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"Genetic and process engineering approaches for improving lipid productivity in the non-conventional oleaginous yeast *Cutaneotrichosporon oleaginosus*"

Tesi di laurea sperimentale

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"Statutory Declaration"

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Abstract

Microbial oils are getting increasing attention as possible alternative to plant-based oil, in the substitution process of fossil fuels. However, several aspects need to be optimized in order to obtain economical competitive oil with the desired composition. In this work, two different approaches were used to achieve the industrial goal. The first one was based on the genetic engineering of *C. oleaginous* to increase the lipid productivity and to modify the composition of the synthesised triglycerides (TAG). An *agrobacterium* mediated transformation was used to overexpress diacylglycerol transferase (DGA1), the enzyme responsible for the final step of the TAG synthesis, and a Δ9-desaturase, which is believed to catalyse the conversion of stearic acid (C18:0) into oleic acid (C18:1). The selection of positive single colonies and the screening of the obtained mutants confirmed the success of the modification. The second approach aimed to study the influence on the *C. oleaginous'* growth and lipid profile of several volatile fatty acids (VFAs), a possible feedstock that can be produced via waste-steam treatment. In this regard, fed-batch fermentations using glucose and synthetic mixtures of acetic acid and VFAs as carbon sources were carried on in a 1-L scale bioreactors. The simultaneous use of acetic acid and secondary acids showed a positive effect on the yeast growth, increasing the accumulation of TAG. Furthermore, it was demonstrated how the different carbon sources can influence the lipid composition.

Abstract

Gli oli microbici stanno ricevendo sempre più attenzioni come possibile alternativa agli oli vegetali, nel processo di sostituzione dei combustibili fossili. Tuttavia, diversi aspetti necessitano di essere ottimizzati al fine di ottenere oli economicamente competitivi e con caratteristiche chimico-fisiche desiderate. In questa ricerca, sono stati utilizzati due differenti approcci per poter realizzare l'obiettivo preposto. Il primo, si è basato sull'ingegnerizzazione genetica del lievito *C. oleaginous*, al fine di incrementare la produttività di lipidi e modificare la composizione dei trigliceridi (TAG) sintetizzati. Un protocollo basato su una trasformazione genetica mediata da Agrobacterium è stato utilizzato per sovraesprimere la diacilglicerol trasnferasi (DGA1), l'enzima responsabile dell'ultimo step della sintesi dei TAG, e la Δ9-desaturasi, l'enzima che catalizza la conversione dell'acido stearico (C18:0) in acido oleico (C18:1). La selezione di colonie positive e l'analisi dei mutanti ottenuti ha confermato la buona riuscita della trasformazione. Il secondo approccio ha mirato a studiare l'influenza sulla crescita e sul profilo di lipidi accumulati da *C. oleaginous* da parte di diversi acidi grassi volatili (VFAs), una materia prima ottenibile da trattamenti di scarti industriali. A questo proposito, sono state utilizzate fermentazioni fed-batch su scala da 1-L basate su glucosio e miscele sintetiche di acido acetico e di VFAs come fonte di carbonio. L'utilizzo simultaneo di acido acetico e acidi secondari ha mostrato come sia possibile stimolare il metabolismo microbico al fine di incrementare l'accumulo di oli e ottenere una composizione chimica lipidica desiderata.

Contents

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Abbreviations

AA, acetic acid

ACL, ATP citrate lyase

ATMT, *agrobacterium* mediated transformation

BA, butyric acid

DGA1, diacylgylerol synthetase

FAs, fatty acids

GHG, greenhouse gases

PA, propionic acid

TAGs, triacylglycerol

VFAs, volatile fatty acids

wt%, weight percentage

YPD, yeast pepton dextrose

1 Introduction

1.1 Depletion of fossil fuels and climate change

It is a well-known fact that fossil fuel are the primary source for energy as well as the starting material for the chemical industry, supplying more than 80% of the world energy demand[1] and almost the totality of the building blocks for chemicals[2] . Due to the intensive usage, to the constant growth of the world population and to the development of countries, the daily consumption of these sources is increasing and it is leading to their depletion[3]. Furthermore, it is largely recognized that fossil fuels are the main sources for "Greenhouse gas" (GHG) emissions[4,5,6] and that the increasing GHG levels in the atmosphere is likely to be the responsible for climate change[7]. The substitution of the fossil fuels in the modern society is not only a necessity due to their depletion but it became mandatory after that several international agreements, such as the Paris one[8], have been reached in order to limit the climate change. It is therefore necessary to find new sources able to replace the fundamental role that fossil fuels have in our society, possibly decreasing the environmental impact of the anthropogenic activities.

1.2 Biodiesel and oleochemicals

A rising possible solution has been identified in plant oils. Indeed, plants provides lipids as renewable sources, while consuming greenhouse gases during the growth. The produced oil can thus be used as Biodiesel or as starting materials for the oleochemical industry[9,10].

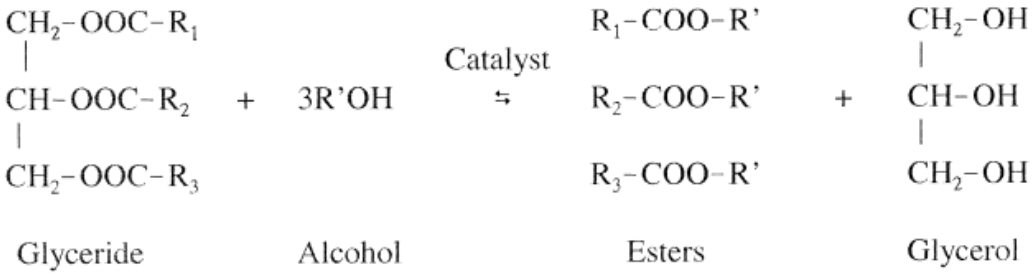


Figure 1 Transesterification of Triglycerides with alcohol[10]

Biodiesel can be produced by the transesterification of plant oil with alcohol, usually methanol or ethanol. This reaction, often catalysed by acids, bases or enzymes, leads to a mixture of methyl/ethyl esters, which is called biodiesel (figure 1). Biodegradability and a minor load of emission produced compared to fossil fuels make this renewable fuel very attractive[11]. Biodiesel production is experiencing a high growth due to its environmental friendly characteristics and to the international legislations that induce the substitution of fossil fuels with renewable ones (figure 2)[12,13].

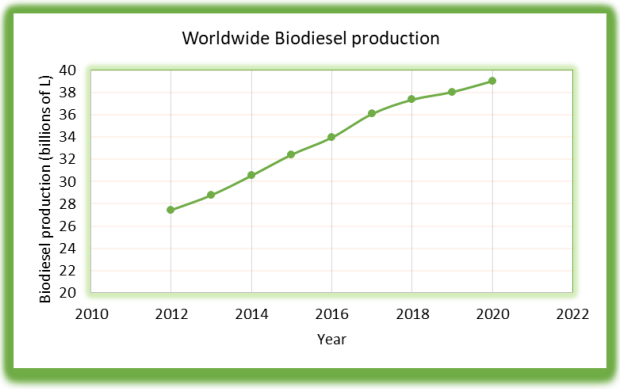


Figure 2 Worldwide Biodiesel production[14]

Plant-based oils could also be used to substitute fossil fuels in niche area of the petrochemical industry. The possibility of producing biolubricants, biosurfactants or biopolymers has been shown[9]. Lubricants market accounts for substantial quantities: in 2004, 37,4 million of tons of lubricants were consumed worldwide and it is estimated that 50% of these chemicals end in the environment, acting as a source of pollution[15]. The substitution of fossil fuel-based lubricants with biolubricants could also provide biodegradable products[16]. For what concern surfactants, their production is mainly based on paraffin, olefins and other petrochemical-based products, which represents toxic substances for human and for the environment. Since the global demand for surfactants is expected to raise up to 460'000 tonnes by 2020, and nowadays only 5% of the entire market is covered by biosurfactants, the utilization of the more environmental friendly biosurfactants could represent a green industry goal[17]. Plant-based oil can be used for other industrial purposes. Indeed, the non-edible castor oil can be used to produce: polyurethane with elastomeric properties[18]; polyurethane for adhesive application for wood[19]; polymers for coating[20]. It is true that the variety of products that can be obtained from vegetable oils is just a small percentage of the petrochemical production[21,22], but the substitution of the fossil fuels based products with plant oils one would be just a step forward into a more sustainable future. However, the use of plant-based oils places several problems. First of all plants require large area to be grown and given to the earth limited space and to the growing demand of food, plant-based oil production will lead to food competition[23]. Another important aspect is the seasonal production of plants that accounts for a discontinuous production. These characteristics lead to low yield production and thus high cost oil, that makes plant-based oils not competitive with fossil fuels industry. Although the price of petroleum and other fossil fuels will increase due to their depletion, making the plants-based oils more competitive, they might still not be the solution from an environmental point of view. In fact it has been shown that Biodiesel and other oleochemical substances can actually increase GHG emissions, affecting even more negatively the environment[24,25]. The converting of already existing plants cultivation or forests is in fact a high carbon emission releasing activity that requires years

before repaying the carbon debt. Fargione et al.[24] have observed that countries like Brazil, Malayisia or U.S.A., which have converted their forest or other cultivated lands to produce crop-based biofuels, have produced an amount of CO_2 from 17 to 420 times more than the annual GHGs emission that the biofuels produced can actually prevent. Therefore, the production of chemicals or fuels from vegetable oil would be related to waste biomass or abandoned agricultural fields[24], not supplying the world energy and chemicals demand.

1.3 Oleaginous yeast as possible answer

In this context, it became necessary to find a way of producing oils that can overcome all the previous described setbacks. Yeast capable of accumulating lipids over 20% of their dried weight are called "oleaginous"[26]. In literature it has been reported the ability of some strains to accumulate lipids up to 70-80% of their dried weight (table 1)[27,28].

Table 1 Lipid accumulation percentage of oleaginous yeast[28,29]

Lipid accumulation percentage of oleaginous yeast on glucose medium	
Cryptococcus curvatus	58
Cryptococcus albidus	65
Candida sp.	42
Lipomyces starkeyi	63
Rhodotorula glutinis	72
Rhodotorula graminis	36
Rhizopus arrhizus	57
Trichosporon pullulans	65
Yarrowia lipolytica	36

Past studies have shown how it is possible to optimize the fermentation conditions to improve the oil production, reaching a dried biomass content of 185 g/L, using O_2-enriched air flow[30], or lipid productivity of 0.995 g/Lh, using partial recycling cultures[31]. The use of yeasts can also account for higher lipid content/area than plants and it is not linked to seasonal production. Furthermore, since they do not require large surfaces such plants do, the problem of food competition can be avoided[32]. The cost of yeast oils though depends on several parameters and thus microbial oil can be advantageous or not compared to plant-based oil. When glucose is used as carbon source, the oil cost are estimated to be 5,5 \$/Kg[33], not economically competitive compared to palm oil, which costs 0,5 \$/kg[34]. While using economical feedstock such as waste steam from brewer industry can highly decrease the cost, down to 0,111 \$/Kg of oil[35]. Moreover, LCA assessment from Masri et al.[36] demonstrated how the microbial-based oil can account for low GHGs emissions (3,56 Kg CO_2-eq for 1 Kg of produced oil). It is thus essential to identify the most promising strain and to optimize the

substrates required for the fermentation, as well as operating conditions and downstream processes in order to make the use of oleaginous yeast industrially competitive to plant oil and to reduce the amount of GHG emissions produced.

1.4 *Cutaneotrichosporon oleaginosus*, a promising yeast for microbial oil production

Cutaneotrichosporon oleaginosus (*C.oleaginosus*), known also as *Candida Curvata*[37], *Trichosporon oleaginosus*[38], *Apiotrichum curvatum*[39], *Cryptococcus curvatus*[40] and *Trichosporon cutaneum*[41], is an interesting oleaginous yeast. *C.oleaginosus* has been reported to be one of the highest accumulating oil strain, with the ability to accumulate up to 60% of its dried weight, compared to 38% of the oleaginous yeast model *Yarrowia lipolytica*[28]. *C.oleaginosus* has been proven to be a good candidate for *de novo* lipid production, not requiring to be grown on lipid in order to accumulate them, differently from other yeast such as *Yarrowia lipolytica*, which often act as *ex novo* lipid producer[27,42]. Due to the latter and to other several characteristics, *C.oleaginosus* appears to be an interesting target of study for oil production via yeast. An overview of the main relevant parameters for fermentation, as well as the main metabolic features, is given below in order to better illustrate the possibility of development of *C.oleaginosus* as microbial oil producer.

1.4.1 Growth substrates

The high growth rate and the possibility of using conventional substrates such as glucose, sucrose, lactose, as well as unconventional ones, like pentose sugars, for *de novo* lipid production give the opportunity to consider *C.oleaginosus* as a candidate for microbial oil production[37,43,44]. Several carbon sources obtainable from the treatment of by-products of industrial processes or from renewable biomasses can be used as feedstock for *C.oleaginosus.* Below few examples to display the potential of this yeast are showed:

- The worldwide production of lignocellulosic material accounts for 2’000 billion of tons and it represents the 50% (wt%) of the overall biomass production[45,46,47]. Considering that in many agricultural activities the percentage of by-product obtained is remarkable (straw represents the 35% (wt%) of over ground biomass of wheat crop[47]), lignocellulosic material could act as feedstock for carbon source for yeast. In fact the hydrolysis of Xylan, one class of polymers that can compose up to 30 wt% of lignocellulose[48], produces xylose[49] which has been showed to act as possible carbon source for *C.oleaginosus*[50]. Often, the use of this non-conventional carbon sources is prevented by the presence of growth inhibitors. In fact, cereal straw hydrolysates contain chemicals, such as furfural, 5-hydroxymethyl-furfural (HMF), VFAs (short chain fatty acids with six or fewer carbon atoms[51]), and the same xylose, known to

suppress the growth of yeast. However, Yu et al. have shown how *C.oleaginosus* can grow even in presence of these compounds[52]. Yu et al. studied the influence of this growth inhibitors on 5 different yeast strains. *C.oleaginosus* showed the best growth in the non-detoxified hydrolysate, consuming most of these unconventional carbon sources.

- Another interesting substrate is N-acetylglucosamine. This monosaccharide is the building block for the second most abundant carbohydrate on the hearth, chitin[53]. Every year 100 billion of tons of chitin are produced by living organisms such as fungi or crustaceans[54]. *C.oleaginosus* has been shown to use N-acetylglucosamine as carbon source, making it an abundant renewable resource[50].
- Crude glycerol is the main by-product of the Biodiesel manufacture, obtained in a 10% (wt %) of Biodiesel. As the Biodiesel yearly productivity increases, glycerol does as well, reaching 300,000 m^3 in 2015[55]. Several researches demonstrated the use of glycerol as potential carbon source for *C.oleaginosus*[35,56], making it another promising low-cost feedstock.
- The ability of *C.oleaginosus* to grow on lactose gives the possibility to use whey permeate, a by-product of the cheese manufacture, as carbon source. Whey permeate contains about 45 g/L of lactose. Its use as feedstock for *C.oleaginosus* led to efficient processes, reaching a productivity of almost 1 g/Lh[31], and provides once again an economic and renewable substrate.

1.4.2 Lipid composition and dependence on growth parameters

Lipid composition defines the use of the oil in industry. According to their composition they can be used as lubricants, surfactants, detergents, plasticizers, paints, drugs or starting material for second generation biofuels[57,58,59]. It has been reported that the accumulated oil in *C.oleaginosus* are composed of triacylglycerols (TAGs) for the 88% (wt%) and that the fatty acids (FAs) composition resembles Cocoa butter's one[60], as shown in Table 2.

Table 2 Relative content of fatty acids of C. oleaginosus[60]
C16:0 palmitic acid, C16:1 palmitoleic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid, C18:3 α-linoleic acid

Relative content of fatty acids (wt%)						
C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Others
29,7 ± 1,1	0,8 ± 0,1	6,1 ± ,01	48,3 ± 0	9,8 ± 0,8	0,3 ± 0	4,6 ± 0,7

A high content of TAGs is essential in order to replace plant oil with yeast oil in Biodiesel production and to produce the previously described oleochemicals. The FAs composition depends on the growth conditions and several parameters can affect it.

- Temperature can be easily manipulated and its effects are largely known. As the temperature decreases, the FAs composition move toward longer and higher unsaturated chain[61], in order to maintain membrane fluidity[62].
- Oxygen can influence the composition of FAs altering the saturation degree. From the literature, it is reported that oxygen content and percentage of double bonds in the aliphatic chains are proportional[63]. An overview of the desaturase mechanism clarifies the oxygen effect (figure 3). The enzyme responsible for the saturation degree of FAs chains are fatty acids-desaturase. The overall process requires a desaturase enzyme, a short electron transport chain and O_2, as the oxidant agent and as electron acceptor. Thus since oxygen acts as a reactant, accepting the electrons furnished by the oxidized substrate, the more oxygen there is the higher is the unsaturated lipids content[64].

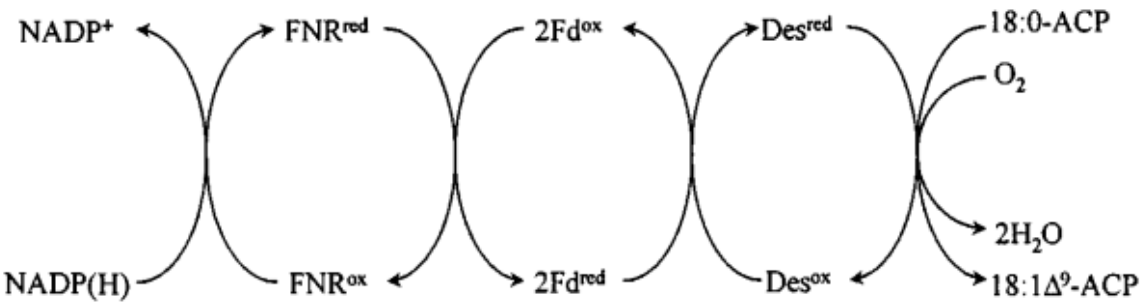


Figure 3 Δ^9-18:0-ACP desaturase electron transport chain in nonphotosynthetic tissues
ACP: acyl carrier protein[64]

- Although the effect of pH on the amount of lipid accumulated and biomass produced has been sufficiently studied[56], the influence on FAs is not completely clear and it is probably the function of several parameters[37]. Chi et al.[65] studied the composition of TGAs at pH values of 6, 7, 8 and 9 (table 3). They observed that as the pH increases, the percentage of saturated FAs increases as well. However, the highest value of C18:2 was obtained at pH 9. A brief explanation was given considering the slow growth observed at such an high pH: they state that C18:2 concentration experiences a maximum at the beginning of the growth and then it decreases, as confirmed by other research[66]. Yet, even if the pH clearly affects the FAs composition, and even if *C. oleaginosus* shows a wide range of pH tolerance, a better comprehension of the pH influence would be required.

Table 3 FAs profile of C.oleaginosus as function of pH[66]
MUFA: mono unsaturated fatty acids; PUFA: polyunsaturated fatty acids

wt%	pH 6	pH 7	pH 8	pH 9
C16:0	16,5	17,2	14,5	24,1
C16:1	0,2	0,2	0,1	0,5
C18:0	16,8	19,0	24,6	18,2
C18:1	46,5	46,2	43,8	36,3
C18:2	14,7	12,7	11,1	16,3
C18:3	1,8	1,6	2,3	3,2
C22:2	1,8	1,2	0,9	0,0
Saturated	34,3	37,3	40,8	42,8
MUFA	46,7	46,3	43,9	36,8
PUFA	18,3	15,6	14,4	19,5

- Substrates used as carbon source can account for different lipid composition too. Liu et al.[67] studied the influence of VFAs on the FAs composition. Synthetic mixtures of acetic, propionic and butyric acids at different compositions were fed to *C.oleaginosus* and the results were reported in figure 4.

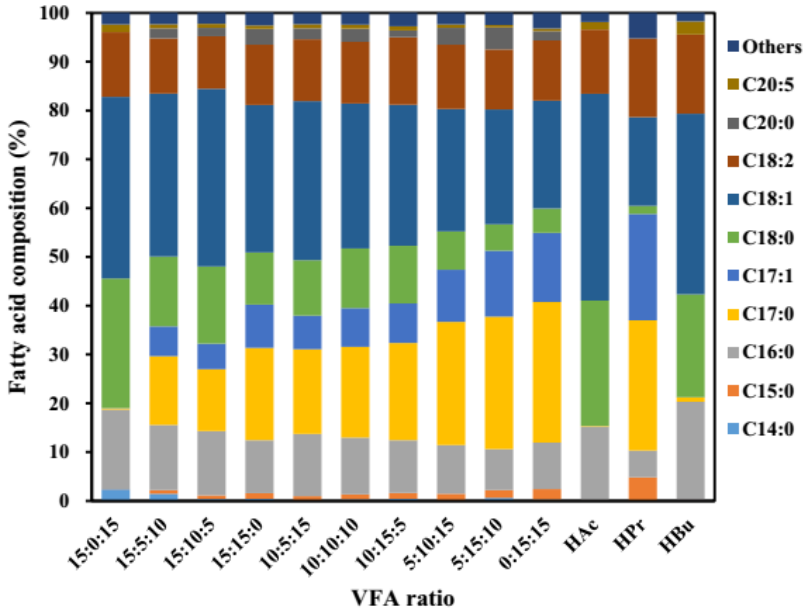


Figure 4 FAs composition obtained feeding with different synthetic mixtures of VFAs[67]
VFA ratio expressed as HAc:HPr:HBu

Apparently, the number of carbon atoms in the principal aliphatic chain influences the FAs composition. Propionic acid, an odd-numbered carbon acid, appeared to increase the concentration of odd-numbered FAs. Acetic and butyric acids, both even-numbered carbon acids, behave similarly, favouring the production of even-numbered FAs.

- According to the fermentation mode it is possible to observe variation in the lipid composition. In particular, continuous culture of *C.oleaginosus* showed a more constant profile in lipid composition than fed-batch one, given that the steady state could be maintained indefinitely[44]. Batch or Fed-batch mode, instead, accounts for a higher lipid composition variability. As already stated, the percentage of C18:2 increases at the beginning of the growth and subsequently decrease in favour of C18:0 and C18:1. Other variation in the FAs profile were observed as in table 4.

Table 4variation in the fatty acids profile according to time[66]

Time	Fatty acids composition (wt%)				
(h)	C16:0	C16:1	C18:0	C18:1	C18:2
8	18	2	5	34	40,5
24	13	1	5	33	48
32	13	Trace	14	37	36
52	18	Trace	16	50	16

Apart from the parameters described above, other factors can affect lipid composition, such as carbon-nitrogen ratio[68,69]. All these growth conditions can be optimized in order to increase the lipid percentage and to promote the production of a particular type of FAs rather than others[44,70]. However, reaching the highest lipid percentage does not mean having the highest productivity too[44]. Often, high lipid percentage is linked to low dried biomass, resulting in lower productivity[71]. Thus, it is necessary to evaluate all the possible factors that can affect yeast growth in order to achieve the best lipid productivity together with the desired composition.

1.4.3 Biochemistry of oleaginous yeast and genetic modification of *C.oleaginosus*

In order to optimize the fermentation and the lipid composition it is necessary to comprehend the mechanisms that regulates the TGA synthesis and the accumulation of lipids. The ability of oleaginous yeast to accumulate lipids was related to the enzyme "ATP : citrate lyase" (ACL)[72]. High activity of ACL was reported for oleaginous yeast, while for non-oleaginous ones the activity is low or almost null[73]. However, this enzyme does not operate at all conditions and most of the time is necessary to stimulate the lipid accumulating process. During the last years, the mechanism of lipid accumulation was deeply studied and it was shown how to efficiently trigger it. In fact, the use of nutrient limiting media was shown to be an efficient method to trigger oil accumulation, in particular nitrogen-limiting media. Yet, as long as lipid accumulation will be bonded to nutrient limiting media, the cell growth will be suppressed as well, leading to lower biomass production[42]. Below, it is

described the mechanism reported from Ratledge[72], explaining the lipid accumulation via nutrient depletion.

The use of nutrient-limiting media guarantees the depletion of at least one of the essential elements required for the growth. In the case of nitrogen-limiting media, once the nitrogen is depleted the cells stop growing since there are no more nutrients available. The lack of nitrogen affects the enzyme "AMP deaminase" by slowly decreasing its intracellular concentration. Indeed, when the nitrogen from the culture is depleted, the yeasts start an internal process to produce ammonia, leading to the consumption of AMP deaminase (figure 5).

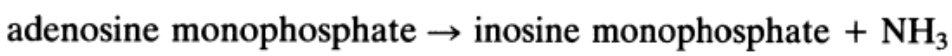


Figure 5 nitrogen production from AMP deaminase

"AMP deaminase" acts as activator of the enzyme isocitrate dehydrogenase (ICDH), responsible for the isocitrate metabolism (figure 6).

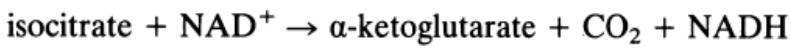


Figure 6 isocitrate metabolism

Consequently, the absence of AMP deaminase affects ICDH, leading to the accumulation of isocitrate and of citrate, due to the equilibrium reaction catalysed by aconitase (figure 7).

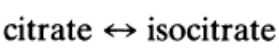


Figure 7 citrate/isocitrate equilibrium

Subsequently, citrate exits the mitochondria and enters the cytoplasm, and then it is transformed to Acetyl-CoA by ACL, starting the fatty acids synthesis (figure 8).

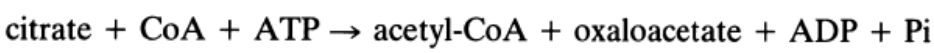


Figure 8 production of Acetyl-CoA from citrate via ACL

Successively the "fatty acid synthase complex" (FAS) acts producing palmitoyl-CoA and then Acyl-CoA. The latter is used to produce TGAs through the Kennedy pathway[74]. During this process the amount of reducing power required is partially furnished by the production of NADPH via oxaloacetate cleavage, previously produced from citrate. Concluding, since the absence of nitrogen will suppress the growth of cells, the higher amount of carbon source will not be used from the cell to reproduce but will act as supply for the synthesis of citrate and the accumulation of TGAs. In this regard it is important to optimize the carbon-nitrogen ratio to obtain the best conditions for lipid accumulation[68]. In figure 9 an overview of the TGAs accumulation pathway is presented.

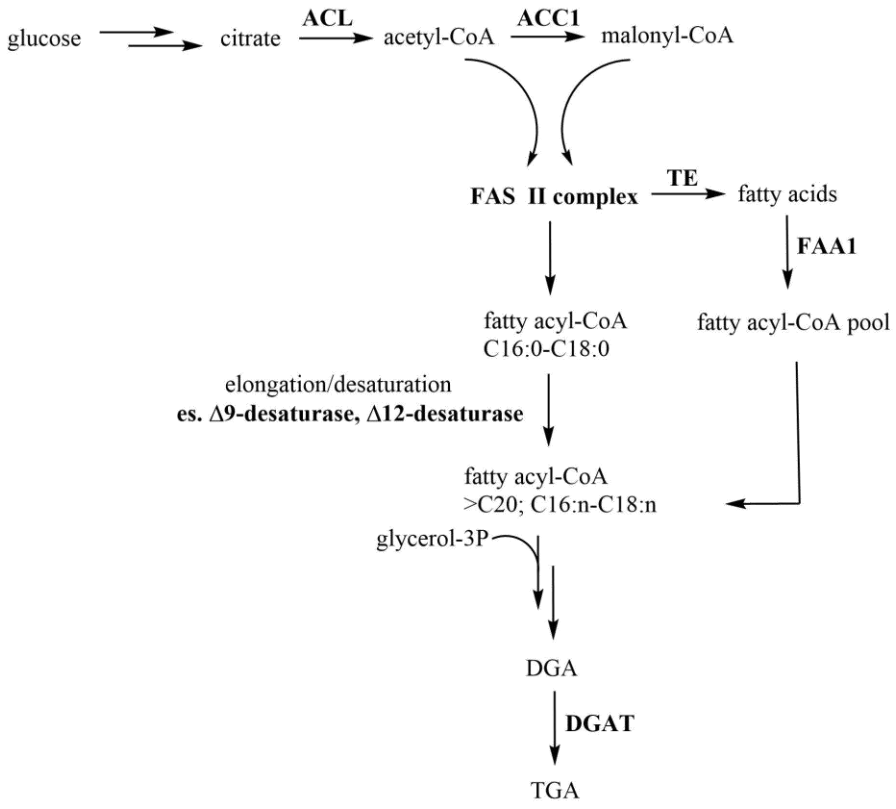


Figure 9 TGA accumulation pathway in oleaginous yeasts
ACL: ATP citrate lyase; ACC1: acetyl-CoA carboxylase; TE: thioesterase; FAA1: fatty acyl-CoA synthetase; DGAT: diaciglycerol transferase; glycerol-3P: glycerol-3-phosphate; DGA: diacylglycerol[74]

Understanding the biochemistry of oleaginous yeast, it is possible to develop strategies to increase the lipid accumulation and obtain a tailor-made FAs composition. Genetic engineering approaches have been studied on yeasts and have succeed in producing mutant with modified metabolism in common species such as *Yarrowia lipolytica*[75,76,77]. Overexpression of genes or the insertion of new metabolic pathway gave successful results. Tai et al. studied the effect of the insertion of genes ACC1 and DGA1 in *Yarrowia* in a 2-L bioreactor fermentation of 120 h. They observed a fivefold increase in the lipid content compared to the control, achieving a percentage of 61,7% (wt%). However genetic modification of *C.oleaginosus* has proven to be a difficult challenge[37] and only few researches were reported. Koivuranta et al.[78] succeeded in the insertion via electroporation of an heterologous metabolic pathway from *Yarrowia L.* to enhance TAG production. Recently an "agrobacterium mediated transformation (ATMT)" protocol was developed by Goerner et al. to insert expression cassettes, using the homologous promotor of glyceraldehyde-3-phosphate (GPD) dehydrogenase gene and hygromicin B as selection marker[42]. The GPD gene promoter was used to enable strong expression of the heterologous gene[79]. The purpose of the research was to insert a new metabolic pathway for the synthesis of plant and marine derived Omega-3 fatty acid. Considering that modified

microorganisms via ATMT are transformed randomly, since the amount of genomic copies and the location of insertion can vary, it is necessary to screen the positive colonies in order to recognize the most promising one. The mutants were screened in different media: rich medium (YPD); nitrogen-limiting media, using glucose or xylose as carbon source; phosphorous-limiting media, using N-acetylglucosamine as carbon source. They confirmed the excellence ability of nitrogen-limiting media in triggering lipid accumulation, achieving a 57% (wt%) of lipid content after 168 h in 500 mL baffled shaking flasks using xylose as carbon source. The genetic modification was correctly performed, observing a variation in the fatty acids profile. In particular, it was observed an increase in α-linoleic acid (ALA, C18:3) and the production of the non-native eicosadienoic acid (EDA, C20:2) and eicosatrienoic acid (ETE, C20:3). However, the nitrogen-limiting media appeared to suppress the expression of the inserted genes, restricting the maximum obtainable percentage of EDA at 4,3% (wt%) (xylose as carbon source, 168h), compared to 17% (wt%) in YPD. They concluded that the GPD promoter is upregulated in nitrogen-rich media. Thus, to observe the genomic modification it would be necessary to trigger the lipid accumulation without using nutrient-limiting media.

1.5 Aim of the research and structure of the project

From the previous considerations, it is possible to deduce how *C.oleaginosus* represents an interesting platform for the sustainable production of lipid for several industrial uses. Yet, many aspects need to be clarified and optimized in order to achieve better oil yields, tailor made production and decrease the cost of manufacture. In this research, two different approaches were used: the first one was a bioprocess engineering approach, while the second was a genetic engineering approach; both aimed to increase lipid production and to modify the FAs composition.

1.5.1 Bioprocess engineering approach

The effect of the feedstock for the yeast fermentation was previously discussed. This research wanted to go deeper in understanding the influence of the carbon source on the composition of the FAs. To do so, the yeast cultures were carried on in semi fed-batch fermenter, feeding the yeast with 5 different acids. Four of them were never analysed in a *C.oleaginosus* culture. The names of this acid were not revealed for secrecy reasons and they were labelled with a code. Acid 1 (A1) was characterized by a linear aliphatic chain with an even number of carbon atoms and the presence of a double bond. Acid 2 (A2) was characterized by a branched aliphatic chain with an odd number of carbon atoms. Acid 3 (A3) was characterized by a branched aliphatic chain with an even number of carbon atoms. The remaining two acids, propionic and butyric acid, were already studied from Liu et al.[67]. Synthetic mixtures of the acids were prepared, together with acetic acid and glucose as carbon sources. The use

of synthetic mixture of acetic acid and glucose was previously optimized by Masri et al.[36] and the potential use of this mixture for fermentation with oleaginous yeast is explained below. It was already described how it is possible for *C.oleaginosus* to grow on VFAs. The use of VFAs as feedstock is interesting from an economical and environmental point of view. In particular, acetic acid can not only be produced starting from fossil fuels[80,81] but also through more environmental friendly approaches such as acidogenic fermentation of waste steam[51] or microorganism mediated conversion of CO[81], making it a promising feedstock. Additionally, acetic acid was proven to trigger lipid accumulation. Gong et al.[71] studied the lipid production in a 3-L stirred tanked reactor, using a continuous mode. They reached a lipid content of 66% (wt%) in *C.oleaginosus,* optimising the dilution rate of acetic acid. Acetic acid is believed to be taken up from cells and converted directly to acetyl-CoA[82], triggering the lipid accumulation by quickly providing an intermediate for the TGA synthesis via the following reaction (figure 10):

$$\text{acetate} + \text{ATP} + \text{CoASH} \leftrightarrow \text{acetylCoA} + \text{AMP} + \text{Ppi}$$

Figure 10 acetate uptake via cytosolic acetyl-CoA synthetase in yeast[83]

However, few setbacks needed to be overcome. First of all, acetic acid is poisonous for yeast. Indeed, Gong et al. observed a maximum in the lipid accumulation, obtaining a decrease in the lipid content as the concentration of acetic acid was raised. Furthermore, Masri et al.[36] showed that acetic acid as sole carbon source enhances lipid accumulation but not biomass growth. The use of glucose together with acetic acid proved to be an efficient way to guarantee the simultaneous growth of cells and TGA accumulation. The poisonous action of the acid was instead reduced by using a fed-batch mode, allowing the continuous feeding while keeping the concentration at non-dangerous level. In this regard, pH affects the influence of the acid on the yeast growth. In fact, the use of acetic acid at pH lower than its pKa (4,75) can result in a poisonous effect for the yeasts, since that once the acid enters into the cell protons are released, leading to an alteration of the metabolic functions[82]; at higher pH, the undissociated fraction is much lower, resulting in a less poisonous effect. Neutral pH was proven to be effective in acetic acid uptake without compromising the growth[69]. Additionally, even if glucose showed the repression of several metabolic pathway[84], the ability of *C.oleaginosus* to metabolise acetic acid in presence of glucose was shown[71] and confirmed by Masri et al.[36]. Hence, mixture of glucose and acetic acid allow to avoid the use of nutrient-limiting media, increasing lipid productivity. In fact, usually microbial oil production are based on a two-steps process: the first step is required to grow the biomass in nutrient-rich media, while the second step is used to enhance lipid production

via nutrient-limiting media[33]. Merging the two steps it is possible to reduce the fermentation time, enhancing the lipid productivity[36].

To assess the effect of carbon source on FAs composition and lipid production, experiments were performed in a 1 L bioreactor. Gravimetric analysis, spectrophotometric analysis and chromatographic analysis were performed in order to establish the influence of the different feedstock on the cell growth and lipid composition.

1.5.2 Genetic engineering approach

The second part of the research was based on the modification of the wild type strain in order to enhance lipid productivity and increase the content of unsaturated FAs. To do so, two different plasmids were inserted via ATMT, using the protocol optimized by Goerner et al.[42]. The first plasmid contained a gene coding for a Δ9-desaturase (for secrecy reason the name of the gene was not revealed). The second plasmid contained the gene DGA1, coding for the diacyl-glycerol acyltransferase[85], and the desaturase gene. The desaturase is believed to catalyse the reaction which transforms stearic acid (C18:0) in oleic acid (C18:1), inserting a double bond at the position 9 of the aliphatic chain (figure 11).

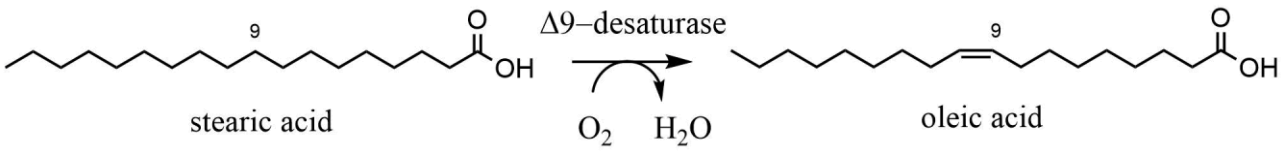


Figure 11 conversion of stearic acid to oleic acid catalysed by a desaturase

Successful studies on desaturase genes were previously done. The overexpression of a Δ9-desaturase in plants and algae was studied. Genetic modification of the green alga *Chlamydomonas reinhardtii* led to an increase in the oleic acid (C18:1) and linoleic acid (C18:2), and surprisingly to a rise in the amount of FAs, 28% (wt%) more that the wild type control[86]. The insertion of a Δ12-desaturase, responsible for the conversion of oleic acid (C18:1) to linoleic acid (C18:2), was studied in both yeasts and fungus[87]. In *S. cerevisiae*, the expression of this heterogeneous gene led to the synthesis of the not-natural linoleic acid. In the fungus *A. oryzae* it was observed an increase in the linoleic acid content, together with a decrease in stearic and oleic acid. No studies were reported about the overexpression of the Δ9-desaturase in *C. oleaginous*. For what concerns DGA1 gene, it is believed to be one of the key enzyme for the TAG synsthesis[88,89]. Acyl-CoA DAG acyl transferase acts on the last step of the TAG synthesis (figure 9), performing the last acylation step[85]. Several researches demonstrated how the overexpression of DGA1 in yeast can enhance the lipid production. Genetically modified *Yarrowia lypolitica* from Tai et al.[75] was able to accumulate lipid for 34% of its dried

weight, compared to 8% of the control. Excellent results were obtained also from Kamisaka et al.[90], increasing the percentage of accumulate lipid by *Saccharomyces cerevisiae* of almost the double, going from 8,8% (wt%) of the wild type to 15% (wt%) of the mutant. Since no data were reported for the overexpression of DGA1 in *C.oleaginosus,* and given the promising results of the previous researches, it was believed that an overexpression of this gene could have led to successful results.

After the transformation, the positive colonies were identified and grown in shaking flasks. Gravimetric analysis, spectrophotometric analysis and chromatographic analysis were performed in order to characterize the lipid produced from the mutant, using the wild type as control.

2 Results and discussion

In order to clarify the following pages, the "results and discussion" section was divided according to the two different approaches and each of them was discussed separately.

2.1 Genetic engineering approach

After having performed the transformation via Agrobacteria, the colonies were selected using Hygromicin B and successively confirmed by PCR. Of all the grown colonies, 14 were found to be positive and were further analysed (table 5).

Table 5 Positive mutants

Δ9-desaturase gene – mutant Label code: D	Δ9-desaturase + DGA1 inserted genes – mutant Label code: E
D3	E1
D4	E2
D5	E4
D6	E5
D7	E11
D25	E16
D30	E20

Since the ATMT produces random transformation, it was necessary to analyse the mutants in order to identify the best performing ones. The screening was conducted in 500 mL baffled shaking flasks, taking samples every 24 h until 96 h. For each mutant, three biological replicants were analysed in order to assess the reproducibility of the new strain. The analysis on the mutants were conducted as listed in table 6.

Table 6 analysis performed on the screening samples

Time	Analysis
24 h	Dried biomass, O.D. measurement
48 h	Dried biomass, O.D. measurement
72 h	Dried biomass, O.D. measurement, Lipid extraction (if confirmed by Nile Red analysis), FAME (fatty acids methyl esters) profile

96h	Dried biomass, O.D. measurement, Lipid extraction, FAME (fatty acids methyl esters) profile

For a better description of the analysis, see the "experimental details" section. FAME analysis allowed to identify the FAs produced from the mutants and monitor the effect of the inserted genes. Nile Red analysis was used as a first quantitative control to identify the peak of lipid production, as described from Sitepu et al.[91]. The gravimetric lipid extraction was then performed for all the 96 h samples, and for the 72 h samples in case the Nile Red analysis confirmed a peak in the lipid production.

2.1.1 O.D. (600 nm) profile

O.D. (600 nm) measurements were used as qualitative way to assess the growth of the mutants. Based only on the O.D. values, it was easily appreciable how the random mutagenesis produced a larger variety of mutants for the Δ9-desaturase gene (D mutants) than for the Δ9-desaturase+DGA1 genes combination (E mutants). From a first sight (figures 12 and 13), it was possible to notice how the D mutants show the highest O.D. values (23,8 ± 1,1 of D3, compared to 19,5 ± 0,2 of E1) as well as the lowest (12,3 ± 0,2 of D30 compared to 14,5 ± 0,7 of E20). Differently from wild type and from the E mutants, most of the D mutants (D3, D4, D6, D7, D25) did not reach the stationary phase at 72 h, but it took instead 96 h to do it (figure 11). This suggestion was confirmed running another screening with only the selected mutants until 120h, in order to confirm the previous hypothesis. Even though the O.D. measurements can be considered as a qualitative analysis, it was possible to assess that the genetic modifications led to changes in the growth profiles. D3, D6, D25, D30, E2, E11, E16, E20 appeared to be statistically different from the wild type (p value < 0,05, table 7). Only for two mutants (D30, E16) the modifications produced less competitive yeasts, while for most of the mutants the growth was almost comparable with the wild type, if not higher. In particular, for the Δ9-desaturase gene mutants, the new strains could be divided in three categories: D3, D6 and D25 which showed a higher growth compared to the wild type; D4, D5, D7 that showed a growth similar to the wild type (statistically not different, p value > 0,05, table 7); D30, which grew slower than the wild type. Similarly, it was possible to divide the E mutants in three categories: E2, which showed an higher growth compared to the wild type; E1, E4 and E5 which showed a similar growth compared to the wild type (statistically non different, p value > 0,05, table 7); E11, E16 and E20, which grew slower than the wild type.

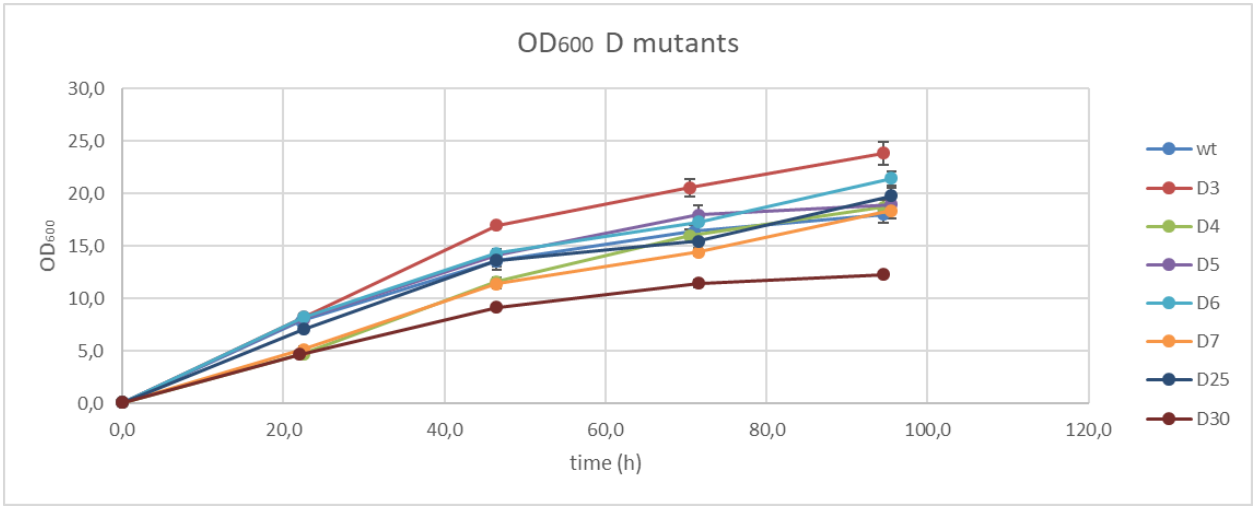


Figure 12 O.D. 600 of D mutants
error bars calculated as standard deviation of the three biological replicates

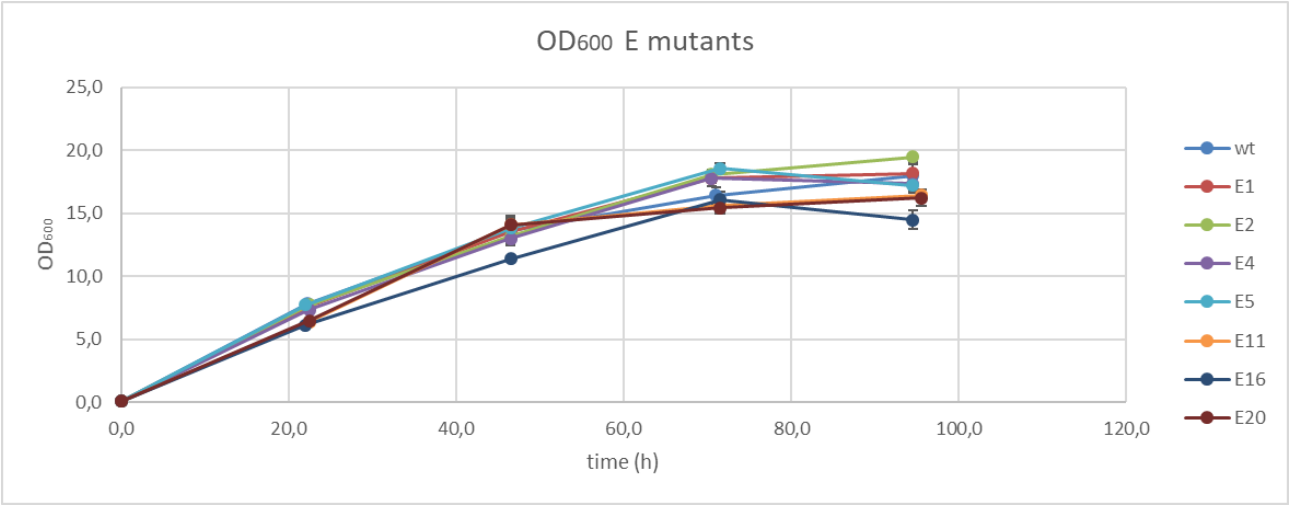


Figure 13 O.D. 600 of E mutants
error bars calculated as standard deviation of the three biological replicates

Table 7 p values for O.D. measurements

P values						
D3	D4	D5	D6	D7	D25	D30
4,33E-05	0,267	0,142	4,45E-04	0,561	0,0162	3,26E-06
E1	E2	E4	E5	E11	E16	E20
0,812	6,08E-03	0,270	0,339	0,0161	4,29E-04	0,0153

2.2.1 Dried Biomass profile

Differently from the O.D. measurements, the dried biomass analysis showed that the highest growth value belonged to wild type, 9,0 ± 0,4 g/L (figure 13). D mutants confirmed the wide variety of grow ability, going from a maximum of 8,8 ± 0,3 g/L for D5, to a minimum of 5,3 ± 0,1 g/L for D30 (figure 14). The considerations previously done for O.D. measurements could be applied in this case as well. In fact, the E mutants showed a similar growth ability: most of them reached 8,0 g/L of biomass after 96 h (figure 14). The growth range went from a maximum of 8,1 ± 0,1 g/L for E1, to 6,6 ± 0,1 g/L for E16. Differently from the O.D. values then, where it was possible to observe big differences with the wild type only for D30 and E16, the dried biomass weights outlined a better grow for the wild type rather than the mutants, suggesting that the genetic modification decreased the ability of yeast to build up biomass (figures 14 and 15). Except for D3, D5 and D6 indeed, all the other D mutants were statistically different from the wild type (p value < 0,05), thus showing a lower growth (table 8). According to this results, it was possible to divide the mutants in three categories: D3, D5 and D6, apparently had an ability of producing biomass similar to that of wild type (statistically not different, p value > 0,05, table 8); D4, D7 and D25, grew slower than the wild type, producing less biomass; D30 grew much slower than all the mutants and the wild type. For what concerns the E mutants, it was possible to divide them in three categories: E1, E2, E4 and E5, which grew slower than the wild type; E11, E16 and E20 which grew much slower than the wild type.

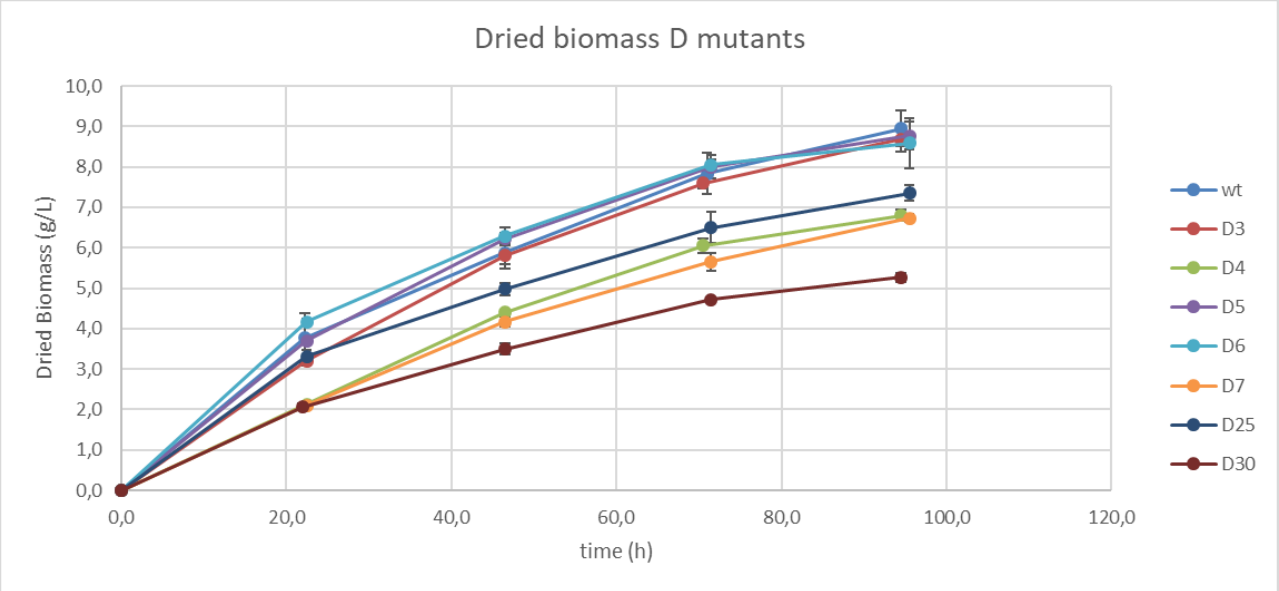


Figure 14 Dried biomass of D mutants
error bars calculated as standard deviation of the three biological replicates

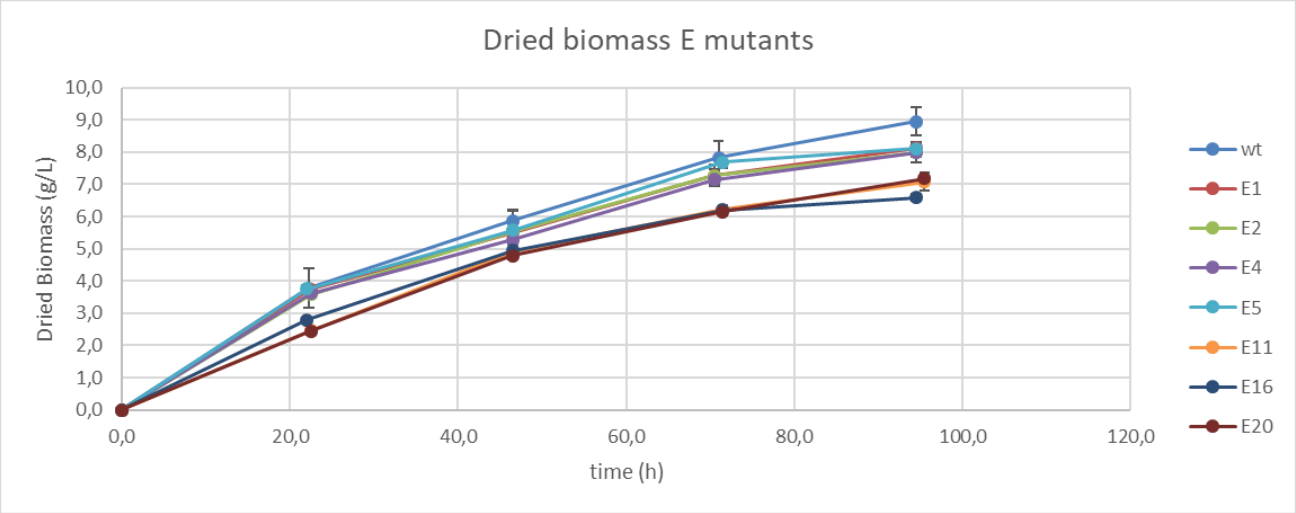


Figure 15 Dried biomass of E mutants
error bars calculated as standard deviation of the three biological replicates

Table 8 p values of dried biomass

P values						
D3	D4	D5	D6	D7	D25	D30
0,375	7,72E-05	0,544	0,330	6,06E-05	5,58E-04	2,07E-06
E1	E2	E4	E5	E11	E16	E20
0,0153	0,0114	7,68E-03	0,0145	2,70E-04	3,97E-05	2,89E-04

2.2.2 Nile Red analysis

The Nile Red analysis allowed to have an approximately evaluation of the lipid content. Since that the wild type strain is known to reach a peak in the lipid production after 96 h in the same conditions used for this screening, the Nile Red analysis was mainly used to confirm the peaks in the lipid production at 96 h for the mutants. The data were showed in orange, as the ratio between the O.D. values (grey line) and the fluorescence (blue bar). The peak in the lipid production was confirmed for all the D mutants (figures 16-30). The non-linear profile for D30 was attributed to the systematic and instrumental errors of the Nile Red analysis and it was not further investigated (figure 22). The E mutants were instead characterized by three different accumulation lipid profiles: E1 and E2 apparently reached the maximum lipid content after 96 h (figures 23 and 24); E4, E5, E11 and E20 seem to present a peak after 72 h, with a decrease in the lipid content for the 96 h sample, (figures 25,26,27 and 29); E16 seems to reach lipid production peak at 48 h, keeping the content constant until 72 h and then starting to consume the lipids (figure 28). Similarly to D30, E2 presents an unpredictable profile that has been attributed to systematic and instrumental errors and that it has not been further investigated. The wild type was confirmed to have a peak at 96 h (figure 30). After the

results obtained from the Nile Red analysis, it was decided to perform the lipid extraction on all the 96 h samples and on the 72 h samples which presented a peak in the fluorescence/OD value.

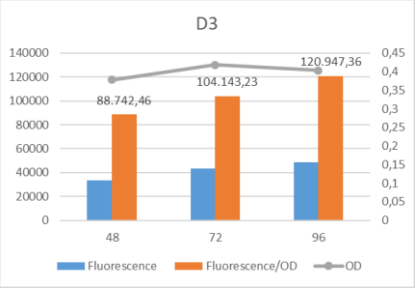


Figure 16 Nile Red analysis D3

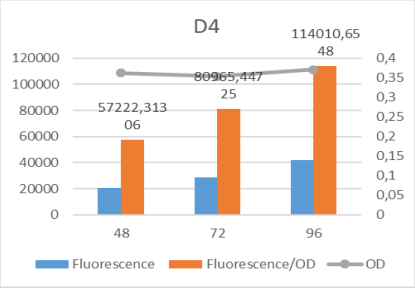


Figure 17 Nile Red analysis D4

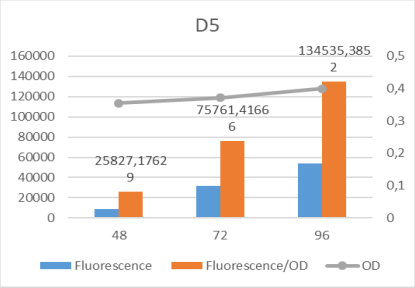


Figure 18 Nile Red analysis D5

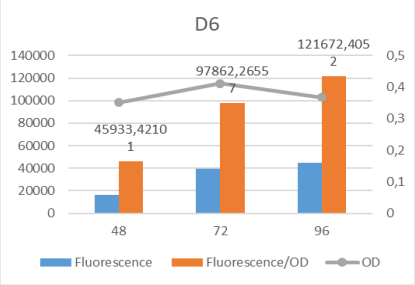


Figure 19 Nile Red analysis D6

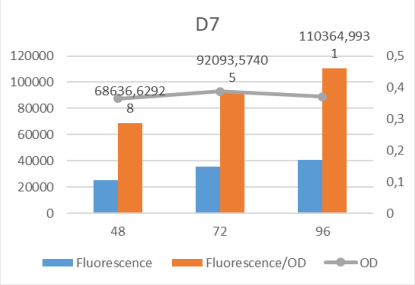


Figure 20 Nile Red analysis D7

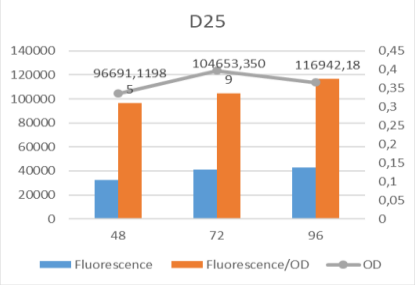


Figure 21 Nile Red analysis D25

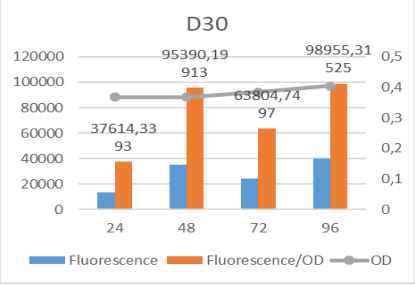


Figure 22 Nile Red analysis D30

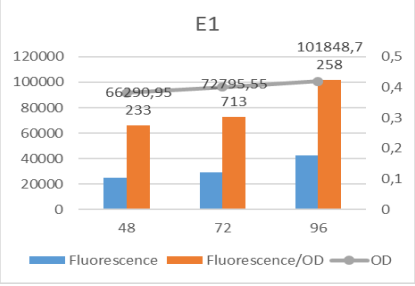


Figure 23 Nile Red analysis E1

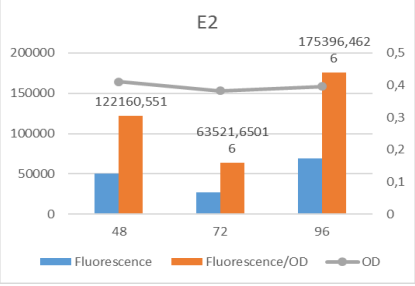


Figure 24 Nile Red analysis E2

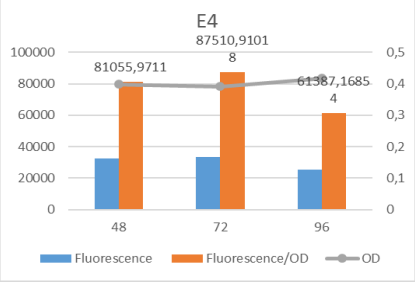


Figure 25 Nile Red analysis E4

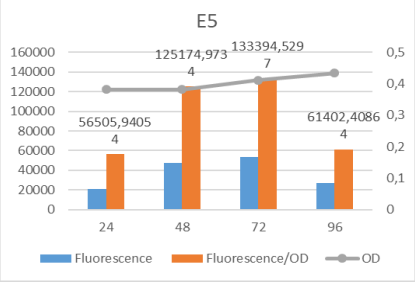


Figure 26 Nile Red analysis E5

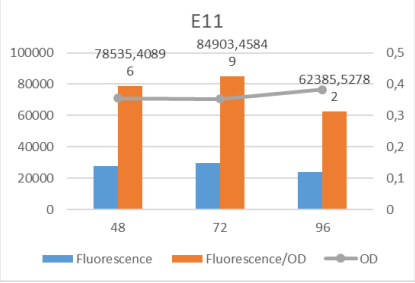


Figure 27 Nile Red analysis E11

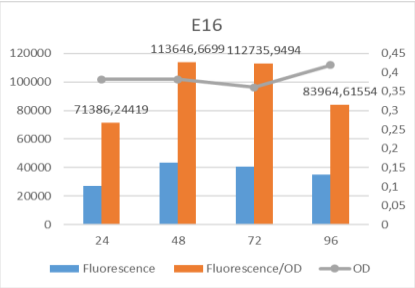


Figure 28 Nile Red analysis E16

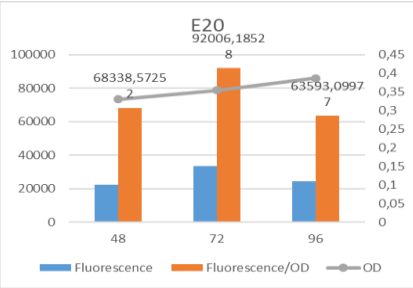


Figure 29 Nile Red analysis E20

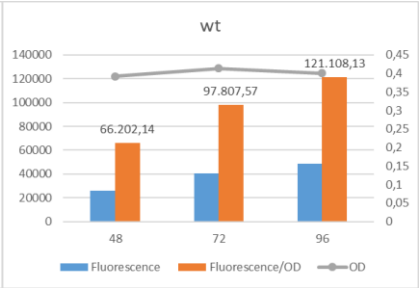


Figure 30 Nile Red analysis wild type strain

2.2.3 Lipid content profile

The lipid extraction was performed as reported from Folch et al.[92] (for more details, see "experiment details" section). The accumulated lipid percentage was calculated as the ratio between the lipid content and the dried biomass. In figure 31, the values of the lipid content for the D samples were shown. It could be observed that all the mutants were producing the same or lower amount of lipids compared to the wild type. In particular, D3, D5 and D6, respectively 6,2 ± 0,3 g/L, 6,3 ± 0,3 g/L and 6,3 ± 0,2 g/L, were not statistically different from the wild type strain (p values > 0,5, table 9), which accumulated an amount of 6,5 ± 0,3 g/L. The other mutants, D4, D7, D25 and D30 instead, showed a decrease in the lipid content, suggesting that the mutation led to a lower ability of the new strain to accumulate lipids with respect to the wild type strain. The lipid accumulation profile of the D mutants was in line with the ability to produce biomass. Indeed, it was previously noticed that D3, D5 and D6 showed a similar growth rate to the wild type, while the other mutants appeared to grow slower. These data were in contrast with the ones reported from Hwangbo et al.[86], who genetically modified the green alga *Chlamydomonas reinhardtii*, overexpressing a Δ9-desaturase gene. They observed not only a modification in the FAs composition, as expected from the overexpression of a desaturase coding gene, but also the increase of total fatty acid, by a 28% (wt%) compared to the wild type.

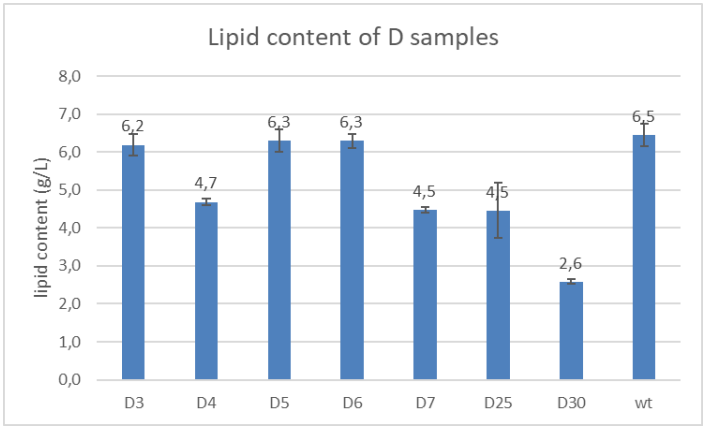


Figure 31 Lipid content of D samples
error bars calculated as standard deviation of the three biological replicates

Table 9 p values of lipid content of D samples

p values of lipid content of D samples

D3	D4	D5	D6	D7	D25	D30
0,245	2,49E-05	0,485	0,426	1,20E-05	0,0449	8,41E-08

The lipid percentage ($g/g_{biomass}$) profile was similar to the lipid content (figure 32). As previously observed, D3, D5 and D6 were statistically non different from the wild type strain (p values > 0,05, table 10). The lipid percentage ($g/g_{biomass}$) of D4 ,68,9 ± 1 % (wt%), was comparable to the wild type strain 72,6 ± 2,6 (wt%) (p value > 0,05), differently from the lipid content which was much lower (4,7 ± 0,1 g/L compared to 6,5 ± 0,3 g/L). Thus, in general it is possible to assess that the mutation led to three different lipid accumulation profiles. The first one was characterized by a similarity to the wild type, as seen for D3, D5 and D6 (statistically not different, p value >0,05, table 9 and 10). The second led to a slowdown in the metabolism, which led to a decreased ability to build up biomass and lipid but maintaining lipid percentage ($g/g_{biomass}$) constant, such as D4. The third observed behaviour was instead characterized by a lower growth rate and an even higher decrease in the ability of accumulating lipid, as in the case of D7, D25 and D30.

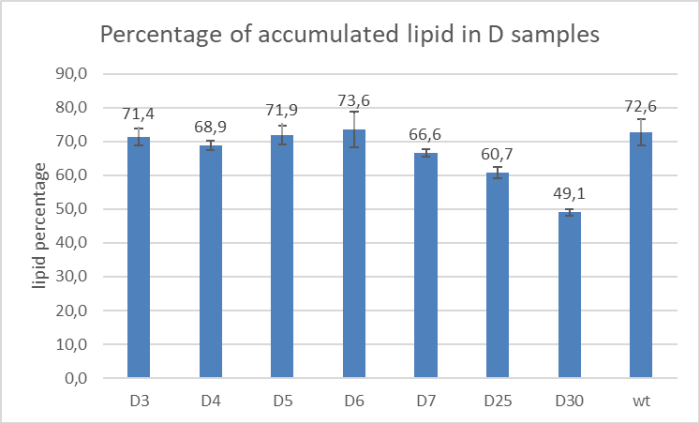


Figure 32 Accumulated lipid percentage of D samples
error bars calculated as standard deviation of the three biological replicates

Table 10 p values of accumulated lipid percentage in D samples

p values for the percentage of accumulated lipid in D samples

D3	D4	D5	D6	D7	D25	D30
0,635	0,160	0,799	0,764	0,0370	1,59E-03	2,03E-05

Similarly for the D mutants, none of the E mutants showed a higher lipid content compared to the wild type. At the first sight it could noticed how the lipid content increased as the time did. In fact, the lipid content at 96 h were higher than the one at 72 h. In contrast with the Nile Red test's results which suggested that E4, E5, E11, E16 and E20 had a peak in the lipid concentration at 72 h. However,

it is likely that the several factors which affect the Nile Red analysis, such as the variable diffusion across the membrane[91], led to these inconsistent results. Hence, it could be concluded that the gravimetric analysis presented more reliable data than the semi-quantitative Nile Red analysis. The gravimetric lipid analysis confirmed the observation previously done: in this case also E2, E4 and E5 appeared to be the mutants with the best ability to accumulate lipids (figure 33). In particular, E2, E4 96 h and E5 96 h were not statistically different from the wild type (p value > 0,05, table 10). E11, E16 and E20 showed a lower cell dried weight than wild type strain and accumulate less lipid as well. E1 showed a lower content of lipid compared to E2, E4 and E5, even though it had a similar growth ability.

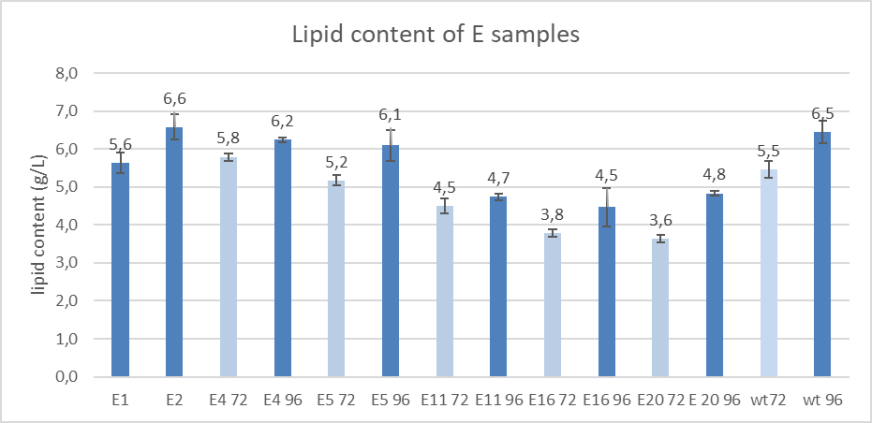


Figure 33 lipid content of E samples
blue: 96 h samples; light blue: 72 h samples
error bars calculated as standard deviation of the three biological samples

Table 11 p values of lipid content in E samples

p values of lipid content of E samples

E1	E2	E4 72	E4 96	E5 96	E11 96	E16 96	E 20 96
5,25E-03	0,574	0,0268	0,146	0,179	3,10E-05	1,33E-04	1,34E-05

Looking at figure 34, it was not surprising to notice that the percentage of the accumulated lipid for mutants such as E2 and E4 was higher than the wild type strain. Indeed, while the cell dried weight was lower, the lipid content was comparable to the wild type strain, resulting in a higher accumulated lipid percentage. Their lipid accumulation was determined approximately at 80% of their dried weight (figure 33). Statistical analysis confirmed the significative differences between E2, 82 ± 3% (wt%), E4 72 h, 81 ± 2% (wt%), and the wild type strain, 72,6 ± 2,6% (wt%) (p value > 0,05, table 11). E4 showed a higher lipid percentage ($g/g_{biomass}$) at 72 h than at 96 h. This could be due to an increase in the biomass accumulation rate after 72 h, a decrease in the lipid synthesis rate or a partial consumption of the accumulated lipid, even though the latter hypothesis was unlikely since the lipid content increased with time. Three mutants appeared to be much less competitive than the wild type: E11,

E16, E20. E1 and E5 had a lower growth ability than the wild type, but a similar accumulating lipid capacity: the lipid percentages ($g/g_{biomass}$) were not statistically different, as well as the lipid content for E5 (see table 10 and 11). Hence, E2 and E4 appeared to be the most interesting mutants, showing an increase in the percentage of accumulated lipid of almost 10% compared to the wild type. E4 also, appeared to accumulate lipid faster than E2, reaching an 81% (wt%) of accumulated lipid in 72h. It is true that the mutation led to a reduced growth ability, considering the lower cell dried weight of the mutants compared to the wild type. Moreover, it seemed that the mutants were more prone to convert the carbon source in lipids, rather than in biomass.

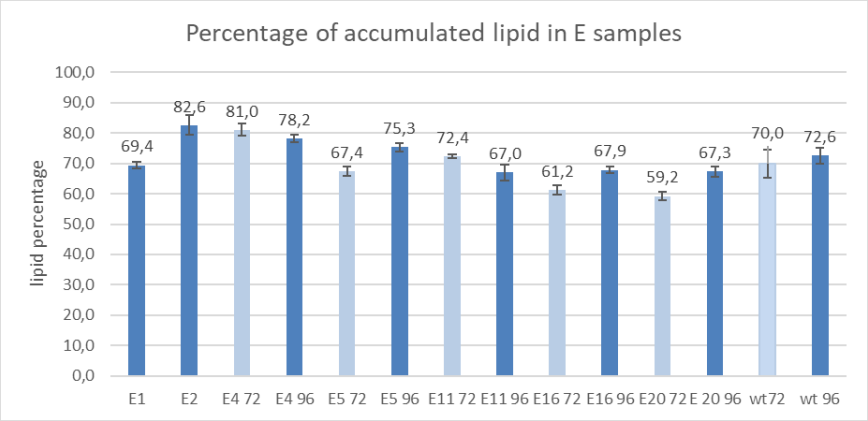


Figure 34 percentage of accumulated lipid in E samples
blue: 96 h samples; light blue: 72 h samples
error bars calculated as standard deviation of the three biological samples

Table 12 p values of percentage of accumulated lipid in E samples

p values for the percentage of accumulated lipid in E samples

E1	E2	E4 72	E4 96	E5 96	E11 96	E16 96	E 20 96
0,216	3,12E-03	0,0104	0,0491	0,288	0,0610	0,0824	0,0624

Below, the accumulated lipid in some of the screened mutants and the wild type strain at 72 h were shown (figure 35). The lipid droplets were easily recognizable. It was noticeable how the lipid droplets covered almost the entire surface of the cell. From a morphologic point of view, it was difficult to identify differences between the mutants and the wild type strain. In figure 36, Nile Red was used as dye to better locate the lipid droplets.

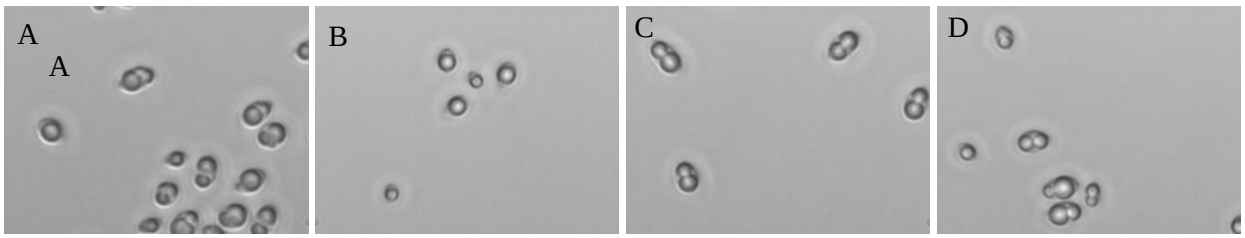


Figure 35 A: wild type strain at 72 h; B: D3 mutant at 72 h; C: E2 mutant at 72 h; D: E4 mutant at 72 h

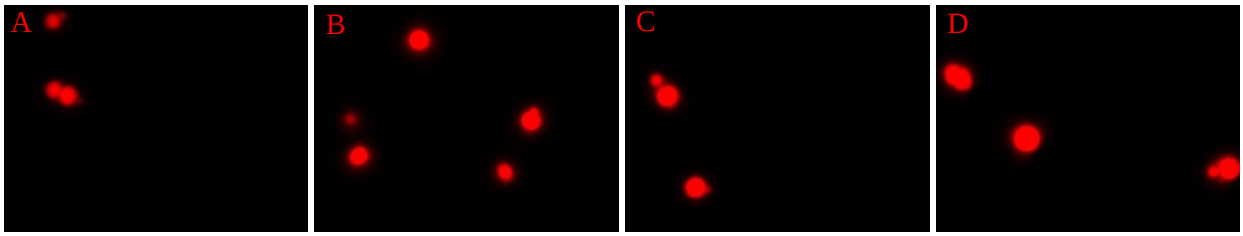


Figure 36 A: wild type strain at 72 h; B: D3 mutant at 72 h; E2 mutant at 72h; E4 mutant at 72 h; Nile Red was used as fluorescent dye

After a brief panoramic on the obtained data, it appeared that the transformation led to interesting results. It was seen that most of the mutants did not show any improvement in the lipid production. In most of them actually the growth ability appeared to be compromised, showing a lower content in cell dried weight compared to the wild type strain. However, mutants such as E2 and E4 were able to accumulate lipid more than the wild type, diverting the carbon uptake from the cell growth to the lipid accumulation. These observations were in line with the data reported from Tai et al.[75], which modified *Yarrowia lypolitica* by overexpressing the DGA1 gene. They reported an increase in the lipid accumulation percentage, from 10% of the cell dried weight for the wild type strain to 33% of the mutants. However, as in the current research, they noticed a decrease in the dried biomass weight. It is important to underline that if the effect of the mutation was limited to an accumulation of lipids, the cell dried weight should be the same for both the wild type strain and the mutants. Though, the lower cell dried weight of the mutants suggested that the mutation partially compromised the growth of the yeast. A reasonable explanation could be given considering that DGA1 catalyses the acylation of diacylglycerol[93]. Diacylglycerol though, is not only required for the TGA synthesis, but also for the phospholipid synthesis. Hence, the overproduction of TGA subtracts the reactant for the phospholipid synthesis, compromising the growth of the yeasts depriving them of one of the essential components required to grow. However, the same explanation could not be applied to the D mutants, which showed a decrease in the accumulation of dried biomass but were modified only by an overexpression of the desaturase. Additionally, similar genetic modification on the oleaginous yeast *Rhodosporidium toruloides*[94] and on *Saccharomyces cerivisiae*[90] showed different results. In fact, the overexpression of DGA1 gene in these two types of yeasts did not lead to a decrease in the cell dried weight. Instead, they observed that the mutants accumulated cell dried weight similar to the wild type strain, but were characterized by a higher percentage of lipids.

In table 13 an overview of the mutants and their respective lipid production is given. Lipid productivity was calculated using the following formula:

$$lipid\ productivity = \frac{lipid\ content\ (\frac{g}{L})}{time\ (h)}$$

The yield of lipid production was calculated as following:

$$yield\ \% = \frac{lipid\ content\ \left(\frac{g}{L}\right) * 100}{carbon\ source\ content\ (\frac{g}{L})}$$

The carbon source in the minimal nitrogen media was glucose 30 g/L, (see experimental details section). E2 (blue) and E4 (light blue) were identified as the most productive mutants. The lipid percentages ($g/g_{biomass}$), obtained with a limiting-nitrogen media with a C/N ~ 320, for E2 96 h and E4 72 were respectively 82,6 ± 3,3% and 81,0 ± 2,0% (wt%). Comparing these results with the one reported in the literature, it was possible to assess that they were some of the highest values ever reached using genetically modified yeast. Zhang et al.[94] registered an increase in the accumulated lipid of *Rhodosporidium toruloides*, from 30% (wt%) of the wild type strain to 60% (wt%). They simultaneous overexpressed ACC1 and DGA1 genes and they studied the lipid production in a limiting-nitrogen media with a C/N ratio of ~340 and with glucose as carbon source in shaking flasks. The same genetic modifications were studied from Tai et al.[75] on *Yarrowia lipolytica*, reaching a value of 40% (wt%) in the accumulated lipid, compared to 9% (wt%) of the wild type strain, in shaking flasks containing media with C/N ratio of 20 and glucose as carbon source. Friedlander et al.[95] enhanced the ability of *Yarrowia lipolytica* to accumulate lipid, inserting DGA1 and DGA2 and deleting the homologous lipid regulator TGL3; in this way, they obtained a lipid percentage of 77% (wt%) in a batch mode fermentation on a 1-L scale. Furthermore, the high level of lipid percentage ($g/g_{biomass}$) obtained in E2 and E4 overcame the low dried biomass built up by the mutants in order to achieve a higher productivity than the wild type strain. In particular, the highest productivity was reached by E4 72h, given its ability to accumulate lipid faster than the other mutants, with a value of 0.080 ± 0.001 g/Lh compared to 0.076 ± 0.003 g/Lh of the wild type, after 72 h. However, the productivities obtained in this experiment are still lower than most of the value reported in the literature for shaking flasks fermentation of *C.oleaginosus*. Ryu et al.[35] reached a productivity of 0,152 g/Lh optimizing the media by adjusting the concentration of carbon source. Chi et al.[65] obtained a productivity of 0,51 g/Lh optimizing the pH and the concentration of acetate. Consequently, it would be necessary to study the mutants under different conditions to assess the best growth parameters and obtain the highest productivity. As it is shown above, the highest productivity is reached at 72 h for all the E mutants. However, the more time the fermentation is conducted, the higher is the carbon source consumption and thus the yield ($g_{lipid}/g_{biomass}$). Hence, if the productivity

and the yield would keep the same profile in a hypothetical scale-up of the process, it would be necessary to find an economical optimum according to the time in order to have the highest lipid production and the lowest waste of carbon source. Regarding the yield, the obtained values were in line with the one reported in the literature[35,44,95].

Table 13 Summary table
E2 (blue) and E4 (light blue) are highlighted as the most promising mutants
error bars calculated as standard deviation of the three biological replicates

Mutants	time (h)	dried biomass (g/L)	lipid content (g/L)	lipid percentage %	productivity (g/Lh)	yield % (g lipid/g substrate)
D3	96	8,7 ± 0,3	6,2 ± 0,3	71,4 ± 2,5	0,064 ± 0,003	20,6 ± 0,9
D4	96	6,8 ± 0,1	4,7 ± 0,1	68,9 ± 1,4	0,049 ± 0,001	15,6 ± 0,3
D5	96	8,8 ± 0,1	6,3 ± 0,3	71,9 ± 2,7	0,066 ± 0,003	21,0 ± 1,0
D6	96	8,6 ± 0,6	6,3 ± 0,2	73,6 ± 5,3	0,066 ± 0,002	21,0 ± 0,6
D7	96	6,7 ± 0,1	4,5 ± 0,1	66,6 ± 1,0	0,047 ± 0,002	14,9 ± 0,2
D25	96	7,4 ± 0,2	4,5 ± 0,7	60,7 ± 1,7	0,046 ± 0,008	14,9 ± 2,4
D30	96	5,3 ± 0,1	2,6 ± 0,1	49,1 ± 1,0	0,027 ± 0,001	8,6 ± 0,2
E1	96	8,1 ± 0,1	5,6 ± 0,3	69,4 ± 1,1	0,059 ± 0,003	18,8 ± 0,9
E2	96	8,0 ± 0,3	6,6 ± 0,3	82,6 ± 3,3	0,069 ± 0,003	21,9 ± 1,1
E4	72	7,2 ± 0,2	5,8 ± 0,1	81,0 ± 2,0	0,080 ± 0,001	19,3 ± 0,3
E4	96	8,0 ± 0,1	6,2 ± 0,1	78,2 ± 1,2	0,065 ± 0,001	20,8 ± 0,2
E5	72	7,7 ± 0,1	5,2 ± 0,1	67,4 ± 1,6	0,072 ± 0,002	17,2 ± 0,4
E5	96	8,1 ± 0,1	6,1 ± 0,4	75,3 ± 1,4	0,064 ± 0,004	20,3 ± 1,4
E11	72	6,2 ± 0,1	4,5 ± 0,2	72,4 ± 0,7	0,063 ± 0,003	15,0 ± 0,7
E11	96	7,1 ± 0,3	4,7 ± 0,1	67,0 ± 2,6	0,049 ± 0,001	15,8 ± 0,3
E16	72	6,2 ± 0,1	3,8 ± 0,1	61,2 ± 1,5	0,053 ± 0,001	12,6 ± 0,3
E16	96	6,6 ± 0,1	4,5 ± 0,5	67,9 ± 1,1	0,047 ± 0,005	14,9 ± 1,7
E20	72	6,2 ± 0,2	3,6 ± 0,1	59,2 ± 1,3	0,051 ± 0,001	12,1 ± 0,3
E20	96	7,2 ± 0,2	4,8 ± 0,1	67,3 ± 1,7	0,050 ± 0,001	16,1 ± 0,2
wt	72	7,8 ± 0,5	5,5 ± 0,2	70,0 ± 4,6	0,076 ± 0,003	18,2 ± 0,8
wt	96	9,0 ± 0,4	6,5 ± 0,3	72,6 ± 2,6	0,067 ± 0,003	21,5 ± 1,0

2.2.4 FAs composition

In order to assess the influence of the genetic modifications on the FAs composition, the accumulated TAGs were esterified with methanol and subsequently analysed at GC (for more details see experimental details section).

It was easily noticeable that the insertion of a desaturase gene did not change the FAs profile dramatically in the D mutants (figure 37 and table 14). It was possible to divide the mutants in two categories: D3, D5 and D6 which resembled the wild type strain profile; D4, D7, D25 and D30 which showed the highest differences in the FAs profile. The first category of mutants was the one

characterized by the best lipid production and the best growth. In particular they were not statistically different from the wild type, both in terms of accumulated lipid and cell dried weight. This suggested that the successful overexpression of desaturase gene, with the consequential variation in the FAs profile, affected the yeast growth and metabolism. Indeed, the second category of mutants appeared to have a lower ability to accumulate biomass (D4, D7, D25 and D30) and lipid (D7, D25 and D30).

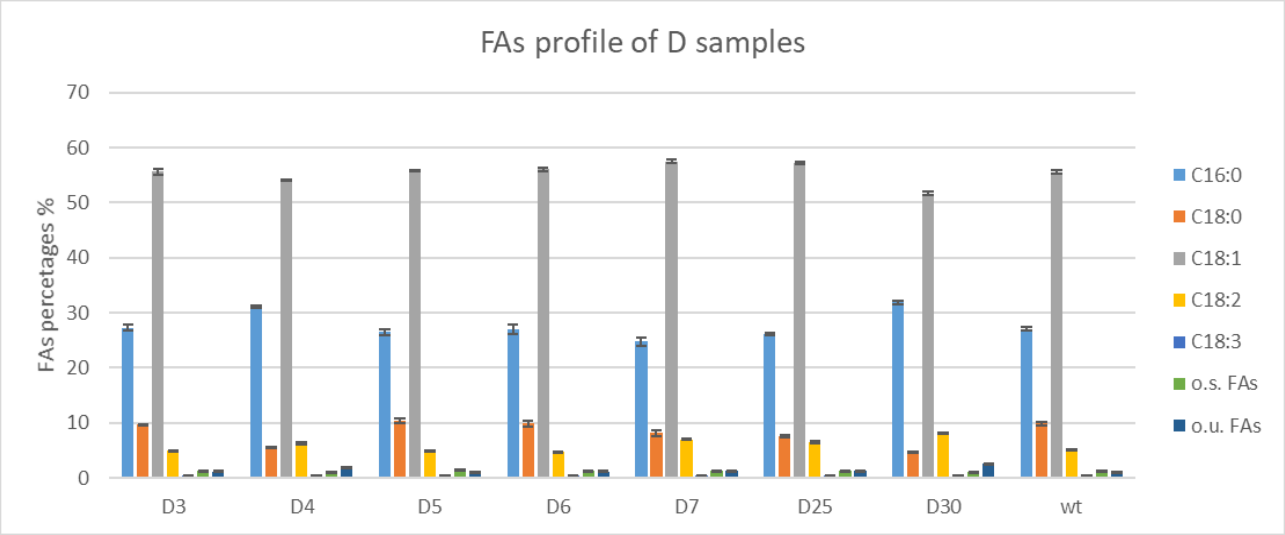


Figure 37 FAs profile of D samples
o.s. FAs: other saturated FAs; o.u. FAs: other unsaturated FAs
error bars calculated as standard deviation of the three biological replicates

Table 14 FAs profile of D samples, values expressed as percentages

	D3		D4		D5		D6		D7		D25		D30		wt	
C16:0	27,3	± 0,5	31,1	± 0,2	26,5	± 0,5	27,0	± 0,8	24,8	± 0,7	26,1	± 0,2	31,8	± 0,4	27,1	± 0,4
C18:0	9,6	± 0,1	5,6	± 0,1	10,4	± 0,5	9,8	± 0,6	8,1	± 0,5	7,6	± 0,1	4,7	± 0,1	9,9	± 0,2
C18:1 (oleat)	55,6	± 0,5	54,1	± 0,1	55,8	± 0,1	56,0	± 0,2	57,5	± 0,4	57,2	± 0,3	51,7	± 0,3	55,5	± 0,3
C18:2	5,0	± 0,1	6,2	± 0,2	4,9	± 0,1	4,7	± 0,1	7,0	± 0,1	6,5	± 0,2	8,2	± 0,1	5,0	± 0,2
C18:3	0,368	± 0,008	0,49	± 0,01	0,362	± 0,005	0,35	± 0,01	0,479	± 0,006	0,36	± 0,01	0,539	± 0,005	0,38	± 0,02
others saturated	1,27	± 0,02	1,1	± 0,1	1,40	± 0,03	1,3	± 0,1	1,33	± 0,04	1,29	± 0,01	1,0	± 0,1	1,33	± 0,03
others unsaturated	1,20	± 0,02	1,9	± 0,1	1,04	± 0,04	1,2	± 0,1	1,3	± 0,1	1,38	± 0,03	2,6	± 0,1	1,1	± 0,1

In figure 38 the variation's percentage of the FAs profile for the mutants which showed a higher expression of the desaturase gene is presented. The variation's percentage was calculated with the following formula:

$$variation's\ percentage\ = composition\ of\ mutant - composition\ of\ wild\ type$$

Figure 38 showed some general common features in the FAs profile. In all the mutants a decrease in C18:0 and an increase in C18:2 were observed. In fact, as already stated, the activity of the overexpressed desaturase gene is believed to focus on the insertion of a double bond in C18:0, leading to C18:1. Thus it was expected a decrease in the concentration of C18:0. However, not all the mutations led to the same pattern. D4 and D30 apparently showed a higher expression of the

desaturase gene, with a decrease in C18:0 from 9,9% of the wild type to 5,6% and 4,7% respectively. This was linked to a lower C18:1 content, which then resulted in a higher C18:2 and C18:3 content. In D4 and D30 also an increase in C16:0 was found. Differently, D7 and D25 were characterized by a more modest decrease in C18:0, from 9,9% of the wild type to 8,1% and 7,7%. Further, C18:1 showed an increase, as well as C18:2. In contrast with D4 and D30, D7 and D25 reported a lower content of C16:0. According to the previous observation it seemed that the desaturase experienced the highest level of expression in D4 and D30 mutants. This hypothesis could be confirmed running a RT-qPCR.

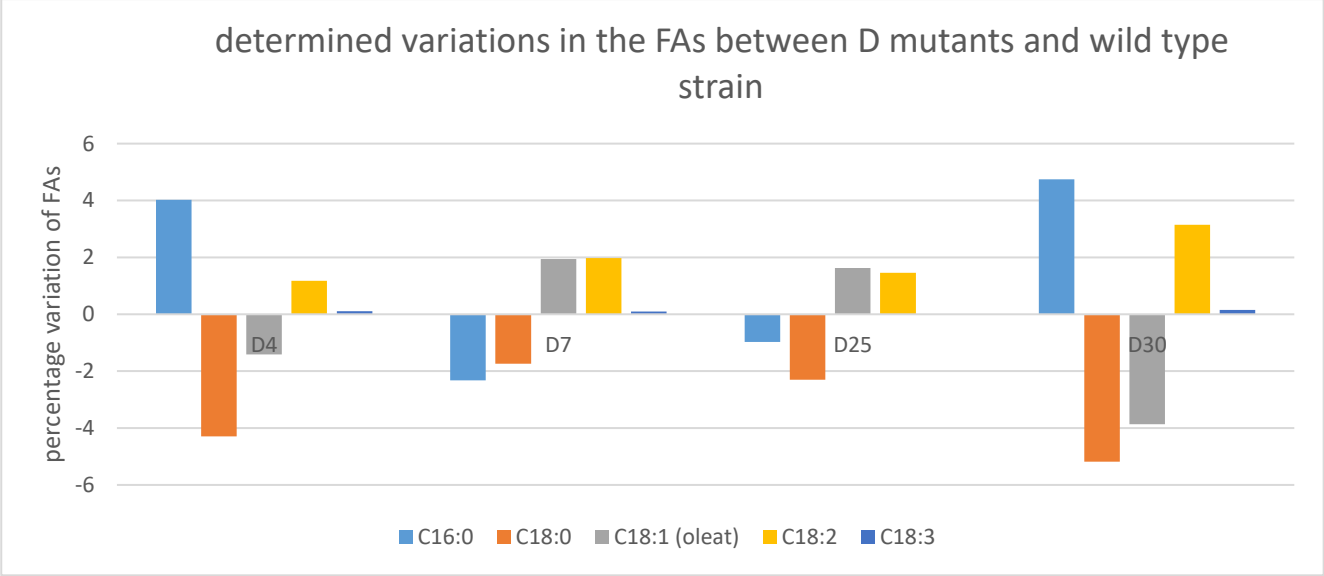


Figure 38 Differences in FAs profile between D mutants and wild type strain

Similarly to the D mutants, the E ones did not show any dramatic change (figure 39 and table 15). Even if the differences in the FAs profile were not so considerable, statistical analysis confirmed that only E5 could be considered significative not different from the wild type strain (p value > 0,05, table 16). Though, the expression of the desaturase gene was much more limited compared to the D mutants. In fact, the highest variation observed in the E mutants was of 2 % in C16:0 for E16 96h. This value was less than half observed for D30, which showed an increase in C16:0 of 5%. Furthermore, the mutant which showed the highest lipid accumulation, E2, was not the one which showed the highest difference in FAs profile, which instead is E16 96 (figure 39). This was curious since the plasmid containing the desaturase gene and the DGA1 gene should be inserted in the yeast cell as a single DNA fragment, maintaining the same quantity of the two overexpressed genes inside the mutant. Additionally, E2 showed a profile different from most of the mutants, which was characterized by the decrease in C16:0 and an increase in C18:0. An analogous profile was observed from Tai et al.[75]. A simple explanation could be given by saying that the overexpression of DGA1 acts pulling the FAs synthesis, speeding up the process of elongase. However, the complexity of the

TGAs synthesis, which is characterized by several factors that interacts between them, suggests that the reason could actually be more profound[72]. E4 96 h presented a similar profile to E2, but only after that it reached the peak in the lipid production (72 h, 81% (wt%)). Curiously, E4 72 h resembled the FAs profile of wild type strain (only C18:2 composition was statistically different). Moreover, the E4 FAs profile changed dramatically according to time. In fact, for most of the mutants, the distributions of the FAs were similar for the 72 h and the 96 h samples (figure 40). Instead, E4 was characterized by a deep change in the FAs profile from 72 h to 96 h (figure 40). This difference could support the hypothesis that E4 started to consume the internal lipids after 72 h. A previous research showed that, according to the type of microorganism, it is possible that the internal lipid consumption process starts to decrease the percentage of the saturated FAs (C16:0), thus increasing the percentages of the unsaturated fatty acids (C18:1, C18:2)[96]. Hence, it was possible to classify the mutants according to three different behaviours: E5, which was characterized by a similarity to the wild type; E4 72 h, E11, E16, E20, which resembled the composition observed in D4 and D30 (increase in C16:0 and C18:2, decrease in C18:0 and C18:1); E2 and E4 96h, which showed a decreases in C16:0 and C18:2 and an increase in C18:0 and C18:1.

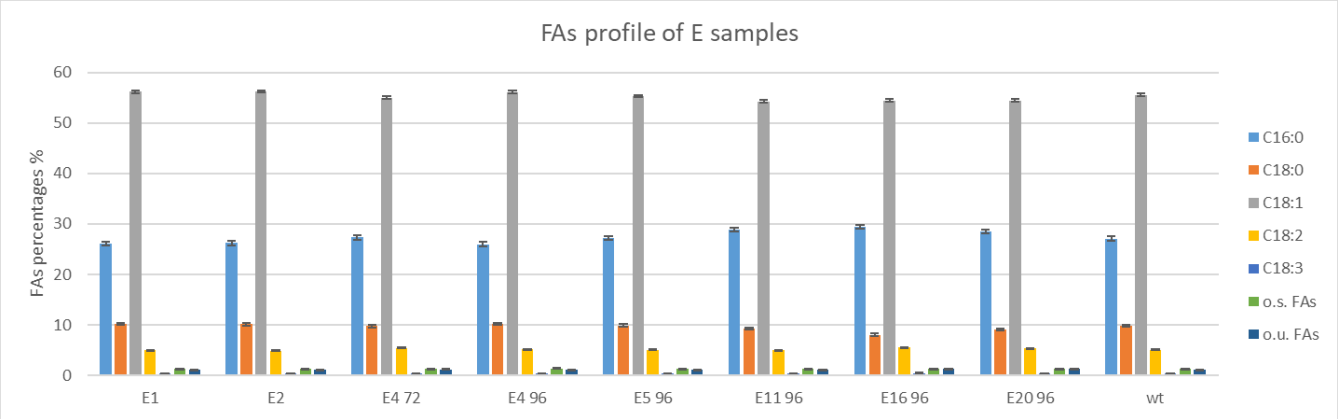


Figure 39 FAs profile of E mutants
error bars calculated as standard deviation of the three biological replicants

Table 15 FAs profile of E samples

	E1	E2	E4 72	E4 96	E5 96	E11 96	E16 96	E20 96	wt
C16:0	26,1 ± 0,5	26,2 ± 0,2	27,3 ± 0,1	26,0 ± 0,2	27,2 ± 0,2	28,9 ± 0,1	29,41 ± 0,04	28,5 ± 0,1	27,1 ± 0,4
C18:0	10,2 ± 0,2	10,1 ± 0,1	9,8 ± 0,1	10,17 ± 0,03	9,9 ± 0,3	9,3 ± 0,1	8,03 ± 0,04	9,1 ± 0,2	9,9 ± 0,2
C18:1 (oleat)	56,2 ± 0,1	56,3 ± 0,3	55,0 ± 0,1	56,1 ± 0,2	55,3 ± 0,2	54,28 ± 0,03	54,47 ± 0,01	54,5 ± 0,2	55,5 ± 0,3
C18:2	5,0 ± 0,1	4,9 ± 0,2	5,46 ± 0,03	5,21 ± 0,03	5,09 ± 0,04	5,02 ± 0,05	5,5 ± 0,1	5,3 ± 0,1	5,0 ± 0,2
C18:3	0,38 ± 0,01	0,37 ± 0,02	0,467 ± 0,003	0,407 ± 0,004	0,396 ± 0,004	0,417 ± 0,001	0,479 ± 0,002	0,42 ± 0,01	0,38 ± 0,03
others saturated	1,4 ± 0,1	1,38 ± 0,01	1,25 ± 0,01	1,400 ± 0,005	1,38 ± 0,02	1,30 ± 0,01	1,25 ± 0,01	1,32 ± 0,01	1,33 ± 0,03
others unsaturated	1,06 ± 0,01	1,1 ± 0,1	1,20 ± 0,01	1,11 ± 0,01	1,09 ± 0,03	1,20 ± 0,01	1,33 ± 0,01	1,24 ± 0,02	1,13 ± 0,06

Table 16 p values for FAs composition of E samples

	E1	E2	E4 72	E4 96	E5	E11 96	E16 96	E20 96
C16:0	0,0115	7,69E-03	0,337	1,71E-03	0,713	2,75E-05	2,21E-05	3,40E-04
C18:0	0,0624	0,115	0,570	0,0255	0,722	9,77E-03	2,21E-06	2,30E-03
C18:1	0,0191	0,0208	0,0480	0,0281	0,380	3,19E-04	6,45E-04	2,25E-03
C18:2	0,835	0,187	5,19E-04	0,0530	0,544	0,758	2,94E-04	6,22E-03

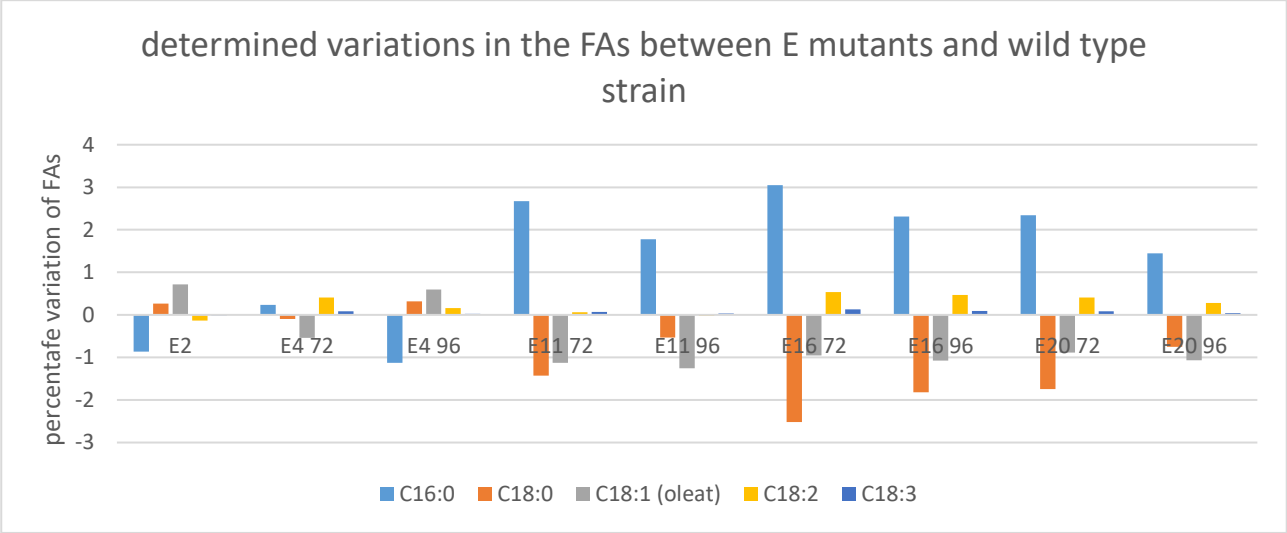


Figure 40 percentage variation of FAs between the E mutants and the wild type strain

Data reported in the literature suggested the complexity of the interaction between the expression/overexpression of heterologous gene in microorganism and lipid accumulation. Sakuradani et al.[87] studied the expression of a Δ12-desaturase, responsible for the insertion of the double bond in C18:1 to produce C18:2, in the yeast *S. cerevisiae* and fungus *A. oryzae*. In particular, the modified fungus appeared to have a variation in the FAs profile similar to the one observed for most of the mutants in this experiment: the expression of the desaturase led to a decrease in C18:0 and C18:1, to favour the production of C18:2; differently from the current study though, they observed almost no change in C16:0. *S. cerevisiae*, instead, showed a pattern which was different from any of the screened mutants: increase in C16:0, C18:0 and C18:2 together with a decrease in C18:1 content. The insertion of a Δ9-desaturase in *S. cerevisiae* returned a new pattern, where C16:0 and C18:1 increased, while C18:0 decreased[97]. The comparison between the value obtained in this research and the data reported in the literature cleared how the TAGs synthesis is a complex process, affected by the type of microorganism and the strain. Additionally, given the diversity of FAs profile observed in this experiment, it was realized how important would it be to proceed with further analysis to better understand the influence of the genetic modification.

2.2 Bioprocess engineering approach

As already explained, the bioprocess engineering approach was based on the use of 1-L scale bioreactor to assess the influence of different carbon sources on the TGAs composition and lipid production. Glucose, xylose and acetic acid were used as main carbon source. To study the influence of secondary acids on yeast growth, synthetic mixtures of acetic acid and secondary acids were prepared. During the experiments, the following analysis were conducted to monitor the growth trend: O.D. (600 nm) measurements; dried biomass; lipid extraction. The FAs profile was studied to assess the influence of the different carbon sources. Due to secrecy reasons, the name of the acids used as carbon source was not revealed and it was referred at them with alphanumeric codes. For more information about the methodic see "experimental details" section.

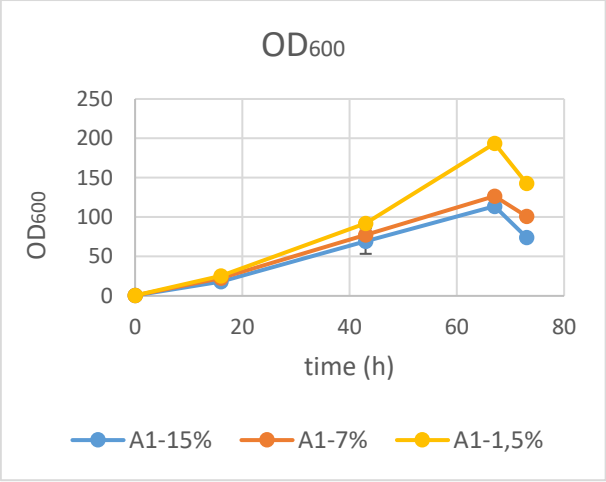
2.2.1 Influence of acid concentration and fed mode

At the beginning, a first experiment was performed to study the effect of the concentration of the acids on the yeast growth together with the fed mode. For this experiment, it was used an odd-numbered acid containing a double bond that was labelled as A1. It is known indeed that acetic acid can be poisonous for *C. oleaginous* but no studies were conducted to assess the effect of A1 on this type on yeast. In this regard, the bioreactors were fed with 3 different solutions of A1. Two different fed modes were analysed, as reported in table 17. One bioreactor was fed with a solution of A1 in acetic acid (AA) at a concentration of 1,5% (wt%). The synthetic mixture was fed to the bioreactor using an automatic pH-stat control. The remaining two bioreactors were fed using an automatic pH-stat control based on a pure acetic acid solution during the non-working hours, while during the working hours the yeast were fed with synthetic mixtures of A1 in water (90 g/L) using a manual pH-stat control. The aqueous solutions of A1 were added so to have the concentrations of 7% and 15% in acetic acid (wt%).

Table 17 fermentation details

Acid concentration in acetic acid (wt%)	Note
A1-1,5%	The bioreactor was fed with a solution of A1 in AA at 1,5% (wt%).
A1-7%	The bioreactor was fed with an aqueous solution of A1 (90g/L) + pure acetic acid. The resulting concentration of A1 in AA was 7% (wt%).
A1-15%	The bioreactor was fed with an aqueous solution of A1 (90g/L) + pure acetic acid. The resulting concentration of A1 in AA was 15% (wt%).

O.D. (600 nm) measurements (figure 41) and dried biomass analysis (figure 42) clearly showed the poisonous effect of A1 on the yeast growth. It was noticeable how, as A1 concentration increased, the growth was suppressed. After 73 h, A1-1,5% produced 85 g/L of biomass, compared to 73 g/l of A1-7% and 56 g/L of A1-15%. Furthermore, the dried biomass indicated the better and faster growth of the yeast fed with A1-7% and A1-1,5%. In particular, if the yeast fed with A1-15% reached a stationary phase, for the other two synthetic mixtures this was not observed. The higher growth was believed to be linked also to the fed mode. In the bioreactor fed with A1-7%, the amount of A1 required was added within 50 h. Hence, after 50 h, the bioreactor was fed with pure acetic acid. In the bioreactor fed with A1-15% instead, A1 was added until 70 h. Indeed, A1-7% bioreactor experienced an increase in the growth rate after having stopped to add A1 (instantaneous growth rate at 16-43 h= 0,3 g/Lh, instantaneous growth rate at 43-67 h= 0,71 g/Lh), compared to A1-15% which reached the stationary phase (instantaneous growth rate at 16-43 h= 0,72 g/Lh, instantaneous growth rate at 43-67 h= 0,67 g/Lh). Secondly, the A1-1,5% bioreactor probably showed a higher growth also thanks to the fed mode that allowed to dilute the addition of A1 in time. In the O.D. measurements it was experienced a decrease from 67 h to 73 h, probably since the measurement was performed only after having stopped the fermentation, raising the temperature at 80 °C and decreasing the pH at 4,5, thus killing the yeasts.



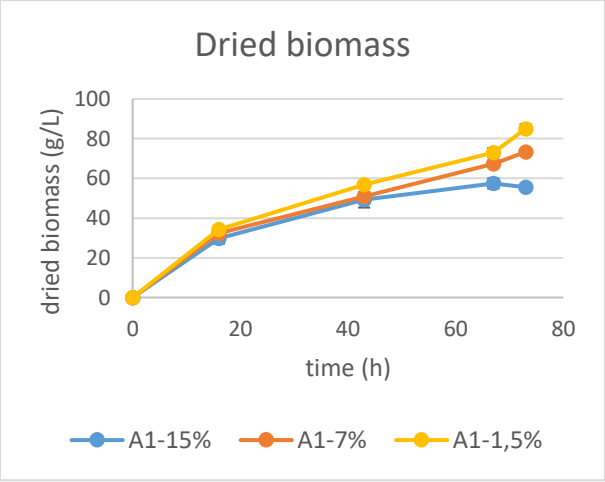


Figure 41 O.D. (600 nm) of A1-15, A1-7 and A1-1,5 % (wt%) *Figure 42 Dried biomass of A1-15, A1-7 and A1-1,5% (wt%) error bars calculated as standard deviation of analytical replicates*

Surprisingly both A1-7% and A1-1,5% experienced an increase in growth after 67 h. In particular, instantaneous growth rate of A1-1,5% went from 0,7 g/Lh in the interval 43-67 h, to 2 g/Lh between 67-73h. Similarly, A1-7% went from 0,7 g/Lh to 1 g/Lh. The reasons behind the increase in the growth rates might not be the same for the two bioreactors. In the case of the bioreactor fed with A1-7% the increase in the growth may be due to the depletion of A1 present in the mixture. In A1-7%,

A1 was stopped of being added after approximately 50 h. Hence, once the acid was partially consumed, the yeast growth experienced a boost. To understand the increment in the growth rate for A1-1,5% instead, it is necessary to look at the lipid content and lipid percentage graphs (figures 43 and 44). As it was shown, after having reached a peak in the lipid content of 63 g/L, equivalent to 88 % ($g_{lipid}/g_{biomass}$), the lipid profile decreased to 59 g/L and 70% ($g_{lipid}/g_{biomass}$) (table 18). Hence, it might be possible that the accumulated lipids were partially consumed, leading to an acceleration in the growth. The consumption of the internal lipids can be linked to the depletion of the sugars (figure 45 and 46), as reported from Zhu et al.[98] and from Papanikolau et *al.*[99] However, the sugars were consumed in A1-15% and A1-7% too but no decrease in lipid content was observed for them. In this regard the high lipid percentage reached from the yeasts may had acted as an important factor. Hence, it appeared that the yeasts, after having consumed all the glucose and xylose in the media, given the high percentage of lipid inside the cell body, preferred to degrade the accumulated lipids instead of up taking the acetic acid. Nevertheless, Masri et al.[36] performed a similar fed-batch fermentation using glucose and acetic acid as carbon source. They obtained 85% ($g_{lipid}/g_{biomass}$) of accumulated lipid but they did not observed the consumption of lipid even after the depletion of the sugars. Surprisingly, even though the different bioreactors reported different growth profiles, the sugar depletion profiles were very similar for all of them. This suggested that the differences in the produced biomass and lipids were linked to the ability of the yeast to uptake acetic acid and A1. Furthermore, despite glucose often suppresses the diauxic growth[84], it was observed the simultaneous uptake of glucose, xylose and acetic acid from the yeast. Similar results were obtained also from Yaguchi et al.[100], who reported the concurrent consumption of aromatic compounds together with glucose and xylose from *C.oleaginous*, even if sugars were preferentially up taken. Gong et al.[71] reported also the simultaneous consumption of sugars and acetate in *C.oleaginous* fermentation.

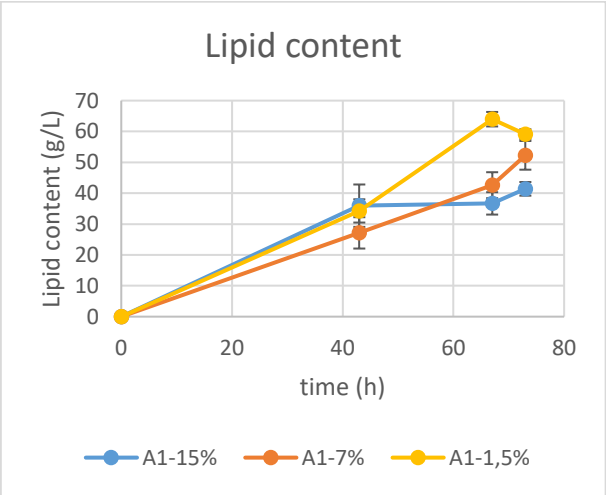


Figure 43 Lipid content of A1-15, A1-7, A1-1,5% (wt%)

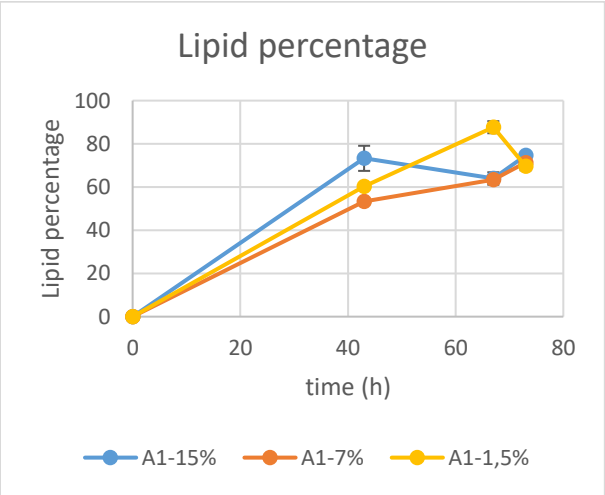


Figure 44 Lipid percentage of A1-15, A1-7,A1-1,5 % (wt%)

error bars calculated as standard deviation of analytical replicates

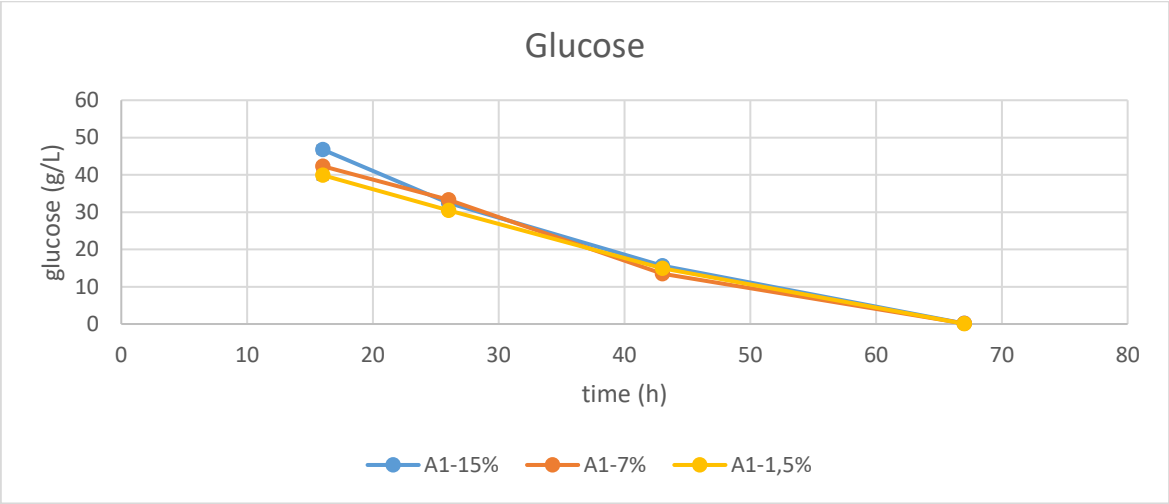


Figure 45 Glucose profile of A1-15, A1-7, A1-1,5 % (wt%)
error bars calculated as standard deviation of analytical replicates

Figure 46 Xylose profile of A1-15, A1-7, A1-1,5 % (wt%)
error bars calculated as standard deviation of analytical replicates

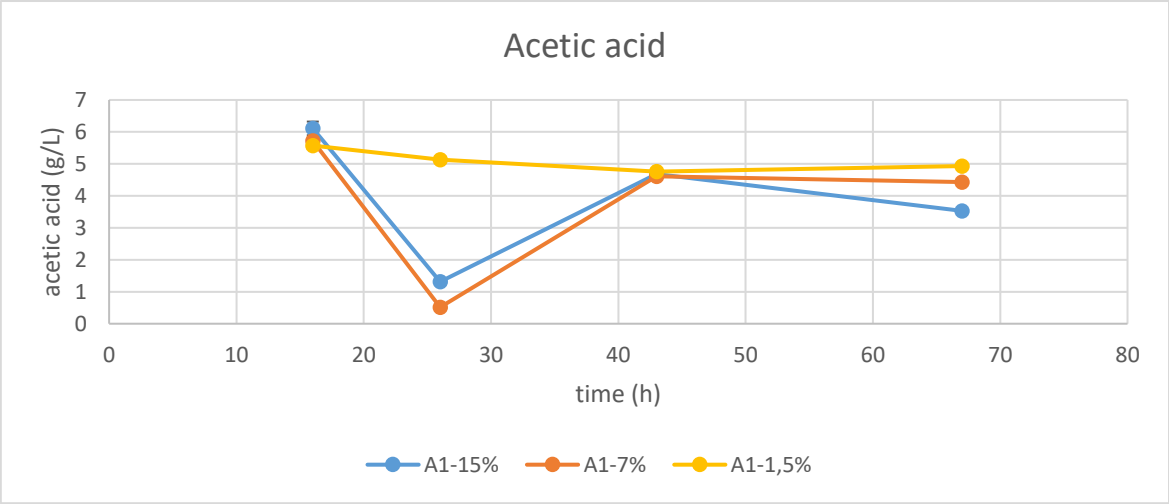


Figure 47 Acetic acid profile of A1-15, A1-7, A1-1,5 % (wt%)
error bars calculated as standard deviation of analytical replicates

As already stated, A1 appeared to be more poisonous than acetic acid. Interestingly, at 43 h the highest lipid percentage ($g_{lipid}/g_{biomass}$) and productivity were reached from A1-15%, (73% (wt%) and 0.84 g/Lh, table 18). Yet, the poisonous effect slowed down the growth, leading the yeast into a stationary phase. Thus, the best configuration was the one used for A1-1,5%, resulting in a productivity of 0,95 g/Lh at 67h, with a lipid content of 64 g/L and a built up biomass of 73 g/L. The decrease in acetic acid content observed for A1-15% and A1-7% at 26 h was explained considering that during that time-lapse the automatic pH control was switched off to in favour of the manual pH control, thus shifting from pure acetic acid to the aqueous solution of A1. Considering this, it was once again confirmed that yeast preferred to use acetic acid as carbon source rather than A1, probably due to its poisonous effect. The analysis of A1 profile according to time could confirm this hypothesis. The main data obtained from the fermentation were summarised in table 18. The productivity was calculated as the ratio between the lipid content and the time required to reach it. The yield was calculated with the following formula:

$$yield\ \% = \frac{lipid\ content * volume * 100}{carbon\ source\ consumed\ (glucose + xylose + AA + secondary\ acid)}$$

Table 18 apparently reported discrepancies between the acid consumptions, the lipid content and the dried biomass. However, analysing the results it was possible to confirm the observations previously done. The maximum lipid content was obtained from A1-1,5%, while the highest consumption of acid was reported for A1-7%. Since the amount of sugars consumed were the same (figures 45 and 46) it meant that there was an accumulation in A1-7%, otherwise it could not be explainable how the yeast could have consumed a higher amount of carbon source producing a lower amount of biomass and lipids compared to A1-1,5%. This phenomenon was explained considering that the A1 added during the manual pH-stat mode acted only as a pH regulator, while the yeast kept on consuming

acetic acid. This observation was already done analysing the acetic acid profile (figure 47). The A1 accumulated inside the bioreactor might had led to the lower yield compared to A1-1,5%.

Table 18 summary table
error bars calculated as standard deviation of analytical replicants

	A1-15%	A1-7%	A1-1,5%
time (h)	Dried Biomass (g/L)		
43	49,2 ± 3,9	50,9 ± 1,2	56,9 ± 0,1
67	57,4 ± 2,6	67,3 ± 0,6	73,0 ± 2,3
73	55,5 ± 0,5	73,3 ± 0,6	85,0 ± 2,3
	Lipid content (g/L)		
43	36,0 ± 6,9	27,1 ± 5,1	34,3 ± 3,8
67	36,7 ± 3,6	42,6 ± 4,2	64,0 ± 2,3
73	41,4 ± 2,2	52,3 ± 4,6	59,1 ± 1,6
	Lipid percentage %		
43	73,3 ± 5,8	53,3 ± 1,2	60,2 ± 0,1
67	63,9 ± 2,9	63,3 ± 0,5	87,7 ± 2,8
73	74,6 ± 0,7	71,3 ± 0,6	69,6 ± 1,9
	Productivity (g/Lh)		
43	0,84 ± 0,16	0,63 ± 0,12	0,80 ± 0,09
67	0,55 ± 0,05	0,53 ± 0,18	0,95 ± 0,04
73	0,57 ± 0,03	0,72 ± 0,06	0,81 ± 0,02
	acid consumed (g)		
	A1 AA	A1 AA	A1 AA
	18,7 102,5	10 131,5	2,1 137
	final yield wt%		
	23,3	27,4	32,2

After this first experiment it was decided to use an automatic pH-stat control to feed the different synthetic mixtures of the acids to the yeast. Furthermore, it was decided to fed the acid in a solution at 5% (wt%) in acetic acid. This value was identified since, as observed, high concentration of A1 could be poisonous for the yeast and since the effect of the other acids on *C. oleaginous* growth were not known.

2.2.2 Study of the influence of several acids on *C. oleaginous* fermentation

The experiments were performed during two different weeks. In each experiment, one bioreactor was fed with pure acetic acid as control. Table 19 summarises the acids used with their principal characteristics.

Table 19 Synthetic mixtures used as feedstock

Acid	Concentration in acetic acid (wt%)	Characteristics
A1	5	Linear odd-numbered aliphatic chain with a double bond
A2	5	Branched odd-numbered aliphatic chain
A3	5	Branched even-numbered aliphatic chain
Propionic acid (PA)	5	Linear odd-numbered aliphatic chain
Butyric acid (BA)	5	Linear even-numbered aliphatic chain

In the experiment where A1, A2 and A3 were analysed, there was a problem with the aeration and the growth was slow down at the beginning. Hence, the experiment was conducted for more time than usual (80 h compared to 70 h). This feature needs to be kept in mind because the differences between the obtained values might be due also to the experimental conditions. Thus, a more reliable analysis will be based on the identification of the differences between the acids and their respective blank. Due to time reasons, it was not possible to repeat the experiments.

The first experiment was conducted with A1-5%, A2 and A3. From the O.D. (600 nm) measurements and the dried biomass it was possible to have a first impression of how the different acids influenced the yeast growth (figure 48 and 49). Apparently, small quantities of A2 allowed *C. oleaginous* to grow better than with pure acetic acid. After 80 h, yeast fed with A2 reached 90,7 ± 0,9 g/L of biomass compared to 83,2 ± 2,3 g/L using only acetic acid (AA1). Yeast fed with A1-5% grew similarly to the one fed with acetic acid: 83,2 ± 2,3 g/L of biomass were obtained feeding AA1 compared to 85,3 ± 3,1 g/L of A1-5%. However, AA1, A1-5% and A2 were not statistically different (p value > 0,05, table 20). Instead, A3 appeared to slow down the growth. The highest dried biomass value was 72,5 ± 0,6 g/L. Moreover, A3 dried biomass profile resembled the one of A1-15%: they both reached a stationary phase after 70h. Hence, this observation suggested that A3 was more poisonous than the other acids. This is likely to be related to the higher lipophilic character and pKa of A3 compared to the other acids. Thus, resulting in a higher content of undissociated A3 in the yeast cells, that leads to a higher release of protons inside the cell with a higher poisonous effect[101].

Figure 48 O.D. measurement of AA1, A1-5%,A2 and A3 error bars calculated as standard deviation of three analytical replicates

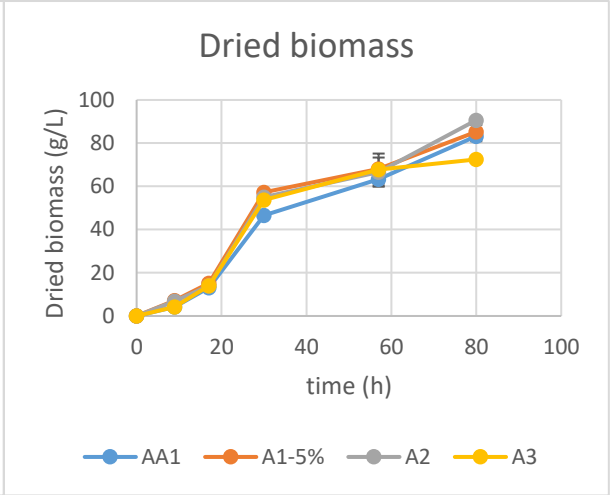


Figure 49 Dried Biomass of AA1, A1-5%,A2 and A3

The lipid content and lipid percentage graphs confirmed the growth profile previously observed (figures 50 and 51). A2 appeared to have a positive influence on the yeast metabolism compared to acetic acid, reaching the maximum lipid content and percentage ($g_{lipid}/g_{biomass}$) at 80 h, 63,2 ± 3,5 g/L and 69,7 ± 0,7%, vs 50,4 ± 0,9 g/L and 60,6 ± 1,7% of AA1. A3 confirmed to slow down the metabolism of the yeast, decreasing the lipid to 43,0 ± 2,7 g/L, while A1-5% profile resembled AA1 ones, 53,9 ± 2,0 g/L. Both A3 and A1-5% reached a lipid percentage ($g_{lipid}/g_{biomass}$) similar to the control, 59,3 ± 0,5% and 63,3 ± 2,3%. Statistical analysis confirmed the significative differences between A2, A3 and AA1 (table 20). The lipid percentage ($g_{lipid}/g_{biomass}$) values observed in this experiment were lower than the highest one obtained in the previous one (70% vs 88%). This could be explained considering that for A1-1,5% the highest value was actually reached as a peak in the lipid production, followed by a decrease probably due to an internal consumption of lipids. Given that the peak was reached at 67h, and that in this experiment there was a time lapse of almost 24 h between the third (57 h) and the fourth point (80h), it might be possible that the yeast reached a peak in the lipid production and that it was not measured. However, the values of the lipid content were

not so different: for both the experiments the maximum value was of 64 g/L, even if in the case of A1-1,5% it was reached after 67 h and for A3 was reached after 80 h.

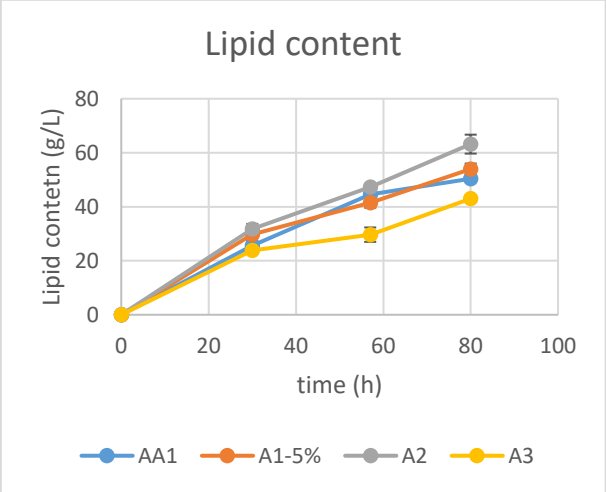


Figure 50 Lipid content of AA1, A1-5%,A2 and A3 error bars calculated as standard deviation of three analytical replicates

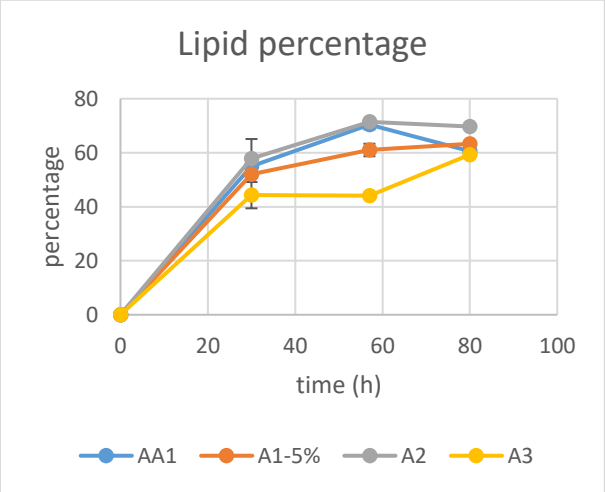


Figure 51 Lipid percentage of AA1, A1-5%,A2 and A3

Figure 52, 53 and 54 report the concentration profiles of glucose, xylose and acetic acid. Apparently, small quantities of acids together with acetic acid speeded up the consumption of the sugars. In fact, glucose was not completely consumed only in the control (pure acetic acid). Furthermore, the diauxic growth looked faster in the case of A1-5% and A2, compared to AA1 and A3, since the xylose was completely consumed only for the first two. However, analysing the acetic acid profiles a different explanation could be given. Indeed, the concentration of the acetic acid was not the same through the different bioreactors. Thus, since the pH was the same for all the fermenters, it meant that the acid responsible of the control of the pH was the secondary one, as observed in the first study. This was probably due to the preference of the yeast to metabolize acetic acid rather than the secondary acid. Hence, if the pH was regulated by the secondary acid, which was probably up taken by the yeast slower than acetic acid, there was also an accumulation of this acid in the bioreactor, as previously speculated. Concluding, since the main role of pH regulator belonged to the slow-metabolised acids, less acetic acid was fed to the bioreactor and this could explain why the sugars concentration for A1-5% and A2 decreased faster than for AA1 and also why the concentration of acetic acid in A1-5% and A2 was lower. For what concern A3, considering the lower dried biomass and lipid content values reached compared to the other acids, it is likely that the slower up take of the sugars was due to the higher poisonous effect. This hypothesis was also in line with the sugar profiles of the first screening. In fact, it was observed a complete consumption of the sugars at 70 h, hence faster than pure acetic acid, but slower than A1-5% and A2. This was consistent since the higher content of acetic acid present when feeding with AA1 competed with the uptake of sugars, while the lower content of acetic

acid in A1-5% rather than A1-1,5% resulted in a faster uptake of glucose and xylose. Hence, on the long time it might be possible to have an accumulation of the secondary acid and then a slow down in the yeast growth, which could also results in the death of the cells. However, it was not clear why the lipid percentage of AA1 decreased after 63 h even if the sugars were not completely depleted.

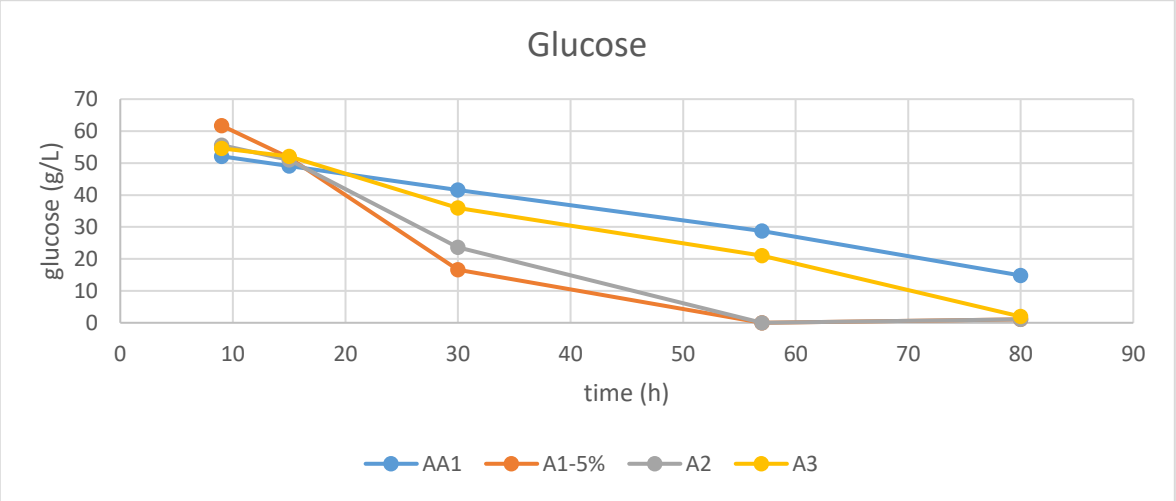


Figure 52 glucose profile of AA1, A1-5%, A2 and A3
error bars calculated as standard deviation of three analytical replicates

Figure 53 xylose profile of AA1, A1-5%, A2 and A3
error bars calculated as standard deviation of three analytical replicates

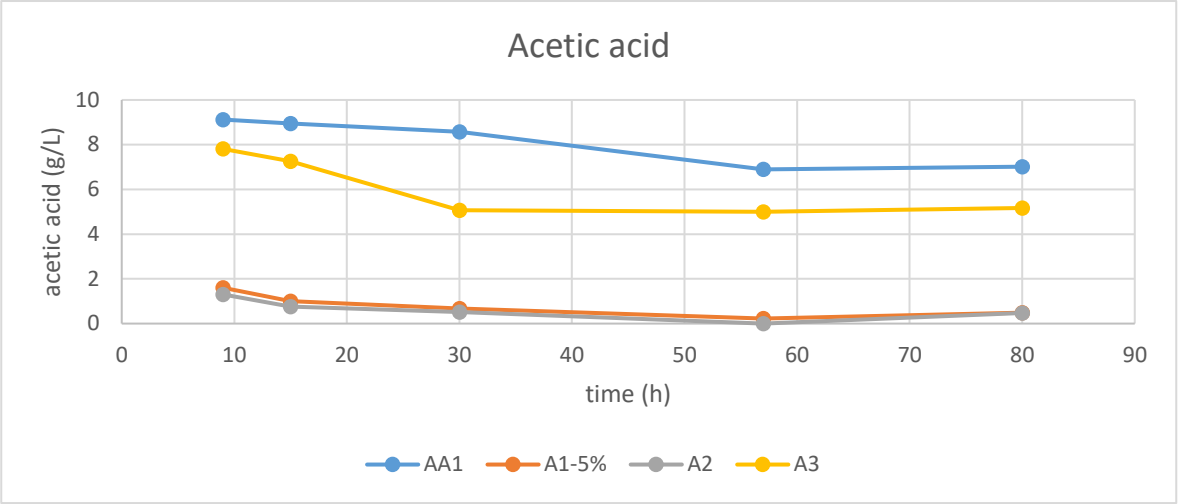


Figure 54 acetic acid profile of AA1, A1-5%, A2 and A3
error bars calculated as standard deviation of three analytical replicates

Table 20 p values of AA1, A1-5%, A2 and A3 (AA1 used as control)

	p values		
	A1-5%	A2	A3
dried biomass	0,536	0,053	0,025
lipid content	0,051	0,004	0,011
lipid percentage	0,309	0,020	0,430

The profiles observed for the lipid accumulation were consistent with the literature. Masri et *a*l.[36] reported a constant growth of the lipid content, but showed also a temporary stationary phase in the lipid percentage from 66 to 90 h. Subsequently they registered another increase in the lipid percentage at 96 h. Thus, it would be interesting to conduct a fermentation up to 120 h to confirm the possibility of increasing the accumulated lipid.

In the second experiment only 2 acids were analysed (PA and BA) together with the control, since the fourth bioreactor was highly contaminated.

Similarly to A1-5% and A2, PA and BA showed a growth comparable to the one of pure acetic acid, AA2 (figures 55 and 56). The dried biomass values reached at 70 h were almost the same for all of the bioreactors: 73,6 ± 3,8 g/L for AA2, 71,5 ± 0,5 g/L for PA and 71,8 ± 3,4 g/L for BA (not statistically different from AA2, p values > 0,05). However, if BA and PA probably reached the stationary phase at 70 h, AA2 appeared to still grow. The results were consistent with what observed for A3, that probably caused a slowdown in the yeast growth, making it enter in the stationary phase.

Figure 55 O.D. measurement of AA2, PA and BA

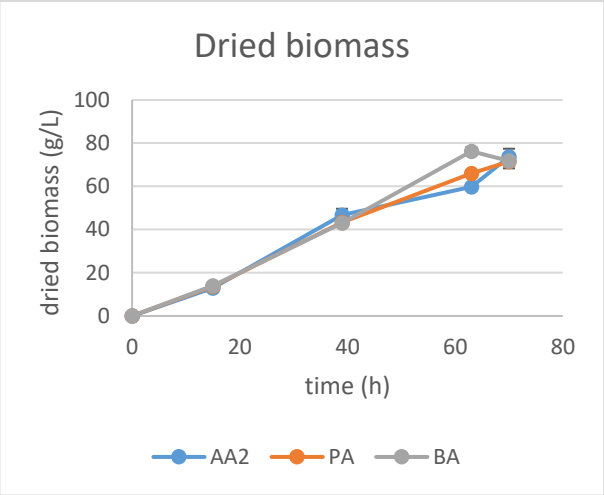


Figure 56 Dried biomass of AA2, PA and BA

error bars calculated as standard deviations of analytical replicates

The lipid content and percentage graphs (figures 57 and 58) of the three screened acids were very similar to the profile observed for AA1, A1-5% and A2. Even if the lipid content increased during time, the lipid percentage ($g_{lipid}/g_{biomass}$) entered a stationary phase or either a partial decrease. As already explained, this was thought to be due to the depletion of sugars (figure 59 and 60). The lipid content reported for AA2 at 70 h is 48,4 ± 1,6 g/L, considerably similar to the one obtained with AA1 at 80 h, 50,4 ± 0,9 g/L. In this fermentation though, the highest lipid content was obtained with AA2, since PA and BA reported 43,3 ± 1,7 and 45,6 ± 1,8 g/L respectively, less than the 63,3 ± 2,3 g/L reached from A2. However, lipid percentages ($g_{lipid}/g_{biomass}$) were much more similar: 65,9 ± 3,4 % for AA2, 60,6 ± 0,4 % for PA and 63,6 ± 3,0% for BA, compared to 60% of AA1 and A3, 63,3 ± 2,3 of A1-5% and 69,7 ± 0,7% of A2. Moreover, statistical analysis confirmed the non-significative differences between AA2, PA and BA (table 21). PA was considered significative different from AA2 only for the lipid content, even if it was not significative different from BA. Hence the influence of PA and BA on the yeast growth was more limited compared to A1, A2 and A3. This might be due to the similarity between PA, BA and AA as chemicals. Moreover, the static profiles observed for lipid contents and lipid percentages ($g_{lipid}/g_{biomass}$) were similar to what reported from Masri et al[36], even though in this fermentation the constant lipid percentage profile was reached after 40 h instead of 60 h. Neither in this fermentation it was observed a lipid percentage value as high as the one obtained with A1-1,5% (88%). In the previous fermentation the highest lipid percentage value was 70% and it was speculated that the peak in the lipid production was not observed due to the big time lapse between the 57 h and 80 h sampling. However, this time the lipid profile clearly showed differences compared to what observed with the A1-1,5% and the time lapse between the 63 h and 70 h samples was not big enough to reach a peak in the lipid production and then decrease to the previous value.

Thus, it was reasonable to think that the higher concentration in the secondary acid (5% vs 1,5%) and its possible accumulation during time, led to a poisonous effect that suppressed the yeast growth and lipid accumulation. Indeed, Fontanille et al.[102] reported that concentration higher than 4 g/L of PA and BA might be inhibit the growth of *Yarrowia L*. Another possible explanation for the lower lipid content is that the concentration of the secondary acid was not the ideal one. Liu et al.[67] analysed the growth of *C. oleaginous* in media with concentration of AA, BA and PA up to 15 g/L. They showed that some combinations of acetic acid and the secondary acid could result in positive synergistic effect. In fact, they observed a boost in the biomass and lipid production when using AA (15 g/L) and BA (15 g/L), similarly to what it was observed when feeding with A1-1,5%. Using this combination, they obtained higher lipid content, dried biomass and yield than with the sole acetic acid. In this experiment instead, the best data were produced when feeding with pure acetic acid. Hence, it might be worth to analyse better the effect of the concentration for all the screened acids, in order to find the best conditions, starting from determining their concentration profile according to time.

Differently from the previous experiment though where it was observed that the sugars were up taken slower when fed with acetic acid, this time the control bioreactor showed the same sugars profiles as the bioreactors fed with the synthetic mixtures of acids. Furthermore, the average concentration of AA2, about 1 g/L (figure 61), was much lower than the concentration monitored with AA1 (averagely 7,5 g/L). Moreover, the acetic acid concentration in AA2 was lower than the one in BA. Hence, it was not possible to explain how the pH could be the same for both the bioreactor if the concentration of the acids in AA2 was lower than the one in BA, since there were no secondary acids that could act as pH control. The most likely hypothesis was that the pH-meter of the bioreactor was not calibrated properly. This could justify both the faster uptake of sugars, since there was less acetic acid, and the different acetic acid profile observed. Previous data from Masri et al.[36] confirmed that a concentration of acetic acid lower than 1 g/L is not consistent with a pH of 6.5. The profile of acetic acid concentration in PA and BA instead were consistent with the literature, since it was reported from Liu et al.[67] that *C. oleaginous* metabolises BA faster than PA. Hence, PA accumulated inside the bioreactor acting as pH control acid and leading to a reduction in the acetic acid. Consequently, it was obtained a higher concentration of acetic acid while feeding with BA rather than with PA.

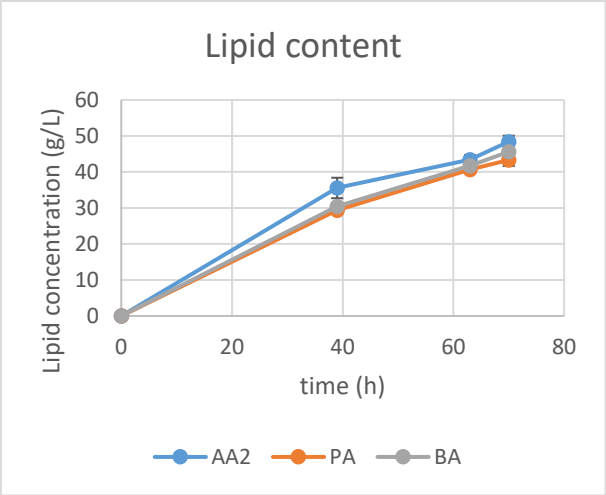


Figure 57 Lipid content of AA2, PA and BA

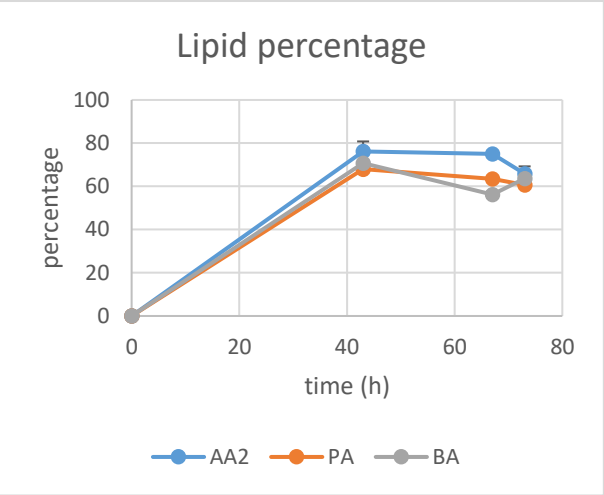


Figure 58 Lipid percentage of AA2, PA and BA

error bars calculated as standard deviations of analytical replicates

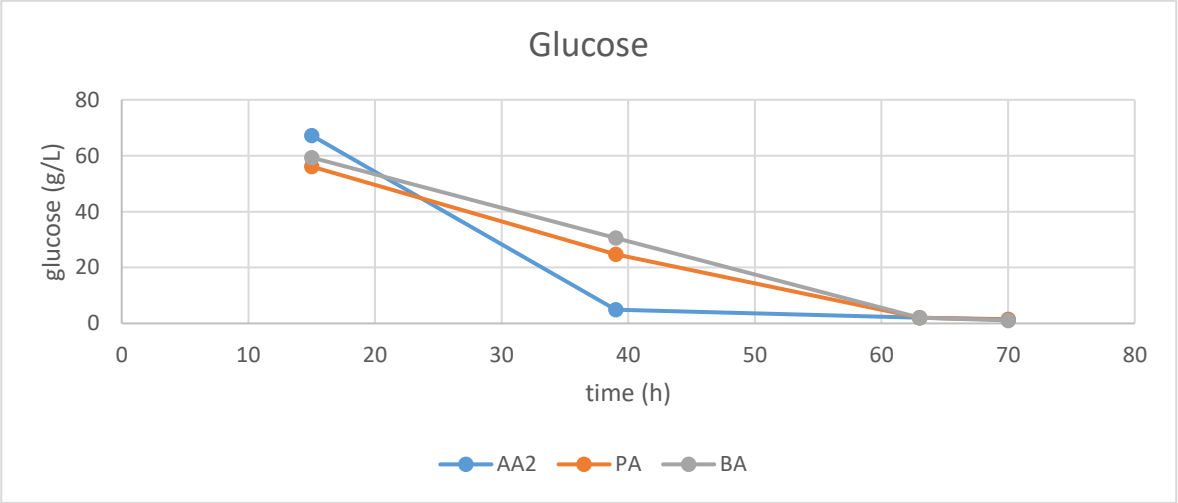


Figure 59 Glucose profile of AA2, PA and BA

error bars calculated as standard deviations of analytical replicates

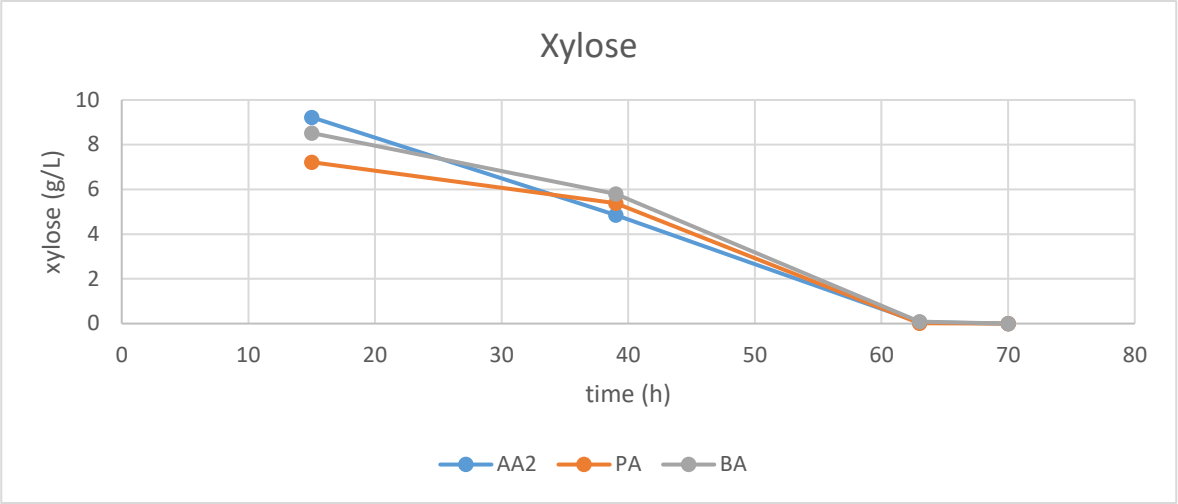


Figure 60 Xylose profile of AA2, PA and BA
error bars calculated as standard deviations of analytical replicates

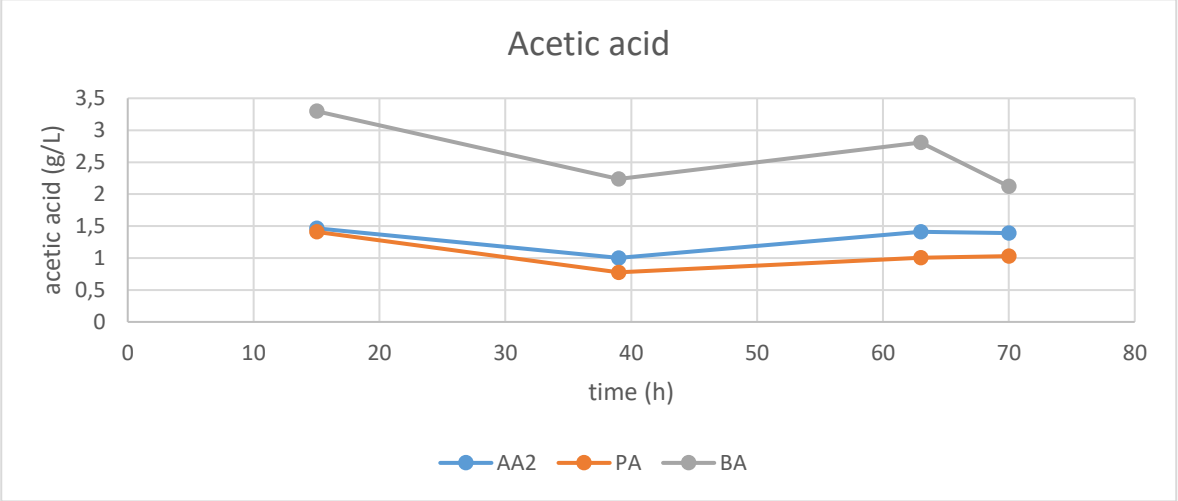


Figure 61 Acetic acid profile of AA2, PA and BA
error bars calculated as standard deviations of analytical replicates

Table 21 p values for PA and BA, using AA2 as control

	p value		
	PA	BA	PA vs BA
dried biomass	0,512	0,655	0,923
lipid content	0,020	0,115	0,186
lipid percentage	0,164	0,561	0,298

The obtained values were in line with what reported from Masri et al.[36] and confirmed the potential of the co-fermentation using glucose and acetic acid as carbon sources. Of all the screened acids, A2 apperared to have the best influence on the yeast growth and lipid accumulation, resulting in a synergistic positive effect. The highest productivity was obtained at 30 h with a value of 1,06 ± 0,06 g/hL, even though at this time the lipid content was still low. Thus, at the end of the fermentation (80 h), the co-feeding of acetic acid and A2 resulted in a lipid productivity of 0,79 ± 0,04 g/Lh, with a

lipid content of 63,2 ± 3,5 g/L and a biomass of 90,7 ± 0,9 g/L. The sole acetic acid feeding resulted in a productivity of 0,63 ± 0,01 g/hL, with a lipid percentage($g_{lipid}/g_{biomass}$) of 60,6 ± 1,7% and a dried biomass of 83,2 ± 2,3 g/L (similar results were obtained for AA2, but at 79 h). Masri et al. reported a productivity of 0,65 g/Lh, with a lipid percentage ($g_{lipid}/g_{biomass}$) of 75 % and a biomass of 75 g/L at 84 h. However, carrying on the fermentation up to 250 h, they obtained surprisingly values, reaching a productivity of 0,8 g/Lh, with a dried biomass of 240 g/L and a lipid percentage ($g_{lipid}/g_{biomass}$) of 87,6 %. Meesters et al.[66] obtained a productivity of 0,59 g/Lh, a lipid percentage ($g_{lipid}/g_{biomass}$) of 25% and a dried biomass of 118 g/L after 50 h, using a two-step 2-L scale fed-batch fermentation with glycerol as carbon source. Chi et al.[65] used a 1-L scale fermentation to study the optimization of pH and acetate condition on the lipid accumulation, obtaining a peak of 0,73 g/Lh in productivity. After 190 h they reached a dried biomass of 168 g/L, with a lipid percentage ($g_{lipid}/g_{biomass}$) of 75 %. The highest value of productivity obtained in this research was also better than one of the highest values reported in the literature. In fact, Ykema et al.[31] observed a lipid productivity of 0,995 g/Lh with 91 g/L of biomass and 30% of lipid percentage ($g_{lipid}/g_{biomass}$). The values obtained in this experiment were higher than the one in the literature also because it was avoided the use of a two-step fermentation. It is important to underline that to calculate the productivity values, no dead times were taken in account. The effect of the secondary acid reflected also in the obtained yield. The average values were around 18% (wt%). The best combination of acetic acid and secondary acid, A2, led to a 24% (wt%) of yield after 80 h, compared to 17% (wt%) of the control. In the first fermentation, the control was also the experiment which led to the lowest yield. The obtained yields were similar to the values reported in the literature. Masri et al.[36] reported a yield of 24% after 120h, which was raised at 39% (wt%) after 240 h. Christophe et al.[103] observed a yield of 15% when feeding with only acetate in a two-step fermentation. Beligon et al.[104] summarised the yield values for acetate fermentation with *C. oleaginous*, reporting an average value of 16%. The data collected in this research were consistent also with what observed from Liu et al.[67], who used synthetic mixtures of AA and PA or BA. Indeed, they observed that the lowest yield belonged to the mixture with AA and PA, while the highest was obtained using BA. They suggested that BA has the highest number of electrons available per gram of carbon, resulting in the highest theoretical growth yield coefficient and thus highest lipid content. However, it is likely that the several factors contributed to the different growth rate, lipid content and yield. In fact, the influence of the acids might be due to a combination of toxicity together with the electrons/carbon atoms that they can supply. The amount of acids consumed from the yeast during the fermentation confirmed what previously observed: AA1 consumed more acetic acid, leading to a not complete depletion of the sugars; A2 was likely to be metabolised easier than A1 since, considering the same amount of sugars uptaken from the yeast in

the two fermenters, the amount of acetic acid in A1-5% is higher than in A2. Thus, it is likely that A1 accumulated in the bioreactor, not acting as carbon source and decreasing the yield. The lower amount of PA consumed compared to BA could be due to a combination of higher toxicity and lower growth theoretical yield. The data obtained for PA and A3 suggested a similar toxic effect on *C. oleaginous.*

	AA1	A1-5%	A2	A3		AA2	PA	BA
time (h)	Dried biomass (g/L)				time (h)	Dried biomass (g/L)		
30	46,6 ± 0,5	57,3 ± 0,2	55,0 ± 0,3	53,7 ± 1,0	39	46,8 ± 2,9	43,3 ± 0,1	43,1 ± 0,4
57	63,3 ± 2,0	68,1 ± 0,8	66,6 ± 6,7	67,7 ± 7,5	63	59,8 ± 1,0	66,0 ± 1,1	76,2 ± 1,9
80	83,2 ± 2,3	85,3 ± 3,1	90,7 ± 0,9	72,5 ± 0,6	70	73,6 ± 3,8	71,5 ± 0,5	71,8 ± 3,4
	Lipid content					Lipid content (g/L)		
30	25,6 ± 1,2	29,8 ± 2,4	31,8 ± 1,8	23,8 ± 0,8	39	35,6 ± 2,8	29,4 ± 0,4	30,5 ± 0,6
57	44,5 ± 1,6	41,5 ± 1,9	47,3 ± 0,9	29,7 ± 0,4	63	43,4 ± 1,3	40,6 ± 0,6	41,8 ± 1,3
80	50,4 ± 0,9	53,9 ± 2,0	63,2 ± 3,5	43,0 ± 2,7	70	48,4 ± 1,6	43,3 ± 1,7	45,6 ± 1,8
	Lipid percentage (wt%)					Lipid percentage (wt%)		
30	55,0 ± 0,6	52,0 ± 0,2	57,8 ± 0,3	44,3 ± 0,8	43	76,1 ± 4,7	68,0 ± 0,1	70,7 ± 0,7
57	70,4 ± 2,2	61,0 ± 0,7	71,4 ± 7,2	44,1 ± 4,9	67	75,0 ± 1,2	63,5 ± 1,1	56,2 ± 1,3
80	60,6 ± 1,7	63,3 ± 2,3	69,7 ± 0,7	59,3 ± 0,5	73	65,9 ± 3,4	60,6 ± 0,4	63,6 ± 3,0
	Productivity (g/Lh)					Productivity (g/Lh)		
30	0,85 ± 0,04	0,99 ± 0,08	1,06 ± 0,06	0,79 ± 0,03	39	0,91 ± 0,07	0,75 ± 0,01	0,78 ± 0,01
57	0,78 ± 0,03	0,73 ± 0,03	0,83 ± 0,02	0,52 ± 0,01	63	0,69 ± 0,02	0,65 ± 0,01	0,66 ± 0,02
80	0,63 ± 0,01	0,67 ± 0,03	0,79 ± 0,04	0,54 ± 0,03	70	0,69 ± 0,02	0,62 ± 0,02	0,65 ± 0,03
	Acid consumed (g)					Acid consumed (g)		
	AA	A1 AA	A2 AA	A3 AA		AA	PA AA	BA AA
	207,0	9,9 188,9	7,6 145,3	6,6 124,9		178,5	8,7 166	6,2 119
	Yield (wt%)					Yield (wt%)		
	16,5	17,3	24,4	18,7		16,0	14,6	19,4

2.2.3 Fatty acids profile

In order to evaluate the differences in the FAs profile obtained when using the synthetic mixtures and the sole acetic acid, the accumulated TAGs were esterified with methanol and subsequently analysed at GC (for more details see experimental details section). Since the use of the synthetic mixtures led to the synthesis of fatty acids which were not contained in the mixture used as reference, it was not possible to perform a quantitative analysis. Consequently, the work focused on recognising the differences in the FAs profile when using sole acetic acid and when using synthetic mixtures. The FAs not present in the reference mixture were highlighted and labelled as “new peak”. It was speculated about their nature, but it was not possible to confirm their chemical formula. Below, it was

analysed the FAs profile of acetic acid and then it was compared to the synthetic mixtures. It is noticeable that since the internal standard was nonadecanoic acid (C19:0), it is possible that the presence of C19:X (X≥0) was due to the internal standard and not to the influence of the secondary acid.

The use of acetic acid as carbon source triggered the production of not recognizable peaks (figures 62 and 63), but few hypotheses can be made. Peak 1 can be associated to C13:X (X≥0). Peaks 2,3,4 to C15:X. Peak 8 to C19:X. Peaks 11 to C23:X. Peaks 13-15 to C>C24:X. Peaks 9,10,12 appeared as small shoulder of C18:3, C20:0 and C24:0 respectively and it was difficult to state what they could be. Considering that Liu et al[67] did not report the production of C19:0 in *C.oleaginous* when using acetic acid as only carbon source, and considering that odd-numbered fatty acids (C15:X, C17:X) were produced in low amount, it was likely that the nonadecanoic acid (10 min) detected by the GC came from the internal standard.

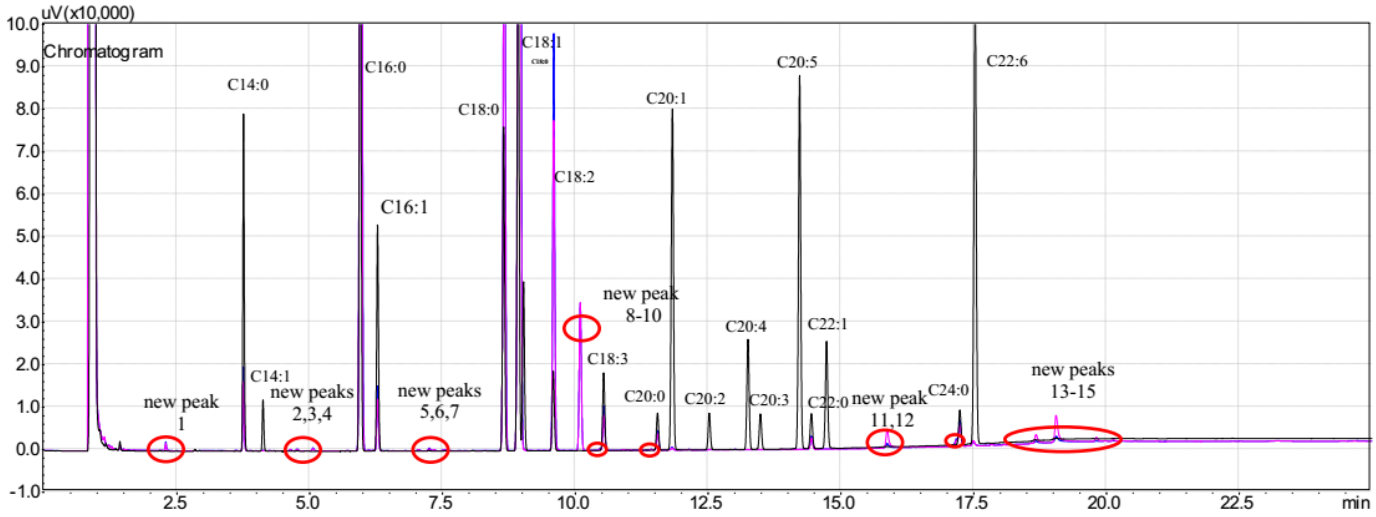


Figure 62 FAs profile obtained when feeding with AA1
Black: marine oil; Rose and blue: AA1

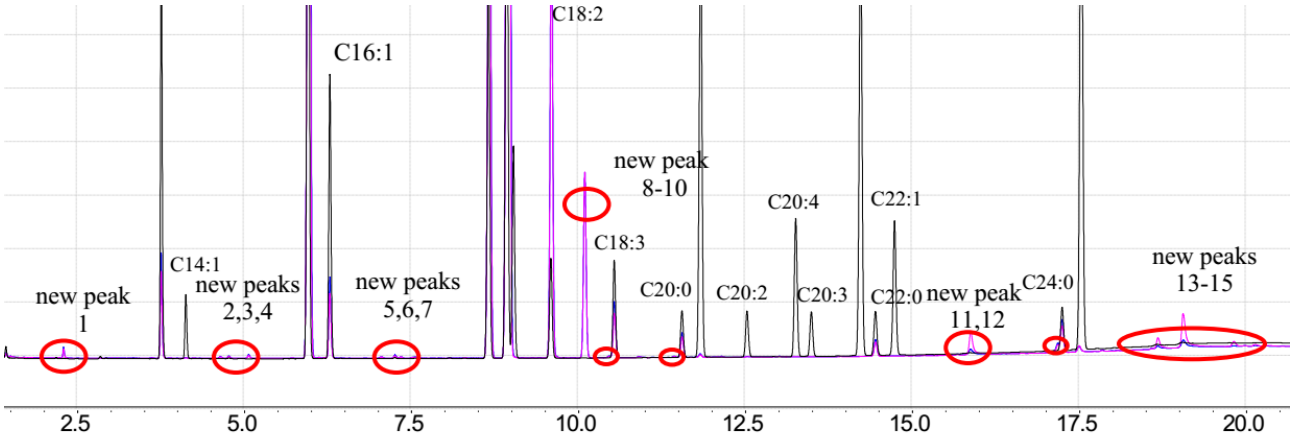


Figure 63 zoom of the FAs profile obtained when feeding with AA1
Black: marine oil; Rose and blue: AA1

In figures 64 and 65, the FAs profile of the TGAs synthesised when using A1-5% is presented. The FAs profile resembled the one of pure acetic acid. The percentage composition might be different, but it was not possible to state the quantitative variations. Hence, it was not possible to assess how the double bond of A1 influenced the accumulated lipids.

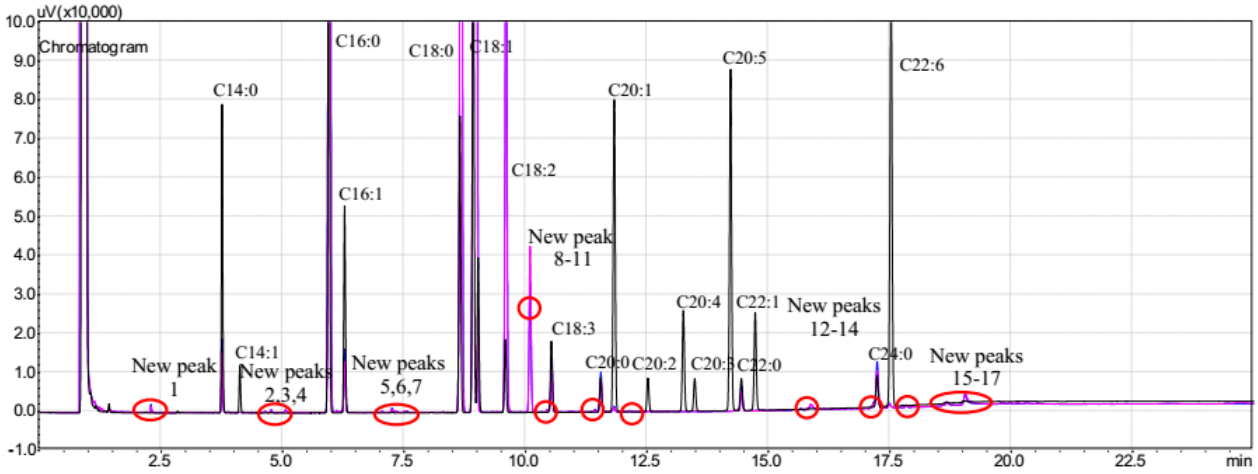


Figure 64 FAs profile obtained when feeding with A1-5%
Black: marine oil; Rose and blue: A1-5%

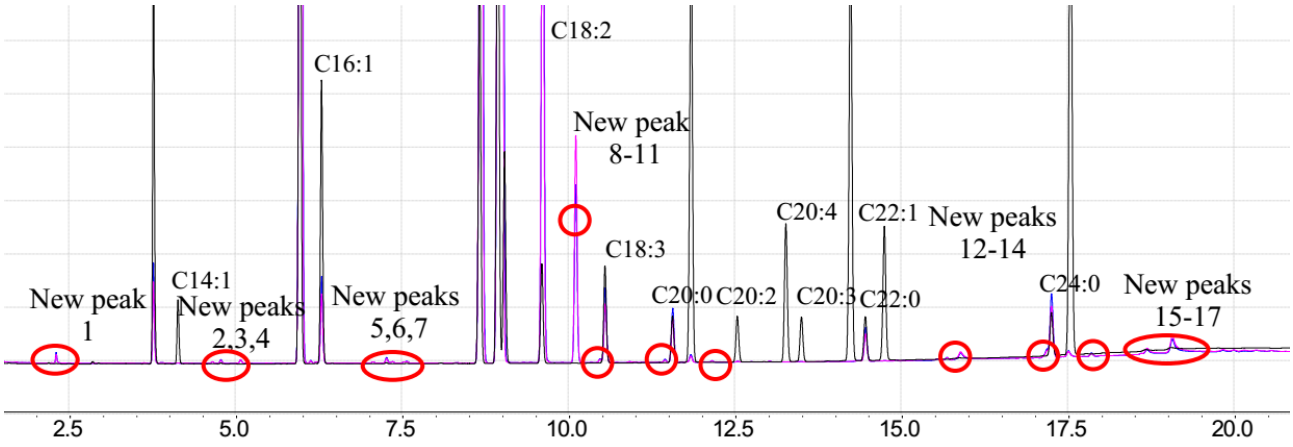


Figure 65 zoom of the FAs profile obtained when feeding with A1-5%
Black: marine oil; Rose and blue: A1-5%

The FAs profile of the TGAs produced when feeding with A2 is presented in figures 66 and 67. Similar to A1, the profile resembled what observed with pure acetic acid. However, peak 5, probably characterized by a formula such as C17:X, was in higher quantity than when using only AA.

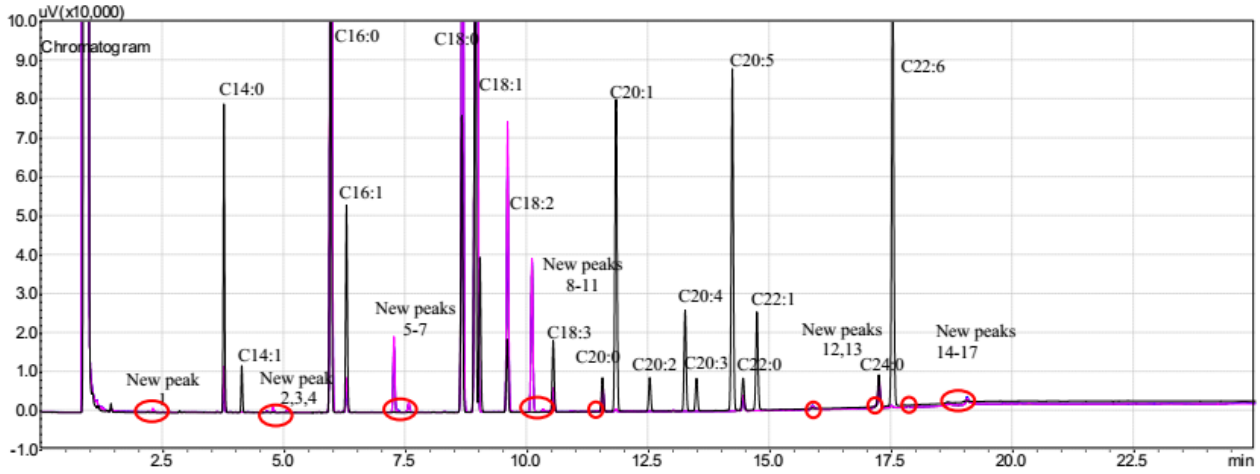


Figure 66 FAs profile obtained when feeding with A2
Black: marine oil; Rose and blue: A2

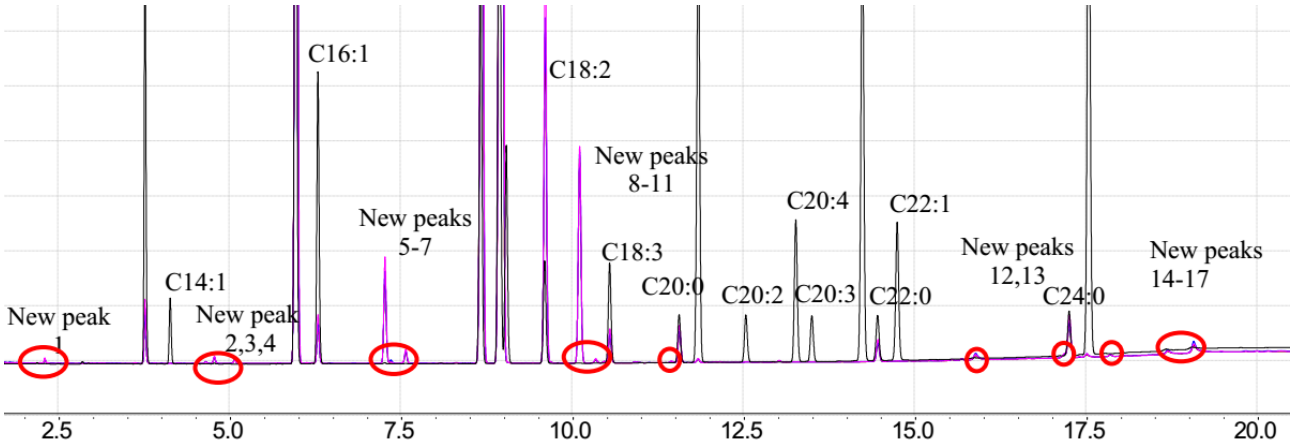


Figure 67 zoom of the FAs profile obtained when feeding with A2
Black: marine oil; Rose and blue: A2

Figures 68 and 69 reported the FAs profile obtained when feeding with A3. Surprisingly, the bigger differences compared to acetic acid were obtained when using this acid, which was also the most poisonous one. A3 appeared to enhance the accumulation of long chain FAs. Indeed, peaks with larger areas than when using pure acetic acid at elution time higher than the one for C22:X were obtained. In particular, peaks 13-15 probably belong to C23:X class of fatty acids. While peaks 16-20 are likely to have a number of carbon atoms higher than 23. This phenomenon was explained considering that A3 was the acid with the longest aliphatic chain between the screened acid. Hence, if the chain was uptaken without being degraded, it could have triggered the synthesis of FAs with high carbon atom numbers. However, Christophe et al.[83] suggested that VFAs with longer chain than acetic acid were first degraded and then released in the cytoplasm, ready to be used as building blocks for the TGAs.

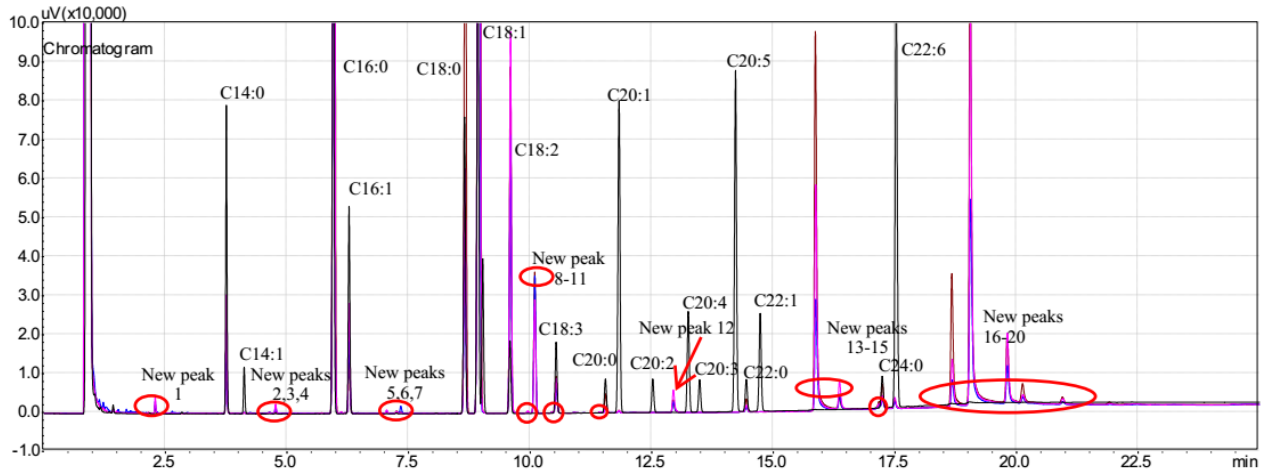


Figure 68 FAs profile obtained when feeding with A3
Black: marine oil; Rose and blue: A3

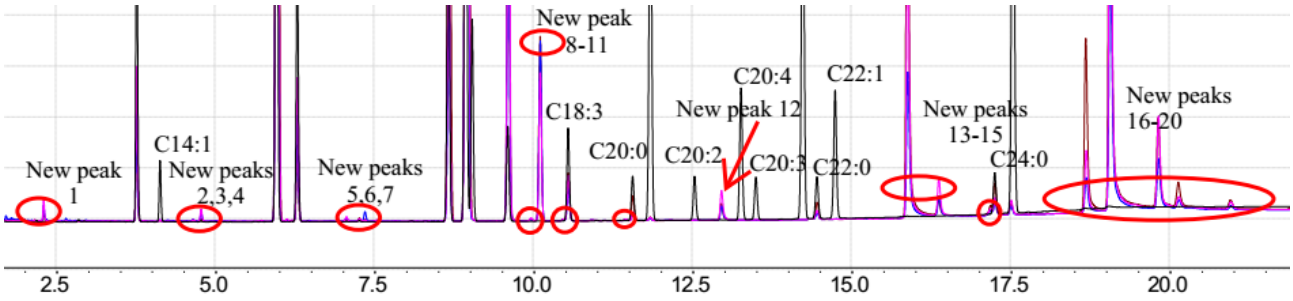


Figure 69 zoom of the FAs profile obtained when feeding with A3
Black: marine oil; Rose and blue: A3

The FAs profile obtained when feeding with PA is presented in figures 70 and 71. These results were in line with what reported from Liu et al.[67]. The odd-numbered chain of PA enhanced the production of odd-numbered FAs such as C15:X (peaks 2-4), C17:X (peaks 6,7) and probably C19:X (peaks 8-11). The production of FAs with carbon atom numbers higher than C20 is similar to the case of sole AA. Liu et al. did not report the presence of C19:0 when using PA as carbon source for *C. oleaginous*' fermentation. Yet, given the high content of odd-numbered fatty acids, it is believed that the C19:X detected partially belonged to the accumulated lipid.

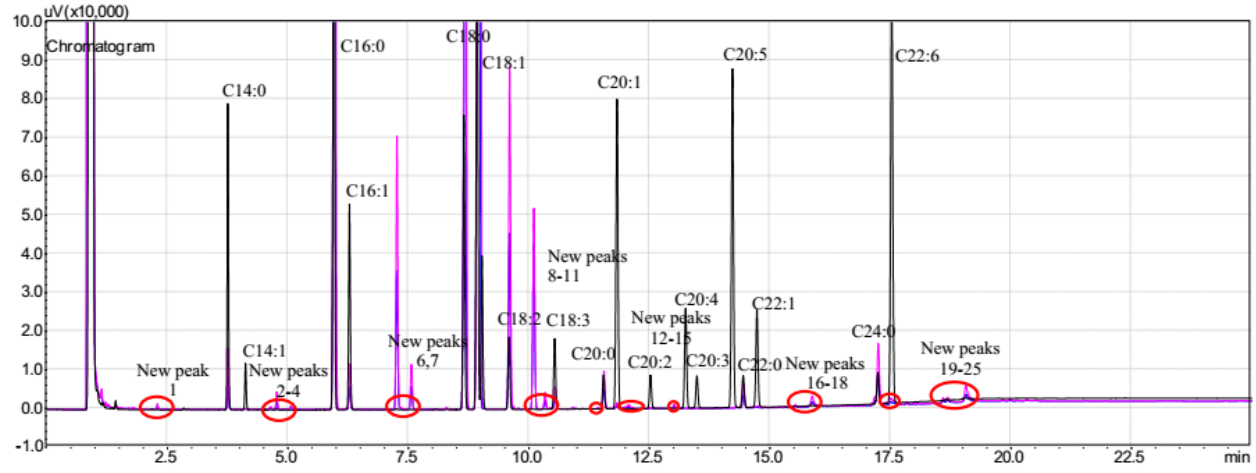


Figure 70 FAs profile obtained when feeding with PA
Black: marine oil; Rose and blue: PA

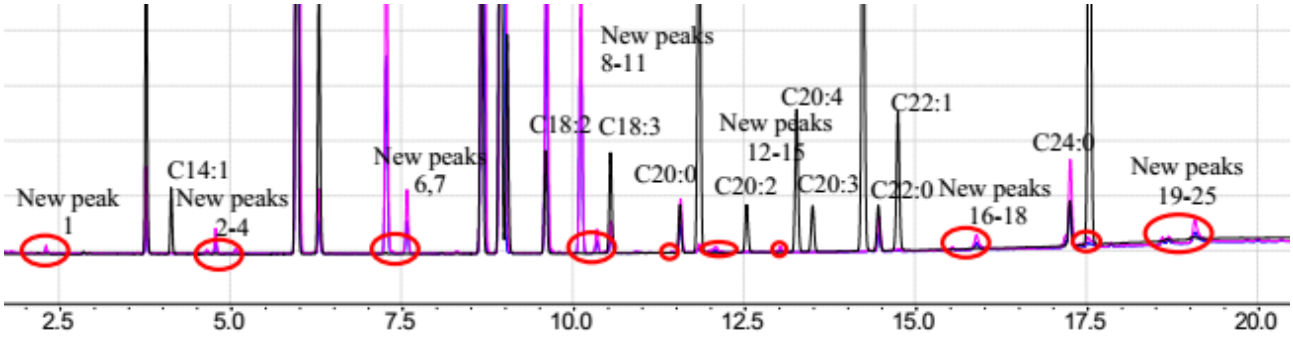


Figure 71 zoom of the FAs profile obtained when feeding with PA
Black: marine oil; Rose and blue: PA

The use of BA confirmed to not particularly affect the FAs profile compared to the sole acetic acid. The same observation was previously done from Liu et al[67]. This could be due to the similar chemical formulas of BA and AA, since they both have an even-numbered carbon chain. Hence, butyric acid differs from acetic acid because each mole of BA gives two mole of acetylCoA, compared to one mole from the sole acetic acid[83].

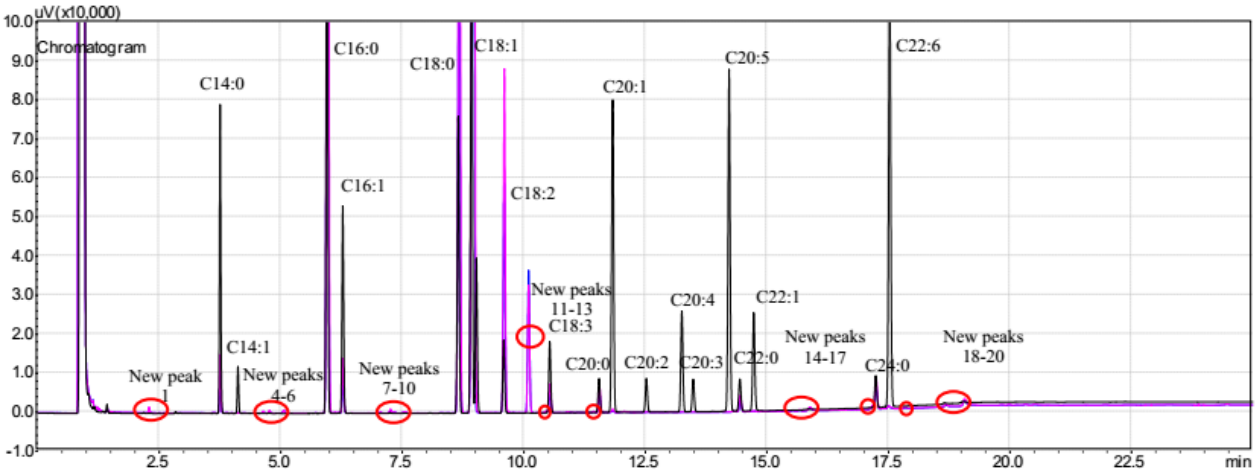


Figure 72 FAs profile obtained when feeding with BA
Black: marine oil; Rose and blue: BA

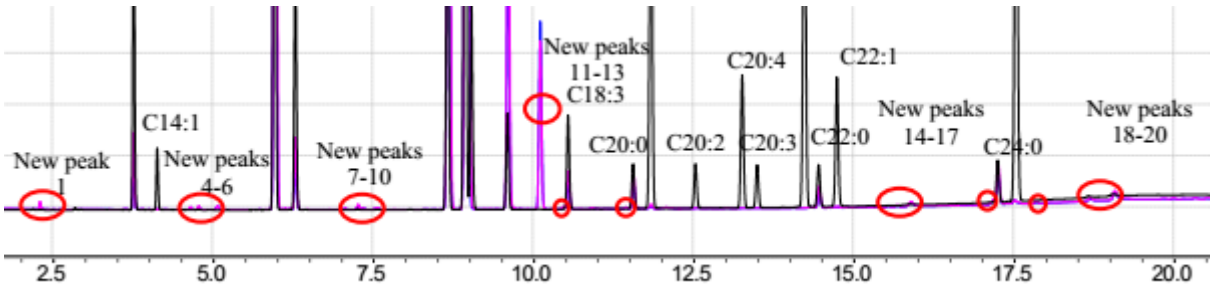


Figure 73 zoom on the FAs profile obtained when feeding with BA
Black: marine oil; Rose and blue: BA

In order to identify the not-recognized peaks and to assess the presence of C19:0, a second FAME analysis was conducted. Dodecanoate (C12:0) was used as internal standard, while Supelco Fatty acids mixture was used to confirm the observations previously done. Nonethless, the reference mixture allowed to confirm the presence of the sole C15:X and C17:X, since the Supelco mixture did not contain nonadecanoic acid. However, the eventual disappearance of the peak at 10 min, identified as C19:0, was used as reliable proof of the natural production of this fatty acids.

As it is possible to notice in figure 74, the use of the sole acetic acid did not trigger the production of C19:0. In fact, it was not observed the peak at 10 min. Moreover, the small peak at 7,4 min confirmed to be C17:0. This data were in line with what reported from Liu et al.[67].

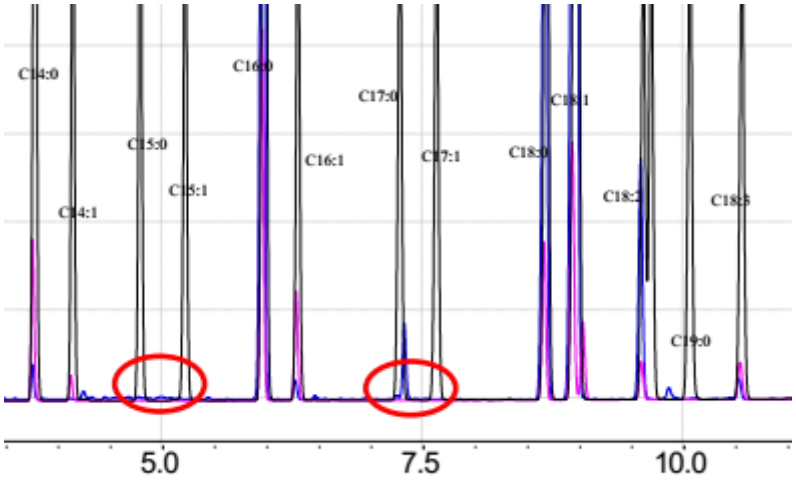


Figure 74 FAs profile obtained when feeding with AA
Black: Supelco mix; Purple: Marine Oil mix; Blue: AA

A2, characterized by an odd-numbered chain, was confirmed to enhance the production of C15:X, C17:X and C19:X (figure 75). The peak at 7,4 and 7,5 min were identified as C17:0 and C17:1, while the peak at 10 min, previously not observed with only acetic acid, was labelled as C19:0.

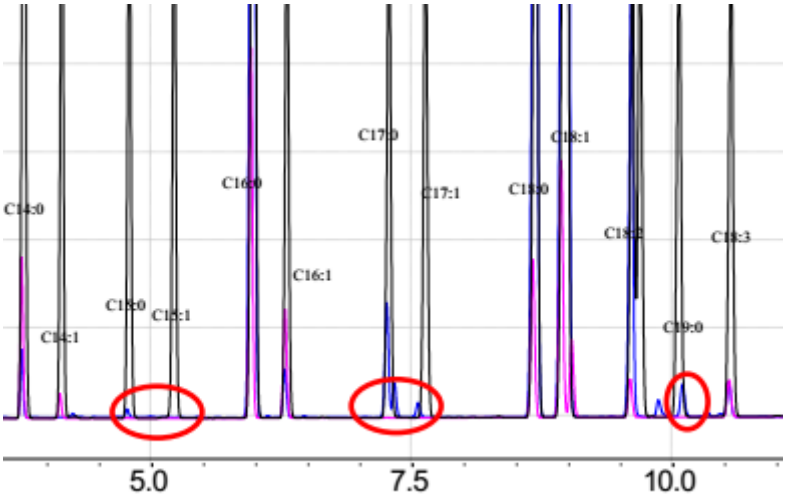


Figure 75 FAs profile obtained whe feeding with A2
Black: Supelco mix; Purple: Marine Oil mix; Blue: A2

Once again, the relation between the number of the carbon atoms in the VFAs used as feeding and the composition of the produced FAs was confirmed. A3, given its even-numbered chain, influenced the lipid composition in a similar way to acetic acid.

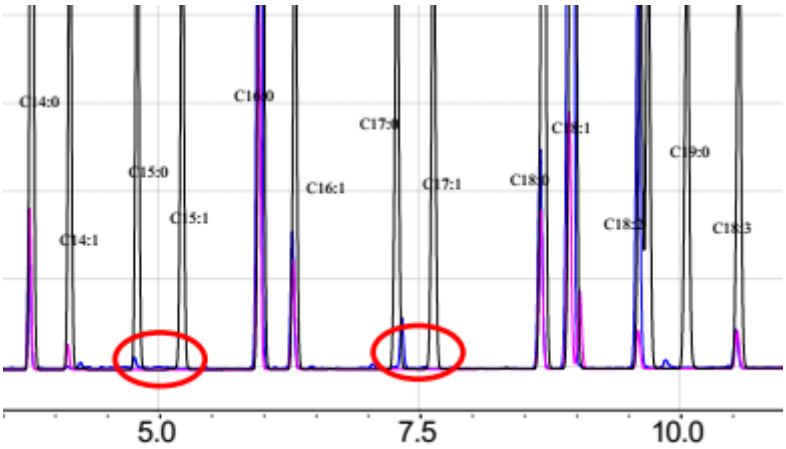


Figure 76 FAs profile obtained when feeding with A3
Black: Supelco mix; Purple: Marine Oil mix; Blue: A3

Not surprisingly, it was confirmed that PA stimulated the production of C15:0, C17:0, C17:1 and C19:0 (figure 77). Yet, this results were in contrast with what stated from Liu et al[67],who did not observed the presence of nonadecanoic acid.

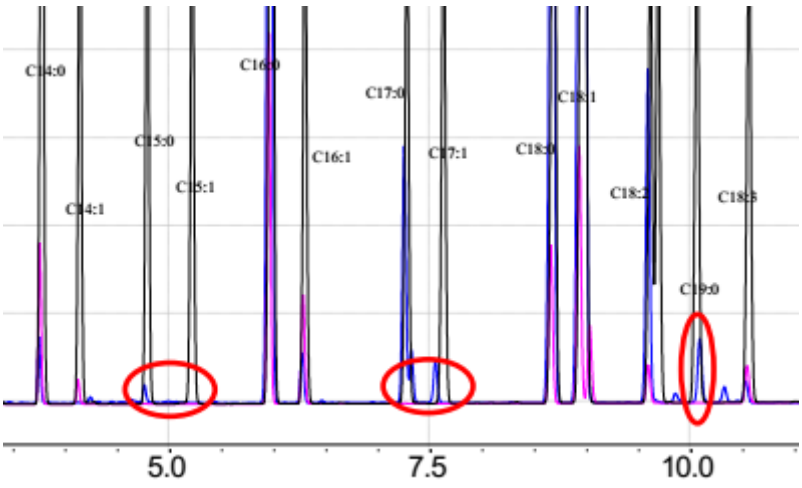


Figure 77 FAs profile obtained when feeding with PA
Black: Supelco mix; Purple: Marine Oil mix; Blue: PA

Butyric acid revealed to have a similar profile as acetic acid, given the even-numbered aliphatic chain. The disappearance of the peak at 10 min confirmed that the amount of nonadecanoic acid precedently detected belonged to the internal standard.

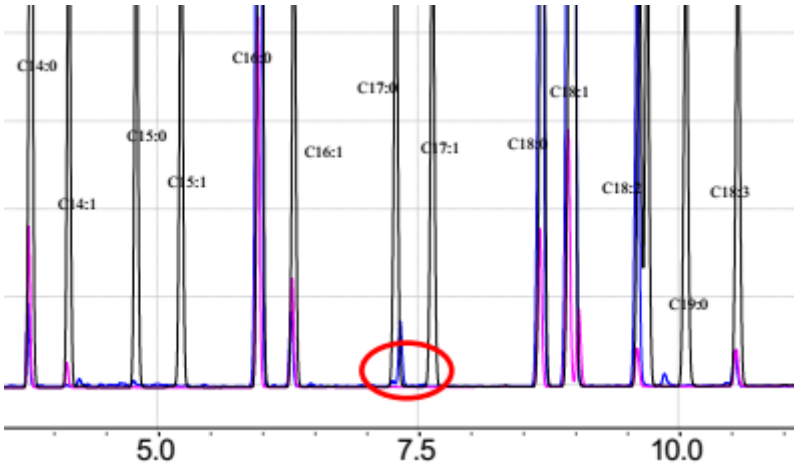


Figure 78 FAs profile obtained when feeding with BA
Black: Supelco mix; Purple: Marine Oil mix; Blue: BA

3 Experimental Methods

3.1 Materials

Table 22 List of materials used

Chemical	Supplier	Chemical	Supplier
Acetosyringone	Carl Roth	Supelco Mixture	Sigma Aldrich
Agar, Agar Kobe I	Carl Roth	Tryptone	Carl Roth
Agarose Basic	Panreac Applichem	Xylose	Panreac Applichem
Ammonium sulphate	Carl Roth	Yeast extract	Carl Roth
Ampicillin	Carl Roth	Zinc chloride	Carl Roth
Antifoam 204	Merck	Zinc sulphate	Carl Roth
β-glucosidase	Novozymes	Phusion DNA polymerase	Fermentas
Boric acid	Carl Roth	Instrumet & software	Model
Calcium chloride	Sigma Aldrich	Balance	ohaus pioneer
Cefotaxime	Panreac Applichem	Bioreactor	DASGIP (1L), Eppendorf
Cellic Ctec 2	Novozymes	Centrifuge	Beckmann Avanti JXN-26
Cellic Htec	Novozymes	Centrifuge	Eppendorf 5424 R
Cobal nitrate	Sigma Aldrich	Centrifuge	Eppendorf 5920R
Cupper Sulphate	VWR	Enspire Multilabe 2300	PerkinElmer
Glucose	Carl Roth	Freezer	New Brunswick Innova U725
Glyceril trinonadecanoate	Sigma Aldrich	GC	GC-2010 Plus, Shimadzu
Glycerol	Carl Roth	Gel Doc XR+	BioRad
Hygromicin B	Panreac Applichem	Homogeneizer	Mulsiflex C3, Avestine
Iron chloride	Carl Roth	Hybond N+ membrande	GE Healthcare
Iron Sulphate	Merck	Image Lab	BioRad
Kanamycin	Carl Roth	HPLC	Agilent 1100
Marine oil (#35066)	Restek	Lyophilizer	Christ alpha 2-4 LD Plus
Manganese sulphate	Sigma Aldrich	Microscope	Axio Lab A1, Zeiss
Mannase	Clariant	Microscope software	Zen 2 lite
MES buffer	Sigma Aldrich	PCR thermocycler	Mastercycler, Eppendorf
Methanol	Carl Roth	Shaker	New Brunswick Innova 44R
Nile Red	Merck	snapgene	GSL biotech
Potassium phosphate	Carl Roth	spectrophotometer	Genesys 10S, Thermoscientific
Potassium phosphate dibasic	Carl Roth	Tm calculator	Life technologies, Carlsbad
Protease	BASF	Yeast DNA kit extraction	Thermoscientific
Sodium chloride	Carl Roth	Strain	Supplier
Sodium methoxide	Sigma Aldrich	*C. oleaginous*	DSMZ, Braunschweig
Sodium molibdate	Carl Roth	*A. tumefaciens*	ATCC, Manassas

3.2 Methods

3.2.1 Genetic engineering approach

3.2.1.1 Strains and media

C. oleaginosus (strain not revealed for secrecy reasons) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ) (Braunschweig, Germany). *Agrobacterium tumefaciens* (strain not revealed) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Media for cultivation, transformation and lipid production were LB medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L); YPD medium (glucose, 20 g/L; tryptone, 20 g/L; yeast extract, 10 g/L) and minimal N medium (glucose 30 g/L; yeast extract, 0.75 g/L; $(NH_4)_2SO_4$, 1,2 μg/L; $MgSO_4*7H_2O$, 1.5 g/L; KH_2PO_4, 0.4 g/L; $CaCl_{2*}H_2O$, 0.22 g/L; $ZnSO_4*7H_2O$, 0.55 μg/L; $ZnCl_2*4H2O$, 24.2 μg/L; $CuSO_4*5H_2O$, 25 μg/L; $FeSO_4*7H_2O$, 25 μg/L) were used. Media for the Agrobacterium tumefaciens mediated transformation were liquid induction medium with acetosyringone (L-IMAS) (K_2HPO_4, 2.05 g/L; KH_2PO_4, 1.45 g/L; NaCl, 0.15 g/L; $MgSO_4*7H2O$, 0.5 g/L; $CaCl_2*2H_2O$, 67.0 mg/L; 2- 4-morpholineethanesulfonic acid monohydrate (MES), 7.8 g/L; glucose, 1.8 g/L; acetosyringone, 39.24 mg/L; $FeSO_4*7H_2O$, 2.5 mg/L; $(NH_4)_2SO_4$, 0.5 g/L; glycerol 5% (v/v); trace elements solution (100 mg Na_2MoO_4, $MnSO_4 \cdot H2O$, $ZnSO_4*7H_2O$, $CuSO_4*5H_2O$, H_3BO_3 in 1 L ddH20, 5% (v/v), pH 5.6) and solid induction medium with acetosyringone (S-IMAS) (equivalent to L-IMAS, without glucose and supplemented with 18 g/L agar).

3.2.1.2 Molecular biology techniques

PCR amplification for cloning was performed using Phusion DNA polymerase (Fermentas, Thermo Scientific,Waltham, MA, USA). Primers for PCR were designed using the Software SnapGene (GSL Biotech). Vendor recommendation for PCR conditions were followed. A high primer concentration was used (1 μM). Annealing temperatures were estimated in the web interface Tm calculator from Life technologies (Carlsbad, CA, USA). All designed primers and genes were synthesized by Eurofins Genomics (Ebersberg, Germany). Analysis of DNA fragments was performed by electrophoresis on a 1% wt/vol agarose gel, at 110 V for 45 min. Yeast DNA Extraction kit from Thermo Scientific (Waltham, MA, USA) was used for genomic DNA extraction.

3.2.1.3 Fluorescence photographs

Fluorescence and brightfield microscopy photographs were obtained using an Axio Lab A1 Microscope with Cam ICm1 (Zeiss, Oberkochen, Germany). Images were processed with Zen 2 lite. For imaging of fluorescence colonies in plates, the Gel Doc XR+ system from BioRad (Hercules, California, USA) was used. Images were edited with Image Lab from BioRad.

3.2.1.4 Mutagenesis and screening of evolved *C. oleaginosus*

Genetically modified *A. tumefaciens* required for the transformation was obtained from previous works conducted by Pariya Shaigani. *A. tumefaciens* mediated transformation was performed as reported from Goerner et al.[42]. In particular, an overnight culture (LB medium supplemented with 30 μg/mL kanamycin at 28 °C, 120 rpm) was used to inoculate (OD600) a 10 mL shake flask culture with L-IMAS medium and cultivated at 28 °C for 6 hours. Next, *A. tumefaciens* was mixed at an equal ratio with *C. oleaginosus* cells resuspended in L-IMAS medium at OD600 0.5. Therefore, an overnight culture of *C. oleaginosus* in YPD (28 °C, 120 rpm) was centrifuged (10 000g) and re-suspended in an appropriate volume of L-IMAS medium. 100 μl of the *A. tumefacien*s and *C. oleaginosus* cell mixture were plated on top of an Amersham Hybond-N+ blotting membrane that was placed on S-IMAS agar plates. The plates were incubated at 24 °C for 48 hours and subsequently the membranes were transferred to YPD agar plates supplemented with 200 μg/mL hygromycin B and 300 μg/mL cefotaxime and incubated at 28 °C for 5 days.

3.2.1.5 Culture and selection

After transformation single colonies were picked and transferred to YPD agar plates supplemented with 300 μg/mL cefotaxime and 100 μg/mL hygromycin B. Subsequently, single colonies were inoculated and grown overnight (28°C, 120 rpm) into YPD supplemented with 300 μg/mL. cefotaxime. After having confirmed that the culture was axenic, 1 mL was taken and treated to prepare

a glycerol stock. To do so, the solution was centrifuged (6 000g, 1 min), the supernatant was discarded and the pellets were washed with water. Then, the centrifugation was repeated and, once the supernatant was discarded, the pellets were resuspended in YPD. Finally, 800 μL were mixed with 200 μL of an 80% solution of glycerol in water and then stocked at -80°C. The rest of the culture was centrifuged (10 000 g, 1 min) and the supernatant was discarded. DNA was extracted from the pellets to confirm the presence of the inserted genes via PCR.

3.2.1.6 Screening of mutants

To identify the best lipid producer, the positive mutants were streaked from glycerol stock to YPD agar plates supplemented with 300 μg/mL cefotaxime and 100 μg/mL hygromycin B. Subsequently, single colonies were streaked to YPD agar plates supplemented with 300 μg/mL cefotaxime. After this, the screening analysis were carried out in 500 mL baffled shake flasks at 28 °C, 120 rpm, for 5 days in biological triplicate. Cultivation media (150 mL) used was nitrogen limitation medium with glucose. Cultivation was started by inoculation from an overnight culture (YPD medium supplemented with 300 μg mL-1 cefotaxime) at O.D. (600 nm) 0.1 in YPD medium. 15 mL samples were taken after 24, 46, 72 and 96 hours to determine the O.D. (600 nm), dried cell mass, lipid content and fatty acid composition.

3.2.1.7 Dried cell mass and lipid yield

Cellular dry weight was determined by centrifugation of the cells at 12 000 g for 10 min at 4 °C. Subsequently, the cell pellets were washed with double distilled water and dried in a lyophilizer. The cellular total lipid was determined by extraction with chloroform and methanol using a modified protocol from Folch et al.[92] In particular, 4 mL of culture were mixed with 1 mL of Folch solution in a glass test tube and shaken for 1.5 h at 175 rpm, room temperature, with 1 g of glass beads (diameter =2,7 mm) and 5 glass beads (diameter = 5 mm). Subsequently, other 3 mL of Folch solution were added and the solution was shaken for other 30 min. Then the glass test tubes were centrifuged at 1000 rpm for 1 min. The chloroform layer was collected in a pre-weighted glass vial. 3 mL of water layer were removed and 4 mL of Folch solution were added into the glass test tubes. The tubes were shaken for 1 h, 175 rpm and room temperature. Then the glass beads were removed by filtration and, after centrifugation at 1000 rpm for 1 min, the chloroform was collected and then evaporated.

3.2.1.8 Fatty acid composition analysis

For the fatty acid analysis, 10 mg of the dried biomass were collected. The cell biomass were lysed using a sonicating bath for 40 min and then the lipids were directly converted into fatty acid methyl esters (FAME) by methanol transesterification according to the protocol of Griffiths et al.[105] FAMEs were analysed on GC, with flame ionisation detector. 1 µl sample was applied by AOC-20i auto injector (Shimadzu) into a ZB-WAX column (30 m, 0.32 mm ID; 0.25 µm df; phenomenex (Torrance, CA, USA)). The initial column temperature was 150 °C (maintained for 1 min). A temperature gradient was applied from 150 °C– 240 °C (5 °C min-1), followed by 6 min maintenance at 240 °C. Fatty acids were identified according to retention times of authentic standards (marine oil mixture). Quantitative analysis was performed using glycerol trinonadecanoate as internal standard.

3.2.1.9 Nile Red analysis

Nile Red analysis was performed according to the protocol of Sitepu et al.[91] using direct flourescence measurements at 530 nm and emission 590 nm filters on a EnSpire Multilabel 2300 plate reader, from PerkinElmer, USA . In particular, screening samples at 48, 72 and 96 h were collected. The O.D. (600 nm) was adjusted to 1 using double distilled water. Then, 200 μL of the culture were mixed with 25 μL DMSO in a black 96-well plate with clear bottom. The O.D. was measured. Subsequently 25 μL of a 100 μg/mL Nile red solution in DMSO were added by pipetting up and down each well to complete a volume of 250 μL. Fluorescence was measured on the microtiter plate reader, using excitation 530 nm and emission 590 nm filters on a microtiter plate reader.

3.2.2 Bioengineering approach

3.2.2.1 Strains and preculture

C. oleaginosus (strain will not be reported for secrecy reasons) was cultivated in baffled shaking flasks containing YPD media broth (10 g /L yeast extract, 20 g /L peptone, and 20 g/L glucose) containing antibiotics (10 mg/L ampicillin, 10 mg/L kanamycin). The yeast was incubated in a rotary shaker at 120 rpm and 28°C for 2 days and was then was used as the inoculum.

3.2.2.2 Media

Medium for the experiment section 2.2.1 was as follow: yeast extract 1 g/L; pepton 10 g/L; NaH_2PO_4 1,5 g/L; $MgSO_4$ 2,5 g/L; $(NH_4)_2SO_4$ 0,5 g/L; $CaCl_2$ 1,5 g/L; CH_3COONa 5,4 g/L; glucose 60 g/L; xylose 5 g/L; $FeCl_3*6H_2O$, 25 μg/L; $ZnSO_4*7H_2O$, 1 μg/L; $MnSO_4*5H_2O$, , 24 μg/L; $CuSO_4*5H_2O$ 25 μg/L; $Co(NO_3)_2*6H_2O$, 3 μg/L. Medium for the experiments section 2.2.2 was as follow: yeast extract 1,5 g/L; pepton 8 g/L; NaH_2PO_4 2,5 g/L; $MgSO_4$ 2 g/L; $(NH_4)_2SO_4$ 0,5 g/L; $CaCl_2$ 1 g/L;

CH_3COONa 0,41 g/L; glucose 70 g/L; xylose 10 g/L; $FeCl_3*6H_2O$, 25 μg/L; $ZnSO_4*7H_2O$, 1 μg/L; $MnSO_4*5H_2O$, , 24 μg/L; g/L ; $CuSO_4*5H_2O$ 25 μg/L; $Co(NO_3)_2*6H_2O$, 3 μg/L.

3.2.2.3 Bioreactors

For cultivation of *C. oleaginosus*, a DASGIP four parallel bioreactor system with a working volume of 1 L was used. The temperature was set to 28°C. The pressure was at the atmospheric value. At the beginning of each fermentation the pH was raised to 8,3, using NaOH 3M, and then adjusted to 6,5 ± 0,02 with acetic acid 100% (wt%). Stirring (800 rpm), oxygen ratio (21–100%) and aeration (8.0–1.5 vvm), were regulated automatically to maintain dissolved oxygen at a pO2 of 50% or more. An antifoam agent was used to prevent foam. The values are presented as averages, and each point was analysed in triplicate. Error bars are calculated as standard deviation of analytical replicates.

3.2.2.4 Analysis

Sugars consumption (glucose and xylose) and acetic acid concentration were analysed by high-performance liquid chromatograph with a Rezex ROA-Organic Acid (Aminex HPX 87H) column. Biomass growth was monitored by measuring the O.D. (600 nm), using the gravimetric method (two samples of 5 mL were dried in a lyophilizer). For lipid extraction, cell destruction was performed with a high-pressure homogenizer on a 10 mL sample. Then, gravimetric lipid quantification was carried out using the Bligh-Dyer method[106]. After lyophilisation, the lipid were extracted using 5 mL of Folch solution. The sample was left to shake at room temperature and 200 rpm for 1,5 h. The chloroform layer was collected and the extraction was repeated other two times. For the fatty acid analysis, 2-5 mg of the extracted oil were collected. Lipids were directly converted into fatty acid methyl esters (FAME) by methanol transesterification according to the protocol of Griffiths et al.[105] FAMEs were analysed on a GC as described in section 3.2.8. Fatty acids were identified according to retention times of authentic standards (marine oil mixture, supelco mixture). Glyceril tridodecanoate and glyceril nonadecanoate were used as internal standard.

3.2.2.5 After-fermentation lipid extraction

The extraction process was conducted as described from Masri et al.[36]. The initial fermentation was carried out as described previously. Yeast growth was terminated by stopping aeration. At this point, the temperature of the fermenter was increased to 45°C, the pH was adjusted to 4.5. Cell lysis was

initiated by adding mix 1 (mannanase, Cellic Ctec2, Cellic Htec, and b-glucosidase). After 20 h of treatment, the reaction conditions were modified; the pH was adjusted to 7.0, and the temperature was changed to 37°C. Next, commercial protease preparation was added to break down cellular proteins and induce demulsification to assist with lipid release. Thereafter, biomass was subjected to centrifugation (9 000 g, 30 min), in which the upper layer fraction contained the released lipid in a surprisingly pure form. Subsequently the lower layer was removed and the upper layer was centrifuged (12 000 g, 30 min). The lipids were obtained as top layer.

3.2.3 Statistical analysis

Statistical analysis were conducted using excel operators. F-test was used to compare the variances of the two samples. Then a two tails t-test assuming equal or different variances was conducted, considering an α-value of 0,05.

4 Conclusion and further work

4.1 Genetic modification approach

In this study it was successfully demonstrated how to genetically modify *C. oleaginous* to enhance lipid production and obtain a tailor-made composition. The use of an optimized protocol based on an *agrobacterium* mediated transformation allowed the insertion of a heterologous cassettes to overexpress a diacylglycerol transferase (DGA1) and a Δ9-desaturase. The selection of the positive single colonies and their analysis in minimal nitrogen media allowed to assess the carried-out modifications. The overexpression of the desaturase gene led to several new FAs profile, suggesting a not trivial interactions between the elongase/desaturase process and the genetic modifications. However, most of the positive mutants showed an increase in palmitic acid (C16:0) and linoleic acid (C18:2), together with a decrease in stearic acid (C18:0) and oleic acid (C18:1), suggesting a higher desaturase activity. The insertion of the DGA1 instead, led to an increase in the accumulated lipid, with the highest reached value of 82,6%, compared to 72,6% of the wild type strain. Interestingly, the simultaneous overexpression of the diacylglycerol transferase and desaturase apparently suppress the desaturase activity. In fact, E2 and E4 were able to accumulate high quantity of lipid (E2 = 82,6 % and E4 72 h = 81%). Yet, they did not show significative differences in the FAs profile compared to the wild type. Similar observations are valid also for the mutants which expressed high differences in the FAs profile from the wild type. E11, E16 and E20 indeed, were able to overexpress the desaturase gene but apparently not the DGA1 gene. Furthermore, the mutants which showed the highest expression of the inserted desaturase appeared to have a compromised metabolism, building up less biomass. This suggested that the introduced genes were placed in region of the genome highly express and vital for the yeast metabolism, which is likely since *Agrobacterium* mediated transformation is known to cause random insertion of the exogenous plasmid inside the new genome[107] . Moreover, comparing the differences observed between the mutants and the wild type strain with data reported for other researches based on genetic modification, it appeared that the values obtained were more modest than the one reported in the literature. A previous research conducted from Gorner et al. based on the genetic modification of *C.oleaginosus* via ATMT, which used the same promoter used in this experiment (glyceraldehyde-3-phosphate dehydrogenase,GPD), showed that this promoter was actually suppressed in minimal N media[42]. They observed an increase in the target FAs of 4 times when using YPD, compared to minimal N media. Hence, further works to assess a better comprehension of the conducted modifications would require the selection of the best performing mutants and the fermentation of this new strain in nitrogen rich media, possibly scaling

up the process. The use of fed-batch fermentation on a 1 L-scale based on glucose and acetic acid could allow the simultaneous lipid and biomass production, permitting the monitoring of the modifications while avoiding the use of nutrient limiting media. Additionally, once the required analysis would confirm the efficacy of the overexpression of the DGA1, it would necessary to study the interaction between the simultaneous overexpression of the diacylglycerol transferase and the desaturase. To do so, the overexpression of the sole DGA1 could give the required information to assess if the contemporary insertion of the two genes leads to a negative synergy, as observed in this study. Moreover, the application of the widely studied push-pull strategy could result in a further increase in the lipid content. It was shown how the overexpression of both ACC1, responsible for the upstream triglycerides' pathway, and DGA1, responsible for the downstream triglycerides' pathway, led to a positive synergistic effect. In fact, the simultaneous overexpression of these two genes could allow to maintain a balance in the up-stream and down-stream pathway of the TGAs synthesis, avoiding any possible intermediate accumulation. Concluding, this study showed the efficacy of the modern genetic techniques in optimizing the microbial oil production, however several analysis and further improvements are required to produce oil sufficiently economical and environmentally sustainable to compete with plant-based oil.

4.2 Bioengineering approach

In this research it was demonstrated how the use of VFAs can influence the yeast growth and the composition of the accumulated TGAs. An initial experiment to assess the effects of the concentration of the acids on *C.oleaginous* revealed the importance of the ratio between the acetic acid and the secondary acid. Indeed, optimizing the concentration of A1, it was possible to obtain high lipid percentage ($g_{lipid}/g_{biomass}$) and yield (88% of accumulated lipid at 67 h and an overall yield of 32%). A subsequent analysis with several acids showed how different carbon sources can account for different growth and lipid accumulation rate. At a concentration of 5% (wt%), A2 appeared to be the most promising acid. In fact, its combination with acetic acid and glucose led to a lipid productivity of 1,06 g/Lh (30 h), with a lipid percentage ($g_{lipid}/g_{biomass}$) of 70% and a dried biomass of 90 g/L at 80 h. Yet, a deeper study about the influence of the concentration of the secondary acids on the yeast growth would be required. In fact, as already said, in the current research it was probably not used the best ratio between acetic acid and the secondary acid. The optimization of this parameter appeared to be essential to guarantee an efficient growth and lipid accumulation. Several researches reported how the acids' toxicity[67] and their theoretical growth yield coefficient[67] influence the yeast metabolism and growth. Hence, it would be necessary to conduct several fermentation maintaining

the secondary acid constant and changing the concentration. Moreover, it was confirmed that the carbon source can influence the composition of the accumulated lipid. A3 appeared to highly trigger the accumulation of long chain FAs. Also, our data supported the observations reported from Liu et al[67], which suggested that odd-numbered VFAs enhanced the accumulation of odd-numbered FAs. In fact, A2 and PA enhanced the production of C17:X and C19:0. However, the relation between the feeding with the secondary acid and the obtained FAs is not completely clear and further analysis would be required. Further, it was not possible to observe if the double bond inside the aliphatic chain can be directly inserted in the synthetized TGAs or if it is saturated before acting as building block for the lipid accumulation. Firstly, it would be necessary to use a FAs reference mixture that could guarantee the identification of the unknown peaks and also their quantification. Furthermore, it would be necessary to monitor the concentration of the secondary acid, in order to confirm the observations about their toxicity and to control the rate of uptake. Concluding, yeast fermentation fed with acetic acid and glucose confirmed to be a good strategy to trigger lipid accumulation while guaranteeing yeast growth, as previously showed from Masri et al[36]. The collected data supported the potential of oleaginous yeast for an economical and environmental sustainable lipid production, yet several parameters need to be studied to decrease the cost of manufacturing and to control the lipid composition.

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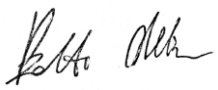

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