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(54) Title: PROCESS FOR THE BIOLOGICAL PRODUCTION OF N-BUTANOL WITH HIGH YIELD

(57) Abstract: The present invention provides a method for the biological production of n-butanol at high yield from a fermentable carbon source. In one aspect of the present invention, a process for the conversion of glucose to n-butanol is achieved by the use of a recombinant organism comprising a host C. acetobutlicum transformed i) to eliminate the butyrate pathway ii) to eliminate the acetone pathway iii) to eliminate the lactate pathway and iv) to eliminate the acetate pathway. In another aspect of the present invention, the hydrogen flux is decreased and the reducing power redirected to n-butanol production by attenuating the expression of the hydrogenase gene. Optionally the n-butanol produced can be eliminated during the fermentation by gas striping and further purified by distillation.

Process for the biological production of n-Butanol with high yield

FIELD OF INVENTION

The invention comprises a process for the bioconversion of a fermentable carbon source to n-butanol at high yield by a metabolically engineered microorganism.

BACKGROUND OF THE INVENTION

n-Butanol is a colorless, neutral liquid of medium volatility with restricted miscibility (about 7-8%) in water, but freely miscible with all common solvents such as glycols, ketones, alcohol, aldehydes, ethers, and aromatic and aliphatic hydrocarbons. n-Butanol is used i) to make other chemicals, ii) as a solvent and iii) as an ingredient in formulated products such as cosmetics. The major uses of n-butanol as a feed-stock are in the synthesis of acrylate/methacrylate esters, glycol ethers, n-Butyl acetate, amino resins and n-Butylamines. Currently more than 9 millions tons of n-Butanol are consumed annually in the world.

More recently it has been shown that n-butanol is a better biofuel than ethanol due to lower vapour pressure, higher energy content (closer to that of gasoline) and lesser susceptibility to separation in the presence of water. Furthermore, n-butanol can be blended at higher concentrations than ethanol for use in standard vehicle engines and it does not require automakers to compromise on performance to meet environmental regulations; it is also suitable for transport in pipelines and as a result it has the potential to be introduced into gasoline quickly and avoid the need for additional large-scale supply infrastructures.

n-butanol can be produced as an acetone/n-butanol/ethanol (ABE) mixture by the fermentation of carbohydrate by solventogenic *Clostridia*. The ABE fermentations are biphasic. During the first acidogenic phase, high growth rate is accompanied by acetic and butyric acids production. In the second solventogenic phase growth rate decrease and the solvents (ABE) are produced with the concomitant consumption of the organic acids produced in the first phase. Carbon dioxide and hydrogen are produced throughout the fermentation.

The biological production of n-butanol, presented in figure 1, requires the formation of butyryl-CoA as an intermediate which can be reduced, depending on the physiological conditions, by two different bi-functional aldehyde-alcohol dehydrogenases encoded by *adhE1* and *adhE2*. Butyryl-CoA can also be converted to butyric acid by a phospho-transbutyrylase and a butyrate kinase encoded respectively by the *ptb* and *buk* genes. Acetone is produced from aceto-acetyl-CoA (an intermediate in the production of butyryl-CoA) by a CoA-transferase and an acetoacetate decarboxylase encoded respectively by the *ctfAB* and *adc* genes. Hydrogen is produced by an iron only hydrogenase encoded by the *hydA* gene. When cultures are performed in the presence of

carbon monoxide, a hydrogenase inhibitor, n-butanol, ethanol and lactate are the main fermentation products. Lactate is produced from pyruvate by a lactate dehydrogenase encoded by the *ldh* gene.

Clostridium acetobutylicum strains with an inactivated *buk* gene (obtained by single crossing over with a non-replicable plasmid) have already been described in the article (Green et al., 1996). The non-replicable vector pJC4BK, with a 0.8 kb internal buk fragment was integrated into the chromosomal buk gene which leaded to an inactivation of the endogenous gene. The obtained strain was named "mutant PJC4BK"from the name of the plasmid. As precised in this article, this gene integration did not completely eliminate enzyme activity nor butyrate formation due to the instability of this type of gene inactivation that can reverse to wild type by plasmid excision. This mutant strain was then used in several studies (Green and Bennett, 1998; Desai and Harris, 1999; Harris et al., 2000).

Traditionally, the commercial ABE fermentation was conducted only in a batch mode due to continuous cultures instability of the producing *Clostridia*. Several solvent yielding fermentation processes have been described. These processes yield n-butanol, acetone and ethanol in a ratio of 6:3:1. Solvent yields of 29-34% (18-25% for n-butanol only) of fermentable carbon source have been reported in the literature. A total solvent concentration of 16-24 g/l and a n-butanol concentration of 10-14 g/l is generally the limit due to toxicity of n-butanol produced. However, these low titers of solvent no longer seem to be an economical limitation to the process as it has recently been demonstrated that solvents can be recovered during fermentation by the use of the "low cost" gas striping technology.

The problem to be solved by the present invention is to obtain a stable mutant strain with no butyrate kinase activity , that could be cultureD for several generations without any possibility of reversion to the wild type genotype. This strain would be useful for the biological production of n-butanol at high yield, from an inexpensive carbon substrate such as glucose or other sugars, by genetically stable cultures of *Clostridia*. The number of biochemical steps to inactivate and the complexity of the regulation of the metabolism necessitate, for an industrial feasible process of n-butanol production, the use of a metabolically engineered whole cell catalyst.

SUMMARY OF THE INVENTION

Applicants have solved the stated problem and the present invention provides a method for bioconverting a fermentable carbon source to n-butanol as a major product by

genetically stable cultures of *Clostridia*. Glucose is used as a model substrate and recombinant *Clostridium acetobutylicum* is used as the model host. In one aspect of this invention, a stable recombinant *C. acetobutylicum* unable to metabolize butyryl-CoA to butyrate is constructed by deleting the gene coding for the butyrate kinase (*buk*). In another aspect of this invention, a recombinant *C. acetobutylicum* unable to produce acetone is constructed by deleting the genes coding for the CoA-transferase (*ctfAB*). In a further aspect of this invention a recombinant strain unable to produce lactate is constructed by deleting the gene coding for the lactate dehydrogenase (*ldh*). Furthermore, a recombinant *C. acetobutylicum* unable to produce acetate is constructed by deleting the genes coding for the phosphotransacetylase and/or acetate kinase (*pta* and *ack*). In a final aspect of this invention, the flux of hydrogen production is decreased and then the flux of reducing equivalent redirected toward n-butanol production by attenuating the gene encoding the hydrogenase (*hydA*).

The present invention may be generally applied to include any carbon substrate that is readily converted to acetyl-coA.

Accordingly it is an object of the present invention to provide a recombinant organism, useful for the production of n-butanol comprising: (a) at least deletion of one of the two genes involved in the conversion of butyryl-CoA to butyrate and (b) at least deletion of one of the two genes encoding the CoA-transferase activity. Optionally the recombinant organism may comprise i) inactivating mutations in endogenous genes selected from the group consisting of: (a) a gene encoding a polypeptide having lactate dehydrogenase activity (b) a gene encoding a polypeptide having phospho-transacetylase or actate kinase activity and ii) attenuation in a gene encoding a polypeptide having hydrogenase activity.

In another embodiment the invention provides a stable process for the production of n-butanol at high yield from a recombinant organism comprising: (a) contacting the recombinant organism of the present invention with at least one carbon source selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and single-carbon substrates whereby n-butanol is produced; optionally (b) recovering the n-butanol during the production through a step of gas striping and (c) purifying n-butanol from the condensate by distillation.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawing which is incorporated in and constitutes a part of this specification exemplifies the invention and together with the description, serve to explain the principles of this invention.

Figure 1 depicts the genetic engineering of central metabolism in the development of a butanol production system from carbohydrates.
1 : Pyruvate-ferredoxin oxydoreductase ; 2 : Thiolase ; 3 : β-Hydroxybutyryl-CoA dehydrogenase ; 4 : Crotonase ; 5 : Butyryl-CoA dehydrogenase ; 6 : Lactate dehydrogenase ; 7 : Phospho-transacetylase ; 8 : Acetate kinase ; 9 : Acetaldehyde deshydrogenase ; 10 : Ethanol dehydrogenase ; 11 : CoA transférase (Acetoacetyl-CoA :acetate/butyrate : CoA transferase) ; 12 : Acetoacetate decarboxylase ; 13 : Phospho-transbutyrylase ; 14 : Butyrate kinase ; 15 : Butyraldehyde-Butanol dehydrogenase ; 16 : hydrogenase.

DETAILED DESCRIPTION OF THE INVENTION

As used herein the following terms may be used for interpretation of the claims and specification.

The term “microorganism” refers to all kind of unicellular organisms, including prokaryotic organisms like bacteria, and eukaryotic organisms like yeasts.
The expression “appropriate culture medium” refers to a culture medium adapted for the used microorganism as it is well known by the man skilled in the art.

The term "carbon substrate" or "source of carbon" means any carbon source capable of being metabolized by a microorganism wherein the substrate contains at least one carbon atom. In particular it may be glucose, sucrose, mono- or oligosaccharides, starch or its derivatives, glycerol, and their mixtures thereof.

The term “attenuation” refers to a decreased expression of a gene or a decreased activity of the protein, product of the gene. The man skilled in the art knows numerous means to obtain this result, and for example:

- Introduction of a mutation into the gene, decreasing the expression level of this gene, or the level of activity of the encoded protein.
- Replacement of the natural promoter of the gene by a low strength promoter, resulting in a lower expression.
- Use of elements destabilizing the corresponding messenger RNA or the protein.
- Deletion of the gene if no expression is needed.

The term "deleted gene" means that a substantial part of the coding sequences of said gene was removed. Preferably, at least 50% of the coding sequence was removed, and more preferably at least 80%.

In the description of the present invention, enzymes are identified by their specific activities. This definition thus includes all polypeptides that have the defined specific activity also present in other organisms, more particularly in other microorganisms. Often enzymes with similar activities can be identified by their grouping to certain families defined as PFAM or COG.

PFAM (protein families database of alignments and hidden Markov models; http://www.sanger.ac.uk/Software/Pfam/) represents a large collection of protein sequence alignments. Each PFAM makes it possible to visualize multiple alignments, see protein domains, evaluate distribution among organisms, gain access to other databases, and visualize known protein structures.

COGs (clusters of orthologous groups of proteins; http://www.ncbi.nlm.nih.gov/COG/) are obtained by comparing protein sequences from 43 fully sequenced genomes representing 30 major phylogenic lines. Each COG is defined from at least three lines, which permits the identification of former conserved domains.

The means of identifying homologous sequences and their percentage homologies are well known to those skilled in the art, and include in particular the BLAST programs, which can be used from the website http://www.ncbi.nlm.nih.gov/BLAST/ with the default parameters indicated on that website. The sequences obtained can then be exploited (e.g., aligned) using, for example, the programs CLUSTALW (http://www.ebi.ac.uk/clustalw/) or MULTALIN (http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl), with the default parameters indicated on those websites

Using the references given on GenBank for known genes, those skilled in the art are able to determine the equivalent genes in other organisms, bacterial strains, yeasts, fungi, mammals, plants, etc. This routine work is advantageously done using consensus sequences that can be determined by carrying out sequence alignments with genes derived from other microorganisms, and designing degenerate probes to clone the corresponding gene in another organism. These routine methods of molecular biology are well known to those skilled in the art, and are described, for example, in Sambrook *et al.* (Molecular Cloning: a Laboratory Manual. 2nd ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York, 1989.).

The present invention provides a method for the fermentative batch or continuous production of n-butanol by culturing a microorganism in an appropriate culture medium comprising a carbon source and the simultaneous recovery of n-butanol from the culture

medium wherein at least one gene involved in butyrate formation is deleted in the microorganism.

A specific embodiment of the invention provides a method wherein the microorganism is modified to be unable to convert butyryl-CoA to butyrate due to the deletion of at least one gene encoding for phospho-transbutyrylase (*ptb*) or butyrate kinase (*buk*). Deletion of genes in *Clostridia* can be done using the method recently described in patent application PCT/EP2006/066997 allowing the i) replacement of the gene to delete with an erythromycin resistance gene and ii) removal of the erythromycin resistance gene with a recombinase.

In another embodiment of the invention, the microorganism is unable to produce acetone due to an attenuation or a deletion of at least one of the gene involved in acetone formation. Preferably, this gene is encoding for the enzyme CoA-transferase (*ctfAB*) or acetoacetate decarboxylase (*adc*). Deletion of one of these genes can be done using the method recently described in patent application PCT/EP2006/066997.

In a further embodiment of the invention, the microorganism used in the method of the invention is unable to produce lactate. In particular this can be due to a deletion of the gene *ldh* encoding for lactate dehydrogenase. Deletion of *ldh* can be done using the method recently described in patent application PCT/EP2006/066997.

In another embodiment, the microorganism is modified in such a way to be unable to produce acetate. This result can be achieved by deletion of at least one involved in acetate formation. Preferably this gene is selected among the group consisting of the genes encoding for phospho-transacetylase (*pta*) or acetate kinase (*ack*). Deletion of one of these genes can be done using the method recently described in patent application PCT/EP2006/066997.

An embodiment of the invention also provides a microorganism with a decreased flux of hydrogen production and then a redirection of the flux of reducing equivalent toward n-butanol production; this can be done by attenuating the gene encoding the hydrogenase (*hydA*), an enzyme that provides a sink for reducing equivalent in the form of hydrogen production. Attenuation of *hydA* can be done by replacing the natural promoter by a low strength promoter or by element destabilizing the corresponding messenger RNA or the protein. If needed, complete attenuation of the gene can also be achieved by a deletion of the corresponding DNA sequence.

Preferably, the used microorganism is selected among the group consisting of *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* or *C. saccharobutylicum.*

In another embodiment of the invention, the culture is continuous and stable.

In another embodiment, the method according to the invention comprises the following steps:

(a) contacting a microorganism with at least one carbon source selected from the group consisting of glucose, xylose, arabinose, sucrose, monosaccharides, oligosaccharides, polysaccharides, cellulose, xylan, starch or its derivatives and glycerol, whereby n-butanol is produced

(b) Recovering the n-butanol during the fermentation by gas striping and

(c) Isolation of n-butanol from the condensate by distillation.

Those skilled in the art are able to define the culture conditions for the microorganisms according to the invention. In particular the clostridia are fermented at a temperature between 20°C and 55°C, preferentially between 25°C and 40°C, and more specifically about 35°C for *C. acetobutylicum*.

The fermentation is generally conducted in fermentors with an inorganic culture medium of known defined composition adapted to the bacteria used, containing at least one simple carbon source, and if necessary a co-substrate necessary for the production of the metabolite.

The invention is also related to the microorganism as described previously. Preferably, this microorganism is selected among the group consisting of *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* or *C. saccharobutylicum.*

EXAMPLE 1

Construction of strains unable to produce butyrate: Clostridium acetobutylicum Δcac1515 Δupp Δbuk

To delete the *buk* gene, the homologous recombination strategy described by Croux & Soucaille (2006) in patent application PCT/EP2006/066997 is used. This strategy allows the insertion of an erythromycin resistance cassette, while deleting most of the gene concerned. The *buk* deletion cassette in pCons::upp was constructed as follows.

Table 1 : primers sequences

Name		Primer sequences
Buk 1	SEQ ID N°1	aaaaggatcc**tagtaaaagggagtgtacgaccagtg**
Buk 2	SEQ ID N°2	ggggtcgcgaaaaaaggggg**gattattagtaatctatacatgttaacattcctccac**
Buk 3	SEQ ID N°3	ccccctttttcgcgacccc**acttcttgcacttgcagaaggtggac**
Buk 4	SEQ ID N°4	aaaaggatcc**tctaaattctgcaatatatgccccccc**
Buk 0	SEQ ID N°5	ataacaggatatatgctctctgacgcgg
Buk 5	SEQ ID N°6	gatcatcactcattttaaacatggggcc

Two DNA fragments surrounding *buk* were PCR amplified with the Pwo polymerase with total DNA from *C. acetobutylicum* as template and two specific couples of olignonucleotides. With the couples of primers BUK 1-BUK 2 and BUK 3–BUK 4, two DNA fragments were respectively obtained. Both primers BUK 1 and BUK 4 introduce a BamHI site while primers BUK 2 and BUK 3 have a complementary region which introduces a NruI site. DNA fragments BUK 1-BUK 2 and BUK 3–BUK 4 were joined in a PCR fusion experiment with primers BUK 1 and BUK 4 and the resulting fragment was cloned in pCR4-TOPO-Blunt to yield pTOPO :buk. At the unique StuI site of pTOPO :buk, an antibiotic resistance MLS gene with FRT sequences on both sides was introduced from the StuI fragment of pUC18-FRT-MLS2. The BUK deletion cassette obtained after BamHI digestion of the resulting plasmid was cloned into pCons::upp at the BamHI site to yield the pREPΔBUK::upp plasmid.

The pREPΔBUK::upp plasmid was used to transform by electroporation *C. acetobutylicum* MGCΔ*cac15*Δ*upp* strain. After selection on Petri plate for clones resistant to erythromycin (40 µg/ml), one colony was cultured for 24 hours in liquid synthetic medium with erythromycin at 40 µg/ml and 100 µl of undiluted culture was plated on RCA with erythromycin at 40 µg/ml and 5-FU at 400 µM. Colonies resistant to both erythromycin and 5-FU were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml to select clones where 5-FU resistance is also associated with thiamphenicol sensitivity. The genotype of clones resistant to erythromycin and sensitive to thiamphenicol was checked by PCR analysis (with primers BUK 0 and BUK 5 located outside of the *buk* deletion cassette). The Δ*cac15*Δ*upp*Δ*buk::mls*^{R} strain which have lost pREPΔbuk::upp was isolated.

The Δ*cac15*Δ*upp*Δ*buk::mls*^{R} strain was transformed with pCLF1.1 vector expressing the *Flp1* gene encoding the Flp recombinase from *S. cerevisiae*. After transformation and selection for resistance to thiamphenicol (50 µg/ml) on Petri plate, one colony was cultured on synthetic liquid medium with thiamphenicol at 50 µg/ml and appropriate dilutions were plated on RCA with thiamphenicol at 50 µg/ml. Thiamphenicol resistant clones were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml. The genotype of clones with erythromycin sensitivity and thiamphenicol resistance was checked by PCR analysis with primers BUK 0 and BUK 5. Two successive 24 hours cultures of the Δ*cac15*Δ*upp*Δ*buk* strain with erythromycin sensitivity and

thiamphenicol resistance were carried out in order to lose pCLF1.1. The Δ*cac15*Δ*upp*Δ*buk* strain which has lost pCLF1.1 was isolated according to its sensitivity to both erythromycin and thiamphenicol.

EXAMPLE 2

Construction of strains unable to produce butyrate and acetone: C. acetobutylicum Δcac1515 Δupp Δbuk ΔctfAB

To delete the *ctfAB* genes, the homologous recombination strategy described by Croux & Soucaille (2006) in patent application PCT/EP2006/066997 is used. This strategy allows the insertion of an erythromycin resistance cassette, while deleting most of the genes concerned. The *ctfAB* deletion cassette in pCons::upp was constructed as follows.

Table 2 : primers sequences

Name		Primer sequences
Ctf 1	SEQ ID N°7	aaaaggatcc**cagacactataatagctttaggtggtacccc**
Ctf 2	SEQ ID N°8	ggggaggcctaaaaaggggg**attataaaaagtagttgaaatatgaaggtttaaggttg**
Ctf 3	SEQ ID N°9	ccccctttttaggcctcccc**atatccaatgaacttagacccatggctg**
Ctf 4	SEQ ID N°10	aaaaggatcc**gtgttataatgtaaatataaataaataggactagaggcg**
Ctf 0	SEQ ID N°11	taccaccttctttcacgcttggctgcgg
Ctf 5	SEQ ID N°12	tatttaaagaggcattatcaccagagcg

Two DNA fragments surrounding *ctfAB* were PCR amplified with the Pwo polymerase with total DNA from *C. acetobutylicum* as template and two specific couples of olignonucleotides. With the couples of primers CTF 1-CTF 2 and CTF 3–CTF 4, two DNA fragments were respectively obtained. Both primers CTF 1 and CTF 4 introduce a BamHI site while primers CTF 2 and CTF 3 have a complementary region which introduces a StuI site. DNA fragments CTF 1-CTF 2 and CTF 3–CTF 4 were joined in a PCR fusion experiment with primers CTF 1 and CTF 4 and the resulting fragment was cloned in pCR4-TOPO-Blunt to yield pTOPO :CTF. At the unique StuI site of pTOPO :CTF, an antibiotic resistance MLS gene with FRT sequences on both sides was introduced from the StuI fragment of pUC18-FRT-MLS2. The UPP deletion cassette obtained after BamHI digestion of the resulting plasmid was cloned into pCons::upp at the BamHI site to yield the pREPΔCTF::upp plasmid.

The pREPΔCTF::upp plasmid was used to transform by electroporation *C. acetobutylicum* MGCΔ*cac15*Δ*upp*Δ*buk* strain. After selection on Petri plate for clones resistant to

erythromycin (40 µg/ml), one colony was cultured for 24 hours in liquid synthetic medium with erythromycin at 40 µg/ml and 100 µl of undiluted culture was plated on RCA with erythromycin at 40 µg/ml and 5-FU at 400 µM. Colonies resistant to both erythromycin and 5-FU were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml to select clones where 5-FU resistance is also associated with thiamphenicol sensitivity. The genotype of clones resistant to erythromycin and sensitive to thiamphenicol was checked by PCR analysis (with primers CTF 0 and CTF 5 located outside of the *ctfAB* deletion cassette). The Δ*cac15*Δ*upp*Δ*buk* Δ*ctfAB::mls*^{R} strain which have lost pREPΔCTF::upp was isolated.

The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB::mls*^{R} strain was transformed with pCLF1.1 vector expressing the *Flp1* gene encoding the Flp recombinase from *S. cerevisiae*. After transformation and selection for resistance to thiamphenicol (50 µg/ml) on Petri plate, one colony was cultured on synthetic liquid medium with thiamphenicol at 50 µg/ml and appropriate dilutions were plated on RCA with thiamphenicol at 50 µg/ml. Thiamphenicol resistant clones were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml. The genotype of clones with erythromycin sensitivity and thiamphenicol resistance was checked by PCR analysis with primers CTF 0 and CTF 5. Two successive 24 hours cultures of the Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB* strain with erythromycin sensitivity and thiamphenicol resistance were carried out in order to lose pCLF1.1. The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB* strain which has lost pCLF1.1 was isolated according to its sensitivity to both erythromycin and thiamphenicol.

EXAMPLE 3

Construction of strains unable to produce butyrate, acetone and lactate: C. acetobutylicum Δcac1515 Δupp Δbuk ΔctfAB Δldh

To delete the *ldh* gene, the homologous recombination strategy described by Croux & Soucaille (2006) in patent application PCT/EP2006/066997 is used. This strategy allows the insertion of an erythromycin resistance cassette, while deleting most of the genes concerned. The *ldh* deletion cassette in pCons::upp was constructed as follows.

Table 3 : primers sequences

Name		Primer sequences
Ldh 1	SEQ ID N°13	AAAAGGATCCGCTTTAAAATTTGGAAAGAGGAAGTTGTG
Ldh 2	SEQ ID N°14	GGGGAGGCCTAAAAAGGGGGTTAGAAATCTTTAAAAATTTC TCTATAGAGCCCATC
Ldh 3	SEQ ID N°15	CCCCCTTTTTTAGGCCTCCCCGGTAAAAGACCTAAACTCCAAGG GTGGAGGCTAGGTC
Ldh 4	SEQ ID N°16	AAAAGGATCCCCCATTGTGGAGAATATTCCAAAGAAGAAAA TAATTGC
Ldh 0	SEQ ID N°17	CAGAAGGCAAGAATGTATTAAGCGGAAATGC
Ldh 5	SEQ ID N°18	CTTCCCATTATAGCTCTTATTCACATTAAGC

Two DNA fragments surrounding *ldh* (*CAC267*) were PCR amplified with the Pwo polymerase with total DNA from *C. acetobutylicum* as template and two specific couples of olignonucleotides. With the couples of primers LDH 1-LDH 2 and LDH 3–LDH 4, 1135 bp and 1177 bp DNA fragments were respectively obtained. Both primers LDH 1 and LDH 4 introduce a BamHI site while primers LDH 2 and LDH 3 have a complementary region which introduces a StuI site. DNA fragments LDH 1-LDH 2 and LDH 3–LDH 4 were joined in a PCR fusion experiment with primers LDH 1 and LDH 4 and the resulting fragment was cloned in pCR4-TOPO-Blunt to yield pTOPO :LDH. At the unique StuI site of pTOPO :LDH, an antibiotic resistance MLS gene with FRT sequences on both sides was introduced from the 1372 bp StuI fragment of pUC18-FRT-MLS2. The UPP deletion cassette obtained after BamHI digestion of the resulting plasmid was cloned into pCons::upp at the BamHI site to yield the pREPΔLDH::upp plasmid.

The pREPΔLDH::upp plasmid was used to transform by electroporation *C. acetobutylicum* MGCΔ*cac15*Δ*upp*Δ*buk*Δ*ctfAB* strain. After selection on Petri plate for clones resistant to erythromycin (40 µg/ml), one colony was cultured for 24 hours in liquid synthetic medium with erythromycin at 40 µg/ml and 100 µl of undiluted culture was plated on RCA with erythromycin at 40 µg/ml and 5-FU at 400 µM. Colonies resistant to both erythromycin and 5-FU were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml to select clones where 5-FU resistance is also associated with thiamphenicol sensitivity. The genotype of clones resistant to erythromycin and sensitive to thiamphenicol was checked by PCR analysis (with primers LDH 0 and LDH 5 located outside of the *ldh* deletion cassette). The Δ*cac15*Δ*upp*Δ*buk* Δ*ctfAB* Δ*ldh::mls*^{R} strain which have lost pREPΔLDH::upp was isolated.

The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh::mls*^{R} strain was transformed with pCLF1.1 vector expressing the *Flp1* gene encoding the Flp recombinase from *S. cerevisiae*. After transformation and selection for resistance to thiamphenicol (50 µg/ml) on Petri plate, one colony was cultured on synthetic liquid medium with thiamphenicol at 50 µg/ml and appropriate dilutions were plated on RCA with thiamphenicol at 50 µg/ml. Thiamphenicol resistant clones were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml. The genotype of clones with erythromycin sensitivity and thiamphenicol resistance was checked by PCR analysis with primers LDH 0 and LDH 5. Two successive 24 hours cultures of the Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB* Δ*ldh* strain with erythromycin sensitivity and thiamphenicol resistance were carried out in order to lose pCLF1.1. The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh* strain which has lost pCLF1.1 was isolated according to its sensitivity to both erythromycin and thiamphenicol.

EXAMPLE 4

Construction of strains unable to produce butyrate, acetone, lactate and acetate: C. acetobutylicum Δcac1515 Δupp Δbuk ΔctfAB Δldh Δpta-ack

To delete the *pta* and *ack* genes, the homologous recombination strategy described by Croux & Soucaille (2006) in patent application PCT/EP2006/066997 is used. This strategy allows the insertion of an erythromycin resistance cassette, while deleting most of the genes concerned. The *pta-ack* deletion cassette in pCons::upp was constructed as follows.

Table 4 : primers sequences

Name		Primer sequences
PA 1	SEQ ID N°19	aaaaggatcc**tattataacagtcaaacccaataaaatactggg**
PA 2	SEQ ID N°20	ggggaggcctaaaaaggggg**ttaatccatttgtattttctcccttcataatgcc**
PA 3	SEQ ID N°21	ccccctttttaggcctcccc**tttattttgcatgcttatataataaattatggctgcg**
PA 4	SEQ ID N°22	aaaaggatcc**gcttttccttctttttacaagatttaaagcc**
PA 0	SEQ ID N°23	cacttttatttatcaagctgtaggcc
PA 5	SEQ ID N°24	tataccttttgaacctaggaaaggc

Two DNA fragments surrounding *pta-ack* were PCR amplified with the Pwo polymerase with total DNA from *C. acetobutylicum* as template and two specific couples of oligonucleotides. With the couples of primers PA 1-PA 2 and PA 3–PA 4, two DNA fragments were respectively obtained. Both primers PA 1 and PA 4 introduce a BamHI site

while primers PA 2 and PA 3 have a complementary region which introduces a StuI site. DNA fragments PA 1-PA 2 and PA 3–PA 4 were joined in a PCR fusion experiment with primers PA 1 and PA 4 and the resulting fragment was cloned in pCR4-TOPO-Blunt to yield pTOPO :PA. At the unique StuI site of pTOPO :PA, an antibiotic resistance MLS gene with FRT sequences on both sides was introduced from the StuI fragment of pUC18-FRT-MLS2. The UPP deletion cassette obtained after BamHI digestion of the resulting plasmid was cloned into pCons::upp at the BamHI site to yield the pREPΔPA::upp plasmid.

The pREPΔPA::upp plasmid was used to transform by electroporation *C. acetobutylicum* MGCΔ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh* strain. After selection on Petri plate for clones resistant to erythromycin (40 µg/ml), one colony was cultured for 24 hours in liquid synthetic medium with erythromycin at 40 µg/ml and 100 µl of undiluted culture was plated on RCA with erythromycin at 40 µg/ml and 5-FU at 400 µM. Colonies resistant to both erythromycin and 5-FU were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml to select clones where 5-FU resistance is also associated with thiamphenicol sensitivity. The genotype of clones resistant to erythromycin and sensitive to thiamphenicol was checked by PCR analysis (with primers PA 0 and PA 5 located outside of the *pta-ack* deletion cassette). The Δ*cac15*Δ*upp*Δ*buk* Δ*ctfAB*Δ*ldh*Δ*pta-ack::mls*^{R} strain which have lost pREPΔPA::upp was isolated.

The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh* Δ*pta-ack::mls*^{R} strain was transformed with pCLF1.1 vector expressing the *Flp1* gene encoding the Flp recombinase from *S. cerevisiae*. After transformation and selection for resistance to thiamphenicol (50 µg/ml) on Petri plate, one colony was cultured on synthetic liquid medium with thiamphenicol at 50 µg/ml and appropriate dilutions were plated on RCA with thiamphenicol at 50 µg/ml. Thiamphenicol resistant clones were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml. The genotype of clones with erythromycin sensitivity and thiamphenicol resistance was checked by PCR analysis with primers PA 0 and PA 5. Two successive 24 hours cultures of the Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh*Δ*pta-ack* strain with erythromycin sensitivity and thiamphenicol resistance were carried out in order to lose pCLF1.1. The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh*Δ*pta-ack* strain which has lost pCLF1.1 was isolated according to its sensitivity to both erythromycin and thiamphenicol.

EXAMPLE 5

Construction of strains with lower hydrogen production: C. acetobutylicum Δcac1515 Δupp Δbuk ΔctfAB Δldh ΔhydA

To delete the *hydA* gene, the homologous recombination strategy described by Croux & Soucaille (2006) in patent application PCT/EP2006/066997 is used. This strategy allows the insertion of an erythromycin resistance cassette, while deleting most of the genes concerned. The *hydA* deletion cassette in pCons::upp was constructed as follow.

Table 5 : primers sequences

Name		Primer sequences
Hyd 1	SEQ ID N°25	AAAA*GGATCC*GCCTCTTCTGTATTATGCAAGGAAAGC AGCTGC
Hyd 2	SEQ ID N°26	GGGG*AGGCCT*AAAAAGGGGGTATATAAAATAAATGTG CCTTAACATC
		TAAGTTGAGGCC
Hyd 3	SEQ ID N°27	CCCCCTTTTT*AGGCCT*CCCCGTTTATCCTCCCAAAATGT AAAATATAA
		TTAAAATATATTAATAAACTTCGATTAATAAACTTCG
Hyd 4	SEQ ID N°28	AAAA*GGATCC*CCTTTTAGCGTATAAAGTTTTATATAGC TATTG
Hyd 0	SEQ ID N°29	CATGTTCTATTGTTACTATGGAAGAGGTAGTAG
Hyd 5	SEQ ID N°30	GCAGTTATTATAAATGCTGCTACTAGAGC

Two DNA fragments surrounding *hydA* (*CAC028*) were PCR amplified with the Pwo polymerase with total DNA from *C. acetobutylicum* as template and two specific couples of olignonucleotides. With the couples of primers HYD 1-HYD 2 and HYD 3–HYD 4, 1269 bp and 1317 bp DNA fragments were respectively obtained. Both primers HYD 1 and HYD 4 introduce a BamHI site while primers HYD 2 and HYD 3 have a complementary region which introduces a StuI site. DNA fragments HYD 1-HYD 2 and HYD 3–HYD 4 were joined in a PCR fusion experiment with primers HYD 1 and HYD 4 and the resulting fragment was cloned in pCR4-TOPO-Blunt to yield pTOPO :HYD. At the unique StuI site of pTOPO :HYD, an antibiotic resistance MLS gene with FRT sequences on both sides was introduced from the 1372 bp StuI fragment of pUC18-FRT-MLS2. The UPP deletion cassette obtained after BamHI digestion of the resulting plasmid was cloned into pCons::upp at the BamHI site to yield the pREPΔHYD::upp plasmid.

The pREPΔHYD::upp plasmid was used to transform by electroporation *C. acetobutylicum* MGCΔ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh* strain. After selection on Petri plate for clones resistant

to erythromycin (40 µg/ml), one colony was cultured for 24 hours in liquid synthetic medium with erythromycin at 40 µg/ml and 100 µl of undiluted culture was plated on RCA with erythromycin at 40 µg/ml and 5-FU at 400 µM. Colonies resistant to both erythromycin and 5-FU were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml to select clones where 5-FU resistance is also associated with thiamphenicol sensitivity. The genotype of clones resistant to erythromycin and sensitive to thiamphenicol was checked by PCR analysis (with primers HYD 0 and HYD 5 located outside of the *hydA* deletion cassette). The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh*Δ*hydA::mls*^{R} strain which have lost pREPΔHYD::upp was isolated.

The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh*Δ*hydA::mls*^{R} strain was transformed with pCLF1.1 vector expressing the *Flp1* gene encoding the Flp recombinase from *S. cerevisiae*. After transformation and selection for resistance to thiamphenicol (50 µg/ml) on Petri plate, one colony was cultured on synthetic liquid medium with thiamphenicol at 50 µg/ml and appropriate dilutions were plated on RCA with thiamphenicol at 50 µg/ml. Thiamphenicol resistant clones were replica plated on both RCA with erythromycin at 40 µg/ml and RCA with thiamphenicol at 50 µg/ml. The genotype of clones with erythromycin sensitivity and thiamphenicol resistance was checked by PCR analysis with primers HYD 0 and HYD 5. Two successive 24 hours cultures of the Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh*Δ*hydA* strain with erythromycin sensitivity and thiamphenicol resistance were carried out in order to lose pCLF1.1. The Δ*cac15*Δ*upp*Δ*buk*Δ*ctfAB*Δ*ldh*Δ*hydA* strain which has lost pCLF1.1 was isolated according to its sensitivity to both erythromycin and thiamphenicol.

EXAMPLE 6

Batch fermentation of n-butanol producing strains.

Strains were initially analyzed in anaerobic flask cultures in the synthetic medium described by Soni et al (Soni et al, 1987, *Appl. Microbiol.Biotechnol.* **27**:1-5) supplemented with 2.5 g/l of ammonium acetate. An overnight culture at 35°C was used to inoculate a 30 ml culture to an OD600 of 0.05. After incubation of the culture for 3 days at 35°C, glucose, organic acids and solvents were analyzed by HPLC using a Biorad HPX 97H column for the separation and a refractometer for the detection.

Strains with the correct phenotype were subsequently tested under production conditions in 300 ml fermentors (DASGIP) using an anaerobic batch protocol.

For this purpose the fermentor was filled with 250 ml of synthetic medium, sparged with nitrogen for 30 min and inoculated with 25 ml of preculture to an optical density (OD600nm) between 0.05 and 0.1.

The temperature of the culture was maintained constant at 35 °C and the pH was permanently adjusted at 5.5 using an NH_4OH solution. The agitation rate was maintained at 300 rpm during the fermentation.

EXAMPLE 7

Continuous fermentation of n-butanol producing strains.

The best n-butanol producing strain was analyzed in chemostat cultures in the synthetic medium described by Soni et al (Soni et al, 1987, *Appl. Microbiol.Biotechnol.* **27**:1-5). An overnight culture at 35°C was used to inoculate a 300 ml fermentors (DASGIP) using an anaerobic chemostat protocol.

For this purpose the fermentor was filled with 250 ml of synthetic medium, sparged with nitrogen for 30 min and inoculated with 25 ml of preculture to an optical density (OD600nm) between 0.05 and 0.1. After 12 hours of batch culture at 35 °C, pH 5.5 (regulated using an NH_4OH solution) and an agitation rate of 300 rpm, the fermentor was continuously fed with oxygen free synthetic medium at a dilution rate of 0.05 h-1 while the volume was kept constant by sequential removal of fermentated medium. Stability of the culture was followed by products analysis using the HPLC protocol previously described.

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Continuous acetone butanol fermentation : influence of vitamins on the metabolic activity of *Clostridium acetobutylicum*.
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CLAIMS

1) A method for the production of n-butanol by culturing a microorganism in an appropriate culture medium comprising a source of carbon and recovery of n-butanol from the culture medium, wherein at least one gene involved in butyrate formation is deleted in the microorganism.

2) A method according to claim 1 wherein the deleted gene is at least one of the following genes:
   - *ptb* encoding phospho-transbutyrylase
   - *buk* encoding butyrate kinase.

3) A method according to claim 1 or 2 wherein at least one gene involved in acetone formation is attenuated in the microorganism.

4) A method according to claim 3 wherein at least one of the following genes is deleted:
   - *ctfAB* encoding CoA-transferase
   - *adc* encoding aceto-acetate decarboxylase.

5) A method according to any one of claims 1 to 4 wherein the microorganism is modified to be unable to produce lactate.

6) A method according to claim 5 wherein the *ldh* gene is deleted.

7) A method as claimed in any one of claims 1 to 6 wherein the microorganism is modified to be unable to produce acetate.

8) A method as claimed in claim 7 in which at least one gene involved in acetate formation is deleted.

9) A method according to claim 10 wherein the deleted gene is selected among the following :
   - *pta* encoding phospho-transacetylase
   - *ack* encoding acetate kinase.

10) A method as claimed in any one of claims 1 to 9, wherein the hydrogen flux is decreased and the reducing power redirected to butanol production.

11) A method as claimed in claim 10 wherein the *hydA* gene is attenuated.

12) A method according to anyone of claims 1 to 11 wherein the microorganism is selected among the group consisting of *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* or *C. saccharobutylicum.*

13) A method according to anyone of claims 1 to 14 wherein the culture is continuous and stable.

14) A method according to claim 13 comprising the following steps:

a) Fermentation of the microorganism producing n-butanol

b) Elimination of n-butanol during the fermentation by gas striping.

c) Isolation of n-butanol from the condensate by distillation.

15) A microorganism as defined in any one of claims 1 to 12.

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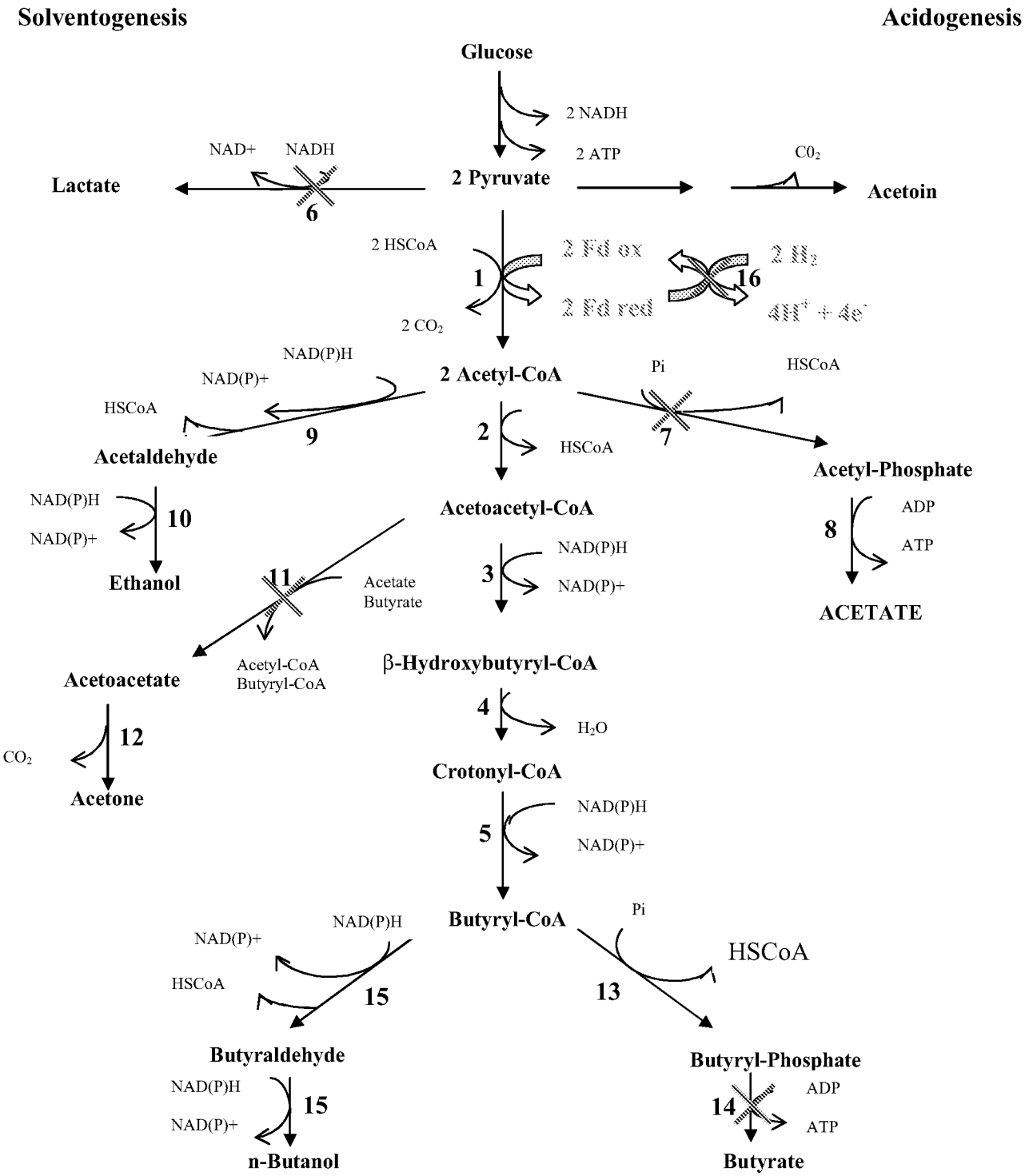
Figure 1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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/067993

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/16 C12N1/15

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 C12R

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 practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GREEN EDWARD M ET AL: "GENETIC MANIPULATION OF ACID FORMATION PATHWAYS BY GENE ACTIVATION IN CLOSTRIDIUM ACETOBUTYLICUM ATCC 824" MICROBIOLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, READING, GB, vol. 142, no. 8, 1996, pages 2079-2086, XP001248643 ISSN: 1350-0872 cited in the application abstract page 2080, column 1, paragraph 2 - page 2085, column 1, paragraph 2	1,2,12, 15
Y		2-4,13, 14

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2007	25/10/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, Ad

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/067993

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARRIS LATONIA M ET AL: "Characterization of recombinant strains of the Clostridium acetobutylicum butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition?" BIOTECHNOLOGY AND BIOENGINEERING, vol. 67, no. 1, 5 January 2000 (2000-01-05), pages 1-11, XP002443069 ISSN: 0006-3592 cited in the application abstract	1,2,12, 15
Y	SONI B K ET AL: "CONTINUOUS ACETONE-BUTANOL FERMENTATION INFLUENCE OF VITAMINS ON THE METABOLIC ACTIVITY OF CLOSTRIDIUM-ACETOBUTYLICUM" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 27, no. 1, 1987, pages 1-5, XP008081386 ISSN: 0175-7598 cited in the application abstract	13
Y	US 2005/089979 A1 (EZEJI THADDEUS C [US] ET AL) 28 April 2005 (2005-04-28) paragraph [0005] - paragraph [0037]	13,14
Y	TUMMALA SESHU B ET AL: "Transcriptional analysis of product-concentration driven changes in cellular programs of recombinant Clostridium acetobutylicum strains." BIOTECHNOLOGY AND BIOENGINEERING, vol. 84, no. 7, 30 December 2003 (2003-12-30), pages 842-854, XP002443071 ISSN: 0006-3592 page 843, column 1 - page 844, column 2, paragraph 2	3,4
Y	TUMMALA SESHU B ET AL: "Design of antisense RNA constructs for downregulation of the acetone formation pathway of Clostridium acetobutylicum." JOURNAL OF BACTERIOLOGY, vol. 185, no. 6, March 2003 (2003-03), pages 1923-1934, XP002443072 ISSN: 0021-9193 page 1924, column 1, paragraph 2 - page 1931, column 1, last paragraph; tables 1,4 page 1933, column 1	3,4

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/067993

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DESAI RUCHIR P ET AL: "Antisense RNA strategies for metabolic engineering of Clostridium acetobutylicum" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 3, March 1999 (1999-03), pages 936-945, XP002443073 ISSN: 0099-2240 abstract page 937, column 1, paragraph 2 - page 943, column 2, paragraph 2	2
A	WOODS D R: "The genetic engineering of microbial solvent production" TRENDS IN BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 13, no. 7, July 1995 (1995-07), pages 259-264, XP004207180 ISSN: 0167-7799 the whole document	1-4, 12-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/067993

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1 (in part), 2-4, 12-15 (in part)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1 (in part), 2-4, 12-15 (in part)

   A method for the production of n-butanol by culturing a microorganism in culture medium, wherein at least one gene involved in butyrate formation is deleted in the microorganism and at least one gene involved in acetone formation is attenuated.

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2. claims: 1 (in part), 5, 6 and 12-15 (in part)

   A method for the production of n-butanol by culturing a microorganism in culture medium, wherein at least one gene involved in butyrate formation is deleted in the microorganism and wherein the microorganism is modified to be unable to produce lactate

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3. claims: 1 (in part), 7-9 and 12-15 (in part)

   A method for the production of n-butanol by culturing a microorganism in culture medium, wherein at least one gene involved in butyrate formation is deleted in the microorganism and wherein the microorganism is modified to be unable to produce acetate.

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4. claims: 1 (in part), 10, 11 and 12-15 (in part).

   A method for the production of n-butanol by culturing a microorganism in culture medium, wherein at least one gene involved in butyrate formation is deleted in the microorganism and wherein the hydrogen flux is reduced in the microorganism.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/067993

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005089979 A1	28-04-2005	NONE	