure the malonyl-coenzyme A reductase activity, the supernatant was diluted 1:50 in TM buffer (50 mM Tris/Cl, 1 mM $MgCl_2$, pH 8.1). 30 μl of the diluted or undiluted (for detecting the methylmalonyl-coenzyme A reductase activity) supernatant were pipetted to 500 μl of HIPS buffer (100 mM HEPES/NaOH, 5 mM $MgCl_2$, 1 mM dithioerythritol, containing 0.5 mM NADPH).

**[0596]** In a first batch, the reaction was started by adding malonyl-coenzyme A, the final concentration being 0.5 mM. The drop in the NADPH absorption at 365 nm was determined. The enzyme activity determined was 15.5 μmol/min/mg protein (15.5 U/mg).

**[0597]** In a second batch, the reaction was started by adding methylmalonyl-coenzyme A (from Fluka, Article No.: 67767), the final concentration being 2.0 mM. The drop in the NADPH absorption at 365 nm was determined. The enzyme activity determined was 0.24 μmol/min/mg protein (0.24 U/mg).

**[0598]** It can be seen from these results that the polypeptide which codes for the DNA sequence with the SEQ ID No 03 catalyzes both the conversion of malonyl-CoA and of methylmalonyl-coenzyme A.

**[0599]** 1 mol of NADPH was oxidized per mole of malonyl-CoA or methylmalonyl-Coa employed. From this it can be concluded that the enzymatic reaction leads to the corresponding semialdehyde.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 22

<210> SEQ ID NO 1
<211> LENGTH: 2214
<212> TYPE: DNA
<213> ORGANISM: Corynebacterium glutamicum ATCC 13032

<400> SEQUENCE: 1

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ggtgccgccg cgatttaccc tccaggaacc gtcatcgcgg agtcggcgat cgatctgatc 2160

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<210> SEQ ID NO 2
<211> LENGTH: 737
<212> TYPE: PRT
<213> ORGANISM: Corynebacterium glutamicum ATCC 13032

<400> SEQUENCE: 2

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Glu Ala Gln Ala Ala Gly His Pro Val Asp Ser Leu Pro Gly Gln Lys
50 55 60

Pro Phe Met Arg Gly Pro Tyr Pro Thr Met Tyr Thr Asn Gln Pro Trp
65 70 75 80

Thr Ile Arg Gln Tyr Ala Gly Phe Ser Thr Ala Ala Glu Ser Asn Ala
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Phe Tyr Arg Arg Asn Leu Ala Ala Gly Gln Lys Gly Leu Ser Val Ala
100 105 110

Phe Asp Leu Ala Thr His Arg Gly Tyr Asp Ser Asp Asn Glu Arg Val
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Val Gly Asp Val Gly Met Ala Gly Val Ala Ile Asp Ser Ile Leu Asp
130 135 140

Met Arg Gln Leu Phe Asp Gly Ile Asp Leu Ser Ser Val Ser Val Ser
145 150 155 160

Met Thr Met Asn Gly Ala Val Leu Pro Ile Leu Ala Phe Tyr Ile Val
165 170 175

Ala Ala Glu Glu Gln Gly Val Gly Pro Glu Gln Leu Ala Gly Thr Ile
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Gln Asn Asp Ile Leu Lys Glu Phe Met Val Arg Asn Thr Tyr Ile Tyr
195 200 205

Pro Pro Lys Pro Ser Met Arg Ile Ile Ser Asn Ile Phe Glu Tyr Thr
210 215 220

Ser Leu Lys Met Pro Arg Phe Asn Ser Ile Ser Ile Ser Gly Tyr His
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Ile Gln Glu Ala Gly Ala Thr Ala Asp Leu Glu Leu Ala Tyr Thr Leu
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Ala Asp Gly Ile Glu Tyr Ile Arg Ala Gly Lys Glu Val Gly Leu Asp
260 265 270

Val Asp Lys Phe Ala Pro Arg Leu Ser Phe Phe Trp Gly Ile Ser Met
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Tyr Thr Phe Met Glu Ile Ala Lys Leu Arg Ala Gly Arg Leu Leu Trp
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Ser Glu Leu Val Ala Lys Phe Asp Pro Lys Asn Ala Lys Ser Gln Ser

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Val Tyr Asn Asn Val Ala Arg Thr Ala Ile Glu Ala Met Ala Ala Thr
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Gln Gly His Thr Gln Ser Leu His Thr Asn Ala Leu Asp Glu Ala Leu
        355                 360                 365

Ala Leu Pro Thr Asp Phe Ser Ala Arg Ile Ala Arg Asn Thr Gln Leu
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Leu Leu Gln Gln Glu Ser Gly Thr Val Arg Pro Val Asp Pro Trp Ala
385                 390                 395                 400

Gly Ser Tyr Tyr Val Glu Trp Leu Thr Asn Glu Leu Ala Asn Arg Ala
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Arg Lys His Ile Asp Glu Val Glu Glu Ala Gly Gly Met Ala Gln Ala
            420                 425                 430

Thr Ala Gln Gly Ile Pro Lys Leu Arg Ile Glu Glu Ser Ala Ala Arg
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Thr Gln Ala Arg Ile Asp Ser Gly Arg Gln Ala Leu Ile Gly Val Asn
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Arg Tyr Val Ala Glu Glu Asp Glu Glu Ile Glu Val Leu Lys Val Asp
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Asn Thr Lys Val Arg Ala Glu Gln Leu Ala Lys Leu Ala Gln Leu Lys
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Ala Glu Arg Asn Asp Ala Glu Val Lys Ala Ala Leu Asp Ala Leu Thr
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Ala Ala Ala Arg Asn Glu His Lys Glu Pro Gly Asp Leu Asp Gln Asn
        515                 520                 525

Leu Leu Lys Leu Ala Val Asp Ala Ala Arg Ala Lys Ala Thr Ile Gly
    530                 535                 540

Glu Ile Ser Asp Ala Leu Glu Val Val Phe Gly Arg His Glu Ala Glu
545                 550                 555                 560

Ile Arg Thr Leu Ser Gly Val Tyr Lys Asp Glu Val Gly Lys Glu Gly
                565                 570                 575

Thr Val Ser Asn Val Glu Arg Ala Ile Ala Leu Ala Asp Ala Phe Glu
            580                 585                 590

Ala Glu Glu Gly Arg Arg Pro Arg Ile Phe Ile Ala Lys Met Gly Gln
        595                 600                 605

Asp Gly His Asp Arg Gly Gln Lys Val Val Ala Ser Ala Tyr Ala Asp
    610                 615                 620

Leu Gly Met Asp Val Asp Val Gly Pro Leu Phe Gln Thr Pro Ala Glu
625                 630                 635                 640

Ala Ala Arg Ala Ala Val Asp Ala Asp Val His Val Val Gly Met Ser
                645                 650                 655

Ser Leu Ala Ala Gly His Leu Thr Leu Leu Pro Glu Leu Lys Lys Glu
            660                 665                 670

Leu Ala Ala Leu Gly Arg Asp Asp Ile Leu Val Thr Val Gly Gly Val
        675                 680                 685

Ile Pro Pro Gly Asp Phe Gln Asp Leu Tyr Asp Met Gly Ala Ala Ala
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Ile Tyr Pro Pro Gly Thr Val Ile Ala Glu Ser Ala Ile Asp Leu Ile
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Glu

<210> SEQ ID NO 3
<211> LENGTH: 1071
<212> TYPE: DNA
<213> ORGANISM: Sulfolobus tokodaii

<400> SEQUENCE: 3

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atagctgata tggaaataaa accaactgat cctaagttaa tggatgatgt agacataata 240

ttttctccat tacctcaagg tgctgctggc ccagtagaag aacaatttgc aaaagaagga 300

ttccctgtga ttagtaattc accagatcat agatttgatc ctgatgttcc cttattggtt 360

cctgaactaa atcctcatac tattagctta attgatgagc aaagaaaaag aagagaatgg 420

aaaggattta tagtaactac accactatgc acagcccagg gtgcagcaat accattaggt 480

gctatattta aagattataa gatggatgga gcatttataa ctactattca atcgctatct 540

ggtgccggtt atccaggaat accatcatta gatgtagtag ataatatctt gcctttaggt 600

gatggatacg atgccaagac gataaaagag atcttcagaa ttttaagcga agttaagaga 660

aatgtagatg aacctaaatt agaagatgta agcttagcag caacaactca tagaatagct 720

actatacatg gtcattatga agtactatat gtatcgttca aagaggaaac tgctgctgaa 780

aaagttaagg agactttaga aaactttaga ggggaaccac aagatctaaa attaccaact 840

gcaccttcaa agccaattat cgttatgaat gaggatacaa gacctcaagt ctattttgat 900

agatgggctg gggatattcc aggaatgagt gtagttgtag gtagattaaa gcaagtgaat 960

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<210> SEQ ID NO 4
<211> LENGTH: 356
<212> TYPE: PRT
<213> ORGANISM: Sulfolobus tokodaii

<400> SEQUENCE: 4

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Ala Tyr Leu Ala Gly Lys Gly Ser Val Gly Lys Pro Tyr Gly Glu Val
35 40 45

Val Arg Trp Gln Thr Val Gly Gln Val Pro Lys Glu Ile Ala Asp Met
50 55 60

Glu Ile Lys Pro Thr Asp Pro Lys Leu Met Asp Asp Val Asp Ile Ile
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Phe Ser Pro Leu Pro Gln Gly Ala Ala Gly Pro Val Glu Glu Gln Phe
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Asp Pro Asp Val Pro Leu Leu Val Pro Glu Leu Asn Pro His Thr Ile
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Ser Leu Ile Asp Glu Gln Arg Lys Arg Arg Glu Trp Lys Gly Phe Ile
    130                 135                 140

Val Thr Thr Pro Leu Cys Thr Ala Gln Gly Ala Ala Ile Pro Leu Gly
145                 150                 155                 160

Ala Ile Phe Lys Asp Tyr Lys Met Asp Gly Ala Phe Ile Thr Thr Ile
                165                 170                 175

Gln Ser Leu Ser Gly Ala Gly Tyr Pro Gly Ile Pro Ser Leu Asp Val
            180                 185                 190

Val Asp Asn Ile Leu Pro Leu Gly Asp Gly Tyr Asp Ala Lys Thr Ile
        195                 200                 205

Lys Glu Ile Phe Arg Ile Leu Ser Glu Val Lys Arg Asn Val Asp Glu
    210                 215                 220

Pro Lys Leu Glu Asp Val Ser Leu Ala Ala Thr Thr His Arg Ile Ala
225                 230                 235                 240

Thr Ile His Gly His Tyr Glu Val Leu Tyr Val Ser Phe Lys Glu Glu
                245                 250                 255

Thr Ala Ala Glu Lys Val Lys Glu Thr Leu Glu Asn Phe Arg Gly Glu
            260                 265                 270

Pro Gln Asp Leu Lys Leu Pro Thr Ala Pro Ser Lys Pro Ile Ile Val
        275                 280                 285

Met Asn Glu Asp Thr Arg Pro Gln Val Tyr Phe Asp Arg Trp Ala Gly
    290                 295                 300

Asp Ile Pro Gly Met Ser Val Val Val Gly Arg Leu Lys Gln Val Asn
305                 310                 315                 320

Lys Arg Met Ile Arg Leu Val Ser Leu Ile His Asn Thr Val Arg Gly
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Tyr Ile Glu Lys
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<210> SEQ ID NO 5
<211> LENGTH: 1293
<212> TYPE: DNA
<213> ORGANISM: Rhodobacter sphaeroides

<400> SEQUENCE: 5

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atcgcgggct ccgacgcgtc gggcatcgtc tgggcggtgg gcgacaaggt caagcgctgg    360

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aacggcggcg acccgatgtt ctcgcccacc cagcggatct ggggctacga gacgccggac    480

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<211> LENGTH: 430
<212> TYPE: PRT
<213> ORGANISM: Rhodobacter sphaeroides

<400> SEQUENCE: 6

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Asp Gln Ala Met Gln Ile Glu Val Val Glu Thr Pro Ser Ile Asp Ser
50 55 60

His Glu Val Leu Val Leu Val Met Ala Ala Gly Val Asn Tyr Asn Gly
65 70 75 80

Ile Trp Ala Gly Leu Gly Val Pro Val Ser Pro Phe Asp Gly His Lys
85 90 95

Gln Pro Tyr His Ile Ala Gly Ser Asp Ala Ser Gly Ile Val Trp Ala
100 105 110

Val Gly Asp Lys Val Lys Arg Trp Lys Val Gly Asp Glu Val Val Ile
115 120 125

His Cys Asn Gln Asp Asp Gly Asp Asp Glu Glu Cys Asn Gly Gly Asp
130 135 140

Pro Met Phe Ser Pro Thr Gln Arg Ile Trp Gly Tyr Glu Thr Pro Asp
145 150 155 160

Gly Ser Phe Ala Gln Phe Thr Arg Val Gln Ala Gln Gln Leu Met Lys
165 170 175

Arg Pro Lys His Leu Thr Trp Glu Glu Ala Ala Cys Tyr Thr Leu Thr
180 185 190

Leu Ala Thr Ala Tyr Arg Met Leu Phe Gly His Lys Pro His Asp Leu
195 200 205

Lys Pro Gly Gln Asn Val Leu Val Trp Gly Ala Ser Gly Gly Leu Gly
210 215 220

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Val Ile Ser Glu Glu Asp Lys Arg Asp Phe Val Met Gly Leu Gly Ala
                245                 250                 255

Lys Gly Val Ile Asn Arg Lys Asp Phe Lys Cys Trp Gly Gln Leu Pro
            260                 265                 270

Lys Val Asn Ser Pro Glu Tyr Asn Glu Trp Leu Lys Glu Ala Arg Lys
        275                 280                 285

Phe Gly Lys Ala Ile Trp Asp Ile Thr Gly Lys Gly Ile Asn Val Asp
    290                 295                 300

Met Val Phe Glu His Pro Gly Glu Ala Thr Phe Pro Val Ser Ser Leu
305                 310                 315                 320

Val Val Lys Lys Gly Gly Met Val Val Ile Cys Ala Gly Thr Thr Gly
                325                 330                 335

Phe Asn Cys Thr Phe Asp Val Arg Tyr Met Trp Met His Gln Lys Arg
            340                 345                 350

Leu Gln Gly Ser His Phe Ala Asn Leu Lys Gln Ala Ser Ala Ala Asn
        355                 360                 365

Gln Leu Met Ile Glu Arg Arg Leu Asp Pro Cys Met Ser Glu Val Phe
    370                 375                 380

Pro Trp Ala Glu Ile Pro Ala Ala His Thr Lys Met Tyr Lys Asn Gln
385                 390                 395                 400

His Lys Pro Gly Asn Met Ala Val Leu Val Gln Ala Pro Arg Thr Gly
                405                 410                 415

Leu Arg Thr Phe Ala Asp Val Leu Glu Ala Gly Arg Lys Ala
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<210> SEQ ID NO 7
<211> LENGTH: 1233
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 7

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gacggccacg ccgtcggtcc ctggaatccg cagctcagca acgaacaact gctgcgcggc    240

atgcgggcga tgctcaagac ccgcctgttc gacgcgcgca tgctcaccgc gcaacggcag    300

aaaaagcttt ccttctatat gcaatgcctc ggcgaggaag ccatcgccac cgcccacacc    360

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aacaaccagt gggcgatctc caccttccag gccatcgccg gcggcgaagg caccaccttc    780

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taccgccccg ccgacgactg gaccaacttc ccgctgggcg acccgatcgc ccgcctgaag 1020

cggcacatga tcggcctcgg catctggtcg gaggaacagc acgaagccac ccacaaggcc 1080

ctcgaagccg aagtactggc cgcgcagaaa caggcggaga gccatggcac cctgatcgac 1140

ggccgggtgc cgagcgccgc cagcatgttc gaggacgtct atgcagaact gccggagcac 1200

ctgcgccggc aacgccagga gctcggggta tga 1233

<210> SEQ ID NO 8
<211> LENGTH: 1049
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 8

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ccaaccccta cgacgccaag ggcctgctga tcgcctgcat cgagaacgac gacccggtga 540

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tgacgccctg gtccaagcat ccggccagcc aggtgccgga cggctactac aaggtgccgc 660

tggacaaggc ggcgatcgtc cgccccggcg cggcgctgac cgtgctgacc tacggcacca 720

tggtctacgt ggcccaggcc gcggccgacg agaccggcct ggacgccgag atcatcgacc 780

tgcgcagcct ctggccgctg gacctggaaa ccatcgtcgc ctcggtgaag aagaccggcc 840

gctgcgtcat cgcccacgag gcgacccgca cctgcgggtt cggcgccgag ctgatgtcgc 900

tggtgcagga gcactgcttc caccacctgg aggcgccgat cgagcgcgtc accggttggg 960

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cggcattcaa gcgtgtgatg gaggtctga 1049

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<211> LENGTH: 924
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 9

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gtgcgcgcct acgacacccc gcagggcacg gcgatcttcc gcctgcaggc gcataccgac 180

cggctgttcg actccgcgca catcatgaac atgcagatcc cgtacagccg cgacgagatc 240

aacgaggcga cccgcgccgc cgtgcgcgag aacaacctgg aaagcgccta tatccgcccg 300

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aaggtgcgca ccagttcctt cacccgccac cacgtcaaca tctcgatgac ccgcgccaag 480

tccaacggcg cctacatcaa ctcgatgctg gccctccagg aagcgatctc cggcggcgcc 540

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<210> SEQ ID NO 10
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Acinetobacter calcoaceticus

<400> SEQUENCE: 10

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tcccaaatgg ggcaattggg ttttatggga atgctggtga gtgagaaatg gggcggatca 180

aatacaggaa atttagctta tgtgctggca cttgaagaaa tcgctgccgc agatggtgcg 240

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gcgggcgtga ttattgtatt tgctgtgaca gatccgaatg cagggaaaaa agggctgagt 540

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ggtttacatg cgtcagatac gtgccaaatt gctttaacgg atgttcgagt acatcacagc 660

ttaatgcttg gtcaggaagg tgagggacta aaaatagcat tgtctaatct ggaaggtggc 720

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tatttaaaag actttcccat cgagcgaatt tatcgtgatg cacgtatttg ccagatttat 1080

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<210> SEQ ID NO 11
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<212> TYPE: DNA
<213> ORGANISM: Acinetobacter calcoaceticus

<400> SEQUENCE: 11

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gacgatttag aaaatgatca aacgattggt tgtatcgtcc ttacaggttc agaaaaagcc 180

tttgccgcag gtgcggatat caaagaaatg gcagaattaa cttttccaaa tatttatttt 240

gatgattttt ttagtcttgc agatcgtatt gcacagcgtc gtaagccttt aattgccgca 300

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tgtgccgaca atgccaagtt tgcactacca gaagtaactt taggtgtcat tcctggtatt 420

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actgcacggc aaatgcaggc tgctgaggca gaacaaagtg gtttggtggc acgcgttttt 540

agtaaagaag aacttttaga acaaacctta caggctgccg aaaaaatagc ggaaaaatca 600

cgggtatcta ccataatgat taaagagtca attaatcgag cttttgaagt gagtttagca 660

gagggtttac gttttgagcg ccgaatgttc cattcagttt ttgcgacctt agatcagaaa 720

gaaggcatgc aagcatttat tgataaacgt ccagcccaat ttaaacatca ataa 774

<210> SEQ ID NO 12
<211> LENGTH: 1029
<212> TYPE: DNA
<213> ORGANISM: Acinetobacter calcoaceticus

<400> SEQUENCE: 12

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caagttgagg attggcaagg tgatgtaaat gttcaggcca tattaattaa atcaaatagt 180

cctaaagcat tttgtgcagg tggtgatatt cgctatcttt atgaaagtta taaaagtgga 240

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ctgattgatt atcatgttcc gagtcagaat tttgagcgac tagaaaatgc attcagccaa 600

tcacagaact tagataaatt tcatattcag aagattattt ctgcttatat ctccagccct 660

gttcagagtg aactcagtct atggcttgaa gccattcgtc agcattttgg tcttaaaaat 720

gtgcaagata tcgaagaaag tttgaaaaat gaacaagatc ccaactatca agtatggaca 780

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<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 13

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<210> SEQ ID NO 14
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 14

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<210> SEQ ID NO 15
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 15

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<210> SEQ ID NO 16
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 17

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<210> SEQ ID NO 18
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 18

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<210> SEQ ID NO 19
<211> LENGTH: 6960
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic plasmid polynucleotide

<400> SEQUENCE: 19

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aggagctggc aactatgtcg atggccgatc gtgatggcgt gatctggtat gacggtgaac 180

tggtgcagtg gcgcgacgcg accacgcacg tgctgaccca taccctgcac tatggaatgg 240

gcgtgttcga gggcgtgcgc gcctacgaca ccccgcaggg cacggcgatc ttccgcctgc 300

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tgaaggtcca tgtgatcatc gccgcctgga gctggggcgc ctacatgggc gaggaagccc 540

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<210> SEQ ID NO 20
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic plasmid polynucleotide

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**1**. A cell which has been genetically modified in comparison with its wild type in such a way that it is capable of forming more 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid in comparison with its wild type.

**2**. The cell as claimed in claim **1**, where the formation of 3-hydroxyisobutyric acid or of polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via methylmalonate semialdehyde as precursor.

**3**. The cell as claimed in claim **2**, where the cell is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via succinyl-coenzyme A as intermediate.

**4**. The cell as claimed in claim **3**, where the cell features an activity of an enzyme $E_1$, which catalyzes the conversion of succinyl-coenzyme A into methylmalonyl-coenzyme A, which is increased in comparison with its wild type.

**5**. The cell as claimed in claim **4**, where the enzyme $E_1$ is a methylmalonyl-coenzyme A mutase (EC 5.4.99.2).

**6**. The cell as claimed in claim **3**, where the cell features an activity of at least one of the following enzymes $E_2$ to $E_4$ which is increased in comparison with its wild type:

of an enzyme $E_2$, which catalyzes the conversion of methylmalonyl-coenzyme A into methyl malonate;

of an enzyme $E_3$, which catalyzes the conversion of methyl malonate into methylmalonate semialdehyde;

of an enzyme $E_4$ which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyrate.

**7**. The cell as claimed in claim **6**, where the enzyme

$E_2$ is a methylmalonyl-coenzyme A hydrolase (EC 3.1.2.17),

$E_3$ is an aldehyde dehydrogenase (EC 1.2.1.3) or an aldehyde oxidase (EC 1.2.3.1) and

$E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**8**. The cell as claimed in claim **3**, where the cell features an activity of at least one of the following enzymes $E_4$, $E_5$, $E_4$ and $E_7$ which is increased in comparison with its wild type:

of an enzyme $E_6$, which catalyzes the conversion of (R) methylmalonyl-coenzyme A into (S) methylmalonyl-coenzyme A;

of an enzyme $E_7$, which catalyzes the conversion of (S) methylmalonyl-coenzyme A into propionyl-coenzyme A;

of an enzyme $E_5$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonate semialdehyde;

of an enzyme $E_4$, which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyric acid.

**9**. The cell as claimed in claim **8**, where the enzyme

$E_6$ is a methylmalonyl-coenzyme A epimerase (EC 5.1.99.1)

$E_7$ is a methylmalonyl-coenzyme A decarboxylase (EC 4.1.1.41),

$E_5$ is a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27), and

$E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**10**. The cell as claimed in claim **3**, where the cell features an activity of at least one of the following enzymes $E_4$, $E_5$ and $E_7$ which is increased in comparison with its wild type:

of an enzyme $E_7$, which catalyzes the conversion of methylmalonyl-coenzyme A into propionyl-coenzyme A;

of an enzyme $E_5$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonate semialdehyde;

of an enzyme $E_4$, which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyric acid.

**11**. The cell as claimed in claim **10**, where the enzyme

$E_7$ is a methylmalonyl-coenzyme A decarboxylase (EC 4.1.1.41),

$E_5$ is a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27), and

$E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**12**. The cell as claimed in claim **3**, where the cell features an activity of at least one of the following enzymes $E_{28}$ and $E_{46}$ which is increased in comparison with its wild type:

of an enzyme $E_{46}$, which catalyzes the conversion of L-glutamate into 2-oxoglutarate;

of an enzyme $E_{28}$, which catalyzes the conversion of 2-octoglutarate into succinyl-coenzyme A.

**13**. The cell as claimed in claim **12**, where the enzyme

$E_{46}$ is a glutamate synthase (EC 1.4.1.13 or EC 1.4.1.14), a glutamate dehydrogenase (EC 1.4.1.2, EC 1.4.1.3 or EC 1.4.1.4) or an aspartate transaminase (EC 2.6.1.1 or EC 2.6.1.2) and

$E_{28}$ is a 2-oxoglutarate synthase (EC 1.2.7.3).

**14**. The cell as claimed in claim **2**, where the cell is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via propionyl-coenzyme A as intermediate.

**15**. The cell as claimed in claim **14**, where the cell features an activity of at least one of the following enzymes $E_4$, $E_5$ and $E_{47}$ to $E_{52}$ which is increased in comparison with its wild type:

of an enzyme $E_{47}$, which catalyzes the conversion of acetyl-coenzyme A into malonyl-coenzyme A;

of an enzyme $E_{48}$, which catalyzes the conversion of malonyl-coenzyme A into malonate semialdehyde;

of an enzyme $E_{49}$, which catalyzes the conversion of malonate semialdehyde into 3-hydroxypropionate;

of an enzyme $E_{50}$, which catalyzes the conversion of 3-hydroxypropionate into 3-hydroxypropionyl-coenzyme A;

of an enzyme $E_{51}$, which catalyzes the conversion of 3-hydroxypropionyl-coenzyme A into acryloyl-coenzyme A;

of an enzyme $E_{52}$, which catalyzes the conversion of acryloyl-coenzyme A into propionyl-coenzyme A;

of an enzyme $E_5$, which catalyzes the conversion of propionyl-coenzyme A into methylmalonate semialdehyde;

of an enzyme $E_4$, which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyrate.

**16**. The cell as claimed in claim **15**, where the enzyme

$E_4$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35),

$E_5$ is a methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27),

$E_{47}$ is a malonyl-coenzyme A decarboxylase (EC 4.1.1.9), a malonate coenzyme A transferase (EC 2.8.3.3), a methylmalonyl-coenzyme A carboxytransferase (EC 2.1.3.1) or an acetyl-coenzyme A carboxylase (EC 6.4.1.2),

$E_{48}$ is a malonate-semialdehyde dehydrogenase (EC 1.2.1.18),

$E_{49}$ is a 3-hydroxypropionate dehydrogenase (EC 1.1.1.59),

$E_{50}$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

$E_{51}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17) and

$E_{52}$ is an acyl-coenzyme A dehydrogenase (EC 1.3.99.3).

**17**. The cell as claimed in claim **2**, where the cell is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via acryloyl-coenzyme A as intermediate.

**18**. The cell as claimed in claim **17**, where the cell features an activity of at least one of the following enzymes $E_{10}$ to $E_{12}$, $E_{56}$, $E_{72}$ and $E_{73}$ which is increased in comparison with its wild type:

of an enzyme $E_{72}$, which catalyzes the conversion of beta-alanine into beta-alanyl-coenzyme A,

of an enzyme $E_{73}$, which catalyzes the conversion of beta-alanyl-coenzyme A into acrylyl-coenzyme A,

of an enzyme $E_{56}$, which catalyzes the conversion of acrylyl-coenzyme A into methylmalonyl-coenzyme A,

of an enzyme $E_{10}$, which catalyzes the conversion of methylmalonyl-coenzyme A into methyl malonate;

of an enzyme $E_{11}$, which catalyzes the conversion of methyl malonate into methylmalonate semialdehyde;

of an enzyme $E_{12}$, which catalyzes the conversion of methylmalonate semialdehyde into 3-hydroxyisobutyric acid.

**19**. The cell as claimed in claim **18**, where the enzyme

$E_{72}$ is a coenzyme A transferase (EC 2.8.3.1) or coenzyme A synthetase, preferably a coenzyme A transferase,

$E_{73}$ is a beta-alanyl-coenzyme A ammonia-lyase (EC 4.3.1.6),

$E_{56}$ is a crotonyl-coenzyme A decarboxylase

$E_{10}$ is a methylmalonyl-coenzyme A hydrolase (EC 3.1.2.17),

$E_{11}$ is an aldehyde dehydrogenase (EC 1.2.1.3) or an aldehyde oxidase (EC 1.2.3.1) and

$E_{12}$ is a 3-hydroxyisobutyrate dehydrogenase (EC 1.1.1.31) or a 3-hydroxyacyl-coenzyme A dehydrogenase (EC 1.1.1.35).

**20**. The cell as claimed in claim **1**, where the formation of 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid takes place via 3-hydroxybutyryl-coenzyme A as precursor.

**21**. The cell as claimed in claim **20**, where the cell is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via isobutyryl coenzyme A as intermediate.

**22**. The cell as claimed in claim **21**, where the cell features an activity of at least one of the following enzymes $E_{76}$ to $E_{79}$, $E_{60}$, $E_{61}$ and $E_8$ which is increased in comparison with its wild type:

of an enzyme $E_{76}$, which catalyzes the conversion of pyruvate into 2-acetolactate;

of an enzyme $E_{77}$, which catalyzes the conversion of 2-acetolactate into 2,3-dihydroxyisovalerate;

of an enzyme $E_{78}$, which catalyzes the conversion of 2,3-dihydroxyisolvalerate into 2-oxoisovalerate;

of an enzyme $E_{79}$, which catalyzes the conversion of 2-oxoisovalerate into isobutyryl-coenzyme A;

of an enzyme $E_{60}$, which catalyzes the conversion of isobutyryl-coenzyme A into methacrylyl-coenzyme A;

of an enzyme $E_{61}$, which catalyzes the conversion of methacrylyl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**23**. The cell as claimed in claim **22**, where the enzyme

$E_8$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

$E_{76}$ is an acetolactate synthase (EC 2.2.1.6),

$E_{77}$ is a dihydroxyisovalerate dehydrogenase (EC 1.1.1.86),

$E_{78}$ is a 2,3-dihydroxyisovalerate dehydratase (EC 4.2.1.9),

$E_{79}$ is a 2-oxoisovalerate dehydrogenase (EC 1.2.1.25 or EC 1.2.4.4),

$E_{60}$ is an acyl-coenzyme A dehydrogenase (EC 1.3.99.3), a butyryl-coenzyme A dehydrogenase (EC 1.3.99.2) or a 2-methylacyl-coenzyme A dehydrogenase (EC 1.3.99.12), and

$E_{61}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17).

**24**. The cell as claimed in claim **21**, where the cell features an activity of at least one of the following enzymes $E_8$, $E_{60}$ to $E_{61}$ and $E_{79}$ to $E_{80}$ which is increased in comparison with its wild type:

of an enzyme $E_{80}$, which catalyzes the conversion of L-valine into 2-oxoisovalerate;

of an enzyme $E_{79}$, which catalyzes the conversion of 2-oxoisovalerate into isobutyryl-coenzyme A;

of an enzyme $E_{60}$, which catalyzes the conversion of isobutyryl-coenzyme A into methacrylyl-coenzyme A;

of an enzyme $E_{61}$, which catalyzes the conversion of methacrylyl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**25**. The cell as claimed in claim **24**, where the enzyme

$E_8$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4),

$E_{60}$ is an enoyl-coenzyme A hydratase (EC 4.2.1.17),

$E_{61}$ is an acyl-coenzyme A dehydrogenase (EC 1.3.99.3), a butyryl-coenzyme A dehydrogenase (EC 1.3.99.2) or a 2-methylacyl-coenzyme A dehydrogenase (EC 1.3.99.12),

$E_{79}$ is a 2-oxoisovalerate dehydrogenase (EC 1.2.1.25 or EC 1.2.4.4), and

$E_{80}$ is an amino acid transferase (EC 2.6.1.42).

**26**. The cell as claimed in claim **20**, where the cell is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via 3-hydroxybutyryl-coenzyme A as intermediate.

**27**. The cell as claimed in claim **26**, where the cell an activity of at least one of the following enzymes $E_8$, $E_{53}$, $E_{54}$ and $E_{82}$ which is increased in comparison with its wild type:

of an enzyme $E_{53}$, which catalyzes the conversion of acetyl-coenzyme A into acetoacetyl-coenzyme A;

of an enzyme $E_{54}$, which catalyzes the conversion of acetoacetyl-coenzyme A into 3-hydroxybutyryl-coenzyme A;

of an enzyme $E_{81}$, which catalyzes the conversion of 3-hydroxybutyryl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**28**. The cell as claimed in claim **27**, where the enzyme

$E_8$ is a 3-hydroxyisobutyryl-coenzyme A hydrolase (EC 3.1.2.4)

$E_{53}$ is a β-kethothiolase (EC 2.3.1.9),

$E_{54}$ is an acetoacetyl-coenzyme A reductase (EC 1.1.1.36), and

$E_{82}$ is an isobutyryl-coenzyme mutase (EC 5.4.99.13).

**29**. A method of preparing a genetically modified cell which is capable of forming 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid via methylmalonate semialdehyde or 3-hydroxybutyryl-coenzyme A, as precursors, comprising the method step of increasing, in the cell, the activity of at least one of the enzymes mentioned in claim **2**.

**30**. A cell obtainable by a method as claimed in **29**.

**31**. A method of producing 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid, comprising the method step of bringing a cell as claimed in claim **1** into contact with a nutrient medium comprising, as carbon source, carbohydrates, glycerol, carbon dioxide, methane, methanol, L-valine or L-glutamate under conditions under which 3-hydroxyisobutyric acid or polyhydroxyalkanoates based on 3-hydroxyisobutyric acid are formed from the carbon source, and, if appropriate, purification of the 3-hydroxyisobutyric acid from the nutrient medium.

**32**. A method of preparing methacrylic acid or methacrylic esters, comprising the method steps

IA) preparation of 3-hydroxyisobutyric acid by a method as claimed in claim **31** and, if appropriate, neutralization of the 3-hydroxyisobutyric acid,

IB) dehydration of the 3-hydroxyisobutyric acid with formation of methacrylic acid and, if appropriate, esterification methacrylic acid.

**33**. A method of preparing methacrylic acid or methacrylic esters, comprising the method steps

IIA) preparation of polyhydroxyalkanoates based on 3-hydroxybutyric acid by a method as claimed in claim **31**,

IIB) cleavage of the polyhydroxyalkanoates based on 3-hydroxyisobutyric acid with formation of 3-hydroxyisobutyric acid and, if appropriate, neutralization of the 3-hydroxyisobutyric acid,

IIC) dehydration of the 3-hydroxyisobutyric acid with formation of methacrylic acid and, if appropriate, esterification of the methacrylic acid.

**34**. A method of preparing polymethacrylic acid or polymethacrylic esters, comprising the method steps

IIIA) preparation of methacrylic acid by a method as claimed in claim **32**,

IIIB) free-radical polymerization of the methacrylic acid,

it being possible, if appropriate, to esterify at least in part the carboxyl groups of the methacrylic acid or the carboxylate group of the methacrylate before or after the free-radical polymerization reaction.

**35**. An isolated DNA, which is selected from the following sequences:

a) a sequence as shown in SEQ ID No 03,

b) an intron-free sequence which is derived from a sequence as specified in a) and which codes for the same protein or peptide as the sequence as shown in SEQ ID No 03,

c) a sequence which codes for a protein or peptide which comprises the amino acid sequence as shown in SEQ ID No 04,

d) a sequence with at least 80% identity with a sequence as specified in a) to c),

e) a sequence which hybridizes, or, taking into consideration the degeneration of the genetic code, would hybridize, with the counter strain of a sequence as specified in any of groups a) to d),

a derivative of a sequence as specified in any of groups a) to e), obtained by substitution, addition, inversion and/or deletion of one or more bases, and

g) a sequence which is complementary to a sequence as specified in any of groups a) to f).

**36**. A vector, comprising a DNA sequence as specified in any of groups a) to f), as defined in claim **35**.

**37**. (canceled)

**38**. A transformed cell, obtainable by transformation with a vector as claimed in claim **36**.

**39**. An isolated polypeptide which features the amino acid sequence with the SEQ ID No 04 or an amino acid sequence obtained when no more than 10 amino acids in SEQ ID No 04 are deleted, inserted, substituted or else added to the C and/or N terminus of the amino acid sequence with the SEQ ID No 04.

**40**. A method of preparing polymethacrylic acid or polymethacrylic esters, comprising the method steps

IIIA) preparation of methacrylic acid by a method as claimed in claim **33**,

IIIB) free-radical polymerization of the methacrylic acid,

it being possible, if appropriate, to esterify at least in part the carboxyl groups of the methacrylic acid or the carboxylate group of the methacrylate before or after the free-radical polymerization reaction.

**41**. The cell as claimed in claim **22**, where the cell features an activity of at least one of the following enzymes $E_8$, $E_{60}$ to $E_{61}$ and $E_{79}$ to $E_{80}$ which is increased in comparison with its wild type:

of an enzyme $E_{80}$, which catalyzes the conversion of L-valine into 2-oxoisovalerate;

of an enzyme $E_{79}$, which catalyzes the conversion of 2-oxoisovalerate into isobutyryl-coenzyme A;

of an enzyme $E_{60}$, which catalyzes the conversion of isobutyryl-coenzyme A into methacrylyl-coenzyme A;

of an enzyme $E_{61}$, which catalyzes the conversion of methacrylyl-coenzyme A into 3-hydroxyisobutyryl-coenzyme A;

of an enzyme $E_8$, which catalyzes the conversion of 3-hydroxyisobutyryl-coenzyme A into 3-hydroxyisobutyrate.

**42**. A cell obtained by the method as claimed in **29**.

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