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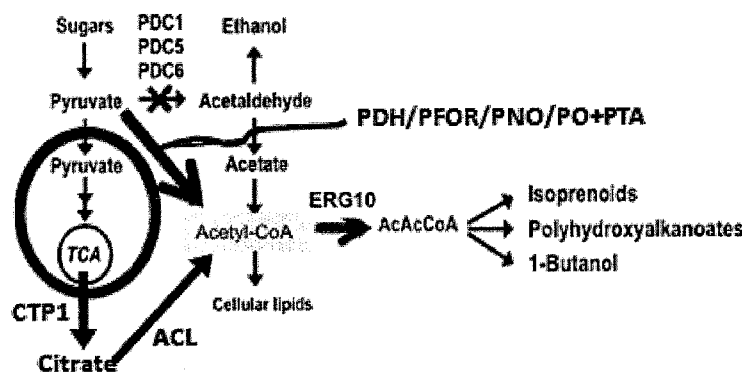
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[Continued on next page]

(54) Title: ENGINEERING OF ACETYL-CoA METABOLISM IN YEAST

Figure 2:



(57) Abstract: The invention relates to engineering of acetyl-CoA metabolism in yeast and in particular to production of acetyl-CoA in a non-ethanol producing yeast lacking endogenous gene(s) encoding pyruvate decarboxylase and comprising a heterologous pathway for synthesis of cytosolic acetyl-CoA.



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ENGINEERING OF ACETYL-CoA METABOLISM IN YEAST

Field of the Invention

The present invention relates to the development of metabolic engineering of microorganisms. More specifically this invention relates to a non-ethanol producing yeast strain, for instance of the yeast *Saccharomyces cerevisiae*, that can produce cytosolic acetyl-Coenzyme A (CoA), such as by converting glucose to acetyl-CoA in the cytosol.

Background of the Invention

With the requirements for development of sustainable solutions for provision of fuels, chemicals and materials, there is much focus on biotechnology, as this may serve as one of the pillars underlying a modern, sustainable society. Biotechnology has been used for generations in the production of fermented beverages and food products, and in the last 60 years for the production of antibiotics, food ingredients and feed additives. In recent years, several new processes for production of chemicals that can be used for polymer production have been introduced, and the production of bioethanol for fuel use has increased rapidly. Currently, there is extensive research on the development of novel cell factories for the production of chemicals and novel fuels, and it is expected that this will lead to implementation of several new biotech processes in the coming years.

The core of this development is the design and construction of cell factories that can ensure efficient conversion of the raw material to the product of interest. Traditionally, microorganisms that naturally produce a desired molecule were identified and then improved through classical strain engineering based on mutagenesis and screening. This has been a very effective approach and has resulted in low-cost production processes for many different chemicals, e.g. penicillin, citric acid and lysine. With the introduction of genetic engineering and methods for detailed analysis of cellular metabolism it became possible to use a more directed approach to improve cell factories, generally referred to as metabolic engineering. Today metabolic engineering has evolved into a research field that encompasses detailed metabolic analysis with the objective to identify targets for metabolic engineering and the implementation of metabolic engineering strategies for improvement and/or design of novel cell factories. In recent years, synthetic biology has emerged as another research field that originally aimed at

reconstruction of small, artificial biological systems, e.g. assembling a new biological regulon or oscillators that can be used to regulate gene expression in response to a specific input. But synthetic biology also includes the synthesis of DNA and complete chromosomes as illustrated in a recent work on reconstruction of a complete bacterial chromosome.

Summary of the Invention

A primary object of the present invention is to generate a yeast platform cell factory with increased cytosolic acetyl-CoA (AcCoA) supply, by introduction of steps for the direct conversion of pyruvate to AcCoA. The yeast platform cell factory of the present invention can efficiently convert pyruvate to acetyl-CoA and this feature is combined with elimination of pyruvate decarboxylase activity, resulting in an efficient cytosolic acetyl-CoA producer that cannot produce ethanol. This will result in high cytosolic levels of the important precursor acetyl-CoA.

Acetyl-CoA metabolism is highly compartmentalized in eukaryotic cells as this metabolite is used for metabolism in the cytosol, mitochondria, peroxisomes and the nucleus. Acetyl-CoA serves as a key precursor metabolite for the production of important cellular constituents such as fatty acids, sterols, and amino acids as well as it is used for acetylation of proteins. Besides these important functions it is also precursor metabolite for many other biomolecules, such as polyketides, isoprenoids, 3-hydroxypropionic acid, 1-butanol and polyhydroxyalkanoids, which encompass many industrially relevant chemicals. The yeast *Saccharomyces cerevisiae* is a very important cell factory as it is already widely used for production of biofuels, chemicals and pharmaceuticals, and there is therefore much interest in developing platform strains of this yeast that can be used for production of a whole range of different products. It is however a problem that such a platform cell factory for efficient production of cytosolic acetyl-CoA is not as efficient as needed for good industrial application. Our invention is a multiple gene modification approach of the yeast generating higher yield of acetyl-CoA, by combining pathways for direct conversion from pyruvate to acetyl-CoA together with elimination of ethanol production.

An aspect of the embodiments relates to a yeast lacking any endogenous gene encoding pyruvate decarboxylase or comprising disrupted gene or genes encoding pyruvate decarboxylase. The yeast also comprises at least one heterologous pathway for synthesis of cytosolic acetyl-

Coenzyme A (CoA). The at least one heterologous pathway comprises at least one heterologous gene encoding a respective enzyme involved in synthesis of acetyl-CoA, with the proviso that the at least one heterologous gene is not a heterologous gene encoding a pyruvate formate lyase.

Another aspect of the embodiments relates to a method of producing acetyl-CoA comprising culturing a yeast according to above in culture conditions suitable for production of cytosolic acetyl-CoA from the yeast.

A further aspect of the embodiments relates to use of a yeast according to above for the production of cytosolic acetyl-CoA.

Yet another aspect of the embodiments relates to a method of producing a yeast suitable for the production of cytosolic acetyl-CoA. The method comprises deleting or disrupting any endogenous gene encoding pyruvate decarboxylase in the yeast. The method also comprises introducing, into the yeast, at least one heterologous pathway for synthesis of cytosolic acetyl-CoA. The at least one heterologous pathway comprises at least one heterologous gene encoding a respective enzyme involved in synthesis of acetyl-CoA, with the proviso that the at least one heterologous gene is not a heterologous gene encoding a pyruvate formate lyase.

Brief Description of the Drawings

Figure 1 provides a simplified overview of acetyl-CoA metabolism in *S. cerevisiae*. Acetyl-CoA is key metabolite in three different compartments: the cytosol (marked as ~ ~ ~), the mitochondria (marked as • • •), and the peroxisomes (marked as - - -). There is no direct transport of the acetyl-CoA between the three compartments, and the biosynthesis of acetyl-CoA in the three compartments involves different metabolic pathways. In the mitochondria, acetyl-CoA is formed from pyruvate by the pyruvate dehydrogenase complex. In the cytosol, acetyl-CoA is formed from acetate by acetyl-CoA synthase. In the peroxisomes, acetyl-CoA can be formed from both acetate (also by acetyl-CoA synthase, not shown) and from fatty acids by beta-oxidation. In the mitochondria, the primary fate of acetyl-CoA is oxidation via the tricarboxylic acid (TCA) cycle. Acetyl-CoA in the peroxisomes can via the glyoxylate cycle (GYC) be converted to C₄ organic acids (malic and succinic acid) that can be transferred to the mitochondria for oxidation via malic enzyme and the TCA cycle. The primary fate of acetyl-CoA in the cytosol is to serve as precursor for cellular lipids (fatty acids and ergosterol). Many industrially interesting biotechnological products are derived from acetyl-CoA and the

biosynthesis of most of these occurs in the cytosol. A platform yeast cell factory for all these products should therefore redirect carbon towards the acetyl-CoA in the cytosol.

Figure 2 provides an overview of the strategy that can be used in the invention. The normal route for conversion of pyruvate to acetaldehyde and further into acetyl-CoA in the cytosol is blocked through deletion of the three structural genes encoding pyruvate decarboxylase activity in yeast (*PDC1*, *PDC5* and *PDC6*). This strain is auxotrophic for C₂ carbon sources such as acetate or ethanol, and it has been shown that this requirement is solely to fulfill the need for acetyl-CoA in the cytosol (required for production of cellular lipids). By removing pyruvate decarboxylase activity the yeast cells also cannot produce ethanol from sugars, such as glucose. To re-install a cytosolic route for production of acetyl-CoA several different strategies can be used: 1) expression of cytosolic pyruvate dehydrogenase (PDH); 2) expression of pyruvate ferredoxin oxidoreductase (PFOR); 3) expression of pyruvate oxidase (PO) and phosphate acetyltransferase (PTA); and 4) expression of ATP-citrate lyase (ACL). The strains with the novel pathways for production of cytosolic acetyl-CoA can e.g. be used for production of fatty acids, 3-hydroxypropionic acid, isoprenoids, polyhydroxyalkanoates and 1-butanol.

Figure 3 shows the PO/PTA pathway.

Figure 4 shows that expression of the PO/PTA pathway leads to production of acetyl-CoA in the cytosol, as assessed via growth in glucose medium. A) Selection of optimal PO. PDC- strains were transformed with either an empty plasmid (GM2), or plasmids containing PTA from *Salmonella enterica* (PTAse) and PO from either *Lactobacillus plantarum* (POlp), *Streptococcus pneumonia* (POsp) or *Aerococcus viridians* (POav). B) Selection of optimal PTA. PDC- strains were transformed with either an empty plasmid (GM2), or plasmids containing PO from *Aerococcus viridians* (POav) and PTA from either *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 (isoform 1 is PTAse, isoform 2 is PTAsc), *Escherichia coli* (PTAec) or *Methanosarcina thermophila* (PTAmt). All strains were grown on SD-ura medium with glucose. Improved growth of the strains on glucose was used as an indicator of pathway functionality.

Figure 5 shows co-expression of PFOR with either an empty HIS plasmid (GM1-04) or a plasmid containing ferredoxin and ferredoxin reductase (Fdx) or flavodoxin and ferredoxin reductase (Fld). Growth on 2% glucose in YPD media is used as an indicator of pathway functionality.

Figure 6 shows that expression of the ACL pathway leads to production of acetyl-CoA in the cytosol through growth in either 2% raffinose (A) or 0.2% glucose medium (B). mACL is ATP-Citrate Lyase from *Mus musculus*; CTP and MDH are Citrate Transporter and Malate Dehydrogenase, respectively, from *Saccharomyces cerevisiae*; RtME is Malic Enzyme from *Rhodospiridium toruloides*.

Figure 7 shows that co-expression of the PDH genes from *Azotobacter vinelandii*, but not from *S. cerevisiae*, together with lplA leads to production of acetyl-CoA in the cytosol. The addition of exogenous lipoic acid may further enhance production of acetyl-CoA.

Figure 8 shows the plasmid pAK02 described in example 3.

Figure 9 shows the plasmid pKB01 described in example 3.

Figure 10 shows the plasmid pKB02 described in example 3.

Figure 11 shows the plasmid pYZ02 described in example 5.

Figure 12 shows the plasmid pYZ03 described in example 5.

Detailed Description of the Invention

The invention herein relies, unless otherwise indicated, on the use of conventional techniques of biochemistry, molecular biology, microbiology, cell biology, genomics and recombinant technology.

To facilitate understanding of the invention, a number of terms are defined below.

As used in the description of the invention and the appended claims, the singular forms “a,” “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Also as used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

As used herein the term “recombinant” when used means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems.

As used herein, the term “overproducing” is used in reference to the production of acetyl-CoA in a host cell and indicates that the host cell is producing more acetyl-CoA by virtue of the introduction of recombinant/heterologous nucleic acid sequences encoding polypeptides that alter the host cell’s normal metabolic pathways or as a result of other modifications (e.g., altering the expression of one or more endogenous polynucleotides) as compared with, for example, the host cell that is not modified/transformed with the recombinant polynucleotides as described herein.

As used herein, the terms “protein” and “polypeptide” refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The terms “increase,” “increasing,” “increased,” “enhance,” “enhanced,” “enhancing,” and “enhancement” (and grammatical variations thereof), as used herein, describe an elevation in, for example, the production of acetyl-CoA in a microorganism (e.g., a yeast). This increase can be observed by comparing said increase in a microorganism transformed with, for example, recombinant polynucleotides encoding a cytosolic pyruvate dehydrogenase (PDH) complex and one or more recombinant polynucleotide sequences encoding polypeptides responsible for synthesis and attachment of the lipoyl group to the microorganism not transformed with recombinant polynucleotides.. Thus, as used herein, the terms “increase,” “increasing,” “increased,” “enhance,” “enhanced,” “enhancing,” and “enhancement” (and grammatical variations thereof), and similar terms indicate an elevation of at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%,

70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 200%, 300%, 400%, 500% or more, or any range therein, as compared to a control.

As used herein, the terms “reduce,” “reduced,” “reducing,” “reduction,” “diminish,” “suppress,” and “decrease” (and grammatical variations thereof), describe, for example, a decrease in the pyruvate decarboxylase activity in a microorganism (e.g., a yeast having deletions in the polynucleotides *PDC1*, *PDC5* and *PDC6*) as compared to a control (e.g., a yeast not having said deletions in the polynucleotides *PDC1*, *PDC5* and *PDC6*). Thus, as used herein, the terms “reduce,” “reduces,” “reduced,” “reduction,” “diminish,” “suppress,” and “decrease” and similar terms mean a decrease of at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 200%, 300%, 400%, 500% or more, or any range therein, as compared to a control.

The term “overexpress,” “overexpresses” or “overexpression” as used herein refers to higher levels of activity of a gene (e.g. transcription of the gene); higher levels of translation of mRNA into protein; and/or higher levels of production of a gene product (e.g., polypeptide) than would be in the cell in its native (or control (e.g., not transformed with the particular heterologous or recombinant polypeptides being overexpressed)) state. These terms can also refer to an increase in the number of copies of a gene and/or an increase in the amount of mRNA and/or gene product in the cell. Overexpression can result in levels that are 25%, 50%, 100%, 200%, 500%, 1000%, 2000% or higher in the cell, as compared to control levels.

A “heterologous” or a “recombinant” nucleotide sequence is a nucleotide sequence not naturally associated with a host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring nucleotide sequence. A heterologous gene may optionally be codon optimized for expression in yeast according to techniques well known in the art and as further described herein. A heterologous gene also encompasses, in some embodiments, an endogenous gene controlled by a heterologous promoter and/or control elements to achieve an expression of the gene that is different from, typically higher, i.e. so-called overexpression, than normal or baseline expression of the gene in a yeast comprising the endogenous gene under control of wild type (endogenous) promoter and control elements.

A “native” or “wild type” nucleic acid, nucleotide sequence, polypeptide or amino acid sequence refers to a naturally occurring or endogenous nucleic acid, nucleotide sequence,

polypeptide or amino acid sequence. Thus, for example, a "wild type mRNA" is an mRNA that is naturally occurring in or endogenous to the organism. A "homologous" nucleic acid sequence is a nucleotide sequence naturally associated with a host cell into which it is introduced.

Also as used herein, the terms "nucleic acid," "nucleic acid molecule," "nucleotide sequence" and "polynucleotide" refer to RNA or DNA that is linear or branched, single or double stranded, or a hybrid thereof. The term also encompasses RNA/DNA hybrids. When dsRNA is produced synthetically, less common bases, such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others can also be used for antisense, dsRNA, and ribozyme pairing. For example, polynucleotides that contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression. Other modifications, such as modification to the phosphodiester backbone, or the 2'-hydroxy in the ribose sugar group of the RNA can also be made.

As used herein, the term "nucleotide sequence" refers to a heteropolymer of nucleotides or the sequence of these nucleotides from the 5' to 3' end of a nucleic acid molecule and includes DNA or RNA molecules, including cDNA, a DNA fragment or portion, genomic DNA, synthetic (*e.g.*, chemically synthesized) DNA, plasmid DNA, mRNA, and anti-sense RNA, any of which can be single stranded or double stranded. The terms "nucleotide sequence" "nucleic acid," "nucleic acid molecule," "oligonucleotide" and "polynucleotide" are also used interchangeably herein to refer to a heteropolymer of nucleotides. Nucleic acid molecules and/or nucleotide sequences provided herein are presented herein in the 5' to 3' direction, from left to right and are represented using the standard code for representing the nucleotide characters as set forth in the U.S. sequence rules, 37 CFR §§1.821 - 1.825 and the World Intellectual Property Organization (WIPO) Standard ST.25.

As used herein, the term "gene" refers to a nucleic acid molecule capable of being used to produce mRNA, antisense RNA, miRNA, anti-microRNA antisense oligodeoxyribonucleotide (AMO) and the like. Genes may or may not be capable of being used to produce a functional protein or gene product. Genes can include both coding and non-coding regions (*e.g.*, introns, regulatory elements, promoters, enhancers, termination sequences and/or 5' and 3' untranslated regions). A gene may be "isolated" by which is meant a nucleic acid that is substantially or essentially free from components normally found in association with the nucleic acid in its

natural state. Such components include other cellular material, culture medium from recombinant production, and/or various chemicals used in chemically synthesizing the nucleic acid.

A “disrupted gene” as defined herein involves any mutation or modification to a gene resulting in a partial or fully non-functional gene and gene product. Such a mutation or modification includes, but is not limited to, a missense mutation, a nonsense mutation, a deletion, a substitution, an insertion, and the like. Furthermore, a disruption of a gene can be achieved also, or alternatively, by mutation or modification of control elements controlling the transcription of the gene, such as mutation or modification in a promoter and/or enhancement elements. In such a case, such a mutation or modification results in partially or fully loss of transcription of the gene, i.e. a lower or reduced transcription as compared to native and non-modified control elements. As a result a reduced, if any, amount of the gene product will be available following transcription and translation.

The objective of gene disruption is to reduce the available amount of the gene product, including fully preventing any production of the gene product, or to express a gene product that lacks or having lower enzymatic activity as compared to the native or wild type gene product.

“Introducing” in the context of a yeast cell means contacting a nucleic acid molecule with the cell in such a manner that the nucleic acid molecule gains access to the interior of the cell. Accordingly, polynucleotides and/or nucleic acid molecules can be introduced yeast cells in a single transformation event, in separate transformation events. Thus, the term “transformation” as used herein refers to the introduction of a heterologous nucleic acid into a cell. Transformation of a yeast cell can be stable or transient.

“Transient transformation” in the context of a polynucleotide means that a polynucleotide is introduced into the cell and does not integrate into the genome of the cell.

By “stably introducing” or “stably introduced” in the context of a polynucleotide introduced into a cell, it is intended that the introduced polynucleotide is stably incorporated into the genome of the cell, and thus the cell is stably transformed with the polynucleotide.

“Stable transformation” or “stably transformed” as used herein means that a nucleic acid molecule is introduced into a cell and integrates into the genome of the cell. As such, the integrated nucleic acid molecule is capable of being inherited by the progeny thereof, more particularly, by the progeny of multiple successive generations. “Genome” as used herein

includes the nuclear genome. Stable transformation as used herein can also refer to a nucleic acid molecule that is maintained extrachromasomally, for example, as a minichromosome.

Transient transformation may be detected by, for example, an enzyme-linked immunosorbent assay (ELISA) or Western blot, which can detect the presence of a peptide or polypeptide encoded by one or more nucleic acid molecules introduced into an organism. Stable transformation of a cell can be detected by, for example, a Southern blot hybridization assay of genomic DNA of the cell with nucleic acid sequences which specifically hybridize with a nucleotide sequence of a nucleic acid molecule introduced into an organism (e.g., a yeast). Stable transformation of a cell can be detected by, for example, a Northern blot hybridization assay of RNA of the cell with nucleic acid sequences which specifically hybridize with a nucleotide sequence of a nucleic acid molecule introduced into a yeast or other organism. Stable transformation of a cell can also be detected by, e.g., a polymerase chain reaction (PCR) or other amplification reaction as are well known in the art, employing specific primer sequences that hybridize with target sequence(s) of a nucleic acid molecule, resulting in amplification of the target sequence(s), which can be detected according to standard methods. Transformation can also be detected by direct sequencing and/or hybridization protocols well known in the art.

In some embodiments, *Saccharomyces cerevisiae* can be a host for carrying out the invention, as it is a popular host in basic and applied research apart from being a good ethanol producer, a precursor of esters and specifically of fatty acid ethyl esters. In addition, other yeast cells useful with the present invention include, but are not limited to, other *Saccharomyces* species, *Hansenula polymorpha*, *Kluyveromyces* species, *Pichia* species, *Candida* species, *Trichoderma* species, *Yarrowia lipolytica*, etc.

In industry, there is much interest in applying a limited number of platform cell factories for production of a wide range of fuels and chemicals as this allows for flexible use of production facilities, which are very capital intensive. One of these platform cell factories is the yeast *Saccharomyces cerevisiae*, which is widely used for the production of beer, bread, wine, bioethanol, nutraceuticals, chemicals and pharmaceuticals. These platform cell factories can efficiently convert raw materials, today typically glucose/fructose derived from starch or sucrose, but in the future also pentoses derived from lignocellulose, into so-called precursor metabolites can then be further converted into a product of interest. One of these precursor metabolites is acetyl-CoA, that is used as precursor for the production of a wide range of industrially very

interesting products (see Figure. 1). Several of these products are produced by pathways that drain acetyl-CoA from the cytosol and in connection with reconstruction of synthetic pathways it is generally desirable to position these pathways in this compartment as this will minimize issues related to product secretion. As illustrated in Figure. 1, acetyl-CoA is, however, produced and used in several different cellular compartments, i.e. the cytosol, mitochondria and the peroxisomes, and in *S. cerevisiae* it cannot be transported directly between the different compartments (*S. cerevisiae* holds all the components of the carnitine transport system, but it cannot synthesize carnitine and in industrial fermentations it would be too expensive to add this component to the medium). In yeast, acetyl-CoA in the cytosol is produced from acetate that is derived from acetaldehyde, that is formed by de-carboxylation of pyruvate. Acetaldehyde can also be converted to ethanol by alcohol dehydrogenase, and during growth on glucose the majority of the glycolytic flux is directed towards ethanol due to the so-called Crabtree effect in yeast. Besides the main alcohol dehydrogenase (Adh1p) there are several alcohol dehydrogenases in *S. cerevisiae* that can catalyze the conversion of acetaldehyde to ethanol, and it is therefore inherently difficult to eliminate ethanol production in yeast. The only strategy that has worked so far is removing pyruvate decarboxylase activity through deletion of all three genes that encode for this activity, but this results in a strain that will require supplementation of acetate to the medium in order to meet the requirement for acetyl-CoA in the cytosol (needed for biosynthesis of fatty acids and ergosterol). Obviously, such a strain would be problematic to serve as a platform cell factory for acetyl-CoA derived products.

In the invention herein the normal route for conversion of pyruvate to acetyl-CoA through acetaldehyde in the cytosol is blocked through deletion of the three structural genes encoding pyruvate decarboxylase activity in yeast (*PDC1*, *PDC5* and *PDC6*). This strain is auxotrophic for C₂ carbon sources such as acetate or ethanol, and it has been shown that this requirement is solely to fulfill the need for acetyl-CoA in the cytosol (required for production of cellular lipids). By removing pyruvate decarboxylase activity, the yeast cells can also not produce ethanol from glucose.

However, in the invention herein we have generated a yeast platform cell factory that can efficiently convert pyruvate to acetyl-CoA and combine this feature with elimination of pyruvate de-carboxylase activity, thereby establishing an efficient cytosolic acetyl-CoA producer that overproduces acetyl-CoA and at the same time cannot produce ethanol.

It is difficult to introduce direct pathways from pyruvate to acetyl-CoA efficiently into non-ethanol producing strains. However, the inventors of the present invention have identified several possible routes for this introduction that enable efficient introduction of a route from pyruvate to acetyl-CoA in a non-ethanol producing yeast strain. Strategies for reconstructing a synthetic pathway from pyruvate to acetyl-CoA, leading to a cell factory for overproducing acetyl-CoA, are described below:

1) Pyruvate oxidase (PO) and phosphate acetyltransferase (PTA)

PO (EC 1.2.3.3) is a tetrameric flavoenzyme, each subunit contains one tightly and noncovalently bound FAD, thiamin diphosphate (TPP), and Mg^{2+} for anchoring the diphosphate moiety of TPP. In the presence of phosphate and oxygen, PO catalyzes the conversion of pyruvate to acetyl-phosphate, hydrogen peroxide and carbon dioxide. The acetyl-phosphate produced in this reaction is then converted to acetyl-CoA by PTA. There are two classes of PTA enzyme, class I enzymes are about 350 residues in length, class II enzymes are about double this size. Class I enzymes share end-to-end homology with the C-terminal domain of class II enzymes. PO has been found in many species, PO genes from several organisms may be used, including *Lactobacillus plantarum*, *Streptococcus pneumoniae*, *Aerococcus viridans*. In some embodiments, the PTA enzyme can be a class I enzyme from *E. coli*, and/or is a class II enzyme from *Salmonella enterica*. Mutants of them can also be created for better catalysis efficiency. Hydrogen peroxide is produced in the conversion of PO. Due to its stress to cells, a catalase from yeast (or another organism) can be expressed or overexpressed to convert any hydrogen peroxide produced by PO to oxygen and water.

Accordingly, in a further embodiment, the invention provides a method of producing a microorganism (e.g., yeast) having increased production of cytosolic acetyl-CoA, comprising introducing into a microorganism (e.g., a yeast) (a) a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) a polynucleotide encoding a polypeptide having the enzyme activity of pyruvate oxidase (PO) (e.g., PO from *Lactobacillus plantarum*, *Streptococcus pneumoniae*, and/or *Aerococcus viridians*), preferably *Aerococcus viridians*; and (c) a polynucleotide encoding a polypeptide having the enzyme activity of phosphate acetyltransferase (PTA) (e.g., from *E. coli*, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* LT2 and/or *Methanosarcina thermophila*), thereby producing a stably transformed microorganism having increased cytosolic acetyl-CoA

production. In a further embodiment, the microorganism can additionally comprise a polynucleotide encoding a polypeptide having the enzyme activity of a catalase. In some embodiments, catalase can be from the microorganism being modified to have increased production of cytosolic acetyl-CoA (e.g., endogenous) (e.g., yeast catalase). In representative embodiments, the catalase can be overexpressed.

In an additional embodiment, the invention further provides a yeast having reduced pyruvate decarboxylase activity and increased acetyl-CoA production, comprising (a) a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) a polynucleotide encoding a polypeptide having the enzyme activity of pyruvate oxidase (PO) (e.g., PO from *Lactobacillus plantarum*, *Streptococcus pneumoniae*, and/or *Aerococcus viridians*), preferably *Aerococcus viridians*; and (c) a polynucleotide encoding a polypeptide having the enzyme activity of phosphate acetyltransferase (PTA) (e.g., from *E. coli*, *Salmonella enterica subsp. enterica serovar Typhimurium LT2* and/or *Methanosarcina thermophila*). In a further embodiment, the modified yeast can additionally comprise a polynucleotide encoding a polypeptide having the enzyme activity of a catalase. In some embodiments, catalase can be from yeast. In representative embodiments, the catalase can be overexpressed.

The ability of the PO/PTA pathway (see Figure 3) to supplement the acetyl-CoA requirement of PDC-negative strains was tested and the resulting data is shown in Figure 4. Plasmids containing different combinations of PO and PTA from different sources were transformed into a PDC-negative strain and assessed for growth on SD-ura medium containing glucose. It was shown that strains containing PO from *Aerococcus viridians* (POav) and PTA from any of the sources examined grew significantly better than the control harbouring the empty plasmid (GM2), suggesting the PO/PTA pathway to be functional.

2) Pyruvate ferredoxin oxidoreductase (PFOR)

Expressing a bacterial pyruvate ferredoxin oxidoreductase (PFOR). Bacterial PFORs are usually homodimers and contain one to three iron-sulfur clusters. Like PDH, PFOR catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA using TPP as a cofactor. In contrast to PDH, the electron acceptor is ferredoxin. By co-expression of ferredoxin and ferredoxin reductase from *E. coli*, the electrons can be further transferred to NADP⁺ resulting in the

formation of NADPH. This reaction is reversible, involves the formation of a radical, and the enzyme is sensitive to oxygen. An exception is PFOR isolated from *Desulfovibrio africanus*, which shows an unusual high stability towards oxygen in vitro and is therefore considered as a good candidate for expression in yeast. The genes were originally cloned into plasmids under constitutively active promoters (pTEF1 or pPGK1) as described in example 2. In addition to testing *fdx* with *fpr*, the use of flavodoxin (*fldA*) instead of *fdx* was also tested. Testing of the function of PFOR together with *fldA* and *fpr* in a *Δpdc1Δpdc5Δpdc6* mutant showed it to grow better on glucose compared to the deletion mutant transformed with an empty plasmid (Figure 5).

In some aspects of the invention a method of producing a microorganism having increased production of cytosolic acetyl-CoA is provided, the method comprising introducing into a microorganism (e.g., a yeast): (a) a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) a polynucleotide encoding a polypeptide having the enzyme activity of pyruvate ferredoxin oxidoreductase (PFOR) (e.g., *pfor* from *Desulfovibrio africanus*); and/or (c) one or more recombinant polynucleotides encoding a ferredoxin or a flavodoxin polypeptide and a ferredoxin reductase polypeptide (e.g., *fdx* and *fpr* from *E. coli* or *fldA* and *fpr* from *E. coli*), preferably a flavodoxin polypeptide and a ferredoxin reductase polypeptide, thereby producing a stably transformed microorganism having increased cytosolic acetyl-CoA production.

In still another aspect, the invention provides a yeast having reduced pyruvate decarboxylase activity and increased acetyl-CoA production, comprising (a) a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) a polynucleotide encoding a polypeptide having the enzyme activity of pyruvate ferredoxin oxidoreductase (PFOR) (e.g., *pfor* from *Desulfovibrio africanus*); and/or (c) one or more recombinant polynucleotides encoding a ferredoxin or a flavodoxin polypeptide and a ferredoxin reductase polypeptide (e.g., *fdx* and *fpr* from *E. coli* or *fldA* and *fpr* from *E. coli*), preferably a flavodoxin polypeptide and a ferredoxin reductase polypeptide.

3) ATP-citrate lyase (*ACL*)

ATP-citrate lyase catalyses the conversion of citrate (derived from the mitochondria and transported to the cytosol) to oxaloacetate and acetyl-CoA. *S. cerevisiae* does not naturally

contain this enzyme. Therefore, ATP-citrate lyase of a different organism, e.g. *Rhodospiridium toruloides*, mouse or human have to be heterologously expressed. To increase the production of citrate in the mitochondria, the pyruvate dehydrogenase subunit mutant Pda1 (S313A) with a mutated phosphorylation site (Oliveira et al, 2012, Molecular Systems Biology 8, 623) can be overexpressed together with the pyruvate transporters Mpc1, Mpc2 and CIT1. In order to recycle the oxaloacetate produced by ATP-citrate lyase in the cytosol, *S. cerevisiae* malate dehydrogenase Mdh3 without its peroxisomal targeting signal and NADP⁺-dependent malic enzyme, e.g. from *R. toruloides* can also be expressed.

Therefore, in some aspects of the invention, a method of producing a microorganism having increased production of cytosolic acetyl-CoA, comprising introducing into a microorganism (e.g., a yeast) (a) a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); and (b) a polynucleotide encoding a polypeptide having the enzyme activity of ATP-citrate lyase (from, e.g., *Rhodospiridium toruloides*, mouse or human). In a further embodiment, the method further comprises introducing (a) a polynucleotide encoding a polypeptide pyruvate dehydrogenase subunit mutant Pda1 (i.e., mutated phosphorylation site); (b) a polynucleotide encoding a polypeptide having the enzyme activity of one or more pyruvate transporters (e.g., Mpc1, Mpc2); and polynucleotide encoding a polypeptide having the enzyme activity of citrate synthase CIT1. In some embodiments, the pyruvate dehydrogenase subunit mutant polypeptide, the one or more pyruvate transporters and the citrate synthase can be overexpressed. In still further embodiments, the method further comprises introducing (a) malate dehydrogenase Mdh3 (absent its peroxisomal targeting signal) and NADP⁺-dependent malic enzyme (from, e.g., *Rhodospiridium toruloides*). In addition, the native citrate transporter from yeast (CTP1) might be overexpressed to improve transport of citrate from the mitochondria to the cytosol. Figure 6 demonstrates functionality of the ACL pathway. PDC-negative strains were transformed with a plasmid encoding ACL from *Mus musculus* (mACL), CTP1 and MDH3 (without peroxisomal targeting signal) from *Saccharomyces cerevisiae* and ME from *Rhodospiridium toruloides* (RtME) and grown on solid media containing either 2% raffinose or 0.2% glucose. The strains containing the former ACL pathway exhibited significantly better growth compared to the control strain.

4) Pyruvate dehydrogenase (PDH)

Expressing a cytosolic pyruvate dehydrogenase (PDH) complex. In *Escherichia coli*, PDH is an enzyme complex consisting of three enzymes: pyruvate dehydrogenase/decarboxylase (E1), dihydrolipoamide transacetylase (E2), and lipoamide dehydrogenase (E3). The octahedral core of the complex is formed by 24 E2 moieties, which can bind varying amounts of E1 and E3 dimers. As prosthetic groups, thiamine pyrophosphate (TPP) is bound to E1, lipoic acid to E2 and flavin adenine dinucleotide (FAD) to E3. E1 catalyses the binding of pyruvate to the TPP cofactor and CO₂ is released. The resulting hydroxyethyl group is transferred to the lipoamide disulfide of E2, thereby being oxidized to an acetyl group, which is finally coupled to coenzyme A. E3 then catalyses the reoxidation of the lipoamide disulfide using NAD⁺ as electron acceptor. *S. cerevisiae* is not able to produce the cofactor lipoic acid in the cytosol. Besides expressing *E. coli* genes encoding enzymes E1-E3 (*aceE*, *aceF*, and *lpd*), it is therefore necessary to express genes responsible for the attachment and activation of the lipoyl group, which belong to the *de novo* pathway (*lipA* and *lipB*) in *E. coli*. This pathway is initiated with octanoic acid which is available as metabolite in the yeast cytosol. As an alternative, the salvage pathway that involves attachment of externally supplied lipoic acid through lipoateprotein ligase encoded by *lplA* will be expressed.

Yet another alternative strategy includes targeting the yeast PDH complex to the cytosol by removing the mitochondrial target signals of the different subunits. Genes that need to be modified and expressed include *PDA1* (encoding the E1 α subunit), *PDB1* (encoding E1 β), *LAT1* (encoding E2), *LPD1* (encoding E3) and *PDX1* (encoding protein X). This could either be combined with one of the *E. coli* lipoic acid synthesis and attachment pathways or alternatively, the yeast *de novo* pathway (it does not seem to contain a salvage pathway) can be targeted to the cytosol as well. In this latter case, at least four additional genes are also modified (*LIP2*, *LIP3*, *LIP5*, and *GCV3*).

The PDH from *Azotobacter vinelandii* is an enzyme complex including three enzymes: pyruvate dehydrogenase/decarboxylase (E1p), dihydrolipoamide transacetylase (E2p), and lipoamide dehydrogenase (E3). This complex is the smallest one among the PDH family with 0.7 MDa, but its catalysis efficiency is as good as the larger one in *E. coli*. The PDH from *Enterococcus faecalis* is an enzyme complex including four enzymes: pyruvate dehydrogenase/decarboxylase alpha subunit (E1 α), pyruvate dehydrogenase/decarboxylase (E1 β), dihydrolipoamide transacetylase (E2) and lipoamide dehydrogenase (E3). This enzyme is

active in aerobic and anaerobic condition. Compared with the PDH from *E. coli* and *A. vinelandii*, this enzyme has lower sensitivity to the NADH/NAD⁺ ratio. The reaction mechanism is the same as for PDH in *E. coli*. For functional expression of these enzymes, the attachment and activation of the lipoyl group is also necessary. Besides expressing the PDH complex of *A. vinelandii*, the *de novo* pathway (*lipA* and *lipB*) is expressed. Another enzyme (acyl-acyl carrier protein synthetase) that can convert octanoic acid to octanoyl-ACP which is the substrate of *de novo* pathway is also expressed. There are two types ACP among different species. Most of the species have a type II ACP, but it is type I in yeast. Considering the substrate specificity of enzymes, type II ACP protein from *E. coli* (*acpP*) is co-expressed.

Accordingly, one aspect of the invention provides a method of producing a microorganism having increased production of cytosolic acetyl-CoA, comprising introducing into a microorganism (e.g., a yeast) (a) a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) one or more recombinant polynucleotides encoding subunits of a cytosolic pyruvate dehydrogenase (PDH) complex (e.g., *E. coli* E1 (*aceE*), E2 (*aceF*), E3 (*lpd*), or *A. vinelandii* pyruvate dehydrogenase/decarboxylase (E1p), dihydrolipoamide transacetylase (E2p) and lipoamide dehydrogenase (E3), or *E. faecalis* pyruvate dehydrogenase/decarboxylase alpha subunit (E1 α), pyruvate dehydrogenase/decarboxylase (E1 β), dihydrolipoamide transacetylase (E2) and lipoamide dehydrogenase (E3)), preferably *A. vinelandii* pyruvate dehydrogenase/decarboxylase (E1p), dihydrolipoamide transacetylase (E2p) and lipoamide dehydrogenase (E3); and (c) one or more recombinant polynucleotides encoding polypeptides responsible for synthesis and/or attachment of the lipoyl group (e.g., *E. coli* *lipA* and *lipB* or *E. coli* *lplA*), thereby producing a stably transformed microorganism having increased cytosolic acetyl-CoA production.

A further aspect of the invention provides a method of producing a microorganism having increased production of cytosolic acetyl-CoA, comprising introducing into a microorganism (e.g., a yeast) (a) a deletion of the endogenous polynucleotide sequences encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) one or more recombinant polynucleotides encoding subunits of a yeast pyruvate dehydrogenase (PDH) complex, wherein the yeast pyruvate dehydrogenase complex subunits are modified by removing the mitochondrial targeting sequences (e.g., *PDA1* (encoding E1 α), *PDB1* (encoding E1 β), *LAT1* (encoding E2), *LPD1* (encoding E3) and *PDX1* (encoding protein X)); and either (c) one or more

recombinant polynucleotide sequences encoding polypeptides responsible for attachment and activation of the lipoyl group derived from a heterologous host (e.g., *E. coli lipA* and *lipB* or *E. coli lplA*) or (d) one or more recombinant polynucleotide sequences encoding yeast polypeptides responsible for attachment and activation of the lipoyl group (*LIP2*, *LIP3*, *LIP5* and *GCV3*) modified by removal of the mitochondrial targeting sequence to produce a stably transformed microorganism having increased cytosolic acetyl-CoA production, thereby producing a stably transformed microorganism having increased production of cytosolic acetyl-CoA.

In addition, microorganisms produced by the methods of this invention are provided. Accordingly, some aspects of the invention provide a yeast having reduced pyruvate decarboxylase activity and increased acetyl-CoA production, comprising (a) a deletion of the endogenous polynucleotide sequences encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) one or more recombinant polynucleotides encoding subunits of a cytosolic pyruvate dehydrogenase (PDH) complex (e.g., *E. coli* E1 (*aceE*), E2 (*aceF*), E3 (*lplD*), or *A. vinelandii* pyruvate dehydrogenase/decarboxylase (E1p), dihydrolipoamide transacetylase (E2p) and lipoamide dehydrogenase (E3), or *E. faecalis* pyruvate dehydrogenase/decarboxylase alpha subunit (E1 α), pyruvate dehydrogenase/decarboxylase (E1 β), dihydrolipoamide transacetylase (E2) and lipoamide dehydrogenase (E3)), preferably *A. vinelandii* pyruvate dehydrogenase/decarboxylase (E1p), dihydrolipoamide transacetylase (E2p) and lipoamide dehydrogenase (E3); and (c) one or more recombinant polynucleotides encoding proteins responsible for synthesis and/or attachment of the lipoyl group (e.g., *E. coli lipA* and *lipB* or *E. coli lplA*).

In other embodiments, a yeast having reduced pyruvate decarboxylase activity and increased acetyl-CoA production is provided that comprises (a) a deletion of the endogenous polynucleotide sequences encoding pyruvate decarboxylase (PDC) (e.g., a deletion of *PDC1*, *PDC5* and *PDC6*); (b) one or more recombinant polynucleotides encoding subunits of a yeast pyruvate dehydrogenase (PDH) complex, wherein the yeast pyruvate dehydrogenase complex subunits are modified by removing the mitochondrial targeting sequences (e.g., *PDA1* (encoding E1 α), *PDB1* (encoding E1 β), *LAT1* (encoding E2), *LPD1* (encoding E3) and *PDX1* (encoding protein X)); and either (c) one or more recombinant polynucleotide sequences encoding proteins responsible for attachment and activation of the lipoyl group derived from a heterologous host (e.g., *E. coli lipA* and *lipB* or *E. coli lplA*) or (d) one or more recombinant polynucleotide

sequences encoding yeast proteins responsible for attachment and activation of the lipoyl group (*LIP2*, *LIP3*, *LIP5* and *GCV3*) modified by removal of the mitochondrial targeting sequence. Figure 7 demonstrates functionality of the PDH pathway. PDH genes from either *A. vinelandii* (Figure 7A and B) or *S. cerevisiae* (mitochondrial targeting signal removed; Figure 7 C and D), as well as *lplA* gene (for lipoic acid salvage) were cloned into a plasmid and introduced into a PDC-negative strain. Strains were grown on glucose with (Figure 7 B and D) or without (Figure 7 A and C) lipoic acid supplement. Strains containing PDH genes from *A. vinelandii* exhibited improved growth compared to control strains, demonstrating pathway functionality.

An aspect of the embodiments relates to a yeast lacking any endogenous gene encoding pyruvate decarboxylase or comprising disrupted gene or genes encoding pyruvate decarboxylase. The yeast also comprises at least one heterologous pathway for synthesis of cytosolic acetyl-CoA. The at least one heterologous pathway comprises at least one heterologous gene encoding a respective enzyme involved in synthesis of acetyl-CoA, with the proviso that the at least one heterologous gene is not a heterologous gene encoding a pyruvate formate lyase.

In an embodiment, the at least one heterologous gene encodes a respective enzyme involved in the conversion of pyruvate into cytosolic acetyl-CoA.

In an embodiment, the yeast lacks endogenous genes encoding PDC1, PDC5 and/or PDC6 or comprises disrupted genes encoding PDC1, PDC5 and/or PDC6, preferably lacks endogenous genes encoding PDC1, PDC5 and PDC6, or comprises disrupted genes encoding PDC1, PDC5 and PDC6, to block conversion of pyruvate to ethanol.

In an embodiment, the yeast further comprises a heterologous gene encoding a pyruvate oxidase, and a heterologous gene encoding a phosphate acetyltransferase.

In a particular embodiment, the heterologous gene encoding the pyruvate oxidase is selected from the group consisting of a gene encoding a *Lactobacillus plantarum* pyruvate oxidase, a gene encoding a *Streptococcus pneumonia* pyruvate oxidase and a gene encoding an *Aerococcus viridians* pyruvate oxidase, preferably *A. viridians* pyruvate oxidase. In this particular embodiment, the heterologous gene encoding the phosphate acetyltransferase is selected from the group consisting of a gene encoding an *Escherichia coli* phosphate acetyltransferase, a gene encoding a *Salmonella enterica* phosphate acetyltransferase and a gene encoding a *Methanosarcina thermophila* phosphate acetyltransferase.

In a particular embodiment, the yeast further comprises a gene encoding a catalase, preferably a gene adapted for overexpression of *Saccharomyces cerevisiae* CTA1.

In an embodiment, the yeast further comprises a heterologous gene encoding a pyruvate ferredoxin oxidoreductase, a heterologous gene encoding a ferredoxin reductase, and a heterologous gene encoding a ferredoxin reductase substrate selected from the group consisting of ferredoxin and flavodoxin.

In a particular embodiment, the heterologous gene encoding the pyruvate ferredoxin oxidoreductase is a gene encoding a *Desulfovibrio africanus* pyruvate ferredoxin oxidoreductase, preferably *D. africanus* pfor. In this particular embodiment, the heterologous gene encoding the ferredoxin reductase is a gene encoding an *Escherichia coli* ferredoxin reductase, preferably *E. coli* fpr. In this particular embodiment, the heterologous gene encoding the ferredoxin reductase is a heterologous *E. coli* gene encoding said ferredoxin reductase substrate, preferably *E. coli* fdx or *E. coli* fldA, more preferably *E. coli* fldA.

In an embodiment, the yeast further comprises a heterologous gene encoding an adenosine triphosphate (ATP)-citrate lyase.

In a particular embodiment, the heterologous gene encoding the ATP-citrate lyase selected from the group consisting of a gene encoding a *Rhodospiridium toruloides* ATP-citrate lyase, a gene encoding a *Mus musculus* ATP-citrate lyase and a gene encoding a human ATP-citrate lyase, preferably a gene encoding a *M. musculus* ATP-citrate lyase.

In a particular embodiment, the yeast further comprises a heterologous gene encoding a pyruvate dehydrogenase subunit mutant having a mutated phosphorylation site, a gene encoding a pyruvate transporter, and a gene encoding a citrate synthase.

In a particular embodiment, the heterologous gene encoding said pyruvate dehydrogenase subunit mutant is a gene encoding a *Saccharomyces cerevisiae* pyruvate dehydrogenase subunit mutant having a mutated phosphorylation site, preferably *S. cerevisiae* Pda1 having a mutated phosphorylation site. In this particular embodiment, the gene encoding the pyruvate transporter is a gene encoding a *S. cerevisiae* pyruvate transporter, preferably *S. cerevisiae* Mpc1 or Mpc2. In this particular embodiment, the gene encoding said citrate synthase is a gene encoding a *S. cerevisiae* citrate synthase, preferably *S. cerevisiae* CIT1.

In a particular embodiment, the yeast further comprises a heterologous gene encoding a malate dehydrogenase lacking any peroxisomal targeting signal, and a heterologous gene encoding a nicotinamide adenine dinucleotide phosphate (NADP⁺)-dependent malic enzyme.

In a particular embodiment, the heterologous gene encoding the malate dehydrogenase lacking any peroxisomal targeting signal is a gene encoding a *Saccharomyces cerevisiae* malate dehydrogenase lacking any peroxisomal targeting signal, preferably *S. cerevisiae* Mdh3 lacking any peroxisomal targeting signal. In this particular embodiment, the heterologous gene encoding said NADP⁺-dependent malic enzyme is a gene encoding a *Rhodospiridium toruloides* NADP⁺-dependent malic enzyme.

In a particular embodiment, the yeast further comprises a gene adapted for overexpression of a citrate transporter, preferably a *Saccharomyces cerevisiae* citrate transporter, more preferably *S. cerevisiae* CTP1.

In an embodiment, the yeast comprises heterologous genes encoding a cytosolic pyruvate dehydrogenase complex, and heterologous genes encoding respective enzymes involved in attachment and activation of lipoyl groups to said cytosolic pyruvate dehydrogenase complex.

In an embodiment, the heterologous genes encoding the cytosolic pyruvate dehydrogenase complex are selected from the group consisting of genes encoding an *Escherichia coli* cytosolic pyruvate dehydrogenase complex, preferably *E. coli* aceE, aceF and lpd, genes encoding a *Saccharomyces cerevisiae* pyruvate dehydrogenase complex but lacking mitochondrial target signal (MTS), preferably *S. cerevisiae* PDA1, PDB1, LAT1, LPD1 and PDX1 lacking MTS, genes encoding an *Azotobacter vinelandii* pyruvate dehydrogenase complex, preferably *A. vinelandii* aceE_{av}, aceF_{av} and lpdA_{av}, and genes encoding an *Enterococcus faecalis* pyruvate dehydrogenase complex, preferably *E. faecalis* pdhA, pdhB, aceF_{ef} and lpdA_{ef}, more preferably, the pyruvate dehydrogenase complex are an *A. vinelandii* pyruvate dehydrogenase complex. In this particular embodiment, the heterologous genes encoding respective enzymes involved in attachment and activation of lipoyl groups are selected from the group consisting of genes encoding *Escherichia coli* lipoic acid synthetase and lipoic acid synthetase and/or lipoate-protein ligase, preferably *E. coli* lipA, and lipB and/or lplA, and genes encoding *Saccharomyces cerevisiae* LIP2, LIP3, LIP5 and GCV3 but lacking MTS.

Another aspect of the embodiments relates to a method of producing acetyl-CoA comprising culturing a yeast according to any of the embodiments in culture conditions suitable for production of cytosolic acetyl-CoA from the yeast.

In an embodiment, the method further comprises collecting the acetyl-CoA or a compound generated by the yeast from the acetyl-CoA from a culture medium in which the yeast is cultured and/or from the yeast. The compound is selected from a group consisting of fatty acids, 3-hydroxypropionic acid, isoprenoids, polyhydroxyalkanoates, 1-butanol and polyketides.

A further aspect of the embodiments relates to use of a yeast according to any of the embodiments for the production of cytosolic acetyl-CoA.

Yet another aspect of the embodiments relates to a method of producing a yeast suitable for the production of cytosolic acetyl-CoA. The method comprises deleting or disrupting any endogenous gene encoding pyruvate decarboxylase in the yeast. The method also comprises introducing, into the yeast, at least one heterologous pathway for synthesis of cytosolic acetyl-CoA. The at least one heterologous pathway comprises at least one heterologous gene encoding a respective enzyme involved in synthesis of acetyl-CoA, with the proviso that said at least one heterologous gene is not a heterologous gene encoding a pyruvate formate lyase.

As would be recognized by the skilled person, it is clear that the various modifications of the microorganisms of this invention described herein can be combined in any useful manner to produce a microorganism having increased production of cytosolic acetyl-CoA as well as other useful phenotypic and genetic traits. Thus, a microorganism (e.g., a yeast) having a deletion of the endogenous polynucleotides encoding pyruvate decarboxylase (PDC) can be further modified to comprise any of the polynucleotides described herein or those otherwise known in the art to provide useful traits.

The invention will now be described with reference to the following examples. It should be appreciated that these examples are not intended to limit the scope of the claims to the invention, but are rather intended to be exemplary of certain embodiments. Any variations in the exemplified methods that occur to the skilled artisan are intended to fall within the scope of the invention.

EXAMPLES

In the examples below references are made to several primers, these primers are to be found in Table 1.

EXAMPLE 1.

Deletion of the three structural genes encoding pyruvate decarboxylase activity in yeast (PDC1, PDC5 and PDC6).

PDC1, *PDC5* and *PDC6* were deleted using a bipartite strategy (Erdeniz et al., 1997). Two overlapping fragments of the *kanMX* resistance marker cassette flanked by *loxP* sites were amplified via PCR from plasmid pUG6 (Güldener et al., 1996) using primers 13-16. Sequences upstream and downstream of the individual genes were amplified using primers 1-12. Due to overlapping ends (introduced through the primer sequences) the *PDC*-upstream fragments could be fused to the 5' *kanMX* fragment and the 3' *kanMX* fragment to the individual *PDC*-downstream fragments by fusion PCR using the outer primers for amplification. The two overlapping PCR fragments thus generated for each gene deletion were transformed into yeast using the lithium acetate method (Gietz and Woods, 2002). After each gene deletion, the *kanMX* marker cassette was looped out via Cre recombinase mediated recombination between the two flanking *loxP* sites using plasmid pUC47 or pUG62 as described previously (Güldener et al., 1996).

PDC1, *PDC5*, and *PDC6* were consecutively deleted in two different background strains: CEN.PK 113-5D (*MATa ura3-52*) and CEN.PK 110-10C (*MATa his3-Δ1*). This resulted in construction of strains YMZ-C1 (*MATa ura3-52 pdc1Δ pdc5Δ pdc6Δ*), and YMZ-A3 (*MATa his3-Δ1 pdc6Δ*). Strains YMZ-C1 and YMZ-A3 were crossed to generate YMZ-E1 (*MATa ura3-52 his3-Δ1 pdc1Δ pdc5Δ pdc6Δ*).

EXAMPLE 2.

Expressing pyruvate oxidase (PO) and phosphate acetyltransferase (PTA) in cytosol

Genes of PTA from *E. coli* (*eutD*) and from *S. enterica* (PTA^{R252H} and PTA^{G273D}) were codon optimized for expression in yeast and synthesized by GenScript (Piscataway, NJ, USA). The gene sequences with introduced restriction sites can be found in Table 2. *eutD* and *pta* were digested with BamHI/NheI and cloned into the pSP-GM1 generating plasmid pZJ08 (*eutD*), pZJ09 (*pta*^{R252H}) and pZJ10 (*pta*^{G273D}). Genes of PO from *L. plantarum*, *S. pneumoniae*, *A. viridans* were

codon optimized for expression in yeast and synthesized by GenScript (Piscataway, NJ, USA). The gene sequences with introduced restriction sites can be found in Table 2. The catalase gene from yeast itself (*CTA1*) was amplified from yeast genome, the *TPH* promoter and *FBA1* terminator were amplified, three fragments were cloned into pZJ08, pZJ09 and pZJ10 generating plasmids pZJ11, pZJ12 and pZJ13. The PO genes were cut with *NotI*/*PacI* and cloned into pZJ11, pZJ12 and pZJ13 generating serial plasmids pZJ14 (*eutD-catA1-PO_{LP}*), pZJ15 (*eutD-catA1-PO_{SP}*), pZJ16 (*eutD-catA1-PO_{AV}*), pZJ17 (*pta^{R252H}-catA1-PO_{LP}*), pZJ18 (*pta^{R252H}-catA1-PO_{SP}*), pZJ19 (*pta^{R252H}-catA1-PO_{AV}*), pZJ20 (*pta^{G273D}-catA1-PO_{LP}*), pZJ21 (*pta^{G273D}-catA1-PO_{SP}*) and pZJ22 (*pta^{G273D}-catA1-PO_{AV}*). These plasmids were transformed into strain YMZ-E1 from example 1 to get strain YMZ-E1-14, YMZ-E1-15, YMZ-E1-16, YMZ-E1-17, YMZ-E1-18, YMZ-E1-19, YMZ-E1-20, YMZ-E1-21 and YMZ-E1-22. After evaluating the cell growth, the best (YMZ-E1-19) one was selected as the template for genomic integration. The expression cassette in pZJ19 was amplified using primers 13-14 and cloned into vector pXI-5 by CPEC cloning. The integration construct was separated from the vector backbone by *XbaI* restriction and transformed into YMZ-E1 generate YMZ-E1-19.

EXAMPLE 3.

Expressing a bacterial pyruvate ferredoxin oxidoreductase (PFOR)

The gene *pfor* from *Desulfovibrio africanus* was codon optimised for expression in yeast and synthesized by GenScript. This gene was flanked by restriction sites for *SpeI* and *SacI*. The gene sequence can be found in Table 2. For expression in episomal plasmids, *pfor* was digested with *SpeI*/*SacI* and cloned into pSP-GM1 yielding pAK02 (Figure. 8).

The genes *fldA* and *fpr* were amplified by PCR using *E. coli* DH5 α genomic DNA as a template and primers 17-20. The *NotI*/*SacI* restricted *fldA* fragment and *BamHI*/*XhoI* restricted *fpr* fragment were cloned into pIYC04 generating pKB01 (Figure. 9).

The *E. coli* gene coding for *fdx* was PCR-amplified using *E. coli* DH5 α genomic DNA as a template and primers 60/61. This fragment was then restricted with *NotI*/*SacI* and cloned into pKB01 restricted with the same enzymes instead of *fldA*, yielding pKB02 (Figure. 10).

EXAMPLE 4.

Expressing ATP-citrate lyase (ACL) in the cytosol

This example describes the alternative route to acetyl-CoA via the pyruvate dehydrogenase complex in the mitochondria and ATP citrate lyase in the cytosol. Pyruvate dehydrogenase subunit Pda1, mutant Pda1 (S313A) with a mutated phosphorylation site, pyruvate transporters Mpc1 (Seq ID P53157) and Mpc2 (Seq ID P38857) and citrate synthase (CIT1, Seq ID NP_014398) were overexpressed. This resulted in plasmids pPDH-B and pPDH-B' (containing the mutant Pda1) constructed according to Shao et al, 2009 and Zhou et al, 2012.

In order to establish cytosolic acetyl-CoA production, ATP citrate lyase was expressed for converting citrate to acetyl-CoA and oxaloacetate with consumption of one molecule of ATP. In order to recycle oxaloacetate to pyruvate, malate dehydrogenase gene *MDH3* from *S. cerevisiae* (Seq ID P32419) (without its peroxisomal targeting signal) and NADP⁺-dependent malic enzyme (eg. from *Rhodospiridium toruloides*, Seq ID M7WHN9) were cloned into expression vector pYX212 generating pCoA4. Citrate transporter (CTP1) from *S. cerevisiae* was also cloned into the same vector (Seq ID P38152). The ATP citrate lyase gene was selected from different organisms, such as *Rhodospiridium toruloides* (Seq ID M7WHC9), human (Seq ID P53396), mouse (Seq ID Q3V117), etc. The pathways were constructed according to Shao et al, 2009 and Zhou et al, 2012.

The cytosolic alternative acetyl-CoA pathway (pCoA4) and mitochondrial citrate synthesis pathway (pTDHB') were introduced into YMZ-E1. These two pathways increased the growth of *Saccharomyces cerevisiae* YMZ-E1 in raffinose media. Then cytosolic alternative acetyl-CoA pathway (pCoA4) and mitochondrial citrate synthesis pathway (pTDHB') were also introduced into *Saccharomyces cerevisiae* YMZ-E1 evolved for better growth (EXAMPLE 6) and a *pdcl pdc5 pdc6 mth1* strain (Oud et al. 2012). The combined alternative acetyl-CoA pathway improved the growth of both strains.

EXAMPLE 5.

Expressing a pyruvate dehydrogenase (PDH) complex in the cytosol

The following genes were codon optimised for expression in yeast and synthesized by GenScript (Piscataway, NJ, USA): *aceE*, *aceF*, *lpd*, and *lplA*. The gene sequences including introduced restriction sites can be found in Table 2. For expression from episomal plasmids, *aceE* was restricted with *SacI/NotI* and cloned into vector pSP-GM1 (Chen et al., 2012a). *LplA* was cut with *BamHI/XhoI* and cloned into the same vector generating plasmid pYZ02 (Figure.

11). *AceF* was restricted with *SacI/NotI* and cloned into vector pIYC04 (Chen et al., 2012b). *Lpd* was cut with *BamHI/SaII* and cloned into the same vector generating plasmid pYZ03 (Figure. 12). For integration into the genome, the *aceE-lplA* cassette including the bidirectional P_{TEFI} - P_{PGKI} promoter was amplified via PCR from pSP-GM1-*aceE-lplA* in two fragments using primers 23-26 and cloned into vector pXI-3 (Mikkelsen et al., 2012) by Circular Polymerase Extension Cloning (CPEC) (Quan and Tian, 2011). The *aceF-lpd* cassette including the bidirectional P_{TEFI} - P_{PGKI} promoter was amplified from pSP-GM1-*aceE-lplA* using primers 27-28 and cloned into vector pXI-5 (Mikkelsen et al., 2012) by CPEC cloning. Both integration constructs were separated from the vector backbone using *NotI* or *XbaI* restriction and transformed into strain YMZ-E1 from example 1. After each integration, strains that had lost the *Kluyveromyces lactis* (*Kl*) *URA3* marker via recombination between its flanking direct repeats were selected on medium containing 5-fluoroorotic acid. This resulted in strain YMZ-E1-ecPDH.

For genomic integration of the *E. coli* lipolic acid synthesis and attachment pathway *lipA* and *lipB* were amplified from *E. coli* genomic DNA using PCR primers 29-32. The bidirectional P_{TEFI} - P_{PGKI} promoter was PCR amplified from pSP-GM1 using primers 33/34. All three fragments were cloned into pXI-2 (Mikkelsen et al., 2012) by USER cloning (Nour-Eldin et al., 2006). The integration construct was separated from the vector backbone by *NotI* restriction and transformed into YMZ-E1-ecPDH to generate YMZ-E1-ecPDHlip.

To target the yeast PDH complex and the *de novo* lipolic acid synthesis and attachment pathway into the cytosol the following genes were amplified from yeast genomic DNA using primers 35-52: *PDA1*, *PDB1*, *LAT1*, *LPD1*, *PDX1*, *LIP2*, *LIP3*, *LIP5*, and *GVC3*. Two genes at a time were cloned together with the P_{TEFI} - P_{PGKI} promoter (amplified from pSP-GM1 using primers 33/34) into different integration plasmids via Uracil Specific Excision Reagent (USER) cloning: *PDA1* and *LPD1* into pX-2, *PDB1* and *PDX1* into pX-3 (Mikkelsen et al., 2012), *LIP2* and *LIP-3* into pXI-2, and *LIP5* and *GVC3* into pXI-3. *LAT1* was cloned together with the P_{TEFI} promoter (amplified from pSP-GM1 using primers 33/53) into pX-4 (Mikkelsen et al., 2012). The *PDA1-LPD1*, *PDB1-PDX1* and *LAT1* integration constructs were separated from the vector by *NotI* restriction and consecutively integrated into the genome of YMZ-E1 to yield strain YMZ-E1-scPDH. After each integration, strains that had lost the *KlURA3* marker via recombination between its flanking direct repeats were selected on medium containing 5-

fluoroorotic acid. Likewise, the *LIP2-LIP3* and *LIP5-GCV3* constructs were integrated into YMZ-E1-scPDH to generate YMZ-E1-scPDHlip. To combine the yeast PDH complex with the *E. coli de novo* lipoleic acid synthesis and attachment pathway, the *lipA-lipB* integration construct (see above) was integrated into YMZ-E1-scPDH to generate YMZ-E1-scPDHclip.

Genes of PDH from *A. vinelandii* were codon optimized for expression in yeast and synthesized by GenScript (Piscataway, NJ, USA): *aceE_{av}*, *aceF_{av}*, and *lpdA_{av}*. The gene sequences with introduced restriction sites can be found in Table 2. For expression from episomal plasmids, *aceE_{av}* was restricted with *PacI/NotI* and cloned into vector pSP-GM1 generating plasmid pZJ01. *aceF_{av}* was restricted with *SacI/NotI* and cloned into vector pIYC04. *lpdA_{av}* was cut with *BamHI/SalI* and cloned into the same vector generating plasmid pZJ02. For integration into the genome, the *aceE_{av}* cassette with *TEF1* promoter and *ADHI* terminator was PCR amplified from pSP-GM1-*aceE_{av}* in two fragments using primers 1-2 and cloned into vector pXI-3 by CPEC cloning. The *aceF_{av}-lpdA_{av}* cassette including the bidirectional P_{TEF1}-P_{PGK1} promoter was amplified from pSP-GM1-*aceF_{av}-lpdA_{av}* using primers 3-4 and cloned into vector pXI-5 by CPEC cloning. Both integration constructs were separated from the vector backbone using *NotI* or *XbaI* restriction and transformed into strain YMZ-E1 from example 1. After integration, strains that had lost the *Kluyveromyces lactis* (KI) *URA3* marker via recombination between its flanking direct repeats were selected on medium containing 5-fluoroorotic acid. This resulted in strain YMZ-E1-*av*PDH.

Genes of PDH from *E. faecalis* were codon optimized for expression in yeast and synthesized by GenScript (Piscataway, NJ, USA): *pdhA*, *pdhB*, *aceF_{ef}*, and *lpdA_{ef}*. The gene sequences with introduced restriction sites can be found in Table 2. For expression from episomal plasmids, *pdhA* was restricted with *SacI/NotI* and cloned into vector pSP-GM1. *pdhB* from *E. coli* was cut with *BamHI/XhoI* and cloned into the same vector generating plasmid pZJ03. *aceF_{ef}* was restricted with *SacI/NotI* and cloned into vector pIYC04. *lpdA_{ef}* was cut with *BamHI/XhoI* and cloned into the same vector generating plasmid pZJ04. For integration into the genome, the *pdhA-pdhB* cassette including the bidirectional P_{TEF1}-P_{PGK1} promoter was PCR amplified from pSP-GM1-*pdhA-pdhB* in two fragments using primers 5-8 and cloned into vector pXI-3 by CPEC cloning. The *aceF_{ef}-lpdA_{ef}* cassette including the bidirectional P_{TEF1}-P_{PGK1} promoter was amplified from pSP-GM1-*aceF_{ef}-lpdA_{ef}* using primers 9-10 and cloned into vector pXI-5 by CPEC cloning. Both integration constructs were separated from the vector backbone

using *NotI* or *XbaI* restriction and transformed into strain YMZ-E1 from example 1. After integration, strains that had lost the *Kluyveromyces lactis* (*Kl*) URA3 marker via recombination between its flanking direct repeats were selected on medium containing 5-fluoroorotic acid. This resulted in strain YMZ-E1-*efPDH*. For genomic integration of the *E. coli* lipoic acid synthesis and attachment pathway *lipA* and *lipB* were amplified from *E. coli* genomic DNA using primer numbers 29-32. *lipA* was digested with *NotI/PacI* and cloned into the pSP-GM1, *lipB* was cut with *BamHI/XhoI* and cloned into the same vector generating plasmid pZJ05. The acyl-acyl carrier protein synthetase from *Vibrio harveyi* (*aasS*) and *acpP* from *E. coli* was digested with *NotI/PacI* and *BamHI/XhoI* respectively and cloned into pSP-GM1 to generate plasmid pZJ06. The directional expression cassette in pZJ06 was amplified and cut with *BspEI/SgrAI* and cloned into pZJ05 generating plasmid pZJ07. The four gene expression cassette was cloned into pXI-2 by USER cloning. The integration construct was separated from the vector backbone by *XbaI* restriction and transformed into YMZ-E1-*avPDH* and YMZ-E1-*efPDH* to generate YMZ-E1-*avPDHlip* and YMZ-E1-*efPDHlip*, respectively.

EXAMPLE 6.

Strain cultivation and evolution

PDC deletion strains containing the introduced pyruvate to AcCoA conversion system, according to the examples above, were initially cultivated on yeast extract peptone dextrose (YPD) liquid media in either shake-flasks or tubes and their growth was compared to strains containing the PDC deletion alone to evaluate system function.

Next, strains were evolved to increase growth on glucose as the sole carbon source in minimal media. This involved two phases. In the first phase, strains were cultivated in shake flasks in YP medium (10 g/L yeast extract, 20 g/L peptone) containing 1.4 % glucose and 0.6 % ethanol. Strains were serially transferred every 48 hours or 24 hours, and the ethanol concentration was gradually decreased until glucose became the sole carbon source in the media. The growth rate of the strains was occasionally determined to evaluate their adaptation level of glucose tolerance.

Once fast-growing, glucose tolerant strains were obtained in YPD media, they were further evolved for growth on minimal media. The strains were cultivated in minimal media

(Verduyn et al., 1992) containing 2 % glucose and serially transferred every 24 hours. The growth rate of strains was occasionally determined to evaluate their adaptation level.

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Table 1. Primer sequences.

Primer #	Primer name	Sequence 5'-3'	SEQ ID NO
1	pdc1-up-fw	TACTTGCTATCGTTCAACAC	1
2	pdc1-up-rev	CAGCGTACGAAGCTTCAGTGCGTGAGGTTATGAGTAG	2
3	pdc1-dw-fw	GTGATATCAGATCCACTAGTACCAACGCTAAGCAATAAG	3
4	pdc1-dw-rev	CCTTGGTTCCACTAATTCATC	4
5	pdc5-up-fw	TTAGGCATAATCACCGAAGA	5
6	pdc5-up-rev	CAGCGTACGAAGCTTCAGGAGAGGAAAGGACTTACTACA	6
7	pdc5-dw-fw	GTGATATCAGATCCACTAGTTCTGTCCTGTCTTCCAG	7
8	pdc5-dw-rev	GGTGCTCTACTGGTGATT	8
9	pdc6-up-fw	ACATCTTCCAAGCATCTCAT	9
10	pdc6-up-rev	CAGCGTACGAAGCTTCAGGAATCGCACCATATCCCTTA	10
11	pdc6-dw-fw	GTGATATCAGATCCACTAGCGTTATCGCCGTGAATTAC	11
12	pdc6-dw-rev	TTGGTTGTAGATGGTGGTG	12

13	kanMX _1_fw	CTGAAGCTTCGTACGCTG	13
14	kanMX _1_rev	TCACCATGAGTGACGACTGA	14
15	kanMX _2_fw	TTCCAACATGGATGCTGAT	15
16	kanMX _2_rev	CTAGTGGATCTGATATCAC	16
17	fpr- BamHI- fw	GTTGTTGGATCCCAGGAGAAAAACATGGCTGA	17
18	fpr - XhoI- rev	GTTGTTCTCGAG CGTTTATCGATAAGTAACCGCT	18
19	fldA- NotI-fw	GTTGTTGCGGCCGCGAGGTTATTTCACTCATGGCT	19
20	fldA- SacI-rev	GTTGTTGAGCTCCATCACATCAGGCATTGAGA	20
23	aceE- lplA1	CGCTTATTTAGAAAGTGTCAACAACGTATCTACAGCTCTCAG GCTAATCTA	21
24	aceE- lplA2	GAAAGCATAGCAATCTAATCTAAGTTTAAATTACAACGCAA AACAATGTCAGA	22
25	aceE- lplA3	GCGTTGTAATTAAAACTTAG	23
26	aceE- lplA4	TCCTTCCTTTTCGGTTAGAGCGGATCTATCTTACAGCACCA GC	24
27	aceF_lp d_1	CGCTTATTTAGAAAGTGTCAACAACGTATCTACAGAGCTCTT ACATGACT	25

28	aceF_lp d_2	TCCTTCCTTTTCGGTTAGAGCGGATACCTACTTCTTCTTGGC T	26
29	G1- lipA-fw	AGCGATACGUATGAGTAAACCCATTGTGATGGAA	27
30	G1- lipA-rv	CACGCGAUTTACTTAACTTCCATCCCTTTCGC	28
31	G2- lipB-fw	ATCAACGGGUATGTATCAGGATAAAATTCTTGTCCG	29
32	G2- lipB-rv	CGTGCGAU TTAAGCGGTAATATATTCGAAGTCC	30
33	P1- TEF1	ACGTATCGCUTTGTAAATTA AAACTTAGATTAGATTGCTATG	31
34	P2- PGK1	ACCCGTTGAUTTGT TTTATATTTGTTGTAAAAAGTAGATAA TTAC	32
35	G1- PDA1- fw	AGCGATACGUATGGCAACTTTAAAAACA ACTGATAAG	33
36	G1- PDA1- rv	CACGCGAUTTAATCCCTAGAGGCAAAACCTTG	34
37	G2- LPD1- fw	ATCAACGGGUATGGTAGTCATCATCGGTGGTGGC	35
38	G2- LPD1- rv	CGTGCGAUTCAACAATGAATAGCTTTATCATAGGC	36
39	G1- PDB1- fw	AGCGATACGUATGTCATCAACAAAGACGATGACCG	37

40	G1-PDB1-rv	CACGCGAUTTATTCAATTGACAAGACTTCTTTGAC	38
41	G2-PDX1-fw	ATCAACGGGUATGTCAGCTAAATTACTTGCTGTAAAGACA	39
42	G2-PDX1-rv	CGTGCGAUTCAAAATGATTCTAACTCCCTTACG	40
43	G1-LAT1-fw	AGCGATACGUATGTACGCATCGTACCCAGAGCA	41
44	G1-LAT1-rv	CACGCGAUTCACAATAGCATTTCCAAAGGATT	42
45	G1-LIP2-fw	AGCGATACGUATGTTCAATGTCTGTAGGCGACAATG	43
46	G1-LIP2-rv	CACGCGAUTCACGGATTCTTTTTCAAATATTG	44
47	G2-LIP3-fw	ATCAACGGGUATGGTGGGACAGCGAAACCTCATA	45
48	G2-LIP3-rv	CGTGCGAUTTATGTGTAAGTGTCAATATTCTCCAAA	46
49	G1-LIP5-fw	AGCGATACGUATGAATGCATTGAATACTGATTCAGATAAC G	47
50	G1-LIP5-rv	CACGCGAUTTATTTTCATGTTTCTTTTCTTCAAAACG	48
51	G2-GCV3-fw	ATCAACGGGUATGACTTCCCAACATGAGTGGATAGC	49

52	G2- GCV3- rv	CGTGCGAUTCAGTCATCATGAACCAGTGTCTTT	50
53	TEF1- fw	CGTGCGAUGCACACACCATAGCTTCAAAATG	51
54	G1- FDH1- fw	AGCGATACGUATGTCGAAGGGAAAGGTTTGTG	52
55	G1- FDH1- rv	CACGCGAUTTATTTCTTCTGTCCATAAGCTCTGG	53
56	G1- Crpfl- fw	AGCGATACGUATGTTAACACCCTTAAGCTATCCTATC	54
57	G1- Crpfl-rv	CACGCGAUTTACATGGTGTCTGGAAGGTG	55
58	G2- CrpflA- fw	ATCAACGGGUATGTTGAAGGCTGCGTTGC	56
59	G2- CrpflA- rv	CGTGCGAUTCACTCGGCGCAGATGACG	57
60	fdx- Not1-fw	ATCGAAGCGGCCGCAAAACAATGCCAAAGATTGTTATTTTG C	58
61	fdx- SacI-rev	ATCGTCGAGCTCTTAATGCTCACGCGCATG	59
62	fdx-F1	CAACAACGTATCTACCAACGGAATGCGTGCGATTTAATGCT CACGCGCATGGTTGATAG	60
63	f1dA-F1	CAACAACGTATCTACCAACGGAATGCGTGCGATTCAGGCA TTG AGAATTTCGTCGAGATG	61

64	FdR-R1	CTTTTCGGTTAGAGCGGATGAATGCACGCGTTACCAGTAAT GCTCCGCTGTCATA	62
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Table 2. Codon optimized gene sequences (start and stop codons are underlined)

aceE	GAATTCGCATAGCAATCTAATCTAAGTTTAAATTACAAGCGGCC GCAAAACA <u>AT</u> GTCAGAAAGATTCCCAAACGATGTCGATCCAATAGAAACAA GAGACTGGTTACAAGCAATAGAAAGTGCATAAGAGAAGAAG GTGTTGAAAGAGCACAAATATTTGATCGATCAATTGTTAGCCGAA GCTAGAAAAGGTGGTGTCAATGTTGCTGCAGGTACTGGTATCTC TAACTACATCAACACAATACCAGTTGAAGAACAACCAGAATAC CCTGGTAATTTGGAATTAGAAAGAAGAATTAGATCAGCAATCA GATGGAACGCCATTATGACCGTTTTGAGAGCTTCCAAAAAGGA TTTGGAATTAGGTGGTCATATGGCAAGTTTTCAATCTTCAGCCA CTATATACGATGTATGTTTCAACCACTTTTTTCAGAGCAAGAAAC GAACAAGATGGTGGTGACTTAGTATACTTTCAAGGTCATATTTT TCCAGGTGTCTATGCAAGAGCCTTCTTGGAAGGTAGATTAAACAC AAGAACAATTGGATAACTTCAGACAAGAAGTACATGGTAACGG TTTATCCAGTTATCCACACCCTAAATTGATGCCAGAATTTTGGC AATTCCTACTGTCTCTATGGGTTTAGGTCCTATAGGTGCAATC TACCAAGCCAAATTCTTGAAGTATTTGGAACATAGAGGTTTGAA GGATACATCTAAGCAAACCGTTTACGCATTTTTGGGTGACGGTG AAATGGACGAACCAGAATCAAAAGGTGCTATAACTATCGCAAC AAGAGAAAAGTTGGATAATTTGGTATTCGTCATTAATTGCAACT TGCAAAGATTAGACGGTCCTGTTACTGGTAACGGTAAAATTATA AACGAATTGGAAGGTATCTTTGAAGGTGCTGGTTGGAATGTAA TCAAGGTCATGTGGGGTTCTAGATGGGATGAATTGTAAAGAAA AGACACTTCTGGTAAATTGATCCAATTGATGAACGAAACAGTC GATGGTGACTATCAAACCTTTAAATCAAAGGATGGTGCATACG TTAGAGAACATTTCTTTGGTAAATATCCAGAAACCGCCGCTTTA GTAGCTGATTGGACTGACGAACAAATCTGGGCATTGAATAGAG GTGGTCATGATCCAAAGAAAATATATGCAGCCTTTAAAAAGGC TCAAGAAACCAAGGGTAAAGCTACTGTTATATTAGCACACACA ATCAAAGGTTACGGTATGGGTGACGCTGCAGAAGGTAAAAATA TCGCACATCAAGTCAAAAAGATGAACATGGATGGTGTAGACA CATCAGAGACAGATTCAATGTTCCAGTATCCGATGCTGACATCG AAAAATTGCCATACATCACATTTCTGAAGGTAGTGAAGAACA TACCTACTTGCACGCACAAAGACAAAAATTGCATGGTTATTTGC CATCTAGACAACCTAACTTTACTGAAAAGTTGGAATTACCATCA TTGCAAGATTTCCGGTGCTTTGTTGGAAGAACAATCTAAGGAAAT CTCAACTACAATTGCCTTTGTAAGAGCTTTGAACGTCATGTTGA AAAATAAGTCCATTAAGGATAGATTGGTTCCTATCATTGCCGAC GAAGCTAGAACTTTTGGTATGGAAGGTTTGTTCAGACAAATTGG	63
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TATATACAGTCCAAATGGTCAACAATATACACCTCAAGATAGA GAACAAGTTGCCTATTACAAAGAAGACGAAAAGGGTCAAATAT TGCAAGAAGGTATAAACGAATTAGGTGCAGGTTGTTCTGGTT GGCCGCTGCAACTTCTTACTCAACAAACAACCTACCAATGATCC CTTTCTACATCTATTACAGTATGTTTCGGTTTCCAAAGAATTGGT GACTTGTGCTGGGCCGCTGGTGACCAACAAGCTAGAGGTTTCTT GATAGGTGGTACATCTGGTAGAACCACCTTGAATGGTGAAGGT TTACAACATGAAGATGGTCATTCCCACATTCAAAGTTTGACCAT TCCAAACTGTATATCATATGATCCTGCTTACGCATATGAAGTCG CTGTTATAATGCATGACGGTTTAGAAAGAATGTACGGTGAAAA GCAAGAAAACGTTTACTACTACATCACAAACCTTGAATGAAAAC TATCACATGCCAGCCATGCCTGAAGGTGCTGAAGAAGGTATCA GAAAAGGTATCTATAAGTTAGAAACAATCGAAGGTTCCAAGGG TAAAGTTCAATTGTTAGGTTCCGGTAGTATTTTAAGACATGTTA GAGAAGCAGCCGAAATATTGGCTAAAGATTACGGTGTTGGTTC TGACGTTTATTCCGTAACCAGTTTCACTGAATTAGCAAGAGATG GTCAAGACTGCGAAAGATGGAATATGTTGCACCCATTAGAAAC TCCAAGAGTTCCTTACATTGCTCAAGTAATGAACGATGCCCCTG CTGTTGCATCTACTGACTATATGAAATTGTTTGCCGAACAAGTC AGAACATACGTTCCAGCTGATGACTATAGAGTCTTAGGTACCG ATGGTTTCGGTAGATCTGACTCAAGAGAAAATTTGAGACATCA CTTTGAAGTTGATGCATCATATGTTGTAGTCGCTGCATTGGGTG AATTAGCCAAGAGAGGTGAAATTGATAAAAAGGTTGTAGCCGA CGCAATAGCAAAGTTCAACATAGACGCTGACAAGGTAAACCCT AGATTAGCCTGA GAGCTCTTAATTAACAATTCTTCGCCAGAGGAATTC	
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aceF	GAATTCGCATAGCAATCTAATCTAAGTTTAAATTACAAGCGGCC GCAAAACA <u>ATGGCAATAGAAATCAAAGTTCAGACATAGGTGCAGACGAAG</u> TAGAAATAACAGAAATCTTAGTCAAAGTTGGTGACAAGGTAGA AGCTGAACAATCTTTGATTACTGTTGAAGGTGACAAAGCATCCA TGGAAGTTCCAAGTCCTCAAGCTGGTATAGTAAAGGAAATTAA AGTTTCTGTAGGTGACAAGACCCAACTGGTGCCTTAATCATGA TTTTTGATTGAGCTGACGGTGCTGCAGACGCCGCTCCAGCACAA GCCGAAGAAAAGAAAGAAGCAGCCCCAGCTGCAGCCCCTGCTG CAGCCGCTGCAAAAGATGTCAATGTTTCTGATATCGGTTTCAGAC GAAGTAGAAGTCACTGAAATTTTGGTTAAAGTAGGTGACAAGG TAGAAGCAGAACAAATCTTTAATTACAGTCGAAGGTGACAAAGC ATCTATGGAAGTCCCAGCCCCCTTTGCTGGTACAGTTAAAGAAA TCAAGGTCAATGTTGGTGACAAGGTTTCTACCGGTTTCATTGATT ATGGTTTTTCGAAGTAGCAGGTGAAGCAGGTGCCGCTGCACCAG CCGCTAAACAAGAAGCTGCCCCCTGCTGCAGCCCCAGCTCCTGCT GCAGGTGTTAAGGAAGTAAACGTCCCAGATATTGGTGGTGACG AAGTTGAAGTAACCGAAGTCATGGTCAAGGTAGGTGACAAGGT TGCCGCTGAACAATCCTTAATTACTGTAGAAGGTGACAAAGCT AGTATGGAAGTTCAGCTCCTTTTGCAGGTGTTGTAAAAGAATT GAAGGTCAACGTTGGTGACAAAGTTAAGACTGGTTCCTTAATA ATGATCTTCGAAGTTGAAGGTGCTGCCCCAGCTGCAGCCCCCTGC AAAACAAGAAGCTGCAGCCCCAGCTCCTGCTGCGAAGGCTGAA GCACCAGCCGCTGCACCTGCCGCTAAAGCTGAGGGTAAATCTG AATTTGCCGAAAATGATGCTTATGTTTCATGCAACACCATTGATT AGAAGATTAGCAAGAGAATTTGGTGTTAACTTGGCTAAAGTAA AGGGTACCGGTAGAAAGGGTAGAATTTTAAGAGAAGATGTCCA AGCATATGTTAAAGAAGCCATAAAGAGAGCTGAAGCTGCCCCA GCTGCTACAGGTGGTGGTATACCTGGCATGTTGCCATGGCCTAA AGTTGACTTTTCTAAGTTCGGTGAAATTGAAGAAGTTGAATTGG GTAGAATACAAAAGATTTCTGGTGCAAATTTGTCAAGAACTG GGTCATGATACCACATGTTACTCACTTTGATAAAACAGACATCA CCGAATTGGAAGCCTTTAGAAAACAACAAAATGAAGAAGCCGC TAAGAGAAAGTTAGATGTTAAGATCACACCAGTCGTTTTTATTA TGAAGGCAGTTGCAGCCGCTTTGGAACAAATGCCTAGATTCAA CTCTTCATTATCTGAAGACGGTCAAAGATTGACCTTGAAGAAAT ACATCAACATCGGTGTAGCTGTCGATACTCCAAACGGTTTGGTA GTCCCTGTTTTTAAAGACGTCAATAAGAAAGGTATCATCGAATT GTCCAGAGAATTAATGACAATCAGTAAAAAGGCTAGAGATGGT AAATTAAGTGCAGGTGAAATGCAAGGTGGTTGTTTTACAATATC CAGTATCGGTGGTTTGGGTACTACACATTTTCGCACCAATAGTAA ATGCACCTGAAGTCGCCATCTTAGGTGTTTCTAAATCAGCTATG GAACCAGTATGGAACGGTAAAGAATTTGTCCCAAGATTGATGT TGCCTATTTCTTAAGTTTCGATCACAGAGTTATAGATGGTGCT GACGGTGCCAGATTCATCACCATCATCAACAACACTTTATCCGA TATTAGAAGATTAGTCATGTA <u>A</u>	64
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	GAGCTCTTAATTAACAATTCTTCGCCAGAGGAATTC	
lpd	GAATTCATTATCTACTTTTTACAACAAATATAAAACAAGGATCC AAAACA ATGTCAACAGAAATCAAGACTCAAGTCGTAAGTATTAGGTGCCG GTCCAGCAGGTTACTCCGCAGCATTAGATGTGCAGACTTAGGT TTGGAAACAGTAATAGTCGAAAGATACAACACCTTGGGTGGTG TTTGTTTAAACGTAGGTTGCATCCCATCTAAAGCATTGTTACAT GTTGCCAAGGTAATTGAAGAAGCCAAAGCATTGGCAGAACACG GTATAGTTTTTGGTGAACCTAAGACCGATATCGACAAGATCAG AACTTGGAAGGAAAAGGTTATTAATCAATTGACTGGTGGTTTA GCTGGTATGGCAAAGGGTAGAAAAGTTAAGGTTGTAAACGGTT TGGGTAAATTCACAGGTGCAAATACCTTAGAAGTCGAAGGTGA AAACGGTAAAACAGTTATAAATTTTCGATAACGCTATTATAGCTG CAGGTTCCAGACCAATCCAATTGCCATTCATTCCTCATGAAGAT CCTAGAATTTGGGATAGTACAGACGCTTTGGAATTAAAGGAAG TACCAGAAAGATTGTTAGTCATGGGTGGTGGTATCATTGGTTTG GAAATGGGTACCGTTTACCACGCTTTAGGTTCCCAAATTGATGT CGTTGAAATGTTTGACCAAGTTATACCTGCCGCTGATAAAGACA TCGTTAAGGTTTTTACTAAGAGAATAAGTAAAAAGTTCAATTTG ATGTTAGAAACCAAGGTCCTGCCGTTGAAGCTAAAGAAGATG GTATCTATGTTACAATGGAAGGTAAAAAGGCCCCAGCTGAACC TCAAAGATACGATGCTGTCTTGTTGCAATCGGTAGAGTTCCAA ATGGTAAAAATTTGGACGCTGGTAAAGCCGGTGTAGAAGTCGA TGACAGAGGTTTTATTAGAGTAGATAAGCAATTGAGAACTAAC	65

	GTCCACATATTTTCGCAATAGGTGACATCGTTGGTCAACCTAT GTTAGCCCAAAAGGTGTACATGAAGGTCACGTCGCAGCCGAA GTTATTGCTGGTAAAAAGCATTACTTCGATCCAAAGGTTATTCC TTCTATAGCTTACACTGAACCAGAAGTAGCATGGGTCGGTTTGA CAGAAAAAGAAGCCAAAGAAAAAGGGTATTTTCATATGAACTGC TACATTTCCCTTGGGCTGCATCTGGTAGAGCAATCGCCTCAGATT GTGCTGACGGTATGACCAAGTTAATCTTCGATAAGGAATCTCAT AGAGTTATCGGTGGTGCAATTGTAGGTACTAATGGTGGTGAATT GTTAGGTGAAATAGGTTTGGCTATCGAAATGGGTTGCGATGCC GAAGACATTGCTTTAACTATACATGCACACCCAACATTGCATGA ATCAGTTGGTTTAGCTGCTGAAGTATTTGAAGGTAGTATAACCG ATTTGCCTAACCTAAAGCCAAGAAGAAGTAG GTCGACATGGAACAGAAGTTGATTTCCGAAGGAATTC	
lplA	GAATTCATTATCTACTTTTACAACAAATATAAAAACAAGGATCC AAAACA ATGTCCACCTTGAGATTATTGATTAGTGACTCCTATGACCCATG GTTCAACTTAGCCGTCGAAGAATGTATCTTCAGACAAATGCCAG CTACCCAAAGAGTTTTATTTTTGTGGAGAAATGCAGATACAGTT GTAATTGGTAGAGCCCAAATCCATGGAAGGAATGTAACACTA GAAGAATGGAAGAAGACAATGTTAGATTGGCTAGAAGATCTTC AGGTGGTGGTGCAGTATTCCATGATTTGGGTAACACTTGCTTTA CATTATGGCTGGTAAACCTGAATATGACAAGACCATCTCTACT TCAATAGTATTGAACGCCTTGAACGCTTAGGGTGTCTCCGCCGA AGCTAGTGGTAGAAATGATTTGGTCGTTAAGACTGTTGAAGGT GACAGAAAAGTATCCGGTAGTGCATATAGAGAAACCAAGGATA GAGGTTTTTCATCACGGTACTTTGTTATTGAATGCCGATTTGTCC AGATTAGCTAACTACTTGAACCCAGACAAAAAGAAATTAGCTG CAAAGGGTATCACATCTGTAAGATCAAGAGTCACAACTTGAC CGAATTATTGCCTGGTATAACACATGAACAAGTTTGTGAAGCTA TCACCGAAGCATTTTTCGCCCACTATGGTGAAAGAGTTGAAGCA GAAATCATCTCTCCAAATAAGACTCCAGATTTGCCTAACTTTGC TGAAACATTGCAAGACAATCCAGTTGGGAATGGAATTTTGGT CAAGCACCTGCCTTCTCACATTTGTTGGATGAAAGATTCACTTG GGGTGGTGTGCAATTGCATTTGACGTTGAAAAAGGTCACATA ACTAGAGCACAAGTTTTTACAGATTCTTTGAACCCAGCCCCTTT GGAAGCATTGGCAGGTAGATTGCAAGGTTGTTTATACAGAGCC GATATGTTACAACAAGAATGCGAAGCATTGTTGGTTGACTTCCC TGAACAAGAAAAGGAATTGAGAGAATTGAGTGCCTGGATGGCT GGTGCTGTAAGATAG CTCGAGTAAGCTTGGTACCGCGGCTAGCTAAGAATTC	66

<i>aceE_{av}</i>	ATAAGAATGCGGCCGCATGGAACAAGACATGCAAGACATGCAA GACTTGGACCCAATCGAAACTCAAGAATGGTTGGACTCTTTGG AATCTGTTTTGGACCACGAAGGTGAAGAAAGAGCTCACTACTT GTTGACTAGAATGGGTGAATTGGCTACTAGAACTGGTACTCAAT TGCCATACGCTATCACTACTCCATACAGAAACACTATCCCAGTT ACTCACGAAGCTCACATGCCAGGTGACTTGTTTCATGGAAAGAA GAATCAGATCTTTGGTTAGATGGAACGCTTTGGCTACTGTTATG AGAGCTAACAAGAAGGACCCAGACTTGGGTGGTCACATCTCTA CTTTCGCTTCTTCTGCTACTTTGTACGACATCGGTTTCAACTACT TCTTCCAAGCTCCAAGTGTGAACACGGTGGTGACTTGATCTAC TTCCAAGGTCACGCTGCGCCAGGTGTATACGCAAGAGCTTTCTT GGAAGGTAGAATCTCTGAAGCTCAATTGCAACAATTCAGACAA GAAGTTGACGGTGACGGTTTGTCTTCTTACCCACACCCACACTT GATGCCAGACTTCTGGCAATTCCCAACTGTTTCTATGGGTTTGG GTCCAATCCAAGCTATCTACCAAGCTAGATTCATGAAGTACTTG GAACACAGAGGTTTCATCCCAGCGGGTAAGCAGAAGGTGTGGT GCTTCATGGGCGACGGTGAATGTGACGAACCAGAATCTTTGGG TGCTATCTCTTTGGCTGGTAGAGAAAAGTTGGACAACCTTGATCT TCGTTATCAACTGTAACCTTGCAAAGATTGGACGGTCCAGTTAGA GGTAACGGTAAGATCATCCAAGAACTCGAGGGTGTGTTTCAGAG GGGCTCAATGGAACGTTAACAAGGTGTTTGGGGTAGATTCTG GGACCCATTGTTGCTAAGGACCACGCTGGTTTGTGCAACAAA GAATGGACGAAGTTGTTGACGGTGACTACCAAACTACAAGGC TAAGGACGGTGCTTTCGTTAGAAAGCACTTCTTCGGTGCTAGAC CAGAATTGTTGGAATTGGTTAAGGACATGTCTGACGAAGACAT CTGGAAGTTGAACAGAGGTGGTCACGACCCATACAAGGTTTAC GCTGCTTACCACCAAGCTGTTAACCACCAAGGTCAACCATCTGT TATCTTGGCTAAGACTATCAAGGGTTACGGTACTGGTGCTGGTG AAGCTAAGAACATCGCTCACAACGTTAAGAAGGTGATGTAGA AAGCTTGAAGTTGTTCCGTGACAAGTTCGACGTTCCATTGAAGG ACGAAGAATTGGAAGACTTGCCATTCTACAGACCAGACGAAAA CTCTCCAGAAATGAAGTACTTGAGATCTAGAAGAGAAGCTTTG GGTGGTTTCGTTCCACAAAGAAGAAGAAAGTCTATCTCTATCCC AACTCCACCATTGGACTCTTTGAAGGCTATCTTGGACGGTACTG GTGACAGAGAAATCTCTACTACTATGGCTTTCGTTAGAATCTTG GCTCAATTGGTTAAGGACAAGGAATTGGGTCTAGAATCGTTCC AATCATCCCAGACGAAGCTAGAAGTTTCGGTATGGAAGGTATG TTCAGACAATTGGGTATCTACTCTTCTGTTGGTCAATTGTACGA ACCAGTTGACAAGGACCAAGTTATGTTCTACAGAGAAGACAAG AAGGGTCAAATCTTGGAAGAAGGTATCAACGAAGCTGGTGCTA TGTCTTCTTGGATCTCTGCTGCTACTGCTTACTCTAACCACAACC AACCAATGTTGCCATTCTACGTTTTCTACTCTATGTTCCGTTTCC AAAGAATCGGTGACTTGGCTTGGGCTGCTGGTGACTCGCAAGC GAGAGGCTTCTTGATCGGTGGCACTGCTGGTAGAACTACTTTGA ACGGTGAAGGTTTGCAACACGAAGACGGTCACTCTCACATCTT	67
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	GGCTTCTACTATCCCAAACCTGTAGAACTTACGACCCAACTTACG CTTACGAAATGGCTGTTATCATCAGAGAAGGTATCAGACAAAT GACTGAAGAACAACAAAACGTTTTCTACTACATCACTGCTATGA ACGAAGCTTACACTCAACCAGCTATGCCAGAAGGTGCTGAAGC TGGTATCGTTAAGGGTATGTACTTGTGGAAGAAGACAAGAGA GACGCTGCTCACCACGTTCAATTGTTGGGTTCTGGTACTATCTT GAGAGAAGTTAGAGAAGCTGCTAAGATCTTGAGAGAAGACTAC AACGTTGCTGCTGACGTTTGGTCTGTTACTTCTTTCAACGAATTG AGAAGAAACGGTTTGGCTGTTGAAAGAAGAAACAGATTGCACC CAGAACAAAAGCCAGAACAATCTTACGTTGAACAATGTTTGAA CGGTAGAAAGGGTCCAGTTGTTGCTTCTACTGACTACATGAAGT TGTTGCTGACCAAATCAGACAATGGGTTCCATCTAGAGAATAC AAGGTTTTGGGTACTGACGGTTTCGGTAGATCTGACACTAGAAA GAAGTTGAGACACTTCTTCGAAGTTGACAGATACTGGGTTGTTT TGGCTGCTCTCGAGGCTCTCGCTGACAGAGGCGACATCGAAGC TAAGGTTGTTGCTGAAGCTATCGCTAAGTTTCGGTATCGACCCAG ACAAGAGAAACCCATTGGACTGTTAATTAAATTAAGG	
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<i>aceF_{av}</i>	ATAAGAATGCGGCCGCATGTCTGAAATCATCAGAGTTCCAGAC ATCGGTGGTGACGGTGAAGTTATCGAATTGTTGGTTAAGACTGG TGACTTGATCGAAGTAGAGCAAGGCTTGGTTGTTTTGGAGTCTG CTAAGGCTTCTATGGAAGTTCCATCTCCAAAGGCTGGTGTGTT AAGTCTGTTTCTGTAAAGTTGGGTGACAAGTTGAAGGAAGGTG ACGCTATCATCGAATTGGAACCAGCTGCTGGTGCTGCTGCTGCT CCAGCTGAAGCTGCTGCTGTTCCAGCTGCTCCAACCTCAAGCTGT TGACGAAGCTGAAGCTCCATCTCCAGGTGCTTCTGCTACTCCAG CTCCAGCTGCTGCTTCTCAAGAAGTTAGAGTTCCAGACATCGGT TCTGCTGGTAAGGCTAGAGTTATCGAAGTTTTGGTTAAGGCTGG TGACCAAGTTCAAGCGGAGCAGTCGTTGATCGTGCTAGAGTCT GACAAGGCTTCTATGGAAATCCCATCTCCAGCTTCTGGTGTGTT TGAATCTGTTGCTATCCAATTGAACGCTGAAGTTGGTACTGGTG ACTTGATCTTGACTTTGAGAACTACTGGTGCTCAAGCTCAACCA ACTGCTCCAGCTGCTGCTGCTGCTGCTTCTCCAGCTCCAGCTCC ATTGGCTCCAGCTGCTGCTGGTCCACAAGAAGTTAAGGTTCCAG ACATCGGTTCTGCTGGTAAGGCTAGAGTTATCGAAGTTTTGGTT AAGGCTGGTGACCAAGTTCAAGCGGAGCAGTCGTTGATCGTGC TAGAGTCTGACAAGGCTTCTATGGAAATCCCATCTCCAGCTGCT GGTGTGTTGAATCTGTTGCTGTTCAATTGAACGCTGAAGTTGG TACTGGTGACCAAATCTTGACTTTGAGAGTTGCTGGTGCTGCTC CAGCTGCTGCTCCAGCTCCAGCTGCTGCTCCAGCTAAGCCAGCT GCTGCTCCAGCTGCTGCTGCTCCAGCTCCAGCTCCAGTTGGTGC TCCATCTAGAAACGGTGCTAAGGTTACGCTGGTCCAGCTGTTA GACAATTGGCTAGAGAATTCGGTGTTGAATTGGCTGCTATCAAC TCTACTGGTCCAAGAGGTAGAATCTTGAAGGAAGACGTTCAAG CTTACGTTAAGGCTATGATGCAAAAGGCTAAGGAAGCTCCAGC TGCTGGTGCTGCTTCTGGTGCTGGTATCCCACCAATCCCACCAG TTGACTTCGCTAAGTACGGTGAAATCGAAGAAGTTCCAATGACT AGATTGATGCAAATCGGTGCTACTAACTTGACACAGATCTTGGTT GAACGTTCCACACGTTACTCAATTCGAATCTGCTGACATCACTG AATTGGAAGCTTTCAGAGTTGCTCAAAAGGCTGTTGCTGAAAA GGCTGGTGTAAAGTTGACTGTTTTGCCATTGTTGTTGAAGGCTT GTGCTTACTTGTGAAGGAATTGCCAGACTTCAACTCTTCTTTG GCTCCATCTGGTCAAGCTTTGATCAGAAAGAAGTACGTTACAT CGGTTTCGCTGTTGACACTCCAGACGGTTTGTGGTTCCAGTTA TCAGAAACGTTGACCAAAGTCTTTGTTGCAATTGGCTGCTGAA GCTGCTGAATTGGCTGAAAAGGCTAGATCTAAGAAGTTGGGTG CTGACGCTATGCAAGGCGCGTGCTTCACCATCTCTTCTTTGGGT CACATCGGCGGCACTGCGTTCCTCCAATCGTTAACGCTCCAGA AGTTGCTATCTTGGGTGTTTCTAAGGCTTCTATGCAACCAGTTT GGGACGGTAAGGCTTTCCAACCAAGATTGATGTTGCCATTGTCT TTGTCTTACGACCACAGAGTTATCAACGGTGCTGCTGCTGCTAG ATTCATAAGAGATTGGGTGACTTGTTGGCTGACATCAGAGCTA TCTTGTTGTAAAGAGCTCG	68
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<i>lpdA_{av}</i>	CGGGATCCATGTCTCAAAAGTTTCGACGTTATCGTTATCGGTGCT GGTCCAGGTGGTTACGTTGCTGCTATCAAGTCTGCTCAATTGGG TTTGAAGACTGCTTTGATCGAAAAGTACAAGGGTAAGGAAGGT AAGACTGCTTTGGGTGGTACTTGTGTTGAACGTTGGTTGTATCCC ATCTAAGGCTTTGTTGGACTCTTCTTACAAGTTCCACGAAGCTC ACGAATCTTTCAAGTTGCACGGTATCTCTACTGGTGAAGTTGCT ATCGACGTTCCAACATGATCGCTAGAAAGGACCAAATCGTTA GAAACTTGAAGTGGTGGTGTGCTTCTTTGATCAAGGCTAACGGT GTTACTTTGTTTGAAGGTCACGGTAAGTTGTTGGCTGGTAAGAA GGTTGAAGTTACTGCTGCTGACGGTCTTCTCAAGTTTTGGACA CTGAAAACGTTATCTTGGCTTCTGGTTCTAAGCCAGTTGAAATC CCACCAGCTCCAGTTGACCAAGACGTTATCGTTGACTCTACTGG TGCTTTGGACTTCCAAAACGTTCCAGGTAAGTTGGGTGTTATCG GTGCTGGTGTATCGGTTTGGAAATTGGGTTCTGTTTGGGCTAGA TTGGGTGCTGAAGTTACTGTTTTGGAAGCTATGGACAAGTTCTT GCCAGCTGTTGACGAACAAGTTGCTAAGGAAGCTCAAAGATC CTACTAAGCAAGGTCTCAAGATCTTGCTCGGTGCTAGAGTTAC TGGTACTGAAGTTAAGAACAAGCAAGTTACTGTAAAGTTCGTTG ACGCTGAAGGTGAAAAGTCTCAAGCTTTCGACAAGTTGATCGTT GCTGTTGGTAGAAGACCAGTTACTACTGACTTGTGTTGGCTGCTGA CTCTGGTGTACTTTGGACGAAAGAGGTTTCATCTACGTTGACG ACTACTGTGCTACTTCTGTTCCAGGTGTTTACGCTATCGGTGAC GTTGTTAGAGGTGCTATGTTGGCTCACAAGGCTTCTGAAGAAGG TGTTGTTGTTGCTGAAAGAATCGCTGGTCACAAGGCTCAAATGA ACTACGACTTGATCCCAGCTGTTATCTACACTCACCAGAAATC GCTGGTGTGTTGTAAGACTGAACAAGCTTTGAAGGCTGAAGGTG TTGCTATCAACGTTGGTGTGTTTCCCATTCGCTGCTTCTGGTAGAG CTATGGCTGCTAACGACACTGCTGGTTTTCGTTAAGGTTATCGCT GACGCTAAGACTGACAGAGTTTTGGGTGTTACGTTATCGGTCC ATCTGCTGCTGAATTGGTTCAACAAGGTGCTATCGCTATGGAAT TCGGTACTTCTGCTGAAGACTTGGGTATGATGGTTTTCGCTCAC CCAGCTTTGTTCGGAGGCTCTCCATGAGGCTGCGCTCGCTGTTTC TGGCCACGCTATCCACGTTGCTAACAGAAAGAAGTAAGTTCGAC GTCGG	69
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<i>pdhA</i>	CGAGCTCATGGCTAAGGCTAAGAAGCAAAAGCCAATCGACTTC AAGGAATTGATGGCTAAGGTTGACGCTGACTTCCCAACTTTCCA AATCTTGGACCAAGACGGTAAGATCGTTAACGAAGACTTGGTT CCAGACTTGTCTGACGAAGAATTGGTTGAATTGATGACTAGAAT GGTTTGGTCTAGAGTTTTGGACCAAAGATCTACTGCTTTGAACA GACAAGGTAGATTGGGTTTCTTCGCTCCAAGTCTGGTCAAGAA GCTTCTCAATTGGCTTCTCAATTCGCTATGGAAAAGGAAGACTA CTTGTGTCAGGTTACAGAGACGTTCCACAATTGGTTCAACACG GTTTGCCATTGAGAGAAGCTTTCTTGTGGTCTAGAGGTCACGTT GCTGGTAACTACTACGCTGAAGACTTGAACGCTTTGCCACCACA AATCATCATCGGTGCTCAATACATCCAAGCTGCTGGTGGTTGCTT TGGGTTTGAAGAAGAGAGGTAAGGAAAACGTTGTTTTCACTTA CACTGGTGACGGTGGTTCTTCTCAAGGTGACTTCTACGAAGCTA TCAACTTCGCTGGTGCTTACCAAGCTAACGGTGTTTTCATCATC CAAAACAACGGTTTCGCTATCTCTACTCCAAGAGAAAAGCAAA CTGCTGCTAAGACTTTGGCTCAAAAGGCTGTTGCTGCTGGTATC CCAGGTATCCAAGTTGACGGTATGGACCCATTGGCTGTTTACGC TATCGCTAAGGAAGCTAGAGACTGGTCTGCTGCTGGTAACGGT CCAGTTCTCATAGAACTCTCACTTACAGATACGGTCCACACAC TTTGTCTGGTGACGACCCAAGTACAGATCTAAGGAAATG GACGACGAATGGGTTCAAAAGGACCCATTGACTAGATTCAGAA AGTACCTCACTGACAAGGGCTTGTGGTCTGAAGCGAAGGAAGA AGAAATCATCGAAAAGACTAAGGAAGAAATCAAGGTTGCTATC GCTGAAGCTGACAAGGCTCCAAAGCAAAAGGTTTCTGACTTCTT GAAGAACATGTTTCGAAGTTCAACCACAACTATCAAGGAACAA ATCGCTTTCTACGAAGCTAAGGAATCTAAGTAAGCGGCCGCTA AACTAT	70
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<i>pdhB</i>	CGGGATCCATGGCTCAAAAGACTATGATCCAAGCTATCACTGAC GCTTTGGCTTTGGAATTGGAAAAGGACGAAAACGTTTTGATCTT CGGTGAAGACGTTGGTAACAACGGTGGTGTTCAGAGCTACT GAAGGTTTGCAAGAAAAGTTCGGTGAAGACAGAGTTTTCGACA CTCCATTGGCTGAATCTGGTATCGGTGGTTTGGCTTTCGGTTTG GCTTTGCAAGGTTACAGACCAGTTCAGAAATCCAGTTCTTTGG TTTCGTCTTCGAAGTGTTTCGACGAAATCGTTGGTCAAATGGCTA GAACTAGATACAGAATGGGTGGTACTAGAAACATGCCAATCAC TGTTAGAGCTCCATTCGGTGGTGGTGTTCACACTCCAGAATTGC ACTCTGACAACTTGGAAGGTTTGATCGCTCAATCTCCAGGTGTT AGAGTTGTTATCCCATCTAACCATAACGACGCTAAGGGTTTGTT GATCTCTTCTATCAGATCTAACGATCCAGTTGTATACCTGGAAC ACATGAAGTTGTACAGATCTTTCAGAGAAGAAGTTCCAGACGA AGCTTACGAAGTTCCATTGGACAAGGCTGCTGTTACTAGAGAA GGTACTGACGTTTCTATCATCACTTACGGTGCTATGGTTAGAGA AGCTATCAAGGCTGCTGACTCTTTGGCTAAGGACAACATCTCTG CTGAAATCATCGACTTGAGAACTGTTGCTCCATTGGACGTTGAA ACTATCATCAACTCTGTTGAAAAGACTGGTAGAGTTGTTGTTGT TCAAGAAGCTCAAAAGCAAGCTGGTGTGGTGCTATGGTTGTTT CTGAAATCTCTGAGCGTGCTGTGCTATCTTTGGAAGCTCCAATC GGTAGAGTTTCTGCTCCAGACACTATCTTCCCATTCGGTCAAGC TGAAAACATCTGGTTGCCAAACGCTAAGGACATCGAAGCTAAG GCTAGAGAAATCGTTGAATTCTAACTCGAGCGG	71
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<i>aceF_{ef}</i>	CGAGCTCATGGCTTACCAATTCAAGTTGCCAGACATCGGTGAAG GTATCGCTGAAGGTGAAATCGTTAAGTGGTTCGTTAAGCCAGGT GACACTATCAACGAAGACGACACTTTGTTGGAAGTTCAAAACG ACAAGTCTGTTGAAGAAATCCCATCTCCAGTTACTGGTACTGTT AAGAACATCGTTGTTCCAGAAGGTACTGTTGCTAACGTAGGCG ACGTGCTAATCGAAATCGACGCTCCAGGTCACGAAGACAACGA CGCTGCTCCAGCTGCTCCAGCTCAAGAACAACCTCCAGCTCAAC CAGCTGCTGTTCCAACACTACTGAAGCTGCTGGTGGTTTCTTCCAA TTCAAGTTGCCAGACATCGGTGAAGGTATCGCTGAAGGTGAAA TCGTTAAGTGGTTCGTTAAGGCTGGTGACACTATCAACGAAGAC GACTCTTTGTTGGAAGTTCAAAACGACAAGTCTGTTGAAGAAAT CCCATCTCCAGTTACTGGTACTGTTAAGAACATCGTTGTTCCAG AAGGTACTGTTGCTAACGTAGGCGACGTCCTCGTTGAAATCGAC GCTCCAGGTCACAACTCTGCTGCTCCATCTGTTGCTGCTCCAGC TACTGACGCTCCAAAGGCTGAAGCTTCTGCTCCAGCTGCTTCTA CTGGTGTGTTGCTGCTGCTGACCCAAACAAGAGAGTTTTGGCT ATGCCATCTGTTAGACAATACGCTAGAGAAAAGGACGTTGACA TCACTCAAGTTACTGCTACTGGTAAGGGTGGTAGAGTTATCAAG GCTGACATCGACGCTTTCGTTTCTGGTGGTTCTCAAGCTGCTCC AGCTACTGAAGCTGCTGCTACTGAAGCTGCTCCAAAGGCTGAA GCTGCTGCTCCAAAGGCTGCTCCAAAGGCTTTCATTCTGACTT GGGTGAAATGGAACTAGAGAAAAGATGACTCCAAGTAGAAA GGCTATCGCTAAGGCTATGGTTAACTCTAAGCACACTGCTCCAC ACGTTACTTTGCACGATGAAGTTGAAGTAAGCAAGTTGTGGGA CCACAGAAAGAAGTTCAAGGACGTTGCTGCTGCTAACGGTACT AAGTTGACTTTCTTGCCATACGTTGTTAAGGCTTTGACTTCTACT GTTCAAAAGTTCCCAATCTTGAACGCTTCTATCGACGACGCTGC TCAAGAAATCGTTTACAAGAATACTTCAACATCGGTATCGCTA CTGACACTGACCACGGTTTGTACGTTCCAAACGTTAAGAACGCT AACACTAAGTCTATGTTGCTATCGCTGACGAAATCAACGAAA AGGCTGCTTTGGCTATCGAAGGTAAGTTGACTGCTCAAGACATG AGAGACGGTACTATCACTATCTCTAACATCGGTTCTGTTGGTGG TGGTTGGTTCACTCCAGTTATCAACTACCCAGAAGTTGCTATCT TGGGTGTTGGTACTATCGCTCAAGAACCAGTTGTTAACGCTGAC GGTGAAATCGTTGTTGGTAGAATGATGAAGTTGTCGCTCAGCTT CGACCACAGAATAGTTGACGGTGCTACTGCTCAAAGGCTATG AACACATCAAGAGATTGTTGGCTGACCCAGAATTGTTGTTGAT GGAAGGTTAAGCGGCCGCTAAACTAT	72
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<i>lpdA_{ef}</i>	CGGGATCCATGGTTGTTGGTGACTTCGCTATCGAATTGGACACT GTTGTTATCGGTGCTGGTCCAGGTGGTTACGTTGCTGCTATCAG AGCTGCTGAAATGGGTCAAAAGGTTGCTATCATCGAAAGAGAA TACATCGGCGGCGTTTGCCTCAACGTTGGTTGTATCCCATCTAA GGCTTTGATCGCTGCTGGTCACCACTACCAAGAAGCTCAAGACT CTTCTACTTTTCGGTGTACTGCTAAGGGTGTGAATTGGACTTC GCTAAGACTCAAGACTGGAAGGACAACACTGTTGTTAAGTCTTT GACTGGTGGTGTGGTATGTTGTTGAAGAAGCACAAAGGTTGAA ATCATCGAGGGCGAAGCGTTCTTCGTTGACGAGAACACTTTGA GAGTTATCCACCCAGACTCTGCTCAAACCTACTCTTTCAACAAC GCTATCGTTGCTACTGGTTCTAGACCAATCGAAATCCCAGGTTT CAAGTTCGGTGGTAGAGTTTTGGACTCGACTGGTGGTCTCAACT TGAAGGAGGTTCCAAAGAAGTTCGTTATCATCGGTGGTGGTGT ATCGGTGCTGAATTGGGTGGTGTACGCTAACTTGGGTCTGA AGTTACTATCTTGAAGGTTCTCCATCTATCTTGCCAACCTACG AAAAGGACATGGTTAAGGTTGTTACTGACCACTTCAAGAAGAA GAACGTTACTATCGTTACTTCTGCTATGGCTAAGGAAGCTGTTG ACAACGGTGACTCTGTTACTGTAAAGTACGAAGTTAACGGTAA GGAAGAATCTGTTGAAGCTGACTACGTTATGGTTACTGTTGGTA GAAGACCAAACTGACGACTTGGGTTTGAACAAGCTGGTGT TGAAATCGGTGAAAGAGGTTTGATCCCAGTTGACAACCAAGGT AGAACTAACGTTAAGAACATCTTCGCTATCGGTGACATCGTTCC AGGTGCTGCTTTGGCTCACAAGGCTTCTTACGAAGCTAAGATCG CTGCTGAAGCTATCTCTGGTAAGAAGGTTGCTGTTGACTACAAG GCTATGCCAGCTGTTGCTTTCACTGACCCAGAATTGGCTTCTGT TGGTATGACTGTTGCTGAAGCTAAGGAAGCTGGTATCGAAGCT AAGGGTTACAAGTTCCCATTCGCTGGTAACGGTAGAGCTATCTC TTTGGACAAGACTGAAGGTTTCATGAGATTGGTTACTACTGTTG AAGACAACGTTATCATCGGTGCTCAAATCGCTGGTGTGGTGCT TCTGACATGATCTCTGAATTGGCTTTGGCTATCGAATCTGGTAT GAACGCGGAAGACATCGCTCTCACTATCCACCCACACCCATCTT TGGGTGAAATCACTATGGACACTGCTGAATTGGCTTTGGGTTTG CCAATCCACATCTAAGTCGACGTCGG	73
<i>acpP</i>	CGGGATCCATGAGCACTATCGAAGAACGCGTTAAGAAAATTAT CGGCGAACAGCTGGGCGTTAAGCAGGAAGAAGTTACCAACAAT GCTTCTTTTCGTTGAAGACCTGGGCGCGGATTCTCTTGACACCGT TGAGCTGGTAATGGCTCTGGAAGAAGAGTTTGATACTGAGATT CCGGACGAAGAAGCTGAGAAAATCACCACCGTTCAGGCTGCCA TTGATTACATCAACGGGCCACCAGGCGTAACTCGAGCGG	74

<i>aasS</i>	ATAAGAATGCGGCCGCATGAACCAGTATGTAAATGATCCATCC AATTATCAGTTACTAATTAATAAACTTGCTGTTTTCCCCAGTTGCT TTCAACCCAGAGCAAGAAATCGTCTACGCTAACCACCGCCGTC ATAGCTACAAAACGTTTCATGATCGTGTAAGACAGTTTGCCAAC GCGCTTACCAAAATGGGCGTGAAAAAAGGCGATACGGTTGCTG TGATGGATTACGACTCCCACCGTTACCTTGAGTGTTACTTTGCT ATCCCAATGATTGGTGCCAAGCTGCACATGATTAACGTTTCGTTT GTCTCCAGAACAGATCCTTTACACCATTGATCATGCCGAAGATG ACATCATTCTTATTCATGAAGAGTTTCTACCAATCTTAGACCAG ATCAAAGGACGCATTGATACGGTCACTCGCTACGTTGTGTTACG TGATGATGAAGAGTGTGAGTATGAACGCCTTCTTGAACAGGAA AGCACGGAATACAATTTCCAGATTTTCGATGAAAACACGGTGG CGACAACCTTTCTACACCACGGGTACTACAGGATTTCCAAAAGG CGTTTTCTTCACGCATCGTCAACTTGTTCTTCATACTATGGGTAT ATTAAGCACAATCGGTACCAACGCTTCACAAGGTCGATTGCATC AAGGTGACATCTACATGCCGATTACGCCGATGTTTCATGTCCAT GCTTGGGGGCTTCCATATATGGCAACCATGCTTGGTGTTAAGCA AGTCTACCCGGGTAAAGTATGTTCCAGATGTTTTGCTTAACCTGA TTGAGCAAGAGAAGGTGACGTTCTCACACTGTGTGCCGACTATT TTGCATCTACTTCTGAGCTCTCCAAAGTCGAAGGCGATGGACTT TTCTGGGTGGAAAGTCGTCATTGGTGGTGCTGCGCTACCAAAAG CATTATGTAAATCAGCTCTAGAACGTGATATCGACGTATTTGCT GGTTACGGCATGAGTGAGACTGGACCTATTCTTTCTATCGTCCA ATTGACGCCTGAGCAATTAGAGTTAGATGTTCGACCAACAAGCG GAATATCGCTCGAAGACAGGTAAGAAAGTTGCGCTTGTAGAAG CGTATATTGTGGATGAGGATATGAACAAACTGCCTCATGATGG CGAAACCGCTGGTGAAATTGTTGTTTCGTGCACCTTGGTTAACAC CTAACTACTACAAAGACAACAAAACTCTAAAGCACTATGGCG TGGCGGTTACCTGCACACAGGTGATGTGGCGCATATTGATGAC GAAGGCTTTATCAAGATCACTGACCGCGTAAAAGATATGATTA AGATATCTGGTGAGTGGGTAAAGCTCTTTAGAGCTTGAAGACATT CTCCACCAGCATCAGAGTGTTTCCGAAGTTGCGGTGATTGGTAT GCCGCACAACAAGTGGGGTGAAGTGCCGTTAGCTTTGGTGACA TTGAAAGAAGACGCTCAAGTCACAGAAAAGGAACTGTTAGGTT TTGCGAAAGACTTCATCAATAAAGGGATTCTTGCTAGAGAAGC ATTACTACTTAAAGTGAAGATAGTGGACGAGATTGCTAAGACC AGCGTTGGTAAAGTGGATAAGAAAGAACTGCGTAAACTTCATC TGTAATTAATTAAGG	75
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Pfor	GTCGCTTATGCTATGTCTGAAGTCGCCGCTATCTATCCTATTACC CCATCTTCAACTATGGGTGAAGAAGCAGATGACTGGGCTGCAC AAGGTAGAAAGAATATTTTTGGTCAAACCTTGACTATAAGAGA AATGCAATCAGAAGCTGGTGCCGCTGGTGCTGTTTCATGGTGCAT TAGCAGCCGGTGCTTTGACTACAACCTTCACTGCATCCCAAGGT TTGTTGTTGATGATCCCAAACATGTACAAAATTAGTGGTGAATT ATTGCCTGGTGTATTTACGTTACCGCTAGAGCAATCGCTGCAC ATGCATTGTCTATTTTCGGTGACCACCAAGACATATATGCCGCT AGACAAACAGGTTTTGCAATGTTAGCCTCCAGTTCTGTACAAGA AGCTCATGATATGGCTTTAGTTGCACACTTGGCAGCCATTGAAT CTAACGTTCCTTTTATGCATTTCTTTGATGGTTTCAGAACATCAC ACGAAATACAAAAGATTGAAGTCTTAGATTACGCCGACATGGC TTCCTTAGTAAATCAAAAAGCCTTGGCTGAGTTTAGAGCAAAG AGTATGAATCCAGAACATCCTCACGTTAGAGGTACTGCTCAAA ACCCAGATATATACTTTCAAGGTAGAGAAGCTGCAAATCCTTAT TACTTGAAAGTACCAGGTATCGTTGCCGAATACATGCAAAAGG TAGCTTCTTTAACAGGTAGATCATAAAGTTGTTTGATTATGTT GGTGACCTGACGCCGAAAGAGTAATTGTTTCAATGGGTTTCATC CTGTGAAACTATCGAAGAAGTTATTAATCATTGGCCGCTAAGG GTGAAAAGATCGGTTTAATTAAAGTAAGATTGTACAGACCATT CGTTTCTGAAGCATTTTTTCGCAGCCTTGCTGCATCAGCCAAAG TCATTACTGTATTAGATAGAACAAGGAACCAGGTGCTCCAGG TGACCCATTATATTTGGACGTATGCTCTGCTTTTGTGAAAGAG GTGAAGCAATGCCAAAAATTTGGCCGGTAGATACGGTTTGGG TTCTAAAGAATTTTCTCCTGCAATGGTTAAGTCTGTCTATGATA ATATGTCAGGTGCTAAAAAGAACCATTTTACAGTTGGTATAGA AGATGACGTCACAGGTACTTCTTTGCCAGTTGATAATGCTTTTCG CAGACACTACACCTAAAGGTACTATTCAATGTCAATTTTGGGGT TTAGGTGCAGATGGTACAGTTGGTGCCAATAAGCAAGCTATAA AGATTATAGGTGACAACACTGACTTGTTTGCTCAAGGTTACTTC TCTTATGATTCCAAAAAGAGTGGTGGTATCACCATTTCTCATT GAGATTCGGTGAAAAGCCAATCCAATCACTTACTTAGTAAAC AGAGCAGATTACGTTGCCTGTCACAACCCTGCTTACGTCGGTAT CTATGATATTTTGAAGGTATCAAGGACGGTGGTACATTCGTTT TAAATTCTCCTTGGTCTTCTTTGGAAGATATGGACAAACATTTG CCTTCAGGTATAAAGAGAACCATCGCTAATAAGAAATTGAAGT TCTACAACATCGATGCTGTCAAGATCGCAACAGACGTAGGTTTA GGTGGTAGAATTAACATGATCATGCAAACCGCATTTTTCAAATT GGCCGGTGTCTTGCCATTCGAAAAGGCAGTTGATTTGTTGAAAA AGTCTATACACAAAGCCTACGGTAAAAAGGGTGAAAAGATTGT TAAGATGAATACCGATGCCGTTGATCAAGCTGTAACCTCCTTAC AAGAGTTTAAATATCCTGATAGTTGGAAGGACGCCCCAGCTGA AACCAAGGCTGAACCTATGACTAATGAATTTTTCAAGAACGTTG TCAAGCCAATATTGACTCAACAAGGTGACAAATTGCCTGTTTCT GCTTTTGAAGCAGACGGTAGATTCCCATTTGGGTACATCACAATT CGAAAAGAGAGGTGTTGCAATCAACGTCCCTCAATGGGTTCCA	76
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	GAAAATTGTATTCAATGCAACCAATGTGCTTTTGTGTTGTCCTCA TTCTGCTATTTTACCAGTTTGGCAAAAGAAGAAGATTAGTCG GTGCACCAGCCAATTTACAGCCTTGGAAGCTAAGGGTAAAGA ATTAAAGGGTTACAAGTTTAGAATACAAATCAATACCTTGGATT GCATGGGTTGTGGTAACTGCGCTGACATTTGCCACCTAAAGAA AAGGCATTAGTAATGCAACCATTGGATACTCAAAGAGACGCTC AAGTTCCTAATTTGGAATACGCTGCAAGAATACCAGTFAAATCT GAAGTCTTACCTAGAGATTCTTGAAGGGTAGTCAATTCCAAGA ACCATTGATGGAATTTTCTGGTGCTTGTTTCAGGTTGCGGTGAAA CTCCTTATGTCAGAGTAATTACACAATTGTTTCGGTGAAAGAATG TTCATCGCTAATGCAACTGGTTGTTTCATCCATTTGGGGTGCCTCT GCTCCTTCAATGCCATACAAAACAAATAGATTGGGTCAAGGTC CAGCTTGGGGTAACTCCTTATTTGAAGATGCCGCTGAATATGGT TTTGGTATGAACATGTCTATGTTTCGCCAGAAGAACACATTTGGC TGATTTGGCAGCCAAGGCTTTGGAATCCGATGCAAGTGGTGAC GTTAAAGAAGCATTACAAGGTTGGTTGGCCGGTAAAAATGATC CAATCAAATCTAAGGAATACGGTGACAAATTGAAAAAGTTATT GGCTGGTCAAAAGGATGGTTTATTGGGTCAAATTGCTGCAATGT CTGATTTGTACACTAAAAAGTCAGTTTGGATCTTCGGTGGTGAC GGTTGGGCATACGACATCGGTTATGGTGGTTTAGATCATGTTTT GGCTTCTGGTGAAGATGTTAATGTCTTCGTAATGGACACTGAAG TTTATTCAAACACCGGTGGTCAAAGTTCTAAAGCTACTCCAACA GGTGCAGTTGCCAAATTTGCTGCTGCTGGTAAAAGAACAGGTA AAAAGGATTTGGCAAGAATGGTTATGACTTATGGTTACGTTTAT GTCGCTACTGTTTCTATGGGTACAGTAAGCAACAATTCTTGAA GGTTTTGAAGGAAGCTGAATCTTTCCAGGTCCATCTTTGGTTA TTGCTTATGCAACCTGTATCAACCAAGGTTTAAGAAAGGGTATG GGTAAATCCCAAGATGTCATGAATACTGCTGTAAAAAGTGGTT ACTGGCCTTTGTTTCAGATATGATCCAAGATTAGCCGCTCAAGGT AAAAACCCATTTCAATTAGATTCCAAGGCACCTGACGGTAGTGT CGAAGAATTTTGTATGGCCCAAAATAGATTCGCTGTATTAGATA GATCATTTCCAGAAGACGCTAAAAGATTGAGAGCACAAGTTGC CCATGAATTGGATGTCAGATTCAAAGAATTAGAACACATGGCA GCCACTAATATTTTGAATCCTTCGCCCCTGCTGGTGGTAAAGC CGATGGTAGTGTTGACTTTGGTGAAGGTGCTGAATTTTGTACAA GAGATGACACCCCTATGATGGCAAGACCAGATTCAGGTGAAGC CTGCGACCAAAACAGAGCAGGTACATCAGAACAACAAGGTGAC TTATCCAAGAGAACCAAGAAATGA	
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<i>eutD</i> <i>pta</i> ^{R252H}	CGGGATCCATGTCTAGAATAATAATGTTGATCCCTACTGGTACT TCCGTCGGTTTGACCTCCGTATCCTTGGGTGTAATAAGAGCAAT GGAAAGAAAGGGTGTAGATTATCAGTCTTTAAACCAATCGCT CAACCTAGAGCAGGTGGTGACGCCCCAGACCAAACCTACAACCA TTGTTAGAGCTAATTCTACATTACCAGCTGCAGAACCTTTGAAG ATGTCTCATGTTGAATCATTGTTGTCTTCAAACCAAAAAGGATGT TTTGATGGAAGAAATCATCGCTAACTACCATGCAAACACTAAA GACGCTGAAGTTGTCTTAGTAGAAGGTTTGGTTCCTACAAGAAA GCACCAATTCGCACAATCTTTGAACTACGAAATAGCAAAAACC TTGAACGCCGAAATAGTATTCGTTATGTCACAAGGTACAGATAC CCCAGAACAATTGAACGAAAGAATCGAATTGACTAGATCCAGT TTCGGTGGTGCAAAGAATACTAACATAACAGGTGTTATTATTAA TAAGTTGAACGCTCCAGTTGATGAACAAGGTAGAACTAGACCT GACTTGTCTGAAATATTCGATGACTCTTCAAAGGCTCAAGTTAT TAAGATCGATCCAGCAAAGTTACAAGAATCCAGTCCATTACCT GTCTTGGGTGCTGTACCTTGGTCTTTCGATTTGATTGCCACAAG AGCTATAGACATGGCTAGACATTTGAATGCAACCATAATCAAC GAAGGTGACATAAAGACAAGACACGTCAAATCCGTAACCTTTT GTGCTAGAAGTATCCACATATGTTGGAACACTTCAGAGCCGGT TCTTTGTTAGTTACATCAGCTGATAGACCTGACGTCTTAGTAGC CGCTTGTGTTGGCAGCCATGAATGGTGTGAAATTGGTGCATTGT TATTGACTGGTGGTTATGAAATGGATGCCAGAATATCTAAGTTA TGCGAAAGAGCATTGTCACCGGTTTGCCAGTTTTTCATGGTCAA TACTAACACATGGCAAACCTTCTTTGTCATTGCAATCTTTTAATTT GGAAGTTCCTGTGATGACCATGAAAGAATCGAAAAGGTTCAA GAATACGTGCGCAATTACGTAAACGCTGAATGGATTGAATCCTT AACCGCAACTTCTGAAAGATCAAGAAGATTGAGTCCACCTGCT TTCAGATACCAATTGACAGAATTGGCAAGAAAGGCCGGTAAAA GAGTAGTTTTACCAGAAGGTGACGAACCTAGAACAGTTAAAGC TGCAGCCATCTGTGCAGAAAGAGGTATTGCCACCTGCGTTTTGT TGGGTAACCCAGATGAAATTAACAGAGTTGCTGCATCACAAGG TGTCGAATTGGGTGCAGGTATCGAAATTGTTGATCCTGAAGTCG TAAGAGAATCTTACGTTGCTAGATTAGTCGAATTGAGAAAATC AAAGGGTATGACTGAACCAGTTGCAAGAGAACAATTGGAAGAT AACGTTGTCTTGGGTACATTAATGTTGGAACAAGATGAAGTAG ACGGTTTAGTTTCTGGTGCTGTCCACACTACAGCAAACACCATA AGACCACCTTTACAATTGATCAAAACTGCTCCAGGTTCTTCATT GGTATCCAGTGTTTTCTTTATGTTGTTGCCTGAACAAGTATACGT TTACGGTGACTGCGCAATTAATCCAGACCCTACAGCCGAACAA TTGGCTGAAATAGCAATCCAATCCGCTGATAGTGCTATCGCATT TGGTATTGAACCAAGAGTTGCAATGTTATCCTATAGTACAGGTA CCTCCGGTGCTGGTAGTGATGTCGAAAAAGTTAGAGAAGCCAC TAGATTGGCTCAAGAAAAGAGACCAGATTTGATGATTGACGGT CCTTTGCAATACGATGCCGCTGTAATGGCCGACGTTGCTAAATC CAAGGCTCCAAATAGTCCTGTAGCAGGTAGAGCCACTGTTTTTA TTTTCCAGATTTGAACACTGGTAACACCCTTATAAGGCCGTT	77
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	CAAAGATCTGCTGACTTAATTTCAATAGGTCCAATGTTGCAGGG TATGAGAAAACCTGTTAACGATTTGTCCAGAGGTGCTTTGGTCG ATGACATAGTATACACCATCGCTTTGACTGCAATTCAAGCCTCT CAACAACAACAATAAGCTAGCTAG	
<i>pta</i> ^{G273} <i>D</i>	CGGGATCCATGTCTAGAATAATAATGTTGATCCCTACTGGTACT TCCGTCGGTTTGACCTCCGTATCCTTGGGTGTAATAAGAGCAAT GGAAAGAAAGGGTGTAGATTATCAGTCTTTAAACCAATCGCT CAACCTAGAGCAGGTGGTGACGCCCCAGACCAAACCTACAACCA TTGTTAGAGCTAATTCTACATTACCAGCTGCAGAACCTTTGAAG ATGTCTCATGTTGAATCATTGTTGTCTTCAAACCAAAAGGATGT TTTGATGGAAGAAATCATCGCTAACTACCATGCAAACACTAAA GACGCTGAAGTTGTCTTAGTAGAAGGTTTGGTTCCTACAAGAAA GCACCAATTCGCACAATCTTTGAACTACGAAATAGCAAAAACC TTGAACGCCGAAATAGTATTCGTTATGTCACAAGGTACAGATAC CCCAGAACAATTGAACGAAAGAATCGAATTGACTAGATCCAGT TTCGGTGGTGCAAAGAATACTAACATAACAGGTGTTATTATTAA TAAGTTGAACGCTCCAGTTGATGAACAAGGTAGAACTAGACCT GACTTGTCTGAAATATTCGATGACTCTTCAAAGGCTCAAGTTAT TAAGATCGATCCAGCAAAGTTACAAGAATCCAGTCCATTACCT GTCTTGGGTGCTGTACCTTGGTCTTTTCGATTTGATTGCCACAAG AGCTATAGACATGGCTAGACATTTGAATGCAACCATAATCAAC GAAGGTGACATAAAGACAAGACACGTCAAATCCGTAACCTTTT GTGCTAGAAGTATCCACATATGTTGGAACACTTCAGAGCCGG ATCTTTGTTAGTTACATCAGCTGATAGACCTGACGTCTTAGTAG CCGCTTGTTTGGCAGCCATGAATGGTGTGAAATTGGTGCATTG TTATTGACTGGTGGTTATGAAATGGATGCCAGAATATCTAAGTT ATGCGAAAGAGCATTTGCCACCGTTTGCCAGTTTTTCATGGTCA	78

	ATACTAACACATGGCAAACCTTCTTTGTCATTGCAATCTTTTAATT TGGAAAGTTCCTGTCGATGACCATGAAAGAATCGAAAAGGTTCA AGAATACGTCGCCAATTACGTAAACGCTGAATGGATTGAATCC TTAACCGCAACTTCTGAAAGATCAAGAAGATTGAGTCCACCTG CTTTCAGATACCAATTGACAGAATTGGCAAGAAAGGCCGGTAA AAGAGTAGTTTTACCAGAAGGTGACGAACCTAGAACAGTTAAA GCTGCAGCCATCTGTGCAGAAAGAGGTATTGCCACCTGCGTTTT GTTGGGTAAACCCAGATGAAATTAACAGAGTTGCTGCATCACAA GGTGTGCAATTGGGTGCAGGTATCGAAATTGTTGATCCTGAAGT CGTAAGAGAATCTTACGTTGCTAGATTAGTCGAATTGAGAAAA TCAAAGGGTATGACTGAACCAGTTGCAAGAGAACAATTGGAAG ATAACGTTGTCTTGGGTACATTAATGTTGGAACAAGATGAAGTA GACGGTTTAGTTTTCTGGTGCTGTCCACACTACAGCAAACACCAT AAGACCACCTTTACAATTGATCAAAACTGCTCCAGGTTCTTCAT TGGTATCCAGTGTTTTCTTTATGTTGTTGCCTGAACAAGTATACG TTTACGGTGACTGCGCAATTAATCCAGACCCTACAGCCGAACA ATTGGCTGAAATAGCAATCCAATCCGCTGATAGTGCTATCGCAT TTGGTATTGAACCAAGAGTTGCAATGTTATCCTATAGTACAGGT ACCTCCGGTGCTGGTAGTGATGTGCAAAAAGTTAGAGAAGCCA CTAGATTGGCTCAAGAAAAGAGACCAGATTTGATGATTGACGG TCCTTTGCAATACGATGCCGCTGTAATGGCCGACGTTGCTAAAT CCAAGGCTCCAAATAGTCCTGTAGCAGGTAGAGCCACTGTTTTT ATTTTCCCAGATTTGAACACTGGTAACACCACTTATAAGGCCGT TCAAAGATCTGCTGACTTAATTTCAATAGGTCCAATGTTGCAGG GTATGAGAAAACCTGTAAACGATTTGTCCAGAGGTGCTTTGGTC GATGACATAGTATACACCATCGCTTTGACTGCAATTCAAGCCTC TCAACAACAACAATAAGCTAGCTAG	
<i>PO_{AV}</i>	ATAAGAATGCGGCCGCATGTCCGACAACAAGATCAACATTGGT TTGGCAGTTATGAAAATATTGGAATCCTGGGGTGCTGACACTAT CTACGGTATCCCTTCAGGTACTTTGTCTTCATTAATGGATGCTAT GGGTGAAGAAGAAAACAACGTAAAGTTCTTGCAAGTTAAGCAT GAAGAAGTCGGTGCAATGGCTGCAGTTATGCAATCCAAATTCG GTGGTAATTTGGGTGTAACAGTTGGTTCCGGTGGTCCAGGTGCT AGTCACTTGATTAACGGTTTATACGATGCCGCTATGGACAATAT TCCAGTTGTCGCTATATTGGGTTCAAGACCTCAAAGAGAATTGA ACATGGATGCATTCCAAGAATTGAACCAAAACCCAATGTACGA TCATATTGCCGTATACAATAGAAGAGTTGCCTATGCTGAACAAT TGCCTAAATTAGTTGATGAAGCAGCCAGAATGGCAATCGCCAA GAGAGGTGTCGCCGTATTAGAAGTCCCTGGTGACTTCGCTAAG GTAGAAATTGATAACGACCAATGGTACTCCAGTGCTAATTCCTT GAGAAAATATGCACCAATAGCCCCTGCTGCACAAGATATCGAC GCCGCTGTAGAATTGTTGAACAACAGTAAGAGACCTGTTATCTA CGCAGGTATTGGTACTATGGGTCACGGTCCAGCCGTCCAAGAA TTAGCTAGAAAAATCAAGGCACCTGTAATTACTACAGGTAAAA ATTTCGAAACATTGCAATGGGATTTTGAAGCATTGACAGGTTCA	79

	ACCTATAGAGTTGGTTGGAAACCAGCCAACGAAACTATTTTGG AAGCAGATACAGTCTTATTCGCCGGTTCTAATTTTCCTTTCTCAG AAGTTGAAGGTACTTTTAGAAACGTCGATAACTTCATCCAAATA GATATAGACCCAGCTATGTTAGGTAAAAGACATCACGCTGATG TTGCAATTTTGGGTGACGCCGGTTTGGCTATCGATGAAATCTTG AATAAGGTTGATGCTGTCTGAAGAATCTGCATGGTGGACAGCCA ATTTGAAGAACATTGCAAACCTGGAGAGAATACATCAACATGTT GGAAACCAAGGAAGAGGGTGACTTGCAATTCTACCAAGTTTAC AACGCTATAAATAACCATGCTGATGAAGACGCAATCTATTCTAT AGATGTTGGTAACTCTACCCAAACTTCAATTAGACATTTGCACA TGACCCCAAGAATATGTGGAGAACCTCACCTTTATTTGCCACT ATGGGTATCGCTATTCCAGGTGGTTTGGGTGCTAAAAACACTTA TCCTGACAGACAAGTTTGGGAATATTATAGGTGACGGTGCATTCT CTATGACATACCCAGACGTAGTTACCAACGTTAGATATAATATG CCTGTTATCAACGTCGTATTTTCAAACACAGAATACGCTTTCAT TAAAAATAAGTATGAAGATACCAATAAGAACTTGTTTGGTGTG GACTTCACTGATGTAGACTACGCTAAAATAGCTGAAGCACAAG GTGCAAAGGGTTTTACCGTTTCTAGAATCGAAGATATGGACAG AGTTATGGCCGAAGCTGTCTGCAGCCAATAAGGCTGGTCATACT GTTGTCATAGACTGTAAGATCACACAAGATAGACCAATTCCTGT TGAAACATTGAAGTTGGACTCCAAGTTGTACAGTGAAGATGAA ATTAAAGCTTACAAGGAAAGATACGAAGCTGCAAATTTGGTTC CTTTTAGAGAATACTTGGAAGCAGAAGGTTTAGAATCTAAATAT ATTAAGTAAATTAATTAAGG	
<i>PO_{SP}</i>	ATAAGAATGCGGCCGCATGACACAAGGTAAAATCACTGCCTCC GCCGCAATGTTGAATGTATTAAAGACCTGGGGTGTGACACAA TCTACGGTATTCCAAGTGGTACATTGTCTTCATTAATGGATGCA TTGGCCGAAGATAAGGACATAAGATTTTTACAAGTAAGACATG AAGAAACCGGTGCCTTGGCTGCAGTTATGCAGGCTAAGTTCGG TGGTAGTATTGGTGTAGCAGTTGGTTCTGGTGGTCCAGGTGCCA CTCATTTGATTAATGGTGTATATGATGCCGCTATGGACAATACA CCATTTTGGCTATATTGGGTTCAAGACCTGTTAACGAATTGAA CATGGATGCATTCCAAGAATTGAACCAAAACCCAATGTACAAC GGTATAGCTGTATACAACAAGAGAGTTGCTTATGCAGAACAAAT TGCCTAAAGTCATCGATGAAGCTTGTAGAGCAGCCATTTCCAAA AAGGGTCCAGCAGTTGTCTGAAATACCTGTAACTTTGGTTTCCA AGAAATCGATGAAAACCTCATACTACGGTTCCGGTAGTTACGAA AGATCCTTTATCGCCCCAGCTTTGAATGAAGTTGAAATCGACAA GGCAGTCGAAATTTTAAATAACGCCGAAAGACCAGTTATATAT GCTGGTTTTCGGTGGTGTCAAAGCAGGTGAAGTAATTACAGAAT TGTCTAGAAAGATTAAAGCCCCAATAATCACTACAGGTAAAAA TTTCGAAGCTTTCGAATGGAACCTACGAAGGTTTAACCGGTTTCAG CTTATAGAGTTGGTTGGAAACCTGCCAATGAAGTAGTTTTTGAA GCTGATACTGTCTTGTCTTAGGTTCCAACCTTGCATTCGCCGAA GTATACGAAGCTTTTAAGAACACAGAAAAATTCATACAAGTTG	80

	ATATCGACCCATATAAAATTGGGTAAAAGACATGCATTGGATGC CAGTATATTAGGTGACGCTGGTCAAGCTGCAAAGGCAATCTTA GATAAAGTTAATCCAGTCGAATCTACACCTTGGTGGAGAGCTA ACGTTAAAAATAACCAAACTGGAGAGACTACATGAATAAGTT GGAAGGTAAAACTGAGGGTGAATTGCAATTGTACCAAGTTTAC AACGCTATTAACAAACACGCCGATCAAGACGCTATCTATTCTTT GGATGTCGGTTCAACCACTCAAACATCCACCAGACATTTGCACA TGACACCTAAAAATATGTGGAGAACTTCACCTTTGTTTGCAACA ATGGGTATAGCCTTACCAGGTGGTATCGCCGCTAAAAAGGATA CCCCTGACAGACAAGTTTGAACATTATGGGTGACGGTGCTTTT AATATGTGCTACCCAGATGTAATCACTAACGTTCAATACGACTT GCCAGTTATTAATTTGGTATTTTCTAACGCTGAATACGGTTTTAT TAAAAATAAGTACGAAGATACAAATAAGCATTGTTCGGTGTT GATTTACCAACGCAGACTACGGTAAAATTGCTGAAGCACAAAG GTGCCGTTGGTTTTACTGTTCGATAGAATCGAAGATATTGACGCA GTCGTAGCCGAAGCTGTTAAGTTGAATAAGGGTGGTAAACCG TTGTCATTGATGCTAGAATAACTCAACATAGACCATTGCCTGTC GAAGTATTGGAATTAGACCCAAAGTTACACAGTGAAGAAGCTA TTAAAGCTTTTAAAGAAAAGTACGAAGCAGAAGAATTGGTTCC TTTATAGATTGTTCTTAGAAGAAGAAGGTTTACAATCTAGAGCTA TTAAAT<u>AA</u>TTAATTAAAG	
<i>PO_{LP}</i>	ATAAGAATGCGGCCGCATGGTAATGAAACAAACAAAGCAAAC AAACATCTTAGCAGGTGCCGCCGTTATCAAGGTATTGGAAGCA TGGGGTGTAGACCACTTATATGGTATTCCAGGTGGTTCCATCAA TAGTATTATGGATGCCTTGTCGCTGAAAGAGACAGAATACATT ACATCCAAGTCAGACACGAAGAAGTAGGTGCAATGGCTGCAGC CGCTGATGCCAAGTTGACTGGTAAAATAGGTGTTTGTTCGGTT CAGCTGGTCCAGGTGGTACACATTTGATGAATGGTTTATATGAT GCAAGAGAAGACCACGTTTCTGTCTTGGCTTTAATTGGTCAATT CGGTACTACTGGTATGAACATGGATACTTTCCAAGAAATGAAC GAAAACCCAATCTATGCAGATGTTGCCGACTACAATGTAACAG CTGTTAACGCAGCCACCTTGCTCATGTCATCGATGAAGCAATT AGAAGAGCCTACGCTACCAAGGTGTCGCTGTTGTCCAAATTCC AGTAGATTTGCCTTGGCAACAAATACCAGCAGAAGACTGGTAT GCATCTGCCAATTCATACCAAAACCCCTTTGTTACCAGAACCTGA TGTCOAAGCTGTAAGTATGATGACCCAAACTTTGTTAGCTGCAG AAAGACCATTAATCTATTACGGTATCGGTGCTAGAAAAGCTGG TAAAGAATTGGAACAATTGTCCAAGACATTGAAGATCCCATTG ATGAGTACCTATCCTGTAAAGGGTATTGTCGCCGATAGATATCC AGCTTACTTGGGTCTGCTAATAGAGTTGCACAAAAACCTGCCA ACGAAGCTTTGGCACAAGCCGATGTAGTTTTGTTTCGTTGGTAAC AACTACCCATTTCGCAGAAGTTTCAAAGGCCTTCAAGAACACTA GATACTTTTTGCAAATAGATATCGACCCTGCAAAGTTGGGTAAA AGACATAAGACTGATATAGCTGTCTTGGCTGACGCACAAAAGA CATTGGCCGCTATATTAGCTCAAGTATCCGAAAGAGAAAGTAC	81

ACCATGGTGGCAAGCCAACTTAGCTAATGTTAAGAACTGGAGA GCATATTTGGCCTCCTTAGAAGATAAACAAGAAGGTCCATTGC AAGCCTACCAAGTATTGAGAGCTGTTAATAAGATCGCTGAACC TGATGCAATCTATAGTATAGATGTTGGTGACATCAATTTGAACG CTAACAGACATTTGAAGTTGACACCATCTAACAGACACATCACT TCAAACCTTATTCGCAACAATGGGTGTTGGTATTCCAGGTGCTAT AGCAGCCAAGTTGAACTACCCTGAAAGACAAGTTTTTAACTTA GCTGGTGACGGTGGTGCATCTATGACCATGCAAGACTTGGCTAC TCAAGTACAATACCATTTGCCTGTTATAAAATGTCGTATTCACAA ACTGCCAATACGGTTTTATCAAGGATGAACAAGAAGACACCAA CCAAAACGATTTTCATAGGTGTTGAATTCAATGATATCGACTTCT CTAAGATCGCTGACGGTGTTTCATATGCAAGCATTGAGAGTCAAC AAGATCGAACAATTGCCAGATGTTTTTGAACAAGCCAAAGCTA TTGCACAACACGAACCTGTTTTGATCGATGCTGTCATTACCGGT GACAGACCATTACCTGCAGAAAAATTGAGATTAGATTCTGCCA TGTCTTCAGCTGCAGACATTGAAGCCTTTAAGCAAAGATATGAA GCTCAAGATTTGCAACCATTGTCAACATACTTAAAGCAATTTCGG TTTGATGACTTACAACATCAAATTGGTCAAGGTGGTTTTTAAAT TAATTAAGG	
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hACL- opt from human	ATGTCCGCAAAAGCCATTTCCGAACAAACTGGTAAAGAATTAT TATACAAGTTCATCTGCACAACCTCAGCCATACAAAACAGATTC AAGTATGCAAGAGTTACTCCAGATACAGACTGGGCCAGATTGT TACAAGATCATCCTTGGTTGTTATCACAAAACCTGGTTGTCAAG CCTGACCAATTGATTAAAAGACGTGGTAAATTGGGTTTAGTAG GTGTTAATTTGACATTAGATGGTGTAAAGTCCTGGTTGAAGCCA AGATTAGGTCAAGAAGCAACCGTCGGTAAAGCCACTGGTTTCT TGAAAAATTTCTTGATCGAACCATTTCGTACCTCATTCTCAAGCT GAAGAATTTTACGTTTGTATATACGCAACTAGAGAAGGTGACT ATGTCTTGTTTCATCACGAAGGTGGTGTGATGTAGGTGACGTA GACGCCAAAGCTCAAAAGTTGTTAGTTGGTGTTCGATGAAAAAT TGAACCCAGAAGACATTAAAAAGCATTTGTTGGTTCACGCCCCCT GAAGATAAAAAGGAAATATTGGCTTCTTTTATCTCAGGTTTGT TAATTTCTACGAAGATTTGTACTTCACTTACTTGGAATTAACC CATTGGTAGTTACAAAGGATGGTGTATACGTTTTGGACTTAGCT GCAAAGGTCGATGCAACAGCCGACTACATTTGTAAAGTAAAGT GGGGTGACATAGAATTTCCACCTCCATTCGGTAGAGAAGCATA TCCAGAAGAAGCCTACATTGCTGATTTGGACGCCAAATCCGGT GCTAGTTTGAAGTTAACCTTGTTGAACCCTAAAGGTAGAATCTG GACTATGGTTGCAGGTGGTGGTGCCTCAGTCGTATATTCCGATA CTATTTGCGACTTGGGTGGTGTAAACGAATTAGCTAACTACGGT GAATACAGTGGTGCACCATCTGAACAACAAACCTATGATTACG CTAAGACTATCTTGAGTTTAATGACAAGAGAAAAGCATCCTGA TGGTAAAATTTTGATCATCGGTGGTCTATCGCTAACTTCACAA ACGTCGCCGCTACCTTCAAAGGTATAGTAAGAGCAATCAGAGA TTACCAAGGTCCATTGAAGGAACACGAAGTAACCATTTTTGT GAAGAGGTGGTCCTAACTACCAAGAAGGTTTAAGAGTCATGGG TGAAGTAGGTAAACTACAGGTATCCCAATTCATGTATTTGGTA CTGAACTCACATGACTGCTATTGTTGGTATGGCATTAGGTCAT AGACCAATACCTAATCAACCTCCAACCTGCTGCTCACACAGCTAA TTTCTTGTTAAACGCATCTGGTTCAACATCCACCCCAGCCCCAT CAAGAACAGCTAGTTTCTCTGAATCAAGAGCTGATGAAGTTGCT CCAGCTAAGAAAGCAAAACCAGCCATGCCTCAAGACTCTGTTC CATCACCTAGATCCTTGCAAGGTAAAAGTACCACTTTGTTTTCA AGACATACAAAGGCAATTGTTTGGGGTATGCAAACCAGAGCCG TCCAAGGCATGTTGGATTTGACTATGTTTGTTCAGAGATGAA CCATCCGTTGCTGCAATGGTCTATCCTTTTACTGGTGACCATAA ACAAAAGTTCTACTGGGGTCACAAGGAAATATTAATCCCAGTTT TAAAAACATGGCCGATGCTATGAGAAAGCATCCTGAAGTTGA TGTATTGATTAACCTTCGCAAGTTTAAGATCAGCCTATGATTCAA CTATGGAACTATGAACTACGCTCAAATCAGAACTATTGCTATC ATTGCAGAAGGTATCCCAGAAGCATTGACAAGAAAATTAATTA AAAAGGCAGATCAAAAGGGTGTAACCATAATCGGTCCAGCAAC TGTTGGTGGTATCAAACCTGGTTGTTTTAAGATTGGTAATACAG GTGGCATGTTGGATAACATATTGGCTTCAAAATTGTATAGACCA GGTTCGGTCGCATACGTATCCAGAAGTGGTGGTATGAGTAACG	82
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Claims

1. A yeast, wherein
said yeast lacks any endogenous gene encoding pyruvate decarboxylase or comprises disrupted gene or genes encoding pyruvate decarboxylase; and
said yeast comprises at least one heterologous pathway for synthesis of cytosolic acetyl-Coenzyme A (CoA), said at least one heterologous pathway comprises at least one heterologous gene encoding a respective enzyme involved in synthesis of acetyl-CoA, with the proviso that said at least one heterologous gene is not a heterologous gene encoding a pyruvate formate lyase.
2. The yeast according to claim 1, wherein said at least one heterologous gene encodes a respective enzyme involved in the conversion of pyruvate into cytosolic acetyl-CoA.
3. The yeast according to claim 1 or 2, wherein said yeast lacks endogenous genes encoding PDC1, PDC5 and/or PDC6 or comprises disrupted genes encoding PDC1, PDC5 and/or PDC6, preferably lacks endogenous genes encoding PDC1, PDC5 and PDC6, or comprises disrupted genes encoding PDC1, PDC5 and PDC6, to block conversion of pyruvate to ethanol.
4. The yeast according to any of the claims 1 to 3, further comprising:
a heterologous gene encoding a pyruvate oxidase; and
a heterologous gene encoding a phosphate acetyltransferase.
5. The yeast according to claim 4, wherein
said heterologous gene encoding said pyruvate oxidase is selected from the group consisting of a gene encoding a *Lactobacillus plantarum* pyruvate oxidase, a gene encoding a *Streptococcus pneumonia* pyruvate oxidase and a gene encoding an *Aerococcus viridians* pyruvate oxidase, preferably *A. viridians* pyruvate oxidase; and
said heterologous gene encoding said phosphate acetyltransferase is selected from the group consisting of a gene encoding an *Escherichia coli* phosphate acetyltransferase, a gene encoding a *Salmonella enterica* phosphate acetyltransferase and a gene encoding a *Methanosarcina thermophila* phosphate acetyltransferase.

6. The yeast according to claim 5, further comprising a gene encoding a catalase.
7. The yeast according to claim 6, wherein said gene encoding said catalase is a gene adapted for overexpression of *Saccharomyces cerevisiae* CTA1.
8. The yeast according to any of the claims 1 to 3, further comprising
a heterologous gene encoding a pyruvate ferredoxin oxidoreductase;
a heterologous gene encoding a ferredoxin reductase; and
a heterologous gene encoding a ferredoxin reductase substrate selected from the group consisting of ferredoxin and flavodoxin.
9. The yeast according to claim 8, wherein
said heterologous gene encoding said pyruvate ferredoxin oxidoreductase is a gene encoding a *Desulfovibrio africanus* pyruvate ferredoxin oxidoreductase, preferably *D. africanus* pfor;
said heterologous gene encoding said ferredoxin reductase is a gene encoding an *Escherichia coli* ferredoxin reductase, preferably *E. coli* fpr; and
said heterologous gene encoding said ferredoxin reductase is a heterologous *E. coli* gene encoding said ferredoxin reductase substrate, preferably *E. coli* fdx or *E. coli* fldA, more preferably *E. coli* fldA.
10. The yeast according to any of the claims 1 to 8, further comprising a heterologous gene encoding an adenosine triphosphate (ATP)-citrate lyase.
11. The yeast according to claim 10, wherein said heterologous gene encoding said ATP-citrate lyase selected from the group consisting of a gene encoding a *Rhodospiridium toruloides* ATP-citrate lyase, a gene encoding a *Mus musculus* ATP-citrate lyase and a gene encoding a human ATP-citrate lyase, preferably a gene encoding a *M. musculus* ATP-citrate lyase.
12. The yeast according to claim 10 or 11, further comprising:

a heterologous gene encoding a pyruvate dehydrogenase subunit mutant having a mutated phosphorylation site;

a gene encoding a pyruvate transporter; and

a gene encoding a citrate synthase.

13. The yeast according to claim 12, wherein

said heterologous gene encoding said pyruvate dehydrogenase subunit mutant is a gene encoding a *Saccharomyces cerevisiae* pyruvate dehydrogenase subunit mutant having a mutated phosphorylation site, preferably *S. cerevisiae* Pda1 having a mutated phosphorylation site;

said gene encoding said pyruvate transporter is a gene encoding a *S. cerevisiae* pyruvate transporter, preferably *S. cerevisiae* Mpc1 or Mpc2; and

said gene encoding said citrate synthase is a gene encoding a *S. cerevisiae* citrate synthase, preferably *S. cerevisiae* CIT1.

14. The yeast according to any of the claims 10 to 13, further comprising:

a heterologous gene encoding a malate dehydrogenase lacking any peroxisomal targeting signal; and

a heterologous gene encoding a nicotinamide adenine dinucleotide phosphate (NADP⁺)-dependent malic enzyme.

15. The yeast according to claim 14, wherein

said heterologous gene encoding said malate dehydrogenase lacking any peroxisomal targeting signal is a gene encoding a *Saccharomyces cerevisiae* malate dehydrogenase lacking any peroxisomal targeting signal, preferably *S. cerevisiae* Mdh3 lacking any peroxisomal targeting signal; and

said heterologous gene encoding said NADP⁺-dependent malic enzyme is a gene encoding a *Rhodospiridium toruloides* NADP⁺-dependent malic enzyme.

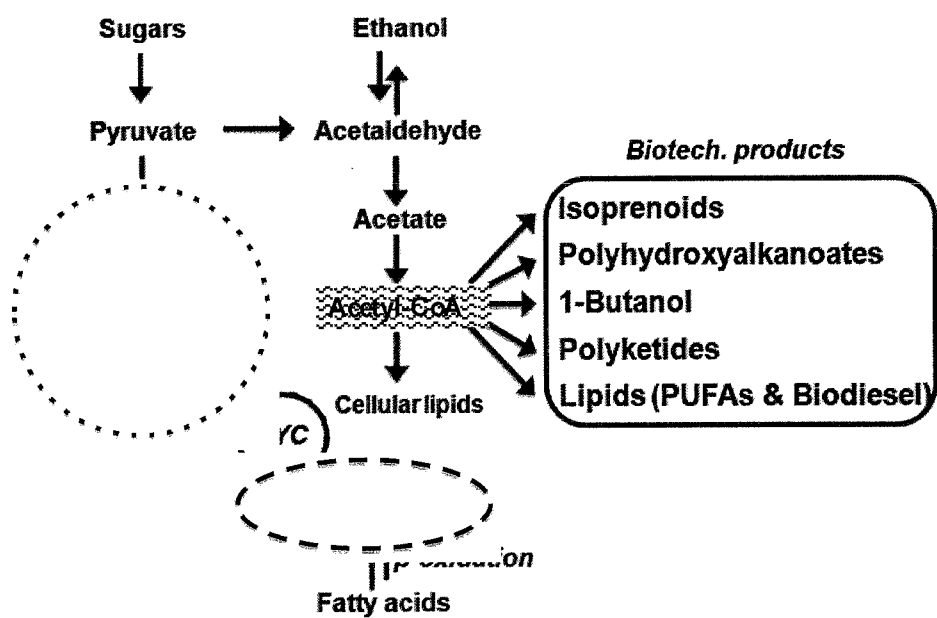
16. The yeast according to claim 14 or 15, further comprising a gene adapted for overexpression of a citrate transporter, preferably a *Saccharomyces cerevisiae* citrate transporter, more preferably *S. cerevisiae* CTP1.

17. The yeast according to any of the claims 1 to 3, further comprising:
heterologous genes encoding a cytosolic pyruvate dehydrogenase complex; and
heterologous genes encoding respective enzymes involved in attachment and activation of lipoyl groups to said cytosolic pyruvate dehydrogenase complex.
18. The yeast according to 17, wherein
said heterologous genes encoding said cytosolic pyruvate dehydrogenase complex are selected from the group consisting of genes encoding an *Escherichia coli* cytosolic pyruvate dehydrogenase complex, preferably *E. coli* aceE, aceF and lpd, genes encoding a *Saccharomyces cerevisiae* pyruvate dehydrogenase complex but lacking mitochondrial target signal (MTS), preferably *S. cerevisiae* PDA1, PDB1, LAT1, LPD1 and PDX1 lacking MTS, genes encoding an *Azotobacter vinelandii* pyruvate dehydrogenase complex, preferably *A. vinelandii* aceE_{av}, aceF_{av} and lpdA_{av}, and genes encoding an *Enterococcus faecalis* pyruvate dehydrogenase complex, preferably *E. faecalis* pdhA, pdhB, aceF_{ef} and lpdA_{ef}, more preferably, said pyruvate dehydrogenase complex are an *A. vinelandii* pyruvate dehydrogenase complex; and
said heterologous genes encoding respective enzymes involved in attachment and activation of lipoyl groups are selected from the group consisting of genes encoding *Escherichia coli* lipoic acid synthetase and lipoic acid synthetase and/or lipoate-protein ligase, preferably *E. coli* lipA, and lipB and/or lplA, and genes encoding *Saccharomyces cerevisiae* LIP2, LIP3, LIP5 and GCV3 but lacking MTS.
19. A method of producing acetyl-Coenzyme A (CoA) comprising culturing a yeast according to any of the claims 1 to 18 in culture conditions suitable for production of cytosolic acetyl-CoA from said yeast.
20. The method according to claim 19, further comprising collecting said acetyl-CoA or a compound generated by said yeast from said acetyl-CoA from a culture medium in which said yeast is cultured and/or from said yeast, wherein said compound is selected from a group consisting of fatty acids, 3-hydroxypropionic acid, isoprenoids, polyhydroxyalkanoates, 1-butanol and polyketides.

21. Use of a yeast according to any of the claims 1 to 18 for the production of cytosolic acetyl-Coenzyme A (CoA).
22. A method of producing a yeast suitable for the production of cytosolic acetyl-Coenzyme A (CoA) comprising:
- deleting or disrupting any endogenous gene encoding pyruvate decarboxylase in said yeast;
 - and
 - introducing, into said yeast, at least one heterologous pathway for synthesis of cytosolic acetyl-CoA, said at least one heterologous pathway comprises at least one heterologous gene encoding a respective enzyme involved in synthesis of acetyl-CoA, with the proviso that said at least one heterologous gene is not a heterologous gene encoding a pyruvate formate lyase.

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Figure 1:



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Figure 2:

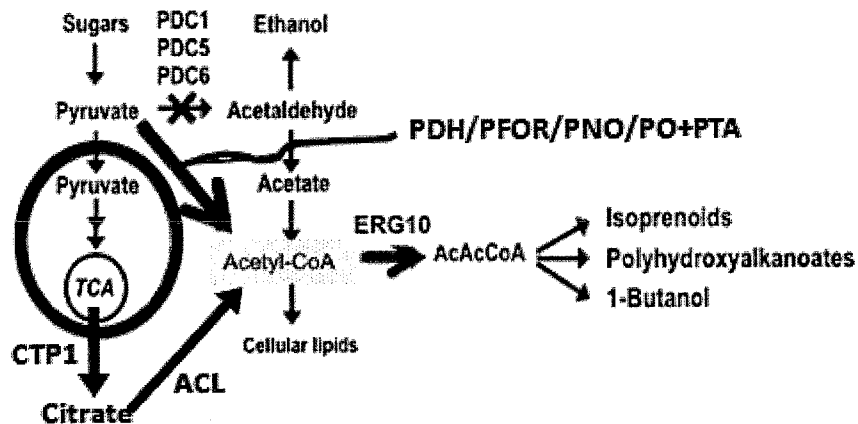


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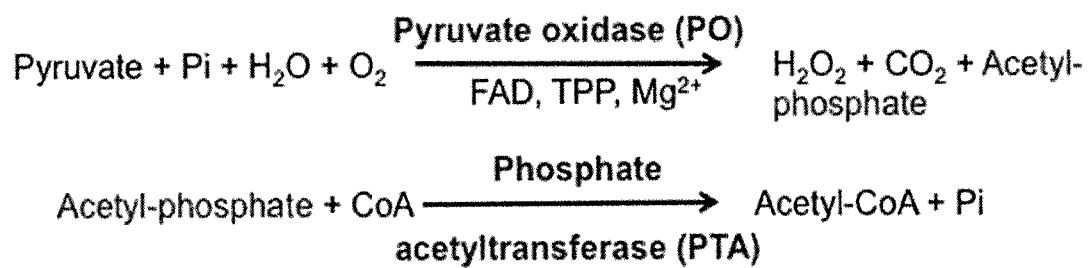
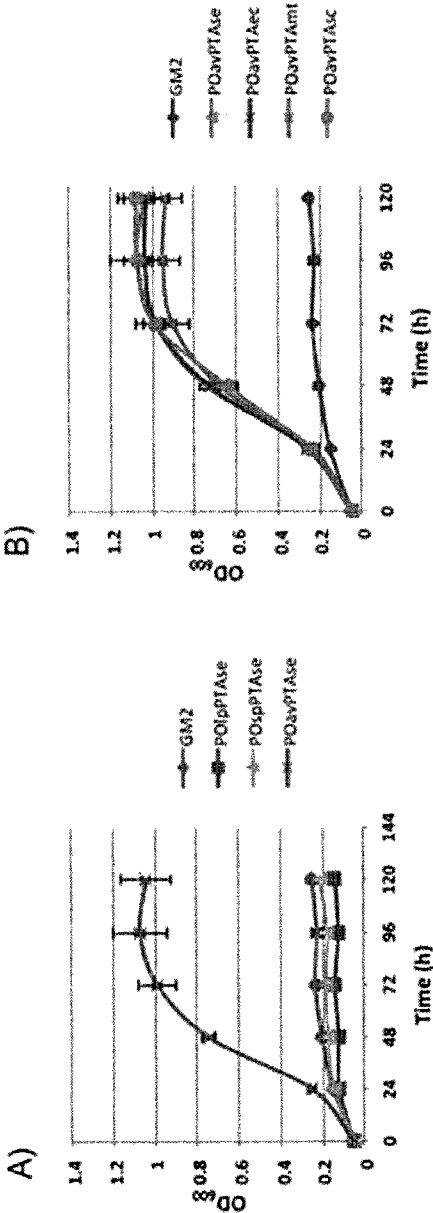
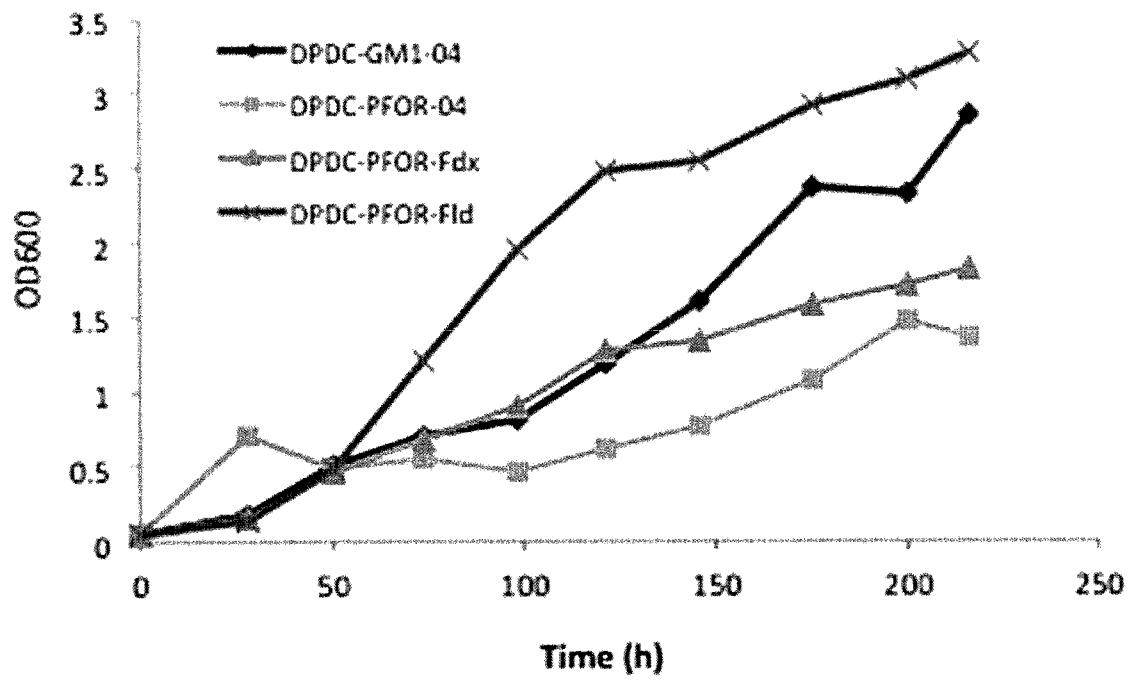


Figure 4:



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Figure 5:



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Figure 6:

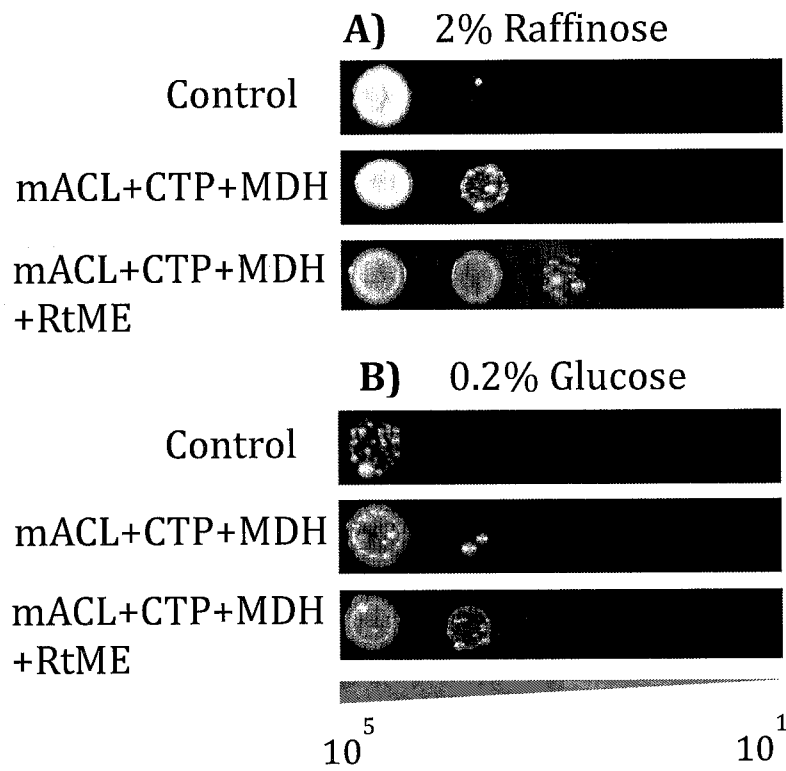
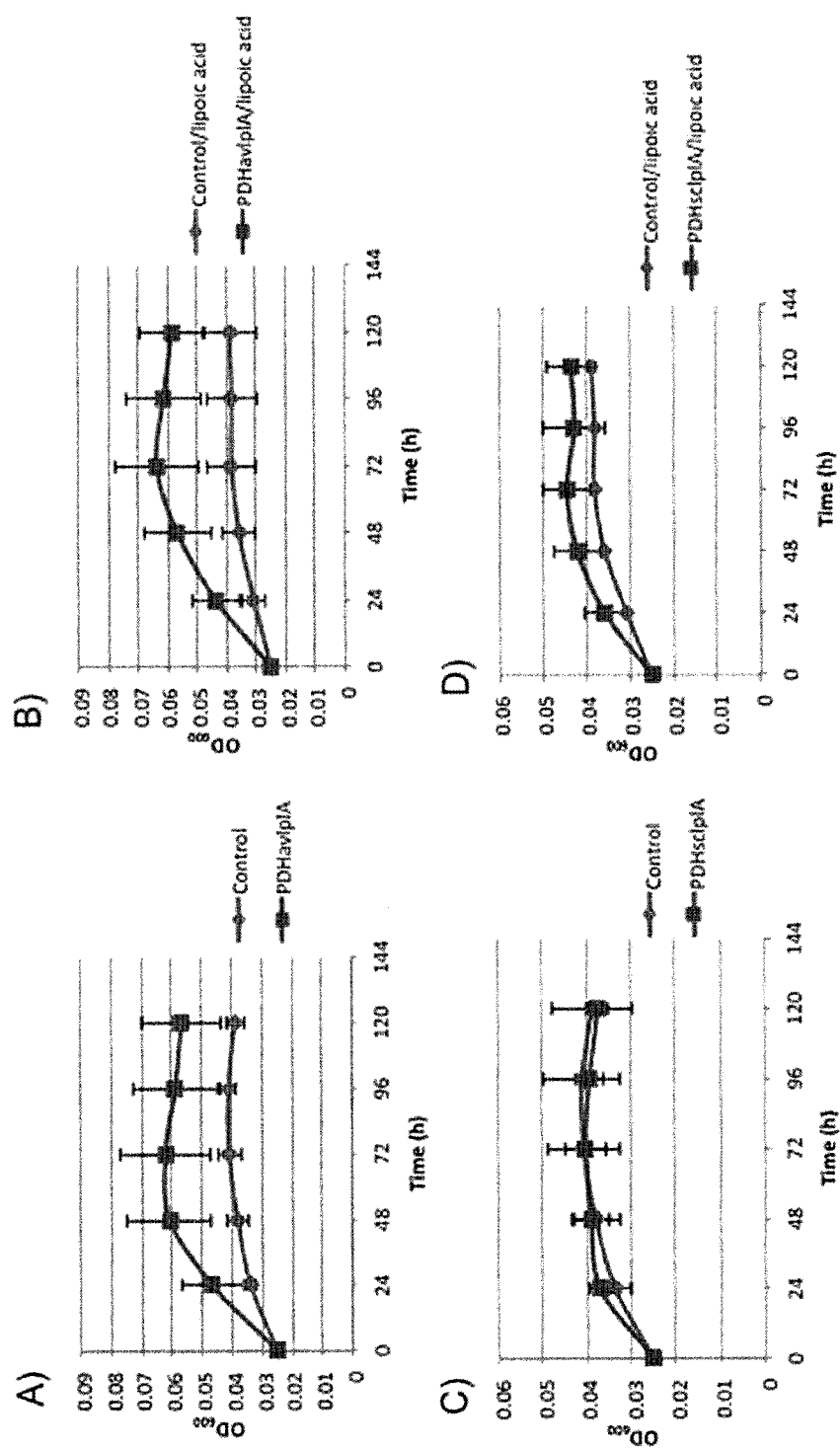
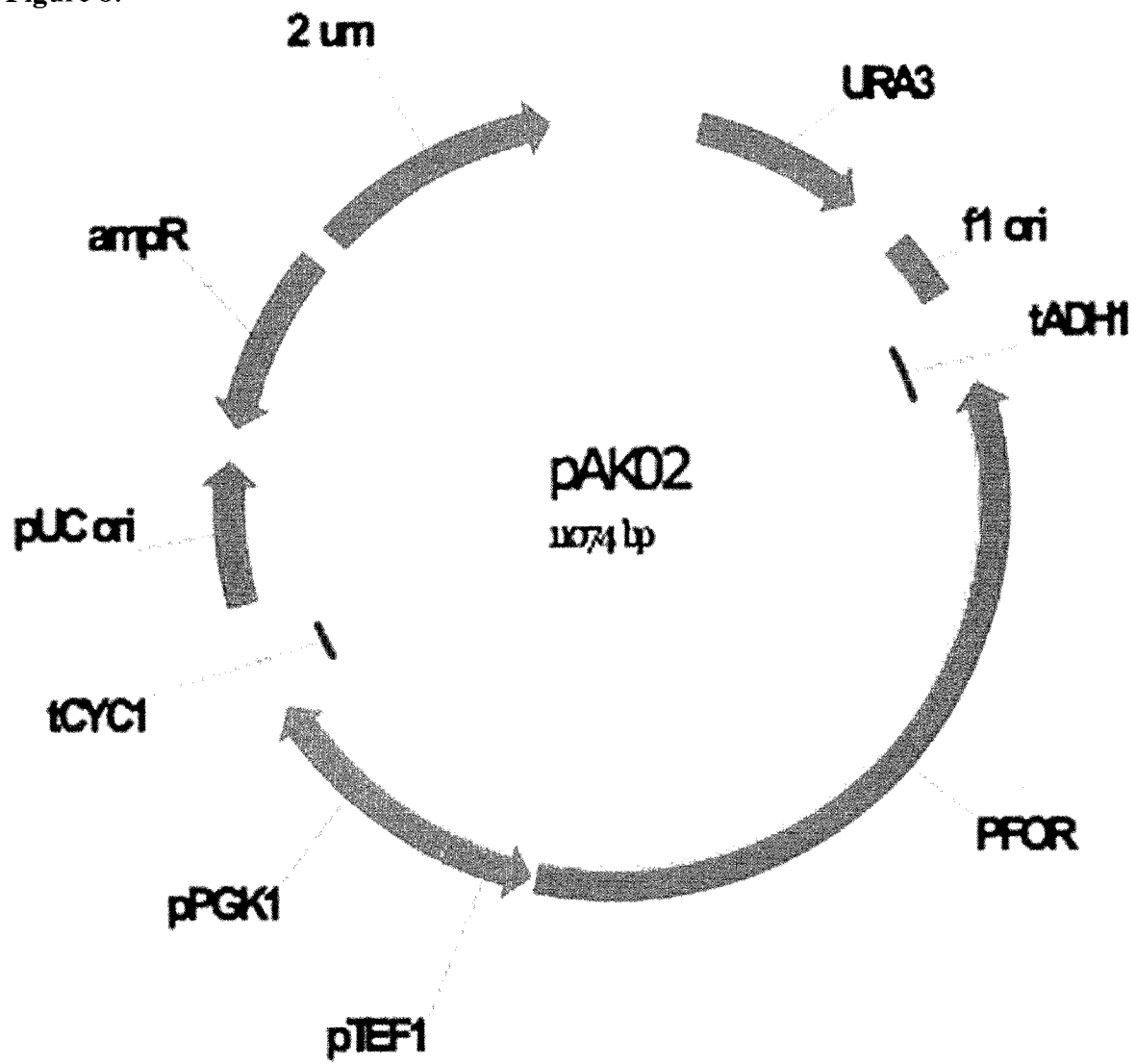


Figure 7:



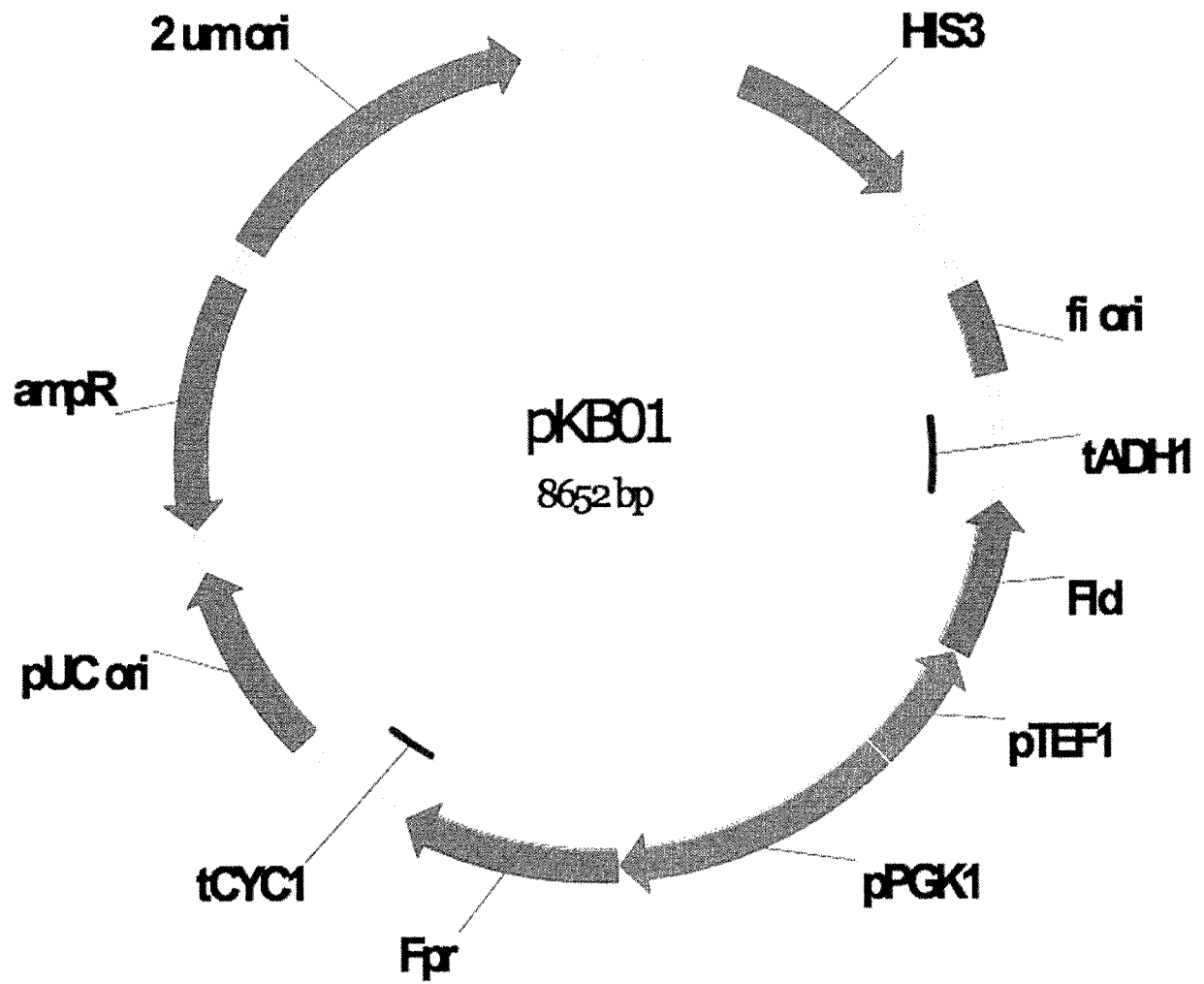
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Figure 8:



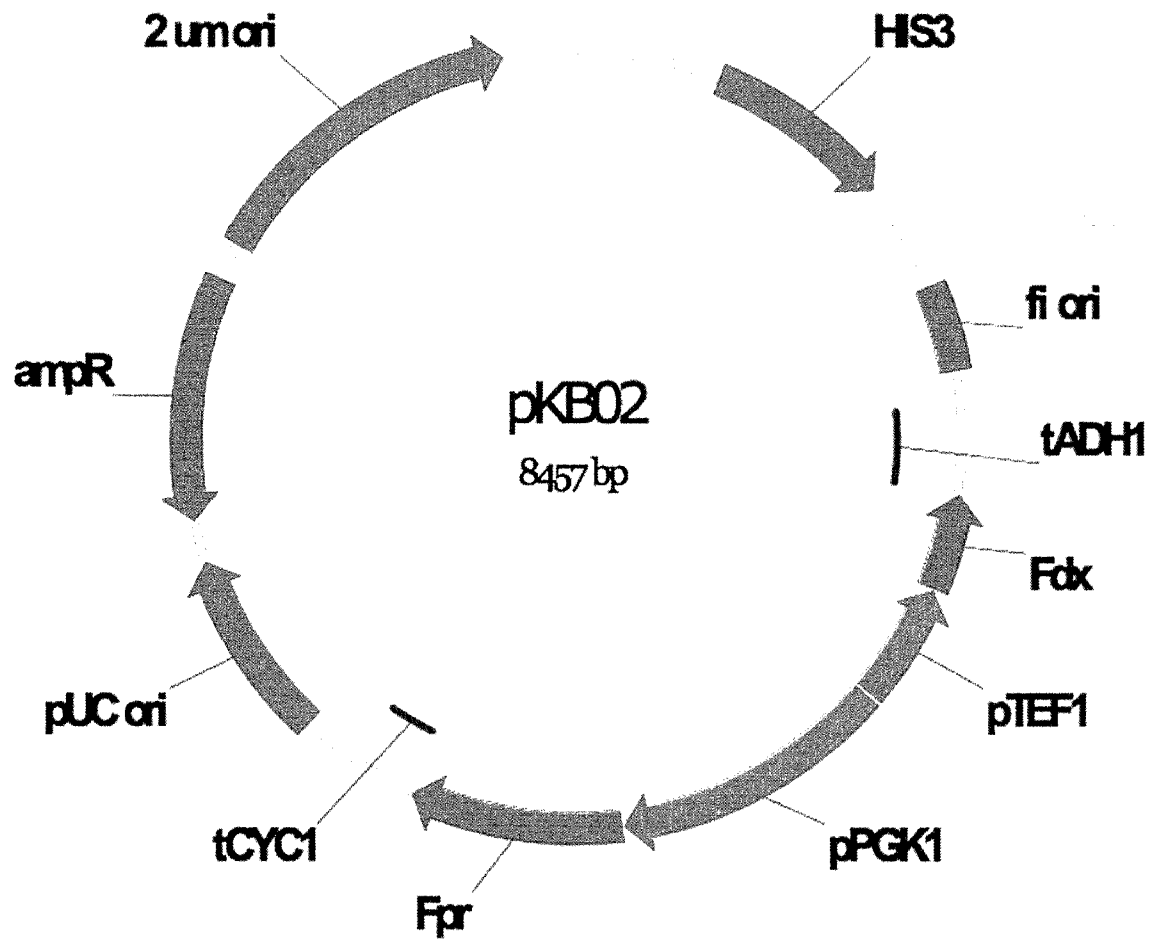
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Figure 9:



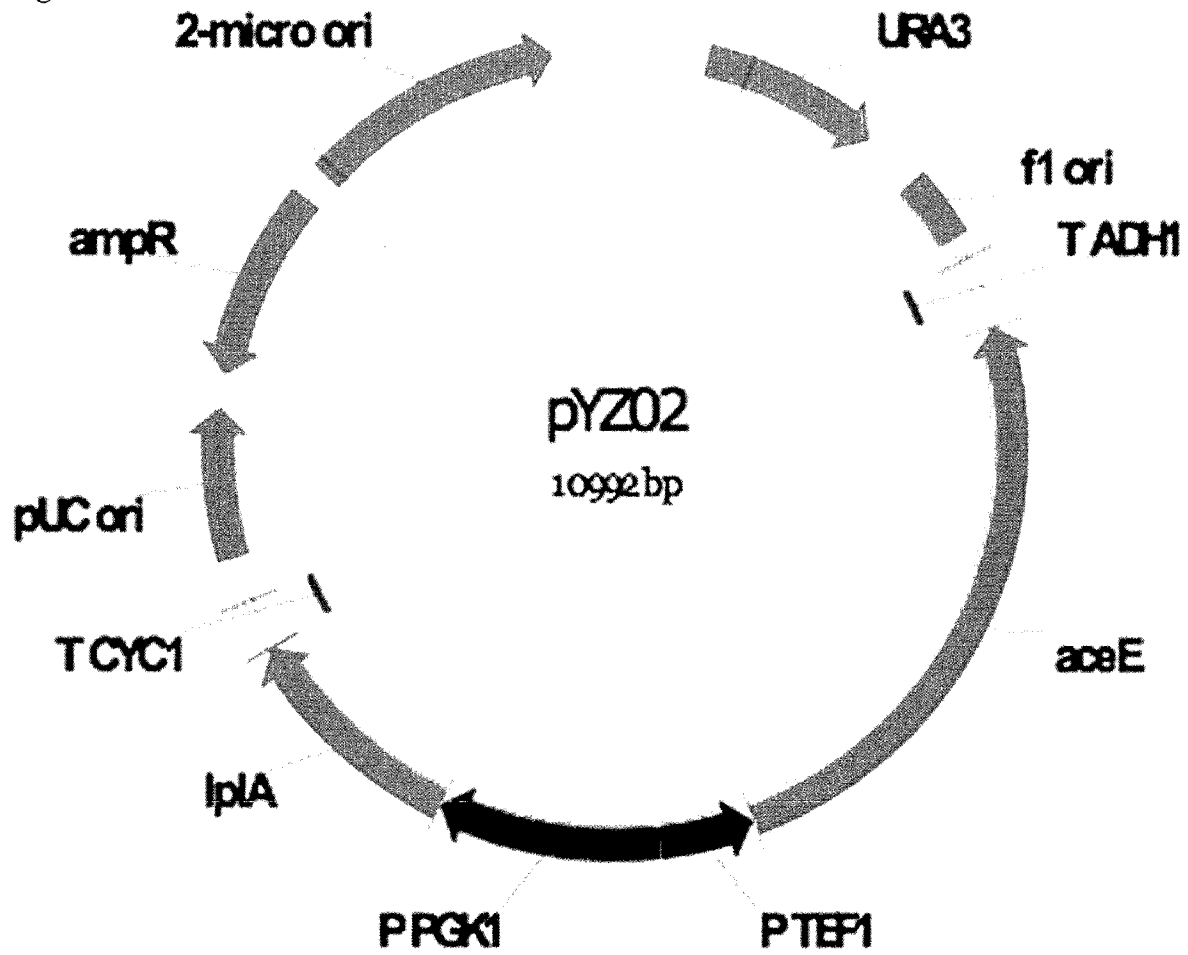
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Figure 10:



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Figure 11:



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Figure 12:

