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Study of lipid production by the Zygomycete *Mortierella isabellina* during growth on blends of cellulosic sugars

M.Sc.-Thesis

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Η παρούσα ερευνητική μελέτη εκπονήθηκε στα εργαστήρια Μικροβιολογίας και Βιοτεχνολογίας Τροφίμων καθώς και στο Εργαστήριο Χημείας και Ανάλυσης Τροφίμων του Γεωπονικού Πανεπιστημίου Αθηνών, στα πλαίσια του Π.Μ.Σ. «Επιστήμης και Τεχνολογίας Τροφίμων και Διατροφής του Ανθρώπου». Πρώτα από όλους θέλω να ευχαριστήσω θερμά τον δάσκαλο μου Αναπληρωτή Καθηγητή Γ.Π.Α. κύριο Σεραφείμ Παπανικολάου για την εμπιστοσύνη που έδειξε στο πρόσωπο μου, την καθοδήγηση και την άψογη συνεργασία μας κατά τη διάρκεια της μελέτης αυτής.

Επιπλέον, θα ήθελα να εκφράσω τις θερμές μου ευχαριστίες στον κύριο Αθανάσιο Μαλλούχο Λέκτορα Γ.Π.Α για την συνεργασία μας, για την πολύτιμη βοήθεια του, την συνεχή καθοδήγηση και τις χρήσιμες συμβουλές του κατά την διάρκεια της πειραματικής διαδικασίας και της συγγραφής της μελέτης.

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Table of Contents

Abstract

Aim of the present study was to investigate the potential of growth and lipid production by an oleaginous fungus (Zygomycetes strain) namely, *Mortierella isabellina* (ATHUM 2935), to accumulate Single Cell Oil (SCO) rich in Polusatturated Fatty Acids (PUFAs), especially, γ- linolenic acid (GLA), an unsaturated fatty acid of nutritional and medical importance. The aforementioned microorganism was cultivated on sugar-based renewable carbon sources, that are the main products from hydrolysis of lignocellulosic residues. Particularly, the substrates used were glucose and xylose, either as sole carbon source or as blends in various ratio at initial total sugar concentration of 80 g/L. Experiments were conducted in shake-flasks and the biochemical behavior (biomass production, accumulation of total lipid, substrate uptake, fatty acid composition of fungal oil) was studied when the microorganism was cultivated under nitrogen limited conditions, in order to direct the microbial metabolism towards the synthesis of intra-cellular lipid (initial molar C/N equal to 204). The microorganism presented notable growth in all substrates, while lipid accumulation was overall satisfactory. The differences observed in the process of lipid accumulation as related to the carbon sources used for *Mortierella isabellina* were attributed to the different metabolic pathways involved in the assimilation of the different substrates. Therefore, the various carbon sources/mixtures were channeled, at different extent, to storage lipid or to lipid-free biomass formation. Specifically, in the case of glucose used as sole carbon source (80 g/L) *Mortierella isabellina* produced 28 g/L biomass after 332 h of cultivation while 13.6 g/L lipid were produced after 499h of cultivation. The corresponding lipid in total dry biomass at this point ($Y_{L/X}$) was equal to 51.1 % (w/w). On the contrary, when xylose was used as sole carbon source (80 g/L) lower final concentrations as far as both growth (20.7 g/L biomass) and lipid accumulation (7.71 g/L) were concerned after 332h. However, the growth of the strain was notable with simultaneous production of noticeable amounts of lipids, also rich in GLA, in all glucose-xylose blends with maximum biomass production ranging from 20.5 to 27.9 g /L. Especially, *Mortierella isabellina* strain ATHUM 2935 grew remarkably on glucose-xylose blend 60:20 (g/L) and produced 27.9 g/L biomass with a lipid content of 12.8 g/L at this point. In this trial, the overall highest lipid production was achieved (15.59 g/L) after 280h of cultivation. As far as GLA production was concerned, the mixture of glucose: xylose 60:20 (in g /L) proved to be the most promising substrate among others tested for the accumulation of the aforementioned FA, reaching the maximum value of 520 mg /L about 280 h after inoculation. Satisfactory results were shown from the cultivation of the microorganism on the glucose-xylose blend (40:40 g/L), where the produced biomass value was 23.0 g/L, containing 8.7 g/L of lipid. Furthermore, the xylose - glucose mixture (60:20 g/L) was proven to be an adequate substrate for *Mortierella isabellina* by producing fair amounts of biomass (20.5 g/L) with notable lipid content (12.3 g/L). Moreover, substrates containing xylose either as the sole carbon source or as a mixture (80 g/L and 60:20 g/L), induced the production of xylitol, reaching a highest value of 23.5 g/L in trials with xylose employed as sole carbon source. Analysis of intra-cellular lipid showed that the most predominant component in the mycelium was oleic acid in the range of 33-55% of the total fatty acids while

the microorganism showed satisfactory capability to produce γ-linolenic acid (GLA), regardless of the substrate employed. GLA (C18:3n- 6) was present in all growth steps in the reserve cellular lipids, in concentrations between 0.73 – 5.41 % w/w. Furthermore, fraction analysis of total lipids revealed that mycelia contained higher amounts of neutral lipids (N) than polar lipids (G+S, P). Additionally, the estimation of the total amount of phospholipids through the phosphorus determination revealed notable differences between the phospholipid content which was affected not only by the duration of the particular fermentation but also from the substrate used. Moreover, the analysis of the total sugar content in lipid samples showed that the sugar content, contained at glycolipid and sphingolipid fractions was not constant since it reflects the biosynthesis variation of these lipid classes during the fermentations. Last but not least, further analysis via thin layer -chromatography (TLC) on silica gel G was conducted to determine the relative composition of the major lipid classes of the aforementioned microorganism. The non-polar lipids identified were: predominantly triacylglycerols (TAG) and to a lesser degree sterol esters (CE), free fatty acids (FFA), sterols (CL) and monoacylglycerols (MAG). Additionally, the following polar lipids were also identified: phosphatidyl-choline (PC), monogalactosyldiacylglycerol (MGDG) and cerebrosides.

1.1.Production of single cell oil

1.1.1. Oleaginous microorganism

The amounts of lipids in microorganisms may vary from less than 1% to over 80% of the cell biomass (Ratledge & Wynn, 2002).The extent of lipid accumulation in these microorganisms is determined by the genetic profile, as maximum attainable lipid contents can vary enormously among species and even among individual strains. It has been known that some microorganisms have a greater propensity to accumulate substantial amounts of oil, sometimes up to and even in excess of 70% of their biomass weight. Those microorganisms that could accumulate lipid to more than about 20% of their biomass were termed as *oleaginous microorganisms* (Thorpe & Ratledge, 1972). Oil obtained from microorganisms is also called as single cell oil (SCO). It is being synthesized with high purification and less expensive than agricultural and animal sources (Ratledge, 2004; Cohen & Ratledge, 2005). The term single cell oil formulated by Ratledge as an obvious parallel to the term Single Cell Protein (SCP, i.e. production of edible protein in the form of microorganisms) (Moreton, 1988).

A major sector of lipid biotechnology centers on the ability of these oleaginous microorganisms to convert various natural substances into these microbial lipophilic compounds. There are many oleaginous heteroptrophic microorganisms (microalgae, yeasts, fungi and bacteria) as it is shown in table 1.1.1. that used, or considered for use, as sources for SCO production.

Table 1.1.1. Oil content of some microorganisms

Microorganisms	**Oil content (% dry wt)**
Microalgae	
Botryococcus braunii	25–75
Cylindrotheca sp.	16–37
Nitzschia sp.	45–47
Schizochytrium sp.	50-57
Yeasts	
Cryptococcus albidus	65
Candida curvata	58
Lipomyces starkei	64
Rhodotorula glutinis	72
Bacteria	
Arthrobacter sp.	>40
Acinetobacter calcoaceticus	27-38
Rhodococcus opacus	24-25
Bacillus alcalophilus	18-24
Fungi	
Aspergillus oryzae	57
Mortierella isabellina	86
Humicola lanuginosa	75
Mortierella vinacea	66

*(adapted by Meng *et al.*, 2009)

The microbial oils are of a potential industrial and financial interest due to their specific characteristics (Ratledge, 1992, 1994; Certik and Shimizu, 1999; Papanikolaou *et al.,* 2001).These oils have long been considered as alternative oil sources, specifically as regards lipids rarely found in the plant or animal kingdom[i.e. lipids containing rare polyunsaturated fatty acids (PUFAs) or cocoa-butter equivalents](Dyal *et al.,* 2005; Dyal & Narine, 2005, Papanikolaou *et al.,* 2003; Fakas *et al.,* 2009; Kenny *et al.,* 2000; Ratledge, 2005). SCO is mainly composed of triacylglycerols -TAGs- with a fatty acid composition rich in C16 and C18, namely palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1) and linoleic acids (18:2) (Ratlledge & Wynn, 2002; Li *et al.,* 2007; Meng *et al.,* 2009).

Microalgae biomass is rich in ω-3 and ω-6 fatty acids, essential amino acids, like leucine, isoleucine, valine, etc. The accumulated oil in almost all microalgaes is mainly triglyceride (>80%), with a fatty acid profile rich in C16 and C18, showing Δ9,Δ12 and Δ15 desaturation (Papanikolaou *et al.,* 2002). These microorganisms grow rapidly and many are exceedingly rich in oil while they double their biomass within 24 h. The average lipid content of algal cells varies between 1% and 70%, but some of them can reach 90% of dry weight under certain conditions. Although microalgae may accumulate high amounts of microbial lipids but they cannot compete with other oleaginous microorganisms, such as, yeasts and fungi because their cultivation requires a big area and long fermentation duration (Meng *et al.,* 2009).

Bacteria may achieve high growth rates (huge biomass production only needs 12–24 h) and easy culture method. Some of them are capable of synthesizing remarkably high amounts of fatty acids (up tow70% of the cellular dry weight) from simple carbon sources like glucose under growth-restricted conditions and accumulate them intracellularly as TAGs . However, the majority of bacteria strains are generally not oil producers (Meng *et al.,* 2009).

Yeasts and fungi (especially molds) are considered as favorable oleaginous microorganisms since 1980s. Table 1.1.2. demonstrates some of these microorganisms as appropriate cell factories for the production of SCO. Yeast strains (e.g. *Rhodosporidium* sp., *Rhodotorula* sp., and *Lipomyces* sp.) can accumulate intracellularly around 70% (w/w) of SCO (Li *et al.,* 2007; Meng *et al.,* 2009). The most efficient oleaginous yeast *Cryptococcus curvatus* can accumulate storage lipid up to >60% of dry weight, when growing under N-limiting conditions. Oleaginous yeasts and molds accumulate triacylglycerols rich in polyunsaturated fatty acids or having specific structure (Kavadia *et al,* 2001; Papanikolaou & Aggelis, 2010, 2011). Fungi present advantages of simultaneously utilizing multiple carbon sources (glucose and xylose) as well as better tolerance to inhibitors [furfural, hydroxymethylfurfural (HMF), and aromatic compounds) in lignocellulosic hydrolysates] (Ruan *et al.,* 2012, 2013). The lipid content in oleaginous fungi ranges from 21% to 74%, which is also comparable to most oleaginous yeasts and microalgae (Nascimento *et al.,* 2014).

Table 1.1.2. SCO production from oleaginous microorganisms, carbon sources and cultivation modes

Microorganism	Carbone source	Total dry weight (g/L)	Oil content (%w/w)	Cultivation	Reference
Fungal species					
Cunnigamella echinulata	Glucose	15	46	shake flask	Fakas *et al*.,2009a
Cunnigamella echinulata	Starch	13.5	28	shake flask	Papanikolaou *et al*.,2007a
Mortierella isabellina	Glucose	27	44.6	shake flask	Fakas *et al*.,2009a
Mortierella isabellina	Starch	10.4	36	shake flask	Papanikolaou *et al*.,2007a
Mucor sp	Tapioca starch	28	17.8	shake flask	Ahmed *et al.*, 2006
Mortierella ramanniana	Glucose	62	46.1	Scale up- batch bioriactor	Hiruta et al., 1997
Yeast species					
Yarrowia lipolitica	Glucose	9.2	25	single- stage continous	Aggelis & Komaitis, 1999
Yarrowia lipolitica	Grude glycerol	8.1	43	single- stage continous	Papanikolaou & Aggelis, 2002
Yarrowia lipolitica	Stearin	15.2	52	shake flask	Papanikolaou *et al.*, 2007b
Candida sp. 107	Glucose	18.1	37.1	single- stage continous	Grill *et al*., 2007
Candida curvata	Lactose	18	31	single- stage continous	Evans & Ratledge, 1983
Candida curvata	Xylose	15	37	single- stage continous	
Apiotrichum curvatum	Whey	21.6	36	batch	Ykema *et al*., 1988
Cryptococcus curvatus	Glycerol	118	25	fed-batch	Meesters *et al.*, 1996
Lipomyces starkey	Glucose & sewage sludge	9.4	68	shake flask	Angerbauer *et al*., 2008
Lipomyces starkey	Glucose & xylose	20.5	61.5	shake flask	Zhao *et al*., 2008
Lipomyces starkey	Glucose	153	54	fed-batch	Yamauchi *et al.*, 1983
Trichosporon fermentans	Molasses	36.4	35.3	shake flask	Zhu *et al*., 2008
	Fructose	21.5	40.7	shake flask	
	Lactose	16.9	49.6	shake flask	
Rhodotorulaa glutinis	Monosodium glutamate wastewater	25	20	shake flask	Xue *et al*., 2008
Rhodosporidium toruloides	Glucose	106.5	67.5	fed-batch	Li *et al.*, 1986

*(adapted by: Koutinas & Papanikolaou, 2011)

Fungi, especially Zygomycetes strains, are considered as favorable oleaginous microorganisms and potential producers of single cell oil (SCO) containing γ-linolenic acid (GLA, $^{\Delta 6,9,12}$C18:3), a polyunsaturated fatty acid (PUFA) of crucial dietary and pharmaceutical importance (Ratledge & Wynn, 2002 , 2006). One of the oleaginous fungi, *Mortierella isabellina* is capable of accumulating considerable amount of lipids, up to 80% of cell biomass (Chatzifragkou *et al.,* 2010). *M. isabellina* could be cultivated on several substrates, including monomer sugars (Chatzifragkou *et al.*, 2010), glycerol (Fakas *et al.*, 2008), as well as

lignocellulosic biomass (rice hull and corn stover) hydrolysates (Ruan et al., 2012). Moreover, Zheng *et al.* (2012), demonstrated that *Mortierella isabellina* had best performance on lipid production among 5 oleaginous fungi growing on dilute sulfuric acid pretreated wheat straw. These features, together with good tolerance to inhibitors derived from lignocellulosic materials (Zeng *et al.*, 2012), suggested that *Mortierella isabellina* could be a good candidate for lipid production from low cost renewable feedstock. Although *M. isabellina* is able to assimilate various hexoses and pentose sugars, its efficiency for xylose utilization is much lower than for glucose (Chatzifragkou *et al.*, 2010; Fakas *et al.*, 2009). Glucose as a model substrate has been studied for microbial lipid production by this strain, with lipid yield on glucose consumed around 0.22 g/g (Chatzifragkou *et al.*, 2010; Papanikolaou *et al.*, 2004). Previous reports showed that the achieved lipid yield from Xylose was much lower than that achieved when glucose was used as substrate. Fakas *et al.* (2008) has reported an overall lipid yield of 0.113 g/g by growing *M. isabellina* on xylose medium with carbon to nitrogen ratio (C/N) from 78 to 285. SCO could be used not only for value –added applications (food additives) but also for commodity uses such us biodiesel production. The possibility of SCO usage as a starting material of second generation biodiesel represents an interesting alternative since concern is mounting over the use of plant oils for biodiesel production (Li *et al.*, 2007, 2010; Xue *et al.*, 2006; Zhao *et al.*, 2008; Ahmed *et al.*, 2009) Indeed, this approach represents a very interesting alternative to the use of plant oils for bio-diesel production because oleaginous microorganisms can transform a variety of substrates to SCO, which can then be transformed to bio-diesel. However, the production cost of SCO are still high, with serious efforts being made to reduce it by using waste materials as substrates, either in submerged or in solid state fermentation systems (Papanikolaou *et al.*, 2003; Xue *et al.*, 2006); Papanikolaou & Aggelis, 2002; Koutinas & Papanikolaou, 2011; Ahmed *et al.*, 2006)

1.1.2. **Substrates for the production of SCO**

A remarkable plethora of substrates have been utilized for the production of SCO, such as pure sugars, sugar –based renewable materials or sugar enriched wastes, vegetable oils, crude waste industrial hydrophobic materials(e.g. industrial fatty acids, waste fats, crude fish oils etc), pure fatty acids or glycerol (Aggelis *et al.*, 1996; Papanikolaou *et al.*, 2001, 2002, 2004a, 2004b, 2007; Fakas *et al.*, 2006, 2007, 2008a, 2008b, 2009a, 2009b; Papanikolaou & Aggelis, 2002, 2003a, 2003b). The strains that are illustrated in Table 1.2. and as many others are capable of producing large amounts of intracellular lipid, which can be useful for the valorization of these substrates with high sugar content, by converting them into fatty compounds of particular composition (e.g., PUFAs, cocoa–butter like lipids) (Ratledge & Wynn, 2002; 2006, Papanikolaou *et al.*, 2010). The most preferable carbon sources are the wastes or/ and byproducts as they present very low cost (sometimes this cost may be zero or even negative). Waste use, however, presents serious problems, as their availability may be restricted (locally or temporally), while their fermentation performance may vary from batch to batch (Ratledge, 1988). In addition, wastes may contain some other non-assimilable polysaccharides that need to be hydrolyzed, either chemically or enzymatically, so as to produce assimilable sugars , a fact increases the fermentation cost. The efficiency and rates of microbial oil production from carbohydrate

sources and industrial systems for its exploitation have been discussed in wide-ranging reviews and articles, which also emphasized the paucity information on filamentous fungi (Ratledge, 1982; Peters, 2007; Fakas *et al.*, 2008a, 2008b).Various types of carbohydrates, which are raw materials deriving from agro-industrial activities are used for SCO production, in liquid or solid cultures, with variable success. These materials include sucrose and molasses from sugarcane and sugar beet, hydrolyzed starch products such as glucose and dextrins, fructose, whey (a by-product of cheese production), and, various natural hydrolysates (e.g., tomato waste hydrolysate, rice straw hydrolysate, etc.).

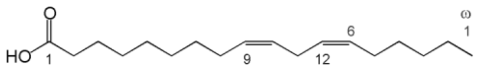
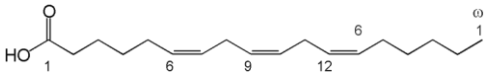
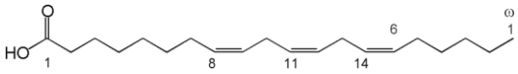
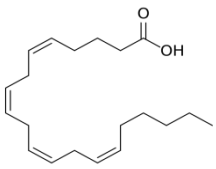
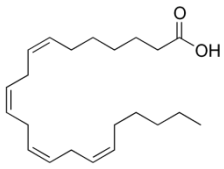
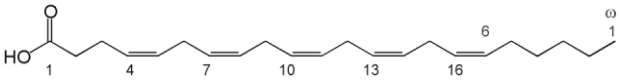
Sugar and polysaccharides that have been usually employed for SCO production are glucose, xylose, fructose, galactose, lactose, maltose, sucrose, starch, and pectin. The efficiency of each sugar is largely dependent on the micro-organism used and the culture conditions employed. It seems thus that the identification of suitable sugar substrates for lipid accumulation is more or less based on a trial and error approach. Glucose is the most common substrate used in SCO fermentation and is often used as a comparison basis to evaluate the performance of other carbon substrates. This is because almost all the oleaginous micro-organisms are capable of assimilating glucose and transform it to SCO. Several Zygomycetes, belonging to the genera *Cunninghamella*, *Mortierella*, *Mucor*, *Rhizopus*, and *Zygorhynchus*, were grown on glucose and *Cunninghamella echinulata* and *Mortierella isabellina* were selected as the most potent producers of GLA-rich SCO (Kavadia *et al.*, 2001). Papanikolaou *et al.*, (2004) cultivated *M. isabellina* on media having glucose as carbon source and after a prolonged fermentation time (250 h) 35.9 g/l of biomass containing 50% lipid were produced. However, the GLA content of the produced oil was less than 4% (801 mg/L). On the other hand, cultivation of *C. echinulata* on a glucose medium yielded less amounts of biomass (5.5 g/L) as compared to *M. isabellina*, but the lipid content in biomass of *C. echinulata* was more or less the same (47%) as in *M. isabellina* (Gema *et al.*, 2002). The GLA content, however, of the *C. echinulata* SCO produced was 14% (720 mg/L). Concerning yeasts, feb-batch cultivation of *Rhodosporidium toruloides* in 15-liter bioreactors yielded in very high biomass amounts (106.5 g/L), which contained 67.5% (w/w) lipid, while the produced SCO was destined for biodiesel production (Li *et al.*, 2007). Glucose is probably the most suitable substrate for SCO production and the obvious choice for the evaluation of the performance of oleaginous micro-organisms. Among waste materials**,** lignocelluloses biomass (e.g. xylose, mannose, galactose, cellobiose) is of great importance because it is produced in enormous quantities annually, as a result of land cultivation (Lee *et al.*, 1999). Xylose is the second most abundant sugar of lignocellulosic biomass, being produced during the dilute acid hydrolysis treatment of this biomass (Lee *et al.*, 1999). Evans and Ratledge (1983) who used the yeast *Candida curvata* showed that xylose is a very efficient substrate, as the lipid accumulation on xylose being almost 50% (w/w). Recently, two Zygomycetes, namely *C. echinulata* and *M. isabellina*, were grown on xylose and glucose and accumulated was almost 60% (w/w) of lipid in their biomass (Fakas *et al.*, 2009). Xylose may be considered as one of the model substrates for SCO production, since the pathways involved in the assimilation of xylose are different from the pathway of glucose assimilation, which is the best-studied substrate for SCO production. The stoichiometry of glucose and similar sugars such as lactose, fructose, etc metabolism is about 1.1 moles of acetyl-CoA per 100 g of glucose

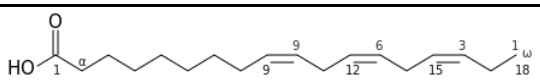
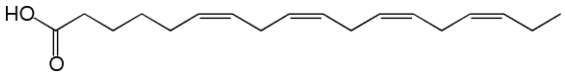
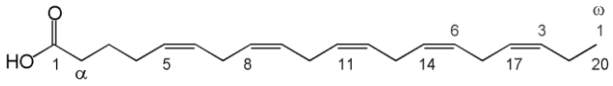
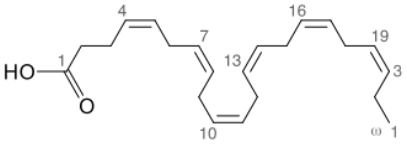
utilized. As far as xylose is concerned, this compound could be either metabolized through the *phosphoketolase reaction*, which is the most efficient pathway yielding 1.2 moles of acetyl-CoA per 100 g of xylose utilized, or the *pentose phosphate pathway*, where 1 mole of acetyl-CoA is formed per 100 g of xylose utilized (Papanikolaou & Agellis, 2011a, 2011b). Therefore, if 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. This value is somewhat higher concerning the fermentation of xylose (around 0.34 g/g), assuming that oleaginous microorganisms utilize exclusively the phosphoketolase pathway for xylose assimilation (Evans & Ratledge, 1984).

1.2. Microbial polyunsaturated fatty acids - PUFAS

Polyunsaturated fatty acids (PUFAs) are functional lipids: aliphatic, long chain hydrocarbons that have two or more Z (cis) double bonds separated from each other by a single methylene group (Hamilton, 1992). The most important polyene acids fall into families the members of which are biosynthetically related. The two most common families namely omega-3 (ω/*n*-3) and omega-6 (ω/*n*-6) are detailed in Table 1.2.1 and constitute the category of Essential fatty acids, or EFAs that humans and other animals must ingest. Such fatty acids are required by the body but cannot be synthesized *de novo*.

Table 1.2.1.The *n*-6 and *n*-3 families of polyene acids.

Common Name	Lipid name	Chemical name	Chemical structure
***n*-6 family**			
Linoleic acid	18:2 (n-6)	*all-cis*-9,12-octadecadienoic acid	
γ-linolenic acid (GLA)	18:3 (n-6)	*all-cis*-6,9,12-octadecatrienoic acid	
Dihomo-γ-linolenic acid(DGLA)	20:3 (n-6)	*all-cis*-8,11,14-eicosatrienoic acid	
Arachidonic acid (AA)	20:4(n-6)	*all-cis*-5,8,11,14-eicosatetraenoic acid	
Adrenic acid	22:2(n-6)	*all-cis*-7,10,13,16-docosatetraenoic acid	
Docosapentaenoic acid	22:5(n-6)	*all-cis*-4,7,10,13,16-docosapentaenoic acid	
***n*-3 family**			

α-linolenic acid (ALA)	18:3(n-3)	*all-cis*-9,12,15-octadecatrienoic acid	
Stearidonic acid (SDA)	18:4(n-3)	*all-cis*-6,9,12,15-octadecatraenoic acid	
Eicosatetraenoic acid (ETA)	20:4(n-3)	*all-cis*-8,11,14,17-eicosateetraenoic acid	
Eicosapentaenoic acid (EPA)	20:5(n-3)	*all-cis*-5,8,11,14,17-eicosapentaenoic acid	
Docosahexaenoic acid (DHA)	22:6(n-3)	*all-cis*-4,7,10,13,16,19-docosahexaenoic acid	

Polyunsaturated fatty acids (PUFAs) have been widely incorporated into several industrial sectors. The 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 can present an enormous financial interest (Ratledge & Wynn, 2002). The PUFA production yield in oleaginous microorganisms is variable depending on individual species and stages of growth and development or, in turn, on their genetic background and regulatory modes at enzymatic and transcriptional levels (Laoteng & Certik, 2010). Various oleaginous Zygomycetes , especially Mucorales fungi have been used in order to produce lipids rich in polyunsaturated fatty acids **(PUFA)** of medical and dietetical interest, such as γ-linolenic (GLA, $^{\Delta 6,9,12}$C18:3) dihomo-γ-linolenic acid (DHGLA, $^{\Delta 8,11,14}$C20:3), arachidonic acid (ARA, $^{\Delta 5,8,11,14}$C20:4) and docosahexaenoic (EPA, $^{\Delta 5,8,11,14,17}$C20:5) (Aggelis et al., 1988; Kendrick and Ratledge, 1992; Certik et al., 1997; Chen and Liu, 1997; Certik and Shimizu, 1999, 2000; Kavadia et al., 2001; Papanikolaou et al., 2004; Ratledge & Wynn, 2006; Sakuradani & Shimizu, 2009). As a general remark, the oil accumulated by the oleaginous fungi is more unsaturated than that of the oleaginous yeasts (Ratledge & Wynn, 2006; Cetrik & Shimizu, 1999; Dyal & Narine, 2005;). This is the main reason for which the oleaginous molds have been principally used in order to produce lipids rich in medically and nutritionally important PUFAs, while few yeast strains have been recorded to be capable to synthesize such types of PUFAs (Ratledge & Wynn, 2006; Cetrik & Shimizu, 1999). The unique characteristic of these oleaginous species is the high accumulation of intracellular lipids in form of triaclyglycerols (TAG) as storage molecules (Ratledge & Wynn, 2002; Ratledge 2004). Other components presented in non-negligible quantities are free-fatty acids, other neutral lipids (such as monoacylglycerols, diacylglycerols and steryl-esters), sterols and polar fractions (*e. g.* phospholipids, sphingolipids, glycolipids) (Fakas *et al.*, 2006). Lipid accumulation and PUFA synthesis of oleaginous fungi are complex and closely associated with cell physiology and environmental adaptation. The Biochemistry of

the lipid accumulating process or oleaginicity in these microorganisms has been intensively reviewed by Ratledge (2002, 2004), Papanikolaou and Aggelis, (2011 a,b) it will be analyzed extensively in the next chapter.

1.2.1. *γ-linolenic acid* (GLA, $^{\Delta 6,9,12}$C18:3)

The γ-linolenic acid (GLA, $^{\Delta 6,9,12}$C18:3) comprised of 18 carbon atoms and three double bonds is also known as gamolenic acid. Recent research suggests that gamma (γ) -linolenic acid is unique among the *n*-6 polyunsaturated fatty acid (PUFA) family members as it ameliorates many health problems. As regards PUFAs produced by microbial means, γ-linolenic acid (GLA) is of great pharmaceutical interest, due to its beneficiary effects against various pathological conditions such as rheumatoid arthritis, multiple sclerosis, and skin disorders (Horrobin, 1992) and, principally, against various types of cancer (Kenny *et al.*, 2000).

Over the past several years extensive research has been made for the microbial production of PUFA and especially for the γ -linolenic acid. GLA is found naturally in varying extends in the fatty acid fraction of some plant seed oil but GLA- containing SCO seems like the most potent source of pure GLA, because most GLA containing plant oils (e.g. Borage oil) also contain high amounts of other PUFAs, especially linoleic acid, which hinder GLA purification (Ratledge & Wynn 2002). Research groups dealing with the production of PUFAs, usually prefer to work with strains capable of producing lipids rich in GLA (Papanikolaou *et al.*, 2004; Fakas *et al.*, 2006, 2007).

Zygomycetes, often cited in the literature for their competence in producing GLA, encompass the order Mucorales (Shaw, 1966; Aggelis *et al.*, 1987; Certik *et al.*, 1997; Kavadia *et al.*, 2001; Fakas *et al.*, 2009; Papanikolaou *et al.*, 2004). Thus, it is no surprise that a Mucorales fungus, namely *Mucor circineloides*, was the producer of the first commercial GLA-containing SCO (Ratledge, 2005). The oil accumulated by the oleaginous fungi is more unsaturated than that of the yeasts and this is the main reason for which the oleaginous molds have been principally used in order to produce lipids rich in polyunsaturated fatty acids (PUFAs) of medical and dietetical interest like GLA (Papanikolaou & Aggelis, 2011). A property related with the oleaginous Zygomycetes is that strains, which produce lipids rich in GLA**,** accumulate low quantities of SCO and vice versa (Kavadia *et al.*, 2001). It may be assumed that strains, which produce lipids with low GLA content, should accumulate high quantities of lipid in order to synthesize the quantity of GLA indispensable for their membrane function (Kavadia et al., 2001). Potent GLA producers are found in various Mucorales genera, such as the already mentioned *Mucor* (Aggelis *et al.*, 1987; Aggelis *et al.*, 1988), *Mortierella* (Aggelis *et al.*, 1987; Nakahara *et al.*, 1992), *Cunninghamella* (Gema *et al.*, 2002; Fakas *et al.*, 2008), *Thamnidium* (Stredansky *et al.*, 2000; Papanikolaou *et al.*, 2010), etc. Among these genera, *Mortierella* fungi have been the micro-organism of choice by many researchers, and a process for GLA production using *M. ramanniana* was developed in Japan (Nakahara *et al.*, 1992).

In this way, up to date, much effort has been put to the development of economical bioprocesses for GLA production (Certik *et al.*, 2006; Fakas *et al.*, 2008). It can be realized, however, that designing a successful bioprocess for GLA production requires profound knowledge of the regulation of the metabolic

pathways that lead to GLA formation. To this end, there has been some evidence showing that GLA is synthesized mostly during the growth phase (Fakas *et al.*, 2007), indicating a correlation between GLA synthesis and mycelia growth. This conclusion, however, was drawn from submerged cultures, where mycelia age is hard to be defined due to the formation of mycelia agglomerates comprising mycelia having different ages. In solid state cultures, however, fungal growth takes place mostly in the substrate surface, where mycelia of different ages can be discriminated. This discrimination then allows for the investigation of the potential correlation between GLA synthesis and mycelia growth.

1.3.Biosynthesis of microbial lipids

Lipids may be relatively simple molecules, as for example the fatty acids themselves, or more complex containing phospho or sulpho groups, amino acids, peptides and their derivatives, sugars and even oligosaccharides. The diversity of lipids signifies a diversity of function (Ratledge & Wilkinson, 1988; Wynn *et al.* 1999). Lipids can act as storage materials in animals, plants and microbial cells, where the lipids typically occur in the form of triacylglycerols in eukaryotic cells and they are also responsible for the structure of cell membranes where the lipids mainly answer as the amphiphilic (glycerol-)phospholipids. Lipids occur in fungi not only as major constituents of membrane systems but also as cell wall components, as storage material (reserve lipid) in abundant and readily observed lipid bodies and, in some cases, as extracellular products (Ratledge & Wilkinson, 1988).

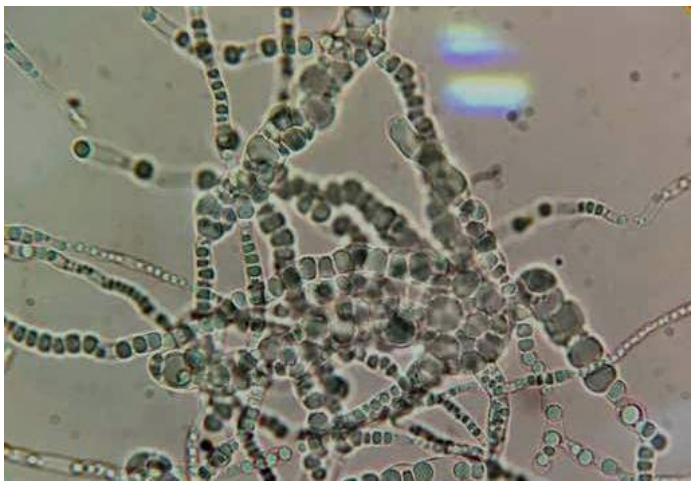


Figure 1.3.1. Lipid storage droplets of an oleaginous fungus, *Mortierella isabellina* (present study).

It is very important for the further development of the SCO processes to understand how microorganisms synthesize their fatty acids and how they are able to accumulate so much oil. In recent years studies on metabolism of lipid synthesis has created more interest, because the wide range of lipid accumulation from 20->70% in different microorganisms. As mentioned above, all living organisms must synthesize a minimum amount of lipid for their membranes and other structural and functional role. Biosynthetic pathway of lipids in most oleaginous microorganisms is same as in non-oleaginous microorganisms. But the presence of certain key enzymes plays an important role in the production of PUFAs,

where these enzymes are not found in non-oleaginous microorganisms (Ratledge, 1992a, 1992b). Several reviews have been published regarding accumulation of lipid from oleaginous microorganisms (Certik & Shimizu 1999; Moreton 1988; Ratledge, 1988, 2004). The mechanism of lipid overproduction in oleaginous microorganisms appears in those involving both the physiology and genetics (Ratledge, 1988).

Fundamental physiological requirement for lipid overproduction in these organisms is excess carbon and deficiency of nutrient, generally limited nitrogen in the growth medium. During these conditions, several physiological and metabolic changes were observed (Ratledge, 1988). When organisms grown in nitrogen limitation media, beyond 70% oil accumulation observed in oleaginous fungus whereas non-oleaginous fungus do not accumulate lipid. Instead those non-oleaginous microorganisms either tend to cease further proliferation or production lipid in their cell. The physiological condition which leads to lipid accumulation is that the organisms is grown in medium such that the supply of nitrogen is quickly exhausted but carbon supply stays in excess.

Various reasons have been proposed as to how oleaginous organisms may achieve this conversion of carbon substrate into lipid (Botham & Ratledge, 1989) such as:

- ✓ Glucose (or other substrate used) is taken up by the potential lipid accumulator regardless of the lack of supply of other important nutrients such as nitrogen source, to the cell. Thus an oleaginous organism may continue to assimilate glucose even though it can no longer generate new cells despite of nitrogen absence to synthesize proteins or nucleic acids. Glucose (substrate) is then converted into triacylglycerols (TAG) within the cell as discrete oil droplets (Ratledge, 2004; Meng *et al.,* 2009) whereas in other microbial cells, glucose may be converted into polysaccharides or metabolites like citric acid, etc. by the same supplement then, the organisms which did not accumulate any such materials would have the tendency to curtail glucose transport to the cell (Papanikolaou & Aggelis, 2010).
- ✓ The rate of lipid biosynthesis is much higher in these organisms than in non-oleaginous ones in which case, recognized key enzymes of fatty acid biosynthesis (Acetyl-CoA carboxylase and fatty acid synthase complex), would be much active in oleaginous microorganisms. This does not appear to be the case of non-oleaginous microorganisms.

1.3.1. **Fatty acid biosynthesis**

The synthesis of fatty acids in most fungi requires a sustainable production of acetyl- CoA and NADPH, as both are required for fatty acid elongation. Increased production of these two products can lead to a fungus becoming oleaginous (Ratledge, 2004). The formation of acetyl-CoA has been attributed to the presence of ATP: citrate lyase (ATP-CL), which is in enzymatic complex that is considered to be the most important factor to account for the oleaginicity of the various microorganisms, being absent in the non-oleaginous microbial cells (Ratledge, 2004). The reaction catalyzed through the use of ATP-CL is depicted as follows (reaction No.1)

$$Citrate + CoA + ATP \longrightarrow acetyl\text{ - }CoA + oxaloacetate + ADP + Pi \quad (1)$$

Citric acid must be made readily available in the cytosol of the cell where fatty acid sythesis occurs. Citric acid is synthesized as part of the tricarboxylic acid (TCA) cycle within the mitochondrion of the cell. The feature that is unique to the oleaginous microorganisms, and which allows citric acid accumulation, is that the activity of isocitrate dehydrogenase as a component of the TCA cycle is dependent on the presence of AMP; no such dependency occurs with the enzyme from non-oleaginous microorganisms (Ratledge, 2004). The concentration of AMP itself is regulated by the activity of AMP deaminase (reaction No. 2):

$$AMP \longrightarrow inosine5' \text{-}monophosphate + NH_3 \quad (2)$$

It is this enzyme whose activity is up-regulated at the onset of nitrogen limitation in the growth medium of the oleaginous microorganism possibly as a means of trying to scavenge additional ammonium ions from intracellular materials. The sequence of events leading to the formation of acetyl-CoA are described as follows (Ratledge, 2004):

- At the onset of nitrogen exhaustion, oleaginous cells show an increased activity of AMP deaminase, which is up to fivefold greater than in cells before N limitation.
- The increased activity of AMP deaminase decreases the cellular content of AMP, including its content in the mitochondrion.
- The reduction of AMP in the mitochondrion stops isocitrate dehydrogenase from working, as in oleaginous cells, this enzyme is strictly dependent on AMP for activity.
- As a result, isocitrate cannot be metabolized; it is, thus, accumulated and is then readily equilibrated with citric acid (via aconitase).
- Citrate therefore accumulates in the mitochondrion.
- An efficient citrate efflux system exists in the mitochondrial membrane for the export of citrate (in exchange for malate).
- Citrate enters the cytosol and is cleaved by ACL to give acetyl-CoA and oxaloacetate.
- The acetyl-CoA is used for fatty acid biosynthesis.
- The oxaloacetate is converted via malate dehydrogenase to malate, which is then used as the counterion in the citrate efflux system. The sequence of events is shown diagrammatically in Scheme 1.3.1. (Ratledge, 2004; Papanikolaou & Aggelis, 2011a).

The first reaction of fatty acid biosynthesis after acetyl- CoA generation, is catalyzed by a biotin-dependent acetyl- CoA carboxylase (Ratledge, 1988). Acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC) to yield malonyl-CoA in an irreversible reaction as follows (reaction No.3):

$$Acetyl\text{-}CoA + HCO_3 + ATP \longrightarrow Malonyl\text{-}CoA + Pi \quad (3)$$

This reaction is considered as the restricting step for fatty acid biosynthesis. Acetyl- and malonyl-CoA are then transferred to an acyl-carrier protein (ACP) to yield acetyland malonyl-ACP. Then, fatty acid synthetase (FAS) a multi-enzymatic complex adds sequentially acetyl units to malonyl- ACP, till a sixteen carbon atom chain is formed. This chain is either cleaved to palmitic acid or transferred to CoA to yield palmitoyl-CoA. 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. The reaction series can be summarized as follows (reaction No.4) (Papanikolaou & Aggelis, 2011):

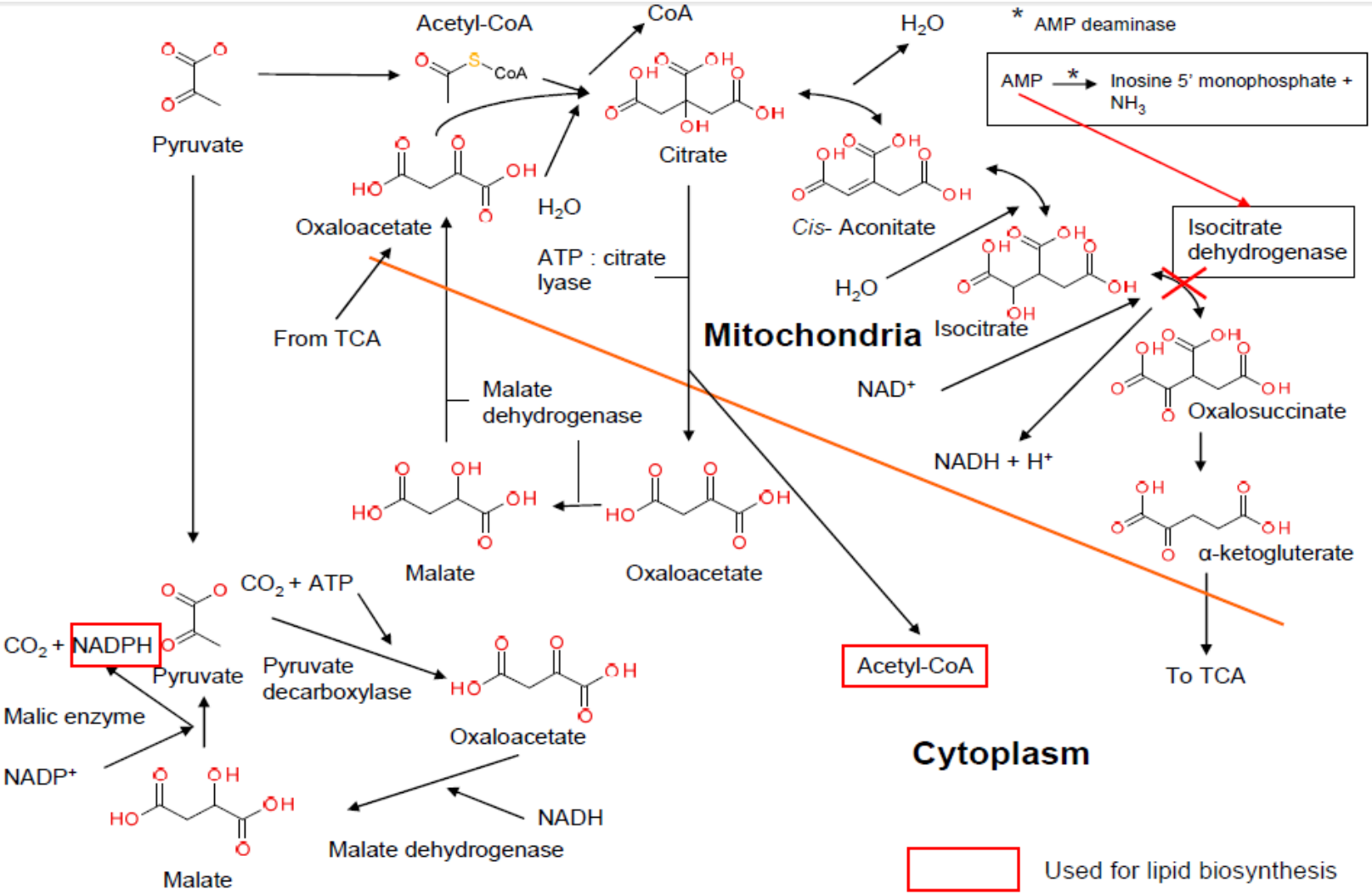
Acetyl-CoA + 7malonyl-CoA +14NADPH ⟶ Palmitoyl-CoA + 7CO2 +14NADP + 7 CoASH + 6H2O (4)

Palmitoyl-CoA is usually further elongated to stearoyl-CoA, which is the major product of fatty acid biosynthesis in most cells. Citric acid is an activator of ACC (Gill &Ratledge 1973), while long chain acyl-CoA esters inhibit ACC. These esters also inhibit FAS, but to a lesser degree than ACC.

It must be remembered that the fatty acids, are highly reduced materials and to achieve their synthesis as a ready supply of reductant as NADPH is essential. The synthesis of 1 mol of a C18 fatty acid requires 16 mols NADPH to be provided as 2 mols NADPH are needed to reduce each 3-keto-fatty acyl group arising after every condensation reaction of acetyl- CoA with malonyl-CoA as part of the standard fatty acid synthetase complex into the saturated fatty acyl chain, which then undergoes a further cycle of chain lengthening. (Meng *et al.,* 2009). The major supplier of NADPH for fatty acid biosynthesis is now considered to be malic enzyme (reaction No.5):

$$\textit{Malate} + NADP^{+} \longrightarrow \textit{pyruvate} + CO_2 + \textit{NADPH} \quad (5)$$

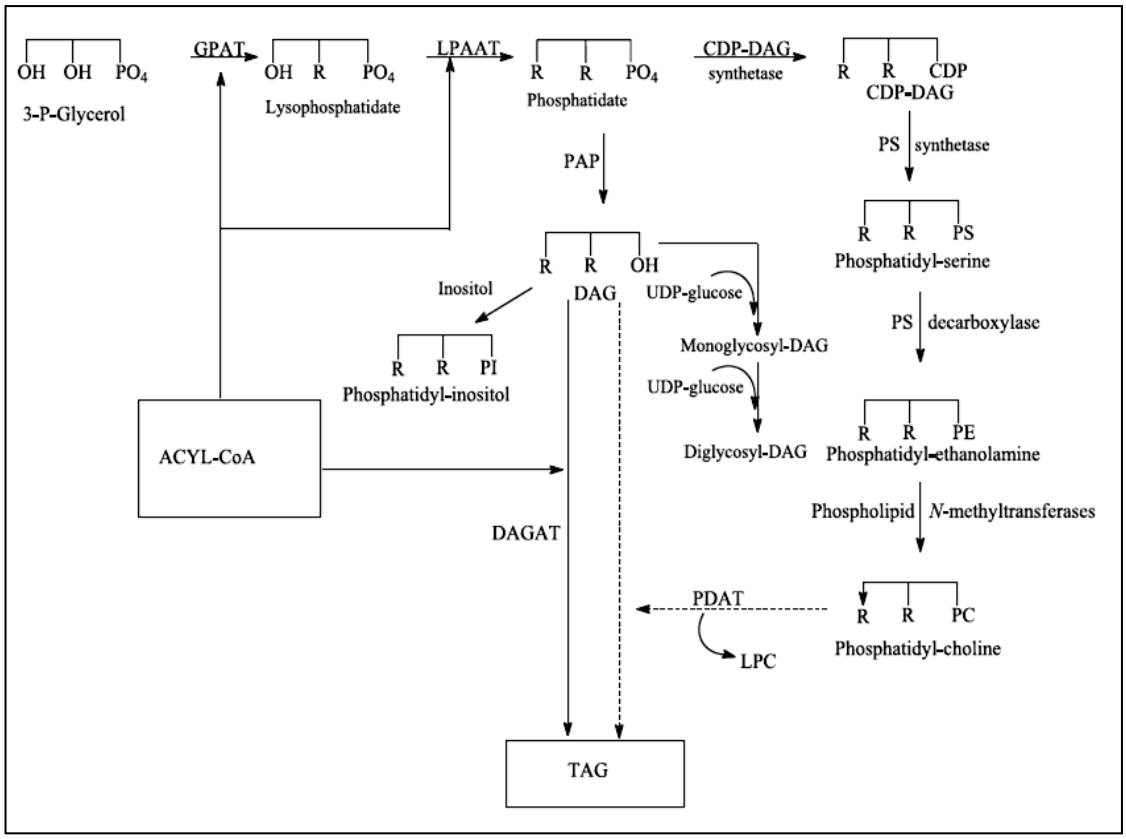
Malic enzyme activity has been found in most oleaginous microorganisms where it is proposed to form an integrated metabolon complex that combines with ACL and fatty acid synthase (FAS) to ensure a direct channelling of acetyl-CoA into fatty acids, which are finally esterified with glycerol into triacylglycerols and incorporated via the endoplasmic recticulum into fatty acid droplets (Ratledge, 2004).



Scheme 1.3.1. The pathways associated with oil accumulation in oleaginous fungi. *Enzymes*: pyruvate decarboxylase; malate dehydrogenase; malic enzyme; pyruvate dehydrogenase; citrate synthase; ATP:citrate lyase; *Net carbon balance*: pyruvate → acetyl-CoA + CO_2. The regulation of AMP deaminase by AMP and the presence of ATP : citrate lyase and malic enzyme are thought to be indicative of oleaginous organism leading to the accumulation of acetyl-CoA and NADPH, which is funnelled predominantly into the FAS complex.

1.3.2. Biosynthesis of acylglycerols

The biosynthesis of acylglycerols in microbial cells is shown in Scheme 1.3.2. (adapted by Fakas *et al*., 2009). After the biosynthesis of intra-cellular fatty-CoA esters, an esterification with glycerol takes place in order for the reserve lipids to be stocked in the form of TAGs. This synthesis in oleaginous microorganisms is conducted by virtue of the so-called pathway of α- glycerol phosphate acylation (Ratledge, 1988; Fakas *et al.*, 2009; Papanikolaou & Aggelis, 2011a). In this metabolic pathway, free fatty acids are activated by coenzyme A and are subsequently used for the acylation of the glycerol backbone to synthesize TAGs. In the first step of TAGs assembly, glycerol-3-phosphate (G-3-P) is acylated by G-3-P acyltransferase (GAT) at the sn-1 position to yield 1-acyl-G-3-P (lysophosphatidic acid- LPA), which is then further acylated by lysophosphatidic acid acyltransferase (also named 1-acyl-G-3-P) acyltransferase – AGAT) in the sn-2 position to yield phosphatidic acid (PA). This is followed by dephosphorylation of PA by phosphatidic acid phosphohydrolase (PAP) to release diacylglycerol (DAG). In the final step DAG is acylated either by diacylglycerol acyltransferase of phosphatidic diacylglycerol acyltransferase to produce TAGs (Ratledge, 1988; Fakas *et al.*, 2009). As far as the structure of the microbial TAGs produced is concerned, although their final position could theoretically be a random substitution of acyl-CoA groups into glycerol in the case of oleaginous microorganisms that have been examined, the glucerol sn-2 position is almost occupied by unsaturated fatty acids (Ratledge, 1988; Papanikolaou & Aggelis, 2011a).



Scheme 1.3.2. Major routes for lipid biosynthesis in microorganisms. DAG: Diacylglycerol; TAG: Triacylglycerol; CDP-DAG: Cytidine diphosphate-diacylglycerol. *Enzymes:* DAGAT: DAG acyltransferase; GPAT: 3-P-glycerol acyltransferase, LPAAT: lysophosphatidate acyltransferase, PAP: phosphatidate phosphatase, PDAT: acyltransferase, PI synthetase: phosphatidylinositol synthetase; PS synthetase: phosphatidyl-serine synthetase; PS decarboxylase: phosphatidyl-serine decarboxylase.

Phosphatidate is the precursor of the major lipid fractions, namely neutral lipids, exemplified by triacylglycerol (TAG) and diacylglycerol (DAG), glycolipids (mono- and di-glycosyl-DAG), and phospholipids (phosphatidylethanolamine (PE), phosphatidyl-choline (PC), etc. Glycolipids, sphingolipids, and phospholipids comprise the polar lipids. Phosphatidate is produced by the sequential acylation of 3-P-glycerol by acyltransferases, which transport the fatty esters of CoA. Phosphatidate may react with cytidine triphosphate (CTP) to yield cytidine triphosphate-diacylglycerol (CDP-DAG), which is the precursor of phospholipids. Phospholipids are the major structural lipids in eukaryotes, and thus these lipids reside in cell membranes. Usually, phospholipids are the minor lipid components. In fungi, the major phospholipid classes are PC and PE, while phosphatidyl-inositol (PI) and phosphatidyl-serine (PS) are found in lesser amounts (Fakas *et al.*, 2006).

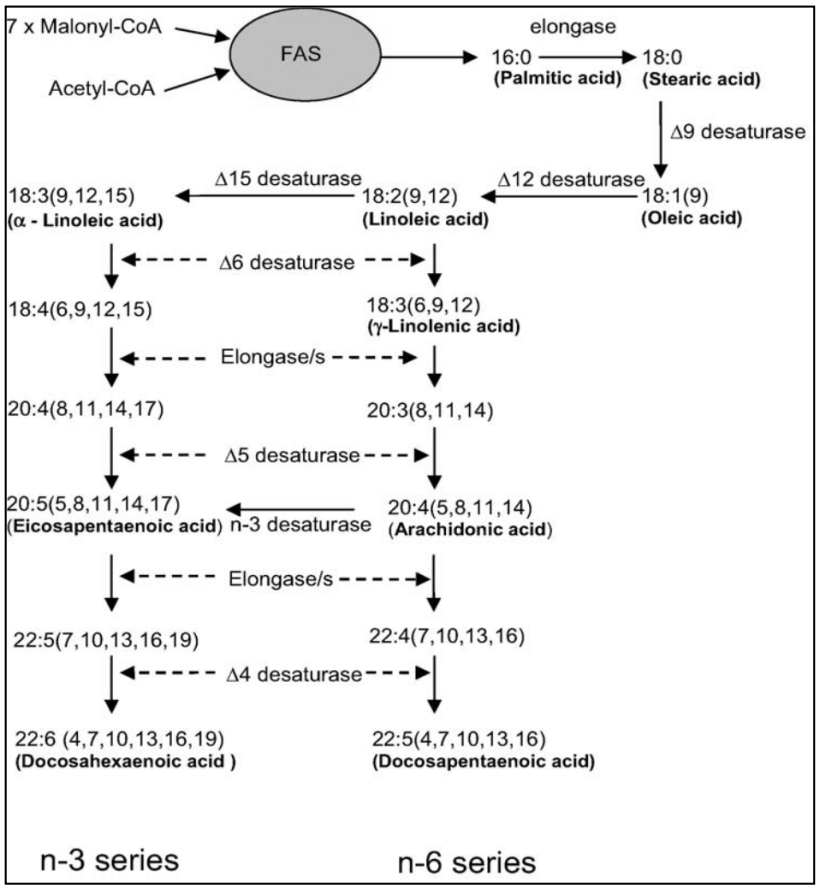
Alternatively, phosphatidate may be hydrolyzed by a phosphatase to produce diacylglycerol (DAG), which may then be acylated to triacylglycerol (TAG). In oleaginous micro-organisms, TAG is the major neutral lipid, while DAG, monoacylglycerol (MAG), and occasionally free fatty acids (FFA), are minor components (Certik and Shimizu 2003; Papanikolaou & Aggelis, 2011a). In fact, the presence of high quantities of DAG, MAG, and FFAS is indicative of lipid deterioration, which may come from the unwanted lipase action during extraction and analysis. TAG is the major storage lipid in fungal cells and is found in lipid bodies. It should be mentioned that fatty acids of TAG are not randomly distributed on the glycerol backbone; instead, they are stereo-specifically distributed: saturated fatty acids occupy the sn-1 position, while their unsaturated counterparts occupy the central (sn-2) position (De Bell & Jack 1975).

Glycolipids comprise the major polar lipids in many micro-organisms. Glycolipids are produced by glycosylation of DAG, usually with galactose, but glucose and mannose may also be used. Whatever the sugar used, it needs to be activated by UTP, thus it is the UDP-sugar derivative that reacts with DAG to yield mono-glycosylglycerol. DAG may be sequentially glycosylated by the same or a different sugar to produce di- and tri-glycosylated derivatives; glycolipids containing four or more sugars are rare among microorganisms. The physiological role of glycolipids is obscure, but it seems that they are implicated in PUFAs biosynthesis (playing a role analogous to phospholipids), while some researchers assume that they function are storage lipids. The reason for this obscurity is that glycolipids have been overlooked in most studies in microbial lipids, possibly because of the inherent difficulties in their analysis.

1.3.3. Biosynthesis of polyunsaturated fatty acids (PUFAs)

The biosynthesis of polyunsaturated fatty acids in microbial cells is shown in Scheme 1.3.3. (adapted by Ratledge, 2004). PUFAs are synthesized from palmitic (C16:0) and stearic acids (C18:0), which are transformed to palmitoleic ($^{\Delta 9}$C16:1) and oleic ($^{\Delta 9}$ C18:1) acid, respectively, by the introduction of a double bond between the eighth and ninth carbon atoms (Ratledge & Wynn 2002). Further desaturation requires the esterification of oleic acid with a phospholipid, usually PC (Certik & Shimizu 1999). The phospholipid-bound oleic acid is then transformed to linoleic acid ($^{\Delta,9,12}$C18:2) and thence to linolenic acid ($^{\Delta 6,9,12}$C18:3),

which in most microorganisms occurs as the a-isomer ($^{\Delta 9,12,15}$C18:3). However, some micro-organisms, generally members of the Zygomycetes, synthesize GLA ($^{\Delta 6,9,12}$C18:3), which is the gamma isomer of linolenic acid. Whatever the isomer synthesized, chain elongation follows and then further desaturation to yield a 20-carbon PUFA; a similar reaction sequence is elaborated to produce the very long chain PUFAs (Certik and Shimizu 1999). After their synthesis, PUFAs are cleaved from phospholipids by a phospholipase and channelled to an acyl-CoA pool found in cytosol (Ratledge and Wynn 2002). This pool then supplies PUFAs to various biosynthetic reactions. Indeed, PUFAs may be used to acylate DAG to produce PUFA-rich TAG. PUFA-rich TAG, however, may also be produced via re-acylation reactions, which involve acyl exchange between a PUFA-containing phospholipid and TAG.



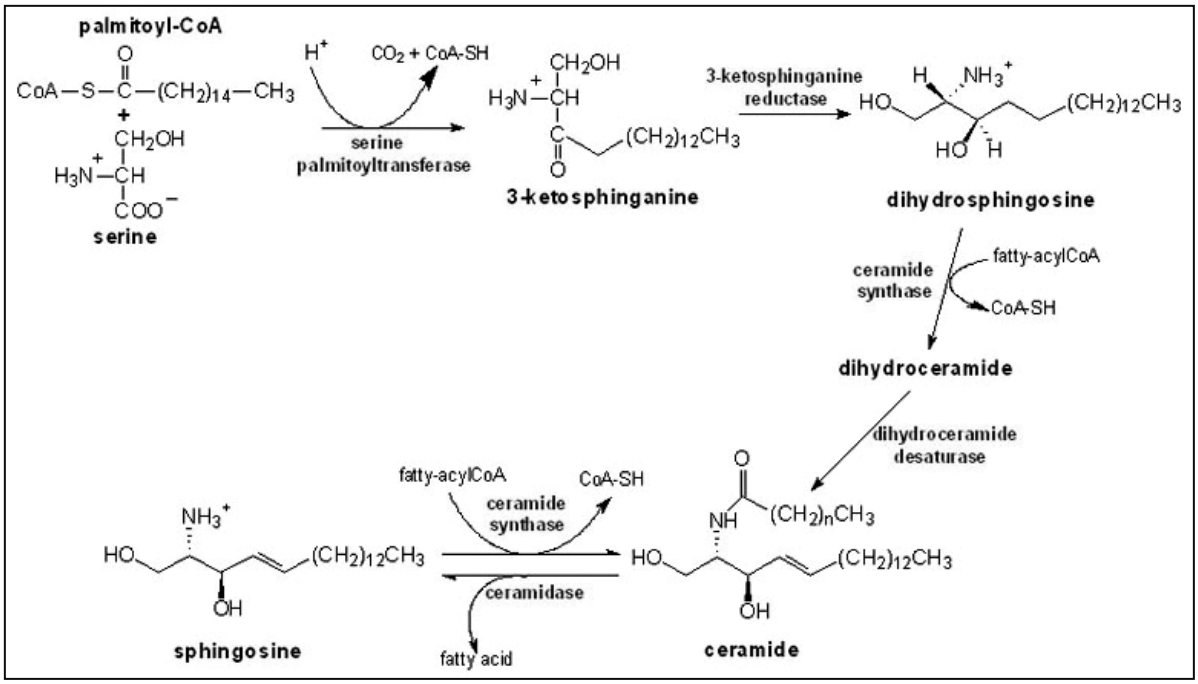
Scheme 1.3.3. Pathways for the formation of PUFA in microorganisms using the "conventional" FAS route. Fatty acids are synthesised from acetyl-CoA and malonyl-CoA using the FAS complex of enzymes. The saturated fatty acid, stearic acid is then successively desaturated and elongated through a series of reactions leading to the formation of various PUFAs. PUFAs fall into two categories, the n-3 and n-6 series, depending on the position of the final double bond nearest the terminal methyl group (Ratledge, 2004)

1.3.4. Biosynthesis of sterols

Fungal sterols are structural lipids that reside in cell membranes. Squalene is the biosynthetic precursor of sterols, which, in fungal cells, is transformed to lanosterol. In most fungi, lanosterol is transformed to ergosterol via a sequence of reactions which may differ among various fungi. Sterols are stored in fungal cells as their fatty acid esters (steryl esters). Sterol composition of fungal membranes affects their fluidity and permeability, as well as the function of membrane proteins.

1.3.5. Biosynthesis of sphingolipids

Sphingolipids represent another class of lipid that, like the phospholipids, are composed of a polar head and two non-polar tails and are frequently found in biological membranes. An 18-carbon amino alcohol, sphingosine, forms the backbone of these lipids rather than glycerol. Sphingolipids typically found in very small quantities in microbial lipids. Their biosynthesis starts with a condensation reaction between palmitoyl-CoA and serine yielding 3-keto-dihydrosphingosine, which is then transformed to phytosphingosine. Phytosphingosine then reacts with a fatty acid, usually having 26 carbon atoms, to produce ceramides. Fatty acid composition of sphingolipids is distinct, as they may contain hydroxylated, long chain (C30-C40) fatty acids. Little is known about the physiological role of sphingolipids, though it seems that they are involved in various cellular processes, such as resistance to various shocks (temperature, pH) (Daum *et al.*, 1998).

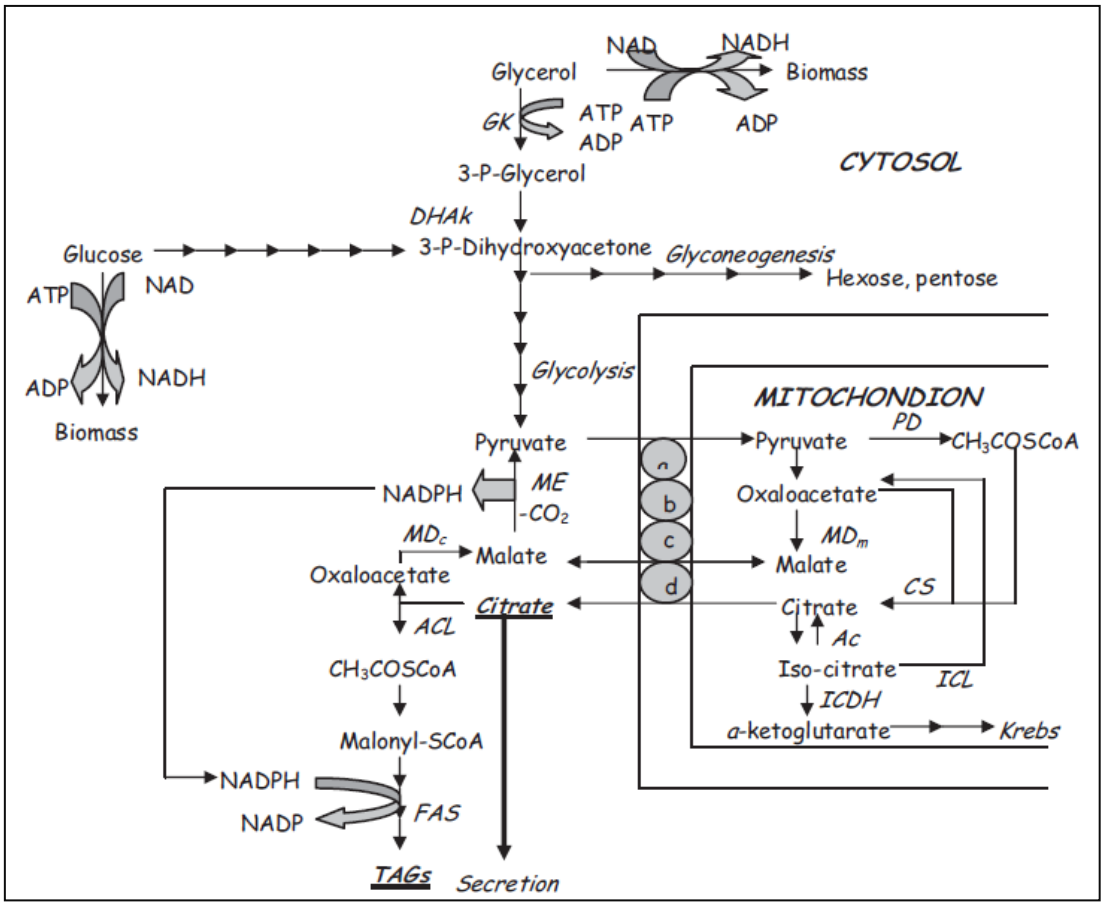


Scheme 1.3.5. *De novo* biosynthesis of sphingolipids (adapted by http://themedicalbiochemistrypage.org/sphingolipids.php)

1.4. Lipid accumulation from fermentation of sugars and related substrates

De novo accumulation of cellular lipids is an anabolic biochemical process in which, by virtue of quasi-inverted *β*-oxidation reaction series, acetyl-CoA issued by the intermediate cellular metabolism, generates the synthesis of intra-cellular fatty acids. Fatty acids will be then esterified in order to synthesize structural (phospholipids, sphingolipids, etc) and reserve lipids (TAGs & SEs) see section of biosynthesis for more details) (Ratledge & Wynn, 2002; Papanikolaou & Aggelis, 2009, 2011). In oleaginous microorganisms in which de novo lipid accumulation is consulted , acetyl-CoA that constitute the precursor of intra-cellular fatty acids, derives from breakdown of citric acid under some circumstances cannot be catabolized through the reactions performed in the Krebs cycle, but it is accumulate inside the mitochondria.

This occurs when its concentration becomes higher than a critical value resulting in citric acid transportation into the cytosol (Ratledge & Wynn, 2002; Wynn & Ratledge, 2006; Papanikolaou & Aggelis, 2011).



Scheme 1.4.1. Pathways involved in the breakdown of glucose by microbial strains capable of producing SCO and / or citric acid in nitrogen – limited conditions. FFA: free fatty acids; TRSP: citric acid transporting system; a,b,c: system transporting pyruvic acid from cytosol to mitochondrion and inversely; d: system transporting citic and malic acid from cytosol to mitochondrion and inversely; *Enzymes:* ACL:ATP-citrate lyase; FAS: fatty acid Synthetase; ICDH: iso-citrate dehydrogenase; MC_c: malate dehydrogenase (cytoplasmic; MD_m: malate dehydrogenase (mitochondrial); ME: NADPH+-malic enzyme; PD: pyruvate dehydrogenase; CS; citate Synthetase; ICL: iso-citrate lyase; EMP: Embden-Mayerhoff-Parnas pathway (adapted by Papanikolaou & Aggelis, 2009).

The Key step for citric acid accumulation inside the mitochondrion matrix is the change of intracellular concentration of various metabolites, conducted after exhaustion of some nutrients (mainly nitrogen) in the culture medium (Ratledge & Wynn, 2002; Wynn & Ratledge, 2006). This exhaustion provokes a rapid decrease of the concentration of intra-cellular AMP, since by virtue of AMP- desaminase, the microorganism cleaves AMP into IMP and NH_4^+ ions in order to utilized nitrogen, in the form of NH_4^+ ions, as a complementary nitrogen source, necessary for synthesis of cell material (Evans & Ratledge, 1985).

The excessive decrease of intra-cellular AMP concentration alters the Krebs cycle function: the activity of both NAD^+ & $NADP^+$- isocitrate dehydrogenases enzymes responsible for the transformation of iso-citric to α-ketoglutaric acid, lose their activity since they are allosterically activated by intra-cellular AMP, and this events results in the accumulation of citric acid inside the mitochondrion (Evans & Ratledge, 1985). When the concentration of citric becomes higher than a critical value, it is secreted into the cytosol.

Finally in the case of lipogenous (lipid accumulating) microorganisms, cytosolic citric acid is cleaved by ATP- citrate lyase (ACL), the key enzyme of lipid accumulation process in the oil-bearing microorganisms, in acetyl-CoA and oxaloacetate, with Acetyl- CoA being converted, by an inversion of *β*-oxidation process, to cellular fatty acids. In contrast, non –oleaginous microorganisms (e.g. *Aspergillus niger* strains) secrete the accumulated citric acid into the culture medium instead of accumulating significant quantities of reserve lipid or other compounds like polysaccharides (Luckner, 1990). Schematically, the inter mediate cellular metabolism resulting in the synthesis of either citric acid or storage lipid is presented in Scheme 1.4.1. (adapted by Papanikolaou & Aggelis, 2009). After the biosynthesis of intra-cellular fatty-CoA esters, an esterification with glycerol takes place in order for the reserve lipids to be stocked in the form of TAGs.(*see :Biosynthesis of acylglycerols)*

1.5.Lipid analysis

The greater cell-size and complexity of fungi, which distinguish them from prokaryotes, is accompanied by a corresponding diversity of lipid components. The analysis of lipids can be undertaken to answer at least four questions at both the qualitative and quantitative level:

a. what fatty acids are present in oil
b. what different classes are present
c. what is the fatty acid composition of each separate lipid class, and
d. since lipids generally contain two or three acyl chains, how are these associated in individual lipid molecule.

Lipids are hydrophobic or amphipathic small molecules. A subdivision into two broad classes, as simple and complex lipids, is convenient for chromatography purposes. *Simple lipids* are defined as those that on hydrolysis yield at most two types of primary product per mole; *complex lipids* yield three or more primary hydrolysis products per mole. The *complex lipids* are best considered in terms of either the Glycerophospholipids (or simply if less accurately as phospholipids), which contain a polar phosphorus moiety and a glycerol backbone, or the glycolipids and sphingolipids, which contain a polar carbohydrate moiety, as these are more easily analyzed separately. Alternatively, the terms "neutral" and "polar" lipids are used to define these groups, but are less exact (Christie, 2003).

1.5.1. Fungal Lipids

The amounts and types of lipid at individual fungus vary not only from one organism to another but also with age, stages of development, nutrition and environmental conditions (Weete & Weber, 1980). The lipid content of fungal species can be manipulated by varying culture conditions, therefore, the records of total lipid content are of limited value unless the parameters of growth are specified. More information is available on lipid composition than on any other aspect of fungal lipids, largely because of the ease of preparation and analysis of total lipid samples. The lipid fractions from a variety of moulds, showed wide range of values for the contents of both polar and neutral lipids.

1.5.1.1.Acylglycerols

Under suitable conditions, acylglycerols are the major storage lipids of many filamentous fungi. The portion of these compounds varies greatly during fungal life-cycles and from species to species at corresponding stages of development, as well as being affected by environmental factors such as temperature and nutritients. Times of synthesis of triacylglycerols often correspond with the formation of reproductive structures. Triacylglycerols (TAGs) are the major lipids as in the mycelium of the most fungi (80-90% of the reserve lipid), which are generally considered as storage lipids that may be used for energy and carbon skeletons during growth and development (Ratledge & Wilkinson, 1988). These are composed of three fatty acids attached to a three carbon properties vary enormously, from hard waxy solids at room temperature (fats) to translucent liquids (oils) (Christie, 2003).

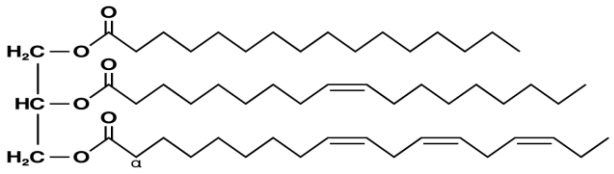


Figure 1.5.1. Unsaturated Triacylglycerol (TAG). Left part : glycerol, right part from top to the bottom: palmitic acid, oleic acid and α-linolenic acid

1.5.1.2. Glycerophospholipids

Phospholipids are important structural components of biological membranes. They have been implicated in the active transport of ions across membranes and are also essential for the activity of some membrane-bound enzymes (Sancholle *et al.*, 2003; Cohen, 2011)

1.5.1.2.1. The major phospholipids

Phospholipids are a class of lipids that consist of two fatty acyl molecules esterified at the *sn*-1 and *sn*-2 positions of glycerol, and contain a head group linked by a phosphate residue at the *sn*-3 position. There is a diversity of phospholipid composition in microorganisms. The relative abundance of phospholipids as a fraction of total lipids for several microorganisms is illustrated in the table 1.5.1. These data adapted by Cerv, (1993) and were obtained under varying experimental conditions, so at least a part of the reason for the different levels is probably due to that fact. Among Various fungi, these are a considerable variability in the components of the phospholipid fraction, illustrated in the table 1.5.1. Usually, in most fungi, phosphatidyl-choline (PC), closely followed by phosphatidyl-ethanolamine (PE), are the major components, with smaller amounts of phosphatidyl-inositol (PI), although in some cases PE may be the most abundant (Cerv, 1993; Ratledge & Wilkinson, 1988).

Table 1.5.1. Phospholipid Content of several Oleaginous Microorganisms

Microorganism	Phospholipid content (% w/w of major lipids)
Bacteria	
Arthrobacter AK19	6.7
Yeasts	
Cryptococcus terricolus	1.8
Endomycopsis vernalis	4
Lipomyces lipofer	14
Lipomyces starkey	15
Phodotorula glutinis	2
Fungus	
Cladosporium herbarum	7.4
Fusarium oxysporum	28
Mortierella isabellina	**7**
Penicillium lilacinum	6.4
Pythium irregular	17.8
Phizopus oryzae	11.8

**Adapted by Cerv, 1993*

Phosphatidylserine (PS) accounts for between 5- 16% of total phospholipid in most classes of fungi as it is showed in the table 1.5.2. Diphosphadidylglycerol (DPG) usually accounting for 2-5% of lipid phosphorus, is a fairly constant fungal component. Phosphatidylglycerol (PG), the characteristic phospholipid class of chloroplasts, appeared to be between 3- 13% in oleaginous fungi as it is showed in the table 1.5.2. In view of its key position in lipid biosynthesis, the lack of records of phosphatidic acid (PA) in published data on fungal phospholipids (Ratledge & Wilkinson, 1988) seems more likely to indicate different metabolic states at the time of harvesting, rapid turnover, or merely the procedures of separation and detection than the actual absence of PA.

Table 1.5.2. Phospholipid Composition of Several Fungi

	Relative % phospholipid components					
Fungus	PC	PE	PS	PI	CL	PG
Mortierella isabellina	42	17	1	11	-	-
Penicillium lilacimun	21	22	5	2	5	6
Cladosporium herbarum	50	16	1	-	18	13
Pellicularia practicola	52	20	5	3	2	3
Fusarium oxysporum	25	18	16	20	2	5

**Adapted by Cerv, 1993*

From the limited reports of the occurrence of lysophosphatidylcholine (lysoPC) and lysoPE, it is impossible to determine whether they occur significantly in normal fungal metabolism or reflect phospholipase activity during lipid extraction. As in other eukaryotes, fungal phospholipids are normally the major components of membrane and other cell organelles. There have been various claims of storage phospholipids in such structures. That the phospholipid of fungal cell may not all be located in membranes had already been suggested first by Illingworth *et al.*, (1973), who observed that ascosporogenesis in *Saccharomyces cerevisiae* was accompanied by an increase in phospholipid content, 171% greater that could be accounted for by membrane development.

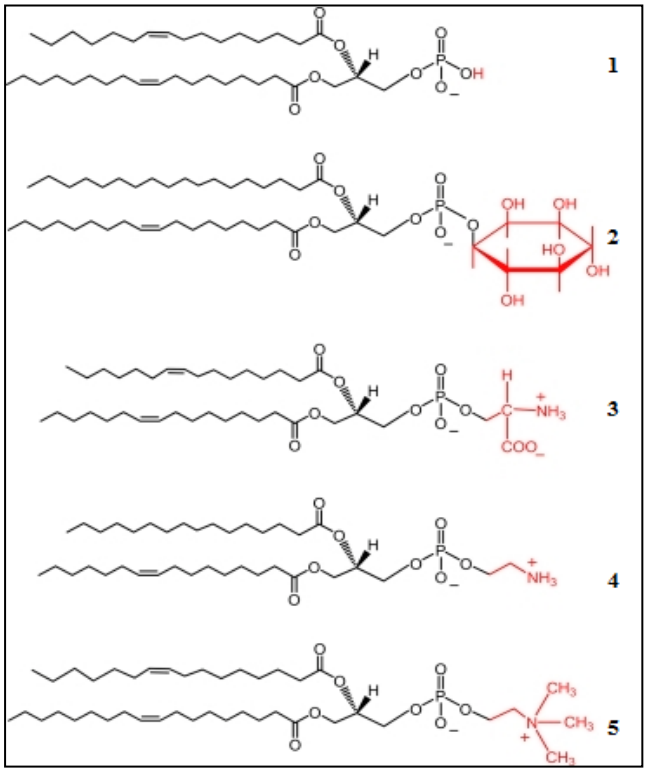


Figure 1.5.2. Structures of major glycerophospholipids. Head groups legend, 1: PA (Phosphatidic acid), 2: PI (Phosphatidyl- inositol), 3: PS (Phosphatidyl-serine), 4: PE (Phosphatidyl-ethanolamine), 5: PC (Phosphatidyl-choline) (adapted by Herry *et al.*, 2012).

1.5.1.3. Glycolipids and sphingolipids

1.5.1.3.1. Nitrogen- free glycolipids

The nitrogen- free glycolipids of filamentous fungi comprise *hydroxyl-acid glycosides*, *acylated sugar alcohols and sugars alcohols*, *glycosyldiacylglycerols* and *polyphenol glycosides*. The hydroxyl-acid glycosides are compounds in which long- chain hydroxyl fatty acids are linked to a carbohydrate moiety and have been identified especially in yeast rather than in filamentous fungi. Acylated sugar alcohols and sugars alcohols have been found in a number of filamentous fungi according to Ratledge & Wilkinson, (1988). Among these are acylglucoses, acylmanitol and acyltrehalose.

Regarding to glycosyldiacylglycerols, glycolipids of this type are common in bacteria and green plants and particularly important in chloroplast, but these have been recognized in a few fungi so far. Diglucosyldiacylglycerol was reported from *Aspergillus niger* (Hackett & Brennan, 1976). Digalactosyldiacylglycerol (DGDG) accompanied by monogalactosyldiacylglycerol (MGDG) was also reported from *A. niger*. Polyphenol glycosides are even less well known than other nitrogen –free glycolipids. Although present in extremely small amounts, they are of particular interest on account of their possible involvement in sugar transport across membranes and in glycoprotein synthesis as demonstrated in bacteria (Brennan & Losel, 1978).

1.5.1.3.2. Sphingolipids and glycolsphingolipids

The small number of fungi which have been sufficiently investigated have yielded in extensive series of ceramides, in which long chain alcohol (C_{14} to C_{22}) sphingosine type bases are esterified with 2-hydroxy fatty acids or normal amide-linked fatty acids as it is showed in the figure 1.5.3., and glycosylceramides or glycophosphosphingolipids, in which sugars and oligosaccharides (typically β-linked galactose (GalCer) or glucose (GlcCer))are attached to ceramides via phosphoinositol bridge. Because of the small amounts generally present (0.2%-0.7% of mycelia dry weight) and the risks of alteration during extraction, the complex sphingolipids are probably frequently overlooked, modified or lost during extraction procedures (Ratledge & Wilkinson, 1988). Detailed analyses of based composition and fatty acids ceramides and cerebrosides revealed a complex series of compounds, in spite of indication of differential destruction of bases during hydrolysis of the sphingolipids. The general structure suggested was 2-amino-1,3,4-trihydroxy-n-alkane or isoalkane. Variations occur in the type of base gives rise to a very large range of homologous ceramides and glycophopshosphingolipids, often referred to as mycoglycolipids in fungi. The fatty acids of fungal ceramides and cerebrosides are commonly 2- hydroxyl fatty acids of chain lengths up to $C_{24:0}$, as well as normal fatty acids of composition similar to those of the acylglycerols fractions. Sphingolipids are assumed to be the fungal membrane components.

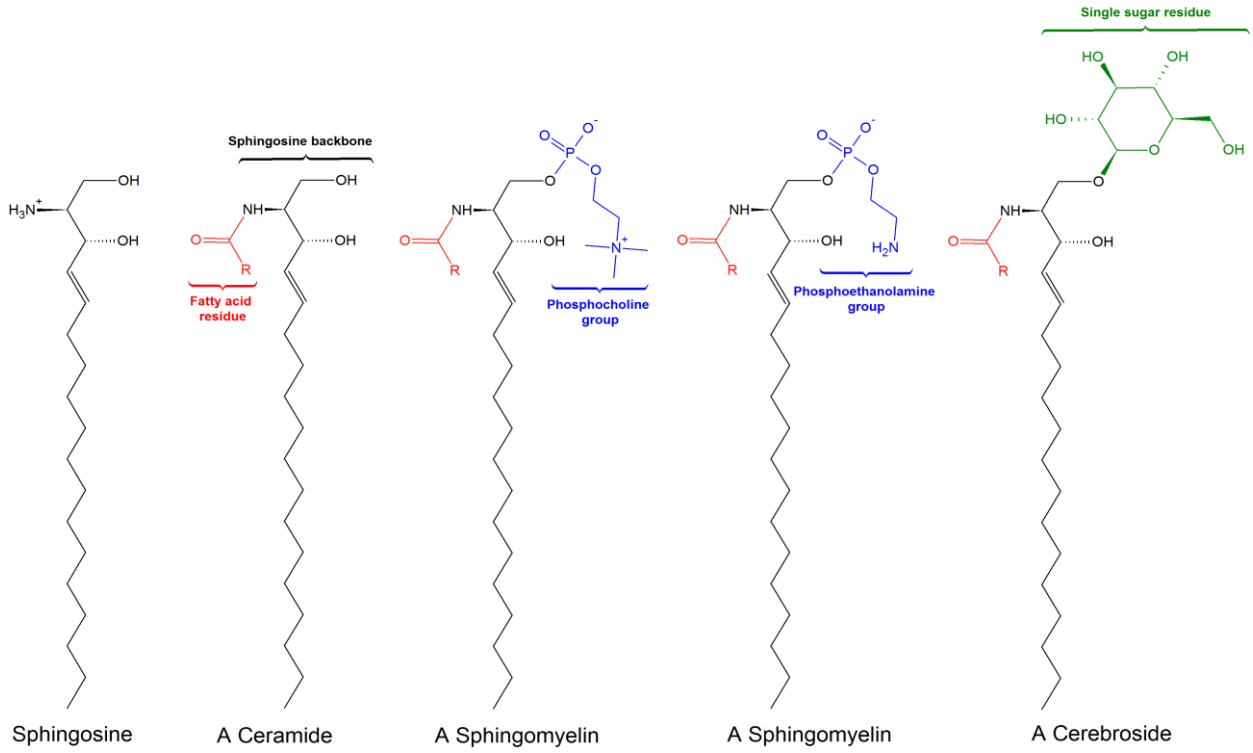


Figure *1.5.3.* General Structure sphingolipids. *Ceramide* :The common lipid component of glycosphingolipids, composed of a long-chain amino alcohol (sphingosine) and an amide-linked fatty acid. *Sphingosine*: Long-chain amino alcohol (adapted by https://en.wikipedia.org/wiki/Sphingolipid).

1.5.1.4. Sterols

Given the required aerobic conditions, most fungi can synthesize sterols. Sterols occur in fungal cell as free sterols, esters and sterol glycosides or in other bound forms. As in other eukaryotes, such evidence as is available indicates that the free sterols in fungi are largely membrane components which may stabilize

other membrane lipids, thus controlling permeability by affecting internal viscosity and molecular motion of lipids in the membrane. They may also serve as precursors of steroid hormones involved in the sexual reproduction of some fungi (Ratledge & Wilkinson, 1988). The Zygomycetes differ from other classes of 'lower' fungi in producing ergosterol generally as a major sterol accompanied by 22-dihydroergosterol and smaller amounts of cholesterol. Any generalization is, however, premature in view of the widely varying sterol composition encountered in fungi of differing taxonomic affinity, which is likely to be accompanied by major differences in their biosynthetic pathways.

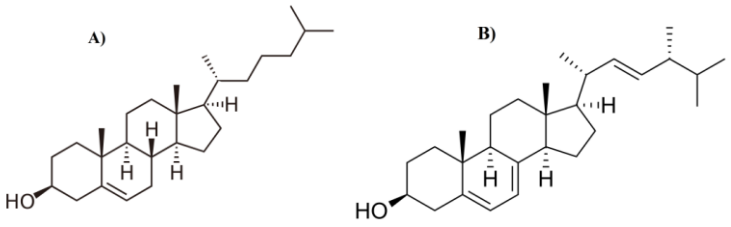


Figure 1.5.4. General Structure of sterols. A): Cholesterol, B): Ergosterol

1.6. Thin Layer Chromatography

Chromatography is the method of separating a mixture into its various components. It makes use of heterogeneous equilibrium established during the flow of a solvent called mobile phase through a fixed (stationary) phase to separate two or more components from material carried by the solvent. The most common form of chromatography is absorption chromatography (Tswett, 1903; Kuhn & Lederer, 1931). Here the stationary phase is a solid such as alumina or silica gel. The sample substance to be separated is applied at one end of this stationary phase, and the mobile phase is allowed to flow through. A polar compound (one with high chromatographic polarity) is one that is held by the stationary phase, whereas a non polar substance tends to move forward in the mobile phase. In TLC capillary action in the finely divided stationary phase causes the mobile phase to move. Separation occurs when one substance in a mixture is more strongly adsorbed by the stationary phase than the other components in the mixture. Since the adsorption is essentially a surface phenomenon, the degree of separation is dependent on the surface area of the adsorbent available; hence the emphasis on a small particle size of the adsorbent. Thin layer Chromatography (TLC) is one of the most frequently described separation techniques in qualitative as well as quantitative analysis. Historically, the work of Stahl, *Thin Layer Chromatography, (*1965) and Kirchner's, *Thin Layer Chromatography, (*1978) among others gave the real motivation to development in this field. Thin layer chromatography can be used not only to simply check the purity of a substance but also to attempt to separate and identify the components in a mixture, or to obtain a quantitative analysis of one or more of the components present. The reasons for the wide use of TLC technique are many, including ease of use, wide applications to a greater number of different samples, high sensitivity, speed of separation and relatively low cost (Touchstone, 1992). The increasing commercial availability of pre-coated TLC plates has significantly improved the achievable reproducibility of separation that was quite limited in the past when

home-made TLC plates were primarily used. Additionally, the availability of many different absorbent materials including high-performing silica, bonded phases and impregnated layers have increased the versatility of HPTLC for numerous and quick separations particularly in the lipid field. Thus, methods of thin-layer chromatography (TLC) and its refined version high-performance thin-layer chromatography (HPTLC) are even nowadays indispensable tools of modern analytical chemistry (Peterson & Cummings, 2006). Although there were (and still are) several potential concerns against the wider application of TLC and HPTLC (e.g. the lower chromatographic resolution in comparison to HPLC and the potential oxidation of the analyte caused by exposition to atmospheric oxygen), there are many advantages that make TLC clearly competitive to liquid chromatography (Fushs *et al*., 2009).

1.6.1. General procedure

Thin Layer chromatography (TLC) has long been used for the separation of lipid mixtures into their components lipid classes (complex lipid mixtures as well as for individual lipids) (Marinetti, 1967; Kurt, 1968; Touchstone, 1992; Christie & Han, 2010). Although lipid analysis by TLC may be carried out by various techniques, one underling procedure is common to all. Basically, a lipid mixture is applied to an absorbent which is coated in a thin layer on a support (stable layer of suitable size). Silica gel is the most commonly used adsorbent for the TLC analysis of lipids. The point of sample application is termed "origin". Samples are applied as discrete spots or as narrow streaks (by hand or with an instrument for automatic sample application), 1.5 to 2 cm from the bottom of the plate/ "origin", in a solvent (frequently Chloroform or Chloroform methanol 1:1 v/v) by means of a micropipette or microsyringe (Christie & Han, 2010). The plate is placed in the mobile phase in a glass developing tank usually lined with filter paper on three sides and the paper is wetted with the developing solvent to saturate the atmosphere. The tank should be prepared 1 hour before immersion of the chromatoplate .The developing solvent moves up the plate by capillary action taking the various compounds present in the lipid extract (or in any other mixture) up to different distances from the origin. The distances depend upon the chemical structure of the compounds, the nature of adsorbent, and the composition of the developing solvent. In a given solvent system each lipid component has a characteristic mobility which can be described as its R_f value. The R_f value is defined as the distance travelled by the component divided by the distance travelled by the solvent front, both distances being measured from the origin. In this way different classes of lipids arise to different levels and form "spots" or "bands". The amount of developing solvent (only pure solvents) should be about 0.75-1.0 cm deep (Skipski, *et al.,* 1967). When the solvent nears the top of the plate, the plate is removed from the tank, dried in air (in a Foam hood) or in a stream of nitrogen (depending on whether it is intended to recover the lipids for further analysis) to remove the solvent. The plates can then be stored in a desiccator for several days, but before use should be prewashed with a mixture of solvents. For neutral lipids the solvents that can be used is chloroform-methanol (4:1 or 2:1, v/v) and for polar lipids acetone /petroleum ether (1:3v/v). The prewashing is necessary to remove the organic material present in the silica gel to the uppermost edge of the plates otherwise, impurities of silica gel would contaminate the areas of hydrocarbons and cholesterol esters.

Usually the solvent is allowed to run overnight or longer. The plates are activated at temperatures above 100°C for 30-60 min just before application of the lipid samples (Hamilton & Hamilton, 1992). Because lipids are generally colorless, the separated lipid components have to be rendered visible by chemical reagents. Reference or standard compounds are applied to the same chromatoplate with the mixture of unknown compounds. Each compound present in the lipid mixture is carried the same distance as the respective reference compound permitting identification of the compounds present in the lipid mixture (lipid extract).

1.6.2. The Stationary phase

Nowadays, there are many different stationary phases commercially available that are potentially useful in the lipid field. The most common support is a glass plate of 20x20 cm or 20x 10cm .It is resistant to the acids and alkalis used in developing solvents or detection sprays and its rigidity makes it suitable for densitometry. Aluminum foil and plastic sheets are also widely used to support the adsorbent and have the advantage that they can be cut easily to make small plates if required. These non-glass supports are available only as pre –coated plates. Among the different adsorbents that have been used for the analysis of lipids [alumina (Aluminium oxide), Kieselguhr (diatomaceous earth based on silicaceous material), cellulose etc.], silica gel 60 (the 60 denotes the pore size) is by far the most commonly used adsorbent (Marinetti, 1967; Kurt, 1968; Touchstone, 1992; Fush *et al*., 2010; Christie & Han, 2010). At a given humidity the amount of water adsorbed by the silica gel increases as pore size decreases. The water content of silica gel determines the polarity of the absorbent and hence its activity and chromatographic properties. For good separations by silica gel the water content of the gel must be carefully controlled. The silica gel on TLC plates is normally activated by heating plates before use at temperatures above 100^{o}C for 30-60 minutes or as specified to remove water (Hamilton & Hamilton, 1992). Silica gel can be modified with various compounds to attain the separation of lipid components not resolved well by chromatography on standard silica gel (Fush *et al*., 2010; Christie & Han, 2010). Among the different modifications of the stationary phase, silver nitrate and boric acid impregnations are most popular. $AgNO_3$ is primarily used to separate lipids with different fatty acyl compositions based on the degree of unsaturation (Wang *et al*., 2003) and in contrast, boric acid (H_3BO_3) is primarily useful for the detection of the different isomers of DAG as well as the separation of isomeric PL (Fush *et al*., 2010) Depending on the type of the binder there are two different types of silica gel. The one that contain $CaS0_4$ as a binder to ensure adhesion of the layer to the plate (Silica Gel G) and silica gel which do not contain $CaSO_4$ binder or any organic binders (Silica Gel H). The type of the binder is sometimes very important.

1.6.3. The mobile phase

In its normal form, silica gel is a polar adsorbent. Consequently, polar lipids are more tightly adsorbed than non polar lipids due to polar interactions. This is frequently termed normal - phase TLC. In the separation of lipids using silica gel the most non-polar lipids therefore migrate at the fastest rates (high

R_f values) and the polar lipids at the slowest rates (low R_f values) (Hamilton & Hamilton, 1992) as listed to the tables 1.6.1., 1.6.2., respectively.

Table 1.6.1. R_f values of neutral lipid classes separated on silica gel 60 in various solvent systems

Lipids	1	2	3
Hydrocarbons	0.95	-	-
Trialkylglyceryl esters	0.90	-	-
Steryl esters	0.90	0.94	0.94
Wax esters	0.90	0.92	0.94
Dialkyl monoacylglycerols	0.70	-	-
Fatty acid methyl esters	0.65	0.81	0.94
Alkyl diacylglycerols	0.55	-	-
Triacylglycerols	0.35	0.73	0.86
Fatty acids	0.18	0.33	0.39
Fatty alcohols	0.15	0.28	0.29
Sterols	0.10	0.24	0.24
1,3-diacylglycerols	0.08	0.24	0.26
1,2-diacylglycerols	0.08	0.21	0.24
Monoacylglycerols	0.00	0.03	0.03
Complex polar lipids	0.00	0.00	0.00

Solvent systems:
1. Petroleum ether (b.p. 60-70^oC : diethyl ether : glacial acetic acid (90:10:1 v/v)
2.Hexane : diethyl ether : glacial acetic acid (80:20:2 v/v)
3. Hexane : diethyl ether : glacial acetic acid (70:30:3 v/v)
*R_f values represent relative migration only, whereas absolute values depend on various environmental parameters (e.g. temperature, humidity) which may vary depending on location.

*adapted by Hamilton & Hamilton, 1992

Table 1.6.2. R_f values of polar lipid classes (Phospholipids & Sphingolipids) in two different solvent systems

Lipid classes	1	2
Phosphatidylethanolamine (PE)	0.79	0.55
Phosphatidyl (Monomethylethanolamine)	0.71	0.41
Phosphatidylglycerol	0.60	0.50
Phosphatidyl (Dimethylethanolamine)	0.58	0.56
Phosphatidic Acid	0.55	0.05
Phosphatidylinositol (PI)	0.39	0.10
Phosphatidylcholine (PC)	0.34	0.30
Phosphatidylserine (PS)	0.33	0.12
Cerebrosides	0.94	0.55
Sphingosine	0.28	0.75
Sphingomyelin	0.28	0.13
Lyso-Phosphatidylglycerol	0.54	0.20
Lyso-Phosphatidylethanolamine	0.45	0.20
Lyso-Phosphatidic Acid	0.40	0.01
Lyso-Phosphatidylinositol	0.29	0.03
Lyso-Phosphatidylcholine	0.22	0.08
Lyso-Phosphatidylserine	0.18	0.02

Solvent systems:
1. Chloroform: methanol: water (65:25:4 v/v)
2.Chloroform: methanol: ammonium hydroxide (65:25:4 v/v)
*R_f values represent relative migration only, whereas absolute values depend on various environmental parameters (e.g. temperature, humidity) which may vary depending on location.

*adapted by *www.avantilipids.com*

By increasing the polarity of the developing solvent system the R_f values of the component can be increased, so the choice of a suitable solvent system is critical in the separation of the different lipid classes.

The solvent systems used to separate simples like neutral lipids most commonly contain non-polar solvents like hexane, diethyl ether and acetic or formic acid in various proportions (Hamilton & Hamilton, 1992), although other solvent systems have also been used as listed in the table below. If samples remain behind on the starting line, a more strongly elutive solvent is chosen and from the other hand, if they travel with the solvent front a more weakly elutive solvent is used. The addition of a small amount of acid (e.g. 1% acetic acid), often causes a considerable increase in elusive power (Kurt, 1968). Although lipid classes are given a single R_f value in the bibliography some classes like triacylglycerols or steryl esters are better represented by a range of values since in natural samples they frequently exhibit more than one band and this is due to their partial resolution into molecular species on the basis of fatty acid composition (Hamilton & Hamilton, 1992). The types of polar lipids present in a lipid extract depends very much on its source and there are very obvious differences exist between plant, animal and microbial lipids (Hamilton & Hamilton, 1992). On account of the large differences which exist in polarity among the classes of polar lipids, there is no single TLC system which can completely separate all of them by development in one dimension. For these reason, a complete separation of the polar lipids can be achieved by two-dimensional TLC. Most of the TLC systems described for the separation of polar lipids are based on the use of chloroform and methanol as major components of the developing solvent system (Kurt, 1968; Skipski, *et al.*, 1964; Lepage, 1964; Ruiser *et al.*, 1970; Touchstone, 1992; Fush *et al*., 2010; Christie & Han, 2010). Because of the fact that commercial standards are not available for every complex lipid, the use of specific staining reagents is therefore of particular use for the identification and characterization of polar lipids separested by TLC.

Table 1.6.3. Some of the solvent systems used in the separation of lipids

Neutral lipid separation	**Volumes**
Hexane: diethyl ether: glacial acetic acid	(90:10:1, v/v)
Hexane: diethyl ether: glacial acetic acid	(70:30:1 or 3 v/v)
Hexane: diethyl ether: glacial acetic acid	(75:25:1 , v/v)
Hexane: diethyl ether :glacial acetic acid	(80:20:2, v/v)
Petroleum ether: diethyl ether: glacial acetic acid	(90:10:1 v/v)
Petroleum ether: diethyl ether: glacial acetic acid	(70:30:1 or 2 v/v)
Phospholipids, Sphingolipids & Glycolipids separation *	**Volumes**
Chloroform: methanol: 7M ammonia	(65:25:4, v/v)
Chloroform: methanol: ammonia (28%)	(65: 25: 5 or 4 v/v)
Chloroform: methanol: water	(65:25:4, v/v)
Chloroform: methanol: glacial acetic acid: water	(25:15:4:2, v/v)
Chloroform: methanol: glacial acetic acid: water	(60:50:1:4, v/v)

*One-Dimentional Separation

References: Mangold & Malins, 1960; Lepage, 1964; Riuser *et al.*, 1970; Pucsok *et al*., 1988; Pernes *et al.*, 1989; Aloisi *et al*., 1991; Touchstone , 1992, 1995; Sowa & Subbaiah, 2004; Fakas *et al*., 2007 Fush *et al*., 2010

1.6.4 Application of the sample and the standards:

The quality of lipid analysis by TLC depends greatly on the careful application of the sample mixture to the adsorbent. In most cases samples are applied as spots, 2-3 mm or narrow steaks of 5-10 mm in

diameter, 1,5 -2 cm from the bottom edge of the plate “origin” (Skipski, *et al.,* 1967; Christie & Han, 2010). Whenever possible, it is better to apply samples, as well as standards, dissolved in a solvent with low-developing action (as non-polar a solvent as possible) (Skipski, *et al.,* 1967; Marinetti, 1967; Kurt, 1968; Touchstone, 1992; Hamilton & Hamilton, 1992; Christie & Han, 2010) unless sample solubility requires the use of Chloroform. A mixture of chloroform-methanol, 2:1 (v/v) also can be used (Pernes *et al.,* 1989). Lipids appear to be more stable to autoxidation on thin-layer adsorbents than has generally been believed. Nonetheless, it is advantageous to add antioxidants such as BHT at a level of 0.01% to the sample, to the eluting solvent or to the spray reagents (BHT migrates with the solvent front even in non-polar systems) to protect the lipids during subsequent analyses (Christie & Han, 2010).

Application of samples (and standards) is performed either by manual with sharp micropipettes (volume of 5, 10, and 25 µl) / special (Hamilton) microsyringes, 10-50 µl (Skipski, *et al.,* 1967) or with automatic sample applicators. The Linomat sample applicator of Camag has been developed to perform high-precision narrow band application of samples for precise qualitative and quantitative analysis. The spray-on technique allows a large volume to be concentrated into a narrow band (Touchstone, 1992). The samples have to be applied as quickly as possible because silica gel is a hygroscopic material and adsorption of water from air proceeds rapidly (Skipski, *et al.,* 1967).

Although lipid classes can be identified by reference to published R_f values the application of authentic lipid standards , either as a mixture or individually, alongside the lipid being analysed, greatly aids in the identification of the components present in the lipid sample. Within the laboratory, the Rf values of lipid classes in a given solvent system are not always constant due to many factors (Hamilton & Hamilton, 1996). By routinely analyzing lipid standards alongside samples such variations can be taken into account.
The standards employed depend on the nature of the sample being analyzed. In the description of the separation of neutral lipids, the term "neutral lipids" includes all lipids containing neither phosphorus nor sugars in their molecules. Therefore, free fatty acids, which, of course, are not neutral compounds from the chemical point of view, are included in the group of "neutral lipids"(Skipski, *et al.,* 1967). The neutral lipid classes which are most commonly encountered in microbial oil, such as triacylglycerols, diacylglycerols, cholesteryl esters, cholesterol and free fatty acids, are all commercially available either individually or as components of standard mixtures (Sigma Chemical Co.) The most abundant phospholipid classes can also be purchased by the same supplier as can some glycolipids or sphingolipids. A complete migration standard solution can be a mixture of CE (1 mg/ml), TG (1 mg/ml), 1,3DG (0.85 mg/ml), 1,2DG (0.15 mg/ml), FC (I mg/ml), oleic acid as non-esterified fatty acid (1 mg/ml), MG (2 mg/ml), phosphatidyl ethanolamine (PE; 1 mg/ml), phosphatidylcholine (PC; 2 -mg/ml) (Pernes *et al.,* 1989).

For qualitative work the optimum concentration of the standard compound is 2-5 mg/ml (depending upon the type of standard), which permits application of the required amount in a volume of 5-10 µl. The concentration of sample should be higher in the range of 20-30 mg/ml. However, the nature of the experiment and the type of lipid studied often makes it necessary to deviate from these concentration limits (Skipski, *et al.,* 1967).

1.6.5. Detection systems

It is a major advantage of TLC that the separated lipid fractions can be easily visualized by binding to a dye. Apart from the general methods of detection there is also a number of specific reactions. An important advantage of thin –layer chromatography is that it enables aggressive spraying reagents to be used for the detection of lipids. For example, unsaturated lipids can be located without spraying, on layers containing a fluorescence indicator, by examination under ultraviolet light (Kurt, 1968). The limits of detection of lipids are much lower on a thin layer than on a paper. A lot of different reagents are nowadays available often even as ready-made spray agents (Wang *et al.,* 2003). These agents can be sorted according to their specificity and if they are destructive or non-destructive.

1.6.5.1. Non-destructive, non-specific

One of the most frequently used methods is the exposure of the developed TLC plate to iodine vapors that forms a non-covalent, brown complex with lipids. Unfortunately, however, completely saturated lipids can hardly be stained, while the iodine cannot be completely removed from highly unsaturated lipids (containing e.g. arachidonoyl residues) because the iodine is chemically bound to the double bonds (Vioque *et al.,* 1962; Touchstone, 1992). Staining with 2,7-dichlorofluorescein or rhodamine 6G (Marinetti, 1964) provides yellow or pink spots, respectively, if the TLC plate is illuminated with UV light. The rhodamine is particularly useful when alkaline solvent systems have been used and 2,7-dichlorofluorescein is to be preferred with acidic solvents due to the stabilities of the dyes (Fush *et al*., 2010). Both dyes can be easily removed if the polarity of the solvent is changed or the lipid (with the bound dye) is passed over a short column. This is also true for the dye primuline (Skipski, 1975; White *et al*.,1998) that can be used in a similar way and gives sensitivities in the low nanomole range that is comparable to rhodamine (Kishimoto *et al.,* 2001). Remarkably, this darkening is dependent on the composition of the solvent system and seems to require the presence of aromatic hydrocarbons such as toluene (Fush *et al*., 2010).

1.6.5.2. Destructive, non-specific

Spraying the complete TLC plate with a corrosive reagent and charring the plate to render the lipids visible is a very common method (Fush *et al*., 2010). 50% sulfuric acid either in methanol or water is a typically used solvent system and the plate is heated afterwards to about 120 ◦C for approximately 1 h. Although mechanistic details are so far widely unknown, it should be noted that saturated and unsaturated lipids require different times to be completely reduced to carbon. The intensities of these black spots can be also quantitatively analyzed using specialized equipment or software. A lot of different reagents such as phosphomolybdic acid (20%) in ethanol (Christie & Han, 2010), potassium dichromate (5%) in 40% sulfuric acid or a 3–6% solution of cupric acetate in 8–10% phosphoric acid were also indicated to be potentially useful (Hamilton & Hamilton, 1996; Fush *et al*., 2010).

1.6.5.3. Destructive, specific

These reagents and the methods for using them are usually more complicated than the non specific stains. They generally involve a chemical or chemicals in the reagent reacting with specific groups in the lipids that result in the lipid being stained or made visible in some way. A survey of the most frequently used reagents is summarized in table 1.6.4. A detailed survey of staining agents is also available from the excellent internet sides*:* *www.cyberlipid.org*, *www.avantilipids.com* & *www.emdchemicals.com.*

Table 1.6.4. Specific detection reagents for the analysis of individual lipid classes

Lipid class	Reagents	Results
Cholesterol and cholesteryl esters	Acidic ferric chloride in n-butanol H_2SO_4 – CH_3COOH (1:1 v/v)	Red to violet spots are generated. The reaction is faster with free cholesterol than with esters (Dudzinski, 1967). Does also react with free fatty acids.
Free fatty acids	2,7 Dichlorofluorescein	Rose color after a few minutes in UV light (Kurt, 1968)
Phospholipids	Molybdic oxide(MoO_3)/molybdenum	Phospholipids form blue spots on a white background (Dittmer & Lester, 1964)
Phospholipids containing choline	Dragendorff reagent (bismuth nitrate ($Bi(NO_3)_3$ + KI)	PC, LPC and SM become detectable as orange-red spots (Wagner *et al.*, 1961)
Phospholipids containing free amino	Ninhydrin solution	PE, PS and the corresponding lysolipids are detected as red-violet spots (Valls *et al.*, 2002; Marinetti, 1964)
Glycolipids	α-Naphthol/sulfuric acid Orcinol/sulfuric acid	All glycoplipids (including cerebrosides) are characterized by blue-purple spots on white background. Although cholesterol is also reactive, it provides a red spot (Kawanami, 1967; Neskovic, *et al.*, 1972; Skipski, *et al.*, 1967)
Sphingolipids	Sodium hypochlorite/benzidine reagent	Blue spots with a white background are generated with all lipids containing a secondary amine group (Skipski, *et al.*, 1967)

2. Materials and methods

2.1. Biological material

For this study was used one strain of the oleaginous fungus *Morterella isabelina* (ATHUM 2935), which was provided by the department of Biotechnology of Agricultural University of Athens. The microorganism was preserved in PDA (Potato Dextrin Agar) (T=6 ± 1 °C), which prepared using the manufacturer's instructions (40 g/L). For sterilization, the PDA was autoclaved at 121°C for 25 minutes. In order to maintain the viability of the strain, the microorganism was sub-cultured every three months. Prior to any inoculation in liquid growth medium (pre-culture and main culture) the strains were regenerated (under sterile conditions) so as the inoculums would be five days old.

2.2. Culture medium

All the experiments were performed in 250-ml Erlenmeyer flasks, containing 50 ml of growth medium. Commercial glucose and xylose were used as carbon sources in five ratios (40:40, 60:20, 20:60, 0:80 and 80:0 g/L) at initial total sugar concentration of 80g/L , in order to study the effect of the substrate (and specifically on lipid and biomass production) on the metabolism of *Mortierella isabellina* ATHUM 2935. The salt composition of the media used according to Kavadia *et al*. (2001), is presented in Table 2.2.1. The nitrogen sources used were ammonium sulfate $(NH4)_2SO_4$ and yeast extract at 0.5 g/L respectively. The initial pH of the medium was equal to 6.0±1. In all the fermentations the initial C/N ratio was approximately 205 (mol/mol).

Before any inoculation the growth medium was sterilized at 121^{o}C for 25 min and inoculated with 1 ml of spore suspension (~2×10^5 spores, which were produced by growing the strain on PDA). The culture was incubated in an orbital shaker at 180 rpm and the incubation temperature was 28±1^{o}C. Flasks were periodically removed from the incubator for processing the analytical methods, described in the following sections.

Table 2.2.1 Composition of mineral salts solution of the media (g/L)

Compound	Concentration (g/L)
KH_2PO_4	7.0
Na_2HPO_4	2.0
$MgSO_4•7H_2O$	1.5
$CaCl_2•2H_2O$	0.1
$FeCl_3•6H_2O$	0.08
$ZnSO_4•7H_2O$	0.001
$CuSO_4•5H_2O$	0.0001
$Co(NO3)_3•H_2O$	0.0001
$MnSO_4•5H_2O$	0.0001

2.3. Analysis

2.3.1. Determination of pH

The pH value of the fermentation medium was monitored in all experiments, and the measurement was conducted by using a selective pH meter (Jenway 3020 Germany apparatus). The pH of the medium was maintained at the desired value aseptically by adding into the flasks, when necessary, NaOH 1M. The exact volume needed for pH correction was evaluated by measuring the volume of the solution required for pH correction in one (at least) flask.

2.3.2. Total sugar determination (of the media)

The reducing sugars (glucose and xylose) concentration was determined either by High Performance Liquid Chromatography (HPLC) analysis or the DNS method (Miller, 1959).

2.3.2.1. Determination of sugars with HPLC

Glucose and xylose were determined with High Performance Liquid Chromatography (HPLC) analysis. The supernatant of the fermentation medium was centrifuged and filtered with 0.2 μm filter. The injection 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 Aminex HPX - 87*H* (Biorad) (300 mm x 7.8 mm), the mobile phase was 0.005 M H_2SO_4, the column temperature was 65°C and the flow rate was 0.6 mL/ min. The data acquisition software used was Empower.

2.3.2.2. Determination of sugars with DNS method

The reducing sugars (glucose and xylose) concentration was determined according to DNS method (Miller, 1959) and was expressed as glucose equivalent. The method is based on the reduction of dinitro 3, 5 salicilic acid to 3-amino-5-nitro salicylic acid in alkaline environment and on the simultaneous oxidization of glucose towards gluconic acid.

For the analysis 0.5 mL of the fermentation medium and glucose standard 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 concentration was expressed as grams of glucose / liter. The calibration curve is shown in Figure 2.3.1.

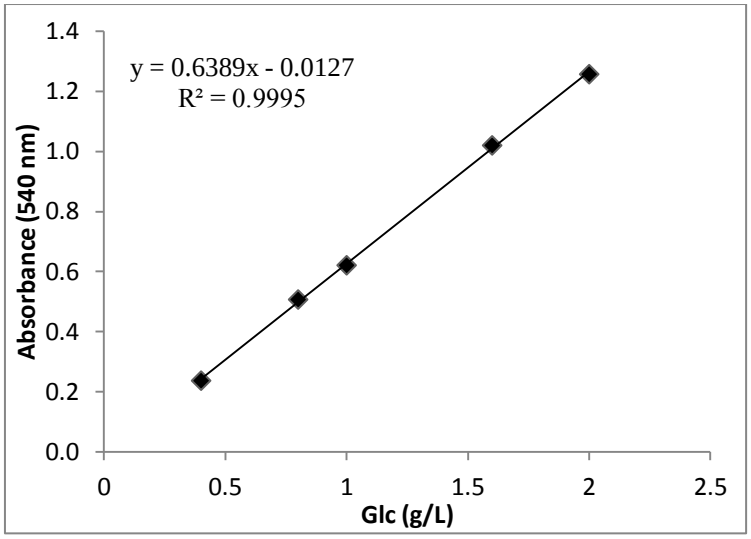


Figure 2.3.1 Standard curve for glucose with the DNS method

2.3.3. Biomass determination

Fungal cell mass was harvested by filtration using a 0.09 mm stainless-steel sieve and washed with distilled. Total biomass concentration (X g/L) was determined gravimetrically from dry weight at temperature *T*=100 °C until constant weight (usually within ~18-24 h).

2.3.4. Analysis of the intracellular oil

For the analysis of the intracellular oil, a number of experimental points from each fermentation were chosen and studied both qualitatively and quantitatively.

2.3.5. Lipid extraction

Total lipid content of *Mortierella isabellina* ATHUM 2935 was estimated according to Folch method, (1957). Total intracellular lipids were extracted with a mixture of chloroform and methanol 2:1 (*v/v*) and after 3 days (at least) the solvent mixture was completely evaporated (Büchi Rotavapor R-114) and lipids were determined gravimetrically (quantitative analysis).

2.3.6. Determination of phospholipid fraction

The quantity of the phospholipid content was determined in order to evaluate the quality of the single cell oil produced by *Mortierella isabellina* ATHUM 2935. To examine the quantity of the phosphorus in the oil produced by this strain, a phosphorus assay was used for the determination of phosphorus content in the lipid samples (Rouser *et al.*, 1970*).*

The lipid samples were transferred into clean glass tubes and the solvent was completely evaporated under a stream of nitrogen. Then, 0.65 ml of concentrated perchloric acid (70%) was added in the tubes. The lipids were digested by heating for about 30 min (until the yellow color disappeared) in the heated metal block with the upper one half of each tube extending outside of the block to prevent loss of perchloric acid fumes. When the samples were cooled, the following reagents were added to the tubes: 3.3 ml water, 0.5 ml of ammonium molybdate solution (2.5 g in 100 ml water) and 0.5 ml of ascorbic acid solution (10 g in 100

ml water). The additions were made rapidly and accurately with pipettes and the tube contents were mixed after each addition with a Vortex. Color was developed by heating for 5 min in a boiling water bath and the optical density values of the samples (including the standards) were read against blank at 800nm in a Hitachi U-2000 spectrophotometer equipped with a digital readout. The phosphorus content of the samples was calculated based on the standard curves of KH_2PO_4 (monopotassium phosphate): dilute 10 times a stock solution of KH_2PO_4 (439 mg/L, i.e. 100 μg P/ml). The standard solutions (1 to 5 μg P/tube) were diluted in 3.3 ml water and 0.65 ml perchloric acid. The digestion of the standards was not necessary before adding reagents. The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25.

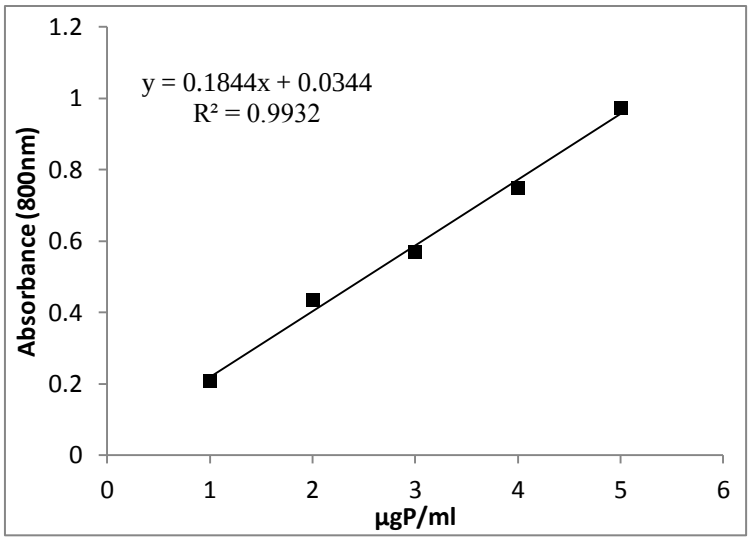


Figure 2.3.2 Standard curve of KH_2PO_4

2.3.7. Determination of sugar content in lipid fractions

Total sugars in lipids have generally been determined by the Phenol- Sulfuric Acid method (Dubois, 1956). In this method, the concentrated sulfuric acid breaks down any polysaccharides, oligosaccharides, and disaccharides to monosaccharides. Pentoses (5-carbon compounds) are then dehydrated to furfural, and hexoses (6-carbon compounds) to hydroxymethyl furfural. These compounds then react with phenol to produce a yellow gold color that can be measured spectrophotometrically. However this method gives sufficient results only in water soluble glycolipid hydrolysates and with intact acyl esters glycolipids. It was found to give inconsistent and irreproducible results when applied to intact microbial oil. In this study, a modification of this method was used in the lipid samples for the determination of total sugar content in glycolipid and sphingolipid fractions (Kushwaha & Kates, 1980).

An aliquot of lipid solutions containing at least 30-60 μg sugar (as hexose) were transferred into glass tubes and the solvent was evaporated to dryness under a stream of nitrogen. Then, 2 ml of water and 1 ml of phenol solution 5% w/w in deionized distilled H_2O were added to the residue. The additions were made accurately with pipettes and the tube contents were mixed gently by hand, making sure that the film of lipid at the bottom of the tube was undisturbed. After that, 5 ml of concentrated sulfuric acid (H_2SO_4) was added with 5-ml pipette, rapidly and directly in the center of the tube. Then, the samples were heated for 5

minutes in a boiling water bath. After the boiling, the samples were allowed to cool for 30 minutes and the absorbance of the gold color (including the standard solutions of glucose) was determined at 490 nm in a Hitachi U-2000 spectrophotometer against a reagent blank. The sugar content of the samples was calculated based on the standard curves of glucose (100mg/L). The standard solutions containing 20, 40, 60, 80 and 100 μg glucose were diluted in water in a total volume of 2 ml.

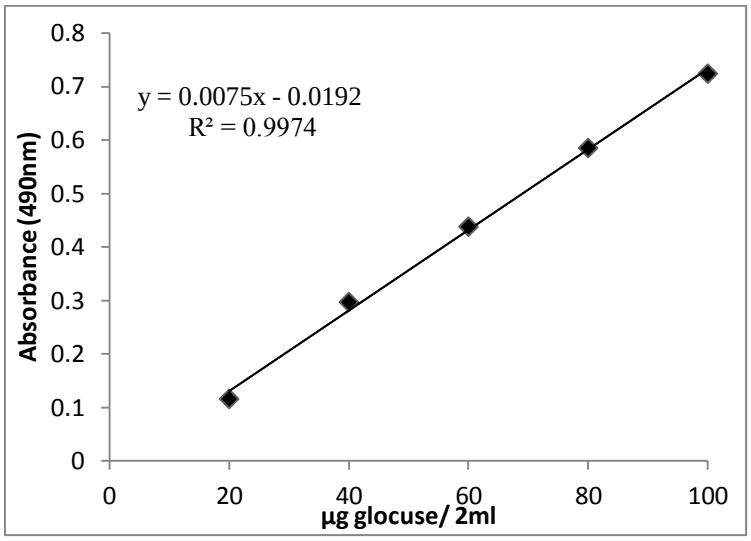


Figure 2.3.3 Standard curve of glucose

2.3.8. Fractionation of lipid classes

Three major lipid classes of microbial oil were fractionated by silicic acid column chromatography (Rouser & Kritchevshy, 1967; Latge & De Bievre, 1980).

For the preparation of the sample, a known amount of lipids was dissolved in 25 ml Folch solution, transferred in a separatory funnel and purified with 5 ml of 0,88% KCl. Then, the lipid sample was dried with a small amount of sodium sulfate anhydrous (Na_2SO_4) (for the absorption of any remaining water) and concentrated to dry weight in a rotary evaporator and the remaining lipid sample (approx. 100 mg) was dissolved in 2 ml of eluting solvent (dichloromethane) and stored in a glass vial until the fractionation. For the preparation of the column (25mm×100 mm), silicic acid was activated by heating at 120^{o}C overnight and again for 1 hour immediately before the preparation of the column. An amount of silicic acid (tenfold the amount of the sample) was dissolved in dichloromethane and poured in to the column (2.2 cm i. d). Then, the lipid sample was quantitatively transferred to the column. Successive applications of dichloromethane, acetone, and methanol produced fractions containing neutral lipids (NL), glycolipids plus sphingolipids (G + S), and phospholipids (P), respectively. Neutral lipids were eluted with 100 ml of dichloromethane, glycolipids plus sphingolipids with 100 ml of acetone and phospholipids with 100 ml of methanol. All the elutions were controlled at a flow rate of 0.5ml-1.0ml/min. The eluted solvents were collected in a weighed flask. The fractions thus obtained were evaporated in a rotary vacuum evaporator and dried under reduced pressure before being weighed. The percentages of these fractions were determined and finally the lipid fractions of known concentration were stored in vials at −4°C. All three lipid fractions were analyzed by TLC using various solvent systems.

2.3.9. Thin layer chromatography (TLC) Analysis of fungal lipid fractions

Plates

Glass precoated ready-made silica-gel G plates for the development of the neutral lipid fraction and Aluminum sheets silica- gel 60 for phospholipid (P) and glycolipid plus sphingolipid (G+S) fraction, from Merck, Darmstadt F.R.G., 20 x 20 cm x 0.25 mm were used. All plates were prewashed by running them with a mixture of solvents. For neutral lipids the solvents were chloroform-methanol (4:1 or 2:1, v/v) and for polar lipids acetone /petroleum ether (1:3v/v). The plates were activated before use by heating for 30 min in an oven at 100°C.

Solvents

Chloroform, methanol, n-hexane, diethyl ether, glacial acetic acid and ammonium solution (25%) were of analytical grade and purchased from Sigma- Aldrich. The separation of neutral lipid (NL) fraction was carried out with n-hexane /diethyl ether/glacial acetic acid (70:30:1, v/v/v). For the polar fractions [glycolipids plus sphingolipids (G+S), phospholipids (P)] analysis, chloroform/methanol/ammonia (25%) (65:25:4, v/v/v) was used. After the development the plates were placed in the fume hood for about 30 minutes until dryness before the visualization procedure.

Lipid standards

The identification of lipid classes were made with authentic standards in specific concentrations. All lipids standards were obtained from Sigma- Aldrich. The Standard solutions were prepared by dissolving one of each individual pure reference compound in chloroform-methanol (l:l, v/v). From these stock solutions, mixtures of working solutions were prepared according to the following table 2.3.1.

Table 2.3.1 Standard solutions (Stock & Working) for neutral lipid fraction and polar lipid fraction

	Type	Standard	Stock solution (mg/ml)	Working solution (mg/ml)
Neutral	**MAG**	Monononadecanoin	5	2
	CE	Cholesteryl linoleate	10	1
	TAG	Glyceryl trioleate	10	2
	CL	Cholesterol	10	1
	FFA	Oleic acid	40	2
Polar	**PS**	L-α-Phosphatidyl-L-serine	10	1
	PE	3-sn-Phosphatidylethanolamine	10	1
	PC	L-α-Phosphatidylcholine	10	1.6

Application of the standards & samples

Standard and sample solutions were applied automatically with the Linomat IV sample applicator of Camag using a microsyringe 100µl (Camag, Muttenz, Switzerland). The origin was either at 2 cm or at 1cm from the lower edge of the silica gel layer.

Development

TLC developments were carried out in all-glass chromatography chambers at room temperature. The chromatography was stopped when the solvent front was 1 cm from the top edge of the plate. For the one dimensional TLC separation technique of polar lipid fractions (P & G+S) the plates were developed to a height of 8 cm above the origin (ca 30 min) and for the neutral lipid fraction to a height of 16 cm above the origin (ca 45 min)

Detection systems of separated lipids

After completion of TLC separation, the bands were located by 50% aqueous sulfuric acid [H_2SO_4:H_2O 1:1 (v/v)] for the quantitative determination of all lipid classes. The reagent remains stable for several weeks when stored in a laboratory refrigerator. After spraying the plates with the detection reagent, they were dried in the fume hood for 15-30 minutes and subsequently were placed in the oven at 110-120 °C for 30 minutes (Touchstone, 1992)

For the detection of lipids containing phosphorus (phospholipid fraction) the molybdenum blue reaction according to Dittmer & Lester (1964) was used. The reagent composition was:

- Solution A. Molybdic oxide (4 g) was dissolved by boiling gently in 70% sulfuric acid (100 ml)
- Solution B. Powdered molybdenum (0.18 g) was added to solution A (50 ml) and the mixture refluxed for 1 h, and then cooled.
- Solution C: Solutions A and B were mixed 1:1 (v/v).

The final spray reagent was prepared by adding 2.5 ml of solution C with 5ml water and 7.5ml ethanol. When TLC plates were sprayed lightly with the reagent, phospholipids appeared within 10 minutes as blue spots on a white background.

For the qualitative determination of glycolipids the detection reagent α-naphthol was used. This reagent reacts with the sugar moieties of glycolipids. 0.5g α-naphthol dissolved in a solution (1:1 v/v) of methanol and water. After spraying with α-naphthol, the plates were dried at room temperature and then sprayed with H_2SO_4: H_2O (95:5 v/v).Heating at 120^{o}C reveals glycolipids as purple-blue spots and other complex lipids as yellow spots.

2.3.10. Transesterification

The methyl esters of fatty acids were prepared according to AFNOR (1959) method and as described in Fakas *et al.* (2006). This procedure includes two stages. The first stage is performed in alkaline environment whereas the second stage in acidic environment. During the first stage the nucleophilic substitution at the molecule of triglycerides and phospholipids takes place, resulting 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.

At the evaporation/boiling flask - including the gravimetrically determined total lipids – 10 ml of sodium methoxide solution [CH_3O-Na+; 1% of sodium metal in a 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 100 °C for 20 min. Following, an amount of hydrochloride 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 (acidification). 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 hexane is added and methyl-esters are extracted into hexane after strong stirring. Finally, the water phase is discarded and a small amount of anhydrous sodium sulfate (Na_2SO_4) is added to the organic phase (for the absorption of any remaining water).

2.3.11. Gas Chromatography (GC) analysis - FAMEs determination

The extracted lipids were converted to their fatty acid methyl-esters (FAMEs) as described above and analyzed (qualitative analysis) in a gas chromatograph (Fisons GC 8000series) equipped with an FID according to Fakas et al. (2006). 1 μL of methyl-esters sample was injected. The carrier gas (He) flow rate was set at 1.38 mL/ min. The column used was a fused silica WCOT: CP-Sill 88 (Chrompack, 50 m x 0.32 mm 0.20 μm). The analysis was carried out at 200°C with the injector and detector at 250°C. FAMEs were identified by comparison with authentic standards. Results were expressed as percentages of peak areas. The degree of unsaturation (Δ/mole) in the extracted lipid was calculated according to Kates & Baxter (1962). The degree of unsaturation (Δ/mole) = 1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100.

3. Results

Lipids produced from microbial sources attract a potential industrial and financial interest due to their specific characteristics. Especially, various oleaginous Zygomycetes have been employed for the biotechnological production of lipids, rich in PUFAs of pharmaceutical and nutritional interest, such as GLA, during their growth on a variety of carbon sources. Consequently, the present study was focused on the potential of growth and lipid production by an oleaginous fungus strain named, *Mortierella isabellina* strain ATHUM 2935, cultivated on two renewable carbon sources namely xylose and glucose, abundant sugars of lignocellulosic biomass, each one as sole carbon source and in blends in batch-flask cultures at initial concentrations of 80 g/L. Additionally, in order to make a step forward to elucidate the single cell oil produced by *Mortierella isabellina* on chemical basis, the chromatographic analysis of the intracellular lipids can be undertaken to reveal which different classes are present in the fungus oil.

The biochemical behavior (biomass production, accumulation of total lipid, substrate uptake, fatty acid composition of fungal oil) was studied when the aforementioned microorganism was cultivated under nitrogen limited conditions, on the following substrates:

- 80 g/L glucose,
- 80 g/L xylose
- 40 g/L glucose : 40 g/L xylose
- 60 g/L glucose : 20 g/L xylose
- 60 g/L xylose : 20 g/L glucose

Mortierella isabellina strain ATHUM 2935 was kept on potato dextrose agar (PDA) at 4^{o}C. The salt composition of the medium used, was according to Kavadia et al., (2001). The nitrogen sources used, were $(NH_4)2SO_4$ and yeast extract at 0.5 g/l, respectively. The initial pH value of all the mediums was equal to 6.0±1. In all the fermentations the initial C/N ration was adjusted to 205 (mol/mol), in order to direct the microbial metabolism towards the synthesis of intracellular lipid (Evans & Ratledge, 1985; Papanikolaou *et al*. 2008, 2009; Koutinas and Papanikolaou 2011; Papanikolaou 2012). All the experiments were performed in 250-ml conical flasks, containing 50 ml of growth medium, sterilized at 121 ^{o}C for 20 min and inoculated with 1 ml of spore suspension (2×10^5 spores). The cultures were incubated in a orbital shaker at a rate of 180 rpm and the incubation temperature was 28± 1 ^{o}C.

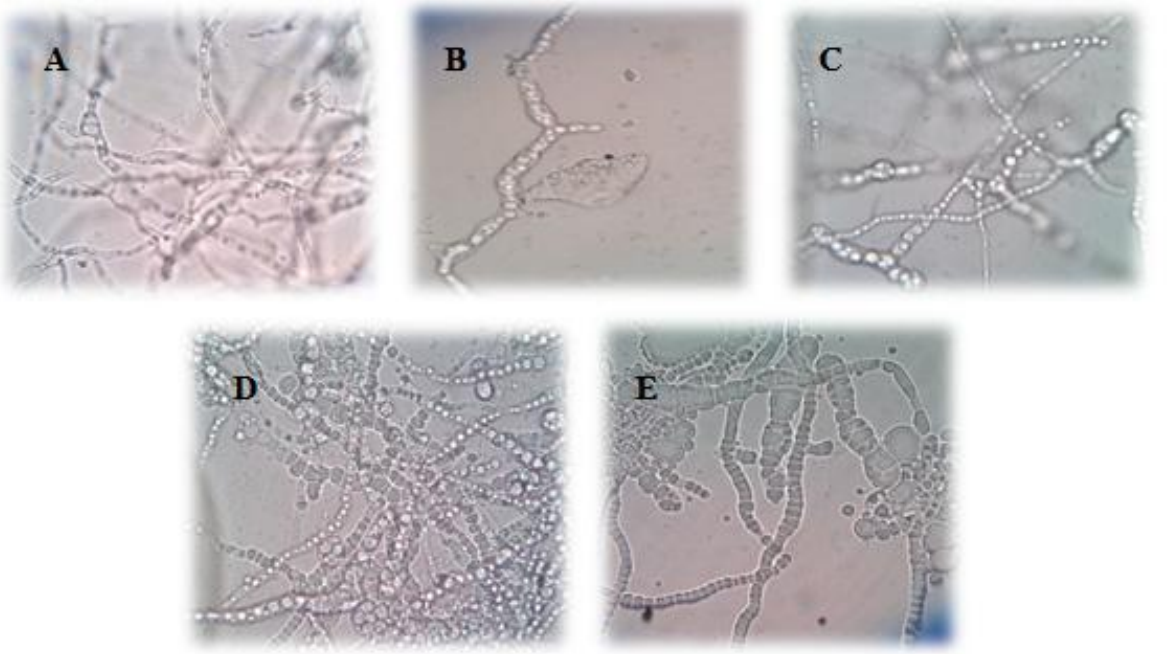
In order to examine the biochemical behavior in these higher substrate concentrations, kinetic experiments were conducted on media that were produced and the following parameters were evaluated:

- pH of the medium
- Glucose, xylose and total sugars consumption (Glc, Xyl & Total sugars , g/L)
- Biomass (in dry cell weight) and total lipid production (X and L, g/L)
- Xylitol production from xylose (xylitol, g/L)

During the microbial growth, the following parameters were calculated:

- Biomass yield on glucose consumed (g formed /g glucose consumed –$Y_{X/Glc}$)

- Biomass yield on xylose consumed (g formed /g xylose consumed –$Y_{X/Xyl}$)
- Biomass yield on total sugars consumed (g formed/ g total sugars consumed – $Y_{X/S}$)
- Lipid yield on total sugars consumed (g formed/ g total sugars consumed – $Y_{L/S}$)
- Lipid in total dry biomass ($Y_{L/X}$ %, w/w)



Scheme 3.1. Oil accumulation of *Mortierella isabellina* ATHUM 2935 growing on commercial glucose medium in nitrogen-limited conditions, A) 28h, B) 45h, C) 90h, D) 120h, E) 379h of cultivation (from present study).

3.1. Cultivation of *Mortierella isabellina* in commercial glucose (S_0= 80g/L)

3.1.1. Growth characteristics and lipid production

Kinetics of biomass and lipid production were conducted in nitrogen-limited media having glucose as the sole carbon source at initial concentration of 80 g/L and an initial C/N ratio adjusted at approx. 205 mol/mol. Up to 40–45 h after inoculation, the fungus consumed all of the available nitrogen and then, reserve lipid was synthesized in distinct oil droplets, visible in microscope. Low organic acid amounts were produced, since pH value of the growth medium presented a small decrease in all growth steps (final pH = 5.7). *Mortierella isabellina* strain ATHUM 2935 grew in full aerobic conditions, and presented appreciable cell growth related to the initial sugar concentration of 80 g/L as it is shown at figure 3.1.1.

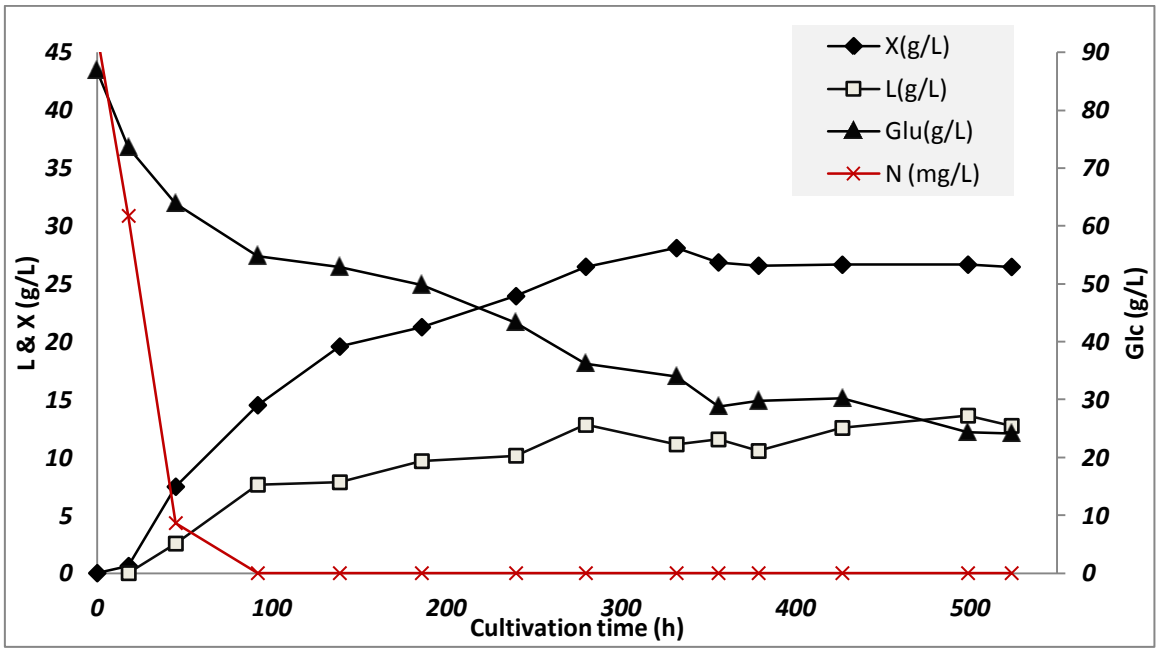


Figure 3.1.1 Kinetics of glucose consumption (Glc g/L), nitrogen consumption (mg/L), biomass (X, g/L) and single cell oil (L, g/L) production of *Mortierella isabellina* ATHUM 2935 growing on commercial glucose medium in nitrogen-limited conditions.

The maximum biomass concentration was achieved after 332h of cultivation and it was remarkably high (up to 28 g/L). Large amounts of glucose remained unconsumed into the growth medium (24.1 g/L) after 524 h of cultivation while no degradation (turnover) of the accumulated lipid occurred during stationary phase. In similar experiments though, cultures of various oleaginous molds on glucose at high initial C/N (e.g. >90) showed that the assimilation rate of the sugar at the late growth steps was decreased and some quantities of sugar remained unconsummated into the growth medium (Certik & Shimizu, 2000; Gema *et al*., 2002). The lipid accumulation started a while before nitrogen exhaustion. The oil content when the maximum biomass achieved (28 g/L) was 39.6% ($Y_{L/X}$ %, w/w), meaning that, 11 g/L of single cell oil was produced while the amount of produced oil wasn't the maximum at growth step. During the cultivation, single cell oil concentrations (in g/L) significantly increased in glucose medium, due to the notable biomass

production. The maximum oil production (L*max*) 13.6 g/L was achieved at 499h after inoculation and at this growth step the fermented mass was 26.7 g/L.

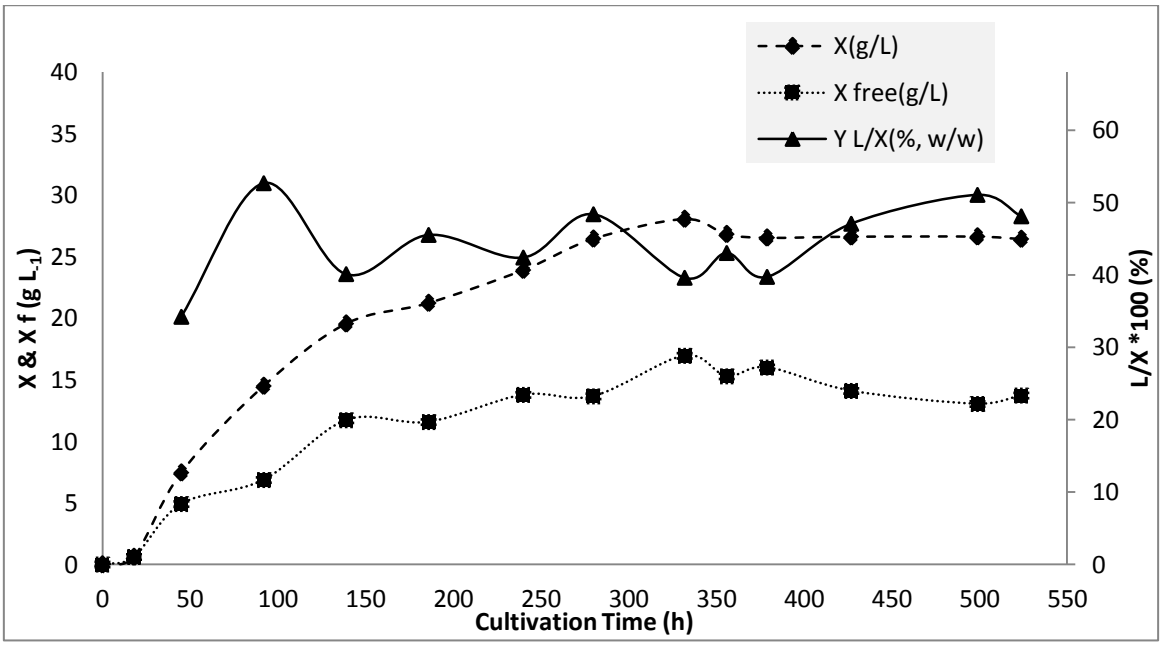


Figure 3.1.2 Representation of total biomass (X, g/L), lipid in dry weight ($Y_{L/X}$ %, w/w) and lipid free biomass (X free, g/L) during growth of *Mortierella isabellina* ATHUM 2935

Although, low quantities of fat-free biomass were produced, high lipid accumulation occurs inside the mycelia mass. The kinetic in Figure 3.1.2 showed that the lipid content in total dry biomass ($Y_{L/X}$ %, w/w) varied from 34.2% to 60 % during growth of *Mortierella isabellina* strain ATHUM 2935 in glucose. More specifically, the lipid content in fermented mass increased rapidly after nitrogen exhaustion and reached its maximum value of 60 %, meaning that, 9.6 g/L of single cell oil was produced, at 92h after inoculation. After that, the oil content remained almost constant (45-47%) during the cultivation time between 139h and 379h while at 499h reached the second maximum value 51.1 % resulting in a maximum lipid quantity (L*max*) of 13.6 g/L.

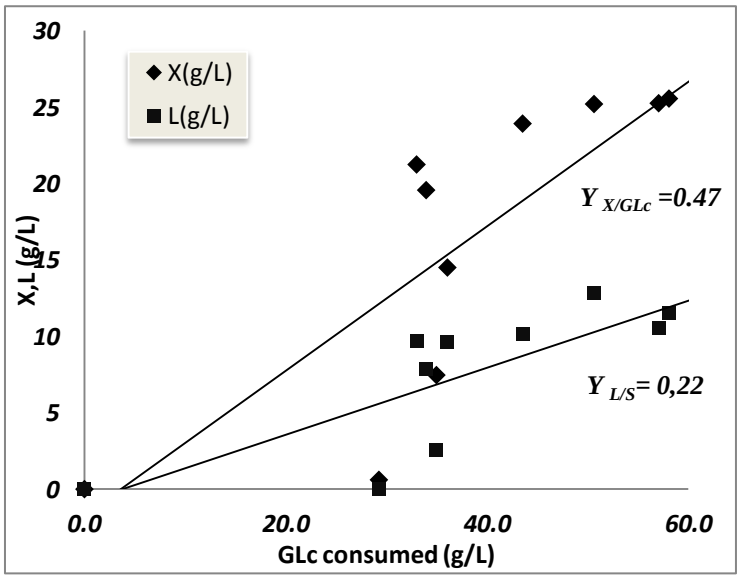


Figure 3.1.3 Biomass (X, g/L) and storage lipid (l, g/L) produced by *Mortierella isabellina* ATHUM 2935 as function of glucose (GLc, g/L) consumed at initial glucose concentration of 80 g/L.

Total biomass and lipid yield on glucose consumed ($Y_{X/Glc}$ and $Y_{L/Glc}$) presented constant and remarkable values (0.47 and 0.22 g/g respectively) at the initial sugar concentration of 80 g/L in the growth medium as it is shown in figure 3.1.3. It is noted that for various sugars, polysaccharides and poly alcohols utilized as carbon and energy source, values between 0.18 and 0.22 g of lipid synthesized per gram of carbon substrate consumed are considered as optimal for all types of oleaginous microorganisms and culture modes (Ratledge, 1994).

3.1.2. Cellular lipid analysis

The fatty acid composition of intra-cellular lipids was analyzed by GC at various cultivation times as it is showed in table 3.1.4. and figure 3.1.3. The length of the fatty acid carbon chain ranged from 14 to 20 for this fungal strain. There weren't any significant differences between the fatty acid compositions in the total cellular lipid of *Mortierella isabellina* strain ATHUM 2935 during the cultivation. The major fatty acids were palmitic (C16:0), stearic (C18:0), oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2) and γ-linolenic ($^{\Delta 6,9,12}$C18:3 , GLA) acids. Oleic acid was the predominant cellular fatty acid with the percentage of 45–53% of the total fatty acids throughout the cultivation times which were selected. Palmitic and linoleic acid were found in significant quantities into the reserve lipids while palmitoleic and stearic acid were detected in low amounts. Taking stearic and palmitoleic acid for example, the lowest content 2.58% and 1.78% observed at 524h and 379h after inoculation, respectively. The concentration of linoleic acid was at the higher level at the end of growth when the oil content in total biomass was still high (43.6%).

Table 3.1.4 Fatty acid composition (% w/w) of *Mortierella isabellina* ATHUM 2935 at various growth phases. IU: The degree of unsaturation (Δ/mole) in the extracted lipid = (1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100).

Carbon source		**Glucose 80 (g L^{-1})**				
Cultivation time (h)		45	186	280	379	524
Fatty acids	**Structure**					
Myristic	**C14:0**	0.71	0.61	1.27	0.86	0.65
Palmitic	**C16:0**	**25.63**	**20.11**	**28.07**	**22.37**	**19.78**
Palmitoleic	**$^{\Delta 9}$C16:1**	2.09	1.93	4.05	1.78	2.58
Stearic	**C18:0**	4.57	3.23	5.19	6.29	2.56
Oleic	**$^{\Delta 9}$C18:1**	**50.42**	**52.96**	**45.78**	**49.17**	**53.24**
Linoleic	**$^{\Delta 9,12}$C18:2**	**9.83**	**13.61**	**7.76**	**12.16**	**14.98**
γ- Linolenic	**$^{\Delta 6,9,12}$C18:3**	2.90	3.28	1.72	2.86	3.35
Arachidic	**C20:0**	1.22	1.52	0.73	0.91	1.18
Others		2.63	2.74	3.08	3.61	1.69
IU		0.81	0.92	0.71	0.84	0.96

Moreover, *Mortierella isabellina* ATHUM 2935 showed a low capability to produce GLA on glucose. Although , GLA($^{\Delta 6,9,12}$C18:3) was presented in all growth steps in the reserve cellular lipids at concentration between 1.32% and 3.35 %, this low concentration of GLA indicated that *M. isabellina*

ATHUM 2935 did not exhibit significant D6 desaturase activity in all growth steps. The highest GLA content of 3.35 % was achieved at 524h, towards the end of fermentation. On the contrary, in other Zygomycetes cultivated on glucose or soluble starch (e.g. *M. circinelloides, C. echinulata, Z. moelleri, R. stolonifer*) the fatty acid composition of cellular lipid remarkably varied as function of the fermentation time, since the microorganisms used tended to produce lipid with low GLA content when large amounts of fat were accumulated and vice versa (Aggelis *et al*., 1988, 1990; Chen and Chang, 1996; Kavadia et al., 2001; Fakas *et al.*, 2009; Papanikolaou *et al.*, 2004). Accordingly, the unsaturation index (UI) indicated that as the concentrations of GLA and linoleic acid raised, the lipid composition of the micro-organism became more unsaturated.

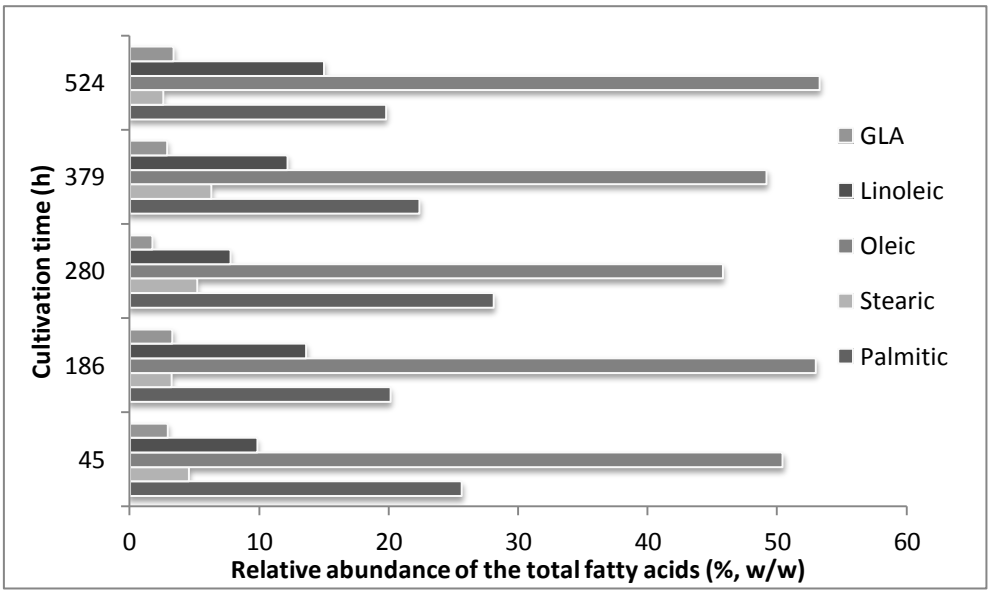


Figure 3.1.3 Fatty acid composition of *Mortierella isabellina* ATHUM 2935 lipid

3.1.3. Fractionation of accumulated oil

Fractionation of accumulated oil of *Mortierella isabellina* ATHUM 2935 into neutral (N), glycolipids plus sphingolipids (G + S) and phospholipids (P), during lipid accumulation phases at this experiment with glucose as sole carbon source revealed that the fraction of neutral lipids (N) was the major constituent of total lipids as it is shown in Table 3.1.5. At the beginning of growing (92h) showed that the percentage of neutral (N) fraction in total lipid was 87.5% which indicated that most of the accumulated lipid proceeds as reserve lipid at this growth step, while the G+ S content was somehow lower (11.3%), but P content was much lower, being 1.2%. There was a slight increase in G + S and P content during 186h of cultivation equal to 0.3% respectively, while the neutral lipid content remained almost constant. P content, however, decreased slightly 0.7% while G + S content showed the greatest decrease 2.3% after 280 h of growth.

Table 3.1.5 Percentages (in%, w/w) of neutral (N), sphingolipid and glycolipid (G + S) and phospholipid (P) fractions during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

Time (h)	Fraction	Percentage %	Recovery %
92	N	87.5	
	P	1.2	103
	G+S	11.3	
186	N	87.0	
	P	1.5	105
	G+S	11.6	
280	N	89.9	
	P	0.8	101
	G+S	9.3	

NL: neutral lipids, P: phospholipids, G+S: glycolipids and sphingolipids

3.1.5. Quantitative determination of phospholipids

In this fermentation, the determination of the phospholipid content was a necessity to grade the quality of the single cell oil produced by *Mortierella isabellina* ATHUM 2935 growing in commercial glucose 80 g/L under nitrogen limited conditions and examine the quantity of the phosphorus in the oil produced by this strain. The quantitative determination of phospholipids was based on the determination of phosphorus content in the lipid samples according to Rouser *et al*., (1970*).* The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. The lipid samples were chosen from three different growth phases (92, 240 and 379h of cultivation) and prepared in appropriate concentrations, as it is shown in Table 3.1.6.

Table 3.1.6 Quantitative composition of Phosphorus (in μg/ g Lipid) and phospholipids (in mg/g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

Time (h)	**Total lipids (g/L)**	**Sample: crude (mg/mL)**	*μg Phopsphorus /g Lipid*	*mg Phospholipids /g lipid*	*Phospholipids (g / L)*
92	9.64	25.35	0.250	**6.25**	0.060
240	10.15	26.27	0.407	**10.16**	0.103
379	10.56	25.45	0.157	**3.93**	0.041

The phospholipid fraction during lipid accumulation phases did not remain constant as it is observed in Table 3.1.6. and Figure 3.1.4. During the growth phase (92h) the phospholipid content achieved the value of 6.25mg. The greatest content of phospholipids was noted in the middle of the growth (240h after cultivation) and it was equal to 10.16 mg/g lipid with a phosphorus content 0.407 μg/g lipid. After 379h of cultivation the phospholipids content decreased to 3.93 mg/g lipid.

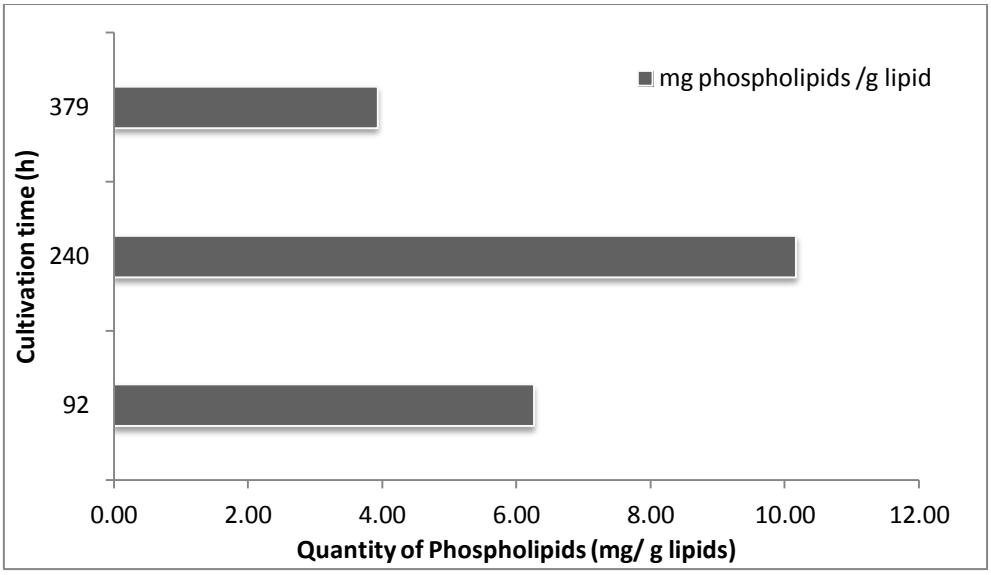


Figure 3.1.4 Quantity of phospholipid fraction during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

3.1.5. Quantitative determination of sugar content in lipid fractions

The constituent hexoses were determined as glucose from a modification of phenol sulfuric method according to Kushwaha & Kates, (1980). The sugar content of the samples was calculated based on the standard curves of glucose and was expressed in mg glucose /g lipid. The lipid samples were chosen from three different growth phases (92, 240 and 379h), it was found that the sugar content, contained at glycolipid and sphingolipid fractions mostly, during the fermentation was not constant (Table 3.1.7.). The results showed an increase in sugar content until 240h of cultivation. The greatest quantity of sugars was noted in the middle of the growth (240h of cultivation) and it was equal to 98.9 mg glucose/ g Lipid. Between 240h and 379 of cultivation, a significant decreased was observed, equal to 64.6.

Table 3.1.7 Sugar content (in mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*mg glucose /g Lipid*
92	9.64	25.35	81.5
240	10.15	26.27	98.9
379	10.56	25.45	34.3

3.2. Cultivation of *Mortierella isabellina* in commercial xylose (S_0= 80g/L)

3.2.1. Growth characteristics and lipid production

Growth of *Mortierella isabellina* ATHUM 2935 on xylose as the sole carbon source at initial concentration of 80 g/L and with initial C/N ratio adjusted at 205 under nitrogen-limited conditions resulted in the accumulation of significant quantities of biomass and oil as it is shown in Figure 3.2.1. NH_4^+ was exhausted from the medium within 45h after inoculation and reserve lipid was synthesized in distinct oil droplets, as it was expected. Low organic acid amounts were produced, since pH value of the growth medium presented a little drop in all growth steps (final pH = 5.4). *Mortierella isabellina* strain ATHUM 2935 grew in full aerobic conditions, and presented noticeable cell growth related to the initial sugar

concentration of 80 g/L as it is showed in Figure 3.2.1. During the cultivation, single cell oil concentrations (in g/L) significantly increased in xylose medium, due to the notable biomass production.

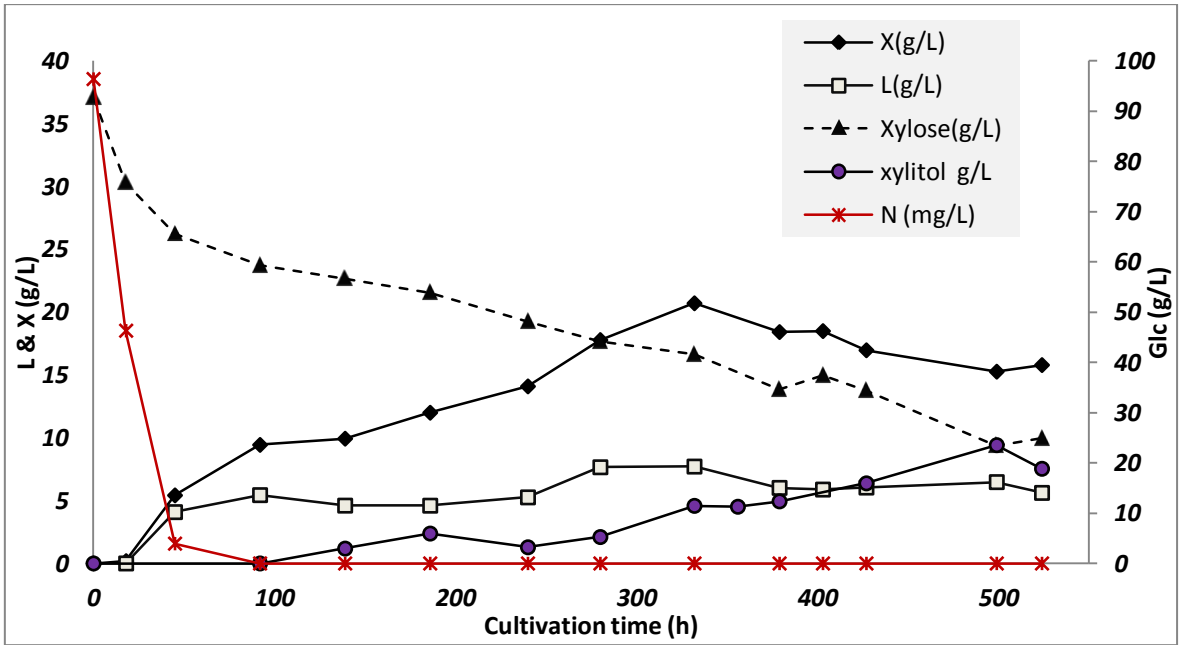


Figure 3.2.1 Kinetics of sugar consumption (Xyl g/L), nitrogen consumption (mg/L), biomass (X, g/L) ,single cell oil (L, g/l) production and xylitol (xylitol, g/L) of *Mortierella isabellina* ATHUM 2935

Commercial xylose was an appropriate substrate for lipid production for this microorganism, resulting in a maximum lipid quantity (L*max*) of 7.7 g/L at 332h after inoculation as it is showed in figure 3.2.1. Culture of *Mortierella isabellina* ATHUM 2935 on this substrate was also accompanied by noticeable biomass production. The maximum biomass production achieved at the same time (332h) and it was remarkably high (20 g/L) (Figure 3.2.1). Large amounts of xylose remained unconsumed into the growth medium (24.9 g/L). In the current study an interesting finding was observed. The cultivation of *Mortierella isabellina* ATHUM 2935 on xylose, favored the production of xylitol, a five carbon sugar alcohol that occurs widely in nature but it is also a normal intermediate in human metabolism. The maximum concentration of xylitol was 23.5 g/L during the end of fermentation (Figure 3.2.1.). The secretion of xylitol can account for the relatively lower final concentrations of produced biomass and cellular lipids by the strain during growth on xylose, as compared with the performance of the micro-organism during growth on glucose. Among microorganisms, yeasts are the best xylitol producers, particularly those belonging to the genus *Candida* (Winkelhausen & Kuzmanova, 1998).

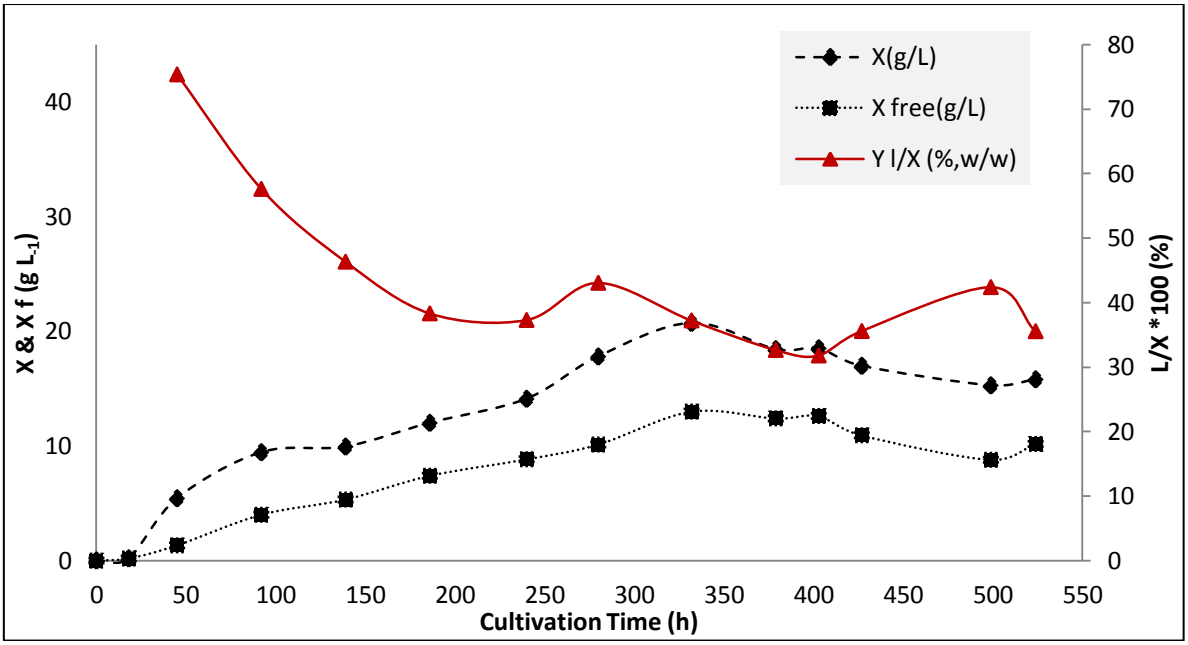


Figure 3.2.2 Representation of total biomass (X, g/L),), lipid in dry weight ($Y_{L/X}$ %, w/w) and lipid free biomass (X free, g/L) during growth of *Mortierella isabellina* ATHUM 2935

Although, low quantities of fat-free biomass were produced, high lipid accumulation occurs inside the mycelia mass. The lipid content in total dry biomass ($Y_{L/X}$ %, w/w) varied from 25% to 73.9 % during growth of *Mortierella isabellina* ATHUM 2935 in xylose (figure 3.2.2.). More specifically, after 45h of cultivation *M. isabellina* produced 5.5 g/L biomass with oil content 73.9%, meaning that, 4.07 g/L of single cell oil was produced. This lipid content was the maximum during the cultivation. In the period between 45 and 186h the oil content decreased rapidly and reached 34.9% in total biomass, meaning that, 3.9 g/L of single cell oil was produced. The maximum oil production 7.7 g/l was achieved at 330 h corresponding to 32.7% lipid content. From this point until the end of fermentation there was a significant decrease about 17% to the oil content in total biomass.

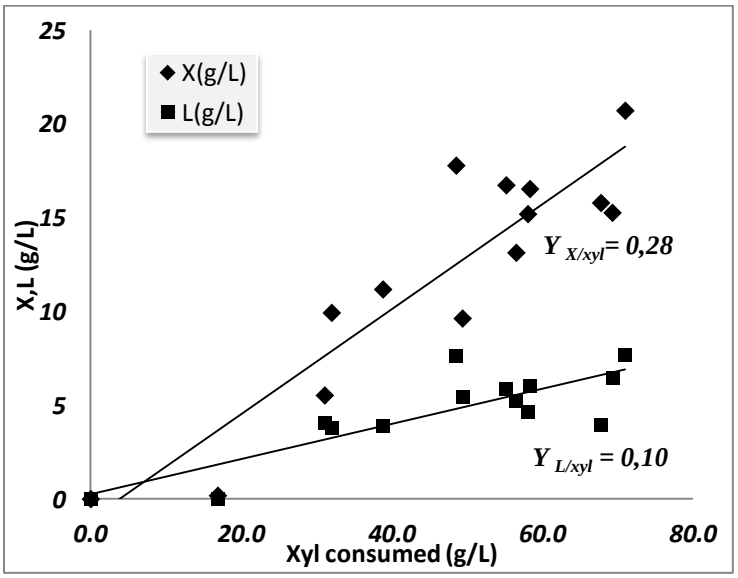


Figure 3.2.3 Biomass (X, g/L) and storage lipid (l, g/L) produced by *Mortierella isabellina* ATHUM 2935 as function of xylose (Xyl, g/L) consumed at initial xylose concentration of 80 g/L

Total biomass and lipid yield on xylose consumed ($Y_{X/Xyl}$ and $Y_{L/Xyl}$) were low and constant (0.28 and 0.10 g/g respectively) at the initial sugar concentration of 80 g/L in the growth medium as it is shown in Figure 3.2.3. It is noted that for various sugars, polysaccharides and poly alcohols utilized as carbon and energy source, values between 0.18 and 0.22 g of lipid synthesized per gram of carbon substrate consumed are considered as optimal for all types of oleaginous microorganisms and culture modes (Ratledge, 1994). According to Ratledge (1988), the metabolism of xylose for SCO production happens through the phosphoketolase pathway resulting in 34% conversion rate which is higher than glucose conversion from glucolytic pathway .The cultivation of *Cunninghamella echinulata* on xylose obtained $Y_{oil/xyl}$ 10% better than the $Y_{oil/Glc}$ (Fakas et al., 2009). However, a relatively lower xylose conversion rate ($Y_{oil/xyl}$ and $Y_{x/xyl}$) by *M. isabellina* is consistent with previous reports (Fakas *et al*., 2009; Ruan *et al*., 2012) implying *M. isabellina* metabolized C5 sugars through the pentose phosphate pathway (Zeng *et al.*, 2013; Papanikolaou & Aggelis, 2011).

3.2.2. Cellular lipid analysis

Mortierella isabellina ATHUM 2935 culture was not accompanied by remarkable fatty acid composition changes of the storage lipid, regardless of the fermentation time. The fatty acid profiles are shown in the table and figure 3.2.4. The length of the fatty acid carbon chain ranged from 14 to 20 for these fungal strain and the major fatty acids were palmitic (C16:0), stearic (C18:0), oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2) and γ-linolenic ($^{\Delta 6,9,12}$C18:3 , GLA) acids. It was observed that the dominant intracellular fatty acid was oleic acid with the percentage of 36–41% w/w of the total fatty acids during the growth on xylose. Palmitic acid was found to be the second most abundant fatty acid in the range of 23- 25.9 % w/w. Linoleic and stearic acid were found in significant quantities into the reserve lipids (were present to a lesser degree than oleic and palmitic) while palmitoleic acid was detected in traces.

Table 3.2.1 Fatty acid composition (% w/w) of *Mortierella isabellina* ATHUM 2935 at various growth phases. IU: The degree of unsaturation (Δ/mole) in the extracted lipid = (1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100).

Carbon source		**Xylose 80 (g L^{-1})**				
Cultivation time (h)		186	280	379	427	524
Fatty acids	**Structure**					
Myristic	**C14:0**	0.85	0.74	nd	0.85	0.79
Palmitic	**C16:0**	**25.90**	**23.18**	**25.68**	**24.45**	**23.44**
Palmitoleic	**$^{\Delta 9}$C16:1**	0.50	0.43	0.48	0.63	0.50
Stearic	**C18:0**	**16.00**	**15.29**	**15.28**	**13.66**	**12.62**
Oleic	**$^{\Delta 9}$C18:1**	**36.77**	**37.94**	**38.17**	**37.29**	**41.21**
Linoleic	**$^{\Delta 9,12}$C18:2**	**12.54**	**13.62**	**13.11**	**13.55**	**13.69**
γ- Linolenic	**$^{\Delta 6,9,12}$C18:3**	**4.42**	**5.09**	**4.92**	**5.14**	**5.41**
Arachidic	**C20:0**	1.37	1.51	1.90	1.41	1.29
Others		1.64	1.89	0.47	3.01	1.07
IU		0.76	0.81	0.80	0.80	0.85

Stearic acid was found to be the third most abundant fatty acid in the range of 12- 16 % w/w. Stearic acid percentage was highest almost in the middle of the growth (16% w/w), decreasing thereafter to 12,6% (w/w). These significant quantities of stearic into the reserve lipids of *Mortierella isabellina* ATHUM 2935 obtained on xylose could be considered unfamiliar in comparison with the amounts of stearic reported in the literature (Gao *et al.*, 2013; Zeng *et al.*, 2013). As it is shown in Table 3.2.1 and Figure 3.2.4, oleic, linoleic and γ-linolenic acid contents increased, with a concomitant decrease in stearic content during the fermentation. Moreover, *M. isabellina* ATHUM 2935 showed satisfactory capability to produce γ-linolenic acid (GLA) on xylose. GLA content in xylose grown mycelia was somewhat higher than that in mycelia grown on glucose or in mediums containing a blend of xylose and glucose. According to Fakas *et al.*, (2009), these discrepancies could be attributed to the different metabolic pathways implicated in the assimilation of each carbon source utilized as a substrate. In this fermentation, GLA($^{\Delta 6,9,12}$C18:3) was presented in all growth steps in the reserve cellular lipids at concentration between 4.42% and 5.41 % with the highest GLA content of 5.41% achieved at 524h, towards the end of fermentation. Accordingly, the unsaturation index (UI) indicated that as the concentrations of GLA and linoleic acid raised, the lipid composition of the micro-organism became more unsaturated.

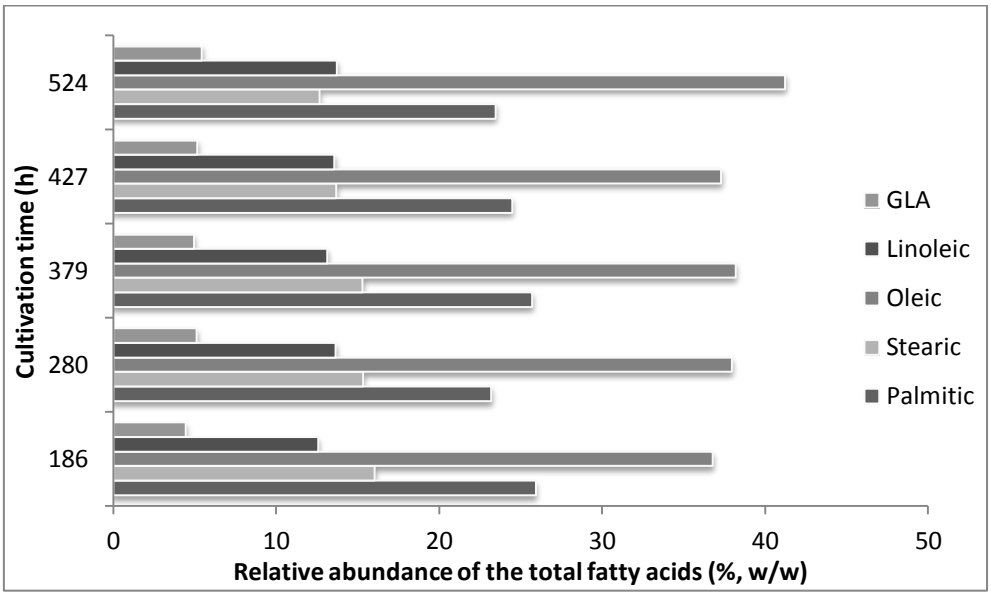


Figure 3.2.4 Fatty acid composition of *Mortierella isabellina* ATHUM 2935 lipid produced in commercial xylose at various cultivation times and at initial concentration of 80 g/L

3.2.3. Fractionation of accumulated oil

The fractionation of accumulated oil of *Mortierella isabellina* strain ATHUM 2935 into neutral (N), glycolipids plus sphingolipids (G + S) and phospholipids (P), during lipid accumulation phases using xylose as sole carbon source revealed that the neutral lipid (N) fraction was the major constituent of total lipids as it is shown in Table 3.2.2. At the beginning of the growth (92h) showed that the percentage of neutral (N) fraction in total lipid was 83.9% which indicated that most of the accumulated lipid proceeds as reserve lipid at this growth step, while the G+ S content was somehow lower (10.2%), but P content was much lower,

being 5.9% . Fractionation of lipids at 139 h showed that neutral lipid and G + S content has a noticeable decrease equal to 4.6%, and 2.2% respectively, while P content showed a significant increase around to 6.8%. Moreover, the neutral and G + S content increased again 6.1% and 3% , respectively, while P content showed the greatest decrease equal to 9% after 240 h of growth.

Table 3.2.3 Percentages (in%, w/w) of neutral (N), sphingolipid and glycolipid (G + S) and phospholipid (P) fractions during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on xylose as sole carbon source at initial concentration of 80 g/L .

Time (h)	Fractions	Percentage %	Recovery %
45	N	83.9	
	P	5.9	96
	G+S	10.2	
139	N	79.3	
	P	12.7	102
	G+S	8.0	
240	N	85.4	
	P	3.6	108
	G+S	11.0	

NL: neutral lipids, P: phospholipids, G+S: glycolipids and sphingolipids

3.2.5. Quantitative determination of phospholipids

In order to grade the quality of the oil produced by *Mortierella isabellina* ATHUM 2935 growing in commercial xylose 80 g/L under nitrogen limited conditions and examine the quantity of the phosphorus in the oil produced by this strain, the knowledge of the phospholipid content was also a necessity. The quantitative determination of phospholipids was based on the determination of phosphorus content in the lipid samples according to Rouser *et al.*, (1970). The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. The lipid samples were chosen from three different growth phases) and before the application of the method, were redefined in appropriate concentrations, as it is shown in table 3.2.4.

Table 3.2.4 Quantitative composition of Phosphorus (in μg/ g Lipid) and phospholipids (in mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*μg Phopsphorus /mg Lipid*	*mg Phospholipids /g lipid*	*Phospholipids (g / L)*
45	4.09	20.66	0.157	**4.19**	0.017
240	5.26	23.16	0.167	**5.91**	0.031
379	4.66	23.31	0.237	**10.39**	0.048

The quantity of phosphorus and therefore the phospholipid content during the three different lipid accumulation phases (45, 240 and 379 h of cultivation showed a significant increase as it is observed in the table 3.2.4. and figure 3.2.4. The first 45 h of cultivation, the phospholipid fraction achieved the value of 4.19 mg. There was a slight increase in P content during 240 h of cultivation equal 5,19 mg, while it was

observed a greatest increase equal to 10.39 mg with a phosphorus content of 0.237μg/mg lipid after 379 h of growth.

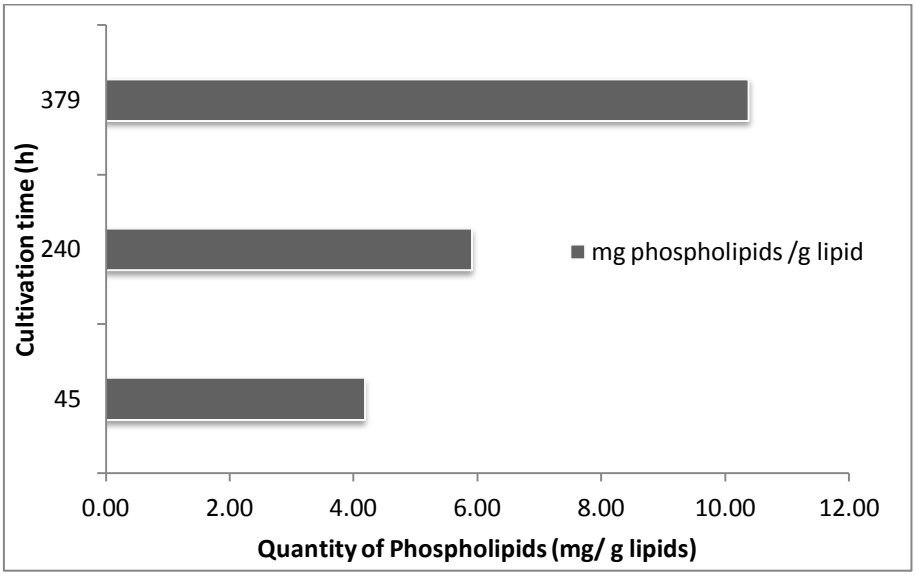


Figure 3.2.5 Quantity of phospholipid fraction during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

3.2.5. Quantitative determination of sugar content in lipid fractions

The lipid samples were chosen from three different growth phases (45, 240 and 379 h) and before the application of the method, were redefined in appropriate concentrations. As the results, it was found that the sugar content, contained at glycolipid and sphingolipid fractions mostly, during the fermentation wasn't constant as it is shown in table 3.2.5. The results showed an increase in sugar content during the cultivation time and the greatest quantity of sugars was noted near to the end of the growth (379h of cultivation) equal to 95.3 mg glucose/ g Lipid.

Table 3.2.5 The sugar content (in g/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*mg glucose /g Lipid*
45	4.09	20.66	23.0
240	5.26	23.16	82.2
379	4.66	23.31	95.3

3.3. Cultivation of *Mortierella isabellina* in a carbon blend of commercial glucose & xylose (S_0= 40:40 g/L Glc:Xyl)

3.3.1. Growth characteristics and lipid production

In order to examine the oil accumulation process in *Mortierella isabellina*, a kinetic experiment was conducted in nitrogen-limited media having as substrate a blend of commercial glucose and xylose in the same proportion (40:40 g/L) at initial concentration of 80 g/L and with initial C/N ratio adjusted at 205 mol/mol. Unexceptionally, there was an almost complete exhaustion of nitrogen source in the fermentation medium occurring approximately at 45 h after inoculation, and the lipid accumulation was enhanced

immediately, synthesized distinct oil droplets, visible in microscope. And in this fermentation, low organic acid amounts were produced, since pH value of the growth medium presented a little drop in all growth steps (final pH = 4.7). Data concerning growth and lipid production (related to the initial sugar concentration of 80 g/L) by *Mortierella isabellina* ATHUM 2935 in this carbon blend utilized as substrate is depicted in the figure 3.4.1. The microorganism grew significantly well on this commercial glucose & xylose blend. Although, low quantities of fat-free biomass were produced, high lipid accumulation occurs inside the mycelia mass. The maximum biomass production of 23.1 g/L was achieved after 379h of cultivation with an oil content in total dry biomass ($Y_{L/X}$ %, w/w) of 47.2%, meaning that, 8.74 g/L SCO was produced and this was the maximum lipid quantity (L max) which was achieved in this fermentation. Large amounts of the total sugars remained unconsumed into the growth medium (22.8 g/L).

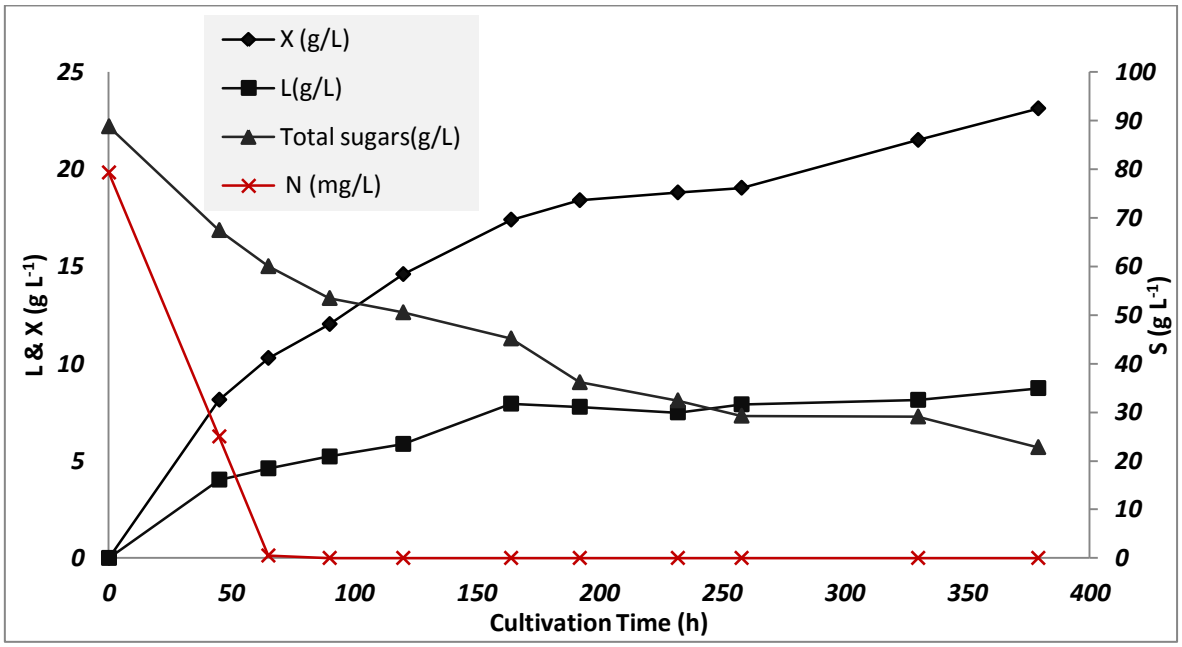


Figure 3.3.1 Kinetics of sugars consumption (S total, g/L), biomass (X, g/L) and single cell oil (L, g/l) production of *Mortierella isabellina* ATHUM 2935 growing on a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L)

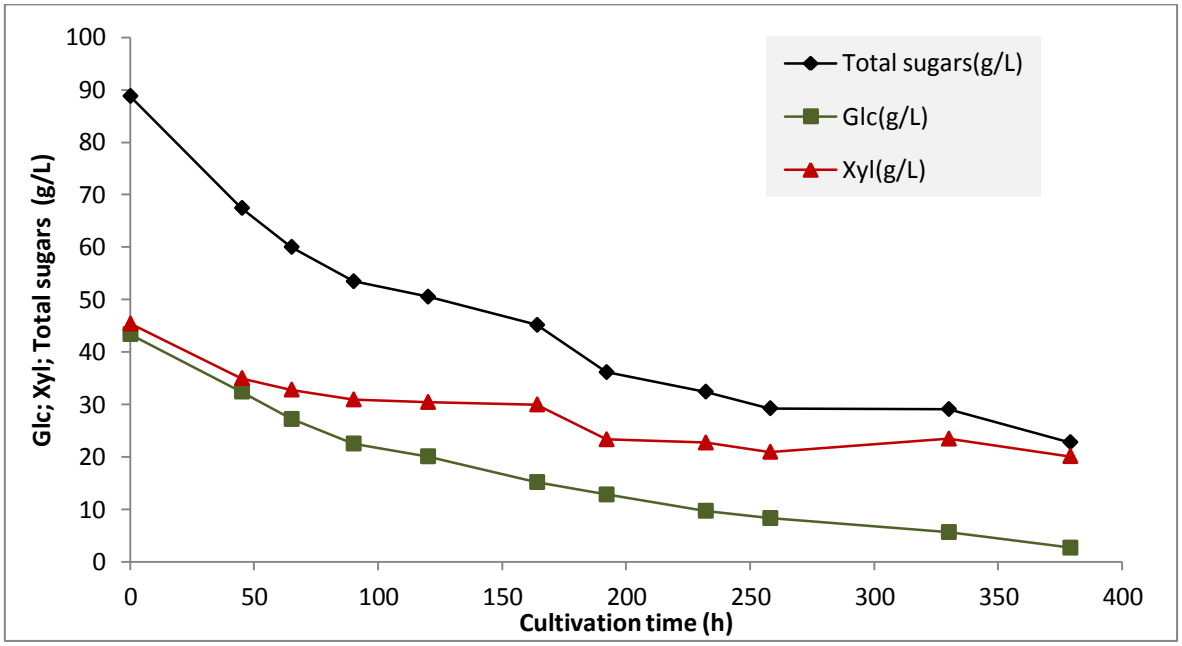


Figure 3.3.2 Kinetics of total sugars consumption (S total, g/L), glucose consumption (Glc, g/L), xylose consumption (Xyl, g/l), of *Mortierella isabellina* ATHUM 2935 growing on a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L).

As regards the consumption of substrate, it is observed that (figure 3.3.2.),in the first 65h of inoculation, the microorganism started the simultaneous assimilation of both sugars occurred regardless of the initial concentration of the sugars adjusted into the medium, with almost the same rate, 0.36 g/L/h and 0.28g/L/h, respectively. After 90 hours there was a constant rate of glucose consumption with a concomitant decrease in the rate of xylose consumption (0.13 g/L/h) reaching the rate of 0.08 g/L/h after 258h of growth. This resulted in the consumption of nearly all the glucose (2.67 g/L) while significant amounts of xylose (20.1 g/L) remained unconsumed at the end of fermentation.

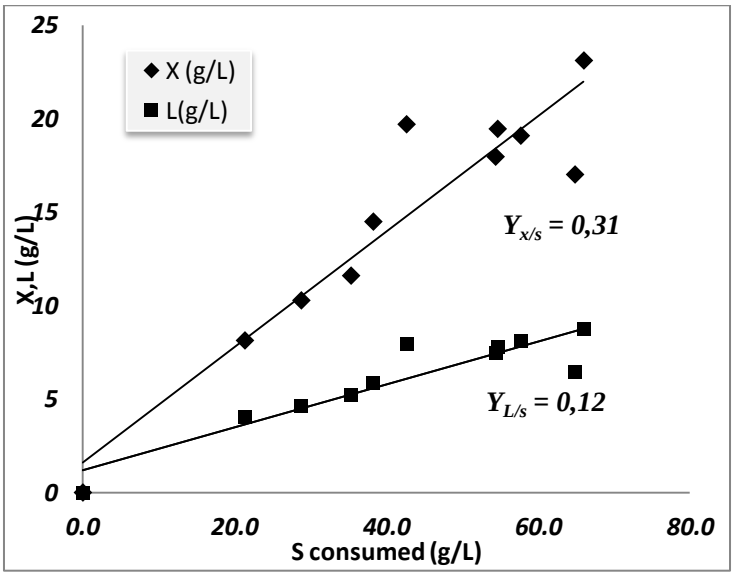


Figure 3.3.3 Biomass (X, g/L) and storage lipid (L, g/L) produced by *Mortierella isabellina* ATHUM 2935 as function of the carbon blend, glucose and xylose (S, g/L) consumed

Total biomass and lipid yield on this carbon blend of glucose and xylose consumed ($Y_{X/S}$ and $Y_{L/S}$) presented constant and remarkable values (0.31 and 0.12 g/g respectively) at the initial sugar concentration of 80 g/L in the growth medium as it is shown in figure 3.3.3.

3.3.2. Cellular lipid analysis

The fatty acid profiles are shown in table 3.3.1 and figure 3.3.5. The length of the fatty acid carbon chain ranged from 14 to 20 for these fungal strains and the major fatty acids were palmitic (C16:0), stearic (C18:0), oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2) and γ-linolenic ($^{\Delta 6,9,12}$C18:3 , GLA) acids. The most predominant component in the mycelium was oleic acid in the range of 33–48% w/w of the total fatty acids during the growth on a medium containing a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L) at initial concentration of 80 g/L under nitrogen limited conditions. Palmitic acid was found to be the second most abundant fatty acid with the percentage of 21-23% w/w. The results showed that linoleic and stearic acid were found in significant quantities into the reserve lipids while palmitoleic acid was detected in traces.

Table 3.3.1 Fatty acid composition (% w/w) of *Mortierella isabellina* ATHUM 2935 at various growth phases. IU: The degree of unsaturation (Δ/mole) in the extracted lipid = (1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100).

Carbon blend		**Glucose : xylose (40:40 g L^{-1})**				
Cultivation time (h)		45	120	232	330	379
Fatty acid	**Structure**					
Myristic Acid	**C14:0**	0.43	0.78	0.66	0.70	0
Palmitic	**C16:0**	**22.48**	**23.86**	**21.52**	**22.83**	**21.87**
Palmitoleic	**$^{\Delta 9}$C16:1**	0.63	0.07	0.53	0.69	0
Stearic	**C18:0**	**14.94**	**15.48**	**11.63**	**7.95**	**8.53**
Oleic	**$^{\Delta 9}$C18:1**	**33.26**	**39.22**	**44.00**	**46.54**	**48.52**
Linoleic	**$^{\Delta 9,12}$C18:2**	**9.31**	**11.86**	**12.62**	**14.61**	**15.56**
γ- Linolenic	**$^{\Delta 6,9,12}$C18:3**	**2.76**	**3.39**	**3.53**	**3.84**	**4.05**
Arachidic Acid	**C20:0**	0.27	1.26	1.39	1.36	0
Others		4.84	4.07	4.12	1.48	1.47
IU		0.65	0.73	0.80	0.88	0.92

It was observed a significant decrease (7%) in the stearic acid content during the fermentation while the higher percent (14.95%) achieved at the beginning of the growth (45h) when the lipid content in the total biomass was almost 50%. As it is shown in Table 3.3.1 the oleic acid was the major fatty acid in microbial oil at all growth stages although its percentage decreased almost 15% as oil accumulation progressed. It was observed small increases 6,25% and almost 2% in linoleic and γ-linolenic acid contents near the end of growth, respectively. *Mortierella isabellina* ATHUM 2935 showed lower capability to produce γ-linolenic acid (GLA) on a medium containing xylose and glucose in equal proportion than on xylose as a sole carbon source. Although, GLA($^{\Delta 6,9,12}$C18:3) was presented in all growth steps in the reserve cellular lipids , the concentration range between 2.76% and 4.05 % with the highest GLA content of 4.05 % achieved at 379 h, towards the end of fermentation. Accordingly, the unsaturation index (UI) indicated that as the concentrations of GLA and linoleic acid raised, the lipid composition of the micro-organism became more unsaturated.

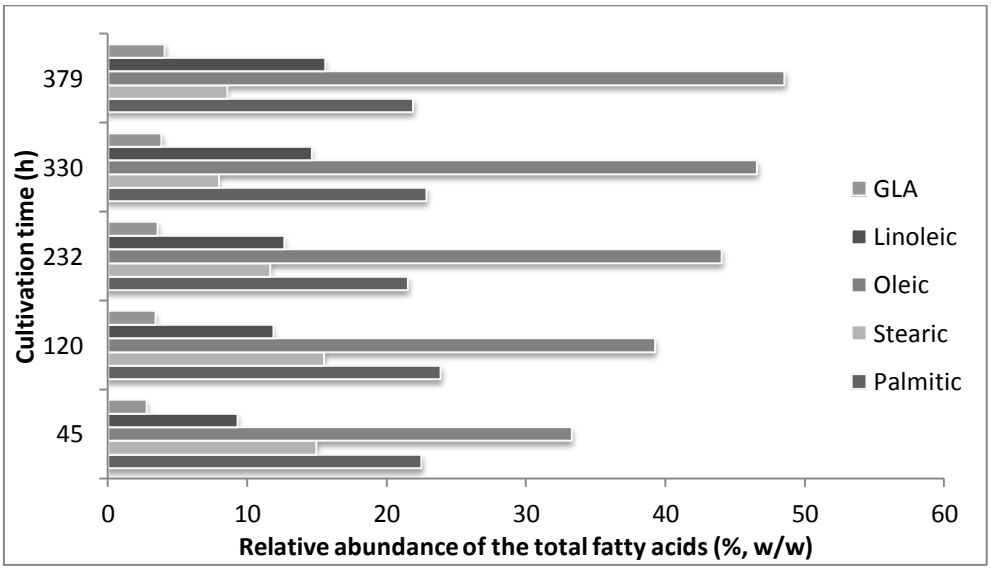


Figure 3.3.5 Fatty acid composition of the *Mortierella isabellina* ATHUM 2935 lipid produced in a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L)

3.3.3. Fractionation of accumulated oil

The fractionation of accumulated oil of *Mortierella isabellina* ATHUM 2935 into neutral (N), glycolipids plus sphingolipids (G + S) and phospholipids (P), during lipid accumulation phases using a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L) as carbon source revealed that the neutral lipid (N) fraction was the major constituent of total lipids as it is shown in the table 3.3.2. It is worth mentioning that the neutral lipid (N) content showed a significant increase of 7.1% after 120h, and a further increase occurred at 232h of growth equal to 2.4%. However, the percentage of G + S and P fractions was high at the beginning of growth but declined thereafter (table 3.3.2.), with an overall decrease of 8.8 and 0.8, respectively.

Table 3.3.2 Percentages (in%, w/w) of neutral (N), sphingolipid and glycolipid (G + S) and phospholipid (P) fractions during lipid accumulation phases of *Mortierella isabellina* ATHUM

Time (h)	Fractions	Percentage%	Recovery %
65	N	83.6	107
	P	3.4	
	G+S	13.1	
120	N	90.7	85
	P	1.7	
	G+S	7.6	
232	N	93.1	88
	P	2.6	
	G+S	4.3	

NL: neutral lipids, P: phospholipids, G+S: glycolipids and sphingolipids

3.3.4. Quantitative determination of phospholipids

The quantitative determination of phospholipids was based on the determination of phosphorus content in the lipid samples according to Rouser *et al.,* (1970*).* The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. The lipid samples produced by *Mortierella isabellina* ATHUM 2935 growing in on a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L) at initial concentration of 80 g/L under nitrogen limited conditions were chosen from three different growth phases, as it is shown in table 3.3.3.

Table 3.3.3 Quantitative composition of Phosphorus (in μg/ g Lipid) and phospholipids (in mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*μg Phopsphorus /mg Lipid*	*mg Phospholipids /g lipid*	*Phospholipids (g / L)*
45	4.03	26.17	0.077	**1.91**	0.008
120	5.86	22.25	0.513	**12.82**	0.075
232	7.49	25.15	0.176	**4.40**	0.033

The phospholipid content during three different lipid accumulation phases (45, 120 and 232 h of cultivation) did not remain constant as it is observed in table 3.3.3 and figure 3.3.6. At early growth, 45h of cultivation it was observed that the phospholipid content was at the lowest quantity of 1.91 mg. The greater quantity of phospholipid was noted after 120h of cultivation and it was equal to 12.82 mg with a phosphorus content 0.513 μg/mg lipid. There was a notable decrease after that equal to 8.42 mg in 232 h of cultivation.

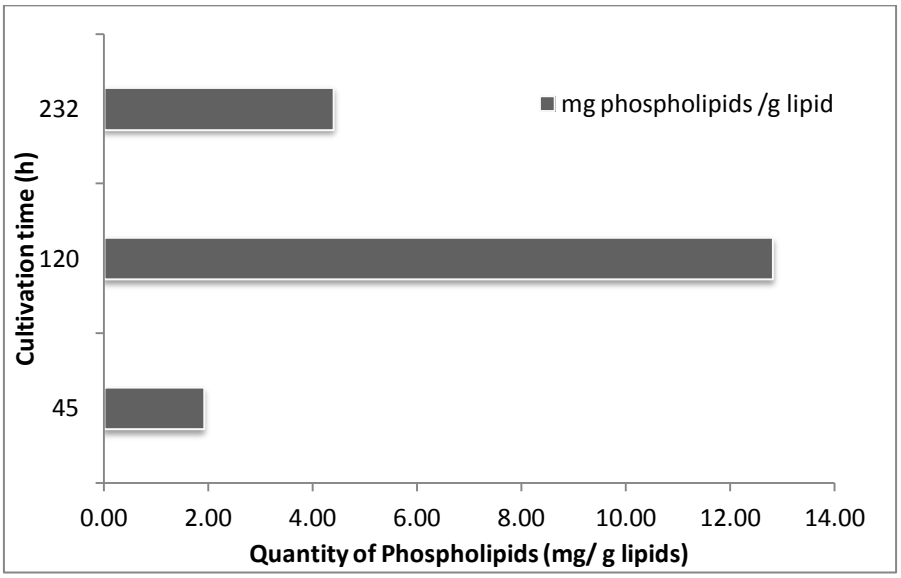


Figure 3.3.6 Quantity of phospholipid fraction during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L)

3.3.5. Quantitative determination of sugar content in lipid fractions

The lipid samples were chosen from three different growth phases (45, 120 and 232 h) and before the application of the method, were redefined in appropriate concentrations. As the results, it was found that the sugar content, contained at glycolipid and sphingolipid fractions mostly, during the fermentation wasn't constant as it is shown in table 3.3.4.below. The results showed a twofold increase in sugar content between the beginning of the growth (45h) and 120h after cultivation. The greatest quantity of sugars was achieved in this point and it was equal to the value of 103.3 mg glucose/ g Lipid, followed by a notable decrease of 64.6 mg, 232h after cultivation.

Table 3.3.4 The sugar content (in g/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 on a carbon blend of commercial glucose (40 g/L) & xylose (40 g/L)

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*mg glucose /g Lipid*
45	4.03	26.17	51.4
120	5.86	22.25	103.3
232	7.49	25.15	38.1

3.4. Cultivation of *Mortierella isabellina* in a carbon blend of commercial glucose & xylose (S_0= 60:20 g/L Glc:Xyl)

3.4.1. Growth characteristics and lipid production

Kinetics of biomass and lipid production were conducted in nitrogen-limited media having as substrate a blend of commercial glucose (60 g/L) and xylose (20 g/L) at initial concentration of 80 g/L and with initial C/N ratio adjusted at 205. Up to 40–43 h after inoculation, the fungus consumed all of the available nitrogen and then, reserve lipid was synthesized in distinct oil droplets. Low organic acid amounts were produced, since pH value of the growth medium presented a little drop in all growth steps (final pH = 5.6). Data concerning growth and lipid production by *Mortierella isabellina* ATHUM 2935 in this blend of glucose and xylose utilized as substrate are depicted in figure 3.4.1. As it is shown, the microorganism grew particularly well on this blend, achieving satisfactory growth and lipid production related to the initial sugar concentration of 80 g/L. The maximum biomass concentration achieved after 397 h of cultivation and it was remarkably high (up to 27.9 g/l) with a lipid content in total dry biomass ($Y_{L/X}$ %, w/w) of 46.1% w/w, meaning that 12.84 g/L SCO was produced at this growth step. During the cultivation, single cell oil concentrations (in g/L) significantly increased in this medium, due to the notable biomass production. Under these conditions lipid accumulation reached the value of 58 % in fungal biomass ($Y_{L/X}$ %, w/w), corresponding to the maximum oil production (L*max*) of 15.59 g/l at 280h after inoculation and at this growth step the fermented mass was 27 g/L .

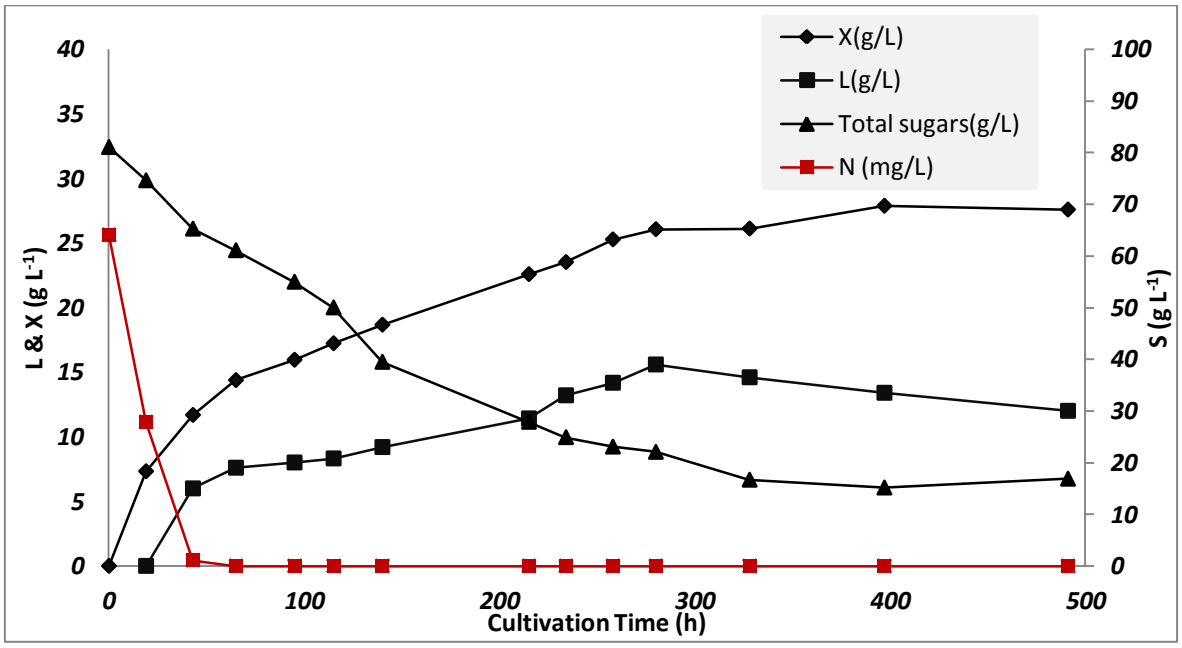


Figure 3.4.1 Kinetics of sugars consumption (S total, g/L), nitrogen (mg/L), biomass (X, g/L) and single cell oil (L, g/l) production of *Mortierella isabellina* ATHUM 2935 growing on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

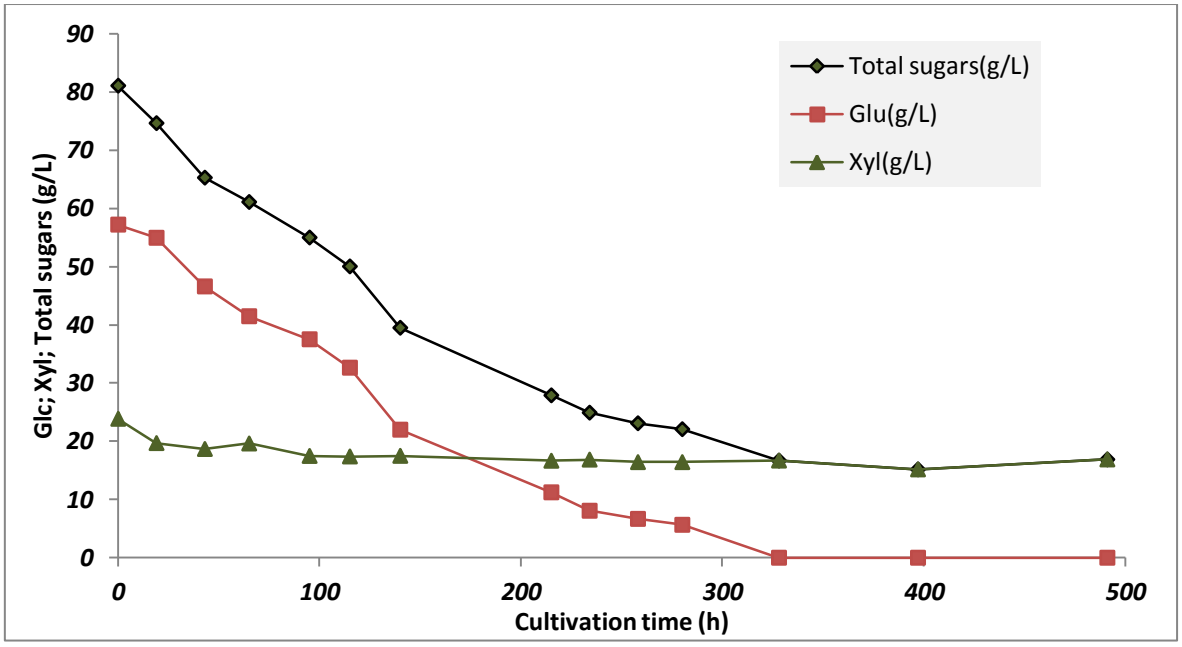


Figure 3.4.2 Kinetics of total sugars consumption (S total, g/L), glucose consumption (Glc, g/L), xylose consumption (Xyl, g/l) of *Mortierella isabellina* ATHUM 2935 growing on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

Concerning the uptake of individual sugars in this fermentation with glucose and xylose used as carbon blend- substrate, it must be stressed that *Mortierella isabellina* ATHUM 2935 showed preference for consumption of glucose. Glucose was noticeably faster consumed as compared to xylose from the beginning of growth as it is shown in Figure 3.4.2. Glucose consumption was almost complete (97 %, wt/wt) after 280h of cultivation, while until the end of growth significant amounts of xylose remained unconsumed into the growth medium (16.9 g/L). It should be noted that no degradation (turnover) of the accumulated lipid occurred during stationary phase.

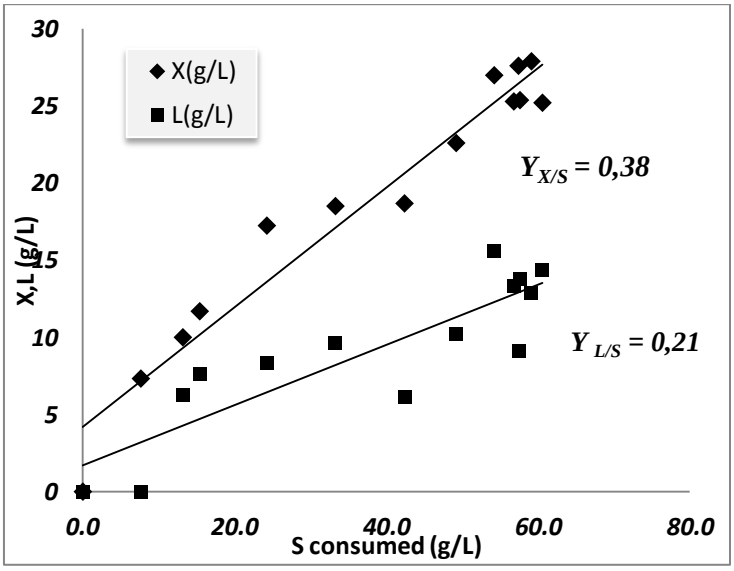


Figure 3.4.3 Biomass (X, g/L) and storage lipid (l, g/L) produced by *Mortierella isabellina* ATHUM 2935 as function of the carbon blend, glucose and xylose (S, g/L) consumed

Total biomass and lipid yield on this carbon blend of glucose and xylose consumed ($Y_{X/S}$ and $Y_{L/S}$) presented constant and remarkable values (0.38 and 0.21 g/g respectively) at the initial sugar concentration of 80 g/L in the growth medium as it is shown in the Figure 3.4.3.

3.4.2. Cellular lipid analysis

The fatty acid composition of intra-cellular lipids was analyzed by GC at various cultivation times as it is shown in table3.4.1. and figure 3.4.4. The length of the fatty acid carbon chain ranged from 14 to 20 for these fungal strain and the major fatty acids were palmitic (C16:0), stearic (C18:0), oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2) and γ-linolenic ($^{\Delta 6,9,12}$C18:3 , GLA) acids comprising over 90% of total fatty acids. Oleic acid was the dominant cellular fatty acid with the percentage of 38–49.9% w/w of the total fatty acids during the growth on a medium containing a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L) at initial concentration of 80 g/L under nitrogen limited conditions. The second most abundant fatty acid was the palmitic acid with the percentage of 18-28% w/w. The results showed that linoleic acid was found in significant quantities into the reserve lipids while palmitoleic and stearic acid was detected in low amounts.

Table 3.4.1 Fatty acid composition in the total cellular lipid (% *w/w*) of *Mortierella isabellina* ATHUM 2935 at various growth phases, cultivated on medium containing a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L). IU: The degree of unsaturation (Δ/mole) in the extracted lipid = (1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100).

Carbon blend		**Glucose : xylose (60:20 g L^{-1})**						
Cultivation time (h)		65	95	140	215	280	328	491
Fatty acids	**Structure**							
Myristic	**C14:0**	0.83	0.57	1.08	0.72	0.57	0.66	nd
Palmitic	**C16:0**	**27.88**	**28.41**	**26.41**	**21.46**	**18.62**	**19.55**	**25.07**
Palmitoleic	**$^{\Delta 9}$C16:1**	0.82	0.43	1.96	2.02	1.42	2.20	2.56
Stearic	**C18:0**	4.98	4.65	3.36	3.01	2.77	3.29	2.64
Oleic	**$^{\Delta 9}$C18:1**	**38.38**	**44.08**	**42.12**	**47.75**	**49.99**	**49.55**	**44.88**
Linoleic	**$^{\Delta 9,12}$C18:2**	**7.51**	**12.17**	**11.76**	**14.16**	**14.91**	**13.73**	**13.61**
γ- Linolenic	**$^{\Delta 6,9,12}$C18:3**	**2.11**	**2.87**	**3.23**	**3.22**	**3.33**	**2.94**	**2.97**
Arachidic	**C20:0**	1.00	1.40	3.36	1.34	1.45	1.81	nd
Others		16.47	5.42	6.72	6.31	6.93	6.26	8.27
IU		0.61	0.77	0.77	0.88	0.91	0.88	0.84

At all growth stages oleic acid was the major fatty acid found in microbial oil, while its percentage increased as oil accumulation progressed with a small decrease 5% near the end of the fermentation. Also, it was observed a significant increase 7% in linoleic acid contents near the middle of the fermentation which followed by a small decrease 1.3% near the end of growth. *Mortierella isabellina* ATHUM 2935 showed low capability to produce γ-linolenic acid (GLA) on a medium containing bigger quantity of glucose (60g/L) than on xylose (20g/L). GLA($^{\Delta 6,9,12}$C18:3) was presented in all growth steps in the reserve cellular lipids with a percentage of 2.1- 3.3 % w/w similar to those which observed when glucose used as a sole carbon source at initial concentration of 80g/L. The highest GLA content of 3.33 % achieved at 280h after inoculation. Accordingly, the unsaturation index (UI) indicated that as the concentrations of GLA and linoleic acid raised, the lipid composition of the micro-organism became more unsaturated.

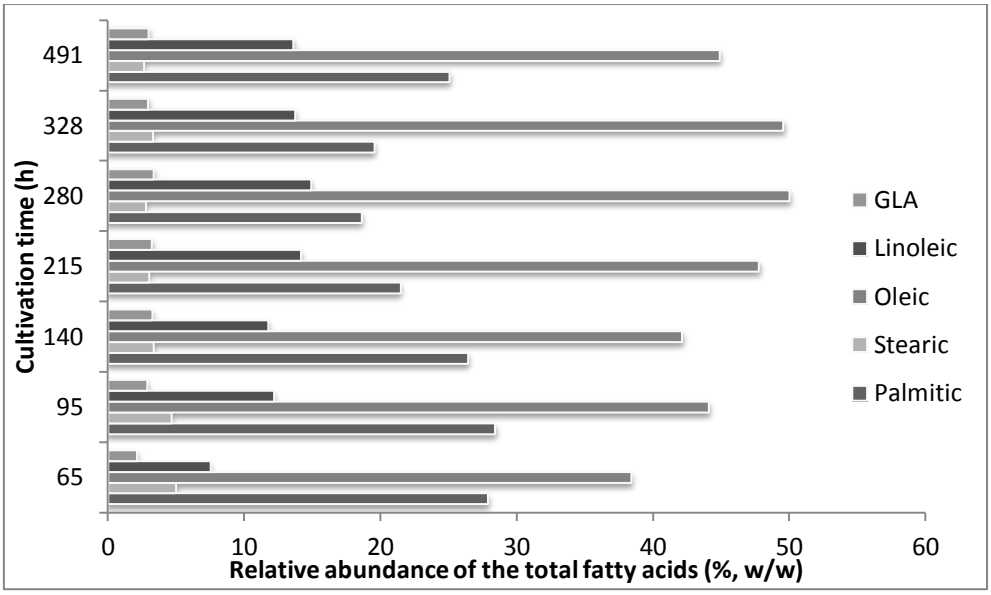


Figure3.4.4 Fatty acid composition of the *Mortierella isabellina* ATHUM 2935 lipid produced in a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L).

3.4.3. Fractionation of accumulated oil

The fractionation of accumulated oil of *Mortierella isabellina* strain ATHUM 2935 into neutral (N), glycolipids plus sphingolipids (G + S) and phospholipids (P), during lipid accumulation phases using a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L) as carbon source revealed that the neutral lipid (N) fraction was the major constituent of total lipids as it is shown in the Table 3.4.2. At early growth (43h) showed that the percentage of neutral (N) fraction in total lipid was 87.3% which indicated that most of the accumulated lipid proceeds as reserve lipid at this growth step, while the G+ S and P content was somehow the same 6.5% & 6.2% , respectively. The neutral lipid (N) and G+ S content remained almost constant after 234h and only in P content there was a slight decrease of 1%. Fractionation of lipids at 397 h showed that the neutral content showed a significant decrease equal to 14% while in the other two fractions (G+ S & P) was observed a significant increase of 11.6 % and 2.1%, respectively.

Table 3.4.2 Percentages (in%, w/w) of neutral (N), sphingolipid and glycolipid (G + S) and phospholipid (P) fractions during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

Time (h)	Fraction	Percentage %	Recovery %
43	N	87.3	
	P	6.5	103
	G+S	6.2	
234	N	87.8	
	P	5.5	105
	G+S	6.7	
397	N	72.9	
	P	8.8	101
	G+S	18.3	

3.4.4. Quantitative determination of phospholipids

The quantitative determination of phospholipids was based on the determination of phosphorus content in the lipid samples according to Rouser *et al.*, (1970*)*. The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. The lipid samples produced by *Mortierella isabellina* ATHUM 2935 growing in on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L) at initial concentration of 80 g/L under nitrogen limited conditions were chosen from three different growth phases, as it is shown in table 3.4.3.

Table 3.4.3 Quantitative composition of phosphorus (in μg/ g Lipid) and phospholipids (in mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

Time (h)	**Total lipids (g/L)**	**Sample: crude (mg/mL)**	*μg Phopsphorus /mg Lipid*	*mg Phospholipids /g lipid*	*Phospholipids (g / L)*
43	7.65	25.47	0.322	**8.05**	0.062
234	14.36	25.67	0.233	**5.84**	0.084
397	12.84	26.62	0.462	**11.55**	0.148

The phospholipid content during three different lipid accumulation phases (43, 234 and 397 h of cultivation) did not remain constant as it is observed in the table 3.4.3. and the figure 3.4.5. At early growth, 43h of cultivation it was observed that the phospholipid content was at a high quantity of 8.05 mg/g lipid. The greater quantity of phospholipid was noted after 397 h of cultivation and it was equal to 11.55 mg with a phosphorus content 0.462 μg/mg lipid. There was a notable decreased in 234 h of cultivation, in which the quantity of phospholipid fraction received the value of 5.84mg.

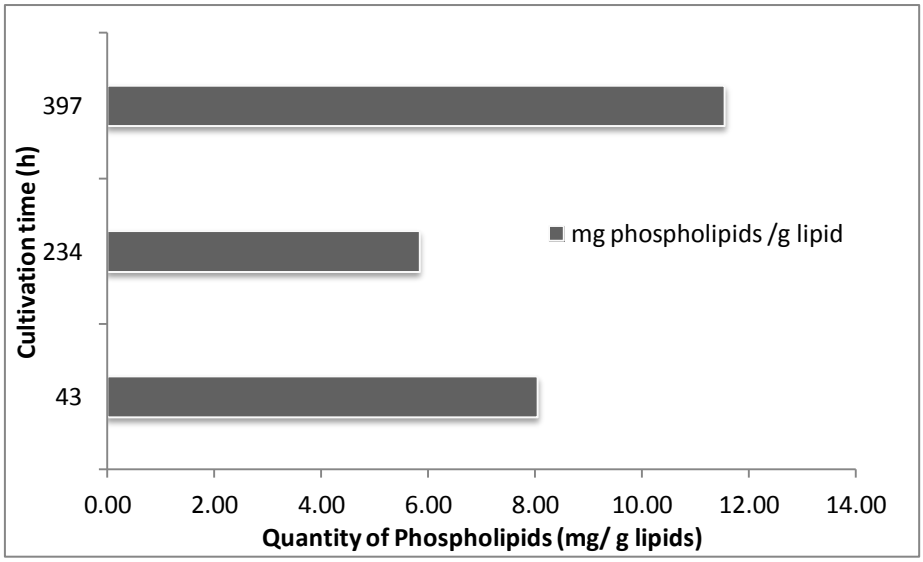


Figure 3.4.5 Quantity of phospholipid fraction during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

3.4.5. Quantitative determination of sugar content in lipid fractions

The lipid samples were chosen from three different growth phases (43, 234 and 397 h) and before the application of the method, were redefined in appropriate concentrations. As the results, it was found that the sugar content, contained at glycolipid and sphingolipid fractions mostly, during the fermentation was not constant as it is shown in table 3.4.3. The results showed a notable decrease in sugar content during the cultivation time and the greatest quantity of sugars was noted at the beginning of the growth (43h of cultivation) equal to 103.8 mg glucose/ g Lipid. Near to the end of fermentation (397h) the sugar content had a six-fold decrease, achieved the value of 16.0 mg glucose/ g Lipid.

Table 3.4.3 The sugar content (in g/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*mg glucose /g Lipid*
43	7.65	25.47	103.8
234	14.36	25.67	23.3
397	12.84	26.62	16.0

3.5. Cultivation of *Mortierella isabellina* in a carbon blend of commercial glucose & xylose (S_0= 20:60 g/L Glc:Xyl)

3.5.1. Growth characteristics and lipid production

In order to examine the oil accumulation process in higher substrate concentrations, a kinetic experiment was conducted in nitrogen-limited media having as substrate a blend of commercial xylose (60 g/L) and glucose (20 g/L) .In this case the initial concentration of reducing sugars was 80 g/L and the initial C/N ratio equal to 205. Onset of lipid accumulation as distinct oil droplets occurred directly after complete exhaustion of ammonium nitrogen from the fermentation medium (approximately 43 h after inoculation). Low organic acid amounts were produced, since pH value of the growth medium presented a little drop in all growth steps (final pH = 5.6). Data concerning growth and lipid production by *Mortierella isabellina* ATHUM 2935 in this blend of xylose and glucose utilized as substrate are depicted in figure 3.5.1. As it is showed, the microorganism presented a satisfactory microbial growth on this carbon blend. Although, low quantities of fat-free biomass were produced, high lipid accumulation occurs inside the mycelia mass. The microorganism exhibited the maximum biomass concentration of 20.5 g/L after 258 h of cultivation with a simultaneous maximum oil production (L *max*) of 12.28 g/L.

The cellular lipid content in total dry biomass ($Y_{L/X}$ %, w/w) at this growth step reached 59.8 % w/w. *Mortierella isabellina* on this blend of xylose and glucose, benefited the production of xylitol, reaching a maximum concentration of 17.2 g/L, as it is shown in figure 3.5.1. However, the amount of accumulated xylitol remained lower than that achieved on the substrate with xylose as the sole carbon source, indicating in accordance with the relevant literature (Kim et al. 2009), that xylitol production could be significantly

altered by the presence of glucose in the fermentation medium. Specifically, xylitol accumulation began only after glucose was depleted from the culture medium, revealing an obstructive role of glucose on glucose–xylose mixtures, in terms of xylitol secretion. Furthermore, as it is shown in figure 3.5.1., there was a small consumption of produced xylitol occurred at the end of the fermentation, a time point in which xylose was almost depleted from the culture medium.

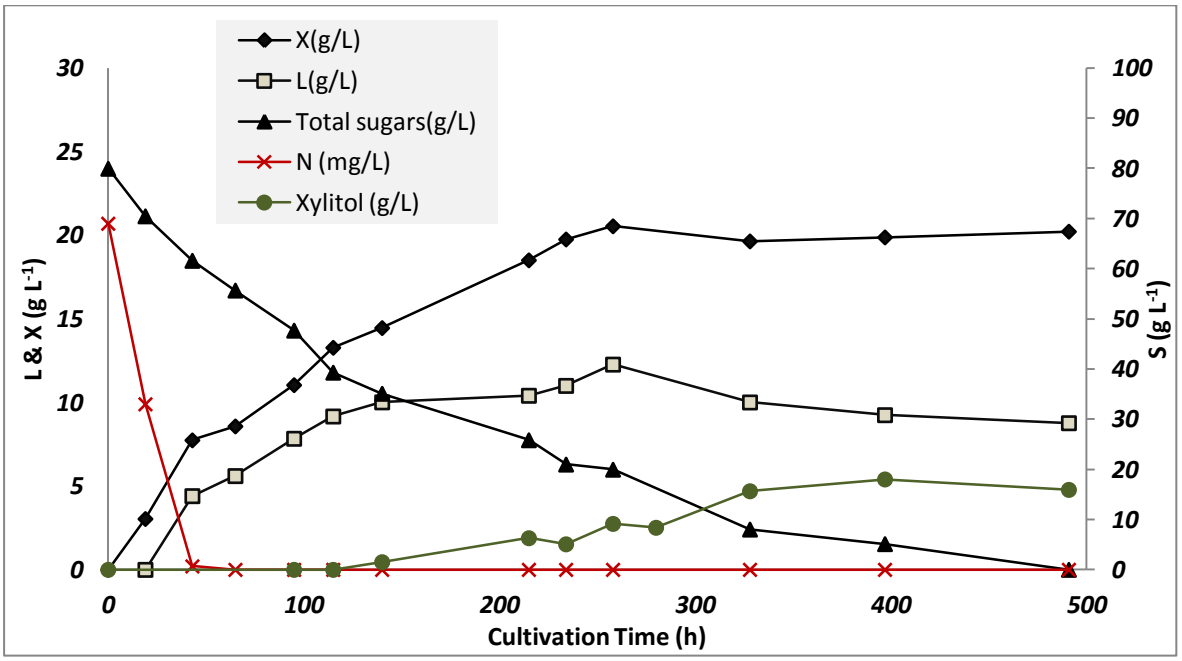


Figure 3.5.1 Kinetics of sugars consumption (S total, g/L),nitrogen consumption (mg/L), biomass (X, g/L) single cell oil (L, g/l) and xylitol (xylitol, g/L) production of *Mortierella isabellina* ATHUM 2935 growing on a carbon blend of commercial xylose (60 g/L) & glucose (20 g/L)

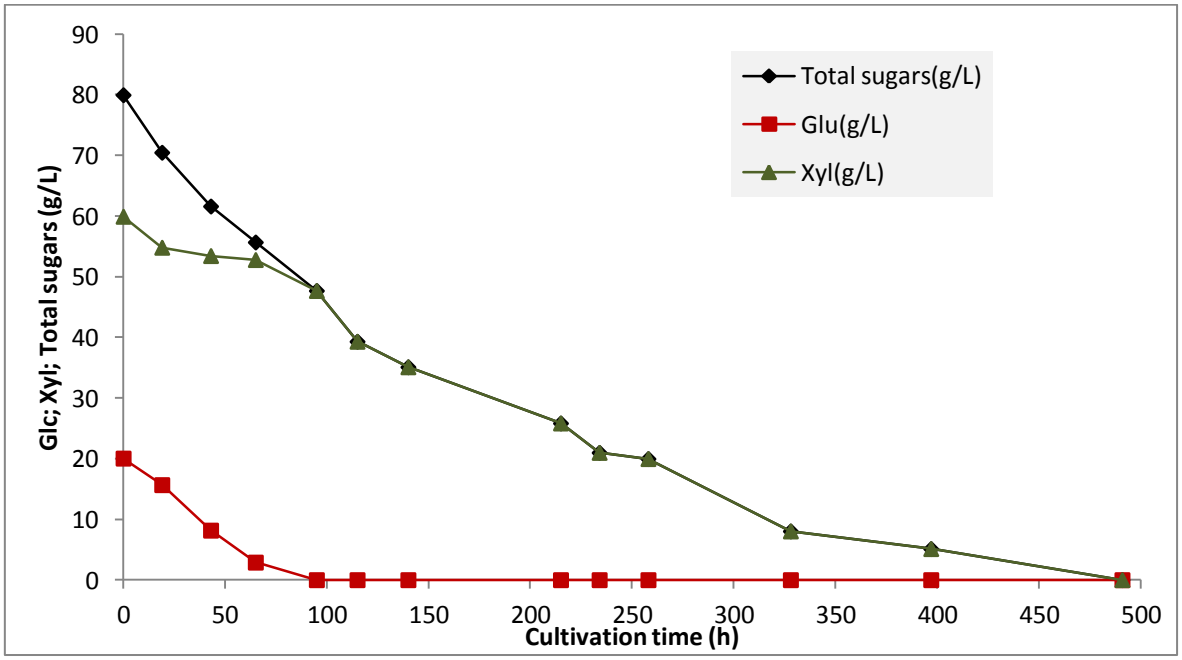


Figure 3.5.2 Kinetics of total sugars consumption (S total, g/L), glucose consumption (Glc, g/L), xylose consumption (Xyl, g/l), biomass (X, g/L) and single cell oil (L, g/l) production of *Mortierella isabellina* ATHUM 2935 growing on a carbon blend of commercial xylose (60 g/L) & glucose (20 g/L)

Concerning the uptake of individual sugars in this fermentation with glucose and xylose used as carbon blend- substrate, it must be stressed that *Mortierella isabellina* ATHUM 2935 showed preference for consumption of glucose. Glucose was noticeably faster consumed as compared to xylose from the beginning of growth as it is shown in figure 3.5.2. Glucose consumption was almost complete (97 %, wt/wt) after 65h of cultivation and in this case xylose was almost totally exhausted in the culture medium after 491h.

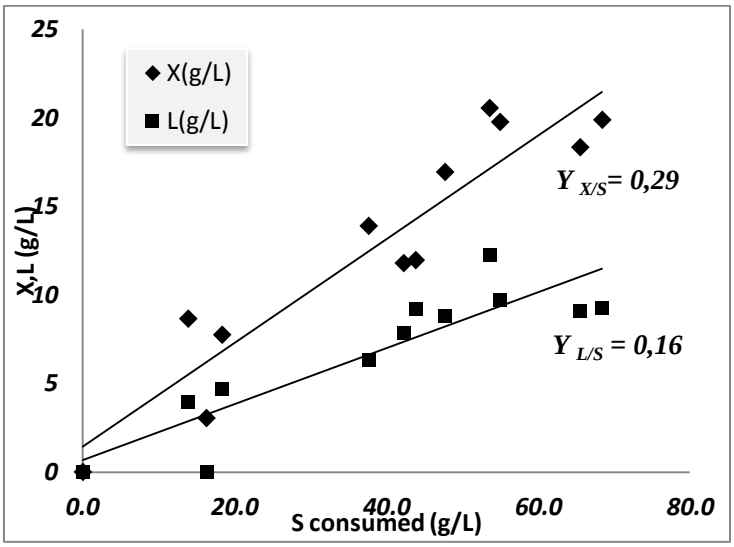


Figure 3.5.2 Biomass (X, g/L) and storage lipid (l, g/L) produced by *Mortierella isabellina* ATHUM 2935 as function of the carbon blend, glucose and xylose (S, g/L) consumed

Total biomass and lipid yield on this carbon blend of glucose and xylose consumed ($Y_{X/S}$ and $Y_{L/S}$) presented constant and remarkable values (0.29 and 0.16 g/g respectively) at the initial sugar concentration of 80 g/L in the growth medium as it is shown in figure 3.5.2.

3.5.2. Cellular lipid analysis

The fatty acid composition of intra-cellular lipids was analyzed by GC at various cultivation times as it is shown in table 3.5.1. and figure 3.5.3. The length of the fatty acid carbon chain ranged from 14 to 20 for these fungal strain and the major fatty acids were palmitic (C16:0), stearic (C18:0), oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2) and γ-linolenic ($^{\Delta 6,9,12}$C18:3 , GLA) acids comprising over 90% of total fatty acids. Oleic acid was the dominant cellular fatty acid in the range of 38.9–49.88% w/w of the total fatty acids during the growth on a medium containing a carbon blend of commercial glucose (20 g/L) & xylose (60 g/L) at initial concentration of 80 g/L under nitrogen limited conditions. The second most abundant fatty acid was the palmitic acid with the percentage of 22.5-28.9 % w/w. The results showed that linoleic and stearic acid were found in significant quantities into the reserve lipids while palmitoleic acid was detected in traces.

Table 3.5.1 Fatty acid composition in the total cellular lipid (% *w/w*) of *Mortierella isabellina* ATHUM 2935 at various growth phases, cultivated on medium containing a carbon blend of commercial glucose (20 g/L) & xylose (60 g/L). IU: The degree of unsaturation (Δ/mole) in the extracted lipid = (1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100).

Carbon blent		**Glucose : xylose (60:20 g L^{-1})**						
Cultivation time (h)		65	95	140	215	280	328	491
Fatty acids	**Structure**							
Myristic	**C14:0**	0.73	0.96	0.79	1.33	1.35	1.24	1.06
Palmitic	**C16:0**	**24.63**	**27.54**	**26.15**	**28.99**	**28.44**	**29.38**	**22.50**
Palmitoleic	**$^{\Delta 9}$C16:1**	0.96	0.70	0.61	1.25	1.04	1.01	1.38
Stearic	**C18:0**	**9.03**	**8.81**	**10.35**	**6.14**	**6.07**	**6.18**	**4.80**
Oleic	**$^{\Delta 9}$C18:1**	**49.88**	**39.91**	**44.20**	**38.97**	**39.85**	**42.92**	**47.30**
Linoleic	**$^{\Delta 9,12}$C18:2**	**10.62**	**11.66**	**9.37**	**11.81**	**12.75**	**11.90**	**14.22**
γ- Linolenic	**$^{\Delta 6,9,12}$C18:3**	**1.94**	**3.14**	**2.27**	**3.74**	**3.91**	**3.32**	**3.95**
Arachidic	**C20:0**	0.94	0.78	1.25	nd	0.65	0.76	1.08
Others		1.27	6.50	5.00	7.77	5.93	3.28	3.71
IU		0.78	0.73	0.70	0.75	0.78	0.78	0.89

At all growth stages oleic acid was the major fatty acid found in microbial oil, while its percentage decreased as oil accumulation progressed with a small increase about 5% near the end of the fermentation. Also, it was observed a significant increase about 4 % in linoleic acid contents near the end of the fermentation. *Mortierella isabellina* ATHUM 2935 showed low capability to produce γ-linolenic acid (GLA) on a medium containing a carbon blend of glucose (20g/L) and xylose (60g/L). GLA ($^{\Delta 6,9,12}$C18:3) was presented in all growth steps in the reserve cellular lipids while its percentage increased as oil accumulation progressed. The percentage varied inter 1.94- 3.95 % w/w with the highest GLA content of 3.95 % achieved at 491h ,near the end of the growth. Accordingly, the unsaturation index (UI) indicated that as the concentrations of GLA and linoleic acid raised, the lipid composition of the micro-organism became more unsaturated.

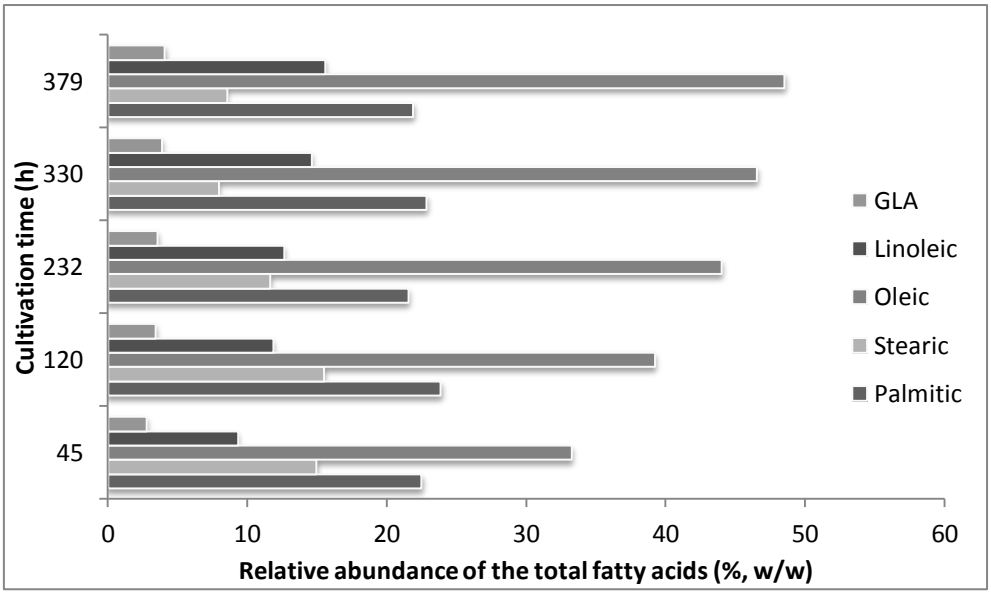


Figure 3.5.3 Fatty acid composition of the *Mortierella isabellina* ATHUM 2935 lipid produced in a carbon blend of commercial glucose (20 g/L) & xylose (60 g/L)

3.5.3. Fractionation of accumulated oil

The fractionation of accumulated oil of *Mortierella isabellina* ATHUM 2935 into neutral (N), glycolipids plus sphingolipids (G + S) and phospholipids (P), during lipid accumulation phases using a carbon blend of commercial xylose (60 g/L) & glucose (20 g/L) as carbon source revealed that the neutral lipid (N) fraction was the major constituent of total lipids as it is shown in table 3.5.2. At early growth (43h) showed that the percentage of neutral (N) fraction in total lipid was low and equal to 78.4 %, while the G+ S and P content was noticeable high and almost the same, 10% & 11% , respectively. The neutral lipid (N) content, 234h of growth showed a significant increase of 8.1%, while a slight decrease was observed at 397h of growth equal to 2.5%. However, the percentage of P fraction was high at the beginning of growth but declined thereafter as it is shown in Table 3.5.2 with an overall decrease of 5%. As for the G + S content was also high at the beginning of growth but a significant decrease equal to 4.1% was observed at 234h of growth and then increased again to the same percentage as at the early growth.

Table 3.5.2 Percentages (in%, w/w) of neutral (N), sphingolipid and glycolipid (G + S) and phospholipid (P) fractions during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial xylose (60 g/L) & glucose (20 g/L)

Time (h)	Fractions	Percentage %	Recovery %
43	N	78.4	
	P	10.0	99
	G+S	11.6	
234	N	87.5	
	P	5.9	100
	G+S	6.6	
397	N	85.0	
	P	5.0	88
	G+S	10.0	

NL: neutral lipids, P: phospholipids, G+S: glycolipids and sphingolipids

3.5.4. Quantitative determination of phospholipids

The quantitative determination of phospholipids was based on the determination of phosphorus content in the lipid samples according to Rouser *et al.*, (1970). The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. The lipid samples produced by *Mortierella isabellina* ATHUM 2935 growing in on a carbon blend of commercial xylose (60 g/L) & glucose (20 g/L) at initial concentration of 80 g/L under nitrogen limited conditions were chosen from three different growth phases, as it is shown in table 3.5.3.

Table 3.5.3 Quantitative composition of Phosphorus (in μg/ g Lipid) and phospholipids (in mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial xylose (60 g/L) & glucose (20 g/L)

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*μg Phopsphorus /mg Lipid*	*mg Phospholipids /g lipid*	*Phospholipids (g / L)*
43	4.7	24.52	0.833	**20.83**	0.098
234	9.7	25.72	0.300	**7.50**	0.073
397	9.25	25.67	0.185	**4.63**	0.043

The quantity of phosphorus and therefore the phospholipid content during the three different lipid accumulation phases (43, 234 and 379h of cultivation) showed a significant decrease as it is observed in Table 3.5.3 and Figure 3.5.4. The first 43h of cultivation, the phospholipid fraction achieved the greatest value of 20.83 mg. During the cultivation a notable decrease was noted, equal to 13.33 and 16.2mg in 234 and 397h, respectively.

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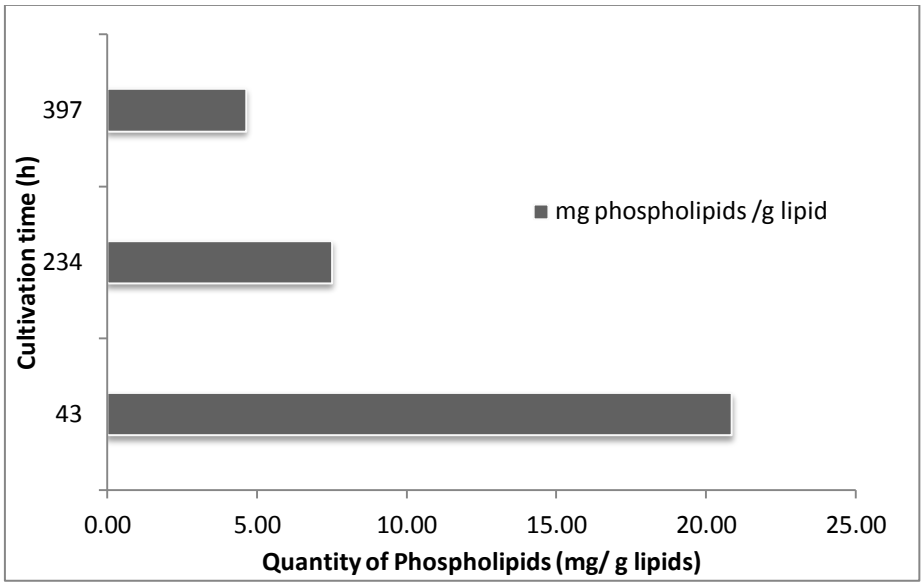

Figure 3.5.4 Quantity of phospholipid fraction during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

3.5.5. Quantitative determination of sugar content in lipid fractions

The lipid samples were chosen from three different growth phases (45, 120 and 232 h) and before the application of the method, were redefined in appropriate concentrations. As the results, it was found that the sugar content, contained at glycolipid and sphingolipid fractions mostly, during the fermentation was not constant (Table 3.5.3) The results showed a decrease in sugar content during the cultivation time and the greatest quantity of sugars was noted at the beginning of the growth (43h of cultivation) and it was equal to 106.4 mg glucose/ g Lipid. Near to the end of fermentation (397h) the sugar content had a six-fold decrease, achieved the value of 12.4 mg glucose/ g Lipid.

Table 3.5.3 The sugar content (in g/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on a carbon blend of commercial glucose (60 g/L) & xylose (20 g/L)

Time (h)	Total lipids (g/L)	Sample: crude (mg/mL)	*mg glucose /g Lipid*
43	4.7	24.52	106.4
234	9.7	25.72	25.7
397	9.25	25.67	12.4

3.6. Thin -layer Chromatography

The application of thin layer chromatography (TLC) to the separation of neutral and polar lipid fractions of *Mortierella isabellina* ATHUM 2935 lipids and their sub-fractions into different components was tested in this study and the results are illustrated in the following sections.

3.6.1. Neutral lipids

Sterol esters, sterols, fatty acids, acylglycerols (mono-, di- and tri) and similar lipids can be separated through adsorption TLC into compound classes of different polarities, according to the type and the number of their functional groups (Stahl, 1969). One dimensional TLC with the stated solvents from materials and methods*: n*-hexane /diethyl ether/ glacial acetic acid (70:30:1, v/v/v), leads to complete separation of the fungus neutral lipid fraction as it is shown in the following figures. Possible traces of polar lipids remain at the origin. The identification of neutral lipid classes was made with authentic standards in specific concentrations as it is shown in table 3.6.1.

Table 3.6.1 Standard solutions (Stock & Working) for neutral lipid fraction.

	Type	Standard	Stock solution (mg/ml)	Working solution 1 (mg/ml)	Working solution 2 (mg/ml)
Neutral	**MAG**	Monononadecanoin	5	1	2
	CE	Cholesteryl linoleate	10	0.5	1
	TAG	Glyceryl trioleate	10	6	2
	CL	Cholesterol	10	0.5	1
	FFA	Oleic acid	40	1	2

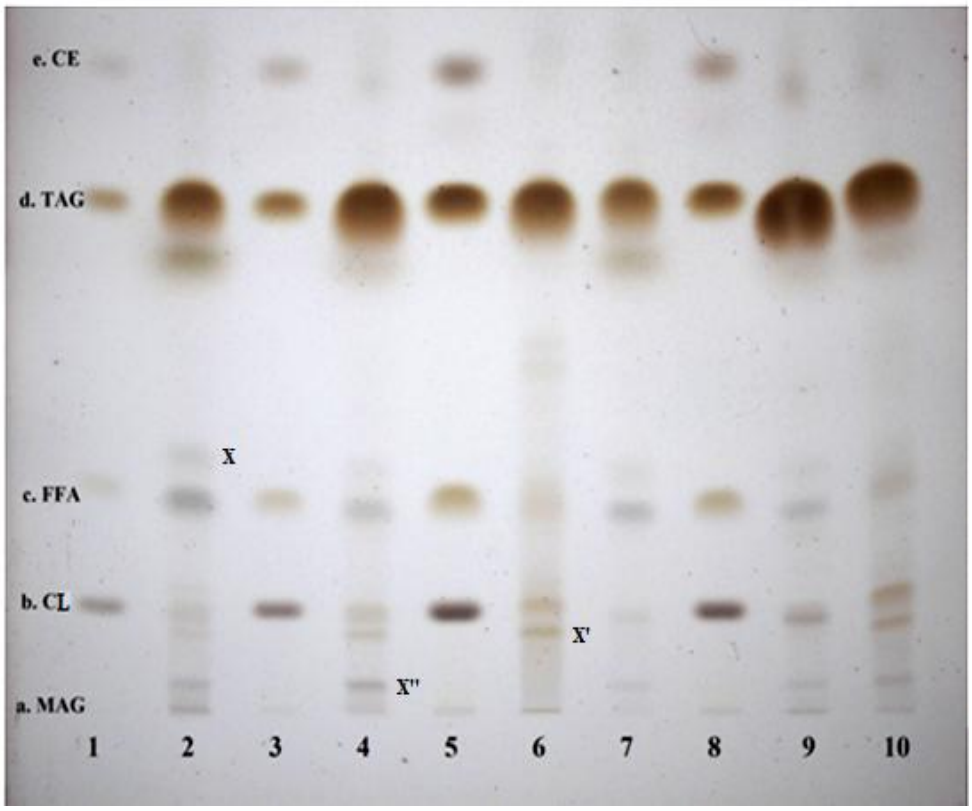

Figure 3.6.1 Separation of neutral lipids into classes by absorption-TLC: Sequence: bands of working solution: 1, 2μl; 3, 5μl; 5, 15μl ,8, 10μl: a. Monononadecanoin, R_f (0.03); b. Cholesterol R_f (0,13); c. Oleic acid R_f (0,29); d. Glyceryl

trioleate R_f (0,63)*; e.* Cholesteryl linoleate R_f (0,78) ; 2, 4, 6, 7, 9, 10 : neutral fraction samples, 10μl of each applied. Layer: 2,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: *n*-hexane /diethyl ether/glacial acetic acid (70:30:1, v/v/v); time of run 45 min (16cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: H_2SO_4:H_2O 1:1 (v/v) at 110-120 °C for 30 min.

Table 3.6.2 Sample application conditions on chromatoplates

Band	Sample	Spot Volume (μl)	mg/ml
1	working solution 1	*2*	***
2	neutral fraction (43h): Glc-Xyl (60:20 g/L)	*10*	25.85
3	working solution 1	*5*	***
4	neutral fraction (234 h): Glc-Xyl (60:20 g/L)	*10*	32
5	working solution 1	*15*	***
6	neutral fraction (397 h): Glc-Xyl (60:20 g/L)	*10*	30.55
7	neutral fraction (43h): Xyl - Glc (60:20 g/L)	*10*	29.15
8	working solution 1	*10*	***
9	neutral fraction (234h): Xyl - Glc (60:20 g/L)	*10*	37.72
10	neutral fraction (397h): Xyl - Glc (60:20 g/L)	*10*	38.88

***The concentration of each substance is given in the table of standards

The neutral lipid fractions from different fermentations, their spot volumes (μl) and the concentrations of each sample (mg/ml) are described in Table 3.6.2. As it is observed in Figure 3.6.1 the identification of the lipid classes of the *Mortierella isabellina* ATHUM 2935 neutral lipid fractions were accomplished by comparison with the available authentic standards. The aforementioned neutral lipids were derived from three different stages of development (43, 234 and 397h) and two different substrates used in fermentations. These fractions consisted predominantly of triacylglycerols (TAG) as it is shown in the figure 3.6.1. and to a lesser degree of other neutral compounds like sterol esters (CE), free fatty acids (FFA), sterols (CL) and monoacylglycerol (MAG).

Three bands X and X', X'' in the lipid samples were not identified. The X band probably corresponds to saturated free fatty acids (FFA), because they migrate faster (slighter adsorption) than the unsaturated homologues. This claim also supported by the fact that saturated lipids usually give less intensive spots than unsaturated lipids with this detection system (Skipski, et al., 1967). The X' and X'' band probably corresponds to 1,2- and 1,3- diacylglycerol (DAG) which is usually present in the fungus oil. However, the particular authentic standard was not available.

According to Hamilton & Hamilton, (1992), the double bands of TAG appearing in all the chromatoplates are due to their partial resolution into molecular species on the basis of fatty acid composition. Furthermore, according to Ratledge & Wilkinson, (1988), Zygomycetes differ from other classes of 'lower' fungi in producing ergosterol generally as a major sterol accompanied by dihydroergosterol and smaller amounts of cholesterol. In a given solvent system each lipid component has a characteristic mobility which can be described by its R_f value. In this solvent system the compounds like MAG, CL, FFA were more tightly adsorbed and therefore migrated at the slowest rates with low R_f values, equal to 0.03, 0.13, 0.29, respectively. On the contrary, compounds like TAG and CE migrated at the fastest rates with high R_f values, equal to 0.63 (upper band) - 0.55 (lower band), 0.78, respectively. The intensity of each band is analogues to the quantity of each substance. Thus, we can claim as shown in figure 3.6.1. that the amounts of the different neutral lipid classes were affected by the stages of development and the

different substrates that were used in this study. However, this claim should be supported by a quantitative analysis in a future work. According to Ratledge & Wilkinson, (1988), the times of synthesis of triacylglycerols often correspond with the formation of reproductive structures. However, triacylglycerols (TAGs) constitute the major lipids in the mycelium of the most fungi (80-90% of the reserve lipid), which are generally considered as storage lipids during growth and development.

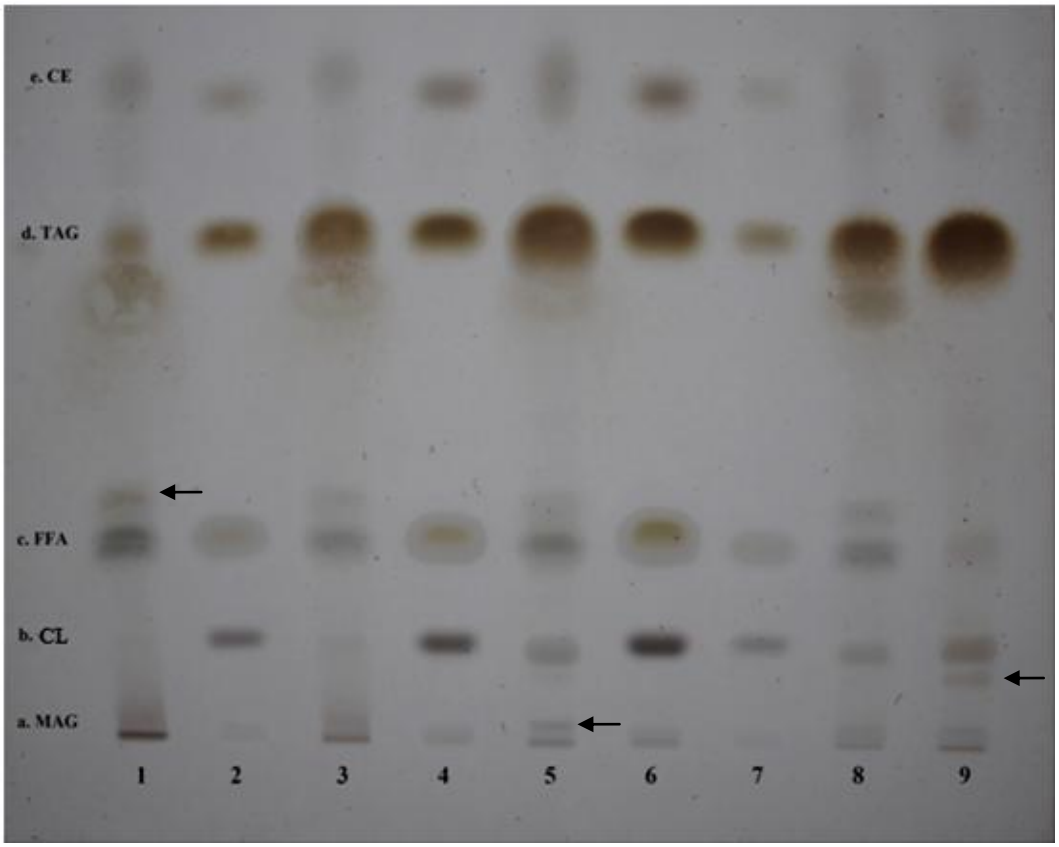

Figure 3.6.2 Separation of neutral lipids into classes by absorption-TLC: Sequence: bands of working solution: 2, 5µl; 4, 10µl; 6, 20µl; 7, 2µl: a. Monononadecanoin R_f (0.03); b. Cholesterol R_f (0.29); c. Oleic acid R_f (0.26); d. Glyceryl trioleate R_f (0.63)*; e.* Cholesteryl linoleate R_f (0.78) ; 1, 3, 5, 8, 9: neutral fraction samples, 10 µl of each applied. Layer: 2,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: *n*-hexane /diethyl ether/glacial acetic acid (70:30:1, v/v/v); time of run 45 min (16cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: H_2SO_4:H_2O 1:1 (v/v) at 110-120 °C for 30 min. Some bands in the lipid samples have not been identified and are shown with an arrow.

Table 3.6.3 Sample application conditions on chromatoplates

Band	Sample	Spot Volume (µl)	mg/ml
1	neutral fraction (45h): Xylose (80 g/L)	10	40.03
2	working solution 1	5	***
3	neutral fraction (139 h): Xylose (80 g/L)	10	38.05
4	working solution 1	10	***
5	neutral fraction (240 h): Xylose (80 g/L)	10	42.10
6	working solution 1	20	***
7	working solution 1	2	***
8	neutral fraction (92h): Glucose (80 g/L)	10	36.53
9	neutral fraction (240 h): Glucose (80 g/L)	10	38.87

***The concentration of each substance is given in the table of standards

One dimensional thin-layer chromatography of the neutral lipid fraction of *Mortierella isabellina* revealed the presence of at least five classes (CE, TAG, FFA, CL, MAG) which correspond to the authentic

standards as it is shown in figure 3.6.2. Triacylglycerols (TAGs) constitute the major lipid compounds in the mycelium in all growth phases when *Mortierella isabellina* ATHUM 2935 cultured in commercial glucose and xylose at initial concentration of 80 g/L as it is shown in the figure 3.6.2. Compounds like cholesterol esters (CE), free fatty acids (FFA), sterols (CL) and monoacylglycerol (MAG) were also present in the chromatoplate but to a lesser amount. All of these compounds were present throughout growth as it is observed in Figure 3.6.2. As it is shown in Figure 3.6.2 the sterols and sterol esters are easily detected through the characteristic colors they yield on heating after having been sprayed with H_2SO_4:H_2O 1:1 (v/v). These compounds turn red, then red –violet and finally brown. On the contrary, the polyunsaturated compounds like TAGs appear as brown-pink spots but most compounds may be subsequently charred, yielding black-brown spots like FFA.

The R_f values were identical to those reported above (fig 3.6.2.). According to the intensity of the bands, during the growth phases, the pattern of the components seems to be essentially different especially in the case of TAGs. The intensity of 1, 3, 5 bands was analogues to the quantity of TAGs in the different growth phases (45, 139 and 240h) of the mycelium when xylose was used as substrate leading to the conclusion that the synthesis of triacylglycerols (TAGs) increased during the growth. The same pattern occurred when glucose was used as substrate as it is shown in the figure 3.6.2.

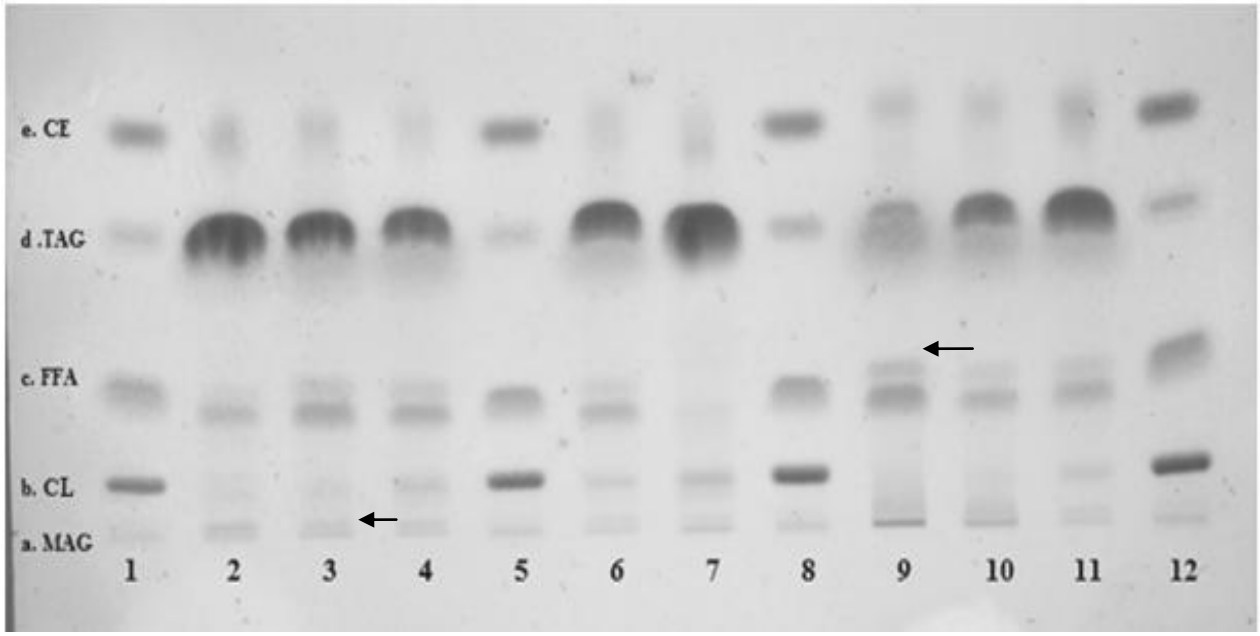

Figure 3.6.3 Separation of neutral lipids into classes by absorption-TLC: Sequence: bands of working solution: 1, 8μl; 5, 10μl; 8, 12μl; 12, 15μl: a. Monononadecanoin R_f (0.03) ; b. Cholesterol R_f (0.13); c. Oleic acid R_f (0.29); d. Glyceryl trioleate R_f (0.63)*; e.* Cholesteryl linoleate R_f (0.78); 2, 3, 4, 6, 7, 9, 10, 11 : neutral fraction samples, 10 μl of each applied. Layer: 1,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: *n*-hexane /diethyl ether/glacial acetic acid (70:30:1, v/v/v); time of run 35 min (8cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: H_2SO_4:H_2O 1:1 (v/v) at 110-120 °C for 30 min. Some bands in the lipid samples have not been identified and are shown with an arrow.

Table 3.6.4 Sample application conditions on chromatoplates

Band	Sample	Spot Volume (μl)	mg/ml
1	working solution 2	8	**
2	neutral fraction (65h): Glc-Xyl (40:40 g/L)	10	41.55
3	neutral fraction (120 h): Glc-Xyl (40:40 g/L)	10	39.13
4	neutral fraction (232 h): Glc-Xyl (40:40 g/L)	10	40.00
5	working solution 2	10	**
6	neutral fraction (92h): Glucose (80 g/L)	10	36.53
7	neutral fraction (240 h): Glucose (80 g/L)	10	38.87
8	working solution 2	12	**
9	neutral fraction (45h): Xylose (80 g/L)	10	40.03
10	neutral fraction (139 h): Xylose (80 g/L)	10	38.05
11	neutral fraction (240 h): Xylose (80 g/L)	10	42.10
12	working solution 2	15	**

**The concentration of each substance is given in the table of standards

As it is observed in Figure 3.6.3 the identification of the lipid classes of the *Mortierella isabellina* strain ATHUM 2935 neutral lipid fractions were accomplished by comparison with the available authentic standards. The aforementioned neutral lipids were derived from different stages of development and three different substrates used in the fermentations as it is shown in the Table 3.6.4 triacylglycerols (TAGs) constitute the major lipid compounds in the mycelium of *Mortierella isabellina* ATHUM 2935, while compounds like sterol esters (CE), free fatty acids (FFA), sterols (CL) and monoacylglycerol (MAG) were present to a lesser amount. All of these compounds were present throughout the growth. The double bands of TAGs in Figure 3.6.3 are not so clear because the neutral lipid fractions were allowed to run in a half distance (8cm) than usual (16cm). The Rf values were identical to those reported above (Figure 3.6.1).

Differences in lipid fractions were observed also during the different growth phases. The pattern of the components seems to be essentially different not only during the different growth phases but also between the different substrates especially in the case of TAGs. The intensity of 2, 3, 4 bands was analogues to the quantity of TAGs in the different growth phases (65, 120 and 232h) of the mycelium when the blend of glucose (40 g/L) and xylose (40 g/L) was used as substrate. In this case it was observed that the synthesis of triacylglycerols (TAGs) slightly decreased during the growth. On the contrary, the quite opposite pattern occurred when glucose and xylose were used solely as substrates as it is shown in the figure 3.6.3.

3.6.2. Polar lipids

One dimensional TLC of polar lipids [glycolipids including sphingolipids (G+S), phospholipids (P)] of *Mortierella isabellina* ATHUM 2935 with the stated solvents from materials and methods: chloroform/ methanol/ ammonia (25%) (65:25:4, v/v/v), leads to a separation of the fungus polar lipids as it is shown in the following figures. The identification of phospholipid fraction was made with authentic standards in specific concentrations as it is shown in the table 3.6.5. and a specific detection system, the molybdenum blue reaction according to Dittmer & Lester (1964). For the qualitative determination of glycolipids the detection reagent α-naphthol was used.

3.6.2.1. Phospholipid fraction

Table 3.6.5 Standard solutions (Stock & Working) for phospholipid fraction.

	Type	Standard	Stock solution (mg/ml)	Working solution (mg/ml)
Polar	**PS**	L-α-Phosphatidyl-L-serine	10	1
	PE	3-sn-Phosphatidylethanolamine	10	1
	PC	L-α-Phosphatidylcholine	10	1.6

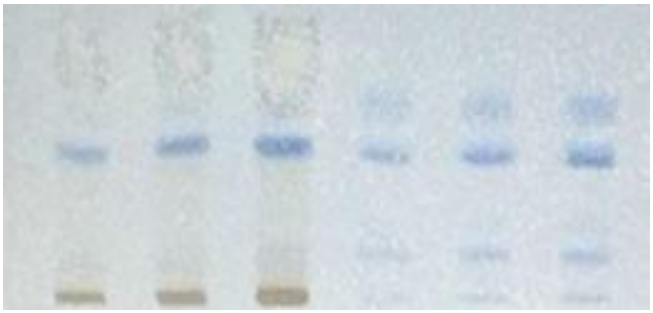

Figure: Separation of phospholipids by absorption TLC and the positive test of phospholipids with a specific detection system, the molybdenum blue reaction according to Dittmer & Lester (1964). Neutral lipids and glycolipids do not give positive test. Phospholipids, however, show up immediately as blue spots on a white or light blue-gray background. Layer: 1,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: chloroform/methanol/ammonia (25%) (65:25:4, v/v/v); time of run 23 min (8cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: Dittmer–Lester molybdenum blue reagent**.**

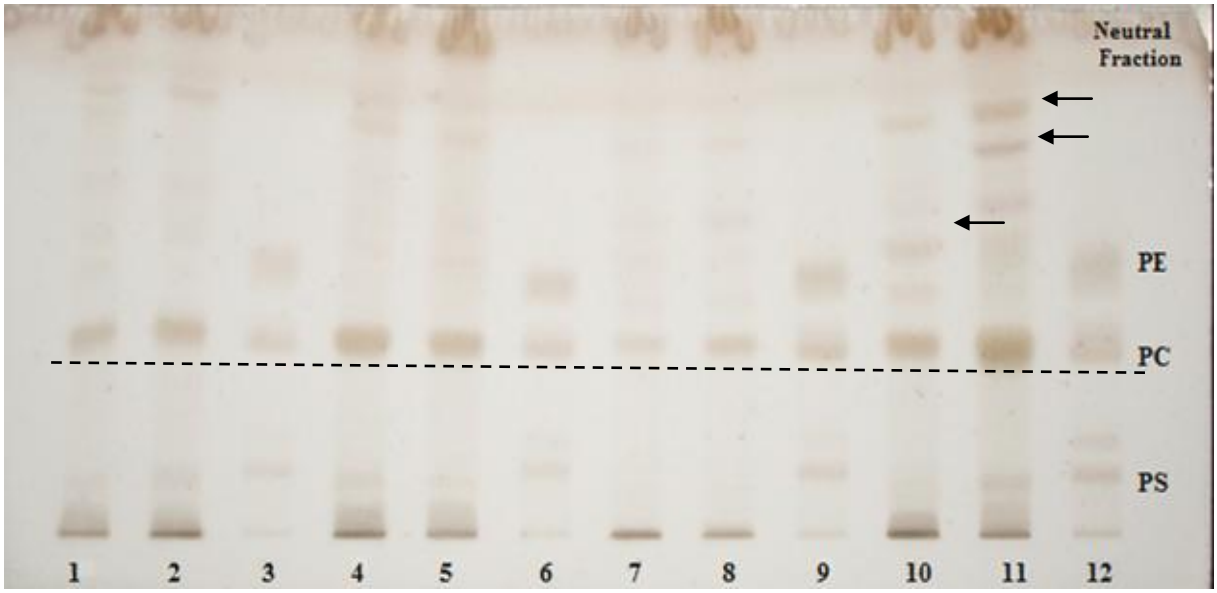

Figure 3.6.4 Separation of phospholipids into classes by absorption-TLC: Sequence: bands of phospholipid fraction samples: 1, 2, 4, 5, 7, 8, 10 , 30µl of each aplied; bands of working solution: 3, 4µl; 6, 6µl; 9, 8µl; 9, 10µl; 12, 10µl : (contains L-α-Phosphatidyl-L-serine, 3-sn-Phosphatidylethanolamine, L-α-Phosphatidylcholin); Layer: 1,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: chloroform/methanol/ammonia (25%) (65:25:4, v/v/v); time of run 33 min (8cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: H_2SO_4:H_2O 1:1 (v/v) Some bands in the lipid samples have not been identified and are shown with an arrow.

Table 3.6.6. Sample application conditions on chromatoplates

Band	Sample	Spot Volume (μl)	mg/ml
1	Phospholipid fraction (232 h): Glc-Xyl (40:40 g/L)	30	1.2
2	Phospholipid fraction (92h): Glucose (80 g/L)	30	1.6
3	working solution	4	*
4	Phospholipid fraction (240 h): Glucose (80 g/L)	30	2.6
5	Phospholipid fraction (280 h): Glucose (80 g/L)	30	1.8
6	working solution	6	*
7	Phospholipid fraction (45h): Xylose (80 g/L)	30	1.6
8	Phospholipid fraction (139 h): Xylose (80 g/L)	30	2.4
9	working solution	8	*
10	Phospholipid fraction (240 h): Xylose (80 g/L)	30	3.6
11	-	-	-
12	working solution	10	*

*The concentration of each substance is given in the table of standards

The phospholipid fractions from different fermentations, their spot volumes (μl) and the concentrations of each sample (mg/ml) are described in the table 3.6.6. As it is observed in Figure 3.6.4 the use of three authentic standards (L-α-Phosphatidyl-L-serine, L-α-Phosphatidylcholine3-sn, Phosphatidylethanolamine) revealed the presence of two phospholipid components in the lipid samples. In most fungi, phosphatidyl-choline (PC) is the major component in phospholipid fraction (Ratledge & Wilkinson, 1988) and usually accounting for 42% of total phospholipid of *Mortierella isabellina* according to Cerv, (1993). The results coincided with the literature since the phospholipid fractions of *Mortierella isabellina* lipids consisted predominantly of phosphatidyl-choline (PC) with a R_f value equal to 0.38 while the bands of phosphatidyl-ethanolamine (PE) and phosphatidyl-serine (PS) did not appear on the chromatoplate in all stages of the development. As it is observed in Figure 3.6.4 the neutral lipids that appeared near the top of the solvent front, is due to incomplete separation during column chromatographic separation of lipids. The pattern of PC seemed to be the same not only during the different growth phases but also between the different substrates. The intensity of all bands was analogues to the quantity of PC in the different growth phases of the mycelium and between the different substrates concluding that the synthesis of PC did not remain constant during the growth as it is shown in the Figure 3.6.4.

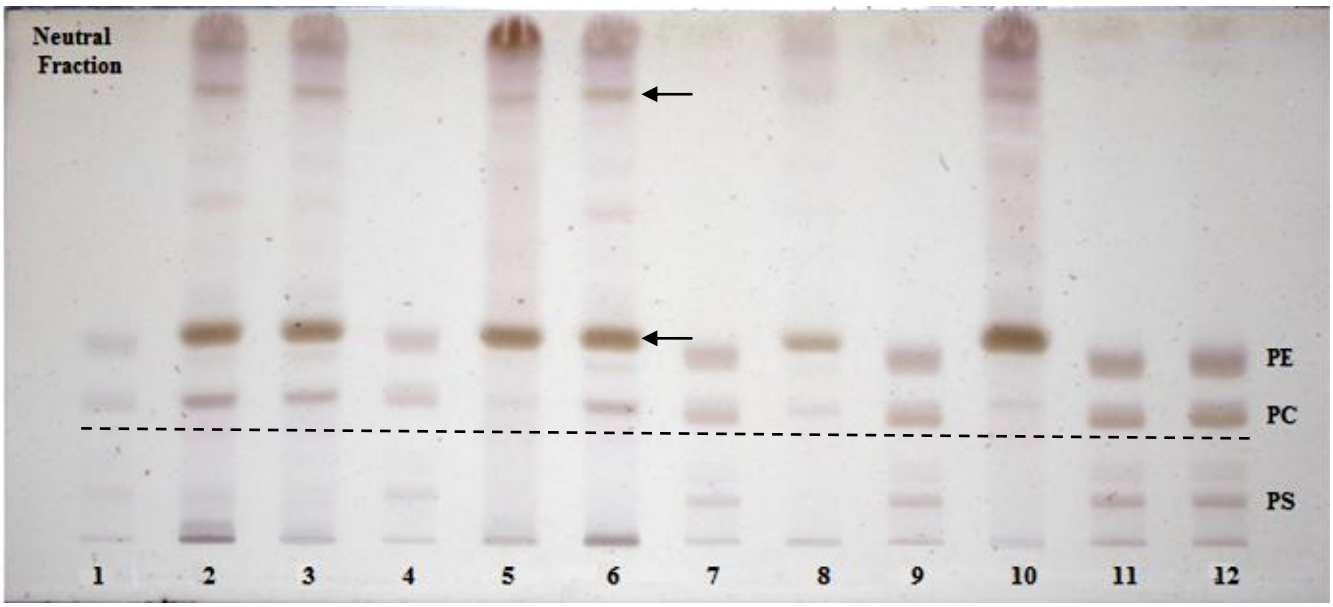

Figure 3.6.5 Separation of phospholipids into classes by absorption-TLC: Sequence: bands of phospholipid fraction samples: 2, 3, 5, 6, 8, 10 , 20μl of each applied; bands of working solution: 1, 4μl; 4, 6μl; 7, 8μl; 9, 10μl; 11, 12μl; 12, 14μl : (contains L-α-Phosphatidyl-L-serine, 3-sn-Phosphatidylethanolamine, L-α-Phosphatidylcholine); Layer: 1,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: chloroform/methanol/ammonia (25%) (65:25:4, v/v/v); time of run 23 min (8cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: H_2SO_4:H_2O 1:1 (v/v) Some bands in the lipid samples have not been identified and are shown with an arrow.

Table 3.6.7 Sample application conditions on chromatoplates

Band	Sample	Spot Volume (μl)	mg/ml
1	working solution	4	*
2	Phospholipid fraction (43h): Glc-Xyl (60:20 g/L)	20	4.2
3	Phospholipid fraction (234 h): Glc-Xyl (60:20 g/L)	20	3.8
4	working solution	6	*
5	Phospholipid fraction (397 h): Glc-Xyl (60:20 g/L)	20	5.25
6	Phospholipid fraction (43h): Xyl - Glc (60:20 g/L)	20	3.2
7	working solution	8	*
8	Phospholipid fraction (237h): Xyl - Glc (60:20 g/L)	20	5.5
9	working solution	10	*
10	Phospholipid fraction (397h): Xyl - Glc (60:20 g/L)	20	5.4
11	working solution	12	*
12	working solution	14	*

*The concentration of each substance is given in the table of standards

A notable different pattern was observed in the figure… the use of three available standards (L-α-Phosphatidyl-L-serine, L-α-Phosphatidylcholine3-sn, Phosphatidylethanolamine) revealed the presence of phosphatidyl-choline (PC) in the lipid samples of *Mortierella isabellina* ATHUM 2935 and another compound that appeared above the Phosphatidylethanolamine standard in a greater amount than PC. On the contrary, there was observed a lack of PE and PS in all the lipid samples. The neutral fraction that appeared near the top of the chromatoplate, is due to the possible extent of contamination across the fractions during the transfer steps in the fractionation process. The phospholipid fractions of *Mortierella isabellina* lipids consisted predominantly of the undetected compound which according to literature R_f is probably Phosphatidyl (Dimethylethanolamine) (PDME). The PDME is one of the basic intermediates in the reaction of PC biosynthesis in fungi according to Vance, (1996). The R_f values of PS, PC and PE standards at the given solvent system were 0.11, 0.27, 0.36, respectively while the R_f values of PDME was 0.40.

Notable differences in phospholipid fractions were observed during the growth phases of *Mortierella isabellina* strain ATHUM 2935 cultivated in two different substrates (table). The pattern of the phospholipid components changed in the case of PC, when a blend of glucose (60 g/L) and xylose (20 g/L) was used as substrate for the mycelium growth. The intensity of 2, 3, 5 bands which is analogues to the quantity of PC, in the different growth phases (43, 234 and 397h) revealed a decrease of PC between the 43h and 234h of cultivation ,while the lack of PC was observed after 397h of cultivation. On the contrary, the amount of the undetected compound remained almost constant during this fermentation. When the blend of xylose (60 g/L) and glucose (20 g/L) was used as substrate, the intensity of 6, 8, 10, bands revealed a notable decrease in the amount of PC during the growth phases as it is shown in the figure 3.6.5. While the amount of the undetected compound decreased 237h of cultivation and after that a notable increase was observed near the end of growth (397h of cultivation).

3.6.2.2. Glycolipid plus sphingolipid fraction

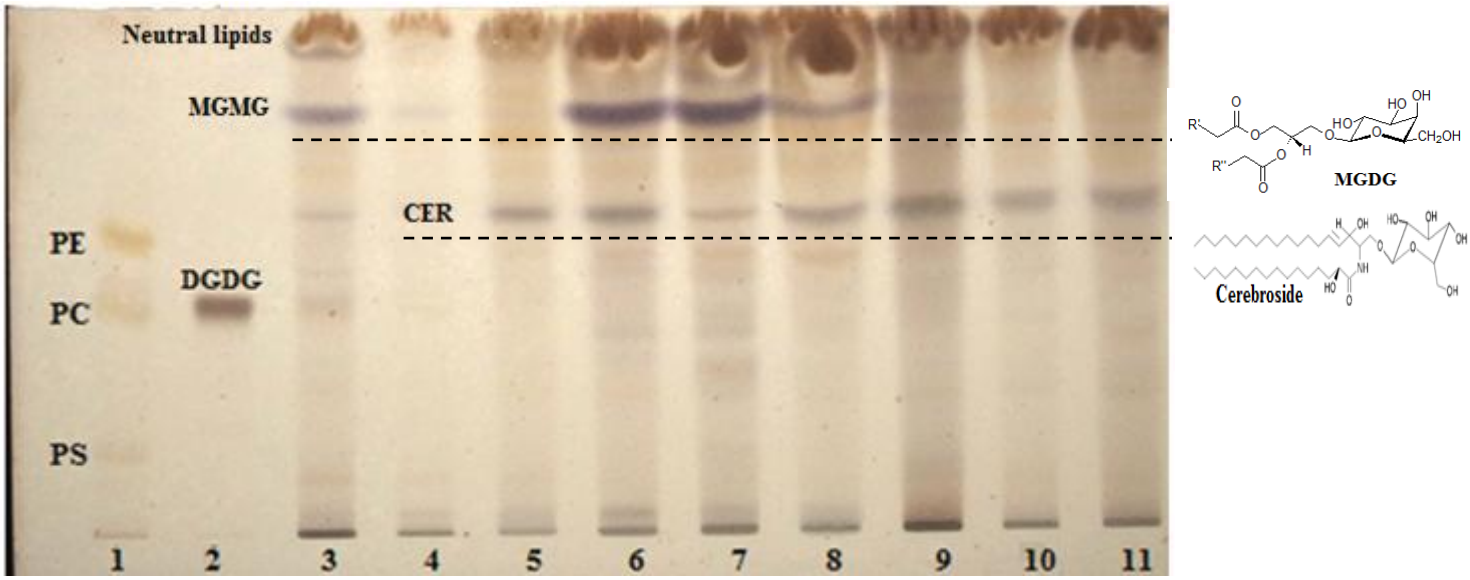

Figure 3.6.6 Separation of polar lipids into classes by absorption-TLC: Sequence: 1. phospholipid working solution: (contains L-α-Phosphatidyl-L-serine, 3-sn-Phosphatidylethanolamine, L-α-Phosphatidylcholin), 8µl; 2. Digalactosyldiacylglycerol (DGDG), 1mg/ml, 6µl; bands of polar fraction: 3, 30µl; 4, 10µl; bands of glycolipid plus sphingolipid fraction: 5-11, 30 µl of each applied; Layer: 1,0 cm origin, Camag, Silica gel G, activated at 100°C for 1 h. Solvents: chloroform/methanol/ammonia (25%) (65:25:4, v/v/v); time of run 36 min (8cm above the origin); intermediate drying at room temperature for 15-30 min Detection system: *a*-Naphthol + H_2SO_4:H_2O 95:5 (v/v)

Table 3.6.8 Sample application conditions on chromatoplates

Band	Sample	Spot Volume (µl)	mg/ml
1	Phospholipid working solution	8	*
2	Digalactosyldiacylglycerol (DGDG)	6	1
3	Combined fraction **(65h): Glc-Xyl (40:40 g/L)	30	26.8
4	Combined fraction **(120h): Glc-Xyl (40:40 g/L)	10	22
5	G+S fraction (232h): Glc-Xyl (40:40 g/L)	30	7.2
6	G+S fraction (92h): Glucose (80g/L)	30	15.4
7	G+S fraction (240h): Glucose (80g/L)	30	15.4
8	G+S fraction (280h): Glucose (80g/L)	30	21
9	G+S fraction (45h): Xylose (80g/L)	30	17
10	G+S fraction (139h): Xylose (80g/L)	30	13.6
11	G+S fraction (240h): Xylose (80g/L)	30	18.6

*The concentration of each substance is given in the table of standards
**Combined fractions of glycolipids /sphingolipids (G+S) + phospholipids (P)

Glycolipid plus sphingolipid fraction was analyzed to ascertain the extent of separation of these components in lipids of *Mortierella isabellina* ATHUM 2935. The G+S fractions from different fermentations, their spot volumes (µl) and the concentrations of each sample (mg/ml) are described in table 3.6.8. As it is observed in the figure the neutral lipids that appeared near the top of the solvent front, is due to incomplete separation during column chromatographic separation of lipids. The use of Digalactosyldiacylglycerol (DGDG) as glycolipid standard revealed the absent of this particular component in all the fractions as it is shown in the Figure 3.6.6. The qualitative determination of glycolipids was affected by the use of the detection reagent *α*-naphthol combined with literature R_f values. This specific detection reagent causes all glycolipids including cerebrosides to appear as blue-purple spots on white

background. Other polar lipids appear as yellow spots while the sterols give gray-red spots (Kawanami, 1967; Neskovic, *et al.,* 1972; Skipski, *et al.,* 1967). As it is observed from figure 3.6.6 the major glycolipid identified (tentative identification) was the Monogalactosyldiacylglycerol (MGDG) with R_f value equal to 0.80 in the bands 3, 6, 7, 8, which correspond to fermentations conducted with either a blend of glucose (40 g/L) plus xylose (40 g/L) or glucose solely as substrates. This particular component appeared also in band 9, but not in bands 10, 11 which correspond to fermentations xylose solely was used as substrate. The second most abundant group of glycosphingolipids which appeared in all the bands are cerebrosides (tentative identification)) with R_f value equal to 0.60 as it is shown in figure 3.6.6.

4. Discussion

A major sector of lipid biotechnology centers on the ability of various oleaginous microorganisms to convert various natural substances into microbial lipophilic compounds. The microbial oils present a potential industrial and financial interest due to their specific characteristics (Ratledge, 1992, 1994; Certik & Shimizu, 1999; Papanikolaou *et al.,* 2001).These oils have long been considered as alternative oil sources, specifically as regards lipids rarely found in the plant or animal kingdom. (Dyal *et al.,* 2005; Dyal & Narine, 2005, Papanikolaou *et al.,* 2003; Fakas *et al.,* 2009; Kenny *et al.,* 2000; Ratledge, 2005). However, the production costs of SCO are still high, with serious efforts being made to reduce these costs by using waste materials as substrates, either in submerged or in solid state fermentation systems (Papanikolaou *et al.,* 2003; Xue *et al.,* 2006; Papanikolaou & Aggelis, 2002; Ahmed *et al.,* 2006). Fungi, especially Zygomycetes strains, are considered as favourable oleaginous microorganisms and potential producers of single cell oil (SCO) containing g-linolenic acid (GLA, $^{\Delta 6,9,12}$C18:3), a polyunsaturated fatty acid (PUFA) of crucial dietary and pharmaceutical importance (Ratledge & Wynn, 2002 , 2006; Fakas *et al.,* 2009; Papanikolaou *et al.,* 2004). One of the oleaginous fungi, *Mortierella isabellina* is capable of accumulating considerable amount of lipids, up to 80% of cell biomass (Chatzifragkou *et al.,* 2010). *M. isabellina* could be cultivated on various substrates, including monomer sugars (Chatzifragkou *et al.,* 2010), glycerol (Fakas *et al.,* 2008), as well as lignocellulosic biomass (rice hull and corn stover) hydrolysates (Ruan *et al.,* 2012). These features, together suggested that *M. isabellina* could be a good candidate for lipid production from low cost renewable feedstock.

Consequently, the present study was focused on the potentiality of growth and lipid production by an oleaginous fungus strain named, *Mortierella isabellina* strain ATHUM 2935 cultivated on two renewable carbon sources namely xylose and glucose, abundant sugars of lignocelluloses' biomass, solely and in mixtures in batch-flask cultures at initial concentrations of 80 g/L. The biochemical behavior (biomass production, accumulation of total lipid, substrate uptake, fatty acid composition of fungal oil) was studied when the aforementioned microorganism was cultivated under nitrogen limited conditions in order to direct the microbial metabolism towards the synthesis of intra-cellular lipid.

Mortierella isabellina ATHUM 2935, presented noticeable biomass production in all substrates used in this study. Growth data for all substrates are presented in Table 4.1. Unexceptionally, there was an almost complete exhaustion of nitrogen source in the fermentation medium occurring approximately at 43- 45 h after inoculation and the lipid accumulation was simultaneously enhanced. The differences that were objerved in the process of lipid accumulation as related to the carbon sources used for *Mortierella isabellina* ATHUM 2935 were attributed to the different metabolic pathways involved in the assimilation of the above substrates. Therefore, the various carbon sources/mixtures were channeled, at different extent, to storage lipid or to lipid-free biomass formation

Table 4.1 Kinetic data of *Mortierella isabellina* ATHUM 2935 cultivation on glucose, xylose or mixtures as carbon source, in shake-flask experiments.

Substrate (g/L)		Time (h)	Glc_r (g/L)	Xyl_r (g/L)	X (g/L)	L (g/L)	Xylitol (g/L)	$Y_{L/X}$ (%,w/w)	GLA (mg/L)
Glucose	**Xylose**								
		332**	37,2		**28**	11.2	-	39.6	193
80	**-**	499*	29,2		26.7	**13.6**	-	51.1	415
		524	24.1	-	26.5	11.5	-	43.6	385
		332**/*		35,7	**20.7**	**7.7**	11.0	37.2	379
-	**80**	499 º		25.4	15.3	6.5	**23.5**	42.4	334
		524	-	24.9	15.8	3.9	21.2	25	211
40	**40**	379**/*	2.6	20.1	**23.1**	**8.7**	-	47.2	352
		280*	5.6	20.4	27	**15.6**	-	57.8	520
60	**20**	397**	0	17,9	**27.9**	12.8	-	46.1	376
		491	0	16.9	27.6	9.1	-	32.9	270
		258**/*	0	19.6	**20.5**	**12.3**	9.2	59.8	481
20	**60**	397 º	0	5.1	19.9	9.3	**17.2**	46.5	309
		491	0	0	20.2	8.8	13.9	43.4	348

Glc_r, remaining glucose, Xyl_r, remaining xylose , X, total dry biomass , L, lipid accumulation , $Y_{X/L}$, lipid in total dry biomass (%, w/w), GLA, γ-linolenic acid

*Representation when the maximum lipid accumulation was achieved.

**Representation when maximum biomass was achieved

º Representation when maximum xylitol was achieved.

As it is noted in Table 4.1 and Figure 4.1, commercial glucose was the most appropriate substrate for biomass production for the aforementioned microorganism, resulting in a maximum biomass production corresponding to 28g/L, after 332 h of cultivation. At the end of fermentation, large amounts of glucose remained unconsumed into the growth medium (24. 14 g/L) while no degradation (turnover) of the accumulated lipid occurred during stationary phase. Moreover, intracellular oil was typically accumulated after depletion of the nitrogen source from the culture medium, resulting in a the maximum lipid quantity (L*max*) of 13,61 g/L with the corresponding lipid in total dry biomass $Y_{X/L}$, equal to 51.1 % (w/w). The cellular lipids of Mortierella isabellina were found to be particularly rich in GLA at this point, reaching the quantity of 415 mg /L of medium. Moreover, the lipid yield, $Y_{L/Glc}$ on glucose consumed was around to 0.22 (g/g) and it was the maximum of all the fermentations (Figure 4.1.). Furthermore, on xylose-based media, the fungal strain was capable of producing satisfactory amounts of biomass, 20.7 g /L, while sufficient amounts of cellular lipids were also produced , with a the maximum lipid quantity (L*max*) of 7.71g /L and at this growth step, the corresponding $Y_{L/X}$ value was equal to 37.2 (w/w). As far as GLA production was concerned on the xylose-based medium (80 g/L),reached the maximum value of 379 mg/L about 332h after inoculation. The lipid yield, $Y_{L/Xyl}$ on xylose consumed was around to 0.10 (g/g), the lowest of all the fermentations (Figure 4.2.). And at this fermentation, large amounts of xylose remained unconsumed into the growth medium (24.9 g/L).

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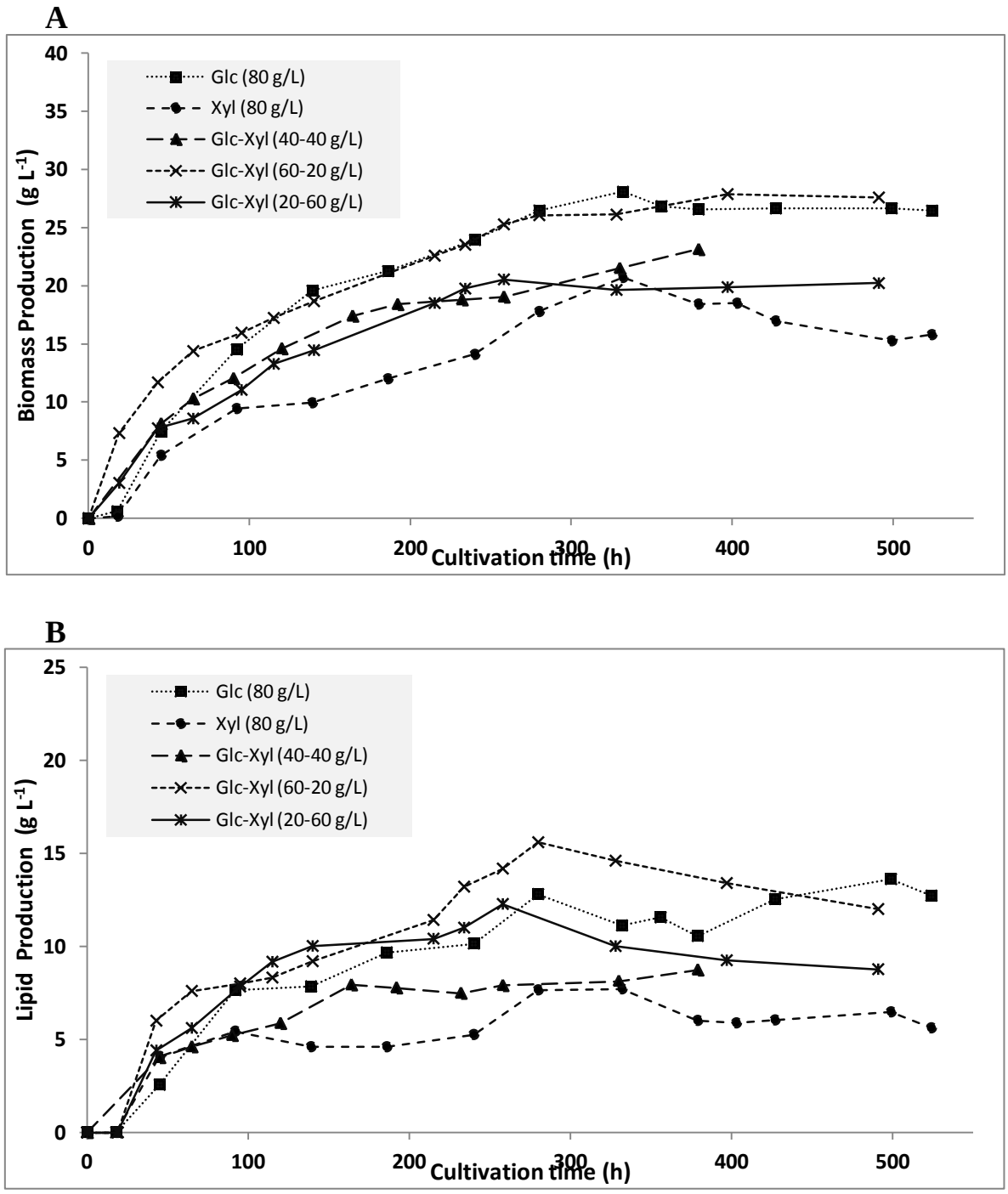

Figure 4.1 Growth kinetics (A) and lipid production kinetics (B) of *Mortierella isabellina* ATHUM 2935 cultivated on glucose and xylose, solely and in mixtures in batch-flask cultures at initial concentrations of 80 g/L. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, $(NH_4)_2SO_4$=0.5 g/L , yeast extract=0.5 g/L , initial pH=6.0±1, initial C/N ratio 205 (mol/mol), incubation temperature *T*=28 ^oC, full aerobic conditions**.**

On the other hand, an interesting finding that was observed in this study was that the cultivation of *Mortierella isabellina* ATHUM 2935 on xylose, favored the production of xylitol, a five carbon sugar alcohol occurs widely in nature but it is also a normal intermediate in human metabolism. The maximum concentration of xylitol was 23.5 g/L during the end of fermentation. The secretion of xylitol can account for the relatively lower final concentrations of produced biomass and cellular lipids by the strain during growth on xylose, as compared with the performance of the microorganism during growth on glucose. Regarding the fungi, the first significant report was by Dahiya, (1991) about *Petromyces albertensis*. This fungus

accumulated 39.8 g/l of xylitol when cultured for 10 d on 100 g/L xylose. Another zygomycetes *Thamnidium elegans* when cultivated on xylose (100 g/L) favoured the production of xylitol, reaching a maximum concentration of 31. 3 g/L (Zikou *et al.*, 2013). In general, among microorganisms, the yeasts are considered to be the best xylitol producers particularly those belonging to the genus *Candida* (Winkelhausen & Kuzmanova, 1998) and therefore, the majority of publications deal with them. *Mortierella isabellina* ATHUM 2935 was tested for its ability to grow and accumulate intracellular oil during its cultivation on glucose–xylose mixtures, under nitrogen-limited conditions. Data obtained from the kinetics are presented also in table 4.1. As it was observed, the strain grew particularly well in all the glucose–xylose blends, with maximum biomass production ranging from 20.5 to 27.9 g /L as it is listed in the table, while only in one of the fermentations (60 g/L xylose :20 g/L glucose) total substrate assimilation occurred. *Mortierella isabellina* ATHUM 2935, when cultured on the carbon blend of commercial glucose (60 g/L) & xylose (20 g/L) produced significant amounts of SCO, reaching up to 15.5 g/L about 280h after inoculation, and this was the maximum lipid quantity (L*max*) which was achieved in this study. As far as GLA production was concerned, the mixture of glucose: xylose 60:20 (in g /L) proved to be the most promising substrate among others tested for the accumulation of the aforementioned FA, reaching the maximum value of 520 mg /L about 280 h after inoculation. The corresponding $Y_{L/X}$ value at this growth step was equal to 57.8 (w/w). The lipid yield, $Y_{L/S}$ on this carbon blend consumed was around to 0.21 (g/g), the second maximum yield in this study. In the other two fermentations: 40 g/L glucose: 40 g/L xylose & 60 g/L xylose: 20 g/L glucose, the microorganism proved capable of producing noticeable amounts of SCO, with maximum lipid quantities (L*max*) equal to 8.74 g/L and 12.28 g/L, accompanied by sufficient values of lipid in dry weight ($Y_{L/X}$), 47.2% and 59.8% (w/w), respectively and GLA production ath this points, reaching the value of 352 mg/L and 482mg/L, respectivelly. The lipid yields ($Y_{L/S}$) on these carbon blends were also satisfactory and equal to 0.12 (g/g) and 0.16 (g/g), respectively. Furthermore, it should be mentioned that xylitol production took place only in the mixture with 60 g/L xylose. It was observed that in mixtures in which xylose was in shortage (xylose < 60g/L, i.e. glucose– xylose 60–20(g/L)], there wasn't any xylitol production. However, the amount of accumulated xylitol remained lower than that achieved on the substrate with xylose as the sole carbon source, indicating in accordance with the relevant literature (Kim et al. 2009), that xylitol production could be significantly altered by the presence of glucose in the fermentation medium. Specifically, xylitol accumulation began only after glucose was depleted from the culture medium, revealing an obstructive role of glucose on glucose–xylose mixtures, in terms of xylitol secretion. There was a small consumption of produced xylitol occurred at the end of the fermentation, a time point in which xylose was almost depleted from the culture medium. However, the above mentioned polyol was not reconsumed by the fungus, in favour of lipid production. Regarding xylose catabolism by yeast and fungal strains, after sugar transport across the cell membrane via facilitative diffusion or active transport, xylose is subjected to a two-step reduction and oxidation (Winkelhausen & Kuzmanova 1998). Firstly, xylose is reduced to xylitol by either NADH- or NADPH-dependent xylose reductase. Then, xylitol is secreted from the cell or oxidized to xylulose by a NAD- or NADP-dependent xylitol dehydrogenase, followed by its phosphorylation to yield in

the synthesis of xylulose-5-P. This intermediate will either be directly cleaved into glycerinaldehyde- 3-P and acetyl-P (the so-called 'phosphoketolase reaction]) or will enter into the pentose phosphate pathway (Jeffries 1983; Winkelhausen & Kuzmanova 1998; Sonderegger *et al.* 2004; Papanikolaou & Aggelis 2011a).

Concerning the uptake of individual sugars in the fermentations with glucose and xylose used as carbon blend- substrate: 60 g/L glucose: 20 g/L xylose & 60 g/L xylose: 20 g/L glucose, it must be stressed that *Mortierella isabellina* ATHUM 2935 showed preference for consumption of glucose. Glucose was noticeably faster consumed as compared to xylose from the beginning of growth. Especially in the trial with the blend of xylose- glucose (60:20 g/L), glucose consumption was almost complete (97 %, wt/wt) from the first 65h of cultivation while the catabolism of xylose started after the depletion of glucose. In some cases, the existence of glucose in the fermentation medium as cosubstrate has been reported to partially inhibit xylose consumption, while the catabolism of the latter starts after the preferred sugar (i.e., glucose) is depleted (Winkelhausen & Kuzmanova 1998; Stulke & Hillen 1999; Kim *et al.* 2009; Zikou *et al.,* 2013). On the contrary, as regards the consumption of substrate in the fermentation with equal portions of the two sugars (40 g/L glucose: 40 g/L xylose) it is observed that, the microorganism started the simultaneous assimilation of both sugars occurred regardless of the initial concentration of the sugars adjusted into the medium, with almost the same rate at the first 90 hours of cultivation. However, after that it is observed a notable decrease in the rate of xylose consumption. This resulted in the consumption of nearly all the glucose (2.67 g/L) while significant amounts of xylose (20.1 g/L) remained unconsumed at the end of fermentation.

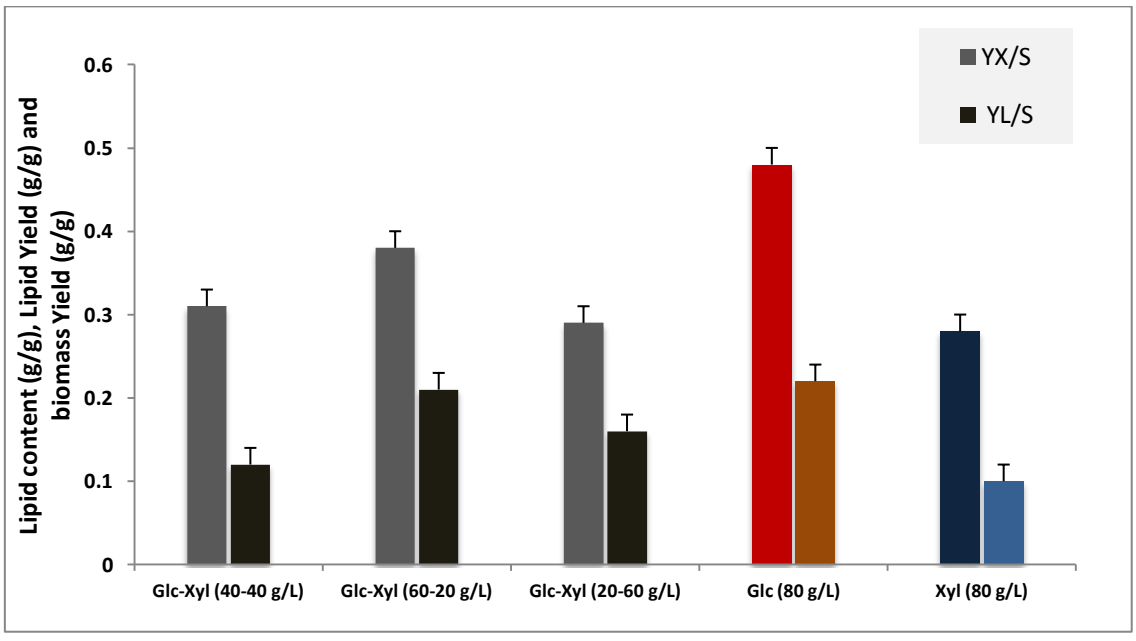

Figure 4.2 Total biomass and lipid yield ($Y_{X/S}$ *and* $Y_{L/S}$) on glucose and xylose, solely and in mixtures in batch-flask cultures at initial concentrations of 80 g/L. Culture conditions: growth on 250-mL flasks at 180 ± 5 rpm, $(NH_4)_2SO_4$=0.5 g/L , yeast extract=0.5 g/L , initial pH=6.0±1, initial C/N ratio 205 (mol/mol), incubation temperature *T*=28 ^oC, full aerobic conditions**.**

According to Ratledge, (1988), the maximum theoretical $Y_{L/Glc}$ may reach a value of 31%, provided that all the glucose consumed would be channeled to lipid biosynthesis. However, reported conversion values

seldom exceed 20%, showing that this value may be considered as a conversion threshold for glucose (Fakas *et al.,* 2009). Although *M. isabellina* is able to assimilate various hexose and pentose sugars, its efficiency for xylose utilization is much lower than for glucose (Chatzifragkou *et al.,* 2010; Fakas *et al.,* 2009). Glucose as a model substrate has been studied for microbial lipid production by this strain, with lipid yield on glucose consumed around 0.22 g g^{-1} (Chatzifragkou *et al.,* 2010; Papanikolaou *et al.,* 2004). Previous reports showed that the achieved lipid yield from xylose was much lower than that of achieved by using glucose as substrate. Fakas *et al. (*2008) has reported an overall lipid yield of 0.113 g g^{-1} by growing *M. isabellina* on xylose medium with carbon to nitrogen ratio (C/N) from 78 to 285. In this study, *Mortierella isabellina* converted glucose to lipids with an overall $Y_{L/Clc}$ equal to 22% (or 0.22 g/g), converging to the reported threshold. Conversely, the yield $Y_{L/Xyl}$ was equal to 0. 10 (g/g) obtained in *Mortierella isabellina* xylose fermentation (figure 4.2) was lower than the one of $Y_{L/Glc}$ indicating that this fungus probably metabolized xylose through the pentose phosphate pathway and not through the pathway of phosphoketolase, which is more efficient than the glucolytic pathway (Evans & Ratledge, 1984). According to Ratledge, (1988), the theoretical $Y_{L/Xyl}$ is 34%, on the condition that xylose is converted to lipid through the phosphoketolase reaction. The yield $Y_{L/Xyl}$ obtained for *Mortierella isabellina* ATHUM 2935 was much lesser than the $Y_{L/Glc}$, further supporting the view that xylose was metabolized through the pentose phosphate pathway.

The analysis of the lipids produced by *Mortierella isabellina* ATHUM 2935 was conducted during the cultivation time in all fermentations, to monitor possible alterations of the lipid profile of the micro-organism. The most abundant fatty acids of fungi tend to be of carbon chain-lengths 16 and 18, with varying degrees of unsaturation .In this study, it was observed that, the major fatty acids were palmitic (C16:0), stearic (C18:0), oleic ($^{\Delta 9}$C18:1), linoleic ($^{\Delta 9,12}$C18:2) and γ-linolenic ($^{\Delta 6,9,12}$C18:3 , GLA) acids. The most predominant component in the mycelium was oleic acid in the range of 33-55% of the total fatty acids. Palmitic acid was found to be the second most abundant fatty acid with the percentage of 18-29% w/w. The results showed that linoleic and stearic acid were found in noticeable quantities into the reserve lipids while palmitoleic acid was detected in traces. As it was observed, the cultivation of *Mortierella isabellina* n ATHUM 2935 on xylose, favored the production of stearic acid. *Mortierella isabellina* ATHUM 2935 showed satisfactory capability to produce γ-linolenic acid (GLA), regardless of the substrate employed. GLA content in xylose grown mycelia was somewhat higher than that in mycelia grown on glucose or in mediums containing a blend of xylose and glucose. According to Fakas *et al.,* (2009), these discrepancies could be attributed to the different metabolic pathways implicated in the assimilation of each carbon source utilized as a substrate. GLA was found in contents between 0.73 – 5.41 % during the growing steps of the fermentations. During cultivation of the strain in xylose or in glucose–xylose mixtures, the percentage of oleic acid was found to be lower, as compared to those obtained during cultivation on glucose as the sole carbon source. The highest quantity of GLA achieved in the current investigation was 520 mg/L that is a rather satisfactory value compared with the literature; concerning other micro-organisms grown in flasks, C. echinulata CCRC 31840 produced 964 mg/L of GLA under optimized conditions (Chen & Chang 1996).

Fakas *et al.* (2009a) demonstrated that the strain C. echinulata ATHUM 4411 cultivated on xylose, accumulated 1119 mg /L of GLA, while growth on tomato waste hydrolysate yielded 800 mg /L (Fakas et al. 2008b). Mortierella isabellina growing on lactose-enriched cheese whey produced GLA to ~300 mg /L (Vamvakaki *et al.* 2010). *Cunninhamella echinulata* produced ~500 mg /L on media based on waste molasses, while significant decolorization-detoxification of the medium was observed together with GLA production (Chatzifragkou *et al.* 2010). The highest quantity of GLA reported in the literature has been obtained by a mutant of *Mortierella ramanniana* (strain MM15-1) cultivated on media containing extremely high initial sugar amounts (initial glucose at 300 g/L), and a concentration of ~5550 mg of GLA per l of culture medium was reported when growth was carried out on a specific type of bioreactor (Hiruta *et al.* 1997).

The fractionation of cellular lipids was conducted for *Mortierella isabellina* ATHUM 2935, in order to investigate the possible impact of the different carbon sources used upon the lipid classes. From the results obtained at table 4.2. it can be noted that, mycelia of *Mortierella isabellina* contained significant higher amounts of neutral lipids than polar lipids. More specifically, the fraction of neutral lipids (N) was the major constituent in the range of 72.9 - 93.1% of the total lipids. The fractionation during the growth of microorganism showed that the percentages changed in favor of neutral lipids (N). More specifically, the percentage of neutral lipids (N) increased nearly in all the experiments, except from the fermentation of commercial glucose (60 g/L) & xylose (20 g/L) in which the neutral lipid content showed a significant decrease equal to 14% after 397 days of growth. The percentage of P fraction showed an increase at the middle of growth but declined slightly therafter, when glucose (80 g/L) was used as sole carbon source. The same happened when xylose (80 g/L) was used as carbon source but it is worth mentioning that in this case, P content showed the greatest decrease equal to 9% after 240 h of growth. The quite opposite occurred in glucose–xylose mixtures (40:40 & 60:20) as it is shown in table 4.2. On the contrary, as regards the P content of cellular lipid in the fermentation using xylose (60 g/L) & glucose (20 g/L), it was observed that, the percentage of P fraction was high at the beginning of growth but significantly decreased with an overall decrease of 5% after 397h of cultivation. The percentage of G+ S fraction showed a significant increase equal to 11.6 % was observed when mixture of glucose (60 g/L) and xylose (20 g/L) were used. On the contrary, as it is shown in the table 4.2. when glucose (80 g/L) was used as sole carbon source, there was a slight increase in G + S content at the middle of the growth, while a significant decrease occurred equal to 2.3%, thereafter, while, the quite opposite occurred when xylose (80 g/L) and xylose & glucose (20:60 g/L) were used as carbon sources. However, when glucose and xylose used in equal portions (40:40 g/L) the percentage of G + S was high at the beginning of growth but declined thereafter, with an overall decrease of about 8.8 %. Generally, it can be said that, the increase of neutral lipid fraction indicated that reserve lipid accumulated, despite the high nitrogen concentration in all the growth mediums, while the increase of G + S and P content most probably reflected the biosynthesis of fat-free biomass occurring after lipid accumulation inside the mycelia mass since both fractions are characteristic components of cell membranes (Fakas et al. 2007, 2009a).

Table 4.2 Percentages (in%, w/w) of neutral (N), sphingolipid and glycolipid (G + S) and phospholipid (P) fractions during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on glucose and xylose-based media, solely or in mixtures.

Substrate (g/L)	Time (h)	Fraction	Percentage %
Glucose: Xylose (40:40)	**65**	N	83.6
		P	3.4
		G+S	13.1
	120	N	90.7
		P	1.7
		G+S	7.6
	232	N	93.1
		P	2.6
		G+S	4.3
Glucose 80	**92**	N	87.5
		P	1.2
		G+S	11.3
	186	N	87.0
		P	1.5
		G+S	11.6
	280	N	89.9
		P	0.8
		G+S	9.3
Xylose 80	**45**	N	83.9
		P	5.9
		G+S	10.2
	139	N	79.3
		P	12.7
		G+S	8.0
	240	N	85.4
		P	3.6
		G+S	11.0
Glucose: Xylose (60:20)	**43**	N	87.3
		P	6.5
		G+S	6.2
	234	N	87.8
		P	5.5
		G+S	6.7
	397	N	72.9
		P	8.8
		G+S	18.3
Xylose :Glucose (60:20)	**43**	N	78.4
		P	10.0
		G+S	11.6
	234	N	87.5
		P	5.9
		G+S	6.6
	397	N	85.0
		P	5.0
		G+S	10.0

NL: neutral lipids, P: phospholipids, G+S: glycolipids and sphingolipids

The estimation of the total amount of phospholipids in oil produced by *Mortierella isabellina* ATHUM 2935 was a necessity before the chromatographic separation by TLC. This prevents the inevitable loss of phospholipid molecules on adsorbents and thus allows accurate estimation of the ratio of phosphorus to phospholipids in the samples. Moreover, the knowledge of the phospholipid content was a necessity to grade the quality of the oil produced by this particular strain growing on xylose and glucose solely and in mixtures at initial concentrations of 80 g/L and examine the quantity of the phosphorus in the oil produced by this strain. The quantitative determination of phospholipids was based on the determination of phosphorus content in the lipid samples according to Rouser *et al.,* (1970*).* The amount of phospholipids was calculated on a molar basis from the amount of P and on a weight basis after multiplying the amount of P by 25. Three different lipid samples were chosen from every fermentation and the aggregated results illustrated in the Table 4.3 and Figure 4.3.

Table 4.3 Amount of Phosphorus (in μg/ g Lipid) and phospholipids (in mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on glucose and xylose solely and in mixtures at initial concentration of 80 g/L .

Substrate(g/L)	Time (h)	Total lipids (g/L)	μg Phopsphorus /mg Lipids	mg Phospholipids /g lipid	Phospholipids (g / L)
Glucose (80)	92	9.64	0.250	**6.25**	0.060
	240	10.15	0.407	**10.16**	0.103
	379	10.56	0.157	**3.93**	0.041
Xylose (80)	45	4.09	0.167	**4.19**	0.017
	240	5.26	0.237	**5.91**	0.031
	379	4.66	0.415	**10.38**	0.048
Glucose :xylose (40:40)	45	4.03	0.077	**1.91**	0.008
	120	5.86	0.513	**12.82**	0.075
	232	7.49	0.176	**4.40**	0.033
Glucose :Xylose (60:20)	43	7.65	0.322	**8.05**	0.062
	234	14.36	0.233	**5.83**	0.084
	397	12.84	0.462	**11.55**	0.148
Xylose :Glucose (60:20)	43	4.7	0.833	**20.83**	0.098
	234	9.7	0.300	**7.50**	0.073
	397	9.25	0.185	**4.63**	0.043

The quantity of phosphorus and therefore the amount of phospholipids was affected not only by the duration of the particular fermentation but also from the substrate used. Some significant differences between the phospholipid content and the fermentations can be discerned in Table 4.3 and in Figure 4.3.

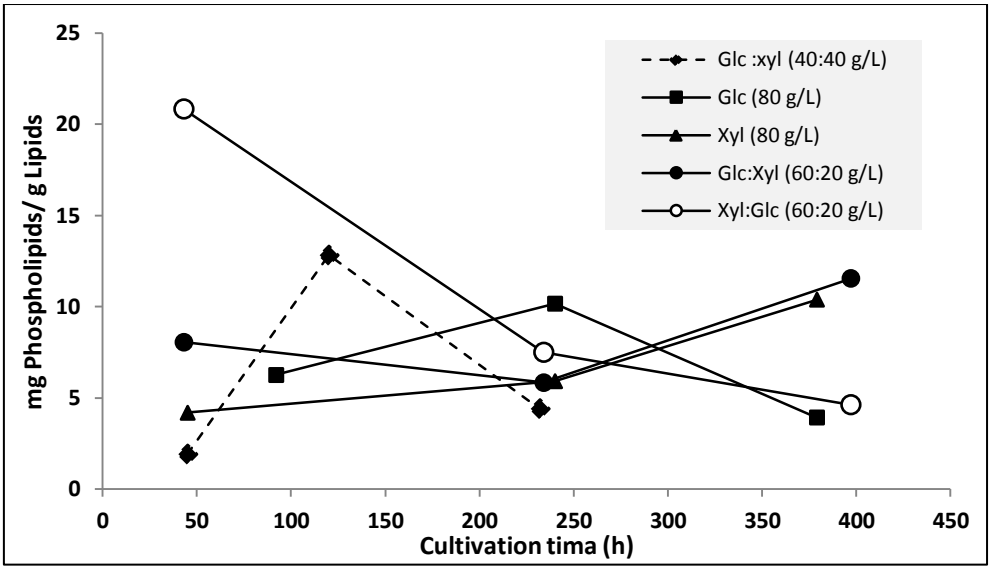

Figure 4.3 Phospholipid content (mg Phospholipids /g lipid) of *Mortierella isabellina* ATHUM 2935 cultivated on glucose and xylose, solely and in mixtures in batch-flask cultures at initial concentrations of 80 g/L.

In total, the quantity of phospholipids ranged from 3.93 mg/g lipid to 20.83 mg/ g lipid. The greatest amount of 20.83 mg phospholipids/ g lipid achieved at the beginning of the growth (43h) when *Mortierella isabellina* strain ATHUM 2935 cultivated on the carbon blend of commercial xylose (60 g/L) & glucose (20 g/L) and the lowest amount of 3.93 mg after 379h of cultivation on glucose as sole carbon source. More specifically, the amount of phospholipids increased during the cultivation when commercial xylose (80 g/L) and a blend of glucose (60 g/L) & xylose (20 g/L) were used, achieved the maximum amount of 10.38mg/ g lipid and 10.55mg/g lipid near the end of fermentation, respectively. On the contrary, when the blend of commercial xylose (60 g/L) & glucose (20 g/L) was used as substrate, a notable decrease was observed during the cultivation and it was equal to 16.2 mg/g lipid near the end of the fermentation. On the other hand, when glucose (80 g/L) solely and the blend of commercial glucose and xylose in the same proportion (40:40 g/L) was used, a similar change was presented on amount of phospholipids. More specifically, the phospholipid fraction in both experiments, increased until the middle (240h) and the early middle (120h) of the growth achieved the maximum value of 10.16 mg/ g lipid and 12.82 mg/g lipid, respectively, while decreased by the end of fermentations (table 4.3., figure 4.3.)

In order to make a step forward to elucidate the single cell oil produced by *Mortierella isabellina* ATHUM 2935 on chemical basis, analysis of total sugar content in crude lipid samples was performed. The constituent hexoses were identified as glucose from a modification of phenol sulfuric method according to Kushwaha & Kates, (1980). The sugar content of the samples was calculated based on the standard curves of glucose and was expressed in mg glucose /g lipid. As it was expected, the sugar content of glycolipid and sphingolipid fractions was not constant since it reflects the biosynthesis variation of these lipid classes during the fermentations.

Table 4.4 Sugar content in crude lipid samples (mg/ g Lipid) during lipid accumulation phases of *Mortierella isabellina* ATHUM 2935 cultivated on glucose and xylose solely and in mixtures at initial concentration of 80 g/L .

Substrate(g/L)	Time (h)	Total lipids (g/L)	mg Glucose /g Lipid
Glucose (80)	92	9.64	**81.45**
	240	10.15	**98.85**
	379	10.56	**34.31**
Xylose (80)	45	4.09	**23**
	240	5.26	**82.2**
	379	4.66	**95.3**
Glucose :xylose (40:40)	45	4.03	**51.42**
	120	5.86	**103.32**
	232	7.49	**38.12**
Glucose :Xylose (60:20)	43	7.65	**103.8**
	234	14.36	**22.3**
	397	12.84	**16**
Xylose :Glucose (60:20)	43	4.7	**106.4**
	234	9.7	**25.7**
	397	9.25	**12.4**

In total, the sugar content in crude lipid samples ranged from 12.4 mg/g lipid to 106 mg/ g lipid. These two extreme values achieved when the blend of xylose (60 g/L) & glucose (20 g/l) was used as substrate, respectively. Generally, the sugar content decreased significantly during the cultivation when blends of xylose and glucose used as substrates (60:20 g/L & 20: 60 g/L) as it is shown in table 4.4. with an overall decrease of about 90 mg/ g lipid in both cases. As far as, the sugar content when glucose (80 g/L) solely and the blend of commercial glucose and xylose in the same proportion (40:40 g/L) were used, a notable increase was observed until the middle (240h) and the early middle (120h) of the growth . Afterwards, there was a decrease in both experiments until the end of fermentation as it is shown in table 4.4 and figure 4.4. Finally, when xylose was used solely at the initial concentration of 80 g/L, a significant increase in the sugar content was observed during the cultivation, reaching the value of 95.3 mg/g lipid near the end of fermentation (379h).

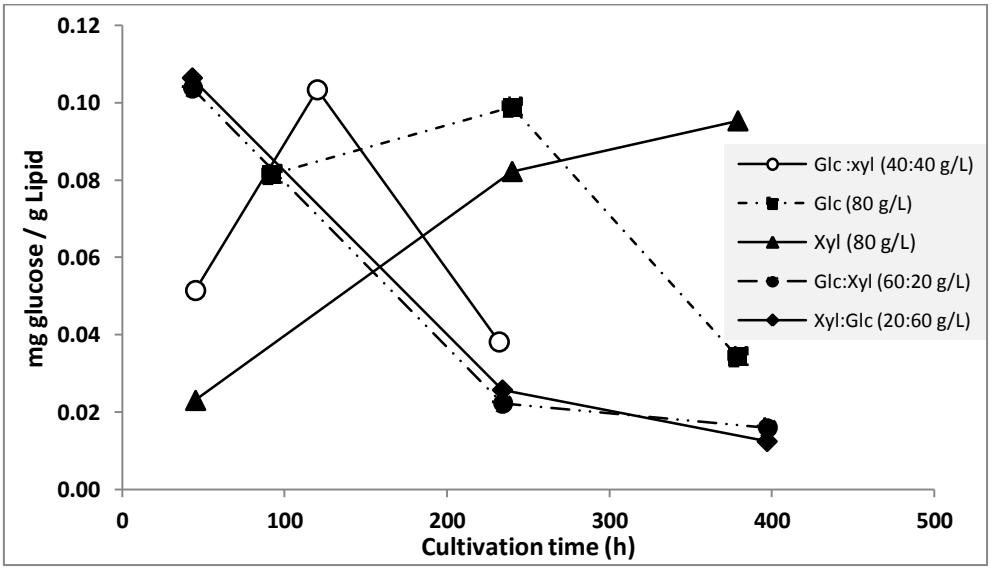

Figure 4.4 Sugar content (mg glucose/ g lipid) in crude lipid samples of *Mortierella isabellina* ATHUM 2935 cultivated on glucose and xylose, solely and in mixtures in batch-flask cultures at initial concentrations of 80 g/L.

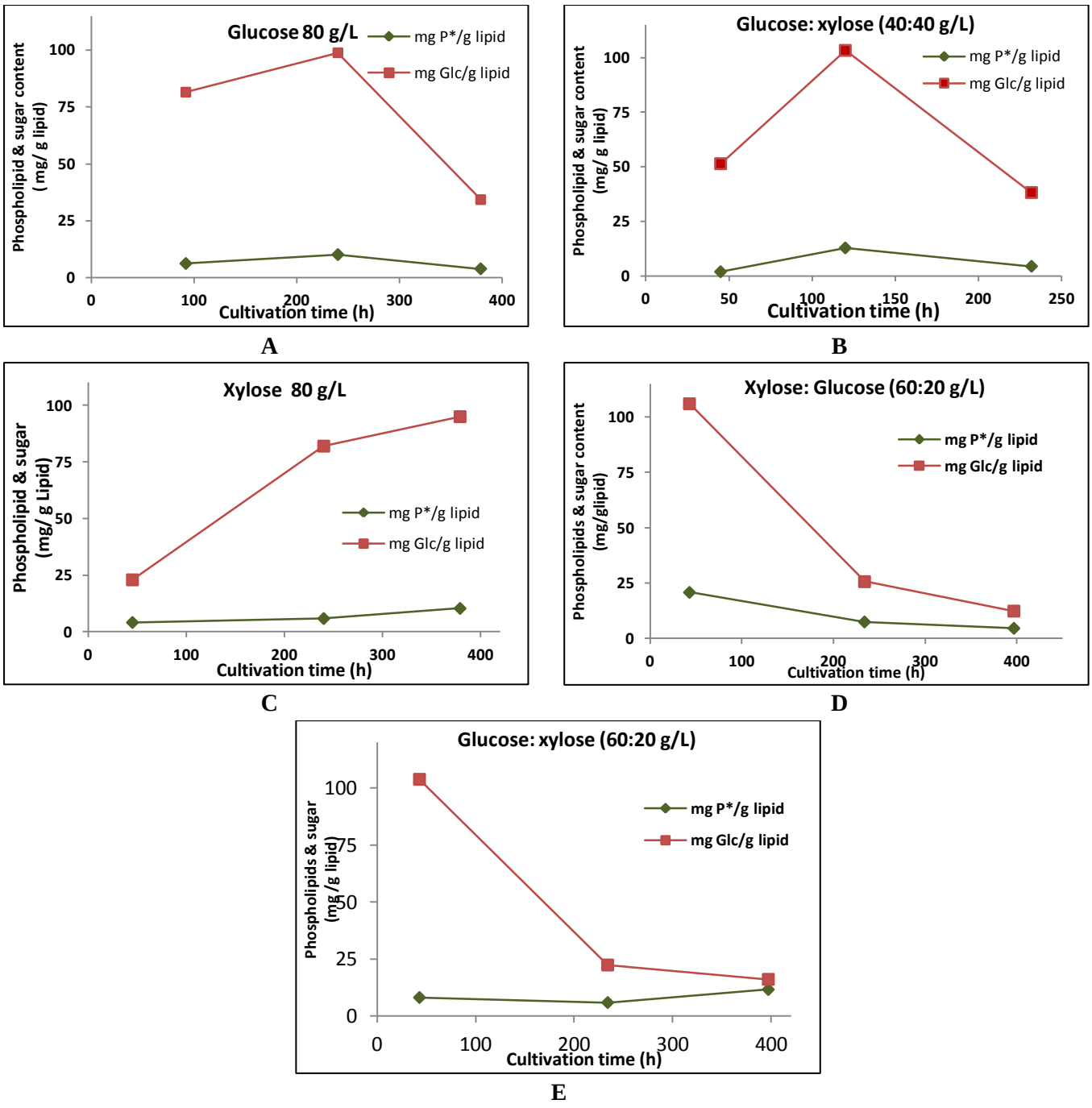

Figure 4.5 Sugar content (mg glucose/ g lipid) and phospholipid content (mg Phospholipids /g lipid) in contrast in different substrates : A) Glucose (80 g/L) B) Glucose : Xylose (40:40 g/L) C) Xylose (80 g/L) D) Xylose: Glucose (60:20 g/L) and E) Glucose: Xylose (60:20 g/L).

Comparing the above results, it seems that the phospholipids and sugar content showed the same pattern when *Mortierella isabellina* strain ATHUM 2935 cultivated on commercial glucose (80 g/L) and on a carbon blend of commercial glucose and xylose (40:40 g/L). More specifically, both phospholipids (mg/g lipid) and sugar content (mg/g lipid) had a notable increase near the middle of cultivation and then decreased until the end of growth. On the contrary, both phospholipids and sugar content showed a notable increase during growth when the aforementioned microorganism cultivated on commercial xylose (80 g/L), while the

exact opposite pattern for both phospholipids and sugar content appeared when a blend of commercial xylose and glucose (60:20 g/L) were used, as it is shown in the figure 4.5. However, the pattern of phospholipids and sugar content was not the same when a blend of commercial glucose and xylose (60:20 g/L) was used as substrate, since there was a notable increase in phospholipids content while the sugar content showed a significant decreased during the growth as it is showed in the figure 4.5. In conclution, it can be said that both the phospholipids and sugar content were affected not only by the duration of the particular fermentation but also from the substrate used.

Thin layer Chromatography (TLC) is one of the most frequently used separation techniques in qualitative as well as quantitative analysis. TLC has long been used for the separation of lipid mixtures into their components lipid classes (complex lipid mixtures as well as for individual lipids) (Marinetti, 1967; Kurt, 1968; Touchstone, 1992; Christie & Han, 2010). In order to make a step forward to elucidate the single cell oil produced by *Mortierella isabellina* ATHUM 2935 on chemical basis, the chromatographic analysis of the intracellular lipids can be undertaken to reveal what different classes are present in the fungus oil. The analysis via thin layer -chromatography (TLC) on silica gel G was conducted to determine the relative composition of the major lipid classes of the aforementioned microorganism. The non polar lipids identified were: predominantly triacylglycerols (TAG) and to a lesser degree sterol esters (CE), free fatty acids (FFA), sterols (CL) and monoacylglycerols (MAG). Additionally, the following polar lipids were also identified: phosphatidyl-choline (PC), monogalactosyldiacylglycerol (MGDG) and cerebrosides. Further experimental work is needed in order to explore the effect of substrate on the composition of the various lipid classes using quantitative TLC. In addition, the separation of a lipid class into individual molecular species in its native form has to be performed in order that biosynthesis or metabolism of every part of the molecule can be studied.

Overall, the fungus *Mortierella isabellina* ATHUM 2935 proved to be a promising microorganism during its cultivation on glucose and xylose substrates, principal sugars of hydrolysed lignocellulosic biomass, yielding significant amounts of intracellular oil, rich in GLA. By taking into account that microbial lipid production still represents an expensive process, the successful biotransformation of sugar-based lignocellulosic residues as substrates represents an attractive alternative.

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