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(54) Title: PRODUCTION OF 2-KETO-3-DEOXY-D-GLUCONIC ACID IN FILAMENTOUS FUNGI

(57) **Abstract:** A recombinant filamentous fungi that includes reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity as compared to the filamentous fungi not transformed to have reduced KDG aldolase enzyme activity is provided. Also provided is a method of producing KDG

PRODUCTION OF 2-KETO-3-DEOXY-D-GLUCONIC ACID IN FILAMENTOUS FUNGI

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority under 35 U.S.C. §119(e) of U.S. Serial No. 62/672,719, filed May 17, 2018, the entire contents of which is incorporated herein by reference in its entirety.

INCORPORATION OF SEQUENCE LISTING

[0002] The material in the accompanying sequence listing is hereby incorporated by reference into this application. The accompanying sequence listing text file, name BP17000_1WO_Sequence_Listing, was created on May 14, 2019, and is 18 kb. The file can be accessed using Microsoft Word on a computer that uses Windows OS.

FIELD OF THE INVENTION

[0003] The present invention relates generally to transformed filamentous fungi and more specifically to methods for producing 2-Keto-3-Deoxy-Gluconate (KDG).

BACKGROUND OF THE INVENTION

[0004] The use of keto sugar intermediates to produce furan derivatives such as Furan Dicarboxylic Acid (FDCA), offers an important alternative to the use of 5-hydroxymethylfurfural (HMF) that is obtained from fructose. One of the advantages of certain sugar keto-intermediates is that they have a higher propensity than fructose to acquire a furanose conformation, which is advantageous for further catalytic steps that lead to FDCA or other furan-derivatives. The percentage of furanose conformation that several keto sugar-derivatives have in solution has been previously described (WO 2016/141148). Among them, 2-Keto-3-Deoxy-D-Gluconic acid (KDG) is an interesting intermediate because it forms a furanose ring more efficiently than fructose; and because it can be produced from gluconate by a single enzymatic step using a gluconate dehydratase (Matsubara et al. (2014)). Furthermore, as shown in Figure 1 and Figure 2, KDG is a normal intermediate during gluconate catabolism in microorganisms that use the non-phosphorylative Entner-Doudoroff pathway (Conway, T. (1992)); from hexuronates catabolism (Suvorova et al. (2011)); or from degradation of pectin and alginates (Hobbs JK. (2016)).

SUMMARY OF THE INVENTION

[0005] The present invention is based on the discovery that filamentous fungi can be used for producing 2-Keto-3-Deoxy-D-Gluconic acid (KDG) and other sugar acid derivatives via fermentation processes.

[0006] Accordingly, the present disclosure provides a genetically modified filamentous fungi that includes reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity as compared to the wild type filamentous fungi.

[0007] In one embodiment, the filamentous fungi is an *Aspergillus* strain.

[0008] In another embodiment, the filamentous fungi is an *Aspergillus niger* strain.

[0009] In a further embodiment, the *Aspergillus niger* strain is NRRL 322, NRRL 328, NRRL 566, NRRL 599 or NRRL 2270.

[0010] In one embodiment, the genetic modification results in reduced endogenous KDG aldolase enzyme activity.

[0011] In an additional, the genetic modification is a mutation.

[0012] In certain embodiments, the mutation is a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and/or a deletion mutation.

[0013] In a further embodiment, the mutation is in the promoter region, the 3' untranslated region, the 5' untranslated region and/or a regulatory sequence of a 2-Keto-3-Deoxy-Gluconate (KDG) aldolase gene.

[0014] In one embodiment, the mutation is in a nucleic acid sequence encoding a polypeptide having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

[0015] In an additional embodiment, the mutation is a deletion of a gene having the nucleic acid sequence of SEQ ID NO:2.

[0016] In a further embodiment, the mutation is in a nucleic acid sequence encoding a KDG aldolase enzyme comprising the amino acid sequence of SEQ ID NO:1.

[0017] In another embodiment, the mutation inhibits the expression of the polypeptide comprising SEQ ID NO:1.

[0018] In a further embodiment, the enzyme having reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity has the classification of EC4.1.2.51.

[0019] The disclosure also provides methods of producing 2-Keto-3-Deoxy-Gluconate (KDG). The methods include transforming a filamentous fungi with a nucleic acid sequence; and culturing the transformed filamentous fungi in a culture medium, wherein the level of KDG produced in the transformed filamentous fungi is higher than the level of KDG accumulated in the filamentous fungi not transformed with the nucleic acid sequence, thereby producing KDG.

[0020] In one embodiment, the nucleic acid sequence inactivates a gene encoding a KDG aldolase enzyme, reduces expression of a gene encoding a KDG aldolase enzyme or inhibits expression of a gene encoding a KDG aldolase enzyme. In one embodiment, the nucleic acid sequence integrates into the filamentous fungi genome, and or the nucleic acid sequence introduces a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and a deletion mutation.

[0021] In another, embodiment, the filamentous fungi is an *Aspergillus* strain.

[0022] In an additional embodiment, the filamentous fungi is an *Aspergillus niger* strain. In another embodiment, the nucleic acid sequence of the gene encoding the KDG aldolase enzyme comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:2.

[0023] In a further embodiment, the amino acid sequence of the KDG aldolase enzyme comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1. In another embodiment, nucleic acid sequence comprises SEQ ID NO:8.

[0024] In a further embodiment, the *Aspergillus niger* strain is NRRL 322, NRRL 328, NRRL 566, NRRL 599 or NRRL 2270.

[0025] In an additional embodiment, KDG is converted to 5-hydroxymethylfuranoic acid (HMFA) and/or Dicarboxylic acid (FDCA).

[0026] The disclosure additionally provides a DNA construct that includes SEQ ID NO:8.

[0027] In one embodiment, the DNA construct comprises a heterologous nucleic acid sequence.

[0028] The disclosure further provides an expression vector for expressing 2-Keto-3-Deoxy-Gluconate (KDG) aldolase, wherein the expression vector includes a DNA sequence encoding a polypeptide having the amino acid sequence comprising SEQ ID NO:1.

[0029] In one embodiment, the expression vector expresses a polypeptide comprising SEQ ID NO:1.

[0030] The disclosure also provides an expression vector for expressing 2-Keto-3-Deoxy-Gluconate (KDG) aldolase, wherein the expression vector includes a DNA sequence substantially similar to SEQ ID NO:2.

[0031] The disclosure additionally provides a method of decreasing D-glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in a filamentous fungi. The method includes transforming a filamentous fungi with a nucleic acid sequence; and culturing the transformed filamentous fungi in a culture medium, wherein the level of D-glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate, in the transformed filamentous fungi, is decreased from the level of glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in a filamentous fungi not transformed with the nucleic acid sequence, thereby decreasing D-glyceraldehyde from2-Keto-3-Deoxy-Gluconate.

[0032] In one embodiment, the nucleic acid sequence inactivates a gene encoding a KDG aldolase enzyme, reduces expression of a gene encoding a KDG aldolase enzyme or inhibits expression of a gene encoding a KDG aldolase enzyme. In one embodiment, the nucleic acid sequence integrates into the filamentous fungi genome, and or the nucleic acid sequence introduces a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and a deletion mutation.

[0033] In an additional embodiment, the filamentous fungi is an *Aspergillus* strain.

[0034] In another embodiment, the filamentous fungi is an *Aspergillus niger* strain.

[0035] In a further embodiment, the *Aspergillus niger* strain NRRL 322, NRRL 328, NRRL 566, NRRL 599 or NRRL 2270.

[0036] In certain embodiments, the nucleic acid sequence of the gene encoding the KDG aldolase enzyme comprises SEQ ID NO:2.

[0037] In another embodiment, the amino acid sequence of the KDG aldolase enzyme comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

[0038] Other aspects and advantages of the invention will be apparent from the following description and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] **Figure 1** shows a summary of the metabolic pathways that some microorganisms utilize to metabolize different sugars, and use KDG as an intermediate.

[0040] **Figure 2** shows a summary of the metabolic pathways and enzymes that some microorganisms utilize to metabolize Glucose and Gluconate, and use KDG as an intermediate.

[0041] **Figure 3** shows Glucose and Gluconate assimilation in *Aspergillus niger* based on analysis of its genome (Flipphi et al. 2009) and the KDG pathway proposed by Elzainy et al. 1973. As reported for other microorganism (van Dijken et al. 2002), another possible pathway where γ-gluconolactone is transported into the cell first, and then converted into gluconate, is indicated by dotted arrows.

[0042] **Figures 4 A-B** show **(A)** Proposed D-Galacturonate degradation pathway in filamentous fungi (Martens-Uzunova, E. and Shcaap, PJ. 2008) and **(B)** Differences between 2-keto-3-deoxy-D-gluconate and 2-keto-3-deoxy-L-galactonate. The position of hydroxyl-group different on both molecules is indicated by the dotted box.

DETAILED DESCRIPTION OF THE INVENTION

[0043] The present invention is based on the seminal discovery that filamentous fungi can be used for producing 2-Keto-3-Deoxy-D-Gluconic acid (KDG) and other sugar acid derivatives via fermentation processes.

[0044] Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular methods, compositions, and experimental conditions described, as such methods, compositions, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0045] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. The definitions set forth below are for understanding of the disclosure but shall in no way be considered to supplant the understanding of the terms held by those of ordinary skill in the art.

[0046] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" include one or more procedures/methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0047] With the goal of producing KDG and other sugar acids derivatives via fermentation processes, filamentous fungi like *Aspergillus* and *Trichoderma* strains are attractive production hosts, due to their wide use at the commercial level, for the production of enzymes and chemical compounds like gluconic, citric and itaconic acids. In this sense, gluconic acid-overproducing strains from *Aspergillus niger* were envisioned to be a good starting point to develop KDG and KDG-derivatives commercial processes, due to the fact that gluconate can be easily converted into KDG via gluconate-dehydratase enzymes. Furthermore, *Aspergillus. niger* may already be able to produce KDG as a normal intermediate during gluconate metabolism. However, there are conflicting reports on how *Aspergillus. niger* metabolizes gluconate: Müller, HM. (1984 and 1985) reported that certain *Aspergillus. niger* strains use the traditional pentose pathway by first phosphorylating gluconate to form gluconate-6-phosphate. In contrast, Elzainy et al. (1973) and Allam et al. (1975) have shown that other strains of *Aspergillus niger* catabolize D-gluconate by dehydrating it first to form KDG, which is subsequently cleaved by a KDG-aldolase, producing D-glyceraldehyde and pyruvate. These two products are further metabolized via the glycolytic pathway. More recently, the genome analysis of *Aspergillus niger* and other *Aspergillus* species, did not reveal any evidence for the presence of a KDG-forming pathway (Pel et al. 2007; Flipphi et al. 2009). Furthermore, these authors proposed several putative gluconokinases that could be responsible for converting gluconate into gluconate-6-phosphate for its assimilation via the normal pentose pathway.

[0048] Accordingly, the disclosure provides a genetically modified filamentous fungi that includes reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity as compared to the wild type filamentous fungi.

[0049] Filamentous fungi are eukaryotic organisms that include *Aspergillus* and, specifically, *Aspergillus niger*. Specific examples of *Aspergillus niger* strains include NRRL 322, NRRL 328, NRRL 566, NRRL 599 or NRRL 2270.

[0050] The filamentous fungi of the present invention are genetically modified to reduce KDG- aldolase enzyme activity. One method of genetically modifying the fungi is by mutation wherein the organisms genome is altered such that KDG- aldolase enzyme activity is reduced. Mutations include a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and/or a deletion mutation.

[0051] Mutations occur anywhere along the genome. Specifically, mutations may be in the promoter region, the 3' untranslated region, the 5' untranslated region and/or a regulatory sequence of a gene. A regulatory sequence is a segment of a nucleic acid molecule which is capable of increasing or decreasing the expression of specific genes within an organism.

[0052] In the present invention, the mutation can be in a nucleic acid sequence encoding a polypeptide having at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

[0053] Additionally, the mutation can be a deletion of a gene having the nucleic acid sequence of SEQ ID NO:2. The mutation can also be in a nucleic acid sequence encoding a KDG aldolase enzyme comprising the amino acid sequence of SEQ ID NO:1. Additionally, the mutation can reduce or inhibit the expression of the polypeptide comprising SEQ ID NO:1.

[0054] Further, in the present invention, the enzyme having reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity has the classification of EC4.1.2.51.

[0055] The disclosure also provides methods of producing 2-Keto-3-Deoxy-Gluconate (KDG). The method includes transforming a filamentous fungi with a nucleic acid sequence; and culturing the transformed filamentous fungi in a culture medium, wherein the level of KDG produced in the transformed filamentous fungi is higher than the level of KDG accumulated in the filamentous fungi not transformed with the nucleic acid sequence, thereby producing KDG.

[0056] In one embodiment, the nucleic acid sequence inactivates a gene encoding a KDG aldolase enzyme, reduces expression of a gene encoding a KDG aldolase enzyme or inhibits expression of a gene encoding a KDG aldolase enzyme. In one embodiment, the nucleic acid sequence integrates into the filamentous fungi genome, and or the nucleic acid sequence introduces a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and a deletion mutation.

[0057] In one embodiment the insertion and integration of the nucleic acid into the filamentous fungi genome can inactivate a gene encoding a KDG aldolase enzyme or reduce or inhibit the expression of a gene encoding a KDG aldolase enzyme.

[0058] The filamentous fungi may be an *Aspergillus* strain and, specifically, an *Aspergillus niger* strain. Additionally, the *Aspergillus niger* strain can be NRRL 322, NRRL 328, NRRL 566, NRRL 599 or NRRL 2270.

[0059] In the present invention, the nucleic acid sequence of the gene encoding the KDG aldolase enzyme comprises at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:2.

[0060] Additionally, the amino acid sequence of the KDG aldolase enzyme comprises at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

[0061] In the present invention, the nucleic acid sequence that integrates into the filamentous fungi genome comprises SEQ ID NO:8.

[0062] The KDG that is produce can be further modified and converted into other furan derivatives. The KDG can be converted to 5-hydroxymethylfuranoic acid (HMFA) and/or Dicarboxylic acid (FDCA) through chemical methods known in the art.

[0063] The disclosure additionally provides a DNA construct that includes SEQ ID NO:8. In one embodiment, the DNA construct comprises a heterologous nucleic acid sequence. A heterologous nucleic acid sequence is a nucleic acid sequence derived from another organism or cell type.

[0064] The disclosure provides an expression vector for expressing 2-Keto-3-Deoxy-Gluconate (KDG) aldolase, wherein the expression vector includes a DNA sequence encoding a polypeptide having the amino acid sequence comprising SEQ ID NO:1. The expression vector can express a polypeptide comprising SEQ ID NO:1. The disclosure also provides an expression vector for expressing 2-Keto-3-Deoxy-Gluconate (KDG) aldolase, wherein the expression vector includes a DNA sequence substantially similar to SEQ ID NO:2

[0065] The invention provides methods of amplifying a nucleic acid encoding a polypeptide comprising amplification of a template nucleic acid with an amplification primer sequence pair capable of amplifying a nucleic acid sequence of the invention, or fragments or subsequences thereof.

[0066] The invention provides expression cassettes comprising a nucleic acid of the invention or a subsequence thereof. In one aspect, the expression cassette can comprise the nucleic acid that is operably linked to a promoter. Optionally, the promoter can be a fungal, yeast, viral, bacterial, mammalian, plant, synthetic or hybrid promoter. The promoter can be a constitutive promoter. In another aspect, the promoter can be an inducible promoter. In one aspect, the promoter can be a tissue-specific promoter or an environmentally regulated or a developmentally regulated promoter. In one aspect, the expression cassette can further comprise a plant or plant virus expression vector.

[0067] The invention provides cloning vehicles comprising an expression cassette (e.g., a vector) of the invention or a nucleic acid of the invention. The cloning vehicle can be a viral vector, a plasmid, a phage, a phagemid, a cosmid, a fosmid, a bacteriophage or an artificial chromosome. The viral vector can comprise an adenovirus vector, a retroviral vector or an adeno-associated viral vector. The cloning vehicle can comprise a bacterial artificial chromosome (BAC), a plasmid, a bacteriophage P1-derived vector (PAC), a yeast artificial chromosome (YAC), or a mammalian artificial chromosome (MAC).

[0068] The invention provides transformed cells comprising a nucleic acid of the invention or an expression cassette (e.g., a vector) of the invention, or a cloning vehicle of the invention. In one aspect, the transformed cell can be a bacterial cell, a mammalian cell, a fungal cell, a yeast cell, an insect cell or a plant cell.

[0069] The invention provides methods of producing a recombinant polypeptide comprising the steps of: (a) providing a nucleic acid of the invention operably linked to a promoter; and (b) expressing the nucleic acid of step (a) under conditions that allow expression of the polypeptide, thereby producing a recombinant polypeptide. In one aspect, the method can further comprise transforming a host cell with the nucleic acid of step (a) followed by expressing the nucleic acid of step (a), thereby producing a recombinant polypeptide in a transformed cell.

[0070] The invention provides methods of editing of an organisms genome. One such method is the use of CRISPR. CRISPR-Cas9 is a unique technology that enables the editing parts of the genome by removing, adding or altering sections of the DNA sequence. Specifically, delivering the Cas9 nuclease complexed with a synthetic guide RNA (gRNA) into a cell enables the cell's genome can be cut at a desired location, allowing existing genes to be removed and/or new ones added.

[0071] The invention provides methods for modifying codons in a nucleic acid encoding a polypeptide; the method comprising the following steps: (a) providing a nucleic acid of the invention; and, (b) identifying a codon in the nucleic acid of step (a) and replacing it with a different codon encoding the same amino acid as the replaced codon, thereby modifying codons in a nucleic acid.

[0072] The invention provides methods of generating variants of the nucleic acids of the invention. These methods can be repeated or used in various combinations to generate polypeptides having an altered or different activity encoded by the template or wild type nucleic acid. These methods also can be repeated or used in various combinations, e.g., to generate variations in gene/message expression, message translation or message stability. In another aspect, the genetic composition of a cell is altered by, e.g., modification of a homologous gene ex vivo, followed by its reinsertion into the cell.

[0073] In one aspect, the term “variant” refers to polynucleotides or polypeptides of the invention modified at one or more base pairs, codons, introns, exons, or amino acid residues (respectively) yet still retain the biological activity of a KDG of the invention. Variants can be produced by any number of means included methods such as, for example, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, Gene Site Saturation Mutagenesis (GSSM), synthetic ligation reassembly (SLR), and any combination thereof.

[0074] A nucleic acid of the invention can be altered by any means. For example, random or stochastic methods, or, non-stochastic, or "directed evolution," methods, see, e.g., U.S. Pat. No. 6,361,974. Methods for random mutation of genes are well known in the art, see, e.g., U.S. Pat. No. 5,830,696. For example, mutagens can be used to randomly mutate a gene. Mutagens include, e.g., ultraviolet light or gamma irradiation, or a chemical mutagen, e.g., mitomycin, nitrous acid, photoactivated psoralens, alone or in combination, to induce DNA breaks amenable to repair by recombination. Other chemical mutagens include, for example, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid. Other mutagens are analogues of nucleotide precursors, e.g., nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. These agents can be added to a PCR reaction in place of the nucleotide precursor thereby mutating the sequence. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used.

[0075] Any technique in molecular biology can be used, e.g., random PCR mutagenesis, see, e.g., Rice (1992) Proc. Natl. Acad. Sci. USA 89:5467-5471; or, combinatorial multiple cassette mutagenesis, see, e.g., Crameri (1995) Biotechniques 18:194-196. Alternatively, nucleic acids, e.g., genes, can be reassembled after random, or "stochastic," fragmentation, see, e.g., U.S. Pat. Nos. 6,291,242; 6,287,862; 6,287,861; 5,955,358; 5,830,721; 5,824,514; 5,811,238; 5,605,793. In alternative aspects, modifications, additions or deletions are introduced by error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, Gene Site Saturation Mutagenesis (GSSM), synthetic ligation reassembly (SLR), recombination, recursive sequence recombination, phosphothioate-modified DNA mutagenesis, uracil-containing template mutagenesis, gapped duplex mutagenesis, point mismatch repair mutagenesis, repair-deficient host strain mutagenesis, chemical mutagenesis, radiogenic mutagenesis, deletion mutagenesis, restriction-selection mutagenesis, restriction-purification mutagenesis, artificial gene synthesis, ensemble mutagenesis, chimeric nucleic acid multimer creation, and/or a combination of these and other methods.

[0076] The invention also provides methods for modifying the polypeptides of the invention by either natural processes, such as post-translational processing (e.g., phosphorylation, acylation, etc.), or by chemical modification techniques, and the resulting modified polypeptides. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphatidylinositol, cross-linking cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristolyation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. See, e.g., Creighton, T. E., Proteins—Structure and Molecular Properties 2nd ed., W.H. Freeman and Company, New York (1993); Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983).“Fragments” as used herein are a portion of a naturally occurring protein which can exist in at least two different conformations. Fragments can have the same or substantially the same amino acid sequence as the naturally occurring protein. “Substantially the same” means that an amino acid sequence is largely, but not entirely, the same, but retains at least one functional activity of the sequence to which it is related, e.g., only has conservative amino acids substitutions. Fragments which have different three dimensional structures as the naturally occurring protein are also included. An example of this is a “pro-form” molecule, such as a low activity proprotein, that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

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[0078] The term "substantially similar" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nucleic acid or the amino acid sequence. With particular reference to nucleic acid sequences, the term "substantially similar" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially similar" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

[0079] The term “codon optimized” refers to a nucleic acid coding region that has been adapted for expression in the cells of a given host by replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that host.

[0080] The disclosure additionally provides a method of decreasing D-glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in a filamentous fungi. The method includes transforming a filamentous fungi with a nucleic acid sequence; and culturing the transformed filamentous fungi in a culture medium, wherein the level of D-glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate, in the transformed filamentous fungi, is decreased from the level of glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in a filamentous fungi not transformed with the nucleic acid sequence, thereby decreasing D-glyceraldehyde from 2-Keto-3-Deoxy-Gluconate.

[0081] In one embodiment, the nucleic acid sequence inactivates a gene encoding a KDG aldolase enzyme, reduces expression of a gene encoding a KDG aldolase enzyme or inhibits expression of a gene encoding a KDG aldolase enzyme. In one embodiment, the nucleic acid sequence integrates into the filamentous fungi genome, and or the nucleic acid sequence introduces a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and a deletion mutation.

[0082] The insertion and integration of the nucleic acid into the filamentous fungi genome can inactivate a gene encoding a KDG aldolase enzyme or reduce or inhibit the expression of a gene encoding a KDG aldolase enzyme.

[0083] The filamentous fungi may be an *Aspergillus* strain and, specifically, an *Aspergillus niger* strain. Additionally, the *Aspergillus niger* strain can be NRRL 322, NRRL 328, NRRL 566, NRRL 599 or NRRL 2270.

[0084] The present invention provides, the nucleic acid sequence of the gene encoding the KDG aldolase enzyme comprises SEQ ID NO:2.

[0085] Additionally, the amino acid sequence of the KDG aldolase enzyme comprises at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

[0086] The invention provides an isolated, synthetic or recombinant nucleic acid comprising a nucleic acid sequence having at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to invention sequences. For example, such sequences include SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:8. The invention further includes variants/modifications of the foregoing sequences.

[0087] Methods disclosed in the present invention and/or methods known to one of ordinary skill in the art may be used to isolate nucleic acids having a sequence with at least 99%, at least 98%, at least 97%, at least 95%, at least 90%, at least 80% or at least 70% homology to a nucleic acid sequence as set forth in SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:8, sequences substantially identical thereto, or fragments comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900 or more consecutive bases thereof, and the sequences complementary to any of the foregoing sequences. Homology may be measured using an alignment algorithm. For example, the homologous polynucleotides may have a coding sequence which is a naturally occurring allelic variant of one of the coding sequences described herein. Such allelic variants may have a substitution, deletion or addition of one or more nucleotides when compared to a nucleic acid sequence as set forth in SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:8, or sequences complementary thereto.

[0088] Another aspect of the invention comprises polypeptides having at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to invention sequences, e.g., SEQ ID NO:1. The invention further includes variants/modifications of the foregoing sequence discussed below.

[0089] Additionally, the methods disclosed in the present invention may be used to isolate nucleic acids which encode polypeptides having at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, or at least 70% homology to a polypeptide having a sequence as set forth in SEQ ID NO:1, sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 250, 300 or more consecutive amino acids thereof as determined using a sequence alignment algorithm (e.g., such as the FASTA version 3.0t78 algorithm with the default parameters).

[0090] Another aspect of the invention is an isolated or purified polypeptide comprising a sequence as set forth in SEQ ID NO:1, sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 250, 300 or more consecutive amino acids thereof. As disclosed in the present invention and/or known to one of ordinary skill in the art, such polypeptides may be obtained by inserting a nucleic acid encoding the polypeptide into a vector such that the coding sequence is operably linked to a sequence capable of driving the expression of the encoded polypeptide in a suitable host cell. For example, the expression vector may comprise a promoter, a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

[0091] As discussed below, significant KDG aldolase enzymatic activity was measured in cell extracts from 5 different *Aspergillus niger* strains. Furthermore, after purifying this putative KDG-aldolase, the structural gene for such an enzyme was identified and inactivated, and it was demonstrated that an *Aspergillus niger* strain carrying this KDG-aldolase deletion was able to accumulate KDG. This indicates the importance of eliminating or attenuating the KDG-aldolase activity for KDG overproduction.

[0092] In sum, KDG aldolase activity was measured in 5 different *Aspergillus niger* strains.

[0093] An enzyme with KDG aldolase activity was purified from one of the strains; and based on LC-MS/MS based proteomic, protein G3Y6P2 from *Aspergillus niger* ATCC 1015 was identified (SEQ ID NO:1). This protein sequence was annotated as EHA21505 in GenBank, and is coded by ASPNIDRAFT_214527 (or Aspni5_214527). The protein sequence showed that it was a previously unknown enzyme, with unknown function, annotated in GenBank as Hypothetical protein or Dihydrodipicolinate synthase family protein. Compared with the 2-keto-3-deoxy-L-galactonate aldolase from *Aspergillus niger,* EHA21505 has 43% identity. As shown on Table 5, Blast searches indicated that this enzyme is well conserved in other *Aspergillus niger* close relatives.

[0094] Based on gene EHA21505 DNA sequence (SEQ ID: 2), a deletion cassette was constructed where the EHA21505 coding sequence was replaced by the Hygromycin B antibiotic resistance marker (SEQ ID: 8). This cassette was used to delete the gene in a lab strain described below.

[0095] Compared with the wild type strain, the *Aspergillus niger* strain containing the deletion of EHA21505 showed 7x less KDG-aldolase activity.

[0096] On solid media containing gluconate as the only carbon source, the KDG-aldolase deleted strain showed impaired growth.

[0097] In liquid cultures with gluconate as the only carbon source, the deleted strain accumulated a significant amount of KDG, while the wild type strain under the same conditions, did not accumulated any KDG.

[0098] An *E.coli* codon-optimized gene was synthesized and cloned in an expression vector. Cell-extracts from an *E.coli* strain carrying this plasmid showed KDG-aldolase activity.

[0099] In addition to the possible use of KDG to produce FDCA and other furan-derivatives, KDG could also be used in a similar application to gluconate and other sugar acids, for example: (1) as a chelating agent for detergents and beverages; (2) for anti-scaling applications; (3) as a de-icer; (4) as a corrosion-inhibitor; (5) as an additive to enhance metal and amine removal in refinery desalting processes, etc. Furthermore, because KDG and its dehydration derivatives will have at least 2 carbonyl groups, it may have better properties than gluconate and other sugar acids for current and future applications.

[00100] The following examples are provided to further illustrate the advantages and features of the present invention, but are not intended to limit the scope of the invention. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those of skill in the art may alternatively be used.

EXAMPLES

EXAMPLE 1

Growth of *Aspergillus niger* strains and preparation of cell-extracts

[00101] *Aspergillus niger* strains NRRL 322, NRRL 328, NRRL 566, NRRL 599, and NRRL 2270 (NRRL Culture Collection) were sporulated on Potato Dextrose Agarose plates and then spores from each strain were inoculated into 50mL of potato dextrose broth in a 250mL flask. The flasks were incubated in a shaker incubator at 30°C at 150rpm and grown for up to 72 hours. Mycelia were harvested by centrifugation, then resuspended in ~40 mL of Czapek Dox broth with sucrose excluded and 5% sodium gluconate as a the sole carbon source. The flasks were returned to the shaker incubator at 30°C and 150rpm and incubated for up to 48 hours. Cells were then lysed via sonication to generate cell lysates. Lysates were buffer exchanged into 200mM sodium phosphate buffer pH 8.0 using gel filtration on PD-10 columns to reduce background absorbance.

EXAMPLE 2

KDG Aldolase Assay of *Aspergillus niger* lysates

[00102] Lysates were then tested using a lactate dehydrogenase coupled reaction to monitor NADH loss spectrophotometrically at 340nm. The assay mixture was prepared with 0.2 mM NADH, 4U/mL lactate dehydrogenase, 10 mM Na Phosphate pH 7.0, and an appropriate volume of lysate. The mixture was warmed to 30°C and the reaction was initiated by addition of KDG-Li salt (Sigma p/n 12271) at 10 mM. Reactions were monitored in a cuvette and a reading was taken every 30 seconds for 2 minutes. Strain NRRL 328 had the highest KDG aldolase activity (See Table 1) and was selected for further growth and purification.

Table 1. KDG Aldolase Activity of *Aspergillus niger* lysates

	Decrease in A340nm per minute
No KDG	0.00562
No NADH	0.00058
No Enzyme	0.00088
Strain NRRL 322	0.01634
Strain NRRL 328	0.02392
Strain NRRL 566	0.01086
Strain NRRL 599	0.00696
Strain NRRL 2270	0.0197

EXAMPLE 3

Purification of an *Aspergillus niger* KDG aldolase

[00103] *Aspergillus niger* strain 328 was inoculated into 200mL PD broth in 4 x 1L flasks and grown at 30°C and 240rpm for 46 hours. Mycelia were harvested by centrifugation and then resuspended in 200mL Czapek Dox broth with 5% Na gluconate as the sole carbon source and returned to the incubator for 44 hours. Mycelia were harvested and sonicated to prepare a lysate. The sample was heat treated for 15 minutes at 60°C and the precipitated protein was removed by centrifugation at 10,000 g for 15 minutes at 4 °C. A volume of 200mL supernatant was mixed with an equal volume of cold acetone (-15°C) and mixed gently for 10 minutes at 4 °C. The precipitate was removed via centrifugation and discarded and another 200mL of cold acetone was added to form a second precipitate.

[00104] This second precipitate was dried, then resuspended in buffer and loaded onto two 5mL DEAE fast flow columns setup in tandem. The mobile phase was 10 mM Potassium Phosphate pH 7.7 and proteins were eluted with a gradient from 0 to 0.5M NaCl over 40 column volumes. The OD was monitored at 280nm and fractions were collected and assayed for KDG dehydratase activity as described previously. The most active fractions were then pooled for proteomic analysis.

EXAMPLE 4

Identification of a KDG aldolase via LC-MS/MS Proteomics

[00105] Samples from the original lysate, the 2nd acetone precipitate, and the most active fractions from the DEAE column were analysed for protein content via trypsin digest and LC-MS/MS. The sample containing the most active fractions contained an enriched abundance of peptides for a protein annotated in the *Aspergillus niger* genome database, as an uncharacterized protein with accession number G3Y6P2 (SEQ ID NO:1). Upon further investigation, another annotation for same sequence was dihydropicolinate synthase in PFAM, a class of enzymes that catalyzes the condensation of L-aspartate-4-semialdehyde and pyruvate to 4-hydroxy-tetrahydropicolinic acid. It was noted that the reverse of this reaction is quite similar to the activity that had been observed where cleavage of KDG resulted in production of pyruvate. Upon further investigation, protein G3Y6P2, had ~76% sequence identity with a putative 4-hydroxy-2-oxoglutarate aldolase from *Aspergillus udagawae*. To further verify that this was the protein sequence associated with the KDG aldolase activity, the protein was re-purified using a similar protocol where the acetone precipitation was excluded to improve the purification yield and a higher resolution MonoQ column was used instead of a DEAE column. The most active fractions were analyzed on an SDS-PAGE gel and the prominent band in the active fractions was excised. A trypsin digest and LC-MS/MS confirmed that the prominent band was in fact G3Y6P2.

[00106] **SEQ ID NO:1**

>tr|G3Y6P2|G3Y6P2_ASPNA Uncharacterized protein OS=Aspergillus niger (strain ATCC 1015 / CBS 113.46 / FGSC A1144 / LSHB Ac4 / NCTC 3858a / NRRL 328 / USDA 3528.7) GN=ASPNIDRAFT_214527 PE=3 SV=1

MAITPRPLKPGIYVPTVAFFTSPNEDLDLSTTALHATYLAQAGVTGLVVQGSNGEAV
HLSREERNLITSTTRRALDSHAPSAPLIVGCGAASTRETIQLCQDAAASGGDYTLILP
PSYYKSLLSPKDLLDHFRLVASASPIPILVYNFPGASSGLDLSSDDILALAEHPNIVGV
KLTCGNTGKLARIAAAAEPGFMTFGGSADFTLQTLVAGGHGVIGGVANLIPRLSVRV
MELYQAGQVEEAQRLQAIVARADWQAIKGGFVAVKSALQTYRGYGALPRRPCVVP
SEAQATAWKDSFAEAMELERQLEKQA

[00107] **SEQ ID NO:2**

[00108] ATGGCCATTACACCCCGCCCCCTCAAACCCGGCATCTACGTCCCA
ACCGTCGCCTTCTTCACCTCTCCCAACGAAGACCTCGACCTCTCCACCACCGC
CCTCCACGCCACCTACCTCGCTCAAGCCGGCGTCACCGGTCTAGTCGTGCAAG
GCAGCAACGGTGAAGCCGTCCACCTCAGCCGCGAAGAGCGCAACCTCATCACT
TCCACCACCCGTCGCGCTCTCGACTCTCACGCCCCCTCCGCCCCGCTCATCGT

CGGCTGCGGCGCCGCCTCCACCCGCGAGACCATCCAGCTGTGCCAAGACGCC
GCCGCCTCCGGAGGCGACTATACCCTGATCCTCCCTCCCTCCTACTACAAATC
CCTCCTCTCTCCCAAGGACCTTCTTGATCACTTCCGCCTCGTCGCCTCCGCCTC
CCCCATCCCCATCCTGGTGTACAACTTCCCCGGCGCCTCTTCGGGCCTGGACC
TCTCCTCCGACGACATCCTCGCCTTGGCGGAGCACCCCAACATCGTCGGCGTG
AAGCTGACCTGTGGAAACACGGGTAAACTGGCGCGCATTGCCGCCGCCGCCG
AACCCGGTTTCATGACCTTTGGTGGTTCCGCTGATTTCACTCTCCAGACGCTGG
TGGCAGGCGGTCATGGAGTGATTGGCGGCGTGGCGAACCTGATCCCTCGTTTG
AGTGTGCGCGTGATGGAGCTGTATCAGGCGGGACAGGTCGAAGAGGCCCAGC
GGTTGCAGGCCATTGTAGCGCGTGCGGACTGGCAGGCTATCAAGGGTGGTTTT
GTAGCGGTGAAGAGTGCGTTGCAGACGTACCGCGGATACGGAGCATTGCCGA
GACGGCCGTGTGTGGTGCCGTCAGAGGCGCAGGCGACGGCGTGGAAGGATTC
TTTTGCGGAGGCTATGGAGCTGGAGAGACAGTTAGAGAAGCAGGCCTAG

EXAMPLE 5

Expression of putative KDG Aldolase EHA21505 in *E. coli.*

[00109] To ensure the proper expression of the *Aspergillus niger* KDG aldolase in *E.coli*, an *E. coli* codon-optimized gene encoding protein G3YP2, referred to here as EHA21505 or 21505, was obtained (SEQ ID NO: 3) (Genewiz, South Plainfield, NJ).

[00110] **SEQ ID NO:3**

ATGGCAATTACCCCTCGCCCGCTGAAGCCGGGCATTTACGTGCCGACCGTTGC
CTTTTTCACCAGCCCGAATGAGGACCTGGACCTGAGCACCACCGCACTGCATG
CAACCTATCTGGCACAGGCAGGTGTGACCGGCCTGGTTGTGCAGGGTAGCAAT
GGTGAAGCCGTGCATCTGAGCCGTGAGGAGCGTAACCTGATTACAAGCACCAC
CCGCCGTGCACTGGATAGCCATGCCCCGAGTGCCCCGCTGATCGTTGGTTGC
GGTGCAGCAAGCACCCGCGAAACCATTCAGCTGTGTCAAGATGCAGCCGCCAG
TGGCGGCGACTATACTCTGATCCTGCCGCCGAGCTACTACAAAAGCCTGCTGA
GTCCGAAGGATCTGCTGGACCATTTTCGCCTGGTTGCCAGCGCAAGCCCGATT
CCGATTCTGGTGTATAACTTTCCGGGCGCCAGTAGCGGTCTGGACCTGAGTAG
CGATGATATTCTGGCACTGGCAGAGCATCCGAACATTGTGGGCGTGAAACTGA
CCTGCGGTAACACAGGCAAACTGGCACGTATCGCAGCCGCAGCAGAACCGGG
TTTTATGACCTTTGGCGGTAGTGCCGACTTTACCTTACAGACCCTGGTTGCCGG
TGGTCATGGTGTGATTGGCGGCGTGGCAAATCTGATTCCGCGCCTGAGCGTTC
GTGTTATGGAGCTGTACCAGGCAGGTCAGGTGGAAGAAGCCCAGCGTCTGCAG
GCCATTGTGGCACGTGCCGACTGGCAGGCCATTAAAGGCGGTTTTGTGGCCGT
GAAAAGCGCCCTGCAGACCTACCGCGGTTATGGTGCACTGCCGCGTCGTCCGT
GTGTGGTGCCTAGCGAAGCACAGGCCACCGCATGGAAAGATAGCTTTGCCGAG
GCTATGGAACTGGAACGCCAGCTGGAAAAACAAGCCTAA

[00111] This gene was cloned in the NcoI-HindIII sites of expression vector pTrcHis2B (Thermo Fisher Scientific, Waltham, MA). This plasmid was named pTrc-An21505.

[00112] Plasmid pTrc-An21505 was transformed into BL21 *E. coli* competent cells (EMD Millipore; Billerica, MA), following the protocol provided by the vendor. The BL21 strain containing plasmid was named *E.coli* 21505. Vector pTrcHis2 was also transformed into BL21 as a control. To evaluate KDG aldolase activity in *E.coli* the mentioned 2 strains were inoculated into shake flasks containing auto-induction liquid media Studier ZYM-5052 (Teknova; Hollister, CA). Flasks were incubated in a shaking incubator for 24 hours at 30°C and 110 rpm. After cultivation, cultures were centrifuged at 12500g for 20 minutes and the pellets were stored at -80°C overnight. To prepare cell- extracts, pellets were thawed and lysed in the following solution: 7 ml per gram of 10mM sodium phosphate buffer, pH 7.3, mixed with 7.5kU rLysozyme per ml of buffer, 1ul benzonase nuclease (EMD Millipore, P/N 70746) per ml of buffer. The mixtures were incubated in a water bath for 10 minutes at 60°C then centrifuged at 10000g for 20 minutes. The lysates were tested for KDG aldolase activity using the method previously described, with modifications for a plate-based assay. Lysates were buffer exchanged in 10mM sodium phosphate buffer (pH 7.7) using a PD-10 column. The assay reactions were monitored in a 96-well Costar plate and a reading was taken every 30 seconds for 20 minutes. Lysate from *Aspergillus niger* strain NRRL 566 was included in the assay as a positive KDG aldolase activity control. As shown in Table 2, the *E. coli* strain expressing the 21505 gene (BL21 + pTrc-An21505 lysate) had higher activity than the *E. coli* empty vector strain (BL21 + pTrcHis2b) and background controls. The *E. coli* empty vector strain (BL21 + pTrcHis2b) had minimal activity only slightly above the "No KDG" background control. The *Aspergillus niger* NRRL 566 positive control had the highest KDG aldolase activity.

Table 2. KDG Aldolase Activity in *Aspergillus niger* and *E. coli* lysates with, and without the 21505 gene

Description	Decrease in Absorbance at 340 nm per minute
No KDG added	0.00296
No NADH added	0.00071
No cell lysate added	0.00198
A. niger NRRL 566 lysate	0.01440
BL21 + pTrc-An21505 lysate	0.00851
BL21 + pTrcHis2b lysate	0.00323

EXAMPLE 6

Inactivation of EHA21505 gene in *Aspergillus niger*

[00113] Preparation of deletion cassette

[00114] A deletion cassette was constructed so that the DNA can integrate into the genome at the 21505 locus, thus substituting the 21505 gene with the hygromycin B resistance gene. Regions approximately 1000 bp upstream and downstream of 21505 were cloned and ligated to the pBluescriptSK+ plasmid (Agilent; Santa Clara, CA). The region upstream of gene 21505 was cloned using the following primers:

[00115] **SEQ ID NO:4**

5'-CTTGATATCGAATTCGACGAGGTGGGATTATTGCTG-3' and

[00116] **SEQ ID NO: 5**

5'-CATCGTTTGCATCATCAGGGGATGGG-GAGAATGCG-3', which was designed to introduce an EcoRI restriction site. The downstream region of 21505 was cloned using the following primers:

[00117] **SEQ ID NO:6**

5'-CACGGCTCAGACTCTCCCACATCTTCTACATACCCATC-3' and

[00118] **SEQ ID NO:7**

5'-CTAGTGGATCCCCCGGGCGC-CTCATATTCCTCGATGC-3'. This primer also introduced an XmaI restriction site. A gene for hygromycin B resistance, using the *Aspergillus* *niger* ubiquitin promoter and ubiquitin terminator, was placed between the upstream and downstream fragments (Punt, et al. 1987). The deletion cassette comprising of the two DNA fragments and the hygromycin B resistance gene was assembled via overlap PCR using In-Fusion HD Cloning Plus kit (Clontech; Mountain View, CA).

[00119] The DNA sequence of the complete deletion cassette is shown below:

[00120] **SEQ ID NO:8**

CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCA
GCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGA
ATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATT
AAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATG
GCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTA
AAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGA
AAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGC
GCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCG
CCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGC
AACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGC
GAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCC
AGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTA
TAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCT
TGATATCGAATTCGACGAGGTGGGATTATTGCTGGCCAGCGGGCGGCGCGTCG
AGGTCCGCCGGCGCAGGGGATGATGAGACGAGAGTTTGTAGGGCGTGGCAGC
TGAGGAGGACGAGTTCTCCGTCGTGGTGGTGTCCAGCAGATCGGGGGAGCTTT
TGCGACGGCGCGATGACGAGTGAGTCTGGAGACGAGCTCGCTTTGCAGGAGG
CTCGGAGTCCGGCTGGTCGGGGGAATGGCCGTGATGAGGATCTCGTCGCAGG
GAGACACGGACAGGCATCAGGGTGGTGGAGGTCATTCGGGAGGATCGAGGAG
GGGAGAGGAATTGAGACGTGGGGGGAAAGAAAGGTCAGGAGGGATGCGTGCA
CGGCATGTCAGGGCCAGTCGGGGGCAATTCGGAGGATTGAAGTTGGCAGGAG
GTTTTTTGGGCGAAGCTGAAGCACTCGAGAATCGCAGCTGCAGGTCTGGATGT
GTTCCGGATTGGGAGGAGTTGAAAGTTGTATCTTTACGGATACCGCAGCAGGTA
TGTCTCAGTACCTGACAGGACAGGTGTTACCGCCAGGATGTATGATACCTGTCA
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TACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTC
ATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGC
GCACATTTCCCCGAAAAGTGCCAC

[00121] Before transformation into *Aspergillus niger*, the deletion cassette was amplified by PCR with iProof polymerase (Bio-Rad; Hercules, CA).

[00122] *Aspergillus niger* Transformation

[00123] Transformations were performed according to the method of Yelton, et al. (1984) with some modifications. $1x10^6$ NRRL 566 spores/mL were used to inoculate Aspergillus Complete Medium (ACMU2; 1% yeast extract, 1% bacto peptone, 2.5% glucose, 11mM KH2PO4, 7mM KCl, 2mM MgSO4, 5mM uridine, 5mM uracil, 1x Hutner's trace elements) and grown in a shaking incubator for 17 hours at 30°C and 250rpm. Mycelia was collected via miracloth filtration, washed with osmotic medium (OM; 1.2M MgSO4, 10mM sodium phosphate, pH 5.8), and transferred to a 1L flask to be combined with a variation of the enzyme cocktail described by de Bekker, et al. (2010). The cocktail comprised of 0.15 U/mL chitinase from *Streptomyces griseus* (Sigma, P/N C6137), lysing enzymes from *Trichoderma harzianum* (Sigma, P/N L1412), β-glucuronidase from *Helix pomatia* (Sigma P/N G1512), and OM at 8mL per gram of biomass. Protoplasts were generated by placing the culture in a shaking incubator for 2.75 hours at 30°C and 100 rpm. Protoplasts were then combined with an equal volume of ST buffer (0.6M sorbitol, 100mM Tris-HCl, pH7.5). The mixture was centrifuged at 4000g for 15 minutes and the resulting protoplast layer was then transferred to a new bottle. The pellet was suspended in the residual buffer and the procedure was repeated to collect any remaining protoplasts. The protoplasts were then washed twice using cold STC buffer (1M sorbitol, 10mM Tris-HCl, pH 7.5, 10mM $CaCl_2$) and a centrifuge speed of 4100g for 15 minutes. A protoplast suspension was made with 10% total volume of PTC (60% PEG 4000, 50mM $CaCl_2$, 10mM Tris-HCl pH 7.5), 10% total volume of 200mM aurintricarboxylic acid (Gielesen, B. 2013) and 80% total volume protoplast suspension mixed with STC to bring the final concentration to $4x10^7$ protoplasts/mL.

[00124] The PCR-amplified knockout cassette DNA was mixed with the protoplast suspension in a round bottom tube, and incubated on ice for 20 minutes. PTC (60% w/v PEG 4000; 10mM Tris-HCl, pH 7.5; 50mM $CaCl_2$) was slowly added and the tubes were incubated at room temperature for 10 minutes. Cold STC was then added and mixed, followed by a centrifugation at 3220g for 10 minutes at 4°C. The pellet was suspended in residual supernatant and plated onto selection plates (PDA, 1.2M sorbitol, hygromycin 500 µg/ml, 5mM uridine, 5mM uracil). After 5 days incubation at 30°C, hygromycin resistant transformants were transferred to secondary selection plates (PDA, hygromycin 500 µg/ml, 5mM uridine, 5mM uracil) and incubated for 4 days at 30°C.

[00125] Thirty-four fungal colonies from the secondary selection plates were analyzed by PCR for the deletion of the 21505 gene using Phire Plant Direct PCR Master Mix (ThermoFisher, Waltham, MA). Primers targeted to regions upstream and downstream of the 21505 gene, 5'-TCGTTGCTTTCCATCTGTCC-3' (SEQ ID NO:9) and 5'-GTGGGGTGGAGTTTGATTCG-3' (SEQ ID NO:10), respectively, produced a 3.1 kb product when the knockout cassette was present and a 1.2 kb product when the 21505 gene was still present. A second PCR was performed to test for the correct targeted integration location of the knockout cassette. Primers targeted the hygromycin B resistance marker, 5'-CGATCAGAAACTTCTCGACAGAC-3' (SEQ ID NO:11), and the region upstream of the 21505 flanking region, 5'-GATGTGATAGTGGGGGTGGAATC-3' (SEQ ID NO:12), producing a 2.3 kb product. Four colonies showed the insertion of the knockout cassette in the targeted location to confirm the deletion of the 21505 gene and three isolates were selected for further analysis.

EXAMPLE 7

Measurement of KDG-aldolase activity in *Aspergillus niger* knockout strain

[00126] Cell lysates from wild type *Aspergillus niger* strain and three knockout strain isolates were prepared using a bead beater and lysis buffer (Zymo Research, Irvine, CA, P/N D6001), and tested for KDG aldolase activity using the method previously described for the *E. coli* 21505 activity assay described in Example 5. As shown in Table 3, wild type *Aspergillus niger* strain NRRL 566 had visible KDG aldolase activity (See Table 3) while the knockout strains only showed absorbance values similar to the "No KDG" background absorbance.

Table 3. KDG Aldolase Activity of *Aspergillus niger* parent and knockout strains

	Decrease in Absorbance at 340 nm per minute
No KDG	0.00116
No NADH	0.00008
No Enzyme	0.00091
Wild-type Parent Strain	0.01716
Knockout Strain Isolate A	0.00179
Knockout Strain Isolate B	0.00151
Knockout Strain Isolate C	0.00140

EXAMPLE 8

Quantification of KDG

[00127] Samples of culture supernatants were analyzed for Gluconate and KDG content using a Dionex ICS-5000 Ion Chromatography System. The instrument was equipped with a Dionex IonPac AS11-HC column and an AG11 guard column. The following potassium hydroxide gradient generated by the eluent generator at 2 mL/minute was utilized to separate KDG from gluconate for quantitation: 5 mM to 20 mM in 2 minutes, 20 mM to 50 mM in 1 minute, 50 mM for 2 minutes. The column was re-equilibrated to 5 mM for 3 minutes. Suppressed conductivity was used for gluconate and KDG detection utilizing Dionex ASRS-Ultra 4 mm AutoSuppressor at 248 mA in recycle mode. The conductivity cell was set to 35 °C, the detector compartment to 15 °C and the column compartment to 30 °C. Solid standards for gluconate and KDG were dissolved in water to 20 mM and combined to a stock concentration of 10 mM each. The stock standard solution was further diluted with water to yield the following concentrations: 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 mM. Injection volume was 10 µL for all samples and standards. All fermentation samples were diluted 1:50 in water prior to injection. Baseline separated peaks for gluconate and KDG eluted from the column at 2.28 minutes and 2.52 minutes respectively. Peak integration was achieved by applying software specific algorithm. Sample concentrations were determined based on standard curve fit.

EXAMPLE 9

Production of KDG by *Aspergillus niger* strains

[00128] The three isolates containing the 21505 knockout were grown in gluconate-based media and evaluated along with the parent strain to determine if KDG was accumulated. For such a purpose, spores from the *Aspergillus niger* parent strain NRRL 566 and three different isolates of the 21505 knockout strain were used to inoculate starter cultures of potato dextrose broth, 5mM uridine, and 5mM uracil in shake flasks. The flasks were grown for 48 hours in a shaking incubator at 30°C and 220rpm. The biomass growth was then transferred to Czapek Dox broth with 3% sodium gluconate as the sole carbon source in shake flasks. The cultures were grown in a shaking incubator for 4 days at 30°C and 220rpm. Culture broths were then collected and centrifuged at 7500g for 20 minutes to remove any residual biomass. Concentrations of Gluconate and KDG in the supernatants were determined as described in Example 8. As shown in Table 4 below, only the 3 knockout strains accumulated KDG in significant amounts, indicating that gene EHA21505 is involved in KDG metabolism in *Aspergillus niger*.

Table 4. KDG Accumulation in cultures of *Aspergillus niger* strains

Sample	KDG (mM)
Growth Media	0
Lysate from Knockout isolate A	11.4
Lysate from Knockout isolate B	10.2
Lysate from Knockout isolate C	10.8
Lysate from A. niger NRRL 566 wild-type	0

Table 5 BLAST Results obtained 07-19-2017 using sequence EHA21505.

Annotation	**Identity**	**Accession**
Hypothetical protein ASPNIDRAFT_214527 [Aspergillus niger ATCC 1015]	100%	EHA21505
Unnamed protein product [Aspergillus niger]	99%	CAL00542
Dihydrodipicolinate synthase-like protein [Aspergillus niger CBS 513.88]	99%	XP_001398069
Hypothetical protein ASPBRDRAFT_195814 [Aspergillus brasiliensis CBS 101740]	98%	OJJ71649.1
Dihydrodipicolinate synthetase family protein [Aspergillus kawachii IFO 4308]	98%	GAA89280
Dihydrodipicolinate synthetase family protein [Aspergillus luchuensis	98%	GAT28880
Hypothetical protein ASPTUDRAFT_56088 [Aspergillus tubingensis CBS 134.48]	98%	OJI84140
hypothetical protein ASPFODRAFT_45842 [Aspergillus luchuensis CBS 106.47]	98%	OJZ86393
dihydrodipicolinate synthetase family protein [Aspergillus niger]	98%	GAQ43461
Hypothetical protein ASPCADRAFT_46975 [Aspergillus carbonarius ITEM 5010]	90%	OOF96363
Hypothetical protein ASPACDRAFT_1889470 [Aspergillus aculeatus ATCC 16872]	81%	XP_020054543
Hypothetical protein ASPACDRAFT_1889470 [Aspergillus aculeatus ATCC 16872	81%	OJJ98203
Hypothetical protein ARAM_004705 [Aspergillus rambellii]	72%	KKK21095
Hypothetical protein ASPWEDRAFT_44050 [Aspergillus wentii DTO 134E9]	72%	OJJ32031

Conserved hypothetical protein [Aspergillus terreus NIH2624]	77%	XP_001210210
Conserved hypothetical protein [Aspergillus terreus NIH2624	77%	EAU38770
Putative Dihydrodipicolinate synthetase family protein [Aspergillus calidoustus]	74%	CEL08355
Hypothetical protein AOCH_003138 [Aspergillus ochraceoroseus]	72%	KKK19949
Dihydrodipicolinate synthetase family protein [Aspergillus clavatus NRRL 1]	76%	XP_001276612
Dihydrodipicolinate synthetase family protein [Aspergillus clavatus NRRL 1]	76%	EAW15186
Hypothetical protein PENSUB_13354 [Penicillium subrubescens]	72%	OKO90637
Probable 4-hydroxy-2-oxoglutarate aldolase, mitochondrial [Aspergillus udagawae]	76%	GAO82082
Putative 4-hydroxy-2-oxoglutarate aldolase, mitochondrial [Aspergillus cristatus]	71%	ODM19761
Hypothetical protein AN1503.2 [Aspergillus nidulans FGSC A4]	74%	XP_659107
Probable 4-hydroxy-2-oxoglutarate aldolase, mitochondrial	74%	Q5BD77
Hypothetical protein AN1503.2 [Aspergillus nidulans FGSC A4]	74%	EAA63816
Dihydrodipicolinate synthase-like protein AN1503 (DHDPS-like protein)[Aspergillus nidulans FGSC A4]	74%	CBF84998
Probable 4-hydroxy-2-oxoglutarate aldolase, mitochondrial [Aspergillus lentulus]	76%	GAQ11949
Dihydrodipicolinate synthetase family protein [Aspergillus ruber CBS 135680]	71%	EYE96024
4-hydroxy-2-oxoglutarate aldolase [Rasamsonia emersonii CBS 393.64]	70%	XP_013330421
4-hydroxy-2-oxoglutarate aldolase [Rasamsonia emersonii CBS 393.64]	70%	KKA23809
Dihydrodipicolinate synthetase family protein [Aspergillus fischeri NRRL 181]	75%	XP_001262053
Dihydrodipicolinate synthetase family protein [Aspergillus fischeri NRRL 181]	75%	EAW20156
Dihydrodipicolinate synthetase family protein [Aspergillus fumigatus Af293]	76%	XP_747309
Dihydrodipicolinate synthetase family protein [Aspergillus fumigatus Af293]	76%	EAL85271

Dihydrodipicolinate synthetase family protein [Aspergillus fumigatus A1163]	76%	EDP48804
Dihydrodipicolinate synthetase [Aspergillus fumigatus var. RP-2014]	76%	KEY78362
Hypothetical protein ASPGLDRAFT_42370 [Aspergillus glaucus CBS 516.65]	70%	OJJ88774
Dihydrodipicolinate synthetase family protein [Aspergillus fumigatus Z5]	75%	KMK56661
Dihydrodipicolinate synthase-like protein [Byssochlamys spectabilis No. 5]	68%	GAD94194
Hypothetical protein ASPVEDRAFT_125872 [Aspergillus versicolor CBS 583.65]	73%	OJI97841
Hypothetical protein ASPSYDRAFT_143055 [Aspergillus sydowii CBS 593.65]	73%	OJJ63118
Dihydrodipicolinate synthetase family protein [Aspergillus parasiticus SU-1]	75%	KJK62017
Dihydrodipicolinate synthase-like protein [Aspergillus oryzae RIB40]	75%	XP_001817680
dihydrodipicolinate synthetase family protein [Aspergillus flavus NRRL3357]	75%	XP_002372806
unnamed protein product [Aspergillus oryzae RIB40]	75%	BAE55678
dihydrodipicolinate synthetase family protein [Aspergillus flavus NRRL3357]	75%	EED57194
dihydrodipicolinate synthase/N-acetylneuraminate lyase [Aspergillus oryzae 3.042]	75%	EIT74147
dihydrodipicolinate [Aspergillus oryzae 100-8]	75%	KDE76740
dihydrodipicolinate synthetase [Aspergillus oryzae]	75%	OOO13100
Hypothetical protein PENDEC_c013G06529 [Penicillium decumbens]	68%	OQD73952
Dihydrodipicolinate synthetase family protein [Aspergillus flavus AF70]	75%	KJJ31403
dihydrodipicolinate synthase-like protein [Aspergillus bombycis]	74%	OGM50862
Dihydrodipicolinate synthase-like protein [Aspergillus nomius NRRL 13137]	75%	XP_015403196
Dihydrodipicolinate synthase-like protein [Aspergillus nomius NRRL 13137]	75%	KNG82273
Aldolase-type TIM barrel [Penicillium griseofulvum]	70%	KXG52230
Putative Dihydrodipicolinate synthetase family protein [Penicillium brasilianum]	69%	CEJ57806

Putative 4-hydroxy-2-oxoglutarate aldolase, mitochondrial [Penicillium brasilianum]	69%	OOQ89866
Aldolase-type TIM barrel [Penicillium camemberti]	70%	CRL20929
Hypothetical protein PENSOL_c027G11035 [Penicillium solitum]	70%	OQD94259
hypothetical protein PENNAL_c0010G10993 [Penicillium nalgiovense]	70%	OQE91236
Hypothetical protein ASPZODRAFT_133064 [Penicilliopsis zonata CBS 506.65]	69%	OJJ46083
hypothetical protein ACN42_g11115 [Penicillium freii]	69%	KUM56111
Aldolase-type TIM barrel [Penicillium italicum]	69%	KGO71495

EXAMPLE 10

[00129] To improve KDG formation in *A. niger,* a gluconate dehydratase activity may need to be enhanced. This can be accomplished by expressing a heterologous gluconate dehydratase in *A. niger*, or by enhancing the expression of an endogenous (native) gluconate dehydratase already present in the *Aspergillus* strain. However, endogenous gluconate dehydratases in filamentous fungi have not been identified. Nevertheless, the existence of such an enzymatic activity is essential for the Nonphosphorylated gluconate degradative pathway present in *A. niger* and many other microorganisms. So far, only a few gluconate dehydratases have been characterized in bacteria and archaea; and it has been found that they belong to the ILVD/EDD protein family (COG0129). This family includes dihydroxy acid dehydratases (DHADs) which are involved in short-chain amino acid biosynthesis, and catalyse the dehydration of 2,3-dihydroxy-3-methylbutanoate. DHADs are commonly named IlvD in bacteria or ILV-3 in fungi. COG0129 also contains sugar acid dehydratases, which are involved in the metabolism of hexose and pentose sugars, and include D-xylonate dehydratases, L-arabinonate dehydratase, D-gluconate dehydratases and 6-phosphogluconate dehydratases (EDD).

[00130] One way to predict the function of a gene in a particular genome, is to look for homologs of a gene for which the activity has been demonstrated in a close relative. For example, to predict gene functions in Aspergillus, very often the known genes from *Saccharomyces*, *Neurospora* or *Penicillium* are utilized for such a purpose. In this sense, Oliver et al., (2012) characterized the DHADs from *Aspergillus fumigatus* and found that this fungus, has 4 homologs (AfIlv3A, AfIlv3B, AfIlv3C and AfIlv3D) of the *Saccharomyces cerevisiae* ILV-3 for which, its function as a DHAD has been clearly demonstrated. Using gene deletions, Oliver et al., found that AfIlv3A was the real DHAD in A. fumigatus. No function for AfIlv3B, AfIlv3C and AfIlv3D was predicted by Oliver et al. Importantly, this publication also showed that *Aspergillus nidulans* had also four ILV-3 homologs, while *Aspergillus niger* had seven homologs (Anig1-7). Based on this report and others, it is proposed that the *A. niger* Anig5 (XP_001397198), Anig6 (XP_001392062) and Anig7 (XP_001394885) are good candidates to be sugar acid dehydratases. In particular, Anig5 which has 60% identity, 74% similarity to the *Achromobacter xylosoxidans* D-gluconate dehydratase *gnaD* (Kim & Lee. 2008). Due to the reannotation and renaming of genes in GenBank, other annotation/names for Anig5, Anig6 and Anig7 are given in the table below. One skilled in the art may determine that the names used to annotate the genome of the *A. niger* strain CBS 513.88 may be different in other *A. niger* strains.

Table 6 Annotations in *A.niger*

Annotations in *A.niger* CBS513.88, accordingly Oliver et al., 2012	Other Annotations
Anig5	XP_001397198; ANI_1_908134; NT_166530; Gene ID 4988271.
Anig6	XP_001392062; ANI_1_2174064; NT_166523; Gene ID 4982256.
Anig7	XP_001394885: ANI_1_2454094; NT_166526; Gene ID 4985143.

[00131] Although the present invention has been described in terms of specific exemplary embodiments and examples, it will be appreciated that the embodiments disclosed herein are for illustrative purposes only and various modifications and alterations might be made by those skilled in the art without departing from the spirit and scope of the invention as set forth in the following claims.

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All references cited herein, including those below and including but not limited to all patents, patent applications, and non-patent literature referenced below or in other portions of the specification, are hereby incorporated by reference herein in their entirety.

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What is claimed is:

1. A genetically modified filamentous fungi having reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity as compared to a wild type filamentous fungi.

2. The filamentous fungi claim of 1, wherein the filamentous fungi is an *Aspergillus* strain.

3. The filamentous fungi claim of 2, wherein the filamentous fungi is an *Aspergillus niger* strain.

4. The filamentous fungi claim of 3, wherein the *Aspergillus niger* strain is selected from the group consisting of NRRL 322, NRRL 328, NRRL 566, NRRL 599 and NRRL 2270.

5. The filamentous fungi of claim 1, wherein the genetic modification results in reduced endogenous KDG aldolase enzyme activity.

6. The filamentous fungi of claim 1, wherein the genetic modification is a mutation.

7. The filamentous fungi of claim 6, wherein the mutation is selected from the group consisting of a frame shift mutation, a substitution mutation, an insertion mutation, a loss of function mutation, a gain of function mutation, an inactivation mutation, a translocation mutation and a deletion mutation.

8. The filamentous fungi of claim 6, where in the mutation is in the promotor region, the 3' untranslated region, the 5' untranslated region and/or a regulatory sequence of a 2-Keto-3-Deoxy-Gluconate (KDG) aldolase gene.

9. The filamentous fungi of claim 6, wherein the mutation is in a nucleic acid sequence encoding a polypeptide having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

10. The filamentous fungi of claim 6, wherein the mutation is a deletion of a gene having the nucleic acid sequence of SEQ ID NO:2.

11. 11 The filamentous fungi of claim 6, wherein the mutation is in a nucleic acid sequence encoding a KDG aldolase enzyme comprising the amino acid sequence of SEQ ID NO:1.

12. The filamentous fungi of claim 6, wherein the mutation inhibits expression of the polypeptide comprising SEQ ID NO:1.

13. The filamentous fungi of claim 1, wherein the enzyme having reduced 2-Keto-3-Deoxy-Gluconate (KDG) aldolase enzyme activity has the classification of EC4.1.2.51.

14. A method of producing 2-Keto-3-Deoxy-Gluconate (KDG) or a furan derivative comprising:

transforming a filamentous fungi with a nucleic acid sequence; and

culturing the transformed filamentous fungi in a culture medium, wherein the level of KDG or a furan derivative accumulated in the transformed filamentous fungi is higher than the level of KDG or a furan derivative accumulated in the filamentous fungi not transformed with the nucleic acid sequence, thereby producing KDG or a furan derivative.

15. The method of claim 14, wherein the nucleic acid sequence inactivates a gene encoding a KDG aldolase enzyme, reduces expression of a gene encoding a KDG aldolase enzyme or inhibits expression of a gene encoding a KDG aldolase enzyme.

16. The method of claim 14, wherein the filamentous fungi is an *Aspergillus* strain.

17. The method of 16, wherein the filamentous fungi is an *Aspergillus niger* strain.

18. The method of claim 14, wherein the nucleic acid sequence of the gene encoding the KDG aldolase enzyme comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:2.

19. The method of claim 14, wherein the amino acid sequence of the KDG aldolase enzyme comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

20. The method of claim 14, wherein the nucleic acid sequence comprises SEQ ID NO:8.

21. The method of claim 17, wherein the *Aspergillus niger* strain is selected from the group consisting of NRRL 322, NRRL 328, NRRL 566, NRRL 599 and NRRL 2270.

22. The method of claim 14, further providing the conversion of KDG to 5-hydroxymethylfuranoic acid (HMFA) and/or dicarboxylic acid (FDCA).

23. A method of decreasing D-glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in a filamentous fungi comprising:

transforming a filamentous fungi with a nucleic acid sequence; and culturing the transformed filamentous fungi in a culture medium, wherein the level of D-glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in the transformed filamentous fungi is decreased from the level of glyceraldehyde production from 2-Keto-3-Deoxy-Gluconate in a filamentous fungi not transformed with the nucleic acid sequence, thereby decreasing D-glyceraldehyde from 2-Keto-3-Deoxy-Gluconate.

24. The method of claim 23, wherein the nucleic acid sequence inactivates a gene encoding a KDG aldolase enzyme, reduces expression of a gene encoding a KDG aldolase enzyme or inhibits expression of a gene encoding a KDG aldolase enzyme.

25. The method of claim 23, wherein the filamentous fungi is an *Aspergillus* strain.

26. The method of claim 23, wherein the filamentous fungi is an *Aspergillus niger* strain.

27. The method of claim 26, wherein the *Aspergillus niger* strain is selected from the group consisting of NRRL 322, NRRL 328, NRRL566, NRRL 599 and NRRL 2270.

28. The method of claim 23, wherein the nucleic acid sequence of the gene encoding the KDG aldolase enzyme comprises SEQ ID NO:2.

29. The method of claim 23, wherein the amino acid sequence of the KDG aldolase enzyme comprises at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more, or complete sequence identity to SEQ ID NO:1.

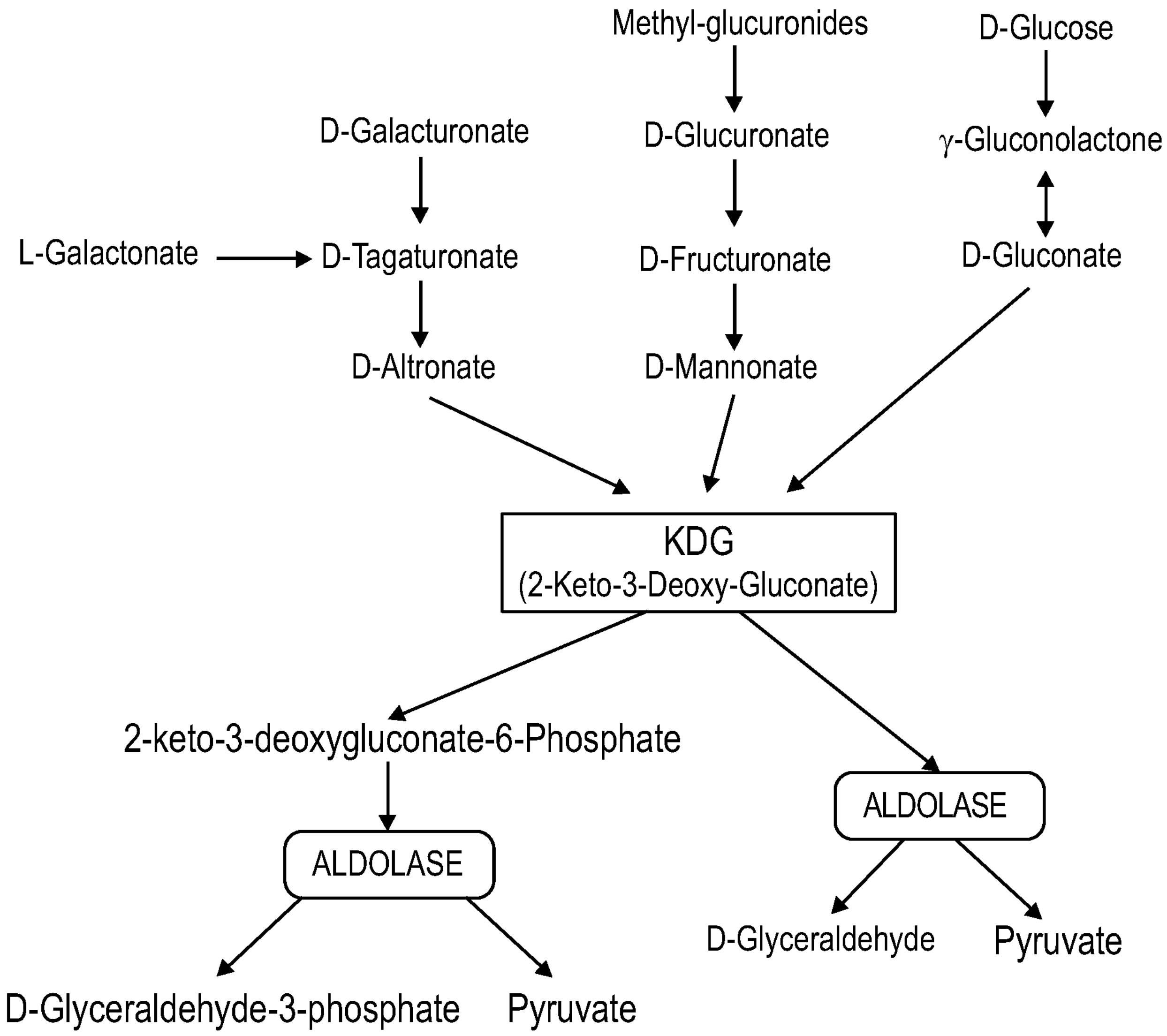
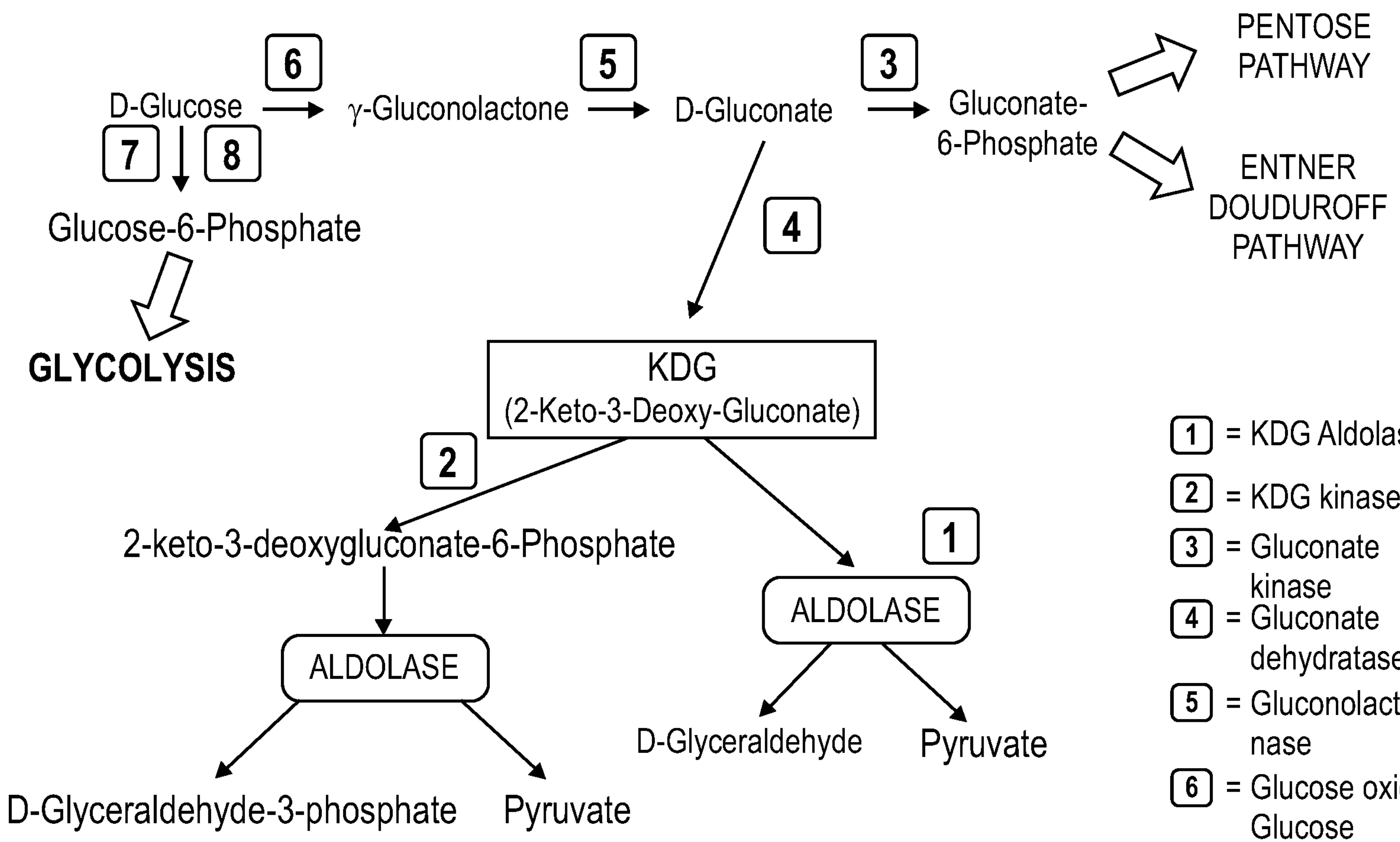

Methyl-glucuronides
D-Glucose
D-Galacturonate
D-Glucuronate
γ-Gluconolactone
L-Galactonate
D-Tagaturonate
D-Fructuronate
D-Gluconate
D-Altronate
D-Mannonate
KDG
(2-Keto-3-Deoxy-Gluconate)
2-keto-3-deoxygluconate-6-Phosphate
ALDOLASE
ALDOLASE
D-Glyceraldehyde
Pyruvate
D-Glyceraldehyde-3-phosphate
Pyruvate

FIGURE 1



1 = KDG Aldolase
2 = KDG kinase
3 = Gluconate kinase
4 = Gluconate dehydratase
5 = Gluconolactonase
6 = Glucose oxidase, Glucose dehydrogenase
7 = Glucokinase
8 = Phosphotransferase system (PTS).

FIGURE 2

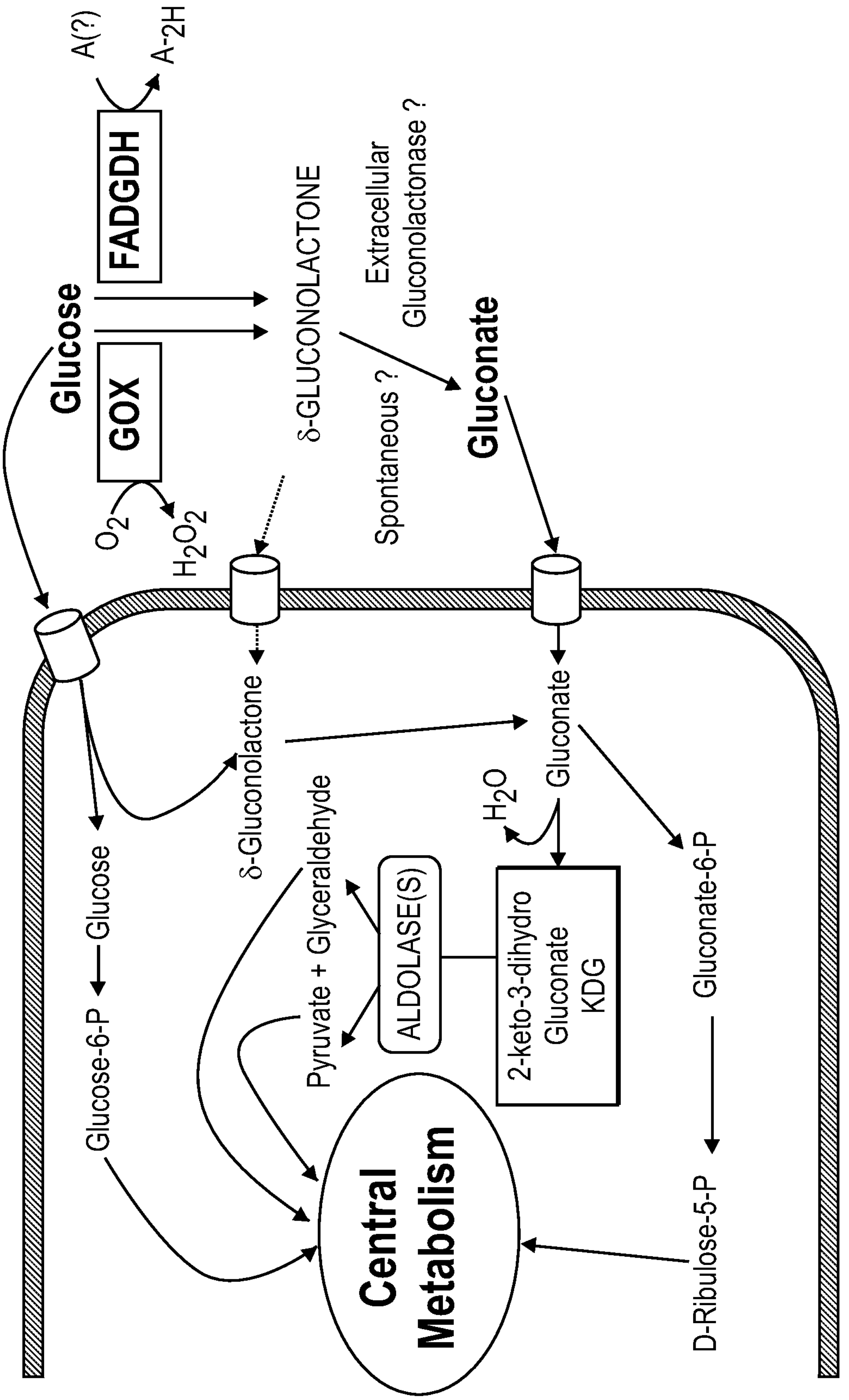

Glucose
GOX
FADGDH
O_2
H_2O_2
A(?)
A_{-2H}
δ-GLUCONOLACTONE
Extracellular
Gluconolactonase ?
Spontaneous ?
Gluconate
Glucose-6-P
Glucose
δ-Gluconolactone
Gluconate
H_2O
Pyruvate + Glyceraldehyde
ALDOLASE(S)
2-keto-3-dihydro
Gluconate
KDG
Gluconate-6-P
Central
Metabolism
D-Ribulose-5-P

FIGURE 3

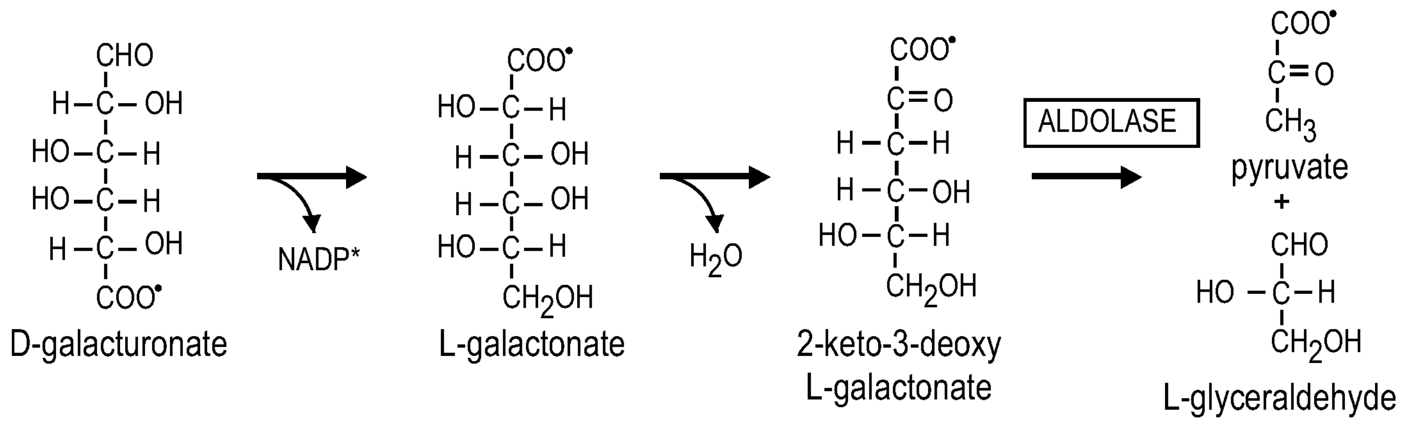


FIGURE 4A

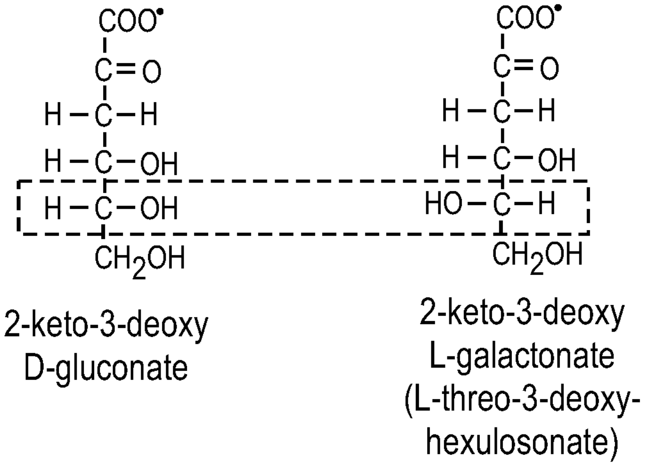


FIGURE 4B