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(54) Title: BUTANOL PRODUCTION IN A EUKARYOTIC CELL

(57) Abstract: The present invention relates to a eukaryotic cell capable of producing butanol, wherein at least one nucleotide sequence encoding an enzyme that is capable of catalysing the conversion from acetaldehyde to ethanol is inactivated. The invention further relates to a process for the production of butanol, and the use of butanol as a chemical or as a fuel.

BUTANOL PRODUCTION IN A EUKARYOTIC CELL

The present invention relates to a eukaryotic cell capable of producing butanol and a process for the production of butanol wherein a eukaryotic cell according to the present invention is used.

Background

Butanol is an important chemical and is suitable as an alternative engine fuel having improved properties over ethanol. Butanol also finds use as a solvent for a wide variety of chemical and textile processes, in the organic synthesis of plastics, as a chemical intermediate and as a solvent in the coating and food and flavour industry. Butanol can be produced from biomass (biobutanol) as well as fossil fuels.

The fermentation of carbohydrates to acetone, butanol, and ethanol by solventogenic *Clostridia*, the classical ABE fermentation, is well known since decades. However, *Clostridia* are sensitive to oxygen, and therefore *Clostridia*-fermentations need to be operated under strict anaerobic conditions, which makes it difficult to operate such fermentations on a large scale. In addition, Clostridia are sensitive to bacteriophages, causing lysis of the bacterial cells during fermentation. Since Clostridia fermentations are carried out at neutral pH, sterile conditions are essential to prevent contamination of the fermentation broth by eg. lactic acid bacteria, which lead to high costs for fermentations on an industrial scale (Zverlov *et al.* Appl. Microbiol. Biotechnol. Vol. 71, p. 587-597, 2006, Spivey, Process Biochemistry November 1978).

Alternative butanol-producing microorganisms are known from WO2007/041269. WO2007/041269 discloses a recombinant microorganism, for instance a yeast such as *Saccharomyces cerevisiae*, which is transformed with at least one DNA molecule encoding a polypeptide that catalyses one of the reactions of the butanol pathway. However, the amount of butanol produced in a genetically modified *Saccharomyces* strain disclosed in WO 2007/041269 was only between 0.2 to 1.7 mg/l, which is a factor of about 1000-10.000 lower than the amount of butanol produced in a classical ABE fermentation.

The aim of the present invention is the provision of a yeast cell which produces a higher amount of butanol than is known from the state of the art.

Summary of the invention

The aim of the invention is achieved by a recombinant eukaryotic cell capable of producing butanol, wherein the cell has been genetically modified such that the cell is capable of producing at least a two times higher amount of butanol, compared to a eukaryotic cell capable of producing butanol which does not comprise the genetic modification.

In another aspect the invention relates to a process for the production of butanol, comprising fermenting a eukaryotic cell according to present invention in a suitable fermentation medium, and optionally recovering butanol.

In a third aspect the invention relates to a fermentation medium comprising butanol and ethanol.

In a fourth aspect the present invention relates to the use of butanol obtained in the process according to the present invention as a chemical agent or as a fuel.

Detailed description

The present invention relates to a recombinant eukaryotic cell capable of producing butanol, wherein the cell has been genetically modified such that the cell is capable of producing at least a two times higher amount of butanol, compared to a eukaryotic cell capable of producing butanol which does not comprise the genetic modification.

Preferably, the genetic modification in the recombinant eukaryotic cell according to the present invention comprises an inactivation of a nucleotide sequence encoding an enzyme capable of catalysing the conversion of acetaldehyde to ethanol.

Preferably, a eukaryotic cell according to the present invention is a cell wherein at least one nucleotide sequence encoding an enzyme capable of catalysing the conversion of acetaldehyde to ethanol is inactivated.

Preferably, the eukaryotic cell according to the present invention produces a reduced amount of ethanol, preferably at least a 10%, preferably at least a 15%, 20% or at least a 25% reduced amount of ethanol, compared to a eukaryotic cell wherein the at least one nucleotide sequence encoding an enzyme catalysing the conversion from acetaldehyde to ethanol is active (not inactivated). Usually a eukaryotic cell comprising a

genetic modification comprising the inactivation of at least one enzyme capable of catalysing the conversion of acetaldehyde to ethanol, is able to produce an amount of ethanol, for instance about 1%, 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60% or more of the amount of ethanol produced by the eukaryotic cell not comprising that genetic modification, but usually below 90%.

Surprisingly, it was found that the eukaryotic cell according to the present invention produces an at least two times, preferably at least three times higher amount of butanol, compared to a cell wherein the at least one nucleotide sequence encoding an enzyme capable of catalysing the conversion from acetaldehyde to ethanol is active (not inactivated).

As used herein, a recombinant eukaryotic cell is defined as a cell which contains a nucleotide sequence and/or protein, or is transformed or genetically modified with a nucleotide sequence that does not naturally occur in the yeast, or it contains additional copy or copies of an endogenous nucleic acid sequence (or protein), or it contains a mutation, deletion or disruption of an endogenous nucleic acid sequence. Preferably a recombinant eukaryotic cell according to the present invention comprises an inactivation of an enzyme capable of catalysing the conversion of acetaldehyde to ethanol. Inactivation of a nucleotide sequence encoding an enzyme, which is capable of catalysing the conversion of acetaldehyde to ethanol, such as an enzyme having alcohol dehydrogenase activity, may be achieved by mutation, deletion or disruption of (part of) a nucleotide sequence encoding an alcohol dehydrogenase, for instance by the method disclosed by Gueldener *et. al.* 2002, Nucleid Acids Research Vol. 30, No. 6, e23.

Any nucleotide sequence encoding an enzyme that is capable of catalysing the conversion of acetaldehyde to ethanol may be inactivated, preferably a nucleotide sequence is inactivated that encodes an alcohol dehydrogenase.

Any suitable nucleotide sequence encoding an enzyme having alcohol dehydrogenase activity present in a eukaryotic cell may be inactivated. A eukaryotic cell may comprise one or more enzyme(s) having alcohol dehydrogenase activity. Preferably, a nucleotide sequence encoding an enzyme which is capable of catalysing the conversion of acetaldehyde to ethanol is inactivated in the cell which has the highest contribution to the conversion of acetaldehyde to ethanol in a eukaryotic cell according to the present invention. In the event a eukaryotic cell comprises multiple nucleotide sequences encoding an enzyme which is capable of catalysing the conversion of

acetaldehyde to ethanol, one, two, three or more of these nucleotide sequences may be inactivated.

A eukaryotic cell according to the present invention may be any suitable microbial cell, preferably a yeast or filamentous fungus. Preferably, a eukaryotic cell belongs to a genus of *Pichia, Kluyveromyces, Saccharomyces, Yarrowia, Aspergillus, Penicillium, Trichosporon, Trichoderma* or *Rhizopus*. A more preferred eukaryotic cell belongs to a species *Aspergillus niger, Penicillium chrysogenum, Trichoderma reesii, Pichia stipidis, Kluyveromyces lactis, Yarrowia lipolytica, Brettanomyces bruxellensis, Zygosaccharomyces bailii.* Preferably, a eukaryotic cell according to the present invention is a yeast cell, preferably a *Saccharomyces* sp., preferably a *Saccharomyces cerevisiae*.

In the event a eukaryotic cell according to the present invention is a *Saccharomyces cerevisiae*, a nucleotide sequence encoding an alcohol dehydrogenase that is inactivated preferably is a nucleotide sequence encoding an alcohol dehydrogenase 1 (ADH1) and/or an alcohol dehydrogenase 2 (ADH2).

Preferably, a eukaryotic cell according to the present invention naturally produces alcohol, for instance ethanol fermentation. A group of eukaryotic cells which is naturally able to produce ethanol is for instance yeast.

Preferably, a eukaryotic cell capable of producing butanol comprises one or more enzymes that produce acetoacetyl-CoA, 3-hydroxybutyryl-CoA, crotonyl-CoA, butyryl-CoA, butyrylaldehyde and butanol. A eukaryotic cell capable of producing butanol according to the present invention preferably is a cell which comprises one or more introduced nucleotide sequence(s) encoding a polypeptide that catalyses the conversion of:

a) acetyl-CoA to acetoacetyl-CoA;

b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA;

c) 3-hydroxybutyryl-CoA to crotonyl-CoA;

d) crotonyl-CoA to butyryl-CoA

e) butyryl-CoA to butyraldehyde;

f) butyraldehyde to 1-butanol.

Suitable enzymes that catalyse the formation of these products are for instance acetyl-CoA acetyltransferase or thiolase (E.C. 2.3.1.9) (SEQ ID NO:15), 3-hydroxybutyryl-CoA dehydrogenase (E.C. 1.1.1.1.57) (SEQ ID NO:16), 3-hydroxybutyryl-CoA dehydratase (E.C. 4.2.1.55) (SEQ ID NO:17), butyryl-CoA dehydrogenase

(E.C.1.3.99.2) (SEQ ID NO:18), alcohol/aldehyde dehydrogenase (E.C.1.1.1.1./E.C.1.2.1.10) (SEQ ID NO:19), and NAD(P)H-dependent butanol dehydrogenase (E.C.1.1.1.-) (SEQ ID NO:20). The enzymes of the butanol pathway may be homologous and/or heterologous to the eukaryotic cell, preferably at least one of the enzymes is a heterologous enzyme. The enzymes may for instance be derived from a *Clostridium sp.* for instance *Clostridium acetobutylicum* or *Clostridium beijerinckii*.

A suitable method for genetically modifying a eukaryotic cell for producing butanol is for instance disclosed in WO2007/041269.

Definitions

The term “butanol” refers to n-butanol, i.e. 1-butanol.

The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain.

The term "heterologous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein that does not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in which it is present, or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which it is introduced, but have been obtained from another cell or synthetically or recombinantly produced.

The term “gene”, as used herein, refers to a nucleic acid sequence containing a template for a nucleic acid polymerase, in eukaryotes, RNA polymerase II. Genes are transcribed into mRNAs that are then translated into protein.

The term "nucleotide sequence" as used herein, includes reference to a deoxyribonucleotide or ribonucleotide polymer, i.e. a polynucleotide, in either single-or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e. g., peptide nucleic acids). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence

thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skilled in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including among other things, simple and complex cells.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation.

In another aspect, the invention relates to a process for the production of butanol, comprising fermenting a eukaryotic cell according to the present invention in a suitable fermentation medium, wherein butanol is produced, and optionally recovery of butanol.

The fermentation medium used in the process for the production of butanol may be any suitable fermentation medium which allows growth of a eukaryotic cell. The essential elements of the fermentation medium are known to the person skilled in the art and may be adapted to a eukaryotic cell selected.

The fermentation process for the production of butanol according to the present invention may be an aerobic or an anaerobic fermentation process.

An anaerobic fermentation process is herein defined as a fermentation process run in the absence of oxygen or in which substantially no oxygen is consumed, preferably less than 5, 2.5 or 1 mmol/L/h, and wherein organic molecules serve as both electron donor and electron acceptors. The fermentation process according to the

present invention may also first be run under aerobic conditions and subsequently under anaerobic conditions.

The fermentation process may also be run under oxygen-limited, or micro-aerobical, conditions. Alternatively, the fermentation process may first be run under aerobic conditions and subsequently under oxygen-limited conditions. An oxygen-limited fermentation process is a process in which the oxygen consumption is limited by the oxygen transfer from the gas to the liquid. The degree of oxygen limitation is determined by the amount and composition of the ingoing gasflow as well as the actual mixing/mass transfer properties of the fermentation equipment used. Preferably, in a process under oxygen-limited conditions, the rate of oxygen consumption is at least 5.5, more preferably at least 6 and even more preferably at least 7 mmol/L/h.

The process for the production of butanol according to the present invention may be run at any suitable temperature, preferably between 10 and 45 degrees Celsius, preferably between 15 and 40, preferably between 20 and 35, or between 25 and 40 degrees Celsius.

The process for the production of butanol according to the present invention may be carried out at any suitable pH value, for instance between 2 and 9, preferably between 2,5 and 8. The pH in the fermentation medium preferably has a value of below 7, 6, preferably below 5,5, preferably below 5, preferably below 4,5, preferably below 4, preferably below pH 3,5 or below pH 3,0, preferably above pH 2,5. An advantage of carrying out the fermentation process at these low pH values is that growth of contaminant bacteria in the fermentation medium may be prevented.

Preferably, the process for the production of butanol further comprises recovery of butanol from the fermentation medium. Recovery of butanol from the fermentation medium may be performed by known methods in the art, for instance by distillation, vacuum extraction, solvent extraction, or pervaporation. Preferably, butanol produced in the process according to the present invention is purified.

In another aspect the present invention relates to the use of butanol obtainable by the process of the invention as a chemical or as a (bio)fuel.

Examples of the use of butanol as a chemical are the use of butanol as a solvent, for instance in the organic chemistry, or as a raw material for the production of butyl esters or ethers, for instance butyl acrylate. Alternatively, butanol produced by a process according to the present invention may be used as a fuel, for instance as an additive to fuels such as gasoline or diesel.

The present invention also relates to a process for the production of butanol, using a eukaryotic cell as butanol producer, whereby at least one enzyme catalysing the conversion of acetaldehyde to ethanol is inactivated. Preferred embodiments of the eukaryotic cell capable of producing butanol and the enzyme are as defined herein above.

The invention is further illustrated by the following non-limiting examples.

Figure 1: A schematic presentation of the constructs pRS415TH and pRS416CBB.

EXAMPLES

General

- oligonucleotides were synthesized by Invitrogen (Carlsbad CA, US).
- DNA sequencing was performed at SEQLAB (Göttingen, Germany) or by Baseclear (Leiden, The Netherlands)
- Restriction enzymes were supplied by Invitrogen or New England Biolabs.
- Used strains: Escherichia coli DH10B electromax competent cells (Invitrogen). Protocol is delivered by manufacturer.

Example 1

Cloning of the butanol biosynthesis genes for overexpression in *Saccharomyces*

Construction of expression plasmids pBOL009 and pBOL012 for 6 Butanol genes was performed as follows

The six *Clostridium acetobutylicum* genes involved in butanol biosynthesis from Acetyl-CoA as listed below were codon pair optimized for *S. cerevisiae* as described in WO2008/000632:

SEQ ID NO. 22: Codon pair optimised (CPO) *thi*L gene (counterclockwise)

SEQ ID NO. 23: Codon pair optimised *hbd* gene

SEQ ID NO. 25: Codon pair optimised *crt* gene (counterclockwise)

SEQ ID NO. 26: Codon pair optimised *bcd* gene

SEQ ID NO. 21: Codon pair optimised *adh*E gene

SEQ ID NO. 24: Codon pair optimised *bdh*B gene

The expression constructs were synthesized at DNA2.0 (Menlo Park CA, USA). Two single-copy expression shuttle vectors, pRS415 and pRS416 derived (Sirkoski R.S. and Hieter P. Genetics, 1989, 122(1):19-27, were created each expressing 3 of the butanol biosynthesis genes.

The *thi*L gene and the *hbd* gene were synthesized as one fragment expressed from the bi-directional GAL1-10 promoter and terminated by the GAL1-10 terminators. The *crt* and *bcd* were expressed from a similar construct. The *adh*E gene was synthesized between the GAL7 promoter and terminator as well as *bdh*B, resulting in 2 different constructs.

A schematic presentation of the constructs is shown in Figure 1. The sequence listings of the promoters and terminators are as follows: SEQ ID NO 27: Gal 1 promoter; SEQ ID NO 28: Gal 1 terminator; SEQ ID NO 29: Gal7 promoter; SEQ ID NO. 30: Gal 7 terminator; SEQ ID NO 31: Gal 10 terminator, counterclockwise; SEQ ID NO 32: Gal 10 promoter, counterclockwise.

Construction of pRS415THE (adhE, thiL and hbd)

The first expression construct was created after an *Apa*I / *Not*I restriction enzyme double digest of the pRS415 vector (LEU) and subsequently ligating in this vector an *Apa*I / *Asc*I restriction fragment consisting of *adh*E combined with a *Asc*I /*Not*I restriction fragment containing the *thiL/hbd* fragment. After this triple ligation the ligation mix is used for transformation of *E. coli* DH10B (Invitrogen) resulting in construct pRS415THE (also named pBOL009)

Construction of pRS416CBB (bdhB, crt and bcd)

The second expression vector was created after a *Bam*HI / *Not*I restriction enzyme double digest of the pRS416 vector (URA) and subsequently ligating in this vector a *Bam*HI / *Asc*I restriction fragment consisting of the *bdh*B gene combined with an *Asc*I /*Not*I restriction fragment containing the *crt/bcd* fragment. After this triple ligation, the ligation mix is used for transformation of *E. coli* DH10B (Invitrogen) resulting in construct pRS416CBB (also named pBOL012)

Example 2

Construction of *adh1/adh2* delta strain BLT011

In order to obtain an *adh1/adh2* delta strain of *S. cerevisae*, the 2 genes were sequentially disrupted using the Cre-lox system.

The *S. cerevisiae adh1* deleted strain was produced by first performing a PCR on plasmid pUG6 (Güldener et al., 1996 Nucleic Acids Res. 24(13):2519-24) with the following primer pair:

P1: 5'-atatttcaagctataccaagcatacaatcaactatctcatataccagctgaagcttcgtacgc-3'

P2: 5'-acttatttaataataaaaatcataaatcataagaaattcgcgcataggccactagtggatctg-3'.

The resulting 1.4 kb fragment, containing the KanMX marker which confers resistance to G418, was used to transform *S. cerevisiae* CEN.PK113-6B (*MATA ura3-52 leu2-112 trp1-289*). After transformation the strains were plated on YPEG (10 g l^{-1} yeast extract (BD Difco), 20 g l^{-1} peptone (BD Difco)), 10 g l^{-1} ethanol, 10 g l^{-1} glycerol) with 200 mg/ml Geneticin (G418). In resistant transformants, correct integration was verified by PCR using 2 primer pairs:

P3: 5'-acggccttccttccagttac-3' with

P4: 5'-cgcacgtcaagactgtcaag-3', and

P5: 5'-ccggtagaggtgtggtcaat-3' with

P6: 5'-tcgtatgtgaatgctggtcg-3'.

The resulting strain was named RWB068 (*MATA ura3-52 leu2-112 trp1-289 adh1::Kanlox*).

RWB068 was transformed with pSH47 (Güldener et al., 1996 Nucleic Acids Res. 24(13):2519-24) and transformants were plated on MYEG (mineral medium (Verduyn et al, 1992), 10 g l^{-1} ethanol, 10 g l^{-1} glycerol) with leucine and tryptophan. One transformant was resuspended in YP with 1% galactose and incubated for 2 hours at 30℃ and an estimated 200 cells were plated on YPEG. Of the resulting colonies 100 were tested for loss of the G418 marker ($G418^S$) and loss of the pSH47 plasmid (ura^-). Of the 25 colonies that were both ura- and $G418^S$, four were restreaked and tested by PCR for correct excision of the G418 marker, using primer pair

P7: 5'-acggccttccttccagttac-3' and

P8: 5'-ccggtagaggtgtggtcaat-3'.

The resulting strain was named RWB067 (*MATA ura3-52 leu2-112 trp1-289 adh1::lox*).

RWB067 was then transformed with a disruption fragment obtained by a PCR on pUG6 with primer pair

P9: 5'-aagcatacaatcaactatcaactattaactatatcgtaatacacacagctgaagcttcgtacgc-3'

P10: 5'-tgataatgaaaactataaatcgtaaagacataagagatccgcttagcataggccactagtggatctg-3'.

Transformants were selected as above for ADH1 and correct integration verified with the following 2 primer pairs

P11: 5'-gccaagaactctaaccagtc-3' with

P12: 5'-cgcacgtcaagactgtcaag-3', and

P13: 5'-ggagacgattcagaggagca-3' with

P14: 5'-tcgtatgtgaatgctggtcg-3'.

A correct transformant was chosen and named BLT011 (*MATA ura3-52 leu2-112 trp1-289 adh1::lox adh2::Kanlox*).

Example 3

Construction of the butanol producing strains BLT012 and BLT060 (*adh1 adh2* delta)

To construct the butanol producing strains with or without deletion of the genes adh1/adh2 plasmids pBOL009, pBOL012 from example 1 and YEplac112 (empty plasmid (2µ TRP1) (Gietz & Sugino (1988) Gene 74:527-34)) were transformed to *S. cerevisiae* CEN.PK113-6B, and BLT011.

Transformants were plated on Yeast Nitrogen Base (YNB) w/o AA (Difco) + 2% glucose. In total 10 transformants of each plasmid combination were checked for the presence of the correct plasmid. Correct transformants were given the names BLT060 (= CEN.PK113-6B with pRS415THE, pRS416CBB YEplac110), and BLT012 (= BLT011 with pBOL009, pBOL012 YEplac110).

Example 4

Butanol and ethanol production in adh1/adh2 delta strain

Strains BLT060, and BLT012 as prepared in Example 3 were grown in Verduyn medium (Verduyn *et al.* (1992) Yeast 8: 501-517) in which the ammonium sulphate is replaced by 2 g/l ureum and which further contains 0.5% wt glucose and 4 wt.% galactose. Cells were grown in 100 ml shake flasks containing 50 ml of medium for 72 hours at 30°C at 180 rpm in a rotary shaker.

The butanol concentration was determined in the supernatant of the culture. Samples were analysed on a HS-GC equipped with a flame ionisation detector and an automatic injection system. Column J&W DB-1 length 30 m, id 0.53 mm, df 5 µm. The following conditions were used: helium as carrier gas with a flow rate of 5 ml/min. Column temperature was set at 110°C. The injector was set at 140°C and the detector performed at 300°C. The data was obtained using Chromeleon software. Samples were heated at 60°C for 20 min in the headspace sampler. One (1) ml of the headspace volatiles were automatically injected on the column.

1-Butanol production of the various strains was as follows:

BLT060: 42 mg/l

BLT012: 143 mg/l

The ethanol concentration was determined in the supernatant of the culture. Samples were analysed using 500 MHz ^{1}H NMR in stopped-flow mode (Bruker BEST system). To the samples was added 10% of a standard solution containing an accurate amount of maleic acid (20,0 g/l). From the integrals of the methyl resonances of ethanol and the olefinic resonances of maleic acid, the exact amount of ethanol present could be calculated.

Ethanol production of the strains was as follows:

BLT060: 10.8 g/l

BLT012: 8.1 g/l

CLAIMS

1. A recombinant eukaryotic cell capable of producing butanol, wherein the cell has been genetically modified such that the cell is capable of producing at least a two times higher amount of butanol, compared to a eukaryotic cell capable of producing butanol which does not comprise the genetic modification.

2. A recombinant eukaryotic cell capable of producing butanol, wherein at least one a nucleotide sequence encoding an enzyme that is capable of catalysing the conversion from acetaldehyde to ethanol is inactivated.

3. A eukaryotic cell according to claim 2, wherein the enzyme is an alcohol dehydrogenase.

4. A eukaryotic cell according to any one of the claims 1 to 3, which is a yeast or a filamentous fungus.

5. A eukaryotic cell according to any one of the claims 1 to 4, which is a *Saccharomyces* sp., preferably a *Saccharomyces cerevisiae.*

6. A eukaryotic cell according to claim 5, wherein the enzyme that is capable of catalysing the conversion from acetaldehyde to ethanol is alcohol dehydrogenase 1 (ADH1) and/or alcohol dehydrogenase 2 (ADH2).

7. A eukaryotic cell according to any one of the claims 1 to 6, wherein the cell comprises one or more nucleotide sequence(s) encoding a polypeptide that catalyses the conversion of:

a) acetyl-CoA to acetoacetyl-CoA;

b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA;

c) 3-hydroxybutyryl-CoA to crotonyl-CoA;

d) crotonyl-CoA to butyryl-CoA;

e) butyryl-CoA to butyraldehyde;

f) butyraldehyde to 1-butanol.

8. A process for the production of butanol, comprising fermenting a eukaryotic cell according to any one of the claims 1 to 6 in a suitable fermentation medium, wherein butanol is produced.

9. A process according to claim 8, further comprising recovery of butanol from the fermentation medium.

10. A fermentation medium comprising butanol and ethanol, obtainable by the process according to claim 8 or 9.

11. Use of butanol obtainable by the process according to claim 8 or 9 as a chemical or as a (bio)fuel.

12. Process for the production of butanol, using a eukaryotic cell as butanol producer, whereby at least one enzyme catalysing the conversion of acetaldehyde to ethanol is inactivated.

13. Process according to claim 12, wherein the enzyme is an alcohol dehydrogenase.

AMENDED CLAIMS

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1. A recombinant eukaryotic cell capable of producing butanol, wherein at least one a nucleotide sequence encoding an enzyme that is capable of catalysing the conversion from acetaldehyde to ethanol is inactivated.

2. A eukaryotic cell according to claim 1, wherein the enzyme is an alcohol dehydrogenase.

3. A eukaryotic cell according to claim 1 or 2, which is a yeast or a filamentous fungus.

4. A eukaryotic cell according to any one of the claims 1 to 3, which is a *Saccharomyces* sp., preferably a *Saccharomyces cerevisiae.*

5. A eukaryotic cell according to claim 4, wherein the enzyme that is capable of catalysing the conversion from acetaldehyde to ethanol is alcohol dehydrogenase 1 (ADH1) and/or alcohol dehydrogenase 2 (ADH2).

6. A eukaryotic cell according to any one of the claims 1 to 5, wherein the cell comprises one or more nucleotide sequence(s) encoding a polypeptide that catalyses the conversion of:
a) acetyl-CoA to acetoacetyl-CoA;
b) acetoacetyl-CoA to 3-hydroxybutyryl-CoA;
c) 3-hydroxybutyryl-CoA to crotonyl-CoA;
d) crotonyl-CoA to butyryl-CoA;
e) butyryl-CoA to butyraldehyde;
f) butyraldehyde to 1-butanol.

7. A process for the production of butanol, comprising fermenting a eukaryotic cell according to any one of the claims 1 to 6 in a suitable fermentation medium, wherein butanol is produced.

8. A process according to claim 7, further comprising recovery of butanol from the fermentation medium.

9. A fermentation medium comprising butanol and ethanol, obtainable by the process according to claim 7 or 8.

10. Use of butanol obtainable by the process according to claim 7 or 9 as a chemical or as a (bio)fuel.

11. Process for the production of butanol, using a eukaryotic cell as butanol producer, whereby at least one enzyme catalysing the conversion of acetaldehyde to ethanol is inactivated.

12. Process according to claim 11, wherein the enzyme is an alcohol dehydrogenase.

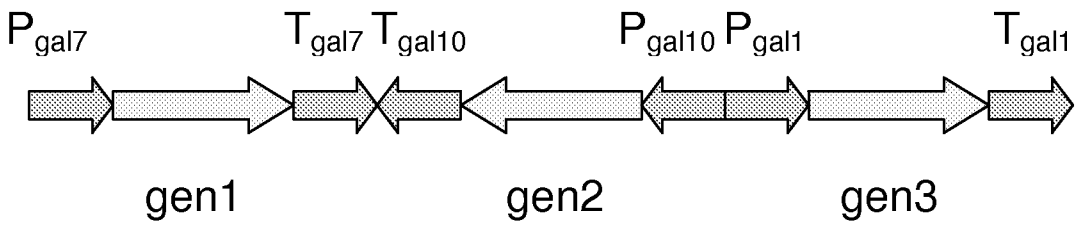


Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/059118

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/16 C12N15/81 C12N9/02

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)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/041269 A (DU PONT [US]; DONALDSON GAIL K [US]; HUANG LIXUAN LISA [US]; MAGGIO-HA) 12 April 2007 (2007-04-12) see the whole document	1,4-11

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 September 2008	18/11/2008

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/059118

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007041269 A	12-04-2007	CA 2619458 A1	12-04-2007
		EP 1929025 A2	11-06-2008
		US 2008182308 A1	31-07-2008