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(71) Applicants: **SILICOLIFE LDA**. [PT/PT]; Rua do Canastreiro, 15, 4715-387 Braga (PT). **UNIVERSIDADE DO MINHO** [PT/PT]; Largo do Paço, 4704-553 Braga (PT).

(72) Inventors: **DE ALMEIDA PEREIRA DA ROCHA, Isabel Cristina**; Quinta da Veiga CCI 101 Crespos, 4710-864 Braga (PT). **DE ALMEIDA PEREIRA DA ROCHA, Miguel Francisco**; Rua da Fábrica, 237, 4715-027 Braga (PT). **ARAÚJO FERREIRA, Ana Sofia**; Rua Trás-o-rio, 22, 4705-629 Sequeira, Braga (PT). **LIU, Filipe Alexandre Wang**; 5118 S Dorchester Ave, #306, Chicago, IL 60615 (US). **DA SILVA PEREIRA, Rui Miguel Pinheiro**; Rua da Ribeira, 146 Candoso S. Martinho, 4835-386 Guimarães (PT). **CARVALHO VILAÇA, Paulo Ricardo**; SilicoLife Lda. Rua do Canastreiro 15, 4715-387 Braga (PT). **DE PINHO SOARES, Simão Pedro**; SilicoLife Lda. Rua do Canastreiro 15, 4715-387 Braga (PT).

(74) Agent: **SCHULZ JUNGHANS PATENTANWÄLTE PARTGMBB**; Großbeerenstr. 71, 10963 Berlin (DE).

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(54) Title: METHOD FOR N-BUTANOL PRODUCTION USING HETEROLOGOUS EXPRESSION OF ANAEROBIC PATHWAYS

(57) Abstract: The present invention relates to a method for the production of n-butanol using a transgenic cell with heterologous expression of 2-hydroxyglutarate dehydrogenase, glutaconate-CoA transferase, (R)-2-hydroxyglutaryl-CoA dehydrogenase, glutaryl CoA dehydrogenase, trans-2-enoyl-CoA reductase (NAD⁺) and bifunctional aldehyde / alcohol dehydrogenase (NAD⁺).



Method for n-butanol production using heterologous expression of anaerobic pathways.

The present invention relates to a method for the production of n-butanol using a transgenic cell capable of heterologous expression of 2-hydroxyglutarate dehydrogenase, glutaconate-
5 CoA transferase, (R)-2-hydroxyglutaryl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, *trans*-2-enoyl-CoA reductase (NAD⁺) and bifunctional aldehyde / alcohol dehydrogenase (NAD⁺).

DescriptionBackground of the invention

10 n-butanol occurs naturally as a minor product of the fermentation of sugars and other carbohydrates and is present in many foods and beverages. It is also a permitted artificial food flavouring in the United States. n-butanol can be used as a drop-in chemical key raw material in the production of cleansing agents, paints, coatings, plasticizers and adhesives; it also acts as the precursor in manufacturing acetates, acrylate, glycol ethers and solvents. It
15 is further used as a drop-in chemical replacement of petroleum-based n-butanol in almost all applications. Its use as an additive has resulted in increasing need from the pharmaceutical industry. It is also regarded as a potential bio-fuel with improved properties when compared with bio-ethanol.

Until the mid-1940's, n-butanol was produced predominantly through fermentation using a
20 process called Acetone-Butanol-Ethanol (ABE) fermentation with clostridia bacteria. Recent advances in the fields of biotechnology and bioprocessing have resulted in a renewed interest in the fermentation production of chemicals and fuels, including n-butanol. With continuous fermentation technology, n-butanol can be produced at higher yields, concentrations and production rates. Advanced technology in metabolic engineering and
25 synthetic biology has also improved the development of heterologous metabolic pathways in well-characterized microbial hosts for n-butanol fermentation. The rapidly expanding genomic information, molecular biology techniques, and high-throughput tools resulted in a significant progress in constructing non-native organisms for the production of fuel-grade compounds beyond the scope of what native organisms can produce. The n-butanol process still has to
30 overcome some important milestones, which include: more microorganisms and pathways able to overproduce n-butanol, microbial tolerance to n-butanol concentrations, improved yields, and specificity of n-butanol production vs. co-products such as acetone, increased productivity and finally the use of flexible feedstocks.

The objective of this invention is to provide means and methods that allow for improved n-butanol production.

This objective is attained by the subject-matter of the independent claims of the present specification.

5 Summary of the invention

A first aspect of the invention relates to a method for production of n-butanol, wherein a transgenic cell heterologously expresses each of the following enzymes:

- a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- 10 c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.) and
- 15 f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

and is grown in a medium comprising a metabolic precursor of 2-oxoglutarate.

A second aspect of the invention relates to a cell heterologously expressing each of the above-mentioned enzymes.

Another aspect of the invention relates to a plurality of plasmids comprising genes encoding
20 the above-mentioned enzymes.

Certain aspects of the invention may be summarized as a novel and unexpectedly advantageous combination of a first set of three reactions capable of producing glutaconate, namely 2-hydroxyglutarate dehydrogenase *hgdH*, glutaconate-CoA transferase *gctAB*, and (R)-2-hydroxyglutaryl-CoA dehydratase *hgdABC*, with the last three steps common to the
25 clostridial pathway (butanol's native producers) through an enzyme never used before to the production of butanol, namely glutaryl-CoA dehydrogenase (encoded by the gene *gcdH*).

Terms and definitions

The term *hgdH* in the context of the present specification relates to 2-hydroxyglutarate
30 dehydrogenase, EC 1.1.99.2.

The term *gctAB* in the context of the present specification relates to glutaconate-CoA transferase subunits A and B, EC 2.8.3.12.

The term *hgdABC* in the context of the present specification relates to (R)-2-hydroxyglutaryl-CoA dehydrogenase subunits A, B and C, EC 4.2.1.167.

The term *gcdH* in the context of the present specification relates to glutaryl-CoA dehydrogenase, EC 1.3.8.6.

- 5 The term *ter* in the context of the present specification relates to *trans*-2-enoyl-CoA reductase (NAD⁺), EC 1.3.1.44.

The terms *adhE*, *adhE1* or *adhE2* in the context of the present specification relate to bifunctional aldehyde / alcohol dehydrogenase (NAD⁺), EC 1.1.1.11/1.2.1.3.

- 10 The term *bp* in the context of the present specification is an abbreviation for base pairs, while *kbp* is an abbreviation for kilo base pairs.

Amino acid sequences are given from amino to carboxyl terminus. Capital letters for sequence positions refer to L-amino acids in the one-letter code (Stryer, Biochemistry, 3rd ed. p. 21). Lower case letters for amino acid sequence positions refer to the corresponding D- or (2R)-amino acids.

- 15 In the context of the present specifications the terms *sequence identity* and *percentage of sequence identity* refer to the values determined by comparing two aligned sequences. Methods for alignment of sequences for comparison are well-known in the art. Alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2:482 (1981), by the global alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Nat. Acad. Sci. 85:2444 (1988) or by computerized implementations of these
20 algorithms, including, but not limited to: CLUSTAL, GAP, BESTFIT, BLAST, FASTA and TFASTA. Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology-Information (<http://blast.ncbi.nlm.nih.gov/>).

- 25 One example for comparison of amino acid sequences is the BLASTP algorithm that uses the default settings: Expect threshold: 10; Word size: 3; Max matches in a query range: 0; Matrix: BLOSUM62; Gap Costs: Existence 11, Extension 1; Compositional adjustments: Conditional compositional score matrix adjustment. One such example for comparison of nucleic acid sequences is the BLASTN algorithm that uses the default settings: Expect
30 threshold: 10; Word size: 28; Max matches in a query range:0; Match/Mismatch Scores:1.-2; Gap costs: Linear. Unless stated otherwise, sequence identity values provided herein refer to the value obtained using the BLAST suite of programs (Altschul et al., J. Mol. Biol. 215:403-410 (1990)) using the above identified default parameters for protein and nucleic acid comparison, respectively.

In the context of the present specification, the terms *anaerobic* or *aerobic* refer to a culture or growth condition, wherein the amount of dissolved oxygen is null in the case of anaerobic conditions and >10% of saturation for aerobic conditions. An *anaerobic bacterium* does not require oxygen for growth. *Strictly anaerobic bacteria* require oxygen-free conditions for survival, while *facultatively anaerobic bacteria* can grow under either oxygen-enriched or oxygen-free conditions.

In the context of the present specification, the term *heterologous* refers to a gene or protein derived from a source other than the host species whereas *homologous* refers to a gene or protein derived from the host microbial organism.

10 In the context of the present specification, the term *plasmid* or *plasmids* refer to a small (1,5 to 15kb, particularly 2-10kb), circular piece of double-stranded DNA comprising an origin of replication operable in a host cell, and a selection marker gene.

In the context of the present specification, the term *codon-optimized* as it refers to genes or coding regions of nucleic acid molecules for transformation of specific hosts, refers to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA. Each codon is recognized by a transfer RNA (tRNA) that translates the codon to an amino acid. There are bioinformatic methods available that search for the most prevalent tRNAs of a host organism for each codon and optimize the codons with respect to the host organism thereby potentially increasing the expression rate.

Detailed description of the invention

In a bioinformatics approach, possible heterologous pathways to produce n-butanol in a transgenic bacterial cell were proposed using enumeration methodologies based on (Liu, F. *et al.* (2015) *Computer Methods and Programs in Biomedicine*, 118(2), pp. 134–146). The solutions obtained were analyzed computationally using a proprietary digital platform from *SilicoLife*. The ranking and evaluation process involved diverse criteria such as novelty, size of pathway, n-butanol yield, conservation of number of carbon atoms. *OptFlux* (Rocha, I. *et al.* (2010), *BMC Systems Biology*, 4(1), p. 45) and a proprietary digital platform from *SilicoLife* were used to perform all simulations. Flux Balance Analysis (FBA) and variants were used as simulation methods. The most promising pathway was translated into laboratory experiments and optimized.

A first aspect of the invention relates to a method for production of n-butanol, wherein a transgenic cell heterologously or endogenously, particularly heterologously, expresses each of the following enzymes:

35 a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);

- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- 5 e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.) and
- f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

and is grown in a medium comprising a metabolic precursor of 2-oxoglutarate.

In certain embodiments, n-butanol is extracted from said medium. In certain embodiments, n-
10 butanol is extracted from said medium via distillation.

In certain embodiments, said metabolic precursor of 2-oxoglutarate is selected from glucose, glycerol, glutamate or acetate.

In certain embodiments, the transgenic cell is a bacterium or a yeast cell.

In certain embodiments, the bacterium or the yeast cell is selected from genera *Escherichia*,
15 *Corynebacterium*, *Ralstonia*, *Clostridium*, *Pseudomonas*, *Lactobacillus*, *Lactococcus*,
Acidaminococcus, *Fusobacterium*, *Peptoniphilus*, *Saccharomyces*, *Streptomyces*
Lactobacillus, *Pichia*, *Kluyveromyces*, *Yarrowia*, or *Staphylococci*, particularly *Escherichia coli*.

In certain embodiments of the method of the invention, the protein *hgdH* is encoded by a
20 gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of
the method of the invention, said *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%,
95% or >95% identical, with respect to its amino acid sequence, to *hgdH* of *Acidaminococcus fermentans*. In certain embodiments of the method of the invention, *hgdH* is at least 60%,
65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 1 and has a catalytic
25 activity of at least 75% of the activity of SEQ NO 1.

In certain embodiments of the method of the invention, the protein *gctAB* is encoded by a
gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of
the method of the invention, said *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%,
95% or >95% identical, with respect to its amino acid sequence, to *gctAB* of
30 *Acidaminococcus fermentans*. In certain embodiments of the method of the invention,
subunit A of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical
to SEQ NO 2 and has a catalytic activity of at least 75% of the activity of SEQ NO 2. In
certain embodiments of the method of the invention, subunit B of *gctAB* is at least 60%, 65%,

70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 3 and has a catalytic activity of at least 75% of the activity of SEQ NO 3.

In certain embodiments of the method of the invention, the A subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain
5 embodiments of the method of the invention, said *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdA* of *Clostridium symbiosum*. In certain embodiments of the method of the invention, *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 4 and has a catalytic activity of at least 75% of the activity of SEQ NO 4.

10 In certain embodiments of the method of the invention, the B subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the method of the invention, said *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdB* of *Clostridium symbiosum*. In certain embodiments of the method of the invention, *hgdB* is at
15 least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 5 and has a catalytic activity of at least 75% of the activity of SEQ NO 5.

In certain embodiments of the method of the invention, the C subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain
20 embodiments of the method of the invention, said *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdC* of *Acidaminococcus fermentans*. In certain embodiments of the method of the invention, *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 6 and has a catalytic activity of at least 75% of the activity of SEQ NO 6.

In certain embodiments of the method of the invention, the protein *gcdH* is encoded by a
25 gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the method of the invention, said *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gcdH* of *Pseudomonas aeruginosa*. In certain embodiments of the method of the invention, *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 7 and has a catalytic
30 activity of at least 75% of the activity of SEQ NO 7.

In certain embodiments of the method of the invention, the protein *ter* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the method of the invention, said *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *ter* of *Treponema denticola*. In
35 certain embodiments of the method of the invention, *ter* is at least 60%, 65%, 70%, 75%,

80%, 85%, 90%, 95% or >95% identical to SEQ NO 8 and has a catalytic activity of at least 75% of the activity of SEQ NO 8.

In certain embodiments of the method of the invention, the protein *adhE1* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the method of the invention, said *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE1* of *Clostridium acetobutylicum*. In certain embodiments of the method of the invention, *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 9 and has a catalytic activity of at least 75% of the activity of SEQ NO 9.

10 In certain embodiments of the method of the invention, the protein *adhE2* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the method of the invention, said *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE2* of *Clostridium acetobutylicum*. In certain embodiments of the method of the invention, *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 13 and has a catalytic activity of at least 75% of the activity of SEQ NO 13.

In certain embodiments, said transgenic cell comprises one or more plasmids encoding said heterologously expressed enzymes under control of a promoter sequence operable in said cell, particularly a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.

In certain embodiments, said fermentation step is performed under anaerobic conditions at 25 to 37°C, particularly at 30°C.

In certain embodiments, the medium comprises 8-12 g.L⁻¹ glucose, 8-10 g.L⁻¹ dibasic sodium phosphate dihydrate, 6-8 g.L⁻¹ monobasic potassium phosphate, 0.5-0.7 g.L⁻¹ sodium chloride, 1.2-1.5 g.L⁻¹ magnesium sulphate, 0.03-0.05 g.L⁻¹ calcium chloride dihydrate, 0.8-1.2 g.L⁻¹ ammonium chloride, and 8-12 mmol.L⁻¹ sodium bicarbonate, 0.1-0.15 μ g.L⁻¹ selenium, 0.08-0.12 μ g.L⁻¹ nickel, 0.7-0.9 μ g.L⁻¹ molybdenum, ampicillin, spectinomycin, and kanamycin and neutral pH, particularly pH 6.8 - 7.3.

In certain embodiments, said plasmid comprises a lac, tac or T7 promoter, and the expression of said heterologous genes is induced by adding Isopropyl β -D-1-thiogalactopyranosid (IPTG) to the medium, particularly 0.1-1 mmol.L⁻¹ IPTG, more particularly 0.5 mmol.L⁻¹ IPTG. In certain embodiments, a T7-RNA-polymerase is under control of a lac promoter and when IPTG is added, the T7-RNA-polymerase is expressed and transcribes the protein under control of a T7 promoter.

In certain embodiments, said plasmid comprises a *trp* promoter, and the expression of heterologous genes is induced by adding 3-b-indoleacrylic acid to the medium, at concentrations ranging from 10 $\mu\text{g}\cdot\text{mL}^{-1}$ to 100 $\mu\text{g}\cdot\text{mL}^{-1}$.

In certain embodiments, said plasmid comprises a λP_L promoter, and the expression of heterologous genes is induced by increasing the temperature to 42 °C.

A second aspect of the invention relates to a transgenic cell, wherein each of the following enzymes are expressed:

- a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- 10 c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.); and
- f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1*
15 and *adhE2* (EC 1.1.1.11 / 1.2.1.3.).

In certain embodiments of the transgenic cell of the invention, at least 4 of said enzymes are expressed heterologously. In certain embodiments of the transgenic cell, 5 or 6 enzymes are expressed heterologously.

In certain embodiments, the cell is selected from genera *Escherichia*, *Corynebacterium*,
20 *Ralstonia*, *Clostridium*, *Pseudomonas*, *Lactobacillus*, *Lactococcus*, *Acidaminococcus*,
Fusobacterium, *Peptoniphilus*, *Saccharomyces*, *Streptomyces*, *Lactobacillus*, *Pichia*,
Kluyveromyces, *Yarrowia*, or *Staphylococci*, particularly *Escherichia coli*.

In certain embodiments of the transgenic cell of the invention, the protein *hgdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments
25 of the transgenic cell of the invention, said *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdH* of
Acidaminococcus fermentans. In certain embodiments of the transgenic cell of the invention, *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO:
1 and has a catalytic activity of at least 75% of the activity of SEQ NO 1.

30 In certain embodiments of the transgenic cell of the invention, the protein *gctAB* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments
of the transgenic cell of the invention, said *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gctAB* of

Acidaminococcus fermentans. In certain embodiments of the transgenic cell of the invention, subunit A of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 2 and has a catalytic activity of at least 75% of the activity of SEQ NO 2. In certain embodiments of the transgenic cell of the invention, subunit B of *gctAB* is at least
5 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 3 and has a catalytic activity of at least 75% of the activity of SEQ NO 3.

In certain embodiments of the transgenic cell of the invention, the A subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the transgenic cell of the invention, said *hgdA* is at least 60%, 65%,
10 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdA* of *Clostridium symbiosum*. In certain embodiments of the transgenic cell of the invention, *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 4 and has a catalytic activity of at least 75% of the activity of SEQ NO 4.

In certain embodiments of the transgenic cell of the invention, the B subunit of the protein
15 *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the transgenic cell of the invention, said *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdB* of *Clostridium symbiosum*. In certain embodiments of the transgenic cell of the invention, *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to
20 SEQ NO 5 and has a catalytic activity of at least 75% of the activity of SEQ NO 5.

In certain embodiments of the transgenic cell of the invention, the C subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the transgenic cell of the invention, said *hgdC* is at least 60%, 65%,
25 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdC* of *Acidaminococcus fermentans*. In certain embodiments of the transgenic cell of the invention, *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 6 and has a catalytic activity of at least 75% of the activity of SEQ NO 6.

In certain embodiments of the transgenic cell of the invention, the protein *gcdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments
30 of the transgenic cell of the invention, said *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gcdH* of *Pseudomonas aeruginosa*. In certain embodiments of the transgenic cell of the invention, *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 7 and has a catalytic activity of at least 75% of the activity of SEQ NO 7.

35 In certain embodiments of the transgenic cell of the invention, the protein *ter* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of

the transgenic cell of the invention, said *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *ter* of *Treponema denticola*. In certain embodiments of the transgenic cell of the invention, *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 8 and has a catalytic activity of at least 75% of the activity of SEQ NO 8.

In certain embodiments of the transgenic cell of the invention, the protein *adhE1* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the transgenic cell of the invention, said *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE1* of *Clostridium acetobutylicum*. In certain embodiments of the transgenic cell of the invention, *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 9 and has a catalytic activity of at least 75% of the activity of SEQ NO 9.

In certain embodiments of the transgenic cell of the invention, the protein *adhE2* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium. In certain embodiments of the transgenic cell of the invention, said *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE2* of *Clostridium acetobutylicum*. In certain embodiments of the transgenic cell of the invention, *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 13 and has a catalytic activity of at least 75% of the activity of SEQ NO 13.

In certain embodiments, said cell comprises the sequences for said heterologously expressed enzymes under control of a promoter sequence operable in said cell. In certain embodiments, the promoter is a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.

A third aspect of the invention relates to a medium for n-butanol production comprising 8-12 g.L⁻¹ glucose, 8-10 g.L⁻¹ dibasic sodium phosphate dihydrate, 6-8 g.L⁻¹ monobasic potassium phosphate, 0.5-0.7 g.L⁻¹ sodium chloride, 1.2-1.5 g.L⁻¹ magnesium sulphate, 0.03-0.05 g.L⁻¹ calcium chloride dihydrate, 0.8-1.2 g.L⁻¹ ammonium chloride, and 8-12 mmol.L⁻¹ sodium bicarbonate, 0.1-0.15 μ g.L⁻¹ selenium, 0.08-0.12 μ g.L⁻¹ nickel, 0.7-0.9 μ g.L⁻¹ molybdenum, ampicillin, spectinomycin, and kanamycin and neutral pH, particularly pH 6.8 - 7.3.

A fourth aspect of the invention relates to a plurality of plasmids comprising genes encoding

- a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);

- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.) and
- f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.).

5 In certain embodiments, each plasmid in said plurality of plasmids comprises more than one of said genes and each of said plasmids comprises a different selection marker. In certain embodiments, the plurality of plasmids consists of three plasmids, each encoding two of said genes.

10 In certain embodiments, each plasmid independently of each other comprises a promoter sequence operable in a desired target cell, particularly a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.

In certain embodiments, one plasmid comprises the genes encoding *gctAB* and *hgdH* and a gene for spectinomycin resistance and having the size of about 6.5 kbp, wherein particularly the one plasmid further comprises a T7 promoter sequence. In certain embodiments, one
15 plasmid has the sequence SEQ NO 10.

In certain embodiments, one plasmid comprises the genes encoding *hgdABC* and *gcdH* and a gene for kanamycin resistance and having the size of about 8.3 kbp, wherein particularly the one plasmid further comprises a T7 promoter sequence. In certain embodiments, one plasmid has the sequence SEQ NO 11.

20 In certain embodiments, one plasmid comprises the genes encoding *adhE1* or *adhE2* and *ter* and a gene for ampicillin resistance and having the size of about 9.1 kbp, wherein particularly the one plasmid further comprises a T7 promoter sequence. In certain embodiments, one plasmid has the sequence SEQ NO 12.

25 A fifth aspect of the invention relates to a kit or set of parts comprising the said transgenic cell or said plasmids and said medium.

Wherever alternatives for single separable features such as, for example, an isotype protein or coding sequence, an organism genus or a concentration of a chemical are laid out herein as "embodiments", it is to be understood that such alternatives may be combined freely to form discrete embodiments of the invention disclosed herein.

30 The invention is further illustrated by the following examples and figures, from which further embodiments and advantages can be drawn. These examples are meant to illustrate the invention but not to limit its scope.

Items

1. A method for production of n-butanol, wherein a transgenic cell heterologously or endogenously, particularly heterologously, expressing each of the following enzymes:
 - a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
 - 5 b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
 - c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
 - d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
 - e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.); and
 - 10 f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

is grown in a medium comprising a metabolic precursor of 2-oxoglutarate.
2. The method according to item 1, wherein n-butanol is extracted from said medium.
3. The method according to item 1 or 2, wherein said metabolic precursor of 2-oxoglutarate is selected from glucose, glycerol, glutamate or acetate.
- 15 4. The method according to any one of items 1 to 3, wherein the transgenic cell is a bacterium or a yeast cell.
5. The method according to item 4, wherein the bacterium or the yeast cell is selected from genera *Escherichia*, *Corynebacterium*, *Ralstonia*, *Clostridium*, *Pseudomonas*, *Lactobacillus*, *Lactococcus*, *Acidaminococcus*, *Fusobacterium*, *Peptoniphilus*, *Saccharomyces*, *Streptomyces*, *Lactobacillus*, *Pichia*, *Kluyveromyces*, *Yarrowia*, or *Staphylococci*, particularly *Escherichia coli*.
- 20 6. The method according to any one of the preceding items, wherein
 - a. the protein *hgdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdH* of *Acidaminococcus fermentans*, more particularly *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 1 and has a catalytic activity of at least 75% of the activity of SEQ NO 1 and/or
 - 25 b. the protein *gctAB* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gctAB* of *Acidaminococcus fermentans*, more particularly subunit A
 - 30

of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 2 and has a catalytic activity of at least 75% of the activity of SEQ NO 2 and/or subunit B of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 3 and has a catalytic activity of at least 75% of the activity of SEQ NO 3 and/or

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c. the A subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdA* of *Clostridium symbiosum*, more particularly *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 4 and has a catalytic activity of at least 75% of the activity of SEQ NO 4 and/or

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d. the B subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdB* of *Clostridium symbiosum*, more particularly *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 5 and has a catalytic activity of at least 75% of the activity of SEQ NO 5 and/or

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e. the C subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdC* of *Acidaminococcus fermentans*, more particularly *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 6 and has a catalytic activity of at least 75% of the activity of SEQ NO 6 and/or

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f. the protein *gcdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gcdH* of *Pseudomonas aeruginosa*, more particularly *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 7 and has a catalytic activity of at least 75% of the activity of SEQ NO 7 and/or

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g. the protein *ter* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *ter* of *Treponema denticola*, more particularly *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 8 and has a catalytic activity of at least 75% of the activity of SEQ NO 8 and/or

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- 5 h. the protein *adhE1* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE1* of *Clostridium acetobutylicum*, more particularly *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 9 and has a catalytic activity of at least 75% of the activity of SEQ NO 9 and/or
- 10 i. the protein *adhE2* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE2* of *Clostridium acetobutylicum*, more particularly *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 13 and has a catalytic activity of at least 75% of the activity of SEQ NO 13.
7. The method according to any one of the preceding items, wherein said transgenic cell comprises one or more plasmids encoding said heterologously expressed enzymes under control of a promoter sequence operable in said cell, particularly a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.
- 15 8. The method according to any one of the preceding items, wherein said fermentation step is performed under anaerobic conditions at 25 to 37°C, particularly at 30°C.
9. The method according to any one of the preceding items, wherein the medium comprises 8-12 g.L⁻¹ glucose, 8-10 g.L⁻¹ dibasic sodium phosphate dihydrate, 6-8 g.L⁻¹ monobasic potassium phosphate, 0.5-0.7 g.L⁻¹ sodium chloride, 1.2-1.5 g.L⁻¹ magnesium sulphate, 0.03-0.05 g.L⁻¹ calcium chloride dihydrate, 0.8-1.2 g.L⁻¹ ammonium chloride, and 8-12 mmol.L⁻¹ sodium bicarbonate, 0.1-0.15 μ g.L⁻¹ selenium, 0.08-0.12 μ g.L⁻¹ nickel, 0.7-0.9 μ g.L⁻¹ molybdenum, ampicillin, spectinomycin, and kanamycin and neutral pH, particularly pH 6.8 - 7.3.
- 20 10. The method according to any one of the preceding items 7 to 9, wherein said plasmid comprises
- a. a lac, tac or T7 promoter, and the expression of said heterologous genes is induced by adding IPTG (Isopropyl β -D-1-thiogalactopyranosid) to the medium, particularly 0.1-1 mmol.L⁻¹ IPTG, more particularly 0.5 mmol.L⁻¹ IPTG;
- 30 b. a trp promoter, and the expression of heterologous genes is induced by adding 3-b-indoleacrylic acid to the medium, at concentrations ranging from 10 μ g.mL⁻¹ to 100 μ g/m.L⁻¹;
- c. a λP_L promoter, and the expression of heterologous genes is induced by increasing the temperature to 42 °C.
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11. A transgenic cell, wherein the following enzymes are expressed:

- a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.); and
- f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

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wherein at least 4 enzymes are expressed heterologously, particularly 5 or 6 enzymes are expressed heterologously.

12. The cell according to item 11, wherein the cell is selected from genera *Escherichia*, *Corynebacterium*, *Ralstonia*, *Clostridium*, *Pseudomonas*, *Lactobacillus*, *Lactococcus*, *Acidaminococcus*, *Fusobacterium*, *Peptoniphilus*, *Saccharomyces*, *Streptomyces*, *Lactobacillus*, *Pichia*, *Kluyveromyces*, *Yarrowia*, or *Staphylococci*, particularly *Escherichia coli*.

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13. The cell according to item 11 or 12, wherein

- a. the protein *hgdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdH* of *Acidaminococcus fermentans*, more particularly *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 1 and has a catalytic activity of at least 75% of the activity of SEQ NO 1 and/or
- b. the protein *gctAB* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gctAB* of *Acidaminococcus fermentans*, more particularly *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 2 and has a catalytic activity of at least 75% of the activity of SEQ NO 2 and/or subunit B of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 3 and has a catalytic activity of at least 75% of the activity of SEQ NO 3 and/or

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- 5 c. the A subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdA* of *Clostridium symbiosum*, more particularly *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 4 and has a catalytic activity of at least 75% of the activity of SEQ NO 4 and/or
- 10 d. the B subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdB* of *Clostridium symbiosum*, more particularly *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 5 and has a catalytic activity of at least 75% of the activity of SEQ NO 5 and/or
- 15 e. the C subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdC* of *Acidaminococcus fermentans*, more particularly *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 6 and has a catalytic activity of at least 75% of the activity of SEQ NO 6 and/or
- 20 f. the protein *gcdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gcdH* of *Pseudomonas aeruginosa*, more particularly *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO
- 25 7 and has a catalytic activity of at least 75% of the activity of SEQ NO 7 and/or
- 30 g. the protein *ter* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *ter* of *Treponema denticola*, more particularly *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 8 and has a catalytic activity of at least 75% of the activity of SEQ NO 8 and/or
- 35 h. the protein *adhE1* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE1* of *Clostridium acetobutylicum*, more particularly *adhE1* is at

least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 9 and has a catalytic activity of at least 75% of the activity of SEQ NO 9 and/or

- 5 i. the protein *adhE2* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE2* of *Clostridium acetobutylicum*, more particularly *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 13 and has a catalytic activity of at least 75% of the activity of SEQ NO 13.

10 14. The cell according to any one of the items 11 to 13, wherein said cell comprises the sequences for said heterologously expressed enzymes under control of a promoter sequence operable in said cell, particularly a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.

15 15. A medium for n-butanol production comprising 8-12 g.L⁻¹ glucose, 8-10 g.L⁻¹ dibasic sodium phosphate dihydrate, 6-8 g.L⁻¹ monobasic potassium phosphate, 0.5-0.7 g.L⁻¹ sodium chloride, 1.2-1.5 g.L⁻¹ magnesium sulphate, 0.03-0.05 g.L⁻¹ calcium chloride dihydrate, 0.8-1.2 g.L⁻¹ ammonium chloride, and 8-12 mmol.L⁻¹ sodium bicarbonate, 0.1-0.15 μ g.L⁻¹ selenium, 0.08-0.12 μ g.L⁻¹ nickel, 0.7-0.9 μ g.L⁻¹ molybdenum, ampicillin, spectinomycin, and kanamycin and neutral pH, particularly pH 6.8 - 7.3.

20 16. A plurality of plasmids comprising genes encoding

- a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- 25 e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.); and
- f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

particularly wherein each plasmid in said plurality of plasmids comprises more than one of said genes and each of said plasmids comprises a different selection marker, more particularly wherein the plurality of plasmids consists of three plasmids, each encoding two of said genes.

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17. A plurality of plasmids according to item 16, comprising the following constructs:

- a. a plasmid comprising the genes encoding *gctAB* and *hgdH* and a gene for spectinomycin resistance and having the size of about 6.5 kbp;
 - b. a plasmid comprising the genes encoding *hgdABC* and *gcdH* and a gene for kanamycin resistance and having the size of about 8.3 kbp;
 - 5 c. a plasmid comprising the genes encoding *adhE1* or *adhE2* and *ter* and a gene for ampicillin resistance and having the size of about 9.1 kbp.
18. A plurality of plasmids according to item 17, wherein said plurality comprises the following constructs:
- a. a plasmid having the sequence SEQ NO 10;
 - 10 b. a plasmid having the sequence SEQ NO 11;
 - c. a plasmid having the sequence SEQ NO 12.

Brief description of the figures

- Fig. 1 The proposed biosynthetic pathway to produce n-butanol from 2-oxoglutarate in *E. coli* with indication of the reactions catalysed by the enzymes. *hgdH* – 2-hydroxyglutarate dehydrogenase; *gctAB* – glutaconate-CoA transferase;
- 15 *hgdABC* - 2-hydroxyglutaryl-CoA dehydratase; *gcdH* – glutary-CoA dehydrogenase; *ter* - *trans*-2-enoyl-CoA reductase, *adhE1/adhE2* - aldehyde dehydrogenase and alcohol dehydrogenase 1 and 2.
- Fig. 2 Construction of the recombinant plasmid pCDFDuet_*gctAB_hgdH*.
- 20 Fig. 3 Construction of the recombinant plasmid pRSFDuet_*gcdH_hgdABC*.
- Fig. 4 Construction of the recombinant plasmid pETDuet_*adhE1_ter*.
- Fig. 5 Construction of the recombinant plasmid pETDuet_*adhE2_ter_opt*

Examples

Materials and Methods:

25 Cloning procedure

E. coli NEB 5-alpha cells were used for gene cloning and vector propagation. These strains were cultured in LB medium (10 g.L⁻¹ of peptone; 5 g.L⁻¹ yeast extract and 5 g.L⁻¹ of NaCl) with the appropriate antibiotics concentration. The solid version of this medium included 15 g.L⁻¹ agar. All cultivations were performed at 37 °C and, in the case of liquid cultures, under shaking

30 conditions (200 rpm).

For long-term storage, glycerol was added to a final concentration of 30 % to overnight cultures in selective media and kept in a -80 °C freezer.

The genes used in this study were amplified by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA) in a LifeECO Thermal Cycler (Bioer Technology, Zehjiang, China). All primers were purchased from Metabion (Munich, Germany). DNA fragments were purified using DNA Clean and Concentrator DNA Kit (Zymo Research, Irvine, USA).

Plasmids were extracted using Plasmid Miniprep kit (Zymo Research). All digestions were performed using the appropriate FastDigest® restriction endonucleases (Thermo Scientific). Ligations were performed with T4 DNA Ligase (Thermo Scientific) and transformed by heat-shock in chemically competent cells *E. coli* NEB 5-alpha (New England BioLabs, Massachusetts, USA). The success of ligation was checked through Colony PCR using DreamTaq (Thermo Scientific) and further confirmed by sequencing (StabVida, Lisbon, Portugal). Protocols were performed in accordance with manufacturer's instructions.

hgdH, *gcdH*, *hgdABC* and *gctAB* genes were codon-optimized through ATGenium for *E. coli*, synthesized and cloned in vector pHTP0 by NZYTech (Lisbon, Portugal). A optimized codon-sequence of *adhE2* and *ter_opt* were synthesized by ATG:biosynthetics (Freiburg, Germany) and cloned in pUC-derivative plasmids.

Plasmid construction

Compatible vectors pETDuet, pCDFDuet and pRSFDuet (Novagen, Darmstadt, Germany) were used to provide individual expression of each protein under the control of the T7lac promoter and a ribosome-binding site (RBS).

Sources of the cloned genes are shown in table 1.

Table 1: Sources of n-Butanol Pathway Genes and their sequences

Name	Abbreviation	Reference	Microorganism	Type
2-Hydroxyglutarate dehydrogenase	<i>hgdH</i>	EC 1.1.99.2 NCBI GI: >gb CP001859.1 :1104274 -1105269 NCBI GeneID: Acfer_0977	<i>Acidaminoco ccus fermentans</i> ATCC 25085	NS AS NS CO
		EC 2.8.3.12 <i>gctA</i> NCBI GI >gi 284047386:2019003-2019965 NCBI GeneID: Acfer_1820 <i>gctB</i>	<i>Acidaminoco ccus fermentans</i> ATCC 25085	NS (A) AS (A) NS CO (A) NS (B) AS (B)

		NCBI GI: >gi 284047386:2018200-2019000 NCBI geneID: Acfer_1819		NS CO (B)
(R)-2-hydroxyglutaryl-CoA dehydrogenase subunits A and B	<i>hgdAB</i>	EC 4.2.1.- NCBI GI: >AF123384.1:1241-2683 NCBI geneID: AF123384	<i>Clostridium symbiosum</i> ATCC 14940	NS (A) AS (A) NS CO (A) NS (B) AS (B) NS CO (B)
(R)-2-hydroxyglutaryl-CoA dehydrogenase Subunit C	<i>hgdC</i>	EC 4.2.1.167 NCBI GI: >gi 284047386:2015608-2016390 NCBI GeneID: Acfer_0168	<i>Acidaminococcus fermentans</i> ATCC 25085	NS (C) AS (C) NS CO (C)
Glutaryl-CoA dehydrogenase	<i>gcdH</i>	EC 1.3.8.6 NCBI GI: >NP_249138.1 NCBI GeneID: PANN_05040	<i>Pseudomonas aeruginosa</i> PAO1	NS AS NS CO
<i>trans</i> -2-enoyl-CoA reductase (NAD ⁺)	<i>ter</i>	EC 1.3.1.44 NCBI GI: >AE017226.1:636109-637302 NCBI GeneID: TDE_0597	<i>Treponema denticola</i> ATCC 35405	NS AS
Bifunctional Aldehyde / Alcohol dehydrogenase (NAD ⁺)	<i>adhE</i>	EC 1.1.1.11 / 1.2.1.3 NCBI GI: >CP002661.1:33722-36298 NCBI GeneID: <i>adhE</i> ATCC ID: DSM 1731	<i>Clostridium acetobutylicum</i> pSMBa - DSM 1731	NS AS

A – alpha subunit ; B – beta subunit B; C – gamma subunit C; NS – Nucleic Acid Sequence; AS – Amino acid Sequence; CO – Codon Optimized

5 The plasmid pCDFDuet (Novagen) was used to clone the codon-optimized genes encoding the first two reactions of the proposed pathway (*gctAB* and *hgdH*). *hgdH* was amplified using the primers *hgdH_fw* and *hgdH_rev* with flanking restriction sites for *KpnI* and *XhoI* and cloned into pCDFDuet. The PCR product for *gctAB*, amplified using primers *gctAB_fw* and *gctAB_rev*, was restricted and ligated into *BamHI* and *HindIII* restriction sites of the previous
10 construction. Colony PCR with appropriate primers was used to find successful clones and the final plasmid was sent for sequencing to confirm the sequence was correct.

The plasmid pRSFDuet (Novagen) was used to clone the codon optimized genes *hgdABC* and *gcdH*, corresponding to the two intermediate steps of the proposed pathway. *gcdH* was amplified using the primers *gcdH_fw* and *gcdH_rev* with restriction sites to *NdeI* and *XhoI*
15 and cloned in pRSFDuet. Then, *hgdABC* was inserted in the previous construction. This

gene was amplified using primers *hgdABC_fw* and *hgdABC_rev* with restriction sites for *SacI* and *NotI*, respectively. Colony PCR with appropriate primers was used to find successful clones and the final plasmid was sent for sequencing to confirm the sequence was correct.

The plasmid pETDuet (Novagen) was used to clone the genes *adhE1* and *ter*, corresponding to the last two genes of the proposed pathway. The *adhE1* gene was amplified from template
5 plasmid pmTA1 (Nielsen, *et al.* (2009), *Metabolic engineering*. Elsevier, 11(4–5), pp. 262–73.) using primers *adhE1_fw* and *adhE1_rev* with restriction sites for *EcoRI* and *NotI*, respectively. The synthetic gene *ter* (ATG:biosynthetics, Freiburg, Germany) was amplified using primers *ter_fw* and *ter_rev*; restricted and ligated into *NdeI* and *XhoI* restriction sites of
10 the previous construction pETDuet_*adhe1*, resulting in the plasmid pETDuet_*adhE1_ter*. Colony PCR with appropriate primers was used to find successful clones and the final plasmid was sent for sequencing to confirm the sequence was correct.

Finally, the plasmid pETDuet (Novagen) was used to clone the genes *adhE2* and *ter_opt*, corresponding to the last two genes of the proposed pathway. The codon-optimized synthetic
15 gene *ter_opt* (ATG:biosynthetics, Freiburg, Germany) gene was directly digested with *NdeI* and *KpnI* and cloned in the respective restriction sites of pETDuet. The codon-optimized synthetic gene *adhE2* (ATG:biosynthetics, Freiburg, Germany) was restricted and ligated into *SacI* and *HindIII* restriction sites of the previous construction pETDuet_*ter*, resulting in the plasmid pETDuet_*adhE2_ter_opt*.

20 In Table 2, the primers used in this study for PCR amplification are shown.

Table 2. Sequences of primers used in the cloning procedures of this study (*restriction sites are underlined). fw-forward; rev - reverse

Primer	Sequence	SEQ NO	Restriction Sites *
<i>adhE1_fw</i>	CC <u>GAAATTC</u> ATGAAAGTCACAACAGTAAAGG	17	<i>EcoRI</i>
<i>adhE1_rev</i>	CCGCGGCCG <u>C</u> TTAAGGTTGTTTTTAAACAATT	18	<i>NotI</i>
<i>ter_fw</i>	CC <u>CATATG</u> ATTGTAAAACC	19	<i>NdeI</i>
<i>ter_rev</i>	CC <u>CTCGAG</u> TTAAATC	20	<i>XhoI</i>
<i>hgdABC_fw</i>	CCGAGCTC <u>A</u> TGAGTATCTATACCCTGGGC	21	<i>SacI</i>
<i>hgdABC_rev</i>	CCGCGGCCG <u>C</u> TTATTTTTGCATCTCCAAAAC	22	<i>NotI</i>
<i>gcdH_fw</i>	CC <u>CATATG</u> GCAACCAAAGCAAG	23	<i>NdeI</i>
<i>gcdH_rev</i>	CC <u>CTCGAG</u> TCAAAGAACGCTTGAATACC	24	<i>XhoI</i>
<i>hgdH_fw</i>	CCG <u>G</u> TACCATGAAAGTGCTGTGCTACGG	25	<i>KpnI</i>

<i>hgdH_rev</i>	CCCTCGAGTTATTTGATTTTGTTCGGGC	26	<i>XhoI</i>
<i>gctAB_fw</i>	CCGGATCCATGAGCAAAGTCATGACCC	27	<i>BamHI</i>
<i>gctAB_rev</i>	CCAAGCTTTTATTTGGCTTCAGTTGGAAC	28	<i>HindIII</i>

The success of the plasmid constructions was confirmed by sequencing the regions of interest with the appropriate primers. In Fig. 2-4 and table 3 the plasmids used or constructed in this study, as well as the respective major features are shown.

5 Table 3: Plasmids used in this study

Plasmid	Construct	Source
pETDuet	ColE1(pBR322) ori, lacI, double T7lac, AmpR	Novagen
pCDFDuet	CloDF13 ori, lacI, double T7lac, StrepR	Novagen
pRSFDuet	RSF ori, lacI, double T7lac, KanR	Novagen
pETDuet_adhE1_ter	pETDuet carrying <i>adhE1</i> from <i>C. acetobutylicum</i> and <i>ter</i> from <i>T. denticola</i>	This study
pCDFDuet_gctAB_hgdH	pCDFDuet carrying codon-optimized <i>gctAB</i> and <i>hgdH</i> from <i>A. fermentans</i>	This study
pRSFDuet_gcdH_hgdABC	pRSFDuet carrying codon-optimized <i>hgdC</i> from <i>A. fermentans</i> ; <i>hgdAB</i> from <i>Clostridium symbiosum</i> and <i>gcdH</i> from <i>Pseudomonas aeruginosa</i>	This study
pETDuet_adhe2_ter_opt	pETDuet carrying codon-optimized <i>adhE2</i> from <i>C. acetobutylicum</i> and <i>ter</i> from <i>T. denticola</i>	This study
pmTA1	Gmr, lacI, taclac: thil, <i>adhE</i>	Nielsen, D. R. <i>et al.</i> (2009) Elsevier, 11(4–5), pp. 262–73.

Bacterial strains

E. coli K12 MG1655 (DE3) and *E. coli* BL21 (DE3) were used as hosts for gene expression under control of T7 promoter. BUT_OXG1 and BUT_OXG2 strains were obtained by transforming *E. coli* BL21 (DE3) and *E. coli* K12 MG1655 (DE3), respectively, with pCDFDuet_gctAB_hgdH; pRSFDuet_gcdH_hgdABC and pETDuet_adhE1_ter by electroporation. The control strains Control_OXG1 and Control_OXG2 were obtained, by transforming, respectively, *E. coli* BL21 (DE3) and *E. coli* K12 MG1655 (DE3) with the plasmids expressing only the last five enzymes of the pathway (pRSFDuet_gcdH_hgdABC and pETDuet_adhE1_ter). Electrocompetent cells were prepared using the protocol developed by (Dower, et al. (1988), *Nucleic Acids Research*, 16(13), pp. 6127–6145) and transformed using 0.1 cm-gap electroporation cuvettes at a voltage of 1.8 KV. Positive transformants were isolated in LB (containing 10 g.L⁻¹ of peptone; 5 g.L⁻¹ yeast extract and 5 g.L⁻¹ of NaCl) agar (15 g.L⁻¹) plates, containing the appropriate antibiotic concentrations (50 µg.mL⁻¹ ampicillin, 50 µg.mL⁻¹ spectinomycin and 30 µg.mL⁻¹ kanamycin) and incubated at 37 °C, overnight. To confirm the success of the transformation, a few transformant colonies were cultivated in LB medium with antibiotics, overnight. After, plasmids were extracted and digested with appropriate restriction enzymes. The correct fragment lengths were confirmed by running the digestion in a 1 % (w/v) agarose gel.

BUT_OXG3 was constructed in the same fashion described above but expressing codon-optimized sequences of *ter* from *Treponoema denticola* and *adhE2* from *Clostridium acetobutylicum*.

Table 4. List of strains and genomic DNA used or engineered for this study

Strains	Relevant genotype	Source
<i>E. coli</i> K12 MG1655 (DE3)	F - λ - ilvGrfb- 50 rph- 1 λ(DE3)	Nielsen, <i>et al.</i> (2009), <i>Metabolic engineering</i> . Elsevier, 11(4–5), pp. 262–73.)
<i>E. coli</i> BL21 (DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHlo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5	New England Labs
BUT_OXG1	<i>E. coli</i> BL21 DE3 pETDuet_adhE1_ter; pCDFDuet_gctAB_hgdH; pRSFDuet_gcdH_hgdABC	This study
BUT_OXG2	<i>E. coli</i> K12 MG1655 DE3 pETDuet_adhE1_ter; pCDFDuet_gctAB_hgdH; pRSFDuet_gcdH_hgdABC	This study
Control_OXG1	<i>E. coli</i> BL21 DE3 pETDuet_adhE1_ter; pRSFDuet_gcdH_hgdABC	This study

Control_OXG2	<i>E. coli</i> K12 MG1655 DE3 pETDuet_adhE1_ter; pRSFDuet_gcdH_hgdABC	This study
BUT_OXG3	<i>E. coli</i> K12 MG1655 DE3 pETDuet_adhE2_ter_opt; pCDFDuet_gctAB_hgdH; pRSFDuet_gcdH_hgdABC	This study

Table 4 summarizes the strains of *E. coli* used or engineered for this study.

Enzymatic assays

5 Table 5 lists the enzymatic reactions and table 6 lists the enzymatic assays for the heterologous enzymes in n-butanol production. The stated reactions are the basis for any reactivity quantities stated herein, unless explicitly stated otherwise.

Table 5: Enzymatic reactions.

Reaction	Enzyme	EC No.	Code	Equation number
2-oxoglutarate + NADH + H ⁺ = (S)-2-hydroxyglutarate + NAD ⁺	2-hydroxyglutarate dehydrogenase (NADH)	1.1.99.2	<i>hgdH</i>	Eq. i
acetyl-CoA + (S)-2-hydroxyglutarate = acetate + (R)-2-hydroxyglutaryl-CoA	glutaconate CoA-transferase	2.8.3.12	<i>gctAB</i>	Eq. ii
(R)-2-hydroxyglutaryl-CoA = (E)-glutaconyl-CoA + H ₂ O	(R)-2-hydroxyglutaryl-CoA dehydratase	4.2.1.167	<i>hgdA</i> <i>BC</i>	Eq. iii
(E)-glutaconyl-CoA = crotonyl-CoA + CO ₂	glutaryl-CoA dehydrogenase (ETF)	1.3.8.6	<i>gcdH</i>	Eq. iv
crotonyl-CoA + NADH + H ⁺ = butanoyl-CoA + NAD ⁺	<i>trans</i> -2-enoyl-CoA reductase (NAD ⁺)	1.3.1.44	<i>ter</i>	Eq. v
butanal + NAD ⁺ + H ₂ O = butanoyl-CoA + NADH + H ⁺	aldehyde dehydrogenase (NAD ⁺)	1.2.1.3	<i>adhE</i>	Eq. vi
n-butanol + NAD ⁺ = butanal + NADH + H ⁺	alcohol dehydrogenase (NAD ⁺)	1.1.1.1	<i>adhE</i>	Eq. vii

The above named compound are also referred to as the following synonyms:

2-oxoglutarate: 2-Oxopentanedioic acid; 2-Ketoglutaric acid; alpha-Ketoglutaric acid; 2-Oxoglutaric acid; Oxoglutaric acid; 2-oxopentanedioate; 4-carboxy-2-oxobutanoate; 2-ketoglutarate; 2-oxopentanedioic acid; α -ketoglutarate.

2-hydroxyglutarate: 2-hydroxypentanedioate.

- 5 2-hydroxyglutaryl-CoA: 3'-phosphoadenosine 5'-{3-[(3R)-4-[[3-({2-[(4-carboxy-2-hydroxybutanoyl)sulfanyl]ethyl}amino)-3-oxopropyl]amino)-3-hydroxy-2,2-dimethyl-4-oxobutyl]dihydrogen diphosphate} 2-hydroxyglutaryl-coenzyme A.

- 10 Glutaconyl-CoA: 5-[2-[3-[[4-[[[5-(6-aminopurin-9-yl)-4-hydroxy-3-phosphonoxyoxolan-2-yl]methoxy-hydroxyphosphoryl]oxy-hydroxyphosphoryl]oxy-2-hydroxy-3,3-dimethylbutanoyl]amino]propanoylamino]ethylsulfanyl]-5-oxopent-3-enoic acid

Crotonyl-CoA: E)-but-2-enoyl-CoA; Crotonoyl-CoA; trans-But-2-enoyl-CoA; trans-butyr-2-enoyl-CoA.

Butanoyl-CoA: butyryl-CoA; butanoyl-coenzyme A; Butyryl-coenzyme A.

Butanal: Butyraldehyde; 1-Butanal; Butaldehyde; Butyl aldehyde; n-Butanal.

- 15 n-Butanol: Butan-1-ol; Butalcohol; Butanol; 1-Butanol; Butyl alcohol; Butyl hydrate; Butylic alcohol; Butyralcohol; Butyric alcohol; Butyryl alcohol; n-Butyl alcohol; 1-Hydroxybutane; n-Propylcarbinol; 1-butyl alcohol.

20 Table 6: Enzymatic Assays used for individual enzyme activities used in pathway for n-butanol production

Enzyme	EC No.	Assay Reference
2-hydroxyglutarate dehydrogenase	1.1.99.2	Kranendijk M. <i>et al.</i> , J. Inherit Metab. Dis. (2009), 32(6):713-19.
glutaconate CoA-transferase	2.8.3.12	Charrier C. <i>et al.</i> Microbiology (2006), 152, 179–185.
(R)-2-hydroxyglutaryl-CoA dehydratase	4.2.1.167	Schweiger G. <i>et al.</i> Arch. Microbiol. (1984), 137: 302- 307.
glutaryl-CoA dehydrogenase (ETF)	1.3.8.6	Estelmann S. <i>et al.</i> FEBS J. (2014()), 4: 5120-5131.
trans-2-enoyl-CoA reductase (NAD ⁺)	1.3.1.44	Bond-Watts B. <i>et al.</i> Nature Chem. Biol. (2011), 7: 22-27.
aldehyde dehydrogenase (NAD ⁺)	1.2.1.3	Guro S. <i>et al.</i> Alcohol. (1990), 7(5):397-401.

alcohol dehydrogenase (NAD⁺) 1.1.1.1 Guro S. *et al.* Alcohol. (1990), 7(5):397-401.

The above named enzymes are also referred to as the following synonyms:

2-hydroxyglutarate dehydrogenase: L-2-hydroxyglutarate dehydrogenase; L-alpha-hydroxyglutarate dehydrogenase; alpha-hydroxyglutarate dehydrogenase; alpha-hydroxyglutarate oxidoreductase; (S)-2-hydroxyglutarate:acceptor 2-oxidoreductase; alpha-ketoglutarate reductase; hydroxyglutaric dehydrogenase; L-2-hydroxyglutaric acid dehydrogenase.

glutaconate CoA-transferase: (E)-glutaconate CoA-transferase; glutaconate CoA-transferase; Acetyl-CoA:(E)-glutaconate CoA-transferase.

10 (R)-2-hydroxyglutaryl-CoA dehydratase : (R)-2-hydroxyglutaryl-CoA hydro-lyase ((E)-glutaconyl-CoA-forming).

glutaryl-CoA dehydrogenase: glutaryl-coenzyme A dehydrogenase; Glutaryl-CoA dehydrogenase.

15 trans-2-enoyl-CoA reductase: mitochondrial 2-trans-enoyl-CoA/ACP reductase; NADPH-dependent trans-2-enoyl-CoA reductase; 2-trans enoyl-ACP(CoA) reductase; trans-2-enoyl-CoA reductase (NADPH); mitochondrial 2-trans-enoyl-thioester reductase.

20 bifunctional aldehyde / alcohol dehydrogenase: aldehyde dehydrogenase; aldehyde reductase; aldehyde/alcohol dehydrogenase; aliphatic alcohol dehydrogenase; ethanol dehydrogenase; NAD⁺-dependent alcohol dehydrogenase; NAD-dependent alcohol dehydrogenase; NAD-specific aromatic alcohol dehydrogenase; NADH-alcohol dehydrogenase; NADH-aldehyde dehydrogenase; NADH-dependent alcohol dehydrogenase.

Butanol production experiments in complex medium

25 The strains BUT_OXG1 and BUT_OXG2 were cultivated in Terrific Broth (TB) medium supplemented with glucose, glutamate, riboflavin and iron (III) citrate according to composition shown in table 7. The pH of this medium was 7.2 ± 0.2 at 25°C.

Table 7: Medium composition of Terrific Broth.

Component	Amount per Liter	Unit
Tryptone	12	g
Yeast extract	24	g
Glycerol	4	mL

Monobasic potassium phosphate	2.31	g
Dibasic potassium phosphate	12.54	g
Glutamate	0.468	g
Riboflavin	0.07529	g
Iron (III) citrate	0.525	g
Glucose	10	g

5

A single colony was picked from Luria-Bertani (LB) plates and inoculated in 10 mL of LB medium (Table 8).

Table 8: LB medium composition.

Component	Amount per Liter	Unit
Tryptone	10	g
Yeast extract	5	g
Sodium Chloride	10	g

10 Cultivation was performed with the addition of suitable antibiotics according to the employed plasmids (50 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ spectinomycin, and 30 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin). The pre-cultures were grown aerobically on a rotary shaker at 37 °C and 200 rpm, overnight.

500 mL shake flasks with 100 mL of TB medium, containing appropriate antibiotics, were inoculated with pre-cultures to obtain an initial optical density OD_{600} of 0.1. Cultivation was
 15 carried on a rotary shaker at 200 rpm at 37 °C. The butanol production genes were induced by the addition of 0.1, 0.5 or 1 $\text{mmol}\cdot\text{L}^{-1}$ isopropyl 1-thio- β -D-galactopyranoside (IPTG) to the culture medium when an optical density OD_{600} of 0.4-0.5 was reached.

To promote butanol production, after induction, the cells were switched to anaerobic conditions by transferring 60 mL of culture to 120 mL sealed serum flasks. The culture was
 20 supplemented with 600 μL of a 0.01 M stock solution of sodium bicarbonate to achieve a final concentration of 10 $\text{mmol}\cdot\text{L}^{-1}$, since it reduces long lag phases in *E. coli* anaerobic growth (Hornsten (1995), *Bioprocess Engineering*, 12, pp. 157–162.).

The cultures were incubated at 30 °C and 180 rpm, for 96 hours. Samples of culture broth were collected at time 0, during induction time and at 96 h. All the experiments were
 25 performed in triplicate and the samples were analysed by High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).

Butanol production experiments in defined medium

The strains BUT_OXG1 and BUT_OXG2 were cultivated in High Density Medium (HDM) adapted from (Sivashanmugam, A. *et al.* (2009), 18(1), pp. 936–948.), supplemented with a
 30 solution of amino acids, extra glutamate, riboflavin and iron citrate (III), according to table 9. The pH of the medium was adjusted to 7.1 using 2 $\text{mol}\cdot\text{L}^{-1}$ NaOH.

Table 9: Medium composition of HDM, adapted from (Sivashanmugam (2009) ibid)

Component	Amount per Liter	Unit
Glucose	10	g
Dibasic sodium phosphate dihydrate	8.89	g
Monobasic potassium phosphate	6.8	g
Sodium chloride	0.58	g
Magnesium sulphate	1.35	g
Calcium chloride dihydrate	0.038	g
Ammonium chloride	1	g
Trace metals	250	μL
Vitamins BME100x	250	μL
Amino acid mix	2	g
Glutamate	0.468	g
Riboflavin	0.07529	g
Iron (III) citrate	0.525	g

The trace metals solution contained (per liter): FeSO₄.7H₂O (30 mg); ZnSO₄.7H₂O (45 mg); CaCl₂.2H₂O (45 mg); MnCl₂.2H₂O (100 mg); CoCl₂.6H₂O (30 mg); CuSO₄.5H₂O (30 mg); Na₂MoO₄.2H₂O (40 mg); H₃BO₃ (10 mg); KI (10 mg) and Na₂EDTA (1.5 g). The amino acid mix contained 1 g of adenine and 4 g of arginine, aspartate, glutamate, histidine, isoleucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine. The vitamin BME 100 x solution (Sigma Aldrich, St. Louis, MO, USA) contained (per liter): D-biotin (0.1 g); choline chloride (0.1 g); folic acid (0.1 g); myo-inositol (0.2 g); niacinamide (0.1 g); D-pantothenic acid.½Ca (0.1 g); riboflavin (0.01 g); thiamine.HCl (0.1 g) and NaCl (8.5 g).

For the pre-cultures, a single colony was picked from Luria-Bertani (LB) plates and inoculated in 10 mL of LB medium. Cultivation was performed with the addition of suitable antibiotics according to the employed plasmids (50 μg.mL⁻¹ ampicillin, 50 μg.mL⁻¹ spectinomycin, and 30 μg.mL⁻¹ kanamycin). The pre-cultures were grown aerobically on a rotary shaker at 37 °C and 200 rpm, overnight. Cells were washed and harvested by centrifugation (10 min at 3000×g). Afterwards, an appropriate volume of pre-culture was transferred to 500 mL shake flasks with 100 mL of HDM medium, containing the appropriate antibiotics, yielding an initial OD₆₀₀ of 0.1. This culture was cultivated on a rotary shaker at 200 rpm at 37 °C. The butanol production genes were induced with 0.1, 0.5 or 1 mmol.L⁻¹ isopropyl 1-thio-β-D-galactopyranoside (IPTG) at an OD₆₀₀ of 0.4-0.5.

After induction, 60 mL of the culture were transferred to 120 mL sealed serum flasks to promote butanol production under anaerobic conditions. The culture was supplemented with 600 μL of a 0.01 mol.L⁻¹ stock solution of sodium bicarbonate to achieve a final concentration of 10 mmol.L⁻¹ (to reduce lag phases in *E. coli* anaerobic growth (Hornsten, 1995, *Bioprocess Engineering*, 12, pp. 157–162)). Selenium, nickel and molybdenum are part of

the formate hydrogen lyase (FHL) complex, which is induced under anaerobic conditions. For this reason, 60 μL of a solution of extra trace metals (NiCl_2 ($1.7 \text{ mg}\cdot\text{L}^{-1}$); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ ($14.5 \text{ mg}\cdot\text{L}^{-1}$); $4\text{H}_2\text{O Na}_2\text{SeO}_3$ ($2.4 \text{ mg}\cdot\text{L}^{-1}$)) was supplied to the medium.

5 The cultures were incubated at 30°C and 180 rpm, for 96 hours. Samples of supernatant were collected at time 0, induction time and 96 h. All the experiments were performed in triplicate and the samples were analysed by GC.

Analytical methods

Samples were centrifuged at $6000\times g$ for 10 min to separate cells from the medium. Afterwards, the supernatant was filtered with a $0.22 \mu\text{m}$ pore filter membrane to glass vials and stored at -20°C until analysed.

10 Butanol concentration was quantified by a Gas Chromatograph GP-9000 system (Chrompack) with a Meta-WAX capillary column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$) equipped with a flame ionization detector (FID); helium was used as carrier gas with a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. The filtered supernatant ($900 \mu\text{L}$) was mixed with $100 \mu\text{L}$ of a $5 \text{ g}\cdot\text{L}^{-1}$ solution of isobutanol, the internal standard, yielding a final concentration of $0.5 \text{ g}\cdot\text{L}^{-1}$, and $1 \mu\text{L}$ of this mixture was injected. The temperature of injector and detector were maintained at 250°C . The column was initially at 50°C , heated to 177.5°C at a $5^\circ\text{C}\cdot\text{min}^{-1}$ rate and then heated to 230°C at $10^\circ\text{C}\cdot\text{min}^{-1}$, which was held for 15 minutes. A calibration curve was obtained by injecting standards with several concentrations of butanol and a fixed concentration of

15 internal standard ($0.5 \text{ g}\cdot\text{L}^{-1}$ of isobutanol). Butanol concentration was calculated by comparing the ratio between its peak area and internal standard peak area with calibration curves.

All cell optical density measurements at 600 nm (OD_{600}) were performed using the spectrophotometer Ultrospec 10 from Biochrom (Cambridge, UK).

25 Example 1:

Preparation of a n-butanol Producing Microbial Organism Having a Pathway coupling the enzymes Glutaryl-CoA dehydrogenase and *trans*-2-enoyl-CoA reductase in complex medium.

This example describes the generation of a microbial organism capable of producing n-butanol from 2-oxoglutarate in complex medium. *Escherichia coli* is used as target organism to engineer the butanol pathway shown in Fig. 1., where glutaryl-CoA dehydrogenase activity was coupled to enzymes activities of 2-hydroxyglutarate dehydrogenase, glutaconate-CoA transferase, 2-hydroxyglutaryl-CoA dehydratase, *trans*-2-enoyl-CoA reductase, aldehyde dehydrogenase and alcohol dehydrogenase. The resulting genetically engineered strains of *E.*

30

coli, BUT_OXG1 and BUT_OXG2, were used for butanol production by cultivation in Terrific Broth (TB) medium. Butanol production 96h after inoculation is shown in Table 10.

Table 10: Butanol production in TB medium 96 h after inoculation.

Butanol (mg.L ⁻¹)		
IPTG (mmol.L ⁻¹)	BUT_OXG1	BUT_OXG2
1	4.5 ± 0.1	16.07 ± 2.3
0.5	6.8 ± 0.46	24.05 ± 4.6
0.1	3.2 ± 0.05	7.25 ± 0.8

5

Example 2:

Preparation of a Producing Microbial Organism Having a Pathway coupling the enzymes glutaryl-CoA dehydrogenase and *trans*-2-enoyl-CoA reductase capable to produce n-butanol from 2-oxoglutarate in defined medium.

- 10 This example describes the generation of a microbial organism capable of producing butanol from 2-oxoglutarate in a defined medium. *Escherichia coli* is used as target organism to engineer the butanol pathway shown in Fig. 1., where glutaryl-CoA dehydrogenase activity was coupled to enzymes activities of 2-hydroxyglutarate dehydrogenase, glutaconate-CoA transferase, 2-hydroxyglutaryl-CoA dehydratase, *trans*-2-enoyl-CoA reductase, aldehyde
- 15 dehydrogenase and alcohol dehydrogenase. Butanol production 96h after inoculation is shown in Table 11.

Table 11: Butanol production in defined medium 96 h after inoculation.

Butanol (mg.L ⁻¹)		
IPTG (mmol.L ⁻¹)	BUT_OXG1	BUT_OXG2
1	7.0 ± 0.59	59.95 ± 6.14
0.5	29.04 ± 1.72	75.32 ± 4.21
0.1	5.6 ± 0.05	20.96 ± 2.86

Example 3:

Negative control for the n-butanol Producing Microbial Organism Having a Pathway where glutaryl-CoA dehydrogenase activity was coupled only to enzymes activities of 2-hydroxyglutaryl-CoA dehydratase, *trans*-2-enoyl-CoA reductase, alcohol dehydrogenase and aldehyde dehydrogenase.

This example describes the generation of a microbial organism incapable of producing butanol from 2-oxoglutarate. This example is considered as negative control since the absence of coupled enzymes will lead to an n-butanol unproductive microbial organism. Butanol production 96h after inoculation is shown in Table 12. The method detection limit is 3 mg.L⁻¹.

Table 12: Butanol production in TB and defined medium 96 h after inoculation from a strain lacking *hgdH* and *gctAB*. n.d.: not detectable.

Strain	Butanol final titer (mg.L ⁻¹)	
	TB	HDM
Ct_OXG1	n.d.	n.d.
Ct_OXG2	n.d.	n.d.

Example 4:

Preparation of a n-butanol producing Microbial Organism Having a Pathway where glutaryl-CoA dehydrogenase activity was coupled to enzymes activities of 2-hydroxyglutaryl-CoA dehydratase, *trans*-2-enoyl-CoA reductase, alcohol dehydrogenase and aldehyde dehydrogenase in aerobic conditions.

This example describes the generation of a microbial organism incapable of producing butanol from 2-oxoglutarate. This example is considered as negative control since the absence of anaerobic conditions will lead to an n-butanol unproductive microbial organism. Butanol production 96h after inoculation is shown in Table 13. The method detection limit is 3 mg.L⁻¹.

Table 13: Butanol production under aerobic conditions in TB and HDM medium 96 h after inoculation. n.d.: not detectable.

Strain	Butanol final titer (mg.L ⁻¹)	
	TB	HDM
BUT_OXG1	n.d.	n.d.
BUT_OXG2	n.d.	n.d.

Example 5:

5 **Optimizing the n-butanol production**

Three factors were changed to increase n-butanol production.

In the first task, the switch to serum bottles was delayed by 4 and 12 h after IPTG induction. By doing so, the butanol titer was increased by 1.6-fold (to 129±8 mg.L⁻¹ for 12h delay).

Secondly, alcohol dehydrogenase 1 (*adhE1*) was replaced by alcohol dehydrogenase 2
 10 (*adhE2*). Reportedly, the protein-product of *adhE2* has more activity in *E. coli*. A codon-optimized sequence of the *adhE2* gene (WT: SEQ NO 14, codon-optimized: SEQ NO 15) and of the gene encoding the *trans*-2-enoyl-reductase (*ter*) (SEQ NO 16) were cloned and expressed in *E. coli* obtaining BUT_OXG3 strain. These two genes were the only ones that
 15 were not codon-optimized in the previous engineered strains. The maximum butanol titer obtained in the experiments with the codon-optimized strains was 172±2 mg.L⁻¹ (with a switch to serum bottles 12 h after IPTG induction).

Thirdly, the medium was supplemented with extra glutamate (2 g.L⁻¹) at the anaerobic switch
 20 moment. The conditions of this last experiment were the following: the working volume was reduced from 60 mL to 40 mL and the switch to anaerobic conditions was 4 h after the IPTG induction. The maximum butanol titer obtained in this experiment was 187±2 mg.L⁻¹.

Claims

1. A method for production of n-butanol, wherein a transgenic cell heterologously expressing each of the following enzymes:
 - a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
 - 5 b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
 - c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
 - d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
 - e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.); and
 - 10 f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

is grown in a medium comprising a metabolic precursor of 2-oxoglutarate.
2. The method according to claim 1, wherein n-butanol is extracted from said medium.
3. The method according to claim 1 or 2, wherein said metabolic precursor of 2-oxoglutarate is selected from glucose, glycerol, glutamate or acetate.
- 15 4. The method according to any one of claims 1 to 3, wherein the transgenic cell is a bacterium or a yeast cell.
5. The method according to claim 4, wherein the bacterium or the yeast cell is selected from genera *Escherichia*, *Corynebacterium*, *Ralstonia*, *Clostridium*, *Pseudomonas*, *Lactobacillus*, *Lactococcus*, *Acidaminococcus*, *Fusobacterium*, *Peptoniphilus*, *Saccharomyces*, *Streptomyces*, *Lactobacillus*, *Pichia*, *Kluyveromyces*, *Yarrowia*, or *Staphylococci*, particularly *Escherichia coli*.
- 20 6. The method according to any one of the preceding claims, wherein
 - a. the protein *hgdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdH* of *Acidaminococcus fermentans*, more particularly *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 1 and has a catalytic activity of at least 75% of the activity of SEQ NO 1 and/or
 - 25 b. the protein *gctAB* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gctAB* of *Acidaminococcus fermentans*, more particularly subunit A
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of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 2 and has a catalytic activity of at least 75% of the activity of SEQ NO 2 and/or subunit B of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 3 and has a catalytic activity of at least 75% of the activity of SEQ NO 3 and/or

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c. the A subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdA* of *Clostridium symbiosum*, more particularly *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 4 and has a catalytic activity of at least 75% of the activity of SEQ NO 4 and/or

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d. the B subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdB* of *Clostridium symbiosum*, more particularly *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 5 and has a catalytic activity of at least 75% of the activity of SEQ NO 5 and/or

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e. the C subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdC* of *Acidaminococcus fermentans*, more particularly *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 6 and has a catalytic activity of at least 75% of the activity of SEQ NO 6 and/or

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f. the protein *gcdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gcdH* of *Pseudomonas aeruginosa*, more particularly *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 7 and has a catalytic activity of at least 75% of the activity of SEQ NO 7 and/or

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g. the protein *ter* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *ter* of *Treponema denticola*, more particularly *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 8 and has a catalytic activity of at least 75% of the activity of SEQ NO 8 and/or

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- 5 h. the protein *adhE1* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE1* of *Clostridium acetobutylicum*, more particularly *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 9 and has a catalytic activity of at least 75% of the activity of SEQ NO 9 and/or
- 10 i. the protein *adhE2* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE2* of *Clostridium acetobutylicum*, more particularly *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 13 and has a catalytic activity of at least 75% of the activity of SEQ NO 13.
7. The method according to any one of the preceding claims, wherein said transgenic cell comprises one or more plasmids encoding said heterologously expressed
15 enzymes under control of a promoter sequence operable in said cell, particularly a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.
8. The method according to any one of the preceding claims, wherein said fermentation step is performed under anaerobic conditions at 25 to 37°C, particularly at 30°C.
9. The method according to any one of the preceding claims, wherein the medium
20 comprises 8-12 g.L⁻¹ glucose, 8-10 g.L⁻¹ dibasic sodium phosphate dihydrate, 6-8 g.L⁻¹ monobasic potassium phosphate, 0.5-0.7 g.L⁻¹ sodium chloride, 1.2-1.5 g.L⁻¹ magnesium sulphate, 0.03-0.05 g.L⁻¹ calcium chloride dihydrate, 0.8-1.2 g.L⁻¹ ammonium chloride, and 8-12 mmol.L⁻¹ sodium bicarbonate, 0.1-0.15 µg.L⁻¹ selenium, 0.08-0.12 µg.L⁻¹ nickel, 0.7-0.9 µg.L⁻¹ molybdenum, ampicillin, spectinomycin, and
25 kanamycin and neutral pH, particularly pH 6.8 - 7.3.
10. The method according to any one of the preceding claims 7 to 9, wherein said plasmid comprises
- a. a lac, tac or T7 promoter, and the expression of said heterologous genes is induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranosid) to the medium,
30 particularly 0.1-1 mmol.L⁻¹ IPTG, more particularly 0.5 mmol.L⁻¹ IPTG;
- b. a trp promoter, and the expression of heterologous genes is induced by adding 3-b-indoleacrylic acid to the medium, at concentrations ranging from 10 µg.mL⁻¹ to 100 µg/m.L⁻¹;
- c. a λP_L promoter, and the expression of heterologous genes is induced by
35 increasing the temperature to 42 °C.

11. A transgenic cell, wherein the following enzymes are expressed:

- a. 2-hydroxyglutarate dehydrogenase *hgdH* (EC 1.1.99.2.);
- b. glutaconate-CoA transferase *gctAB* (EC 2.8.3.12);
- c. (R)-2-hydroxyglutaryl-CoA dehydratase subunits A, B and C *hgdABC* (EC 4.2.1.167);
- d. glutaryl CoA dehydrogenase *gcdH* (EC 1.3.8.6.);
- e. *trans*-2-enoyl-CoA reductase (NAD⁺) *ter* (EC 1.3.1.44.); and
- f. a bifunctional aldehyde / alcohol dehydrogenase (NAD⁺) selected from *adhE1* and *adhE2* (EC 1.1.1.11 / 1.2.1.3.);

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wherein at least 4 enzymes are expressed heterologously, particularly 5 or 6 enzymes are expressed heterologously.

12. The cell according to claim 11, wherein the cell is selected from genera *Escherichia*, *Corynebacterium*, *Ralstonia*, *Clostridium*, *Pseudomonas*, *Lactobacillus*, *Lactococcus*, *Acidaminococcus*, *Fusobacterium*, *Peptoniphilus*, *Saccharomyces*, *Streptomyces*, *Lactobacillus*, *Pichia*, *Kluyveromyces*, *Yarrowia*, or *Staphylococci*, particularly *Escherichia coli*.

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13. The cell according to claim 11 or 12, wherein

- a. the protein *hgdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdH* of *Acidaminococcus fermentans*, more particularly *hgdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 1 and has a catalytic activity of at least 75% of the activity of SEQ NO 1 and/or
- b. the protein *gctAB* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gctAB* of *Acidaminococcus fermentans*, more particularly *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 2 and has a catalytic activity of at least 75% of the activity of SEQ NO 2 and/or subunit B of *gctAB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 3 and has a catalytic activity of at least 75% of the activity of SEQ NO 3 and/or

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- 5 c. the A subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdA* of *Clostridium symbiosum*, more particularly *hgdA* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 4 and has a catalytic activity of at least 75% of the activity of SEQ NO 4 and/or
- 10 d. the B subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdB* of *Clostridium symbiosum*, more particularly *hgdB* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 5 and has a catalytic activity of at least 75% of the activity of SEQ NO 5 and/or
- 15 e. the C subunit of the protein *hgd* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *hgdC* of *Acidaminococcus fermentans*, more particularly *hgdC* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 6 and has a catalytic activity of at least 75% of the activity of SEQ NO 6 and/or
- 20 f. the protein *gcdH* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *gcdH* of *Pseudomonas aeruginosa*, more particularly *gcdH* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO
- 25 7 and has a catalytic activity of at least 75% of the activity of SEQ NO 7 and/or
- 30 g. the protein *ter* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *ter* of *Treponema denticola*, more particularly *ter* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 8 and has a catalytic activity of at least 75% of the activity of SEQ NO 8 and/or
- 35 h. the protein *adhE1* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE1* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE1* of *Clostridium acetobutylicum*, more particularly *adhE1* is at

least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 9 and has a catalytic activity of at least 75% of the activity of SEQ NO 9

- 5 i. the protein *adhE2* is encoded by a gene derived from a strictly or facultatively anaerobic bacterium, particularly said *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical, with respect to its amino acid sequence, to *adhE2* of *Clostridium acetobutylicum*, more particularly *adhE2* is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or >95% identical to SEQ NO 13 and has a catalytic activity of at least 75% of the activity of SEQ NO 13.

- 10 14. The cell according to any one of the claims 11 to 13, wherein said cell comprises the sequences for said heterologously expressed enzymes under control of a promoter sequence operable in said cell, particularly a T7 promoter, a lac promoter, a trp promoter, a tac promoter or a λP_L promoter.

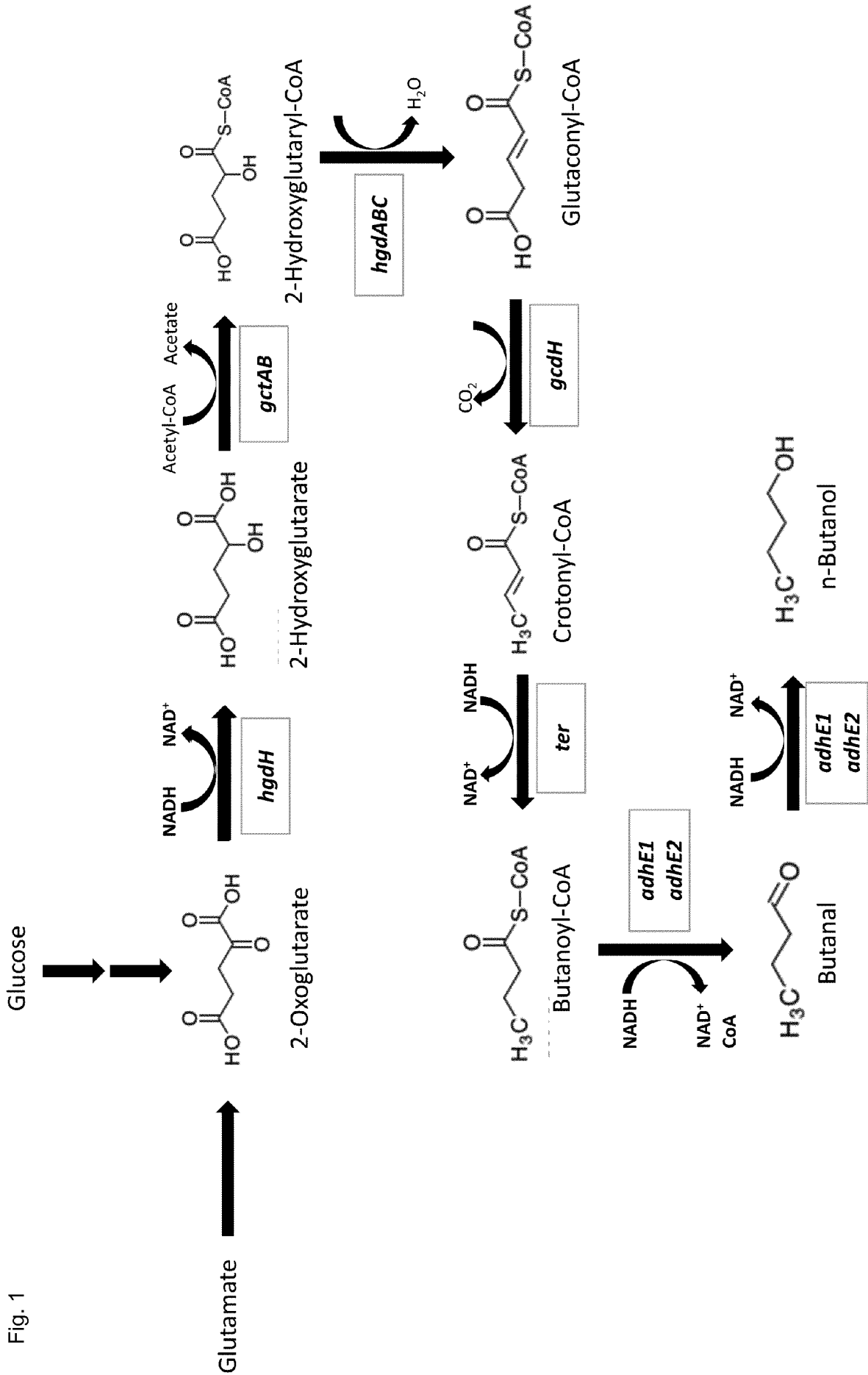


Fig. 2

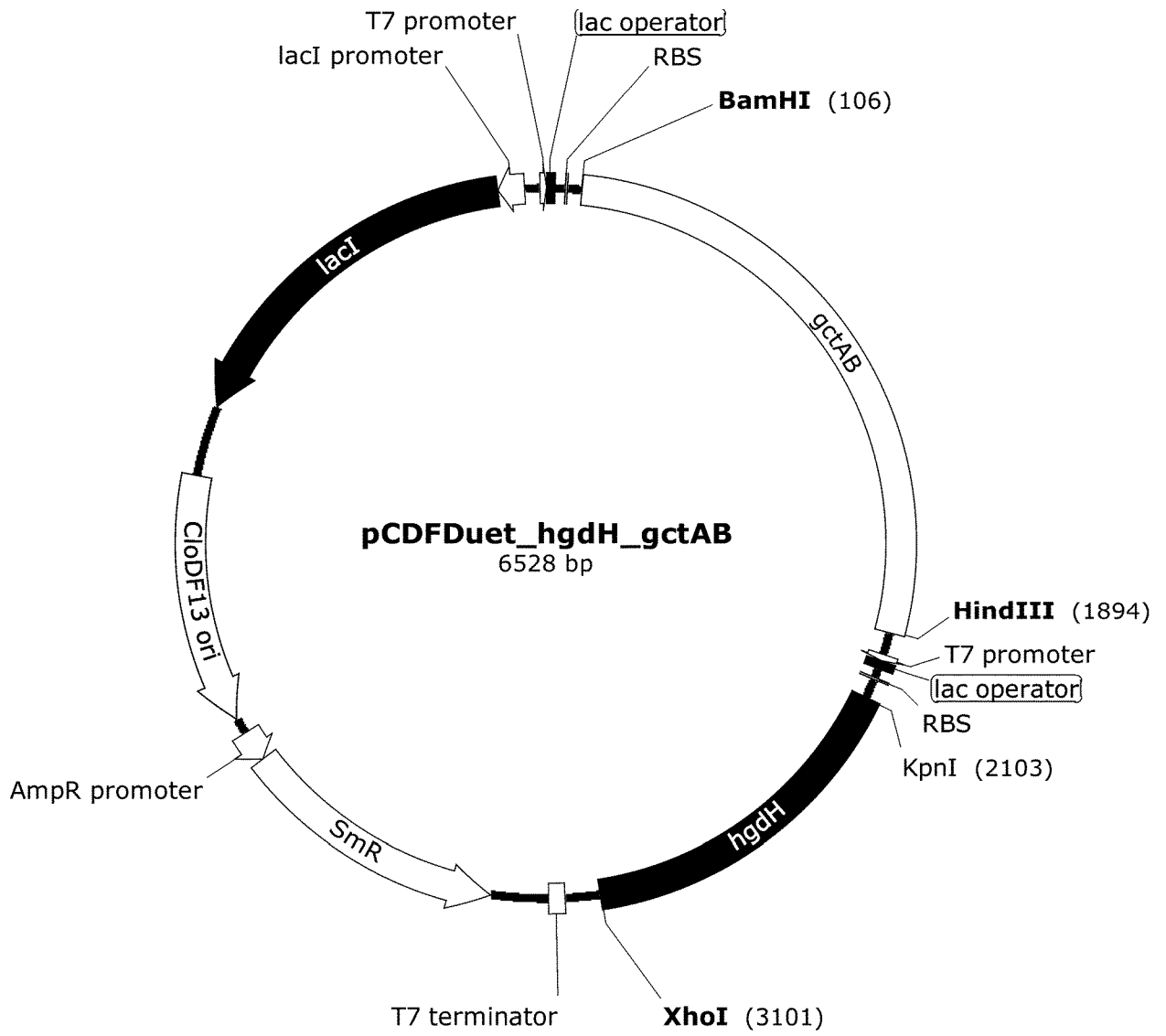


Fig. 3

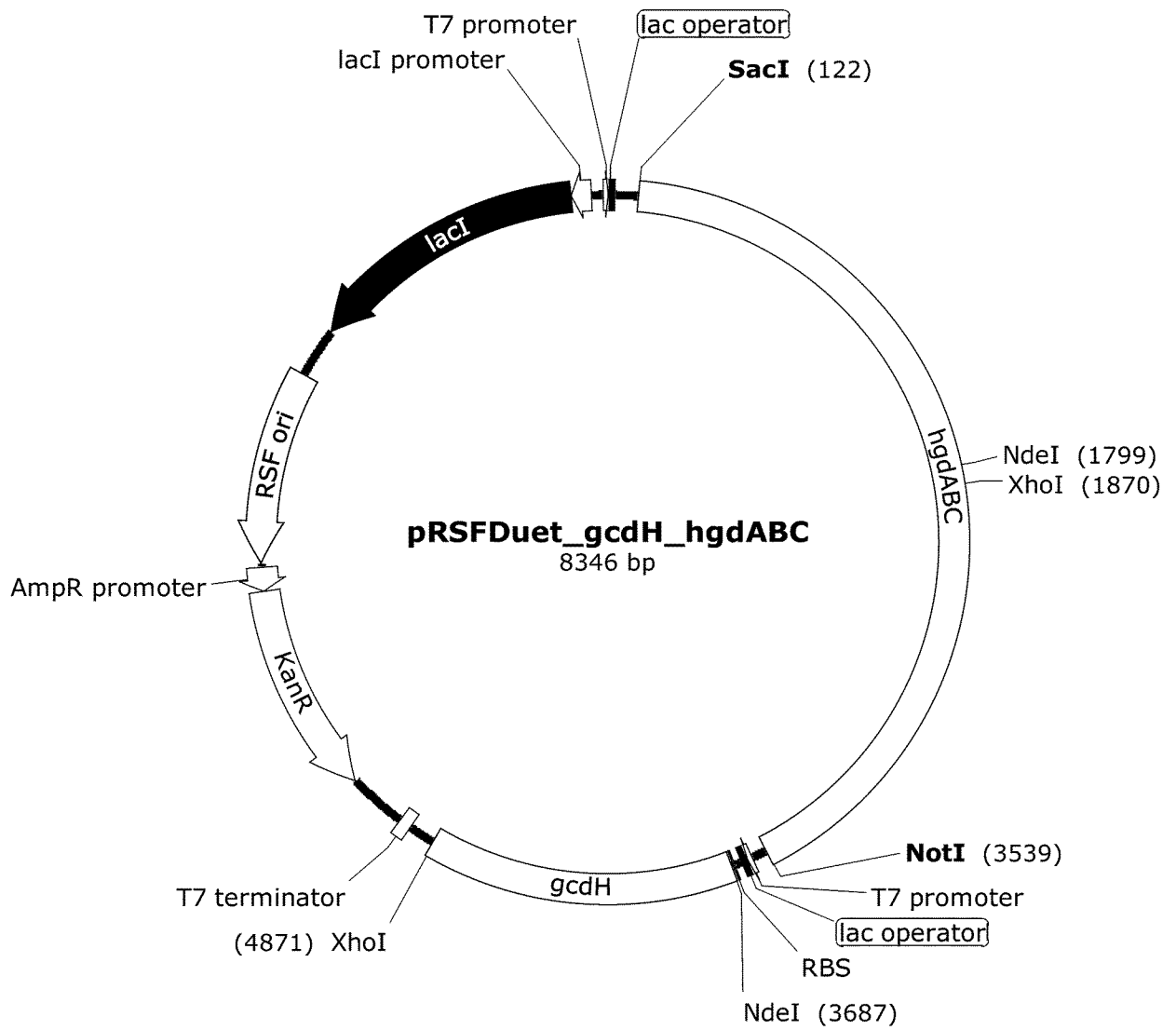


Fig. 4

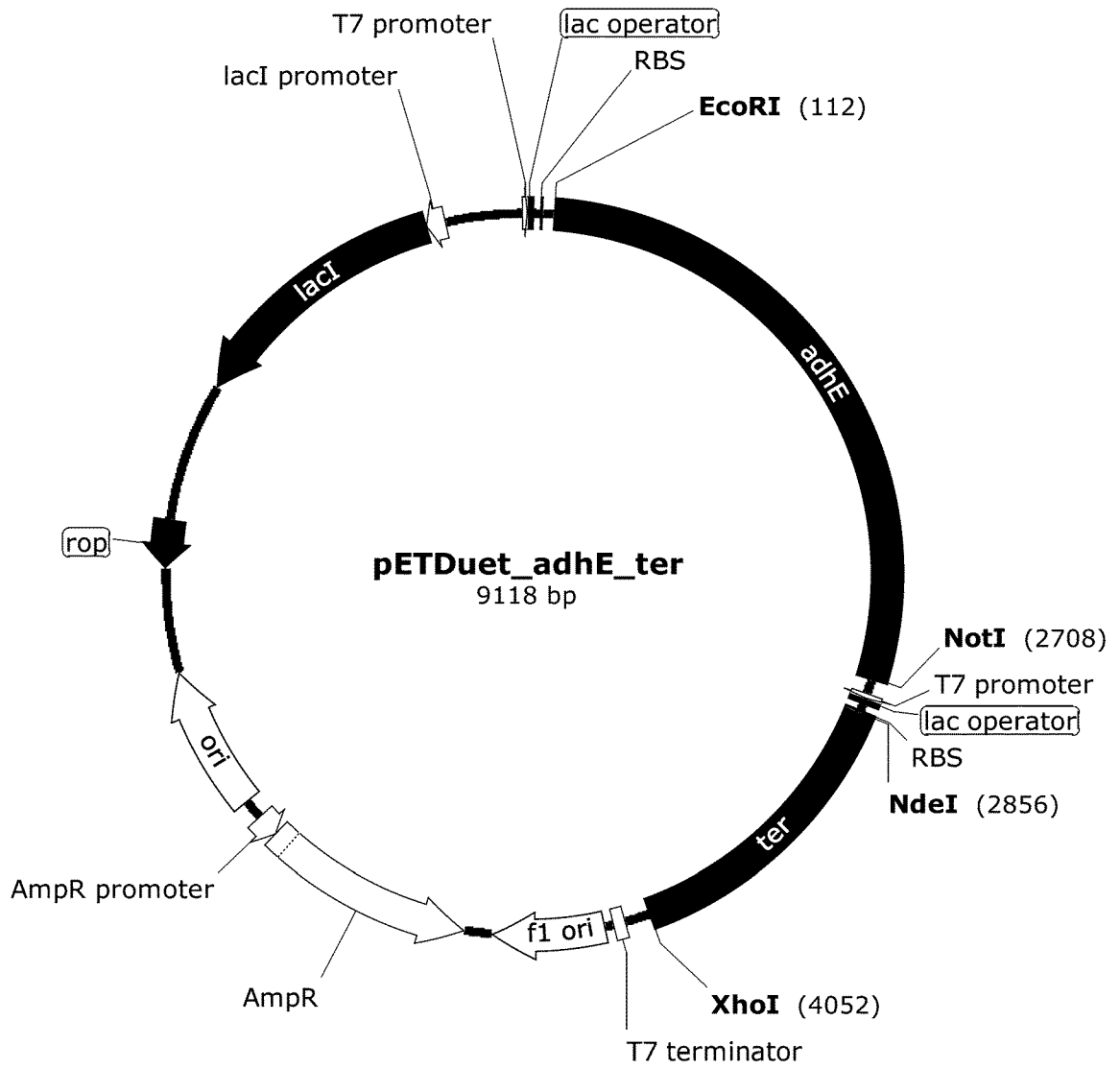
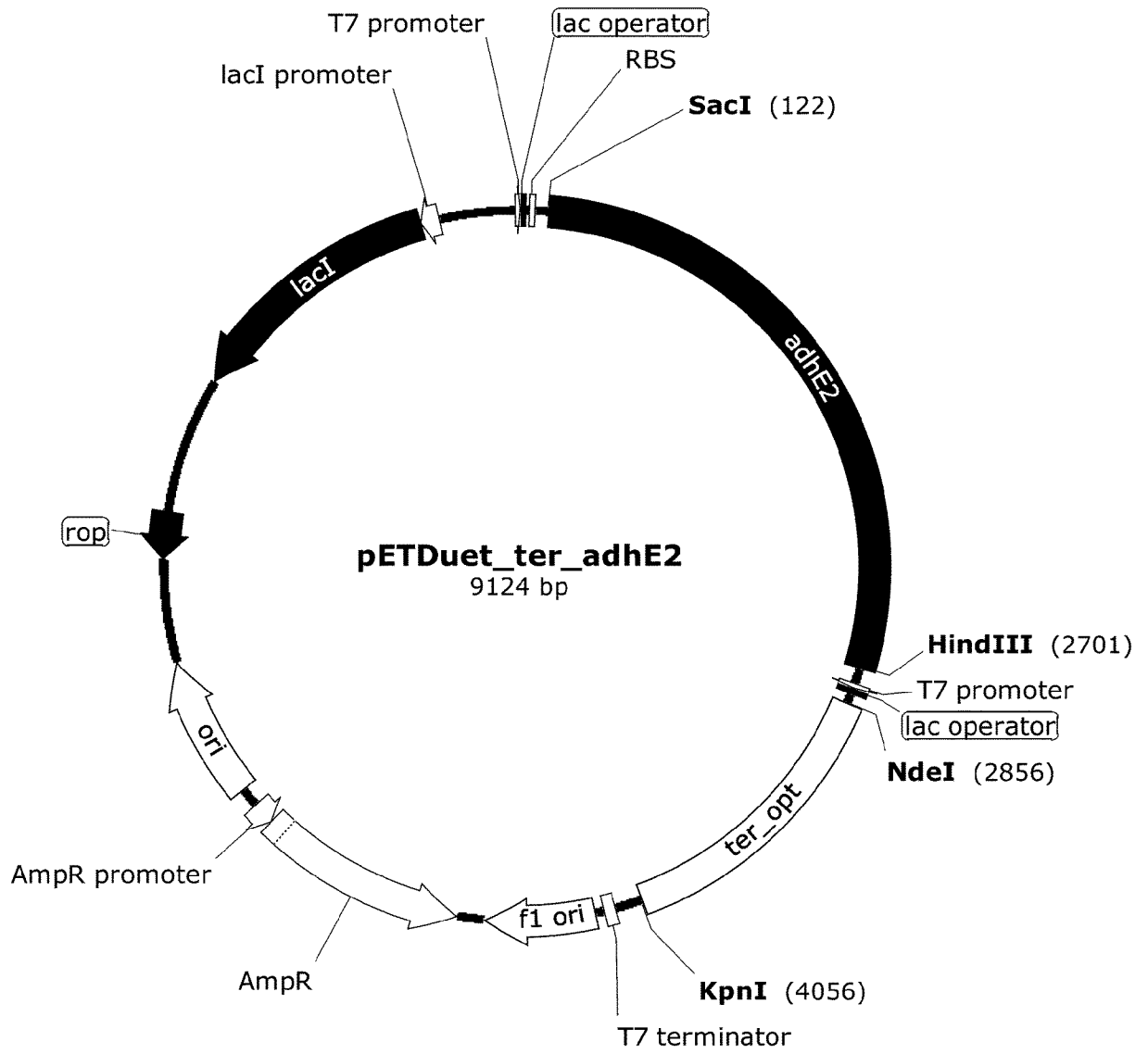


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/057944

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/16
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12P
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/138977 A2 (OHIO STATE INNOVATION FOUNDATION [US]) 17 September 2015 (2015-09-17) abstract page 1, lines 24-27 page 2, line 31 - page 3, line 10 example 1	1-14
A	----- SCHADEWEG VIRGINIA ET AL: "Increasing n-butanol production with Saccharomyces cerevisiae by optimizing acetyl-CoA synthesis, NADH levels and trans-2-enoyl-CoA reductase expression.", BIOTECHNOLOGY FOR BIOFUELS 2016, vol. 9, 2016, page 257, XP002787788, abstract page 4 ----- -/--	1-14

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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- "E" earlier application or patent but published on or after the international filing date
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Date of the actual completion of the international search 13 May 2019	Date of mailing of the international search report 24/05/2019
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Chavanne, Franz

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/057944

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MINGRUI YU ET AL: "Effects of different replicons in conjugative plasmids on transformation efficiency, plasmid stability, gene expression and n-butanol biosynthesis in", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 93, no. 2, 4 December 2011 (2011-12-04), pages 881-889, XP035001292, ISSN: 1432-0614, DOI: 10.1007/S00253-011-3736-Y abstract page 883 pages 885-887</p> <p style="text-align: center;">-----</p>	1-14
A	<p>LODER ANDREW J ET AL: "Alcohol Selectivity in a Synthetic Thermophilic n-Butanol Pathway Is Driven by Biocatalytic and Thermostability Characteristics of Constituent Enzymes", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 81, no. 20, October 2015 (2015-10), pages 7187-7200, XP002787789, abstract page 7189 page 7193 table 1</p> <p style="text-align: center;">-----</p>	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/057944

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015138977	A2	NONE	17-09-2015