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(54) **Title:** ENGINEERED MICROBES AND METHODS FOR MICROBIAL OIL PRODUCTION

(57) **Abstract:** Some aspects of this invention provide engineered microbes for oil production. Methods for microbe engineering and for use of engineered microbes are also provided herein. In some embodiments, microbes are provided that are engineered to modulate a combination of rate-controlling steps of lipid synthesis, for example, a combination of a step generating metabolites, acetyl-CoA, ATP or NADPH for lipid synthesis (a push step), and a step sequestering a product or an intermediate of a lipid synthesis pathway that mediates feedback inhibition of lipid synthesis (a pull step). Such push-and-pull engineered microbes exhibit greatly enhanced conversion yields and TAG synthesis and storage properties.



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ENGINEERED MICROBES AND METHODS FOR MICROBIAL OIL PRODUCTION

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119 to U.S. provisional patent
5 application U.S.S.N. 61/548,901, filed October 19, 2011; and U.S. provisional patent
application U.S.S.N. 61/663,263, filed June 22, 2012, both entitled “*Engineered Microbes
and Methods for Microbial Oil Production*,” the entire contents of each of which are
incorporated herein by reference.

GOVERNMENT SUPPORT

This invention was made with government support under Grant No. DE-AR0000059
awarded by the US Department of Energy. The government has certain rights in this
invention.

BACKGROUND

Sustainably produced biofuels are an alternative to fossil fuels and may help to
alleviate the depletion of easily accessible fossil fuel stocks while avoiding fossil fuel-
associated pollution and greenhouse gas emission, thus satisfying a rising demand for
affordable energy in a sustainable way. The development of methods and oil-producing
20 organisms suitable for the efficient conversion of carbon sources to lipids is prerequisite for
widespread implementation of microbial biofuel production.

SUMMARY OF CERTAIN ASPECTS OF THE INVENTION

Microbial oil production by heterotrophic organisms is a most promising path for the
25 cost-effective production of biofuels from renewable resources provided high conversion
yields can be achieved. The key to cost-effective microbial oil production from renewable
feedstocks is a high carbohydrate to oil conversion yield. Metabolic engineering has emerged
as the enabling technology applied to this end and numerous examples exist of successful
pathway engineering that markedly improved the performance of microbial biocatalysts in
30 the synthesis of chemical, pharmaceutical and fuel products.

Prior efforts at engineering microbes for oil production have focused on amplifying
presumed rate-controlling steps in the fatty acid synthesis pathway. One significant drawback

of such amplifications is that increasing carbon flux into fatty acid synthesis pathways increases the level of saturated fatty acids in the cell, which activate a potent negative feedback loop of fatty acid biosynthesis.

Some aspects of this disclosure provide a strategy for microbe engineering that combines the amplification of upstream, metabolite-forming pathways, also referred to herein as “push” metabolic pathways, with a similar increase in the flux of downstream, product-sequestering pathways, also referred to herein as “pull” pathways. Some aspects of this invention provide that a balanced combination of push-and-pull modifications in a microbe results in large carbon flux amplifications into lipid synthesis pathways without significant departures of the concentrations of intermediate metabolites from their homeostatic physiological levels, thus avoiding feedback inhibition of lipid synthesis.

Some aspects of this disclosure relate to the recognition that the effects of single-branch modifications, e.g., push-only modifications or pull-only modifications, on conversion efficiency are typically limited because of compensatory regulation of synthesis yield in the cell, and that a concerted modulation of push and pull steps of lipid biosynthesis in microbial cells results in a surprising synergistic effect on lipid production.

For example, some aspects of this disclosure provide genetically modified oleaginous microbes comprising a combination of modifications in their lipid biosynthetic pathways, also referred to herein as push-pull modifications. In some embodiments, a microbe provided herein comprises a modification effecting an increased production of metabolites or intermediates required for lipid synthesis and a modification resulting in sequestration of a product of lipid synthesis, for example, of triacylglycerol into a storage form lipid within the cell, thus alleviating feedback inhibition of lipid synthesis by some of its products, e.g., by fatty acids or diacylglycerols. In some embodiments, the combination of modifications of the metabolic push and metabolic pull pathways results in a synergistically increased lipid production. In some embodiments, the push modification results in an increased level of lipid-synthesis building blocks, or metabolites for lipid synthesis, in the cell. In some embodiments, the pull modification alleviates feedback inhibition on lipid synthesis.

Some aspects of this invention provide microbes that comprise genetic modifications simultaneously affecting a push- and a pull pathway of lipid biosynthesis. For example, push and pull modifications were introduced into a model microbe for oil production, the oleaginous yeast *Yarrowia lipolytica*. The overexpression of diacylglycerol acyltransferase

(*DGA1*), the final step of the triglyceride (TAG) synthesis pathway, was investigated as an exemplary pull modification. DGA1 overexpression resulted in a 4-fold increase in lipid production over control microbes, to a lipid content of 33.8% of dry cell weight (DCW). The overexpression of acetyl-CoA carboxylase (*ACC1*), the first committed step of fatty acid synthesis, was investigated as an exemplary push modification. ACC1 overexpression increased lipid content 2-fold over control, to a lipid content of 17.9%. Simultaneous coexpression of *ACC1* and *DGA1* from a tandem gene expression construct was investigated as an exemplary push-pull modification. Simultaneous ACC1 and DGA1 overexpression further increased lipid content to 41.4%, demonstrating synergistic effects of *ACC1* + *DGA1* coexpression.

The lipid production characteristics of the *ACC1* + *DGA1* transformant were explored in a 2-L bioreactor fermentation, achieving 61.7% lipid content after 120 hr. The overall yield and productivity were 0.195 g/g and 0.143 g/L/hr, respectively, while the maximum yield and productivity were 0.270 g/g and 0.253 g/L/hr during the lipid accumulation phase of the fermentation. This work demonstrates the excellent capacity for lipid production by the oleaginous yeast *Y. lipolytica* and the effects of metabolic engineering of two important steps of the lipid synthesis pathway, which acts to divert flux towards the lipid synthesis and creates driving force for TAG synthesis.

Some aspects of this invention provide a novel overexpression platform for use in oil-producing microbes, for example, in oleaginous yeast. Some aspects of this invention provide expression constructs comprising a promoter driving transcription of a transcript that comprises a coding sequence and an intron, for example, a translation elongation factor-1 α (TEF) promoter positioned upstream of a nucleic acid sequence comprising an intron and a coding sequence. Some aspects of this disclosure provide that such intron-containing expression constructs are capable of increasing expression at least 17-fold over the intronless TEF promoter.

Some aspects of this disclosure provide an isolated oleaginous cell comprising a genetic modification that increases expression of a DGA1 gene product. In some embodiments, the isolated oleaginous cell further comprises a genetic modification that increases expression of an ACC1 gene product. In some embodiments, the isolated oleaginous cell further comprises a genetic modification that increases expression of an SCD gene product. In some embodiments, the isolated oleaginous cell further comprises a genetic

modification that increases expression of an ACL gene product. In some embodiments, the genetic modification comprises a nucleic acid construct that increases the expression of the gene product, the nucleic acid construct comprising (a) an expression cassette comprising a nucleic acid sequence encoding the gene product under the control of a suitable homologous or heterologous promoter, and/or (b) a nucleic acid sequence that modulates the level of expression of the gene product when inserted into the genome of the cell. In some embodiments, the promoter is an inducible or a constitutive promoter. In some embodiments, the promoter is a TEF promoter. In some embodiments, the expression construct further comprises an intron. In some embodiments, the intron is downstream of the transcription initiation site. In some embodiments, the intron is within the nucleic acid sequence encoding the gene product. In some embodiments, the nucleic acid construct inhibits or disrupts the natural regulation of a native gene encoding the gene product resulting in overexpression of the native gene. In some embodiments, inhibition or disruption of the natural regulation of the native gene is mediated by deletion, disruption, mutation and/or substitution of a regulatory region, or a part of a regulatory region regulating expression of the gene. In some embodiments, the gene product is a transcript. In some embodiments, the gene product is a protein. In some embodiments, the nucleic acid construct is inserted into the genome of the cell. In some embodiments, the increased expression of the gene product confers a beneficial phenotype for the conversion of a carbon source to a lipid, e.g., a fatty acid, fatty acid derivative and/or triacylglycerol (TAG) to the cell. In some embodiments, the beneficial phenotype comprises a modified fatty acid profile, a modified TAG profile, an increased fatty acid and/or triacylglycerol synthesis rate, an increase conversion yield, an increased triacylglycerol accumulation in the cell, and/or an increased triacylglycerol accumulation in a lipid body of the cell. In some embodiments, the synthesis rate of a fatty acid or a TAG of the cell is at least 2-fold increased as compared to unmodified cells of the same cell type. In some embodiments, the synthesis rate of a fatty acid or a TAG of the cell is at least 5-fold increased as compared to unmodified cells of the same cell type. In some embodiments, the synthesis rate of a fatty acid or a TAG of the cell is at least 10-fold increased as compared to unmodified cells of the same cell type. In some embodiments, the cell converts a carbon source to a fatty acid or a TAG at a conversion rate within the range of about 0.025g/g to about 0.32g/g (e.g., g lipid produced/ g carbon source consumed). In some embodiments, the cell converts a carbon source to a fatty acid or a TAG at a conversion rate of at least about

0.11g/g. In some embodiments, the cell converts a carbon source to a fatty acid or a TAG at a conversion rate of at least about 0.195g/g. In some embodiments, the cell converts a carbon source to a fatty acid or a TAG at a conversion rate of at least about 0.24g/g. In some
5 embodiments, the cell converts a carbon source to a fatty acid or a TAG at a conversion rate
of at least about 0.27g/g. In some embodiments, the cell comprises a lipid body or vacuole.
In some embodiments, the cell is a bacterial cell, an algal cell, a fungal cell, or a yeast cell.
In some embodiments, the cell is an oleaginous yeast cell. In some embodiments, the cell is a
Y. lipolytica cell.

Some aspects of this disclosure provide a culture comprising an oleaginous cell, e.g.,
10 an oleaginous cell as described herein. In some embodiments, the culture further comprises a
carbon source. In some embodiments, the carbon source comprises a fermentable sugar. In
some embodiments, the fermentable sugar is a C6 sugar. In some embodiments, the carbon
source comprises glucose. In some embodiments, the carbon source comprises an organic
acid. In some embodiments, the organic acid is acetic acid. In some embodiments, the acetic
15 acid is at a concentration of at least 5% vol/vol, at least 10% vol/vol, at least 15% vol/vol, at
least 20% vol/vol, at least 25% vol/vol, or at least 30% vol/vol. In some embodiments, the
carbon source comprises acetate. In some embodiments, the acetate is at a concentration of at
least 1% vol/vol. In some embodiments, the acetate is at a concentration of at least 2%
vol/vol. In some embodiments, the acetate is at a concentration of at least 3% vol/vol. In
20 some embodiments, the acetate is at a concentration of at least 4% vol/vol. In some
embodiments, the acetate is at a concentration of at least 5% vol/vol. In some embodiments,
the culture comprises glycerol. In some embodiments, the glycerol is at a concentration of
about 2% vol/vol. In some embodiments, the culture comprises a dissolved oxygen level of
at least 5%, at least 10%, at least 15%, or at least 20%. In some embodiments, the culture
25 exhibits a pH within the range of pH7.0 to pH7.5. In some embodiments, the culture
comprises ammonium sulfate. In some embodiments, the culture comprises ammonium
sulfate and acetic acid at a ratio of 1:2. In some embodiments, the culture exhibits a lipid titer
between 5 g/l and 60 g/l. In some embodiments, the culture exhibits a lipid production
between 0.04 g/l/h and 0.60 g/l/h. In some embodiments, the culture exhibits a maximum
30 lipid productivity of between 0.1 g/l/h and 1 g/l/h.

Some aspects of this disclosure provide a method comprising contacting a carbon
source with an isolated oleaginous cell, the cell comprising a genetic modification that

increases expression of a DGA1 gene product; and incubating the carbon source contacted with the cell under conditions suitable for at least partial conversion of the carbon source into a fatty acid or a triacylglycerol by the cell. In some embodiments, the oleaginous cell further comprises a genetic modification that increases expression of an ACC1 gene product. In some embodiments, the oleaginous cell further comprises a genetic modification that increases expression of an SCD gene product. In some embodiments, the oleaginous cell further comprises a genetic modification that increases expression of an ACL gene product. In some embodiments, the isolated oleaginous cell is an engineered isolated oleaginous cell as described herein. In some embodiments, the carbon source comprises a fermentable sugar.

10 In some embodiments, the carbon source comprises glucose. In some embodiments, the carbon source comprises acetate. In some embodiments, the acetate is at a concentration of at least 1% vol/vol, of at least 2% vol/vol, of at least 3% vol/vol, of at least 4% vol/vol, or of at least 5% vol/vol. In some embodiments, the carbon source comprises acetic acid. In some embodiments, the acetic acid is at a concentration of at least 5% vol/vol, at least 10% vol/vol, at least 15% vol/vol, at least 20% vol/vol, at least 25% vol/vol, or at least 30% vol/vol. In some embodiments, the method comprises contacting the cell with dissolved oxygen at a level of at least 5%, at least 10%, at least 15%, or at least 20%. In some embodiments, the contacting and/or the incubating is performed at a pH within the range of pH7.0 to pH7.5.

20 70. The method of any one of claims 53-69, wherein the method comprises contacting the cell with ammonium sulfate. In some embodiments, the method comprises contacting the cell with ammonium sulfate and acetic acid at a ratio of 1:2. In some embodiments, the method further comprises contacting the cells with glycerol. In some embodiments, the method comprises contacting the cells with glycerol at a concentration of about 2% vol/vol. In some embodiments, the carbon source contacted with the isolated oleaginous cell is

25 incubated in a reactor. In some embodiments, the carbon source is contacted with the isolated oleaginous cell and incubated for conversion of the carbon source to a fatty acid or a triacylglycerol in a fed batch process. In some embodiments, the carbon source is contacted with the isolated oleaginous cell and incubated for conversion of the carbon source to a fatty acid or a triacylglycerol in a continuous process. In some embodiments, the method further

30 comprises contacting an additional amount of the carbon source or an amount of an additional carbon source with the carbon source contacted with the isolated oleaginous cell one or more times during the incubating step. In some embodiments, the fatty acid or the triacylglycerol

is extracted from the carbon source contacted with the isolated oleaginous cell by solvent extraction. In some embodiments, the solvent extraction comprises a chloroform methanol extraction. In some embodiments, the solvent extraction comprises a hexane extraction. In some embodiments, the fatty acid or the triacylglycerol is separated from the carbon source contacted with the isolated oleaginous cell and subsequently refined by transesterification.

Some aspects of this disclosure provide a method comprising modifying the fatty acid profile, the triacylglycerol profile, the fatty acid synthesis rate, the triacylglycerol synthesis rate, the extent of fatty acid derivative accumulation, the rate of fatty acid derivative secretion, the rate of carbohydrate to fatty acid or fatty acid derivative conversion, and/or the efficient yield of carbohydrate to fatty acid or fatty acid derivative conversion in an oleaginous cell by increasing in the cell the expression of a DGA1 gene product. In some embodiments, the method further comprises increasing in the cell the expression of an ACC1 gene product, of an SCD gene product, and/or of an ACL gene product. In some embodiments, the extent of fatty acid derivative accumulation is the extent of fatty acid derivative accumulation in a lipid body. In some embodiments, the fatty acid derivative is a triacylglycerol. In some embodiments, modifying the fatty acid profile, the triacylglycerol profile, the fatty acid synthesis rate, the triacylglycerol synthesis rate, the extent of fatty acid derivative accumulation, the rate of fatty acid derivative secretion, the rate of carbohydrate to fatty acid or fatty acid derivative conversion, and/or the efficient yield of carbohydrate to fatty acid or fatty acid derivative conversion in the oleaginous cell comprises increasing the fatty acid synthesis rate, the triacylglycerol synthesis rate, the extent of fatty acid derivative accumulation, the rate of fatty acid derivative secretion, the rate of carbohydrate to fatty acid or fatty acid derivative conversion, and/or the efficient yield of carbohydrate to fatty acid or fatty acid derivative conversion in the oleaginous cell. In some embodiments, modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 2-fold. In some embodiments, modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 3-fold. In some embodiments, modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 4-fold. In some embodiments, modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least

5-fold. In some embodiments, the cell is a yeast cell. In some embodiments, the yeast cell is a *Yarrowia* sp. cell. In some embodiments, the oleaginous yeast is *Y. lipolytica*.

Some aspects of this disclosure provide an isolated nucleic acid molecule comprising:

a) a nucleotide sequence that encodes SEQ ID NO: 2 (*Y. lipolytica* DGA1), or b) a nucleotide

sequence that is at least 85% identical to the nucleotide sequence of a). In some

embodiments, the nucleotide sequence that encodes SEQ ID NO:2 comprises SEQ ID NO: 1.

Some aspects of this disclosure provide an expression cassette comprising an isolated nucleic acid molecule as described herein and a heterologous promoter. In some embodiments, the

promoter is a constitutive promoter or an inducible promoter. In some embodiments, the

heterologous promoter is a Translation Elongation Factor (TEF) promoter. In some

embodiments, the heterologous promoter comprises an intron. In some embodiments, the

heterologous promoter further comprises a start codon. In some embodiments, the intron is

downstream of the translation start site of the nucleotide sequence that encodes SEQ ID NO:

2. Some aspects of this disclosure provide a vector comprising an expression cassette as

described herein. Some aspects of this disclosure provide a cell comprising an expression

cassette as described herein or at least a part of a vector described herein.

The subject matter of this application may involve, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of a single system or article.

Other advantages, features, and uses of the invention will be apparent from the detailed description of certain non-limiting embodiments, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Overview of the principal metabolic pathways for lipid synthesis in *Y. lipolytica*.

Figure 2. Enzyme activity of β -galactosidase under different promoters after 50 hours of culture.

Figure 3. Bioreactor fermentation of ACC1+DGA1 transformant (MTYL065).

Figure 4. Fatty Acid Profile (FAP) of ACC1 + DGA1 transformant in 2-L bioreactor fermentation.

Figure 5. Fatty acid profile of ACC + DGA transformant grown in 2-L bioreactor with acetate as carbon source.

Figure 6. Fatty acid production by ACC +DGA1 *Y. lipolytica* on acetate.

Figure 7. Combinatorial expression construction scheme. Cloning path and strategy for construction of plasmids containing a combination of lipid accumulation gene targets. These plasmids were then transformed into *Y. lipolytica* for study of lipid accumulation.

Figure 8. Transcriptional expression of TEFin-DGA cassette when integrated via pMT053 compared to pMT092.

Figure 9. Relative lipid productivity and yield among *Y. lipolytica* strains expressing combinatorial constructs, as measured by total fatty acid content normalized to the control strain. The presence (+) or absence (-) of a transformed overexpression cassette for the corresponding gene target are indicated below the graph. The productivity (light grey bars) was calculated as relative lipid accumulation within the first 100 hours of culture. Yield calculations were made by dividing lipid accumulation by sugar consumed. The C/N ratio of the media was 20. Results are averaged values across multiple experiments.

Figure 10. Transcriptional expression of target genes in the strain MTYL089. Expression is internally normalized to actin expression, and compared against a control (MTYL038) strain and was taken after 66 hours of growth.

Figure 11. Batch bioreactor fermentation of strain MTYL089, overexpression ACC, D9, ACL12 and DGA. C/N molar ratio was 100. All sampling was performed in triplicate.

Figure 12. Microscopy of strain MTYL089 at the end of 2-L fermentation. Normal light microscopy (image left) shows that a majority of the cells are in the yeast form, and contain large vacuoles. Fluorescence microscopy (image right) indicates that these vacuoles are composed of neutral lipids.

Figure 13. Comparison of fatty acid profiles between strains MTYL065 and MTYL089. Fatty acid profiles taken from final time point in the respective fermentations, normalizing to the total fatty acid content.

Figure 14. Trends of nitrogen (mM), non-lipid and lipid titers (g/L).

Figure 15. Trends of lipid titer (g/L), lipid content (%) and C/N ratio. Primary axis shows lipid titer and lipid content as % of dry cell weight. Secondary axis shows C/N ratio. C/N ratio drops at the end due to the rapid consumption of acetate.

Figure 16. Top - 'Floating cells' seen under oil immersion microscope (100X) without fluorescence. Below – Same frame under fluorescence shows the bright red lipid body

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS OF THE INVENTION

Liquid biofuels are a promising alternative to fossil fuels that can help ease concerns about climate change and smoothen supply uncertainties(1). Biodiesel, jet oil and other oil-derived fuels in particular are necessary for aviation and heavy vehicle transport. They are presently produced exclusively from vegetable oils, which is a costly and unsustainable path(2). An attractive possibility is the non-photosynthetic conversion of renewable carbohydrate feedstocks to oil(3). For biodiesel, a transition from vegetable oil to microbial oil production for the oil feedstock presents numerous additional advantages: adaptability to diverse feedstocks, flexibility in land requirements, efficient process cycle turnover, and ease of scale-up(4). The biological platforms for microbial production are also more genetically tractable for further optimization.

The key to a cost-effective microbial technology for the conversion of carbohydrates to oils is a high carbohydrate to oil conversion yield. Metabolic engineering has emerged as the enabling technology applied to this end and numerous examples exist of successful pathway engineering that markedly improved the performance of microbial biocatalysts in the synthesis of chemical, pharmaceutical and fuel products(5). Prior efforts at engineering microbes with high lipid synthesis have focused on amplifying presumed rate-controlling steps in the fatty acid synthesis pathway(6). These efforts, however, have produced mixed results, presumably because modulating fatty acid flux gave rise to the levels of saturated fatty acids, which are potent allosteric inhibitors of fatty acid biosynthetic enzymes providing a negative feedback loop for the fatty acid biosynthesis(7). Here we describe an approach that combines the amplification of upstream, metabolite-forming pathways with a similar increase in the flux of downstream, metabolite-consuming pathways. When balanced, this push-and-pull strategy can achieve large flux amplifications without significant departures of the concentrations of intermediate metabolites from their homeostatic physiological levels.

The oleaginous yeast *Yarrowia lipolytica* is an attractive candidate for microbial oil production, which has also demonstrated usefulness in a wide range of other industrial applications: citric acid production, protein production (e.g., proteases and lipases), and bioremediation (8-10). With a fully sequenced genome and a growing body of genetic engineering tools, engineering of *Y. lipolytica* can be achieved with relative ease (11). *Y. lipolytica* also has been found to be robust in culture, able to grow on a variety of substrates, and has been used for lipid production on agro-industrial residues, industrial

glycerol, and industrial fats (12-14). It has excellent lipid accumulation capacity, commonly accumulating up to 36% of its dry cell weight (DCW) in lipids (15).

The metabolic pathways for *de novo* lipid synthesis in *Y. lipolytica* are beginning to be fully mapped out, and a current model of lipid synthesis is illustrated in **Figure 1**: Glucose entering glycolysis enters the mitochondria as pyruvate for use in the TCA cycle; however, excess acetyl-coA is transported from the mitochondria to the cytosol via the citrate shuttle. Cytosolic acetyl-CoA is then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC) as the first step of fatty acid synthesis. After fatty acid synthesis, triacylglycerol (TAG) synthesis follows the Kennedy pathway, which occurs in the endoplasmic reticulum (ER) and lipid bodies. Acyl-CoA is the precursor used for acylation to the glycerol-3-phosphate backbone to form lysophosphatidic acid (LPA), which is further acylated to form phosphatidic acid (PA). PA is then dephosphorylated to form diacylglycerol (DAG) and then a final acylation occurs by diacylglycerol acyltransferase (DGA) to produce TAG.

Transport of acetyl-CoA from the mitochondria to the cytosol is carried out by the ATP-citrate lyase (ACL)-mediated cleavage of citrate via the citrate shuttle yielding Acetyl-CoA and Oxaloacetate (OAA). Acetyl-CoA carboxylase (ACC) then catalyzes the first committed step towards lipid biosynthesis, converting cytosolic acetyl-CoA into malonyl-CoA, which is the primary precursor for fatty acid elongation. Completed fatty acyl-CoA chains are then transported to the endoplasmic reticulum (ER) or lipid body membranes for the final assembly of triacylglycerol (TAG) via the Kennedy pathway. Over 80% of the storage lipids produced in *Y. lipolytica* are in the form of TAG (16). Cytosolic OAA is converted to malate by malic dehydrogenase and transported back into the mitochondria to complete the citrate shuttle cycle. Reducing equivalents in the form of NADPH is provided either by the pentose phosphate pathway or by malic enzyme in the transhydrogenase cycle. In *Y. lipolytica*, high PPP flux and ineffectual malic enzyme overexpression suggest that the former is the primary source for NADPH(4, 17).

Intracellular lipid accumulation can occur via two methods: *de novo* lipid synthesis or *ex novo* incorporation of exogenous fatty acids and lipids. Lipid accumulation most commonly occurs when nutrient supply is exhausted in the presence of excess carbon. In culture, this state typically coincides with the onset of the stationary phase. In practice, the most commonly used limiting-nutrient is nitrogen, as it is easily controllable in media compositions (15). Despite these inducible conditions, lipid synthesis pathways are highly

regulated in order for the organism to balance cell growth with energy storage. For example, ACC alone is regulated at multiple levels and by multiple factors (7).

This tight regulation was circumvented in certain cases. By eliminating peroxisomal oxidation pathways and engineering glycerol metabolism, *Y. lipolytica* was able to achieve 40%-70% lipids through *ex novo* lipid accumulation (18, 19). Coexpression of $\Delta 6$ - and $\Delta 12$ -desaturase genes allowed for significant production of γ -linolenic acid (GLA) (20). However, engineering lipid biosynthesis pathways in *Y. lipolytica* is still relatively unexplored and strategies are still being developed for effective engineering of the lipid production pathways to maximize output.

Some aspects of this disclosure provide engineered microbes for the production of biofuel or biofuel precursor. The term "biofuel" refers to a fuel that is derived from a biological source, such as a living cell, microbe, fungus, or plant. The term includes, for example, fuel directly obtained from a biological source, for example, by conventional extraction, distillation, or refining methods, and fuel produced by processing a biofuel precursor obtained from a biological source, for example by chemical modification, such as transesterification procedures. Examples of biofuels that are directly obtainable are alcohols such as ethanol, propanol, and butanol, fat, and oil. Examples of biofuels that are obtained by processing of a biofuel precursor (e.g., a lipid), are biodiesel (e.g., produced by transesterification of a lipid), and green diesel/modified oil fuels (e.g., produced by hydrogenation of an oil). Biodiesel, also referred to as fatty acid methyl (or ethyl) ester, is one of the economically most important biofuels today and can be produced on an industrial scale by transesterification of lipids, in which sodium hydroxide and methanol (or ethanol) reacts with a lipid, for example, a triacylglycerol, to produce biodiesel and glycerol.

Feedstocks for industrial-scale production of biodiesel include animal fats, vegetable oils, palm oil, hemp, soy, rapeseed, flax, sunflower, and oleaginous algae. In other approaches, biomass is converted by a microbe into a biofuel precursor, for example, a lipid, that is subsequently extracted and further processed to yield a biofuel. The term "biomass" refers to material produced by growth and/or propagation of a living cell or organism, for example, a microbe. Biomass may contain cells, microbes and/or intracellular contents, for example cellular fatty acids and TAGS, as well as extracellular material. Extracellular material includes, but is not limited to, compounds secreted by a cell, for example, secreted fatty acids or TAGs. Important types of biomass for biofuel production are algal biomass and

plant-derived biomass, for example, corn stover and wood fiber. In some embodiments, biomass for biofuel or biofuel precursor production may comprise plant derived sugars, for example, sugarcane or corn derived sugars.

Some aspects of this disclosure relate to the engineering and development of a microbial source of lipids, useful, for example, for economically viable, industrial-scale biodiesel production. The term “lipid” refers to fatty acids and their derivatives. Accordingly, examples of lipids include fatty acids (FA, both saturated and unsaturated); glycerides or glycerolipids, also referred to as acylglycerols (such as monoglycerides (monoacylglycerols), diglycerides (diacylglycerols), triglycerides (triacylglycerols, TAGs, or neutral fats); phosphoglycerides (glycerophospholipids); nonglycerides (sphingolipids, sterol lipids, including cholesterol and steroid hormones, prenol lipids including terpenoids, fatty alcohols, waxes, and polyketides); and complex lipid derivatives (sugar-linked lipids or glycolipids, and protein-linked lipids). Lipids are an essential part of the plasma membrane of living cells and microbes. Some cells and microbes also produce lipids to store energy, for example in the form of triacylglycerols in lipid bodies, lipid droplets, or vacuoles.

Some aspects of this invention relate to engineered microbes for biofuel or biofuel precursor production. In some embodiments, the microbes provided herein are engineered to optimize their lipid metabolism for lipid production. The term “lipid metabolism” refers to the molecular processes that involve the creation or degradation of lipids. Fatty acid synthesis, fatty acid oxidation, fatty acid desaturation, TAG synthesis, TAG storage and TAG degradation are examples of processes that are part of the lipid metabolism of a cell. Accordingly, the term “fatty acid metabolism” refers to all cellular or organismic processes that involve the synthesis, creation, transformation or degradation of fatty acids. Fatty acid synthesis, fatty acid oxidation, TAG synthesis, and TAG degradation are examples of processes are part of the fatty acid metabolism of a cell.

The term “triacylglycerol” (TAG, sometimes also referred to as triglyceride) refers to a molecule comprising a single molecule of glycerol covalently bound to three fatty acid molecules, aliphatic monocarboxylic acids, via ester bonds, one on each of the glycerol molecule’s three hydroxyl (OH) groups. Triacylglycerols are highly concentrated stores of metabolic energy because of their reduced, anhydrous nature, and are a suitable feedstock for biodiesel production.

Many cells and organisms store metabolic energy in the form of fatty acids and fatty acid derivatives, such as TAGs. Fatty acids and their derivatives, such as TAGs, provide an ideal form to store metabolic energy. The energy contained in the C–C bonds can be efficiently released by β -oxidation, a reaction formally equivalent to the reverse of fatty acid biosynthesis, but mediated and regulated by different enzymes constituting a different molecular pathway. Microbes can derive fatty acids from external supply, endogenous turnover, and de novo synthesis. Some aspects of this invention relate to the identification of a microbe for biofuel or biofuel precursor production based on the microbe's ability to synthesize and store fatty acids or fatty acid derivatives, such as TAGs, efficiently from an externally supplied carbon source.

Natural fatty acid molecules commonly have an unbranched, aliphatic chain, or tail, of 4 to 28 carbon atoms. Fatty acids are referred to as "saturated", if all carbon atoms of the aliphatic chain are connected via a C-C single bond, or as "unsaturated", if two or more carbon atoms are connected via a C-C double bond. Unsaturated fatty acids play important roles in the regulation of membrane fluidity, cellular activity, metabolism and nuclear events governing gene transcription.

The spectrum of fatty acids in yeast consists mostly of C16 and C18 fatty acids, for example palmitic acid (C16), palmitoleic acid (C16), stearic acid (C18) and oleic acid (C18). Palmitic acid is an unbranched, saturated fatty acid, with an aliphatic chain of 16 carbon atoms (carbon atoms/unsaturated bonds: 16.0). Stearic acid is an unbranched, saturated fatty acid with an aliphatic chain of 18 carbon atoms (18.0). Palmitoleic acid is a monounsaturated fatty acid with an aliphatic chain of 16 carbon atoms (16.1). Oleic acid is a monounsaturated fatty acid with an aliphatic chain of 18 carbon atoms (18.1). Minor fatty acid species in yeast include C14 and C26 fatty acids, which play essential functions in protein modification or as components of sphingolipids and GPI anchors, respectively.

De novo synthesis of fatty acids utilizes substantial amounts of metabolites, acetyl-CoA, ATP and NADPH, and thus competes with other cellular processes that are dependent on these compounds. NADPH is required for two reduction steps in the fatty acid elongation cycle, linking fatty acid synthesis to the metabolic state of the cell and results in fatty acid synthesis being restricted to conditions of high energy load of the cells, indicated by increased ATP/AMP ratio, elevated reduction equivalents and elevated acetyl-CoA pool. Almost all subcellular organelles are involved in fatty acid metabolism, indicating that

maintenance of fatty acid homeostasis requires regulation at multiple levels. Lipid synthesis steps that generate metabolites, acetyl-CoA, ATP, or NADPH for lipid biosynthesis are sometimes referred to herein as “push steps” of lipid synthesis. The amplification of a process that increases the production of a metabolites, acetyl-CoA, ATP, or NADPH for lipid synthesis in a cell, for example, by overexpressing a gene product mediating such a metabolite-producing process, is sometimes referred to herein as a “push modification.”

Most organisms, including yeast, are able to synthesize fatty acids de novo from a variety of carbon sources. In an initial step, acetyl-CoA is carboxylated by the addition of CO₂ to malonyl-CoA, by the enzyme acetyl-CoA carboxylase (ACC; encoded by ACC1 and Hfa1 in yeast). Biotin is an essential cofactor in this reaction, and is covalently attached to the ACC apoprotein, by the enzyme biotin:apoprotein ligase (encoded by BPL1/ACC2 in yeast). ACC is a trifunctional enzyme, harboring a biotin carboxyl carrier protein (BCCP) domain, a biotin-carboxylase (BC) domain, and a carboxyl-transferase (CT) domain. In most bacteria, these domains are expressed as individual polypeptides and assembled into a heteromeric complex. In contrast, eukaryotic ACC, including mitochondrial ACC variants (Hfa1 in yeast) harbor these functions on a single polypeptide. Malonyl-CoA produced by ACC serves as a two carbon donor in a cyclic series of reactions catalyzed by fatty acid synthase, FAS, and elongases.

The immediate product of de novo fatty acid synthesis are saturated fatty acids. Saturated fatty acids are known to be the precursors of unsaturated fatty acids in eukaryotes, including yeast. Unsaturated fatty acids are generally produced by desaturation of C-C single bonds in saturated fatty acids by specialized enzymes, called desaturases. The control mechanisms that govern the conversion of saturated fatty acids to unsaturated fatty acids are not well understood. In eukaryotes, unsaturated fatty acids play important roles in the regulation of membrane fluidity, cellular activity, metabolism and nuclear events that govern gene transcription. Typically, about 80% of yeast fatty acids are monounsaturated, meaning that they contain one unsaturated bond in their aliphatic chain.

Fatty acids are potent inhibitors of fatty acid synthesis and the feedback inhibition of fatty acid synthesis by fatty acids is a major obstacle in engineering microbes for oil production. Some aspects of this disclosure are based on the recognition that while push modifications of lipid synthesis are typically unable to override fatty acid-mediated feedback inhibition of lipid synthesis, a combination of a push modification (e.g., ACC1

overexpression) with a pull modification (e.g., DGA1 overexpression), can efficiently bypass the feedback inhibition, thus fully realizing the increased carbon flux to the lipid synthesis pathway, for example, in TGAs stored in a lipid body or vacuole of the cell.

Some aspects of this disclosure provide strategies for engineering microbes for oil production. In some embodiments, such strategies employ genetic engineering of oleaginous microbes, for example, *Y. lipolytica*, to simultaneously amplify a push- and a pull-step of lipid synthesis. As disclosed herein, significant increases of lipid production in oleaginous yeast host cells were achieved using these strategies.

Some aspects of this disclosure are based on the recognition that push-an-pull modifications, for example, simultaneous amplification of metabolic steps that produce metabolites for lipid synthesis pathways and of metabolic steps that sequester synthesis products mediating feedback inhibition of lipid synthesis, result in a significant increase of carbon flux into lipid synthesis pathways as compared to the engineering of push-only or pull-only modifications. Some aspects of this invention relate to the surprising discovery that the overexpression of a DGA1 gene product in an oleaginous microbe, for example, *Yarrowia lipolytica*, results in a marked increase in carbon flux into lipid synthesis pathways, and that simultaneous overexpression of an ACC1 gene product synergizes with DGA1 overexpression, resulting in a push-and-pull modified microbe with greatly enhanced carbon flux properties that is suitable for industrial-scale oil production from a variety of carbon substrates.

The discovery that a balanced modulation of metabolite-generating steps (e.g., production of malonyl-CoA) as well as product-sequestering metabolic steps (e.g., acylation of diacylglycerols to triacylglycerols) in a microbe results in a significant increase of net carbon flux into lipid synthesis has major implication for processes aiming to convert renewable carbon sources into biofuel or biofuel precursors with the help of engineered cells. Based on some aspects of this invention it is now possible to modify the lipid synthesis metabolism of a microbe, for example an oleaginous yeast such as *Y. lipolytica*, in a way that confers highly desirable phenotypes for industrial-scale carbohydrate to biofuel or biofuel precursor conversion, such as remarkable increases in fatty acid synthesis, TAG synthesis, and fatty acid and TAG storage in lipid bodies or vacuoles.

According to some aspects of this invention, modifying the lipid metabolism in a microbe in accordance with methods provided herein, for example by simultaneously

overexpressing a gene product mediating a metabolite-generating (push) step and a gene product mediating a product-sequestering (pull) step of lipid synthesis, allows for the generation of a microbe optimized for use in biofuel or biofuel precursor production processes. Some aspects of this invention provide strategies and methods for engineering the fatty acid metabolism in a microbe by simultaneously amplifying a push step and a pull step of lipid biosynthesis, resulting in increased synthesis rate and accumulation of fatty acids and fatty acid derivatives in the microbe.

Some aspects of this invention provide methods that include genetic modifications resulting in the modulation of the expression and/or activity of gene products regulating the lipid metabolism of microbes for biofuel or biofuel precursor production. Such genetic modifications according to some aspects of this invention are targeted to increase carbohydrate to fatty acid and/or TAG conversion in order to optimize the modified microbe for large-scale production of lipids from a carbon source, for example, a carbohydrate source. Some modifications provided according to some aspects of this invention, for example, overexpression, knockout, knock-down, activation and/or inhibition of specific gene products, may be effected alone or in combination, and/or in combination with other modifications known to those of skill in the art. The term “modification” refers to both genetic manipulation, for example, overexpression, knockout, knock-down, activation and/or inhibition of specific gene products, and non-genetic manipulation, for example, manipulation of the growth media, substrate, substrate pretreatment, pH, temperature, conversion process, etc.

A modification of gene expression, also referred to herein as a modulation of gene expression, can be a disruption or inhibition of the natural regulation of expression, an overexpression, an inhibition of expression, or a complete abolishment of expression of a given gene. The insertion of a heterologous promoter upstream of a native gene sequence, for example the native DGA1 or ACC1 gene sequence, or the deletion of regulatory sequences within a promoter, for example regulatory sequences that mediate the feedback inhibition of the DGA1 or ACC1 gene by saturated fatty acids, are examples of a disruption or inhibition of the natural regulation of expression. Strategies for the modulation of gene expression may include genetic alterations, for example by recombinant technologies, such as gene targeting or viral transductions, or non-genetic alterations, for example environmental alterations known to result in the up- or down-regulation of gene expression, or transient delivery of

modulators, for example drugs or small RNA molecules to the target cells. Methods for genetic and non-genetic alterations of microbes are well known to those of skill in the art, and are described, for example, in J. Sambrook and D. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press; 3rd edition (January 15, 2001); David C.

- 5 Amberg, Daniel J. Burke; and Jeffrey N. Strathern, *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, Cold Spring Harbor Laboratory Press (April 2005); John N. Abelson, Melvin I. Simon, Christine Guthrie, and Gerald R. Fink, *Guide to Yeast Genetics and Molecular Biology, Part A, Volume 194* (Methods in Enzymology Series, 194), Academic Press (March 11, 2004); Christine Guthrie and Gerald R. Fink, *Guide to Yeast*
10 *Genetics and Molecular and Cell Biology, Part B, Volume 350* (Methods in Enzymology, Vol 350), Academic Press; 1st edition (July 2, 2002); Christine Guthrie and Gerald R. Fink, *Guide to Yeast Genetics and Molecular and Cell Biology, Part C, Volume 351*, Academic Press; 1st edition (July 9, 2002); Gregory N. Stephanopoulos, Aristos A. Aristidou and Jens Nielsen, *Metabolic Engineering: Principles and Methodologies*, Academic Press; 1 edition
15 (October 16, 1998); and Christina Smolke, *The Metabolic Pathway Engineering Handbook: Fundamentals*, CRC Press; 1 edition (July 28, 2009), all of which are incorporated by reference herein.

The term “overexpression”, as used herein, refers to an increased level of expression of a given gene product in a given cell, cell type or cell state, as compared to a reference cell,
20 for example, a wild type cell of the same cell type or a cell of the same cell type but lacking a specific modification, for example, a genetic modification. Forced, continuous expression of the DGA1 and/or ACC1 gene in *Y. lipolytica* cells exhibiting concentrations of saturated fatty acids that would inhibit DGA1 or ACC1 gene expression in wild-type cells is an example of gene overexpression.

25 Some aspects of this invention provide a method for the manipulation of the activity of a diacylglycerol acyltransferase 1 (DGA1) gene product in a microbe for biofuel or biofuel precursor production. The DGA1 gene encodes an acyltransferase that catalyzes the terminal step of triacylglycerol (TAG) formation, acylating diacylglycerol using acyl-CoA as an acyl donor. The result of this acyltransferase reaction are triacylglycerols, which do not exhibit
30 the same inhibitory feedback effect on fatty acid synthesis as fatty acids themselves. TAGs are typically stored in lipid bodies or vacuoles in lipid producing cells. In some embodiments, the manipulation is an overexpression. In some embodiments, the

manipulation is effected by contacting a microbe for biofuel or biofuel precursor production with an expression construct comprising a nucleic acid coding for a DGA1 gene product, for example, a DGAT2 protein, operably linked to a heterologous promoter, for example, a constitutive or an inducible promoter. In some embodiments, the nucleic acid coding for a DGA1 gene product comprises the coding sequence of SEQ ID NO: 1. In some
 5 embodiments, the DGA1 is *Y. lipolytica* DGA1, for example, *Y. lipolytica* DGA1 comprising the amino acid sequence of SEQ ID NO: 2. In some embodiments, the microbe is *Y. lipolytica*. In some embodiments, manipulation of the activity of a DGA1 gene product in a microbe is effected to confer a beneficial phenotype for large-scale carbohydrate to lipid
 10 conversion, for example increased lipid synthesis rate, increased carbohydrate to lipid conversion efficiency, increased lipid storage and, increased growth rate, increased tolerance to elevated concentrations of a carbon source or a lipid product. DGA1 gene and gene product sequences are well known to those of skill in the art. Exemplary, representative gene and gene product sequences can be found under entry XM_504700 in the NCBI database
 15 (www.ncbi.nlm.nih.gov).

Non-limiting examples of suitable sequences of DGA1 nucleic acid and protein sequences are provided below. Additional suitable DGA1 sequences, including sequences from other species, will be apparent to those of skill in the art, and the invention is not limited in this respect.

20 >gil50554582|reflXM_504700.1| Yarrowia lipolytica YALI0E32769p (YALI0E32769g)
 mRNA, complete cds

ATGACTATCGACTCACAATACTACAAGTCGCGAGACAAAAACGACACGGCACCCAAAATCGCGGGAATCCGATAT
 GCCCCGCTATCGACACCATTACTCAACCGATGTGAGACCTTCTCTCTGGTCTGGCACATTTTCAGCATTCCCACT
 25 TTCTCACAATTTTCATGCTATGCTGCGCAATTCCACTGCTCTGGCCATTTGTGATTGCGTATGTAGTGTACGCT
 GTTAAAGACGACTCCCCGTCCAACGGAGGAGTGGTCAAGCGATACTCGCCTATTTCAAGAACTTCTTCATCTGG
 AAGCTCTTTGGCCGCTACTTCCCCATAACTCTGCACAAGACGGTGGATCTGGAGCCACGCACACATACTACCCT
 CTGGACGTCCAGGAGTATCACCTGATTGCTGAGAGATACTGGCCGCAGAACAAGTACCTCCGAGCAATCATCTCC
 ACCATCGAGTACTTTCTGCCCCCTTCATGAAACGGTCTCTTTCTATCAACGAGCAGGAGCAGCCTGCCGAGCGA
 30 GATCCTCTCCTGTCTCCCGTTTCTCCCAGCTCTCCGGTTTCTCAACCTGACAAGTGGATTAACCACGACAGCAGA
 TATAGCCGTGGAGAATCATCTGGCTCCAACGGCCACGCCTCGGGCTCCGAACCTTAACGGCAACGGCAACAATGGC
 ACCACTAACCGACGACCTTTGTCTCGTCCGCCTCTGCTGGCTCCACTGCATCTGATTCCACGCTTCTTAACGGGTCC
 CTCAACTCCTACGCCAACCAGATCATTGGCGAAAACGACCCACAGCTGTGCGCCACAAAACCTCAAGCCCACTGGC
 AGAAAATACATCTTCGGCTACCACCCCCACGGCATTATCGGCATGGGAGCCTTTGGTGGGAATTGCCACCGAGGGA
 35 GCTGGATGGTCCAAGCTCTTTCCGGGCATCCCTGTTTCTCTTATGACTCTCACCAACAACCTCCGAGTGCCTCTC

TACAGAGAGTACCTCATGAGTCTGGGAGTCGCTTCTGTCTCCAAGAAGTCCTGCAAGGCCCTCTCAAGCGAAAC
 CAGTCTATCTGCATTGTCGTTGGTGGAGCACAGGAAAGTCTTCTGGCCAGACCCGGTGTTCATGGACCTGGTGCTA
 CTCAAGCGAAAGGGTTTTGTTGACTTGGTATGGAGGTCGAAATGTCGCCCTTGTTCCTCATCATGGCCTTTGGT
 GAGAACGACCTCTATGACCAGGTTAGCAACGACAAGTCGTCCAAGCTGTACCGATTCCAGCAGTTTGTCAAGAAC
 5 TTCCTTGGATTACCCCTTCCTTTGATGCATGCCCAGGCGTCTTCAACTACGATGTCGGTCTTGTCCCCTACAGG
 CGACCCGTCAACATTGTGGTTGGTTCCCCCATTGACTTGCCTTATCTCCCACACCCACCGACGAAGAAGTGTC
 GAATACCACGACCGATACATCGCCGAGCTGCAGCGAATCTACAACGAGCACAAGGATGAATATTTTCATCGATTGG
 ACCGAGGAGGGCAAAGGAGCCCCAGAGTTCCGAATGATTGAGTAA (SEQ ID NO: 1)

10 >gil50554583|reflXP_504700.1| YALIOE32769p [*Yarrowia lipolytica*]

MTIDSQYYKSRDKNDTAPKIAGIRYAPLSTPLLNRCEFTSLVWHIFS IPTFLTIFMLCCAIPLLWPFVIAYVVYA
 VKDDSPSNGGVVKRYSPISRNFIFWKLFGRYFPITLHKTVLDLEPTHTYYP LDVQEYHLIAERYWPQNKYLRAIIS
 TIEYFLPAFMKRSLSINEQEQAERDPLLSPVSPSSPGSQPKWINHDSRYSRGESSGSGNHASGSELNGNGNG
 TTNRRPLSSASAGSTASDSTLLNGSLNSYANQIIIGENDPQLSPTKLKPTGRKYIFGYHPHGIIGMGAFFGGIATEG
 15 AGWSKLFPGIPVSLMTLTNNFRVPLYREYLSLGVASVSKKSKALLKRNSICIVVGAQESLLARPGVMDLVL
 LKRKGFVRLGMEVGNVALVPIMAFGENDLYDQVSNKSSKLYRFQQFVKNFLGFTLPLMHARGVFNYDVGLVPYR
 RPNIVVVGSPIDLPYLPHTDEEVSEYHRYIAELQRIYNEHKDEYFIDWTEEGKGAPEFRMIE (SEQ ID
 NO: 2)

20 Some aspects of this invention provide a method for the manipulation of an acetyl-
 CoA carboxylase (ACC) gene product in a microbe for biofuel or biofuel precursor
 production, for example, in *Y. lipolytica*. ACC gene products mediate the conversion of
 acetyl-CoA, the main C2-precursor in fatty acid synthesis, to malonyl-CoA, which is
 considered the first committed step in fatty acid synthesis and has been suggested to also be
 25 the rate-limiting step in fatty acid synthesis (see Cao Y, Yang J, Xian M, Xu X, Liu W.
 Increasing unsaturated fatty acid contents in *Escherichia coli* by coexpression of three
 different genes. *Appl Microbiol Biotechnol.* 2010). In some embodiments, ACC activity
 manipulation is ACC overexpression. In some embodiments, the manipulation is effected by
 contacting a microbe for biofuel or biofuel precursor production with an expression construct
 30 comprising a nucleic acid coding for an ACC gene product, for example, an ACC1 protein,
 operably linked to a heterologous promoter, for example, a constitutive or an inducible
 promoter. In some embodiments, the nucleic acid coding for an ACC gene product
 comprises the coding sequence of SEQ ID NO: 3. In some embodiments, the ACC gene
 product is an ACC1 protein comprising the amino acid sequence of SEQ ID NO: 4. In some
 35 embodiments, ACC overexpression in a microbe increases fatty acid synthesis rate and/or

confers a beneficial phenotype for large-scale carbohydrate to biofuel or biofuel precursor conversion, for example increased lipid synthesis rate, increased carbohydrate to lipid conversion efficiency, increased lipid storage and, increased growth rate, increased tolerance to concentrations of a substance, e.g. a carbon source, a biofuel or biofuel precursor, or a toxic substance. ACC gene and gene product sequences are well known to those of skill in the art. Exemplary, representative gene and gene product sequences can be found under the entry for GeneIDs: 855750 and 2909424, or under the entry NC_006069 in the NCBI database (www.ncbi.nlm.nih.gov).

Non-limiting examples of suitable sequences of ACC nucleic acid and protein sequences are provided below. Additional suitable ACC sequences, including sequences from other species, will be apparent to those of skill in the art, and the invention is not limited in this respect.

ACC encoding nucleic acid sequence:

atgcgactgcaattgaggacactaacacgtcgggtttttcaggtgagtaaacgacggtggccgtggccacgacagc
cgaggcgtcacgatgggcccagacgagcacattctcgccgccacaacctcgccagcacaagaaactaaccagtat
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5 cgaaactggctctgctttccctctgcgagcaatgatcaacaacgtctctgggttacgttgtcagctctgagctgta
cgctgaggccaagaacgacaaggccagtggttttcaagtctctgggcaagcccggctccatgcacatgcggtc
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cgatctcatgacttgcaacgagctgattctcgatgaggactctggcgagctgcaggaggtgaaccgagagcccg
10 cgccaacaacgtcggatggttgctggaagtttgaggccaagacccccgagtagcctcgaggccgatctttcat
cgtggtggccaacgatataccttccagattggttcgtttggccctgctgaggaccagttcttcttcaaggtgac
ggagctggctcgaaaagctcggatttccctcgaatctatctgtctgccaactctggtgctcgaatcggcattgctga
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caccctgagctcttggccaccctcaagcccgacactgttgtcaccactgagattgaggaggaggggtcccaacgg
15 cgtggagaagcgtcatgtgatcgactacattgtcggagagaaggacggctctcgagtcgagtgctcgcggggctc
tggtctcattgcaggcgccacttctcgagcctacaaggatatttctactctcactcttgtcacctgtcgatccgt
tggtatcggtgcttaccttgttcgtcttgggtcaacgagccatccagattgagggccagcccatcattctcactgg
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20 atacatccctgcttctcgaggtcttccagtgcttctccctcacaagaccgatgtgtgggatcgagacgtgac
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agctcgtcttggcggcattcccttcgggtgtcattgggtgtcgagactgcgaccgtcgacaataactaccctgccga
tcccgccaacccggactctattgagatgagcacctctgaagccggccagggttggtagcccaactcggccttcaa
25 gacctctcaggccatcaacgacttcaaccatgggtgaggcgcttctctcatgattcttgcctaactggcgaggctt
ttctgggtggtcagcgagacatgtacaatgaggttctcaagtacggatctttcattgttgatgctctggttgacta
caagcagcccatcatggtgtacatccctcccaccgggtgagctgcgaggtggttcttgggttggttgacccac
catcaactcggacatgatggagatgtacgctgacgtcgagtcctcgaggtggtgtgctggagcccaggggaatggt
cggtatcaagtaccgacgagacaagctactggacaccatggctcgtctggatcccagtagtactctctcaagaa
30 gcagcttgaggagctctccgattctgaggagctcaagggtcaagctcagcgtgcgagagaagtctctcatgccat
ctaccagcagatctccgtgcagtttggcgacttgcatgaccgagctggccgaatggaggccaagggtgtcattcg
tgaggctcttgtgtggaaggatgctcgtcgattcttcttctggcgaatccgacgacgattagtcgaggagtacct
cattaccaagatcaatagcattctgccctcttgactcggcttgagtgctcgtcgaatcaagtcgtggaagcc
35 tgccactcttgatcagggtctgaccgggtgttgccgagtggtttgacgagaactctgatgccgtctctgctcg
actcagcgagctcaagaaggacgcttctgcccagtcggttgccttctcaactgagaaaggacccgacaggggtactct
ccagggcataagcaggctctcgcttctcttctgaggctgagcgggtgagctgctcaaggggttggtga (SEQ
ID NO: 3)

>gil50548503|reflXP_501721.1| YALIOC11407p [Yarrowia lipolytica]

MRLQLRTLRRFFSMASGSSTPDVAPLVDPNIHKGLASHFFGLNSVHTAKPSKVKEFVASHGGHTVINKVLIANN
 GIAAVKEIRSVRKWAYETFGDERAISFTVMATPEDLAANADYIRMADQYVEVPGGTNNNNYANVELIVDVAERFG
 VDAVWAGWGHAASENPLLPESLAASPRKIVFIGPPGAAMRSLGDKISSTIVAQHAKVPCIPWSGTGVDEVVVDKST
 5 NLVSVSEEVYTKGCTTGPKQGLEKAKQIGFVMIKASEGGGGKGIRKVEREEDFEAAHQVEGEIPGSPIFIMQL
 AGNARHLEVQLLADQYGNNISLFGDCSVQRRHQKIIIEAPVTVAGQQTFTAMEKAAVRLGKLVGYVSAGTVEYL
 YSHEDDKFYFLELNPRLQVEHPTTEMVTGVNLPAAQLQIAMGIPLDRIKDIRLFYGVNPHTTTTPIDFDFSGEDAD
 KTQRRPVPRGHTTACRITSEDPGEGFKPSGGTMHELNFRSSSNVWGYFSVGNQGGIHSFSDSQFGHIFAFGENRS
 ASRKHMVVALKELSIRGDFRTTVEYLIKLLLETPDFEDNTITTWLDELISNKLTAERPDSFLAVVCGAATKAHRA
 10 SEDSIATYMASLEKGQVPARDILKTLFPVDFIYEGQRYKFTATRSEDSTYTLFINGSRCDIGVRPLSDGGILCLV
 GGRSHNVYWKEEVGATRLSVDSKTCLELVENDPTQLRSPSPGKLVKFLVENGHDHVRANQPYAEIEVMKMYMTLTA
 QEDGIVQLMKQPGSTIEAGDILGILALDDPSKVKHAKPFEGQLPELGPPTLSGNKPHQRYEHCQNVLHNILLGFD
 NQVVMKSTLQEMVGLLRNPELPYLQWAHQVSSLHTRMSAKLDATLAGLIDKAKQRGGEFPAKQLLRALEKEASSG
 EVDALFQQTLAPLFDLAREYQDGLAIHELQVAAGLLQAYYDSEARFCGPNVRDEDVILKLREENRDSLKVVMAQ
 15 LSHSRVGAKNNLVLALLDEYKVADQAGTDSASNHVAKYLRPVLRKIVELESRASAKVSLKAREILIQCALPSL
 KERTDQLEHILRSSVVESRYGEVGLHRTPRADILKEVVDISKYIVFDVLAQFFAHDDPWIVLAALEYIIRACKA
 YSILDINYHQSDLPVISWRFRLPTMSSALYNSVSSGSKTPTSPSVSRADSVSDFSYTVERDSAPARTGAIVA
 VPHLDDLEDALTRVLENLPKRGAGLAISVGASNKSAAASARDAAAAAASSVDTGLSNICNMIGRVDESDDDDTL
 IARISQVIEDFKEDFEACSLRRIITFSFGNSRGTYPKYFTFRGPAYEEDPTIRHIEPALAFQLELARLSNFDIKPV
 20 HTDNRNIHVYEATGKNAASDKRFFTRGIVRPGRLRENIPTSEYLISEADRLMSDILDALLEVIGTTNSDLNHIFIN
 FSAVFALKPEEVEAAFGGFLERFGRRLWRLRVTGAEIRMMVSDPETGSAPPLRAMINNVSGYVVQSELYAEAKND
 KGQWIFKSLGKPGSMHMRSINTPYPTKEWLQPKRYKAHLMGTTYCYDFPELFRQSIESDWKKYDGKAPDDLMTCN
 ELILDEDSGELQEVNREPGANNVGMVAWKFEAKTPEYPRGRSFIVVANDITFQIGSFGPAEDQFFFKVTELARKL
 GIPRIYLSANS GARIGIADELVGKYKVAWNDETDP SKGFKYLYFTPESLATLKPDTVVTTEIEEEGPNGVEKRHV
 25 IDYIVGEKDGLGVECLRGSLIAGATSRAYKDIFTTLTLVTCRSVGIGAYLVRLGQRAIQIEGQPIILTGAPAINK
 LLGREVYSSNLQLGGTQIMYNNGVSHLTARDDLNGVHKIMQWLSYIPASRGLPVPVLPKTDVWDRDVTQFQVRG
 EQYDVRWLISGRTLEDGAFESGLFDKDSFQETLSGWAKGVVVGARLGGIPFGVIGVETATVDNTTPADPANPDS
 IEMSTSEAGQVWPNSAFKTSQAINDFNHGEALPLMILANWRGFSGGQRDMYNEVLKYGSFIVDALVDYKQPIMV
 YIPPTGELRGGSWVVVDPTINSMMEMYADVESRGGVLEPEGMVGIKYRRDKLLDTMARLDPEYSSLKKQLEESP
 30 DSEELKVKLSVREKSLMPIYQQISVQFADLHDRAGRMEAKGVIREALVWKDARRFFFFWRIRRRRLVEEYLITKINS
 ILPSCTRLECLARIKSWKPATLDQGS DRGVAEWF DENS DAVSARLSELKKDASAQSFASQLRKDRQGT LQGMKQA
 LASLSEAERAELLKGL (SEQ ID NO: 4).

Some aspects of this invention provide a method for the manipulation of the activity
 35 of a stearoyl-CoA-desaturase (SCD) in a microbe for biofuel or biofuel precursor production.
 SCD is a $\Delta 9$ desaturase that inserts a double bond between C9 and C10 of stearic acid
 coupled to CoA, a key step in the generation of desaturated fatty acids and their derivatives,
 as described in more detail elsewhere herein. In some embodiments, the manipulation is an

overexpression. In some embodiments, the manipulation is effected by contacting a microbe for biofuel or biofuel precursor production with an expression construct comprising a nucleic acid coding for a SCD gene product, for example, a SCD protein, operably linked to a heterologous promoter, for example, a constitutive or an inducible promoter. In some
 5 embodiments, the nucleic acid coding for an SCD gene product comprises the coding sequence of SEQ ID NO: 5. In some embodiments, the SCD is *Y. lipolytica* SCD, for example, *Y. lipolytica* SCD comprising the amino acid sequence of SEQ ID NO: 6. In some embodiments, the microbe is *Y. lipolytica*. In some embodiments, manipulation of the activity of a SCD in a microbe is effected to confer a beneficial phenotype for large-scale
 10 carbohydrate to lipid conversion, for example increased lipid synthesis rate, increased carbohydrate to lipid conversion efficiency, increased lipid storage and, increased growth rate, increased tolerance to elevated concentrations of a carbon source or a lipid product. Stearoyl-CoA Desaturase gene and gene product sequences are well known to those of skill in the art. Exemplary, representative gene and gene product sequences can be found under the
 15 entry for GeneID: 852825 in the NCBI database (www.ncbi.nlm.nih.gov).

Non-limiting examples of suitable sequences of SCD nucleic acid and protein sequences are provided below. Additional suitable SCD sequences, including sequences from other species, will be apparent to those of skill in the art, and the invention is not limited in this respect.

20
 >gil50548052|reflXM_501496.1| *Yarrowia lipolytica* YALI0C05951p (YALI0C05951g)
 mRNA, complete cds

ATGGTGAAAAACGTGGACCAAGTGGATCTCTCGCAGGTCGACACCATTGCCTCCGGCCGAGATGTCAACTACAAG
 GTCAAGTACACCTCCGGCGTTAAGATGAGCCAGGGCGCCTACGACGACAAGGGCCGCCACATTTCCGAGCAGCCC
 25 TTCACCTGGGCCAACTGGCACCAGCACATCAACTGGCTCAACTTCATTCTGGTGATTGCGCTGCCTCTGTCGTCC
 TTTGCTGCCGCTCCCTTCGTCTCCTTCAACTGGAAGACCGCCGCGTTTGCTGTGGCTATTACATGTGCACCGGT
 CTCGGTATCACCGCCGGCTACCACCGAATGTGGGCCCATCGAGCCTACAAGGCCGCTCTGCCCGTTTGAATCATC
 CTTGCTCTGTTTGGAGGAGGAGCTGTGAGGGCTCCATCCGATGGTGGGCCTCGTCTCACCGAGTCCACCACCGA
 TGGACCGACTCCAACAAGGACCCTTACGACGCCCGAAAGGGATTCTGGTTCTCCCACTTTGGCTGGATGCTGCTT
 30 GTGCCCAACCCCAAGAACAAGGGCCGAAGTACATTTCTGACCTCAACAACGACTGGGTTGTCCGACTCCAGCAC
 AAGTACTACGTTTACGTTCTCGTCTTCATGGCCATTGTTCTGCCACCCCTCGTCTGTGGCTTTGGCTGGGGCGAC
 TGGAAAGGGAGGTCTTGTCTACGCCGGTATCATGCGATACACCTTTGTGCAGCAGGTGACTTTCTGTGTCAACTCC
 CTTGCCCCACTGGATTGGAGAGCAGCCCTTCGACGACCGACGAAGTCCCCGAGACCACGCTCTTACCGCCCTGGTC
 ACCTTTGGAGAGGGCTACCACAAGTCCACCACGAGTTCCCTCGGACTACCGAAACGCCCTCATCTGGTACCAG
 35 TACGACCCACCAAGTGGCTCATCTGGACCCTCAAGCAGGTTGGTCTCGCCTGGGACCTCCAGACCTTCTCCAG

AACGCCATCGAGCAGGGTCTCGTGCAGCAGCGACAGAAGAAGCTGGACAAGTGGCGAAACAACCTCAACTGGGGT
 ATCCCCATTGAGCAGCTGCCTGTCATTGAGTTTGAGGAGTTCCAAGAGCAGGCCAAGACCCGAGATCTGGTTCTC
 ATTTCTGGCATTGTCCACGACGTGTCTGCCTTTGTCGAGCACCACCCTGGTGGAAAGGCCCTCATTATGAGCGCC
 GTCGGCAAGGACGGTACCGCTGTCTTCAACGGAGGTGTCTACCGACACTCCAACGCTGGCCACAACCTGCTTGCC
 5 ACCATGCGAGTTTTCGGTCATTTCGAGGCGGCATGGAGGTTGAGGTGTGGAAGACTGCCCAGAACGAAAAGAAGGAC
 CAGAACATTGTCTCCGATGAGAGTGGAAACCGAATCCACCGAGCTGGTCTCCAGGCCACCCGGGTCGAGAACCCC
 GGTATGTCTGGCATGGCTGCTTAG (SEQ ID NO:5)

>gil50548053|reflXP_501496.1| YALI0C05951p [*Yarrowia lipolytica*]

10 MVKNVDQVDLSQVDTIASGRDVNYKVKYTSQVKMSQAYDDKGRHISEQPFTWANWHQHINWLNFLVIALPLSS
 FAAAPFVSFNWKTAAFAVGYYMCTGLGITAGYHRMWAHRAKALPVRIILALFGGGAVEGSIRWWASSHRVHHR
 WTDNKNPDYDARKGFWFSEHFGWMLLVNPNKNGRTDISDLNNDWVRLQHKYYVYVLVFMIAIVLPTLVCGFGWGD
 WKGGGLVYAGIMRYTFVQQVTFVNSLAHWIGEQQPFDDRRTPRDHALTALVTFGEGYHNFHHEFPSDYRNALIWYQ
 YDPTKWLIIWTLKQVGLAWDLQTFSQNAIEQGLVQQRQKLDKWRNNLNWGIPIEQLPVIEFEFQEQAQTRDLVL
 15 ISGIVHDVSAFVEHHPGGKALIMSAVGKDGTAVFNGGVYRHSNAGHNLLATMRVSVIRGGMEVEVWKTQNEKKD
 QNIVSDESGNRIHRAGLQATRVENPGMSGMAA (SEQ ID NO:6)

Some aspects of this invention provide a method for the manipulation of the activity
 of an ATP-citrate lyase (ACL) in a microbe for biofuel or biofuel precursor production. ACL
 20 provides cytosolic acetyl-CoA by cleaving citrate which is shuttled out of the mitochondria as
 a product of the TCA cycle. Cleaving citrate into oxaloacetate and acetyl-CoA, ACL gene
 products provide an acetyl-CoA substrate for ACC, which then mediates the conversion of
 acetyl-CoA, the main C2-precursor in fatty acid synthesis, to malonyl-CoA, which is
 considered the first committed step in fatty acid synthesis, as described in more detail
 25 elsewhere herein. In some embodiments, an ACL gene product is a protein composed of two
 subunits encoded by separate genes. In some embodiments, an ACL gene product is
 composed of two subunits encoded by the same gene. In some embodiments, the
 manipulation is an overexpression. In some embodiments, the manipulation is effected by
 contacting a microbe for biofuel or biofuel precursor production with an expression construct
 30 comprising a nucleic acid coding for an ACL gene product, for example, an ACL protein,
 operably linked to a heterologous promoter, for example, a constitutive or an inducible
 promoter. In some embodiments, the nucleic acid coding for an ACL gene product comprises
 the coding sequences of SEQ ID NO: 7 and SEQ ID NO: 9. In some embodiments, the ACL
 is *Y. lipolytica* ACL, for example, *Y. lipolytica* ACL comprising the amino acid sequences of
 35 SEQ ID NO: 8 and SEQ ID NO: 10. In some embodiments, the microbe is *Y. lipolytica*. In

some embodiments, manipulation of the activity of a ACL in a microbe is effected to confer a beneficial phenotype for large-scale carbohydrate to lipid conversion, for example increased lipid synthesis rate, increased carbohydrate to lipid conversion efficiency, increased lipid storage and, increased growth rate, increased tolerance to elevated concentrations of a carbon source or a lipid product. ATP-citrate lyase gene and gene product sequences are well known to those of skill in the art. Exemplary, representative gene and gene product sequences can be found under the entry for GeneID: 2912101 and 2910381 in the NCBI database (www.ncbi.nlm.nih.gov).

Non-limiting examples of suitable sequences of ACL nucleic acid and protein sequences are provided below. Additional suitable ACL sequences, including sequences from other species, will be apparent to those of skill in the art, and the invention is not limited in this respect.

ATP Citrate Lyase (*Yarrowia lipolytica*) subunit 1, ACL1 DNA

YALI0E34793g
XM_504787

atgtctgccaacgagaaacatctcccgattcgacgcccctgtgggcaaggagcaccgacctacgagctcttccat
aaccacacacgatctttcgtctatggtctccagcctcgagcctgccagggtatgctggacttcgacttcacatctgt
aagcgagagaacccctccgtggccggtgtcatctatcccttcggcgccagttcgtcaccaagatgtactggggc
accaaggagactcttctccctgtctaccagcaggtcgagaaggccgctgccaaagcaccgaggtcgatgtcgtg
gtcaactttgcctcctctcgatccgtctactcctctaccatggagctgctcgagtaccccgagttccgaaccatc
gccattattgccgaggggtgtccccgagcgacgagcccgagagatcctccacaaggcccagaagaagggtgtgacc
atcattgggtcccgctaccgtcggaggtatcaagcccgggtgcttcaagggttgaaacaccggaggtatgatggac
aacattgtgcctccaagctctaccgaccgggtccgttgctacgtctccaagtccggaggaatgtccaacgag
ctgaacaacattatctctcacaccaccgacggtgtctacgagggtattgctattgggtggtagccgataccctggt
actaccttcattgaccatatacctgcgatacagggccgaccccaagtgtgaagatcatcgctcctccttggtgaggtt
gggtggtgttgaggagtaccgagtcacgaggtgttaagaacggccagatcaagaagcccatcgctcgcttggggc
attggtacttggtgcctccatgttcaagactgaggttcagttcggccacgcccgtccatggccaactccgacctg
gagactgccaaaggctaagaacgccgccatgaagtctgctggcttctacgtccccgataccttcgaggacatgcc
gaggtccttgccgagctctacgagaagatggtcgccaagggcgagctgtctcgaatctctgagcctgaggtcccc
aagatccccattgactactcttggggcccaggagcttgggtcttatccgaaagcccgtgctttcatctccactatt
tccgatgaccgaggccaggagcttctgtacgctggcatgccatttccgaggttttcaaggaggacattgggtatc
ggcggtgtcatgtctctgctgtggttccgacgacgactccccgactacgcctccaagtttcttgagatgggtctc
atgcttactgctgaccacggtcccgccgtatccggtgcatgaacaccattatcaccacccgagctggtaaggat
ctcattttcttccctgggtgctggtctcctgaccattgggtaccgattcggaggtgctcttgacgggtgctgccacc
gagttcaccactgcctacgacaagggtctgtccccccgacagttcgttgataccatgcgaaagcagaacaagctg

attcctggtattggccatcgagtcgaagtcctcgaacaacccccgatttccgagtcgagcttgtcaaggactttgtt
aagaagaacttccccccaccagctgctcgactacgcccttgctgtcgaggaggtcaccacctccaagaaggac
aacctgattctgaacgttgacggtgctattgctgtttcttttgcgatctcatgcgatcttgcggtgcctttact
gtggaggagactgaggactacctcaagaacggtgttctcaacggtctgttcggttctcggtcgatccattggtctc
5 attgcccaccatctcgatcagaagcgactcaagaccggtctgtaccgacatccttgggacgatatcacctacctg
gttggccaggaggctatccagaagaagcgagtcgagatcagcgccggcgacgtttccaaggccaagactcgatca
tag (SEQ ID NO: 7)

ATP Citrate Lyase (*Yarrowia lipolytica*) subunit 1, ACL1 Protein

10 YALI0E34793p
XP_504787

MSANENISRFDAPVGKEHPAYELFHNHTRSFVYGLQPRACQGMDFDFICKRENPSVAGVIYPFGGQFVTKMYWG
TKETLLPVYQQVEKAAAKHPEVDVVNFASSRSVYSSTMELLEYPQFRTIAIIAEGVPERRAREILHKAQKKGVT
IIGPATVGGIKPGCFKVGNTGGMDNIVASKLYRPGSVAYVSKSGGMSNELNNIIISHTTDGVYEGIAIGGDRYPG
15 TTFIDHILRYEADPKCKIIVLLGEVGGVEEYRVIEAVKNGQIKKPIVAWAIGTCASMFKTEVQFGHAGSMANS DL
ETAKAKNAAMKSAGFYVPDTFEDMPEVLAELYEKMVAKGELSRISEPEVPKIPIDYSWAQELGLIRKPAAFISTI
SDDRGQELLYAGMPISEVFKEDIGIGVMSLLWFRRLPDYASKFLEMVLMLTADHGPAVSGAMNTIIITRAGKD
LISSLVAGLLTIGTRFGGALDGAATEFTTAYDKGLSPRQFVDTMRKQNKLIPIGIGHRVKSRNNPDFRVELVKDFV
KKNFPSTQLLDYALAVEEVTTSKKDNLIILNVDGAIIVSFVDLMRSCGAFTVEETEDYLKNGVLNGLFVLGRSIGL
20 IAHHLDDQKRLKTGLYRHPWDDITYLVGQEIQKKRVEISAGDVSKAKTRS (SEQ ID NO: 8)

ATP Citrate lyase (*Yarrowia lipolytica*) subunit 2, ACL2 DNA

YALI0D24431g
XM_503231

25 atgtcagcgaaatccattcacgaggccgacggcaaggccctgctcgacacttttctgtccaaggcgcccggtgtgg
gccgagcagcagcccatcaacacgtttgaaatgggcacaccaagctggcgtctctgacgttcgaggacggcggtg
gcccccgagcagatcttcgccgcccgtgaaaagacctaccctggctgctggagtccggcgccaagtttgtggcc
aagcccgaccagctcatcaagcgacgaggcaaggccggcctgctggtactcaacaagtcgtgggaggagtgcaag
ccctggatcgccgagcgggccgccaagcccatcaacgtggagggcattgacggagtgtgcgaacgttcctggtc
30 gagccctttgtgccccacgaccagaagcacgagtactacatcaacatccactccgtgagagggcgactggatc
ctcttctaccacgagggaggagtcgacgtcggcgacgtggacgccaaggccgccaagatcctcatccccgttgac
attgagaacgagtaccctccaacgccacgctcaccaaggagctgctggcacacgtgcccaggaccagcaccag
accctgctcgacttcatcaaccggctctacgccgtctacgtcgatctgcagtttacgtatctggagatcaacccc
ctgggtcgtgatccccaccgcccaggcgctcgaggtccactacctggatcttgccggcaagctcgaccagaccgca
35 gagtttgagtgcggccccaagtgggctgctgcgcggtccccgcgctctgggcccaggctcgtcaccattgacgcc
ggctccaccaaggtgtccatcgacgccggccccgccatggtcttccccgctcctttcggtcgagagctgtccaag
gaggaggcgtagattgcggagctcgattccaagaccggagcttctctgaagctgactgttctcaatgccaagggc

cgaatctggacccttgtggctggtggaggagcctccgtcgtctacgccgacgccattgcgtctgccggctttgct
gacgagctcgccaactacggcgagtactctggcgtcccaacgagacccagacctacgagtacgcaaaaccgta
ctggatctcatgacccggggcgacgctcaccgagggcaaggtactgttcattggcggaggaatcgccaacttc
accaggttggatccaccttcaagggcatcatccgggccttcgggactaccagtcttctctgcacaaccacaag
5 gtgaagatttacgtgcgacgaggcgggtcccaactggcaggagggtctgcggttgatcaagtcggctggcgacgag
ctgaatctgcccattggagatttacggccccgacatgcacgtgtcgggtattgttcctttggctctgcttgaaag
cggcccaagaatgtcaagccttttggcaccggaccttctactgaggcttcactcctctcgaggttaa (SEQ
ID NO: 9)

10 ATP Citrate lyase (*Yarrowia lipolytica*) subunit 2, ACL2 Protein

YALI0D24431p

XP_503231

MSAKSIHEADGKALLAHFLSKAPVWAEQQPINTFEMGTPKLASLTFEDGVAPEQIFAAAEKTYPWLLESGAKFVA
KPDQLIKRRGKAGLLVLNKSWECKPWIAERAAKPINVEGIDGVLRTFLVEPFVPHDQKHEYYINIHSVREGDWI
15 LFYHEGGVDVGDVDAKAAKILIPVDIENEYPSNATLTKELLAHVPEQHQTLTLLDFINRLYAVYVDLQFTYLEINP
LVVIPTAQGVEVHYLDLAGKLDQTAEFECGPKWAAARSPAALGQVVTIDAGSTKVSIDAGPAMVFPAPFGRELSK
EEAYIAELDSKTGASLKLTVLNAKGRIWTLVAGGGASVYADAIASAGFADELANYGEYS GAPNETQTYEYAKTV
LDLMTRGDAHPEGKVLFIGGGIANFTQVGSTFKGIIRAFRDYQSSLHNHVKIYVRRGGPNWQEGLRLIKSAGDE
LNLPMEIYGPD MHVSGIVPLALLGKRPNVKNPFGTGPSTEASTPLGV (SEQ ID NO: 10)

20

Some aspects of this invention provide oleaginous microbes for oil production comprising any of the modifications described herein, for example, a DGA1 modification as described herein, an ACC1 modification as described herein, and/or an SCD modification as described herein. In some embodiments, a modified oleaginous microbe is provided that
25 comprises a push modification as described herein and a pull modification as described herein. In some embodiments, the push modification comprises overexpression of an ACC1 gene product. In some embodiments, the pull modification comprises overexpression of a DGA1 and/or an SCD gene product.

Some aspects of this invention provide nucleic acids coding for a gene product
30 conferring a required and/or desired phenotype for biofuel or biofuel precursor production to a microbe, for example, *Y. lipolytica*. In some embodiments, the nucleic acid is a nucleic acid derived from *Y. lipolytica*. In some embodiments, the nucleic acid encodes a DGA1 gene product, for example, a DGA1 protein. In some embodiments, the nucleic acid encodes an ACC1 gene product, for example, an ACC1 protein. In some embodiments, the nucleic acid

encodes a desaturase, for example a $\Delta 9$ desaturase. In some embodiments, the nucleic acid encodes *Y. lipolytica* $\Delta 9$ desaturase (SCD). In some embodiments, a nucleic acid is provided that encodes a combination of gene products, for example in multiple cistrons, comprising a gene product the overexpression of which represents a push modification of lipid biosynthesis (e.g., an ACC1 gene product), and a gene product the overexpression of which represents a pull modification of lipid biosynthesis (e.g., a DGA1 and/or SCD gene product).

The term "nucleic acid" refers to a molecule comprising multiple linked nucleotides. "Nucleic acid" and "nucleic acid molecule" are used interchangeably and refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms also include polynucleosides (i.e., a polynucleotide minus a phosphate) and any other organic base containing nucleic acid. The organic bases include adenine, uracil, guanine, thymine, cytosine and inosine. The nucleic acids may be single or double stranded. The nucleic acid may be naturally or non-naturally occurring. Nucleic acids can be obtained from natural sources, or can be synthesized using a nucleic acid synthesizer (i.e., synthetic). Isolation of nucleic acids are routinely performed in the art and suitable methods can be found in standard molecular biology textbooks. (See, for example, Maniatis' Handbook of Molecular Biology.) The nucleic acid may be DNA or RNA, such as genomic DNA, mitochondrial DNA, mRNA, cDNA, rRNA, miRNA, PNA or LNA, or a combination thereof, as described herein. Non-naturally occurring nucleic acids such as bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs) can also be used in accordance with some aspects of this invention.

Some aspects of this invention relate to the use of nucleic acid derivatives. The use of certain nucleic acid derivatives may increase the stability of the nucleic acids of the invention by preventing their digestion, particularly when they are exposed to biological samples that may contain nucleases. As used herein, a nucleic acid derivative is a non-naturally occurring nucleic acid or a unit thereof. Nucleic acid derivatives may contain non-naturally occurring elements such as non-naturally occurring nucleotides and non-naturally occurring backbone linkages. Nucleic acid derivatives according to some aspects of this invention may contain backbone modifications such as but not limited to phosphorothioate linkages, phosphodiester modified nucleic acids, combinations of phosphodiester and phosphorothioate nucleic acid, methylphosphonate, alkylphosphonates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl

esters, methylphosphorothioate, phosphorodithioate, p-ethoxy, and combinations thereof. The backbone composition of the nucleic acids may be homogeneous or heterogeneous.

Nucleic acid derivatives according to some aspects of this invention may contain substitutions or modifications in the sugars and/or bases. For example, some nucleic acid derivatives may include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position (e.g., an 2'-O-alkylated ribose group). Nucleic acid derivatives may include non-ribose sugars such as arabinose. Nucleic acid derivatives may contain substituted purines and pyrimidines such as C-5 propyne modified bases, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, 2-thiouracil and pseudoisocytosine.

In some embodiments, a nucleic acid may comprise a peptide nucleic acid (PNA), a locked nucleic acid (LNA), DNA, RNA, or a co-nucleic acids of the above such as DNA-LNA co-nucleic acid.

As used herein the term "isolated nucleic acid molecule" refers to a nucleic acid that is not in its natural environment, for example a nucleic acid that has been (i) extracted and/or purified from a cell or microbe, for example, a bacteria or yeast, by methods known in the art, for example, by alkaline lysis of the host cell and subsequent purification of the nucleic acid, for example, by a silica adsorption procedure; (ii) amplified *in vitro*, for example, by polymerase chain reaction (PCR); (iii) recombinantly produced by cloning, for example, a nucleic acid cloned into an expression vector; (iv) fragmented and size separated, for example, by enzymatic digest *in vitro* or by shearing and subsequent gel separation; or (v) synthesized by, for example, chemical synthesis. In some embodiments, the term "isolated nucleic acid molecule" refers to (vi) an nucleic acid that is chemically markedly different from any naturally occurring nucleic acid. In some embodiments, an isolated nucleic acid can readily be manipulated by recombinant DNA techniques well known in the art.

Accordingly, a nucleic acid cloned into a vector, or a nucleic acid delivered to a host cell and integrated into the host genome is considered isolated but a nucleic acid in its native state in its natural host, for example, in the genome of the host, is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a small percentage of the

material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein.

Some aspects of this invention relate to nucleic acids encoding a gene product conferring a required or desirable phenotype to a microbe for biofuel or biofuel precursor production which are linked to a promoter or other transcription activating element. In some embodiments, the nucleic acid encoding the gene product and linked to a promoter is comprised in an expression vector or expression construct. As used herein, the terms "expression vector" or "expression construct" refer to a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host microbe, for example, an oleaginous yeast. In some embodiments, the expression vector may be part of a plasmid, virus, or nucleic acid fragment. In some embodiments, the expression vector includes the coding nucleic acid to be transcribed operably linked to a promoter. A promoter is a nucleic acid element that facilitates transcription of a nucleic acid to be transcribed. A promoter is typically located on the same strand and upstream (or 5') of the nucleic acid sequence the transcription of which it controls. In some embodiments, the expression vector includes the coding nucleic acid to be transcribed operably linked to a heterologous promoter. A heterologous promoter is a promoter not naturally operably linked to a given nucleic acid sequence. For example, the DGA1 gene in *Y. lipolytica* is naturally operably linked to the *Y. lipolytica* DGA1 gene promoter. Any promoter other than the wildtype *Y. lipolytica* DGA1 gene promoter operably linked to the DGA1 gene, or parts thereof, for example in an expression construct, would, therefore, be a heterologous promoter in this context. For example, a TEF1 promoter linked to a nucleic acid encoding a DGA1 gene product is a heterologous promoter in the DGA1 context.

In some embodiments, the expression vector includes a coding nucleic acid, for example, a nucleic acid encoding a DGA1, ACC1, and/or SCD gene product, operably linked to a constitutive promoter. The term "constitutive promoter" refers to a promoter that allows for continual transcription of its associated gene. In some embodiments, the expression vector includes a coding nucleic acid, for example, a nucleic acid encoding a DGA1, ACC1, and/or SCD gene product, operably linked to an inducible promoter. The term "inducible promoter", interchangeably used herein with the term "conditional promoter", refers to a promoter that allows for transcription of its associated gene only in the presence or absence of biotic or

abiotic factors. Drug-inducible promoters, for example tetracycline/doxycycline inducible promoters, tamoxifen-inducible promoters, as well as promoters that depend on a recombination event in order to be active, for example the cre-mediated recombination of loxP sites, are examples of inducible promoters that are well known in the art.

5 Some aspects of this disclosure relate to the surprising discovery that overexpression of a given gene product from a heterologous promoter in oleaginous microbes can be significantly enhanced by including an intron in the respective expression construct. Some aspects of this disclosure provide an intron-enhanced constitutive promoter for gene overexpression in oleaginous microbes and expression constructs and vectors comprising this
10 intron-enhanced promoter. In some embodiments, an intron-enhanced TEF promoter is provided, that comprises a TEF promoter sequence, a transcription start site, an intronic sequence downstream of the transcription start site, and a coding nucleic acid sequence, for example, a nucleic acid sequence encoding a DGA1, ACC1 and/or SCD gene product. In some embodiments, the intron is positioned downstream of the translation start site, yet
15 within the open reading frame of the gene sequence, e.g., after the start codon, but before the termination site of the nucleic acid sequence encoding the gene product. In some embodiments, the intron is positioned immediately downstream of the translation start site, e.g., an ATG start codon, yet upstream of the remainder of the coding sequence. For illustration purposes, a non-limiting, exemplary structure of an intron-enhanced expression
20 construct is provided as follows:

5'--TEF promoter -- transcription start site -- intron -- DGA1 coding sequence--3'. Another non-limiting, exemplary structure of an intron-enhanced expression construct is provided as follows:

5'--TEF promoter -- transcription start site -- start codon -- intron -- DGA1 coding sequence -
25 - stop codon -- 3'. Expression constructs for ACC1 and SCD gene products would have the DGA1 coding sequence substituted for an ACC or SCD coding sequence, respectively.

Suitable TEF promoter sequences as well as suitable intron sequences will be apparent to those of skill in the art. Some intron-less TEF promoter sequences are disclosed, for example, in U.S. Patent #6,265,185. Some exemplary, representative sequences are
30 provided below. However, it will be understood that the invention is not limited in this respect.

Exemplary TEF promoter sequence:

agagaccgggttgccggcgcatTTgtgtcccaaaaaacagccccaattgcccccaattgaccccaaattgacccagtagcgggccc
 ccccggcgagagcccccttctccccacatatcaaacctccccgggtcccacacttgccgttaaggcgtaggggtactgcagtctgga
 atctacgcttggtcagactttgtactagtttcttctgtctggccatccgggtaacctatgccggacgcaaaatagactactgaaaatttttgc
 5 tttgtggtgggacttttagccaagggtataaaagaccaccgtccccgaattaccttctctctctctctctcttctgtaactcacacc
 gaaatcgtaagcatttctctctgagtataagaatcattcaaa (SEQ ID NO:11)

Exemplary intron sequence:

gtgagtttcagaggcagcagcaattgccacgggctttgagcacacggccgggtgtggtccattcccatcgacacaagacgccacgt
 catccgaccagcacttttgcagtactaaccgcag (SEQ ID NO: 12)

Exemplary TEF promoter-intron sequence comprising a start codon (ATG) between
 the promoter and the intron sequences:

agagaccgggttgccggcgcatTTgtgtcccaaaaaacagccccaattgcccccaattgaccccaaattgacccagtagcgggccc
 ccccggcgagagcccccttctccccacatatcaaacctccccgggtcccacacttgccgttaaggcgtaggggtactgcagtctgga
 atctacgcttggtcagactttgtactagtttcttctgtctggccatccgggtaacctatgccggacgcaaaatagactactgaaaatttttgc
 15 tttgtggtgggacttttagccaagggtataaaagaccaccgtccccgaattaccttctctctctctctctcttctgtaactcacacc
gaaatcgtaagcatttctctctgagtataagaatcattcaaaATGgtgagtttcagaggcagcagcaattgccacgggctttgagca
cacggccgggtgtggtccattcccatcgacacaagacgccacgtcatccgaccagcacttttgcagtactaaccgcag (SEQ
 ID NO: 13)

Methods to deliver expression vectors or expression constructs into microbes, for
 example, into yeast cells, are well known to those of skill in the art. Nucleic acids, including
 expression vectors, can be delivered to prokaryotic and eukaryotic microbes by various
 methods well known to those of skill in the relevant biological arts. Methods for the delivery
 of nucleic acids to a microbe in accordance to some aspects of this invention, include, but are
 not limited to, different chemical, electrochemical and biological approaches, for example,
 25 heat shock transformation, electroporation, transfection, for example liposome-mediated
 transfection, DEAE-Dextran-mediated transfection or calcium phosphate transfection. In
 some embodiments, a nucleic acid construct, for example an expression construct comprising
 a combination of DGA1, ACC1, and/or SCD encoding nucleic acid sequences, is introduced
 into the host microbe using a vehicle, or vector, for transferring genetic material. Vectors for
 30 transferring genetic material to microbes are well known to those of skill in the art and
 include, for example, plasmids, artificial chromosomes, and viral vectors. Methods for the
 construction of nucleic acid constructs, including expression constructs comprising

constitutive or inducible heterologous promoters, knockout and knockdown constructs, as well as methods and vectors for the delivery of a nucleic acid or nucleic acid construct to a microbe are well known to those of skill in the art, and are described, for example, in J. Sambrook and D. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press; 3rd edition (January 15, 2001); David C. Amberg, Daniel J. Burke; and Jeffrey N. Strathern, *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, Cold Spring Harbor Laboratory Press (April 2005); John N. Abelson, Melvin I. Simon, Christine Guthrie, and Gerald R. Fink, *Guide to Yeast Genetics and Molecular Biology, Part A, Volume 194* (Methods in Enzymology Series, 194), Academic Press (March 11, 2004); Christine Guthrie and Gerald R. Fink, *Guide to Yeast Genetics and Molecular and Cell Biology, Part B, Volume 350* (Methods in Enzymology, Vol 350), Academic Press; 1st edition (July 2, 2002); Christine Guthrie and Gerald R. Fink, *Guide to Yeast Genetics and Molecular and Cell Biology, Part C, Volume 351*, Academic Press; 1st edition (July 9, 2002); Gregory N. Stephanopoulos, Aristos A. Aristidou and Jens Nielsen, *Metabolic Engineering: Principles and Methodologies*, Academic Press; 1 edition (October 16, 1998); and Christina Smolke, *The Metabolic Pathway Engineering Handbook: Fundamentals*, CRC Press; 1 edition (July 28, 2009), all of which are incorporated by reference herein.

In some embodiments, the native promoter of a gene encoding a gene product conferring a required or desirable phenotype to a microbe, for example, the native DGA1, ACC1, or SCD promoter, is modified in the microbe to alter the regulation of its transcriptional activity. In some embodiment, the modified promoter exhibits an increased transcriptional activity as compared to its unmodified counterpart. The term “modified promoter”, as used herein, refers to a promoter the nucleotide sequence of which has been artificially altered. Nucleotide deletion(s), insertion(s) or mutation(s), alone or in combination, are examples of such artificial alterations. Artificial promoter alterations can be effected in a targeted fashion, for example by homologous recombination approaches, such as gene targeting, knockout, knock in, site-directed mutagenesis, or artificial zinc finger nuclease-mediated strategies. Alternatively, such alterations may be effected by a random or quasi-random event, such as irradiation or non-targeted nucleotide integration and subsequent selection. Promoter modifications, in general, are fashioned in order to modulate the transcriptional activation properties of the respective promoter. For example, the disruption or deletion of a regulatory element mediating the repression of a DGA1, ACC1, or SCD

promoter in response to elevated intracellular fatty acid levels would lead to continued transcriptional activation of the respective gene even under conditions of elevated intracellular fatty acid levels. Similarly, the insertion of a constitutively active transcriptional activator element into a conditional promoter region may effect overexpression of the
5 respective gene under normally inhibitive conditions. Methods for the targeted disruption of a native promoter, for example, a native DGA1, ACC1, or SCD promoter, in a microbe, for example, for targeted disruption resulting in an increased transcription rate, are well known to those of skill in the art.

Some aspects of this invention relate to engineering of a microbe, for example, *Y. lipolytica*, to exhibit a required and/or desirable phenotype for large-scale production of a biofuel or biofuel precursor. Some aspects of this invention relate to the metabolic engineering of the lipid synthesis pathway in order to yield a microbe optimized for biofuel production. Some aspects of this invention relate to metabolic engineering that comprises a combination of genetic modifications modulating the expression of genes regulating carbon
15 flux into a lipid synthesis pathway in order to yield a microbe optimized for biofuel production. In some embodiments, the combination of genetic modifications includes a push modification and a pull modification. In some embodiments, the push modification comprises a genetic modification that increases the level of metabolites, acetyl-CoA, ATP, or NADPH for lipid synthesis in a cell, for example, overexpression of an ACC1 gene product.
20 In some embodiments, the pull modification is a genetic modification that decreases the level of a product or intermediary of lipid synthesis that exhibits a feedback inhibitory function, for example, a fatty acid. In some embodiments, the pull modification comprises overexpression of a DGA1 and/or an SCD gene product.

Some aspects of this invention provide methods to greatly increase the efficiency of *Y. lipolytica* mediated carbon source to lipid conversion by modulating *Y. lipolytica*'s native lipid metabolism. Remarkably and unexpectedly, combinations of push-and-pull modifications of lipid metabolism according to some methods provided by this invention confers significantly increased carbon flux to lipid synthesis pathways as compared to individual modifications modulating only push or pull processes, respectively.

30 Some aspects of this invention relate to a microbe engineered and/or optimized for large-scale biofuel or biofuel precursor production. In some embodiments, an engineered microbe is provided that has been manipulated by a method or using a nucleic acid or protein

provided by some aspects of this invention, for example, an expression construct or a combination of expression constructs as provided herein, resulting in the overexpression of a combination of a gene product mediating a push process of lipid synthesis (e.g., an ACC1 product), and a gene product mediating a pull process of lipid synthesis (e.g., a DGA1 and/or SCD gene product). In some embodiments, an engineered microbe is provided, that overexpresses a push-and-pull combination of gene products that, according to some aspects of this invention, confers a required and/or desirable phenotype for biofuel or biofuel precursor production to the microbe. In some embodiments, a microbe comprising an increased DGA1, ACC1, and/or SCD gene product activity is provided. In some embodiments, the microbe exhibits an increased fatty acid synthesis rate, an increased TAG storage, and/or an additional required or desirable trait.

The term “increased synthesis rate” or “increased rate of synthesis” as used herein in the context of microbial lipid synthesis, e.g., in the context of a fatty acid synthesis rate of an oil-producing microbe described herein, refers to a rate of synthesis in an engineered microbe that is increased as compared to the corresponding rate of synthesis in a wild-type microbe of the same species. E.g., an increased rate of TAG synthesis in an engineered *Y. lipolytica* microbe described herein refers to rate of lipid synthesis that is increased as compared to the rate of TAG synthesis in a wild-type *Y. lipolytica*. In some embodiments, an increased rate of lipid synthesis, e.g., of TAG or of total lipid synthesis, refers to a rate of fatty acid synthesis of a culture of cells, e.g., of a culture of engineered microbes. In some embodiments, an increased rate of lipid synthesis is a rate of lipid synthesis, e.g., of TAG synthesis or total lipid synthesis of at least 0.01g/L/h (grams of lipid per liter of culture per hour), at least 0.004g/L/h, at least 0.05g/L/h, at least 0.1g/L/h, at least 0.14g/L/h, at least 0.15g/L/h, at least 0.2g/L/h, at least 0.3g/L/h, at least 0.4g/L/h, at least 0.5g/L/h, at least 0.6g/L/h, at least 0.7g/L/h, at least 0.8g/L/h, at least 0.9g/L/h, at least 1g/L/h, at least 2g/L/h, at least 3g/L/h, at least 4g/L/h, at least 5g/L/h, at least 6g/L/h, at least 7g/L/h, at least 8g/L/h, at least 9g/L/h, at least 10g/L/h, or at least 25g/L/h.

In some embodiments, the rate of synthesis in this context is the rate of synthesis measured over a complete run of a bioreactor, e.g., calculating the rate of synthesis from the total amount of lipid, e.g., TAG, synthesized over the total time that the bioreactor was run or the total time lipid production was measured over. This type of synthesis rate is also referred to herein sometimes as “total lipid productivity” or “overall lipid productivity,” and it is

typically provided in g/L/h (grams of lipid produced per liter of culture medium per run time in hours). In some embodiments, an engineered microbe is provided, e.g., an engineered *Y. lipolytica* that overexpresses an ACC1 gene product, a DGA1 gene product, and/or an SCD gene product, that exhibits at least a 5-fold increase, at least a 6-fold increase, at least a 7-fold increase, at least an 8-fold increase, at least a 9-fold increase, at least a 10-fold increase, at least a 12-fold increase, at least a 10-fold increase, at least a 12.5-fold increase, at least a 15-fold increase, at least a 20-fold increase, at least a 30-fold increase, at least a 40-fold increase, at least a 50-fold increase, at least a 60-fold increase, at least a 70-fold increase, at least an 80-fold increase, at least a 90-fold increase, at least a 100-fold increase, at least a 500-fold increase, or at least a 1000-fold increase in total lipid productivity as compared to a wild-type microbe, e.g., a wild-type *Y. lipolytica*.

In some embodiments, an increased rate of total lipid synthesis or an increased total lipid productivity is at least 0.01g/L/h, at least 0.004g/L/h, at least 0.05g/L/h, at least 0.1g/L/h, at least 0.14g/L/h, at least 0.15g/L/h, at least 0.2g/L/h, at least 0.3g/L/h, at least 0.4g/L/h, at least 0.5g/L/h, at least 0.6g/L/h, at least 0.7g/L/h, at least 0.8g/L/h, at least 0.9g/L/h, at least 1g/L/h, at least 2g/L/h, at least 3g/L/h, at least 4g/L/h, or at least 5g/L/h.

In some embodiments, the rate of synthesis is the maximum rate of synthesis, or the peak rate of synthesis, measured, e.g., under optimal growth conditions and exposure to nutrients. This type of synthesis rate is also referred to herein sometimes as “maximum lipid productivity”. In some embodiments, an increased maximum rate of lipid synthesis is a rate of lipid synthesis, e.g., of TAG synthesis, of at least 0.2g/L/h, at least 0.3g/L/h, at least 0.4g/L/h, at least 0.5g/L/h, at least 0.6g/L/h, at least 0.7g/L/h, at least 0.8g/L/h, at least 0.9g/L/h, at least 1g/L/h, at least 2g/L/h, at least 3g/L/h, at least 4g/L/h, at least 5g/L/h, at least 6g/L/h, at least 7g/L/h, at least 8g/L/h, at least 9g/L/h, at least 10g/L/h, or at least 25g/L/h.

In some embodiments, the engineered microbe is an oleaginous yeast, for example, *Y. lipolytica*. In some embodiments, an engineered yeast provided by this invention exhibits one or more highly desirable and unexpected phenotypic characteristics, for example: increased carbon to oil conversion rate or efficiency, increased lipid accumulation in a lipid body.

In some embodiments, an engineered microbe, for example, an engineered yeast, provided by aspects of this invention exhibits a carbon to oil conversion rate, also referred to herein as “lipid yield,” within the range of about 0.02g/g (g oil, lipid, or TAG produced/ g

carbon, e.g., glucose, acetate, or acetic acid consumed) to about 0.3 g/g. In some embodiments, the engineered microbe, for example, the engineered yeast, provided by aspects of this invention exhibits a carbon to oil conversion rate of about 0.010g/g, about 0.02g/g, about 0.025g/g, about 0.03g/g, about 0.04g/g, about 0.05g/g, about 0.06g/g, about 0.07g/g, about 0.075g/g, about 0.08g/g, about 0.09g/g, about 0.1g/g, about 0.11g/g, about 0.12g/g, about 0.13g/g, about 0.14g/g, about 0.15g/g, about 0.16g/g, about 0.17g/g, about 0.18g/g, about 0.19g/g, about 0.2g/g, about 0.21g/g, about 0.22g/g, about 0.23g/g, about 0.24g/g, about 0.25g/g, about 0.26g/g, about 0.27g/g, about 0.28g/g, about 0.29g/g, about 0.3g/g, about 0.31g/g, about 0.32g/g, or approaching theoretical values. In some embodiments, the engineered microbe, for example, the engineered yeast, provided by aspects of this invention exhibits a carbon to oil conversion rate of at least about 0.010g/g (g lipid produced/ g carbon, e.g., glucose, acetate, or acetic acid consumed), at least about 0.02g/g, at least about 0.025g/g, at least about 0.03g/g, at least about 0.04g/g, at least about 0.05g/g, at least about 0.06g/g, at least about 0.07g/g, at least about 0.075g/g, at least about 0.08g/g, at least about 0.09g/g, at least about 0.1g/g, at least about 0.11g/g, at least about 0.12g/g, at least about 0.13g/g, at least about 0.14g/g, at least about 0.15g/g, at least about 0.16g/g, at least about 0.17g/g, at least about 0.18g/g, at least about 0.19g/g, at least about 0.2g/g, at least about 0.21g/g, at least about 0.22g/g, at least about 0.23g/g, at least about 0.24g/g, at least about 0.25g/g, at least about 0.26g/g, at least about 0.27g/g, at least about 0.28g/g, at least about 0.29g/g, at least about 0.3g/g, at least about 0.31g/g, at least about 0.32g/g, or approaching theoretical values.

The term “lipid titer” as used herein in the context of microbial lipid synthesis, e.g., in the context of a fatty acid synthesis by an oil-producing microbe described herein, refers to an amount of lipid synthesized per volume of a microbial culture comprising the oil-producing microbe. In some embodiments, an engineered microbe, e.g., an engineered *Y. lipolytica* microbe described herein, can achieve or does achieve a lipid titer of at least 1g/L (grams of lipid per liter of microbial culture), at least 2g/L, at least 3g/L, at least 4g/L, at least 5g/L, at least 6g/L, at least 7g/L, at least 8g/L, at least 9g/L, at least 10g/L, at least 15g/L, at least 20g/L, at least 25g/L, at least 30g/L, at least 40g/L, at least 50g/L, at least 60g/L, at least 70g/L, at least 80g/L, at least 90g/L, at least 100g/L, at least 200g/L, or at least 250g/L.

In some embodiments, an engineered microbe as provided herein exhibits an increased lipid titer during carbon to oil conversion. The term “increased lipid titer” as used

herein in the context of microbial lipid synthesis, e.g., in the context of a fatty acid synthesis by an oil-producing microbe described herein, refers to an amount of lipid synthesized per volume of a microbial culture comprising the oil-producing microbe that is increased as compared to the corresponding lipid titer of a wild-type microbe of the same species and under the same conditions (e.g., in the same growth medium, with the same C/N ratio, the same amount of oxygen, the same pH, the same nutrients, and so forth). For example, an increased lipid titer achieved by an engineered *Y. lipolytica* microbe described herein refers to a lipid titer that is increased as compared to the lipid titer that can be achieved by a wild-type *Y. lipolytica* under identical conditions. In some embodiments, an increased lipid titer refers to a lipid titer of at least 1g/L (grams of lipid per liter of microbial culture), at least 2g/L, at least 3g/L, at least 4g/L, at least 5g/L, at least 6g/L, at least 7g/L, at least 8g/L, at least 9g/L, at least 10g/L, at least 15g/L, at least 20g/L, at least 25g/L, at least 30g/L, at least 40g/L, at least 50g/L, at least 60g/L, at least 70g/L, at least 80g/L, at least 90g/L, at least 100g/L, at least 200g/L, or at least 250g/L.

Some aspects of this invention provide engineered microbes for oil production that can use a variety of carbon sources, including, but not limited to fermentable sugars, for example, C6 sugars, such as glucose, and organic acids, e.g., acetic acid, and/or their salts, e.g., acetate.

Some aspects of this invention relate to cultures of genetically modified microbes provided herein. In some embodiments, the culture comprises a genetically modified microbe provided herein and a medium, for example, a liquid medium. In some embodiments, the culture comprises a genetically modified microbe provided herein and a carbon source, for example, a fermentable carbohydrate source, or an organic acid or salt thereof. In some embodiments, the culture comprises a genetically modified microbe provided herein and a salt and/or buffer establishing conditions of salinity, osmolarity, and pH, that are amenable to survival, growth, and/or carbohydrate to biofuel or biofuel precursor conversion by the microbe. In some embodiments, the culture comprises an additional component, for example, an additive. Non-limiting examples of additives are nutrients, enzymes, amino acids, albumin, growth factors, enzyme inhibitors (for example protease inhibitors), fatty acids, lipids, hormones (e.g., dexamethasone and gibberellic acid), trace elements, inorganic compounds (e.g., reducing agents, such as manganese), redox-regulators (e.g., antioxidants), stabilizing agents (e.g., dimethylsulfoxide), polyethylene glycol, polyvinylpyrrolidone (PVP),

gelatin, antibiotics (e.g., Brefeldin A), salts (e.g., NaCl), chelating agents (e.g., EDTA, EGTA), and enzymes (e.g., cellulase, dispase, hyaluronidase, or DNase). In some embodiments, the culture may comprise a drug inducing or inhibiting transcription from a conditional or inducible promoter, for example doxycycline, tetracycline, tamoxifen, IPTG, hormones, or metal ions.

While the specific culture conditions, for example, the concentration of the carbon source, will depend upon the respective engineered microorganism to be cultured, general methods and culture conditions for the generation of microbial cultures are well known to those of skill in the art, and are described, for example, in J. Sambrook and D. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press; 3rd edition (January 15, 2001); David C. Amberg, Daniel J. Burke; and Jeffrey N. Strathern, *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, Cold Spring Harbor Laboratory Press (April 2005); John N. Abelson, Melvin I. Simon, Christine Guthrie, and Gerald R. Fink, *Guide to Yeast Genetics and Molecular Biology, Part A, Volume 194* (Methods in Enzymology Series, 194), Academic Press (March 11, 2004); Christine Guthrie and Gerald R. Fink, *Guide to Yeast Genetics and Molecular and Cell Biology, Part B, Volume 350* (Methods in Enzymology, Vol 350), Academic Press; 1st edition (July 2, 2002); and Christine Guthrie and Gerald R. Fink, *Guide to Yeast Genetics and Molecular and Cell Biology, Part C, Volume 351*, Academic Press; 1st edition (July 9, 2002), all of which are incorporated by reference herein. For oil production, the cultures of engineered microbes described herein are cultured under conditions suitable for oil accumulation, as known in the art.

In some embodiments, the genetically modified microbe exhibits a growth advantage over wild type microbes of the same kind and/or over other microbes, for example, microbes commonly found to contaminate microbial cultures for carbon source to biofuel or biofuel precursor conversion. In some embodiments, the growth and/or proliferation advantage of an engineered microbe provided by aspects of this invention translates into the possibility of using non-sterile culturing and fermentation conditions for biofuel or biofuel precursor production, because the problem of culture overgrowth by contaminating microbes is mitigated or completely abolished. In some embodiments, an engineered microbe provided by aspects of this invention is cultured under non-sterile conditions for biofuel or biofuel precursor production. For example, in some embodiments, non-sterilized feedstock, non-

sterilized culture media, non-sterilized supplements, or a non-sterilized bioreactor (e.g. an open reactor under non-sterile conditions) is used for biofuel or biofuel precursor production.

A variety of different microbes can be genetically modified according to some aspects of this invention and used for industrial-scale biofuel or biofuel precursor production, for example, microbes from various sources of yeast, such as oleaginous yeast, bacteria, algae and fungi. Non-limiting examples of suitable yeast cells are cells from *Yarrowia lipolytica*, *Hansenula polymorpha*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *S. bayanus*, *S. K. lactis*, *Waltomyces lipofer*, *Mortierella alpine*, *Mortierella isabellina*, *Hansenula polymorpha*., *Mucor rouxii*, *Trichosporon cutaneu*, *Rhodotorula glutinis* *Saccharomyces diastasicus*, *Schwanniomyces occidentalis*, *S. cerevisiae*, *Pichia stipitis*, and *Schizosaccharomyces pombe*. Non-limiting examples of suitable bacteria are *Bacillus subtilis*, *Salmonella*, *Escherichia coli*, *Vibrio cholerae*, *Streptomyces*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas sp*, *Rhodococcus sp*, *Streptomyces sp*, and *Alcaligenes sp*. Non-limiting examples of suitable fungal cells can, for example, be cultured from species such as *Aspergillus shirousamii*, *Aspergillus niger* and *Trichoderma reesei*. Non-limiting examples of suitable algal cells are cells from *Neochloris oleoabundans*, *Scenedesmus obliquus*, *Nannochloropsis sp.*, *Dunaliella tertiolecta*, *Chlorella vulgaris*, *Chlorella emersonii*, and *Spirulina maxima*.

Some aspects of this invention provide methods for the production of biofuel or biofuel precursors using genetically modified microbes provided herein. In some embodiments, methods for biofuel or biofuel precursor production on an industrial scale are provided.

A variety of carbon sources can be converted into a biofuel or biofuel precursor using a method and/or a genetically modified microbe provided herein. In some embodiments, the carbon source comprises a carbohydrate. Sugars, starches, and fibers are non-limiting examples of carbohydrate sources suitable for conversion methods provided herein. According to some aspects of this invention, a carbohydrate source may comprise a refined and/or unrefined sugar, starch, and/or fiber, or a combination of any of these. Non-limiting examples of sugars are fermentable sugars, such as glucose, fructose, sucrose, xylose, and lactose. Non-limiting examples of starches are amylase and amylopectin. Non-limiting examples of fibers are plant fibers, such as cellulose, hemicellulose and wood fibers. Some aspects of this invention relate to the use of industrial byproducts, intermediates, or waste

products, for example raw plant extracts, molasses, stover, or sewage as a carbon source. In some embodiments, the carbon source is derived from algae. In some embodiments, algal biomass is produced specifically for use as a carbon source in microbe-mediated biofuel or biofuel precursor production.

5 In some embodiments, methods for the production of biofuel or biofuel precursor are provided that include the use of a cheap, abundant, and readily available carbon source feedstock as the carbon source. In some embodiments, cellulose or hemicellulose is used as the carbon source. In some embodiments, the cellulose or hemicellulose is derived from industrial by- or waste products. In some embodiments, the cellulose or hemicellulose is
10 derived directly from plant or algal biomass. Plant or algal biomass is one of the most abundant feedstocks and comprises a significant amount of non-fermentable sugars and fibers, for example, cellulose and hemi-cellulose. In some embodiments, biomass feedstock is pretreated to convert a non-fermentable sugar or fiber into a fermentable sugar, thus making them available for microbe growth and microbe-mediated biofuel or biofuel precursor
15 production. In some embodiments, the pretreatment of biomass feedstock includes depolymerizing cellulose and/or hemicellulose components to monomeric sugars using a pretreatment method known to those of skill in the art, for example, a dilute acid or ammonia fiber expansion (AFEX) method (see, e.g., Yang B, Wyman CE. *Dilute acid and autohydrolysis pretreatment*. Methods Mol Biol. 2009;581:103-14; Balan V, Bals B,
20 Chundawat SP, Marshall D, Dale BE, *Lignocellulosic biomass pretreatment using AFEX* Methods Mol Biol. 2009;581:61-77). Other methods for depolymerization of biomass polymers to monomeric sugars are well known to those of skill in the art and are contemplated to be used in some embodiments of this invention.

In some embodiments, a biomass feedstock containing non-fermentable sugars is
25 pretreated using a dilute acid method to depolymerize a non-fermentable sugar to a monomeric, fermentable sugar. In some embodiments, biomass is treated with dilute sulphuric acid at moderately mild temperatures for a defined period of time. For example, in some embodiments, the biomass is treated with about 0.5%, about 1%, about 2%, about 3%, about 4%, about 5%, or about 6% sulphuric acid. In some embodiments, the biomass is
30 treated at about 30°C, at about 37°C, at about 40°C, at about 50°C, at about 60°C, at about 70°C, at about 80°C, at about 90°C, at about 100°C, at about 110°C, at about 120°C, at about

130°C, at about 140°C, at about 150°C, at about 175°C, at about 200°C, or at above about 200°C.

In some embodiments, the resulting hydrolysate contains insoluble lignin and solubilized cellulosic and hemicellulosic polymers. The latter products can be further treated to generate hexose and pentose sugars such as glucose and xylose monomers by methods well known to those of skill in the art, for example, by treatment with cellulase or other hydrolyzing enzymes. In some embodiments, the pretreatment of non-fermentable sugars with dilute acid results in the generation of by-products that include toxic compounds which inhibit growth, decrease viability, and/or inhibit biofuel or biofuel precursor production of microbes not engineered according to aspects of this invention. In some embodiments, the pre-treated feedstock is washed, supplemented with media supporting microbial growth and biofuel or biofuel precursor production, and/or over-limed for detoxification.

In some embodiments, a biomass feedstock containing non-fermentable sugars is pretreated using an AFEX method to depolymerize a non-fermentable sugar to a monomeric, fermentable sugar. In some embodiments, biomass is treated with liquid ammonia at high temperature and pressure for a defined period of time. In some embodiments, biomass is treated for about 10 minutes, about 20 minutes, about 30 minutes, about 40 minutes, about 50 minutes, about 60 minutes, about 70 minutes, about 80 minutes, about 90 minutes, or longer. In some embodiments, biomass is treated at about 30°C, at about 37°C, at about 40°C, at about 50°C, at about 60°C, at about 70°C, at about 80°C, at about 90°C, at about 100°C, at about 110°C, at about 120°C, at about 130°C, at about 140°C, at about 150°C, at about 175°C, at about 200°C, or at above about 200°C. In some embodiments, the AFEX pretreatment results in the conversion of crystalline cellulose contained in the feedstock into an amorphous, fermentable form. In some embodiments, the AFEX pre-treated biomass feedstock does not contain significant amounts of toxic byproducts that inhibit microbial growth and/or biofuel or biofuel precursor production, and is used without prior detoxification for microbial biofuel or biofuel precursor production.

In some embodiments, biomass feedstock, with or without pre-treatment, is treated with an enzyme that hydrolyzes or depolymerizes sugar polymers, for example, with a cellulase or hemicellulase enzyme. In some embodiments, the feedstock is contacted with the enzyme in a liquid phase and incubated at a temperature allowing for the enzyme to catalyze a depolymerization or hydrolyzation reaction for a time sufficient to hydrolyze or

depolymerize a significant amount of the non-fermentable sugar or fiber in the biomass feedstock. In some embodiments, the liquid phase of the feedstock contacted with the enzyme, which contains the soluble, fermentable sugar fraction, is separated from the solid phase, including non-fermentable sugars and fibers, after incubation for hydrolyzation and depolymerization, for example, by centrifugation. In some embodiments, the liquid fraction of the feedstock is subsequently contacted with a microbe, for example, a microbe provided by aspects of this invention, for conversion to biofuel or biofuel precursor. In some embodiments, enzymatic conversion of non-fermentable sugars or fiber occurs in a consolidated bioprocess, for example, at the same time and/or in the same reactor as microbial conversion of the produced fermentable sugars to biofuel or biofuel precursor. In some embodiments, the enzymatic conversion is performed first, and the feedstock contacted with enzyme is subsequently contacted with the microbe for biofuel or biofuel precursor production. In some embodiments, enzymatic and microbial conversion are performed at the same time and in the same reactor.

In some embodiments, an engineered microbe as provided herein, for example, a *Yarrowia lipolytica* overexpressing a DGA1, ACC1, and/or SCD gene product, is grown on acetate as the main carbon source. In some embodiments, the microbe is grown in a solution of acetic acid with a concentration of about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10% vol/vol, about 20 % vol/vol, about 25 % vol/vol, or about 30 % vol/vol. In some embodiments, the acetate concentration is between about 3%-10% wt/vol. In some embodiments, cell cultures comprising genetically modified microbes as provided herein that are cultured on acetate or acetic acid as the main carbon source are contacted, or "spiked," with glycerol. In some embodiments, the genetically modified microbes are intermittently contacted with glycerol. In some embodiments, the microbes are continuously or semi-continuously contacted with glycerol. In some embodiments, the microbes are contacted with glycerol at a concentration of about 0.5%, about 1%, about 2%, about 3%, about 4%, or about 5% vol/vol. Contacting the engineered microbes provided herein with glycerol provides metabolites for the production of TAGs, as well as reducing moieties for the production of fatty acids from carbohydrates. In some embodiments, glycerol spiking is performed in biofuel or biofuel precursor production methods using a carbon source other than acetate, for example, any carbon source described herein.

In some embodiments, an engineered microbe as provided herein, for example, a *Yarrowia lipolytica* overexpressing a DGA1 gene product, and/or optionally, and ACC1 and/or SCD gene product, is grown on a carbon source, e.g., on acetate or acetic acid, that is replenished during the growth process or culture period, e.g., by contacting the microbe with an additional amount of the carbon source, or with an amount of an additional carbon source, after a period of time in culture, e.g., after 8 hours, after 24 hours, or after 48 hours. In some embodiments, an engineered microbe as provided herein is grown initially, e.g., for the first 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, or 72 hours in a culture medium that comprises a low carbon to nitrogen (C/N) ratio, e.g., a C/N ratio of about 10, of about 20, of about 25, of about 30, of less than 30, of less than 25, or of less than 20. In some embodiments, a low C/N ratio is achieved by supplementing the culture media with a nitrogen source, e.g., with ammonia, to achieve the desired C/N ratio. In some embodiments, e.g., in embodiments, where carbon source, e.g., acetate or acetic acid, is fed into a culture of oil-producing microbes as described herein, the carbon source is supplemented with a nitrogen source, e.g., ammonia. In some embodiments, the supplementation with a nitrogen source is ceased after an initial period of time in culture, e.g., after for the first 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, or 72 hours in culture, thus allowing the engineered microbes in culture to consume the nitrogen source, which, in turn, results in an increase of the C/N ratio. This shift in C/N ratio can be enhanced or sped up by feeding additional carbon source into the culture that is not supplemented with a nitrogen source, e.g., by feeding acetic acid or acetate that is not supplemented with ammonia or any other nitrogen source. In some embodiments, the optimal C/N ratio for oil production by an engineered microbe described herein is within the range of 80-120.

In some embodiments, fermentation processes for large-scale microbe-mediated carbohydrate to lipid conversion may be carried out in bioreactors. As used herein, the terms “bioreactor” and “fermentor”, which are interchangeably used, refer to an enclosure, or partial enclosure, in which a biological and/or chemical reaction takes place, at least part of which involves a living organism or part of a living organism. A “large-scale bioreactor” or “industrial-scale bioreactor” is a bioreactor that is used to generate a product, for example a biofuel or biofuel precursor, for example a fatty acid and/or TAG, on a commercial or quasi-commercial scale. Large scale bioreactors typically have volumes in the range of liters, hundreds of liters, thousands of liters, or more.

A bioreactor in accordance with aspects of this invention may comprise a microbe or a microbe culture. In some embodiments, a bioreactor may comprise a spore and/or any kind of dormant cell type of any isolated microbe provided by aspects of this invention, for example, in a dry state. In some embodiments, addition of a suitable carbohydrate source to such bioreactors may lead to activation of the dormant cell, for example to germination of a yeast spore, and subsequent conversion of the carbohydrate source, at least in part, to a biofuel or biofuel precursor.

Some bioreactors according to aspects of this invention may include cell culture systems where microbes are in contact with moving liquids and/or gas bubbles. Microbes or microbe cultures in accordance with aspects of this invention may be grown in suspension or attached to solid phase carriers. Non-limiting examples of carrier systems include microcarriers (e.g., polymer spheres, microbeads, and microdisks that can be porous or non-porous), cross-linked beads (e.g., dextran) charged with specific chemical groups (e.g., tertiary amine groups), 2D microcarriers including cells trapped in nonporous polymer fibers, 3D carriers (e.g., carrier fibers, hollow fibers, multicartridge reactors, and semi-permeable membranes that can comprising porous fibers), microcarriers having reduced ion exchange capacity, encapsulation cells, capillaries, and aggregates. Carriers can be fabricated from materials such as dextran, gelatin, glass, and cellulose.

Industrial-scale carbohydrate to lipid conversion processes in accordance with aspects of this invention may be operated in continuous, semi-continuous or non-continuous modes. Non-limiting examples of operation modes in accordance with this invention are batch, fed batch, extended batch, repetitive batch, draw/fill, rotating-wall, spinning flask, and/or perfusion mode of operation.

In some embodiments, bioreactors may be used that allow continuous or semi-continuous replenishment of the substrate stock, for example a carbohydrate source and/or continuous or semi-continuous separation of the product, for example a secreted lipid, an organic phase comprising a lipid, and/or cells exhibiting a desired lipid content, from the reactor.

Non-limiting examples of bioreactors in accordance with this invention are: stirred tank fermentors, bioreactors agitated by rotating mixing devices, chemostats, bioreactors agitated by shaking devices, airlift fermentors, packed-bed reactors, fixed-bed reactors, fluidized bed bioreactors, bioreactors employing wave induced agitation, centrifugal

bioreactors, roller bottles, and hollow fiber bioreactors, roller apparatuses (for example benchtop, cart-mounted, and/or automated varieties), vertically-stacked plates, spinner flasks, stirring or rocking flasks, shaken multiwell plates, MD bottles, T-flasks, Roux bottles, multiple-surface tissue culture propagators, modified fermentors, and coated beads (e.g.,
5 beads coated with serum proteins, nitrocellulose, or carboxymethyl cellulose to prevent cell attachment).

Bioreactors and fermentors according to aspects of this invention may, optionally, comprise a sensor and/or a control system to measure and/or adjust reaction parameters. Non-limiting examples of reaction parameters are: biological parameters, for example growth rate,
10 cell size, cell number, cell density, cell type, or cell state, chemical parameters, for example pH, redox-potential, concentration of reaction substrate and/or product, concentration of dissolved gases, such as oxygen concentration and CO₂ concentration, nutrient concentrations, metabolite concentrations, glucose concentration, glutamine concentration, pyruvate concentration, apatite concentration, concentration of an oligopeptide, concentration
15 of an amino acid, concentration of a vitamin, concentration of a hormone, concentration of an additive, serum concentration, ionic strength, concentration of an ion, relative humidity, molarity, osmolarity, concentration of other chemicals, for example buffering agents, adjuvants, or reaction by-products, physical/mechanical parameters, for example density, conductivity, degree of agitation, pressure, and flow rate, shear stress, shear rate, viscosity,
20 color, turbidity, light absorption, mixing rate, conversion rate, as well as thermodynamic parameters, such as temperature, light intensity/quality etc.

Sensors able to measure parameters as described herein are well known to those of skill in the relevant mechanical and electronic arts. Control systems able to adjust the parameters in a bioreactor based on the inputs from a sensor as described herein are well
25 known to those of skill in the art of bioreactor engineering.

The type of carbon source to be employed for conversion to a biofuel or biofuel precursor according to aspects of this invention depends on the specific microbe employed. Some microbes provided by aspects of this invention may be able to efficiently convert a specific carbohydrate source, while a different carbohydrate source may not be processed by
30 the same microbe at high efficiency or at all. According to aspects of this invention, the oleaginous yeast *Y. lipolytica*, for example, can efficiently convert sugars, such as glucose,

fructose, sucrose, and/or lactose, and carbohydrate sources high in sugars, for example molasses, and plant fibers into fatty acids and their derivatives.

In some embodiments, a biofuel or biofuel precursor, for example, a fatty acid or a triacylglycerol, generated from a carbon source feedstock is secreted, at least partially, by a
5 microbe provided by aspects of this invention, for example, an oleaginous yeast, such as a *Y. lipolytica* cell. In some embodiments, a microbe provided by aspects of this invention is contacted with a carbohydrate source in an aqueous solution in a bioreactor, and secreted biofuel or biofuel precursor forms an organic phase that can be separated from the aqueous phase. The term organic phase, as used herein, refers to a liquid phase comprising a non-
10 polar, organic compound, for example a fatty acid, TAG, and/or other non-polar lipid. And organic phase in accordance to this invention might further contain a microbe, a carbohydrate, or other compound found in other phases found in a respective bioreactor. Methods useful for industrial scale phase separation are well known to those of ordinary skill in the art. In some embodiments, the organic phase is continuously or semi-continuously
15 siphoned off. In some embodiments, a bioreactor is employed, comprising a separator, which continuously or semi-continuously extracts the organic phase.

In some embodiments, a biofuel or biofuel precursor is accumulated in cells according to aspects of this invention. In some embodiments, cells that have accumulated a desirable amount of biofuel or biofuel precursor, are separated continuously or semi-continuously from
20 a bioreactor, for example, by centrifugation, sedimentation, or filtration. Cell separation can further be effected, for example, based on a change in physical cell characteristics, such as cell size or density, by methods well known to those skilled in the art. The accumulated biofuel or biofuel precursor can subsequently be extracted from the respective cells using standard methods of extraction well known to those skilled in the art, for example, solvent
25 hexane extraction. In some embodiments, microbial cells are collected and extracted with 3 times the collected cell volume of hexane. In some embodiments, the extracted biofuel or biofuel precursor are further refined. In some embodiments, a biofuel precursor, for example a triacylglycerol is converted to a biofuel, for example, biodiesel, using a method well known to those of skill in the art, for example, a transesterification procedure.

30 The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope

of the invention. Accordingly, it will be understood that the example section is not meant to limit the scope of the invention.

EXAMPLES

5 Example 1.

Materials and Methods

Yeast strains, growth, and culture conditions

The *Y. lipolytica* strains used in this study were derived from the wild-type
 10 *Y. lipolytica* W29 strain (ATCC20460). The auxotrophic *Po1g* (Leu⁻) used in all transformations was obtained from Yeastern Biotech Company (Taipei, Taiwan). All strains used in this study are listed in Table 1.

15 Table 1. Total fatty acid content, yield and distribution for *Y. lipolytica* strains. Total fatty acid content is given as means \pm S.D. (n = 3) for a 50 mL culture after 100 hrs (C/N molar ratio of 20). Fatty acid profiles are given as percent of fatty acid of total fatty acids, with error less than 2.5%.

	Biomass (g DCW)	Lipid Content (%)	Oil Yield (g/g glucose)	Fatty Acid (Fractional Abundance %)				
				C16	C16:1	C18:0	C18:1	C18:2
Control (MTYL037)	10.49	8.77 \pm 0.37	2.28	20.5	3.9	24.0	43.0	8.7
<i>ACC1</i> (MTYL040)	6.04	17.9 \pm 1.13	6.25	20.3	3.5	23.4	41.7	11.0
<i>DGA1</i> (MTYL053)	7.53	33.8 \pm 0.55	9.36	18.3	2.6	34.1	38.6	6.4
<i>ACC1+DGA1</i> (MTYL065)	8.60	41.4 \pm 1.90	11.4	16.0	2.3	32.8	44.9	4.0

Media and growth conditions for *Escherichia coli* have been previously described by
 20 Sambrook et al.(21), and those for *Y. lipolytica* have been described by Barth and Gaillardin(11). Rich medium (YPD) was prepared with 20 g/L Bacto peptone (Difco Laboratories, Detroit, MI), 10 g/L yeast extract (Difco), 20 g/L glucose (Sigma-Aldrich, St. Louis, MO). YNB medium was made with 1.7 g/L yeast nitrogen base (without amino acids) (Difco), 0.69 g/L CSM-Leu (MP Biomedicals, Solon, OH), and 20 g/L glucose. Selective

YNB plates contained 1.7 g/L yeast nitrogen base (without amino acids), 0.69 g/L CSM-Leu, 20 g/L glucose, and 15 g/L Bacto agar (Difco).

Shake flask experiments were carried out using the following medium: 1.7 g/L yeast nitrogen base (without amino acids), 1.5 g/L yeast extract, and 50 g/L glucose. From frozen stocks, precultures were inoculated into YNB medium (5 mL in Falcon tube, 200 rpm, 28°C, 24 hr). Overnight cultures were inoculated into 50 mL of media in 250 mL Erlenmeyer shake flask to an optical density (A_{600}) of 0.05 and allowed to incubate for 100 hours (200 rpm, 28°C), after which biomass, sugar content, and lipid content were taken and analyzed.

Bioreactor scale fermentation was carried out in a 2-liter baffled stirred-tank bioreactor. The medium used contained 1.5 g/L yeast nitrogen base (without amino acids and ammonium sulfate), 2 g/L ammonium sulfate, 1 g/L yeast extract, and 90 g/L glucose. From a selective plate, an initial preculture was inoculated into YPD medium (40 mL in 250 mL Erlenmeyer flask, 200 rpm, 28°C, 24 hr). Exponentially growing cells from the overnight preculture were transferred into the bioreactor to an optical density (A_{600}) of 0.1 in the 2-L reactor (2.5 vvm aeration, pH 6.8, 28°C, 250 rpm agitation). Time point samples were stored at -20°C for subsequent lipid analysis. Sugar organic acid content was determined by HPLC. Biomass was determined by determined gravimetrically from samples dried at 60°C for two nights.

Plasmid Construction

Standard molecular genetic techniques were used throughout this study (21). Restriction enzymes and Phusion High-Fidelity DNA polymerase used in cloning were obtained from New England Biolabs (Ipswich, MA). Genomic DNA from yeast transformants was prepared using Yeastar Genomic DNA kit (Zymo Research, Irvine, CA). All constructed plasmids were verified by sequencing. PCR products and DNA fragments were purified with PCR Purification Kit or QIAEX II kit (Qiagen, Valencia, CA). Plasmids used are described in Table 3. Primers used are described in Table 4.

Plasmid pMT010 was constructed by amplifying the translation elongation factor-1 α (TEF) promoter region (Accession number: AF054508) from *Y. lipolytica* Po1g genomic DNA using primers MT078 and MT079. The amplicon was inserted between *SalI* and *KpnI* sites of the starting vector, pINA1269, also known as pYLEX1, obtained from Yeastern

Biotech Company (Taipei, Taiwan). Also included in the reverse primer MT079 were *MluI* and *NsiI* sites to add restriction sites to the multi-cloning site.

Plasmid pMT015 was constructed by amplifying from *Y. lipolytica* Po1g genomic DNA the TEF promoter and the 5' coding region containing the ATG start codon and 113 bp of the endogenous intron (Accession number: CR382129). Primers MT118 and MT122 were used for this amplification and inserted between *SalI* and *MluI* sites of pMT010. For cloning purposes, some of the intron was omitted so that the *SnaBI* restriction site could be incorporated. Cloning a gene into this plasmid thus requires the omission of the gene's ATG start codon, addition of TAACCGCAG to the beginning of the 5' primer, and blunt-end ligation at the 5' end.

Plasmid pMT025 was constructed by amplifying the *LacZ* gene, encoding β -galactosidase, from *E. coli* and inserting it into the *PmlI* and *BamHI* sites of starting vector pINA1269 using primers MT170 and MT171. Plasmid pMT038 was constructed by amplifying the *LacZ* gene and inserting it into the *MluI* and *NsiI* sites of pMT010 using primers MT168 and MT169. Since *LacZ* contains multiple *MluI* sites, *AscI* was used as the 5' restriction site on MT168 which has a matching overhang. Plasmid pMT037 was constructed by amplifying *LacZ* gene and inserting it into the *SnaBI* and *NsiI* sites of pMT015. Primers MT172 and MT169 were used, where forward primer MT127 omits the ATG start codon of *LacZ* and instead begins with the sequence TAACCGCAG which completes the intron sequence of pMT015.

Plasmid pMT013 was constructed by amplifying the *ACC1* gene from *Y. lipolytica* Po1g genomic DNA (Accession Number: XM_501721) and inserting it into the *MluI* and *NsiI* sites of pMT010 using primers MT080 and MT081. Plasmid pMT040 was constructed by amplifying the *ACC1* gene and its terminator from pMT013 using primers MT222 and MT137 and inserting this into starting vector pINA1269 digested with *PmlI* and *ClaI*.

Plasmid pMT053 was constructed by amplifying the *DGA1* gene from *Y. lipolytica* Po1g genomic DNA (Accession Number: XM_504700) using primers MT271 and MT272. The amplified gene was digested with *NsiI* and was inserted into PMT015 in the same manner as in the construction of pMT037.

To produce a single plasmid which could express both *ACC1* and *DGA1*, a promoter-gene-terminator cassette was amplified from pMT053 using primers MT220 and MT265. This was then digested with *DpnI* and *AseI* and inserted into pMT040 which was digested

with *NruI* and *AseI* resulting in tandem gene construct pMT065. The *AseI* restriction site was selected to facilitate selection, as it resides within the Ampicillin resistance marker. Because *NruI* is a blunt end restriction site, insertion of the amplicon does not increase the total number of *NruI* sites to facilitate progressive insertions.

Plasmids were linearized with either *NotI* or *SacII* and chromosomally integrated into *Polg* according to the one-step lithium acetate transformation method described by Chen et al.(22). Transformants were plated on selective media and verified by PCR of prepared genomic DNA. Verified transformants were then stored as frozen stocks at -80°C and on selective YNB plates at 4°C.

RNA isolation and transcript quantification

The yeast strains were grown on YNB for 24 hr, harvested, frozen with liquid nitrogen and kept at 80°C. Samples were crushed in liquid nitrogen, and total RNA was isolated from *Y. lipolytica* with the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase during the isolation step according to the manufacturer's instructions. The isolated RNA was quantified by spectrophotometry at 260 nm. qRT-PCR analyses were carried out using iScript One-step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA). Relative quantification was based on the $2^{\Delta CT}$ method using ACT1, encoding for actin, as an internal control. Samples were analyzed in triplicate.

β -galactosidase assay

LacZ enzyme activity was measured using the β -gal assay kit from Sigma-Aldrich. Cells were resuspended in PBS buffer and lysed by vortexing with 500 μ m glass beads (Sigma-Aldrich) for 2 minutes. 40 μ L of the cell lysate was transferred into 340 μ L reaction mix containing 47 mg/mL ONPG, 0.6 M Na₂HPO₄-7H₂O, 0.4 M NaH₂PO₄-H₂O, 0.1 M KCl, 0.01 M MgSO₄-7H₂O. Reaction was incubated at 37°C for color evolution to occur, and was finally quenched using 500 μ L 1 M Sodium Carbonate. Absorbance was then measured with a spectrophotometer at 420 nm. Enzymatic units are calculated based on enzyme activity divided by incubation time and dry cell weight.

Lipid Analysis

Total lipids were extracted using the procedure by Folch et al (23). A measured quantity of cell biomass (roughly 1 mg) was suspended in 1 mL of chloroform:methanol (2:1) solution and vortexed for 1 hour. After centrifugation, 500 μ L was transferred to 125 μ L saline solution. The upper aqueous layer was removed and the bottom layer was evaporated and resuspend in 100 μ L hexane. Samples were then stored at -20°C until transesterification.

Transesterification of total lipid extracts was performed by adding 1 mL 2% (wt/vol) sulfuric acid in methanol to each sample. Samples were then incubated at 60°C for 2 hours. After that the samples were partially evaporated, and the fatty acid methyl esters (FAME) were extracted by adding 1 mL hexane and vortexing for 10 min. 800 μ L of this hexane was then transferred into glass vials for GC analysis.

GC analysis of FAMEs was performed with a Bruker 450-GC instrument equipped with a flame-ionization detector and a capillary column HP-INNOWAX (30 m x 0.25 mm). The GC oven conditions were as follows: 150°C (1 min), a 10 min ramp to 230°C, hold at 230°C for 2 min. The split ratio was 10:1. Fatty acids were identified and quantified by comparison with commercial FAME standards normalized to methyl tridecanoate (C13:0). Total lipid content was calculated as the sum of total fatty acid contents for five FAMEs: methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2) (Sigma-Aldrich). The addition of tridecanoic acid to the chloroform-methanol extraction fluid was used as the internal standard, which was carried through the entire analysis procedure and transesterified into its methyl ester.

Results and Discussion

Establishing a high expression platform utilizing the expression-enhancing intron of

Translation Elongation Factor-1 α

In *Y. lipolytica*, a number of promoters are available for gene expression, including inducible and constitutive ones (24). The TEF promoter was originally identified as being a strong constitutive promoter; however, subsequent cloning and characterization resulted in lower expression relative to the inducible XPR2 promoter (25). More recently, the hybrid hp4d promoter has become popular for its strong quasi-constitutive expression (26), and has been used in a number of applications requiring high protein expression (20, 27, 28).

Analysis of the genomic sequence for TEF reveals the presence of a 122-bp spliceosomal intron immediately after the start codon in the 5' region of the open reading frame. Promoter-proximal spliceosomal introns have often been found to dramatically affect expression of their corresponding genes in a variety of organisms (29). We speculated that the strong expression of TEF was dependent on this intron, and that stronger expression could be achieved by including the intron along with the promoter in the expression vector. Indeed, the initial screening and isolation of the TEF promoter likely relied on the intron enhancement for enrichment in cDNA libraries, a feature which would not have been noticed once the intron was spliced.

Plasmids pMT025, pMT037 and pMT038 were constructed expressing LacZ to compare the relative expression of three promoters: synthetic hybrid promoter (php4d), TEF promoter without intron (pTEF), and TEF promoter with intron (pTEFin). Remarkably, the TEFin promoter exhibits a 17-fold increase in expression over the intronless TEF promoter, and a 5-fold increase in expression over the hp4d promoter after 50 hrs of culture.

The intron enhancement observed in other systems varies wildly: from only 2-fold in human cells and yeast to over 1000-fold in maize (30, 31). Introns are believed to enhance gene expression in a number of ways: by containing regulatory elements, facilitating mRNA export, and increasing transcription initiation rates (29). Intronic genes, as a group, tend to exhibit higher levels of expression relative to non-intronic genes. For example, in *S. cerevisiae*, intronic genes only represent less than 4% of the total gene count, yet account for 27% of the total RNA generated in the cell (32). The genome *Y. lipolytica* contains introns in 10.6% of its genes, compared to only 4.5% in *S. cerevisiae* (33). Enlisting this endogenous process to enhance our own desired genes represents a simple means for maximizing expression, applicable to a broad range of eukaryotic organisms. For example, there is high sequence homology of splice sequences among hemiascomycetous yeast (34). While research continues to elucidate more about the function, evolution, and purpose of introns, the utilization of introns for biotechnological purposes is a relatively untapped opportunity.

Overexpression of ACC1 and DGA1 leads to significant increases in lipid accumulation

The use of the TEF promoter along with its expression-enhancing intron provides an excellent platform for high gene expression in *Y. lipolytica*. We therefore used this for overexpression of *DGA1* (pMT053), which has been shown to be important in lipid

accumulation in both *Y. lipolytica* and *S. cerevisiae* (19, 35). *ACC1*, already having two endogenous promoter-proximal introns, was not cloned with the *TEF_{in}* promoter. Instead it was cloned with *TEF* and *hp4d* promoters (pMT013 and pMT040, respectively). Growth rates and lipid production was relatively similar between the two (data not shown), so *hp4d-ACC1* was used for lipid experiments and for the tandem gene construction of *ACC1+DGAI* (pMT065). The *hp4d* promoter also was selected to minimize the possibility of homologous recombination of the two parallel gene cassettes in the *ACC1+DGAI* construct. Simultaneous coexpression of two genes in *Y. lipolytica* using tandem gene construction has been successfully performed elsewhere (20).

The effect of *ACC1* and *DGAI* overexpression on lipid production was first assessed in shake flask experiments. The control *LacZ* strain only produced 8.77% (g/g DCW) lipids, which is similar to wild-type performance in shake flasks with glucose as sole substrate (36). *ACC1* and *DGAI* transformants both outperformed the control, accumulating 17.9% and 33.8% lipid content, respectively. *DGAI* in particular exhibited almost twice as much lipid accumulation as *ACC1*, almost 4-fold over the control. The biomass generated from the control was significantly higher than the other strains, suggesting that the expression of *ACC1* and *DGAI* may perturb the growth potential of *Y. lipolytica*. Overall oil yields were relatively low in comparison to a theoretical maximum yield of 0.32 g/g (37). There were also slight shifts in the fatty acid profile, with *ACC1* producing significantly more linoleic acid and *DGAI* maintaining a higher proportion of stearic acid. The proportion of oleic acid stayed relatively flat across all transformants.

Improving upon both single gene transformants, *ACC1+DGAI* was able to achieve 41.4% lipid content, a 4.7-fold improvement over the control. The biomass production was improved over the single transformants, but still less than the control. Oil yield improved proportionally, to 0.114 g/g, or 35% of theoretical yield.

In eukaryotic organisms, overexpression of ACC has been met with only limited improvement of lipid production. Heterologous expression of *ACC1* from the oleaginous fungus *Mucor rouxii* in the non-oleaginous yeast *Hansenula polymorpha* was only able to achieve a 40% increase in total fatty acid content, from 3.8% to 5.3% (38). In plants, overexpression of *Arabidopsis ACC1* has led to dramatic increases in enzyme activity, but to no more than 30% increase in final lipid content (39, 40). It is suspected that improvements have been limited because of the strong metabolic and regulatory control maintained over this

enzyme in eukarotes. ACC expression and activity is influenced by numerous transcription factors, protein kinases, and metabolites (41). For example, in *Candida (Yarrowia) lipolytica*, the accumulation of acyl-CoA in acetyl-CoA synthetase mutants led to an 8-fold decrease in ACC activity (42). Nonetheless, *Y. lipolytica* might represent a regulatory exception in eukaryotic organisms, lending much to its oleaginous nature, as here we achieve a 2-fold increase in lipid content through overexpression of endogenous *ACC1*.

The role of DGA has only recently been found to be important for lipid synthesis. In *Y. lipolytica*, DGA1p predominantly localizes to the membrane surface of lipid bodies and acts in concert with triglyceride lipase (TGL3) to balance TAG flux in and out of lipid bodies (16). One thus expects the storage of TAGs to rest heavily on the relative activity (and abundance) of DGA1p with respect to its TGL3p counterpart. It has also been hypothesized that DGA diverts flux away from phospholipid synthesis, and thus creates a driving force for lipid synthesis as more flux is required to produce the still necessary phospholipids (6). Consequently, overexpression of *DGA1* has led to marked effects on lipid accumulation. *DGA1* overexpression in an oleaginous $\Delta snf2$ mutant led to accumulation of up to 27% lipid content in *S. cerevisiae*, a 2.3-fold increase (35). In plants, *Arabidopsis* *DGAT* overexpression led to a 20-fold increase in lipid content in the leaves, and two-fold overall (43).

The balance between fatty acid and TAG synthesis pathways revolves around the acyl-CoA intermediates, since they function as both product and feedback inhibitors in the fatty acid (upstream) pathway and primary precursors in the TAG (downstream) pathway. Up-regulation of the upstream pathway increases the throughput of fatty acid synthesis and, for ACC in particular, diverts flux away from any pathways which would compete for cytosolic acetyl-CoA. Up-regulation of the downstream pathway creates a driving force by depleting acyl-CoA intermediates and increasing the rate of storage of TAG in lipid bodies. However, when modulated individually, they can lead to imbalances which can produce adverse effects on cell metabolism and growth.

By coexpressing *ACC1* and *DGA1*, both upstream and downstream pathways are simultaneously up-regulated, which leads to increased lipid production without perturbing intermediate-mediated regulation. It also combines high flux through lipid synthesis from ACC with the driving force provided by the sequestration of TAG into lipid bodies by DGA. The result is a synergistic increase in lipid accumulation, almost 5-fold greater than the control. Indeed, coupling overproduction and driving forces with a metabolic sink has

become a very powerful strategy in recent efforts of metabolic engineering, particularly for biofuels (44-46).

Fermentation performance of the ACC1 + DGA1 transformant

To further characterize the ACC1+DGA1 transformant (MTYL065) and explore its lipid accumulation characteristics, large-scale fermentation was conducted using a 2-L stirred-tank bioreactor. Glucose concentration was increased and ammonium sulfate concentration was reduced to achieve a C/N molar ratio of 100. Optimal C/N molar ratios for lipid accumulation typically range from 80-120 (15).

Glucose was fully consumed over the course of the 120 hour fermentation, with final biomass reaching 28.49 g/L (**Figure 3**). Final lipid content was 61.7% of DCW, a 50% increase in lipid accumulation compared to the shake flask experiment. This compares favorably with other sugar fermentations (28, 47-49), as well as values found in *ex novo* lipid accumulation schemes (19, 50). Overall yield and productivity was 0.195 g/g and 0.143 g/L/hr, respectively; however, during maximum lipid production observed between 70-100 hours, a yield of 0.270 g/g, and a productivity of 0.253 g/L/hr were reached (Table 2). Almost all biomass produced during this phase can be accounted for by the increase in lipid content. The overall and maximum yields achieved are 60.9% and 84.3% of the theoretical yield for TAG synthesis.

Table 2. Yield and Productivity calculations for 2-L bioreactor fermentation of ACC1+DGA1 transformant. Yield is calculated by grams lipids produced divided by grams glucose consumed. Productivity calculated by concentration of lipids produced per hour. Maximum values calculated from the time points between 70-100 hrs.

Oil Yield (g/g glucose)	
Overall	0.195
Maximum	0.270
Oil Productivity (g/L/hr)	
Overall	0.143
Maximum	0.253

The fatty acid profile changed dramatically during the scale up (**Figure 4**). Relative depletion of stearic acid and enrichment of palmitic acid and oleic acid was observed, with

oleic acid ultimately comprising 49.3% of total fatty acids. The ratio between oleic and stearic acids steadily increased throughout the fermentation (data not shown), finally ending in a ratio of 4.6. This is a dramatic change from the ratio of 1.3 seen in shake flask experiments. A similar experiment was performed with acetate as the carbon source. The fatty acid profile was analyzed at 134 hrs and the results are shown in **Figure 5**.

High relative oleate concentrations (up to 58.5%) has also been observed in other 2-L fermentations (51), and is more similar to profiles of other oleaginous yeasts that accumulate more than 50% lipid content(15). In conditions of rapid lipid production, oleic acid might be more rapidly stored and easier to accumulate, as *DGA1p* is known to have varying specificities for different acyl-CoA; in *S. cerevisiae*, C18:1 is the most preferred substrate, having twice the activity of C18:0 (52). Furthermore, the high oleic acid concentration might also be a response to the higher aeration rate achieved in the bioreactor and not easily seen in shake flask fermentations.

The high lipid content, yield, and productivity seen in the *ACC1+DGA1* strain demonstrate the innate capacity of *Y. lipolytica* to accommodate high flux through the lipid synthesis pathway. With further modifications and process optimization, *Y. lipolytica* with engineered lipid synthesis pathways can yield promising breakthroughs in the robust, efficient *de novo* synthesis of lipids

Table 3. Strains and plasmids used in this study

Strains (host strain)	Genotype or plasmid	Source
<i>E. coli</i>		
DH5 α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Invitrogen
pINA1269	<i>JMP62-LEU</i>	Yeastern
pMT010	<i>pINA1269 php4d::TEF</i>	This Example
pMT015	<i>pINA1269 php4d::TEF_{in}</i>	This Example
pMT025	<i>hp4d-LacZ</i>	This Example
pMT038	<i>YTEF-LacZ</i>	This Example
pMT037	<i>YTEF_{in}-LacZ</i>	This Example
pMT013	<i>YTEF-ACC1</i>	This Example
pMT040	<i>php4d-ACC1</i>	This Example
pMT053	<i>YTEF_{in}-DGA</i>	This Example
pMT065	<i>php4d-ACC1 + YTEF_{in}-DGA</i>	This Example
<i>Y. lipolytica</i>		
Polg	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2	Yeastern

MTYL038	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-LacZ-LEU2	This Example
MTYL037	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEFin-LacZ-LEU2	This Example
MTYL040	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 hp4d- <i>ACC1</i> -LEU2	This Example
MTYL053	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEFin- <i>DGA1</i> -LEU2	This Example
MTYL065	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 hp4d- <i>ACC1</i> + TEFin- <i>DGA1</i> -LEU2	This Example

Table 4: Primers used in this study

Primer	Description	Sequence	SEQ ID
PCR			
MT078	TEF	<i>GACTGTCGACAGAGACCGGGTTGGCGGCGCAT</i> <i>TTGTG</i>	14
MT079	TEF	<i>GACTGGTACCTCAAGATGCATAGCACGCGTT</i> <i>TTGAATGATTCTTATACTC</i>	15
MT118	TEFin	<i>GGCAGTCGACAGAGACCGGGTTGGCGGC</i>	16
MT122	TEFin	<i>TTATTCACGCGTGTAGATACGTACTGCAAAAAGT</i> <i>GCTGGTCGGA</i>	17
MT170	LacZ	<i>AATGACCATGATTACGGATTCACTGG</i>	18
MT171	LacZ	<i>CTAGGTGGATCCTTATTTTTGACACCAGACCAAC</i> <i>TGGTAA</i>	19
MT172	LacZ	<i>TAACCGCAGACCATGATTACGGATTCACTGGCC</i>	20
MT173	LacZ	<i>CTAGGTATGCATATGACCATGATTACGGATTAC</i> <i>TGG</i>	21
MT174	LacZ	<i>CTTACAGGTACCTTATTTTTGACACCAGACCAAC</i> <i>TGGTAA</i>	22
MT080	TEF-ACC	<i>GACTACGCGTCACAATGCGACTGCAATTGA</i>	23
MT081	TEF-ACC	<i>TAGCATGCATTACAAACCCCTTGAGCAGCT</i>	24
MT222	Hp4d-ACC	<i>AATGCGACTGCAATTGAGGACACTAA</i>	25
MT137	Hp4d- <i>ACC1</i>	<i>CGTTGAATCGATTTCGGACACGGGCATCTCAC</i>	26
MT271	DGA	<i>TAACCGCAGACTATCGACTCACAATACTACAAGT</i> <i>CGCG</i>	27
MT272	DGA	<i>CTAGGTATGCATTTACTCAATCATTCGGAACT</i> <i>CTGGG</i>	28
MT220	NruI AseI	<i>CCCGGCAACAATTAATAGACTGGAT</i>	29
MT265	NruI AseI	<i>TTCGGACACGGGCATCTCAC</i>	30
RT-PCR			
	Actin		
	Actin		
	LacZ		

LacZ
 ACC1
 ACC1
 DGA
 DGA

Production of Lipids from Acetate using Engineered ACC + DGA strain

Yarrowia lipolytica naturally grows on the organic acid acetate. Acetate is an attractive substrate as it can be produced at high yields by homoacetogenic organisms from carbon dioxide or carbon monoxide via non-photosynthetic carbon fixation pathways. Acetate enters cellular metabolic pathways in the form of acetyl-coA, which is the main precursor for lipid synthesis via acetyl-coA carboxylase (ACC). As such, the ACC + DGA strain looked to be a promising candidate for lipid production on acetate substrate, since there is a direct overexpression of an acetyl-CoA utilizing enzyme coupled with the strong driving force for lipid accumulation.

A 2-liter bioreactor fermentation was performed using the ACC+DGA strain with acetate as carbon substrate (**Figure 6**). At high oxygen content, it was observed that acetate would be rapidly consumed with low biomass generation, so the fermentation was conducted under both nitrogen and oxygen-limited conditions to maximize lipid production and yield.

Final titer of oil production was 5.5 g/L after 130 hours, which constitutes 62% of the total 8.9 g/L biomass dry cell weight. The overall lipid yield on acetate was 0.152 g oil/g acetate. During the lipid production phase (between 90 - 130 hrs), maximum lipid production was achieved at a yield of 0.27 g oil/g acetate, which is 96% of the theoretical maximum yield.

A comparison of the fermentation characteristics to that on glucose show that despite lower biomass yields and growth rates, lipid accumulation and maximum lipid yield were commensurate.

Table 5. Comparison of fermentation on glucose and acetate.

	Glucose	Acetate
Exponential Growth Rate	0.0982 hr ⁻¹	0.0368 hr ⁻¹
Overall Biomass Yield	0.316 g/g	0.246 g/g
Overall Lipid Yield	0.195 g/g	0.152 g/g
Lipid Production Phase	70 - 120 hrs	90 - 130 hrs
Maximum Lipid Yield	0.249 g/g	0.270 g/g
Maximum Productivity	0.258 g/L/hr	0.103 g/L/hr
Final Lipid Titer	17.6 g/L	5.5 g/L

Conclusions

Lipid biosynthesis is a tightly regulated metabolic pathway. For industrially-relevant applications for the production of lipids, thoughtful metabolic engineering is necessary to maximize yields and productivity. The use of the oleaginous yeast *Y. lipolytica* benefits from having high capacity for lipid accumulation and tools for engineering the lipid metabolic pathway. Here we show that the intron-enhanced co-overexpression of two important genes in the lipid synthesis pathway, *ACC1* and *DGA1*, provides driving force towards the production of lipids even under moderate C/N ratios. As the two enzymes carry out the first and last steps of lipid synthesis, the simultaneous push and pull of carbon flux towards TAG allows for enhanced production with minimal intermediate accumulation, which can lead to inhibition. The resulting *ACC1+DGA1* strain was able to accumulate up to 62% of its DCW as lipids through *de novo* synthesis at an overall volumetric productivity of 0.143 g/L/hr.

The concepts of (a) strong overexpression of pathway genes, (b) balance of upstream and downstream pathways, (c) diversion of flux towards desired pathways, and (d) driving forces towards the final product, are prominent strategies in the practice of metabolic engineering, where metabolic networks are engineered and optimized for the generation of desirable products. Implementation of these concepts with respect to lipid accumulation may readily extend to a number of biological platforms, including microalgae. These strategies will be foundational in enabling the technologies of robust, efficient, commodity-scale production of biologically-derived chemicals and fuels. Their application to lipid biosynthesis opens the path for microbial oil overproduction and cost-effective biofuel manufacturing.

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Example 2.

Combinatorial Expression of Lipid Synthesis Genes in *Yarrowia lipolytica*

The study of cellular metabolism can be elucidated using metabolic engineering.

- 20 Metabolic engineering is the use of recombinant DNA technologies to manipulate metabolic pathways in organisms (Bailey 1991). Through manipulation and engineering of specific metabolic networks, controlling factors and rate-limiting steps can be identified and elaborated. Building upon existing knowledge and tools for a specific organism or pathway, one can evaluate how novel perturbations can be used to redirect and control the generation
- 25 of desired products.

- Lipid biosynthesis is an excellent pathway for study using for metabolic engineering, having wide applications ranging from health, cancer and medicine, to biochemicals and biofuels production (Beopoulos et al. 2011; Courchesne et al. 2009; Kohlwein and Petschnigg 2007). The physiology, enzymology, and metabolism for lipid biosynthesis in a wide range of
- 30 organisms, from bacteria to humans, have been extensively studied, forming a strong knowledge base for both comparative and exploratory analysis (Kurat et al. 2006; Ohlrogge and Jaworski 1997). Lipid metabolism plays an integral role in numerous aspects of cell physiology, from cell growth and proliferation to energy storage and metabolism (Kohlwein and Petschnigg 2007; Tehlivets et al. 2007). To utilize these pathways for both medicinal and
- 35 industrial purposes, it is important to understand which perturbations have the greatest impact on the overall process.

The oleaginous yeast *Yarrowia lipolytica* stands as an excellent model organism to study lipid metabolism. As an oleaginous yeast, *Y. lipolytica* can naturally accumulate up to

36% lipids in carbon rich environments (Beopoulos et al. 2009). These lipids are stored in the form of triacylglycerides (TAG) in lipid bodies. It is one of the most extensively studied 'non-conventional' yeast species, with a sequenced genome and a range of genetic tools available (Barth and Gaillardin 1997). It has been used in a number of industrial applications and has been viewed as a model organism for protein secretion, hydrophobic substrate utilization, lipid metabolism, and mitochondrial respiration (Beckerich et al. 1998; Beopoulos et al. 2009; Coelho et al. 2010; Kerscher et al. 2002). While *Y. lipolytica* naturally accumulates large quantities of lipids, a number of engineering efforts have been successful in further increasing or otherwise improving its lipid accumulation characteristics (Beopoulos et al. 2008; Chuang et al. 2010; Dulermo and Nicaud 2011; Zhang et al. 2011). However the number and variety of genetic manipulations examined has remained relatively limited towards this end and the potential for *Y. lipolytica* as a platform for lipid overproduction remains relatively unexplored.

A number of interesting gene targets have been linked to lipid accumulation through a variety of approaches and strategies (Courchesne et al. 2009). Acetyl-coA carboxylase (ACC) is generally known as the rate-limiting step in fatty biosynthesis, controlling the flux entering the pathway. It is responsible for producing malonyl-coA, which can be utilized in fatty acid elongation. ACC utilizes cytosolic acetyl-coA as its main metabolic precursor. The enzyme that supplies cytosolic acetyl-coA in most eukaryotes is ATP citrate lyase (ACL). ACL cleaves citrate, which has been shuttled out of the mitochondria as a product of the TCA cycle, to form acetyl-coA and oxaloacetate. After fatty acid production is completed with the fatty acid synthase complex, acyl-coA molecules can be further manipulated through elongation and desaturation at the endoplasmic reticulum. These processes help modify the chemical properties of the acyl-coA chains to facilitate storage or utilization in other metabolic pathways. Enzymes such as $\Delta 9$ -desaturase (D9) convert stearyl-coA molecules into oleoyl-coA molecules, which seem to be very important in both lipid regulation and metabolism (Dobrzyn and Ntambi 2005). The final step in lipid assembly and storage is the conversion of diacylglycerol (DAG) into TAG via the enzyme diacylglycerol acyltransferase (DGA). This step occurs at both the endoplasmic reticulum and on the surface of lipid bodies, with the latter establishing a dynamic equilibrium of TAG assembly and degradation depending on the energy needs of the organism (Athenstaedt et al. 2006). In *Y. lipolytica*, a number of DGA genes have been identified that perform this function (Zhang et al. 2011).

These enzymatic steps exhibit an interesting relationship to lipid accumulation. ACC controls flux entering lipid synthesis, and overexpression of ACC in the bacteria *Escherichia coli* resulted in 6-fold increase in fatty acid synthesis (Davis et al. 2000). The citrate shuttle, which is under control of ACL, is differentially observed in oleaginous fungi compared to non-oleaginous fungi, and is speculated as a necessary pathway for high flux into the lipid biosynthesis pathway (Boulton and Ratledge 1981; Vorapreeda et al. 2012). It is also thought that deactivation of ACL leads to citrate accumulation and secretion, an undesirable phenomenon in lipid production (Papanikolaou and Aggelis 2002; Papanikolaou et al. 2002). D9 has been implicated in cancer metabolism, being upregulated in mammalian tumor cells. It is potentially a strong positive regulator of lipogenesis and facilitates the lipid production necessary for rapid growth found in cancer cells (Dobrzyn and Ntambi 2005; Hulver et al. 2005; Ntambi and Miyazaki 2004). DGA is the final committed step for lipid storage, and overexpression of DGA in a *S. cerevisiae* Δ snf2 mutant resulted in dramatic increases in lipid accumulation (Kamisaka et al. 2007). While these results have produced interesting results and implications, analysis of their contributions within a single model organism can allow us to systematically identify how they can contribute and cooperate to achieve the increased lipid production.

Here we look at the impact of several important genes involved in lipid biosynthesis and explore their contributions towards increasing lipid accumulation in the oleaginous yeast *Y. lipolytica*. By overexpression of gene targets, both individually and in combination, we can explore how genes can positively impact flux through lipid biosynthesis pathway. Furthermore we investigate the lipid production performance of two candidate strains to elucidate the importance of balanced metabolic flux within the cell to achieve high productivity.

Materials and Methods

Yeast strains, growth, and culture conditions

The *Y. lipolytica* strains used in this study were derived from the wild-type *Y. lipolytica* W29 strain (ATCC20460). The auxotrophic Po1g (Leu-) used in all transformations was obtained from Yeastern Biotech Company (Taipei, Taiwan). All strains used in this study are listed in Table 6. Constructed plasmids were linearized with SacII and chromosomally integrated into Po1g according to the one-step lithium acetate transformation method

described by Chen et al. (Chen et al. 1997). MTYL transformants were named after the numbering of their corresponding integrated plasmids, with the exception of MTYL088 and MTYL089. For construction of strains MTYL088 and MTYL089, strains MTYL078 and MTYL079 underwent two additional rounds of transformation: (1) transformed with URA KO cassette on selective 5-FOA to knock out endogenous URA; (2) transformed with PMT092 linearized with SacII. Transformants were plated on selective media and verified by PCR of prepared genomic DNA. Verified transformants were then stored as frozen glycerol stocks at -80°C and on selective YNB plates at 4°C. A summary of the designed overexpression of genes for each strain is described in Table 7.

Media and growth conditions for *Escherichia coli* have been previously described by Sambrook et al. (Sambrook and Russell 2001), and those for *Y. lipolytica* have been described by Barth and Gaillardin (Barth and Gaillardin 1997). Rich medium (YPD) was prepared with 20 g/L Bacto peptone (Difco Laboratories, Detroit, MI), 10 g/L yeast extract (Difco), 20 g/L glucose (Sigma-Aldrich, St. Louis, MO). YNB medium was made with 1.7 g/L yeast nitrogen base (without amino acids) (Difco), 0.69 g/L CSM-Leu (MP Biomedicals, Solon, OH), and 20 g/L glucose. Selective YNB plates contained 1.7 g/L yeast nitrogen base (without amino acids), 0.69 g/L CSM-Leu, 20 g/L glucose, and 15 g/L Bacto agar (Difco). Shake flask experiments were carried out using the following medium: 1.7 g/L yeast nitrogen base (without amino acids), 1.5 g/L yeast extract, and 50 g/L glucose. From frozen stocks, precultures were inoculated into YNB medium (5 mL in Falcon tube, 200 rpm, 28°C, 24 hr). Overnight cultures were inoculated into 50 mL of media in 250 mL Erlenmeyer shake flask to an optical density (A₆₀₀) of 0.05 and allowed to incubate for 100 hours (200 rpm, 28°C), after which biomass, sugar content, and lipid content were taken and analyzed.

Bioreactor scale fermentation was carried out in a 2-liter baffled stirred-tank bioreactor. The medium used contained 1.7 g/L yeast nitrogen base (without amino acids and ammonium sulfate), 2 g/L ammonium sulfate, 1 g/L yeast extract, and 90 g/L glucose. From a selective plate, an initial preculture was inoculated into YPD medium (40 mL in 250 mL Erlenmeyer flask, 200 rpm, 28°C, 24 hr). Exponentially growing cells from the overnight preculture were transferred into the bioreactor to an optical density (A₆₀₀) of 0.1 in the 2-L reactor (2.5 vvm aeration, pH 6.8, 28°C, 250 rpm agitation). Time point samples were stored at -20°C for subsequent lipid analysis. Sugar organic acid content was determined by HPLC.

Biomass was determined by determined gravimetrically from samples washed and dried at 60°C for two nights.

Genetic Techniques

Standard molecular genetic techniques were used throughout this study (Sambrook and Russell 2001). Restriction enzymes and Phusion High-Fidelity DNA polymerase used in cloning were obtained from New England Biolabs (Ipswich, MA). Genomic DNA from yeast transformants was prepared using Yeastar Genomic DNA kit (Zymo Research, Irvine, CA). All constructed plasmids were verified by sequencing. PCR products and DNA fragments were purified with PCR Purification Kit or QIAEX II kit (Qiagen, Valencia, CA). Plasmids used are described in Table 6. Primers used are described in Table 9.

Deletion of URA3

To increase the availability of markers in strains of *Y. lipolytica*, the gene encoding for uracil prototrophy, orotidine-5'-phosphate decarboxylase (URA3, Accession Number: AJ306421), was amplified and used as the basis of a knockout cassette for the generation of URA auxotrophic strains. Upstream and downstream sequences of the URA open reading frame were amplified using primer pairs MT310 - MT311 and MT312 - MT313, respectively. The primers are designed such that the two amplicons carry 23 bp overlapping region. Upon purification of the two amplicons, both products are mixed and a PCR is performed using the primers MT310 and MT313 to produce a 456 bp amplicon fusing the upstream and downstream amplicons. This DNA was purified and subsequently transformed into *Polg*. Transformed cells were then plated on a selective media plate containing uracil and 5-Fluoroorotic Acid (5-FOA). Colonies which grew were replated on 5-FOA plates to reselect for URA auxotrophy, and verified by PCR of prepared genomic DNA. The resulting Δ LEU2 Δ URA3 strain was named MTYL100.

Plasmid construction

The construction of plasmids pMT010, pMT015, pMT038, pMT040, pMT013 and pMT065 were described above, and are similar to methods described here. Plasmid pMT047 expressing ATP:citrate lyase subunit 1 was constructed by amplifying the ACL1 gene from *Y. lipolytica* *Polg* genomic DNA (Accession Number: XM_504787) and inserting it into the MluI and NsiI sites of pMT010 using primers MT252 and MT253. Plasmid pMT049

expressing ATP:citrate lyase subunit 2 was likewise constructed by amplifying the ACL2 gene from *Y. lipolytica* Po1g genomic DNA (Accession Number: XM_503231) and inserting it into the MluI and NsiI sites of pMT010 using primers MT254 and MT255. Plasmid pMT061 expressing Delta-9 fatty acid desaturase (D9) was amplified from Po1g genomic DNA (Accession Number: XM_501496) and inserted into pINA1269 under control of the php4d promoter using the restriction sites PmlI and BamHI with primers MT283 and MT284.

To produce plasmids that express multiple genes (pMT050, pMT065, pMT066, pMT073, pMT074, pMT075, pMT078, pMT079), a promoter-gene-terminator cassette was amplified from one plasmid using primers MT220 and MT265. This was then digested with DpnI and AseI and inserted into a second plasmid that was digested with NruI and AseI resulting in tandem gene construct pMT065. The AseI restriction site was selected to facilitate selection, as it resides within the ampicillin resistance marker. Because NruI is a blunt end restriction site, ligation of the insertion does not increase the total number of NruI sites, thus enabling iterative insertions using the same process. The overall sequence and scheme used for the combinatorial construction of these plasmids is described in **Figure 7**. For the construction of a complementary vector, pACYCDUET-1 was selected as the complementary shuttle vector, as it utilizes a different backbone, selective marker and origin of replication. The upstream sequences of LIP2 (Accession Number: XM_500282) was amplified from *Y. lipolytica* Po1g genomic DNA using the primer pairs MT316 - MT317 and integrated into pDUET using the restriction sites BamHI and EcoRI. The downstream sequence of LIP2 was amplified using primer pairs MT318 - MT319 and integrated into pDUET using the restriction sites KpnI and AvrII. The selective marker for yeast uracil prototrophy was amplified from the plasmid JMP62-URA using primers pairs MT314 - MT315 and integrated into pDUET using the restriction sites PvuI and KpnI. The resulting plasmid pMT091 contained a multi-cloning site flanked by upstream and downstream LIP2 sequences and a URA3 marker. Digestion with the restriction enzyme SacII linearizes the plasmid and separates the integration vector from the plasmid backbone, minimizing the integration of foreign and unnecessary DNA. Use of the complementary plasmid requires construction of the expression cassette on the original pINA1269 backbone and then transferring the cassette over to pMT091 using restriction enzyme subcloning. The large multi-cloning site and differential antibiotic marker facilitate the cloning and selection process.

RNA isolation and transcript quantification

Shake flask cultures grown for 42 hrs were collected and centrifuged for 5 min at 10,000g. Each pellet was resuspended in 1.0 ml of Trizol reagent (Invitrogen) and 100 μ L of acid-washed glass beads were added (Sigma-Aldrich). Tubes were vortexed for 15 min at 4°C for cell lysis to occur. The tubes were then centrifuged for 10 min at 12,000g at 4°C and the supernatant was collected in a fresh 2-mL tube. 200 μ L chloroform was then added and tubes were shaken by hand for 10 seconds. The tubes were again centrifuged for 10 min at 12,000g at 4°C. 400 μ L of the upper aqueous phase was transferred to a new tube, and an equal volume of phenol-chloroform-isoamyl alcohol (pH 4.7) (Ambion, Austin, TX) was added. Tubes were again shaken by hand for 10 seconds and centrifuged for 10 min at 12,000g at 4°C. 250 μ L of the upper phase was transferred to a new tube with an equal volume of cold ethanol and 1/10th volume sodium acetate (pH 5.2). Tubes were chilled at -20°C for thirty minutes to promote precipitation. Tubes were then centrifuged for 5 min at 12,000g, washed twice with 70% ethanol, dried in a 60°C oven and finally resuspended in RNase free water. RNA quantity was analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and samples were stored in -80°C freezer. qRT-PCR analyses were carried out using iScript One-step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) using the Bio-Rad iCycler iQ Real-Time PCR Detection System. Fluorescence results were analyzed using Real-time PCR Miner and relative quantification and statistical analysis was determined with REST 2009 (Qiagen) using actin as the reference gene and MTYL038 as the reference strain (Zhao and Fernald 2005). Samples were analyzed in quadruplicate.

Lipid extraction and quantification

Total lipids were extracted using the procedure by Folch et al (Folch et al. 1957). A measured quantity of cell biomass (roughly 1 mg) was suspended in 1 mL of chloroform:methanol (2:1) solution and vortexed for 1 hour. After centrifugation, 500 μ L was transferred to 125 μ L saline solution. The upper aqueous layer was removed and the bottom layer was evaporated and resuspend in 100 μ L hexane. Samples were then stored at -20°C until transesterification.

Transesterification of total lipid extracts was performed by adding 1 mL 2% (wt/vol) sulfuric acid in methanol to each sample. Samples were then incubated at 60°C for 2 hours. After that the samples were partially evaporated, and the fatty acid methyl esters (FAME) were extracted by adding 1 mL hexane and vortexing for 10 min. 800 µL of this hexane was then transferred into glass vials for GC analysis.

GC analysis of FAMES was performed with a Bruker 450-GC instrument equipped with a flame-ionization detector and a capillary column HP-INNOWAX (30 m x 0.25 mm). The GC oven conditions were as follows: 150°C (1 min), a 10 min ramp to 230°C, hold at 230°C for 2 min. The split ratio was 10:1. Fatty acids were identified and quantified by comparison with commercial FAME standards normalized to methyl tridecanoate (C13:0). Total lipid content was calculated as the sum of total fatty acid contents for five FAMES: methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2) (Sigma-Aldrich). The addition of tridecanoic acid to the chloroform-methanol extraction fluid was used as the internal standard, which was carried through the entire analysis procedure and transesterified into its methyl ester.

Results & Discussion

Complementary vector construction allows for alternative method for expression of DGA.

In order to provide an alternative and complementary method for expressing genes in *Y. lipolytica*, the URA marker was knocked out of the parent strain Po1g. As part of the design of a complementary integration vector, the extracellular lipase LIP2 gene was selected as the docking site, as this gene is well characterized and likely has a negligible or neutral effect on de novo lipid synthesis and accumulation. Thus in the process of integrating the complementary vector, LIP2 will be knocked out. Upon construction of the complementary vector for gene expression, it was necessary to examine whether the expression of a gene would be affected depending on which transformation vector was used. To test this, the gene encoding for diacylglycerol acyltransferase (DGA) was cloned into both pMT015 and pMT091 vectors. While the expression cassette was the same in both vectors, the docking site was different: a pBR322 docking site for pMT015 (and all pINA1269-based vectors), and the LIP2 gene for pMT091. Upon transformation of these vectors and verification of genomic integration, RT-PCR of extracted RNA was performed on both strains, examining the overexpression of DGA in both cases relative to a control strain (MTYL038). As shown in

Figure 8, both strains exhibit 32-fold increases in DGA expression relative to MTYL038 control strains. These results validate the use of pMT091 as a complementary vector and LIP2 as an alternative docking site for gene expression, with no detectable interference from possible epigenetic phenomena.

5

Full survey of combinatorial constructs identifies improved strains with select genes.

In order to investigate the contributions and interactions of the gene targets, a survey was performed across various intermediate gene expression combinations, testing for the lipid production capabilities of the transformed strains. Table 7 describes the 13 constructed strains and their corresponding gene up-regulation. Lipid measurements were all performed after 100 hrs of culture in order to compare lipid productivity rather than merely lipid accumulation. For industrial purposes, overall productivity is a more important measurement than total lipid content, as a slow growing strain producing high yields might still be less useful than a fast growing, moderately high yielding strain. The results of the complete survey of lipid productivities and yields is depicted in **Figure 9**.

Examining strains containing only single gene overexpressions (MTYL040, MTYL053, MTYL050, MTYL061), ACC and DGA have clear improvements in both productivity and yield. ACL and D9 did not have any significant increases in either productivity or yield. These results indicate that for *Y. lipolytica*, ACC and DGA exhibit control over lipid biosynthesis and are rate-limiting steps, while ACL and D9 do not exhibit similar phenomena.

The effects of ACC and DGA were discussed in depth above, but DGA creates driving force by sequestering lipids and depleting acyl-coA intermediates, while ACC increases yields by diverting flux towards lipid synthesis and mobilizing the cytosolic acetyl-coA pool more rapidly. When the two genes are combined in strain MTYL065, they produce a synergistic response by establishing a push-and-pull dynamic within the lipid synthesis pathway, with acyl-coA as the balanced intermediate.

When combined with other genes, the gene D9 was able to confer slight benefits to the lipid productivity. For example, MTYL069, overexpressing D9 and DGA, had higher lipid productivities than MTYL053, which contained only DGA overexpression. Likewise, MTYL066, overexpressing ACC and D9, had higher lipid productivities than MTYL040, overexpressing ACC alone. However, MTYL073, overexpressing ACC+D9+DGA, exhibited

lower lipid productivity than MTYL065. There were no significant differences between MTYL089 and its D9-lacking variant, MTYL088. While some benefits were observed for accumulation and productivity, the benefits for yield were not significant. The observation that D9 only improves lipid production in combination with other genes seems to suggest that D9 does not have strong regulatory or rate-limiting control over the lipid synthesis process, but its enzymatic action provides favorable conditions magnifying the effect of other genes. As a membrane-associated enzyme on the lipid body membrane and endoplasmic reticulum, D9 is upregulated during lipid accumulation phases (Morin et al. 2011). Many lipid synthesis enzymes have been found to have the highest specificities for oleate, which is the product of D9 desaturation (Oelkers et al. 2002). This is also demonstrated in the observation that *Y. lipolytica* grows very rapidly on oleate as a carbon source and has extensively been studied growing off of this substrate (Beopoulos et al. 2008; Fickers et al. 2005). Consequently, an increased concentration of oleate, while not specifically driving lipid production or yield, transforms the fatty acid pool to be more rapidly sequestered. This ultimately results in faster rates of lipid accumulation without increases in yield, as increased sequestration will only occur in situations where lipid synthesis has already upregulated by other manipulations.

In contrast, when combining ACL overexpression with other genes, lipid productivity tended to decrease. MTYL078 and MTYL079 exhibited no significant increases in lipid production, despite overexpressing ACC and/or D9. MTYL088, harboring ACC+ACL+DGA, increased lipid production over control, but did not exhibit any lipid production improvements over MTYL065. These results indicate that while ACL may be affecting the distribution of carbon flux throughout the metabolic network, the overexpression of ACL, whether independently or in combination with other lipogenic improvements, does not significantly promote lipid production and in most cases lowers lipid productivity. This is similar to the observation that while ATP citrate lyase is an enzyme differentially expressed in oleaginous yeast compared to non-oleaginous yeast, the activity of the gene in various organisms has no correlation with the measured oleaginicinity (Boulton and Ratledge 1981).

Another observation from the lipid survey is that expression of DGA along with multiple other targets typically resulted in similar responses, both in productivity and yield. While it is possible that there is some saturation in expression or activity occurring in these constructs, this plateaued response may also have been due to a limitation of the experiment

used, as the measured characteristic in this survey was initial overall productivity, rather than stationary phase lipid accumulation or productivity. Furthermore, while strain MTYL065 clearly demonstrated the strongest productivity, the yield was relatively similar to many of the plateaued strains. This suggests that while all of these strains successfully divert flux towards lipids, giving increased yields, MTYL065 is exceptional in its balance of upstream and downstream pathways to achieve both high productivity and yield. These results highlight the importance of balancing perturbations to the metabolic flux network in order to achieve optimal productivities and yields, which is a common theme in metabolic engineering for growth-coupled products (Feist et al. 2010; Tyo et al. 2007).

RT-PCR analysis of full construct shows overexpression in MTYL089.

To explore the plateaued region of the lipid survey, the strain MTYL089, overexpressing ACC+D9+ACL12+DGA, was further investigated. The strain was constructed from the transformation of two plasmids, pMT079 and pMT092, into the Δ LEU and Δ URA MTYL100 background strain of *Y. lipolytica*. The use of two plasmids was primarily due to plasmid size considerations, as pMT079 already included four tandem expression cassettes and was 23 kb in length. PCR of genomic DNA confirmed the successful integration of both plasmids into the strain, with confirmation of correct integration of each individual expression cassette. RT-PCR analysis of the completed and verified strain relative to the control strain confirmed proper transcriptional overexpression of all five genes (**Figure 10**). The strong overexpression of DGA, which was the only gene under TEF_{in} expression, demonstrates the enhancement characteristics of the spliceosomal intron even with the potential competition from numerous cassettes utilizing the same promoter. ACC, which was under control of the intronless TEF promoter, showed the lowest expression. The two subunits of ACL, also under control of a TEF promoter, exhibited higher expression than D9, which was under control of an hp4d promoter. Since sampling occurred well after exponential growth phase (where hp4d can exhibit quasi-growth dependent expression (Madzak et al. 2000)), the constitutive expression of the hp4d and TEF all seem to be relatively close to each other. These results show sufficient overexpression of the targeted genes.

2-L Fermentation of MTYL089 demonstrates strong lipid accumulation capacity.

After verification of gene expression in the strain MTYL089, the lipogenic performance of the strain was tested in a 2-L bioreactor fermentation. The C/N ratio of the media was adjusted to 100 to help promote lipid accumulation. The C/N ratio determines the amount of excess carbon available in the fermentation once nitrogen has been depleted, and often requires delicate balancing to optimize lipid production over citrate production (Beopoulos et al. 2009). **Figure 11** shows the time profile for the duration of the batch fermentation. After 185 hrs of fermentation, all 90 g/L of glucose is consumed, yielding 26 g/L of biomass (dry cell weight) and a remarkable 76.8 % lipid content for a productivity of 0.109 g lipids/L/hr. The overall yield of lipids on glucose was 0.227 g lipids/g glucose, which is 70% of the theoretical maximum yield. During the lipid accumulation phase, from 66 to 185 hrs, a maximum in lipid productivity and yield were achieved, at 0.154 g lipids/L/hr and 0.277 g lipids/g glucose, respectively, with the yield increasing to 85% of the maximum theoretical yield.

Microscopy of the cell culture, shown in **Figure 12**, at the end of the fermentation shows that all the cells contain large vacuoles that occupy virtually the entire volume of the cell. Nile red staining and fluorescence indicates that the vacuoles are predominantly composed of neutral lipids. Even the few hyphal cells, which typically are less productive (Coelho et al. 2010), exhibit significant lipid accumulation producing multiple lipid bodies along the length of the cell.

Despite the dramatic accumulation of lipids observed in the culture, a number of characteristics were found to be undesirable, particularly in comparison with previous work using MTYL065. Firstly, there was a significant amount of citrate production occurring concomitantly with lipid production beginning at 75 hrs. Citrate is an intermediate of the lipid biosynthesis pathway, utilizing ATP citrate lyase for the enzymatic conversion to oxaloacetate and the lipogenic precursor acetyl-coA. Despite the overexpression of both ACL enzymes in the MTYL089 strain, the accumulation of citrate was still observed. It is possible that the C/N ratio was too high, which has been shown to lead to citrate production instead of lipid production (Beopoulos et al. 2009); however, our results show both citrate and lipids being produced simultaneously. This coupled production of both products differs from a discrete lipid production phase followed by a citrate production phase observed in experiments with strain MTYL065. Furthermore, the C/N ratio was matched to the batch

fermentation of MTYL065, which did not show this large amount of citrate production. This indicates that it is more likely that inadequate amounts of ATP generation under the fermentation conditions, combined with high upstream flux into the pathway, led to accumulation of the intracellular citrate pool, ultimately leading to secretion. Additionally, a significantly lower productivity was observed in MTYL089. Since aeration is kept constant throughout the fermentation, oxygen-limited growth is expected in the latter stages of the fermentation. However, the onset of linear growth occurred much earlier in this fermentation than with MTYL065, occurring only after one day when the biomass concentration reached approximately 6 g/L. The earlier onset of linear growth resulted in a longer fermentation time, and thus lower productivity despite the higher lipid content. Because the aeration was also matched to the fermentation conditions of MTYL065, it is likely that the metabolic changes of MTYL089 are putting greater limitations on growth. On the other hand, MTYL089 exhibited better overall lipid yield, 0.227 g/g compared to 0.195 g lipid/g glucose in MTYL065. It also ended the fermentation with a higher titer, 20.2 g/L lipids, compared to 17.6 g/L.

Table 8 summarizes the comparison of key performance characteristics between 2-L fermentations of strain MTYL065 and MTYL089. Comparison of the fatty acid profiles (**Figure 13**) indicates only slight changes in the distribution of fatty acids between the strains, having stronger preference for the monounsaturated fatty acids palmitoleate and oleate. The additional effects of ACL and D9 overexpression appear to increase the flux towards lipid synthesis, but at a considerable cost to growth rate. Additionally, since there is no matching increase in ATP generation by the cell metabolism, citrate is secreted as a byproduct rather than utilized in the lipid synthesis pathway. Since lipid synthesis and storage can strongly compete with growth for resources, tight regulation is normally necessary to manage this activity (Tehlivets et al. 2007). Overexpression of these four gene targets has unlocked a great deal of this regulation, and as a result we are observing strong competition between prioritization of cellular growth and lipid production. As a common theme in metabolic engineering, maximizing production of a particular product often requires balancing the flux towards the desired product and the overall health and growth of the organism. The contrasts between MTYL065 and MTYL089 clearly demonstrate this need, with MTYL065 exemplifying more optimized flux through the lipid synthesis pathway.

Conclusion

While the components of the lipid biosynthesis are well-understood, there is still a great deal unknown about how gene perturbations, particularly in combination, affect the capacity and flux through this pathway. By studying the oleaginous yeast *Y. lipolytica*, we are able to utilize a host organism with natural capacity for lipid production to study the extent to which metabolic engineering can improve lipid productivity and yield. We examine four gene targets - ACC, D9, ACL, DGA - and are able to achieve a remarkable 76.8% lipid content in a 2-L bioreactor with a strain carrying all four target overexpressions. By further investigation of these gene targets through combinatorial overexpression, we were able to rank the positive impact of these genes on lipid production, with DGA and ACC being strong positive contributors, D9 making slight contributions only when combined with other genes, and finally ACL making no significant positive contributions. We were also able to explore possible interactions between the individual effects, identifying the strongest synergistic interaction between ACC and DGA. The production of microbial lipids has a wide range of uses, and has fast gained attention for its utilization in the production of biodiesel. Metabolic engineering of the central pathways of lipid synthesis will be critical in providing success in enabling these future technologies and processes.

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Table 6. Strains and plasmids used in this study

Strains (host strain)	Genotype or plasmid	Source
<i>E. coli</i>		
NEB10 β	<i>araD139 Δ(ara-leu)7697 fhuA lacX74 galK (ϕ80 Δ(lacZ)M15) mcrA galU recA1 endA1 nupG rpsL (StrR) Δ(mrr-hsdRMS-mcrBC)</i>	New England Biolabs
pINA1269	<i>JMP62-LEU</i>	Yeastern
pDUET1	<i>pACYCDUET-1</i>	Novagen
JMP-URA	<i>JMP62-URA</i>	(Nicaud et al. 2002)
pMT010	<i>pINA1269 php4d::TEF</i>	Example 1
pMT015	<i>pINA1269 php4d::TEF_{in}</i>	Example 1
pMT038	<i>YTEF-LacZ</i>	Example 1
pMT013	<i>YTEF-ACC1</i>	Example 1
pMT040	<i>php4d-ACC1</i>	Example 1
pMT047	<i>YTEF-ACL1 (LEU)</i>	This Example

pMT049	YTEF-ACL2 (LEU)	This Example
pMT050	YTEF-ACL1 + YTEF-ACL2 (LEU)	This Example
pMT053	YTEFin-DGA	Example 1
pMT061	php4d-D9 (LEU)	This Example
pMT065	php4d- <i>ACC1</i> + YTEFin-DGA	Example 1
pMT066	YTEF-ACC + YLEX-D9 (LEU)	This Example
pMT073	YTEF-ACC + YLEX-D9 + YTEFin-DGA (LEU)	This Example
pMT074	YTEF-ACC + YTEF-ACL1 (LEU)	This Example
pMT075	YTEF-ACC + YLEX-D9 + YTEF-ACL1 (LEU)	This Example
pMT078	YTEF-ACC + YTEF-ACL1 + YTEF-ACL2 (LEU)	This Example
pMT079	YTEF-ACC + YLEX-D9 + YTEF-ACL1 + YTEF-ACL2 (LEU)	This Example
pMT091	pACYC Lip2 KO plasmid (URA)	This Example
pMT092	YTEFin-DGA (URA)	This Example
<i>Y. lipolytica</i>		
Polg	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2	Yeastern
MTYL038	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-LacZ-LEU2	Example 1
MTYL040	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 hp4d- <i>ACC1</i> -LEU2	Example 1
MTYL050	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACL1 + TEF-ACL2	This Example
MTYL053	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 YTEFin- <i>DGA1</i> -LEU2	Example 1
MTYL065	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 hp4d- <i>ACC1</i> + YTEFin- <i>DGA1</i> -LEU2	Example 1
MTYL066	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACC + hp4d-D9	This Example
MTYL073	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACC + hp4d-D9 + YTEFin-DGA1	This Example
MTYL078	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACC + TEF-ACL1 + TEF-ACL2	This Example
MTYL079	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACC + hp4d-D9 + TEF-ACL1 + TEF-ACL2	This Example
MTYL088	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACC + TEF-ACL1 + TEF-ACL2 + YTEFin-DGA	This Example
MTYL089	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-ACC + hp4d-D9 + TEF-ACL1 + TEF-ACL2 + YTEFin-DGA	This Example
MTYL092	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 LIP2::YTEFin-DGA	This Example

Table 7. List of all strains examined for lipid accumulation, labeled with the presence (+) or absence (-) of overexpression cassettes for the four targets: acetyl-coA carboxylase (ACC), diacylglycerol acyltransferase (DGA), ATP: citrate lyase (ACL12), and $\Delta 9$ -desaturase (D9).

Plasmid	ACC	DGA	ACL	D9	Number of Targets
MTYL038	-	-	-	-	0
MTYL040	+	-	-	-	1
MTYL053	-	+	-	-	1
MTYL050	-	-	+	-	1
MTYL061	-	-	-	+	1
MTYL065	+	+	-	-	2
MTYL078	+	-	+	-	2
MTYL066	+	-	-	+	2
MTYL069	-	+	-	+	2
MTYL073	+	+	-	+	3
MTYL079	+	-	+	+	3
MTYL088	+	+	+	-	3
MTYL089	+	+	+	+	4

- 5 Table 8. Comparison of fermentation characteristics between strains MTYL065 and MTYL089. C/N ratio of both fermentations were 100, performed in 2-L bioreactors. Glucose reactor initially charged with 90 g/L glucose.

	MTYL065	MTYL089
Overall Biomass Yield	0.316 g/g	0.295 g/g
Overall Lipid Yield	0.195 g/g	0.227 g/g
Overall Productivity	0.143 g/L/hr	0.109 g/L/hr
Lipid Production Phase	70 - 120 hrs	66 - 185 hrs
Maximum Lipid Yield	0.249 g/g	0.277 g/g
Maximum Productivity	0.178 g/L/hr	0.154 g/L/hr
Final Lipid Titer	17.6 g/L	20.2 g/L

Table 9. Primers used in this study. Relevant restriction sites are in bold.

Primer	Description	Sequence	SEQ ID
<i>PCR</i>			
MT252	ACL1	<i>CTTACAACGCGTATGTCTGCCAACGAGAACATCTCC</i>	31
MT253	ACL1	<i>CTAGGTATGCATCTATGATCGAGTCTTGGCCTTGGA</i>	32
MT254	ACL2	<i>CTTACAACGCGTATGTCAGCGAAATCCATTACGA</i>	33
MT255	ACL2	<i>CTAGGTATGCATTTAAACTCCGAGAGGAGTGGAAGC</i>	34
MT283	D9	<i>AATGGTGAAAAACGTGGACCAAGTG</i>	35
MT284	D9	<i>CTCACAGGATCCCTAAGCAGCCATGCCAGACATACC</i>	36
MT220	Cassette	<i>CCCGGCAACAATTAATAGACTGGAT</i>	37
MT265	Cassette	<i>TTCGGACACGGGCATCTCAC</i>	38
<i>RT-PCR</i>			
MTR001	Actin	<i>TCCAGGCCGTCCTCTCCC</i>	39
MTR002	Actin	<i>GGCCAGCCATATCGAGTCGCA</i>	40
MTR031	DGA	<i>AACGGAGGAGTGGTCAAGCGA</i>	41
MTR032	DGA	<i>TTATGGGGAAGTAGCGGCCAA</i>	42
MTR033	D9	<i>GACCGACTCCAACAAGGACCCTT</i>	43
MTR034	D9	<i>GGGTTGGGCACAAGCAGCAT</i>	44
MTR041	ACC	<i>GCTTCTCACGGAGGTCATACAGT</i>	45
MTR042	ACC	<i>CTGCGGCAATACCGTTGTTAG</i>	46
MTR068	ACL1	<i>CCTCCTTGGTGAGGTTGGTGGT</i>	47
MTR069	ACL1	<i>GCGACGATGGGCTTCTTGATC</i>	48
MTR070	ACL2	<i>CCTTCAAGGGCATCATCCGG</i>	49
MTR071	ACL2	<i>CGCCTCGTCGCACGTAAATCT</i>	50

Example 3.

5

Materials and Methods***Yeast strains, growth, and culture conditions***

The ACC+DGA1 transformant strain of *Yarrowia lipolytica* (MTLY065), as discussed in Example 1, was employed in the experiments described in this section. YPD media was prepared as described in Example 1. The media constituents used in the bioreactor were yeast nitrogen base (without amino acids and ammonium sulfate) (Amresco), yeast extract (Difco), ammonium sulfate (Macron Chemicals), sodium acetate (Macron Chemicals) and acetic acid (Sigma Aldrich, St. Louis, MO). Bioreactor runs were carried out in 2L baffled stirred tank reactors. The inoculum for the bioreactor was prepared as described in Example 1.

15

Bioreactor operation: acetate, acetic acid, and ammonium sulfate feeding

The initial media composition in the reactor was: 30 g/L sodium acetate, 2.5 g/L yeast extract, 4.25 g/L yeast nitrogen base and 2.4 g/L ammonium sulfate. The yeast extract and yeast nitrogen base concentrations used in the experiments described in this section were higher as compared to those discussed in Example 1. A carbon to nitrogen ratio (C/N) of 20 was chosen to provide sufficient nitrogen for biomass production. A constant dissolved oxygen level of 20% was maintained at all times in the reactor using cascade control. A pH setpoint of 7.3 was used.

In order to generate high cell densities, which would then serve as the platform for lipid accumulation, a strategy was devised and implemented in which the carbon source of 30 g/L of sodium acetate was supplemented by feeding acetic acid. The use of acetic acid had several advantages, in that it served the dual purpose of providing a source of carbon to the growing and dividing cells and at the same time provided pH control. In order to maintain a low C/N ratio for the initial period of growth, a nitrogen source was fed concurrently with the acetic acid. The initial period of growth involved feeding 15 g/L ammonium sulfate per liter of 30% (vol/vol) acetic acid. The subsequent feed contained pure 100% acetic acid (and no ammonium sulfate), thus creating an increase in the C/N ratio as the nitrogen was consumed by the cells and depleted from the medium, resulting in improved lipid accumulation.

The working volume at the beginning of the run was 1.6 L. 1L of the acid and ammonium sulfate solution was added over the first 60 h. Around 200 ml of pure acetic acid was added over the subsequent 40 h. Culture volume was not removed at any point other than the sampling done every 24 h for the purpose of measurements. To compensate for the additional volume entering the reactor, liquid was allowed to evaporate. The high aeration rate (2.5 vvm) led to sufficient evaporation, which helped in maintaining the volume of the reactor. Even with evaporation, the volume of the broth did go up slightly to around 1.8L during the run.

Optical density (OD) of the culture broth was measured every 24 hours. Samples were stored at -20° C for lipid analysis. HPLC analysis yielded acetate levels in the reactor.

Ammonium was measured with an YSI 7100 ammonium electrode (YSI Life Sciences). Biomass was determined as discussed in Example 1.

Lipid analysis

The lipid analysis involved a direct transesterification protocol, which was adapted from US Patent 7,932,077 and Griffiths et al. LIPIDS (2010) 45:1053–1060, the entire contents of each of which are incorporated herein by reference. The protocol employed 0.5 N sodium methoxide and 18 M sulfuric acid (Sigma Aldrich, St. Louis, MO). Sodium methoxide was generated in-house using sodium hydroxide (Macron Chemicals) and methanol (Sigma Aldrich, St. Louis, MO). 500 µl of the 0.5 N sodium methoxide was first added to 1 mg of the pelleted cell sample followed by vortexing for 1 hour. This was followed by addition of 40 µl sulfuric acid and 500 µl hexane followed by another vortex step for 30 min to dissolve the transesterified methyl esters into the hexane. The reaction mixture was centrifuged for 1 min at 8000 rpm, and 800 µl of the upper hexane phase were transferred into GC vials and analyzed in a GC-FID. The GC column, operating method, and final analysis followed the methods discussed in Example 1, except that glyceryl triheptadecanoate was used as the control instead of tridecanoic acid.

Results and Discussion

The results of the bioreactor run are summarized in Table 10.

Table 10. Results of the bioreactor run with acetate

Lipid titer	55 g/L
Lipid content	67 %
Overall lipid productivity	0.56 g/L/h
Maximum lipid productivity	1 g/L/h
Overall lipid yield (g lipid/ g acetate)	0.16 g/g
Maximum lipid yield	0.24 g/g

Figures 14 and 15 show trends of nitrogen, non-lipid and lipid titers, lipid content and C/N ratios during a representative acetate bioreactor run. The non-lipid biomass went up for the initial period of growth, when nitrogen was fed into the reactor. After 48h, nearly all the biomass increase was due to lipid accumulation. In this period, the cells synthesized lipids at a yield of 0.24 g/g and at a rate of 1 g/L/h to produce a final lipid titer of 55 g/L, which is at least 10-fold higher than the titers achieved in the experiments described in Example 1. The final lipid content and overall lipid yield, though, were very similar to the lipid content and overall lipid yield in Example 1. These numbers depend mostly on the microbial strain

employed. Almost 70% of the lipid was found to be oleate, which is consistent with the finding described in Example 1.

The run discussed here was aimed at maximizing lipid production using the ACC+DGA1 mutant through the adoption of above mentioned operating strategies and optimal process conditions. A comparison of the results of this run with those shown in Example 1 is been shown in table 11 below. A titer improvement of 10 fold and overall productivity improvement of 14 fold is seen between the acetate runs.

Table 11. Comparison of the run on acetate with the runs shown in example 1

	Glucose (example 1)	Acetate (example 1)	Acetate (this section)
Lipid titer	17.6 g/L	5.5 g/L	55 g/L
Overall lipid productivity	0.14 g/L/h	0.04 g/L/h	0.56 g/L/h
Max. lipid productivity	0.26 g/L/h	0.10 g/L/h	1 g/L/h
Overall lipid yield	0.19 g/g	0.15 g/g	0.16 g/g
Maximum lipid yield	0.25 g/g	0.27 g/g	0.24 g/g

Cells with high lipid content (greater than 80%) on centrifugation float at the top instead of settling down. These cells appear to be filled with oil under microscope. **Figure 16** shows such cells under an oil immersion microscope (100x) when stained with Nile Red. The bright spots are the lipid vacuoles.

All publications, patents and sequence database entries mentioned in the specification herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

EQUIVALENTS AND SCOPE

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above description, but rather is as set forth in the appended claims.

In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant

to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present
5 in, employed in, or otherwise relevant to a given product or process.

Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, *etc.*, from one or more of the claims or from relevant portions of the description is introduced into another claim. For example, any claim that is dependent on
10 another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are
15 included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

Where elements are presented as lists, *e.g.*, in Markush group format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It is also noted that the term “comprising” is intended to be open
20 and permits the inclusion of additional elements or steps. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, steps, *etc.*, certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, steps, *etc.* For purposes of simplicity those embodiments have not been specifically set forth *in haec verba*
25 herein. Thus for each embodiment of the invention that comprises one or more elements, features, steps, *etc.*, the invention also provides embodiments that consist or consist essentially of those elements, features, steps, *etc.*

Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the
30 understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates

otherwise. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower
5 limit of the range.

In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are given, any value within the range may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or
10 methods of the invention, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.

CLAIMS

1. An isolated oleaginous cell, comprising a genetic modification that increases expression of a DGA1 gene product.
- 5 2. The isolated oleaginous cell of claim 1, further comprising a genetic modification that increases expression of an ACC1 gene product.
3. The isolated oleaginous cell of claim 1 or claim 2, further comprising a genetic modification that increases expression of an SCD gene product.
4. The isolated oleaginous cell of any of claims 1-3, further comprising a genetic
10 modification that increases expression of an ACL gene product.
5. The isolated oleaginous cell of any one of claims 1-4, wherein the genetic modification comprises a nucleic acid construct that increases the expression of the gene product, the nucleic acid construct comprising
 - (a) an expression cassette comprising a nucleic acid sequence encoding the gene
15 product under the control of a suitable homologous or heterologous promoter, and/or
 - (b) a nucleic acid sequence that modulates the level of expression of the gene product when inserted into the genome of the cell.
6. The isolated oleaginous cell of claim 5, wherein the promoter is an inducible or a constitutive promoter.
- 20 7. The isolated oleaginous cell of claim 5 or claim 6, wherein the promoter is a TEF promoter.
8. The isolated oleaginous cell of any one of claims 5-7, wherein the expression construct further comprises an intron.
9. The isolated oleaginous cell of claim 8, wherein the intron is downstream of the
25 transcription initiation site.

10. The isolated oleaginous cell of claim 8 or claim 9, wherein the intron is within the nucleic acid sequence encoding the gene product.

11. The isolated oleaginous cell of any of claims 1-10, wherein the nucleic acid construct inhibits or disrupts the natural regulation of a native gene encoding the gene product resulting
5 in overexpression of the native gene.

12. The isolated oleaginous cell of claim 11, wherein inhibition or disruption of the natural regulation of the native gene is mediated by deletion, disruption, mutation and/or substitution of a regulatory region, or a part of a regulatory region regulating expression of the gene.

13. The isolated oleaginous cell of any of claims 1-12, wherein the gene product is a
10 transcript.

14. The isolated oleaginous cell of any of claims 1-13, wherein the gene product is a protein.

15. The isolated oleaginous cell of any of claims 5-14, wherein the nucleic acid construct is inserted into the genome of the cell.

16. The isolated oleaginous cell of any of claims 1-15, wherein the increased expression of
15 the gene product confers a beneficial phenotype for the conversion of a carbon source to a fatty acid, fatty acid derivative and/or triacylglycerol (TAG) to the cell.

17. The isolated oleaginous cell of claim 16, wherein the beneficial phenotype comprises a modified fatty acid profile, a modified TAG profile, an increased fatty acid and/or triacylglycerol synthesis rate, an increase conversion yield, an increased triacylglycerol
20 accumulation in the cell, and/or an increased triacylglycerol accumulation in a lipid body of the cell.

18. The isolated oleaginous cell of claim 16 or 17, wherein the synthesis rate of a fatty acid or a TAG of the cell is at least 2-fold increased as compared to unmodified cells of the same cell type.

19. The isolated oleaginous cell of claim 18, wherein the synthesis rate of a fatty acid or a TAG of the cell is at least 5-fold increased as compared to unmodified cells of the same cell
25 type.

20. The isolated oleaginous cell of claim 18, wherein the synthesis rate of a fatty acid or a TAG of the cell is at least 10-fold increased as compared to unmodified cells of the same cell type.
21. The isolated oleaginous cell of any of claims 16-20, wherein the cell converts a carbon
5 source to a fatty acid or a TAG at a conversion rate within the range of about 0.025g/g to about 0.32g/g (g TAG produced/ g Glucose consumed).
22. The isolated oleaginous cell of claim 21, wherein the cell converts a carbon source to a fatty acid or a TAG at a conversion rate of at least about 0.11g/g.
23. The isolated oleaginous cell of claim 22, wherein the cell converts a carbon source to a
10 fatty acid or a TAG at a conversion rate of at least about 0.195g/g.
24. The isolated oleaginous cell of claim 23, wherein the cell converts a carbon source to a fatty acid or a TAG at a conversion rate of at least about 0.24g/g.
25. The isolated oleaginous cell of claim 24, wherein the cell converts a carbon source to a fatty acid or a TAG at a conversion rate of at least about 0.27g/g.
- 15 26. The isolated oleaginous cell of any one of claims 1-25, wherein the cell comprises a lipid body or vacuole.
27. The isolated oleaginous cell of any one of claims 1-26, wherein the cell is a bacterial cell, an algal cell, a fungal cell, or a yeast cell.
28. The isolated oleaginous cell of any of claims 1-27, wherein the cell is an oleaginous
20 yeast cell.
29. The isolated oleaginous cell of any of claims 1-28, wherein the cell is a *Y. lipolytica* cell.
30. A culture, comprising the oleaginous cell of any of claims 1-29.
31. The culture of claim 30, further comprising a carbon source.
32. The culture of claim 31, wherein the carbon source comprises a fermentable sugar.
- 25 33. The culture of claim 32, wherein the fermentable sugar is a C6 sugar.

34. The culture of claim 33, wherein the carbon source comprises glucose.

35. The culture of any of claims 31-34, wherein the carbon source comprises an organic acid.

36. The culture of claim 35, wherein the organic acid is acetic acid.

37. The culture of claim 36, wherein the acetic acid is at a concentration of at least 5%
5 vol/vol, at least 10% vol/vol, at least 15% vol/vol, at least 20% vol/vol, at least 25% vol/vol,
or at least 30% vol/vol.

38. The culture of any one of claims 31-37, wherein the carbon source comprises acetate.

39. The culture of claim 38, wherein the acetate is at a concentration of at least 1% vol/vol.

40. The culture of claim 38, wherein the acetate is at a concentration of at least 2% vol/vol.

10 41. The culture of claim 38, wherein the acetate is at a concentration of at least 3% vol/vol.

42. The culture of claim 38, wherein the acetate is at a concentration of at least 4% vol/vol.

43. The culture of claim 38, wherein the acetate is at a concentration of at least 5% vol/vol.

44. The culture of any of claims 30-43, wherein the culture comprises glycerol.

45. The culture of claim 44, wherein the glycerol is at a concentration of about 2% vol/vol.

15 46. The culture of any one of claims 30-45, wherein the culture comprises a dissolved oxygen
level of at least 5%, at least 10%, at least 15%, or at least 20%.

47. The culture of any one of claims 30-46, wherein the culture exhibits a pH within the
range of pH7.0 to pH7.5.

20 48. The culture of any one of claims 30-47, wherein the culture comprises ammonium
sulfate.

49. The culture of claim 48, wherein the culture comprises ammonium sulfate and acetic acid
at a ratio of 1:2.

50. The culture of any one of claims 30-49, wherein the culture exhibits a lipid titer between 5 g/l and 60 g/l.

51. The culture of any one of claims 30-50, wherein the culture exhibits a lipid production between 0.04 g/l/h and 0.60 g/l/h.

5 52. The culture of any one of claims 30-51, wherein the culture exhibits a maximum lipid productivity of between 0.1 g/l/h and 1 g/l/h.

53. A method, comprising

10 contacting a carbon source with an isolated oleaginous cell, the cell comprising a genetic modification that increases expression of a DGA1 gene product; and
 incubating the carbon source contacted with the cell under conditions suitable for at least partial conversion of the carbon source into a fatty acid or a triacylglycerol by the cell.

54. The method of claim 53, wherein the oleaginous cell further comprises a genetic modification that increases expression of an ACC1 gene product.

15 55. The method of claim 53 or claim 54, wherein the oleaginous cell further comprises a genetic modification that increases expression of an SCD gene product.

56. The method of any of claims 53-55, wherein the oleaginous cell further comprises a genetic modification that increases expression of an ACL gene product.

20 57. The method of any one of claims 53-56, wherein the isolated oleaginous cell is the isolated oleaginous cell of any of claims 1-27.

58. The method of any one of claims 53-57, wherein the carbon source comprises a fermentable sugar.

59. The method of claim 58, wherein the carbon source comprises glucose.

60. The method of any of claims 53-59, wherein the carbon source comprises acetate.

25 61. The method of claim 60, wherein the acetate is at a concentration of at least 1% vol/vol.

62. The method of claim 60, wherein the acetate is at a concentration of at least 2% vol/vol.

63. The method of claim 60, wherein the acetate is at a concentration of at least 3% vol/vol.

64. The method of claim 60, wherein the acetate is at a concentration of at least 4% vol/vol.

65. The method of claim 60, wherein the acetate is at a concentration of at least 5% vol/vol.

5 66. The method of any one of claims 53-65, wherein the carbon source comprises acetic acid.

67. The method of claim 66, wherein the acetic acid is at a concentration of at least 5% vol/vol, at least 10% vol/vol, at least 15% vol/vol, at least 20% vol/vol, at least 25% vol/vol, or at least 30% vol/vol.

10 68. The method of any one of claims 53-67, wherein the method comprises contacting the cell with dissolved oxygen at a level of at least 5%, at least 10%, at least 15%, or at least 20%.

69. The method of any one of claims 53-68, wherein the contacting and/or the incubating is performed at a pH within the range of pH7.0 to pH7.5.

15 70. The method of any one of claims 53-69, wherein the method comprises contacting the cell with ammonium sulfate.

71. The method of claim 70, wherein the method comprises contacting the cell with ammonium sulfate and acetic acid at a ratio of 1:2.

72. The method of any of claims 53-71, wherein the method further comprises contacting the cells with glycerol.

20 73. The method of claim 72, wherein the method comprises contacting the cells with glycerol at a concentration of about 2% vol/vol.

74. The method of any one of claims 53-73, wherein the carbon source contacted with the isolated oleaginous cell is incubated in a reactor.

75. The method of any of claims 53-74, wherein the carbon source is contacted with the isolated oleaginous cell and incubated for conversion of the carbon source to a fatty acid or a triacylglycerol in a fed batch process.

5 76. The method of any of claims 53-75, wherein the carbon source is contacted with the isolated oleaginous cell and incubated for conversion of the carbon source to a fatty acid or a triacylglycerol in a continuous process.

77. The method of any of claims 53-76, further comprising contacting an additional amount of the carbon source or an amount of an additional carbon source with the carbon source contacted with the isolated oleaginous cell one or more times during the incubating step.

10 78. The method of any of claims 53-77, wherein the fatty acid or the triacylglycerol is extracted from the carbon source contacted with the isolated oleaginous cell by solvent extraction.

79. The method of claim 78, wherein the solvent extraction comprises a chloroform methanol extraction.

15 80. The method of claim 78, wherein the solvent extraction comprises a hexane extraction.

81. The method of any of claims 53-80, wherein the fatty acid or the triacylglycerol is separated from the carbon source contacted with the isolated oleaginous cell and subsequently refined by transesterification.

20 82. A method, comprising modifying the fatty acid profile, the triacylglycerol profile, the fatty acid synthesis rate, the triacylglycerol synthesis rate, the extent of fatty acid derivative accumulation, the rate of fatty acid derivative secretion, the rate of carbohydrate to fatty acid or fatty acid derivative conversion, and/or the efficient yield of carbohydrate to fatty acid or fatty acid derivative conversion in an oleaginous cell by increasing in the cell the expression of a DGA1 gene product.

25 83. The method of claim 82, further comprising increasing in the cell the expression of an ACC1 gene product.

84. The method of claim 82 or claim 83, further comprising increasing in the cell the expression of an SCD gene product.

85. The method of any claim 82-84, further comprising increasing in the cell the expression of a ACL gene product.

5 86. The method of any one of claims 82-85, wherein the extent of fatty acid derivative accumulation is the extent of fatty acid derivative accumulation in a lipid body.

87. The method of any one of claims 82-86, wherein the fatty acid derivative is a triacylglycerol.

10 88. The method of any one of claims 82-87, wherein modifying the fatty acid profile, the triacylglycerol profile, the fatty acid synthesis rate, the triacylglycerol synthesis rate, the extent of fatty acid derivative accumulation, the rate of fatty acid derivative secretion, the rate of carbohydrate to fatty acid or fatty acid derivative conversion, and/or the efficient yield of carbohydrate to fatty acid or fatty acid derivative conversion in the oleaginous cell comprises increasing the fatty acid synthesis rate, the triacylglycerol synthesis rate, the extent of fatty acid derivative accumulation, the rate of fatty acid derivative secretion, the rate of carbohydrate to fatty acid or fatty acid derivative conversion, and/or the efficient yield of carbohydrate to fatty acid or fatty acid derivative conversion in the oleaginous cell.

15 89. The method of claim 88, wherein modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 2-fold.

90. The method of claim 88, wherein modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 3-fold.

25 91. The method of claim 88, wherein modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 4-fold.

92. The method of claim 88, wherein modifying the efficiency of carbohydrate to fatty acid or fatty acid derivative conversion of the cell comprises increasing the efficiency of conversion by at least 5-fold.
93. The method of any one of claims 82-92, wherein the cell is a yeast cell.
- 5 94. The method of claim 93, wherein the yeast cell is a *Yarrowia* sp. cell.
95. The method of claim 94, wherein the oleaginous yeast is *Y. lipolytica*.
96. An isolated nucleic acid molecule comprising:
a) a nucleotide sequence that encodes SEQ ID NO: 2 (*Y. lipolytica* DGA1), or
b) a nucleotide sequence that is at least 85% identical to the nucleotide sequence of a).
- 10 97. The isolated nucleic acid molecule of claim 96, wherein the nucleotide sequence that encodes SEQ ID NO:2 comprises SEQ ID NO: 1.
98. An expression cassette, comprising the isolated nucleic acid molecule of claim 96 or 97 and a heterologous promoter.
99. The expression cassette of claim 98, wherein the promoter is a constitutive promoter or
15 an inducible promoter.
100. The expression cassette of claim 99, wherein the heterologous promoter is a Translation Elongation Factor (TEF) promoter.
101. The expression cassette of any of claims 98-100, wherein the heterologous promoter comprises an intron.
- 20 102. The expression cassette of any of claims 98-101, wherein the heterologous promoter further comprises a start codon.
103. The expression cassette of claim 102, wherein the intron is downstream of the translation start site of the nucleotide sequence that encodes SEQ ID NO: 2.
104. A vector comprising the expression cassette of any one of claims 98-103.

105. A cell comprising the expression cassette of any of claims 98-104 or at least a part of the vector of claim 104.

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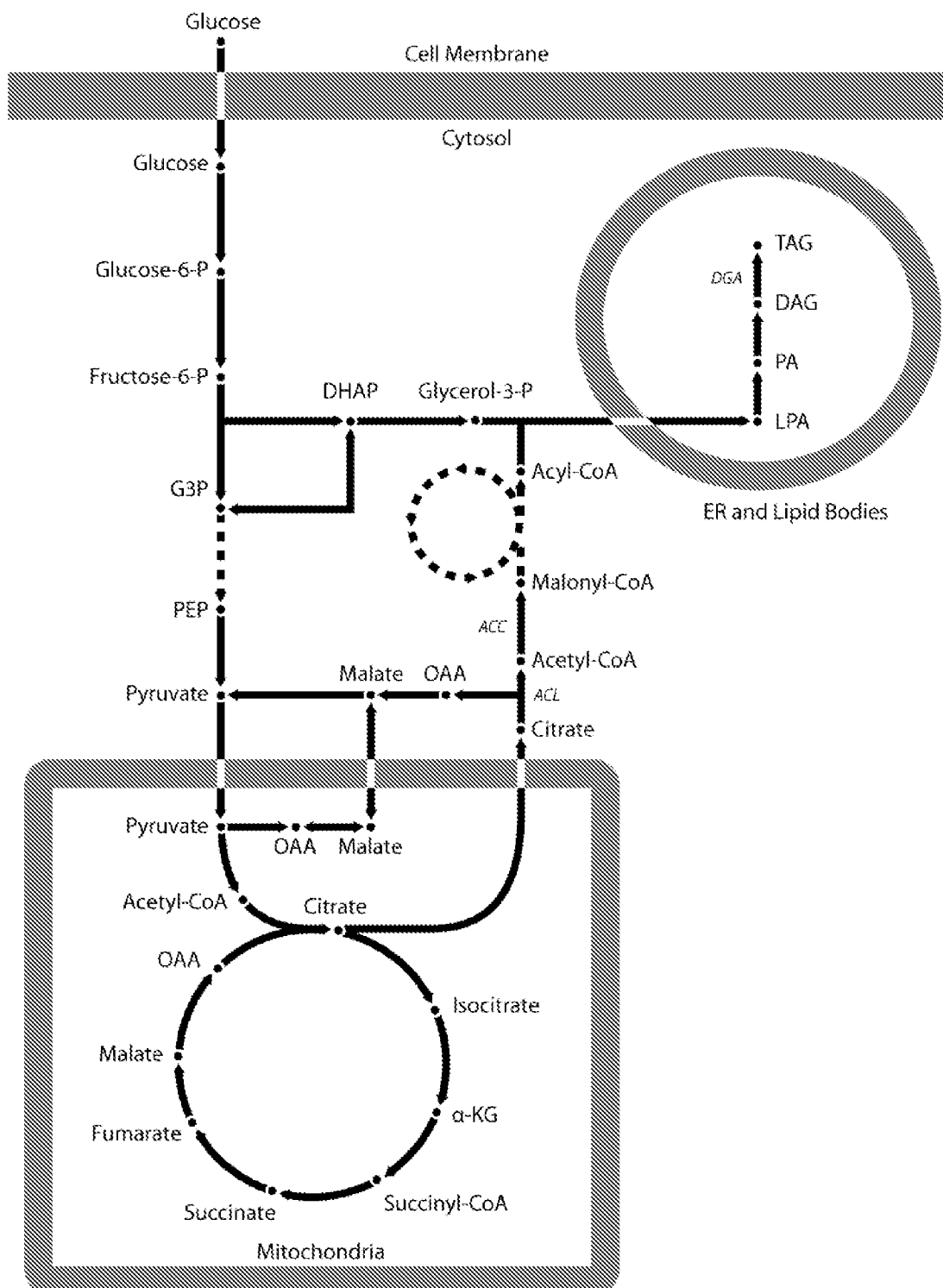


Figure 1

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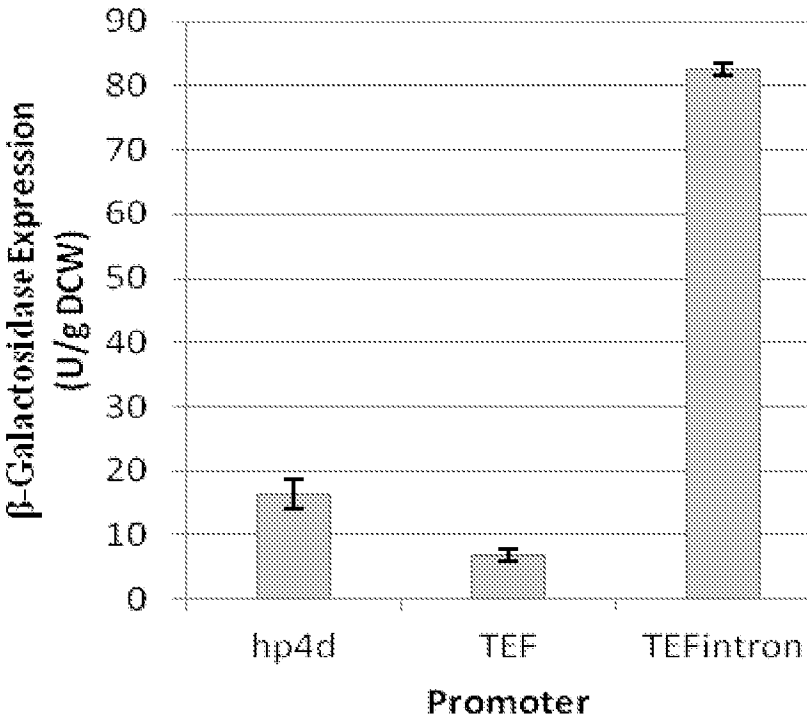


Figure 2

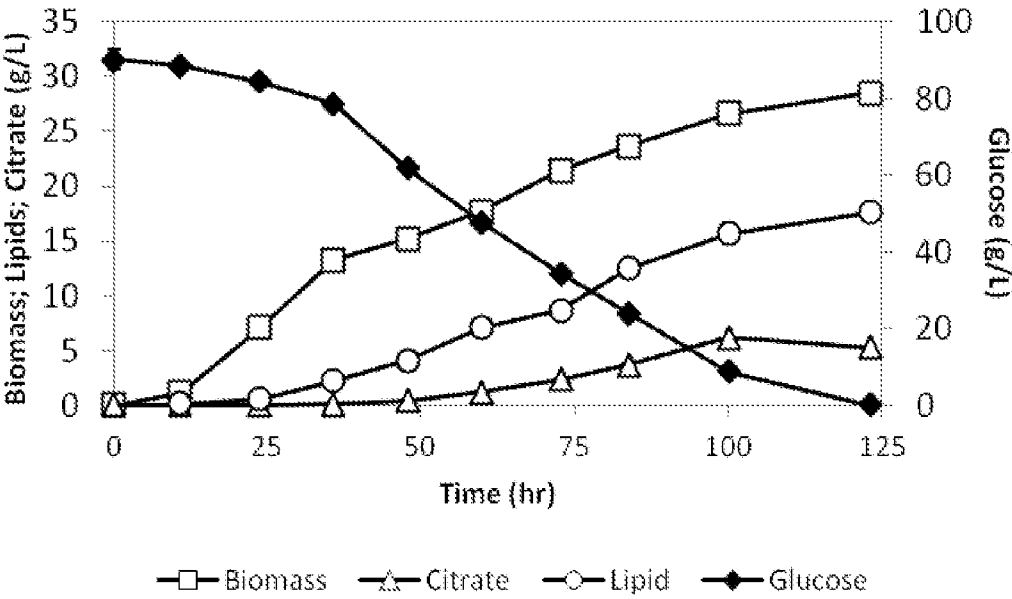


Figure 3

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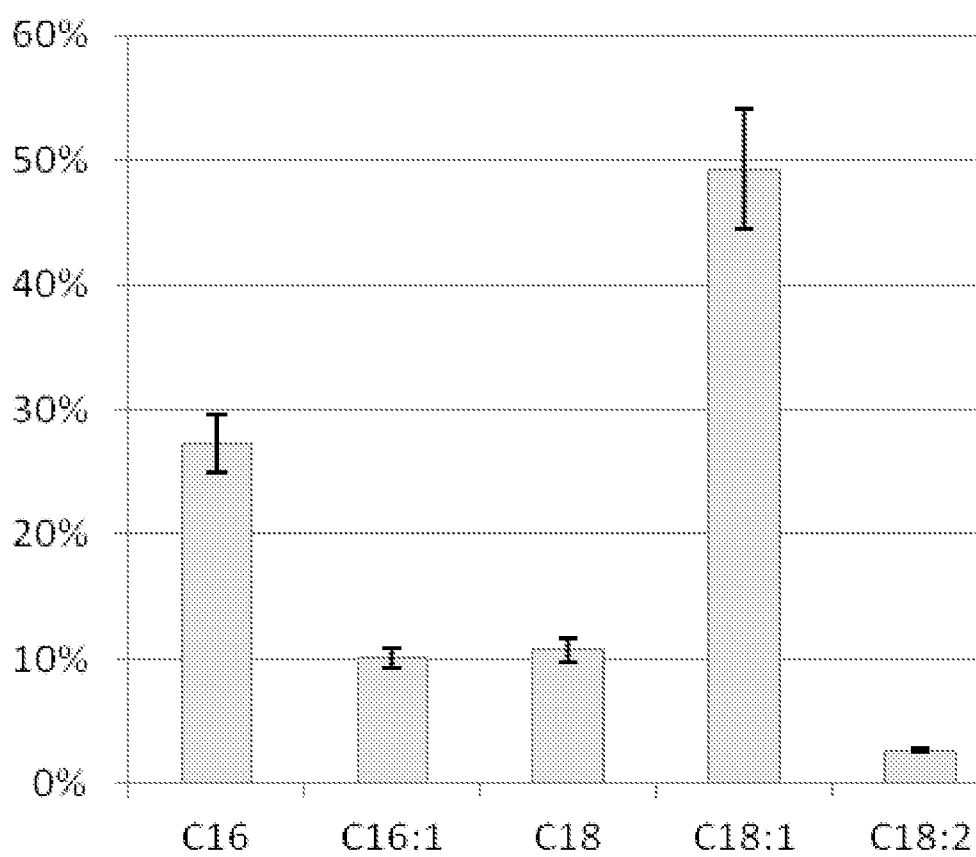


Figure 4

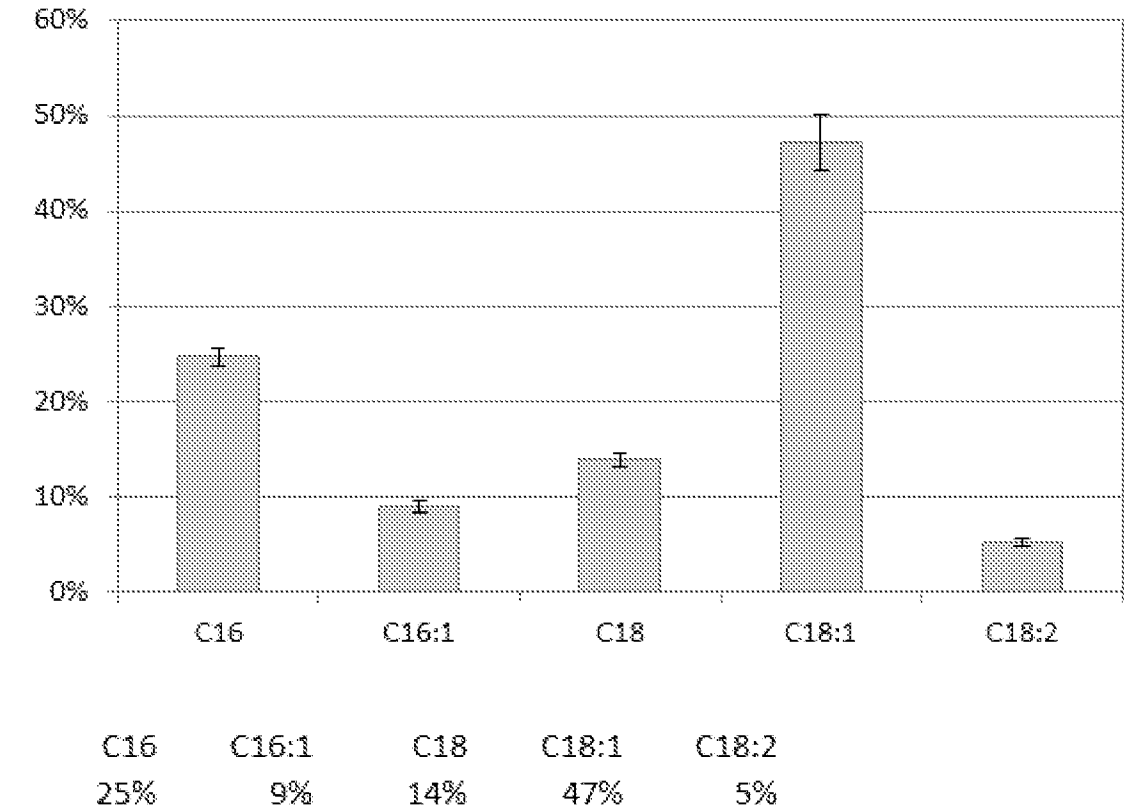


Figure 5

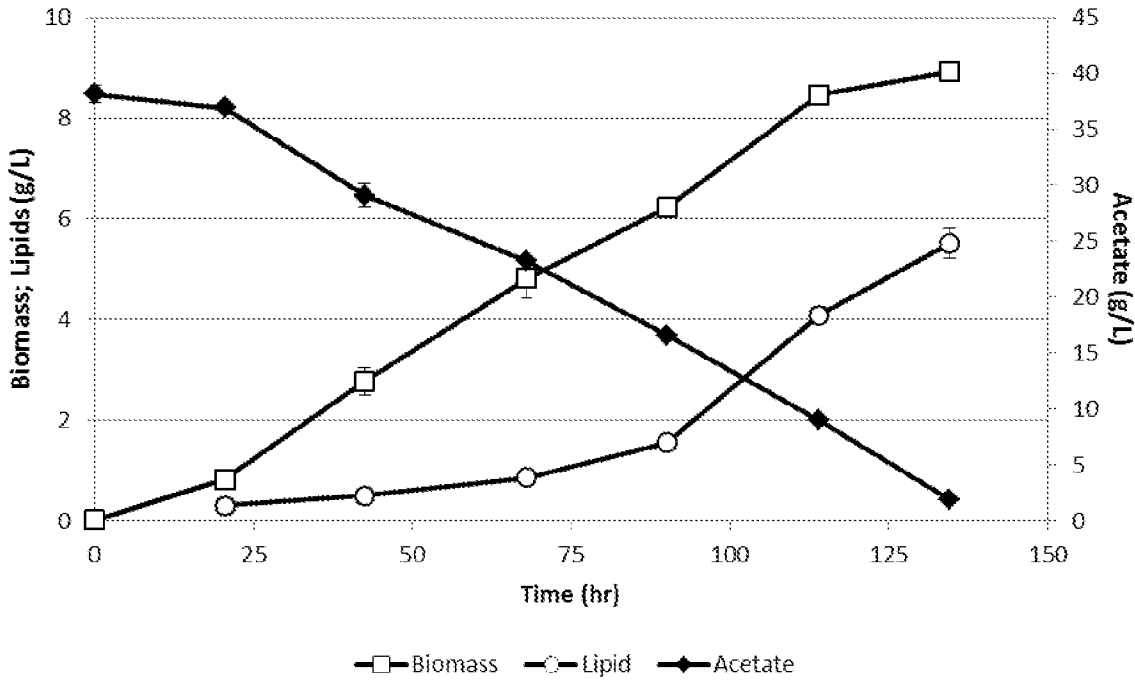


Figure 6

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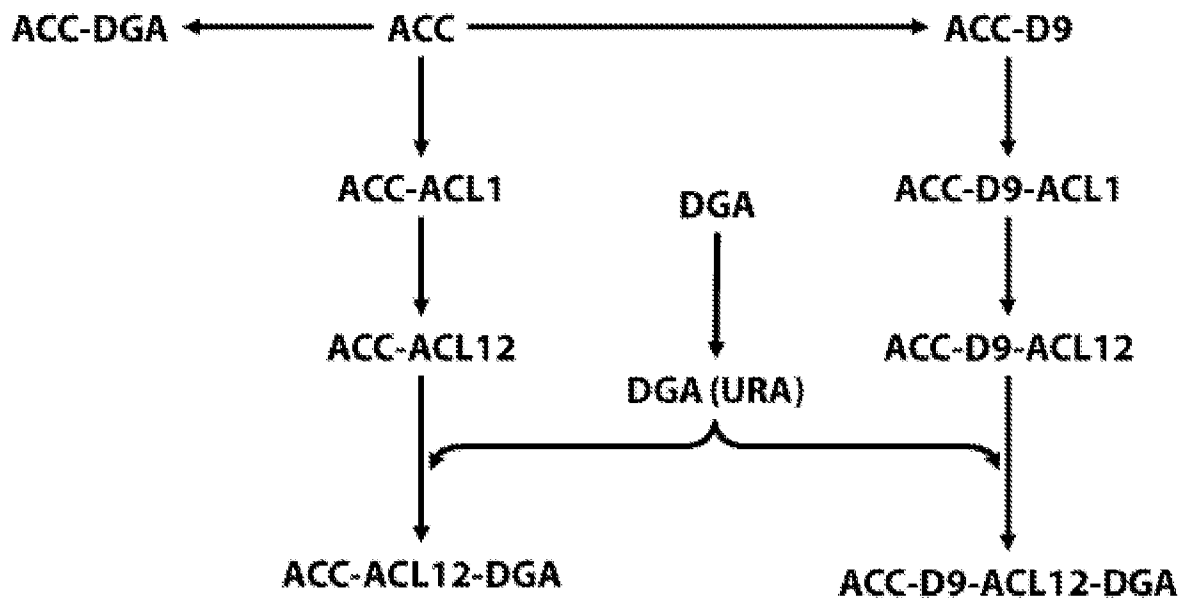


Figure 7

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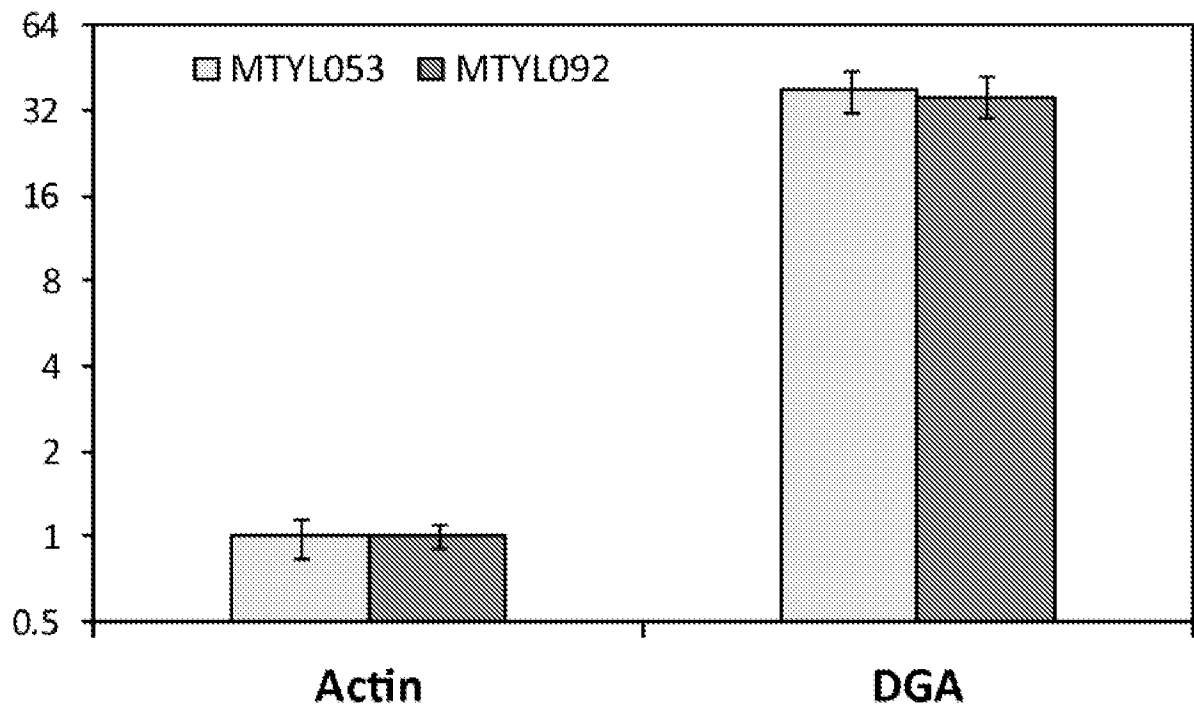


Figure 8

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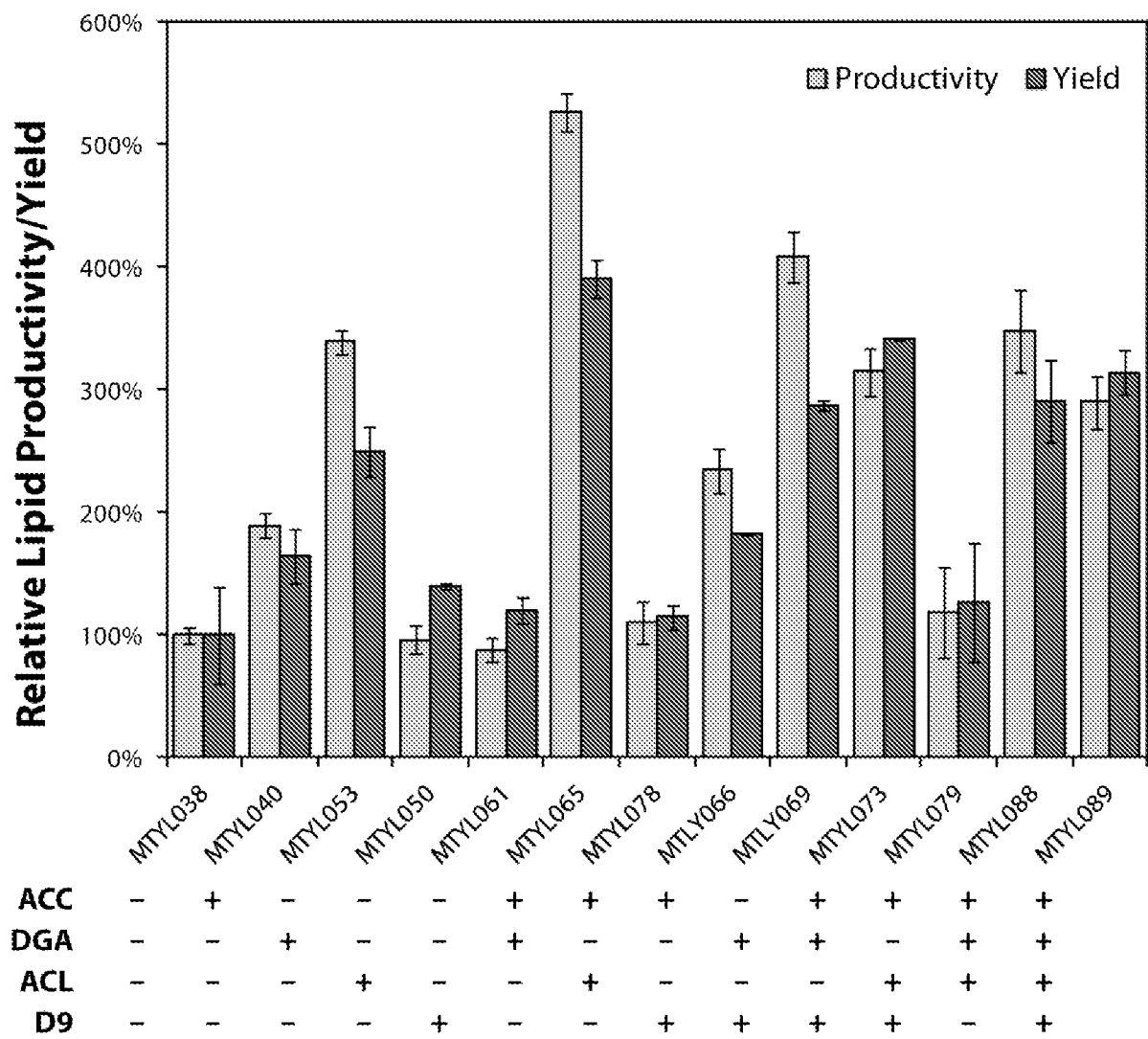


Figure 9

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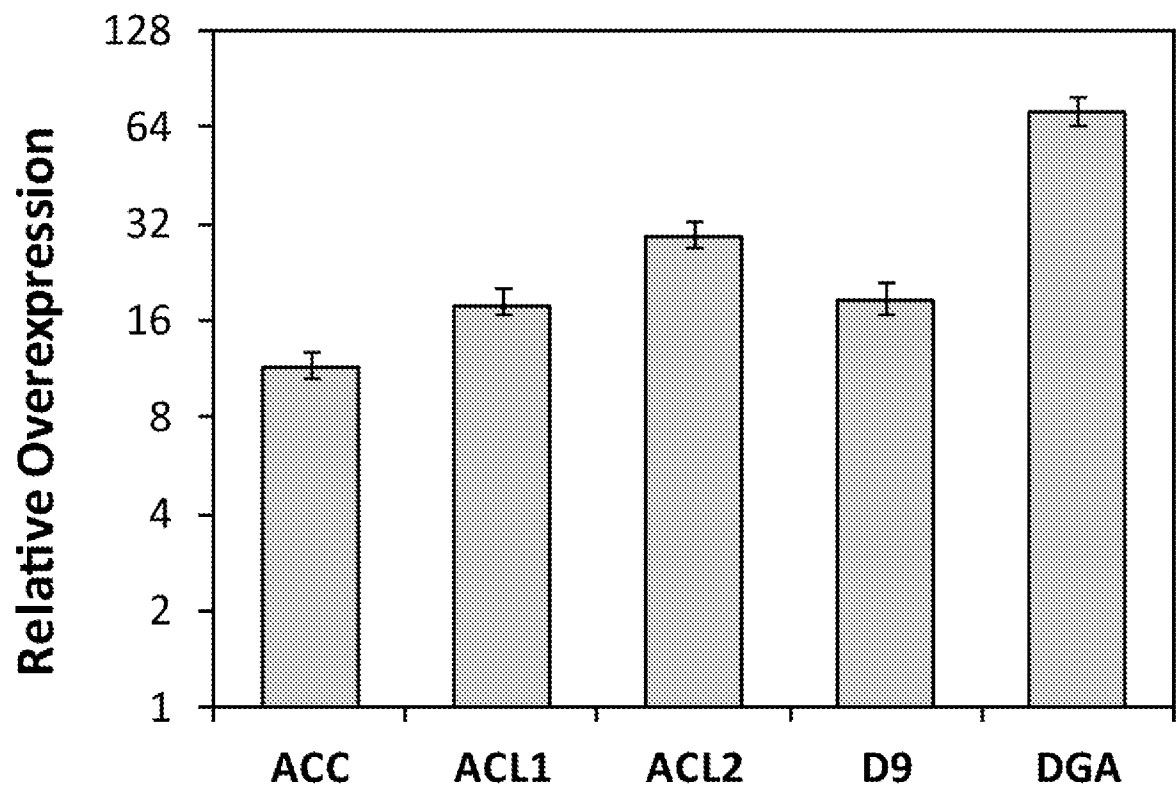


Figure 10

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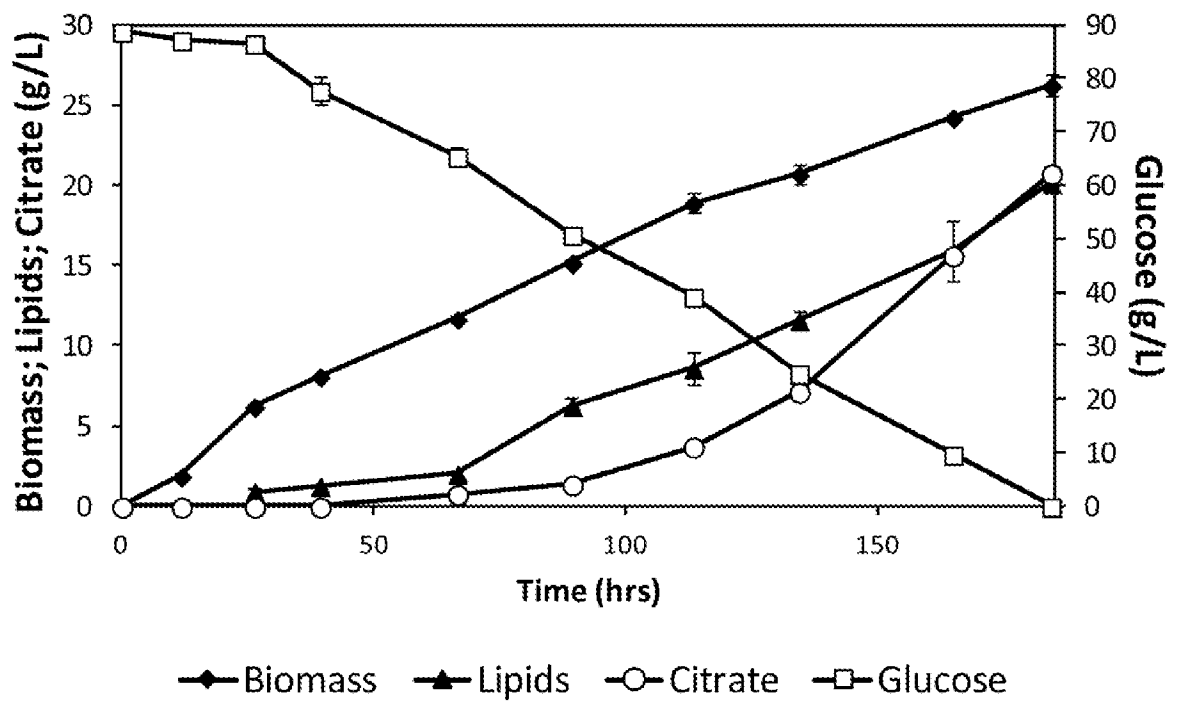


Figure 11

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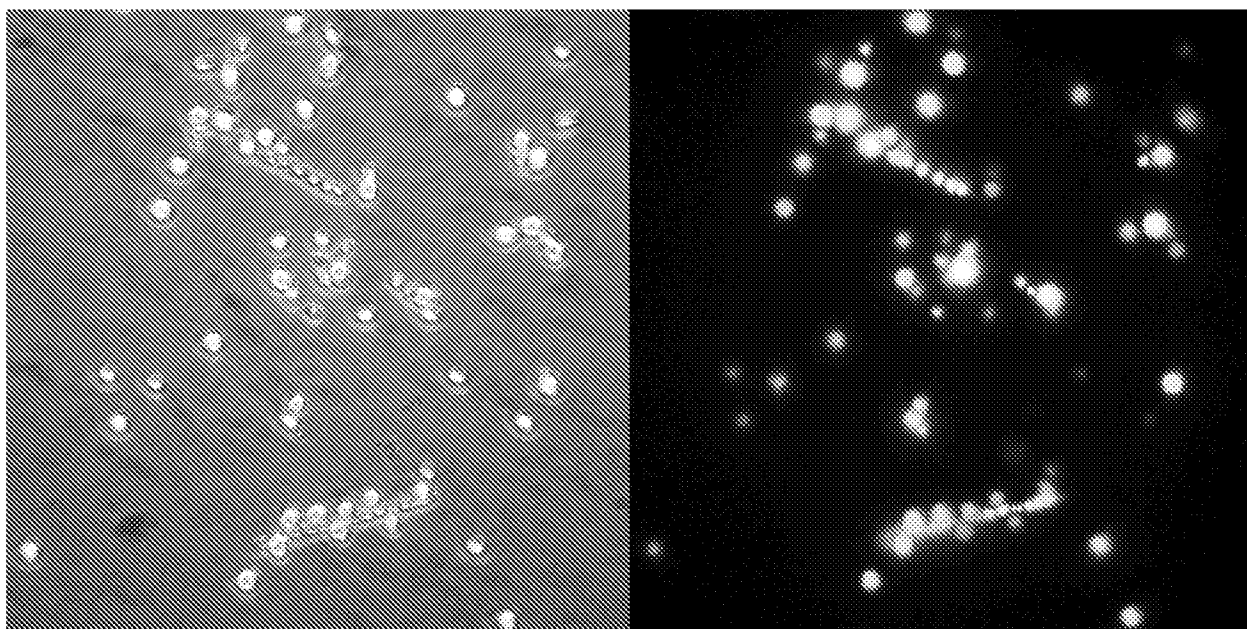


Figure 12

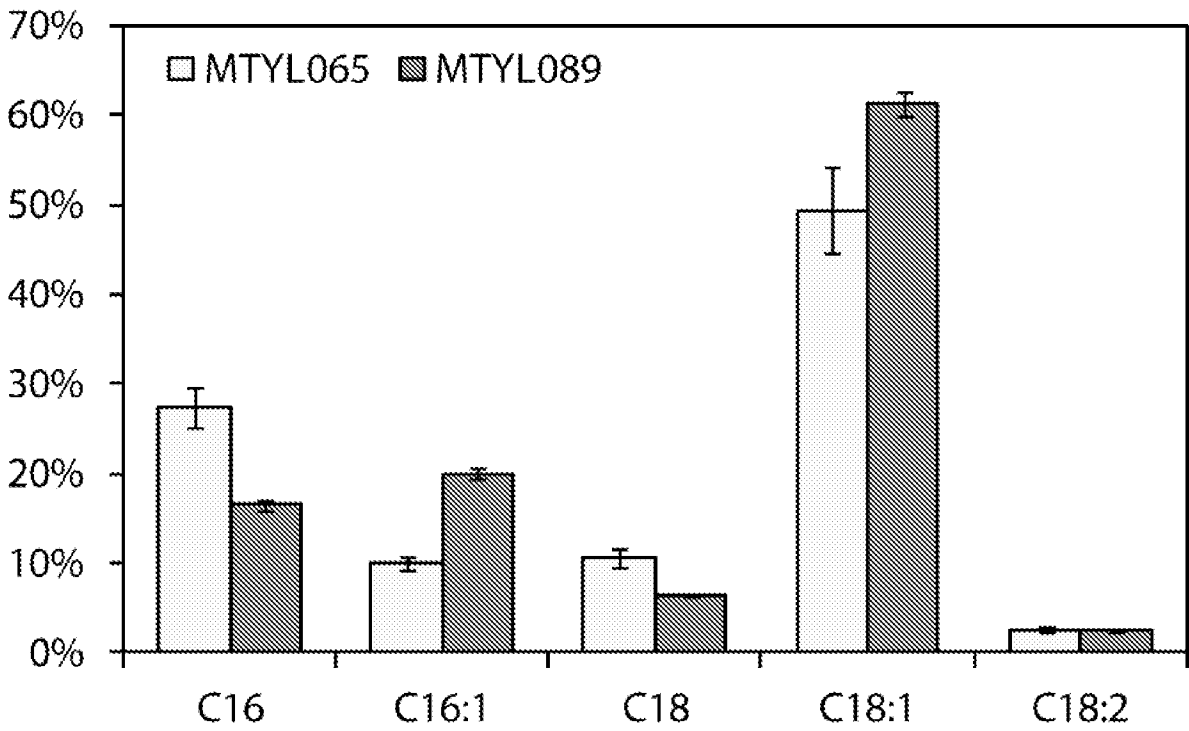


Figure 13

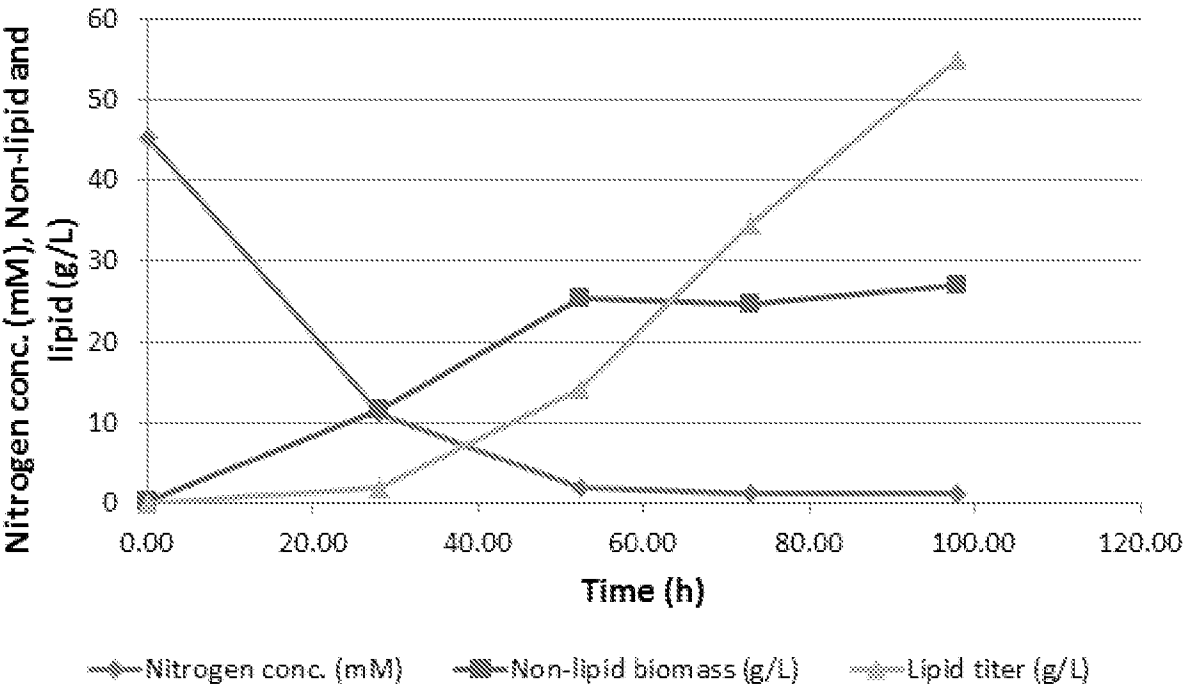


Figure 14

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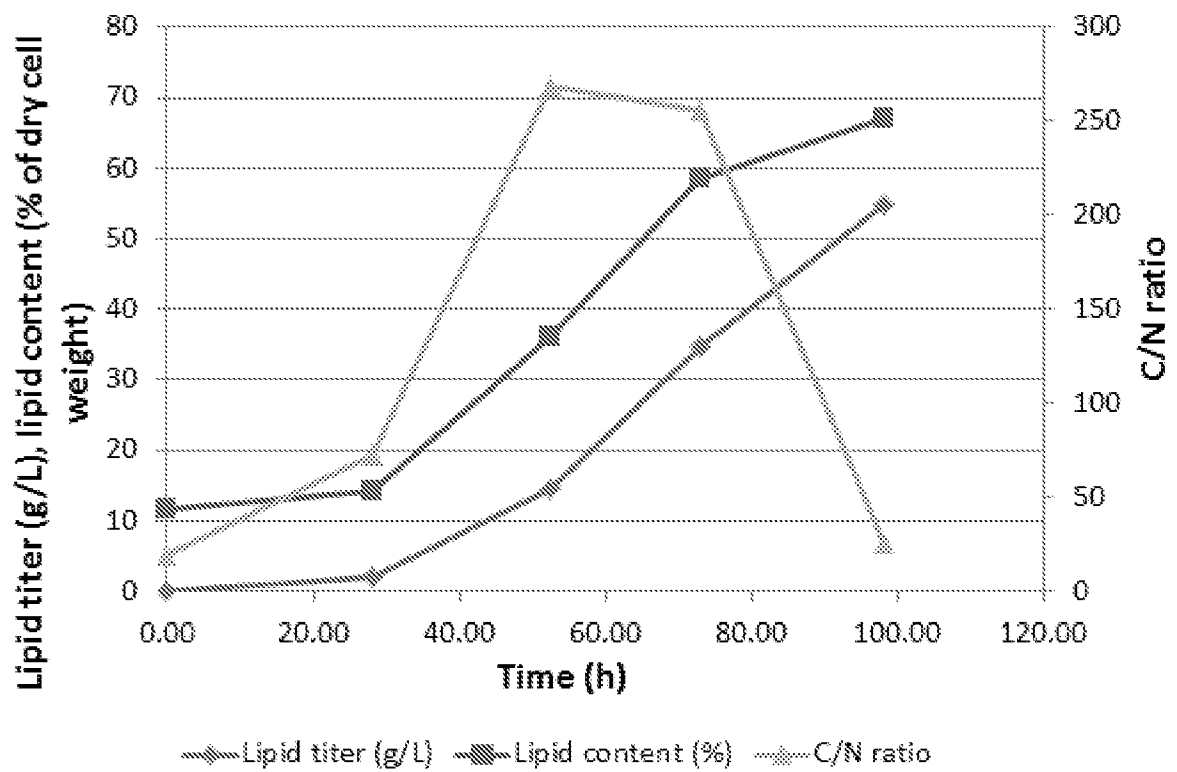


Figure 15

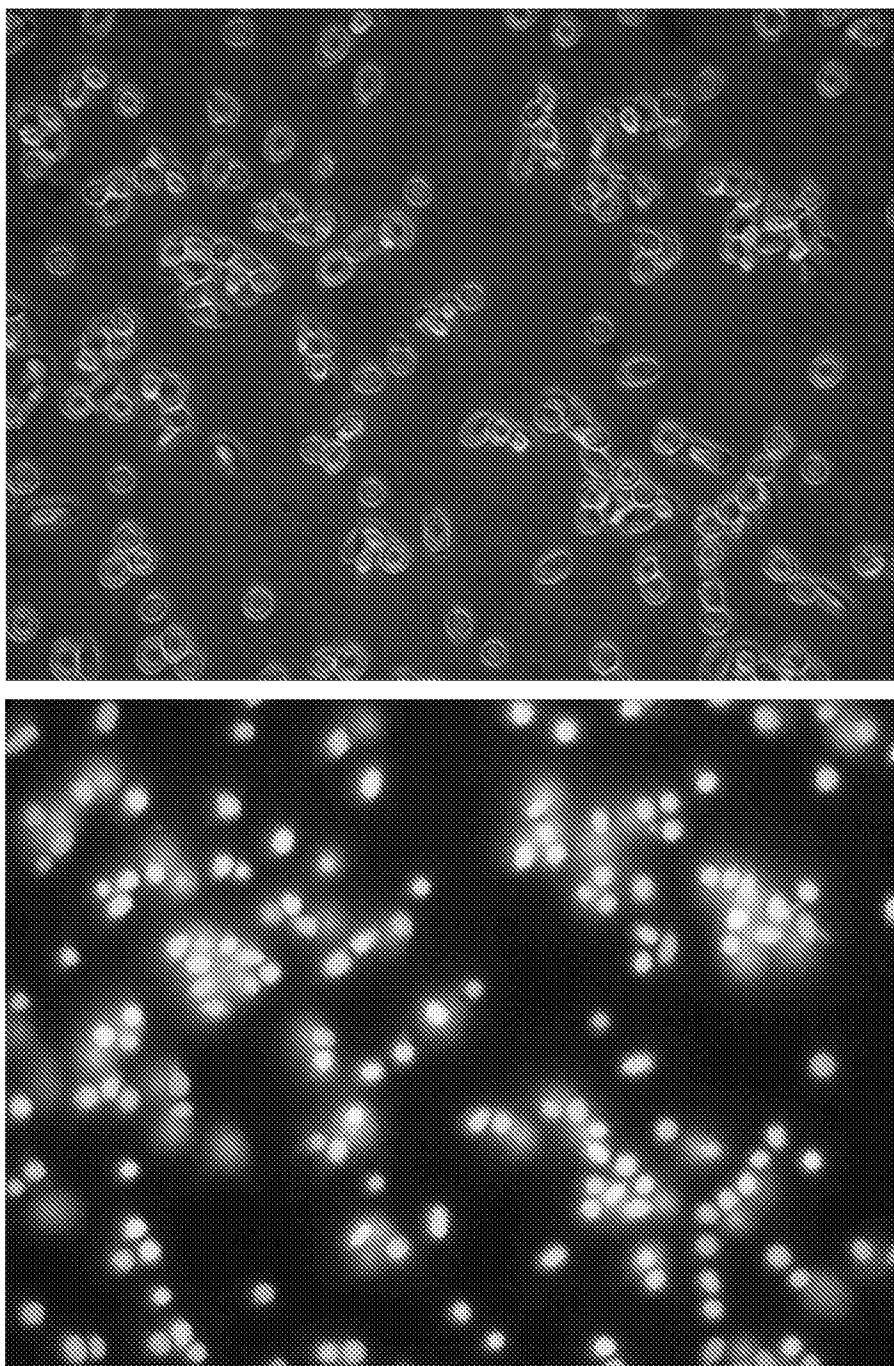


Figure 16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/61101

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 9/10; C12P 7/64 (2012.01)

USPC - 435/193, 435/134

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 435/193, 435/134

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

USPC: 435/193, 134, 183, 171, 41 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic data bases: PatBase, Google Scholar

Search terms: oleagenous yeast, Yarrowia lipolitica, diacylglycerol acyltransferase (e.g. DGAT1 or DGAT2), stearyl-CoA desaturase (synonym: delta 9 desaturase), acetyl-coenzyme A carboxylase (ACC1), expression vector or cassette, promoter, constitutive, inducible,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/0104674 A1 (YADAV et al.) 23 April 2009 (23.04.2009). Especially para [0312], [0338], [0468], [0472], [0473], [0474].	1, 3/(1), 53, 55/(1), 82 and 84/(1)
X	US 2006/0051847 A1 (GUNNARSSON et al.) 9 March 2006 (09.03.2006). Especially para [0195], [0265], [0266], [0410], [0416].	1-3/(1,2), 53-55/(1,2) and 82-84/(1,2)
X	US 2006/0160193 A1 (YADAV et al.) 20 July 2006 (20.07.2006). Especially SEQ ID NOs: 30, 31; para [0175], [0176], [0191], [0235].	96-100

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 December 2012 (16.12.2012)

Date of mailing of the international search report

09 JAN 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

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PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/61101

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

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

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

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

3. ☐ Claims Nos.: 4-52, 56-81, 85-95, 101-105
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.