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Getting Lipids for Biodiesel Production from Oleaginous Fungi

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1. Introduction

Biomass-based biofuel production represents a pivotal approach to face high energy prices and potential depletion of crude oils reservoirs, to reduce greenhouse gas emissions, and to enhance a sustainable economy (Zinoviev et al., 2010). Microbial lipids can represent a valuable alternative feedstock for biodiesel production, and a potential solution for a bio-based economy.

Nowadays, the production of biodiesel is based mostly on plant oils, even though animal fats, and algal oils can also be used. In particular, soybean, rapeseed, and palm oils are adopted as the major feedstock for biodiesel production. They are produced on agricultural land, opening the debate on the impact of the expansion of bioenergy crop cultures, which displace land from food production. Furthermore, their price restricts the large-scale development of biodiesel to some extent.

In order to meet the increasing demand of biodiesel production, other oil sources have been explored. Recently, the development of processes to produce single cell oil (SCO) by using heterotrophic oleaginous microorganisms has triggered significant attention (Azocar et al., 2010). These organisms accumulate lipids, mostly consisting of triacylglycerols (TAG), that form the storage fraction of the cell. The occurrence of TAG as reserve compounds is widespread among all eukaryotic organisms such as fungi, plants and animals, whereas it has only rarely been described in bacteria (Meng et al., 2009). In fact, bacteria generally accumulate polyhydroxyalkanoates as storage compound and only few bacterial species, belonging to the actinobacterial genera *Mycobacterium*, *Streptomyces*, *Rhodococcus* and *Nocardia* produce relevant amounts of lipids (Alvarez & Steinbuchel, 2002).

Among heterotrophic microorgansisms, oleaginous fungi, including both molds and yeasts, are increasingly been reported as good TAG producers. This chapter will focus on current knowledge advances in their metabolism, physiology, and in the result achieved in strain improvement, process engineering and raw material exploitation.

2. Ecology of oleaginous fungi

Oleaginous microorganisms are able to accumulate lipids above the 20% of their biomass, on dry basis. Several species of yeasts and filamentous fungi are regarded as oleaginous, since they have the capability to synthetize and accumulate high amounts of TAG within their cells, up to 70% of the biomass weight. These lipids have similar composition and energy

value to plant and animal oils, but their production do not compete for food resources, in particular if it is based on inexpensive carbon sources, such as raw materials, by-products, and surplus. Furthermore, fungal SCO have a short process cycle, and their production is not subjected to seasonal and cyclical weather variations.

The study of oleaginous yeasts has a long history: their ability to accumulate lipids has been known from the 70s, but only in the last years the attention has been focused on exploitation of SCO for biodiesel production. The yeasts represent a part of the microbiota in all natural ecosystems, such as soils, freshwaters and marine waters, from the ocean surface to the deep sea. Widely distributed in the natural environment, they colonize also more extreme environments, such as low temperatures, low oxygen availabilities, and oceanic waters (Butinar et al., 2007). Approximately 1500 species of yeasts belonging to over 100 genera have been described so far (Satyanarayana & Kunze, 2010). Although the vast majority of yeasts are beneficial to human life, only a few are opportunistic human pathogens. As a whole, they play a pivoltal role in the food chain, and in the carbon, nitrogen and sulphur cycles. Among the huge number of species that have been described, only 30 are able to accumulate more than 25% of their dry weight as lipids (Beopoulos et al., 2009b).

Basidiomycetous yeasts strongly prevail among oleaginous yeasts, representing most of all the strains identified as lipid producers, even though some important oleaginous species have been identified among Ascomycota as well (e.g. *Yarrowia lipolytica*).

The most deeply investigated oleaginous yeasts belong to the genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporodium*, *Cryptococcus*, and *Lypomyces* (Ageitos et al., 2011; Li et al., 2008; Rossi et al., 2009). *Yarrowia lipolytica*, previously referred to as *Candida lipolytica*, is a good candidate for single-cell oil production (Beopoulos et al., 2009a; Beopoulos et al., 2009b). *Yarrowia* are hemiascomycetous dimorphic fungi that belong to the order *Saccharomycetales*. They are able to degrade hydrophobic substrates such as n-paraffins and oils very efficiently and this physiological feature prompted the scientific community to explore several biotechnological applications (Bankar et al., 2009). The common habitats of these fungi are oil-polluted environments and foods such as cheese, yogurt, kefir, shoyu, meat, and poultry products. Despite *Y. Lipolytica* is distantly related to the conventional yeast *Saccharomyces cerevisiae,* the genome displays an expansion of protein families and genes involved in hydrophobic substrate (such as alkanes and lipids) utilization. Wild-type strains accumulate up to 38% of dry weight (DW) as lipids. Albeit the levels are lower than those of other oleaginous yeasts, it became a model organism because it can be subjected to genetic and metabolic engineering, having be developed a reliable and versatile system for disruption, cloning and expression of target genes.

Within the *Candida* genus, *Candida curvata* (Holdsworth & Ratledge, 1991) also referred as *Apiotrichum curvatum* and *Candida freyschussii* (Amaretti et al., 2011) synthetize and store significant amount of lipids. *Candida* comprises an extremely heterogeneous group of Ascomycota that can all grow with yeast morphology, classified in 150 heterogeneous species, among which only a minority have been implicated in human diseases, since approximately 65% of *Candida* species are unable to grow at 37°C, then they can not be successful pathogens or commensals of humans (Calderone, 2002). Therefore, most of the species can be exploited for biotechnological applications, despite of unwarranted negative public perceptions.

Lipomyces spp. present a great propensity to accumulate triacylglycerols. This genus belongs to the *Saccharomycetales* order and represents a unique branch in the evolution of the

ascomycetes (van der Walt, 1992). *Lipomyces* are true soil inhabitants and have a worldwide distribution. The oleaginous species *Lipomyces starkeyi* has the capability to accumulate over 70% of its cell biomass as lipid under defined culture conditions, and can produce lipid on xylose, ethanol, and L-arabinose, or using a mixture of glucose and xylose (Zhao et al., 2008), as well as other wastes (Angerbauer et al., 2008).

Cryptococcus curvatus is a yeast with industrial potential as single-cell oil because it can grow and accumulate lipid on a very broad range of substrates. It requires minimal nutrients for growth, accumulating up to 60% of its cellular dry weight (DW) as intracellular lipid (Meesters et al., 1996; Zhang et al., 2011). Yeasts of the *Cryptococcus* genus are widely distributed in nature and may be isolated from various substrates such as air, soil, bird excreta, water, animal surfaces and mucosae, leaves, flowers, and decomposing wood. Most species are considered as free-living (non-symbiotic) and only a few have medical importance being responsible for disease in man and animals (*C. neoformans* and *C. gattii*). *C. curvatus* is recognized as an opportunistic pathogen of animals, including humans (Findley et al., 2009).

Species belonging to the genus *Rhodosporidium*, and to its asexual counterpart *Rhodotorula*, have been claimed as oleaginous yeasts. They belong to one of the three main lineages of the *Basidiomycota*, the *Pucciniomycotina*. *Rhodotorula* is a common environmental inhabitant. The synthesis of different commercially important natural carotenoids by yeast species belonging to the genus *Rhodotorula* has led to consider these microorganisms as a potential pigment sources. Within this genus, the mesophilic red yeast *Rhodotorula glutinis* is able to synthetize and store lipids also growing on glycerol, whereas the psychrophilic species *Rhodotorula glacialis,* that are not red yeasts, accumulates lipids in a range of temperature between -3 and 20°C (Amaretti et al., 2010). The red yeast *Rhodosporidium toruloides* is an oleaginous mesophilic species. *Rhodosporidium* are able to carry out a number of diverse biochemical reactions such as biodegradation of epoxides, biphenyls and oxiranes (Smit, 2004), biosynthesis of carotenoids (de Miguel et al., 1997) and other types of biotransformations, but a major biotechnological exploitation is associated to their ability to convert glycerol and lignocellulosic biowastes into lipids (Hu et al., 2009; Yu et al., 2011). Among the oily yeasts, two novel species of the anamorphic basidiomycetous genus *Trichosporon* have been recently identified (*T. cacaoliposimilis* and *T. oleaginosus*) (Gujjari et al., 2011), despite lipid accumulation has not yet explored in the perspective of biodiesel production. *Trichosporon* are basidiomycetous yeasts widely distributed in nature, consisting of soil- and water-associated species, predominantly found in environmental substrates, such as decomposing wood. They present distinct morphological characteristics of budding yeast cells and true mycelia that disarticulate to form arthroconidia. Some species are causative agents of diseases in man and cattle. They can occasionally belong to the gastrointestinal microbiota of humans as well as transiently colonize the skin and respiratory tract.

Exploitation of oleaginous filamentous fungi for biodiesel production has a more recent history, which, with few exceptions, derives from studies focused to poly-unsaturated fatty acid production (PUFA), such as arachidonic acid and γ-linolenic acid. The most relevant example of this biotechnological application is represented by exploitation of *Mortierella alpina* to produce oils containing n-1, n-3, n-4, n-6, n-7, and n-9 PUFAs (Sakuradani et al., 2009). Among the major lipid producers there is *Mucor circinelloides,* a zygomycete fungus, which is emerging as opportunistic pathogen in immunocompromised patients (Li et al., 2011). *M. circinelloides* has been used for the first

commercial production of microbial lipids (Ratledge, 2004). Lipid accumulation in *M. circinelloides* has been extensively studied (Wynn et al., 2001), and its TAG have been proposed as feedstock for producing biodiesel by direct transformation of its lipids (Vicente et al., 2009). *M. circinelloides* represents an outstanding model within the *Zygomycota* phylum, based on the availability of an efficient transformation procedure (Gutierrez et al., 2011) and on the whole sequence of genome (http://genome.jgi-psf.org/Mucci2/Mucci2.home.html). Also the phylogenetically related *Umbelopsis isabellina* has emerging as a promising species to convert biomass residues to biodiesel precursors (Meeuwse et al., 2011a). To the best of our knowledge, limited are the attempts to get lipids with *Aspergillus oryzae* that, conversely, is extensively studied as lipase producer to carry out transesterification of TAG (Adachi et al., 2011).

3. Biochemistry of lipid accumulation

Lipid accumulation in oleaginous yeasts and molds has been demonstrated to occur when a nutrient in the medium (e.g. the nitrogen or the phosphorus source) becomes limited and the carbon source is present in excess. Nitrogen limitation is the most efficient condition for inducing lipogenesis. During the growth phase, nitrogen is necessary for the synthesis of proteins and nucleic acids, while the carbon flux is distributed among energetic and anabolic processes yielding carbohydrates, lipids, nucleic acids and proteins. When nitrogen gets limited, the growth rate slows down and the synthesis of proteins and nucleic acids tends to cease. In non-oleaginous species, the carbon excess remains unutilized or is converted into storage polysaccharides, while, in oleaginous species, it is preferentially channeled toward lipid synthesis, leading to the accumulation of TAG within intracellular lipid bodies (Ratledge & Wynn, 2002; Granger at al., 1993).

The biochemical pathway of lipid biosynthesis is not very different among eukaryotic organisms and does not differ in oleaginous and non-oleaginous fungi. The ability to accumulate high amounts of lipid depends mostly on the regulation the biosynthetic pathway and the supply of the precursors (i.e. acetyl-CoA, malonyl-CoA, and glycerol-3-phosphate) and the cofactor NADPH.

Most information were obtained from the model yeast *Saccharomyces cerevisiae* (Kohlwein, 2010), that does not accumulate lipids, and *Yarrowia lipolytica*, that represent a model for bio-oil production and is suitable for genetic manipulation (Beopoulos et al., 2009b).

3.1 Fatty acids biosynthesis and modifications

De novo synthesis of fatty acids (FA), the first step of lipid accumulation, is carried out in the cytosol by fatty acids synthetase (FAS) complex. In yeasts, FAS bears phosphopantheteine transferase activity to activate its acyl carrier protein (ACP) by loading the coenzyme pantothenate. FAS is a multimer of 6 α and 6 β subunits encoded by *fas2* and *fas1*, respectively, each subunit containing four functional domains. Therefore, FAS consists in a α6β6 molecular complex of 2.6 MDa with 48 functional centers that catalyze all reactions required for synthesis of fatty acids through cycles of multistep reactions. FAS firstly loads acetyl-CoA on its β-ketoacyl-ACP synthase (KS), then it exherts β-ketoacyl-ACP reductase (KR), β-hydroxyacyl-ACP dehydratase (DH), and enoyl-ACP reductase (EAR) activities. This set of reactions is repeated cyclically seven times to yield palmitoyl-ACP (Fig. 1) (Tehlivets et al, 2007).

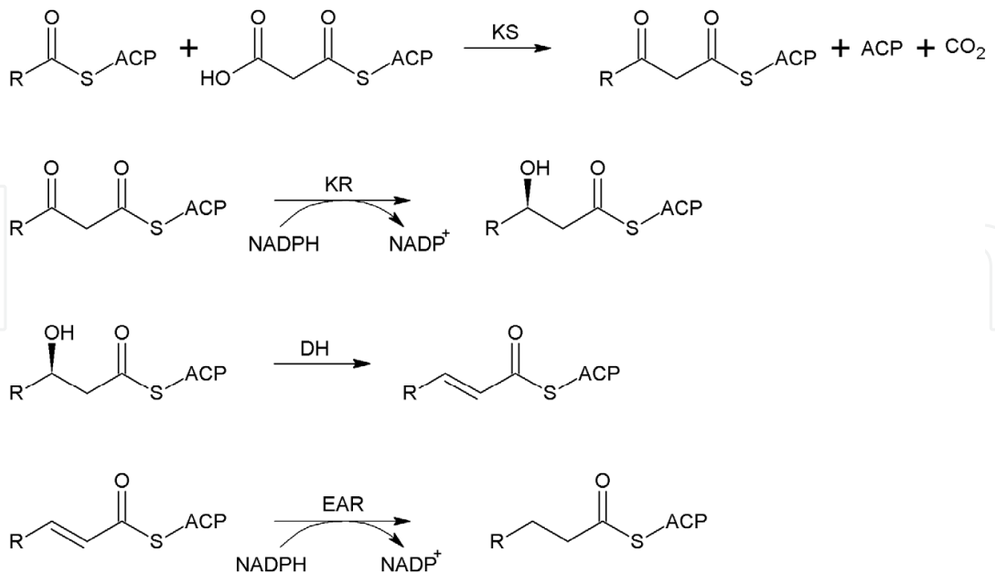

Fig. 1. Reactions occurring sequentially in fatty acid synthetase: condensation of acyl-ACP and malonyl-ACP mediated by KS, NADPH-dependent reduction of the keto group to a hydroxyl group by means of KR, dehydration to create a double bond with DH and reduction of the double bond by means of EAR. R = H, $CH_3(CH_2)_{2n;}$ n_{max}=7.

The biosynthesis of FA requires the constant supply of acetyl-CoA as initial biosynthetic unit and of malonyl-CoA as the elongation unit, supplying two carbons at each step. Non-oleaginous yeasts receive acetyl-CoA mostly from glycolysis. In oleaginous yeasts, acetyl-CoA is mostly provided by the cleavage in the cytosol of citrate, which accumulated as a consequence of nitrogen limitation (Ratledge, 2002) (Fig. 2). In fact, lipid accumulation by oleaginous fungi does not occur under balanced nutrient conditions.

In oleaginous yeasts, nitrogen limitation activates AMP-deaminase (Ratledge & Wynn, 2002), which supply ammonium to the nitrogen-starved cell. As a consequence, mitochondrial AMP concentration decreases, causing isocitrate dehydrogenase activity to drop. The TCA cycle is then blocked at the level of isocitrate, which accumulates and equilibrates with citrate through aconitase. Excess of citrate from TCA cycle is exported out of the mitochondrion via the malate/citrate antiport. Cytosolic ATP-citrate lyase (ACL) cleaves citrate to give oxaloacetate and acetyl-CoA (Fig. 2).

ACL represents one of the key enzymes that contribute to the oleaginous trait of yeasts, whereas its activity is negligible in non-oleaginous species. ACL is composed of two subunits, encoded by *ACL1* and *ACL2* and is negatively regulated by exogenous FA.

Malonyl-CoA is produced from acetyl-CoA by acetyl-CoA carboxylase (ACC) that condensate an acetyl-CoA unit with bicarbonate:

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \leftrightarrows \text{malonyl-CoA} + \text{ADP} + \text{Pi}$$

ACC is also a key enzyme in *de novo* FA synthesis, since *ACC1* mutants became FA auxotrophs or maintain low levels of ACC activity (Tehlivets et al., 2007). ACC1 undergoes allosteric activation by citrate. Furthermore the transcription of *FAS1*, *FAS2*, and *ACC1* is coordinately regulated, being negatively regulated by FA.

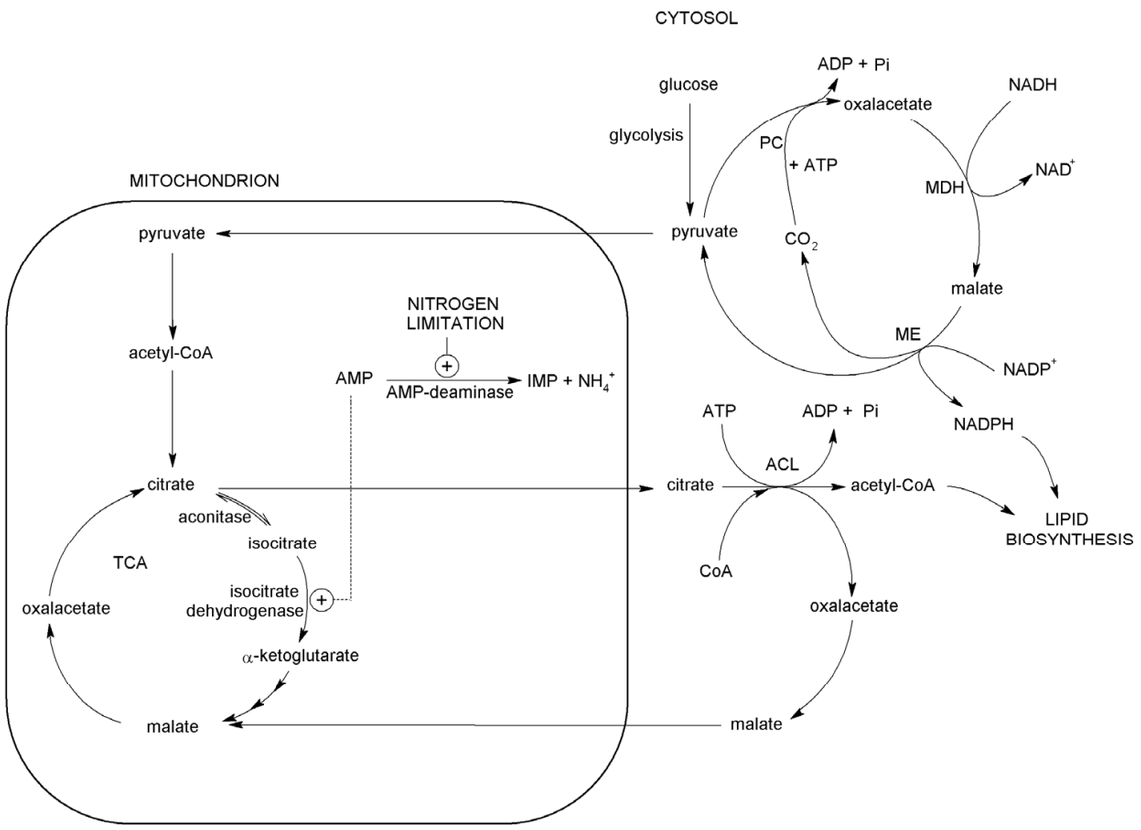

Fig. 2. Lipid biosynthesis from excess of citrate as a consequence of nitrogen limitation. Adapted from Ratledge, 2004.

Cytosolic NADPH is required for KR and EAR functions of FAS. For each elongation step of the acyl chain, two molecules of NADPH are required. One of the major sources of cytosolic NADPH are the pentose phosphate pathway and the transhydrogenase cycle, which transforms NADH into NADPH through the activity of pyruvate carboxylase (PC), malate dehydrogenase (MDH), and malic enzyme (ME), catalyzing the following reactions:

$$\text{Pyruvate} + CO_2 + \text{ATP} \leftrightarrows \text{oxaloacetate} + \text{ADP} + \text{Pi} \quad \text{(PC)}$$

$$\text{Oxaloacetate} + \text{NADH} \leftrightarrows \text{Malate} + NAD^+ \quad \text{(MDH)}$$

$$\text{Malate} + NADP^+ \leftrightarrows \text{pyruvate} + CO_2 + \text{NADPH} \quad \text{(ME)}$$

$$\text{NADH} + NADP^+ \leftrightarrows \text{NADPH} + NAD^+$$

ME has been found in several oleaginous fungi and it has been regarded as a key enzyme involved in lipid accumulation (Ratledge, 2002). In *Mortierella circinelloides*, overexpression of ME enhanced lipid accumulation (Zhang et al., 2011), whereas overexpression of the ME homologous in *Yarrowia lipolytica* did not result in yield improvement.

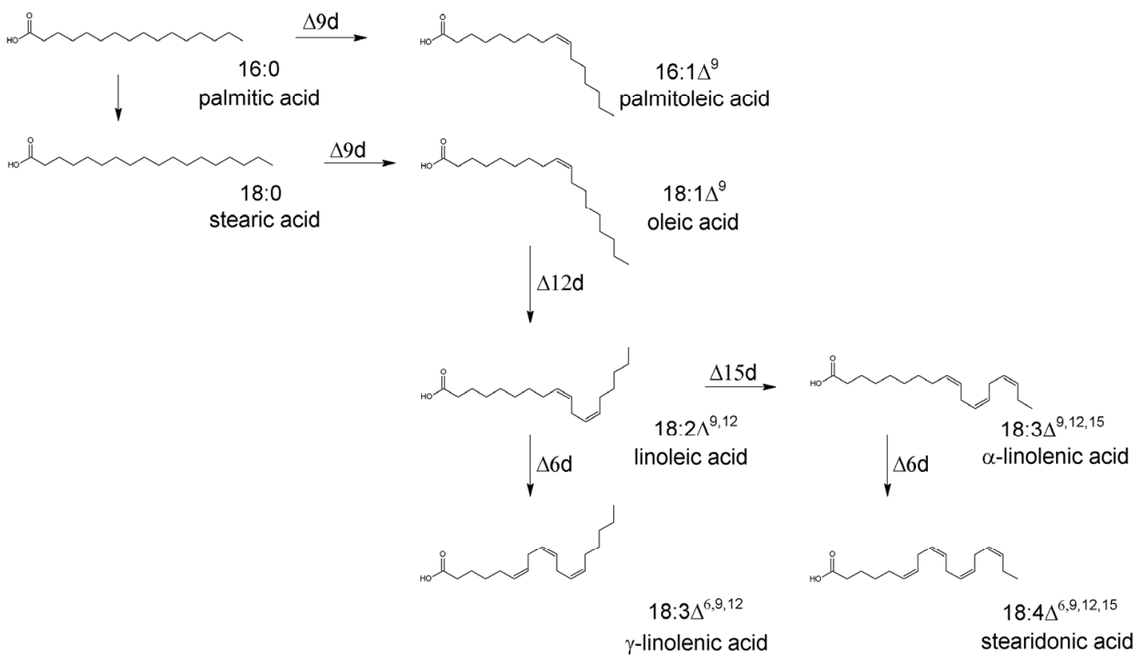

Fig. 3. Biosynthesis of poly-unsaturated fatty acid. Δ9d, Δ12d and Δ15d are the most common desaturases which are present in the endoplasmic reticulum (Ratledge 2004).

The final products of FAS are myristic or palmitic acids, depending on the yeast species. Reactions resulting in further elongation or desaturation occur in the endoplasmic reticulum (ER). Elongation reactions are catalyzed by elongases (such as malonyl-palmitoil transacylase, MPT) organized in a complex that requires malonyl-CoA provided by ACC. Desaturations are introduced by ER desaturases, hydrophobic membrane-bound proteins. The most common desaturases are Δ9, which inserts the first double bond onto palmitic and/or stearic acids, and Δ12, which catalyzes the insertion of the second unsaturation into oleic acid to produce linoleic acid. Δ6 and Δ15 desaturase activities have been recently described in in psychrophilic oleaginous yeasts, based on production of γ and α-linolenic acids, respectively (Fig. 3).

3.2 Biosynthesis of triacyl-glycerol

The fatty acyl-CoA produced by *de novo* synthesis are esterified with glycerol or sterols to produce triacyl-glycerol (TAG) and steryl-esters (SE), respectively. In oleaginous fungi, the neutral lipids SE and TAG are store inside the lipid bodies (LB). TAG are mostly formed by consecutive acylation of glycerol-3-phosphate (G3P), carried out by diverse acyl transferases. G3P is formed from glycerol by glycerol kinase or can be synthesized from dihydroxyacetone phosphate (DHAP) by G3P dehydrogenase, in a reversible reaction. *S. cerevisiae* can use both G3P and DHAP as acyl-group acceptor. The addition of the first acyl group leads to 1-acyl G3P, also named lysophosphatidic acid (LPA). LPA can also be formed by reduction of acyl-DHAP, carried out by a NADPH dependent reductase. A second acyltransferase loads an other acyl group, producing 1,2-diacyl G3P (phosphatidic acid, PA). Phosphate is removed from PA by phosphatidate phosphatase isoenzymes, generating diacylglycerol (DAG). DAG can be the direct precursor of TAG, or can be channeled toward phospholipids biosynthesis (Fig. 4).

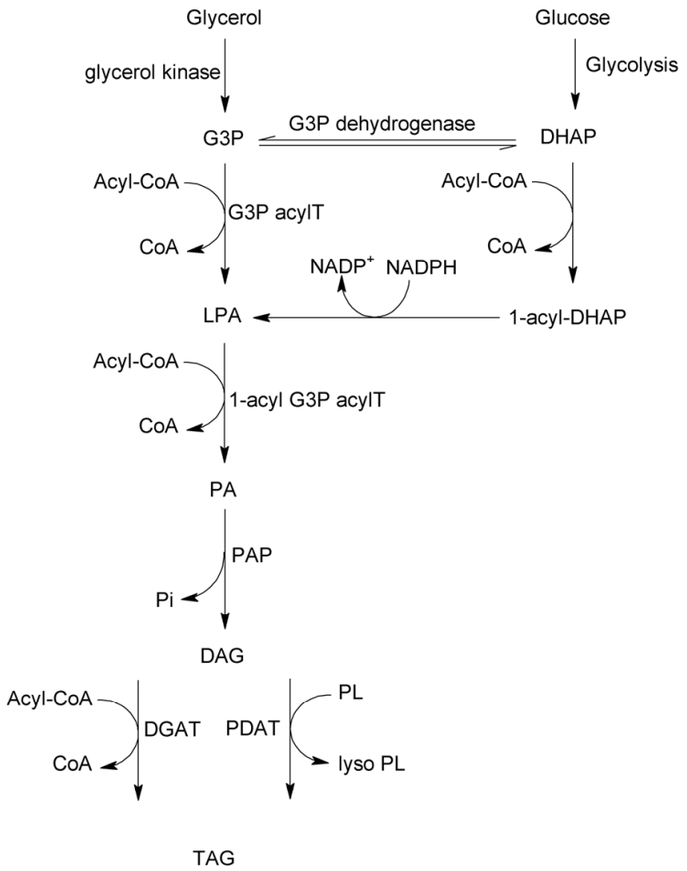

Fig. 4. *de novo* synthesis of TAG (adapted from Czabany et al., 2007)

The last step of *de novo* synthesis of TAG can be carried either by using diverse acyl donors, such as acyl-CoA or with phospholipids. In the former case, DAG acyl transferases (DGAT), which are integral proteins of the ER, can directly load the third Acyl-CoA. A DGAT enzyme is present in *S. cerevisiae* and *Y. lipolytica* and is mostly active during the stationary phase, although it is expressed also during the exponential phase. A second DGAT, more active during the exponential growth phase, has been identified in *Y. lipolytica.* In *S. cerevisiae* the phospholipid:DAG acyltransferase (PDAT) is localized in the ER, whereas in *Y. lipolytica* it is present both in the ER and in the surface of LB (Fig. 4).

3.3 Biogenesis of lipid bodies

In eukaryotes, neutral lipids are stored in specialized compartments known as lipids bodies (LB). They are assembled at a specialized subdomain of the ER where most biosynthetic enzymes and structural proteins are located (Waltermann et al., 2005). The neutral lipids do not fit among phospholipids and are thus deposited between the two leaflets of the membrane bilayer. However, substantial amounts of neutral lipids cannot be incorporated into membrane bilayer of ER and ongoing neutral lipid synthesis leads to the formation of a bud which buds off of the ER as a mature LB after reaching the critical size (Fig. 5).

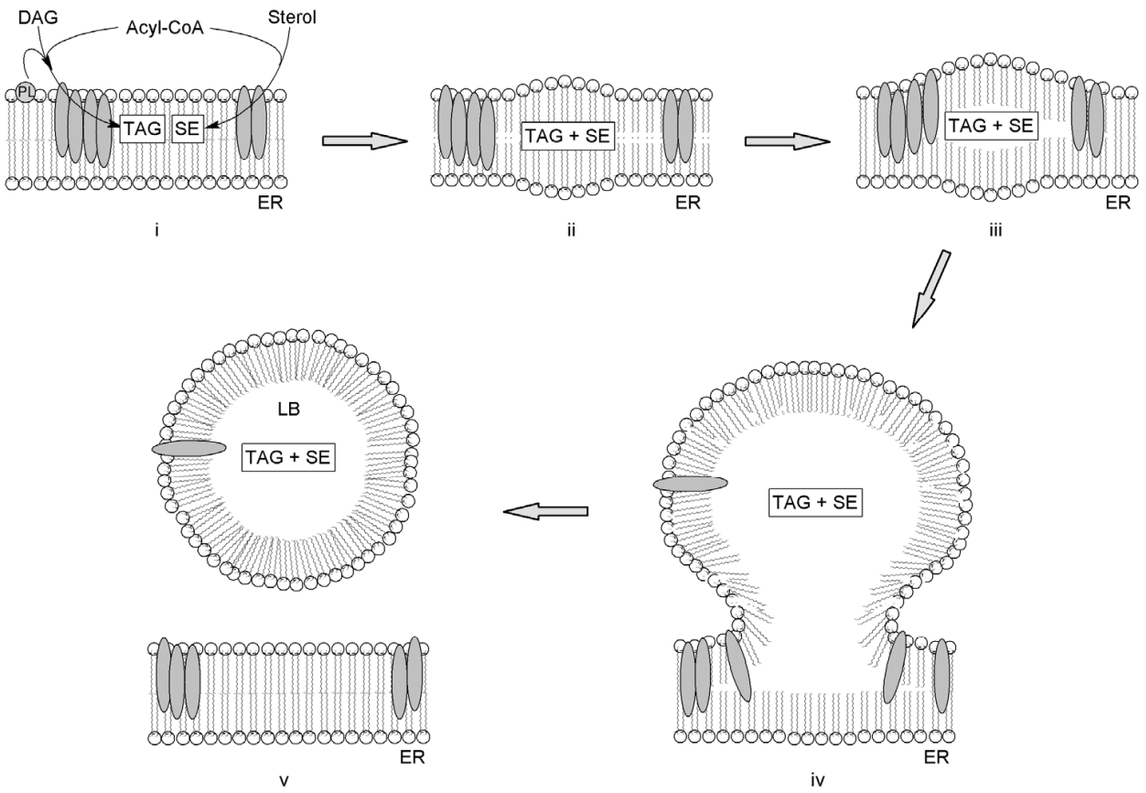

Fig. 5. Model of lipid bodies biogenesis from the membrane of the ER. TAG and SE accumulate between the two leaflets of the phospholipid bilayer (i to iii). The micro-droplet generated (iii, iv) evolve to lipid bodies (v) (Figure adapted from Czabany et al., 2007).

In most oleaginous yeasts, the neutral lipids of LB consist mostly of TAG (up to 90% or more) whereas a small fraction is represented by steryl esters. The presence of significant quantity of free fatty acids (FFA) within LP has been reported only for *Y. lipolytica*. In *S. cerevisiae*, which accumulates less than 15% lipids of its biomass, LB contain similar amounts of TAG and SE.

The core of LB, consisting of neutral lipids is surrounded by a phospholipid monolayer where several proteins are embedded. These proteins exert a key role in lipid metabolism, biosynthesis, and substrate trafficking. Upon requirement, storage lipids are mobilized from this compartment by triacylglycerol lipases and steryl ester hydrolases. The respective degradation products serve as energy sources and/or building blocks for membrane formation. In fact, FA hydrolyzed from TAG or SE are either channeled to the peroxisome, where β-oxidation takes place, or to phospholipid biosynthesis.

4. Metabolic engineering of oleaginous yeasts

The availability of genome data and genetic tools, such as the possibility to integrate homologous or heterologous genes, opened up the possibility to use metabolic engineering to understand the molecular mechanisms involved in lipid accumulation or to increase the yield of stored lipids in *S. cerevisiae* and *Y. lipolytica*. Whereas *S. cerevisiae*

has been used mostly as a model to investigate and understand the lipid metabolism, in *Y. lipolytica* several attempts have been done in order to address the carbon flux toward TAG production and accumulation. Similar approaches are precluded to other oleaginous fungi since they lack genome information and the necessary tools for gene manipulation and strain improvement.

In *Y. lipolytica*, the role of glycerol-3-phosphate (G3P) in triacylglycerol (TAG) biosynthesis and accumulation has been investigated (Beopoulos et al., 2008). In this yeast G3P is formed from glycerol by the glycerol kinase encoded by *GUT1*, or it is synthetized from dihydroxyacetone phosphate (DHAP) by the G3P dehydrogenase (*GPD1*). The antagonist reaction, which produces DHAP from G3P, is carried out in competition by a second isoform of the G3P dehydrogenase, encoded by *GUT2*. In order to force the conversion of DHAP into G3P, the gene *GPD1* was over-expressed and the gene *GUT2* was deleted.

A diverse strategy to increase lipid accumulation was based on the disruption of the β-oxidative metabolism, through the deletion of the 6 *POX* genes (*POX1 to POX6*) that encode the peroxisomal acyl-coenzyme oxidases (Mlickowa et al., 2004a; Mlickowa et al., 2004b; Beopoulos et al., 2008). As a whole, the best results in terms of percentage of lipids per dry biomass, were reached coupling the increased level of G3P with the disactivation of the β-oxidation pathway (65%) (Dulermo et al., 2011).

Metabolic engineering strategies have been recently exploited to expand the range of substrates used by oleaginous fungi, also through functional expression of heterologous genes. Recently, it has been found that inulin is a good material for bio-productions (Chi et al., 2009). In order to make the oleaginous yeast *Y. lipolytica* able to accumulate lipids on inulin containing materials, the *Kluyveromyces marxianus* exo-inulinase gene (*INU1*) was heterologously expressed on a high copy plasmid (Zhao et al., 2010). The inulinase was efficiently secreted by *Y. lipolytica*, and inulin was hydrolyzed, assimilated and converted into TAG.

5. Cultivation condition of oleaginous yeasts

Lipid accumulation by oleaginous yeasts depends mostly on nutrient limitation conditions when excess carbon is present in the medium. Nutrient limitation prevents cells from being generated, while the carbon excess is converted into storage TAG. Published studies reports that phosphorus, magnesium, zinc, or iron limitation lead to lipid accumulation in model oleaginous yeasts (Hall & Ratledge, 1977; Beopoulos et al., 2009; Wu et al., 2010). However, nitrogen limitation is the most efficient form of nutrient limitation for lipogenesis induction, leading to the highest values of substrate/lipid conversion yield and lipid content within biomass (Hall & Ratledge, 1977; Wynn et al., 2001). Thus, nitrogen limitation is commonly used to induce lipogenesis in oleaginous fungi and the utilization of cultural media with appropriate C/N ratio is crucial to maximize lipid production.

Several studies focused on determining the optimal composition of cultural media for oleaginous fungi with the aim to optimize the performance of lipid-producing bioprocesses. The effect of the C/N ratio on lipid metabolism has been investigated for a number of oleaginous yeasts and molds, such as *Y. lipolytica* and many oleaginous species of *Rhodotorula*, *Candida*, *Apiotrichum/Cryptococcus*, *Mortierella* (Hall & Ratledge, 1977; Papanikolau et al., 2003; Granger et al., 1992; Wu et al., 2010; Park et al., 1990; Jang et al., 2005; Amaretti et al., 2010), and has been mathematically modeled for some of these

organisms (Ykema et al., 1986; Granger et al., 1993; Economou et al., 2011). *Y. lipolytica* is the oleaginous microorganism for which information about the metabolic response to different C/N ratios is most abundant (Beopoulos et al., 2009a), particularly due to the availability of molecular tools for genetic engineering of this organism. Therefore, *Y. lipolytica* is regarded as a model organism for microbial oil production and the main traits of its metabolism can be used to give a general description of the metabolic response to different C/N ratios in the majority of oleaginous yeasts. With the increase of the C/N ratio, different metabolic behaviors were observed in *Y. lipolytica*: i) growth with mobilization of storage lipids, ii) growth of fat-free biomass, iii) growth with accumulation of lipids, and iv) growth with lipid accumulation and production of organic acids (Fig. 6).

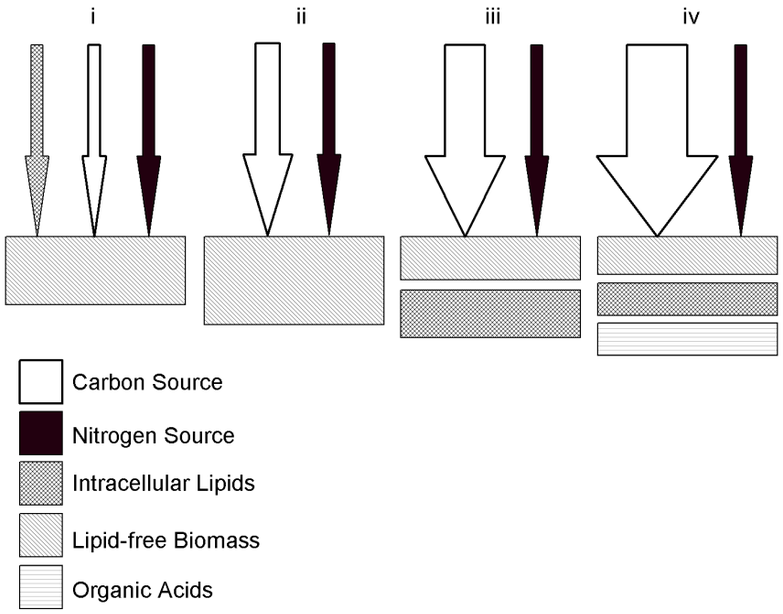

Fig. 6. Metabolic activity of oleaginous fungi (e.g. *Y. lipolitica*) as a function of carbon flow rate for a fixed nitrogen flow rate. Arrows indicate the consumption of nitrogen and carbon sources by the cells; squares indicate production rate. The dimension of arrows and squares is proportional to flow. (adapted from Beopoulos et al., 2009a)

If the medium is carbon limited or when the extracellular carbon supply gets exhausted, previously stored intracellular lipid can be mobilized and utilized by oleaginous microorganisms to sustain cells generation and production of lipid-free biomass (Park et al., 1990) (Fig. 6, i). If the medium is balanced and/or furnishes just the right amount of carbon flow to satisfy the growth need, balanced growth occurs without any accumulation of storage lipids (Fig. 6, ii). In conditions of carbon excess, a part of the carbon flow, which is proportional to nitrogen availability (Granger et al., 1993), is directed toward cells generation, whereas the carbon exceeding growth needs is channeled to the production of storage lipids (Fig. 6, iii). In some oleaginous fungi, the presence of a large carbon excess leads to the production of great amounts of organic acids, such as pyruvic acid and diverse TCA-cycle intermediates, at the expenses of lipid accumulation (Fig. 6, iv). In these latter conditions, *Y. lipolytica* produces citric acid (Levinson et al., 2007) but other oleaginous yeasts have never been reported to behave this way.

6. Batch, fed-batch and fermentation processes

Batch, fed batch, and continuous modes of culture have been developed to culture oleaginous microorganisms. Lipid production in batch cultures is carried out in a cultural medium with a high initial C/N ratio, the carbon source being present in an adequate excess with respect to the nitrogen source. In fact, in this condition, the flow of carbon utilization is limited only by the substrate uptake system of the cell, while the changes in nitrogen concentration determine the passage from a phase of balanced growth to a phase of lipid accumulation, causing the process to proceed through two phases. As nitrogen is consumed from the culture the C/N ratio tends to increase, but growth remains exponential and balanced until nitrogen is not the limiting substrate. During the growth phase, the carbon flow is mostly channeled to satisfy the growth need, therefore growth is balanced and lipid-free biomass is mostly produced (Fig. 6 ii). As nitrogen concentration becomes limiting, the growth rate and the carbon flow toward biomass generation decrease, while lipid production is triggered, resulting in a shift of microbial metabolism into the lipogenic phase (Fig. 6 iii, Fig. 7).

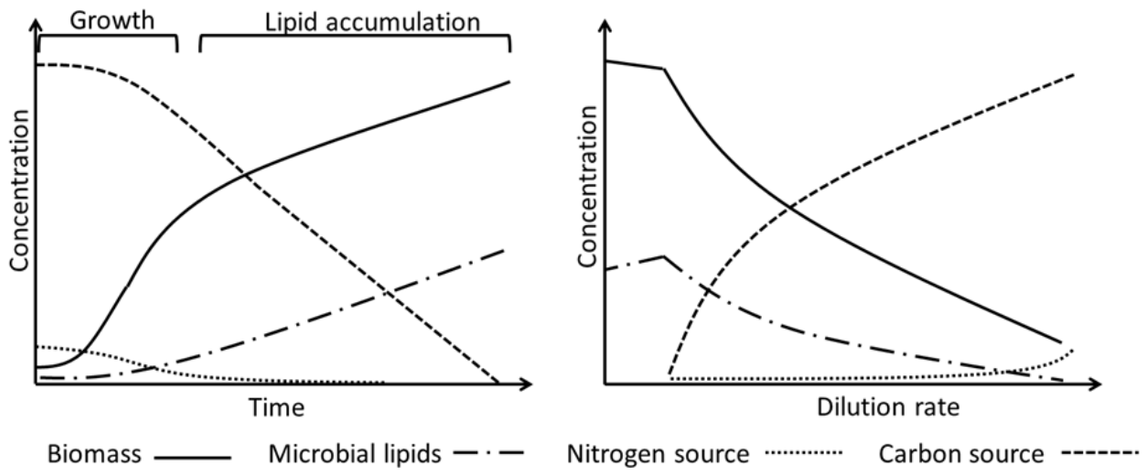

Fig. 7. Modeling and prediction of the timecourse of a batch fermentation (left) and the steady-state values of a continuous process (right) for microbial production of lipids. Axis are in arbitrary scales.

In batch cultures the initial C/N ratio of the cultural medium has a pivotal role in determining the bioprocess performance. In fact, both the rate and the yield of lipid production depend by the C/N ratio, which affects the duration of the exponential phase and the amount of biomass produced during growth. With a fixed carbon concentration, higher amounts of lipid-free biomass produced during the growth phase correspond to higher lipid production rates during the lipogenic phase, but to lower amounts of lipid content within cells and lipid/substrate conversion yields. Therefore, the initial C/N ratio needs to be optimized to maximize lipid productivity in batch cultures. The optimal C/N value is always high (e.g. in the range between 80 and 350 mol/mol) and strongly depends on the microorganism, the medium composition, the carbon source (e.g. glucose, glycerol, etc.), and the nitrogen source (e.g. diverse organic or inorganic sources). The minimal C/N ratio suitable for lipid accumulation can be estimated as $(Y_{X/S} \cdot q)^{-1}$, where $Y_{X/S}$ is the biomass/carbon source yield coefficient under conditions of carbon limitations (C-mol/C-

mol) and q is the nitrogen/carbon content of biomass (N-mol/C-mol) (Ykema et al., 1986). However, it should be considered that extremely high C/N ratios may cause the production of unwanted byproducts, such as organic acids (Fig. 6 iv), or may lead to severe nitrogen deficiency, causing a rapid decrease in cells viability.

Unlike batch processes, in fed-batch mode, nutrients are fed into the bioreactor in a controlled manner, with the purpose to monitor and control the specific growth rate and the flows of nitrogen and carbon utilization. Through the judicious management of the feeding rate and composition, it is possible to control the C/N ratio within the culture and maintain the oleaginous microorganism in the optimal metabolic status, as appropriate, first for the growth phase, and later for the lipogenic phase. The lipogenic phase is the most extensive, corresponding to lipid production under nitrogen limitation, with constant C/N ratio, preventing loss of viability and acids production (Beopoulos et al., 2009a).

In continuous cultures, at the steady state, the assimilation of C and N sources and the microbial growth occur at constant rates, which ultimately depend by the dilution rate (D). The concentration of the substrates within the bioreactor is steady and depends by the dilution rate as well, the actual C/N ratio of the culture remaining constant unlike in batch cultures. Likewise in batch cultures, in continuous cultures the C/N ratio of the fresh medium needs to be higher than $(Y_{X/S} \cdot q)^{-1}$ to obtain some lipid accumulation (Ykema et al., 1986). However, at the steady-state with this medium, the C/N ratio within the culture is higher than in the fresh medium, due to nitrogen consumption. The extent of substrates utilization, and also the biomass and lipid concentrations are the highest at low D values and decrease with the increase of D (Fig. 7). While low D values promote lipid production and a more complete substrate utilization, on the contrary, the volumetric productivity of continuous processes is positively affected by the increase of the dilution rate (Ykema et al., 1986; Meeuwse et al., 2011b). Therefore, both the C/N content of the medium and the dilution rate need to be thoroughly tuned to maximize lipid productivity of continuous processes.

7. Substrates and raw material

The demand for the inexpensive production of biofuels has intensified due to increasing concerns of climate change, depletion of petroleum-based fuels, and environmental problems. In a market economy, corporations aim to maximize profit, seeking the most competitive feedstock. To produce single-cell oils for biodiesel production, the carbon source has necessarily to be cheap and available in large quantities. Therefore, while the first investigations on oleaginous fungi most commonly employed glucose as carbon source, nowadays the production of single-cell oils is predominantly addressed to transformation of raw materials, by-products and surplus.

Glucose is the carbon source most commonly employed for growth of oleaginous fungi and lipid production (Boulton & Ratledge, 1984; Hansson & Dostalek, 1986; Hassan et al., 1993; Heredia & Ratledge, 1988; Jacob, 1991; Jacob, 1992; Johnson et al., 1992; Li et al., 2007; Pan et al., 1986; Ratledge, 2004; Rau et al., 2005; Saxena et al., 2008; Zhao et al., 2008). High glucose concentrations enhance the carbon flow that is directed toward TAG production, thus improving lipid production in several yeasts. However, growth of some yeasts (e.g. *R. toruloides)* is inhibited by high concentration of glucose, (Li et al., 2007). Furthermore, in batch cultures, initial glucose concentration also affects the fatty acids composition of the lipids (Amaretti et al., 2010).

Carbon sources other than glucose, such as xylose (Chistopher et al., 1983; Heredia & Ratledge, 1988;), lactose (Christopher et al., 1983; Daniel et al., 1999;), arabinose, mannose (Hansson & Dostalek, 1986), mannitol (Hansson & Dostalek, 1986), ethanol (Chistopher et al., 1983; Eroshin & Krylova, 1983), have been also investigated in the 80s and 90s for the production of microbial lipids.

Albeit glucose is a very good carbon source for lipid production with oleaginous fungi, molasses, which carbohydrate fraction is mainly composed of sucrose, glucose, and fructose, do not represent a promising raw material for lipid production, since they are characterized by a high nitrogen content which delays the unbalanced growth, where number of cells can not augment anymore and lipids are accumulated (Johnson et al., 1995).

Carbons sources obtained from ligno-cellulosic biomasses represent one of the most important potential to produce biodiesel. In fact, several waste biomasses containing forest residues, agricultural residues, food wastes, municipal wastes, and animal wastes can be utilized for the production of lignocellulosic based microbial lipids. Microbial oil production from sulphuric acid treated rice straw hydrolysate (SARSH) by the yeast *Trichosporon fermentans* pointed out the difficulty to perform the process of lipid accumulation in presence of the inhibitory compounds released during hydrolysis, such as acetic acid, furfural, 5-hydroxymethylfurfural, and water soluble lignin (Huang et al., 2009). Selected strains were able to grow on xylose and glucose (Zhu et al., 2008), but the crude hydrolyzate did not result an optimal substrate for a high yield process of lipid production. Cellulose and hemicellulose are generally hardly hydrolyzed and assimilated by yeasts, while they can be degraded and used as carbon source by filamentous fungi. A screening of endophytic fungi from the oleaginous plants was the selection of strains belonging to the genera *Microsphaeropsis, Phomopsis, Cephalosporium, Sclerocystis* and *Nigrospora* that simultaneously accumulated lipids (21.3 to 35.0% of dry weight) and produced cellulase (Peng & Chen, 2007). Albeit these strains could be exploited as microbial oil producers by utilising straw as substrate, they have never been claimed again as a SCO producers on lingo-cellulosic biomass. Attempts to carry out lipid production in Solid State Fermentation (SSF) on wheat straw have been performed exploiting a cellulolytic strain of *Aspergillus oryzae* (Lin et al., 2010). This strain is able to use cellulose as substrate and accumulate lipids in a low cost fermentation system on this abundant cellulosic by-product.

Other complex matrices have been used, such as solids from wheat bran fermentation (Jacob 1991), sewage sludge (Angerbauer et al., 2008), wastewaters of animal fat treatment (Papanikolaou et al., 2002), whey derivatives (Ykema et al., 1989; Vamvakaki et al., 2010), olive oil mill wastewaters (Yousuf et al., 2010), and tomato waste hydrolysate (Fakas et al., 2008).

Nowadays, lipid production with oleaginous yeasts is focused on selection and development of yeasts as converters of glycerol into lipid for biodiesel production, since it is the major side-product of the biodiesel production process. The biotransformation of glycerol into TAG is therefore regarded as a promising way to decrease the cost of biodiesel process through simultaneous reutilization of its major byproduct. In general, for every 100 kg of biodiesel produced, approximately 10 kg of crude glycerol are created. Crude glycerol is a mixture of glycerol (65–85%, w/w), methanol, and soap, and contains macro elements such as calcium, potassium, magnesium, sulfur and sodium. In order to minimize unknown variables introduced through the use of crude glycerol, several studies to determine whether or not glycerol could be used as substrate or co-substrate for growth have been conducted using purified glycerol.

A deep characterization of lipid accumulation on glycerol has been carried out with *Yarrowia lipolytica*, that is able to metabolize several important industrial and agro-industrial

by-products such as raw glycerol, producing large amounts of SCO and organic acids (Papanikolaou et al., 2003; Papanikolaou & Aggelis, 2002; Rymowicz et al., 2010; Rywinska et al., 2009). Biochemistry of lipid production on glycerol has been investigated in this organism: glycerol passes into the microbial cell by facilitated diffusion and the conversion is carried out via phosphorylation pathway, with direct phosphorylation to G3P and subsequent dehydrogenation. Recently, *Y. lipolytica* has been subjected to targeted and purposeful alteration of G3P shuttle pathway to better utilize glycerol for lipid production. In the genetically manipulated strains, lipid accumulation resulted from a complex interrelation between different processes in diverse cell compartments, such as lipid synthesis in the cytosol, location and storage in ER and LB, mobilization and degradation processes (Dulermo & Nicaud, 2011).

Pure glycerol supported growth and lipid accumulation of *Rhodotorula glutinis* and *Candida freyschussii* (Easterling et al., 2009; Amaretti et al., 2011), being used as sole carbon and energy source or in addition to xylose or glucose. The diverse composition of the medium affected not only the lipid/biomass yield, but also the TAG composition, in terms of ration of saturated, monounsaturated, and polyunsaturated fatty acids (Easterling et al., 2009).

Attempts to convert crude glycerol into lipids have been successfully performed exploiting the oleaginous yeast *Cryptococcus curvatus* (Liang et al., 2010). Different processes have been developed with very efficient yields and productivities. In a 12 days two-stage fed-batch where raw glycerol was fed, the biomass density and the lipid content reached 32.9 g/l and 52%, respectively. Methanol of crude glycerol did not pose a significant inhibitory effect even though it was existent in the bioreactor. Lipid accumulated by *C. curvatus* on glycerol presented high amount of monounsaturated fatty acid, turning out as excellent substrate for transformation into biodiesel.

8. Conclusions and perspectives

Oleaginous fungi, and particularly yeasts, are very efficient in the accumulation of intracellular TAG and it is expected that they will be exploited by the biofuel industry in the future. Nonetheless, the costs of microbial lipids are still too high in order to compete with plant oils for biodiesel manufacturing. Cheap carbon sources have necessarily to be used as carbon sources for the cultivation of these microorganisms and the performance of the bioprocess has to be further improved in terms of both the yield and the productivity. The exploration of the natural biodiversity is a promising strategy to identify novel oleaginous species that assimilate and get fat on agro-industrial residues, particularly the lingo-cellulosic biomass and crude glycerol from biodiesel industry. Further approaches combining genomic, transcriptomic, metabolomics, and lipidomic techniques will undoubtedly provide deeper information of lipid production by oleaginous fungi. A metabolic engineering approach is very promising, but it is still precluded for the most oleaginous species, for which genome disclosure has not been accomplished and genetic tools are not available yet.

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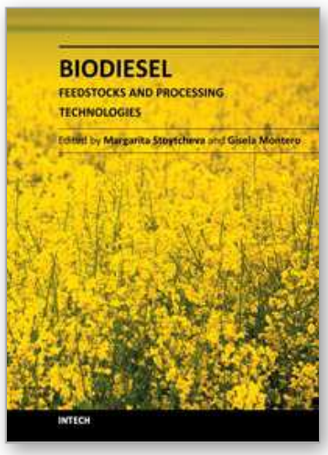

Biodiesel - Feedstocks and Processing Technologies

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The book "Biodiesel: Feedstocks and Processing Technologies" is intended to provide a professional look on the recent achievements and emerging trends in biodiesel production. It includes 22 chapters, organized in two sections. The first book section: "Feedstocks for Biodiesel Production" covers issues associated with the utilization of cost effective non-edible raw materials and wastes, and the development of biomass feedstock with physical and chemical properties that facilitate it processing to biodiesel. These include Brassicaceae spp., cooking oils, animal fat wastes, oleaginous fungi, and algae. The second book section: "Biodiesel Production Methods" is devoted to the advanced techniques for biodiesel synthesis: supercritical transesterification, microwaves, radio frequency and ultrasound techniques, reactive distillation, and optimized transesterification processes making use of solid catalysts and immobilized enzymes. The adequate and up-to-date information provided in this book should be of interest for research scientist, students, and technologists, involved in biodiesel production.

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