Genetically modified fungi and their use in lipid production

Field of the Invention

The present invention relates to fungi, which have been genetically modified to enhance their lipid production. The invention also relates to a method for preparing the fungi, and to expression cassettes for genetic modification of the fungi. The invention further relates to a method of producing lipids by these fungi. The lipids produced are useful in manufacturing biofuels, lubricants and functional fatty acids. The invention thus also provides a method for producing biofuels and lubricants. Still further the invention provides a new enzyme protein that is useful in the methods, and a nucleic acid encoding it. Even further the invention relates to a recombinant *Cryptococcus* cell, and a method for its construction.

Background of the Invention

Biofuels are current favorites to be the next generation transportation fuels. They are produced from renewable biological sources such as vegetable oils and animal fats. They are biodegradable, non-toxic and have a low emission profile. Due to the limited sources of biodiesel raw materials such as rape seed oil, soy bean oil or palm oil, it is of importance to expand biodiesel raw materials to non-food materials like microbes. The benefits of using microbes for production of oils are: they are affected neither by seasons nor by climates, they are able to produce high lipid contents, and the oils can be produced from a wide variety of sources with short production times, especially from residues with abundant nutrition. Microbiologically produced lipids may also be used e.g. for the production of functional fatty acids.

A few fungal species accumulate remarkable amounts of lipid in the cells. It has been observed that lipids accumulate in these so called oleaginous fungi under nitrogen limited conditions, which has resulted in a hypothesis for effective lipid accumulation (Review Ratledge and Wynn 2002 and references thenceforth). Nitrogen limitation causes activation of the AMP deaminase which utilizes AMP to produce NH_4. The decrease in AMP concentration inhibits the activity of mitochondrial isocitrate dehydrogenase (IDH) which is part of the mitochondrial tricarboxylic (TCA) cycle. Reduction in IDH activity results in equilibration of isocitrate to citrate by aconitase. Produced citrate is transferred to the cytosol where it is converted with Coenzyme A (CoA) to acetyl-CoA by ATP:citrate lyase (ACL) with ATP hydrolysis.

Cytosolic acetyl-CoA can be further used in fatty acid synthesis. A comprehensive review on fatty acid synthesis and elongation in yeast, especially in *Saccharomyces cerevisiae*, is that of Tehlivets *et al.*, 1997. In the first step of fatty acid synthesis acetyl-CoA is carboxylated by the addition of carbon dioxide to malonyl-CoA by the enzyme acetyl-CoA carboxylase in an ATP demanding reaction. In the following reactions by the fatty acid synthase systems acyl and malonyl moieties from acyl-CoA and malonyl-CoA, respectively, are tranfered to acyl carrier proteins (ACPs), after the acyl chain, typically initiated by acetyl-CoA, is condensated with malonyl-ACP followed by reduction of the 3-ketoacyl-ACP to 3-hydroxyacyl-ACP, dehydration to enoyl-ACP, and a second reduction to a saturated acyl-chain that is extended by two carbon atoms. These synthesis steps are usually repeated seven times resulting in palmitoyl ACP (C16:0). Palmitic acid and intermediates of the fatty acid synthesis after hydrolysed to acyl-CoAs by hydrolase/ thioesterase, can be further modified by different elongases and desaturases to different length acyl-chains with or without double bonds. In one cycle of fatty acid synthesis two NADPHs are required in the reduction steps. Acyl-CoAs can be further synthesised to triacylglycerols.

Triacylglycerol synthesis starts from glycerol-3-phosphate or dihydroxyacetone phosphate which is acylated (dihydroxyacetone-phosphate also reduced) to 1-acyl-glycerol-3-phosphate which is further acylated to phosphatidic acid. Phosphatidic acid can be further dephosphorylated to diacylglycerol. Diacylglycerol is further acylated to triacylglycerol mainly by acyl-CoA:diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT) utilizing acyl-CoA or phosphatidylcholine, respectively, as acyl donors. The triacylglycerol pathway in yeast *S. cerevisiae* is described in more detail in a mini-review of Sorger and Daum 2003.

Phospholipid:diacylglycerol acyltransferase (PDAT) encoding genes originating from *S. cerevisiae* and *Yarrowia lipolytica* have been expressed in yeasts *S. cerevisiae* and *Y. lipolytica* to enhance their triacylglycerol production (WO00/60095 and WO2005/003322, respectively). WO2009/126890 provides systems for producing engineered oleaginous yeast or fungi that express caroteinoids. Oleaginy is promoted e.g. by increased or heterologous expression of DGAT or PDAT, whereas reducing the activity of PDC is expected to promote oleaginy.

Methods of manufacturing biodiesel and other oil-based compounds using glycerol as an energy source in fermentation of oil-bearing microorganisms have been described e.g. in US2009/0004715. Methods of producing lipid-based biofuels from cellulose containing feedstock by heterotrophic fermentation of microorganisms have been described in US2009/0064567. Both publications focus on the use of algae as lipid producers. No details are given. WO2007/136762 provides genetically engineered microorganisms that produce desired products from the fatty acid biosynthetic pathway.

With the above-described triacylglycerol production pathway high triglyceride yields indicated as triglyceride production per used carbon source cannot be achieved or triacylglycerol production per cell biomass cannot be significantly enhanced. In general, lipids especially triglycerides are produced when nitrogen becomes a growth limiting factor at the late logarithmic or early stationary growth phase resulting in a low triglyceride production rate compared e.g. to yeast ethanol production. Additionally, the need of several carbons and reduced cofactors in synthesis of triacylglycerol result in low yield per used carbon. The present invention uses another route for microbial lipid production. In the present invention microbial lipid production rate and yields are enhanced, and the need of reduced cofactors from the outside of the lipid pathway is decreased. The present invention further provides lipid production that is not linked to nitrogen limitation.

Summary of the Invention

The present invention is based on the use of a pyruvate dehydrogenase bypass route for producing cytosolic acetyl-CoA. The invention makes use of an active cytosolic pathway for acetyl-CoA production, which proceeds via the enzymatic reaction catalyzed by pyruvate decarboxylase (PDC). This pathway is known in Crabtree-positive yeast *S. cerevisiae*, where it is essential for cytosolic acetyl-CoA production, but it has not been characterized in oleaginous yeasts and oleaginous filamentous fungi. In oleaginous fungi another cytosolic pathway for acetyl-CoA production, proceeding via the reaction catalyzed by pyruvate dehydrogenase (PDH) is well known and which has been shown to be essential for cytosolic acetyl-CoA production from pyruvate. This pathway operates via mitochondria and in this pathway a higher fraction of carbon is lost than in the pathway via pyruvate decarboxylase. In the pyruvate decarboxylase pathway, exploited in this invention, a higher fraction of carbon is directed to triacylglycerol and it is not dependent on mitochondrial enzyme

activities. Further, it produces NAD(P)H which is needed in the following fatty acid synthesis. The pyruvate dehydrogenase bypass route optionally together with an enhanced diacylglycerol acyltransferase activity as provided by the invention removes many bottlenecks in microbial lipid production.

The invention is directed to genetically modified fungal cells that have been modified to enhance the expression of a nucleic acid encoding PDC, ALD, ACS and/or DAT, and to methods of constructing them.

In particular the invention is directed to a genetically modified oleaginous fungal cell comprising at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS).

The invention is further directed to a genetically modified fungal cell comprising:

a) a nucleic acid with modified expression encoding a pyruvate decarboxylase (PDC) enzyme, and

b) at least one nucleic acid with modified expression encoding an enzyme selected from the group consisting of acetaldehyde dehydrogenase (ALD), acetyl-CoA synthetase (ACS) and diacylglycerol acyltransferase (DAT).

The invention is also directed to a genetically modified fungal cell comprising:

a) at least one nucleic acid with modified expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS), and

b) a nucleic acid with modified expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

The invention is also directed to a method of producing lipids comprising cultivating the genetically modified fungal cell according to the invention in a medium containing carbon and nitrogen sources, and recovering the lipids produced.

The invention is further directed to a method of producing biofuel, or lubricant said method comprising cultivating the genetically modified fungal cell according to the invention in a medium containing carbon and nitrogen sources, and recovering the lipids produced, and optionally esterifying said lipids to obtain biodiesel or lubricant, or hydrogenizing the lipids to obtain renewable diesel or lubricant.

The invention is still further directed to methods of preparing i.e. constructing a genetically modified fungal cell of the invention, said methods comprising transforming a fungal cell with

at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS); or with

a) a nucleic acid with enhanced expression encoding a pyruvate decarboxylase (PDC) enzyme, and

b) at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of acetaldehyde dehydrogenase (ALD), acetyl-CoA synthetase (ACS) and diacylglycerol acyltransferase (DAT); or with

a) at least one nucleic acid with modified expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS), and

b) a nucleic acid with modified expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

In addition the invention is directed to an expression cassette comprising

a) at least one nucleic acid with modified expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS), and

b) a nucleic acid with modified expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

Conveniently the genetically modified fungal cells are constructed according to the invention by transforming the fungal cell with the nucleic acid(s) encoding said enzyme(s) resulting in enhanced enzyme activity of said enzyme.

Still further the invention is directed to an enzyme protein having phosholipid:diacylglycerol acyltransferase (PDAT) activity and at least 40% sequence identity to SEQ ID NO:52, or an enzymatically active fragment or variant thereof, and to an isolated nucleic acid molecule selected from the group consisting of: a) a nucleic acid encoding said protein, b) a nucleic acid comprising the nucleotide sequence of SEQ ID NO:53 or SEQ ID NO:93, c) a complementary strand of a) or b), and d) a sequence that is degenerate as a result of the genetic code to anyone of sequences a) - c).

The invention is additionally directed to a genetically modified fungal cell comprising a nucleic acid with modified expression encoding said PDAT, and to a method of producing lipids, comprising cultivating said genetically modified fungal cell in a medium containing carbon and nitrogen sources, and recovering the lipids produced.

Still further the invention is directed to the use of a genetically modified fungal cell of the invention for producing lipids, biofuels, biodiesel, renewable diesel or lubricants. The use for producing lipids includes e.g. the use for producing precursors of fatty acids e.g. of functional fatty acids, and for producing the fatty acids or functional fatty acids.

As a still further aspect, the invention is directed to a genetically modified *Cryptococcus* cell, which has been modified to enhance the expression of a heterologous nucleic acid, and to a method of constructing the cell, said method comprising transforming a *Cryptococcus* cell with a nucleic acid encoding a heterologous protein.

Specific embodiments of the invention are set forth in the dependent claims. Other objects, details and advantages of the present invention will become apparent from the following drawings, detailed description and examples.

Brief Description of the Drawings

Figure 1 shows probable metabolic routes for cytosolic acetyl-CoA production via the pyruvate dehydrogenase route in grey, and via the pyruvate dehydrogenase bypass route i.e. the pyruvate decarboxylase route in black.

Figure 2 shows the metabolic route for triacylglycerol production.

Figure 3 is a diagram depicting plasmid pKK81.

Figure 4 is a diagram depicting plasmid pKK82.

Figure 5 is a diagram depicting plasmid pKK86.

Figure 6 is a diagram depicting plasmid pKK95.

Figure 7 is a diagram depicting plasmid pKK85.

Figure 8 is a diagram depicting plasmid pKK75.

Figure 9 is a diagram depicting plasmid pKK94.

Figure 10 is a diagram depicting plasmid pKK96.

Figure 11 is a diagram depicting plasmid pKK98.

Figure 12 is a diagram depicting plasmid pKK101.

Figure 13 is a diagram depicting plasmid pKK102.

Detailed Description of the Invention

The presumed pyruvate dehydrogenase (PDH) pathway for producing cytosolic acetyl-CoA is shown in grey in Figure 1. First cytosolic pyruvate is transported into the mitochondria where it is oxidatively decarboxylated to acetyl-CoA and carbon dioxide by the pyruvate dehydrogenase complex. The resulting acetyl-CoA is then entering the tricarboxylic (TCA) cycle by citrate synthase (CS) which catalyses the condensation reaction of the two-carbon acetate residue from acetyl-CoA and a molecule of four-carbon oxaloacetate (OAA) to form the six-carbon citrate. Citrate is then isomerised to isocitrate which is the substrate for mitochondrial isocitrate dehydrogenase (IDH). Under limited nitrogen supply AMP deaminase activity increases leading to production of IMP and ammonium from AMP. The decrease in the amount of AMP results in decrease in mitochondrial isocitrate dehydrogenase (IDH) activity, whereby the amount of citrate in the mitochondria increases. The mitochondrial citrate is transported into the cytosol, where ATP:citrate lyase (ACL) converts citrate, ATP and CoA into acetyl-CoA and oxaloacetate. The oxaloacetate is degraded by malate dehydrogenase (MDH) to malate, which in turn is converted to pyruvate and carbon dioxide by malic enzyme (MAE) under production of NADPH, which is an important cofactor in fatty acid synthesis. In this invention cytosolic acetyl-CoA is produced via a pyruvate dehydrogenase bypass pathway, which is shown in black in Figure 1. This pathway is also called the pyruvate decarboxylase pathway. In this pathway pyruvate is decarboxylated to acetaldehyde and carbon dioxide by pyruvate decarboxylase (PDC). Acetaldehyde is further oxidised to acetate by $NADP^+$ (or NAD^+) -dependent acetaldehyde dehydrogenase (ALD). Acetate is then converted to acetyl-CoA by acetyl-CoA synthetase (ACS) with ATP and Coenzyme A. In this cytosolic pathway [pyruvate + CoA + ATP + $NAD(P)^+$ = Acetyl-CoA + CO_2 + NAD(P)H + AMP + PPi + H^+] the overall acetyl-CoA yield is higher than in the pyruvate dehydrogenase –ATP:citrate lyase pathway [pyruvate + CoA+ ATP + NAD^+ (in mitochondria) + oxaloacetate (in mitochondria) = Acetyl-CoA + CO_2 + ADP + Pi + NADH (in mitochondria) + H^+ + oxaloacetate (in cytosol)].

Cytosolic acetyl-CoA is used by NADPH-dependent fatty acid synthase (FAS) and other enzymes for the production of acyl-CoA esters of different length, which then are attached to for example glycerol through the triglyceride metabolic pathway shown in Figure 2. In the last step of this pathway an acyl group from acyl-CoA or from a phospholipid is attached to the diacylglyc-

erol by acyl-CoA:diacylglycerol acyltransferase (DGAT) or phospholipid:diacylglycerol acyltransferase (PDAT).

In the present invention triacylglycerol production of fungi will be enhanced by overexpressing at least one gene, which encodes an enzyme involved in the pyruvate decarboxylate pathway for converting pyruvate to acetyl-CoA as shown in Figure 1, together with a gene, which encodes an enzyme that catalyses the acylation of diacylglycerol to triacylglycerol as shown in Figure 2.

Lipid production including triacylglycerol production in the cell is a highly NADPH demanding process. E.g. in production of 1 mole of oleic acid (9-octadecenoic acid) 17 mole of NADPH is needed. NADPH produced by malic enzyme has been proposed to be the main source for NADPH needed in fatty acid synthesis. Said NADPH production occurs totally outside the cytosolic acetyl-CoA production pathway resulting in the consumption of extra carbons in NADPH production, even though the reaction of the malic enzyme is linked to the degradation of the oxaloacetate produced by ATP:citrate lyase. In the present invention fatty acid and further triacylglycerol production is connected directly to NADPH cofactor production by NADP -dependent acetaldehyde dehydrogenase thus reducing the need to produce NADPH outside the triglyceride production pathway resulting in an increased triacylglycerol yield. The $NADP^+$ -dependent acetaldehyde dehydrogenase produces simultaneously one NADPH and one acetate molecule from NADP+ and acetaldehyde resulting in production of one mole of NADPH per one mole of pyruvate. This means that half of the NADPH molecules needed in the fatty acid synthesis are produced simultaneously with the cytosolic acetyl-CoA production. This simultaneous NADPH production with lower carbon loss during production of cytosolic acetyl-CoA results in a better yield in fatty acid production following also better yield in triacylglycerol production. In this invention only one carbon is lost from the carbon skeleton downstream of pyruvate prior to cytosolic acetyl-CoA. Additionally, no side reactions are needed to cleave metabolites further outside the triglyceride production pathway. The production of cytosolic acetyl-CoA via the pyruvate dehydrogenase bypass completes the existing pyruvate dehydrogenase pathway for cytosolic acetyl-CoA production.

Triacylglycerols and other lipids are naturally produced in fungi via the pyruvate dehydrogenase pathway during growth, but the main triacylglycerol and lipid accumulation occurs when excess citrate will be available after

nitrogen limitation in the late stage of cultivation. This triacylglycerol production in late logarithmic or stationary phases of cultivation results in low triacylglycerol production rates, especially at the early stage of cultivation. In this invention expression of the pyruvate dehydrogenase bypass catalyzed by PDC, ALD, and ACS results in a situation where triacylglycerol accumulation is not linked to nitrogen limitation thus allowing enhanced triacylglycerol production during cultivation resulting in a better triacylglycerol production rate. The earlier triacylglycerol production is further enhanced by expressing an acyltransferase such as a phospholipid:diacylglycerol acyltransferase (PDAT) encoding gene e.g. under a constitutive promoter thus increasing triacylglycerol concentration at the expense of phospholipids.

Contrary to oleaginous yeasts and moulds like *Cryptococcus curvatus* and *Mucor circinelloides, S. cerevisiae* lacks the pyruvate dehydrogenase route for acetyl-CoA production. In this Crabtree-positive yeast cytosolic acetyl-CoA, and further fatty acids and triacylglycerols, are produced only via pyruvate dehydrogenase bypass. The essential role of the pyruvate dehydrogenase bypass including the enzymes pyruvate decarboxylate (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS) in cytosolic acetyl-CoA and further in lipid production and the role of ALD in the generation of reducing equivalents (NADH and NADPH) in *S. cerevisiae* has been described for example in Flikweert *et al*. 1996, Pronk *et al* 1996, Saint-Prix *et al* 2004). In US2009/0053797 expression of endogenous NADP-dependent acetaldehyde dehydrogenase (*ALD6*) gene and native or modified endogenous *ACS1* gene or *Salmonella enterica* acetyl-CoA synthetase (*ACS1*) gene in *S. cerevisiae* resulted in an increased concentration of cytosolic acetyl-CoA in the production of isoprenoids. Shiba *et al*., 2007 found that overexpression of *ALD6* and *ACS1* in *S. cerevisiae* increased cytosolic acetyl-CoA derived amorphadiene overproduction, whereas overexpression of *ACS2* with *ALD6* did not. The acetyl-CoA synthetase isoforms *ACS1* and *ACS2* behave differently in *S. cerevisiae*: *ACS1* gene has been shown to be under glucose repression whereas *ACS2* gene has been shown to be constitutively expressed and co-regulated with structural genes of fatty acid biosynthesis (van den Berg *et al* 1996, Hiesinger *et al*. 1997).

The essential role of the PDH bypass in *S. cerevisiae* has been described: Deletion of three structural genes for pyruvate decarboxylase (*PDC1, PDC5* and *PDC6*) results in loss of growth in a defined glucose medium

(Flikweert et al 1996, Pronk et al. 1996), and deletion of two acetyl-CoA synthetase encoding genes ACS1 and ACS2 is lethal on all carbon sources (Takahashi et al 2006 and van den Berg et al 1996), indicating that there is no alternative route for cytosolic acetyl-CoA production in *S. cerevisiae.* The enhanced expression of this pathway has been found to induce cytosolic acetyl-CoA production in *S. cerevisiae* (e.g. US2009/0053797 and WO2008/080124). However, the existence and functionality of the pyruvate dehydrogenase bypass pathway in oleaginous yeasts or moulds has not been described in the literature.

PDC has been characterised e.g. from the *Rhizopus oryzae*, which in addition to lipids produced ethanol (Skory 2003). Also, ALD and ACS have been characterised from some of the oleaginous fungi e.g. *A. nidulans* (Flipphi et al. 2001, Connerton et al. 1990). Still, it has also been shown that deletion of the only cytoplasmic ACS encoding gene from *A. nidulans* had no effect on growth on glucose (Sandeman et al. 1989). Instead, it has been shown in several articles that the cytosolic acetyl-CoA for lipid production will be produced via pyruvate dehydrogenase and ATP:citrate lyase (ACL) in oleaginous fungi (Wynn et al 2001, Boulton and Ratledge 1981). ATP:citrate lyase has been shown to be absent from the non-oleaginous yeasts (Boulton and Ratledge 1981). E.g. ACL is absent from the sequenced members of the *Saccharomycotina* with the exception of *Y. lipolytica* which is oleaginous yeast. The essential role of ACL for cytosolic acetyl-CoA production has also been shown at a functional level by deteting the *acl* gene from *A. nidulans.* This deletion strain could not grow in the absence of external sources of cytoplasmid acetyl-CoA, which strongly suggests that ACL activity is required to generate cytoplasmic acetyl-CoA. This also indicates the absence of any pyruvate dehydrogenase bypass pathway, which could compensate *acl* deletion (Hynes and Murray 2010). The production of cytosolic acetyl-CoA via ATP:citrate lyase in oleaginous fungi is also suggested in the patent applications WO2005/003322 and US2006/094087, where diacylglycerol transferase encoding genes have been expressed to enhance triacylglycerol production.

The term “pyruvate dehydrogenase bypass” refers to an alternative route to the pyruvate dehydrogenase reaction for the conversion of pyruvate to acetyl-CoA. The pyruvate dehydrogenase bypass comprises the enzymes pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-

CoA synthetase (ACS). It has been shown in literature that the pyruvate dehydrogenase bypass is not essential in Crabtree-negative yeasts.

In the present context the corresponding genes encoding the enzyme proteins are indicated in italics.

The term "PDC" refers to pyruvate decarboxylase enzyme (EC 4.1.1.1). This enzyme catalyses the thiamine pyrophosphate- and Mg^{2+} -dependent decarboxylation of pyruvate to acetaldehyde and carbon dioxide. A preferred PDC is one of a Crabtree-positive organism. Preferably the PDC is a fungal PDC, especially of a Crabtree-positive fungus, such as *S. cerevisiae* e.g. PDC1 of *S. cerevisiae* (GenBank accession number CAA54522, version number CAA54522.1). According to one embodiment of the invention the PDC1 contains the amino acid sequence of SEQ ID NO:95, and/or is encoded for example by a polynucleotide containing the nucleotide sequence of SEQ ID NO:94, SEQ ID NO:96 or SEQ ID NO:97.

The term "ALD" refers to acetaldehyde dehydrogenase enzyme (EC 1.2.1.5 and EC 1.2.1.4). This enzyme catalyses the reaction where acetaldehyde is oxidized to acetate, and NAD^+ or $NADP^+$ -cofactor is reduced to NADH or NADPH, respectively. $NADP^+$ -specific ALDs are preferred. In the present invention the ALD is preferably a fungal ALD, more preferably of *S. cerevisiae*, and most preferably it is *S. cerevisiae* ALD6, which is encoded for example by a polynucleotide of SEQ ID NO:48 or 49, and/or comprises the amino acid sequence of SEQ ID NO:47. Further, the ALD is preferably a cytosolic ALD. ALD6 is cytosolic. Cytosolic ALD can be modified from mitochondrial isoforms of ALD by removing the mitochondrial targeting signal from the originally mitochondrial ALD by genetic engineering. The cleavage site of the mitochondrial targeting signal can be decided e.g. with programs designed for this purpose such as MITOPROT. Examples of mitochondrial ALD, which can be modified to be cytosolic are *S. cerevisiae* ALD4 and ALD5 encoding genes. Suitable ALD encoding genes can be found from databases e.g. KEGG Enzyme database and Brenda with EC numbers 1.2.1.4 and 1.2.1.5. Table 1 contains examples of $NAD(P)^+$ dependent acetaldehyde dehydrogenases.

Table 1. NAD(P)$^+$ –dependent acetaldehyde dehydrogenase enzymes

Organism	Accession number of the amino acid sequence	Version number of the amino acid sequence	Database
Saccharomyces cerevisiae	AAB68304	AAB68304.1	GenBank
Saccharomyces cerevisiae	DAA07732	DAA07732.1	GenBank
Saccharomyces cerevisiae	DAA11133	DAA11133.1	GenBank
Aspergillus niger	A2QMA4	A2QMA4.1	TrEMBL
Aspergillus niger	A2QiG1	A2QiG1.1	TrEMBL
Aspergillus niger	A5AAZ8	A5AAZ8.1	TrEMBL
Aspergillus niger	A2Q9V7	A2Q9V7.1	TrEMBL
Aspergillus fumigatus	Q4WQP1	Q4WQP1.1	TrEMBL
Pichia angusta	Q12648	Q12648	Swiss-Prot
Pichia stipitis	A3M013	A3M013.2	TrEMBL
Candida dubliniensis	B9W6J2	B9W6J2.1	TrEMBL
Candida glabrata	CAG59952	CAG59952.1	GenBank
Kluyveromyces lactis	CAH00079	CAH00079.1	Genbank
Lachancea thermotolerans	CAR23570	CAR23570.1	Genbank
Burkholderia xenovorans	Q13WK4	Q13WK4.1	TrEMBL
Vibrio harveyi	Q56694	Q56694.1	Swiss-Prot
Mus musculus	P47739	P47739.1	Swiss-Prot
Mus musculus	Q80VQ0	Q80VQ0.1	Swiss-Prot
Rattus norvegicus	P11883	P11883.3	Swiss-Prot
Rattus norvegicus	Q5XI42	Q5XI42.1	Swiss-Prot
Canis lupus familiaris	A3RF36	A3RF36.1	Swiss-Prot
Bos taurus	P30907	P30907.2	Swiss-Prot
Bos taurus	Q1JPA0	Q1JPA0.1	Swiss-Prot
Homo sapiens	P30838	P30838.2	Swiss-Prot
Homo sapiens	P43353	P43353.1	Swiss-Prot

The term “cytosolic” refers to a fluid component of the cytoplasm excluding organelle and other suspended intracellular structures.

The term “ACS” refers to acetyl-CoA synthetase enzyme (EC 6.2.1.1). The enzyme catalyses the reaction where acetyl-CoA is formed from

acetate and CoA with hydrolysis of ATP. Preferably the ACS is a fungal ACS, more preferably *S. cerevisiae* ACS, especially *S. cerevisiae* ACS2. Preferably the ACS encoding gene to be expressed is a gene, which is not under glucose repression and/or which gene product is not subject to post-translational regulation e.g. acetylation, in the original species. In a particular embodiment the ACS contains the sequence of SEQ ID NO:50. Several yeasts such as *Candida albicans* have genes similar to *S. cerevisiae ACS2*, which most likely are not under post-translational regulation. This abolishes the need to modify the existing gene. According to one embodiment ACS2 is encoded by a polynucleotide having the sequence of SEQ ID NO:51 or 92.

The term "DAT" refers to diacylglycerol acyltransferase enzyme (EC 2.3.1.X). The enzyme catalyses a reaction where an acyl group is transferred to 1,2-diacylglycerol to the position sn-3. DAT includes both acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) and phospholipid:diacylglycerol acyltransferase (PDAT). Preferably the DAT is PDAT.

The term "PDAT" refers to phospholipid:diacylglycerol acyltransferase enzyme (EC 2.3.1.158). The enzyme catalyses a reaction where an acyl group from a phospholipid is transferred to 1,2-diacylglycerol to the position sn-3 via an acyl-CoA-independent mechanism. Preferably the PDAT is of fungal origin, and more preferably of an oleaginous fungus. In one preferred embodiment the PDAT is a *Rhizopus oryzae* PDAT, and most preferably it is encoded by a polynucleotide that contains the sequence of SEQ ID NO:53 or 93. Preferably the PDAT contains an amino acid sequence having at least 40% identity to SEQ ID NO:52, or an enzymatically active fragment or variant thereof.

In particular preferred embodiments of the invention the encoded enzyme comprises an amino acid sequence with a sequence identity of at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99% or 100% to SEQ ID NO:95, 47, 50, or 52, or an enzymatically active fragment or variant thereof.

Percent identity of amino acid sequences can conveniently be computed using BLASTP version 2.2.23 software with default parameters. Sequences having an identities score and a positives score of a given percentage, using the BLASTP version 2.2.23 algorithm with default parameters, are considered to be that percent identical or homologous (Altschul *et al*. 1997).

It is well known that deletion, addition or substitution of one or a few amino acids does not necessarily change the catalytic properties of an enzyme protein. Therefore the invention also encompasses variants and fragments of

the given amino acid sequences having the stipulated enzyme activity. The term "variant" as used herein refers to a sequence having minor changes in the amino acid sequence as compared to a given sequence. Such a variant may occur naturally e.g. as an allelic variant within the same strain, species or genus, or it may be generated by mutagenesis or other gene modification. It may comprise amino acid substitutions, deletions or insertions, but it still functions in substantially the same manner as the given enzymes, in particular it retains its catalytic function as an enzyme.

A "fragment" of a given protein sequence means part of that sequence, i.e. a sequence that has been truncated at the N- and/or C-terminal end. It may for example be the mature part of a protein comprising a signal sequence, or it may be only an enzymatically active fragment of the mature protein.

The term "lipid" refers to a group of organic compounds that are relatively or completely insoluble in water but soluble in nonpolar organic solvents. These properties are a result of long hydrocarbon tails, which are hydrophobic in nature. The term thus encompasses fats, oils, waxes, fatty acids, fatty acid derivatives, like phospholipids, glycolipids, acylglycerids such as monoglycerides, diglycerides, and triglycerides and terpenoids such as carotenoids and steroids.

The term "fatty acid" refers to a compound obtainable via condensation of malonyl coenzyme A units by a fatty acid synthase system. They may be saturated or unsaturated. "Functional fatty acid" refers to a fatty acid compound having at least one functional group e.g. a hydroxyl (-OH) or carboxyl (-COOH) group within the fatty acid and being responsibe for the characteristic chemical reactions of those molecules.

The term "fatty acid derivative" refers to a compound having at least one esterified fatty acyl group. Fatty acid derivatives include e.g. phospholipids, glycolipids and acylglycerides.

The term "acylglyceride" is synonymous with "acylglycerol" and refers to a compound having a glycerol moiety with one or several hydroxyl groups esterified to a fatty acid.

The terms "monoglyceride" and "monoacylglycerol" refer to a glyceride where one fatty acid residue has been esterified to a glycerol molecule. The fatty acid residue in the monoacylglycerol can be a short or long chain fatty acid with or without double bonds.

The terms “diglyceride” and “diacylglycerol” and “DAG” refer to glyceride where two fatty acid residues have been esterified to a glycerol molecule. Fatty acid residues in diacylglycerol can be short or long chain fatty acids with or without double bonds.

The terms “triglyceride” and “triacylglycerol” and “TAG” refer to a glyceride where three fatty acid residues have been esterified to a glycerol molecule. Fatty acid residues in triacylglycerol can be short or long chain fatty acids with or without double bonds. Triacylglycerol is the major acylglycerol group in oleaginous fungi.

The term “acyl-CoA” refers to a fatty acid residue, which has been esterified to a CoA molecule. Fatty acid residues in acyl-CoA can be short or long chain fatty acids with or without double bonds.

The term “phospholipids” refers to any lipid containing a diglyceride combined with a phosphate group and a simple organic molecule such as choline or ethanolamine.

The term “glycolipid” refers to a lipid attached with a carbohydrate.

The term “fat” refers to a group of organic compounds that are relatively or completely insoluble in water but soluble in nonpolar organic solvents and which are solids at normal room temperature.

The term “oil” refers to a group of organic compounds that are relatively or completely insoluble in water but soluble in nonpolar organic solvents and which are liquids at normal room temperature.

Generally fats and oils are triesters of glycerol and fatty acids.

The term “wax” refers to a compound that may contain long-chain alkanes, esters, polyesters and hydroxyl esters of long-chain primary alcohols and fatty acids.

The term “terpenoid” refers to a compound formally derived from hydrocarbon isoprene.

The term “steroid” refers to a terpenoid lipid compound having a sterane core and additional functional groups. Sterols are special forms of steroids, with a hydroxyl group at the atom C-3 and a skeleton derived from cholestane.

The term “carotenoids” refers to a compound belonging to the category of tetraterpenoids. Structurally they are in the form of a polyene chain, which is sometimes terminated by rings.

In the present invention fungal cells are genetically modified to express particular enzymes. The cells can be genetically modified to produce increased levels of lipid by transforming them with nucleic acids that have been modified to enhance the expression of nucleic acids encoding at least one of PDC, ALD and ACS, together with a nucleic acid that has been modified to enhance the expression of a nucleic acid encoding DAT so as to allow overexpression of the enzymes. A "genetically modified" organism or cell is an organism or cell that comprises an expression modified nucleic acid. It may be a recombinant organism or cell, or a host organism or cell, or a mutant.

"Nucleic acid" is a macromolecule comprising a chain of monomeric nucleotides i.e. a polynucleotide. It can be e.g. DNA such as cDNA or genomic DNA or mRNA, and it can be e.g. recombinantly or synthetically produced, double or single stranded, encompassing both sense and antisense strands.

"Recombinant" nucleic acid refers to an artificial combination of at least two otherwise separated sequences, i.e. to a not naturally occurring combination of nucleic acids.

"Nucleic acid with modified expression" as used herein denotes nucleic acids that are foreign or exogenous to the host meaning that they are not naturally found in said host. The term also includes nucleic acids that are endogenous i.e. naturally found in the host, but which are produced in an unnatural amount e.g. as multiple copies, or nucleic acids that differ in sequence from the naturally occurring nucleic acids but encode the same type of protein. Further, the term includes nucleic acids comprising at least two nucleotide sequences that do not occur in the same relationship to each other in nature, such as e.g. an endogenous protein encoding sequence that is operably linked to a transcriptional control element e.g. a promotor and/or terminator in a way that does not occur in nature. Said promotor and/or terminator can be of endogenous or exogenous origin. High copy number plasmids comprising the protein encoding nucleotide sequence are also considered nucleic acids with modified expression. The above mentioned expression modified nucleic acids encompass recombinant nucleic acids, herein also called heterologous nucleic acids. Alternatively the expression modified nucleic acid can be a mutated nucleic acid. "Modified expression" in this context is used only in the meaning of over-expression i.e. "enhanced expression". The enhanced expression results in overproduction of the expressed protein in the modified organism compared to that in an unmodified organism.

In one embodiment of the invention the nucleic acid with modified expression contains an exogenous gene encoding PDC, ALD, ACS or DAT derived from another organism. The genes encoding PDC, ALD or ACS can be obtained from yeast such as *Saccharomyces cerevisiae, Candida glabrata, Dekkera bruxellensis, Kluyveromyces lactis, Kluyveromyces marxianus, Pichia pastoris, Pichia angusta, Pichia stipitis, Zygosaccharomyces rouxii, Issatchenkia terricola, Debaryomyces hansenii, Candida angusta* and *Pichia guilliermondii*, and the DAT can be obtained from yeast or filamentous fungi such as *Saccharomyces cerevisiae, Candida glabrata, Zygosaccharomyces rouxii, Lachancea thermotolerans, Ashbya gossypii, Cryptococcus curvatus, Cryptococcus albidus, Lipomyces lipofer, Lipomyces starkeyi, Lipomyces tetrasporus, Rhodosporidium toruloides, Rhodotorula glutinis, Rhodotorula graminis, Yarrowia lipolytica, Aspergillus nidulans, Aspergillus oryzae, Fusarium oxysporum, Humicola lanuginose, Mortierella alpina, Mortierella vinacea, Mucor circinelloides, Mucor plumbeus, Penicillium spinulosum,* and *Rhizopus oryzae.* In another embodiment the enzyme genes to be expressed are endogenous genes, the promotor of which is replaced with another promotor, preferably a constitutive promotor, or the existing promotor is modified to become a constitutive promotor.

Proteins or polynucleotides ”derived from”, “originated from” or “obtained from” a particular organism encompass products isolated from said organism, as well as modifications thereof. A protein derived from a particular organism may be a recombinantly produced product, which is identical to, or a modification of the naturally occurring protein. The protein may also be modified e.g. by glycosylation, phosphorylation or other chemical modification. Products derived from the particular organism also encompass mutants and natural variants of the products, where one or more nucleic acid and/or amino acid is deleted, inserted and/or substituted.

Expression of any combination of the genes of the pyruvate dehydrogenase bypass route may be linked to expression of the DAT encoding gene. The expression of the DAT encoding gene may for example be combined to the expression of an ALD encoding gene, or an ACS encoding gene, or a PDC encoding gene. In another embodiment both ASC and ALD, or both PDC and ACS, or PDC and ALD, are overexpressed, and in still another all PDC, ALD and ACS are overexpressed together with the DAT encoding gene. In one specific embodiment of the invention the expression of *S. cerevisiae*

ALD encoding gene *ALD6* is linked to the expression of a PDAT encoding gene.

Alternatively expression of PDC may be combined with expression of ACS and/or ALD thus providing the combinations PDC+ALD, PDC+ACS and PDC+ALD+ACS. These combinations may be expressed with or without further expressing a gene encoding DAT, such as PDAT.

"Fungal" "fungus" and "fungi" as used herein refers to yeast and filamentous fungi i.e. moulds. A genetically modified fungal cell is also referred to as host cell.

The yeast host cell may be selected for example from the genera *Cryptococcus, Candida, Galactomyces, Hansenula, Lipomyces, Rhodosporidium, Rhodotorula, Trichosporon* and *Yarrowia*. Preferably the yeast host cell is selected from the group consisting of *Candida sp., Cryptococcus curvatus, Cryptococcus albidus, Galactomyces geotrichum, Hansenula ciferri, Lipomyces lipofer, Lipomyces ssp., Lipomyces starkeyi, Lipomyces tetrasporus, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulans* and *Yarrowia lipolytica.* In one embodiment the yeast is selected from the phylum *Basidiomycota,* which includes *Cryptococcus*, *Rhodotorula* and *Rhodosporidium*. Most preferably it is *Cryptococcus curvatus*.

The filamentous fungus host cell may be selected from the genera *Aspergillus, Cunninghamella, Fusarium, Glomus, Humicola, Mortierella, Mucor, Penicillium, Pythium* and *Rhizopus.* Preferably the filamentous fungus is selected from the group consisting of *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus niger, Cuninghamella japonica, Fusarium oxysporum, Glomus caledonius, Humicola lanuginose, Mortierella isabellina, Mortierella pusilla, Mortierella vinacea, Mucor circinelloides, Mucor plumbeus, Mucor ramanniana, Penicillium lilacinum, Penicillium spinulosum, Pythium ultimum* and *Rhizopus oryzae.* According to one embodiment the filamentous fungus belongs to subphylum *Mucoromycotina,* which includes *Mucor* and *Mortierella*. Most preferably it is *Mucor circinelloides.*

According to one preferred embodiment the fungal host cell is an oleaginous fungus. The term "Oleaginous fungi" refers to yeasts or filamentous fungi, which accumulate at least 10%, 12.5%, 15%, 17.5%, preferably at least 20% or even at least 25% (w/w) of their biomass as lipid. They may even accumulate at least 30%, 40%, 50%, 60%, 70%, 80% (w/w) or more of their biomass as lipids. The biomass is usually measured as cell dry weight (CDW).

Oleaginous fungi are found e.g. in genera *Cryptococcus, Candida, Galactomyces, Hanseluna, Lipomyces, Rhodosporidium, Rhodotorula, Trichosporon, Yarrowia, Aspergillus, Cunninghamella, Fusarium, Glomus, Humicola, Mortierella, Mucor, Penicillium, Pythium* and *Rhizopus,* and especially in species *Candida sp., Cryptococcus curvatus, Cryptococcus albidus, Galactomyces geotrichum, Hansenula ciferri, Lipomyces lipofer, Lipomyces ssp., Lipomyces starkeyi, Lipomyces tetrasporus, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulans, Yarrowia lipolytica, Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus niger, Cuninghamella japonica, Fusarium oxysporum, Glomus caledonius, Humicola lanuginose, Mortierella isabellina, Mortierella pusilla, Mortierella vinacea, Mucor circinelloides, Mucor plumbeus, Mucor ramanniana, Penicillium lilacinum, Penicillium spinulosum, Pythium ultimum* and *Rhizopus oryzae.* In one embodiment it is a Crabtree-negative oleaginous yeast, and in another embodiment it is a Crabtree-positive filamentous fungus. In still another embodiment the filamentous fungus is Crabtree-negative, or the yeast is Crabtree-positive. *Saccharomyces* yeasts including *S. cerevisiae* are not oleaginous fungi. A “Crabtree-positive” organism is one that is capable of producing ethanol in the presence of oxygen, whereas a “Crabtree-negative” organism is not.

According to one preferred embodiment the host cell is a *Cryptococcus*, and especially *Cryptococcus curvatus*. Genetically modified *Cryptococcus curvatus* strains have not been described in literature (Meesters et al. 1997). Routinely, in yeast expression systems *S. cerevisiae* promoters and terminators, original genes without codon optimisation or codon-optimised for *S. cerevisiae* are used. In this invention we showed that it is possible to express genes successfully in *C. curvatus* when using endogenous promoters and terminators i.e. which originate from the species wherein the expression cassette will be transformed, and an expressed gene that is codon-optimised according to codon usage of the species wherein the expression cassette will be transformed, or its close relative, which has a genome that is known at a level where codon-optimisation is possible. Such a close relative is e.g. *Ustilago maydis*. In a preferred embodiment the promotors are constitutive promotors, especially from the glycolysis pathway.

Promoters and terminators of oleaginous fungi might contain sites for different regulatory elements and transcription factors than the promoters and terminators of *S. cerevisiae* due to the different nature of the strains: *S.*

cerevisiae can grow and produce ethanol under anaerobic conditions whereas *C. curvatus* does not produce ethanol at all, and lipids it produces under aerobic conditions. Also the codon usage differs in *S. cerevisiae* and in *C. curvatus*: E.g. *S. cerevisiae* codons and genome are adenine and thymine rich, whereas the ratio of guanine and cytosine is much higher in *C. curvatus* (Meesters et al. 1997).

It is also important to use endogenous promoters and terminators with resistance markers to detect transformants after transformations. Due to the fact that the genome of *C. curvatus* is not known different procedures described in this invention can be carried out to clone endogenous promoters and terminators. Preferably constitutive promoters are used.

The fungal cell can be genetically modified by transforming it with a heterologous nucleic acid that encodes a heterologous protein. "Heterologous" in this context means not naturally occurring. In one embodiment, the cell is transformed with a heterologous nucleic acid that encodes at least one of PDC, ALD and ACS, and a heterologous nucleic acid comprising a nucleic acid that encodes DAT, operably linked to allow expression of the genes encoding said enzymes. DNA isolation, enzymatic treatment and genetical modifications may be carried out using standard molecular biology methods as described e.g. Sambrook and Russell (2001). The promoter and terminator regions of the genes of interest can be cloned e.g. from a yeast or filamentous fungus strain of interest by polymerase chain reaction (PCR) using gene specific oligonucleotides designed based on known published gene sequences of strains of the same species or other species or gene sequences of the strain of interest. Oligonucleotides designed based on the sequence of the strain of interest are preferred.

New gene fragments from yeast and filamentous fungus species or strains with unknown genomic sequences can be cloned by PCR by using degenerative primers. The term "degenerative primer" refers to mixtures of similar kinds of synthesied primers differing from each other by one nucleotide. Degenerative primers for cloning of specific gene fragments from desired species or strains are designed based on known characterised or putative gene sequences.

A person skilled in art can use known characterised gene sequences as templates in a Blast search to find out other characterised or putative gene sequences. Another possibility is to search specific gene sequences by

enzymes names from databases containing genomic sequences of species from different genome sequencing projects. These kinds of databases are found for example, but not excluding, in Broad Institute's Fungal Genome Initiative sequence projects. In the searches of specific gene sequences species that are closely related to the yeast and filamentous fungus species of interest are preferred. After a set of specific gene sequences has been found nucleotide sequence alignments are carried out with appropriate programs e.g. Clustal W, resulting in a consensus sequence, which is used in designing degenerative primers. Designed degenerative primers are used in a PCR reaction with DNA of the yeast or filamentous fungus strain of interest as a template. Resulting PCR fragments are gel isolated and sequenced directly or after being cloned into plasmids. Detected sequences are used in Blast searches to confirm that right gene fragments have been cloned.

An unknown promoter and/or terminator region of a gene of interest can be cloned by a chromosome walking method. The term "chromosome walking" refers to sequential isolation of clones carrying overlapping sequences of a known gene region and an unknown sequence of an adjacent gene region produced by ligation-mediated PCR amplification method (Mueller and Wold 1989). Gene specific oligonucleotides corresponding to a known sequence of a gene of interest are designed and used in PCR reactions with linker specific oligonucleotides. The known sequence of the gene of interest may originate e.g. from a sequence of a gene fragment generated in a PCR reaction with degenerative oligos or from a gene sequence published in sequence databases. The resulting PCR fragments are gel isolated and sequenced directly or after being cloned into plasmids. Detected sequences are used in Blast searches to confirm that right gene fragments have been cloned. If needed, the chromosome walking experiment will be repeated so many times that desired length of promoter or terminator region has been cloned. The existence of the sequence of the promoter or terminator region of the desired gene is confirmed by usual bioinformatics methods e.g. with multiple sequence alignment.

A strain specific gene fragment containing the promoter and/or terminator region of the gene of interest can also be cloned by conventional library screening methods described e.g. in Sambrook and Russell (2001). The sequences of the oligonucleotides used in PCR reactions to clone desired promoter or terminator regions can also contain sequences of restriction sites

of specific restriction enzymes in addition to the gene specific sequence. The PCR fragment containing the desired promoter or terminator will be cloned into plasmid e.g. pBluescript and sequenced.

Promoters used in expression cassettes can be promoters of constitutively expressed genes e.g. of 3-phosphoglycerate dehydrogenase (PGK), triose phosphate isomerase (TPI) or enolase (ENO). Alternatively, the promoter used in the expression cassette can be a promoter of a gene, which is expressed under specific cultivation conditions.

"Expression cassette" as used herein refers to a nucleic acid construct that comprises a transcription initiation or transcription control sequence, e.g. a promotor, operably linked to a coding region for the protein to be transcribed, and preferably a transcription termination region. In addition it conveniently comprises one or more marker regions, i.e. regions encoding a selection marker.

Genes to be expressed can be cloned directly from the desired species or strains by conventional molecular biology methods e.g. by using PCR. More preferably the genes to be expressed are synthesised using a codon optimised nucleotide sequence based on the known codon usage of the host strain. If the codon usage of the host strain or host species is not known, the gene to be expressed can be codon optimised based on the known codon usage of a closely related species of the host strain. Alternatively, the gene to be expressed can be synthesised according to a known amino acid sequence by translating the amino acid sequence into a DNA sequence, preferably into a codon-optimised DNA sequence. The term "codon optimization" refers to an optimization of a synthetic nucleotide sequence encoding expressed genes to enhance gene product production in a host strain. Codon optimization can occur by replacing existing codons of the original gene by the codons used more often in the host strain. In codon optimisation also internal TATA-boxes, chi-sites, ribosomal entry sites, AT-rich or GC-rich sequence stretches, repeated sequences, RNA secondary structures and cryptic splice donor and acceptor sites can be avoided. Additionally in codon optimisation sequences of restriction sites of specific restriction enzymes can be avoided. Preferably, in a synthesised gene sequence the CTG codon will be avoided due to its different coding in different fungi (leucine or serine).

A nucleic acid that is "degenerate as a result of the genetic code" to a given sequence, means that it contains one or more different codons, but

encodes for the same amino acids. A “polynucleotide” as used herein may be a single or double stranded polynucleic acid. The term encompasses genome DNA, cDNA and RNA.

A “synthetic gene” or “synthetic nucleotide sequence” is an artificially designed gene or sequence, which has been synthesised into a physical DNA sequence.

The gene to be expressed is cloned between a promoter and terminator. An expression cassette of the gene to be expressed with promoter and terminator, and a marker gene can be transformed. The marker gene can be under its own promoter and terminator, but preferably it is under a functional promoter and terminator from another species, or more preferably under a functional promoter and terminator of the host strain. Markers to be used can be antibiotic markers like genes for hygromycin, geneticin and cerulenin resistances or other dominant marker like the melibiase gene. Additionally genes of the amino acid synthesis can be used as markers with auxotrophic fungi. The gene to be expressed can be transformed into the yeast or filamentous fungus as a plasmid to produce epitopic transformants, or as a DNA fragment containing the expression cassette to produce transformants with the expression cassette integrated into the genome of the host strain.

Expression cassettes containing the marker gene and gene to be expressed can be transformed in the same DNA fragment, or the expression cassettes of marker gene and gene to be expressed can be in separate DNA fragments. Transformation methods contain chemical, protoplast, electroporation methods. Transformants can be selected based on their ability to grow on a medium (solid or liquid) containing antibiotics, or a medium lacking some essential component e.g. an amino acid, or transformants can be selected based on different phenotype such as colour reaction of the transformants in specific conditions.

The DNA level of the transformants can be characterised by PCR or by Southern analysis using conventional molecular biology methods. Additionally enzyme activities of the expressed gene can be assayed as indicated in the example 27 or as described e.g. in Dahlqvist *et al.* 2000 and Postma *et al.* 1989.

The gene to be expressed can be an endogenous gene, whereby the promoter region can be replaced with a promoter of a constitutively expressed gene, or with a promoter of a gene, which is expressed under specific

cultivation conditions. Additionally, expression of an endogenous gene can be enhanced by classical mutagenesis.

The genetically modified fungi of the present invention are capable of producing increased levels of lipids, and especially of triacylglycerols. The increase may be at least a 1.5, 3, 5 or 10 fold increase in lipid or triacylglycerol concentration in transformants compared to the unmodified host strain during cultivation. Alternatively, it may be at least a 1.5, 3, 5 or 10 fold increase in lipid or triacylglycerol yield per used carbon source (e.g. glucose) in transformants compared to the unmodified host strain. It may also refer to a 1.5, 3, 5 or 10 fold increase in lipid or triacylglycerol production rate (mg/l/h) compared to the unmodified host strain. This increase in lipid or triacylglycerol production can be detected either intracellularly or in the amount of lipids and triacylglycerols in culture medium.

The genetically modified fungi are cultivated in a medium containing appropriate carbon and nitrogen sources together with other optional ingredients like yeast extract, peptone, minerals and vitamins, such as KH_2PO_4, Na_2HPO_4, $MgSO_4$, $CaCl_2$, $FeCl_3$, $ZnSO_4$, citric acid, $MnSO_4$, $CoCl_2$, $CuSO_4$, Na_2MoO_4, $FeSO_4$, H_3BO_4, D-biotin, CaPantothenate, nicotinic acid, myoinositol, thiamine, pyridoxine, *p*-aminobenzoic acid. The invention works at a wide range of C/N ratios about from 20 to 160 under microaerobic (50 ml medium in 250 ml flask with 100 rpm shaking) and aerobic (50 ml medium in 250 ml flask with 250 rpm shaking or 1-2 vvm in bioreactors) conditions from the beginning of the cultivation to the end of cultivation as far as up to at least 8 days. The host cells used are preferably such that are able not only to use hexoses, such as glucose, but also pentoses such as xylose, and arabinose, or even glycerol as carbon source. Preferably the carbon source is a hexose and/or pentose sugars containing material such as cellulose or hemicellulose. The genetically modified host cells are preferably grown on agricultural or industrial waste materials e.g. cellulose or hemicellulose containing materials, which makes the lipid production economically and environmentally beneficial.

After cultivation, the yeast or filamentous fungal cells are normally separated from the culture medium. Lipids are recovered from the cells typically by using non-polar organic solvents, such as hexane. Prior to extraction, cells can be dried and/or disrupted. Alternatively, the lipids can be recovered directly from the culture medium, which of course is an advantage. This is especially convenient when the host cell is *Cryptococcus*, such as *C. curvatus*.

Preferably lipid extraction is carried out to obtain lipids which mainly contain triacylglycerol (TAG). The lipid fraction can also contain mono- and diglycerides, free fatty acids, phospholipids, glycolipids and other lipids.

The lipids, and especially the TAG and the fatty acids, obtained can be used for preparing biofuels and lubricants. Said lipids may be directly used as biofuel or lubricant, but usually they are further processed to e.g. biodiesel or renewable diesel and/or lubricant formulations. "Biofuel" as used herein refers to fuel that has been at least partially biologically produced.

"Biodiesel" consists essentially of fatty acid methyl esters and is typically produced by transesterification in which the acylglycerides are converted to fatty acid methyl esters. According to EU directive 2003/30/EU biodiesel refers to a methyl-ester produced from vegetable oil or animal oil, of diesel quality to be used as biofuel. More broadly, biodiesel refers to long-chain alkyl esters, such as methyl, ethyl or propyl esters, from vegetable oil or animal fat of diesel quality.In the present content biodiesel can also be produced from fungal lipids.

"Renewable diesel" refers to fuel which is produced by hydrogen treatment (hydrogenation or hydroprocessing) of lipids of animal, vegetable or fungal origin, or their mixtures. Renewable diesel can be produced also from waxes derived from biomass by gasification and Fischer-Tropsch synthesis. Optionally, in addition to hydrogen treatment, isomerization or other processing steps can be performed. In hydrogen treatment, acylglycerides are converted to corresponding alkanes (paraffins). The alkanes (paraffins) can be further modified by isomerization or by other process alternatives. These processes can also produce hydrocarbons which are suitable for jet fuel or gasoline applications.

"Lubricant" refers to a substance, such as grease, lipid or oil that reduces friction when applied as a surface coating to moving parts. Two other main functions of a lubricant are heat removal and dissolving impurities. Applications of lubricants include, but are not limited to uses in internal combustion engines as engine oils, additives in fuels, in oil-driven devices such as pumps and hydraulic equipment, or in different types of bearings. Typically lubricants contain 75-100% base oil and the rest is additives. In the present invention at least part of the base oil is of fungal origin. Viscosity index is used to characterise base oil. Typically high viscosity index is preferred.

Lubricants or at least the base oil for lubricants may be prepared in the same way as described above for the biofuels. Usually conventional additives such as viscosity modifyers, antioxidants, pour point modifyers etc. are added to the base oil to obtain the lubricant.

The lipids, and especially the TAG and the fatty acids obtained can also be used for precursors for functional fatty acids. Said lipids are further processed to release fatty acyls which can be used in the production of functional fatty acids, like dicarboxylic acids and epoxides, which can be further converted into products like polyesters, polyurethane, coatings and resins.

The following examples are provided to illustrate the invention, but are not intended to limit the scope thereof. The enzyme names used are based on sequence homology.

Example 1A: Cloning of *Cryptococcus curvatus TEF* (*CcTEF1*) promoter and terminator region

A genomic ~800 bp fragment of the *C. curvatus TEF* gene was amplified by PCR with degenerative primers identified as SEQ ID NO:1 (Yeast TEF1), and SEQ ID NO:2 (Yeast TEF4), using *C. curvatus* (C-01440, VTT Culture Collection) genomic DNA as the template. The degenerative primers were designed based on a consensus sequence of the putative *TEF1* genes of *Ustilago maydis, Candida guilliermondii* and *Candida tropicalis*. The detected genomic fragment was sequenced.

Genomic fragments containing the *CcTEF1* promoter region were obtained with ligation-mediated PCR amplification (Mueller, P.R. and Wold, B. 1989). A mixture of a linker identified as SEQ ID NO:3 (PCR linker I), and a linker identified as SEQ ID NO:4 (PCR linker II) was ligated to *Pvu*II digested *C. curvatus* genomic DNA with T4 DNA ligase (New England BioLabs). Samples of the ligation mixtures were used as templates for 50 µl PCR reactions containing 0.1 µM of a primer identified as SEQ ID NO: 3 (PCR linker I), and 1 µM of a primer identified as SEQ ID NO:5 (CC_TEF2). The reaction mixture was heated at 94°C for 3 minutes after 2 U of Dynazyme EXT was added. The reactions were cycled 30 times as follows: 1 minute at 94°C, 2 minutes at 60°C and 2 minutes at 72°C, with final extension of 10 minutes at 72°C. A diluted sample of this first PCR-amplification was used as the template in a nested PCR reaction (50 µl) containing 0.05 µM of a primer identified as SEQ ID NO:3 (PCR Linker I), and 0.5 µM of a primer identified as SEQ ID NO:6 (CC_TEF1). The reaction mixture was heated at 94°C for 3 minutes after 2 U of Dynazyme

EXT was added. The reactions were then cycled 30 times as follows: 1 minute at 94°C, 2 minutes at 60°C and 2 minutes at 72°C, with final extension of 10 minutes at 72°C.

A ~800 bp fragment was isolated and sequenced. Nested primers identified as SEQ ID NO:7 (CC_TEF6), and SEQ ID NO:8 (CC_TEF5) were designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ ID NO:3 (PCR linker I), and a linker identified as SEQ ID NO:4 (PCR linker II) similarly as above except that *Nru*I–digested *C. curvatus* DNA was used. A ~2000 bp PCR fragment was isolated and sequenced.

The *C. curvatus* TEF1 promoter was PCR amplified using primers identified as SEQ ID NO:9 (CC_TEF10), and SEQ ID NO:10 (CC_TEF11) and the *C. curvatus* genomic DNA as the template. A PCR fragment was digested with *Sac*II and *Xba*I. A 1276 bp fragment was gel isolated and ligated to a *Sac*II and *Xba*I -digested pBluescript KS- plasmid (Stratagene). The resulting plasmid was designated pKK58. Plasmid pKK58 contains *C. curvatus TEF1* promoter.

A genomic fragment containing the *CcTEF1* terminator region was obtained with a ligation-mediated PCR amplification with *C. curvatus TEF1* gene specific oligonucleotides identified as SEQ ID NO:11 (CC_TEF3), and SEQ ID NO:12 (CC_TEF4) together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II) similarly as above except that *Nru*I-digested *C. curvatus* DNA was used. A ~1600 bp PCR fragment was isolated and sequenced.

The *C. curvatus* TEF1 terminator was PCR amplified using primers identified as SEQ ID NO:13 (CC_TEF7) and SEQ ID NO:14 and the *C. curvatus* genomic DNA as the template. A PCR fragment was digested with *Xma*I and *Eco*RI. A 358 bp fragment was gel isolated and ligated to *Xma*I and *Eco*RI-digested pBluescript KS- plasmid. The resulting plasmid was designated pKK55. Plasmid pKK55 contains the *C. curvatus TEF1* terminator.

Example 1B: Cloning of *Cryptococcus curvatus TPI* (*CcTPI1*) promoter and terminator region

A genomic fragment of the *C. curvatus TPI1* gene was amplified by PCR from genomic *C. curvatus* (C-01440, VTT Culture Collection) DNA with degenerative primers identified as SEQ ID NO:15 (Yeast TPI5), and SEQ ID NO:16 (Yeast TPI8). The degenerative primers were designed based on a

consensus sequence of the *TPI1* genes of *Ustilago maydis* and *Cryptococcus neoformans*. A ~800 bp genomic fragment was isolated and sequenced.

A genomic fragment containing the *CcTPI1* promoter region was obtained with a ligation-mediated PCR amplification with *TPI1* gene specific oligonucleotides identified as SEQ ID NO:17 (CC_TPI2), and SEQ ID NO:18 (CC_TPI1), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Eco*RV-digested *C. curvatus* DNA was used. A ~1300 bp PCR fragment was isolated and sequenced.

The *C. curvatus TPI1* promoter was PCR amplified by using primers identified as SEQ ID NO:19 (CC_TPI7) and SEQ ID NO:20 (CC_TPI_9) and the *C. curvatus* DNA as the template. A PCR fragment was digested with *BamH*I and *Sbf*I. A 851 bp fragment was gel isolated and ligated to a *BamH*I and *Pst*I –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK63. Plasmid pKK63 contains the *C. curvatus TPI1* promoter.

A genomic fragment containing the *CcTPI1* terminator region was obtained with a ligation-mediated PCR amplification with *TPI1* gene specific oligonucleotides identified as SEQ ID NO:21 (CC_TPI 4) and SEQ ID NO:22 (CC_TPI3), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Eco*RV-digested *C. curvatus* DNA was used. A ~1200 bp PCR fragment was isolated and sequenced.

The *C. curvatus TPI1* terminator was PCR amplified by using primers identified as SEQ ID NO:23 (CC_TPI5) and SEQ ID NO:24 (CC_TPI6) and the *C. curvatus* genomic DNA as the template. A PCR fragment was digested with *Xba*I and *Bam*HI. A 361 bp fragment was gel isolated and ligated to a *Xba*I and *Bam*HI –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK61. Plasmid pKK61 contains *C. curvatus TPI1* terminator.

Example 1C: Cloning of *Cryptococcus curvatus ENO* (*CcENO1*) promoter and terminator region

A genomic fragment of the *C. curvatus ENO1* gene was amplified by PCR from genomic *C. curvatus* (C-01440, VTT Culture Collection) DNA with degenerative primers identified as SEQ ID NO:25 (YeastENO5) and SEQ ID NO:26 (YeastENO10). The degenerative primers were designed based on a consensus sequence of *ENO1* genes of *Ustilago maydis* and *Cryptococcus neoformans*. A ~1000 bp genomic fragment was isolated and sequenced.

A genomic fragment containing the *CcENO1* promoter region was obtained with a ligation-mediated PCR amplification with *ENO1* gene specific oligonucleotides identified as SEQ ID NO:27 (CC_ENO2) and SEQ ID NO:28 (CC_ENO1), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Pvu*II-digested *C. curvatus* DNA was used.

A ~600 bp fragment was isolated and sequenced. Nested primers identified as SEQ ID NO:29 (CC_ENO5) and SEQ ID NO:30 (CC_ENO6) were designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II) similarly as above except that *Ssp*I-digested *C. curvatus* DNA was used. A ~2000 bp PCR fragment was isolated and sequenced.

The *C. curvatus ENO1* promoter was PCR amplified by using primers identified as SEQ ID NO:31 (CC_ENO9) and SEQ ID NO:32 (CC_ENO10) and the *C. curvatus* genomic DNA as the template. A PCR fragment was digested with *Eco*RI. A 1214 bp fragment was gel isolated and ligated to a *Eco*RI-digested pBluescript KS- plasmid. The resulting plasmid was designated pKK74. Plasmid pKK74 contains the *C. curvatus ENO1* promoter.

A genomic fragment containing the *CcENO1* terminator region was obtained with a ligation-mediated PCR amplification with *ENO1* gene specific oligonucleotides identified as SEQ ID NO:33 (CC_ENO4) and SEQ ID NO:34 (CC_ENO3), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Nru*I-digested *C. curvatus* DNA was used. A ~1100 bp PCR fragment was isolated and sequenced.

The *C. curvatus ENO1* terminator was PCR amplified by using primers identified as SEQ ID NO:35 (CC_ENO7) and SEQ ID NO:36 (CC_ENO8), and the *C. curvatus* genomic DNA as the template. A PCR fragment was digested with *Hind*III. A 375 bp fragment was gel isolated and ligated to a *Hind*III-digested pBluescript KS- plasmid. The resulting plasmid was designated pKK60. Plasmid pKK60 contains the *C. curvatus ENO1* terminator.

Example 1D: Cloning of *C. curvatus GPD* (*CcGPD1*) terminator region

A genomic fragment containing the *CcGPD1* terminator region was obtained with a ligation-mediated PCR amplification with *GPD1* gene specific oligonucleotides identified as SEQ ID NO:37 (CC_GPD3) and SEQ ID NO:38 (CC_GPD4), together with oligonucleotides identified as SEQ ID NO:3 (PCR

Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Ssp*I-digested *C. curvatus* DNA was used. A ~1800 bp fragment was isolated and partially sequenced. *GPD1* gene specific oligonucleotides were designed according to the *C. curvatus GPD1* gene (GenBank Accession number AF126158, version number AF126158.1) sequence.

The *C. curvatus GPD1* terminator was PCR amplified by using primers identified as SEQ ID NO:39 (CC_GPD6) and SEQ ID NO:40 (CC_GPD7), and the *C. curvatus* genomic DNA as the template. A PCR fragment was digested with *Xba*I and *Bam*HI. A 336 bp fragment was gel isolated and ligated to a *Xba*I and *Bam*HI-digested pBluescript KS- plasmid. The resulting plasmid was designated pKK54. Plasmid pKK54 contains *C. curvatus GPD1* terminator.

Example 2A: Cloning of the *E. coli* hygromycin resistance gene; construction of a plasmid (pKK76) having the *E. coli* hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator

The *E. coli* hygromycin (*hph*) gene, that confers resistance to hygromycin B, was PCR amplified using primers identified as SEQ ID NO:41 (Hph 5) and SEQ ID NO:42 (Hph 3), and the plasmid pRLMEX30 (Mach *et al.* 1994) DNA as the template. A PCR fragment was digested with *Spe*I. A 1048 bp fragment was gel isolated and ligated to *Spe*I-digested pBluescript KS- plasmid and sequenced. The resulting plasmid was designated pKK52.

Plasmid pKK58 was digested with *Sac*II and *Sbf*I. A 1272 bp fragment was gel isolated. Plasmid pKK52 was digested with *Sbf*I. A 1034 bp fragment was gel isolated. Plasmid pKK58 contains the *C. curvatus TEF1* promoter and plasmid pKK61 contains the *C. curvatus TPI1* terminator. The 1272 bp fragment originating from plasmid pKK58 and the 1034 bp fragment originating from the pKK52 plasmid were ligated to a 3285 bp fragment obtained by digesting a plasmid designated as pKK61 with *Sac*II and *Sbf*I. The resulting plasmid was designated pKK76. Plasmid pKK76 contains the *E. coli* hygromycin gene under the control of the *C. curvatus TEF1* promoter and the *C. curvatus TPI1* terminator.

Example 2B: Cloning of the *E. coli* G418 resistance gene; construction of a plasmid (pKK67) having the *E. coli* geneticin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator

The *E. coli* G418 resistance gene was PCR amplified using primers identified as SEQ ID NO:43 (Kan 5) and SEQ ID NO:44 (Kan 3), and the plasmid pPIC9K (Invitrogen) DNA as the template. A PCR fragment was digested with *Spe*I. A 838 bp fragment was gel isolated and ligated to *Spe*I-digested pBluescript KS- plasmid and sequenced. The resulting plasmid was designated pKK51.

Plasmid pKK58 was digested with *Sac*II and *Sbf*I. A 1272 bp fragment was gel isolated. Plasmid pKK51 was digested with *Sbf*I. A 824 bp fragment was gel isolated. The 1272 bp fragment originating from pKK58 plasmid and the 824 bp fragment originating from pKK51 plasmid were ligated to a 3285 bp fragment obtained by digesting a plasmid designated pKK61 with *Sac*II and *Sbf*I. Plasmid pKK58 contains the *C. curvatus TEF1* promoter and plasmid pKK61 contains the *C. curvatus TPI1* terminator. The resulting plasmid was designated pKK67. Plasmid pKK67 contains the *E. coli* G418 resistance gene under the control of the *C. curvatus TEF1* promoter and the *C. curvatus TPI1* terminator.

Example 2C: Cloning of *S. cerevisiae* cerulenin resistance gene; construction of a plasmid (pKK91) having the *S. cerevisiae* cerulenin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator

The *S. cerevisiae* cerulenin resistance gene was PCR amplified using primers identified as SEQ ID NO:45 (CERR 5) and SEQ ID NO:46 (CERR 3), and the plasmid pSH47Y DNA as the template. Plasmid pSH47Y contains the cerulenin resistance gene from the plasmid pCR1 (Nakazawa *et al.* 1993). A PCR fragment was digested with *Sbf*I. A 1685 bp fragment was gel isolated and ligated to *Pst*I-digested pBluescript KS- plasmid and sequenced. The resulting plasmid was designated pKK80.

Plasmid pKK80 was digested with *Pst*I. A 1685 bp fragment was gel isolated and ligated to a 4557 bp fragment obtained by digesting a plasmid designated as pKK67 with *Sbf*I. Plasmid pKK67 contains the *E. coli* G418 resistance gene linked to a *C. curvatus TEF1* promoter and *C. curvatus TEF1* terminator. The resulting plasmid was designated pKK91. Plasmid pKK91 con-

tains the *S. cerevisiae* cerulenin resistance gene under the control of the *C. curvatus TEF1* promoter and the *C. curvatus TPI1* terminator.

Example 3A. Construction of a plasmid (pKK81, Fig. 3) containing the hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator

The plasmid UmALD (Geneart AG, Germany) contains a *S. cerevisiae* ALD6 (SEQ ID NO:47) encoding gene which has been codon optimized according to *Ustilago maydis* yeast codon usage (SEQ ID NO:48) with flanking *Sbf*I restriction sites. The plasmid RoALD (Geneart AG, Germany) contains a *S. cerevisiae* ALD6 (SEQ ID NO:47) encoding gene which has been codon optimized according to *Rhizopus* oryzae filamentous fungus codon usage (SEQ ID NO:49) with flanking *Sbf*I restriction sites and an *E. coli* kanamycin resistance gene. Plasmid UmALD was digested with *Sfi*I. A 1554 bp fragment was gel isolated and ligated to a 2258 bp fragment obtained by digesting a plasmid RoALD with *Sfi*I. The resulting plasmid was designated as pKK50. The plasmid pKK50 contains a *S. cerevisiae* ALD6 (SEQ ID NO:47) encoding gene which has been codon optimized according to *Ustilago maydis* yeast codon usage (SEQ ID NO:48) with flanking *Sbf*I restriction sites and an *E. coli* kanamycin resistance gene.

Plasmid pKK74 was digested with *Eco*RI and *Sbf*I. A 1204 bp fragment was gel isolated. Plasmid pKK55 was digested with *Eco*RI and *Sbf*I. A 347 bp fragment was gel isolated. The 1204 bp fragment originating from plasmid pKK74 and the 347 bp fragment originating from plasmid pKK55 were ligated to a 2961 bp fragment obtained by digesting a plasmid designated pKK74 with *Eco*RI. Plasmid pKK74 contains *C. curvatus ENO1* promoter and plasmid pKK55 contains *C. curvatus TEF1* terminator. The resulting plasmid was designated as pKK77pre.

Plasmid pKK50 was digested with *Sbf*I. A 1514 bp fragment was gel isolated and ligated to a 4517 bp fragment obtained by digesting plasmid pKK77pre with *Sbf*I. The resulting plasmid was designated as pKK77. The plasmid pKK77 contains the *S. cerevisiae ALD6* encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator.

Plasmid pKK76 was digested with *Bam*HI and *Xmn*I. A 2652 bp fragment was gel isolated and ligated to a 6031 bp fragment obtained by digesting plasmid pKK77 with *Bam*HI. The plasmid pKK76 contains the *E. coli*

hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator. The resulting plasmid was designated as pKK81 (Fig 3).

Example 3B. Generation of a genetically modified *C. curvatus* (Y23/81) with an integrated ALD6 encoding gene and hygromycin resistance gene by transforming wild-type *C. curvatus* with digested plasmid pKK81 (Fig 3, Ex. 3A)

Plasmid pKK81 was restricted with *Not*I and *Psp*OMI, and the resulting linear DNA was used to transform a wild-type *C. curvatus* strain ATCC 20509 designated as Y23 by electroporation using a standard electroporation method.

The transformed cells were screened for hygromycin resistance. Several hygromycin-resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of *C. curvatus* with *Not*I and *Psp*OMI cut pKK81 and containing a *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator were designated as Y23/81-8, Y23/81-51, Y23/81-59, Y23/81-66 and Y23/81-69.

Example 4A. Construction of a plasmid (pKK82, Fig. 4) containing the G418 resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator

Plasmid pKK67 was digested with *Bam*HI and *Xmn*I. A 2442 bp fragment was gel isolated and ligated to a 6031 bp fragment obtained by digesting a plasmid pKK77 with *Bam*HI. The plasmid pKK67 contains the *E. coli* G418 resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the plasmid pKK77 contains *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator. The resulting plasmid was designated as pKK82 (Fig 4).

Example 4B. Generation of a genetically modified *C. curvatus* (Y23/82) with an integrated ALD6 encoding gene and G418 resistance gene by transforming wild-type *C. curvatus* with digested plasmid pKK82 (Fig 4, Ex. 4A)

Plasmid pKK82 was restricted with *Not*I and *Psp*OMI, and the resulting linear DNA was used to transform wild-type *C. curvatus* strain ATCC 20509 designated as Y23 by electroporation. The transformed cells were screened for

G418 resistance. Several G418-resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of *C. curvatus* with *Not*I and *Psp*OMI cut pKK82 and containing *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator were designated as Y23/82-1, Y23/82-2, Y23/82-4 and Y23/82-13.

Example 5A. Construction of a plasmid (pKK86, Fig. 5) containing the hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the *S. cerevisiae* ACS2 encoding gene under the control of the *CcTPI1* promoter and the *CcENO1* terminator

Plasmid pKK63 was digested with *Bam*HI and *Sbf*I. A 851 bp fragment was gel isolated and ligated to a 3295 bp fragment obtained by digesting plasmid pKK60 with *Bam*HI and *Sbf*I. The plasmid pKK63 contains the *CcTPI1* promoter and the plasmid pKK60 contains the *CcENO1* terminator. The resulting plasmid was designated as pKK78pre.

The plasmid UmACS contains the *S. cerevisiae* ACS2 (SEQ ID NO:50) encoding gene which has been codon optimized according to *Ustilago maydis* yeast codon usage (SEQ ID NO:51) with flanking *Sbf*I restriction sites. Plasmid UmACS was digested with *Sbf*I and *Dra*I. A 2060 bp fragment was gel isolated and ligated to a 4146 bp fragment obtained by digesting plasmid pKK78pre with *Sbf*I. The resulting plasmid was designated as pKK78.

Plasmid pKK76 was digested with *Bam*HI and *Dra*I. A 2652 bp fragment was gel isolated and ligated to a 6206 bp fragment obtained by digesting plasmid pKK78 with *Bam*HI. The plasmid pKK76 contains the *E. coli* hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator. The resulting plasmid was designated as pKK86 (Fig 5).

Example 5B. Generation of a genetically modified *C. curvatus* (Y23/86) with an integrated ACS2 encoding gene and hygromycin resistance gene by transforming wild-type *C. curvatus* with digested plasmid pKK86 (Fig 5, Ex. 5A)

Plasmid pKK86 was restricted with *Not*I and *Psp*OMI, and the resulting linear DNA was used to transform a wild-type *C. curvatus* strain ATCC 20509 designated as Y23 by electroporation. The transformed cells were screened for hygromycin resistance. Several hygromycin-resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of *C. curvatus* with *Not*I and *Psp*OMI cut pKK86 and containing

S. cerevisiae ACS2 encoding gene under the control of the *CcTPI1* promoter and the *CcENO1* terminator were designated as Y23/86-86, Y23/86-92, Y23/86-93, Y23/86-98 and Y23/86-100.

Example 6A. Construction of a plasmid (pKK95, Fig 6) containing the cerulenin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the *R. oryzae* PDAT encoding gene under the control of the *CcTPI1* promoter and the *CcGPD1* terminator

Plasmid pKK54 was digested with *Kpn*I and *Sbf*I. A 394 bp fragment was gel isolated and ligated to a 3742 bp fragment obtained by digesting plasmid designated as pKK63 with *Kpn*I and *Sbf*I. The plasmid pKK54 contains the *CcGPD1* terminator and the plasmid pKK63 contains the *CcTPI1* promoter. The resulting plasmid was designated as pKK93pre. Plasmid UmPDAT was digested with *Sbf*I. A 1844 bp fragment was gel isolated and ligated to a 4136 bp fragment obtained by digesting plasmid pKK93pre with *Sbf*I. The plasmid UmPDAT contains a *R. oryzae* PDAT (SEQ ID NO:52) encoding gene which has been codon optimized according to *U. maydis* yeast codon usage (SEQ ID NO:53) with flanking *Sbf*I restriction sites. The resulting plasmid was designated as pKK93.

The plasmid pKK93 was digested with *Eco*RI and *Dra*I. A 3029 bp fragment was gel isolated and ligated to a 6242 bp fragment obtained by digesting plasmid pKK91 with *Eco*RI. The plasmid pKK91 contains the *S. cerevisiae* cerulenin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator. The resulting plasmid was designated as pKK95 (Fig 6).

Example 6B. Generation of a genetically modified *C. curvatus* (Y23/95) with an integrated PDAT encoding gene and cerulenin resistance gene by transforming wild-type *C. curvatus* with digested plasmid pKK95 (Fig 6, Ex. 6A)

Plasmid pKK95 was restricted with *Eco*RV, and the resulting linear DNA was used to transform a wild-type *C. curvatus* strain ATCC 20509 designated as Y23 by electroporation. The transformed cells were screened for cerulenin resistance. Several cerulenin-resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of *C. curvatus* with *Eco*RV cut pKK95 and containing the *R. oryzae* PDAT encoding gene under the control of the *CcTPI1* promoter and the *CcGPD1* terminator

were designated as Y23/95-87, Y23/95-98, Y23/95-99, Y23/95-104 and Y23/95-109.

Example 7A. Construction of a plasmid (pKK85, Fig 7) containing the hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator and the *S. cerevisiae* ACS2 encoding gene under the control of the *CcTPI1* promoter and the *CcENO1* terminator

Plasmid pKK77 was digested with *Eco*RI and *Xmn*I. A 3073 bp fragment was gel isolated and ligated to a 6206 bp fragment obtained by digesting plasmid pKK78 with *Eco*RI. The plasmid pKK77 contains the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator and the plasmid pKK78 contains the *S. cerevisiae* ACS2 encoding gene under the control of the *CcTPI1* promoter and the *CcENO1* terminator. The resulting plasmid was designated as pKK84. The plasmid pKK76 was digested with *Bam*HI and *Xmn*I. A 2652 bp fragment was gel isolated and ligated to a 9279 bp fragment obtained by digesting pKK84 with *Bam*HI. The plasmid pKK76 contains the hygromycin resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator. The resulting plasmid was designated as pKK85 (Fig 7).

Example 7B. Generation of a genetically modified *C. curvatus* (Y23/85) with an integrated ALD6 encoding and ACS2 encoding genes and hygromycin resistance gene by transforming wild-type *C. curvatus* with digested plasmid pKK85 (Fig 7, Ex. 7A)

Plasmid pKK85 was restricted with *Not*I and *Psp*OMI, and the resulting linear DNA was used to transform wild-type *C. curvatus* strain ATCC 20509 designated as Y23 by electroporaton. The transformed cells were screened for hygromycin resistance. Several hygromycin-resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of *C. curvatus* with *Not*I and *Psp*OMI cut pKK85 and containing the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator and the *S. cerevisiae* ACS2 encoding gene under the control of the *CcTPI1* promoter and the *CcENO1* terminator were designated as Y23/85-119, Y23/85-125, Y23/85-128, Y23/85-129 and Y23/85-139.

Example 8. Generation of a genetically modified *C. curvatus* (Y23/81/95) with an integrated ALD6 encoding and PDAT encoding genes and hygromycin and cerulenin resistance genes by transforming genetically modified strain Y23/81-51 (Ex. 3B) with plasmid pKK95 (Fig. 6, Ex. 6A)

Plasmid pKK95 was restricted with *Eco*RV and the resulting linear DNA was used to transform a genetically modified strain Y23/81-51 by electroporation. The transformed cells were screened for cerulenin and hygromycin resistance. Several cerulenin and hygromycin resistance colonies were analysed at DNA level by PCR. The transformants originating from the transformation of genetically modified strain Y23/81-51 with *Eco*RV cut pKK95 and containing the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator and the *R. oryzae* PDAT encoding gene under the control of the *CcTPI1* promoter and the *CcGPD1* terminator were designated as Y23/81/95-18 and Y23/81/95-42.

Example 9. Generation of a genetically modified *C. curvatus* (Y23/85/95) with an integrated ALD6, ACS2 and PDAT encoding genes and hygromycin and cerulenin resistance genes by transforming genetically modified strain Y23/85-128 (Ex. 7B) with plasmid pKK95 (Fig 6, Ex. 6A)

Plasmid pKK95 was restricted with *Eco*RV and the resulting linear DNA was used to transform a genetically modified strain Y23/85-128 by electroporation. The transformed cells were screened for cerulenin and hygromycin resistance. Several cerulenin and hygromycin resistance colonies were analysed at DNA level by PCR. The transformants originating from the transformation of genetically modified strain Y23/85-128 with *Eco*RV cut pKK95 and containing the *S. cerevisiae* ALD6 encoding gene under the control of the *CcENO1* promoter and the *CcTEF1* terminator, the *S. cerevisiae* ACS2 encoding gene under the control of the *CcTPI1* promoter and the *CcENO1* terminator and *R. oryzae* PDAT encoding gene under the control of the *CcTPI1* promoter and the *CcGPD1* terminator were designated as Y23/85/95-4 and Y23/85/95-68.

Example 10. Cloning of *Mucor circinelloides TPI (McTPI1)* promoter and terminator region

A genomic fragment of the *M. circinelloides TPI1* gene was amplified by PCR from genomic *M. circinelloides f. griseocyanus* (D-82202, VTT Culture Collection) DNA with degenerative primers identified as SEQ ID NO:54

(Mould TPI1) and SEQ ID NO:55 (Mould TPI3). The degenerative primers were designed based on a consensus sequence of *TPI1* genes of *Rhizopus oryzae, Fusarium oxysporum, Aspergillus fumigatus, A. terreus*and *A. nidulans*. A ~400 bp genomic fragment was isolated and sequenced.

A genomic fragment containing the *McTPI1* promoter region was obtained with a ligation-mediated PCR amplification with *TPI1* gene specific oligonucleotides identified as SEQ ID NO:56 (MC_TPI2) and SEQ ID NO:57 (MC_TPI1), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Ssp*I-digested *M. circinelloides* DNA was used. A ~1500 bp PCR fragment was isolated and sequenced.

The *M. circinelloides TPI1* promoter was PCR amplified by using primers identified as SEQ ID NO:58 (MC_TPI7) and SEQ ID NO:59 (MC_TPI_8), and the *M. circinelloides* genomic DNA as the template. A PCR fragment was digested with *Pst*I and *Bam*HI. A 1251 bp fragment was gel isolated and ligated to a *Pst*I and *Bam*HI –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK56. Plasmid pKK56 contains the *M. circinelloides TPI1* promoter.

A genomic fragment containing the *McTPI1* terminator region was obtained with a ligation-mediated PCR amplification with *TPI1* gene specific oligonucleotides identified as SEQ ID NO:60 (MC_TPI4) and SEQ ID NO:61 (MC_TPI3), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Nru*I-digested *M. circinelloides* DNA was used. A ~1500 bp PCR fragment was isolated and partially sequenced.

The *M. circinelloides TPI1* terminator was PCR amplified by using primers identified as SEQ ID NO:62 (MC_TPI5) and SEQ ID NO:63 (MC_TPI6), and the *M. circinelloides* genomic DNA as the template. A PCR fragment was digested with *Xba*I and *Bam*HI. A 347 bp fragment was gel isolated and ligated to a *Xba*I and *Bam*HI-digested pBluescript KS- plasmid. The resulting plasmid was designated pKK57. Plasmid pKK57 contains the *M. circinelloides TPI1* terminator.

Example 11. Cloning of *Mucor circinelloides TEF (McTEF1)* promoter and terminator region

A genomic fragment of the *M. circinelloides TEF1* gene was amplified by PCR from genomic *M. circinelloides f. griseocyanus* (D-82202, VTT

Culture Collection) DNA with degenerative primers identified as SEQ ID NO:64 (Mould TEF1) and SEQ ID NO:65 (Mould TEF4). The degenerative primers were designed based on a consensus sequence of *TEF1* genes of *Rhizopus oryzae, Fusarium oxysporum, Aspergillus terreus* and *A. nidulans*. A ~600 bp genomic fragment was isolated and sequenced.

A genomic fragment containing the *McTEF1* promoter region was obtained with a ligation-mediated PCR amplification with *TEF1* gene specific oligonucleotides identified as SEQ ID NO:66 (MC_TEF2) and SEQ ID NO:67 (MC_TEF1), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Ssp*I-digested *M. circinelloides* DNA was used. A ~500 bp PCR fragment was isolated and sequenced. Nested primers identified as SEQ ID NO:68 (MC_TEF6) and SEQ ID NO:69 (MC_TEF5) were designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as above except that *Hae*III-digested *M. circinelloides* DNA was used. A ~1500 bp PCR fragment was isolated and sequenced.

The *M. circinelloides TEF1* promoter was PCR amplified by using primers identified as SEQ ID NO:70 (MC_TEF9) and SEQ ID NO:71 (MC_TEF10), and the *M. circinelloides* genomic DNA as the template. A PCR fragment was digested with *Hind*III and *Pst*I and a 1387 bp fragment was gel isolated and ligated to a *Hind*III and *Pst*I –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK64. Plasmid pKK64 contains the *M. circinelloides TEF1* promoter.

A genomic fragment containing the *McTEF1* terminator region was obtained with a ligation-mediated PCR amplification with *TPI1* gene specific oligonucleotides identified as SEQ ID NO:72 (MC_TEF4) and SEQ ID NO:73 (MC_TEF3), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Ssp*I-digested *M. circinelloides* DNA was used. A ~1100 bp PCR fragment was isolated and sequenced. Nested primers identified as SEQ ID NO:74 (MC_TEF8) and SEQ ID NO:75 (MC_TEF7) were designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as above except that *Hpa*I-digested *M. circinelloides* DNA was used. A ~1200 bp PCR fragment was isolated and sequenced.

The *M. circinelloides TEF1* terminator was PCR amplified by using primers identified as SEQ ID NO:76 (MC_TEF11) and SEQ ID NO:77 (MC_TEF12), and the *M. circinelloides* genomic DNA as the template. A PCR fragment was digested with *Xma*I and *Eco*RI and a 389 bp fragment was gel isolated and ligated to a *Xma*I and *Eco*RI –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK65. Plasmid pKK65 contains the *M. circinelloides TEF1* terminator.

Example 12. Cloning of *Mucor circinelloides PGK (McPGK1)* promoter region

A genomic fragment of the *M. circinelloides PGK1* gene was amplified by PCR from genomic *M. circinelloides f. griseocyanus* (D-82202, VTT Culture Collection) DNA with degenerative primers identified as SEQ ID NO:78 (Mould PGK4) and SEQ ID NO:79 (Mould PGK2). The degenerative primers were designed based on a consensus sequence of *PGK1* genes of *Rhizopus oryzae, Fusarium oxysporum, Aspergillus fumigatus, A. oryzae* and *A. nidulans*. A ~250 bp genomic fragment was isolated and sequenced.

A genomic fragment containing the *McPGK1* promoter region was obtained with a ligation-mediated PCR amplification with *PGK1* gene specific oligonucleotides identified as SEQ ID NO:80 (MC_PGK2) and SEQ ID NO:81 (MC_PGK1), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Ssp*I-digested *M. circinelloides* DNA was used. A ~1300 bp PCR fragment was isolated and sequenced. Nested primers identified as SEQ ID NO:82 (MC_PGK4) and SEQ ID NO:83 (MC_PGK3) were designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as above except that *Hae*III-digested *M. circinelloides* DNA was used. A ~600 bp PCR fragment was isolated and sequenced.

The *M. circinelloides PGK1* promoter was PCR amplified by using primers identified as SEQ ID NO:84 (MC_PGK5) and SEQ ID NO:85 (MC_PGK6), and the *M. circinelloides* genomic DNA as the template. A PCR fragment was digested with *Sac*II and *Xba*I and a 1291 bp fragment was gel isolated and ligated to a *Sac*II and *Xba*I –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK62. Plasmid pKK62 contains the *M. circinelloides PGK1* promoter.

Example 13. Cloning of *Mucor circinelloides GPD (McGPD1)* promoter region

A genomic fragment containing the *McGPD1* promoter region was obtained with a ligation-mediated PCR amplification with *Mucor circinelloides* (Syn. *racemosus*) *GPD1* gene (GenBank accession number AJ293012, version number AJ293012.1) specific oligonucleotides identified as SEQ ID NO:86 (MC_GPD2) and SEQ ID NO:87 (MC_GPD1), together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as in Example 1A except that *Eco*RV-digested *M. circinelloides* (D-82202, VTT Culture Collection) DNA was used. A ~500 bp PCR fragment was isolated and sequenced. Nested primers identified as SEQ ID NO:88 (MC_GPD10) and SEQ ID NO:89 (MC_GPD9) were designed and used in a ligation-mediated PCR amplification together with oligonucleotides identified as SEQ ID NO:3 (PCR Linker I) and SEQ ID NO:4 (PCR Linker II), similarly as above except that *Stu*I-digested *M. circinelloides* DNA was used. A ~1400 bp PCR fragment was isolated and sequenced.

The *M. circinelloides GPD1* promoter was PCR amplified by using primers identified as SEQ ID NO:90 (MC_GPD11) and SEQ ID NO:91 (MC_GPD12), and the *M. circinelloides* genomic DNA as the template. A PCR fragment was digested with *Eco*RI and *Hind*III and a 1440 bp fragment was gel isolated and ligated to a *Eco*RI and *Hind*III –digested pBluescript KS- plasmid. The resulting plasmid was designated pKK59. Plasmid pKK59 contains the *M. circinelloides GPD1* promoter.

Example 14A: Cloning of *E. coli* hygromycin resistance gene; construction of a plasmid (pKK69) having the *E. coli* hygromycin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator

Plasmid pKK62 was digested with *Sac*II and *Sbf*I. A 1286 bp fragment was gel isolated. Plasmid pKK52 was digested with *Sbf*I. A 1034 bp fragment was gel isolated. The 1286 bp fragment originated from plasmid pKK62 and the 1034 bp fragment originated from plasmid pKK52 were ligated to a 3268 bp fragment obtained by digesting plasmid pKK57 with *Sac*II and *Sbf*I. Plasmid pKK52 contains the *E. coli* hygromycin resistance gene, plasmid pKK62 contains the *M. circinelloides PGK1* promoter and plasmid pKK57 contains the *M. circinelloides TPI1* terminator. The resulting plasmid was designated pKK69. Plasmid pKK69 contains the *E. coli* hygromycin resistance gene

under the control of the *M. circinelloides PGK1* promoter and the *M. circinelloides TPI1* terminator.

Example 14B: Cloning of *S. cerevisiae* cerulenin resistance gene; construction of a plasmid (pKK92) having the *S. cerevisiae* cerulenin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator

Plasmid pKK80 was digested with *Pst*I. A 1685 bp fragment was gel isolated. Plasmid pKK80 contains the *S. cerevisiae* cerulenin resistance gene. The 1685 bp fragment was ligated to a 4554 bp fragment obtained by digesting plasmid pKK69 with *Sbf*I. Plasmid pKK69 contains the *M. circinelloides PGK1* promoter and the *M. circinelloides TPI1* terminator. The resulting plasmid was designated pKK92. Plasmid pKK92 contains the *S. cerevisiae* cerulenin resistance gene under the control of the *M. circinelloides PGK1* promoter and the *M. circinelloides TPI1* terminator.

Example 15A. Construction of a plasmid (pKK75, Fig 8) containing the hygromycin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator and the *S. cerevisiae ALD6* gene under the control of the *McTPI1* promoter and the *McTEF1* terminator

Plasmid pKK56 was digested with *Bam*HI and *Pst*I. A 1251 bp fragment was gel isolated. Plasmid pKK56 contains the *McTPI1* promoter. Plasmid RoALD was digested with *Sbf*I. A 1514 bp fragment was gel isolated. The plasmid RoALD contains the *S. cerevisiae* ALD6 (SEQ ID NO:47) encoding gene which has been codon optimized according to *Rhizopus oryzae* filamentous fungus codon usage (SEQ ID NO:49) with flanking *Sbf*I restriction sites. The 1251 bp fragment originating from the plasmid pKK56 and the 1514 bp fragment originating from the plasmid RoALD were ligated to a 3321 bp fragment obtained by digesting plasmid pKK65 with *Bam*HI and *Sbf*I. Plasmid pKK65 contains the *McTEF1* terminator. The resulting plasmid was designated as pKK73. Plasmid pKK73 contains the *S. cerevisiae* ALD6 encoding gene under the control of the *M. circinelloides TPI1* promoter and the *M. circinelloides TEF1* terminator.

Plasmid pKK69 was digested with *Bam*HI and *Xmn*I. A 2652 bp fragment was gel isolated and ligated to a 6086 bp fragment obtained by digesting plasmid pKK73 with *Bam*HI. Plasmid pKK69 contains the *E. coli* hygromycin resistance gene under the control of the *M. circinelloides PGK1* pro-

moter and the *M. circinelloides TPI1* terminator. The resulting plasmid was designated as pKK75 (Fig 8).

Example 15B. Generation of a genetically modified *Mucor circinelloides* (M22/75) with an integrated ALD6 encoding gene and a hygromycin resistance gene by transforming wild-type *M. circinelloides* with digested plasmid pKK75 (Fig 8, Ex. 15A)

Plasmid pKK75 was restricted with *Kpn*I and *Not*I. A 5866 bp fragment was gel isolated and used to transform a wild-type *M. circinelloides* strain (D-82202, VTT Culture Collection) designated as M22, using a *Mucor* protoplast transformation method (Wolff et. al. 2002). The transformed cells were screened for hygromycin resistance. Several hygromycin-resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of the wild-type *M. circinelloides* strain with *Kpn*I and *Not*I cut pKK75 and containing the *S. cerevisiae* ALD6 encoding gene under the control of the *McTPI1* promoter and the *McTEF1* terminator were designated as M22/75-80 and M22/75-86.

Example 16A. Construction of a plasmid (pKK94, Fig 9) containing the hygromycin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator and the *S. cerevisiae* ALD6 encoding gene under the control of the *McTPI1* promoter and the *McTEF1* terminator and the *S. cerevisiae* ACS2 encoding gene under the control of the *McTEF1* promoter and the *McTPI1* terminator

Plasmid pKK57 was digested with *Pst*I. A 351 bp fragment was gel isolated and ligated to a 4326 bp fragment obtained by digesting plasmid pKK64 with *Pst*I. The plasmid pKK57 contains the *McTPI* terminator and the plasmid pKK64 contains the *McTEF* promoter. The resulting plasmid was designated pKK90Pre. Plasmid RoACS was digested with *Sbf*I. A 2060 bp fragment was gel isolated and ligated to a 4677 bp fragment obtained by digesting plasmid pKK90Pre with *Sbf*I. The plasmid RoACS contains the *S. cerevisiae* ACS2 (SEQ ID NO:50) encoding gene which has been codon optimized according to *Rhizopus oryzae* filamentous fungus codon usage (SEQ ID NO:92) with flanking *Sbf*I restriction sites. The resulting plasmid was designated as pKK90.

Plasmid pKK75 was digested with *Kpn*I followed by removal of the 3' overhangs by T4 DNA polymerase and each of the 4 dNTPs. *Kpn*I

(blunt) -digested plasmid pKK75 was digested with *Not*I and a 5866 bp fragment was gel isolated. The plasmid pKK75 contains the *E. coli* hygromycin resistance gene under the control of the *M. circinelloides PGK1* promoter and the *M. circinelloides TPI1* terminator and *S. cerevisiae* ALD6 encoding gene under the control of the *M. circinelloides TPI1* promoter and the *M. circinelloides TEF1* terminator. The 5866 bp fragment originating from plasmid pKK75 was ligated to a 6698 bp fragment obtained by digesting plasmid pKK90 with *Sma*I and *Not*I. The resulting plasmid was designated as pKK94 (Fig 9).

Example 16B. Generation of a genetically modified *Mucor circinelloides* (M22/94) with an integrated ALD6 and ACS2 encoding genes and a hygromycin resistance gene by transforming wild-type *M. circinelloides* with digested plasmid pKK94 (Fig 9, Ex. 16A)

Plasmid pKK94 was restricted with *Kpn*I and *Sac*I. A 9701 bp fragment was gel isolated and used to transform a wild-type *M. circinelloides* strain (D-82202, VTT Culture Collection) designated as M22, using the transformation method described in Example 15B. The transformed cells were screened for hygromycin resistance. Several hygromycin resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of a wild-type *M. circinelloides* strain with *Kpn*I and *Sac*I cut pKK94 and containing the *S. cerevisiae* ALD6 encoding gene under the control of the *McTPI1* promoter and the *McTEF1* terminator and *S. cerevisiae* ACS2 encoding gene under the control of the *McTEF1* promoter and the *McTPI1* terminator were designated as M22/94-12, M22/94-16 and M22/94-24.

Example 17A. Construction of a plasmid (pKK96, Fig 10) containing the hygromycin gene under the control of the *McPGK1* promoter and the *McTPI1* terminator and the *S. cerevisiae* ACS2 encoding gene under the control of the *McTEF1* promoter and the *McTPI1* terminator

Plasmid pKK94 was digested with *Bsr*GI and a 9683 bp fragment was gel isolated and self ligated. The resulting plasmid was designated as pKK96 (Fig 10). The plasmid pKK96D contains the *S. cerevisiae* ACS2 encoding gene under the control of the *McTEF1* promoter and the *McTPI1* terminator.

Example 17B. Generation of a genetically modified *Mucor circinelloides* (M22/96) with an integrated ACS2 encoding gene and hygromycin resistance gene by transforming wild-type *M. circinelloides* with digested plasmid pKK96 (Fig 10, Ex. 17A)

Plasmid pKK96 was restricted with *Kpn*I and *Sac*I. A 6824 bp fragment was gel isolated and used to transform the wild-type *M. circinelloides* strain (D-82202, VTT Culture Collection) designated as M22, using the transformation method described in Example 15B. The transformed cells were screened for hygromycin resistance. Several hygromycin resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of the wild-type *M. circinelloides* strain with *Kpn*I and *Sac*I cut pKK96D and containing the *S. cerevisiae* ACS2 encoding gene under the control of the *McTEF1* promoter and the *McTPI1* terminator were designated as M22/96-1 and M22/96-6.

Example 18A. Construction of a plasmid (pKK98) containing the cerulenin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator and the *R. oryzae* PDAT gene under the control of the *McGPD1* promoter and the *McTEF1* terminator

Plasmid pKK59 was digested with *Sbf*I and *Xba*I. A 1467 bp fragment was gel isolated and ligated to a 3309 bp fragment obtained by digesting plasmid pKK65 with *Sbf*I and *Xba*I. The plasmid pKK59 contains the *McGPD1* promoter and the plasmid pKK65 contains the *McTEF1* terminator. The resulting plasmid was designated as pKK88. Plasmid RoPDAT was digested with *Sbf*I. A 1844 bp fragment was gel isolated and ligated to a 4776 bp fragment obtained by digesting plasmid pKK88 with *Sbf*I. The plasmid RoPDAT contains the *R. oryzae* PDAT (SEQ ID NO:52) encoding gene, which has been codon optimized according to *R. oryzae* codon usage (SEQ. ID. NO 93) with flanking *Sbf*I restriction sites. The resulting plasmid was designated as pKK97.

Plasmid pKK97 was digested with *Eco*RI. A 3659 bp fragment was gel isolated and ligated to a 6239 bp fragment obtained by digesting plasmid pKK92 with *Eco*RI. The plasmid pKK92 contains the *S. cerevisiae* cerulenin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator. The resulting plasmid was designated as pKK98 (Fig 11).

Example 18B. Generation of a genetically modified *Mucor circinelloides* (M22/98) with an integrated PDAT encoding gene and cerulenin resistance gene by transforming wild-type *M. circinelloides* with digested plasmid pKK98 (Fig 11, Ex. 18A)

Plasmid pKK98 was restricted with *Apa*I and *Sac*II. A 7029 bp fragment was gel isolated and used to transform the wild-type *M. circinelloides* strain (D-82202, VTT Culture Collection) designated as M22, using the transformation method described in Example 15B. The transformed cells were screened for cerulenin resistance. Several cerulenin resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of the wild-type *M. circinelloides* strain with *Apa*I and *Sac*II cut pKK98 and containing the *R. oryzae* PDAT encoding gene under the control of the *McGPD1* promoter and the *McTEF1* terminator were designated as M22/98-16.

Example 19. Generation of a genetically modified *Mucor circinelloides* (M22/75/98) with integrated ALD6 and PDAT encoding genes and hygromycin and cerulenin resistance genes by transforming genetically modified strain M22/75-86 (Ex 15B) with digested plasmid pKK98 (Fig 11, Ex. 18A)

Plasmid pKK98 was restricted with *Apa*I and *Sac*II. A 7029 bp fragment was gel isolated and used to transform the genetically modified strain M22/75-86, using the transformation method described in Example 15B. The transformed cells were screened for cerulenin resistance. Several cerulenin resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of the recombinant M22/75-86 strain with *Apa*I and *Sac*II cut pKK98 and containing the *R. oryzae* PDAT encoding gene under the control of the *McGPD1* promoter and the *McTEF1* terminator and the *S. cerevisiae* ALD6 encoding gene under the control of the *McTPI1* promoter and the *McTEF1* terminator were designated as M22/75/98-7 and M22/75/98-9.

Example 20. Generation of a genetically modified *Mucor circinelloides* (M22/94/98) with integrated ALD6, ACS2 and PDAT encoding genes and hygromycin and cerulenin resistance genes by transforming genetically modified strain M22/94-31 (Ex 16B) with digested plasmid pKK98 (Fig 11, Ex. 18A)

Plasmid pKK98 was restricted with *Apa*I and *Sac*II. A 7029 bp fragment was gel isolated and used to transform the genetically modified strain M22/94-31, using the transformation method described in Example 15B. The transformed cells were screened for cerulenin resistance. Several cerulenin resistant colonies were analysed at DNA level by PCR. The transformants originating from the transformation of the recombinant M22/94-31 strain with *Apa*I and *Sac*II cut pKK98 and containing the *R. oryzae* PDAT encoding gene under the control of the *McGPD1* promoter and the *McTEF1* terminator, the *S. cerevisiae* ALD6 encoding gene under the control of the *McTPI1* promoter and the *McTEF1* terminator and the *S. cerevisiae* ACS2 encoding gene under the control of the *McTEF1* promoter and the *McTPI1* terminator were designated as M22/94/98-19 and M22/94/98-22.

Example 21. Lipid extraction and total lipid and triglyceride concentration measurements

A lipid extraction method was modified from the protocol of Folch *et al.*, 1957. 0.5 to 2 ml of cell culture was taken into an Eppendorf tube. The sample was centrifuged and the supernatant discarded. The pellet was placed rapidly in liquid nitrogen and stored at -80ºC. Alternatively, filamentous fungal cells of 2 to 12 ml culture broth were collected by vacuum filtration through disks of glass microfiber filters (Whatman, England). After washing twice with distilled water, biomass was removed from the filter using a clean spatula and put into 2 ml microfuge tubes, which were placed rapidly in liquid nitrogen and stored at -80°C. In a homogenisation step the frozen pellet was suspended in 500 µl of ice-cold methanol with 0.1% BHT (2,6-Di-*tert*-butyl-4-methylphenol) and homogenised with a Mixer Mill homogenizator with 5-mm zirconium oxide and 3-mm yttrium stabilized zirconium oxide balls (Retsch) at 25 Hz for 5 min. After homogenisation 1000 µl of chloroform was added and homogenisation repeated. After re-homogenisation 300 µl of 20mM acetic acid was added and the sample vortexed for 10 min. After vortexing the sample was centrifuged 13 000 rpm for 5 min at RT. The lower phase was recovered and 1000 µl of

chloroform was added to the remaining phase, vortexed and recentrifuged. The lower phases were combined into pre-weighed 2 ml microfuge tubes, and dried, after which the total lipid content of the sample was determined by gravimetry. Then the lipid sample was redissolved in 1.5 ml of chloroform:methanol (2:1) + 0.1% BHT and stored at -20°C. For triacylglycerol analysis 100 to 1500 µl of chloroform:methanol extracted lipids were evaporated and re-dissolved in 200 - 1000 µl of isopropanol. Triacylglycerols were measured enzymatically from the samples by using the Konelab Triglycerides Kit (Thermo Scientific, Finland) and Cobas Mira automated analyser (Roche) or a microtitre-plate reader (Varioskan, Thermo Electron Corporation). This lipid extraction and total lipid and triglyceride concentration measurements methods were used in the following examples if not otherwise indicated.

Example 22. Microaerobic shake flask characterization of strains Y23/81-51 and Y23/81-66 (Ex. 3B), Y23/86-86 and Y23/86-92 (Ex. 5B), Y23/95-87 and Y23/95-109 (Ex. 6B) and Y23/85/95-4 (Ex. 9), in glucose medium with C/N ratio of 20

Transformants were separately cultivated in 50 ml of culture medium “glucose CN20” (pH 5.5, 20 g glucose, 0.3 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* 7H_2O, 4.0 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 100 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control.

Lipid extraction and triacylglycerol concentration measurement were carried out as described in Example 21. Cell dry weight was determined by centrifuging 1 ml of the culture broth in pre-dried, pre-weighed Eppendorf tubes. After washing with 1 ml of distilled water the cell pellet was dried at 100°C for 24 hours and weighed again. HPLC analyses for sugars were conducted with a Waters 2690 Separation Module and Water System Interfase Module liquid chromatography coupled with a Waters 2414 differential refractometer and Waters 2487 dual absorbance detector. The liquid chromatography columns were a 100 X 7.8 mm Fast Acid Analysis column from Bio-Rad and a 300 X 7.8 mm Aminex HPX-87H column from Bio-Rad. The columns were equilibrated with 2.5 mM H_2SO_4 in water at 55°C and samples were elut-

ed with 2.5 mM H_2SO_4 in water at 0.5 ml/min flow rate. Data acquisition was done using Waters Millennium software. This HPLC method was used in all appropriate Examples.

After 48 hours cultivation (Table 2A), when 4 to 8 g/l glucose was left, Y23/81, Y23/86, Y23/95 and Y23/85/95 transformants produced 12, 22, 15 and 24% more triacylglycerols with higher rate, respectively, than the control strain in glucose medium. Triacylglycerol yields per used glucose were also better up to 10% with the transformants compared to the control strain. Additionally Y23/85/95 transformant had 13 % higher triacylglycerol yield on biomass than the control strain (12.9 and 11.4 % TAG yield on biomass, respectively). In particular, after 24 hours of cultivation (Table 2B), the strains expressing ALD or ACS alone, or ALD and ACS together, had enhanced production of triacylglycerols, measured as concentration (g/l) and as yield per biomass and used glucose, with higher rate (mg/l/h) compared to the control strain.

Table 2A. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yield (%) per used glucose after 48 hours microaerobic cultivation in glucose medium with C/N ratio of 20

Strain	TAG (g/l)	Yield TAG (% used glucose)	TAG mg/l/h
Control	0.72	5.88	15.0
Y23/81 (ALD)	0.81	5.90	16.8
Y23/86 (ACS)	0.88	6.00	18.3
Y23/95 (PDAT)	0.82	6.02	17.2
Y23/85/95 (ALD+ACS+PDAT)	0.89	6.46	18.5

Table 2B. Triacylglycerol (TAG) concentration (g/l) and yield (%) per biomass (CDW) and used glucose after 24 hours microaerobic cultivation in glucose medium with C/N ratio of 20

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	0.22	5.15	4.29	9.1
Y23/81-66 (ALD)	0.26	6.09	4.86	10.8
Y23/85-125 (ALD+ACS)	0.31	7.01	5.49	12.8
Y23/86-86 (ACS)	0.30	6.76	5.26	12.5

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production, and triacylglycerol yields from used glucose or per dry weight in cultivation with low C/N ratios.

Example 23. Aerobic shake flask characterization of strains Y23/81-8, 51, 59, 66 and 69 (Ex. 3B), Y23/85-119, 125, 128, 129 and 139 (Ex. 7B), Y23/86-86, 92, 93, 98 and 100 (Ex. 5B), Y23/95-99 and 104 (Ex. 6B), Y23/81/95-42 (Ex. 8) and Y23/85/95-4 and 68 (Ex. 9), in glucose medium with C/N ratio of 65

Transformants were separately cultivated in 50 ml of Yeast culture medium II (pH 5.5, 20 g glucose, 0.3 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* 7H_2O, 0.6 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction and triacylglycerol measurements as described in Example 21.

After 44 hours cultivation (Table 3A) transformants Y23/81, Y23/85, Y23/86, Y23/95, Y23/81/95 and Y23/85/95 had 4, 3, 6, 1, 12 and 4% better triacylglycerol yields per used glucose, respectively, than the control strain. Additionally, transformants Y23/81/95 and Y23/85/95 had 7-8% higher triacylglycerol yield on biomass than the control strain (42.8, 43.0 and 40.0 % TAG yield

[/ CDW], respectively). After 25 hours cultivation (Table 3B), the strains expressing ALD or ACS alone, or ALD and ACS together, had enhanced production of triacylglycerols, measured as concentration (g/l) and as yield per biomass and used glucose.

Table 3A. Maximal triacylglycerol (TAG) yield (%) per used glucose after 44 hours cultivation in glucose medium with C/N ratio of 65

Strain	Yield TAG (% used glucose)
Control	16.6
Y23/81 (ALD)	17.3
Y23/85 (ALD+ACS)	17.2
Y23/86 (ACS)	17.6
Y23/95 (PDAT)	16.9
Y23/81/95 (ALD+PDAT)	18.6
Y23/85/95 (ALD+ACS+PDAT)	17.4

Table 3B. Triacylglycerol (TAG) concentration (g/l) and yield (%) per biomass (CDW) and used glucose after 25 hours cultivation in glucose medium with C/N ratio of 65

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	1.01	21.7	10.3	40.5
Y23/81-51, 66 (ALD)	1.15	26.4	12.5	46.0
Y23/85-125, 129 (ALD+ACS)	1.14	27.2	13.3	45.4
Y23/86-92, 100 (ACS)	1.18	22.4	12.0	47.1

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production and triacylglycerol yields from used glucose or per dry weight.

Example 24. Aerobic shake flask characterization of strains Y23/81-66 (Ex. 3B), Y23/85-125 (Ex. 7B), Y23/86-92 (Ex. 5B), Y23/95-98 (Ex. 6B) and Y23/81/95-18 (Ex. 8) in glucose medium with C/N ratio of 103

Transformants were separately cultivated in 50 ml of Yeast culture medium IV (pH 5.5, 20 g glucose, 0.15 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* $7H_2O$, 0.45 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction and triacylglycerol measurements as described in Example 21.

After 35 hours cultivation, when 6-8 g/l glucose was left, transformants Y23/81-66, Y23/85-125, Y23/86-92, Y23/95-98 and Y23/81/95-18 had 3, 6, 9, 11 and 11 % higher triacylglycerol titre and rate than the control strain, respectively. The transformants Y23/81-66, Y23/85-125, Y23/86-92, Y23/95-98 and Y23/81/95-18 had also 11, 19, 7, 20 and 30% better triacylglycerol yields per used glucose, respectively, than the control strain. Additionally Y23/81/95-18 had 24% higher yield on biomass than the control strain (49.4 and 40.0 % TAG yields on biomass, respectively).

Table 4. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yield (%) per used glucose after 35 hours cultivation in glucose medium with C/N ratio of 103

Strain	TAG (g/l)	Yield TAG (% used glucose)	TAG mg/l/h
Control	2.00	16.2	57.1
Y23/81-66 (ALD)	2.05	18.0	58.7
Y23/85-125 (ALD+ACS)	2.11	19.3	60.3
Y23/86-92 (ACS)	2.17	17.4	61.9
Y23/95-98 (PDAT)	2.22	19.4	63.5
Y23/81/95-18 (ALD+PDAT)	2.22	21.1	63.5

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production and triacylglycerol yield from used glucose or per dry weight with high C/N ratios.

Example 25. Aerobic shake flask characterization of strains M22/75-86 (Ex. 15B), M22/96-1 (Ex. 17B), M22/94-24 (Ex. 16B), M22/75/98-9 (Ex. 19) and M22/94/98-19 (Ex. 20), in glucose medium with C/N ratio of 40

Transformants were separately cultivated in 50 ml of mould C/N 40 medium (pH 5.5, 20 g glucose, 1.4 g yeast extract, 2.5 g KH_2PO_4, 0.3 g $(NH_4)_2SO_4$, 10 mg $ZnSO_4$* $7H_2O$, 2 mg $CuSO_4$*$5H_2O$, 10 mg $MnSO_4$, 0.5 g $MgSO_4$* 7 H_2O, 0.1 g $CaCl_2$, 20 mg $FeCl_3$* $6H_2O$ per litre). Each flask (250 ml) was inoculated with 1 * 10^7 spores. The cultivations were maintained at a temperature of 25°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Mucor circinelloides* wild type strain M22 was used as a control. Cell dry weight was determined by vacuum filtration through disks of glass microfiber filters (Whatman, England) of 2 to 24 ml culture broth. After washing twice with distilled water, biomass was removed from the cloth using clean spatula and transferred to pre-dried, pre-weighed 2 ml microfuge tubes in which the mycelia were dried at 100 °C for 48 hours and weighed after cooling in a dessicator. HPLC analysis was carried out as described in Example 22. Lipid extraction and total lipid and triacylglycerol measurements as described in Example 21.

After 46 hours cultivation (Table 5) transformants M22/75-86, M22/96-1, M22/94-24, M22/75/98-9 and M22/94/98-19 produced 21, 9, 18, 91 and 55% more triacylglycerol with higher rate than the control strain, respectively. The transformant M22/75-86, M22/96-1., M22/94-24, M22/75/98-9 and M22/94/98-19 had also 39 (20), 23 (10), 18 (13), 127 (57) and 80 (25)% higher triacylglycerol yield on used glucose (on biomass) than the control strain, respectively. Additionally the transformants M22/75/98-9 and M22/94/98-19 had higher total lipid concentration (0.93-1.09 g/l) and rate (20-24 mg/l/h) with yields on biomass (26.1-31.4 %) and on used glucose (6.29-7.52 %) than the control strain (0.71 g/l, 15 mg/l/h, 24.6% and 4.09%, respectively).

Table 5. Triacylglycerol (TAG) and total lipid concentrations (g/l), rates (mg/l/h) and yields (%) per biomass (CDW; cell dry weight) and used glucose after 46 hours cultivation in glucose medium with C/N ratio of 40

Strain	TAG g/l	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	0.33	11.5	1.92	7.2
M22/75-86 (ALD)	0.40	13.8	2.67	8.8
M22/96-1 (ACS)	0.36	12.6	2.36	7.9
M22/94-24 (ALD+ACS)	0.39	13.0	2.27	8.5
M22/75/98-9 (ALD+PDAT)	0.63	18.1	4.35	13.7
M22/94/98-19 (ALD+ACS+PDAT)	0.51	14.4	3.46	11.1

Strain	Lipid g/l	Yield lipid (% CDW)	Yield lipid (% used glucose)	Lipid mg/l/h
Control	0.71	24.6	4.09	15
M22/75-86 (ALD)	0.76	26.1	5.04	17
M22/96-1 (ACS)	0.70	24.2	4.53	15
M22/94-24 (ALD+ACS)	0.71	23.5	4.10	15
M22/75/98-9 (ALD+PDAT)	1.09	31.4	7.52	24
M22/94/98-19 (ALD+ACS+PDAT)	0.93	26.1	6.29	20

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol and total lipid concentrations and rates of production and triacylglycerol and total lipid yields from used glucose or per dry weight.

Example 26. Aerobic shake flask characterization of strains M22/75-86 (Ex. 15B), M22/96-1 (Ex. 17B), M22/94-24 (Ex. 16B), M22/75/98-9 (Ex. 19) and M22/94/98-19 (Ex. 20), in glucose medium with C/N ratio of 66

Transformants were separately cultivated in 50 ml of mould C/N 66 medium (pH 5.5, 20 g glucose, 1.0 g yeast extract, 2.5 g KH_2PO_4, 0.1 g $(NH_4)_2SO_4$, 10 mg $ZnSO_4$* $7H_2O$, 2 mg $CuSO_4$*$5H_2O$, 10 mg $MnSO_4$, 0.5 g $MgSO_4$* 7 H_2O, 0.1 g $CaCl_2$, 20 mg $FeCl_3$* $6H_2O$ per litre). Each flask (250 ml) was inoculated with 1 * 10^7 spores. The cultivations were maintained at a temperature of 25°C with shaking at 250 rpm. Samples for cell dry weight meas-

urement, lipid extraction, enzyme activity measurement and HPLC analysis were withdrawn periodically during cultivation. *Mucor circinelloides* wild type strain M22 was used as a control. Cell dry weight was determined as described in Example 25 and HPLC analysis was carried out as described in Example 22. Lipid extraction and total lipid and triacylglycerol measurements as described in Example 21.

After 93 hours cultivation, when 2-6 g/l glucose was left, transformants M22/75-86, M22/96-1, M22/94-24, M22/75+98-9 and M22/94+98-19 had produced 34, 30, 45, 186 and 214% more triacylglycerols with higher rate than the control strain. The transformants M22/75-86, M22/96-1, M22/94-24, M22/75+98-9 and M22/94+98-19 also had 63 (24), 68 (30), 62 (43), 229 (72) and 102 (104)% higher triacylglycerol yield on used glucose (on biomass) than the control strain. Additionally, the transformants produced more lipids (0.89-2.29 g/l) with higher rates (9.6-24.6 mg/l/h) and with higher yields on used glucose (6.90-17.68%) and on biomass (37.2-57.7%) than the control strain (0.71 g/l, 7.6 mg/l/h, 4.82% and 30.2%, respectively).

Table 6. Triacylglycerol (TAG) and total lipid concentrations (g/l), rates (mg/l/h) and yields (%) per biomass (CDW; cell dry weight) and used glucose after 93 hours cultivation in glucose medium with C/N ratio of 66.

Strain	TAG g/l	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	0.44	18.6	2.97	4.7
M22/75-86 (ALD)	0.59	23.0	4.84	6.4
M22/96-1 (ACS)	0.57	24.1	4.99	6.1
M22/94-24 (ALD+ACS)	0.64	26.6	4.82	6.9
M22/75/98-9 (ALD+PDAT)	1.26	31.9	9.76	13.6
M22/94/98-19 (ALD+ACS+PDAT)	1.38	37.9	9.00	14.8

Strain	Lipid g/l	Yield lipid (% CDW)	Yield lipid (% used glucose)	Lipid mg/l/h
Control	0.71	30.2	4.8	7.6
M22/75-86 (ALD)	0.96	37.2	7.8	10.3
M22/96-1 (ACS)	0.89	37.3	7.7	9.6
M22/94-24 (ALD+ACS)	0.91	37.6	6.9	9.8
M22/75/98-9 (ALD+PDAT)	2.29	57.7	17.7	24.6
M22/94/98-19 (ALD+ACS+PDAT)	2.00	55.1	13.1	21.5

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol and total lipid concentrations and rates of production and triacylglycerol and total lipid yields from used glucose or per dry weight with high C/N ratios.

Example 27. Microaerobic shake flask characterization of strains Y23/81-51 and Y23/81-66 (Ex. 3B), Y23/85-125 and Y23/85-128 (Ex. 7B), Y23/86-86 and Y23/86-92 (Ex. 5B), Y23/95-87 and Y23/95-109 (Ex. 6B) and Y23/85/95-4 (Ex. 9), in xylose medium with C/N ratio of 20

Transformants were separately cultivated in 50 ml of culture medium “xylose CN20” (pH 5.5, 20 g xylose, 0.3 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* $7H_2O$, 4.0 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was

inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 100 rpm. Samples for cell dry weight measurement, lipid extraction, enzyme activity measurement and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control.

Lipid extraction and triacylglycerol concentration measurements were carried out as described in Example 21. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22.

At the end of cultivation samples for acetaldehyde dehydrogenase and acetyl-CoA synthetase activity measurements were taken. Cells were harvested by centrifugation and washed once with 100 mM Tris-HCl pH 7.0. Washed cells were stored at -20°C. Frozen cells were melted and washed once with 100 mM Tris-HCl pH 7.0 and suspended in 100 mM Tris-HCl pH 7.0, 1 mM DTT buffer containing EDTA-free protease inhibitors (Roche, USA). Cell disruption was carried out with 0.5 mm diameter glass beads (Sigma Chemicals Co, USA) in a Fast Prep homogenizer (Thermo Scientific, USA). Cell debris was removed by centrifugation and supernatant was used in enzyme activity measurements. Acetaldehyde dehydrogenase and acetyl-CoA synthetase activity measurements were carried out with a Konelab Arena automatic analyzer (Thermo Scientific, Finland). The acetaldehyde dehydrogenase reaction mixture contained (final concentration) 50 mM potassium phosphate pH 7.0, 15 mM pyrazole, 0.4 mM DTT, 10 mM $MgCl_2$, 0.4 mM NADP and cell extract. The reaction was started with 0.1 mM acetaldehyde. The formation of NADPH was followed at 340 nm. One unit was defined as the amount of formation of 1 μmol of NADPH per min. The acetyl-CoA synthetase reaction mixture contained (final concentration) 100 mM Tris-HCl pH 7.5, 10 mM L-malate pH 7.5, 0.2 mM Coenzyme A, 8 mM ATP, 1 mM NAD, 10 mM $MgCl_2$, 3 U/ml malate dehydrogenase, 0.4 U/ml citrate synthase and cell extract. The reaction was started with 100 mM potassium acetate. The formation of NADH was followed at 340 nm. One unit was defined as the amount of formation of 1 μmol of NADH per min.

After 24 hours cultivation the transformants Y23/81, Y23/85, Y23/86, Y23/95 and Y23/85/95 produced 13- 31% more triacylglycerol with 5-38% higher yield on biomass than the control strain. The transformants Y23/81, Y23/86, Y23/95 and Y23/85/95 also had 4-12% higher yields on used xylose than the control strain. The transformants having ALD (ACS) encoding gene

expressed had 21.5 to 42.7 (1.5 to 1.9) times higher ALD (ACS) activity than the control strain.

Table 7. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yields (%) per biomass (CDW; cell dry weight) and used xylose after 24 hours microaerobic cultivation in xylose medium with C/N ratio of 20

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used xylose)	TAG mg/l/h
Control	0.16	4.33	3.46	6.8
Y23/81 (ALD)	0.20	5.98	3.61	8.2
Y23/85 (ALD+ACS)	0.18	4.54	3.46	7.3
Y23/86 (ACS)	0.21	5.33	3.86	8.9
Y23/95 (PDAT)	0.20	5.55	3.68	8.4
Y23/85/95 (ALD+ACS+PDAT)	0.20	5.55	3.72	8.2

Table 8. Relative acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS) activities in microaerobic cultivation in xylose with C/N ratio of 20 compared to the control strain ALD and ACS activities

Strain	ALD	ACS
Control	1	1
Y23/81 (ALD)	42.7	1.2
Y23/85 (ALD+ACS)	27.3	1.9
Y23/86 (ACS)	4.3	1.5
Y23/95 (PDAT)	2.3	1.2
Y23/85/95 (ALD+ACS+PDAT)	21.5	1.5

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production and triacylglycerol yields from used xylose or per dry weight with low C/N ratios. Additionally, the example shows that acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS) enzymes are expressed in active forms.

Example 28. Aerobic shake flask characterization of strains Y23/81-8 and 59 (Ex. 3B), Y23/85-119 and 128 (Ex. 7B), Y23/86-86 and 92 (Ex. 5B), Y23/95-99 and 109 (Ex. 6B), Y23/81/95-18 and 42 (Ex. 8) and Y23/85/95-4 and 68 (Ex. 9), in xylose medium with C/N ratio of 103

Transformants were separately cultivated in 50 ml of Yeast xylose culture medium IV (pH 5.5, 20 g xylose, 0.15 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* $7H_2O$, 0.45 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction and triacylglycerol measurements as described in Example 21.

After 52 hours cultivation the transformants Y23/81, Y23/85, Y23/86, Y23/95, Y23/81/95 and Y23/85/95 had produced 23, 19, 50, 52, 40 and 75% more triacylglycerol than the control strain. The transformants Y23/81, Y23/85, Y23/86, Y23/95, Y23/81/95 and Y23/85/95 had also 35, 47, 59, 60, 103 and 111 % higher triacylglycerol yield on biomass and 11, 11, 15, 28, 66 and 63% higher triacylglycerol yield on used xylose than the control strain. Additionally the transformants Y23/81, Y23/85, Y23/86, Y23/95, Y23/81/95 and Y23/85/95 had higher lipid concentration (2.40-2.90 g/l) and rate (46-56 mg/l/h) with higher yield on biomass (56.5-78.8%) than the control strain (2.35 g/l, 45 mg/l/h and 52.2%, respectively). The transformants Y23/81/95 and Y23/85/95 had also higher lipid yield on used xylose (29.0-31.6%) than the control strain (25.6%).

Table 9. Triacylglycerol (TAG) and total lipid concentrations (g/l), rates (mg/l/h) and yields (%) per biomass (CDW; cell dry weight) after 52 hours cultivation in xylose medium with C/N ratio of 103. Triacylglycerol yield (%) from used xylose is also indicated.

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used xylose)	TAG mg/l/h
Control	0.60	13.3	6.52	19.0
Y23/81 (ALD)	0.83	17.9	7.23	26.4
Y23/85 (ALD+ACS)	0.79	19.6	7.26	25.0
Y23/86 (ACS)	0.90	21.1	7.52	28.5
Y23/95 (PDAT)	0.91	21.3	8.37	28.8
Y23/81/95 (ALD+PDAT)	0.84	27.0	10.8	26.6
Y23/85/95 (ALD+ACS+PDAT)	1.05	28.0	10.7	33.3

Strain	Lipid (g/l)	Yield lipid (% CDW)	Lipid mg/l/h
Control	2.35	52.2	45
Y23/81 (ALD)	2.90	62.3	56
Y23/85 (ALD+ACS)	2.53	62.9	49
Y23/86 (ACS)	2.58	60.6	50
Y23/95 (PDAT)	2.40	56.5	46
Y23/81/95 (ALD+PDAT)	2.45	78.8	47
Y23/85/95 (ALD+ACS+PDAT)	2.85	76.0	55

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol and total lipid concentrations and rates of production and triacylglycerol and total lipid yields per dry weight and triacylglycerol yields from used xylose with high C/N ratios.

Example 29. Aerobic shake flask characterization of strains M22/75-80 (Ex. 15B), M22/96-6 (Ex. 17B), M22/98-16 (Ex. 18B), M22/94-16 (Ex. 16B), M22/75/98-7 (Ex. 19) and M22/94/98-22 (Ex. 20), in xylose medium with C/N ratio of 66

Transformants were separately cultivated in 50 ml of mould xylose C/N 66_medium (pH 5.5, 20 g xylose, 1.0 g yeast extract, 2.5 g KH_2PO_4, 0.1 g $(NH_4)_2SO_4$, 10 mg $ZnSO_4$* $7H_2O$, 2 mg $CuSO_4$*$5H_2O$, 10 mg MnSO4, 0.5 g $MgSO_4$* 7 H_2O, 0.1 g $CaCl_2$, 20 mg $FeCl_3$* $6H_2O$ per litre). Each flask (250 ml) was inoculated with 1 * 10^7 spores. The cultivations were maintained at a temperature of 25°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction, enzyme activity measurement and HPLC analysis were withdrawn periodically during cultivation. *Mucor circinelloides* wild type strain M22 was used as a control. Cell dry weight was determined as described in Example 25, and HPLC analysis was carried out as described in Example 22. Lipid extraction and total lipid and triacylglycerol measurements as described in Example 21.

After 143 hours cultivation the transformants M22/75-80, M22/96-6, M22/98-16, M22/75/98-7 and M22/94/98-22 had higher TAG concentration (0.33-0.48 g/l TAG) compared to the control strain (0.28 g/l TAG). The all transformants had also higher TAG yield (%) per biomass (13.71-17.78%) than the control strain (12.15%) and the transformants M22/96-6, M22/98-16, M22/75/98-7 and M22/94/98-22 had also higher TAG yield (%) per used xylose (4.83 - 6.23%) than the control strain (3.97%). Additionally the transformants M22/75/98-7 and M22/94/98-22 had higher lipid concentration (0.79-1.13 g/l), rate (9.2-10.8 mg/l/h) and lipid yields on biomass (48.9-55.6%) and on used xylose (17.1-18.3%) than the control strain (0.92 g/l, 6.4 mg/l/h, 40.0% and 13.0%, respectively).

Table 10. Triacylglycerol (TAG) and total lipid concentrations (g/l), rates (mg/l/h) and yields (%) per biomass (CDW; cell dry weight) and used xylose after 143 hours cultivation in xylose medium with C/N ratio of 66

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used xylose)	TAG mg/l/h
Control	0.28	12.2	3.97	1.9
M22/75-80 (ALD)	0.33	14.8	3.45	2.3
M22/96-6 (ACS)	0.33	15.3	5.40	2.3
M22/94-16 (ALD+ACS)	0.27	13.7	3.67	1.9
M22/98-16 (PDAT)	0.48	13.7	4.83	3.3
M22/75/98-7 (ALD+PDAT)	0.48	17.8	6.23	3.3
M22/94/98-22 (ALD+ACS+PDAT)	0.45	16.1	5.31	3.1

Strain	Lipid (g/l)	Yield lipid (% CDW)	Yield lipid (% used xylose)	Lipid mg/l/h
Control	0.92	40.0	13.0	6.4
M22/75-80 (ALD)	1.00	44.8	10.5	7.0
M22/96-6 (ACS)	0.90	41.5	14.7	6.3
M22/94-16 (ALD+ACS)	0.82	41.6	11.1	5.7
M22/98-16 (PDAT)	1.62	46.4	16.4	11.3
M22/75/98-7 (ALD+PDAT)	1.32	48.9	17.1	9.2
M22/94/98-22 (ALD+ACS+PDAT)	1.54	55.6	18.3	10.8

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol and total lipid concentrations and rates of production and triacylglycerol and total lipid yields from used xylose or per dry weight with high C/N ratios.

Example 30. Production of triacylglycerol or lipid by strains of *C. curvatus* modified by addition of genes encoding ALD and PDAT (Y23/81/95-18, Ex. 8) or ALD and PDAT and ACS (Y23/85/95-4 Ex. 9) in high cell density cultures grown on glucose with C/N ratio of 80

Transformants (Y23/85/95-4 and Y23/81/95-18) were separately cultivated in Multifors bioreactors (max. working volume 500 ml, Infors HT,

Switzerland) at pH 4.0, 30°C, in 500 ml medium containing 90 to 96 g glucose, 2.56 g $(NH_4)_2SO_4$, 1.2 g KH_2PO_4, 0.3 g $Na_2HPO_4 \cdot 2H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 6H_2O$, 5.26 mg citric acid$\cdot H_2O$, 5.26 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $MnSO_4 \cdot 4H_2O$, 0.5 mg $CoCl_2 \cdot 6H_2O$, 0.26 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $Na_2MoO_4 \cdot 2H_2O$, 1.4 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg H_3BO_4, 0.05 mg D-biotin, 1.0 mg CaPantothenate, 5.0 mg nicotinic acid, 25 mg myoinositol, 1.0 mg thiamine·HCl, 1.0 mg pyridoxine·HCl and 0.2 mg *p*-aminobenzoic acid per litre. The pH was maintained constant by addition of 1 M KOH or 1 M H_2PO_4. Cultures were agitated at 1000 rpm (2 Rushton turbine impellors) and aerated at 2 volumes air per volume culture per minute (vvm). Clerol FBA 3107 antifoaming agent (Cognis, Saint-Fargeau-Ponthierry Cedex France, 1 ml l^{-1}) was added to prevent foam accumulation. Bioreactors were inoculated to initial OD_{600} of 0.5 to 4.0 with cells grown in the same medium (substituting 1.5 g urea per litre for $(NH_4)_2SO_4$ and omitting the $CaCl_2 \cdot 6H_2O$) in 50 ml volumes in 250 ml flasks at 30°C with shaking at 200 rpm for 24 to 42 h. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as the control. For measurement of the yield of triacylglycerol on glucose or biomass, some control cultures contained 58 to 134 g glucose l^{-1}.

Lipid extraction and triacylglycerol concentration measurements were carried out as described in Example 21. Cell dry weight was determined by centrifuging 0.5 to 2.0 ml culture broth in pre-dried, pre-weighed 2 ml microfuge tubes. After washing twice with 1.8 ml distilled water, the cell pellet was dried at 100°C for 48 h and weighed after cooling in a dessicator. HPLC analyses were carried out as described in Example 22.

Table 11 shows that a transformant containing the genes for ALD and PDAT produced 23% more triacylglycerol than Y23, with a 24 % increase in the yield on glucose consumed when cells were cultivated to high cell density in bioreactor cultures. A transformant containing the genes for ALD, ACS and PDAT produced 15% more triacylglycerol than Y23, with a 12% increase in the yield on glucose consumed.

Table 11. Triacylglycerol produced in pH controlled bioreactor culture of Y23 and transformants of Y23 expressing ALD+PDAT or ALD+ACS+PDAT, with glucose as carbon source and C/N 80. Data is the average of 2 (transformants) or 4 to 9 (Y23) cultures ± standard error of the mean. Percentage increase is shown in parenthesis

Strain	TAG (g/l)	Yield TAG (% glucose consumed)
Y23	17.3 ± 0.3	18.8 ± 1.0
Y23/81/95-18 (ALD+PDAT)	21.3 ± 0.8 (23%)	23.3 ± 1.0 (24%)
Y23/85/95-4 (ALD+ACS+PDAT)	19.9 ± 0.1 (15%)	21.1 ± 0.1 (12%)

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and triacylglycerol yields from used glucose in high cell density cultures.

Example 31. Production of triacylglycerol or lipid by strains of *C. curvatus* modified by addition of genes encoding ALD and PDAT (Y23/81/95-18, Ex. 8) or ALD and PDAT and ACS (Y23/85/95-4, Ex. 9) in high cell density cultures grown on xylose with C/N ratio of 80

Transformants (Y23/85/95-4 and Y23/81/95-18) were separately cultivated in Multifors bioreactors as described in Example 30. The medium contained 92 to 118 g xylose per litre, instead of glucose. Bioreactors were inoculated to initial OD_{600} of 17 to 24 with cells grown in low nitrogen medium with glucose as carbon source in the Multifors bioreactors at 30°C, as described for lipid production in Example 30. Alternatively, some cultures of Y23 were inoculated with cells grown in flasks, as described in Example 30, to initial OD_{600} 0.2 to 0.5. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as the control.

Lipid extraction and total lipid and triacylglycerol concentration measurements were carried out as described in Example 21. Cell dry weight was determined as described in Example 30. HPLC analyses were carried out as described in Example 22.

Table 12 shows that a transformant containing the genes for ALD and PDAT produced 7% more triacylglycerol than Y23, with a 17% increase in the yield on xylose consumed and a 3% increase in the yield on biomass when cells were cultivated to high cell density in bioreactor cultures. A transformant containing the genes for ALD, ACS and PDAT produced only 1% more triacylglycerol than Y23, but showed 13% increase in the yield on xylose consumed and 4% increase in yield on biomass.

Table 12. Triacylglycerol produced in pH controlled bioreactor culture of Y23 and transformants of Y23 expressing ALD+PDAT or ALD+ACS+PDAT, with xylose as carbon source and C/N 80. Data is the average of 2 (transformants) or 4 (Y23) cultures, ± standard error of the mean. Percentage increase is shown in parenthesis.

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% xylose consumed)
Y23	17.9 ± 1.4	46.9 ± 4.0	17.2 ± 1.2
Y23/81/95-18 (ALD+PDAT)	19.1 ± 0.3 (7%)	48.4 ± 5.7 (3%)	20.2 ± 0.5 (17%)
Y23/85/95-4 (ALD+ACS+PDAT)	18.1 ± 0.3 (1%)	49.0 ± 3.4 (4%)	19.5 ± 0.0 (13%)

Table 13 shows that a transformant containing the genes for ALD and PDAT produced 10% more lipid than Y23, with a 21 % increase in the yield on xylose consumed and a 2% increase in the yield on biomass when cells were cultivated to high cell density in bioreactor cultures. A transformant containing the genes for ALD, ACS and PDAT produced 3% more lipid than Y23, with 16% higher yield on xylose consumed and a 3% increase in yield on biomass.

Table 13. Lipid produced in pH controlled bioreactor culture of Y23 and transformants of Y23 expressing ALD+PDAT or ALD+ACS+PDAT, with xylose as carbon source and C/N 80. Data is the average of 2 (transformants) or 4 (Y23) cultures, ± standard error of the mean. Percentage increase is shown in parenthesis.

Strain	Lipid (g/l)	Yield lipid (% CDW)	Yield lipid (% xylose consumed)
Y23	20.5 ± 1.3	55.9 ± 7.2	19.8 ± 2.5
Y23/81/95-18 (ALD+PDAT)	22.5 ± 0.6 (10%)	57.0 ± 1.9 (2%)	23.8 ± 0.8 (21%)
Y23/85/95-4 (ALD+ACS+PDAT)	21.2 ± 0.1 (3%)	57.5 ± 1.0 (3%)	22.9 ± 0.3 (16%)

This example shows that expression of *ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol and total lipid concentrations and triacylglycerol and total lipid yields from used xylose or per dry weight in high cell density cultures.

Example 32. Production of triacylglycerol or lipid by strain of *M. circinelloides* modified by addition of genes encoding ALD and PDAT and ACS (M22/94/98-19, Ex. 20) in pH controlled bioreactor cultures grown on glucose with C/N ratio of 60

Transformant (M22/94/98-19) was cultivated in Braun Biostat® CT bioreactors (2.5 max working volume, B. Braun Biotech International, Sartorius AG, Germany) at pH 5.0, 30°C, in 1.0 to 1.2 l medium containing 53 g glucose, 1.57 g $(NH_4)_2SO_4$, 2.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 6H_2O$, 5.26 mg citric acid$\cdot H_2O$, 5.26 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $MnSO_4 \cdot 4H_2O$, 0.5 mg $CoCl_2 \cdot 6H_2O$, 0.26 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $Na_2MoO_4 \cdot 2H_2O$, 1.4 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg H_3BO_4, 0.005 mg D-biotin, and 0.05 mg thiamine·HCl per litre. The pH was maintained constant by addition of 1 M KOH or 1 M H_2PO_4. Cultures were agitated at 600 rpm (2 Rushton turbine impellors) and aerated at 1 volume air per volume culture per minute (vvm). Polypropylene glycol (mixed molecular weights containing Fluka P1200, Fluka P2000 and Henkel Performance Chemicals Foamaster in a ratio of 4:4;1, 1 ml l^{-1}) was added to prevent foam accumulation. Bioreactors were inoculated to an initial biomass concentration of approximately 100 mg l^{-1} with mycelia grown in the same medium

with the following modifications: 15 g glucose l^{-1}, enough $(NH_4)_2SO_4$ to provide a C/N ratio of 16.2, and additionally 4.0 g agar l^{-1}. Pre-cultures were grown in 50 ml volumes in 250 ml flasks at 30°C with shaking at 200 rpm for 42 to 72 h. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. Biomass was separated from the culture supernatant by filtration under vacuum. *Mucor circinelloides* wild type strain M22 was used as the control.

Lipid extraction and total lipid and triacylglycerol concentration measurements were carried out as described in Example 21. Cell dry weight was determined as descriped in Example 25, except that disposable cleaning cloth (X-tra, 100% viscose household cleaning cloth, Inex Partners Oy, Helsinki) was used in vacuum filtration. HPLC analyses were carried out as described in Example 22.

Table 14 shows that a transformant containing the genes for ALD, ACS and PDAT produced 17% more triacylglycerol than Y23, with 8% increase in the yield on glucose consumed, when cells were cultivated in bioreactor cultures.

Table 14. Triacylglycerol produced in pH controlled bioreactor cultures of M22 and transformant of M22 expressing ALD+ACS+PDAT, with glucose as carbon source and C/N 60. Percentage increase is shown in parenthesis.

Strain	TAG (g/l)	Yield TAG (% glucose consumed)
M22	9.8	21.9
M22/94/98-19 (ALD+ACS+PDAT)	11.5 (17%)	23.6 (8%)

Table 15 shows that a transformant containing the genes for ALD, ACS and PDAT produced 44% more lipid than M22, with 55% higher yield on glucose consumed and 26% increase in yield of lipid on biomass, when cells were cultivated in bioreactor cultures.

Table 15. Lipid produced in pH controlled bioreactor cultures of M22 and transformant of M22 expressing ALD+ACS+PDAT, with glucose as carbon source and C/N 60. Percentage increase is shown in parenthesis.

Strain	Lipid (g/l)	Yield lipid (% CDW)	Yield lipid (% glucose consumed)
M22	10.4	59.4 ± 2.8	19.7
M22/94/98-19 (ALD+ACS+PDAT)	14.9 (44%)	75.4 ± 3.5 (26%)	30.5 (55%)

This example shows that expression of *ALD6, ACS2* and *PDAT* genes enhanced triacylglycerol and total lipid concentrations and triacylglycerol and total lipid yields from used glucose or total lipid yield per dry weight in high cell density cultures.

Example 33. Production of triacylglycerol or lipid by strain of *M. circinelloides* modified by addition of genes encoding ALD and PDAT and ACS (M22/94/98-19, Ex. 20) in pH controlled bioreactor cultures grown on xylose with C/N ratio of 60

Transformant (M22/94/98-19) was cultivated in Braun Biostat CT bioreactors as described in Example 32. The medium contained 44 to 56 g xylose per litre, instead of glucose cultures were supplemented with 1 g peptone per litre and the $(NH_4)_2SO_4$ concentration was reduced to 1.12 g per litre. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Mucor circinelloides* wild type strain M22 was used as the control.

Lipid extraction and total lipid and triacylglycerol concentration measurements were carried out as described in Example 21. Cell dry weight was determined as described in Example 32. HPLC analyses were carried out as described in Example 22.

Table 16 shows that a transformant containing genes for ALD, ACS and PDAT produced 11% more triacylglycerol than M22, with 10% increased yield on xylose consumed and 9% increased yield on biomass in pH controlled bioreactor cultures.

Table 16. Triacylglycerol produced in pH controlled bioreactor cultures of M22 and transformant of M22 expressing ALD+ACS+PDAT, with xylose as carbon source and C/N 60. Percentage increase is shown in parenthesis.

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% xylose consumed)
M22	5.6	46.5 ± 2.7	22.9
M22/94/98-19 (ALD+ACS+PDAT)	6.2 (11%)	50.9 ± 1.4 (9%)	25.1 (10%)

Table 17 shows that a transformant containing the genes for ALD and ACS and PDAT produced 24% more lipid than M22 and the yield of lipid on biomass was 22% higher than in M22, when mycelia were grown on xylose. The yield of lipid on xylose consumed was increased 18%.

Table 17. Lipid produced in pH controlled bioreactor culture of M22 and transformant of M22 expressing ALD+ACS+PDAT, with xylose as carbon source and C/N 60. Percentage increase is shown in parenthesis.

Strain	Lipid (g/l)	Yield lipid (% CDW)	Yield lipid (% xylose consumed)
M22	5.8	49.2 ± 4.6	24.1
M22/94/98-19 (ALD+ACS+PDAT)	7.1 (22%)	58.2 ± 0.6 (18%)	28.7 (19%)

This example shows that expression of *ALD6, ACS2* and *PDAT* genes enhanced triacylglycerol and total lipid concentrations and triacylglycerol and total lipid yields from used xylose or per dry weight in high cell density cultures.

Example 34. Construction of a plasmid containing a marker gene under the control of an endogenous promoter and terminator and a pyruvate decarboxylase (PDC) encoding gene under the control of an endogenous promoter and terminator

A pyruvate decarboxylase (PDC) encoding gene, such as *PDC1* from *S. cerevisiae* (SEQ ID NO:94) which encodes the amino acid sequence of SEQ ID NO:95 is codon optimised. The codon optimised PDC encoding gene

with flanking *Sbf*I restriction sites is digested with *Sbf*I and ligated to a plasmid containing an endogenous promoter and terminator, such as plasmid pKK77pre. The resulting plasmid which contains the PDC encoding gene under the control of the endogenous promoter and terminator will be linearised e.g. with *Bam*HI and ligated with a fragment containing the marker gene under the control of the endogenous promoter and terminator. Such fragment can be obtained e.g. by digesting a plasmid pKK67 with *Bam*HI and *Xmn*I. The resulting plasmid contains the PDC encoding gene under the control of the endogenous promoter and terminator and the marker gene under the control of the endogenous promoter and terminator.

Example 35. Generation of genetically modified strain with an integrated PDC together with ALD6 and/or ACS2 encoding genes and marker genes by transforming genetically modified strains with plasmid containing PDC encoding gene (Ex 34)

The plasmid containing the PDC encoding gene (Ex. 34) is restricted e.g. with *Not*I and *Psp*OMI, and the resulting linear DNA containing the PDC encoding gene under the control of the endogenous promoter and terminator and the marker gene under the control of the endogenous promoter and terminator is used to transform e.g. a genetically modified strain Y23/81-51 (Ex. 3B), Y23/86-92 (Ex. 5B) or Y23/85-128 (Ex. 7B) by electroporation or a genetically modified strain M22/75-86 (Ex. 15B), M22/96-1 (Ex. 17B) or M22/94-31 (Ex 16B) using the transformation method described in Example 15B. The transformed cells are screened e.g. for antibiotic resistance. Several transformed colonies are analysed at DNA level by PCR.

Example 36. Aerobic shake flask characterization of strains harbouring PDC together with ALD6 and/or ACS2 encoding genes and marker genes in glucose or xylose medium with different C/N ratios

Transformants are separately cultivated in 50 ml of culture medium such as described in Examples 22, 23, 24, 27 or 28. Each flask (250 ml) is inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations are maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis are withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 is used as a control. Cell dry weight is determined and HPLC analysis is carried out as described in Example 22. Lipid extraction

and triacylglycerol measurements as described in Example 21. Alternatively transformants are separately cultivated in 50 ml of culture medium such as described in Examples 25, 26 or 29. Each flask (250 ml) is inoculated with 1 * 10^7 spores. The cultivations are maintained at a temperature of 25°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis are withdrawn periodically during cultivation. *Mucor circinelloides* wild type strain M22 is used as a control. Cell dry weight is determied as described in Example 25 and HPLC analysis is carried out as described in Example 22. Lipid extraction and triacylglycerol measurements as described in Example 21.

The transformants harbouring PDC together with ALD6 and/or ACS2 encoding genes produce more triacylglycerol than the control strain. Additionally the transformants harbouring PDC together with ALD6 and/or ACS2 encoding gene have higher triacylglycerol yield on used carbon than the control strain.

Example 37A. Construction of a plasmid (pKK101) containing the G418 resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator and the *S. cerevisiae* PDC1 encoding gene under the control of the *CcTPI1* promoter and the *CcTEF1* terminator

The plasmid Y4_TPIp-PDC-TEFt (Geneart AG, Germany) contains a *S. cerevisiae* PDC1 (SEQ ID NO:95) encoding gene which has been codon optimized according to *Ustilago maydis* yeast codon usage (SEQ ID NO:96) with the *CcTPI1* promoter and the *CcTEF1* terminator. Plasmid Y4_TPIp-PDC-TEFt was digested with *Eco*RI. A 2893 bp fragment was gel isolated and ligated to a 5381 bp fragment obtained by digesting a plasmid designated pKK67 (Ex. 2B) with *Eco*RI. The resulting plasmid was designated as pKK101 (Fig. 12). The plasmid pKK101 contains a *S. cerevisiae* PDC1 encoding gene under the control of the *CcTPI1* promoter and the *CcTEF1* terminator and the *E. coli* G418 resistance gene under the control of the *CcTEF1* promoter and the *CcTPI1* terminator.

Example 37B. Generation of genetically modified strains with an integrated PDC encoding gene and G418 resistance gene by transforming wild-type *C. curvatus* and genetically modified strains Y23/81-66 (Ex. 3B), Y23/85-125 (Ex. 7B), Y23/86-86 (Ex. 5B), Y23/95-99 (Ex. 6B), Y23/81/95-18 (Ex. 8) and Y23/85/95-4 (Ex. 9) with digested plasmid pKK101 (Fig 12, Ex. 37A)

Plasmid pKK101 was restricted with *Sac*II and *Psp*OMI, and the resulting linear DNA was used to transform wild-type *C. curvatus* strain ATCC20509 designated as Y23 and genetically modified strains Y23/81-66, Y23/85-125, Y23/86-86, Y23/95-99, Y23/81/95-18 and Y23/85/95-4 by electroporation. The transformed cells were screened for G418 resistance. Several G418 resistance colonies were analysed at DNA level by PCR. The transformants originating from the transformation of wild-type *C. curvatus* strain ATCC20509 designated as Y23 and genetically modified strains Y23/81-66, Y23/85-125, Y23/86-86, Y23/95-99, Y23/81/95-18 and Y23/85/95-4 with *Sac*II and *Psp*OMI cut pKK101 and containing the *S. cerevisiae* PDC1 encoding gene under the control of the *CcTPI1* promoter and the *CcTEF1* terminator were designated as Y23/101-55, Y23/101-57, Y23/101-59, Y23/81/101-4, Y23/85/101-13, Y23/85/101-14, Y23/85/101-19, Y23/86/101-23, Y23/95/101-1, Y23/95/101-2, Y23/81/95/101-20, Y23/85/95/101-7 and Y23/85/95/101-8.

Example 38A. Construction of a plasmid (pKK102) containing the cerulenin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator and the *S. cerevisiae* PDC1 encoding gene under the control of the *McPGK1* promoter and the *McTEF1* terminator

The plasmid M22_PGKp-PDC-TEFt (Geneart AG, Germany) contains a *S. cerevisiae* PDC1 (SEQ ID NO:95) encoding gene which has been codon optimized according to *Rhizopus oryzae* filamentous fungus codon usage (SEQ ID NO:97) with the *McPGK1* promoter and the *McTEF1* terminator. Plasmid M22_PGKp-PDC-TEFt was digested with *Sal*I. A 3363 bp fragment was gel isolated and ligated to a 6239 bp fragment obtained by digesting a plasmid designated pKK92 (Ex. 14B) with *Sal*I. The resulting plasmid was designated as pKK102 (Fig. 13). The plasmid pKK102 contains a *S. cerevisiae* PDC1 encoding gene under the control of the *McPGK1* promoter and the *McTEF1* terminator and the *S. cerevisiae* cerulenin resistance gene under the control of the *McPGK1* promoter and the *McTPI1* terminator.

Example 38B. Generation of a genetically modified *Mucor circinelloides* (M22/94/102) with integrated ALD6, ACS1 and PDC1 engoding genes and hygromycin and cerulenin resistance genes by transforming genetically modified strain M22/94-12 (Ex. 16B) with digested plasmid pKK102 (Fig 13, Ex. 38A)

Plasmid pKK102 was restricted with *Sac*II and *Psp*OMI, and the resulting linear DNA was used to transform the genetically modified strain M22/94-12, using the transformation method described in Example 15B. The transformed cells were screened for cerulenin resistance. Several cerulenin resistant colonies were analysed at DNA level by PCR. The transformant originating from the transformation of the genetically modified strain M22/94-12 with *Sac*II and *Psp*OMI cut pKK102 and containing the *S. cerevisiae* PDC1 encoding gene under the control of the *McPGK1* promoter and the *McTEF1* terminator was designated as M22/94/102-31.

Example 39. Aerobic shake flask characterization of strains Y23/101-59 (Ex. 37B), Y23/81/101-4 (Ex. 37B), Y23/85/101-14 (Ex. 37B), Y23/86/101-23 (Ex. 37B), Y23/95/101-2 (Ex. 37B), Y23/81/95/101-20 (Ex. 37B) and Y23/85/95/101-9 (Ex. 37B) in glucose medium with C/N ratio of 153

Transformants were separately cultivated in 50 ml of Glucose-CN153 medium (pH 5.5, 30 g glucose, 0.15 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* $7H_2O$, 0.45 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction and triacylglycerol measurements as described in Example 21. Lipid extractions from the culture medium i.e. from the supernatant samples recovered after centrifugation of the cell lipid extraction samples or dry weight measurement samples were carried out as follows. To 150 µl of supernatant sample 150 µl of 0.9% NaCl and 150 – 1500 µl of chloroform:methanol (2:1) was added. The sample was vortexed 2 minutes and sample was incubated at room temperature at 30 min. After incubation sample was centrifuged 10 000

rpm for 3 min at RT. The lower phase was recovered into microfuge tubes, dried and redissolved in 1.5 ml of chloroform:methanol (2:1) and stored at -20°C prior triacylglycerol measurements as described in Example 21. Alternatively, dried lipid pellet was redissolved directly in isopropanol and triacylglycerol was measured as described in Example 21.

After 47 hours cultivation (Table 18), when 17 to 18 g/l glucose was left, Y23/101-59, Y23/85/101-14, Y23/86/101-23, Y23/95/101-2, Y23/81/95/101-20 and Y23/85/95/101-8 transformants produced 52, 78, 9, 27, 32 and 48% more triacylglycerol with higher rate, respectively, than the control strain in glucose medium. Also triacylglycerol yields on biomass and per used glucose were 33 to 83% and 35 to 85% higher in transformants Y23/101-59, Y23/85/101-14, Y23/86/101-23, Y23/95/101-2, Y23/81/95/101-20 and Y23/85/95/101-8 than the control strain, respectively.

Table 18. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yield (%) per biomass (CDW) and used glucose in the yeast cells after 47 hours cultivation in glucose medium with C/N ratio of 153

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	1.30	22.5	10.9	28
Y23/101-59 (PDC)	1.98	37.1	16.3	42
Y23/85/101-14 (PDC+ALD+ACS)	2.31	41.1	20.2	49
Y23/86/101-23 (PDC+ACS)	1.42	29.8	15.4	30
Y23/95/101-2 (PDC+PDAT)	1.65	30.9	14.7	35
Y23/81/95/101-20 (PDC+ALD+PDAT)	1.72	36.4	16.4	37
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	1.93	36.6	18.1	41

After 94 hours cultivation (Table 19A), when 6 to 11 g/l glucose was left, Y23/101-59, Y23/85/101-14, Y23/81/95/101-20 and Y23/85/95/101-8 transformants produced 5, 27, 8 and 12% more triacylglycerol with higher rate, respectively, than the control strain in glucose medium. Also triacylglycerol yields on biomass and per used glucose were 9 to 33% and 7 to 38% higher in transformants Y23/101-59, Y23/81/101-4, Y23/85/101-14, Y23/86/101-23,

Y23/95/101-2, Y23/81/95/101-20 and Y23/85/95/101-8 than the control strain, respectively. Additionally, triacylglycerol concentration in the culture medium in the cultivations with the transformants Y23/81/101-4 and Y23/86/101-23 was 525 and 350% higher, respectively, than in the cultivation with the control strain (Table 19B). Additionally, the total triacylglycerol yields per used glucose calculated from the intracellular triacylglycerol concentration and the triacylglycerol concentration detected from the culture medium were 13 to 38% higher with the transformants than with the control strain.

Table 19A. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yield (%) per biomass (CDW) and used glucose in the yeast cells after 94 hours cultivation in glucose medium with C/N ratio of 153

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	4.06	43.4	17.4	43
Y23/101-59 (PDC)	4.28	51.6	19.9	46
Y23/81/101-4 (PDC+ALD	3.39	55.7	18.6	36
Y23/85/101-14 (PDC+ALD+ACS)	5.17	57.9	22.7	55
Y23/86/101-23 (PDC+ACS)	4.00	53,4	21.1	43
Y23/95/101-2 (PDC+PDAT)	3.50	47.5	20.1	37
Y23/81/95/101-20 (PDC+ALD+PDAT)	4.38	62.0	24.0	47
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	4.55	54.4	21.5	48

Table 19B. Triacylglycerol (TAG) concentration (g/l) in the culture medium and calculated total TAG concentration (g/l), rate (mg/l/h) and yield (%) per used glucose in cultivation after 94 hours cultivation in glucose medium with C/N ratio of 153

Strain	TAG (g/l)	Total TAG (g/l)	Yield total TAG (% used glucose)	total TAG mg/l/h
Control	0.04	4.10	17.6	44
Y23/101-59 (PDC)	0.01	4.29	20.0	46
Y23/81/101-4 (PDC+ALD	0.25	3.63	19.9	39
Y23/85/101-14 (PDC+ALD+ACS)	0.03	5.20	22.8	55
Y23/86/101-23 (PDC+ACS)	0.18	4.18	22.1	44
Y23/95/101-2 (PDC+PDAT)	0.01	3.51	20.2	37
Y23/81/95/101-20 (PDC+ALD+PDAT)	0.03	4.42	24.2	47
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	0.04	4.6	21.7	49

This example shows that expression of *PDC1, ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production and triacylglycerol yields from used glucose or per dry weight in the yeast cell and/or in the culture medium with high C/N ratio in different stages of the cultivation.

Example 40. Aerobic shake flask characterization of strains Y23/101-55 (Ex. 37B), Y23/81/101-4 (Ex. 37B), Y23/85/101-19 (Ex. 37B), Y23/86/101-23 (Ex. 37B), Y23/95/101-2 (Ex. 37B) and Y23/85/95/101-7 (Ex. 37B) in glucose medium with C/N ratio of 28

Transformants were separately cultivated in 50 ml of Glucose-CN28 medium (pH 5.5, 30 g glucose, 0.3 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* 7H_2O, 4.0 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus glucose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis

were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction from the yeast cells and triacylglycerol measurements were carried out as described in Example 21 and lipid extraction from the culture medium as described in Example 39.

After 46 hours cultivation (Table 20A), when 12 to 14 g/l glucose was left, Y23/101-55, Y23/81/101-4, Y23/85/101-19, Y23/86/101-23, Y23/95/101-2 and Y23/85/95/101-7 transformants produced 18, 17, 20, 24, 7 and 17% more triacylglycerol with higher rate, respectively, than the control strain in glucose medium. Also triacylglycerol yields on biomass and per used glucose were 10 to 35% and 7 to 26% higher with the transformants than the control strain, respectively. After 72 and 94 hours cultivation triacylglycerol concentration in the culture medium with the transformants Y23/81/101-4 and Y23/86/101-23 was 195 and 56% and 35 and 30% higher, respectively, than in the cultivations with the control strain (Table 20B). Additionally, yield of triacylglycerol detected in culture medium per used glucose was 57 to 206% and 36 to 56% higher with the transformants Y23/81/101-4 and Y23/86/101-23 than with the control after 72 and 94 hours cultivation, respectively.

Table 20A. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yield (%) per biomass (CDW) and used glucose in the yeast cells after 46 hours cultivation in glucose medium with C/N ratio of 28

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used glucose)	TAG mg/l/h
Control	3.10	46.6	20.6	67
Y23/101-55 (PDC)	3.67	51.2	24.4	80
Y23/81/101-4 (PDC+ALD	3.64	53.5	22.1	79
Y23/85/101-19 (PDC+ALD+ACS)	3.71	53.8	23.2	81
Y23/86/101-23 (PDC+ACS)	3.84	63.1	26.0	83
Y23/95/101-2 (PDC+PDAT)	3.31	54.7	23.3	72
Y23/85/95/101-7 (PDC+ALD+ACS+PDAT)	3.64	58.5	26.3	79

Table 20B. Triacylglycerol (TAG) concentration (g/l) and yield (%) per used glucose in the culture medium after 72 and 94 hours cultivation in glucose medium with C/N ratio of 28

	72 h		94 h	
Strain	TAG (g/l)	Yield TAG % (/used glucose)	TAG (g/l)	Yield TAG (% used glucose)
Control	0.85	3.19	2.79	9.84
Y23/81/101-4 (PDC+ALD)	2.51	9.75	4.35	15.4
Y23/86/101-23 (PDC+ACS)	1.15	5.00	3.62	13.4

This example shows that expression of *PDC1, ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production and triacylglycerol yields from used glucose or per dry weight in the yeast cell and/ or in the culture medium with low C/N ratio at different stages of the cultivation.

Example 41. Aerobic shake flask characterization of strains Y23/101-57 (Ex. 37B), Y23/81/101-4 (Ex. 37B), Y23/85/101-13 (Ex. 37B), Y23/86/101-23 (Ex. 37B), Y23/95/101-1 (Ex. 37B), Y23/81/95/101-20 (Ex. 37B) and Y23/85/95/101-8 (Ex. 37B) in xylose medium with C/N ratio of 103

Transformants were separately cultivated in 50 ml of Yeast culture medium IV (pH 5.5, 20 g xylose, 0.15 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* 7H_2O, 0.45 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus xylose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction from the yeast cells and triacylglycerol measurements were carried out as described in Example 21 and lipid extraction from the culture medium as described in Example 39.

After 47 hours cultivation (Table 21) Y23/101-57, Y23/81/101-4, Y23/85/101-13 and Y23/95/101-1 transformants produced 12, 18, 4 and 5% more triacylglycerol with higher rate, respectively, than the control strain in xylose medium. Also triacylglycerol yields on biomass and per used xylose were up to 28% and 16% higher with the transformants Y23/101-57, Y23/81/101-4, Y23/85/101-13, Y23/86/101-23 and Y23/95/101-1 than the control strain, respectively. Also total lipid concentration, rate and yields on biomass and per used xylose were 8 to 29%, 8 to 29%, 19 to 44% and 11 to 33% higher with the transformants than the control strain, respectively, after 47 hours cultivation in xylose medium.

Table 21. Triacylglycerol (TAG) and total lipid concentrations (g/l), rates (mg/l/h) and yields (%) per biomass (CDW) and used xylose in the yeast cells after 47 hours cultivation in xylose medium with C/N ratio of 103

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used xylose)	TAG mg/l/h
Control	1.90	38.5	16.0	41
Y23/101-57 (PDC)	2.12	44.7	18.0	45
Y23/81/101-4 (PDC+ALD	2.24	49.2	18.6	48
Y23/85/101-13 (PDC+ALD+ACS)	1.98	44.3	18.1	42
Y23/86/101-23 (PDC+ACS)	1.85	47.7	18.3	39
Y23/95/101-1 (PDC+PDAT)	2.00	38.5	16.5	43

Strain	Lipid (g/l)	Yield lipid (% CDW)	Yield lipid (% used xylose)	Lipid mg/l/h
Control	2.40	48.5	20.2	51
Y23/101-57 (PDC)	2.75	57.9	23.3	59
Y23/81/101-4 (PDC+ALD	2.70	59.3	22.4	57
Y23/85/101-13 (PDC+ALD+ACS)	2.60	58.1	23.7	55
Y23/86/101-23 (PDC+ACS)	2.70	69.7	26.8	57
Y23/95/101-1 (PDC+PDAT)	3.10	59.6	25.6	66

After 94 hours cultivation (Table 22A) Y23/101-57, Y23/81/101-4, Y23/85/101-13, Y23/86/101-23, Y23/95/101-1, Y23/81/95/101-20 and

Y23/85/95/101-8 transformants produced 7, 9, 8, 7, 6, 6 and 4% more triacylglycerol with higher rate, respectively, than the control strain in xylose medium. Also triacylglycerol yields on biomass and per used xylose were 4 to 25% and 5 to 17% higher with the transformants than the control strain, respectively. Triacylglycerol concentration in the culture medium with the transformants Y23/81/101-4 and Y23/86/101-23 was 222 and 156% higher, respectively, than in the cultivations with the control strain (Table 22B). Additionally, the total triacylglycerol yields per used xylose calculated from the intracellular triacylglycerol concentration and the triacylglycerol concentration detected from the culture medium were 7 to 17% higher with the transformants than with the control strain.

Table 22A. Triacylglycerol (TAG) concentration (g/l), rate (mg/l/h) and yield (%) per biomass (CDW) and used xylose in the yeast cells after 94 hours cultivation in xylose medium with C/N ratio of 103

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used xylose)	TAG mg/l/h
Control	4.50	72.0	23.9	48
Y23/101-57 (PDC)	4.82	79.4	25.6	51
Y23/81/101-4 (PDC+ALD	4.92	84.2	26.1	52
Y23/85/101-13 (PDC+ALD+ACS)	4.85	75.1	25.7	52
Y23/86/101-23 (PDC+ACS)	4.82	88.5	25.6	51
Y23/95/101-1 (PDC+PDAT)	4.79	79.8	25.7	51
Y23/81/95/101-20 (PDC+ALD+PDAT)	4.78	89.7	27.9	51
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	4.66	80.4	25.2	50

Table 22B. Triacylglycerol (TAG) concentration (g/l) in the culture medium and calculated total TAG concentration (g/l), rate (mg/l/h) and yield (%) per used xylose in cultivation after 94 hours cultivation in xylose medium with C/N ratio of 103

Strain	TAG (g/l)	Total TAG (g/l)	Yield total TAG (% used xylose)	total TAG mg/l/h
Control	0.09	4.59	24.3	49
Y23/101-57 (PDC)	0.07	4.89	26.0	52
Y23/81/101-4 (PDC+ALD	0.29	5.22	27.7	55
Y23/85/101-13 (PDC+ALD+ACS)	0.10	4.94	26.2	53
Y23/86/101-23 (PDC+ACS)	0.23	5.05	26.8	54
Y23/95/101-1 (PDC+PDAT)	0.07	4.86	26.1	52
Y23/81/95/101-20 (PDC+ALD+PDAT)	0.09	4.86	28.4	52
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	0.10	4.76	25.7	51

This example shows that expression of *PDC1, ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol and total lipid concentrations and rates of production and triacylglycerol and total lipid yields from used xylose or per dry weight in the yeast cell and/ or in the culture medium with high C/N ratio in different stages of the cultivation.

Example 42. Aerobic shake flask characterization of strains Y23/101-57 (Ex. 37B), Y23/85/101-13 (Ex. 37B) and Y23/81/95/101-20 (Ex. 37B) in xylose medium with C/N ratio of 20

Transformants were separately cultivated in 50 ml of Xylose-CN20 medium (pH 5.5, 20 g xylose, 0.3 g $(NH_4)_2SO_4$, 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4 * 2 H_2O, 1.5 g $MgSO_4$* 7H_2O, 4.0 g Yeast extract, 51 mg $CaCl_2$, 8 mg $FeCl_3$* 6 H_2O and 0.1 mg $ZnSO_4$* 7 H_2O per litre). Each flask (250 ml) was inoculated to an OD_{600} of 0.3 with cells grown on yeast peptone plus xylose plates. The cultivations were maintained at a temperature of 30°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type

strain Y23 was used as a control. Cell dry weight was determined and HPLC analysis was carried out as described in Example 22. Lipid extraction from the yeast cells and triacylglycerol measurements were carried out as described in Example 21 and lipid extraction from the culture medium as described in Example 39.

After 94 hours cultivation (Table 23A) transformants Y23/101-57 and Y23/85/101-13 produced 9 and 5% more triacylglycerol with higher rate, respectively, than the control strain in xylose medium. Also triacylglycerol yields on biomass and per used xylose were 2 to 10% and 4 to 8% higher with the transformants than the control strain, respectively. Triacylglycerol concentration in the culture medium with the transformants Y23/101-57, Y23/85/101-13 and Y23/81/95/101-20 was 163, 163 and 188% higher, respectively, than in the cultivations with the control strain (Table 21B). Additionally, the total triacylglycerol yields per used xylose calculated from the intracellular triacylglycerol concentration and the triacylglycerol concentration detected from the culture medium were 4 to 12% higher with the transformants than with the control strain.

Table 23A. Triacylglycerol (TAG) concentration (g/l) and yield (%) per biomass (CDW) and used xylose in the yeast cells after 94 hours cultivation in xylose medium with C/N ratio of 20

Strain	TAG (g/l)	Yield TAG (% CDW)	Yield TAG (% used xylose)
Control	4.21	68.7	21.1
Y23/101-57 (PDC)	4.57	75.8	22.8
Y23/85/101-13 (PDC+ALD+ACS)	4.41	69.9	22.0

Table 23B. Triacylglycerol (TAG) concentration (g/l) in the culture medium and calculated total TAG concentration (g/l), rate (mg/l/h) and yield (%) per used xylose in cultivation after 94 hours cultivation in xylose medium with C/N ratio of 20

Strain	TAG (g/l)	Total TAG (g/l)	Yield total TAG (% used xylose)	total TAG mg/l/h
Control	0.08	4.28	21.4	46
Y23/101-57 (PDC)	0.21	4.78	23.9	51
Y23/85/101-13 (PDC+ALD+ACS)	0.21	4.61	23.1	49
Y23/81/95/101-20 (PDC+ALD+PDAT)	0.23	4.45	22.3	47

This example shows that expression of *PDC1, ALD6, ACS2* and *PDAT* genes in different combinations enhanced triacylglycerol concentrations and rates of production and triacylglycerol yields from used xylose or per dry weight in the yeast cell and/or in the culture medium with low C/N ratio.

Example 43. Aerobic shake flask characterization of strains M22/94-16 (Ex. 16B) and M22/94/102-31 (Ex. 38B), in xylose medium with C/N ratio of 21

Transformants were separately cultivated in 50 ml of mould C/N 21 medium (pH 5.5, 10 g glucose, 1.4 g yeast extract, 2.5 g KH_2PO_4, 0.3 g $(NH_4)_2SO_4$, 10 mg $ZnSO_4$* $7H_2O$, 2 mg $CuSO_4$*$5H_2O$, 10 mg $MnSO_4$, 0.5 g $MgSO_4$* 7 H_2O, 0.1 g $CaCl_2$, 20 mg $FeCl_3$* $6H_2O$ per litre). Each flask (250 ml) was inoculated with 1 * 10^7 spores. The cultivations were maintained at a temperature of 28°C with shaking at 250 rpm. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Mucor circinelloides* wild type strain M22 was used as a control. Cell dry weight was determined as described in Example 25 and HPLC analysis was carried out as described in Example 22. Lipid extraction and total lipid and triacylglycerol measurements as described in Example 21.

After 96 hours cultivation (Table 24) transformants M22/94-16 and M22/94/102-31 produced 18 and 30% more triacylglycerol with higher rate, respectively, than the control strain in xylose medium. Also triacylglycerol yields

on biomass and per used xylose were 10 to 34% and 1 to 24% higher with the transformants than the control strain, respectively. Also total lipid concentration and yield on biomass were 15 to 16% and 6 to 33% higher in the transformants than in the control.

Table 24. Triacylglycerol (TAG) and total lipid concentrations (g/l), rates (mg/l/h) and yields (%) per biomass (CDW; cell dry weight) and used xylose after 96 hours cultivation in xylose medium with C/N ratio of 21

Strain	TAG g/l	Yield TAG (% CDW)	Yield TAG (% used xylose	TAG mg/l/h
Control	0.60	34.9	13.9	6
M22/94-16 (ALD+ACS)	0.71	38.4	14.0	7
M22/94/102-31 (PDC+ALD+ACS)	0.78	53.2	17.2	8

Strain	Lipid g/l	Yield lipid (% CDW)	Yield lipid (% used xylose	Lipid mg/l/h
Control	0.81	47.6	18.9	8
M22/94-16 (ALD+ACS)	0.94	50.5	18.5	10
M22/94/102-31 (PDC+ALD+ACS)	0.93	63.5	20.5	10

This example shows that expression of *PDC1, ALD6* and *ACS2* genes in different combinations enhanced triacylglycerol and total lipid concentrations and rates of production and triacylglycerol and total lipid yields from used xylose or per dry weight in the yeast cell culture mewith low C/N ratio.

Example 44. Production of triacylglycerol or lipid by strains of *C. curvatus* modified by addition of genes encoding PDC and ALD and PDAT and ACS (Y23/85/95/101-8, Ex. 37B) in high cell density cultures grown on glucose with C/N ratio of 28

Transformant (Y23/85/95/101-8) was cultivated in Multifors bioreactors (max. working volume 500 ml, Infors HT, Switzerland) at pH 4.0, 30°C, in 500 ml medium containing 90 to 96 g glucose, 6.74 g $(NH_4)_2SO_4$, 1.2 g

KH_2PO_4, 0.3 g $Na_2HPO_4 \cdot 2H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 6H_2O$, 5.26 mg citric acid$\cdot H_2O$, 5.26 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $MnSO_4 \cdot 4H_2O$, 0.5 mg $CoCl_2 \cdot 6H_2O$, 0.26 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $Na_2MoO_4 \cdot 2H_2O$, 1.4 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg H_3BO_4, 0.05 mg D-biotin, 1.0 mg CaPantothenate, 5.0 mg nicotinic acid, 25 mg myoinositol, 1.0 mg thiamine·HCl, 1.0 mg pyridoxine·HCl and 0.2 mg *p*-aminobenzoic acid per litre. The pH was maintained constant by addition of 1 M KOH or 1 M H_2PO_4. Cultures were agitated at 1000 rpm (2 Rushton turbine impellors) and aerated at 2 volumes air per volume culture per minute (vvm). Clerol FBA 3107 antifoaming agent (Cognis, Saint-Fargeau-Ponthierry Cedex France, 1 ml l^{-1}) was added to prevent foam accumulation. Bioreactors were inoculated to initial OD_{600} of 0.5 to 4.0 with cells grown in the same medium (substituting 1.5 g urea per litre for $(NH_4)_2SO_4$ and omitting the $CaCl_2 \cdot 6H_2O$) in 50 ml volumes in 250 ml flasks at 30°C with shaking at 200 rpm for 24 to 42 h. Samples for cell dry weight measurement, lipid extraction and HPLC analysis were withdrawn periodically during cultivation. *Cryptococcus curvatus* wild type strain Y23 was used as the control.

Lipid extraction and triacylglycerol concentration measurements were carried out as described in Example 21. Lipid extraction from the culture medium was carried out as described in Example 39. Cell dry weight was determined as described in Example 30. HPLC analyses were carried out as described in Example 22.

Table 25A shows that a transformant containing the genes PDC, ALD, ACS and PDAT produced 58% more triacylglycerol than Y23, with a 67% and 185% increase in the yields on glucose consumed and on biomass, respectively. Additionally, the transformant containing the genes PDC, ALD, ACS and PDAT produced 20% more triacylglycerol than Y23, with a 111% increase in the yield on glucose consumed in the culture medium (Table 25B).

Table 25A. Triacylglycerol produced in pH controlled bioreactor culture of Y23 and transformant of Y23 expressing PDC+ALD+ACS+PDAT, with glucose as carbon source and C/N 28. Data is the average of 2 cultures ± standard error of the mean. Percentage increase is shown in parenthesis.

Strain	TAG (g/l)	Yield TAG (% glucose consumed)	Yield TAG (% per CDW)
Y23	1.30 ± 0.06	1.22 ± 0.01	2.67 ± 0.14
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	2.05 ± 0.34 (+58%)	2.04 ± 0.22 (+67%)	4.95 ± 0.33 (+185%)

Table 25B. Triacylglycerol produced in the culture medium in pH controlled bioreactor culture of Y23 and transformant of Y23 expressing PDC+ALD+ACS+PDAT, with glucose as carbon source and C/N 28. Data is the average of 2 cultures ± standard error of the mean. Percentage increase is shown in parenthesis.

Strain	TAG (g/l)	Yield TAG (% glucose consumed)
Y23	0.15 ± 0.04	0.18 ± 0.03
Y23/85/95/101-8 (PDC+ALD+ACS+PDAT)	0.18 ± 0.00 (+20%)	0.38 ± 0.07 (+111%)

This example shows that expression of *PDC1, ALD6, ACS1* and *PDAT* enhanced triacylglycerol concentrations and triacylglycerol yields from used glucose or per dry weight in high cell density cultures in the yeast cell and/ or in the culture medium.

Sequences used:

SEQ ID NOs: 1 and 2 correspond to primers YeastTEF1 and YeastTEF4, respectively, used to isolate genomic fragment of the *C. curvatus TEF* gene.

SEQ ID NOs: 3 and 4 correspond to primers PCR linker I and PCR linker II, respectively, used in chromosome walk experiments.

SEQ ID NOs: 5, 6, 7 and 8 correspond to primers CC_TEF2, CC_TEF1, CC_TEF6 and CC_TEF5 respectively, used to isolate genomic

fragments of the *C. curvatus* TEF promoter region in chromosome walk experiments.

SEQ ID NOs: 9 and 10 correspond to primers CC_TEF10 and CC_TEF11, respectively, used to isolate promoter of the *C. curvatus TEF* gene.

SEQ ID NOs: 11 and 12 correspond to primers CC_TEF3 and CC_TEF4, respectively, used to isolate genomic fragment of the *C. curvatus* TEF terminator region in chromosome walk experiments.

SEQ ID NOs: 13 and 14 correspond to primers CC_TEF7 and CC_TEF8, respectively, used to isolate terminator of the *C. curvatus TEF* gene.

SEQ ID NOs: 15 and 16 correspond to primers Yeast TPI5 and Yeast TPI8, respectively, used to isolate genomic fragment of the *C. curvatus TPI* gene.

SEQ ID NOs: 17 and 18 correspond to primers CC_TPI2 and CC_TPI1, respectively, used to isolate genomic fragment of the *C. curvatus* TPI promoter region in chromosome walk experiments.

SEQ ID NOs: 19 and 20 correspond to primers CC_TPI7 and CC_TPI9, respectively, used to isolate promoter of the *C. curvatus TPI* gene.

SEQ ID NOs: 21 and 22 correspond to primers CC_TPI4 and CC_TPI3, respectively, used to isolate genomic fragment of the *C. curvatus* TPI terminator region in chromosome walk experiments.

SEQ ID NOs: 23 and 24 correspond to primers CC_TPI5 and CC_TPI6, respectively, used to isolate terminator of the *C. curvatus TPI* gene.

SEQ ID NOs: 25 and 26 correspond to primers YeastENO5 and YeastENO10, respectively, used to isolate genomic fragment of the *C. curvatus ENO* gene.

SEQ ID NOs: 27, 28, 29 and 30 correspond to primers CC_ENO2, CC_ENO1, CC_ENO5 and CC_ENO6, respectively, used to isolate genomic fragments of the *C. curvatus* ENO promoter region in chromosome walk experiments.

SEQ ID NOs: 31 and 32 correspond to primers CC_ENO9 and CC_ENO10, respectively, used to isolate promoter of the *C. curvatus ENO* gene.

SEQ ID NOs: 33 and 34 correspond to primers CC_ENO4 and CC_ENO3, respectively, used to isolate genomic fragment of the *C. curvatus* ENO terminator region in chromosome walk experiments.

SEQ ID NOs: 35 and 36 correspond to primers CC_ENO7 and CC_ENO8, respectively, used to isolate terminator of the *C. curvatus ENO* gene.

SEQ ID NOs: 37 and 38 correspond to primers CC_GPD3 and CC_GPD4, respectively, used to isolate genomic fragment of the *C. curvatus* GPD terminator region in chromosome walk experiments.

SEQ ID NOs: 39 and 40 correspond to primers CC_GPD6 and CC_GPD7, respectively, used to isolate terminator of the *C. curvatus GPD* gene.

SEQ ID NOs: 41 and 42 correspond to primers Hph 5 and Hph 3, respectively, used to isolate *E. coli* hygromycin gene.

SEQ ID NOs: 43 and 44 correspond to primers Kan 5 and Kan 3, respectively, used to isolate *E. coli* G418 resistance gene.

SEQ ID NOs: 45 and 46 correspond to primers CERR 5 and CERR 3, respectively, used to isolate *S. cerevisiae* cerulenin resistance gene.

SEQ ID NO: 47 corresponds to the amino acid sequence of the *S. cerevisiae ALD6* gene, with GenBank accession number AAB68304 (version number AAB68304.1).

SEQ ID NO: 48 corresponds to *S. cerevisiae* ALD6 protein encoding DNA codon optimized according to *Ustilago maydis* –fungus codon usage.

SEQ ID NO: 49 corresponds to *S. cerevisiae* ALD6 protein encoding DNA codon optimized according to *Rhizopus oryzae* –filamentous fungus codon usage.

SEQ ID NO: 50 corresponds to the amino acid sequence of the *S. cerevisiae ACS2* gene, with GenBank accession number CAA97725 (version number CAA97725.1.

SEQ ID NO: 51 corresponds to *S. cerevisiae* ACS2 protein encoding DNA codon optimized according to *Ustilago maydis* –fungus codon usage.

SEQ ID NO: 52 corresponds to the amino acid sequence of the *Rhizopus oryzae PDAT* gene, encoded by gene with locus number RO3G_07851.3 in Broad Institute *Rhizopus oryzae* database.

SEQ ID NO: 53 corresponds to *Rhizopus oryzae* PDAT protein encoding DNA codon optimized according to *Ustilago maydis* –fungus codon usage.

SEQ ID NOs: 54 and 55 correspond to primers Mould TPI1 and mould TPI3, respectively, used to isolate genomic fragment of the *Mucor circinelloides TPI* gene.

SEQ ID NOs: 56 and 57 correspond to primers MC_TPI2 and MC_TPI1, respectively, used to isolate genomic fragment of the *Mucor circinelloides* TPI promoter region in chromosome walk experiments.

SEQ ID NOs: 58 and 59 correspond to primers MC_TPI7 and MC_TPI8, respectively, used to clone promoter of the *Mucor circinelloides TPI* gene.

SEQ ID NOs: 60 and 61 correspond to primers MC_TPI4 and MC_TPI3, respectively, used to isolate genomic fragment of the *Mucor circinelloides* TPI terminator region in chromosome walk experiments.

SEQ ID NOs: 62 and 63 correspond to primers MC_TPI5 and MC_TPI6, respectively, used to clone terminator of the *Mucor circinelloides TPI* gene.

SEQ ID NOs: 64 and 65 correspond to primers Mould TEF1 and Mould TEF4, respectively, used to isolate genomic fragment of the *Mucor circinelloides TEF* gene.

SEQ ID NOs: 66, 67, 68 and 69 correspond to primers MC_TEF2, MC_TEF1, MC_TEF6 and MC_TEF5, respectively, used to isolate genomic fragments of the *Mucor circinelloides* TEF promoter region in chromosome walk experiments.

SEQ ID NOs: 70 and 71 correspond to primers MC_TEF9 and MC_TEF10, respectively, used to clone promoter of the *Mucor circinelloides TEF* gene.

SEQ ID NOs: 72, 73, 74 and 75 correspond to primers MC_TEF4, MC_TEF3, MC_TEF8 and MC_TEF7, respectively, used to isolate genomic fragments of the *Mucor circinelloides* TEF terminator region in chromosome walk experiments.

SEQ ID NOs: 76 and 77 correspond to primers MC_TEF11 and MC_TEF12, respectively, used to clone terminator of the *Mucor circinelloides TEF* gene.

SEQ ID NOs: 78 and 79 correspond to primers Mould PGK4 and Mould PGK2, respectively, used to isolate genomic fragment of the *Mucor circinelloides* PGK gene.

SEQ ID NOs: 80, 81, 82 and 83 correspond to primers MC_PGK2, MC_PGK1, MC_PGK4 and MC_PGK3, respectively, used to isolate genomic fragments of the *Mucor circinelloides* PGK promoter region in chromosome walk experiments.

SEQ ID NOs: 84 and 85 correspond to primers MC_PGK5 and MC_PGK6, respectively, used to clone promoter of the *Mucor circinelloides PGK* gene.

SEQ ID NOs: 86, 87, 88 and 89 correspond to primers MC_GPD2, MC_GPD1, MC_GPD10 and MC_GPD9, respectively, used to isolate genomic fragment of the *Mucor circinelloides* GPD promoter region in chromosome walk experiments.

SEQ ID NOs: 90 and 91 correspond to primers MC_GPD11 and MC_GPD12, respectively, used to clone promoter of the *Mucor circinelloides GPD* gene.

SEQ ID NO: 92 corresponds to *S. cerevisiae* ACS2 protein encoding DNA codon optimized according to *Rhizopus oryzae* –fungus codon usage.

SEQ ID NO: 93 corresponds to *Rhizopus oryzae* PDAT protein encoding DNA codon optimised according to *Rhizopus oryzae* –fungus codon usage.

SEQ ID NO: 94 corresponds to *S. cerevisiae* PDC1 protein encoding DNA, with GenBank accession number X77316 (version number X77316.1).

SEQ ID NO: 95 corresponds to the amino acid sequence of the *S. cerevisiae PDC1* gene, with GenBank accession number CAA54522 (version number CAA54522.1.

SEQ ID NO: 96 corresponds to *S. cerevisiae* PDC1 protein encoding DNA codon optimized according to *Ustilago maydis* –fungus codon usage.

SEQ ID NO: 97 corresponds to *S. cerevisiae* PDC1 protein encoding DNA codon optimized according to *Rhizopus oryzae* –fungus codon usage.

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Claims

1. A genetically modified oleaginous fungal cell comprising at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS).

2. The genetically modified oleaginous fungal cell of claim 1, wherein the encoded PDC is a PDC of a Crabtree-positive organism.

3. The genetically modified oleaginous fungal cell of claim 2, wherein the encoded PDC is a PDC of *Saccharomyces cerevisiae*, preferably PDC1 of *S. cerevisiae*.

4. The genetically modified oleaginous fungal cell of any one of claims 1-3, wherein the encoded ALD is a cytosolic ALD.

5. The genetically modified oleaginous fungal cell of claim 4, wherein the encoded ALD is a fungal ALD, preferably *Saccharomyces cerevisiae* ALD6.

6. The genetically modified oleaginous fungal cell of any one of claims 1-5, wherein the nucleic acid encoding ACS is a gene that is not under glucose repression or its gene product is not subject to post-translational regulation.

7. The genetically modified oleaginous fungal cell of claim 6, wherein the encoded ACS is a fungal ACS, preferably *Saccharomyces cerevisiae* ACS2.

8. The genetically modified oleaginous fungal cell of any one of claims 1-7 further comprising a nucleic acid with enhanced expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

9. The genetically modified oleaginous fungal cell of claim 8, wherein the DAT encoding nucleic acid encodes a phosholipid:diacylglycerol acyltransferase (PDAT).

10. The genetically modified oleaginous fungal cell of claim 9, wherein the encoded DAT is a fungal DAT, preferably of *Rhizopus oryzae*, and most preferably it encodes a phosholipid:diacylglycerol acyltransferase (PDAT) having at least 40% sequence identity to SEQ ID NO:52, or an enzymatically active fragment or variant thereof.

11. The genetically modified oleaginous fungal cell of any one of claims 1-10, which is:

a yeast cell selected from the genera *Cryptococcus, Candida, Galactomyces, Hansenula, Lipomyces, Rhodosporidium, Rhodotorula, Trichosporon* and *Yarrowia,* preferably from the group consisting of *Candida sp., Cryptococcus curvatus, Cryptococcus albidus, Galactomyces geotrichum, Hansenula ciferri, Lipomyces lipofer, Lipomyces ssp., Lipomyces starkeyi, Lipomyces tetrasporus, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulans* and *Yarrowia lipolytica*, or

a filamentous fungal cell selected from the genera *Aspergillus, Cunninghamella, Fusarium, Glomus, Humicola, Mortierella, Mucor, Penicillium, Pythium* and *Rhizopus*, preferably from the group consisting of *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus niger, Cuninghamella japonica, Fusarium oxysporum, Glomus caledonius, Humicola lanuginose, Mortierella isabellina, Mortierella pusilla, Mortierella vinacea, Mucor circinelloides, Mucor plumbeus, Mucor ramanniana, Penicillium lilacinum, Penicillium spinulosum, Pythium ultimum* and *Rhizopus oryzae.*

12. The genetically modified oleaginous fungal cell of claim11, which is from the genera *Cryptococcus* or *Mucor*, preferably it is a cell of *Cryptococcus curvatus* or *Mucor circinelloides*.

13. A method of producing lipids, comprising cultivating a genetically modified oleaginous fungal cell according to any one of claims 1-12 in a medium containing carbon and nitrogen sources, and recovering the lipids produced.

14. The method of claim13, wherein the lipids are recovered from the culture medium.

15. The method of claim 13 or 14, wherein lipids comprising acylglycerols, preferably triacylglycerols (TAG) are produced.

16. The method of any one of claims 13-15, wherein the carbon source is a hexose or pentose sugars containing material.

17. The method of claim13, comprising producing precursors for functional fatty acids.

18. A method of producing biofuel, or lubricant, said method comprising cultivating a genetically modified oleaginous fungal cell according to anyone of claims 1-12 in a medium containing carbon and nitrogen sources, and recovering the lipids produced, and optionally esterifying said lipids to ob-

tain biodiesel or lubricant, or hydrogenizing the lipids to obtain renewable diesel or lubricant.

19. A method of preparing an oleaginous fungal cell of any one of claims 1-12, said method comprising transforming an oleaginous fungal cell with at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS).

20. A genetically modified oleaginous fungal cell comprising:

a) a nucleic acid with enhanced expression encoding a pyruvate decarboxylase (PDC) enzyme, and

b) at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of acetaldehyde dehydrogenase (ALD), acetyl-CoA synthetase (ACS) and diacylglycerol acyltransferase (DAT).

21. The genetically modified oleaginous fungal cell of claim 20, wherein the encoded PDC is a PDC of a Crabtree-positive organism.

22. The genetically modified oleaginous fungal cell of claim 21, wherein the encoded PDC is a PDC of *Saccharomyces cerevisiae*, preferably PDC1 of *S. cerevisiae*.

23. The genetically modified oleaginous fungal cell of any one of claims 20-22 comprising a nucleic acid with enhanced expression encoding ALD.

24. The genetically modified oleaginous fungal cell of claim 23, wherein the encoded ALD is a cytosolic ALD.

25. The genetically modified oleaginous fungal cell of claim 24, wherein the encoded ALD is a fungal ALD, preferably *Saccharomyces cerevisiae* ALD6.

26. The genetically modified oleaginous fungal cell of any one of claims 20-25, wherein the nucleic acid encoding ACS is a gene that is not under glucose repression or its gene product is not subject to post-translational regulation.

27. The genetically modified oleaginous fungal cell of claim 26, wherein the encoded ACS is a fungal ACS, preferably *Saccharomyces cerevisiae* ACS2.

28. The genetically modified oleaginous fungal cell of any one of claims 20-27, wherein the DAT encoding nucleic acid encodes a phosholipid:diacylglycerol acyltransferase (PDAT).

29. The genetically modified oleaginous fungal cell of claim 28, wherein the encoded DAT is a fungal DAT, preferably of *Rhizopus oryzae*, and most preferably it encodes a phosholipid:diacylglycerol acyltransferase (PDAT) having at least 40% sequence identity to SEQ ID NO:52, or an enzymatically active fragment or variant thereof.

30. The genetically modified oleaginous fungal cell of any one of claims 20-29, which is a yeast cell selected from the genera *Cryptococcus, Candida, Galactomyces, Hansenula, Lipomyces, Rhodosporidium, Rhodotorula, Trichosporon* and *Yarrowia,* preferably from the group consisting of *Candida sp., Cryptococcus curvatus, Cryptococcus albidus, Galactomyces geotrichum, Hansenula ciferri, Lipomyces lipofer, Lipomyces ssp., Lipomyces starkeyi, Lipomyces tetrasporus, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulans* and *Yarrowia lipolytica,* or

a filamentous fungal cell selected from the genera *Aspergillus, Cunninghamella, Fusarium, Glomus, Humicola, Mortierella, Mucor, Penicillium, Pythium* and *Rhizopus*, preferably from the group consisting of *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus niger, Cuninghamella japonica, Fusarium oxysporum, Glomus caledonius, Humicola lanuginose, Mortierella isabellina, Mortierella pusilla, Mortierella vinacea, Mucor circinelloides, Mucor plumbeus, Mucor ramanniana, Penicillium lilacinum, Penicillium spinulosum, Pythium ultimum* and *Rhizopus oryzae.*

31. The genetically modified oleaginous fungal cell of claim 30, which is from the genera *Cryptococcus* or *Mucor*, preferably it is a cell of *Cryptococcus curvatus* or *Mucor circinelloides*.

32. A method of producing lipids, comprising cultivating a genetically modified oleaginous fungal cell according to any one of claims 20-31 in a medium containing carbon and nitrogen sources, and recovering the lipids produced.

33. The method of claim 32, wherein the lipids are recovered from the culture medium.

34. The method of claim 32 or 33, wherein lipids comprising acylglycerols, preferably triacylglycerols (TAG) are produced.

35. The method of any one of claims 32-34, wherein the carbon source is a hexose or pentose sugars containing material.

36. The method of claim 32, comprising producing precursors for functional fatty acids.

37. A method of producing biofuel, or lubricant, said method comprising cultivating a genetically modified oleaginous fungal cell according to anyone of claims 20-31 in a medium containing carbon and nitrogen sources, and recovering the lipids produced, and optionally esterifying said lipids to obtain biodiesel or lubricant, or hydrogenizing the lipids to obtain renewable diesel or lubricant.

38. A method of preparing an oleaginous fungal cell of any one of claims 20-31, said method comprising transforming a fungal cell with

a) a nucleic acid with enhanced expression encoding a pyruvate decarboxylase (PDC) enzyme, and

b) at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of acetaldehyde dehydrogenase (ALD), acetyl-CoA synthetase (ACS) and diacylglycerol acyltransferase (DAT).

39. A genetically modified fungal cell comprising:

a) at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS), and

b) a nucleic acid with enhanced expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

40. The genetically modified oleaginous fungal cell of claim 39, wherein the encoded PDC is a PDC of a Crabtree-positive organism.

41. The genetically modified fungal cell of claim 40, wherein the encoded PDC is a PDC of *Saccharomyces cerevisiae*, preferably PDC1 of *S. cerevisiae*.

42. The genetically modified fungal cell of any one of claims 39-41, wherein the encoded ALD is a cytosolic ALD.

43. The genetically modified fungal cell of claim 42, wherein the encoded ALD is a fungal ALD, preferably *Saccharomyces cerevisiae* ALD6.

44. The genetically modified fungal cell of any one of claims 39-43, wherein the nucleic acid encoding ACS is a gene that is not under glucose repression or its gene product is not subject to post-translational regulation.

45. The genetically modified fungal cell of claim 44, wherein the encoded ACS is a fungal ACS, preferably *Saccharomyces cerevisiae* ACS2.

46. The genetically modified fungal cell of any one of claims 39-45, wherein the DAT encoding nucleic acid encodes a phosholipid:diacylglycerol acyltransferase (PDAT).

47. The genetically modified fungal cell of claim 46, wherein the encoded DAT is a fungal DAT, preferably of *Rhizopus oryzae*, and most preferably it encodes a phosholipid:diacylglycerol acyltransferase (PDAT) having at least 40% sequence identity to SEQ ID NO:52, or an enzymatically active fragment or variant thereof.

48. The genetically modified fungal cell of any one of claims 39-47, which is an oleaginous yeast or oleaginous filamentous fungal cell.

49. The genetically modified fungal cell of claim 48, which is a yeast cell selected from the genera *Cryptococcus, Candida, Galactomyces, Hansenula, Lipomyces, Rhodosporidium, Rhodotorula, Trichosporon* and *Yarrowia,* preferably from the group consisting of *Candida sp., Cryptococcus curvatus, Cryptococcus albidus, Galactomyces geotrichum, Hansenula ciferri, Lipomyces lipofer, Lipomyces ssp., Lipomyces starkeyi, Lipomyces tetrasporus, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulans* and *Yarrowia lipolytica*, or

a filamentous fungal cell selected from the genera *Aspergillus, Cunninghamella, Fusarium, Glomus, Humicola, Mortierella, Mucor, Penicillium, Pythium* and *Rhizopus*, preferably from the group consisting of *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus niger, Cuninghamella japonica, Fusarium oxysporum, Glomus caledonius, Humicola lanuginose, Mortierella isabellina, Mortierella pusilla, Mortierella vinacea, Mucor circinelloides, Mucor plumbeus, Mucor ramanniana, Penicillium lilacinum, Penicillium spinulosum, Pythium ultimum* and *Rhizopus oryzae.*

50. The genetically modified oleaginous fungal cell of claim 49, which is from the genera *Cryptococcus* or *Mucor*, preferably it is a cell of *Cryptococcus curvatus* or *Mucor circinelloides*.

51. A method of producing lipids, comprising cultivating a genetically modified fungal cell according to any one of claims 39-50 in a medium containing carbon and nitrogen sources, and recovering the lipids produced.

52. The method of claim 51, wherein the lipids are recovered from the culture medium.

53. The method of claim 51 or 52, wherein lipids comprising acylglycerols, preferably triacylglycerols (TAG) are produced.

54. The method of any one of claims 51-53, wherein the carbon source is a hexose or pentose sugars containing material.

55. The method of claim 51, comprising producing precursors for functional fatty acids.

56. A method of producing biofuel, or lubricant, said method comprising cultivating a genetically modified fungal cell according to anyone of claims 39-50 in a medium containing carbon and nitrogen sources, and recovering the lipids produced, and optionally esterifying said lipids to obtain biodiesel or lubricant, or hydrogenizing the lipids to obtain renewable diesel or lubricant.

57. A method of preparing a fungal cell of any one of claims 39-50, said method comprising transforming a fungal cell with

a) at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS), and

b) a nucleic acid with enhanced expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

58. Expression cassette comprising

a) at least one nucleic acid with enhanced expression encoding an enzyme selected from the group consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS), and

b) a nucleic acid with enhanced expression encoding a diacylglycerol acyltransferase (DAT) enzyme.

59. An enzyme protein having phosholipid:diacylglycerol acyltransferase (PDAT) activity and at least 40% sequence identity to SEQ ID NO:52, or an enzymatically active fragment or variant thereof.

60. An isolated nucleic acid molecule selected from the group consisting of:

a) a nucleic acid encoding the protein of claim 59,

b) a nucleic acid comprising the nucleotide sequence of SEQ ID NO:53 or SEQ ID NO:93,

c) a complementary strand of a) or b), and

d) a sequence that is degenerate as a result of the genetic code to anyone of sequences a) - c).

61. A genetically modified fungal cell comprising a nucleic acid with enhanced expression encoding the enzyme protein of claim 59.

62. A method of producing lipids, comprising cultivating the genetically modified fungal cell of claim 61 in a medium containing carbon and nitrogen sources, and recovering the lipids produced.

63. Use of a genetically modified fungal cell of any one of claims 1-12, 20-31, 39-50 or 61 for producing lipids, precursors of functional fatty acids, functional fatty acids, biofuels, biodiesel, renewable diesel or lubricants.

64. A genetically modified *Cryptococcus* cell, which has been modified to enhance the expression of a heterologous nucleic acid.

65. The genetically modified *Cryptococcus* cell of claim 64, which is *C. curvatus*.

66. A method of constructing a *Cryptococcus* cell of claim 64 or 65, said method comprising transforming a *Cryptococcus* cell with a nucleic acid encoding a heterologous protein.

67. The method of claim 66, comprising transforming a *C. curvatus* cell.

68. The method of claim 66 or 67, wherein a nucleic acid encoding the heterologous protein is transferred under the control of a promotor and terminator of *Cryptococcus*.

69. The method of any one of claims 66-68, wherein the nucleic acid encoding the heterologous protein has been codon optimized to *Cryptococcus*, or its close relative.