Abstract:

An invention relates to recombinant yeast cells producing polylactic acid (PLA), and the uses thereof.

Title: RECOMBINANT YEAST CELLS PRODUCING POLYLACTIC ACID AND USES THEREOF

Figure 1

Lactate \( \rightarrow \) Lactyl-CoA \( \rightarrow \) Lactyl-CoA polymerase \( \rightarrow \) PLA homopolymere

Acetyl-CoA \( \rightarrow \) CoA-SH

ACS

Acetate
The present invention relates to recombinant yeast cells having the ability to produce polylactic acid, and to the uses thereof. The present invention further relates to a method for producing polylactic acid with such recombinant yeast cells, and more particularly homopolymers of polylactic acid, as well as to fermentation broths or cell-free extracts of said recombinant yeast cells comprising polylactic acid.

BACKGROUND

Polylactic acid (PLA) is a biodegradable polyester that may be derived from renewable resources such as corn, rice or other sugar- or starch-producing plants. PLA can be easily processed by extrusion, injection, molding, film formation, etc., allowing a large range of applications, particularly for short-term uses (i.e., food packaging, bags, films, etc). Furthermore, because of the non-toxicity of lactic acid monomers, PLA is one of the most promising biopolymers for medical applications. Due to their excellent biocompatibility and mechanical properties, PLA and their copolymers are becoming widely used in tissue engineering for function restoration of impaired tissues, in drug delivery systems and in various medical implants.

Lactic acid polymers can be synthesized by different processes so as to obtain products with an ample variety of chemical and mechanical properties. More particularly, PLA is mainly synthesized by two methods: the polycondensation of lactic acid (LA), which is carried out in bulk or in solution; or the ring-opening polymerization of lactide (cyclic dimer of lactic acid), which requires catalysts. The direct polycondensation of lactic acid in bulk is not applied on a great scale, because of the competitive reaction of lactide formation and the simultaneously occurring degradation process. The polycondensation of lactic acid in solution gives PLA with molecular weights ranging from the tens to a few hundred thousand g/mol. So far, the synthesis of PLA from lactide is the most effective method of synthesis in industry. However, the use of metal catalysts, cationic catalysts and/or organic catalysts may impact the quality of final product, some catalyst residues being incorporated into the polymer. Moreover, the racemization of part of the lactides during the process may lead to heteropolymers comprising...
both L-lactic acid and D-lactic acid. In addition this chemical polymerization requires high energetic inputs (heat) that bring additional economic and environmental costs.

Recently, alternative biological processes have been developed, wherein prokaryote cells such as bacteria have been engineered for producing microbial LA-based polyesters. This biological production takes advantage of the enzymatic activity of a polyhydroxyalkanoate (PHA) synthase leading to the production of LA-based polyesters. More precisely, the pathway for utilizing lactyl-CoA as a substrate for the production of LA-based polyesters has been developed in bacteria. However, the main polymer produced by this biological process is a copolymer composed of LA and other PHA (hydroxyacids or hydroxyalkanoate such as 3-hydroxybutyrate) monomers. The resulting copolymers, such as P(3HB-co-LA) have limited mechanical and industrial interest. Furthermore, the PLA polymers generated by this method are amorphous and have a low molecular weight (less than 30,000g/mol). Such PLA exhibits poor mechanical properties and may not be easily processed in industrial applications such as injection molding, thermoforming or extrusion. Accordingly, such polymer is of low industrial interest.

The present invention describes novel biological methods and microorganisms for producing PLA. The invention allows effective production of homopolymers of PLA with high molecular weight, on large scale.

SUMMARY OF THE INVENTION

The present invention relates to yeast cells engineered to produce PLA and to the uses thereof. More particularly, the invention relates to recombinant yeast cells that exhibit both lactyl-CoA synthase activity and lactyl-CoA polymerase activity and, optionally, an altered lactic acid (LA) metabolism leading to a reduced consumption of LA as carbon source. Consequently, the invention describes the ability of the aforementioned yeast cells to produce high quantities of PLA, and that the PLA produced may be in the form of homopolymers of high molecular weight. The yeast cells compartmentalisation and its membrane-bound organelles are of particular interest for the production of PLA homopolymers essentially devoid of other hydroxyalkanoate monomers. These cells thus allow the design of improved methods of production of PLA with high mechanical properties and broad industrial utilities.
It is therefore an object of the invention to provide recombinant yeast cells comprising a gene encoding a protein having lactyl-CoA synthase activity and a gene encoding a protein having lactyl-CoA polymerase activity, said recombinant cells having the ability of producing polylactic acid (PLA).

In a particular embodiment, the protein exhibiting lactyl-CoA synthase activity is an acyl-CoA transferase, more preferably a propionyl-CoA transferase (Pctp). In another embodiment, the protein exhibiting lactyl-CoA synthase activity is a ligase, such as acyl-CoA ligase.

Preferably, the protein exhibiting lactyl-CoA polymerase activity is a polyhydroxyalkanoate (PHA) synthase.

In a preferred embodiment, the invention relates to recombinant yeast cells expressing at least a gene encoding a protein having lactyl-CoA synthase activity and a gene encoding a protein having lactyl-CoA polymerase activity, and wherein a lactic acid oxidoreductase activity of the yeast is inactivated.

In a particular embodiment, the invention relates to recombinant yeast cells expressing at least a gene encoding a protein having lactyl-CoA synthase activity and a gene encoding a protein having lactyl-CoA polymerase activity, and wherein a lactic acid oxidoreductase activity of the yeast is inactivated, and wherein the 6 acyl-CoA oxidases have been deleted.

In a particular embodiment, the yeast cell is of the genus of *Yarrowia*, preferably *Yarrowia Upolytica*.

The invention also relates to a method for producing PLA comprising

- culturing recombinant yeast cells of the invention in the presence of lactic acid; and optionally
- recovering PLA produced.

A further object of the invention relates to a composition comprising a cell-free extract of the recombinant yeast cells of the invention.

A further object of the invention relates to a composition comprising dried recombinant yeast cells of the invention.
The invention further relates to the use of recombinant yeast cells of the invention for producing PLA and to a method for obtaining PLA and its uses.

The invention is particularly adapted for the production of homo-polylactic acid, especially poly-D-lactic acid (PDLA). In addition, the method described is particularly advantageous for the production of high molecular weight polymer such as PLA, and more especially PDLA, with average molecular weight (Mw) above 40,000g/mol, such as about 80,000g/mol.

LEGEND TO THE FIGURES

Figure 1: Schematic representation of the enzymatic production of polylactic acid using lactic acid and lactyl-CoA as substrates; CoA-SH: Coenzyme A; ACS: acyl-CoA synthase.

Figure 2: Depiction of the different metabolic pathways leading to production or consumption of acetyl-CoA in Y. lipolytica and are subject of modification; PDH: pyruvate dehydrogenase; OAA: oxaloacetic acid; DGA1, DGA2: acyl-CoA:diacylglycerol acyltransferases; LROI: phospholipid:diacylglycerol acytransferase.

Figure 3: Schematic representation of the different metabolic pathways that are the subjects of expression in Yarrowia lipolytica, for the increase of the cytosolic acetyl-CoA pool. GAPN: glyceraldehyde-3P dehydrogenase; PDH: pyruvate dehydrogenase; PTA: phosphotransacetylase kinase; ACS: acyl-CoA synthetase; ACK: acetate kinase; XPK: phosphoketolase; PPP: pentose phosphate pathway.

Figure 4: Growth curve of the control strain JMY2341 (Figure 4A) and of the lactic acid dehydrogenase YIDLD1 knockout strain ThY1_434 (Figure 4B) in media containing lactate or glucose (positive control). The YIDLD1 disrupted strain is unable to grow on D-lactate and only maintains half of its growth potential (OD600nm) on the equimolar racemic mixture of DL-lactate.

Figure 5: Growth curve of the control strain JMY2341 (Figure 5A) and of the lactic acid oxidoreductase YICYB21 knockout strain ThY1_436 (Figure 5B) on L-lactate. The YICYB21 knockout strain is unable to grow on L-lactic acid.

Figure 6: Schematic illustration of the successive genomic modifications performed in the W29 Y. lipolytica wild type strain, according to a particular embodiment.
Figure 7: NMR spectra of: (1) Soxhlet extracted lipid fraction of ThYl_964 control strain dried biomass and (2) Soxhlet extracted lipid fraction of ThYl_976 strain expressing both lactyl-CoA synthase activity and lactyl-CoA polymerase activity dried biomass. The signals between 5.3 and 5.2 ppm, and between 1.7 and 1.5 ppm correspond to the PLA fraction produced by the recombinant yeast cells; (3) Commercial PLA used as control.

Figure 8: Biomass evolution and substrate consumption of L-lactate, D-Lactate and Glucose during growth of ThYl_976 recombinant yeast strain.

Figure 9: Graphic representation of PLA production from ThYl_976 recombinant yeast cells during culture.

Figure 10: HPLC chromatogram obtained after complete hydrolysis of PLA extracted from ThYl_976 strain and showing that the PLA produced is a homopolymer exclusively composed of monomers of D-lactic acid.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to new recombinant eukaryotic microorganisms engineered for producing polylactic acid. More particularly, the invention relates to recombinant yeast cells, engineered by the inventors, which allow large scale production of high molecular weight (Mw) homopolymers of PLA having remarkable mechanical and physical properties. More particularly, the inventors have discovered that the use of an eukaryotic cell makes it possible to produce PLA homopolymers, devoid of hydroxyalkanoates. Indeed, in yeast there is no free 3-hydroxyalkanoates (3HA) apart from the one under the form of CoA present in the peroxisome. 3HA compounds are produced under the form of ACP (acyl carrier protein) as a transient intermediate during the fatty acid biosynthesis in the confined reaction chambers of the yeast type I fatty acid synthase and thus cannot be liberated in the cytosol and used for polymer production. Said 3HA-CoA production, occurring during the second step of the beta oxidation process, can be abolished by the deletion of the genes encoding the proteins responsible of first or second steps, if PLA homopolymers are produced in the peroxisomes. It is then possible, using yeast cells to produce PLA devoid of other hydroxyalkanoate by producing polymer in a compartment free of them (such as cytosol or mitochondria) or by deleting genes responsible of their synthesis in the peroxisomes. In addition, repurposing yeast organelles, such as peroxisomes and mitochondria, as production compartment can allows to
bypass metabolic cross-talk between the engineered PLA pathway and endogenous pathways. An additional advantage using yeasts, such as of *Yarrowia* genus, is their resistance to acidic environment, as the monomer supply could induce a decrease in pH. More importantly, such acidic environment greatly improves the uptake of the monomer and drastically reduces risk of undesirable culture contamination. Moreover, some yeasts such as *Yarrowia lipolytica* are GRAS (Generally Recognized As Safe) microorganisms. They do not produce any endotoxin. PLA can be purified without any contamination by lipopolysaccharides, which is encountered in bacteria and cause immunogenic reactions. Genes that are added to produce the strain of interest are integrated in the genomic chromosome of the yeast and are thus more stable than expression via plasmids as practiced in bacteria. The invention thus provides competitive alternative methods for PLA production that are less expensive and more efficient than chemical processes.

**Definitions**

The present disclosure will be best understood by reference to the following definitions.

A "polymer" refers to a chemical compound or mixture of compounds whose structure is constituted of multiple units linked by covalent chemical bonds. Within the context of the invention, the term "polymer" includes polymers comprising a single type of repeating unit (i.e., homopolymers) or different types of units (i.e., block copolymers and random copolymers). More particularly, in the context of the invention the terms "homopolymer of PLA" refers to a polymer composed solely either of L- or D-lactic acid units and devoid of any other units (like hydroxyalkanoate or the like). Accordingly, "homopolymers of PLA" designate poly-L-lactic acid (PLLA) or poly-D-lactic acid (PDLA). "Heteropolymers of PLA" designate poly-DL-lactic acid (PDLLA).

In the context of the invention, the terms "lactate" and "lactic acid" are used interchangeably to refer to an organic compound with formula CH3CH(OH)C0₂H or its ionized form. In absence of further indications, these terms designate indistinctly the L-lactic acid, the D-lactic acid and mixture thereof. In the same way, the terms "polylactate" and "polylactic acid" are used interchangeably to refer to a polyester composed essentially of lactic acid units. In absence of further indication, these terms designate indistinctly PLLA, PDLA and PDLLA.
The terms "nucleic acid", "nucleic sequence", "polynucleotide", "oligonucleotide" and "nucleotide sequence" designate a molecule or sequence of deoxyribonucleotides and/or ribonucleotides.

A "gene" designates any nucleic acid molecule comprising an open reading frame encoding a protein. A gene may contain additional sequences such as a promoter, a transcription terminator, a signal peptide, or an intron, for instance.

The term "recombinant" refers to a nucleic acid construct, a vector, a polypeptide or a cell produced by genetic engineering.

In the present description, a "recombinant yeast cell" refers to a yeast cell that has been genetically modified, most often by insertion of a nucleic acid sequence or unit that did not exist naturally in the existing yeast cell, and/or by inactivation of a native nucleic acid sequence or unit. Said nucleic acid sequence or unit may have been inserted or inactivated in said yeast cell or an ancestor thereof, using recombinant DNA technology or random mutagenesis.

In the context of the invention, the terms "variant" or "mutant" are used for designating a functional protein comprising at least one amino acid modification or alteration, i.e., a substitution, insertion, and/or deletion, at one or more (e.g., several) positions compared to a parent protein (i.e., it is mutated at least at one amino acid position) and that presents the desired properties. The variants may be obtained by various techniques well known in the art. In particular, examples of techniques for altering the DNA sequence encoding the wild-type protein, include, but are not limited to, site-directed mutagenesis, random mutagenesis and synthetic oligonucleotide construction. The term "expression", as used herein, refers to any step involved in the production of a polypeptide including, but being not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

In the context of the invention, the "genome of a yeast cell" means all of the genetic material contained in said yeast cell, including the extrachromosomal genetic material contained, for example, in the plasmids, episomes, synthetic chromosomes, etc.

The terms "heterologous sequence" or "heterologous protein" designate a sequence or protein that does not exist in the natural state in the considered microorganism. Conversely, the terms "homologous sequence" and "endogenous sequence", or "homologous protein" and "endogenous
"protein" designate a sequence or protein that does exist in the natural state in the considered microorganism.

In the same way, the terms "endogenous activity" designates an activity that is present in the natural state in the considered microorganism. Conversely, the terms "exogenous activity" designates an activity that is not present in the natural state in the considered microorganism.

Herein, the terms "peptide", "polypeptide" and "protein" are employed interchangeably and refer to a chain of amino acids linked by peptide bonds, regardless of the number of amino acids forming said chain. The amino acids are herein represented by their one-letter or three-letter code according to the following nomenclature: A: alanine (Ala); C: cysteine (Cys); D: aspartic acid (Asp); E: glutamic acid (Glu); F: phenylalanine (Phe); G: glycine (Gly); H: histidine (His); I: isoleucine (ile); K: lysine (Lys); L: leucine (Leu); M: methionine (Met); N: asparagine (Asn); P: proline (Pro); Q: glutamine (Gin); R: arginine (Arg); S: serine (Ser); T: threonine (Thr); V: valine (Val); W: tryptophan (Trp) and Y: tyrosine (Tyr).

As used therein, the terms "inactivated", "inactivation", "altered" or "defective" in relation with a given activity or protein, indicate a reduction or attenuation in the level of said activity or protein in the yeast cell. Such a reduction is typically of about 20%, more preferably 30%, as compared to a wild-type protein in the yeast cell. Reduction may be more substantial (e.g., above 50%, 60%, 70%, 80% or more), or complete (i.e., knock-out). According to the invention, such inactivation may be obtained by various techniques, performed at the level of DNA, mRNA or protein, to inhibit the expression of the corresponding gene (e.g., transcription or translation) or the activity of the protein. At the level of DNA or mRNA, inactivation may be accomplished by, e.g., deletion, insertion and/or substitution of one or more nucleotides, site-specific mutagenesis, ethyl methanesulfonate (EMS) mutagenesis, targeting induced local lesions in genomes (TILLING), knock-out techniques, or gene silencing using, e.g., RNA interference, antisense, aptamers, and the like. Another particular approach is gene inactivation by insertion of a foreign sequence, e.g., through transposon mutagenesis using mobile genetic elements called transposons, which may be of natural or artificial origin. A protein may also be rendered defective by altering its activity, either by altering the structure of the protein, or by expressing in the cell a ligand of the protein, or an inhibitor thereof, for instance. Preferred inactivation methods affect expression and lead to the absence of production of a functional protein in the yeast cell. In particular, defective gene is obtained by deletion, mutation, insertion and/or substitution of one or more nucleotides.
**Biological Polylactic acid synthesis**

Biological production of PLA may occur in two steps. The first one consists in lactic acid activation in lactyl-CoA using CoA donor. In a particular embodiment, illustrated in Figure 1, acetyl-CoA is used as the CoA donor. This step requires a lactyl-CoA synthase such as a CoA transferase such as propionyl-CoA transferase (Pctp). In another embodiment, a ligase may be used, such as acyl-CoA ligase that binds CoA on lactic acid in order to form lactyl-CoA.

The second step is the polymerization of lactyl-CoA in PLA by a lactyl-CoA polymerase such as a PHA synthase.

In order to allow biological production of PLA into yeast cells, the inventors have engineered yeast cells that exhibit both lactyl-CoA synthase activity and lactyl-CoA polymerase activity.

According to the invention, the yeasts may naturally exhibit one of these activities and be genetically modified to have the second activity. For instance, the yeast cell originally exhibits lactyl-CoA synthase activity, and a protein having lactyl-CoA polymerase activity has been genetically introduced into the yeast cell or ancestors thereof, or the inverse. Alternatively, the yeast is originally devoid of both activities, and has been genetically modified for these two activities. Alternatively, or in addition, the yeast originally exhibits at least one of these activities, and has been modified so as to promote and/or enhance the expression of the gene(s) involved.

In accordance with the invention, the genome of the yeast cell or of an ancestor thereof has been modified by the introduction of at least one nucleic sequence coding for at least one enzyme involved in the PLA producing pathway, or a biologically active fragment thereof.

In a particular embodiment, the invention provides a recombinant *Yarwwia*, typically a recombinant *Y. lipolytica*, that exhibits an endogenous lactyl-CoA synthase activity, and that is further engineered for expressing a heterologous protein having a lactyl-CoA polymerase activity.

In another embodiment, the invention provides a recombinant *Yarwwia*, typically a recombinant *Y. lipolytica* that has been engineered for expressing both a heterologous protein having lactyl-CoA polymerase activity and a heterologous protein having lactyl-CoA synthase activity.
In a particular embodiment, the protein having lactyl-CoA synthase activity is CoA transferase. Particularly, the protein having CoA transferase activity is a propionyl-CoA transferase (EC:2.8.3.1) known to be able to activate lactic acid in lactyl-CoA.

For instance, a nucleic acid coding for a propionyl-CoA transferase (Pctp) having an amino acid sequence as set forth in SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4 or SEQ ID No 16, or functional variants thereof having lactyl-CoA synthase activity, has been introduced in the recombinant yeast cell or ancestors thereof.

SEQ ID No 1 / Pctp Clostridium propionicum (Accession number Q9L3F7 / CAB77207.1)

M RKVPI ITADEAAKLI KDGTDTVTSG FVGNAIPEALDRAVEKRFLGETEPKINCTYYVCGSQG NRDG RGAEH F
AHEG LLLKRYIAG HWATVPALG KMAM EM KN EAYNVSQALCH LFRDIASHKPGVFVTKVIGIFTFIDPRNGG
GKVN DITKED IVELVEIKQGEYLFYPAPFHI HVALIRGTYAD ESGNTITEKEVAPLEGTSVCQAVKNSGG IVVVQ
VERVKAGTLDPRHVKPGYIVDYVVPADV PDEHQQSLDCEYDPAKSG EHRPEVGG EPLP LSAAKVIG RRG
AJIELEKDVAVN LGVGAPEYVNASVD EEGIVDFMTLTAESAIGGVPAGGVR FGASYNADALIDQGYQFDYY
DDG GLDLCYLGAECDELGKINGIVNISRFGPRATGCCGFIJNIQTNPKVFFCGTFTAG GLKVKIEDG KVIQVEGK
QKKFLKAVEEQITFENGDVALANKQOVYITERCVFLKEDG LHALSEIAPG IDLQTOIDVMDFAPIDRDANGQIKLM
DAALFAEG LMG LKEM K5

In a particular embodiment, a nucleic acid coding for a variant of the Pctp of SEQ ID No 1 comprising preferably at least one of the mutations selected from VI93A and A243T and having lactyl-CoA synthase activity is used.

SEQ ID No 2 / Pctp Aspergillus nidulans (Accession number Q5B0U7 / AN5833.2)

MTH PQOAVH AASLQNPWPAFWSHHAOQLWHKKPSRAIGRSTKTIASGASHESWSWFDGEGISTTNYCSV
DRHVLK NGNGNVAIWDSAVTG KKEKYTYRQLEDVEVLAVGLEEGVGGVDDVIMPMIPAALIGALAV
ARLGAI HAASFG GFKAASLQAQR IEAARPAI LTASCG IEAGAKPIAYRPVEGAEASSKPEKVLIWQDQLR
WNNPDKLGQGRNN WRLVKSARM RG IRAE PVPRSTGDG LIIYITSGTTG LP KGVRVR EAG GHAVG LSLISKYL
FDI HPGG DTM FCASD IGWVVG HSYI LYAP LLGVATTILTLEGKPGTPDAGTFWRRVVAE HAKANLFTAPTA
LRAl RKED PDN KH FKVAG DNNLNHLR LRLALFLAG ERSE PSIVRAYQD LTTKHAARGALVVD NWWSSESQSPI
SG LALRSAG RVP RESDYVAPLAI RPSGAGLP MPG FDVRVVD EG N EVAGQTMG N IVMATLAPLATAF
TRLN DDER FYGKGLKR FGG RWLDTG DAG MI D OGGYI HVMSRSDIIINVAH RFS TQQGSIEQAI LSH PAI
GEASVVG IPDALKG HLPFAITLQSQSG NSPARPSAE LFNSVN RLVRREQIGAIASLG G M IQG Q G M IPKTRSG
KTLR RVL R ELVEN GARG E F EKEAVPPTVED RGVVEVARE K V R EYFESQSQSP KAKL

SEQ ID N°3 / Pctp Escherichia coli (Accession number A0A0F6C4Y4 / AFJ29290.1)

M KPKVKKP R I NG RVPVLSAQEAVNYI PD EATLCLVLGAG G G I LEATTLITALAD KYKQTQTP R NL S I SP T G L G D
RADRG ISPLAQEG LVKWALCG HWG QSP IR SD L AEQN K I AYNYPQGVLTQTLRAA AH Q PG I S D I G I GTFV
D PRQQGG KLN EVTK HDL KLYE FDN KE YLYKAIAP DIAFI R ATTCDSEGYATFD E V MYLDAVIAQAVHN N
NGG IVM MQVQMVKKATLH PKSVR IPGYLVIDIVVDP DQSQLYG GAPV N R FISG DFTLDDSTKLSPLN Q
RKLVARRALFEM RKGA VG NVVG V I AG I G L V A R E EG CAD DFI LTVETG P I G G I TSQQ I AFGANVNTRAI LD
MTSQDFYH GGG LDVCYL SFAEVDQH GNVGH K FN G K I MGTGG F IDISATS K K I F CG TL T A G S L K T E IADG
PEKLM DERLFI DAAMG F V L P E A A H

SEQ ID N°4 / Pctp Ralstonia etukopha (Accession number Q0K874 / CAJ93797.1)

M KVI TAREA AALVQDGWTASAG FVGAG H AEAVT EALEQOR FLQSG LPR DLTLVYSAQ G Q D RG A R G V N H
FG NAG MTASIVGG HWRSAT L TAL AMAE Q CE Q Y N LPQVLTH LYRIAAGK PGVM TKIG LHTFVD PR TA
QDARYH G GAVN ERARQIAE G KACWVDADVFR G D E YLFYPSFP IHCALI RCTAADARG N LSTH REAFH HE
LLAMAQA AH N S G G I V A Q V E S L V D H HE I L Q A I H V P G ILVDYVVVCDN P AN H QMTF A E S YN
PAYVPWOG EA AVA EA EA P V A A G P LDARTIVORRAV M ELAR RAPRVVN LG V G M PAAVG M LA H AQ LGDFTLTV E AG
PIGGTPADG LSFGAS AYP EA VVDPOAPAQFD FYE GG G IDLAI LG L AEL D G H G N V N S K F G E G E G ASI AG VGG F
INITOSARAVVF MG T LT AG G LE VRAG DGG LQI VREG R V K K I VP EVSH LSFN G PY VAS LG IPVLYI T E R A V FE M
RAGADG EARLTLVEIAPGDVQLRDVLDQCSTP I A V AQD LRE M DAR LFOAG PLH L

SEQ ID N°16 / Pctp Yarrowia lipolytica (Accession number XP_505057.1)

SEDHPAI H PPSE F KD N H PH G F G HL DCLQDYH Q L H K ESI E D PKAFWKKMAN E LISWSTPFETVRS GG FEH
GDVAWFPQEGNLNAS Y CVDR HAFAN PD KPAI IF EAD EPG Q G R I V Ty G E LLRQ V S Q V AA TRLSF G VQKG DT
CPSVT HTLVFR RAGVEN LAWTEG RD FW HH EE V KH R PYLAPVPVASE D P IF LLYT S GSTGT PKG LA H AT G
GYLLGA AL TAK YVFDI H G D DKLFTAG DG W IT G H T YLV G PL M LGATTV E VEGTPAYPFSR Y WD I V D HK
In another embodiment, the protein having lactyl-CoA synthase activity is a CoA ligase (EC:6.2.1), preferably an acyl-CoA ligase.

In a particular embodiment, the protein having lactyl-CoA synthase activity is expressed in the cytosol. In another embodiment, the protein having lactyl-CoA synthase activity is expressed in a subcellular organelle, such as the peroxisome or mitochondria. To this end, a nucleic acid coding for the respective targeting sequence may be added at the 5' or 3' terminus of the sequence of interest (see below).

According to the invention, the recombinant yeast cell may express a heterologous protein having a lactyl-CoA polymerase activity.

In a particular embodiment, the protein having a lactyl-CoA polymerase activity is stereospecific. Advantageously, the protein has a D-lactyl-CoA polymerase activity, leading exclusively to the production of PDLA. In another embodiment, the protein has L-lactyl-CoA polymerase activity, leading exclusively to the production of PLLA.

In a particular embodiment, the lactyl-CoA polymerase presents affinity for short chain length acyl-CoA, comprising from 3 to 7 carbons, preferably from 3 to 5 carbons, more preferably 3 carbons, more preferably lactic acid.

In a particular embodiment, the protein having lactyl-CoA polymerase activity is expressed in the cytosol. In another embodiment, the protein having lactyl-CoA polymerase activity is expressed in a subcellular organelle, such as the peroxisome or mitochondria. To this end, a nucleic acid coding for the respective targeting sequence may be added at the 5' or 3' terminus of the sequence of interest (see below).
Preferably, the recombinant yeast cell expresses a heterologous PHA synthase (PhaCp) as lactyl-CoA polymerase.

In a particular embodiment, the PHA synthase is a class II PHA synthase from *Pseudomonas aeruginosa* PAOl (PaPhaCp).

For instance, the PHA synthase has the amino acid sequence as set forth in SEQ ID N°5, or a variant thereof having lactyl-CoA polymerase activity.

SEQ ID N°5 / PhaClp of *P. aeruginosa* PAOl (Accession number G3XCV5 / NP_253743.1)

Alternatively, a nucleic acid coding for a variant of PHA synthase of *P. aeruginosa* PAOl (SEQ ID N°5) comprising preferably at least one of the mutations selected from E130D; S325T; S477R; S477F; Q481M; Q481K; S482G; L484V and A547V, and having lactyl-CoA polymerase activity is used.

In a particular embodiment, the recombinant yeast cell expresses a PHA synthase with the three following mutations in SEQ ID N°5: E130D; S477F; Q481K.

In another embodiment, the recombinant yeast cell expresses a PHA synthase with the four following mutations in SEQ ID N°5: E130D; S325T; S477R; Q481M (SEQ ID N°11 or gene sequence SEQ ID N°15).

Alternatively or in addition, a nucleic acid coding for a functional mutant of Class I PHA synthase (EC:2.3.1.B3) such as a mutant of PHA synthase from *R. eutropha*, or a nucleic acid coding for a functional mutant of class III PHA synthase (EC:2.3.1.B4) such as a mutant of PHA synthase from *Chromatium vinosum* may be used (such as SEQ ID N°6 or SEQ ID N°7).
SEQ ID N° 6 / PhaClp of *R. eutropha* H16 (Accession number P23608 / CAJ92572.1)

MATG KGAAASTQEG KSQP FKVTGP FDLPATWLESRQWQGTEG NGHAAASG IPG LDALAGVKAAPAQ
LG DIOQRYM KD FSALWQAMAEG KAEATG PLHD RRFAQ DAWRTN LPYR FAAFYLLNARALTE LADAVE
ADAKTRQR IRFAISQWVDAMPAN FLATN PEAQLLI ESGG ESLRAGVR NM M ED LTRGKISQTD ESAFEV
GRNVAVTEGAVVFEN EYFQLQYKPLTDKVRHARP LLVPPCI NKYI LDLPQ ESSLVRHVVEQG HTVFLVS
WRN PDASMAGSTWDDYI EHAAI RAI EVAR DISGQDKI NVLG FCVG GTIVSTALAVLAARG EH PAASVTLLT
TLLD FADTG ILDVFD EG HVQLREATLLG GAGAPCALLRG LE LANTFSFLR PND LVWNYYVNNYLGK NTP
VPFD LLFWN GDATN LPG PWYCWYLRTYLMN ELKVPG KLTVCVPVDLAS VDPTYIYGRS ED HIPWTA
AYASTALLAN KLRFVLGAS G HAGVI N PPAKN KRSHWTN DLPESPO QWLGA G EHHGSWPDWTAWL
AGQAQAKRAAPANYG NARYRAI EAPG RYVKAKA

SEQ ID N° 7 / PhaCp of *C. vinosum* (Accession number Q402A9)

MFPI DLRP DKLTPM LDDSRKLQG QM EN LLNAEAI DTGVP KQAVYSED KLVLYR YDR PEGAP EAPVPLLI
VYALVN RPYMTDI QED RSTI KG LLATGQDVVYL D WGYPDQAD RALTLD DYINGYI DRCVYLR EAH GVD KV
NLLG ICQGGA FSLMYSALH PD KVR N LVMTV PVDKTPD NLLSAVQNVDI DLAVDMG N IPG ELLNWT
FLS KPF S L TGV K KYVN MV DLLD DP DKVKN FLRM EK WFD SP DQ AG E TFRQFI KDFYQ N NG FN NGVV LV G
QEVD LKD ICTPV LN IFALQD HLVP PDASRALKG LTSSPYTELAFPG GHIG IYVS G KAOKEVTA PAG KWL N ER

In a particular embodiment, several copies of a nucleic sequence coding for at least one enzyme involved in the pathway for producing PLA are introduced in the recombinant yeast, particularly, at least two copies.

In a particular embodiment, at least two copies of a nucleic sequence coding for a lactyl-CoA synthase activity, preferably at least two copies of a nucleic sequence coding for a propionyl-CoA transferase, are introduced in the recombinant yeast.

Alternatively or in addition, at least two copies of a nucleic sequence coding for a lactyl-CoA polymerase activity, preferably at least two copies of a nucleic sequence coding for a PHA synthase, are introduced in the recombinant yeast.

In a preferred embodiment, at least two copies of a nucleic sequence coding for a propionyl-CoA transferase and at least two copies of a nucleic sequence coding for a PHA synthase are introduced in the recombinant yeast.
Advantageously, the nucleic sequence(s) introduced in the recombinant yeast is (are) under the control of an inducible or constitutive promoter. Particularly, promoters with different strength are used to control the expression of at least one enzyme. As an example, promoters TEF and 4UAS-TEF are used to control the expression of the nucleic sequences. Preferably, the nucleic sequence coding for a propionyl-CoA transferase and the nucleic sequence coding for a PHA synthase are under the control of the 4UAS-TEF promoter.

It is therefore an object of the invention to provide a recombinant yeast cell, preferably a recombinant *Yarrowia*, typically a recombinant *Y. lipolytica* that has been engineered for expressing both at least one Pctp having the amino acid sequence as set forth in SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°16, or a variant thereof, and at least one PhaCp having the amino acid sequence as set forth in SEQ ID N°5, SEQ ID N°6, SEQ ID N°7 or SEQ ID N°1 1, or a variant thereof.

*Protein targeting in subcellular organelles*

In a particular example, the production of PLA may be confined into specific subcellular organelles of the recombinant yeast cell, such as mitochondria or peroxisome, rather than the cytosol. Such confined production may be useful for favoring the production of PLA with high molecular weight.

The activities required for the PLA production, and the associated proteins, must then be targeted into the subcellular organelle of interest. To this end, specific sequences allowing protein targeting may be added at the 5’ or 3’ terminus of the nucleic acid coding for the proteins of interest.

In a particular embodiment, the PLA production may be confined into peroxisome, by using a Peroxisome Targeting Sequence (PTS) with the proteins that are not expressed or targeted naturally into the peroxisome.

For instance, a nucleic acid coding for a PTS as set forth in SEQ ID N°8 (PTS1), which corresponds to 14 successive amino acids of the isocitrate dehydrogenase (*YALI0C16885p / P41555 - Icllp Isocitrate lyase from Y. lipolytica*) may be used. In another embodiment, the PTS may correspond to a tripeptide such as SKL or AKL or any sequence known by a person skilled in the art. Preferably, the PTS is located at the C-terminus of the protein of interest. In another embodiment, the PTS sequence consists on the N-terminus of the *Y. lipolytica* thiolase.
(YALI0E18568p / Q05493 - Potlp 3-ketoacyl-CoA thiolase) which is cleaved upon peroxisome entry (SEQ ID N°9 - PTS2). PTS2 is preferably located at the N-terminus of the protein of interest. More generally speaking, any PTS known by the person skilled in the art may be used.

SEQ ID N°8: MGAGVTEDFKSKL

SEQ ID N°9: MDRLNNLATQLEQNP

It is therefore an object of the invention to provide a recombinant yeast cell, preferably a recombinant Yarrowia, typically a recombinant Y. lipolytica that has been engineered for expressing at least one Pctp having the amino acid sequence as set forth in SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°16, or a variant thereof, in cytosol and at least one PhaCp having the amino acid sequence as set forth in SEQ ID N°5, SEQ ID N°6, SEQ ID N°7 or SEQ ID N°11, or a variant thereof in peroxisome. In a particular embodiment, the amino acid sequence of the PhaCp further comprises at its C-terminus the amino acid sequence set forth in SEQ ID N°8 or at its N-terminus the amino acid sequence set forth in SEQ ID N°9.

Alternatively, the PLA production may be confined into mitochondria, and more particularly into mitochondrial matrix or mitochondrial intermembrane space, by attaching a Mitochondrial Targeting Sequence (MTS) to the proteins that are not expressed or targeted naturally into the mitochondria. In a particular embodiment, a nucleic acid coding for the MTS as set forth in SEQ ID N°10 may be used, that corresponds to 26 successive amino acids of the malic enzyme (YALI0E18634p / Q6C5F0 - Maelp). This MTS is preferably added at the N-terminus of the protein of interest. More generally speaking, any MTS known by the person skilled in the art may be used.

SEQ ID N°10: MLRLRTMRPTQTSVRAALGPTAAARN

Lactic acid metabolism

According to the invention, the ability of the yeast cell to consume lactic acid as a carbon source may be attenuated or inhibited. Accordingly, the recombinant yeast cell will be solely able to polymerize LA for producing PLA. That may be of particular interest with Y. lipolytica or Saccharomyces cerevisiae that usually consume lactic acid as a carbon source.
To this end, the yeast cell may be modified in order to inactivate its lactic acid degradation pathway. For instance at least one endogenous lactic acid oxidoreductase responsible of converting lactic acid to pyruvate is inactivated.

In the context of the invention, the expression "lactic acid oxidoreductase" is used to designate any enzyme able to produce pyruvate from lactate, and the reverse (EC: 1.1.1.27, EC: 1.1.1.28, EC: 1.1.2.3, EC: 1.1.2.4). More particularly, this expression encompasses lactate dehydrogenase, ferricytochrome, flavocytochrome, and cytochrome oxidoreductase.

Preferably, a D-lactic acid dehydrogenase, that converts D-lactic acid to pyruvate, is inactivated. In a particular embodiment, an endogenous L-lactic acid cytochrome b2 or c oxidoreductase may be maintained, so that the yeast remains able to degrade L-lactic acid. D-lactic-acid (D-LA) stays available for the synthesis of PLA. Such recombinant yeast cell may advantageously be able to produce PDLA.

Alternatively, an endogenous L-lactic acid oxidoreductase is inactivated, whereas an endogenous D-lactic acid oxidoreductase is maintained, so that the yeast remains able to degrade D-lactic acid. L-lactic-acid (L-LA) stays available for the synthesis of PLA. Such recombinant yeast cell may advantageously be able to produce PLLA.

Alternatively, both endogenous D-lactic acid and L-lactic acid oxidoreductases may be inactivated so that D-lactic-acid (D-LA) and L-lactic-acid (L-LA) both stay available for the synthesis of PLA. Such recombinant yeast cell may advantageously be able to produce PDLLA.

In a particular embodiment, D-lactic acid oxidoreductase is D-lactic acid dehydrogenase (DLD), preferably from the protein families GL3C0735 or GL3C0514, such as Dld1lp, Dld2p and Dld3p. In another particular embodiment, the L-lactic acid oxidoreductase is cytochrome oxidoreductase (CYB), preferably from the protein family GL3C0472, such as Cyb2p, Cyb21p and Cyb22p.

Table 1 and Table 2, below, list four putative lactic acid oxidoreductases from Y. lipolytica and S. cerevisiae respectively and their predicted localisation.

Table 1. Putative lactic acid oxidoreductases of Y. lipolytica
<table>
<thead>
<tr>
<th>Name</th>
<th>Family</th>
<th>Protein</th>
<th>Putative function</th>
<th>Accession number</th>
<th>Predicted localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLDLDl</td>
<td>GL3C073</td>
<td>YALI0E03212</td>
<td>D-lactate dehydrogenase</td>
<td>Q6C773</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>YXDLD2</td>
<td>5</td>
<td>YALI0C06446</td>
<td></td>
<td>Q6CCU5</td>
<td></td>
</tr>
<tr>
<td>YXCYB21</td>
<td>GL3C047</td>
<td>YALIOD12661</td>
<td>L-lactic acid dehydrogenase</td>
<td>Q6C9A7</td>
<td>Peroxisome</td>
</tr>
<tr>
<td>YXCYB22</td>
<td>2</td>
<td>YALI0E21307</td>
<td></td>
<td>Q6C538</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Putative lactic acid oxidoreductases of *S. cerevisiae*.

<table>
<thead>
<tr>
<th>Name</th>
<th>Family</th>
<th>Protein</th>
<th>Putative function</th>
<th>Accession number</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScDLD1</td>
<td>GL3C051</td>
<td>SACE0D01650</td>
<td>D-lactate dehydrogenase</td>
<td>P32891</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>ScDLD2</td>
<td>GL3C073</td>
<td>SACE0D01562</td>
<td></td>
<td>P46681</td>
<td></td>
</tr>
<tr>
<td>ScDLD3</td>
<td>5</td>
<td>SACE0E00242</td>
<td></td>
<td>P39976</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>ScCYB2</td>
<td>GL3C047</td>
<td>SACE0M01892</td>
<td>L-lactic acid dehydrogenase</td>
<td>P00175</td>
<td>Mitochondria</td>
</tr>
</tbody>
</table>

In a particular embodiment, the invention provides a recombinant *Y. lipolytica* wherein YLDldlp has been inactivated.

In another particular embodiment, the invention provides a recombinant *Y. lipolytica* wherein YICyb21p has been inactivated.
It is therefore an object of the invention to provide a recombinant \textit{Y. Upolytica} that has been engineered for expressing at least one Pctp having the amino acid sequence as set forth in SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°16, or a variant thereof, in cytosol, at least one PhaCp having the amino acid sequence as set forth in SEQ ID N°5, SEQ ID N°6, SEQ ID N°7 or SEQ ID N°1, or a variant thereof and further comprising at its C-terminus the amino acid sequence set forth in SEQ ID N°8 or at its N-terminus the amino acid sequence set forth in SEQ ID N°9 in order to be addressed in the peroxisome, and that has been further engineered in order to inactivate the lactate dehydrogenase (YIDldlp and/or YICyb21p).

Alternatively, the inventors have discovered that no D-lactate oxidoreductase activity is observed by culturing the recombinant yeast cell in a medium devoid of particular amino acids. For instance, \textit{Y. Upolytica} may be cultured in a culture media devoid of Ala, Arg, Asn, Gin, ile, Leu, Lys, Phe, Pro, Thr and Val to avoid the expression of YIDldlp.

Alternatively, it is possible to inhibit or reduce the consumption of lactic acid by providing other suitable carbon sources to the yeast cell.

In order to improve lactic acid transport into the yeast cells or to reduce its export out of the yeast cells, the cells may be partially inactivated for export or improved for import, preferably D-lactic transport/export.

For instance, the intake of lactic acid into the cells can be increased by overexpressing or overactivating lactate importers, such as specific membrane transporters. Alternatively or in addition, the export of lactic acid out of the cells can be at least reduced by inactivation or deletion of lactic acid exporters.

In an embodiment, the recombinant yeast cell exhibits an exogenous lactic acid racemase activity. For instance, the racemase converts L-lactate into D-lactate or the reverse. In a particular embodiment, the recombinant yeast cell comprises the racemase from \textit{Lactobacillus plantarum} (LpLarAp - EC:5.2.2.1 - M4KD2 / AGE37852).

\textit{Modified cytosolic acetyl-CoA metabolism}
According to the invention, the recombinant yeast cell exhibits lactyl-CoA synthase activity to produce lactyl-CoA from lactic acid. In a particular embodiment, lactyl-CoA synthase activity is performed using a Pctp, which uses acetyl-CoA as CoA donor.

In this context, the production of PLA in the cytosol may be facilitated by enhancing acetyl-CoA availability into the cytosol of the recombinant yeast cells. To this end, three alternative or cumulative ways may be considered: redirecting part of the acetyl-CoA (in majority contained in the mitochondria of yeast cells) from the mitochondria to the cytosol; altering the metabolic pathways that use acetyl-CoA in the cytosol; introducing new metabolic pathways in order to increase cytosolic acetyl-CoA production (Figure 2 and Figure 3).

Some yeasts, such as *Y. lipolytica*, may use cytosolic acetyl-CoA for the synthesis of triacylglycerol (TAG). In such case, TAG production is directly in competition with PLA production. Thus, according to a preferred embodiment, the recombinant yeast cell may be modified to inactivate the TAG synthesis pathway. For instance, in *Y. lipolytica*, the genes *YXDGAI* (YALI0E32769g / XM_504700.1) and/or *YXDGAI* (YALI0F06578g/XM_505086.1) and/or *YXLR0I* (YALI0E16797g / XM_504038.1), encoding endogenous diacylglycerol- transferases (Beopoulos et al., 2008) may be inactivated (Figure 2 point 3).

In addition, some yeasts, such as *Y. lipolytica*, also possess a gene encoding for a citrate synthase (EC:2.3.3.16) that performs the reverse reaction of the one catalyzed by the ATP citrate lyase. Such reaction consumes cytosolic acetyl-CoA and is directly in competition with PLA production. Accordingly, in another preferred embodiment, citrate synthase activity is inactivated in the recombinant yeast cell (Figure 2, point 4).

In addition, in some yeast cells, such as *Y. lipolytica*, most of the cytosolic acetyl-CoA pool comes from the conversion of citrate, produced through the Krebs cycle, into acetyl-CoA by ATP citrate lyase (EC:2.3.3.8). A transmembrane transport mechanism couples malate import to export citrate out of the mitochondria. Accordingly, in a particular embodiment, the recombinant yeast cell of the invention is cultivated under particular conditions that induce a physiological state favorable for citrate export to the cytosol (see Figure 2, point 1). For instance, the culture medium is at least temporarily restricted in specific nutrient(s), such as nitrogen, phosphate and/or oxygen.
Alternatively or in addition, malate synthase activity (EC:2.3.3.9) that drives part of the mitochondrial acetyl-CoA away from citrate production as to be used for malate production (glyoxylate shunt pathway) may be inactivated or attenuated (Figure 2, point 2).

In a particular embodiment, a gene encoding a citrate synthase and/or a gene encoding a malate synthase is inactivated in the recombinant yeast cell of the invention, or ancestor thereof.

In another embodiment, the recombinant yeast cell exhibits an inactivated acyl-CoA:diacylglycerol acyltransferase (DGAT1/2, DGA1/2) and/or phospholipid:diacylglycerol acyltransferase (PDAT, LROI) activity.

As well illustrated in Figure 1, the reaction catalyzed by the Pctp produces acetate in addition to lactyl-CoA. In a particular embodiment, in order to increase the cytosolic production of acetyl-CoA, a heterologous gene coding for an acetyl-CoA synthase also called Acsp (EC:6.2.1) may be introduced in the recombinant yeast cell to convert the acetate back into acetyl-CoA. Alternatively, an endogenous gene coding for an acetyl-CoA synthase can be overexpressed. This reaction can be ADP or AMP dependent according to the enzyme considered (Figure 3, point 5). Accordingly, in a particular embodiment, the recombinant yeast cell further comprises a gene encoding an acetyl-CoA synthase.

In the same way, a heterologous pyruvate dehydrogenase may be introduced in the recombinant yeast cell for the conversion of glycolytic pyruvate into acetyl-CoA in the cytosol (Figure 3, point 6). An enzyme with low sensitivity to high NADH/NAD⁺ ratio is preferably used, such as the EfPdhp (NP_815074.1, NP_815075.1, NP_815076.1, NP_815077.1) from Enterococcus faecalis or any other enzyme known by the person skilled in the art. NADH/NAD⁺ ratio in the cytosol could also be modulated by introduction of a gene coding for a non-phosphorylating glyceraldehyde-3P dehydrogenase that allows generation of NADPH during glycolysis instead of NADH (Figure 3, point 6). Accordingly, in a particular embodiment, the recombinant yeast cell further comprises a gene encoding a pyruvate dehydrogenase, said pyruvate dehydrogenase being expressed at least in the cytosol of the recombinant cell.

Alternatively or in addition, an alternative pathway for cytosolic acetyl-CoA production may be introduced in the yeast cell, by the heterologous expression of a phosphoketolase (EC:4.1.2.22). Phosphoketolase catalyzes the formation of acetyl-P and erythrose-4P from fructose-6P (Meile et al., 2001). In this metabolic pathway, acetyl-P is converted into acetyl-
CoA by the action of a phosphotransacetylase (EC:2.3.1.8) or by the combined action of an acetate kinase (EC:2.7.2.1) and an acetyl-CoA synthase. Erythrose-4P undergoes carbon rearrangement by the action of transketolase and transaldolase activities, both intrinsically expressed in the pentose phosphate pathway of *Y. lipolytica* and thus is made capable of entering glycolysis. In addition, introduction of a gene coding for an heterologous fructose-1,6-biphosphatase (EC:3.1.1.11) allows a complete recycling of erythrose-4P that gives the possibility to convert 100% of the carbon present in the substrate into acetyl-CoA without CO₂ production (Figure 3, point 7).

**Recombinant yeast cell**

It is an object of the invention to provide a recombinant yeast cell exhibiting lactyl-CoA synthase activity, preferably a CoA transferase activity and a lactyl-CoA polymerase activity, and that is able to produce PLA.

Advantageously, the selected yeast cell is originally able to produce and accumulate high concentration of lipids. In a particular embodiment, the yeast cell is selected from GRAS (Generally Regarded As Safe) yeasts. In addition, the selected yeast is advantageously able to grow at low pH.

According to the invention, the yeast cell preferably belongs to *Yarrowia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Pichia*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Hansenula*, *Trichosporon*, *Yamadazyma*, *Cryptococcus*, *Lipomyces*, *Rhodosporidium*, *Rhodotorula*, *Geotrichum*, *Kloeckera*, *Schwanniomyces*, *Brettanomyces*, *Debaryomyces* or *Issatchenkia* genus.

In a particular embodiment, the yeast cell is a *Yarrowia* selected from the group consisting of *Yarrowia bubula*, *Yarrowia deformans*, *Yarrowia lipolytica*, *Yarrowia yakushimensis*, *Yarrowia galli*, *Yarrowia oslonensis*, *Yarrowia hollandica*, *Yarrowia phangngensis*, *Yarrowia alimentaria*, and *Yarrowia porcina*, preferably *Yarrowia lipolytica*.

It is therefore an object of the invention to provide a recombinant *Y. lipolytica* expressing a heterologous Pctp, and a heterologous PhaCp.
In a particular embodiment, the recombinant *Yarrowia lipolytica* expresses a Pctp having an amino acid sequence as set forth in SEQ ID No1, SEQ ID No2, SEQ ID No3, SEQ ID No4, or SEQ ID No16 or functional variants thereof having lactyl-CoA synthase activity.

In a particular embodiment, the recombinant *Y. lipolytica* expresses a PhaCp having an amino acid sequence as set forth in SEQ ID No5, or a functional variant thereof having lactyl-CoA polymerase activity, comprising preferably at least one mutation selected from E130D, S325T, S477F, S477R, Q481K Q481M, S482G, L484V and A547V, or as set forth in SEQ ID No6 or SEQ ID No7.

Advantageously, a lactic acid oxidoreductase activity of the recombinant *Y. lipolytica* has been inactivated. In a particular embodiment, at least YYDldp has been inactivated, so that the degradation pathway of D-lactic acid is suppressed or at least attenuated.

In a particular embodiment, the recombinant *Y. lipolytica* expresses a quadruple variant of PhaCp from *P. aeruginosa* (SEQ ID NoII) and a functional variant of the Pctp from *C. propionicum* (SEQ ID NoI) with the mutation VI93A.

SEQ ID No1 / PhaCp from *P. aeruginosa* PA01 with mutations E130D, S325T, S477R and Q481M

MSQKN NNELPKQAENTLN LN PVIG IRG KDLLT AR MVLLQAVRQP LHSAR HV AH FSLE LKNVLLG QSEL R PG DDD RRFSD PAWSQN PL YKRYMOTY LA WR KELHSW ISHD LSPQD ISRGQFVI NLLTDAMSPTNLSN P AAVKR FFETG GKL LD LG HLAKD LVN NGGM PSQ MD DAFEVG KNL ATTEGAVVF R NDVLEIQYR PITE SVH ERL LLVVPPQI NKFYVF DLSPD KSLAR FCLR N GVQTFIVSWRN PTKSQR EWG LTYYEALKEAI EVVLSIT GSKD LN LLGACSSG ITTATLVG HYV ASG EKVNAFTQLVTVLDFE LNTQVALFADE KTL EAARK RSYQSGVL EG KDMAKVF AWM RPN DLWNYYWN NYLLG NQ PPAFDL LYWN NDTR LPA AL H GEVFELFKSN PLN RPG ALEVSGTP IDLKVCTDFCYCVAG LN D H ITP WECYS KAR LLG G KCE FLSN RG HIMS I LN PPG NP KAR FMTN PELPAE PKAWLEQAG KHADSWWLH WQQWLA E RSG KT KAPASLG NK TYP AG EAAPGTYYH ER

As exposed above, if required, all or part of the heterologous sequences expressed in the recombinant yeast cell may contain a PTS or a MTS in order to be addressed into the peroxisome or mitochondria of the cell. In the case where a targeting signal peptide is already present in the sequence, it could be deleted and/or changed for the protein to be addressed in...
the desired compartment. Particularly, the recombinant yeast cell expresses a heterologous Pctp addressed to cytosol, and a heterologous PhaCp addressed to peroxisome.

In a particular embodiment, the recombinant yeast cell has been deleted for diacylglycerol transferases to avoid TAG (triacylglycerol) production. Accordingly, the present invention provides a recombinant *Yarrowia*, wherein at least one of the genes selected from *YILROL*, *YIDGAl* and *YIDGA2* have been deleted.

In another particular embodiment, the recombinant yeast cell has been deleted for the 6 acyl-CoA oxidases (*YIPOXI*: YALI0E32835g / XM_504703.1, *YIPOX2*: YALI0F10857g / XM_505264.1, *YIPOX3*: YALI0D24750g / XM_503244.1, *YIPOX4*: YALI0E27654g / XM_504475.1, *YIPOX5*: YALI0C23859g / XM_502199.1, *YIPOX6*: YALI0E06567g / XM_503632.1), preventing beta-oxidation (genotype: *poxl-6A*) to avoid incorporation of beta-oxidation derived 3-hydroxy-acyl-CoA. This deletion may be useful for the production of homopolymer of PLA in peroxisome.

It is therefore an object of the invention to provide a recombinant *Y. lipolytica* that has been engineered for expressing at least one Pctp having the amino acid sequence as set forth in SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°16, or a variant thereof, in cytosol, at least one PhaCp having the amino acid sequence as set forth in SEQ ID N°5, SEQ ID N°6, SEQ ID N°7 or SEQ ID N°11, or a variant thereof and further comprising at its C-terminus the amino acid sequence set forth in SEQ ID N°8 or at its N-terminus the amino acid sequence set forth in SEQ ID N°9 in order to be addressed in the peroxisome, and that has been further engineered in order to inactivate both the lactate dehydrogenase (*YIDldlp* and/or *YICyb21p*) and the acyl-CoA oxidases (*YIPOXI*, *YIPOX2*, *YIPOX3*, *YIPOX4*, *YIPOX5*, *YIPOX6*).

In a particular embodiment, the recombinant yeast cell may further express amphiphilic proteins capable of forming micro-compartments that encapsulate and thereby isolate the PLA produced. For instance, the recombinant yeast cell may be further engineered to express phasins that arrange in micro-compartments encapsulating the PLA produced.

In a particular embodiment, the recombinant yeast cell expresses may express a phasine protein as set forth in SEQ N°17 or SEQ N°18. These proteins may contain no peptide signal, a PTS or a MTS in order to be addressed to cytosol, peroxisomes or mitochondria respectively. For
For instance, PTS as set forth in SEQ ID N°8 or SEQ ID N°9 may be used. For instance, MTS as set forth in SEQ ID N°10 may be used.

SEQ ID N°17 / Phalp from *Pseudomonas putida* (Q5QBP4)

MAKVTVKKDDALGTLG  EVRGYARKWLAG  IGAYARVG  QEGSDYFQELVKAG  EGVE  KRG  KKR  IDKE  LDAA

5  NQIDEAAEVESRVG  EVE  IQLD  KIEKAFDARVG  RALN  RLG  IPSKH  DVEALS  IKLEQLH  ELLE  RVAHKP

SEQ ID N°18 / phaPlp from *Rastonia eutropha* H16 (Q0KBV4)

MILTEPVAAQKAN  LETLFG  LTTKAFEGVE  KLVPLN  LQVVTGAEVGVD  NAKKALSADQIE  LLAQAAA

VQPVAS  KTLAYTRH  LYEIASETQF  TKVAEQLAEGSKNVQALVE  NLAKNAPGSESTVAIVKSAISAAN  N

10  AYESVQKATQAVE  IAETN  FOAAATA  AT  KAAQQAS  ATA  RTATA  KKTAA

*Method for producing poly-lactic acid (PLA) using recombinant yeast cells*

The invention further relates to a method for producing PLA by culturing recombinant yeast cells of the invention.

According to the invention, PLA may be produced by culturing such recombinant yeast cells in presence of lactic acid.

In a particular embodiment, the recombinant yeast cells are first cultivated in a fermentation broth devoid of lactic acid. For instance, such fermentation broth comprises low cost carbon source such as wheat bran or refined sugars such as glucose, galactose, xylose, saccharose, glycerol, etc., as a carbon source. Then, the fermentation broth is implemented with lactic acid to allow the production of PLA. This lactic acid can be produced by a microorganism such as lactic bacteria directly in the same fermentation broth. Alternatively, the lactic acid can be produced by a microorganism such as lactic bacteria in a different fermentation broth connected or not with the PLA production fermentation broth.

25 Advantageously, the fermentation broth comprises at least D-LA, in order to produce PDLA.
In a particular embodiment, a D-lactic acid dehydrogenase has been inactivated in the recombinant yeast cell, further containing a PDLA synthase, in order to favor the production of PDLA.

In a particular embodiment, the pH in the medium is controlled in order to increase the uptake of lactic acid from the medium into the cell. Preferably, the pH is maintained between 3 and 7, more preferably between 3 and 4.

Advantageously, the method of the invention leads to the production of at least 0.01 g of PLA / g dry cells, at least 0.02g, 0.03g, 0.04g, 0.05g, 0.1g, 0.2g, 0.5g, or more of PLA / g dry cells. Preferably, the method of the invention leads to the production of at least 1 g of PLA / g dry cells.

In a particular embodiment, the method of the invention comprises the step of recovering PLA of the cells and optionally the step of purifying the extracted PLA.

The PLA may be extracted from the yeast cells by any method known by the skilled person.

In a particular embodiment, the cells are lysed before recovering the PLA. Particularly, the cells may be lysed biologically, by contacting them with an enzyme able to digest their cell walls (e.g. zymolyase). Alternatively, the yeast cells may be lysed mechanically, by breaking their cell walls, for instance using a vortex with glass beads or lysed chemically, for instance by osmotic shock or alkali treatment.

The PLA may be extracted directly from non-lysed cells or after lysis using a solvent extraction process such as Soxhlet extraction (Yamada et al., 2011), maceration in solvent (i.e. chloroform, dichloromethane, acetone, etc.), filtration and precipitation, or ultracentrifugation, etc. After extraction and solubilization, PLA purification may be executed by using the precipitation method according to Matsumoto et al. (2005) or any other method known to the person skilled in the art.

According to the invention, the PLA recovered has advantageously a molecular weight (Mw) greater than 40,000g/mol, 50,000 g/mol, 60,000 g/mol, 70,000 g/mol or 80,000 g/mol.

The invention further provides a fermentation broth comprising such recombinant yeast cells, and/or of a cell-free extract of such recombinant yeast cells, selected from cell supernatant, cell
debris and cell walls extract. The invention further relates to dried recombinant yeast cells, containing PLA in their cytosol and/or peroxisome and/or mitochondria. According to the invention, such fermentation broth and/or dried cells may be used directly as raw material for producing plastic products or the like. For instance, dried cells of the invention may be directly introduced in an extruder, optionally with other polyesters, including other PLA, to provide PLA containing material and/or PLA containing plastic products.

Generally speaking, the present invention proposes to use recombinant yeast cells as described above, for producing PLA, preferably PDLA. The produced PLA interestingly exhibits a high molecular weight (Mw), generally greater than 40,000 g/mol, 50,000 g/mol, 60,000 g/mol, 70,000 g/mol or 80,000 g/mol.

It is therefore an object of the present invention to provide PLA, more particularly PDLA, having a molecular weight (Mw) greater than 40,000 g/mol, 50,000 g/mol, 60,000 g/mol, 70,000 g/mol or 80,000 g/mol.

Further aspects and advantages of the invention will be disclosed in the following examples, which should be considered as illustrative and do not limit the scope of this application.

**EXAMPLES**

**Example 1: Construction of yeast strains unable to assimilate lactic acid**

*Construction of disrupted strains followed by marker excision*

The disruption cassettes were generated by PCR amplification on *Y. lipolytica* genomic DNA. It consists in a first amplification of a promoter region (P) and a termination region (T) of the gene to be deleted. The primers were designed as described by Fickers and coworkers (2003) and/or as described by Beopoulos et al. (2008 and 2011). After a secondary amplification consisting in a fusion of the P and T fragments, the resulting PT cassette was then inserted into the PCR4®Blunt-TOPO vector from Life Technologies (Carlsbad, California). Then the auxotrophic marker URA3ex or LEU2ex was inserted into the PT vector through the *Isce*l specific cloning site to generate the corresponding plasmid (PUT or PLT, respectively). The PUT or PLT disruption cassette was introduced into *Y. lipolytica* by transformation with the
lithium acetate method (Barth et al. 1996). Transformants were selected on the adequate minimal medium. Verification primers coding for specific sequences present in the auxotrophic marker and in the gene were used to verify gene disruption by PCR amplification of the genomic loci. Marker rescue was performed after transformation with the replicative plasmid pUB-Crel as described by Fickers and coworkers (2003).

**Effect of YlDLDl disruption on growth on D-lactate.**

Growth of YlDLDl knockout strain (ThYl_434 - MATA ura3-302 leu2-270 xpr2-322, ku70A, zeta, DLD1::URA3, LEU2ex-) (SEQ ID N°12: YlDLDl gene) was compared to the growth of the host strain (JMY2341 -MATA ura3-302 leu2-270 xpr2-322, KU70::URA3ex, zeta-LEU2ex- ) on YNB medium (1.7g/L YNB, 5g/L NH₄Cl, 50mM phosphate buffer, pH6.8) containing 400mg/L leucine and different carbon sources: 10g/L Glucose, or 10g/L DL-lactate, or 10g/L D-lactate or 10g/L L-lactate (Figures 4A and 4B). Growth on D-, L- and DL-lactate reaches the same OD₆₀₀nm in the host strain while growth of the knockout strain on D-lactate is totally abolished and growth on DL-lactate reaches half of the OD₆₀₀nm of the growth on L-lactate, suggesting that D-lactate is not consumed by the knockout strain and its growth is due to L-lactate consumption.

The YlDLDl disrupted strain can then be modified according to the invention in order to produce PLA, more preferably PLLA.

**Effect of YlCYB21 deletion on growth on L-lactate.**

Growth of YlCYB21 knockout strain (ThYl_436 - MATA ura3-302 leu2-270 xpr2-322, ku70A, zeta, CYB21::LEU2ex, URA3ex-) (SEQ ID N°13: YlCYB21 gene) was compared to growth of the host strain (JMY2341) on YNB medium containing 10g/L L-lactate (Figures 5A and 5B). While the JMY2341 grows on L-lactate, which is completely consumed after 72 hours, the disrupted strain did not consume L-lactate and so, did not grow.

The YlCYB21 knockout strain can then be modified according to the invention in order to produce PLA, more preferably PLLA.

**Example 2: Construction of yeast strains capable of producing PLA**
Cloning and expression of genes under the control of the TEF constitutive promoter

Genes were amplified by PCR and placed under the control of the yeast TEF constitutive promoter (pTEF). Coding gene sequences were then inserted between the BamHI-Avrl sites of the JMP62-pTEF expression vector, containing the URA3ex or LEU2ex selective marker. Plasmids were then digested with NotI restriction enzyme and the released coding gene containing fragment was used to transform the strains by the lithium acetate method (Barth et al. 1996). Transformants were selected by their respective auxotrophy on the adequate minimal medium. Marker rescue was performed after transformation with the replicative plasmid pUB-Crel as described by Fickers and coworkers (2003).

Yeast strain

Y. lipolytica strain used was derived from the strain JMY2159 described in Beopoulos et al., 2014 (QPF). The YIDLDMI gene (SEQ ID N°12) was deleted to produce the strain ThYl_967 using the protocol described in Example 1 and Figure 6. A variant of Pctp from C. propionicum (SEQ ID N°1) with mutation V193A (CpPctp opt V193A - gene: SEQ ID N°14) and a variant of PHA synthase (PhaClp opt E130D S325T S477R Q481M - SEQ ID N°11) from P. aeruginosa PA01 with mutations E130D; S325T; S477R; Q481M (gene: SEQ ID N°15) were expressed under the control of the same TEF promoter. The variant of the Pctp was expressed in the cytosol and the variant of the PhaClp was targeted to the peroxisomes. Finally, the genotype of the ThYl_976 strain used in this example was: MATA ura3-302 leu2-270 xprl-322, poxl-6A, dgalA, IrolA, dga2A, fad2A, ddlA, pTEF-PaPHACl opt E130D, S325T, S477R, Q481M, perox-URA3ex, pTEF-CpPCT opt V193A cyto-LEU2ex as shown in Figure 6.

Culture conditions

The ThYl_976 recombinant yeast cells were first cultured on rich medium (10g/L yeast extract, 10g/L peptone, 10g/L glucose) at 28°C overnight. Cells were then harvested by centrifugation to remove medium and resuspended in controlled medium with L-lactic acid as the sole carbon source and D-lactic acid as synthon for PDLA production (1.7g/L YNB, 2g/L casamino acids, 5g/L NH4Cl, 20g/L DL-lactic acid, 50mM phosphate buffer) with an initial OD600nm around 10. L-lactic acid was added when needed. Alternatively, for enhanced polymer accumulation, cells were resuspended in controlled medium with glucose and L-lactic acid as carbon source and D-lactic acid as synthon for PDLA production (1.7g/L YNB, 2g/L casamino acids, 5g/L NH4Cl,
40g/L glucose, 15g/L L-lactic acid, 5g/L D-lactic acid, 50mM phosphate buffer) with an initial OD600nm around 0.5. The cultures were grown at 28°C, with an agitation of 100 rpm for 150 hours and at indicated time cells were then harvested by centrifugation, washed twice with water and the cell pellet was kept at -80°C until further analysis. Culture media was filtered through a 0.4µm filter and kept at -20°C until analyzed (see Figure 9).

**Polymer extraction**

About 1.5g of lyophilized cells were resuspended in 15mL 100mM Tris, pH8, 0.5mg/mL zymolyase and incubated at 25°C overnight. Cell suspensions were frozen at -80°C and/or freeze dried by lyophilization until polymer extraction. Produced polymer was extracted using a Soxhlet apparatus and chloroform. About 1.5g of dried cells was used and the chamber of the Soxhlet apparatus was filled 10 times before solvent and extracted materials were collected.

Alternatively, after extraction PLA can be precipitated by adding hexane (or cyclohexane) to the mixture.

**Analysis**

Glucose concentration was determined using a YSI 2900 analyser (System C Industrie, St Paul Trois Chateaux, France).

Lactic acid concentration was determined by high-performance liquid chromatography (HPLC) using Thermo Fisher Scientific system (Courtaboeuf, France) equipped with a UV detector at 254nm and a Phenomenex column (Chirex 3126 (D)-penicillamine 150x4.6mm, Le Pecq, France) using 2mM CuSO₄, 15% methanol (v/v) as the mobile phase at 1mL/min.

Polymer composition was determined by NMR on a Bruker Avance II 500 spectrometer. The cells extracts were thoroughly dried, prior to being diluted in CDCl₃ containing 1% TMS (internal standard) and transferred to 5 mm NMR tubes. NMR spectra were recorded at 298 K. Each NMR spectrum was acquired using an excitation flip angle of 30° at a radiofrequency field of 29.7kHz, a relaxation delay of 10 seconds and 2 dummy scans. For each experiment, 16 scans were performed with a repetition delay of 6.5 seconds. PLA concentrations were determined by integration of the specific quadruplet signal at 4.19ppm.
Weight average molecular weight (Mw) and dispersity of the polymer were determined by gel permeation chromatography (GPC) at 20°C using a Shimadzu system (Marne la Vallee, France) equipped with Wyatt detectors (MALLS, Dawn Heleos-II, 18 angles and refractometer at 22°C, Optilab T-rEX, Toulouse, France) with two Agilent columns (PLGel 5um MFXED-C 300x7.5mm). The elution solvent used was dichloromethane. Samples were resuspended in dichloromethane and filtered through a 0.4μm filter. The molecular weight Mw and dispersity were calculated using dn/dc value previously determined and equal to 0.0296.

**PLA purification and composition analysis**

After extraction, PLA was diluted in chloroform and purified by precipitation by adding 10 volumes of hexane (adapted from Yamada et al., 2011). The precipitant was then collected by filtration on PTFE membrane. Complete hydrolysis of the polymer was performed at 180°C and 100 bars during 50 minutes, according to Faisal et al. (2007) in order to identify the isomer of lactic acid which composed the polymer.

**Results**

In a first time, cultures were carried out in DL lactate, using L-lactic acid as the sole carbon source. Yeast growth was measured at an OD of 600nm and substrate concentrations were measured over time. After 120 hours of culture, cells were collected and treated as for to extract PLA. PLA synthesis was demonstrated using NMR, based on the PLA specific signals and quantified using an internal standard (TMS) (Figure 7). The NMR data demonstrate that the ThYl_976 recombinant strain exhibiting both CoA-transferase activity and lactyl-CoA polymerase activity produces PLA, while the strain that does not exhibit the aforementioned activities (ThYl_964, see Figure 6), does not (spectrum 1 vs 2 of Figure 7). The spectrum also shows that the main contaminants of the Soxhlet extractions are fatty acids, primarily constituted of oleic acid, and are retrieved in both extracts in similar proportions. Spectrum 3 shows the specific signals obtained using commercial PLA. The analysis reveals that synthesized polymer is a homopolymer containing 100% of lactic acid monomer. PLA molecular weight Mw and dispersity were quantified using GPC. Produced PLA Mw reached 43,800g/mol after 120 hours of culture with a dispersity of 1.4. When cultured in DL-lactate with L-lactic acid used as the sole carbon source, recombinant cells accumulate 1.9% (g/g) PLA over the duration of the culture with a final biomass concentration around 2.5g/L.
To improve PLA accumulation and biomass production, other carbon sources were tested. Both can be greatly improved using glucose as an additional carbon source. When cultured with DL-lactate and glucose, with L-lactic acid and glucose as carbon source, substrates concentration were followed and added when needed. D-lactic acid concentration was also measured and it slowly decreased over time indicating that it is used for the production of a polymer of PLA (Figure 8).

PLA production started during the exponential phase (first 48 hours) with a productivity of 0.21 mg/g/h. Productivity then increased to 0.33 mg/g/h during the stationary phase. After 150 hours of culture, the total biomass was around 10.5 g dry cell weight per liter and the total PLA production reached 0.5 g/L. Accumulation of PLA was then 4.25% (g/g) (Figure 9).

After extraction, PLA was precipitated and its complete hydrolysis was performed at 180°C, 100 bars for 50 minutes. PLA composition was then determined by HPLC analysis on chiral column. This analysis reveals that the PLA is composed with 100% of D-lactic acid (Figure 10).

Example 3: Improvement of PLA production by adding two copies of genes Pctp opt V193A or PhaCl opt E130D S325T S477R Q481M

Yeast strain construction

*Y. lipolytica* strain used was derived from the strain ThYl_976 described in Example 2 and expressing one copy of a variant of Pctp from *C. propionicum* (SEQ ID N°1) with mutation V193A (CpPctp opt V193A) and one copy of a variant of PHA synthase (PhaClp opt E130D S325T S477R Q481M) from *P. aeruginosa* PAO1 with mutations E130D; S325T; S477R; Q481M (SEQ ID N°ll). A second copy of the gene encoding for the variant of Pctp from *C. propionicum* (SEQ ID N°1) with mutation V193A (CpPctp opt V193A) and/or a second copy of the variant of PHA synthase (PhaClp opt E130D S325T S477R Q481M) from *P. aeruginosa* PAO1 with mutations E130D; S325T; S477R; Q481M (gene SEQ ID N°15) were expressed in the strain ThYl_976 with the same targeting than the first copy (cytosol or peroxisome).
The different strains used in this example were built with one or two copies of Pctp gene and one or two copies of PhaClp gene under the control of the TEF promoter. The genotypes of the strains of this example are described in Table 3.

Table 3: Name and genotype of strains with one or two copies of genes of interest

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of copies of Pctp</th>
<th>Number of copies of PhaClp</th>
<th>Genotype</th>
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<tr>
<td>ThYl_976</td>
<td>1</td>
<td>1</td>
<td>QPF, Adldl, pTEF-PaPHAC opt E130D, S325T, S477R, Q481M, perox-URA3ex, pTEF-CpPCT opt V193A, cyto-LEU2ex</td>
</tr>
</tbody>
</table>

Culture condition, polymer extraction and analysis

The recombinant yeast cells were first cultured on rich medium (10g/L yeast extract, 10g/L peptone, 10g/L glucose) at 28°C overnight. Cells were then resuspended in controlled medium with glucose and L-lactic acid as carbon source and D-lactic acid as synthon for PDLA production (1.7g/L YNB, 2g/L casamino acids, 5g/L NH₄Cl, 40g/L glucose, 10g/L DL-lactic acid, 50mM phosphate buffer) with an initial OD₆₀₀nm around 0.5. The cultures were grown at 28°C, with an agitation of 100 rpm for 5 days, and cells were then harvested by centrifugation, washed twice with water and the cell pellet was kept at -80°C until further analysis. Culture media was filtered through a 0.4µm filter and kept at -20°C until analyzed.
Polymer extraction and NMR analysis were done as described in example 2.

Results

After 5 days of culture, cells were collected and PLA extracted. PLA accumulation (determined by quantitative NMR) in ThYl_1086 was improved by about 42% when two copies of both genes were introduced into the genome compared to the control strain ThYl_976.

Example 4: Improvement of PLA production by using a strong and constitutive promoter (4UAS-TEF)

Cloning and expression of genes under the control of the 4UAS-TEF strong and constitutive promoter and yeast strain construction

Genes were amplified by PCR and placed under the control of the yeast strong and constitutive 4UAS-TEF promoter. The cloning and expression were performed using the same technic described in Example 2.

Y. lipolytica strain used was derived from the strain ThYl_967 described in Example 2. A variant of Pctp from C. propionicum (SEQ ID N°1) with mutation V193A (CpPctp opt V193A) was expressed in the cytosol and a variant of PHA synthase (PhaClp opt E130D S325T S477R Q481M - SEQ ID N°11) from P. aeruginosa PAO1 with mutations E130D; S325T; S477R; Q481M (gene SEQ ID N°15) was expressed in the peroxisome. For both genes, promoter TEF and 4UAS-TEF were used. The different strains used in this example were built with one or two gene under the control of the TEF promoter or the 4UAS-TEF promoter. The genotypes of the strain used in this example were described in the table 4.

Table 4: Name and genotype of strains with genes under the control of different promoters

<table>
<thead>
<tr>
<th>Strain</th>
<th>Promoter used for CpPctp opt V193A</th>
<th>Promoter used for PhaClp opt E130D S325T S477R Q481M</th>
<th>Genotype</th>
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Table 5: Accumulation of PLA determined by quantitative NMR

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<tr>
<th>Strain</th>
<th>PLA accumulation (g/g)</th>
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<tr>
<td>ThYl_976 (control strain)</td>
<td>2.4%</td>
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<tr>
<td>ThYl_1174</td>
<td>3.1% (+28.5% vs control strain)</td>
</tr>
<tr>
<td>ThYl_1156</td>
<td>3.5% (+46.2% vs control strain)</td>
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REFERENCES


CLAIMS

1. A recombinant yeast cell comprising a gene encoding a protein exhibiting lactyl-CoA synthase activity and a gene encoding a protein exhibiting lactyl-CoA polymerase activity, said recombinant cell having the ability of producing polylactic acid (PLA).

2. The recombinant yeast cell of claim 1, wherein the protein exhibiting lactyl-CoA synthase activity is CoA transferase, preferably a propionyl-CoA transferase (Pctp).

3. The recombinant yeast cell of claim 1 or 2, wherein the protein exhibiting lactyl-CoA polymerase activity is a polyhydroxyalkanoate (PHA) synthase.

4. The recombinant yeast cell of any one of claims 1 to 3, comprising at least one nucleic acid sequence encoding a Pctp having an amino acid sequence as set forth in SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°16 or a variant thereof having lactyl-CoA synthase activity.

5. The recombinant yeast cell of any one of claims 1 to 4, comprising at least one nucleic acid sequence encoding a PHA synthase having an amino acid sequence as set forth in SEQ ID N°5, SEQ ID N°6, SEQ ID N°7 or SEQ ID N°11 or a variant thereof having lactyl-CoA polymerase activity.

6. The recombinant yeast cell of any one of claims 1 to 5, wherein at least one lactic acid oxidoreductase activity is inactivated, preferably a gene encoding a lactic acid dehydrogenase is inactivated, more preferably a gene encoding a D-lactic acid dehydrogenase.

7. The recombinant yeast cell of any one of claims 1 to 6, wherein the TAG synthesis pathway is inactivated, preferably wherein at least one gene encoding for an endogenous diacylglycerol-transferase is inactivated.

8. The recombinant yeast cell of any one of claims 1 to 7, wherein at least one citrate synthase activity is inactivated, preferably wherein at least one gene encoding for a citrate synthase is inactivated.

9. The recombinant yeast cell of any one of claims 1 to 8, which is of the genus of Yarrowia, Saccharomyces, Kluyveromyces, Candida, Pichia, Schizosaccharomyces, Zygosaccharomyces, Hansenula, Trichosporon, Yamadazyma, Cryptococcus, Lipomyces, Rhodosporidium,
Rhodotorula, Geotrichum, Kloeckera, Schwanniomyces, Brettanomyces, Debaryomyces or Issatchenkia.

10- The recombinant yeast cell of any one of claims 1 to 9, which is selected from the group consisting Yarrowia bubula, Yarrowia deformans, Yarrowia Upolytica, Yarrowia yakushimensis, Yarrowia galli, Yarrowia oslonensis, Yarrowia hollandica, Yarrowia phangngensis, Yarrowia alimentaria, and Yarrowia porcina, preferably Yarrowia Upolytica.

11- The recombinant yeast cell of any one of claims 1 to 10, which is a Y. Upolytica that has been engineered for expressing at least one Pctp having the amino acid sequence as set forth in SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4, SEQ ID No 16, or a variant thereof, in cytosol, at least one PhaCp having the amino acid sequence as set forth in SEQ ID No 5, SEQ ID No 6, SEQ ID No 7 or SEQ ID No 11, or a variant thereof and further comprising at its C-terminus the amino acid sequence set forth in SEQ ID No 8 or at its N-terminus the amino acid sequence set forth in SEQ ID No 9 in order to be addressed in the peroxisome, and that has been further engineered in order to inactivate the lactate dehydrogenase (YlDldlp and/or YICyb21p).

12- The recombinant yeast cell of claim 11, that has been engineered further engineered in order to inactivate the acyl-CoA oxidases (YIPOX1, YIPOX2, YIPOX3, YIPOX4, YIPOX5, YIPOX6).

13- A method for preparing poly-lactic acid (PLA) comprising

- culturing recombinant yeast cells of any one of claims 1-12 in presence of lactic acid;

and optionally

- recovering PLA.

14- The method of claim 13, wherein the PLA produced is a homo-PLA, preferably poly-D-lactic acid (PDLA), and/or the PLA produced has a molecular weight greater than 40,000g/mol.

15- A fermentation broth comprising recombinant yeast cells of any one of claims 1-12.

16- Use of recombinant yeast cells of any one of claims 1 to 12, for producing PLA, preferably PDLA.

17- Polylactic acid obtainable from the recombinant yeast cell of any one of claims 1 to 12.
FIGURE 3
W29
\[ MATA\, ura3-302\, leu2-270\, xpr2-322 \]
\[ \text{Po1d} \]

(Beopoulos et al 2014)

JMY2159
\[ MATA\, ura3-302\, leu2-270\, xpr2-322,\, pox1-6\Delta,\, dga1\Delta,\, lro1\Delta,\, dga2\Delta, fad2\Delta \text{(QPF)} \]
\[ \text{PUT}ddl1,\, ddl1::\text{URA3ex} \]

ThYl\_964
\[ \text{QPF, ddl1::URA3ex} \]
\[ \text{pUB-Cre1} \]

ThYl\_967
\[ \text{QPF, ddl1}\Delta \]
\[ \text{JMP62 pTEF-PaPHAC opt E130D, S325T, S477R, Q481M, perox-URA3ex} \]

ThYl\_979
\[ \text{QPF, ddl1}\Delta, \text{pTEF-PaPHAC opt E130D, S325T, S477R, Q481M, perox-URA3ex} \]
\[ \text{JMP62 pTEF-CpPCT opt V193A, cyto-LEU2ex} \]

ThYl\_976
\[ \text{QPF, ddl1}\Delta, \text{pTEF-PaPHAC opt E130D, S325T, S477R, Q481M, perox-URA3ex, pTEF-CpPCT opt V193A, cyto-LEU2ex} \]

FIGURE 6
A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/54 C12P7/62 C12P7/56 C12N9/10 C12N1/19
C08G63/08
ADD.
According to International Patent Classification (IPC) into both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12P C08G

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPTENDEX, EMBASE, FSTA, IBM-TDB, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>X</td>
<td>EP 2 471 910 A2 (LG CHEM, LTD.; KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY) 4 July 2012 (2012-07-04) page 2, paragraph 7 - page 3, paragraph 16 page 3, paragraph 20 - page 4, paragraph 48 pages 12-15; example 3; table 6 pages 25-26; sequence 8 pages 43-45; sequence 78 pages 45-46; claims 1-7</td>
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</table>

Relevant to claim No. 1-16

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

* Special categories of cited documents:
- **A** document defining the general state of the art which is not considered to be of particular relevance
- **E** earlier application or patent but published on or after the international filing date
- **L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- **O** document referring to an oral disclosure, use, exhibition or other means
- **P** document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 29 March 2017

Date of mailing of the international search report: 01/06/2017

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer: Fuchs, Ulrike
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<tr>
<td>X</td>
<td>EP 2 377 945 AI (TOYOTA MOTOR CO LTD; NATIONAL UNIVERSITY CORPORATION HOKKAIDO UNIV.) 19 October 2011 (2011-10-19) page 3, paragraph 13 - page 4, paragraph 28 page 5, paragraph 30 - page 8, paragraph 57 pages 8-11; example 1 pages 16-19; sequence 2 pages 27-30; sequence 6 pages 31-32; claims 1, 3-7, 9-12</td>
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<tr>
<td>A</td>
<td>OKINO, S. ET AL.: “Production of D-lactic acid by Corynebacterium glutamicum under oxygen deprivation”, APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 78, no. 3, 10 January 2008 (2008-01-10), pages 449-454, XP019586313, the whole document</td>
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<td>A</td>
<td>WD 2010/081887 AI (FUTERR0 S.A.) 22 July 2010 (2010-07-22) page 3, line 12 - page 4, line 19 pages 18-19; example 10 pages 20-21; claims 1, 2, 12-15</td>
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International application No
PCT/EP2016/081205

INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-16

Remark on Protest

☐ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/21 0 (continuation of first sheet (2)) (April 2005)
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-16

   a recombinant yeast cell comprising a gene encoding a protein exhibiting lactyl-CoA synthase activity and a gene encoding a protein exhibiting lactyl-CoA polymerase activity, having the ability of producing polylactic acid (PLA),
   a method for preparing polylactic acid (PLA) comprising:
   - culturing recombinant yeast cells of any one of claims 1-12 in presence of lactic acid, and optionally
   - recovering PLA,
   a fermentation broth comprising recombinant yeast cells of any one of claims 1-12,
   the use of recombinant yeast cells of any one of claims 1-12 for producing PLA

2. claim: 17

   a polylactic acid obtainable from the recombinant yeast cell of any one of claims 1-12
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<th>Patent family member(s)</th>
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<td>JP 2012531905 A</td>
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