

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 May 2009 (28.05.2009)

PCT

(10) International Publication Number
WO 2009/065780 A1

(51) International Patent Classification:

C12N 15/60 (2006.01) *C12P 7/46* (2006.01)
C12N 9/88 (2006.01)

SAGT, Cornelis, Maria, Jacobus [NL/NL]; Karl Mil-
lockerhof 19, NL-3543 HV Utrecht (NL).

(21) International Application Number:

PCT/EP2008/065588

(74) Agents: **CAZEMIER, Anne Engeline** et al.; DSM In-
tellectual Property, Delft Office (600-0240), P.O. Box 1,
NL-2600 MA Delft (NL).

(22) International Filing Date:

14 November 2008 (14.11.2008)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

07121120.5	20 November 2007 (20.11.2007)	EP
07121117.1	20 November 2007 (20.11.2007)	EP
07121113.0	20 November 2007 (20.11.2007)	EP
08156960.0	27 May 2008 (27.05.2008)	EP
08156961.8	27 May 2008 (27.05.2008)	EP
08156959.2	27 May 2008 (27.05.2008)	EP

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(71) Applicant (*for all designated States except US*): **DSM IP ASSETS B.V.** [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **VERWAAL, René** [NL/NL]; Marsdiep 17, NL-2631 NL Nootdorp (NL). **WU, Liang** [NL/NL]; Sweelinckstraat 36, NL-2625 VK Delft (NL). **DAMVELD, Robbertus, Antonius** [NL/NL]; Slufterhof 17, NL-2651 NK Berkel en Rodenrijs (NL).

Published:

- with international search report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

(54) Title: DICARBOXYLIC ACID PRODUCTION IN EUKARYOTES

(57) Abstract: The present invention relates to a recombinant eukaryotic microbial cell comprising a nucleotide sequence encoding a heterologous enzyme catalysing the conversion from phosphoenolpyruvate to oxaloacetate whereby ATP is generated. The invention further relates to a process for the preparation of a dicarboxylic acid such as succinic acid and fumaric acid, comprising fermenting the eukaryotic microbial cell according to the invention in a suitable fermentation medium.



WO 2009/065780 A1

DICARBOXYLIC ACID PRODUCTION IN EUKARYOTES

5

The present invention relates to a recombinant eukaryotic cell comprising a nucleotide sequence encoding an enzyme that catalyses the conversion of phosphoenolpyruvate to oxaloacetate, and a process for the production of a dicarboxylic acid.

10

The 4-carbon dicarboxylic acids malic acid, fumaric acid and succinic acid are potential precursors for numerous chemicals. For example, succinic acid can be converted into 1,4-butanediol (BDO), tetrahydrofuran, and gamma-butyrolactone. Another product derived from succinic acid is a polyester polymer which is made by linking succinic acid and BDO.

15

Succinic acid is predominantly produced through petrochemical processes by hydrogenation of butane. These processes are considered harmful for the environment and costly. The fermentative production of succinic acid may be an attractive alternative process for the production of succinic acid, wherein renewable feedstock as a carbon source may be used.

20

A number of different bacteria such as *Escherichia coli*, and the rumen bacteria *Actinobacillus*, *Anaerobiospirillum*, *Bacteroides*, *Mannheimia*, or *Succinimonas*, sp. are known to produce succinic acid. Metabolic engineering of these bacterial strains have improved the succinic acid yield and/or productivity, or reduced the by-product formation.

25

WO2007/061590 discloses a pyruvate decarboxylase negative yeast for the production of malic acid and/or succinic acid which is transformed with a pyruvate carboxylase enzyme or a phosphoenolpyruvate carboxylase, a malate dehydrogenase enzyme, and a malic acid transporter protein (MAE).

30

Despite the improvements that have been made in the fermentative production of dicarboxylic acid, there remains a need for improved microorganisms for the fermentative production of dicarboxylic acids.

The aim of the present invention is an alternative eukaryotic microorganism for the production of a dicarboxylic acid.

35

The aim is achieved according to the invention with a recombinant eukaryotic microbial cell comprising a nucleotide sequence encoding an enzyme catalysing the conversion from phosphoenolpyruvate to oxaloacetate whereby ATP is generated,

wherein the enzyme comprises an amino acid sequence which has at least 50% sequence identity with the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 and/or SEQ ID NO: 5.

Preferably, the enzyme has phosphoenolpyruvate carboxykinase activity, preferably the enzyme is a phosphoenolpyruvate (PEP) carboxykinase (E.C. 4.1.1.49). Preferably, the PEP carboxykinase is active under anaerobic or oxygen limited conditions in the presence of a fermentable carbon source or glycerol. A fermentable carbon source may be glucose, fructose, galactose, raffinose, arabinose, or xylose. It was found advantageous that the eukaryotic cell comprises a PEP carboxykinase according to the present invention, since PEP carboxykinase catalysing the conversion from PEP to OAA fixates CO₂ and generates energy in the form of ATP. .

Surprisingly, it was found that a recombinant eukaryotic cell according to the present invention produces an increased amount of dicarboxylic acid, such as succinic acid and fumaric acid as compared to the amount of dicarboxylic acid produced by a wild-type eukaryotic cell. Preferably, a eukaryotic cell according to the present invention produces at least 1.2, preferably at least 1.5, 1.6, 1.8 preferably at least 2 times more of a dicarboxylic acid than a wild-type eukaryotic cell which does not comprise the nucleotide sequence encoding an enzyme catalysing the conversion from phosphoenolpyruvate to oxaloacetate of the invention.

Preferably, a eukaryotic microbial cell according to the present invention expresses a nucleotide sequence encoding an enzyme having PEP carboxykinase activity, preferably a PEP carboxykinase wherein the PEP carboxykinase comprises an amino acid sequence that has at least 55%, preferably at least 60, 65, 70, 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, or 99% sequence identity with the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 and/or SEQ ID NO: 5. Preferably the the PEP carboxykinase comprises SEQ ID NO: 1, SEQ ID NO: 3 and/or SEQ ID NO: 5.

Sequence identity is herein defined as a relationship between two or more amino acid (polypeptide or protein) sequences or two or more nucleic acid (polynucleotide) sequences, as determined by comparing the sequences. Usually, sequence identities or similarities are compared over the whole length of the sequences compared. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences.

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include BLASTP and BLASTN,
5 publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894). Preferred parameters for amino acid sequences comparison using BLASTP are gap open 11.0, gap extension 1, Blosum 62 matrix.

A nucleotide sequence encoding an enzyme expressed in the cell of the invention
10 may also be defined by their capability to hybridise with nucleotide sequences encoding an enzyme having PEP carboxykinase activity of SEQ ID NO.'s: 1, 3 and/or 5, or with the nucleotide sequence encoding an malate dehydrogenase of SEQ ID NO: 14 or with the nucleotide sequence encoding fumarase of SEQ ID NO: 16, under moderate, or preferably under stringent hybridisation conditions. Stringent hybridisation conditions are
15 herein defined as conditions that allow a nucleic acid sequence of at least about 25, preferably about 50 nucleotides, 75 or 100 and most preferably of about 200 or more nucleotides, to hybridise at a temperature of about 65°C in a solution comprising about 1 M salt, preferably 6 x SSC (sodium chloride, sodium citrate) or any other solution having a comparable ionic strength, and washing at 65°C in a solution comprising about 0.1 M
20 salt, or less, preferably 0.2 x SSC or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having about 90% or more sequence identity.

Moderate conditions are herein defined as conditions that allow a nucleic acid
25 sequence of at least 50 nucleotides, preferably of about 200 or more nucleotides, to hybridise at a temperature of about 45°C in a solution comprising about 1 M salt, preferably 6 x SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, preferably 6 x SSC
30 or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours, and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having up to 50% sequence

identity. The person skilled in the art will be able to modify these hybridisation conditions in order to specifically identify sequences varying in identity between 50% and 90%.

A recombinant eukaryotic microbial cell according to the present invention is defined herein as a cell which contains, or is transformed or genetically modified with a nucleotide sequence that does not naturally occur in the eukaryotic cell, or it contains additional copy or copies of an endogenous nucleic acid sequence. A wild-type eukaryotic cell is herein defined as the parental cell of the recombinant cell.

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 "nucleic acid" 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.

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.

The term "enzyme" as used herein is defined as a protein which catalyses a (bio)chemical reaction in a cell.

To increase the likelihood that the introduced enzyme is expressed in active form in a eukaryotic cell of the invention, the corresponding encoding nucleotide sequence may be adapted to optimise its codon usage to that of the chosen eukaryotic host cell. Several methods for codon optimisation are known in the art. A preferred method to optimise codon usage of the nucleotide sequences to the eukaryotic cell according to the present invention is codon pair optimization technology as disclosed in WO2008/000632. Codon-pair optimization is a method for producing a polypeptide in a host cell, wherein the nucleotide sequences encoding the polypeptide have been modified with respect to their codon-usage, in particular the codon-pairs that are used, to obtain improved expression of the nucleotide sequence encoding the polypeptide and/or improved production of the polypeptide. Codon pairs are defined as a set of two subsequent triplets (codons) in a coding sequence.

Usually, a nucleotide sequence encoding an enzyme, such as an enzyme having PEP carboxykinase activity, or any other enzyme disclosed herein is operably linked to a promoter that causes sufficient expression of the corresponding nucleotide sequence in the eukaryotic cell according to the present invention to confer to the cell the ability to produce a dicarboxylic acid.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements (or coding sequences or nucleic acid sequence) in a functional relationship. A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence.

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is structurally

identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences known to one of skilled in the art. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under
5 environmental or developmental regulation.

A promoter that could be used to achieve expression of a nucleotide sequence coding an enzyme, eg. an enzyme having PEP carboxykinase activity may be not native to the nucleotide sequence coding for the enzyme to be expressed, i.e. a promoter that is heterologous to the nucleotide sequence (coding sequence) to which it is operably
10 linked. Preferably, the promoter is homologous, i.e. endogenous to the host cell.

Suitable promoters in eukaryotic host cells are known to the skilled man in the art. Suitable promoters may be, but are not limited to TDH, GPDA, GAL7, GAL10, or GAL 1, CYC1, HIS3, ADH1, PGL, PH05, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI, and AOX1. Other suitable promoters include PDC, GPD1, PGK1, and TEF1.

Usually a nucleotide sequence encoding an enzyme comprises a terminator. Any terminator, which is functional in the cell, may be used in the present invention. Preferred terminators are obtained from natural genes of the host cell. Suitable terminator sequences are well known in the art. Preferably, such terminators are combined with mutations that prevent nonsense mediated mRNA decay in the host cell of the invention
15 (see for example: Shirley et al., 2002, Genetics 161:1465-1482).

In a preferred embodiment, a nucleotide sequence encoding an enzyme, such as an enzyme having PEP carboxykinase activity is overexpressed. It was found that an increased production of malic acid, fumaric acid or succinic acid by the cell may be achieved when the nucleotide sequences are overexpressed.

There are known methods in the art for overexpression nucleotide sequences encoding enzymes. A nucleotide sequence encoding an enzyme may be overexpressed by increasing the copy number of the gene coding for the enzyme in the cell, e.g. by integrating additional copies of the gene in the cell's genome, by expressing the gene from a centromeric vector, from an episomal multicopy expression vector or by
25 introducing an (episomal) expression vector that comprises multiple copies of one or more gene(s). Preferably, overexpression of a nucleotide sequence encoding an enzyme according to the invention is achieved with a (strong) constitutive promoter.

The invention also relates to a nucleotide construct comprising one or more nucleotide sequence(s) selected from SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9 and SEQ ID NO: 10.

A nucleotide sequence encoding an enzyme may be ligated into a nucleic acid construct, for instance a plasmid, such as a low copy plasmid or a high copy plasmid. The eukaryotic cell according to the present invention may comprise a single, but preferably comprises multiple copies of the nucleotide sequence encoding an enzyme that catalyses the conversion of PEP to OAA, for instance by multiple copies of a nucleotide construct.

A nucleic acid construct may be maintained episomally and thus comprises a sequence for autonomous replication, such as an autosomal replication sequence. If the eukaryotic cell is of fungal origin, a suitable episomal nucleic acid construct may e.g. be based on the yeast 2 μ or pKD1 plasmids (Gleer et al., 1991, Biotechnology 9: 968-975), or the AMA plasmids (Fierro et al., 1995, Curr Genet. 29:482-489). Alternatively, each nucleic acid construct may be integrated in one or more copies into the genome of the eukaryotic cell. Integration into the cell's genome may occur at random by non-homologous recombination but preferably, the nucleic acid construct may be integrated into the cell's genome by homologous recombination as is well known in the art.

In a preferred embodiment, a eukaryotic microbial cell according to the present invention comprises an enzyme having PEP carboxykinase activity, wherein the enzyme is a heterologous enzyme, preferably the heterologous enzyme is derived from a bacterium, more preferably the enzyme having PEP carboxykinase activity is derived from *Escherichia coli*, *Mannheimia sp.*, *Actinobacillus sp.*, or *Anaerobiospirillum sp.*, more preferably *Mannheimia succiniciproducens*, *Actinobacillus succinogenes*, or *Anaerobiospirillum succiniciproducens*.

In a preferred embodiment a nucleotide sequence encoding an enzyme having PEP carboxykinase activity in the eukaryotic cell according to the present invention is expressed in the cytosol. Surprisingly cytosolic activity of the enzyme resulted in an increased production of a dicarboxylic acid by the eukaryotic cell.

It was found that a nucleotide sequence encoding an enzyme having PEP carboxykinase activity may comprise a peroxisomal or mitochondrial targeting signal, for instance as determined by the method disclosed by Schlüter et al, Nucleic acid Research 2007, Vol 25, D815-D822.

It was found that PEP carboxykinase derived from *Actinobacillus succinogenes* comprises a peroxisomal targeting signal. Surprisingly it was found that when the peroxisomal targeting signal was replaced with the corresponding motif in the PEP carboxykinase derived from *Mannheimia succiniproduces*, peroxisomal targeting was prevented.

Preferably, a eukaryotic cell according to the present invention expresses a nucleotide sequence encoding an enzyme having PEP carboxykinase, wherein the enzyme is a PEP carboxykinase, comprising an amino acid sequence that has at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, 98, or 99% sequence identity with the amino acid sequence of SEQ ID NO: 3 and/or SEQ ID NO: 5. Preferably, the PEP carboxykinase comprises SEQ ID NO: 3 and or SEQ ID NO: 5.

In one embodiment it may be preferred that the activity of a native or endogenous or homologous enzyme catalysing the conversion of OAA to PEP in the eukaryotic cell according to the present invention is reduced or is completely knocked out. Knocking out or reducing the activity of an enzyme catalysing the conversion of OAA to PEP are known methods to the skilled man in the art. This may for instance be achieved by mutation, disruption or deletion of the nucleotide sequence encoding the enzyme having PEP carboxykinase activity. A reduced activity of a native PEP carboxykinase is preferred in order to prevent the reverse reaction from OAA to PEP to occur.

A eukaryotic microbial cell according to the present invention, preferably is selected from the group consisting of a yeast and a filamentous fungus. A eukaryotic cell preferably belongs to the genera *Saccharomyces*, *Aspergillus*, *Penicillium*, *Pichia*, *Kluyveromyces*, *Yarrowia*, *Candida*, *Hansenula*, *Humicola*, *Torulaspora*, *Trichosporon*, *Brettanomyces*, *Rhizopus*, *Zygosaccharomyces*, *Pachysolen* or *Yamadazyma*. Preferably, the eukaryotic cell belongs to a species *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Saccharomyces bayanus*, *Aspergillus niger*, *Penicillium chrysogenum*, *P. symplissicum*, *Pichia stipidis*, *Kluyveromyces marxianus*, *K. lactis*, *K. thermotolerans*, *Yarrowia lipolytica*, *Candida sonorensis*, *C. glabrata*, *Hansenula polymorpha*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis*, *Rhizopus orizae* or *Zygosaccharomyces bailii*.

Preferably, a eukaryotic cell according to the invention is a yeast, preferably *Saccharomyces cerevisiae*, preferably a *Saccharomyces cerevisiae* comprising one or more of the nucleotide sequences selected from SEQ ID NO: 9 and SEQ ID NO: 10. The eukaryotic cell may also be a filamentous fungus, preferably *A. niger*, preferably *A. niger*

comprising one or more heterologous nucleotide sequences selected from SEQ ID NO: 7, and SEQ ID NO: 8.

In addition to a nucleotide sequence encoding an enzyme having PEP carboxykinase activity, the eukaryotic cell according to the present invention may be further genetically modified or transformed with nucleotide sequences that encode homologous and/or heterologous enzymes that catalyse reactions in the cell resulting in an increased flux towards malic acid, fumaric acid and/ or succinic acid. It may for example be favourable to introduce and/or overexpress nucleotide sequences encoding i) a malate dehydrogenase which catalyses the conversion from OAA to malic acid; ii) a fumarase, which catalyses the conversion of malic acid to fumaric acid; or iii) a fumarate reductase that catalyses the conversion of fumaric acid to succinic acid, depending on the dicarboxylic acid to be produced.

Preferably a eukaryotic cell according to the present invention overexpresses a nucleotide sequence encoding a pyruvate carboxylase (PYC), preferably a pyruvate carboxylase that is active in the cytosol upon expression of the nucleotide sequence, for instance a pyruvate carboxylase comprising an amino acid sequence according to SEQ ID NO: 26. Preferably, an endogenous or homologous pyruvate carboxylase is overexpressed. Surprisingly, it was found that overexpressing an endogenous pyruvate carboxylase resulted in increased succinic acid production levels by the eukaryotic cell according to the present invention comprising a phosphoenolpyruvate carboxykinase as described herein. It was found that the concomitant (over)expression of a pyruvate carboxylase and a phosphoenolpyruvate carboxykinase resulted in surprising increase of succinic acid production levels of at least 1.5 as compared to a eukaryotic cell comprising either pyruvate carboxylase or a phosphoenolpyruvate carboxykinase as described herein.

In another preferred embodiment a cell according to the present invention further comprises nucleotide sequence encoding a malate dehydrogenase (MDH) active in the cytosol upon expression of the nucleotide sequence. A cytosolic MDH may be any suitable homologous or heterologous malate dehydrogenase. Preferably the MDH is a *S. cerevisiae* MDH, such as MDH3 or MDH1. Preferably, the MDH lacks a peroxisomal or mitochondrial targeting signal in order to localize the enzyme in the cytosol. Alternatively, the MDH is *S. cerevisiae* MDH2 which has been modified such that it is not inactivated in the presence of glucose and is active in the cytosol. It is known that the transcription of *MDH2* is repressed and Mdh2p is degraded upon addition of glucose to glucose-starved

cells. Mdh2p deleted for the 12 amino-terminal amino acids is less-susceptible for glucose-induced degradation (Minard and McAlister-Henn, J Biol Chem. 1992 Aug 25;267(24):17458-64). Preferably, a eukaryotic cell according to the present invention comprises a nucleotide sequence encoding a malate dehydrogenase that has at least
5 70%, preferably at least 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, 99% or 100% sequence identity with the amino acid sequence of SEQ ID NO: 14. Preferably, the activity of malate dehydrogenase is increased by overexpressing the encoding nucleotide sequence by known methods in the art..

Preferably, a eukaryotic cell according to the present invention further comprises
10 a nucleotide sequence encoding an enzyme that catalyses the conversion of malic acid to fumaric acid, which may be a heterologous or homologous enzyme. An enzyme that catalyses the conversion of malic acid to fumaric acid, for instance a fumarase, may be derived from any suitable origin, preferably from microbial origin, for instance a yeast such as *Saccharomyces* or a filamentous fungus, such *Rhizopus oryzae*. Preferably, a
15 eukaryotic cell according to the present invention comprises a nucleotide sequence encoding a fumarase that has at least 70%, preferably at least 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, 99% sequence identity with the amino acid sequence of SEQ ID NO: 16, preferably the fumarase comprises SEQ ID NO:16.

Preferably, the enzyme catalysing the conversion of malic acid to fumaric acid is
20 active in the cytosol upon expression of the nucleotide sequence. Cytosolic activity of the enzyme having fumarase activity is preferred for a high productivity of a dicarboxylic acid by the eukaryotic cell. In the invent a nucleotide sequence encoding an enzyme having fumarase activity comprises a peroxisomal or mitochondrial targeting signal (for instance as determined by the method disclosed by Schlüter et al, Nucleic acid Research 2007,
25 Vol 25, D815-D822), it may be preferred to delete said targeting signal to localize an enzyme having fumarase activity in the cytosol. Preferably, a nucleotide sequence encoding an enzyme catalysing the conversion from malic acid to fumaric acid is overexpressed by known methods in the art.

Preferably, the cell according to the present invention is a cell wherein at least
30 one gene encoding alcohol dehydrogenase is not functional. An alcohol dehydrogenase gene that is not functional is used herein to describe a eukaryotic cell, which comprises a reduced alcohol dehydrogenase activity compared to a cell wherein all genes encoding an alcohol dehydrogenase are functional. A gene may become not functional by known methods in the art, for instance by mutation, disruption, or deletion, for instance by the

method disclosed by Gueldener et. al. 2002, Nucleic Acids Research, Vol. 30, No. 6, e23. Preferably, the cell is a *Saccharomyces cerevisiae*, wherein one or more genes *adh1* and/or *adh2*, encoding alcohol dehydrogenase are inactivated.

Preferably, the cell according to the present invention further comprises at least one gene encoding glycerol-3-phosphate dehydrogenase which is not functional. A glycerol-3-phosphate dehydrogenase gene that is not functional is used herein to describe a eukaryotic cell, which comprises a reduced glycerol-3-phosphate dehydrogenase activity, for instance by mutation, disruption, or deletion of the gene encoding glycerol-3-phosphate dehydrogenase, resulting in a decreased formation of glycerol as compared to the wild-type cell.

In another preferred embodiment the recombinant eukaryotic cell according to the present invention comprises at least one gene encoding succinate dehydrogenase that is not functional. A succinate dehydrogenase that is not functional is used herein to describe a eukaryotic cell, which comprises a reduced succinate dehydrogenase activity by mutation, disruption, or deletion, of at least one gene encoding succinate dehydrogenase resulting in a increased formation of succinic acid as compared to the wild-type cell. A eukaryotic cell comprising a gene encoding succinate dehydrogenase that is not functional may for instance be *Aspergillus niger*, preferably an *Aspergillus niger*, wherein one or more genes encoding succinate dehydrogenase, such as *sdhA* and is not functional.

Preferably, a eukaryotic cell according to the present invention comprising any one of the genetic modifications described herein is capable of producing at least 0.3, 0.5, 0.7, g / L succinic acid, preferably at least 1 g / L succinic acid, preferably at least 1.5 preferably at least 2, or 2.5, 4.5 preferably at least 8, 10, 15, or 20 g / L succinic acid but usually below 200 or below 150 g / L.

A preferred eukaryotic cell according to the present invention may be able to grow on any suitable carbon source known in the art and convert it to a desirable dicarboxylic acid as mentioned herein before. The eukaryotic cell may be able to convert directly plant biomass, celluloses, hemicelluloses, pectines, rhamnose, galactose, fucose, maltose, maltodextrines, ribose, ribulose, or starch, starch derivatives, sucrose, lactose and glycerol. Hence, a preferred host organism expresses enzymes such as cellulases (endocellulases and exocellulases) and hemicellulases (e.g. endo- and exo-xylanases, arabinases) necessary for the conversion of cellulose into glucose monomers and hemicellulose into xylose and arabinose monomers, pectinases able to convert

pectines into glucuronic acid and galacturonic acid or amylases to convert starch into glucose monomers. Preferably, the cell is able to convert a carbon source selected from the group consisting of glucose, fructose, galactose, xylose, arabinose, sucrose, lactose, raffinose and glycerol.

5 In another aspect, the present invention relates to a process for the preparation of a dicarboxylic acid, comprising fermenting the eukaryotic cell according to the present invention in a suitable fermentation medium and preparing the dicarboxylic acid. It was found advantageous to use a eukaryotic cell as defined herein above in the process for the production of a dicarboxylic acid such as succinic acid, because most eukaryotic
10 cells do not require sterile conditions for propagation and are insensitive to bacteriophage infections. The process according to the present invention may be run under aerobic and anaerobic conditions. Preferably, the process is carried out under anaerobic conditions or under micro-aerophilic or oxygen limited conditions. An anaerobic fermentation process is herein defined as a fermentation process run in the
15 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.

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
20 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.

25 The process for the production of a dicarboxylic acid according to the present invention may be carried out at any suitable pH between 1 and 9. Preferably, the pH in the fermentation broth is between 2 and 7, preferably between 3 and 5. It was found advantageous to be able to carry out the process according to the present invention at low pH, since this prevents bacterial contamination and less alkaline salts are needed for
30 titration to maintain the pH at a desired level in the process for the production of a dicarboxylic acid.

A suitable temperature at which the process according to the present invention may be carried out is between 5 and 60°C, preferably between 10 and 50°C, more

preferably between 15 and 35°C, more preferably between 18°C and 30°C. The skilled man in the art knows the optimal temperatures for fermenting a specific eukaryotic cell.

The dicarboxylic acid that is produced in the process according to the present invention may be succinic acid, fumaric acid or malic acid, preferably succinic acid.

5 Preferably, the dicarboxylic acid is recovered from the fermentation broth by a suitable method known in the art, for instance by crystallisation, ammonium precipitation or ion exchange technology.

10 Preferably, the dicarboxylic acid that is prepared in the process according to the present invention is further converted into a pharmaceutical, cosmetic, food, feed, or chemical product. Succinic acid may for instance be further converted into a polymer, such as polybutylene succinate (PBS) or other suitable polymers derived therefrom.

The present invention also relates to a fermentation broth comprising a dicarboxylic acid obtainable by the process according to the present invention.

15 The invention relates to a process for the production of a dicarboxylic acid wherein a eukaryotic cell is used as dicarboxylic acid producer, whereby phosphoenolpyruvate carboxykinase is used to increase dicarboxylic acid production, preferably wherein the phosphoenolpyruvate carboxykinase is active in the cytosol. Preferably the phosphoenolpyruvate carboxykinase is a heterologous enzyme preferably derived from *Actinobacillus succinogenes* or *Mannheimia succiniciproducens*.

20

Genetic modifications

Standard genetic techniques, such as overexpression of enzymes in the host cells, genetic modification of host cells, or hybridisation techniques, are known methods in the art, such as described in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, *Cold Spring Harbor Laboratory Press*, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation, genetic modification etc of fungal host cells are known from e.g. EP-A-0 635 574, WO 25 98/46772, WO 99/60102 and WO 00/37671, WO90/14423, EP-A-0481008, EP-A-0635 574 and US 6,265,186.

30

Description of the figures

Figure 1: Map of the the pGBTOP-11 vector used for expression of phosphoenolpyruvate carboxykinase in *A. niger*.

Figure 2: Plasmid map of pGB414SUS-01, encoding PEP carboxykinase from *Actinobacillus succinogenes* for expression in *Saccharomyces cerevisiae*. CPO denotes codon pair optimized.

5

Figure 3: Plasmid map of pGB414SUS-04, encoding PEP carboxykinase from *Mannheimia succiniciproducens* for expression in *Saccharomyces cerevisiae*. CPO denotes codon pair optimized.

10 **Figure 4:** Plasmid map of pDEL-SDHA.

Figure 5: Replacement scheme of *sdhA*.

15 **Figure 6:** Map of pGBTOPAn5, wherein the constitutive promoter *gpdA* drives the expression of PCKa. *GlaA* flanks were used for integration. *E. coli* DNA was removed by *NotI* digestion.

20 **Figure 7:** Map of pGBTOPAn6, wherein the constitutive promoter *gpdA* drives the expression of PCKm. *GlaA* flanks were used for integration. *E. coli* DNA was removed by *NotI* digestion.

Figure 8: Plasmid map of pGBS416PPK-1, encoding PEP carboxykinase from *Actinobacillus succinogenes* for expression in *Saccharomyces cerevisiae*. CPO denotes codon pair optimized.

25

Figure 9: Plasmid map of pGBS416PEK-1, encoding PEP carboxykinase from *Mannheimia succiniciproducens* for expression in *Saccharomyces cerevisiae*. CPO denotes codon pair optimized.

30 **Figure 10:** Plasmid map of pGBS415FUM-3, containing fumarase from *Rhizopus oryzae* (FUMR) and peroxisomal malate dehydrogenase from *Saccharomyces cerevisiae* (MDH3) for expression in *Saccharomyces cerevisiae*. The synthetic gene constructs TDH1 promoter-FUMR-TDH1 terminator and TDH3 promoter-MDH3-TDH3 terminator were cloned into expression vector pRS415. CPO denotes codon pair optimized.

Figure 11: Succinic (dashed lines) and fumaric acid (solid lines) levels in strains SUC-101 (○, empty vectors control), SUC-152 (□, overexpression of PCKa, MDH3, FUMR), SUC-154 (■, PCKm, MDH3, FUMR). All overexpressed genes were codon pair optimized for expression in *S. cerevisiae*. All data represent averages and standard deviations of 3 independent growth experiments of SUC-152 and 2 independent growth experiments of SUC-154 and averages and standard deviations of 6 independent growth experiments of SUC-101.

Figure 12: Plasmid map of pGBS414PPK-3, containing PEP carboxykinase from *Actinobacillus succinogenes* (PCKa) and glycosomal fumarate reductase from *Trypanosoma brucei* (FRDg) for expression in *Saccharomyces cerevisiae*. The synthetic gene constructs TDH1 promoter-PCKa-TDH1 terminator and TDH3 promoter-FRDg-TDH3 terminator were cloned into expression vector pRS414.

Figure 13: Plasmid map of pGBS426PYC-2, containing pyruvate carboxylase from *Saccharomyces cerevisiae* for expression in *Saccharomyces cerevisiae*. The PYC2 coding nucleotide sequence was obtained by PCR using genomic DNA from strain CEN.PK113-5D as template and the PCR product was cloned into expression vector p426GPD.

Figure 14: Plasmid map of pGBS414FRE-1, encoding glycosomal fumarate reductase (FRDg) from *Trypanosoma brucei* for expression in *Saccharomyces cerevisiae*. The synthetic gene construct TDH3 promoter-FRDg-TDH3 terminator was cloned into expression vector pRS414.

Figure 15: Succinic acid levels in strains SUC-226 (□, PCKa, MDH3, FUMR, FRDg), -227 (▲, PYC2, PCKa, MDH3, FUMR, FRDg), SUC-228 (■, PYC2, MDH3, FUMR, FRDg) and SUC-230 (○, MDH3, FUMR, FRDg). Data represents the average of 3 independent growth experiments.

The following examples are for illustrative purposes only and are not to be construed as limiting the invention.

EXAMPLES

Example 1

Cloning of Phosphoenolpyruvate carboxykinase from *Actinobacillus succinogenes* and *Mannheimia succiniciproducens* in *Aspergillus niger*

1.1. Expression constructs

Phosphoenolpyruvate carboxykinase [E.C. 4.1.1.49], GenBank accession number 152977907, from *Actinobacillus succinogenes* was analysed for the presence of signal sequences using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) Bendtsen, J. et al. (2004) Mol. Biol., 340:783-795 and TargetP 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>) Emanuelsson, O. et al. (2007) Nature Protocols 2, 953-971. Analysis as described by Schlüter et al., (2007) NAR, 35, D815–D822 revealed a putative PTS2 signal sequence at position 115-123. The *A. succinogenes* sequence (amino acid SEQ ID NO: 1, nucleotide sequence SEQ ID NO: 2) was modified to resemble the *Mannheimia succiniciproducens* protein sequence by replacing the amino acids EGY at position 120-122 with DAF (amino acid sequence SEQ ID NO: 3; nucleotide sequence SEQ ID NO: 4). Sequence SEQ ID NO: 3 was subjected to the codon-pair method as disclosed in WO2008/000632 for *A. niger*. The resulting sequence SEQ ID NO: 7 was put behind the constitutive GPDA promoter sequence SEQ ID NO: 11, wherein the last 10 nucleotide sequences were replaced with optimal Kozak sequence CACCGTAAA. Convenient restriction sites were added. The resulting sequence was synthesised at Sloning (Puchheim, Germany). The fragment was *Sna*BI, *Sfi*II cloned in the *A. niger* expression vector pGBTOP11 (see Figure 1) using appropriate restriction sites.

Likewise phosphoenolpyruvate carboxykinase [E.C. 4.1.1.49], GenBank accession number 52426348, from *Mannheimia succiniciproducens* was analysed for the presence of signal sequences as described in Schlüter et al., (2007) NAR, 35, D815–D822. The sequence as shown in SEQ ID NO: 5 (nucleotide sequence SEQ ID NO: 6) required no modifications. Subsequently the sequence was subjected to the codon-pair method as disclosed in WO2008/000632 for *A. niger*. The resulting sequence SEQ ID NO: 8 was put behind the constitutive GPDA promoter sequence SEQ ID NO: 11, and convenient restriction sites were added. The resulting sequence was synthesised at Sloning (Puchheim, Germany). The fragment was *Sna*BI, *Sfi*II cloned in *A. niger* expression vector pGBTOP11 (see Figure 1) using appropriate restriction sites. After

cloning of the PCKa gene into pGBTOP11, the vector was renamed pGBTOPAn5 (Figure 6). After cloning of the PCKa gene into pGBTOP11, the vector was renamed pGBTOPAn6 (Figure 7).

5 1.2. Transformation of *A. niger*

A. niger WT-1: This A. niger strain is CBS513.88 comprising deletions of the genes encoding glucoamylase (*glaA*), fungal amylase and acid amylase. A. niger WT-1 was constructed by using the "MARKER-GENE FREE" approach as described in EP 0 635 574 B1.

10 The expression constructs are co-transformed to strain A. niger WT-1 according to the method described by Tilburn, J. et al. (1983) Gene 26, 205-221 and Kelly, J. & Hynes, M. (1985) EMBO J., 4, 475-479 with the following modifications:

Spores are germinated and cultivated for 16 hours at 30 degrees Celsius in a shake flask placed in a rotary shaker at 300 rpm in *Aspergillus* minimal medium (100ml).
15 *Aspergillus* minimal medium contains per litre: 6 g NaNO₃, 0.52 g KCl, 1.52 g KH₂PO₄, 1.12 ml 4 M KOH, 0.52 g MgSO₄·7H₂O, 10 g glucose, 1 g casaminoacids, 22 mg ZnSO₄·7H₂O, 11 mg H₃BO₃, 5 mg FeSO₄·7H₂O, 1.7 mg CoCl₂·6H₂O, 1.6 mg CuSO₄·5H₂O, 5 mg MnCl₂·2H₂O, 1.5 mg Na₂MoO₄·2H₂O, 50 mg EDTA, 2 mg riboflavin, 2 mg thiamine-HCl, 2 mg nicotinamide, 1 mg pyridoxine-HCL, 0.2 mg panthotenic acid, 4 g
20 biotin, 10 ml Penicillin (5000 IU/ml) Streptomycin (5000 UG/ml) solution (Gibco).

- Novozym 234TM (Novo Industries) instead of helicase is used for the preparation of protoplasts;

- After protoplast formation (60-90 minutes), KCl buffer (0.8 M KCl, 9.5 mM citric acid, pH 6.2) is added to a final volume of 45 ml, the protoplast suspension is
25 centrifuged for 10 minutes at 3000 rpm at 4 degrees Celsius in a swinging-bucket rotor. The protoplasts are resuspended in 20 ml KC buffer and subsequently 25 ml of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) is added. The protoplast suspension is centrifuged for 10 minutes at 3000 rpm at 4 degrees Celsius in a swinging-bucket rotor, washed in STC-buffer and resuspended in STC-buffer at a concentration of
30 10E8 protoplasts/ml;

- To 200 microliter of the protoplast suspension, the DNA fragment, dissolved in 10 microliter TE buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) and 100 microliter of PEG solution (20% PEG 4000 (Merck), 0.8 M sorbitol, 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) is added;

- After incubation of the DNA-protoplast suspension for 10 minutes at room temperature, 1.5 ml PEG solution (60% PEG 4000 (Merck), 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) is added slowly, with repeated mixing of the tubes. After incubation for 20 minutes at room temperature, suspensions are diluted with 5 ml 1.2 M sorbitol, mixed by inversion and centrifuged for 10 minutes at 4000 rpm at room temperature. The protoplasts are resuspended gently in 1 ml 1.2 M sorbitol and plated onto solid selective regeneration medium consisting of either *Aspergillus* minimal medium without riboflavin, thiamine.HCL, nicotinamide, pyridoxine, panthotenic acid, biotin, casaminoacids and glucose. In case of acetamide selection the medium contains 10 mM acetamide as the sole nitrogen source and 1 M sucrose as osmoticum and C-source. Alternatively, protoplasts are plated onto PDA (Potato Dextrose Agar, Oxoid) supplemented with 1-50 microgram/ml phleomycin and 1M sucrose as osmoticum. Regeneration plates are solidified using 2% agar (agar No.1, Oxoid L11). After incubation for 6-10 days at 30 degrees Celsius, conidiospores of transformants are transferred to plates consisting of *Aspergillus* selective medium (minimal medium containing acetamide as sole nitrogen source in the case of acetamide selection or PDA supplemented with 1-50 microgram/ml phleomycin in the case of phleomycin selection) with 2% glucose and 1.5% agarose (Invitrogen) and incubated for 5-10 days at 30 degrees Celsius. Single transformants are isolated and this selective purification step is repeated once upon which purified transformants are stored.

1.3. Shake flask growth of *A. niger*

In total 10 transformants are selected for each construct and the presence of the construct is confirmed by PCR using primers specific for the constructs. Subsequently spores are inoculated in 100ml *Aspergillus* minimal enriched medium comprising 100g/l glucose. Strains are grown in an incubator at 250 rotations per minute for four days at 34 degrees Celsius. The supernatant of the culture medium is analysed for oxalic acid, malic acid, fumaric acid and succinic acid formation by HPLC and compared to a non transformed strain.

1.4 HPLC analysis

HPLC is performed for the determination of organic acids and sugars in different kinds of samples. The principle of the separation on a Phenomenex Rezex-RHM-Monosaccharide column is based on size exclusion, ion-exclusion and ion-exchange

using reversed phase mechanisms. Detection takes place by differential refractive index and ultra violet detectors.

Example 2A

5 Cloning of phosphoenolpyruvate carboxykinase from *Actinobacillus succinogenes* or *Mannheimia succiniciproducens* in *Saccharomyces cerevisiae*.

2A.1. Expression constructs

Phosphoenolpyruvate carboxykinase [E.C. 4.1.1.49] GenBank accession number 152977907 from *Actinobacillus succinogenes* was analysed for the presence of signal
10 sequences as described under § 1.1. SEQ ID NO: 3 was subjected to the codon-pair method as disclosed in WO2008/000632 for *S. cerevisiae*. The resulting sequence SEQ ID NO: 9 was put behind the constitutive TDH1 promoter sequence SEQ ID NO: 12 and before the TDH1 terminator sequence SEQ ID NO: 13, and convenient restriction sites were added. The resulting sequence was synthesised at Sloning (Puchheim, Germany).
15 The expression construct pGBS414SUS-01 was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS414 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the phosphoenolpyruvate carboxykinase (origin *Actinobacillus succinogenes*) synthetic gene construct (Figure 2). The ligation mix is used for
20 transformation of *E. coli* DH10B (Invitrogen) resulting in the yeast expression construct pGBS414SUS-01 (Figure 2).

Phosphoenolpyruvate carboxykinase [E.C. 4.1.1.49] GenBank accession number 52426348 from *Mannheimia succiniciproducens* identified and modified as described under § 1.1. SEQ ID NO: 5 was subjected to the codon-pair method as disclosed in
25 WO2008/000632 for *S. cerevisiae*. The resulting sequence SEQ ID NO: 10 was put behind the constitutive TDH1 promoter sequence SEQ ID NO: 12 and before the TDH1 terminator sequence SEQ ID NO: 13, and convenient restriction sites were added. The resulting sequence was synthesised at Sloning (Puchheim, Germany). The expression construct pGBS414SUS-04 was created after a *Bam*HI/*Not*I restriction of the *S.*
30 *cerevisiae* expression vector pRS414 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the phosphoenolpyruvate carboxykinase (origin *Mannheimia succiniciproducens*) synthetic gene construct (Figure 3). The ligation mix is used for

transformation of *E. coli* DH10B (Invitrogen) resulting in the yeast expression construct pGBS414SUS-04 (Figure 3).

2A.2. Transformation and shake flask growth

5 The constructs pGBS414SUS-01 and pGBS414SUS-04 are independently transformed into *S. cerevisiae* strains CEN.PK113-6B (*MATA ura3-52 leu2-112 trp1-289*), RWB066 (*MATA ura3-52 leu2-112 trp1-289 adh1::lox adh2::Kanlox*) and RWB064 (*MATA ura3-52 leu2-112 trp1-289 adh1::lox adh2::lox gpd1::Kanlox*). Transformation mixtures are plated on Yeast Nitrogen Base (YNB) w/o AA (Difco) + 2% glucose
10 supplemented with appropriate amino acids. Transformants are inoculated in Verduyn medium comprising glucose, supplemented with appropriate amino acids (Verduyn et al., 1992, Yeast. Jul;8(7):501-17) and grown under aerobic, anaerobic and oxygen-limited conditions in shake flasks. The medium for anaerobic cultivation is supplemented with 0.01 g/l ergosterol and 0.42 g/l Tween 80 dissolved in ethanol (Andreasen and Stier,
15 1953, J. cell. Physiol, 41, 23-36; Andreasen and Stier, 1954, J. Cell. Physiol, 43: 271-281). All yeast cultures are grown at 30°C in a shaking incubator at 250-280 rpm. At different incubation times, aliquots of the cultures are removed, centrifuged and the medium was analysed by HPLC for formation of oxalic acid, malic acid, fumaric acid and succinic acid as described under section 1.4.

Example 2B

Cloning of phosphoenolpyruvate carboxykinase from *Actinobacillus*

***succinogenes* or *Mannheimia succiniciproducens* in *Saccharomyces cerevisiae*.**

2B.1. Expression constructs

25 In a similar way as disclosed in Example 2A.1 the PCKa gene (SEQ ID NO: 9) was ligated into *S. cerevisiae* expression vector pRS416 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27). The ligation mix was used for transformation of *E. coli* TOP10 cells (Invitrogen) resulting in the yeast expression construct pGBS416PPK-1 (Figure 8).

30 Likewise, the PCKm gene (SEQ ID NO: 10) was ligated into pRS416. The ligation mix was used for transformation of *E. coli* TOP10 cells (Invitrogen) resulting in the yeast expression construct pGBS416PEK-1 (Figure 9).

2B.2. Transformation and microtiterplates (MTP's) growth experiments

The constructs pGBS416PPK-1 and pGBS416PEK-1 were independently transformed into *S. cerevisiae* strain CEN.PK113-5D (*MATA ura3-52*). As negative control, empty vector pRS416 was transformed into strain CEN.PK 113-5D. Transformation mixtures were plated on Yeast Nitrogen Base (YNB) w/o AA (Difco) + 2% glucose. The following numbers of individual transformants were inoculated *in duplo* in 250 microlitres Verduyn medium comprising 2% glucose in 96 deep-well MTP's and pre-cultured at 30 degrees Celsius, 550 rpm, and a humidity of 80% in an Infors Microplate shaking incubator: 12 pGBS416PPK-1 (PCKa), 12 pGBS416PEK-1 (PCKm) and 24 pRS416 empty vector control transformants. After 3 days, 25 microlitres of the pre-culture present in the wells of the MTP's were transferred to new 96 deep-well MTP plates containing Verduyn medium containing glucose and CaCO₃ (end-concentrations: glucose 10%, CaCO₃ 1% w/v in a total volume of 250 microlitres). After 7 days of growth at 30 degrees Celsius, 550 rpm, and a humidity of 80% in an Infors Microplate shaking incubator, the MTP's were centrifuged for 2 minutes at 2000 rpm, 200 microliters of supernatant was harvested using the Multimek 96 (Beckman), and the supernatant was analyzed by HPLC as described in Example 1.4 for the presence succinic acid. The results are shown in Table 2.

Table 1. Effect of insertion of PCKa and PCKm in *S. cerevisiae* on succinic acid production levels, compared to control strain comprising empty vector pRS416 after 7 days of cultivation.

<i>S. cerevisiae</i> strain CEN.PK 113-5D, comprising plasmid:	Succinic acid (mg/L)
pRS416	203±48 (n=48)
pGBS416PPK-1 (PCKa)	259±63 (n=24)
pGBS416PEK-1 (PCKm)	268±49 (n=24)

The results in Table 1 show that introduction and overexpression of phosphoenolpyruvate carboxykinase from *Actinobacillus succinogenes* or *Mannheimia succiniciproducens* resulted in an increased production level of succinic acid in *S. cerevisiae* (1.28 fold, $p=4.92E^{-}$, and 1.32 fold, $p=2.95E^{-6}$ Student's t-test, respectively).

Example 2C

Cloning of phosphoenolpyruvate carboxykinase from *Actinobacillus succinogenes* or *Mannheimia succiniciproducens*, malate dehydrogenase from *Saccharomyces cerevisiae* and fumarase from *Rhizopus oryzae* in *Saccharomyces cerevisiae*.

2C.1. Gene sequences

Phosphoenolpyruvate carboxykinase:

Gene sequences of PEP carboxykinase from *A. succinogenes* (PCKa) and *M. succiniciproducens* (PCKm) were designed and synthesized as described under 2A.1.

Malate dehydrogenase:

Peroxisomal malate dehydrogenase (Mdh3) [E.C. 1.1.1.37], GenBank accession number 1431095, was analysed for peroxisomal targeting in filamentous fungi using the PTS1 predictor <http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp> with the fungi-specific prediction function. The C-terminal amino acids at position 341-343 (SKL) were removed resulting in protein SEQ ID NO: 14. SEQ ID NO: 14 was subjected to the codon-pair method as disclosed in WO2008/000632 for *S. cerevisiae* resulting in SEQ ID NO: 15. The stop codon TGA in SEQ ID NO: 15 was modified to TAAG. The nucleotide sequence SEQ ID NO: 15 containing TAAG as stop codon was synthesized behind the constitutive TDH3 promoter sequence SEQ ID NO: 18 (600 bp upstream of start codon) and before the TDH3 terminator sequence SEQ ID NO: 19 (300 bp downstream of stop codon), and convenient restriction sites were added. The synthetic construct TDH3p-MDH3-TDH3t (SEQ ID NO: 20) was synthesised at Sloning (Puchheim, Germany).

Fumarase:

Fumarase [E.C. 4.2.1.2], GenBank accession number 469103, from *Rhizopus oryzae* was analysed for the presence of signal sequences using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) Bendtsen, J. et al. (2004) Mol. Biol., 340:783-795 and TargetP 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>) Emanuelsson, O. et al. (2007) Nature Protocols 2, 953-971. A putative mitochondrial targeting sequence in the first 23 amino acid of the protein was identified. To avoid potential targeting to mitochondria in *S. cerevisiae*, the first 23 amino acids were removed resulting in SEQ ID NO: 16 and a methionine amino acid was reintroduced. SEQ ID NO: 16 was subjected to

the codon-pair method as disclosed in WO2008/000632 for *S. cerevisiae* delivering nucleotide sequence SEQ ID NO: 17. The stop codon TAA in SEQ ID NO: 17 was modified to TAAG. SEQ ID NO: 17 containing TAAG as stop codon was synthesized behind the constitutive TDH1 promoter sequence SEQ ID NO: 12 and before the TDH1 terminator sequence SEQ ID NO: 13 and convenient restriction sites were added. The synthetic construct TDH1p-FumR-TDH1t (SEQ ID NO: 21) was synthesised at Sloning (Puchheim, Germany).

2C.2. Construction of expression constructs

The expression construct pGBS415FUM-3 (Figure 10) was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS415 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the fumarase (origin *Rhizopus oryzae*) synthetic gene construct (SEQ ID NO: 21). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS415FUM-1. Subsequently, pGBK415FUM-1 was restricted with *Asc*I and *Not*I. To create pGBS415FUM-3, an *Asc*I/*Not*I restriction fragment consisting of peroxisomal malate dehydrogenase from *S. cerevisiae* (*MDH3*) synthetic gene construct (SEQ ID NO: 20) was ligated into the restricted pGBS415FUM-1 vector. The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS415FUM-3 (Figure 10).

Construction of expression constructs pGBS414SUS-01 and pGBS414SUS-04 is described under example 2A.1.

2C.3. *S. cerevisiae* strains

Plasmids pGBS414SUS-01, pGBS415FUM-3 and pRS416 were transformed into *S. cerevisiae* strain CEN.PK113-6B (*MATA ura3-52 leu2-112 trp1-289*) to create strain SUC-152. Plasmids pGBS414SUS-04, pGBS415FUM-3 and pRS416 were transformed into *S. cerevisiae* strain CEN.PK113-6B (*MATA ura3-52 leu2-112 trp1-289*) to create strain SUC-154. A control strain overexpressing only empty vectors (SUC-101) was created by transformation of pRS414, pRS415 and pRS416. All genes were codon pair optimized for expression in *S. cerevisiae*. The expression vectors were transformed into yeast by electroporation. The transformation mixtures were plated on Yeast Nitrogen

Base (YNB) w/o AA (Difco) + 2% glucose. The genes overexpressed in strains SUC-152 and SUC-154 are described in Table 2.

Table 2: Yeast strains constructed for Example 2C.

<u>Name</u>	<u>Background</u>	<u>Plasmids</u>	<u>Genes</u>
SUC-152	CEN.PK113-6B	pGBS414SUS-01 pGBS415FUM-3 pRS416 (empty vector)	PCKa FUMR, MDH3
SUC-154	CEN.PK113-6B	pGBS414SUS-04 pGBS415FUM-3 pRS416 (empty vector)	PCKm FUMR, MDH3
SUC-101	CEN.PK113-6B	pRS414 (empty vector) pRS415 (empty vector) pRS415 (empty vector)	

5

2D.4. Growth experiments and succinic acid and fumaric acid production

Transformants were inoculated in 20 ml pre-culture medium consisting of Verduyn medium (Verduyn et al., 1992, Yeast. Jul;8(7):501-17) comprising 2% galactose (w/v) and grown under aerobic conditions in 100 ml shake flasks in a shaking incubator at 30°C at 250 rpm. After 72 hours, the culture was centrifuged for 5 minutes at 4750 rpm. 1 ml supernatant was used to measure succinic acid levels by HPLC as described in section 1.5. The remaining supernatant was decanted and the pellet (cells) was resuspended in 1 ml production medium. The production medium consisted of Verduyn medium with 10 % galactose (w/v) and 1% CaCO₃ (w/v). The resuspended cells were inoculated in 50 ml production medium in 100 ml shake flasks and grown in a shaking incubator at 30°C at 100 rpm. At various time points, 1 ml sample was taken from the culture. Succinic acid and fumaric acid levels were measured by HPLC as described in section 1.4 (Figure 11).

Strains transformed with empty vectors (control strain) produced up to 0.3 g/L succinic acid (Figure 11, dashed line). Overexpression of PEP carboxykinase from *M. succiniciproducens* (PCKm), peroxisomal malate dehydrogenase (MDH3) from *S. cerevisiae* and fumarase from *R. oryzae* (FUMR) resulted in a production level of 0.9 g/L succinic acid. Overexpression of PEP carboxykinase from *A. succinogenes* (PCKa), MDH3 and FUMR resulted in a succinic acid production level of 1.0 g/L. These results

20

show that when *S. cerevisiae* was transformed with a truncated MDH3 and FUMR in addition to either PCKa or PCKm, a further increased amount of succinic acid was produced as compared to a *S. cerevisiae* overexpressing PCKa or PCKm alone (Table 1).

5 Strains transformed with empty vectors (control strain) produced up to 14 mg/L fumaric acid after 8 days of growth (Figure 11, solid line). Overexpression of PEP carboxykinase from *A. succinogenes* (PCKa), malate dehydrogenase from *S. cerevisiae* (MDH3) and fumarase from *R. oryzae* (FUMR) resulted in maximal production of 55 mg/L fumaric acid after 7 days of growth. Overexpression of PEP carboxykinase from *M.*
10 *succiniciproducens* (PCKm), malate dehydrogenase from *S. cerevisiae* (MDH3) and fumarase from *R. oryzae* (FUMR) resulted in maximal production of 52 mg/L fumaric acid after 8 days of growth.

These data show that overexpression of PCKa or PCKm, MDH3 and FUMR in *S. cerevisiae* resulted in increased fumaric acid production levels as compared to a
15 corresponding wild-type *S. cerevisiae*.

Example 2D

**Cloning of phosphoenolpyruvate carboxykinase from *Actinobacillus succinogenes*, pyruvate carboxylase from *Saccharomyces cerevisiae*, malate
20 dehydrogenase from *Saccharomyces cerevisiae*, fumarase from *Rhizopus oryzae* in *Saccharomyces cerevisiae* and fumarate reductase from *Trypanosoma brucei*.**

2D.1. Gene sequences

Glycosomal fumarate reductase (FRDg) [E.C. 1.3.1.6], GenBank accession number 23928422, from *Trypanosoma brucei* was analysed for peroxisomal targeting in
25 filamentous fungi using the PTS1 predictor <http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp> with the fungi-specific prediction function. The C-terminal amino acids at position 1140-1142 (SKI) were removed from the protein, resulting in SEQ ID NO: 22. SEQ ID NO: 22 was subjected to the codon-pair method as disclosed in PCT/EP2007/05594 for expression in *S.*
30 *cerevisiae*. The resulting sequence SEQ ID NO: 23 was put behind the constitutive TDH3Sc promoter sequence SEQ ID NO: 24 and before the TDH3Sc terminator sequence SEQ ID NO: 25, and convenient restriction sites were added. The stop codon in SEQ ID NO: 23 was modified to TAAG. The resulting sequence was synthesised at Sloning (Puchheim, Germany).

The gene sequence of PEP carboxykinase from *A. succinogenes* was described under 2A.1. Gene sequences of malate dehydrogenase from *S. cerevisiae* and fumarase from *R. oryzae* were described under 2C.1.

Cytoplasmic pyruvate carboxylase from *Saccharomyces cerevisiae* (Pyc2p) [E.C. 6.4.1.1.], GenBank accession number 1041734, SEQ ID NO: 26, is encoded by the nucleotide sequence SEQ ID NO: 27. Genomic DNA from *S. cerevisiae* strain CEN.PK113-5D (*MATA ura3-52*) was used as template to amplify the *PYC2* coding sequence (SEQ ID NO: 29), using primers P1 SEQ ID NO: 28 and P2 SEQ ID NO: 29, and the Phusion DNA polymerase (Finnzymes, Finland) according to manufacturer's instructions. Convenient restriction sites were included in the primers for further cloning purposes.

2D.2. Construction of expression constructs

The expression construct pGBS414PPK-3 (Figure 12) was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS414 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a *Bam*HI/*Not*I restriction fragment consisting of the phosphoenolpyruvate carboxykinase (origin *Actinobacillus succinogenes*) synthetic gene construct (see 2A.1.). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS414PPK-1. Subsequently, pGBK414PPK-1 was restricted with *Asc*I and *Not*I. To create pGBS414PPK-3, an *Asc*I/*Not*I restriction fragment consisting of glycosomal fumarate reductase from *T. brucei* (FRDg) synthetic gene construct (see 2D.1.) was ligated into the restricted pGBS414PPK-1 vector. The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS414PPK-3 (Figure 12).

The expression construct pGBS426PYC-2 (Figure 13) was created after a *Spe*I/*Xho*I restriction of the *S. cerevisiae* expression vector p426GPD (Mumberg *et al.*, Gene. 1995 Apr 14;156(1):119-22) and subsequently ligating in this vector a *Spe*I/*Xho*I restriction fragment consisting of the amplified *PYC2* nucleotide sequence (SEQ ID NO: 29). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS426PYC-2 (Figure 13).

Expression construct pGBS414FRE-1 (Figure 14) was created after a *Bam*HI/*Not*I restriction of the *S. cerevisiae* expression vector pRS414 (Sirkoski R.S. and Hieter P, Genetics, 1989, 122(1):19-27) and subsequently ligating in this vector a

*Bam*HI/*Not*I restriction fragment consisting of the glycosomal fumarate reductase (origin *Trypanosoma brucei*) synthetic gene construct (see 2D.1.). The ligation mix was used for transformation of *E. coli* TOP10 (Invitrogen) resulting in the yeast expression construct pGBS414FRE-1 (Figure 14).

5 Construction of expression construct pGBS415FUM-3 was described under 2C.2.

2D.3. *S. cerevisiae* strains

Strains SUC-226, SUC-227, SUC-228 and SUC-230 were obtained by transformation of different combinations of the plasmids pGBS414FRE-1, pGBS414PPK-
10 3, pGBS415FUM-1, pGBS426PYC-2 and p426GPD into strain CEN.PK113-6B (*MAT*A *ura*3-52 *leu*2-112 *trp*1-289), as depicted in Table 3.

Table 3: Yeast strains constructed for Example 2D.

Name	Background	Plasmids	Genes
SUC-226	CEN.PK113-6B	pGBS414PPK-3 pGBS415FUM-3 p426GPD (empty vector)	PCKa, FRDg FUMR, MDH3
SUC-227	CEN.PK113-6B	pGBS414PPK-3 pGBS415FUM-3 pGBS426PYC-2	PCKa, FRDg FUMR, MDH3 PYC2
SUC-228	CEN.PK113-6B	pGBS414FRE-1 pGBS415FUM-3 pGBS426PYC-2	FRDg FUMR, MDH3 PYC2
SUC-230	CEN.PK113-6B	pGBS414FRE-1 pGBS415FUM-3 p426GPD (empty vector)	FRDg FUMR, MDH3

15 2D.4. Growth experiments and succinic acid production

Growth parameters and sample analysis were performed as described under example 2C.4 with the following modifications: pre-culturing was performed using 2% glucose (w/v) as carbon source. In the production medium 10% glucose (w/v) was used as carbon source.

20 As depicted in Figure 15 strain SUC-230, overexpressing *MDH3*, *FUMR* and *FRDg*, produced up to 3.0 g/L succinic acid. Additional overexpression of *PCKa*

increased succinic acid production up to 3.4 g/L (strain SUC-226), and additional overexpression of *PYC2* increased succinic acid production up to 3.7 g/L (strain SUC-228). Surprisingly, overexpression of both *PCKa* and *PYC2* (SUC-227) resulted in 1.5 increase of succinic acid production levels up to 5.0 g/L, as compared to the effect of PCK and PYC alone. These results show a synergistic effect of combined overexpression of both PEP carboxykinase from *A. succinogenes* (*PCKa*) and pyruvate carboxylase from *S. cerevisiae* (*PYC2*) on succinic acid production levels in *S. cerevisiae*.

Example 3

Inactivation of succinate dehydrogenase encoding genes in *Aspergillus niger*

3.1. Identification

Genomic DNA of *Aspergillus niger* strain CBS513.88 was sequenced and analyzed. Two genes with translated proteins annotated as homologues to succinate dehydrogenase proteins were identified and named *sdhA* and *sdhB* respectively. Sequences of the *sdhA* (An16g07150) and *sdhB* (An02g12770) loci are available on genbank with accession numbers 145253004 and 145234071, respectively. Gene replacement vectors for *sdhA* and *sdhB* were designed according to known principles and constructed according to routine cloning procedures (see figures 4 and 5). The vectors comprise approximately 1000 bp flanking regions of the *sdh* ORFs for homologous recombination at the predestined genomic loci. In addition, they contain the *A. nidulans* bi-directional *amdS* selection marker driven by the *gpdA* promoter, in-between direct repeats. The general design of these deletion vectors were previously described in EP635574B and WO 98/46772.

*3.2. Inactivation of the *sdhA* gene in *Aspergillus niger*.*

Linear DNA of deletion vector pDEL-SDHA (figure 4) was isolated and used to transform *Aspergillus niger* CBS513.88 as described in: Biotechnology of Filamentous fungi: Technology and Products. (1992) Reed Publishing (USA); Chapter 6: Transformation p. 113 to 156. This linear DNA can integrate into the genome at the *sdhA* locus, thus substituting the *sdhA* gene by the *amdS* gene as depicted in figure 6. Transformants were selected on acetamide media and colony purified according to standard procedures as described in EP635574B. Spores were plated on fluoro-acetamide media to select strains, which lost the *amdS* marker. Growing colonies were

diagnosed by PCR for integration at the *sdhA* locus and candidate strains tested by Southern analyses for deletion of the *sdhA* gene. Deletion of the *sdhA* gene was detectable by the ~ 2,2 kb size reduction of DNA fragments (4.6 kb wild-type fragment versus 2.4 kb for a successful deletion of SDHA) covering the entire locus and hybridized to appropriate probes. Approximately 9 strains showed a removal of the genomic *sdhA* gene from a pool of approximately 96 initial transformants.

Strain dSDHA was selected as a representative strain with an inactivated *sdhA* gene. The production of succinic acid by the dSDHA strain was measured as described in Example 4.

Example 4

Cloning of PCKa and PCKm in *A. niger* dSDHA and growth in microtiter plates (MTP's)

A. niger strain dSDHA of example 3.2. was transformed with the expression construct pGBTOPAn5 (Figure 6) comprising PEP carboxykinase from *Actinobacillus succinigenes* (PCKa, SEQ ID NO: 7) and expression construct pGBTOPAn6 (Figure 7) comprising PEP carboxykinase from *Mannheimia succinicproducing* (PCKm, SEQ ID NO: 8) as described in Example 1.1., according to the transformation method as described in Example 1.2.

A. niger transformants were picked using Qpix and transferred onto MTP's containing selective media. After 7 days of incubation at 30 degrees Celsius the biomass was transferred to MTP's containing PDA by hand or colony picker. After 7 days incubation at 30 degrees Celsius, the biomass was sporulated. These spores were resuspended using the Multimek 96 (Beckman) in 100 microlitres minimal enriched Aspergillus medium containing 10% glucose. Subsequently 2 MTP's with 170 microlitres minimal enriched Aspergillus medium containing 10% glucose and 1% CaCO₃ were inoculated with 30 microlitres of the spore suspension. Likewise dSDHA and control *A. niger* strain CBS513.88 were inoculated in the MTP's. These MTP's were incubated for 5 days at 34 degrees Celsius, 550 rpm at 80% humidity. After 5 days 160 microlitres were harvested using the Multimek 96 (Beckman). Succinic acid in the media was measured by HPLC as described in Example 1.4. The results are shown in Table 3.

Table 4. Effect of deletion of succinate dehydrogenase (SDHA) and insertion of PCKa and PCKm in *A. niger* on succinic acid production levels

<i>A. niger</i> strain	Succinic acid mg/l
CBS513.88	38
dSDHA	50
dSDHA, + PCKa	160
d,SDHA, + PCKm	241

5 The results in Table 4 show that insertion of phosphoenol pyruvate carboxykinase from both *A. succinogenes* or from *M. succiniciproducens* increased succinic acid production levels by *A. niger*.

CLAIMS

1. A recombinant eukaryotic microbial cell comprising a nucleotide sequence encoding an enzyme catalysing the conversion from phosphoenolpyruvate to oxaloacetate whereby ATP is generated, wherein the enzyme comprises an amino acid sequence which has at least 50% sequence identity with the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3 and/or SEQ ID NO: 5.
5
2. A cell according to claim 1, wherein the enzyme is a phosphoenolpyruvate carboxykinase.
10
3. A cell according to claim 1 or 2 wherein the enzyme is a heterologous enzyme, preferably derived from a bacterium.
4. A cell according to any one of the claims 1 to 3, wherein the nucleotide sequence is expressed in the cytosol.
15
5. A cell according to any one of the claims 1 to 4, wherein the cell is a yeast, preferably a *Saccharomyces cerevisiae*, or a filamentous fungus such as *Aspergillus niger*.
20
6. A cell according to any one of the claims 1 to 5, wherein the cell overexpresses a nucleotide sequence encoding a pyruvate carboxylase.
7. A cell according to any one of the claims 1 to 6, wherein the cell further comprises a nucleotide sequence encoding a malate dehydrogenase, wherein the malate dehydrogenase is active in the cytosol upon expression of the nucleotide sequence encoding malate dehydrogenase.
25
8. A cell according to any one of the claims 1 to 7, wherein the cell further comprises a nucleotide sequence encoding an enzyme that catalyses the conversion of malic acid to fumaric acid in the cytosol, upon expression of the nucleotide sequence encoding an enzyme that catalyses the conversion of malic acid to fumaric acid
30

9. A cell according to any one of the claims 1 to 8, wherein at least one gene encoding succinate dehydrogenase is not functional.
- 5 10. A cell according to claim 1 to 9 which is an *Aspergillus niger* comprising a nucleotide sequence encoding a phosphoenolpyruvate carboxykinase of SEQ ID NO: 7 and/or SEQ ID NO: 8
- 10 11. A cell according to claim 1 to 9 which is a *Saccharomyces cerevisiae* comprising a nucleotide sequence encoding a phosphoenolpyruvate carboxykinase of SEQ ID NO: 9 and/or SEQ ID NO: 10.
- 15 12. A process for the preparation of a dicarboxylic acid, comprising fermenting the eukaryotic microbial cell according to any one of the claims 1 to 12 in a suitable fermentation medium, and preparing the dicarboxylic acid .
13. A process according to claim 12, wherein the dicarboxylic acid is succinic acid, fumaric acid or malic acid.
- 20 14. A process according to claim 12 or 13, wherein the dicarboxylic acid is further converted into a pharmaceutical, cosmetic, food, feed, or chemical product.
- 25 15. A fermentation broth comprising a dicarboxylic acid obtainable by the process according to claim 12 or 13.

1/15

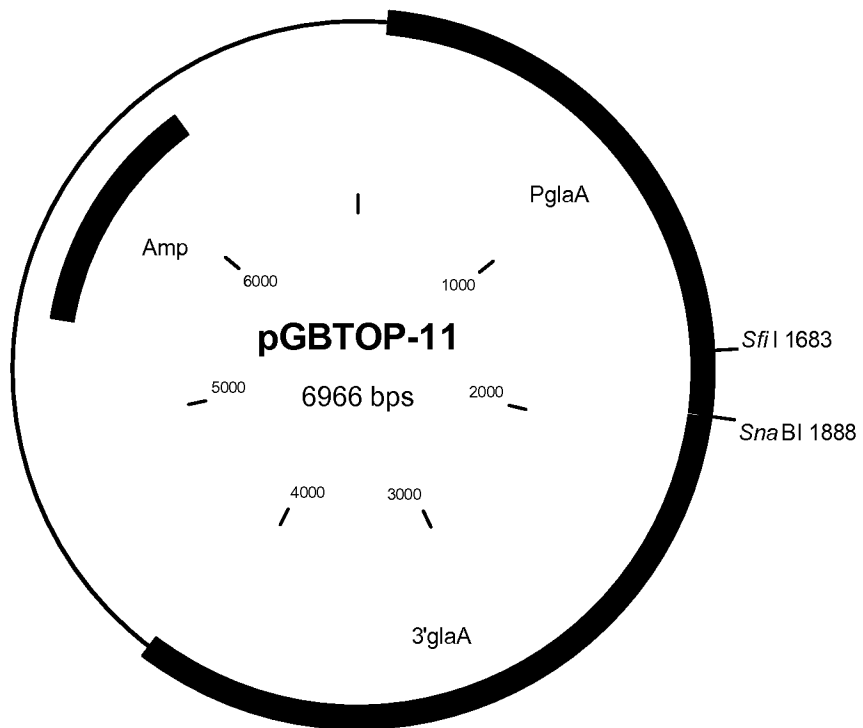


Figure 1

2/15

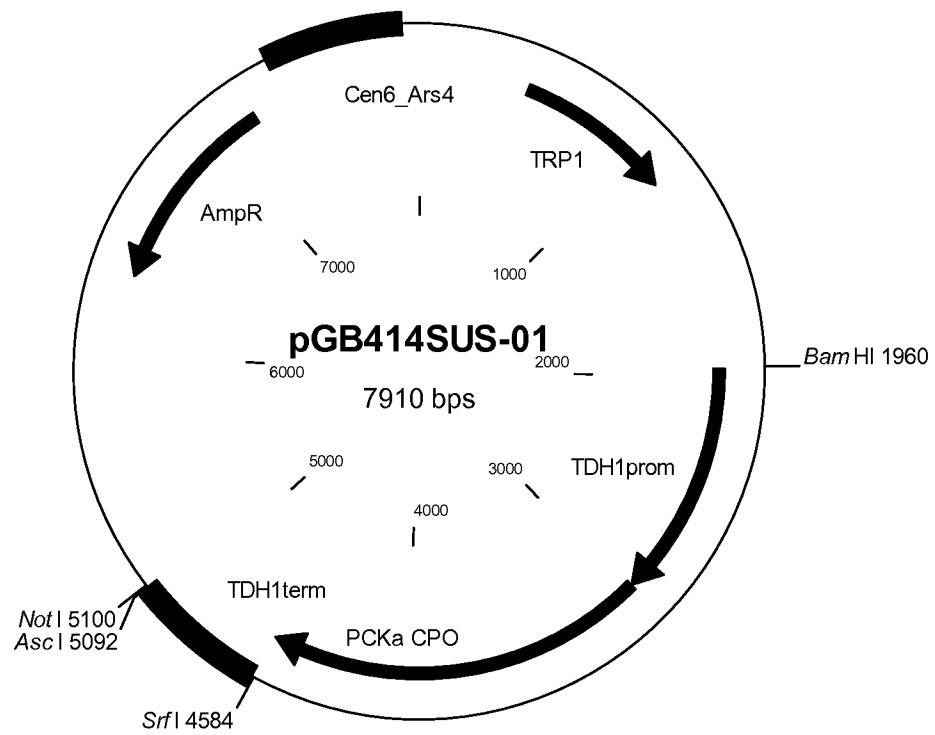


Figure 2

3/15

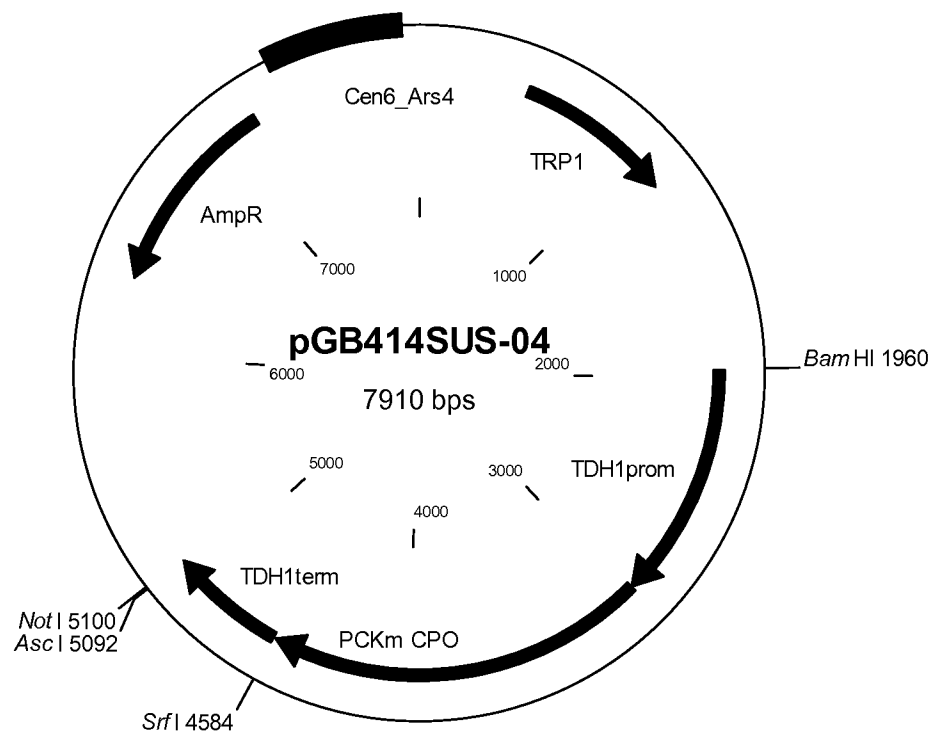


Figure 3

4/15

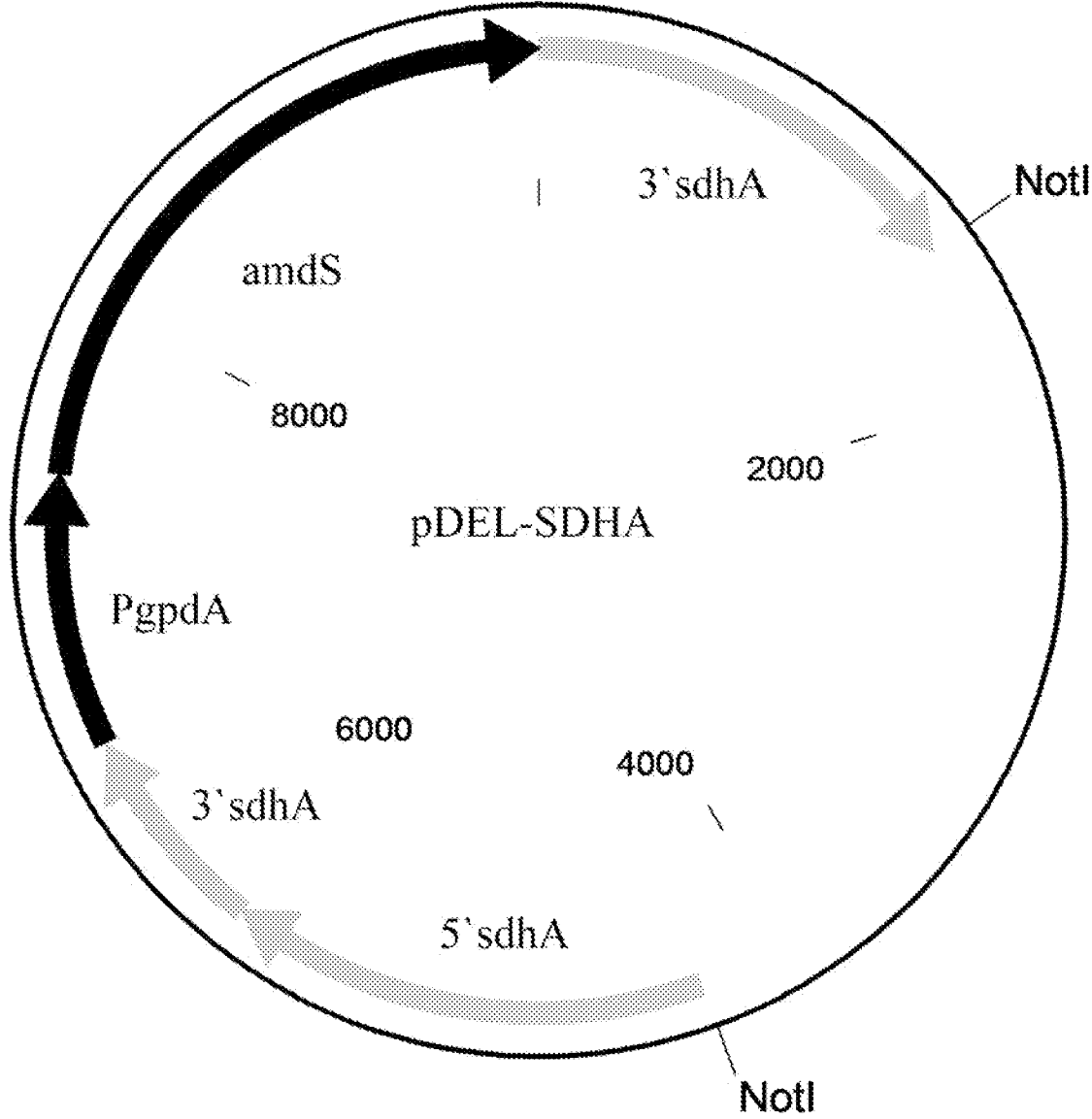


Figure 4

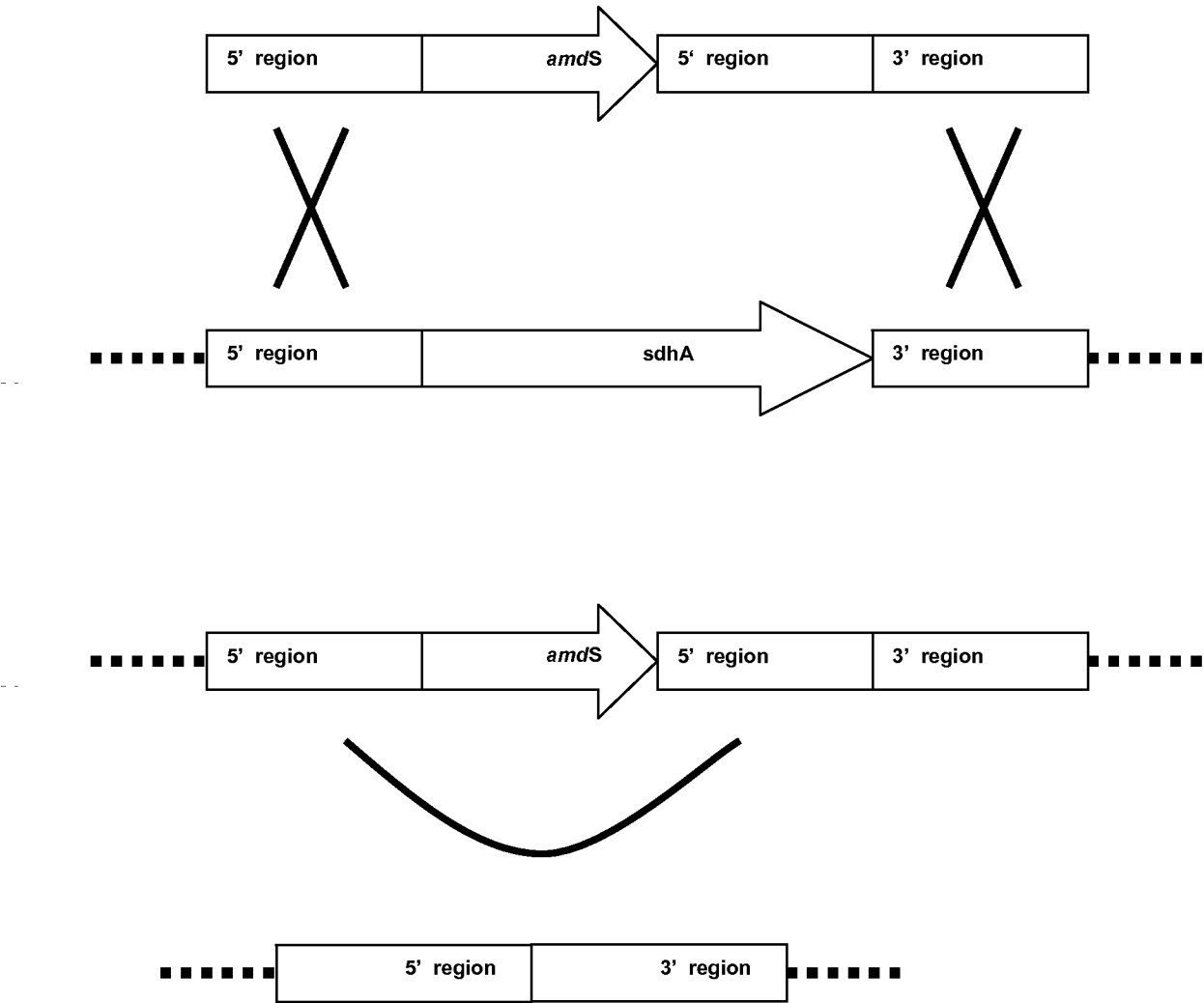


Figure 5

6/15

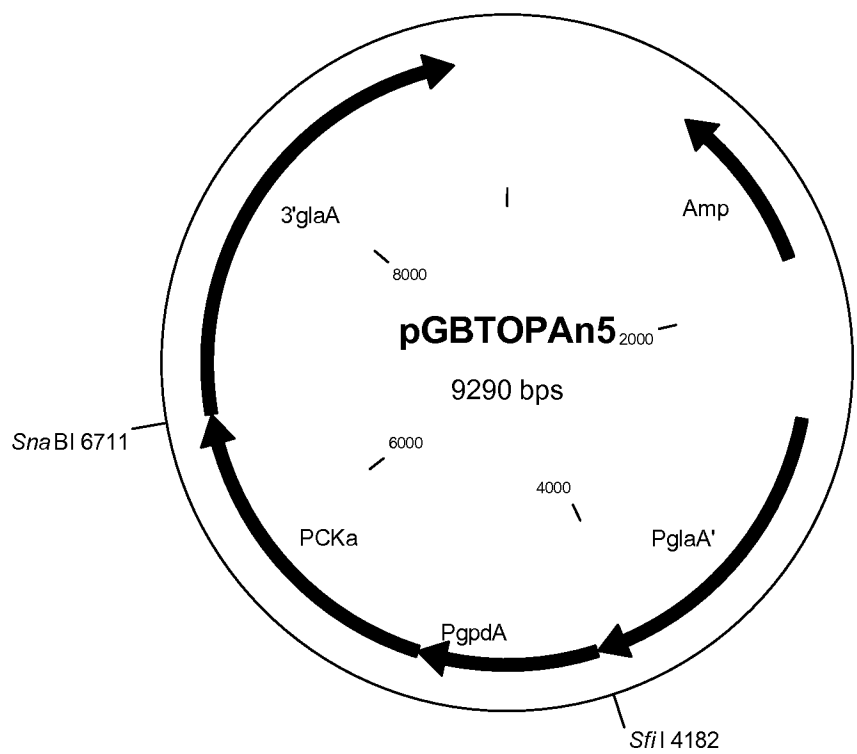


Figure 6

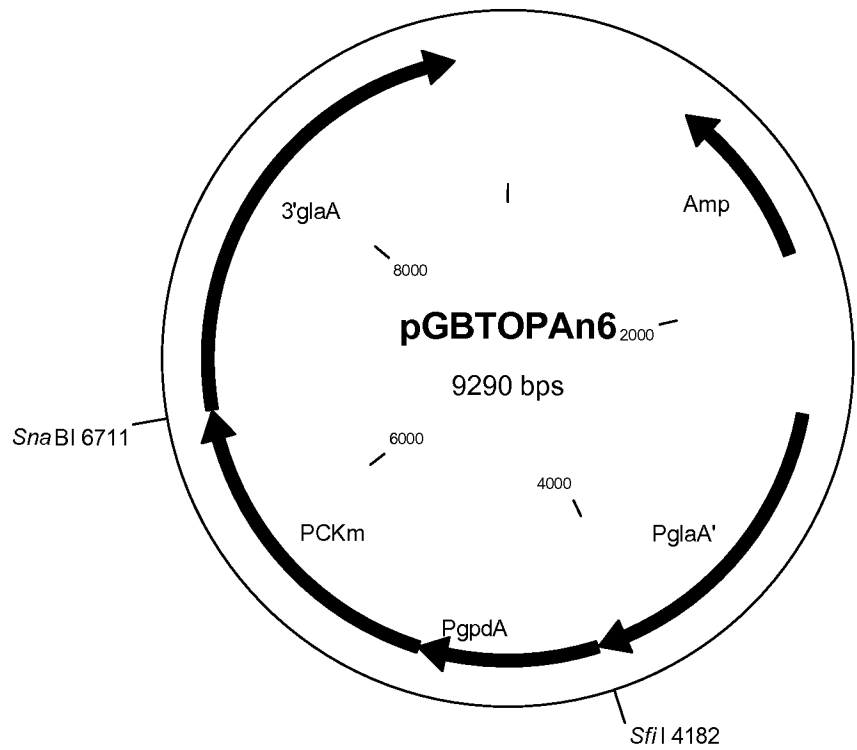


Figure 7

8/15

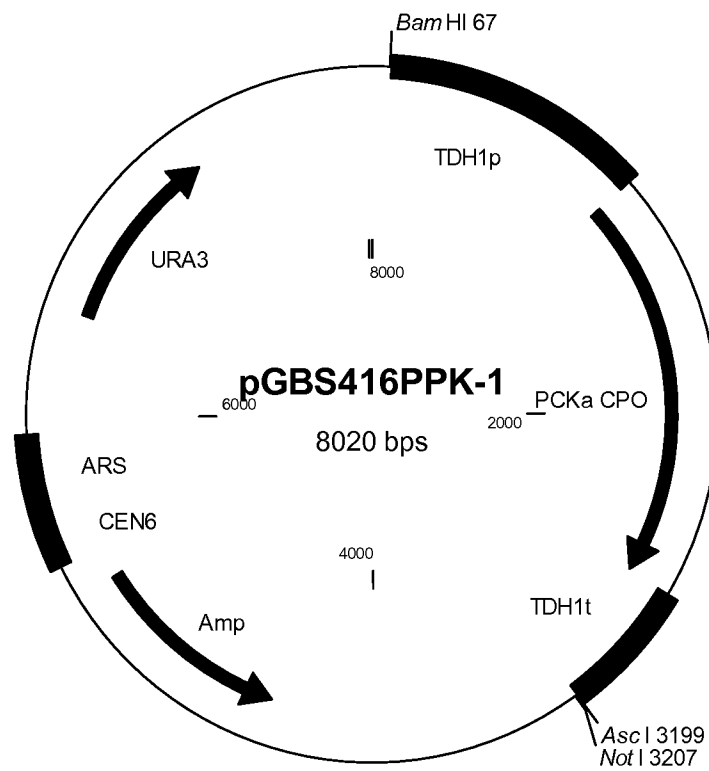


Figure 8

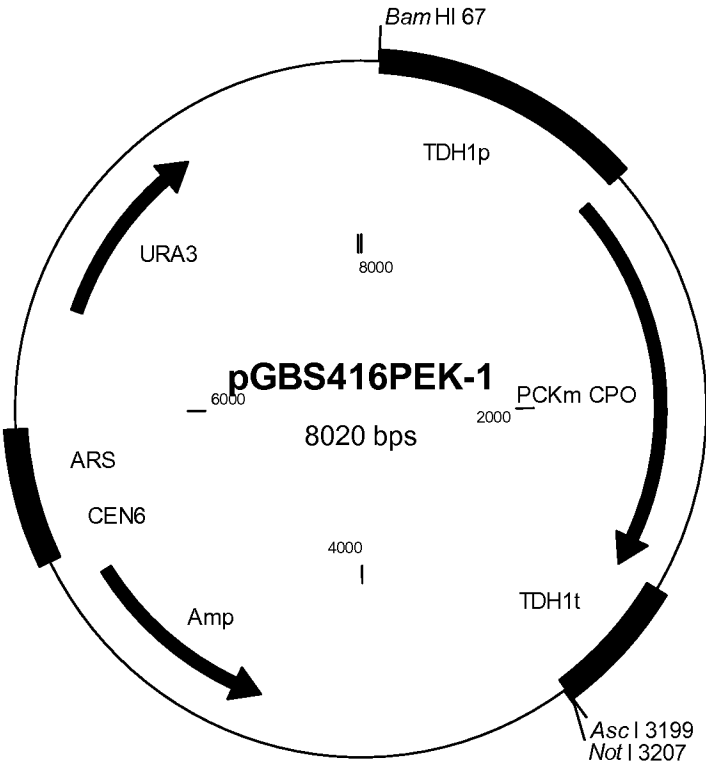


Figure 9

10/15

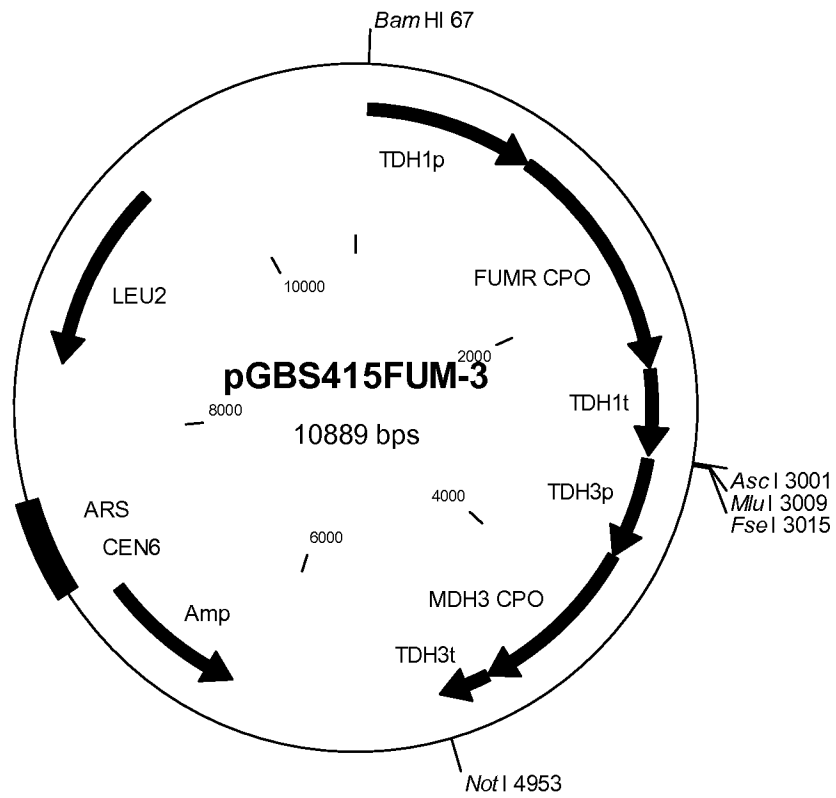


Figure 10

11/15

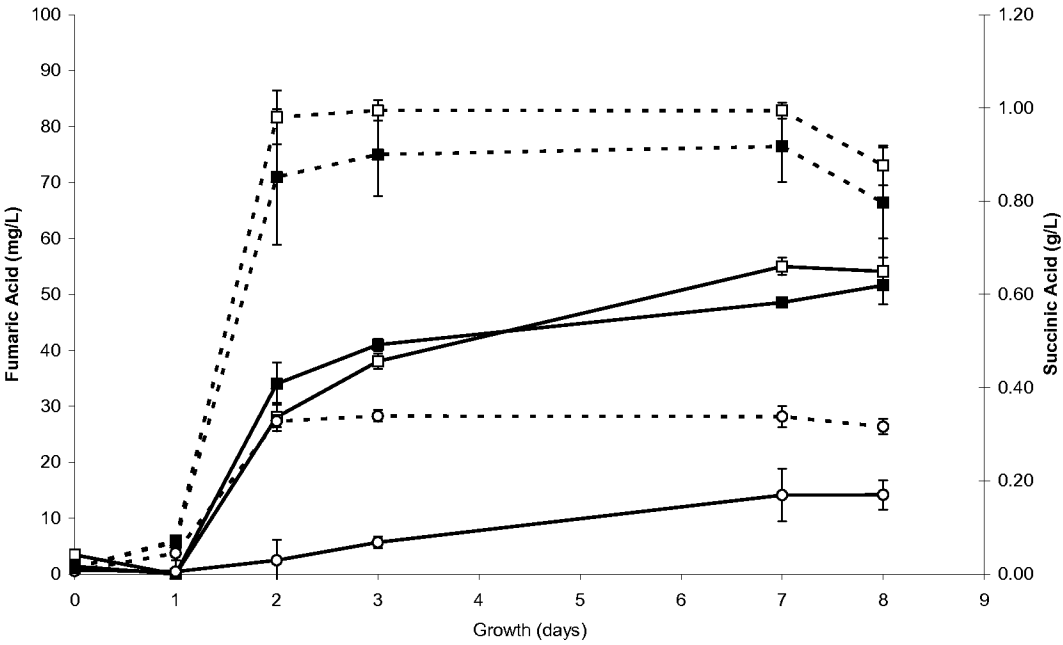


Figure 11

12/15

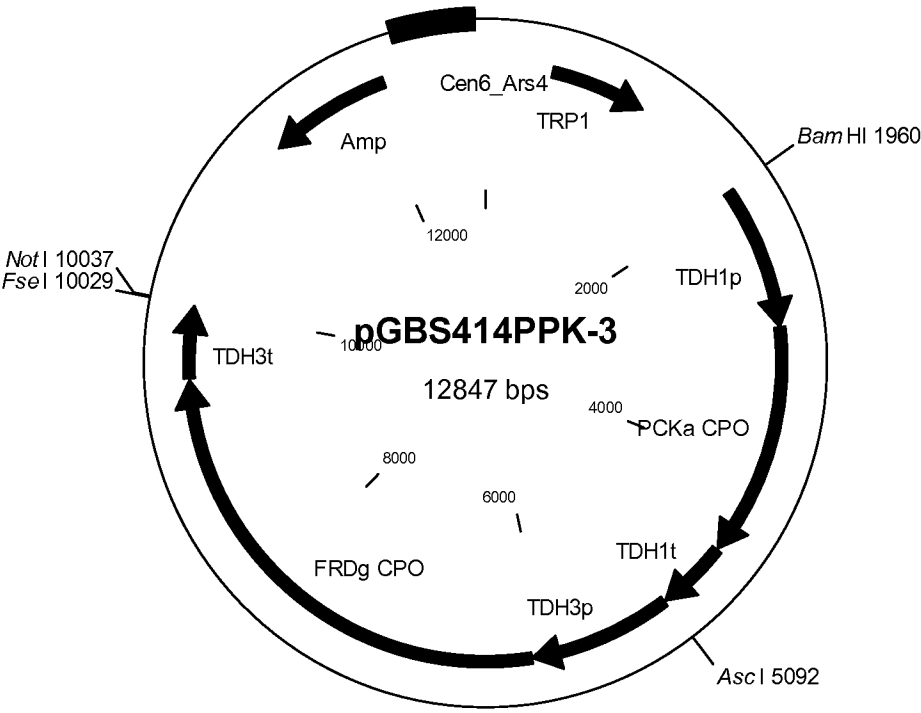


Figure 12

13/15

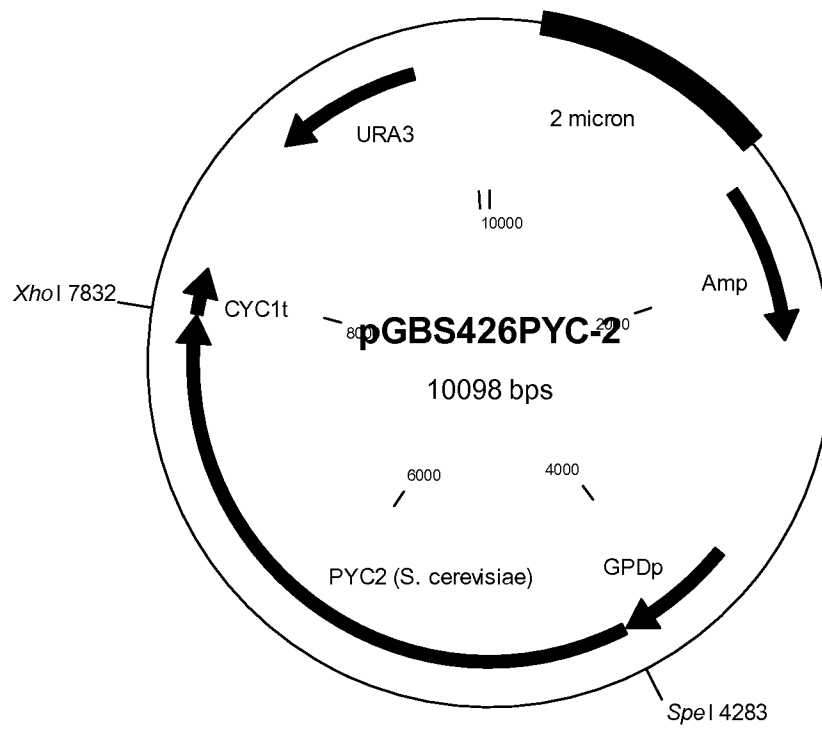


Figure 13

14/15

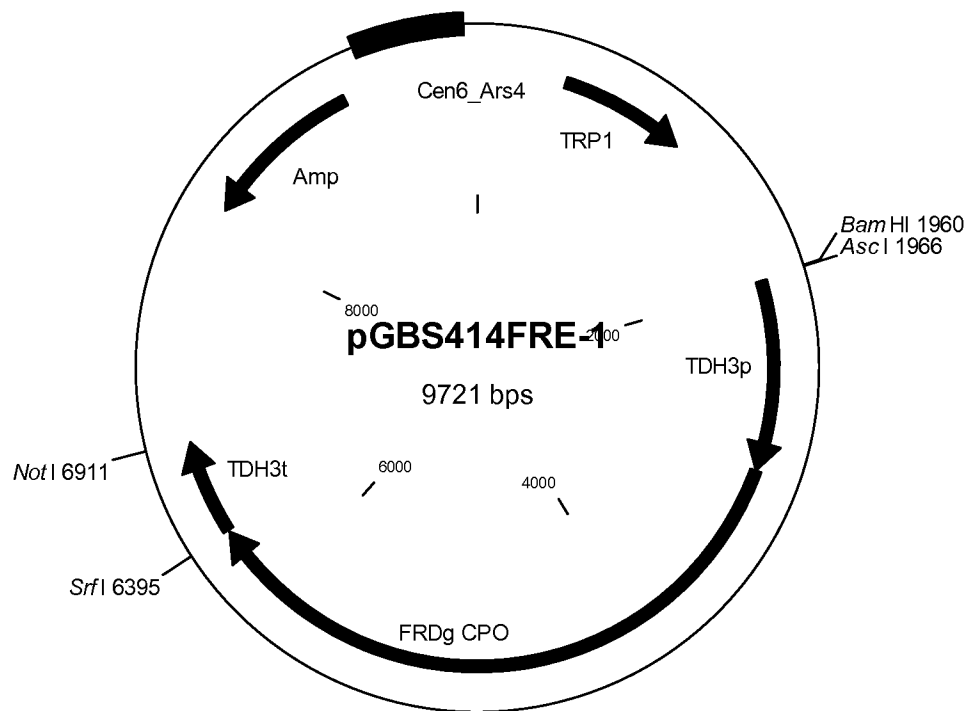


Figure 14

15/15

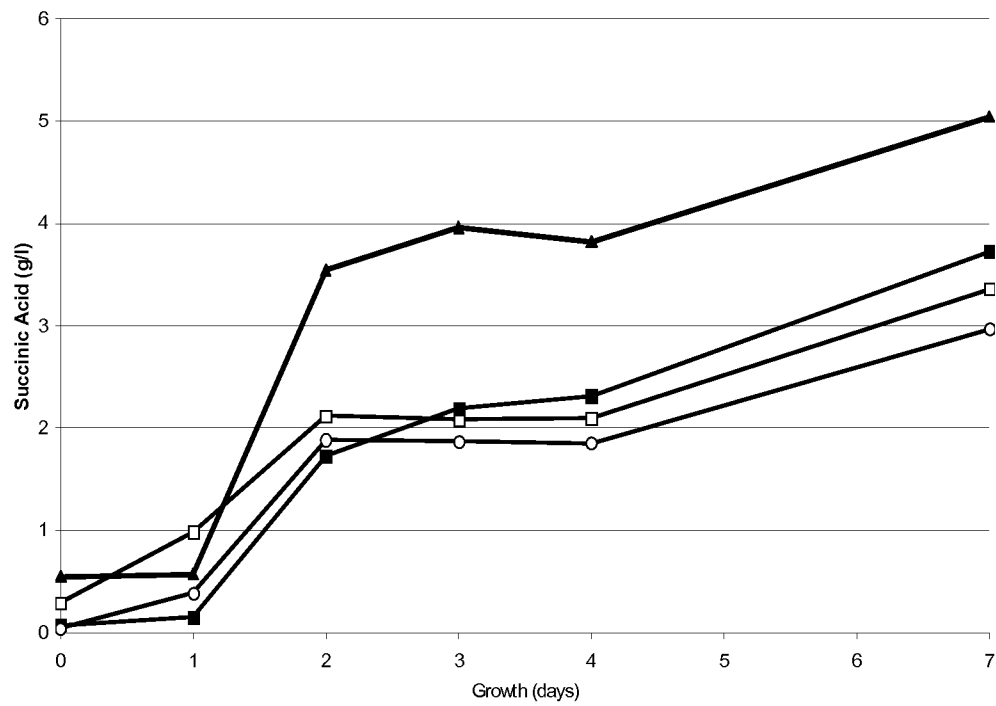


Figure 15

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/065588

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/60 C12N9/88 C12P7/46

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, Sequence Search, 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	JACOB L R ET AL: "Fast high-performance liquid chromatographic purification of <i>Saccharomyces cerevisiae</i> phosphoenolpyruvate carboxykinase." JOURNAL OF CHROMATOGRAPHY, vol. 625, no. 1, 13 November 1992 (1992-11-13), pages 47-54, XP008091044 ISSN: 0021-9673 abstract ----- -/--	1,2,4,5

☒ 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
- *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

2 February 2009

Date of mailing of the international search report

12/02/2009

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

van de Kamp, Mart

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/065588

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HYNES M J ET AL: "Regulation of the acvF gene, encoding phosphoenolpyruvate carboxykinase in the filamentous fungus <i>Aspergillus nidulans</i>" JOURNAL OF BACTERIOLOGY, vol. 184, no. 1, January 2002 (2002-01), pages 183-190, XP002478742 ISSN: 0021-9193 page 184, right-hand column, lines 26-34 abstract	1,2,4,5
A	BAZAES S ET AL: "Comparative kinetic effects of Mn (II), Mg (II) and the ATP/ADP ratio on phosphoenolpyruvate carboxykinases from <i>Anaerobiospirillum succiniciproducens</i> and <i>Saccharomyces cerevisiae</i>." THE PROTEIN JOURNAL, vol. 26, no. 4, June 2007 (2007-06), pages 265-269, XP002478743 ISSN: 1572-3887 abstract	1-3,12
A	JABALQUINTO A M ET AL: "Characterization of the Oxaloacetate Decarboxylase and Pyruvate Kinase-like Activities of <i>Saccharomyces cerevisiae</i> and <i>Anaerobiospirillum succiniciproducens</i> Phosphoenolpyruvate Carboxykinases" JOURNAL OF PROTEIN CHEMISTRY, vol. 18, no. 6, 1 August 1999 (1999-08-01), pages 659-664, XP019284032 ISSN: 1573-4943 abstract	1-3,12
A	WO 2007/061590 A (TATE & LYLE INGREDIENTS AMERIC [US]; WINKLER AARON ADRIAAN [NL]; DE HU) 31 May 2007 (2007-05-31) cited in the application the whole document page 1, line 18 - page 2, line 5 page 3, line 6 - page 4, line 4 page 6, lines 7-15 claims 1,2,9,11	1,12
A	WO 2007/019301 A (UNIV MICHIGAN [US]; ZEIKUS J GREGORY [US]; MCKINLAY JAMES B [US]; LAIV) 15 February 2007 (2007-02-15) the whole document claims 2,6	1,12

-/--

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/065588

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BAUER JET AL: "By-product formation during exposure of respiring <i>Saccharomyces cerevisiae</i> cultures to excess glucose is not caused by a limited capacity of pyruvate carboxylase" FEMS MICROBIOLOGY LETTERS, vol. 179, no. 1, 1 October 1999 (1999-10-01), pages 107-113, XP002478740 ISSN: 0378-1097 abstract	1,12
A	US 2006/246560 A1 (FATLAND-BLOOM BETH [US] ET AL) 2 November 2006 (2006-11-02) the whole document example 6	1,12
A	MILLARD C S ET AL: "Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in <i>Escherichia coli</i> " APPLIED AND ENVIRONMENTAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 62, no. 5, 1 May 1996 (1996-05-01), pages 1808-1810, XP002132795 ISSN: 0099-2240 abstract	1,12
A	LIN H ET AL: "Metabolic engineering of aerobic succinate production systems in <i>Escherichia coli</i> to improve process productivity and achieve the maximum theoretical succinate yield" METABOLIC ENGINEERING, vol. 7, no. 2, 1 March 2005 (2005-03-01), pages 116-127, XP004801711 ISSN: 1096-7176 the whole document	1,9,12
A	SONG ET AL: "Production of succinic acid by bacterial fermentation" ENZYME AND MICROBIAL TECHNOLOGY, vol. 39, no. 3, 3 July 2006 (2006-07-03), pages 352-361, XP005459365 ISSN: 0141-0229 the whole document paragraph 3.4	1,12

-/--

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/065588

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE JONGH W A ET AL: "Enhanced citrate production through gene insertion in <i>Aspergillus niger</i>" METABOLIC ENGINEERING, vol. 10, no. 2, 17 November 2007 (2007-11-17), pages 87-96, XP022510142 ISSN: 1096-7176 the whole document paragraph 4.2-5 figure 6	8
A	DE JONGH W A: "Organic acid production by <i>Aspergillus niger</i>" PHD THESIS, May 2006 (2006-05), pages I-109, XP002445685 BioCentrum-DTU Technical University of Denmark chapter 6,7	8
A	WO 2007/030830 A (GENOMATICA INC [US]; BURGARD ANTHONY P [US]; VAN DIEN STEPHEN J [US]) 15 March 2007 (2007-03-15) page 1, line 31 - page 2, line 3 example 1 claims 1,5,7-9,13,15,27,28	9
A	KUBO Y ET AL: "Effect of gene disruption of succinate dehydrogenase on succinate production in a sake yeast strain" JOURNAL OF BIOSCIENCE AND BIOENGINEERING, vol. 90, no. 6, 2000, pages 619-624, XP003009625 ISSN: 1389-1723 abstract	9
A	GOLDBERG I ET AL: "Organic acids: old metabolites, new themes" JOURNAL OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY, vol. 81, no. 10, October 2006 (2006-10), pages 1601-1611, XP002477014 ISSN: 0268-2575 the whole document page 1605, right-hand column, line 10 - page 1606, right-hand column, line 2	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2008/065588

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2007061590	A	31-05-2007	AU 2006317058 A1 CA 2630333 A1 EP 1954799 A1 KR 20080069678 A US 2008090273 A1	31-05-2007 31-05-2007 13-08-2008 28-07-2008 17-04-2008
WO 2007019301	A	15-02-2007	CA 2618694 A1 CN 101278041 A EP 1922405 A2 KR 20080031504 A	15-02-2007 01-10-2008 21-05-2008 08-04-2008
US 2006246560	A1	02-11-2006	NONE	
WO 2007030830	A	15-03-2007	AU 2006287257 A1 CA 2621562 A1 CN 101300356 A EP 1937821 A2	15-03-2007 15-03-2007 05-11-2008 02-07-2008