ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΤΡΟΦΙΜΩΝ ΚΑΙ ΔΙΑΤΡΟΦΗΣ ΤΟΥ ΑΝΘΡΩΠΟΥ
ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ ΤΡΟΦΙΜΩΝ

Μελέτη της παραγωγής λιπιδίων, πολυσακχαριδίων και λοιπών μεταβολικών προϊόντων βιοτεχνολογικού ενδιαφέροντας κατά την αύξηση ζυμών σε ανανεώσιμα υποστρώματα χαμηλού κόστους

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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ΑΘΗΝΑ

Δεκέμβριος 2014

AGRICULTURAL UNIVERSITY OF ATHENS
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Study of the production of lipids, polysaccharides and other metabolic compounds of biotechnological interest during growth of yeasts on low cost renewable substrates

PhD THESIS

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ATHENS
DECEMBER 2014

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ACKNOWLEDGEMENTS

My sincere and deep gratitude is given to my Savior.

My sincere thanks and appreciation are addressed to The State Scholarships Foundation (I.K.Y.), Athens – Greece, for the financial support of this scientific work.

My sincere and deep gratitude is given to my Thesis supervisor Dr. Seraphim Papanikolaou, who did not only guided me with valuable advices throughout the course of my research work, but also educated me to possess scientific mind of thinking and problem solving. Grateful acknowledgements are also attributed to Dr. Apostolis A. Koutinas and Dr. George Aggelis who contributed for the accomplishment of this Thesis.

I would like to thank Mrs Ourania Kalantzi for laboratory assistance and advices throughout the course of my research work and for have been emotional source as my biological mother during these four years.

I would like to thank Dr. Afroditi Chatzifragkou and Dr. Nikolaos Kopsahelis for laboratory assistance and in article writing. My acknowledgements and thanks are also extended to all the people in the Department of Food science and Human Nutrition, Laboratory of Food Microbiology and Technology that have help me in the way that they will never know.

I would like to thank my Beloved br. Theodore Andoseh who initiated this Thesis in 2010 (Yaounde-Cameroun).

My acknowledgements and thanks to my beautiful parents (Tchakouteu David and Wandji Jeanne), sisters and my brother for their appreciations and everlasting love throughout my life.

I would like to thank all my friends for their encouragements that give me the strength to move on.

I would like to thank my dearly Felix Ebane for his exceptional emotional support.

Sidoine Sadjeu

December 2014

ABSTRACT

The present investigation focused on the potential of several yeast strains to synthesize metabolic compounds of importance for the Industrial Biotechnology. The metabolic compounds the study of which interested the current investigation were principally microbial lipids (called also single cell oils – SCOs), microbial mass and microbial intra-cellular polysaccharides and to lesser extent citric acid, mannitol and enzymes. The carbon sources used from the yeast strains were composed of hydrophilic materials of low (or even negative) value, whereas kinetic and physiological studies related with the microbial growth of the yeast strains were performed and discussed.

In the first part of this study, it was desirable to study the potential of SCO and microbial mass production in some yeast strains growing in media containing glucose as sole carbon source. Firstly, a screening of 6 yeast strains (*Rhodosporidium toruloides* DSM 4444, *Rhodotorula glutinis* NRRL YB-252, *Rhodosporidium toruloides* NRRL Y-27012, *Yarrowia lipolytica* ACA YC 5033, *Lipomyces starkeyi* DSM 70296, *Cryptococcus curvatus* NRLL Y1511) growing on media containing an initial concentration of glucose (Glc_i) adjusted at *c.* 50 g/L under nitrogen-limited conditions in shake-flask experiments was performed. Thereafter, the most promising lipid-producing strain on media composed of glucose, namely *Rhodosporidium toruloides* DSM 4444, was cultivated in shake-flask and batch-bioreactor experiments in order to further demonstrate its capabilities towards SCO production. Initially, the effect of the addition of NaCl in several concentrations upon the production of biomass and SCO by the particular strain was investigated; batch-flask trials with increasing amounts of NaCl revealed the tolerance of the strain against NaCl content up to 6.0% (w/v). However, 4.0% (w/v) of NaCl was found to trigger oil accumulation for the particular strain, by enhancing lipid production yield up to 71.3% (w/w) per dry cell weight. The same amount of NaCl was employed in previously pasteurised batch-flask cultures in order to investigate the potential of performing the fermentation of SCO under non-aseptic conditions; indeed, the combination of NaCl and high glucose concentrations was found to result in very interesting SCO production *Rhodosporidium toruloides* cultures, with suppressed bacterial contamination of under these conditions having been observed. Aseptic batch-bioreactor trials of the yeast in the same media with high glucose content (up to 150 g/L) resulted in satisfactory substrate assimilation, with almost linear kinetic profile for lipid accumulation, regardless of the initial

glucose concentration imposed. Finally, fed-batch bioreactor cultures led to the production of 37.2 g/L of biomass, accompanied by 64.5% (w/w) of lipid yield.

After the successful growth and production of biomass and SCO by *Rhodosporidium toruloides* in glucose-based media containing significant initial quantities of NaCl, the next step was the application of growth of this microorganism on a salty wastewater supplemented with glucose. On the other hand, during the initial screening study on glucose, the employed strain *Yarrowia lipolytica* ACA YC 5033 had presented interesting production of citric acid, while literature suggested the potential of using strains of the species of *Yarrowia lipolytica* in salty media. Therefore, the second part in this chapter referred to the application of the ability of *Rhodosporidium toruloides* DSM 4444 and *Yarrowia lipolytica* ACA YC 5033 yeast strains to grow and produce useful compounds (SCO, yeast biomass, citric acid) in supplemented with glucose wastewaters contain high NaCl concentration like the table olive processing wastewaters (TOPWs), with the perspective of the (partial) replacement of tap water by this salty wastewater in fermentation processes. Therefore, *Rhodosporidium toruloides* DSM 4444 and *Yarrowia lipolytica* ACA YC 5033 were cultivated on TOPW-based media containing 3.0% (w/v) of NaCl enriched with several glucose concentrations (50, 100, 135 g/L) under different culture conditions. The maximum lipid content (9.3 g/L and 58.4% of lipid in dry cell weight – DCW) was achieved during the shake-flask fermentation of *Rhodosporidium toruloides* in nitrogen-limited TOPW-based media with 135 g/L of glucose. An increase up to 13.9 g/L and 73.9% w/w of lipid was achieved during the culture in 2-L bioreactor. Significant SCO production, comparable to the one achieved in aseptic cultures was equally performed in previously pasteurized media. In carbon-limited flask cultures, the highest biomass concentration achieved was 35.7 g/L, with biomass conversion yield per unit of sugar consumed ($Y_{X/Glc}$) being ≈0.36 g/g during cultivation of *Rhodosporidium toroloides* in TOPW-based media with glucose at 100 g/L. Furthermore, maximum biomass of 8.7 g/L with 14.3% w/w of lipid in DCW and citric acid produced up to 43.5 g/L (yield of citric acid per glucose consumed $Y_{Cit/Glc}$=0.44 g/g) were observed during the flask fermentation of *Yarrowia lipolitica* in TOPW-based media with glucose at 100 g/L under nitrogen-limited conditions. In carbon-limited media of *Yarrowia lipolytica* growing in TOPW-based media supplemented with glucose, almost 30 g/L of DCW were produced with $Y_{X/Glc}$≈0.33 g/g. Lipid in DCW and citric acid produced during fermentation in 2-L fermentor were up to 32.9%, w/w and *c.* 68 g/L respectively, whereas similarly with the trials performed

with *Rhodosporidium toruloides*, the efficiency of *Yarrowia lipolytica* growing in TOPW-based media enriched with glucose under non-aseptic conditions was similar to the aseptic exoeriment.

After the study of yeasts strain growing on substrates containing glucose as carbon source, in the next part of this thesid, we hav investigated the potential of biodiesel-derived waste glycerol conversion into metabolic compounds of added-value by yeast strains growing under nitrogen-limited conditions. After a first initial selection of yeast strains cultivated on biodiesel-derived waste glycerol utilized as carbon source under nitrogen-limited conditions (conditions that favor the accumulation of storage lipid by microorganisms) in flask experiments, two microbial species that presented the best performances on the production of lipid from crude glycerol, namely *Lipomyces starkeyi* (strain DSM 70296) and *Rhodosporidium toruloides* (strain NRRL Y-27012) were more profoundly studied concerning the above-mentioned aspect. Significant biomass and SCO production was reported even in cultures with high initial glycerol concentration (i.e. 180 g/L). Lipid quantities of *c.* 12 g/L (lipid in dry cell weight 35-40%), quite high values compared with the literature for oleaginous microorganisms growing on glycerol, were obtained for both *Lipomyces starkeyi* and *Rhodosporidium toruloides.* However, these strains presented presented different kinetic profiles regarding synthesis of intra-cellular polysaccharides (IPS). As far as *Rhodosporidium toruloides* was concerned, it has been seen that during the first growth phases of the microbial growth, and despite the nitrogen presence into the culture medium, significant quantities of intra-cellular polysaccharides were synthesized (at the first growth steps polysaccharides in DCW – $Y_{IPS/X}$≈40% w/w was observed). Thereafter, $Y_{IPS/X}$ values were depleted while in absolute quantities (g/L), after the significant biosynthesis that had occurred at the first growth steps, *c.* 50 h after inoculation and until the end of the culture polysaccharides' concentration remained practically constant, whereas simultaneously significant accumulation of storage lipids (rise of both absolute – g/L and relative values - % in DCW) was observed. On the other hand, *Lipomyces starkeyi* presented a different physiological profile since polysaccharides per unit of DCW remained practically constant with a value *c*. 30% w/w throughout the culture, while in absolute values (g/L), the concentration of polysaccharides constantly increased; *Lipomyces starkeyi* produced a significant quantity of polysaccharides of *c*. 7 g/L. Intra-cellular polysaccharides' production from crude glycerol, has been rarely reported for microorganisms growing on glycerol.

Moreover, very scarce number of reports has indicated the production of SCO by *Rhodosporidium toruloides* and *Lipomyces starkeyi* growing on glycerol. In this part of the thesis, therefore, we report that these yeasts are able to efficiently convert raw glycerol into SCO, while *Lipomyces starkeyi* also synthesizes intra-cellular polysaccharides in remarkable quantities.

In the last part of this investigation and after having performed experiments on glucose- or glycerol-based media, it was desirable to perform trials on media composed from either lactose or sucrose employed as the sole carbon source. The yeast species *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Rhodotorula glutinis* and *Lipomyces starkeyi* cannot easily consume either sucrose or lactose (or both of these carbon sources for wild strains of the species *Yarrowia lipolytica*). Therefore, in this last part of the investigation, the trials were performed exclusively with the strain *Cryptococcus curvatus* NRRL Y-1511, which has never previously been studied in relation with its potential of producing SCO and other metabolic compounds of biotechnological interest (e.g. intra-cellular polysaccharides, extra-cellular enzymes) during growth on these abundant carbon sources. When lactose or sucrose was employed as substrate under nitrogen-limited conditions, the yeast strain accumulated high quantities of IPS at the beginning of fermentation (up to 68% w/w), with IPS values progressively decreasing to 20%, w/w, at the end of the fermentation. Decrease in IPS content and consumption of extra-cellular lactose led to a subsequent rise in lipid accumulation, reaching 29.8% in dry cell weight (DCW) at 80 g/L of initial lactose concentration. Lactose was more favorable substrate for lipid production than sucrose. In nitrogen-excess conditions, IPS were produced in significant quantities despite the continuous presence of nitrogen into the medium. Growth on lactose was not followed by secretion of extra-cellular β-galactosidase. High quantities of extra-cellular invertase were observed during growth on sucrose. The composition of IPS was highly influenced by the sugar used as substrate. Cellular lipids contained mainly palmitic and to lesser extent linoleic and stearic acid. This part of the current thesis is the first report in the literature that demonstrates the interplay between the biosynthesis of intra-cellular total sugars and lipids synthesis for oleaginous yeast strains.

The yeast strains tested in this study represent promising agents for the successful production of metabolic compounds of significance for the Industrial Biotechnology. In several cases, production of metabolic compounds (like lipid in citric acid) were comparable

with the higher ones achieved in the international literature, while growth in agro-industrial wastes not previously employed in the literature (like TOPW-enriched media) or in low-cost carbon sources utilized as substrates was performed, in some cases under non-aseptic conditions. Other metabolic compounds of importance rarely studied during growth of yeast strains, like like microbial intra-cellular polysaccharides, were produced and in some cases in significant quantities.

I-INTRODUCTION

I.1. Oleaginous Microorganisms

I.1.1. Generalities

Oleaginous microorganisms, defined as the microorganisms that are capable of accumulating at least 20% of their biomass as lipid, have been initially studied at the end of World War II (Ratledge and Wynn, 1997). Oleaginicity is a relatively rare characteristic in the microbial world; less than 100 species of yeasts, mold and algae are considered to be oleaginous (Ratledge, 1988; 1997; 2006; Ratledge and Evans, 1989). Theses microorganisms, from the simplest class (bacteria) to the most complex one (fungi) have an important increasing economic and technological interest (Davies and Holdsworth, 1992). Although the production price of the microbial oils, the so-called single-cell oils (SCOs), is higher compared with the traditional utilization of common oils and fats due to the obligatory maintenance of aseptic conditions (Ratledge and Cohen, 2008), several alternative options for large-scale production of SCOs exist, since the price of various naturally occurring lipids and fats of the plant and animal kingdom can tremendously vary (from 0.3 to up to 100 US $ per kg; see: Ratledge and Wynn, 2002). Therefore identification of microorganisms capable of producing in increased quantities lipids with structure and composition similar to that of high-value fats, and subsequent large-scale production of these specialty lipids, can present an enormous financial interest (Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a).

A very large variety of substrates has been used as carbon sources for the oleaginous microorganisms such as analytical-grade or industrially-derived (therefore, of low-cost) sugars or sugar-enriched wastes or residues, polysaccharides, N-acetylglucosamine, hydrolysates of various products or by-products, vegetable oils, pure free fatty acids, fatty acid methyl-esters (FAMEs), fatty byproducts or wastes, n-alkanes, ethanol, glycerol, and organic acids (Ratledge, 1997; Ratledge, 1998; Angerbauer et al., 2008; Papanikolaou and Aggelis, 2009; Papanikolaou and Aggelis, 2010; Subramaniam et al., 2010; Wu et al., 2010).

The oleaginous microorganisms have been used in order to study the more complex aspects of lipid biochemistry. Oleaginous microorganisms produce and accumulate lipids under specific environmental conditions when glucose or similarly metabolized sompounds (e.g. polysaccharides) are used as substrates; in fact, exhaustion of an essential ingredient, and usually nitrogen, is a critical factor in order to initiate lipid accumulation, although mineral depletion (e.g. phosphorus, sulfate) has been shown to cause lipid accumulation in some

organisms (Ratledge, 1988; 1997). On the other hand, as a general remark it should be stressed that when growth is carried out on various hydrophobic substances utilized as substrates (the so-called ‘‘ex novo’’ lipid accumulation), the microbial lipid produced contains lower quantities of triacylflycerols (TAGs) compared with growth elaborated on sugar-based substrates (the so-called ‘‘de novo’’ lipid accumulation) (Koritala et al., 1987; Guo et al., 1999; Papanikolaou et al., 2001; Fakas et al., 2006; 2007; 2008). Additionally, the oil accumulated by the oleaginous fungi is more unsaturated than that of the yeasts (Ratledge and Wynn, 2002; Fakas et al., 2009a; Fakas et al., 2009b).

Non oleaginous organisms are not able to store lipids under any conditions (Botham and Ratledge, 1979; Ratledge and Wynn, 2002). Under nitrogen limiting conditions, non-oleaginous organisms cease cell proliferation and division and may store polysaccharides (Sutherland, 1999; Diamantopoulou et al., 2014), or secrete secondary metabolites of low molecular weight (e.g. citric acid, acetic acid, mannitol, etc) into the medium (Papanikolaou and Aggelis, 2010; 2011a; 2011b).

I.1.2. Oleaginous bacteria

There is a restricted number of oleaginous bacteria that exists, like *Nocardia* sp., *Rhodococcus* sp., *Mycobacterium.* sp. AK 19 and *Arthrobacter* sp. (Ratledge and Boulton, 1985) which can contain more than 500 mg of triacylglycerols per g of biomass (0.5 g per g of dry cell weight). Although *Arthrobacter* can accumulate more than 70% of lipid in dry cell weight, its accumulation rate 0.0025 g_{lip}/g biomass/h is incompatible for industrial production. In the marine environment, there are some other oleaginous bacteria like *Alteromonas* sp., *Shewanella* sp., *Flexibacter* sp. and *Vibrio* sp. (Ratledge and Wilkinson, 1988) which can accumulate large amounts of polyunsaturated fatty acids up to 0.06 g per g of dry cell weight (Table I.1.).

Table I. 1. Maximum total lipid profiles associated with fatty acids observed in different bacteria species (Ratledge and Boulton, 1985)

SPECIES	%lipid [g_{lip}/g_X]	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Arthrobacter AK 19	78	30	14	6	30	T	T
Rhodococcus ruber	19	35	14	5	35	T	T
Rhodococcus opacus	48	26	9	7	33	T	T
Noccardia corallina	13	34	T	15	42	T	T

Note: T=Trace

I.1.3.Oleaginous algae

The number of existing species of algae in the world is estimated between 200,000 and one million. Biodiesel productivity could reach 121,104 Kg/ha per year using a 70% of oil content in algae utilized as feedstock for biodiesel production (Table I.2).

Table I. 2. Oil yield of some biodiesel feedstocks. Source: http://journeytoforever.org/biodiesel_yield.html; Chisti (2007); Mata et al. (2009).

Crop	Oil yield (L/ha /year)	Biodiesel	Productivity	Oil yield (Kg/ha/year)	Biodiesel Productivity (Kg/ha/year)
Rapeseed	1190	862	Sunflower	952	946
Oil palm	5950	4747	Jatropha	1892	656
Corn	172	152	Microalgae[a]	58700	51927
SOYBEAN	446	562	Microalgae[b]	136900	121104

[a]Algae contain 30% oil (/wt) in biomass. [b]Algae contain 70% oil (/wt) in biomass

Oil from algae on 20-30 million acres of marginal land would replace the entire US supply of imported crude oil, leaving 450 million acres of fertile soil in the country entirely for food production (Um and Kim, 2009). An important reason for which microalgae are an attractive source for biodiesel production is that they can perform sequestration of carbon dioxide for growth (Pokoo-Aikins et al., 2009). It is evident that the above-mentioned phenomenon contributes to atmospheric CO_2 emission mitigation. On the other hand, lipids from photosynthetically-grown algae are complex materials that in most cases require hydrolysis to recover the fatty acids – whatever their use is envisaged (Ratledge, 2011).

Moreover, the requirement for making a satisfactory biodiesel is that the lipid produced should be rich in saturated and monounsaturated fatty acids, such as palmitate (16:0), stearate (18:0), and oleate (18:1). Unsaturated fatty acids, particularly the poly-unsaturated ones with three or more double bonds, that are very recently produced as storage lipid materials by oleaginous algae (Makri et al., 2010; Bellou et al., 2012), are undesirable as they readily auto-oxidize making the final biodiesel technically unsatisfactory as well as giving it an unpleasant smell (Ratledge, 2011). Almost all data on lipid productivity are derived from trials on photobioreactors (there is not any outdoor cultivation – it should also be noted that photobioreactors of whatever design are expensive) using constant temperature, constant CO_2 addition and often constant illumination, in order to increase the biomass production due to autotrophic conditions (Ratledge, 2011; Koutinas et al., 2014a; 2014b). Finally, the dark reaction (respiration and metabolism of stored oils) during the night is largely ignored, rendering even less suitable for large-scale biodiesel production in outdoor cultivations (Ratledge, 2011).

Looking for the microalgal strains with the combination of high oil content and a rapid growth rate is the start of biodiesel production. In the world, over 50,000 microalgae species are present in not only aquatic but also terrestrial environments, implying their widespread availability (Richmond, 2004). A limited number, about 4,000 species have been identified, which can be divided into several groups including *Cyanobacteria* (*Cyanophyceae*), green algae (*Chlorophyceae*), diatoms (*Bacillariophyceae*), yellow-green algae (*Xanthophyceae*), golden algae (*Chrysophyceae*), red algae (*Rhodophyceae*), brown algae (*Phaeophyceae*), dinoflagellates (*Dinophyceae*) and "picoplankton" (*Prasinophyceae* and *Eustigmatophyceae*) (Hu et al., 2008). Among these, diatoms and green algae are relatively abundant (Khan et al., 2009). Most common microalgae (*Botryococcus*, *Chlamydomonas*, *Chlorella*, *Dunaliella*, *Neochloris*, etc) have oil levels between 20 and 75% by weight of dry biomass (Table I.3).

In any case, as already previously indicated, photobioreactors of whatever design are too expensive to be used to produce a cheap product. Tubular photobioreactors, though, are used on a commercial scale but only to produce high-value products such as astaxanthin. Costs of such systems are probably in the region of US$ 40 per kg biomass: if an oleaginous alga were then grown similarly giving oil content of *c.* 50% this would mean that the cost per T of oil would be of 80,000 US $ (Ratledge, 2011). This compares to the current prices of

most plant oils of less than 900 US $/T and the price of crude petroleum oil of about US$500/ton; *c*. US$70/barrel).

Table I. 3. Lipid content of many microalgae species (Chisti, 2007; Mata et al., 2009; Sialve et al., 2009).

Microalgae species	Lipid content (% dry weight biomass)	Microalgae species	Lipid content (% dry weight biomass)
Ankistrodesmus sp.	24–31	*Monodus subterraneus*	16
Botryococcus braunii	25–75	*Monallanthus salina*	20–22
Chaetoceros muelleri	33	*Nannochloris sp.*	20–56
Chlamydomonas reinhardtii	21	*Nannochloropsis oculata.*	22–29
Chlorella emersonii	25–63	*Nannochloropsis sp.*	12–53
Chlorella minutissima	57	*Neochloris oleoabundans*	29–65
Chlorella protothecoides	14–57	*Pyrrosia laevis*	69.1
Chlorella sorokiniana	19–22	*Pavlova salina*	30
Chlorella sp.	10–48	*Prostanthera incisa*	62
Chlorella vulgaris	5–58	*Prymnesium parvum*	22-39
Crypthecodinium cohnii	20–51	*Pavlova lutheri*	35
Dunaliella salina	6–25	*Phaeodactylum tricornutum*	18–57
Dunaliella primolecta	23	*Scenedesmus obliquus*	11–55
Dunaliella tertiolecta	16–71	*Skeletonema costatum*	13–51
Dunaliella sp.	17–67	*Scenedesmus dimorphus*	16-40
Euglena gracilis	14-20	*Schizochytrium sp.*	50-77
Ellipsoidion sp.	27	*Thalassiosira pseudonana*	20
Haematococcus pluvialis	25.0	*Isochrysis galbana*	7–40
Isochrysis sp.	7–33	*Zitzschia sp.*	45-47

In conclusion, the interest of studying algae as oleaginous microorganisms is limited, due to the high cost of the culture in infertile reactors with controlled luminosity. In addition, the most important drawback for a commercial use of these lipids is that the sources which accumulate high amounts of cellular lipids contain little polyunsaturated fatty acid (Ratledge, 1994; 2011).

I.1.4. Oleaginous fungi

A significant number (more than 64) of fungal species able to accumulate more than 25% (in mass) of lipids, have been identified and characterized (Ratledge, 1994). Generally, lipids accumulated by fungi have higher unsaturation than those accumulated by yeasts. This is the raison why the studies of lipid accumulation by these microorganisms are mainly focused on the production of polyunsaturated fatty acids (Papanikolaou and Aggelis, 2011a; 2011b). The characteristics of some oleaginous fungi are shown in Table I.4.

Table I. 4. Cellular lipid content and fatty acid composition of some oleaginous fungi (Ratledge, 1994).

	Lipids (%, w/w)	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**others**
Species				Entomopthorales				
Entomopthora cronota	43	31	9	2	14	2	1[a]	12:0(40)
Entomopthora obscura	34	8	37	7	4	/	/	12:0(40)
Species				Mucurales				
Absidia corymbifera	27	1	24	7	46	8	10[a]	
Cunninghamelle japonica	60	/	16	14	48	14	8[a]	
Species				Ascomycetes				
Aspergillus terreurs	57	2	23	/	14	40	21[b]	
Pellicularia practicola	39	/	8	2	11	72	2[b]	9

a: γ-linolenic acid

b: α-linolenic acid

Oleaginous fungi, as potential source of polyunsaturated lipids, show considerable economic interest, which makes them very attractive and interesting studies.

I.1.5. Oleaginous yeasts

The organisms which can store more than 20% of lipid are considered as oleaginous. The oleaginous yeasts are able to synthesize and accumulate lipids up to 72% of the dry mass of the cell. *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus Trichosporon* and *Lipomyces* are essentially oleaginous genera (Ratledge and Tan, 1990). Amongst more than 600 species of yeasts, only 25 of them are known to be oleaginous. In general, yeast lipid synthesized by the de novo mechanism is composed of C16 and C18 fatty acids. Palmitic acid (C16:0) constitutes the 15-25% w/w of total lipids, whilst palmitoleic acid (C16:1) is, in

general, presented in percentages inferior than 5% w/w of total lipids (Ratledge, 1997; Papanikolaou and Aggelis, 2009; 2011b). Likewise, stearic acid (C18:0) is generally a minor component of the yeast lipid (5-8% w/w). Oleic acid (C18:1) is the principal fatty acid accumulated inside the yeast cells (amounts sometimes higher than 70% w/w), while linoleic (C18:2) is found in the second position (Papanikolaou and Aggelis, 2009). In general, the production of yeast lipids containing globally saturated microbial fatty acids, is considered to be the limiting step for the synthesis of microbial analogous of expensive exotic fats (e.g. cocoa-butter) (Ratledge, 1994; Papanikolaou and Aggelis, 2010). Various strategies have been performed in order to alleviate the above disadvantage. The fatty acid profiles of several oleaginous yeasts when de novo lipid accumulation is performed are depicted in Table I.5.

Table I.5. Fatty acid composition of lipid produced by various yeast strains growing on sugars substrates in culture conditions favoring the accumulation of microbial lipid.

Strain	Lipid (% w/w)	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Reference
Candida sp. 107[a)]	37.1	37	1	14	36	7	T.	Gill et al. 1977
Candida sp. 107	n.r.	28	n.r.	8	41	17	17	Davies, 1988
Candida sp.	40.3	23	13	3	54	5	2	Aggelis et al. 1996
Rhodotorula gracilis	41.0	21	T.	13	51	11	3	Choi et al. 1982
Candida curvata[b)]	29.1	36	T.	14	40	7	T.	Evans and Ratledge, 1983
Candida curvata[b)]	28.0	37	T.	10	44	6	T.	Evans and Ratledge, 1983
Apiotrichum curvarum[b)]	31.0	34	T.	10	43	7	2	Hassan et al. 1993
Cryptococcus curvatus[b)]	38.0	24	T.	10	46	9	6	Hassan et al. 1994
Cryptococcus curvatus[b)]	25.0	18	T.	16	50	16	T.	Meesters et al. 1996
Cryptococcus curvatus[b)]	50.0	31	-	22	42	1	n.r	Wu et al. 2010
Cruptococcus albidus	46.3	14	T.	9	53	18	2	Hansson and Dostalek, 1986
Cruptococcus albidus	n.r	20	n.r	11	59	6	6	Davies, 1988
Yarrowia lipolytica	43.2	15	2	11	47	21	3	Papanikolaou and Aggelis, 2002
Yarrowia lipolytica	30	7	12	11	9	57	11	André et al. 2009
Yarrowia lipolytica [a)]	22	3	13	17	6	55	7	Makri et al. 2010
Rhodosporidium toruloides	67.5	20	1	15	47	13	3	Li et al. 2007
Rhodosporidium toruloides	65.2	34	T.	13	48	1	T.	Hu et al. 2009
Rhodosporidium toruloides	62.1	26	2	2	62	62	3	Wu et al. 2010
Rhodosporidium toruloides	55	6	43	T.	16	35	2	Wu et al. 2011
Lipomyces starkeyi	68.0	56	2	14	26	T.	T.	Angerbauer et al. 2008
Lipomyces starkeyi	61.5	37	4	6	49	1	T.	Zhao et al. 2008
Rhodotorula mucilaginosa	48.6	22	2	9	55	11	T.	Zhao et al. 2010
Trichosporon capitatum	37.6	12	1	2	74	9	n.r	Wu et al. 2011
Rhodotorula sp.	22.0	22	1	7	56	12	n.r	Chatzifragkou et al. 2011
Candida oleophila	15.3	13	3	7	66	11	n.r	Chatzifragkou et al. 2011

T. <0.5% w/w; n.r.: not reported.

a) Representation of the neutral fraction of microbial lipids produced. b) *Cryptococcus curvatus* was formely *Candida curvata* and then *Apiotrichum curvatum*; thus these microorganisms in fact are the same species.

The biochemical and physiological level, lipid accumulation was mainly studied in yeast. Furthermore, the possibility to use these yeasts for food or feed, as well as for the production substitute's expensive fats, seems attractive.

I.2. The biochemistry of lipid accumulation and degradation in the oleaginous microorganisms

I.2.1. Substrates used

Lipid accumulation in the oleaginous microorganisms can be performed when hydrophilic (sugars, polysaccharides, glycerol, ethanol, etc) or hydrophobic (e.g. fats, oils, hydrocarbons, etc) substrates are used as carbon sources of the oleaginous microorganisms. In the first case, the de novo lipid accumulation process (through biosynthesis of acetyl-CoA and subsequent quasi-inverted β-oxidation process) is elaborated. In the second case, the ex novo lipid accumulation process is used. With the exception of cellulose (only in a scarce number of reported this source has been used as substrate by the oleaginous microorganism; see: Hui, 2010) and mainly of methanol a very high number of carbon sources have been considered as substrates for the de novo lipid biosynthesis from oleaginous microorganisms (Ratledge and Bouton, 1985).

Sugar-based media such as simple sugars (e.g. glucose and fructose), lactose, sucrose, whey, glucose enriched wastes, molasses, etc (Papanikolaou et al., 2004; Fakas et al., 2007; Fakas et al., 2008; Angerbauer et al., 2008; Chatzifragkou et al., 2010; Papanikolaou et al. 2010; Wu et al., 2011) have been used. Xylose-based media have been recently considered as substrates of noticeable importance due to the abundance of xylose as substrate, deriving after chemical hydrolysis of various lignocellulosic materials (Zhao et al., 2008; Fakas et al., 2009a,b; Huang et al., 2009; Pan et al., 2009; Zikou et al., 2013). In addition, the utilization of sugar-based substrates more complicated compared with glucose, such as polysaccharides (e.g. starch and pectin), has been studied. Although the above substrates are similarly metabolized, in some cases the results that have been achieved, in terms of both lipid and fatty acid composition of the SCO produced, presented notable differences (Papanikolaou et al., 2007). Moreover, in several cases sugar-based or sugar-enriched waste materials (e.g. olive-mill waste-waters containing in several cases increased quantities of glucose) have been used the last years as substrates favoring the de novo lipid accumulation process (Sarris et al., 2011).

Ethanol has been considered as a potential substrate for the de novo lipid biosynthesis of the oleaginous microorganisms by a number of investigators (Eroshin et al., 1983; Ratledge, 1988; Emelyanova, 1997), given that it is considered as a very proper one since no

residual carbon arises from its uses in fermentation processes (Ratledge, 1988). Taking into consideration that ethanol is by far the more reduced substrate by any other considered yet for the process of de novo lipid accumulation, the final stoichiometric balance for SCO synthesis from ethanol could result in a theoretical yield of 0.54 g of lipid per 1 g of ethanol consumed (Ratledge, 1988). Nevertheless such high conversion yields have never been achieved in the literature with the conversion threshold of ethanol into SCO being around 0.31 g/g (Eroshin and Krylova, 1983; Ratledge, 1988, Fakas et al., 2009b).

Glycerol, even though this substrate presents a slightly lower theoretical conversion yield compared with glucose, this carbon source is of substantial and increasing importance due to its appearance into the market volume in continuously growing quantities due to application into an industrial scale of the biodiesel production process. In general, the utilization of glycerol as a microbial substrate, refers mainly to the production of 1,3-propanediol by bacteria (Papanikolaou, 2009), whilst to the best of our knowledge, only the last years an increasing number of reports has been seen so far in which (biodiesel derived waste) glycerol has been utilized as substrate (or co-substrate) by microorganisms in order to produce SCO (Meesters et al., 1996; Papanikolaou and Aggelis, 2002b; Papanikolaou et al., 2003; Chi et al., 2007; Pyle et al., 2008; André et al., 2009, 2010; Liang et al., 2010a,b; Makri et al., 2010; Chatzifragkou et al., 2011a; Ethier et al., 2011; Saenge et al., 2011; Dedyukhina et al., 2012; 2014; Fontanille et al., 2012; Chang et al., 2013; Duarte et al., 2013a;b; Louhasakul and Cheirslip, 2013; Wensel et al., 2014).

Citric acid (Aggelis, 1996), acetic acid (Roux et al., 1995, Botha et al., 1997, Immelman et al., 1997), or other low-molecular weight organic acids (Fei et al., 2011) have been equally considered as substrates for SCO production. Specially, as far as acetic acid is concerned, it is considered as a remarkable pollutant, generated either in the process water of Uranium bleaching or as effluent issued from the Fischer-Tropsch reaction (Du Preez et al., 1997; Botha et al., 1997); the investigations, thus, concerning its biotransformation in SCO are very interesting in both economical and ecological terms.

As previously indicated, during the process of lipid accumulation from oleaginous microorganisms, employed substrates are divided into hydrophilic and hydrophobic ones (as will be described in the following tables). Lipid biosynthesis from sugars and related substrates is called de novo lipid accumulation and lipid accumulation from hydrophobic substrates is called ex novo lipid accumulation.

I.2.2. Biochemistry of de novo lipid accumulation

I.2.2.1. Biosynthesis of the cellular fatty acid

De novo accumulation of cellular lipids is a secondary anabolic activity, conducted after essential nutrient (usually nitrogen) depletion in the medium. Due to this exhaustion, the carbon flow is directed towards the accumulation of intracellular citric acid that is used as acetyl-CoA donor in the cytoplasm. Acetyl-CoA generates cellular fatty acids and subsequently triacylglycerols.

The net product of glycolysis is pyruvic acid, which passes through the mitochondrial membrane to the mitochondrion matrix. Pyruvate-dehydrogenase catalyzes the formation of acetyl-CoA from pyruvic acid, and acetyl-CoA either enters inside the Krebs cycle, or is transported again into the cytoplasm in order to enhance biosynthesis of cellular fatty acids (Davies, 1992; Ratledge, 1994; Ratledge, 1988; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a,b). Since the mitochondrial membrane is not permeable by acetyl-CoA, the transformation of this compound to acetyl-carnitine (catalyzed by carnitine-acyl-transferase). should be necessary for the transport of this unit into the cytosol. Though, the capital role carnitine-acyl-transferase is exactly the opposite, namely the transport of the acetyl-CoA, issued by β-oxidation, inside the mitochondrion matrix. Minimal amounts of acetylcarnitine may pass through the mitochondrion matrix in order to enter the cytosol, and this is the case of de novo lipid biosynthesis of the non-oleaginous microorganisms (Ratledge, 1994; Ratledge, 1997; Ratledge and Wynn, 2002). In the oleaginous microorganisms, acetyl-CoA that constitutes the precursor of intracellular biosynthesis of fatty acids derives from breakdown of citric acid that under some circumstances has been previously accumulated inside the mitochondria and then is transported into the cytosol (for reviews see: Ratledge, 1994;1998; Ratledge and Wynn, 2002; Fakas et al., 2009a; Papanikolaou and Aggelis 2009; 2011a).

The key step for lipid accumulation in the oleaginous microorganisms is the change of intracellular concentration of various metabolites, conducted after exhaustion of some nutrients into the culture medium (Ratledge, 1994; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2009, 2011a). In most of the performed studies, the essential nutrient the depletion of which induced the accumulation of reserve lipid is that of nitrogen, whereas the biochemistry of de novo lipid accumulation of lipid has been completely elucidated only

when extracellular nitrogen in the limiting factor of microbial growth (Davies, 1992, Ratledge, 1997; Papanikolaou and Aggelis, 2009; Fakas et al., 2009b; Papanikolaou and Aggelis, 2010; 2011a; 2011b). Nitrogen exhaustion provokes a rapid decrease of the concentration of intracellular AMP (adenosine monophosphate), since, by virtue of AMP-desaminase, the microorganism cleaves AMP in IMP (inosine monophosphate) and $NH4^+$ ions. The $NH4^+$ ions constitute a complementary nitrogen source, necessary for synthesis of cell material after the extracellular nitrogen limitation (Evans and Ratledge, 1985). The excessive decrease of intra-cellular AMP concentration alters the Krebs cycle function; NAD^+- (and in various cases also $NADP^+$-) isocitrate dehydrogenase, enzyme responsible for the transformation of isocitric to α-ketoglutaric acid, losses its activity, since it is allosterically activated by intracellular AMP (Boulton and Ratledge, 1981) Chen and Chang, 1996). Thus, iso-citric acid is accumulated inside the mitochondrion. This acid is found in equilibrium with citrate (reaction catalyzed by isocitrate acotinase).

When the intra-mitochondrial citric acid concentration reaches a critical value, citrate enters the cytoplasm in exchange with malate (Evans et al., 1983). Finally, citric acid is cleaved by the ATP-citrate lyase (ATP-CL), the enzyme-key of lipid accumulation process in the oil-bearing microorganisms, in acetyl-CoA and oxaloacetate (for reviews see: Ratledge 1988; 1994; Ratledge and Wynn, 2002). Acetyl-CoA, by a quasi-inverted β-oxidation process, will generate the cellular fatty acids (for reviews see: Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2009; 2011a; Fakas et al., 2009b).

NADPH, indispensable fatty acid biosynthesis, is provided by the intermediate cellular metabolism, in which the importance of malic enzyme has been considered as crucial for various oil-bearing microorganisms, specifically in the cases in which NADPH is produced exclusively by virtue of the reaction catalyzed by the above mentioned enzyme under nitrogen limited conditions (Wynn et al., 2001; Ratledge, 2002). Schematically, the intermediate cellular metabolism of the oleaginous microorganisms in which lipid accumulation is performed after nitrogen exhaustion from the medium, is presented in Fig. I.1.

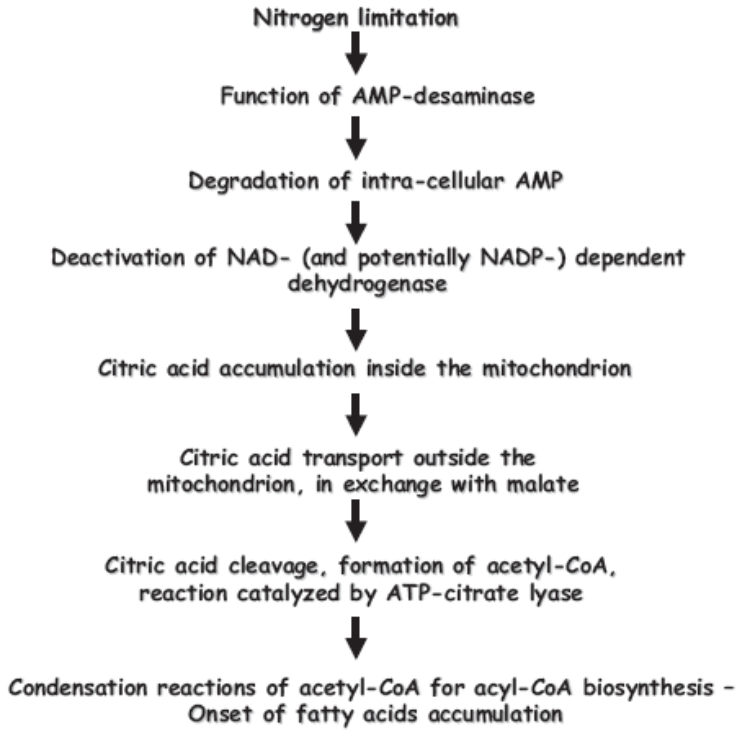
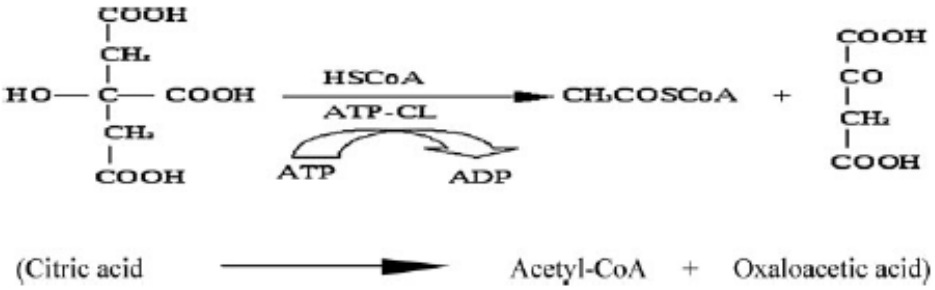


Figure I. 1. Consecutive steps leading to the de novo lipid biosynthesis in oleaginous microorganisms growing under nitrogen-limited conditions (Papanikolaou and Aggelis, 2011a).

ATP-CL is an enzymatic complex that is considered to be the most important factor to account for the oleaginicity of the various microorganisms, being absent in most of the non-oleaginous microbial cells (Ratledge, 1988). Net product of the action of ATP-CL, is acetyl-CoA, that will be further converted into intra-cellular fatty acids. If ATP-CL enzymatic complex does not exist, nitrogen exhaustion leads in the accumulation of citric acid inside the cytoplasm. In this case, citric acid either will be excreted into the culture medium (case of citric acid production by *Aspergillus niger* and *Candida* sp. strains (see: Ratledge, 1994; Papanikolaou and Aggelis, 2009) or will provoke the inhibition of the 6-phosphoro-fructokinase, having as result intracellular accumulation of polysaccharides (case of *Aureobasidium pullulans* - see: Galiotou-Panayotou et al., 1998). The effectuated reaction catalyzed by ATP-CL can be summarized as follows (Davies and Holdsworth, 1992; Fakas et al., 2009a,b; Papanikolaou and Aggelis, 2011a):



Due to the significant biochemical similarity between the intracellular de novo lipid accumulation and the extracellular secretion and production of citric acid, the last years, the yeasts have been divided and classified by various authors as either lipid-accumulating or citric acid-producing ones (Papanikolaou et al., 2002a; 2002b; 2006; 2008a; 2008b; 2009; Anastassiadis et al., 2002). The pattern of intermediate metabolism and de novo lipid biosynthesis is presented in Fig. I.2.

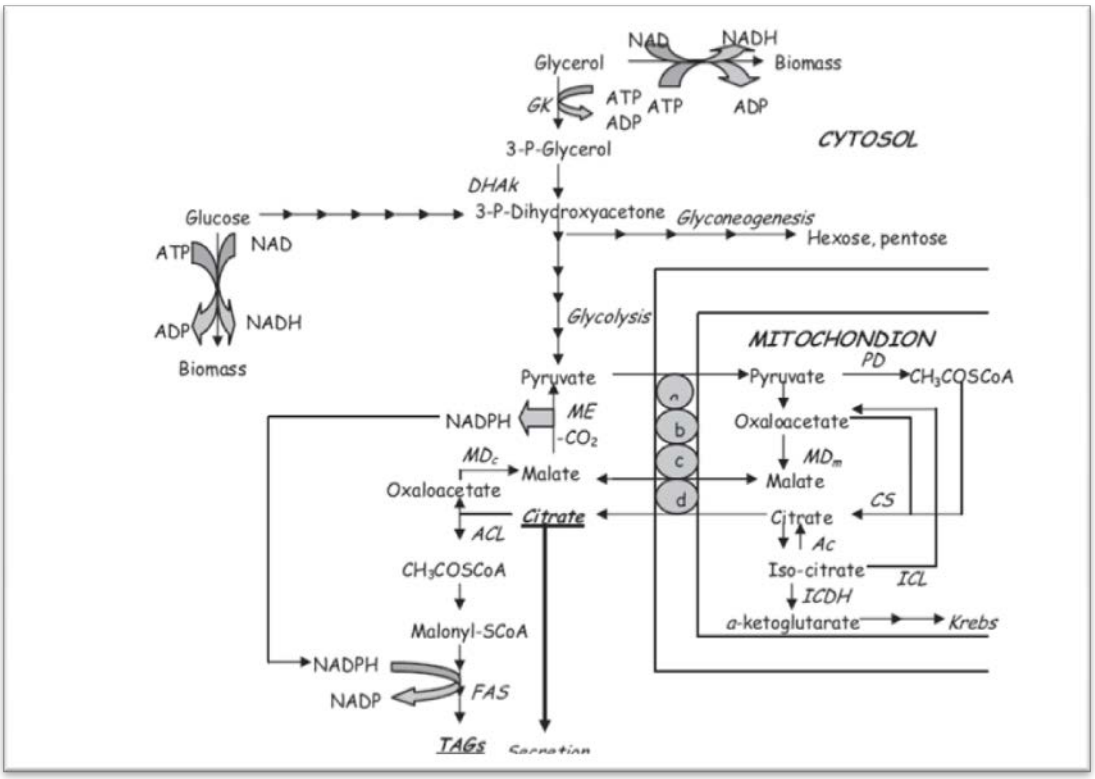


Figure I. 2. Intermediate metabolism in the oleaginous microorganisms.

(a–c) Systems of pyruvate transport from cytoplasm to mitochondrion and inversely for the malate. (d) System of citrate and malate transport between cytoplasm and mitochondrion. Enzymes:Ac, acotinase; ACC, acetyl-CoA carboxylase; ACL, ATP-citratelyase; FAS,fatty acid synthetase; ICDH, iso-citrate dehydrogenase; MDc, malate dehydrogenase (cytoplasmic); MDm, malate dehydrogenase(mitochondrial);

PD, pyruvate dehydrogenase; PFK, phospho-fructo-kinase; PK, pyruvate kinase (see:Ratledge , 1988; Papanikolaou and Aggelis, 2009, adapted).

I.2.2.2. Biosynthesis of fatty acid and triglycerides

The first reaction of fatty acid biosynthesis after acetyl-CoA generation is catalyzed by a biotin-dependent acetyl-CoA carboxylase (Ratledge, 1988). Its action is described as follows:

$$\text{Acetyl-CoA} + HCO_3 + \text{ATP} \longrightarrow \text{Malonyl-CoA} + \text{Pi}$$

This reaction is considered as the restricting step for fatty acid biosynthesis. It is activated by the presence of citric acid in theoleaginous strain *Candida sp*. 107, but not in the non-oleaginous strain *Candida utilis* (Gill et al., 1977). In fact, in the absence of citrate, the above mentioned enzyme exists as multi functional inactive protein with a molecular size of 2.4×10^5 daltons, while the presence of citrate induces enzyme monomers aggregation into an active macro-structural protein with asize of $7–9\times10^6$ daltons (Ratledge, 1988; Papanikolaou and Aggelis, 2011a). Following the generation of malonyl-CoA, the biosynthesis of fatty acids is performed with the aid of the multi-enzymatic complex of fatty acid synthetase (FAS). The effectuated reaction series can be summarized as follows (Davies, 1992; Fakas et al., 2009a; 2009b):

Acetyl-CoA + 7 malonyl-CoA + 14 NADPH $\longrightarrow$ Palmitoyl-CoA + 7 CO2 +14 NADP + 7 CoASH + 6 H2O

Generally, the multi-enzymatic complexes of FAS and ATP-CL are inhibited by the presence of exogenous long aliphatic chains (e.g. fatty acids, n-alkanes, etc) found into the culture medium (Alvarez et al., 1997; Meyer and Schweizer, 1976). However, the last years in investigations performed with the yeast *Yarrowia lipolytica* ACA-DC 50109 in submerged cultures, in which growth was supported by the simultaneous use of stearin (mixtures of free-fatty acids and mainly of C16:0 and C18:0) and industrial glycerol or glucose, extensive studies of the intra-cellular lipid profile of microbial lipid produced, suggested some de novo synthesis of intra-cellular fatty acids, in spite of the presence of long-chain fatty acids found into the culture medium (Papanikolaou et al., 2002a; 2003a; 2003b; 2006); when *Y. lipolytica* ACA-DC 50109 had been cultured on stearin utilized as the sole substrate, no dehydrogenation or elongation reactions were conducted in cellular level, while the microbial

lipid produced was almost completely saturated and was composed mainly of C18:0 fatty acid (Papanikolaou et al., 2002a; 2007). Given that growth of *Y. lipolytica* on glucose or glycerol used as the sole substrate is accompanied by synthesis of a lipid that is globally unsaturated (Papanikolaou et al., 2002a; 2006; 2008a; 2008b), enrichment of the reserve lipid with unsaturated fatty acids during growth on glucose/stearin or glycerol/stearin mixtures (principally C18:1 and C18:2) that occurred, indicates de novo fatty acid biosynthesis despite the presence of long aliphatic chains into the medium. Whatever the origin of the intracellular aliphatic chains, after the biosynthesis of acyl-CoA esters, an esterification with glycerol takes place in order for the lipids reserves to be stocked in the form of TAGs (Ratledge, 1988; Ratledge, 1994).

The steps of TAG assembly through the α-glycerol phosphate acylation pathway, a pathway that is very commonly used in the oleaginous microorganisms, are depicted in Fig.I.3.

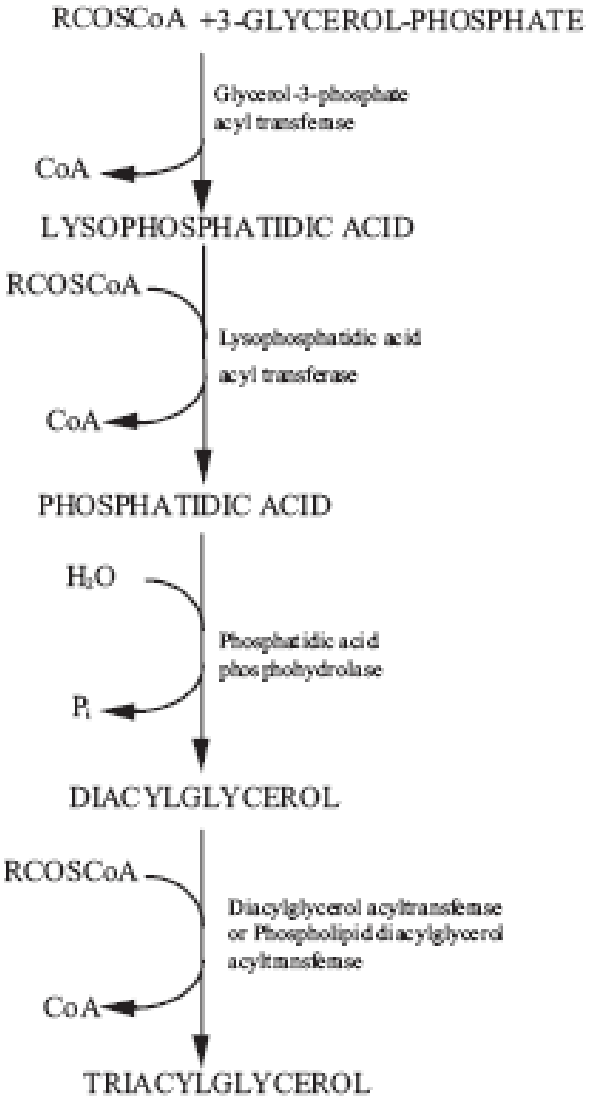


Figure I. 3. **Formation of intracellular triacylglycerols via the pathway of a-glycerol phosphate acylation (Papanikolaou and Aggelis, 2011a).**

I.2.3. Hydrophobic substrates: ex novo lipid biosynthesis

A somehow restricted number of yeast strains have been recorded to be capable of growing on fats and at the same time accumulate significant lipid quantities. These yeasts belong to the genera *Torulopsis* (*T. versatilis*, *Torulopsissp*.), *Candida* [*C. tropicalis, C. guilliermondii, Yarrowia (C.) lipolytica*], *Trichosporon*, *Geortichum* and to the species *Pichia methanolica, Apiotrichum curvatunand, Rhodosporidium toruloides* (Matsuo et al., 1981; Bati et al., 1984; Koritala et al, 1987; Guo et al., 1999; Papanikolaou et al., 2002a; Papanikolaou et al., 2009). It is evident that the number of microorganisms that are capable to consume soaps

and free-fatty acids is higher, since culture on these materials is done regardless of the lipolytic capacity of the microorganism used (Papanikolaou and Aggelis, 2010). In contrast, the microorganisms that are able to proceed with TAG or fatty-esters break-down, should obligatory possess an active lipase system into their enzymatic arsenal (Ratledge and Boulton, 1985; Aggelis et al., 1995a;1995b; Aggelis et al., 1997; Papanikolaou and Aggelis, 2010). As far as the yeast *Y. lipolytica* is concerned, in various reports in the past years it was considered as a non-oleaginous microorganism, since it had been assumed as ineffectual of accumulating significant lipid quantities from sugars or similarly metabolized compounds during submerged growth in nitrogen-limited media (Ratledge, 1994). However, the capacity of at least some *Y. lipolytica* strains to accumulate high lipid quantities (up to 60% w/w, in dry weight) when various fats or oils were used as sole carbon and energy source is out of question indicating the oleaginicity of this microorganism (Bati et al., 1984, Koritala et al, 1987; Aggelis et al., 1997; Papanikolaou et al., 2001; Papanikolaou et al., 2002a; Papanikolaou and Aggelis, 2003a; 2003b; Papanikolaou et al., 2007; Najjar et al., 2011).

The fatty materials utilized as substrate from the oleaginous strains may be vegetable oils (Bati et al., 1984; Glatz et al., 1984; Koritala et al., 1987; Aggelis et al., 1995a; 1995b; Aggelis et al., 1997), fatty esters (methyl-, ethyl-, butyl-, or vinyl-esters of fatty acids) (Matsuo et al., 1981), soap-stocks (Montet et al., 1985), pure free-fatty acids (Gierhart,1984; Mlickova et al., 2004a; 2004b), industrial fats composed of free-fatty acids of animal or vegetable origin (Papanikolaou et al., 2001; Papanikolaou et al., 2002a) and crude fish oils (Guo et al., 1999; Guo et al., 2000; Kinoshita and Ota, 2001). In some cases, n-alkanes can be used as carbon sources, provided that the microorganisms can oxidize these materials into fatty acids in order to be further catabolized (in most cases the alkane mono-oxygenase system is employed – see Fig. I.4.).

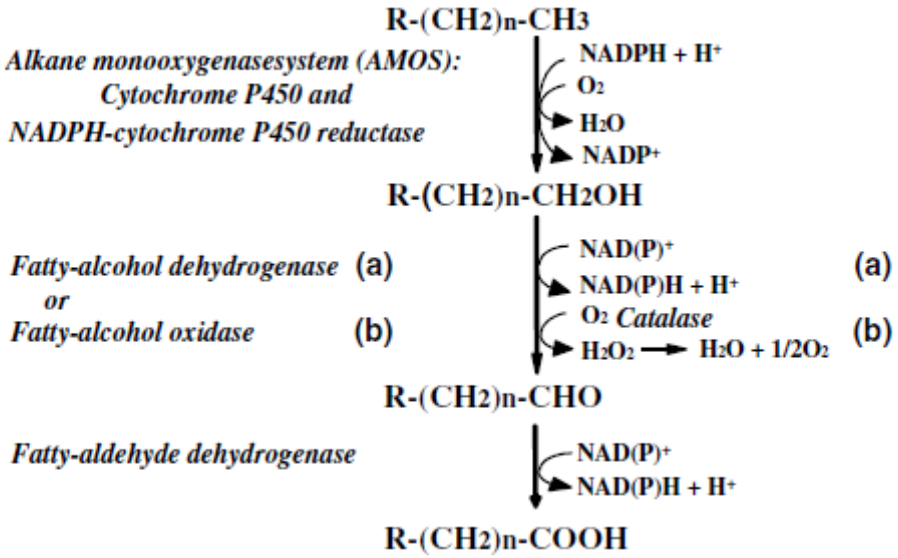


Figure I. 4. Primary or monoterminal alkane oxidation steps. Three enzymatic steps are involved in the bioconversion of n-alkanes to corresponding fatty acids of the same chain-lengths, catalysed by ER resident P450-dependent alkane monooxygenase systems (AMOS), consisting of P450 (ALK genes) as terminal oxidase and its electron transfer component NADPH-dependent P450 reductase to form the corresponding 1-alkanols, and subsequently by fatty-alcohol oxidizing enzymes, either by: (a) hydrogen peroxide-forming fatty-alcohol oxidases (FAOD) in peroxisomes, or: (b) NAD(P)+-dependent fatty alcohol dehydrogenases (FADH) in ER or cytosol, followed by fattyaldehyde dehydrogenases (FALDH) in peroxisomes, ER or cytosol (Fickers et al., 2005).

I.2.3.1.The biochemistry of ex novo lipid accumulation

The free-fatty acids (existed as initial substrate or produced after lipase-catalyzed hydrolysis of the TAGs/fatty esters or n-alkanes oxidation) are incorporated, with the aid of active transport, inside the microbial cell. The incorporated fatty acids will be dissimilated for growth needs [by virtue of the process of β-oxidation, into smaller chain acyl-CoAs and finally acetyl-CoA – reactions catalyzed by the various acyl-CoA oxidases (Aox)], or will be accumulated as storage materials, as they are or after having been subjected to enzyme-catalyzed modifications (e. g. desaturation or elongation reactions) (Papanikolaou et al., 2001; 2002a; 2007; Papanikolaou and Aggelis, 2003a; 2003b). The process of ex novo lipid accumulation is critically influenced by the dissimilation and accumulation rates of the various fatty acids previously incorporated inside the cells or mycelia, and obviously lipid accumulation is a dominant process when the rate of accumulation is significantly higher that the one of dissimilation for growth needs (Guo et al.,1999). The fate of the extra- and intra-

cellular fatty acids during growth of a yeast strain on a blend of fatty acids is depicted in Fig. I.5.

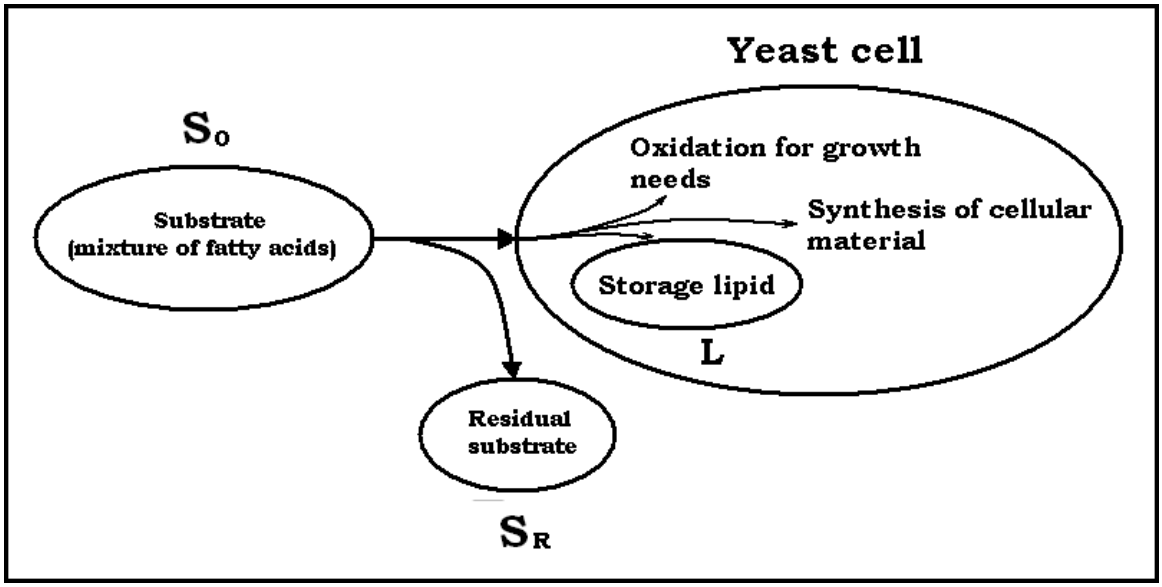


Figure I. 5. Fate of extra-cellular and intra-cellular fatty acids during growth of oleaginous yeasts on a fatty mixture (from Papanikolaou and Aggelis 2003a; 2010).

The dissimilated free-fatty acids will be degraded, by virtue of the process of β-oxidation, into smaller chain acyl-CoAs and acetyl-CoA, reactions catalyzed by the various acyl-CoA oxidases (Aox) (for reviews see: Fickers et al., 2005; Beopoulos et al., 2009), providing, thus, firstly the necessary energy for cell growth and maintenance (channel of acetyl-CoA inside the Krebs cycle), and secondly the formation of organic substances (intermediate metabolites) which constitute the precursors for the synthesis of cellular materials (Ratledge, 1997).

β-Oxidation contributes one mole of NADH and one mole of $FADH_2$ for every 1 mole of acetyl-CoA generated, before the entering of acetyl-CoA inside the Krebs cycle (Ratledge, 1994; Ratledge, 1997), and is depicted in Fig. I.6.

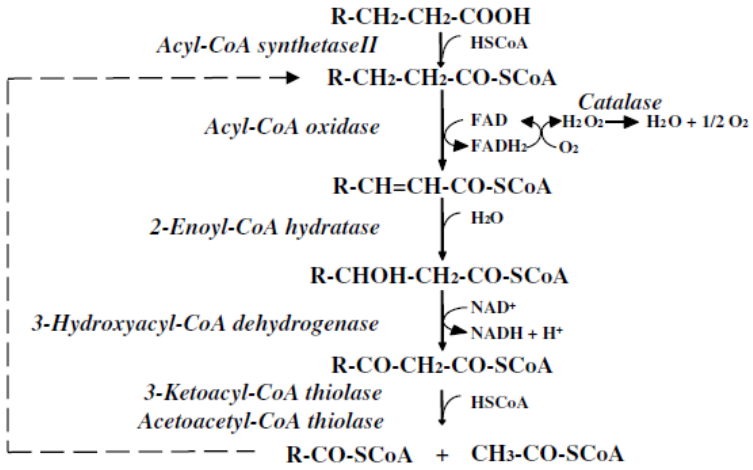


Figure I. 6. Oxidation process of a fatty acid with even number of carbon atoms (from Fickers et al., 2005).

Principal biochemical differences exist between de novo and ex novo lipid biosynthesis; in the later case, lipid accumulation occurs simultaneously with cell growth, being entirely independent from nitrogen exhaustion from the culture medium (Aggelis et al., 1995a; 1995b; Aggelis et al., 1997; Papanikolaou et al., 2001; Papanikolaou et al., 2002a; Papanikolaou et al., 2007; Aggelis and Sourdis, 1997). When fats or other hydrophobic materials are utilized as the sole carbon and energy source, accumulation of reserve lipid is a growth coupled process (Aggelis et al., 1995a; 1995b; Papanikolaou et al., 2001; Papanikolaou et al., 2002a) in which lipid is accumulated simultaneously with lipid-free material formation in the presence of assimilable nitrogen into the culture medium.

Finally, when the extracellular substrates concentration is low, the microorganisms degrade their own lipid reserves, in order to produced they energy that the need for their growth (Hodsworth et al., 1988; Aggelis et al., 1995a). In a series of investigations it has been demonstrated that the phenomenon of cellular lipid degradation in the oleaginous microorganisms was independent of the culture “pre-history” (meaning, in fact, independent of the type of the carbon source assimilated by the microorganism in order to proceed with reserve lipid accumulation), since such turnover has been observed even though SCO accumulation previously occurred through de novo (Fakas et al., 2007; Makri et al., 2010; Vamvakaki et al., 2010) or ex novo mechanism (Aggelis and Sourdis, 1997; Papanikolaou et al., 2001; 2002a; 2007; Papanikolaou and Aggelis, 2003a; 2003b; 2010). In any case, the released cellular fatty acids will be catabolized via the process of β-oxidation and the

produced acetyl-CoA will be further converted via the Krebs cycle and potentially the anaplerotic by-pass of glyoxylic acid (Papanikolaou and Aggelis, 2011a); in the case in which de novo lipid accumulation had been previously performed, during lipid turnover period, sugar (that could potentially exist into the culture medium), is no longer assimilated, while the function of Krebs cycle through the utilization of NAD^+- (and potentially $NADP^+$)-isocitrate dehydrogenase had already been suppressed due to extra-cellular nitrogen limitation (Papanikolaou et al., 2004). In general, the activity of glyoxylic acid by-pass enzymes (carnitine acetyl-transferase and iso-citrate lyase) increases considerably in cells growing on C_2 compounds (e.g. ethanol) or on substrates leading to C_2 unit's formation (i.e. previously accumulated TAGs, extra-cellular TAGs, hydrocarbons or free fatty acids), while this activity is indeed reduced during growth on glucose or other sugars (Papanikolaou and Aggelis, 2010; 2011a). Therefore, even though microbial growth had been performed on sugars or similarly metabolized compounds, SCO turnover should be principally performed through the glyoxylic acid by-pass pathway, while cellular nitrogen (obtainable via AMP-desaminase) should secure the biosynthesis of new lipid-free material. On the other hand, degradation of accumulated lipid when ex novo lipid accumulation had been previously performed is conducted indisputable principally through the glyoxylic acid anaplerotic by-pass pathway; in fact, reserve lipid turnover generally occurs when the extra-cellular flow rate of aliphatic chains is considerably decreased. This fact can be either due to the apparent absence of substrate fat from the culture medium (Aggelis and Sourdis, 1997; Papanikolaou et al., 2011) or due to the presence of fat that is substantially rich in the fatty acid C18:0 and cannot be easily catabolized by the yeast strains due to discrimination against this fatty acid (Papanikolaou et al., 2001; 2002a; 2007; Papanikolaou and Aggelis, 2003a; 2003b; 2010; 2011a).

The implicated schemas of lipid accumulation and degradation when hydrophobic substances are employed as substrates are illustrated in Fig. I.7.

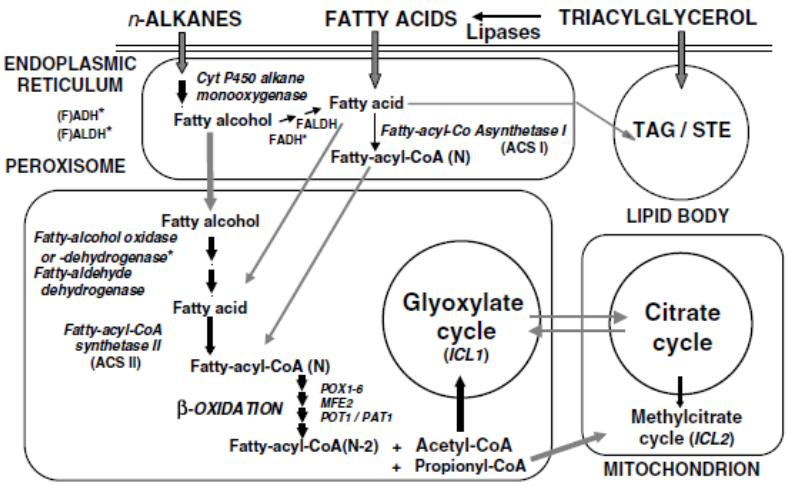


Figure I. 7. Main metabolic pathways and cellular departments implicated on hydrophobic substances (HS) degradation. HS (alkanes, fatty acids, trigacylglycerols – TAG) need to enter the cells via unknown uptake systems (grey arrows, indicating transport processes). Main metabolic flux during alkane oxidation is shown with bold black (enzymatic steps) or grey arrows. Alkanes are first oxidised by P450-dependent alkane monooxygenase systems (AMOS, ALK and CPR genes) in ER and further converted by fatty-alcohol-oxidising enzymes (fatty-alcohol oxidase, FAOD, or -dehydrogenases, FADH, fatty-aldehyde dehydrogenases), FALDH, in two steps into corresponding fatty acids in peroxisomes or in ER (black arrows). Fatty acids are activated either by fatty-acyl-CoA synthetase I (ACS I) in ER or by the peroxisomal fatty-acyl-CoA synthetase II (ACS II) prior to entry into the b-oxidation pathway (genes POX1-6, MFE2, POT1, PAT1). Formed acetyl-CoA, or propionyl-CoA, in case of odd-chain alkanes, enter the glyoxylate-cycle pathway (marker gene ICL1), located in peroxisomes, which interacts with the TCA and methylcitric-acid cycles (marker gene ICL2), located in mitochondria. Fatty acids could also be stored into lipid bodies as TAG or steryl esters (STE). Enzymes and genes are in italics, except enzyme abbreviations. *, FADH is probably absent in particulate fractions, but additional cytosolic (F)ADH and (F)ALDH activities are present (Fickers et al., 2005).

I.3. Technology and potential applications of single cell Oil

I.3.1. General presentation

Two major sectors of lipid biotechnology, the non-conventional biocatalysis and the production of lipid deriving from microbial sources (the SCOs) present a continuous expansion in the last years. In the former sector, hydrolytic enzymes (e.g. lipases or other esterases, glycosidases, etc.) in free and/or immobilized form are used in media presenting a feeble water concentration (organic or non-conventional media), in order to synthesize novel biomolecules of industrial, technological, and medical interest (for reviews see: Buchholz and Bornscheuer, 2005; Adlercreutz, 2008; Metzger and Bornscheuer, 2006; Böttcher et al., 2009). The second sector of lipid biotechnology refers to the production of SCOs. This sector is of particular interest due to the capacity of various microorganisms (principally yeasts, molds, and algae and to lesser extent bacteria) to synthesize lipids with specific structure and/or composition (Davies and Holdsworth, 1992; Ratledge, 1994; Moreton, 1988; Ratledge, 1988). The continuously increasing demand of the 1st generation biodiesel (FAMEs deriving from trans-esterification of principally plant oils) has increased the cost of various food-stuffs, and this situation has led to the necessity of discovery of non-conventional sources of oils, that could be subsequently converted into biodiesel.

The oleaginous microorganisms are considered as potential candidates for the production of this lipid that would result in the generation of the ‘‘2nd generation’’ biodiesel deriving from lipid produced by oleaginous microorganisms growing on wastes or agro-industrial residues like sewage sludge, hemicelluloses hydrolysates, waste glycerol, cheese whey, etc. (Luque et al., 2008; Papanikolaou and Aggelis, 2010), or the “3rd generation” biodiesel deriving from lipid produced by oleaginous micro-algae, with carbon being offered by atmospheric CO_2 sequestration (Chisti, 2007; Luque et al., 2008). In general, the oleaginous yeasts produce lipid containing unsaturated fatty acids similar to that found in common plant oils (Davies and Holdsworth, 1992; Ratledge and Wynn, 2002; Ratledge and Cohen, 2008; Papanikolaou and Aggelis, 2010). Although the production cost of the microbial lipids generally remains higher than that of the conventional vegetable oils (Ratledge and Cohen, 2008; Koutinas et al., 2014b), the production of yeast lipids with composition similarities with high added-value specialty fatty materials (like the cocoa butter or other exotic fats) has been considered as a process potentially economically viable (Moreton, 1988;

Ratledge,1994; Papanikolaou and Aggelis, 2010), specifically if various low or negative cost raw materials (e.g., whey, industrial-crude fatty acids, waste glycerol, xylose, etc.) are utilized as substrates (Ykema et al., 1989; Hassan et al., 1993; Papanikolaou et al., 2001). Yeast lipid presenting composition similarities with the cocoa butter has been produced in industrial scale at the end of 1980s (Davies, 1992). Furthermore, due to the last crisis in the production and price of the various comestible products, the cost of the various plant oils (e.g. rapeseed oil, soybean oil, etc) has considerably increased the last years (Ratledge and Wynn, 2002); this event has resulted in a non-negligible increase of conventional biodiesel production cost. It is evident that considering the production of yeast lipid that will be subsequently converted into biodiesel, only the process of de novo lipid accumulation is concerned, since cultivation in fatty materials is principally performed in order to add value into the fatty material converted, and, therefore, ex novo lipid accumulation is principally performed in order to produce specialty ‘‘tailor-made’’ lipids, like substitutes of cocoa butter of other high-added value exotic fats. In the next chapter the influence of cultivation parameters on yeast lipid accumulation will be discussed.

I.3.2. Factors influencing the accumulation of lipid by oleaginous microorganisms

In the process of de novo accumulation of storage lipid the concentration of the limiting nutrient (nitrogen) frequently determines the quantity of the biomass produced, whilst the concentration of the carbon source (e.g. glucose) found in excess in the growth environment largely determines the amount of accumulated lipid. Therefore, the molar ratio C/N plays a key-role in determining the oil content and biomass density of the oleaginous microorganisms (Davies and Holdsworth, 1992; Ratledge, 1994). The factors influencing the process of SCO production are briefly presented in the following paragraphs.

1) Generally, it is considered that the process of lipid accumulation is induced at molar ratio C/N >20. In some cases cultures in media in which very high initial C/N ratios were imposed (e.g. >70) resulted in decreased fat accumulation, suggesting that optimum initial C/N molar ratios are required for the conversion conducted (case of *Rhodosporidium toruloides* yeast – see Moreton (1988) In contrast, in other cases (e.g., culture of *Lipomyces starkeyi* on glucose), in trials performed with constant initial glucose and decreasing initial nitrogen concentrations, accumulation of storage lipid inside the cells constantly increased even though in some cases

high initial C/N ratios (around 150 moles/moles) were used, for the range of initial nitrogen concentrations tested (Angerbauer et al., 2008).

2) The nitrogen source has been also reported to be of importance for the process of de novo lipid accumulation in yeasts and molds; for instance, in the case of *Cryptococcus albidus* strain CBS 4715, addition of inorganic nitrogen sources (ammonium sulphate or ammonium chloride) favored the accumulation of lipid the yeast cells in comparison with organic nitrogen source (e.g. urea, L-arginine, etc) (Hansson et al., 1986). In contrast, in other cases addition of organic nitrogen sources into the medium has substantially increased the quantity of SCO produced (Evans and Ratledge, 1984a). In *Rhodosporidium toruloides* CBS 14 addition of asparagine instead of ammonium chloride increased the lipid content in dry yeast mass from 18 to 51%, w/w (Evans and Ratledge, 1984b) Further study of this strain in flask or bioreactor trials showed the same trend with a wide range of organic N sources used (Moreton, 1988).

3) The pH and the incubation temperature have been reported as factors that equally have importance in relation with the process of SCO accumulation. Specifically, the incubation temperature critically influenced lipid accumulation in the yeasts *Cryptococcus albidus*, *Apiotrichum curvatum*, *Rhodotorula minuta*, and *Yarrowia lipolytica* (Saxena et al., 1998; Hansson et al., 1986). For the case of *Yarrowia lipolytica* (ex novo lipid accumulation with low-cost saturated free-fatty acids used as substrate), temperatures of 19 and 39^{o}C did not allow high growth, whilst significant growth was observed within the range of 24–33^{o}C (maximum biomass concentration - X_{max} ranging between 7.5–8.7 g/L, μ_{max} 0.26/h). However, fat accumulation was favored only at T=28^{o}C (Papanikolaou et al., 2002a). Similar trend has been observed for *Rhodotorula minuta* (Saxena et al., 1998), while the optimum temperature for biomass and lipid production by *Cryptococcus albidus* was 20^{o}C (Hansson and Dosta´lek, 1986).

4) As far as the pH of the medium is concerned, for the case of *Yarrowia lipolytica* growing on stearin, medium pH was considered as crucial factor for SCO production, since cultures were performed in initial pH values of 5.0–7.0, and substantial growth was observed only at pH 6.0–6.5 whereas fat accumulation was favored only at pH=6.0 (Papanikolaou et al., 2002a). On the other hand, continuous cultures of *A. curvatum* growing on whey at constant D and variations of the pH of the medium resulted in significant and almost unaffected from the pH accumulation of fat for a pH range between 3.5 and 5.5 (Davies, 1988). Apparently

continuous fermentation at pH=3.5 provides considerable advantages since this operation can be feasible with a pasteurized whey feed in industrial scale (Davies, 1988).

5) It has been demonstrated that lipid accumulation was strongly depended on the dilution rate imposed (Brown et al., 1989; Gill et al., 1977) as well as of the molar ratio C/N of the growth medium (Ykema et al., 1986). D of less than 0.06/h were normally required for optimum conversions (Evans and Ratledge, 1983), since the microbial cells need to remain within the chemostat for at least 12–24 h in order to consume the available nitrogen and then convert the remaining sugar to oil and ‘‘fatten’’. Moreover, in a restricted number of reports fed-batch experiments have been performed, remarkably high biomass production (100 g/L) containing various lipid quantities has been observed (Li et al., 2007; Tsakona et al., 2014). Finally, concerning strains of the nonconventional yeast *Yarrowia lipolytica*, cultivation of this microorganism in nitrogen-limited glucose- (or glycerol-) based media resulted in equivocal results; in highly aerated batch bioreactors, moderate quantities of lipids were accumulated inside the yeast cells, whereas nitrogen limitation led also to citric acid biosynthesis (Makri et al., 2010). In contrast, in highly aerated chemostat cultures and moderate dilution rates imposed (e.g. D=0.04/h), lipids were produced in high quantities (e.g. >25% w/w) inside the yeast cells (Aggelis and Komaitis, 1999), whereas in at least one case, a strain of the above-mentioned species produced simultaneously SCO and citric acid in shake-flask nitrogen-limited cultures (André et al., 2009).

I.3.3. Production of cocoa butter substitutes by oleaginous yeasts

I.3.4.1. General presentation

One major industrial application referred to yeast lipid production is that of the synthesis of microbial substitutes of cocoa butter (Ratledge, 1994; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2010). Cocoa butter is commonly used in the food technology and principally in chocolate fabrication process, whereas it is also used in various cosmetology applications. It is principally produced in some of the African and Central American countries such as Ivory Coast, Nigeria, Jamaica, etc. This lipid is mainly composed of TAGs of the type P–O–S and S–O–S (P: palmitic acid, O: oleic acid, S: stearic acid). Oleic acid, hence, is always found esterified in the position sn-2 of glycerol.

Cocoa butter contains 55-67% w/w saturated fatty acid while its composition is dependent of the plant variety and the culture conditions. An average fatty acid profile of this fat is:

-Palmitate C16:0 23–30% w/w

-Oleate C18:1 30–37% w/w

-Stearate C18:0 32–37% w/w

-Linoleate C18:2 2–4% w/w (Ratledge, 1994; Papanikolaou and Aggelis, 2010). From the above-mentioned analysis, the total saturated fatty acid (TSFA) content of this lipid is of ~60-70% w/w, therefore, several strategies in order to produce lipids with enhanced TSFAs should be developed.

The production of cocoa butter substitutes (CBSs), economically viable during the years 1980-1990 (at that time the price of the cocoa butter was > 8.0 US $ per kg), is dependable of the price of this fat. During the years 1990–1994, a significant fall of cocoa butter price (<2.5 US $ per kg) has constituted an enormous disadvantage for the production of various substitutes of this fat (Ratledge et al., 1985; 1994; Ratledge and Wynn, 2002) However, the years after 2000, there has been increment again of its price, due to the prevalence of harmful insects and viruses that have been reported to create several problems on cocoa butter production (Ratledge and Wynn, 2002; Wu et al., 2010; Papanikolaou et al., 2001). Currently, the price of cocoa butter is around 5.0 US $ per Kg, though the tendency of this price, is to present (a remarkably high) increase in the near future (Papanikolaou and Aggelis, 2010). An ‘‘extreme’’ scenario recently presented in both the written and the electronic international press indicates that cocoa butter risks to disappear the next years due to general failure of the cultivation techniques of the cocoa plant, and, thus, the utilization and application of the various CBSs will be generalized for the food industry (Papanikolaou and Aggelis, 2011b). Several strategies have been applied in order to produce a SCO presenting compositional similarities with the cocoa-butter that will be presented below:

I.3.4.2. Utilization of mixtures of plant fats.

Numerous approaches have been conducted, in order to produce lipids having composition similarities with those of the cocoa butter. The first strategy performed referred to the preparation of mixtures of different fats of exotic plants (e.g. mango fat, kokum butter, sal fat) with fractions of palm oil (Lipp and Anklam, 1998; Smit et al., 1992; Kaphueakngam

et al., 2009) in order to create fatty materials with composition and technological properties relatively close to that of cocoa butter. It is noted however, that already the price of some of these exotic fats is remarkably high (Papanikolaou and Aggelis, 2010). In parallel, different biotechnological approaches, either enzymatic or fermentative ones have been already carried out for the production of CBSs (for reviews see: Lipp and Anklam 1998; Papanikolaou and Aggelis, 2010).

I.3.4.3. Utilization of various low-cost fatty materials

The last years, reactions of the above type have been ameliorated, optimized, and carried out in larger scale, with, principally, utilization of various low-cost fatty materials (e.g. lard, pomace olive oil, etc) as substrates in order to create various CBSs (Zhao et al., 2006; Ciftci et al., 2009). Numerous investigators have utilized oleaginous micro- organisms, and principally yeasts, that can be considered as ‘‘perfect’’ candidates for this purpose (Davies and Holdsworth, 1992; Papanikolaou and Aggelis, 2010). The fact that these microorganisms stock their lipids principally in the form of TAGs esterified in the sn-2 position by unsaturated fatty acids, has favored this approach (Ratledge, 1988; Ratledge, 1994). However, the main drawback to alleviate was how to increase the C18:0 and, generally, the TSFA content inside the yeast cells (Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2010), whilst in most instances the oleaginous yeasts accumulate unsaturated fatty acids to more than 65% w/w, in their total lipids. Many strategies concerning the increase of TSFA content in the yeasts have been realized so far and they will be presented in the next paragraphs.

I.3.4.4 Strategies dealing with the production of CBSs by oleaginous yeasts

The first of the approaches carried out in order to produce a microbial analogous of cocoa butter, consisted of a conventional culture of an oleaginous yeast (e.g. *Rhodosporidium toruloides*, *Lipomyces lipofer*, or *Rhodotorula graminis*) on glucose in nitrogen- limited media followed by a separation of the synthesized cellular lipid. The unit operation proposed was either crystallization fractional separation process in order to finally recover the fractions S–U–S (S: saturated fatty acid, U: unsaturated fatty acid) of the produced SCO (Tatsumi et al., 1979). Following this type of operation, TAGs of type P–O–P and P–O–S have been purified and utilized in the fabrication of chocolate, replacing cocoa butter. The result was

revealed satisfactory considering both fusion point and organoleptic properties of the product (Tatsumi et al., 1979). The inconvenient of this approach is the fact that only the 35% w/w, of the produced fat is presented in the form S–U–S (Davies and Holdsworth, 1992). Another strategy used in order to increase the C18:0 amount of the yeast lipids produced, was based on the principle that plants, animals, and microorganisms do not pro- duce their unsaturated fatty acids directly; firstly, a formation of a saturated precursor is performed, and then, by virtue of sequential desaturation reactions, double bonds are introduced firstly in the position Δ_9 and subsequently in the positions Δ_{12} and Δ_{15} (it is noted that the desaturation in the position Δ_{15} is unusual in the oil-bearing yeasts, whereas humans are incapable of de novo introducing double bonds after the 9th carbon in the aliphatic chain) (Ratledge and Wynn, 2002; Moreton, 1985). The acylated groups desaturation pathway is illustrated in Fig. I.8. (Ratledge, 1994).

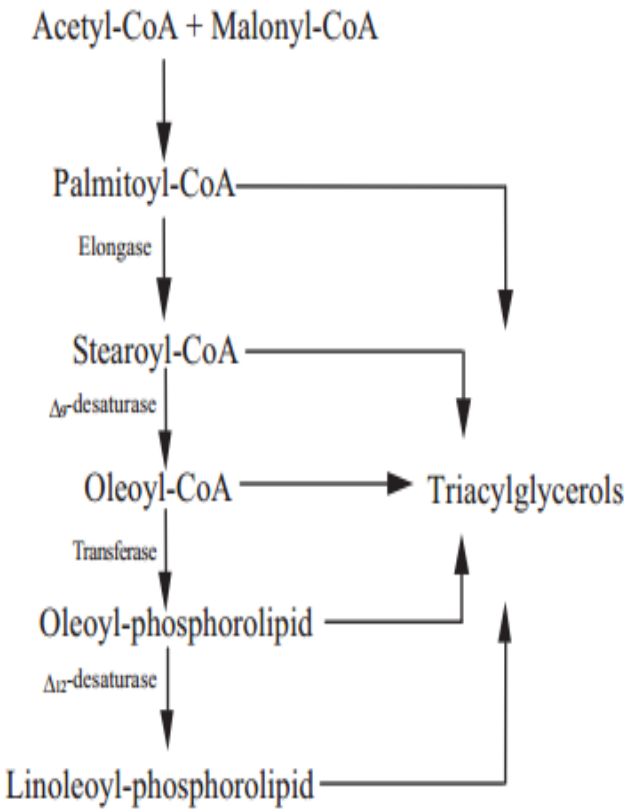


Figure I. 8. Pathways of desaturation of acylated groups in the oleaginous yeasts (from Ratledge, 1994, adapted).

Consequently the desaturation activity could be restricted using various desaturase inhibitors. Sterculic and malvalic acid are cyclopropene fatty acids found in the seed-oil of

different plants of the families Malvaceae and Sterculiaceae like sterculia oil and to lesser extent to kapok oil (Yano et al., 1972). Being fatty acids of 17 or 18 carbons with a cyclopropenic group in the position Δ_9, sterculic and malvalic acid have been reported to inhibit the desaturation activity in various plants and animals. In fact, these cyclopropene fatty acids are structural analogous of the natural substrates for the Δ_9 desaturase enzymes, which carry out the conversion: C18:0 ⟶ C18:1. These substances have been equally utilized in order to modify the accumulated lipid produced by the oleaginous yeasts (Moreton, 1988). Numerous strains have been tested (*Candida* sp. *107, Trichosporon cutaneum*, *Lipomyces starkeyi*, *Rhodosporidium toruloides,* etc). Although in some cases relatively significant quantities of the cyclopropenic inhibitor have been added into the culture medium (e.g. up to 4.0 mL/L of sterculia oil, that indicates presence of more than 2.0 g/L of cyclopropene fatty acids), cell growth and lipid accumulation have not been altered (total biomass always ranging between 6.4 and 12.3 g/L containing fat >25% w/w, inside the yeast cells) (Moreton, 1985). The addition of sterculia oil (containing around 50% w/w, of sterculic acid) into the culture medium resulted in the synthesis of a yeast lipid in which the concentration of the fatty acid C18:0 increased from 3–5% up to 40% w/w (Moreton, 1988).

The utilization of desaturase inhibitors remarkably increases the saturation of the SCO produced, having as a result the synthesis of lipids presenting composition similarities with the cocoa butter. However, this strategy presents two fundamental drawbacks, namely the increased coast of the inhibitors used and the fact that these products may provoke mutagenesis and cancerogenesis (Ratledge, 1994). As far as the second disadvantage is concerned, this is of major importance, taking into consideration the current trends of biotechnology, in which eco-friendly and ‘‘healthy’’ approaches should be considered in order for the mass production of biotechnological products (Papanikolaou and Aggelis, 2010). By taking into consideration that addition of other compounds could potentially interfere with lipid desaturation (Moreton, 1988), in order to alleviate this previously mentioned serious disadvantage, addition into the culture medium of other natural compounds that are completely non-toxic could potentially be envisaged.

I.3.4.5. Use of genetically modified strains

Genetic manipulation strategies based on the destruction of the gene encoding for Δ_9 dehydrogenase, which is the responsible enzyme for the biotransformation of C18:0 into

C18:1, have also been performed (Davies and Holdsworth, 1992; Hassan et al., 1993;1994; 1995). In most of the cases Δ_9-defective mutants of *Apiotrichum curvatum* have been constructed; cellular suspensions of the wild strain were treated with various mutagenic factors like N-methyl-N-nitro-N nitrosoguanidine (MNNG), ethyl-methanesulfonate or UV irradiation (Ykema et al., 1990; Smit et al, 1992; Hassan et al. 1993; 1994; 1995), and then have been grown in a medium supplementary in oleic acid.

The colonies were transferred in a medium without supplementary oleic acid. In general, the formed colonies in the two types of substrates have been screened extensively, and small numbers of colonies of auxotroph mutants in the unsaturated fatty acids (Ufa mutants) have been obtained (Ykema et al., 1990; Smit et al, 1992), since also fatty acid synthetase (FAS) mutants have been created (Ykema et al., 1989; Hassan et al., 1994). In most of the cases, the addition of a C18:1 donor (even in small concentrations) is obligatory, since the Ufa mutants may not be capable at all of synthesizing the above-mentioned fatty acid. The cellular lipids isolated from the Ufa mutants grown in a medium supplementary in oleic acid, have been analyzed and compared with these of the wild strain *A. curvatum*. A correct use of this fatty acid with a sugar as co-substrate, results in the synthesis of a cellular CBS by the various Ufa mutants (Smit et al., 1992).

It is evident that the genetic manipulation of the oleaginous strains has presented very satisfactory results considering both quantity of accumulated fats and cellular lipid composition, since the cellular stearic acid amount increases significantly. However, not any process scale-up has been performed, while, in addition, the wild cultures present higher productivities compared with the mutants.

I.3.4.6. Cultures in conditions of limited oxygen

In order to obtain by a metabolic manipulation what the genetic engineers have realized with the aid of mutations, Davies et al. (1990) have performed cultures of *Apiotrichum curvatum* in conditions of limited oxygen tension during the phase of lipid accumulation. The idea was based on the fact that all of the reactions of desaturation are oxygen dependent (Davies and Holdsworth, 1992). A critical limitation of the oxygenation in the fermentation medium could, hence, decrease the conversion percentage of the reaction of C18:0 into C18:1. In the performed trials, although the percentage of the fatty acid C18:0 into the reserve lipids was not very high, the sum of saturated fatty acids (C16:0, C18:0, C20:0, and C24:0) produced by

Apiotrichum curvatum was in most of the cases higher than 50% w/w, of total lipids. This fact represents, indeed, an interesting result in a double coincidence; firstly because it has been demonstrated with a completely eco-friendly method that the oleaginous yeasts have in some circumstances the tendency to accumulate lipids containing significant quantities of saturated fatty acids. The second reason is that not any expensive or dangerous method was applied (Ratledge, 1994). Oxygen tension decrease had almost not any negative effect upon cell growth (0.4 g of cells were formed per 1 g of lactose consumed) and lipid accumulation (around 37% w/w, of lipids in dry weight were synthesized). In addition, this approach dealing with the production of CBSs by oleaginous yeasts is the only one which has been extrapolated in semi-industrial (500-L bubble column reactors; Davies et al. 1990) and finally industrial (13-m^3 reactors; Davies et al. 1992) scale.

I.3.4. Production of "2nd generation biodiesel" by oleaginous yeasts

I.3.4.1. Generalities

Current industrialization and decrease of petroleum stock have raised the worldwide need for energy generation deriving from various alternative and renewable resources (e.g. biodiesel, biohydrogen, and/or bioethanol) with biodiesel being considered as one of the most important renewable energy sources due to its economic and environmental benefits (Alper and Stephanopoulos, 2009). From 2004 to 2008, the production of biodiesel increased almost four-fold in European Union, the world's biggest biodiesel producer, rising from 1.9 million metric tons in 2004 to 7.7 million metric tons in 2008 (Fig. I.9) (Bozbas, 2008). Thus, the global market for biodiesel is expected for an exponential growth in the coming decade. It is predicted that global biodiesel production would in-crease significantly from 11.1 million metric tons in 2008 to approximately 121million metric tons in 2016. Therefore, the necessity of discovery of novel (non-conventional) sources of oils, which could be subsequently converted into biodiesel is of crucial importance, with the oleaginous microorganisms being considered as potential candidates for the production of this "2nd generation biodiesel" (Li et al., 2007).

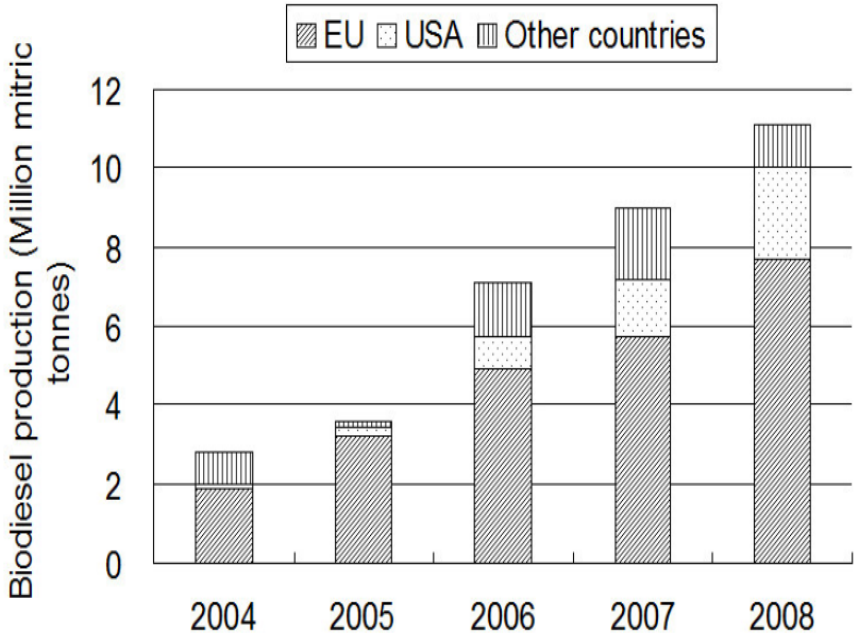


Figure I. 9. The global production of biodiesel from 2004 to 2008. Sources: Biodiesel board (http://www.biodiesel.org), European Biodiesel Board (Http://www.ebb-eu.org) and Emerging Markets Online (http://www.emerging-markets.com/biodiesel/).

Biodiesel has many advantages, such as inherent lubricity, superior flash point, biodegradability, and negligible sulfur content. It is composed of monoalkyl esters with long-chain fatty acids, which can be derived from oil resources in the form of triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), and free fatty acid (FA). The chemical process for biodiesel production is known as transesterification (Fig. I.10), which involves a reaction that replaces the glycerol group of TAG (or DAG, MAG) with a short-chain monohydric alcohol. Conventionally, vegetable oils such as palm oil, soybean oil, rapeseed oil, and sunflower oil have been explored as the feedstock for biodiesel production in many countries. However, vegetable oils are relatively expensive. In addition, being a common food staple, vegetable oils may be subject to shortage if a significant amount of them are dedicated to biodiesel production. Eventually, their supply may not meet the demand as a feedstock for the ever-growing biodiesel industry. Therefore, the exploration of non-vegetable oil production becomes imminently necessary.

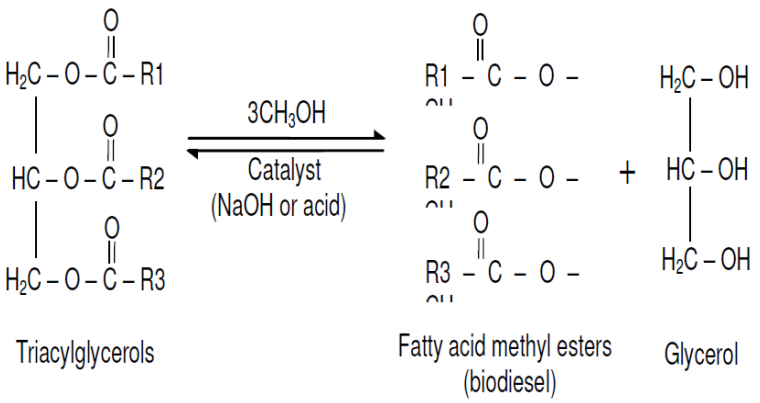


Figure I. 10. **Biodiesel production via transesterification of triacylglycerols (Deng et al., 2009).**

The oleaginous yeasts, with their unicellular form and their higher specific growth rate compared with molds and algae, can be considered as appropriate organisms that could be used for the production of this 2nd generation biodiesel (Papanikolaou and Aggelis, 2010). Biomass and lipid contains for several strains growing in different carbon source for microbial oil production (Xu et al., 2012 modified) is described in table I.6). Microbial oil production by microalgae have been already well discuted in I.1.3. It is important to evaluate the fatty acid compositions of microbial oil in carbon chain length and unsaturation degree. A summary of fatty acid composition of microbial oil derived from various oleaginous microorganisms with representative carbon sources used had been provided in Table I.5 (Papanikolaou and Aggelis, 2011a), with the major fatty acid compositions are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), which is similar to that of vegetable oils. It is also noticed that the fatty acid profile is species dependent, and also depends from the carbon source used, the fermentation time and the incubation temperature.

Table I.6. Biomass and Lipid contains for several strains growing in different carbon source for microbial oil production (Xu et al., 2012 modified).

Species	Carbon source	Biomass (g/L)	Lipid (%, w/w)	Cultivation type	Reference
Conversion of saccharides and energy crops					
Cryptococcus curvatus	Glucose	104.1	82.7	Fed-batch	Zhang et al., 2011
Thamnidium curvatus	Glucose	9.2	64.1	Batch	Papanikolaou et al., 2010
Thamnidium elegans	Fructose	10.1	65.9	Batch	Papanikolaou et al., 2010
Thamnidium elegans	Sucrose	9.6	64.6	Batch	Papanikolaou et al., 2010
Epicoccum purpurascens	Sucrose	15.5	80	A	Koutb & Morsy, 2011
Candida curvatus	Xylose	15.8	26.55	Batch	Evans & Ratledge 1983
Chlorella protothecoides	Cassava starch	15.8	26.5	Batch	Wei et al., 2009
Conversion of C_2 compounds					
Crypthecodinium cohnii	Acetic acid	109	56.0	Fed-batch	De Swaaf et al., 2003
Cryptococcus albidus	Acetic acid	2.85	25.8	Batch	Fei et al., 2011
Cryptococcus albidus	Sodium acetate	2.79	24.5	Batch	Fei et al., 2011
Candida curvata	Ethanol	8.5	30.1	Batch	Evans & Ratled, 1983
Chlorella vulgaris	Sodium acetate	≈4.4	≈19	Batch	Heredia-Arroyo et al., 2011
Conversion of lignocellulosic sugars					
Cryptococcus curvatus	Wheat straw	Batch	17.2	Batch	Yu et al., 2011
Yarrowia lipolytica	B	11.42	58.5	Batch	Tsigie et al., 2011
Mortierella isabellina	Rice hulls	5.6	64.3	Batch	Economou et al., 2011
Mortierella isabellina	Corn fiber	18.2	45.7	Batch	Xing et al., 2010
Aspergillus oryzae	Wheat straw	NA	C	D	Lin et al., 2010
Conversion of glycerol					
Cryptococcus curvatus	Pure glycerol	118	25	Fed-batch	Meesters et al., 1996
Cryptococcus curvatus	Crude glycerol	32.9	52.9	Fed-batch	Liang et al., 2010
Rhodotorula glutinis	Crude glycerol	10.5	60.7	Fed-batch	Saenge et al., 2011
Yarrowia lipolytica	Crude glycerol	8.1	43.2	Continuous	Papanikolaou & Aggelis, 2002
Mortierella isabellina	Crude glycerol	6.2	53.2	Batch	Papanikolaou & Aggelis, 2002
Schizochytrium limacinum	Crude glycerol	13.3	73.3	Batch	Liang et al., 2010

Note: NA = not available. A= Light submerged culture, B= *Sugarcane bagasse*, D= Solid-state fermentation, C=36.6 mg/g dry substrate

Despite the potential difficulties for the conversion several carbon sources into microbial oil (summarized in Table I.7 – see: Xu et al. 2012), converting industrial by-products and wastes containing plenty of organic carbons into lipid is a sustainable option for recycling and conserving resources. Glycerol is a good example fortifying the above-mentioned example; as described previously, glycerol can be utilized by most oleaginous microorganisms with similar lipid yield to that of saccharides (Ratledge, 1988; Papanikolaou and Aggelis, 2011a). With the fast growing of biodiesel industries, it will be largely generated as a by-product of the transesterification process from the local plants, and thus is a promising carbon source for microbial oil production. Besides, other organic wastes, locally obtained from agriculture, fishing, or manufacturing industries, can also be useful carbon sources. However, compared with the biodiesel by-product glycerol, their complicated compositions, especially some hard-to-degrade pollutants or toxics, continue to be a challenge (Xu et al., 2012).

Table I. 7. **Prospects microbial oil for sustainable carbon sources (Xu et al., 2012).**

Carbon source	**Advantages**	**Disadvantages, difficulties and future efforts needed**
Carbon dioxide	Reduction of CO_2 emission; low cost	Low efficiency in CO_2 utilization and conversion into microbial lipids; energy input for CO_2 supply; Smart bioreactor design and microorganism development are needed
Conventional saccharides (glucose, etc)	Effective to cell growth and lipid accumulation; utilization of energy crops at marginal lands can be considered	High cost; competition with food when starch or other compestible sugars are used as feedstocks
C_2 compounds	Available from a variety of biodegradable organic wastes; effective conversion was demonstrated in some oleaginous micro-organisms	Further study is needed for comprehensive demonstration of their potential applications
Lignocellulosic biomass	Abundant and cheap organic sources	Compositional complexity and heterogeneity; Pretreatment leads to inhibitive chemicals; Co-utilization of sugar components, and comprehensive utilization of the lingo cellulosic biomass are expected
Industrial by-products and other organic wastes	Abundant and available from industries; low cost	Especially for organic wastes, the compositional complexity, which is dependent on the industrial process, may lead to difficulties in microbial degradation and lipid production

Although the overall economic feasibility needs to be evaluated further, with the advances in genetic engineering, protein engineering, metabolic engineering, and process improvement, microbial oil from various sustainable carbon sources will become one of the feedstocks for biodiesel production in the future.

I.3.5. Biotechnological production of citric acid, polyols and polysaccharides

I.3.5.1. Citric acid

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid), is an intermediate organic compound (a six-carbon tricarboxylic acid – Fig. I.11) located into the tricarboxylic acid (TCA) cycle, that is employed in all living cells in the biological process in which carbohydrates are oxidized into carbon dioxide.

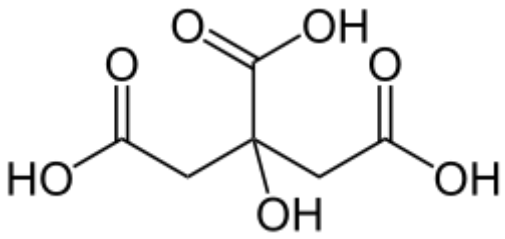


Figure I. 11. **Molecular structure of citric acid (http://en.wikipedia.org/wiki/Citric_acid).**

This organic acid is found naturally in citrus fruits (such as lemon, orange), pineapples, plums and pears, in the seeds of different vegetables, in animal bone, muscle, and blood and crystallized as calcium citrate (Najafpour, 2006; Roukas, 2006). Citric acid is a commodity chemical produced and consumed throughout the world with over 1.6 million tones production in 2007 (Berovic and Legisa, 2007), with China accounting for 35-40% of its worldwide production (Soccol et al., 2006). Citric acid is produced through (principally) submerged fermentations by the mold *Aspergillus niger* (for reviews see: Soccol et al., 2006; Papanikolaou and Aggelis, 2009). Recently various (natural or transformed) *Yarrowia (Candida – Saccharomycopsis – Endomycopsis) lipolytica* strains have also been shown to be capable of producing citric acid when cultivated on various substances. In the later case, media that are employed in order for citric acid to be synthesized include but are not limited to glucose (Briffaud and Engasser, 1979; Rane and Sims, 1993; Moresi, 1996; Papanikolaou et al., 2006; 2009), vegetable or animal fats (Kamzolova et al., 2005), saccharose (for genetically engineered *Yarrowia lipolytica* strains – Foster et al., 2007) and ethanol

(Kamzolova et al., 2003). On the other hand, the last years there has been a continuously increasing number of reports related with the production of citric acid by strains of the above-mentioned species growing on crude glycerol, that is the main by-product of biodiesel production facilities (for reviews see: Papanikolaou and Aggelis, 2009; Rywinska et al., 2013a). Finally, other yeast strains that have been reported capable to synthesize citric acid during growth on sugars in several fermentation configurations belong to the species *Candida oleophila* (Anastassiadis et al., 2002; Anastassiadis and Rehm, 2005) and *Candida guillermondii* (Gutierrez et al., 1993).

On a biochemical basis, the biosynthesis and production of citric acid by citrate-producing fungi and yeasts (as stressed, in most of the cases these yeasts belong to the genus *Candida* sp. and the fungi belong to the genus *Aspergillus* sp.) is a process carried out when extra- and hence intra-cellular nitrogen is depleted (Ratledge, 1994; Papanikolaou and Aggelis, 2011a), while studies of the intracellular enzyme activities and co-enzyme concentrations have somehow identified and clarified the biochemical events leading to citric acid biosynthesis (Morgunov et al., 2004). As previously noted, on a biochemical basis the secretion of citric acid into the growth medium and the biosynthesis and accumulation of reserve lipid by the oleaginous (oil-bearing) yeasts appear of being identical; the step-key of citric acid secretion and/or storage lipid accumulation in microorganisms is the change of intracellular concentration of various metabolites, conducted after exhaustion of some nutrients (principally nitrogen) in the culture medium (Ratledge, 1994; Papanikolaou and Aggelis, 2011a). This exhaustion provokes a rapid decrease of the concentration of intracellular AMP, due to its cleavage by AMP-desaminase. Due to the excessive decrease of intracellular AMP concentration, both NAD^+ and $NADP^+$-isocitrate dehydrogenases, enzymes responsible for the transformation of iso-citric to α-ketoglutaric acid, loss their activity, since they are allosterically activated by intracellular AMP, and this event results in the accumulation of citric acid inside the mitochondrion (Ratledge, 1994; Ratledge and Wynn, 2002).

When the concentration of citric acid becomes higher than a critical value, it is secreted into the cytosol. Finally, in the case of lipid-accumulating microorganisms, cytosolic citric acid is cleaved by ATP-citrate lyase (ATP-CL), the enzyme-key of lipid accumulation process in the oil-bearing microorganisms, in acetyl-CoA and oxaloacetate, with acetyl-CoA being converted, by an inversion of β-oxydation process, to cellular fatty acids. In contrast,

non-oleaginous microorganisms secrete the accumulated citric acid into the culture medium (Papanikolaou and Aggelis, 2011a). The main reactions involved in the synthesis of citric acid in citric acid-producing microorganisms are illustrated in Fig. I.12.

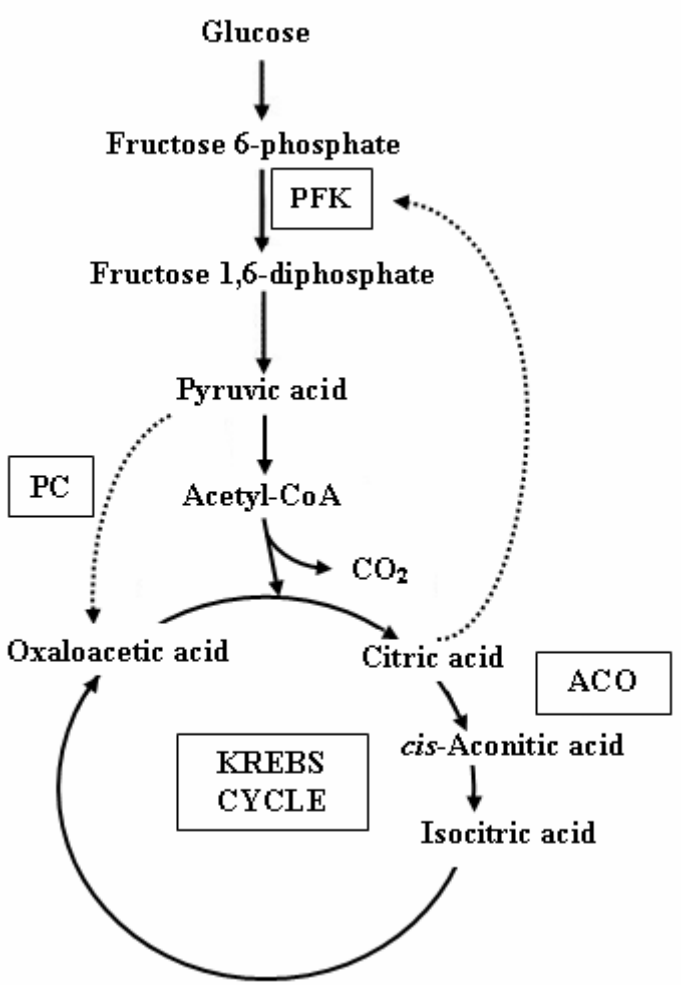


Figure I. 12. **Schematic representation of the main metabolic reactions involved in the production of citric acid by *Aspergillus niger*. PFK: phosphofructokinase, PC: pyruvate carboxylase, ACO: aconitase (Max et al., 2010 adapted).**

Although citric acid can be synthesized through chemical synthesis, there is no chemical method that is superior to microbial fermentation (Roukas, 2006). Citric acid is the main biotechnological product currently synthesized in industrial-scale operations by fermentation (Soccol et al., 2006; Berovic and Legisa, 2007). Yeast (mainly *Yarrowia lipolytica*) strains when compared to *Aspergillus niger* strains present some serious advantages including:

-Rapid growth;

-Short fermentation time leading to high productivity rates;

-Higher tolerance in contamination;

-Capability of metabolizing high initial sugar concentrations;

-Broader spectrum of substrates amenable to be converted into citric acid;

- Insensitivity to substrate variations thus can be used for developing a continuous process

-Greater tolerance for metal ions allowing the use of less refined substrates like wastes (for reviews see: Roukas, 2006; Soccol et al., 2006; Max et al., 2010).

Some experimental results reported so far concerning the production of citric acid by citrate producing microorganisms are illustrated in Table I.8.

Table I. 8. Experimental results concerning citric acid (Cit) production by yeasts and molds during growth on various renewable resources utilized as substrates in submerged cultures.

Strain	Substrate	Cit (g/L)	$Y_{Cit/S}$ (g/g)*
Aspergillus niger UE-1[a]	Hydrolysate starch	74.0	0.49
Aspergillus niger GCMC-7[a]	Cane molasses	113.6	1.00
Aspergillus niger IMI-41874[a]	Wood hemicellulose	27.0	0.45
Aspergillus niger GCM-7[a]	Black strap molasses	85.9	0.65
Aspergillus niger ATTC 914 [a]	Brewery wastes	19.0	0.78
Yarrowia lipolytica A101[a]	Beet molasses	54.0	0.69
Saccharomycopsis lipolytica NRRL Y7576[b]	Glucose	51.5	0.71
Candida guillermondii IMK-1[b]	Galactose	13.5	0.38
Yarrowia lipolytica 187/1[c]	Rapeseed oil	135.1	1.55
Yarrowia lipolytica 1.31[c]	Crude glycerol	124.5	0.62
Yarrowia lipolytica ACA-DC 50109[d]	Crude glycerol	33.6	0.45
Yarrowia lipolytica ACA-DC 50109[e]	Crude glycerol	62.5	0.56

*: $Y_{Cit/S}$: g of citric acid produced per g of carbon substrate consumed (maximum values); in the case of cultures performed on sugar-based substrates, this yield is expressed on the basis of total sugar quantity consumed

[a] Data presented in the review-article by Soccol et al., (2006); [b] Data obtained by other authors and discussed in Papanikolaou et al., (2003); [c] Data obtained by other authors and discussed in Papanikolaou et al., (2008); [d] Data from Papanikolaou et al., (2003); [e] Data from Papanikolaou et al., (2008)

Concerning its applications, this organic acid is used mainly in the food and beverage industry, primarily as acidulate. It is an important chemical used as pharmaceutical as also in other industrial uses such as the manufacture of ink and dyes, in cosmetics, toiletries and in detergents and cleaning products (Najafpour, 2006; Soccol et al., 2006).

I.3.5.2. Polyols

The last years, there has been some research related with the production of polyols (mannitol and erythritol) by *Yarrowia lipolytica* strains cultivated mainly on media composed of pure or raw (mostly biodiesel-derived) glycerol (André et al., 2009; Rywińska and Rymowicz, 2009; Chatzifragkou et al., 2011a; 2011b; Tomaszewska et al., 2012; 2014). Mannitol - a six-carbon sugar alcohol - has several applications in the food, pharmaceutical and medical industries. It is distributed widely in nature, being found in various plants, algae, in the mycelia of various fungi and it is one of the main carbohydrates in mushrooms. It is currently produced industrially by chemical synthesis using hydrogenation of fructose at high temperature and pressure. This process is not very efficient and requires a high purity of substrates. Therefore, microbiological production of mannitol would be an interesting alternative. The formula of mannitol is depicted below.

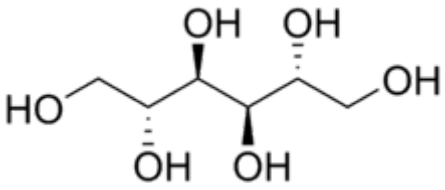


Figure I. 13. Molecular structure of mannitol (http://en.wikipedia.org/wiki/Sugar_alcohol).

Erythritol is a four-carbon sugar alcohol produced in microbiological processes. In comparison to other sugar alcohols used currently as sucrose replacers, erythritol has a much lower energy value (~0.2 kca1/g) than sucrose (4 kcal/g) and other sugar alcohols (~2.4 kcal/g). Moreover, this polyol is non-cariogenic, generally free of gastric side-effects in regular use and its use in food is largely approved (Tomaszewska et al., 2012; 2014). The formula of erythritol is depicted below.

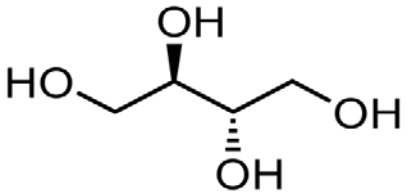


Figure I. 14. **Molecular structure of erythritol (http://en.wikipedia.org/wiki/Sugar_alcohol).**

It is known that osmophilic yeast-like fungi and some bacteria can produce sugar alcohols or their derivatives in response to increased external osmotic pressure (Veiga-da-Cunha et al., 1992). Most yeasts produce trace amounts of one or more sugar alcohols, but more effective biosynthesis has been obtained by species that display a relatively high tolerance for high concentrations of salts or sugars (for review see: Moon et al., 2010). Erythritol is produced by fermentation involving some bacteria such as *Leuconostoc oenos* (only under anaerobic conditions) and yeast-like fungi such as *Trigonopsis variabilis*, *Trichosporon* sp., *Torula* sp., etc. (Veiga-da-Cunha et al., 1992). Substrates such as glucose or fructose seem to favor erythritol production by osmophilic microorganisms and the fermentation process in such substrates has extensively been studied. However only the last years there has been some information is available about the production of this compound by yeasts cultivated in glycerol, although it has been reported that erythritol and mannitol were produced during the production of citric acid from glycerol by *Yarrowia lipolytica* strains (André et al., 2009; Tomaszewska et al., 2012). Erythritol is synthesized from erythrose-4-phosphate, an intermediate in the pentose-phosphate pathway, by dephosphorylation and the subsequent reduction of erythrose. Erythrose reductase (ER), which catalyzes the final step in this pathway, is a key enzyme in the biosynthesis of erythritol, as it is shown in Fig. I.15.

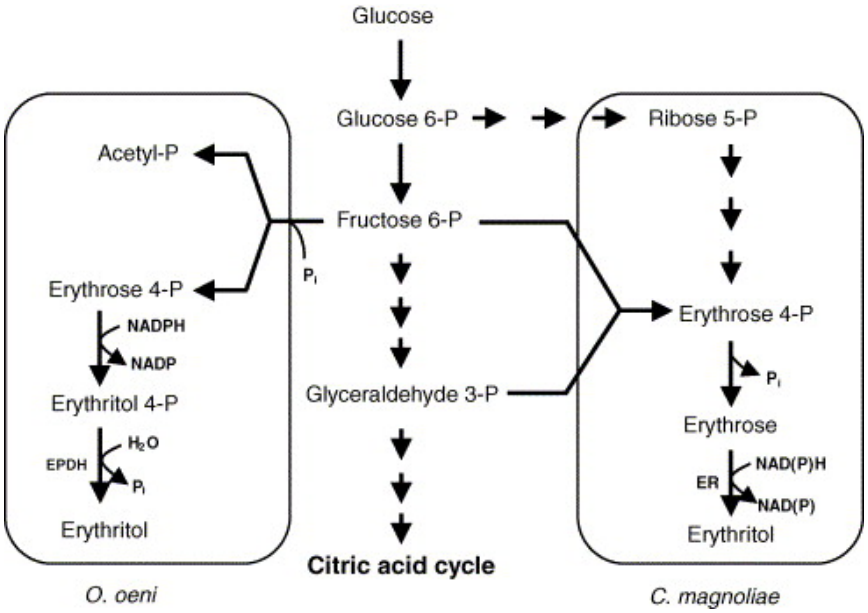


Figure I. 15. Hypothetical pathways of erythritol biosynthesis in yeast and bacteria. EPDH erythritol-4-phosphate dehydrogenase, PK phosphoketolase, E4PK erythrose-4-phosphate kinase, Pi inorganic phosphate, PTase, phosphatase, ER erythrose reductase, G-6-P glucose 6-phoaphate, F-6-P fructose 6-phosphate, F-1,6-P fructose 1,6-diphosphate; GA-3-P, glyceraldehyde 3-phosphate, 1,3-BPG 1,3-diphoglycerate, PG phosphoglycerate. b Pentaketide pathway for melanin biosynthesis in fungi. CoA coenzyme A, TCA tricarboxylic acid (Park et al., 2005, modified).

On the other hand, the biochemical mechanism of mannitol biosynthesis by strains growing on glycerol has not been completely elucidated. It has been postulated that sugar alcohols, including mannitol (and potentially erythritol), can protect various cells from osmotic stress (Kets et al., 1996) which likely occurs due to exposure of strains into relatively high glycerol concentrations into the medium (since in most cases the production of mannitol and erythritol is performed in media presenting high initial glycerol concentrations - Rywińska et al., 2008; André et al., 2009; Rywińska and Rymowicz, 2009; Chatzifragkou et al., 2011; Tomaszewska et al., 2012; 2014).

In few reports, secretion of polyols into the culture medium together with citric acid, when glycerol was utilized as the sole carbon source in nitrogen-limited submerged experiments, has been studied (Rymowicz et al., 2008; André et al., 2009; Rywińska et al., 2009; Rywińska and Rymowicz, 2009). For instance, when mutant *Yarrowia lipolytica* strains (e.g. Wratislavia AWG7 and K1 strains) were used in bioreactor experiments, erythritol was the principal polyol produced, in some cases in significant concentrations (e. g. up to 47 g/L), while mannitol was secreted in small amounts (e. g. up to 9 g/L), while nevertheless citric

acid was the principal metabolite produced (in very high quantities e. g. 87-139 g/L) (Rywińska et al., 2009). Moreover, mannitol in concentrations 4-9 g/L together with erythritol (in concentrations 4-15 g/L) have been produced by another mutant strain (Wratislavia 1.31) in fed-batch bioreactor experiments with glycerol utilized as the sole carbon source (Rywińska and Rymowicz, 2009), while as previously, very high citric acid amounts have been produced (109-148 g/L) with polyols representing only a small portion of total metabolites produced. On the other hand, production of erythritol as sole metabolite, in some cases in indeed very high quantities (e.g. >45 g/L or even >80 g/L) has been reported when crude or pure glycerol has been employed as fermentation substrate by wild or mutant *Y. lipolytica* strains when growth was performed on low pH or addition of NaCl into the medium was performed (Tomaszewska et al., 2012; 2014; Rywińska et al., 2013a; 2013b).

I.3.5.1. Microbial polysaccharides

Polysaccharide is a class of high-molecular-weight carbohydrates (colloidal complexes) which are subjected to breakdown following chemically or enzymatically catalyzed hydrolysis to monosaccharides containing five or six carbon atoms. The polysaccharides are considered to be polymers in which monosaccharides have been glycosidically joined with the elimination of water. A polysaccharide consisting of hexose mono-saccharide units may be represented by the reaction below.

$$n C_6H_{12}O_2 \rightarrow (C_6H_{10}O_5)_n + (n-1)H_2O$$

The term polysaccharide is limited to those polymers which contain 10 or more monosaccharide residues. Polysaccharides such as starch, glycogen and dextran consist of several thousand of D-glucose units. Polymers of relatively low molecular weight, consisting of two to nine monosaccharide residues, are referred to as oligosaccharides. Polysaccharides are often classified on the basis of the number of monosaccharide types present in the molecule. Polysaccharides, such as cellulose or starch, that after extensive chemical or enzymatic hydrolysis are generating only one monosaccharide (e.g. D-glucose) are termed as “homo-polysaccharides” (see i.e. Fig. 1.16).

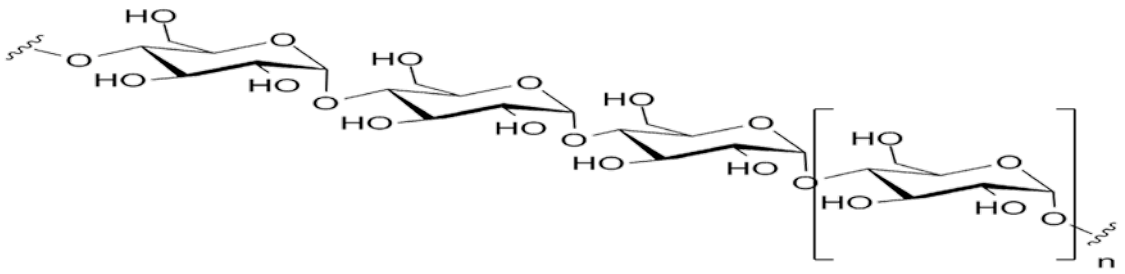


Figure I. 16. Molecular structure of amylase, which is a typical homopolysaccharide (http://en.wikipedia.org/wiki/Polysaccharide)

On the other hand, polysaccharides, such as hyaluronic acid, which after extensive chemical or enzymatic hydrolysis result in the liberation of more than one monosaccharide (e.g. *N*-acetylglucosamine and D-glucuronic acid for the above-mentioned case) are named heteropolysaccharides (Fig. I.17).

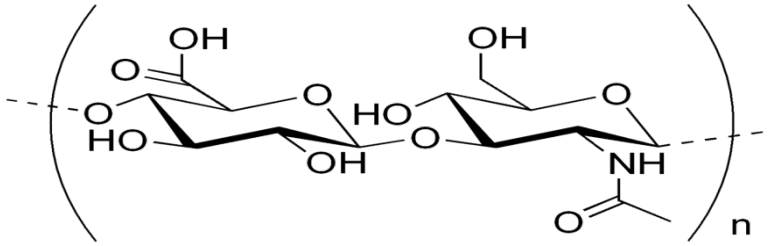


Figure I. 17. **Molecular structure of hyaluronan (also called hyaluronic acid), that is a typical heteropolysaccharide (http://en.wikipedia.org/wiki/ Hyaluronan).**

Many polysaccharides, including starch, cellulose, and pectins, are used in the food and chemical industries, as well as in medicinal applications (Stacey, 1967). Fungi (principally macrofungi and to lesser extent yeasts) have been studied as potential producers of polysaccharides (exopolysaccharides and endopolysaccharides) that in several cases present medicinal and therapeutic actions with antitumor and immunological activities. Polysaccharides which are associated with the above-mentioned activities include but are not limited to hetero-β-glucans (e.g. lentinan, schoziphyllan) (Tang and Zhong, 2002). These β-glucans generally consist of a backbone of glucose residues joined by β-(1,3) glycosidic linkages to which are attached β-(1,6) linked glucose units (Seviour et al., 1992; Fazenda et al., 2008). However, the extent of this branching can vary considerably in several macrofungi (e.g. *Botryosphaeria rhodina*, *Pleurotus eryngii*, *Pleurotus ostreatoroseus*, *Lentinula edodes*), the branching frequency is one single glucose residue attached to one in three backbone

residues, while in others like *Pestalotia* spp. the branching frequency is much higher with three single β-(1,6) linked glucose units attached every five backbone residues (Fazenda et al., 2008).

Fungal polysaccharides are produced in both solid-state and submerged culture systems. Polysaccharide's synthesis is strongly influenced by the culture conditions, especially medium composition (Wu et al., 2006), the knowledge of which is necessary for the direction of fungal metabolism towards the production of the desirable metabolites. Polysaccharide's production is also favored by glucose and organic nitrogen sources (e.g. yeast extract, peptone, corn steep liquor) as it has been reported (Gern et al., 2008; Fang and Zhong, 2002). Likewise, non-nutritional factors such as the initial pH, the incubation temperature and the aeration play an important role upon the biosynthesis of polysaccharides. However, culture conditions that favor the production of biomass may not favor the accumulation of polysaccharides (mainly the exopolysaccharides) and vice-versa (Tang and Zhong, 2002; Diamantopoulou et al., 2012a; 2012b; 2014).

Intra-cellular carbohydrates produced by higher fungi include simple sugars (e.g. fructose), disaccharides (e.g. trehalose), polyalcohol-type compounds (e.g. mannitol) but principally polysaccharides like glycogen, pullulan, β-glycans, etc (Seviour et al., 1992; Galiotou-Panayotou et al., 1998; Fang and Zhong, 2002; Tang and Zhong, 2002; Fazenda et al., 2008; Tang et al., 2008; 2011). Fungal-derived polysaccharides can present potential immuno-modulating, anti-tumor and hypoglycemic activities (Fazenda et al., 2008). Characteristic types of fungi implicated in the above-mentioned bioprocess include but are not limited to *Aureobasidium pullulans*, *Agaricus bisporus*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Cordyceps jiangxiensis*, *Cordyceps pruinosa*, *Ganoderma lucidum*, *Tuber sinense*, etc.

On a biochemical basis, in many reports found into the literature it has been demonstrated that nitrogen depletion into the medium favors the process of accumulation of these compounds (the mechanism is equally similar to that of the accumulation of storage lipids); due to the obligatory cleavage of AMP in order for nitrogen requirements of the cells to be secured, inhibition of the 6-phosphoro-fructokinase (6-PFK) is performed, given that this enzyme (as the mitochondrial NAD^+-isocitrate dehydrogenase) is allosterically activated by the cellular AMP (Galiotou-Panayotou et al., 1998; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a). The above-mentioned situation can have as result the

intra-cellular accumulation of polysaccharides (principally based on 6-phosporo-glucose). The hypothetic scenario of accumulation of intra-cellular polysaccharides is seen in Fig. I.18.

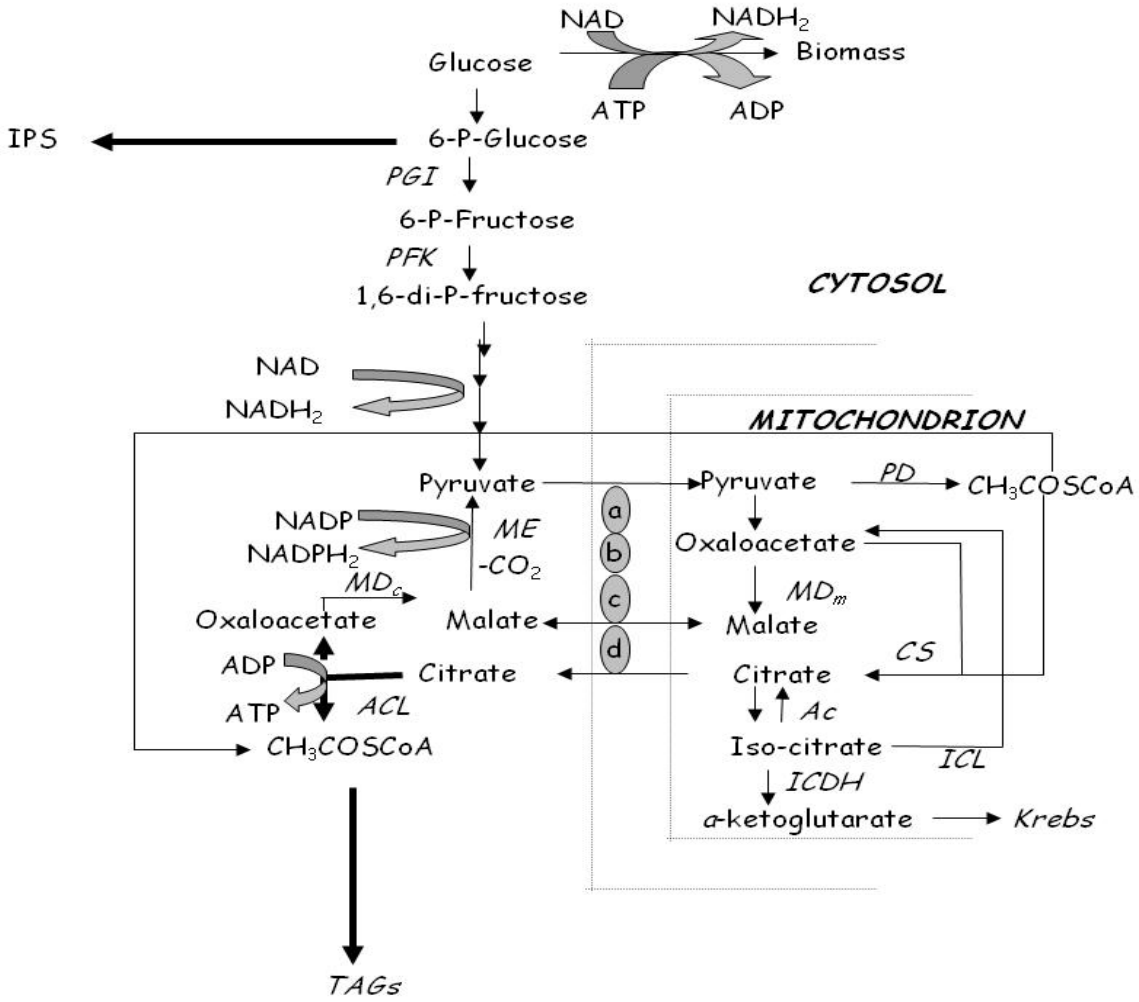


Figure I. 18. **Hypothetical scenario of accumulation of intra-cellular polysaccharides (IPS) and lipids by microorganisms (adapted from: Papanikolaou and Aggelis, 2009; Διαμαντοπούλου, 2012).**

As indicated in the previous paragraphs, the production of intra-cellular polysaccharides (that in several cases present remarkable therapeutic activities) is performed mainly by macrofungi; for instance, the Chinese truffle *Tuber sinense* was cultivated in shake-flask experiments with various sugars (e.g. sucrose, lactose, etc) used as carbon sources and a maximum dry weight (X_{max}) concentration achieved was ~24 g/L while, the total intracellular sugars (ITS_{max}) concentration was ~3 g/L (corresponding to total intracellular sugars per dry weight $Y_{ITS/X}$~13%, w/w) (Tang et al., 2008). *Ganoderma lucidum* 5.616 when cultivated on glucose-based media in shake-flask experiments presented X_{max} and ITS_{max} quantities of 16.7

g/L and 1.19 g/L respectively (Tang and Zhong, 2002). Tang and Zhong (2002) performed kinetic studies with the above-mentioned strain in shake-flask and stirred-tank bioreactor cultures by using several sugars employed at different initial concentrations and in fed-batch culture mode in both flasks and bioreactor X_{max} and ITS_{max} quantities of ~22 g/L and ~2.5 g/L respectively were obtained, while Tang et al. (2011) successfully scaled-up the above-mentioned fed-batch process in large-scale stirred-tank bioreactors (with volumes ranging between 7.5 and 220 L), and X_{max} and ITS_{max} concentrations of ~22 g/L and ~5 g/L respectively were achieved. Xiao et al. (2004) performed submerged cultures of *Cordyceps pruinosa* on media composed of molasses and in shake-flask experiments X_{max} and ITS_{max} quantities of 31.86 g/L and 5.07 g/L were achieved after cultivation of 9 days. In 25-L bioreactor the respective quantities were 29.9 g/L and 9.5 g/L but these values were achieved substantially earlier (within ~2 days). Xiao et al. (2006) after optimization of shake-flask cultures of *Cordyceps jiangxiensis*, reported X_{max} and ITS_{max} values of 24.5 g/L and 8.93 g/L after a cultivation period of 9 days. Diamantopoulou et al. (2012b) performed a detailed kinetic analysis of mycelial biomass and extra- and intra- cellular polysaccharides (EPS, ITS respectively) biosynthesis by a *Volvariella volvacea* selected strain in submerged-static and agitated cultures. Maximum values of 15 g/L of biomass, 0.3 g/L of EPS and 5.5 g/L of ITS were recorded. Agitation did not have severe impact on biomass, EPS and ITS production. Glucose was the major cellular carbohydrate detected as the major building block of the ITS produced. Finally, Diamantopoulou et al. (2014) reported significant production of ITS during growth on several macrofungi on glucose shake-flask or static-flask experiments; an *Agrocybe aegerita* strain was reported to achieve a ITS_{max} concentration of ~5.5 g/L ($Y_{ITS/X}$~60%, w/w) while a *Pleurotus pulmonarius* strain produced ITS to a maximum concentration of ~11 g/L ($Y_{ITS/X}$~48%, w/w) (Diamantopoulou et al., 2014).

I.3.6. Economical consideration and future perspectives

SCO fermentation attracts much interest the last years. Amongst microbial lipids, yeast lipids present importance in both academic and industrial point of view. Due to their unicellular nature and their potentiality to grow on a plethora of hydrophilic or hydrophobic substrates, the oleaginous yeasts are considered as perfect ‘‘tools’’ for studying phenomena of advanced lipid biochemistry and biotechnology (Ratledge, 1994; Papanikolaou and Aggelis, 2010).

The last years, there has been a significant rise in the number of publications in the international literature that deal with the production of oils and fats deriving from microbial sources, mainly in order to utilize these lipids as biodiesel precursors. A great number of scientific works refer to the utilization of yeasts belonging to the species *Lipomyces starkeyi*, *Rhodosporidium toruloides*, *Apiotrichum curvatum* (known also as *Candida curvata* or *Cryptococcus curvatus*), *Rhodotorula* spp., *Trichosporon fermentans* and *Yarrowia lipolytica* cultivated on several types of sugar-based or similarly metabolized raw materials, that include but are not limited to cheese-whey, glucose, glucose syrups, xylose, hemicellolose hydrolysates, molasses, waste glycerol deriving from bio-diesel production, organic acids, etc (Papanikolaou and Aggelis, 2011b; Ratledge, 2011; Papanikolaou, 2012) in order for biodiesel precursors (e.g. TAGs) to be synthesized. However, it needs to be stressed that despite the large quantity of works recently appeared on this issue, and although mass production of yeast lipids amenable for the synthesis of biodiesel attracts currently noticeable interest, specifically at academic level, the production cost of SCOs remains always much higher than that of plant oils (Koutinas et al., 2014a; 2014b). A representative value of yeast SCO produced on 2008 was around 3 US$ per kg (excluding cost of feedstock used for SCO production) while the ones of rapeseed oil, soybean oil and sunflower oil were 1.4-1.5, 1.2-1.3 and 1.8-1.9 US$ per kg respectively (Ratledge and Cohen, 2008). However, the increment of bio-diesel needs, covered through the utilization of edible oils (this is the so-called "1st generation bio-diesel") has resulted in the fact that between 2007 and 2008 there has been a 2-fold increase of the price of conventional plant commodity oils (Ratledge and Cohen, 2008). Continuation of this situation and also severe rise in the price of crude oil (that currently is being reported) could potentially render the utilization of oleaginous yeasts as an economically viable process, in order for biodiesel precursors to be produced (Papanikolaou, 2012; Koutinas et al., 2014a; 2014b).

A major aspect that could also be taken into consideration is that the economics of SCO bio-processes could be further ameliorated by using waste materials as substrates of the oleaginous microorganisms because most of these compounds have a negative value, their direct discharge without previous treatment causes serious environmental problems, while national or European legislation has rendered obligatory the treatment and safe disposal of these materials; for instance, the removal of non-toxic and non-hazardous waste material from the food industry (e.g. expired sugar-rich products, expired breads, concentrated sucrose- or

glucose-based effluents, etc) that will further be subjected to compost processing costs ~0.1-0.5 US $ per Kg of waste, depending on the nature of the food waste discarded (Papanikolaou and Aggelis, 2010; Papanikolaou, 2012; Koutinas et al., 2014a). One can imagine the economic losses for the food-processing facilities, specifically by taking into consideration that ~20%, w/w, of the materials utilized in these facilities finally turns to be converted into residues, and the concomitant economic benefit by the establishment of sugar-based bio-refineries, so as to convert the residual materials into biodiesel precursors (Papanikolaou and Aggelis, 2010; Papanikolaou, 2012). On the other hand, successful utilization of waste streams has as pre-requisite a well-established network providing the waste material into the fermentation plant, and only under this circumstance the economic viability of the bio-refinery proposed could be secured (Koutinas et al., 2014b). Then the produced biomass rich in lipid can be directly trans-esterified to yield the bio-diesel, thus avoiding the oil extraction step, which is one of the most costly steps of the SCO production procedure (Ratledge and Cohen, 2008), while the de-fatted yeast biomass could be used as feed supplement. However, in any case, a major problem that remains to be solved in order to significantly reduce the cost in the fermentation of SCO production is the one of the maintenance of aseptic conditions in the large-scale operations performed.

Another potential issue of the yeast lipids refers to their potentiality of "specialty-type" lipids production, which can possibly replace very expensive lipids found into the plant or animal kingdom. The price of various naturally occurring lipids and fats can tremendously vary (from 0.3 to up to 100 US $ per kg – see: Ratledge and Wynn, 2002). Therefore identification of microorganisms capable of producing in increased quantities lipids with structure and composition similar to that of high-value fats, and subsequent large-scale production of these lipids can present an obvious financial interest, specifically if the starting material used is of low or negative cost. One such case refers to the replacement of the cocoa-butter by yeast lipids (the cocoa-butter substitutes – CBSs). Cocoa-butter presents unique physicochemical characteristics (solid at ambient temperature but liquid at 37°C that of the temperature of mouth) that are mainly due to the fatty acid composition of this fat; as indicated in the previous paragraphs, cocoa-butter is mainly composed of triacylglycerols of the type P-O-S and S-O-S (P: palmitic acid – C16:0, O: oleic acid – $^{\Delta 9}$C18:1, S: stearic acid – C18:0). Oleic acid, hence, is always found esterified in the position sn-2 of glycerol.

The production of CBSs, economically viable during the years 1980-1990 (at that time the price of the cocoa-butter was >8.0 US $ per kg), is obviously dependable of the price and the availability of this fat. Currently, the price of cocoa-butter is ~5.0 US *$* per Kg, though the tendency of this price, is to present (a remarkably significant) increase in the near future; prospective studies that have been recently presented in both the written and the electronic international press indicate that cocoa-butter risks to disappear the next years due to general failure of the cultivation techniques of the cocoa plant, and, thus, the utilization and application of the various CBSs will be generalized for the food industry (Papanikolaou, 2012).

Concluding, it must be pointed out that the production of lipid from microbial sources presents continuous expansion in the last years. Amongst microbial lipids, yeast lipids present a very high importance in both academic and industrial point of view. Due to their unicellular nature and their potentiality to grow on a plethora of hydrophilic or hydrophobic substrates, the oleaginous yeasts are considered as perfect "tools" for studying phenomena of advanced lipid biochemistry and biotechnology (Papanikolaou and Aggelis, 2010; 2011b; Papanikolaou, 2012). The last years, works indicating interesting analogies between lipid metabolism in the yeast cells and the gastro-intestinal tract and vascular system have appeared (Pembroke, 2006). Also, increasing demand and utilization of 1st generation biodiesel has increased the cost of various food-stuffs, and this led to the necessity of discovery of non-conventional sources of oils, that could be subsequently converted into biodiesel. The oleaginous yeasts, which in most cases are categorized as GRAS microorganisms and due to their fast growth rates and their potentiality to grow on a plethora of substrates, are considered perfect candidates for the production of this 2nd generation bio-diesel. Finally, the potential (significant) rise in the price of cocoa-butter or the already existing high price of other saturated exotic fats (e.g. shea butter, sal fat) renders the utilization of several oleaginous yeast strains amenable to produce substitute SCOs of these high-value fats as very promising.

II- MATERIALS AND METHODS

II.1. Characteristics and specifications of carbon sources used in the present investigation

In the present study it was desirable to study the physiological and kinetic behavior of selected yeast strains during growth on several hydrophilic carbon sources employed as substrates. Microbial substrates utilized during the present work were the commercial (low-cost) glucose, employed in several confectionary faciliries, provided by the "Hellenic Industry of Sugar SA" (Thessaloniki, Greece) [purity *c.* 95% w/w impurities composed of maltose (2%, w/w), malto-dextrines (0.5%, w/w), water (1.5%, w/w) and salts (1.0%, w/w)], the commercial lactose deriving from cheese-whey and utilized in various confectionary industries [purity *c.* 94% w/w with impurities being salts (2.5% w/w) and water (3.5% w/w)], the commercial sucrose, supplied by the Hellenic Sugar Industry and produced industrially from sugar beet (*Beta vulgaris*) (purity 99% w/w impurities mainly composed of salts), the biodiesel-derived raw glycerol, the deproteinized and concentrated cheese-whey and, finally, the molasses.

Molasses are the viscous by-product of the sugar cane or sugar beet processing into sugar, containing sugars (44-60% *w/w*) and various minerals (calcium, phosphorus, sodium, chlorine, sulphure<1%, potassium (2-4%) and nitrogen compound (2-6%) (Curtin, 1983; Chen et al., 1993). Due to the high content in sugars, molasses have been used as growth medium for the production of various (high-) added value products through microbial fermentations. The waters remaining after such bio-processes, called molasses wastewaters (MWWs), are characterized by high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values, strong odor and dark brown color (Satyawali and Balakrishnan, 2008). The high molecular weight polymer melanoidin is the dark brown pigment found in the molasses wastewaters (Kumar and Chandra, 2006; Plavšić et al., 2006). The composition of this effluent and its release into the environment without appropriate previous treatment, may lead to eutrophication phenomena in the water. Its dark color hinders photosynthesis by blocking sunlight labeling molasses deleterious to aquatic life (FitzGibbon et al., 1998). Fungi, bacteria and yeasts have been cultivated on molasses either for melanoidin degradation and the reduction of color and of BOD and COD values (Ohmomo et

al., 1988; Kumar and Chandra, 2006) or for the production of added-values metabolites such as ethanol, gluconic acid, citric acid, fructo-oligosaccharides, pullulan, succinic acid, SCO, erythromycin and bacteriocins (Roukas et al., 2006; Nahvi et al., 2002; Baptista et al., 2006; Kopsahelis et al., 2007; 2012; Cáceres-Farfán et al., 2008; Sharma et al., 2008; Zhu et al., 2008; Chatzifragkou et al., 2010; Metsoviti et al., 2011). In a limited number of reports, the products of added-value compounds and the detoxification decolorization of molasses were simultaneously studied (Chatzifragkou et al., 2010; Metsoviti et al., 2011).

As previously indicated, raw beet molasses use in this study were supplied by Hellenic Sugar Industry (Thessaloniki, Greece) and contained *c*. 48-52%, w/v of sucrose (analysis performed by DNS method – see below), 6%, w/v of protein (analysis performed by Lawry method – see below), 5%, w/v of fructose (analysis performed by HPLC – see below) and 14%, w/v of ash.

Cheese whey is the liquid remaining following the precipitation and removal of milk casein during cheese-making. This byproduct represents about 85-95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% of dried extract). Actual market trends point to a gradual increase in cheese production that generates more than 145×10^6 T of liquid whey per year, with 6×10^6 T corresponding to lactose (Castillo, 1990). To make 1 kg of cheese, 9 kg of whey is generated (Kosikowski, 1979). Although several possibilities for cheese-whey exploitation have been assayed over the last 50 years, approximately half of world cheese-whey production is not treated, but is discarded as effluent. Therefore, cheese whey represents an important environmental problem because of the high volumes produced and its high organic matter content, exhibiting a BOD5 = 30000-50 000 ppm and COD =60000-80000ppm (Marwaha and Kennedy, 1988; Gardner, 1989; Kemp and Quickenden, 1989; Mawson, 1994), with lactose being largely responsible for the high BOD and COD, seeing that protein recovery reduces the COD of the whey only by about 10000 ppm (Mawson, 1994). Bioconversion of whey lactose to SCP (single cell protein), ethanol or methane reduces more than 75% of the BOD while producing marketable products, but in most cases the ensuing effluent is not then ready for disposal (Mawson, 1994). Microbial biomass has been produced commercially from whey since the 1940s. The development and operation of several pilot-scale and commercial plants in France, the USA, Germany and Austria has been reported (Sienkiewicz and Riedel, 1990; Mawson, 1994).

Cheese whey used in this study were obtained from the Laboratory of Dairy Science of the Agricultural University of Athens and contained *c.* 80 g/L of lactose and *c.* 9 g/L of proteins, analysis performed as described in II.3

In the current investigation, the effect of the addition of NaCl upon the microbial growth and the production of metabolites was studied. As an application of the results obtained, it was desirable to study the production of microbial metabolites of added-value during growth on a salty wastewater, properly enriched with low-cost glucose. Table olives processing has long been and still remains, a very important tradition and industry in Spain, Greece, Turkey and many other countries around the Mediterranean Basin (Garrido Fernandez et al., 1997).There are three principal types of table olives: green, black and black through oxidation. The residues of the production of table olives, the table olive processing wastewaters (TOPWs), contain large amounts of mineral and organic matter. The latter fraction contains a complex blend of sugars and phenolic compounds, some nitrogenous compounds (especially amino acids), organic acids, tannins, pectins, carotenoids and oil residues (Gracia Gracia, 2006). It is therefore, obvious that these wastewaters are highly polluting and are not simply treated by conventional methods. Many strategies have been studied to reduce the environmental impact of these wastewaters such as washing water re-use, reduction of washing waters and debittering with low-concentration lyes. Any of these approaches has resulted in meeting the needs (Garrido Fermandez et al., 1997). In Greece, the maximum estimation of the production of olives and waste water are 108.2 and 649 tons (Cappelletti et al. (2011).

Although, the optimization of biological treatment of green TOPWs using *Aspergillus niger* have been performed (Ayed et al., 2013), due to the high presence of salt and phenolic compounds, TOWP-based media enriched with other carbon sources have not yet been used for the cultivation of molds, prokaryotic microorganisms, yeast and yeast-like species leading to the remediation of the wastewater, the (partial) replacement of tap waster from TOPW as water in fermentation processes as also to the production of added-value compounds such as yeast and fungal biomass.

TOPWs used in this study were obtained from the Laboratory of Food Microbiology and Biotechnology of the Agricultural University of Athens containing ~0 g/L glucose and ~60 g/L NaCl were immediately frozen at T=20± 2°C in order to preserve their characteristics. To be used in the experiments, TOPWs were de-frozen and the solids removed

by centrifugation (9000 rpm, 15 min, T=21±1°C – Hettich Universal 320R). TOPW phenolic content expressed as gallic acid equivalent was 1.4 g/L. (analysis performed by Slinkard and Singleton, 1977; see below). TOPWs pH was 4.5-4.8. All analyses performed as describe in II.3.

Biodiesel fuels defined as fatty acid methyl esters derived from various renewable lipid resources (e.g. rapeseed oil, soybean oil, palm oil, etc) constitute an alternative type of fuel for various types of diesel engines and heating systems (Hirschmann et al., 2005; Papanikolaou and Aggelis, 2011b). With the obligatory application of biofuels in a large commercial scale in Europe, enormous quantities of glycerol, the principal side-product of biodiesel production process (Papanikolaou and Aggelis, 2011b), are likely to be generated in the near future (Hirschmann et al., 2005). On the other hand, glycerol-containing waters also produced in significant amounts from fat saponification and alcoholic beverage fabrication units (Barbirato et al., 1998; Papanikolaou and Aggelis, 2009). Conversion, thus, of glycerol to various high-value added products attracts much interest. A principal technique of biotechnological valorization of glycerol is related to its biotransformation into 1,3-propanediol, a substance of notable importance for the textile and chemical industry (Zeng and Biebl, 2002; Lee et al., 2004). On the other hand, a phethora of works have appeared the last years in relation with the biotechnological conversions of glycerol into citric acid, SCO, microbial mass, polyfydroxy-alkanoates, etc (Papanikolaou and Aggelis, 2002; 2009; Rymowicz et al., 2006; Kachrimanidou et al., 2013; Rywinska et al., 2013a).

The glycerol feedstock used in the current investigation was biodiesel-derived crude glycerol provided by the “Pavlos N. Pettas Fats and Oils SA”, Patras, Greece, that performed trans-esterification of various blends of edible oils in order to generate biodiesel. The purity of the feedstock was as follows: glycerol 91-93% w/w; impurities composed of: 2-4% w/w water, 2-4% w/w potassium salts, 1% w/w free-fatty acids (Kachrimanidou et al., 2013).
All analyses performed as describe in II.3.

II.2. Microorganisms and culture conditions

The strains used in this study were the following ones: *Yarrowia lipolytica* (ACA-YC 5029 and ACA-YC 5033; isolated from traditional Greek sourdoughs and identified in the Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens) (Papanikolaou et al., 2009), *Cryptococcus curvatus* NRRL Y-1511,

Rhodosporidium toruloides DSM 4444, *Rhodosporidium toruloides* NRRL Y-27012, *Rhodotorula glutinis* NRRL YB-252 and *Lipomyces starkeyi* DSM 70296. The strains NRRL Y were provided by the NRRL culture collection (Peoria, USA), while the strains DSM were provided by the DSMZ culture collection (Leibniz, Germany). All strains were maintained on yeast peptone dextrose agar (YPDA) at T=4°C and sub-cultured every 4 months in order to maintain their viability.

Prior to any inoculation in liquid growth medium (pre-culture and main culture) the strains were regenerated (under sterile conditions) so as the inoculum would be three days old. Following, three 250-mL Erlenmeyer flasks (pre-culture) filled with 50±1 mL of mineral salts medium (for composition of mineral salts solution see Table II.1.) were aseptically inoculated from the principal freshly regenerated strain and were incubated in orbital shaker (for 24±2 hours at 180±5 rpm, 28±1°C; in case of *Lipomyces starkeyi*, the duration of incubation were 48 h). Microscopic observation of the yeasts was carried out in order to verify the purity of the strain. The pre-culture was consisted by 10 g/L of commercial glucose, 0.75 g/L peptone and 0.5 g/L yeast extract. Finally, the inoculation of the main culture (from the principal pre-culture) took place. The inoculation of the culture was done under aseptic conditions only at the trials which included sterile media (sterilization performed at *c*. T=115°C, 20min).

In fermentations that were carried out in 250-mL Erlenmeyer flasks filled with 50 ± 1 mL of growth medium, the inoculation volume was 1 mL (2% v/v inoculum – 10^5-10^7 cfu) of the exponential pre-culture. However, in the case of non-aspetic cultures, the inoculation volume was 3ml. Flasks were incubated in an orbital shaker (New Brunswick Scientific, USA) at an agitation of 180 ± 5 rpm and incubation temperature 28±1°C (and 26±1^{o}C in case of *Rhodosporidium toruloides* strain). The correction of the medium pH was maintained at the desired value by periodically (and aseptically concerning the trials performed under sterile conditions) adding into the flasks, quantities of KOH 5 mol/L or 1 mol/L HCl. The exact base or acid solution volume needed for pH correction was evaluated by measuring the volume of the solution required for pH correction in one (at least) flask. Following, the appropriate volume of base or acid was (aseptically) added in the remaining flasks and the value of the pH reached was verified to be the desired one.

Aseptic and non-aseptic batch fermentations were also conducted in a laboratory scale bioreactor (benchtop bioreactor Labfors 5, INFORS HT), with total volume 2.5 Land working volume 2L, fitted with four probes and two six-bladed turbines. The culture vessel was

inoculated with 150 mL (7.5% v/v inoculum) of exponential pre-culture (see above). The incubation temperature was controlled automatically at 28±1°C (or 26°C for *R. toruloides*). Agitation rate was adjusted to 300 rpm. The pH was automatically controlled at the desired value by adding base quantities of KOH 5 mol/L or 1 mol/L HCl.

All trials were conducted in duplicate and each experimental point of the kinetics was the mean value of two independent determinations.

II.2.1. Glucose-based media as substrates for the production of metabolic compounds by oleaginous microorganisms

The composition of mineral salts composition used in the present investigation is shown in Table II.1.

Table II.1. Composition of mineral salts solution (Papanikolaou et al., 2010).

Compound	Concentration (g/L)
KH_2PO4	7.00
$Na_2HPO_4 \cdot 2H_2O$	2.50
$MgSO_4 \cdot 7H^2O$	1.50
$FeCl_3 \cdot 6H_2O$	0.15
$CaCl_2 \cdot 2H_2O$	0.15
$ZnSO_4 \cdot 7H_2O$	0.02
$MnSO_4 \cdot H_2O$	0.06

Trials were performed either under nitrogen-limited or nitrogen-excess conditions. In nitrogen-limited culture conditions, peptone and yeast extract were used as nitrogen sources in concentrations of 0.75 g/L and 0.5 g/L respectively. In nitrogen-excess media, 5.5 g/L peptone and 0.5 g/L yeast extract were added. Peptone contained *c.* 18%, w/w nitrogen and *c.* 30%, w/w carbon whereas yeast extract contained *c.* 14%, w/w nitrogen and *c*. 12%, w/w carbon. In some cases in which the carbon substrate contained also significant quantities of proteins (e.g. case of growth on cheese-whey and molasses), no supplementary addition of nitrogen sources was performed.

The evolution of the experimental procedure during growth of microorganisms on glucose-based media is presented in the following paragraphs:

II.2.1.1. Initial screening of yeast strains on glucose base media.

Five employed strains (*Rhodosporidium toruloides* DSM 4444, *Rhodotorula glutinis* NRRL YB-252, *Rhodosporidium toruloides* Y-27012, *Yarrowia lipolytica* ACA YC 5033, *Lipomyces starkeyi* DSM 70296) were tested in media composed of glucose at initial glucose concentration (Glc_i) adjusted at *c.* 50 g/L under nitrogen-limited conditions (utilization of peptone at 0.75 g/L and yeast extract at 0.5 g/L; initial molar ration employed at *c.* 100 moles/moles) in order to favor the accumulation of storage lipids and (potentially for the case of *Yarrowia lipolytica*) the secretion of secondary metabolites (i.e. citric acid). The most promising of the tested microorganisms, were further studied.

II.2.1.2. Growth of Rhodosporidium toruloides DSM 444 in glucose-based media for the production of biomass and lipids.

As it will be presented in the "Results and Discussion" section, the strain DSM 4444 was proved to be the most efficient convertor of glucose into SCO. Thus this strain was further studied on media composed of commercial glucose. This strain was initially cultivated in batch-flask trials, in media containing 50 g/L glucose supplemented with increasing NaCl concentrations (0, 10, 15, 25, 40, 60 g/L) in order to identify the potential effect of NaCl addition upon the microbial growth. Cultures were done under nitrogen-limited conditions (carbon-to-nitrogen ratio equal to 106 mol/mol) in order to stimulate lipid accumulation. Moreover, an experiment without NaCl addition was included that served as control. The pH medium was maintained between 5.0 and 6.0, and the incubation temperature was T=26^{o}C. Then the yeast were cultivate in media containing 4% (w/v) of NaCl with Glc_i=50 and 100 g/L. Following, experiment were performed under non-aseptic conditions (thermal treatment of the medium in *c.* T=100°C for 7 min before inoculation, inoculation performed with 3 mL – 6% v/v inoculum of exponential pre-culture, in shake-flacks with Glc_i=50 and 100 g/L glucose in media supplemented with 4% w/v of NaCl). Finally, aseptic batch-bioreactor experiments (total volume 2.5 L; working volume 2.0 L, T=26±1°C, agitation rate 300 rpm, pH=6.0±0.1) were carried out under nitrogen-limited conditions with Glc_i=50, 100 and 150 g/L, inoculated with 150 mL of exponential pre-culture, before finishing with a fed-batch bioreactor trial employed in order to further ameliorate the production of microbial lipid by *Rhodosporidium toruloides* (in all these bioreactor trials, NaCl had been added at 4% w/v).

II.2.1.3. TOPW-based growth media supplemented with commercial glucose for the production of metabolic compounds

After the successful growth and production of biomass and SCO by *Rhodosporidium toruloides* in glucose-based media containing significant initial quantities of NaCl, the next step was the application of growth of this microorganism on salty wastewater supplemented with glucose. Moreover, literature indicates that strains of the yeast *Yarrowia lipolytica* can present growth and production of metabolites on media presenting increased concentrations of NaCl (Tomaszewska et al., 2012; 2014), while, as it will be presented in the section "Results and Discussion", the employed strain *Yarrowia lipolytica* ACA YC 5033 presented interesting production of citric acid during growth on glucose. Therefore, in this part of work, it was desirable to perform trials in salty culture media (TOPW-based growth media) enriched with commercial glucose in order to assess the potential of these microorganisms in this wastewater not previously studied, with the perspective of the (partial) replacement of tap water by this salty wastewater in fermentation processes.

TOPWs, containing 1.4 g/L phenolic compound and 6% w/v of NaCL were diluted in several ratios to yield in liquid media presenting various initial phenolic compound (*c.* 1 g/L and 0.75 g/L) with corresponding value of 3.5% (w/v) and 3.0%(w/v) of NaCL respectively. Commercial glucose (with 95% w/w purity and impurities composed of maltose (2% w/w), malto-dextrines (0.5% w/w), water (1.5% w/w) and salts (0.5% w/w) were added to TOPWs.

The yeast strains *Rhodosporidium toruloides* and *Yarrowia lipolytica* were cultivated in batch flaks cultures under nitrogen limitation and carbon limitation in TOPW-based growth media diluted in several ratios. Non-aseptic shake flasks culture (thermal treatment and inoculation as previously) were also performed in TOPWs containing 3% (w/v) of NaCl enriched with 50 and 100 g/L glucose. Finally, aseptic bioreactor of *Rhodosporidium toruloides* and *Yarrowia lipolytica* (see previously) were carried out under nitrogen limited condition in TOPW-base media containing 3% (w/v) of NaCl, enriched with Glc_i=135 g/L and Glc_i=100 g/L respectively, inoculated with 150 mL of pre-culture.

II.2.2. Raw glycerol growth media for the production of biomass, lipids and intra-cellular polysaccharides as main metabolic products

II.2.2.1. Initial screening of yeast strains on glycerol based media.

Fives yeast strains, namely *Rhodosporidium toruloides* (DSM 4444, NRRL Y-27012) *Cryptococcus curvatus* NRRL Y-27012, *Yarrowia lipolytica* (ACA YC 5033, ACA YC 5029) *Lipomyces starkeyi* DSM 70296, were tested on media composed of glycerol at initial glycerol concentration ($Glol_0$) =30 g/L under nitrogen-limited conditions (utilization of peptone at 0.75 g/L and yeast extract at 0.5 g/L; initial molar ration employed at *c.* 100 mol/mol). The pH was maintained *c* 6.0 and cultures were incubated at T=28±1°C at 180±5. Salts were added having the final concentration mentioned in Table II.1.

II.2.2.2. Growth of Rhodosporidium toruloides NRRL Y-27012 and Lipomyces starkeyi DSM 70296 yeast strain in glycerol for the production of biomass, lipids and intra-cellular polyssacharides.

Shake-flask experiment of *Rhodosporidium turoloides* NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296 strains were carried out asepticaly under nitrogen limitation (0.5 g/L yeast extract and 0.75 g/L peptone used as nitrogen sources) in at increasing $Glol_o$ concentrations (*c.* 30, 50, 95, 120, 185g/L). The pH was maintained *c* 6.0, incubated at T=28±1°C at 180±5. For more information about pH correction and maintenance see paragraph II.2. Salts were added having the final concentration mentioned in Table II.1.

II.2.3. Lactose, sucrose and residues containing these compounds (molasses and cheese-whey) used as substrates by *Cryptococcus curvatus* NRLL Y-1511

II.2.3.1. General consideration

In the third part of this investigation and after having performed experiments on glucose- or glycerol-based media, it was desirable to perform trials on media composed from either lactose or sucrose employed as the sole carbon source. By taking into consideration that, in general, the yeast species *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Rhodotorula glutinis* and *Lipomyces starkeyi* cannot easily consume either sucrose or lactose (or both of these carbon sources for wild strains of the species *Yarrowia lipolytica*), in this last

part of the investigation, the trials were performed exclusively with the strain *Cryptococcus curvatus* NRRL Y-1511. It is noted that the above-mentioned strain (NRRL Y-1511) has never previously been studied in relation with its potential of producing SCO and other metabolic compounds of biotechnological interest (e.g. intra-cellular polysaccharides, extra-cellular enzymes) during growth on these abundant carbon sources.

II.2.3.2. Growth on lactose-based media

Shake-flasks experiments were carried out in commercial lactose and deproteinized and concentrated cheese whey in order to evaluate the ability of *Cryptococcus curvatus* to convert these substrates into biomass, lipids and intra-cellular polysaccharides. The yeast strain was firstly cultivated under nitrogen limited-conditions, three initial lactose concentrations were employed (40, 60 and 80 g/L), whereas extra-cellular nitrogen concentration (0.5 g/L yeast extract and 0.75 g/L peptone) was maintained in all media. The pH was maintained to *c.* 6.0, cultures were incubated at T=28±1°C at 180±5. Salts were added having the final concentration mentioned in Table II.1. Following, the yeast strain *Cryptococcus curvatus* was cultivated under carbon limited-conditions (0.5 g/L yeast extract and 5.5 g/L peptone) in aseptic conditions. After performance assessment of the yeast strain on lactose-based media, a trial was performed using deproteinized and concentrated cheese-whey containing ~80g/L lactose and ~9 g/L protein as substrate. Culture conditions were maintained as in trial with lactose based media.

II.2.3.3. Growth on sucrose-based media

Lastly, *Cryptococcus curvatus* yeast strain was cultivated under nitrogen limited-conditions, using sucrose as the sole carbon source. Two initial sucrose concentrations were employed (40 and 80 g/L), whereas constant extra-cellular nitrogen concentration (0.5 g/L yeast extract and 0.75 g/L peptone) was maintained in all media. As in the trials on lactose, the pH was maintained to the value of *c.* 6.0, and the cultures were incubated at T=28±1°C at 180±5. Salts, as previously, were added at a final concentration mentioned in Table II.1. Following, the yeast strain *Cryptococcus curvatus* was cultivated under carbon limited-conditions (0.5 g/L yeast extract and 5.5 g/L peptone) in aseptic conditions. Maintaining the same culture condition as the trial with sucrose base media, a trial were performed in carbon-limited media (0.5 g/L yeast extract and 5.5 g/L) using molasses (52% sucrose and ~15 g/L

protein) as substrate. Given amounts of commercial sucrose was added to molasses in order to obtained 80 g/L total sugar.

The flow charts of all aforementioned experimental procedures are shown in the following figures:

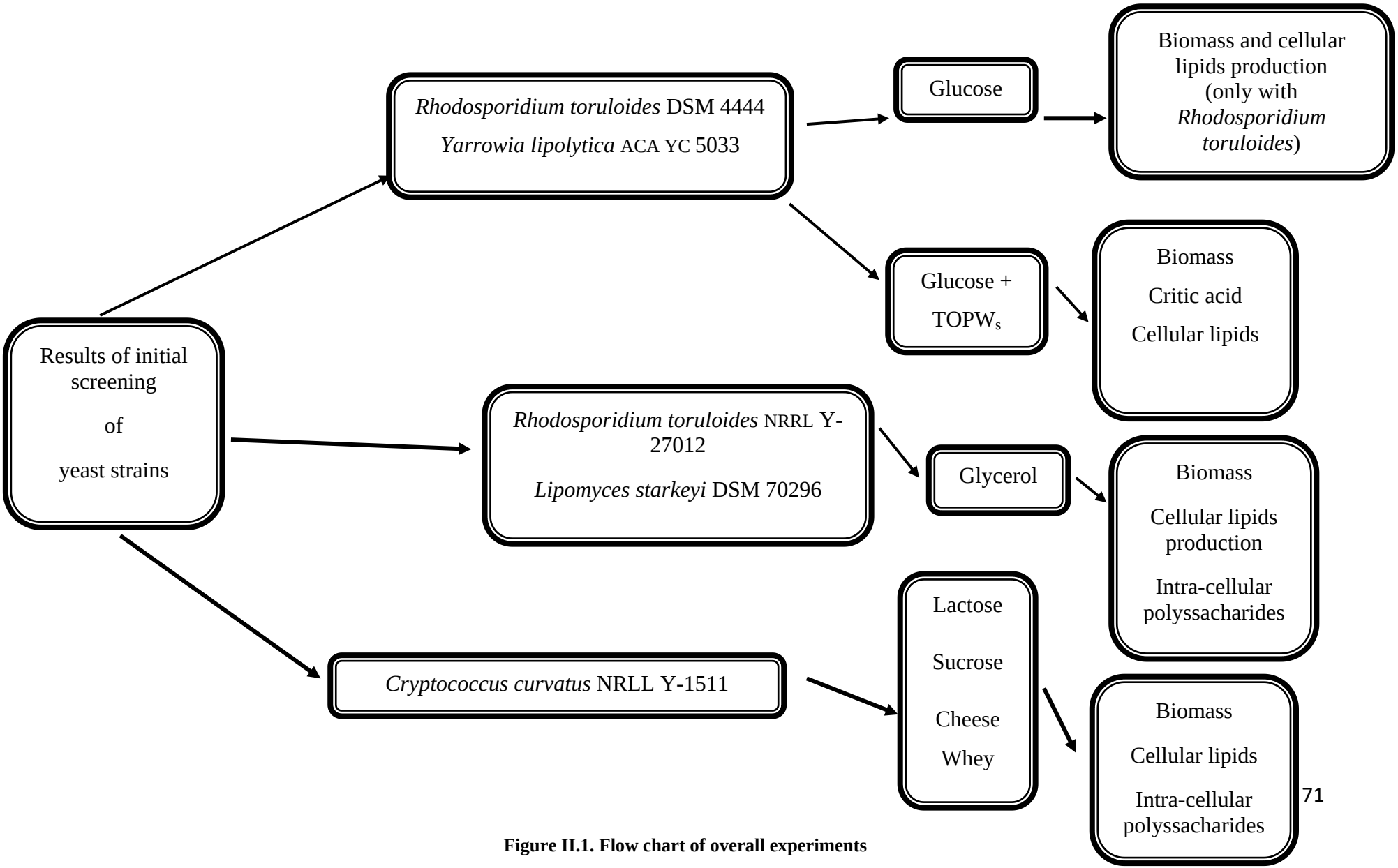


Figure II.1. Flow chart of overall experiments

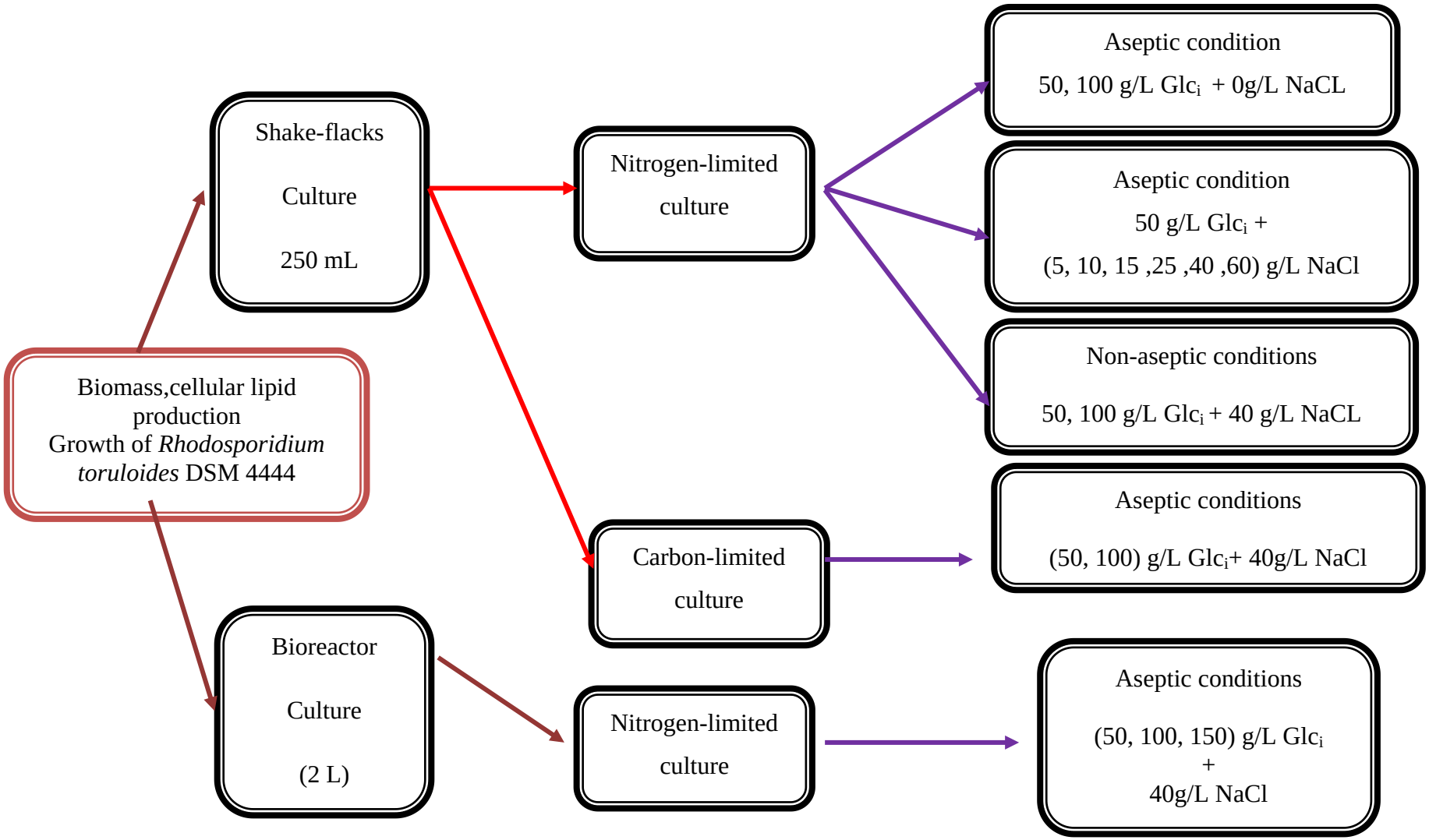


Figure II.2. Flow chart of experiments for the production of biomass and cellular lipids by *Rhodosporidium toruloides* DSM 4444 strains cultivated in glucose on nitrogen and carbon limited media under aseptic and non aseptic culture.

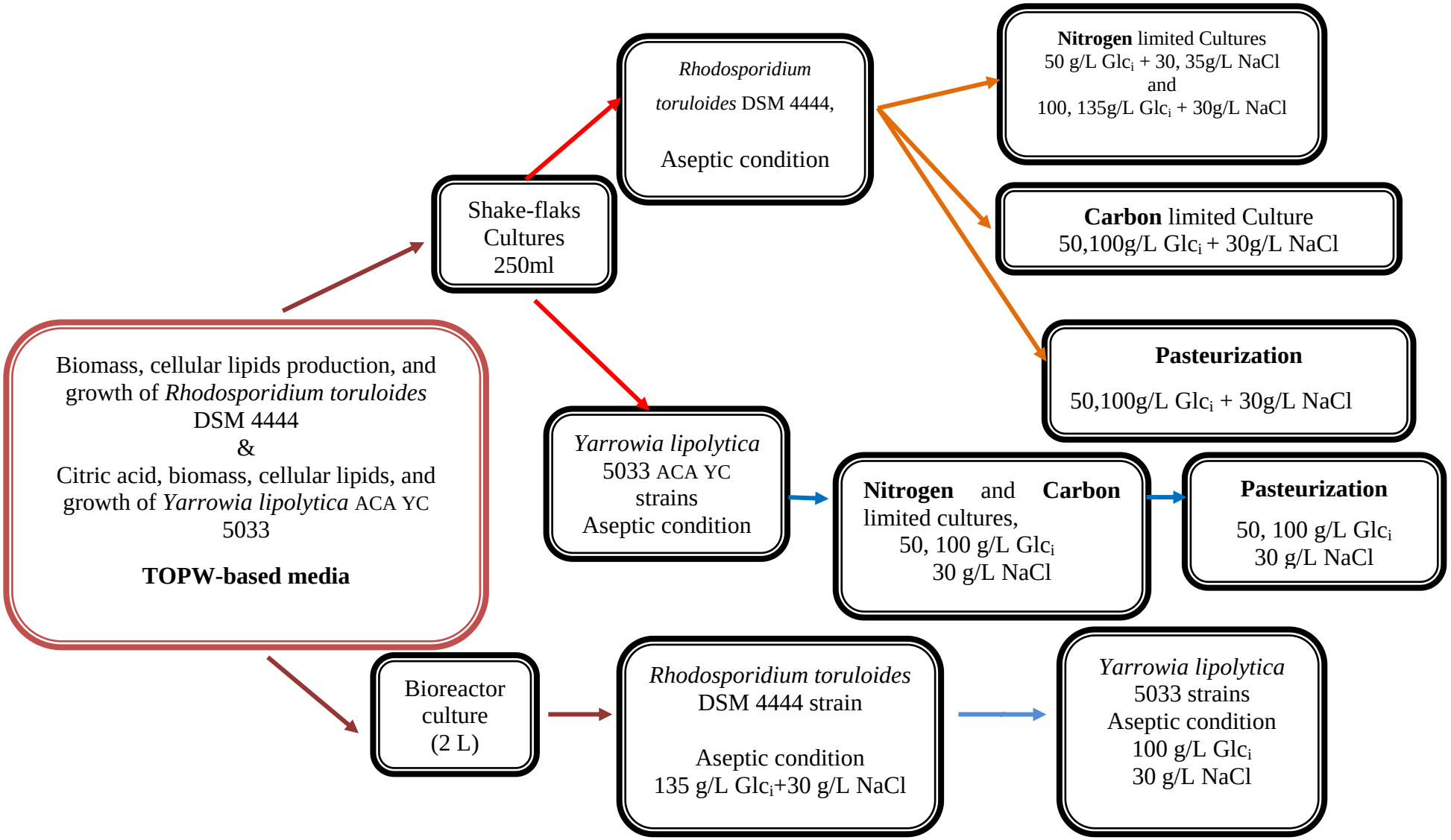


Figure II.3. Flow chart of experiments for the production of biomass, cellular lipids and Citric acid by *Rhodosporidium toruloides* DSM 4444 and *Yarrowia lipolytica* ACA YC 5033 strains cultivated on TOPWS based media in nitrogen and carbon limited media under aseptic and non aseptic culture

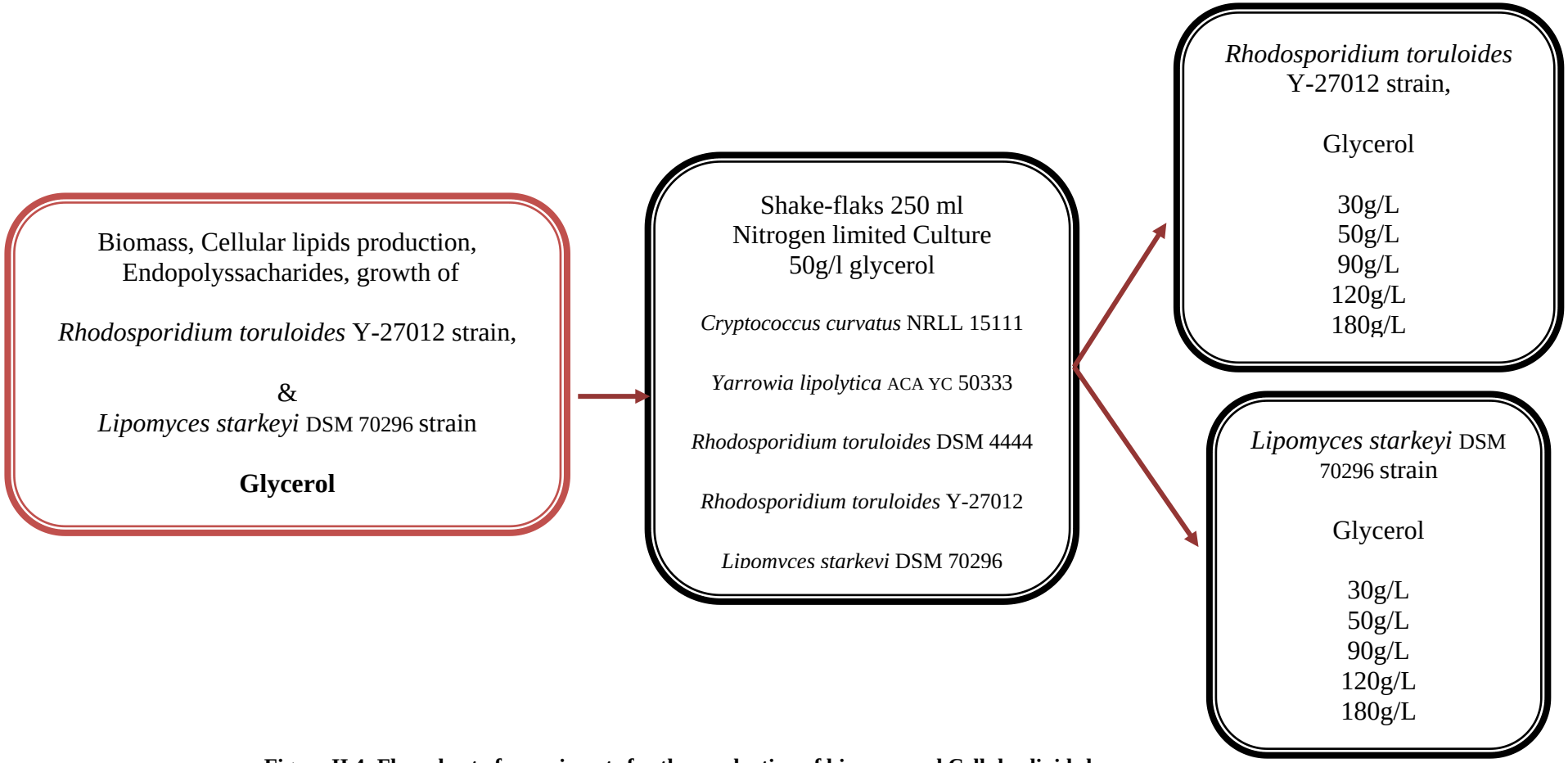


Figure II.4. Flow chart of experiments for the production of biomass and Cellular lipids by *Rhodosporidium toruloides* Y-27012 and *Lipomyces starkeyi* DSM 70296 strains cultivated on glycerol under nitrogen limited media.

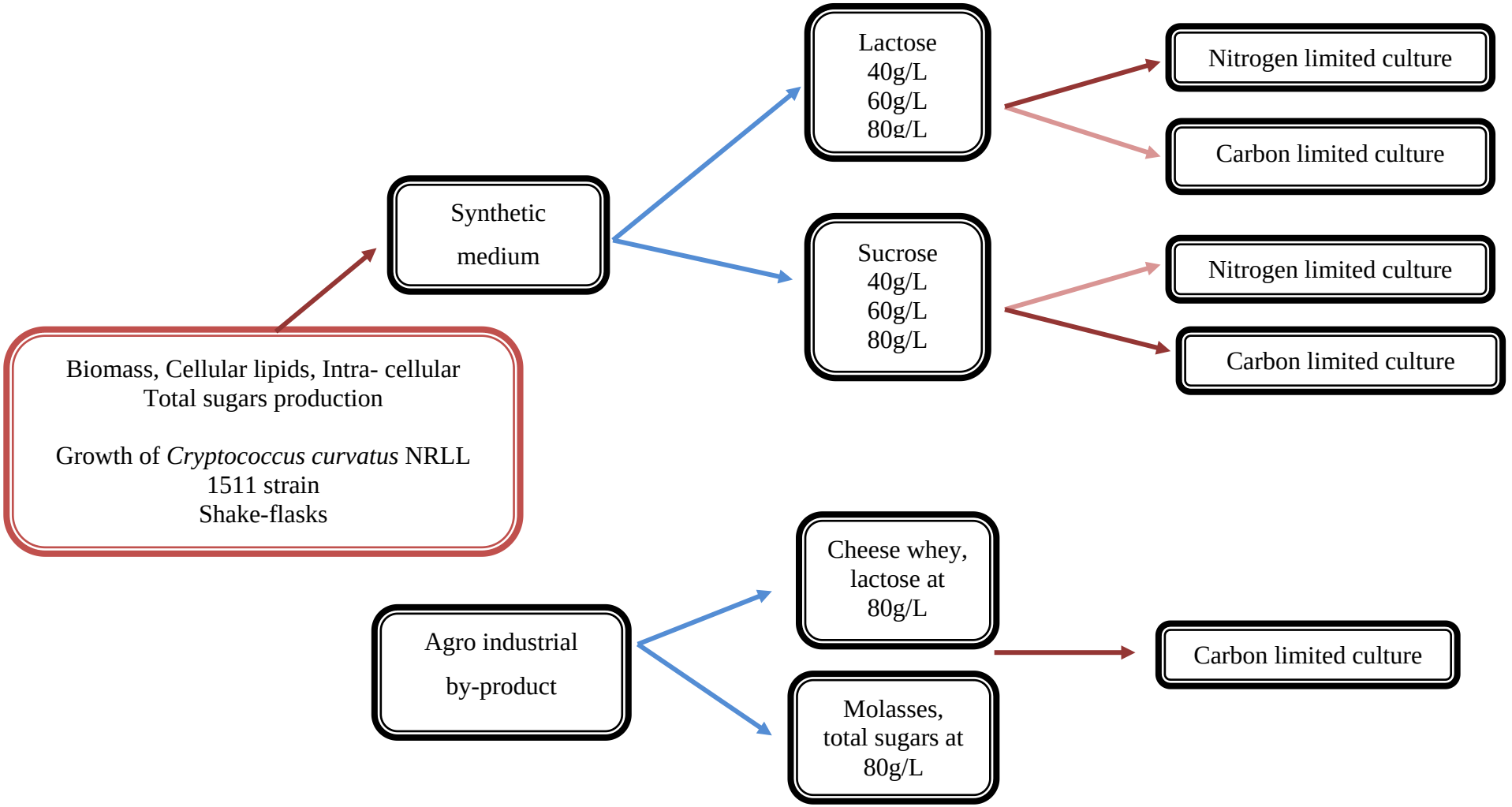


Figure II.5. Flow chart of experiments for the production of biomass, Cellular lipids, Intra- cellular Total sugars production by *Cryptococcus curvatus* cultivated in lactose, sucrose, cheese whey and molasses media.

II.3. Analyses

The flow chart of experimental analyses performed in this study (described as follows) is presented schematically in Fig. II.17.

II.3.1. Determination of pH

For the determination of the medium pH (for the shake-flask experiments) a Jenway pH meter 3020 apparatus was used. The correction of the medium pH was maintained at the desired value by periodically (and aseptically concerning the trials performed under sterile conditions) adding into the flasks, quantities of KOH 5 mol/L or HCl 1 mol/L. The exact base or acid solution volume needed for pH correction was evaluated by measuring the volume of the solution required for pH correction in one (at least) flask. Following, the appropriate volume of base or acid was (aseptically) added in the remaining flasks and the value of the pH reached was verified to be the desired one.

II.3.2. Dissolved oxygen (DO) determination

For the determination of DO of the shake-flask cultures, a Lonibond Sensodirect OXI 200 (Dortmund, Germany) oxygen meter was used. While the shaker being in operation the electrode was set in a flask [and under stable (agitation and temperature) conditions after ~10 min] giving a % DO value. Following, the shaking was terminated and for 30 sec the value of DO was noted every 5 sec showing reduction.

II.3.3. Biomass determination

Yeast cell mass was harvested by centrifugation at Universal 320R-Hettich centrifuge at 9000 rpm for 10 min at T=21±1°C, washed once with distilled water and centrifuged again. Biomass concentration (X, g/L) was determined gravimetrically from dry cell weight (DCW) at temperature T=100°C until constant weight (usually within ~24 h).

II.3.4. Glucose and lactose determination

Glucose and lactose were determined by 3,5-dinitrosalicylic acid (DNS) assay (Miller 1959). 0.5 mL of the sample was mixed with 0.5 mL DNS reagent and were placed in a test tube and stirred at the vortex mixer. Afterwards the whole mixture was placed in boiling water for exactly 5 minutes and left to cool down. Finally, 5 mL distilled water were added to the tube and after stirring, absorbance was measured at 540 nm (Hitachi U-2000

spectrophotometer). The calibration curve in g/L of glucose and lactose is show at the next figures.

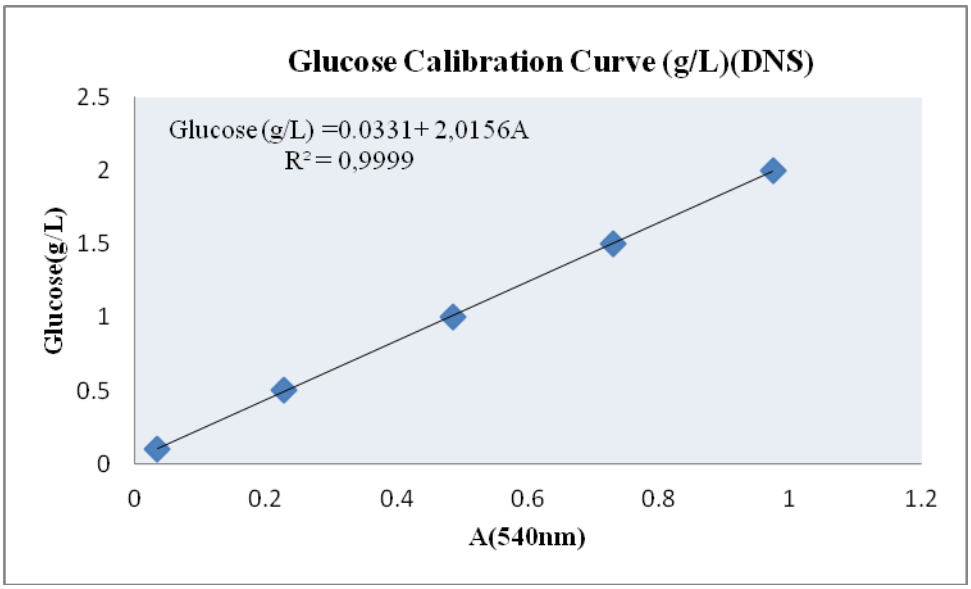


Figure II. 6. Calibration Curve of Glucose (DNS).

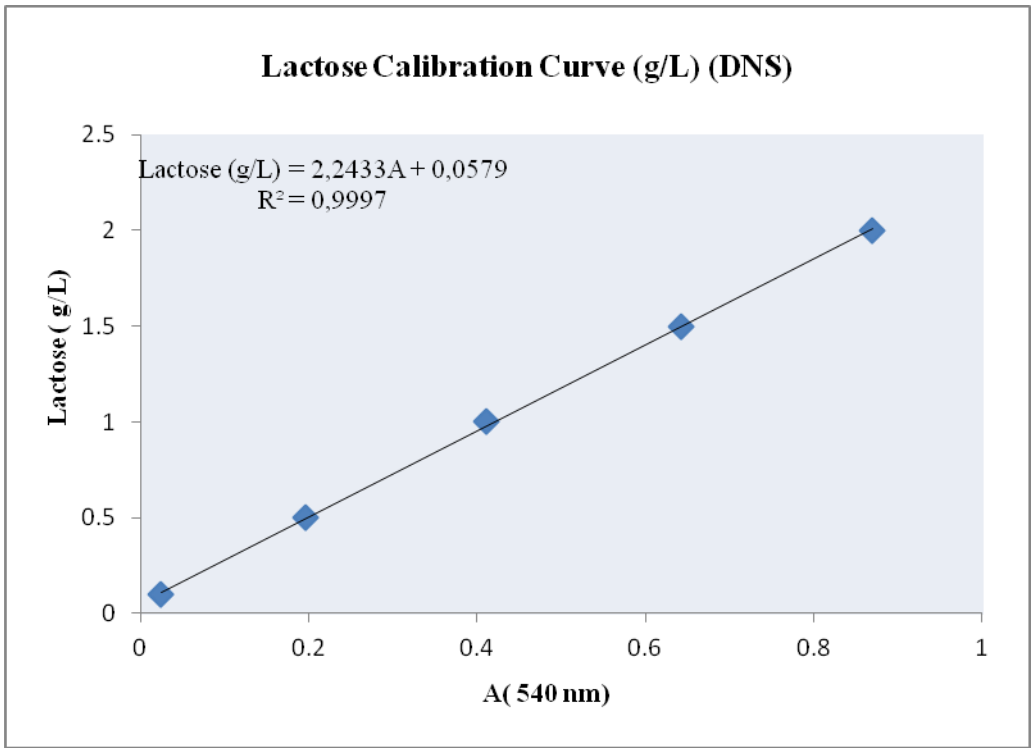


Figure II.7. Calibration curve of lactose (DNS).

II.3.5.Citric acid and glycerol determination

Citric acid and glycerol consumption were determined with the aid of High Performance Liquid Chromatography (HPLC) analysis. The supernatant of the fermentation

medium was centrifuged and filtered with 0.2 µm filter. The inject volume was 20 µL. The HPLC apparatus (Waters Association 600E) was equipped with a UV (Waters 486) and RI (Waters 410) detector. The column used for the separation of compounds was Animex HPX - 87H (Biorad) (30 cm × 7.8 mm), the mobile phase was 0.005 mol/L H_2SO_4, the column temperature was 65°C and the flow rate was 0.6 ml/min. Citric and iso-citric acid were not sufficiently separated and the reported concentration corresponds to the sum of these acids, expressed as total citric acid. The chromatographic HPLC software used was the "Empower Ltd". Calibration curves for the analyzed compounds are presented in the following figures.

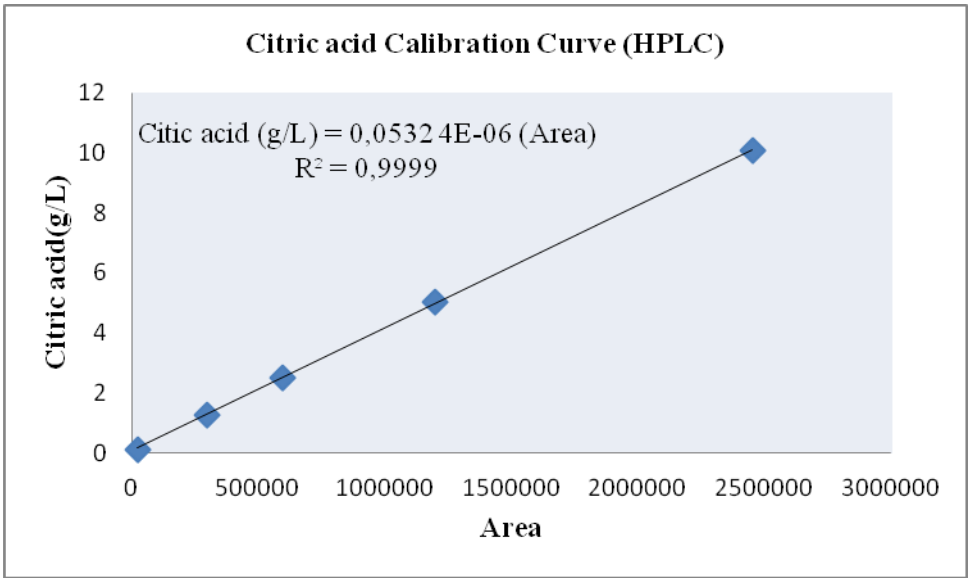


Figure II. 8. Calibration curve of Citric Acid (HPLC)

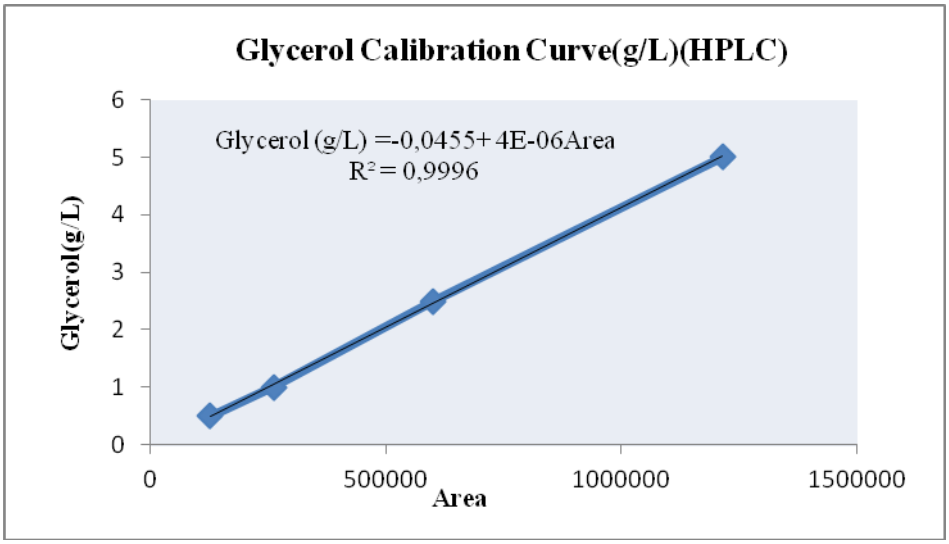


Figure II. 9. Calibration curve of Glycerol

II.3.6. Total sugars determination

For the hydrolysis of sucrose into glucose and fructose [at the experiments including molasses (as they consist of 48-52% sucrose)], sucrose (as total sugars equivalent in sucrose) was determination by the colorimetric method of Dubois et al. (1956). Briefly, 2 mL of culture medium in which cells were previously discarded with the aid of centrifugation were properly diluted and were mixed with a 50% (w/v) phenol solution and 5 mL of concentrated sulfuric acid (37% H_2SO_4). The mixture was vortexed and kept in cooling water for 10 minutes. Absorbance was measured at 490 nm with a Hitachi U-2000 spectrophotometer. Simultaneously, the reducing sugars concentration was determined according to DNS method (Miller, 1959) and was expressed as glucose equivalent.

II.3.7. Intracellular polysaccharides (Intra-cellular total sugars) determination

Total intracellular polysaccharides (IPS, expressed as g/L and % of DCW) were measured based on a modified protocol published by Liang et al. (2009). Briefly, 0.05 g of DCW was acidified by adding 10 mL HCl (2.5 N). The acidified solution was then hydrolyzed at 100°C for 30 min and neutralized to pH 7 with 10 mL of KOH (2.5N), filtered through Whatman filter paper and subjected to reducing sugar content determination (glucose equivalents), according to the 3,5-dinitrosalicylic acid method (Miller, 1959). Total sugar composition of IPS produced after filtration was analyzed with the aid of HPLC (see previously for the conditions of the analyses performed). Calibration curves for the analyzed compounds found into the intra-cellular polysaccharides (glucose, fructose and galactose) are presented in the following figures.

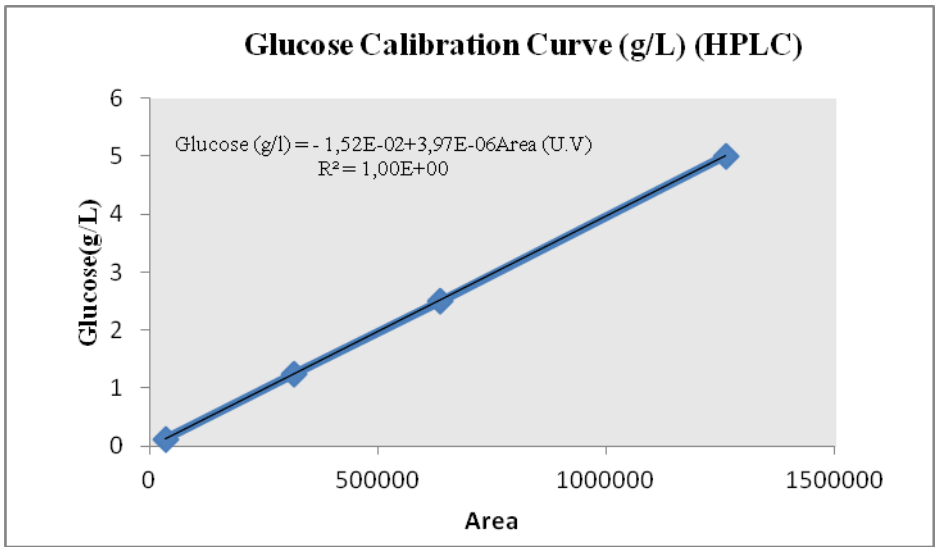


Figure II.10 Calibration Curve of Glucose (g/L) (HPLC)

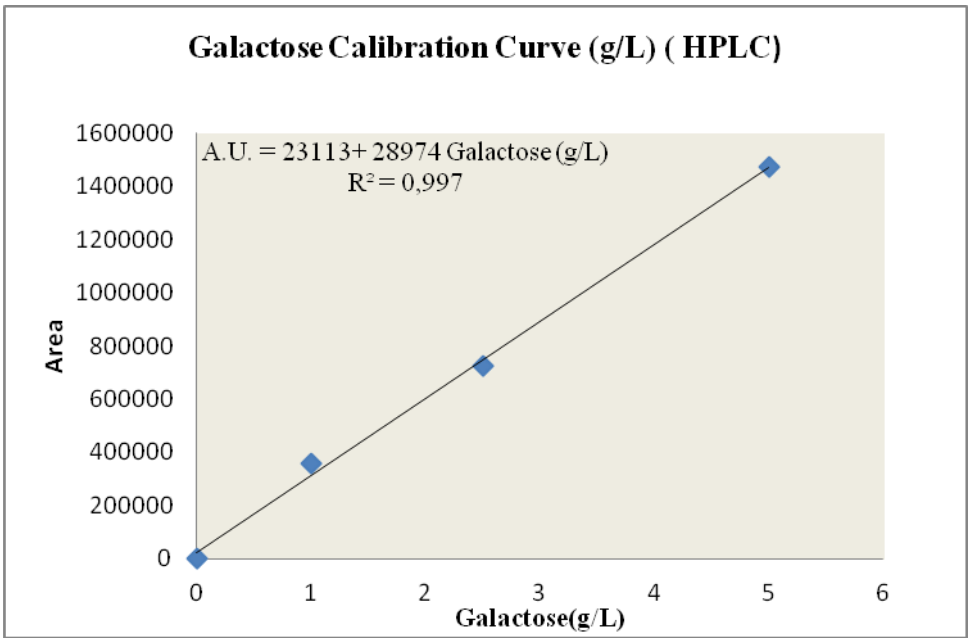


Figure II.11. Calibration Curve of Galactose (HPLC)

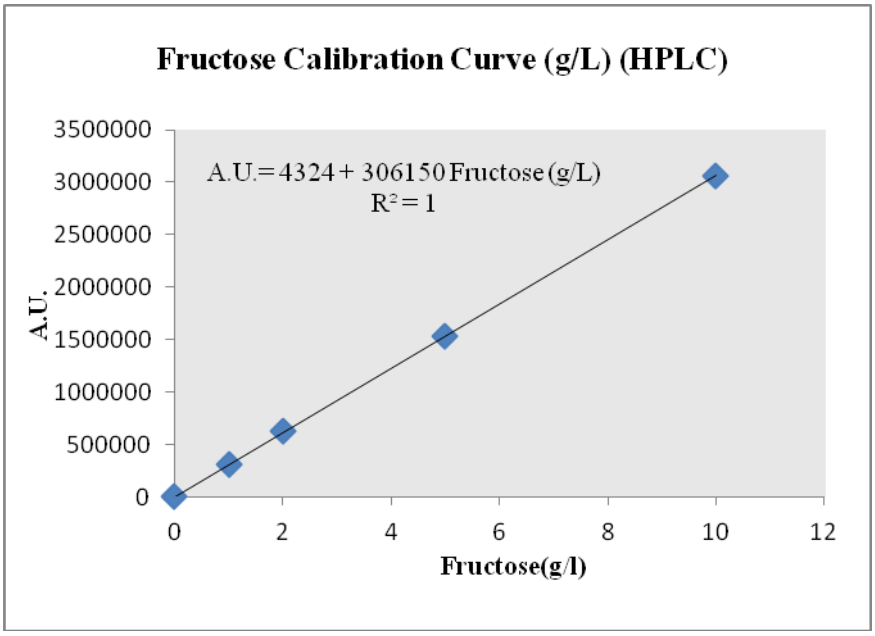


Figure II.12. Calibration Curve of Fructose (HPLC)

II.3.8. Invertase and β-Galactosidase activity determination

Invertase activity (IA, U/mL) was determined using the method of Oliveira et al. (2006), with slight modifications. In particular, 0.1 mL of cell and crude extract was mixed with 0.9 mL of 0.05 M phosphate buffer (Na_2HPO_4-NaH_2PO_4, at pH=7) and 1% (w/v) sucrose. After incubation of the mixture for 30 minutes at 50°C, the produced reducing sugars were determined by DNS method (Miller, 1959). One unit of invertase (IU) is defined as the amount of enzyme which liberated 1 μmole of glucose per minute per mL under the assay conditions. β-Galactosidase activity was measured based on a modified protocol published by Rose and Botstein (1983). Briefly, 0.9 mL of buffer (Na_2HPO_4-NaH_2PO_4, at pH=7) were added to 100 μL of crude extract (culture medium containing cells or supernatant after centrifugation). The mixture was incubated in water bath at T=28°C for 5 min. Then the reaction was initialized by adding 0.2 mL of o-nitrophenyl-β-D-galactosidase (ONPG) and after 30 min, 0.5 mL of Na_2CO_3 was added in order to stop the reaction. The amount of o-nitrophenol released was determined spectophotometrically by measuring the A_{420} of the solution. One enzyme unit (EU) is defined as the quantity of enzyme that catalyzes the liberation of 1 μmole of o-nitrophenol from (ONPG), per minute per mL under the assay conditions. Moreover, besides the assay of β-galactosidase, in some of the performed trials, in order to investigate whether lactose was extra-cellularly hydrolyzed by the microorganism, the culture medium was also analyzed with the aid of HPLC (see previously the conditions of

the analysis) in order to potentially quantify the released galactose and glucose, resulting from the cleavage of lactose.

II.3.9. Total cellular lipids and FAMEs determination

Quantitative and qualitative analysis of total intra-cellular lipids was done. Generally total intracellular lipids were extracted with a mixture of chloroform and methanol 2:1 (v/v) (Folch et al., 1957; Papanikolaou et al., 2001). Solvents were removed at reduced pressure (Büchi Rotavapor R-114) and lipids were determined gravimetrically (quantitative analysis). However, in the case of total lipid production by *Lipomyces starkeyi*, an additional step was necessary; as described by the modified protocol published by Liang et al. (2009), the half of the dry biomass was acidified by adding 10 mL HCl (2.5 N). The acidified solution was then hydrolyzed at 100°C for 30 min and neutralized to pH 7 with 10 ml of KOH (2.5 N). Then the biomass were separated and mixed with a mixture of chloroform and methanol as describe by Papanikolaou et al. 2001. Solvents were removed as describe before.

Lipids were converted to their fatty acid methyl-esters (FAMEs) and analyzed in gas chromatograph-flame ionization detector (GC-FID) apparatus (Fisons 8000 series) according to Fakas et al. (2006). FAMEs were identified by comparison with authentic standards. Because of the fact that fatty acids are not highly volatile and in order to be detected in GC analysis, their transformation to the respective volatile methyl esters is necessary. The procedure of free fatty acids transformation into the respective methyl-esters is called trans esterification and at the present study was done according to AFNOR (1959) method and as described in Fakas et al. (2006). This procedure includes two stages (Fig. 2.10.). The first stage is under alkaline environment whereas the second stage is under acidic environment. During the first stage the nucleophilic substitution at the molecule of triglycerides and phospholipids, resultσ in the formation of the respective methyl-esters of fatty acids. Simultaneously the, already existing, free fatty acids are transformed to the respective soaps reacting with sodium methoxide solution. At the second stage, soaps are converted into the respective methyl-esters.

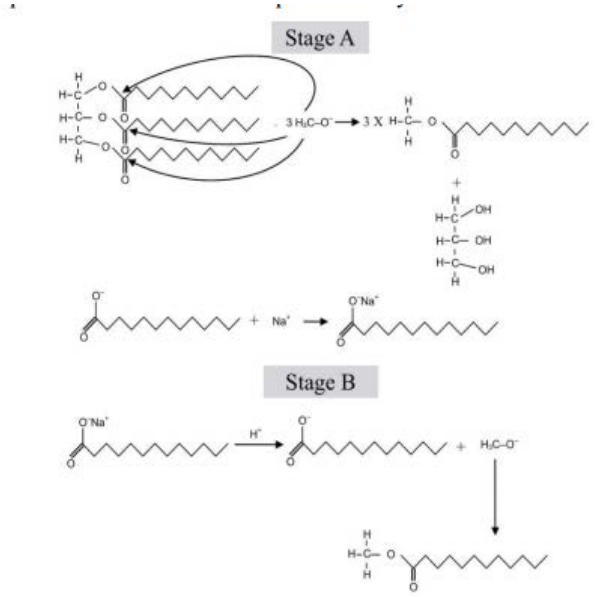


Figure II. 13. Transesterification of free fatty acids, Stage A: under alkaline environment; Stage B: under acidic environment (Μακρή, 2011).

At the evaporation/boiling flask - including the gravimetrically determined total lipids - 10 mL of sodium methoxide solution [CH_3ONa^+; 1% of sodium metal in mixture of methanol and benzene (70:30 v/v) and phenolphthalein] and some boiling chips are added. The flask is then placed on a heating mantle with a vertical condenser attached at 80 °C for 20 min. Following, an amount of hydrocloride methanol (CH_3OH-HCl; 20 mL acetyl chloride mixed with 250-mL of methanol) is added until the solution solvent turns from pink to white. Boiling continues for 20 more minutes.

The addition of an amount of deionized water terminates the reaction. Finally the solution is transferred into a separator funnel where 6 mL of (n-) hexane is added and methyl-esters are extracted into hexane after strong stirring. Finally, the water phase is discarded and a small amount of sodium sulfate anhydrous (Na_2SO_4) is added to the organic phase (for the absorption of any remaining water).For the analysis of FAMEs at the GC with an FID detector, 1 μL of methyl-esters sample was injected. The carrier gas (He) flow was 1.38 mL/min, H_2 60 kPa and O_2 110 kPa. The stationary phase was Fused silica WCOT: CP-Sill 88 (50 m x 0.32 mm and DF=0.20 μm film thickness). The column temperature was stable at 200 °C for 20 min.

II.3.10. Phenolic compounds concentration determination

Phenolic compounds concentration in the culture media was determined according to Folin -Ciocalteu (FC) method measured at 750 nm and expressed as gallic acid equivalent (Slinkard and Singleton, 1977) (the calibration curve in g/L of gallic acid is show at Fig. II.15.). This method is based on the breakup of total phenols under alkaline environment with the addition of FC reagent which is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PM_{12}O_{40}$). The FC reagent is simultaneously reduced towards the respective oxides WsO_{23} and Mo_8O_{23} which are responsible for the blue colour resulting. In a test tube, 0.2 mL of supernatant (after appropriate dilution), 10.8 mL of distilled water, 8 mL of anhydrous sodium carbonate (Na_2CO_3; 75 g/L) solution and 1 mL of FC reagent. After two hours at room temperature, the absorbance was measured at 750 nm (Hitachi U-2000 spectrophotometer). In all trials, initial phenolic compounds concentration was determined after sterilization (when sterilization occurred).

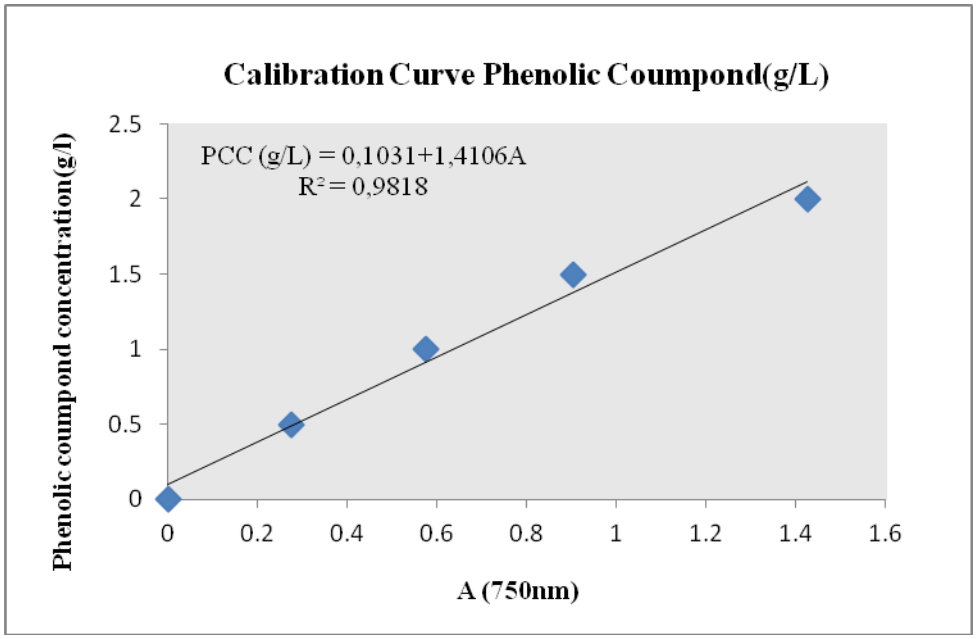


Figure II. 14. Calibration curve of phenolic compounds

II.3.11. NaCl determination

The amount of sodium chloride (NaCl) present in the sample was determined by a argentometry method, in which the sample solution is titrated against a solution of silver nitrate of known concentration (Yoder and Lester, 1919). 10 mL of sample were mixed with 90 mL of water and 1 mL of chloride. Afterwards, the dilution samples containing 1 mL of

chloride were titrated with a $AgNO_3$ solution (0.1N), the titration stopped when the mixture has got a red color. The volume of reactant $AgNO_3$ consumed is measured and used to calculate the concentration of NaCl present in the sample

II.3.12. Protein determination

Protein concentration were determined by Lowry methods assay (Lowry, 1951).1ml of the sample was mixed with 5ml $CaSO_4$ and were placed in a test tube and stirred at the vortex mixer. Afterwards the whole mixture was let in Room temperature for 20 min. Then 0.5ml of Folin reagent were added to the mixture, after vortex, the mixture were let ones again for 30 min in RT, the absorbance was measured at 540 nm (Hitachi U-2000 spectrophotometer). The calibration curve in g/L of Bovine Serum Albumin is show at the next figure.

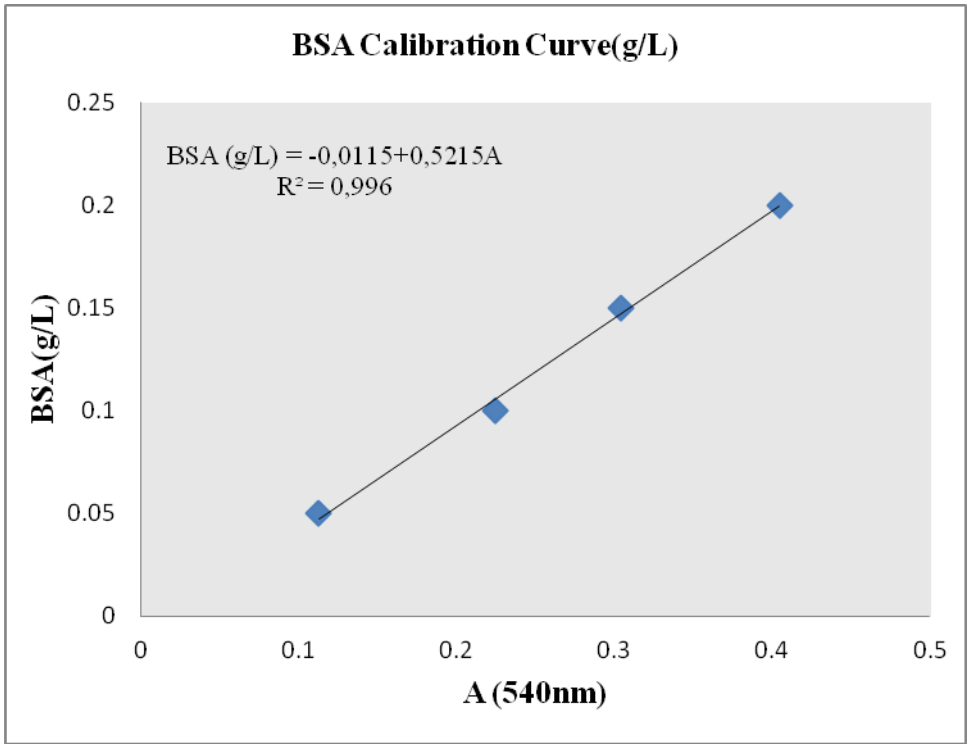


Figure II.15. Calibration curve of Bovine Serum Albumin

II.3.13. Free Amino Nitrogen determination

Free amino nitrogen (FAN) concentration in liquid samples was determined by the ninhydrin colorimetric method promulgated in the European Brewery Convention (Lie, 1973; Kachrimanidou et al., 2013). Samples were initially centrifuged at 5000 rpm for 10 min in order to collect the supernatant). In 2 mL of diluted sample, 1 mL of colour reagent was added in to each test tube. Then the mixture was transfer into a boiling water bath for exactly 16

minutes. After being cooled down for 20 minutes in a 20°C water bath, 5 mL of dilution reagent was added to each test tube. The absorbance was measured at 570 nm (Hitachi U-2000 spectrophotometer). Readings below 1.5 are recommended as the most accurate.

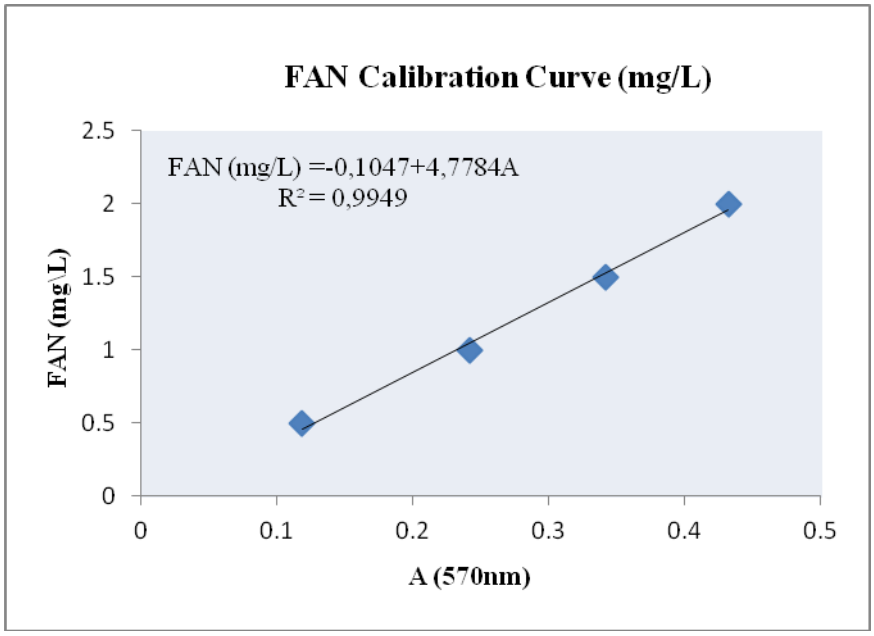


Figure II.16. Calibration curve of FAN

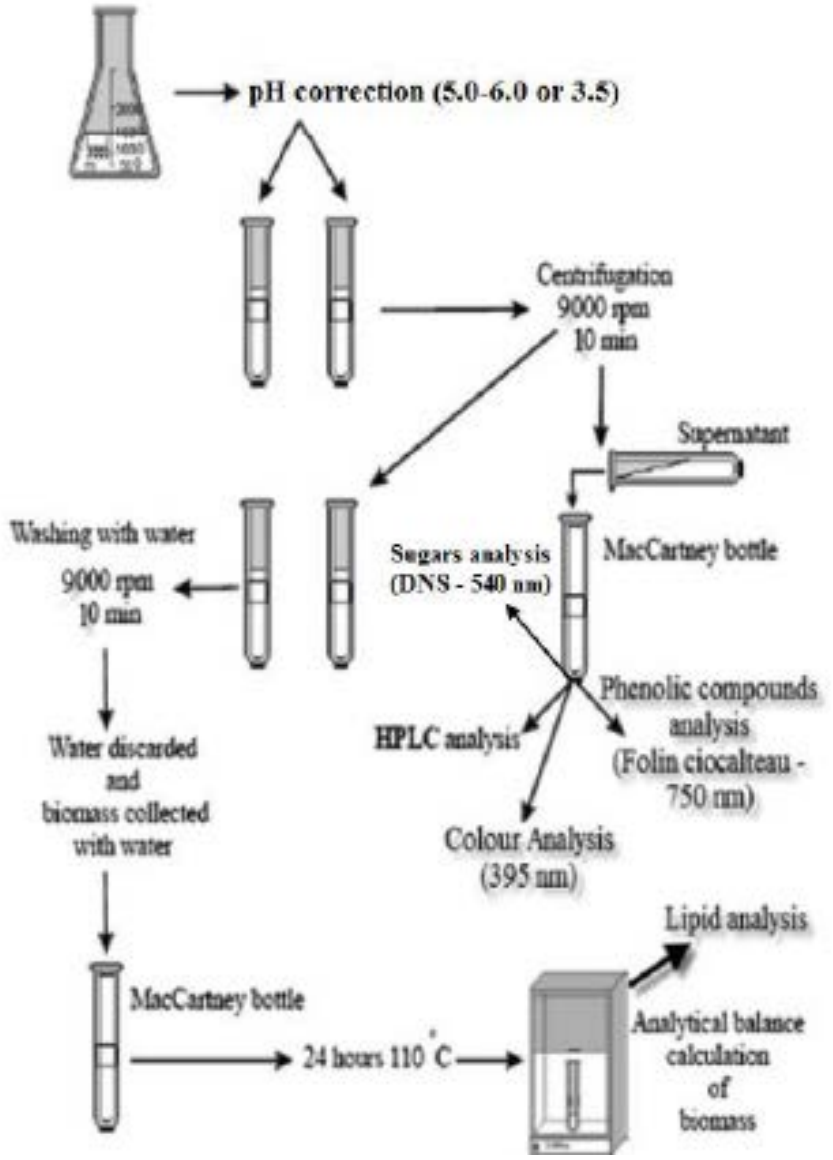


Figure II. 17. Flow chart of experimental analytical methods. Biomass harvesting by centrifugation and gravimetrical determination from dry weight, supernatant photometric (color, phenolic compounds and sugars) and chromatographic (total citric acid, glucose and FAMEs) analysis.

III-RESULTS AND DISCUSSION

III.1. Utilization of glucose as carbon source for the production of microbial lipid and other useful microbial compounds: impact of NaCl quantity upon the synthesis of cellular lipid; application of the process on a wastewater containing high NaCl quantities

In the first part of this study, it was desirable to study the potential single cell oil (SCO) production in some yeast strains growing in media containing glucose as sole carbon source. Firstly, a screening of 6 yeast strains growing on media containing an initial concentration of glucose (Glc_i) adjusted at *c.* 50 g/L under nitrogen-limited conditions in shake-flask experiments was performed. Thereafter, the most promising lipid-producing strain on media composed of glucose, namely *Rhodosporidium toruloides* DSM 4444, was cultivated in shake-flask and batch-bioreactor experiments in order to further demonstrate its capabilities towards SCO production. Lipid production was optimized as regards the initial concentration of NaCl added into the medium and the Glc_i concentration. Trials under non-aseptic conditions (in previously pasteurized media) were equally performed. Finally, fed-batch bioreactor trials under optimized cultures were performed.

After the successful growth and production of biomass and SCO by *Rhodosporidium toruloides* in glucose-based media containing significant initial quantities of NaCl, the next step was the application of growth of this microorganism on a salty wastewater supplemented with glucose. Moreover, literature indicates that strains of the yeast *Yarrowia lipolytica* can present growth and production of metabolites on media presenting increased concentrations of NaCl (Tomaszewska et al., 2012; 2014), during the initial screening study on glucose, the employed strain *Yarrowia lipolytica* ACA YC 5033 presented interesting production of citric acid. Therefore, the second part in this chapter referred to the application of the ability of *Rhodosporidium toruloides* DSM 4444 and *Yarrowia lipolytica* ACA YC 5033 yeast strains to grow and produce useful compounds (SCO, yeast biomass, citric acid) in wastewaters contain high NaCl concentration like the TOPWs (table olive processing wastewaters), with the perspective of the (partial) replacement of tap water by this salty wastewater in fermentation processes.

III.1.1. Introduction

Environmental concerns against petroleum-based energy sources and commercial products have driven scientific research towards alternative resources, as means of disengagement from fossil oil (Meng et al., 2009). Biodiesel consists one of the major renewable transportation fuels, deriving by trans-esterification process of long chain fatty acids of plant or animal origin. However, constant rising demand of biodiesel production competes with the availability of existing raw materials and as a result, other non-conventional oil resources are explored, mainly of non-edible nature (Vincente et al., 2009). In this light, scientific interest on microbial lipids as alternative source of oil has gain momentum the last decades.

Microbial oil production can be carried out by a number of heterotrophic (principally yeasts and fungi) or phototrophic (algae) organisms which are found to accumulate oil up to 80% of their dry weight (Ratledge, 1997; Papanikolaou and Aggelis, 2011a). This lipid, namely SCO, is mainly composed of neutral fractions (principally triacylglycerols-TAGs and to lesser extent steryl-esters) (Papanikolaou and Aggelis, 2010). Storage lipids of the oleaginous microorganisms, unable to integrate into phospholipid bilayers, cluster to form the hydrophobic core of the lipid bodies or oil bodies or lipid droplets (Papanikolaou and Aggelis 2010).

Generally, microorganisms that can accumulate more than 20% (w/w) of oil in their cells are classified as “oleaginous” (Ratledge, 1997). It has been well established in international literature that when culture is performed in media in which sugars or similarly metabolized compounds (e.g. polysaccharides, glycerol, etc) are used as carbon sources by the oleaginous microorganisms, the conditions required to trigger lipid production are usually met in a culture environment with carbon excess and (at least) one essential nutrient depletion (usually nitrogen) (Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2010; 2011a). This is the so-called de novo lipid accumulation process. On the other hand, lipid accumulation from hydrophobic substances (like oils or fats, fatty acids-FAs, fatty acid methylesters-FAMEs, soap-stocks, etc) used as the sole carbon and energy source (the so-called ex novo lipid accumulation) is performed by virtue of a radically different biochemical mechanism; culture triacylglycerols are hydrolyzed with the aid of (extra-cellular or cell-bounded) lipases and the liberated fatty acids (FAs) are incorporated inside the microbial cells or mycelia with

various incorporation rates (Aggelis and Sourdis, 1997; Papanikolaou et al., 2001). Substrate FAs are either assimilated for growth needs or become a substrate for intra-cellular bio-transformations. In the *ex novo* lipid accumulation process, lipid production is a growth associated process occurring simultaneously with cell growth, being entirely independent from nitrogen exhaustion from the culture medium (Aggelis and Sourdis, 1997; Papanikolaou et al., 2001; Papanikolaou and Aggelis, 2010; 2011a; 2011b).

As indicated in the previous paragraphs, SCOs could constitute the starting material for the synthesis of the "2nd generation biodiesel" (produced by virtue of the utilization of heterotrophic microorganisms that previously had accumulated lipid through fermentation of abundant sugar-based residues and renewable materials) or the "3rd generation bio-diesel" (produced via direct CO_2 sequestration through the use of autotrophic oleaginous algae) (Papanikolaou and Aggelis, 2011b; Papanikolaou, 2012). Obviously, concerning the 2nd generation biodiesel, only the conversion of sugars or related compounds in lipid is concerned; the ex novo lipid production process mainly refers to the utilization of low-cost hydrophobic materials as substrates, that through fermentation with the oleaginous microorganisms would be "up-graded", in order to finally present a "better" structure and/or composition compared with the initial fatty material (e.g. production of equivalents of exotic fats like cocoa-butter) (Gierhart, 1984a; Papanikolaou et al., 2001; Papanikolaou and Aggelis, 2010; 2011b). On the other hand, microbial oils synthesized through the de novo biochemical mechanism are mainly composed of saturated and unsaturated long chain fatty acids of 16 or 18 carbon atoms, and are generally regarded as acceptable raw material for biodiesel production (Vincente et al., 2009). However, the feasibility of sustainable bioprocess development for microbial oil production is mainly determined by the cost of both raw materials and fermentation process (Ykema et al., 1988; Koutinas et al., 2014a; 2014b). For this reason, numerous efforts are made to optimize microbial lipid production, through investigation and assessment of culture parameters such as culture conditions, substrate origin, as well as strain selection and development.

Our society currently faces the twin challenges of resource depletion and waste accumulation leading to rapidly escalating raw material costs and increasingly expensive and restrictive waste disposal legislation. The variety of food processes used in the food and drink industry globally generates food supply chain waste on a multi tons scale every year (Pfaltzgraff et al., 2013). Amongst others, the objectives of valorization regarding food

processing and/or agro-industrial by-products and waste streams, is based on the recovery of fine chemicals, as well as the production of (high) added-value metabolites though the implementation of chemical and biotechnological processes (Federico Federico et al., 2009; Papanikolaou and Aggelis, 2010; Pfaltzagraff et al., 2013). Table olives processing has long been and still remains, a very important traditional and industrial process in Spain, Greece, Turkey and many other countries around the Mediterranean Basin (Garrido Fernandez et al., 1997). There are three principal types of table olives: green, black and black through oxidation. These table olive processing wastewaters (TOPWs) contain large amounts of mineral and organic matter. The latter fraction contains a complex consortium of sugars an phenolic compounds, some nitrogenous compounds (especially amino acids), organic acids, tannins, pectins, carotenoids and oil residues (Gracia Gracia, 2006). It is therefore, obvious that these waste waters are highly polluting and are not simply treated by conventional methods.

Many strategies have been studied to reduce the environmental impact of these wastewaters such as washing water re-use, reduction of washing waters and debittering with low-concentration lyes. Any of these approaches has resulted in meeting the needs (Garrido Fermandez et al., 1997). In Greece, the preparation of natural black olives either in brine or in dry salt had developed a similar fashion to elsewhere from small family businesses to cooperative and artificial factories (Garrido Fernandez et al., 1997). The following table shows the estimated wastewater production in many countries.

Table olives production and estimated wastewater (average 2003-2009) (1,000 tons)

	Countries										
	Spain	Egypt	Turkey	Syria	Greece	Marocco	Algeria	Argentina	USA	Italy	Others
Production	503.3	299.6	230.8	143.3	108.2	98.3	81.9	80.8	79.7	65	249
Estimated wastewater (min)	252	150	115	72	54	49	41	40	40	32	125
Estimated wastewater (max)	3020	1798	1385	860	649	590	491	485	478	390	1494

(from Cappelletti et al. 2011)

Although, in a relatively scarce number of reports the optimization of biological treatment of green table olive processing wastewaters using *Aspergillus niger* has been performed (Ayed et al., 2013), TOPW-based media enriched with other low-cost carbon sources have not yet been used for the cultivation of molds, prokaryotic microorganisms, yeast and yeast-like species leading to potentially some partial detoxification (e.g. removal of phenolic compounds) of the waste, replacement of the typically used tap water in the biotechnological industry with the TOPW and also production of added-value compounds such as yeast or fungal biomass, citric acid and/or SCO.

Oleaginous yeasts species can be considered as advantageous microorganisms, compared to fungal and algal species, due to their relatively fast specific growth and their ability to assimilate a wide range of carbon sources (Papanikolaou and Aggelis, 2011b). Yeast strains belonging to the genera of *Cryptococcus* sp., *Lipomyces* sp., *Rhodotorula* sp., *Rhodosporidium* sp, *Trichosporon* sp. and *Yarrowia* sp. and. are among those reported as possible biodiesel producers (Papanikolaou and Aggelis, 2002; Galafassi et al., 2012). Among those, *Rhodosporidium toruloides* Y4 has been reported capable of producing 106.5 g/L of biomass containing 67.5% (w/w) of oil, during cultivation in a 15-L bioreactor, under fed-batch mode (Li et al., 2007), designated as the highest oil production from the particular strain so far. In terms of fatty acid composition, cultivation conditions and substrate have been found to influence the distribution of individual fatty acids of microbial oils. Nevertheless, they are of unsaturated nature, with oleic acid often being the predominant fatty acid in the total oil composition.

The objective of the study was to study the potential SCO production in some yeast strains growing in media containing glucose as sole carbon source. The most promising microorganism, namely *Rhodosporidium toruloides* DSM 4444, was further studied and SCO production by this microorganism was optimized, Finally, TOPWs supplemented with glucose were used as substrates by *Rhodosporidium toruloides* DSM 4444 and *Yarrowia lipolytica* ACA YC 5033 in order for the production of added-value compounds on this waste to be performed.

III.1.2. Results

III.1.2.1. Initial screening of yeast strains on glucose base media.

Five employed strains were tested on media composed of glucose at 50 g/L under nitrogen-limited conditions (utilization of peptone at 0.75 g/L and yeast extract at 0.5 g/L; initial molar ration employed at *c.* 100 mol /mol) in order to favor the accumulation of storage lipids and (potentially for the employed *Yarrowia lipolytica* strains) the secretion of secondary metabolites (e.g. citric acid, mannitol, etc) useful for the Food Industry. The obtained results of the performed trials as regards biomass and lipid production of the screened strains are illustrated in Table III.1.

Table III. 1. Experimental results originated from kinetics of yeast strains grown on glucose in shake-flask experiments. Representations of biomass (X, g/L), lipid (L, g/L), glucose consumed (Glc_{cons}, g/L), fermentation time (h) and lipid in dry biomass ($Y_{L/X}$, % w/w) when the maximum quantity of lipids in dry cell weight ($Y_{L/X}$, % w/w) and the maximum concentration of biomass (X, g/L). Culture conditions: growth on 250-ml flasks at 180±5 rpm, T=28±1°C, initial glucose concentration (Glc_i) ~50 g/L, initial molar ratio C/N~100 moles/moles, initial pH=6.1±0.1, pH ranging between 5.0 and 6.0, oxygen saturation higher than 40% (v/v) for all growth phases. Each experimental point is the mean value of two independent measurements (SE<10%).

Yeast strains	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)
Rhodosporidium toruloides DSM 4444	168	48.8	8.9	4.9	55.1
Rhodotorula glutinis NRRL YB-252	192	45.0	11.9	3.0	25.3
Rhodosporidium toruloides NRRL Y-27012	168	40.0	11.4	4.5	39.5
Yarrowia lipolytica ACA YC 5033	24	06.0	4.8	0.7	14.5
Lipomyces starkeyi DSM 70296	96	50.0	13.6	3.9	29.0

As far as the yeast strains that were screened in this part of the work were concerned, the yeast *Lipomyces starkeyi* DSM 70296 presented interesting biomass formation (13.6 g/L) with a concomitant dry biomass yield per unit of glucose consumed ($Y_{X/Glc}$) of *c.* 0.27 g/g. Furthermore the higher lipid production per unit of DCW ($Y_{L/X}$ =55.1% w/w – see Table III.1) were reached by *Rhodosporidium toruloides* DSM 4444 with a satisfactory biomass production (X≈8.9 g/L; $Y_{X/Glc}$≈0.18 g/g). Moreover, remarkable biomass production (X>11 g/L; $Y_{X/Glc}$≈0.27 g/g) followed by moderate accumulation of lipid during growth on glucose in

shake-flask experiments was observed for the strains *Rhodotorula glutinis* NRRL YB-252 and *Rhodosporidium toruloides* NRRL Y-27012. What was interesting in the current submission and coincided with previous information concerning the growth of several strains of the species *Yarrowia lipolytica* on hydrophilic carbon sources (e.g. glycerol, glucose, etc) (Makri et al., 2010; Sarris et al. 2011; Chatzifragkou et al., 2011a; Papanikolaou et al., 2013) was related with the fact that $Y_{L/X}$ values in this microorganism, at the first stages of the culture increased, despite the fact that nitrogen was found in excess into the growth medium ($Y_{L/X}$ reached to *c.* 14% w/w for the strain ACA YC 5033. Thereafter, cellular lipids were subjected to some biodegradation, with this biodegradation being accompanied by secretion into the medium of citric acid. Patters of changes of biomass (g/L), citric acid (g/L) and lipid in biomass (%, w/w) for *Yarrowia lipolytica* ACA-YC 5033 yeast strain are illustrated in Fig. III.1..

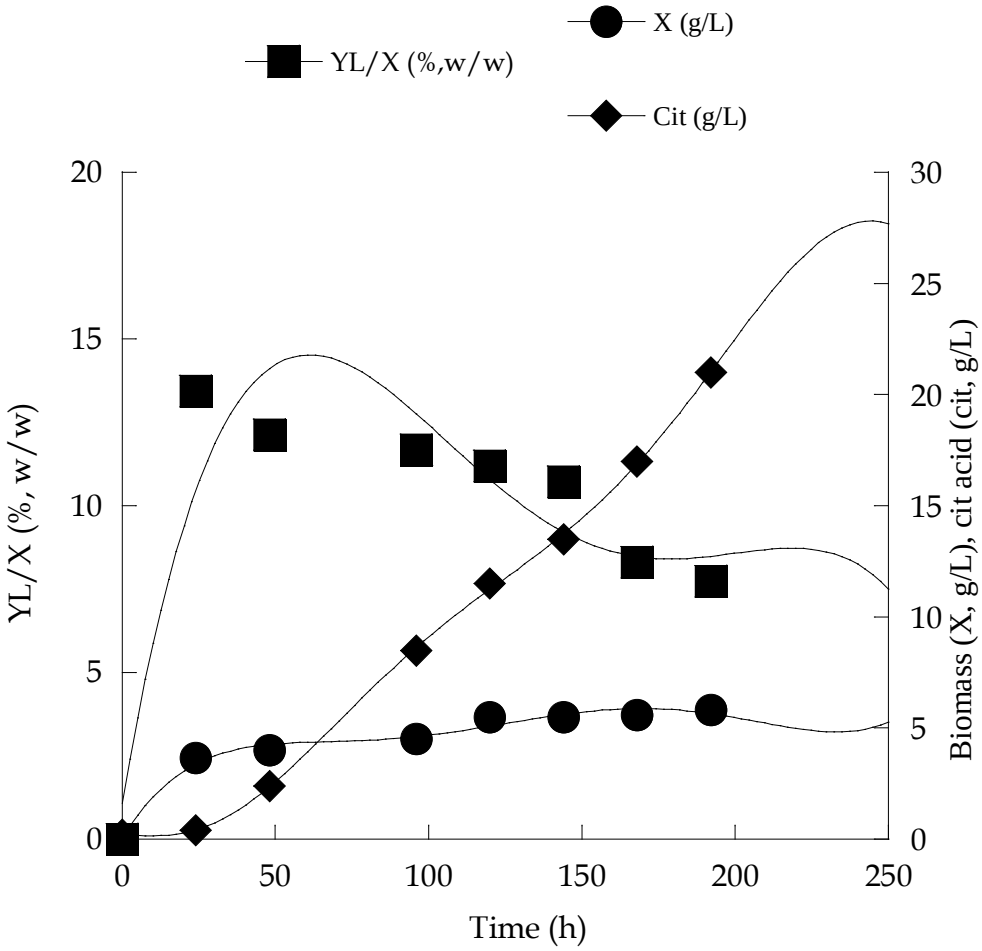


Figure III. 1. Kinetics of biomass produced (X, g/L), citric acid produced (Cit, g/L) and intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$, % w/w) of *Yarrowia lipolytica* ACA-YC 5033 during growth on glucose, at initial concentration (Glc_i) of *c.* 50 g/L, under nitrogen-limited conditions. Culture conditions: growth on 250-ml flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=28±1°C. Each point is the mean value of two independent measurements (SE<10%).

In agreement with the recently appeared literature (Papanikolaou et al., 2002b; 2009; Rymowicz et al., 2006; 2010; Sarris et al., 2011), in the current investigation and during growth of the strain *Yarrowia lipolytica* ACA-YC 5033 on glucose under nitrogen limitation, citric acid was produced almost exclusively at the stationary growth phase of the microbial fermentation, while, as previously stressed, Cit secretion coincided with significant decrease in the quantity of lipids produced per unit of biomass synthesized (Makri et al., 2010; Sarris et al., 2011). In the strain *Yarrowia lipolytica* ACA-YC 5033 the maximum quantity of citric acid produced was 26.7 g/L and the concomitant conversion yield of citrate produced per unit of glucose consumed ($Y_{Cit/Glc}$) was 0.53 g/g .

From all the above mentioned analysis it can be summarized that the most potential candidate for SCO production from glucose is the microorganism *Rhodosporidium toruloides* DSM 4444. The production of SCO by this microorganism will be studied and optimized in the next paragraphs.

III.1.2.2. Effect of NaCl concentration on Rhodosporidium toruloides cultures

The yeast strain *Rhodosporidium toruloides* DSM 4444 was cultivated in batch-flask trials, in media containing 50 g/L of glucose supplemented with increasing NaCl concentrations. Cultures were done under nitrogen limited conditions (carbon-to-nitrogen ratio equal to 106 mol/mol) in order to stimulate lipid accumulation. Moreover, an experiment without NaCl addition was included that served as control. The obtained results as regards the impact of NaCl upon the physiological behavior of *Rhodosporidium toruloides* are depicted in Table III.2.

Table III. 2. Quantitative data of *Rhodosporidium toruloides* DSM 4444 originated from kinetics in media with six different initial NaCl concentrations with the same initial glucose concentration (50 g/L). Representation of consumed glucose (Glc_{cons}, g/L), produced biomass (X, g/L), lipid content (L, g/L) and lipid in dry weight ($Y_{L/X}$, % w/w) when the maximum quantity of lipid (in g/L) was achieved. Culture

conditions: growth in 250-ml flasks at 185 rpm, initial pH=6.0±0.1, DO>20% (v/v), incubation temperature T=26 °C. Each experimental point is the mean value of two independent measurements.

Entry	NaCl (%, w/v)	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)
1	0.0	168	48.8	8.9	4.9	55.1
2	0.5	168	43.6	8.7	5.4	62.1
3	1.0	144	48.7	8.2	5.1	62.2
4	1.5	144	44.5	8.5	5.3	62.4
5	2.5	120	47.9	8.9	5.4	60.6
6	4.0	192	48.6	9.4	6.7	71.3
7	6.0	168	30.0	6.3	2.8	44.4

Generally, it should be stressed that only at increased initial NaCl concentration (e.g. NaCl at 60 g/L or higher) some negative effect upon the biomass and lipid produced was reported (Table III.2.). On the other hand, for NaCl concentrations varying from 0.5 to 2.5% (w/v), microbial growth as well as lipid production was maintained in similar levels; specifically, 88-97% of initial glucose concentration was consumed within 120-168 h of fermentation, whereas biomass production ranged between 8.2 and 8.9 g/L. In terms of lipid production, cells accumulated 60.6-62.4 (%, w/w) of oil. However, initial NaCl concentration of 4.0% (w/v) was found to positively affect biomass and, specifically, lipid production, yielding 9.4 and 6.7 g/L respectively. Consequently, lipid quantity per DCW increased to 71.3% (w/w). Higher NaCl additions exerted inhibitory effects on yeast growth, as only 6.3 g/L of biomass were synthesized, with concomitant impact on lipid production and yield (Table III.2). On the other hand, for all the above-mentioned trials, the yield of total biomass produced per glucose consumed ($Y_{X/Glc}$) was *c.* 0.20 g/g, ranging between 0.19 and 0.21 g/g.
Taking into account the satisfactory performance of the strain at NaCl supplementation of 4.0% (w/v), subsequent batch-flasks cultures were carried out with the same NaCl addition and increased glucose concentration (Glc_i≈100 g/L). In the same manner, a control experiment without NaCl addition was included. In the absence of NaCl, elevated glucose concentrations prolonged the course of the fermentation up to 433 h. However, at that time, *c.* 88% of initial carbon source concentration (≈93 g/L) was finally consumed by the strain, leading to 17.5 g/L of biomass. Biomass production per substrate consumption yield ($Y_{X/Glc}$) was equal to *c.* 0.19 g/g, the same value as in trials with Glc_i=50 g/L, indicating absence of substrate inhibition for the particular strain in these conditions. Furthermore, the increased

carbon-to-nitrogen ratio (C/N=211 mol/mol) seemed to enhance lipid production in terms of absolute values, reaching a maximum SCO production of 8.1 g/L. On the other hand, and despite the significant increase of lipid in absolute values compared with the respective trial in which Glc_i was adjusted to *c*. 50 g/L (see Table III.1. entry 1), total lipid in DCW ($Y_{L/X}$) value was lower than the one obtained in the trial with Glc_i=50 g/L (*c*. 46% against 55.1% w/w). The presence of 4.0% (w/v) NaCl in media with 100 g/L of glucose, extended the fermentation duration to more than 500 h, a time point in which *c*. 80% of the initial glucose was consumed. Biomass synthesis was slightly reduced to 16.1 g/L compared with the culture with $Glc_i \approx 100$ g/L and no NaCl addition was performed. Nonetheless, $Y_{X/Glc}$ value was unaffected (0.20 g/g). On the other hand, lipid in terms of both absolute (g/L) and relative (% in DCW) values was noticeably higher compared with the equivalent experiment ($Glc_i \approx 100$ g/L) in which no NaCl addition occurred (L=9.2 g/L, $Y_{L/X}$=57.1% w/w). Surprisingly enough, *Rhodosporidium toruloides* accumulated oil in an almost linear manner, whereas shortly after virtual exhaustion of the assimilable nitrogen from the culture medium (i.e. *c.* 50 h after inoculation) lipid in DCW ($Y_{L/X}$, in % w/w) almost reached its plateau (see Fig. III.2.).

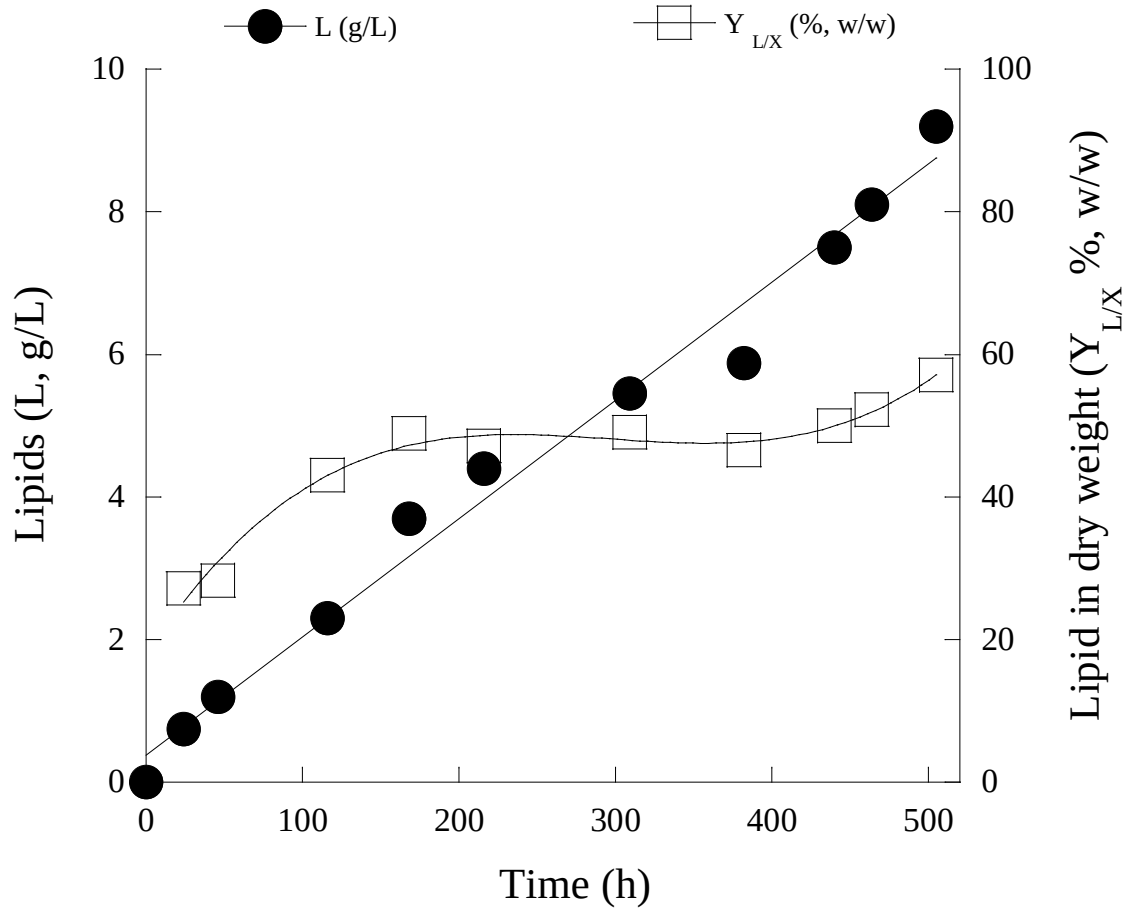


Figure III. 2. Kinetics of lipid production (g/L) and lipid in dry cell weight ($Y_{L/X,}$ % w/w) during growth of *Rhodosporidium toruloides* DSM 4444 on glucose-based media (100 g/L) supplemented with 4% (w/v) NaCl. Culture conditions as in Table III.2.

Table III.3. shows the FA profiles of *Rhodosporidium toruloides* cellular lipids, during growth on media with increasing NaCl concentrations. In every case, the predominant fatty acid of the yeast was oleic (C18:1), followed by palmitic (C16:0) stearic (C18:0) and linoleic acid (C18:2). The implementation of NaCl did not seem to affect the amounts of individual fatty acids in the composition of the accumulated lipids. On the contrary, the unsaturated nature of lipids increased during the course of fermentation, as indicated by the SFA/UFA ratio. This fact could be mainly attributed to the increased amounts of the unsaturated oleic and linoleic acids and the declining percentage of stearic acid that occurred in late exponential, as well as in stationary growth phase.

Table III. 3. Fatty acid composition of cellular lipids of *Rhodosporidium toroloides* DSM 4444 on glucose-based media (Glc_i=50 g/L), containing various NaCl concentrations. Very early (VE) growth phase is that in which the incubation time is between 20-30 h. Early (E) growth phase is that in which the incubation time is between 50-70 h. Late growth phase is that in which incubation time is *c.* 150 h. Culture conditions as in Table III.2.

		Fatty acid composition (%, w/w)									
NaCl (%, w/v)	Growth phase	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	*SFA/ UFA
0.0	VE	1.1	28.2	0.8	11.5	48.6	7.7	2.1	-	-	0.69
	E	0.8	24.1	1.5	9.9	51.2	9.1	2.4	-	-	0.54
	L	0.6	22.2	1.4	7.1	54.5	11.5	3.0	-	-	0.43
0.5	VE	1.4	27.5	1.2	10.0	49.0	8.6	2.1	-	-	0.68
	E	1.2	25.5	0.8	12.5	48.8	7.8	2.3	0.3	0.7	0.67
	L	1.1	24.6	1.2	10.0	49.0	10.4	2.8	0.6		0.57
1.0	VE	1.6	23.8	0.9	16.7	45.7	6.6	-	-	4.3	0.87
	E	1.6	25.5	1.1	10.9	49.3	8.1	2.3	0.3	0.2	0.63
	L	1.0	23.3	0.8	8.6	52.2	11.2	2.2	0.2	0.2	0.50
1.5	VE	1.1	26.8	-	10.0	51.3	8.6	1.9	-	-	0.61
	E	1.2	25.8	0.5	11.2	50.7	7.3	2.1	0.3	0.5	0.56
	L	0.9	24.5	1.2	9.8	51.5	8.0	2.6	-	-	0.56
2.5	VE	1.2	26.9	1.2	10.5	51.3	7.0	1.6	-	-	0.63
	E	1.1	25.8	0.7	9.6	50.5	9.0	2.3	0.2	0.3	0.59
	L	0.9	24.0	1.5	9.0	53.0	8.0	2.7	-	-	0.52
4.0	VE	1.4	23.4	0.4	15.5	47.2	9.7	2.6	0.4	0.4	0.68
	E	1.2	26.3	0.4	10.3	50.4	8.2	2.4	-	0.5	0.62
	L	0.8	25.5	1.5	8.5	51.8	8.9	2.8	-	-	0.54

III.1.2.3. Trials of Rhodosporidium toruloides on previously pasteurized media supplemented with NaCl

Based on evidence of the tolerance against noticeable amounts of NaCl (e.g. 4.0% w/v) shown by the employed yeast strain, it was decided to further investigate the stability of microbial growth and lipid production under pasteurized conditions and assess whether the presence of 4.0% (w/v) NaCl could reduce the probability of culture contamination. It is evident that a successful accomplishment of SCO production in not previously sterilized media can reduce the cost of the process when a scale-up of the bio-process is envisaged. To this end, batch-flask trials were carried out with *c.* 50 and 100 g/L of glucose as substrate, in

media supplemented with 4% (w/w) NaCl. At Glc_i≈50 g/L, substrate exhaustion occurred around 160 h after inoculation, yielding 11.7 g/L of biomass production (Table III.4.).

Table III. 4. Quantitative data of *Rhodosporidium toruloides* DSM 4444 originated from kinetics in previously sterilized and pasteurized media, supplemented with 4.0% (w/v) NaCl and 50 and 100 g/L initial glucose concentration. Representation of initial glucose (Glc_i, g/L), consumed glucose (Glc_{cons}, g/L), produced biomass (X, g/L), produced lipid (L, g/L) and lipid in dry weight (%, w/w). Culture conditions: growth in 250-mL flasks at 185 rpm, initial pH=6.0±0.1, DO>20% (v/v), incubation temperature T=26 °C. Each experimental point is the mean value of two independent measurements.

Media heat-treatment	Glc_i (g/L)	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%,w/w)
Sterilized	≈50	192	48.6	9.4	6.7	71.3
Pasteurized	≈50	160	48.0	11.7	5.9	50.4
Sterilized	≈100	505	93	16.1	9.2	57.1
Pasteurized	≈100	311	80	17.9	9.1	50.8

However, lipid accumulation was lower than in the experiment in which the medium had been previously subjected to heat sterilization (L=5.9 g/L against 6.7 g/L). Microscopy observations revealed the presence of bacterial contamination (rods), accounting for *c.* 8% of the total microbial population. When higher initial glucose concentrations were employed in previously pasteurized media, equally some bacterial contamination occurred (*c.* 5% of the total microbial population). As in the trial with Glc_i≈50 g/L, glucose assimilation in the previously pasteurized medium was more rapid than in the aseptic fermentation, possibly due to this contamination. Equally, biomass formation was enhanced in the pasteurized medium in comparison with the aseptic culture, reaching a DCW value of 17.9 g/L that contained 9.1 g/L of oil (lipid in DCW of *c.* 51% w/w) while in the aseptic culture the respective values were for DCW 16.1 g/L and for lipid 9.2 g/L (see Table III.4.). The kinetics of biomass produced, lipid accumulated and glucose assimilated for the trials with Glc_i≈100 g/L are seen in Fig. III.3..

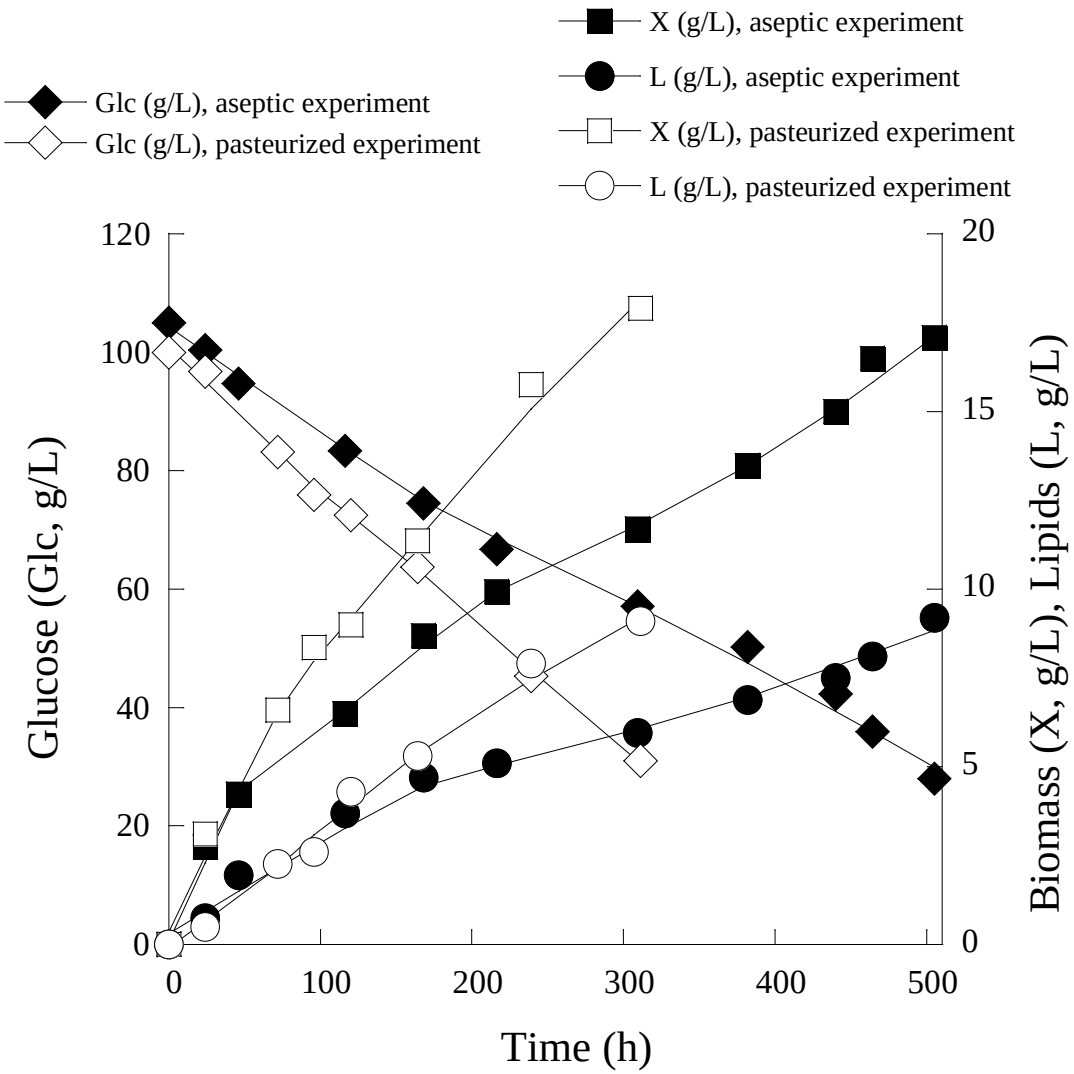


Figure III. 3. Kinetics of residual glucose, biomass production and lipid accumulation (cycles), during growth of *Rhodosporidium toruloides* DSM 4444 in previously sterilized or pasteurized media supplemented with 100 g/L of glucose and 4% (w/v) NaCl. Culture conditions as in Table III.3.

Table III.5. shows the FA profiles of the produced cellular lipids for the previously pasteurized media, in which Glc_i concentration was adjusted to *c*. 50 and 100 g/L and constant NaCl quantity added. Despite the fact that the cultures were not axenic (as stated, some contamination by bacilli existed), the FA composition presented significant similarities with the trials in which growth and lipid accumulation occurred in previously sterilized media (see and compare Tables III.3. and III.5.). In any case, lipid produced through the non-aseptic experiments was rich in the FA C18:1, constituting, thus, a perfect fatty material amenable to be converted into biodiesel (Papanikolaou and Aggelis, 2009; 2011b).

Table III. 5. Fatty acid composition of cellular lipids of *Rhodosporidium toroloides* DSM 4444 growing in previously pasteurized media presenting increasing initial glucose (Glc_i) concentration and constant NaCl concentration. For Glc_i≈50 g/L, very early (VE) growth phase is that in which the incubation time is between 10-20 h and late growth phase is that in which incubation time is *c.* 150 h. For Glc_i≈100 g/L, very early (VE) growth phase is that in which the incubation time is between 20-30 h and late growth phase is that in which incubation time is *c.* 300 h. Culture conditions as in Table III.4.

Glc_i (g/L)	Growth Phase	C14:0	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 9,12,15}$C18:3
	VE	0.6	26.4	0.7	8.4	54.4	5.9	2.3
≈50	L	1.1	23.0	0.5	7.9	57.4	6.9	2.6
	VE	1.2	24.8	-	9.6	52.9	6.5	2.7
≈100	L	1.6	22.0	1.5	8.2	55.0	6.8	2.2

III.1.2.4. Batch–bioreactor cultures of Rhodosporidium toruloides

The next step in the experimental process involved the realization of batch-bioreactor cultures of the yeast with increasing glucose concentrations and NaCl supplementation, aiming to promote, if possible, lipid production. Previously sterilized fermentation media containing *c.* 50, 100 and 150 g/L of glucose and 4.0% (w/v) NaCl were used in bench top bioreactor cultures. Table III.6. summarizes the quantitative data of *Rhodosporidium toruloides* trials in bioreactor experiments.

Table III. 6. Quantitative data of *Rhodosporidium toruloides* DSM 4444 originated from kinetics in media containing 50, 100 and 150 g/L glucose and 4% (w/v) NaCl in batch-bioreactor cultures. A batch-flask trial was also included as control. Representation of initial glucose (Glc_i, g/L) consumed glucose (Glc_{cons}, g/L), produced biomass (X, g/L), produced lipid (L, g/L), lipid in dry weight ($Y_{L/X}$, % w/w), lipid yield per consumed substrate ($Y_{L/S}$, g/g) and biomass yield per consumed substrate ($Y_{X/S}$, g/g). Bioreactor culture conditions: agitation speed 200-500 rpm, pH=6.0±0.1, DO>20% (v/v), temperature T=26 °C. Batch-flask culture conditions as in Table III.2. Each experimental point is the mean value of two independent measurements.

Culture mode	Glc_i (g/L)	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%,w/w)	$Y_{L/S}$ (g/g)	$Y_{X/S}$ (g/g)
Batch-flask	≈50.0	192	50.0	9.4	6.7	71.3	0.13	0.19
Batch-bioreactor	≈50.0	72	44.5	12.7	8.1	63.8	0.18	0.29
	≈100.0	160	90.9	25.2	14.2	56.3	0.16	0.28
	≈150.0	312	110.3	36.2	23.6	65.1	0.21	0.33

At 50 g/L of glucose, the strain exhibited rapid substrate assimilation within 72 h. Biomass was notably enhanced, as opposed to the respective batch-flask experiment, yielding 12.7 g/L with 8.1 g/L of oil. Increasing amounts of carbon source did not seem to drastically affect microbial metabolism, while in all cases, it is interesting to indicate that glucose was linearly consumed (Fig. III.4.). In particular, the more the Glc_i concentration (and, hence, the initial molar ratio C/N of the medium) increased, the more the glucose consumption rate (r_{Glc}, expressed as $r_{Glc} = -\frac{\Delta Glc}{\Delta t}$) decreased; for Glc_i adjusted at *c.* 50 r_{Glc} was =0.62 g/L/h decreasing to 0.54 g/L/h for Glc_i≈100 g/L. Finally, at Glc_i≈150 g/L, r_{Glc} value eventually dropped to 0.34 g/L/h (Fig. III.4.). During these trials, lipid accumulation process demonstrated remarkable stability; in accordance with the trial performed in shake flasks and as depicted in Fig. III.4., the evolution of lipids' kinetic profile was almost linear, regardless of the applied initial glucose concentration. Maximum biomass production was achieved at 150 g/L of glucose equal to 34.1 g/L, containing 65.1% (w/w) of oil. It is interesting to indicate that under the present culture conditions, growth was not inhibited by the increment of Glc_i concentration up to the threshold of 150 g/L; this assumption can be justified by the fact that the yields $Y_{X/Glc}$ and $Y_{L/Glc}$ presented their higher values at the trial in which the concentration of carbon substrate had been adjusted at *c.* 150 g/L (=0.33 and 0.21 g/g respectively), being clearly the higher ones obtained in all of the previously performed trials (including fermentations in shake-flasks or bioreactor). In addition, specifically the yield $Y_{L/Glc}$ value obtained in the bioreactor experiment with Glc_i≈150 g/L (=0.21 g/g) was a value very close to the maximum potentially achievable one of 0.22-0.23 g per g of consumed sugar that has been achieved so far in the international literature (Ratledge, 1988; Ykema et al., 1988; Ratledge and Wynn, 2002; Papanikolaou et al., 2011a), suggesting, once more, the absence of inhibitory phenomena of increased Glc_i concentrations upon the growth of *Rhodosporidium toruloides* under the present culture conditions.

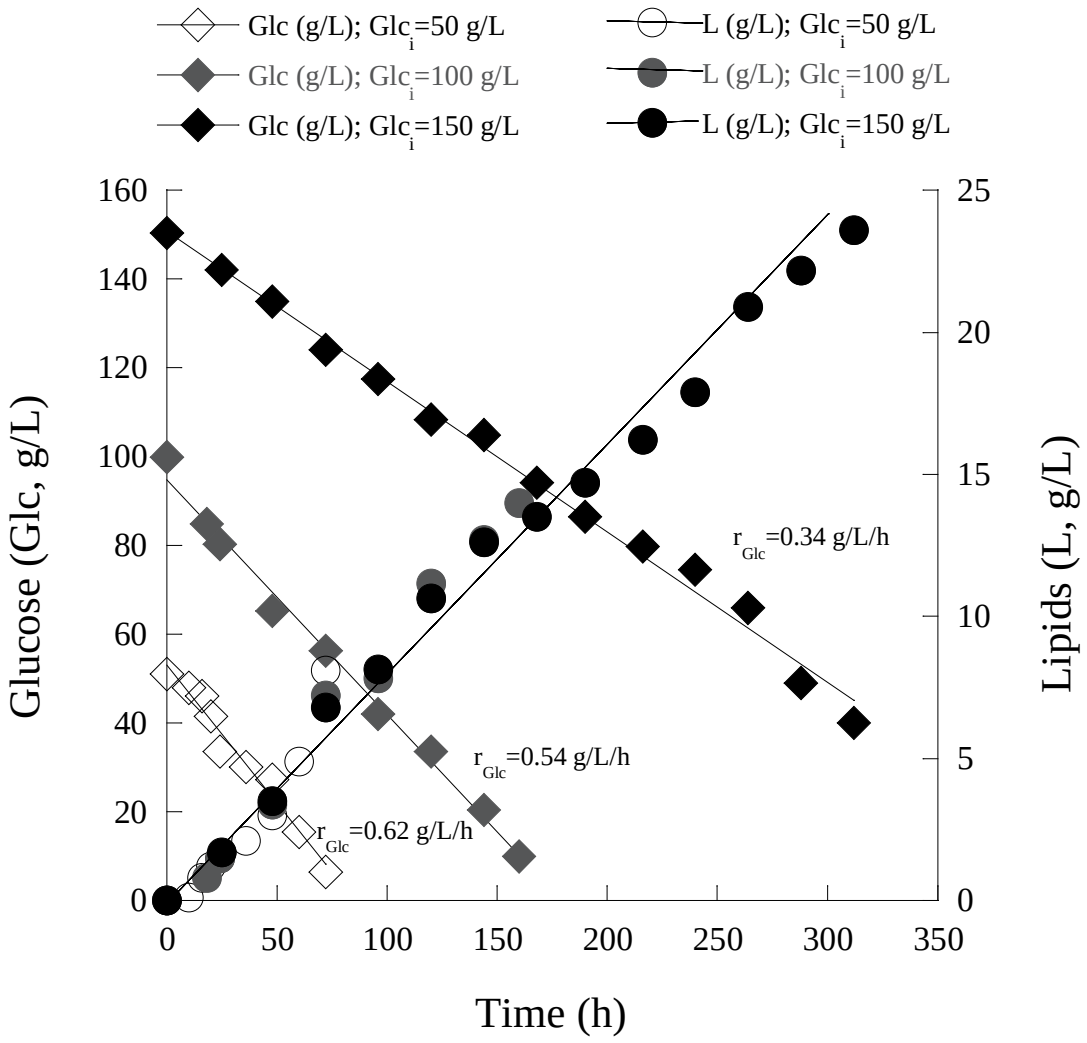


Figure III. 4. Kinetics of residual glucose and lipid accumulation during batch-bioreactor cultures of *Rhodosporidium toruloides* DSM 4444 in media containing 50 g/L, 100 g/L and 150 g/L of glucose, supplemented with 4.0% (w/v) of NaCl. Culture conditions as in Table III.6.

Table III.7. shows the FA profiles of *Rhodosporidium toruloides* cellular lipids, during growth on bioreactor cultures in media presenting increasing Glc_i concentrations and constant NaCl quantity added. As in the shake-flask trials, the predominant FA of the yeast was the C18:1, followed by the FAs C16:0, C18:0 and C18:2. Moreover, as in the case of the trials with the increasing initial NaCl quantities into the medium, the increment of Glc_i concentration did not seem to have serious impact upon the FA composition of the strain, while cellular FAs were slightly more saturated at the beginning of the fermentation (Table III.7.). Once again, one can observe that the SCO produced by *Rhodosporidium toruloides* cultivated in bioreactor experiments constitutes a perfect starting fatty material amenable to be converted into biodiesel (Papanikolaou and Aggelis, 2009; 2011b; Koutinas et al., 2014a).

Table III. 7. Fatty acid composition of cellular lipids of *Rhodosporidium toroloides* DSM 4444 cultivated in batch-bioreactor experiments in media presenting increasing initial glucose (Glc_i) concentration and constant NaCl concentration. For Glc_i≈50 g/L, very early (VE) growth phase is that in which the incubation time is between 10-20 h, early (E) growth phase is that in which the incubation time is between 30-40 h and late growth phase is that in which incubation time is *c.* 70 h. For Glc_i≈100 g/L, very early (VE) growth phase is that in which the incubation time is between 20-30 h, early (E) growth phase is that in which the incubation time is between 50-80 h and late growth phase is that in which incubation time is *c.* 150 h. Culture conditions as in Table III.6.

Glc_i (g/L)	Growth Phase	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
≈50	VE	1.5	25.2	-	12.0	50.2	8.4	2.0
	E	1.0	23.8	1.1	10.1	54.7	9.3	-
	L	1.2	22.1	1.5	10.8	55.7	6.5	1.4
≈100	VE	-	23.8	-	12.2	53.7	6.7	1.5
	E	-	22.1	2.5	11.9	55.4	5.8	2.0
	L	-	20.9	3.0	9.1	54.9	8.9	2.5

III.1.2.5. Fed- batch bioreactor culture of Rhodosporidium toruloides

In an attempt to further investigate lipid production potential of the yeast *Rhodosporidium toruloides* and to reduce the time of the fermentation (as seen in the previous paragraph, the more the Glc_i concentration increased, the more the time of the fermentation rose) fed-batch cultures were performed in bench top bioreactor, in media containing 4.0% (w/v) NaCl. Trials were initiated batch-wise (Glc_i≈50 g/L) and when the glucose level dropped below 10 g/L, a volume of concentrated glucose solution was aseptically introduced to the culture. In every case, it was desirable to maintain the feeding of glucose to concentrations ≤50 g/L, in order to increase the uptake rate of glucose. During 272 h of the fermentation, feeding pulses were done twice resulting in the total consumption of 127 g/L of glucose (Fig III.5.).

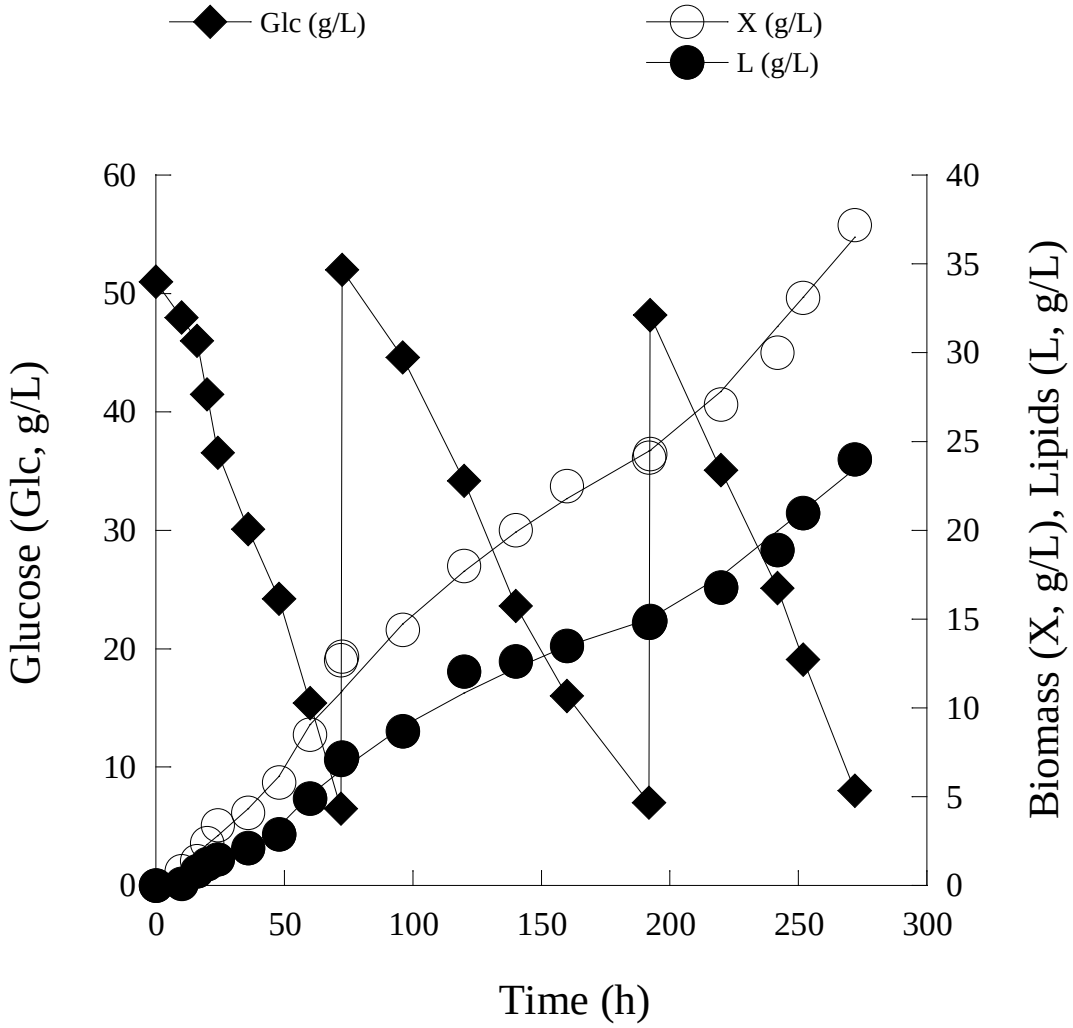


Figure III. 5. Kinetics of residual glucose, biomass production and lipid accumulation during fed-batch bioreactor cultures of *R. toruloides* DSM 4444, in media supplemented with 4.0% (w/v) NaCl. Culture conditions as in Table III.6.

Maximum biomass achieved was 37.2 g/L containing 64.5% (w/w) of storage lipids (L_{max}≈24 g/L). Furthermore, overall yields for lipid and biomass production per consumed substrate in fed-batch process were 0.21 and 0.33 g/g, respectively (Fig. III.6.). Compared to the batch-bioreactor cultures with high initial glucose concentration (Glc_i≈150 g/L), both biomass formation and lipid accumulation were slightly improved during the fed-batch culture mode, whereas the fermentation was accomplished more rapidly in the later case than in the former one, thus the lipid volumetric productivity achieved in the fed-batch experiment was improved compared with the batch process presenting high Glc_i concentration (0.088 against 0.075 g/L/h). Finally, it is interesting to indicate the constancy of the conversion of glucose into total biomass and SCO during the fed-batch bioreactor culture of *Rhodosporidium*

toruloides, where the global yield of biomass produced per unit of glucose consumed throughout the fermentation was 0.31 g/g, while the respective one of SCO produced per unit of glucose consumed was 0.21 g/g (Fig. III.6.).

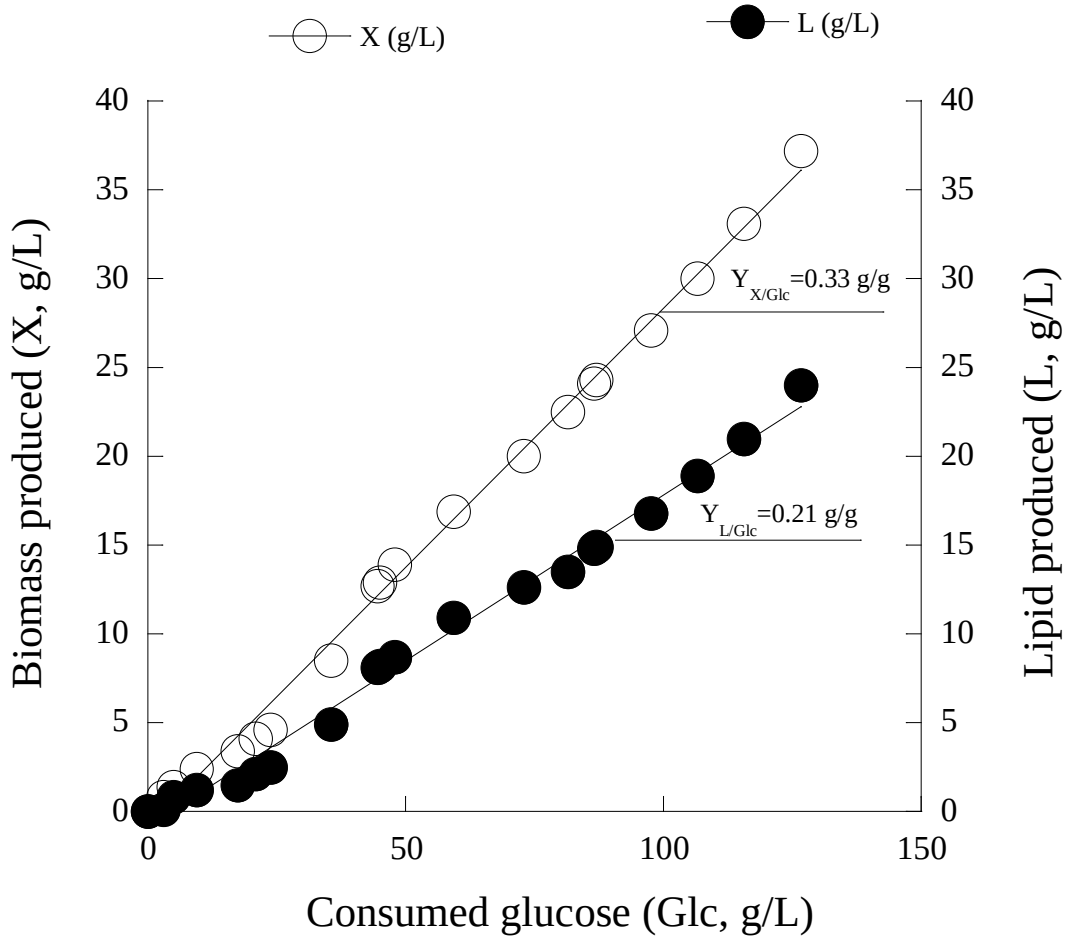


Figure III. 6. Representation of biomass production yield (squares) and lipid production yield (cycles) per substrate consumed during fed-batch bioreactor cultures of *R. toruloides* DSM 4444, in media supplemented with 4.0% (w/v) NaCl. Culture conditions as in Table III.6.

The yeast strain *Rhodosporidium toruloides* DSM 4444 was found capable of growing in media supplemented with NaCl and presented sufficient lipid production. Based on the results of the present study, this particular feature of the strain combined with its tolerance against relatively high amounts of glucose could denote the feasibility of microbial oil production without the need of stringent sterile conditions.

The third step of this study is an application of the capability of the above-mentioned strain to grow on salty media, in an agro-industrial residue, that of table olive processing wastewater (TOPW) which contains high NaCl concentrations. Likewise, literature indicates that strains of the yeast *Yarrowia lipolytica* can also present growth and production of metabolites on media presenting increased concentrations of NaCl (Tomaszewska et al., 2012; 2014). Thus, besides *Rhodosporidium toruloides,* trials using this wastewater as fermentation medium, were also performed with *Yarrowia lipolytica* ACA YC 5033, that as previously was demonstrated, is a microorganism capable to convert glucose into biomass and citric acid under nitrogen-limited conditions. In any case, in order to enhance the production of metabolites by both *Rhodosporidium toruloides* and *Yarrowia lipolytica*, TOPWs were supplemented with low-cost commercial glucose, in order to demonstrate the potential of (partial) replacement of the tap water with TOPW in the fermentations of SCO and citric acid production.

III.1.2.6. Rhodosporidium toruloides DSM 4444 *yeast strain growing on TOPW-based media enriched with glucose and presenting different initial NaCl concentrations*

The yeast strain *Rhodosporidium toruloides* was firstly cultivated under nitrogen-limited media (employment of 0.5 g/L of yeast extract and 0.75 g/L of peptone employed as nitrogen source), in order to choose the appropriate NaCl concentration in which the strain can growth and maintain significant cell growth and lipid production. TOPW-based media (containing initially 6.0% w/v of NaCl) were diluted in order to obtain diluted TOPWs with 3.0% and 3.5% (w/v) of NaCl (these diluted media contained 0.78 and 0.93 g/L phenolic compounds respectively). The production of biomass indicated that the TOPWs with 3.0% w/v of NaCl were the more suitable media for the growth of *Rhodosporidium toruloides* (13.6 g/L instead to 12.9 g/L for the trial with 3.5% w/v of NaCl). Likewise, glucose assimilation in the trial with 3.0% (w/v) of NaCl was higher than the trial with 3.5% (w/v) of NaCl. Additional, biomass productivity were important than the ones with 3.5% (w/v) of NaCl, respectively. Therefore, TOPW-based media containing 3.0% (w/v) of NaCl (and 0.78 g/L of phenolic compounds) was chosen for the rest of the experiments.

III.1.2.7. Trials with Rhodosporidium toruloides DSM 4444 and Yarrowia lipolytica ACA YC 5033 growing on TOPW-based media, enriched with increasing initial glucose concentration.

In order to evaluate the consequence of the increment of the initial glucose concentration on lipid production, *Rhodosporidium toruloides* was cultivated batch-flask trials in TOPW-based media containing 3.0% (w/v) of NaCl enriched with Glc_i at concentrations adjusted at *c.* 50, 100 and 135 g/L. The kinetics of glucose consumption (Glc, g/L), cell biomass production (X, g/L), total lipid content (L, g/L) was studied during the fermentations (Table III.8.).

Table III. 8. Kinetics of *Rhodosporidium toruloides* DSM 4444 grown on TOPWs enriched with 50, 100 and 135 g/L glucose under nitrogen-limited conditions. Representations of total biomass (X, g/L), maximum total cellular lipid (L_{max}, g/L) and consumed glucose (Glc_{cons}, g/L). Culture conditions: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

Glc_i (g/L)	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	$Y_{X/Glc}$ (g/L)
50	168	50.0	13.6	4.7	39.0	0.27
100	456	100.4	18.6	6.5	45.0	0.18
135	312	83.1	16.0	9.3	58.4	0.19

The yeast was able to grow in TOPW based-media containing high initial glucose concentrations and accumulated non-negligible quantities of cellular lipids. The fermentation time was significantly increased (from 168 to 456 h) during the fermentation on TOPW based-media enriched with Glc_i adjusted at 100 g/L compared to the trial with Glc_i≈50 g/L. Moreover, the increase in the initial glucose concentration, and thus the increment of carbon-to-nitrogen (C/N) ratio into the medium, had a positive effect upon both the production of total biomass and the accumulation of intra-cellular lipids; indeed, the obtained percentage of accumulated oil rose from 39.0 to 45.0% w/w, with the Glc_i increment from 50 to 100 g/L respectively. Furthermore, despite the fact that only *c.* 62% w/w of the initial substrate was consumed during the trial with Glc_i≈135 g/L, one can easily notice a decrease in the production of total biomass (X=16 g/L) as compared with the trial with Glc_i=100 g/L (X=18.6 g/L); on the other hand, increased lipid accumulation occurred (L_{max}=9.3 g/L corresponding to $Y_{L/X}$≈58% w/w) as compared with the trials with both Glc_i≈50 g/L and Glc≈100 g/L. It must be noted however, that as far as the trial with Glc≈135 g/L was concerned, further incubation

of the microorganism (after 312 h), did not improve the assimilation of glucose and, hence, the production of both DCW and SCO. Finally, biomass production per unit of substrate consumed ($Y_{X/Glc}$) was ≈0.18 g/g for both trials with Glc_i ≈100 and 135 g/L, while it was *c.* 0.27 g/g for the trial with Glc_i≈50 g/L, indicating potential substrate inhibition for the particular strain in these conditions.

The yeast *Yarrowia lipolitica* ACA-YC 5033 was also cultivated on TOPW based-media enriched with higher initial glucose concentrations. Unlike cellular lipid biosynthesis that was a relatively marginal event during growth of *Yarrowia lipolytica* on glucose-enriched TOPWs (as it was previously seen – see Table III.1.), HPLC analysis showed a remarkable citric acid biosynthesis during the culture on this glucose-enriched wastewater. In nitrogen-limited fermentations performed, a regular drop of pH was observed in all trials as a consequence of citric acid synthesis. In all cases, the pH into the culture medium was corrected and maintained within the range of 5.2-6.0. Whereas the duration of the fermentation increased (to 1.9 fold-times) during the culture in TOPWs enriched glucose added at 100 g/L as compared with the trial in which TOPWs had been enriched glucose added at 50 g/L (Table III.9.), it must be pointed out that substrate (glucose) assimilation rate, expressed as $r_{Glc} = -\frac{\Delta Glc}{\Delta t}$, remained almost constant with the Glc_i increment into the medium; for Glc_i adjusted at *c.* 50 g/L, r_{Glc} was =0.265 g/L/h while at Glc_i≈100 g/L, r_{Glc} value was 0.275 g/L/h (these results are in disagreement with the results achieved with *Rhodosporidium toruloides* cultivated under nitrogen-limited conditions).

Table III. 9. Kinetics of *Yarrowia lipolytica* ACA-YC 5033 grown on TOPWs enriched with 50 and 100 g/L glucose under nitrogen-limited condition. Representation of total biomass (X, g/L), total cellular lipid (L, g/L) and consumed substrate (Glc_{cons}, g/L) when the maximum concentration of citric acid (Cit_{max}, g/L) was achieved. Culture condition: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

Glc_i (g/L)	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	Cit_{max} (g/L)
50	192	50.9	7.9	0.7	9.1	20.4
100	360	99.0	8.7	1.1	14.3	43.5

In the trials performed with diluted TOPWs enriched with glucose, total biomass, lipid content and particularly citric acid biosynthesis seemed to be affected by the initial glucose concentration added into the media (Table III. 9.). Specifically, with the rise of Glci concentration into the medium, some slight increase in the production of lipids by *Yarrowia lipolytica* was observed ($Y_{L/X}$=14.3% w/w for Glc_i≈100 g/L against 9.1% w/w for Glc_i≈50 g/L). Finally, a considerable increase of the amount of citric acid in trial with TOPWs enriched 100 g/L (Cit_{max}=43.5 g/L) was observed compared to 20.4 g/L obtained in trial with TOPWs enriched with glucose at 50 g/L. Equally, a slight increase in the value of the conversion yield of citrate produced per glucose consumed was observed when glucose concentration increased from 50 to 100 g/L (from 0.40 to 0.44 g/g).

III.1.2.8. Trials with Rhodosporidium toruloides DSM 4444 and Yarrowia lipolytica ACA-YC 5033 growing on TOPW-based media, enriched with glucose under carbon-limited conditions

As a next step, trials were carried out, using the same initial glucose concentrations (50 and 100 g/L), but in this time cultivations were performed under carbon-limited conditions (extra-cellular nitrogen concentration was 0.5 g/L of yeast extract and 5.5 g/L of peptone). As anticipated, carbon-limited culture conditions had a positive impact on DCW formation, reaching up to 35.7 g/L ($Y_{X/Glc}$≈0.36 g/g) at the end of the fermentation during *Rhodosporidium toruloides* growth in culture with Glc_i≈100 g/L and 16.4 g/L ($Y_{X/Glc}$≈0.36 g/g) in TOPWs enriched with 50 g/L of glucose, while in both instances restricted lipid accumulation occurred (Table III.10.).

Table III. 10. Kinetics of *Rhodosporidium toruloides* DSM 4444 and *Yarrowia lipolytica* 5033 grown on TOPWs enriched with 50 and 100 g/L glucose under carbon-limited condition. Representations of total maximum biomass (X_{max}, g/L), maximum total cellular lipid (L_{max}, g/L), maximum total citric acid (Cit_{max}, g/L) and consumed substrate (Glc_{cons}, g/L). Culture condition: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

Yeast strain	Glc_i (g/L)	Time (h)	Glc_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	Cit_{max} (g/L)
Rhodosporidium toruloides DSM	50	144	45.5	16.4	2.2	13.5	Traces
4444	100	192	99.1	35.7	5.0	14.0	Traces
Yarrowia lipolytica ACA-YC 5033	50	192	48.0	16.2	0.3	1.9	0.9

	100	312	94.5	29.0	0.5	1.7	2.1

The corresponding maximum biomass values for *Rhodosporidium toruloides* grown in nitrogen-limited media were 18.6 and 13.6 g/L. Moreover, growth of *Yarrowia lipolytica* in carbon-limited media showed that DCW production was remarkably higher in as compared with the trial performed under nitrogen limitation (see Tables III.9. and III.10.). Specifically, biomass concentration increased 2-fold times in trial with TOPWs enriched with glucose at 50 g/L, reaching up to X_{max}=16.2 g/L, whereas it increased 3.3-fold times in the trial with glucose added at a concentration of *c*. 100 g/L.

III.1.2.9. Growth of Rhodosporidium toruloides and Yarrowia lipolytica in TOPW-based media enriched with glucose under non-aseptic conditions

In order to evaluate if the stability of the microbial growth and the production of SCO under non-aseptic conditions could be achieved, as demonstrates in the previous sub-chapters, previously pasteurized TOPW-based media supplemented with glucose were used as carbon source (Glc_i≈50 and Glc_i≈100 g/L). Total lipid production was *c.* 17% lower during growth in TOPWs enriched 50 g/L glucose in previously pasteurized than in aseptic conditions with corresponding value of L_{max} being =3.9 g/L instead of 4.7 g/L obtained in the axenic culture (Fig III.7.). When higher initial glucose concentrations were employed, although biomass formation was slightly enhanced (X_{max}=20 g/L) in comparison with the ones reached during culture in TOPWs enriched 100 g/L glucose trials in aseptic conditions, the same concentration of oil as in the axenic culture (L_{max}=6.5 g/L corresponding to lipid in DCW of 32.5% w/w) was obtained.

Similarly with the feature of *Rhodosporidium toruloides*, during the non aseptic culture of the employed strain of *Yarrowia lipolytica*, a negligible variation was observe in relation with the values of maximum DCW, citric acid and total cellular lipid produced compared to the trial performed under previously aseptic conditions. Despite the slight increase of the biomass produced in the previously pasteurized medium, the maximum value citric acid produced (=40.2 g/L) were indeed similar to the maximum value obtained under aseptic conditions (=43.5 g/L). These differentiations between the results achieved in the trials obtained in previously pasteurized compared with the previously sterilized media are attributed to the slight bacterial contamination (presence of bacilli) into the culture medium in the “pasteurized” experiments.

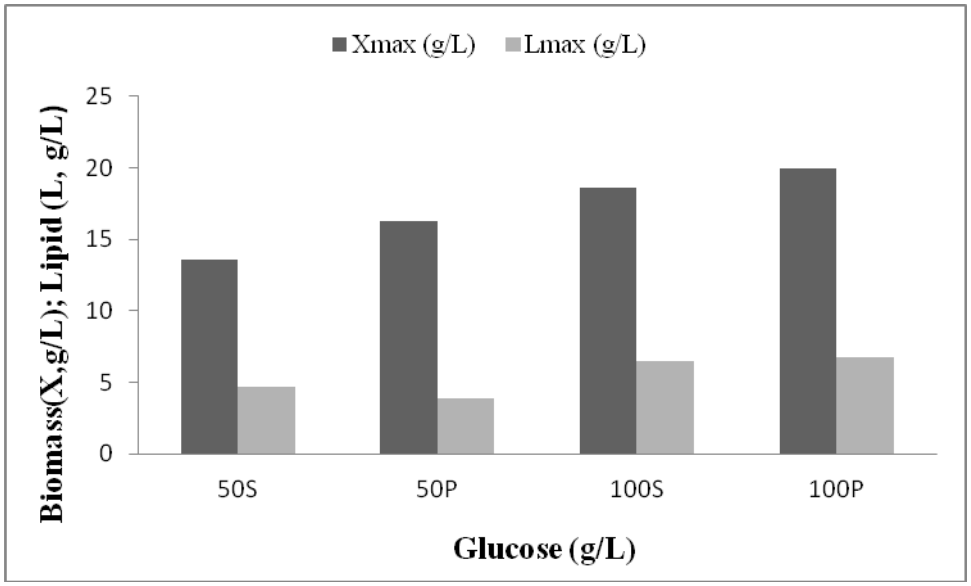


Figure III. 7. Results of *Rhodosporidium toruloides* DSM 4444 originated from kinetic studies on TOPW-based media enriched with glucose at initial concentration adjusted at 50 and 100 g/L in previously sterilized (S) and pasteurized (P) media under nitrogen-limited conditions. Representation of the biomass (X, g/L) when maximum lipid production (L_{max}, g/L) was observed. Culture condition: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

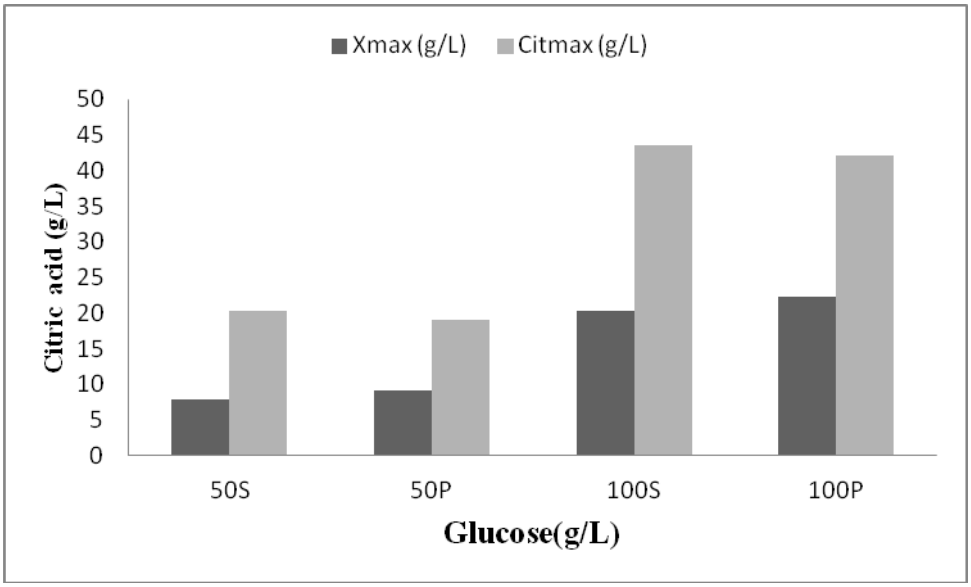


Figure III. 8. Results of *Yarrowia lipolytica* ACA-YC 5033 originated from kinetic studies on TOPW-based media enriched with glucose at initial concentration adjusted at 50 and 100 g/L in previously sterilized (S) and pasteurized (P) media under nitrogen-limited conditions. Representation of the biomass (X, g/L) when maximum citric acid production (Cit_{max}, g/L) was observed. Culture condition: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

III.1.2.10. *Batch-bioreactor culture of Rhodosporidium toruloides in TOPW-based media enriched with glucose under nitrogen-limited conditions*

In order to further demonstrate the potential of SCO production by *Rhodosporidium toruloides* in glucose-enriched TOPWs, a batch culture in a 2-L fermentor under nitrogen-limited conditions employed as in the shake-flasks was assessed; as in the trial in shake flasks, dilution of TOPWs was done aiming to obtain a 3.0% (w/v) of NaCl into the culture medium, while glucose was added in order to result in an initial glucose concentration of *c.* 135 g/L. As shown in Fig. III.9. considerable reduction of the fermentation time to 216 h instead of 312 h compared to the corresponding value in the shake flasks with Glc_i=135 g/L was observed.

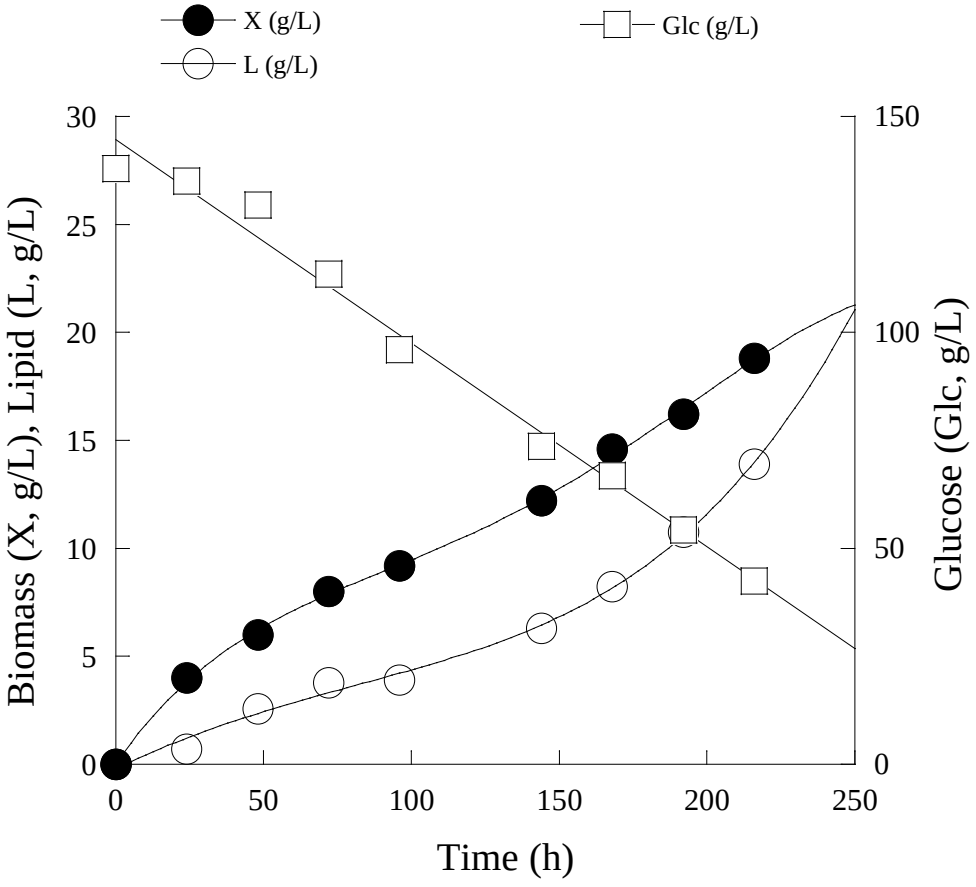


Figure III. 9. Kinetics of *Rhodosporidium toruloides* DSM 4444 grown on TOPW-based nitrogen-limited media containing 3.0% (w/v) of NaCl enriched with 135 g/L glucose in sterile culture under. Representation of the biomass (X, g/L) and lipid production (L, g/L) and glucose assimilated (Glc, g/L) as function of the fermentation time. Culture condition: growth on 2-L fermentor at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

Moreover a considerable enhancement of both the dry cell biomass (X_{max}=18.8 g/L) and lipid production (L=13.9 g/L; $Y_{L/X}$=73.9%, w/w) was observed as compared to the corresponding shake-flask experiment (see Table III.8.). Another interesting feature of the microbial metabolism was related with the fact that glucose was consumed almost linearly from the culture medium, with substrate (glucose) assimilation rate, expressed as $r_{Glc} = -\frac{\Delta Glc}{\Delta t}$, obtaining the value of 0.42 g/L/h, while in the bioreactor experiment it seems that further incubation would result in even higher production of DCW and SCO.\.

III.1.2.11. Fed-batch culture of Yarrowia lipolytica in TOPW-based media enriched with glucose under nitrogen-limited conditions

In order to further demonstrate the potential of citric acid production by *Yarrowia lipolytica* in glucose-enriched TOPWs, a fed-batch culture in a 2-L fermentor under nitrogen-limited conditions employed. As in the trial in shake flasks, dilution of TOPW was done in order to obtained 3.0% (w/v) of NaCL and glucose were added giving an initial glucose concentration of 100 g/L. Fermentation in bioreactor seemed to improve the amount of lipid and citric acid produced. A maximum value of total lipid per DCW and citric acid were 32.9%, w/w (at around 160 h after inoculation) and 68.1 g/L (at around 320 h after inoculation) respectively (Fig. III.10.). Furthermore at 144 h of the experiment in the bioreactor, almost the total glucose concentration of *c.* 90g/L had been assimilated with a substrate consumption rate, expressed as $r_{Glc} = -\frac{\Delta Glc}{\Delta t}$, being equal to 0.62g/L/h, being 2.3 fold-times higher than the corresponding value of 0.27g/L/h obtained in shake-flasks experiment with TOPWs enriched 100 g/L glucose. After a twice feeding pulse with a sterilized glucose solution, 155 g/L of glucose had been consumed with the substrate consumption progressively decreasing to 0.49 g/L/h with a final yield $Y_{Cit/Glc}$ being =0.44 g/g.

Generally, the cultures of both *Rhodosporidium toruloides* and *Yarrowia lipolytica* did not decrease the concentration of NaCl. Moreover, the fermentation performed with the aid of the yeast *Rhodosporidium toruloides* did not result in remarkable removal of the phenolic compounds from the growth medium. In contrast, the fermentation performed with the aid of *Yarrowia lipolytica* resulted in a decrease of the phenolic content of the waste to a value of 14-18% w/w.

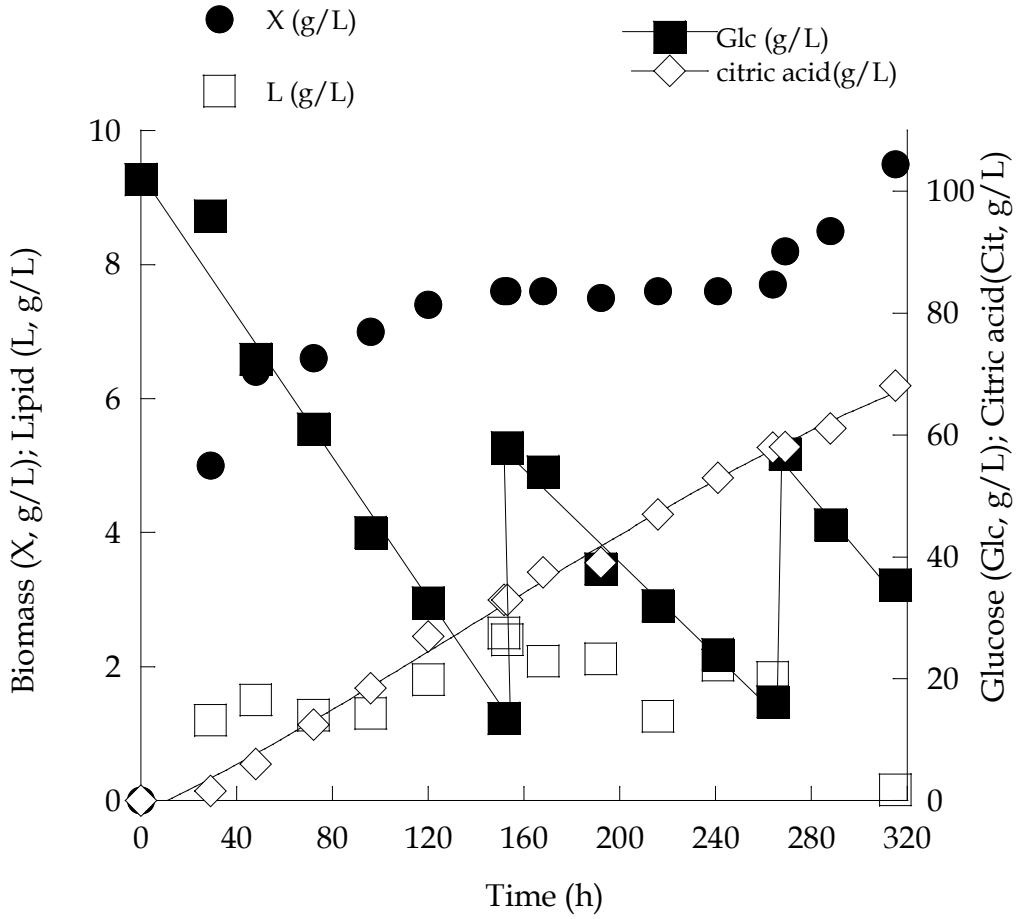


Figure III. 10. Kinetics of *Yarrowia lipolytica* ACA-YC 5033 grown on TOPWs based nitrogen-limited media containing 3.0% (w/v) of NaCl enriched with 135 g/L glucose in aseptic experiments. Representation of the biomass (X, g/L), lipid (L, g/L) and citric acid (Cit, g/L) production and glucose assimilated (Glc, g/L) as function of the fermentation time. Culture condition: growth on 2-L fermentor at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=26°C. Each point is the mean value of two independent measurements.

Fatty acid analyses of total cellular lipid produced at the different stages of the fermentation of *Rhodosporidium toruloides* demonstrated that the main fatty acids (FAs) detected were oleic (C18:1), palmitic (C16:0), palmitoleic (C16:1), arachidic (C20:0) as well as stearic acid (C18:0).

Table III. 11. Fatty acid composition of total cellular lipids of *Rhodosporidium toruloides* DSM 4444 at early exponential (EE), late exponential (LE) and stationary growth phase (S), on TOPW based media

containing 3.0%(v/w) of NaCl supplemented with different initial glucose concentrations(Glc_i adjusted at 50, 100 and 135 g/L). Culture conditions as in Table III.2. and III.4.

Glucose (g/L)	Growth phase	C16:0	C16:1	C18:0	C18:1	C20:0
Nitrogen-limited conditions						
50	EE	19.7	21.1	5.9	33.3	20.0
	LE	23.6	8.7	8.5	49.3	9.9
	S	24.6	6.3	9.0	50.2	9.9
100	EE	23.4	4.2	6.6	50.8	15.0
	LE	24.4	6.9	9.6	48.5	10.6
	S	26.5	3.3	9.9	49.9	10.4
135	EE	23.9	3.8	15.9	39.2	17.2
	LE	27.3	5.5	9.9	47.2	10.1
	S	30.6	2.9	11.1	47.4	8.0
Non-aseptic conditions						
50	EE	21.9	16.2	5.7	44.8	11.4
	LE	24.4	6.6	8.3	51.3	9.4
	S	24.8	5.6	8.0	52.8	8.8
100	EE	23.9	15.5	4.2	44.7	11.7
	LE	27.0	4.8	8.5	50.6	9.1
	S	27.3	5.3	9.6	51.0	6.8

The fatty acid composition *of Yarrowia lipolytica* presented similarities with that of *Rhodosporidium toruloides* (Table III.12.). Again, the main FAs detected were oleic (C18:1), palmitic (C16:0), palmitoleic (C16:1), arachidic (C20:0) and stearic acid (C18:0).

Table III. 12. Fatty acid composition of total cellular lipids of *Yarrowia lipolytica* ACA-YC 5033 at early exponential (EE), late exponential (LE) and stationary growth phase (S), on TOPW based media containing 3.0%(v/w) of NaCl supplemented with different initial glucose concentrations(Glc_i adjusted at 50 and 100 g/L). Culture conditions as in Table III.3.

Nitrogen-limited conditions						
Glucose (g/L)	Growth phase	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9}$C20:0
50	EE	20.4	4.1	8.1	51.5	15.9
	LE	20.2	4.4	15.0	51.6	8.8
	S	20.2	4.3	15.5	49.4	10.6
100	EE	19.4	3.9	10.7	48.6	17.4
	LE	20.3	5.9	8.4	54.3	11.1
	S	14.8	10.5	6.5	58.0	10.2
Non-aseptic conditions						
50	EE	21.3	3.6	8.6	50.9	15.6
	LE	21.2	4.1	8.3	51.5	14.9
	S	20.3	4.8	14.9	49.5	10.5
100	EE	18.6	4.2	11.3	50.1	15.8
	LE	20.4	4.9	7.9	54.9	11.9
	S	14.8	10.2	6.9	58.9	9.2

III.1.3. Discussion

The oleaginous nature of several *Rhodosporidium toruloides* strains has been a topic of interest for many studies in the international literature. Culture parameters such as the origin of carbon or nitrogen sources, the nutrient limitation and the feeding strategy have been assessed for the optimization of SCO production by the particular yeast. Lipid accumulation by *Rhodosporidium toruloides* has been shown to improve in the presence of an organic nitrogen source (Evans and Ratledge, 1984b). Although nitrogen limitation has long been recognized as a determinant factor for de novo lipid synthesis in oleaginous microorganisms (Papanikolaou and Aggelis, 2011b), phosphorus- and sulfate-limitation conditions have been also investigated as lipid inducing factors for *Rhodosporidium toruloides* strains (Wu et al., 2010; 2011). In terms of carbon sources, strains of the particular yeasts are reported to withstand carbon-rich media and under certain conditions, to promote high density cell cultures (Li et al., 2007). On the other hand, in earlier studies, strains of *Rhodosporidium toruloides* have been flask-cultured in media composed of pure stearic acid or blends of pure stearic acid, glucose and glycerol (in these media the ex novo lipid accumulation mechanism was partially or completely employed in order for SCO to be synthesized – Papanikolaou and Aggelis, 2011a) and tailor-made lipids presenting structural and compositional similarities with the cocoa-butter have been synthesized (Gierhart, 1984). Likewise, equally in early studies, strains of this species had been cultivated on glucose-based media in which Δ9 and Δ12 natural or artificial desaturase inhibitors had been added into the culture medium (e.g. sterculic acid, malvanic acid, cis-methylen-octadecenoïc acid, etc) in order to suppress the desaturation reaction inside *Rhodosporidium toruloides* cells, so as finally to synthesize lipids presenting compositional similarities with the cocoa-butter (Moreton, 1985; Moreton and Clode, 1985). Finally, more recently, Zhu et al. (2012) carried out a study based on genomic sequencing of *Rhodosporidium toruloides*, in an attempt to unravel lipid accumulation process on a genetic level.

One major objective of the study was to identify the effect of NaCl addition into the culture medium upon the process of lipid accumulation of *Rhodosporidium toruloides* DSM 4444. To this end, batch-flask cultures of the microorganism were performed in media containing increasing amounts of NaCl up to 6.0% (w/v). The particular strain can be categorized as halotolerant, due to its ability to grow sufficient in the presence or absence of

salt (Margesin and Schinner, 2001). Furthermore, NaCl concentrations up to 4.0% (w/v) were found to promote optimum growth of the yeast and thus, according to Larsen (1986) the strain can be designated as moderate halotolerant. This feature is commonly encountered in microorganisms isolated from marine environments, possessing unique adaptation mechanisms in high salinity conditions (Zaky et al., 2014). As far as yeast species are concerned, osmotolerant strains of the genera *Saccharomyces* sp., *Pichia* sp., *Debaryomyces* sp., *Yarrowia* sp. and *Rhodotorula* sp. have been isolated from such environments (Kutty and Philip, 2008). *Debaryomyces* sp. strains have been widely used as model microorganism for the investigation of biochemical events associated with intra-cellular defense mechanisms against sodium toxicity (Prista et al., 2005). Apparently, halotolerant microorganisms can follow different strategies in order to adjust to high saline environments. These include membrane interactions to maintain intra-cellular sodium in low concentrations (sodium excluders) or in other cases, high intra-cellular salt concentrations are required for preserving normal enzymatic activities (sodium includers) (Rengipipat et al., 1988). In any case, major part of the mechanisms involved in osmotic balance regulation for halotolerant microorganisms represents the production and accumulation of solutes, such as glycerol, trehalose, mannitol and erythritol (Breuer and Harms, 2006). Under this optic, the last years there has been a number of reports in which the polymorphic yeast *Yarrowia lipolytica* has been cultivated in media composed of high initial concentrations of (pure or biodiesel-derived) glycerol supplemented with increased initial concentrations of NaCl, and enhancement of production of erythritol, the concentration of which in some cases reached in indeed very high levels (e.g. >45 g/L or even *c*. 200 g/L) has been reported when crude or pure glycerol has been employed as fermentation substrate by wild or mutant *Yarrowia lipolytica* strains (Tomaszewska et al., 2012; 2014; Rywińska et al., 2013a; 2013b). Likewise, besides mannitol and erythritol, due to their high osmotic tolerance, halotolerant yeasts have been proposed as candidates for bioethanol production (Obara et al., 2012), enzyme production (Zhang et al., 2009; Chen et al., 2010) as well as xylitol (Misra et al., 2012). However, none of these microorganisms has ever been reported as oleaginous. In our study, the yeast *Rhodosporidium toruloides* DSM 4444 accumulated more than 55% (w/w) of oil in dry cell weight during its cultivation in the absence of NaCl, proving its oleaginous nature. Surprisingly enough, the addition of NaCl not only did not suppress microbial growth, but on the contrary was found to enhance lipid accumulation, as such was the case in media

containing 4.0% (w/v) NaCl. Under these conditions, lipid accumulation increased 36.7% compared to the control experiment. This observation is reported for the first time for an oleaginous yeast strain, while a positive correlation between salt and lipid production has been shown for oleaginous microalgae strains belonging to the genera of *Dunaliella* sp. and *Nannochloropsis* sp. (Takagi and Yoshida, 2006; Su et al., 2010).

The ability of salt tolerance combined with lipid production enhancement can offer a significant biotechnological advantage for the particular strain, enabling the possibility of utilizing salty or brackish water instead of tap water during its cultivation. Furthermore, valorization of salty waste water streams deriving from fisheries or olive production establishments could be another prospect, as these wastes can serve as fermentation media being often rich in nutrient sources, with the additional benefit of waste bio-remediation. This application was performed in the current investigation, with the partial replacement of water by TOPWs in order for SCO or microbial mass to be produced by *Rhodosporidium toruloides.* Indeed, significant quantities of SCO (under nitrogen-limited conditions) and non-negligible quantities of microbial biomass (under carbon-limited conditions) have been produced by virtue of the above-mentioned application. Unfortunately, the yeast *Rhodosporidium toruloides* was not revealed capable to reduce the phenolic content of the diluted TOPWs. In contrast, the yeast *Yarrowia lipolytica*, that equally was cultivated on this salty wastewater, besides the significant production of citric acid (under nitrogen-limited conditions) and the interesting production of yeast biomass (under carbon-limited conditions) removed some quantities of the phenolic compounds of the culture medium, in accordance with the results reported for the growth of this species on olive-mill wastewaters (Sarris et al., 2011).

Another aspect of the study was the realization of microbial lipid production in the presence of NaCl under pasteurized conditions. In batch-flask trials with 50 or 100 g/L of initial glucose concentration, signs of bacterial contamination were noted, despite the use of a more concentrated inoculum. However, bacterial presence was less than 5% of the total microbial population and was not found to be detrimental for yeast growth and lipid formation. In the case of microbial conversions, pasteurized grape must has been used for alcoholic fermentation (Sarris et al., 2009), while the effect of pasteurized whey-based medium on propionic acid production has also been evaluated (Anderson et al., 1986). Additionally, the application of completely non-aseptic conditions has been tested for

microbial solvent production such as ethanol, butanol and 1,3-propanediol (Chatzifragkou et al., 2011; Chen et al., 2013; Metsoviti et al., 2013; Sarris et al., 2013), as means of energy and operation cost reduction. However, in all of the above-mentioned cases (e.g. production of bio-ethanol, butanol, 1,3-propanediol), it was the main metabolic compound-target (the alcohol) that was synthesized rapidly and in high concentrations that hindered the microbial contamination with undesirable microorganisms, fact that does not happen during the SCO fermentation. Recently, non-aseptic fermentations concerning the production of SCO by the fungi *Cunninhgamella echinulata* and *Thamnidium elegans* cultivated on biodiesel-derived glycerol have been performed in media supplemented with essential oils and/or antibiotics (Moustogianni et al., 2015). In any case, the current submission is one of the first in the literature that deals with the production of SCO under non-aseptic conditions, and based on the findings of this study, the realization of microbial lipid production without stringent sterility measures could be, under certain conditions, feasible.

During batch-bioreactor trials, the yeast *Rhodosporidium toruloides* exhibited notable tolerance against high substrate concentrations. The particular strain grew satisfactorily with a similar metabolic profile in media containing up to 150 g/L of glucose, while of importance was the almost linear profile of lipid accumulation, regardless of the initial substrate concentration. Tolerance against high substrate (e.g. sugar or glycerol) input is essential, in order to achieve high-density cultures that have been proved as effective cultivation strategy in the case of microbial lipid production. In a similar manner, Li et al. (2007) demonstrated that the yeast *Rhodosporidium toruloides* Y4 grew well in media containing glucose up to 150 g/L, a fact directly correlated with the tolerance of the yeast against osmotic stress. On the other hand, for several oleaginous yeasts, substrate (e.g. sugar) concentrations at *c.* 100 g/L have been reported as threshold, above which microbial cell growth was often repressed significantly (Li et al., 2007; Zhu et al., 2008; Zhang et al., 2011). For glycerol utilized as the sole substrate, in several cases the upper tolerance threshold for oleaginous yeast strains can be lower; for instance, the classical oleaginous strain *Cryptococcus curvatus* ATCC 20509, when it was flask cultured on various initial (crude or pure) glycerol concentrations presented significantly decreased microbial growth when the initial glycerol concentration was added in quantities >60 g/L (Meesters et al., 1996; Liang et al., 2010). In contrast, oleaginous fungi seem more resistant in high initial substrate (sugar or glycerol) quantities, since initial concentrations ranging between 80-100 g/L have been considered as ideal ones in order to

significantly enhance the process of lipid accumulation for the species *Mortierella isabellina*, *Thamnidium elegans* and *Cunninghamella echinulata* (Papanikolaou et al., 2004; 2010; Fakas et al., 2009b; Chatzifragkou et al., 2011; Zikou et al., 2013). In any case, in order to overcome substrate inhibition phenomena, fed-batch cultivation strategies are preferred, during which substrate is introduced between long time intervals and in a discontinuous manner (Meesters et al., 1996; Liang et al., 2010; Tsakona et al., 2014). In our case, fed-batch cultivations led to somehow faster substrate assimilation, which in turn affected positively biomass formation and lipid accumulation.

Rhodosporidium toruloides DSM 4444 presented remarkable cell growth and lipid production in both shake-flask and bioreactor experiments. As indicated in the previous paragraphs, strains of this particular species have been employed already from early studies in order for SCO production to be performed. As indicated, initially (mid 80s) strains of this species had been employed as microbial cell factories in order to produce lipids that mimicked the composition of cocoa-butter (Gierhart, 1984a; 1984b; Moreton, 1985; Moreton and Clode, 1985; reviewed in Papanikolaou and Aggelis, 2010; 2011b). The last decade, with the potentiality of the replacement of edible oils by non-conventional ones (e.g. yeast oils) as starting materials in order for biodiesel precursors to be synthesized has been assessed, and *Rhodosporidium toruloides* has been considered as a microorganism of importance amenable to be used in the conversion of low-cost hydrophilic materials (*e.g.* lignocellulosic sugars, glucose, glycerol, etc) into SCO. Characteristic results concerning production of lipid in several fermentation configurations are depicted in Table III.13.

Table III. 13. Maximum biomass and lipid content of some *Rhodosporidium toruloides* strain during growth under various fermentation configurations.

Strain	Substrate	Culture mode	X (g/L)	$Y_{L/X}$ (% w/w)	Reference
*R. toruloides**	Pure stearic acid	Shake flasks	11.7	35.0	Gierhart (1984a)
*R. toruloides**	Glucose/pure stearic acid	Shake flasks	9.8	46.1	Gierhart (1984b)
R. toruloides CBS14	Glucose	Shake flasks	12.3	30.8	Moreton (1985)
R. toruloides CBS14	Glucose	Shake flasks	8.0	42.5	Moreton (1988)
”	Fructose	Shake flasks	7.9	25.3	”
”	Glycerol (pure)	Shake flasks	5.81	34.6	”
”	Glucose	Batch bioreactor	12.5	42.9	”

”	Fructose	Batch bioreactor	8.7	39.8	”
R. toruloides CBS14	Xylose	Batch bioreactor	8.3	42.2	Moreton (1988)
R. toruloides AS2.1389	Glucose	Shake flasks	18.3	76	Li et al. (2006)
R. toruloides Y4	Glucose	Fed-batch bioreactor	106.5	67.5	Li et al. (2007)
R. toruloides Y4				62.2	Wu et al. (2010)
R. toruloides Y4	Jerusalem artichoke extracts	Shake flasks	25.5	40	Zhao et al. (2010)
”	Jerusalem artichoke extracts	Batch bioreactor	4.00	43	Zhao et al. (2010)
”	Jerusalem artichoke hydrolysates	Fed-batch bioreactor	70.1	56.5	Zhao et al. (2010)
R. toruloides Y4				58.3	Wu et al. (2011)
R. toruloides CBS14	Glucose	Fed-batch bioreactor	47	75	Wiebe et al. 2012
R. toruloides AS2.1389	Glycerol	Shake flasks	19.2	47.7	Xu et al. (2012)
R. toruloides AS2.1389	Glycerol	Batch bioreactor	26.7	69.5	Xu et al. (2012)
R. toruloides Y4	Glycerol	Batch bioreactor	35.3	46	Uçkun Kiran et al. (2013)
R. toruloides 2F5	Inulin	Shake-flasks	15.8	62.1	Wang et al. (2014)
R. toruloides 2F5	Inulin	Batch bioreactor	15.6	70.4	Wang et al. (2014)
***R. toruloides* DSM 444**	**Glucose**	**Fed-batch bioreactor**	**37.2**	**64.5**	**Present study**
***R. toruloides* DSM 444**	**TOPW-Glucose**	**Batch bioreactor**	**18.8**	**73.9**	**Present study**

*: No indication of the strain

In the current investigation and specifically in the bioreactor experiments, conversion yields of the order of *c.* 0.21 g of lipid produced per g of glucose consumed have been seen. The stoichiometry of glucose (and similar sugars such as lactose, fructose, etc) metabolism indicates that about 1.1 moles of acetyl-CoA are generated from 100 g of glucose (Ratledge, 1988; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a). Assuming that all the acetyl-CoA produced is channeled towards lipid synthesis, the maximum theoretical yield of SCO produced per glucose consumed is around 0.32 g/g (Ratledge, 1988; Ratledge and Wynn, 2002). This value concerning the fermentation of xylose ranges between 0.31-0.34 g/g), while with reference to glycerol, the maximum theoretical yield of SCO is around 0.30 g/g (Papanikolaou and Aggelis, 2011a). However, even under ideal conditions for SCO production (e.g. highly aerated bioreactor cultures) lipid yield on glucose consumed can rarely be higher than 0.22-0.23 g/g (Ykema et al., 1988; Ratledge and Wynn, 2002; Ratledge and Cohen, 2008). It may be assumed therefore that in the current investigation one of the highest

conversion yields of SCO produced per sugar consumed has been achieved. As previously indicated (Ratledge and Wynn, 2002; Ratledge and Cohen, 2008) cultivation in highly aerated bioreactors was considered as an important prerequisite in order to achieve such high conversion yields. However, in some cases, in shake-flask experiments equally high lipid yields can be achieved; in trials with *Thamnidium elegans* grown on sucrose in shake flasks, the conversion yield of lipid produced per sugar consumed was *c.* 0.24 g/g, while utilization of other sugars (glucose or fructose) equally resulted in exceptional conversion yields, i.e. >0.20 g/g (Papanikolaou et al., 2010). Likewise, maximum conversion yields of the same magnitude compared with growth of *Thamnidium elegans* on sucrose (*c.* 0.23 g/g) have been reported for *Cunninghamella echinulata* cultivated on xylose in shake-flask experiments (Fakas et al., 2009b).

Fatty acid analysis was carried out in lipids produced during *Rhodosporidium toruloides* batch-flask cultures with increasing NaCl content. The main fatty acids detected in yeast oil composition were oleic (C18:1), palmitic (C16:0), linoleic (C18:2) as well as stearic acid (C18:0). Interestingly, during growth on TOPW-based media, also the FA C20:0, in some cases in non-negligible quantities, appeared. NaCl presence did not affect the concentration of individual fatty acids, whereas microbial lipid became generally more unsaturated, during lipid accumulation phase. This is attributed to the increase of oleic acid as major constituent of accumulated triglycerides (Meesters et al. 1996). The distribution of *Rhodosporidium toruloides* fatty acids is similar to oil profiles obtained by other oleaginous yeasts (Li et al., 2007; Zhao et al., 2008). Recent studies have shown the suitability of microbial oil as starting material for biodiesel production, through its direct transformation from microbial biomass (Liu and Zhao, 2007; Vicente et al., 2009). On the other hand, as previously indicated, in early reports the addition of natural or chemically synthesized compounds (e.g. sterculic acid, malvanic acid, cis-methylen-octadecenoïc acid, etc), was performed in glucose-based nitrogen-limited cultures of *R. toruloides*. The above-mentioned compounds are Δ9 and Δ12 desaturase inhibitors, while in the quantities of these compounds added into the media, whereas growth and lipid production was not altered, the cells indeed accumulated lipids containing significant quantities of the FA C18:0, presenting thus composition similarities with the cocoa-butter (Moreton, 1985; Moreton and Clode, 1985).

Besides the cultures of *Rhodosporidium toruloides*, the ability of the yeast *Yarrowia lipolitica* to grow in a salty wastewater, namely glucose-enriched TOPW, has been revealed

for the first time in the international literature through this study; the microorganism during its culture under nitrogen-limited conditions presented a noticeable production of citric acid; maximum citric acid quantity achieved was of 68.1 g/L with a simultaneous yield of citrate produced per unit of glucose consumed ($Y_{Cit/Glc}$) of *c.* 0.44 g/g being reached. These results are of interest; values of yield of citric acid produced per substrate consumed reported in the literature by strains growing on sugars (or similarly metabolized compounds such as glycerol) range between 0.40 and 0.80 g/g (Rane and Sims, 1993; 1996; Anastassiadis et al., 2002; Morgunov et al., 2004; Anastassiadis and Rehm, 2005; Moeller et al., 2007; Papanikolaou et al., 2008a; 2008b; André et al., 2009; Makri et al., 2010). Only in a very limited number of reports this conversion yield was >0.90 g/g (case of mutant *Yarrowia lipolytica* strain growing on waste biodiesel-derived glycerol – see: Morgunov et al. 2013. Papanikolaou et al., 2013). Likewise, Cit_{max} quantities of c. 68 g/L are equally of interest. An overview of the experimental results achieved concerning citric acid-producing yeasts when grown under various fermentation configurations including the current investigation is presented in Table III.14.

The main cellular fatty acid of *Yarrowia lipolytica* lipid produced during all trials was the C18:1. In general, the distribution of intra-cellular fatty acids in *Yarrowia lipolytica* strains growing on glucose or other hydrophilic compounds (glycerol or ethanol) seems to be strain-dependent, while other parameters influencing the FA composition of *Yarrowia lipolytica* yeasts are the initial substrate concentration, the fermentation time, the dilution rate and the addition of exogenous natural compounds (like oregano oil) (Aggelis and Komaitis, 1999; Papanikolaou et al., 2008b; 2009; 2013; André et al., 2009; Makri et al., 2010; Chatzifragkou et al., 2011a).

Table III. 14. Experimental results of citric acid-producing yeasts during growth under various fermentation configurations

Strain	Substrate	culture mode	Cit (g/L)	$Y_{Cit/S}$ (g/g)	Reference
Yarrowia lipolytica ATCC 20346	Glucose	Fed-batch culture, bioreactor	50.0-69.0	0.52	Moresi (1994)
Saccharomycopsis lipolytica NRRL Y7576	Glucose	Batch culture, bioreactor	51.5	0.71	Klasson et al. (1989)
Candida lipolytica Y1095	Glucose	Fed–batch culture bioreactor	13.6-78.5	0.79	Rane and Sims (1993)
Candida lipolytica Y 1095	Glucose	Continuous culture recycling, bioreactor	40.0-50.0	0.72	Rane and Sims (1995)
Yarrowia lipolytica ACA-DC 50109	Raw glycerol	Batch culture, flacks	35.1	0.44	Papanikolaou et al. (2002b)
Yarrowia lipolytica 187/1	Rapeseed oil	Fed-batch culture, bioreactor	135.1	1.55	Kamzolova et al. (2005b)
Yarrowia lipolytica ACA-DC 50109	Raw glucose	Batch culture, flacks	42.9	0.56	Papanikolaou et al. (2006)
Yarrowia lipolytica Wratislavia 1.31	Raw glycerol	Batch culture bioreactor	124.5	0.62	Rymowicz et al. (2006)
Yarrowia lipolytica Wratislavia AWG7			88.1	0.46	
Yarrowia lipolytica Wratislavia K1			75.7	0.40	
Yarrowia lipolytica ACA-DC 50109	Raw glycerol	Batch culture, flacks	62.5	0.56	Papanikolaou et al. (2008b)
Yarrowia lipolytica A-101-1.22	Raw glycerol	Batch culture bioreactor	112.0	0.60	Rymowicz et al. (2010)
Yarrowia lipolytica ACA-YC 5033	Raw glycerol	Batch culture, flasks	50.1	0.44	André et al. (2009)
Yarrowia lipolytica A-101	Raw glycerol	Batch culture bioreactor	0.43	66.8	Rywińska et al. (2010a)
Yarrowia lipolytica Wratislavia K1	Pure glycerol		53.3	0.34	
	Raw glycerol		36.8	0.25	
Yarrowia lipolytica Wratislavia AWG7	Raw glycerol	Fed-batch culture bioreactor	157.5	0.58	Rywińska et al. (2010b)
Yarrowia lipolytica Wratislavia 1.31			155.2	0.55	
Yarrowia lipolytica NG40/UV7	Pure glycerol	Fed-batch culture bioreactor	115.0	0.64	Morgunov et al. (2013)
Yarrowia lipolytica JMY1203	Raw glycerol	Batch culture, flasks	57.7	0.92	Papanikolaou et al. (2014)
Yarrowia lipolytica ACA-YC 5033	OMW-based media	Batch culture, flasks	47.1	0.67	Sarris et al. (2013)
		Batch culture, flasks pasteurized	15.5	0.68	
		Batch culture bioreactor	13.9	0.58	
***Yarrowia lipolytica* ACA-YC 5033**	**TOPW-based media**	**Batch culture, flasks**	**43.5**	**0.43**	**Present study**
		Batch culture bioreactor	**68.1**	**0.43**	

III.2. Utilization of biodiesel-derived crude glycerol for the production of microbial lipid and other useful microbial compounds by oleaginous microorganisms: strain selection and impact of substrate concentration on the fermentation efficiency

After the study of some yeasts strain growing on substrates containing glucose as carbon source, in the second part of this study, we are investigated the potential of biodiesel derived waste glycerol conversion into metabolic compounds of added-value by yeast strains growing under nitrogen-limited conditions. After a first initial selection of yeast strains cultivated on biodiesel-derived waste glycerol utilized as carbon source under nitrogen-limited conditions (conditions that favor the accumulation of storage lipid by microorganisms) two microbial species that presented the best performances on the production of lipid from crude glycerol, namely *Lipomyces starkeyi* (strain DSM 70296) and *Rhodosporidium toruloides* (strain NRRL Y-27012) were more profoundly studied concerning the above-mentioned aspect.

III.2.1. Introduction

The worldwide decrease of petroleum feedstocks and the concomitant rise of price of crude oil have rendered as a very important priority for the scientific community the discovery of "new" and "renewable" types of energy sources, with biodiesel being considered as one of the most important ones (Papanikolaou and Aggelis, 2009; Wen et al., 2009a). Biodiesel is prepared through *trans*-esterification of conventional or non-conventional (*e.g.* microbial) oils and fats with short chain alcohols (principally methanol and to lesser extent ethanol or butanol) (Papanikolaou and Aggelis, 2009; 2011a). Fat splitting in order for biodiesel generation to be performed, results in the synthesis of concentrated glycerol-containing water as the principal side-product of this process. With the production of 10 kg of bio-diesel deriving from trans-esterification of various oils, 1 kg of (pure) glycerol is generated (Rivaldi et al., 2009; Wen et al., 2009a). The forthcoming significant rise in the production of biodiesel and the concomitant glycerol over-production and disposal is likely to cause very important environmental problems in the near future. Likewise, significant quantities of glycerol-containing water can also be generated through bioethanol and/or alcoholic beverages production units; for instance, during the typical bioethanol production process, ethanol is separated via distillation while the liquid fraction of the remaining material (the so-called thin stillage) contains *c.* 2% w/v of glycerol (Yazdani and Gonzalez, 2007). Likewise, liquid waste streams containing high levels of glycerol (glycerol quantities of 55-90% w/v) are generated in the various oleochemical facilities employing transformations of vegetable or animal fats, and this additional surplus will inevitably negatively affect the price of glycerol (Yazdani and Gonzalez, 2007; Papanikolaou and Aggelis, 2009). Finally, opportunities for even larger presence of glycerol feedstocks into the market volume exist due to the very high intra-cellular accumulation of glycerol (in quantities up to *c.* 85% w/w) in several algal species like *Dunaliella* sp. (Clomburg and Gonzalez, 2012). In some of the above-mentioned cases, quantities of glycerol into the water up to 7.8 mol/L (equivalent to *c.* 720 g of glycerol per L in water) can occur (Clomburg and Gonzalez, 2012). It may be assumed therefore, that conversion of this low- or even negative value product to higher added-value compounds by the means of chemical or fermentation technology currently attracts high and continuously increasing interest; as far as the biotechnological conversions are concerned, in several cases, prokaryotic microorganisms have been implicated in the

fermentative conversion of (crude) glycerol into compounds like bioalcohols (principally 1,3-propanediol and to lesser extent 2,3-butanediol, ethanol and butanol), poly-(hydroxylakanoates), biosurfactants, dihydroxyacetone and succinic acid (Yazdani and Gonzalez, 2007; Celińska and Grajek, 2009; Clomburg and Gonzalez, 2012). Likewise, a significant number of reports, principally appeared the last years, indicates the potentiality of eukaryotic microorganisms (yeasts, molds and heterotrophically grown algae) to convert (crude) glycerol into a plethora of metabolic compounds of potentially high value like microbial lipids (called also single cell oils – SCOs) citric acid, microbial mass, enzymes and polyols (for reviews see: Rivaldi et al., 2009; Papanikolaou and Aggelis, 2009; 2011b; Wen et al., 2009a; 2009b; Rywińska et al., 2013a; Abghari and Chen, 2014).

SCOs, lipids that are produced by the so-called oleaginous microorganisms, present a very high interest for the Industrial Biotechnology, since they can either be used as replacements of very high-added value oils and fats rarely found in the plant or animal kingdom, or they can constitute the precursors of the synthesis of the "2nd" or "3rd" generation biodiesel (Papanikolaou and Aggelis 2009; 2011a; 2011b; Abghari and Chen, 2014). Although (crude, biodiesel derived) glycerol has been used in many reports as substrate for the production of SCOs by several eukaryotic microbial strains (Meesters et al., 1996; Papanikolaou and Aggelis, 2002; Chi et al., 2007; Pyle et al., 2008; André et al., 2009, 2010; Liang et al., 2010a; 2010b; Makri et al., 2010; Chatzifragkou et al., 2011a; 2011b; Ethier et al., 2011; Saenge et al., 2011; Dedyukhina et al., 2012; 2014; Fontanille et al., 2012; Chang et al., 2013; ; Duarte et al., 2013a, 2013b; Kitcha and Cheirslip 2013; Louhasakul and Cheirslip, 2013; Wensel et al., 2014), or as substrate for the production of metabolic compounds (like citric acid, acetic acid, polyols, etc) by yeast strains (Papanikolaou et al., 2002, 2008, 2013; Rymowicz et al., 2008, 2009; André et al., 2009; Rywińska et al., 2009, 2011; Rywińska and Rymowicz, 2010; Chatzifragkou et al., 2011a; 2011b; Kamzolova et al., 2011; Morgunov et al., 2013; Petrik et al., 2013), data concerning growth and lipid production by strains of the yeasts *Rhodosporidium toruloides* and *Lipomyces starkeyi* growing on glycerol are indeed scarce in the international literature (Moreton, 1988; Xu et al., 2012; Uçkun Kiran et al., 2013).

III.2.2. Results

III.2.2.1. Initial screening of yeast strains on crude glycerol-based media.

In the first part of this work, the six employed strains were tested on media composed of crude glycerol at $Glol_0$ concentration adjusted at *c.* 30 g/L under nitrogen-limited conditions (utilization of peptone at 0.75 g/L and yeast extract at 0.5 g/L; initial molar ration employed at *c.* 100 mol/mol) in order to favor the accumulation of storage lipids and (potentially for the employed *Yarrowia lipolytica* strains) the secretion of secondary metabolites (e.g. citric acid, mannitol, etc) useful for the Food Industry. The obtained results of the performed trials as regards biomass and lipid production of the screened strains are illustrated in Table III.15.

Table III. 15. Experimental results originated from kinetics of yeast strains grown on crude glycerol in shake-flask experiments. Representations of biomass (X, g/L), lipid (L, g/L), glycerol consumed ($Glol_{cons}$, g/L), fermentation time (h) and lipid in dry biomass ($Y_{L/X}$, % w/w) when the maximum quantity of lipids in dry cell weight ($Y_{L/X}$, % w/w) (a) and the maximum concentration of biomass (X, g/L) (b) respectively, were achieved. Culture conditions: growth on 250-ml flasks at 180±5 rpm, T=28±1°C, initial glycerol concentration ($Glol_0$) ~30 g/L, initial molar ratio C/N~100 moles/moles, initial pH=6.1±0.1, pH ranging between 5.0 and 6.0, oxygen saturation higher than 40% (v/v) for all growth phases. For the trial performed with *Lipomyces starkeyi*, biomass produced was subjected to acidification before extraction of total lipids. Each experimental point is the mean value of two independent measurements.

Yeast strain		Time	$Glol_{cons}$ (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)
Cryptococcus curvatus NRLL Y-1511	a,b	195	25.7	11.8	1.48	12.5
Yarrowia lipolytica ACA YC 5029	A	22	5.9	3.1	0.28	9.0
	B	95	29.1	5.5	0.21	3.8
Yarrowia lipolytica ACA YC 5033	A	26	8.5	4.1	0.86	20.9
	B	190	31.1	6.1	0.27	4.4
Rhodosporidium toruloides DSM 4444	a,b	168	25.5	8.9	2.22	24.9
Rhodosporidium toruloides NRRL Y-27012	a,b	92	30.4	11.0	2.83	25.7
Lipomyces starkeyi DSM 70296	A	168	21.0	9.0	2.95	32.7
	B	192	22.1	9.3	1.10	11.8

Indeed, as it has already been previously reported for several types of strains of the yeast *Yarrowia lipolytica* (Papanikolaou et al. 2002; 2008a; 2013; Makri et al., 2010; Chatzifragkou et al., 2011b) batch cultures on glycerol, despite the significant nitrogen

limitation imposed, were not accompanied by remarkable accumulation of lipids per unit of biomass. What was interesting in the current submission and coincided with previous information concerning the growth of several strains of the species *Yarrowia lipolytica* on hydrophilic carbon sources (e.g. glycerol, glucose, etc) (Makri et al., 2010; Sarris et al., 2011; Chatzifragkou et al., 2011b; Papanikolaou et al., 2013) was related with the fact that $Y_{L/X}$ values at the first stages of the culture increased, despite the fact that nitrogen was found in excess into the growth medium ($Y_{L/X}$ reached to *c.* 21% w/w for the strain ACA YC 5033). Thereafter, cellular lipids were subjected to biodegradation, with this biodegradation being accompanied by secretion into the medium of citric acid (for the strain ACA-YC 5033) and mixture of citric acid and mannitol (for the strain ACA-YC 5029). Patters of changes of biomass (g/L), citric acid (g/L) and lipid in biomass (%, w/w) for the strain ACA-YC 5033 are illustrated in Fig. III.11., in which, in agreement with the current literature (Papanikolaou et al., 2002, 2009; Rymowicz et al., 2006, 2010; Sarris et al., 2011) citric acid was produced almost exclusively at the stationary growth phase of the microbial fermentation, while, as previously stressed, its secretion coincided with significant decrease in the quantity of lipids produced per unit of biomass DCW (Makri et al., 2010; Sarris et al., 2011).

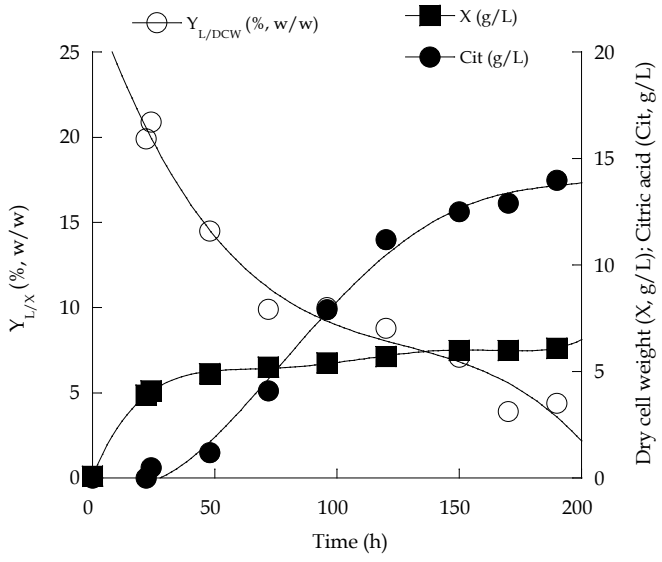


Figure III. 11. Kinetics of biomass produced (X, g/L), citric acid produced (Cit, g/L) and intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$, % w/w) of *Yarrowia lipolytica* ACA-YC 5033 during growth on crude glycerol, at initial glycerol concentration ($Glol_0$) of *c.* 30 g/L, under nitrogen-limited conditions. Culture conditions: growth on 250-mL flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and

6.0, DOT>20% (v/v), incubation temperature T=28±1°C. Each point is the mean value of two independent measurements.

As far as the other yeast strains that were screened in this part of the work were concerned, the yeast *Cryptococcus curvatus* NRRL Y-1511 presented interesting biomass formation (11.8 g/L) with a concomitant dry biomass yield per unit of glycerol consumed ($Y_{X/Glol}$) of *c.* 0.46 g/g. Nevertheless, despite the significant nitrogen limitation imposed, this microorganism accumulated under the present culture conditions relatively low quantities of total lipids ($Y_{L/X}$=12.5% w/w – see Table III.15.). Moreover, the microorganism *Rhodosporidium toruloides* DSM 4444 presented a satisfactory biomass production (X_{max}≈9 g/L; $Y_{X/Glol}$≈0.25 g/g) but, on the other hand, it accumulated moderate quantities of lipid ($Y_{L/X}$=24.9% w/w). Higher quantities of total biomass (X=11.0 g /L; $Y_{X/Glol}$≈0.36 g/g) were produced by the strain *Rhodosporidium toruloides* NRRL Y-27012, that accumulated slightly higher quantities of total lipids compared with the strain DSM 4444 ($Y_{L/X}$=25.7% w/w). Likewise, the microorganism *Lipomyces starkeyi* DSM 70296 produced remarkable quantities of total biomass (X=9.0 g/L; $Y_{X/Glol}$≈0.43 g/g) that contained interesting quantities of microbial lipids ($Y_{L/X}$=32.7% w/w; L=2.95 g/L). Interestingly, in the latter fermentation, although some non-negligible quantities of glycerol remained unconsumed into the growth medium (at the fermentation time t=192 h, Glol≈6 g/L), the microorganism proceeded at a significant degradation of its previously accumulated lipids resulting in a further increase of the biomass value achieved (Table III.15.).

Besides the studied strains of *Yarrowia lipolytica*, in the other yeasts screened (*Cryptococcus curvatus*, *Rhodosporidium toruloides* and *Lipomyces starkeyi*), the pH of the culture medium presented an insignificant drop (initial pH=6.0±0.1, final pH=5.7±0.1), indicating small secretion of organic acids into the culture medium. Indeed, HPLC analysis performed into the supernatant at the end of culture of *Cryptococcus curvatus*, *Rhodosporidium toruloides* and *Lipomyces starkeyi*, showed that citric acid in indeed small concentrations (0.8±0.2 g/L) was the sole extra-cellular metabolite detected. It is evident that unlike the two *Yarrowia lipolytica* strains tested in which glycerol was mainly converted into citric acid (for the strain ACA-YC 5033) and blends of citric acid and mannitol (for the strain ACA-YC 5029), in all other microorganisms studied (*Cryptococcus curvatus*, *Rhodosporidium toruloides* and *Lipomyces starkeyi*) the carbon flow was mainly channeled towards the synthesis of DCW (that presented maximum absolute values that were 45%-93%

higher than the maximum obtained one of 6.1 g/L for the strain ACA YC 5033) and lipid (that presented maximum absolute values that were 72%-343% higher than the maximum obtained one of 0.86 g/L for the strain ACA YC 5033) (see values in Table III.15.).

II.2.2.2. Trials with Rhodosporidium toruloides NRRL Y-27012 and Lipomyces starkeyi DSM 70296 growing at increasing initial glycerol concentration media.

As seen in the previous paragraph, the microorganism *Rhodosporidium toruloides* NRRL Y-27012 presented clearly higher biomass production and relatively higher lipid accumulation compared to *Rhodosporidium toruloides* DSM 4444 during shake-flask cultures on media composed of crude glycerol. Likewise, *Lipomyces starkeyi* DSM 70296 presented appreciable biomass and lipid production during its cultivation on crude glycerol. In a next step therefore, it was desirable to enhance the production of SCO in these microorganisms, hence, *Rhodosporidium toruloides* NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296 were cultivated on media containing higher $Glol_0$ concentrations by maintaining the same initial nitrogen quantity employed (utilization of peptone at 0.75 g/L and yeast extract at 0.5 g/L), in order to formulate culture media presenting higher excess of carbon, and, therefore, to favor the process of lipid accumulation (Ratledge, 1997; Papanikolaou and Aggelis, 2011a). As indicated in the section "Materials and Methods", 10% v/v inoculum was used in these fermentations, the results of which are shown in Tables III.16. (for *Rhodosporidium toruloides*) and III.17. (for *Lipomyces starkeyi*).

Table III. 16. Experimental results originated from kinetics of the yeast *Rhodosporidium toruloides* NRRL Y-27012 grown on crude glycerol in shake-flask experiments. Representation of fermentation time (h) in which biomass (X, g/L), lipid (L, g/L), glycerol consumed ($Glol_{cons}$, g/L), lipid in dry biomass ($Y_{L/X}$, % w/w) and yield of lipid produced per unit of glycerol consumed ($Y_{L/Glol}$, g/g) were achieved when the maximum quantity of lipids in dry cell weight ($Y_{L/X}$, % w/w) was obtained. Culture conditions: growth on 250-mL flasks at 180±5 rpm, T=28±1°C, various initial glycerol concentrations employed, initial pH=6.1±0.1, pH ranging between 5.0 and 6.0, oxygen saturation higher than 20% (v/v) for all growth phases, inoculation volume 10% (v/v). Each experimental point is the mean value of two independent measurements.

$Glol_0$ (g/L)	Time (h)	$Glol_{cons}$ (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	$Y_{X/Glol}$ (g/L)	$Y_{L/Glol}$ (g/L)
≈50	195	47.6	16.7	7.89	47.2	0.35	0.17
≈95	288	79.0	23.8	11.19	47.0	0.30	0.14
≈120	395	115.9	30.1	12.04	40.0	0.26	0.10

≈180	360	79.1	16.2	8.80	54.3	0.20	0.11

Table III. 17. Experimental results originated from kinetics of the yeast *Lipomyces starkeyi* DSM 70296 grown on crude glycerol in shake-flask experiments. Representation of fermentation time (h) in which biomass (X, g/L), lipid (L, g/L), glycerol consumed ($Glol_{cons}$, g/L), lipid in dry biomass ($Y_{L/X}$, % w/w) and yield of lipid produced per unit of glycerol consumed ($Y_{L/Glol}$, g/g) were achieved when the maximum quantity of lipids in dry cell weight ($Y_{L/X}$, % w/w) was obtained. Culture conditions: growth on 250-ml flasks at 180±5 rpm, T=28±1°C, various initial glycerol concentrations employed, initial pH=6.1±0.1, pH ranging between 5.0 and 6.0, oxygen saturation higher than 20% (v/v) for all growth phases, inoculation volume 10% (v/v). Each experimental point is the mean value of two independent measurements.

$Glol_0$ (g/L)	Time (h)	$Glol_{cons}$ (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	$Y_{X/Glol}$ (g/g)	$Y_{L/Glol}$ (g/g)
≈50	144	46.7	11.2	3.70	33.0	0.24	0.08
≈100	336	105.1	23.3	8.16	35.0	0.22	0.08
≈120	470	115.1	34.4	12.34	35.9	0.30	0.11
≈180	310	91.1	18.2	6.74	37.0	0.20	0.07

As far as the microorganism *Rhodosporidium toruloides* NRRL Y-27012 was concerned, its cultivation on high $Glol_0$ concentration media with simultaneous constant initial nitrogen availability clearly enhanced the production of total biomass and lipid up to the $Glol_0$ concentration adjusted at *c*. 120 g/L. Significant quantities of lipids (L≈12 g/L) were produced in the trial with $Glol_0$≈120 g/L (see Table III.16.). However, it is interesting to indicate that within the range of $Glol_0$ concentrations tested 50-120 g/L, although in absolute values the quantities of produce X and L increased with the increment of $Glol_0$ concentration, lipid in dry weight ($Y_{L/X}$) values decreased. Moreover, another interesting feature of the microbial behavior was related with the fact that growth was not ceased in trial in which a very high $Glol_0$ concentration was tested ($Glol_0$≈180 g/L). Even at this trial, non-negligible production of X and L was observed (interestingly, at this very high $Glol_0$ concentration tested, the $Y_{L/X}$ value was the highest one obtained in this study by this microorganism being of *c*. 54% w/w), while remarkable assimilation of glycerol occurred (360 h after inoculation *c.* 45% w/w of glycerol had been consumed by the microorganism – further incubation did not improve the assimilation of glycerol). However, it must be stressed that apparently substrate inhibition occurred due to the increasing $Glol_0$ concentrations imposed, since the more $Glol_0$ concentration into the medium increased, the more the yield values of DCW and total lipid produced per unit of glycerol consumed ($Y_{X/Glol}$ and $Y_{L/Glol}$) decreased (see Table III.16).

Specifically, the $Y_{L/Glol}$ yield value, while at $Glol_0 \approx 50$ g/L it was found to be =0.17 g/g (a value close to the maximum achievable one of 0.21 g/L – see Papanikolaou and Aggelis 2011a), this value decreased to *c.* 0.10-0.11 g/g when the $Glol_0$ concentration rose to *c.* 120 or 180 g/L (Table III.16.). Concerning the $Y_{X/Glol}$ yield value, it clearly dropped from 0.36 to 0.20 g/g with the rise of glycerol concentration from 50 to 180 g/L (Table III.16), clearly demonstrating the negative effect of the high glycerol concentration on the biosynthetic ability of *R. toruloides* NRRL Y-27012.

As in the case of the microorganism *Rhodosporidium toruloides* NRRL Y-27012, the microorganism *Lipomyces starkeyi* DSM 70296 presented significantly increased production of biomass and lipid with increment of $Glol_0$ concentration until an initial substrate concentration adjusted to *c.* 120 g/L (see Table III.17.). Significant quantities of lipids ($L \approx 12.5$ g/L) and total biomass (X=34.4 g/L), the highest values reported in this investigation, were produced by *Lipomyces starkeyi* in the trial with $Glol_0 \approx 120$ g/L (see Table III.17.).

The production of lipid in absolute values (g/L) clearly increased with the rise of $Glol_0$ concentration into the medium (up to the value of *c.* 120 g/L), whereas in accordance with most of the findings of the international literature (Papanikolaou and Aggelis, 2011b), $Y_{L/X}$ equally increased with the increase of glycerol concentration. Moreover, in contrast to the trials performed with *Rhodosporidium toruloides* NRRL Y-27012, it seems that yields obtained by *Lipomyces starkeyi* DSM 70296 were improve by the increase of $Glol_0$ concentration up to the threshold of 120 g/L; this statement can be justified by the fact that the yields $Y_{X/Glol}$ and $Y_{L/Glol}$ presented their higher values at the trial in which the concentration of carbon substrate had been adjusted at *c.* 120 g/L (=0.30 and 0.11 g/g respectively), being clearly higher than the ones obtained in the trials with $Glol_0 \approx 50$ and 100 g/L. Finally, as it was observed in *Rhodosporidium toruloides* NRRL Y-27012, the strain *Lipomyces starkeyi* DSM 70296 presented appreciable biomass production, noticeable glycerol assimilation and non-negligible lipid production in a fermentation in which a significantly high $Glol_0$ concentration had been added into the medium (*c.* 180 g/L). However, in any case, the values of the yields $Y_{X/Glol}$ and $Y_{L/Glol}$ were the lower ones for this microorganism at this initial substrate concentration within the range of $Glol_0$ concentrations tested in the current study, fact that suggests substrate inhibition due to this very high $Glol_0$ quantity found into the medium. It should also be pointed out that in this trial, in accordance with the results obtained for

Rhodosporidium toruloides, further incubation after 310 h did not improve glycerol assimilation and biomass and lipid production by *Lipomyces starkeyi*.

In the trials performed with both *Rhodosporidium toruloides* and *Lipomyces starkeyi* it was also desirable to study besides the production of lipid, also the production of intra-cellular polysaccharides (IPS) as function of the fermentation time. The obtained results of biomass (g/L), lipid (g/L), IPS (g/L), glycerol (g/L) $Y_{L/X}$ (% w/w) and $Y_{IPS/X}$ (% w/w) in one $Glol_0$ concentration selected are seen for *Rhodosporidium toruloides* (Figs III. 12a; b) and *Lipomyces starkeyi* (Figs III.13a; b). As it may be seen, different kinetic profiles as regards the production of IPS and lipids are seen for the above-mentioned microorganisms; for the case of *Rhodosporidium toruloides*, intra-cellular polysaccharides in significant quantities were principally synthesized at the early growth steps (see Figs III. 12a; b).

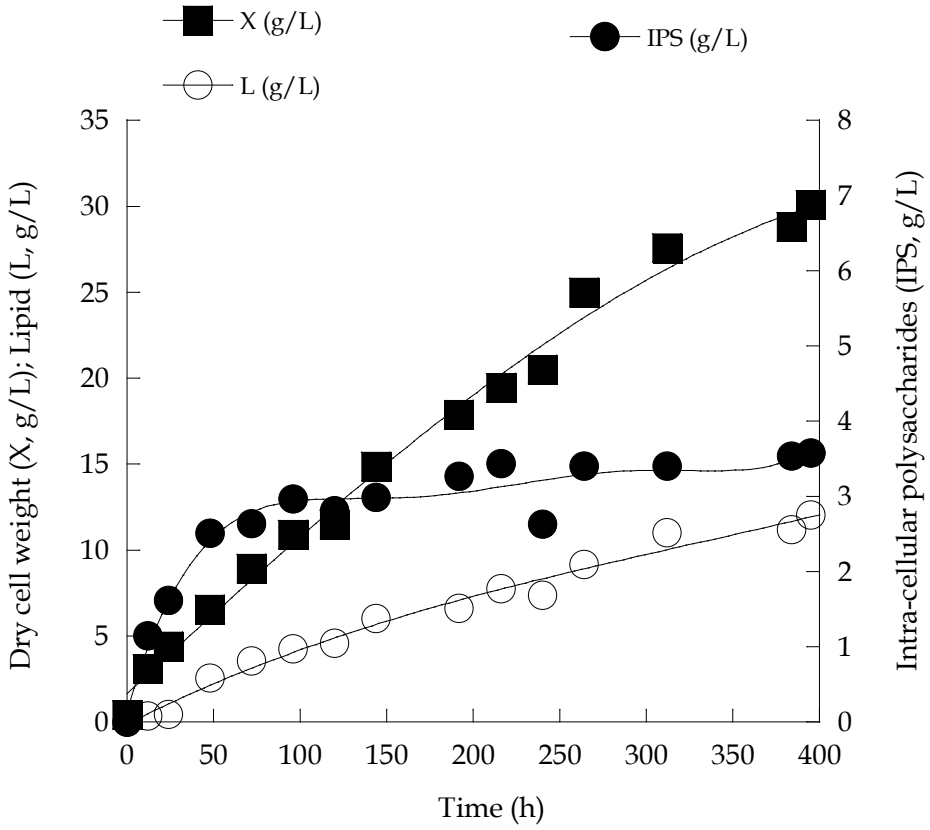


Fig. III.12a

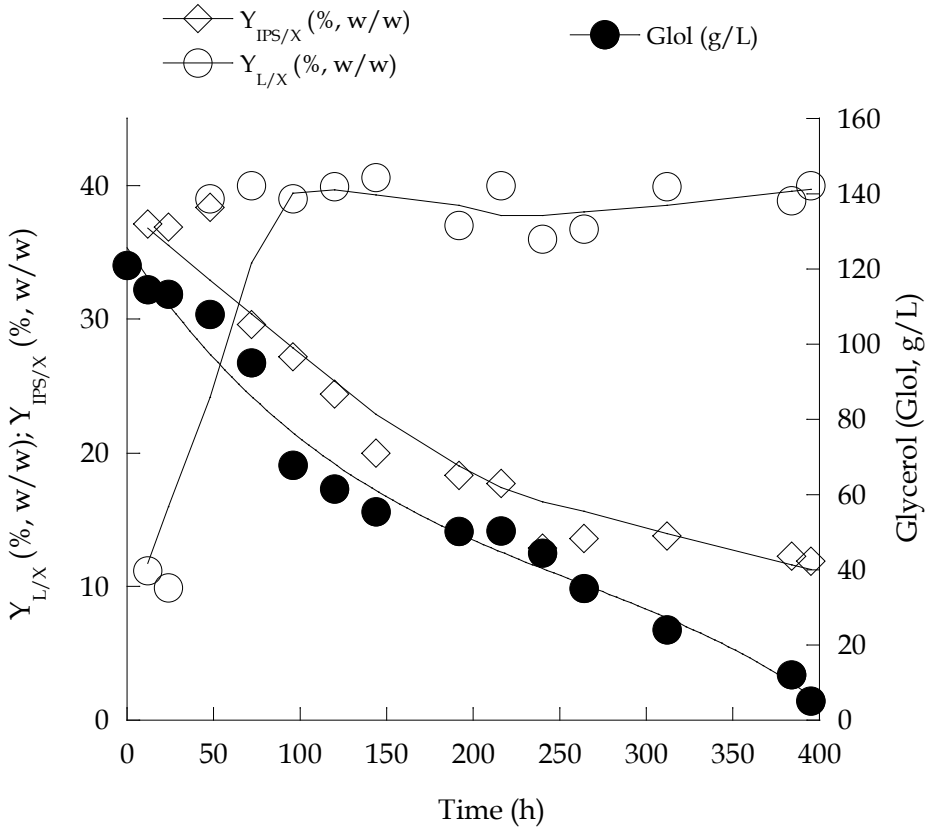


Fig. III.12b

Figure III. 12. Kinetics of biomass produced (X, g/L), lipid produced (L, g/L), intra-cellular polysaccharides produced (IPS, g/L) (a), remaining glycerol (Glol, g/L), intra-cellular polysaccharides produced per unit of dry weight ($Y_{IPS/X}$, % w/w) and intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$, % w/w) of *Rhodosporidium toruloides* NRRL Y-27012 during growth on crude glycerol, at initial glycerol concentration ($Glol_0$) of c. 120 g/L, under nitrogen-limited conditions. Culture conditions: growth on 250-ml flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=28±1°C. Each point is the mean value of two independent measurements.

It is interesting to indicate that significant quantities in IPS per unit of biomass (i.e. $Y_{IPS/X}$≈40% w/w) were synthesized at the initial growth phases (up to 50 h after inoculation) period in which nitrogen was found into the growth medium (extra-cellular nitrogen analysis not presented). Thereafter, $Y_{IPS/X}$ quantities constantly decreased (final $Y_{IPS/X}$ value ≈12% w/w, after *c.* 400 h of culture) while in absolute values (g/L), after the significant biosynthesis that had occurred at the first growth steps, *c.* 50 h after inoculation and until the end of the culture the concentration of intra-cellular polysaccharides remained practically constant. On the other hand, after virtual assimilable nitrogen depletion from the medium (*c.* 50 h after

inoculation) significant quantities of lipids were produced with the threshold of lipid in biomass ($Y_{L/X}$) being of *c.* 40% w/w throughout the culture (see Fig.III.12b). On the other hand, *Lipomyces starkeyi* presented a different kinetic profile; IPS per unit of X remained practically constant with a value *c.* 30% w/w throughout the culture, while in absolute values (g/L), the concentration of intra-cellular polysaccharides constantly increased until the end of the culture (with a significant IPS quantity ≈7 g/L at *c.* 350 h after inoculation). Likewise, total lipids in both absolute (g/L) and relative (%, w/w) values constantly increased, with significant onset of lipid production being given after nitrogen limitation, until the virtual glycerol depletion from the growth medium (see Figs III.13a; b).

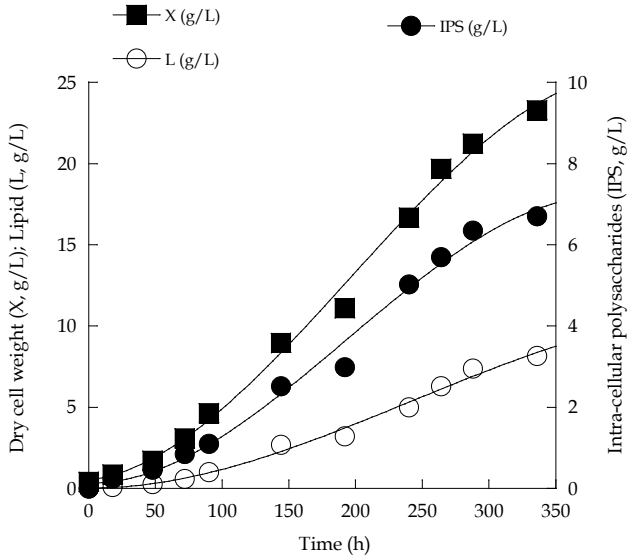


Fig. III.13a

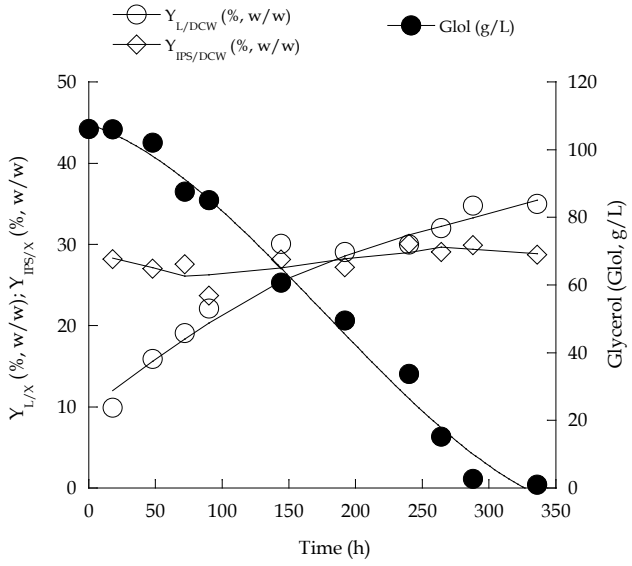


Fig. III.13b

Figure III. 13. Kinetics of biomass produced (X, g/L), lipid produced (L, g/L), intra-cellular polysaccharides produced (IPS, g/L) (a), remaining glycerol (Glol, g/L), intra-cellular polysaccharides produced per unit of dry weight ($Y_{IPS/X}$, % w/w) and intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$, % w/w) of *Lipomyces starkeyi* DSM 70296 during growth on crude glycerol, at initial glycerol concentration ($Glol_0$) of *c.* 100 g/L, under nitrogen-limited conditions. Culture conditions: growth on 250-ml flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DOT>20% (v/v), incubation temperature T=28±1°C. Each point is the mean value of two independent measurements.

II.2.2.3. Fatty acid composition of lipids produced by Rhodosporidium toruloides NRRL Y-27012 and Lipomyces starkeyi DSM 70296.

Total lipids of *Rhodosporidium toruloides* NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296 cultivated on several $Glol_0$ concentrations were converted into their respective FAMEs and were analyzed at the early and the late growth steps of the cultures, and the results are presented in Tables III.18 (*Rhodosporidium toruloides*) and III.19 (*Lipomyces starkeyi*). From the obtained results it can be seen that the fatty acid (FA) composition of the cellular lipids presented differentiations as function of the fermentation time and the $Glol_0$ concentration employed for both the microorganisms studied; for the case of *Rhodosporidium toruloides*, higher quantities of unsaturated FAs (in % w/w) and mainly of C18:1 and C18:2 were detected at the early compared with the late growth phases.

Table III. 18. Fatty acid composition of cellular lipids of *Rhodosporidium toruloides* NRRL Y-27012, during growth on media composed of crude glycerol added at various initial glycerol ($Glol_0$) concentrations and constant initial nitrogen, under nitrogen-limited conditions. Early growth phase is that in which incubation time is between 24-72 h. Late growth phase is that in which incubation time is c. 100 h (for $Glol_0$≈30 g/L) and c. 200-250 h (forThe higher $Glol_0$ concentrations). Culture conditions as in Tables III.13 and III.14.

$Glol_0$ (g/L)	Growth phase	C16:0	C18:0	C18:1	C18:2
≈30	Early	23.0	10.5	55.5	13.0
	Late	31.0	8.0	50.2	10.0
≈50	Early	21.8	14.4	53.9	7.1
	Late	31.4	13.4	48.7	6.0
≈95	Early	23.3	11.1	54.1	5.1
	Late	34.1	13.0	50.1	1.9
≈180	Early	30.4	13.4	47.0	5.3
	Late	35.4	12.2	44.0	2.4

Table III. 19. Fatty acid composition of cellular lipids of *Lipomyces starkeyi* DSM 70296, during growth on media composed of crude glycerol added at various initial glycerol ($Glol_0$) concentrations and constant initial nitrogen, under nitrogen-limited conditions. Early growth phase is that in which incubation time is between 24-72 h. Late growth phase is that in which incubation time is *c.* 150 h (for $Glol_0$≈30 g/L) and c. 200-250 h (for the higher $Glol_0$ concentrations). Culture conditions as in Tables III.13 and III.15.

$Glol_0$ (g/L)	Growth phase	C16:0	C18:0	C18:1	C18:2
≈30	Early	35.8	8.8	46.1	5.3
	Late	30.9	7.9	52.1	5.6
≈50	Early	32.8	8.4	52.3	2.6
	Late	28.0	7.1	57.3	7.7
≈120	Early	33.1	8.0	53.1	4.4
	Late	26.1	7.0	57.0	9.7

Likewise, the obtained results indicated that the more the $Glol_0$ concentration into the medium increased, the more the quantity of saturated FAs (mainly C16:0 and lesser extent C18:0) rose into the storage lipids of *Rhodosporidium toruloides* (Table III.18).

On the other hand, different kinetic profile of the FA composition of the lipids of *Lipomyces starkeyi* has been identified, both as regards the fermentation time and the $Glol_0$ concentration into the medium; cellular lipids of *L. starkeyi* were more saturated at the early growth steps than the late ones, whereas the more the $Glol_0$ concentration into the medium increased the more the cellular FAs were unsaturated (Table III.19). In any case, lipid of both *Rhodosporidium toruloides* and *Lipomyces starkeyi* was mainly composed of the FAs C16:0 and $^{\Delta 9}$C18:1 and to lesser extent of the FAs C18:0 and $^{\Delta 9,12}$C18:2, constituting, thus, in accordance with the literature (Li et al., 2007; Zhao et al., 2008; Xu et al., 2012), a perfect material amenable to be converted into 2nd generation biodiesel.

III.2.3. Discussion

Yeast strains belonging to the species *Yarrowia lipolytica*, *Cryptococcus curvatus*, *Rhodosporidium toruloides* and *Lipomyces starkeyi* were initially screened in nitrogen-limited media composed of biodiesel-derived waste glycerol (at $Glol_0$ concentration adjusted at *c.* 30 g/L) as regards their potential to produce (mostly) SCO and probably other valuable metabolites such as citric acid, polyols, etc. The last few years, a continuously increasing number of researchers are interested in screening studies related with the selection of yeast

strains capable to proceed on glycerol conversion into metabolites of significance for the Industrial Biotechnology, like pigments, yeast biomass, organic acids, polyols, ribonucleotides, SCO, etc (Chatzifragkou et al., 2011b; Rivaldi et al., 2012; Taccari et al., 2012; Duarte et al., 2013a; Juszczyk et al., 2013; Kitcha and Cheirslip, 2013; Petrik et al., 2013). On the other hand, many screening studies of (mainly) yeasts capable to produce SCO growing on several carbon sources (e.g. glycerol, molasses, lignocellulosic sugars, *etc*) have been performed the last years and a large number of strains have been tested, due to the continuously increasing interest of the scientific community towards the discovery of new strains capable to store in high quantities SCOs, amenable to be converted into 2nd generation biodiesel (Duarte et al., 2013a; Sitepu et al., 2013; 2014; Vieira et al., 2014). In the present research paper we have decided to test a relatively small number of strains, so as to include in here data concerned the effect of glycerol concentration on yeast growth and lipid production that would be of interest for researchers and engineers. On the other hand, in the current investigation we have included *Rhodosporidium toruloides* and *Lipomyces starkeyi* yeasts, since strains of these species have presented excellent capacities of lipid production during growth on hydrophilic carbon sources (in some cases glycerol was amongst the substrates tested) (Moreton, 1988; Xu et al., 2012; Uçkun Kiran et al., 2013; Vieira et al., 2014; Yang et al., 2014).

In the current study, from this initial screening test, the microorganism *Cryptococcus curvatus* NRLL Y-1511 was revealed capable to produce non-negligible quantities of DCW (*c.* 12 g l^{-1}) with a $Y_{X/Glol}$ value ≈0.46 g/g, that constitutes a quite interesting value reported for the production of yeast biomass from crude glycerol-based media (Chatzifragkou et al., 2011b; Taccari et al., 2012; Juszczyk et al., 2013). It is interesting to indicate that despite the nitrogen-limited conditions imposed, the above-mentioned microorganism did not produce significant quantities of SCO, in contrast to the results reported for other strains of the same species (*i.e. C. curvatus* ATCC 20509). This strain (ATCC 20509), has been revealed capable to produce remarkable quantities of SCO during growth on media composed of (pure or crude) glycerol; specifically, when cultivated in fed-batch mode with pre-treated crude glycerol employed as substrate, this strain produced *c.* 22 g/L of lipid with a concomitant $Y_{L/X}$ value of *c*. 49% w/w (Cui et al., 2012). During growth on hydrolyzed spent yeast waste blended with glycerol ATCC 20509 strain presented a lipid production of c. 19 g/L with the respective $Y_{L/X}$ value being *c*. 38% w/w (Ryu et al., 2013). In fed-batch bioreactor trails

performed with different feeding strategies, the above-mentioned strain managed to produce SCO ranging between 13.7 and 17.4 g/L with respective $Y_{L/X}$ values 44.6 and 52.9% w/w (Liang et al. 2010a). Finally, the same strain (ATCC 20509) when cultivated in fed-batch cultures with pure glycerol employed as substrate produced *c.* 30 g/L of lipid with $Y_{L/X}$≈25% w/w (Meesters et al., 1996).

The two strains of *Yarrowia lipolytica* employed in the current investigation (ACA-YC 5029 and ACA-YC 5033) produced some quantities of citric acid during their growth on glycerol. Both the maximum quantity of citric acid produced and the conversion yield of citric acid produced per glycerol consumed are (relatively or much) lower than the respective values reported in the international literature for flask or bioreactor cultures of *Yarrowia lipolytica* strains cultivated on glycerol (maximum values ranging between 50 and 155 g/L with concomitant yields 0.55 to 0.90 g/g have been already seen in the literature) (Rymowicz et al., 2006; 2010; Papanikolaou et al., 2008a; 2013; Rywińska and Rymowicz, 2010; Rywińska et al., 2010; 2011; Kamzolova et al., 2011; Morgunov et al., 2013). Moreover, an interesting result associated with the growth of the strain ACA-YC 5033 cultivated on crude glycerol was that this strain secreted in non-negligible quantities mannitol into the growth medium (up to 6.0 g/L). This polyol was synthesized almost in equal quantities with citric acid (citric acid produced of *c.* 7.0 g/L). No other polyols (i.e. erythritol) were produced. In a relatively scarce number of reports, secretion of polyols into the culture medium together with citric acid, when glycerol was utilized as the sole carbon source in nitrogen-limited submerged experiments, has been presented (Rymowicz et al., 2008; Rywińska and Rymowicz, 2009). On the other hand, production of erythritol, in some cases in indeed very high quantities (e.g. >45 g/L or even >80 g/L) has been reported when crude or pure glycerol has been employed as fermentation substrate by wild or mutant *Yarrowia lipolytica* strains (Tomaszewska et al., 2012; Rywińska et al., 2013b).

The two *Yarrowia lipolytica* strains employed in this investigation, during their growth on a substrate in which the *de novo* lipid accumulation mechanism was employed (glycerol) under nitrogen-limited conditions, in accordance with several reports presented in the international literature for various strains of this species (André et al., 2009; Papanikolaou and Aggelis, 2009; Papanikolaou et al., 2009; 2013; Makri et al., 2010; Sarris et al., 2011) did not present the typical feature of the classical oleaginous microorganisms; in the first growth step, and during the balanced growth phase (nitrogen-excess conditions) both strains presented

some accumulation of storage lipid (in the case of the strain ACA-YC 5033 the value of $Y_{L/X}$ was >20% w/w, that is considered as the value-threshold that categorizes a microorganism as "oleaginous" – Ratledge, 1997; Papanikolaou and Aggelis, 2009). Thereafter and despite significant presence of glycerol into the medium and progressive and finally almost complete exhaustion of nitrogen from the medium, $Y_{L/X}$ values were depleted while simultaneously low-molecular weight metabolites (citric acid and mannitol) were secreted into the medium. The concentration of available nitrogen can be considered of importance for the process of SCO production in *Yarrowia lipolytica* yeast, since, as it has been demonstrated in the literature (Fontanille et al., 2012; Papanikolaou et al., 2013), some quantities of nitrogen seem indispensable for lipid accumulation, whereas when nitrogen concentration drops below a threshold value, secondary metabolites, and notably citric acid, are produced, while lipid biodegradation is observed (Papanikolaou et al., 2013). Likewise, in agreement with the current investigation and unlike the typical oleaginous strains, during growth on glycerol also other *Yarrowia lipolytica* strains (e.g. strain ATCC 20460) have been reported to present increased $Y_{L/X}$ at the beginning of the culture ($Y_{L/X}$≈32% w/w 48 h after inoculation) (Sestric et al., 2014), while a restricted number of strains of this species have been reported to present high accumulation of lipid (e.g. L>10 g/L with simultaneous $Y_{L/X}$>30% w/w) during growth on glycerol in batch or fed-batch fermentations (Fontanille et al., 2012; Celińska and Grajek, 2013).

Rhodosporidium toruloides NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296 when cultivated on several $Glol_0$ concentrations under constant nitrogen availability presented significant DCW and SCO production; interestingly both strains presented remarkable biomass production and glycerol assimilation even in very high $Glol_0$ concentrations added into the medium ($Glol_0$≈180 g/L), although a conversion yield reduction due to the high $Glol_0$ concentration was observed. In similar types of experiments, the strain *Cryptococcus curvatus* ATCC 20509, when it was flask-cultured on various initial (crude or pure) glycerol concentrations presented significantly decreased microbial growth when $Glol_0$ concentration was added in quantities >60 g/L (Meesters et al., 1996; Liang et al., 2010a). For this reason, and in order to increase the quantity of glycerol assimilated by the strain so as to enhance the production of SCO, fed-batch fermentation strategies have been employed (Meesters et al., 1996; Liang et al., 2010a; Cui et al., 2012). Other yeast strains presented similar physiological behavior with the strains that were investigated in the current study; for instance, the strain

Yarrowia lipolytica ACA-DC 50109 presented significant biomass and citric acid formation and noticeable glycerol assimilation in media in which the $Glol_0$ concentration was adjusted to *c*. 170 g/L (Papanikolaou et al., 2008a). Likewise, in a recent development a newly isolated *Metschnikowia pulcherrima* strain when cultivated in flasks was revealed capable to present biomass production and glycerol assimilation even in media containing extremely high $Glol_0$ concentrations of *c*. 250 g/L (Santamauro et al., 2014). As far as oleaginous fungi are concerned, strains belonging to the genus *Mortierella* have been revealed capable to present remarkable growth, SCO production and glycerol assimilation in media with $Glol_0$ concentration adjusted to values >100 g/L with little inhibition being observed due to increment of glycerol concentration into the medium (Papanikolaou et al., 2008; Dedyukhina et al., 2012); for instance, two *Mortierella alpina* strains cultivated on media in which increasing $Glol_0$ concentrations had been used, presented their highest DCW and glycerol assimilation values for $Glol_0$ concentration values >110 g/L (Dedyukhina et al., 2012).

Rhodosporidium toruloides NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296 presented noticeable production of DCW and microbial lipids during growth on biodiesel-derived glycerol. The conversion of (pure or crude) glycerol into SCO has rarely been seen in *Rhodosporidium toruloides* strains (Moreton 1988; Xu et al., 2012; Uçkun Kiran et al., 2013) whereas the current study is the first report in the literature that deals with the production of SCO by *Lipomyces starkeyi* growing on glycerol-based media. The reported maximum values of SCO produced for both strains (>12 g/L) as well as the concomitant $Y_{L/X}$ values (35-47% w/w) are amongst the highest ones reported for shake-flask fermentations of microorganisms growing on glycerol, while they can compare favorable with many of the results achieved in bioreactor experiments. Summarizing results concerning DCW and SCO production by several oleaginous microorganisms cultivated on glycerol-based media in various fermentation configurations and their comparison with the present investigation are illustrated in Table III.20.

Table III. 20. Experimental results of microbial strains cultivated on glycerol-based media and producing microbial lipid during growth under various fermentation configurations and their comparisons with the present study.

Strain	Culture mode	X (g/L)	$Y_{L/X}$ (% w/w)	Reference
1) Yeasts				
Cryptococcus albidus CBS 4517 ¶	Shake flasks	1.4	43.8	Hansson and Dostálek, (1986)
Rhodosporidium toruloides CBS 14 ¶	Shake flasks	5.8	34.6	Moreton, (1988)
Cryptococcus curvatus ATCC 20509 ¶	Fed-batch bioreactor	118.0	25.0	Meesters et al., (1996)
Yarrowia lipolytica ACA-DC 50109 ‡	Single stage continuous	8.1	43.0	Papanikolaou and Aggelis, (2002)
Yarrowia lipolytica ACA-DC 50109 ‡ a	Shake flasks	11.4	29.8	Papanikolaou et al., (2003)
Yarrowia lipolytica ACA-DC 50109 ¶	Fed Batch bioreactor	4.7	23.1	Makri et al., (2010)
Cryptococcus curvatus ATCC 20509 ‡	Fed Batch bioreactor	32.9	52.9	Liang et al., (2010a)
Rhodotorula glutinis TISTR 5159	Shake flasks	5.5	35.2	Saenge et al., (2011)
Cryptococcus curvatus ATCC 20509 ‡	Fed Batch bioreactor	22.0	49.0	Cui et al., (2012)
Yarrowia lipolytica MUCL 28849 ¶	Fed Batch bioreactor	42.2	38.2	Fontanille et al., (2012)
Yarrowia lipolytica MUCL 28849 ¶ b	Fed Batch bioreactor	41.0	34.6	Fontanille et al., (2012)
Rhodosporidium toruloides AS2.1389 ‡	Shake flasks	19.2	47.7	Xu et al., (2012)
Rhodosporidium toruloides AS2.1389 ‡	Batch bioreactor	26.7	69.5	Xu et al., (2012)
Yarrowia lipolytica A10 ¶	Fed Batch bioreactor	23.0	12.9	Celińska and Grajek (2013)
Yarrowia lipolytica NCYC 3825 ¶ c	Fed Batch bioreactor	42.0	30.9	Celińska and Grajek, (2013)
Candida sp. LEB-M3 ‡	Shake flasks	19.7	50.2	Duarte et al., (2013b)
Kodamaea ohmeri BY4-523 ‡	Shake flasks	10.3	53.3	Kitcha and Cheirslip, (2013)
Trichosporanoides spathulata JU4-57 ‡	Shake flasks	17.1	43.4	Kitcha and Cheirslip, (2013)
Trichosporanoides spathulata JU4-57 ‡	Fed Batch	13.8	56.4	Kitcha and Cheirslip, (2013)

	bioreactor			
Yarrowia lipolytica TISTR 5151 ‡ d	Batch bioreactor	5.5	50.8	Louhasakul and Cheirslip, (2013)
Yarrowia lipolytica JMY1203 ‡ e	Shake flasks	3.2	30.7	Papanikolaou et al., (2013)
Cryptococcus curvatus ATCC 20509 ¶ f	Shake flasks	50.4	37.7	Ryu et al., (2013)
Rhodosporidium toruloides Y4 ¶	Batch bioreactor	35.3	46.0	Uçkun Kiran et al., (2013)
Yarrowia lipolytica Q21 ‡	Shake flasks	3.85	22.1	Poli et al., (2014)
Metschnikowia pulcherrima	Shake flasks	7.4	40.0	Santamauro et al., (2014)
Yarrowia lipolytica ATCC 20460 ‡	Shake flasks	11.6	31.0	Sestric et al., (2014)
2) Fungi and micro-algae				
Schizochytrium limacinum SR21 ¶	Shake flasks	14.4	44.8	Chi et al., (2007)
Schizochytrium limacinum SR21 ‡	Shake flasks	18.0	50.6	Chi et al., (2007)
Mortierella isabellina ATHUM 2935 ‡	Shake flasks	8.5	51.7	Papanikolaou et al., (2008a)
Cunninghamella echinulata ATHUM 4411 ‡	Shake flasks	7.8	25.6	Fakas et al., (2009b)
Aspergillus niger LFMB 1 ‡	Shake flasks	5.4	57.4	André et al., (2010)
Aspergillus niger NRRL 364 ‡	Shake flasks	8.2	41.4	André et al., (2010)
Schizochytrium limacinum SR21 ‡	Shake flasks	13.1	73.3	Liang et al., (2010b)
Thamnidium elegans CCF 1465 ‡	Shake flasks	16.3	71.1	Chatzifragou et al., (2011b)
Schizochytrium limacinum SR21 ‡	Single stage continuous	≈11	50.2	Ethier et al., (2011)
Mortierella ramanniana MUCL 9235 ¶	Shake flasks	7.0	53.1	Bellou et al., (2012)
Mortierella ramanniana MUCL 9235 ¶	Batch bioreactor	9.7	32.7	Bellou et al., (2012)
Cunninghamella echinulata ATHUM 4411 ¶	Shake flasks	6.9	25.1	Bellou et al., (2012)
Cunninghamella echinulata ATHUM 4411 ¶	Batch bioreactor	4.2	15.4	Bellou et al., (2012)
Mortierella alpina LPM 301 ¶	Shake flasks	28.6	33.4	Dedyukhina et al., (2012)
Mortierella alpina NRRL-A-10995 ¶	Shake flasks	26.7	35.4	Dedyukhina et al., (2012)
Schizochytrium sp. S31 ¶	Batch bioreactor	≈40	49.1	Chang et al., (2013)
Mortierella alpina LPM 301 ‡ g	Shake flasks	15.6	33.3	Dedyukhina et al., (2014)
Mortierella alpina NRRL-A-10995 ‡ g	Shake flasks	20.5	31.9	Dedyukhina et al., (2014)

Lipomyces starkeyi DSM 70296 ‡	Shake flasks	34.4	35.9	Present study
Rhodosporidium toruloides NRRL Y-27012 ‡	Shake flasks	30.1	40.0	Present study

‡: Utilization of crude glycerol
¶: Utilization of pure glycerol
a: Utilization of blend of glycerol with saturated free-fatty acids
b: Utilization of blend of glycerol with volatile fatty acids
c: Strain genetically modified, over-expressing glycerol dehydratase and a wide spectrum of alcohol oxidoreductases
d: Utilization of blend of glycerol with decanter effluent from palm oil mill
e: Strain genetically modified, lacking in the function of methyl-citrate cycle
f: Utilization of blend of glycerol with spent yeast lysate
g: Utilization of biodiesel waste containing blend of glycerol with saturated and unsaturated free-fatty acids

Two different physiological patterns as regards the accumulation of storage materials have been seen in *Rhodosporidium toruloides* NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296; as far as *Rhodosporidium toruloides* was concerned, it has been seen that during the first growth phases of the microbial growth, and despite the nitrogen presence into the culture medium, significant quantities of intra-cellular polysaccharides were synthesized (at the first growth steps $Y_{IPS/X}$≈40% w/w was observed). Thereafter, $Y_{IPS/X}$ values were reduced while in absolute quantities (g/L), after the significant biosynthesis that had occurred at the first growth steps, *c*. 50 h after inoculation and until the end of the culture the concentration of intra-cellular polysaccharides remained practically constant, whereas simultaneously significant accumulation of storage lipids (rise of both L and $Y_{L/X}$ values) was observed. Similar feature with the interplay of polysaccharides and lipids biosynthesis of *Rhodosporidium toruloides* has been reported for an oleaginous *Chlorella* sp. strain growing autotrophically under constant illumination conditions in an open-pond simulating photo-bioreactor (Bellou and Aggelis, 2012). On the other hand, *Lipomyces starkeyi* presented a different physiological profile since IPS per unit of DCW remained practically constant with a value *c*. 30% w/w throughout the culture, while in absolute values (g/L), the concentration of intra-cellular polysaccharides constantly increased (a significant quantity of *c*. 7 g/L was reported at the end of the culture). Worth-mentioning was the fact that high quantities of IPS ($Y_{IPS/X}$≥30% w/w) were produced even at the early growth steps for both microorganisms tested. This is rather unusual as result; in a biochemical basis, the events that lead to IPS and/or storage lipid production are theoretically triggered after nitrogen depletion from the culture medium, which leads to rapid decrease of intra-cellular AMP, resulting in intra-mitochondrial accumulation of citric acid, since the enzyme-key of NAD^+-isocitrate dehydrogenase is allosterically activated by the presence of AMP, that now is found in low concentrations (Ratledge, 1997; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a). Citric acid, after exceeding a critical value, is excreted to the cytoplasm. Thereafter, citric acid will be cleaved to acetyl-CoA and oxaloacetate, reaction catalyzed by ATP-citrate lyase (ATP-CL) and the produced acetyl-CoA will constitute the precursor of the synthesis of cellular acyl-CoAs, reactions catalyzed by the FA-synthetase complex. Finally, the synthesized acyl-CoAs will be stored in the cytoplasm mostly in the form of triacylglycerols

(TAGs) formed presumably through the Kennedy pathway (Ratledge and Wynn, 2002; Fakas et al., 2009a; Papanikolaou and Aggelis 2011a).

When microorganisms grow on sugars under nitrogen-limited conditions, in the imbalanced growth phase (absence of nitrogen from the medium) and in the absence of ATP-CL enzymatic complex, citric acid will be either excreted into the culture medium (case of many *Yarrowia lipolytica* strains – see i.e.: Papanikolaou et al., 2002; 2008a; 2008b; 2009, 2013), or will provoke the inhibition of 6-phosphoro-fructokinase (6-PFK). The above-mentioned event, in association with the decreased activity of 6-phosphoroglucose isomerase (PGI) will result in intra-cellular accumulation of polysaccharides (Galiotou-Panayotou et al., 1998; Zhong and Tang, 2004). On the other hand, the IPS biosynthetic pattern from glycerol revealed in this paper presents a specific interest; passive diffusion is used for glycerol uptake, and therefore the uptake rate of this sugar-alcohol depends on IPS concentration into the growth medium (Papanikolaou and Aggelis, 2011a). Therefore, the fact that intra-cellular polysaccharides are accumulated for both *Rhodosporidium toruloides* and *Lipomyces starkeyi* inside the cells in the early growth steps, period in which glycerol is found in the medium in high concentrations, suggests that glycerol uptake rate occasionally exceeds the catabolic capacity of the cell and therefore the high glycerol quantities, which cross the plasma membrane, are used for IPS synthesis. Later, for the case of *Rhodosporidium toruloides*, when glycerol concentration in the growth medium drops, IPS in DCW values significantly decrease, suggesting possible IPS use for generating energy. Biosynthesis of other cellular compounds, i.e. reserve lipid, also might occur using IPS as an intra-cellular substrate, together with the assimilated extra-cellular glycerol. In contrast, as far as *Lipomyces starkeyi* is concerned, this microorganism constantly produces intra-cellular polysaccharides, since their absolute values constantly increase, indicating that the enzymes of gluconeogenesis (e.g. fructose biphosphate alsolase, fructose biphosphatase, etc) were active in both the balanced and the imbalanced growth phases. Likewise, several higher fungi like *Flammulina velutipes*, *Pleurotus pulmonarius*, *Morchella esculenta* and *Volvariella volvacea* have been reported to produce significant quantities of IPS during balanced growth phase (Diamantopoulou et al., 2012, 2014). In any case, the production of IPS by oleaginous yeasts (and in significant quantities, *c*. 7 g/L, like in the current investigation) has rarely been reported, whereas the current investigation is amongst the first ones in the international literature that deals with the conversion of waste glycerol into intra-cellular polysaccharides**.**

The analysis of the FA composition of SCOs produced by both *Rhodosporidium toruloides* and *Lipomyces starkeyi* showed differentiations as function of both the fermentation time and the $Glol_0$ concentration employed; for *Rhodosporidium toruloides*, higher quantities of unsaturated FAs were detected at the early compared with the late growth phases, while the more the $Glol_0$ concentration into the medium increased, the more the quantity of saturated FAs increased. For *Lipomyces starkeyi*, cellular lipids were more saturated at the early growth steps than the late ones, whereas the more the $Glol_0$ concentration into the medium increased the more the cellular FAs were unsaturated. In all cases and in accordance with the literature in which *Lipomyces starkeyi* and *Rhodosporidium toruloides* were cultivated on sugar-based or similarly metabolized materials (implication of the de novo lipid accumulation mechanism), mainly the FAs C16:0 and C18:1 and to lesser extent of the FAs C18:0 and C18:2 were synthesized, indicating that SCOs produced by the above-mentioned microorganisms can constitute perfect precursors for the synthesis of 2nd generation biodiesel (Li et al., 2007; Zhao et al., 2008; Xu et al., 2012). Differences in the FA composition of the storage lipids produced by *Lipomyces starkeyi* and *Rhodosporidium toruloides* seem to be strain dependent; neither the fermentation time nor the increasing in the concentration of glycerol (or glucose) in the culture medium have any systematic common effect on the modification of cellular FAs in the cells of oleaginous yeasts (Papanikolaou and Aggelis, 2009; Papanikolaou et al., 2002, 2009, Makri et al., 2010; Chatzifragkou et al., 2011a). Likewise, there is no systematic effect concerning the trend on the composition of cellular FAs due to the addition of natural compounds into the culture medium, since in other cases the synthesis of saturated FAs is favored (Moreton, 1988; Chatzifragkou et al., 2011a) whilst in other ones mostly the unsaturated FAs are predominant into the cellular lipids (Sarris et al., 2011). On the other hand, utilization of fatty materials and partial or complete implication of the ex novo mechanism for the synthesis of cellular lipids results in the production of intra-cellular fatty materials that present, in general, composition similarities with the fats that had been used as substrates (Papanikolaou et al., 2003; 2011; Papanikolaou and Aggelis 2011a). It is evident that the ex novo mechanism of lipid accumulation presents fundamental differences with that of the de novo, whereas in the former case, fermentation of the fatty material is performed mainly in order to “up-grade” and “ameliorate” the quality of the hydrophobic carbon source employed as substrate (Papanikolaou et al. 2003; 2011; Papanikolaou and Aggelis 2011a).

In conclusion, 6 yeast strains were screened towards their ability to assimilate crude glycerol, waste deriving from biodiesel production units, and produce metabolic compounds of significance for the Industrial Biotechnology. Two of the tested strains, *Rhodosporidium toruloides* NRRL Y-27012 and *Lipomyces starkeyi* DSM 70296 were revealed as satisfactory candidates of glycerol consumption and during growth on high $Glol_0$ content media presented noticeable production of biomass and microbial lipid. SCO production comparable to some of the highest ones in the international literature for microorganisms growing on glycerol was reported. A scarce number of reports have indicated the production of SCO by *Rhodosporidium toruloides* growing on crude glycerol, whilst this is the first report that deals with the conversion of this residue to SCO by *Lipomyces starkeyi*. Finally, this is one of the first reports in the international literature that indicates the conversion of glycerol into intra-cellular polysaccharides.

III.3. Utilization of lactose, sucrose and residues containing the above-mentioned materials for the production of microbial lipid and other useful microbial compounds

In the third part of this investigation and after having performed experiments on glucose- or glycerol-based media, it was desirable to perform trials on media composed from either lactose or sucrose employed as the sole carbon source. As indicated in the previous paragraphs, the yeast species *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Rhodotorula glutinis* and *Lipomyces starkeyi* cannot easily consume either sucrose or lactose (or both of these carbon sources for wild strains of the species *Yarrowia lipolytica*). Therefore, in this last part of the investigation, the trials were performed exclusively with the strain *Cryptococcus curvatus* NRRL Y-1511, which has never previously been studied in relation with its potential of producing SCO and other metabolic compounds of biotechnological interest (e.g. intra-cellular polysaccharides, extra-cellular enzymes) during growth on these abundant carbon sources.

III.3.1. Introduction

The production and study of lipids produced, the single cell oils (SCOs) represents a major challenges in the industrial biotechnology (Ratledge, 1997; Papanikolaou and Aggelis, 2011a; 2011b). When sugars or related substrates are used as substrates of the oleaginous microorganisms, lipid accumulation is initiated after virtual exhaustion of nitrogen (or potentially other nutrients besides carbon) from the medium. Nitrogen exhaustion leads to a rapid decrease of the concentration of cellular AMP, which is further cleaved in order for nitrogen to be offered to the microorganism. Cellular AMP concentration decrease alters the Krebs cycle function resulting in the accumulation of intra-mitochondrial citric acid. When the concentration of citric acid inside the mitochodria becomes higher than a critical value, it is secreted inside the cytoplasm. Then, citric acid is cleaved by ATP-citrate lyase, enzyme-key showing the oleaginous character of the microorganisms, into acetyl-CoA and oxaloacetate, and acetyl-CoA, by virtue of the action of fatty acid synthetase generates cellular fatty acids and subsequently triacylglycerols (TAGs) (Ratledge, 1997; Papanikolaou and Aggelis, 2010; 2011a; 2011b). In the non-lipid producing microorganisms, nitrogen exhaustions provokes secretion of the previously hyper-synthesized citric acid into the growth medium (case of the fungus *Aspergillus niger* and many of the strains of the yeast *Yarrowia lipolytica*) or results in a block in the level of 6-phospho-fructokinase, leading in the accumulation of (intra-cellular) polysaccharides (Galiotou-Panayotou et al., 1998). On the other hand, lipid accumulation from hydrophobic substances (like oils or fats, free fatty acids, soap-stocks, etc) used as the sole carbon and energy source (ex novo lipid accumulation) is performed by virtue of a radically different biochemical mechanism; culture triacylglycerols are hydrolyzed with the aid of (extra-cellular or cell-bounded) lipases and fatty acids (FAs) are incorporated inside the microbial cells or mycelia with various incorporation rates (Aggelis and Sourdis,1997; Papanikolaou et al., 2001; Papanikolaou and Aggelis, 2010; 2011a). Substrate FAs are either assimilated for growth needs or become a substrate for intra-cellular bio-transformations. In the ex novo lipid accumulation process, lipid production is a growth associated process occurring simultaneously with cell growth, being entirely independent from nitrogen exhaustion from the culture medium (Papanikolaou and Aggelis, 2010; 2011a).

Several studies have been performed employing *Cryptococcus curvatus* (formerly *Candida curvata* or *Apiotrichum curvatum*) strains for the production of SCO in different fermentation configurations and culture media, comprising as carbon source pure or raw glycerol (Meesters et al., 1996; Liang et al., 2010a; Chatzifragkou et al., 2011b), glucose (Zhang et al., 2011), lignocellulosic sugars (Gong et al., 2013), N-acetylglucosamine (Wu et al., 2010), waste spent yeast (Ryu et al., 2013), whey permeate (Davies, 1988; Ykema et al., 1989; 1990; Davies et al., 1990; Daniel et al., 1998; 1999) and other carbohydrate-rich materials (Bednarski et al., 1986; Vega et al., 1988). Despite the significant quantity of published information concerning the potential of *Cryptococcus curvatus* strains to produce biomass, little information is available so far related with the potential of the above-mentioned strains to produce intra-cellular polysaccharides (IPS), as well as the observed interactions between the accumulation of IPS and storage lipids.

The aim of the current part of this investigation was to perform physiological and kinetic investigations on a rarely studied *Cryptococcus curvatus* strain (NRRL Y-1511) in relation with IPS potential to convert low-cost sugars (*e.g.* commercial sucrose and cheese-whey lactose) into microbial biomass, IPS and cellular lipids. The interplay between the synthesis of IPS and lipids as well as the secretion of enzymes implicated in the break-down of the available carbon sources (sucrose and lactose) was also studied. Special attention was paid to the composition of accumulated IPS and their relation to the utilized substrate. Finally, the physiological behavior of the strain cultured on residues containing the above-mentioned sugars (i.e. molasses and cheese-whey) was evaluated.

III.3.2. Results

III.3.2.1. Growth of Cryptococcus curvatus on lactose-based media.

Cryptococcus curvatus was firstly cultivated under nitrogen limited-conditions, using commercial lactose as the sole carbon source. In order to evaluate the influence of the carbon source concentration and the nitrogen limitation on lipid productivity, three initial lactose concentrations were employed ($Lac_{i,}$ of 40, 60 and 80 g/L), whereas the extra-cellular nitrogen availability was maintained the same in all media with 0.5 g/L yeast extract and 0.75 g/L peptone employed as nitrogen sources. The kinetics of lactose consumption (Lac, g/L), cell biomass production (X, g/L), total lipid content (L, g/L) and intra-cellular total sugars

(IPS, g/L) was studied during the fermentations and the obtained results are listed in Table III.21. and Fig. III.14.

Table III. 21. Quantitative data of *Cryptococcus curvatus* NRLL Y-1511 originated from kinetics on lactose in nitrogen-limited and nitrogen-excess media at three initial lactose concentrations (40, 60, 80 g/L) and constant initial nitrogen concentration. Two different points in the fermentation are represented: (a) when the maximum quantity of intra-cellular total sugars per DCW ($Y_{IPS/X}$ %, w/w) was observed; (b) when the maximum quantity of lipid per DCW ($Y_{L/X}$ %, w/w) was observed. Fermentation time (h) and quantities of DCW (X, g/L), total lipid (L, g/L), intra-cellular polysaccharides (IPS, g/L) and lactose consumed (Lac_{cons}, g/L) are also given for all fermentation points. Culture condition: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DO>20% (v/v), incubation temperature T=28 °C. Each point is the mean value of two independent measurements.

Nitrogen-limited media								
Lac_i (g/L)	C/N ratio (mol/mol)	Time (h)	Lac_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	IPS (g/L)	$Y_{IPS/X}$ (%, w/w)
≈40 *a*	≈90	23	6.1	4.9	0.16	3.2	2.9	60.3
b		168	28.9	10.5	1.31	12.5	3.2	30.8
≈60 *a*	≈135	20	11.6	4.3	0.24	5.5	2.9	67.6
b		336	42.3	13.7	2.53	18.3	3.2	23.4
≈80 *a*	≈180	24	11.7	4.0	0.23	5.7	2.7	68.1
b		528	49.2	14.5	4.30	29.7	2.8	19.4
Nitrogen-excess media								
≈40 *a*	≈17	92	43.4	19.7	0.93	4.7	9.0	45.9
b		24	13.1	6.8	0.45	6.6	2.9	42.1
≈60 *a*	≈25	92	41.7	20.0	0.26	1.3	8.3	41.5
b		24	12.7	5.9	0.23	3.9	9.4	33.7
≈80 *a*	≈34	265	78.2	27.7	0.14	0.5	11.0	41.2
b		25	10.2	6.0	0.13	2.1	1.8	30.0

a: Representation when maximum value of intra-cellular total sugars in DCW (%, w/w) was achieved

b: Representation when maximum value of lipid in DCW (%, w/w) was achieved

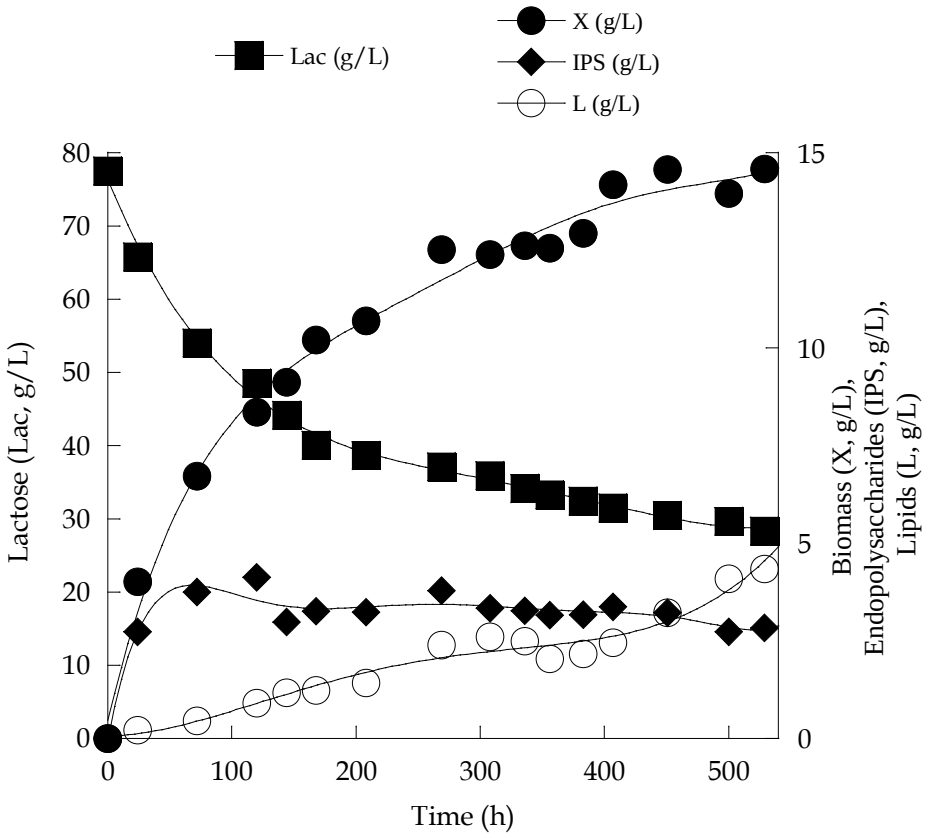


Figure III. 14. Kinetics of lactose (Lac, g/L), biomass (X, g/L) (a), intra-cellular polysaccharides produced per unit of dry weight ($Y_{IPS/X}$ %, w/w), intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$ %, w/w) by *Cryptococcus curvatus*, during growth on cheese-whey containing *c.* 80 g/L of total sugars under nitrogen-excess conditions. Culture conditions: growth on 250-mL flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DO>20 % (v/v), incubation temperature T=28°C. Each point is the mean value of two independent measurements.

The yeast was able to consume lactose and produce lipid and IPS during its growth (Fig. III.14.), while a very interesting feature of the yeast metabolism was related with the fact that IPS were accumulated in significant quantities at the early growth steps (in the presence of nitrogen), indicating that IPS accumulation occurred under balanced growth conditions. On the other hand, the increase in the Lac_i concentration, and thus the increment of carbon-to-nitrogen (C/N) ratio, had a clear positive effect on the accumulation of intra-cellular lipids. Indeed, the obtained percentage of accumulated oil rose from 12.5% to 29.8% on DCW basis when the Lac_i concentration was elevated from 40 to 80 g/L. Likewise, a small positive effect in terms of IPS accumulation by the yeast strain was observed during IPS growth at increasing

initial C/N ratio media; maximum IPS on DCW basis increased from 60.3% to 68.1% with Lac_i increment from 40 to 80 g/L. As previously stressed, *Cryptococcus curvatus* cultivated on lactose-based nitrogen-limited media produced elevated IPS quantities (in terms of both absolute and relative values) at the first hours of the fermentation, suggesting significant production of IPS during active growth (i.e. in the presence of nitrogen), while IPS in DCW values were progressively reduced during the course of the bioprocess (Fig. III.15).

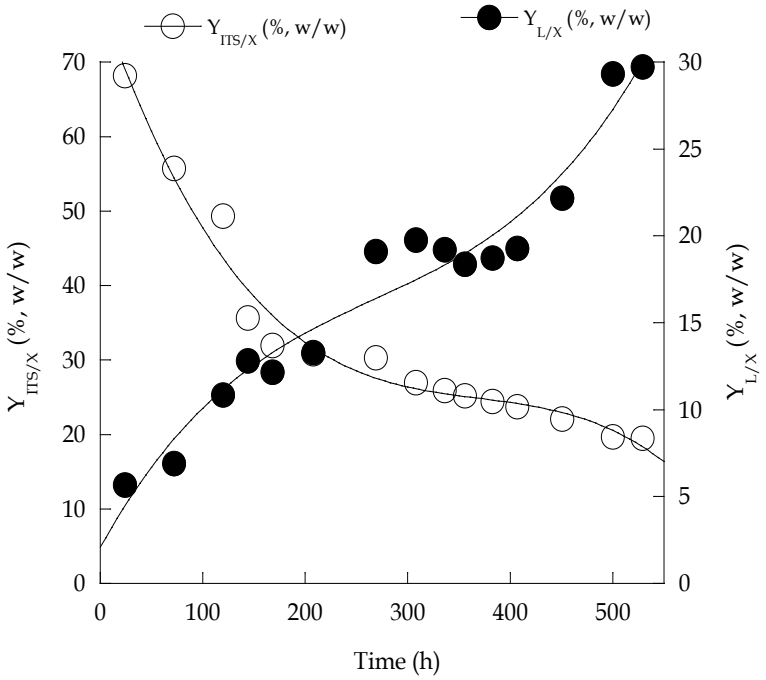


Figure III. 15. Kinetics of intra-cellular polysaccharides produced per unit of dry weight ($Y_{IPS/X}$ %, w/w) and intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$ %, w/w) of *Cryptococcus curvatus*, during growth on lactose, at initial lactose concentration (Lac_i) of 80 g/L, under nitrogen-limited conditions. Culture conditions as in Fig. III.14. Each point is the mean value of two independent measurements.

Moreover, in order to have a more global idea of the cellular metabolism of the particular strain, in the above-mentioned experiment (Lac_i≈80 g/L under nitrogen-limited conditions) assay of the protein content in the dry microbial biomass was performed; at the initial growth stages in which the microorganism synthesized significant quantities of total intra-cellular sugars ($Y_{IPS/X}$ ranging between 55 and 68% w/w), microbial protein in DCW was *c.* 10-15% w/w. Thereafter, microbial protein in DCW increased to *c*. 25% w/w, before decreasing again at a point which virtual lipid accumulation occurred (at fermentation time ranging between 400 and 500 h after inoculation) to *c.* 10-12% w/w. On the other hand, in

trials with Lac_i≈40 g/L, the IPS content in DCW ($Y_{IPS/X}$) was found equal to *c.* 60% (w/w) during the first 24 h, while at the end of the fermentation the IPS was gradually reduced in half ($Y_{IPS/X}$≈30%). The physiological events related with the production of storage materials by *Cryptococcus curvatus* cultivated on lactose in nitrogen-limited conditions are evolving as follows: initially, during balanced growth phase, lactose was assimilated with a relatively high assimilation rate, while intra-cellular total carbohydrates in significant quantities (in terms mainly of $Y_{IPS/X}$) were produced. Thereafter, a significant reduction of the value of the yield $Y_{IPS/X}$ was observed, while concomitantly a significant increment of the quantity of accumulated lipids in absolute (g/L) and relative (%, in DCW – $Y_{L/X}$) values was observed (see Fig. III.15). Similar trends regarding the evolution of $Y_{IPS/X}$ and $Y_{L/X}$ values were observed in all other trials performed with lactose under nitrogen-limited conditions.

As a next step, cultures were carried out using the same Lac_i concentrations (*c.* 40, 60 and 80 g/L), under nitrogen-excess conditions, whereas extra-cellular nitrogen concentration was maintained the same (0.5 g/L yeast extract and 5.5 g/L peptone), aiming to evaluate the effect of increasing lactose concentration on biomass production and IPS concentration. As shown in Table III.21., during growth of *Cryptococcus curvatus* the produced biomass was enhanced two-fold as compared to the respective values achieved under nitrogen-limited conditions. Specifically, maximum biomass (X_{max}) values of 19.7, 20.0 and 27.7 g/L were noted in cultures with initial lactose concentrations of 40, 60 and 80 g/L, respectively, while the corresponding maximum biomass values in nitrogen-limited media were 10.5, 13.7 and 14.5 g/L (Table III.21.). Likewise, the assimilation rate of lactose was much higher in the carbon-limited cultures as compared with the nitrogen-limited ones; for instance, when Lac_i concentration was adjusted to ≈80 g/L, in the nitrogen-excess trial almost all of the available sugar quantity was assimilated by the microorganism within *c.* 300 h, whereas in the respective nitrogen-limited one, only 49.2 g/L (*c.* 65% w/w) were assimilated within 528 h. On the other hand, in the nitrogen-excess experiments, and despite the fact that almost all of the available lactose quantity had been assimilated by the microorganism, some substrate inhibition exerted due to the increasing Lac_i quantity seemed to occur; by taking into consideration the biomass yield on lactose consumed for the above-mentioned trials performed, when Lac_i was adjusted at *c.* 40 g/L the yield $Y_{X/Lac}$ was ≈0.45 g/g, while the respective value at Lac_i≈80 g/L was only 0.36 g/g. Moreover, total cellular lipids, as was expected from the literature (Papanikolaou and Aggelis, 2011a) were not produced in high

quantities (lipid in biomass ranging between 0.5-6.6% w/w). However, interestingly, it appears that higher quantities of lipid in biomass were produced at the early growth step, decreasing afterwards.

As initially observed during nitrogen-limited trials, the interesting feature of the microbial metabolism related with the significant production of IPS despite the presence of nitrogen into the medium was confirmed; specifically, IPS quantities in DCW ranging between 37.6-45.9% were observed in all of the nitrogen-excess cultures realized (see Table III.21.). Additionally, a slight decrease in the IPS per DCW values, parallel to the increment of Laci concentrations was observed; for $Lac_i \approx 40$ g/L, IPS relative quantities were equal to 46% (w/w), whilst their percentage was dropped to 35% during trials with Laci≈80 g/L, indicating a decrease of around 12% (Table III.21.). However, despite the reduction of the IPS relative quantity with Lac_i increase, a quite impressive maximum IPS quantity of ≈11 g/L was seen at the trial with $Lac_i \approx 80$ g/L. At $Lac_i \approx 80$ g/L and nitrogen-excess conditions) assay of the protein content in the dry microbial biomass was performed; under these culture conditions and in contrast to the fluctuations of cellular protein content observed during the nitrogen-limited experiment, it was seen that during all fermentation steps of the culture, microbial protein in DCW remained practically constant to a value of *c.* 23-28% w/w.

Extracellular β-galactosidase activity of the yeast strain *Cryptococcus curvatus* was monitored during its growth on lactose in nitrogen-excess and nitrogen limited conditions in order to observe the course of lactose hydrolysis by the strain. However, the enzyme in question was not detected in crude extracts of the cultures, in both nitrogen-excess and nitrogen-limited experiments regardless of the Lac_i concentration imposed into the culture. In order to investigate whether cell-bounded β-galactosidase activity occurred, assay of the enzyme was performed also with the presence of cells in the assay mixture, and equally no β-galactosidase activity was detected. By taking into account the absence of monosaccharides (i.e. glucose and galactose) in the culture medium, as designated by HPLC analysis, it can be concluded that the yeast lacked the existence of β-galactosidase in free and cell-bounded form.

Analysis of the monosaccharide's composition of the synthesized IPS was carried out during cultivation of the yeast strain in lactose-based media. The principal monosaccharides that composed the IPS were glucose and galactose (Table III.22.). In the case of nitrogen-limited cultures, the concentration of glucose into the cellular total carbohydrates (in %, w/w)

was reduced during the course of the fermentation, whereas the opposite trend was observed for galactose. When nitrogen-excess conditions were applied, glucose and galactose concentrations were almost stable for low lactose concentrations (i.e. $Lac_i \approx 40$ g/L). In $Lac_i \approx 80$ g/L, small quantities of fructose were also detected in the composition of the accumulated IPS towards the beginning and the middle of the fermentation, indicating de novo synthesis of fructose by the microorganism, while fructose concentration into the IPS was negligible at the end of the culture. Finally, at $Lac_i \approx 80$ g/L under nitrogen-excess conditions, glucose concentration into the IPS constantly increased during the course of the fermentation while the opposite trend was reported for galactose (Table III.22.).

Table III. 22. Sugar composition of intra-cellular total sugars (IPS) produced by *Cryptococcus curvatus* NRRL Y-1511, during its growth in nitrogen- and carbon-limited media containing different initial amounts of lactose. Culture conditions as in Table III.21.

Lactose (g/L)	Time (h)	Glucose (%, w/w)	Fructose (%, w/w)	Galactose (%, w/w)
Nitrogen-limited media				
≈40	24	100	-	-
	120	57.8	-	42.2
	168	56.5	-	43.5
≈60	20	64.8	-	35.2
	269	36.4	-	63.6
	384	33.3	-	66.7
≈80	120	54.4	-	45.6
	264	35.9	-	64.2
	528	35.5	-	64.5
Nitrogen-excess media				
≈40	24	52.9	-	47.1
	72	50.9	-	49.1
	96	52.0	-	48.0
≈80	48	48.4	6.6	45.0
	144	50.0	4.4	46.6
	216	58.8	4.3	36.9
	336	60.4	-	39.6

III.3.2.2. Growth of Cryptococcus curvatus strain NRRL Y-1511 on sucrose-based media

Cryptococcus curvatus was cultivated on sucrose-based media (initial sucrose, Su_i, concentrations adjusted at *c*. 40 and 80 g/L), under nitrogen-limited and nitrogen-excess

conditions, with the initial concentrations of yeast extract and peptone being the same as in the respective trials with lactose utilized as microbial substrate. This set of experiments was carried out in order to determine the suitability of sucrose as carbon source for the particular strain, and monitor the patterns of biomass, SCO and IPS production.

Table III. 23. Quantitative data of *Cryptococcus curvatus* NRLL Y 1511 originated from kinetics on sucrose in nitrogen- and carbon-limited media at two initial sucrose concentrations (40 and 80 g/L) and constant initial nitrogen concentration. Two different point of fermentation are represented: (a) when the maximum quantity of intra-cellular total sugars per DCW ($Y_{IPS/X}$ %, w/w) was observed; (b) when the maximum quantity of lipid per DCW ($Y_{L/X}$ %, w/w) was observed. Fermentation time (h) and quantities of DCW (X, g/L), total lipid (L, g/L), intra-cellular total sugars (IPS, g/L) and sucrose consumed (Su_{cons}, g/L) are also given for all fermentation points. Culture condition: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DO>20% (v/v), incubation temperature T=28 °C. Each point is the mean value of two independent measurements.

Nitrogen-limited media								
Su_i (g/L)	C/N ratio (mol/mol)	Time (h)	Su_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	IPS (g/L)	$Y_{IPS/X}$ (%, w/w)
≈40 *a*	90	24	4.4	5.5	0.13	2.4	2.8	50.9
b		240	38.2	12.3	0.64	5.2	3.1	25.2
≈80 *a*	180	24	20.8	5.1	0.58	11.3	3.5	68.6
b		408	69.5	17.5	3.30	18.8	3.6	20.6
Nitrogen-excess media								
≈40 *a*	17	25	8.0	7.2	0.18	2.5	3.0	42.2
b		96	38.2	19.3	0.63	3.3	6.8	35.2
≈80 *a*	34	265	78.2	27.7	0.61	2.2	10.9	41.2
b		25	10.3	6.0	0.29	4.9	1.8	30.0

a: Representation when maximum value of intra-cellular total sugars in DCW (%, w/w) was achieved

b: Representation when maximum value of lipid in DCW (%, w/w) was achieved

The kinetics of growth of *Cryptococcus curvatus* under nitrogen-limited conditions showed that the yeast in question was able to consume sucrose in the same way as lactose and produce biomass, SCO and IPS as major metabolic products (Table III.23.). Although a slight elevation of DCW production was observed on sucrose, the kinetics of growth under nitrogen-limited conditions on both sucrose and lactose were essentially similar. At initial sucrose concentrations of 80 g/L, maximum biomass values of 17.5 g/L were obtained, while the

respective value of biomass during growth of the strain on lactose-based media was equal to 14.5 g/L. However, sucrose was found to be a less adequate substrate for SCO production by *Cryptococcus curvatus*, as maximum relative lipid concentration was 18.8% in trials with initial sucrose concentration of 80 g/L compared to $Y_{L/X}$≈30% obtained in lactose-based cultures. As far as intra-cellular polysaccharide content was concerned, the accumulation of IPS followed the same interesting feature already observed during cultivation of the strain in lactose; $Y_{IPS/X}$ quantities were remarkably high at the initial steps of the fermentation, and then progressively decreased (from 68.6% w/w in the first 24 hours to 20.6% w/w at the end of the cultivation). This trend coincided with a concomitant rise in the value of $Y_{L/X}$, and hence in lipid accumulation process, although as previously stressed, sucrose was a less adequate substrate compared to lactose concerning lipid production for the employed *C. curvatus* strain (Table III.23.). Cultivation of the yeast on sucrose in nitrogen-excess media had, as anticipated, a negative impact on intra-cellular lipid production. Sucrose catabolism was principally channeled towards biomass production, reaching up to 27.7 g/L in trials with 80 g/L of initial sucrose concentration. As in trials on lactose, despite the fact that almost all available sucrose had been assimilated rapidly by the microorganism, partial substrate inhibition due to the increasing Su_i quantity was exerted. By taking into consideration the DCW yield on sucrose consumed, when Su_i was adjusted at *c*. 40 g/L the yield $Y_{X/Su}$ was ≈0.51 g/g, while the respective value at Su_i≈80 g/L was only 0.35 g/g. Moreover, as was observed in the nitrogen-excess lactose-based experiments, trials on sucrose under nitrogen-excess cultivations were accompanied by significant IPS biosynthesis, with IPS relative quantities remaining stable during all growth phases of the yeast, ranging between 35-45% w/w (Table III.23.).

Extracellular invertase activity (IA) of the yeast *Cryptococcus curvatus* was monitored during growth on sucrose-based media, in order to observe the course of sucrose hydrolysis by the strain. Maximum invertase activity ($IA_{max)}$ was reached slightly latter after almost complete hydrolysis of the disaccharide sucrose into reducing sugars, and was depended both on the initial sucrose concentration into the medium and on the culture conditions imposed (nitrogen-excess or nitrogen-limited). In trials with Su_i≈40 g/L, IA_{max} was found to be slightly enhanced in nitrogen-excess media, compared to nitrogen-limited ones, yielding thus 1.7 against 1.2 U/mL, respectively. In cultures with Su_i≈80 g/L in nitrogen-excess media, IA obtained its maximum value, equal to 2.9 U/mL, whereas the respective value of IA during

growth in nitrogen-limited was lower (1.8 U/mL; see Figs III.16a and III.16b). Generally, invertase was found to be active when sucrose was in excess in the fermentation medium, while its activity was reduced only after complete hydrolysis of sucrose into reducing sugars (glucose and fructose).

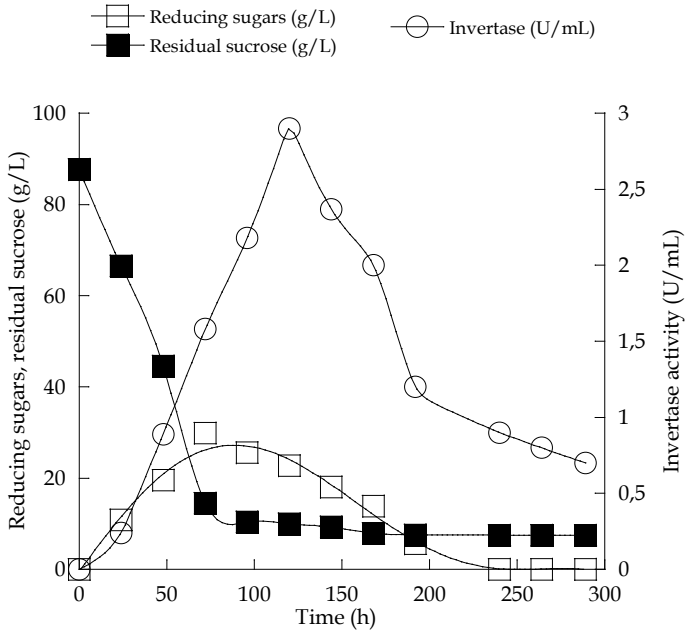


Fig. III.16a

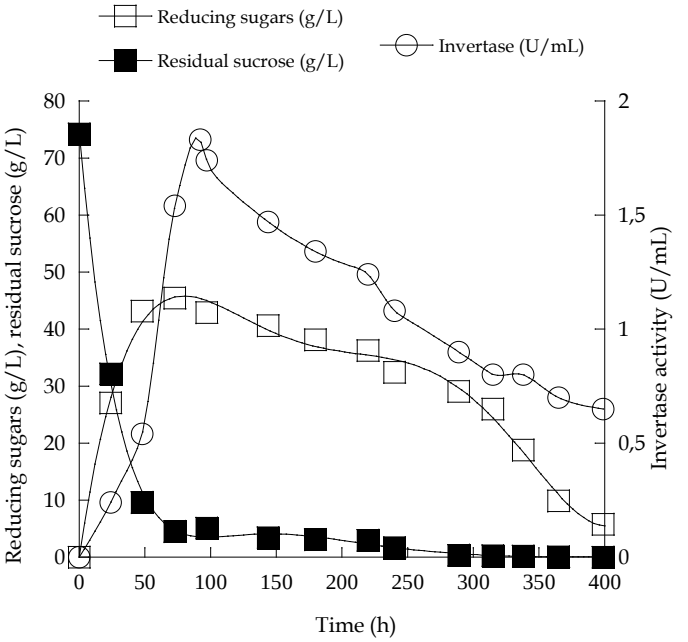


Fig. III.16b

Figure III. 16. Kinetics of invertase enzyme activity (U/mL), residual sucrose (g/L) and reducing sugars (g/L) by *Cryptococcus curvatus*, during growth on 80 g/L of sucrose under nitrogen-excess (a) and nitrogen-limited (b) conditions. Culture conditions: growth on 250-mL flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DO>20% (v/v), incubation temperature T=28 °C. Each point is the mean value of two independent measurements.

Analysis of the monosaccharides composition during growth on sucrose-based media indicated that the principal sugars that composed the intra-cellular total carbohydrates were glucose and fructose (Table III.24.).

Table III. 24. Sugar composition of intra-cellular total sugars (IPS) produced by *Cryptococcus curvatus* NRRL Y-1511, during IPS growth in nitrogen-limited and nitrogen-excess media containing different initial amounts of sucrose. Culture conditions as in Table III.23.

Sucrose (g/L)	Time (h)	Glucose (%, w/w)	Fructose (%, w/w)
Nitrogen-limited media			
≈40	48	84.8	15.2
	120	84.5	15.5
	240	88.3	11.7
≈80	48	91.4	8.6
	269	89.9	10.1
	408	88.6	11.4
Nitrogen-excess media			
≈40	48	89.2	10.8
	72	88.7	11.3
	96	85.5	14.5
≈80	48	93.5	6.5
	144	91.5	8.5
	216	87.9	12.1
	264	87.5	12.5

In contrast to the trials performed on lactose, cultures on saccharose were accompanied by accumulated IPS mainly composed of glucose (concentration >80% w/w of total IPS) regardless of the carbon or nitrogen limitation imposed or the fermentation time. No other sugars besides glucose or fructose were identified as IPS building blocks, indicating that galactose, that was detected in significant quantities during growth on lactose, can only be found as IPS building block through the ex novo biosynthetic mechanism. Moreover, small

differentiations in the sugar composition of the produced IPS as function of the culture time were recorded; in most of the performed trials, the trend was that glucose concentration slightly decreased with the fermentation time, while fructose slightly increased, but, as previously stressed, in all cases the concentration of glucose was very high in the IPS produced.

III.3.2.3.Growth of Cryptococcus curvatus strain NRRL Y-1511 on cheese whey and molasses.

Further trials were performed using cheese-whey or molasses as substrates, in order to evaluate the physiological behavior of the strain on residues often utilized for SCO production. The employed cheese-whey contained *c.* 80 g/L lactose and *c.* 9 g/L protein, while water was added into the molasses to yield in a final concentration of total sugars and proteins of *c.* 80 and *c.* 18 g/L, respectively. As far as culture on cheese-whey was concerned, the overall fermentation time was noticeably reduced to 216 h, as compared to the respective trial with commercial lactose in synthetic medium (more than 260 h of fermentation duration) (see Tables III.21. and III.25.).

Table III. 25. Quantitative data of *Cryptococcus curvatus* NRRL Y-1511 during growth on cheese-whey and molasses under nitrogen-excess culture conditions (initial total sugars into the medium TS_0≈80 g/L). Two different points of fermentation are represented: (a) when the maximum quantity of lipid per DCW ($Y_{L/X}$ %, w/w) was observed; (b) when the maximum quantity of IPS (g/L) was observed. Fermentation time (h), consumed substrate (TS_{cons}, g/L) and quantities of intra-cellular polysaccharides (IPS, g/L), total lipids (L, g/L) and residual protein (Pr, g/L) are also given for all fermentation points. Culture conditions: growth on 250-mL conical flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DO>20% (v/v), incubation temperature T=28 °C. Each point is the mean value of two independent measurements.

Substrate	Time (h)	TS_{cons} (g/L)	X (g/L)	L (g/L)	$Y_{L/X}$ (%, w/w)	IPS (g/L)	$Y_{IPS/X}$ (%, w/w)	Pr (g/L)
Cheese-whey	72 *a*	24.8	20.3	1.88	9.3	5.0	24.8	4.3
	216 *b*	71.6	38.5	1.43	3.7	11.1	28.8	1.5
Molasses	120 *a*	33.8	17.8	0.71	4.0	5.8	32.6	15.4
	287 *b*	75.6	35.7	0.61	1.7	9.9	27.8	8.4

a: Representation when maximum value of lipid in DCW (%, w/w) was achieved

b: Representation when maximum quantity of IPS (g/L) was achieved

Biomass production was significantly enhanced during growth of yeast in cheese-whey, reaching 38.5 g/L at the end of the fermentation, while DCW yield on lactose equivalent (=total sugars + protein) consumed was ≈0.48 g/g. Lipids and IPS were produced by the strain in similar quantities as those obtained during cultivation on commercial lactose under nitrogen-excess conditions. Lipid in DCW ($Y_{L/X}$, % w/w) was always lower than 10% w/w, while non-negligible quantities of intra-cellular polysaccharides were produced despite the nitrogen-excess conditions, indicating once again the potential of the strain to produce high IPS quantities during IPS balanced growth phase. Significant quantities of IPS, the highest in the current report (11.1 g/L) were recorder during growth on cheese-whey.

The kinetics in the trial performed on cheese-whey under nitrogen-excess conditions is presented in Figs III.17a-c. As previously indicated, the highest DCW production (*c.* 39 g/L) was recorded during growth on this residue, while an interesting result achieved was associated with the fact that lactose consumption under nitrogen-excess conditions was almost linear, with lactose consumption rate ($r_{Lac} = -\frac{\Delta Lac}{\Delta t}$) being ≈0.31 g/L/h ($r^2$ for the linear regression was ≈0.97). Likewise, as in the previous trials performed under nitrogen-excess conditions, lipid in dry weight ($Y_{L/X}$) presented somehow more elevated values at the beginning of the fermentation (Fig. III.17b). Finally, worth mentioning was the fact that the quantity of FAN into the medium was almost negligible (*c.* 20 mg/L) already from the first 24 hours after inoculation, while protein was not depleted from the growth medium but at the end of culture (*i.e.* more than 150 h after inoculation) suggesting that the rate of proteolysis was the similar to the rate of amino-acids assimilation by *Cryptococcus curvatus* (Fig. III.17c).

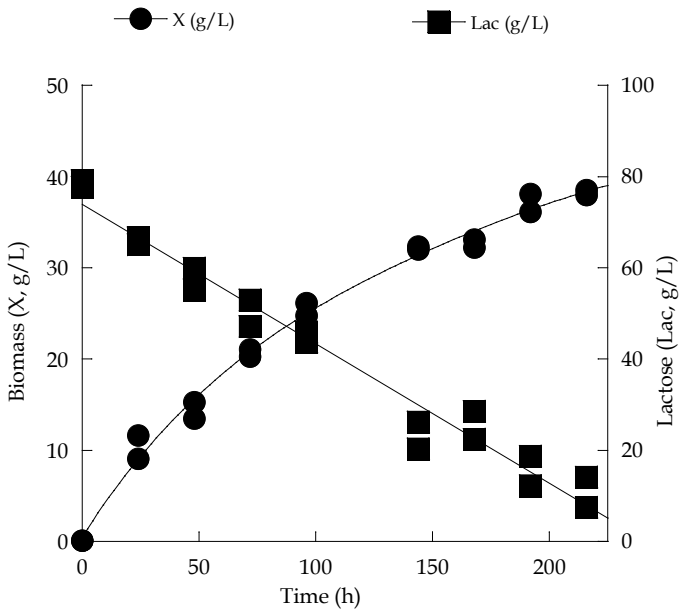


Fig. III.17a

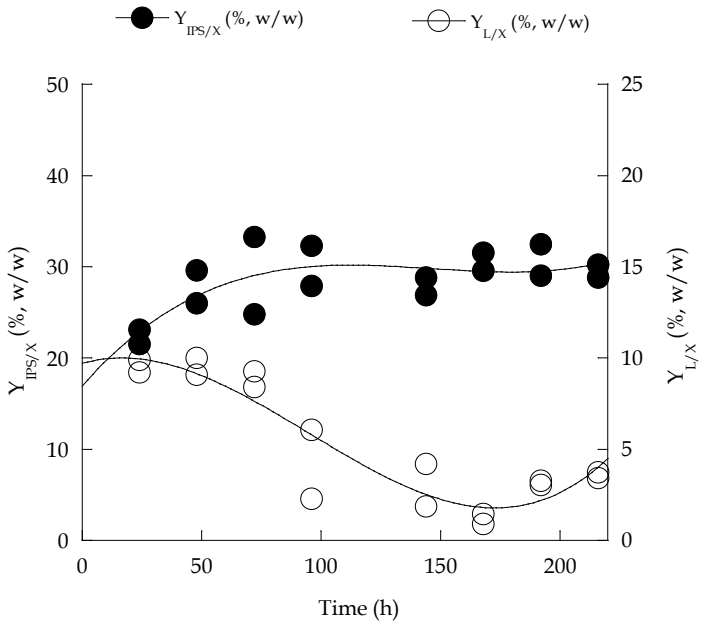


Fig. III.17b

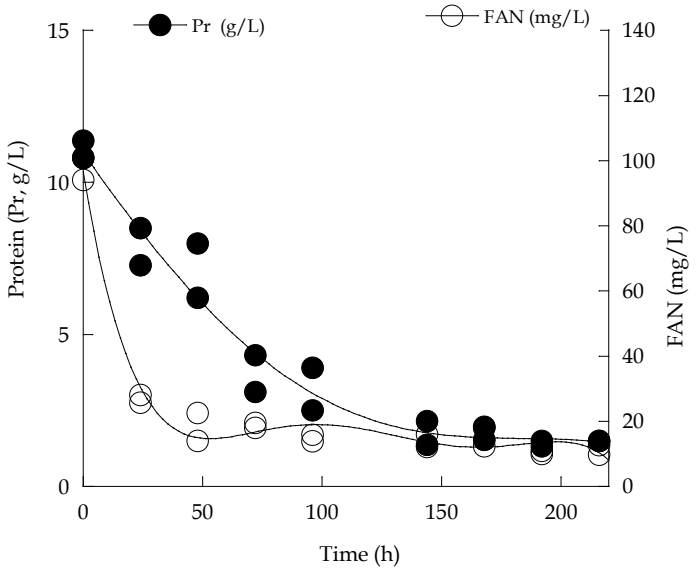


Fig. III.17c

Figure III. 17. Kinetics of lactose (Lac, g/L), biomass (X, g/L) (a), intra-cellular polysaccharides produced per unit of dry weight ($Y_{IPS/X}$ %, w/w), intra-cellular lipids produced per unit of dry weight ($Y_{L/X}$ %, w/w) (b), extra-cellular protein (Pr, g/L) and free amino-nitrogen (FAN, mg/L) into the medium (c) by *Cryptococcus curvatus*, during growth on cheese-whey containing *c.* 80 g/L of total sugars under nitrogen-excess conditions. Culture conditions: growth on 250-mL flasks at 185 rpm, initial pH=6.0±0.1, pH ranging between 5.2 and 6.0, DO>20 % (v/v), incubation temperature T=28 °C. Each point is the mean value of two independent measurements.

Lastly, cultivation of *Cryptococcus curvatus* on molasses was employed, in media containing *c.* 80 g/L of initial total sugar concentration and *c.* 18 g/L of protein. As presented in Table III.25., this substrate had a positive impact on biomass formation, reaching up to *c.* 36 g/L at the end of the fermentation. In terms of lipid production and IPS content, lipids were only detected in traces ($Y_{L/X}$ ranging between 1.7 and 4.0% w/w), as anticipated in conditions of nitrogen excess, whereas high quantities of IPS (*c.* 10 g/L), but slightly lower compared to those on synthetic medium (IPS≈11 g/L), were synthesized. Dry biomass yield on sucrose equivalent (=total sugars + protein) consumed was *c.* 0.43 g/g, slightly lower than the maximum $Y_{X/Su}$ recorded in this investigation (≈0.51 g/g) potentially due to substrate inhibition by the increased initial total sugars concentration into the growth medium. Finally, the kinetics of FAN and protein presented similar profiles to those on cheese-whey.

III.3.2.4 Fatty acid composition of Cryptococcus curvatus NRRL Y-1511 cellular lipids.

As far as total cellular lipids of the yeast were concerned, they were analyzed in terms of fatty acid (FA) composition during early, final exponential phase, and stationary phases of the microbial growth in nitrogen limited condition; and during the exponential and stationary phase in carbon limited media. The main FAs detected were oleic (C18:1), palmitic (C16:0), linoleic acid (C18:2), as well as stearic acid (C18:0). As depicted in Tables III.27. and III.28., the concentration of the individual FAs did not seem to chance significantly, regardless of the carbon source and the cultivation conditions imposed. However, the course of the fermentation was found to have an impact upon the concentrations of some major FAs. For instance, in the case of lactose-based media under nitrogen-limited conditions, the quantity of palmitic and linoleic acid was reduced in parallel to the evolution of the fermentation and the entrance of the culture to the stationary growth phase. A similar trend was observed for stearic acid, while the exact opposite behavior was noted in the case of oleic acid. Equal trend is observed in sucrose-based cultures under nitrogen-limited conditions, whereas for both sugars in carbon-limited media there was not any clear trend of evolution between the major fatty acids present as function of the fermentation time. Between nitrogen-excess and nitrogen-limited cultures presenting the same initial carbohydrate concentration, the FA composition presented similarities for the trials on lactose. In contrast, nitrogen-excess cultures on sucrose utilized as the sole substrate were accompanied by higher production of the FA $^{\Delta 9,12}$C18:2 and lower production of the FA $^{\Delta 9}$C18:1 as compared with the respective nitrogen-limited trials presenting similar initial sucrose concentration.

Table III. 26. Fatty acid composition of intracellular lipids of *Cryptococcus curvatus* NRRL Y-511, during growth on lactose-based media, under nitrogen- and carbon-limited conditions. Culture conditions as in Table III.21.

Fatty acid content (%, w/w)									
Nitrogen-limited media									
Lactose (g/L)	Time (h)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:1
40	48	-	24.0	-	13.6	46.4	12.6	3.4	-
	144	0.3	19.0	0.5	11.8	56.3	10.4	1.7	-
	192	0.3	20.0	0.3	14.5	53.8	9.0	2.1	-

60	72	-	22.4	-	14.3	51.3	12.0	-	-
	192	0.3	20.3	0.3	14.5	54.2	8.6	1.8	-
	264	0.3	18.2	0.3	13.2	57.1	9.1	1.8	-
	360	0.3	16.8	0.3	13.5	57.5	9.6	2.0	-
80	72	-	25.3	-	13.1	47.2	11.2	3.2	-
	192	0.3	18.9	0.3	15.0	54.3	9.2	2.0	-
	456	0.3	16.0	0.3	11.8	59.6	10.1	1.9	-
Nitrogen-excess media									
40	24	-	25.3	-	14.2	47.4	13.1	-	-
	72	3.1	28.4	-	6.7	45.1	14.1	2.6	-
	96	0.9	14.3	0.9	3.3	62.1	17.9	0.6	-
80	48	1.4	21.4	-	15.3	50.5	11.4	-	-
	168	-	26.4	-	8.2	53.7	11.7	-	-
	240	1.2	24.2	1.5	7.3	49.5	15.3	0.6	0.4
	336	-	26.2	-	11.2	48.7	13.9	-	-

Table III. 27. Fatty acid composition of intracellular lipids of *Cryptococcus curvatus* NRRL Y-511, during growth on sucrose-based media, under nitrogen- and carbon-limited conditions. Culture conditions as in Table III.23.

Fatty acid content (%, w/w)										
Nitrogen-limited media										
Sucrose (g/L)	Time (h)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1
40	48	-	28.3	-	18.4	43.3	10.0	-	-	-
	144	0.6	21.4	-	13.1	54.5	9.3	1.1	-	-
	216	0.6	20.3	0.5	14.2	53.2	8.9	1.2	1.1	-
80	48	-	22.5	-	16.8	49.9	10.6	-	0.2	-
	192	0.3	18.1	0.3	14.2	56.2	8.4	1.7	0.8	-
	336	0.6	18.6	0.6	11.1	56.6	10.2	1.7	0.5	0.1
Nitrogen-excess media										
40	72	1.3	23.5	2.2	13.3	42.6	13.2	2.3	1.6	-
	148	1.2	25.4	0.8	9.2	49.5	12.7	1.2	-	-
	214	0.8	24.2	1.1	9.0	48.5	14.1	1.5	0.8	-
80	72	1.3	24.9	-	17.7	38.6	15.3	2.2	-	-
	148	1.5	22.5	1.1	9.3	41.3	22.4	1.9	-	-
	264	-	22.9	-	10.7	40.6	25.8	-	-	-

III.3.3. Discussion

The potential of the yeast *Cryptococcus curvatus* to successfully transform sugar-based substrates into yeast biomass, lipid, IPS and enzymes was investigated in the current study. The produced biomass was enriched with lipids and/or intracellular polysaccharides, depending on the cultivation conditions imposed. Apart from synthetic media, yeast performance was further validated in cultures of molasses and cheese-whey, two important representatives of sugar-based food industry by-products. During cultivation in synthetic media, the patterns of major metabolites were affected not only by the type and concentration of the carbon source, but also by the cultivation conditions. In particular, lactose-based media were positively associated with microbial oil production by the yeast *Cryptococcus curvatus*, under nitrogen-limited conditions, yielding *c.* 30% (w/w) at initial lactose concentration of 80 g/L (L=4.3 g/L). In contrast, growth on sucrose under nitrogen-limited conditions was not accompanied by such high accumulation of storage lipid as compared with growth on lactose

($Y_{L/X}$≈19% w/w, SCO=3.3 g/L). Lactose or cheese-whey is considered as appropriate substrate for the production of lipid by *Cryptococcus curvatus* strains (most cultures have been performed with the strain ATCC 20509). For instance, Daniel et al. (1998) reported a production of SCO of 20 g/L and *c.* 59% in biomass during growth on deproteinized cheese-whey. In other culture conditions with the same strain, quantities of 10 g/L and 42% w/w were reported (Daniel et al., 1999). Ykema et al. (1988) reported values of 7.8 g/L and 36% w/w during growth on lactose in batch bioreactor trials, while Brown et al. (1989) indicated lipid production of 8 g/L and 40% of lipid in biomass during growth on lactose on single-stage continuous culture, always with the same strain (ATCC 20509). The same strain when cultivated on fed-batch cultures with pure glycerol employed as substrate produced *c.* 30 g/L of lipid with $Y_{L/X}$≈25% w/w (Meesters et al., 1996). With biodiesel-derived glycerol as substrate, the potentiality of same strain was 13.8 g/L and 44.2% w/w (Liang et al., 2010a). During growth on hydrolyzed spent yeast waste blended with glycerol the strain ATCC 20509 presented a lipid production of *c.* 19 g/L with the respective $Y_{L/X}$ value being ≈38% w/w (Ryu et al., 2013). Significantly high lipid quantities (i.e. $Y_{L/X}$ ≈55-60% w/w) have been achieved by the above-mentioned strain (ATCC 20509) during its cultivation on cellulose in a trial in which simultaneous saccharification and lipid production process occurred (Gong et al., 2013) or during its culture on N-acetylglucosamine (Wu et al., 2010).

Cultivation of the yeast under nitrogen-excess conditions favored the synthesis of biomass that contained restricted quantities of microbial lipids. It is well established in the international literature that the molar C/N ratio represents a key factor for de novo lipid synthesis in oleaginous microorganisms (Davies and Holdsworth, 1992; Ratledge, 1997; Papanikolaou and Aggelis 2010; 2011a). Moreover, in the current investigation, special attention was given in monitoring the biosynthesis of intracellular polysaccharides during cultivation of *Cryptococcus curvatus*. In nitrogen-limited media, IPS relative values were high (up to 68.1% w/w), as a result of cell growth and proliferation during the first hours of the fermentation. However, as cultivation proceeded, IPS relative values were reduced to half, whereas in absolute values, IPS were mainly synthesized at the first growth steps, despite the significant presence of nitrogen into the medium. On the other hand, $Y_{IPS/X}$ reduction was accompanied by a gradual increase in total cellular lipids produced (in both absolute and relative values). Likewise, the cellular protein content was very low at the first growth stages, period in which total intra-cellular sugars in DCW presented their highest values, increased at

the middle steps of the fermentation, decreasing finally at the last fermentation stages, period in which lipid accumulation picked. All these events indicate that the extra-cellular carbon source was mainly transformed into IPS (initially) and IPS and proteins (at the middle steps of the fermentation), and thereafter the intra-cellular total carbohydrates together with the supplementary consumption of the extra-cellular lactose were converted into storage lipids. This interaction between the synthesis of intra-cellular polysaccharides and cellular lipids has never been previously reported for oleaginous yeasts, whereas a similar trend with the current investigation has been reported for an oleaginous *Chlorella* sp. strain growing autotrophically under constant illumination conditions in an open-pond simulating photo-bioreactor (Bellou and Aggelis, 2012). Exactly the inverse trend has been reported for many higher fungi cultivated on glucose in static or agitated liquid cultures with glucose utilized as the sole substrate; edible and medicinal mushrooms like *Pleurotus pulmonarius, Agrocybe aegerita, Ganoderma applanatum* and *Volvariella volvacea* during their growth on glucose have shown a relatively elevated lipid yield $Y_{L/X}$ (reaching in some cases the value of *c.* 20% w/w although the above-mentioned microorganisms are not considered as oleaginous) at the first growth steps, decreasing substantially as the culture proceeded despite significant presence of glucose into the medium, with concomitant significant rise in the biosynthesis of endopolysaccharides (maximum quantities ranging between *c.* 2.5 and 11 g/L with concomitant yields $Y_{IPS/X}$ of 30-60% w/w have been reported at the late growth phase of these cultures) (Diamantopoulou et al., 2012a; 2012b; 2014).

A very important finding of the current investigation was related with the fact that nitrogen-excess cultivation conditions enhanced noticeably the IPS production in *Cryptococcus curvatus*. The events that lead to IPS and storage lipid production are related with the alteration of intra-cellular concentration of various metabolites, triggered after nutrient (mostly nitrogen) limitation in the culture medium (Ratledge, 1997; Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a). Specifically, nitrogen depletion leads to rapid decrease of intracellular AMP, causing alterations to the function of the Krebs cycle and results in intra-mitochondrial accumulation of citric acid, which after exceeding a critical value, is excreted to the cytoplasm. At this point, the action of ATP-citrate lyase (ATP-CL) is crucial, since IPS presence in oleaginous microorganisms will cleavage citric acid and generate cellular fatty acids via quasi-inverted reactions of β-oxidation (Ratledge and Wynn, 2002; Papanikolaou and Aggelis, 2011a). In the absence ATP-CL enzymatic complex, citric

acid is accumulated in the cytoplasm and will be either excreted into the culture medium, or will provoke the inhibition of 6-phosphoro-fructokinase (6-PFK). The above-mentioned event, in association with the decreased activity of 6-phosphoroglucose isomerase (PGI) will result in intracellular accumulation of polysaccharides (Galiotou-Panayotou et al., 1998; Zhong and Tang, 2004). Based on the findings of the current investigation, the remarkable production of IPS under nitrogen-excess conditions denotes potential saturation of the enzymes PFK (Papanikolaou and Aggelis, 2011a) and PGI (Zhong and Tang, 2004), the down-regulation of which is responsible for the accumulation of intra-cellular polysaccharides. Apparently, under nitrogen-excess conditions, as in the present study, the activity of the enzymes 6-PFK and PGI, of primordial importance for the synthesis of IPS, seems not capable to saturate the carbon flow towards the synthesis of pyruvate at the TCA cycle, potentially due to bottleneck in the above-mentioned enzymes, resulting in shift and subsequent accumulation of IPS. Similar features have been reported by several higher fungi like *Flammulina velutipes*, *Pleurotus pulmonarius* and *Morchella esculenta* during cultivation on glucose-based submerged cultures, in which significant accumulation of IPS has been reported in media that did not present nitrogen limitation (Diamantopoulou et al., 2014). In some of the above-mentioned experiments (e.g. utilization of *Flammulina velutipes* fungus) the biosynthesis of IPS was significant, while kinetic analysis demonstrated that IPS production was a perfectly growth associated process (Diamantopoulou et al., 2014), indicating, as the results of the current submission, the potential of IPS biosynthesis in balanced growth phase of several fungi.

The biotechnological interest towards the synthesis of intra-cellular carbohydrates (polysaccharides in most of the cases) lies on their implementation as bioactive compounds, synthesized by higher fungi (mushrooms) belonging to the genus of *Ganoderma sp*. *Lentinula sp*., *Morchella sp*. etc. A number of fungal-derived polysaccharides have been reported to present antitumor and immune-modulating properties (Wasser, 2002; Zhong and Tang, 2004). As far as the IPS produced by yeast strains are concerned, yeast's IPS (principally β-glycans and α-mannans, deriving from cell walls of *Saccharomyces cerevisiae* and *Candida utilis*), have been equally demonstrated to reveal therapeutic (principally antioxidant) properties (Kogan et al., 2008). Moreover, algal species like seaweeds contain cell wall-bounded polysaccharides up to 50% of their DCW that serve as storage and structure molecules, whereas recent studies have revealed the potential of such polysaccharides as biological active

compounds for human health (Zhou et al., 2004). Characteristic results of the production of intra-cellular total carbohydrates production by several types of microorganisms is given in Table III.29. As previously mentioned, higher fungi are considered as most apparent candidates for IPS production; Diamantopoulou et al. (2014) reported that *Pleurotus pulmonarius* accumulated 10.9 g/L of IPS (48.4% w/w in DCW) when cultivated on glucose-based media. In another study, the medicinal mushroom *Cordyceps jiangixiensis* produced 8.9 g/L of IPS (36.6% w/w in DCW) in an optimized medium containing a mixture of molasses and glycerol (Xiao et al., 2014). Furthermore, oleaginous microalgae strains of *Schizochytrium limacinum* have been reported to accumulate up to 33% (w/w in DCW) of IPS during cultivation in glucose or glycerol-based media (Pyle et al., 2008; Liang et al., 2010). The maximum IPS quantities achieved in the current submission (9-11 g/L with $Y_{IPS/X}$≈50-68% w/w) are significant and comparable with the ones reported for *Pleurotus pulmonarius*, *Morchella esculenta* and *Flammulina velutipes* (Diamantopoulou et al., 2014), or even higher compared to *Volvariella volvacea*, *Ganoderma lucidum* and *Tuber sinense* ones (Diamantopoulou et al., 2012a; 2012b; Fang et al., 2012) during growth in shake-flask experiments.

Table III. 28. Experimental results concerning the production of intra-cellular total sugars (IPS) by microbial strains cultivated under various fermentation configurations and their comparisons with the present study.

Microorganism	Species / Genus	IPS (g/L)	$Y_{IPS/X}$ (%, w/w)	Substrate	Cultivation type	Reference
Mushrooms	*Pleurotus pulmonarius*	10.9	48.4	Glucose	Shake flasks	Diamantopoulou et al., (2014)
	Flammulina velutipes	6.7	51.4	Glucose	Shake flasks	Diamantopoulou et al., (2014)
	Agrocybe aegerita	5.5	60.4	Glucose	Static flasks	Diamantopoulou et al., (2014)
	Ganoderma lucidum	6.3	41.4	Potato dextrose and olive oil	Bioreactor	Berovič et al., (2003)
	Vorvariella volvacea	5.6	40.0	Glucose	Shake flasks	Diamantopoulou et al., (2012a)
	Cordyceps jiangxiensis	8.9	36.6	Molasses/Glycerol	Shake flasks	Xia et al., (2006)
	Ganoderma applanatum	*c.* 0.3-0.5	*c.* 5-13%	Glucose	Shake flasks	Lee et al.,

						(2007)
	Chlorella sp.	n.r	35.9	Photo-autotrophy	Bioreactor	Bellou and Aggelis, (2012)
	Nannochloropsis salina	n.r	31.3	Photo-autotrophy	Bioreactor	Bellou and Aggelis, (2012)
Algae	*Schizochytrium limacinum*	n.r.	12.6	Glycerol	Shake flasks	Liang et al., (2010b)
	Schizochytrium limacinum	n.r.	33.4	Glucose	Shake flasks	Pyle et al., (2008)
	Schizochytrium limacinum	n.r.	25.7	Glycerol	Shake flasks	Pyle et al., (2008)
	Cryptococcus curvatus	11.0	41.2	Lactose	Shake flasks	Present study
Yeasts	*Cryptococcus curvatus*	10.9	41.2	Sucrose	Shake flasks	Present study
	Cryptococcus curvatus	11.1	28.8	Cheese-whey	Shake flasks	Present study
	Cryptococcus curvatus	9.9	27.8	Molasses	Shake flasks	Present study

In the current investigation, the composition in individual sugars of the IPS of *Cryptococcus curvatus* was found to reflect the sugar composition of the culture medium. While the principle monosaccharide of IPS was glucose, growth on lactose was followed by synthesis of IPS that contained non-negligible quantities of galactose, whereas growth on sucrose was accompanied by IPS containing besides glucose, also fructose. Glucose has been reported as the principal carbohydrate in the cellular composition of total intra-cellular sugars of higher fungal species, regardless of the substrate utilized, deriving mainly from β-D-glucans (Diamantopoulou et al., 2012a; Smiderle et al., 2012; Diamantopoulou et al., 2014). On the other hand, the reports deriving from the literature demonstrate contradictions concerning the presence of fructose as building block in the cellular polysaccharides of several yeast and fungal strains; in accordance with the current investigation, the presence of fructose in the intra-cellular polysaccharides has been demonstrated in cultures of *Ganoderma applanatum* during all stages of growth, but in significantly lower concentrations than that of glucose (Diamantopoulou et al., 2014). On the other hand, Zhang et al. (2012) reported the presence of several types of monosaccharides (like fucose, rhamnose, galactose, glucose, arabinose, *etc*) but not that of fructose as building blocks of the IPS of the mushroom *Hericium erinaceus* during growth on whey. Likewise, Lee et al. (2007) reported that the intra-cellular polysaccharides of the mushroom *Ganorerma applanatum* were composed of glucose, galactose, mannose and xylose found in various concentrations in the IPS, regardless

of the sugars utilized as individual substrates. In the above-mentioned work, when fructose was used as starting material of the fermentation, no fructose at all was detected in the individual carbohydrates of the IPS of this microorganism (Lee et al., 2007). Moreover, the IPS of the yeast *S. cerevisiae* are mainly composed of glucose and mannose, since in this microorganism the cellular polysaccharides are mainly glucans and mannans (Kogan et al., 2008). Finally, in terms of galactose existence in the IPS composition in *C. curvatus*, the fungus *Phellinus igniarius* has been reported to contain the aforementioned carbohydrate due to the presence of *β*-galactans (Guo et al., 2010). In our investigation, the concentration of all monosaccharide building-blocks of IPS was found to be influenced by the growth stage of the microorganism, as well as the cultivation conditions imposed (in accordance with the results achieved with *Pleurotus pulmonarius, Volvariella volvacea, Morchella esculenta* and *Flammulina velutipes* (Diamantopoulou et al., 2012a; 2014).

During trials on lactose, extra-cellular β-galactosidase activity was not detected, whereas the absence of glucose and galactose in the fermentation medium, as products of lactose hydrolysis, indicated the presence of intra-cellular β-galactosidase in *Cryptococcus curvatus*. Most yeast strains include the particular enzyme in their complex in intra-cellular level, and thus, the whole cell is often utilized as catalytic agent for the hydrolysis of lactose and lactose-based by-products (such as cheese-whey or whey permeate), in an immobilized form (Kosseva et al., 2009). In our study, cheese-whey proved to be a suitable substrate for biomass production; DCW of *c.* 39 g/L with biomass yield produced per lactose equivalent of *c.* 0.48 g/g was produced. The produced yeast biomass was enriched primarily with IPS and to a lesser extent with lipids. Cultivation of the yeast on sucrose-based media resulted in significant biomass production, whereas both IPS and lipids were accumulated in lower amounts as compared to those achieved in lactose under nitrogen-limited conditions. Furthermore, molasses proved to be an adequate substrate for biomass and IPS production. During batch flask fermentations on sucrose-based media, extra-cellular invertase activity was monitored. Maximum activity of the particular enzyme was noted when almost all the available sucrose was hydrolyzed into glucose and fructose; thereafter, a drastic reduction in invertase activity was noted. Invertase is present in the enzymatic complex of some yeast and fungal strains, belonging to the genera of *Saccharomyces* sp., *Candida* sp., *Aspergillus* sp., *Thamnidium* sp., etc both intra-cellularly and extra-cellularly (Gascón and Ottolenghi, 1967; Rubio, 1995; 2006; Alegre et al., 2009; Chatzifragkou et al., 2010; Papanikolaou et al., 2010).

The maximum invertase activities achieved in the current submission are comparable with some of the highest ones in the literature; for instance, the fungus *Thamnidium elegans* produced *c.* 1.4 U/mL of extra-cellular invertase during growth on sucrose, with maximum invertase activity being substantially enhanced with the increment of sucrose into the medium (Papanikolaou et al., 2010). Extra-cellular invertase production of more than 3 U/mL has been reported for *Aspergillus niger* (Gascón and Ottolenghi, 1967; Rubio, 1995; 2006; Alegre et al., 2009; Chatzifragkou et al., 2010; Papanikolaou et al., 2010), while *Cunninghamella echinulata* produced *c.* 0.5 U/mL (Chatzifragkou et al., 2010). Finally, genetically engineered *Yarrowia lipolytica* strains were created in order for improving growth on sucrose for industrial applications (e.g. production of citric acid) and maximum extra-cellular invertase activities of *c.* 4.6 U/mL (Lazar et al., 2013) 3.7 U/mL (Lazar et al., 2011) and 0.84 U/mL (Förster et al., 2007) were reported in several fermentation configurations.

The main FAs detected from the total lipid produced by *Cryptococcus curvatus* were oleic (C18:1), palmitic (C16:0), linoleic acid (C18:2), as well as stearic acid (C18:0). The distribution of the individual FAs seem to have some slight changes related with the carbon source and the cultivation conditions imposed. The FA compositions of cellular lipids produced by other *C. curvatus* strains or even other oleaginous yeast species (e.g. *Y. lipolytica*, *Rhodotorula* sp., *etc*) cultivated on glucose or similarly metabolized compounds (like glycerol or other sugars) can result in differences depending on the microbial growth stage and the initial concentration of substrate in the culture medium. These differences seem to be strain dependent; it appears that neither the fermentation time nor the increasing in the concentration of glycerol (or glucose) in the culture medium have any systematic common effect on the modification of cellular FAs in the cells of oleaginous yeasts (Hassan et al., 1993; Papanikolaou et al., 2002; Papanikolaou et al., 2006; 2009; Papanikolaou and Aggelis, 2009; Makri et al., 2010; Chatzifragkou et al., 2011).

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CONTENTS OF TABLES

CONTENTS OF FIGURES

Publications in Journals or Conferences

1. **Sidoine Sadjeu Tchakouteu**, Afroditi Chatzifragkou, Ourania Kalantzi, Apostolis A. Koutinas, George Aggelis, Seraphim Papanikolaou, Peculiarities of major metabolites biosynthesis by the oleaginous yeast *Cryptococcus curvatus* cultivated on sugar-based substrates. European Journal of Lipid Science and Technology (2015) Accepted manuscript

2. **Sidoine Sadjeu Tchakouteu**, Ourania Kalantzi, Chryssavgi Gardeli, Apostolis A. Koutinas, George Aggelis, Seraphim Papanikolaou, Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation efficiency. Journal of Applied Microbiology (Under revision)

3. **Sidoine Sadjeu Tchakouteu**, Afroditi Chatzifragkou, Ourania Kalantzi, Nikolaos Kopsahelis, Ioannis K. Kookos, George Aggelis, Apostolis A. Koutinas, Seraphim Papanikolaou, Physiological patterns of of major metabolites biosynthesis by *Cryptococcus curvatus* growing on cheese-whey lactose (2014) 3rd International ISEKI Food Conference, pp 138.