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Microbial Oil Production from Yeasts and Moulds

Grown on Agro-Industrial Waste

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List of abbreviations and acronyms

ACL	ATP-citrate lyase
ARA	arachidonic acid
D	dilution rate
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAME	fatty acid methyl esters
FAS	fatty acid synthetase
FFA	free fatty acids
GLA	γ -linoleic acid
MUFA	mono-unsaturated fatty acids
OM	oleaginous moulds
OPE	orange peel extract
OY	oleaginous yeast
PA	palmitoleic acid
PDA	potato dextrose agar
RCW	ricotta cheese whey
SCD	stearoyl-CoA desaturase
SD	standard deviation
TAG	triacylglycerols
YPD	yeast extract-peptone-dextrose

Short abstract

Progressive depletion of fossil fuels and the environmental problems associated with their use, have highlighted the need to find alternative, sustainable and renewable sources of oils for the oleochemical and biodiesel industry. For this purpose, in this PhD thesis project, 27 oleaginous yeast and mould strains were investigated for their ability of growing and accumulating lipids on 3 different renewable agro-industrial waste: glycerol, ricotta cheese whey (RCW) and orange peel aqueous extract (OPE). After an initial screening in shaken flasks, the feasibility of transfer to bioreactor scale was also assessed for the most promising strains. On the basis of the results obtained on each substrate, the yeast and mould strains which showed the highest performances were selected for growing on a 3-L stirred-tank bench-top bioreactor. More specifically, among yeasts, *Cryptococcus laurentii* UCD 68-201 was chosen to investigate a batch fermentation on RCW and *Rhodospiridium toruloides* NRRL 1091 was selected for a batch-fermentation on OPE. Since glycerol individually didn't yielded satisfying results, it was used in combination with OPE in a mixed substrate fed-batch fermentation in order to study if, in combination with other sugars, as observed in other works, was able to stimulate lipid accumulation (Galafassi et al., 2012; Rakicka et al., 2015). *R. toruloides* NRRL 1091 on OPE, yielded $6.5 \pm 0.4 \text{ g L}^{-1}$ of biomass containing the 80% of intracellular lipids, while *C. laurentii* UCD 68-201 on RCW demonstrated the highest performances compared to all the yeasts and moulds investigated in bioreactor on all the substrates in batch mode, with $14.4 \pm 0.1 \text{ g L}^{-1}$ and $69.0 \pm 1.6\%$ of biomass and lipid content, respectively. A fed-batch fermentation was also tested on *R. toruloides* on a mixed substrate OPE-glycerol, achieving at 104 hours of fermentation, a maximum lipid production of $20.6 \pm 0.23 \text{ g L}^{-1}$, with a net improvement of volumetric yield and productivity similar to those got in batch fermentation.

As concerns the investigation on moulds, *Mortierella isabellina* NRRL 1757, having exhibited a considerable adaptability on all the carbon sources tested, was chosen for a comparative study on all the substrates. In bioreactor, it reached $9.9 \pm 0.3 \text{ g L}^{-1}$ of biomass and $37.5 \pm 2.0\%$ of lipid content on OPE and, respectively, 7.3 ± 0.2 and 49.5 ± 0.2 on RCW, while growth on glycerol was considerably lower.

Finally, *Cryptococcus curvatus*, showing genomic similarity with *C. laurentii* and for which whole genome data is available, was chosen as model microorganism to investigate the metabolic step of desaturation of palmitic acid (C16:0) to palmitoleic acid (C16:1). The aim was the increase of total monounsaturated fatty acids, and in particular of C16:1, in order to

improve the properties of the derived biodiesel in terms of oxidative stability and cold temperature behaviour (Pinzi et al., 2009).

Key words: Lipid production; Microbial oil; *Cryptococcus curvatus*; *Rhodospiridium toruloides*; fed-batch; third-generation biodiesel.

Riassunto breve

L'esaurimento progressivo dei combustibili fossili ed i problemi ambientali associati al loro utilizzo hanno evidenziato la necessità di trovare fonti alternative, sostenibili e rinnovabili di oli per l'industria oleochimica e del biodiesel. A questo scopo, in questo progetto di tesi di dottorato, sono stati studiati 27 ceppi di lievito e muffe oleaginosi per la loro capacità di crescita e accumulo di lipidi su 3 diversi scarti/sottoprodotti agroindustriali rinnovabili: glicerolo, scotta (RCW) ed estratto acquoso di buccia d'arancia (OPE). Dopo uno screening iniziale in beuta agitata, è stata valutata anche la fattibilità di trasferimento in bioreattore per i ceppi più promettenti. Sulla base dei risultati ottenuti su ciascun substrato, i ceppi che hanno mostrato le migliori performances sono stati selezionati per la fermentazione in un bioreattore da banco ad agitazione meccanica da 3-L. Più specificamente, tra i lieviti, il *Cryptococcus laurentii* UCD 68-201 è stato selezionato per una fermentazione batch su RCW e *Rodospiridium toruloides* NRRL 1091 su OPE. Poiché il glicerolo individualmente non ha dato risultati soddisfacenti, è stato utilizzato in combinazione con OPE in una fermentazione fed-batch a substrato misto per studiare se, in combinazione con altri zuccheri, come osservato in altri lavori, fosse in grado di stimolare l'accumulo di lipidi (Galafassi et al., 2012; Rakicka et al., 2015). *R. toruloides* NRRL 1091 su OPE, ha prodotto $6.5 \pm 0.4 \text{ g L}^{-1}$ di biomassa contenente l'80% di lipidi intracellulari, mentre *C. laurentii* UCD 68-201 su RCW ha dimostrato le più alte produzioni volumetriche rispetto a tutti i lieviti e muffe studiati in bioreattore in modalità batch, con $14.4 \pm 0.1 \text{ g L}^{-1}$ e $69.0 \pm 1.6\%$ di biomassa e contenuto lipidico, rispettivamente. Inoltre, una fermentazione fed-batch è stata testata su *R. toruloides* su un substrato misto OPE-glicerolo ottenendo, dopo 104 ore di fermentazione, una produzione massima di lipidi di $20.6 \pm 0.23 \text{ g L}^{-1}$, con un netto miglioramento della resa volumetrica e produttività rispetto alla fermentazione svolta in batch con il solo OPE.

Per quanto riguarda lo studio sulle muffe, *Mortierella isabellina* NRRL 1757, avendo esibito una notevole adattabilità su tutte le fonti di carbonio testate, è stata scelta per uno studio comparativo su tutti i substrati. Nel bioreattore, essa ha raggiunto $9.9 \pm 0.3 \text{ g L}^{-1}$ di biomassa e $37.5 \pm 2.0\%$ di contenuto lipidico su OPE e, rispettivamente, 7.3 ± 0.2 e 49.5 ± 0.2 su RCW, mentre la crescita su glicerolo è stata considerevolmente inferiore. Infine, *Cryptococcus curvatus*, mostrando similarità genomica a *C. laurentii* e per il quale l'intero genoma è disponibile, è stato scelto come microrganismo modello per studiare lo step metabolico di desaturazione dell'acido palmitico (C16: 0) ad acido palmitoleico (C16: 1) con l'obiettivo di aumentare il contenuto totale di acidi grassi monoinsaturi. Infatti, è stato osservato che un'alta percentuale di MUFA, e in particolare di acido palmitoleico, migliorerebbe le proprietà del biodiesel derivato in termini di stabilità ossidativa e

comportamento a basse temperature (Pinzi et al., 2009).

Parole chiave: produzione di lipidi; olio microbico; *Cryptococcus curvatus*; *Rhodosporidium toruloides*; fed-batch; biodiesel di terza generazione.

Extended abstract

This PhD project focused on the possibility of using oleaginous fungi for the bioconversion of agro-industrial waste into microbial lipids of industrial interest.

The term “oleaginous” refers to the ability of certain yeasts and moulds of producing and accumulating intracellular lipids, over the 20% of their dry weight, when subjected to an environmental stress such as the lack of a key nutrient (Ageitos et al., 2011).

Studies on oleaginous fungi began in the XX century, in particular in Germany during both World Wars, with the main aim of seeking alternative and cheap sources of mono- (MUFAs) and poly-unsaturated fatty acids (PUFAs) for nutraceutical purposes (Ratledge and Wynn, 2002).

In addition to nutraceutical applications, microbial oils are currently on the spotlight as alternative feedstock to crude and vegetable oils. In fact, the increasing demand of plant oils for the production of biodiesel and lipid derivatives has put in evidence the necessity of looking for new strategies based on non-food crops, agro-industrial waste and renewable resources that do not compete with food and feed production (Ageitos et al., 2011).

In this context, oleaginous fungi could be a valuable alternative since they would fulfil these requirements, with the important advantage of being independent on season and climate factors, having a short process cycle and being easy to handle and scale up. So far, the major obstacle for the commercialization of these oils is the high production costs, depending mainly by the cost of growth medium and the costs associated with the fermentation process itself (Ageitos et al., 2011). The use of agro-industrial waste and by-products as carbon sources for oleaginous fungi is undoubtedly a possible way to lower these costs. The variety of raw materials already investigated for this purpose include molasses, dairy serum, glycerol, organic solids from wheat bran fermentation, general wastewaters, wastewaters of animal fat treatment, olive-oil mill wastewaters and some others studied to a lesser extent (Leiva-Candia et al., 2014).

However, since lipid content and profile differ among species and strongly depend on fermentation medium and conditions, this PhD project wanted first to assess the suitability, in shaken flasks, of three different agro-industrial waste as growth substrate for 27 oleaginous fungi.

Substrates under study were glycerol, ricotta cheese whey (RCW) and orange peel aqueous extract (OPE).

Glycerol is a by-product of several oleochemical processes involving the transesterification of vegetable oils and animal fats (e.g. biodiesel production), and to a lesser extent, of bioethanol and alcoholic beverage industries (Tchakouteu et al., 2015).

RCW is a by-product of dairy industry derived from ricotta cheese production, having the following composition: 4.8-5.0% lactose, 1.0-1.3% salts, 0.15-0.22% proteins, 0.20-0.25% organic acids and 0.20% fats (Sansonetti et al., 2009). Although resembling cheese whey, the addition of organic acids and salts make it a less suitable substrate for animal feed causing additional costs for the dairy industry due to the need of its disposal.

Orange peel is a widespread waste deriving from juice manufacturing, the production of which amounts to tons per day. After juice extraction the residual peel accounts for 50% (w/w) of the fruit, and apart from some applications as a source of essential oils or animal feed, most of this waste remains unused (Balu et al., 2012). In this project, an aqueous extract of de-oiled orange peels was used as substrate, consisting mainly of glucose, sucrose and fructose.

Finally, through metabolic engineering techniques, the possibility of modifying the lipid profile, increasing the ratio of MUFAs over saturated fatty acids (SFAs), was explored. In fact, a high percentage of MUFAs, and in particular of oleic and palmitoleic acid, should improve the properties of the derived biodiesel in terms of oxidative stability and cold temperature behavior (Pinzi et al., 2009).

Glycerol

Glycerol is probably one of the most common substrates taken into account for lipid production, due to its widespread diffusion and ease of preparation of growth media based on it. Furthermore, it is the main by-product of biodiesel production, so its recycling represents the valorization of a by-product of the same production processes, thus contributing to lower production costs (Leiva-Candia et al., 2014).

It is important to note that, even if the uptake of glycerol was inefficient in most of the strains, this carbon source was conducive to a high lipid content in *Rhodospiridium torouloides* NRRL Y1091 (64%), *Cryptococcus curvatus* NRRL Y-1511 (60%) and *Lypomyces starkeii* NRRL 11557 (46%). A possible explanation is that glycerol may be incorporated directly into triacylglycerols (TAG) synthesis or that it can stimulate their synthesis, being therefore important during lipid production phase, more than in growth phase (Galafassi et al., 2012).

Among moulds, *Mortierella isabellina* proved to be the best lipid producer, with a volumetric lipid production of 3.10 g L⁻¹, corresponding to the 27.63% of his dry weight. Although yields of both biomass and lipid should be theoretically similar using glycerol or glucose as carbon source, experimentally, the use of glycerol brought to unsatisfactory result. It has been supposed that this could be due to a poor regulation of the enzymes involved in glycerol assimilation (Papanikolaou et al., 2008).

RCW

Despite the high nutritional value of RCW, only few studies have been conducted on this medium. Most of research has focused instead on cheese whey, which, however, differs from RCW because of its lower concentration of salts and organic acids and higher content of proteins (Takakuwa and Saito, 2010). On RCW, *C. curvatus* NRRL Y1511 and *Cryptococcus laurentii* UCD 68-201 were the most performing yeasts with a lipid volumetric production of 6.83 and 5.52 g L⁻¹, respectively. It is interesting to note that while this matrix strongly favoured lipid accumulation in the above-mentioned strains, it was not conducive to the growth of other screened yeasts, resulting in low lipid production probably due to their inability of using lactose, which is the main C source in this matrix.

Conversely, all moulds achieved a good growth on this effluent ranging from 5.85 g L⁻¹ for *Chaetomium* sp. LAS_G to 12.84 g L⁻¹ for *M. isabellina*. In addition, in the latter, lipid content reached the 35% confirming *M. isabellina* and RCW as a promising combination substrate-strain.

OPE

OPE sugar composition, consisting mainly of glucose, sucrose and fructose, ensured an appreciable growth for all the yeasts screened. Among them, *R. toruloides* NRRL 1091 emerged as a promising lipid producer, with a remarkable volumetric lipid production of 5.7 g L⁻¹, corresponding to 77% of lipid content.

As concerns moulds, *M. isabellina* reconfirmed itself as a really promising strain, with lipid production amounting to 3.1 g L⁻¹. Considering the appreciable results obtained also on the other media under study, *M. isabellina* resulted as the most versatile and efficient strain of the screening, able to achieve high growth and lipid production both on glycerol and sugar-based complex media.

Comparison of lipid profiles

For all strains, C 16 and C 18 were, in general, the most abundant fatty acids and, among them, oleic acid (C 18:1) was the predominant one.

Among the substrates investigated, glycerol led to a higher relative content of linoleic acid (C18:2), except for *R. toruloides*, *Yarrowia lipolytica* and *Aspergillus tubingensis*, but this did not affect the content of other polyunsaturated fatty acids (PUFA), being below 2.6% in every production. In view of a potential application of these oils for biodiesel production, the low content in PUFA is an important parameter to consider. According to the European Standard UNE-EN 14214, limit of cetane number and iodine value (correlated with combustion quality and formation of combustion deposits, respectively) are satisfied only when PUFA are below the 30% of total lipids (Ramos et al., 2009). On the other hand, a

content in mono-unsaturated fatty acids (MUFA) higher than 50%, is associated with a good quality biodiesel. This was accomplished by *Y. lipolytica* when grown on glycerol (65%), *C. curvatus*, *C. laurentii*, *Limpomyces starkeii*, *M. isabellina* and *Mucor racemosus* on RCW, and by *C. curvatus*, *R. toruloides*, *Rhodotorula glutinis* and *A. tubingensis* on OPE.

Transfer to bioreactor scale-yeasts

The feasibility of scale-up to bioreactor scale of fermentation processes previously investigated in flask, was assessed. Based on the results obtained on each substrate, the yeast and mould strain which showed the highest levels of biomass and lipid production, consistent with a suitable fatty acid profile for biodiesel production, were selected for growing on a 3-L bench-top bioreactor. More specifically, among yeasts, *C. laurentii* UCD 68-201 was chosen to investigate a batch fermentation on RCW and *R. toruloides* NRRL 1091 was selected for a batch-fermentation on OPE and for a fed-batch fermentation on a mixed glycerol-OPE substrate.

Transfer of *C. laurentii* to bioreactor scale, led to significantly improved yields, achieving a volumetric biomass and lipid production of 14.4 g L⁻¹ and 9.9 g L⁻¹, respectively, after 60 hours of fermentation, while, in flask, the maximum biomass production was reached at 72 hours with 7.3 g L⁻¹ and similar lipid content.

R. toruloides batch fermentation on OPE resulted in a volumetric biomass concentration of 6.53±0.4 g L⁻¹ biomass at 36 hours with an intracellular lipid content of about 80%. Such high lipid accumulation values, exceeding 70%, were also observed in fed-batch fermentations of *R. toruloides* on glucose (Wiebe et al., 2012). Compared to flask fermentations, the peak of production was considerably anticipated, from 72 to 36 hours, thus significantly increasing biomass and lipid productivity.

Fed-batch fermentation on a mixed orange-peel and glycerol substrate

Since glycerol individually didn't yielded satisfying results, it was used in combination with OPE in an mixed substrate fed-batch fermentation in order to study if, in combination with other sugars, it was able to stimulate lipid accumulation (Galafassi et al., 2012; Rakicka et al., 2015).

R. toruloides NRRL 1091, able to achieve a satisfactory growth on OPE and, at the same time, to accumulate 64% of intracellular lipids when grown on glycerol, was selected for this purpose. Although improvements are still needed, concerning for example the dilution rate and concentration of feeding media, 20.5 g L⁻¹ of volumetric concentration of lipids were produced in 104 hours, with a remarkable increase in production rates compared to results obtained on the two individual substrates

Transfer to bioreactor scale-moulds

Among the mould group, *M. isabellina* NRRL 1757, having exhibited a considerable adaptability on all the carbon sources tested, was chosen for a comparative study on all the substrates. However, the process transfer from shaken flask to stirred tank bioreactor was unsatisfactory, leading to greatly reduced growth, especially on glycerol and RCW. This behaviour was already observed by Chatzifragkou et al. (2010) who noticed growth inhibition at high aeration and agitation rates. For this reason, in the experiment with *M. isabellina*, the agitation rate was decreased from 600 to 450 rpm with respect to yeast fermentations, but further agitation decrease was not possible due to the formation of pellets, which prevented the sampling, and the proper transfer of nutrients. It should be noted, however, that the relative concentrations of the fatty acids produced were similar on all substrates tested, both in flask and in bioreactor, thus showing that, unlike other strains (Donot et al., 2014), the fatty acid profile of *M. isabellina* is less influenced by the carbonaceous source and conditions used.

Metabolic engineering of an oleaginous model strain

During the last period of this PhD project, *C. curvatus* was chosen as model microorganism to investigate the metabolic step of desaturation of palmitic acid (C16:0) to palmitoleic acid (C16:1) with the aim of increasing total MUFA content. Although *C. laurentii* was slightly more versatile on both RCW and OPE, more genetic information is available for *C. curvatus*, which, anyhow, share with the first one a high genetic similarity. Furthermore, so far, most of metabolic engineering tools have been developed for the modulation of *Y. lipolytica* lipid pathway (Beopoulos et al., 2009), an Ascomycota yeast, while just few attempts have been made on Basidiomycota, to which *Cryptococcus* belongs. The interest on Basidiomycota is supported by the evidence that, in comparison with Ascomycota, they can metabolize a broader range of carbon sources and even recalcitrant natural substrates and xenobiotics (Johnson, 2013) without any supplementation of vitamins (Sitepu et al., 2014).

To achieve higher yields of C16:1, a heterologous expression of two different Δ^9 -desaturase, acting mainly on C16:0, deriving from *Caenorhabditis elegans* (Fat-5) and *Saccharomyces cerevisiae* (ole1p) was attempted in *C. curvatus*.

The modified strains obtained showed a 5% increase, with both selected genes, of C 16:1 content on total lipids, compared to the wild-type (WT). Nevertheless, this difference was appreciable only within the first 12 hours of fermentation, before the beginning of lipid accumulation, when the lipid composition analysed represented essentially cell membrane composition. When lipid accumulation started, the effect of the genes inserted, was hidden by the higher relative accumulation of C 18:1, thus indicating a lower efficiency of the

heterologous desaturases specific for C16:0, with respect to the endogenous desaturase specific for C18:0.

It is worth noting that, although it was not possible to see the effect of *ole1p* and *Fat-5* on C16:1 content during lipid accumulation phase, the concentration of C16:0 was anyway 15% lower in modified strains and C18:0 higher than in the wild type strain. This result indicates that the transformation influenced somehow the pool of C16:0, but some regulatory mechanisms probably took place to restore a possibly more functional lipid profile to the cell

CHAPTER 1

INTRODUCTION

In recent years, depletion of crude oil resources derived mainly by the growth in global energy market together with the rising demand of edible plant oils for the oleochemical, nutraceutical and biodiesel industry, have highlighted the need for different oil sources.

In this regard, the use of microbial oils as alternative feedstock, could have many advantages, being renewable, sustainable and non-competitive with food or arable lands (Beopoulos et al., 2011).

The potential of certain microorganisms to accumulate high amounts of lipids was discovered already at the end of XIX centuries when Lindner observed that the yeast *Metschnikowia pulcherrima* could accumulate intracellular fat globules. Then, during World Wars I and II the interest on microbial oils continued to grow, mainly with the aim of looking for alternative sources for food and fuel uses at a time when supplying was difficult. Such microorganisms, capable of accumulating lipids over 20% of their dry weight, were thus defined as “oleaginous” and all throughout the twentieth century to the present day, the studies on them continued with the aim of elucidating the metabolic, abiotic and genetic factors leading to lipid accumulation (Sitepu et al., 2014), as it will be discussed in the next chapters.

In particular, two major research branches developed over the years: nutraceutical sector, whose primary interest is to understand and modulate the specific steps leading to the production of polyunsaturated fatty acids (PUFAs) with high added-value such as α - and γ -linolenic acid, eicosapentaenoic acid or docosahexaenoic acid; biofuel sector, mainly focused on lowering production costs, through the investigation of low-cost substrates and of strains capable of metabolizing complex carbon sources and achieving high yields on them (Ochsenreither et al., 2016). In fact, while the production of PUFAs is economically competitive (i.e., commercialization of microbial oils enriched in arachidonic acid from *Mortierella alpina* (Béligon et al., 2016), the production of biodiesel from oleaginous microorganisms is still not economically feasible unless specific strategies to lower process costs are adopted (Koutinas et al., 2014).

In this thesis, a particular attention will be given to the latter and most of the discussion will be addressed to the use of fungal oils for biodiesel production.

Biodiesel, a mixture of fatty acid methyl esters (FAMES) currently derived for the 95% from vegetable oils has become an attractive alternative source of energy thanks to its environmental benefits: it is renewable and biodegradable, it contributes no net carbon dioxide or sulphur to the atmosphere and emits less gaseous pollutants than normal diesel. However, the current “food vs fuel” debate, along with the high cost of biodiesel from edible crops, the raw material of which amounts to about 75% of total costs, has become one of the major obstacles for its development and wide application. Adoption of animal fats, used frying oils and waste oils from restaurants as feedstock is a good strategy to reduce the cost. However, these limited resources cannot meet realistic needs for clean renewable fuels (Canakci and Sanli, 2008).

To overcome this obstacle, different sources have been explored, among which, one of the most promising, are microbial oils. In the context of a circular economy and of sustainability, the exploration of low-cost feedstock, such as lignocellulose-based carbohydrates or agro-industrial waste as nutritional sources for the growth of oleaginous microorganisms, could allow to have remarkable amounts of oil to be used as complement, at least at first, to vegetable oils, with several advantages over the latter. In fact, microbial oils can be produced all over the year, in a short time and with production rates up to 100× that of plant oils in liters/hectare/year (Sitepu et al., 2014).

The variety of raw materials already investigated include molasses, dairy serum, glycerol, solids from wheat bran fermentation, general wastewaters, wastewaters of animal fat treatment, olive oil mill wastewaters and still others studied to a lesser extent. However, the variability between different species and strains and on different substrates does not allow to predict precisely the final yield and lipid profile, which is why numerous screening studies are still ongoing (Ageitos et al., 2011).

In the following chapter, an overview on oleaginous yeasts (OYs) and moulds (OMs), biochemistry of lipid accumulation and fatty acid composition will be given, followed by general extraction methods used for microbial oils and their applications.

Chapters 3-8 will discuss, instead, the aim of the project, methodology used and results obtained during the PhD course.

CHAPTER 2

STATE OF THE ART

2.1 Overview on oleaginous microorganisms

The term “oleaginous” refers to the ability of certain microorganisms of producing and accumulating intracellular lipids, over the 20% of their dry weight, when subjected to an environmental stress such as the lack of a key nutrient (Ageitos et al., 2011).

Various species belonging to yeasts, moulds, bacteria and microalgae show this ability, although presenting significant differences in metabolism, composition and amount of lipids produced (Garay et al., 2014). As a consequence, the prospects for potential applications vary according to the specific characteristics of every microorganism.

In general, bacteria are not good lipid producer, the average oil content being between 20-40% on total dry weight, even if they can produce some interesting lipoids such as poly-b-hydroxy-butyrate and -alkanoates as storage polymers (Bugnicourt et al., 2014).

Contrariwise, yeasts exhibit several advantages over other oleaginous microorganisms, having a higher growth rate compared to algae, associated with levels of lipid accumulation up to 80% of their dry weight. Moreover, they can grow on a wide variety of different, low-cost, carbon sources and be more easily scaled-up than microalgae (Ageitos et al., 2011).

The cultivation of microalgae in photobioreactors in fact, besides being quite expensive, poses problems of design for large-scale productions, while cultivation in outdoor ponds, marine lagoons or in brackish water, albeit reducing plant costs, does not allow the achievement of high biomass and lipid yields, with the additional disadvantages of requiring large acreages, high concentrations of dissolved CO₂, being influenced by climate and photoperiod, having no control of growth conditions, and with a greater probability of contamination by bacteria and predatory protozoa (Sitepu et al., 2014).

For these reasons fungi have been among the most investigated microorganisms over the years for the commercial production of microbial oils and they will be also the focus of this PhD thesis.

More specifically, yeasts have been studied, mainly, for the production of oils for biodiesel, having a lipid profile characterized by a high concentration of monounsaturated (MUFAs) and saturated fatty acids (SFAs), very similar to that one of vegetable oils (Sitepu et al., 2014). Moulds, instead, which in many cases are able to produce high value-added PUFAs, have been addressed mostly to studies in the nutraceutical field (Ochsenreither et al., 2016).

2.2 History of oleaginous yeasts and moulds

Studies on fungal lipids began in 1878 when Nägeli and Loew (1878) reported the fatty acid composition of *Saccharomyces cerevisiae*. About 20 years later, Lindner, describing an unknown species of yeast which he named *Torula pulcherrima* (now renamed as *Metschnikowia pulcherrima*), observed the presence, inside the cell, of a highly refractile globule apparently rich in fat, which can be considered as the first evidence of lipid accumulation process in a microorganism (Kluyver et al., 1953).

Applications of these oils began to be investigated in Germany during World Wars I and II as alternative feedstock for food and energy uses in a country that had been denied access to such goods (Ratledge and Wynn, 2002). During these years, Lindner's studies on the OY *Guehomyces pullulans* (Lindner, 1922; Sitepu et al., 2014) allowed to understand some of the mechanisms underlying lipid accumulation, identifying in the limitation of nitrogen one of the factors triggering such metabolism. Some years later, two abandoned breweries were reconverted to microbial oil factories using *G. pullulans* as producer and sulphite waste liquor from the cellulose industry as carbon source. The high costs associated to a non-optimized production process led to the failure of the factories after only 2 years but this experience led to the identification of other key parameters for the fermentation process: abundant supply of a carbon source, abundant aeration and use of a young, actively dividing inoculum (Woodbine, 1959).

In the 1950s, the interest in microbial oils spread even in other countries, such as the United Kingdom and USA but, as had already happened in Germany, it declined rapidly due to the economic recovery and development of agriculture after the II World War which made microbial oils no longer needed and expensive compared to vegetable oils (Ratledge and Wynn, 2002).

A renewed interest was observed again in the 1980s when the target shifted from generic surrogates of vegetable oils to high value MUFAs and PUFAs for nutraceutical purposes, uncommon in nature and with beneficial effects on health (Ratledge and Wynn, 2002).

In 1985, a microbial oil rich in γ -linoleic acid (GLA; 18:3 n-6) by *Mucor circinelloides*, was actually commercialized as a substitute of evening primrose oil (Ratledge, 2004) followed by dietary supplements enriched in arachidonic acid (ARA; 20:4 n-6) from *Mortierella alpina* that are still on the market (Bélignon et al., 2016).

Since the 1990s, many researchers focused also on docosahexaenoic acid (DHA; 22:6 n-3) production, a PUFA produced mainly by filamentous fungi, because it had been seen that the association of DHA and ARA in infant dietary supplementations, could improve their

nutritional quality. As estimated, the production of DHA oil reached 2000 tonnes in 2013 (produced however not from fungal strains but from microorganism belonging to the genus *Labyranathula*) and in 2012 ARA oil obtained approval for sale in EU (Ratledge, 2013).

Finally, another interesting combination studied was the one between DHA and eicosapentaenoic acid (EPA; 20:5 n-3) for the prevention of various cardiac problems.

For this purpose, DuPont company (Wilmington, DE, USA) developed a genetically-modified *Yarrowia lipolytica* strain which recently obtained FDA approval (Ratledge, 2013).

In addition, microbial oils are currently on the spotlight also as alternative feedstock to vegetable oils for biodiesel production. In fact, the increasing demand of plant oils for the production of biodiesel, from 15,000 barrels per day in 2000 to 289,000 in 2008 (Atabani et al., 2012), has put in evidence the necessity of looking for new strategies based on non-food crops, agro-industrial waste and renewable sources that do not compete with food and feed production.

So far, the major obstacle for the commercialization of these oils has been the high production costs, however, the rising price of crude oil and vegetable oils themselves has allowed to re-evaluate the production from fungal oils (Ageitos et al., 2011).

Since 75-80% of the cost of raw materials for biodiesel production derives from the substrate of growth used (Koutinas et al., 2014), to make the process more economically competitive, many efforts focused on using low-cost substrates and on looking for high-producing microorganisms able to metabolize them.

2.3 Taxonomic diversity of oleaginous fungi

From an ecological point of view, many oleaginous fungi have been isolated from relatively dry, nutrient-poor habitats such as plant surfaces. Therefore, accumulation of storage lipids, rather than storage carbohydrates, would probably be an adaptation strategy to survive in desiccating conditions. Moreover, a relevant characteristic of OYs, is their versatility of metabolizing a variety of carbon sources, which would give them an advantage for the utilization of sugars deriving from degradation of lignocellulosic breakdown (Sitepu et al., 2014).

The group of oleaginous fungi presents a remarkable taxonomic diversity, including species belonging to the phylum of Zygomycota, Ascomycota and Basidiomycota.

Although quantity and quality of the lipids produced are dependent on species, time and fermentation parameters, there are some common features common to members belonging to each phylum.

Zygomycota are versatile filamentous fungi with the ability of producing a wide range of enzymes for the assimilation of complex substrates, such as starch, cellulose, phytic acid, and proteins, thus allowing the cultivation on low-cost waste substrate, without need of pretreatment (Ferreira et al., 2013). Among them, *Mortierella*, and *Mucor* genera are among the most known for their lipid accumulation ability and for their tendency to produce high value-added PUFA, such as GLA, EPA and ARA (André et al., 2009; Bélignon et al., 2016). Examples already mentioned include the production of a primrose oil substitute rich in GLA from *Mucor circinelloides* and dietary supplements enriched in arachidonic from *Mortierella alpina* (Ratledge, 2004).

Among Ascomycota, *Yarrowia lipolytica* is undoubtedly the most widely studied, from a metabolic, fermentative, genetic and metabolic engineering point of view, and it can be considered a model microorganism for lipid accumulation (Beopoulos et al., 2009, 2008; Sabirova et al., 2011; Tai and Stephanopoulos, 2013; Zhu and Jackson, 2015). Other known and widely studied members of Ascomycota include species belonging to *Candida*, *Geotrichum*, *Lipomyces*, and *Aspergillus* genera and some other studied to a lesser extent (Papanikolaou and Aggelis, 2011a).

While Ascomycota microorganisms present high lipid accumulation rates, recent studies have focused mainly on Basidiomycota, able to degrade recalcitrant natural substrates and xenobiotics (Johnson, 2013), to metabolize a broader array of carbon sources than Ascomycota, and to grow easily without any supplementation of vitamins (Sitepu et al., 2014).

Concerning Basidiomycota, numerous studies on various agro-industrial waste have led to good results in species such as: *Rhodosporidium toruloides* on dried sorghum stalks (Matsakas et al., 2015), pre-treated wheat straw (Yu et al., 2011), cassava starch (Wang et al., 2012), Jerusalem artichoke extract (Zhao et al., 2010) and crude glycerol (Xu et al., 2012); *Rhodotorula glutinis* on monosodium glutamate wastewater (Xue et al., 2008), palm oil effluent (Saenge et al., 2011a), hydrolyzed tree leaves and corn stalk (Dai et al., 2007) and crude glycerol (Saenge et al., 2011b; Yen et al., 2012); *Cryptococcus curvatus* on sweet sorghum (Liang et al., 2012), municipal wastewater (Chi et al., 2011), wheat straw hydrolysate (Yu et al., 2011), organic waste from the brewery industry (Ryu et al., 2013), beet molasses and cheese whey medium (Takakuwa and Saito, 2010) and glycerol (Meesters et al., 1996).

These are only few examples, but new promising species continue to emerge from environmental screening studies.

It has been estimated that about 3–10% of randomly selected yeasts screened are oleaginous. Thus, considering that about 1600 yeast species are currently known, 50 to 160 of them may be oleaginous. Taking into account that several new yeast species are discovered every year, the number of OYs is expected to increase (Sitepu et al., 2014).

2.4 Biochemistry of lipid accumulation

All microorganisms can synthesize lipids, albeit only oleaginous strains can accumulate intracellular lipids over the 20% w/w of their dry weight (Papanikolaou and Aggelis, 2011a). A few model microorganisms have been taken into account for the study of lipid metabolism, although, with the current interest in controlling and modulating lipid accumulation and the specific fatty acids produced through metabolic engineering, the variety of models is increasing.

The most widely studied models are *S. cerevisiae*, a non-OY, which allowed the definition of metabolic steps involved in lipid anabolism and catabolism and *Y. lipolytica*, an OY, which allowed the understanding of the mechanisms underlying oleaginicacy (Rossi et al., 2010).

Although the metabolic steps are essentially the same between non-oleaginous and oleaginous-yeasts, in the latter the presence of accessory enzymes and different regulatory mechanisms lead them to a different response under the same culture conditions.

Depending on the substrate used for growth, lipid production may occur through two different pathways named *de novo* synthesis and *ex novo* accumulation, as discussed in the next paragraphs.

2.4.1 *Ex novo* accumulation

Ex novo lipid accumulation occurs when a OY is grown on hydrophobic substrates such as fatty acids, triacylglycerols or, occasionally, alkanes, which are incorporated in an unchanged or modified form within the cell (Beopoulos et al., 2009).

Unlike the lipid pathway that is generally mentioned (*de novo* synthesis), accumulation doesn't depend on the limitation of some nutrient factor, but it occurs during primary anabolic growth. In fact, *ex novo* accumulation can be considered as an imbalance between fat uptake and assimilation rate, so that when the uptake is higher than the assimilation rate, lipids are accumulated inside the cell. This also means that, when fats in the medium are going to finish and the uptake is lower than the assimilation rate, the cell will promptly degrade storage lipids to maintain its growth needs (Papanikolaou and Aggelis, 2003).

Depending on the substrate used, this pathway may require the hydrolysis of the hydrophobic substrate followed by the transport of the released fatty acids in the form of CoA-thioesters into the endoplasmic reticulum where they proceed with the same steps that will be described for the *de novo* synthesis to generate neutral lipids (Beopoulos et al., 2011).

In order to have an effective uptake of the substrate, the presence of specialized enzymes for the hydrolysis (lipases), solubilization and transport of hydrophobic compounds inside the cell is crucial, as it can be found in species such as *Pichia jadinii*, *Candida rugosa*, *Y. lipolytica*, and *Torulopsis colliculosa* (Beopoulos et al., 2011).

The specificity of these enzymes also reflects in a greater affinity for some fatty acids rather than others, meaning that the bioconversion of fat in microbial oils must be quantitatively studied in order not to risk that the microorganism begins unexpectedly to use reserve lipids even if lipids in the growth medium have not yet exhausted (Papanikolaou and Aggelis, 2003).

Applications of the *ex novo* lipid production include the valorization of fatty waste such as animal fat, a by-product of the meat industry (Papanikolaou et al., 2007a, 2002, 2001), vegetable oils (Aggelis and Sourdis, 1997; Bati et al., 1984) or fish oil (Gou and Ota, 2000; Kinoshita and Ota, 2001).

In view of an industrial application, however, it must be taken into account that *ex novo* accumulation, compared to *de novo* synthesis, leads to a significantly lower quantity of (triacylglycerols) TAGs with respect to total accumulated lipids. Conversely, free-fatty acids are produced in higher amounts, meaning that, although both pathways convey into the same step within the ER, some biochemical differences between them exist (Papanikolaou and Aggelis, 2011a).

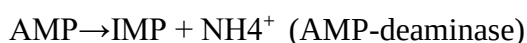
2.4.2 *De novo* synthesis

De novo synthesis is a secondary anabolic metabolism occurring when an OY is grown on a sugar-based media characterized by an excess of carbon source and limitation of another essential compound for growth. Substrates investigated are several and include simple sugars such as glucose (Evans and Ratledge, 1983; Li et al., 2007; Pan et al., 2009), fructose (Evans and Ratledge, 1983; Lazar et al., 2014), xylose (Dai et al., 2007; Gong et al., 2012; Hu et al., 2011; Tapia et al., 2012), whey (Carota et al., 2017; Seo et al., 2014; Takakuwa and Saito, 2010) molasses (Karatay and Dönmez, 2010; Takakuwa and Saito, 2010), or by polysaccharides such as starch (Gen et al., 2014; Tanimura et al., 2014; Wang et al., 2012; Zhu et al., 2003) or pectin (Papanikolaou et al., 2007b; Wang et al., 2015).

Although some publications investigate the influence of phosphorus (Wu et al., 2010), sulphate (Wu et al., 2011) or iron (Hassan et al., 1996) limitation on lipogenesis, nitrogen deficiency is the most studied and generally the most efficient condition to induce such metabolism (Rossi et al., 2010). For this reason, the discussion will focus on nitrogen limitation.

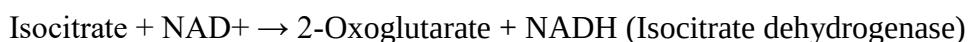
The main difference between non-OYs and OYs is that the first react to the exhaustion of nitrogen stopping cell proliferation and diverting, in some cases, the left carbon source to form polysaccharides, while OYs divert carbon to form storage lipids (Ratledge, 2004).

As can be seen in Fig. 2.1, one of the first features underlying a different response is the presence, in OYs, of a AMP-deaminase activated by nitrogen exhaustion, which catalyses the following reaction (Ratledge, 2004):



AMP-deaminase acts as a scavenger of ammonium ions, hydrolysing AMP to IMP to compensate for the lack of nitrogen.

The rapid drop in AMP concentration affects the tricarboxylic acid cycle inhibiting isocitrate dehydrogenase, that, in OYs, is allosterically activated by AMP (Papanikolaou and Aggelis, 2011a), responsible for the transformation of isocitric to α -ketoglutaric acid:



The resulting excess isocitrate is then converted back to citrate by isocitrate aconitase:



When citrate exceeds a certain threshold concentration in the mitochondria, it is exported into the cytoplasm via the malate/citrate antiport.

In the cytoplasm, citric acid is cleaved by ATP-citrate lyase (ACL) in oxaloacetate and acetyl-CoA, through the following reaction:



ACL is a fundamental enzyme for lipid accumulation, highly active in OY, but absent in the majority of non-oleaginous species. Its function is to provide, on the one end, the acetyl-CoA pool used to build up the fatty acid chain and, on the other, the oxaloacetate used to generate a continuous supply of reducing power in the form of NADPH for the condensation of acetyl-CoA to the growing fatty acid chain (Garay et al., 2014).

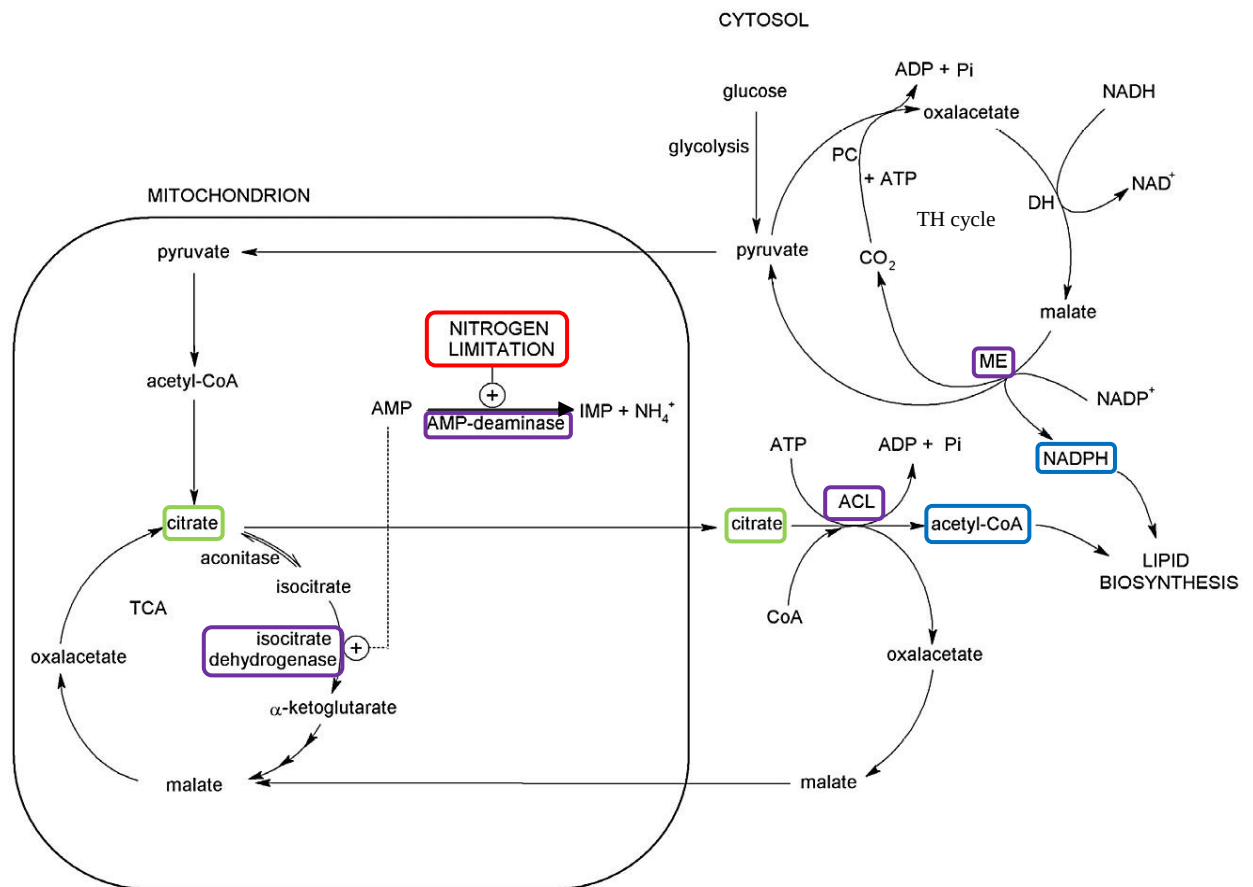
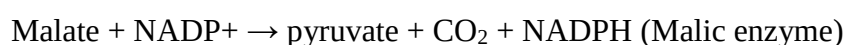
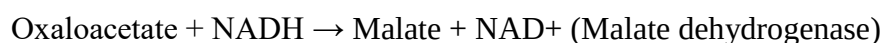


Figure 2.1. De novo lipid synthesis pathway, as a result of nitrogen limitation (red box), inducing the activation of an AMP-deaminase which acts as a scavenger of nitrogen from AMP. Decrease in AMP causes the inhibition of isocitrate dehydrogenase, one of the enzymes of tricarboxylic acid cycle (TCA), with a consequent accumulation of citrate. When citrate reaches a threshold concentration value in the mitochondria, it is exported into the cytosol where, by means of an ATP-citrate lyase (ACL), and is hydrolysed to provide acetyl CoA, the basic unit for the construction of lipids. In parallel, malic enzyme (ME), provides NADPH, the reducing power essential for the lipid synthesis reaction. Adapted from Rossi et al. (2010).

The last mentioned reaction is part of the transhydrogenase cycle which converts NADH into NADPH through the activity of 3 consecutive enzymes: pyruvate carboxylase, malate dehydrogenase and malic enzyme, catalysing, in the order, the following reactions (Garay et al., 2014):



The activity of these three enzymes is believed to be among the essential factors determining the extent of lipid accumulation ability in oleaginous fungi.

Once acetyl-CoA and NADPH pool have been produced, the cell can proceed with the synthesis of fatty acids.

First of all, acetyl-CoA is carboxylated to malonyl-CoA by means of a cytoplasmatic acetyl-CoA carboxylase (ACC) considered as another key enzyme in lipid synthesis, since ACC overexpression leads to an increase in lipid accumulation, while its disruption causes a lethal phenotype (Hasslacher et al., 1993).

Malonyl-CoA can now enter the cytosolic fatty acid synthetase (FAS) complex where, by consecutive cycles of condensation with acetyl-CoA, is elongated until 14 (myristic acid) or 16 (palmitic acid) atoms of carbon, depending on the species (Rossi et al., 2010).

Once fatty acyl chains have been produced, they are released by FAS complex and are transported to the ER membrane where they undergo further modifications, such as elongation and desaturation and finally, esterification with glycerol or sterols to produce TAG and steryl-esters, respectively. TAG and steryl-esters are now ready to be stored inside specialized compartments called lipid bodies (LB) or lipid droplets.

LB biogenesis is a topic still under study, but the most shared model (shown in Fig. 2.2), predicts that neutral lipids (composed in fungi by 90% of TAG and 10% of steryl-esters) start to accumulate between the two membrane leaflets of the ER in some specialized domains where most lipid biosynthetic enzymes and structural proteins are also located (Hashemi and Goodman, 2015). As lipids accumulate, a protrusion is formed between phospholipid bilayers and structural proteins, called PAT (from the initials of perilipin, adipocyte differentiation-related protein, 67 and TIP47) are recruited in the outer leaflet. When the protrusion reaches a limit size and the phospholipid bilayer lose stability, the outer layer buds off to give origin to a LB (Garay et al., 2014).

LBs are not simply passive lipid containers; conversely, they are highly dynamic specialized compartments where a continuous turnover of lipids occurs, depending on the needs of the cell. As lipid accumulation/degradation proceed, LB can increase/decrease in size by means of different possible mechanisms, as shown in Fig. 2.3.

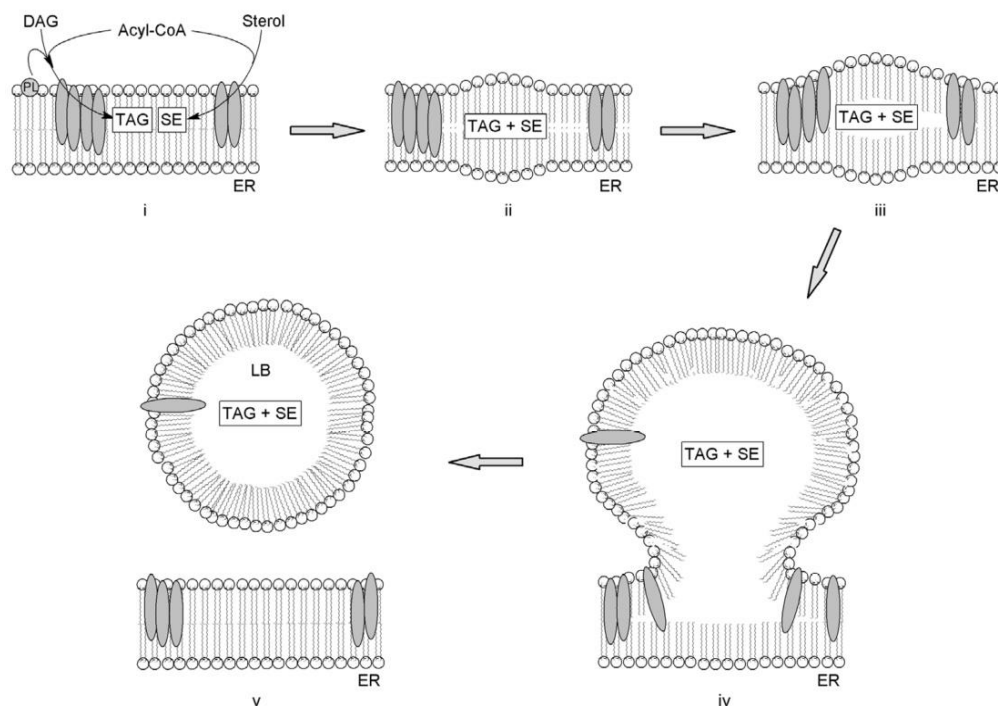


Figure 2.2. Model of lipid bodies biogenesis from the membrane of the ER. TAG and SE accumulate between the two leaflets of the phospholipid bilayer (i to iii). The micro-droplet generated (iii, iv) evolve to lipid bodies (v). From Rossi et al. (2010).

On LB membrane, apart from structural proteins, there are also many proteins involved in lipid metabolism, such as diacylglycerol acyltransferases catalysing, directly in situ, the incorporation of Acyl-CoA into diacylglycerol to form triglycerides (Fujimoto et al., 2008). Live imaging has demonstrated that fusion between LBs is another possible way, with a similar mechanism to vesicular fusion (Boström et al., 2007).

Another possible mechanisms include the formation of bridges between endoplasmic reticulum and LB, which have recently been visualized (Wilfling et al., 2013) or a communication between the two compartments thanks to a continuous physical contact between them, or the presence of specific soluble carriers for the transport of lipids (Uittenbogaard et al., 2013). Alternatively, the existence of repeated cycle of fusion and fission has also been proposed (Gibbons et al., 2000).

2.4.3 Factors influencing lipid accumulation

The type and amount of lipids that a microorganism can produce depend both on the microorganism itself and on the conditions used for cultivation.

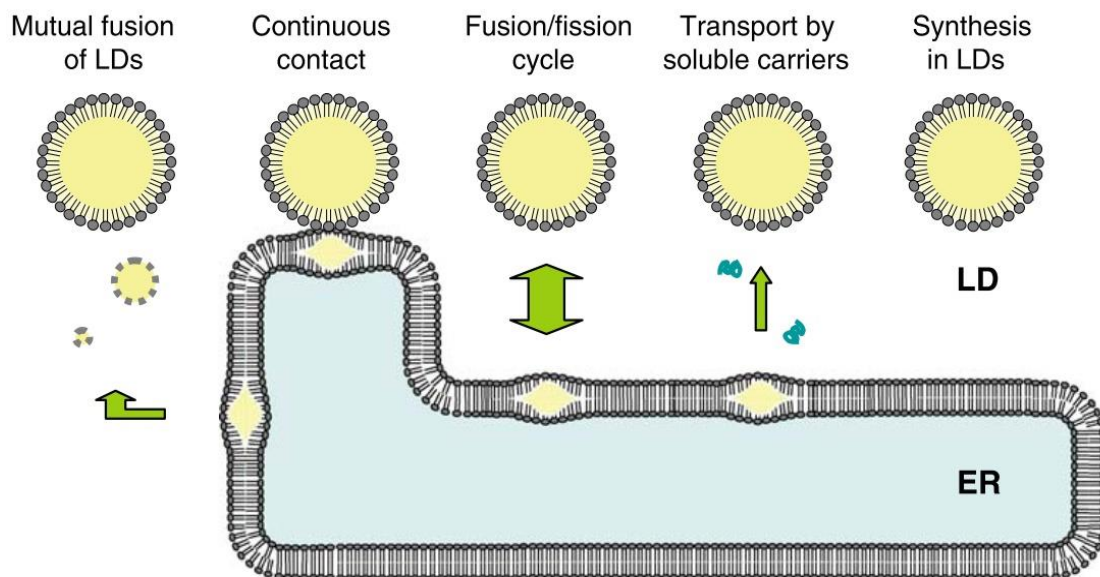


Figure 2.3. Possible mechanisms of lipids exchange between endoplasmic reticulum (ER) and LB, allowing turnover of lipids. From Fujimoto et al. (2008).

Among the conditions, the most influential and cited ones include carbon and nitrogen source and relative concentration, pH, temperature, aeration and feeding strategies, albeit it is not possible to generalize for all the microorganisms, because each of them can be influenced, or not, by the change of these factors.

2.4.3.1 Carbon source type and concentration

A wide variety of carbon sources have been tested for the production of microbial oils and it has been seen that not only the nature, but also the concentration, can influence biomass growth and the quality and quantity of lipids produced (Donot et al., 2014).

By stoichiometric calculations, the maximum theoretical yields of lipids and biomass obtainable on different substrate have been calculated, as reported in Tab. 2.1 (Bommareddy et al., 2015; Papanikolaou and Aggelis, 2011a; Wang et al., 2015). Such theoretical yields, however, only serve to have an idea of the maximum yield obtainable for each substrate, but experimental studies have shown that it is not possible to predict the actual behaviour of each microorganism without testing it.

Studies on *Cunninghamella echinulata* and *Mortierella isabellina* on different sugar-based media, such as glucose, sucrose and fructose, which apparently should lead to similar yields, have shown how much the carbon source used can influence the final result (Chatzifragkou et al., 2010).

Table 2.1. Maximum theoretical yields of TAG and cell mass on different substrates. Cell mass yield is based on the empirical molecular formula for a unit of yeast cell mass $C_6H_{10}NO_3$ (Cooney et al., 1977), thus maximum cell mass yield expressed as $[g\ g^{-1}\ substrate]$ is 0.79 times the value expressed as $[C\ mol/ Cmol\ substrate]$

Carbon source	Maximum yield of TAG [g g ⁻¹ substrate]	Maximum yield of TAG [C mol/ Cmol substrate]	Maximum cell mass yield [C mol/ Cmol substrate]
Glucose	0.30	0.58	0.67
Glycerol	0.32	0.63	0.73
Xylose	0.34	0.64	0.77
Arabinose	0.27	0.53	0.63
Galacturonate	0.26	0.51	0.61

In this set of experiments, for example, sucrose led to a significantly higher lipid-free biomass production in *C. echinulata* (19.6 g L⁻¹, compared to 16.7 g L⁻¹ on fructose and 12.9 g L⁻¹ on glucose), while glucose was the most effective sugar for *M. isabellina* (13.2 g L⁻¹, compared to 5.2 g L⁻¹ on sucrose and 12.1 g L⁻¹ on fructose). Furthermore, while the volumetric concentration of lipids in *C. echinulata* was similar across all three substrates (between 3.1 and 3.9 g L⁻¹), in *M. isabellina* glucose resulted in 9.9 g L⁻¹, fructose in 7.4 g L⁻¹ and sucrose in only 0.5 g L⁻¹ of lipids produced.

Another study conducted on *R. toruloides* on different carbon sources, namely glucose, arabinose and xylose, showed how the yield obtained on a mixed substrate containing all three sugars was lower than expected by the results obtained by fermentation on individual sugars (Wiebe et al., 2012).

Another carbon source widely used is glycerol, and looking at theoretical yields, it seems an even more promising substrate than the expensive glucose. However, experimental studies have shown how in the reality, yields are much variable depending on the strain used. *C. curvatus* ATCC 20509 was able to effectively convert it, reaching a high cell density of 118 g L⁻¹ in bioreactor, accompanied by 25% of intracellular lipids (Meesters et al., 1996), while *R. glutinis*, even with optimized conditions, did not achieve a lipid yield higher than 0.067 (g g⁻¹ glycerol) (Saenge et al., 2011b). A study on different strains of *Y. lipolytica*, which should be one of the most suitable species to be grown on glycerol, ranged between 0.004 a 0.1 (g g⁻¹ glycerol) lipid yield, thus showing that there is a different response also among strains of the same species (André et al., 2009).

The number of examples that can be reported is really extensive, but it is already clear how much variability of response exists, which means that beyond estimations and calculations, it is always necessary to test a strain to understand its real capacity.

2.4.3.2 Nitrogen source and concentration

As said previously, lipid accumulation is induced by the lack of a key nutrient, usually represented by nitrogen.

In *de novo* synthesis, the concentration of initial nitrogen is usually related to the biomass produced during the first exponential growth phase (Papanikolaou and Aggelis, 2011a).

Several nitrogen sources, both organic and inorganic, have been tested until now, including $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , $(\text{NH}_4)_2\text{HPO}_4$, NH_4NO_3 , NaNO_3 , urea, yeast extract, peptone, L-arginine, corn gluten, corn steep, whey concentrate and tomato waste hydrolysate (Donot et al., 2014). In a study conducted on 17 OY species to compare the effect of different organic (glutamate, urea or arginine) and inorganic (NH_4Cl) nitrogen sources, only strains of *R. toruloides* (CBS 14, IFO0559, CBS 6016) and *Trichosporon cutaneum* showed a preference for organic nitrogen with a significant increase in intracellular lipid content. All the other species, including *Candida curvata* D, *Candida utilis* NCYC 359, *Lipomyces lipofer* CBS 944 and CBS 5482, *Lipomyces starkeyi* CBS 1807, CBS 1809 and CBS 6047, *R. glutinis* NCYC 59, *Rhodotorula graminis* CBS 5811 and NCYC 502, instead, showed no significant differences among different nitrogen sources. The explanation of a different response with *R. toruloides* was specifically studied in the case of glutamate, suggesting a higher lipid accumulation as a result of the induction of a glutamate dehydrogenase by glutamate itself, which would lead to a concentration of intracellular NH_4 higher than that one obtained through the metabolism of NH_4Cl (Evans and Ratledge, 1984).

In another study on *C. echinulata*, the comparison of several inorganic sources (NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{HPO}_4$, NH_4NO_3 , NaNO_3) with organic sources (lysine, urea, alanine, glutamine, glutamate, a mixture of amino acids and corn steep) demonstrated that, although inorganic N-sources resulted in higher growth, organic sources led to increased oil accumulation (Certik et al., 1999). Similarly, other fermentations experiments on *C. echinulata* proved how organic nitrogen from tomato waste hydrolysate could improve the uptake of glucose and lead to a higher lipid and biomass production, indicating a close interrelation between the metabolism of C and N. However, when glucose was substituted with glycerol, no differences were detected between organic and inorganic nitrogen (Fakas et al., 2008).

Since nowadays agro-industrial waste are among the most investigated substrates, nature and concentration of the carbon and nitrogen source should be investigated case by case, making a first feasibility evaluation based on literature, and in any case testing experimentally as growth medium in order to have an idea of its suitability.

2.4.3.3 Carbon/nitrogen ratio

The carbon to nitrogen ratio (C/N) in the growth medium is considered as the most influencing factor for lipid accumulation (Moreton, 1988).

In fact, C/N ratio must be such that, in the first exponential phase of growth, carbon and nitrogen may sustain a fast and abundant biomass increase and in a second stage, after exhaustion of nitrogen, carbon may be sufficient for the production of lipids. For this reason, an optimal C/N ratio should be at least equal 20 to effectively induce lipid accumulation (Papanikolaou and Aggelis, 2011b).

However, it has also been seen that an optimum C/N ratio cannot be defined *a priori*, but it depends on both the microorganism and the specific nitrogen and carbon sources used.

In *R. toruloides* Y4, when grown on glucose as carbon source and $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source, a C/N ratio equal to 70 seemed to be a good compromise between growth and lipid accumulation, with lower ratios leading to lower lipid accumulation and higher ratios causing poor growth and incomplete use of the carbon source (Zhao et al., 2011).

In *L. starkeyi* DSM 70296, through fed-batch experiments on a mixed glucose-xylose medium, the relationship between cell growth and lipid content at constant (50) and increasing C/N ratios from 50 to 900 was evaluated. The results showed an increased cell and lipid productivity at increasing C/N ratios, but a higher lipid content percentage when C/N ratio was kept constant, suggesting that there is a fine balance between growth and lipid accumulation (Anschau et al., 2014). Likewise, *L. starkeyi* grown on digested sewage sludge achieved a lipid content of 68% (w/w) at a C/N ratio of 150 compared to 40% in the presence of a C/N ratio of 60 (Angerbauer et al., 2008).

A C/N ratio of about 50 was required instead for *C. curvatus* in batch and fed batch fermentations when grown on glucose (Hassan et al., 1996), of 70 when grown on glycerol (Meesters et al., 1996) and of 35 when grown on the organic waste from the brewery industry (Ryu et al., 2013).

As it can be seen, while for some microorganisms very high C/N are required, for others, less stringent conditions are needed. In fact, the formulation of a growth medium with high C/N, does not give the certainty of obtaining a high lipid accumulation but, on the contrary,

it can lead to significant quantities of unconsumed sugars, without tendency of being consumed (Papanikolaou and Aggelis, 2011b).

These studies have highlighted how lipid accumulation is critically influenced by the specific sugar uptake rate (q_S) inside the cells which doesn't depend only on the nature of the carbon source, but also of the nitrogen source, as seen in the previous paragraph for *C. echinulata* grown on tomato waste hydrolysate (Fakas et al., 2008). It has been observed that decreased q_S values result in increased total biomass (g L^{-1}) and total biomass yield (g g^{-1} substrate) values, with concomitant lower lipid production. This can be explained thinking that, when the sugar is rapidly assimilated by the microorganism, the intracellular C/N ratio is perceived as higher compared with cultures presenting low q_S values (Papanikolaou and Aggelis, 2011b). This is why apparently similar experiments for carbon source and conditions used can lead to significant differences. Therefore, it can be concluded that, when different combinations of carbon and nitrogen sources are used, the evaluation of C/N ratio is not sufficient to understand if a substrate is suitable for lipid accumulation, but the actual availability and rate of uptake of each nutrient must be individually evaluated.

2.4.3.4 Cultivation strategies

Feeding strategy is a factor that deeply affects fermentation kinetics. Three different modes of culture are commonly used: batch, fed-batch and continuous mode.

Usually, batch cultivation is the first approach to study the behaviour of a microorganism on a certain substrate, since, with respect to other strategies, it depends on less variables. It allows to investigate how different nutrients are metabolized, to determine the most favourable growth conditions in terms of pH, aeration, temperature, agitation and dissolved oxygen. However, when the purpose of the fermentation is the production of a specific compound, batch operation is more suitable for products deriving from primary anabolic processes, while fed-batch or continuous cultivation for products deriving from secondary metabolism. Talking about *de novo* lipid synthesis, it is known that the concentration of intracellular lipids is dependent on the biomass produced during the exponential phase which in turn depends on the initial concentration of carbon source in the growth medium. In this regard, several issues can arise. A high concentration of carbon source is needed to sustain a high biomass and lipid production but, if excessive, it may be inhibitory for the microorganism. Furthermore, optimum conditions for growth may be different from those required for lipid accumulation (Beopoulos et al., 2009).

In fed-batch cultivation the batch is prolonged by feeding of nutrients along the time, thus overcoming substrate inhibition or catabolite repression. This strategy has proved to be, for

most of the microorganisms investigated, the most effective in increasing both cell density and intracellular lipids. The possibility of changing the composition of the feeding, in fact, may allow to reach, in a first phase, a high biomass through a medium containing a balanced amount of nitrogen and carbon source and, in a second phase, lipid accumulation through a continuous supply of carbon but limited nitrogen. In different cases, this strategy has led to impressive yields, with high cell density and significant lipid content (Christophe et al., 2012).

In *R. toruloides* Y4, batch cultivations on glucose showed a repression when the sugar was over 90 g L⁻¹, resulting, for this concentration, in 18.6 g L⁻¹ of biomass and 8.63 g L⁻¹ of lipids after 120 h of fermentation. Fed-batch mode, starting with 60 g L⁻¹ of glucose in the medium and continuing with 4 repeated feeding of a glucose solution without any addition of nitrogen, significantly improved productions, resulting in 106.5 g L⁻¹ of dry biomass and 67.5% (w/w) of lipid content after only 134 hours (Li et al., 2007). Lorenz et al. (2017) developed with *R. glutinis* a two-stage fed-batch process, split into a growth phase and lipogenesis phase. The first one was carried out under nutrient rich conditions, with a feeding solution containing 400 g L⁻¹ of glucose and a C/N equal to 15 that was added to the initial medium. During the lipogenesis phase, a highly concentrated sucrose solution (800 g L⁻¹) without any addition of nitrogen was fed. Final yields were 106 g L⁻¹ of biomass and 63% of lipid content, associated to a high lipid-substrate yield of 0.18 g g⁻¹ in only 84 h.

Another study on *L. starkeyi* DSM 70296 aimed at assessing the feasibility of using hemicellulose hydrolysate as growth substrate, compared batch, fed-batch, repeated fed-batch and continuous cultures productivity. Highest productivity was achieved by fed-batch, resulting in 82.4 g L⁻¹ of biomass and a lipid content of 38.6 g L⁻¹ (46.9%) with a biomass productivity and lipid productivity of 0.597 and 0.280 g L⁻¹ h⁻¹ respectively. In comparison, batch mode resulted in higher biomass productivity (0.704 g L⁻¹ h⁻¹) but lower lipid productivity (0.224 g L⁻¹ h⁻¹) and with a final biomass concentration of about 35 g L⁻¹, due to the fact that, in batch mode, too high concentrations of sugars may inhibit growth. In continuous culture, yields were similar to those ones obtained in fed-batch, demonstrating that for this strain, both fed-batch and continuous cultivation modes can be suitable for further development (Anschau et al., 2014).

In fed-batch cultivation, feeding can be added through 2 different strategies: intermittent or continuous one. In the first case, a certain amount of medium is added at regular time intervals or when some nutrient decreases below a certain threshold. This is the case of *R.*

toruloides Y4 fed-batch fermentation mentioned above, where substrate concentration was always maintained above 20 g L⁻¹ by adding feeding medium (Li et al., 2007).

When feeding is added in continuous way, usually peristaltic pumps are used and, as for continuous cultivation, a feed flow rate must be determined in order not to have a situation of nutrient accumulation or deficiency. An example of fermentation kinetics with intermittent or continuous feeding is represented in Fig. 2.4.

A comparison between intermittent and continuous feeding is rarely discussed and it is difficult to compare fermentations from different publications as the variables are numerous.

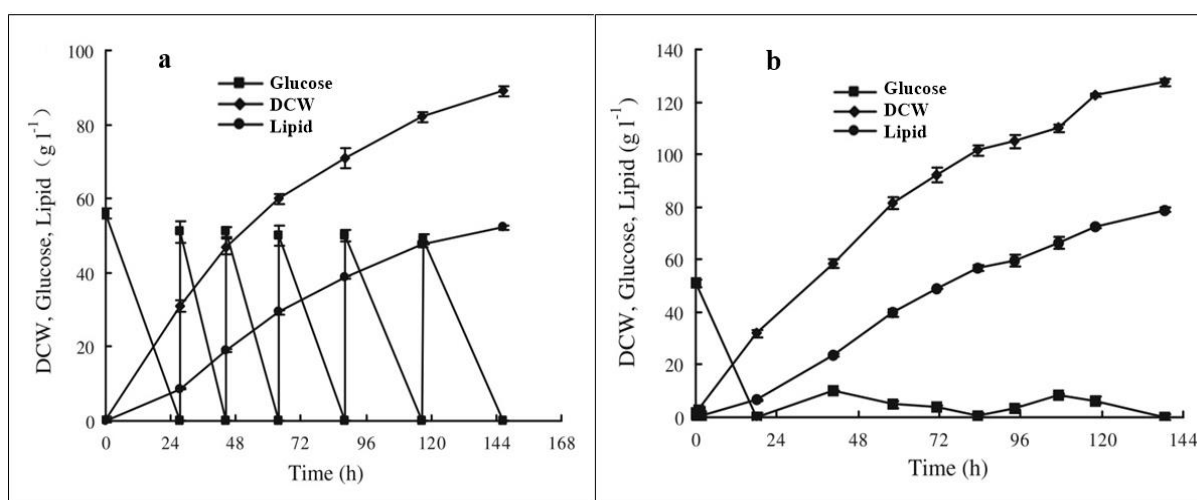


Figure 2.4. Example of fermentation kinetics of glucose consumption, dry cell weight (DCW) production and lipid accumulation in intermittent (a) and continuous (b) fed-batch feeding. From Zhao et al. (2011).

Lorenz et al. (2017) tried to answer to this question, finding out that in *R. glutinis* there were no differences in lipid content and biomass production between different feeding modes, but there was a higher accumulation of glycerol as by-product of lipid metabolism when feeding was intermittent.

An important parameter to take into consideration in continuous fed-batch feeding, as mentioned above, is feeding flow rate, usually expressed as dilution rate (*D*) that is the flow of medium per time, over the volume of culture in the bioreactor.

D value lower than 0.06 h⁻¹ is normally required for optimum conversions. Usually, at high *D*, the higher concentration of available sugars per time results in an increased biomass yield but a decreased lipid production (Papanikolaou and Aggelis, 2011b). For low *D* and consequent lower specific growth rates with intermediate C/N ratios, lipid and biomass concentrations obtained are higher than those obtained with higher *D* (Christophe et al., 2012). Therefore, the optimization of the process involves determination of the optimal *D* with an optimal intermediate C/N ratio.

2.4.3.5 Other factors (pH, temperature, inoculum density, dissolved oxygen concentration)

Other cultivation conditions such as dissolved oxygen, temperature, inoculum density and pH can also influence cell growth and oil accumulation, although, also in this case, parameters must be optimized for each strain.

In general, oxygen concentration in the culture medium is directly correlated with both accumulation and degree of unsaturation of fatty acids produced (Li et al., 2008). Fermentations on *R. glutinis* demonstrated that high dissolved oxygen concentration could improve cell density and lipid accumulation from 20 to 40% and from 110 g L⁻¹ to 185 g L⁻¹, respectively (Pan et al., 1986). In *Rhodotorula gracilis*, faster growth rates were observed increasing aeration, but without any change in lipid production rate, thus leading to a decreasing percentage in lipid content. Furthermore, over 234 µM, high oxygenation also increased PUFAs (C18:2, C 18:3) over other fatty acids (C16:0, C18:0, C18:1)(Choi et al., 1982), probably due to the dependence of desaturases from oxygen (Meesapyodsuk and Qiu, 2012). Similarly, in *C. curvatus*, an increase in fatty acid unsaturation and decrease in lipid yield on substrate was observed in low oxygen conditions (Davies et al., 1990). In other works done on *R. toruloides*, it was observed that, rather than a control on agitation rate, a constant oxygen saturation (50%) led to an oil composed for 90% of oleic and palmitic suitable for biodiesel production, while a constant aeration rate at 1.5 vvm led to a high percentage of SFAs, similar to cocoa butter composition. (Bommareddy et al., 2015).

Medium pH is a less investigated factor. Usually, pH-controlled fermentations are performed in a range varying between 5 and 6, that are typical values used for yeast cultivation. However, some yeasts can be influenced more than others by pH variation, also resulting in significant different yields. This is the case of *R. glutinis* IIP30, which, grown on glucose-based medium, achieved 66% lipid content (w/w) on dry weight at pH 4 and 12, 48 and 44%, at pH 3, 5, 6, respectively, albeit with a cell lipid fatty acid composition substantially unchanged (Johnson et al., 1992). Studies on *C. curvatus*, instead, produced different results depending on the substrate used for growth. When cultivated on whey, it was not affected by variations of pH in a range between 3.5 and 5.5, resulting in similar yields (Papanikolaou and Aggelis, 2011b). On the other hand, when *C. curvatus* was cultivated on the organic waste of brewery industry at various pHs (4.8, 5.8, and 6.8) differences on biomass and lipid productivities were observed. Highest lipid productivity was achieved at pH 5.8 while highest cell density at pH 6.8 (Ryu et al., 2013).

Zhu and collaborators (2008) observed that *Trichosporon fermentans* could grow on a wide range of pHs, from 4 to 10 with an optimum at 6.5. Although with differences in growth rate,

intracellular percentage content was always higher than 52% and a maximum value of 62% was reached at pH 6.5. On *R. toruloides* NCYC 921, a dual-stage pH control fed-batch cultivation strategy was tested: when the growth phase was conducted at pH 4.0 and lipid accumulation phase at pH 5.0, biomass, total fatty acid and productivities were significantly improved comparing with fed batch cultivations carried out at fixed medium pH (4 or 5) (Dias et al., 2015).

There are just few studies investigating the effect of temperature on lipid accumulation. Zhang et al. (2014) observed that a condition of irradiation and high temperature (30 °C) could favour *R. glutinis* growth, while dark/low-temperature (24 °C) could enhance lipid content. Conversely, in *T. fermentans* a range of temperatures between 20 and 35 °C was tested, showing that 25 °C is the optimal temperature for growth and lipid accumulation, even if only growth rates were significantly affected by temperature, while lipid content was always over 54%, except at 35 °C where it was significantly lower (Zhu et al., 2008). However, unlike the previous work where two differentiated temperatures were used for different fermentation phases, in this case the work is summarized in the simple search for optimum growth temperature, without being closely related to lipid accumulation metabolism. The parameter that is more affected by temperature, instead, is lipid composition since several desaturases are up-regulated at low temperatures as adaptation strategy (Aguilar and De Mendoza, 2006; Bellou et al., 2012; Kamisaka et al., 2014; Michinaka et al., 2003).

2.5 Fatty acid composition in yeasts and moulds

In oleaginous fungi, TAGs and sterol esters are the primary neutral lipids accumulated inside LBs (Garay et al., 2014). Although fatty acids in microbial lipids range from lauric (C12:0) to docosaheptaenoic acid (C22:6), palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids are usually the most represented, at various extent, within the same fermentation over time (Subramaniam et al., 2010).

Some examples of typical microbial and plant oil compositions are listed in Tab. 2.2, even if it must be taken into account that the conditions of growth can influence significantly the fatty acid composition as well as the saturation degree (Bellou et al., 2016; Zhang et al., 2014; Zhu et al., 2008).

Table 2.2. Examples of fatty acid profiles of oleaginous yeasts and moulds, compared to plant oils and animal fats.

	Major fatty acids (%)						
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	References
Yeasts							
<i>C. curvatus</i>	32	1	8	48	9	-	(Thiru et al., 2011)
<i>C. curvatus</i>	18	Traces	16	50	16	-	(Meesters et al., 1996)
<i>Cryptococcus terricola</i>	3	3	5	73	14	1	(Tanimura et al., 2014)
<i>L. starkeyi</i>	34	6	5	51	3	0.12	(Ratledge and Wynn, 2002)
<i>L. starkeyi</i>	36	6	10	41.5	2.5	-	(Wang et al., 2014)
<i>R. glutinis</i>	17	1	4	46	18	4	(Saenge et al., 2011b)
<i>R. graminis</i>	21	-	7	42	17	3	(Galafassi et al., 2012)
<i>R. toruloides</i>	20	1	15	47	13	3.5	(Li et al., 2007)
Moulds							
<i>C. echinulata</i>	13	1	2	45.5	16	19.5	(Chatzifragkou et al., 2010)
<i>C. echinulata</i>	19	1.5	9	35	18.5	15	(Bellou et al., 2012)
<i>Mortierella alpina</i>	19	-	8	28	9	8	(Ratledge and Wynn, 2002)
<i>M. isabellina</i>	20-27	1-4	2-6	44-54	4-18	3-8	(Ratledge and Wynn, 2002)
<i>M. isabellina</i>	28	3	3	53	7	3	(Chatzifragkou et al., 2010)
<i>Mortierella ramanniana</i>	21	1	6	49	16	4	(Bellou et al., 2012)
<i>M. circinelloides</i>	22	1	5	38	10	15	(Ratledge and Wynn, 2002)
<i>Mucor moelleri</i>	20.5	2	7	40	11	18	(Bellou et al., 2012)
Plant oils							
Palm	32-59	-	1-8	27-52	5-14	Traces	(Christophe et al., 2012)
Sunflower	3-10	-	1-10	14-65	20-75	Traces	(Christophe et al., 2012)
Soya	7-14	-	1-6	19-30	44-62	4-11	(Christophe et al., 2012)
Rapeseed	3-14	-	0.9-2	56-64	22-26	8-10	(Christophe et al., 2012)
Corn	12	-	0.9	25	61	0.48	(Christophe et al., 2012)
Animal fats							
Tallow	24-32	-	20-25	37-43	2-3	-	(Canakci and Sanli, 2008)
Yellow grease	23	4	13	44	7	1	(Canakci and Sanli, 2008)
Brown grease	23	3	12.5	43	12	1	(Canakci and Sanli, 2008)

On the basis of data shown in Tab. 2.2 (e.g. *C. echinulata*, *M. alpina*, *M. circinelloides* and *M. moelleri*) moulds tend to produce a greater amount of PUFAs and have received, therefore, a greater attention from the nutraceutical industry. Yeasts, instead, even without producing high value added fatty acids could be used as alternative substrates for the biodiesel and oleochemical industry, thanks to their fatty acid composition. Moreover, they usually have the advantage of having shorter production times than moulds and of being simple to cultivate.

2.5.1 Biodiesel

Biodiesel, a mixture of FAMES currently derived for the 95% from vegetable oils, has become an attractive alternative source of energy thanks to its environmental benefits: it is renewable and biodegradable, it contributes no net carbon dioxide or sulphur to the atmosphere and emits less gaseous pollutants than normal diesel (Gui et al., 2008).

Biodiesel is obtained from extracted oils or directly on cell biomass, by acid transesterification, using a strong acid such as HCl or H₂SO₄ or by alkaline esterification, using NaOH or KOH, together with an alcohol that can be methanol or ethanol. Base-catalyzed transesterification is the most commonly used method in commercial biodiesel production processes due to its cost-effectiveness, as the reaction is carried out under low temperature and pressure environment, at a high conversion rate with no intermediate steps. Acid-catalyzed esterification, instead, is less used due to the slow reaction rate, need for high temperatures, pressure conditions and higher solvent to oil molar ratio. The only problem with base-catalyzed transesterification is that, if a high content of free fatty acids (FFAs) is present, the reaction of the latter with the alkaline catalysis can lead to the formation of soaps which reduce biodiesel yield (Thliveros et al., 2014).

Alternatively, methods based on enzymatic reactions involving the use of lipases have also been investigated which constitute, undoubtedly, a more environmentally sustainable alternative, though more expensive (Hama and Kondo, 2013).

Various specifications that a biodiesel fuel must fulfil are described in biodiesel standards, such as the American Society for Testing and Materials (ASTM) D6751, in the United States and the EN 14214, in Europe (Tab 2.3). While part of these specifications depends on fuel quality issues, such as completeness of the transesterification reaction or storage conditions, several parameters are directly influenced by the fatty acid composition of the oil used. The latter include cetane number, kinematic viscosity, oxidative stability, and low-temperatures properties such as the cloud point or cold-filter plugging point (Knothe, 2008).

The most common oils actually used for biodiesel production include: i) edible vegetable oils, such as rapeseed, soybean, peanut, coconut and palm oil; ii) non-edible vegetable oils such as jatropha and Karanja; iii) waste or recycled oils such as cooking and frying oil and animal fats such as tallow, yellow grease, chicken fat and by-products from fish oil. These oils include, though in different proportions, mainly palmitic, stearic, oleic, linoleic and linolenic acid (Atabani et al., 2012) which are also the same fatty acids produced by oleaginous fungi.

Although the most commonly used feedstock are vegetable oils, the growing debate about the use of edible oils and arable land for biofuel production and the rising price of oil have led to the need of seeking for alternative sources. Animal waste and recycled oils partially respond to this need, but they are not in sufficient quantities to support this growing market. Adoption of animal fats, used frying oils and waste oils from restaurants as feedstock is a good strategy to reduce the cost. However, these limited resources cannot meet realistic needs for clean renewable fuels (Christophe et al., 2012). In this context, yeast oils could be a valuable alternative to vegetable oils, since they would fulfil these requirements, with the important advantage of being independent on season and climate factors, having a short process cycle and being easy to handle and scale up (Ageitos et al., 2011).

Due to the antagonistic effect of certain FAME compositional upon fuel properties, it is not possible to define a specific fatty acid composition that is optimum with respect to all important properties. The most important variables to take into consideration to assess the suitability of an oil are, as mentioned above, the cetane number, viscosity, cold flow, oxidative stability, and lubricity.

Tab. 2.3. European Biodiesel Standards EN 14214 for Vehicle Use and EN 14213 for Heating Oil Use. From Knothe (2006).

Property	Test method	Limits		Unit
		EN 14214	EN 14213	
Ester content	EN 14103	96.5 min	96.5 min	% (mol/mol)
Density; 15°C	EN ISO 3675, EN ISO 12185	860–900	860–900	kg/m ³
Viscosity; 40°C	EN ISO 3104, ISO 3105	3.5–5.0	3.5–5.0	mm ² /s
Flash point	EN ISO 3679	120 min	120 min	°C
Sulfur content	EN ISO 20846; EN ISO 20884	10.0 max	10.0 max	mg/kg
Carbon residue (10% distillation residue)	EN ISO 10370	0.30 max	0.30 max	% (mol/mol)
Cetane number	EN ISO 5165	51 min	—	
Sulfated ash	ISO 3987	0.02 max	0.02 max	% (mol/mol)
Water content	EN ISO 12937	500 max	500 max	mg/kg
Total contamination	EN 12662	24 max	24 max	mg/kg
Copper strip corrosion (3h, 50°C)	EN ISO 2160	1	—	degree of corrosion
Oxidative stability, 110°C	EN 14112	6.0 min	4.0 h	h
Acid value	EN 14104	0.50 max	0.50 max	mg KOH/g
Iodine value	EN 14111	120 max	130 max	g I ₂ /100 g
Linolenic acid content	EN 14103	12.0 max	—	% (mol/mol)
Content of FAME with ≥4 double bonds		1 max	1 max	% (mol/mol)
Methanol content	EN 14110	0.20 max	—	% (mol/mol)
MAG content	EN 14105	0.80 max	0.80 max	% (mol/mol)
DAG content	EN 14105	0.20 max	0.20 max	% (mol/mol)
TAG content	EN 14105	0.20 max	0.20 max	% (mol/mol)
Free glycerine	EN 14105, EN 14106	0.020 max	0.02 max	% (mol/mol)
Total glycerine	EN 14105	0.25 max	—	% (mol/mol)
Group I metals (Na + K)	EN 14108, EN 14109	5.0 max	—	mg/kg
Group II (Ca + Mg)	prEN 14538	5.0 max	—	mg/kg
Phosphorus content	EN 14107	10.0 max	—	mg/kg
Cold filter plugging point	EN 116	—	—	°C
Pour point	ISO 3016	—	0 max	°C
Heating value	DIN 51900-1			
	DIN 51900-2			
	DIN 51900-2	—	35 min	MJ/kg

Of these five, the two properties that are generally considered as most critical and are more affected by fatty acid composition are cold flow and oxidative stability. Several researchers have investigated the influence of different fatty acids on these two properties and, in principle, a lipid profile can be assumed as suitable if it has got relatively low levels of SFAs (to minimize cold flow problems), low levels of PUFAs (to minimize oxidative instability), and high levels of MUFAs (Hoekman et al., 2012). Since also chain length is an important parameter, some other studies have concluded that palmitoleic acid and oleic acid provide the best compromise between oxidative stability and cold flow, without excessive reduction of cetane number (Knothe, 2008).

As it can be seen in Tab. 2.2, most of OYs can produce high amounts of oleic acid, between 40 and 50% of total fatty acids produced, proving to be promising feedstock for biodiesel production. Palmitoleic acid, instead, is poorly found in nature, as can be also observed from the composition of vegetable oils.

In order to modify the lipid composition of a microorganism, different approaches can be investigated, including the cultivation under different conditions (e.g. lower temperature), or the modification, through metabolic engineering of some of the enzymes involved in lipid accumulation, such as desaturases.

2.5.2 Pharmaceutical and nutraceutical industry

Since the beginning of studies on microbial oils, moulds have always attracted a great interest in the nutraceutical sector thanks to their ability to produce significant amounts of PUFAs, such as GLA and ARA (Subramaniam et al., 2010).

PUFAs are involved in many different physiological functions in the human body not only as structural components of membrane phospholipids but also as precursors of the eicosanoids. Eicosanoids are hormone-like substances involved in the cardiovascular system, the immune system, the central nervous system, the brain and in inflammatory reactions (Ochsenreither et al., 2016).

DHA, an omega-3 fatty acid, is a major structural component of the grey matter of the brain and the eye retina and an important component of heart tissue. For this reason, DHA is also used as supplement in infant formulas for proper development of the brain and eye. ARA, an omega-6 fatty acid, is the most abundant PUFA in humans, present mainly associated with membrane phospholipids in organs, muscle and blood tissue. ARA, together with DHA, are important in brain development of infants. ARA is also a direct precursor of a number of eicosanoids regulating lipoprotein metabolism, blood rheology, leucocyte function and platelet activation (Ward and Singh, 2005).

Several strains belonging to *Mortierella* genus are excellent producers of oils containing high concentration of PUFAs. The fungal genus *Mortierella* is divided in two subgenera: *Mortierella* (*M. alpina*, *Mortierella hyaline*, *Mortierella elongata*) and *Micromucor* (*M. isabellina*, *Mortierella ramanniana*, *Mortierella vinacea*) (Asadi et al., 2015).

Species belonging to the subgenus *Micromucor* accumulate high proportions of linoleic acid (up to 25%) and GLA (up to 31%). Among them, *M. isabellina* can produce the highest amount of C18 PUFAs and it is also able to reach a high volumetric concentration of biomass and lipids content when cultivated on sugar-based media (Asadi et al., 2015; Chatzifragkou et al., 2010; Papanikolaou et al., 2007b).

The best producer of C20 PUFAs, *M. alpina*, belongs instead to *Mortierella* subgenus and it is able to produce remarkable concentrations of ARA. As well as *M. isabellina*, even *M. alpina* can grow on various carbon sources. Some examples include cultivation on glucose and yeast extract, resulting in 10.3 g of ARA per 100 g of dry biomass and 38% lipid content in 10 days of fermentation, or cultivation on soluble starch as carbon source achieving 14.3 g ARA per 100 g of dry biomass and 66.4% of total PUFAs (Stressler et al., 2013).

Furthermore, several *M. alpina* strains are already commonly used for commercial production of ARA rich single-cell oil, such as the ARASCO product, which contains 40-50% ARA and minimal amounts of other long chain PUFAs (Asadi et al., 2015).

In addition to these remarkable results, other promising fermentation processes were set up by Hiruta et al. (1997) with *M. ramanniana*, reaching 31.3 g L⁻¹ of lipids containing 5.6 g L⁻¹ of ARA on glucose, or *C. echinulata* producing 47% of intracellular lipids, composed for the 14% by GLA when grown on glucose (Gema et al., 2002) or *M. circinelloides* whose oil rich in GLA was commercialized in 1985 (Ratledge, 2004).

2.5.3 Oleochemical industry

Basic oleochemicals include fatty acids, fatty alcohols and methyl ester which can have various applications in different industries, such as the production of soaps, detergents, cosmetic additives, pheromones and flavors in place of vegetable oils or animal fats (Ochsenreither et al., 2016). Moreover, glycerol obtained as by-product by the hydrolysis of TAG could be recycled for the cultivation of OYs themselves or used as cosmetic additive; it could be also converted through green chemical reactions into a wide range of chemicals, such as dichloropropanol, epichlorohydrin, acrolein, 1,3-propanediol, polyhydroxybutyrate and succinic acid (Koutinas et al., 2014).

2.6 Cell disruption and extraction methods

In general, downstream processing costs are one of the factors that most affect the economic feasibility of microbial oil production on an industrial scale. One of the most important steps is cell disruption because efficiency of cell disruption directly influences all subsequent downstream operations and overall extraction efficiencies. A wide variety of cell disruption procedures, involving cold, heat or chemical, enzymatic and mechanical breakage, have been developed. The extraction phase can be performed simultaneously or after cell disruption. Extraction process also depends on lipid application. For example, in food industry, toxic solvent cannot be used in order to prevent solvent residues in food or contaminations with heavy metals (Ochsenreither et al., 2016) and toxic compounds or solvents should be avoided to develop an environmentally sustainable process.

Furthermore, some methods that can give high yields on a laboratory scale may be unsuitable on an industrial scale because too expensive.

2.6.1 Mechanical disruption methods

Cell disruption by mechanical stress can be achieved by cavitation, impingement at high pressure, abrasion or ultrasound. These methods are more suitable for industrial applications because they are easy to control and, compared to enzymes for example, are much less influenced by the type of microorganism under study. Moreover, not requiring any chemicals, they are suitable also for food industry.

The only major disadvantage is that these processes generate very high heat and therefore require cooling systems to avoid damaging heat-sensitive lipids (Lee et al., 2012).

Bead milling, homogenization and ultrasound are the most common used. In bead milling, cells are disrupted by the impact of grinding beads. Disruption efficiency is further depending on bead size and type, agitator velocity, flow rate, cell concentrations, bead loading and microorganism. To recover microbial oils, solvent extraction is generally performed subsequently to the bead milling process (Ochsenreither et al., 2016).

During the homogenization process, biomass is forced under high pressure through an orifice and cell disruption efficiency is dependent on the pressure, number of passes and organisms used (Clarke et al., 2010). It has been successfully used for yeast, algae and bacterial species, while for filamentous fungi it has proved to be unsuitable due to blocking and clogging by mycelium and fungal pellets (Middelberg, 1995). Since homogenization was originally applied to form emulsions, the mixture of water contained in cells with intracellular lipids, can give origin to very stable emulsions which are not easy to break.

Ultrasound, using frequencies around 25 kHz, causes cell disruption through cavitation. However, this process generates high temperatures which need to be lowered and it also promotes radical formation, resulting in chemical reactions which can cause a damage to lipids. To achieve an efficient cell disruption, optimization of sonication time, cell density and power input has to be performed (Ochsenreither et al., 2016).

2.6.2 Non-mechanical Disruption Methods

These methods require less energy with respect to mechanical methods, but they are usually more expensive, thus representing a limitation for industrial application (Klimek-Ochab et al., 2011). They include physical, chemical and enzymatic methods.

Among physical methods, decompression, microwave-treatment, pulsed electrical fields, and freeze-drying have been investigated.

Cell disruption by decompression is performed through the use of pressurized supercritical gas and subsequent release of the pressure. The gas enters the cells, expands upon pressure release and causes cell disruption due to the high pressure. It is a gentle technique, minimizing chemical and physical stresses as well as heat development and has the potential to be applied to larger scales (Ochsenreither et al., 2016). Different gases can be used, such as nitrogen (Lee et al., 2012) or carbon dioxide (Lin et al., 1992, 1991). However, it is not as effective for some of the microbial cells, including yeast, of which the cell walls are extremely robust and rigid (Lin et al., 1992).

Microwaves, by heating and consequent expansion of water, cause the explosion of cells, thus allowing the release of their intracellular content. The combination of microwave treatment with solvent extraction, called microwave-assisted solvent extraction, represents a quick and inexpensive method of lipid extraction, as amounts of solvents can be reduced and yield can be increased compared to a simple thermal treatment (Motasemi and Ani, 2012). Microwaves, being already used in some industrial productions, have proved to be suitable for large scale productions.

Some attempts have been done also with the aim of performing direct transesterification of yeast wet cells to produce biodiesel (Cui and Liang, 2014), performing at the same time cell disruption, transesterification reaction and extraction of FAMES produced, with satisfying results. However, as for ultrasound disruption, a particular attention to the conditions used must be taken, for the development of heat and free-radicals might damage lipids.

Pulse electric field is a method already used for DNA transformation experiments, based on the formation of pores in cell membrane (Lee and Tai, 1999). However, cell suspension must

be free of ions, making washing steps and/or deionization steps, and the release of intracellular compounds - when cell start to break - causes an increase of conductivity which decreases the efficiency of the method (Ochsenreither et al., 2016).

Freeze-drying or simply drying is usually coupled to one of the other methods, because it helps to weaken the cells but is not always highly efficient.

2.6.3 Chemical disruption methods

Chemical disruption methods include a wide variety of compounds, such as antibiotics, chelating agents, chaotropes, detergents, solvents, alkalis, and acids with different selectivity, efficiency and mode on action to the respective cell wall components of different microorganisms. However, many of these treatments cannot be used for food productions.

The use of solvents is probably the most common method used on a laboratory scale and in oleochemical industry, because it allows to simultaneously perform cell breakage and extraction and to recycle part of the solvents used. The first protocols have been developed by Bligh and Dyer (1959) and Folch et al. (1957) but they involved the use of large amounts of organic hazardous solvents, like chloroform and methanol. Therefore, methods using less toxic solvents, like hexane/isopropanol, have been developed (Ochsenreither et al., 2016)

2.6.4 Enzymatic disruption methods

Lytic enzymes have several advantages over other disruption methods, such as mild reaction conditions, less energy demand, substrate specificity, environmental sustainability and safety for food productions. However, due to the high specificity of lytic enzymes, different cocktails need to be optimized for different microorganisms. Some studies have been performed on yeasts, demonstrating that it is a feasible process (Jin et al., 2012). Application on large scale is possible but depends heavily on the costs.

2.6.5 Extraction methods

Extraction can be performed by means of solvents or enzymes. The choice of solvent depends on the polarity of lipids to be extracted. Most common protocols are those ones developed by Bligh and Dyer (1959) and Folch et al. (1957), already mentioned in *Chemical disruption methods* paragraph. In fact, storage lipids, like TAG, are neutral lipids and can be extracted with rather non-polar solvents, such as chloroform or hexane, while more polar lipids, like phospholipids and glycolipids, are dissolved more easily in polar solvents, such as alcohols. From this it follows that, mixtures of solvents, such as methanol and hexane, can enhance the extraction efficiency and separating TAG from other membrane lipids (Atabani et al., 2012).

2.7 Low-cost feedstock for fungal oil production

Until now, the major obstacle for the commercialization of microbial oils in the oleochemical and biodiesel industry, as well as in several cases including the nutraceutical field, has derived from the high production costs, depending mainly by the cost of growth medium and the costs associated with the fermentation process itself (Ageitos et al., 2011).

Koutinas et al. (2014) estimated that the unitary cost of purified microbial oil production from glucose is \$3.4/kg for an annual production capacity of 10000 t microbial oil and zero cost of glucose. If a cost of glucose of \$400/t was considered, the cost of microbial oil production and conversion to biodiesel was estimated at \$5.5/kg oil and \$5.9/kg biodiesel, respectively.

However, they also estimated that, if the cost of production of microbial oil could be halved, then microbial oil could potentially compete with vegetable oils for biodiesel production. In this regard, the use of agro-industrial waste and by-products as carbon sources for OF can undoubtedly be a possible way to lower these costs. The variety of raw materials already investigated include molasses, dairy serum, glycerol, solids from wheat bran fermentation, general wastewaters, wastewaters of animal fat treatment, lignocellulosic substrates, olive oil mill wastewaters and some others studied to a lesser extent, part of which is listed in Tab. 2.4.

The use of complex substrates obviously requires greater effort than using simple sugar-based media, such as glucose or fructose, since it is necessary to investigate the wide microbial diversity in order to find the microorganisms more suitable to grow on that specific medium. As already mentioned, the combination of both substrate and microorganism has an important effect not only on the lipid and biomass yield, but also on fatty acid composition of the produced microbial oil. For this reason, it is important to study how each microorganism responds to the substrate under study, also in terms of the quality of the oil produced and thus of the biodiesel obtained.

Table 2.4. Lipid content and dry cell weight production of different oleaginous species grown on agro-industrial waste

Strain	Lipid content (%)	Biomass (g L ⁻¹)	T (°C)	t (h)	pH	C source	References
<i>Aspergillus oryzae</i>	25	7	30	144	6	Wheat straw (bran, orange peel, apple peel, sugar cane bagasse)	Hui et al., 2010a
<i>C. echinulata</i>	48	18	28	280	6	Tomato waste	Fakas et al., 2008
	28	14	28	120	6	Soluble starch	Papanikolau et al. 2007b
	10	4	28	120	6	Pectin	Papanikolau et al. 2007b
	5	1	28	120	6	Lactose	Papanikolau et al. 2007b
	25	8	28	300	6	Glycerol	Fakas et al., 2009
<i>L. starkeyi</i>	68	9.4	-	-	-	Sewage sludge	Angerbauer et al. 2008
	47	17.2	28	192	6-8	Corn cob	Huang et al., 2014
<i>M. isabellina</i>	12	-	28	212	6	Pear pomace	Fakas et al., 2009
	36	10	28	170	6	Soluble starch	Papanikolau et al. 2007b
	24	8	28	120	6	Pectin	Papanikolau et al. 2007b
	37	9	28	120	6	Lactose	Papanikolau et al. 2007b
	17	23	28	120	6	Cheese whey	Vamvakaki et al., 2010
	53	6.2	28	264	6	Glycerol	Fakas et al., 2009
<i>M. moelleri</i>	54.4	4.25	28	168	6	OMW (50%)	Bellou et al., 2014
<i>R. glutinis</i>	11.78	17.04	-	-	-	Corn stalk	Dai et al., 2007
	28.59	16.56	-	-	-	Tree leaves	Dai et al., 2007
	5.74	3.58	-	-	-	Rice straw	Dai et al., 2007
	20	25	30	120	5.5	Monosodium glutamate wastewater	Xue et al., 2008
	42	25.0	30	120	4	Molasses+glu+sucre	Johnson et al., 1995
<i>T. fermentans</i>	40.1	-28.6	25	190	6.5	Rice straw	Huang et al., 2009
	35.3	-36.4	25	170	6	Cane molasses	Zhu et al., 2008
<i>Thamnidium elegans</i>	64.1	3.75	28	72	6	OMW (50%)	Bellou et al., 2014
<i>Y. lipolytica</i>	38.15	42	30	48	5.6	Glycerol	Fontanille et al., 2012
	44	15	28	120	6	Animal fats	Papanikolau et al. 2002
	40	9	28	240	6	Industrial fats+glycerol	Papanikolau et al. 2002

CHAPTER 3

AIM OF THE PROJECT

Growth in global energy demand, depletion of crude oil resources and price imbalance created by the rising development of vegetable oil market for the manufacturing of biodiesel, specific lipid derivatives (e.g. lubricants) and dietary supplements, have given rise to heated debates on land-use practices and to environmental concerns about oil production strategies (Ageitos et al., 2011). Therefore, the investigation of alternative sources of oil represents a challenge that needs to be undertaken in order to develop industrial processes that are sustainable from an environmental, social and economic point of view. To this aim, the exploitation of oleaginous yeasts and filamentous fungi for the synthesis of microbial oils presents a great industrial interest, although the high costs associated with the production process have always been a bottleneck to their broader commercialization, unless different strategies are adopted. Possible approaches include the use of low cost substrates, screening of high lipid-producing strains, optimization of fermentation conditions and genetic improvement of promising strains.

In this context, the present research project aimed at developing fermentative processes based on the use of low-cost substrates for the production of fungal oils to be exploited primarily in the biodiesel industry. To achieve that, the project has been subdivided into 4 phases, whose specific aims were:

- Screening of 27 strains belonging to yeasts and moulds on three different agro-industrial waste (glycerol, OPE and RCW) so to select the most efficient combinations species-substrate to be brought forward in successive stages. In fact, most of the studies concerning oleaginous microorganisms, have focused on synthetic and high-cost growth substrates (e.g. glucose-based media) on which it is possible to modulate more finely and with less interference different parameters such as the C/N ratio and the type of C and N source, depending on the selected microorganism. Although these studies have been, and still are, of fundamental importance for the identification of promising species and of the conditions that can most promote lipid production, it has become essential to investigate also low-cost substrates in view of a cost-effective industrial-scale transfer.

Although numerous waste and by-products have been explored for this purpose, it is important to continue investigating for different reasons. The optimal substrate, in fact, should be produced in sufficient quantities to be able to meet the demand for oils, supplied

continuously throughout the year (or different seasonal waste may alternate during the year to have continuity) and be currently under-valorized so as to increase the global process sustainability.

Obviously, the choice of the most suitable substrate can not be absolute, but strongly depends on the local market and production activities.

In this project, OPE and RCW have been selected for investigation since they represent two widespread (especially in the Mediterranean area) and scarcely-valorized waste, in addition to being poorly studied for lipid accumulation purposes, despite their high nutritional value and appreciable sugar content.

Therefore, the screening performed on them has been necessary, on the one hand, to assess the potential of growth and lipid accumulation of the strains under study and, on the other hand, to assess the suitability of these substrate for supporting lipid production.

Glycerol, instead, a by-product of biodiesel industry itself, has been used principally as a reference to literature being, probably, the most widely investigated waste for lipid accumulation, and also to evaluate its ability to support oil production in view of a mixed substrate fermentation, since it represents a high concentrated carbon source and could, therefore, allow high production levels.

- Characterization of lipid profile by gas chromatography in order to assess the most suitable application of the oil produced.

It is known that the specific composition of microbial oils is strongly dependant on the strain and substrate used, in addition to fermentation conditions. Although for some strains the influence of different variables have been studied, a model that can be applied to all microorganisms does not exist and, even for known strains, it is often not possible to predict the behaviour on a substrate that has never been studied before. Knowing the specific lipid composition is of fundamental importance, since it determines the potential application of the oils produced. In fact, generally speaking, the predominance of SFA and MUFA is desirable for the biodiesel industry, while the presence of a significant concentration of PUFA, and in particular of omega-3 and omega-6, is interesting from a nutraceutical point of view. Among the strains investigated in this project, little information is available regarding the lipid profile of species such as *Aspergillus tubingensis*, *Cryptococcus albidus* or *Mucor racemosus* and also for what concerns widely known strains, such as *M. isabellina*, *Y. lipolytica* or *R. toruloides*, there is a lack of information about the lipid profile produced in response to complex and poorly studied substrates.

- Transfer of the fermentations from flask to stirred-tank bioreactor (STR) in order to evaluate the effect of mechanical agitation on microorganisms and to study the process in controlled pH, agitation and aeration conditions. In this regard, filamentous fungi present probably the greatest challenge since, compared to yeasts, are more sensitive to mechanical stress and, at the same time, cause more rheological and technical problems.
- Development of a fed-batch strategy in bioreactor to improve cell density and total lipid production. The concentration and composition of the growth medium that is fed into the reactor must in fact first ensure, in a first phase, high biomass production and, in a second phase, stimulate and maintain lipid production condition over time. For this purpose, aim of this part of the project was the evaluation of different substrate formulations in order to improve yields and productivities with respect to batch fermentations.
- Metabolic engineering of *Cryptococcus curvatus*, a Basidiomycota yeast chosen as model strain, with the purpose of modulating the content in MUFA produced and investigating the metabolic step of desaturation of palmitic acid to palmitoleic acid. In fact, it has been seen that a high percentage of MUFA and in particular of oleic and palmitoleic acid should improve the properties of the derived biodiesel in terms of oxidative stability and cold temperature behaviour. Furthermore, most of metabolic engineering studies, concerning lipid metabolism, has been developed for *S. cerevisiae*, a non-OY, and *Y. lipolytica*, an oleaginous Ascomycota, while only few studies involved basidiomycetes, which are, however, more versatile for metabolising different carbonaceous sources and thus, more promising for the valorization of agro-industrial waste.

CHAPTER 4

GENERAL MATERIALS AND METHODS

Part of this chapter has been published in Material and Methods section of the following publication:

Carota, E., Crognale, S., D'Annibale, A., Gallo, A.M., Stazi, S.R., Petruccioli, M., 2017. A sustainable use of Ricotta Cheese Whey for microbial biodiesel production. *Sci. Total Environ.* 584, 554–560. doi:<http://dx.doi.org/10.1016/j.scitotenv.2017.01.068>

4.1 Microorganisms and culture media

4.1.1. Yeast microbial strains and maintenance

The strains screened on RCW, glycerol and OPE were obtained from various culture collections or were isolated from environmental matrices and chosen on the basis of their reported lipid accumulation ability on synthetic media. *C. rugosa* NRRL Y-95, *C. curvatus* NRRL Y-1511, *L. starkeii* NRRL 11557, *R. toruloides* NRRL Y- 1091, *R. toruloides* NRRL Y-17902, *T. fermentans* NRRL Y-1492, *Y. lipolytica* NRRL YB-423, *Y. lipolytica* NRRL Y-1095 and *Y. lipolytica* NRRL Y-7208 were provided by the ARS Culture Collection (NRRL, Peoria, IL). *Cryptococcus albidus* UCD 68-150, *C. albidus* UCD 68-174, *C. laurentii* UCD 68-201, *R. glutinis* UCD 68-255 and *Rhodotorula minuta* UCD 68-280 were obtained from the UCD Collection (Davis, California), while *R. glutinis* DBVPG 3853 from DBVPG Collection (Perugia, Italy). *Pichia guilliermondii* 1067 and *Pichia anomala* AN/4 were a kind gift of Prof. Cardinali (University of Perugia; Italy). *Pichia membranifaciens* 6C1 was isolated from olive brine (Crognale et al., 2012) and identified on the basis of ITS sequence (GenBank Accession number JN900498). During the study, the strains were maintained on potato dextrose agar (PDA) slants at 4 °C and sub-cultured every month.

4.1.2 Mould strains and maintenance

Aspergillus tubingensis NRRL 4700, *C. echinulata* NRRL 3655, *M. isabellina* NRRL 1757, *M. circinelloides* NRRL 3631, *Mucor miehei* NRRL 3169 and *Mucor racemosus* UCD 71-20 were obtained from the ARS (NRRL, Peoria and Davis, California) and UCD (Davis, California) Culture Collections. *A. tubingensis* E1 (Accession number KF482450) was isolated from wood chips (Barontini et al., 2014), while *Mucor moelleri* LAS_E (Accession number KY678773) and *Chaetomium* sp. LAS_G (Accession number KY688070) were

isolated from As- and Hg-polluted soils collected from the Pestarena decommissioned gold mine site (Crognale et al., 2017). Identification was performed on the basis of ITS sequence and, for *Chaetomium* sp. LAS_G, on the basis of 18S rDNA

During the study, the strains were maintained on potato dextrose agar (PDA) slants at 4 °C and sub-cultured every month.

4.1.3 Culture media

RCW-based media preparation is described in paragraph 5.2.3 “Growth media” and in 7.2.2 “Growth media”. Glycerol and OPE-based media preparation is described in paragraph 6.2.2.1 “Glycerol” and 6.2.2.2 OPE, respectively and in 7.2.2 “Growth media”.

4.2 Culture conditions

4.2.1. Shaken flask experiments

The microorganisms used in this project were firstly screened in shaken flasks to select the best strain in terms of biomass and lipid production. Regardless of the strain, each inoculum was obtained by suspending 72-h-old PDA slants with sterile physiological solution. Inocula were added to 250-mL Erlenmeyer flasks containing 50 ml of growth medium to yield an initial value of optical density of 600 (OD₆₀₀) equal to 0.2. After inoculation, flasks were incubated at 30 °C in an orbital shaker (185 rpm) for 5 days. Samples were collected on a daily basis. All experiments were performed in triplicate.

4.2.2. Batch bioreactor experiments

Bioreactor experiments were performed in a 3-L jacketed bench-top stirred tank reactor (STR) (Applikon, Schiedam, NL) filled with 2 L of medium. The bioreactor was endowed with a top stirrer bearing two six-blade Rushton-type turbines (diameter 4.5 cm, blade width 1.4 cm, blade length 1.4 cm) and three baffles (width 1.4 cm). Air was introduced through a perforated pipe sparger located under the bottom turbine. The top plate was equipped with the following probes: dissolved oxygen and pH sensors (Applikon) and a PT 100 temperature sensor. Standard bioprocess conditions were as follows: impeller speed, between 500 and 700 rpm (impeller tip speed = 141 cm s⁻¹); aeration rate, 1.5 vvm; temperature, 30 °C; initial dissolved oxygen concentration, 100% of saturation. Silicon Antifoam 289 (0.5 mL L⁻¹) (Sigma Chemical Co., St Louis, MO, USA) was added before inoculation and an additional 1 mL L⁻¹ was added when needed. The fermentation parameters (temperature, pH and dissolved oxygen) were monitored in bioreactors by an ADI 1030 (Applikon) adaptive/PID digital controller. Pre-inoculum was grown at 30 °C in shaken flasks on Potato Dextrose Broth medium for 24 h, under orbital shaking (185 rpm) and added to the reactor so as to

yield an initial value of OD600 equal to 0.2. Reactor experiments were performed in duplicate and culture samples collected every 12 h. Besides yields ($Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$), kinetic parameters, such as specific growth rate (μ) and lipid and biomass production rates (r_P and r_X , respectively) and N and total sugars consumption rates (r_N and r_S , respectively) were calculated, as described in subsection 4.3.

4.3 Determination of yields and rates

The specific growth rate (μ) was calculated according to the following

Equation

$$\mu = \frac{1}{X} \cdot \frac{\delta X}{\delta t} \quad (1)$$

where X is the biomass concentration (g L^{-1}) at time t (h).

The biomass yield ($Y_{X/S}$) and product yield ($Y_{P/S}$) were calculated according to Eqs. (2) and (3), respectively:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (2)$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (3)$$

where ΔX is the amount of biomass and ΔP is the product (either as total lipids or biodiesel calculated as the amounts of total FAMES), respectively, and ΔS is the amount of substrate consumed. The volumetric growth and production rates (r_X and r_P , respectively) were calculated according to Eqs. (4) and (5) by relating the amounts of biomass and biodiesel, respectively, to the time required to attain the lipid production peak (Δt):

$$r_X = \frac{\Delta X}{\Delta t} \quad (4)$$

$$r_P = \frac{\Delta P}{\Delta t} \quad (5)$$

Substrate (r_S) and nitrogen (r_N) consumption rates were calculated by Eq. (6) by relating the amounts of total sugars and nitrogen consumed, respectively, to the time required to attain the lipid production peak (Δt):

$$r_{[S,N]} = \frac{\Delta[S,N]}{\Delta t} \quad (6)$$

4.4 Analytical methods

4.4.1 Analysis on the supernatant

Supernatants were recovered after centrifugation at 8000 x g for 10 minutes.

Total sugars content was determined by using the phenol-sulphuric acid method (Dubois et al., 1956). COD and TOC were determined according to Standard Methods (APHA, 2005).

Ashes were determined gravimetrically after 12-h ignition at 550 °C in a muffle furnace.

Nitrogen was determined by a modified Kjeldahl method (Domini et al., 2009). Digestions were carried out in a batch microwave digestion system (MarsXpress, CEM, Matthews, NC, USA) at 500 W power, 200 °C for 10 min by adding a mixture of 37% HCl (Carlo Erba Reagenti, Milan, Italy) and 30% H₂O₂ (Merck KGaA, Darmstadt, Germany) to 1 mL sample. Afterwards, nitrogen present in the form of ammonium was determined spectrophotometrically at 650 nm using the nitroprusside method described by (Anderson and Ingram, 1993).

4.4.2 Analysis on cell biomass

Cell biomass was collected from 5 mL-samples in pre-weighed Falcon tubes. The suspension was centrifuged at 8000 x g for 10 min and washed 3 times with distilled water. Dry cell weight was determined gravimetrically after lyophilisation for 48 h for yeasts and 72 hours for filamentous fungi.

Determination of total lipids was performed according to the method by Izard and Limberger, (2003) on fresh biomass pellet deriving from 2 ml of cell suspension, after centrifugation.

Yeast cells were stained with Sudan Black B followed by counterstaining with Safranin to detect the presence of intracellular storage lipids as described by Ravikumar et al. (2012).

Characterisation of lipid profiles was performed by a direct transesterification on lyophilised cells (Schutter and Dick, 2000) to obtain FAMES, which were analysed by a Master GC gas chromatograph (DANI Instrument SpA, Cologno Monzese, Italy) equipped with a Rxi-5MS (Restek, Germany) capillary column (0.25 mm id × 30 m length). FAMES were eluted by using the following program: isothermal at 89 °C for 2 min; temperature gradient from 89 to 280 °C at a 6 °C min⁻¹; hold at 280 °C for 5 min. The temperatures of the injector and flame ionization detector were set at 280 and 300 °C, respectively. Each FAME was identified by comparing its retention time with that of authentic standards contained in the FAME Mix C8-C24 (Sigma Aldrich, 18918-1AMP, USA). For quantification purposes, an internal standard (i.e., methyl nonadecanoate) was added to each sample prior to the transesterification.

4.5 Statistical analyses

The experiments in flasks were carried out with three independent replicates, while experiments in bioreactor in duplicate. The data were analyzed by one-way or two-way analysis of variance (ANOVA test), and the means were compared by Tukey's test using SigmaStat Software version 3.5.

CHAPTER 5

A SUSTAINABLE USE OF RICOTTA CHEESE WHEY FOR MICROBIAL BIODIESEL PRODUCTION

A slightly modified version of this chapter has been published as:

Carota, E., Crognale, S., D'Annibale, A., Gallo, A.M., Stazi, S.R., Petruccioli, M., 2017. A sustainable use of Ricotta Cheese Whey for microbial biodiesel production. *Sci. Total Environ.* 584, 554–560. doi:<http://dx.doi.org/10.1016/j.scitotenv.2017.01.068>

5.1 Introduction

RCW is a by-product of dairy industry derived from ricotta cheese production. During this process, the whey is heated at 80–90 °C and generally added with organic acids and salts to induce the denaturation and consequent precipitation of whey proteins. The curd thus obtained is allowed to cool and filtered to separate the solid part (ricotta) from the liquid waste which is referred to as RCW (Lavarda, 1972). Although the large majority of RCW is produced in the Mediterranean basin (about 1 Mton per year only in Italy) (Sansone et al., 2009), the manufacturing of ricotta cheese is also widespread in USA, where this product is often referred to as ricottone. Therefore, the technical hurdles related to either upgrading or disposal of this dairy by-product are not only a regional issue. The typical composition of RCW is 4.8–5.0% lactose, 1.0–1.3% salts, 0.15–0.22% proteins, 0.20–0.25% organic acids and 0.20% fats with a COD ranging between 50.000 and 80.000 mg L⁻¹ (Sansone et al., 2009). However, despite the presence of residual nutrients, the aforementioned presence of additives impairs its nutritional value and, consequently, its profitable use as a feed for livestock, causing additional costs for the dairy industry due to the need of its disposal. Moreover, RCW might cause a considerable environmental impact in case of an inappropriate disposal procedure.

Despite the appreciable sugar content in RCW which makes it a putative candidate as a growth medium in microbial production processes, only few studies have been conducted for this purpose. These studies include bio-ethanol production by *Kluyveromyces marxianus* (Sansone et al., 2009; Zoppellari and Bardi, 2013) and lactic acid production by mixed and pure bacterial cultures (Secchi et al., 2012). Most of research has focused instead on cheese whey, which, however, differs substantially from RCW, due to its lower concentration of salts and organic acids and higher content of whey proteins (Takakuwa and Saito, 2010;

Ykema et al., 1988). Microbial biodiesel production is among the most promising upgrading options of low cost feedstocks characterized by a high content in carbohydrates associated with low nitrogen content. In this respect, RCW seems to meet these nutritional requirements. Biodiesel is a mixture of fatty acids alkyl monoesters derived from renewable resources such as higher plant oils, or, to a lesser extent, animal fats and waste cooking oil (Srivastava and Prasad, 2000) produced from transesterification reactions of TAG. Compared to conventional diesel, it has several advantages, being non-toxic, renewable and biodegradable. However, the rising costs of plant oils, from which the large majority of biodiesel is derived (around 95% of total world production), and the increasing demand for biofuels have given rise to concerns about land-use practices, increase of food price and oil production strategies (Atabani et al., 2012). Therefore, the use of microbial oils might represent a promising alternative to mitigate the problems associated with the “food vs. fuel” issue. Several microorganisms, belonging to yeasts, moulds and microalgae, have the reported ability to accumulate intracellular lipids up to 70% (w/w) of their dry weight. The microbial fatty acid composition is often similar to that of vegetable oils used for biodiesel (Christophe et al., 2012). Among them, OY seem to be more suitable for an industrial production, being easy to cultivate, fast-growing and receptive to scale-up (Li et al., 2008). Lipid accumulation in OY occurs through two different mechanisms depending on the nature of growth medium. “Ex-novo” synthesis is observed on hydrophobic substrates, while “de novo” synthesis occurs on sugar-based media in concomitance with a shortage of a key element, usually nitrogen. In this study, the adequacy of RCW as a growth medium for the production of microbial oil was assessed. In fact, apparently the chemical composition of RCW characterized by high C/N ratios might be compatible with microbial “de novo synthesis” of lipids (Pirozzi et al., 2013)

The exploitation of RCW in this direction would be in line with sustainability since it would enable a partial replacement of edible oils as feedstock for biodiesel, giving back land use to food crops. Furthermore, in view of a possible development of a circular economy (Cicatiello et al., 2016), RCW upgrading would be beneficial for both dairy industries, due to a reduction of production costs, and for the environment since the spent medium derived from fermentation, would have a negligible organic load.

To this aim, a screening was initially performed with several OYs belonging to well-known lipid-producing species, some of which previously isolated from dairy products (Corbo et al., 2001), to assess their respective abilities to grow and produce lipids on that dairy by-product and to determine productivities. The fatty acid methyl ester compositions derived from transesterification of lipids produced by some selected strains were analysed and

compared with well-established feedstocks for biodiesel production. The most promising strain was then tested in a stirred tank reactor in view of a preliminary assessment of the process scale transfer feasibility.

5.2 Materials and methods

5.2.1 Culture conditions

Conditions used for shaken flask and bioreactor experiments are described in section 4.1.1. “Shaken flask experiments” and 4.1.2 “Bioreactor experiments”, respectively.

5.2.2 Microbial strains and maintenance

Microorganisms used in this study are listed in paragraph 4.1.1.2 “*Yeast microbial strains and maintenance*”

5.2.3. Growth medium

RCW was collected from a cheese processing plant (Formaggi Boccea S.r.l., Rome, Italy) and stored at $-20\text{ }^{\circ}\text{C}$ until used. RCW had the following characteristics (g L^{-1}): dry weight, 48.2 ± 4.10 ; Chemical Oxygen Demand (COD), 43.5 ± 3.8 ; Total Organic Carbon (TOC), 16.3 ± 1.4 ; lactose, 40.2 ± 0.8 ; galactose, 1.6 ± 0.2 , total nitrogen, 0.053 ± 0.04 ; protein, 0.008 ± 0.001 ; C/N, 307; ash, 4.5 ± 0.4 . Initial pH was 5.8. After thawing, the RCW was centrifuged ($8000 \times g$, 15min), two-fold diluted with deionized water, added with $(\text{NH}_4)_2\text{SO}_4$ so as to reach a C/N ratio of 55 and finally its pH was adjusted to 5.5 with 0.1 M NaOH.

5.2.4 Determination of yields and rates

Yields and rates were calculated using formulas mentioned in Section 4.2

5.2.5 Analytical methods

Cell biomass, total lipids and nitrogen quantification, COD and TOC determination and lipid profiles characterisation were determined as described in section 4.3

The concentrations of lactose and galactose were determined by ion-moderated partitioning chromatography in a Varian HPLC system equipped with an Aminex HPX 87-P column (Biorad Laboratories, Milan, IT). Samples were eluted with Milli-Q water (flow rate 0.6 mL min^{-1}) at $65\text{ }^{\circ}\text{C}$ and the elution profile was monitored by an IR4 refractive index detector (Varian, Sunnyvale, CA).

5.3 Results and discussion

5.3.1 Screening of yeast strains

The objective of this study was to investigate the feasibility of a second- generation biodiesel production by OYs grown on a dairy wastewater byproduct (i.e., RCW), for which, as opposed to cheese whey, there is a limited amount of information as a feedstock (Castanha et al., 2014; Pirozzi et al., 2013). Table 5.1 comparatively reports biomass and lipid productions and related yields calculated at the time (t) of maximal lipid accumulation.

Regardless of the strain under study, the duration of the production process was brief with best lipid accumulation being observed between 48 and 96 h. Although both initial C/N ratio and absolute N amounts in the RCW-based medium might be putatively conducive to substantial yeast growth (Beopoulos et al., 2011), only 4 out the 18 strains under study were able to produce a biomass higher than 3 g L⁻¹. The strains that met this requirement were *R. glutinis* UCD 68-255, *T. fermentans* NRRL Y-1492 and two species belonging to the genus *Cryptococcus* (i.e., *C. curvatus* and *C. laurentii*). The *Cryptococcus* and *Trichosporon* genera encompass several species with lactose-assimilating ability.

C. curvatus was able to produce 10.77 g L⁻¹ of biomass and 6.83 g L⁻¹ of intracellular lipids which amounted to 63% of biomass dry weight, while *C. laurentii* achieved a slightly lower production level (5.06 g L⁻¹) but with a higher yield with respect to biomass, amounting to 70%. The ability of the former and the latter strain to use RCW as a growth substrate was confirmed by marked reduction in COD (86.7 and 77.9%, respectively) (data not shown).

The other strains showed greater difficulties in adapting to this substrate but some of them, despite a low growth, were able to accumulate high percentages of total lipids with respect to biomass. In particular, $Y_{P/X}$ values in *L. starkeii* and *R. toruloides* NRRL Y-17902 amounted to 0.63 and 0.79 although their biomass productions were 0.79 and 0.64 g L⁻¹, respectively (Table 5.1). The failure of these strains to substantially grow on RCW might be due to their weak or absent lactose- assimilating ability. In this respect, it may be noted here that lactose- assimilating capacity in yeasts is not very widespread (Frengova et al., 2004) and, for some species, such as *L. starkeii* and *Kluyveromyces marxianus*, this feature has been shown to be strain-dependent (Naumov, 2006; Slodki and Wickerham, 1966). However, irrespective of their growth abilities on RCW, the large majority of the strains under study met the requirement of oleaginicacy accumulating intracellular lipids to larger than 20% of their cellular dry mass (Ratledge and Wynn, 2002) with the only exceptions of *P. membranifaciens* 6C1, *C. rugosa* NRRL Y-95 and *R. glutinis* UCD 68-225.

Table 5.1. Biomass (X) and lipid (P) productions, yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) and time of maximal lipid accumulation (t) obtained for each of the 18 yeast strains grown on RCW-based medium.

	X (g L ⁻¹)	P (g L ⁻¹)	$Y_{X/S}$	$Y_{P/X}$	$Y_{P/S}$	t (h)
<i>C. rugosa</i> NRRL Y-95	1.74±0.01 ^{fg}	0.30±0.01 ^{ab}	0.13±0.01 ^c	0.17±0.01 ^{ab}	0.02±0.00 ^a	72
<i>C. albidus</i> UCD 68-150	2.00±0.07 ^g	0.72±0.10 ^d	0.15±0.01 ^{cd}	0.36±0.06 ^{def}	0.06±0.01 ^c	72
<i>C. albidus</i> UCD 68-174	0.71±0.13 ^a	0.35±0.02 ^{ab}	0.06±0.01 ^{ab}	0.49±0.04 ^{fgh}	0.03±0.00 ^{ab}	48
<i>C. curvatus</i> NRRL Y-1511	10.77±0.21 ^l	6.83±0.14 ^f	0.84±0.04 ^h	0.63±0.02 ^{hi}	0.53±0.02 ^e	72
<i>C. laurentii</i> UCD 68-201	7.28±0.10 ^k	5.06±0.28 ^e	0.52±0.02 ^g	0.70±0.05 ^{ij}	0.36±0.03 ^d	72
<i>L. starkeii</i> NRRL 11557	0.79±0.10 ^a	0.50±0.07 ^{bcd}	0.08±0.01 ^{ab}	0.63±0.14 ^{hi}	0.05±0.01 ^{bc}	96
<i>P. anomala</i> AN/4	1.50±0.06 ^{def}	0.34±0.01 ^{ab}	0.10±0.01 ^{bc}	0.23±0.01 ^{abc}	0.02±0.00 ^a	72
<i>P. guilliermondii</i> 1067	0.74±0.00 ^a	0.35±0.02 ^{ab}	0.05±0.00 ^a	0.48±0.03 ^{fg}	0.02±0.00 ^a	72
<i>P. membranifaciens</i> 6C1	2.71±0.20 ^h	0.34±0.05 ^{ab}	0.31±0.03 ^f	0.13±0.03 ^a	0.04±0.01 ^{abc}	72
<i>R. glutinis</i> DBVPG 3853	0.95±0.05 ^{ab}	0.31±0.04 ^{ab}	0.07±0.01 ^{ab}	0.32±0.06 ^{cde}	0.02±0.00 ^a	72
<i>R. glutinis</i> UCD 68-255	3.03±0.15 ⁱ	0.27±0.01 ^a	0.21±0.01 ^e	0.09±0.01 ^a	0.02±0.00 ^a	72
<i>R. minuta</i> UCD 68-280	0.66±0.03 ^a	0.37±0.03 ^{ab}	0.06±0.00 ^{ab}	0.56±0.07 ^{ghi}	0.03±0.00 ^{ab}	72
<i>R. toruloides</i> NRRL Y-17902	0.64±0.03 ^a	0.51±0.01 ^{bcd}	0.05±0.00 ^a	0.79±0.05 ^j	0.04±0.00 ^{abc}	96
<i>R. toruloides</i> NRRL 1091	0.86±0.07 ^{ab}	0.41±0.03 ^{ab}	0.06±0.01 ^a	0.48±0.07 ^{fg}	0.03±0.00 ^{ab}	72
<i>T. fermentans</i> NRRL Y-1492	3.50±0.22 ^j	0.69±0.06 ^{cd}	0.32±0.03 ^f	0.20±0.03 ^{abc}	0.06±0.01 ^c	48
<i>Y. lipolytica</i> NRRL Y-1095	1.56±0.08 ^{ef}	0.39±0.02 ^{ab}	0.18±0.01 ^{de}	0.25±0.03 ^{abcd}	0.05±0.00 ^{bc}	72
<i>Y. lipolytica</i> NRRL Y-7208	1.35±0.15 ^{cde}	0.38±0.01 ^{ab}	0.18±0.02 ^{de}	0.28±0.02 ^{bcde}	0.05±0.01 ^{bc}	72
<i>Y. lipolytica</i> NRRL YB-423	1.15±0.19 ^{bc}	0.38±0.04 ^{ab}	0.08±0.01 ^{ab}	0.33±0.09 ^{de}	0.03±0.00 ^{ab}	72

Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same letters in apex near the values indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values (column).

Thus, on the basis of the performance observed in shaken cultures, the determination of the lipid profiles was limited to some selected strains. Fig. 5.1 A, reporting the percent concentrations of each identified fatty acid with respect to total FAME, shows that in all the strains considered, the main fatty acids were palmitic (C16:0), oleic (C18:1 Δ 9), linoleic (C18:2 Δ 9,12) and stearic (C18:0) acids, while the concentrations of PUFAs, such as linolenic (C18:3 Δ 9,12,15), eicosadienoic (C20:2 Δ 11,14) and arachidonic (20:4 Δ 5,8,11,14) acids, were either negligible or even absent.

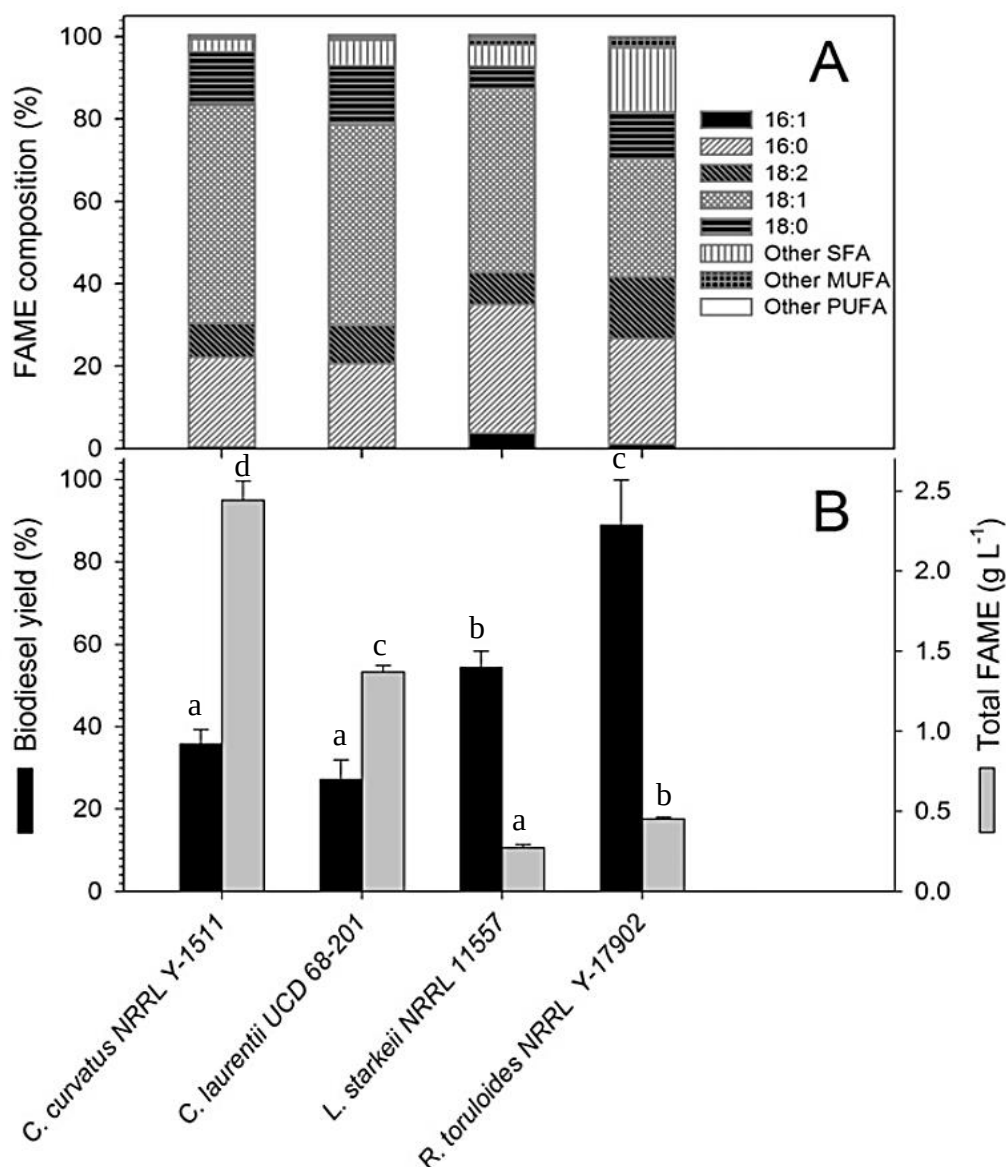


Figure 5.1. Percent compositions of fatty methyl esters (FAMES) derived from lipids produced by *C. curvatus* NRRL Y-1511, *C. laurentii* UCD 68-201, *L. starkeii* NRRL 11557 and *R. toruloides* Y-17902 grown in shaken flask on the RCW-based medium (A) and biodiesel yields (%) and FAME amounts obtained from lipid transesterification and referred to unit volume of culture broth (B). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Abbreviations: 16:1, palmitoleic acid; 16:0, palmitic acid; 18:2, linoleic acid; 18:1 oleic acid; 18:0, stearic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values (Biodiesel yield and FAME).

The FAME profiles of *C. curvatus*, *C. laurentii* and *L. starkeii* were rather similar while that of *R. toruloides* greatly differed from the others due to its higher content in total SFAs (52.6%) and PUFAs (15%), as shown in Fig. 5.1A.

Despite the lower biodiesel yields of *C. curvatus* and *C. laurentii* ($35.77 \pm 3.51\%$ and $27.1 \pm 4.80\%$, respectively) with respect to those ones of *R. toruloides* and *L. starkeii* ($88.90 \pm 11.20\%$ and $54.32 \pm 3.99\%$, respectively) (Fig. 5.1B), their volumetric lipid productions, lipid accumulation capacities and specific fatty acid profiles, were deemed to be suitable for subsequent transfer of the process to the reactor scale.

Although *C. curvatus* exhibited better production properties than *C. laurentii*, the latter was selected since a scant amount of information is currently available regarding its use for biodiesel production.

In fact, *C. curvatus* NRLL Y-1511 is widely recognized as a good lipid producer and this attitude has been already tested on several low-cost substrates, including biodiesel-derived glycerol (Tchakouteu et al., 2015; Thiru et al., 2011), beet molasses (Takakuwa and Saito, 2010), olive mill wastewater (Yousuf et al., 2010), hydrolysates of sweet sorghum bagasse and wheat straw (Liang et al., 2012; Yu et al., 2014) and cheese whey (Seo et al., 2014). On the last substrate, which is highly related to that used in the present study, in particular, a maximum lipid productivity of $4.68 \text{ g L}^{-1} \text{ d}^{-1}$ was obtained. Conversely, the lipid-producing capacity of *C. laurentii* has not been investigated on whey-based media with a sole exception where another strain of this species was tested on cheese whey supplemented with sugarcane molasses (Castanha et al., 2014). In addition, this species deserves particular attention due to its remarkable ability to use a variety of carbon sources and to its tolerance to potential inhibitors of yeast growth (Sitepu et al., 2014). In the present study, lipid volumetric production and productivity of *C. laurentii* (5.06 g L^{-1} and $1.7 \text{ g L}^{-1} \text{ d}^{-1}$) were significantly higher than those reported (2.96 g L^{-1} and $0.10 \text{ g L}^{-1} \text{ d}^{-1}$) in the aforementioned study of Castanha et al. (2014) and these parameters were further improved at the reactor scale.

A typical fermentation of *C. laurentii* in RCW medium in shaken flask is reported in Fig. 5.2 and shows that maximal lipid accumulation took place after 24 h from the depletion of N from the growth medium. Taking into account the lipid production peak (5.06 g L^{-1}) and the relative biodiesel yield (27.1%), the volumetric production of FAME derived from lipid transesterification was estimated to amount to 1.37 g L^{-1} (Fig. 5.1B).

5.3.2. Reactor experiments with *C. laurentii*

To assess the feasibility of upscaling, the production process with *C. laurentii* was performed in a stirred tank reactor, as shown in Fig. 5.3. In STR, nitrogen starvation occurred after only

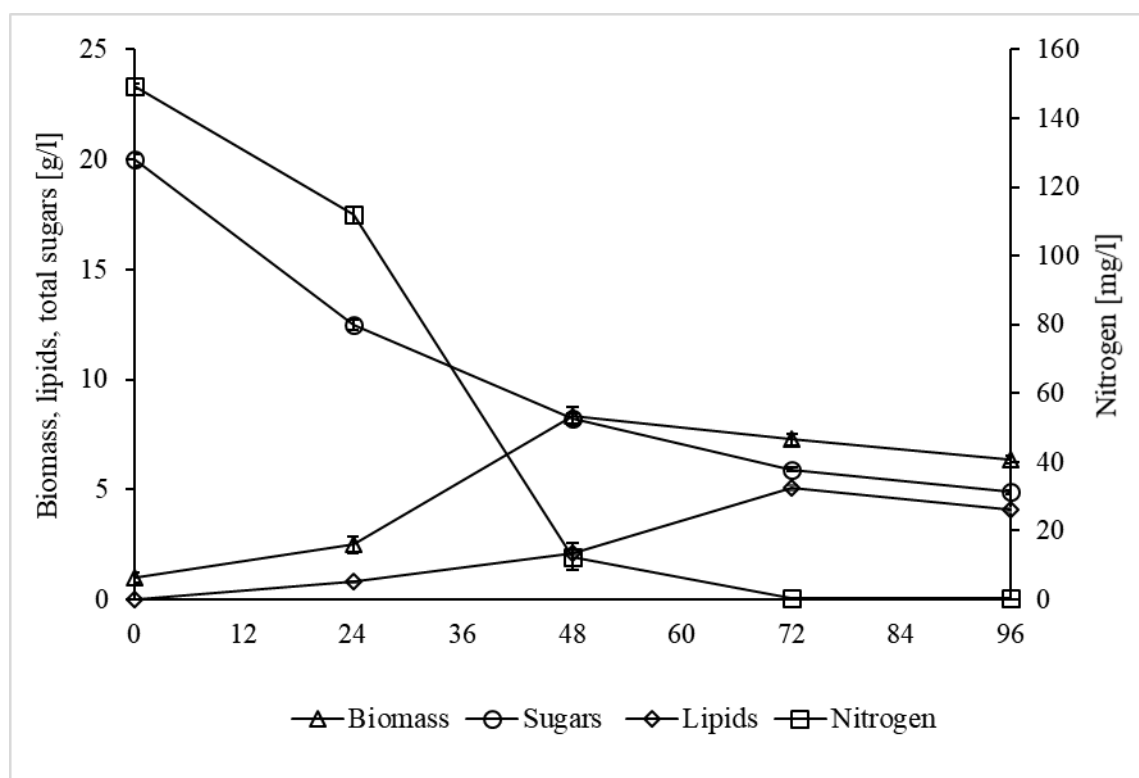


Figure 5.2. Time courses of lipid production, residual nitrogen, total sugar concentrations and biomass production, in *C. laurentii* UCD 68-201 cultures grown in 250 ml Erlenmeyer flasks at 185 rpm. Data are the means $\pm$ standard deviations of duplicate.

36 h, thus resulting in an anticipated peak of lipid production (9.93 g L^{-1} at 60 h). The lipid production peak coincided with the occurrence of maximal biomass production (14.37 g L^{-1}). The decrease in total sugars concentration proceeded at an almost linear rate in the early 48 h to dramatically decline thereafter (Fig. 5.3A). As a consequence, the oxygen percent saturation, after a significant drop in the same time interval, tended to rise again to reach values which approached initial ones.

Conversely, the pH did not significantly change throughout the process as shown in Fig. 5.3B. Noteworthy, at the lipid production peak, the soluble COD was reduced by 83.4% reaching values as low as 3600 mg L^{-1} (data not shown). Table 5.2 shows that at the time of maximal lipid accumulation, biodiesel yield amounted to $32.64 \pm 3.24\%$; on this basis, the maximal volumetric amounts of total FAMEs derived from transesterification were estimated to be 3.24 g L^{-1} . As a result of this calculation, $Y_{P/X}$ and $Y_{P/S}$ were 0.23 ± 0.01 and 0.17 ± 0.01 , respectively.

Therefore, on the basis of these results, the process transfer from shaken flask to the STR was successful leading to remarkably improved biomass and lipid productions; on a volumetric basis, the amounts of FAMEs derived from STR were found to be 2.4-fold higher than in shaken cultures. Moreover, the attainment of the lipid peak was anticipated with respect to shaken flask thus resulting in higher productivity. This anticipation observed in

the STR was due to a better mass transfer of both O₂ and nutrients thus leading to an earlier occurrence of nitrogen starvation in bioreactor, an event known to trigger lipid accumulation in OYs. In particular, when nitrogen depletion takes place, the residual carbon in the medium is readily converted to storage lipids (Ratledge, 2002; Papanikolaou and Aggelis, 2011). Noteworthy, the productivity observed in the present study was higher than that reported for *C. laurentii* DMKU-AmC14 on a glycerol-based medium (Polburee et al., 2015). Although a marked reduction in organic load was observed in the spent medium in concomitance with the lipid production peak, residual COD values were well above the regulatory standards for effluent discharge into receiving water bodies. However, treatment costs of this spent medium in a wastewater treatment plant would be significantly lower than those for RCW since, in the absence of other critical parameters (e.g., toxic pollutants, chromophoric substances etc.), they mostly depend on residual COD rather than on the volumes conferred. As concerns the composition of lipids produced on STR, the determination of the fatty acid profile of *C. laurentii* (Tab. 5.2) indicated the predominance of 16- and 18-carbon chain SFAs and MUFAs in agreement with Castanha et al. (2014) and this was associated with a low content in PUFAs. Noteworthy, the FAME profile of lipids obtained in the bioreactor significantly differed from that obtained in shaken flask with a concomitant increase in linoleic acid (23.7 vs. 8.3%) and decrease in total SFAs (27.9 vs. 38.2%). In this respect, dissolved oxygen levels in the medium can markedly modify the fatty acid profile produced by oleaginous microorganisms (Laoteng et al., 2011) and a decrease in unsaturated fatty acids concomitant to the reduction of available oxygen was observed in *Saccharomyces cerevisiae* (Bardi et al., 1999) and *Apiotrichum curvatum* (Davies et al., 1990) cultures. This is not surprising since oxygen acts as the terminal electron acceptor in reactions catalyzed by fatty acid desaturases which play a key role in the synthesis of MUFAs and PUFAs (Lee et al., 2016); moreover, an oxygen- induced increase in the transcripts levels of desaturase genes was observed in *Mucor rouxii* cultures (Ruenwai et al., 2010). Although an increase in PUFA has been shown to be detrimental to the oxidative stability of the biodiesel (Jakeria et al., 2014), in the present study, the reactor- induced change led to a FAME composition which substantially resembled that of the *Jatropha* oil (Thiru et al., 2011), the use of which is well established in biodiesel production. It has been reported that oil sources containing high amounts of oleic acid and long chain SFAs would be ideal candidates for biodiesel purposes, because this composition positively impacts on biodiesel performance parameters, such as cetane number, kinematic viscosity, melting point, oxidative stability and heat of combustion (Knothe, 2005). In this respect, the FAME composition of *C. laurentii* appears to meet these requirements.

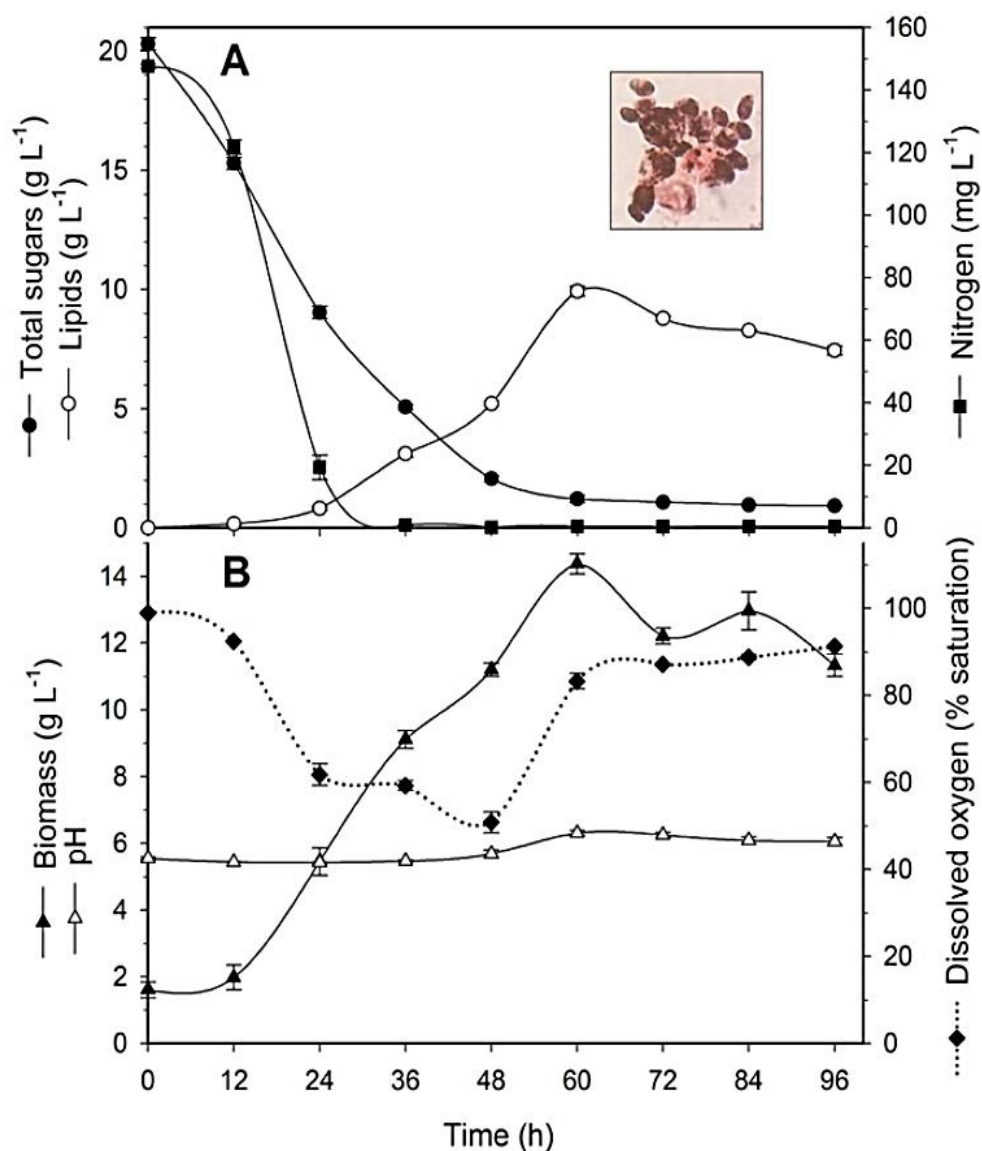


Figure 5.1 Time courses of lipid production and residual nitrogen and total sugar concentrations (Plot A) and biomass production, pH and dissolved oxygen (Plot B) in *C. laurentii* UCD 68-201 cultures grown in a 3-L stirred tank reactor (impeller speed, 600 rpm; aeration rate, 1.5 vvm). Data are the means $\pm$ standard deviations of duplicate reactor experiments. Inset in plot A contains a micrograph of 72-h-old cells stained according to Ravikumar et al. (2012) to detect the presence of storage lipid bodies.

Table 5.2

Performance indicators of biodiesel production process in STR by *C. laurentii* including yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) and biodiesel and biomass production rates (r_P and r_X , respectively), N and total sugars consumption rates (r_N , r_S) specific growth rate (μ) and percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

Parameter	Dimension unit	Value
Yields §		
Biodiesel yield	(%)	32.64±3.24
$Y_{P/X}^\dagger$	(g g ⁻¹)	0.23±0.01
$Y_{P/S}^\dagger$	(g g ⁻¹)	0.17±0.01
$Y_{X/S}$	(g g ⁻¹)	0.65±0.02
Rates §		
$r_P^\dagger$	(g L ⁻¹ d ⁻¹)	1.3±0.09
r_S	(g L ⁻¹ h ⁻¹)	0.31±0.00
r_N	(mg L ⁻¹ h ⁻¹)	2.49±0.00
r_X	(g L ⁻¹ h ⁻¹)	0.24±0.00
μ	(h ⁻¹)	0.02 ± 0.00
Lipid profile		
Palmitoleic acid	††	
	(%)	0.39±0.05
Palmitic acid	(%)	18.53±1.24
Linoleic acid	(%)	23.47±1.01
Oleic acid	(%)	47.16±0.97
Stearic acid	(%)	5.45±0.28
Other SFA	(%)	3.93±0.73
Other MUFA	(%)	0.3±0.07
Other PUFA	(%)	0.77±0.14
Total SFA	(%)	27.91±2.25
Total MUFA	(%)	47.85±1.09
Total PUFA	(%)	24.24±1.15

In conclusion, on the one hand, the screening performed with a variety of strains belonging to well known lipid-accumulating species confirmed that whey-related substrates, such as RCW, are often not adequate to support yeast growth since the large majority of carbon in this by-product is found as lactose, the assimilation of which is not widespread among yeasts. Thus, a profitable use of this dairy by-product as a feedstock for microbial production processes involving yeasts would require its pre-treatment either by acid hydrolysis and subsequent pH correction or enzymatic hydrolysis with β -galactosidase which is commercially available at low costs. This might be beneficial to lipid production processes involving *L. starkeii* and *R. toruloides*, which despite a limited growth on RCW, maintained a significant lipid accumulation capacity and yielded FAME profiles which were compatible with good biodiesel properties; studies are underway to assess this hypothesis. On the other

hand, the successful transfer of the lipid production process of *C. laurentii* grown on RCW from shaken flask to STR offers wide and promising perspectives of further improvements.

CHAPTER 6

EFFECT OF MIXED GLYCEROL-ORANGE PEEL EXTRACT SUBSTRATE ON YEASTS LIPID ACCUMULATION

This chapter is part of a draft that will be published as:

Carota, E., D'Annibale, A., Crognale, S., Gallo, A.M., Petruccioli, M., 2017. Effect of mixed glycerol-orange peel extract substrate on yeasts lipid accumulation.

6.1 Introduction

The development of processes for the production of third-generation biodiesel derived from microbial oils is currently under the spotlight as a more sustainable alternative to plant oil-based fuel. In order to develop an economically feasible process, low cost substrates might be used for the growth of microorganisms and high concentrations of biomass and intracellular lipids should be achieved (Li et al., 2008). Although there are many microorganisms able to accumulate oils under specific cultivation conditions (e.g. algae, bacteria and fungi), they present different prospects of application depending on fatty acid composition, oil yield, lipid volumetric productivity and ability of metabolizing complex or unusual substrates (Ageitos et al., 2011). Yeasts and moulds, thanks to their high versatility in metabolizing different carbon sources and to their fast growth, are considered as favourable oleaginous microorganisms for industrial applications (Meng et al., 2009).

For more than a century, numerous synthetic and industrial waste-based substrates have been evaluated for their ability to support the growth of oleaginous fungi and yeasts, and among them, probably glycerol is one of those that has attracted the most attention.

Glycerol, a by-product of biodiesel production itself, represents an interesting compound as main carbon source or as supplement to stimulate lipid accumulation. In fact, being a precursor for TAG synthesis (Ratledge, 2004), it may be able to promote the synthesis of lipids since it could be integrated directly into TAG. Successful fermentations based on glycerol as sole carbon source have been set up for *C. curvatus* (Meesters et al., 1996; Rakicka et al., 2015) but in several other studies, glycerol has led to low biomasses and yield coefficients, poor lipid conversion yields and/or extended process times needed (Karamerou et al., 2015; Lorenz et al., 2017; Saenge et al., 2011b). One possible explanation could be the lack for specific glycerol carrier systems in yeasts, leading to an insufficient glycerol utilization (Bellou et al., 2016; Makri et al., 2010). However, other studies have also observed that glycerol, in combination with other sugars, could promote a higher lipid content with respect to the use of individual substrates (Galafassi et al., 2012).

To this aim, different media formulations have been tested including the sequential use in fed-batch mode of molasses to stimulate biomass growth and glycerol to favour lipid production in *Y. lipolytica* (Rakicka et al., 2015); the supplementation of a glucose-based medium with glycerol (5 g L⁻¹) in *R. graminis* yielding to an increased lipid productivity with respect to the only glucose-based medium (Galafassi et al. 2011) or the use of a glucose-xylose-glycerol (10:20:30) blend in *R. toruloides* where, despite the occurrence of catabolite repression, an increased lipid yield was observed compared to the only glucose-based medium (Bommareddy et al. 2015).

In the same way, in the perspective of waste valorization, the sugar based medium could be replaced by an agro-industrial sugary waste, that, in our study, was represented by orange peels. Orange peel, in fact, is a widespread waste deriving from juice manufacturing, the production of which amounts to tons per day. After juice extraction the residual peel accounts for 50% (w/w) of the fruit, and apart from some applications as source of essential oils or animal feed, most of this waste remains unused (Balu et al., 2012). In the context of microbial oil production, OPE has been poorly studied as growth substrate (Gema et al., 2002; Park et al., 2014) although it contains easily assimilable sugars such as glucose, sucrose and fructose, along with all the major minerals necessary for the growth of fungi, thus avoiding the addition of supplements and reducing the costs of preparation of the growth medium (Torrado et al., 2011). Raw orange peels also contain D-limonene, a volatile compound constituting citrus peel oil, which is reported to have an inhibitor effect on yeast fermentation (Wilkins et al., 2007). However, having D-limonene a wide range of commercial uses (e.g., flavoring agent, solvent, moistener of water-free cleansers, cancer-preventing agent), different recovery approaches have been developed. The limonene-free residual powder thus obtained, is usually dried 400 °C and destined only to animal feed (Santi et al., 2014), remaining for the most part not valorized and needing appropriate disposal.

Aim of the present study was to develop an OY-based lipid production process using glycerol and OPE as low-cost substrates for lipid production.

For this purpose, 18 yeast strains were first screened in shaken flask to investigate their ability of metabolizing and producing lipids on the two individual substrates. After that, on the basis of the performances demonstrated, one strain was selected for transfer to bioreactor scale, first in batch mode and then in dual- and single-stage fed-batch using a mixed glycerol-OPE substrate.

In fact, the formulation of such mixed-substrate would allow, on the one hand, to have all the nutrients of the OPE which can ensure a fast growth phase, on the other hand, glycerol

addition would increase the total carbon concentration in the growth medium and it should stimulate, as mentioned above, the synthesis of TAG.

6.2 Materials and methods

6.2.1 Culture conditions

6.2.1.1 Shaken flask screening

Conditions used for shaken flask screening are described in section 4.1.1 “Shaken flask experiments”.

6.2.1.2 Batch bioreactor experiments

Conditions used for batch bioreactor experiments are described in section 4.1.2 “Bioreactor experiments”.

6.2.1.3 Fed-batch bioreactor experiments

Fed-batch fermentations were carried out in a jacketed 3-L Applikon (Schiedam, The Netherlands) bioreactor, equipped with DO and pH probes. Initial working volume was 1.2 L, to arrive, after the feeding phase, to a final volume of 2.2 L. Agitation rate was maintained between 500 and 700 rpm in order to maintain DO above 30%. Aeration was set at 1.5 vvm and temperature at 30 °C.

Pre-inoculum was grown for 24 hours on PDB medium, in shaken flasks at 185 rpm and 30°C. Inoculum culture (10%, v/v) was used at a concentration resulting in an initial OD₆₀₀ of 1.0.

In the first trial, a dual-stage feeding was investigated. At the beginning, 1.2 L of OPE supplemented with (NH₄)₂SO₄ to have an initial C/N ratio of 15, were used for the initial growth phase. First feeding (24-72 hours) was a solution composed by 20% (v/v) of glycerol and 80% (v/v) OPE, having 269.5 g L⁻¹ of total carbon source, with a C/N adjusted to 20 via defined amount of (NH₄)₂SO₄. D was 0.01 h⁻¹. Second feeding (72-108 hours) was formulated with the same composition of first feeding except for the C/N ratio increased to 80 by addition of (NH₄)₂SO₄, as before. Same D was used.

In the second trial, a single-stage feeding strategy was tested. During the initial growth phase, the growth medium used was the same as for the first trial. Feeding was composed of 15% (v/v) glycerol and 85% (v/v) OPE, C/N ratio 20, supplied from 22 to 72 hours with a D= 0.02 h⁻¹.

6.2.2 Microbial strains and maintenance

Microorganisms used in this study are listed in paragraph 4.1.1.2 “*Yeast microbial strains and maintenance*”

6.2.2 Growth media

6.2.2.1 Glycerol

Glycerol-based medium used for screening experiment contained (g L^{-1}): glycerol, 20; $(\text{NH}_4)_2\text{SO}_4$, 0.5; yeast extract (containing 10% total nitrogen), 0.5; NaH_2PO_4 , 3.0; KH_2PO_4 , 0.7. After sterilization in autoclave, 0.1% of filter sterilized mineral salt solution 1000x was added, containing per litre: 4.4 g ZnSO_4 , 1.1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.315 g CuSO_4 , 1.47 g CaCl_2 , 1 g FeSO_4 and adjusted to pH 5.5.

6.2.2.2 OPE

Air-dried orange peels were crushed using a blender and subjected to extraction at 100°C in distilled water for 1 hour to obtain two extracts: OPE150 (150 g L^{-1} orange peels in water), used for screening experiments and batch fermentation; OPE200 (200 g L^{-1}), used for fed-batch fermentations in bioreactor. OPE150, after centrifugation and filtration, had a dry weight of $25.43 \pm 0.54 \text{ g L}^{-1}$ composed of $39.85 \pm 0.15\%$ total carbon and $1.32 \pm 0.02\%$ total nitrogen determined by elemental analyser, while OPE200 had a dry weight of $36.81 \pm 0.24 \text{ g L}^{-1}$ with the same C and N percentage composition.

For screening experiments and for batch fermentation, OPE150 was supplemented with a small amount of $(\text{NH}_4)_2\text{SO}_4$, passing from a C/N ratio of 31 to 25, in order to ensure a rapid initial growth and allow the microorganisms to adapt quickly to the new substrate after inoculation. In fed-batch mode, different C/N ratios, as described above, were used.

Sugar composition of OPE, determined by enzymatic kits (Megazyme International Ireland Ltd, Wicklow, Ireland) was the following (g L^{-1}): OPE150 5.68 ± 0.17 glucose, 5.98 ± 0.21 sucrose, 5.04 ± 0.17 fructose, 1.08 ± 0.22 mannose and 1.78 ± 0.14 pectin; OPE200 8.08 ± 0.44 glucose, 8.54 ± 0.37 sucrose, 7.22 ± 0.20 fructose, 1.50 ± 0.15 mannose and 2.47 ± 0.24 pectin.

6.2.3 Determination of yields and rates

Yields and rates calculation are reported in paragraph 4.2 “Determination of yields and rates”.

6.2.4 Analytical methods

Methods concerning the determination of volumetric biomass and lipid production, quantification of total nitrogen, GC characterization of FAME and HPLC characterization of OPE medium, are described in paragraph 4.3 “Analytical methods”.

6.2.4.1 *Glucose, fructose, sucrose, maltose and glycerol*

During fermentation, substrate consumption was assessed by means of enzymatic kits. Specifically, glucose, fructose and sucrose were analyzed using the SUCROSE, D-FRUCTOSE and D-GLUCOSE kit (Megazyme International Ireland Ltd, Wicklow, Ireland), following the procedure described by the company in the data booklet at this link: https://secure.megazyme.com/files/Booklet/K-SUFRG_DATA.pdf

Maltose was determined by means of Maltose/Sucrose/D-Glucose Assay Kit (Megazyme International Ireland Ltd, Wicklow, Ireland), whose data booklet can be found at this link https://secure.megazyme.com/files/Booklet/K-MASUG_DATA.PDF.

Glycerol was quantified by K-GCROLGK kit (Megazyme International Ireland Ltd, Wicklow, Ireland), whose entire assay procedure is reported in the data booklet: https://secure.megazyme.com/files/Booklet/K-GCROLGK_DATA.pdf

6.2.4.2 Pectin

Pectin was indirectly quantified by galacturonic acid assay (Taylor and Buchanan-Smith, 1992) using pectin as standard for calibration curve in a range between 10 and 200 µg/ml. Orange peels are composed of 15-25% (w/w) of pectin (Grohmann et al., 1995) and in the mild conditions used here for extraction, a small percentage was expected in the final OPE. Galacturonic acid, which should constitute about the 85% of pectin, was used also, as comparison, for the construction of a calibration curve, resulting in a response equal to 90% with respect to that one given by pectin.

Concentrated sulphuric acid (1.5 ml) and 0.1% (w/v) carbazole in absolute ethanol reagent (50 µl) were added to 100 µl sample and mixed. Samples were then incubated at 60 °C in a water bath for 1 h and cooled to room temperature in another water bath. The absorbance of the pink-to-red-coloured sample was read at 530 nm. Since carbohydrates produce a brown colour in presence of concentrated sulfuric acid, for each sample a blank was prepared by adding to the sample absolute ethanol without carbazole, and the absorbance was subtracted to the absorbance of the sample containing carbazole. Calibration curve was made by using galacturonic acid (0-0.5 g L⁻¹) as standard.

6.2.5 Empirical equations for the calculation of saponification, iodine value and cetane number

Empirical equations for the calculation of saponification (SV) and iodine value (IV) developed by (Kalayasiri et al., 1996) and of cetane number (CN) (Krisnangkura, 1986) were used to evaluate the suitability of lipids produced for biodiesel, using as reference the Standard EN14214.

SV (mg g^{-1}) represents the number of milligrams of potassium hydroxide required to saponify 1g of fat. It does not fall under the requirements of but its value is used for the estimation of IV and CN (Wakil et al., 2015).

Iodine value (IV) is empirically determined by measuring the amount of I_2 that reacts by addition to carbon-carbon double bonds, thus it is directly related to the degree of unsaturation. A maximum value of 120 g $\text{I}_2/100$ g of biodiesel is accepted by EN14214 Standard.

CN is an inverse function of a fuel's ignition delay and it is considered as a measure of a fuel's autoignition quality characteristics. Since biodiesel is mainly composed of long-chain hydrocarbon groups, with virtually no branching or aromatic structures, this value is typically higher than in petroleum diesel. Increase of unsaturation degree leads to decreasing CN. The requirement for EN14214 Standard is a value higher than 51 (Hoekman et al., 2012).

Formula used for estimation were as follows:

$$\text{SN} = 100 \times \sum \frac{560 \times A_i}{\text{MW}_i}$$
$$\text{IV} = 100 \times \sum \frac{254 \times D \times A_i}{\text{MW}_i}$$

where A_i represents the percentage of each component, D the number of double bond and MW_i the molecular mass of each component.

$$\text{CN} = 46.3 + \frac{5458}{\text{SN}} - (0.225 \times \text{IV})$$

6. 3. Results and discussion

In order to establish an economically competitive productive process of microbial oils, it is essential to identify one or more strains showing a high growth and lipid production rate, and a suitable substrate, easily assimilable and cheap. After that, it is important to define the most suitable and productive conditions for growth and lipid production.

In this work, 18 yeast strains known to be oleaginous by literature, were first screened in shaken flask on two different agro-industrial waste, glycerol and OPE, in order to select the most efficient substrate-strain combinations on which the optimization of cultural conditions should be attempted. On the strains that stood out for their growth and production ability on each of the two substrates, a GC characterization of the specific fatty acids produced was also performed in order to verify the suitability of the oils produced for the production of biodiesel. On the basis of the results obtained, one strain was thus selected to investigate the technical feasibility of the scale transfer to bioreactor, first in batch mode and then in fed-batch.

6. 3.1 Screening on glycerol

The set of results concerning growth and lipid accumulation of all strains on glycerol is reported in Tab. 6.1.

Glycerol is probably one of the most common substrates taken into account for lipid production, due to its widespread diffusion and ease of preparation of growth media based on it. Furthermore, it is the main by-product of biodiesel production, so its recycling represents the valorization of a by-product of the same production processes, thus contributing to lower production costs within the context of circular economy (Leiva-Candia et al., 2014).

The highest biomass volumetric production was reached by *T. fermentans* NRRL Y-1492, in spite of a low lipid production, equal to a lipid content of only 7% with respect to cell dry weight. Conversely, *Y. lipolytica* NRRL YB-423, *Y. lipolytica* NRRL Y-1095, *R. toruloides* NRRL Y-1091 and, to a lesser extent, *C. curvatus* NRRL Y-1511 were able to convey a higher portion of carbon to the production of intracellular lipids, while reaching lower biomass values. In particular, the highest lipid yield value $Y_{P/S}$ was achieved by *Y. lipolytica* NRRL YB-423 equal to $0.34 \pm 0.02 \text{ g g}^{-1}$ which corresponded to the maximum conversion yield achievable on glycerol (Ratledge, 2002). However, also *Y. lipolytica* NRRL Y-1095, *R. toruloides* NRRL Y-1091 and *C. curvatus* NRRL Y-1511, demonstrated a good capacity in converting glycerol to storage lipids, with a $Y_{P/S}$ of 0.19, 0.16 and 0.13 g g^{-1} , respectively. Nevertheless, comparing the yields obtained in this screening with the theoretical $Y_{X/S}$ and $Y_{P/S}$ (0.57 and 0.32 g g^{-1} substrate, respectively; Bommarreddy et al., 2015) it can be noted that the uptake of glycerol or its conversion was poorly efficient in most of the strains used in spite of a C/N ratio of 45 which is within the values usually used to stimulate lipid accumulation, but not too high to be limiting for OYs (Papanikolaou and Aggelis, 2011b). However, this is in line with other screening experiments performed on glycerol

(Chatzifragkou et al., 2011; Raimondi et al., 2014). In fact, although in theory yields of lipids ($Y_{P/S}$) obtained using glycerol as carbon source should be comparable or even higher to yields obtained on easily metabolizable substrates such as glucose, experimentally, they usually range between 0.08 and 0.12 with only few exceptions (Raimondi et al., 2014). It has been supposed that this could be due to a poor regulation of the enzymes involved in glycerol uptake (Papanikolaou et al., 2008).

Table 6.1. Biomass (X) and lipid (P) productions, yield parameters ($Y_{X/S}$, $Y_{L/X}$, $Y_{P/S}$) calculated at time of maximal lipid accumulation (t) obtained for each of the 18 yeast strains grown on glycerol-based medium.

Yeasts	X (g L ⁻¹)	P (g L ⁻¹)	$Y_{X/S}$	$Y_{P/X}$	$Y_{P/S}$	t(h)
<i>C. rugosa</i> NRRL Y-95	3.79±0.15 ^d	0.35±0.00 ^{bc}	0.45±0.03 ^d	0.09±0.00 ^{ab}	0.04±0.00 ^{bc}	72
<i>C. albidus</i> UCD 68-150	0.53±0.19 ^a	0.09±0.01 ^a	0.08±0.01 ^a	0.17±0.05 ^{cd}	0.01±0.00 ^a	72
<i>C. albidus</i> UCD 68-174	0.91±0.06 ^{ab}	0.13±0.01 ^a	0.13±0.01 ^{ab}	0.15±0.02 ^{bc}	0.02±0.00 ^{ab}	72
<i>C. curvatus</i> NRRL Y-1511	1.46±0.19 ^{bc}	0.87±0.01 ^{de}	0.22±0.03 ^c	0.60±0.07 ⁱ	0.13±0.00 ^e	72
<i>C. laurentii</i> UCD 68-201	0.67±0.14 ^a	0.13±0.02 ^a	0.10±0.01 ^a	0.19±0.01 ^{cd}	0.02±0.00 ^{ab}	72
<i>L. starkeii</i> NRRL 11557	0.93±0.30 ^{ab}	0.43±0.09 ^{bc}	0.13±0.01 ^{ab}	0.46±0.06 ^h	0.06±0.00 ^{cd}	96
<i>P. anomala</i> AN/4	3.27±0.81 ^d	0.61±0.16 ^{cd}	0.45±0.05 ^d	0.19±0.00 ^{cd}	0.08±0.01 ^d	72
<i>P. Guilliermondii</i> 1067	1.22±0.20 ^{bc}	0.30±0.04 ^{bc}	0.15±0.02 ^{abc}	0.24±0.02 ^{de}	0.04±0.00 ^{bc}	96
<i>P. membranifaciens</i> 6C1	1.93±0.23 ^c	0.07±0.00 ^a	0.28±0.02 ^c	0.04±0.01 ^a	0.01±0.00 ^a	72
<i>R. toruloides</i> NRRL Y-1091	1.72±0.02 ^{bc}	1.11±0.04 ^{ef}	0.25±0.02 ^c	0.64±0.03 ⁱ	0.16±0.01 ^f	72
<i>R. toruloides</i> NRRL Y-17902	0.88±0.08 ^a	0.21±0.02 ^{ab}	0.12±0.01 ^{ab}	0.24±0.00 ^{de}	0.03±0.00 ^{ab}	72
<i>R. glutinis</i> DBVPG 3853	1.38±0.06 ^{bc}	0.46±0.05 ^c	0.21±0.02 ^c	0.34±0.02 ^{fg}	0.07±0.02 ^d	72
<i>R. glutinis</i> UCD 68-255	3.27±0.16 ^d	0.35±0.03 ^{bc}	0.27±0.03 ^c	0.11±0.01 ^{abc}	0.03±0.00 ^{ab}	72
<i>R. minuta</i> UCD 68-280	1.50±0.19 ^{bc}	0.43±0.03 ^{bc}	0.21±0.03 ^c	0.29±0.06 ^{ef}	0.06±0.00 ^{cd}	72
<i>T. fermentans</i> NRRL Y-1492	6.50±0.50 ^e	0.43±0.08 ^b	0.56±0.05 ^e	0.07±0.01 ^{ab}	0.04±0.00 ^{bc}	72
<i>Y. lipolytica</i> NRRL Y-1095	3.89±0.30 ^d	1.39±0.08 ^f	0.54±0.05 ^e	0.36±0.04 ^{fg}	0.19±0.01 ^g	72
<i>Y. lipolytica</i> NRRL Y-7208	1.24±0.19 ^{bc}	0.50±0.08 ^c	0.18±0.02 ^{bc}	0.41±0.00 ^{gh}	0.07±0.01 ^d	72
<i>Y. lipolytica</i> NRRL YB-423	3.81±0.65 ^d	2.42±0.23 ^g	0.53±0.03 ^e	0.63±0.05 ⁱ	0.34±0.02 ^h	72

$Y_{X/S}$ =biomass yield; $Y_{P/X}$ =specific lipid yield; $Y_{P/S}$ =lipid yield referred to consumed glycerol. Volumetric lipid amount (P) was calculated according to Izard and Limberger (2003) method. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P\leq 0.05$). Same letters in apex near the values indicate lack of statistically significant difference ($P\leq 0.05$) within the same group of values (column).

Higher production yields have usually been observed when high cell density cultures involving fed-batch feeding processes are used (Kim et al., 2000; Meesters et al., 1996).

It is important to note that, although the uptake of glycerol was inefficient in most of the strains, this carbon source was conducive to a high lipid content in *R. toruloides* NRRL Y-1091 (64%), *C. curvatus* NRRL Y-1511 (60%) and *L. starkeii* NRRL 11557 (46%), suggesting that it may be incorporated directly into TAG synthesis or that it can stimulate their synthesis, being therefore important during lipid production phase, more than in the growth phase (Galafassi et al., 2012). This is why in some fed-batch studies, the approach is to use an easily assimilable sugar-based substrate during the exponential growth phase and a glycerol-based substrate during the lipid accumulation phase (Rakicka et al., 2015).

6.3.2 Screening on OPE

Values concerning growth and lipid accumulation of all strains on OPE-based medium and derived yield parameters calculated at time of maximal lipid accumulation are reported in Tab. 6.2.

Looking at the results, it is immediately evident that OPE sugar composition, consisting mainly of glucose, sucrose and fructose, ensured a higher biomass production with respect to glycerol-based medium. Among these strains, only *C. laurentii* proved to be able to convert efficiently the carbon source both into biomass and intracellular lipids, reaching a lipid yield on substrate ($Y_{P/S}$) equal to 0.25 g g^{-1} and a lipid volumetric production of 4.51 g L^{-1} . However, although the production of a high initial biomass is a major requirement for a high lipid production, *R. toruloides* NRRL Y-1091 managed to produce a remarkable higher quantity of lipids (5.74 g L^{-1}) with respect to other strains, demonstrating a higher ability in balancing the production of biomass and lipids by investing more energies in the second one. It also reached the highest lipid content of all strains (77%), thus offsetting the less biomass produced.

It is possible that with different growth conditions and, in particular, with a different C/N ratio, other species could have conveyed a greater amount of carbon source to lipid production rather than biomass. However, it is also true that C/N ratio in orange peels used was 31 and although a small amount of initial inorganic nitrogen was added, it did not significantly alter the C/N ratio that was already low for the nature of the substrate used. Most importantly, *C. laurentii* and *R. toruloides* have demonstrated greater versatility to adapt to less stringent conditions than those usually used to induce lipid accumulation and have also confirmed the concept of finding the right microorganism-substrate combination leading to maximum exploitation of both of them.

Table 6.2. Biomass (X) and lipid (P) productions, yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) calculated at time of maximal lipid accumulation (t) obtained for each of the 18 yeast strains grown on OPE-based medium.

Yeasts	X (g L ⁻¹)	P (g L ⁻¹)	$Y_{X/S}$	$Y_{P/X}$	$Y_{P/S}$ §	t (h)
<i>C. rugosa</i> NRRL Y-95	4.61±0.10 ^{bc}	1.62±0.09 ^{abc}	0.31±0.01 ^{bc}	0.35±0.01 ^{de}	0.11±0.01 ^{bc}	72
<i>C. albidus</i> UCD 68-150	6.06±0.19 ^{de}	1.51±0.08 ^{abc}	0.34±0.02 ^{cd}	0.25±0.02 ^{bc}	0.09±0.01 ^{ab}	72
<i>C. albidus</i> UCD 68-174	9.31±0.29 ^g	3.83±0.33 ^{ef}	0.46±0.02 ^f	0.41±0.05 ^{ef}	0.20±0.02 ^f	72
<i>C. curvatus</i> NRRL Y-1511	10.85±0.36 ^h	1.74±0.06 ^{abc}	0.57±0.04 ^g	0.15±0.00 ^a	0.09±0.01 ^{ab}	96
<i>C. laurentii</i> UCD 68-201	9.68±0.37 ^g	4.51±0.02 ^f	0.53±0.02 ^g	0.47±0.02 ^{gh}	0.25±0.00 ^g	72
<i>L. starkeii</i> NRRL 11557	9.33±0.64 ^g	1.90±0.05 ^{abc}	0.57±0.04 ^g	0.20±0.01 ^{ab}	0.12±0.00 ^{bc}	72
<i>P. anomala</i> AN/4	10.55±0.41 ^h	2.95±0.24 ^d	0.58±0.02 ^g	0.28±0.03 ^c	0.16±0.01 ^{de}	72
<i>P. Guilliermondii</i> 1067	6.16±0.06 ^{de}	1.84±0.15 ^{abc}	0.34±0.01 ^{cd}	0.30±0.02 ^{cd}	0.10±0.01 ^{ab}	72
<i>P. membranifaciens</i> 6C1	4.13±0.34 ^b	1.61±0.15 ^{abc}	0.26±0.02 ^b	0.39±0.00 ^{ef}	0.10±0.01 ^{ab}	72
<i>R. toruloides</i> NRRL 1091	7.44±0.03 ^f	5.74±0.05 ^g	0.40±0.00 ^e	0.77±0.01 ^j	0.31±0.00 ^h	72
<i>R. toruloides</i> NRRL Y-17902	4.68±0.21 ^{bc}	2.04±0.12 ^{bc}	0.27±0.01 ^b	0.44±0.01 ^{fgh}	0.12±0.01 ^{bc}	72
<i>R. glutinis</i> DBVPG 3853	6.84±0.16 ^{ef}	3.33±0.38 ^{de}	0.38±0.01 ^{de}	0.49±0.07 ^{hi}	0.19±0.02 ^{ef}	72
<i>R. glutinis</i> UCD 68-255	3.21±0.01 ^a	1.33±0.01 ^a	0.17±0.00 ^a	0.41±0.00 ^{efg}	0.07±0.00 ^a	72
<i>R. minuta</i> UCD 68-280	10.04±0.61 ^{gh}	3.16±0.36 ^{de}	0.59±0.04 ^g	0.31±0.02 ^{cd}	0.19±0.02 ^{ef}	96
<i>T. fermentans</i> NRRL Y-1492	7.18±0.48 ^f	1.19±0.09 ^a	0.42±0.03 ^{ef}	0.17±0.02 ^a	0.07±0.01 ^a	72
<i>Y. lipolytica</i> NRRL Y-1095	5.38±0.05 ^{cd}	2.14±0.14 ^c	0.34±0.00 ^{cd}	0.40±0.02 ^{ef}	0.14±0.01 ^{cd}	72
<i>Y. lipolytica</i> NRRL Y-7208	6.15±0.22 ^{de}	1.67±0.20 ^{abc}	0.41±0.02 ^{ef}	0.27±0.01 ^c	0.11±0.02 ^{bc}	72
<i>Y. lipolytica</i> NRRL YB-423	3.21±0.07 ^a	1.76±0.07 ^{abc}	0.17±0.00 ^a	0.55±0.01 ⁱ	0.10±0.00 ^{ab}	72

$Y_{X/S}$ =biomass yield; $Y_{P/X}$ =specific lipid yield; $Y_{P/S}$ =lipid yield referred to consumed sugars; § Calculated by relating total lipids, determined according to Izard and Limberger (2003), to the amounts of total sugars consumed. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same letters in apex near the values indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values (column).

In recent years, *R. toruloides* has emerged as a promising strain able to metabolize a wide range of carbonaceous and agro-industrial waste. Interesting results have been obtained on substrates such as sugarcane juice (Soccol et al., 2017), sugarcane bagasse hemicellulosic hydrolysate without nutrient supplementation (Bonturi et al., 2017), Jerusalem artichoke (Zhao et al., 2010), crude glycerol (Xu et al., 2012), cassava starch (Wang et al., 2012) and molasses (Vieira et al., 2014), as well as on all carbohydrates typical of plant hydrolysate, including glucose, sucrose, xylose, fructose, galactose and maltose (Wiebe et al., 2012).

A foreseeable result was therefore predictable by this species, though the screening allowed to select, between two strains of *R. toruloides* tested, the most suitable one. Furthermore, orange peels were never been tested for this strain, as well as substrates with such an amount of organic nitrogen and a low C/N ratio.

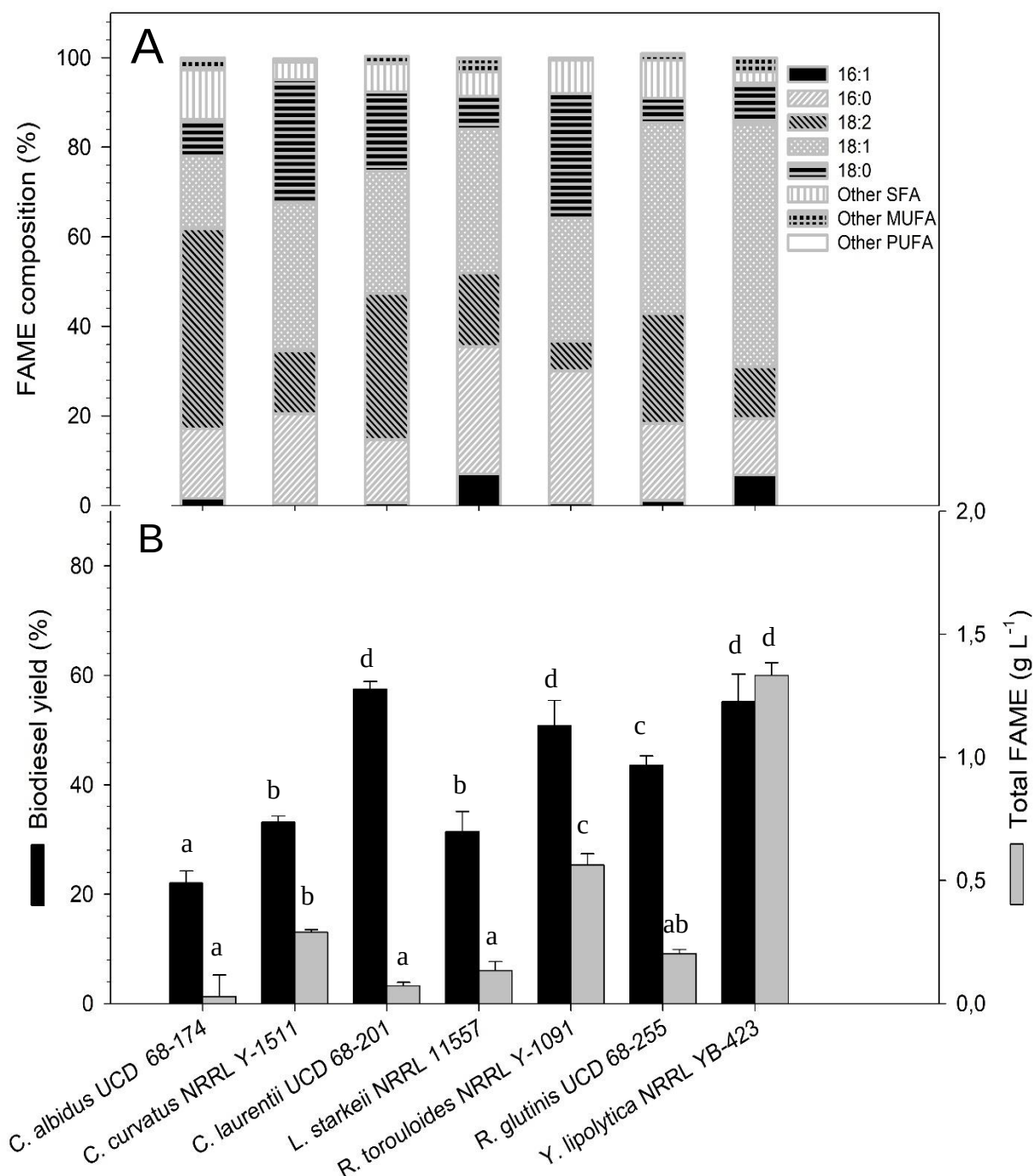


Figure 6.1. Percent compositions of fatty methyl esters (FAMES) derived from lipids produced by 7 selected strains grown in shaken flask on glycerol (A) and biodiesel yields (%) and FAME amounts obtained from lipid transesterification (B). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Abbreviations: 16:1, palmitoleic acid; 16:0, palmitic acid; 18:2, linoleic acid; 18:1 oleic acid; 18:0, stearic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

6.4 Comparison of lipid profiles

Lipid profile characterization on selected strains, showing better performances with respect to the other tested, was considered important to better elucidate the nature of the accumulated lipids and also to understand whether particular characteristics of the composition of the oils could be dependent on the substrate used.

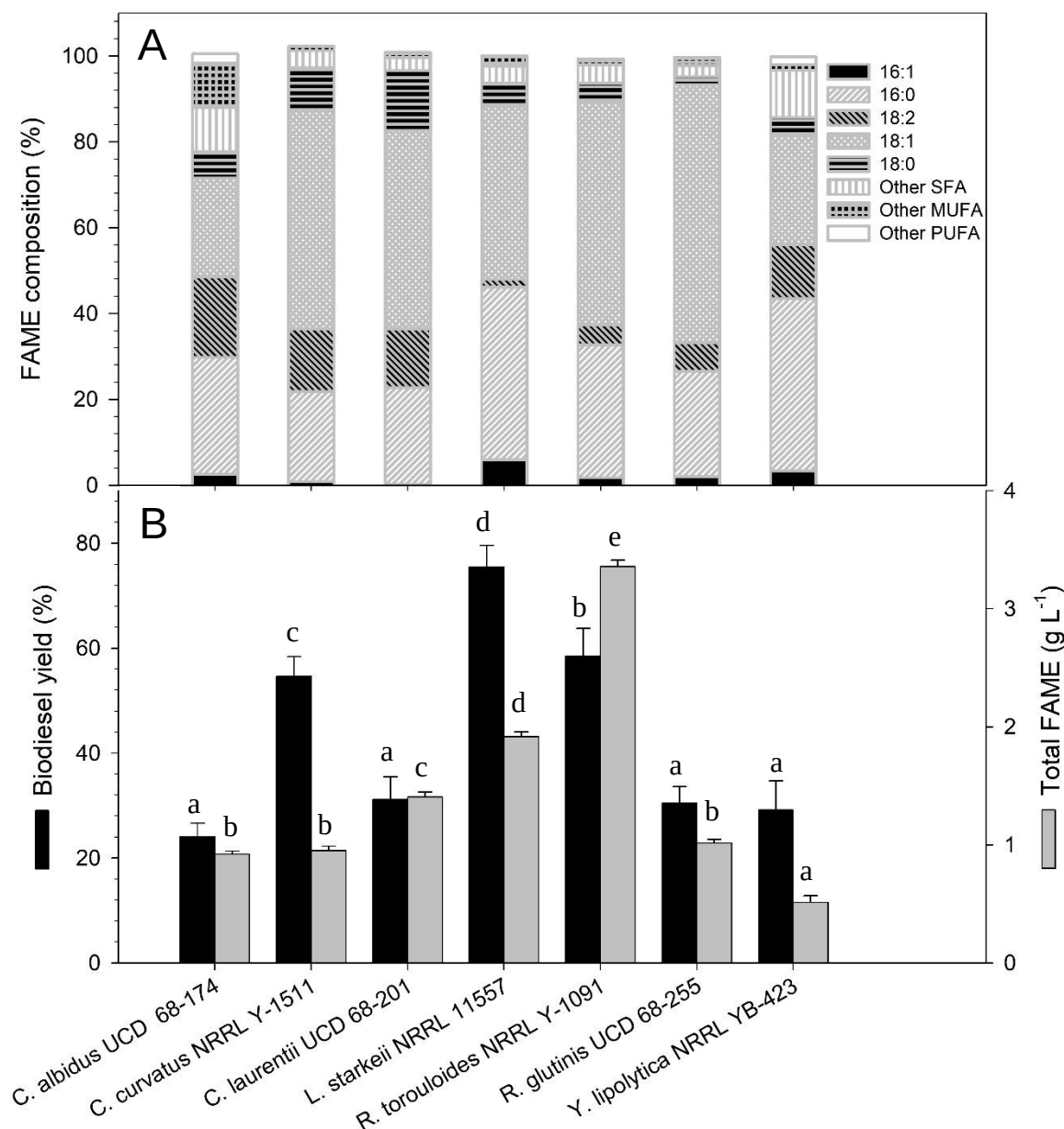


Figure 6.2. Percent compositions of (FAME) derived from lipids produced by 7 selected strains grown in shaken flask on OPE (A) and biodiesel yields (%) and FAME amounts obtained from lipid transesterification (B). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values (Biodiesel yield and FAME).

Moreover, the transesterification reaction, indispensable for analyzing fatty acids through gas-chromatography, also gave an idea of the real yield in biodiesel of the microbial oils studied, which may depend, for example, on the presence of an excessive amount of free fatty acids (Patel et al., 2017).

As shown in Fig. 6.1 and 6.2 for all strains, C 16 and C 18 were the most abundant fatty acids and, among them, oleic acid (C 18:1) was the major one, although some evident differences can be observed. Glycerol led to a significantly higher relative content of C18:2 with respect to OPE in *C. albidus* UCD 60-174, *C. laurentii* UCD 68-201 and in *R. glutinis* UCD 68-255 at the expense of C 18:1 and C 16:0. More in general, C16:0 was produced, for all strains, in higher amounts when they were grown on OPE. C18:0 was also present in different amounts among strains and between substrates but not with a specific correlation. Moreover, except for C 18:2, only small amounts of other PUFA were detected (below 2.6%) in all the strains. In view of a potential application of these oils for biodiesel production, the low content in PUFA is an important parameter to consider. According to the European Standard UNE-EN 14214, limit of cetane number and iodine value (correlated with combustion quality and formation of combustion deposits, respectively) are satisfied only when PUFA are below the 30% of total lipids (Ramos et al., 2009). On the other hand, a content in MUFA higher than 50% is associated with a good quality biodiesel.

Therefore, with the exception of fatty acid profile observed in *C. albidus* UCD 68-174 and *C. laurentii* UCD 68-201 on glycerol, all the other strains fulfilled the requirements defined for PUFA content by the European Standard UNE-EN 14214. Moreover, in all strains grown on OPE, except for *C. albidus* UCD 68-174, MUFA content was equal or higher than 50%, thus indicating a potential high quality of the derived biodiesel. Conversely, on glycerol, the percentage content in MUFA was lower than 50%, finding apparently in line with the literature, although in other works carried out on glycerol MUFA content was over the 50% of total FAME (André et al., 2009; Tchakouteu et al., 2015).

Concerning values of total FAME production, as already mentioned, the highest performances were achieved for all strains on OPE. Biodiesel yield, instead, varied without a precise trend on the two substrates and between different species, except for *C. albidus* UCD 68-174 in which FAME content remained constant at $22\pm 2\%$ and for *R. toruloides* NRRL1091 where a slightly increased amount was observed (from $51\pm 5\%$ on glycerol to $58\pm 5\%$ on OPE).

For *C. curvatus* NRRL Y-1511 and *L. starkeii* NRRL 11557, biodiesel yield was higher on OPE while for *C. laurentii* UCD 68-201, *R. glutinis* UCD 68-255 and *Yarrowia lipolytica* NRRL YB-423, it was higher on glycerol medium.

Unfortunately, it is difficult to understand the reason behind these differences or make comparisons with other works since biodiesel yield is a poorly studied parameter and in most of the studies, total lipids produced are directly quantified as FAME.

6.5 Transfer to bioreactor scale

On the basis of the results obtained by screening on glycerol- and OPE-based media and taking into account the specific composition of lipids produced and the biodiesel yield achieved for each strain, *R. toruloides* NRRL Y-1091 was selected to evaluate the fermentative process transfer from flask to a conventional 3-L stirred-tank benchtop bioreactor. In fact, *R. toruloides* emerged from the other strains for the highest volumetric production of lipids, achieving on OPE $5.74 \pm 0.05 \text{ g L}^{-1}$ and demonstrating superior efficiency in converting the carbon source into lipids, with a lipid yield $Y_{P/S}$ of 0.31 g g^{-1} . OPE was chosen as the reference substrate for this selection since, on glycerol, volumetric biomass and lipid productions were significantly lower. *C. laurentii* UCD 68-201 also demonstrated a remarkable ability of producing lipids on OPE but it was not further investigated in this study for the not so good performance on glycerol, since our purpose was not just a fermentation in bioreactor in batch mode, but also a fed-batch using a mixed substrate OPE-glycerol. In fact, OPE, while having the advantage of containing easily assimilable sugars, mineral salts and vitamins that foster yeast growth, contains a relative diluted concentration of carbon source, which would not allow to achieve high cell density cultivations. Glycerol, instead, being a pure carbon source, represents a great way to accurately regulate the C/N ratio of the medium and, at the same time, to increase the content of the carbon source. Furthermore, as already mentioned, it seems that glycerol, in combination with sugars, can promote lipid accumulation (Galafassi et al., 2012; Rakicka et al., 2015).

Previous studies in bioreactor with *R. toruloides* have demonstrated the ability of the species to obtain high-density cultivation using glucose as carbon source (Li et al., 2007; Zhao et al., 2011), which is, however, an expensive carbon source. Satisfactory scale-up on a pilot scale has also been achieved using sugarcane juice as growth medium (Soccol et al., 2017), a low-cost substrate, widespread in sugarcane producing countries, such as Brazil, India or China, but to a lesser extent in Europe (Zhao and Li, 2015), where the same circular economy principles would indicate other local waste.

The approach used in the present work at first involved a batch cultivation in bioreactor of *R. toruloides* on OPE, which also represents the substrate used in the initial growth phase of fed-batch fermentations, in order to estimate fermentation kinetic parameters. Then, fed-

batch cultivations were planned on the basis of the uptake rate of sugars observed and the effect of different C/N ratios and different glycerol concentrations.

6.5.1 Batch cultivation of *R. toruloides* NRRL 1091 on OPE.

The time courses of biomass and lipid production and of sugars consumption are shown in Fig. 6.3A, together with time course of nitrogen consumption and dissolved oxygen concentration, calculated as % of saturation with respect to the initial time of fermentation.

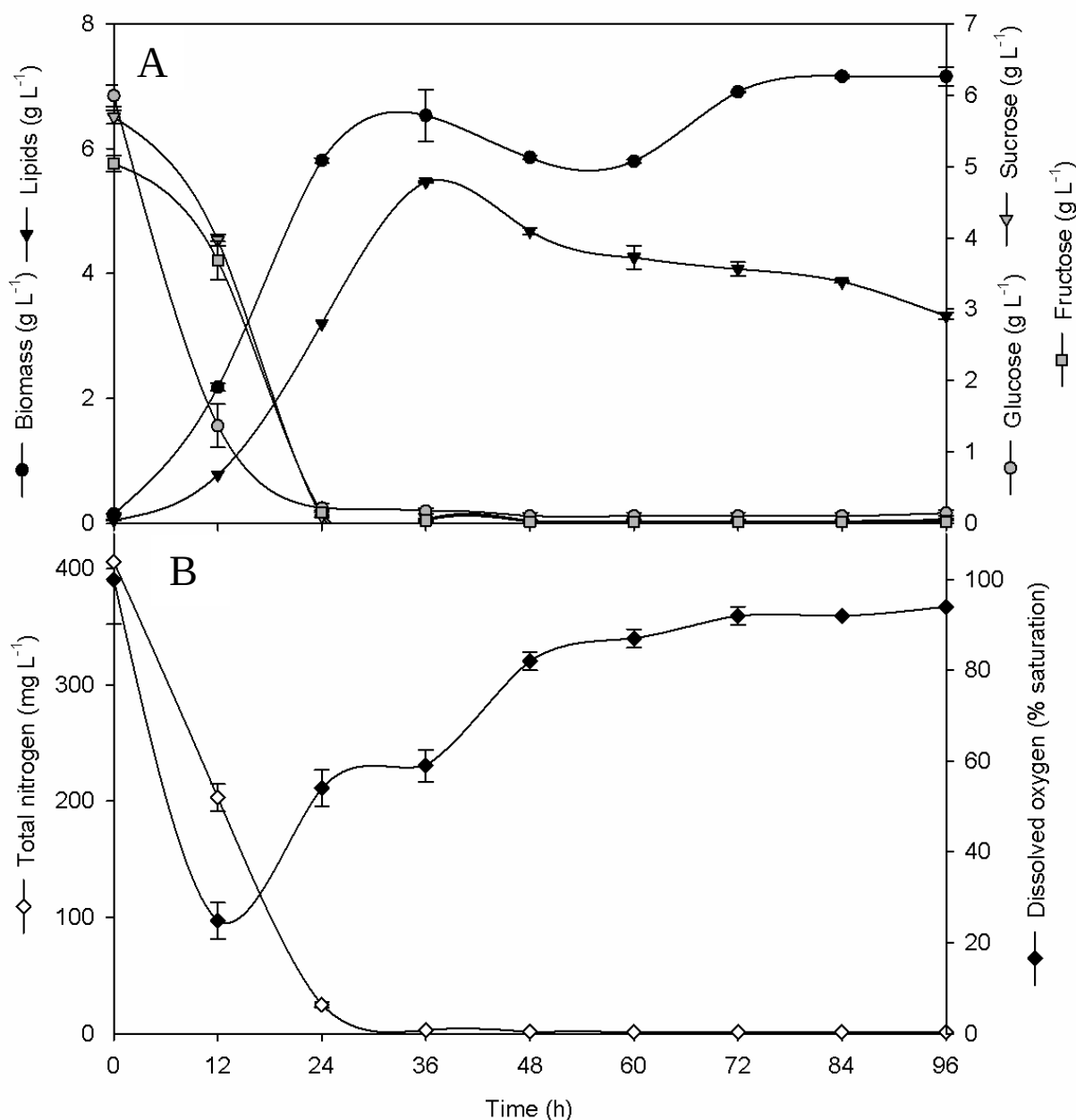


Fig. 6.3 Time course of lipid and biomass production and glucose, sucrose and fructose consumption (plot A) by *R. toruloides* grown under batch fermentation on OPE; in plot B, consumption of nitrogen and dissolved oxygen (% saturation). Data are the means $\pm$ standard deviations of duplicate reactor experiments.

In Tab. 6.3, kinetic parameters calculated at the time of maximum lipid production and fatty acid composition of the oil produced are reported.

Despite a low initial inoculum, equal to an optical density of 0.2, the growth rate was very high already in the early fermentation times, reaching a value of 0.18 g/(l·h), and a corresponding biomass concentration of 6.53 ± 0.4 g L⁻¹ at 36 hours.

Table 6.3. Performance indicators of *R. toruloides* in batch-cultivation, including yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) and biodiesel and biomass production rates (rp and rX, respectively), N and total sugars consumption rates (rN, rS) and percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

Parameter	Dimension unit	Batch fermentation values
Yields §		
Biodiesel yield	(%)	36.90±3.58
$Y_{P/X}^{\dagger}$	(g g ⁻¹)	0.27±0.04
$Y_{P/S}^{\dagger}$	(g g ⁻¹)	0.12±0.01
$Y_{X/S}$	(g g ⁻¹)	0.43±0.04
Rates §		
rP [‡]	g L ⁻¹ h ⁻¹	0.06±0.00
rS	g L ⁻¹ h ⁻¹	0.47±0.00
rN	mg L ⁻¹ h ⁻¹	8.22±0.09
rX	g L ⁻¹ h ⁻¹	0.18±0.00
Lipid profile	†‡	
Palmitoleic acid	(%)	1.13±0.11
Palmitic acid	(%)	20.94±1.21
Linoleic acid	(%)	25.34±0.67
Oleic acid	(%)	42.06±1.17
Stearic acid	(%)	3.84±0.12
Other SFA	(%)	4.04±0.35
Other MUFA	(%)	2.38±0.30
Other PUFA	(%)	0.28±0.04
Total SFA	(%)	28.81±1.39
Total MUFA	(%)	45.57±0.82

§Data are the means ± standard deviations of duplicate reactor experiments; †Calculated on the basis of the biodiesel yield; ‡Data are the means ± standard deviations of 4 chromatographic runs (2 technical replicates for each reactor experiment); ‡Predominant fatty acids are listed as a function of increasing retention time.

Rapid exponential phase was also accompanied by a net decrease in dissolved oxygen, reaching about the 20% at 12 hours and slowly increasing again during lipid accumulation phase to return close to the initial fermentation values during the stationary phase, as a clear signal of the end of any fermentative process, except for the maintenance of cell themselves. Likewise, lipid production also started very early, indicating that the initial amount of nitrogen supplied by both the protein component contained in OPE and by $(\text{NH}_4)_2\text{SO}_4$ addition was rapidly depleted already within the first 12 hours. Such a fast nitrogen source consumption pattern is not surprising since it has been already described in other studies on *R. toruloides* strains (Li et al., 2007; Socol et al., 2017). At 24 hours, lipid content was already 55% on total dry weight and at 36 hours it reached 80%, of which 37% was converted into biodiesel through transesterification reaction, as detected by GC. Such high lipid accumulation values, exceeding 70%, were also observed in fed-batch fermentations of *R. toruloides* on glucose (Wiebe et al., 2012).

Compared to flask fermentations, the peak of production in bioreactor was considerably anticipated (from 72 to 36 hours), thus significantly increasing biomass and lipid productivity and indicating that the best agitation in bioreactor and consequently better exchange of nutrient and oxygen elements had a positive contribution to the outcome of fermentation.

Unlike flask experiment where only total sugars were determined, in bioreactor each sugar was also determined individually, except for maltose, which constituted a minority fraction of total sugars and pectin, that was not consumed.

Although glucose, fructose and sucrose were all rapidly metabolized at a rate of $0.47 \text{ g L}^{-1} \text{ h}^{-1}$ considered together, a sequential utilization emerged, with a quick exhaustion of glucose within the first 12 hours and of the other residual sugars at 24 hours.

Concerning the fatty acid profile of lipids produced, a difference was observed between flask and bioreactor experiments. C18:1 and C16:0 contents were both 10% lower in bioreactor, in favour of a 20% increase of C18:2, thus resulting in an oil with a higher content in PUFA and less suitable for biodiesel production. However, since this change of composition was influenced by the different fermentation conditions, before going to investigate the reasons for that change, it was decided to check first the lipid profile obtained in fed-batch mode; feeding strategy, in fact, is more interesting for lipid production and can also affect the lipid profile.

6.5.2 Fed-batch fermentations

In fed-batch cultivations, some changes were done in order to speed up the initial batch phase and taking into account that the purpose of the initial batch phase was to foster a fast growth and not lipid accumulation. For this reason, a higher inoculum density ($OD=1$) and a more concentrated extract (OPE200) were used, and the initial C/N ratio was decreased from 25 to 15 by adding a higher amount of $(NH_4)_2SO_4$, since in the previous batch fermentation, nitrogen was exhausted within the first 24 hours. Furthermore, dissolved oxygen in the medium was not just monitored, but controlled and kept over the 20% of saturation by increasing the agitation rate, when necessary, in order to prevent it from being a limiting factor.

The configuration of bioreactor, bio-controller, peristaltic pumps used for both pH control (pump A) and addition of feeding solutions (pump B) in a continuous mode, is shown in Fig. 6.4.

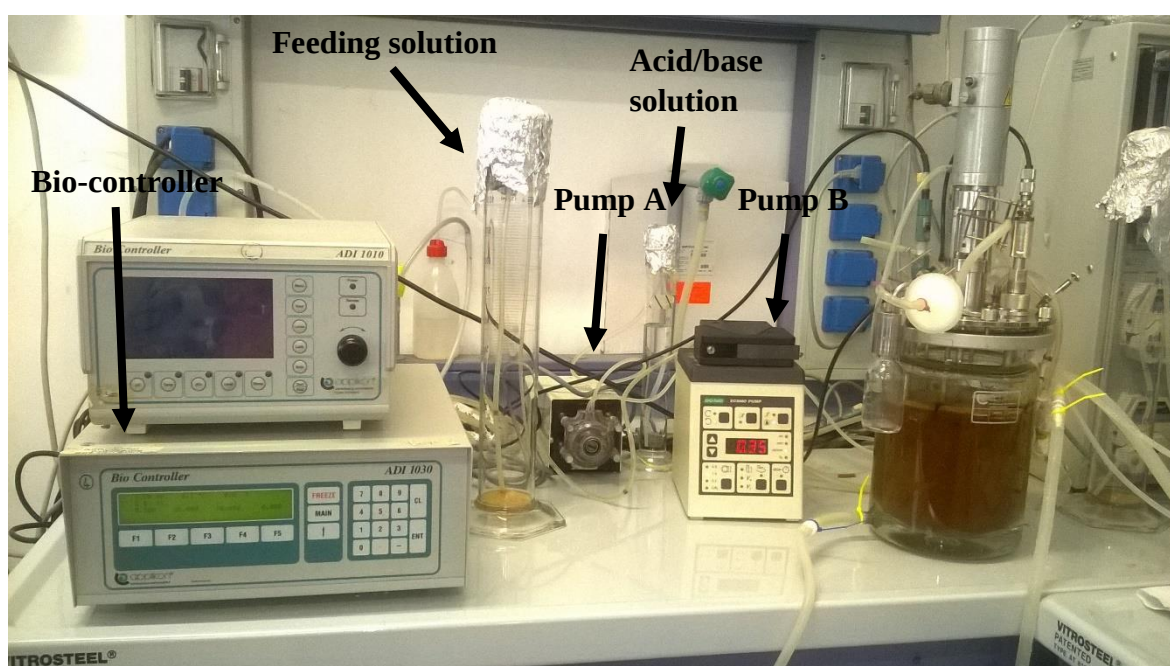


Figure 6.2. Configuration of bioreactor, bio-controller, peristaltic pumps, feeding solution and acid/base solution for pH correction during fermentation.

6.5.2.1 First fed-batch trial

Aim of this experiment was to evaluate the response of *R. toruloides* to 3 different C/N ratios, achieved by changing the composition of feeding solution. More specifically, the initial batch phase was performed on 1.2 l of OPE200 supplemented with $(NH_4)_2SO_4$ to reach a C/N ratio 15. After 24 hours, when residual sugars were about 5 g L^{-1} , the culture was fed with a solution containing 80% (v/v) OPE and 20% (v/v) glycerol, with 20 C/N ratio, using

a peristaltic pump in order to extend the yeast exponential phase and achieve a higher cell density. For both feeding phases, D was set at 0.1.

Time courses of biomass and lipids production and substrate consumption are plotted in Fig. 6.5.

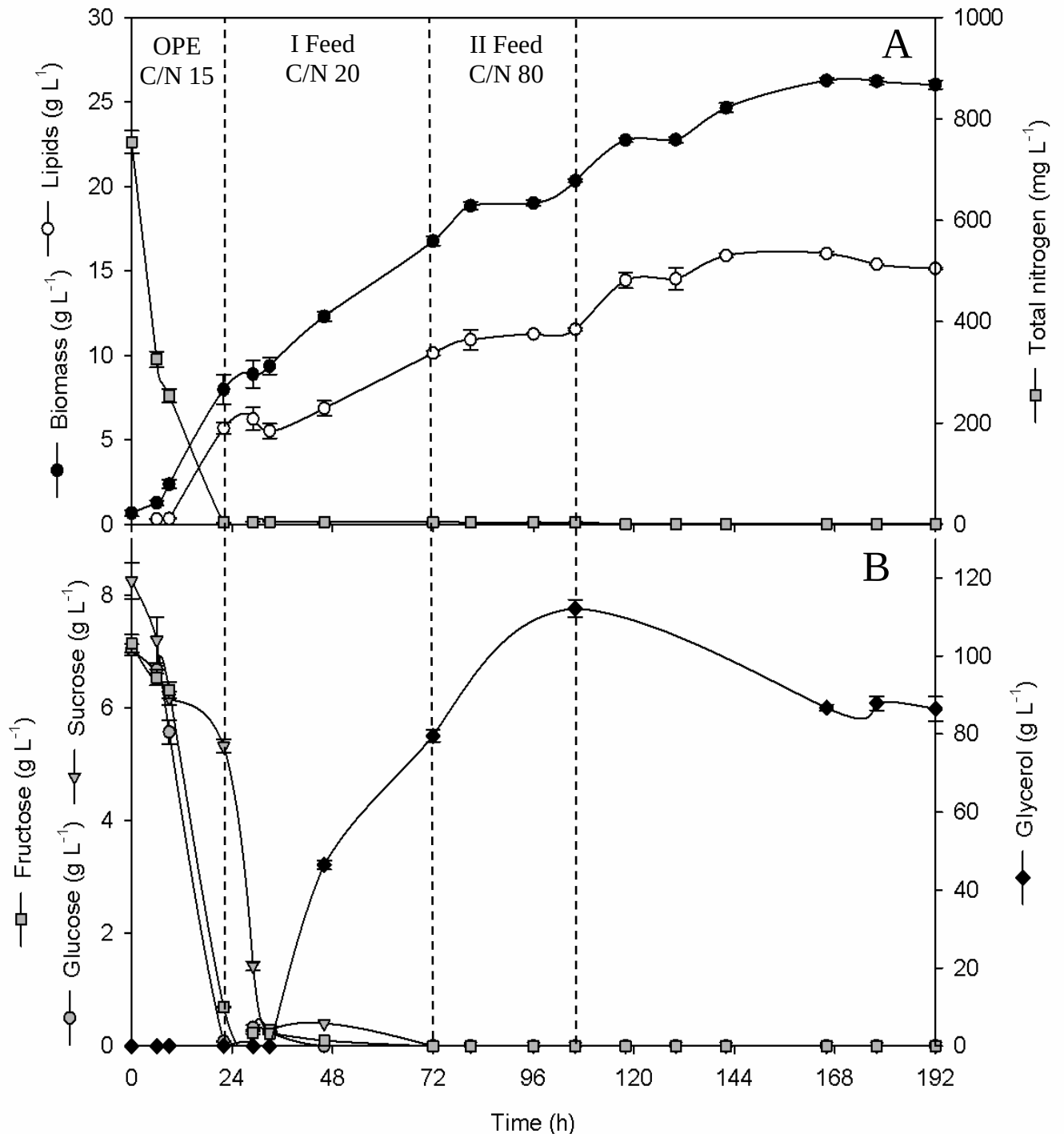


Figure 6.5. *R. toruloides* fed-batch fermentation (first fed-batch trial) time courses of lipid and biomass production and nitrogen consumption in plot A; glucose, sucrose fructose and glycerol consumption in plot B. Data are the means $\pm$ standard deviations of duplicate reactor experiments. Between 0-24 hours growth medium was composed by the only OPE, C/N ratio 15. I feed: feeding solution composed by 20% glycerol and 80% OPE, C/N ratio 20. II feed: 20% glycerol and 80% OPE, C/N ratio 80. After 108 hours no more feeding was added.

The increase of inoculum density and extract concentration led, already within the first 24 hours, to $7.99 \pm 0.89 \text{ g L}^{-1}$ of biomass production with the concomitant exhaustion of glucose.

Sucrose, as previously observed, was consumed more slowly and after consumption of the other two sugars, which induced a very early lipid accumulation, equal to 70% (w/w) on dry weight, thus showing faster kinetics than batch fermentations. A really similar pattern of sugar utilization has also been observed in other species belonging to *Rhodospiridium* genus (Patel et al., 2015), and anyway its slower utilization is predictable since it requires one more step, with respect to glucose and fructose, involving invertase production and disaccharide hydrolysis.

Unexpectedly, the rapid depletion of nitrogen before the onset of feeding promoted lipid accumulation which reached the highest value of the entire fermentation time, equal to $71 \pm 2\%$ on cell dry weight.

During the first feeding phase, biomass further increased, reaching $16.78 \pm 0.27 \text{ g L}^{-1}$ at 72 hours. At the same time, also lipid accumulation continued, though lipid content on cell dry weight slightly decreased to $60 \pm 6\%$ (w/w). With respect to the initial growth phase, biomass productivity decreased from $0.30 \text{ g/(l}\cdot\text{h)}$ to $0.18 \text{ g/(l}\cdot\text{h)}$ and lipids productivity decreased from $0.24 \text{ g/(l}\cdot\text{h)}$ to $0.09 \text{ g/(l}\cdot\text{h)}$. Unfortunately, an aspect that clearly emerges from plot in Fig. 5 is that glycerol, up to 48 hours, instead of its utilization for favouring biomass and/or lipid production, was not used at all by the yeast and increased linearly with the feeding process. After 48 hours, the slope of this trend slightly decreases, indicating that *R. toruloides* slowly started to activate the metabolic machinery for glycerol utilization (consumption rate $0.24 \text{ g L}^{-1} \text{ h}^{-1}$); most likely the sugars supplied by OPE200 were no longer able to satisfy the demand for a growing cell density. This is plausible since from 48 hours of fermentation onwards, no more glucose and fructose were detected in the medium and also sucrose reached 0.4 g L^{-1} , to reach values close to 0 g L^{-1} at 72 hours, meaning that biomass was able to metabolize all sugars supplied with the feeding.

Diauxic growth has already been observed in *R. toruloides* cultivated on a mixed glucose-glycerol substrate, where glycerol was consumed only after glucose exhaustion (Bommareddy et al., 2015). However, despite of the observed catabolite repression, in that study, the addition of glycerol significantly enhanced the lipid yield on glucose and lipid productivity. In the same study, it was also observed that sugars were consumed when more nitrogen was available, while glycerol was consumed during nitrogen limitation.

In line with these observations, in the second feeding phase of our fermentation at a lower nitrogen concentration and a C/N ratio of 80, a higher consumption rate of glycerol was

observed, which significantly increased after the end of the second feeding phase, when no nitrogen source has been supplied anymore.

However, in spite of the partial use of glycerol during this phase, both biomass and lipid productivity decreased to the same extent. Therefore, lipid content remained constant at 60% (w/w), but a general slowdown in productions was observed, indicating that this C/N ratio was able to maintain the culture at a steady state preventing cell death, but was not conducive to lipid production.

After the end of the second feeding phase, despite a constant biomass productivity, an increase in lipid productivity (from 0.04 to 0.07 g L⁻¹ h⁻¹), compared to the second feeding phase, was observed. This was probably triggered by the complete exhaustion of nitrogen, which promoted a better utilization of glycerol for lipid production, with a consumption rate increased to 0.43 g L⁻¹ h⁻¹. Despite that, 80 g L⁻¹ of glycerol remained unconsumed.

In Tab 6.4, fatty acid composition along fermentation time is reported in order to understand if lipids were suitable for biodiesel production in every phase of fermentation. These data show an increase along time of C 18:1 and C18:0 content, at the expense of C16:0 and C18:2.

Tab. 6.4 Fatty acid composition of *R. toruloides* fed-batch fermentation, experiment 1. No other MUFA or PUFA were detected.

Cultivation time (h)	C16:1	C16:0	C18:2	C18:1	C18:0	Other SFA
22	0.63±0.03 ^a	32.18±0.23 ^b	13.50±0.07 ^b	43.98±0.10 ^a	6.06±0.26 ^a	3.65±0.17 ^c
72	0.58±0.09 ^a	26.20±2.74 ^a	10.29±0.37 ^a	50.95±2.02 ^b	9.04±1.00 ^b	2.93±0.56 ^b
106	0.65±0.04 ^a	27.40±2.38 ^a	9.83±0.04 ^a	50.58±0.16 ^b	9.03±0.30 ^b	2.51±0.04 ^b
166	0.66±0.08 ^a	24.28±1.34 ^a	8.84±2.99 ^a	54.67±1.22 ^c	9.73±0.28 ^b	1.78±0.20 ^a

Data are the means ± standard deviations of 6 chromatographic runs (3 technical replicates for each reactor experiment). Multiple pair-wise comparisons of percentage concentration of each fatty acid along time were performed by the Tukey test (P≤0.05). Same letters indicate lack of statistically significant difference (P≤0.05).

Tab. 6.5. Estimated values of iodine value (IV) and cetane number (CN) of oils produced by *R. toruloides* in fed-batch fermentation, experiment 1 along time

Cultivation time (h)	IV (g I ₂ /100 g)	CN
22	64.63	58.22
72	65.04	58.31
106	63.94	58.55
166	65.83	58.20
Standard EN14214	120 max	51 min

However, despite the differences mentioned in fatty acids composition over time, according to the estimated value in Tab. 6.5, FAMES produced at any fermentation time meets the parameters of the European Standard.

6.5.2.2 Second fed-batch trial

Taking into account the results obtained in the previous fed-batch experiment, a new fed-batch fermentation was planned, whose time course is plotted in Fig. 6.6. This time, only one feeding solution was supplied, with a C/N ratio 20, since it demonstrated to be more suitable than 80, inducing a higher biomass and lipid productivity.

Although a so low C/N ratio shouldn't induce lipid accumulation, a similar response has been seen in another study on *R. toruloides* grown in fed-batch mode on Jerusalem artichoke extract or hydrolysate (42 and 18 C/N ratio, respectively); the highest lipid content (56.5%, w/w) on cell dry weight was observed, in fact, when the culture was fed with the hydrolysate (Zhao et al., 2010).

Additional modification with respect to previous fed-batch included a lower glycerol concentration in the feeding solution (15 and 85%,v/v, of glycerol and OPE200, respectively) and a shorter feeding phase obtained by increasing the D to 0.02 h⁻¹.

As a matter of fact, in the first trial, the decrease in biomass productivity was certainly due to an insufficient sugar intake since, in this phase, *R. toruloides* did not metabolize the carbon sources deriving from glycerol. For this reason, the increase of OPE200 percentage in the feeding substrate in the second trial, should overcome this limitation.

During first batch phase (0 - 22h), biomass and lipid productivity reached similar values to the first trial, being 0.30 and 0.20 g L⁻¹ h⁻¹, respectively. Glucose and fructose, as previously observed, were almost completely consumed at 22 hours, while sucrose was consumed more slowly. When feeding was started, both biomass and lipids continued to be produced but at a slower rate with respect to the first batch phase, reaching a volumetric concentration of 14.84±0.06 and 10.43±0.15 g L⁻¹, respectively.

Even though the faster inflow of nutrients (until 72 hours) should have favoured faster kinetics of growth and lipid accumulation were really similar between the two experiments. The only difference was a lower accumulation of unused glycerol in the medium and a better metabolization of it already at 48 hours, with a consumption rate equal to 0.67 g L⁻¹ h⁻¹

A significant difference can be noted, instead, after the end of the feeding phase probably because of a sudden change in conditions, without passing through an intermediate stage where the nitrogen was limited but still present, thus causing a higher stress for the microorganism. During this final phase, lipid productivity increased from 0.12 to 0.17 g L⁻¹ h⁻¹ (at 0-72 and 72-104 h, respectively) returning to values close to the initial ones.

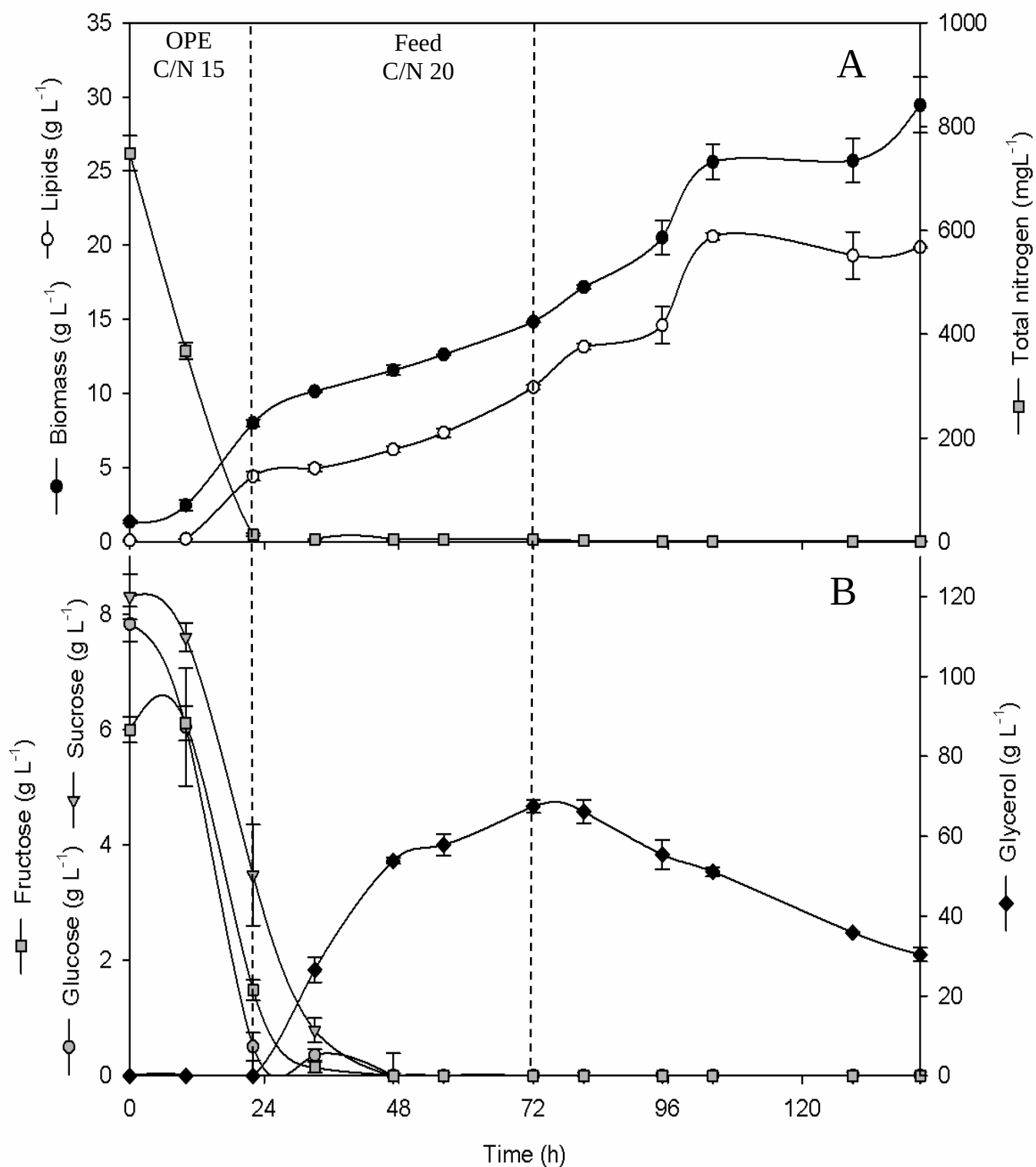


Figure 6.6. *R. toruloides* fed-batch fermentation (second fed-batch trial) time courses of lipid and biomass production and nitrogen consumption in plot A; glucose, sucrose fructose and glycerol consumption in plot B. Data are the means $\pm$ standard deviations of duplicate reactor experiments. Between 0-22 hours growth medium was composed by the only OPE, C/N ratio 15. Feed: feeding solution composed by 20% glycerol and 80% OPE, C/N ratio 20. After 72 hours no more feeding was added to the bioreactor.

Table 6.5 Fatty acid composition of *R. toruloides* fed-batch fermentation, experiment 2. No other MUFA or PUFA were detected.

Cultivation time (h)	C16:1	C16:0	C18:2	C18:1	C18:0	Other SFA
22	0.36±0.07 ^a	27.14±0.55 ^b	13.82±0.25 ^c	49.29±0.43 ^a	7.00±0.25 ^a	2.52±0.20 ^a
72	0.53±0.06 ^b	24.17±0.46 ^a	9.74±0.74 ^a	55.15±0.69 ^b	8.27±0.40 ^b	2.15±0.08 ^a
104	0.52±0.02 ^b	23.45±0.06 ^a	10.11±0.70 ^b	54.99±0.92 ^b	8.82±0.25 ^b	2.11±0.05 ^a
141	0.55±0.03 ^b	23.65±0.10 ^a	10.67±0.44 ^b	54.66±0.73 ^b	8.45±0.21 ^b	2.02±0.01 ^a

Data are the means ± SD of 6 chromatographic runs (3 technical replicates for each reactor experiment). Multiple pair-wise comparisons of percentage concentration of each fatty acid along time were performed by the Tukey test ($P \leq 0.05$). Same letters indicate lack of statistically significant difference ($P \leq 0.05$).

At 104 hours, corresponding to the peak of maximum lipid production, $25.62 \pm 1.2 \text{ g L}^{-1}$ of biomass and $20.6 \pm 0.23 \text{ g L}^{-1}$ of lipids were produced, with a net improvement compared to previous experiments, both in batch and fed-batch.

As concerns the profile of fatty acids produced over time, reported in Tab. 6.5, the composition and trend were really similar to those observed in experiment 1, thus in agreement with the requirements of the Standard EN14214.

A comparative table of performance indicators of biodiesel production among batch and fed-batch experiments 1 and 2, is reported below (Tab. 6.6), including the overall yields, fatty acids profiles and production rates calculated at the time of maximum lipid production.

Looking at Tab. 6, one of the major improvements was undoubtedly biodiesel yield which passed from a 37% to about 90% when glycerol was added in the culture medium.

It would be interesting to compare these data with those ones obtained by other works but unfortunately, biodiesel yield is a parameter rarely taken into account. This is due to the fact that many works estimate lipid concentration directly as FAME, without quantifying them before the transesterification reaction. The net improvement could be due to the fact that glycerol, although having had some difficulties to be metabolized, contributed to increasing the proportion of TAG on total lipids, thus improving the efficiency of the conversion reaction.

Looking at biodiesel and biomass yields ($Y_{P/S}$, $Y_{X/S}$) on substrate, an enhancement was also observed, reaching in experiment 2, values really close to the theoretical ones.

With respect to other works, production rate of lipids (r_P , considered as FAME) was lower, being 0.38 when *R. toruloides* Y4 was grown in fed-batch on Jerusalem artichoke hydrolysates (Zhao et al., 2010) and 0.54 when the same strain was fed with glucose (Li et al., 2007).

Table 6.6. Comparison of performance indicators of biodiesel production process in STR by *R. toruloides* in batch and fed-batch experiments, including yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) and biodiesel and biomass production rates (r_P and r_X , respectively), N and total sugars consumption rates (r_N , r_S) and percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

Parameter	Dimension unit	Batch fermentation values	Fed-batch 1 fermentation values	Fed-batch 2 fermentation values
Yields §				
Biodiesel yield	(%)	36.90±3.58 ^a	93.54±1.48 ^c	88.00±2.68 ^b
$Y_{P/X}†$	(g g ⁻¹)	0.27±0.04 ^a	0.57±0.01 ^b	0.71±0.00 ^c
$Y_{P/S}†$	(g g ⁻¹)	0.12±0.01 ^a	0.16±0.00 ^b	0.31±0.00 ^c
$Y_{X/S}$	(g g ⁻¹)	0.43±0.04 ^b	0.28±0.00 ^a	0.44±0.00 ^b
Rates §				
$r_P†$	g L ⁻¹ h ⁻¹	0.06±0.00 ^a	0.09±0.00 ^a	0.17±0.00 ^b
r_S	g L ⁻¹ h ⁻¹	0.47±0.00 ^a	0.57±0.00 ^b	0.56±0.00 ^b
r_N	mg L ⁻¹ h ⁻¹	8.22±0.09 ^a	16.14±3.20 ^b	23.37±2.10 ^c
r_X	g L ⁻¹ h ⁻¹	0.18±0.00 ^a	0.16±0.00 ^a	0.25±0.00 ^b
Lipid profile	†‡			
Palmitoleic acid	(%)	1.13±0.11 ^c	0.66±0.08 ^b	0.52±0.02 ^a
Palmitic acid	(%)	20.94±1.21 ^a	24.28±1.34 ^b	23.45±0.06 ^b
Linoleic acid	(%)	25.34±0.67 ^b	8.84±2.99 ^a	10.11±0.70 ^a
Oleic acid	(%)	42.06±1.17 ^a	54.67±1.22 ^b	54.99±0.92 ^b
Stearic acid	(%)	3.84±0.12 ^a	9.73±0.28 ^c	8.82±0.25 ^b
Other SFA	(%)	4.04±0.35 ^b	1.93±0.30 ^a	2.11±0.05 ^a
Other MUFA	(%)	2.38±0.30	-	-
Other PUFA	(%)	0.28±0.04	-	-

§Data are the means±standard deviations of duplicate reactor experiments; †Calculated on the basis of the biodiesel yield; ‡Data are the means ± standard deviations of 6 chromatographic runs (2 technical replicates for each reactor experiment); ‡Predominant fatty acids are listed as a function of increasing retention time. Multiple pair-wise comparisons among yields, rates and single fatty acids of the three different fermentation trials were performed by the Tukey test ($P \leq 0.05$). Same letters indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values (row)

This means that, although the efficiency of conversion of the carbonaceous source into lipids was high, the uptake was slow.

However, this was an expected result, since during feeding phase, biomass and lipid productivity were always lower than in the batch phase. Therefore, this strain of *R. toruloides*, had showed the ability to assimilate sugars but the rate at which they were fed was not enough. A higher D should be used in future experiments or the process of extraction from orange peels should be improved, so as to have a more concentrated substrate.

Furthermore, it has been demonstrated that the rate of uptake of the carbon source is also dependent on the nature of the nitrogen source used (Papanikolaou and Aggelis, 2011b); therefore, by choosing a different compound than $(\text{NH}_4)_2\text{SO}_4$, a better uptake of glycerol could be achieved.

Finally, comparing the fatty acid profiles obtained in the various fermentations, it can be noted that they are really similar when fed-batch strategy was adopted, as already discussed, but significantly different in batch mode. In particular, in the latter, C 18:2 was significantly higher while C18:1 and C18:0 were lower. This could be due to the fact that in batch mode, aeration and agitation rate were kept constant and during exponential phase, dissolved oxygen drastically dropped to values even below 20%. Also in other works on *R. toruloides*, it was observed that oxygen limitation significantly influenced the composition of SFAs and unsaturated fatty acids: oxygen controlled conditions resulted in a fatty acid compositions that was more suitable for biodiesel conversion (Bommareddy et al., 2015).

CHAPTER 7

BIOCONVERSION OF AGRO-INDUSTRIAL WASTE INTO OILS BY FILAMENTOUS FUNGI

A modified version of this chapter has been submitted to Process Safety and Environmental Protection journal (Elsevier) as:

Carota, E., Crognale, S., D'Annibale, A., Petruccioli, M., 2017. Bioconversion of agro-industrial waste into oils by filamentous fungi.

7.1 Introduction and study objectives

Oleaginous filamentous fungi include species belonging predominantly to Zygomycota such as *Mucor*, *Mortierella* and *Cunninghamella* genera (Ferreira et al., 2013) and to a lesser extent to Ascomycota, such as *Aspergillus* genus (Hui et al., 2010a; Venkata Subhash and Venkata Mohan, 2011). These fungi are widely known in industrial food fermentative processes for their ability to produce interesting metabolites such as organic acids, ethanol, and intracellular high-value lipids or to produce enzymes that facilitate the assimilation of various complex substrates, often not metabolizable by bacteria or yeasts, such as cellulases, amylases, lipases, pectinase, phytases and proteases, thus representing a valuable way to valorize agro-industrial waste (Ferreira et al., 2013).

Furthermore, Zygomycota are valuable sources of industrially interesting lipids for food industry, such as linoleic and γ -linolenic acids (ω -6) or eicosapentanoic acid and docosahexaenoic acid (long-chain ω -3). In addition to these high-value added fatty acids, they can also produce more common fatty molecules such as palmitic, palmitoleic, oleic and stearic acids that could be used as renewable and sustainable feedstock for biodiesel or other oleochemicals production. (Bellou et al., 2012).

However, a disadvantage of filamentous fungi with respect to other microorganisms, when cultivated in submerged cultures, is that they can assume different morphologic forms depending on culture conditions, e.g. inoculum size and medium composition, that often significantly affect process performances. Usually the pellet-mode is preferred for industrial applications because it poses less rheological and technical problems (Gibbs et al., 2000), but it is not easy to manage fungal morphology during fermentation processes.

Several strains have been investigated so far on different waste, such as *Mortierella isabellina*, *Thamnidium elegans*, *Cunninghamella elegans* and *Mortierella ramanniana* on raw glycerol (Bellou et al., 2012; Fakas et al., 2009; Papanikolaou et al., 2008; Silva et al., 2012); *M. isabellina* on glucose derived from maize starch hydrolysate (Zhu et al., 2003),

corn stove (Zhang and Hu, 2014), whey treated with lactase, or sweet sorghum (Economou et al., 2010); several strains belonging to *Cunninghamella* genus on wood hydrolysates and combinations of various agro-industrial waste (e.g. potato peelings, potato pulp, coffee bean slurry) (Lunin et al., 2013); *Microsphaeropsis* sp. on steam-exploded wheat straw mixed with wheat bran (Peng and Chen, 2008); *M. circinelloides* on corn thin stillage derived from corn ethanol production (Mitra et al., 2012) or *C. echinulata* on orange peel in solid state fermentation (Gema et al., 2002). Fewer studies have been conducted instead on *Aspergillus* genus which is more interesting for lipase production to catalyze the transesterification of triacylglycerols (Adachi et al., 2011), rather than for lipid production, since low levels of accumulation, below 25%, have been obtained so far (Hui et al., 2010b; Venkata Subhash and Venkata Mohan, 2011).

In this study, 9 strains of filamentous fungi belonging to the genera *Aspergillus*, *Cunninghamella*, *Mortierella* and *Mucor* were investigated for their ability to produce intracellular lipids when grown on three different widespread agro-industrial waste, containing different carbon sources: glycerol, OPE and RCW.

Glycerol is a by-product of several oleo chemical processes involving the transesterification of vegetable oils and animal fats (e.g. biodiesel production), and to a lesser extent, of bioethanol and alcoholic beverage industries (Tchakouteu et al., 2015).

RCW is a by-product of dairy industry derived from ricotta cheese production composed, on average, of 4.8-5.0% lactose, 1.0-1.3% salts, 0.15-0.22% proteins, 0.20-0.25% organic acids and 0.20% fats. (Sansonetti et al., 2009).

Orange peel is a widespread waste deriving from juice manufacturing, containing, as main sugars, glucose, fructose and sucrose (Balu et al., 2012). In this project, aqueous extract of de-oiled orange peels, was used as growth substrate for submerged fermentations.

In a first phase, the ability of assimilating and accumulating lipids on the above-mentioned substrates were assessed in shaken flasks. Based on biomass and lipids yields and rates obtained, the most versatile strain was further investigated in stirred-tank bioreactor in order to evaluate the technical feasibility of a first scale-up process.

Except for glycerol, which has been studied more extensively, OPE and RCW have never been tested in submerged fermentations involving filamentous fungi. To our knowledge, only two works have been performed on whey, in one of which an enzymatic pre-treatment was performed in order to release *D*-galactose and *D*-glucose, which can be more easily metabolized.

Since this is a preliminary study to assess the suitability of the above mentioned substrates and the quantity and quality of lipids produced, the possible industrial applications will be discussed on the basis of specific fatty acids produced.

7.2 Materials and methods

7.2.1 Microbial strains, maintenance and inoculum preparation

Microbial strains used in this study are listed in “General Materials and Methods” chapter, paragraph 4.1.2 “Mould strains and maintenance”.

Pre-inocula for shaken flask and bioreactor experiments were prepared by growing 10^6 spores mL^{-1} in 250-ml flasks containing 50 ml of PDB medium, at 30 °C for 3 days in shaken culture (185 rpm).

7.2.2 Growth media

Air-dried orange peels were crushed using a blender and then subjected to extraction at 100 °C in distilled water for 1 hour. The aqueous extract thus obtained, after centrifugation and filtration through Whatman filter n. 41, had a dry weight of $25.43 \pm 0.54 \text{ g L}^{-1}$ composed of $39.85 \pm 0.15\%$ total carbon and $1.32 \pm 0.02\%$ total nitrogen determined by elemental analyser. A small amount of $(\text{NH}_4)_2\text{SO}_4$, was added after autoclave sterilization, passing from a C/N ratio of 31 to 25, in order to ensure a rapid initial growth and allow the microorganisms to adapt quickly to the new substrate after inoculation. Substrate composition, determined by enzymatic kits (Megazyme International Ireland Ltd, Wicklow, Ireland), was as follows (g L^{-1}): glucose, 5.68 ± 0.17 ; sucrose, 5.98 ± 0.21 ; fructose, 5.04 ± 0.17 ; mannose, 1.08 ± 0.22 and pectin, 1.78 ± 0.14 .

Glycerol-based medium had the following composition (g L^{-1}): glycerol, 20; $(\text{NH}_4)_2\text{SO}_4$, 0.5; yeast extract (containing 10% total nitrogen) 1.0; NaH_2PO_4 , 3.0 and KH_2PO_4 , 0.7. After sterilization in autoclave, 0.1% of filter sterilized 1000 X concentrated mineral salt solution was added; this solution at pH 5.5 contained (g L^{-1}): ZnSO_4 , 4.4; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.1; CuSO_4 , 0.315; CaCl_2 , 1.47; FeSO_4 1.0.

RCW was collected from a cheese processing plant (Formaggi Boccea S.r.l., Rome, Italy) and stored at -20 °C until used. RCW had the following characteristics (g L^{-1}): dry weight, 48.2 ± 4.10 ; lactose, 40.2 ± 0.8 ; galactose, 1.6 ± 0.2 , total nitrogen, 0.053 ± 0.04 ; protein, 0.008 ± 0.001 ; C/N, 307; ash, 4.5 ± 0.4 . Initial pH was 5.8. After thawing, the RCW was centrifuged ($8000 \times g$, 15min), two-fold diluted with deionized water, added with $(\text{NH}_4)_2\text{SO}_4$ so as to reach a C/N ratio of 25 and finally its pH was adjusted to 5.5 with 0.1 M NaOH.

7.2.3 Culture conditions

7.2.3.1 *Shaken flask experiments*

All experiments in shaken culture were performed in 250-mL Erlenmeyer flasks containing 45 mL of growth medium inoculated with 5 mL of pre-inoculum. Cultures were incubated in an orbital shaker (185 rpm) at 30 °C for 5 d. Samples were collected on a daily basis for analysis.

7.2.3.2 *Bioreactor experiments*

Bioreactor fermentations were carried out as described in section 4.1.3 "Batch bioreactor experiments", using a fixed agitation speed at 450 rpm.

7.2.4 Analytical methods

Dry weight was determined gravimetrically after vacuum filtering the biomass, washing it with sterile water three times and freeze-drying for 72 hours. The other methods used are described in section 4.2 "Analytical methods". Quantification of glycerol in flask and in bioreactor experiments and quantification of glucose, fructose, sucrose, maltose in bioreactor experiments involving OPE as growth medium, were performed by enzymatic kits as described in section "6.2.4.1 Glucose, fructose, sucrose, maltose and glycerol". Quantification of pectin in OPE medium was performed according to section 6.2.4.2 "Pectin".

7.2.5 Determination of yields and rates

Yields and rates calculation are reported in paragraph 4.2 "Determination of yields and rates".

7.3 Results and discussion

Results obtained using the three substrates will be first discussed separately and then a comparison among them will be done in order to evaluate the versatility of the different strains on different carbon sources.

7.3.1 Screening of fungal strains on glycerol

Values of growth in shaken flask on glycerol, including volumetric biomass (X) and lipid (P) production at the time of maximum lipid accumulation (t), together with yields of biomass on substrate ($Y_{X/S}$), of lipids ($Y_{P/X}$) on biomass and of lipids on substrate ($Y_{L/S}$) are reported in Tab. 7.1.

Except for *Chaetomium* sp. LAS_G, which poorly metabolized glycerol leaving most of it unconsumed in the medium, all the other strains were able to widely grow on it, with the highest values of biomass produced by *M. circinelloides* and *C. echinulate*, which also showed a high conversion yield $Y_{X/S}$. Nevertheless, lipid accumulation was not stimulated as expected, except for *M. isabellina*, *M. moelleri* and *M. racemosus*, which exceeded 20% of intracellular lipid accumulation with 1.99 ± 0.07 , 2.10 ± 0.20 and 2.33 ± 0.21 g L⁻¹ of lipids produced, respectively. All the other species resulted in lower percentage lipid content, below the threshold according to which it is possible to speak of lipid accumulation (Rossi et al., 2010).

Table 7.1

Volumetric biomass (X) and lipid (P) productions and yield parameters ($Y_{X/S}$, $Y_{L/X}$, $Y_{P/S}$) calculated at the time of maximal lipid accumulation (t) obtained for each of the 9 filamentous fungi strains grown on glycerol-based medium.

Mould	X (g L ⁻¹)	P (g L ⁻¹)	t (h)	$Y_{X/S}$	$Y_{P/X}$	$Y_{L/S}$
<i>A. tubingensis</i> E1	10.82 ± 0.12^{de}	2.00 ± 0.17^d	96	0.54 ± 0.05^{bc}	0.19 ± 0.02^c	0.10 ± 0.02^e
<i>A. tubingensis</i> NRRL 4700	9.64 ± 0.83^{cd}	1.39 ± 0.12^c	96	0.54 ± 0.09^{bcd}	0.14 ± 0.03^d	0.08 ± 0.01^a
<i>C. echinulata</i> NRRL 3655	11.66 ± 0.14^e	2.12 ± 0.18^{de}	96	0.58 ± 0.06^{cd}	0.18 ± 0.02^d	0.11 ± 0.02^f
<i>Chaetomium</i> sp. LAS_G	3.45 ± 0.13^a	0.28 ± 0.02^a	96	0.36 ± 0.04^a	0.08 ± 0.01^b	0.03 ± 0.00^c
<i>M. isabellina</i> NRRL 1757	7.24 ± 0.37^b	1.99 ± 0.07^d	72	0.36 ± 0.03^a	0.28 ± 0.02^f	0.10 ± 0.01^f
<i>M. circinelloides</i> NRRL 3631	12.07 ± 0.24^e	0.43 ± 0.03^{ab}	72	0.60 ± 0.06^d	0.04 ± 0.00^a	0.02 ± 0.00^b
<i>M. miehei</i> NRRL 3169	6.46 ± 0.88^b	0.65 ± 0.01^b	96	0.36 ± 0.05^a	0.10 ± 0.02^b	0.04 ± 0.00^d
<i>Mucor moelleri</i> LAS_E	8.77 ± 0.61^c	2.10 ± 0.20^{de}	96	0.44 ± 0.07^{ab}	0.24 ± 0.04^e	0.11 ± 0.01^f
<i>M. racemosus</i> UCD 71-20	10.16 ± 0.65^d	2.33 ± 0.21^e	96	0.51 ± 0.08^{bcd}	0.23 ± 0.04^e	0.12 ± 0.00^g

$Y_{X/S}$ =biomass yield; $Y_{P/X}$ =specific lipid yield; $Y_{P/S}$ =lipid yield referred to consumed substrate, calculated by relating total lipids, determined according to Izard and Limberger (2003), to the amounts of total glycerol consumed. Data are the mean $\pm$ SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same superscript letters indicate the lack of statistically significant difference ($P \leq 0.05$) within column means.

These results are in line with other flask screening works performed on filamentous fungi. Using a similar concentration of glycerol used in our work, 6.9 g L⁻¹ of biomass and 25.1% of lipids were obtained in *C. echinulata* and 7.0 g L⁻¹ with 53.1% intracellular lipids in *Mortierella ramanniana*. As a difference, in our study, the time of maximum lipid accumulation was anticipated at 72-96 hours, depending on the strain (Bellou et al., 2012). When 100 g L⁻¹ of glycerol were used, similar yields were obtained anyway, resulting in 8.2

g L⁻¹ of biomass and 19.5% lipid content in *C. echinulata*, 6.2 g L⁻¹ and 53.2% in *M. isabellina* and 7.9 g L⁻¹ and 19.5% in *Mucor* sp. (Fakas et al., 2009).

As already observed in yeasts, low yields at high concentration of glycerol could be due to an inefficient uptake of glycerol (Papanikolaou et al., 2008) or to inhibition when glycerol is used over a certain limit concentration (Fakas et al., 2008; Saenge et al., 2011b).

7.3.2 Screening of fungal strains on OPE

Performances of biomass and lipids produced at the time of maximum lipid accumulation and conversion yields are reported in Tab. 7.2.

Table 7.2. Volumetric biomass (X) and lipid (P) productions and yield parameters (Y_{X/S}, Y_{L/X}, Y_{P/S}) calculated at the time of maximal lipid accumulation (t) obtained for each of the 9 filamentous fungi strains grown on OPE medium.

Mould	X (g L ⁻¹)	P (g L ⁻¹)	t (h)	Y _{X/S}	Y _{P/X}	Y _{L/S}
<i>A. tubingensis</i> E1	11.23±0.13 ^f	1.82±0.08 ^c	96	0.57±0.01 ^d	0.16±0.01 ^a	0.09±0.00 ^c
<i>A. tubingensis</i> NRRL 4700	11.41±0.25 ^f	1.80±0.16 ^c	48	0.56±0.02 ^d	0.16±0.01 ^a	0.09±0.00 ^c
<i>C. echinulata</i> NRRL 3655	4.08±0.03 ^b	1.08±0.04 ^b	48	0.27±0.00 ^b	0.26±0.01 ^{bc}	0.07±0.00 ^b
<i>Chaetomium</i> sp. LAS_G	5.03±0.04 ^c	1.19±0.27 ^b	72	0.26±0.00 ^b	0.24±0.06 ^{bc}	0.06±0.01 ^b
<i>M. isabellina</i> NRRL 1757	11.21±0.35 ^f	3.10±0.16 ^e	72	0.58±0.04 ^d	0.28±0.02 ^c	0.16±0.01 ^f
<i>M. circinelloides</i> NRRL 3631	8.63±0.04 ^e	1.81±0.15 ^c	72	0.47±0.00 ^c	0.21±0.02 ^{ab}	0.10±0.01 ^{de}
<i>M. miehei</i> NRRL 3169	11.05±0.01 ^f	2.23±0.02 ^d	72	0.58±0.00 ^d	0.20±0.00 ^{ab}	0.12±0.00 ^e
<i>Mucor moelleri</i> LAS_E	5.45±0.14 ^d	1.01±0.08 ^b	96	0.28±0.01 ^b	0.18±0.00 ^a	0.05±0.00 ^{ab}
<i>M. racemosus</i> UCD 71-20	2.59±0.23 ^a	0.54±0.20 ^a	96	0.16±0.03 ^a	0.21±0.02 ^{ab}	0.03±0.00 ^a

Y_{X/S}=biomass yield; Y_{P/X}=specific lipid yield; Y_{P/S}=lipid yield referred to consumed sugars, calculated by relating total lipids, determined according to Izard and Limberger (2003), to the amounts of total sugars consumed. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test (P≤0.05). Same superscript letters indicate lack of statistically significant difference within column means.

Among the strains tested, *A. tubingensis* E1, *A. tubingensis* NRRL 4700, *M. isabellina* and *M. miehei* showed a remarkable growth on OPE, demonstrating their capability of efficiently metabolizing sugars present in orange peels, including mainly glucose, sucrose and fructose. Among them, *M. isabellina* emerged from the other strains for the highest lipid production, achieving 3.10±0.16 g L⁻¹ at 72 hours, and the highest conversion yield Y_{L/S} equal to 0.17±0.00 g g⁻¹ of substrate. Previous screenings performed using commercial sugars as substrate for lipid production, already suggested the potential of *M. isabellina* on a substrate

such as OPE, being able to metabolize glucose, fructose and sucrose, corresponding to the main sugars contained in our extract. Results obtained with *C. echinulata*, instead, were more disappointing, since, in the same study, biomass and lipid production on the above mentioned sugars were even higher than those obtained for *M. isabellina* (Chatzifragkou et al., 2010). Anyway, different strains and culture conditions were used, therefore it is not possible to properly compare the results obtained.

7.3.3 Screening of fungal strains on RCW

Biomass and lipid production results obtained at the time of maximum lipid accumulation, are reported in Tab. 7.3.

Main carbon sources contained in RCW-based medium were lactose (20 g L⁻¹) and a small amount of galactose (0.4 g L⁻¹). Unlike a previous screening performed by our group on yeasts on the same substrate (Carota et al., 2017), where only a few species were able to metabolize lactose and galactose, in this case a satisfying substrate utilization was observed for all the fungi tested except for *Chaetomium* sp. LAS_G, resulting only in 3.95±0.88 g L⁻¹ of biomass produced after 96 hours of fermentation.

Table 7.3. Volumetric biomass (X) and lipid (P) productions and yield parameters (Y_{X/S}, Y_{L/X}, Y_{P/S}) calculated at the time of maximal lipid accumulation (t) obtained for each of the 9 filamentous fungi strains grown on RCW medium.

Mould	X (g L ⁻¹)	P (g L ⁻¹)	t (h)	Y _{X/S}	Y _{P/X}	Y _{L/S}
<i>A. tubingensis</i> E1	10.01±0.93 ^d	1.86±0.03 ^b	96	0.58±0.09 ^c	0.19±0.02 ^{bc}	0.11±0.01 ^{cd}
<i>A. tubingensis</i> NRRL 4700	5.85±0.58 ^b	0.75±0.0 ^a	72	0.30±0.05 ^{ab}	0.13±0.02 ^a	0.04±0.00 ^a
<i>C. echinulata</i> NRRL 3655	6.49±0.66 ^{bc}	1.09±0.07 ^a	72	0.35±0.06 ^{ab}	0.17±0.03 ^b	0.06±0.01 ^{abc}
<i>Chaetomium</i> sp. LAS_G	3.95±0.31 ^a	0.98±0.02 ^a	96	0.26±0.03 ^a	0.25±0.02 ^d	0.06±0.00 ^{abc}
<i>M. isabellina</i> NRRL 1757	11.89±0.71 ^e	4.49±0.05 ^d	72	0.59±0.06 ^c	0.38±0.03 ^f	0.22±0.01 ^d
<i>M. circinelloides</i> NRRL 3631	7.61±0.17 ^{cd}	1.60±0.16 ^b	72	0.52±0.04 ^{abc}	0.21±0.03 ^c	0.11±0.02 ^{bcd}
<i>M. miehei</i> NRRL 3169	7.64±0.16 ^{cd}	0.77±0.02 ^a	72	0.51±0.05 ^{abc}	0.10±0.00 ^a	0.05±0.01 ^{ab}
<i>Mucor moelleri</i> LAS_E	6.30±0.71 ^{bc}	0.85±0.09 ^a	72	0.36±0.04 ^{ab}	0.13±0.03 ^a	0.05±0.01 ^{ab}
<i>M. racemosus</i> UCD 71-20	8.68±0.66 ^d	2.63±0.07 ^c	72	0.56±0.08 ^{bc}	0.30±0.03 ^e	0.17±0.02 ^d

Y_{X/S}=biomass yield; Y_{P/X}=specific lipid yield; Y_{P/S}=lipid yield referred to consumed sugars, calculated by relating total lipids, determined according to Izard and Limberger (2003), to the amounts of total sugars consumed. Data are the mean± SD of three replicates. Multiple pair-wise comparisons were performed by the Tukey test (P≤0.05). Same letters in apex near the values indicate lack of statistically significant difference within column means.

Among the other strains, *M. isabellina* was again the most performing, resulting in 11.89 ± 0.71 and 4.49 ± 0.50 g L⁻¹ of volumetric biomass and lipids produced after 72 hours of fermentation. Although only few studies have been conducted on a similar substrate, such as simple lactose (Zhang and Hu, 2014) or cheese whey (Demir et al., 2013), *M. isabellina* has re-confirmed in this screening its capacity to hydrolyse and use lactose, even without pre-treatment with β -galactosidase. Furthermore, it has also demonstrated to channel equally the carbon source both to biomass and lipid production, thus yielding the highest percentage lipid content on dry weight (38% w/w) compared to the other substrates tested.

Anyway, it must be considered that, in this screening only a standard culture condition was tested. Therefore, the strains that have been able to efficiently metabolize RCW, OPE and glycerol and to convey the carbon source more towards biomass synthesis, may have the potential to result in a significantly higher lipid production, if placed in different conditions.

7.3.4 Comparison of lipid profiles

Taking into account the results obtained in shaken flask on all substrates, three strains were selected for a further characterization of the lipid produced by GC, on the basis of their versatility in metabolizing different carbon sources. Percent compositions of fatty methyl esters (FAMEs) and biodiesel yields obtained after transesterification of total lipids produced, are represented in Fig. 7.1.

A. tubingensis was chosen because it was able to efficiently convert all the substrates used in this study. Although lipid accumulation levels were not among the highest, it appears to be possible to increase them, probably by modulating growth conditions. Furthermore, only few studies have been performed on the fatty acid profile of this strains, therefore it was interesting to see if it could produce some interesting fatty acids.

M. isabellina was evidently the strain that showed, on average, the highest accumulation capacity on the different substrates and which also reached the highest values of volumetric lipid production, with 4.49 ± 0.50 g L⁻¹ on RCW and 3.10 ± 0.16 on OPE.

M. racemosus, instead, was in an intermediate position between the other two strains, having demonstrated good performances of both growth and lipid accumulation, especially on glycerol and RCW.

Fatty acids produced were predominantly between C16 and C18, as usually expected in oleaginous fungi (Rossi et al., 2010). The degree of unsaturation, however, proved to be very variable depending on the substrate used. In *A. tubingensis*, C18:2 content (with respect to total FAME) passed from 32% on glycerol, to 11% on OPE and 44% on RCW, at the expense principally of oleic acid, which dropped from 53% on OPE to 15% on RCW.

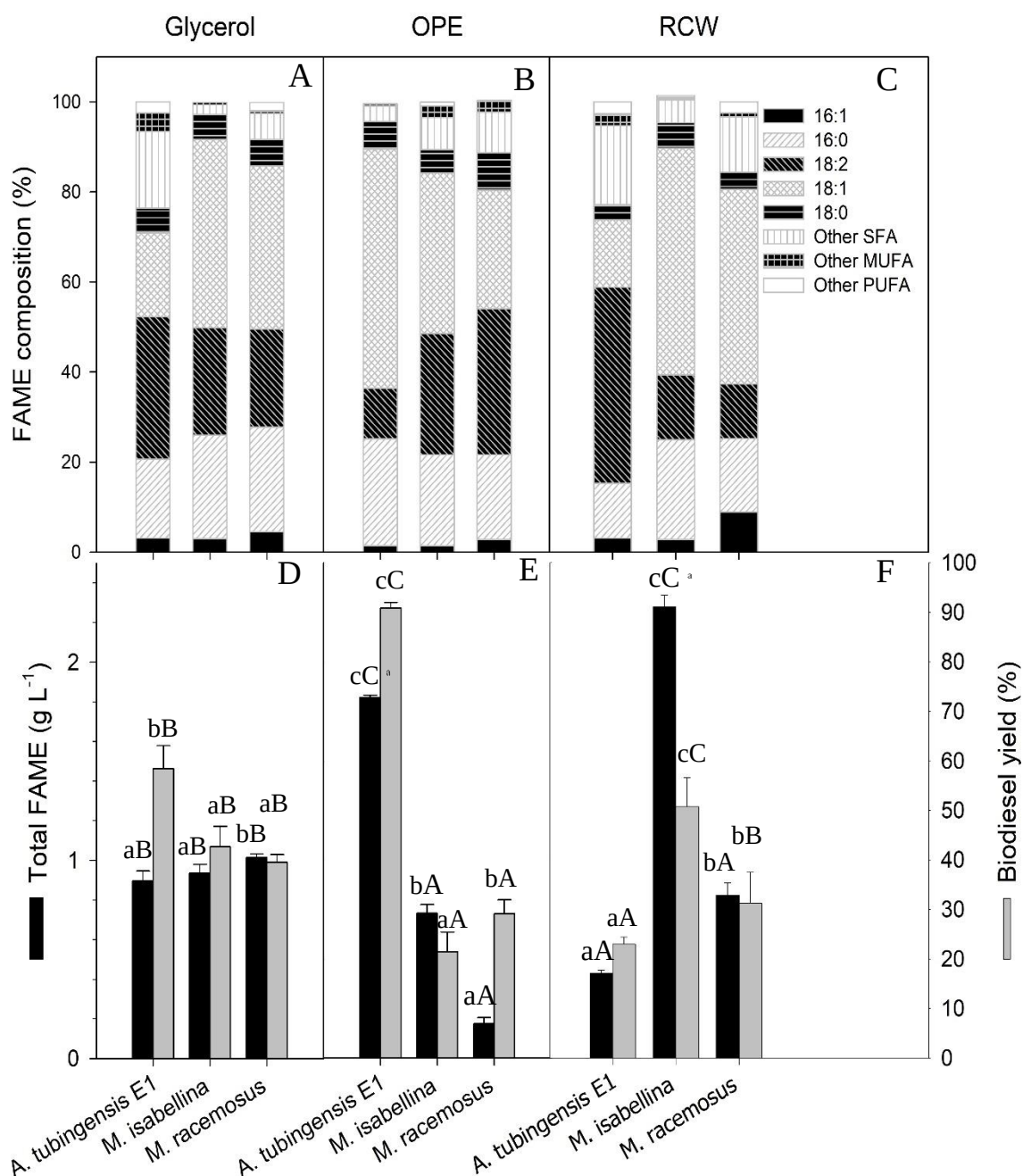


Figure 7.1 Percent compositions of fatty methyl esters (FAMES) derived from lipids produced by 3 selected strains grown in shaken flask on glycerol (A), OPE (B) and RCW (C). Corresponding biodiesel yields after transesterification (%) and total FAME determined by gas-chromatography are reported (D: glycerol; E: OPE; F: RCW). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Abbreviations: 16:1, palmitoleic acid; 16:0, palmitic acid; 18:2, linoleic acid; 18:1 oleic acid; 18:0, stearic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). Same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$). Lowercase letters are referred to the comparison of values (FAME or biodiesel yield) obtained on each substrate individually; uppercase letter are referred to the comparison of values obtained by the same strain n different substrates.

Other PUFA produced, especially when *A. tubingensis* was grown on glycerol, included γ -linolenic acid, representing about 2.5% of total lipids.

Although this fatty acid has a remarkable interest in food industry as food additive for infant formula or for the treatment of eczema, rheumatoid arthritis, multiple sclerosis and premenstrual tension, there are microorganisms that can synthesize significantly higher amounts, around 20% of total lipids (Béligon et al., 2016). On the other hand, a lipid profile so variable and with a high percentage of PUFA is not suitable for biodiesel industry as well, being SFAs and MUFAs preferred (Patel et al., 2017).

As concerns *M. isabellina*, a more stable lipid profile was observed, with a content in oleic acid between 35% and 50% and a degree of poly-unsaturation never exceeding the 28%. Even in this case, on one hand, fatty acids of particular interest for the nutraceutical sector have not been detected, but, on the other hand, this oil, and in particular the composition obtained on glycerol and RCW, can be used for the production of biodiesel or surfactants. *M. racemosus*, likewise, showed a lipid profile very similar to that one from *M. isabellina* and, unfortunately, no high value-added fatty acids were detected for this strain.

Therefore, since no compounds of particular industrial interest were detected *M. isabellina*, showing better performances of growth and lipid production, this fungus was selected for a comparative scale-up to stirred-tank bioreactor (STR) scale on all three substrates in order to investigate the response of the microorganism to different carbon sources, but equal growth conditions.

7.3.5 Comparative scale-up of *M. isabellina* to stirred-tank bioreactor on glycerol, OPE and RCW.

M. isabellina NRRL 1757, having exhibited a considerable adaptability on all the carbon sources tested, was chosen for a comparative study on all substrates. Values of growth and fermentation performances are reported in Tab. 7.4.

Transfer to bioreactor, thanks probably to the high agitation rate which prevented from the formation of large cell aggregates, was not so problematic as expected. However, lower biomass than those obtained in flask, were achieved, probably due to the mechanical stress deriving from agitation. The same behavior was already observed by other authors (Chatzifragkou et al., 2010; Koike et al., 2001) who noticed in *Mortierella* growth inhibition at high aeration and agitation rates. In this study, an agitation rate of 450 rpm was used in order to avoid growth inhibition, but further agitation decrease was not possible due to the formation of pellets, which prevented the sampling, and the proper mass transfer of nutrients. It should be noted, however, that the concentrations of the fatty acids produced were similar

on all substrates tested, both in flask and in bioreactor, thus showing that, unlike other strains (Donot et al., 2014), the fatty acid profile of *M. isabellina* is less influenced by the carbonaceous source than the culture conditions used. Furthermore, the reduced biomass was offset by a higher lipid content, amounting to 35%, 34% and 50% with respect to cell dry weight on glycerol, OPE and RCW, respectively.

Biodiesel yields were not really high, but this problem could be due to a greater resistance of a filamentous fungus to break and release intracellular lipids with protocols commonly used for yeasts. Certainly, future improvements will involve not only the optimization of fermentation conditions, but also of downstream extraction processes.

In Fig. 7.2, an example of fermentation kinetics of *M. isabellina* on OPE is given.

Unlike other substrates that contained only one carbon source and where consumption was linear and without particular problems, in OPE a sequential and incomplete utilization of sugars was observed. While glucose, as expected, was quickly metabolized within the first 24 hours, and fructose within 36 hours, sucrose was metabolized much slower, and completely exhausted only after 72 hours. However, this is in line with other studies on different strains of *M. isabellina*, where sucrose was metabolized less efficiently than other sugars (Chatzifragkou et al., 2010; Zhang and Hu, 2014). From a certain point of view this phenomenon, if properly managed, could allow to maintain a sort of reserve sugars to be used later, during lipid accumulation phase, that is what actually happened in our fermentation. Pectin instead, as already observed in yeasts, was not metabolized at all, remaining unconsumed in the medium.

Certainly, these are just preliminary studies and it will be necessary to continue investigating cultural conditions and strategies to achieve higher lipid yields in shorter fermentation times. However, it is important to understand the potentials of oleaginous fungi, especially using agro-industrial waste, in order to develop processes that can be both environmentally and economically sustainable. In this study, for example, in order to maximize the potential of a versatile strain such as *M. isabellina*, a possible improvement could be the formulation of a mixed substrate, whereby the total carbon source can be more concentrated, thus favoring the achievement of higher yields. For example, OPE could be extracted directly in RCW, which would, on the one hand, enrich RCW with additional sugars, mineral salts, and vitamins and, on the other, it would save the water otherwise needed to extract orange peels, making the process more sustainable and less expensive.

Table 7.4. Comparison among performance indicators of biodiesel production process in STR by *M. isabellina* on glycerol, OPE and RCW, including: yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$); lipids and biomass production rates (rP and rX, respectively); N and total sugars consumption rates (rN, rS); percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

Parameter	Dimension unit	Glycerol	OPE	RCW
Volumetric productions				
Biomass [‡]	g L ⁻¹	4.6±0.4 ^a	11.1±0.8 ^c	7.3±0.2 ^b
Lipids* [†]	g L ⁻¹	1.6±0.1 ^a	3.72±0.22 ^b	3.62±0.06 ^b
t	(h)	84	72	96
Yields §				
Biodiesel yield	(%)	47.3±5.0 ^b	50.8±6.2 ^b	20.0±2.00 ^a
$Y_{P/X}$ [‡]	(g g ⁻¹)	0.35±0.04 ^a	0.34±0.04 ^a	0.49±0.05 ^b
$Y_{P/S}$ [‡]	(g g ⁻¹)	0.11±0.01 ^a	0.20±0.02 ^b	0.26±0.02 ^b
$Y_{X/S}$ [‡]	(g g ⁻¹)	0.30±0.02 ^a	0.58±0.06 ^c	0.49±0.02 ^b
Rates §				
rP [‡]	g L ⁻¹ h ⁻¹	0.02±00 ^a	0.05±00 ^b	0.04±0.00 ^b
rS [‡]	g L ⁻¹ h ⁻¹	0.15±0.01 ^a	0.24±0.01 ^b	0.14±0.01 ^a
rX [‡]	g L ⁻¹ h ⁻¹	0.04±0.00 ^a	0.14±0.01 ^b	0.07±0.00 ^a
μ [‡]	g L ⁻¹ h ⁻¹	0.012±0.001 _a	0.014±0.001 _a	0.010±0.001 _a
Lipid profile ^{†‡}				
Palmitoleic acid	(%)	2.4±0.5 ^a	2.0±0.1 ^a	2.7±0.4 ^a
Palmitic acid	(%)	23.3±3.1 ^b	24.13±1.13 ^b	20.33±1.49 ^a
Linoleic acid	(%)	15.2±3.4 ^a	13.9±1.5 ^a	27.8±1.9 ^b
Oleic acid	(%)	51.9±2.7 ^b	49.8±4.3 ^b	41.2±1.3 ^a
Stearic acid	(%)	5.0±0.4 ^a	4.6±0.4 ^a	4.0±0.2 ^a
Other SFA	(%)	1.5±0.1 ^a	4.3±0.4 ^c	2.8±0.5 ^b
Other MUFA	(%)	0.50±0.1 ^a	0.80±0.19 ^a	1.02±0.16 ^a
Other PUFA	(%)	0.10±0.0 ^a	0.44±0.1 ^c	0.23±0.1 ^b

[‡]Data are the means ±SD of duplicate reactor experiments; *calculated on the basis of lipids determined by colorimetric method (Izard and Limberger, 2003); [†]data are the means ± standard deviations of 4 determinations (2 technical replicates for each reactor experiment); ^{‡‡}predominant fatty acids are listed as a function of increasing retention time. Multiple pair-wise comparisons among yields, rates and single fatty acids were performed by the Tukey test ($P \leq 0.05$). Same superscript letters indicate lack of statistically significant difference within row means.

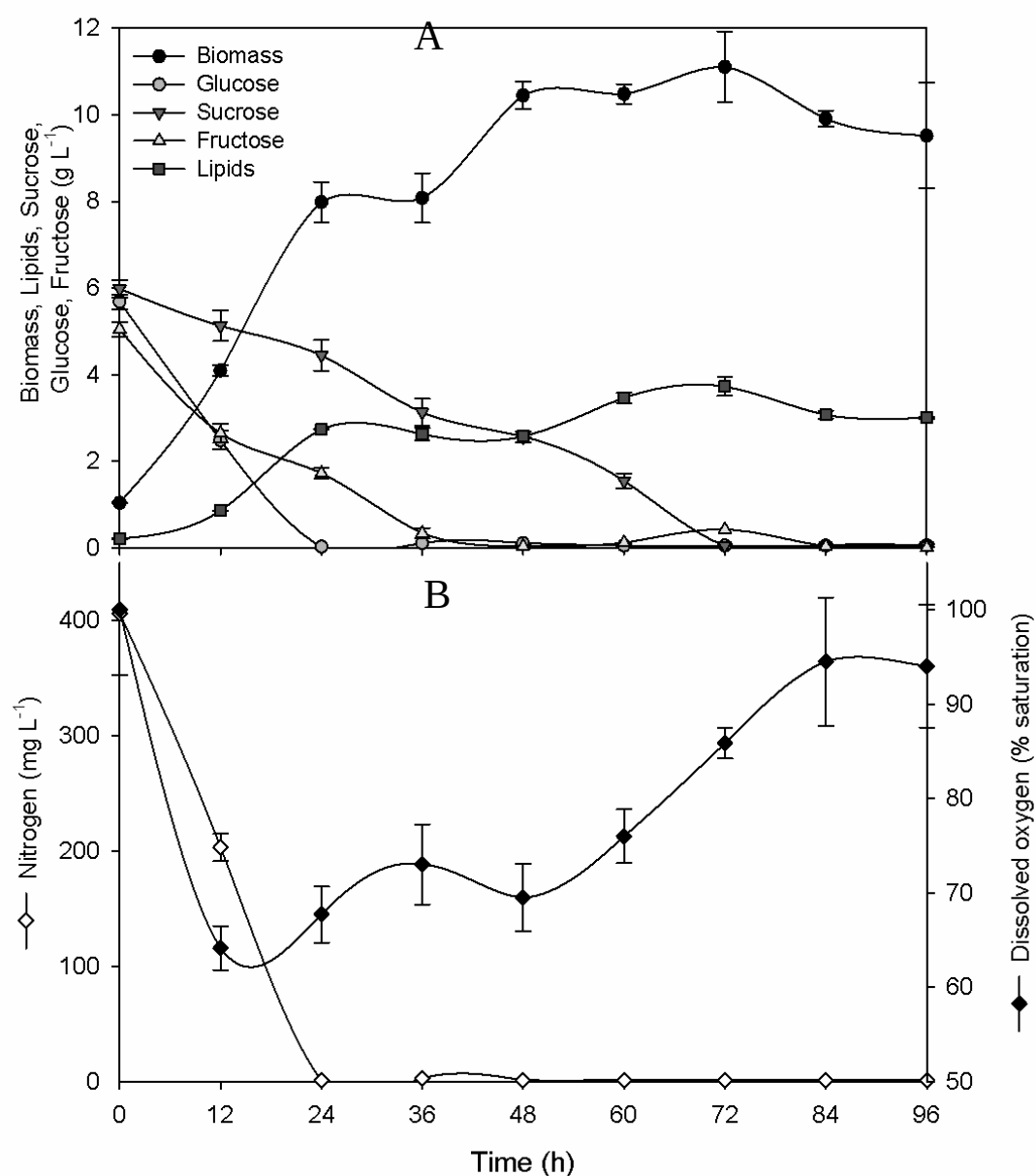


Figure 7.2. *M. isabellina* batch fermentation kinetics on OPE. Time courses of lipid and biomass production and glucose, sucrose, fructose consumption are represented in plot A; nitrogen consumption and trend of dissolved oxygen saturation are reported in plot B. Data are the means $\pm$ standard deviations of duplicate reactor experiments.

CHAPTER 8

MODULATION OF PALMITOLEIC ACID CONTENT IN *CRYPTOCOCCUS CURVATUS* THROUGH METABOLIC ENGINEERING

8.1 Introduction

Microbial oils are on the focus as alternative to crude and vegetable oils in the oleo chemical, biodiesel and nutraceutical industry. Numerous oleaginous microorganisms have been investigated so far and among them, yeasts show particular advantages over bacteria or microalgae. In fact, under the appropriate conditions, they can convert a broad range of carbonaceous sources into microbial oils, achieving a lipid content over 70% (w/w) of their dry weight in addition to a relative ease of cultivation and high growth rate (Ageitos et al., 2011).

Microbial lipids produced mainly consist of triacylglycerols (TAG), whose quantity and composition depend on the strain, fermentation conditions, and growth medium chosen. Albeit with significant differences, the most represented fatty acids include palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acid, thus resembling in composition, common vegetable oils (Papanikolaou and Aggelis, 2011b).

In addition to these common fatty acids, some plants and microorganisms can also produce unusual fatty acids with industrial potential. Among them, GLA (18: 3 n-6), ARA (20: 4 n-6) and DHA (22: 6 n-3) have probably received the greater attention thanks to studies that identified their beneficial effects on human health. Others, such as palmitoleic acid (PA, 16: 1, n-9), have been less investigated while presenting interesting applicative potentials.

PA is a MUFA derived from palmitic acid by the enzyme $\Delta 9$ -desaturase by introducing a double-bond between carbons 9 and 10 of the saturated Acyl-CoA-C16:0 chain, as shown in Fig. 8.1 where the main metabolic steps discussed in this chapter are shown. This desaturation is catalysed by a $\Delta 9$ -desaturase domain that uses O_2 and reduces equivalents obtained from NADH via a short electron transport chain consisting of NADH, NADH-dependant cytochrome b5-reductase, and cytochrome b5. The vast majority of desaturases are endoplasmic reticulum (ER) membrane-bound proteins characterized by three conserved histidine-rich motifs and by four stretches of hydrophobic residues that are predicted to form two membrane traversing loops, each consisting of two transmembrane segments. The conserved histidine residues are important for coordinating two iron atoms at the active site

on the cytosolic face of the ER while the hydrophobic residues form two membrane regions that anchor the protein into the lipid bilayer (Watts and Browse, 2000). The proposed model for a mouse $\Delta 9$ -desaturase showing the above described characteristics is represented in Fig. 8.2.

Various studies have shown the role of PA in different physiological processes of mammals.

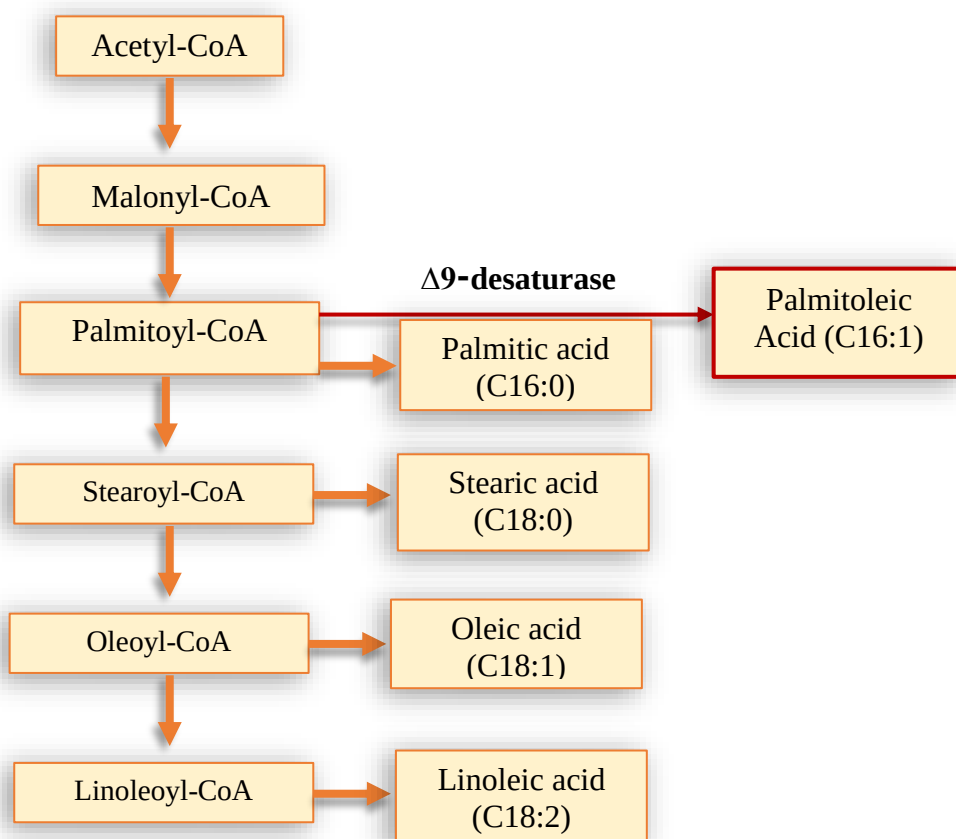


Figure 8.1 Main metabolic steps involved in fatty acid elongation and desaturation in oleaginous yeasts as discussed in this study

Administration of PA to mice with type 2 diabetes demonstrated to reduce insulin resistance, body weight, hyperglycemia, and hypertriglyceridemia (Yang et al., 2011). A protective function on rat pancreatic β -cells has also been observed, leading to increased cell viability, thus highlighting PA is an interesting compound in the treatment of diabetes type 1 and 2 (Welters et al., 2006). Clinical trials in hypercholesterolemic men demonstrated that a diet rich in PA can lower plasma total cholesterol levels, decreasing major cardiovascular disease risk factor (Garg et al., 2003; Griel et al., 2008). Furthermore, PA is reported to reduce bacterial proliferation caused by *Streptococcus pyogenes* and *Staphylococcus aureus* in skin infections (Georgel et al., 2005).

As concerns the biofuel field, studies aimed at the optimization of biodiesel properties highlighted an improvement of kinematic viscosity properties, especially at low

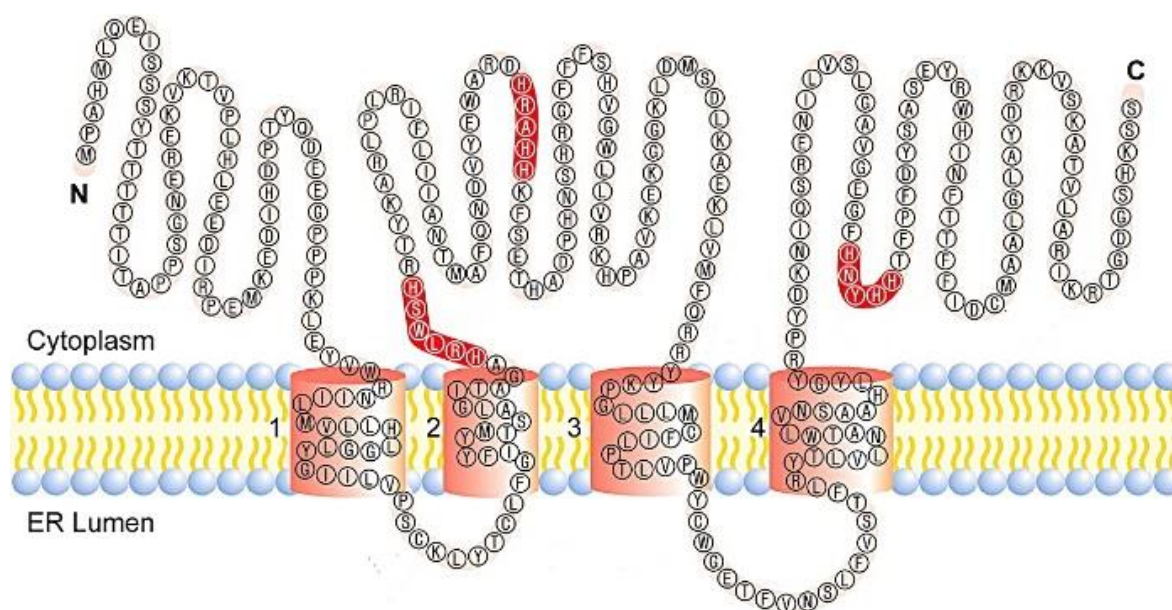


Fig. 8.2. Proposed model for the mouse membrane bound $\Delta 9$ -desaturase SCD-1. The red highlighted residues represented the conserved histidine regions. N and C represent NH_2 and COOH termini, respectively. Image adapted from Paton and Ntambi (2009)

temperatures, when biodiesel was derived from oils with a high content in palmitoleic acid (C16:1) rather than oleic acid (C18:1) (Knothe, 2008).

Moreover, PA could be a valid precursor for 1-octen used in oleo chemical industry as co-monomer for the production of linear low density polyethylene, plasticizers, and lubricants (Wu et al., 2012). Unfortunately PA natural sources such as the plants *Kermadecia sinuate*, cat's claw, macadamia nuts, and sea buckthorn, are generally expensive, with limited availability or with poor agronomic characteristics (Wu et al., 2012).

Some attempts to produce PA via fermentative processes have been made mainly with *Saccharomyces cerevisiae*, characterized by a natural high percentage content of this fatty acid in cell membrane due to a Δ -9 desaturase that can efficiently desaturase both C16:0 and C18:0. However, the non-oleaginous nature of the yeast is an obstacle for achieving high levels of productions and even through genetic manipulation (Runguphan and Keasling, 2014; Tang et al., 2013) or optimization of fermentation parameters (Kolouchová et al., 2015), yields obtained are not comparable to those obtained in oleaginous microorganisms. Alternatively, transgenic oilseed crops with increased PA content have been created, including tobacco, petunia, tomato, eggplant, *Arabidopsis* and soybeans plants (Wu et al., 2012). For this purpose, different $\Delta 9$ -desaturase genes from various eukaryotic organisms (mammalian and yeasts) having a relative high PA content, have been isolated, cloned and expressed in plants, resulting in transgenic plants with an increased PA content (Grayburn et al., 1992; Grayburn and Hildebrand, 1995; Polashock et al., 1992; Xue et al., 2013).

However, the use of transgenic plants, as feedstock for the oleochemical and biodiesel industry, is under debate, first because of the concern on GMO's crops in general and secondly because of the concern of replacing food crops with industrial crops (Chapotin and Wolt, 2007; Chin et al., 2014; Tenenbaum, 2008).

For these reasons, this work focused on the genetic manipulation of an oleaginous yeast, *Cryptococcus curvatus*, as platform for the production of PA.

C. curvatus, has attracted industrial interest due to its ability of growing at high cell densities with minimal nutritional requirements (Hassan et al., 1996) and producing high amounts of intracellular lipids on a variety of different carbon sources, such as glycerol (Meesters et al., 1996; Thiru et al., 2011), whey (Daniel et al., 1999), mixed substrate of beet molasses and cheese whey (Takakuwa and Saito, 2010), pear juice (Hassan et al., 1995), acetate (Gong et al., 2015).

Moreover, it is a good starting point for PA production for the high content in palmitic acid (C16:0), ranging between 20 and 32% on total lipids produced (Evans and Ratledge, 1983; Liang et al., 2010; Meesters et al., 1996).

Although *C. laurentii* was slightly more versatile on both RCW and OPE (chapter 5 and 6, respectively), more genetic information is available for *C. curvatus*. Furthermore, so far, most of metabolic engineering tools have been developed for the modulation of *Y. lipolytica* lipid pathway (Beopoulos et al., 2009), an Ascomycota yeast, while just few attempts have been made on Basidiomycota, to which *Cryptococcus* belongs. The high guanine and cytosine content in the genome of the latter, implies in fact additional problems in expressing heterologous genes (Scholtmeijer et al., 2001).

The interest on Basidiomycota is supported by the evidence that, in comparison with Ascomycota, they can metabolize a broader range of carbon sources and even recalcitrant natural substrates and xenobiotics (Johnson, 2013) without any supplementation of vitamins (Sitepu et al., 2014).

To achieve higher yields of C16:1, heterologous expression of two different $\Delta 9$ -desaturases, acting mainly on C16:0, deriving from *Caenorhabditis elegans* (Fat-5) and *S. cerevisiae* (Ole1p) was attempted in *C. curvatus*. In fact, although $\Delta 9$ -desaturases exist in almost all organisms as the most important desaturases in unsaturated fatty acid biosynthesis (Gostinčar et al., 2010), the specificity of substrate on which they can act, is species dependant, ranging from C12 to C19 saturated fatty acids (Martin et al., 2002).

In *C. curvatus*, specificity is mainly for C18:1 (Meesters et al., 1997), while the aforementioned isoform Fat-5 is specific only for C16:0 showing nearly undetectable activity on the common substrate stearic acid (Watts and Browse, 2000) and among the

fungal desaturases reported so far, Ole1p from *S. cerevisiae* is the only one preferring C16:0 to C18:0 (Hildebrand and Thoguru, 2011; Stukeys et al., 1990).

Two different desaturases have been selected because in *S. cerevisiae* (Martin et al., 2002) and in all organisms from bacteria to humans in general (Aguilar and De Mendoza, 2006), it has been observed that unsaturation is a highly regulated step. Unsaturated fatty acids have crucial roles in membrane biology and signalling processes in organisms for instance the ratio of unsaturated fatty acids in cell membrane strongly influences fluid properties (Martin et al., 2002). For this reason, we wanted to investigate *C. curvatus* response to two desaturases that, in the microorganisms from which they originate exhibit different specificity and regulation.

Furthermore, the genes encoding these desaturases, were expressed in *C. curvatus* under the control of two different strong constitutive promoters, glyceraldehyde-3-phosphate dehydrogenase (GPD) and translation elongation factor 1 alpha (TEF) promoters, known to induce very strong protein expression in order to assure high level expression under different growth conditions.

8.2 Materials and methods

8.2.1 Growth media and culture conditions

C. curvatus ATCC20509 was used for genetic manipulation studies, since its full genome is known (Close and Ojumu, 2016), and because it shows high lipid productivity on glycerol (Meesters et al., 1996).

For long term storage, overnight cell suspensions grown in YPD (Duchefa, Haarlem, The Netherlands) were maintained in 20% glycerol at -80 °C. During the study, the yeast was maintained on YPD-agar plates pH 5.5 at 4 °C and transferred to fresh plates every four weeks. For expression experiments, *C. curvatus* was pre-cultured overnight in shaken flasks at 30 °C and 250 rpm on YPD medium, pH 5.5. Part of this suspension, after a washing step in sterile water, was used to inoculate 1 L Erlenmeyer flasks (with 250 ml medium), in order to have an initial optical density (OD) of 1.0. Growth medium was the same used by Meesters et al. (1996) composed of a minimal medium supplemented with 16 g L⁻¹ of glycerol as carbon source and 0.27 g L⁻¹ of NH₄Cl as nitrogen source. pH 5.5. The minimal medium contained (g L⁻¹): KH₂PO₄ 2.7; Na₂HPO₄ 0.95; MgSO₄ · 7H₂O 0.2; yeast extract 0.1; EDTA 0.1; pH 5.5, supplemented with a 100 × spores stock solution, which contained (g L⁻¹): CaCl₂·2H₂O 4; FeSO₄·7H₂O 0.55; citric acid ·H₂O 0.52, ZnSO₄·7H₂O 0.10; MnSO₄ ·H₂O 0.076; 100µl 18M H₂SO₄. Growth conditions were the same used for pre-inoculum.

8.2.2 Genetic manipulation of *C. curvatus*

8.2.2.1 Plasmids

All the transformation vectors used for *C. curvatus* were derived from a common plasmid, pCGBleG previously built up for other studies on the backbone of pMTL23 (Chambers et al., 1988) which carries an extensive polylinker and the ampicillin resistance gene for *E. coli*.

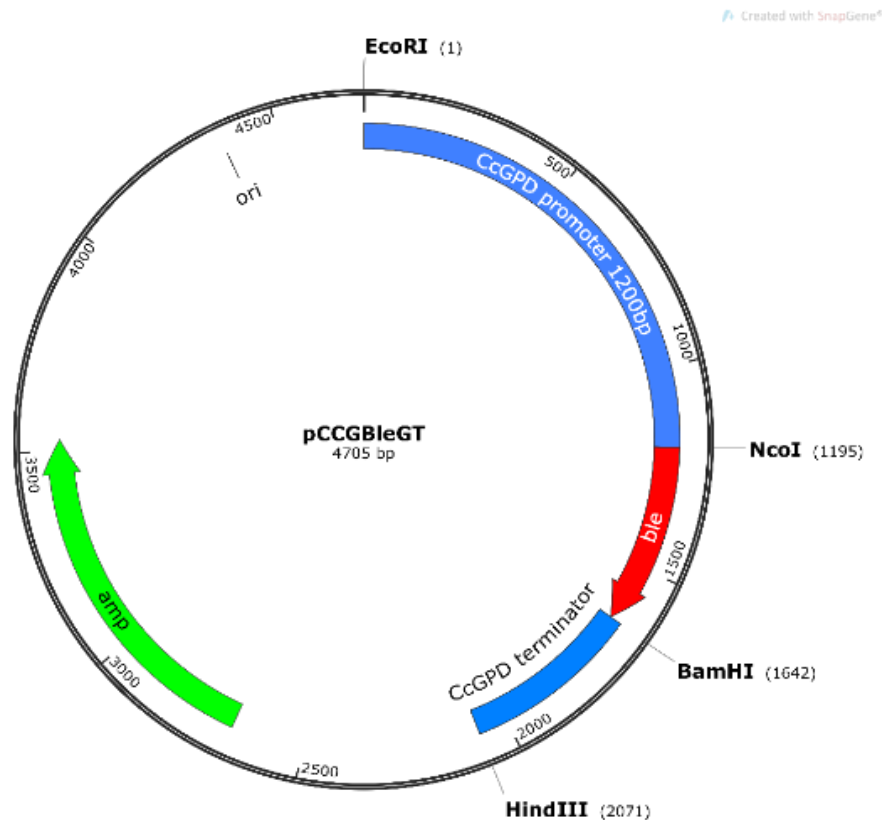


Figure 8.3 pCCGBleG plasmid backbone. Highlighted features include a 1200 bp GPD promoter isolated from *C. curvatus* (CcGPD promoter), zeocin resistance gene (*ble*) and GPD terminator isolated from *C. curvatus* (CcGPD terminator) and ampicillin resistance gene for *E. coli*.

In pCGBleG, the GPD promoter (GPDp) was inserted as an EcoRI / NcoI fragment (the NcoI restriction site was created at the end of promoter by using a degenerate primer), the Zeocin resistance gene (*ble*) was inserted between this NcoI site and a BamHI site and the GPD terminator was inserted as a BamHI / HindIII fragment. See Fig. 8.3. For the graphic presentation.

The TEF promoter (TEFp), was isolated from *C. curvatus* WT using PCR amplification with PCR primers designed on the basis of the genome sequence. Specifically, TEFp forward (fw) primer (TEFp fw 1) was chosen on a region 1067 bp upstream TEF start codon and it was extended at 5' end to include a EcoRI site. TEFp reverse (rv) primer (TEFp rev 2) was designed in the region surrounding the start codon of the gene, substituting two base pairs

(bp) in order to introduce a NcoI restriction site to facilitate subsequent cloning and to ensure the correct reading frame of the gene. Since within TEFp sequence a NcoI restriction site is present on position 595, an overlapping PCR was performed to substitute one base and thus remove the NcoI site. For this purpose two additional primers, between position 591 and 610 were designed (TEFp Rv1 and TEFp Fw 2) in order to generate 2 fragments (TEFp1 and TEFp2), having an overlapping region of 20 nucleotides. TEFp1 and TEFp2 PCR products were first run and extracted from the gel, by using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and ligated by overlapping PCR following Urban et al. (1997) protocol (A scheme of the overlapping PCR is shown in Fig. 8.4). The obtained TEF promoter fragment was inserted into pCR-Blunt™ vector (Invitrogen) and cloned into *E. coli* DG1 competent cells. Successively, plasmids were extracted by GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and checked by digestion with EcoRI and NcoI restriction enzymes in order to verify the correct insertion of EcoRI and NcoI site at the beginning and at the end of the promoter and the removal of NcoI site within the sequence.

TEF original sequence is shown in Fig. 8.5, while modified sequence is reported in Results and discussion section.

TEFp fragment obtained was finally ligated into pCGBleG deprived of GPD promoter by digestion with the same enzymes, and cloned again into *E. coli* DG1 competent cells in order to obtain a high plasmid DNA concentration. The new plasmid was called pCTBleG.

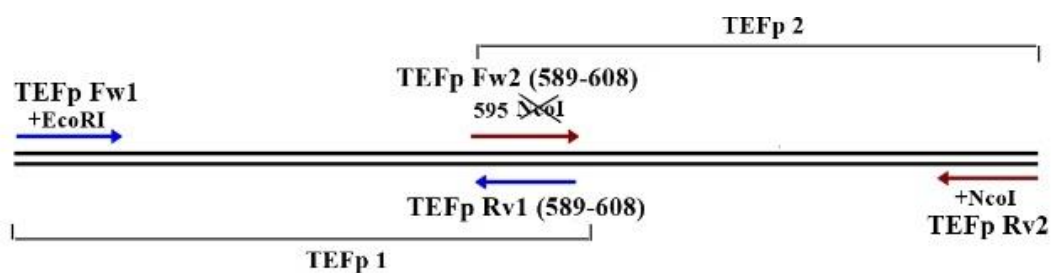


Figure 8.4. Scheme of changes made to TEF promoter with the aim of introducing EcoRI and NcoI restriction sites at the beginning and at the end of promoter region. Scheme of primers used for overlapped PCR is also shown.

CAGATGAAAAGTGCAGCGCAAGAGAGGTTGAGCAGTGTGATCAGATCTTGC	50
AGCTAACAGCGTTAGACGGGGGGCGGTTTGTGCTCGCAATGCTCAGCCC	100
AGATGCAAGATGATGAGACGAAAGCCGAGTGGACGATGAGTGAGGACTCA	150
CCCAGGAAAAATTTTGTGGCGGCGCCTGGGCGGGGCATGGACCGGGCGGA	200
AGATTGCAACTGGCGAGCTGCTCGGCGAGGTTCCGCTCATACCGCTCGCTC	250
AGCTCCCTCTCTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCT	300
CATCAAATTAACGTGAGGGGATCGGATAAACATTGCGCCAAATCACCCCG	350
TACGGTGAGCAAGCCGACGTCAGGCTGCCACACAAGGGCCCTCTAGCGGC	400
GTTATAGACCCCGCCACACGTCATAACACCATAACGCCGTGACTGGGCTT	450
TGAGTAGACAAAGGCAATTCAGGGTCTGTTGGAGGGGCGAGCTCCAGTGC	500
CAGCGCACTGTAAATCCGCGTGCGATCCGCTTTCTCTCTTCACTCCCAC	550
CCTCTCTCTCTCCTCCAAAAACAGTGAGTAGCACCACGACAA	600
CAATGGTGGTCGCCAAAGAGGCAAAGACAGCGAGCGCACTGCGACCGACA	650
ACGACCTGCCCCTCATCGACTCGAACAACCTGCAGCACCATCGCTCTCGT	700
CATCGCCGCGCGCCACTTGCCCTGCACTCCTTCCCCCACCCTCATCCA	750
TCCCATCCTTTCTCTCGACCATGCAAGGCCACCTGCCTCGAGTGCATGC	800
GCTCTTGCGGATGCTCGCGCATTGGTATCGTGGTTCGTCTGCATCGTCGTC	850
AAACAGACGGAGCACGACGACCGTGACGAATTGGATGGCACAAGGGTCC	900
ATGTCAAATTTGACCTCCCGCTTCCACCTTCAACCTTCCTCCATCCAACA	950
TCCTCCGCCTTTGACTCGCAATGCGCATGCTGTCTTTGTCTCCCCACTCC	1000
CACCATTGACTGCCACTCTCAGAGAATGTCGACTAACATTGCAGACAAAA	1050
CCTATTCAAAATG	1070

Figure 8.5. *C. curvatus* TEF promoter region. NcoI site within sequence is highlight in green, while start codon for TEF gene in red.

8.2.2.2 Gene optimization

C. curvatus genome is characterized by a high G+C content (Meesters et al., 1997), therefore codon optimization of the gene sequences Fat-5 and Ole1p was necessary in order to allow a correct and complete expression of the nucleotide sequence (Scholtmeijer et al., 2001), changing the coding nucleotides on the basis of *C. curvatus* codon usage (Table 8.1). Synthetic genes were synthesized by GenScript Corporation (Piscataway, NJ), with the specifications of introducing a NcoI restriction site at the beginning of the gene, a BamHI site at the end and avoiding, within the sequence, EcoRI, NcoI, BamHI and HindIII restriction sites, without changing amino acid sequence, in order to facilitate cloning. Fat-5 and Ole1p amino acid sequences sent to the company are reported in Fig. 8.6 and 8.7, respectively. Genes, received in pUC57 plasmids were first cloned into *E. coli* DG1 competent cells by thermal shock (Sambrook et al., 1989) and, the day after, plasmids were extracted using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The purified plasmid DNA was digested with NcoI and BamHI restriction enzymes and run on 1% (w/v) agarose electrophoretic gel in order to isolate Fat-5 and Ole1p corresponding bands by using GeneJET Gel Extraction Kit (Thermo Fisher Scientific). The same protocol was used for pCGBleG plasmid in order to remove the ble gene from the backbone.

Table 8.1. *C. curvatus* codon usage frequency for each amino acid (Aa). Coding regions are composed of 61.92% GC. In each codon, 1st letter GC 58.70% 2nd letter GC 43.69% 3rd letter GC 83.36%. From Codon Usage Database (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=57679&aa=3&style=N>), extracted by NCBI-GenBank.

Codon	Aa	Frequency	Codon	Aa	Frequency
UUU	F	0.23	UAU	Y	0.05
UUC	F	0.77	UAC	Y	0.95
UUA	L	0	UAA	Stop	0.6
UUG	L	1	UAG	Stop	0.4
CUU	T	0.12	CAU	H	0.01
CUC	T	0.4	CAC	H	0.99
CUA	T	0	CAA	Q	0.01
CUG	T	0.04	CAG	Q	0.99
AUU	I	0.2	AAU	N	0.03
AUC	I	0.8	AAC	N	0.97
AUA	M	0.01	AAA	K	0.03
AUG	M	0.99	AAG	K	0.97
GUU	V	0.19	GAU	D	0.01
GUC	V	0.7	GAC	D	0.99
GUA	V	0	GAA	E	0.08
GUG	V	0.11	GAG	E	0.92
UCU	S	0.1	UGU	C	0.07
UCC	S	0.27	UGC	C	0.93
UCA	S	0.03	UGA	W	0.03
UCG	S	0.53	UGG	W	0.97
CCU	P	0.13	CGU	R	0.28
CCC	P	0.65	CGC	R	0.59
CCA	P	0.09	CGA	R	0.01
CCG	P	0.12	CGG	R	0.04
ACU	T	0.07	AGU	S	0.01
ACC	T	0.3	AGC	S	0.06
ACA	T	0.03	AGA	R	0.04
ACG	T	0.04	AGG	R	0.03
GCU	A	0.24	GGU	G	0.27
GCC	A	0.54	GGC	G	0.67
GCA	A	0.05	GGA	G	0.04
GCG	A	0.16	GGG	G	0.02

Genes and plasmid were then ligated and cloned again into *E. coli* DG1 competent cells and plated on agarose LB medium supplemented with Ampicillin. Growing colonies were screened by PCR using the proper combination of GPD or TEF Fw1 primers and Fat-5 rv or Ole1p rv primers in order to verify the correct insertion of the genes (see table 8.2 for the primers used). Plasmids containing the construct GPD-Ole1p and GPD-Fat-5 were called pCGOle1G and pCGFat5G while plasmid carrying the construct TEFp-Fat-5 pCTFat5G.

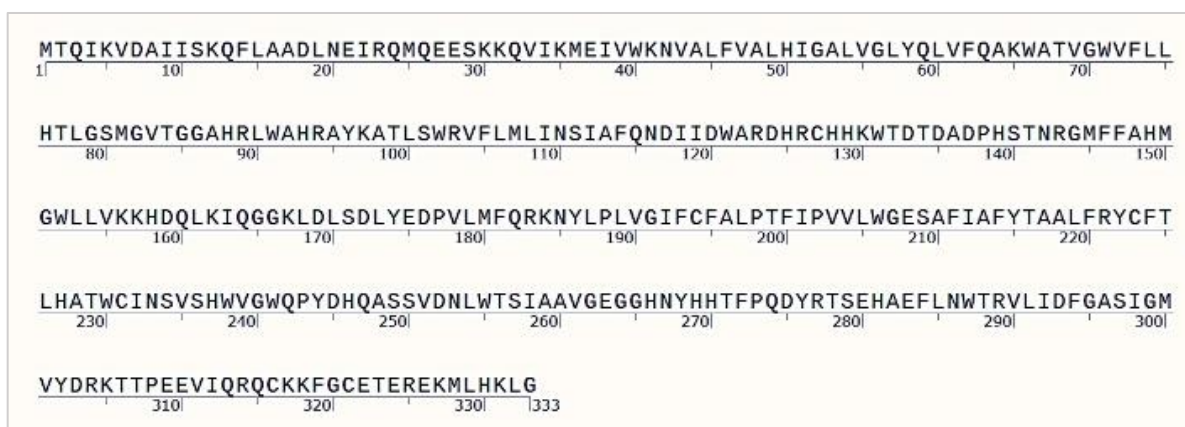


Figure 8.6. Fat-5 $\Delta 9$ -desaturase from *C. elegans* amino acid sequence.

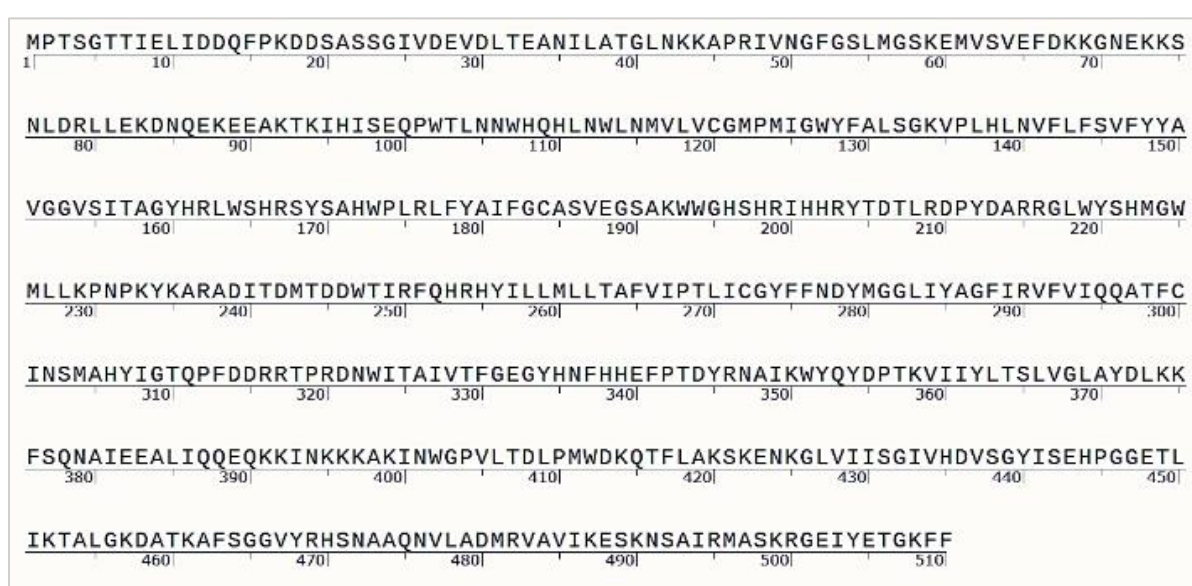


Figure 8.7. Ole1p $\Delta 9$ -desaturase from *S. cerevisiae* amino acid sequence

8.2.2.3 *C. curvatus* transformation

Electroporation was used on *C. curvatus* competent cells to allow the entry of DNA.

In order to make cells competent, one colony of *C. curvatus* was first grown in YPD medium overnight, at 30°C and 250 rpm. 1 ml of this pre-culture was used to inoculate 50 ml of YPD medium in a 250 ml Erlenmeyer flask and grown for few hours until reaching an OD of about 1. The cells were collected into 50-ml polypropylene tube by centrifugation at 3500 rpm, 4°C, for 10 minutes. The pellet was resuspended in 50 mM sodium phosphate buffer (PB) pH 7.5 supplemented with 25 mM dithiothreitol (DTT) and incubated at 37°C for 12 minutes in a water bath. Cells were then placed on ice and centrifuged again at 3500 rpm, 4°C, for 10 minutes and washed twice with cold 10 mM Tris/HCl buffer, pH 7.5, supplemented with 270 mM sucrose and 1 mM MgCl₂. The final pellet was resuspended in a small volume of the same buffer and used for electroporation step.

50 µl of cell suspension in sucrose was placed into chilled 0.2 cm gap width electroporation cuvettes together with 5-10 µl of plasmid DNA to be inserted, for a total amount between 500-1000 ng depending on the experiment. To improve transformation efficiency, plasmid DNA was first linearized with EcoRI restriction enzyme and salts were removed by means of Micro Bio-Spin™ 6 Columns (Bio-Rad). The mixture of cells and DNA was pulsed at 0.8 kV, 1000 Ohm and 25 µFarad in a Bio-Rad Gene Pulser and immediately resuspended in 1 ml of YPD medium. Cells were allowed to recover for 2 hours at 30 °C without shaking prior to plating on YPD agar plates supplemented with 400 µg/ml Zeocin. The gene of interest, was co-transformed with pCGBleG or pCTBleG. This enabled a first selection of transformants on antibiotic containing plates after which the transformants can be screened for insertion of the desaturase gene. This is an efficient approach since it has been established that the frequency of co-transformation is about 50% (Mach, 2004).

After 4 days, resistant colonies were plated on a fresh plate, containing the same amount of zeocin in order to avoid apparent resistant colonies derived from transient expression of the resistant marker. Finally, growing colonies from the second transfer were screened by PCR. DNA from the colonies was extracted by using a fast protocol. Picked colonies were resuspended in 24 µl lysis buffer (50 mM PB, 1 mM EDTA, 5% glycerol, pH 7.4), incubated at 90 °C for 15 minutes and 2 µl of the suspension were directly used for PCR amplification. In Tab. 8.2, all the primers used in this study are listed.

Table 8.2. List of primers used in this study. Lower case letter represents modification with respect to WT nucleotide sequence, in order to introduce or remove restriction sites. For ITS 2 region, ITS86F and ITS 4 were used (Op De Beeck et al., 2014)(Vancov and Keen, 2009).

Gene, marker	Forward primer	Reverse primer	Fragment lenght
Ble	CGGAGCGGTCTGAGTTCTG	GCGGAAACCATGCTACAACC	411
GPDp	GTCATCATCTCGGCCCTTC	-	
TEFp1	cgaattcCAGATGAAAAGTGCAG CGCAAG	CACCATTGCCtTGGTTGTCG	615
TEFp2	CGACAACCAaGGCAATGGTG	CCCATggTGAATAGGTTTTGTCTGC	477
Ole1p	GCTCCCCGCATCGTCAACG	GATGAAGCCGGCGTAGATGAG	735
Fat-5	CGCCAGATGCAGGAAGAGTC	AAGAGGGCGGCGGTGTAG	592

8.2.3 Fatty acid analysis

Acid catalysed esterification directly on freeze-dried biomass was used to analyse fatty acid methyl esters (FAME) by GC. 15% H₂SO₄ in methanol and chloroform were added to 10 mg of biomass and kept at 85 °C for 4 hours on a heating plate with mixing. Lipids dissolved in

chloroform layer were subsequently washed with water and after centrifugation and separation of the phases, Na₂SO₄ was added to the chloroform layer in order to remove residual water. A Thermo Scientific FOCUS GC was used to analyse FAME obtained. Methyl benzoate was used as internal standard to quantify oil content and Supelco FAME Mix C8-C24 (Supelco Analytical) was used for the identification of peaks on the basis of retention time.

8.3 Results and discussion

8.3.1 $\Delta 9$ -desaturases sequence analysis

Amino acid comparison between different desaturases isolated from different organisms didn't show any particular pattern correlating with substrate specificity, but rather indicating phylogenetic proximity, as visible in Fig. 8.8. In fact, desaturases such as Ole1p from *S. cerevisiae* and Fat-5 from *C. elegans* having higher specificity for C16:0 than most of $\Delta 9$ -desaturase identified until now (Stukey et al., 1989; J L Watts and Browse, 2000), are located in different and distant subgroups, while within the same subgroups, different desaturases from the same microorganism, such as in *Fistulifera solaris* and *C. elegans* or from the same phylogenetic division (Ascomycota and Basidiomycota) can be predominantly observed.

The deduced amino acid sequence of Fat-5 (NCBI Reference Sequence: NP_507482.1) showed 34% homology with Ole1p (GenBank: AAA34826.1) and 34% with CCd9 (GenBank: CAA71448.1), while Ole1p and CCd9 were 47% identical. In contrast, due to codon usage differences, the nucleotide sequence of *C. elegans* Fat-5 (NCBI Reference Sequence: NC_003283.11) showed no significant similarity with Ole1p from *S. cerevisiae* (NCBI Reference Sequence: NC_001139.9) and $\Delta 9$ -desaturase from *C. curvatus* (CCd9) ATCC 20509 (GenBank: Y10421.1).

Yeast membrane bound desaturases, such as the $\Delta 9$ -desaturases studied in this work, although presenting significant differences in sequences, maintain commonly conserved domains. Highlighted in Fig. 8.9. “#” is the location of the eight conserved histidine residues grouped in three regions (His-regions) associated with the diiron-oxo moieties (Mitchell and Martin, 1995) and conserved in mammals, fungi, insects, higher plants and cyanobacteria membrane desaturases. Associated with these regions, three long hydrophobic domains spanning the membrane bilayer twice are usually found, allowing the anchorage of the protein to the membrane (Shanklin et al., 1994). Highlighted with “*” symbol is the position of the conserved amino acids that correspond to a heme-binding pocket common to cytochrome b5 peptides. Surprisingly this domain is not present in the *C. elegans* Fat-5.

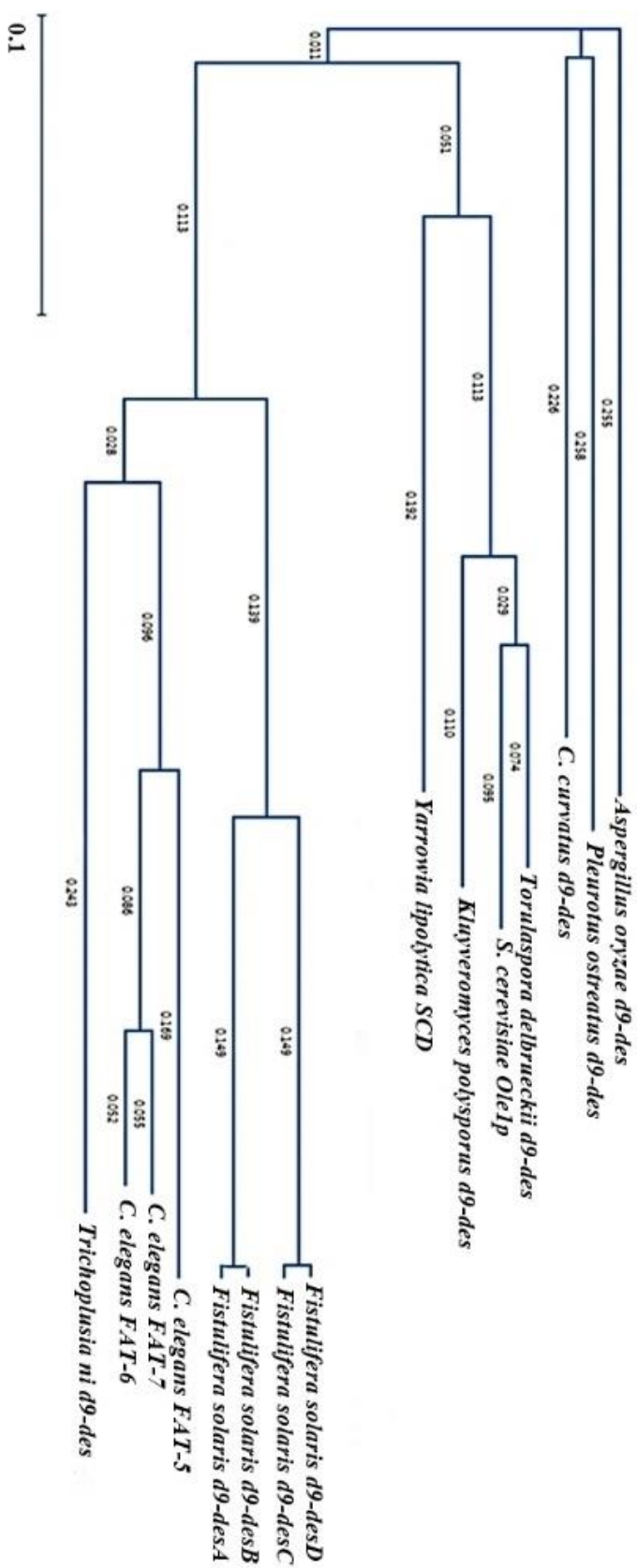


Figure 8.8. Homology tree on the basis of amino acid sequence of different delta 9-desaturases enzymes specific for C18:0, C16:0 or with similar affinity for both substrates. No substrate-specific homology was found but only homology based on phylogenetic proximity. In *C. elegans*, FAT-6 and FAT-7 readily act on C18:0, while Fat-5 acts on C16:0. *S. cerevisiae* Ole1p can desaturate both C16:0 and C18:0. All the other desaturases have higher affinity for C18:0.

Fat-5 1 MTQIKV-DAT-----I---SKQFLAAD-----
 Ole1p 1 MPTSGTTIEL-----I---DDQFPKDDSSASSGIVDEVLDLSEA
 d9des 1 MSASTKQASTTVAQPSGKPVTNVIDPERDDFTVPD-----

Fat-5 19 -----LNEI
 Ole1p 35 NILATGLNKKAPRIVNGFGSLMGSKEMVSVEFDKKGNEKKSNDRLLEKD
 d9des 36 -----NYV-----TRTVENM

Fat-5 23 RQMQEESKKQVIKMEIVW--KNVA-----LFVALHIGALVGLYQLV--F
 Ole1p 85 NQEKEEAKTKIHISEQPWTLNNWHQHNLNWLNMVLVCGMPMIGWYFALSGK
 d9des 46 K-----MLPPVTWRNLHKNIQWISFLALTIPPAMAIYGLC--T

Fat-5 63 -QAKWATVGWVFL LHTLGSMGVTGGAHRLWAHRAAYKATLSWRVFLMLINS
 Ole1p 135 VPLHLNVFLFSVFEYAVGGVSITAGYHRLWSHRSYSAHWPLRLFYALFGC
 d9des 82 VPVQTKTFIWSVYYFITGLGITAGYHRLWAHRSYNASKPLQYFLALCGA
 # #

Fat-5 112 IAFQNDIIDWARDHRCHHKWTDTDADPHSTNRGMFFAHMGWLLVKKHDQL
 Ole1p 185 ASVEGSAKWWGHSRHRIHHRYTDTLRDPYDARRGLWYSHMGWMLLKPNPKY
 d9des 132 GSVQGSIRWWSRGHRAHHRYTDTKLDPYSAHEGFWHAHMGWMLIKPRGKI
 # ##

Fat-5 162 KIQGGKLDLSDLYEDPVIMFQRKNYLPLVGIFCFALPTFIPVVLWGESAFA
 Ole1p 235 K---ARADITDMTDDWTIRFQHRHYILLMLLTAFVIPTLICGYFFNDYMG
 d9des 182 ---GVADISDL SKNPVVKWQHNNYVALLFMGLAFPTLVAGLGWGDWVG

Fat-5 212 IAFYTAALFRYCF TLHATWCINSVSHWVGWQPYDHQASSVDNLWTSIAAV
 Ole1p 282 GLIY-AGFIRVFVLIQQATFCINSMAHYIGTQPFDDRRTPRDNWITAIVTF
 d9des 228 GLFF-AGAARLVFVHHSFVCVNSLAHWLGETPFDNKHTPKDHFITAIVTV

Fat-5 262 GEGGHNYHHTFPQDYRTSEHAEFLNWTRVLIDFGASIGMVYDRKTTPEEV
 Ole1p 331 GEGYHNFHHEFPTDYRNAIKWYQYDPTKVIIYLTSLVGLAYDLKKFSQNA
 d9des 277 GEGYHNFHHQFPMDFRNAIKWYQYDPTKWFIIWTMAQLGLASHLKKFPDNE
 # ##

Fat-5 312 IQRQ-----CKK-----FGCETER-EKML--
 Ole1p 381 IEEALIQQEQKKINKKKAKINWGPVLTDLPMWDKQTF LAKSKENKGLVII
 d9des 327 IKKGQYTMKLMQLQESEQLEWPKHSNDLPVISWEDFQAESKT-RALIAV

Fat-5 330 ---HKLGL-----
 Ole1p 431 SGIVHDVSGYISEHPPGETLIKTALGKDATKAFSGGVYRHSNAAQNVLAD
 d9des 376 HGFTHDCSSFIEDHPGGAHLIKRAIGTDSSTAFFGGVYDHSNAAHNILAM
 *** **

Fat-5 -----
 Ole1p 481 MRVAVIKES-----KNSAIRMASKRGEI
 d9des 426 MRVGVLDGGMEVEHLKVGRLTRSNSADSLVSDAPSSASTVSASSILSEV

```

Fat-5      -----
Ole1p    504 YETGKFF-----
d9des     476 EDMDKPERKPSTSRSPRCPTPGRSPCLLRSSIASSARPPRSAQDSSRASR

Fat-5      -----
Ole1p      -----
d9des     526 APPRPASHGQRALTLLSRLRAFSVEHPGFH

```

Fig. 8.9. Homology of $\Delta 9$ -desaturase amino acid sequences from *C. elegans* (Fat-5), *S. cerevisiae* (Ole1p) and WT *C. curvatus* ATCC 20509 (d9des). “#”, highlight the location of the three Histidine rich regions highly conserved among membrane bound desaturase.

“*” symbol indicate the position of conserved amino acids forming a heme-binding pocket common to cytochrome b5 peptides.

A significant difference between yeast (including *C. curvatus* and *S. cerevisiae*) and *C. elegans* $\Delta 9$ -desaturases is the presence of a cytochrome b5 domain at C-terminal end, missing in all *C. elegans* desaturases and, in general, in mammalian stearyl-CoA desaturase (SCD) proteins. However, this does not seem to be an obstacle to the correct heterologous expression of these genes between mammals and yeasts. For example, heterologous expression of $\Delta 9$ -desaturases lacking b5 domain, such as rat SCD-1 in *S. cerevisiae*, was able to rescue fatty acid auxotrophy of the *ole1* mutant indicating that the rat desaturase enzyme could interact successfully with yeast components of the desaturase system (Jennifer L. Watts and Browse, 2000).

The difference in domains of the two desaturases inserted in this work in *C. curvatus*, one comprising cytochrome b5 and the other without, may allow to understand whether these heterologous proteins, and in particular Fat-5, can interact to the same extent with yeast cellular components.

8.3.2 TEF promoter isolation and nucleotide sequence modifications

Overexpression of heterologous enzymes in yeasts to form a new metabolic pathway usually requires endogenous promoters because promoter architecture in eukaryotes is complex and transcriptional responses is difficult to predict (Weingarten-Gabbay and Segal, 2014). In this study, two widely used strong constitutive promoters, GPDp and TEFp, isolated from *C. curvatus* ATCC 20509 were compared for their ability to regulate the two genes under study. The sequence of the TEF gene was taken from the genome sequence JGI fungal genomic resource (<https://genome.jgi.doe.gov/Crycu1/Crycu1.home.html>). Subsequently, a region of about 1000 bp upstream of the start codon was selected as described in the “Materials and methods” section. PCR amplification products TEFp1 and TEFp2 and TEFp, modified fragment after overlapping PCR, are shown in Fig. 8.10. Original TEFp fragment containing

NcoI restriction site and modified TEFp fragment, after cloning into pCR™-Blunt vector (Invitrogen) were digested with NcoI in order to check the correct substitution of the base and, in the latter, no internal digestion was observed, confirming the removal of NcoI site. The final sequence obtained in which some bases have been modified to facilitate cloning is shown in Fig 8.11.

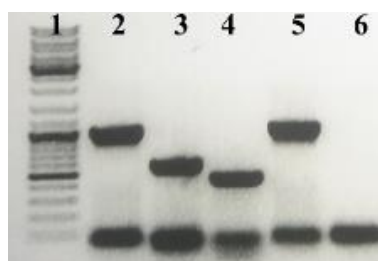


Figure 8.10. TEFp modification by overlapping PCR. Line 1: GeneRuler DNA Ladder Mix 100-10000bp (Thermo Fisher Scientific). Line 2: product of TEFp Fw1 and TEFp Rv 2 primers on *C. curvatus* WT genome. Line 3: TEFp 1 fragment. Line 4: TEFp2 fragment. Line 5: TEFp obtained by overlapping PCR of TEFp1 and TEFp2. Line 6: Negative control of all primers with sterile water.

8.3.3 Control of TEFp functionality

```

cGAAttcTGCAGCGCAAGAGAGGTTGAGCAGTGTGATCAGATCTTGCAGCT 50
AACAGCGTTAGACGGGGGGCGGTTTGTCTGCTCGCAATGCTCAGCCAGAT 100
GCAAGATGATGAGACGAAAGCCGAGTGGACGATGAGTGAGGACTCACCCA 150
GGAAAAATTTTGTGGCGGCGCCTGGGCGGGGCATGGACCGGGCGGAAGAT 200
TGCAACTGGCGAGCTGCTCGGCAAGGGTCCGCTCATACCGCTCGCTCAGCT 250
CCCTCTCTCCTTCCTTCCTTCTTTCTTCCCTCCTGCCCTCCTGGCATC 300
AAATTAACGTGAGGGGATCGGATAAACATTGCGCCAAATCACCCCGTACG 350
GTGAGCAAGCCGACGTCAGGCTGCCACACAAGGGCCCTCTAGCGGCGTTA 400
TAGACCCCGCCACACGTCATAACACCATAACGCCGTGACTGGGCTTTGAG 450
TAGACAAAGGCAATTCAGGGTCTGTTGGAGGGGCGAGCTCCAAGTGCCAGC 500
GCACTGTAAATCCGCGTGCATCCGCTTTCTCTCTTCACTCCCACCCTC 550
TCTCTCTCCTCCAAAAACAGTGAGTAGCACCAACGACAACCAaGGCAAT 600
GGTGGTGCCCAAAGAGGGCAAAGACAGCGAGCGCACTGCGACCGACAACGA 650
CCTGCCCGTCATCGACTCGAACAACCTGCAGCACCATCGCTCTCGTCATC 700
GCCGCGCGCCACTTGCCCTGCACTCCTTCCCCCACCCTCATCCATCCC 750
ATCCTTTCTCTCGACCATGCAAGGCCACCTGCCTCGAGTGCATGCGCTC 800
TTGGCGATGCTCGCGCATTGGTATCGTGGTCGTCTGCATCGTCGTCAAAC 850
AGACGGAGCACGACGACCGTGACGAATTGGATGGCACAAAGGGTCCATGT 900
CAAATTTGACCTCCCGCTTCCACCTTCAACCTTCTCCATCCAACATCCT 950
CCGCCTTTGACTCGCAATGCGCATGCTGTCTTTGTCTCCCACTCCCACC 1000
ATTGACTGCCACTCTCAGAGAATGTCGACTAACATTGCAGACAAAACCTA 1050
TTCAcCATGGG 1061

```

Figure 8.11. TEFp final sequence in which some bases have been modified to facilitate cloning. EcoRI and NcoI added sites are highlighted in red, while NcoI site removed by site-directed mutagenesis is highlighted in green.

After ligation of TEFp into pCTBleG expression vector, and PCR control by using TEFp Fw2 and Ble Rv primers, its functionality was assessed by transformation of *C. curvatus* and cultivation of yeast cells on YPD agar medium supplemented with Zeocin 400 µg/ml to verify that the Zeocin resistance gene ble was actually expressed. A comparison between transformants carrying the construct GPD-Ble and TEF-Ble was performed and, in order to

evaluate the initial cell density, cell suspension after electroporation was plated on YPD agar medium without antibiotic supplementation, too (Fig. 8.12).

The number of growing cells on YPD agar medium supplemented with Zeocin was significantly lower than that one observed on YPD agar medium without antibiotic addition, demonstrating that Zeocin had an inhibiting effect on cells that did not integrate the resistance gene for Zeocine. *C. curvatus* WT was not able to grow at all (plate not shown).

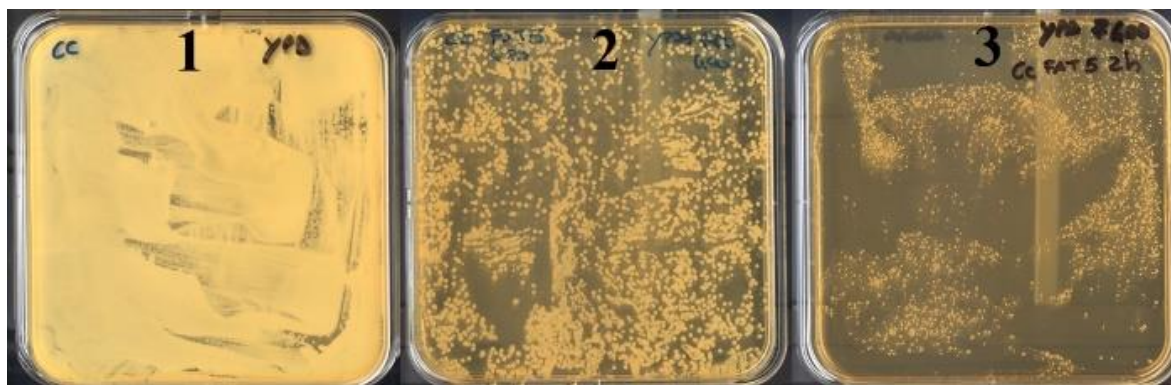


Figure 8.12. Comparison between transformants carrying the constructs GPD-Ble and TEF-Ble on YPD+Zeocin (2 and 3, respectively) and TEF-Ble on YPD (1).

Moreover, a high number of colonies was able to grow either when *C. curvatus* integrated the construct pCGBleG, or pCTBleG demonstrating that both promoters can induce gene expression.

8.3.4 Comparison of Ole1p and Fat-5 expression under TEF promoter control

To further investigate the *in vivo* function of Ole1 and Fat-5, pCGOle1G, pCGFat5G *C. curvatus* transformants were cultivated in shaken flask, on glycerol medium, in limiting nitrogen conditions to stimulate lipid accumulation. Fatty acids profiles were analysed by GC every 24 hours (Fig. 8.13).

In the initial growth phase TEF-Ole1p and TEF-Fat-5 showed to contain 3.52 ± 0.23 and $4.34 \pm 0.29\%$ of C 16:1 respectively that, compared to the WT profile where C16:1 represented only $1.02 \pm 0.00\%$, was a statistically significant increase. This increase was appreciable only before the beginning of lipid accumulation, when the lipid composition analysed represents essentially cell membrane composition.

During the lipid accumulation phase the C16:1 content decreased to standard WT level however in both type of transformants the concentration of C16:0 was 8% lower, with respect to WT strain after 8 hours of fermentation and 19 and 14% lower for TEF-Fat-5 and TEF-Ole1p transformants respectively, after 72 hours of fermentation.

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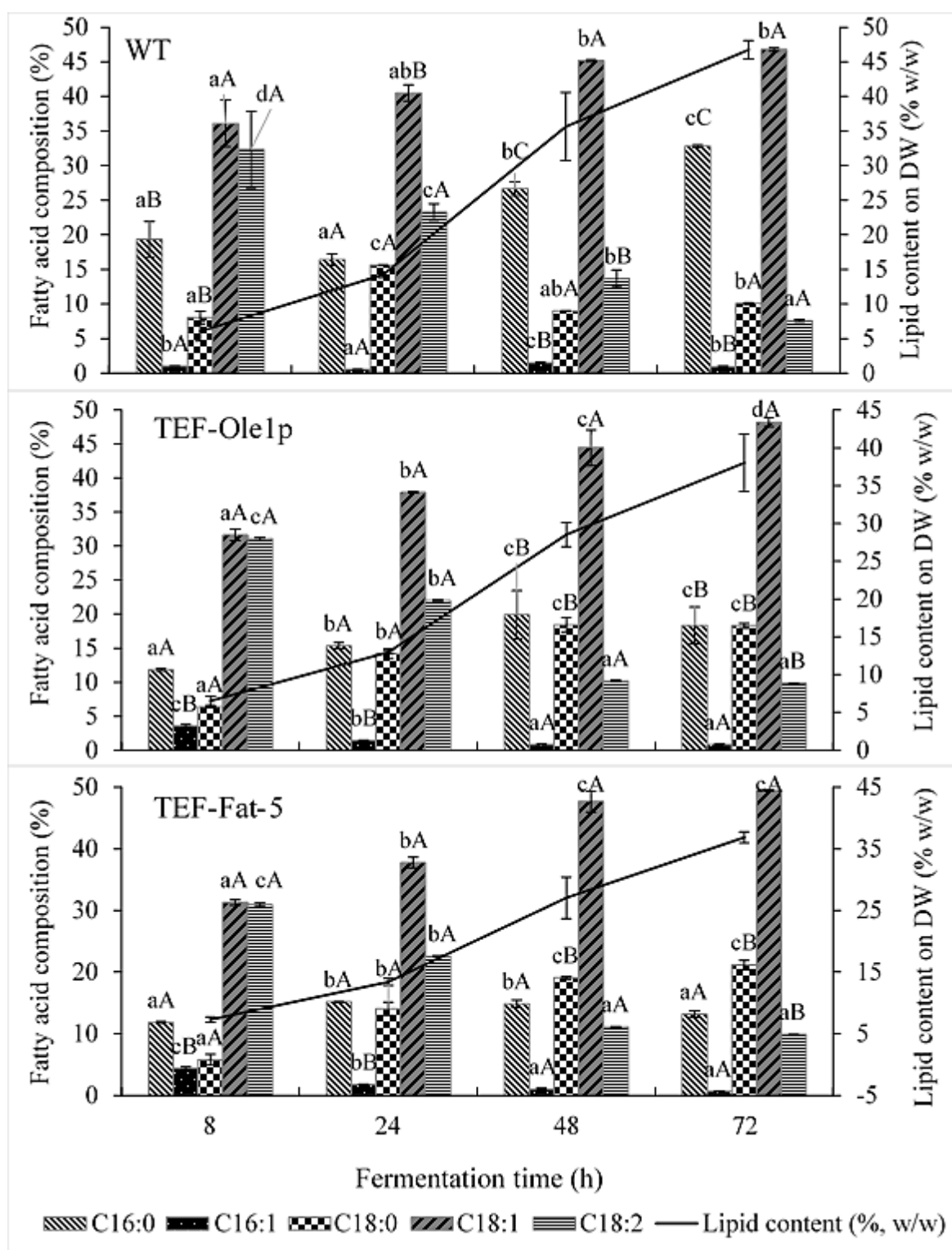


Figure 8.13 Comparison of WT, TEF-Ole1p and TEF-Fat-5 transformants lipid profile at different fermentation times. Total lipid percentage content with respect to dry weight is also represented in order to evaluate if fermentation phases are unmodified or if there was a delay in lipid accumulation. Multiple pair-wise comparisons among fatty acid percentage on total lipids were performed by the Tukey test. Same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values. Lowercase letters are referred to comparisons of individual fatty acids (C16:0, C16:1, C18:0, C18:1 and C18:2) of the same strain along fermentation time. Uppercase letters are referred to comparisons of individual fatty acids, at the same fermentation time, among different strains (WT, TEF-Ole1p and TEF-Fat-5 transformants).

The C16:0 lower content was largely compensated, in both transformants, by a 10% increase of C18:0 content. This result indicates that introduction of the Fat-5 or Ole-1p desaturase gene influenced somehow ratio of C16:0 to C18:0 which is determined by the final step of fatty acid biosynthesis (elongase). Apparently this increase in C18:0 is not significantly converted into C18:1 and C18:2 respectively although a small increase in both is observed. Unfortunately, there are only few studies concerning the metabolic step of desaturation of C16:0 to C16:1. Native Ole1p in *S. cerevisiae* is able to produce high amounts of C16:1, reaching the 42% of total lipids (Stukey et al., 1989) as well as Fat-5 when expressed in a yeast ole-1 mutant (Jennifer L. Watts and Browse, 2000). However, it must be taken into account that *S. cerevisiae* is not a lipid producer, thus it is not possible to predict the effect of these genes during lipid accumulation.

A possible explanation for low C16:1 accumulation in the *C. curvatus* transformants could be due to the acyl transferases involved in the transport of fatty acids from the endoplasmic reticulum to the lipid bodies. In *C. curvatus* acyl-transferase probably have low affinity for the unusual fatty acids, such as C16:1, as already seen in other microorganisms (Beopoulos et al., 2014). The small effect on C18:1 and C18:2 can be the result of the high regulation of $\Delta 9$ -desaturase activity in general (Aguilar and De Mendoza, 2006).

In fact, it has been shown in the literature that, in order to produce tailor-made fatty acids, the expression of the appropriate fatty-acid-modifying enzyme alone is not sufficient to shift lipid metabolism towards the accumulation of the desired fatty acid and a standard biotechnological approach is to express the fatty-acid-specific TAG acyltransferase (Beopoulos et al., 2014; Napier, 2007).

In an additional experiment, the effect of another promoter driving Fat-5 gene was analysed. Expression analysis have shown that the expression levels of TEF gene are higher than those of GPD gene from the beginning of the fermentation and that the expression of the GPD gene increases over time (data not shown), and it was therefore interesting to see if this could favour a C16:1 production in late fermentation phases.

8.3.5 Comparison of Fat-5 expression under GPD and TEF promoter control

Fatty acids profile resulting from cultivation of transformants of *C. curvatus* with pCTFAT5G and pCGFAT5G are shown in Fig. 8.14.

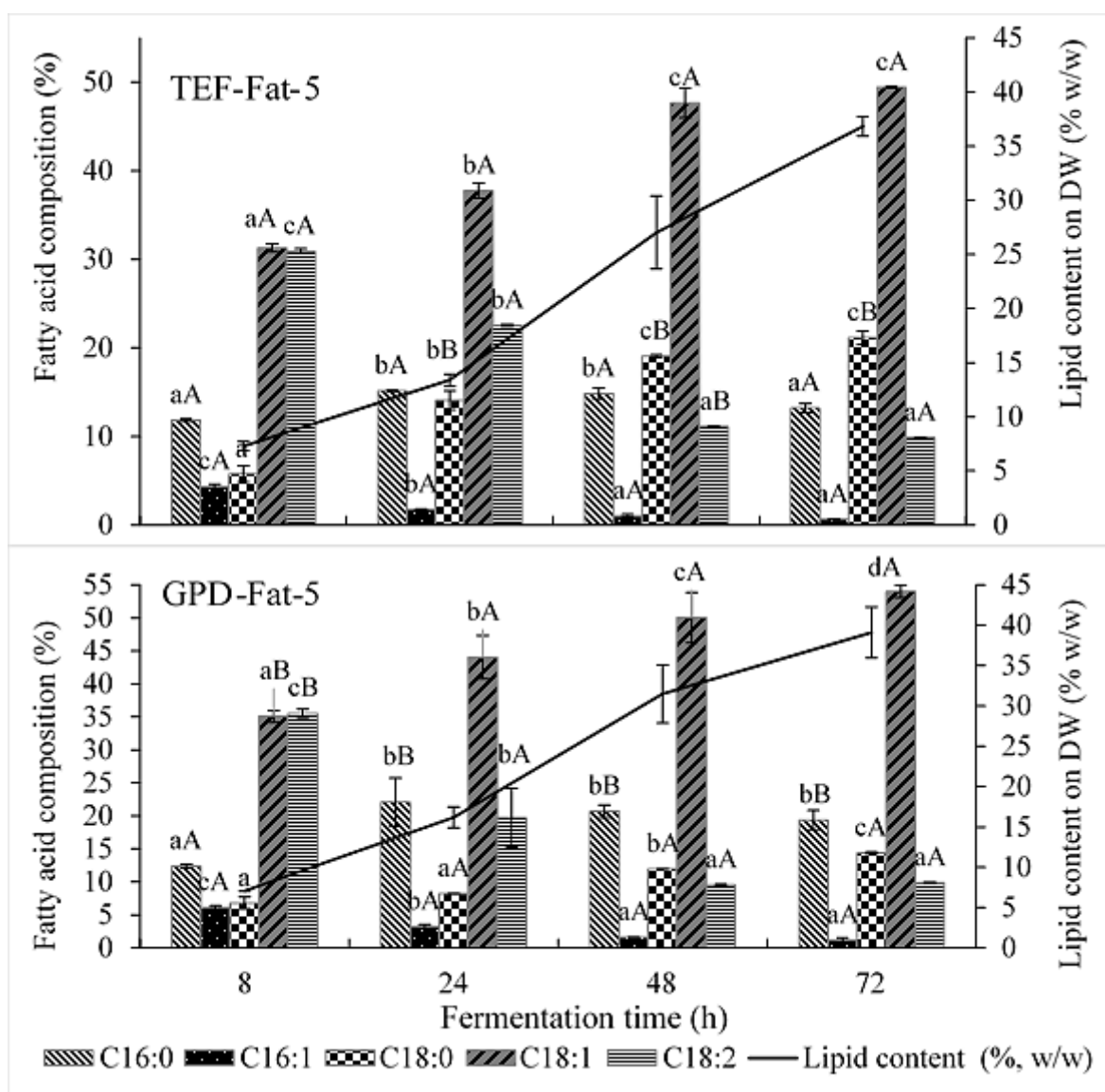


Figure 8.14. Comparison of TEF-Fat-5 and GPD-Fat-5 transformants lipid profile at different fermentation times. Total lipid percentage content with respect to dry weight is also represented in order to evaluate if fermentation phases are unmodified or if there was a delay in lipid accumulation. Multiple pair-wise comparisons among fatty acid percentage on total lipids were performed by the Tukey test. Same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values. Lowercase letters are referred to comparisons of individual fatty acids (C16:0, C16:1, C18:0, C18:1 and C18:2) of the same strain along fermentation time. Uppercase letters are referred to comparisons of individual fatty acids, at the same fermentation time, among different strains (TEF-Fat-5 and GPD-Fat-5 transformants).

As seen before, C16:1 was produced only within the first 8 hours of fermentation and decreased when lipid accumulation started. The only difference was that, under the control

of GPD promoter, a slightly higher content in C16:1 was observed, amounting to $6.12 \pm 0.26\%$ that represents a 6 times increase with respect to WT.

However, unlike the TEF-Fat-5 transformant, the GPD-Fat-5 transformant did not show the large shift in C16:0 / C18:0 ratio.

It is also to be noted that, as already observed in the complementation of *S. cerevisiae ole1* mutant with *C. elegans* Fat-5 (Jennifer L. Watts and Browse, 2000) the cytochrome b5 domain lacking Fat-5, is able to interact with *C. curvatus* desaturase system.

8.3.6 Comparison of GPD-Fat-5 expression at 30 and 20 °C

A final attempt to improve C16:1 production was made by changing cultural conditions. It is known that low temperatures can lead to higher production of unsaturated fatty acids as adaptation strategy to maintain membrane fluidity (Aguilar and De Mendoza, 2006; Bellou et al., 2012; Kamisaka et al., 2014; Michinaka et al., 2003; Suutari et al., 1990). Furthermore, a specific study on C16:1 production at low temperatures was performed in *S. cerevisiae* (Kamisaka et al., 2014), leading to a higher C16:1 content when *S. cerevisiae* was cultivated at 20 °C, together with 2 g L^{-1} of methionine, even if the reason of the effect of methionine on PA was not clear.

Fatty acids profiles obtained in GPD-FAT5 transformants grown at 30 and 20 °C in comparison with WT grown at 20 °C, are shown in Fig. 8.15. In WT strain, low temperatures led to a significant increase in C18:2 after 72h, rising from $7.61 \pm 0.19\%$ when grown at 30 °C to $16.62 \pm 0.10\%$, but no significant changes were observed in C16:1 content. In transformants bearing the insert GPD- Fat-5, instead, an increase in C16:1 was detected at 20 °C, arriving to $9.11 \pm 0.75\%$ at 8 hours of fermentation, although this value decreased with the progress of fermentation, arriving to $3.36 \pm 0.12\%$ at 72 hours.

Although there was no delay or decrease in the levels of lipid accumulation when *C. curvatus* WT was grown at 20 °C instead of its optimal temperature 30 °C, a decrease in percentage lipid content on dry weight (w/w) was observed in GPD-Fat-5 mutant which did not exceed the 25%, with respect to 50% produced by WT strain.

In conclusion overexpression of a heterologous palmitoleic acid specific $\Delta 9$ -desaturase gene in *C. curvatus* leads to a 2-4 fold increase of palmitoleic acid in the membrane lipids but not to an increase of this fatty acid in the accumulated lipids. In addition there is a major effect (50% reduction) on the extent of lipid accumulation at 20 °C.

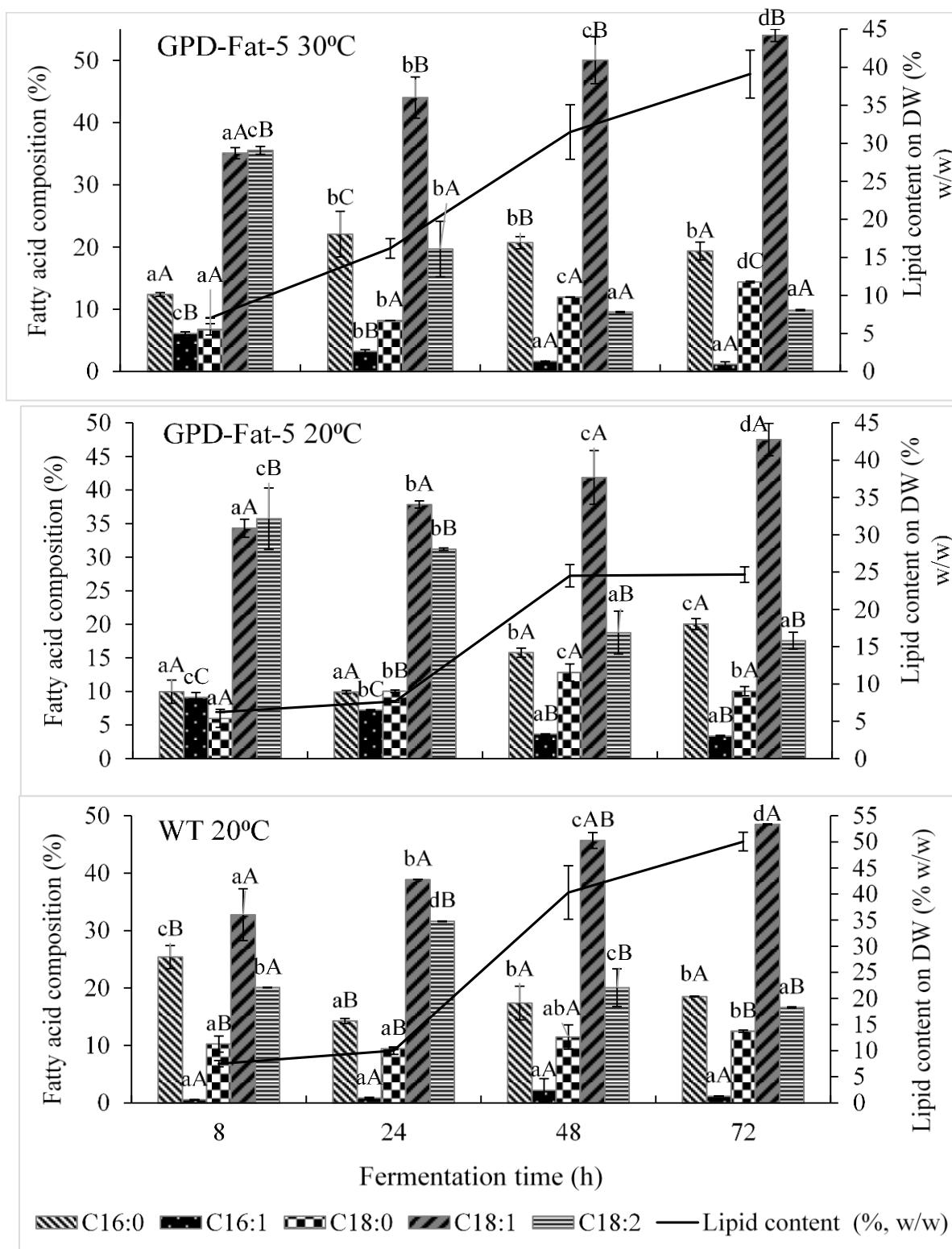


Figure 8.15. Comparison of lipid profiles produced by GPD-Fat-5 mutants grown at 30 and 20 °C in comparison with WT grown at 20 °C, at different fermentation times. Multiple pair-wise comparisons among fatty acid percentage on total lipids were performed by the Tukey test. Same letters above the bars indicate lack of statistically significant difference ($P \leq 0.05$) within the same group of values. Lowercase letters are referred to comparisons of individual fatty acids (C16:0, C16:1, C18:0, C18:1 and C18:2) of the same strain along fermentation time. Uppercase letters are referred to comparisons of individual fatty acids, at the same fermentation time, among different strains (TEF-Fat-5 and GPD-Fat-5 transformant grown at 20 and 30 °C and WT grown at 20 °C).

Although these results are not entirely satisfactory in creating a palmitoleic producing yeast strain, they are in line with studies carried out on transgenic plants where heterologous genes coding for $\Delta 9$ -desaturases have been introduced. $\Delta 9$ -desaturases alone, either from animals, yeast or 16:1-high accumulators, generally only produce C16:1 content below 10%, a much lower value than the one observed in the micro-organisms from which they are isolated (Wu et al., 2012).

Therefore, the regulation of metabolic pathways should be studied in the context of the whole cell rather than at the single pathway level because many mechanisms influence the production of each fatty acid. Further studies will involve the KO of genes coding for enzymes that are part of the lipid pathway and strictly related to C16:0 production step, such as the gene for the WT $\Delta 9$ -desaturase, in order to evaluate the individual effect of heterologous desaturases

An attempt of knocking out the elongase involved in the elongation of C16: 0 to C18: 0 was also made, but even by supplementing the growth medium with C18:0, no knock out strains were obtained, indicating that this step could be strictly necessary for the survival of the cell. Overexpression of endogenous acyl-transferases could be investigated, in order to favour transportation of an unusual fatty acid, such as C16:1 to lipid bodies. Successfully results have been obtained with this strategy for the production and accumulation of ricinoleic acid in *Yarrowia lipolytica* (Beopoulos et al., 2014).

Another option could be the isolation of acyl-transferases from plants or from other microorganism, but studies are still ongoing in order to understand what specific amino acids determine their specificity (Cao, 2011). Furthermore, these enzymes have shown different substrate specificities depending on which microorganism was used as host for heterologous expression making understanding even more complex.(Kalscheuer et al., 2004; Lager et al., 2013).

CHAPTER 9

CONCLUSIONS AND FUTURE PERSPECTIVES

Nowadays, there is a great interest in the valorization of organic waste by means of biorefineries. In particular, the possibility of finding alternative, renewable and sustainable energy and biofuel sources is a problem that needs to be solved in relatively short time due to the fast depletion of crude oil and to the increasing needs of the world's population. In this context, this research project has sought to respond to these needs by investigating the potential oleaginous fungi on different agro-industrial by-products/waste such as glycerol, orange peels and RCW. Although currently the production of microbial oils is relegated to the nutraceutical sector, where high added-value fatty acids allow for economic return, in the future, the possibility of using these oils for the production of biodiesel will be undoubtedly evaluated. Since these processes take a long time to be optimized, it is important to be prepared and aware of all the different possibilities. In fact, although oleaginous microorganisms have been known for a long time, the great variability of response they have to different fermentation conditions, require an immense screening and optimization work. The real applicative effects are also demonstrated by the fact that oil companies, including Eni S.p.A, are investing in research on microbial oils. The union of different research lines, both from private and from the academia, will bring us to be prepared for when the real need will arise.

This PhD project focused on the possibility of using oleaginous yeasts and moulds for the bioconversion of agro-industrial waste into microbial lipids of industrial interest. Specifically, the possibility of using lipids produced as feedstock for biodiesel production was assessed. Substrates under study were glycerol, OPE and RCW, on which 18 yeast and 9 mould strains were first screened in shaken flasks and then transferred to bioreactor scale by using different strategies.

From the results obtained, it can be concluded that:

- RCW, although not being metabolized by most of the yeasts screened, strongly favored the growth and lipid production by *C. curvatus* and *C. laurentii*. Furthermore, the fermentation experiment in bioreactor with *C. laurentii* demonstrated that a scale-up is feasible and that controlled conditions can significantly improve production yields: 14.4 g L⁻¹ of biomass and 9.9 g L⁻¹ of lipids were produced, the last of which were characterized by a similar composition to *Jatropha* oil, a well established feedstock for biodiesel production.

- OPE, thanks to its easily assimilable sugar content, managed to support the growth of most of the yeasts tested, among which *R. toruloides* emerged for the remarkably high lipid content, achieving in bioreactor 5.2 g L⁻¹ in only 36 hours of fermentation.
- Screening of yeasts on glycerol resulted in unsatisfactory biomass production but, despite that, in high lipid content for some strains. This observation represented, therefore, the starting point to formulate a mixed substrate composed of glycerol and OPE, and to develop a fed-batch strategy. Using this strategy, *R. toruloides* achieved, after 104 hours of fermentation, 25.6 and 20.6 g L⁻¹ of biomass and lipids produced, respectively, with a net improvement compared to simple OPE batch fermentation.
- Screening on filamentous fungi demonstrated that, compared with yeasts, they are more versatile in metabolizing different carbon sources, since satisfying values of growth were obtained on all substrates tested, despite lower lipid contents on dry weight. Among the strains tested, *M. isabellina* was the most versatile and performing one, at least in the conditions we used for flask screening. However, transfer to bioreactor scale showed that mechanical agitation, suggested also by other authors (Chatzifragkou et al., 2010; Koike et al., 2001), is probably not the best strategy to grow this strain, since values of biomass produced were lower compared to those obtained in flask, even if with a higher lipid content.
- Metabolic engineering experiments, performed on *C. curvatus* with the aim of improving palmitoleic acid content in the accumulated oil, demonstrated that it is possible to insert and express heterologous genes in *C. curvatus* as evidenced by the acquisition of antibiotic resistance, used as a marker gene, permanently. However, being lipid metabolism finely regulated, C16:1 production was observed only during exponential phase, while it was detected at low levels during lipid accumulation phase.

Since part of this study was only preliminary and regulation mechanisms of lipid metabolism have not yet been fully understood, future perspectives will be focused on the following crucial steps:

- the poor growth achieved by most of the strains on RCW, suggests, as future improvement, a pre-treatment by acid hydrolysis and subsequent pH correction or enzymatic hydrolysis with β -galactosidase in order to release glucose, thus favouring the assimilation of sugars even by those microorganisms that are not able to metabolize lactose. In particular, this might improve lipid production in strains such as *L. starkeii* and *R. toruloides*, which despite a limited growth on RCW, maintained a significant lipid accumulation capacity.
- Results obtained from fed-batch fermentation of *R. toruloides* on a mixed glycerol-OPE substrate, although being quite satisfactory, demonstrated that improvements are still

possible and needed. For example, it has been observed that the rate of uptake of the carbon source is also dependent on the nature of the nitrogen source used (Papaikolaou and Aggelis, 2011). Therefore, by choosing a different compound than $(\text{NH}_4)_2\text{SO}_4$, a better uptake of glycerol could be achieved. Other improvements could be the optimization of the dilution rate and concentration of the various components of the feeding solution, which may provide more favorable conditions to lipid accumulation.

- As concerns filamentous fungi, since there are only few studies on agro-industrial waste, it will be important to continue investigating their response to different and complex carbon sources, but above all, to improve cultural conditions to achieve higher lipid yields in shorter fermentation times. Different reactor systems (e.g. air-lift) will be also investigated in order to determine the best production strategy.

- Low C16:1 production obtained in metabolic engineering experiments on *C. curvatus*, demonstrated that lipid metabolism is finely regulated and that, probably, different modifications need to be done in order to increase significantly lipid composition. Therefore, further studies will involve the knock-out of genes downstream of the C16:0 metabolic step, such as WT $\Delta 9$ desaturation, in order to evaluate the isolated effect of heterologous desaturases. Furthermore, overexpression of endogenous acyl-transferases should be attempted in order to favor transportation of an unusual fatty acid to lipid bodies. Once the fermentation conditions will be implemented, an economic feasibility study will be performed in order to evaluate total costs and possible further improvements.

- Alternative and more sustainable extraction and esterification methods will be tested with the aim of reducing the not negligible environmental impact of these steps.

- The possibility of reuse of waste deriving from the fermentation itself (biomass and exhausted substrate) will also be evaluated in order to make the whole process zero impact.

The development of a circular, sustainable and zero-impact industrial process requires the integration of several activities that must be evaluated both individually and as part of the whole production process. Once this has been planned, also in relation to the local reality in which the process is to be placed, it is important to obtain the consent of the public opinion and the support of the companies that can take part in this value chain. As concerns the production of microbial oils, the transfer to the industrial scale has already been accomplished for some specific high value-added products in the nutraceutical field; therefore, the possibility of achieving the same also in the biofuel field seems plausible and no doubt promising.

The study carried out in this project, albeit still on a laboratory scale, having taken into account waste that are easily available even at the local level and currently poorly exploited, is perfectly integrated in this context. The final future perspective is therefore that of pursuing this line of research up to the development, hopefully, of an industrial process.

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List of papers

Scientific Publications produced during the PhD course

1. Carota, E, Crognale, S, D'Annibale, A, Petruccioli, M. Bioconversion of agro-industrial waste into microbial oils by filamentous fungi. Process. Saf. Environ. Prot. Submitted.
2. Carota, E, Crognale, S, D'Annibale, A, Gallo, A.M, Stazi, SR, Petruccioli, M, 2017. A sustainable use of Ricotta Cheese Whey for microbial biodiesel production. Sci. Total Environ. 584–585, 554-560. doi:10.1016/j.scitotenv.2017.01.068.

Poster/Oral Communications to Congresses

1. Carota E, Springer J, Crognale S, D'Annibale A, Weusthuis RA, Eggink G, Petruccioli M, 2017. *Cryptococcus curvatus*: a versatile yeast for the bioconversion of agro-industrial waste into microbial oils of industrial interest. XXXII SIMGBM Congress, University of Palermo, September 17-20, 2017.
2. Carota E, Petruccioli M, Gallo AM, Crognale S, D'Annibale A, 2016. Bioconversion Of Citrus Waste Into Lipids By Zygomycetes. Conference Abstract Book-9th International Conference on Environmental Engineering And Management – Iceem09”, Bologna (Italy), 6 - 9 September 2017.
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