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(71) **Demandeur/Applicant:**
EVONIK DEGUSSA GMBH, DE

(72) **Inventeurs/Inventors:**
HAAS, THOMAS, DE;
POTTER, MARKUS, CN;
DEMLER, MARTIN, DE;
ECKL, EVA-MARIA, DE;
BECK, SIMON, DE

(74) **Agent:** MARKS & CLERK

(54) **Titre : CULTURE AUTOTROPHIQUE**
(54) **Title: AUTOTROPHIC CULTIVATION**

(57) **Abrégé/Abstract:**
The invention relates to a method comprising the method steps: A) propagating cells that have been genetically modified in such a manner that they have a reduced polyhydroxyalkanoate synthesis compared to their wild type, in a medium under autotrophic conditions to form a cell density of X cells/litre; B) diluting at least one part of the cells to a cell density of 0.001 X to 0.5 X, preferably 0.01 X to 0.3 X, particularly preferably 0.05 X to 0.2 X in a medium; and C) propagating the at least one part of the cells under autotrophic conditions.

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(71) Anmelder *(für alle Bestimmungsstaaten mit Ausnahme von US)*: **EVONIK INDUSTRIES AG** [DE/DE]; Rellinghauser Straße 1-11, 45128 Essen (DE).

(72) Erfinder; und
(71) Anmelder *(nur für US)*: **HAAS, Thomas** [DE/DE]; Backenkamp 9, 48161 Münster (DE). **PÖTTER, Markus** [DE/CN]; 658 Gaojing Road, House #85, Shanghai 201702 (CN). **DEMLER, Martin** [DE/DE]; Barkenberger Allee 102, 46286 Dorsten (DE). **ECKL, Eva-Maria** [DE/DE]; Uranusweg 22, 45770 Marl (DE). **PRZYBILLA, Simon** [DE/DE]; Kerkheideweg 21, 48163 Münster (DE).

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(54) **Title**: AUTOTROPHIC CULTIVATION

(54) **Bezeichnung** : AUTOTROPHE KULTIVIERUNG

(57) **Abstract**: The invention relates to a method comprising the method steps: A) propagating cells that have been genetically modified in such a manner that they have a reduced polyhydroxyalkanoate synthesis compared to their wild type, in a medium under autotrophic conditions to form a cell density of X cells/litre; B) diluting at least one part of the cells to a cell density of 0.001 X to 0.5 X, preferably 0.01 X to 0.3 X, particularly preferably 0.05 X to 0.2 X in a medium; and C) propagating the at least one part of the cells under autotrophic conditions.

(57) **Zusammenfassung**: Gegenstand der Erfindung ist ein Verfahren umfassend die Verfahrensschritte A) Vermehren von Zellen, welche derart gentechnisch verändert wurden, dass sie verglichen zu ihrem Wildtyp eine verminderte Polyhydroxyalkanoat-Synthese aufweisen, in einem Medium unter autotrophen Bedingungen bis zu einer Zelldichte X Zellen/Liter, B) Verdünnen mindestens eines Teils der Zellen auf eine Zelldichte von 0,001 X bis 0,5 X, bevorzugt 0,01 X bis 0,3 X, besonders bevorzugt 0,05 X bis 0,2 X in einem Medium und C) Vermehren des mindestens einen Teils der Zellen unter autotrophen Bedingungen.

# Autotrophic cultivation

## Field of the invention

The invention relates to a method comprising an autotrophic cultivation of cells.

## Prior art

In the preparation of products by fermentation, efforts are always made to avoid, or to reduce to a minimum, the formation of undesired by-products as far as possible. Generally, the product yield may be thus increased and also the purification of the end product may be simplified.

Cells which use inorganic carbon as carbon source to synthesize organic molecules are known, particularly in the form of hydrogen-oxidizing bacteria and acetogenic organisms.
Organisms which produce polyhydroxyalkanoate use 3-hydroxyalkanyl-CoA as precursor. This metabolic product may serve as an excellent starting substance for metabolism to other, high-quality organic substances, particularly by artificially inserted metabolic pathways.
In particular, the hydrogen-oxidizing bacterium *Cupriavidus necator* has in recent years been characterized and investigated in some detail.
In Vollbrecht et al., *Excretion of Metabolites by Hydrogen Bacteria I. Autotrophic and Heterotrophic Fermentations*, European J. Appl. Microbiol. Biotechnol. 6, 145-155 (1978), the formation of by-products by various *Cupriavidus necator* strains was analyzed.

It was an object of the invention to provide a method which reduces by-product formation in fermentation processes and thus increases the yield of monomer units of the polyhydroxyalkanoate reduced in the biosynthesis.

## Description of the invention

It has been found that, surprisingly, the method described hereinafter is able to achieve the stated object.

The present invention therefore provides a method in which the precultivation, which serves particularly to generate cell mass, is already carried out under autotrophic conditions, followed

by a further autotrophic cultivation, which is used for product formation preferably across at least part of the time period of the cultivation.

An advantage of the present invention is that undesired by-products, such as, for example, acetate, lactate, pyruvate, succinate, alanine, valine, butanol, butyrate, malate, acetone, 2-methylpropionic acid or 3-methylbutyrate, more particularly acetate, pyruvate and acetone, are formed in a distinctly reduced manner.

Therefore, the present invention advantageously increases the space-time yield.

A further advantage of the present invention is that the desired product can be isolated more easily.

A further advantage of the present invention is that it is possible to operate completely independently of complex carbon sources, such as, for example, sugar.

A further advantage of the present invention is that carbon dioxide is bound, which might otherwise be problematic as a greenhouse gas.

The method according to the invention comprises the method steps of:

A) propagating cells which have been genetically modified in such a way that they have a reduced polyhydroxyalkanoate synthesis compared to their wild type, in a medium under autotrophic conditions up to a cell density of X cells/liter,

B) diluting at least one portion of the cells to a cell density of 0.001 X to 0.5 X, preferably 0.01 X to 0.3 X, particularly preferably 0.1 X to 0.2 X, in a medium and

C) propagating the at least one portion of the cells under autotrophic conditions.

The term "under autotrophic conditions" in connection with the present invention is to be understood to mean cultivation conditions in which the carbon source available to the cells contains inorganic carbon to an extent of at least 90% by weight, based on carbon atoms.

The term "wild type" of a cell preferably denotes a cell whose genome is present in a state as has arisen naturally by evolution. The term is used both for the whole cell and for individual genes. The term "wild type", therefore, particularly does not include those cells or genes whose gene sequences have been at least partially modified by man by means of recombinant techniques.

The term "reduced polyhydroxyalkanoate synthesis compared to the wild type" in connection with the present invention is to be understood to mean that the cells under consideration form less polyhydroxyalkanoate per cell than the wild type when cultivated under identical conditions to the wild type.

Unless otherwise stated, the percentages (%) given are percentages by mass.

The cells used in the method according to the invention are genetically modified cells, i.e. recombinant cells. They can be prokaryotes or eukaryotes. They can be mammalian cells (such as human cells), plant cells or microorganisms such as yeasts, fungi or bacteria, with microorganisms being particularly preferred and bacteria and yeasts being most preferred. It is preferred according to the invention that the cells used are selected from acetogenic bacteria or hydrogen-oxidizing bacteria.

The term "hydrogen-oxidizing bacterium" is to be understood to mean a bacterium which is capable of chemolithoautotrophic growth and able to construct carbon skeletons having more than one carbon atom from $H_2$ and $CO_2$ in the presence of oxygen, in which the hydrogen is oxidized and the oxygen is used as terminal electron acceptor. According to the invention, it is possible to use either those bacteria which are naturally hydrogen-oxidizing bacteria or else bacteria which have become hydrogen-oxidizing bacteria by genetic modification, such as, for example, an *E. coli* cell which, as a result of recombinant insertion of the necessary enzymes, has been enabled to construct carbon skeletons having more than one carbon atom from $H_2$ and $CO_2$ in the presence of oxygen, in which the hydrogen is oxidized and the oxygen is used as terminal electron acceptor. Preferably, the hydrogen-oxidizing bacteria used in the method according to the invention are those which are already hydrogen-oxidizing bacteria as the wild type.

Hydrogen-oxidizing bacteria preferably used according to the invention are selected from the genera *Achromobacter*, *Acidithiobacillus*, *Acidovorax*, *Alcaligenes*, *Anabena, Ancyclobacter*, *Aquifex*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Cupriavidus*, *Derxia*, *Helicobacter*, *Herbaspirillum*, *Hydrogenobacter*, *Hydrogenobaculum*, *Hydrogenophaga*, *Hydrogenophilus*, *Hydrogenothermus*, *Hydrogenovibrio*, *Ideonella sp. O1*, *Kyrpidia*, *Metallosphaera*, *Mycobacterium*, *Nocardia*, *Oligotropha*, *Paracoccus*, *Pelomonas*, *Polaromonas*, *Pseudomonas*, *Pseudonocardia*, *Rhizobium*, *Rhodococcus*, *Rhodopseudomonas*, *Rhodospirillum*, Seliberia, *Streptomyces*, *Thiocapsa*, *Variovorax*, *Xanthobacter*, *Wautersia,* wherein *Cupriavidus* is particularly preferred,

particularly from the species *Cupriavidus necator* (alias *Ralstonia eutropha*, *Wautersia eutropha*, *Alcaligenes eutrophus*, *Hydrogenomonas eutropha)*, *Achromobacter ruhlandii*, *Acidithiobacillus ferrooxidans*, *Acidovorax facilis*, *Alcaligenes hydrogenophilus*, *Alcaligenes latus*, *Anabena cylindrica*, *Anabena oscillaroides*, *Anabena sp.*, *Anabena spiroides*, *Ancyclobacter vacuolatus*, *Aquifex aeolicus*, *Aquifex pyrophilus*, *Arthrobacter strain 11X*, *Bacillus schlegelii*, *Bradyrhizobium japonicum*, *Derxia gummosa*, *Escherichia coli*, *Heliobacter pylori*, *Herbaspirillum autotrophicum*, *Hydrogenobacter hydrogenophilus*, *Hydrogenobacter*

*thermophilus*, *Hydrogenobaculum acidophilum*, *Hydrogenophaga flava*, *Hydrogenophaga palleronii*, *Hydrogenophaga pseudoflava*, *Hydrogenophaga taeniospirales*, *Hydrogeneophilus thermoluteolus*, *Hydrogenothermus marinus*, *Hydrogenovibrio marinus*, *Ideonella sp. O-1*, *Kyrpidia tusciae*, *Metallosphaera sedula*, *Myobacterium gordonae*, *Nocardia autotrophica*, *Oligotropha carboxidivorans*, *Paracoccus denitrificans*, *Pelomonas saccharophila*, *Polaromonas hydrogenivorans*, *Pseudomonas hydrogenovora*, *Pseudomonas thermophila*, *Rhizobium japonicum*, *Rhodococcus opacus*, *Rhodopseudomonas palustris*, *Seliberia carboxydohydrogena*, *Thiocapsa roseopersicina*, *Variovorax paradoxus*, *Xanthobacter autrophicus*, *Xanthobacter flavus*, wherein *Cupriavidus necator* is particularly preferred, particularly from the strains *Cupriavidus necator* H16, *Cupriavidus necator* H1 or *Cupriavidus necator* Z-1.

Particular preference is given to using the strain *Cupriavidus necator* H 16 PHB-4, DSM 541.

The cells used according to the invention have a reduced polyhydroxyalkanoate synthesis compared to their wild type owing to at least one genetic modification. In this connection, it is particularly preferred that the cells used according to the invention do not synthesize detectable amounts of polyhydroxyalkanoate. The preferred polyhydroxyalkanoate, whose synthesis is decreased, is polyhydroxybutyrate.

The reduced polyhydroxyalkanoate synthesis compared to the wild type can be achieved preferably by a genetic modification, and so the decreased activity, compared to the wild type of the cell, of at least one enzyme which catalyzes the conversion of 3-hydroxyalkanyl-coenzyme A to polyhydroxyalkanoate, preferably 3-hydroxybutyryl-coenzyme A to polyhydroxybutyrate. The expression used "decreased activity of an enzyme" is accordingly understood to mean preferably activity decreased by a factor of at least 0.5, particularly preferably at least 0.1, further preferably at least 0.01, still further preferably at least 0.001 and most preferably at least 0.0001. The expression "decreased activity" also includes no detectable activity ("activity of zero"). The decrease in the activity of a certain enzyme can be effected, for example, by targeted mutation or by other measures known to those skilled in the art for decreasing the activity of a certain enzyme.

Methods for decreasing enzymatic activities in microorganisms are known to those skilled in the art, which include insertion of foreign DNA into the gene coding for the target enzyme, deletion of at least parts of the gene coding for the target enzyme, point mutations in the gene sequence coding for the target enzyme, RNA interference (siRNA), antisense RNA or modification (insertion, deletion or point mutations) of regulatory sequences such as promoters and terminators or of ribosomal binding sites which flank the gene coding for the target enzyme.

Foreign DNA in this context is understood to mean any DNA sequence which is "foreign" to the

gene (and not to the organism), i.e. endogenous DNA sequences can also function as "foreign DNA" in this context.

The enzyme which the conversion of 3-hydroxyalkanyl-coenzyme A to polyhydroxyalkanoate is preferably a polyhydroxyalkanoate synthase, particularly preferably a polyhydroxybutyrate synthase. This enzyme is particularly preferably encoded by the phbC or phaC genes, in which phaC is particularly preferred.

In method step A), the method is under autotrophic conditions, therefore under conditions in which the carbon source available to the cells contains inorganic carbon to an extent of at least 90% by weight, preferably 95% by weight, particularly preferably 99% by weight, based on carbon atoms. The inorganic carbon used in method step A) according to the invention is supplied particularly in the form of substances selected from the group comprising, preferably consisting of, carbonates, carbon dioxide and carbon monoxide, with carbon dioxide being particularly preferred. In method step A), it is also possible to use mixtures of carbon sources.

In the event that hydrogen-oxidizing bacteria are used in the method according to the invention, preference is given to carrying out the method in the presence of hydrogen. More particularly, in method steps A) and C), the medium containing the cells is contacted with a gas containing $H_2$, $CO_2$ and $O_2$,

preferably in a weight ratio of 20 to 95, to 2 to 45, to 1 to 35, more particularly 70 to 95, to 4 to 15, to 1 to 10.

In method step A), the cells are propagated preferably by at least 10-fold, particularly preferably by at least 100-fold, particularly by at least 1000-fold, based on the cell count per volume of the total culture.

The cell density X in method step A is preferably at least $1x10^5$, particularly preferably at least $1x10^7$, particularly at least $1x10^8$ cells/ml of total culture.

In method step B) of the method according to the invention, the cells are diluted with medium. The medium used can be the same medium as used in method step 1, but it can also be a different medium.

In method step C) of the method according to the invention, the cells are propagated under autotrophic conditions, therefore under conditions in which the carbon source available to the

cells contains inorganic carbon to an extent of at least 90% by weight, preferably 95% by weight, particularly preferably 99% by weight, based on carbon atoms. The inorganic carbon used in method step C) according to the invention is supplied particularly in the form of substances selected from the group comprising, preferably consisting of, carbonates, carbon dioxide and carbon monoxide, with carbon dioxide being particularly preferred. In method step C), it is also possible to use mixtures of carbon sources.

Method step C) is advantageously and thus preferably utilized across at least part of the time period for the production of the product.

In a preferred embodiment of the method according to the invention, target products which are formed via the intermediate 3-hydroxyalkanyl-CoA, particularly 3-hydroxybutyryl-coenzyme A, are synthesized by the cells in method step C).

The term "intermediate" in this context defines a chemical compound which can be converted to the desired product by an enzymatic pathway using at least one enzyme, in which those "chemical compounds" either having or not having coenzyme A thioester functionalization are to be regarded as equivalent, and thus thioester-forming or thioester-cleaving enzymes are not included.

It may be advantageous if the cells have been altered by genetic modification according to the desired target product such that the formation of the target product by the cells is enhanced.

In the context of the present invention, a preferred target product is 2-hydroxyisobutyric acid.

For this target product, it is preferable if the cell used in the method according to the invention has an increased activity, compared to the wild type thereof, of enzyme $E_1$, which catalyzes the conversion of 3-hydroxybutyryl-coenzyme A to 2-hydroxyisobutyryl-coenzyme A. Enzyme $E_1$ is preferably a hydroxyisobutyryl-CoA mutase, an isobutyryl-CoA mutase (EC 5.4.99.13) or a methylmalonyl-CoA mutase (EC 5.4.99.2), preferably in each case a coenzyme B12-dependent mutase.

Enzyme $E_1$ is preferably such enzymes that can be isolated from the microorganisms Aquincola tertiaricarbonis L108, DSM18028, DSM18512, Methylibium petroleiphilum PM1, Methylibium sp. R8, Xanthobacter autotrophicus Py2, Rhodobacter sphaeroides (ATCC 17029), Nocardioides sp. JS614, Marinobacter algicola DG893, Sinorhizobium medicae WSM419, Roseovarius sp. 217, Pyrococcus furiosus DSM 3638, particularly preferably the coenzyme B12-dependent mutase described in PCT/EP2007/052830, and also enzymes which, in at least one part of their sequence, have a sequence identity to the amino acid sequence of the small or large subunit of the mutase described in PCT/EP2007/052830 (accession number DQ436457.1 and DQ436456.1) of at least 60%, preferably at least 80%, particularly preferably at least 95%,

especially preferably at least 99% at the amino acid level, determined by the blastp algorithm with an expect threshold of 10, a word size of 3, a blosum62 matrix with gap costs of existence: 11 and extension: 1 and a conditional compositional score matrix adjustment.

The term "increased activity of an enzyme", as has been used above in connection with enzyme $E_1$ and is used in the following comments below in connection with enzymes $E_2$ etc., is preferably understood to mean increased intracellular activity.

The following comments regarding the increase in enzyme activity in cells apply both to the increase in activity of enzyme $E_1$ and to all enzymes mentioned below whose activity may possibly be increased.

In principle, an increase in the enzymatic activity can be achieved by increasing the copy number of the gene sequence(s) coding for the enzyme, by using a strong promoter, by altering the codon usage of the gene, by increasing in various ways the half-life of the mRNA or of the enzyme, by modifying the regulation of expression of the gene or by using a gene or allele coding for a corresponding enzyme with increased activity and by combining these measures as appropriate. In particular, the gene encoding enzyme $E_x$ is thus overexpressed, which leads to an increased activity via overexpression. Cells genetically modified according to the invention are generated, for example, by transformation, transduction, conjugation, or a combination of these methods, with a vector containing the desired gene, an allele of this gene or parts thereof and a promoter enabling the gene to be expressed. Heterologous expression is achieved in particular by integrating the gene or alleles into the chromosome of the cell or an extrachromosomally replicating vector.

An overview of the options for increasing enzyme activity in cells is given for pyruvate carboxylase by way of example in DE-A-100 31 999, which is hereby incorporated by way of reference and whose disclosure forms part of the disclosure of the present invention regarding the options for increasing enzyme activity in cells.

Expression of the enzymes or genes specified above and all enzymes or genes specified below is detectable with the aid of 1- and 2-dimensional protein gel separation and subsequent optical identification of the protein concentration in the gel using appropriate evaluation software. If the increase in an enzyme activity is based exclusively on an increase in expression of the corresponding gene, the increase in said enzyme activity can be quantified in a simple manner by comparing the 1- or 2-dimensional protein separations between wild type and genetically modified cells. A customary method of preparing protein gels in the case of coryneform bacteria and of identifying said proteins is the procedure described by Hermann et al. (Electrophoresis, 22:1712.23 (2001)). The protein concentration may likewise be analyzed by Western blot hybridization using an antibody specific for the protein to be detected (Sambrook et al.,

Molecular Cloning: a laboratory manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA, 1989) and subsequent optical evaluation using appropriate software for determination of 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 well-described methods of reporter gene assays (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 determined by various described methods (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). If no specific methods for determining the activity of a particular enzyme are stated in the comments below, the increase in the enzyme activity and also the decrease in an enzyme activity are preferably determined 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., Journal of Bacteriology 183: 2151-2155 (2001).

If the increase in the enzyme activity is accomplished by mutation of the endogenous gene, such mutations can either be generated in a non-directed manner according to classical methods, for example, by UV radiation or by chemicals which cause mutation, or specifically by means of genetic engineering methods such as deletion(s), insertion(s) and/or nucleotide substitution(s). Modified cells are obtained by these mutations. Particularly preferred mutants of enzymes are also particularly those enzymes which are no longer, or at least less compared to the wild type enzyme, subject to feedback inhibition.

If the increase in the enzyme activity is accomplished by increasing the synthesis of an enzyme, the copy number of the relevant genes, for example, is increased or the promoter and regulatory region or the ribosomal binding site, which is located upstream of the structural gene, is mutated. Expression cassettes which are incorporated upstream of the structural gene have a similar effect. Additionally, by means of inducible promoters, it is possible to increase expression at any desired time. Furthermore, however, so-called "enhancers" can also be assigned to the enzyme gene as regulatory sequences, which likewise cause increased gene expression via improved interaction between RNA polymerase and DNA. Expression is also improved by measures to prolong the lifetime of the mRNA. Moreover, enzyme activity is also intensified by preventing the degradation of the enzyme protein. Here, the genes or gene constructs are present either in plasmids of different copy number or are integrated in the chromosome and amplified. Alternatively, moreover, overexpression of the relevant genes can

be achieved by modification of the medium composition and culturing. Those skilled in the art find directions for this, inter alia, 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 US 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)) and in well-known genetics and molecular biology textbooks. The measures described above, like the mutations, also result in genetically modified cells.

To increase the expression of the particular genes, episomal plasmids, for example, are used. In principle, as plasmids or vectors, all embodiments available to those skilled in the art for this purpose are possible. Such plasmids and vectors can, for example, be inferred from the brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D. M. (1985), DNA cloning: a practical approach, Vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. und Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.

The plasmid vector which contains the gene to be amplified is then transferred into the desired strain by conjugation or transformation. The method of conjugation is described, for example, in Schäfer et al., Applied and Environmental Microbiology 60: 756-759 (1994). Methods for transformation 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). By homologous recombination by means of a "cross-over" event, the resulting strain comprises at least two copies of the gene concerned.

The wording "an increased activity, compared to the wild type thereof, of an enzyme $E_x$" used above and in the explanations below should preferably always be understood to mean an activity of the particular enzyme $E_x$ increased by a factor of at least 2, particularly preferably at least 10, further preferably at least 100, still further preferably at least 1000 and most preferably at least 10 000. Furthermore, the cell according to the invention which has "an increased activity, compared to the wild type thereof, of an enzyme $E_x$" in particular also includes a cell, the wild type of which has no or at least no detectable activity of this enzyme $E_x$, and which only displays detectable activity of this enzyme $E_x$ after increasing the enzyme activity, for example,

by overexpression. In this connection, the term "overexpression" or the wording "increase in expression" used in the explanations below also includes the case that a starting cell, for example a wild-type cell, displays no or at least no detectable expression and detectable synthesis of the enzyme $E_x$ is only induced by recombinant methods.

It may furthermore be advantageous when the cells used in the method according to the invention have an increased activity, compared to the wild type thereof, of an enzyme $E_2$ which catalyzes the conversion of acetoacetyl-coenzyme A to 3-hydroxybutyryl-coenzyme A.
The enzyme $E_2$ is preferably an enzyme selected from the group comprising:
a 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35),
an acetoacetyl-coenzyme A reductase (EC 1.1.1.36),
a long chain 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.211) and
a 3-hydroxybutyryl-coenzyme A dehydrogenase (EC 1.1.1.157).
This enzyme is preferably encoded by the genes selected from the group consisting of phaB, phbB, fabG, phbN1, phbB2 or, wherein particular preference is given to phaB, phbB. 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 the nucleotide sequence database of the European Molecular Biology Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK).

The method according to the invention preferably has a method step D) for purification of the target product.

The present invention is illustratively described in the examples which follow without any intention of limiting the invention, whose scope is determined by the entire description and the claims, to the embodiments referred to in the examples.

Examples:

*Comparison of heterotrophic/autotrophic cultivation versus autotrophic/autotrophic cultivation of* Cupriavidus necator *H 16 PHB-4 DSM 541*

Two *Cupriavidus necator* H 16 PHB-4 DSM 541 were used:
*Cupriavidus necator* H 16 PHB-4 DSM 541 and

*Cupriavidus necator* H16 PHB-4 pBBR1MCS-2::icmA-icmB(lac) (referred to hereinafter as RITA).

The RITA strain is transformed with an expression vector for icmA and icmB from *Aquincola tertiaricarbonis*. Detailed generation of the strain is described in example 2 of WO2009156214.

The method used the Vollbrecht medium, consisting of 2 g/l $(NH4)_2HPO_4$, 2.1 g/l $KH_2PO_4$, 0.2 g/l $MgSO_4$, 0.01 g/l $CaCl_2$, 6 mg/l $FeCl_3$, 0.05 mg/l Titriplex III, 0.02 mg/l $FeSO_4$ x 7 $H_2O$, 1 µg/l $ZnSO_4$ x 7 $H_2O$, 0.3 µg/l $MnCl_2$ x 4 $H_2O$, 3 µg/l $H_3BO_3$, 2 µg/l $CoCl_2$ x 6 H2O, 0.1 µg/l $CuCl_2$ x 2 $H_2O$, 0.2 µg/l $NiCl_2$ x 6 $H_2O$ and 0.3 µg/l $Na_2MO4$ x 2 $H_2O$.

In the case of plasmid-bearing strains, 1 ml of kanamycin of concentration 300 µg/ml was added in method step A) and, in addition, 120 µg/l of coenzyme B12 were added in method step C).

The pH was adjusted to 6.8 with NaOH (aq). 10 g/l of fructose were added to the heterotrophic preculture.

Heterotrophic preculture (but method step A) heterotrophic, not in accordance with the invention):

The preculture was carried out in two batches for each strain in 500 ml shake flasks, each containing 50 ml of medium. The preculture was inoculated with a single colony from an agar plate. The incubation was carried out at 30°C and 150 rpm for 28.5 hours.

The cultures were centrifuged at 4500 x g for 10 min.

The pellets were resuspended in 2 ml of medium and supplied to the autotrophic main culture (see relevant passage).

Autotrophic preculture (method step A):

The preculture was carried out in 250 ml pressure-resistance coated Schott flasks, each containing 50 ml of medium. The flask closure had a gas-supply frit, an outgoing-air filter and a sampling tube.

Prior to the inoculation, the flasks were flushed 3 times with $N_2$ and 3 times with oxyhydrogen. The experiment took place at 0.8 bar elevated pressure, 28°C and 150 rpm. Gas was supplied using oxyhydrogen comprising 4% $O_2$, 6% $CO_2$ and 90% $H_2$. The preculture was inoculated with a single colony from an agar plate. The cultivation took 143 hours.

The cultures were centrifuged at 4500 x g for 10 min. The pellets were resuspended in 2 ml of medium and supplied to the autotrophic main culture (see relevant passage).

Autotrophic main culture (method step C):

The main culture was carried out in pressure-resistance coated 250 ml Schott flasks containing 50 ml of medium. The flask closure had a gas-supply frit, an outgoing-air filter and a sampling tube.

Prior to the inoculation, the flasks were flushed 3 times with $N_2$ and 3 times with oxyhydrogen. The experiment took place at 0.8 bar elevated pressure, 28°C and 150 rpm for each double-batch strain. Inoculation is carried out to a start OD of 0.5. Gas was supplied using oxyhydrogen comprising 4% $O_2$, 6% $CO_2$ and 90% $H_2$. Sampling took place after 25 hours. To this end, the cultures were centrifuged at 4500 x g for 10 min and the supernatant was analyzed by means of NMR.

Measured values:

| Experiment | 3HB [mg/L] | 2HIB [mg/L] | Acetate [mg/L] | Acetone [mg/L] |
|---|---|---|---|---|
| DSM 541 heterotrophic/autotrophic | 0 | 0 | 10 | 0 |
| DSM 541 autotrophic/autotrophic | 29 | 0 | 4 | 1 |
| RITA heterotrophic/autotrophic | 0 | 5 | 14 | 0 |
| RITA autotrophic/autotrophic | 0 | 18 | 0 | 0 |

These experiments show that the method according to the invention increases the yield of 3-hydroxybutyrate (3HB) or of the substance directly derived therefrom, 2-hydroxyisobutyric acid (2-HIB). The accumulatively reduced by-product formation thus favors the increase in 3HB and 2-HIB.

Claims

1. A method comprising the method steps of

A) propagating cells which have been genetically modified in such a way that they have a reduced polyhydroxyalkanoate synthesis compared to their wild type, in a medium under autotrophic conditions up to a cell density of X cells/liter,

B) diluting at least one portion of the cells to a cell density of 0.001 X to 0.5 X, preferably 0.01 X to 0.3 X, particularly preferably 0.05 X to 0.2 X, in a medium and

C) propagating the at least one portion of the cells under autotrophic conditions.

2. The method as claimed in claim 1,
characterized in that
the cells used are selected from acetogenic bacteria or hydrogen-oxidizing bacteria.

3. The method as claimed in claim 1 or 2,
characterized in that
the polyhydroxyalkanoate, whose synthesis is decreased, is polyhydroxybutyrate.

4. The method as claimed in at least one of the preceding claims,
characterized in that
the cell has a reduced activity, compared to wild type, of polyhydroxybutyrate synthase encoded by the genes phbC or phaC.

5. The method as claimed in at least one of the preceding claims,
characterized in that
the carbon source available to the cells contains carbon dioxide to an extent of at least 90% by weight, based on carbon atoms.

6. The method as claimed in at least one of the preceding claims,
characterized in that
hydrogen-oxidizing bacteria are used and, in method steps A) and C), the medium is contacted with a gas containing $H_2$, $CO_2$ and $O_2$ in a weight ratio of 20 to 95, to 2 to 45, to 1 to 35, more particularly 70 to 95, to 4 to 15, to 1 to 10 n.

7. The method as claimed in at least one of the preceding claims,
characterized in that
the cells synthesize in method step C) target product which is formed via the intermediate 3-hydroxyalkanyl-CoA, particularly 3-hydroxybutyryl-coenzyme A.

8. The method as claimed in claim 7,
characterized in that
2-hydroxyisobutyric acid is synthesized by the cells in method step C) and the cells have an increased activity, compared to the wild type thereof, of enzyme $E_1$, which catalyzes the conversion of 3-hydroxybutyryl-coenzyme A to 2-hydroxyisobutyryl-coenzyme A.

9. The method as claimed in at least one of the preceding claims,
characterized in that
the cells used in the method according to the invention have an increased activity, compared to the wild type thereof, of an enzyme $E_2$ which catalyzes the conversion of acetoacetyl-coenzyme A to 3-hydroxybutyryl-coenzyme A.

10. The method as claimed in at least one of the preceding claims,
characterized in that
it comprises method step
D) purifying the target product.