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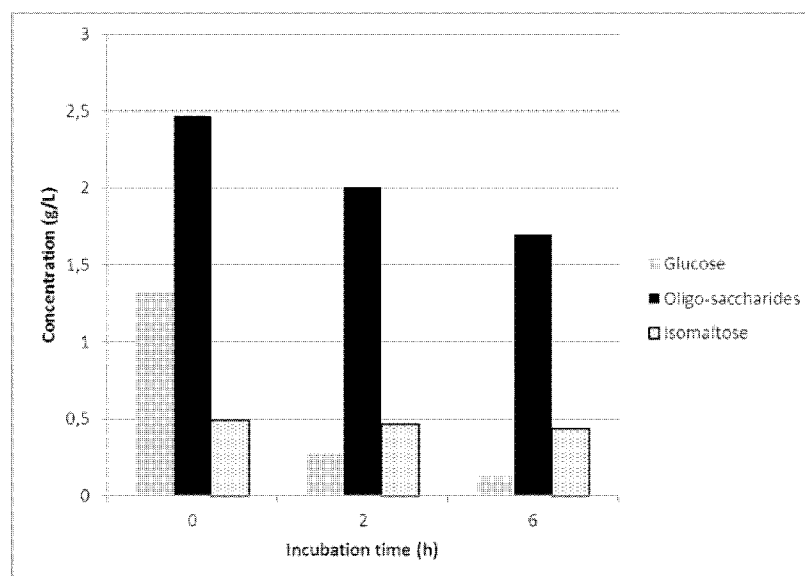


Fig. 6

(57) Abstract: The invention relates to a process for the production of a compound of interest, which process comprises fermenting a first cell capable of producing the compound of interest in a suitable fermentation medium under conditions which allow for production of the compound of interest, wherein the fermentation medium comprises disaccharides and/or oligosaccharides which are not completely fermentable by the first cell; and wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the disaccharides and/or the oligosaccharides; and, optionally, recovering the compound of interest from the reaction medium. The invention also relates to a fermentation broth obtainable by such a process.

FERMENTATION PROCESS

Field of the invention

5 The present invention relates to a process for the production of a compound of interest by fermentation. The invention also relates to an end-of-fermentation broth, for example one obtainable by a process of the invention.

Background to the invention

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 Dicarboxylic acids such as malic acid, fumaric acid and succinic acid, are important compounds which are used in the food industry for the preparation and preservation of food, in the medical industry for the formulation of medical products, building blocks in (bio)polymers and other industrial uses. To meet the increasing need
15 for dicarboxylic acids, more efficient and cost effective production methods are being developed.

 Several processes for the production of a dicarboxylic acid are known. Traditionally, dicarboxylic acids are made by fermentation of bacteria at neutral pH, for example described in US 5,573,931. Also other microorganisms like yeasts have been
20 employed for the production of dicarboxylic acids at low pH, which has the advantage of producing the acid directly (WO2010/003728).

 In order to realize an economically viable process, fast and efficient production of the dicarboxylic acid is required. State of the art fermentation-based succinic acid production makes use of industrial feed-stocks. In some instances, industrial feedstocks
25 are used that contain high fractions of fermentable sugars, such as glucose, fructose, maltose or sucrose. Examples of such feedstocks are sucrose-based raw materials (e.g., cane or beet molasses) or high quality dextrose obtained from starch hydrolysates. In said cases, the fermentable sugars are readily converted into succinic acid via microbial fermentation and low amounts of residual sugars are present in the
30 end-of-fermentation broth. In other instances, however, industrial feed-stocks such as certain starch-hydrolysates are used which contain non-fermentable disaccharides and oligosaccharides, the quantity of such disaccharides and oligosaccharides being dependent on the grade of hydrolysate. These disaccharides and oligosaccharides

cannot be consumed by the micro-organisms used in fermentation processes and thereby limit the potential fermentation yield. More importantly, disaccharides and oligosaccharides present in the process following fermentation have a negative effect on the recovery yield and product quality.

5

Summary of the invention

The present invention relates to a process for the production of a compound of interest. The process is a fermentation process, for example a process for the fermentative production of a dicarboxylic acid such as succinic acid. In the process of the invention, fermentation is carried out in the presence of an enzyme, so as to reduce the amount of residual sugar present at the end of fermentation.

The use of an enzyme in the process enables non-fermentable disaccharides and/or oligosaccharides present in the feedstock, for example a DE-95 type of glucose syrup, to be hydrolyzed during fermentation towards fermentable sugars. Such fermentable sugars may in turn be converted towards a desired end-product, for example a dicarboxylic acid. In this way, a higher product yield may be achieved in fermentation. In addition, a higher yield in down-stream processing and/or a higher product quality may be realized.

Accordingly, the invention relates to a process for the production of a compound of interest, which process comprises fermenting a first cell capable of producing the compound of interest in a suitable fermentation medium under conditions which allow for production of the compound of interest,

wherein the fermentation medium comprises disaccharides and/or oligosaccharides which are not completely fermentable by the first cell; and

wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the disaccharides and/or the oligosaccharides;

and, optionally, recovering the compound of interest from the reaction medium.

The invention also relates to:

- an end-of-fermentation broth comprising a compound of interest obtainable by a process according to such a method; and
- an end-of-fermentation broth comprising a compound of interest and a lower amount of non-fermentable disaccharides and/or oligosaccharides than present at the beginning of fermentation.

Brief description of the drawings

Figure 1 sets out the plasmid map of pSUC223.

Figure 2 sets out a schematic depiction of the principle of *PDC6* replacement by a *FRD1* expression cassette by direct integration and in vivo recombination of the split Cre-recombinase construct. Expression of Cre-recombinase is regulated by the GAL1 promoter.

Figure 3 sets out a plasmid map of pSUC228.

Figure 4 shows the effect of incubation of fermentation supernatant without addition of Amigase Mega on individual sugar concentrations. All sugar concentrations are calculated back to glucose concentration.

Figure 5 shows the effect of addition of Amigase Mega to a fermentation supernatant on individual sugar concentrations. All sugar concentrations are calculated back to glucose concentration.

Figure 6 shows the effect of addition of Amigase Mega to a fermentation broth (including yeast cells) on individual sugar concentrations. All sugar concentrations are calculated back to glucose concentration.

Figure 7 shows the effect of incubating succinic fermentation supernatant with 5 g/l cellobiose and acid tolerant β -1,4-glucosidase.

Figure 8 shows the effect of incubating MilliQ water + 80 g/l succinic acid (pH 3) with 5.5 g/l cellobiose and acid tolerant β -1,4-glucosidase.

Figure 9 shows stability of sugars after 19 hrs incubation (no enzyme). MQ EoF: MilliQ water + succinic acid, SUC EoF: succinic fermentation.

Description of the sequence listing

SEQ ID NO: 1 sets out the *Escherichia coli* fumarase fumB amino acid sequence.

SEQ ID NO: 2 sets out the complete nucleic acid sequence of plasmid pSUC223.

5 SEQ ID NO: 3 sets out the amino acid sequence of *FRD1* (encoded by *YEL047c*) lacking the first 19 amino acids.

SEQ ID NO: 4 sets out the nucleic acid sequence of the synthetic TDH3p-FRD1-TDH3t gene.

10 SEQ ID NO: 5 sets out the nucleic acid sequence of primer 1 (TDH3p FW with PDC6 5' overhang).

SEQ ID NO: 6 sets out the nucleic acid sequence of primer 2 (TDH3t REV with overhang to pSUC228 Cre-1).

SEQ ID NO: 7 sets out the nucleic acid sequence of primer 3 (pSUC228 Cre-1 FW with overhang TDH3t).

15 SEQ ID NO: 8 sets out the nucleic acid sequence of primer 4 (DBC-03373, pSUC228 Cre-1 REV).

SEQ ID NO: 9 sets out the complete nucleic acid sequence of plasmid pSUC228.

20 SEQ ID NO: 10 sets out the nucleic acid sequence of primer 5 (DBC-03374, pSUC225 Cre-2 FW).

SEQ ID NO: 11 sets out the nucleic acid sequence of primer 6 (pSUC225 Cre-2 REV with PDC6 3' overhang).

25 SEQ ID NO: 12 sets out the glycosomal *Trypanosoma brucei* fumarate reductase (FRDg) amino acid sequence lacking a 3 amino acid C-terminal targeting signal.

SEQ ID NO: 13 sets out the *Rhizopus oryzae* fumarase amino acid sequence, lacking the first 23 N-terminal amino acids.

30 SEQ ID NO: 14 sets out the *Actinobacillus succinogenes* phosphoenolpyruvate carboxykinase amino acid sequence, with EGY to DAF modification at position 120 – 122.

SEQ ID NO: 15 sets out the *Saccharomyces cerevisiae* peroxisomal malate dehydrogenase (Mdh3) amino acid sequence, lacking the C-terminal peroxisomal targeting sequence (amino acids SKL).

SEQ ID NO: 16 sets out the *Saccharomyces cerevisiae* pyruvate carboxylase amino acid sequence.

SEQ ID NO: 17 sets out the *Kluyveromyces lactis* isocitrate lyase amino acid sequence.

5 SEQ ID NO: 18 sets out *Saccharomyces cerevisiae* peroxisomal malate synthase (Mls1) amino acid sequence, lacking the 3 C-terminal peroxisomal targeting sequence.

SEQ ID NO: 19 sets out the amino acid sequence of a dicarboxylic acid transporter from *Aspergillus niger*.

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Detailed description of the invention

Throughout the present specification and the accompanying claims, the words
15 "comprise", "include" and "having" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to
20 one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

The invention relates to a process for the production of a compound of interest, which process comprises fermenting a first cell capable of producing the compound of interest in a suitable fermentation medium under conditions which allow for production
25 of the compound of interest,

wherein, the fermentation medium comprises disaccharides and/or oligosaccharides which are not completely fermentable by the first cell; and

wherein the fermentation is carried out, at least in part, in the presence of an enzyme which is capable of hydrolyzing, at least in part, the disaccharides and/or the oligosaccharides;
30

and, optionally, recovering the compound of interest from the reaction medium.

The invention relates to a process for the production of any compound of interest. The process comprises fermenting a first cell capable of producing the compound of interest in a suitable fermentation medium under conditions which allow for production of the compound of interest.

5 The fermentation medium is one which comprises disaccharides and/or oligosaccharides which are not completely fermentable by the first cell. That is to say, the first cell is a cell capable of producing the compound of interest, but typically not capable of fermentation of all of the disaccharides and/or the oligosaccharides in the fermentation medium. However, the first cell may be modified according to the
10 invention as described herein, so that it is capable of fermentation of a greater fraction of the disaccharides and/or the oligosaccharides that it otherwise would be.

A typical fermentation process in which a feedstock is used which comprises non-fermentable disaccharides and/or oligosaccharides will result in an amount of residual sugar being present in the fermentation broth at the end of fermentation. That
15 is to say, residual sugar is typically present in the form of non-fermentable disaccharides and/or oligosaccharides.

Herein, an oligosaccharide is a saccharide polymer comprising a small number, typically from three to about 10 or more of simply sugars (monosaccharides).

Herein, a disaccharide is composed of two monosaccharides.

20 Disaccharides and/or oligosaccharides are non-fermentable in the sense intended in the invention if (in the absence of enzyme according to the invention) they are not available to the first cell in a form in which that cell can convert the sugars to the compound of interest.

Example of non-fermentable disaccharides may include isomaltose and
25 cellobiose.

According to the invention, the process is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the non-fermentable disaccharides and/or oligosaccharides.

Thus, in a process of the invention, the fermentation broth at the end of
30 fermentation will comprise less residual sugar (than would be present for an equivalent process carried out in the absence of the enzyme).

The enzyme may be introduced into the fermentation in any convenient fashion.

In the invention, therefore, enzyme *per se* is added to the fermentation medium. That is to say, the enzyme, for example in the form of a suitably formulated composition may be added directly to the fermentation medium.

Alternatively, or in addition, the fermentation process of the invention may be carried out in the presence of a second cell, different to the first cell, which is capable of expressing the enzyme.

Alternatively, or in addition, the fermentation process of the invention may be carried out wherein the first cell is modified so that it is capable of expressing the enzyme to a greater degree than an unmodified form of the cell.

Any combination of these approaches could be used. Expression of the enzyme by a second cell or by a modified first cell may be inducible.

In process of the invention, the enzyme may be present for only a portion of the fermentation period or for the entire course of fermentation.

In the process of the invention, the production phase may preferably be preceded by a biomass formation phase for optimal biomass production. When such a process is carried out, the enzyme may be present for the entire or part of the production phase.

Enzyme may also be present in the biomass formation phase.

If the process of the invention is carried out where enzyme is present only for a portion of the fermentation period, the enzyme is preferably present for up to about the final 20% of the fermentation period, for example up to about the final 15% of the fermentation period, such as up to about the final 10% of the fermentation period (the percentage expressed being in terms of the total fermentation period, for example the total production period).

In the process of the invention, the enzyme may be any enzyme capable of hydrolysing a disaccharide and/or an oligosaccharide. Typically, a suitable enzyme may be an amylase such as an α -amylase, a β -amylase or a gluco-amylase, a pullulanase, an α -glucosidase, a β -glucosidase or a trehalase.

Amylase is any glycoside hydrolase which is capable of acting on α -1,4-glycosidic bonds. The amylase may be an α -amylase (EC 3.2.1.1), a β -amylase (EC 3.2.1.2) or a glucoamylase (EC 3.2.1.3).

A suitable pullulanase (EC 3.1.41) may be a type I pullulanase, which specifically attacks α -1,6 linkages, or a type II pullulanase, which is also able to hydrolyze α -1,4 linkages.

A suitable enzyme may be added in any suitable amount. The skilled person
5 will readily be able to determine an appropriate dosage of enzyme in order to reduce the amount of residual sugar, for example dependent on the specific enzyme being used.

The invention also relates to an end-of-fermentation broth comprising a compound of interest obtainable by a process of the invention. The invention also
10 relates to an end-of-fermentation broth comprising a compound of interest and a lower amount of non-fermentable disaccharides and/or oligosaccharides than present at the beginning of fermentation, for example as obtained by a process of the invention. Such an end-of-fermentation broth may comprise an amount of non-fermentable disaccharides and/or oligosaccharides of less than 5g/L disaccharides and/or
15 oligosaccharides, such as less than 4g/L disaccharides and/or oligosaccharides, for example less than 3g/L disaccharides and/or oligosaccharides, less than 2g/L disaccharides and/or oligosaccharides or less.

The compound of interest in the method according to the invention can be any biological compound. In particular, the compound of interest may be a dicarboxylic
20 acid, for example succinic acid.

The compound of interest may be the cells themselves, i.e. biomass, in which case cells may be recovered from the medium following cultivation according to the method of the invention.

The biological compound may be biomass (i.e. the cells in the fermentation
25 themselves) or any biopolymer or metabolite. The biological compound may be encoded by a single polynucleotide or a series of polynucleotides composing a biosynthetic or metabolic pathway or may be the direct result of the product of a single polynucleotide or products of a series of polynucleotides. The biological compound may be native to the host cell or heterologous.

The term "heterologous biological compound" is defined herein as a biological
30 compound which is not native to the cell; or a native biological compound in which structural modifications have been made to alter the native biological compound.

The term "biopolymer" is defined herein as a chain (or polymer) of identical, similar, or dissimilar subunits (monomers). The biopolymer may be any biopolymer. The biopolymer may for example be, but is not limited to, a nucleic acid, polyamine, polyol, polypeptide (or polyamide), or polysaccharide.

5 The biopolymer may be a polypeptide. The polypeptide may be any polypeptide having a biological activity of interest. The term "polypeptide" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The polypeptide may be a pharmaceutical polypeptide and/or a bioactive. Polypeptides further include naturally occurring allelic and engineered variations
10 of the above- mentioned polypeptides and hybrid polypeptides. The polypeptide may be native or may be heterologous to the host cell. The polypeptide may be a collagen or gelatin, or a variant or hybrid thereof. The polypeptide may be an antibody or parts thereof, an antigen, a clotting factor, an enzyme, a hormone or a hormone variant, a receptor or parts thereof, a regulatory protein, a structural protein, a reporter, or a transport protein, protein involved in secretion process, protein involved in folding process, chaperone,
15 peptide amino acid transporter, glycosylation factor, transcription factor, synthetic peptide or oligopeptide or intracellular protein. The intracellular protein may be an enzyme such as, a protease, ceramidases, epoxide hydrolase, aminopeptidase, acylases, aldolase, hydroxylase, aminopeptidase, lipase. The polypeptide may be an enzyme secreted extracellularly. Such enzymes may belong to the groups of oxidoreductase, transferase,
20 hydrolase, lyase, isomerase, ligase, catalase, cellulase, chitinase, cutinase, deoxyribonuclease, dextranase, esterase. The enzyme may be a carbohydrase, e.g. cellulases such as endoglucanases, β -glucanases, cellobiohydrolases or β -glucosidases, hemicellulases or pectinolytic enzymes such as xylanases, xylosidases, mannanases, galactanases, galactosidases, pectin methyl esterases, pectin lyases, pectate lyases, endo
25 polygalacturonases, exopolygalacturonases rhamnogalacturonases, arabanases, arabinofuranosidases, arabinoxylan hydrolases, galacturonases, lyases, or amylolytic enzymes; hydrolase, isomerase, or ligase, phosphatases such as phytases, esterases such as lipases, proteolytic enzymes, oxidoreductases such as oxidases, transferases, or isomerases. The enzyme may be a phytase. The enzyme may be an aminopeptidase,
30 asparaginase, amylase, a maltogenic amylase, carbohydrase, carboxypeptidase, endo-protease, metallo-protease, serine-protease, catalase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase,

glucoamylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, protein deaminase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, galactolipase, polyphenoloxidase, ribonuclease, transglutaminase, or glucose oxidase, hexose oxidase, monooxygenase. The enzyme may
5 catalyse a non-naturally occurring conversion, for example an enzyme suitable for use in a biocatalysis reaction.

The enzyme may be involved in the synthesis of a primary or secondary metabolite

According to the present invention, a polypeptide or enzyme also can be a product as described in WO2010/102982. According to the present invention, a polypeptide can
10 also be a fused or hybrid polypeptide to which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding one polypeptide to a nucleic acid sequence (or a portion thereof) encoding another polypeptide.

Techniques for producing fusion polypeptides are known in the art, and include,
15 ligating the coding sequences encoding the polypeptides so that they are in frame and expression of the fused polypeptide is under control of the same promoter(s) and terminator. The hybrid polypeptides may comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be heterologous to the host cell. Example of fusion polypeptides and signal
20 sequence fusions are for example as described in WO2010/121933.

The biopolymer may be a polysaccharide. The polysaccharide may be any polysaccharide, including, but not limited to, a mucopolysaccharide(e. g., heparin and hyaluronic acid) and nitrogen-containing polysaccharide (e.g., chitin). In a more preferred option, the polysaccharide is hyaluronic acid.

25 The polynucleotide of interest according to the invention may encode an enzyme involved in the synthesis of a primary or secondary metabolite, such as organic acids, carotenoids, (beta-lactam) antibiotics, and vitamins. Such metabolite may be considered as a biological compound according to the present invention.

The term "metabolite" encompasses both primary and secondary metabolites; the
30 metabolite may be any metabolite, such as organic acids, carotenoids, (beta-lactam) antibiotics, and vitamins. Such metabolites may be considered as a biological compound according to the present invention.

Metabolites include citric acid, gluconic acid, itaconic acid, a dicarboxylic acid such as fumaric acid, succinic acid, malic acid, or adipic acid, lactic acid or a keto acid such as levulinic acid.

The metabolite may be encoded by one or more genes, such as in a biosynthetic or metabolic pathway. Primary metabolites are products of primary or general metabolism of a cell, which are concerned with energy metabolism, growth, and structure. Secondary metabolites are products of secondary metabolism (see, for example, R. B. Herbert, *The Biosynthesis of Secondary Metabolites*, Chapman and Hall, New York, 1981).

Primary metabolites are for example intermediates of the main metabolic pathways such as the glycolytic pathway or the TCA cycle. The primary metabolite may be, but is not limited to, an amino acid, fatty acid, such as a polyunsaturated fatty acid, nucleoside, nucleotide, sugar, triglyceride, or vitamin.

The secondary metabolite may be, but is not limited to, an alkaloid, coumarin, flavonoid, polyketide, quinine, steroid, peptide, or terpene. The secondary metabolite may be an antibiotic, antifeedant, attractant, bacteriocide, fungicide, hormone, insecticide, or rodenticide. Antibiotics include cephalosporins, beta-lactams and macrolides, such as erythromycin.

Examples of β -lactams are clavulanic acid, penicillin (e.g. penicillin G, penicillin V or 6-aminopenicillanic acid) and semi synthetic penicillins such as amoxicillin and cephalosporins such as cephalosporin C. The β -lactam may be an N-acylated derivative of β -lactam intermediates such as 7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (7ACCCA), 7-aminocephalosporanic acid (7-ACA), 7-amino-3-chloro-3-cephem-4-carboxylic acid (7-ACCA), 7-aminodesacetoxycephalosporanic acid (7-ADCA), 7-aminodesacetylcephalosporanic acid (7-ADAC), 7-amino-3-[(Z/E)-1-propen-1-yl]-3-cephem-4-carboxylic acid (7-PACA) and the like. The acyl group at the 7-amino position is preferably adipic acid yielding the corresponding adipoyl derivate as disclosed in WO 93/05158, WO 93/08287 or WO 2004/106347. Alternative suitable side chains have been disclosed in WO 95/04148, WO 95/04149, WO 96/38580, WO 98/48034 and WO 98/48035.

Further examples of secondary metabolites are adipoyl-7-ADCA, adipoyl-7-ACA, adipoyl-7-ADAC, adipoyl-7-ACCA, adipoyl-7-PACA or adipoyl-7-ACCCA, most preferred is adipoyl-7-ADCA.

The biological compound may also be the product of a selectable marker. A selectable marker is a product of a polynucleotide of interest which product provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Selectable markers include, but are not limited to, *amdS* (acetamidase), *argB* (ornithinecarbamoyltransferase), *bar* (phosphinothricinacetyltransferase), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), *trpC* (anthranilate synthase), *ble* (phleomycin resistance protein), *hyg* (hygromycin), NAT or NTC (Nourseothricin) as well as equivalents thereof.

A suitable first cell for use in the method of the invention may be any wild type strain producing a compound of interest. Furthermore, a suitable first cell may be a strain which has been obtained and/or improved by subjecting a parent or wild type strain of interest to a classical mutagenic treatment or to recombinant nucleic acid transformation. Thus, a cell suitable for use in the method of the invention may already be capable of producing the compound of interest. However, the cell may also be provided with a homologous or heterologous expression construct that encodes a polypeptide involved in the production of the compound of interest.

The person skilled in the art will be aware of methods for modification of a cell, such as a microbial cell, such that it is capable of production of the polypeptide involved in the production of the compound of interest.

As used herein, a genetically modified cell, such as a genetically modified yeast, is defined as a cell which contains, or is transformed or genetically modified with or a nucleotide sequence or polypeptide that does not naturally occur in the cell, or it contains additional copy or copies of an endogenous nucleic acid sequence, or it contains a deletion or disruption of an endogenous or homologous nucleotide 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 organism 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.

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, 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 extend 1, Blosum 62 matrix.

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.

There are various means available in the art for overexpression of nucleotide sequences encoding enzymes in a yeast in the process of the invention. In particular, 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 introducing an
5 (episomal) expression vector that comprises multiple copies of the gene. Preferably, overexpression of the enzyme according to the invention is achieved with a (strong) constitutive promoter.

Suitable promoters in fungal cells are known to the skilled man in the art. Suitable promoters may be, but are not limited to, TDH1, TDH3, GAL7, GAL10, GAL1,
10 CYC1, HIS3, ADH1, PH05, ADC1, ACT1, TRP1, URA3, LEU2, ENO1, TPI1, AOX1, PGL, GPDA and GAPDH. Other suitable promoters include PDC1, GPD1, PGK1, and TEF1.

A gene 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 fungal cell
15 according to the present invention may comprise a single copy, but preferably comprises multiple copies of a gene, 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 autonomously replicating sequence
20 and a centromere (Sikorski and Hieter, 1989, *Genetics* 122, 19–27). 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 fungal cell. Integration into the cell's
25 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.

Typically, this may involve the use of an expression construct (or nucleic acid construct) which is isolated from a naturally occurring gene or which has been modified
30 to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. Such an expression cassette/construct typically may contain all the control sequences required for expression of a coding

sequence, wherein said control sequences are operably linked to said coding sequence.

The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the production of a polypeptide.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of mRNA and / or a polypeptide, either *in vitro* or in a host cell. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, Shine-Delgarno sequence, optimal translation initiation sequences (as described in Kozak, 1991, J. Biol. Chem. 266:19867-19870), a polyadenylation sequence, a pro-peptide sequence, a pre-pro-peptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. Control sequences may be optimized to their specific purpose.

A first cell suitable for use in the method according to the invention may be a prokaryotic cell. Preferably, the prokaryotic cell is a bacterial cell. The term "bacterial cell" includes both Gram-negative and Gram-positive microorganisms. The bacterium may be an Actinomycete.

Suitable bacteria may be selected from *e.g.* *Escherichia*, *Anabaena*, *Caulobacter*, *Gluconobacter*, *Rhodobacter*, *Pseudomonas*, *Paracoccus*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Rhizobium* (*Sinorhizobium*), *Flavobacterium*, *Klebsiella*, *Enterobacter*, *Lactobacillus*, *Lactococcus*, *Methylobacterium*, *Staphylococcus* or Actinomycetes such as *Streptomyces* and *Actinoplanes* species. Preferably, the bacterial cell is selected from the group consisting of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. megaterium*, *B. halodurans*, *G. oxydans*, *Caulobacter crescentus* CB 15, *Methylobacterium extorquens*, *Rhodobacter sphaeroides*, *Pseudomonas zeaxanthinifaciens*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Paracoccus denitrificans*, *E. coli*, *C. glutamicum*, *Staphylococcus carnosus*, *Streptomyces lividans*, *Streptomyces clavuligerus*, *Sinorhizobium meliloti* and *Rhizobium radiobacter*.

The first cell suitable for use in the method according to the invention may be a eukaryotic host cell. Preferably, the eukaryotic cell is a fungal or an algal cell. The

eukaryotic cell may be a fungal cell, for example a yeast cell, such as *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* strain. More preferably from *Kluyveromyces lactis*, *S. cerevisiae*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Issatchenkia orientalis* (also known as *Candida krusei*) and *Pichia pastoris*.

5 The eukaryotic cell may be a filamentous fungal cell. Filamentous fungi include all filamentous forms of the subdivision *Eumycota* and *Oomycota* (as defined by Hawksworth *et al.*, In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex
10 polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. Filamentous fungal strains include, but are not limited to, strains of *Acremonium*, *Agaricus*, *Aspergillus*, *Aureobasidium*, *Chrysosporium*, *Coprinus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Phanerochaete*,
15 *Pleurotus*, *Schizophyllum*, *Talaromyces/Rasamsonia*, *Thermoascus*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.

Preferred filamentous fungal cells belong to a species of an *Acremonium*, *Aspergillus*, *Chrysosporium*, *Myceliophthora*, *Penicillium*, *Talaromyces/Rasamsonia*, *Thielavia*, *Fusarium* or *Trichoderma* genus, and most preferably a species of
20 *Aspergillus niger*, *Acremonium chrysogenum*, *Acremonium alabamense*, *Aspergillus awamori*, *Aspergillus sojae*, *Aspergillus fumigatus*, *Rasamsonia emersonii*, *Aspergillus oryzae*, *Chrysosporium lucknowense*, *Fusarium oxysporum*, *Myceliophthora thermophila*, *Trichoderma reesei*, *Thielavia terrestris*, *Penicillium chrysogenum* or *Penicillium citrium*. A more preferred host cell belongs to the genus *Aspergillus*, more
25 preferably the host cell belongs to the species *Aspergillus niger*. When the host cell according to the invention is an *Aspergillus niger* host cell, the host cell preferably is CBS 513.88, CBS124.903 or a derivative thereof.

Several strains of filamentous fungi are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche
30 Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL), and All-Russian Collection of Microorganisms of Russian Academy of Sciences, (abbreviation in Russian - VKM, abbreviation in English -

RCM), Moscow, Russia. Useful strains in the context of the present invention may be *Aspergillus niger* CBS 513.88, CBS124.903, *Aspergillus oryzae* ATCC 20423, IFO 4177, ATCC 1011, CBS205.89, ATCC 9576, ATCC14488-14491, ATCC 11601, ATCC12892, *P. chrysogenum* CBS 455.95, *P. chrysogenum* Wisconsin54-1255(ATCC28089), *Penicillium citrinum* ATCC 38065, *Penicillium chrysogenum* P2, *Thielavia terrestris* NRRL8126, *Rasamsonia emersonii* CBS 124.902, *Acremonium chrysogenum* ATCC 36225 or ATCC 48272, *Trichoderma reesei* ATCC 26921 or ATCC 56765 or ATCC 26921, *Aspergillus sojae* ATCC11906, *Myceliophthora thermophila* C1, Garg 27K, VKM-F 3500 D, *Chrysosporium lucknowense* C1, Garg 27K, VKM-F 3500 D, ATCC44006 and derivatives thereof.

A fungal cell in a process as disclosed herein may be any suitable wild type or recombinant or genetically modified fungal cell, in particular a recombinant or genetically modified yeast cell.

A genetically modified fungal cell may comprise a genetic modification of a gene selected from the group consisting of a gene encoding a pyruvate carboxylase, a phosphoenolpyruvate carboxykinase, a malate dehydrogenase, a fumarase, a fumarate reductase, an isocitrate lyase, a malate synthase and a dicarboxylic acid transporter.

Thus, a genetically modified yeast cell suitable for use in the process of the invention, may comprise any suitable genetic modifications, such as deletions or disruptions, and insertions of homologous or heterologous nucleotide sequences. A yeast cell suitable for use in the process of the invention may be 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 a dicarboxylic acid such 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.

Thus, in the invention a genetically modified yeast cell may be used. Preferably, a yeast cell used in the process according to the present invention comprises genetic modifications according to the preferred embodiments as described herein below.

A recombinant fungal cell may comprise a genetic modification with a pyruvate carboxylase (PYC), that catalyses the reaction from pyruvate to oxaloacetate (EC 6.4.1.1). The pyruvate carboxylase may for instance be active in the cytosol upon expression of the gene. For instance the fungal cell overexpresses a pyruvate carboxylase, for instance an endogenous or homologous pyruvate carboxylase is overexpressed.

A host cell according to the present invention may be genetically modified with a pyruvate carboxylase which has at least 80, 85, 90, 95, 99 or 100% sequence identity with amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO: 16.

Preferably the genetically modified yeast cell expresses a nucleotide sequence encoding a phosphoenolpyruvate (PEP) carboxykinase in the cytosol. Preferably a nucleotide sequence encoding a phosphoenolpyruvate (PEP) carboxykinase is overexpressed. The PEP carboxykinase (EC 4.1.1.49) preferably is a heterologous enzyme, preferably derived from bacteria, more preferably the enzyme having PEP carboxykinase activity is derived from *Escherichia coli*, *Mannheimia sp.*, *Actinobacillus sp.*, or *Anaerobiospirillum sp.*, more preferably *Mannheimia succiniciproducens*. A gene encoding a PEP carboxykinase may be overexpressed and may be expressed and active in the cytosol of a fungal cell. Preferably, a yeast cell according to the present invention is genetically modified with a PEP carboxykinase which has at least 80, 85, 90, 95, 99 or 100% sequence identity with amino acid sequence of SEQ ID NO: 14.

In one embodiment a fungal cell is further genetically modified with a gene encoding a malate dehydrogenase (MDH) active in the cytosol upon expression of the gene. Cytosolic expression may be obtained by deletion of a peroxisomal targeting signal. The malate dehydrogenase may be overexpressed. A cytosolic MDH may be any suitable homologous or heterologous malate dehydrogenase, catalyzing the reaction from oxaloacetate to malate (EC 1.1.1.37), for instance derived from *S. cerevisiae*.

Preferably, the MDH is *S. cerevisiae* MDH3, more preferably one which has a C-terminal SKL deletion such that it is active in the cytosol. Preferably, a yeast cell according to the present invention comprises a nucleotide sequence encoding a malate dehydrogenase that has at least 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: 15.

In another embodiment a fungal cell of the present disclosure is further genetically modified with a gene encoding a fumarase, that catalyses the reaction from malic acid to fumaric acid (EC 4.2.1.2). A gene encoding 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*, or a bacterium such a *Escherichia coli*. A fungal cell of the present disclosure may overexpress a nucleotide sequence encoding a fumarase. The fumarase may be active in the cytosol upon expression of the nucleotide sequence, for instance by deleting a peroxisomal targeting signal. It was found that cytosolic activity of a fumarase resulted in a high productivity of a dicarboxylic acid by the fungal cell.

Preferably, a yeast in the process according to the present invention overexpresses a nucleotide sequence encoding a fumarase that has at least 70%, preferably at least 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, or 99% or 100% sequence identity with the amino acid sequence of SEQ ID NO: 13.

In another embodiment the fungal cell is genetically modified with any suitable heterologous or homologous gene encoding a NAD(H)-dependent fumarate reductase, catalyzing the reaction from fumarate to succinate (EC 1.3.1.6). The NADH-dependent fumarate reductase may be a heterologous enzyme, which may be derived from any suitable origin, for instance bacteria, fungi, protozoa or plants. A fungal cell of the present disclosure comprises a heterologous NAD(H)-dependent fumarate reductase, preferably derived from a *Trypanosoma* sp, for instance a *Trypanosoma brucei*. In one embodiment the NAD(H)-dependent fumarate reductase is expressed and active in the cytosol, for instance by deleting a peroxisomal targeting signal. The fungal cell may overexpress a gene encoding a NAD(H)-dependent fumarate reductase.

Preferably, a yeast cell in the process according to the present invention is genetically modified with a NAD(H)-dependent fumarate reductase, which has at least 80, 85, 90, 95, 99 or 100% sequence identity with the amino acid sequence of SEQ ID NO: 12.

In another embodiment, a genetically modified yeast in the process according to the invention expresses a nucleotide sequence encoding a dicarboxylic acid transporter protein. Preferably the dicarboxylic acid transporter protein is overexpressed. A

dicarboxylic acid transporter protein may be any suitable homologous or heterologous protein. Preferably the dicarboxylic acid transporter protein is a heterologous protein. A dicarboxylic acid transporter protein may be derived from any suitable organism, preferably from yeast or fungi such as *Schizosaccharomyces pombe* or *Aspergillus niger*. Preferably, a dicarboxylic acid transporter protein is a dicarboxylic acid transporter/malic acid transporter protein, eg. from *Aspergillus niger* which has at least 80, 85, 90, 95 or 99% or 100% sequence identity with the amino acid sequence of SEQ ID NO: 19.

A genetically modified fungal cell may further comprise a genetic modification with a gene encoding an isocitrate lyase (EC 4.1.3.1), which may be any suitable heterologous or homologous enzyme. The isocitrate lyase may for instance be obtained from *Kluyveromyces lactis* or *Escherichia coli*.

A first cell in the process according to the present invention is genetically modified with a isocitrate lyase which has at least 80, 85, 90, 95, 99 or 100% sequence identity with amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO: 17.

A genetically modified fungal cell may further comprise as genetic modification with a malate synthase (EC 2.3.3.9). The malate synthase may be overexpressed and / or active in the cytosol, for instance by deletion of a peroxisomal targeting signal. In the event the malate synthase is a *S. cerevisiae* malate synthase, for instance the native malate synthase is altered by the deletion of the SKL carboxy-terminal sequence.

A first cell in the process according to the present invention is genetically modified with a malate synthase which has at least 80, 85, 90, 95, 99 or 100% sequence identity with amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO: 18.

In another embodiment, a recombinant fungal cell in the process for producing a dicarboxylic acid disclosed herein comprises a disruption of a gene encoding an enzyme of the ethanol fermentation pathway. A gene encoding an enzyme of an ethanol fermentation pathway, may be pyruvate decarboxylase (EC 4.1.1.1), catalyzing the reaction from pyruvate to acetaldehyde, or alcohol dehydrogenase (EC 1.1.1.1), catalyzing the reaction from acetaldehyde to ethanol. Preferably, a fungal cell in the process as disclosed herein comprises a disruption of one, two or more genes encoding an alcohol dehydrogenase. In the event the fungal cell is a yeast, e.g. *S.*

cerevisiae, the yeast preferably comprises a disruption of an alcohol dehydrogenase gene *adh1* and / or *adh2*.

Alternatively or in addition, the yeast in the process of the invention 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 a wild-type cell.

Preferably, a genetically modified yeast in the process according to the present invention overexpresses a nucleotide sequence encoding a PEP carboxykinase, a nucleotide sequence encoding a malate dehydrogenase, a nucleotide sequence encoding a fumarase, a nucleotide sequence encoding a NAD(H) dependent fumarate reductase, and/or a nucleotide sequence encoding a malic acid transporter protein, preferably wherein the enzymes are active in the cytosol. Preferred embodiments of the enzymes are as described herein above.

Cytosolic expression of the enzymes described above may be obtained by deletion of a peroxisomal or mitochondrial targeting signal. The presence of a peroxisomal or mitochondrial targeting signal may for instance be determined by the method disclosed by Schlüter *et al.*, Nucleic Acid Research 2007, 35, D815-D822.

In the method of the invention, the cells are cultivated in a reaction medium and under conditions suitable for production of the compound of interest. Typically, in the method of the invention the cells may be cultivated at a large-scale (including, for example, in the form of a continuous, batch or fed-batch cultivations) in a suitable reaction medium and under conditions allowing the compound of interest to be produced and/or isolated. The amount of reaction medium may be maintained at an approximately constant volume in the method of the invention. That is to say, evaporated reaction medium may be replenished so that the volume of reaction medium remains approximately constant.

The cultivation takes place in a suitable reaction medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e. g., Bennett, J. W. and LaSure, L., eds., *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be

prepared using published compositions (e. g., in catalogues of the American Type Culture Collection).

A process for producing a dicarboxylic acid may be carried out at any suitable temperature. A suitable temperature may for instance be between about 10 and about 40 degrees Celsius, for instance between about 15 and about 30 degrees Celsius, in particular at about 30°C.

If the compound of interest is secreted into the reaction medium, the compound can be isolated directly from the reaction medium. If the compound of interest is not secreted, it can be isolated from, for example, cell lysates.

The compound of interest may be isolated by methods known in the art. For example, the compound of interest may be isolated from the reaction medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation. The isolated compound of interest may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (e. g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e. g., ammonium sulfate precipitation), or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989). In some applications the compound of interest may be used without substantial isolation from the culture broth; separation of the reaction medium from the biomass may be adequate.

In a preferred embodiment the process for the production of a dicarboxylic acid further comprises recovering the dicarboxylic acid. Recovery of the dicarboxylic acid may be carried out by any suitable method.

In one embodiment, a dicarboxylic acid that is produced in a process as disclosed herein is recovered from the fermentation medium. Recovery of a dicarboxylic acid may be carried out by any suitable method known in the art, for instance by crystallization, ammonium precipitation, ion exchange technology, centrifugation or filtration or any suitable combination of these methods.

In a preferred embodiment, the recovery of dicarboxylic acid comprises crystallizing the dicarboxylic acid and forming dicarboxylic acid crystals. Preferably, the crystallizing of dicarboxylic acid comprises removing part of the fermentation medium, preferably by evaporation, to obtain a concentrated medium.

In a preferred embodiment the process according to the present invention comprises recovering a dicarboxylic acid which is succinic acid and wherein the recovering comprises crystallizing succinic acid from an aqueous solution having a pH of between 1 and 5 and comprising succinic acid, comprising evaporating part of the aqueous solution to obtain a concentrated solution, lowering the temperature of the concentrated solution to a value of between 5 and 35 degrees Celsius, wherein succinic acid crystals are formed. Preferably, the crystallizing comprises bringing the temperature of the concentrated medium to a temperature of between 10 and 30 degrees Celsius, preferably between 15 and 25 degrees Celsius. Preferably, the fermentation medium has a pH of between 1.5 and 4.5, preferably between 2 and 4.

It has been found that crystallizing succinic acid at higher temperatures such as between 10 and 30 degrees Celsius results in succinic acid crystals with a lower amount of impurities such as organic acid, protein, color and/or odor, than succinic acid crystals that were crystallized at a low temperature of below 10 degrees.

Another advantage of crystallizing succinic acid at a higher temperature is that it requires a lower amount of energy for cooling the aqueous solution as compared to a process wherein crystallizing succinic acid is carried out below 10 or 5 degrees Celsius, resulting in a more economical and sustainable process.

Preferably, the crystallizing of succinic acid comprises a step of washing the succinic acid crystals. Succinic acid may be crystallized directly from the fermentation medium having a pH of between 1 and 5 to a purity of at least 90% w/w, preferably at least 95, 96, 97, or at least 98%, or 99 to 100%w/w.

Preferably, the recovery of the dicarboxylic acid, preferably succinic acid, comprises removing the biomass from the fermentation medium and crystallizing the dicarboxylic acid, preferably crystallizing as described herein above. Preferably, the removing of biomass is carried out by filtration.

In a preferred embodiment, the process for the production of a dicarboxylic acid further comprises using the dicarboxylic acid in an industrial process. An industrial process for a dicarboxylic acid may be the application as a cosmetic additive, deicing agent, food additive or as a building block for (bio)polymers.

In a preferred embodiment, the fermentation medium comprises an amount of succinic acid of between 1 and 150 g/l, preferably between 5 and 100 g/l, more preferably between 10 and 80 g/l or between 15 and 60 g/l of succinic acid.

In another aspect the present invention relates to a process for crystallizing succinic acid from an aqueous solution having a pH of between 1 and 5 and comprising succinic acid, comprising removing part of the aqueous solution by evaporation to obtain a concentrated solution, and bringing the temperature of the concentrated solution to a value of between 10 and 30 degrees Celsius, wherein succinic acid crystals are formed. Preferably, the crystallizing comprises bringing the temperature of the concentrated solution between 15 and 25 degrees Celsius, preferably between 18 and 22 degrees Celsius. . Preferably, the aqueous solution has a pH of between 1.5 and 4.5, preferably between 2 and 4. The aqueous solution may be any suitable solution comprising succinic acid. The aqueous solution may comprise soluble constituents and insoluble constituents and, such as (fragments of) microbial cells, protein, plant biomass lignocellulose, cellulose and the like. Preferably the aqueous solution is a fermentation medium, preferably a fermentation medium obtainable by a process for the production of a dicarboxylic acid as described herein.

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 98/46772, WO 99/60102 and WO 00/37671, WO90/14423, EP-A-0481008, EP-A-0635 574 and US 6,265,186.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

The present invention is further illustrated by the following Examples:

EXAMPLES

Example 1: Construction of yeast strain SUC-947

5 The *Saccharomyces cerevisiae* strain SUC-632 was constructed as described in WO2013/004670. *Saccharomyces cerevisiae* strain SUC-632 was constructed as described in WO2013/004670. Strain SUC-632 was used as a starting point to construct strain SUC-947. Strain SUC-708 is a mutant of strain SUC-632
10 obtained by classical strain improvement.

A fumarase gene of *E. coli* (*fumB*) was transformed to strain SUC-708 as described below. SEQ ID NO: 1 describes the fumarase (*fumB*) protein sequence from *Escherichia coli* (E.C. 4.2.1.2, UniProt accession number P14407). The gene sequence was codon pair optimized for expression in *S. cerevisiae* as disclosed in patent
15 application WO2008/000632. The stop codon TAA was modified to TAAG. Expression of the *FUM_01* gene is controlled by the *TDH1* promoter (600 bp directly before the start codon of the *TDH1* gene) and the *TDH1* terminator (300 bp directly after the stop codon of the *TDH1* gene). The *TDH1* promoter and *TDH1* terminator sequences controlling expression of *FUM_01* are native sequences derived from *Saccharomyces*
20 *cerevisiae* S288C. The synthetic promoter-gene sequence including appropriate restriction sites was synthesized by GenArt (Regensburg, Germany). This synthetic fragment is part of plasmid pSUC223 (Figure 1), whose sequence is described in SEQ ID NO: 7. Plasmid pSUC223 contains a KanMX marker with allows for selection for growth in the presence of G418. The KanMX marker, flanked by lox66 and lox71 sites
25 (Albert *et al.*, Plant Journal, 7(4), 649-659), can be removed by the action of Cre-recombinase, as described by Gueldender *et al.*, (Nucleic Acids Res. 1996 Jul 1;24(13):2519-24). The TDH1p-fumB-TDH1t and the lox66-KanMX-lox71 sequences were flanked by sequences that allow integration by double cross-over at the YPRCtau3 locus, which is located on chromosome XVI.

30 Plasmid pSUC223 was restricted using restriction enzymes *Apa*LI and *Xho*II. A 5.408 bp fragment containing the 5' YPRCtau3 flank, the synthetic *fumB* construct, the KanMX selection marker flanked by lox66 and lox71 sites and the 3' YPRCtau3 flank was excised from an agarose gel and purified using the ZymocleanTM Gel DNA

Recovery Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions.

The fragment was transformed to strain SUC-708. Transformants were selected on Yeast Extract BactoPeptone (YEP) 2% galactose plates supplemented with 200 µg G418/milliliter for selection of transformants containing the KanMX marker, yielding multiple transformants. Presence of the introduced *fumB* gene was confirmed by PCR using primer sequences that can anneal to the coding sequences of the ORF encoded by SEQ ID NO: 2. One of the correct transformants, SUC-708 FUM_01 #3, was named SUC-813.

Fumarate reductase *FRD1* of *S. cerevisiae* encoded by *YEL047C*, E.C. 1.3.1.6, UniProt accession number P32614, was analyzed for the presence of signal sequences using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen *et al.*, 2004, Mol. Biol., 340:783-795) and TargetP 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>) (Emanuelsson *et al.*, 2007, Nature Protocols 2, 953-971). The *S. cerevisiae* *Frd1* protein has multiple possible localizations. It contains a predicted 19 amino acid signal peptide. After removal of the putative SP, the predicted tendency for cytosolic localization was drastically increased. The variant omitting the 19 aa signal peptide is described in SEQ ID NO: 3, which was subjected to the codon-pair method as disclosed in WO2008/000632 for expression in *S. cerevisiae*. The stop codon TAA was modified to TAAG. Expression of the *FRD1* gene is controlled by the TDH3 promoter (600 bp directly before the start codon of the TDH3 gene) and the TDH3 terminator (300 bp directly after the stop codon of the TDH3 gene). The TDH3 promoter and TDH3 terminator sequences controlling expression of *FRD1* are native sequences derived from *Saccharomyces cerevisiae* S288C. The synthetic promoter-gene sequence including appropriate restriction sites was synthesized by DNA2.0 (Menlo Park, CA, USA) and is described in SEQ ID NO: 4.

The modified *FRD1* gene was transformed into strain SUC-813 as depicted in Figure 2. The modified *FRD1* gene replaced the *PDC6* gene in strain SUC-813 using the "split Cre recombinase integration" or "direct Cre recombinase integration" (DCI) approach as described in PCT/EP2013/055047. PCR fragments were generated using Phusion DNA polymerase (New England Biolabs, USA) according to manufacturer's instructions. PCR fragment 1 was generated by using the primer sequences described in SEQ ID NO: 5 and SEQ ID NO: 6, using a cloning plasmid of DNA 2.0 containing

SEQ ID NO: 4 as template. SEQ ID NO: 5 contains a 66 bp overlap with the 5' region of the *PDC6* gene, directly located before the start codon of the *PDC6* gene. PCR fragment 2 was generated by using the primer sequences described in SEQ ID NO: 7 and SEQ ID NO: 8 using plasmid pSUC228 (Figure 3, SEQ ID NO: 9) as template.

5 Plasmid pSUC228 is a modified version of pSUC227, which is described in PCT/EP2013/055047. Plasmid pSUC227 contains a KanMX marker, which is replaced by a nourseothricin (natMX4) marker (Goldstein and McCusker, Yeast. 1999 Oct;15(14):1541-53) resulting in pSUC228. PCR fragment 3 was generated by using the primer sequences described in SEQ ID NO: 10 and SEQ ID NO: 11, using

10 pSUC225 (described in PCT/EP2013/055047) as template. SEQ ID NO: 11 contains a 64 bp overlap with the 3' region of the *PDC6* gene, directly located after the stop codon of the *PDC6* gene.

The size of the PCR fragments was checked with standard agarose electrophoresis techniques. PCR amplified DNA fragments were purified using the

15 Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions.

Yeast transformation was done by a method known by persons skilled in the art. *S. cerevisiae* strain SUC-813 was transformed with purified PCR fragments 1, 2 and 3. PCR fragment 1 contained an overlap with PCR fragment 2 at its 3' end. PCR

20 fragment 3 contained an overlap with PCR fragment 2 at its 5' end. PCR fragment 2 contained an overlap at its 5' end with PCR fragment 1 and at its 3' end with PCR fragment 3, such that this allowed homologous recombination of all three PCR fragments (Figure 2). The 5' end of PCR fragment 1 and the 3' end of PCR fragment 3 were homologous to the *PDC6* locus and enabled integration of all three PCR

25 fragments in the *PDC6* locus. This resulted in one linear fragment consisting of PCR fragments 1 to 3 integrated in the *PDC6* locus (Figure 2). This method of integration is described in patent application WO2013/076280.

Transformation mixtures were plated on YPD-agar (per liter: 10 grams of yeast extract, 20 grams per liter peptone, 20 grams per liter dextrose, 20 grams of agar)

30 containing 100 µg nourseothricin (Jena Bioscience, Germany) per ml. After three to five days of growth at 30°C, individual transformants were re-streaked on fresh YPD-agar plates containing 100 µg nourseothricin per ml.

Subsequently, the marker cassette and Cre-recombinase are effectively out-recombination by the method described in PCT/EP2013/055047, resulting in replacement of the *PDC6* gene by SEQ ID NO: 4 and leaving a *lox72* site as a result of recombination between the *lox66* and *lox71* sites. Due to the activity of Cre-recombinase, the KanMX marker flanked by *lox66* and *lox71* sites, which was introduced into genomic DNA to create strain SUC-813 was also efficiently out-recombined. The resulting markerfree strain was named SUC-947. Strain SUC-947 was able to grow on YPD-agar plates, but unable to grow on YPD-agar plates supplemented with either 200 µg G418/ml or 100 µg/ml nourseothricin or both, confirming out-recombination of both the KanMX and the natMX4 marker.

Example 2: Effect of addition of an amylo-glucosidase to a succinic acid fermentation on the sugar level end of fermentation

The yeast strain SUC-947 constructed as described above, was cultivated in shake-flask (50 ml) for 3 days at 30°C and 280 rpm. The medium was based on Verduyn *et al.* (Verduyn C, Postma E, Scheffers WA, Van Dijken JP. Yeast, 1992 Jul;8(7):501-517), with modifications in the carbon and nitrogen sources, as described herein below.

Table 1. Preculture medium composition

Raw material		Concentration (g/kg)
Galactose	$C_6H_{12}O_6 \cdot H_2O$	20.0
Urea	$(NH_2)_2CO$	2.3
Potassium dihydrogen phosphate	KH_2PO_4	3.0
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.5
Trace element solution ^a		1
Vitamin solution ^b		1
Chalk	$CaCO_3$	1

^aTrace elements solution

Component	Formula	Concentration (g/kg)
EDTA	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	15.00
Zincsulphate $\cdot 7H_2O$	$ZnSO_4 \cdot 7H_2O$	4.50
Manganezechloride $\cdot 2H_2O$	$MnCl_2 \cdot 2H_2O$	0.84

Cobalt (II) chloride . 6H ₂ O	CoCl ₂ . 6H ₂ O	0.30
Copper (II) sulphate . 5H ₂ O	CuSO ₄ . 5H ₂ O	0.30
Sodium molybdenum . 2H ₂ O	Na ₂ MoO ₄ . 2H ₂ O	0.40
Calciumchloride . 2H ₂ O	CaCl ₂ . 2H ₂ O	4.50
Ironsulphate . 7H ₂ O	FeSO ₄ . 7H ₂ O	3.00
Boric acid	H ₃ BO ₃	1.00
Potassium iodide	KI	0.10

^bVitamin solution

Component	Formula	Concentration (g/kg)
Biotin (D-)	C ₁₀ H ₁₆ N ₂ O ₃ S	0.05
Ca D(+) panthothenate	C ₁₈ H ₃₂ CaN ₂ O ₁₀	1.00
Nicotinic acid	C ₆ H ₅ NO ₂	1.00
Myo-inositol	C ₆ H ₁₂ O ₆	25.00
Thiamine chloride hydrochloride	C ₁₂ H ₁₈ Cl ₂ N ₄ OS . xH ₂ O	1.00
Pyridoxol hydrochloride	C ₈ H ₁₂ ClNO ₃	1.00
p-aminobenzoic acid	C ₇ H ₇ NO ₂	0.20

Subsequently, ~3 ml of the content of the shake-flask was transferred into a seed fermenter (starting volume 0.3 kg, 1% inoculation strength), which contained the medium set out in Table 2.

Table 2. Medium composition of the seed fermenter

Raw material		Concentration (g/kg)
Ammonium sulphate	(NH ₄) ₂ SO ₄	1.0
Potassium dihydrogen phosphate	KH ₂ PO ₄	10
Magnesium sulphate	MgSO ₄ . 7H ₂ O	5.0
Trace element solution		8.0
Vitamin solution		8.0

The pH was controlled at 5.0 by addition of ammonia (10 wt%). Temperature was controlled at 30°C. pO₂ was controlled at 25% (relative to air saturation) by adjusting the stirrer speed. Total airflow applied was 18 NL/h. Glucose concentration was kept limited by controlled feed to the fermenter (exponent of 0.15 was applied).

After 68 hours of fermentation, 80 g of culture broth of the seed fermenter was transferred to a production fermenter (starting volume 0.4 kg), with the medium set out in Table 3.

Table 3. Medium composition of the production fermenter

Raw material		Concentration (g/kg)
Urea	$(\text{NH}_2)_2\text{CO}$	1.0
Potassium dihydrogen phosphate	KH_2PO_4	1.5
Magnesium sulphate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
Iron sulphate. $7\text{H}_2\text{O}$	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.006
Chalk	CaCO_3	12.5
Biotin	$\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$	0.001

No pH control was applied, as the added CaCO_3 initially buffered the medium at the pH of 5-5.5. As result of natural acidification the pH dropped towards 3 at the end of fermentation. Temperature was controlled at 30°C. Total gasflow applied was 12 NL/h, 50% air and 50% CO_2 . Glucose concentration was kept limited by controlled feed (glucose concentration in feed was 423 g/kg) to the fermenter (0-9h: 5.6 ml/h g/L/h; >9h: feed in ml/h = $(0.0003 \cdot t)^2 - 0.1028 \cdot t + 7.8991$)^{4/5}). When necessary, these rates were adjusted accordingly.

Table 4. Typical composition DE-95 grade of starch hydrolysate on dry-matter basis

Compound	% on dry matter
Glucose	94,8
Fructose	0,2
Maltose	1,5
Isomaltose	1,3
DP>2	2
Other	0,2

After 48 hours glucose feed was stopped and 0,5 g of formulated solution of Amigase Mega (formulation in glycerol, containing approximately 89.5% of glucan 1,4- α -glucosidase; DSM Food Specialties, Delft, Netherlands) was added to the fermentation (final concentration 0,1 mg Amigase Mega solution/ ml fermentation broth) and incubated for 6 hours.

Results

As a first test to demonstrate the effectiveness of the enzyme, Amigase Mega was added to the cell-free supernatant (final concentration 0,1 mg/ml supernatant) of the same fermentation (before actual addition of Amigase Mega to the fermentation
5 broth). This supernatant was incubated separately at 30°C for 6 hours. As a negative control, no enzyme was added to the supernatant.

Total sugar (after addition of strong acid to break all glycosidic bonds) and individual sugars were analyzed with HPLC or NMR.

In case no enzyme was added to the supernatant no conversion of sugars could
10 be observed, demonstrating that individual sugars were stable at these conditions (see Figure 4).

However, when Amigase Mega was added to the supernatant a clear decrease in oligo-saccharides (DP>2) and an increase in glucose could be observed in time (see Figure 5). No conversion of isomaltose was seen, indicating that this applied enzyme
15 had no significant pullulanase activity (hydrolysis of alpha 1,6 glycosidic bonds). Total sugar analysis confirmed that the total amount of sugars before and after enzyme addition was constant (4,4 g/L).

Subsequently, the enzyme Amigase Mega was added to the fermentation (including yeast cells) directly.

Total sugar analysis before and after incubation with Amigase Mega showed a
20 clear reduction in total sugar concentration (from 4,4 to 2,5 g/L), demonstrating that the applied enzyme made more sugars available for fermentation (see Figure 6). This extra sugar could yield about 2 g/L more succinic acid (taking into account a stoichiometric yield of 1,12 g/g).

Second, as a consequence of this lower sugar concentration at the end of the
25 fermentation process, a higher recovery yield of succinic acid and a lower color formation in the end product can be realized. Typically, a 3% increase in recovery yield can be achieved when this sugar concentration at the end of fermentation can be realized. This is possible because a larger fraction of the mother liquor from the
30 crystallizer can be recycled back into the process to get to the same sugar levels in the mother liquor (determining the quality of the end-product).

Example 3: Effect of the addition of an acid tolerant β -1,4-glucosidase to a succinic acid fermentation on the sugar level end of fermentation

Succinic yeast strains were cultivated as described above in Example 2. To
5 cell-free supernatant 5 g/l cellobiose (Sigma Aldrich) was added along with 0.175 mg/ml
of acid tolerant β -1,4-glucosidase from *Talaromyces emersonii*, heterologously
expressed in *Aspergillus niger* (EBA4 see patent: WO2011/098577). Alternatively, 80
g/l of succinic acid (Sigma Aldrich) was dissolved in MilliQ water, adjusted to pH 3 with
carbonate, 5.5 g/l cellobiose (Sigma Aldrich) was added along with 0.175 mg/ml of acid
10 tolerant β -1,4-glucosidase described above. Both solutions were incubated at 30°C
under gentle shaking.

Samples were taken at $t = 0$ (before adding enzyme) and $t = 19$ hours.
Individual sugars were analyzed with High Performance Anion Exchange - Pulsed
Amperometric Detection (HPAEC-PAD) analysis. In case no enzyme was added to the
15 supernatant and MilliQ + succinic acid solution, no conversion of cellobiose could be
observed, demonstrating that this sugar is stable at these conditions (see Fig. 9). When
acid tolerant β -1,4-glucosidase was added all cellobiose was converted to glucose
within the 19 hour incubation. Conversion of cellobiose to glucose was complete in both
cell-free supernatant (see Fig. 7) and MilliQ + succinic acid solution (see Fig. 8),
20 indicating that the addition of acid tolerant β -1,4-glucosidase to fermentation can be
used to make unfermentable (cellobiose) sugars available for fermentation (glucose).
As a consequence of this lower sugar concentration at the end of the fermentation
process, a higher recovery yield of succinic acid and a lower color formation in the end
product can be realized.

CLAIMS

1. A process for the production of a compound of interest, which process comprises fermenting a first cell capable of producing the compound of interest in a suitable fermentation medium under conditions which allow for production of the compound of interest,
5 wherein the fermentation medium comprises disaccharides and/or oligosaccharides which are not completely fermentable by the first cell; and wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the disaccharides and/or oligosaccharides;
10 and, optionally, recovering the compound of interest from the reaction medium.
- 15 2. A process according to claim 1, wherein the enzyme is added directly to the fermentation medium.
3. A process according to claim 1 or 2, wherein the fermentation is carried out in the presence of a second cell, different to the first cell, which is capable of expressing the enzyme.
20
4. A process according to any one of the preceding claims, wherein the first cell is modified so that it is capable of expressing the enzyme to a greater degree than an unmodified form of the cell.
25
5. A process according to any one of the preceding claims, wherein the enzyme is present for a portion of the fermentation period or for the whole of the fermentation period.
- 30 6. A process according to any one of the preceding claims, wherein the enzyme is an amylase.

7. A process according to any one of the preceding claims, wherein the first cell is a fungal cell.
8. A process according to claim 7, wherein the fungal cell is a yeast cell, such as a *Saccharomyces cerevisiae* cell.
9. A process according to claim 7 or 8, wherein the fungal cell is a genetically modified fungal cell which comprises a genetic modification of a gene selected from the group consisting with a gene encoding a pyruvate carboxylase, a phosphoenolpyruvate carboxykinase, a malate dehydrogenase, a fumarase, a fumarate reductase, an isocitrate lyase, a malate synthase and a dicarboxylic acid transporter.
10. A process according to any one of the preceding claims, wherein the fermentation is carried out at a pH at from about pH1.0 to about pH5.5.
11. A process according to any one of the preceding claims, wherein the fermentation is carried out at a temperature of about 30°C.
12. A process according to any one of the preceding claims, wherein the compound of interest is a dicarboxylic acid, such as succinic acid, fumaric acid, malic acid or adipic acid.
13. An end-of-fermentation broth comprising a compound of interest obtainable by a process according to any one of the preceding claims.
14. An end-of-fermentation broth comprising a compound of interest and a lower amount of non-fermentable disaccharides and/or oligosaccharides than present at the beginning of fermentation.
15. An end-of-fermentation broth according to claim 14, wherein the amount of non-fermentable disaccharides and/or oligosaccharides is less than 5g/L disaccharides and/or oligosaccharides.

16. An end-of-fermentation broth according to an one of claims 13 to 15 obtainable by a process according to any one of claims 1 to 13.

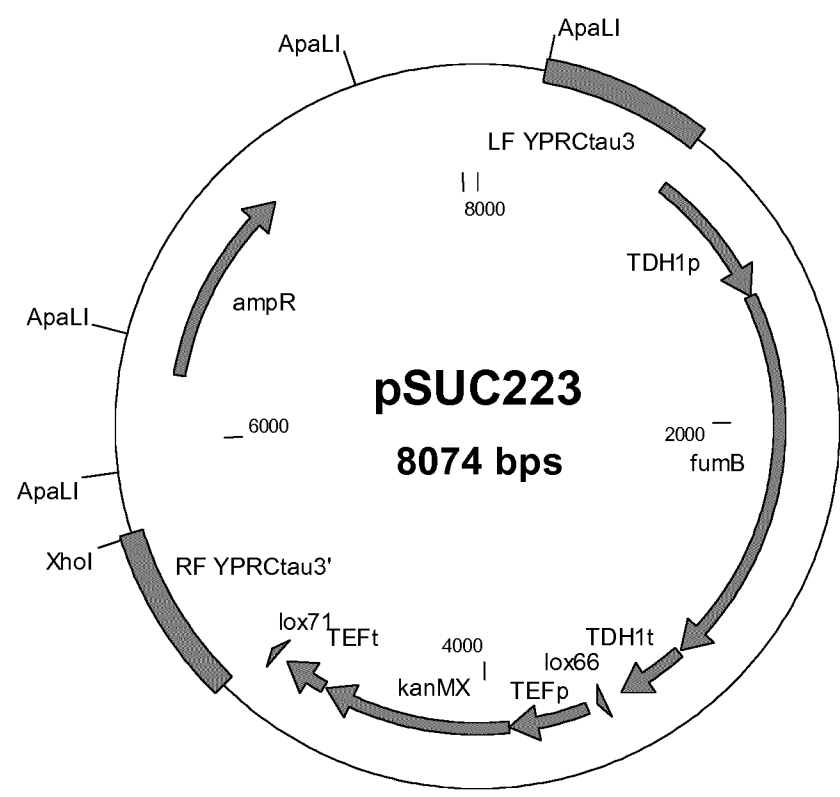


Fig. 1

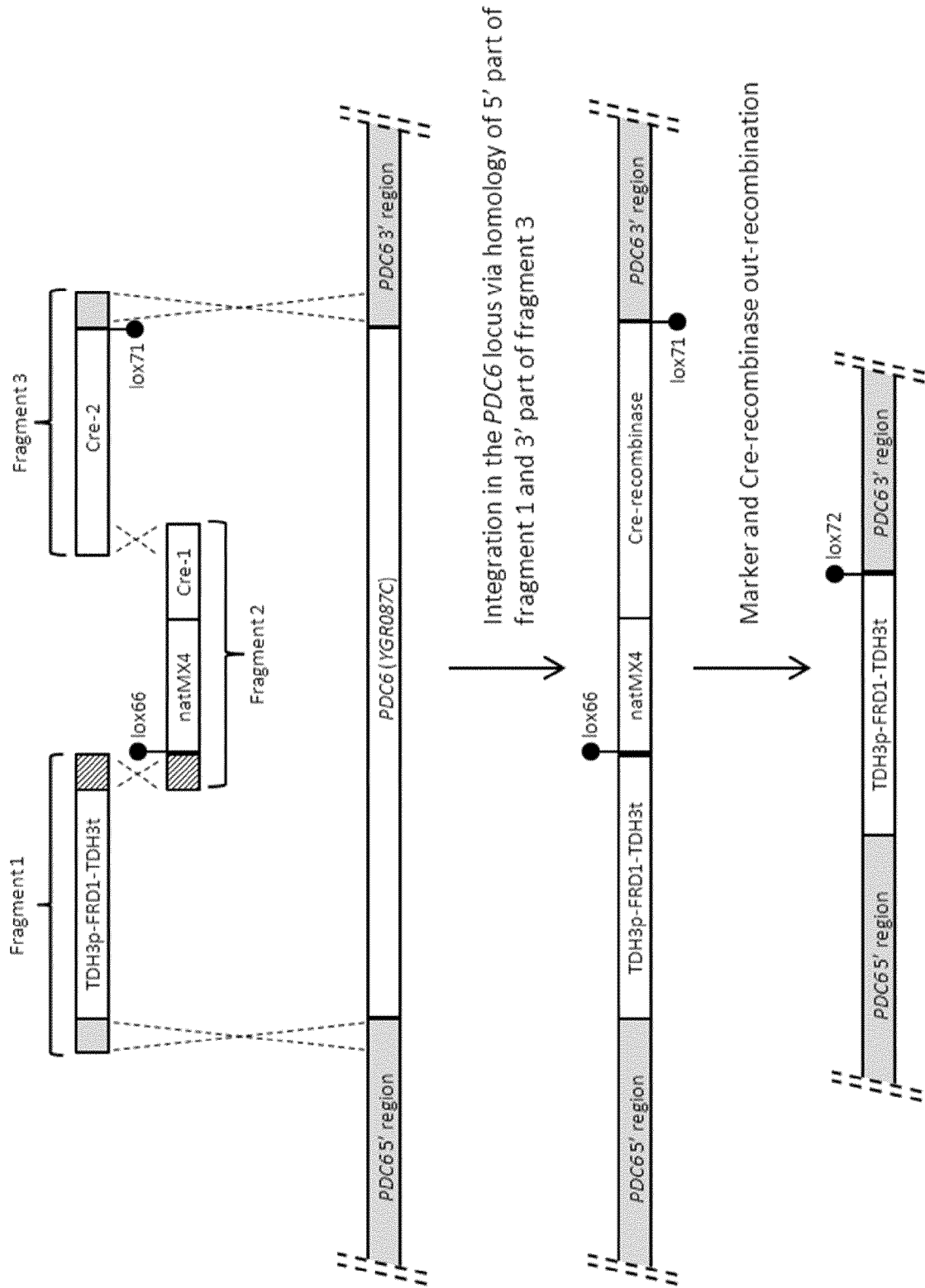


Fig. 2

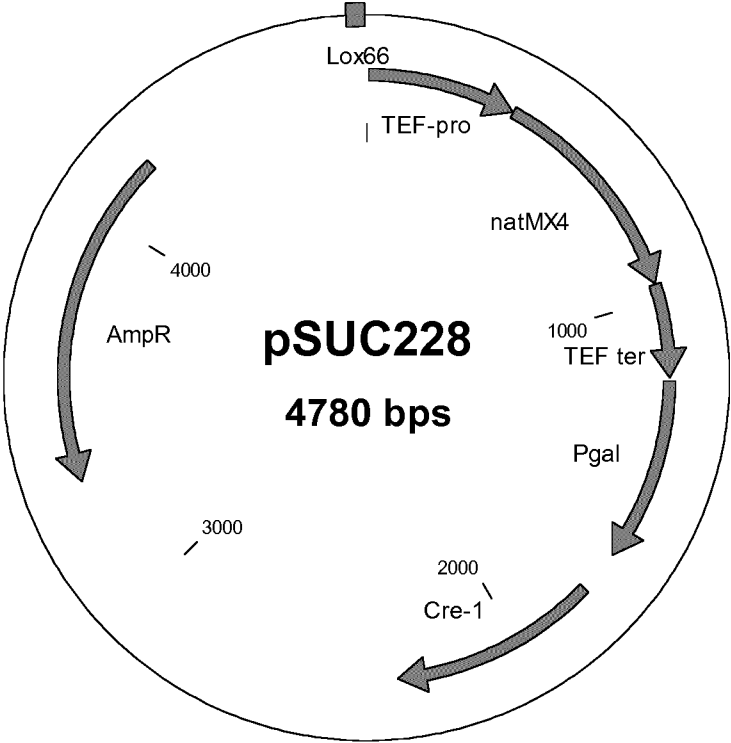


Fig. 3

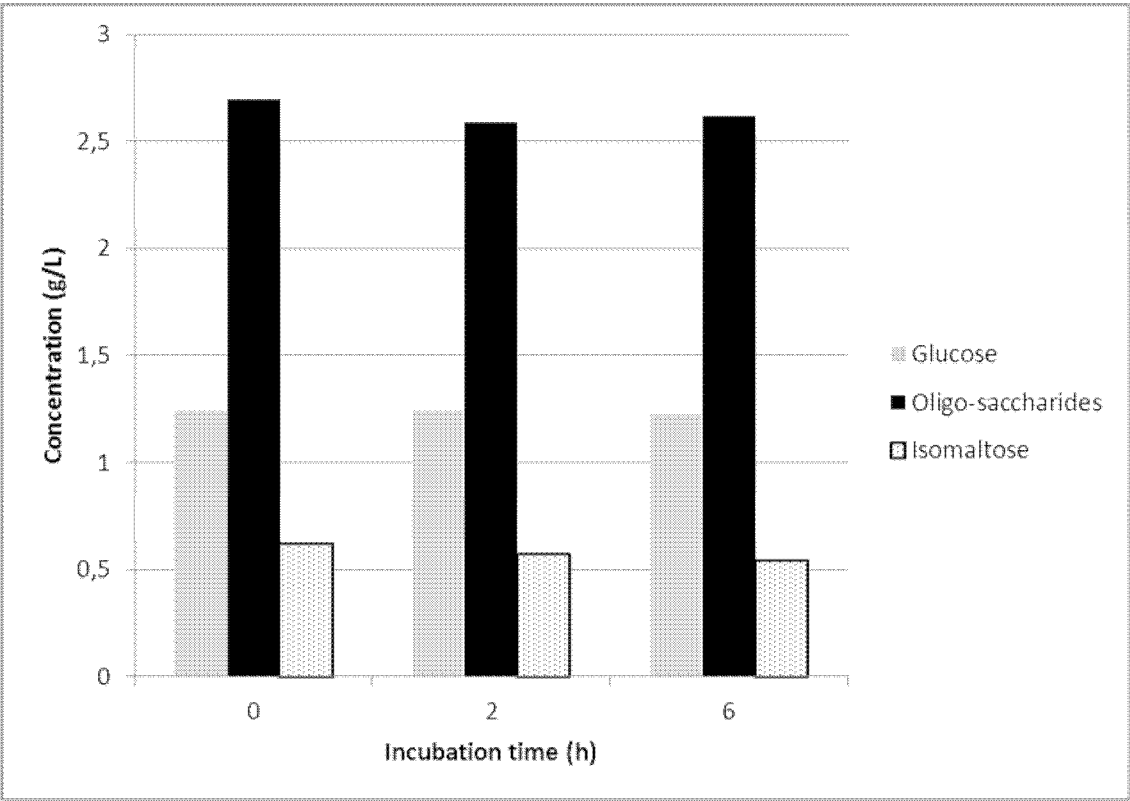


Fig. 4

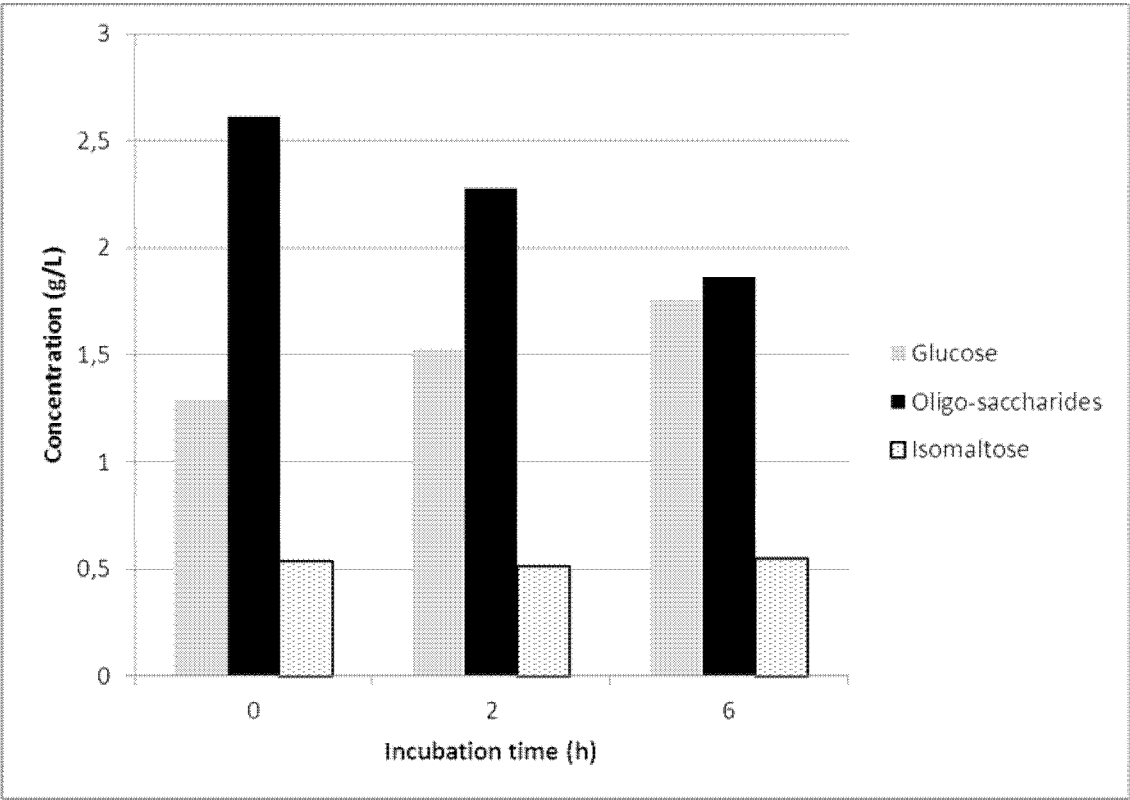


Fig. 5

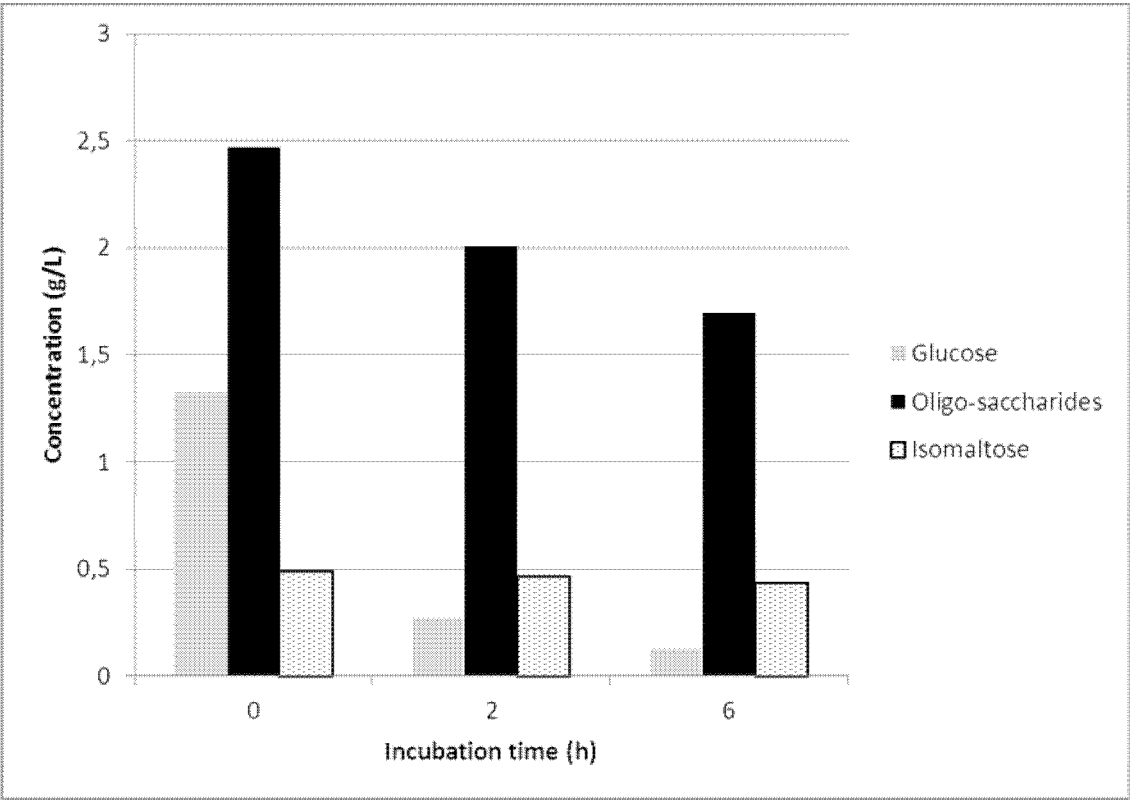


Fig. 6

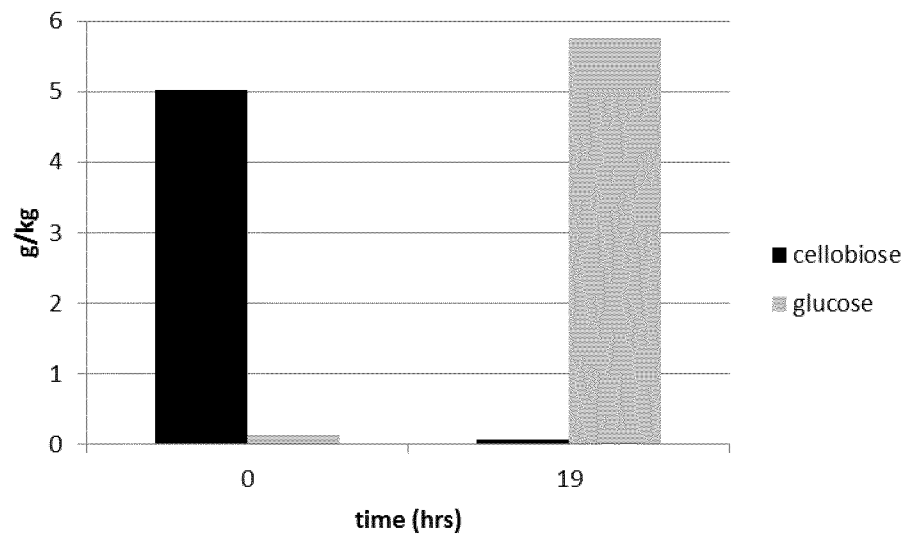


Fig. 7

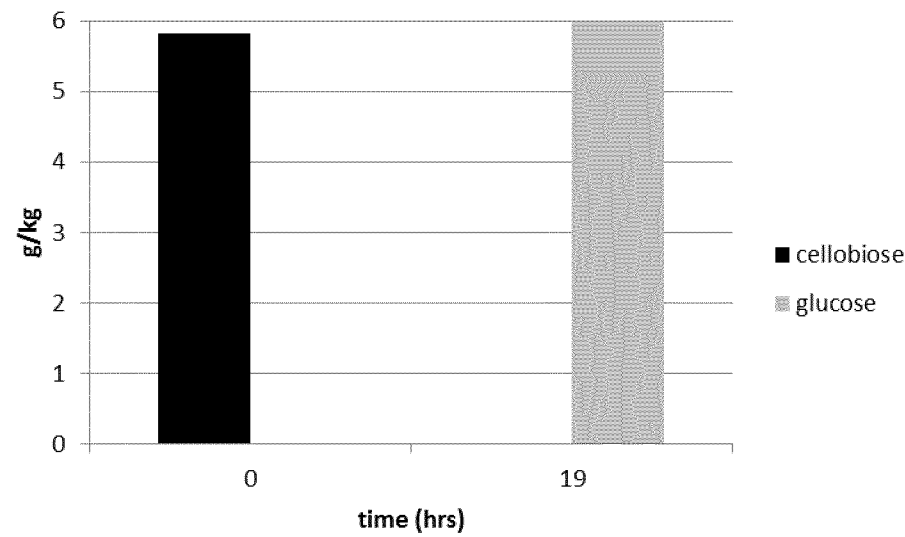


Fig. 8

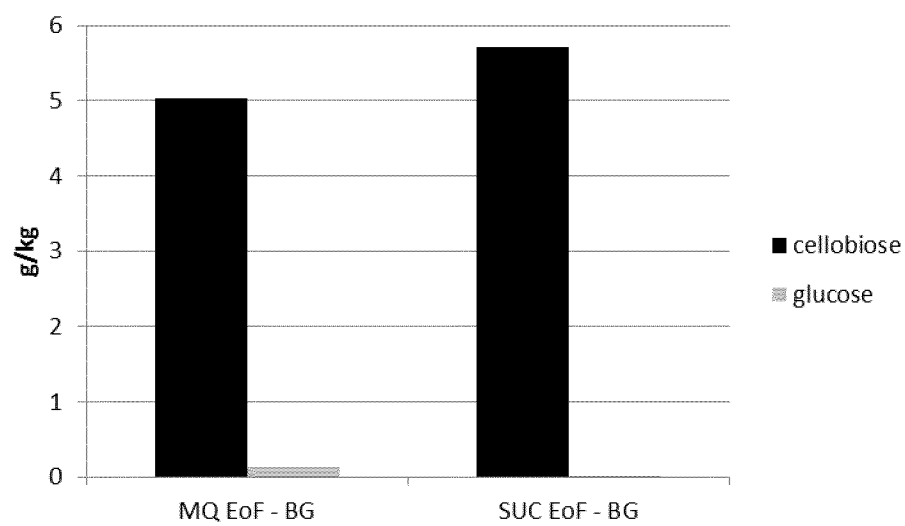


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/063792

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/46 C12P19/02 C12P39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, COMPENDEX, FSTA, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	CHINH-NGHIA NGUYEN ET AL: "Pilot scale simultaneous saccharification and fermentation at very high gravity of cassava flour for ethanol production", INDUSTRIAL CROPS AND PRODUCTS, vol. 56, 2014, pages 160-165, XP002754888, * See page 160 (Abstract) *	1-16



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

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

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"&" document member of the same patent family

Date of the actual completion of the international search

8 August 2016

Date of mailing of the international search report

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Korsner, Sven-Erik

INTERNATIONAL SEARCH REPORT

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International application No
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International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International application No

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