

MICROORGANISMS WITH IMPROVED 1,3-PROPANEDIOL AND BUTYRIC ACID PRODUCTION

5 The present invention concerns a new mutant strain of *Clostridium acetobutylicum* able to produce 1,3-propanediol and comprising attenuated glycerol kinase activity. In addition, the present invention concerns a consortium of *Clostridium* species comprising at least said mutant strain and at least one other *Clostridia* chosen among *C. sporogenes* and *C. sphenoides*. This modified strain may be adapted for growth and for the production of 1,3-propanediol in an appropriate culture medium with high glycerol content. The invention
10 also relates to a method for the production of 1,3-propanediol and butyric acid, by culturing at least this mutant strain in an appropriate culture medium.

BACKGROUND OF THE INVENTION

15 1,3-propanediol (PDO), also called trimethylene glycol or propylene glycol, is one of the oldest known fermentation products. It was originally identified as early as 1881 by August Freund in a glycerine fermented culture containing *Clostridium pasteurianum*. PDO is a typical product of glycerine fermentation and has been found in anaerobic conversions of other organic substrates. Only very few organisms, all of them bacteria,
20 are able to form it. They include bacteria of the genera *Klebsiella* (*K. pneumoniae*), *Enterobacter* (*E. agglomerans*), *Citrobacter* (*C. freundii*), *Lactobacilli* (*L. brevis* and *L. buchneri*) and *Clostridia* of the *C. butyricum* and the *C. pasteurianum* group.

PDO, as a bifunctional organic compound, may potentially be used for many synthesis reactions, in particular as a monomer for polycondensations to produce
25 polyesters, polyethers, polyurethanes, and in particular, polytrimethylene terephthalate (PTT). These structure and reactivity features lead to several applications in cosmetics, textiles (clothing fibers or flooring) or plastics (car industry and packing or coating).

PDO can be produced by different chemical routes but they generate waste stream containing extremely polluting substances and the cost of production is high. Thus,
30 chemically produced PDO cannot compete with the petrochemically available diols like 1,2-ethanediol, 1,2-propanediol and 1,4-butanediol. To increase this competitiveness, in 1995, DuPont started a research program for the biological conversion of glucose to PDO. Although this process is environmentally friendly it has the disadvantage to i) use vitamin B12, a very expensive cofactor and ii) be a discontinuous process due to the instability of
35 the producing strain.

Due to the availability of large amounts of glycerol produced by the bio-diesel industry, a continuous, vitamin-B12-free process with a higher carbon yield would on the contrary, be advantageous.

It is known in the art that PDO can be produced from glycerine, an unwanted by-product in particular from the biodiesel production that contains roughly 80-85% of glycerol mixed with salts and water.

5 *C. butyricum* was previously described as being able to grow and produce PDO from glycerol contained in industrial glycerine in batch and two-stage continuous fermentation (Papanikolaou *et al.*, 2000). However, at the highest glycerol concentration, the maximal PDO titer obtained was 48.1 g.L^{-1} at a dilution rate of 0.02 h^{-1} , meaning a productivity of $0.96 \text{ g.L}^{-1}.\text{h}^{-1}$. The cultures were conducted with a maximum concentration of glycerol originating from glycerine in the fed medium of 90 g.L^{-1} and in the presence of
10 yeast extract, a costly compound containing organic nitrogen that is known by the man skilled in the art to help increase bacterial biomass production.

Application WO 2006/128381 discloses the use of this glycerol for the production of PDO with batch and fed-batch cultures using natural PDO producing organisms such as *Klebsiella pneumoniae*, *C. butyricum* or *C. pasteurianum*. Furthermore, the medium
15 used in WO 2006/128381 also contains yeast extract. As described in this patent application, the maximal productivity reached was comprised between 0.8 and $1.1 \text{ g.L}^{-1}.\text{h}^{-1}$.

The performance of a *C. acetobutylicum* strain modified to contain the vitamin B12-independent glycerine-dehydratase and the PDO-dehydrogenase from *C. butyricum*, called "*C. acetobutylicum* DG1 pSPD5" strain has been described in Gonzalez-Pajuelo *et al.*, 2005. This strain originally grows and produces PDO in a fed medium containing up to 120 g.L^{-1} of pure glycerol. In addition, analyses in a fed medium containing a maximum of 60 g.L^{-1} of pure glycerol or glycerol contained in industrial glycerine did not point out to any differences. These results have been obtained in presence of yeast extract. Moreover, no test was performed with concentrations of glycerol originating from industrial glycerine
20 higher than 60 g.L^{-1} . When comparing a wild-type *C. butyricum* to the modified microorganism "*C. acetobutylicum* DG1 pSPD5", a globally similar behaviour was observed.

In patent application WO 2010/128070 the inventors described a process to adapt the strain of *C. acetobutylicum* DG1 pSPD5 such as described in Gonzalez-Pajuelo *et al.*
30 (2005) to grow in a medium with a high concentration of glycerol contained in industrial glycerine and without yeast extract. The resulting population of *C. acetobutylicum* DG1 pSPD5 adapted strains was able to produce PDO in medium containing up to 120 g.L^{-1} of glycerol contained in industrial glycerine with a titer up to 53.5 g.L^{-1} of PDO, a yield up to 0.53 g.g^{-1} and productivity up to $2.86 \text{ g.L}^{-1}.\text{h}^{-1}$. In patent application WO 2012/062832, the
35 inventors described the isolation of clone from another population of *C. acetobutylicum* DG1 pSPD5 adapted strains obtained by the same process as described in WO 2010/128070 patent application. This second population was able to produce PDO in medium containing around 105 g.L^{-1} of glycerol contained in industrial glycerine with a titer

up to 50.45 g/L⁻¹ of PDO, a yield up to 0.53 g.g⁻¹ and productivity up to 3.18 g.L⁻¹.h⁻¹ whereas isolated clone was able to produce PDO in medium containing around 105 g.L⁻¹ of glycerol contained in industrial glycerine with a titer up to 51.30 g/L⁻¹ of PDO, a yield up to 0.50 g.g⁻¹ and productivity up to 3.05 g.L⁻¹.h⁻¹.

5 Butyrate (also named Butyric Acid or butanoic acid, abbreviated BA) is a carboxylic acid with the structural formula CH₃CH₂CH₂-COOH, which is volatile, colourless, oily liquid with an unpleasant odour, miscible in both water and solvents, such as ethanol and ether. Butyrate is used for a variety of applications including i) the fabrication of other chemicals, ii) as feed additive in animal nutrition iii) as a food flavouring, or food supplement, and iv) 10 in pharmaceutical and cosmetic compositions. In particular, butyrate may be used as a feedstock in the production of thermoplastic polymers, such as cellulose acetate butyrate, one of the most commercially important mixed esters. Indeed, cellulose acetate butyrate is often present in paints and polymers. Butyrate is also a precursor to butanol, a biofuel and possible gasoline replacement for internal combustion.

15 Currently, production of butyrate on an industrial scale is mainly performed using chemical synthesis, for example via the oxidation of n-butyl alcohol or butyraldehyde with oxygen or organic oxidants. Alternatively, butyrate may be produced by extraction from butter, which contains an estimated 2-4% of butyrate, though this method is costly and difficult. Butyrate may also be produced as a metabolite in *Clostridium* strains during 20 fermentation under anaerobic conditions. More specifically, butyrate may be produced as an acetate/butyrate mixture, which is an intermediate in biphasic acetone/n-butanol/ethanol (ABE) fermentation. Production of acetate/butyrate occurs in the first acidogenic phase under high growth rate conditions, and is then consumed in the second solventogenic phase, in which growth rate decreases and ABE solvents are produced. 25 Carbon dioxide and hydrogen are produced throughout the fermentation process.

In the present patent application, the inventors have noticed that a strain of *Clostridium acetobutylicum* DG1 pSPD5 able to produce PDO in presence of glycerol mutated for having an attenuated glycerol kinase activity provides an improved production of PDO and BA compared to the performances of a non-mutated producer strain. Further 30 improvement of production of PDO and BA may be obtained when the mutant strain according to the invention is further carrying an attenuated hydrogenase activity and/or an enhanced glycerol uptake facilitator permease activity.

Thus, using the mutant strain of *Clostridium* according to the invention conducts to an improved production of PDO and BA, compared with using a non-mutated *C. acetobutylicum* DG1 pSPD5 strain just adapted for producing PDO in presence of 35 glycerol.

BRIEF DESCRIPTION OF THE INVENTION

The present invention concerns a mutant strain of *Clostridium acetobutylicum* expressing the *dhaB1*, *dhaB2* and *dhaT* genes from *C. butyricum* and comprising attenuated glycerol kinase activity.

Particularly, in the mutant stain according to the present invention, the glycerol kinase whose activity is attenuated is encoded by the gene *glpK* at locus CA_C1321 in the *C. acetobutylicum* genome ATCC824.

In a particular embodiment of the invention, the mutant strain according to the invention comprises at least one of the following mutations in the gene *glpK* at locus CA_C1321:

- a. Mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine,
- b. Mutation of the codon at nucleotide positions 1461486 to 1461488 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid histidine in position 22 to be replaced with tyrosine,
- c. Mutation of the codon at nucleotide positions 1462818 to 1462820 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid valine in position 466 to be replaced by methionine

In a preferred embodiment of the invention, the mutant strain according to the invention comprises the combination of the three mutations.

The mutant strain of the invention may also further comprise attenuated hydrogen dehydrogenase activity, preferably inactivated hydrogen dehydrogenase activity. In a particular embodiment of the invention, said hydrogenase is encoded by the gene *hydA* at locus CA_C0028 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

The mutant strain of the invention may also further comprise enhanced glycerol uptake facilitator permease activity. In a particular embodiment of the invention, said glycerol uptake facilitator protein is encoded by the gene *glpF* at locus CA_C0770 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

The mutant strain of the invention may also further comprise attenuated electron transfer flavoprotein subunit alpha activity. In a particular embodiment of the invention, said electron transfer flavoprotein subunit alpha is encoded by the gene *etfA* at locus CA_C2709 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

The present invention also concerns a consortium of *Clostridia species* comprising the mutant strain of *C. acetobutylicum* according to the invention as disclosed above.

In a particular embodiment of the invention, this consortium comprises the mutant strain *Clostridium acetobutylicum* as defined above and at least one strain of *Clostridium sporogenes* and/or at least one strain of *Clostridium sphenoides*.

The present invention also concerns a method for the production of PDO and BA comprising culturing the mutant strain *Clostridium acetobutylicum* or a consortium comprising said mutant strain as defined above, in an appropriate culture medium comprising at least glycerine and recovering the PDO and/or BA produced from the culture medium.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention concerns a mutant strain of *Clostridium acetobutylicum* able to produce PDO and comprising attenuated glycerol kinase activity, compared to a non-mutated strain. In the present invention, a glycerol kinase is an enzyme also named ATP:glycerol 3-phosphotransferase or glycerokinase GK.

Particularly, in the mutant strain according to the present invention, the glycerol kinase (SEQ ID NO: 1) whose activity is attenuated is encoded by the gene *glpK* at locus CA_C1321 (SEQ ID NO: 2) in the *C. acetobutylicum* genome ATCC824. In the mutant strain according to the present invention, the glycerol kinase activity is attenuated compared to a non-mutated strain, in particular compared to a strain in which the gene *glpK* is not mutated.

As mentioned herein, the *C. acetobutylicum* genome ATCC824 corresponds to the 3940880 bp circular DNA available under the access number Genbank AE001437 (AE007513-AE007868), version AE001437.1 as *Clostridium acetobutylicum* ATCC 824, complete genome.

In a particular embodiment of the invention, the mutant strain according to the invention comprises at least one of the following mutations in the gene *glpK* at locus CA_C1321:

- a. Mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine, leading to a mutated protein with SEQ ID NO: 3 and mutated gene with SEQ ID NO: 4,
- b. Mutation of the codon at nucleotide positions 1461486 to 1461488 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid histidine in position 22 to be replaced with tyrosine, leading to a mutated protein with SEQ ID NO: 5 and mutated gene with SEQ ID NO: 6,

- c. Mutation of the codon at nucleotide positions 1462818 to 1462820 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid valine in position 466 to be replaced by methionine, leading to a mutated protein with SEQ ID NO: 7 and mutated gene with SEQ ID NO: 8.

5 In an advantageous embodiment of the present invention, the mutant strain comprises at locus CA_C1321, at least mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine (SEQ ID NO: 3 and SEQ ID NO: 4).

10 In a preferred embodiment of the present invention, the mutant strain comprises at locus CA_C1321, at least:

- (i) mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine, and
- 15 (ii) mutation of the codon at nucleotide positions 1461486 to 1461488 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid histidine in position 22 is to be replaced by with tyrosine, leading to the mutated protein with SEQ ID NO: 9 and to the mutated gene with SEQ ID NO: 10.

20 In another preferred embodiment of the present invention, the mutant strain comprises at locus CA_C1321, at least:

- (i) mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine, and
- 25 (ii) mutation of the codon at nucleotide positions 1462818 to 1462820 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid valine in position 466 to be replaced by methionine, leading to the mutated protein with SEQ ID NO: 11 and to the mutated gene with SEQ ID NO: 12.

30 In another preferred embodiment of the present invention, the mutant strain comprises at locus CA_C1321, at least:

- (i) mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine, and
- 35 (ii) mutation of the codon at nucleotide positions 1461486 to 1461488 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid histidine in position 22 is to be replaced by with tyrosine, and

(iii) mutation of the codon at nucleotide positions 1462818 to 1462820 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid valine in position 466 to be replaced by methionine, leading to the mutated protein with SEQ ID NO: 13 and to the mutated gene with SEQ ID NO: 14.

In all the above-mentioned particular and preferred embodiments, each of the mutation are preferably the following:

- G replaced with A, at nucleotide position 1462461 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437,
- C replaced with T at nucleotide position 1461486 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437;
- G replaced with A at nucleotide position 1462818 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

The mutant strain of the invention may also further comprise attenuated hydrogen dehydrogenase activity, preferably inactivated hydrogen dehydrogenase activity. In a particular embodiment of the invention, said hydrogenase (SEQ ID NO: 15) is encoded by the gene *hydA* (SEQ ID NO: 16) at locus CA_C0028 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

In a preferred embodiment of the present invention, the mutant strain comprises at locus CA_C0028, at least mutation of the codon at nucleotide positions 39233 to 39235 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having a stop codon leading to a shorter protein of 209 amino acids instead of 582 amino acid of SEQ ID NO: 17, preferably by deletion of C at nucleotide position 39233 (mutated gene of SEQ ID NO: 18).

The mutant strain of the invention may also further comprise enhanced glycerol uptake facilitator permease activity. In a particular embodiment of the invention, said glycerol uptake facilitator protein (SEQ ID NO: 19) is encoded by the gene *glpF* (SEQ ID NO: 20) at locus CA_C0770 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

In a preferred embodiment of the present invention, the mutant strain comprises at locus CA_C0770, at least mutation of the codon at nucleotide positions 892875 to 892877 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid methionine in position 13 to be replaced with isoleucine (mutated protein of SEQ ID NO: 21), preferably by replacement of G at nucleotide position 892875 with A, C or T, more preferably with A (mutated gene of SEQ ID NO: 22).

The mutant strain of the invention may also further comprise attenuated electron transfer flavoprotein subunit alpha activity. In a particular embodiment of the invention, said electron transfer flavoprotein subunit alpha (SEQ ID NO: 23) is encoded by the gene

effA (SEQ ID NO: 24) at locus CA_C2709 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

In a preferred embodiment of the present invention, the mutant strain comprises at locus CA_C2709, at least mutation of the codon at nucleotide positions 2833384 to 2833386 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid isoleucine in position 105 to be replaced with valine (mutated protein of SEQ ID NO: 25), preferably by replacement of A at nucleotide position 2833384 with G (mutated gene of SEQ ID NO: 26).

The mutant strain *C. acetobutylicum* according to the present invention is modified to be able to produce 1,3-propanediol when culturing in an appropriate culture medium in presence of glycerol. This mutant *C. acetobutylicum* strain is modified to contain the 1,3-propanediol operon from *C. butyricum*: the vitamin B12-independent glycerol-dehydratase encoded by *dhaB1* (SEQ ID NO: 27) and *dhaB2* (SEQ ID NO: 28) genes and the PDO-dehydrogenase encoded by *dhaT* (SEQ ID NO: 29) gene by introducing extra copies of the 1,3-propanediol operon from *C. butyricum* either over-expressed by a plasmid or integrated into the chromosome of the microorganism. For example, the pSPD5 plasmid can be used for an over-expression of the 1,3-propanediol operon. This mutant *C. acetobutylicum* strain is called *C. acetobutylicum DG1 pSPD5* and has been fully described in Gonzalez-Pajuelo *et al.*, 2005 and in González-Pajuelo *et al.*, 2006 which are incorporated herein by reference.

Method for directing the glycerol metabolism towards production of PDO are known in the art (see for instance WO 2006/128381, González-Pajuelo *et al.*, 2006). Also as for example, strains of *C. acetobutylicum* genetically modified for the production of PDO from glycerol as sole source of carbon are known in the art and disclosed, particularly in applications WO 2001/04324 and WO 2010/128070.

In an advantageous embodiment, the mutant strain of the present invention is a strain *C. acetobutylicum* which is adapted for growth and production of PDO from a culture medium with high glycerol content and specifically with a high concentration of glycerol contained in industrial glycerine and without any organic nitrogen source.

A “*C. acetobutylicum* adapted”, “*C. acetobutylicum* previously adapted”, or “*C. acetobutylicum* being adapted” means a *C. acetobutylicum* which is modified to be able to grow on high concentration of industrial glycerine.

For example, the *C. acetobutylicum* strain may be adapted to grow in a culture medium with high glycerol content and specifically with a high concentration of glycerol originating from industrial glycerine by a selection pressure culturing process as disclosed in WO 2010/128070 patent application. The adaptation of the strain *C. acetobutylicum* is

preferably carried out by an anaerobic continuous process which is a technique well known by the skilled person. Among the particulars of this process known by the one skilled in the art, it may be for example mentioned that fed medium is added to the fermenter continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously removed from the system. The rate of nutrient exchange is expressed as the dilution rate. Hence the dilution rate is applied to the culture medium, takes into consideration maximum growth rate of the microorganism and impacts the rate of intake and withdrawal of the medium. Said adaptation of the producing microorganism is obtained by culturing the microorganism on a culture medium comprising high industrial glycerine content (comprised between 90 and 120 g/L, and preferably of about 105 g/L) at a low dilution rate (comprised between 0.005 and 0.1 h⁻¹, preferably between 0.005 and 0.02 h⁻¹), and selecting the adapted microorganism able to grow on the culture medium having high concentration of glycerol originating from industrial glycerine.

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The expression "genetically modified" means that the strain has been transformed in the aim to change its genetic characteristics. Endogenous genes can be attenuated, deleted, or over-expressed, or preferably exogenous genes can be introduced, carried by a plasmid, or integrated into the genome of the strain, to be expressed into the cell.

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The term "plasmid" or "vector" as used herein refers to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules.

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Any standard techniques of mutagenesis and/or gene replacement in *Clostridium*, such as disclosed in application WO 2008/040387 which contents are incorporated herein by reference, may be used for adapting the *C. acetobutylicum* strain for growth and production of 1,3-propanediol from a culture medium with high glycerol content and specifically with a high concentration of glycerol originating from industrial glycerine.

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The man skilled in the art knows how to manage each of these experimental conditions, and to define the culture conditions for the Clostridia strains used according to the invention. In particular Clostridia strains are fermented at a temperature between 20°C and 60°C, preferentially between 25°C and 40°C for *C. acetobutylicum*.

The activity of each enzyme mentioned herein which is modified in the mutant strain of the invention is intended to mean the specific activity.

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In addition, the activity of each enzyme which is modified in the mutant strain of the invention is defined compared to a non-mutated strain or wild-type strain of *Clostridium acetobutylicum*.

The terms “enhanced”, “expressing,” “overexpressing,” or “overexpression” of a protein of interest, such as an enzyme, refer herein to a significant increase in the expression level and/or activity of said protein in a microorganism, as compared to the parent microorganism in which the expression level and/or activity of said protein of interest is not modified, especially not genetically modified. In contrast, the terms “attenuated”, “attenuating” or “attenuation” of a protein of interest refer to a significant decrease in the expression level and/or activity of said protein in a microorganism, as compared to the parent microorganism in which the expression level and/or activity of said protein of interest is not modified, especially not genetically modified. The complete attenuation of the expression level and/or activity of a protein of interest means that expression and/or activity is abolished; thus, the expression level of said protein is null. Where the “enhanced”, “expressing,” “overexpressing,” “overexpression” “attenuated”, “attenuating” or “attenuation” is/are due to a mutation in the corresponding gene or protein of interest, the modification of the expression level and/or activity of said gene or protein of interest is compared to the expression level and/or activity of the same non-mutated gene or protein of interest.

The term “expression level”, as applied herein, refers to the amount (e.g. relative amount, concentration) of a protein of interest (or of the gene encoding said protein) expressed in a microorganism, which is measurable by methods well-known in the art, such as by Western Blot-Immunoblot (Burnette, 1981), Enzyme-linked immunosorbent assay (e.g. ELISA) (Engvall and Perlman, 1971), or quantitative proteomics (Bantscheff *et al.*, 2007).

Modulating the expression level of one or more proteins may occur by altering the expression of one or more endogenous genes that encode said protein within the microorganism.

Thus, in addition to the mutations, especially to the specific mutations disclosed above for modulating the *glpK* gene expression, the *hydA* gene expression, the *effA* gene, and/or the gene *glpF*, classical means well known by the skilled person in the art may be used for further attenuating, abolishing or overexpressing the gene expression of the above-mentioned enzyme as described above in the mutant strain of the invention.

The expression level of endogenous genes coding for enzymes having a particular activity can notably be over-expressed, attenuated, or abolished in the recombinant microorganism according to the invention. Such modifications can be performed, for example, by genetic engineering, by adaptation, wherein a microorganism is cultured in that apply a specific stress on the microorganism and induce mutagenesis, and/or by forcing the development and evolution of metabolic pathways by combining directed mutagenesis and evolution under specific selection pressure.

The term "endogenous gene" refers herein to a gene that is naturally present in a microorganism.

5 An endogenous gene can notably be overexpressed or attenuated by introducing heterologous sequences which favor upregulation or downregulation, respectively, in addition to endogenous regulatory elements. Additionally, or alternatively, endogenous regulatory elements may themselves be replaced with appropriate heterologous sequences modulating gene expression. Additionally, or alternatively, one or more supplementary copies of the endogenous gene may be introduced chromosomally (i.e. into the chromosome) or extra-chromosomally (e.g. into a plasmid or vector) within the microorganism. Thus, the microorganism comprises several copies of a gene that are homologous to one another. When one or more supplementary copies of the endogenous gene are expressed extra-chromosomally, they may be carried on different types of plasmid, as detailed above for the introduction of extra-chromosomal exogenous genes.

10 Another way to overexpress or attenuate the expression of an endogenous gene is to exchange its promoter (i.e. wild-type promoter) with a stronger or weaker promoter, respectively. Promoters suitable for such purposes may be homologous (i.e. originating from the same species) or heterologous (i.e. originating from a different species), and are well known in the art. Indeed, the skilled person can easily select an appropriate promoter for modulating the expression of an endogenous gene.

20 Endogenous gene expression levels, or the activity of the encoded protein, can also be increased or attenuated by introducing mutations into the coding sequence of a gene or into non-coding sequences. These mutations may be synonymous, when no modification in the corresponding amino acid occurs, or non-synonymous, when the corresponding amino acid is altered. Synonymous mutations do not have any impact on the function of translated proteins but may have an impact on the regulation of the corresponding genes or even of other genes, if the mutated sequence is located in a binding site for a regulator factor. Non-synonymous mutations may have an impact on the function or activity of the translated protein as well as on regulation depending the nature of the mutated sequence. In particular, mutations in non-coding sequences may be located upstream of the coding sequence (i.e. in the promoter region, in an enhancer, silencer, or insulator region, in a specific transcription factor binding site) or downstream of the coding sequence. Mutations introduced in the promoter region may be in the core promoter, proximal promoter or distal promoter. Mutations may be introduced by site-directed mutagenesis using, for example, Polymerase Chain Reaction (PCR), by random mutagenesis techniques for example via mutagenic agents (Ultra-Violet rays or chemical agents like nitrosoguanidine (NTG) or ethylmethanesulfonate (EMS)) or DNA shuffling or error-prone PCR or using culture conditions that apply a specific stress on the microorganism and induce mutagenesis. The insertion of one or more supplementary

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nucleotide in the region located upstream of a gene can notably modulate gene expression.

In a second aspect, the present invention concerns a consortium of *Clostridia* comprising the PDO and BA producing mutant strain according to the invention as disclosed above.

In a particular embodiment of the invention, this consortium comprises the mutant strain *Clostridium acetobutylicum* as defined above and at least one strain of *Clostridium sporogenes* and/or at least one strain of *Clostridium sphenoides*.

Preferably, the consortium according to the present invention comprises from 89 % to 98% of *C. acetobutylicum*, from 1 % to 10 % of *C. sporogenes*, and/or from 1 % to 10 % of *C. sphenoides* considering that the totality of the cells contained in the culture corresponds to 100 %. More preferably, the consortium according to the present invention comprises from 89 % to 95% of *C. acetobutylicum*, from 3 % to 7 % of *C. sporogenes*, and/or from 1 % to 5 % of *C. sphenoides* considering that the totality of the cells contained in the culture corresponds to 100 %.

The consortium according to the present invention preferably comprises the PDO and BA producing mutant strain *C. acetobutylicum* which is previously modified for growth and production of PDO from a culture medium in presence of glycerol. All the preferred embodiment concerning the mutant strain *C. acetobutylicum* mentioned above also apply *mutatis mutandis* to this specific embodiment.

In a third aspect, the present invention concerns a method for the production of PDO and BA comprising culturing the mutant strain *Clostridium acetobutylicum* or a consortium comprising said mutant strain as defined above, in an appropriate culture medium comprising at least glycerol and recovering the PDO and/or the BA produced from the culture medium.

In the methods of the invention, the production is advantageously done in a batch, fed-batch or continuous process. Culturing microorganisms at industrial scale for the production of PDO and BA is known in the art, particularly disclosed in WO2010/128070 and WO2011/042434, which content are incorporated herein by reference.

In the methods according to the invention, the PDO and/or BA obtained, may be further purified after recovering.

Methods for recovering and eventually purifying PDO or BA from a fermentation medium are known to the skilled person. PDO may be isolated by distillation. In most embodiments, PDO is distilled from the fermentation medium with a by-product, such as acetate, and then further purified by known methods. A particular purification method is disclosed in applications WO2009/068110 and WO 2010/037843, which content is incorporated herein by reference.

BA may be isolated by distillation as disclosed in patent application EP350355 or by liquid-liquid extraction as disclosed for instance in WO2017/013335 using solvent preferably chosen among carboxylic acid such as pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid or nonanoic acid or ketone such as methyl isobutyl ketone, methyl isoamyl ketone, methyl heptyl ketone, methyl nonyl ketone.

In a preferred embodiment of the method for the production of PDO and BA according to the present invention, glycerol concentration is from 90 to 120 g/L in the culture medium, preferably about 105 g/L.

An "appropriate culture medium" or a "culture medium" refers to a culture medium optimized for the growth of the *Clostridium* strains or consortium and the synthesis of product of interest by the cells. The fermentation process is generally conducted in reactors with a synthetic, particularly inorganic, culture medium of known defined composition adapted to the *Clostridium* species used and containing glycerol.

The term "synthetic medium" means a culture medium comprising a chemically defined composition on which organisms are grown. In the culture medium of the present invention, glycerol is advantageously the single source of carbon. Nevertheless, the culture medium may comprise a carbohydrate in addition to the glycerol as carbon source. The carbohydrate is selected in the group consisting of xylose, glucose, fructose and sucrose or combination thereof.

In a particular embodiment, glycerol is added to the medium in the form of glycerine composition comprising at least 50% of glycerol, preferably at least 85% of glycerol.

Advantageously, the glycerine used in the culture medium of the invention is industrial glycerine. "Industrial glycerine" means a glycerine product obtained from an industrial process without substantial purification. Industrial glycerine can also be designated as "raw glycerine". Industrial glycerine comprises more than 70% of glycerol, preferably more than 80%, water and impurities such as mineral salts and fatty acids. The maximum content of glycerol in industrial glycerine is generally 90%, more generally about 85%.

The method for the production of PDO and BA according to the present invention may thus be carried out by using glycerol provided by industrial glycerine comprising more than 70% of glycerol.

Industrial processes from which industrial glycerine is obtained are, inter alia, manufacturing methods where fats and oils, particularly fats and oils of plant origin or fats and oils of animal origin or used cooking oils, are processed into industrial products such as detergent or lubricants. In such manufacturing methods, industrial glycerine is

considered as a by-product. In a particular embodiment, the industrial glycerine is a by-product from biodiesel production and comprises known impurities of glycerine obtained from biodiesel production, comprising about 80 to 85 % of glycerol with salts, methanol, water and some other organic compounds such as fatty acids. Industrial glycerine obtained from biodiesel production may be further subjected to an acidification step to eliminate fatty acids. The man skilled in the art knows technology of acidification and is able to define the best conditions of acidification according to the glycerine used.

The method for the production of PDO and BA according to the present invention may thus be carried out by using industrial glycerine which is a by-product of biodiesel production.

The concentration of glycerol used in the method of the invention is more than 90 g.L⁻¹ of glycerol in the fed medium. In preferred embodiments, the concentration is comprised between 90 and 200 g.L⁻¹ of glycerol, more particularly between 90 and 140 g/L of glycerol, preferably about 120 g.L⁻¹ of glycerol and more preferably about 105 g.L⁻¹ of glycerol contained into the glycerine solution.

Preferably, the culture medium is a synthetic medium without addition of organic nitrogen. The method for the production of PDO and BA, according to the present invention may thus be carried out by using a culture medium which is exempt of organic nitrogen, and preferably using a synthetic medium without addition of organic nitrogen, more preferably without yeast extract.

Such culture media are disclosed in the art, particularly in patent applications WO 2010/128070 and WO 2011/042434, which contents are incorporated herein by reference.

In a particular embodiment, the method for the production of PDO and BA according to the present invention comprises culturing said mutant strain *Clostridium acetobutylicum* as defined above with at least one strain of *Clostridium sporogenes* and/or at least one strain of *Clostridium sphenoides*, in an appropriate culture medium comprising at least glycerol as carbon source and recovering the PDO and/or the BA produced from the culture medium.

EXAMPLES

The present invention is further defined in the following examples. It should be understood that these examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From above disclosure and these examples, the person skilled in the art can make various changes of the invention to adapt it to various uses and conditions without modifying the essential means of the invention.

Example 1: Identification of mutations present in one clone strayed from the Type 174P population

Clone isolation

5

Clone isolation was performed on agar plates starting from continuous culture of the population strain *Clostridium acetobutylicum* DG1 pSPD5 Type 174P which is a population overexpressing the 1,3-propanediol operon from *C. butyricum* and adapted for growth and production of PDO from a culture medium with a high concentration of industrial glycerine as described in patent application WO2012/062832A1. The synthetic medium used for isolation on agar plate contained per liter of deionized water: agar, 25g; commercial glycerine, 30g; KH₂PO₄, 0.5g; K₂HPO₄, 0.5g; MgSO₄, 7H₂O, 0.2g; CoCl₂ 6H₂O, 0.01g; acetic acid, 99.8%, 2.2ml; NH₄Cl, 1.65g; MOPS, 23.03g, biotin, 0.16mg; *p*-amino benzoic acid, 32mg; FeSO₄, 7H₂O, 0.028g; resazurin, 1mg and cysteine, 0.5g. The pH of the medium was adjusted to 6.5 with NH₄OH 6N.

15

Isolated clones were considered pure after three subsequent subcultures on agar plates. One of them, named clone **PD0557Vc05** was then transferred into liquid synthetic medium. Subsequently, growing liquid culture was conserved on glycerine 20% at -80°C until characterization.

20

DNA isolation

Genomic DNA was extracted from 16 ml of continuous culture of the clone PD0557Vc05 using the Genomic DNA buffer set and Genomic tip 500/G kits from Qiagen (QIAGEN SA, COURTABOEUF, France). Bacterial samples were prepared according to protocol described in the QIAGEN Genomic DNA Handbook (Sample Preparation and Lysis Protocol for Bacteria), followed by Genomic-tip Protocol (Protocol for Isolation of Genomic DNA from Blood, Cultured Cells, Tissue, Yeast, or Bacteria)

25

Sequencing analysis

Genome of the clone PD0557Vc05 was sequenced using the Genome Sequencer Illumina HiSeq technology. The sequencing project was performed by GATC (GATC Biotech AG, Jakob-Stadler-Platz 7, 78467 Konstanz, Germany) with sequence mode Illumina HiSeq sequencing, standard genomic library, read length 2 x 150 bp, run type: paired end, read length: 2 x 150 bp, sequenced reads 11,589,200, sequenced bases 3,476,760,000.

35

Bioinformatic analysis

The bioinformatics analysis was performed by GATC Biotech AG using the GATC VARIANT ANALYSIS V1.2 workflow and reference database ASM876v1. Variant analysis was performed by GATC Biotech AG: SNP and InDel calling is done using GATK's Haplotype Caller [McKenna *et al.*, 2010; De Pisto *et al.*, 2011]; variants detected are annotated based on their gene context using snpEff (Cingolani *et al.*, 2012). SnpEff software is available for instance on the website <http://snpeff.sourceforge.net>. Several metrics, that are used to evaluate the quality of a variant, are annotated using GATK's VariantAnnotator module; customised filters are applied to the variants to filter false positive variants using GATK's VariantFiltration module.

Validation of the identified mutations

To confirm the presence of identified mutations in PD0557Vc05, PCR reactions and Sanger sequencing were done on DNA for each gene or DNA region of interest, using PCR and Sanger sequencing oligonucleotides described in Table 1.

5

Table 1: Oligonucleotides sequences designed for PCR and Sanger sequencing of mutated genes identified in the sequencing analysis; CA_C1321, CA_C0028, CA_C0770 and CA_C2709 genes

Gene name	Oligonucleotides for PCR reactions	Oligonucleotides for Sanger sequencing
CA_C1321 (<i>glpK</i>)	SEQ ID NO: 30 ggagcaacatgtgtatcaacaacc SEQ ID NO: 31 GCATCCAATAACACCTGCTCC	SEQ ID NO: 30 ggagcaacatgtgtatcaacaacc SEQ ID NO: 31 GCATCCAATAACACCTGCTCC SEQ ID NO: 32 ggcaaagtcctatgaaccg
CA_C0028 (<i>hydA</i>)	SEQ ID NO: 33 catgttctattgttactatggaagaggtagtag SEQ ID NO: 34 GCAGTTATTATAAATGCTGCTACT AGAGC	SEQ ID NO: 33 catgttctattgttactatggaagaggtagtag SEQ ID NO: 35 cgtgaggttgacctccaccatttatacatcc SEQ ID NO: 34 GCAGTTATTATAAATGCTGCTACTA GAGC SEQ ID NO: 36 GTGGACAATGTTCTAGAAGAG
CA_C0770	SEQ ID NO: 37 atgcattatagtttagctg SEQ ID NO: 38 CGTATTTATGTTAACACAGG	SEQ ID NO: 39 CTGTGACAAATCCATTTATATG SEQ ID NO: 38 CGTATTTATGTTAACACAGG
CA_C2709 (<i>etfA</i>)	SEQ ID NO: 40 gaagttaaaggacagggagaag SEQ ID NO: 41 GCAAATGCCTGAGCAATTCC	SEQ ID NO: 40 gaagttaaaggacagggagaag SEQ ID NO: 41 GCAAATGCCTGAGCAATTCC

Based on both Illumina and Sanger sequencing analysis performed on PD0557Vc05, several mutations were identified as described in table 2.

- 5 **Table 2:** Mutations identified in PD0557Vc05 clone. Genes and corresponding protein sequences are presented with their respective SEQ ID NO.

Protein names	Gene name	Uniprot ref	Protein sequences	DNA sequences	Protein sequence modification	Protein sequences	DNA sequences
Glycerol kinase (EC 2.7.1.30) (ATP:glycerol 3-phosphotransferase) (Glycerokinase) (GK)	glpK CA_C1321	Q97JG4	SEQ ID NO: 1	SEQ ID NO: 2	H22Y A347T V466M	SEQ ID NO: 13	SEQ ID NO: 14
Hydrogene dehydrogenase	hydA CA_C0028	Q7D472	SEQ ID NO: 15	SEQ ID NO: 16	I210* (stop codon)	SEQ ID NO: 17	SEQ ID NO: 18
Glycerol uptake facilitator protein, permease	CA_C0770	Q97KZ6	SEQ ID NO: 19	SEQ ID NO: 20	M13I	SEQ ID NO: 21	SEQ ID NO: 22
Electron transfer flavoprotein subunit alpha (Alpha-ETF) (Electron transfer flavoprotein large subunit) (ETFLS)	etfA CA_C2709	P52039	SEQ ID NO: 23	SEQ ID NO: 24	I105V	SEQ ID NO: 25	SEQ ID NO: 26

Example 2: Characterization of the mutations identified in genes *glpK* and *hydA*

10

A. Characterisation of proteins glycerol kinase wild type (GlpK) and mutants (GlpK*)

15

Three different point mutations were identified in *glpK* gene in the clone PD0557Vc05 leading to the mutant protein GlpK* with H22Y, A347T and V466M (table 2 above). In order to better characterize the effect of the mutations, we tested them individually or in

combination. Therefore, the alleles coding for the mutated glycerol kinase were cloned into the expression plasmid pPAL7 (Biorad®) and the plasmids obtained were transformed into *E. coli* strain BL21(DE3)star to purify each mutated protein and perform biochemical experiments.

5

Table 3: strains constructed and used for kinetic parameters determination of glycerol kinase mutants from PD0557Vc05

Strain Number	Name	Uniprot Ref	protein sequence	gene sequence
1	GlpK wt	Q97JG4	SEQ ID NO: 1	SEQ ID NO: 2
2	GlpK*(H22Y/A347T/V466M)	-	SEQ ID NO: 13	SEQ ID NO: 14
3	GlpK*(H22Y)	-	SEQ ID NO: 5	SEQ ID NO: 6
4	GlpK*(A347T)	-	SEQ ID NO: 3	SEQ ID NO: 4
5	GlpK*(V466M)	-	SEQ ID NO: 7	SEQ ID NO: 8
6	GlpK*(H22Y/A347T)	-	SEQ ID NO: 9	SEQ ID NO: 10
7	GlpK*(A347T/V466M)	-	SEQ ID NO: 11	SEQ ID NO: 12

10 Overproduction of the proteins

Cultures of the seven strains 1 to 7 for the overproduction of the proteins were realized in a 2 L Erlenmeyer flask, using LB broth (Bertani, 1951) that was supplemented with 5 g/L glucose and 100 mg/L of ampicillin. An overnight culture was used to inoculate a 500 mL culture to an OD_{600nm} of about 0.15. The culture was first kept on a shaker at 37°C and 200 rpm until OD_{600nm} was about 0.5 and then the culture was moved on a second shaker at 25°C and 200 rpm until OD_{600nm} was 0.6 – 0.8 (about one hour), before induction with 500 µM IPTG. The culture was kept at 25°C and 200 rpm until OD_{600nm} was around 4, and then it was stopped. Cells were centrifuged at 7000 rpm, 5 minutes at 4°C, and then stored at -20°C. This protocol was the same for strains 1 to 7.

20

Purification of the proteins

Step 1: Preparation of cell-free extracts.

5 For each strain, about 250 mg of *E. coli* biomass was suspended in 40 ml of 100 mM potassium phosphate pH 7.6, and a protease inhibitor cocktail. The cells suspension (15 ml per conical tube) was sonicated on ice (Bandelin sonoplus, 70 W) in a 50 ml conical tube during 8 cycles of 30 sec with 30 sec intervals. After sonication, cells were incubated for 30 min at room temperature with 5 mM MgCl₂ and 1U/ml of DNaseI. Cells debris were
10 removed by centrifugation at 12000g for 30 min at 4°C.

Step 2: Affinity purification

The proteins were purified from the crude cell-extract by affinity on a Profinity column (BIORAD, Bio-Scale Mini Profinity exact cartridge 5 ml) according to the protocol
15 recommended by the manufacturer. The crude extract was loaded on a 5 ml Profinity exact cartridge equilibrated with 100 mM potassium phosphate pH 7.6. The column was washed with 10 column volumes of the same buffer and incubated 45 min with 100 mM potassium phosphate pH 7.6, 100 mM fluoride at room temperature. The protein was eluted from the column with 2 column volumes of 100 mM potassium phosphate pH 7.6.
20 The tag remained tightly bound to the resin and the purified protein was released. The fractions containing the protein were pooled, concentrated. Protein concentration was measured using the Bradford protein assay.

Determination of kinetic parameters of GlpK wild type and mutants

25 Glycerol kinase activity (GLPK) that catalyses the following reaction: Glycerol + ATP => Glycerol-3-Phosphate + ADP, was determined by measuring the ADP appearance by enzymatic coupling to NADH oxidation. The reaction mixture (1mL) containing 50 mM Sodium bicarbonate pH 9, 2 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 5 mM MgCl₂, 5 Units pyruvate kinase, 5 units lactate dehydrogenase was incubated for 5 min at
30 30°C. Then, 0.01-50 mM glycerol was added to start the reaction. The substrate glycerol was omitted from the blank. The oxidation of NADH was monitored at 30°C by absorbance at 340 nm on a spectrophotometer ($\lambda_{340} = 6290 \text{ M}^{-1} \text{ cm}^{-1}$).

One unit of enzyme activity was defined as the amount of enzyme catalyzing the decrease of 1 μmol of NADH per min. Kinetic parameters were determined with Sigmaplot by fitting
35 to the Michaelis-Menten equation. The specific activity and kinetic parameters of the purified enzymes towards glycerol are provided in Table 4.

Table 4: catalytic performances (Specific activity and Kinetic) of the wild type and GlpK mutants towards glycerol

Catalytic performance on glycerol	GlpK wt	GlpK* A347T	GlpK* H22Y	GlpK* V466M	GlpK* (H22Y/A347 T)	GlpK* (A347T/V466M)	GlpK* (H22Y/A347 T /V466M)
Km (mM)	0.054 ± 0.001	0.26 ± 0.031	0.070 ± 0.004	0.058 ± 0.003	0.62 ± 0.05	0.48 ± 0.048	0.039 ± 0.002
Vm (nmol/min/mg)	87734 ± 499	6977 ± 155	91725 ± 1186	60399 ± 537	9784 ± 195	6267 ± 175	1404 ± 13
Kcat (min ⁻¹)	4933 ± 28	392.3 ± 8.7	5158 ± 67	3396 ± 30	545 ± 11	352.4 ± 9.8	79.0 ± 0.7
Kcat/km (mM ⁻¹ s ⁻¹)	1520 ± 53	24.9 ± 3.5	1229 ± 93	973 ± 52	14.7 ± 1.5	12.31 ± 1.6	33.9 ± 2.3

5 Each simple mutation decreases either the specific activity (A347T and V466M) or the affinity (A347T) towards the substrate glycerol. This conclusion is also true for the double mutants, which have reduced catalytic activity and affinity (H22Y/A347T and V466M/A347T).

10 The triple mutant GlpK* combining all the mutations (A347T/V466M/H22Y) shows a strong drop of the catalytic efficiency with a value of 33,9 mM⁻¹s⁻¹ compared to 1520 mM⁻¹s⁻¹ for the wild type protein.

The conclusion is that whatever the mutation or the combination of them, the catalytic activity of glycerol kinase is reduced more or less drastically. Therefore, the strain PD0557Vc05 has a reduced GlpK catalytic activity.

15

B. Characterization of the HydA mutation

The more relevant mutation identified in the *hydA* gene lead to the integration of a stop codon at the amino acid position 210 as disclosed in the HydA protein alignment below:

20 HydA protein alignment: CA_C0028: HydA wild-type protein and CA_C0028*(I210*): truncated HydA*(I210*)

CA_C0028 (161)

STCSIQFIKKDGGQRAVGTVDDVCLDDSTCLLCGQCVIACPVAALKEKSHI

CA_C0028*(I210*) (161)

STCSIQFIKKDQGRAVGTVDVCLDDSTCLLCGQCVIACPVAALKKNPI—

In order to characterize the phenotype of the mutation, we measured the Hydrogen and Carbone dioxide production of a fermentation of the PD0557Vc05 clone during a continuous culture (Table 5). Fermentation gas samples were taken in Supel™-Inert Multi-Layer bags and analyzed by Quad-Lab laboratory. H₂ and CO₂ were quantified by μ GC/ μ TCD in accordance with NFX 20-303.

Table 5: Hydrogen production by PD0557Vc05 in continuous culture. Limit of quantification about 0.0005%; Error: 15%

Gaz reactor analysis		
Sample	H ₂ (v/v%)	CO ₂ (v/v%)
PD0557Vc05	0.0520	98.3028

These data show that the clone PD0557Vc05 does not produce hydrogen anymore but exclusively carbon dioxide. This confirms the absence of HydA activity. This result has been validated by proteomic analysis performed on samples (cell extracts) of cultures of the clone. The HydA protein is not detected at all.

In conclusion, the clone PD0557Vc05 has no hydrogenase activity anymore.

Example 3: Effect of GlpK* and HydA truncation on the PDO and BA production by two producer strains; *C. acetobutylicum* DG1 pSPD5 and PD0001VE05c08

In order to confirm the effect of the mutations identified in clone PD0557Vc05 on the production of PDO/BA (see Example 2), we introduced the *glpK** and *hydA** alleles, alone or in combination into two producer strains that are not modify on these genes.

The two recipient strains chosen for the genetic modifications were:

- DG1 pSPD5: *C. acetobutylicum* strain DG1 pSPD5 described in publication Gonzalez-Pajuelo et al., 2006
- PD0001VE05c08: *C. acetobutylicum* strain DG1 pSPD5 isolated clone and adapted on high concentrations of glycerine as described in patent application WO2012/062832

Mutations in genes *glpK** (triple mutant) and *hydA** identified in strain PD0557Vc05 described above were introduced into the DG1 pSPD5 strain, giving rise to strains 8, 9 and 10 or in PD0001VE05c08 giving rise to strains 11, 12 and 13.

5 Strains 8 to 10 were cultivated in continuous culture conditions described in Gonzalez-Pajuelo et al., 2006, and strains 11 to 13 were cultivated as described in patent application WO2012/062832.

10 **Table 6:** Level of performances improvement of the *C. acetobutylicum* DG1 pSPD5 and PD0001VE05c08 strains carrying *glpK** and/or *hydA** mutations. Q 1,3 PDO / BA corresponds to PDO /ABH volumetric productivity

Strain	Genetic modification	Q 1,3 PDO improvement (%)	Q BA improvement (%)
Reference DG1 pSPD5	-	-	-
8	<i>glpK</i> *(H22Y/A347T/V466M) <i>hydA</i> wt	+5 %	+2 %
9	<i>glpK</i> wt <i>hydA</i> *(I210*)	+3 %	+4 %
10	<i>glpK</i> *(H22Y/A347T/V466M) <i>hydA</i> *(I210*)	+ 7 %	+ 6 %
Reference PD0001VE05c08	-	-	-
11	<i>glpK</i> *(H22Y/A347T/V466M) <i>hydA</i> wt	+7 %	+2 %
12	<i>glpK</i> wt <i>hydA</i> *(I210*)	+4 %	+6 %
13	<i>glpK</i> *(H22Y/A347T/V466M) <i>hydA</i> *(I210*)	+ 9 %	+ 7 %

15 Upon mutations of *glpK* and *hydA* genes leading respectively to the decrease of the glycerol kinase activity with *GlpK** (H22Y/A347T/V466M) and to the suppression of the hydrogenase activity with *HydA** (I210*), productivities of both PDO and BA are improved.

Example 4: Performances of PD0557Vc05 in a continuous culture with high concentrations of raw glycerine

5 **Bacterial strains:**

- PD0001VE05c08: *C. acetobutylicum* strain DG1 pSPD5 isolated clone and adapted on high concentrations of glycerine as described in patent application WO2012/062832
- PD0557Vc05: *C. acetobutylicum* strain DG1 pSPD5 isolated clone from a continuous culture of *C. acetobutylicum* strain DG1 pSPD5 Type 174P on high concentrations of raw industrial glycerine as described in patent application WO2012/062832A1.

10 **Culture media:**

15 The synthetic medium used for clostridia batch cultivations contained per liter of tap water: glycerol, 30g; KH₂PO₄, 0.5g; K₂HPO₄, 0.5g; MgSO₄, 7H₂O, 0.2g; CoCl₂ 6H₂O, 0.01g; H₂SO₄, 0.1ml; NH₄Cl, 1.5g; biotin, 0.16mg; p-amino benzoic acid, 32mg and FeSO₄, 7H₂O, 0.028g. The pH of the medium was adjusted to 6.3 with NH₄OH 3N. Commercial glycerol purchased from SDS Carlo_Erba (purity 99%) was used for batch cultivation. The feed medium for continuous cultures contained per liter of tap water : glycerol from raw glycerine, 105g; KH₂PO₄, 0.45g; K₂HPO₄, 0.45g; MgSO₄, 7H₂O, 0.2g; CoCl₂ 6H₂O, 0.013g; biotin, 0.08mg; p-amino benzoic acid, 16mg; FeSO₄, 7H₂O, 0.04g; anti-foam, 0.05ml; ZnSO₄, 7H₂O, 8mg; CuCl₂, 2H₂O, 4mg; MnSO₄, H₂O, 20mg. Medium pH was adjusted between 3,5 and 4 with H₂SO₄ 96%. Raw glycerine, from the transesterification process for biodiesel, was provided by different providers (Avril, Carotech Berhad) and had purity comprised between 80 and 86 % (w/w). These glycerines were blended and pretreated by acidification.

25 **Experimental set-up:**

30 Continuous cultures were performed in a 5L bioreactor Tryton (Pierre Guerin, France) with a working volume of 2000 mL. The culture volume was kept constant at 2000 mL by automatic regulation of the culture level. Cultures were stirred at 200 RPM, the temperature was set to 35°C and pH maintained constant at 6.5 by automatic addition of NH₄OH 5.5 N. To create anaerobic conditions, the sterilized medium in the vessel was flushed with sterile O₂-free nitrogen for one hour at 60°C and flushed again until 35°C was attained (flushing during 2 hours). The bioreactor gas outlet was protected from oxygen by a pyrogallol arrangement (Vasconcelos *et al.*, 1994). After sterilization the feed medium

35

was also flushed with sterile O₂-free nitrogen until room temperature was attained and maintained under nitrogen to avoid O₂ entry.

Batch and continuous cultures process:

5 A culture growing in a 100 mL Penicillin flask on synthetic medium (the same as described above for batch culture but with addition of acetic acid, 2.2 g.L⁻¹ and MOPS, 23.03 g.L⁻¹) taken at the end of exponential growth phase was used as inoculum (5% v/v).

10 Cultures were first grown batchwise. At the early exponential growth phase we performed a pulse of glycerol from raw glycerine: For the pulse, synthetic medium (the same as described for feed culture) with 105 g.L⁻¹ of glycerol from raw glycerine was added at a static flow rate during 3 hours (i.e. an addition of 18 g.L⁻¹ of glycerol). Then the growth continued batchwise and before the end of the exponential growth phase the continuous feeding started with a dilution rate of 0.035 h⁻¹. The feed medium contains 105 g.L⁻¹ of glycerol from raw glycerine. 6-13 days after inoculation of the bioreactor and after 4
15 residence times (RT) the dilution rate was increased from 0.035 h⁻¹ to 0.070 h⁻¹ in five days. After that, stabilization of the culture was followed by PDO production and glycerol consumption using the HPLC protocol described below. Particularly we waited until the concentration of residual glycerine was as low as possible.

20 The overall performances of PD0557Vc05 are presented in table 7 below and compared with performances of PD0001VE05c08 obtained with culture media, batch and continuous process described in B60. These process conditions start at a dilution rate of 0.025 h⁻¹ and reached only a dilution rate of 0.06 h⁻¹. PD0001VE05c08 washout with process conditions described in this example and applied for the mutated strain PD0557Vc05 with higher dilution rate (start dilution rate at 0.035 h⁻¹ and reached a dilution rate at 0.07 h⁻¹).

25

Analytical procedures:

30 Cell concentration was measured turbidimetrically at 620 nm and correlated with cell dry weight determined directly. Glycerol, 1,3-propanediol, ethanol, lactate, acetic and butyric acids concentrations were determined by HPLC analysis. Separation was performed on a Biorad Aminex HPX-87H column and detection was achieved by refractive index. Operating conditions were as follows: mobile phase sulphuric acid 0.5 mM; flow rate 0.5 ml/min, temperature, 25°C.

Table 7: Performances of the *C. acetobutylicum* PD0001VE05c08 and PD0557Vc05 isolated clones grown in continuous culture (mean data from 2 chemostats). The feed medium contained 105 g.L⁻¹ of glycerol from raw glycerine, dilution rate was 0.025 h⁻¹ to 0.06h⁻¹ for PD0001VE05c08 and 0.035 h⁻¹ to 0.07h⁻¹ for PD0557Vc05. Values correspond to the average of samples analyzed after culture stabilization at the maximum dilution rate obtained.

	PDO/BA Production performances for PD0001VE05c08	PDO/BA Production performances for PD0557Vc05
Feed glycerine (g.l ⁻¹)	104	105
1,3-propanediol (g.l ⁻¹)	50.4	49.2
Butyric acid (g.l ⁻¹)	11.4	10.8
Y1,3-PDO (g.g ⁻¹)	0.49	0.47
Q1,3-PDO (q.l ⁻¹ .h ⁻¹)	2.99	3.40
Y1,3-BA (g.g ⁻¹)	0.11	0.11
Q1,3-BA (q.l ⁻¹ .h ⁻¹)	0.67	0.75
Dilution rate (h ⁻¹)	0.059	0.068
Residual glycerine (g.l ⁻¹)	3.6	4.4
Biomass (g.l ⁻¹)	1.99	2.28
Acetic acid (g.l ⁻¹)	2.29	1.85

Y1,3-PDO / BA: PDO / BA yield (g/g of glycerol engaged)

Q1,3 PDO / BA: PDO / BA volumetric productivity

10

These results show that the clone PD0557Vc05 carrying mutations on *glpK* and *hydA* genes described and characterized in examples above produces PDO and BA with higher productivities than clone PD0001VE05c08. The gain is significant as it is above 10% more productivity for both products (data in bold).

15

An important fact mentioned above is that the strain PD0001VE05c08 was not able to reach the dilution rate of 0.07 h⁻¹ on the contrary of PD0557Vc05 strain, confirming the positive impact of the claimed mutations on the growth rate and PDO and BA productivity of bacteria.

Moreover, when culture conditions described in WO2012/062832 were applied to PD0557Vc05 strain, the performances of said strain were better than PD0001VE05c08 ones (data not shown).

5 **Example 5: Performances of microbial consortium comprising strains PD0557Vc05, C. sporogenes and C. sphenoides in continuous culture with high concentrations of raw glycerine**

10 In order to improve the production of PDO and BA with the new strain PD0557Vc05, we decided to recreate a consortium of bacteria that has previously shown improvement on such production (not disclosed data). The consortium was therefore reconstituted by addition to PD0557Vc05, of microorganisms' *C. sphenoides* and *C. sporogenes*. This new consortium named "PD0557Vc05 consortium" was created during the first days of a continuous culture as describe below in section "batch and continuous cultures process".

15

Culture media:

The synthetic media used for clostridia batch cultivations contained per liter of tap water: glycerol, 30g; KH₂PO₄, 0.5g; K₂HPO₄, 0.5g; MgSO₄, 7H₂O, 0.2g; CoCl₂ 6H₂O, 0.01g; H₂SO₄, 0.1ml; NH₄Cl, 1.5g; biotin, 0.16mg; p-amino benzoic acid, 32mg and FeSO₄, 7H₂O, 0.028g. The pH of the medium was adjusted to 6.3 with NH₄OH 3N. Commercial glycerol purchased from SDS Carlo_Erba (purity 99%) was used for batch cultivation. The feed medium for continuous cultures contained per liter of tap water: glycerol from raw glycerine (purity comprised between 80 and 86% w:w), 105g; KH₂PO₄, 0.45g; K₂HPO₄, 0.45g; MgSO₄, 7H₂O, 0.2g; CoCl₂ 6H₂O, 0.013g; biotin, 0.08mg; p-amino benzoic acid, 16mg; FeSO₄, 7H₂O, 0.04g; anti-foam, 0,05ml; CuCl₂, 2H₂O, 2mg; MnSO₄, H₂O, 20mg. Medium pH was adjusted between 3.5 and 4 with H₂SO₄ 96%. Glycerine was pretreated by acidification.

Experimental set-up:

30 Continuous cultures were performed in a 5L bioreactor Tryton (Pierre Guerin, France) with a working volume of 2000 mL. The culture volume was kept constant at 2000 mL by automatic regulation of the culture level. Cultures were stirred at 200 RPM, the temperature was set to 35°C and pH maintained constant at 6.5 by automatic addition of NH₄OH 5.5 N. To create anaerobic conditions, the sterilized medium in the vessel was
35 flushed with sterile O₂-free nitrogen for one hour at 60°C and flushed again until 35°C was attained (flushing during 2 hours). The bioreactor gas outlet was protected from oxygen by a pyrogallol arrangement (Vasconcelos *et al.*, 1994). After sterilization the feed medium

was also flushed with sterile O₂-free nitrogen until room temperature was attained and maintained under nitrogen to avoid O₂ entry.

Batch and continuous cultures process:

5 The PD0557Vc05 culture growing in a 100 mL Penicillin flask on synthetic medium (the same as described above for batch culture but with addition of acetic acid, 2.2 g.L⁻¹ and MOPS, 23.03 g.L⁻¹) taken at the end of exponential growth phase was used as inoculum (5% v/v).

10 Cultures were first grown batchwise. At the early exponential growth phase we performed a pulse of glycerol from raw glycerine. For the pulse, synthetic medium (the same as described for feed culture) with 105 g.L⁻¹ of glycerol from raw glycerine was added at a static flow rate during 3 hours (i.e. an addition of 18 g.L⁻¹ of glycerol). Then the growth continued batchwise and before the end of the exponential growth phase the continuous feeding started with a dilution rate of 0.035 h⁻¹. The feed medium contained 105 g.L⁻¹ of glycerol from raw glycerine. 8 days after inoculation of the bioreactor, microbial
15 consortium of *C. sporogenes* and *C. sphenoides* was added to have respectively an optical density (620 nm) of 0.009 uOD and 0.317 uOD in the bioreactor. After culture stabilization, the dilution rate was increased from 0.035 h⁻¹ to 0.070 h⁻¹. After that, stabilization of the culture was followed by 1,3-propanediol production, butyric acid
20 production and glycerol consumption using the HPLC protocol described below. Particularly we waited until the concentration of residual glycerine was as low as possible. The overall performances of PD0557Vc05 consortium are presented in Table 8 and compared with performances of PD0557Vc05 without *C. sporogenes* and *C. sphenoides*.

25 **Analytical procedures:**

Cell concentration was measured turbidimetrically at 620 nm and correlated with cell dry weight determined directly. Glycerol, 1,3-propanediol, ethanol, lactate, acetic and butyric acids concentrations were determined by HPLC analysis. Separation was performed on a Biorad Aminex HPX-87H column and detection was achieved by refractive index.
30 Operating conditions were as follows: mobile phase sulphuric acid 0.5 mM; flow rate 0.5 ml/min, temperature, 25°C.

Table 8: Performances of the *C. acetobutylicum* PD0557Vc05 and of the PD0557Vc05 consortium grown in continuous culture. The feed medium contained 105 g.L⁻¹ of glycerol from raw glycerine, dilution rate was 0.07h⁻¹. Values correspond to the average of samples analyzed after culture stabilization at dilution rate of 0.07h⁻¹.

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	PDO/BA Production performances for PD0557Vc05	PDO/BA Production performances for PD0557Vc05 consortium
Feed glycerine (g.l ⁻¹)	105	105
1,3-propanediol (g.l ⁻¹)	49.2	51.4
Butyric acid (g.l ⁻¹)	10.8	11.2
Y1,3-PDO (g.g ⁻¹) engaged	0.47	0.49
Q1,3 PDO (g.l ⁻¹ .h ⁻¹)	3.40	3.51
Y1,3-BA (g.g ⁻¹) engaged	0.11	0.11
Q1,3 BA (g.l ⁻¹ .h ⁻¹)	0.75	0.77
Dilution rate (h ⁻¹)	0.068	0.066
Residual glycerine (g.l ⁻¹)	4.4	2.4
Biomass (g.l ⁻¹)	2.28	2.12
Acetic acid (g.l ⁻¹)	1.85	2.44

Y1,3-PDO: PDO / BA yield (g/g of glycerol engaged)

Q1,3 PDO: PDO / BA volumetric productivity

10 These results show that the PD0557Vc05 consortium has better production performances both on PDO and BA than the PD0557Vc05 strain (data in bold). Indeed, the PD0557Vc05 consortium shows higher PDO and BA titers, higher yield of PDO and less residual glycerine, which means a clear overall improvement of the technology.

15 **Microbial consortium quantification**

DNA isolation

20 Genomic DNA was extracted from 1 mL of culture, homogenized and transferred to 2 mL tube with Glass Bead Tube Kit, 0.1 mm, using a Precellys®24 lyser/homogeniser (Bertin Technologies, Saint Quentin Yvelines, France). Precellys®24 settings were: 6500 rpm, 20 sec cycle duration, 5 sec delay time between cycles, for 2 total cycles. After Precellys extraction, the lysate was centrifuged at 10,000 g for 10 min and the supernatant used for total genomic extraction using the NucleoSpin® Gel and PCR Clean-up from Macherey (Macherey Nagel, Hoerd, France)

PD0557Vc05 consortium quantification by quantitative PCR

The recent advances in molecular techniques such as PCR technology enable rapid, specific, and sensitive detection and the identification of potential microorganisms in different type of environments as for instance in this patent application, culture broth from a fermentative production.

Absolute quantification in samples was determined by quantitative PCR (qPCR) using the Sso Advanced Universal SYBR Green Supermix (Bio-rad Mitry Mory, France). The qPCR was performed on a Bio-Rad C1000™ Thermal Cycler equipped with a CFX96™ Real-Time System (Bio-Rad Mitry Mory, France).

Reactions mixtures consisted of 1× Sso Advanced Universal SYBR Green Supermix (Bio-Rad Mitry Mory, France), 6 µL of each forward (F) and reverse (R) primers (1 µM), 2 µL of diluted sample (2 ng/µL from Nanodrop measure) and nuclease free water to reach a final volume of 20 µL. Amplification was achieved according to the following thermal cycling program: initial melting at 98°C for 2 min. (1 cycle) followed by 40 cycles of melting at 98°C for 10 sec, annealing of primers and elongation at 60°C for 30 sec. (Melt Curve 65 to 95°C, increment 0.5°C every 5 sec).

A standard curve was determined for each microorganism using Cq values of serial dilutions of genomic DNA at known concentrations. The Cq value for each well (standard curve and samples) was determined by the CFX Manager™ 3.1 software. The samples were plotted against the standard curve to determine abundance of nucleic acid. Absolute quantification were based on one *gapC* gene copy per cell for *C. acetobutylicum*, one *cpn60* gene copy per cell for *C. sphenoides* and one *tpi* gene copy per cell for *C. sporogenes*. For the purposes of calculation, nucleic acid extractions were assumed to be perfect, because no measurement of extraction efficiency is available.

Abundance of each microorganism in samples was determined by quantitative PCR (qPCR) using the Sso Advanced Universal SYBR Green Supermix (Bio-rad Mitry Mory, France) on a Bio-Rad C1000™ Thermal Cycler equipped with a CFX96™ Real-Time System. The *gapC* gene based primers used to target *C. acetobutylicum* were *gapC_F*, 5'-TGCTGCTGTAAGTATCATC-3' (SEQ ID NO: 42) and *gapC_R*, 5'-GTTGGAAGTGGAACTCTT-3' (SEQ ID NO: 43). The *cpn60* gene based primers used to target *C. sphenoides* were *cpn60_F*, 5'-TTATATGTGCACCGATATG-3' (SEQ ID NO: 44) and *cpn60_R*, 5'-GAGAAGTCTTGCGCCGGAC-3' (SEQ ID NO: 45). The *tpi* gene based primers used to target *C. sporogenes* were *tpi_F*, 5'-CCAGCGGTATTAGAAGAA-3' (SEQ ID NO: 46) and *tpi_R*, 5'-GTCCTATAATTACATAATGAACTC-3' (SEQ ID NO: 47).

In the table below, are given percentages of representation of the different species present in the PD0557Vc05 consortium considering that the totality of the cells contained in the culture corresponds to 100 %.

- 5 **Table 9:** PD0557Vc05 microbial consortium composition after culture stabilization at dilution rate of 0.070h^{-1} .

Sample	<i>C. acetobutylicum</i>	<i>C.sphenoïdes</i>	<i>C.sporogenes</i>
Established permanent state culture	89.67% - 94.79%	1.42% - 4.52%	3.79% - 6.67%

NON PATENT REFERENCES

- 5 - Bantscheff M, Schirle M, Sweetman G, Rick J, and Kuster B. (2007), *Analytical and Bioanalytical Chemistry*, vol. 389(4): 1017–1031.
- Bertani G. (1951), *J Bacteriol.* 62: 293-300.
- Burnette WN (1981). *Analytical Biochemistry*, 112(2): 195-203.
- 10 - DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, McKenna A, Fennell TJ, Kernytzsky AM, Sivachenko AY, Cibulskis K, Gabriel SB, Altshuler D, and Daly MJ. (2011), *Nat Genet*, 43:491–498.
- Engvall E and Perlman P (1981), *Immunochemistry*, 8: 871-874.
- 15 - González-Pajuelo M, Meynial-Salles I, Mendes F, Andrade JC, Vasconcelos I, and Soucaille P. 2005. *Metabolic Engineering*, 7: 329–336.
- González-Pajuelo M, Meynial-Salles I, Mendes F, Soucaille P. and Vasconcelos I. (2006). *Applied and Environmental Microbiology*, 72: 96-101.
- McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytzsky A, Garimella K, Altshuler D, Gabriel S, Daly M, and DePristo MA. (2010), *Genome Research*, 20(9):1297–1303.
- 20 - Papanikolaou S, Ruiz-Sanchez P, Pariset B, Blanchard F and Fick M. (2000), *Journal of Biotechnology*, 77: 191-208.
- Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X and Ruden DM. (2012), *Fly*, 6(2): 80-92
- 25 - Vasconcelos I, Girbal L, Soucaille P., (1994), *Journal of Bacteriology*, 176(5): 1443-1450.

CLAIMS

1. A mutant strain of *Clostridium acetobutylicum* expressing *dhaB1*, *dhaB2* and *dhaT* genes from *C. butyricum* and comprising attenuated glycerol kinase activity.
2. The mutant strain according to claim 1, wherein said glycerol kinase is encoded by the gene *glpK* at locus CA_C1321 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.
3. The mutant strain according to claim 2 comprising at least one of the following mutations in the gene *glpK* at locus CA_C1321:
- a. Mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine,
 - b. Mutation of the codon at nucleotide positions 1461486 to 1461488 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid histidine in position 22 to be replaced with tyrosine,
 - c. Mutation of the codon at nucleotide positions 1462818 to 1462820 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid valine in position 466 to be replaced by methionine.
4. The mutant strain according to claim 3, comprising at least at locus CA_C1321, the mutation of the codon at nucleotide positions 1462461 to 1462463 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437, for having amino acid alanine in position 347 to be replaced with threonine.
5. The mutant strain according to anyone of the preceding claims, further comprising attenuated hydrogen dehydrogenase activity, preferably inactivated hydrogen dehydrogenase activity.
6. The mutant strain according to claim 5, wherein said hydrogenase is encoded by the gene *hydA* at locus CA_C0028 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.
7. The mutant strain according to claim 6 comprising at least deletion of the nucleotide C at the nucleotide position 39233 in the gene *hydA* at locus CA_C0028 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.

8. The mutant strain according to anyone of the preceding claims, further comprising enhanced glycerol uptake facilitator permease activity.
- 5 9. The mutant strain according to claim 8, wherein said glycerol uptake facilitator protein is encoded by the gene *glpF* at locus CA_C0770 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.
- 10 10. The mutant strain according to claim 9 comprising at least replacement of the nucleotide G at the nucleotide position 892875 in the gene *glpF* at locus CA_C0770 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.
11. The mutant strain according to anyone of the preceding claims, further comprising attenuated electron transfer flavoprotein subunit alpha activity.
- 15 12. The mutant strain according to claim 11, wherein said electron transfer flavoprotein subunit alpha is encoded by the gene *effA* at locus CA_C2709 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.
- 20 13. The mutant strain according to claim 12 comprising at least replacement of the nucleotide T at the nucleotide position 2833384 in the gene *effA* at locus CA_C2709 in the *C. acetobutylicum* genome ATCC824, Genbank AE001437.
- 25 14. A consortium of *Clostridium* comprising the mutant strain according to anyone of claims 1 to 13.
- 30 15. The consortium of *Clostridium* according to claim 14 comprising the mutant strain *Clostridium acetobutylicum* as defined in anyone of claims 1 to 13 and at least one strain of *Clostridium sporogenes* and/or at least one strain of *Clostridium sphenoides*.
- 35 16. A method for the production of 1,3-propanediol and butyric acid comprising culturing the mutant strain as defined in anyone of claims 1 to 13 or a consortium as defined in claim 14, in an appropriate culture medium comprising at least glycerol and recovering the 1,3-propanediol and/or the butyric acid produced from the culture medium.
17. The method for the production of 1,3-propanediol and butyric acid according to claim 16, wherein 1,3-propanediol and/or butyric acid produced is further purified.

18. The method for the production of 1,3-propanediol and butyric acid according to anyone of claims 16 to 17, wherein glycerol concentration in the culture medium is from 90 to 120 g/L, preferably about 105 g/L.
- 5 19. The method for the production of 1,3-propanediol and butyric acid according to anyone of claims 16 to 18, wherein the culture medium is exempt of organic nitrogen, and preferably is a synthetic medium without addition of organic nitrogen, more preferably without yeast extract.