

Yeast: A new oil producer?

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Abstract: *The increasing demand of plant oils or animal fat for biodiesel and specific lipid derivatives for the oleochemical field (such as lubricants, adhesives or plastics) have created price imbalance in both the alimentary and energy field. Moreover, the lack of non-edible oil feedstock has given rise to concerns on land-use practices and on oil production strategies. Recently, much attention has been paid to the exploitation of microbial oils. Most of them present lipid profiles similar in type and composition to plants and could therefore have many advantages as are no competitive with food, have short process cycles and their cultivation is independent of climate factors. Among microorganisms, yeasts seem to be very promising as they can be easily genetically enhanced, are suitable for large-scale fermentation and are devoid of endotoxins. This review will focus on the recent understanding of yeasts lipid metabolism, the succeeding genetic engineering of the lipid pathways and the recent developments on fermentation techniques that pointed out yeasts as promising alternative producers for oil or plastic.*

Key words: yeast, oleochemistry, microbial oils, lipids, fermentation, *Y. lipolytica*, microorganisms

The contemporary context of petroleum crisis and the raising concern of natural resources depletion, forces research to seek for alternative methods or sources to replace petroleum-derived products. A known outcome of the above is commercialization of biodiesel, an attractive alternative to fuel, having environmental benefits and being produced by renewable resources. Biodiesel is the monakyl ester of fatty acids and is manufactured by their catalytic transesterification, the alkoolysis. The actual feedstock for its production and for other oil-derived products such as oleochemicals and bio-plastics, originates from a variety of biomasses of different sources in the agricultural, forestry and animal field. However, the succeeding demand of plant oils and animal fats raise reasonable concerns on land-use practices and on the environmental impact of oil production strategies. It is therefore necessary to search for a sustainable solution for the oil industry, from renewable raw materials that are not in competition with food, land or water use.

What relates all oil feedstock agricultural sources is the lipid storage molecule of

triacylglycerol (TAG). This is also the storage molecule of the microbial lipid metabolism. Some of these microorganisms, as certain species of yeast, have the ability to store lipids over 20% and up to 70% of their cell dry weight. Their lipid profile differs between species, but is similar in type and composition to the oils and fats produced by most plants and animals. Oils extracted from microorganisms are called single cell oils (SCO) and recently much attention has been paid to these species as sustainable oil producers.

Remarkably, the idea of using microorganisms for SCO production goes back to the first half of the twentieth century. Between the period of the two world wars and especially in Germany, researchers began to explore oleaginous organisms as an alternative to plant oils, which were increasingly in short supply. Unfortunately, these processes were doomed to fail because of the lack of large-scale fermentation technology and the absence of genetic tools. Nevertheless, great advances were made in the identification of oleaginous microorganisms and the evaluation of their oil accumulating capacity.

The development of fermentation technology in the mid-50s allowed to optimize conditions for SCO production and to determine the fatty acid profile of these oils. However, the concurrent explosion of agriculture, rendering the commodities of the field cheaper and plentiful, made the exploitation of SCO economically unrealistic (Wynn and Ratledge, 2005).

Nevertheless, at the beginning of the 80's the interest in the beneficial effects of polyunsaturated fatty acids (PUFAs) in human nutrition opened a new opportunity for SCO production. The common source of PUFAs is fish, which is a scarce resource and requires extensive treatment for the removal of various pollutants such as organo-mercury compounds and dioxanes, or deodorization to remove fish taste and smell. Such PUFAs, as omega-3 and omega-6 fatty acids, were already known to exist in microorganisms SCO. This paved the way of a new commercial adventure for microbial oils. Besides, microorganisms were long used in human nutrition and therefore some of PUFA producers had already the GRAS (Generally Recognized

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As Safe) status. Supported by the advances in genetic screening and large-scale fermentation techniques the first commercialized products hit the market in 1985. The GLA-SCO produced in the UK by John & E. Sturge, (Selby, North Yorkshire) was sold under the name of Oil of Javanicus due to the oriental origins of *Mucor circinelloides*, also known as *Mucor Javanicus*, the producing fungus. During the 6 years of its production, before its cease due to EU regulations and taxes on the sugar cane, a great improvement of cultivation parameters and extraction procedures were gained. All these attempts finally led to the successful large-scale production of arachidonic (ARA, 20:4 n-6) and docosahexanoic acid (DHA, 22:6 n-3) under the commercial names ARASCO and DHASCO by Martek biosciences Co (DSM) in the late 80s. Since then, enormous progress has been achieved from microorganism research on microbial metabolism, genomic, molecular genetics and genetic engineering. This greatly contributed to reconsider microorganism as reliable producers of high-valued molecules not only for nutritional needs but for the oleochemical field as well.

Yeast: a model of choice

Microorganisms are under the scope of biotechnological research as they present many advantages: they present great diversity among them, they grow exponentially fast as they divide once every 20 to 90 min, they are able to utilize cheap substrates, they can be cultivated at large scale, spontaneous mutants can be easily

isolated and strains can be genetically manipulated. Among microorganisms, yeast seem to be the most adapted microorganisms for biotechnological applications as bacteria store excess carbon as polysaccharides, and lipids mostly in form polyhydroxyalkanoates or wax ester, while yeasts accumulate carbon as glycogen and lipids mostly in the form of TAG.

Fewer than 30 of the 600 known species of yeasts are found to be oleaginous. The best known oleaginous yeasts are typically found, but not exclusively, in genera such as *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhizopus*, *Trichosporon*, *Lipomyces* and *Yarrowia* (Ratledge *et al.*, 1990). On average, these yeasts accumulate lipids to levels corresponding to 40% of their biomass. However, under conditions of nutrient limitation (*see below*), these levels may exceed 70% of their cell dry weight (CDW). The lipid contents and fatty acid profiles of some representative oleaginous yeast are presented in *table 1*.

We observe that lipid content and profile differs between species, however it can be acknowledged that the main FA produced by oleaginous yeast are similar to those produced by plants and are mainly consisted by: myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linoleic acid (C18:3).

Incidentally, this is the type of oil composition (and in particular the lipid classes of 16 to 18 carbons) that has been recommended for biodiesel production (Steen *et al.*, 2010). Actually, the most common feedstock used for its production is rapeseed and soybean oil,

having FA profile similar to yeasts. This profile is crucial for the utility of biodiesel as defines the physical and chemical properties of the lipid mixture, *i.e* viscosity, energetic density and melting point. The above specifications refer to the use of biodiesel in standard diesel engines, alone or blended with regular diesel fuel. Otherwise, biodiesel can also be used as a low carbon alternative to heating oil.

Furthermore, the development of genetic tools for yeast engineering coupled with culture strategy give us the ability to control and modulate the fatty acid profile produced. This can broaden the utility of the FA mixture produced and meet the specifications of aviation fuels or the needs of the oleochemical industry. Another advantage is that estimation of quantitative and qualitative productivity of the yeast process is superior to plant cultivation as fermentation is not subjected to climate changes, microbial or insect infections. This could lead to the definition of an industrial process with identical performances around the world. However, a low cost starting material is essential for the economic viability of the process.

To this end, we should acknowledge the yeasts ability to convert renewable carbohydrates (as saccharose or glucose) or industrial by-products such as glycerol or lactose into fatty acids. The major byproduct in conventional biodiesel production (transesterification of TAG) is glycerol: for every tone of biodiesel manufactured, 100 kg of glycerol is produced. As a consequence, the increase in global biodiesel production

Table 1. Lipid accumulation and fatty acid profiles of selected oleaginous yeasts. Data adapted from Ratledge and Tan, 1990.

Species	Lipid content (% CDW)	Major fatty acid residues (% w/w)					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
<i>Cryptococcus curvatus</i>	58	25	Trace	10	57	7	0
<i>Cryptococcus albidus</i>	65	12	1	3	73	12	0
<i>Candida sp 107</i>	42	44	5	8	31	9	1
<i>Lipomyces starkeyi</i>	63	34	6	5	51	3	0
<i>Rhodotorula glutinis</i>	72	37	1	3	47	8	0
<i>Rhodotorula graminis</i>	36	30	2	12	36	15	4
<i>Rhizopus arrhizus</i>	57	18	0	6	22	10	12
<i>Trichosporon pullulans</i>	65	15	0	2	57	24	1
<i>Yarrowia lipolytica</i>	36	11	6	1	28	51	1

C.D.W.; cell dry weight

resulted in a crash to glycerol's market price (Thurmond, 2008). It would be thus a great opportunity for yeasts to use this cheap by-product as starting material to regenerate fatty acids for biodiesel production in a recycling nature process. In order to better understand the potential of yeasts as oil producers, we would try to briefly overview the mechanisms of the cell leading to lipid accumulation. We would also try to provide information about the advances (genetic or fermentation-based) made in order to increase the amount of lipids produced and to modify the FA profile.

An overview of yeasts lipid metabolism

In oleaginous yeasts, lipids may accumulate via two different pathways:

(i) the *de novo* synthesis, which is the synthesis of lipids from the acetyl-CoA and malonyl-CoA building blocks and (ii) the *ex novo* lipid accumulation pathway involving the uptake of fatty acids, oils and TAG from the culture medium and their accumulation in an unchanged or modified form within the cell. The latter requires hydrolysis of the hydrophobic substrate and incorporation of the released fatty acids. In order to do so, yeasts have elaborated sophisticated strategies: for instance, *Yarrowia lipolytica*, a well studied oleaginous yeast, secretes an emulsifying agent to the medium (liposan) to reduce the size of hydrophobic droplets and also secrete lipases for the hydrolysis of external TAG; then it binds the reduce-sized hydrophobic droplets on its cell surface by the formation of

protrusions and incorporates the hydrophobic substrate *via* various transport mechanisms, which unfortunately remain unknown in their majority (Fickers *et al.*, 2005). Once inside the cell, the hydrophobic substrate (HS) can undergo various modification reactions and then is distributed to the various metabolic pathways of the cell as modification, degradation or storage. A schematic representation of this process for the oleaginous yeast *Y. lipolytica* can be found in Figure 1.

Regardless the accumulation pathway, oleaginous yeasts store their lipids mostly in the form of TAG (80-90% of the neutral lipid fraction) and the rest in the form of steryl esters (SE). These storage molecules are not suitable for integration into phospholipid bilayers and are therefore accumulated in a specialized compartment of the cell, the lipid body (LB). This organelle is consisted of the lipid core encased in a phospholipid monolayer within which many proteins with diverse functions are embedded. Recent proteomic studies revealed the important role of these proteins in lipid metabolism (synthesis, storage, trafficking and degradation of lipids) proving that the LB should not be regarded as a simple storage compartment (Athenstaedt *et al.*, 2006; Brown, 2001; Fujimoto *et al.*, 2008; Zweytick *et al.*, 2000).

In oleaginous microorganisms, during *de novo* lipid synthesis the initiation of lipid accumulation is induced by the exhaustion or a limitation of a primary nutrient from the culture medium. Although many nutrients can be limiting, usually nitrogen is used for this purpose, as its supply is the easiest to control. When nitrogen becomes unavailable cell proliferation slows down, as it is an essential nutrient for protein and nucleic acid synthesis. However, the organism continues to assimilate the carbon source (sugars or glycerol) from the medium, which is now channeled towards lipid synthesis. Furthermore, nitrogen limitation provokes the deregulation of the tricarboxylic acid (TCA) cycle, resulting in an overproduction of citrate, the immediate precursor of the acetyl and malonyl-CoA, the building blocks of lipid synthesis. This comes as a consequence of the breakdown of AMP desaminase, an essential cofactor of citrate metabolism, to inosine 5'-monophosphate and ammonium to be used as a source of intracellular nitrogen. By contrast, during

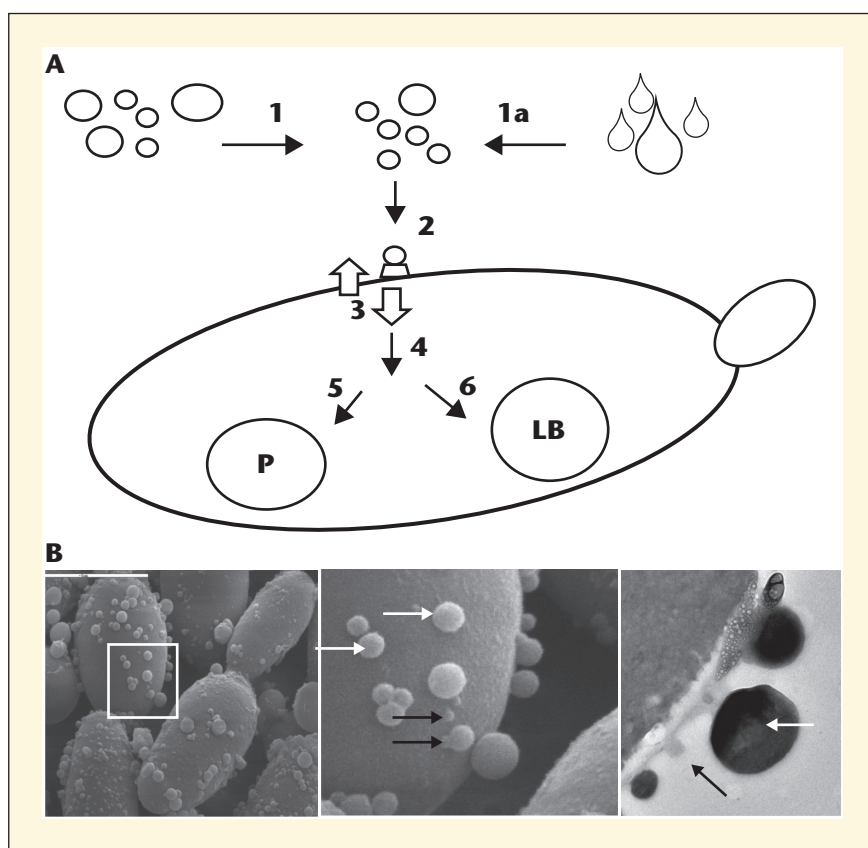


Figure 1. A) Schematic representation hydrophobic substrate (HS) incorporation in the oleaginous yeast *Y. lipolytica*. 1. The size of the hydrophobic droplets is reduced by the secretion of liposan or 1a. FA are cleaved from the glycerol backbone by the secretion of Lip2 TAG lipase; 2. the modified HS can now bind in the cell surface by protrusion formation; 3. The HS enters into the cells via complex transport/ export systems; 4. modification of the HS through several pathways; 5. HS is degraded by β -oxidation in the peroxisomes (P) or 5. stored into lipid bodies as TAG. B) Electronic microscopic picture of *Y. lipolytica* cells cultivated on hydrophobic substrate (oleic acid). White rectangle/ arrows: hydrophobic substrate droplets adhesion on yeasts surface. Black arrows: protrusions; figure adapted from Mlickova *et al.*, 2004.

the *ex novo* pathway, lipid accumulation is independent of the ammonium concentration in the medium. These features are unique to oleaginous species; when non-oleaginous microorganisms are placed in the same nutrient limiting conditions the assimilated carbon is diverted into various polysaccharides, including glycogen, glucans and mannans (Ratledge, 2002).

The acetyl-CoA and malonyl-CoA produced during the *de novo* lipid synthesis are then added up in the growing FA chain by the fatty acid synthase enzymatic complex (FAS). Usually, the FA synthesized by the FAS is between 14 and 16 carbons long. Then, depending on the enzymatic arsenal of each organism desaturation or further elongation reactions can take place. The FAs produced, or assimilated from the medium, are then directed to the storage lipid pathway. There, a number of acyltransferase enzymes esterify them onto the glycerol backbone to produce TAG. However, glycerol in order to be available for TAG synthesis has to be in the form of glycerol-3-phosphate (G3P). Nonetheless, this molecule is easily converted to dihydroxyacetone phosphate (DHAP), resulting in variations of G3P pools. Poor availability of G3P has found to be one of the bottlenecks in neutral lipid synthesis in oleaginous microorganisms (*see below*). In parallel, a small fraction of the FA is esterified in a sterol to produce the steryl esters. This neutral lipid fraction, stored inside the LBs can then be mobilized depending on the energy requirements of the cell.

Upon demand, as a first step of the catabolic reactions, triacylglycerol lipases will hydrolyze the FA from the TAG or the steryl ester. The released FA would be either directed towards phospholipid biosynthesis for membrane formation, or towards the peroxisome where degradation through β -oxidation takes place. This set of reactions, reminiscent of the ones carried by the FAS complex during FA synthesis, break down FA in a spiral, removing 2 carbons (a molecule of acetyl-CoA) from the shortening FA chain in each cycle. The process is supposed to be closed, meaning that the cycle would be repeated until complete breakdown of the FA. However, intermediate FA may eventually leak from this biochemical route. This "malfunction", which can also be induced or stressed-out by genetic modification,

can be biotechnologically exploited for aroma production (cyclization of an FA during its breakdown) or bio-plastic synthesis (block the breakdown at the point of formation of polymerizing intermediates; *see below*).

The first step of β -oxidation is carried out the acyl-CoA oxidase gene family (Aox). Gene representation of this family varies between species: for instance, there is only one gene in *S. cerevisiae*, the bakers yeast, and six different genes in *Y. lipolytica*, an oleaginous yeast. The encoded enzymes have different FA chain length specificities and therefore multiple gene representation implies greater yeast adaptation in utilizing broader FA substrates (Wang *et al.*, 1999). Genetic modification of this gene family can result in specific FA accumulation (as only the FA corresponding to Aox specificity would be catabolized), or into obese and slim yeast phenotypes (*see below*). In general, enhancement or modification of lipid production can be achieved by genetic modification or by altering cultivation conditions. However, for the development of a biotechnological process it is essential to combine both strategies. Here, we will try to briefly present some of the recent advances in these fields.

Yeast cultivation-process development

Yeasts under nutrient limitation undergo three phases of growth: (i) cell proliferation or the exponential growth phase, (ii) a lipid accumulation phase where growth slows down due to nutrient (*i.e.* nitrogen) limitation and lipid synthesis is maximal and (iii) a late accumulation phase where lipids continue to accumulate, but β -oxidation, the catabolic (break down) pathway is active in an effort to remobilize the carbon stored. Finally, cells become unable to produce essential metabolites and most of metabolic activity ceases. The duration of each phase depends on the C/N ratio, in the case of nitrogen limitation. For instance, Granger and colleagues showed that accumulation in *Rodotorula glutinis*, an oleaginous yeast, in batch mode culture with different C/N ratio could result in a 3-fold increase of lipid production (Granger, 1992). In addition, temperature, pH, metal traces and mineral concentrations all influence lipid accumulation in oleaginous yeasts

(Ratledge *et al.*, 2002). Therefore, fine adjustments in culture conditions can be used to up-regulate lipid metabolism: by regulating the quantity of dissolved oxygen, the C/N ratio, the pH and the carbon substrate, yeast accumulation can increase from 37 to 70% of cell dry weight (Bati *et al.*, 1984). All these observations led to more sophisticated fermentation techniques than the batch mode, whereas the C/N ratio is initially fixed and no further modification is allowed during culture. The continuous fermentation mode allowing to have a constant C/N ratio throughout the culture and regulation of substrate concentration leads to the fine tuning of the growth rate: lower growth rates promote more extensive lipid accumulation (Ykema *et al.*, 1986). The fed batch mode, however, allowing the precise control of nutrient and substrate flow rates during fermentation is suggested as the most accurate and reliable approach to use for the control of lipid accumulation (Cescut, 2009). However, one should keep in mind that the efficiency of the procedure should rely on the abundance and cost of the renewable starting materials. Further on, enhancement of lipid accumulation and modulation of lipid profile can be achieved by genetic engineering.

Genetic engineering: towards a yeast cell factory

Improvement of cultures has long relied on classical genetic techniques such as hybridization and mutagenesis followed by selection. Recently, however, it has become a banality to customize production strains dedicated to a specialized purpose (application) by genetic engineering. We should note, nevertheless, that public concern and legal regulation might raise difficulties in the commercial application of GMOs and their products, especially when the desired product is destined for the alimentary field. Even though the recent development of fast and low cost genome sequencing allows new species to be sequenced every day, few oleaginous yeasts genomes have been sequenced and published. Therefore, genetic tools remain scarce or under development for the majority of oleaginous yeasts. Among them, only *Y. lipolytica* was used as a model organism

(Fickers *et al.*, 2005). The availability of its genome was made public throughout the Genolevures consortium (Dujon *et al.*, 2004) and many genetic tools for its modification are available. Therefore, we will often make reference to this organism in this section.

The first attempts at enhancing lipid accumulation were performed by modification of the expression of key enzymes situated in the crossroads of metabolic routes. In *Y. lipolytica* the increase of glycerol-3-phosphate (G3P) pools (G3P provides the glycerol backbone for TAG synthesis) by modifying gene expression of the enzymes leading to its production and/or its degradation, resulted in 3-fold increase in lipid accumulation compared to the wild type strain. The simultaneous abolishment of the β -oxidation by deletion of the Aox encoding genes gave rise to an obese yeast capable of accumulating more than 80% of its cell dry weight in lipids (Beopoulos *et al.*, 2008; Dulerio *et al.*, 2011). The phenotypic profile of the wild type and the obese mutant strain is shown in figure 2. In the same organism and context, overexpression of the acyltransferases involved in the storage lipid pathway resulted in a great increase of TAG content of the cells and in overall lipid accumulation (Beopoulos *et al.*, 2011).

On the other hand, selective expression of these acyltransferases with regard to

their FA specificity leads to customized acylation of TAG. Dupont de Nemours is trying to exploit this pathway by heterologous expression of acyltransferases in *Y. lipolytica* for specific quality oil production (U.S. patent 7465565). Strains presenting great lipid accumulation yields could be easily used for biodiesel production by means of transesterification. The subsequent modification of their lipid profile gives control to the physical properties of the produced fuel.

Yeasts lipid metabolism can be also adapted for aroma production. Safisis, (Lessafre, France) in an already commercialized process, makes use of yeasts β -oxidation in order to produce aromatic compounds such as the peach aromatic additive γ -decalactone from ricinoleic acid (C18:1-OH). This is achieved by taking advantage of the aforementioned induced "malfunction" in the spiral of β -oxidation, releasing a 10 carbon hydroxylated FA, which is readily cyclized in the intracellular pH. A different procedure taking advantage of yeasts oxidation pathways is the production of dicarboxylic acids (DCA), commonly produced by chemical synthesis for nylon, resins, adhesives and biodiesel production. The yeasts *Candida cloacae*, *Candida tropicalis* and *Y. lipolytica* have already been modified in order to induce ω -oxidation that adds a carboxylic moiety in the last carbon of the FA (Eschenfeldt

et al., 2003; Picataggio *et al.*, 1992; Smit *et al.*, 2005; Thevenieau, 2006). The process has been already commercialized, using genetically modified strains of *C. tropicalis* (Cathay Biotech, Shanghai; Cognis, Germany).

Always in the oleochemical field, a promising biotechnological application for yeasts is the production of bio-plastics and more precisely polyhydroxyalkanoates. PHAs are polyesters with interesting thermoplastic and elastomeric properties, often used for synthetic parts manufacture for medical purpose. Their synthesis is achieved by blocking yeasts β -oxidation at the point where the 3-hydroxyl-CoA intermediate is produced and by the heterologous expression of a PHA synthase, a protein found in some bacteria, nonetheless absent in yeasts. The PHA synthase is capable of polymerizing the released hydroxylated CoA from β -oxidation; additionally, by modifying the carbon number of the degrading CoA (i.e. by modulating the Aox expression) one can alter the physical properties of the PHA synthesized. The process has already been successful in *Pichia Pastoris* (Poirier *et al.*, 2002), *S. cerevisiae* (Poirier *et al.*, 2001) and recently in *Y. lipolytica*, whereas the great number of Aox proteins permits fine modulation of the PHA profile (Haddouche *et al.*, 2010; Haddouche *et al.*, 2011).

Furthermore, carotenoid production has been considered in yeasts: carotenoids represent a large group of structurally diverse pigments with their acyl chain ranging from 30 to 50 carbons. They are commercially used as food colorants, nutraceuticals and pharmaceutical purposes. Most of them are commonly produced by chemical synthesis; however, their biosynthesis in yeast hosts by heterologous expression of a geranylgeraldehyde diphosphate synthase seems to give encouraging results (Schmidt-Dannert, 2000). Besides the aforementioned applications the reader is also referred to a recent review presenting novel ideas for the bioconversion of fats and lipids by heterologous gene expression in *Y. lipolytica* (Sabirova *et al.*, 2011).

Future works and perspectives

Re-engineering microbial metabolism to favor oil production for fuel use,

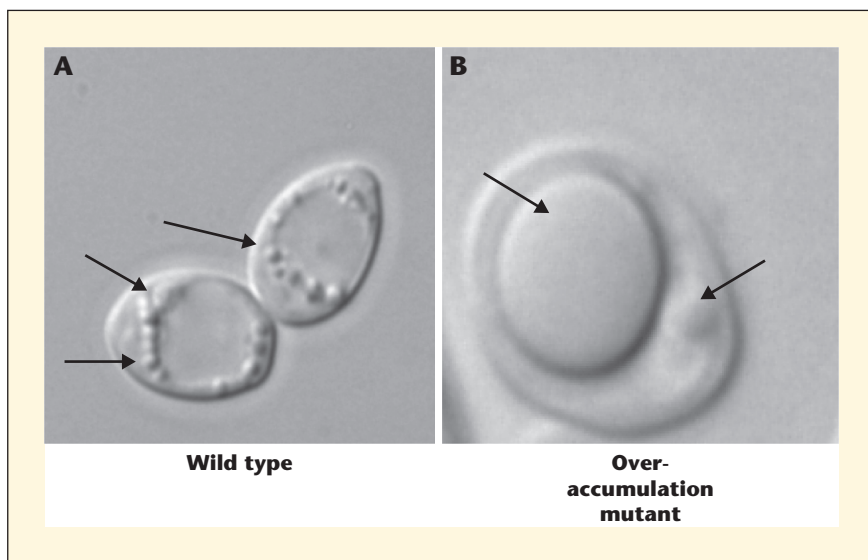


Figure 2. Optical microscopic picture of *Y. lipolytica* wild-type (A) and over-accumulation mutant (B) were lipid content could reach 70% of the yeast cell dry weight (CDW) compared to the 15% for the wild-type in the same conditions (adapted from Beopoulos *et al.*, 2008).

bioplastics or molecules destined for the oleochemical industry seems to be a promising path for future biotechnology applications. However, the efficiency of the procedure should rely on the abundance and cost of the renewable starting materials such as cellulose, glycerol, or even oil waste. An interesting approach would be the use of plant waste as cultivation substrate for oil production by yeasts. This should entail the screening for oleaginous yeast strains tolerant to lignocellulose degradation products (Chen *et al.*, 2009) and evolutionary engineering of yeasts. Such an approach has already been developed in *S. cerevisiae* to improve xylose fermentation for ethanol production (Garcia Sanchez *et al.*, 2010).

Another important issue to address is the extracting procedures of the desired materials. Researchers seek ways to replace traditional, costly and environmentally unfriendly chemical extraction with natural procedures such as regulation of the import/ export FA transport mechanisms in yeasts. Unfortunately, the multiple functions of these proteins, which may also be subject to multiple regulation procedures (*i.e.* regulation at transcriptional and translational levels, post-translational modifications of enzymes, or complex coordination depending on substrate) cause great difficulties in achieving so. However, progress in fed-batch fermentation makes possible not only to real-time monitor and control culture conditions, but to dissociate growth and lipid accumulation as well. Novel studies combining lipidomic, metabolomic and genetic approaches taking advantage of the fed-batch culture will undoubtedly provide a wealth of information about the regulation of lipid metabolism (Morin *et al.*, 2011). The challenge here is to be able to construct a complete genome-scale model of yeasts such as *Yarrowia lipolytica* in order to optimize lipid accumulation (rate of accumulation) and lipid modification (chain length, insaturation, functional group modifications) allowing thus the prediction of the production of any lipid type without affecting yeast lipid homeostasis. It is therefore credible to believe that for the development of a bio-based economy, bypassing the aforementioned concerns on classical production of oil-derived products, microbial lipids could now, more than ever, be consid-

ered as an exploitable feedstock for non-edible oil production.

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