Metabolically Engineered Fungal Cells With Increased Content Of Polyunsaturated Fatty Acids

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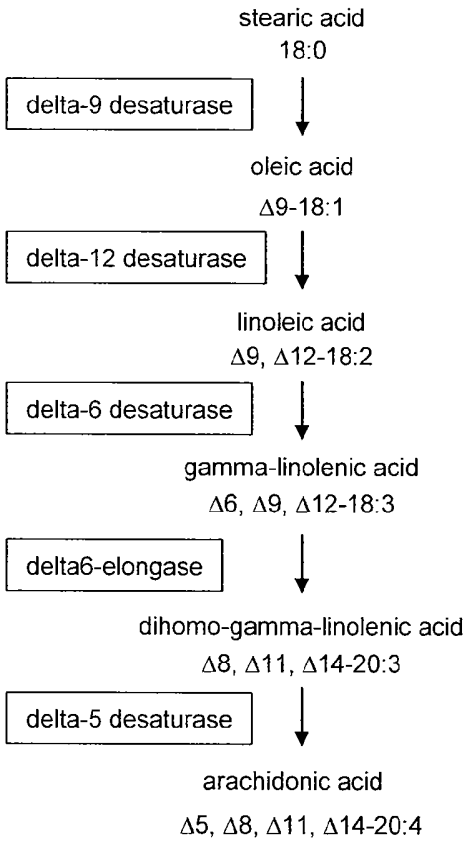

(57) Abstract: This invention relates to the production of fatty acids and particularly to the production of the polyunsaturated fatty acids (PUFAs) arachidonic acid (ARA) and eicosapentaenoic acid (EPA) in genetically engineered fungal cells, in particular, to metabolically engineered Saccharomyces cerevisiae cells with increased content of ARA and EPA. The invention especially involves improvement of the PUFA content in the host organism through various over-expression of e.g. cytochrome b5 and cytochrome b5 reductase involved in fatty acid desaturation, and heterologous expression of cytochrome b5 and cytochrome b5 reductase and expression of heterologous fatty acid synthases.

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METABOLICALLY ENGINEERED FUNGAL CELLS WITH INCREASED CONTENT OF POLYUNSATURATED FATTY ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

Each application, patent, and each document cited in this text, and each of the documents cited in each of these applications, patents, and documents ("application cited documents"), and each document referenced or cited in the application cited documents, either in the text or during the prosecution of the applications and patents thereof, as well as all arguments in support of patentability advanced during prosecution thereof, are hereby incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

This invention relates to the production of fatty acids and particularly to the production of the polyunsaturated fatty acids (PUFAs) gamma-linolenic acid (GLA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA) in genetically engineered fungal cells, and in particular to metabolically engineered Saccharomyces cerevisiae cells with increased content of GLA, ARA and EPA.

BACKGROUND OF THE INVENTION

PUFAs are polyunsaturated fatty acids with a long hydrocarbon chain composed of 18 or more carbon atoms having two or more double bonds and a terminal carboxylate group.

The properties of polyunsaturated fatty acids are highly influenced by the position of the double bond, and one differentiates omega-3 PUFAs, which have the first double bond at the third position counting from the methyl end of the carbon chain, and omega-6 PUFAs, which have the first double bond at the sixth position counting from the methyl end of the carbon chain. Eicosapentaenoic acid (EPA; 5, 8, 11, 4, 17-20:5) belongs to the former group, while, arachidonic acid (ARA; 5, 8, 11, 14-20:4) and gamma-linolenic acid (GLA, 6, 9, 12-18:3) belong to the latter group.

PUFAs are essential for humans, and it has been proven that they have many beneficial effects on human health, including proper development of brain and

visual functions and prevention of disease, such as cardiovascular disease and cancer.

Natural dietary sources of GLA and ARA include animal meats, egg yolks, and shellfish, while the main dietary source of EPA is fish oil. Commercial ARA-containing oils are produced by Martek Biosciences Corporation (Columbia, Md.), while EPA is available commercially as diverse mixtures of docosahexaenoic acid (DHA) and EPA (concentrates), which are produced by distillation of fish oils.

In relation to existing sources of commercial ARA and EPA, a recombinant microbial approach of production has many advantages. Most notably, a microbial process for EPA production circumvents certain problems associated with fish-oil derived products, such as bad taste and contamination by environmental pollutants. In addition, the fatty acid composition of fish oil may vary during the fishing season. Many naturally ARA- and EPA-producing microbes also contain inherent drawbacks, such as low growth rates and difficult-to-control fermentation behaviour. In addition, lack of tools for genetic manipulation of natural producers makes it difficult to alter, e.g., the fatty acid composition in the oil that they produce. The use of a well-characterized microbial host for ARA and EPA production provides solutions for the above-mentioned problems and, importantly, offers possibilities to develop the process further by metabolic engineering.

Fluxome Sciences A/S has recently made use of baker's yeast, Saccharomyces cerevisiae, as a preferred host for production of PUFA-containing oil (PCT/DK2005/000372 and US-2006-0051847-A1). S. cerevisiae is one of the most well-characterized production organisms in biotechnology, and additionally has a long tradition in the manufacturing of food and beverage products. By expression of up to 6 heterologous fatty acid desaturases and elongases, S. cerevisiae was engineered to produce PUFAs from a non-fatty acid carbon source.

S. cerevisiae has previously been used as a platform in which to analyze the function and substrate specificity of various fatty acid desaturases and elongases (e.g. US 6,432,684, PCT/US98/07422, WO 200244320-A, WO 99/27111) by means of adding exogenous fatty acid substrates. Others have utilized S. cerevisiae to reconstitute parts of the pathway towards ARA and EPA with the

intention of transferring this technology to plants (e.g. Domergue et al., 2003, Beaudoin et al., 2000, US 2003/0177508), still making use of an exogenous supplementation of desaturated fatty acid substrate.

In US2006/0094092 and US 60/624812, different approaches to increase the production of ARA and EPA by genetically modified Yarrowia lipolytica are reported. These approaches include deletion of native desaturases and acyltransferases, expression of heterologous acyltransferases and phospholipases, and variation of the types of desaturases and elongases used in the heterologous PUFA pathways. Specifically, the authors of said disclosures express a heterologous Acyl-CoA/lysophosphatidic acid acyltransferase, a heterologous diacylglycerol acyltransferase, a heterologous glycerol-3-phosphate acyltransferase and a heterologous phospholipase C in their production host Yarrowia lipolytica. In addition to phospholipase C, the authors claim a recombinant PUFA-producing Yarrowia lipolytica expressing phospholipase A2, although experimental results on the use of this type of phospholipase are not disclosed. The authors also claim expression of a phospholipid:DAG acyltransferase (PDAT) in Y. lipolytica, and more specifically over-expression of a native PDAT from Y. lipolytica. However, the effect of such an over-expression on the PUFA yield is not described in said disclosures.

Furthermore, several recent disclosures describe specific acyltransferases that can be used for increasing the yield of PUFAs in different production hosts (e.g. WO2004/076617-A2, WO06052807-A2, WO06052824A2, WO06052814A2).

PUFA production pathways have also been introduced into plant production hosts, disclosed e.g. in WO2004/057001 and WO2005/012316. In PCT/EP2005/007754 metabolic engineering methods for increasing the yield of heterologoys PUFAs in transgenic plants are disclosed, including expression of a heterologous phospholipase, a ketoacyl-CoA reductase and a dehydratase involved in fatty acid elongation.

S. cerevisiae has the advantage of being well characterized, safe and easily manipulated by targeted genetic engineering. It is therefore well suited for yield improvement through metabolic engineering. It has been shown that the lipid

content of PUFA-producing S. cerevisiae can be substantially increased by applying metabolic engineering approaches (PCT/DK2005/000372 and US-2006-0051847-A1). Presently, applicants have introduced further genetic modifications into fungal cells that surprisingly and substantially alter the fatty acid composition and increase the yield of GLA, ARA and EPA in the recombinant yeast.

SUMMARY OF THE INVENTION

The present invention relates to the construction and engineering of non-plants more particularly microorganisms, such as fungal cells, for improved PUFA production through overexpression and/or deletion of various endogenous genes, or through expression of heterologous genes.

In particular, the present invention describes methods for improving production of polyunsaturated fatty acids comprising expression of heterologous genes and/or genetic modifications of native genes in a Saccharomyces cerevisiae.

Furthermore, the present invention relates to the construction and engineering of fungal cells for improved production of particular PUFAs, including gamma-linolenic acid, arachidonic acid, and eicosapentaenoic acid.

The present invention particularly relates to improvement of the PUFA content in the host organism through metabolic engineering, e.g. through over-expression of fatty acid synthases, over-expression of enzymes involved in fatty acid desaturation, over-expression or deletion of regulatory proteins, over-expression of acyltransferases and/or lipases, or expression of corresponding heterologous enzymes.

Thus in one aspect, the present invention relates to a method for the production of polyunsaturated fatty acid (PUFA) in a fungal cell comprising at least two desaturases, said method comprises increasing the *in vivo* desaturase efficiency in said cell. One embodiment discloses that particularly over-expression of MCR1 and/or CYB5 increases the *in vivo* desaturase efficiency.

Another aspect present invention relates to fungal cells containing the genetic engineering described in the present invention that yields EPA and ARA in the

ratio of at least 1:1, preferably at least 2:1, most preferably 2.6:1. Such high ratios are important for optimising the commercial potential of large scale PUFA production.

The invention also relates to improvement of the total fatty acid content in the host organism through modification of transcriptional regulation of structural genes involved in phospholipid and fatty acid synthesis.

Thus, in another aspect, the present invention discloses new approaches for increasing the fatty acid content in particular fungal cells. In one embodiment such an increase in the fatty acid content is achieved by over-expression of at least one gene selected from the group consisting of INO2 and INO4. In another embodiment with the same achievement is obtained by deletion of the gene OPI1.

These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

DETAILED DESCRIPTION OF THE INVENTION

It should be understood that any feature and/or aspect discussed above in connection with the methods according to the invention apply by analogy to the uses.

As will be apparent, preferred features and characteristics of one aspect of the invention may be applicable to other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The present inventors have developed improved methods for producing polyunsaturated fatty acids by metabolic engineering of fungal host cells expressing heterologous pathways to GLA, ARA and EPA.

As previously described (PCT/DK2005/000372 and US-2006-0051847-A1), production of ARA in S. cerevisiae can be accomplished by simultaneous expression of heterologous genes encoding proteins with the following activities: delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase (figure 1). Alternatively, production of ARA can be achieved by simultaneous heterologous expression of genes encoding delta-12 desaturase, delta-9 elongase, delta-8 desaturase and delta-5 desaturase (figure 2). As previously described, the yield of ARA can be improved by additionally expressing a heterologous delta-9 desaturase and/or applying various metabolic engineering strategies (PCT/DK2005/000372 and US-2006-0051847-A1).

Furthermore, production of EPA in S. cerevisiae can be achieved by following the same strategies as described above for ARA, and additionally expressing a heterologous gene encoding an omega-3 desaturase (figure 3 and 4).

The present disclosure describes metabolic engineering strategies that further increases the yield of ARA, EPA, and/or intermediates in the pathway to ARA and EPA in a genetically modified fungal host cell, in particular S. cerevisiae, and demonstrates that metabolic engineering can be used to enable commercial production of polyunsaturated fatty acids in S. cerevisiae. Thus, the prior art approaches to produce ARA, EPA, and/or intermediates in the pathway to ARA and EPA in fungal cells are comprehended in the present term "expression of a heterologous pathway in a fungal cell".

This application relates to further genetic modifications into fungal cells that surprisingly and substantially alter the fatty acid composition and increase the yield of GLA, ARA and EPA in the recombinant yeast comprising both prior art disclosed and potential novel heterologous pathways.

In one aspect, the present invention relates to a method for the production of polyunsaturated fatty acid (PUFA) in a fungal cell comprising at least two desaturases, said method comprises increasing the *in vivo* desaturase efficiency in said cell. The in vivo desaturation efficiency is related to the desaturation complex within the cells, and by increasing the effect of the desaturation complex higher yields of PUFAs may be achieved as disclosed in the examples below.

This may for example be achieved by either over-expression or heterologous expression of at least one of the genes selected from the group consisting of MCR1 and CYB5.

In one embodiment of the present aspect, the at least two desaturases are selected from the group consisting of delta-9 desaturase, delta-6 desaturase, delta-12 desaturase, delta-5 desaturase, omega-3 desaturase, delta-4 desaturase and delta-8- desaturase.

The present invention also relates to a method for producing polyunsaturated fatty acids comprising expression of a heterologous pathway in a fungal cell, wherein the heterologous expression further comprises over-expression of at least one of the genes selected from the group consisting of FAS2, LRO1, SPO14, INO2, INO4, MCR1 and CYB5.

It is desirable to provide a method for the production of polyunsaturated fatty acid that has increased fatty acid content in the cells used. Therefore the present inventors here presents a method for the production of polyunsaturated fatty acid (PUFA) in a fungal cell comprising at least two desaturases, said method comprising increasing the fatty acid content in said cell.

Increasing the fatty acid content in cells, such as yeast, may be achieved by over-expression of the at least one gene selected from the group consisting of INO2 and INO4. The skilled artisan would be able to obtain such over expression both by increasing the endogenous expression of INO2 and INO4 by tools available in the art and by e.g. heterologous expression of the genes.

In a another aspect, the present invention relates to a method for producing polyunsaturated fatty acids comprising expression of a heterologous pathway in a fungal cell, wherein the heterologous expression further comprises heterologous expression of at least one of the nucleotide sequences selected from the group consisting of nucleotide sequences encoding cytochrome b5, cytochrome b5 reductase, FAS alpha subunit, FAS beta subunit and FAS (both subunits).

In a further aspect, the present invention relates to a method for producing polyunsaturated fatty acids comprising expression of a heterologous pathway in a fungal cell, wherein the heterologous expression further comprises deletion of OPI1. Deletion of OPI1 also generates metabolic engineered cells that have increased fatty acid content.

Polyunsaturated fatty acid

In the present context the term "polyunsaturated fatty acid" relates to a long hydrocarbon chain composed of 18 or more carbon atoms having at least 2 double bonds and a terminal carboxylate group.

As the skilled artisan would recognise, a fatty acid may be esterified to form triglycerides and/or phospholipids as well as sphingolipids. Thus, in one embodiment the present invention also relates to such esterified products.

Furthermore, the fatty acid product of the present invention can be free fatty acids. Free fatty acids have a free carboxyl group, are not chemically connected to any other compound including triacylglycerides, phospholipids or sphingolipids, and can be present freely in any compartment of the cell.

When applying the specific heterologous genes described above several intermediate products may be formed, and thus such intermediate products are included in the present invention. However, in some or many cases some or all of the intermediate products may be present at low levels that may not be easy to detect.

In the present context these intermediate products could be oleic acid, linoleic acid, gamma-linolenic acid, dihomo-gamma-linoleic acid, eicosadienoic acid, particularly, eicosadienoic acid with double bonds in position 11 and 14, eicosatrienoic acid, particularly, eicosatrienoic acid with double bonds in position 11, 14, and 17, arachidonic acid, alpha-linoleic acid, stearidonic acid, eicosatetraenoic acid, particularly eicosatetraenoic acid with double bonds in position 8, 11, 14 and 17, eicosapentaenoic acid, particularly eicosapentaenoic acid with double bonds in position 5, 8, 11, 14 and 17.

In one preferred embodiment, a method according to the present invention is provided, wherein the polyunsaturated fatty acid comprises at least 3 double bonds, such as 3 double bonds, such as 4 double bonds, such as 5 double bonds or such as 6 double bonds.

In another preferred embodiment the polyunsaturated fatty acid is selected from the group consisting of gamma-linolenic acid, arachidonic acid and eicosapentaenoic acid.

In one preferred embodiment, said polyunsaturated fatty acid is gamma-linolenic acid.

In one preferred embodiment, said polyunsaturated fatty acid is arachidonic acid.

In one preferred embodiment, said polyunsaturated fatty acid is eicosapentaenoic acid.

Oxygen requiring pathway

An oxygen-requiring pathway shall mean that at least one of the enzymes in the pathway requires oxygen to function. For example the expression of nucleic acids coding for desaturase usually leads to a pathway that requires oxygen for activity as desaturases usually are oxygen-requiring enzymes.

Endogenous genes

The genes natively present in the host organism, including genes in the pathway to fatty acids, including the pathway to any desirable PUFA in a cell of the present invention, are in the present context termed endogenous genes.

Heterologous Genes

The technology described within the present invention relates to genetically engineered fungal host cells that produce PUFAs through expression of a heterologous pathway, and that have been further genetically engineered to produce increased amounts of PUFAs.

However, in addition to any heterologous gene with a specific function mentioned in the present application, one or more endogenous genes with the same or

similar function may be present in the chosen fungal host cell. Furthermore, the chosen fungal cell in question may contain endogenous genes that have satisfactory expression for PUFA production at commercially viable yields without the need for heterologous expression of a PUFA pathway. Thus, the technology described within the present invention also relates to such fungal cells, which contain an endogenous pathway for PUFA production.

As described in detail in prior art (PCT/DK2005/000372 and US-2006-0051847-A1), the genetically transformed cells particularly harbour a heterologous pathway from stearic acid to PUFAs by expression of the following heterologous enzymes delta-9 desaturase, delta-12 desaturase, delta-9 elongase, delta-8 desaturase omega-3 desaturase, delta-6 desaturase, delta-6 elongase, delta-5 desaturase, or subsets hereof.

The heterologous genes in the pathway to PUFAs can be chosen among a wide range of described and published sequences, or can be isolated from any living organism, including fungi, plants, animals, algae and marine protists, amoeba and bacteria, that harbours pathways to oleic acid, linoleic acid, alpha-linolenic, gamma-linoleic acids, dihomo-gamma-linolenic acid, arachidonic acid, stearidonic acid, eicosatetraenoic acid or EPA.

In one embodiment, the present invention utilizes the simultaneous heterologous expression of genes encoding delta-12 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase in a microorganism, which leads to the production of PUFAs, and in particular production of arachidonic acid.

Furthermore, it makes use of the simultaneous heterologous expression of genes encoding delta-12 desaturase, omega-3 desaturase, delta-6 desaturase, delta-6 elongase and delta-5 desaturase in a microorganism, which results in production of EPA and other PUFAs.

Heterologous expression of specific genes

By "expression", it is meant the production of a functional polypeptide through the transcription of a nucleic acid segment into mRNA and translation of the mRNA into a protein.

By "heterologous expression", it is generally meant that a nucleic acid, not naturally present in the host genome, is present in the host cell and is operably linked to promoter and terminator nucleic acid sequences in a way so it is expressed in the host cell.

Also, in the present context heterologous expression further relates to the presence of a nucleic acid with a similar function to a naturally present nucleic acid, wherein the expression of said heterologous nucleic acid product changes the fatty acid composition.
For example, expression in yeast of a fungal delta-9 desaturase with different substrate specificity than the native yeast delta-9 desaturase changes the fatty acid composition of yeast.

Said nucleic acid may be contained on an extra chromosomal nucleic acid construct or may be integrated in the host genome. Methods for isolation of nucleic acids for heterologous expression and preferred embodiments of heterologous expression are further described in details below.

By heterologous expression of a pathway is meant that several genes are expressed heterologously, whose gene products constitute steps in a pathway, not naturally present in the host.

In one aspect, the present invention relates to a method for producing polyunsaturated fatty acids comprising expression of a heterologous pathway in a fungal cell, wherein the heterologous expression further comprises heterologous expression of nucleotide sequences encoding at least one of the enzymes selected from the group consisting cytochrome b5, cytochrome b5 reductase, FAS alpha subunit, FAS beta subunit and FAS (both subunits).

The expression of said heterologous genes allows not only the production of omega-6 fatty acids, but also the production of omega-3 fatty acids, simultaneously or not, in host cells that endogenously only produce fatty acids of up to 18 carbon atoms of length with up to one double bond. In particular, the

expression of said heterologous genes allows the production of eicosapentaenoic acid in said host cells.

Furthermore, the expression of said heterologous genes generally improves the production of eicosapentaenoic acid and/or one or more of its intermediate precursors, including arachidonic acid, in a host cell. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

Substrate

The expression of the heterologous pathways described herein generally improves the production of polyunsaturated fatty acids such as arachidonic acid in a host cell and can also lead to improved production of one or more of its intermediate precursors. A general advantage of this method is that it allows the use of non-fatty acid substrates, such as sugars. However, fatty acid-containing substrates, such as oils derived from, for example, plants, animals or microorganisms, can also be used.

The fermentation substrate for the production of PUFAs according to the present aspect maybe any complex medium or defined medium e.g. containing sugars, such as glucose, mannose, fructose, sucrose, galactose, lactose, erythrose, threose, ribose, glyceraldehyde, dihydroxyacetone, ribulose, cellobiose, starch, glycogen, trehalose, maltose, maltotriose, xylose, arabinose, stachyose, raffinose, or non-fermentable carbon sources, such as ethanol, acetate, lactate, or glycerol, or oils, such as oils derived from plants, animals or microorganisms or fatty acids, such as butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, palmitoleic acid, oleic acid, elaidic acid, cis-vaccenic acid, linoleic acid, alpha-linolenic, gamma-linoleic acids, and dihomo-gamma-linolenic acid.

Thus in one embodiment the present invention relates to a method according to the present application, wherein the fungal cell is grown on a non fatty acid substrate. In a particular preferred embodiment such non-fatty acid substrate is

the exclusive carbon source. In one embodiment the fungal cell is cultivated in a myo-inositol deficient medium.

Non fatty acid substrate

In the present context, a "non-fatty acid substrate" relates to any substrate, but not a fatty acid, with two or more carbon atoms, such as but not limited to sugars, such as glucose, mannose, fructose, sucrose, galactose, lactose, erythrose, threose, ribose, glyceraldehyde, dihydroxyacetone, ribulose, cellobiose, starch, glycogen, trehalose, maltose, maltotriose, xylose, arabinose, stachyose, raffinose, or non-fermentable carbon source, such as but not limited to ethanol, lactate, acetate and glycerol.

Exclusive carbon source or exclusive carbon sources

Usually, a living organism needs a supply of many or all of the macroelements such as carbon, sulphur, phosphor, nitrogen, oxygen or hydrogen. An organism may grow on mixtures of different carbon sources, such as a fatty acid substrate and a non-fatty acid substrate. If a substrate or substrates is referred to an exclusive carbon or exclusive carbon sources, it is only that substrate or those substrates that is supplied to the organism as a carbon source. This shall not exclude other macroelements or other nutritional requirements, such as requirements for example for trace elements and vitamins. For example, if a non-fatty acid substrate is exclusively supplied as a carbon source. This means, it is only that non-fatty acid that is supplied as a carbon source without supplying another carbon source. However, this does not exclude other macroelements or other nutritional requirements. An advantage of using for example a sugar, such as glucose or sucrose, or glycerol exclusively as a carbon source can be at least one of the following reasons: lower raw material cost, easier control of the fermentation process, better and constant quality of the produced oil.

In a particular preferred embodiment said non-fatty acid substrate is the exclusive carbon source.

Growth on myo-inositol deficient medium

It is known that in some yeast species grown on media deficient in myo-inositol, the lipid yield is increased. Hence, it is of advantage to grow the genetically modified cells of this invention on a medium that is not supplemented with myo-

inositol such that the lipid and thereby advantageously the PUFA yield are increased.

Thus, a preferred embodiment of the present invention relates to a method according to the present invention, wherein said host cell, such as Saccharomyces cerevisiae, is cultivated in a myo-inositol deficient medium.

Fermentation processes

PUFA containing yeast may be produced using well-known fermentation processes, such as batch, fed-batch or chemostat fermentation. Generally, a fed-batch or chemostat fermentation process is preferred over a batch process, as the high initial glucose concentration in a batch process may cause overflow metabolism and alcoholic fermentation and thereby a low biomass yield. Any process that results in high biomass concentrations is suitable for production of PUFA-containing yeast, since the PUFA-containing lipid is incorporated in the membranes and lipid bodies of the yeast.

Fed-batch processes are often used for production of baker's yeast and may be suitable for production of PUFA containing yeast. In a fed-batch process, a growth substrate is continuously fed into the reactor at a rate which optimally allows full respiratory growth of the yeast cells. The feeding rate can be optimized to allow the cells to grow at a maximal or a high specific growth rate without significant accumulation of by-products such as ethanol, glycerol, acetate, succinate, lactate, or pyruvate. Often, an exponential feed-profile is applied in order to achieve high biomass productivity. Thus, the feeding rate can be pre-set as a linear or exponential rate, or a combination of a linear and exponential rate. Moreover, the feeding rate may be controlled on-line by one or several parameters, such as the concentration of ethanol, glycerol, acetate, succinate, lactate or pyruvate in the fermentation broth, the concentration and rate of formation of biomass in the fermentation broth, the oxygen tension in the fermentation broth, pH of the fermentation broth or the rate of acid or base addition for pH control. Thus, the feeding rate may be controlled by on-line measurements of one or more of these or other parameters, using the on-line measurements for feed-back control of the feeding rate. The feed-back control may be in the form of an algorithm that takes into account the predicted response of the culture to increased feeding rates.

A chemostat process may also suitable for the production of PUFA containing yeast. In a chemostat process, the growth substrate is continuously fed into the reactor, while the working volume of the reactor is kept constant by continuously removing culture broth from the reactor at the same rate as the feeding rate. The culture thereby reaches a steady state, where the concentrations of biomass, substrates and products in the reactor are constant over time. Chemostat processes have the advantages that the process can keep running for a long time (up to several hundred hours) at a high production rate and that the product can be harvested continuously. Furthermore chemostats comprise the advantage that the conditions in the reactor can be precisely controlled and are unchanging over time, such that optimal conditions for biomass and PUFA can be maintained without making use of advanced on-line feed control.

In any fermentation process for production of yeast biomass, it is important to maintain a sufficiently high aeration rate and oxygen mass transfer to allow unrestricted respiration and fully aerobic metabolism. In particular, sufficient oxygen supply is important for the production of PUFAs, since oxygen is a direct substrate in the fatty acid desaturation reactions. Suitably high aeration rates may be achieved for example by sparging the fermentor with air or pure oxygen or a mix of air and pure oxygen at suitable flow rates. Mass transfer may for example be improved by altering the shape, size and number of impellers or by using different size, shape and designs of the fermentation reactor. For example, an air-lift reactor design may be used.

The ratio of carbon to nitrogen in the growth medium of fed-batch and continuous fermentation may be an important parameter for production of fatty acids. Normally, a higher yield of fatty acids on biomass is achieved when the cells are limited for nitrogen than during carbon-limitation. Usually a molar C/N ratio of less than 6 may result in clear carbon-limitation, while a molar C/N ration of more than 40 may result in clear nitrogen-limitation. Generally, a nitrogen limited medium composition is desired for production of fatty acids. However, it is also desired not to have a high residual concentration of the carbon source, especially glucose, in the reactor. High residual glucose concentration may result in fermentative metabolism and formation of by-products such as ethanol.

Therefore, a molar C/N ratio in the medium in between 6 and 40, preferably between 10 and 25, such as a C/N ratio of around 15, is presently preferred. The presently preferred nitrogen sources for production of polyunsaturated fatty acids are easily assimilated sources such as ammonium salts, while the presently preferred carbon source is glucose. Other, less easily assimilated nitrogen sources such as urea or amino acids may also be beneficial for production of polyunsaturated fatty acids, since their uptake rate is lower and their use as nitrogen sources therefore may result in a similar metabolism as during nitrogen limitation by an easily assimilated nitrogen source.

The presently preferred carbon-source for production of polyunsaturated fatty acids is glucose, as yeasts grow at a high specific growth rate and with a high biomass yield using this carbon source. The carbon source may also be other sugars, such as sugars selected from the group consisting of mannose, fructose, sucrose, galactose, lactose, erythrose, threose, ribose, glyceraldehyde, dihydroxyacetone, ribulose, cellobiose, starch, glycogen, trehalose, maltose, maltotriose, xylose, arabinose, stachyose, raffinose, and non-fermentable carbon sources, such as but not limited to ethanol, lactate, acetate and glycerol. Non-fermentable carbon sources have the advantage that they may be easily channelled into the fatty acid bio-synthetic pathway in the form of acetyl-CoA and malonyl-CoA, which are the precursors for fatty acid synthesis. However, the biomass yield on non-fermentable carbon-sources is lower than on sugars. The fermentation process may also utilize several carbon-sources.

It may be beneficial for production of fatty acids and in particular for polyunsaturated fatty acids to supply fatty acids to the growth medium. The fatty acids are preferably in the form of a vegetable oil and are supplied in addition to the carbon source or carbon sources used for growth. Fatty acids from the supplied oil can be taken up and used as substrates in the polyunsaturated fatty acid biosynthetic pathway, resulting in increased polyunsaturated fatty acid percentage in total fatty acid. Furthermore, oil supplementation is likely to increase the total content of fatty acids in the biomass. Fatty acids from the oil supplementation can be taken up by the cell and be incorporated into the biomass, mainly in the form of phospholipids or triacylglycerols. Presently

preferred supplementation oils include Sunflower oil, Safflower oil, Peanut oil, Soybean oil, Olive oil, Cottonseed oil and Canola oil.

Host cell

In the present context the term "host cell" relates to host cells selected from the group consisting of micro-organisms, animals, fungi, bacteria, invertea (insects), plants or protozoa. In particular, it relates to microscopic organisms, including bacteria, viruses, unicellular algae, protozoans and microscopic fungi including yeast.

In a presently preferred embodiment the host cell is a non-plant host cell.

More specifically, the microorganism may be a fungus, and more specifically a filamentous fungus belonging to the genus of Aspergillus, e.g. A. niger, A. awamori, A. oryzae, A. nidulans, a yeast belong to the genus of Saccharomyces, e.g. S. cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum, a yeast belonging to the genus Kluyveromyces, e.g. K. lactis K. marxianus var. marxianus, K. thermotolerans, a yeast belonging to the genus Candida, e.g. C. utilis, C. tropicalis, C.albicans, C. lipolytica, C. versatilis, a yeast belonging to the genus Pichia, e.g. P. stipidis, P. pastoris, P. sorbitophila, or other yeast genus, e.g. Cryptococcus, Debaromyces, Hansenula, Pichia, Yarrowia, Zygosaccharomyces or Schizosaccharomyces. Concerning other microorganisms a non-exhaustive list of suitable filamentous fungi is supplied: a species belonging to the genus Penicillium, Rhizopus, Fusarium, Fusidium, Gibberella, Mucor, Mortierella, Trichoderma, among others

Concerning bacteria a non-exhaustive list of suitable bacteria is given as follows: a species belonging to Bacillus, a species belonging to the genus Escherichia, a species belonging to the genus Lactobacillus, a species belonging to the genus Corynebacterium, a species belonging to the genus Acetobacter, a species belonging to the genus Acinetobacter, a species belonging to the genus Pseudomonas, etc., that are well known in the art.

The preferred microorganisms of the invention may be S. cerevisiae, A. niger, Escherichia coli or Bacillus subtilis.

The constructed and engineered microorganism can be cultivated using commonly known processes, including chemostat, batch, fed-batch cultivations, etc.

Thus, in one preferred embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining heterologous expression of genes encoding various desaturases and elongases in a host cell as described herein, wherein said host cell is selected from the group consisting of plants, micro-organisms, animals, fungi, bacteria, invertea (insects) or protozoa.

In a particular preferred embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining heterologous expression of genes encoding various desaturases and elongases in a host cell as described herein, wherein said host cell is a fungus, and preferably, wherein said fungus is a filamentous fungus or a yeast.

In one embodiment said yeast is selected from the group of the genus Saccharomyces, Kluyveromyces, Candida, Pichia, Cryptococcus, Debaromyces, Hansenula, Yarrowia, Zygosaccharomyces Schizosaccharomyces, Lipomyces.

In a preferred embodiment said yeast is Saccharomyces cerevisiae.

In another embodiment said filamentous fungus is selected from the group of the genus Aspergillus, Penicillium, Rhizopus, Fusarium, Fusidium, Gibberella, Mucor, Mortierella or Trichoderma.

In further embodiment said Aspergillus is selected from the species Aspergillus niger, Aspergillus awamori, Aspergillus oryzae or Aspergillus nidulans. And in a presently most preferred embodiment, said host is Aspergillus niger.

In another preferred embodiment, the present invention relates to a method for producing a polyunsaturated fatty acid comprising combining heterologous expression of genes encoding various desaturases and elongases in a host cell as described herein, wherein said host is a bacterium.

In one embodiment, said bacterium is selected from the group of Bacillus, Escherichia, Lactobacillus, Corynebacterium, Acetobacter, Acinetobacter, or Pseudomonas

In a presently most preferred embodiment said bacterium is Bacillus subtilis.

In another presently most preferred embodiment said one host cell is Escherichia coli.

In a presently preferred embodiment, the present invention relates to a genetically modified Saccharomyces cerevisiae capable of producing polyunsaturated fatty acids with four or more double bonds when grown on a non-fatty acid substrate.

In a presently most preferred embodiment, the present invention relates to a genetically modified Saccharomyces cerevisiae according to the invention, wherein said Saccharomyces cerevisiae is capable of producing polyunsaturated fatty acids with four or more double bonds when grown on a non-fatty acid substrate as the exclusive carbon source.

Metabolic Engineering of microbial metabolism

Metabolic engineering is a term that is used for rational modification of the metabolism of cells with the objective of altering metabolic fluxes, often with the aim to increase yield of a specific product. The concept of metabolic engineering includes analysis of the metabolism at several levels as well as development of hypotheses that can be tested experimentally.

In this context it is important to stress that although rational metabolic engineering strategies can be developed in silico or by means of logical analysis of the metabolism, experimental verification of these strategies may be essential. Microbial metabolism and in particular the regulation of microbial metabolism is far from being fully understood at present, even in a well-characterized microorganism such as S. cerevisiae. In particular, this applies to alteration of the magnitude of metabolic fluxes.

With the current knowledge of microbial metabolism, it can be possible to predict whether a certain change, e.g. deletion of a native gene or expression of a heterologous gene, has a positive or negative effect on the metabolic flux through a specific pathway, and thereby on the resulting yield of the desired product. In contrast, it is generally not possible to predict the magnitude of the change in the flux, due to the complex and largely unknown regulatory mechanisms of the cell. For the same reason, rationally designed metabolic engineering strategies in many cases fail altogether to give the intended effect. Thus, it is known to the person skilled in the art that metabolic strategies always have to be tested experimentally, and in most cases multiple strategies need to be applied in order to achieve the desired effect.

Fatty acid desaturation

Generally in eukaryotes, fatty acids are desaturated by NADH and O2 -dependent membrane-bound multiprotein enzyme complexes (figure 5). These complexes consist of three necessary protein components; a desaturase enzyme, an electron donor such as cytochrome b5 or ferredoxin, and an NADH (or NADPH) -dependent reductase enzyme. The total desaturation reaction (exemplified by desaturation of Stearoyl-CoA (18:0) to form oleoyl-CoA (18:1)) can be written as shown below:

$$\text{Stearoyl-CoA} + \text{NADH} + \text{H+} + \text{O2} \rightarrow \text{Oleoyl-CoA} + \text{NAD+} + 2\ \text{H2O}$$

The introduction of a double bond in the fatty acid chain thus involves oxidation of NADH and reduction of molecular oxygen to form NAD+ one molecule of water. The formation of the double bond involves an intricate system of electron transport through the desaturation complex: electrons are transferred from NADH to the FAD moiety of cytochrome b5 reductase, and further to the heme iron atom of 2 molecules of cytochrome b5 which is then reduced to its ferrous (Fe2+) form. Electrons from the ferrous cytochrome b5 molecules are transported to the non-heme iron atoms of the desaturase enzyme which then are reduced to their ferrous forms. When in its ferrous form, the desaturase binds and activates molecular oxygen and creates a double bond in the fatty acid substrate, with the simultaneous formation of H2O.

Desaturases

Fatty acid desaturase enzymes exist in many varieties. The biochemical best characterized desaturase is the soluble delta-9 desaturases from castor (Ricinus communis). This enzyme has been purified and subjected to resonance Raman spectroscopy analysis (Fox et al. 1994) which allowed the authors to identify the presence of an oxo-bridged diiron cluster in the enzyme and propose a structure for the diiron site along with a molecular mechanism of action for oxygen activation. The two iron atoms are bound at the active site by several histidine and glutamate residues and are in the ferric form connected by an oxo-bridge. The enzyme is in the resting state when the iron atoms are in the ferric form. Following reduction to a di-ferrous state, activation of molecular oxygen is thought to proceed via a bound hydroperoxide intermediate.

Most desaturases, and in particular PUFA desaturases, are membrane-associated and this makes purification and biochemical characterization difficult. However, it is believed that also membrane-bound desaturases are iron-chelating enzymes that have an iron-driven mechanism of action similar to the soluble fatty acid desaturases. The best characterized membrane-associated desaturase is the rat liver delta-9 desaturase. This desaturase exhibits an absorption spectrum that is characteristic of an oxo-bridged diiron cluster. Sequence alignments of the rat liver delta-9 desaturase with other membrane desaturases (delta-9, omega-3 and delta-12) from a variety of organisms revealed three conserved histidine-containing motifs present in all membrane bound desaturases included in the study (Shanklin et al. 1994). Through expression in S. cerevisiae of mutated versions of the rat delta-9 desaturase encoding gene, the inventors of the present invention were able to show that the conserved histidine residues were essential for fatty acid desaturation by the desaturase. The authors suggested that the conserved histidine motifs of membrane-bound desaturases serve as an iron-binding active site close to the membrane surface on the cytosolic side. Later, many other membrane bound desaturases, including PUFA desaturases, have been isolated, and all of them contain histidine-rich motifs associated with metal-chelating properties (reviewed e.g. by Napier et al. 2003).

In one embodiment the present invention relates to a method for the production of PUFA in a fungal cell comprising at least two desaturases selected from the

group consisting of delta-9 desaturase, delta-6 desaturase, delta-12 desaturase, delta-5 desaturase, omega-3 desaturase, delta-4 desaturase and delta-8-desaturase.

In another embodiment the desaturases are delta-9 desaturase and delta-12 desaturase.

In yet an embodiment the desaturases is delta-9 desaturase, delta-12 desaturase and delta-6 desaturase.

In a further embodiment the desaturases are delta-9 desaturase, delta-12 desaturase, delta-5 desaturase and delta-6 desaturase.

In a yet an embodiment the desaturases are delta-9 desaturase, delta-12 desaturase, delta-5 desaturase, delta-6 desaturase and omega-3 desaturase.

Cytochrome b5 as electron donor in the desaturation complex

Cytochrome b5 acts as the electron donor in most eukaryotic desaturation complexes. The main exception to this rule is the plastid and chloroplast desaturases of higher plants, which typically utilize ferredoxin rather than cytochrome b5. Cytochrome b5 binds the iron-carrying prosthetic group heme, which is a central component in the electron transport chain of the desaturation reaction. The importance of cytochrome b5 in the desaturation complex is underscored by the fact that several desaturase enzymes contain a cytochrome b5 subdomain; in these enzymes the desaturase is thus physically linked to cytochrome b5. The cytochrome b5 domain can either be located at the C-terminus or the N-terminus of the desaturase sequence. The positioning of the domain at the C-terminus or N-terminus seems related to the substrate specificity of the enzymes in that the so called front-end desaturases, which create a double bond between the carboxylated carbon and a pre-existing double bond in the fatty acid, typically contain N-terminal cytochrome b5 domains, while other desaturases contain no cytochrome b5 domain or a C-terminal cytochrome b5 domain. The desaturases that do not contain a cytochrome b5 domain utilize the free form of cytochrome b5; this interaction seems to be rather universal since desaturases that are expressed in heterologous hosts usually can function well together with the cytochrome b5 and cytochrome b5 reductase of the host organism. While it

has been shown for several cytochrome b5–containing desaturases that the cytochrome b5 domain is essential for the function of the enzyme (Mitchell et al. 1995, Sayanova et al. 1999), it is not clear to what extent the cytochrome b5–containing desaturases also utilize the free form of cytochrome b5. However, the stoichiometry of the desaturation reaction (figure 5) show that two cytochrome b5 molecules are required in the reaction, thus showing that desaturases that contain a cytochrome b5 domain make use of free cytochrome b5 in the desaturation reaction.

Depending on the type of desaturase enzyme, the necessary cytochrome b5 activity is either accessed in the form of a separately encoded cytochrome b5, or incorporated in the desaturase itself in the form of a cytochrome b5 domain. Thus, delta-12 desaturases and omega-3 desaturases typically do not contain a cytochrome b5 domain, while the so-called front-end desaturases (including delta-9 desaturases, delta-6 desaturase, delta-5 desaturase and delta-8 desaturase) contain a heme-binding cytochrome b5 domain either at the N-terminal or the C-terminal part of the protein.

Cytochrome b5 reductase

Cytochrome b5 reductase is as well as cytochrome b5 an integral part of the desaturation complex, however, in contrast to cytochrome b5, no desaturases have yet been isolated that contain a cytochrome b5 reductase domain. It is therefore likely that a separate cytochrome b5 reductase is essential for any desaturation reaction.

In vivo desaturase efficiency

The distribution of fat between PUFA and saturated fatty acids does not only depend on the amount of desaturase present in the cell. It also depends on how efficient the desaturases work e.g. a certain level of PUFA can be obtained by either increasing the amount of desaturase or by increasing the efficiency of the desaturases present.

In vivo desaturase efficiency is a measure of how good a certain amount of desaturase works. The higher efficiency the more PUFA is created with the same amount of desaturase.

In vivo desaturase efficiency can be estimated by relating the actual *in vivo* desaturation activity to the concentration of desaturase in the cell.

Actual *in vivo* desaturation activity can be measured by analyzing the fatty acid content and composition of the cell, since the cellular content of unsaturated fatty acids directly reflects the actual *in vivo* desaturation activity. The desaturation activity can be expressed as mg unsaturated fatty acids per mg cell dry-weight.

The concentration of a specific desaturase in the cell can be measured indirectly by performing Northern blot analysis or real-time RT PCR. By these methods, it is possible to estimate the mRNA copy number of the desaturase per mg cell dry-weight. Alternatively, if antibodies specific for the desaturases are available, the desaturase protein concentration can be measured directly by Western blot. However, this method is more cumbersome, especially since antibodies for PUFA desaturases are generally not commercially available. The preferred method of measuring the level of desaturases in the cell is therefore to use real-time RT PCR to estimate the mRNA copy number per mg cell dry-weight for each desaturase present in the cell. Furthermore, if two different strains express the same set of desaturases, and each of these desaturases are present in the same gene-copy number, and furthermore are expressed using the same promoter in both strains, it can be considered that the two strains contain an equal number of desaturase mRNA copy number and also the same desaturase protein concentration.

By relating the actual *in vivo* desaturation activity with the desaturase concentration, it is possible to estimate of the desaturase efficiency expressed, for example, as mg unsaturated fatty acids per desaturase mRNA copy number.

The efficiency can be used to assess whether all desaturases are actually actively performing fatty acid desaturation. Thus, one can compare the *in vivo* desaturase efficiency of strains which have the same copy number of desaturases per mg cell dry-weight, but have different levels of other factors necessary for forming a desaturation complex. If the desaturase efficiency is the same in both strains, this indicates that all desaturase copies are active in both strains. In contrast, if the desaturase efficiency is increased in a strain with an increased level of one of the

other factors required for fatty acid desaturation relative to a control strain, this indicates the presence of excess, inactive, desaturase enzyme in the control strain. In this situation, one can deduce that the increased level of other factors has enabled more desaturase molecules to actively perform fatty acid desaturation.

Accordingly, the present invention discloses a method wherein the increased *in vivo* desaturase efficiency can be measured by the following steps:

- providing a first fungal cell population having a genotype comprising at least two genes encoding desaturases

- providing a second fungal cell population with the same genotype as the first fungal cell population and further adding in said cell population at least one modification increasing the *in vivo* desaturase efficiency,

- measuring the fraction of PUFA with 2 or more double bonds as % of total fatty acids produced in both the first and second fungal cell populations,

-identifying a fungal cell population as having an increased *in vivo* desaturase efficiency, if the second cell population compared to the first cell population show an increase in the PUFA fraction (% of PUFA of total fatty acid) by at least 0,5%, such as at least 1%, e.g. at least 1.5%, such as at least 5%, e.g. at least 25%, such as at least 40%, e.g. at least 50%.

In the present context the term "fungal cell population" is to be understood as one or more fungal cells.

For production of polyunsaturated fatty acids at high yields, it is desirable to have a high total rate of fatty acid desaturation in the cell. To achieve this, it is of importance to maintain a high level of the relevant desaturase enzymes, which typically may be achieved by increasing the expression of the genes encoding these relevant desaturases, for example by introducing multiple gene copies and/or by applying strong promoters. However, if the molar concentration of desaturase enzymes increases above the molar concentration of available

cytochrome b5 and cytochrome b5 reductase in the cell, this results in a situation where not all desaturase molecules can find partners to form a desaturation complex and where excess desaturase enzymes will accumulate. Therefore, in order to increase the *in vivo* desaturation efficiency, the level of cytochrome b5 and cytochrome b5 reductase of the desaturation complex needs to be increased.

Sufficient availability of remaining desaturation complex components will allow for all desaturase molecules to be active, which leads to a total increase in the cycle of Fe3+ reduction and reoxidation, and thereby an increase in the total efficiency of fatty acid desaturation. Thus, in an aspect of the present invention, the rate of Fe3+ reduction and reoxidation is increased.

Accordingly, other factors such as the availability of NADH reducing equivalents, the availability of heme as a prosthetic group of cytochrome b5, the availability of iron atoms, the availability of FAD and the availability of molecular oxygen, may also limit the desaturation efficiency. It cannot be easily predicted which of the factors are limiting to the reaction. This however will have to be shown experimentally. The present invention shows that an increased level of cytochrome b5 or cytochrome b5 reductase in the cell results in increased accumulation of unsaturated fatty acids. This shows that both cytochrome b5 and cytochrome b5 reductase to some extent control the rate of fatty acid desaturation in a host cell where several heterologous desaturases are present at high levels. Increasing the level of cytochrome b5 has a larger effect than increasing the level of cytochrome b5 reductase, which implies that cytochrome b5 controls the desaturation rate to a greater extent than cytochrome b5 reductase.

Overexpression of cytochrome b5 reductase in a cell that has an increased level of cytochrome b5 increases the efficiency of desaturation even further. Thus, in a preferred embodiment of the invention, both cytochrome b5 and cytochrome b5 reductase are overexpressed in a PUFA-producing cell.

Cytochrome b5 is encoded by CYB5 and Cytochrome b5 reductase is encoded by MCR1. The endogenous delta-9 desaturase of S. cerevisiae, encoded by OLE1,

contains a C-terminal cytochrome b5 domain and therefore does not require the activity of the cytochrome b5 encoded by CYB5. In contrast, expression of a heterologous delta-12 desaturase or omega-3 desaturase in S. cerevisiae requires the activity of the cytochrome b5 encoded by CYB5, since these enzymes do not contain cytochrome b5 domains. In addition, both the native delta-9 desaturase and the heterologous fatty acid desaturases in the pathway to ARA and EPA (delta-9 desaturase, delta-12 desaturase, delta-6 desaturase, delta-5 desaturase, delta-8 desaturase and omega-3 desaturase) require the activity of a separately encoded cytochrome b5 reductase.

Since production of GLA, ARA or EPA in a fungal cell, and particularly in S. cerevisiae, requires expression of many heterologous fatty acid desaturases, endogenous cytochrome b5 and cytochrome b5 reductase are not sufficiently active to achieve a high flux through the desaturation pathway. Therefore, the genes encoding these proteins can be overexpressed using a strong constitutive promoter such as, e.g., the ADH1 promoter, the TDH3 promoter, the TPI1 promoter or the HXT7 promoter, or a strong inducible promoter such as the GAL1 promoter or the GAL10 promoter. Applicants have found that over-expression of MCR1 in an arachidonic acid producing recombinant S. cerevisiae results in a surprisingly high increase in the percentage of arachidonic acid in total fatty acid. Furthermore, the applicants have found that over-expression of CYB5 results in a substantially increased content of gamma-linolenic acid in a recombinant S. cerevisiae expressing the pathway to arachidonic acid.

It is possible that the heterologous desaturases do not function as efficiently in conjunction with the endogenous cytochrome b5 and cytochrome b5 reductase as they would in conjunction with the corresponding proteins from the donor organism. For example, if the delta-9 desaturase, delta-12 desaturase, delta-6 desaturase and delta-5 desaturase from Mortierella alpina are expressed in a fungal host cell, it may be beneficial to also express the cytochrome b5 and the cytochrome b5 reductase from M. alpina. A non-exhaustive list of heterologous genes encoding cytochrome b5 and cytochrome b5 reductase that could be beneficial for GLA, ARA and EPA production in a fungal host cell, particularly S. cerevisiae, is given in Table 1.

Table 1. Cytochrome b5 and cytochrome b5 reductase genes useful for increasing GLA, ARA and EPA yield in a recombinant fungal cell.

Enzyme	Source	Reference	SEQ ID NO: (nucleotide)	SEQ ID NO: (amino acid)
Cytochrome b5	Mortierella alpina (AB022444)	Kobayashi et al. 1999 J Biochem 125: 1094-1103, JP121873-A	1	2
Cytochrome b5 reductase	Mortierella alpina Cb5R-I	Sakuradani et al. 1999 Appl Environ Microbiol 65: 3873-3879, JP10113181-A	3	4
	Mortierella alpina Cb5R-II	Certik et al. 1999 J Biosci Bioeng 88: 667-671		5

In addition to the sequences listed in Table 1, sequences encoding cytochrome b5 and cytochrome b5 reductase can be obtained from other sources. Useful cytochrome b5 and cytochrome b5 reductase sequences can be derived from any source, for example, isolated from organisms containing highly unsaturated fatty acids. Genes encoding cytochrome b5 and cytochrome b5 reductase can also be identified in available sequence data using bioinformatics. The sequences can be isolated and expressed in the fungal host cell using conventional DNA amplification and cloning techniques, or they can be chemically synthesized de novo. Furthermore, the sequences can be codon-optimized for expression in the host cell.

Thus, in a presently preferred embodiment of the invention, S. cerevisiae MCR1 (SEQ ID NO: 19) is over-expressed in a S. cerevisiae cell expressing a heterologous pathway to polyunsaturated fatty acids such as gamma-linolenic acid, arachidonic acid and/or eicosapentaenoic acid. In particularly preferred

embodiments, MCR1 overexpression results in increased arachidonic acid content to more than 4% of the total fatty acid.

In another preferred embodiment, S. cerevisiae CYB5 (SEQ ID NO: 18) is over-expressed in a S. cerevisiae cell expressing a heterologous pathway to polyunsaturated fatty acids such as gamma-linolenic acid, arachidonic acid and/or eicosapentaenoic acid. In particularly preferred embodiments, CYB5 overexpression results in increased gamma-linolenic acid content to more than 13% of the total fatty acid.

In the present context, "CYB5" is defined as a gene encoding cytochrome b5. Cytochrome b5 is involved in the sterol and lipid biosynthesis pathways and is required in fatty acid desaturation. It catalyses the reaction from reduced cytochrome b5 and Fe3+ to oxidized cytochrome b5 and Fe2+ (Figure 5) More details can be found above under fatty acid desaturation..

In the present context, "MCR1" is defined as a gene encoding cytochrome b5 reductase (EC. 1.6.2.2). This enzyme catalyzes the reaction from NADH and oxidized cytochrome b5 to reduced cytochrome b5 and NAD+ (Figure 5). More details can be found above under fatty acid desaturation.

Fatty acid synthesis

Fatty acid synthase (FAS) catalyzes the synthesis of long-chain saturated fatty acids from acetyl-CoA and malonyl-CoA. FAS consist of a heterohexamer (α6β6) of two multifunctional α and β subunits that are encoded by FAS2 and FAS1 genes, respectively. The FAS1 gene encodes four functional domains- acetyl transferase, enoyl reductase, dehydratase and malonyl/palmitoyl transferase. The FAS2 gene product contains three domains- β-ketoacyl synthase, β-ketoacyl reductase and acyl carrier protein. S. cerevisiae FAS is known to primarily synthesize palmitic acid (16:0) and stearic acid (18:0), palmitic acid being the predominant product. However, applicants have found that the yield of ARA is increased in a S. cerevisiae strain simultaneously overexpressing FAS2 and a heterologous pathway to ARA.

In contrast to S. cerevisiae, other yeasts such as Saccharomyces kluyveri, Candida albicans and Yarrowia lipolytica are known to produce larger amounts of C18 than C16 fatty acids. It has been shown that heterologus expression of the gene coding for the α subunit of FAS from S. kluyveri in S. cerevisiae reduces the amount of C16 fatty acids and increases the amount of C18 fatty acids (Oura et al. 2006 Curr Genet 49: 393-402). It is therefore possible that expression of this gene in S. cerevisiae can increase the flux towards PUFA-production.

The organisms M. alpina and Aspergillus nidulans are also known to produce more C18 fatty acids than C16 fatty acids. A. nidulans contains two functionally distinct FASs: one required for primary fatty acid metabolism and the other required for secondary metabolism. The FAS involved in primary fatty acid metabolism is interesting for heterologus expression in S. cerevisiae since it is possible that the flux towards PUFA-production can be increased by expression of FAS genes from organisms producing more C18 than C16 fatty acids.

A non-exhaustive list of genes encoding FAS that could be beneficial for ARA and EPA production in a recombinant fungal cell, particularly S. cerevisiae, is given in Table 2.

Table 2. FAS genes useful for increasing ARA and EPA yield in a recombinant fungal cell.

Source	Reference	SEQ ID NO: (nucleotide)	SEQ ID NO: (amino acid)
Saccharomyces kluyveri Alpha subunit	Oura et al. 2006 Curr Genet 49: 393-402	6	7
Candida albicans Alpha subunit	Southard et al. 1995 Gene 156: 133-138	8	9

Candida albicans Beta subunit	Jones et al. 2004 Proc Natl Acad Sci USA 101: 7329-7334	10	11
Aspergillus nidulans Alpha subunit	Brown et al. 1996 Proc Natl Acad Sci USA 93: 14873-14877	12	13
Aspergillus nidulans Beta subunit	Brown et al. 1996 Proc Natl Acad Sci USA 93: 14873-14877	14	15
Yarrowia lipolytica Beta subunit	Kottig et al. 1991 *Mol Gen Genet* 226: 310-4	16	17

In addition to the sequences listed in Table 2, sequences encoding FAS α and β subunits can be obtained from other sources. Useful FAS encoding sequences can be derived from any source, for example, isolated from organisms containing highly unsaturated fatty acids. Genes encoding FAS α and β subunits can also be identified in available sequence data using bioinformatics. The sequences can be isolated and expressed in the fungal host cell using conventional DNA amplification and cloning techniques, or they can be chemically synthesized de novo. Furthermore, the sequences can be codon-optimized for expression in the fungal host.

In the present context "FAS1" is defined as a gene encoding fatty acid synthase, beta unit

In the present context "FAS2" is defined as a gene encoding fatty acid synthase, alpha unit

Phospholipid – DAG acyltransferase

Following synthesis of fatty acids by FAS, these are incorporated into lipids via the action of several acyltransferase enzymes. Glycerol-3 phosphate (G3P) or di-

hydroxy acetone phosphate (DHAP) are the precursors for all lipid compounds. A fatty acid chain can be added to either G3P or DHAP by the action of the acyltransferases encoded by GAT1 and GAT2. The products of these reactions are 1-acyl-G3P and 1-acyl-DHAP, respectively. 1-acyl-DHAP can be converted into 1-acyl-G3P by the enzyme encoded by AYR1. A second fatty acid chain is then added to this carbon backbone to form the intermediate phosphatidic acid (PA), which serves as the precursor both for phospholipids via the CDP-DAG pathway and triacylglycerol (figure 13). Phosphatidic acid is de-phosphorylated to form diacylglycerol via the action of the phosphatases encoded by LPP1 and DPP1. Diacylglycerol, in turn, can be further acylated to form triacylglycerol (TAG) or can enter the Kennedy pathway towards aminoglycerophospholipids. The final acylation of diacylglycerol to form TAG can be carried out by several enzymes, including the enzymes encoded by DGA1, ARE1, ARE2 and LRO1. While Dga1p, Are1p and Are2p transfer fatty acid chains from acyl-CoA molecules to diacylglycerol, the acyltransferase encoded by LRO1 transfers the fatty acid from the sn-2 acyl group of the phospholipid phosphatidylcholine (PC). It has been shown (e.g. Domergue et al. 2003) that many PUFA desaturases predominantly desaturate fatty acids in the sn-2 position of PC, and it is therefore likely that this position of PC often contains polyunsaturated fatty acids. Therefore, it is contemplated that over-expression of LRO1 in recombinant fungal cell producing polyunsaturated fatty acids, such as arachidonic acid and eicosapentaenoic acid, will contribute to increased incorporation of polyunsaturated fatty acids into TAG and thereby increased the accumulation of polyunsaturated fatty acids within the cell.

Thus, in one preferred embodiment of the invention, LRO1 is overexpressed in a fungal cell expressing a heterologous pathway to polyunsaturated fatty acids such as gamma-linolenic acid, arachidonic acid and/or eicosapentaenoic acid.

In the present context, "LRO1" is defined as a gene encoding a phospholipid-diacylglycerol acyltransferase (EC 2.3.1.158), which catalyzes the transfer of an acyl chain from phosphatidylcholine to diacylglycerol, resulting in formation of TAG.

Phospholipase D

As mentioned above, the site of fatty acid desaturation is predominantly in the phospholipid fraction of lipids. Conversely, fatty acid elongases generally act on CoA-bound fatty acids, and the full desaturation and elongation pathway towards polyunsaturated fatty acids such as gamma-linolenic acid, arachidonic acid and eicosapentaenoic acid therefore involves fatty acid substrates in different lipid pools. It is contemplated that increased exchange of fatty acids between the phospholipid, TAG, and acyl-CoA pool will enable higher flux through the pathway to polyunsaturated fatty acids. Therefore, it is contemplated that increased expression of Phospholipase D, encoded by SPO14, will increase the flux to polyunsaturated fatty acids. Phospholipase D hydrolyses PC into PA and choline, whereby the PA originating from PC can be converted into the storage compound TAG.

Thus, in a preferred embodiment of the invention, SPO14 is over-expressed in a in a fungal cell expressing a heterologous pathway to polyunsaturated fatty acids such as gamma-linolenic acid, arachidonic acid and/or eicosapentaenoic acid.

In the present context, "SPO14" is a gene encoding Phospholipase D (EC. 3.1.4.4)

Regulation of lipid synthesis

Several genes encoding enzymes of the lipid synthesis pathway are co-regulated by inositol and choline. The INO1 gene encodes inositol 1-phosphate synthase, which converts glucose 6-phosphate to inositol 1-phosphate. The reaction is fundamental for synthesis of inositol containing phospholipids when inositol is absent from the growth medium. Conversely, INO1 gene transcription is repressed when inositol is in the medium. The presence of the lipid precursors repress the primary biosynthetic route of phospholipids and leads to an increased level of phosphatidylinositol (PI) at the expense of a reduced level of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS).

Transcriptional regulation includes the positive regulators of phospholipid biosynthesis INO2 and INO4 as well as the negative regulator OPI1. The gene products exert their effect by controlling expression of transcription of the INO1 and CHO1 structural genes as well as INO2 and OPI1. When inositol and choline

are absent in the growth media, the heterodimeric complex consisting of Ino2 and Ino4 bind the inositol-choline responsive element (ICRE) to activate transcription of INO1, INO2 and OPI1. The reverse occurs when inositol and choline is supplemented to the medium. Under these conditions Opi1 represses the transcription of these genes.

The structural gene ACC1 encoding acetyl-CoA carboxylase, which catalyzes the initial and rate-limiting step in fatty acid synthesis, is also under this coordinated transcriptional control. Thus, transcript levels are repressed in ino2/ino4 mutant strains and derepressed in opi1 mutant strains.

Since Ino2 and Ino4 act as positive regulators of lipid synthesis it is possible that overexpression of the genes encoding these proteins will increase the total lipid synthesis in the strains. Likewise, Opi1 acts as a negative regulator of lipid synthesis and a deletion of the gene encoding Opi1 might result in a higher lipid yield of the mutated strain.

Furthermore, over-expression of INO2 and INO4 in addition to deletion of OPI1 is likely to result in increased phospholid content in relation to the TAG content, which is likely to increase the content of polyunsaturated fatty acids such as gamma-linolenic acid, arachidonic acid and eicosapentaenoic acid in the cell.

Thus, the above described modifications over-expression of INO2, over-expression of INO4 and deletion of OPI1 are likely to increase both the yield of total lipids and the percentage of polyunsaturated fatty acid in total lipid.

In the present context, "INO1" is a gene encoding inositol 1-phosphate synthase (EC. 5.5.1.4), which converts glucose 6-phosphate to inositol 1-phosphate.

In the present context, "CHO1" is a gene encoding phosphatidylserine synthase (EC. 2.7.8.8).

In the present context, "INO2" is a gene encoding a protein which forms one part of a heterodimeric complex that binds to the inositol-choline responsive element.

In the present context, "INO4" is a gene encoding a protein which forms one part of a heterodimeric complex that binds to the inositol-choline responsive element.

In the present context, "OPI1" is a gene encoding a negative regulator of transcription of the INO1, CHO1 and INO2 genes.

The present inventors here presents a method for the production of polyunsaturated fatty acid (PUFA) in a fungal cell comprising increasing the fatty acid content by over-expression of INO2 and/or INO4 and/or deletion of OPI.

Many genes involved in phospholipid biosynthesis are regulated at the level of transcription in response to the lipid precursor inositol. The inositol-responsive genes have in common a repeated regulatory element with the consensus sequence 5'-CATGTGAAAT-3' in their promoter sequences, usually referred to as the inositol-sensitive upstream activation sequence (UASINO). Although inositol is the main effector in transcriptional regulation of the UASINO containing genes (Jesch et al. 2005), another lipid precursor, choline, has additional effects on the transcription of inositol-regulated genes and the UASINO sequence is therefore also sometimes referred to as the inositol/choline-responsive element (ICRE).

The transcriptional factors Ino2 and Ino4 are positive transcriptional activators, which together form a complex that binds to the UASINO element (Greenberg and Lopes 1996, Ambroziak and Henry 1994). Ino2 and Ino4 are therefore thought to control synchronized expression of structural enzymes in phospholipid biosynthesis. In addition to the positive regulators Ino2 and Ino4, many structural genes encoding phospholipid biosynthetic enzymes are regulated by the negative transcriptional regulator Opi1. Even though Opi1 does not directly interact with the UASINO element, it has been shown that the UASINO element is required for repression by Opi1 (Bachhawat et al. 1995).

The Ino2-Ino4 complex binds to the promoter sequences of at least 32 genes in S. cerevisiae (Harbison et al. 2004), while it has been shown that 24 genes are more than 2-fold upregulated in an opi1-deleted strain (Jesch et al. 2005). Among these genes, many are regulated both by the Ino2-Ino4 complex and Opi1. Genes that are regulated both by the Ino2-Ino4 complex and Opi1 and encode structural

enzymes in phospholipid biosynthesis include CDS1, CHO1, INO1, ITR1, OPI3, PSD1 and RMD12, while genes that are regulated by the Ino2-Ino4 complex but are unaffected by Opi1 include ADO1, CKI1, HNM1, OLE1 and SAH1.

In addition to genes involved in phospholipid biosynthesis, Ino2-Ino4 and Opi1 regulate transcription of structural genes in fatty acid synthesis. One example of this is the transcriptional regulation of ACC1 (Hasslacher et al. 1993), encoding acetyl-CoA carboxylase, which is one of the key structural enzymes in fatty acid synthesis. Acetyl-CoA carboxylase catalyzes the caroxylation of acetyl-CoA to form malonyl-CoA, which is the elongation unit used by the fatty acid synthase for synthesis of fatty acids. Acc1 is the only source of malonyl-CoA in the cell, and is therefore a key enzyme in fatty acid synthesis. The importance of Acc1 in regulation of fatty acid synthesis is underscored by this regulation at the transcriptional level, but also by several mechanisms of regulation at the protein level – the Acc1 enzyme is inactivated by phosphorylation by the Snf1 kinase and activated by de-phosphorylation by the protein phosphatase Sit4. Additionally, Acc1 is activated by citrate and inhibited in the presence of fatty acids.

Since Opi1 repress many structural genes in phospholipid biosynthesis, it is anticipated that deleting Opi1 in a PUFA-producing yeast will result in increased lipid content and thereby increased PUFA content. Similarly, since Ino2 and Ino4 activate transcription of many genes in phospholipid biosynthesis, it is anticipated that overexpression of these transcription factors in a PUFA-producing yeast will result in increased lipid content and thereby increased PUFA content. Moreover, both Opi1 and the Ino2-Ino4 complex regulate the transcription of many UASINO containing genes. Therefore it is preferred that Opi1 deletion and overexpression of INO2 and INO4 is combined in a PUFA-producing yeast.

Genetic modification of S. cerevisiae

Genetic modification of S. cerevisiae can be carried out using well-established methods. Thus, various methods are available for the person skilled in the art, allowing deletion and overexpression of native genes, expression of heterologous genes, as well as different types of genetic modifications such as point mutations and gene fusions. Methods for performing the above mentioned modifications in

S. cerevisiae are described in, e.g., Erdeniz et al. (1997) Genome Res 7, 1174-83; Wach et al. (1994) Yeast 10, 1793-1808; Longtine et al. 1998 Yeast 14, 953-961.

Expression of a heterologous gene, such as a heterologous gene encoding cytochrome b5, cytochrome b5 reductase or FAS, in S. cerevisiae is usually carried out either by expression from a plasmid or by integration into the genome. Expression from plasmids can be beneficial because it is a versatile system that can be used in different genetic backgrounds. In addition, it is often possible to achieve high expression of the heterologous gene by using a high-copy, 2μ origin-based plasmid. Expression by genomic integration, on the other hand, often results in a more stable strain that does not easily loose the heterologous gene. To achieve high expression of a heterologous gene integrated into the genome, several copies of the heterologous gene can be integrated and strong yeast promoters can be used to control expression. In addition to the choice of promoter and copy number of the heterologous gene, the position in the genome chosen for integration also influences the expression. Since some regions of the genome are less transcribed than others, it is anticipated that a heterologous gene can be differentially transcribed depending on its location in the genome. Furthermore, heterologous sequences usually contain a different codon usage than S. cerevisiae sequences, which can impair efficient translation of the gene transcript. Optimization of the codon-usage in a heterologous gene can therefore improve expression of the gene. Codon-optimization is done by employing the host-preferred codons, as determined from codons of the highest frequency in highly expressed proteins of the host of interest. The codon-optimized sequence coding for a heterologous polypeptide can then be chemically synthesized in the form of oligonucleotides and be assembled by PCR-techniques.

There are thus several factors, such as gene copy number, plasmid-based or genomic expression, position in the genome, choice of promoter, and codon-usage, which can influence the magnitude of heterologous expression. Tuning the magnitude of the heterologous expression can be important for obtaining the optimal effect of the heterologous gene product, and it is therefore contemplated that it may be beneficial to adjust several of the factors that influence expression.

Also for overexpression of native yeast genes such as LRO1, SPO14, INO2, INO4, CYB5 and MCR1, several methods exists, such as expression with the native promoter from a high-copy plasmid, expression with a strong yeast promoter from a low-copy or high-copy plasmid, or replacement of the native promoter in the genome by a stronger promoter. The magnitude of expression can be tuned by adjusting the described factors such as copy-number and the strength of the chosen promoter.

Native yeast genes, such as OPI, can be deleted using established methods, for example gene deletion or gene disruption using a bi-partite gene targeting substrate Erdeniz et al. (1997) Genome Res 7, 1174-83. Of course, reducing the transcription of the gene (by, e.g. promoter replacement with a weaker promoter) or introducing mutations that destroys or modifies the function of the gene product, or other methods of reducing or abolishing the activity of the gene product, would give similar effects as deletion of the gene.

As previously described (PCT/DK2005/000372 and US-2006-0051847-A1) the heterologous pathway to polyunsaturated fatty acids, such as GLA, ARA and EPA, can be expressed in S. cerevisiae using expression from vectors or integration into the genome.

In a presently preferred embodiment of the invention, the expression of a heterologous pathway to PUFAs in S. cerevisiae further comprises expression of one or several heterologous genes encoding cytochrome b5, cytochrome b5 reductase and FAS by integration into the genome under the control of strong yeast promoters, such as, but not limited to, the ADH1 promoter, the TDH3 promoter, the TPI1 promoter or the HXT7 promoter.

This is anticipated to increase the percentage of ARA in the recombinant S. cerevisiae, so that the percentage of ARA exceeds 4% of the total fatty acid, resulting in for example 4% ARA of total fatty acid, 6% ARA of total fatty acid, 8% ARA of total fatty acid, 10% ARA of total fatty acid, 12% ARA of total fatty acid, 14% ARA of total fatty acid, or more.

Furthermore, for an EPA-producing recombinant S. cerevisiae, the described additional expression of heterologous genes is anticipated to result in an increased content of EPA, such that the percentage of EPA in total fatty acid is at least 3%, for example 4% EPA of total fatty acid, 6% EPA of total fatty acid, 8% EPA of total fatty acid, 10% EPA of total fatty acid, 12% EPA of total fatty acid, 14% EPA of total fatty acid, or more.

In another preferred embodiment, the expression of a heterologous pathway to PUFAs in S. cerevisiae further comprises over-expression of one or several native genes such as LRO1, SPO14, INO2, INO4, CYB5 and MCR1 by replacing the native promoter in the genome with a stronger promoter, such as, but not limited to, the ADH1 promoter, the TDH3 promoter, the TPI1 promoter or the HXT7 promoter.

Over-expression of one ore more of the described native genes increases the percentage of ARA in the recombinant S. cerevisiae to at least 4% of the total fatty acid. Furthermore, combining several over-expressions in a single S. cerevisiae strain is anticipated to increase the percentage of ARA even more, resulting in 6% ARA of total fatty acid, 8% ARA of total fatty acid, 10% ARA of total fatty acid, 12% ARA of total fatty acid, 14% ARA of total fatty acid, or more.

For an EPA-producing recombinant S. cerevisiae, the described over-expression of LRO1, SPO14, INO2, INO4, CYB5 and/or MCR1 is anticipated to result in an increased content of EPA, such that the percentage of EPA in total fatty acid is at least 3%. Furthermore, combining over-expression of several of these genes is anticipated to result in even higher EPA content, such as 4% EPA of total fatty acid, 6% EPA of total fatty acid, 8% EPA of total fatty acid, 10% EPA of total fatty acid, 12% EPA of total fatty acid, 14% EPA of total fatty acid, or more.

Over-expression of one or more of the described native genes is also anticipated to increase the percentage of intermediates in the pathway to ARA and EPA. In particular, over-expression of CYB5 results in increased content of gamma-linolenic acid to at least 13 % of the total fatty acid. Combining CYB5 over-expression with additional over-expression of the described native or heterologous genes is anticipated to increase the GLA content even more, so that GLA

constitutes 15% of total fatty acid, 18% of total fatty acid, 20% of total fatty acid, 25% of total fatty acid, or more.

Furthermore, it is anticipated that the described modifications can increase the lipid content of the recombinant S. cerevisiae, and thereby increase the yield of PUFA. It is therefore anticipated that the described heterologous expression of genes encoding cytochrome b5, cytochrome b5 reductase and/or FAS, over-expression of the native genes LRO1, SPO14, INO2, INO4, CYB5 and/or MCR1, and/or deletion of OPI1, can increase the percentage of PUFA in the yeast dry-weight to more than 0.2 %.

Genetic modification of fungal hosts other than S. cerevisiae

The present invention demonstrates how metabolic engineering can be used in S. cerevisiae to increase fatty acid content and fatty acid desaturation. The invention may as well be used in yeast species in general e.g. such as yeast species selected from the group consisting of Y. lipolytica, K. lactis, S. pombe and S. kluyvery, or other yeasts such as yeasts selected from the genera Kluyveromyces, Candida, Pichia, Cryptococcus, Debaromyces, Hansenula, Yarrowia, Zygosaccharomyces Schizosaccharomyces, Lipomyces.

When incorporating the invention into other yeast types suitable endogenous target genes must be identified as well as a suitable constitutive promoter. The constitutive promoter may be identified by looking into references such as (http://cbi.labri.fr/Genolevures, WO2006125000; WO2006055322)). A regulatory promoter region from a gene with constitutive expression can be cloned and inserted in front of the endogenous target gene to replace the native promoter and mediate stable over-expression (promoter replacement is described in Example 2). e.g. in Y. lipolytica common promoter regions to use for over-expression are TEF, GPD, GPDIN, GPM, FBA and YAT. In hosts that contain the conventional yeast markers, DNA substrates can be amplified by using the described PCR-based gene targeting method (Erdeniz et al, 1997). If the yeast host strain is devoid of markers, genetic modifications can be obtained by using an antibiotic-based technique to isolate and identify transformant cells. Both methods are based on conventional DNA amplification and cloning techniques. The DNA substrates could be designed specifically for each individual host. The

targeted integration of substrates is performed by means of homologous recombination and thus substrates will normally be designed to harbour homology to the endogenous DNA target.

Auxotrophy-based gene targeting

For auxotrophy-based gene-targeting, linear bi-partite PCR-generated gene-targeting substrates are preferentially used. The marker gene is preferentially counter-selectable, allowing for pop-in pop-out recombination, for example the URA3 marker or the TRP1 marker. The counter-selectable URA3 marker that is used for gene targeting in S. cerevisiae can also be used for selection of positive transformants in Y. lipolytica.

Antibiotic resistance-based

Gene targeting in prototrophic yeast strains can be mediated by using antibiotic markers such as the kan^r gene, which renders the yeast transformants resistant to the aminoglycoside antibiotic kanamycin G418. The use of antibiotic markers for identification and isolation of transformants are well known (C. Michels, Genetic Techniques for biological research. Wiley 2002). Targeted integration allowing multiple inserts by re-using a marker system is possible using a system referred to as the loxP-kanMX-loxP system (Güldener et al., 1996). kan^r is flanked by loxP repeats, which again is flanked by sequences homologous to the genetic locus to be disrupted. Positive transformants are isolated on G418-plates. Transformants are furthermore transformed with a plasmid containing the Cre recombinase. Cre-mediated recovery of the kan^r marker is performed upon expression of the Cre recombinase (Wach et al., 1994). The gene of interest is fused to a loxP-kanMX-loxP gene disruption cassette by PCR.

Fatty acid desaturation

The present invention demonstrates methods to increase desaturation of fatty acids in baker's yeast, S. cerevisiae. Desaturation requires the activity of a fatty acid desaturase, cytochrome b5 and cytochrome b5 reductase. NADH-cytochrome b5 reductase is encoded by MCR1 and cytochrome b5 is encoded by CYB5. Recombinant S. cerevisiae cells expressing a heterologous pathway to polyunsaturated fatty acids show increased production of arachidonic acid and gamma-linolenic acid when over-expressing S. cerevisiae CYB5 and MCR1, respectively.

Y. lipolytica

In case it is desirable to introduce the invention into the yeast Y. lipolytica, it is recognizable that Y lipolytica contain both endogenous YALI0D12122g, which is similar to CYB5 and endogenous YALI0D11330g which is similar to MCR1 (http://cbi.labri.fr/Genolevures). Although, the function of the endogenous proteins in Y. lipolytica is not specifically described, it is highly likely that they function as cytochrome b5 and cytochrome b5 reductase in desaturation of fatty acids as Y. lipolytica contain both 18:2 and 18:3 fatty acids. Furthermore is likely, that the activity of native YALI0D12122g and YALI0D11330g of Y. lipolytica can be increased by over-expression. Endogenous YALI0D12122g and YALI0D11330g can be put under the control of a constitutive promoter for over-expression. Furthermore, heterologous CYB5 and MCR1 DNA sequences can be isolated and expressed in Y. lipolytica. CYB5 and MCR1 donor sequences can be obtained from sources such as S. cerevisiae and M. alpina. The genes can be amplified from DNA or cDNA and be introduced into the genome of Y.lipolytica together with a suitable promoter using said methods. Alternatively, they can be chemically synthesized de novo. In addition, the sequences can be codon-optimized for optimal expression in the Y. lipolytica host cell.

Y. lipolytica will most likely display increased levels of desaturation if YALI0D12122g and YALI0D11330g are over-expressed by constitutive promoters as demonstrated in Saccharomyces. Stable genetic integration of promoter sequences and/or heterologous cytochrome b5 and cytochrome b5 reductase can be conducted by the above mentioned auxotrophy-based method where the DNA substrate is cloned by PCR and inserted into the yeast strain by transformation and subsequent homologous recombination integration. Alternatively, stable genetic integration can be conducted by the use of the above described antibiotic resistance-based method.

Fatty acid synthesis

The present invention demonstrates methods to improve the PUFA content in the host organism through metabolic engineering, e.g. through over-expression of fatty acid synthases, or over-expression of an acyltransferase.

LRO1 encodes for an acyltransferase that mediates the esterification of diacylglycerol using phosphatidylcholine as acyl donor. A significant increase in

triglyceride production is expected by over-expressing LRO1. FAS2 encodes the FAS alpha-subunit, and over-expressing FAS2 of the fatty acid synthase complex FAS1/FAS2 increases the content of C18 fatty acids over C16 fatty acids.

Y. lipolytica

In case it is desirable to introduce this aspect of the invention into Y lipolytica it is recognizable that sequence homologues for LRO1 and FAS2 are found in Y. lipolytica as YALI0E16797g and YALI0B19382g respectively (http://cbi.labri.fr/Genolevures). Over-expressing the native lipolytica proteins can possibly increase the protein activity of the corresponding proteins. Consequently, endogenous Y. lipolytica genes can be put under the control of a constitutive promoter for over-expression. Furthermore, heterologous DNA sequences can be isolated and expressed in Y. lipolytica. FAS2 donor sequences can be obtained from other sources such as S. cerevisiae, S. kluyveri, C. albicans, A. nidulans and M. alpine. The sequences can be amplified from DNA or cDNA and be introduced into the genome of Y.lipolytica under the control of a suitable promoter using said methods. Alternatively, they can be chemically synthesized de novo. In addition, the sequences can be codon-optimized for optimal expression in the Y. lipolytica host cell.

Over-expression of LRO1 or FAS2 may lead to elevated PUFA levels in Y. lipolytica. Again, both gene targeting methods might be used for the over-expression of LRO1 and FAS2. The DNA substrates harbouring the promoter sequences can be isolated from Y. lipolytica and inserted at the desired locus in Y. lipolytica by using either of the two gene targeting methods described above. Likewise, heterologous LRO1 and FAS2 can be amplified from other yeasts and integrated in Y. lipolytica by said methods.

Regulation of fatty acids

The present invention demonstrates that metabolic engineering of the regulation mechanism of fatty synthesis increase the fatty acid content in S. cerevisiae. Lipid synthesis is transcriptionally regulated by the positive regulators Ino2 and Ino4 as well as the negative regulator Opi1. Thus, over-expression of the native INO2 and INO4 genes and deletion of the native yeast gene OPI1 is expected to result in a higher lipid yield of the mutated strain.

Y. lipolytica

In case it is desirable to introduce this aspect of the invention into Y it is recognizable that Y. lipolytica exhibit the same type of fatty acid regulation as S. cerevisiae, which corresponds well with the fact that homologues of the regulators in S. cerevisiae can be found in Y. lipolytica (http://cbi.labri.fr/Genolevures). The regulators can also be conserved between the yeast species in respect to function. Consequently, one can transfer the same genetic changes to Y. lipolytica by performing genetic engineering on the genes involved in fatty acid and lipid synthesis regulation. To illustrate this, the homologue of S. cerevisiae INO4 in Y. lipolytica is YALI0C02387g. The function of the protein in Y. lipolytica is unknown, but it is likely that over-expression of this gene will lead to elevated fatty acid synthesis and lipid synthesis activity. In addition, INO2 and INO4 can be amplified from S. cerevisiae where their function is described and introduced into Y. lipolytica by the methods already described. Thus, in order to increase fatty acid synthesis in Y. lipolytica, endogenous YALI0C02387g can be over-expressed by inserting a strong constitutive promoter in front of the gene. Donor sequences can be obtained from sources such as the yeasts S. cerevisiae and M. alpina, or they can be chemically synthesized de novo.

Regulation of fatty acid synthesis can be altered in a Y. lipolytica strain by for instance over-expressing the positive regulator INO4. The substrates for over-expression can be amplified from Y. lipolytica or from another yeast species. Integration of the promoter substrates can be obtained by using the above mentioned auxotrophy-based gene targeting method or by using an antibiotic resistance-based method.

The methods described above for genetic manipulation of S. cerevisiae can with some modifications also be used in other fungi. For example, it has been shown that genomic integration in several different species of Aspergillus can be accomplished using largely the same method as described by Erdeniz et al. (1997). It is likely that this method can also be used for genomic modification of other yeast species, such as Yarrowia lipolytica, Cryptococcus curvatus, Kluyveromyces lactis, Saccharomyces kluyveri and others. Furthermore, plasmid systems designed for Saccharomyce cerevisiae are functional in other similar yeast species. . A plasmid system derived from pARp1 that contains the AMA1

initiating replication sequence from Aspergillus nidulans allows plasmid replication in several different species of Aspergillus (Gems et al., 1991). This shuttle vector containing the replication sequence of Escherichia coli can be used for expression of genes in different species of Aspergillus.

Production of PUFA-containing fungal cells

Recombinant fungal cells containing heterologous pathways to PUFAs, such as GLA, arachidonic acid or EPA, can be grown in batch, fed batch or chemostat cultivation in order to produce high amounts of PUFA-containing biomass. Following harvest of biomass, e.g. by centrifugation or filtration, and possible drying of the biomass to a suitable degree, it can be used as a functional food ingredient, for example as bakers yeast, yeast extract or as a flavour enhancer. The PUFA-containing biomass can also be used directly as a functional food, for example in tablets as an alternative to fish oil capsules.

Thus, the present invention also relates to food products, such as functional food products, wherein said food product has an increased content of polyunsaturated fatty acids when compared to a product produced by a cell, which is not modified for heterologous expression according to the present invention.

Codon usage and optimization

Codon usage usually differs among different species. For the expression of a heterologous protein from an organism that has a different codon usage it is of advantage to alter the codon usage of the heterologous protein to match that of the host cell. Thereby protein expression can be improved. Hence, codon optimization of nucleotide sequences of the corresponding enzymes mentioned in Table 1 and Table 2 and will increase PUFA production.

Thus, a preferred embodiment of the present invention relates to a method according to the present invention, wherein said heterologous nucleotide sequences are codon optimized for expression in Saccharomyces cerevisiae.

PUFA content

In one embodiment, the present invention relates to methods, cells, and compositions relating to an improved polyunsaturated fatty acid content, wherein said heterologous expression, over-expression or deletion increases the content of

each individual specific polyunsaturated fatty acid, particularly ARA and EPA, to more than 3 % of the total fatty acid content, such as 3% of the total fatty acid content, 4% of the total fatty acid content, 5% of the total fatty acid content, 6% of the total fatty acid content, 7% of the total fatty acid content, 8% of the total fatty acid content, 9% of the total fatty acid content, 10% of the total fatty acid, 15% of the total fatty acid content or more.

Thus, in one presently particular preferred embodiment, the method of the invention discloses metabolic engineering which increases the content of arachidonic acid to more than 4 % of the total fatty acid content in the genetically modified fungal cell described herein.

In another particular preferred embodiment, the method of the invention discloses metabolic engineering which increases the content of eicosapentaenoic acid to more than 3 % of the total fatty acid content in the genetically modified fungal cell described herein.

In another particular preferred embodiment, the method of the invention discloses metabolic engineering which increases the content of gamma-linolenic acid to more than 13 % of the total fatty acid content in the genetically modified fungal cell described herein.

In another embodiment the present invention relates methods, cells, and compositions relating to an improved polyunsaturated fatty acid content, wherein said heterologous expression increases the content of each individual specific polyunsaturated fatty acid to more than 0.2% of the biomass dry weight, such as 0.2% of the biomass dry weight, 0.3% of the biomass dry weight, 0.4% of the biomass dry weight, 0.5% of the biomass dry weight, 0.6% of the biomass dry weight, 0.7% of the biomass dry weight, 0.8% of the biomass dry weight, 0.9% of the biomass dry weight, 1% of the biomass dry weight, 2% of the biomass dry weight, 3 of the biomass dry weight, 4% of the biomass dry weight, 5% of the biomass dry weight or more.

In one embodiment, the present invention relates to a *Saccharomyces cerevisiae* comprising EPA and ARA in the ratio of at least 1:1, preferably at least 2:1, most preferably 2.6:1.

In one embodiment of the present invention the metabolic engineered *Saccharomyces cerevisiae* of the present invention comprises at least 0.4 mg EPA pr. gram dry weight cell, such as at least 0.5 mg EPA pr. gram dry weight cell, at least 0,6 mg EPA pr. gram dry weight cell, at least 0.7 mg EPA pr. gram dry weight cell, at least 0,8 mg EPA pr. gram dry weight cell, at least 0.9 mg EPA pr. gram dry weight cell, at least 10.0 mg EPA pr. gram dry weight cell or more,

In one embodiment of the present invention the metabolic engineered *Saccharomyces cerevisiae* of the present invention comprises at least 1,5 mg ARA pr. gram dry weight cell, such as at least 1.6 mg ARA pr. gram dry weight cell, at least 1.7 mg ARA pr. gram dry weight cell, at least 1.8 mg ARA pr. gram dry weight cell, at least 1.9 mg ARA pr. gram dry weight cell, at least 2.0 mg ARA pr. gram dry weight cell, at least 2.1 mg ARA pr. gram dry weight cell or more,

In one embodiment of the present invention the metabolic engineered *Saccharomyces cerevisiae* of the present invention comprises at least 8.0 mg GLA pr. gram dry weight cell, such as at least 8.1 mg GLA pr. gram dry weight cell, at least 8.2 mg GLA pr. gram dry weight cell, at least 8.3 mg GLA pr. gram dry weight cell, at least 8.4 mg GLA pr. gram dry weight cell, at least 8.5 mg GLA pr. gram dry weight cell, at least 8.6 mg GLA pr. gram dry weight cell or more,

In one embodiment of the present invention the metabolic engineered *Saccharomyces cerevisiae* of the present invention comprises at least 24 mg. PUFA pr. gram dry weight cell, such as at least 25 mg PUFA pr. gram dry weight cell, at least 26 mg PUFA pr. gram dry weight cell, at least 27 mg PUFA pr. gram dry weight cell, at least 28 mg PUFA pr. gram dry weight cell, at least 29 mg PUFA pr. gram dry weight cell, at least 30 mg PUFA pr. gram dry weight cell or more,

Vector

Heterologous genes can be expressed in the host organism from extra chromosomal elements. For extra chromosomal expression in e.g. yeast, high

copy number plasmids are preferred. Other yeast vectors include yeast replicating plasmids (YRps), such as the 2μ plasmid, which have a chromosomally derived replicating sequence and are propagated in medium copy-number (20 to 40 copies per cell), and yeast centromere plasmids (Ycps; also known as CEN plasmids), which have both a replication origin and a centromere sequence, ensuring stable segregation. Several yeast expression vectors with differing selection markers can be used in combination when the purpose is to express several heterologous genes. In addition, several heterologous genes can be expressed from the same plasmid, for example using the pESC vectors (Stratagene), which permit simultaneous, inducible expression from the divergent GAL1/GAL10 promoter sequence. A variety of prokaryotic expression systems can be used to express PUFA-synthesizing desaturases and elongases, including the pBR322 plasmid, the pUC plasmids and derivatives thereof. For expression in prokaryotes the heterologous genes can be assembled in an artificial operon, meaning that a single promoter sequence controls the expression of a cluster of genes. Several genes encoding PUFA-synthesizing desaturases and elongases can be fused by PCR and subsequently be subcloned into a bacterial expression vector using standard techniques.

The described heterologous genes encoding, e.g. cytochrome b5, cytochrome b5 reductase and FAS alpha-subunit, can be expressed by means of vectors as described above.

As the skilled addressee would recognise, the individual nucleotide sequences can be expressed either from a single vector or from separate vectors. The skilled artisan is also well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention.

Preferably, the heterologous genes are expressed from several vectors. It can also be advantageous to express one or several heterologous genes in the PUFA pathway from a genomic location.

Once the vector or vectors have been constructed, it may then be introduced into the host cell of choice by methods known to those of ordinary skill in the art including, for example, transfection, transformation and electroporation.

The host cell is then cultured under suitable conditions permitting expression of the genes leading to the improved production of the desired PUFA, which is then recovered and purified.

The genetically modified cell

In another aspect, the present invention relates to a genetically modified cell comprising genomic modifications, a vector or several vectors according to the present invention.

As indicated, a further embodiment of the present invention relates to a genetically modified cell, wherein expression of said isolated nucleotide sequences from said vector results in said cell producing a polyunsaturated fatty acid that is not produced in a wild-type of said host cell.

BRIEF DESCRIPTION OF THE DRAWINGS

The following Detailed Description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, wherein:

Figure 1

Pathway from stearic acid to arachidonic acid, via the delta-6 desaturase/delta-6 elongase route

Figure 2

Pathway from stearic acid to arachidonic acid, via the delta-9 elongase/delta-8 desaturase route

Figure 3

Pathway from stearic acid to eicosapentaenoic acid, via the delta-6 desaturase/delta-6 elongase route

Figure 4

Pathway from stearic acid to arachidonic acid, via the delta-9 elongase/delta-8 desaturase route

Figure 5

Mechanism of fatty acid desaturation.

Figure 6

Promoter replacement using a bi-partite gene targeting substrate

Figure 7

Promoter replacement using a quadruple gene targeting substrate

Figure 8

Plasmid maps over pWAD1 and pWAD2

Figure 9

Plamsmid maps over pWJ716-TD1 and pWJ716-TD2

Figure 10

Method for integration of a heterologous gene into the genome of S. cerevisiae

Figure 11

Yeast vector for expression of genes encoding delta-6 elongase and delta-5 desaturase.

Figure 12

Yeast vector for expression of genes encoding delta-12 desaturase and delta-6 desaturase.

Figure 13

Lipid synthesis pathway.

Figure 14

Gene-targeting strategy for integration of heterologous genes together with a suitable promoter in the genome of S. cerevisiae.

Figure 15

Gene-targeting strategy for integration of M.alpina delta-6 elongase together with the PYK1 promoter in the trp1-289 marker of S. cerevisiae.

Figure 16

Plasmid map of plasmid pSF002

Figure 17

Plasmid map of plasmid pSF003

Figure 18

Plasmid map of pESC-TRP

Figure 19

Plasmid map of pESC-TRP-OtD6D

SEQUENCE ORIGIN

SEQ ID NO: 1 is a nucleotide sequence of from Mortierella alpina encoding a cytochrome b5

SEQ ID NO: 2 is the amino acid sequence encoded by SEQ ID NO: 1

SEQ ID NO: 3 is a nucleotide sequence of from Mortierella alpina encoding a cytochrome b5 reductase

SEQ ID NO: 4 is the amino acid sequence encoded by SEQ ID NO: 3

SEQ ID NO: 5 is a Mortierella alpina amino acid sequence of a cytochrome b5 reductase

SEQ ID NO: 6 is a nucleotide sequence of from Saccharomyces kluyveri encoding a FAS alpha-subunit

SEQ ID NO: 7 is the amino acid sequence encoded by SEQ ID NO: 6

SEQ ID NO: 8 is a nucleotide sequence of from Candida albicans encoding a FAS alpha-subunit

SEQ ID NO: 9 is the amino acid sequence encoded by SEQ ID NO: 8

SEQ ID NO: 10 is a nucleotide sequence of from Candida albicans encoding a FAS beta-subunit

SEQ ID NO: 11 is the amino acid sequence encoded by SEQ ID NO: 10

SEQ ID NO: 12 is a nucleotide sequence of from Aspergillus nidulans encoding a FAS alpha-subunit

SEQ ID NO: 13 is the amino acid sequence encoded by SEQ ID NO: 12

SEQ ID NO: 14 is a nucleotide sequence of from Aspergillus nidulans encoding a FAS beta-subunit

SEQ ID NO: 15 is the amino acid sequence encoded by SEQ ID NO: 14

SEQ ID NO: 16 is a nucleotide sequence of from Yarrowia lipolytica encoding a FAS beta-subunit

SEQ ID NO: 17 is the amino acid sequence encoded by SEQ ID NO: 16

SEQ ID NO: 18 is the S. cerevisiae CYB5 gene, encoding cytochrome b5

SEQ ID NO: 19 is the S. cerevisiae MCR1 gene, encoding cytochrome b5 reductase

SEQ ID NO: 20 is the S. cerevisiae FAS1 gene, encoding FAS beta-subunit

SEQ ID NO: 21 is the S. cerevisiae FAS2 gene, encoding FAS alpha-subunit

SEQ ID NO: 22 is the S. cerevisiae LRO1 gene, encoding phospholipid:diacylglycerol acyltransferase

SEQ ID NO: 23 is the S. cerevisiae SPO14 gene, encoding Phospholipase D

SEQ ID NO: 24 is the S. cerevisiae INO2 gene, encoding a transcription activator

SEQ ID NO: 25 is the S. cerevisiae INO4 gene, encoding a transcription activator

SEQ ID NO: 26 is the S. cerevisiae OPI1 gene, encoding a transcriptional regulator

SEQ ID NO: 27 is the sequence of primer AD-fw

SEQ ID NO: 28 is the sequence of primer T2-2

SEQ ID NO: 29 is the sequence of primer Int3'

SEQ ID NO: 30 is the sequence of primer Int5'

SEQ ID NO: 31 is the sequence of primer AD-rv

SEQ ID NO: 32 is the sequence of primer T-DGA

SEQ ID NO: 33 is the sequence of primer TPI1-fw

SEQ ID NO: 34 is the sequence of primer HX7-fw

SEQ ID NO: 35 is the sequence of primer TPI1-rvP

SEQ ID NO: 36 is the sequence of primer HX7-rvP

SEQ ID NO: 37 is the sequence of primer TPI1-rv

SEQ ID NO: 38 is the sequence of primer HX7-rv

SEQ ID NO: 39 is the sequence of primer Ext 5'

SEQ ID NO: 40 is the sequence of primer Ext 3'

SEQ ID NO: 41 is the sequence of primer CYBU-fw

SEQ ID NO: 42 is the sequence of primer CYBU-rv

SEQ ID NO: 43 is the sequence of primer CYBD-fw

SEQ ID NO: 44 is the sequence of primer CYBD-rv

SEQ ID NO: 45 is the sequence of primer CYB5-C

SEQ ID NO: 46 is the sequence of primer TPI-C

SEQ ID NO: 47 is the sequence of primer MCRU-fw

SEQ ID NO: 48 is the sequence of primer MCRU-rv

SEQ ID NO: 49 is the sequence of primer MCRD-fw

SEQ ID NO: 50 is the sequence of primer MCRD-rv

SEQ ID NO: 51 is the sequence of primer MCR1-C

SEQ ID NO: 52 is the sequence of the yeast ADH1 promoter

SEQ ID NO: 53 is the sequence of the yeast TDH3 promoter

SEQ ID NO: 54 is the sequence of Kluyveromyces lactis URA3

SEQ ID NO: 55 is the sequence of the yeast TPI1 promoter

SEQ ID NO: 56 is the sequence of the yeast HXT7 promoter

SEQ ID NO: 57 is the sequence of the KLURA5 fragment

SEQ ID NO: 58 is the sequence of the KLURA3 fragment

SEQ ID NO: 59 is the sequence of primer GPP1-UP-fw

SEQ ID NO: 60 is the sequence of primer GPP1-UP-rv

SEQ ID NO: 61 is the sequence of primer GSK3-fw

SEQ ID NO: 62 is the sequence of primer SK33-rv-t2

SEQ ID NO: 63 is the sequence of primer GSK3-rv

SEQ ID NO: 64 is the sequence of primer GPP1-D-fw2

SEQ ID NO: 65 is the sequence of primer GPP1-D-rv

SEQ ID NO: 66 is the sequence of primer GPP1-UP-rvMF

SEQ ID NO: 67 is the sequence of primer FAD3-fwMF

SEQ ID NO: 68 is the sequence of primer FAD3-rv

SEQ ID NO: 69 is the sequence of primer F2D-fw

SEQ ID NO: 70 is the sequence of primer F2D-rv

SEQ ID NO: 71 is the sequence of primer F2U-fw

SEQ ID NO: 72 is the sequence of primer F2U-rv

SEQ ID NO: 73 is the sequence of primer ADF2-rv

SEQ ID NO: 74 is the sequence of primer F1D-fw

SEQ ID NO: 75 is the sequence of primer F1D-rv

SEQ ID NO: 76 is the sequence of primer F1U-fw

SEQ ID NO: 77 is the sequence of primer F1U-rv

SEQ ID NO: 78 is the sequence of primer ADF1-rv

SEQ ID NO: 79 is the sequence of primer ADH1ted

SEQ ID NO: 80 is the sequence of primer F1C-rv

SEQ ID NO: 81 is the sequence of primer F2C-rv

SEQ ID NO: 82 is the sequence of primer INO2_up_fw

SEQ ID NO: 83 is the sequence of primer INO2_up_rv

SEQ ID NO: 84 is the sequence of primer INO2_S_fw

SEQ ID NO: 85 is the sequence of primer INO2_S_rv

SEQ ID NO: 86 is the sequence of primer INO2_C

SEQ ID NO: 87 is the sequence of primer INO4_up_fw

SEQ ID NO: 88 is the sequence of primer INO4_up_rv

SEQ ID NO: 89 is the sequence of primer INO4_S_fw

SEQ ID NO: 90 is the sequence of primer INO4_S_rv

SEQ ID NO: 91 is the sequence of primer INO4_C

SEQ ID NO: 92 is the sequence of primer OPI1_up_fw

SEQ ID NO: 93 is the sequence of primer OPI1_up_rv

SEQ ID NO: 94 is the sequence of primer OPI1_D_fw

SEQ ID NO: 95 is the sequence of primer OPI_D_rv

SEQ ID NO: 96 is the sequence of primer LRO1_UP_fw

SEQ ID NO: 97 is the sequence of primer LRO1_UP_rv

SEQ ID NO: 98 is the sequence of primer LRO1_S_fw

SEQ ID NO: 99 is the sequence of primer LRO1_S_rv

SEQ ID NO: 100 is the sequence of primer LRO1-C

SEQ ID NO: 101 is the sequence of primer SPO14_up_fw

SEQ ID NO: 102 is the sequence of primer SPO14_up_rv

SEQ ID NO: 103 is the sequence of primer SPO14_S_fw

SEQ ID NO: 104 is the sequence of primer SPO14_S_rv

SEQ ID NO: 105 is the sequence of primer SPO14_C

SEQ ID NO: 106 is a nucleotide sequence from Aspergillus oryzae encoding a FAS beta-subunit

SEQ ID NO: 107 is the amino acid sequence encoded by SEQ ID NO: 106

SEQ ID NO: 108 is a nucleotide sequence from Aspergillus oryzae encoding a FAS alpha-subunit

SEQ ID NO: 109 is the amino acid sequence encoded by SEQ ID NO: 108

SEQ ID NO: 110 is a nucleotide sequence of from Aspergillus oryzae encoding a NADH-cytochrome b-5 reductase

SEQ ID NO: 111 is the amino acid sequence encoded by SEQ ID NO: 110

SEQ ID NO: 112 is a nucleotide sequence of from Aspergillus oryzae encoding a cytochrome b5

SEQ ID NO: 113 is the amino acid sequence encoded by SEQ ID NO: 112

SEQ ID NO: 114 is the sequence of primer Eloa-fw-MF

SEQ ID NO: 115 is the sequence of primer Elo-T-rv

SEQ ID NO: 116 is the sequence of primer TRP1-5end-fw

SEQ ID NO: 117 is the sequence of primer TRP1-5end-rv

SEQ ID NO: 118 is the sequence of primer TRP1-3end-fw

SEQ ID NO: 119 is the sequence of primer TRP1-3end-rv

SEQ ID NO: 120 is the sequence of primer PYK1-fw

SEQ ID NO: 121 is the sequence of primer PYK1-rv

SEQ ID NO: 122 is the sequence of primer D6E_2212

SEQ ID NO: 123 is the sequence of primer TRP1-C-rv

SEQ ID NO: 124 is the nucleotide sequence of *DCI1* from *S. cerevisiae*

SEQ ID NO: 125 is the amino acid sequence encoded by SEQ ID NO: 124

SEQ ID NO: 126 is the sequence of primer DCI1-UP-fw

SEQ ID NO: 127 is the sequence of primer DCI1-UP-rv

SEQ ID NO: 128 is the sequence of primer DCI1-D-fw

SEQ ID NO: 129 is the sequence of primer DCI1-D-rv

SEQ ID NO: 130 is a codon-optimized nucleotide sequence from Ostreococcus tauri encoding a delta-6 desaturase

SEQ ID NO: 131 is the amino acid sequence encoded by SEQ ID NO: 130

SEQ ID NO: 132 is the sequence of primer D6D-OTi-fw

SEQ ID NO: 133 is the sequence of primer D6D-OTi-rv

SEQ ID NO: 134 is the nucleotide sequence of *FOX2* from *S. cerevisiae*

SEQ ID NO: 135 is the amino acid sequence encoded by SEQ ID NO: 134

SEQ ID NO: 136 is the sequence of primer FOX2-UP-fw

SEQ ID NO: 137 is the sequence of primer FOX2-UP-rv

SEQ ID NO: 138 is the sequence of primer FOX2-D-fw

SEQ ID NO: 139 is the sequence of primer FOX2-D-rv

SEQ ID NO: 140 is the sequence of primer D12Di-fw

SEQ ID NO: 141 is the sequence of primer D12Di-rv

SEQ ID NO: 142 is the nucleotide sequence of *POT1* from *S. cerevisiae*

SEQ ID NO: 143 is the amino acid sequence encoded by SEQ ID NO: 142

SEQ ID NO: 144 is the sequence of primer POT1-UP-fw

SEQ ID NO: 145 is the sequence of primer POT1-UP-rv

SEQ ID NO: 146 is the sequence of primer POT1-D-fw

SEQ ID NO: 147 is the sequence of primer POT1-D-rv

SEQ ID NO: 148 is the sequence of primer D5Di-fw

SEQ ID NO: 149 is the sequence of primer D5Di-rv

SEQ ID NO: 150 is a nucleotide sequence from Ostreococcus tauri encoding a delta 6-desaturase

SEQ ID NO: 151 is the sequence of primer OtD6D-fw

SEQ ID NO: 152 is the sequence of primer OtD6D-rv

SEQ ID NO: 153 is the sequence of primer Ot1T3_fw1

SEQ ID NO: 154 is the sequence of primer Ot1T3_fw2

SEQ ID NO: 155 is the sequence of primer Ot1T3_rv1

SEQ ID NO: 156 is the sequence of primer Ot1T3_rv2

SEQ ID NO: 157 is the sequence of the *S. cerevisiae* PYK1 promoter

SEQ ID NO: 158 is the sequence of primer OPI1_up_fw

SEQ ID NO: 159 is the sequence of primer OPI1_up_rv

SEQ ID NO: 160 is the sequence of primer OPI1_D_fw

SEQ ID NO: 161 is the sequence of primer OPI_D_rv

SEQ ID NO: 162 is the sequence of primer 7001

SEQ ID NO: 163 is the sequence of primer 7002

SEQ ID NO: 164 is the sequence of primer 7003

SEQ ID NO: 165 is the sequence of primer 7004

SEQ ID NO: 166 is the sequence of *S. cerevisiae* INO2 on plasmid pSF003

SEQ ID NO: 167 is the coding sequence of a putative cytochrome b5 from *Y.lipolytica*

SEQ ID NO: 168 is the coding sequence of a putative cytochrome b5 reductase from *Y.lipolytica*

SEQ ID NO: 169 is the coding sequence of a putative phospholipid-DAG acyltransferase from *Y.lipolytica*

SEQ ID NO: 170 is the coding sequence of a putative fatty acid synthase alpha subunit from *Y.lipolytica*

SEQ ID NO: 171 is the coding sequence of a putative Ino4-like transcriptional regulator from *Y.lipolytica*

EXAMPLES

Example 1

General molecular biology methods used in strain construction

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by: Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989). Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art as described by, e.g. Manual of Methods for General Bacteriology (Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E. W., Wood, W.A., Krieg, N.R., and Briggs, G., Eds.) American Society for Microbiology: Washington, D.C. (1994). All chemicals and reagents used for maintenance and growth of cells were obtained from Sigma, DIFCO Laboratories or GIBCO/BRL unless specified otherwise. Restriction enzymes and DNA ligase was purchased from New England Biolabs. All PCR reactions were carried out using the Phusion polymerase (Finnzymes). Oligonucleotides and sequencing services were purchased from MWG Biotech, Ebersberg, Germany. Purification of DNA fragments was carried out using GFX-columns (Amersham) or the QiaexII purification kit (Qiagen).

E. coli DH5α cells were made competent by the Inoue method as described in Sambrook et al., supra. E. coli cells were typically grown at 37°C in Luria Bertani (LB) medium, supplied with 50 mg/l ampicillin where necessary.

Yeast cells were typically grown at 30°C in YPD medium or synthetic complete drop-out medium, and were made competent by a LiAc-based method (Sambrook et al., supra).
Genomic modifications (overexpression and deletion of genes, integration of heterologous genes) were performed by means of homologous recombination using PCR-generated targeting substrates and the K.lactis URA3 gene as a selectable marker, essentially as described in Erdeniz, N., Mortensen, U.H., Rothstein, R. (1997) Genome Res. 7:1174-83. Information on primer design for fusion PCR can be found in the same publication. Generally, fusion of DNA fragments was made possible by using primers with appropriately designed 5'

overhangs for amplification of the original DNA fragments. In all cases, PCR-generated fragments were excised from a 1% agarose gel and purified before proceeding with fusion PCR. Transformants were generally selected on –URA plates, and pop-out of the K.lactis URA3 marker gene was selected for by plating on 5-FOA medium (5-fluoroorotic acid, 750 mg/l). Correct integration of promoters and heterologous genes was verified by PCR, always using one primer annealing to a sequence outside of the target sequence for integration and one primer annealing inside the sequence to be integrated. Gene deletions were also verified by PCR, using primers on both sides of the deleted gene. Generally, PCR-verification of genomic modifications was performed by means of colony-PCR. For colony-PCR, a small amount of cells was dispersed in 10 μl H2O and was placed at -80°C for approximately 30 min, followed by 15 min. incubation at 37°C. The cell suspension was then used as template for PCR.

Methods for combining genetic features by crossing of strains used in Examples are well known and are, e.g., described in: Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1997). Typically, strains of opposite mating types were allowed to mate, diploids were selected and transferred to sporulation medium (20 g/l potassium acetate, 1 g/l glucose, 2.5 g/l yeast extract, pH 7.0) and were allowed to sporulate at 30°C for approximately 3 days. The asci were dissected on a YPD plate using a Singer MSM microscope and micromanipulator dissection microscope. The mating types of the resulting tetrads were scored by replica-plating to a lawn of cells with either a or alpha mating type, incubating at 30°C to allow mating, replica-plating to sporulation medium, and visualizing sporulation by illuminating plates under a 302 nm UV-light source. Auxotrophic markers were scored by replica plating to drop-out plates. Genetic modifications that could not be scored by phenotype were scored by colony-PCR. In general, the same primer sets that were used for verification of genomic integrations or knockouts were also used for colony-PCR scoring of tetrads (see above).

The genetic nomenclature used for describing the genotypes of the strains is as follows: Native yeast genes are written in capital letters, while deleted or mutated native yeast genes are written in small letters. Yeast promoters are indicated by a

small p, for example pADH1, pTDH3 for the ADH1 and TDH3 promoters. Overexpressions of native yeast genes by the promoter-replacement method are indicated by the promoter name followed by the gene name, for example pADH1-FAS1, pTDH3-DGA1 for overexpression of FAS1 with the ADH1 promoter and overexpression of DGA1 with the TDH3 promoter. Disruption of native yeast genes are indicated by a double colon, for example pox1::pTDH3-M. alpina ole1, which means that the POX1 gene has been disrupted and that the TDH3 promoter and the M. alpina ole1 gene has been integrated in its place. Plasmids are written in brackets.

Example 2

Over-expression of yeast genes

Over-expression of native yeasts genes with constitutive yeast promoters is carried out by means of a promoter-replacement method based on a linear, PCR-generated gene-targeting substrate and using K. lactis URA3 as a recyclable marker. An intermediate strain is first generated, where the marker gene is integrated in combination with two copies of the strong constitutive promoter sequence as a direct repeat on each side of the marker gene. The marker gene is then looped out through recombination mediated by the direct repeat, an event which is selected for by plating the intermediate strain on medium containing 5-fluoroorotic acid (5-FOA), which is toxic to cells expressing the URA3 gene. The result is a strain, in which the native promoter has been replaced with the strong constitutive promoter. Integration of the above described promoter sequence and marker gene is directed to the correct location in the genome by means of PCR-generated target sequences.

The above described gene-targeting substrate can be constructed by means of multiple rounds of fusion-PCR. However, to avoid introduction of PCR-generated mutations, it is beneficial to use a bi-partite (figure 6) or even a quadruple gene-targeting substrate (figure 7).

Bi-partite gene targeting substrate

For overexpression with the strong ADH1 and TDH3 yeast promoters, these promoters have been introduced into intermediate working vectors on either side of K.lactis URA3, resulting in the vectors pWAD1, pWAD2, pW-TD1 and pW-TD2 (PCT/DK2005/000372, and figures 8, 9). With these vectors as templates, fragments can be amplified that contain (in the 5′ to 3′ direction) 1) the ADH1 or TDH3 promoter coupled to two thirds of K.lactis URA3 towards the 5′ end, using the primers AD-fw or T2-2 and Int3′, and 2) two thirds of K.lactis URA3 towards the 3′ end coupled to the ADH1 or TDH3 promoter, using the primers Int5′ and AD-rv or T-DGA. Target sequences corresponding to a 300-500 bp sequence upstream of the gene to be overexpressed and a 300-500 bp starting with ATG of the gene to be overexpressed, are amplified from genomic yeast DNA using suitable primers. The reverse primer used for amplification of the upstream target sequence contains a 5′ overhang that allows fusion to fragment 1 described above. The forward primer used for amplification of the target sequence starting with ATG contains a 5′ overhang that allows fusion with fragment 2 described above. Following fusion by PCR of the upstream target sequence with fragment 1, and fusion by PCR of fragment 2 with the target sequence starting with ATG, the two linear substrates as shown in figure 6 are ready for transformation.

Quadruple gene targeting substrate

When using a quadruple gene targeting substrate, a target sequence upstream of the gene to be overexpressed and a target sequence starting with ATG of the gene to be overexpressed are amplified by PCR using genomic yeast DNA as template. Furthermore, the wanted promoter sequence (here the TPI1 promoter or the HXT7 promoter) is amplified by PCR using genomic yeast DNA as template. Two different primer sets are used in the amplification of the promoter sequence. In the first primer set, resulting in promoter fragment 1, the forward primer TPI1-fw or HX7-fw contains a 5′ overhang that allows fusion with the upstream target sequence and the reverse primer TPI1-rvP or HX7-rvP contains a 5′ overhang that allows fusion with the 5′ end of the marker gene K.lactis URA3. In the second primer set, resulting in promoter fragment 2, the forward primer TPI1-fw or HX7-fw contains a 5′ overhang that allows fusion with the 3′ end of K.lactis URA3 and the reverse primer TPI1-rv or HX7-rv contains a 5′ overhang that allows fusion with the target sequence starting with ATG. Promoter fragment 1 and 2 are

identical, except for the end-sequences introduced by the 5′ overhangs of the primers used in amplification. Promoter fragment 1 is fused to the upstream target sequence by PCR and promoter fragment 2 is fused to the target sequence starting with ATG by PCR. These two target sequences are then used for transformation together with the fragments KLURA5, constituting two thirds of K. lactis URA3 towards the 5′ end and KLURA3, constituting two thirds of K.lactis URA3 towards the 3′ end. KLURA5 and KLURA3 are amplified by PCR using a plasmid containing K.lactis URA3 as template and the primers Ext 5′ and Int 3′ for KLURA5 or Int 5′ and Ext 3′ for KLURA3.

Example 3

Integration of heterologous genes into the genome of S. cerevisiae

For integration of heterologous genes into the genome of S. cerevisiae under the control of a suitable yeast promoter, an approach similar to the ones described above is used. K. lactis URA3 is used as a recyclable marker in the integration, and looping out of the marker gene is selected for by plating the intermediate strain on 5-FOA containing medium (figure 10). Targeting of the integration substrate to the desired location in the genome is performed, as described above, by means of PCR-generated target sequences.

Generally, an upstream and a downstream target sequence are amplified by PCR using yeast genomic DNA as template. The primers that are used in the amplification contain suitable 5′ overhang which later allows fusion of the upstream target sequence to the 5´end of the promoter sequence, and fusion of the downstream target sequence to the 3´end of the heterologous gene to be integrated. The heterologous gene is amplified from a suitable source, e.g. a cDNA preparation, by PCR using primers with suitable 5′ overhangs. These overhangs allow fusion of the heterologous gene with the downstream target sequence at the 5′ end of the heterologous gene and fusion with the chosen promoter sequence (for example the TPI1 promoter or the HXT7 promoter) at the 3′ end of the heterologous gene. The chosen promoter sequence is amplified by PCR using yeast genomic DNA as template. The primers used for this amplification contain suitable 5′ overhangs that later allows fusion of the promoter fragment to 1) the

upstream target sequence at the 5′ end of the promoter sequence and to the KLURA5 fragment at the 3′ end of the promoter sequence and 2) the KLURA3 fragment at the 5′ end of the promoter sequence and the downstream target sequence at the 3′ end of the promoter sequence.

Following amplification of these fragments, the upstream target sequence is fused to the promoter fragment by PCR. Furthermore, in a separate reaction, the promoter fragment is fused to the heterologous gene. The promoter-heterologous gene fusion product is further fused to the downstream target sequence by PCR. Finally, a suitable ura3 yeast strain is transformed with 1) the upstream target fragment fused to the promoter fragment, 2) the KLURA5 fragment, 3) the KLURA3 fragment and 4) the promoter-heterologous gene-downstream target fusion product. Transformants are selected on medium lacking uracil, and contain, inserted between the upstream and downstream target sequences, two copies of the chosen promoter as a direct repeat on each side of the marker gene K. lactis URA3, followed by the heterologous gene. Following selection of pop-out recombination on 5-FOA, the resulting ura3 strain contains, inserted between the upstream and downstream target sequences, the chosen promoter followed by the heterologous gene.

Example 4

Description of the plasmids pESC-URA-elo-delta-5, pESC-TRP-delta-12 delta-6 and pESC-LEU-SK33

The plasmid pESC-URA-elo-delta-5 (figure 11) contains a gene encoding delta-6 elongase from Mortierella alpina under the control of the GAL1 promoter and a gene encoding delta-5 desaturase from M. alpina under the control of the GAL10 promoter. Furthermore it contains the yeast URA3 gene for selection in uracil-deficient medium.

The plasmid pESC-TRP-delta-12 delta-6 (figure 12) contains a gene encoding delta-12 desaturase from M. alpina under the control of the GAL1 promoter and a gene encoding delta-6 desaturase from M. alpina under the control of the GAL10

promoter. Furthermore it contains the yeast TRP1 gene for selection in tryptophane-deficient medium.

The plasmid pESC-LEU-SK33 contains a gene encoding omega-3 desaturase from Saccharomyces kluyveri under the control of the GAL1 promoter. It also contains the yeast LEU2 gene for selection in leucine-deficient medium.

Further information on these three plasmids can be found in patent application PCT/DK2005/000372.

Example 5

Chemostat fermentations

Continuous cultivations were performed in Braun Biostat B fermenters (Braun Biotech International). Cells from a 48 h shake flask culture in defined minimal medium (20 g/l glucose, 15 g/L (NH4)2SO4, 1 g/L MgSO4 *7H2O, 14.4 g/L , KH2PO4, /l vitamin solution and 1 ml/l trace metal solution as specified in Example 6) were used for inoculation of 1.0 l medium to an OD600 of 0.2 as measured by using a Hitachi U-1100 spectrophotometer (Tokyo, Japan). The fermentations were carried out at 17 °C and at pH 5.0, controlled by 2M KOH. Foaming was avoided by the addition of 100 µl Antifoam 204 (Sigma-Aldrich, St Louis, Missouri) per liter medium. Aerobic conditions were obtained by sparging the fermentor with sterile air at a flow rate of 1.5-2.5 l/min to ensure that the dissolved oxygen concentration was above 60%. The stirring speed was kept at 800 rpm and the carbon dioxide content of outflowing gas was measured with a Brüel and Kjær acoustic gas analyzer (Brüel & Kjær, Denmark). Following depletion of the carbon source, level controlled continuous fermentation mode at a dilution rate of 0.05 h-1 was applied.

Example 6

Growth media used in chemostat fermentations

The growth medium used in chemostat fermentations contained: 2.5 g/l glucose, 10 g/l galactose, 5 g/l (NH4)2SO4, 3 g/l KH2PO4, 0.5 g/l MgSO4 *7H2O, 1ml/l vitamin solution and 1 ml/l trace metal solution. The vitamin solution contained: 50 mg/L biotin, 1 g/L calcium panthotenate, 1 g/L nicotinic acid, 1 g/L thiamine HCl, 1 g/L pyridoxal HCl and 0.2 g/L para-aminobenzoic acid, while the trace metal solution contained: 15 g/L EDTA, 4.5 g/L ZnSO4·7H2O, 1 g/L MnCl2·2H2O, 0.3 g/L CoCl2·6H2O, 0.4 g/L Na2MoO4·2H2O, 4.5 g/L CaCl2·2H2O, 3 g/L FeSO4·7H2O, 1 g/L H3BO3 and 0.1 g/L KI.

Example 7

HPLC analysis

Glucose, galactose, ethanol, glycerol, acetate, succinate, and pyruvate concentrations in the culture broth were determined by column liquid chromatography (CLC) using a Dionex Summit CLC system (Dionex, Sunnyvale, CA) after removing the cells from the culture broth via centrifugation. An Aminex HPX-87H column (BioRad, Hercules, CA) was used at 60°C with a Waters 410 Differential refractive index detector (Millipore, Milford, MA) and a Waters 486 Tuneable Absorbance Detector (UV detector) set at 210 nm. The two detectors were connected in series. As mobile phase 5 mM H2SO4 was used at a flow rate of 0.6 ml/min.

Example 8

Biomass dry weight determination

The cell dry weight was determined by filtering a known volume of the culture broth through a pre-dried, pre-weighed 0.45 µm Supor membrane (Pall Corporation, Ann Arbor, MI) filter. After washing with 1 volume of distilled water and drying in microwave oven for 15 minutes at 150 W, the filter was weighed again.

Example 9

Total lipid analysis

For analysis of total lipid yield, the biomass was separated through centrifugation for 5 minutes at 5000 rpm. The biomass was re-dissolved in 10 ml distilled water and the resulting cell suspension was broken using the glass bead method to generate cell extract.

The cell extract was prepared by addition of 1 ml glass beads with a particle size of 250-500 µm (Sigma-Aldrich, St Louis, Missouri) to 1 ml cell suspension in a micro tube with screw cap (Sarstedt, Germany). For each cell suspension 6 tubes were processed. The tubes were shaken at level 4 for 20 seconds in a FastPrep FP120 instrument (Qbiogene, France). This was done in total 6 rounds for each tube with a 5 minutes intervening cooling of the tubes on ice after 3 rounds. The cell extracts were combined in 2 ml eppendorf tubes by transferring 600 µl cell extract to generate 3 eppendorf tubes each containing 1.2 ml glass bead free cell extract. 1 ml of the cell extract was transferred into a glass tube with screw cap containing 20 ml chloroform/methanol 2:1. The tube was sparged with nitrogen then closed immediately and placed on a rotary mixer and the total lipid extraction was performed over night. This was done in triplicates. The extract was then filtered through a Whatman filter (Whatman International, England) and the collected solvent was washed with 4 ml 0.9% NaCl and finally dried over nitrogen in pre-weighed 10 ml glass tubes. The tubes with dry lipid fraction were weighed and the lipid yield was determined by calculating the lipid dry weight divided by the dry weight of the biomass in 1 ml of the initial cell suspension.

Example 10

Transesterification of lipids and GC-MS analysis

Dry lipid was generated as for the determination of total lipid (Example 9) and was dissolved in 1 ml toluene and 2 ml 1 % sulphuric acid in methanol was added. The tube was closed after mixing and sparging with nitrogen and left at 50°C over night for transesterification of the lipids. The sample was then washed with 5 ml 5% NaCl solution. Methyl esters were subsequently extracted twice by adding 5

ml hexane, vortexing the sample and collecting the organic upper phase. The organic phase was washed with 4 ml 2% sodium carbonate and the organic phase was collected again. Trace of water phase was removed by adding anhydrous sodium sulphate and filtering the sample through a Whatman filter paper (Whatman International, England) to remove the sodium sulphate. The hexane phase was then dried under a stream of nitrogen. When dry, the sample was redissolved in 0.5 ml of hexane containing 0.01% butylated hydroxytoluene (BHT) (Sigma-Aldrich, St Louis, Missouri) for protection of double bonds was added and the sample was analyzed for methyl esters. The analysis was performed using a gas chromatograph coupled to mass selective detector (GC/MS). The GC/MS system was a Hewlett Packard HP G1723A with a gas chromatograph-quadruple mass selective detector (EI) operated at 70 eV. The column used was Supelco SPTM-2380. The MS was operated in SCAN Mode. The oven temperature was initially 170°C and in the following risen to 220°C at 4°C/min. The final temperature was held for 15 min. The flow through the column was 0.6 ml He/min. Injection volumes were 1 - 5 µl. The injector was driven at split of 100:1 splitless for all analyses. The temperature of the inlet was 300°C, the interface temperature 230 °C, and the quadropule temperature 105°C. Detected fatty acid methyl esters were confirmed with the 1998 NIST Mass Spectral Database, and retention times were confirmed with standard fatty acid methyl esters.

Example 11

Generation of strain FS01467, overexpressing CYB5 and producing GLA and ARA

The native yeast gene CYB5 was overexpressed with the TPI1 promoter using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of CYB5 was amplified from genomic DNA by PCR using the primers CYBU-fw and CYBU-rv and was fused to the TPI1 promoter fragment 1 by PCR. Furthermore a target sequence corresponding to the 5' end of CYB5 was amplified from genomic DNA using the primers CYBD-fw and CYBD-rv. The downstream target sequence was fused to the TPI1 promoter fragment 2 by PCR.

The yeast strain FS01440 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2) was transformed with the two linear fusion substrates described above and additionally the overlapping marker fragments KLURA5 and KLURA3. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Transformants were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-CYB5 and was named FS01455. Correct integration of the TPI1 promoter was verified by PCR using the primers CYB5-C and TPI-C.

The yeast strain FS01455 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-CYB5) was then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain FS01467 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-CYB5 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 12

Generation of strain FS01468, overexpressing MCR1 and producing ARA

The native yeast gene MCR1 was overexpressed with the TPI1 promoter using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of MCR1 was amplified from genomic DNA by PCR using the primers MCRU-fw and MCRU-rv and was fused to the TPI1 promoter fragment 1 by PCR. Furthermore a target sequence corresponding to the beginning of MCR1 was amplified from genomic DNA using the primers MCRD-fw and MCRD-rv. The downstream target sequence was fused to the TPI1 promoter fragment 2 by PCR.

The yeast strain FS01440 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2) was transformed with the two linear fusion substrates described above and additionally the overlapping marker fragments KLURA5 and KLURA3. Transformants were selected on medium lacking uracil and were streak-purified

on the same medium. The resulting strain was named FS01456 and had the genotype MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-K.lactis URA3-pTPI1-MCR1. Correct integration of the TPI1 promoter was verified by PCR using the primers MCR1-C and TPI-C. To select for looping out of the marker gene, FS01456 was then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 and was named FS01457.

The yeast strain FS01457 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain FS01468 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 13

Generation of strain FS01490, overexpressing MCR1 and producing ARA

Strain FS01456 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-K.lactis URA3-pTPI1-MCR1) was crossed with strain FS01408 (MATα ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1). The resulting diploid was allowed to sporulate and the ascospores were dissected. Among the resulting haploid strains, a strain with the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-K.lactis URA3-pTPI1-MCR1 was identified and was named FS01488. In addition, a strain with the genotype MATalpha ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-K.lactis ura3-pTPI1-MCR1 was identified and was named FS01492. To select for looping out of the marker gene K.lactis URA3, FS01488 was transferred to 5-FOA plates and the resulting pop-out recombinants were streak purified on the same medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 and was named FS01494. FS01494 was transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain FS01490

(MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 14

Generation of a S. cerevisiae strain overexpressing MCR1 and producing EPA

To integrate S. kluyveri FAD3 into the genome of S. cerevisiae, a fragment containing the GAL1 promoter fused to S. kluyveri FAD3 was amplified from plasmid pESC-LEU-SK33, using the primers GSK3-fw and SK33-rv-t2. In addition, an upstream targeting sequence was amplified by PCR using genomic yeast DNA as template and using the primers GPP1-UP-fw and GPP1-UP-rv, and a downstream targeting sequence was amplified by PCR using genomic yeast DNA as template and using the primers GPP1-DOWN-fw-2 and GPP1-DOWN-rv. Furthermore a GAL1 promoter fragment was amplified by PCR, using plasmid pESC-LEU-SK33 as template and using primers GSK3-fw and GSK3-rv. The upstream target sequence was then fused to the GAL1 promoter fragment using the primers GPP1-UP-fw and GSK3-rv, while the fragment containing the GAL1 promoter fused to S. kluyveri FAD3 was fused by PCR to the downstream target sequence, using primers GSK3-fw and GPP1-DOWN-rv. The yeast strain FS01458 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-TSC13 pADH1-FAS1) was transformed with the two resulting fusion products and additionally the overlapping marker fragments KLURA5 and KLURA3. Transformants were selected on medium lacking uracil, and popout of the marker gene was selected for by plating on 5-FOA containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 leu2-3 pox1::pTDH3-M.alpina OLE1 pADH1-TSC13 pADH1-FAS1 gpp1::pGAL1-S.kluyveri FAD3 and was named FS01480.

The strain FS01480 was crossed to strain FS01492 (MATalpha ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-K.lactis ura3-pTPI1-MCR1). The resulting diploid was allowed to sporulate and the ascospores were dissected. Among the resulting haploid strains, a strain with the genotype MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-K.lactis URA3-pTPI1-MCR1 gpp1::pGAL1-S.kluyveri FAD3 was

selected and named FS01504. Following looping out of the selection marker in FS01504 on medium containing 5-FOA, strain FS01505 was obtained (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 gpp1::pGAL1-S.kluyveri FAD3).

To replace the GAL1 promoter with the HXT7 promoter in front of S.kluyveri FAD3 in FS01505, promoter replacement with a quadruple gene targeting substrate was carried out according to the procedure described in Example 2. Target sequences upstream and downstream of the GAL1 promoter, respectively, were amplified by PCR using genomic DNA from FS01505 as template and the primer pairs GPP1-UP-fw and GPP1-UP-rvMF for the upstream target and FAD3-fwMF and FAD3-rv for the downstream target. The upstream target was fused to the HXT7 promoter fragment 1, while the downstream target was fused to the HXT7 promoter fragment 2 by PCR. The yeast strain FS01505 was then transformed with the two fusion products, in addition to the two overlapping marker fragments KLURA5 and KLURA3. Transformants were selected on medium lacking uracil and the resulting strain was named FS01510 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 gpp1::pHXT7-Klactis URA3-pHXT7-S.kluyveri FAD3). Popout of the selection marker in FS01510 was selected for by plating the strain on medium containing 5-FOA, and the resulting strain was named FS01511 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 gpp1::pHXT7-S.kluyveri FAD3).

FS01511 was transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain FS01524 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 gpp1::pHXT7-S.kluyveri FAD3 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6])

Furthermore, FS01511 is crossed to a sutaible strain, e.g. FS01408 (MATalpha ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1). The resulting diploid strain is allowed to sporulate and the ascospores are dissected. Among the resulting haploid strains, strains with desired genotypes are selected, for example a strain with the same genotype as FS01511 but without the leu2-3_112 mutation, and a

strain with the same genotype as FS01511 but without the leu2-3_112 mutation and without MCR1 over-expression. These strains are transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, and the influence of MCR1 over-expression on EPA production is investigated. In addition, strains with MATalpha mating type and containing the gpp1::pHXT7-S.kluyveri FAD3 modification are selected and are further used in the construction of new strains.

Example 15

Generation of a recombinant S. cerevisiae strain overexpressing CYB5 and producing EPA

The yeast strain FS01455 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-CYB5) is crossed to a suitable yeast strain with MATalpha mating type and containing the gpp1::pHXT7-S.kluyveri FAD3 modification. The resulting diploid strain is allowed to sporulate and the ascospores are dissected. Among the resulting haploid strains, a strain containing both CYB5 over-expression and the gpp1::pHXT7-S.kluyveri FAD3 modification is selected. The strain is transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, and the influence of CYB5 over-expression on EPA production is investigated.

Example 16

Overexpression of MCR1 results in increased ARA production

In one experiment, the influence of MCR1 overexpression was investigated in a genetic background carrying overexpressions of the native yeast genes FAS1, FAS2 and TSC13 (details about these genetic modifications can be found in co-pending patent application PCT/DK2005/000372). The yeast strain FS01468 carries MCR1 overexpression with the TPI1 promoter in the mentioned genetic background, and in addition expresses the pathway to ARA from multi-copy plasmids. As a reference, strain FS01442 was also analyzed; FS01442 has the same genotype as FS01468 but without MCR1 overexpression.

The strains were grown in chemostat cultivation at 17°C and D=0.05 h-1. At steady state, samples were taken for analysis of fatty acid composition as described in the above Examples.

Table 3. Fatty acid composition (% of total fatty acid) of FS01442 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]) and FS01468 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]) cultivated in chemostat fermentation.

Fatty acid		FS01442 (Reference)	FS01468 (pTPI1-MCR1)
10:0	Capric acid	0.5	
16:0	Palmitic acid	14.5	15.1
16:1	Palmitoleic acid	43.7	37.5
16:2	-	5.0	5.4
16:3			3.2
18:0	Stearic acid	4.7	6.0
18:1	Oleic acid	9.1	10.4
18:2 (n-6)	Linoleic acid (LA)	11.7	9.3
18:3 (n-6)	Gamma-linolenic acid (GLA)	7.4	6.6
20:3 (n-6)	Dihomo-gamma-linolenic acid (DHGLA)	0.3	0.6
20:4 (n-6)	Arachidonic acid (ARA)	0.8	2.2
misc	Other FA's	2.3	3.5
SUM		100.0	100.0
SUM, Saturated FA		19.7	21.1
SUM, MUFA		52.8	47.9
SUM, PUFA (≥2 double bonds)		25.13	27.42

SUM, n-6 PUFA (≥3 double bonds)		8.5	9.5
SUM, n-3 PUFA (≥3 double bonds)		-	-
Lipid content (weight % of dw)		11.3 +/- 0.2	11.8 +/- 1.1

Analysis of the fatty acid composition of the two strains (Table 3) shows that the percentage of ARA was increased more than two fold in FS01468 (overexpressing MCR1) compared to FS01442 (reference strain).

Example 17

Overexpression of CYB5 results in increased fatty acid desaturation and increased gamma-linolenic acid production

To assess the effects of increased cytochrome b5 activity, CYB5 was overexpressed with the TPI1 promoter in a genetic background carrying overexpressions of the native yeast genes FAS1, FAS2 and TSC13. The strain FS01467 overexpresses CYB5 in the mentioned genetic background and also expresses the pathway to ARA from high-copy plasmids. As a reference, the strain FS01442 (same genetic background but without CYB5 overexpression) was also analyzed under the same conditions. The strains were grown in chemostat cultivation at 17°C and D=0.05 h-1, and samples were taken for analysis of fatty acid composition at steady state.

Surprisingly, the results of the fatty acid composition analysis (Table 4) show that overexpression of CYB5 results in increased percentage of C-18 fatty acids at the expense of C-16 fatty acids. In addition, CYB5 overexpression results in increased fatty acid desaturation and thereby substantially increased amounts of linoleic acid (9,12-18:2) and gamma-linolenic acid (6,9,12-18:3).

Table 4. Fatty acid composition (% of total fatty acid) of FS01442 (MATa ura3-52 trp1-289 pADH1-TSC13 pADH1-FAS1 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]) and FS01467 (MATa ura3-52 trp1-289 pADH1-

TSC13 pADH1-FAS1 pADH1-FAS2 pTPI1-CYB5 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]) cultivated in chemostat fermentation.

Fatty acid		FS01442 (Reference)	FS01467 (pTPI1-CYB5)
10:0	Capric acid	0.55	
16:0	Palmitic acid	14.48	12.48
16:1	Palmitoleic acid	43.70	25.21
16:2	-	4.98	8.90
16:3			4.56
18:0	Stearic acid	4.68	7.92
18:1	Oleic acid	9.14	6.44
18:2 (n-6)	Linoleic acid (LA)	11.70	16.84
18:3 (n-6)	Gamma-linolenic acid (GLA)	7.35	13.65
20:3 (n-6)	Dihomo-gamma-linolenic acid (DHGLA)	0.31	0.00
20:4 (n-6)	Arachidonic acid (ARA)	0.79	0.14
misc	Other FA's	2.3	3.87
SUM		100.0	100.0
SUM, Saturated FA		19.71	20.40
SUM, MUFA		52.84	31.65
SUM, PUFA (≥2 double bonds)		25.13	44.08
SUM, n-6 PUFA (≥3 double bonds)		8.45	13.79
SUM, n-3 PUFA (≥3 double bonds)		-	-
Lipid content (weight % of dw)		11.3 +/- 0.2	8.7 +/- 0.5

Example 18

Generation of a S. cerevisiae strain expressing a heterologous cytochrome b5

To isolate a heterologous gene encoding cytochrome b5 from Mortierella alpina (SEQ ID NO 1), cDNA is first prepared from M. alpina.

M. alpina CBS 608.70 is cultivated in 100 ml GY medium (20 g/L glucose, 10 g/L yeast extract pH 6.0) at room temperature for 3 days. Biomass is collected by filtration and total RNA is isolated using Trizol reagent (Gibco BRL). Approximately 5 μg of RNA is used for reverse transcription (Superscript II RT, Invitrogen) using Oligo(dT)12-18 as primer. After first strand cDNA sythesis, complementary RNA is removed by RNAse digestion. The cDNA is then used as template for PCR (Phusion enzyme, Finnzymes), using primers designed to target the cytochrome b5 encoding gene (SEQ ID NO 1) and containing suitable 5' overhangs.

For integration of the M. alpina gene encoding cytochrome b5 into the genome of S. cerevisiae under the control of a strong yeast promoter, the strategy described in example 3 is used. The gene targeting fragments are transformed into a suitable yeast strain, for example FS01396 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2). Following selection on medium lacking uracil, and looping out of the marker on medium containing 5-FOA, the resulting strain carries the M. alpina gene encoding cytochrome b5 integrated in the genome under the control of a strong yeast promoter and is ura3. The strain is then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, and the influence of the heterologous cytochrome b5 on ARA production is evaluated.

Example 19

Generation of a S. cerevisiae strain expressing a heterologous cytochrome b5 reductase

To isolate a heterologous gene encoding cytochrome b5 reductase from Mortierella alpina (SEQ ID NO 3), cDNA is first prepared from M. alpina.

M. alpina CBS 608.70 is cultivated in 100 ml GY medium (20 g/L glucose, 10 g/L yeast extract pH 6.0) at room temperature for 3 days. Biomass is collected by filtration and total RNA is isolated using Trizol reagent (Gibco BRL). Approximately 5 μg of RNA is used for reverse transcription (Superscript II RT, Invitrogen) using Oligo(dT)12-18 as primer. After first strand cDNA sythesis, complementary RNA is removed by RNAse digestion. The cDNA is then used as template for PCR (Phusion enzyme, Finnzymes), using primers designed to target the cytochrome b5 reductase encoding gene (SEQ ID NO 3) and containing suitable 5' overhangs.

For integration of the M. alpina gene encoding cytochrome b5 reductase into the genome of S. cerevisiae under the control of a strong yeast promoter, the strategy described in example 3 is used. The gene targeting fragments are transformed into a suitable yeast strain, for example FS01396 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2). Following selection on medium lacking uracil and looping out of the marker on medium containing 5-FOA, the resulting strain carries the M. alpina gene encoding cytochrome b5 reductase integrated in the genome under the control of a strong yeast promoter and is ura3. The strain is then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, and the influence of the heterologous cytochrome b5 reductase on ARA production is evaluated.

Example 20

Generation of a S. cerevisiae strain overexpressing FAS2 and producing arachidonic acid

FAS2 was over-expressed by replacing its native promoter with the ADH1 promoter, using a bi-partite gene targeting substrate as described in Example 2.

A target sequence upstream of FAS2 was amplified from genomic DNA by PCR using the primers F2U-fw and F2U-rv and a target sequence corresponding to the beginning of FAS2, starting with ATG, was amplified from genomic DNA using the primers F2D-fw and F2D-rv. The ADH1 promoter/K.lactis URA3 fragment 1 was amplified by PCR using the plasmid pWAD2 as template and the primers AD-fw

and Int3´, and was fused to the upstream target sequence by PCR using primers F2U-fw and Int3´. The K. lactis URA3/ADH1 promoter fragment 2 was amplified by PCR using the plasmid pWAD1 as template and the primers Int5´and ADF2-rv, and was fused to the downstream target by PCR using the primers Int5´and F2D-rv.

The yeast strain FS01202 (MATa ura3-52) was transformed with the two linear fusion substrates described above. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Correct integration of the ADH1 promoter was verified by PCR using the primers ADH-ted and F2C-rv. To select for looping out of the marker gene, the transformant was transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 pADH1-FAS2 and was named FS01352. Correct integration of the ADH1 promoter and absence of PCR-generated mutations was verified by sequencing of the modified region.

FS01352 was crossed to FS01269 (MATalpha trp1-289) and the resulting diploid strain was allowed to sporulate and the ascospores dissected. Among the resulting haploid strains, the strain FS01518 (MATa ura3-52 trp1-289 pADH1-FAS2) was selected since it allows transformation of the URA3- and TRP1-based plasmids overexpressing the genes in the ARA pathway. FS01518 was transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain FS01520 (MATa ura3-52 trp1-289 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 21

Generation of a S. cerevisiae strain overexpressing FAS1 and producing arachidonic acid

FAS1 was over-expressed by replacing its native promoter with the ADH1 promoter, using a bi-partite gene targeting substrate as described in Example 2.

A target sequence upstream of FAS1 was amplified from genomic DNA by PCR using the primers F1U-fw and F1U-rv and a target sequence corresponding to the beginning of FAS1, starting with ATG, was amplified from genomic DNA using the primers F1D-fw and F1D-rv. The ADH1 promoter/K.lactis URA3 fragment 1 was amplified by PCR using the plasmid pWAD2 as template and the primers AD-fw and Int3´, and was fused to the upstream target sequence by PCR using primers F1U-fw and Int3´. The K. lactis URA3/ADH1 promoter fragment 2 was amplified by PCR using the plasmid pWAD1 as template and the primers Int5´and ADF1-rv, and was fused to the downstream target by PCR using the primers Int5´and F1D-rv.

The yeast strain FS01202 (MATa ura3-52) was transformed with the two linear fusion substrates described above. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Correct integration of the ADH1 promoter was verified by PCR using the primers ADH-ted and F1C-rv. To select for looping out of the marker gene, the transformant was transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 pADH1-FAS1 and was named FS01351. Correct integration of the ADH1 promoter and absence of PCR-generated mutations was verified by sequencing of the modified region.

FS01351 was crossed to FS01269 (MATalpha trp1-289) and the resulting diploid strain was allowed to sporulate and the ascospores dissected. Among the resulting haploid strains, the strain FS01517 (MATa ura3-52 trp1-289 pADH1-FAS1) was selected since it allows transformation of the URA3- and TRP1-based plasmids overexpressing the genes in the ARA pathway. FS01517 was transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain FS01519 (MATa ura3-52 trp1-289 pADH1-FAS1 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 22

Generation of a S. cerevisiae strain over-expressing FAS1 and FAS2 and producing arachidonic acid

To combine overexpression of FAS1 and FAS2 in one strain, the FAS1 overexpressing strain FS01351 (MATa ura3-52 pADH1-FAS1) was first crossed to the strain FS01269 (MATalpha trp1-289). Diploids were allowed to sporulate, and the asci were dissected into spore tetrads. From the set of haploid strains derived from the cross, a strain with the genotype MATalpha trp1-289 pADH1-FAS1 was selected and named FS01342.

FS01342 (MATalpha trp1-289 pADH1-FAS1) was then crossed to FS01352 (MATa ura3-52 pADH1-FAS2). Following transfer of the diploids to sporulation medium, asci were dissected into ascospore tetrads. From the set of haploid strains derived from the cross, a strain with the genotype MATa ura3 trp1 pADH1-FAS1 pADH1-FAS2 was selected and was named FS01372. FS01372 was transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in strain FS01373 (MATa ura3-52 trp1-289 pADH1-FAS1 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6].

Example 23

Overexpression of FAS2 results in increased ARA production

To assess the effects of overexpressing FAS2, the strain FS01520 overexpressing FAS2 and also expressing the pathway to ARA from high-copy plasmids was analysed in chemostat cultivation. As a reference, the strains FS01324 (no overexpression of FAS1 and FAS2 genes, MATa ura3-52 trp1-289 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]) and FS01373 (overexpression of both FAS1 and FAS2 genes, MATa ura3-52 trp1-289 pADH1-FAS1 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]) were also analyzed under the same conditions. The strains were grown in chemostat cultivation at 17°C and D=0.05 h-1, and samples were taken for analysis of fatty acid composition at steady state.

The results of the fatty acid composition analysis (Table 5) show that overexpression of FAS2 results in increased percentage of C-18 fatty acids at the expense of C-16 fatty acids. In addition, FAS2 overexpression results in increased amounts of 5,8,11,14-eicosatetraenoic acid (ARA).

Table 5. Fatty acid composition (% of total fatty acid) of FS01324 (MATa ura3-52 trp1-289 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]), FS01373 (MATa ura3-52 trp1-289 pADH1-FAS1 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]), and FS01520 (MATa ura3-52 trp1-289 pADH1-FAS2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6 . cultivated in chemostat fermentation.

Fatty acid		FS01324 (Reference)	FS01373 (pADH1-FAS1 pADH1-FAS2)	FS01520 (pADH1-FAS2)
12:0	Lauric acid	0.48	0.53	
14:0	Myristic acid			
16:0	Palmitic acid	15.18	13.22	14.40
16:1	Palmitoleic acid	46.79	47.43	42.87
16:2	-	6.58	5.59	6.21
16:3	-	3.82	3.94	2.35
18:0	Stearic acid	3.51	3.33	5.11
18:1	Oleic acid	7.75	8.95	10.97
18:2(n-6)	Linoleic acid (LA)	8.00	7.52	5.79
18:3(n-6)	Gamma-linolenic acid (GLA)	4.79	5.69	3.31
20:3(n-6)	Dihomo-gamma-linolenic acid (DHGLA)	0.50	0.51	0.59
20:4(n-6)	Arachidonic acid (ARA)	0.80	1.23	1.53
misc	Other FA's	1.8	2.06	5.4
SUM		100	100	100

SUM, Saturated FA	19.18	17.08	19.51
SUM, MUFA	54.54	56.38	53.84
SUM, PUFA (³ 2 double bonds)	24.50	24.48	19.78
SUM, n-6 PUFA (³3 double bonds)	6.09	7.43	5.43
SUM, n-3 PUFA (³3 double bonds)	-	-	-
Lipid content (weight % of dw)	10.4 +/- 1.0	11.7 +/- 1.0	11.7 +/- 5.8

Example 24

Generation of a S. cerevisiae strain overexpressing LRO1 and producing polyunsaturated fatty acids

The native yeast gene LRO1 (SEQ ID NO: 22) was overexpressed with the TPI1 promoter using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of LRO1 was amplified from genomic DNA by PCR using the primers LRO1_UP_fw and LRO1_UP_rv and was fused to the TPI1 promoter fragment 1 by PCR. Furthermore a target sequence corresponding to the 5′ end of LRO1 was amplified from genomic DNA using the primers LRO1_S_fw and LRO1_S_rv. The downstream target sequence was fused to the TPI1 promoter fragment 2 by PCR.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and additionally the overlapping marker fragments KLURA5 end and KLURA3. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Transformants were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-

MCR1 pTPI1-LRO1. Correct integration of the TPI1 promoter was verified by PCR using the primers LRO1-C and TPI-C.

The yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pTPI1-LRO1) was then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in a yeast strain with the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pTPI1-LRO1 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 25

Generation of a S. cerevisiae strain overexpressing SPO14 and producing polyunsaturated fatty acids

The native yeast gene SPO14 (SEQ ID NO: 23) was overexpressed with the TPI1 promoter using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of SPO14 was amplified from genomic DNA by PCR using the primers SPO14_UP_fw and SPO14_UP_rv and was fused to the TPI1 promoter fragment 1 by PCR. Furthermore a target sequence corresponding to the 5′ end of SPO14 was amplified from genomic DNA using the primers SPO14_S_fw and SPO14_S_rv. The downstream target sequence was fused to the TPI1 promoter fragment 2 by PCR.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and additionally the overlapping marker fragments KLURA5 end and KLURA3. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Transformants were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-

MCR1 pTPI1-SPO14. Correct integration of the TPI1 promoter was verified by PCR using the primers SPO14-C and TPI-C.

The yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pTPI1-SPO14) was then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pTPI1-SPO14 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 26

Generation of a S. cerevisiae strain overexpressing INO2 and producing polyunsaturated fatty acids

The native yeast gene INO2 (SEQ ID NO: 24) was overexpressed with the PYK1 promoter using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of INO2 was amplified from genomic DNA by PCR using the primers INO2_UP_fw and INO2_UP_rv and was fused to the PYK1 promoter fragment 1 by PCR. Furthermore a target sequence corresponding to the 5′ end of INO2 was amplified from genomic DNA using the primers INO2_S_fw and INO2_S_rv. The downstream target sequence was fused to the PYK1 promoter fragment 2 by PCR.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and additionally the overlapping marker fragments KLURA5 end and KLURA3. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Transformants were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-mole1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1

pPYK1-INO2. Correct integration of the PYK1 promoter was verified by PCR using the primers INO2-C and PYK-C.

The yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pPYK1-INO2) was then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pPYK1-INO2 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 27

Generation of a S. cerevisiae strain overexpressing INO4 and producing polyunsaturated fatty acids

The native yeast gene INO4 was overexpressed with the HXT7 promoter using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of INO4 (SEQ ID NO: 25) was amplified from genomic DNA by PCR using the primers INO4_UP_fw and INO4_UP_rv and was fused to the HXT7 promoter fragment 1 by PCR. Furthermore a target sequence corresponding to the 5′ end of INO4 was amplified from genomic DNA using the primers INO4_S_fw and INO4_S_rv. The downstream target sequence was fused to the HXT7 promoter fragment 2 by PCR.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and additionally the overlapping marker fragments KLURA5 end and KLURA3. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. Transformants were then transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pHXT7-INO4. Correct integration of the HXT7 promoter was verified by PCR using the primers INO4-C and HXT-C.

The yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pHXT7-INO4) was then transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pHXT7-INO4 [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 28

Generation of a S. cerevisiae strain carrying a deletion of OPI1 and producing polyunsaturated fatty acids

The native yeast gene OPI1 (SEQ ID NO: 26) is deleted using a quadruple gene targeting substrate according to the following procedure:

A target sequence upstream of OPI1 is amplified from genomic DNA by PCR using the primers OPI1_up_fw and OPI1_up_rv and is fused to the two thirds of the K. lactis URA3 gene to the 5′ end by PCR. Furthermore a target sequence corresponding to the downstream region of OPI1 is amplified from genomic DNA using the primers OPI1-D-fw and OPI1_d_rv. The downstream target sequence is fused to the two thirds of the K. lactis URA3 gene to the 3′ end by PCR.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) is transformed with the two linear fusion substrates described above containing the upstream target region and the downstream target region of the gene to be deleted fused to either two thirds of the K. lactis URA3 gene. Transformants are selected on medium lacking uracil and are streak-purified on the same medium. Transformants are transferred to plates containing 5-FOA. Pop-out recombinants are streak-purified on 5-FOA-containing medium. The resulting strain has the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 opi1Δ. Correct deletion of the OPI1 gene is verified by PCR using the primers OPI1_up_fw and OPI1_D_rv.

The yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 opi1Δ) is transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in the yeast strain (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 opi1Δ [pESC-URA-elo-delta-5] [pESC-TRP-delta-12 delta-6]).

Example 29

Generation of a S. cerevisiae strain overexpressing S. kluyveri FAS2 and producing polyunsaturated fatty acids

The FAS2 gene product of S. kluyveri contains three domains- β-ketoacyl synthase, β-ketoacyl reductase and acyl carrier protein. To express S. kluyveri FAS2 in S. cerevisiae, the gene is amplified by PCR using genomic DNA from S. kluyveri as template. The defined primers used for the amplification are designed to match the published sequence encoding FAS2 from S. kluyveri. The amplified gene is then put under the control of a constitutive promoter through integration into the genome of a suitable S. cerevisiae strain, e.g. FS01516 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1), using the strategy described in Example 3. The resulting strain is transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in an arachidonic acid producing strain. Furthermore, in a separate experiment, the strain is transformed with the plasmids pESC-URA-elo-delta-5, pESC-TRP-delta-12 delta-6 and pESC-LEU-SK33, resulting in an eicosapentaenoic acid producing strain.

Example 30

Generation of a S. cerevisiae strain expressing a heterologous FAS from Aspergillus nidulans and producing polyunsaturated fatty acids

A. nidulans produces more C18 than C16 and FAS catalyzes the synthesis of long-chain saturated fatty acids from acetyl-CoA, malonyl-CoA and NADPH. The nucleotide sequences encoding the alpha and beta subunits of FAS in A. nidulans are amplified by PCR using cDNA from A. nidulans as template. The defined

primers used for the amplification are designed to match the published sequences encoding FAS in A. nidulans.

The procedure is as follows:

After cultivation of A. nidulans, biomass is collected by filtration and total RNA is isolated using Trizol reagent (Gibco BRL). Approximately 5 μg of RNA is used for reverse transcription (Superscript II RT, Invitrogen) using Oligo(dT)12-18 as primer. After first strand cDNA sythesis, complementary RNA is removed by RNAse digestion. The cDNA is used as template for PCR using appropriately designed forward- and reverse primers that are complementary to the DNA-sequence encoding the N- and C- termini for FAS in A. nidulans. The resulting fragments of the expected sizes are excised from an agarose gel and purified using GFX-columns (Amersham). The amplified genes are put under the control of constitutive promoters through integration into the genome of a suitable S. cerevisiae strain, e.g. FS01516 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1), using using the strategy described in Example 3. The resulting strain is transformed with the plasmids pESC-URA-elo-delta-5 and pESC-TRP-delta-12 delta-6, resulting in an arachidonic acid producing strain. Furthermore, in a separate experiment, the strain is transformed with the plasmids pESC-URA-elo-delta-5, pESC-TRP-delta-12 delta-6 and pESC-LEU-SK33, resulting in an eicosapentaenoic acid producing strain.

Example 31

Deletion of yeast genes

Deletion of native yeasts genes is carried out by means of a replacement method based on a linear, PCR-generated gene-targeting substrate and using K. lactis URA3 as a recyclable marker. An intermediate strain is first generated, where the marker gene flanked with two direct repeat sequences is integrated at the locus of the gene to be deleted. The marker gene is then looped out through recombination between the two direct repeat sequences flanking K. lactis URA3. This event is selected for by plating the intermediate strain on medium containing 5-fluoroorotic acid (5-FOA), which is toxic to cells expressing the URA3 gene. The

result is a strain, in which the native gene has been replaced with one copy of the direct repeat sequence. Integration of the above described direct repeat sequences and marker gene is directed to the correct location in the genome by means of PCR-generated target sequences.

A target sequence upstream of the gene to be deleted and a target sequence downstream of the gene to be deleted are amplified from genomic yeast DNA. Furthermore, the vector pWJ1042 is used as template to obtain marker fragments for integration. The vector contains the K.lactis URA3 gene flanked by two direct repeat sequences. By using the primers cKL3' and 5'int the two thirds of K.lactis URA3 towards the 5' end are amplified (fragment 1), and using the primers dKL5' and 3'int the two thirds of K.lactis URA3 towards the 3' end are amplified (fragment 2). The reverse primer used for amplification of the upstream target sequence contains a 5' overhang that allows fusion to fragment 1 described above. The forward primer used for amplification of the downstream target sequence contains a 5' overhang that allows fusion with fragment 2 described above. Following fusion by PCR of the upstream target sequence with fragment 1, and fusion by PCR of fragment 2 with the downstream target sequence, the two linear substrates are ready for transformation.
Transformants and pop-out events are selected for as described above.

Example 32Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from Mortierella alpina in species belonging to the genus Aspergillus.

The plasmid that is used in the following examples, is derived from pARp1 that contains the AMA1 initiating replication sequence from Aspergillus nidulans, which also sustains autonomous plasmid replication in A. niger and A. oryzae (Gems et al., 1991). Moreover, the plasmid is a shuttle vector, containing the replication sequence of Escherichia coli, and the inherent difficult transformations in A. niger and A. oryzae can therefore overcome by using E. coli as an intermediate host for the construction of recombinant plasmids. The plasmid contains one or more marker genes to allow the microorganism that harbour them to be selected from those which do not. The selection system can be either based upon dominant

markers e.g. resistance against hygromycin B, phleomycin and bleomycin, or heterologous markers e.g amino acids and the pyrG gene. In addition the plasmid contains promoter- and terminator sequences that allow the expression of the recombinant genes. Suitable promoters are taken from genes of Aspergillus nidulans e.g. alcA, glaA, amy, niaD, and gpdA. Furthermore, the plasmid contains suitable unique restriction sites to facilitate the cloning of DNA fragments and subsequent identification of recombinants. The plasmid used in the following examples contains the strong constitutive gpdA-promoter and auxotropic markers, all originating from A. nidulans; the plasmid containing the gene methG that is involved in methionine biosynthesis, is designated as pAMA1-MET; the plasmid containing the gene hisA that is involved in histidine biosynthesis, is designated as pAMA1-HIS.

The gene encoding delta-6 desaturase from M. alpina is reamplified by PCR from the plasmid pESC-TRP-delta-12 delta-6 (example 4), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-HIS vector that contains the gpdA promoter from Aspergillus nidulans. The resulting plasmid, pAMA1-HIS-delta-6 contains the gene delta-6 desaturase from M. alpina under the control of the gpdA promoter from Aspergillus nidulans.

The sequence of the gene encoding delta-6 desaturase is verified by sequencing of clones of pAMA1-HIS-delta-6.

Example 33

Expression of the pathway to GLA in Aspergillus oryzae.

Aspergillus oryzae strains are transformed with the vector described in example 32. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking histidine. A strain of A. oryzae that is auxotrophic for histidine is transformed with the vector pAMA1-HIS-delta-6 (example 32), yielding the strain FSAN-delta-6.

Example 34

Construction of a fungal vector for expression of the delta-6 elongase from Mortierella alpina and the delta-5 desaturase from M. alpina in species belonging to the genus Aspergillus.

The gene encoding a delta-6 elongase from Mortierella alpina is reamplified by PCR from the plasmid pESC-URA-elo-delta-5 (example 4), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from Aspergillus nidulans. The resulting plasmid, pAMA1-MET-elo contains the delta-6 elongase from M. alpina under the control of the gpdA promoter from A. nidulans.

The gene encoding a delta-5 desaturase from M. alpina is reamplified by PCR from the plasmid pESC-URA- elo-delta-5 (example 4) using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-HIS vector to yield pAMA1-HIS-delta-5. The gpdA promoter and the delta-5 desaturase from M. alpina are reamplified as one fragment by PCR from the plasmid pAMA1-HIS-delta-5 using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pAMA1-MET-elo. The resulting plasmid, pAMA1-MET-elo-delta-5, contains the delta-6 elongase and the delta-5 desaturase from M. alpina that are each under the control of an individual pgdA promoter from A. nidulans. The sequence of the delta-6 elongase and the delta-5 desaturase is verified by sequencing of clones of pAMA1-MET- elo-delta-5.

Example 35

Expression of the pathway to ARA in Aspergillus oryzae.

Aspergillus oryzae strains are transformed with the vectors described in examples 32 and 34. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-HIS-delta-6 (example 32) and pAMA1-MET- elo-delta-5 (example 34), yielding the strain FSAN-delta-6 –elo -delta-5.

Example 36

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and the gene encoding omega-3 desaturase from Saccharomyces kluyveri in species belonging to the genus Aspergillus.

The gene encoding omega-3 desaturase from S. kluyveri is reamplified by PCR from the plasmid pESC-LEU-SK33 (example 4), using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-SK33 contains the gene encoding omega-3 desaturase from S. kluyveri under the control of the gpdA promoter from A. nidulans.

The gpdA promoter and the gene encoding omega-3 desaturase from S. kluyveri are reamplified as one fragment by PCR from the plasmid pAMA1-MET-SK33 using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pAMA1-HIS-delta-6 (example 32). The resulting plasmid, pAMA1-HIS-delta-6 –SK33, contains the genes encoding delta-6 desaturase from M. alpina and omega-3 desaturase from S. kluyveri that are each under the control of an individual pgdA promoter from A. nidulans. The sequence of the gene encoding omega-3 desaturase from S. kluyveri is verified by sequencing of clones of pAMA1-HIS-delta-6 –SK33.

Example 37

Expression of the pathway to EPA in Aspergillus oryzae.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 36. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vector pAMA1-MET- elo-delta-5 (example 34) and the vector pAMA1-HIS-delta-6 –SK33 (example 36), yielding the strain FSAN–elo -delta-5 –delta-6 –SK33.

Example 38

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and the gene encoding cytochrome b5 from Mortierella alpina in species belonging to the genus Aspergillus.

To isolate a heterologous gene encoding cytochrome b5 from Mortierella alpina (SEQ ID NO 1), cDNA is first prepared from M. alpina as described in example 18. The cDNA is then used as template for PCR, using primers designed to target the cytochrome b5 encoding gene (SEQ ID NO 1) and containing suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-cytb5het contains the gene encoding cytochrome b5 from M. alpina under the control of the gpdA promoter from A. nidulans.

The gpdA promoter and the gene encoding cytochrome b5 from M. alpina are reamplified as one fragment by PCR from the plasmid pAMA1-MET-cytb5het using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33 (example 36).

The resulting plasmid, pAMA1-HIS-delta-6 – cytb5het, contain the genes encoding delta-6 desaturase from M. alpina and cytochrome b5 from M. alpina and the resulting plasmid, pAMA1-HIS-delta-6 – SK33-cytb5het, contain the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and cytochrome b5 from M. alpina. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the gene encoding cytochrome b5 from M. alpina is verified by sequencing of clones of pAMA1-HIS-delta-6 – cytb5het and pAMA1-HIS-delta-6 – SK33-cytb5het.

Example 39

Generation of an Aspergillus oryzae strain expressing a cytochrome b5 from M. alpina and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 38. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is transformed with the vector pAMA1-HIS-delta-6 – cytb5het (example 38), yielding the gamma-linolenic acid producing strain FSAN–delta-6 –cytb5het.

Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – cytb5het (example 38), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 –cytb5het.

Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33-cytb5het (example 38), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33-cytb5het.

Example 40

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and S. kluyveri FAS2 in species belonging to the genus Aspergillus.

To express S. kluyveri FAS2 (SEQ ID NO 6) in Aspergillus, the gene is amplified by PCR using genomic DNA from S. kluyveri as template. The defined primers used for the amplification are designed to match the published sequence encoding FAS2 from S. kluyveri and contain suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-FAS2sk contains S. kluyveri FAS2 under the control of the gpdA promoter from A. nidulans.
The gpdA promoter and S. kluyveri FAS2 are reamplified as one fragment by PCR from the plasmid pAMA1-MET- FAS2sk using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33 (example 36). The resulting plasmid, pAMA1-HIS-delta-6 – FAS2sk, contain the genes encoding delta-6 desaturase from M. alpina and S. kluyveri FAS2 and the resulting plasmid, pAMA1-HIS-delta-6 – SK33-FAS2sk, contain the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and S. kluyveri FAS2. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the S. kluyveri FAS2 is verified by sequencing of clones of pAMA1-HIS-delta-6 – FAS2sk and pAMA1-HIS-delta-6 – SK33- FAS2sk.

Example 41

Generation of an Aspergillus oryzae strain expressing S. kluyveri FAS2 and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 38. The transformation of the fungal cell is conducted in accordance with

methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – FAS2sk (example 40), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 – FAS2sk. Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33- FAS2sk (example 40), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33-FAS2sk.

Example 42

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and a cytochrome b5 reductase from Mortierella alpina in species belonging to the genus Aspergillus.

To isolate a heterologous gene encoding cytochrome b5 reductase from Mortierella alpina (SEQ ID NO 3), cDNA is first prepared from M. alpina as described in example 19. The cDNA is then used as template for PCR, using primers designed to target the cytochrome b5 reductase (SEQ ID NO 3) and containing suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-cytb5r contains the gene encoding a cytochrome b5 reductase from M. alpina under the control of the gpdA promoter from A. nidulans.

The gpdA promoter and the gene encoding a cytochrome b5 reductase from M. alpina are reamplified as one fragment by PCR from the plasmid pAMA1-MET-cytb5r using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33

(example 36). The resulting plasmid, pAMA1-HIS-delta-6 – cytb5r, contain the genes encoding delta-6 desaturase from M. alpina and the gene encoding a cytochrome b5 reductase from M. alpina and the resulting plasmid, pAMA1-HIS-delta-6 – SK33- cytb5r, contain the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and the gene encoding a cytochrome b5 reductase from M. alpina. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the gene encoding a cytochrome b5 reductase from M. alpina is verified by sequencing of clones of pAMA1-HIS-delta-6 – cytb5r and pAMA1-HIS-delta-6 – SK33- cytb5r.

Example 43

Generation of an Aspergillus oryzae strain expressing a cytochrome b5 reductase from Mortierella alpina and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 42. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – cytb5r (example 42), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 – cytb5r. Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33- cytb5r (example 42), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33- cytb5r.

Example 44

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and Aspergillus oryzae FAS1 in species belonging to the genus Aspergillus.

To isolate FAS1 from Aspergillus oryzae (SEQ ID NO 106), cDNA is first prepared from Aspergillus oryzae as described for Aspergillus nidulans in example 30. The cDNA is then used as template for PCR, using primers designed to target the A. oryzae FAS1 (SEQ ID NO 106) and containing suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-FAS1ory contains the A. oryzae FAS1 under the control of the gpdA promoter from A. nidulans.

The gpdA promoter and the A. oryzae FAS1 are reamplified as one fragment by PCR from the plasmid pAMA1-MET-FAS1ory r using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33 (example 36). The resulting plasmid, pAMA1-HIS-delta-6 –FAS1ory, contain the genes encoding delta-6 desaturase from M. alpina and the A. oryzae FAS1 and the resulting plasmid, pAMA1-HIS-delta-6 – SK33- FAS1ory, contain the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and the A. oryzae FAS1. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the A. oryzae FAS1 is verified by sequencing of clones of pAMA1-HIS-delta-6 – FAS1ory and pAMA1-HIS-delta-6 – SK33- FAS1ory.

Example 45

Generation of an Aspergillus oryzae strain overexpressing FAS1 and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 44. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-

delta-5 (example 34) and pAMA1-HIS-delta-6 – FAS1ory (example 44), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 – FAS1ory. Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33- FAS1ory (example 44), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33- FAS1ory.

Example 46

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and Aspergillus oryzae FAS2 in species belonging to the genus Aspergillus.

To isolate FAS2 from Aspergillus oryzae (SEQ ID NO 108), cDNA is first prepared from Aspergillus oryzae as described for Aspergillus nidulans in example 30. The cDNA is then used as template for PCR, using primers designed to target the A. oryzae FAS2 (SEQ ID NO 108) and containing suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-FAS2ory contains the A. oryzae FAS2 under the control of the gpdA promoter from A. nidulans.

The gpdA promoter and the A. oryzae FAS2 are reamplified as one fragment by PCR from the plasmid pAMA1-MET-FAS2ory r using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33 (example 36). The resulting plasmid, pAMA1-HIS-delta-6 –FAS2ory, contain the genes encoding delta-6 desaturase from M. alpina and the A. oryzae FAS2 and the resulting plasmid, pAMA1-HIS-delta-6 – SK33- FAS2ory, contain the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and the A. oryzae FAS2. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence

of the A. oryzae FAS2 is verified by sequencing of clones of pAMA1-HIS-delta-6 – FAS2ory and pAMA1-HIS-delta-6 – SK33- FAS2ory.

Example 47

Generation of an Aspergillus oryzae strain overexpressing FAS2 and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 46. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – FAS2ory (example 46), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 – FAS2ory. Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33- FAS2ory (example 46), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33- FAS2ory.

Example 48

Construction of a fungal vector for expression of the genes encoding the delta-6 elongase from Mortierella alpina, the delta-5 desaturase from M. alpina and Aspergillus oryzae FAS2 in species belonging to the genus Aspergillus.

The plasmid, pAMA1-MET-FAS2ory (example 46) contains the A. oryzae FAS2 under the control of the gpdA promoter from A. nidulans. The gpdA promoter and the A. oryzae FAS2 are reamplified as one fragment by PCR from the plasmid pAMA1-MET-FAS2ory r using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmid pAMA1-MET- elo-delta-5 (example 34). The resulting

plasmid, pAMA1-MET- elo-delta-5 –FAS2ory, contains the delta-6 elongase and the delta-5 desaturase from M. alpina and the A. oryzae FAS2. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the A. oryzae FAS2 is verified by sequencing of clones of pAMA1-MET- elo-delta-5 –FAS2ory.

Example 49

Generation of an Aspergillus oryzae strain overexpressing FAS1 and FAS2 and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 44 and 48. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-HIS-delta-6 – FAS1ory (example 44) and pAMA1-MET- elo-delta-5 –FAS2ory (example 48), yielding the arachidonic acid producing strain FSAN–elo -delta-5 – FAS2ory-delta-6 – FAS1ory.

Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-HIS-delta-6 – SK33- FAS1ory (example 44) and pAMA1-MET- elo-delta-5 –FAS2ory (example 48), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 – FAS2ory-delta-6 – SK33- FAS1ory.

Example 50

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and the gene encoding NADH-cytochrome b-5 reductase from Aspergillus oryzae in species belonging to the genus Aspergillus.

To isolate the gene encoding NADH-cytochrome b-5 reductase from Aspergillus oryzae (SEQ ID NO 110), cDNA is first prepared from Aspergillus oryzae as described for Aspergillus nidulans in example 30. The cDNA is then used as

template for PCR, using primers designed to target the the gene encoding NADH-cytochrome b-5 reductase from Aspergillus oryzae (SEQ ID NO 110) and containing suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-cyt5rory contains the the gene encoding NADH-cytochrome b-5 reductase from A. oryzae under the control of the gpdA promoter from A. nidulans.

The gpdA promoter and the gene encoding NADH-cytochrome b-5 reductase from A. oryzae are reamplified as one fragment by PCR from the plasmid pAMA1-MET-cyt5rory using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33 (example 36). The resulting plasmid, pAMA1-HIS-delta-6 – cyt5rory, contains the genes encoding delta-6 desaturase from M. alpina and the gene encoding NADH-cytochrome b-5 reductase from A. oryzae and the resulting plasmid, pAMA1-HIS-delta-6 – SK33- cyt5rory, contains the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and the gene encoding NADH-cytochrome b-5 reductase from A. oryzae. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the gene encoding NADH-cytochrome b-5 reductase from A. oryzae is verified by sequencing of clones of pAMA1-HIS-delta-6 – cyt5rory and pAMA1-HIS-delta-6 – SK33-cyt5rory.

Example 51

Generation of an Aspergillus oryzae strain overexpressing the gene encoding NADH-cytochrome b-5 reductase and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 50. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium

lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – cyt5rory (example 50), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 – cyt5rory. Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33- cyt5rory (example 50), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33- cyt5rory.

Example 52

Construction of a fungal vector for expression of the gene encoding delta-6 desaturase from M. alpina and the gene encoding a cytochrome b5 from Aspergillus oryzae in species belonging to the genus Aspergillus.

To isolate the gene encoding a cytochrome b5 from Aspergillus oryzae (SEQ ID NO 112), cDNA is first prepared from Aspergillus oryzae as described for Aspergillus nidulans in example 30. The cDNA is then used as template for PCR, using primers designed to target the the gene encoding a cytochrome b5 from Aspergillus oryzae (SEQ ID NO 112) and containing suitable 5′ overhangs. The introduction of said restriction sites at the 5′ and 3′ ends of the gene allows ligation of the restricted PCR product into a digested pAMA1-MET vector that contains the gpdA promoter from A. nidulans. The resulting plasmid, pAMA1-MET-cytb5 contains the the gene encoding a cytochrome b5 from A. oryzae under the control of the gpdA promoter from A. nidulans.
The gpdA promoter and the gene encoding a cytochrome b5 from A. oryzae are reamplified as one fragment by PCR from the plasmid pAMA1-MET- cytb5 using forward- and reverse primers, with 5′ overhangs containing suitable restriction sites. The introduction of said restriction sites at the 5′ and 3′ ends of the DNA fragment allows ligation of the restricted PCR product into the digested plasmids pAMA1-HIS-delta-6 (example 32) or pAMA1-HIS-delta-6 –SK33 (example 36). The resulting plasmid, pAMA1-HIS-delta-6 – cytb5, contains the genes encoding delta-6 desaturase from M. alpina and the gene encoding a cytochrome b5 from A. oryzae and the resulting plasmid, pAMA1-HIS-delta-6 – SK33- cytb5, contains

the genes encoding delta-6 desaturase from M. alpina, omega-3 desaturase from S. kluyveri and the gene encoding a cytochrome b5 from A. oryzae. All the genes are under the control of an individual pgdA promoter from A. nidulans. The sequence of the gene encoding a cytochrome b5 from A. oryzae is verified by sequencing of clones of pAMA1-HIS-delta-6 – cytb5 and pAMA1-HIS-delta-6 – SK33- cytb5.

Example 53

Generation of an Aspergillus oryzae strain overexpressing the gene encoding cytochrome b5 and producing polyunsaturated fatty acids.

Aspergillus oryzae strains are transformed with the vectors described in examples 34 and 52. The transformation of the fungal cell is conducted in accordance with methods known in the art, for instance, by electroporation or by conjugation (see, e.g., Sambrook et al., 1989). Transformants are selected on minimal medium lacking methionine and histidine. A strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – cytb5 (example 52), yielding the arachidonic acid producing strain FSAN–elo -delta-5 –delta-6 – cytb5. Furthermore, in a separate experiment, a strain of A. oryzae that is auxotrophic for histidine and methionine is co-transformed with the vectors pAMA1-MET- elo-delta-5 (example 34) and pAMA1-HIS-delta-6 – SK33- cytb5 (example 52), yielding the eicosapentaenoic acid producing strain FSAN–elo -delta-5 –delta-6 – SK33- cytb5.

Example 54

Fermentation with recombinant strains of Aspergillus oryzae in fermentors.

The recombinant strains described in examples 33, 35, 37, 39, 41, 43, 45, 47, 49, 51 and 53 can be grown in fermentors operated as batch, fed-batch or chemostat cultures.

Batch and Fed-batch cultivations

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope thereof.

Example 55

Lipid Extraction

Prior to lipid extraction, the biomass was separated through centrifugation for 5 minutes at 5000 rpm. The biomass was re-dissolved in 10-15 ml distilled water and the resulting cell suspension was broken using the glass bead method to generate cell extract.

The cell extract was prepared by addition of 1 ml glass beads with a particle size of 250-500 µm (Sigma-Aldrich, St Louis, Missouri) to 1 ml cell suspension in a micro tube with screw cap (Sarstedt, Germany). For each cell suspension 2 tubes were processed. The tubes were shaken at level 4 for 20 sec. in a FastPrep FP120 instrument (Qbiogene, France). This was done in a total of 6 rounds for each tube with a 5 minutes intervening cooling of the tubes on ice after 3 rounds. The cell extracts were combined in 2 ml eppendorf tubes by transferring 600 µl cell extract from each of the tubes to a eppendorf tube there by containing 1.2 ml glass bead free cell extract. 1 ml of the cell extract was immediately transferred to a glass tube with screw cap containing 20 ml chloroform/methanol (C:M) (2:1, v/v) and added 100-200µl internal standard (ISTD) (C19:0-, C21:0- or C23:0-TAG, >99%, Nu-Chek-Prep/Larodan)) using a 200µl precision glass pipette (Socorex). Headspace was flushed with nitrogen, closed immediately and placed on a rotary mixer (Multi-Tube Vortexer, DVX-2500) at 2500 rpm for 15min using the pulse function (20 sec.). The extract was then filtered through a Whatman filter (Whatman International, England), the tube and filter unit rinsed in 3x2 ml C:M (2:1, v/v) and the collected solvent was washed with 5.5 ml 0.73% NaCl and dried over nitrogen. Traces of water were removed by adding 1 ml methanol and taking it to complete dryness under nitrogen. Finally 1 ml of toluene was added and the sample stored at -20°C if methylation could not be performed immediately.

Example 56

Fatty acid methylation

Fatty acid methyl esters (FAME) were produced by acidic transmethylation. To the dry lipid dissolved in 1 ml toluene (generated in Example 9), 2 ml 1% sulphuric acid in methanol was added. The tube was closed after mixing and flushing headspace with nitrogen, and left at 50°C over night for transesterification of the lipids. After having reached room temperature the sample was washed with 3 ml saturated NaCl solution containing 0.2% sodium carbonate. FAME were subsequently extracted twice by adding 2 ml heptane, vortexing the sample, centrifugation at 3000 rpm for 2min (4°C) and collecting the organic upper phase. The combined upper phases were dried under a stream of nitrogen (40°C) and traces of water were removed by adding 1 ml methanol and taking it to complete dryness under nitrogen. FAME was dissolved in a suitable volume of heptane (0.2-1.0 ml) containing 0.01% butylated hydroxytoluene (BHT) (Sigma-Aldrich, St Louis, Missouri), transferred to a 2 ml GC-vial with a 200µl insert and FAME analysed using gas-liquid-chromatography (GLC).

Example 57

Gas chromatography with FID detection

FAME were analysed on a gas chromatograph (GC) (GC-2010, Shimadzu) coupled to a mass-selective-detector (MS) (GCMS-QP2010, Shimadzu)) and a flame-ionisation-detector (FID). The GC-MS-FID was operated with a split/splitless auto-injector (AOS-20i, Shimadzu) and GCMSsolution software, Lab solution (version 2.50, Shimadzu).

Sample injection volumes were 1 - 5µl (2-6mg/mL) and the split ratio 10:1 - 50:1 operated at an injector temperature of 250°C. Number of rinses with sample prior to injection was 1 and after injection number of rinses with solvent was 5. Samples were split and components separated in parallel on two identical capillary columns (50mx0.25mmID, 0.25µm film thickness) (CP-Select CB for FAME,

Varian). One column was fitted to a mass spectrometer (MS-quadropole) and one to a flame-ionization-detector (FID) for identification/structural clarification and quantification, respectively. Helium was used as carrier gas and operated at a linear velocity of 36 cm/sec (18 cm/sec pr column). Purge flow was set at 3mL/min.

Based on the highly polar nature of the column coating (100% cyanopropyl) and an optimized temperature programme (see below), FAME were separated according to differences in polarity and boiling point. Oven temperature was initially set at 50°C. Immediately after injection it was increased to 145°C at 30°C/min, then increased to 205°C at 2°C/min and finally increased to 250°C at 20°C/min and kept there for 5min. Total run time was 40.42 min.

The MS was operated in the SCAN mode (46m/z-500m/z) using electronic ionisation (EI) at 70eV, with a scan speed of 1000 amu/sec and scan events every 0.5 sec. Ionsource temperature was set at 105°C and interface temperature at 250°C.
At the FID side helium as used as makeup gas (40mL/min) and air/hydrogen set at 10:1 (400:40ml/min). The detector was set at 275°C with a sampling rate of 40msec.

Along with MS spectra compared with the 1998 NIST Mass Spectral Database, FAME were routinely identified based on relative retention time (RRT) with C18:0 ME (Octadecanoic acid methyl ester) as reference component, using the GCMSsolution software (version 2.50, Shimadzu). RRT were produced and updated using an array of commercially available FAME standards (Sigma, Nu-Chek-Prep, Larodan, Avanti, Matreya). A quantitative FAME standard (GLC 68A, Nu-Chek-Prep) was run routinely to monitor the condition of the columns and over all GC performance.

Example 58

Fatty acid quantification and yield

Quantification was based on FID data auto-integrated by the GCMS solution software and manually corrected for potential artefacts. Amounts of individual fatty acids (FA) and total FA (mg) were calculated based on the ISTD (C19:0-, C21:0- or C23:0-triacylglycerol), added during lipid extraction, and a specific correction factor (e.g. C21:0-TAG = 1.0039). The ISTD was made up in a solution of chloroform:methanol (2:1, v/v) at ca 2.00 mg/mL and the added volume adjusted (100-200µl) to represent 5-10% of total FA. For comparative purposes an allowed total area range was set at 0.5-1.0 million.

FA yield (mg FA/g DW) was determined by calculation based on the ISTD and divided by the dry weight (DW) of the biomass in 1 ml of the initial cell suspension.

Example 59

Codon-optimization and gene assembly of the synthetic Ostreococcus tauri delta 6-desaturase using PCR

Ostreococcus tauri delta 6-desaturase sequence (SEQ ID NO 150) was obtained from a sequence database (Pubmed), based on its accession number AY746357, found in literature. Using a backtranslation tool, the amino acid sequence (SEQ ID NO 131) was translated into a codon-optimized DNA sequence (SEQ ID NO: 130), with the options: standard genetic code, optimization according to S. cerevisiae codon usage, and discarding codons below 50% of theoretical ratio.

The reverse complementing strand of the optimized gene sequence was obtained using a "reverse complement" tool (ex. from Saccharomyces genome database). The sequences of both strands were divided into oligomers 40 bp in size. The oligomers overlapped between the strands, and the outermost oligomers were smaller than 40 bp.

The oligomers were mixed together, and the assembly was done by PCR.

Two outer primers, OtD6D-fw (SEQ ID NO 151) and OtD6D-rv (SEQ ID NO 152), one forward and one reverse, were designed for a final PCR amplification, in which the DNA template was the purified DNA from the previous PCR reaction. These primers introduced NotI and BglII cloning sites upstream and downstream of the start and stop codons, respectively.

The desaturase sequence was cloned into the plasmid pESC-TRP (figure 18), using the same restriction sites, yielding pESC-TRP-OtD6 (figure 19).

The plasmid pESC-TRP-OtD6 was sequenced, and the two point mutations found in the desaturase gene sequence were corrected by site directed mutagenesis, using a multi site-directed mutagenesis kit (QuickChange® Multi Site-Directed Mutagenesis Kit, Stratagene). Two forward (Ot1T3_fw1 and Ot1T3_fw2, SEQ ID NO 153 and SEQ ID NO 154, respectively), and two reverse (Ot1T3_rv1 and Ot1T3_rv2, SEQ ID NO 155 and SEQ ID NO 156, respectively) primers with approximately 45 bp were designed in a way that the point mutation should be close to the middle of the primer, with 10-15 bases of sequence on both sides. Both forward and both reverse primers anneal to the same strand of the template plasmid. The mutagenesis reaction was carried out according to specified instructions.

The repaired delta 6 desaturase sequence was restricted from pESC-TRP-OtD6 with NotI and BglII restriction enzymes and cloned into the vector pESC-TRP-delta-12 delta-6 (figure 12). Correct integration of the gene was verified by restriction reaction using XmaI.

Example 60

Generation of a S. cerevisiae strain overexpressing a gene encoding a delta-6 desaturase from Ostreococcus tauri by deletion of the gene DCI1

In order to integrate the codon-optimized gene encoding O. tauri delta-6 desaturase (SEQ ID NO: 157) into the genome of S. cerevisiae with a constitutive yeast promoter and with simultaneous deletion of DCI1 (SEQ ID NO: 124), a bi-partite gene targeting strategy was used as described in Example 66. The procedure was as follows:

A target sequence upstream of DCI1 was amplified from genomic DNA by PCR using the primers DCI1-UP-fw (SEQ ID NO: 126) and DCI1-UP-rv (SEQ ID NO: 127) and a target sequence downstream of DCI1 was amplified from genomic DNA by PCR using the primers DCI1-D-fw (SEQ ID NO: 128) and DCI1-D-rv (SEQ

ID NO: 129). The ADH1 promoter/K.lactis URA3 fragment 1 was amplified by PCR using the plasmid pWAD2 as template and the primers AD-fw and Int3´, and was fused to the upstream target sequence by PCR using primers DCI1-UP-fw and Int3´. The K. lactis URA3/ADH1 promoter fragment 2 was amplified by PCR using the plasmid pWAD1 as template and the primers Int5´and AD-rv. A gene encoding a delta-6 desaturase from Ostreococcus tauri codon optimized for S. cerevisiae (SEQ ID NO: 130) was amplified from plasmid pESC-TRP-delta-12 delta-6Ot (Example 59) by PCR using the primers D6D-OTi-fw (SEQ ID NO: 132) and D6D-OTi-rv (SEQ ID NO: 133). This DNA fragment was fused to the target sequence downstream of DCI1 by PCR using the primers D6D-OTi-fw and DCI1-D-rv.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and the K. lactis URA3/ADH1 promoter fragment 2. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. To select for looping out of the marker gene, the transformant was transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 dci1::pADH1-OtD6D and was named FS01559.

Example 61

Generation of a S. cerevisiae strain overexpressing a gene encoding a delta-12 desaturase from M. alpina by deletion of the gene FOX2

In order to integrate a gene from M. alpina encoding a delta-12 desaturase into the genome of S. cerevisiae with simultaneous deletion of FOX2, a bi-partite gene targeting strategy was used as described in Example 66. The procedure was as follows:

The native yeast gene FOX2 (SEQ ID NO: 134) was deleted using a bi-partite gene targeting substrate (Example 3) according to the following procedure:

A target sequence upstream of FOX2 was amplified from genomic DNA by PCR using the primers FOX2-UP-fw (SEQ ID NO: 136) and FOX2-UP-rv (SEQ ID NO: 137) and a target sequence downstream of FOX2 was amplified from genomic DNA by PCR using the primers FOX2-D-fw (SEQ ID NO: 138) and FOX2-D-rv (SEQ ID NO: 139). The TDH3 promoter/K.lactis URA3 fragment 1 was amplified by PCR using the plasmid pW-TD2 as template and the primers T2-2 and Int3´, and was fused to the upstream target sequence by PCR using primers FOX2-UP-fw and Int3´. The K. lactis URA3/TDH3 promoter fragment 2 was amplified by PCR using the plasmid pW-TD1 as template and the primers Int5´and T-DGA. A gene encoding a delta-12 desaturase from M. alpina was amplified from the plasmid pESC-TRP-delta-12 delta-6 (Example 4) by PCR using the primers D12Di-fw (SEQ ID NO: 140) and D12Di-rv (SEQ ID NO: 141). This DNA fragment was fused to the target sequence downstream of FOX2 by PCR using the primers D12Di-fw and FOX2-D-rv.

The yeast strain FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and the K. lactis URA3/TDH3 promoter fragment 2. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. To select for looping out of the marker gene, the transformant was transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 fox2::pTDH3-MaD12D and was named FS01557.

Example 62

Generation of a S. cerevisiae strain overexpressing a gene encoding a delta-5 desaturase from M. alpina by deletion of the gene POT1

In order to integrate a gene from M. alpina encoding a delta-5 desaturase into the genome of S. cerevisiae with simultaneous deletion of POT1 (SEQ ID NO: 142), a

bi-partite gene targeting strategy was used as described in Example 66. The procedure was as follows:

A target sequence upstream of POT1 was amplified from genomic DNA by PCR using the primers POT1-UP-fw (SEQ ID NO: 144) and POT1-UP-rv (SEQ ID NO: 145) and a target sequence downstream of POT1 was amplified from genomic DNA by PCR using the primers POT1-D-fw (SEQ ID NO: 146) and POT1-D-rv (SEQ ID NO: 147). The ADH1 promoter/K.lactis URA3 fragment 1 was amplified by PCR using the plasmid pWAD2 as template and the primers AD-fw and Int3´, and was fused to the upstream target sequence by PCR using primers POT1-UP-fw and Int3´. The K. lactis URA3/ADH1 promoter fragment 2 was amplified by PCR using the plasmid pWAD1 as template and the primers Int5´and AD-rv. A gene encoding a delta-5 desaturase from M. alpina was amplified from the plasmid pESC-URA-elo-delta-5 (Example 4) by PCR using the primers D5Di-fw (SEQ ID NO: 148) and D5Di-rv (SEQ ID NO: 149). This DNA fragment was fused to the target sequence downstream of POT1 by PCR using the primers D5Di-fw and POT1-D-rv.

The yeast strain FS01509 (MATalpha ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1) was transformed with the two linear fusion substrates described above and the K. lactis URA3/ADH1 promoter fragment 2. Transformants were selected on medium lacking uracil and were streak-purified on the same medium. To select for looping out of the marker gene, the transformant was transferred to plates containing 5-FOA. Pop-out recombinants were streak-purified on 5-FOA-containing medium. The resulting strain had the genotype MATalpha ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1 pot1::pADH1-MaD5D and was named FS01577.

Example 63

Generation of a S. cerevisiae strain expressing a delta-6 desaturase from Ostreococcus tauri and a delta-5 desaturase from M. alpina

To combine overexpression of a delta-6 desaturase from Ostreococcus tauri and delta-5 desaturase from M. alpina in one strain, the strain FS01559 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 dci1::pADH1-OtD6D) (Example 60) was crossed to the strain FS01577 (MATalpha ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1 pot1::pADH1-MaD5D) (Example 62). Diploids were allowed to sporulate, and the asci were dissected into spore tetrads. From the set of haploid strains derived from the cross, a strain with the genotype MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1 pot1::pADH1-MaD5D dci1::pADH1-OtD6D was selected and named FS01578.

Example 64

Generation of a S. cerevisiae strain expressing a delta-12 desaturase from M. alpina and a delta-5 desaturase from M. alpina

To combine overexpression of a delta-12 desaturase from M. alpina and a delta-5 desaturase from M. alpina in one strain, the strain FS01557 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 fox2::pTDH3-MaD12D) (Example 61) was crossed to the strain FS01577 (MATalpha ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1 pot1::pADH1-MaD5D) (Example 62). Diploids were allowed to sporulate, and the asci were dissected into spore tetrads. From the set of haploid strains derived from the cross, a strain with the genotype MATalpha ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pTPI1-MCR1 pADH1-FAS1 pADH1-FAS2 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D was selected and named FS01580.

Example 65

Generation of a S. cerevisiae strain expressing a delta-6 desaturase from Ostreococcus tauri, a delta-12 desaturase from M. alpina and a delta-5 desaturase from M. alpina

To combine overexpression of a delta-6 desaturase from Ostreococcus tauri, a delta-12 desaturase from M. alpina and delta-5 desaturase from M. alpina in one

strain, the strain FS01578 (MATa ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1 pot1::pADH1-MaD5D dci1::pADH1-OtD6D) (Example 63) was crossed to the strain FS01580 (MATalpha ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pTPI1-MCR1 pADH1-FAS1 pADH1-FAS2 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D) (Example 66). Diploids were allowed to sporulate, and the asci were dissected into spore tetrads. From the set of haploid strains derived from the cross, a strain with the genotype MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::ADH1p-OtD6D was selected and named FS01591. In addition, a strain with the genotype MATalpha ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pTPI1-MCR1 pot1::pADH1-MaD5D dci1::pADH1-OtD6D fox2::pTDH3-MaD12D was selected from the same cross and was named FS01610.

Example 66

Integration of heterologous genes into the genome of S. cerevisiae using a bi-partite gene targeting substrate

For integration of heterologous genes into the genome of S. cerevisiae under the control of a suitable yeast promoter, an approach similar to the ones described above is used. K. lactis URA3 is used as a recyclable marker in the integration, and looping out of the marker gene is selected for by plating the intermediate strain on 5-FOA containing medium (figure 10). Targeting of the integration substrate to the desired location in the genome is performed, as described above, by means of PCR-generated target sequences.

Bi-partite gene targeting substrate

To enable expression of heterologous genes under the control of the strong ADH1 and TDH3 yeast promoters by deletion of native yeast genes, these promoters have been introduced into intermediate working vectors on either side of K.lactis URA3, resulting in the vectors pWAD1, pWAD2, pW-TD1 and pW-TD2 (PCT/DK2005/000372, and figures 8, 9). With these vectors as templates, fragments can be amplified that contain (in the 5′ to 3′ direction) 1) the ADH1 or TDH3 promoter coupled to two thirds of K.lactis URA3 towards the 5′ end, using

the primers AD-fw or T2-2 and Int3', and 2) two thirds of K.lactis URA3 towards the 3' end coupled to the ADH1 or TDH3 promoter, using the primers Int5' and AD-rv or T-DGA. Target sequences corresponding to a 300-500 bp sequence upstream of the native gene to be deleted and target sequences corresponding to a 300-500 bp sequence downstream of the native gene to be deleted, are amplified from genomic yeast DNA using suitable primers. The heterologous gene is amplified from a suitable source, e.g. a cDNA preparation, by PCR using primers with suitable 5' overhangs. These overhangs allow fusion of the heterologous gene with the downstream target sequence at the 5' end of the heterologous gene and fusion with the chosen promoter sequence (the ADH1 promoter or the TDH3 promoter) at the 3' end of the heterologous gene. The reverse primer used for amplification of the upstream target sequence contains a 5' overhang that allows fusion to fragment 1 described above. The forward primer used for amplification of the downstream target sequence contains a 5' overhang that allows fusion with the heterologous gene. Following fusion by PCR of the upstream target sequence with fragment 1, and fusion by PCR of the heterologous gene with the downstream target, these two linear substrates are ready for transformation together with fragment 2 described above as shown in figure 14. Finally, a suitable ura3 yeast strain is transformed with 1) the upstream target fragment fused to fragment 1 described above, 2) fragment 2 described above, 3) the heterologous gene-downstream target fusion product. Transformants are selected on medium lacking uracil, and contain, inserted between the upstream and downstream target sequences, two copies of the chosen promoter as a direct repeat on each side of the marker gene K. lactis URA3, followed by the heterologous gene. Following selection of pop-out recombination on 5-FOA, the resulting ura3 strain contains, inserted between the upstream and downstream target sequences, the chosen promoter followed by the heterologous gene.

Example 67

Shake flask cultivations with glucose as carbon source

The medium for shake flask cultivations contained 20 g/L glucose, 15 g/L (NH4)2SO4, 1 g/L MgSO4·7H2O, 14.4 g/L KH2PO4, 1mL/L vitamin solution and 1 mL/L trace metal solution. The vitamin solution contained: 50 mg/L biotin, 1 g/L

calcium panthotenate, 1 g/L nicotinic acid, 25 g/L myo-inositol, 1 g/L thiamine HCl, 1 g/L pyridoxal HCl and 0.2 g/L para-aminobenzoic acid, while the trace metal solution contained: 15 g/L EDTA, 4.5 g/L $ZnSO_4 \cdot 7H_2O$, 1 g/L $MnCl_2 \cdot 2H_2O$, 0.3 g/L $CoCl_2 \cdot 6H_2O$, 0.4 g/L $Na_2MoO_4 \cdot 2H_2O$, 4.5 g/L $CaCl_2 \cdot 2H_2O$, 3 g/L $FeSO_4 \cdot 7H_2O$, 1 g/L H_3BO_3 and 0.1 g/L KI. The carbon source was autoclaved separately from the other medium components, and the vitamin and trace metal solutions were added following autoclavation by sterile filtration. The pH of the medium was set to pH 6.5 prior to autoclavation.

The yeast was inoculated at an initial cell density of approximately 0.1 (OD600) into 100 ml medium in 500 ml Erlenmeyer shake flasks with 2 baffles and was incubated at 30°C shaking at 150 rpm.

Example 68

Shake flask cultivations with galactose as carbon source

The medium for shake flask cultivations with galactose as carbon source was as described in Example 67, but with 20 g/L galactose as carbon source instead of glucose. The carbon source was autoclaved separately from the other medium components, and the vitamin and trace metal solutions were added following autoclavation by sterile filtration. The pH of the medium was set to pH 6.5 prior to autoclavation.

Cells were pre-grown in 500 ml Erlenmeyer shake flasks with 2 baffles in 100 mL medium containing 5 g/L glucose, 20 g/L galactose and remaining medium components as specified in Example 67. Following depletion of glucose and while in the exponential growth phase on galactose, 1 ml of the pre-culture was transferred to 100 mL medium containing galactose as the sole carbon source in 500 ml Erlenmeyer shake flasks with 2 baffles. The shake flasks were incubated at 30°C with 150 rpm shaking.

Example 69

Growth medium used in chemostat fermentations with glucose as carbon source

For chemostat fermentations with 100 g/L glucose as carbon source, a minimal growth medium with a molar C/N ratio of 15 was used. The medium contained: 100 g/L glucose, 14.8 g/L (NH4)2SO4, 4.6 g/l KH2PO4, 2.8 g/l MgSO4 *7H2O, 2.4 ml/L vitamin solution and 2 ml/L trace metal solution. The vitamin solution contained: 50 mg/L biotin, 1 g/L calcium panthotenate, 1 g/L nicotinic acid, 25 g/L myo-inositol, 1 g/L thiamine HCl, 1 g/L pyridoxal HCl and 0.2 g/L para-aminobenzoic acid, while the trace metal solution contained: 15 g/L EDTA, 4.5 g/L ZnSO4 7H2O, 1 g/L MnCl2·2H2O, 0.3 g/L CoCl2·6H2O, 0.4 g/L Na2MoO4·2H2O, 4.5 g/L CaCl2·2H2O, 3 g/L FeSO4·7H2O, 1 g/L H3BO3 and 0.1 g/L KI.

Example 70

Integration of M. alpina delta-6 elongase gene in the trp-289 locus of S. cerevisiae

In order to integrate the delta-6 elongase encoding gene from M. alpina (MaD6E) into the genome of S.cerevisiae a bi-partite gene targeting strategy was used, using target sequences which directed the gene to the trp1-289 locus of S.cerevisiae (figure 15).

The procedure was as follows:
MaD6E was amplified by PCR using the primers Eloa-fw-MF (SEQ ID NO: 114) and Elo-T-rv (SEQ ID NO: 115) and using the plasmid pESC-URA-elo-delta-5 as template. The primers contained 5′ overhangs to allow fusion to the promoter (pPYK1) and target sequences (TRP1 fragment 1). The marker gene TRP1 was amplified as to separate fragments by PCR using genomic DNA from a tryptophane-prototrophic strain of S.cerevisae as template. TRP1 fragment 1 consisted of 396 bp of the TRP1 upstream region and the first 353 bp of the TRP1 coding sequence, and was amplified using the primers TRP1-5end-fw (SEQ ID NO: 116) and TRP1-5end-rv (SEQ ID NO: 117), while TRP1 fragment 2 consisted of pos 354 to 914 of the TRP1 coding sequence and was amplified using primers TRP1-3end-fw (SEQ ID NO: 118) and TRP1-3end-rv (SEQ ID NO: 119). TRP1-3end-rv contained a 5′ overhang allowing fusion to a PYK1 promoter fragment.

The PYK1 promoter fragment was amplified by PCR using genomic S.cerevisae DNA as template and using the primers PYK1-fw (SEQ ID NO: 120) and PYK1-rv (SEQ ID NO: 121). MaD6E was then fused to TRP1 fragment 1 by PCR using primers Eloa-fw-MF and TRP1-5end-rv, resulting in Fusion fragment B (figure 15), while the PYK1 promoter fragment was fused to TRP1 fragment 2 by PCR using primers TRP1-3end-fw and PYK1-rv, resulting in fusion fragment A (figure 15). The yeast strain FS01591 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::ADH1p-OtD6D) was transformed with fusion fragments A and B, and transformants were selected on medium lacking tryptophane. Correct integration of the substrate was verified by colony PCR using two different primer sets; D6E_2212 (SEQ ID NO: 122) in combination with TRP1-5end-rv and D6E_2212 in combination with TRP1-C-rv (SEQ ID NO: 123). The genotype of the resulting strain FS01613 was MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D.

Example 71

Generation of strains FS01639, FS01640 and FS01641

In order to combine the integrated ARA pathway with the gene encoding S.kluyveri omega-3 desaturase (S.kluyveri FAD3), and to further combine the integrated EPA pathway with other traits (combinations of FAS1 and FAS2 overexpression) a MATalpha strain containing S.kluyveri FAD3 integrated in the gpp1 locus, and furthermore devoid of FAS1 and FAS2 overexpression, was crossed to the above described MATa strain FS01613.

FS01625 (MATalpha ura3-52 trp1-289 pTPI1-MCR1 pox1::pTDH3-M.alpina OLE1 pTPI1-ACC1 fox2::pTDH3-MaD12D dci1::pADH1-OtD6D pot1::pADH1-MaD5D gpp1::pHXT7-K.lactis URA3-pHXT7-S.kluyveri FAD3) was crossed to FS01613 (MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D), the resulting diploid was allowed to sporulate and the ascospores were dissected. Among the resulting haploid strains, the strains

FS01622, FS01623 and FS01627 (for genotypes see table 6) were selected. These strains were transferred to 5-FOA containing medium to select for looping out of the K.lactis URA3 marker, were streak-purified on the same medium and were transformed with empty pESC-URA vectors in order to obtain prototrophic strains.

Table 6. Names and genotypes of haploid strains resulting from the cross between FS01625 and FS01613, names of the corresponding strains following looping out of the K.lactis URA3 marker and names of the corresponding strains following looping out of marker and transformation with the empty vector pESC-URA.

Strain name	Genotype	Strain name after popout	Strain name with empty vector
FS01622	MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI1-MCR1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-K.lactis URA3-pHXT7-S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2	FS01621	FS01639
FS01623	MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI1-MCR1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-K.lactis URA3-pHXT7-S.kluyveri FAD3 pADH1-FAS2	FS01624	FS01640
FS01627	MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI1-MCR1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-K.lactis URA3-pHXT7-S.kluyveri FAD3 pADH1-FAS1	FS01635	FS01641

Example 72

Generation of strain FS01658

To generate the control strain FS01658 without MCR1 overexpression, FS01622 (MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI1-MCR1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-K.lactis URA3-pHXT7-S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2) was crossed to strain FS01620 (MATalpha ura3-52 trp1-289 leu2-3_112 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D). FS01620 had previously been generated from a cross between FS01396 and FS01610. Following sporulation and dissection of the cross FS01622xFS01620, a strain was selected with the genotype MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-K.lactis URA3-pHXT7-S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2 and was named FS01647. The strain was transferred to 5-FOA containing medium to select for looping out of the K.lactis URA3 marker. The resulting strain had the genotype MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7- S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2 and was named FS01648. FS01648 was transformed with an empty pESC-URA vector in order to achieve prototrophy, and the transformed strain was named FS01658.

Example 73

Overexpression of MCR1 results in increased fatty acid desaturation

To investigate the influence of MCR1 overexpression on fatty acid desaturation, the EPA-producing strains FS01658 (MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7- S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2 [pESC-URA]) and FS01639 (MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI1-MCR1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7 -S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2

[pESC-URA]) were compared with respect to fatty acid composition. FS01639 has identical genetic background to FS01658 but additionally overexpresses MCR1 from the TPI1 promoter. The strains were cultivated in triplicate in shake flasks with glucose as carbon source as described in Example 67, and were harvested for fatty acid analysis following depletion of the carbon source. Fatty acids were extracted, analyzed and quantified as described in examples 55-58.

The results of the analysis show that MCR1 overexpression results in an increased percentage of fatty acids with 2 or more double bonds in total fatty acid (Table 7). Specifically, the percentage of linoleic acid is increased from an average of 6.0% of total fatty acid to an average of 10.6 % of total fatty acid.

Table 7. Fatty acid composition (% of total fatty acid) of the EPA-producing strains FS01658 and FS01639 (overexpressing MCR1), analyzed in shake flask cultivations with glucose as carbon source (20 g/L) in triplicate.

Fatty acid		Average FS01658 (Ref)	abs. SD	Average FS01639 (pTPI1-MCR1)	abs. SD
10:0	Capric acid	0.3	0.03	0.5	0.06
12:0	Lauric acid	0.6	0.03	0.8	0.01
14:0	Myristic acid	0.8	0.06	1.3	0.01
16:0	Palmitic acid	13.2	0.86	16.3	0.25
16:1	Palmitoleic acid	34.1	1.10	28.6	0.33
16:2	-	3.0	0.19	4.3	0.15
18:0	Stearic acid	3.6	0.04	3.4	0.07
18:1	Oleic acid	25.8	1.45	14.8	0.22
18:2 (n-6)	Linoleic acid (LA)	6.0	1.00	10.6	0.66
18:3 (n-6)	Gamma-linolenic acid (GLA)	0.1	0.00	0.3	0.01
20:3 (n-6)	Dihomo-gamma-linolenic acid (DHGLA)	0.8	0.03	0.5	0.01
20:4	Arachidonic acid (ARA)	0.4	0.03	0.2	0.01

(n-6)					
18:3 (n-3)	Alpha-linolenic acid (ALA)	1.9	0.29	2.3	0.03
18:4 (n-3)	Stearidonic acid (STA)	0.1			
20:4 (n-3)	Eicosatetraenoic acid	1.1	0.05	1.1	0.01
20:5 (n-3)	Eicosapentaenoic acid (EPA)	0.7	0.07	0.5	0.01
misc	Other FA's	7.6	0.32	12.5	0.97
SUM		100.0		100.0	
SUM, Saturated FA		18.4	0.92	22.1	2.49
SUM, MUFA		59.9	2.41	43.4	10.91
SUM, PUFA (≥2 double bonds)		14.1	1.73	19.8	4.26
SUM, n-6 PUFA (≥3 double bonds)		1.2	0.12	1.0	0.19
SUM, n-3 PUFA (≥3 double bonds)		3.8	0.44	4.0	1.32
Fatty acid content (weight % of dw)		5.58	0.39	6.40	0.28

Example 74

Increased oxygen transfer by using large impellers results in improved omega-3 desaturation

The EPA-producing strain FS01658 was cultivated in controlled chemostat cultivation using two different fermentation set-ups; one fermentor set-up (A) utilized two standard rushton turbine impellers (Ø 5.2 cm) supplied by the fermentor manufacturer (Biostat B, Sartorius), and in the other fermentor set-up (B) the lower rushton turbine was replaced by a custom-made rushton turbine with a larger diameter (Ø 7.2 cm). The fermentors were run in chemostat mode as described in example 5, but at 30°C in stead of 17°C. Sterile air was supplied at a flow rate of 2.3 L/min and the stirring rate was set at 1000 rpm. The medium was a mineral medium with 100 g/l glucose as described in example 69.

The dissolved oxygen concentration was around 42% in fermentor A and around 61% in fermentor B, indicating that the oxygen transfer in the cultivation broth was more efficient in the fermentor utilizing a custom-made large impeller. Biomass samples for lipid analysis were taken at steady state and fatty acids were extracted, analyzed and quantified as described in Examples 55-58.

Analysis of the fatty acid composition (table 8) shows that the percentage of EPA was increased from 0.8 % of total fatty acids in the fermentor A to 1.3% of fatty acids in fermentor B. Moreover, the summed percentage of all omega-3 fatty acids with 3 or more double bonds increased from 6.4% of total fatty acids in the fermentor A to 10.1 % of fatty acids in fermentor B. This indicates that increased oxygen availability in particular affected the activity of the omega-3 desaturase.

Table 8. Fatty acid composition (% of total fatty acid) of FS01658 (MATa ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pox1::pTDH3-M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7- S.kluyveri FAD3 pADH1-FAS1 pADH1-FAS2 [pESC-URA]) cultivated in chemostat fermentation at 30°C in fermentors with standard (A) or custom-made (B) impellers. The values shown are averages of two samples from the same steady state.

Fatty acid (% of total FA)		Fermentor A	SD	Fermentor B	SD
8:0	Caprylic acid				
10:0	Capric acid				
12:0	Lauric acid	0.3		0.1	
14:0	Myristic acid	0.6	0.1	0.6	0.2
16:0	Palmitic acid	14.2	2.1	14.8	2.6
16:1(n-7)	Palmitoleic acid	16.2	1.3	14.8	0.0
16:2(n-4)	-	4.7	0.3	4.3	0.5
18:0	Stearic acid	5.8	0.6	5.8	0.4
18:1(n-9)	Oleic acid	10.5	1.3	10.3	0.2
18:1(n-7)		1.1	0.1	1.0	0.1

18:2(n-6)	Linoleic acid (LA)	29.2	5.3	27.0	1.5
18:3(n-6)	Gamma-linolenic acid (GLA)	0.9	0.1	1.0	0.2
20:2(n-6)	Eicosadienoic acid	0.4	0.1	0.3	0.0
20:3(n-6)	Dihomo-gamma-linolenic acid (DHGLA)	0.6	0.1	0.5	0.0
20:4(n-6)	Arachidonic acid (ARA)	0.7	0.5	0.8	0.1
22:4(n-6)	Docosatetraenoic acid (DTA)				
18:3(n-3)	Alpha-linolenic acid (ALA)	4.4	0.6	7.1	0.3
18:4(n-3)	Stearidonic acid (STA)	0.2	0.0	0.3	0.0
20:3(n-3)	Eicosatrienoic acid (ETA)	0.1		0.2	
20:4(n-3)	Eicosatetraenoic acid	0.9	0.1	1.3	0.2
20:5(n-3)	Eicosapentaenoic acid (EPA)	0.8	0.3	1.3	0.2
Total other fatty acids (%)		8.5	1.0	8.6	0.5
SUM (%)		100		100	
SUM, Saturated FA		20.8	1.8	21.3	2.3
SUM, MUFA		27.8	2.7	26.2	0.3
SUM, PUFA (³ 2 double bonds)		43.0	5.5	44.0	2.0
SUM, n-6 PUFA (³3 double bonds)		2.6	0.3	2.7	0.0
SUM, n-3 PUFA (³3 double bonds)		6.4	0.4	10.1	0.1
FA content in biomass (weight %)		4.3	0.1	3.9	0.1

Example 75

Overexpression of LRO1 results in improved DHGLA and ARA percentage

To investigate the effects of increased phospholipid-DAG acyltransferase activity on the PUFA composition, LRO1 was overexpressed with the PYK1 promoter in a genetic background carrying deletion of POX1, overexpression of M.alpina OLE1 and overexpressions of the native yeast genes FAS1, FAS2 and MCR1. The strain FS01539 overexpresses LRO1 in the mentioned genetic background and also expresses the pathway to ARA from high-copy plasmids. As a reference, the strain FS01490 (same genetic background but without CYB5 overexpression) was also

analyzed under the same conditions. The strains were grown with galactose as carbon in chemostat cultivation as described in Example 5 and using the growth medium described in Example 6. Biomass samples were taken at steady state and lipids were extracted and analyzed as described in Examples 9 and 10.

The results of the fatty acid composition analysis (Table 9) show that overexpression of LRO1 results in increased DHGLA and ARA percentage of total fatty acids.

Table 9. Fatty acid composition (% of total fatty acid) of FS01490 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 [pESCTRP-delta12-delta6] [pESCURA-elo-delta5]) and FS01539 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 pPYK1-LRO1 [pESCTRP-d12d-d6d] [pESC-URA-d6elo-d5d]) cultivated in chemostat fermentation.

Fatty acid		FS01490 (Ref)	FS01539 (pPYK1-LRO1)
16:0	Palmitic acid	15.1	15.4
16:1	Palmitoleic acid	17.2	19.9
18:0	Stearic acid	1.9	1.7
18:1	Oleic acid	24.8	23.8
18:2(n-6)	Linoleic acid (LA)	20.1	18
18:3(n-6)	Gamma-linolenic acid (GLA)	13.6	13
20:3(n-6)	Dihomo-gamma-linolenic acid (DHGLA)	1.7	2.1
20:4(n-6)	Arachidonic acid (ARA)	2.2	3.7
Other FA		3.3	2.4
Total FA		100	100
SUM, Saturated FA		17.0	17.1
SUM, MUFA		42.0	43.7
SUM, PUFA (ω3 2 double bonds)		37.6	36.8

SUM, n-6 PUFA (ᵌ3 double bonds)	17.5	18.8
SUM, n-3 PUFA (ᵌ3 double bonds)	-	-

Example 76

OPI1 deletion

Deletion of the OPI1 gene was carried out as described in Example 31. Target sequences upstream and downstream of OPI1 were amplified from genomic yeast DNA. Primers OPI1_up_fw and OPI1_up_rv were used to amplify a fragment upstream of the OPI1 gene. The upstream fragment was fused with a URA3 marker fragment, fragment 1 (construction of fragment 1 is described in Example 31), using primers OPI1_up_fw and Int3'. Likewise, the downstream fragment of OPI1 was amplified by primers OPI1_D_fw and OPI1_D_rv. The downstream fragment was fused to another URA3 marker fragment, fragment 2 (construction of fragment 2 is described in Example 31) using primers Int5' and OPI1_D_rv. Transformation with the linear substrates, subsequent isolation of transformants and 5-FOA mediated loop-out is described in Example 31. Correct deletion of the OPI1 gene was verified by PCR.

Example 77

Overexpression of FAS2 without overexpression of FAS1 results in increased EPA in total fatty acid

The effect of over-expressing FAS2 in a strain where the EPA-genes have been integrated in the genome was tested in shake flask cultivations. Strains were grown in triplicate in shake flasks with glucose as carbon source as described in Example 67, except that the concentration of glucose was 40g/L instead of 20g/L. Cells were harvested in exponential phase for lipid extraction, and fatty acids were extracted, analyzed and quantified as described in examples 55-58. The strain FS01639 (Mata ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI-MCR1 pox::pTDH3 M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-KLURA-pHXT7-S. kluveri FAD3 pADH1-FAS1 pADH1-FAS2) over-expresses both FAS1 and FAS2. The strain FS01640 over-expresses FAS2 alone

(Mata ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI-MCR1 pox::pTDH3 M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MaD5D dci1::pADH1-OtD6D gpp1::pHXT7-KLURA-pHXT7-S. kluveri FAD3 pADH1-FAS2) and FS01641 (Mata ura3-52 trp1-289-pPYK1-MaD6E-TRP1 pTPI-MCR1 pox::pTDH3 M.alpina OLE1 fox2::pTDH3-MaD12D pot1::pADH1-MAD5D dci1::pADH1-OtD6D gpp1::pHXT7-KLURA-pHXT7-S. kluveri FAD3 pADH1-FAS1) over-expresses FAS1 alone.

Fatty acid		Average FS01639 (pADH1-FAS1 pADH1-FAS2)	abs. SD	Average FS01640 (pADH1-FAS2)	abs. SD	Average FS01641 (pADH1-FAS1)	abs. SD
12:0	Lauric acid	0.5	0.1	0.2	0.0	0.5	0.0
14:0	Myristic acid	1.2	0.0	0.6	0.0	1.0	0.2
16:0	Palmitic acid	19.6	0.7	14.2	1.4	18.3	4.3
16:1	Palmitoleic acid	29.5	1.7	26.9	3.7	27.0	6.7
16:2	-	5.1	0.5	3.6	0.7	6.4	2.2
18:0	Stearic acid	3.5	0.2	4.4	0.1	3.6	0.9
18:1	Oleic acid	15.1	1.2	21.0	3.9	15.1	7.2
18:2(n-6)	Linoleic acid (LA)	9.8	0.7	11.1	3.1	10.6	4.1
18:3(n-6)	Gamma-linolenic acid (GLA)	0.4	0.1	0.3	0.1	2.4	0.6
20:3(n-6)	Dihomo-gamma-linolenic acid (DHGLA)	1.0	0.1	1.3	0.2		
20:4(n-6)	Arachidonic acid (ARA)	0.4	0.0	0.6	0.1		
18:3(n-3)	Alpha-linolenic acid (ALA)	2.6	0.2	3.5	0.7	3.1	1.4
18:4(n-3)	Stearidonic acid (STA)			0.1	0.1	2.7	
20:3(n-3)	Eicosatrienoic acid (ETA)			0.1	0.1		
20:4(n-3)	Eicosatetraenoic acid	1.8	0.2	2.3	0.3		
20:5(n-3)	Eicosapentaenoic acid (EPA)	0.9	0.1	1.6	0.2		
misc	Other FA's	8.7	1.1	8.1	0.6	9.5	1.6
SUM		100		100		100	
SUM, Saturated FA		24.8	0.5	19.4	1.5	23.3	3.5
SUM, MUFA		44.6	2.8	48.0	7.6	42.1	13.9
SUM, PUFA (³2 double bonds)		21.9	1.8	24.6	5.5	25.1	9.2
SUM, n-6 PUFA (³3 double bonds)		1.7	0.2	2.4	0.4	2.4	0.6
SUM, n-3 PUFA (³3 double bonds)		5.3	0.5	7.5	1.3	5.8	2.3
FA content in biomass (weight %)		4.4	0.4	4.4	0.2	4.0	0.0

Table 10. Fatty acid composition (% of total fatty acid) of strains with genes encoding the EPA-pathway integrated in the genome of S. cerevisiae strains and either over-expressing FAS1, FAS2 or the FAS1/FAS2 complex. The FAS1/FAS2

complex is over-expressed in strain FS01639, FAS2 in FS01640 and FAS1 in FS01641. Shake flask cultivation with glucose as carbon source (40g/L) in triplicate at 30°C.

The results of the fatty acid composition analysis (Table 10) showed that over-expression of FAS2 results in increased amounts of ARA and EPA when compared to the strain over-expressing either FAS1 alone or the FAS1/FAS2 complex. Moreover FAS2 over-expression lead to increased percentage of C-18 fatty acids at the expense of C-16 fatty acids as described in Example 23.

Example 78

Deletion of OPI1 results in increased lipid content

Deletion of the negative regulator of fatty acid synthesis Opi1 was analyzed in shake flask cultivations. Strains were grown in triplicate in minimal media with galactose as carbon source as described in example 68, except that the incubation temperature was 17°C instead of 30°C. FS01542 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 opi1::R [pESC-TRP-d12d-d6d] [pESC-URA-d6elo-d5d]) was analyzed using FS01490 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 [pESCTRP-d12d-d6d] [pESCURA-d6e-d5d]) as reference.
The deletion of OPI1 increased the lipid yield to an average of 11.46% of dry weight (absolute SD 1.02%) compared with an average of 7.99% of dry weight (absolute SD 0.51%) for the reference strain.

Example 79

Construction of plasmid pSF002 expressing INO4 from the GAL1 promoter in vector pESC-TRP

The coding sequence of INO4 was isolated by PCR from genomic DNA from yeast strain CEN-PK using gene specific primers 7003 (SEQ ID NO: 164) and 7004 (SEQ ID NO: 165). Forward primer 7003 contains a BamH1 cloning site upstream of the

ATG. Reverse primer 7004 contains a Xho1 cloning site downstream of the stop codon.

The PCR reaction was purified with a PCR clean-up kit (Macherey-Nagel). Vector p-ESC-TRP (Stratagene) and the PCR reaction were digested with restriction enzymes BamH1 and Xho1 (New England Biolabs) in reaction buffer 3, purified from an agarose gel using a gel-extraction clean-up kit (Macherey-Nagel) and ligated together. This produces a plasmid pSF002 (figure 16) with the coding sequence of INO4 under control of the GAL1 promoter and with TRP1 as selectable marker for yeast expression.

Example 80

Construction of plasmid pSF003 expressing INO2 from the GAL1 promoter in vector pESC-URA

The coding sequence of INO2 was isolated by PCR from genomic DNA from yeast strain CEN-PK using gene specific primers 7001 (SEQ ID NO: 162) and 7002 (SEQ ID NO: 163) described below. Forward primer 7001 contains a BamH1 cloning site upstream of the ATG. Reverse primer 7002 contains a Xho1 cloning site downstream of the stop codon.

The PCR reaction contained: 35 microliter water, 10 microliter 5xHF buffer, 1 microliter primer 7001 20 micromolar stock, 1 microliter primer 7002 20 micromolar stock, 2 microliter genomic DNA template at ca 50 nanogram/microliter, 1 microliter Phusion polymerase (Finnzymes) at 2 units/microliter. The PCR program was: 98C 5 minutes, 1 cycle; 98C 30 seconds, 68C 30 seconds, 72C 30 seconds, 10 cycles; 95C 30 seconds, 60C 30 seconds, 72C 30 seconds, 20 cycles; 72C 5 minutes. 1 cycle.

The PCR reaction was purified with a PCR clean-up kit (Macherey-Nagel). Vector p-ESC-URA (Stratagene) and the PCR reaction were digested with restriction enzymes BamH1 and Xho1 (New England Biolabs) in reaction buffer 3, purified from an agarose gel using a gel-extraction clean-up kit (Macherey-Nagel) and ligated together. This produces a plasmid pSF003 (figure 17) with the coding

sequence of INO2 under control of the GAL1 promoter and with URA3 as selectable marker for yeast expression.

Example 81

Transformation of plasmids expressing INO2 and INO4 into yeast strains

Plasmids pSF002 and pSF003 were transformed together into yeast strains FS01494 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1), FS01541 (MATa ura3-52 trp1-289 pox1::pTDH3-M.alpina OLE1 pADH1-FAS1 pADH1-FAS2 pTPI1-MCR1 opi1::R) and FS01567 (MATalpha ura3-52 trp1-289 pox1::pTDH3-M.alpina ole1 pADH1-FAS1 pTPI1-MCR1 pTPI1-ACC1) selecting for growth on SC media lacking tryptophan and uracil.

The same strains were also transformed with empty vectors pESC-URA and pESC-TRP to serve as controls.

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CLAIMS

1. A method for the production of polyunsaturated fatty acid (PUFA) in a fungal cell comprising at least two desaturases, said method comprises increasing the *in vivo* desaturase efficiency in said cell.

2. A method according to claim 1, wherein the at least two desaturases are selected from the group consisting of delta-9 desaturase, delta-6 desaturase, delta-12 desaturase, delta-5 desaturase, omega-3 desaturase, delta-4 desaturase and delta-8- desaturase.

3. A method according to claims 1-2, wherein the at least two desaturases are delta-9 desaturase and delta-12 desaturase.

4. A method according to any of the preceeding claims, wherein the at least two desaturases are delta-9 desaturase, delta-12 desaturase and delta-6 desaturase.

5. A method according to any of the preceeding claims, wherein the at least two desaturases are delta-9 desaturase, delta-12 desaturase, delta-5 desaturase and delta-6 desaturase.

6. A method according to any of the preceeding claims, wherein the at least two desaturases are delta-9 desaturase, delta-12 desaturase, delta-5 desaturase, delta-6 desaturase and omega-3 desaturase.

7. A method according to any of the preceeding claims, wherein the increased *in vivo* desaturase efficiancy may be measured by the following steps:

- providing a first fungal cell population having a genotype comprising at least two genes encoding desaturases

- providing a second fungal cell population with the same genoype as the first fungal cell population and further adding in said cell population at least one modification increasing the *in vivo* desaturase efficiency,

- measuring the fraction of PUFA with 2 or more double bonds as % of total fatty acids produced in both the first and second fungal cell populations,

-identifying a fungal cell population as having an increased *in vivo* desaturase efficiency, if the second cell population compared to the first cell population show an increase in the PUFA fraction (% of PUFA of total fatty acid) by at least 0,5%.

8. A method according to any of the preceeding claims, wherein the rate of Fe3+ reduction and reoxidation is increased.

9. A method according to any of the preceeding claims, comprising over-expression of at least one of the genes selected from the group consisting of MCR1 and CYB5.

10. A method according to any of the preceeding claims, comprising heterologous expression of at least one of the genes selected from the group consisting of MCR1 and CYB5.

11. A method according to any of the preceeding claims, comprising over-expression of at least one of the genes selected from the group consisting of FAS2, INO2, INO4 and LRO1.

12. A method according to any of the preceeding claims, further comprising deletion of OPI1.

13. A Saccharomyces cerevisiae comprising EPA and ARA in the ratio of at least 1:1.

14. A Saccharomyces cerevisiae according to claim 13, comprising at least 0.4 mg EPA pr. gram dry weight cell.

15. A Saccharomyces cerevisiae according to any of claims 13-14, comprising at least 1,5 mg ARA pr. gram dry weight cell.

16. A Saccharomyces cerevisiae according to any of claims 13-15, comprising at least 8.0 mg GLA pr. gram dry weight cell.

17. A Saccharomyces cerevisiae according to any of claims 13-16, comprising at least 24 mg. PUFA pr. gram dry weight cell.

18. A method for the production of polyunsaturated fatty acid (PUFA) in a fungal cell comprising at least two desaturases, said method comprising increasing the fatty acid content in said cell.

19. A method according to claim 18, wherein said method comprises over-expression of the at least one gene selected from the group consisting of INO2 and INO4.

20. A method according to claim 18, wherein said method comprises deletion of the gene OPI1.

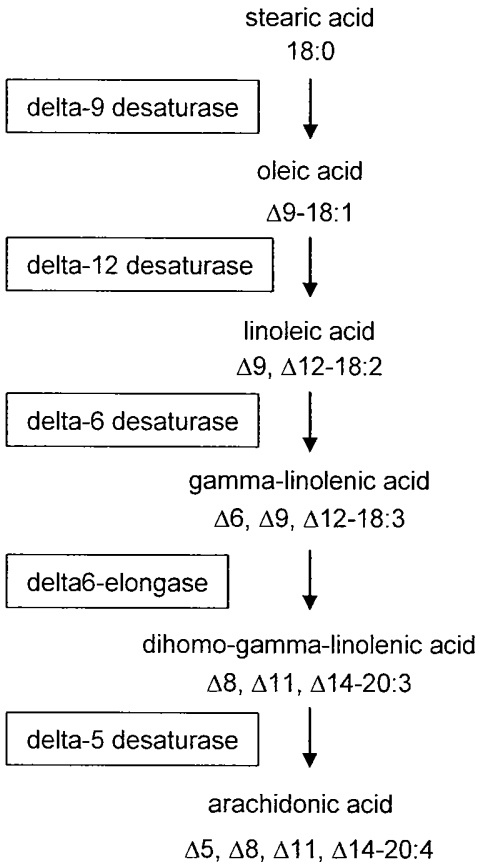
stearic acid
18:0
delta-9 desaturase
oleic acid
Δ9-18:1
delta-12 desaturase
linoleic acid
Δ9, Δ12-18:2
delta-6 desaturase
gamma-linolenic acid
Δ6, Δ9, Δ12-18:3
delta6-elongase
dihomo-gamma-linolenic acid
Δ8, Δ11, Δ14-20:3
delta-5 desaturase
arachidonic acid
Δ5, Δ8, Δ11, Δ14-20:4

Fig. 1

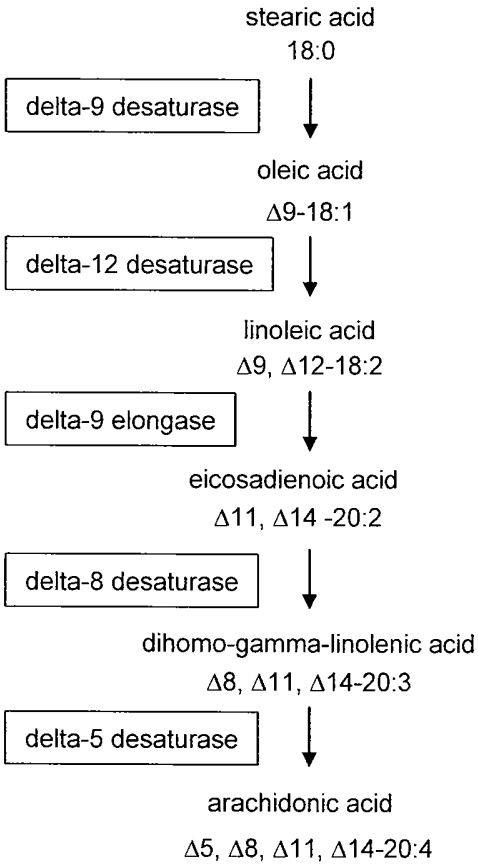
stearic acid
18:0
delta-9 desaturase
oleic acid
Δ9-18:1
delta-12 desaturase
linoleic acid
Δ9, Δ12-18:2
delta-9 elongase
eicosadienoic acid
Δ11, Δ14 -20:2
delta-8 desaturase
dihomo-gamma-linolenic acid
Δ8, Δ11, Δ14-20:3
delta-5 desaturase
arachidonic acid
Δ5, Δ8, Δ11, Δ14-20:4

Fig. 2

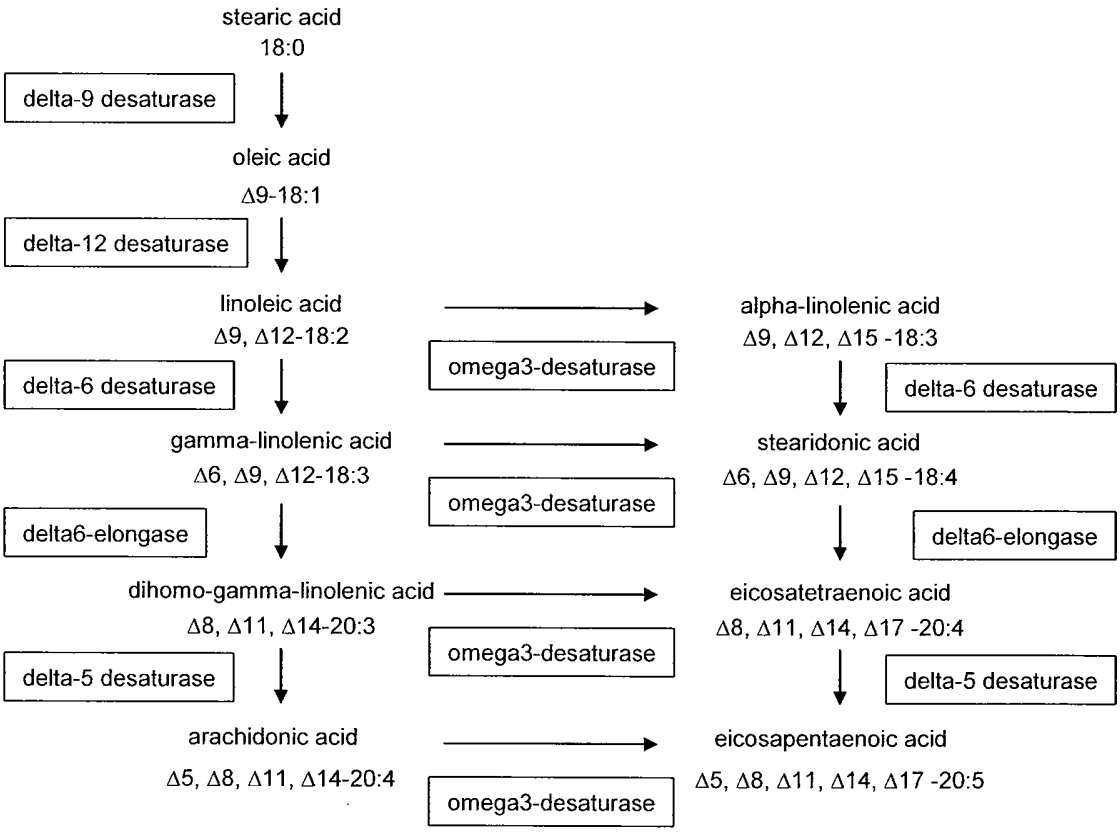
stearic acid
18:0
delta-9 desaturase
oleic acid
Δ9-18:1
delta-12 desaturase
linoleic acid
Δ9, Δ12-18:2
delta-6 desaturase
gamma-linolenic acid
Δ6, Δ9, Δ12-18:3
delta6-elongase
dihomo-gamma-linolenic acid
Δ8, Δ11, Δ14-20:3
delta-5 desaturase
arachidonic acid
Δ5, Δ8, Δ11, Δ14-20:4
omega3-desaturase
omega3-desaturase
omega3-desaturase
omega3-desaturase
alpha-linolenic acid
Δ9, Δ12, Δ15 -18:3
delta-6 desaturase
stearidonic acid
Δ6, Δ9, Δ12, Δ15 -18:4
delta6-elongase
eicosatetraenoic acid
Δ8, Δ11, Δ14, Δ17 -20:4
delta-5 desaturase
eicosapentaenoic acid
Δ5, Δ8, Δ11, Δ14, Δ17 -20:5

Fig. 3

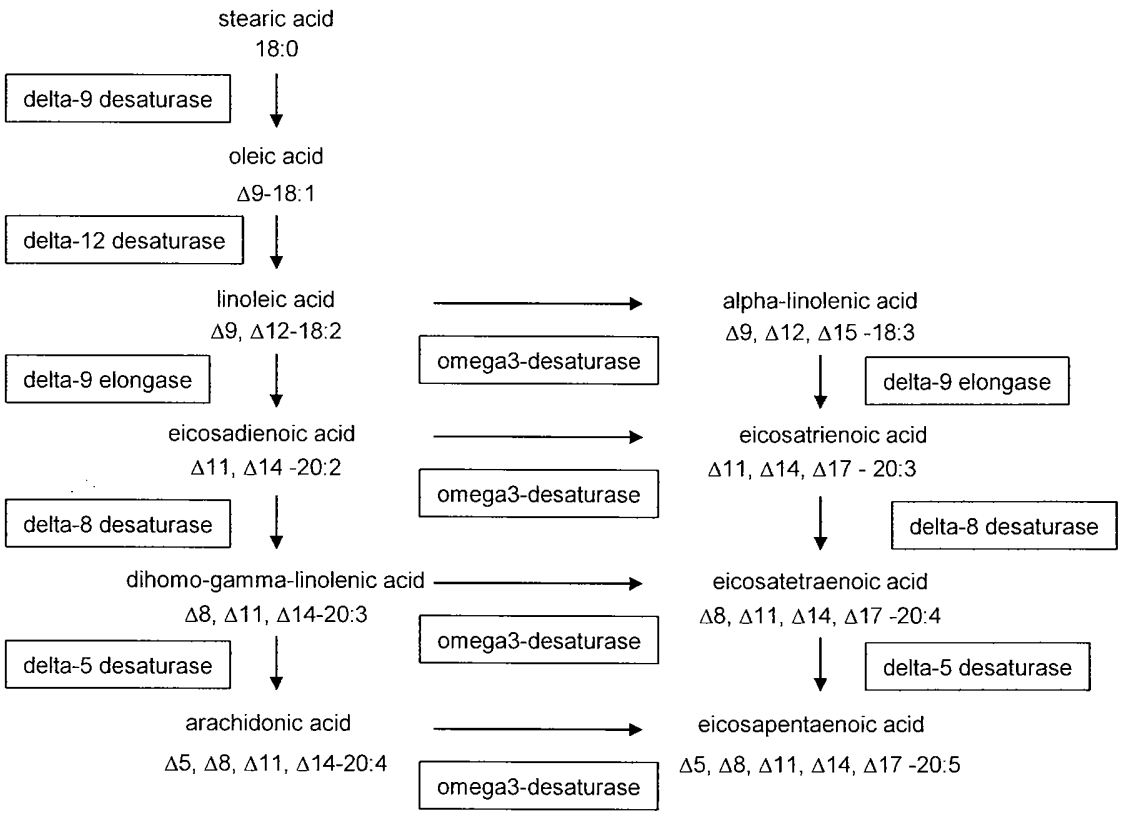
stearic acid
18:0
delta-9 desaturase
oleic acid
Δ9-18:1
delta-12 desaturase
linoleic acid
Δ9, Δ12-18:2
omega3-desaturase
alpha-linolenic acid
Δ9, Δ12, Δ15 -18:3
delta-9 elongase
delta-9 elongase
eicosadienoic acid
Δ11, Δ14 -20:2
omega3-desaturase
eicosatrienoic acid
Δ11, Δ14, Δ17 - 20:3
delta-8 desaturase
delta-8 desaturase
dihomo-gamma-linolenic acid
Δ8, Δ11, Δ14-20:3
omega3-desaturase
eicosatetraenoic acid
Δ8, Δ11, Δ14, Δ17 -20:4
delta-5 desaturase
delta-5 desaturase
arachidonic acid
Δ5, Δ8, Δ11, Δ14-20:4
omega3-desaturase
eicosapentaenoic acid
Δ5, Δ8, Δ11, Δ14, Δ17 -20:5

Fig. 4

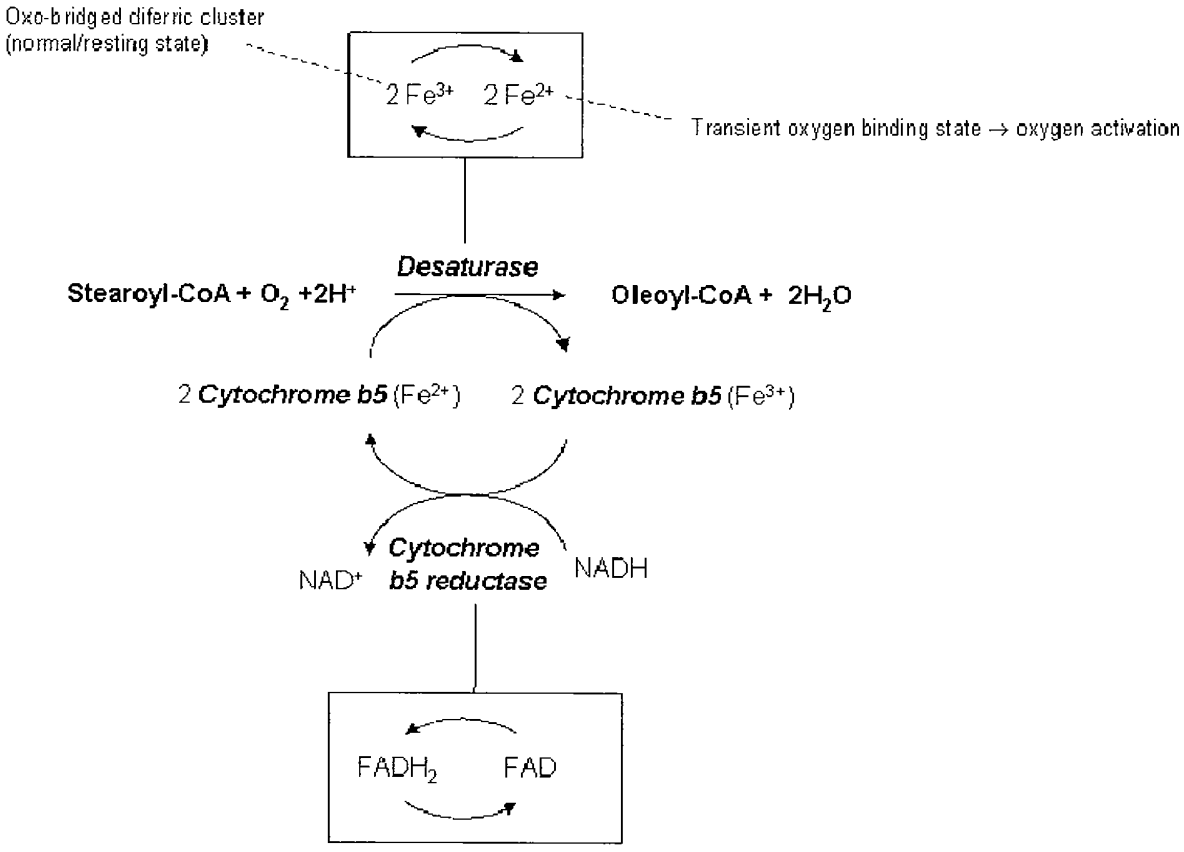
Oxo-bridged diferric cluster
(normal/resting state)
2 Fe3+ 2 Fe2+
Transient oxygen binding state → oxygen activation
Desaturase
Stearoyl-CoA + O2 +2H+
Oleoyl-CoA + 2H2O
2 Cytochrome b5 (Fe2+)
2 Cytochrome b5 (Fe3+)
Cytochrome
b5 reductase
NAD+
NADH
FADH2
FAD

Fig. 5

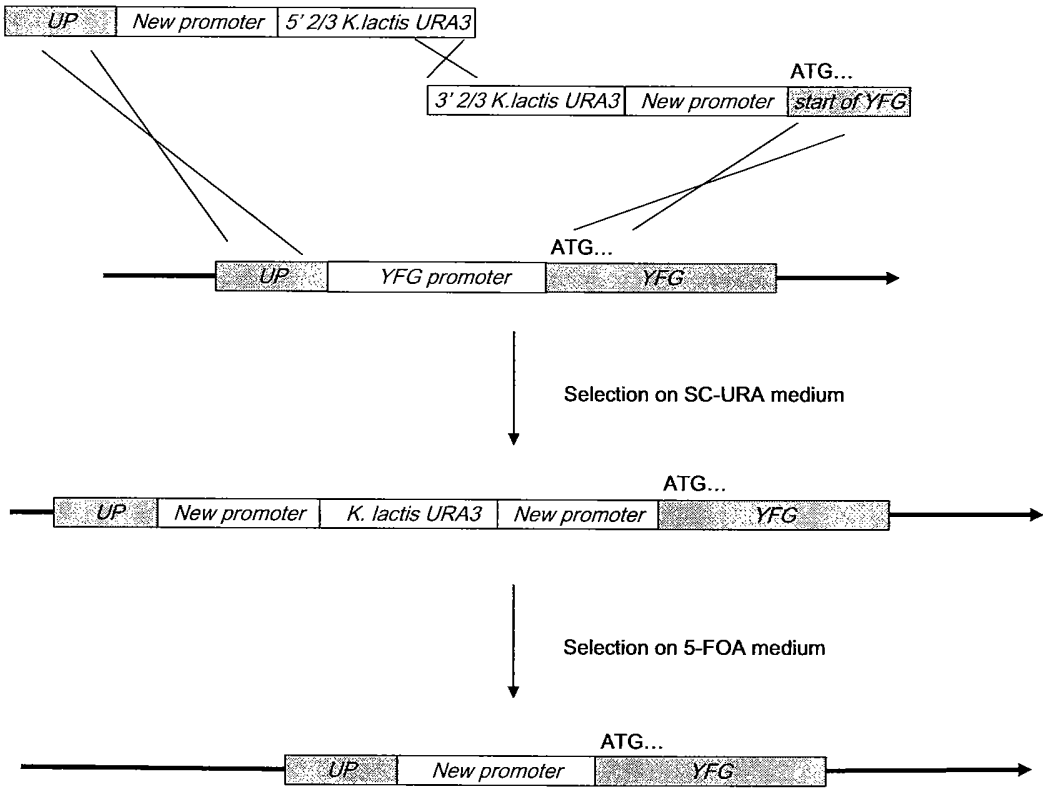
UP
New promoter
5' 2/3 K.lactis URA3
3' 2/3 K.lactis URA3
New promoter
start of YFG
ATG...
ATG...
UP
YFG promoter
YFG
Selection on SC-URA medium
ATG...
UP
New promoter
K. lactis URA3
New promoter
YFG
Selection on 5-FOA medium
ATG...
UP
New promoter
YFG

Fig. 6

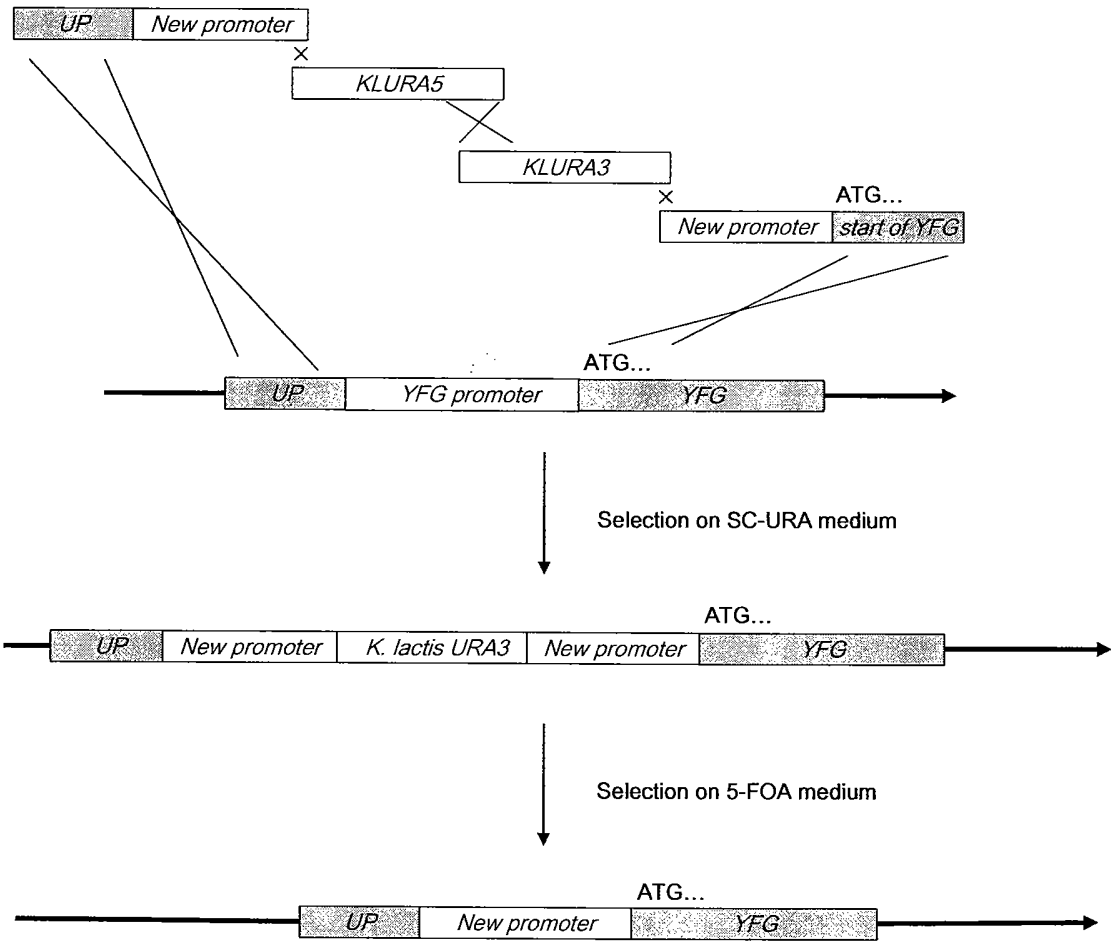
UP
New promoter
KLURA5
KLURA3
ATG...
New promoter
start of YFG
ATG...
UP
YFG promoter
YFG
Selection on SC-URA medium
ATG...
UP
New promoter
K. lactis URA3
New promoter
YFG
Selection on 5-FOA medium
ATG...
UP
New promoter
YFG

Fig. 7

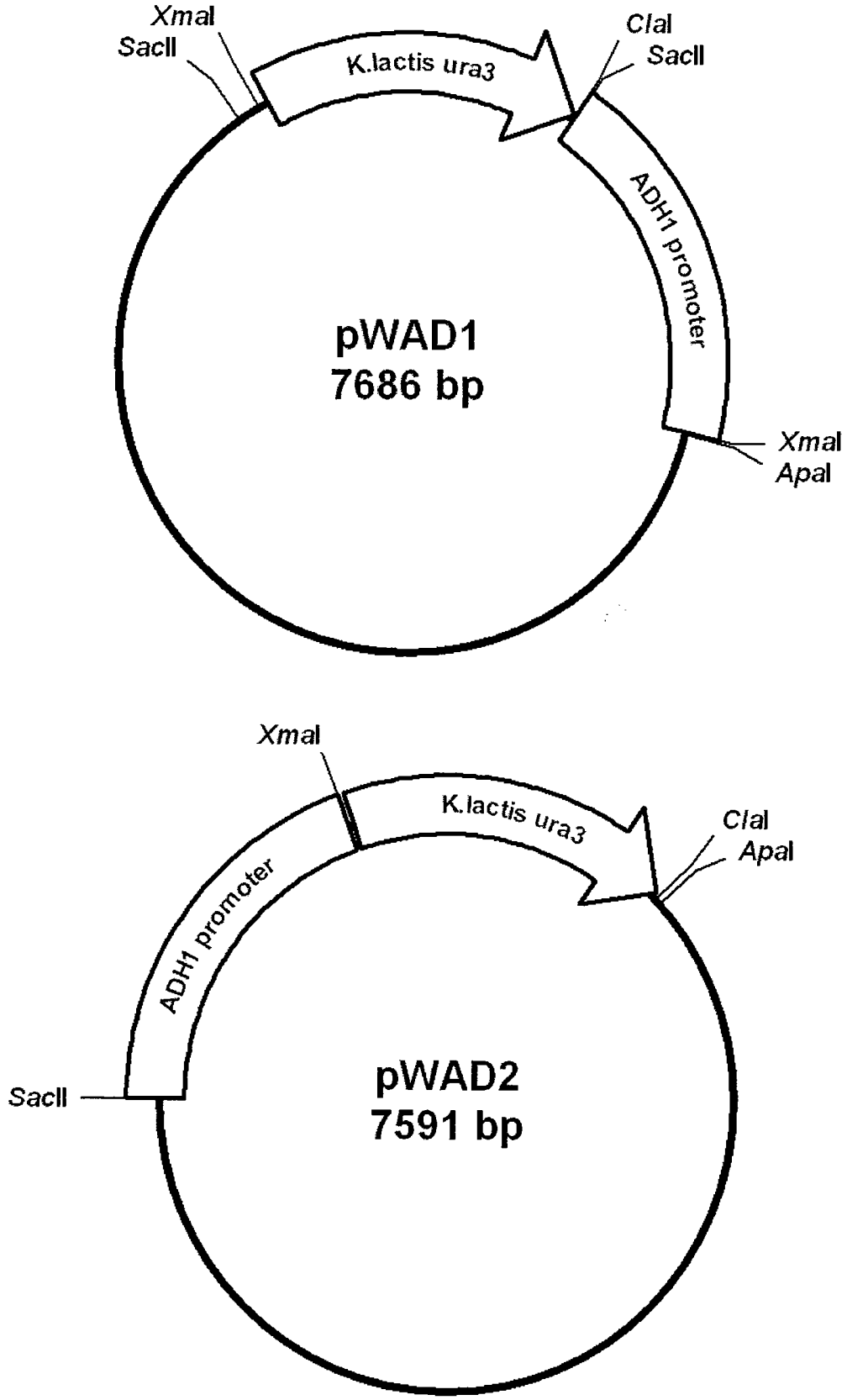
XmaI
SacII
K.lactis ura3
ClaI
SacII
ADH1 promoter
pWAD1
7686 bp
XmaI
ApaI
XmaI
K.lactis ura3
ClaI
ApaI
ADH1 promoter
SacII
pWAD2
7591 bp

Fig. 8

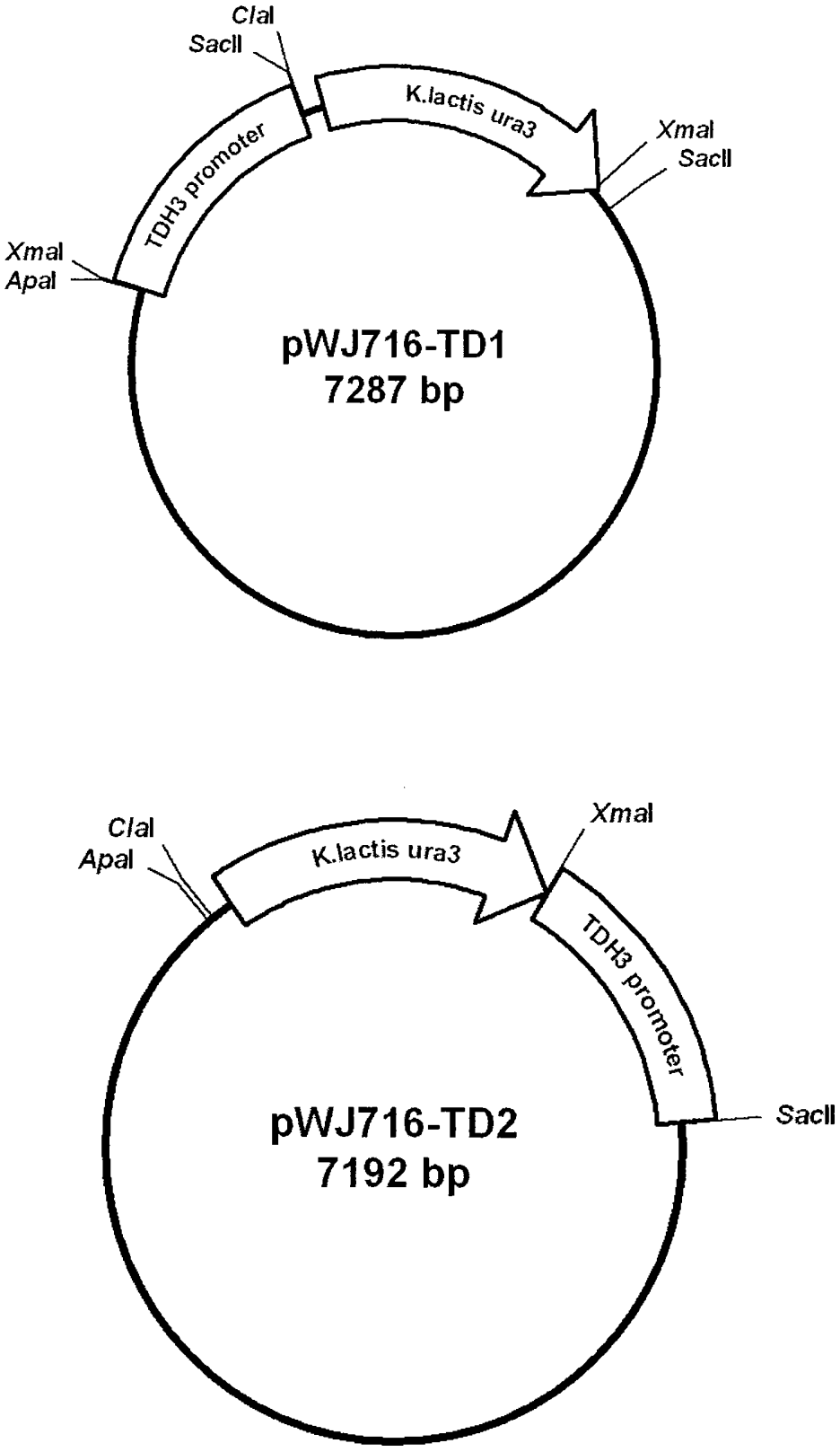
ClaI
SacII
K.lactis ura3
XmaI
SacII
TDH3 promoter
XmaI
ApaI
pWJ716-TD1
7287 bp
ClaI
ApaI
K.lactis ura3
XmaI
TDH3 promoter
SacII
pWJ716-TD2
7192 bp

Fig. 9

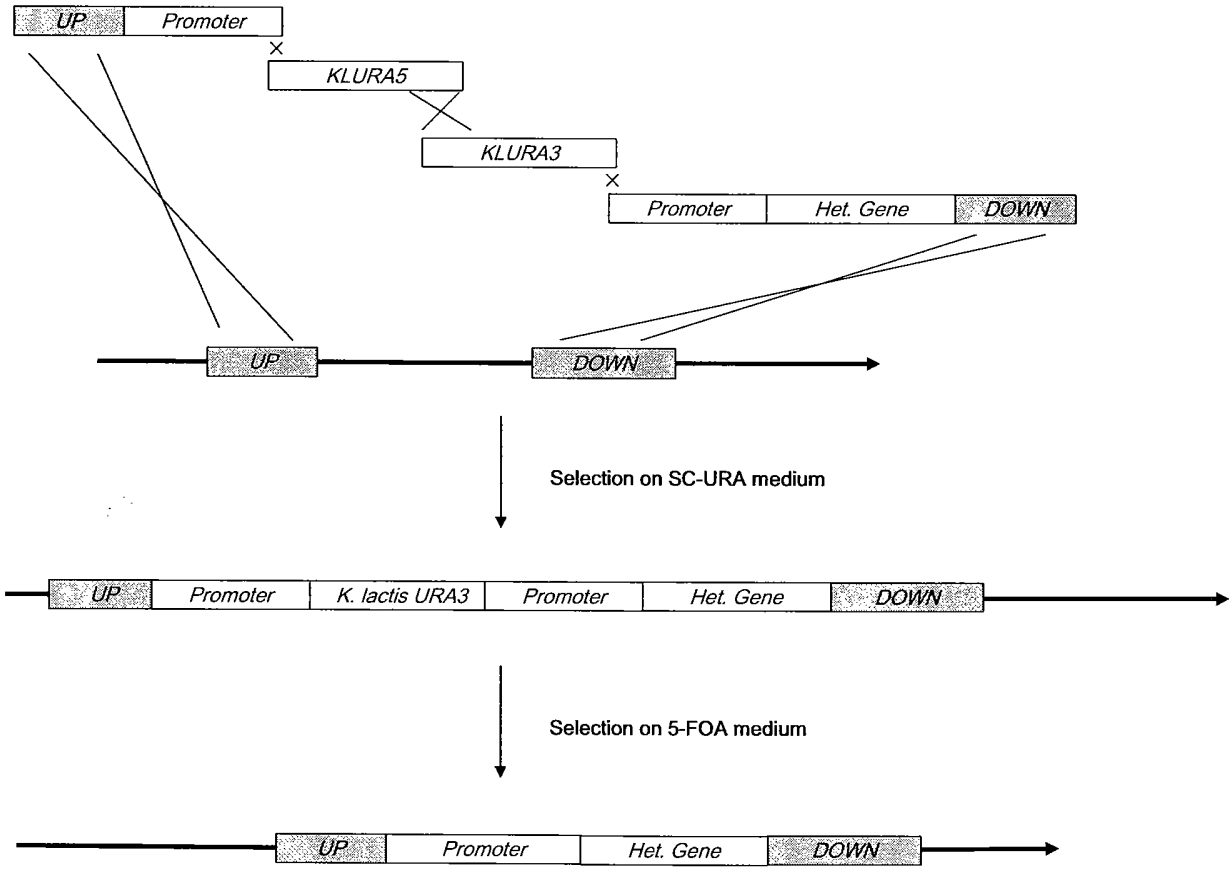
UP
Promoter
KLURA5
KLURA3
Promoter
Het. Gene
DOWN
UP
DOWN
Selection on SC-URA medium
UP
Promoter
K. lactis URA3
Promoter
Het. Gene
DOWN
Selection on 5-FOA medium
UP
Promoter
Het. Gene
DOWN

Fig. 10

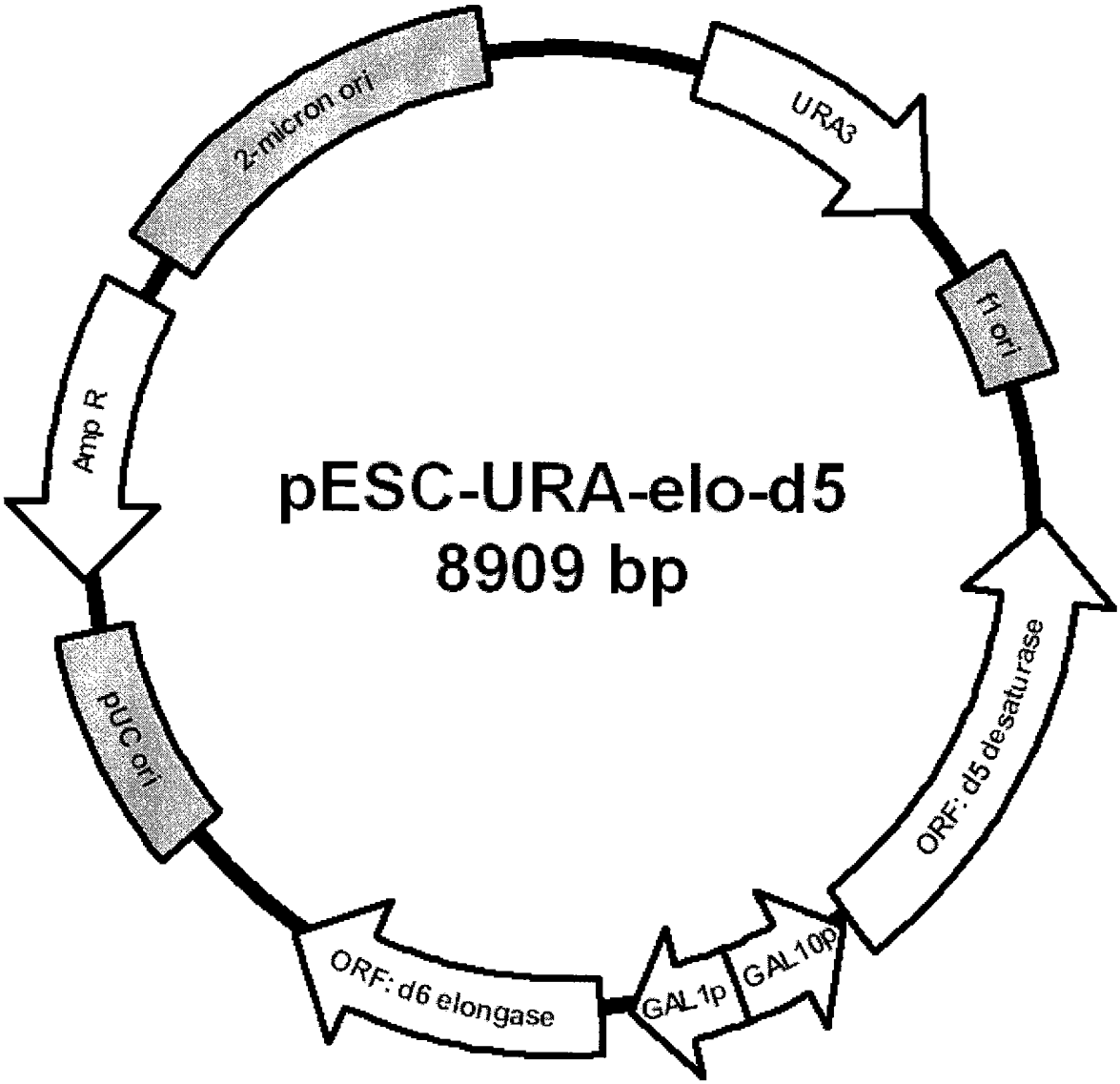
pESC-URA-elo-d5
8909 bp
2-micron ori
URA3
f1 ori
ORF: d5 desaturase
GAL10p
GAL1p
ORF: d6 elongase
pUC ori
Amp R

Fig. 11

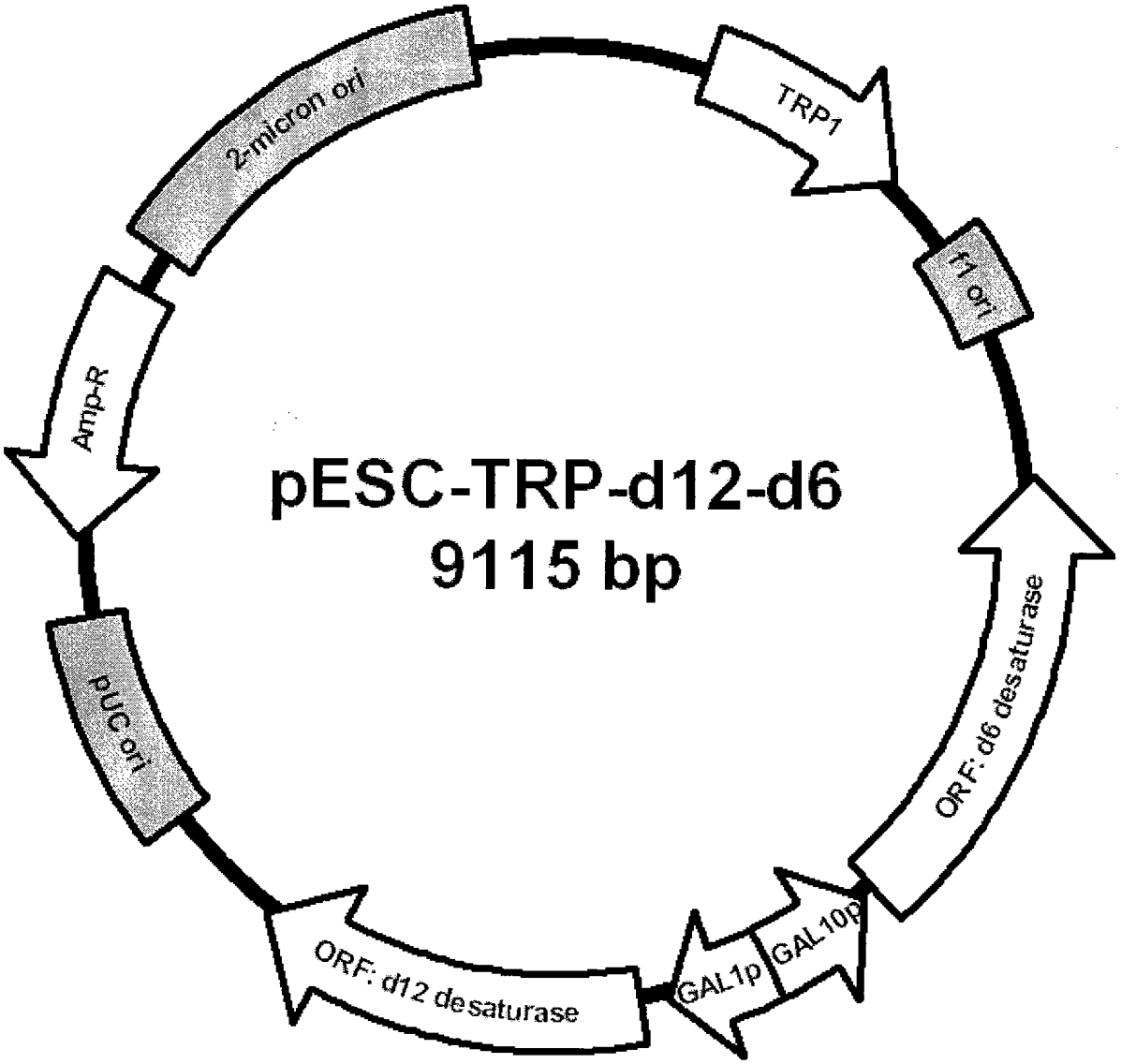
pESC-TRP-d12-d6
9115 bp
2-micron ori
TRP1
f1 ori
ORF: d6 desaturase
GAL10p
GAL1p
ORF: d12 desaturase
pUC ori
Amp-R

Fig. 12

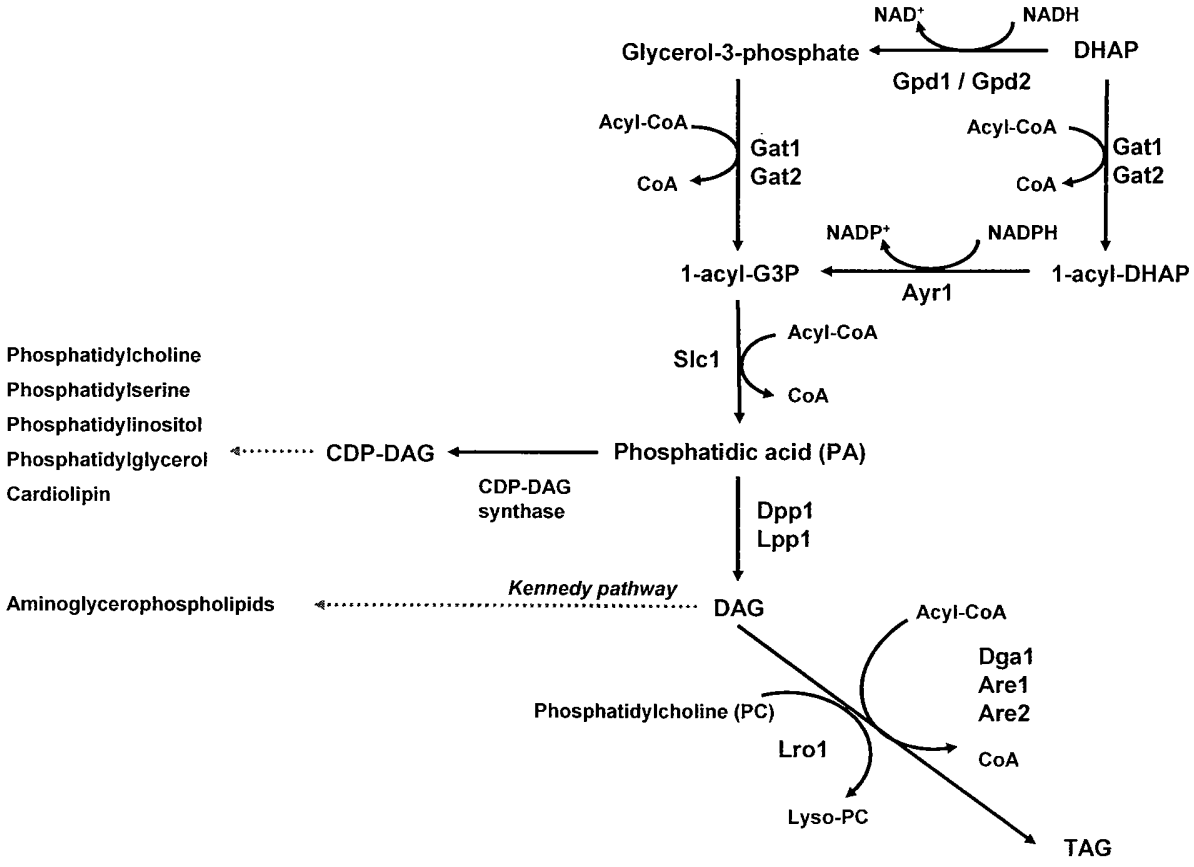
NAD+
NADH
Glycerol-3-phosphate
DHAP
Gpd1 / Gpd2
Acyl-CoA
Gat1
Gat2
CoA
Acyl-CoA
Gat1
Gat2
CoA
NADP+
NADPH
1-acyl-G3P
1-acyl-DHAP
Ayr1
Acyl-CoA
Slc1
CoA
Phosphatidylcholine
Phosphatidylserine
Phosphatidylinositol
Phosphatidylglycerol
Cardiolipin
CDP-DAG
Phosphatidic acid (PA)
CDP-DAG
synthase
Dpp1
Lpp1
Kennedy pathway
Aminoglycerophospholipids
DAG
Acyl-CoA
Dga1
Are1
Are2
Phosphatidylcholine (PC)
Lro1
CoA
Lyso-PC
TAG

Fig. 13

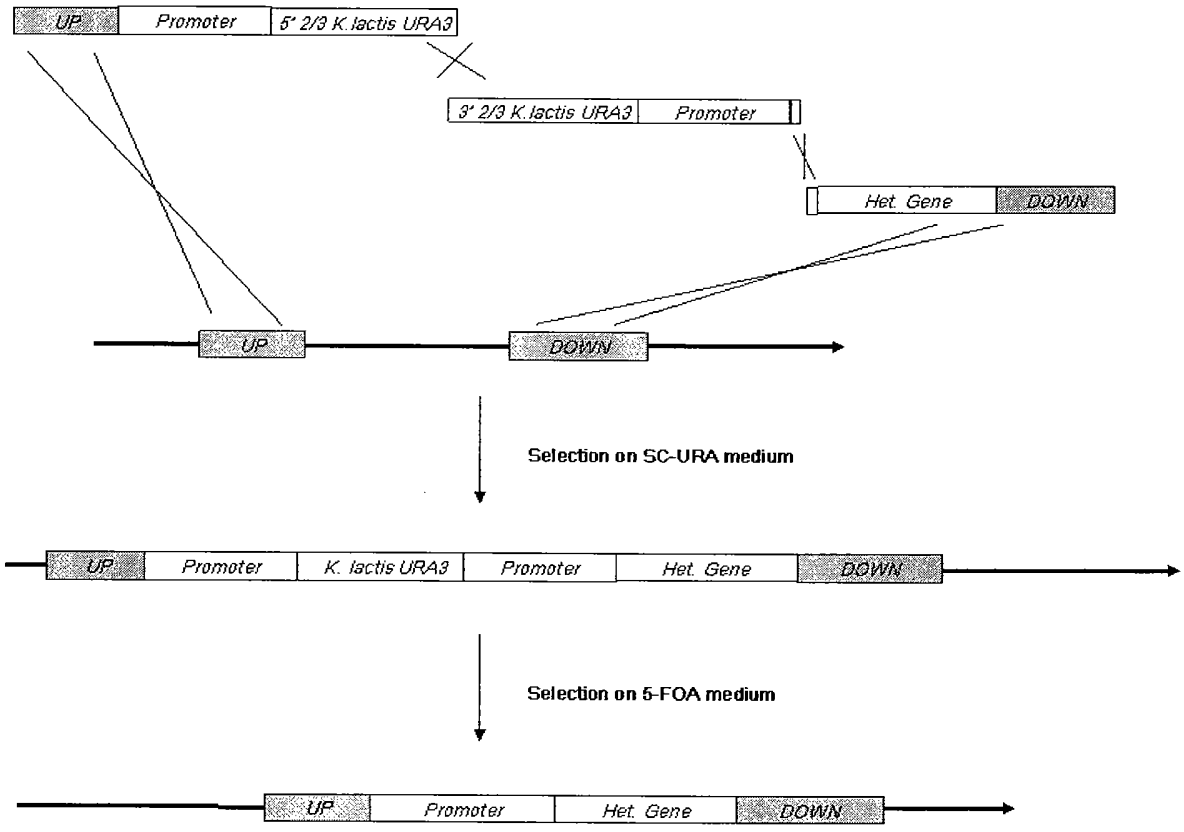
UP
Promoter
5' 2/3 K.lactis URA3
3' 2/3 K.lactis URA3
Promoter
Het. Gene
DOWN
UP
DOWN
Selection on SC-URA medium
UP
Promoter
K. lactis URA3
Promoter
Het. Gene
DOWN
Selection on 5-FOA medium
UP
Promoter
Het. Gene
DOWN

Fig. 14

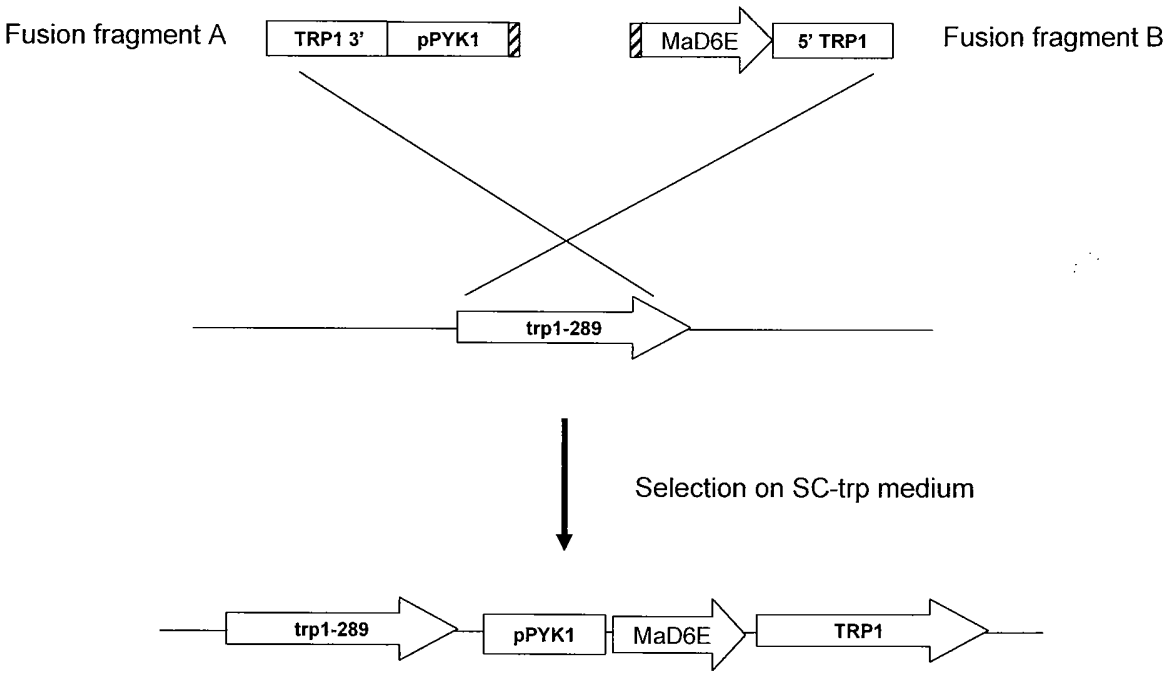
Fusion fragment A
TRP1 3'
pPYK1
MaD6E
5' TRP1
Fusion fragment B
trp1-289
Selection on SC-trp medium
trp1-289
pPYK1
MaD6E
TRP1

Fig. 15

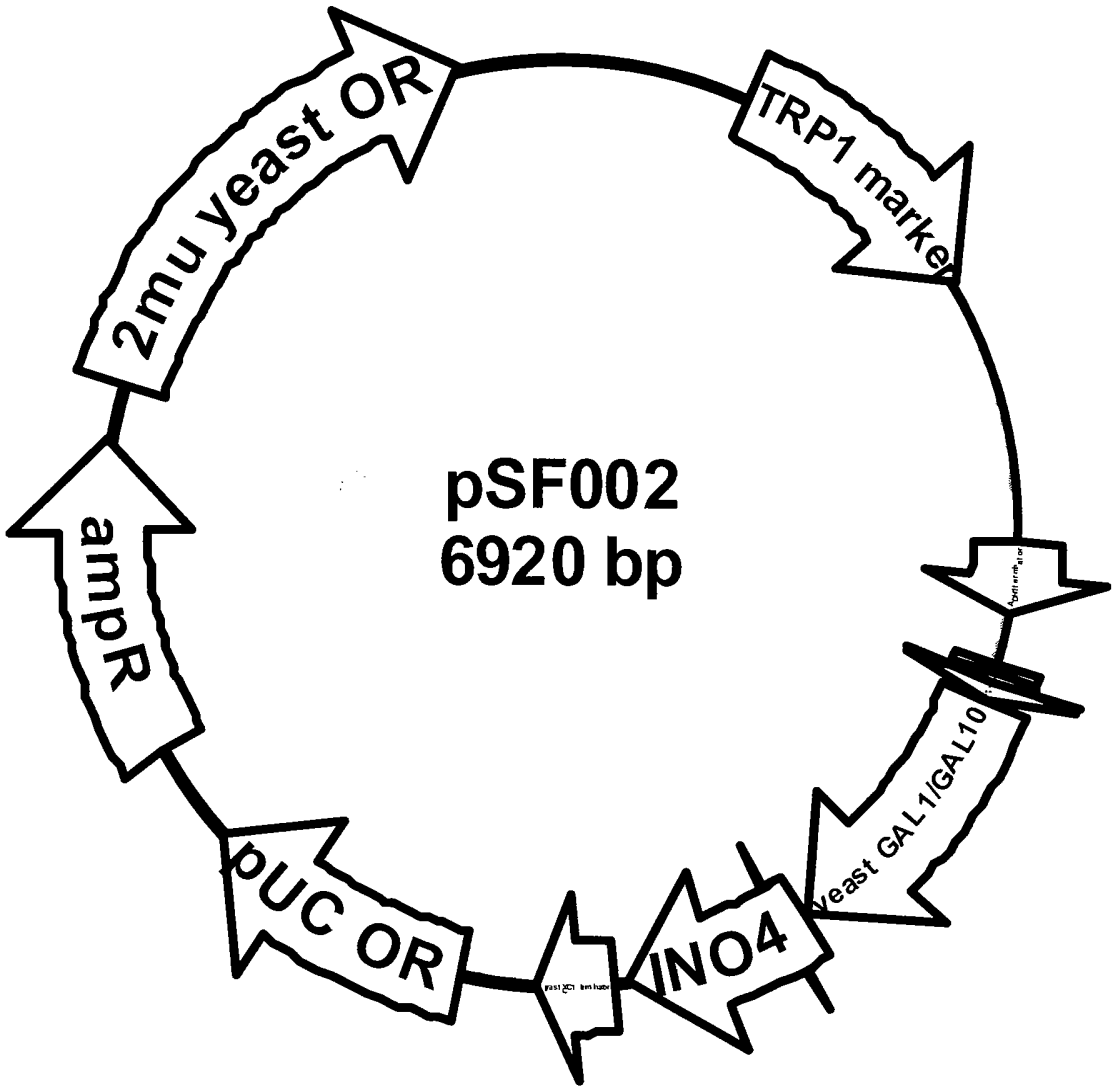
2mu yeast OR
TRP1 marker
pSF002
6920 bp
yeast GAL1/GAL10
INO4
pUC OR
ampR

Fig. 16

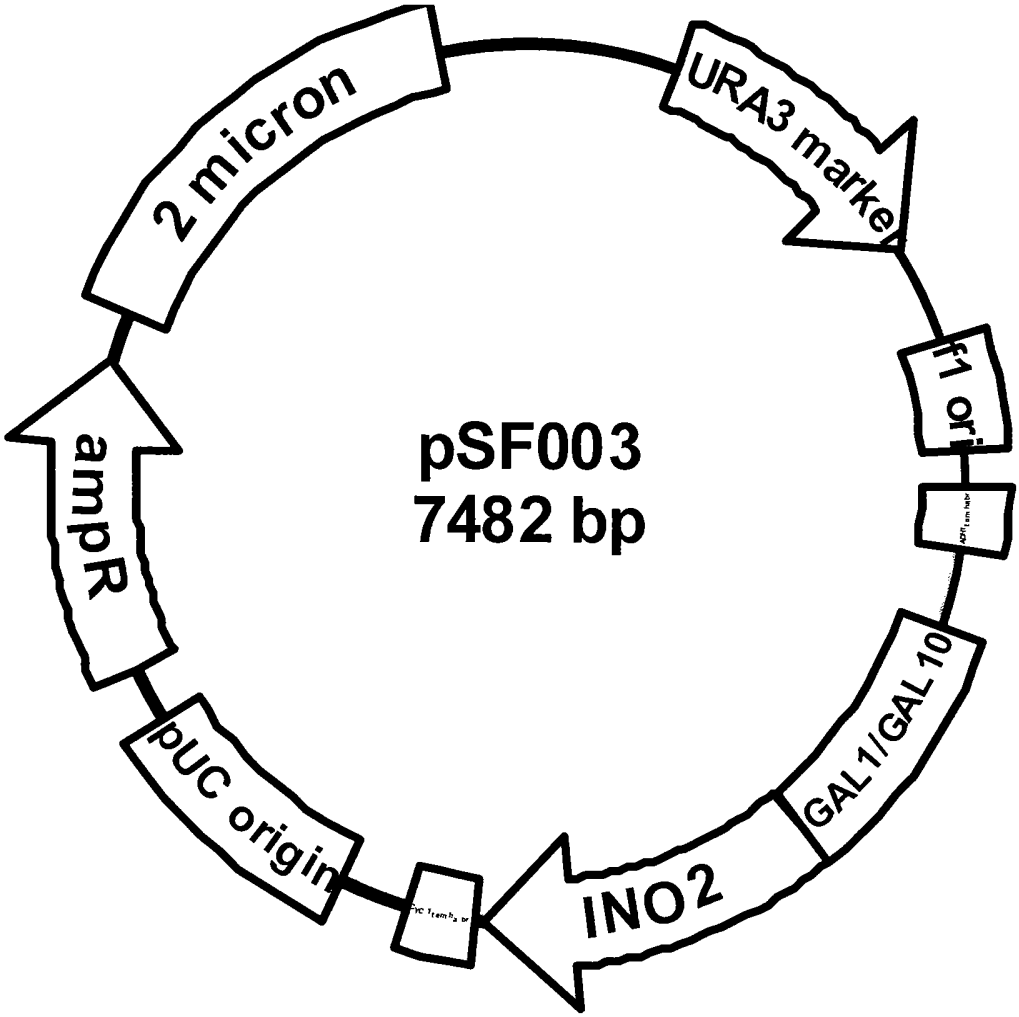
2 micron
URA3 marker
f1 ori
GAL1/GAL10
INO2
pUC origin
ampR
pSF003
7482 bp

Fig. 17

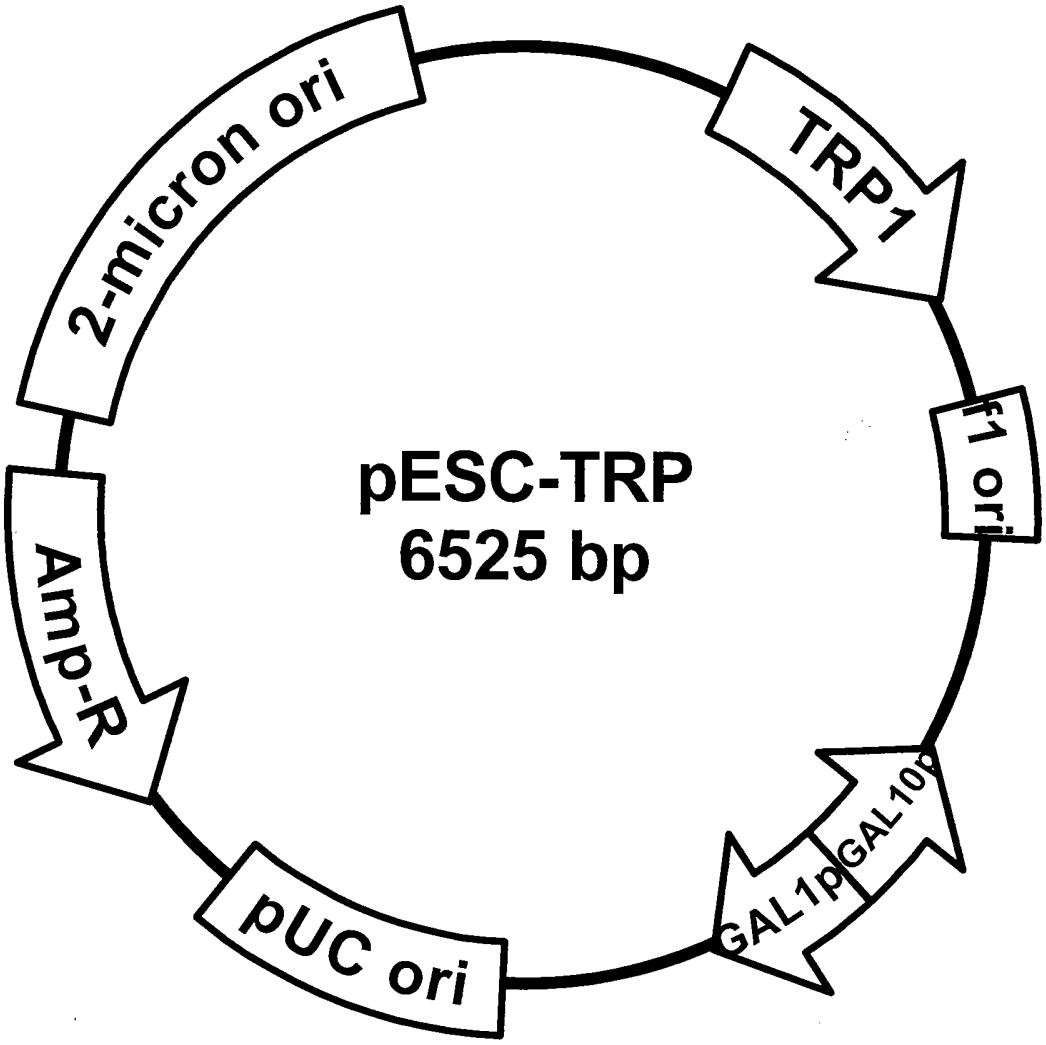
2-micron ori
TRP1
f1 ori
GAL10p
GAL1p
pUC ori
Amp-R
pESC-TRP
6525 bp

Fig. 18

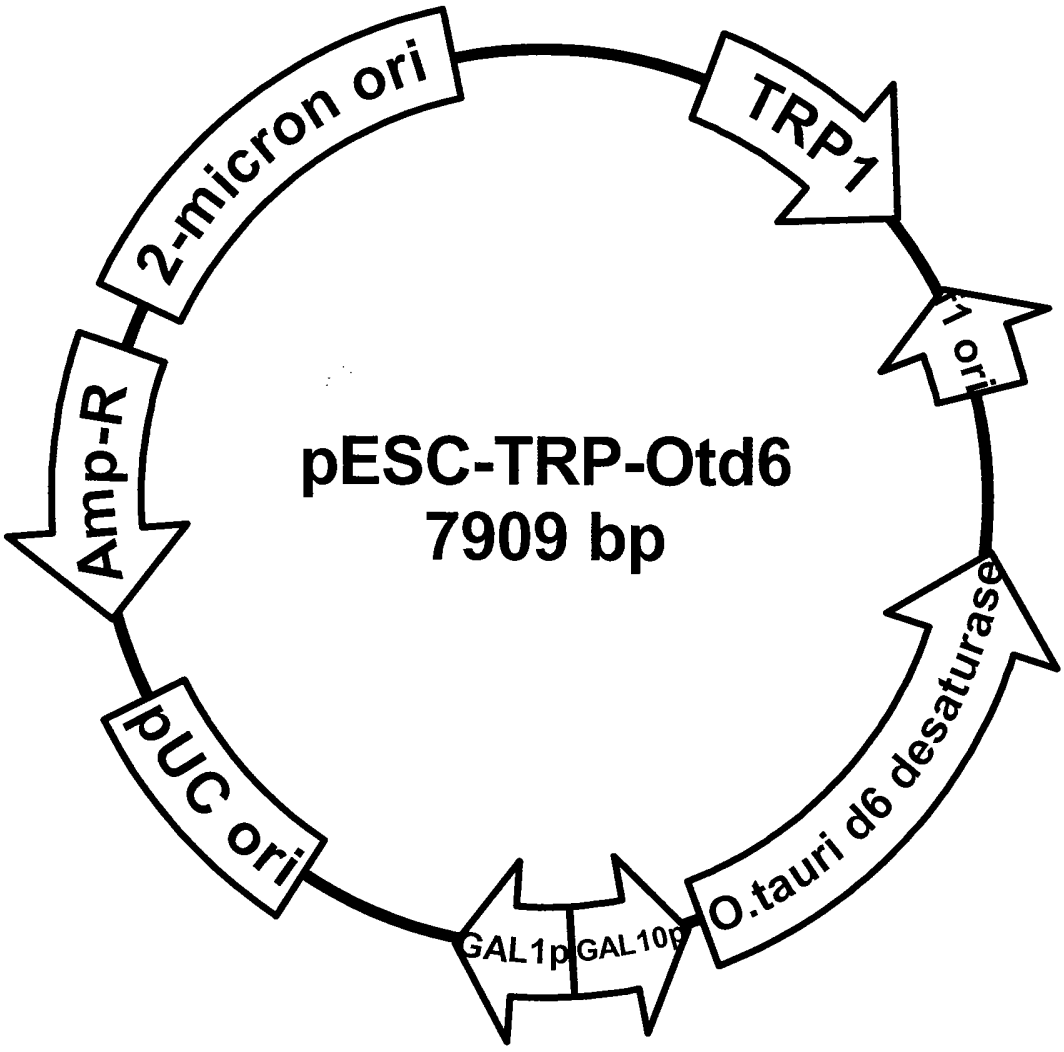
2-micron ori
TRP1
ori
Amp-R
pESC-TRP-Otd6
7909 bp
pUC ori
GAL1p
GAL10p
O.tauri d6 desaturase

Fig. 19