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(54) Title: DIRECT CONVERSION OF SUGARS TO GLYCOLIC ACID

(57) Abstract: According to an example aspect of the present invention, there is provided a eukaryotic host, which has been modified to directly convert sugars to glycolic acid. The whole glycolic acid production pathway is introduced into cytosol. There is also provided a method for producing glycolic acid with such host.



WO 2016/193540 A1

DIRECT CONVERSION OF SUGARS TO GLYCOLIC ACID

FIELD

[0001] The present invention relates to a method for a eukaryotic host and a method
5 for producing glycolic acid using eukaryotic host cells, especially cells of a genetically
modified fungal host.

BACKGROUND

[0002] Glycolic acid is a widely used chemical. It has applications within cosmetic
10 field and organic synthesis of polyglycolic acid and other biocompatible polymers. In
textile industry it is used as a dyeing and tanning agent and in food processing as a
flavouring agent and as a preservative. Traditionally glycolic acid is derived from fossil
sources but it is possible to derive glycolic acid from biomass by using biochemical routes.

[0003] Glycolic acid is the smallest α -hydroxy acid and it is not naturally produced
15 by microorganisms at least in feasible quantities for industrial use. This is different to
lactic acid which is also a small α -hydroxy acid, but this acid is produced naturally by a
large number of microorganisms. To produce glycolic acid from carbohydrates in a
fermentative way using microorganisms requires genetically engineered microorganisms.
Previously *Escherichia coli* strains were described that were engineered to produce
20 glycolic acid from glucose (WO 2007/141316 A2, WO2010/108909 A1 and
WO2011/036213). In these strains the glyoxylate cycle was disrupted by deleting malate
synthase and overexpressing an endogenous glyoxylate reductase so that the glyoxylate
produced by isocitrate lyase could be converted to glycolic acid. Also other modifications
were done to channel more carbon to the glyoxylate cycle or to prevent the utilization of
25 glyoxylic acid. *E. coli* strains engineered in this way were producing glycolic acid from
glucose.

[0004] A characteristic of *E. coli* and bacteria in general is that the inner space of the
cell or the cytosol of the organism is not compartmentalized. This is different in eukaryotic
organisms. Eukaryotic cells, including yeast and filamentous fungi, have different
30 compartments such as vacuoles, mitochondria or peroxisomes that are separated by

membranes. In difference to bacteria the cellular reactions take place in different compartments. For example the reactions of the TCA cycle (Krebs cycle, citric acid cycle) are located in the mitochondria, whereas the different reactions of the glyoxylate cycle are in separate compartments. According to current understanding isocitrate lyase and malate
5 synthase are peroxisomal in filamentous fungi. In yeasts such as *S. cerevisiae* isocitrate lyase occurs in cytosol whereas malate synthase can be cytosolic or peroxisomal depending on sole carbon source. The citrate synthase is located in the peroxisomes and in the mitochondria, the aconitase can be in mitochondria and cytosol and the succinate dehydrogenase is exclusively in mitochondria. The distribution of the enzyme activities to
10 different compartments and inability of the metabolites to freely travel between the compartments makes a fundamental difference to metabolic pathways and consequently the approaches to engineer these pathways by means of genetic engineering are essentially different. It is therefore not clear whether the teaching disclosed in the WO 2007/141316, WO 2010/108909 and WO 2011/036213 can be applied to eukaryotic microorganism.

15 [0005] Using *E. coli* for the production of glycolic acid has several drawbacks. *E. coli* is requiring a complex growth medium which is in general more expensive than growth media for yeast or filamentous fungi. Another drawback is that *E. coli* is operating close to neutral pH. This increases the contamination risk and in the case of acid production it requires base to neutralize the acid. Further, in neutral conditions the
20 produced glycolic acid is in dissociated form, which is a disadvantage in downstream processing.

[0006] WO 2013/050659 concerns a eukaryotic host selected from micro-organisms, and a method for producing glycolic acid using said eukaryotic host cells, especially cells of a genetically modified fungal host. However, with the modifications described in said
25 publication the production of glycolic acid occurs via ethanol.

[0007] From industrial point of view, it is appealing to allow direct conversion of various sugars into glycolic acid.

SUMMARY OF THE INVENTION

[0008] The invention is defined by the features of the independent claims. Some specific embodiments are defined in the dependent claims.

[0009] According to a first aspect of the present invention, there is provided a micro-
5 organism, which is able to convert sugars to glycolic acid.

[0010] According to a second aspect of the present invention, there is provided a sustainable method for direct conversion of sugars derived from biomass to glycolic acid.

[0011] These and other aspects, together with the advantages thereof over known solutions are achieved by the present invention, as hereinafter described and claimed.

10 [0012] The host according to an embodiment of the present invention is mainly characterized by what is stated in the characterizing part of claim 1.

[0013] The method according to an embodiment of the present invention is mainly characterized by what is stated in the characterizing part of claim 14.

[0014] Considerable advantages are obtained by means of the invention. Direct
15 conversion of renewable sugar sources to glycolic acid provides a green alternative and enables an economically competitive production method, because the energy consuming metabolic routes towards glycolic acid are engineered in a way which favors the production towards glycolic acid and inhibit the production of intermediates and/or side products. This enables fast and straight forward production of glycolic acid.

20 [0015] Next, the present technology will be described more closely with reference to certain embodiments.

EMBODIMENTS

[0016] The present technology describes a host and a method for producing glycolic acid directly from various sugars.

[0017] A phrase “direct conversion of sugars to glycolic acid” as used herein is intended to mean that no ethanol is accumulated as an intermediate before glycolic acid production. In other words, cells are able to produce glycolic acid directly from sugars without first converting sugars into ethanol.

[0018] A phrase “genetically modified to express” as used herein covers the cells where a protein-encoding polynucleotide has been transformed in such a manner that the host is capable of producing an active protein or where a promoter region of a cell has been modified to allow or enhance the expression of a heterologous or homologous gene encoding desired enzyme activity.

[0019] As used in the present context the term "identity" refers to the global identity between two amino acid sequences compared to each other from the first amino acid encoded by the corresponding gene to the last amino acid. For the purposes of the present invention identity is preferably determined by means of known computer programs using standard algorithms. An example of such a program is NCBI BLAST; BLASTp (comparison of known protein sequences, amino acids), BLASTn (comparison of nucleic acid sequences), BLASTx (comparison of translated nucleic acid sequences against known protein sequences).

[0020] “An active fragment” means a fragment having all the parts needed for completing the function typical for the protein.

[0021] The term “homologous” means native and term “heterologous” means non-native gene, promotor, terminator etc. to the host cell.

[0022] In this connection term “attenuating” refers to partial or total deletion or knock-out of the said gene, modification of regulatory regions of the said gene in order to decrease its activity or silencing or decreasing the activity of the said gene by any methods without restricting to those mentioned above.

[0023] As used herein, the term “promoter” refers to a sequence located upstream (e.g., 5') to the translation start codon of a structural gene and which controls the start of

transcription of the structural gene. Similarly, the term “terminator” refers to a sequence located downstream (e.g., 3’) to the translation stop codon of a structural gene and which controls the termination of transcription of the structural gene. A promoter or terminator is “operatively linked” to a structural gene if its position in the genome relative to that of the structural gene is such that the promoter or terminator, as the case may be, performs its transcriptional control function.

[0024] FIGURE 1 illustrates the metabolic routes relating to the present invention.

[0025] FIGURE 2 illustrates pUG-lox71-LEU2-lox66 plasmid used for constructing the promoter change cassettes

10 [0026] FIGURE 3 illustrates pUG-lox71-HIS3-lox66 plasmid used for constructing the promoter change cassettes

[0027] FIGURE 4 illustrates p4185 plasmid where *GLYR1* encoding gene is expressed under *S. cerevisiae* *TPII* promoter.

15 [0028] FIGURE 5 is a chart showing the production curves of glycolic acid and ethanol with different strains during 100 hours.

[0029] In one embodiment, a eukaryotic production host is selected from microorganisms, wherein the host is a fungal cell or a filamentous fungus cell, which has been genetically modified by transformation of a protein-encoding polynucleotide, or modification of a promoter region of the host cell, to overexpress gene encoding glyoxylate reductase activity and produce glycolic acid, and thus able to convert C5-C6 sugars directly to glycolic acid.

[0030] According to one embodiment, the host is able to convert C5-C6 sugars to glycolic acid directly without first accumulating ethanol.

25 [0031] According to another embodiment, promoters of the glycolic acid pathway genes aconitase and isocitrate lyase encoding genes of the host are changed under constitutive promoters (such as pTEF1 or pFBA1) to avoid the regulation of transcription caused by the native promoters.

[0032] According to another embodiment, promoters of the extended glyoxylate cycle recycling succinate formed from the isocitrate lyase reaction are changed into

constitutive promoters (such as pTEF1 or pFBA1) to avoid regulation of transcription caused by the native promoters. These genes include cytoplasmic malate dehydrogenase, fumarase and mitochondrial succinate-fumarate transporter.

[0033] Thus, according to a further embodiment one or several of the host's
5 promoters of the glycolic acid pathway encoding genes selected from citrate synthase, aconitase and isocitrate lyase, or the extended glycolic acid pathway encoding genes selected from cytosolic malate dehydrogenase, fumarase and mitochondrial succinate-fumarate transporter, are changed into constitutive promoters.

[0034] According to another embodiment, the host conducts the whole glycolic acid
10 pathway in cytosol. Naturally most of the enzymatic reactions of the pathway are cytosolic but some of the reactions are peroxisomal or mitochondrial, which therefore requires a transport between the organelles and cytosol and for example slows down the production process in the cell.

[0035] According to one embodiment, the host is able to express citrate synthase
15 (*CIT1* or *CIT2*) in cytosol. Naturally, the enzymatic reaction is taking place in the mitochondria or peroxisomes, respectively.

[0036] The eukaryotic host may be any eukaryotic organism, but most usually it is a cell, preferably a micro-organism. In one embodiment the cell is a fungal cell. When compared to for example *E. coli*, fungal cells are generally more tolerant to changing
20 culture conditions such as low pH, lignocellulosic hydrolysates, impurities, degradation products of hydrolytic enzymes and toxins. They also have lower nutritional requirements compared to bacteria most commonly used in industrial processes. Thus fungal production is an economic approach to microbial production of glycolic acid.

[0037] In one embodiment the host is a yeast cell. Yeasts like *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus niger* are naturally operating at very acidic
25 pH. Yeast produced in fermentations is often used as cattle feed which benefits the economics of the process. Bacteria are generally not used as cattle feed but deemed a waste.

[0038] Suitable yeasts are for example the genera *Saccharomyces*, *Kluyveromyces*,
30 *Candida*, *Scheffersomyces*, *Pachysolen* and *Hansenula*. Yeast species of particular interest include *S. cerevisiae*, *S. exiguus* (also known as *Kazachstania exigua*), *K. marxianus*, *K.*

lactis, *K. thermotolerans*, *C. sonorensis*, *C. krusei* (also known as *Issachenkia orientalis* and *Pichia kudriavzevii*), *C. shehatae*, *Pachysolen tannophilus* and *Scheffersomyces stipitis*.

[0039] In one embodiment the host is a filamentous fungus. One particular advantage
5 of using filamentous fungi to produce glycolic acid is that it can be done in a consolidated process, meaning that the fungus produces the enzymes for biomass hydrolysis and ferments the resulting sugars in the same process.

[0040] Suitable filamentous fungi hosts are for example of the genera *Aspergillus*,
Trichoderma, *Monascus*, and *Penicillium*. Fungal species of particular interest include *A.*
10 *niger*, *A. ficuum*, *A. phoenicis*, *T. reesei*, *T. harzianum*, *M. ruber*, and *P. chrysogenum*. Filamentous fungi allow using only partially hydrolysed biomass as a carbon source which is benefit if some lignocellulosic waste is used as a carbon source. *A. niger* is also a well-known citric acid producer and by metabolic engineering it can be made an efficient host for glycolic acid production.

15 [0041] In a preferred embodiment the host includes those of the species *S. cerevisiae*, *S. exiguus*, *K. marxianus*, *K. lactis*, *C. krusei* and *A. niger*.

[0042] The host organism of this invention is genetically modified and may contain
also other genetic modifications than those specifically described herein. Methods for
making modifications of these types are generally well known and are described in various
20 practical manuals describing laboratory molecular techniques.

[0043] In one embodiment of the invention the host is capable of producing glycolic
acid at pH below 6, preferably below 5.5, below 5.0, below 4.5, below 4.0, below 3.5,
below 3.0, below 2.5 and even below 2.0, until pH 1.5. Acidic or mildly acidic culture
conditions reduce risk of contaminations and thus improve the process hygiene and safety.
25 Each contaminated large scale fermentation results in direct loss of money. For the
downstream processing it is of advantage when the glycolic acid is produced at acidic pH.
Glycolic acid has a pKa of 3.83 meaning that below a pH 3.8 it is mainly in the acid form
which facilitates the separation.

[0044] In one embodiment of the invention the host is capable of producing glycolic
30 acid in non-buffered culturing conditions. In other words the strain is tolerant to decreasing
pH during the cultivation process. This simplifies the culturing process and thereby reduces

costs. However, this characteristic naturally does not exclude possibility to regulate the pH conditions using bases (or acids) or even buffering agents.

[0045] In one embodiment the glyoxylate reductase gene is a heterologous gene to a host. Especially when the K_m value of the homologous gene is high it is beneficial to
5 replace the gene by heterologous gene having lower K_m value. The heterologous gene is preferably obtained from a plant source, gene obtained from *Arabidopsis thaliana* (Hoover *et al.* 2007) being the most preferred. It is also possible to use endogenous or native genes for production and modify the cell e.g. by changing its native promoter to a constitutive promoter to overproduce glyoxylate reductase.

10 [0046] In one embodiment the glyoxylate reductase enzyme is characterized by having an EC number EC 1.1.1.79 (NADP+) or EC 1.1.1.26 (NAD+). Enzymes of class EC 1.1.1.79 (glyoxylate:NADP+ reductases, CAS 37250-17-2) catalyze reduction of glyoxylate using NADPH into glycolic acid and NADP or reduces hydroxypyruvate to glycerate. *A. thaliana* glyoxylate reductase designated here as “GLYR1” is one example of
15 this class. Enzymes of class EC 1.1.1.26 (glyoxylate:NAD+ reductases, CAS 9028-32-4) reduces glyoxylate to glycolic acid or hydroxypyruvate to D-glycerate. In the European chemical legislation the enzymes are defined according to the type of reaction they catalyze. Each enzyme is given a systematic name and an IUBMB (International Union of Biochemistry and Molecular Biology) number such as EC 1.1.1.26
20 (www.chem.qmul.ac.uk/iubmb/enzyme/).

[0047] It is understood by the skilled reader that the gene must be operably linked to the sequences regulating the expression of the gene. Two DNA sequences are operably linked when the function of the promoter results in transcription. An operable linkage is a linkage in which a sequence is connected to a regulatory sequence (or sequences) in such a
25 way as to place expression of the sequence under the influence or control of the regulatory sequence.

[0048] In one embodiment the gene is genetically optimized. It is understood by a skilled man that heterologous gene obtained by a different organism may need genetic optimization in order to properly function in the host cell. In one embodiment the
30 heterologous gene is genetically optimized to fit the host systems.

[0049] Standard molecular biology methods can be used in the cloning of glyoxylate reductase or other overexpressed genes. The basic methods used like isolation and enzyme treatments of DNA, *E. coli* transformations made for plasmid constructions, the isolation of the vectors or fragments containing the said gene and amplification of fragments by PCR are described in the standard molecular biology handbooks e.g. Sambrook *et al.* (1989) and Sambrook and Russell (2001). Genetic modification of the host fungus is accomplished in one or more steps via the design and construction of appropriate vectors and transformation of the host fungus with those vectors. Electroporation, protoplast-PEG and/or chemical (such as calcium chloride- or lithium acetate-based) transformation methods can be used.

[0050] The heterologous gene can be stably introduced into the genome of the host cell. Stable transformation is obtained when the expression cassette is integrated to the chromosomal DNA of the host. Targeted integration can be accomplished by designing a vector or fragment having regions that are homologous to the upstream (5'-) and downstream (3'-) flanks of the target locus. The *GLYRI* cassette (including suitable promoters and terminators if different from those of the target gene) and/or selection markers (with suitable promoters and terminators) will reside between the regions that are homologous to the upstream and downstream flanks of the target locus. Stable transformation is preferred as no selection pressure is needed during cultivation but alternatively also episomal plasmids and other non-integrated constructs are within this invention.

[0051] The use of native (homologous to the host cell) or non-native (heterologous to the host cell) promoters and terminators, together with respective upstream and downstream flanking regions, can permit the targeted integration of the *GLYRI* or any other gene mentioned above, or any other gene further described below, into specific loci of the host cell's genome, and for simultaneous integration of the said gene and deletion of a native gene, such as, for example, a malate synthase (e.g. *MLS1* or *DAL7*) encoding gene.

[0052] The exogenous glyoxylate reductase gene may be maintained on a self-replicating plasmid, integrated randomly into the host cell's genome or inserted at one or more targeted locations. Examples of targeted locations include the locus of a gene that is desirably deleted or disrupted, such as the *MLS1* encoding gene in *S. cerevisiae* or in *A. niger*.

[0053] In one embodiment the cell has been further modified to overexpress the gene encoding glyoxylate reductase.

[0054] The overexpressed genes, such as glyoxylate reductase (e.g. *GLYRI* gene from *A. thaliana*), isocitrate lyase, aconitase, citrate synthase, fumarate reductase, acetyl-coenzyme A synthetase or a gene regulating glyoxylate cycle is under the control of a promoter and a terminator, both of which are functional in the modified fungal cell.

[0055] The genetically modified fungus may contain a single copy or multiple copies of the glyoxylate reductase gene (e.g. *GLYRI* gene) or any other gene mentioned above, or any other gene further described below. If multiple copies of the glyoxylate reductase gene are present, from 2 to 10 or more copies may be integrated into the genome, or > 100 copies may be present on self-replicating plasmids. If multiple copies of the glyoxylate reductase gene are integrated into the genome, they may be integrated at a single locus (so they are adjacent each other), or at several loci within the host's genome. It is possible for different glyoxylate reductase genes to be under the control of different types of promoters and/or terminators.

[0056] In one embodiment the production host has been further modified by increasing the flux towards the glyoxylate cycle. The flux towards the cycle can be increased e.g. by overexpressing isocitrate lyase, aconitase, citrate synthase, fumarase, malate dehydrogenase, mitochondrial succinate-fumarate transporter or fumarate reductase. Heterologous fumarate reductase would convert succinate into fumarate, which can be then metabolized further and used again in the glyoxylate cycle. Heterologous fumarate reductase would also by-pass the natural fumarate dehydrogenase which has a dual role in citric acid cycle and in oxidative phosphorylation. Hence, heterologous fumarate reductase would decouple glyoxylate production from oxidative phosphorylation. Increase of the glyoxylate flux may require also reducing activity of the enzymes consuming the intermediates of the cycle, e.g. attenuating expression of malate synthase(s), NAD and/or NADP dependent mitochondrial and/or cytosolic isocitrate dehydrogenase(s). Increase of flux towards the cycle results in more efficient production of glycolic acid.

[0057] The yield of the glycolic acid can be increased by several ways. Increased yield saves the fermentation costs and enhances the down-stream processing.

[0058] In one embodiment the production host has been further modified by modifying the genes regulating the glyoxylate cycle i.e. genes involved in negative regulation of glucose-repressible genes. This can be done for example by activating genes such as *CAT8* in *S. cerevisiae* or in *K. lactis* by mutation or overexpression or by
5 attenuating genes such as *REG1* in *S. cerevisiae*.

[0059] In one embodiment the production host has been further modified by improving NADPH availability. This can be done e.g. by overexpressing cytosolic aldehyde dehydrogenase such as *ALD6* in *S. cerevisiae* or deleting phosphoglucose isomerase gene such as *PGII* in *S. cerevisiae* or *K. lactis*. Glyoxylate reductases are
10 usually NADPH dependent and thus need NADPH for functioning. Glyoxylate reductase can be also NADH dependent and in these cases the improvement of NADPH availability is unnecessary.

[0060] Thus, in one embodiment the cell has been further modified by attenuating genes involved in alcohol production in yeast or production of hydrolytic enzymes in
15 filamentous fungi. Alcohol production competes with the glyoxylate cycle and it can be preferred to decrease it. This can be done e.g. by overexpressing one or more of pyruvate carboxylases such as *PYC2*, *PYC1* in *S. cerevisiae* and acetyl-coenzyme A synthetase genes such as *ACSI* in *S. cerevisiae*; or reducing expression of genes encoding alcohol dehydrogenases in *S. cerevisiae*.

20 [0061] If the host organism chosen for glycolic acid production is capable of further utilizing glycolic acid it is necessary to also attenuate the genes responsible for these enzyme reactions. For example some microbes are known to have glycolate oxidase which oxidases glycolic acid to glyoxylate. In addition glycolic acid production can also be reduced if the host has enzymes other than glyoxylate reductase utilizing glyoxylate.
25 Deletion of malate synthase was already described above as an example how glyoxylate cycle needs to be modified to produce glycolic acid. In addition to malate synthase some fungi are known to have e.g. glyoxylate oxidase, which would need to be deleted in order to produce glycolic acid efficiently.

[0062] In one embodiment the cell has been further modified by any combination of
30 the above described modifications.

[0063] One embodiment of this invention is also a method for producing glycolic acid. In the method a eukaryotic cell encoding glyoxylate reductase is cultured in conditions allowing the expression the glyoxylate reductase gene, producing and secreting the glycolic acid and optionally recovering the glycolic acid from said medium.

5 [0064] In the production process of the invention, the fungus is cultivated in a growth and production medium that includes a carbon source and typical nutrients required by the particular host, including but not limited to a source of nitrogen (such as amino acids, proteins, inorganic nitrogen sources such as ammonia or ammonium salts), and various vitamins and minerals. Alternatively, more than one different carbon source can be
10 used.

[0065] Carbon source contains a sugar which can be hexose or pentose. Hexose can be e.g. glucose, fructose, mannose, or galactose and oligomers of glucose such as maltose, maltotriose, isomaltotriose, starch or cellulose. Examples of pentoses are xylose, xylan or other oligomer of xylose, and preferably also other carbon containing compounds to
15 provide for growth and energy. The medium may also contain ethanol, glycerol, acetate, or amino acids, or any mixture thereof, preferably ethanol or acetate, most suitably ethanol, which further components can also function as carbon sources.

[0066] The carbon substrates may be provided as pure substrates or from complex technical sources. The xylose containing sugars are suitably hydrolysates of plant biomass
20 e.g. hemicellulose-containing biomass, such as lignocellulose. In addition, the medium may consist of or contain complex, poorly defined elements, such as would be present in relatively inexpensive sources like black liquor, corn steep liquor or solids, or molasses. In case of oligomeric sugars, it may be necessary to add enzymes to the fermentation broth in order to digest these to the corresponding monomeric sugar. It is also possible to use
25 production hosts, such as filamentous fungus hosts that secrete hydrolytic enzymes enhancing the production of fermentative sugars.

[0067] Other fermentation conditions, such as temperature, cell density, selection of nutrients, and the like are not considered to be critical to the invention and are generally selected to be suitable for the cell used and to provide an economical process.
30 Temperatures during each of the growth phase and the production phase may range from above the freezing temperature of the medium to about 50°C, although the optimal temperature will depend somewhat on the particular micro-organism. A preferred

temperature, particularly during the production phase, is from about 25 to 35°C.

[0068] The pH of the process may or may not be controlled to remain at a constant pH, but should be between 1.5 and 6.5, depending on the production organism. In one embodiment the culturing pH is below 6, preferably below 5.5, below 5.0, below 4.5, below 4.0, below 3.5, below 3.0, below 2.5 and even below 2.0, until pH 1.5. Depending on the production organism the lower limit of the pH may vary between 1.5 and 4. Preferred pH of the culture media is 1.5 to 5, more preferably 2 to 4 and most preferably 2 to 3. In one embodiment the culture medium contains no buffering agent.

[0069] According to an embodiment of the invention, the pH is controlled to a constant pH of 3.5 to 5.5. Suitable buffering agents for regulating or buffering pH are basic materials that neutralize glycolic acid as it is formed, and include, for example, calcium hydroxide, calcium carbonate, sodium hydroxide, potassium hydroxide, potassium carbonate, sodium carbonate, ammonium carbonate, ammonia, ammonium hydroxide and the like. In general, those buffering agents that have been used in conventional fermentation processes are also suitable here. It is within the scope of the invention, however, to allow the pH of the fermentation medium drop from a starting pH that is typically 6 or higher, to below the pKa of the acid fermentation product, such as in the range of about 3 to about 4.

[0070] The fermentation is conducted aerobically or micro aerobically. If desired, specific oxygen uptake rate, dissolved oxygen level or redox level can be used as a process control. The process of the invention can be conducted continuously, batch-wise, or some combination thereof.

[0071] During the fermentation glycolic acid is excreted out from the cells into the growth medium from which it may be recovered without disrupting the cells.

[0072] In one embodiment of the invention a cell described above is cultured and glycolic acid is recovered. Glycolic acid can be recovered from the fermentation medium by e.g. two-phase liquid-liquid extraction, ion exchange chromatography or reactive extraction or it can be polymerized in the fermentation medium and recovered thereafter.

[0073] One embodiment of the invention is the use of eukaryotic host cells or eukaryotic organisms in production of glycolic acid or as a starting organism for preparation a production host suitable for production of glycolic acid. In preferred

embodiment the eukaryotic organism is a fungal cell, preferably a yeast or filamentous fungus. The eukaryotic organism may be modified as described here and is suitable for the method as described here.

[0074] It is to be understood that the embodiments of the invention disclosed are not limited to the particular structures, process steps, or materials disclosed herein, but are extended to equivalents thereof as would be recognized by those ordinarily skilled in the relevant arts. It should also be understood that terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting.

[0075] Reference throughout this specification to one embodiment or an embodiment means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment. Where reference is made to a numerical value using a term such as, for example, about or substantially, the exact numerical value is also disclosed.

[0076] As used herein, a plurality of items, structural elements, compositional elements, and/or materials may be presented in a common list for convenience. However, these lists should be construed as though each member of the list is individually identified as a separate and unique member. Thus, no individual member of such list should be construed as a de facto equivalent of any other member of the same list solely based on their presentation in a common group without indications to the contrary. In addition, various embodiments and example of the present invention may be referred to herein along with alternatives for the various components thereof. It is understood that such embodiments, examples, and alternatives are not to be construed as de facto equivalents of one another, but are to be considered as separate and autonomous representations of the present invention.

[0077] Furthermore, the described features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. In the following description, numerous specific details are provided, such as examples of lengths, widths, shapes, etc., to provide a thorough understanding of embodiments of the invention. One skilled in the relevant art will recognize, however, that the invention can be practiced without one or more of the specific details, or with other methods, components, materials,

etc. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the invention.

[0078] While the forgoing examples are illustrative of the principles of the present invention in one or more particular applications, it will be apparent to those of ordinary skill in the art that numerous modifications in form, usage and details of implementation can be made without the exercise of inventive faculty, and without departing from the principles and concepts of the invention. Accordingly, it is not intended that the invention be limited, except as by the claims set forth below.

[0079] The verbs “to comprise” and “to include” are used in this document as open limitations that neither exclude nor require the existence of also un-recited features. The features recited in depending claims are mutually freely combinable unless otherwise explicitly stated. Furthermore, it is to be understood that the use of “a” or “an”, that is, a singular form, throughout this document does not exclude a plurality.

15

INDUSTRIAL APPLICABILITY

[0080] At least some embodiments of the present invention find industrial application in biodegradable polymers. Glycolic acid may for example be polymerized to polyglycolic acid (PGA). Glycolic acid is also used as a copolymer with lactic acid. Producing glycolic acid directly from renewable sources is a green alternative to be an industrially feasible production process.

20

ACRONYMS

Genes:

	<i>ACO1</i>	aconitase
25	<i>ICL1</i>	isocitrate lyase
	<i>FUM1</i>	fumarase
	<i>MDH2</i>	cytoplasmic malate dehydrogenase

	<i>SFC1</i>	mitochondrial succinate-fumarate transporter
	<i>CIT2</i>	peroxisomal citrate synthase
	<i>CIT1</i>	mitochondrial citrate synthase
	<i>MLS1</i>	malate synthase
5	<i>GLYR1</i>	glyoxylate reductase (heterologous gene from <i>Arabidopsis thaliana</i>)

Strains:

	H3986	control i.e. the old glycolic acid production strain used for glycolic acid production in WO 2013/050659
10	KL16	$\Delta mls1 \Delta idp2$ + the native promoters of the <i>ACO1</i> , <i>ICL1</i> , <i>MDH2</i> , <i>FUM1</i> and <i>SFC1</i> encoding genes replaced by constitutive promoters
	KL21	KL16 strain as background + p4185 plasmid (<i>GLYR1</i> under <i>TPH1</i> promoter)

EXAMPLE 1

- 15 *K. lactis* strain modified to produce glycolic acid directly from sugars

K. lactis H3976 strain described in WO 2013/050659 was used as a progenitor strain for all further modifications.

- 20 Constitutive *S. cerevisiae* promoters were amplified by PCR with the primer pairs listed in the Table 1. Fragments were cloned between the *SpeI* site of a vector pUG-lox71-LEU2-lox66 with *LEU2* marker gene (Figure 2) or pUG-lox71-LEU2-lox66 (Figure 3) with *HIS3* marker gene containing the selection marker between the loxP sites (Figure 3). Orientation of the ligated insert was checked by digestions and sequenced. The resulting vector was then amplified with flanking primer pairs shown in the Table 2. Each primer has flanks
- 25 ~60-70 bp which overlap with the 5' UTR region of the target gene and the start of the ORF of the gene i.e. they introduce the constitutive promoter in front of the target gene.

K. lactis aconitase (SEQ ID NO: 13) and isocitrate lyase (SEQ ID NO: 16) promoters were changed first. *HIS3* and *LEU2* marker genes were used respectively. Both cassettes contained loxP sites in front and after the marker genes. The loxP sites function as a specific target site for the Cre DNA recombinase enzyme (Güldener et al., 1996). Cre catalyzes DNA recombination leading up to the cleavage of loxP sites. Cre recombinase was expressed in *K. lactis* by transforming a Cre expression plasmid pKINatCre into the yeast cell. The plasmid contained *NAT* marker gene and the transformants were selected on medium containing nourseotricin. Once the *LEU2* and *HIS3* markers had been looped out the plasmid was lost by cultivating the cells on YPD medium without selection.

10

This constructed KL6 strain is further transformed with the glyoxylate reductase expression vector, p4185 (Figure 4) or uracil marker containing *GLYRI* integration fragment and the transformants selected on media containing uracil. Optionally the endogenous glyoxylate reductase encoding gene is put under a constitutive promoter in a similar manner as aconitase and isocitrate lyase genes.

15

Table 1. Primers used for amplifying the *S. cerevisiae* constitutive promoters

Promoter	Primer 1	Primer 2
<i>S. cerevisiae</i> <i>FBA1</i>	pFBA15'frw (SEQ ID NO: 3)	pFBA13'rev (SEQ ID NO: 4)
<i>S. cerevisiae</i> <i>TEF1</i>	pTEF15'frw (SEQ ID NO: 5)	pTEF13'rev (SEQ ID NO: 6)
<i>S. cerevisiae</i> <i>TPI1</i>	pTPI15'frw (SEQ ID NO: 8)	pTPI13'rev (SEQ ID NO: 8)
<i>S. cerevisiae</i> <i>PGK1</i>	pGK15'frw (SEQ ID NO: 9)	pGK13'rev (SEQ ID NO: 10)

EXAMPLE 2

Further modification of *K. lactis* producing glycolic acid directly from sugars

5 The KL6 strain described in Example 1 was further modified by introducing constitutive promoter in front of a truncated cytosolic malate dehydrogenase gene (SEQ ID NO: 19), fumarase encoding gene (SEQ ID NO: 22) and mitochondrial succinate fumarase transporter encoding gene (SEQ ID NO: 25).

10 Constitutive *S. cerevisiae* promoters were amplified by PCR with the primer pairs listed in the Table 1. Fragments were cloned between the *SpeI* site of the vector pUG-lox71-LEU2-lox66 with *LEU2* marker gene (Figure 2) or pUG-lox71-LEU2-lox66 with *HIS3* marker gene (Figure 3) containing the selection marker between the loxP sites. The resulting vector was then amplified with flanking primer pairs shown in the Table 2. Each primer has flanks ~60-70 bp which overlap with the 5' UTR region of the target gene and the start
15 of the ORF of the gene i.e. they introduce the constitutive promoter in front of the target gene.

This strain was further transformed with the glyoxylate reductase expression vector, p4185 containing *URA3* marker. The resulting strain, KL21, was tested for glycolic acid
20 production.

EXAMPLE 3

Comparison of glycolic acid production of strain H3986 and KL21 at pH 5.0:

25 *K. lactis* strain H3986 and KL21 were pregrown in flasks in modified synthetic complete (SC) medium lacking uracil (Sherman F, Fink G, Hicks JB. (1983) Methods in Yeast Genetics. A Laboratory Manual. Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y.). Glucose (20 g L⁻¹) was provided as carbon source. Pregrown cells were used for inoculating bioreactors.

30 *K. lactis* H3986 and KL21 was grown in Biostat Qplus bioreactors (max working volume 1.1 L, Sartorius AG, Germany) in synthetic complete medium lacking uracil with glucose (10 g L⁻¹) at pH 5, 30°C, 600-750 rpm and 0.8 L air per minute (~1 volume air per volume

culture per minute). Clerol antifoaming agent (Cognis, France, 0.08-0.10 $\mu\text{l l}^{-1}$) was used to prevent foam formation. The initial reactor volume was 800 mL. Bioreactors were inoculated to an initial OD_{600} value of 0.23, from pre-cultures in synthetic complete medium (lacking uracil), which had grown overnight in shaken flasks. Cells were collected
 5 from the pre-cultures by centrifugation and re-suspended in a small volume of medium. Cultures were maintained in batch phase for 29.6 h and then fed with 51 g L^{-1} glucose in synthetic complete medium (lacking uracil) at rates between 0.7 and 4.2 g h^{-1} . The feed rate was periodically adjusted to prevent or reduce glucose or ethanol accumulation.

The culture supernatant samples were analysed with Waters Alliance e2695 HPLC system
 10 (Waters, Milford, USA) where the injection volume was 10 μl . An Aminex HPX-87H Organic Acid Column (300 mm $\times$ 7.8 mm) (Bio-Rad, USA) linked to a Fast Acid Analysis Column (100 mm $\times$ 7.8 mm) (Bio-Rad, USA) was used as a stationary phase in the HPLC. Columns were maintained at +55 $^{\circ}\text{C}$ and 5.0 mM H_2SO_4 (Merck KgaA, Germany) was used as an eluent with the flow rate of 0.3 min^{-1} . Waters 2489 dual wavelength UV (210
 15 nm) detector (Waters, Milford, USA) and Waters 2414 differential refractometer (Waters, Milford, USA) were used for the detection of glucose, ethanol and glycolic acid (Table 2).

Table 2. Glycolic acid production by strain H3986 and KL21 in bioreactor.

Time (h)	Final product concentration ($\text{g}_{\text{glycolate}}\text{l}^{-1}$)		Cell growth (μ)		Specific productivity ($\text{mg}_{\text{glycolate}}\text{g}_{\text{cdw}}^{-1}\text{h}^{-1}$)		Product yield ($\text{g}_{\text{glycolate}}\text{g}_{\text{consumed}}\text{glucose}^{-1}$)	
	H3986	KL21	H3986	KL21	H3986	KL21	H3986	KL21
0-21.8	0.00	0.04	0.13	0.12	0.00	1.64	0.00	0.02
98.5-237.9	-	-	-	-	0.29	0.46	0.05	0.05
0-237.9	0.50	0.90	-	-	-	-	0.02	0.03

EXAMPLE 4

Alternative modifications of *K. lactis* strain producing glycolic acid directly from sugars

The strain KL6 described in Example 1 and KL16 described in Example 2 are further modified by introducing a constitutive *S. cerevisiae TDH3* promoter in front of the *K. lactis* cytosolic malate dehydrogenase *MDH2* (SEQ ID NO: 26). This replaces the native
5 *MDH2* promoter function.

The KL6 strain is transformed with cassettes replacing the native promoters of the cytosolic malate dehydrogenase encoding gene (SEQ ID NO: 26), fumarase encoding gene (SEQ ID NO: 22) and mitochondrial succinate fumarase transporter encoding gene (SEQ
10 ID NO: 25) in different combinations.

All constructs are transformed with the glyoxylate reductase expression vector (p4185) or uracil marker containing *GLYRI* integration fragment and the transformants selected on media containing uracil. Optionally the endogenous glyoxylate reductase encoding gene is put under a constitutive promoter in a similar manner as aconitase and isocitrate lyase
15 genes in the Example 1.

EXAMPLE 5

Introducing cytosolic citrate synthase to the *K. lactis* glycolic acid production strain

The *K. lactis* strain KL16 is transformed with a plasmid or a fragment overexpressing
20 cytosolic citrate synthase encoding gene e.g. *K. lactis* citrate synthase (SEQ ID NO: 27). Citrate synthase is targeted to cytosol by deleting or altering the mitochondrial or peroxisomal targeting signal of an endogenous citrate synthase encoding gene or introducing a heterologous citrate synthase gene which is targeted to cytosol naturally or is targeted to cytosol by deleting or altering mitochondrial or peroxisomal targeting signal of
25 the gene e.g. *S. cerevisiae CIT2* (YCR005C).

CITATION LIST

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5 WO 2011/036213

WO 2013/050659

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metabolism in planta. Can. J. Bot. 85: 883–895.
- Sambrook *et al.* (1989)
- 15 Sambrook and Russel (2001)

CLAIMS:

1. A eukaryotic production host selected from microorganisms, wherein the host is a fungal cell or a filamentous fungus cell, which has been genetically modified by transformation of
5 a protein-encoding polynucleotide, or modification of a promoter region of the host cell, to overexpress gene encoding glyoxylate reductase activity and produce glycolic acid, **characterized** in that the host is able to convert C5-C6 sugars directly to glycolic acid.
2. The host of claim 1, **characterized** in that one or several of its promoters of the glycolic
10 acid pathway encoding genes selected from citrate synthase, aconitase and isocitrate lyase, or the extended glycolic acid pathway encoding genes selected from cytosolic malate dehydrogenase, fumarase and mitochondrial succinate-fumarate transporter, are changed into constitutive promoters.
- 15 3. The host of claim 1 or 2, wherein the host is able to express citrate synthase in cytosol.
4. The host of any of the preceding claims, wherein the host is a yeast cell.
5. The host of any of the preceding claims, wherein the host is capable of producing
20 glycolic acid at pH 1.5 to 6.0, preferably at pH 1.5 to 5.
6. The host of any of the preceding claims, wherein the host is capable of producing glycolic acid in non-buffered culturing conditions.
- 25 7. The host of any of the preceding claims, wherein the glyoxylate reductase gene is a heterologous gene.
8. The host of any of the preceding claims, wherein the glyoxylate reductase gene encodes a protein having EC number 1.1.1.79 or 1.1.1.26.
- 30 9. The host of any of the preceding claims, wherein the cell has been genetically modified by
 - a) overexpressing the gene encoding glyoxylate reductase; or
 - b) increasing the flux towards the glyoxylate cycle; or

- c) modifying the genes regulating the glyoxylate cycle; or
- d) improving NADPH availability; or
- e) attenuating genes involved in alcohol production; or
- f) any combination of a) to e).

5

10. The host of claim 9, wherein glyoxylate pathway flux is increased

- a) by overexpressing one or more of the genes selected from isocitrate lyase, fumarate reductase, aconitase, citrate synthase, and acetyl-coenzyme synthetase genes; or
- 10 b) by reducing activity of the enzymes consuming the intermediates of the cycle, e.g. attenuating expression of malate synthase, NAD and/or NADP dependent mitochondrial and/or cytosolic isocitrate dehydrogenase; or
- c) by any combination of a) and b).

- 15 11. The host of claim 9, wherein glyoxylate cycle regulating genes *REG1* is attenuated or *CAT8* is activated.

12. The host of claim 9, wherein alcohol production is reduced by

- a) overexpressing pyruvate carboxylases; or
- 20 b) reducing expression of gene encoding alcohol dehydrogenase, such as alcohol dehydrogenase gene.

13. The host of claim 9, wherein NADPH availability is improved by overexpressing cytosolic aldehyde dehydrogenase gene or deleting phosphoglucose isomerase gene.

25

14. Method of producing glycolic acid, comprising culturing a eukaryotic cell, selected from microorganism cells of any of claims 1 to 13, in a growth and production medium, and optionally recovering the glycolic acid from said medium.

- 30 15. The method of claim 14, wherein the pH of the media is 1.5 to 6; preferably 1.5 to 5, more preferably 2 to 4, and most preferably 2 to 3.

16. The method of claim 14 or 15, wherein the culture medium is not buffered.

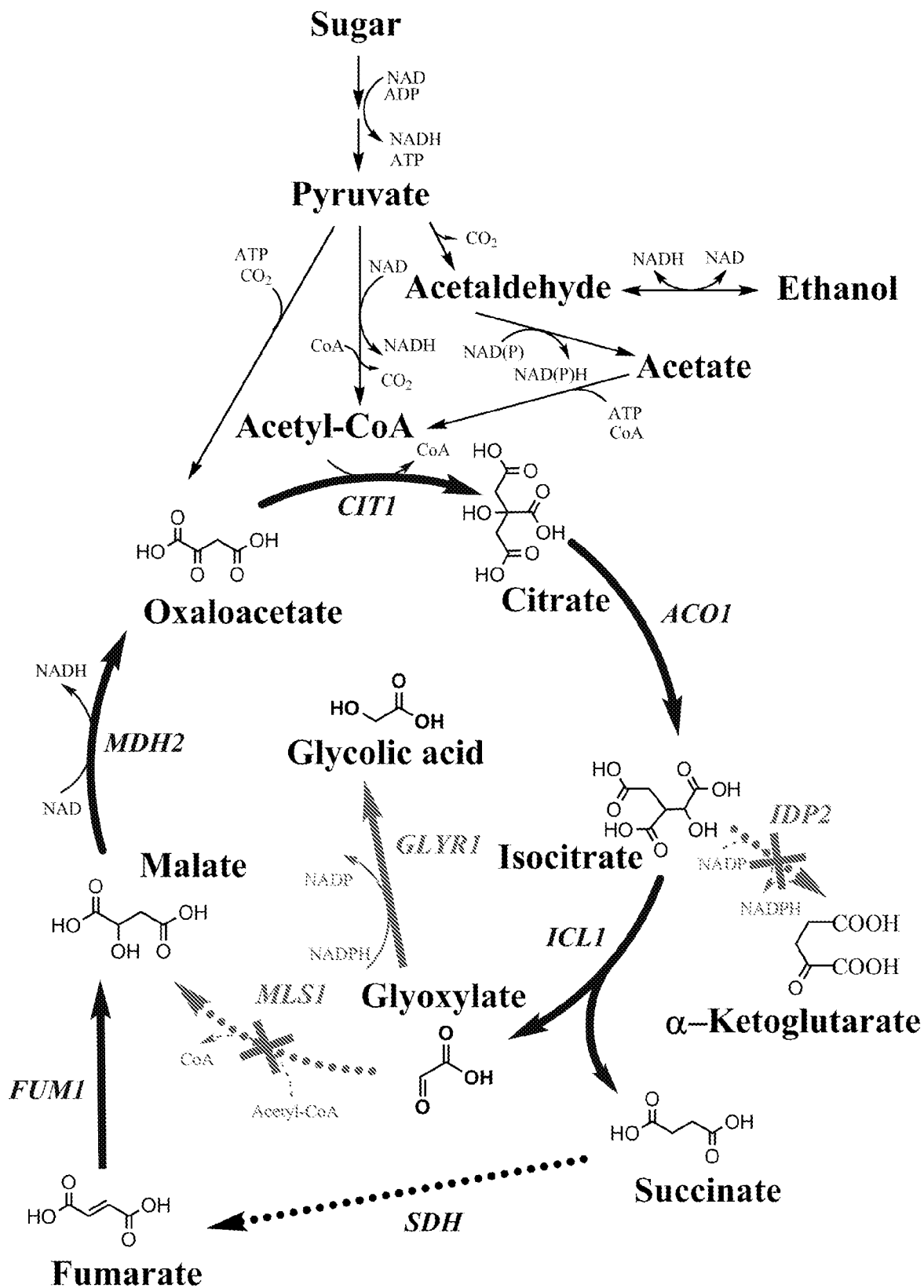


FIG. 1

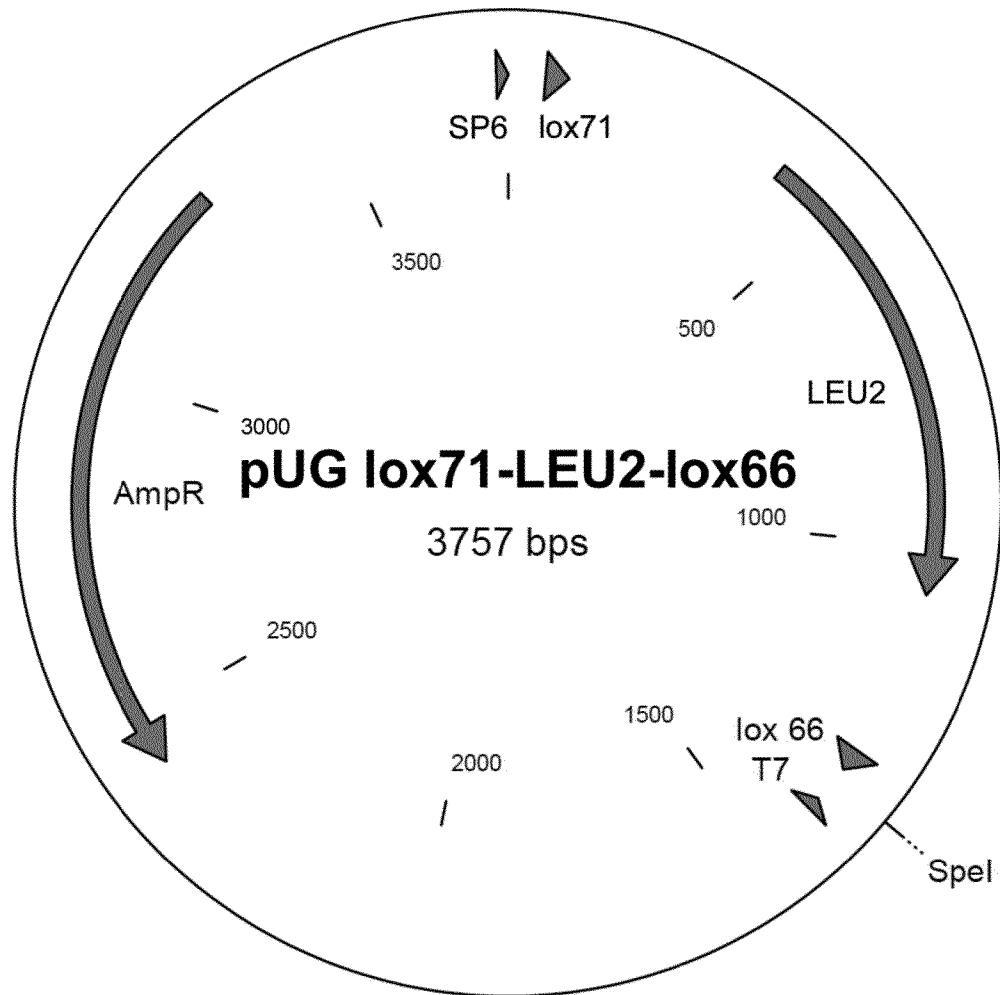


FIG. 2

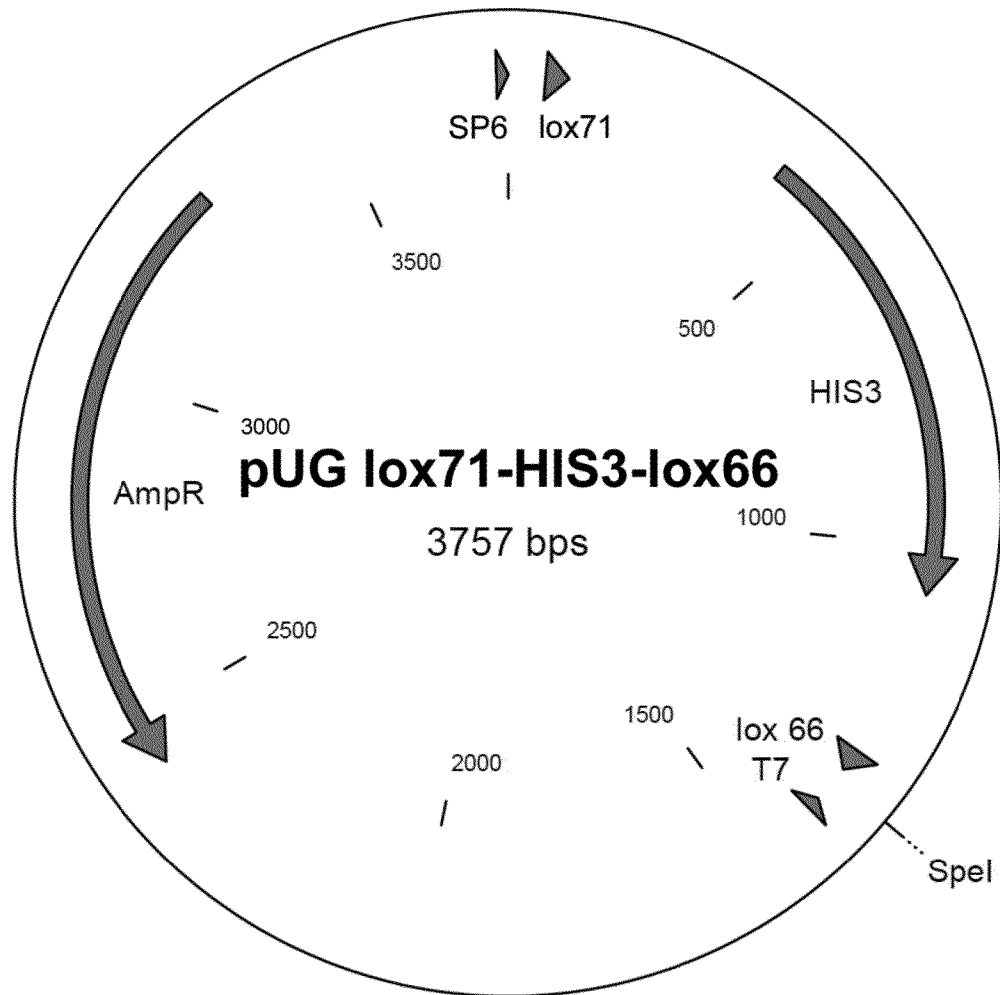


FIG. 3

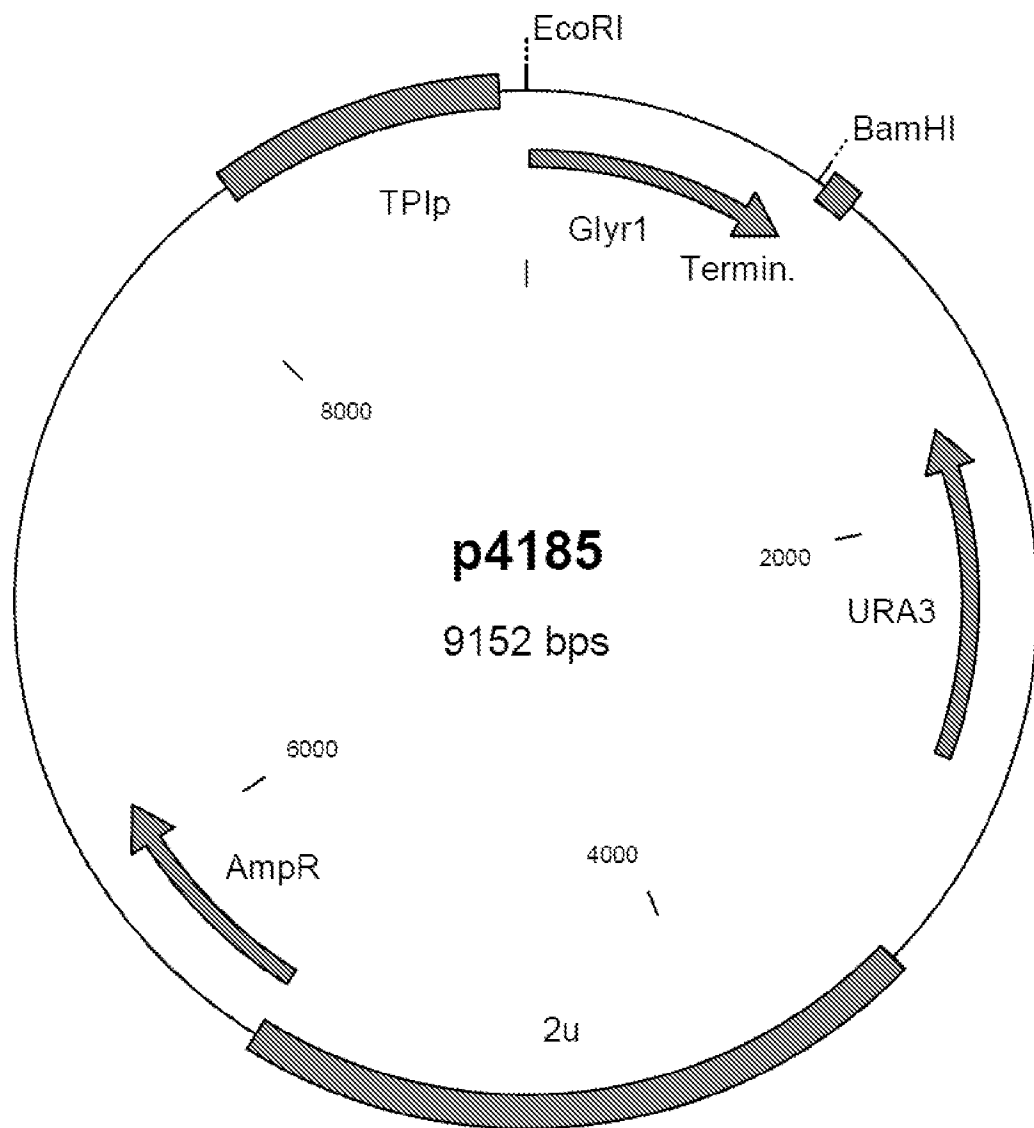


FIG. 4

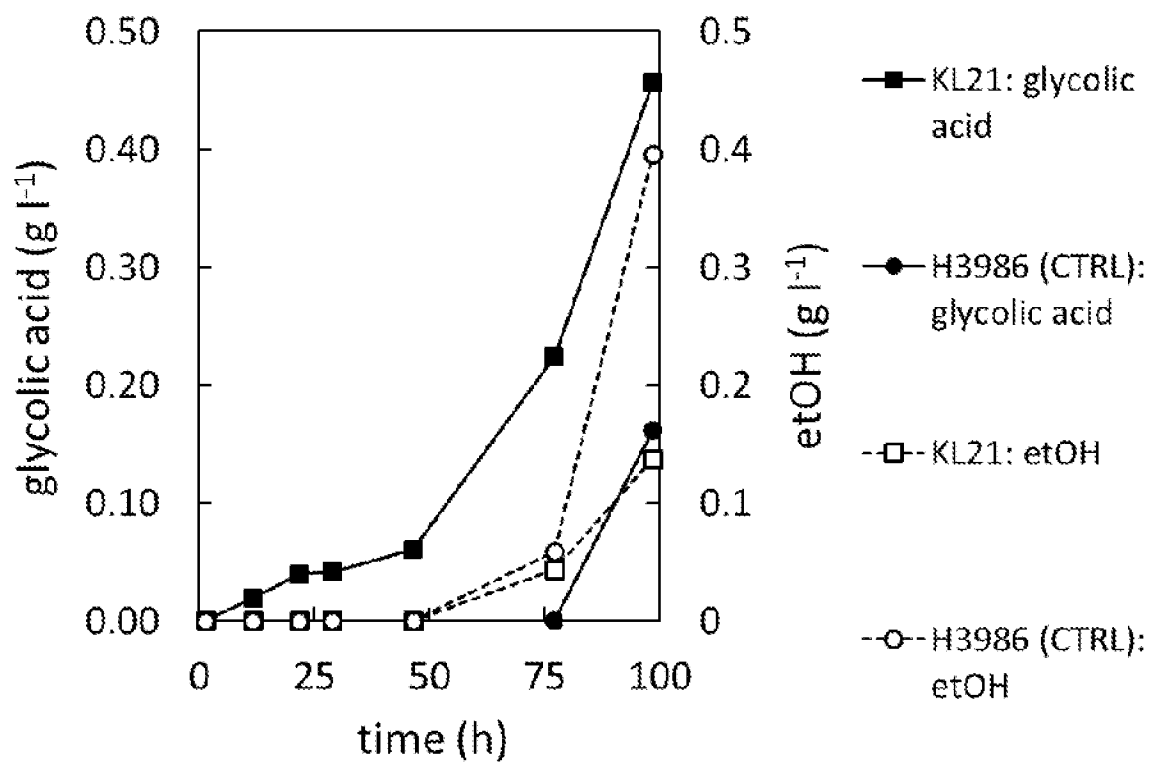


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI2016/050373

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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)

IPC: C12P, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
FI, SE, NO, DK

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

EPODOC, EPO-Internal full-text databases, Full-text translation databases from Asian languages, WPIAP, PRH-Internal, BIOSIS, CAPLUS, COMPDX, EMBASE, MEDLINE, XPESP, XPOAC, XPRD

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013050659 A1 (TEKNOLOGIAN TUTKIMUSKESKUS VTT [FI]) 11 April 2013 (11.04.2013)	1, 2, 4-16
Y	abstract; page 10, line 33 – page 11, line 30; page 13, lines 15-24; page 17, lines 4-16; pages 18-19, example 2; page 21, table 3; page 33, example 14; claims 1-19	3
Y	Koivistoinen, Outi. Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for organic acid production. Thesis for the degree of Doctor of Science, Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki, Finland; VTT Science 43, 2013, ISBN 978-951-38-8100-9 (URL: http://www.vtt.fi/publications/index.jsp) page 47, paragraph 1; page 63, paragraph 4	3

☒ Further documents are listed in the continuation of Box C.
☒ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

18 September 2016 (18.09.2016)

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INTERNATIONAL SEARCH REPORT

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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A	EP 2233562 A1 (METABOLIC EXPLORER SA [FR]) 29 September 2010 (29.09.2010) paragraph [0046]; claims	1-16
A	Zahoor, A. et al. Metabolic engineering of Corynebacterium glutamicum for glycolate production. Journal of Biotechnology, December 2014, Vol. 192, Part B, pages 366-375 abstract; page 367, figure 1	1-16
A	Kunze et al. A central role for the peroxisomal membrane in glyoxylate cycle function. Biochimica et Biophysica Acta, December 2006. Vol. 1763, No 12, p. 1441-1452 the whole document	1-16
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P, A	FR 3028529 A1 (INST NAT DE LA RECH AGRONOMIQUE - INRA [FR]) 20 May 2016 (20.05.2016) page 5, line 11 – page 6, line 15; page 16, lines 19-20; page 34, lines 7-35; claims 11-15	1-16

INTERNATIONAL SEARCH REPORT
Information on Patent Family Members

International application No.
PCT/FI2016/050373

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.....			

International application No.
PCT/FI2016/050373

Form PCT/ISA/210 (patent family annex) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI2016/050373

CLASSIFICATION OF SUBJECT MATTER

IPC

C12P 7/42 (2006.01)

C12N 9/04 (2006.01)

C12N 15/52 (2006.01)

C12N 15/80 (2006.01)

C12N 15/81 (2006.01)

C12N 1/14 (2006.01)