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Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to the development of genetically engineered microorganisms that can produce wax esters in a controllable and economic fashion. More specifically the invention relates to the production of liquid wax esters that can be used for biofuel, lubricants, cosmetics, linoleum, printing inks as well as products related thereto, and for the production of solid wax esters used for candles and polishes as well as products related thereto.

Description of the Related Art

[0002] Fossil fuels, such as coal, oil, and natural gas, have been powering modern society for more than one century. However, fresh discoveries of deposits are on the wane and demands are increasing. The world's demand of fossil fuels will soon outweigh the current supply. An innovative approach offering some solution comes from the biotechnology industries. Efforts have made biodiesel as one of the most thoroughly developed and promising alternative fuels on the market. It works well in conventional diesel engines, with less hazardous emissions, and is consumed at greater than 3.5 billion gallons per year.

[0003] Biodiesel is generally composed of fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEE), and is mostly derived from vegetable oil or animal fat by chemically transesterification with methanol or ethanol. Despite the fact that ethanol-yielded FAEEs have better performances, for cost reasons methanol is the reagent most frequently used for triglyceride transesterification. The current process has several drawbacks, including energy intensiveness, consuming edible feedstocks, difficulty of removal of the catalyst from the product and treatment of toxic waste-water, as well as geographical and seasonal restrictions.

[0004] To overcome the problems related to the use of catalysts people have been exploring new alternatives such as enzymatic conversion using lipases (EC 3.1.1.3, triacylglycerol hydrolases). Lipases can break down neutral lipids such as triglycerides and perform a transesterification reaction in a solvent system (i.e. tert-butanol). Enzymatic production of biodiesel can be carried out at moderate reaction conditions and at a lower alcohol to oil ratio. The main drawbacks with this kind of enzymatic catalysis are the strong inactivation effect caused by alcohols (i.e. methanol) and the high enzyme costs.

[0005] Both chemical and enzymatic transesterification require the use of toxic, petrochemically-derived alcohols and expensive feedstocks. Thus, transesterification-based biodiesel becomes unsustainable when fossil fuel derived products are used. As a result, the current feedstocks for biodiesel are mainly derived from plant oils like rapeseed oil.

[0006] However such plant oils are inherently limited by supply of water and land, and subsequently, they cannot produce enough biofuel without threatening food supplies and/or native biodiversity. Algae are a promising choice as an alternative feedstock. Nevertheless, there are problems with surface usage and oil extraction from algae based production. Everyone agrees that fuels derived from biomass are one of the best alternatives to fossil fuels. Thus, genetically manipulation of microorganisms to produce fatty acid esters, will substantially contribute to produce environmentally friendlier, sustainable, and cost-effective biodiesels.

[0007] In this regard, it was previously shown that an engineered *E. coli* strain expressing the wax synthase (WS) from *Acinetobacter baylyi* ADP1 and ethanol-production genes from *Z. mobilis,* could produce fatty acid ethyl esters by esterifying exogenously added fatty acids (Kalscheuer, Stolting et al. 2006). The research is an excellent demonstration of feasibility for microbial production of fatty acid esters. Recently, researchers from the Keasling group and the company LS9 Inc. (South San Francisco, USA) developed this idea further by constructing an engineered *E. coli* that can produce fatty-acid-derived fuels and chemicals from simple sugars and plant-derived biomass, without the need for fatty acid feeding (Steen, Kang et al. 2010). Production of fatty acid derivatives as biofuels has also been reported in recent patent applications WO2009/009391, WO2007136762 and WO2008119082, all owned by LS9 Inc. Briefly, the metabolically engineered *E. coli* strain was manipulated to be able to produce fatty (acid) esters and derivatives thereof (short and long chain alcohols, hydrocarbons, fatty alcohols, waxes, etc.) through the introduction of several genes encoding for enzymes such as thioesterase, wax synthase, alcohol acyltransferase, alcohol dehydrogenase, and different kinds of fatty alcohol forming acyl-CoA reductases. In U.S. patent publication 2010/0071259, inventors from the same company teach that by adding a mixture of at least two different alcohols to a medium containing the engineered *E. coli* strain that produces fatty esters, at least two different fatty esters could be produced.

[0008] The afore-mentioned biodiesel producing methods are all based on the use of the bacterium *E coli.* However, *E coli* is unable to naturally overproduce the two substrates of biodiesel, fatty acids and alcohol (i.e. ethanol), and this organism is not suitable for large-scale production that often involves harsh environmental conditions. Furthermore, *E. coli* is sensitive to phage contamination often resulting in substantial economic losses. The patents of the prior art

successfully teach several strategies to enhance fatty acids biosynthesis in *E. coli*. Nevertheless, and apart from the drawbacks associated with the use of this host, it should be noted that strategies working in *E. coli* might not be appropriate when applied in other microorganisms.

[0009] A far better choice of microbial cell factory for industrial production of biodiesel would be the yeast *Saccharomyces cerevisiae*. This yeast is already widely used in industry, including for large-scale bioethanol production, but also for a range of specialty chemicals. The development of *S. cerevisiae* as a cell factory for biodiesel production would represent a major contribution as this could represent a plug and play solution where current infrastructures used for production of bioethanol could be used for production of far more valuable biodiesels. In contrast to the insufficient ethanol productivity of *E. coli, S. cerevisiae* is already a good ethanol producer.

[0010] In fact, production of FAEEs and fatty acid isoamyl esters (FAIEs) has been achieved in recombinant *S. cerevisiae* with oleic acid addition by expressing the *A. baylyi* bifunctional WS/DGAT enzyme (Kalscheuer, Luftmann et al. 2004). A recent patent application, namely US patent application 2009/0117629 by Schmidt-dannert and Holtzapple, also describes a method for the production of esters, including isoprenoid wax esters and fatty acid alkyl esters, such as FAME and FAEE, by heterologous expression of *Marinobacter hydrocarbonoclasticus* wax synthase (WS2) in *S. cerevisiae*. The invention is however, limited to the use of specific isolated polynucleotides from *Marinobacter hydrocarbonoclasticus,* and its application in e.g. producing biodiesel). Moreover this method requires exogenous supply of fatty acids as the endogeneous production of fatty acids by yeast is too low to ensure economically viable production of FAEEs.

[0011] A modified strain carrying the genes encoding the wax synthase from *Marinobacter hydrocarbonoclasticus* could be considered a potential host for biodiesel production in yeasts. Nonetheless, while this product is very suitable for the particular purpose it addresses, it is not the ideal option when the synthesis of other esters is desired. The knowledge of the preferred substrates for each wax synthase allows the use of yeast cells in applications other than biodiesel production. Moreover there is still a need for methods and products allowing large-scale production of fatty acid esters.

[0012] Thus it is an object of the present invention to provide an improved fungal cell factory, such as a yeast cell factory that can be used for fermentation based production of FAEEs, that is not dependent on the addition of exogenous fatty acids to the yeast culture and that possess an increased flux towards fatty acid biosynthesis and where high level production of FAEEs is obtained.

SUMMARY OF THE INVENTION

[0013] The above presented problems have now been solved by providing a fungal cell for producing fatty acids as defined in claim 1.

[0014] The invention relates to a *Saccharomyces cerevisiae* cell for overproducing fatty acids. The S. *cerevisiae* cell comprises a mutated acetyl-CoA carboxylase gene encoding a mutated acetyl-CoA carboxylase, in which the serine at position 659 in SEQ ID NO: 16 has been replaced by alanine and the serine at position 1157 in SEQ ID NO: 16 has been replaced by alanine. The acetyl-CoA carboxylase gene is under control of a constitutively expressed promoter.

[0015] Accordingly, a primary object of the present invention is to provide an advance in the microorganism fermentation method for producing wax esters, which include, but is not limited to, the liquid waxes used for biofuel, lubricants, cosmetics, linoleum and printing inks, and the solid waxes used for candles, polishes etc. The fungal cell system and the method disclosed herein combine the expression of different wax synthases with metabolic engineering modifications to ensure a high flux to biosynthesize wax esters. The high flux described herein means at least 2-fold increase in the fatty acids flux compared with flux towards fatty acids in the reference yeast.

[0016] In this respect, before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and to the arrangements of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced and carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein are for the purpose of the description and should not be regarded as limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017]

Figure 1 shows the glycolysis pathway in yeast *Saccharomyces cerevisiae* for producing two direct precursor substrates (ethanol and acyl-CoA) of wax synthase. The glucose, via glycolysis, could be converted to ethanol (1), and acyl-CoA (2), the precursor for fatty acids.

Figure 2 shows the different reactions of the fatty acids consuming pathways.

Figure 3 shows a wax ester (e.g. FAEEs) biosynthesis pathway catalyzed by heterologous wax synthase in *Sac-*

charomyces cerevisiae. The alcohols could be biosynthesized by the production host or heterologous supplemented. The acyl-CoA could be produced via fatty acids biosynthesis by a production host or supplemented heterologously.
Figure 4 shows the vector used for gene expression in the invention herein.
Figure 5 shows GC-MS analysis of heptadecanoic acid ethyl ester produced by wax synthase expressing *S. cerevisiae* CB1 with heptadecanoic acid supplemented. The retention time is 15.53 minutes.
Figure 6 shows a GC-MS analysis of standard heptadecanoic acid ethyl ester. The retention time is 15.62 minutes.
Figure 7 shows the constructed plasmids for expressing WSs from *Acinetobacter baylyi, Marinobacter hydrocarbonociasticus DSM 8798*, *Rhodococcus opacus PD630, Mus musculus C57BL /6,* and *Psychrobacter articus 273-4.*
Figure 8 shows an overview of different metabolic engineering strategies for enhancing fatty acid derivative production in yeast. The heterologous enzymes are shown underlined.
Figure 9 shows the outline of the gene deletion method.
Figure 10 shows biodiesel production in engineered strains.
Figure 11 shows a method for chromosomal integration. The chromosomal integration cassette, obtained by fusion PCR, contains wax synthase controlled by TEF1 or PGK1 and a selectable marker (neo) is delivered to the chromosome. Iterative tandem gene duplication is accomplished by selecting in the plates with higher antibiotics.
Figure 12 shows the effect of different promoter and biodiesel production in plasmid or chromosome integration based strains.
Figure 13 shows the relationship of the concentration of biodiesel production and the concentration of G418.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS THEREOF

[0018] The invention herein relies, unless otherwise indicated, on the use of conventional techniques of biochemistry, molecular biology, microbiology, cell biology, genomics and recombinant technology.
[0019] To facilitate understanding of the invention, a number of terms are defined below.
The term "recombinant" means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems
[0020] The term "overproducing" is used herein in reference to the production of FAEE in a host cell and indicates that the host cell is producing more of the FAEE by virtue of the introduction of nucleic acid sequences encoding different polypeptides involved in the host cell's metabolic pathways or as a result of other modifications as compared with the wild-type or unmodified host cell.
[0021] As used herein, the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.
[0022] As used herein, an "ACBP or acyl-CoA-binding protein" is a small (10 Kd) protein that binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters. The majority of the cellular long-chain acyl-CoA esters are presumed to be sequestered with acyl-CoA binding protein (ACBP).
[0023] Although ACBP occurs as a completely independent protein, intact ACB domains have been identified in a number of large, multifunctional proteins in a variety of eukaryotic species ranging from yeasts and plants to reptiles and mammals. In general ACBP is highly conserved in all eukaryotes. The yeasts homologue of ACBP is known as Acb1p. As used herein, an "Acetyl CoA carboxylase" is a biotin-containnig enzyme that catalyzes the irreversible reaction in which acetyl-CoA is carboxylated to malonyl-CoA, see Figures 1 and 8, which is the precursor of long-chain fatty acyl-CoA. In mammals, two main isoforms of ACC are expressed, ACC1 and ACC2, which differ in both tissue distribution and function. ACC1 is found in the cytoplasm of all cells and encodes acetyl CoA carboxylase in yeast cells.
[0024] As used herein, FAS or a "fatty acid synthases" is an enzymatic system that catalyzes the initiation and elongation of acyl chains and thus plays a key role in fatty acid synthesis from acetyl-CoA and malonyl-CoA. Examples of these enzymes are AccABCD, FabD, FabH, FabG, FabA, FabZ, FabI, FabK, FabL, FabM, FabB, and FabF. In the yeast *Saccharomyces cerevisae,* fatty acids are synthesized by a 2.4Mba multifunctional enzyme complex with two subunits encoded by two unlinked genes FAS1 and FAS 2.
[0025] As used herein, acyl-CoA synthase includes peptides in enzyme classification number EC 2.3.1.86, and are any of various ligases that catalyze the conversion of a fatty acid to acyl-CoA for subsequent β-oxidation.
[0026] As used herein, "glyceraldehyde-3-phosphate dehydrogenase" (GAPDH) catalyzes the reversible interconversion between 1,3-bisphosphoglycerate and d-glyceraldehyde 3-phosphate using either NAD(H) or NADP(H) as a coenzyme. This is the sixth step of the glycolysis (Figure 1) and thus serves to break down glucose for energy and carbon molecules.
[0027] NADPH, a product of the pentose phosphate pathway, functions as a reductant in various synthetic (anabolic) pathways including fatty acid synthesis.
[0028] As used herein, an "acetyl-coenzyme A synthetase" is an enzyme that catalyzes the formation of a new chemical bond between acetate and coenzyme A (CoA), which is a key branching molecule for different metabolic pathways.

[0029] As used herein, "β-oxidation" is the process by which fatty acids in the form of Acyl-CoA molecules are broken down to generate Acetyl-CoA. It is the principal metabolic pathway responsible for the degradation of fatty acids (Figure 2).

[0030] As used herein a "catalytic motif" is a three-dimensional structural unit formed by a particular sequence of amino acids, found in proteins and which is often linked with a particular function. For nucleic acids is a particular, usually short, nucleotide sequence that forms a recognition site usually, to which other proteins bind.

[0031] A peptide of the present invention may be present in an expression vector. The term "expression vector" is defined herein as a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide of the invention, and which is operably linked to additional nucleotides that ensure its expression. Suitable expression vectors include fungal, baculovirus vectors, bacteriophage vectors, plasmids, phagemids, cosmids, fosmids, *Acinetobacter baylyi,* yeast plasmids and any other vectors specific for the hosts of interest. Vectors may be introduced into a host cell using methods that are known in the art such as, calcium phosphate precipitation, electroporation, heat shock, lipofection, microinjection, etc.

[0032] A "fungal cell system" as disclosed herein comprises a fungal cell which has been modified, such as genetically modified, as described herein, and which expresses at least one wax synthase, as exemplified herein. Said wax synthase is introduced into said fungal cell to provide for expression thereof in said fungal cell. The fungal cell system according to the invention hence provides the combination of a modified fungal cell and the expression of a wax synthase in said fungal cell, which allows for an increased metabolic flux towards fatty acid ester biosynthesis in said fungal cell. This advantageous combination is herein referred to as the "fungal cell system".

[0033] As used herein pESC vectors are a series of epitope-tagging vectors designed for expression and functional analysis of eukaryotic genes in the yeast *S. cerevisiae.* These vectors contain the GAL1 and GAL10 yeast promoters in opposing orientation. With these vectors one or two cloned genes can be introduced into a yeast host strain under the control of a repressible promoter Preferably the expression vector of the present invention is a pESC-derived plasmid in which the original promoter have been replaced. (S. Partow et al. 2010)

[0034] As used herein a "promoter" is a DNA sequence that usually precedes a gene in a DNA polymer and provides a site for initiation of the transcription into mRNA. In the present invention we used promoters derived from transcriptional Enhancer Factor 1 (TEF1) and phosphoglycerate kinase (PGK1).(S. Partow et al. 2010).

[0035] As used herein, sequence identity refers to sequence similarity between two nucleotide sequence or two peptide or protein sequences. The similarity is determined by sequence alignment to determine the functional, structural, and/or evolutionary relationships between the sequences. Gaps in either or both sequences are permitted in making successive alignment.

[0036] By two nucleotide sequence or two peptide or protein sequences having an amino acid sequence at least, for example 95% identical to a reference amino acid sequence, is intended that the amino acid sequence of e.g. the peptides is identical to the reference sequence, except that the amino acid sequence may include up to 5 point mutations per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a peptide having an amino acid sequence at least 95% identical to a reference amino acid sequence: up to 5% of the amino acids in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acids in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the amino and/or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

[0037] In the present invention, a local algorithm program is best suited to determine identity. Local algorithm programs, (such as Smith Waterman) compare a subsequence in one sequence with a subsequence in a second sequence, and find the combination of subsequences and the alignment of those subsequences, which yields the highest overall similarity score. Internal gaps, if allowed, are penalized. Local algorithms work well for comparing two multidomain proteins, which have a single domain or just a binding site in common.

[0038] Methods to determine identity and similarity are codified in publicly available programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J et al (1994)) BLASTP, BLASTN, and FASTA (Altschul, S.F. et al (1990)). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S.F. et al, Altschul, S.F. et al (1990)). Each sequence analysis program has a default scoring matrix and default gap penalties. In general, a molecular biologist would be expected to use the default settings established by the software program used.

Fatty Acids' Synthesizing Pathway

[0039] The fatty acids synthesizing pathway includes fatty acid synthase enzymes selected from the group consisting of ACC1 (encoding acetyl-CoA carboxylase), FAS1/FAS 2 (encoding fatty acid synthase), and ACS1 (acetyl coenzyme A synthase) from any species to encode such proteins.

[0040] Fatty acids (FA) play an important role as building blocks of biodiesel. In *S. cerevisiae,* FA is mainly synthesized

in cytosol and limits biodiesel production. A fatty acid overproducing yeast cell will in turn overproduce fatty acids derived esters, e.g. FAEES (biodiesel).

In this embodiment the inventors herein improve the supply of FAEE precursors. Thus an over-expression of the gene coding to acetyl-CoA carboxylase (ACC1) in combination with an increased expression of fatty acid synthetases (FAS1 and FAS 2) yields an increased amount of Malonyl CoA and Fatty Acids, respectively.

[0041] The sources of malonyl-CoA, are generally supposed to be limited, impeding its utility for overproducing FA. The activity of acetyl-CoA carboxylase is highly regulated in *S. cerevisiae:* (1) Transcription of ACC1 is repressed by inositol and choline, as UASINO site was found in the promoter of ACC1 (Chirala, Zhong et al. 1994); (2) Acetyl-CoA carboxylase activity could be directly inactivated by Snf1p through phosphorylating (Shirra, Patton-Vogt et al. 2001).

[0042] For releasing the tight regulation of the ACC1 at the mRNA and protein level, the promoter of ACC1 is replaced. Furthermore, the inventors herein have found that under control of the constitutively expressed promoter, a release of ACC1 phosphorylation sites would provide a further increase towards FAEE biosynthetic flux. For example Ser659Ala and Ser1157Ala could be substituted (SEQ ID NO 16). Thus the inactivation by Snf1 could be avoided. The resulting strain with hyperactive Acc1p would enhance the FA biosynthesis significantly.

[0043] As previously stated, in addition to up-regulated activity of acetyl-CoA carboxylase, fatty acid synthase (FAS) could be over-activated to reinforce the push of hyperactive acetyl-CoA carboxylase. Therefore, FASI and FAS2 would be over-expressed in the engineered strain with hyperactive Acc1p. The combined manipulations would lead a high flux towards fatty acids biosynthesis.

[0044] Another preferred modification aimed at increasing the pool of fatty acids is the over-expression of acetyl coenzyme A synthase. In *S. cerevisiae,* cytosolic acetyl-CoA is produced by decarboxylation of pyruvate to acetaldehyde that is then converted further to acetate and acetyl-CoA (Figure 1; Figure 8), which is used for the synthesis of malonyl-CoA and FA biosynthesis. The supply of acetyl-CoA may become a shortage when the FA biosynthesis ability is severely reinforced. The step for biosynthesizing acetyl-CoA is catalyzed by acetyl-coenzyme A synthetase, which is encoded by two genes, ACS1 and ACS2, in *S. cerevisiae.* Compared to ACS2, ACS1 has been reported to show a considerably higher activity and therefore in this invention, ACS1 has been chosen to be over-expressed.

[0045] The genetically modified yeast cell of the present invention will provide for increased production of FA that is at least 2-fold higher than the amount of the ester produced by a control yeast that is not genetically modified as described herein.

Fatty Acids Consuming Pathways

[0046] Fatty acids are the precursor of Acyl CoA and the production host is engineered to produce fatty acid esters from acyl-CoA and ethanol. That's why it is important to improve the pool of fatty acids by down-regulating the fatty acid-consuming pathways.

Most of the fatty acids are stored in the form of neutral lipids such as triacylglycerols (TAG) and steryl esters, which can constitute up to 70% of the total lipid content of the cell. In *S. cerevisiae,* TAG can be synthesized through two different pathways. As shown in figure 8, one is an acyl-CoA-dependent reaction that is catalyzed by acyl-CoA:diacylglycerol acyltransferase (encoded by DGA1 gene); another is phospholipids (PL) dependent reaction that is catalyzed by lecithin:cholesterol acyltransferase (encoded by the LRO1 gene). Steryl esters are formed from sterols through the action of the enzyme acyl-CoA:sterol acyltransferase (ASAT) which is encoded by ARE1 and ARE2 genes in *S. cerevisiae.*

[0047] Previous studies have shown that the quadruple mutant, *S. cerevisiae* H1246, in which DGA1, LRO1, ARE1 and ARE2 were disrupted, was no longer capable of producing any TAG or steryl esters and had no apparent growth defects under standard conditions (Sandager, Gustavsson et al. 2002). In stationary phase, the quadruple disrupted strain has a 2.5-fold increase in fatty acids. Using Cre-loxP system, the four genes, DGA1, LRO1, ARE1 and ARE2, were disrupted sequentially. The mutant would decrease or abolish the amount of FA converted to neutral lipid production.

[0048] The stored neutral lipids could be hydrolyzed at any moment to yield fatty acids. The liberated fatty acids and free fatty acids could in turn be oxidized to generate energy by β -oxidation. *S. cerevisiae* has only one peroxisomal acyl-CoA oxidase, Pox1 p, which is regarded as being the main enzymatic step controlling the flux through the β-oxidation. Knocking out the endogenous POX1 gene to block fatty acid β -oxidation would be beneficial for the accumulation of lipid.

[0049] Suitable modifications allowing this particular embodiment include deletion of the afore-mentioned key genes: DGA1, LRO1, ARE1, ARE2 and POX1. Thus, a yeast cell with all non-essential fatty acid conversion reactions deleted or attenuated, specifically those related with β-oxidation, synthesis of phospholipids, triacylglycerol and sterol esters, would show a higher production of fatty acids and hence an over-production of FAEE. On the other hand, as reported by several authors a decrease in β-oxidation flux would increase lipid accumulation (Slocombe, Cornah et al. 2009; Steen, Kang et al. 2010).

Carbohydrate Biosynthesis Pathways

[0050] The modified yeast cell with e.g. an enhanced ability to overproduce fatty acids, should need much more NADPH, as two molecules of NADPH are required for each step in the elongation of the growing FA acyl chain (Figure 1).

[0051] Basically, the availability of intracellular NADPH is enhanced by engineering the production host to express an NADH:NADPH transhydrogenase. The expression of one or more NADH:NADPH transhydrogenases results in an increased conversion of the NADH produced in glycolysis to NADPH. Specifically, the authors herein have designed a novel yeast strain expressing a heterologous NADP+ dependent glyceraldehyde-3- phosphate dehydrogenase (GAPN, coded by *gapN* gene) (Figure 8), for augmenting the production of fatty acid derivatives. Heterologous expression of gapN, from *Streptococcus* mutants in yeast provides a further push on FA biosynthesis ability, meanwhile it also lead to a higher ethanol yield, which is another precursor for biodiesel (Figure 8).

Establishment of FAEE Biosynthesis Pathway

[0052] One known method for producing fatty acid esters includes increasing the expression of ester synthases such as wax synthases (EC 2.3.1.75) (Figure 2). A further increase might be obtained by increasing wax synthase's substrate availability e.g. overproducing fatty acids as suggested above.

[0053] A wild-type yeast cell does not have the metabolic machinery for producing FAEEs from fatty acids. Wax synthases, the enzymes catalyzing these reactions, are characteristics of organisms such as *Mycobacterium, Rhodococcus, Acinetobacter,* and *Marinobacter* strains that grow in environments where a carbon source was abundant relative to other nutrients such as phosphorous and nitrogen. The wax synthase sequence usually contains the catalytic motif HHXXXDG, which is reported to be crucial for enzymatic activity.

[0054] Wax synthase activity has never previously been described for yeast. In yeast, it was only shown that polypeptides Eht1p and Eeb1p have medium chain (C4-C8) fatty acid ethyl ester -synthesizing and -degrading activity. However, Kalscheuer et al. (2004) showed for the first time that low wax synthase activity could be detected in wild-type *S. cerevisiae* G175 using palmitoyl-CoA and 1-Hexadecanol as substrates. But no homologous sequence was detected in yeast. In addition, GC/MS analysis of total lipid extracts from wild-type *S. cerevisiae* showed that FAEEs were absent, even when the medium is supplied with fatty acids (oleic acid).

[0055] Therefore, the ability to synthesize long chain fatty acid ethyl esters may exist in yeast and it may be generated by the unspecific activity of Eht1p and Eeb1p, but the activity is very poor, which is not enough to form FAEEs (i.e. long chain fatty acid ethyl ester) from fatty acids in a wild-type yeast.

[0056] In a particular embodiment the inventors herein propose the use of a microbial wax ester synthase/acyltransferase (WS/DGAT) from *Acinetobacter baylyi* ADP1 as this enzyme is known to have the activity for short-chain alcohols and the ability to form FAEEs. It is obtained by expressing the *atfA* gene. This wax synthase may have sequence similarity with the nucleotide sequence of SEQ ID NO 1 (see attached Sequence Listing). However, the wax synthase from *Acinetobacter baylyi* ADP1 is a rather unspecific enzyme with broad spectra of possible substrates, and it was in fact bifunctional in vivo, also acting as a diacylglycerol acyltransferase (DGAT).

[0057] Genes with high homologies to the *Acinetobacter baylyi* ADP1 wax synthase have also been identified in other species. There are three unrelated families of wax synthase found in higher plants, mammals and bacteria. The wax synthase of plants shows no activity for short-chain alcohols.

[0058] Several heterologous wax synthases from other organisms were evaluated (Example 3). As suspected, most wax synthase had the highest activity for acyl-CoAs and alcohols with a chain length from 14 to 18, with a much lower specificity for ethanol. All the detected wax synthase (i.e. wax synthase from *Acinetobacter baylyi* ADP1, *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BL/6, and *Psychrobacter articus* 273-4) have varied activity for ethanol and could lead to the formation of FAEEs. However the enzyme with highest activity for synthesizing FAEEs was found to be wax synthase from *Marinobacter hydrocarbonoclasticus* DSM 8798 (named CB2 in the present application). According to the present invention a yeast cell able to efficiently produce FAEEs that could directly be used as biodiesel should express a wax synthase from *M. hydrocarbonoclasticus* or wax synthase from *Psychrobacter articus* (Table 3).

[0059] Overexpression of native EEb1gene coding for EeB1p, with the ability to synthesize medium chain fatty acid ethyl ester in combination with expression of selected WSs might lead to favorable results with regard to the synthesis of specific FAEEs. For this purpose, a wax synthase with the best-adapted substrate specificity should be chosen.

[0060] On the other hand, the standardization of methods of molecular evolution or protein fusion could help improving the preference of existing WSs for certain substrates, e.g. using error prone PCR, gene shuffling or more directed protein engineering of the WSs. For example it could lead to the identification of WSs with higher specificity for ethanol. The selection of WSs with high activity for ethanol is of course, of crucial importance for designing an effective biodiesel producer as biodiesel is generally composed of fatty acid ethyl esters (FAEE). Said fatty acids have generally a chain-length from 14 to 20 carbon atoms, within the optimal operating range for acyl-CoAs. A recombinant yeast cell expressing

e.g a *M. hydrocarbonoclasticus* is a good choice for designing a FAEEs producer because of its high preference for ethanol.

[0061] The identified broad spectra of possible substrates of different WSs as shown in Table 3 of the invention herein (see below) allows for many biotechnological applications including but not limited to biodiesel production. Depending on the substrate specificity of the wax synthase (WS) enzymes, various mixtures of ester isomers and chain lengths can be generated. These esters relates to liquid wax esters that can be used for biofuel, lubricants, cosmetics, linoleum, printing inks as well as products related thereto, and solid wax esters used for candles, polishes as well as products related thereto. Another exemplary biotechnological application of wax synthase is spermaceti production. Spermaceti is mainly composed by cetyl palmitate and cetyl myristate, and is widely used in cosmetics, pharmacy and also in candles.

[0062] A wax synthase polypeptide of the present invention may be isolated and obtained from other sources including microorganisms isolated from nature. People skilled in the art know how to screen a genomic or cDNA library for this purpose. Once a polynucleotide sequence encoding a polypeptide has been detected it can be isolated or cloned by utilizing techniques, which are well known to those of ordinary skill in the art.

[0063] Here again we have used plasmid pSP-GM2, derived from pESC, which is a common plasmid with high copy number. The original weaker promoters in pESC were exchanged by two strong promoter TEF1 and PGK1, respectively, to construct pSP-GM2. The high copy number and the strong driven by TEF1 ensures high-level expression of the WS. A polynucleotide encoding a wax synthase polypeptide of the present invention may be present in the yeast cell as a vector or integrated into a chromosome (S. Partow et al.).

Enzyme acting as Wax Ester Transporters.

[0064] As mentioned herein, the engineered cell expressing a wax synthase would be able to synthesize fatty acid esters e.g. FAEEs. The transfer of esters to the fermentation medium is dependent on their composition. It decreases drastically with increasing chain length, e.g. from 100% for ethyl hexanoate, to 54-68% for ethyl octanoate and 8-17% for ethyl decanoate. A wax ester transporter would facilitate the release of esters to the fermentation medium.

[0065] In one embodiment the invention herein uses a plant wax ester transporter (Pighin, Zheng et al. 2004). For example, Cer5 from Arabidopsis facilitates the export of very long chain aldehydes, ketones, alcohols, alkanes, esters and other possible fatty acids derivatives.

Strain and polypeptide characterizations

[0066] The wax synthase activity is an important parameter. It is measured according to previous publications (Kalscheuer et al., 2004). Basically, crude extracts are prepared from *S. cerevisiae* strains and added into a reaction system containing [1-14C] palmitoyl-CoA and alcohols with specific chain. The test assays are incubated at 35 °C for 30 min, and stopped by extraction with chloroform/methanol. The extracts are separated by TLC.

[0067] Spots corresponding to waxes are scraped from the plates, and radioactivity is measured by scintillation counting.

[0068] The FAEEs, are detected by GC-MS. Briefly, total lipids are first extracted from *S. cerevisiae* strains, and then run on a TLC plate. Spots corresponding to FAEEs are scraped from the plates, and resolved in chloroform/methanol, which is then measured by GC-MS.

[0069] The genetically modified yeast cells hereby disclosed may be included in a composition further comprising additional components selected from, but not limited to, the group consisting of: buffers; stabilizers; protease-inhibiting agents; hydrolytic enzymes, saccharolytic enzymes; cell membrane- and/or cell wall-preserving compounds, nutritional media appropriate to the cell; and the like.

[0070] For expressing the heterologous sequences, the yeast cells are cultured in a medium supplemented with carbohydrate as the only externally supplied source. Compounds included in this group, but not limited to, are glucose, fructose, galactose, xylose, arabinose, sucrose, maltose, starch, cellulose, and hemicellulose

[0071] In this invention instead of providing the alcohol in the fermentation media as is known in the art e.g. when *E. coli* is used as biodiesel factory, Applicant has developed a genetically engineered microorganism that can produce wax esters in a controllable and economic fashion without the need of fatty acids or ethanol supplementation.
In specific embodiments the carbohydrate concentration in the culture medium is between 20 g/l and 50 g/l. Additional components of the culture media are yeast nitrogen base and CSM -Ura.

[0072] Accordingly, the present invention relates to a fungal cell system for producing fatty acyl ethyl esters (FAEE), said system comprising a fungal cell, and an expression vector encoding at least one wax synthase, wherein the metabolism of said fungal cell is additionally modified, said modification providing for down-regulation, attenuation, deletion and/or over-expression of one or more gene(s) selected from the group consisting of genes encoding one or more enzyme(s) involved in at least one of said fungal cell's fatty acid synthesizing pathways, fatty acid consuming pathways and carbohydrate biosynthesis pathways, and/or selected from the group consisting of genes encoding one or more

enzyme(s) acting as wax ester transporter(s) of said fungal cell. The invention also relates to a fungal cell which is a yeast cell.

[0073] When herein down-regulation, attenuation, deletion and/or over-expression of one or more gene(s) is referred to, this means that the expression/translation/transcription level of the gene or the gene product has been altered in some manner. The manipulation herein could be achieved by medium supplementation, genetic engineering, or synthetic biology. Regulated genes include genes that could be translated into protein, as well as genes that are transcribed into types of RNA that are not translated into protein. Gene regulation could be made by altering the structural or control region, introducing more copy number, deactivating the corresponding repressor gene or activating the inducible gene, increasing the RNA stability of the gene, and combinations thereof.

[0074] Fatty acid ethyl esters (FAEEs) are esterification products of ethanol and fatty acids. Biodiesel is one kind of mixture of wax esters (FAEEs). The biosynthesis of FAEE is catalyzed by wax ester synthase, also called wax synthase (WS). The chain-length and degree of un-saturation and branching of the fatty acid may vary. Generally, this site of the ester is at least 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length and can be mono-, di-, or tri- unsaturated.

[0075] The present invention provides genetically modified yeast cells that have at least one heterologous polynucleotide encoding a polypeptide involved in a FAEE biosynthesis pathway. The present invention also relates to other genetically modified fungal cells, as exemplified herein, that have at least one heterologous polynucleotide encoding a polypeptide involved in a FAEE biosynthesis pathway.

[0076] A fungal cell used in the context of the present invention can be selected from the group of fungal cells consisting of *Saccharomyces, Saccharomyces cerevisae Hansenula polymorpha, Kluyveromyces, Pichia, Candida albicans, Aspergilli, Rhodotorula rubra, Torulopsis, Trichosporon cutaneum, Trichoderma reesei, Apiofrichum curvafum, Yarrowia lipolytica,* and *Cryptococcus curvatus. An example of a fungal cell that can be used is Saccharomyces cerevisiae* CEN.PK113-5D (van Dijken, J. P., et al., 2000).

A modification of the metabolism of a fungal cell according to the present invention can be genetic and effectuated by the introduction of one or more exogenous expression vector(s) into said fungal cell. In the context of the invention, said one or more exogenous expression vector(s) can be a plasmid, or another carrier such as exemplified herein. The vector also comprises a structural gene for selection of transformed cells, such as URA3, HIS3.

[0077] In aspects of the invention, said genetic modification of said fungal cell provides for an increased supply of fatty acyls to the metabolism of said fungal cell. Furthermore, said fungal cell can be genetically modified to stimulate overproduction of fatty acids, as further described herein.

[0078] In aspects of the invention, a modification to a fungal cell system as defined herein is performed to any one or more of the following genes, or its expression products: *ACB1* (ACBP, acyl-CoA-binding protein), *ACC1* (Acetyl-CoA carboxylase), *FAS1, FAS2* (Fatty acid synthase), *gapN* (NADP+ dependent glyceraldehyde-3-phosphate dehydrogenase), *ACS1* (Acetyl-CoA synthetase), *DGA1* (Acyl-CoA:diacylglycerol acyltransferas), *LRO1* (Lecithin: cholesterol acyltransferase), *ARE1, ARE2* (Acyl-CoA:sterol acyltransferase), and *POX1* (Peroxisomal acyl-CoA oxidase).

[0079] In other aspects, optionally in combination with other modifications, said modification to said fungal cell is performed by a knockout/deletion of one or more of the genes *DGA1, LRO1*, *ARE1, ARE2,* and *POX1.*

[0080] According to the invention, a modification to a fungal cell as described herein can also be performed by over-expressing one or more gene product(s) by the introduction of one or more expression vector(s) encoding said one or more gene product(s), said one or more gene product(s) being selected from the group consisting of: acyl-CoA-binding protein, Acetyl-CoA carboxylase (ACC1), NADP+ dependent glyceraldehyde-3-phosphate dehydrogenase, Fatty acid synthases (FAS1, FAS2) and Acetyl-CoA synthetase (ACS1).

[0081] According to the invention, a modification can also provide for an overexpression of ACC1 in combination with an increased expression of FAS1 and FAS2. In some aspects of the invention. the modification of *ACC1* is performed by the introduction of an expression vector and an increased expression of FAS1/FAS2 is performed by replacing the promoter thereof (the promoter of FAS1/FAS2). In one aspect of the invention, the *ACC1* gene is modified by virtue Ser659Ala and Ser1157Ala of said *ACC1 gene* being replaced (SEQ ID NO:16).

[0082] The invention also provides for a fungal cell system as defined herein, wherein said wax synthase encoded by said expression vector is heterologous. In this context, a "heterologous" wax synthase refers to a wax synthase originating from a different organism than the fungal cell used in the fungal cell system.

[0083] A fungal cell system as defined herein can comprise a wax synthase obtained from one or more of the species *Mycobacterium, Rhodococcus, Acinetobacter, Mus Musculus* and/or *Marinobacter.* Furthermore, more specifically, said at least one wax synthase can be selected from the group consisting of *Acinetobacter baylyi ADP1, Marinobacter hydrocarbonoclasticus DSM 8798, Rhodococcus opacus PD630, Mus musculus C57BU6,* and *Psychrobacter articus 273-4.* A gene expressing said wax synthase used herein can be codon optimized and comprise a nucleic acid sequence encoded by any one of SEQ ID NO:1, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 and/or SEQ ID NO 7. Also encompassed by the present invention are nucleic acid sequences having at least 80% identity with the presented sequence, such as approximately at least 80, 82, 85, 87, 90, 92, 95, 97, or 99% identity with the presented sequence. A nucleotide sequence disclosed herein can be from a natural species, a mutated version of a naturally occurring wax synthase, or a redesigned

enzyme produced by protein engineering, the wax synthases being mutated or redesigned still maintaining their activity when expressed. The present invention also relates to a wax synthase having at least 80%, such as at least 80, 82, 85, 87, 90, 92, 95, 97, or 99% in sequence identity with an amino acid sequence corresponding to any of the wax synthases presented herein.

[0084] A wax synthase of the fungal cell system according to the present invention can be encoded by one or more of the following expression vectors pSP-B1, pSP-B2, pSP-B3, pSP-B4 and/or pSP-B5. These expression vectors are further defined herein, e.g. in the experimental section, and in Figure 7. The nucleic acid sequences of the prior mentioned vectors are SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 33, SEQ ID NO 34, and SEQ ID NO 35. The present invention also relates to expression vectors pSP-B1, pSP-B2, pSP-B3, pSP-B4 and/or pSP-B5, wherein certain parts thereof have been slightly modified or parts have been removed, said expression vectors still retaining their activity, as well as expression vectors comprising any one of the sequences SEQ ID NO:30-35.

[0085] According to the invention, said expression vector encoding said one or more wax synthase(s) can be an episomal plasmid (single copy plasmids) or a high-copy plasmid. A single copy plasmid is defined as a plasmid that exists only as one or a few copies in each host. A high copy plasmid is a plasmid which will provide for a longer expression in the host as it will be present in more copies than the single copy plasmid.

[0086] In the context of the present invention, said expression vector encoding said wax synthase can provide for chromosomal integration into the chromosome of said fungal cell. Such an event is further illustrated in Figure 9 and 10 and in the experimental section (Example7).

[0087] To a fungal cell system as defined herein, carbohydrates can be supplied as an external substrate to said fungal cell system for the production of FAEE. Said carbohydrates can be selected from the group consisting of glucose, fructose, galactose, xylose, arabinose, sucrose, maltose, starch, cellulose, and hemicellulose.

[0088] In some aspects of the invention, additionally either or both of the genes Eht1p and Eeb1p of said fungal cell, are overexpressed by said fungal cell. It was shown that Eht1p and Eeb1p have medium chain fatty acid ethyl ester (including ethyl hexanoate)-synthesizing and -degrading activity (Lilly, M., F. Bauer, M. Lambrechts, J. Swiegers, D. Cozzolino, and I. Pretorius. 2006. The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. Yeast 23:641-659.). Eht1 preferred short-chain substrates (highest production was for ethyl butanoate), whereas Eeb1 preferred longer chain substrates (highest production was for ethyl octanoate) (Saerens, S., K. Verstrepen, S. Van Laere, A. Voet, P. Van Dijck, F. Delvaux, and J. Thevelein. 2006. The Saccharomyces cerevisiae EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. Journal of Biological Chemistry 281:4446.).

[0089] The present invention also relates to the use of an fatty acyl ester, such as a fatty acyl ethyl ester (FAEE), produced by a fungal cell system as defined herein as a component in a biofuel, such as biodiesel, a lubricant, cosmetic, linoleum, printing ink, and/or a solid wax ester used for candles and/or polishes.

[0090] The present invention also relates to a composition comprising a fungal cell system as defined herein, said composition further comprising at least one additional component selected from the group consisting of: buffers; stabilizers; protease-inhibiting agents; hydrolytic enzymes, saccharolytic enzymes; cell membrane- and/or cell wall-preserving compounds, nutritional media appropriate to the cell; and the like.

[0091] The present invention also relates to a method for producing fatty acyl ethyl esters (FAEE), said method comprising:

a) providing a fungal cell system as defined herein in a culture broth,
b) adding one or more sources of carbohydrates as an external substrate to said fungal cell system;
c) and wherein said FAEE are thereafter retrieved by extraction from said culture broth.

[0092] Said carbohydrates can be selected from the group consisting of: glucose, fructose, galactose, xylose, arabinose, sucrose, maltose, starch, cellulose, and hemicellulose. Considering the importance of developing second generation processes based on biomass, it will be a promising advantage how the biofuels can be produced from xylose, cellulose, and hemicellulose, which yeast does not naturally consume.

[0093] The invention also relates to a composition comprising a fungal cell which metabolism is modified thereby possessing an increased flux towards fatty acid biosynthesis; and one or more expression vectors encoding one or more wax synthase(s). Such a fungal cell can be any fungal cell as described herein, i.e. *Saccharomyces, Saccharomyces cerevisae Hansenula polymorpha, Kluyveromyces, Pichia, Candida albicans, Aspergilli, Rhodotorula rubra, Torulopsis, Trichosporon cutaneum, Trichoderma reesei, Apiofrichum curvafum, Yarrowia lipolytica,* and *Cryptococcus curvatus.* Furthermore, said wax synthase in such a composition can be selected from *Mycobacterium, Rhodococcus, Acinetobacter, Mus Musculus* and/or *Marinobacter.* A modification of such a fungal cell can be performed in any manner exemplified herein, such as by down-regulation, attenuation, deletion and/or over-expression of one or more gene(s) selected from the group consisting of genes encoding one or more enzyme(s) involved in at least one of said fungal cell's fatty acid synthesizing pathways, fatty acid consuming pathways and carbohydrate biosynthesis pathways, and/or selected

from the group consisting of genes encoding one or more enzyme(s) acting as wax ester transporter(s) Such a fungal cell can be used for producing biofuel esters, such as biodiesel, lubricants, cosmetics, linoleum and printing inks, and/or the solid waxes used for candles and polishes.

[0094] The present invention also relates to a yeast cell having an increased metabolic flux towards fatty acid ester biosynthesis, said yeast cell expressing at least one wax synthase selected from the group consisting of *Acinetobacter baylyi* ADP1, *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BL/6 and *Psychrobacter articus* 273-4 in combination with over-expressing the protein ACBP (acyl-CoA-binding protein). Said yeast cell can for example be *Saccharomyces cerevisae*

Preferred Embodiments

[0095] In a preferred embodiment the invention teaches a method for increasing fatty acid production in yeast cells via over-expression of ACBP.

[0096] *Saccharomyce cerevisae* is the preferred host for carrying out the invention, as it is a popular host in basic and applied research apart from being a good ethanol producer, a precursor of esters and specifically of fatty acid ethyl esters. Nevertheless as previously mentioned herein other fungal cells allowing the present invention are selected from the group consisting of other *Saccharomyces species* as well as other fungi such as, but not limited to, *Hansenula polymorpha, Kluyveromyces, Pichia, Candida albicans, Aspergilli, Rhodotorula rubra, Torulopsis, Trichosporon cutaneum, Trichoderma reesei, Apiofrichum curvafum, Yarrowia lipolytica, Cryptococcus curvatus.*

[0097] In *S. cerevisae,* fatty acids act as a feedback inhibitor of acetyl CoA carboxylase, and also as an inhibitor of fatty acid oxidation in response to increased fatty acid availability. On the other hand we know that the regulatory properties of fatty acids are mediated through their activation to Acyl CoA. This means that in *S. cerevisiae,* fatty acid biosynthesis is inhibited by its product, the acyl-CoA.

[0098] Acyl CoA binding protein (ACBP) can attenuate the inhibitory effect of Acyl CoA by binding long- and medium-chain acyl-CoA esters with very high affinity. Owing to the high affinity of ACBP for Acyl CoA, the intracellular free Acyl CoA concentration is predicted to be very low. It has been demonstrated that overexpression of Acb1p and bovine ACBP in *S. cerevisiae* increased the total acyl-CoA pool size. The inventors herein have developed a yeast cell in which ACBP (acyl CoA binding protein) is over-expressed so that to down-regulate the activity of enzymes involved in the lipid metabolism and in this specific case for deregulating acetyl-CoA carboxylase. Increased ACBP expression is translated in low free Acyl CoA levels and more fatty acid availability.

[0099] As previously explained, the present invention adopted a pESC derived plasmid as the expression vector. The plasmid pSP-GM2 shown in Figure 4, can express two genes simultaneously. In this specific modification, Acb1 is also ligated to plasmid pSP-GM2 and under control of promoter PGK1 (S. Partow et a., 2010).

[0100] Over-expressing ACBP as used herein means altering the rate of transcription, Post-transcription, or translation of the gene encoding the protein as compared with the same rates in the yeast cell without modification.

[0101] Methods of testing for over-expression are well known in the art, for example transcribed RNA levels can be assessed using rtPCR, and protein levels can be assessed using SDS page gel analysis.

[0102] In a further preferred embodiment the invention teaches a system and a method in which a further increase in fatty acid production is obtained by down-regulating, attenuating, deleting or over-expressing additional genes encoding enzymes involved in the fatty acids synthesizing pathway, fatty acid consuming pathways, carbohydrate biosynthesis pathways or enzyme acting as wax ester transporters or a combination thereof. In this regard the genetically modified yeast cell of the present invention may include other modifications in addition to an over-expressed ACBP, but preferably it should contain genes encoding a combination of different wax synthases with high specificity for short-chain alcohols.

[0103] Basically, the genetic modifications may increase the level of enzymes involved in the different biosynthetic pathways, reduce feedback inhibition at different locations in the biosynthesis pathways, affect the availability of different substrates and cofactors used in said pathways, affect expression of genes coding those enzymes, etc.

[0104] Polypeptides according to the invention may be purified and isolated by methods known in the art. In particular, having identified the gene sequence, it is possible to use recombinant techniques to express the genes in the selected suitable host.

EXPERIMENTAL SECTION

EXAMPLE 1

Construction of biodiesel production host Saccharomyces cerevisiae CB1

[0105] In this experiment the wax ester synthase from *A. baylyi* ADP1 was expressed in a laboratory strain *Saccharomyces cerevisiae* CEN.PK113-5D (MAT-alpha ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2) to create biodiesel producer,

Saccharomyces cerevisiae CB1.

[0106] Briefly, cloning and DNA manipulations were all carried out in *E.coli* DH5α and were performed by standard procedures (Sambrook and Russell 2001). The sequence of the gene *atfA* with the reported wax synthase from *Acinetobacter baylyi* ADP1 was optimized for expression in a yeast host. The optimized sequence is given as SEQ ID NO 1, which was based on the published gene sequence (Gene bank accession no. AF529086). It was synthesized and provided by the DNA2.0 Company (Menlo Park, Calif.). SEQ ID NO 1 was amplified using the following oligonucleotides:

5'-CGGGATCCCGCTCGAGATGCGTCCATT-3' (SEQ ID NO 2) introducing BamHI restriction site (underlined) and;
5'-GGGGTACCCCAAGCTTGGGTTAGTTTGCAG-3' (SEQ ID NO 3) introducing HindIII restriction site (underlined).
The BamHI/ HindIII digested DNA sequence was ligated into vector pSP-GM2 (Figure. 4) and under control of the constitutively expressed promoter TEF1, which gave plasmid pSP-B1 (Figure 7A). The cloned sequences were verified by sequencing. The plasmids pSP-GM2 and pSP-B1 were transformed into *S. cerevisiae* CEN.PK113-5D. The resulting strains were named *S. cerevisiae* CB0 and *S. cerevisiae* CB1, respectively. Synthetic minimal dropout (SD) medium lacking uracil was used to select for transformants.

EXAMPLE 2

Characteristics of the recombinant host

[0107] The inoculated transformants *S. cerevisiae* CB0 and *S. cerevisiae* CB1 were cultured to late exponential growth period in 100 mL SD medium lacking uracil and containing 2% (w/v) glucose at 30°C. The cultures were then harvested. Cell-free extracts were prepared using a previously reported fast prep method for enzyme analysis (Hou, Vemuri et al. 2009). The lipid analysis were extracted from the lyophilized cell pellets using the reported method (Gu, Valianpour et al. 2004).

[0108] The wax synthase activities in the transformants were testified in vitro using [1-14C] palmitoyl-CoA and 1-hexadecanol or ethanol as the substrates. Table 1 summarizes the results of enzyme analysis. A low wax synthase activity could be detected in negative control *S. cerevisiae* CB0 using 1-hexadecanol or ethanol as the substrates. In contrast, a significant high wax synthase activity was detected in *S. cerevisiae* CB1.

The lipid extraction was analyzed with Gas Chromatography/ Mass spectroscopy (GC/MS). No FAEEs were detected in the negative control *S. cerevisiae* CB0 even when the cultured medium was supplemented with 0.1% (w/v) free fatty acids, heptadecanoic acid. In contrast, *S. cerevisiae* CB1 could produced FAEEs to a titer of 5.0 mg/L. The heptadecanoic acid ethyl ester was produced by *S. cerevisiae* CB1 when the cultured medium was supplemented with 0.1% (w/v) free fatty acids, heptadecanoic acid (C17), which doesn't synthesized by yeast itself. Taking heptadecanoic acid ethyl ester as an example for the GC/MS results, it eluted at around 15.6 min, and the parent ion mass spectrum of m/z 298 was clearly observed (Figure 5). Additionally, structural confirmation was received by daughter ion scans of m/z 298 (Figure 5). The spectrums are the same as in standard heptadecanoic acid ethyl ester (Figure 6).

Table 1. WS activities in crude extracts of different recombinant *S. cerevisiae*

Strain	Wax synthase activity[a] (pmol [mg cell extract ·min^{-1}])	
	With palmitoyl-CoA and hexadecanol	With palmitoyl-CoA and ethanol
CB0	0.9 ± 0.2	0.67 ± 0.15
CB1	41.6 ± 2.21	4.9 ± 0.55
[a]Data are mean values of two independent experiments ± SD.		

EXAMPLE 3

Evaluation of the substrate preference of different WSs in yeast

[0109]

Table 2. Specific oligonucleotides used for PCR amplification of the synthesized WS sequences

	Primer Sequence 5'→3'	
	Upstream	Downstream
WS from *Marinobacter hydrocarbonoc lasticus* DSM 8798	CGGGATCCCGCTC GAGATGAAGAGATT AGG (SEQ ID NO 8)	GGGGTACCCCAAGCTTGGGTTACTT TCTAGTACG (SEQ ID NO 9)
WS from *Rhodococcus opacus* PD630	CGGGATCCCGCTC GAGTTGACCGACG TGATTAC (SEQ ID NO 10)	GGGGTACCCCAAGCTTGGGTTAGCT AGCCACCACC (SEQ ID NO 11)
WS from *Mus musculus* C57BL/6	CGGGATCCCGCTC GAGATGTTCTGGCC AACC (SEQ ID NO 12)	GGGGTACCCCAAGCTTGGGTTAAAC AATGACCAAC (SEQ ID NO 13)
WS from *Psychrobacter articus* 273-4	CGGGATCCCGCTC GAGATGAGATTACT GACCGCTGT (SEQ ID NO 14)	GGGGTACCCCAAGCTTGGGTTAAG GGGCCAACT (SEQ ID NO 15)

[0110] In this example, except for the wax synthase from *Acinetobacter baylyi* ADP1, four other putative WSs from *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BL/6, and *Psychrobacter articus* 273-4 were optimized for expression in a yeast host. The optimized sequences could be seen in SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 and SEQ ID NO 7. Then they were synthesized by the DNA2.0 Company (Menlo Park, Calif.). These synthesized sequences were PCR amplified by using specific oligonucleotides introducing BamHI and HindIII restriction sites (Table 2 above). The BamHI/HindIII digested DNA sequences were ligated into pSP-GM2 (Figure. 4), respectively, and under control of the constitutively expressed promoter TEF1, which resulted in the plasmids pSP-B2, pSP-B3, pSP-B4 and pSP-B5 (Figure. 7B, 7C, 7D, 7E). Cloned sequences were verified by sequencing. The plasmids pSP-B2, pSP-B3, pSP-B4 and pSP-B5 were transformed into *S. cerevisiae* CEN.PK113-5D to construct *S. cerevisiae* CB2, CB3, CB4 and CB5, respectively. Synthetic minimal dropout (SD) medium lacking uracil was used to select for transformants.

[0111] The cell-free extracts from the constructed recombinant *S. cerevisiae* CB1, CB2, CB3, CB4 and CB5 were prepared as the method described in Example 2. The wax synthase activities in the transformants were testified *in vitro* using alcohols with various chain lengths as substrates. Table 3 summarizes the results of enzyme analysis.

[0112] The substrate profiles in Table 3 show that CB2 and CB5 catalyzed ethanol with a higher activity, which could reduce the formation of byproducts and drive the carbon flux toward the target ethyl esters. Actually, CB2 and CB5 could produce FAEEs at yield of 6.3 mg/L and 2.3 mg/L, which are clearly higher than other wax synthase expressing yeast. Moreover CB2 catalyzed cetyl alcohol (1-Hexadecanol) with a higher activity, which is the choice for constructing the spermaceti producing yeast. Our findings clearly show that the substrate preferences of the different WSs are the instructions for producing certain wax esters.

Table 3. Acyl acceptor specificities with different alcohols in crude extracts of different recombinant *S. cerevisiae*

Acyl acceptor	Wax synthase activity[a] (pmol [mg cell extract ·min^{-1}])				
	CB1	CB2	CB3	CB4	CB5
Ethanol	4.6 ± 0.55	8.1 ± 1.87	2.7 ± 0.37	3.8 ± 0.51	5.9 ± 0.83

(continued)

Acyl acceptor	Wax synthase activity[a] (pmol [mg cell extract ·min^{-1}])				
	CB1	CB2	CB3	CB4	CB5
Butanol	10.8 ± 1.60	14.6 ± 1.75	6.8 ± 0.82	3.5 ± 0.53	4.2 ± 0.46
1-Hexanol	17.3 ± 2.04	33.8± 3.77	16.1 ± 2.29	10.2 ± 1.59	18.7 ± 2.19
1-Octanol	23.0 ± 2.39	45.7± 4.51	32.3± 3.84	22.3 ± 2.44	17.7 ± 1.67
1-Decanol	19.7 ± 3.11	41.1± 4.13	37.3± 3.90	33.5 ± 2.22	27.5 ± 2.50
1-Dodecanol	31.8± 3.48	48.4± 4.56	36.7± 3.78	44.2 ± 3.07	42.8 ± 3.11
1-Tetradecanol	45.0 ± 4.72	49.7± 4.38	33.5± 3.66	35.1 ± 2.87	36.5 ± 3.03
1-Hexadecanol	41.6 ± 2.21	49.0 ± 3.65	28.9 ± 3.29	35.5 ± 2.91	39.1 ± 2.72

[a]Data are mean values of two independent experiments ± SD.

EXAMPLE 4

[0113] Metabolic engineering strategy for enhancing fatty acid biosynthesis-Expression of heterologous NADP+ dependent glyceraldehyde-3-phosphate dehydrogenase In this invention supplying more NADPH is taken as an example to illustrate the metabolic engineering strategy for enhancing fatty acid biosynthesis. To make more NADPH, the heterologous expression of NADP+ dependent glyceraldehyde-3-phosphate dehydrogenase (*gapN,* from *Streptococcus mutants*) is used. The heterologous reaction is listed in Figure 8.

The sequence of *gapN* (from *Streptococcus mutants*) was optimized for expression in a yeast host (SEQ ID NO 17) and was synthesized by DNA2.0 Company (Menlo Park, Calif.). The synthesized sequence was PCR amplified by using specific oligonucleotides: 5'-AAACAA GCGGCCGCACTAGTTTGACAAAAC-3' (SEQ ID NO 18) introducing NotI restriction site (underlined) and 5'- TTAATTAAGAGCTCAGATCTTTATTTGATATCAA-3' (SEQ ID NO 19) introducing SacI restriction site (underlined). The NotI/SacI digested DNA sequences were ligated into pSP-GM2, and transformed into host *S. cerevisiae* strain.

EXAMPLE 5

[0114] Summary of modifications useful for making yeast with increased FA supply for producing wax esters

Table 4. is a summary of modifications to construct engineered yeast cells that can efficiently biosynthesis FA for producing wax esters. The modifications can be combined together.

Enzyme	Sources	Gene
Wax synthase	*Acinetobacter baylyi* ADP1	*atfA*
	Marinobacter hydrocarbonoclasticus DSM 8798	WS2
	Rhodococcus opacus PD630	*atf1*
	Mus musculus C57BL/6	AY611031 and AY611032
	Psychrobacter articus	YP_263530
ACBP (acyl-CoA-binding protein)	*Saccharomyces cerevisiae* CEN.PK113-5D	Acb1
Acetyl-CoA carboxylase	*Saccharomyces cerevisiae* CEN.PK113-5D	Desensitized ACC1
Fatty acid synthase	*Saccharomyces cerevisiae* CEN.PK113-5D	FAS1, FAS2
$NADP^+$ dependent glyceraldehyde-3-phosphate dehydrogenase	*Streptococcus mutants*	*gapN*
Acetyl-CoA synthetase	*Saccharomyces cerevisiae* CEN.PK113	ACS1

(continued)

Enzyme	Sources	Gene
Acyl-CoA:diacylglycerol acyltransferase	*Saccharomyces cerevisiae* CEN.PK113	DGA1
Lecithin:cholesterol acyltransferase	*Saccharomyces cerevisiae* CEN.PK113	LRO1
Acyl-CoA:sterol acyltransferase	*Saccharomyces cerevisiae* CEN.PK113	ARE1, ARE2
Peroxisomal acyl-CoA oxidase	*Saccharomyces cerevisiae* CEN.PK113	POX1

EXAMPLE 6

Fermentation

[0115] After combination of the above engineering strategies, the engineered host yeast holds the ability with increased flux towards FA biosynthesis. After combined wax synthase expression, it produces wax ester without the need for addition of exogenous fatty acids to the culture. In such an example, the engineered wax synthase expressing *S. cerevisiae* with an increased flux towards FA biosynthesis allow for high level production of biodiesel (FAEEs) from the only externally supplied substrate, carbohydrates. For large-scale biodiesel production, the engineered *S. cerevisiae* is cultured in 5 L fermentor. Glucose is continuously fed into the medium, in which maintained a high ratio of C/N. Meanwhile, dodecane (10%, v/v) was overlayed the medium to potentially prevent FAEEs evaporation and facilitate *in-situ* product capture.

EXAMPLE 7

Plasmids Construction for evaluation of five wax ester synthases on FAEE production:

[0116] Briefly, cloning and DNA manipulations were all carried out in *E.coli* DH5α and were performed by standard procedures (Sambrook and Russell 2001). The five sequences of the wax synthase from different species were optimized for expression in a yeast host. Then they were synthesized and provided by the DNA2.0 Company (Menlo Park, Calif.). These five different sequences were amplified using the ligonucleotides primers, respectively (table 2). The five BamHI/HindIII digested DNA sequences were, respectively, ligated into vector pSP-GM2 and under control of the constitutively expressed promoter TEF1, which gave five different plasmids. These plasmids were transformed into *Saccharomyces cerevisiae* CEN.PK113-5D (MAT-alpha ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2) to create five biodiesel producers. The method for yeast transformation is the standard LiAc/SS Carrier DNA/PEG method (Xiao 2006). Synthetic minimal dropout (SD) medium lacking uracil was used to select for transformants.

Gene deletions:

[0117] Shown in figure 9, the five genes (DGA1, LRO1, ARE1, ARE2, POX1) were deleted subsequently in *Saccharomyces cerevisiae* CEN.PK113-5D using the loop-out method with the help of loxP-KanMX-loxP cassette (Xiao 2006).

General description and Method for the chromosomal integration

[0118] Wax synthase from *Marinobacter hydrocarbonoclasticus* DSM 8798 is suggested to have the highest activity for biodiesel production and chosen as the working enzyme. In the deletion strains, the related genes were introduced and constructed the following strains. The ability of biodiesel production was shown in figure 10, and the genotypes of strains were listed in Table 5. The overexpressed ACC1 were released its phosphorylation sites (Ser659Ala and Ser1157Ala), as shown in SEQ ID NO 16.

Table 5. List of strains and their genotypes.

Strain	Genetype or relevant Characteristics
SJ03	*ΔDGA1, ΔLRO1, ΔARE1, ΔARE2,* with wax synthase (*Marinobacter*) overexpressed from plasmid pSP-GM2
SJ04	*ΔDGA1, ΔLRO1, ΔARE1, ΔARE2,* with wax synthase (*Marinobacter*) and Acetyl-CoA carboxylase overexpressed from plasmid pSP-GM2
SJ05	*ΔPOX1,* with wax synthase (*Marinobacter*) overexpressed from plasmid pSP-GM2
SJ06	*ΔPOX1,* with wax synthase (*Marinobacter*) and Acetyl-CoA carboxylase overexpressed from plasmid pSP-GM2
SJ07	*ΔDGA1, ΔLRO1, ΔARE1, ΔARE2, ΔPOX1,* with wax synthase (*Marinobacter*) overexpressed from plasmid pSP-GM2
SJ08	*ΔDGA1, ΔLRO1, ΔARE1, ΔARE2, ΔPOX1,* with wax synthase (*Marinobacter*) and Acetyl-CoA carboxylase overexpressed from plasmid pSP-GM2

EXAMPLE 8

[0119] Although plasmids based methods have been used for biodiesel production, the plasmid is not genetic stable, which contributed the loss in productivity. In this work, we developed a plasmid-free method with high genetic stability and high gene copy expression for biodiesel production. Shown in figure 11, the wax synthase and bacterial neo gene (Neo, G418 resistance gene) were fused together, and integrated into delta sequence of chromosome by yeast transformation. The copies of delta sequence occur in multiple places throughout the yeast genome, and, under the selection of increasing concentration of G418, clones with multiple copies of the inserted gene can be generated. Finally, pathway copy number is stabilized by RAD52 knockout, and the resulting engineered strain requires no selection markers and is unaffected by plasmid instabilities.

[0120] The WS (wax synthase) from *Marinobacter hydrocarbonoclasticus* DSM 8798 were evaluated under control of two different strong promoters, TEF1 and PGK1. Amplified by primer 1 and primer 2 (Table 6), the BamHI/ HindIII digested WS sequence was ligated into vector pSP-GM2 and under control of the constitutively expressed promoter TEF1, which gave plasmid pSP-B2. Using plasmid pSP-B2 as the template, the WS sequence with TEF1 promoter and CYC1 terminator could be amplified with primer 3 and primer 4. The neo gene was amplified from plasmid pJEF1105 (Wang, Wang et al. 1996) with primer 5 and primer 6. Shown in Figure 11, the 5' end of primer 3 and primer 6 are homolog to the delta sequence, which would facilitate the integration; the 5' end of primer 4 is homolog to neo gene and the 5' end of primer 5 is homolog to CYC1 terminator, which would facilitate sequence fusion. The two DNA sequences, TEF1 controlled WS (PCR product 1, figure 11) and neo gene (PCR product 2, figure 11), could be fused together as one by PCR amplification taken these two sequences as the template and primer 3 and 6 as the PCR primers. The fused DNA fragment (PCR product 3, figure 11) be transformed into yeast and selected on the plats with G418 concentration. Similarly, DNA fragment that contained PGK1 controlled WS and neo gene was also constructed and integrated into yeast.

[0121] Shown in Figure 12, the initial results suggests PGK1 controlled WS have a higher productivity and chosen as the choice for selection on the plates with increasing G418 concentration. The colonies selected from the plate with higher concentration of G418 should contain higher copy number of WS, and contribute to a higher biodiesel production. Figure 13 shows the relationship of the concentration of biodiesel production and the concentration of G418. Yield increased remarkably as more G418 was used in the chromosomal evolution until yield stopped increase when the supply of precursors limited the function of wax synthase. The chromosome integration constructed a stable pathway and the production is comparable or higher than those achievable using multicopy plasmids.

Table 6: primers list

	Primer Sequence 5'→3'
Primer 1	CGGGATCCCGCTCGAGATGAAGAGATTAGG (SEQ ID NO:20)
Primer 2	GGGGTACCCCAAGCTTGGGTTACTTTCTAGTACG (SEQ ID NO:21)
Primer 3	GTTGGGATTCCATTGTTGATAAAGGCGcacacaccatagcttcaaaatgtttc (SEQ ID NO:22)
Primer 4	GTGCAATGTAgatcttcgagcgtcccaaaacc (SEQ ID NO:23)
Primer 5	GacgctcgaagatcTACATTGCACAAGATAAAAATATATCATCATGAACAAT (SEQ ID NO:24)

(continued)

	Primer Sequence 5'→3'
Primer 6	GCCTTTATCAACAATGGAATCCCAACCGCCGTCCCGTCAAGTC (SEQ ID NO:25)
Primer 7	ACAACAAATATAAAACAAGCGGCCGCACTATGAAGAGATTAGGTACT C (SEQ ID NO:26)
Primer 8	GGCGAAGAATTGTTAATTAAGAGCTCGGTACCCCAAGCTTGGGTTA (SEQ ID NO:27)
Primer 9	GTTGGGATTCCATTGTTGATAAAGGCGGAAGTACCTTCAAAGAATGG GGTC (SEQ ID NO:28)
Primer 10	CTTGTGCAATGTAGAGCGACCTCATGCTATACCTGAG (SEQ ID NO:29)
Primer 11	ATGAGGTCGCTCTACATTGCACAAGATAAAAATATATCATCATGAAC (SEQ ID NO:30)

Analysis:

[0122] The inoculated transformants of *S. cerevisiae* were cultured to late exponential growth period in 100 mL SD medium lacking uracil and containing 2% (w/v) glucose at 30°C. The cultures were then harvested. Cell-free extracts were prepared using a previously reported fast prep method for enzyme analysis (Hou, Vemuri et al. 2009). The wax synthase activities in the transformants were testified in vitro using [1-14C] palmitoyl-CoA and 1-hexadecanol or ethanol as the substrates (Kalscheuer, Luftmann et al. 2004). ACCase (Acetyl-CoA carboxylase) activity was measured under a fume hood as the incorporation of radioactivity from $NaH_{14}CO_3$ into an acid-stable product, as described previously (Diacovich, Peir et al. 2002). The total lipid were extracted from the lyophilized cell pellets using the reported method (Gu, Valianpour et al. 2004). The putative FAEEs in the total lipid were purified by preparative TLC and detected by GC-MS (Kalscheuer, Luftmann et al. 2004).

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tttttgaaaa agagacaggg tttcgtgaga actgcgttga agcatggtgt ttctctgatc 660

ccagcttacg ctttcgggga aactgatctc tacgatcaac acatattcac accaggtggt 720

tttgtcaata gatttcagaa atggtttcaa aagatggtac acatctaccc atgcgctttc 780

tatggcagag ggctcaccaa aaactcatgg gggctactac cttattcaca gcctgttacc 840

acagtggttg gagaaccttt acctctgcca aagattgaaa acccttccga agagattgtt 900

gcgaagtacc atacactgta catcgatgca cttaggaagc tattcgacca acacaaaact 960

aagtttggta ttagtgaaac ccaagagttg gtcattgttt aa 1002

<210> 7
<211> 1428
<212> DNA
<213> Artificial sequence

<220>
<223> Artificial Sequence, optimized from wax synthase of Psychrobacter articus 273-4

<400> 7

atgagattac tgaccgctgt cgatcaactc tttctattgt tggagagtag aaagcaccca 60

atgcacgttg gtggactgtt cctattcgag cttccagaga atgctgacat tagtttcgtt 120

caccagcttg ttaagcaaat gcaagattcc gacgtaccac caacattccc attcaatcag 180

gttctggaac acatgatgtt ttggaaggag gacaaaaact ttgacgtaga acatcatcta 240

caccatgtgg ctttaccaaa acctgccaga gttagagaat tactcatgta cgtttccagg 300

gaacatggga ggttgctcga tagagcaatg ccactatggg agtgccatgt gatcgaaggt 360

attcaaccag agactgaagg ttctccagag agattcgcat tgtatttcaa gattcatcat 420

tccttagtcg atggtatcgc cgctatgagg ttggtgaaaa agtcattatc acagtcacca 480

aacgaaccag ttacccttcc aatctggtct ttgatggctc accatagaaa ccaaatcgat 540

gccatcttcc caaaggaaag atcagccttg cgtatcttaa aggaacaagt ttctacaatc 600

aagcctgtgt ttactgaact cttgaataac ttcaaaaact acaatgacga tagttacgtc 660

agcacttttg acgctcctag atcaatcctt aaccgtagaa tttctgcctc aagacgtatt 720

gcagcgcagt catacgatat caaaagattc aatgacatag cggagagaat caacatttcc 780

aaaaacgatg tggttttggc agtatgttcc ggtgctatta gaagatacct tatctctatg 840

gatgctttac catcaaaacc tctgatagca ttcgttccta tgtctttgcg aactgatgat 900

agtatagctg gaaaccaatt gagttttgta ctagcgaatc tgggcacaca tttggatgat 960

ccattatcta gaatcaagct cattcatcgt agcatgaaca actctaagag aagattcaga 1020

aggatgaacc aagcacaagt tatcaattac tccatagtat cttacgcatg ggaaggcatt 1080

aacttggcca ctgatctttt ccctaaaaag caagccttta acttaatcat ctctaacgtc 1140

ccaggctcag aaaaaccttt gtattggaat ggtgcaagat tagaatcact atatcctgct 1200

tcaatcgtgt ttaacggaca agctatgaat atcacgcttg catcttactt ggacaagatg 1260

gaattcggta taactgcttg ttctaaagct ctacctcatg tccaagatat gttgatgctt 1320

attgaggaag agctacaact gctggaatct gttagcaagg aactagaatt caatgggatt 1380

acagtaaaag ataagtcaga gaaaaagctg aaaaagttgg ccccttaa 1428

<210> 8
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 8
cgggatcccg ctcgagatga agagattagg 30

<210> 9
<211> 34
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 9
ggggtacccc aagcttgggt tactttctag tacg 34

<210> 10
<211> 33
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 10
cgggatcccg ctcgagttga ccgacgtgat tac 33

<210> 11
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 11
ggggtacccc aagcttgggt tagctagcca ccacc 35

<210> 12
<211> 31
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 12
cgggatcccg ctcgagatgt tctggccaac c 31

<210> 13
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 13
ggggtacccc aagcttgggt taaacaatga ccaac 35

<210> 14
<211> 36
<212> DNA

<213> Artificial sequence

<220>
<223> primer

<400> 14
cgggatcccg ctcgagatga gattactgac cgctgt 36

<210> 15
<211> 33
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 15
ggggtacccc aagcttgggt taaggggcca act 33

<210> 16
<211> 2233
<212> PRT
<213> Artificial sequence

<220>
<223> modified ACC1 gene

<400> 16

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Met Ser Glu Glu Ser Leu Phe Glu Ser Ser Pro Gln Lys Met Glu Tyr
1               5                  10                  15

Glu Ile Thr Asn Tyr Ser Glu Arg His Thr Glu Leu Pro Gly His Phe
            20                  25                  30

Ile Gly Leu Asn Thr Val Asp Lys Leu Glu Glu Ser Pro Leu Arg Asp
        35                  40                  45

Phe Val Lys Ser His Gly Gly His Thr Val Ile Ser Lys Ile Leu Ile
    50                  55                  60
```

```
Ala Asn Asn Gly Ile Ala Ala Val Lys Glu Ile Arg Ser Val Arg Lys
65                  70                  75                  80

Trp Ala Tyr Glu Thr Phe Gly Asp Asp Arg Thr Val Gln Phe Val Ala
                85                  90                  95

Met Ala Thr Pro Glu Asp Leu Glu Ala Asn Ala Glu Tyr Ile Arg Met
            100                 105                 110

Ala Asp Gln Tyr Ile Glu Val Pro Gly Gly Thr Asn Asn Asn Asn Tyr
        115                 120                 125

Ala Asn Val Asp Leu Ile Val Asp Ile Ala Glu Arg Ala Asp Val Asp
    130                 135                 140

Ala Val Trp Ala Gly Trp Gly His Ala Ser Glu Asn Pro Leu Leu Pro
145                 150                 155                 160

Glu Lys Leu Ser Gln Ser Lys Arg Lys Val Ile Phe Ile Gly Pro Pro
                165                 170                 175

Gly Asn Ala Met Arg Ser Leu Gly Asp Lys Ile Ser Ser Thr Ile Val
            180                 185                 190

Ala Gln Ser Ala Lys Val Pro Cys Ile Pro Trp Ser Gly Thr Gly Val
        195                 200                 205

Asp Thr Val His Val Asp Glu Lys Thr Gly Leu Val Ser Val Asp Asp
    210                 215                 220

Asp Ile Tyr Gln Lys Gly Cys Cys Thr Ser Pro Glu Asp Gly Leu Gln
225                 230                 235                 240

Lys Ala Lys Arg Ile Gly Phe Pro Val Met Ile Lys Ala Ser Glu Gly
                245                 250                 255

Gly Gly Gly Lys Gly Ile Arg Gln Val Glu Arg Glu Glu Asp Phe Ile
            260                 265                 270

Ala Leu Tyr His Gln Ala Ala Asn Glu Ile Pro Gly Ser Pro Ile Phe
        275                 280                 285

Ile Met Lys Leu Ala Gly Arg Ala Arg His Leu Glu Val Gln Leu Leu
    290                 295                 300

Ala Asp Gln Tyr Gly Thr Asn Ile Ser Leu Phe Gly Arg Asp Cys Ser
305                 310                 315                 320
```

```
Val Gln Arg Arg His Gln Lys Ile Ile Glu Glu Ala Pro Val Thr Ile
                325                 330                 335

Ala Lys Ala Glu Thr Phe His Glu Met Glu Lys Ala Ala Val Arg Leu
            340                 345                 350

Gly Lys Leu Val Gly Tyr Val Ser Ala Gly Thr Val Glu Tyr Leu Tyr
        355                 360                 365

Ser His Asp Asp Gly Lys Phe Tyr Phe Leu Glu Leu Asn Pro Arg Leu
    370                 375                 380

Gln Val Glu His Pro Thr Thr Glu Met Val Ser Gly Val Asn Leu Pro
385                 390                 395                 400

Ala Ala Gln Leu Gln Ile Ala Met Gly Ile Pro Met His Arg Ile Ser
                405                 410                 415

Asp Ile Arg Thr Leu Tyr Gly Met Asn Pro His Ser Ala Ser Glu Ile
            420                 425                 430

Asp Phe Glu Phe Lys Thr Gln Asp Ala Thr Lys Lys Gln Arg Arg Pro
        435                 440                 445

Ile Pro Lys Gly His Cys Thr Ala Cys Arg Ile Thr Ser Glu Asp Pro
    450                 455                 460

Asn Asp Gly Phe Lys Pro Ser Gly Gly Thr Leu His Glu Leu Asn Phe
465                 470                 475                 480

Arg Ser Ser Ser Asn Val Trp Gly Tyr Phe Ser Val Gly Asn Asn Gly
                485                 490                 495

Asn Ile His Ser Phe Ser Asp Ser Gln Phe Gly His Ile Phe Ala Phe
            500                 505                 510

Gly Glu Asn Arg Gln Ala Ser Arg Lys His Met Val Val Ala Leu Lys
        515                 520                 525

Glu Leu Ser Ile Arg Gly Asp Phe Arg Thr Thr Val Glu Tyr Leu Ile
    530                 535                 540

Lys Leu Leu Glu Thr Glu Asp Phe Glu Asp Asn Thr Ile Thr Thr Gly
545                 550                 555                 560

Trp Leu Asp Asp Leu Ile Thr His Lys Met Thr Ala Glu Lys Pro Asp
                565                 570                 575
```

```
Pro Thr Leu Ala Val Ile Cys Gly Ala Ala Thr Lys Ala Phe Leu Ala
            580                 585                 590

Ser Glu Glu Ala Arg His Lys Tyr Ile Glu Ser Leu Gln Lys Gly Gln
        595                 600                 605

Val Leu Ser Lys Asp Leu Leu Gln Thr Met Phe Pro Val Asp Phe Ile
    610                 615                 620

His Glu Gly Lys Arg Tyr Lys Phe Thr Val Ala Lys Ser Gly Asn Asp
625                 630                 635                 640

Arg Tyr Thr Leu Phe Ile Asn Gly Ser Lys Cys Asp Ile Ile Leu Arg
                645                 650                 655

Gln Leu Ala Asp Gly Gly Leu Leu Ile Ala Ile Gly Gly Lys Ser His
            660                 665                 670

Thr Ile Tyr Trp Lys Glu Glu Val Ala Ala Thr Arg Leu Ser Val Asp
        675                 680                 685

Ser Met Thr Thr Leu Leu Glu Val Glu Asn Asp Pro Thr Gln Leu Arg
    690                 695                 700

Thr Pro Ser Pro Gly Lys Leu Val Lys Phe Leu Val Glu Asn Gly Glu
705                 710                 715                 720

His Ile Ile Lys Gly Gln Pro Tyr Ala Glu Ile Glu Val Met Lys Met
                725                 730                 735

Gln Met Pro Leu Val Ser Gln Glu Asn Gly Ile Val Gln Leu Leu Lys
            740                 745                 750

Gln Pro Gly Ser Thr Ile Val Ala Gly Asp Ile Met Ala Ile Met Thr
        755                 760                 765

Leu Asp Asp Pro Ser Lys Val Lys His Ala Leu Pro Phe Glu Gly Met
    770                 775                 780

Leu Pro Asp Phe Gly Ser Pro Val Ile Glu Gly Thr Lys Pro Ala Tyr
785                 790                 795                 800

Lys Phe Lys Ser Leu Val Ser Thr Leu Glu Asn Ile Leu Lys Gly Tyr
                805                 810                 815

Asp Asn Gln Val Ile Met Asn Ala Ser Leu Gln Gln Leu Ile Glu Val
```

820 825 830

Leu Arg Asn Pro Lys Leu Pro Tyr Ser Glu Trp Lys Leu His Ile Ser
835 840 845

Ala Leu His Ser Arg Leu Pro Ala Lys Leu Asp Glu Gln Met Glu Glu
850 855 860

Leu Val Ala Arg Ser Leu Arg Arg Gly Ala Val Phe Pro Ala Arg Gln
865 870 875 880

Leu Ser Lys Leu Ile Asp Met Ala Val Lys Asn Pro Glu Tyr Asn Pro
885 890 895

Asp Lys Leu Leu Gly Ala Val Val Glu Pro Leu Ala Asp Ile Ala His
900 905 910

Lys Tyr Ser Asn Gly Leu Glu Ala His Glu His Ser Ile Phe Val His
915 920 925

Phe Leu Glu Glu Tyr Tyr Glu Val Glu Lys Leu Phe Asn Gly Pro Asn
930 935 940

Val Arg Glu Glu Asn Ile Ile Leu Lys Leu Arg Asp Glu Asn Pro Lys
945 950 955 960

Asp Leu Asp Lys Val Ala Leu Thr Val Leu Ser His Ser Lys Val Ser
965 970 975

Ala Lys Asn Asn Leu Ile Leu Ala Ile Leu Lys His Tyr Gln Pro Leu
980 985 990

Cys Lys Leu Ser Ser Lys Val Ser Ala Ile Phe Ser Thr Pro Leu Gln
995 1000 1005

His Ile Val Glu Leu Glu Ser Lys Ala Thr Ala Lys Val Ala Leu
1010 1015 1020

Gln Ala Arg Glu Ile Leu Ile Gln Gly Ala Leu Pro Ser Val Lys
1025 1030 1035

Glu Arg Thr Glu Gln Ile Glu His Ile Leu Lys Ser Ser Val Val
1040 1045 1050

Lys Val Ala Tyr Gly Ser Ser Asn Pro Lys Arg Ser Glu Pro Asp
1055 1060 1065

```
Leu Asn  Ile Leu Lys Asp Leu  Ile Asp Ser Asn Tyr  Val Val Phe
    1070                 1075                 1080

Asp Val  Leu Leu Gln Phe Leu  Thr His Gln Asp Pro  Val Val Thr
    1085                 1090                 1095

Ala Ala  Ala Ala Gln Val Tyr  Ile Arg Arg Ala Tyr  Arg Ala Tyr
    1100                 1105                 1110

Thr Ile  Gly Asp Ile Arg Val  His Glu Gly Val Thr  Val Pro Ile
    1115                 1120                 1125

Val Glu  Trp Lys Phe Gln Leu  Pro Ser Ala Ala Phe  Ser Thr Phe
    1130                 1135                 1140

Pro Thr  Val Lys Ser Lys Met  Gly Met Asn Arg Ala  Val Ala Val
    1145                 1150                 1155

Ser Asp  Leu Ser Tyr Val Ala  Asn Ser Gln Ser Ser  Pro Leu Arg
    1160                 1165                 1170

Glu Gly  Ile Leu Met Ala Val  Asp His Leu Asp Asp  Val Asp Glu
    1175                 1180                 1185

Ile Leu  Ser Gln Ser Leu Glu  Val Ile Pro Arg His  Gln Ser Ser
    1190                 1195                 1200

Ser Asn  Gly Pro Ala Pro Asp  Arg Ser Gly Ser Ser  Ala Ser Leu
    1205                 1210                 1215

Ser Asn  Val Ala Asn Val Cys  Val Ala Ser Thr Glu  Gly Phe Glu
    1220                 1225                 1230

Ser Glu  Glu Glu Ile Leu Val  Arg Leu Arg Glu Ile  Leu Asp Leu
    1235                 1240                 1245

Asn Lys  Gln Glu Leu Ile Asn  Ala Ser Ile Arg Arg  Ile Thr Phe
    1250                 1255                 1260

Met Phe  Gly Phe Lys Asp Gly  Ser Tyr Pro Lys Tyr  Tyr Thr Phe
    1265                 1270                 1275

Asn Gly  Pro Asn Tyr Asn Glu  Asn Glu Thr Ile Arg  His Ile Glu
    1280                 1285                 1290

Pro Ala  Leu Ala Phe Gln Leu  Glu Leu Gly Arg Leu  Ser Asn Phe
    1295                 1300                 1305
```

```
Asn Ile  Lys Pro Ile Phe Thr  Asp Asn Arg Asn Ile  His Val Tyr
    1310                1315                1320

Glu Ala  Val Ser Lys Thr Ser  Pro Leu Asp Lys Arg  Phe Phe Thr
    1325                1330                1335

Arg Gly  Ile Ile Arg Thr Gly  His Ile Arg Asp Asp  Ile Ser Ile
    1340                1345                1350

Gln Glu  Tyr Leu Thr Ser Glu  Ala Asn Arg Leu Met  Ser Asp Ile
    1355                1360                1365

Leu Asp  Asn Leu Glu Val Thr  Asp Thr Ser Asn Ser  Asp Leu Asn
    1370                1375                1380

His Ile  Phe Ile Asn Phe Ile  Ala Val Phe Asp Ile  Ser Pro Glu
    1385                1390                1395

Asp Val  Glu Ala Ala Phe Gly  Gly Phe Leu Glu Arg  Phe Gly Lys
    1400                1405                1410

Arg Leu  Leu Arg Leu Arg Val  Ser Ser Ala Glu Ile  Arg Ile Ile
    1415                1420                1425

Ile Lys  Asp Pro Gln Thr Gly  Ala Pro Val Pro Leu  Arg Ala Leu
    1430                1435                1440

Ile Asn  Asn Val Ser Gly Tyr  Val Ile Lys Thr Glu  Met Tyr Thr
    1445                1450                1455

Glu Val  Lys Asn Ala Lys Gly  Glu Trp Val Phe Lys  Ser Leu Gly
    1460                1465                1470

Lys Pro  Gly Ser Met His Leu  Arg Pro Ile Ala Thr  Pro Tyr Pro
    1475                1480                1485

Val Lys  Glu Trp Leu Gln Pro  Lys Arg Tyr Lys Ala  His Leu Met
    1490                1495                1500

Gly Thr  Thr Tyr Val Tyr Asp  Phe Pro Glu Leu Phe  Arg Gln Ala
    1505                1510                1515

Ser Ser  Ser Gln Trp Lys Asn  Phe Ser Ala Asp Val  Lys Leu Thr
    1520                1525                1530

Asp Asp  Phe Phe Ile Ser Asn  Glu Leu Ile Glu Asp  Glu Asn Gly
    1535                1540                1545
```

```
Glu Leu  Thr Glu Val Glu Arg  Glu Pro Gly Ala Asn  Ala Ile Gly
    1550                 1555                 1560

Met Val  Ala Phe Lys Ile Thr  Val Lys Thr Pro Glu  Tyr Pro Arg
    1565                 1570                 1575

Gly Arg  Gln Phe Val Val Val  Ala Asn Asp Ile Thr  Phe Lys Ile
    1580                 1585                 1590

Gly Ser  Phe Gly Pro Gln Glu  Asp Glu Phe Phe Asn  Lys Val Thr
    1595                 1600                 1605

Glu Tyr  Ala Arg Lys Arg Gly  Ile Pro Arg Ile Tyr  Leu Ala Ala
    1610                 1615                 1620

Asn Ser  Gly Ala Arg Ile Gly  Met Ala Glu Glu Ile  Val Pro Leu
    1625                 1630                 1635

Phe Gln  Val Ala Trp Asn Asp  Ala Ala Asn Pro Asp  Lys Gly Phe
    1640                 1645                 1650

Gln Tyr  Leu Tyr Leu Thr Ser  Glu Gly Met Glu Thr  Leu Lys Lys
    1655                 1660                 1665

Phe Asp  Lys Glu Asn Ser Val  Leu Thr Glu Arg Thr  Val Ile Asn
    1670                 1675                 1680

Gly Glu  Glu Arg Phe Val Ile  Lys Thr Ile Ile Gly  Ser Glu Asp
    1685                 1690                 1695

Gly Leu  Gly Val Glu Cys Leu  Arg Gly Ser Gly Leu  Ile Ala Gly
    1700                 1705                 1710

Ala Thr  Ser Arg Ala Tyr His  Asp Ile Phe Thr Ile  Thr Leu Val
    1715                 1720                 1725

Thr Cys  Arg Ser Val Gly Ile  Gly Ala Tyr Leu Val  Arg Leu Gly
    1730                 1735                 1740

Gln Arg  Ala Ile Gln Val Glu  Gly Gln Pro Ile Ile  Leu Thr Gly
    1745                 1750                 1755

Ala Pro  Ala Ile Asn Lys Met  Leu Gly Arg Glu Val  Tyr Thr Ser
    1760                 1765                 1770

Asn Leu  Gln Leu Gly Gly Thr  Gln Ile Met Tyr Asn  Asn Gly Val
```

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     1775                1780                1785

Ser His  Leu Thr Ala Val Asp  Asp Leu Ala Gly Val  Glu Lys Ile
     1790                1795                1800

Val Glu  Trp Met Ser Tyr Val  Pro Ala Lys Arg Asn  Met Pro Val
     1805                1810                1815

Pro Ile  Leu Glu Thr Lys Asp  Thr Trp Asp Arg Pro  Val Asp Phe
     1820                1825                1830

Thr Pro  Thr Asn Asp Glu Thr  Tyr Asp Val Arg Trp  Met Ile Glu
     1835                1840                1845

Gly Arg  Glu Thr Glu Ser Gly  Phe Glu Tyr Gly Leu  Phe Asp Lys
     1850                1855                1860

Gly Ser  Phe Phe Glu Thr Leu  Ser Gly Trp Ala Lys  Gly Val Val
     1865                1870                1875

Val Gly  Arg Ala Arg Leu Gly  Gly Ile Pro Leu Gly  Val Ile Gly
     1880                1885                1890

Val Glu  Thr Arg Thr Val Glu  Asn Leu Ile Pro Ala  Asp Pro Ala
     1895                1900                1905

Asn Pro  Asn Ser Ala Glu Thr  Leu Ile Gln Glu Pro  Gly Gln Val
     1910                1915                1920

Trp His  Pro Asn Ser Ala Phe  Lys Thr Ala Gln Ala  Ile Asn Asp
     1925                1930                1935

Phe Asn  Asn Gly Glu Gln Leu  Pro Met Met Ile Leu  Ala Asn Trp
     1940                1945                1950

Arg Gly  Phe Ser Gly Gly Gln  Arg Asp Met Phe Asn  Glu Val Leu
     1955                1960                1965

Lys Tyr  Gly Ser Phe Ile Val  Asp Ala Leu Val Asp  Tyr Lys Gln
     1970                1975                1980

Pro Ile  Ile Ile Tyr Ile Pro  Pro Thr Gly Glu Leu  Arg Gly Gly
     1985                1990                1995

Ser Trp  Val Val Val Asp Pro  Thr Ile Asn Ala Asp  Gln Met Glu
     2000                2005                2010
```

```
Met Tyr  Ala Asp Val Asn Ala  Arg Ala Gly Val Leu  Glu Pro Gln
    2015                 2020                 2025

Gly Met  Val Gly Ile Lys Phe  Arg Arg Glu Lys Leu  Leu Asp Thr
    2030                 2035                 2040

Met Asn  Arg Leu Asp Asp Lys  Tyr Arg Glu Leu Arg  Ser Gln Leu
    2045                 2050                 2055

Ser Asn  Lys Ser Leu Ala Pro  Glu Val His Gln Gln  Ile Ser Lys
    2060                 2065                 2070

Gln Leu  Ala Asp Arg Glu Arg  Glu Leu Leu Pro Ile  Tyr Gly Gln
    2075                 2080                 2085

Ile Ser  Leu Gln Phe Ala Asp  Leu His Asp Arg Ser  Ser Arg Met
    2090                 2095                 2100

Val Ala  Lys Gly Val Ile Ser  Lys Glu Leu Glu Trp  Thr Glu Ala
    2105                 2110                 2115

Arg Arg  Phe Phe Phe Trp Arg  Leu Arg Arg Arg Leu  Asn Glu Glu
    2120                 2125                 2130

Tyr Leu  Ile Lys Arg Leu Ser  His Gln Val Gly Glu  Ala Ser Arg
    2135                 2140                 2145

Leu Glu  Lys Ile Ala Arg Ile  Arg Ser Trp Tyr Pro  Ala Ser Val
    2150                 2155                 2160

Asp His  Glu Asp Asp Arg Gln  Val Ala Thr Trp Ile  Glu Glu Asn
    2165                 2170                 2175

Tyr Lys  Thr Leu Asp Asp Lys  Leu Lys Gly Leu Lys  Leu Glu Ser
    2180                 2185                 2190

Phe Ala  Gln Asp Leu Ala Lys  Lys Ile Arg Ser Asp  His Asp Asn
    2195                 2200                 2205

Ala Ile  Asp Gly Leu Ser Glu  Val Ile Lys Met Leu  Ser Thr Asp
    2210                 2215                 2220

Asp Lys  Glu Lys Leu Leu Lys  Thr Leu Lys
    2225                 2230
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<210> 17
<211> 1428

<212> DNA
<213> Artificial sequence

<220>
<223> optimized gapN sequence

<400> 17

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aagatatacg aacctgcctc aggcgcagaa ctgggttctg ttcctgcaat gtccactgag 120

gaagtggatt acgtgtatgc ttcagccaaa aaggctcagc ctgcatggag atccctaagt 180

tacattgaaa gagctgccta tttgcataaa gtcgcagaca tattgatgag ggataaagag 240

aagattggcg ctgtgctttc taaggaagtc gctaagggat acaaatctgc agtatctgag 300

gtagttagaa cagcagagat tatcaattac gctgccgagg aaggtcttag aatggaggga 360

gaggtacttg aaggaggatc atttgaagca gcatccaaaa agaagatcgc tgtagtaagg 420

agagaaccag taggccttgt tctagccatc agtcctttca actatccagt caacttagct 480

ggctccaaaa tcgctcctgc cttaatcgct ggtaatgtca ttgctttcaa gccacctact 540

caagggtcta tttcaggttt gttgttggcc gaggcttttg ctgaagcagg tctgccagct 600

ggtgttttca atacaattac aggtagagga tctgaaattg gagactacat tgtcgaacat 660

caagctgtca actttatcaa tttcacaggt tcaacaggaa ttggcgagag aatagggaaa 720

atggcaggta tgcgtccaat catgttagaa ctaggcggga aagactctgc aatcgtgttg 780

gaagatgctg atttggaact taccgccaaa aacatcattg ccggtgcatt cggttattct 840

ggacaaagat gtactgcagt taaacgtgtt ttagtaatgg aatcagtggc agatgaactt 900

gtggaaaaga tcagggaaaa agtccttgca ctgactatcg gtaatccaga agatgatgct 960

gacatcaccc cacttattga cactaagtca gctgattatg ttgaaggttt gatcaatgat 1020

gcaaatgata agggtgccgc tgccttaaca gagatcaaaa gagaaggtaa cttaatctgc 1080

ccaatcctgt ttgataaggt tactactgat atgagattgg cttgggagga accatttggt 1140

cctgttttgc ctatcataag agttacctct gttgaggaag ctatagagat atctaacaaa 1200

tcagaatacg gcttacaagc ctctatcttt actaatgatt tcccaagagc atttggaata 1260

gctgaacaac tagaagtagg tacagttcac attaacaaca aaacccagag aggcacagac 1320

aatttcccat ttctaggggc caaaaagtca ggggctggaa ttcaaggcgt gaaatactcc 1380

attgaagcta tgactacagt gaaaagtgtt gtctttgaca taaagtga 1428

<210> 18
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 1
aaacaagcgg ccgcactagt ttgacaaaac 30

<210> 19
<211> 34
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 19
ttaattaaga gctcagatct ttatttgata tcaa 34

<210> 20
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 20
cgggatcccg ctcgagatga agagattagg 30

<210> 21
<211> 34
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 21
ggggtacccc aagcttgggt tactttctag tacg 34

<210> 22
<211> 53
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 22
gttgggattc cattgttgat aaaggcgcac acaccatagc ttcaaaatgt ttc 53

<210> 23
<211> 32
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 23
gtgcaatgta gatcttcgag cgtcccaaaa cc 32

<210> 24
<211> 52
<212> DNA
<213> Artificial sequence

<220>
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<400> 24
gacgctcgaa gatctacatt gcacaagata aaaatatatc atcatgaaca at 52

<210> 25
<211> 43
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 25
gcctttatca acaatggaat cccaaccgcc gtcccgtcaa gtc 43

<210> 26
<211> 48
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 26
acaacaaata taaaacaagc ggccgcacta tgaagagatt aggtactc 48

<210> 27
<211> 46
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 27
ggcgaagaat tgttaattaa gagctcggta ccccaagctt gggtta 46

<210> 28
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gtatctcagt tcggtgtagg tcgttcgctc caagctgggc tgtgtgcacg aacccccccgt 5880

tcagcccgac cgctgcgcct tatccggtaa ctatcgtctt gagtccaacc cggtaagaca 5940

cgacttatcg ccactggcag cagccactgg taacaggatt agcagagcga ggtatgtagg 6000

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tgaaagtgcg tcttcagagc gcttttggtt ttcaaaagcg ctctgaagtt cctatacttt 8340

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gatcatacta agaaaccatt attatcatga cattaaccta taaaaatagg cgtatcacga 8760

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PATENTKRAV

1. *Saccharomyces cerevisiae*-celle til overproduktion af fedtsyrer, hvilken *S. cerevisiae*-celle omfatter et acetyl-CoA-carboxylasegen, **kendetegnet ved, at**

acetyl-CoA-carboxylasegenet er et muteret acetyl-CoA-carboxylasegen, der koder for en muteret acetyl-CoA-carboxylase, hvor serin i position 659 i SEQ ID NO: 16 er blevet erstattet med alanin, og serin i position 1157 i SEQ ID NO: 16 er blevet erstattet med alanin; og

acetyl-CoA-carboxylasegenet er under kontrol af en konstitutivt udtrykt promoter.

2. *S. cerevisiae*-celle ifølge krav 1, **kendetegnet ved** en ekspressionsvektor, der omfatter det muterede acetyl-CoA-carboxylasegen.

3. *S. cerevisiae*-celle ifølge et hvilket som helst af kravene 1 til 2, **kendetegnet ved:**

overekspression af ACB1 (ACBP, acyl-CoA-bindende protein);

overekspression af FAS1 (fedtsyre-synthase);

overekspression af FAS2 (fedtsyre-synthase);

heterolog ekspression af gapN (NADP+afhængig glyceraldehyd-3-phosphatdehydrogenase); og/eller

overekspression af ACS1 (acetyl-CoA-synthetase).

4. *S. cerevisiae* ifølge et hvilket som helst af kravene 1 til 3, **kendetegnet ved** en eller flere deletioner af gener udvalgt fra gruppen bestående af:

DGA1 (acyl-CoA:diacylglycerolacyltransferase);

LRO1 (lecithin:cholesterolacyltransferase);

ARE1 (acyl-CoA:sterolacyltransferase);

ARE2 (acyl-CoA:sterolacyltransferase); og

POX1 (peroxisomal acyl-CoA-oxidase).

5. *S. cerevisiae*-celle ifølge et hvilket som helst af kravene 1 til 4, **kendetegnet ved et** gen, der koder for en voksestersynthase.

6. *S. cerevisiae*-celle ifølge krav 5, **kendetegnet ved, at** voksestersynthasen er udvalgt fra gruppen bestående af *Acinetobacter baylyi* ADP1, *Marinobacter hydrocarbonoclasticus* DSM 8798, *Rhodococcus opacus* PD630, *Mus musculus* C57BU6 og *Psychrobacter articus* 273-4.

7. *S. cerevisiae*-celle ifølge et hvilket som helst af kravene 1 til 6, **kendetegnet ved, at** *S. cerevisiae*-cellen anvender tilførte carbohydrater som eksternt substrat, hvor carbohydraterne er udvalgt fra gruppen bestående af glucose, fructose, galactose, xylose, arabinose, sucrose, maltose, stivelse, cellulose og hemicellulose.

DRAWINGS

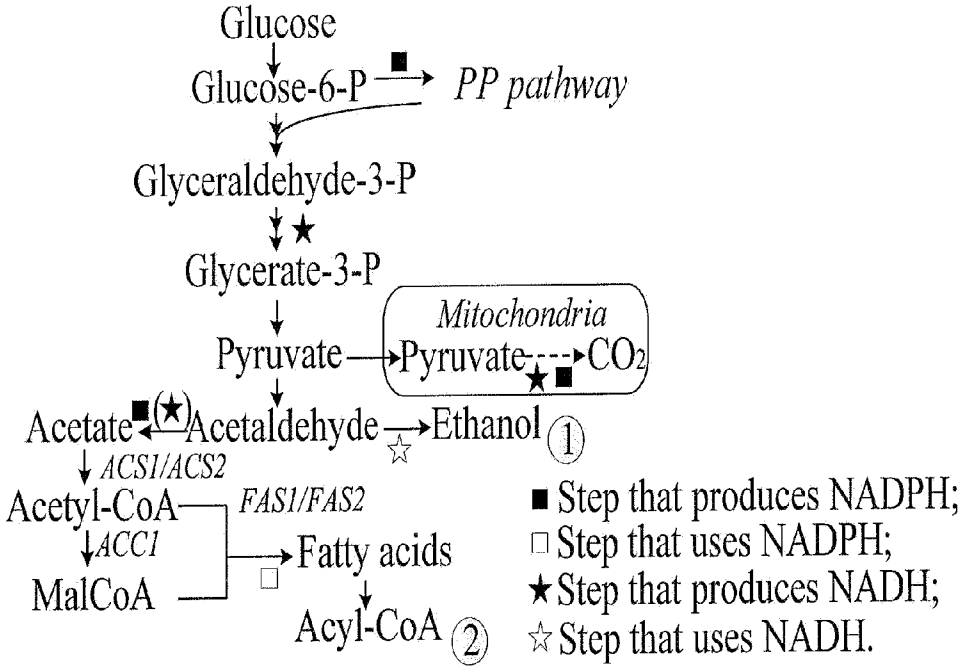


Figure 1

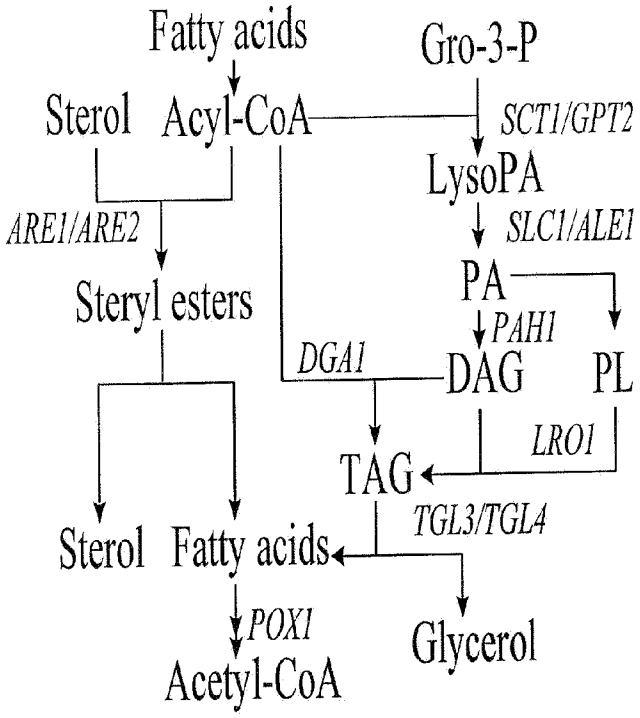

Fatty acids
Gro-3-P
Sterol
Acyl-CoA
SCT1/GPT2
LysoPA
ARE1/ARE2
SLC1/ALE1
PA
Steryl esters
PAH1
DGA1
DAG
PL
LRO1
TAG
TGL3/TGL4
Sterol
Fatty acids
POX1
Glycerol
Acetyl-CoA

Figure 2

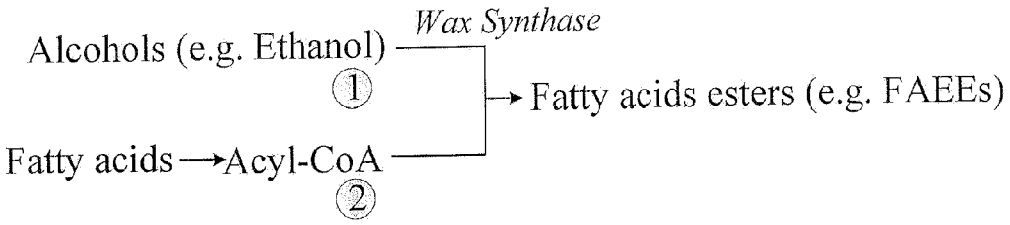
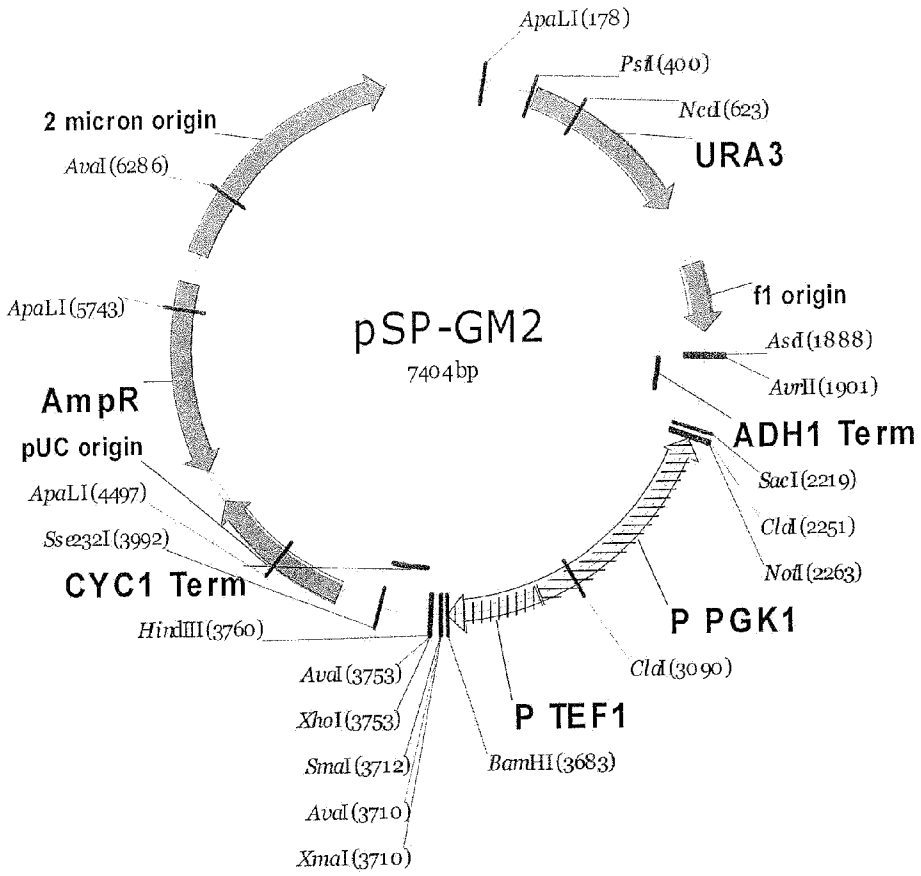

Wax Synthase
Alcohols (e.g. Ethanol)
①
Fatty acids esters (e.g. FAEEs)
Fatty acids → Acyl-CoA
②

Figure 3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PstI(400)
NcoI(623)
URA3
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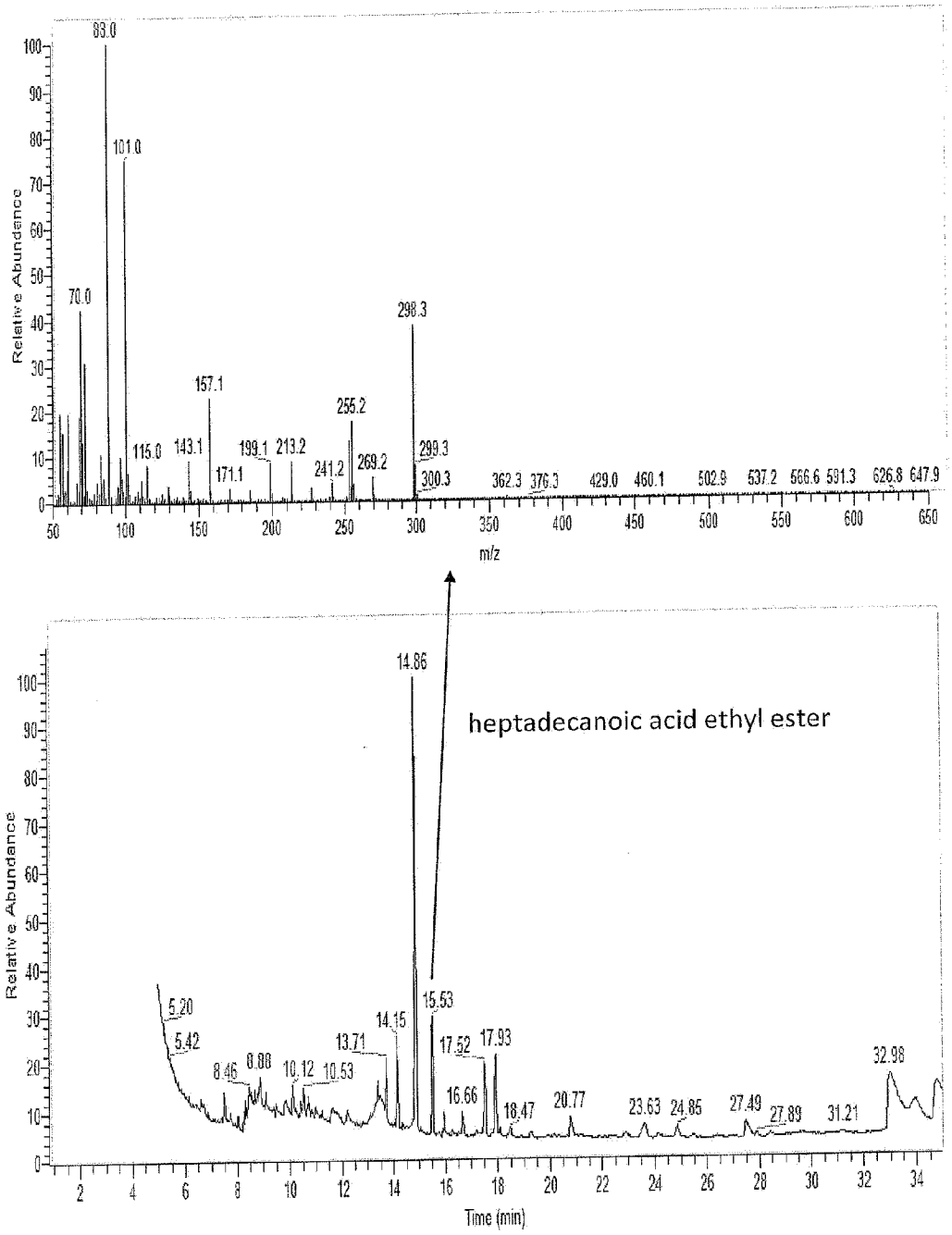
AsiI(1888)
AvrII(1901)
ADH1 Term
SacI(2219)
ClaI(2251)
NotI(2263)
P PGK1
ClaI(3090)
P TEF1
BamHI(3683)
AvaI(3753)
XhoI(3753)
SmaI(3712)
AvaI(3710)
XmaI(3710)
HindIII(3760)
CYC1 Term
Sse232I(3992)
ApaLI(4497)
pUC origin
AmpR
ApaLI(5743)
2 micron origin
AvaI(6286)
pSP-GM2
7404bp

Figure 4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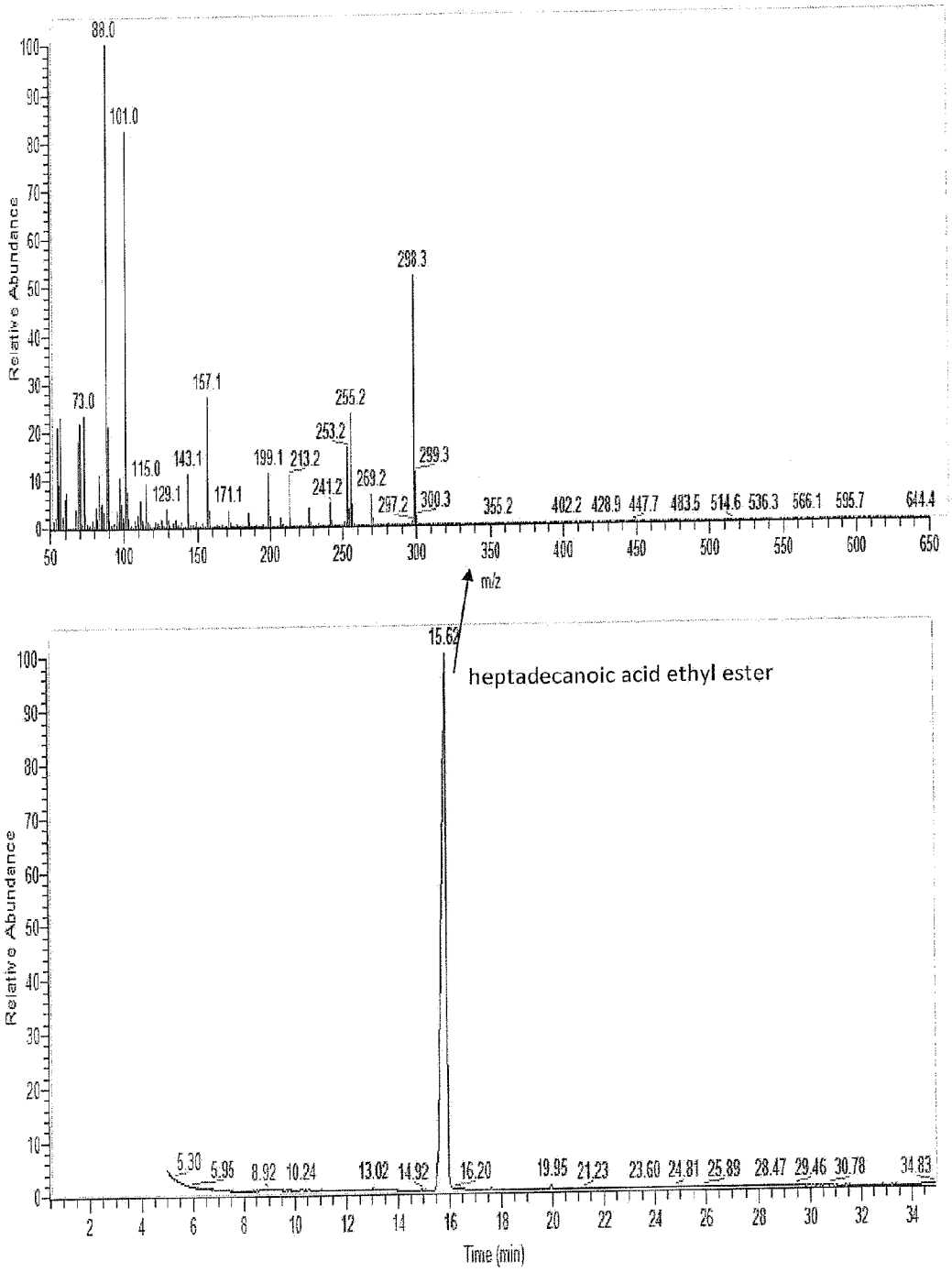
101.0
70.0
298.3
157.1
255.2
115.0
143.1
199.1
213.2
241.2
269.2
299.3
300.3
171.1
362.3
376.3
429.0
460.1
502.9
537.2
566.6
591.3
626.8
647.9
Relative Abundance
m/z
14.86
heptadecanoic acid ethyl ester
5.20
5.42
8.46
8.88
10.12
10.53
13.71
14.15
15.53
16.66
17.52
17.93
18.47
20.77
23.63
24.85
27.49
27.89
31.21
32.98
Relative Abundance
Time (min)

Figure 5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101.0
298.3
157.1
73.0
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253.2
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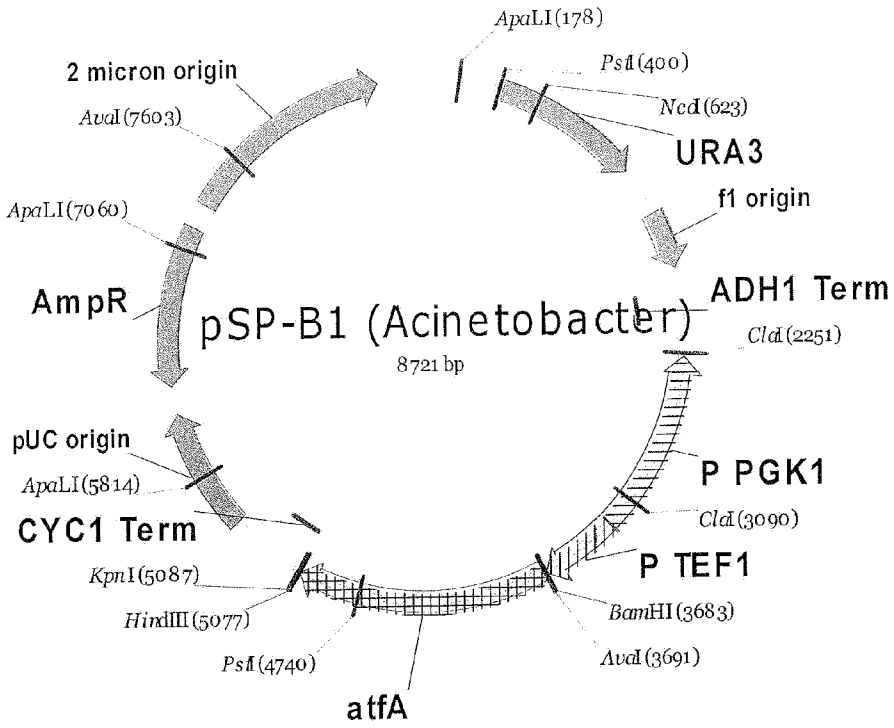
115.0
143.1
199.1
213.2
269.2
129.1
171.1
241.2
297.2
300.3
355.2
402.2
428.9
447.7
483.5
514.6
536.3
566.1
595.7
644.4
Relative Abundance
m/z
15.62
heptadecanoic acid ethyl ester
5.30
5.95
8.92
10.24
13.02
14.92
16.20
19.95
21.23
23.60
24.81
25.89
28.47
29.46
30.78
34.83
Relative Abundance
Time (min)

Figure 6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P PGK1
ClaI(3090)
P TEF1
BamHI(3683)
AvaI(3691)
atfA
PstI(4740)
HindIII(5077)
KpnI(5087)
CYC1 Term
ApaLI(5814)
pUC origin
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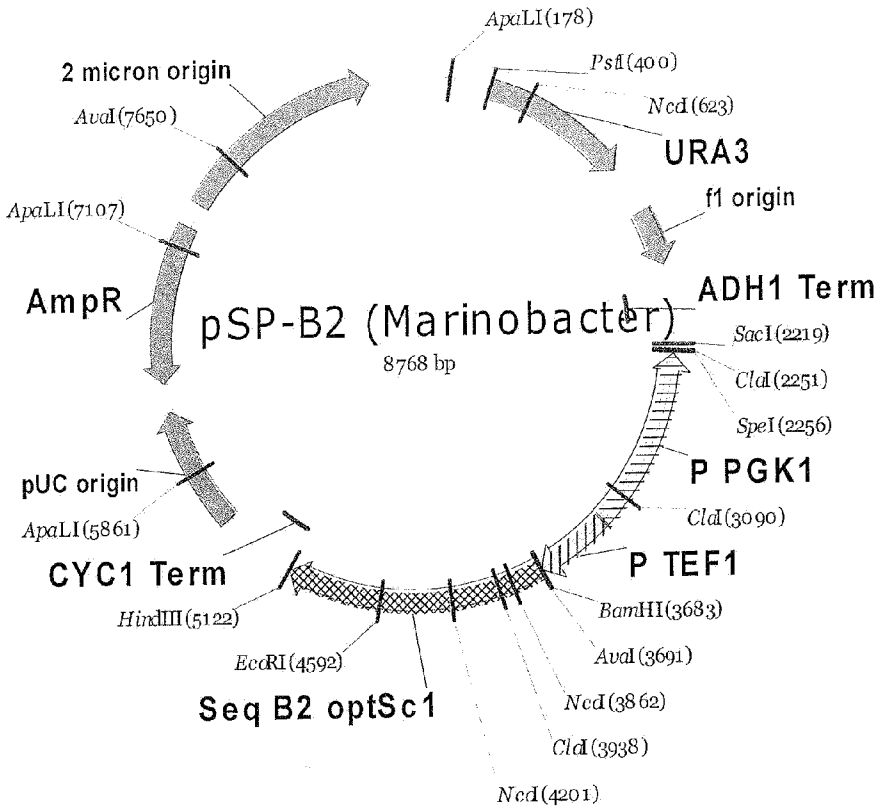
ApaLI(7060)
2 micron origin
AvaI(7603)
pSP-B1 (Acinetobacter)
8721 bp

Figure 7A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f1 origin
ADH1 Term
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SpeI(2256)
P PGK1
ClaI(3090)
P TEF1
BamHI(3683)
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NcoI(3862)
ClaI(3938)
NcoI(4201)
Seq B2 optSc1
EcoRI(4592)
HindIII(5122)
CYC1 Term
ApaLI(5861)
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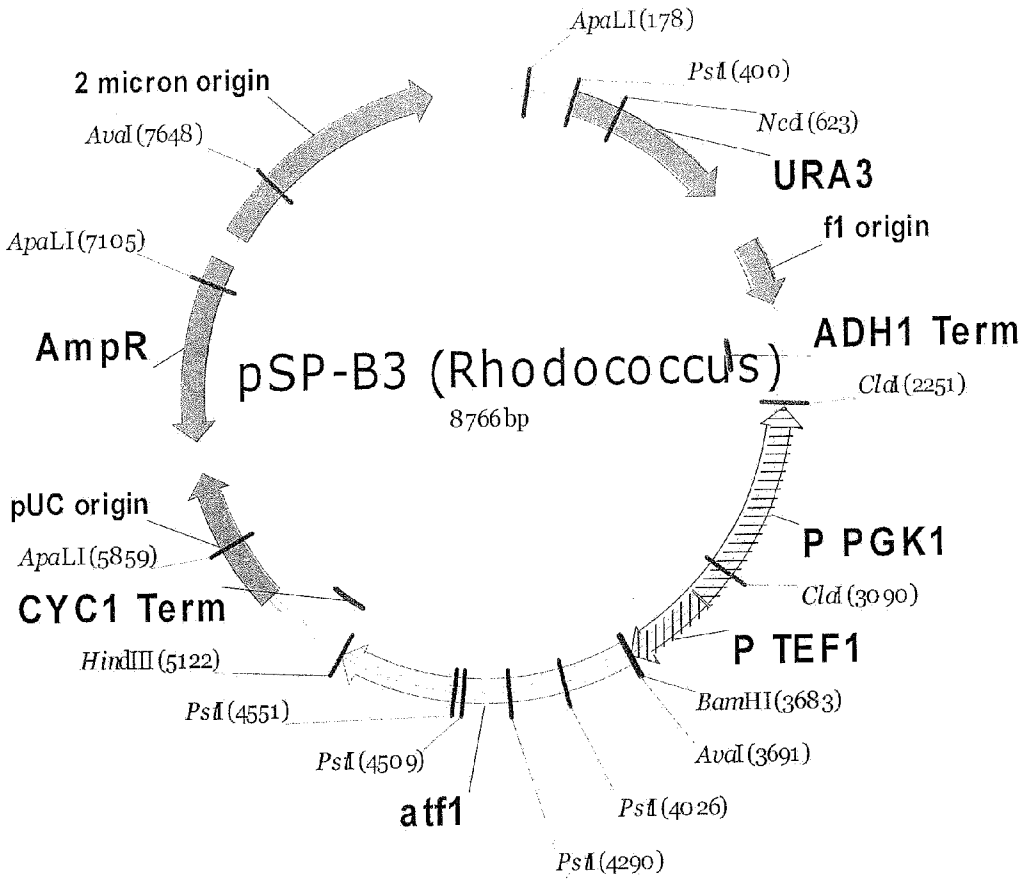
2 micron origin
pSP-B2 (Marinobacter)
8768 bp

Figure 7B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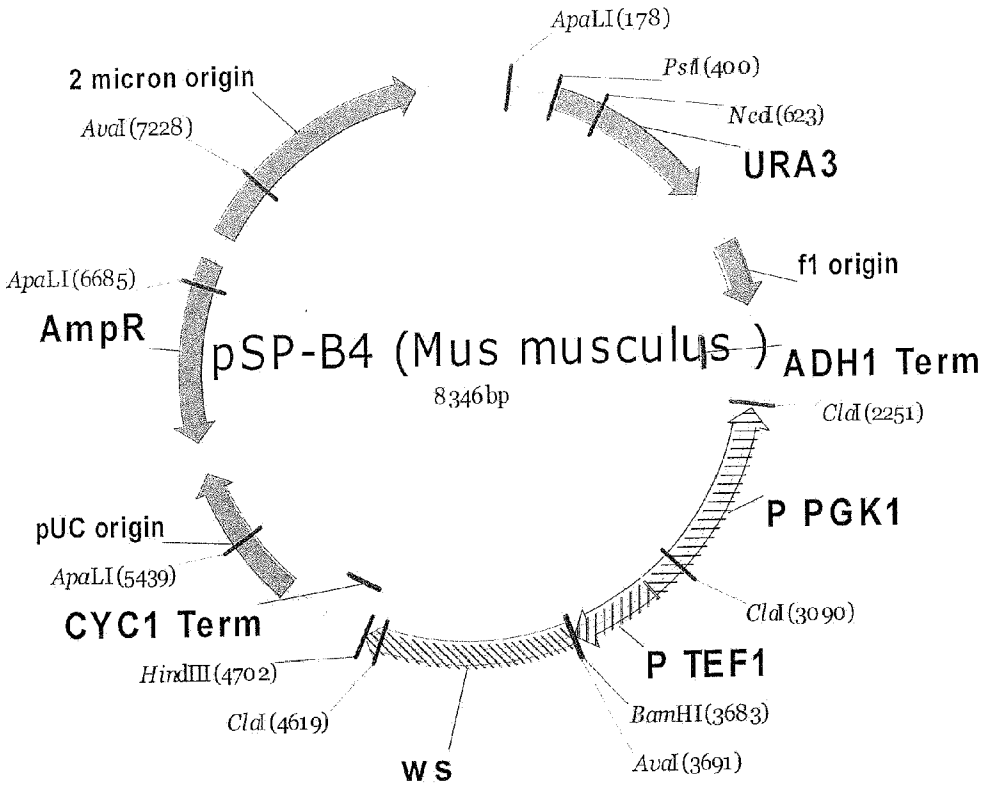
PstI(400)
NcoI(623)
URA3
f1 origin
ADH1 Term
ClaI(2251)
P PGK1
ClaI(3090)
P TEF1
BamHI(3683)
AvaI(3691)
PstI(4026)
PstI(4290)
atf1
PstI(4509)
PstI(4551)
HindIII(5122)
CYC1 Term
ApaLI(5859)
pUC origin
AmpR
ApaLI(7105)
AvaI(7648)
2 micron origin
pSP-B3 (Rhodococcus)
8766bp

Figure 7C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NcoI(623)
URA3
2 micron origin
AvaI(7228)
f1 origin
ApaLI(6685)
AmpR
pSP-B4 (Mus musculus)
8346bp
ADH1 Term
ClaI(2251)
P PGK1
pUC origin
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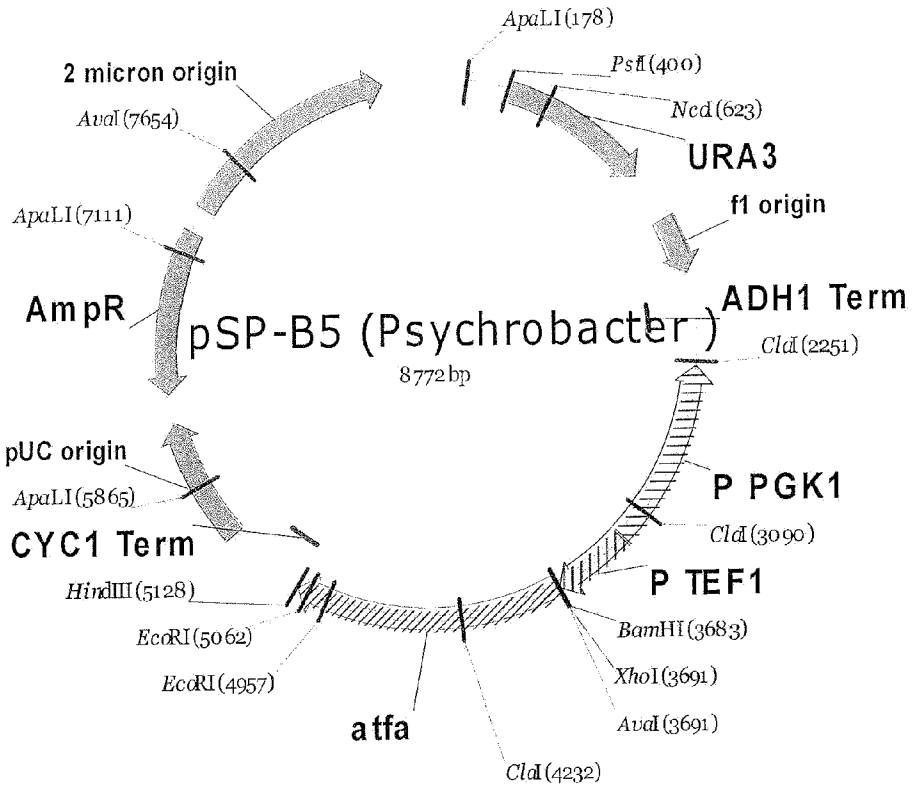
BamHI(3683)
AvaI(3691)
WS

Figure 7D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NcoI(623)
URA3
f1 origin
ADH1 Term
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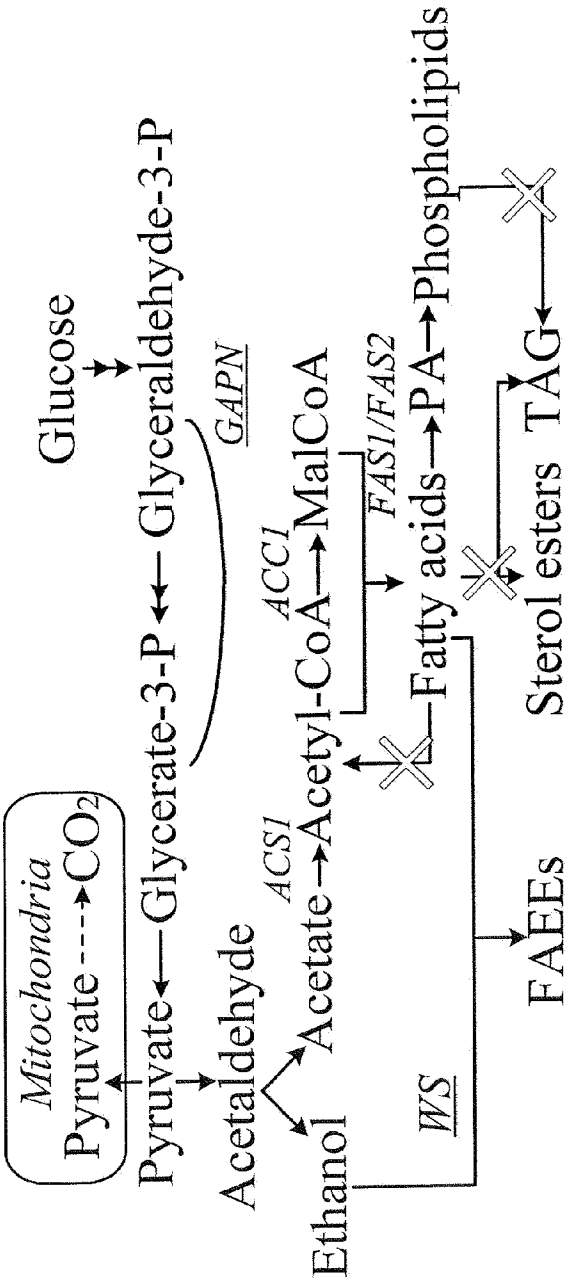
P PGK1
ClaI(3090)
P TEF1
BamHI(3683)
XhoI(3691)
AvaI(3691)
ClaI(4232)
atfa
EcoRI(4957)
EcoRI(5062)
HindIII(5128)
CYC1 Term
ApaLI(5865)
pUC origin
AmpR
ApaLI(7111)
AvaI(7654)
2 micron origin
pSP-B5 (Psychrobacter)
8772 bp

Figure 7E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GAPN
Glycerate-3-P
Pyruvate
Mitochondria
Pyruvate
CO2
Acetaldehyde
Ethanol
ACS1
Acetate
Acetyl-CoA
ACC1
MalCoA
FAS1/FAS2
Fatty acids
PA
Phospholipids
WS
FAEEs
Sterol esters
TAG

Figure 8

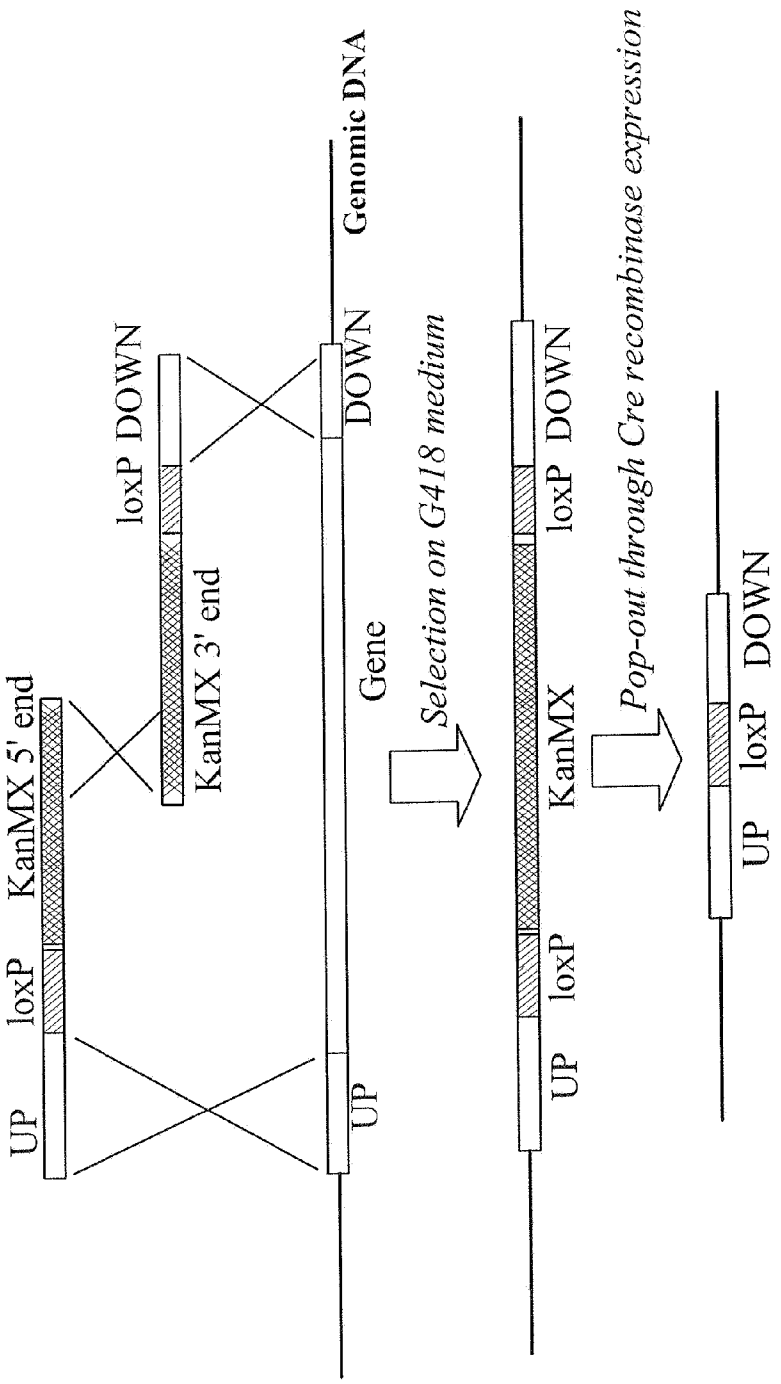
UP
loxP
KanMX 5' end
loxP DOWN
KanMX 3' end
Genomic DNA
DOWN
Gene
UP
Selection on G418 medium
UP
loxP
KanMX
loxP DOWN
Pop-out through Cre recombinase expression
UP
loxP
DOWN

Figure 9

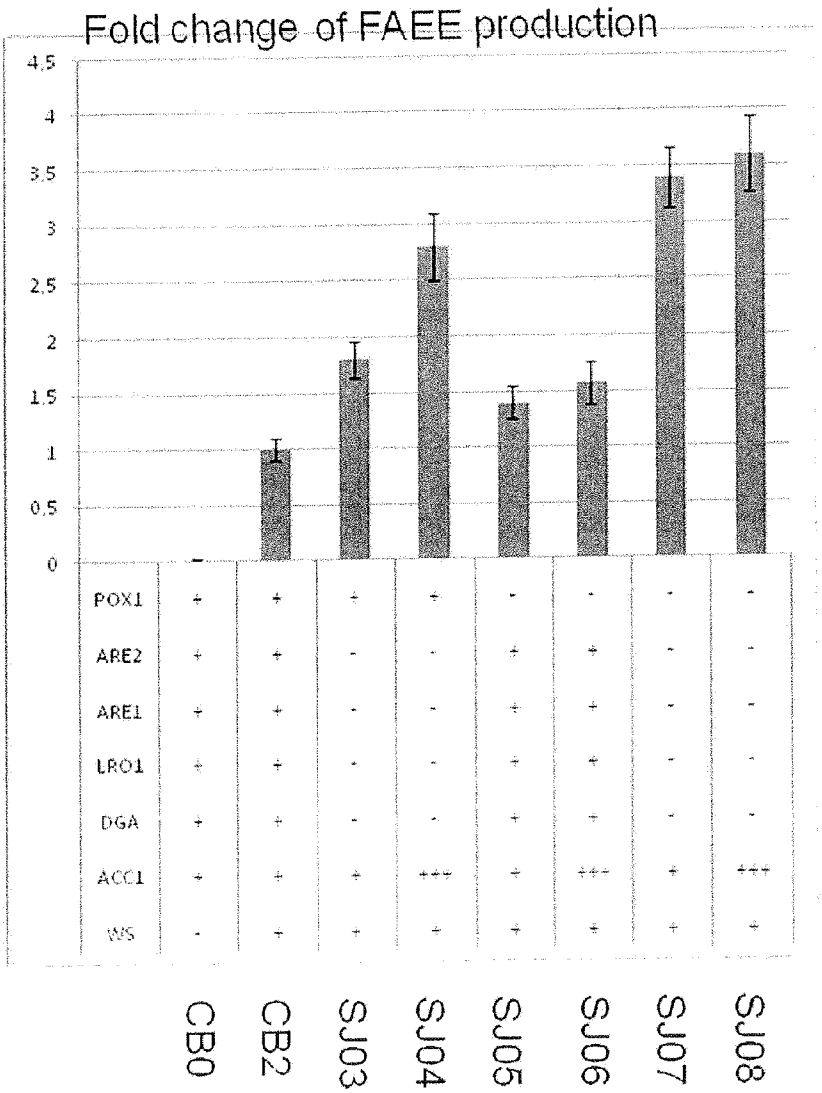
Fold change of FAEE production
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
POX1
ARE2
ARE1
LRO1
DGA
ACC1
WS
CB0
CB2
SJ03
SJ04
SJ05
SJ06
SJ07
SJ08

Figure 10

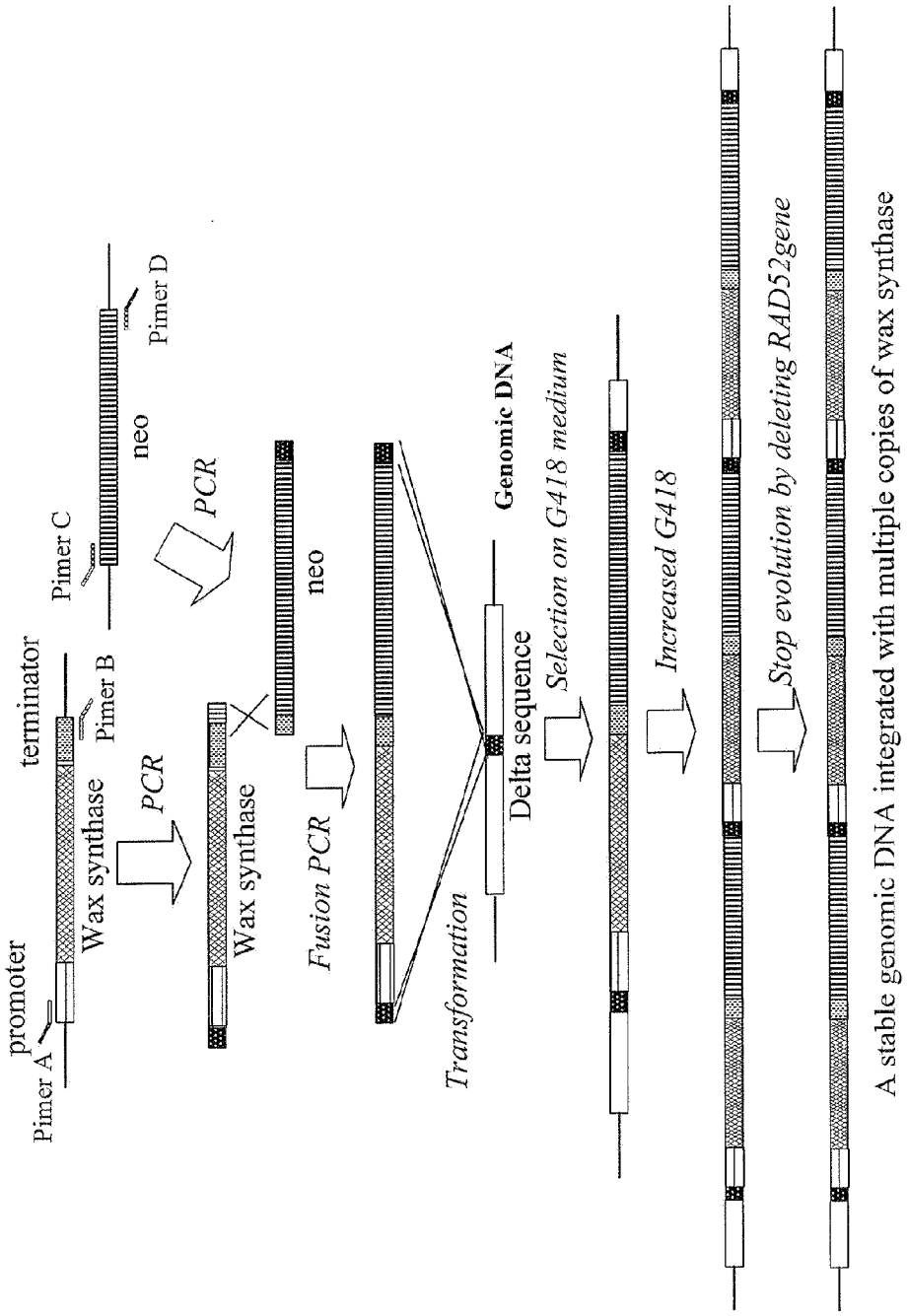
promoter
terminator
Pimer A
Wax synthase
Pimer B
PCR
Wax synthase
neo
Pimer C
neo
Pimer D
PCR
neo
Fusion PCR
Transformation
Genomic DNA
Delta sequence
Selection on G418 medium
Increased G418
Stop evolution by deleting RAD52gene
A stable genomic DNA integrated with multiple copies of wax synthase

Figure 11

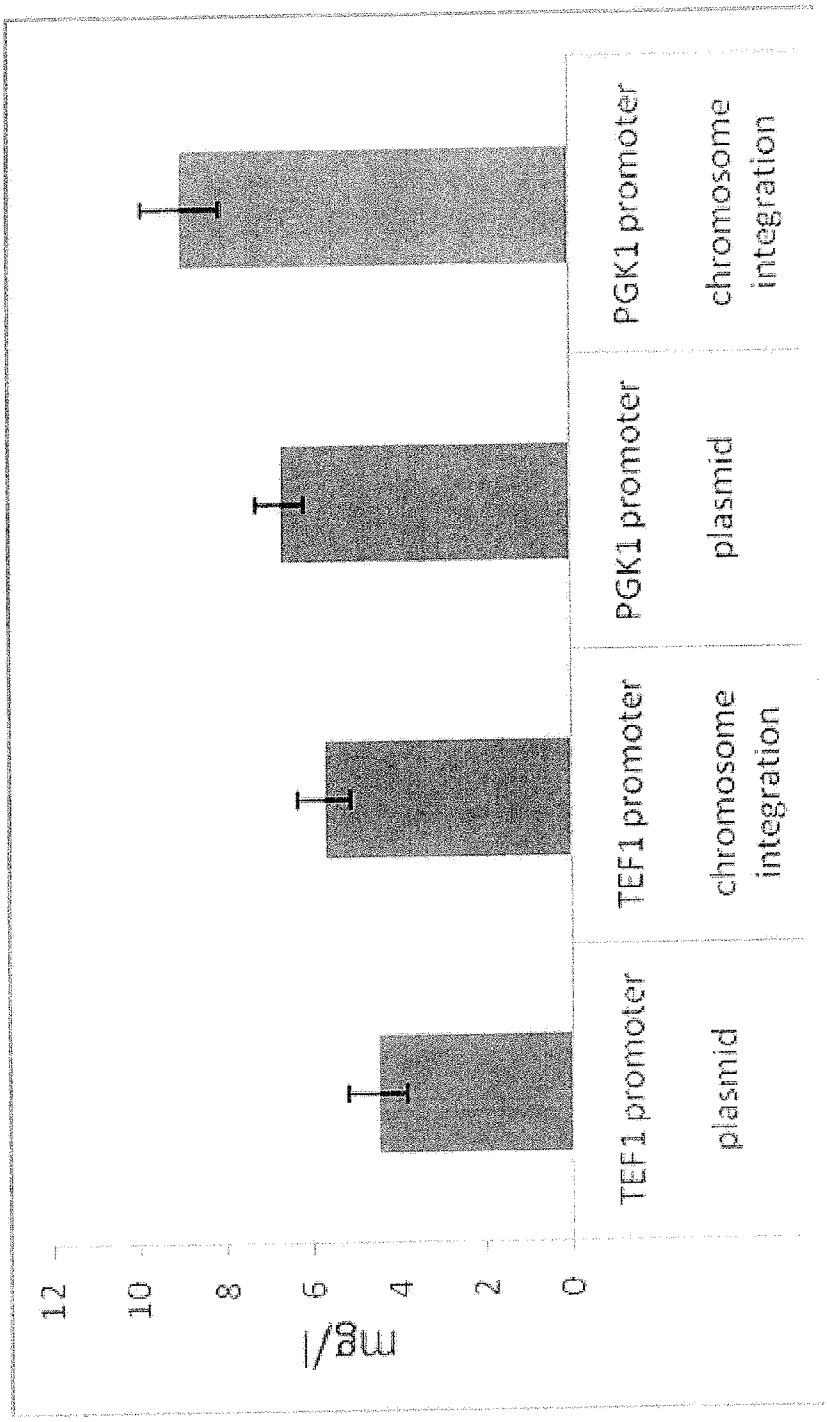
mg/l
0
2
4
6
8
10
12
TEF1 promoter
plasmid
TEF1 promoter
chromosome integration
PGK1 promoter
plasmid
PGK1 promoter
chromosome integration

Figure 12

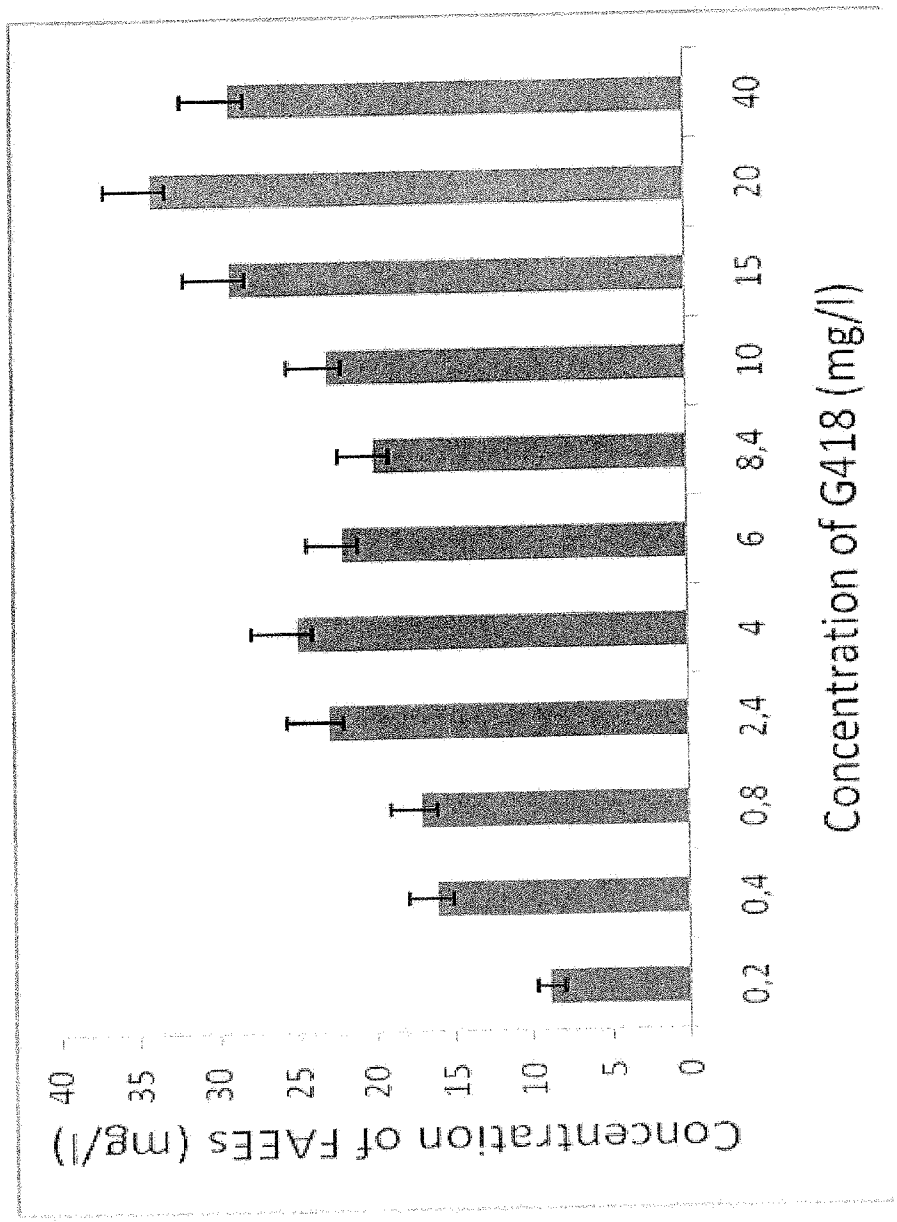
Concentration of FAEEs (mg/l)
0
5
10
15
20
25
30
35
40
Concentration of G418 (mg/l)
0,2
0,4
0,8
2,4
4
6
8,4
10
15
20
40

Figure 13