Polyunsaturated fatty acids synthesized by Zygomycetes grown on glycerol

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Introduction

Oleaginous Zygomycetes have the ability to accumulate large amounts of lipids (namely single cell oils- SCO) rich in polyunsaturated fatty acids (PUFA), which are of significant pharmaceutical and nutritional value [1]. Among PUFA, γ-linolenic acid (GLA) is of particular interest as it is known for its selective killing of tumor cells without harming normal cells. Besides its anticancer properties, GLA is effective against rheumatoid arthritis, retards the development of atherosclerosis and normalizes nerve conduction velocity and sciatic endoneurial blood flow [2]. The production cost of SCO is currently considered high, so substrates for cultivating oleaginous microorganisms of low acquisition cost, such as glycerol produced during biodiesel manufacture, have attracted the interest of several research groups [3, 4, 5]. Lipid biosynthesis in microorganisms has been studied in detail, whereas PUFA biosynthesis in oleaginous microorganisms needs further studying. Fatty acids are the building blocks of all lipids. PUFA synthesis begins from palmitic (C16) and stearic (C18) acids, which are then, respectively, transformed to palmitoleic ($^{\Delta 9}$C16:1) and oleic ($^{\Delta 9}$C18:1) acids by the introduction of a double bond between the eighth and ninth carbon atoms. Further chain elongation and desaturation follows to yield 20-carbon PUFA and similarly the very long chain PUFA [1, 6]. The proportional changes of the major lipid fractions that are implicated in the biosynthesis of PUFA, as well as their fatty acid compositional shifts occurred in several Zygomycetes during their growth on glycerol were studied in this work in order to get an insight of PUFA biosynthesis in Zygomycetes and how this is related to microbial growth.

Materials and Methods

Microorganisms: *Cunninghamella echinulata* ATHUM 4411, *Mortierella ramanniana* MUCL 9235, *Mucor* sp. LGAM 36, *Thamnidium elegans* CCF-1465 and *Zygorhynchus moelleri* MUCL 143. **Culture conditions:** 250 ml Erlenmeyer flasks containing 50 ml of a liquid medium, were cultivated under nitrogen limited conditions and were incubated in a rotary shaker at T=28°C and 180 rpm. Further incubation of cultures of *M. ramanniana* under non growth conditions, at various temperatures, was performed, after addition in the culture medium of 50 g/l glycerol. **Medium:** Glycerol at 25 g/l, supplemented with minerals and yeast extract. **HPLC analysis:** Glycerol was determined in filtered (through 0.2 μm pore size bacteriological filter, Whatman) aliquots of the culture by an HPLC apparatus (Ultimate 3000, Dionex, Germering, Germany) equipped with an HPX-87H column and a R.I. detector. Conditions: eluant H_2SO_4 0.004 N, flow rate 0.9 ml/min, T=55 °C. **Lipid extraction:** According to Folch protocol [7]. **Lipid fractionation:** by using a column of silicic acid activated by heating overnight at 80 °C. Successive applications of dichloromethane, acetone and methanol produced fractions containing neutral lipids (NL), glycolipids plus sphingolipids (G+S) and phospholipids (P), respectively [8]. **GC analysis:** Fatty acid analysis was performed after trans-methylation according to the AFNOR method [9], in an Agilent Technologies 7890 A device equipped with a HP-88 (J&W scientific) column (60 m x 0.25 mm). Conditions: carrier gas helium, flow rate 1 ml/min, oven T=200 °C, injector T=250 °C, detector (FID) T=280 °C.

Table 1: Growth of *Cunninghamella echinulata*, *Mortierella ramanniana*, *Mucor* sp., *Thamnidium elegans* and *Zygorhynchus moelleri* on glycerol and synthesis of lipids.
Culture conditions: growth in flasks, initial substrate concentration 25 g/l, incubation in a rotary shaker at T=28°C and 180 rpm, initial pH 6. Experiments were performed in duplicate.

Strain	Fermentation time (h)	X (g/l)	L/X %	Lipid fractions % of total lipids			Lipid fractions (mg/l)		
				NL	G+S	P	NL	G+S	P
Cunninghamella echinulata	48	0.6	37.9	75.7	22.2	2.1	172.2	50.4	4.8
	96	1.1	34.4	84.1	13.8	2.1	318.2	52.2	8.0
	144	3.9	31.7	84.2	9.8	6.0	1041.0	121.2	74.2
Mortierella ramanniana	48	0.9	23.9	64.3	24.9	10.8	138.4	53.6	23.2
	96	3.6	32.4	85.6	10.1	4.3	998.4	117.8	50.2
	144	7.2	44.1	85.8	11.4	2.8	2724.4	362.0	89.0
Mucor sp.	48	0.4	37.7	68.3	25.0	5.7	103.0	37.8	8.6
	96	0.8	20.4	81.5	12.6	5.9	133.0	20.6	9.6
	144	1.6	20.5	68.7	14.0	17.2	225.4	46.0	56.4
Thamnidium elegans	48	0.6	18.9	66.5	20.9	12.6	75.4	23.8	14.2
	96	1.5	34.2	76.6	16.1	7.3	393.0	82.6	37.4
	144	2.5	37.2	82.2	16.2	1.6	764.4	150.6	14.8
Zygorhynchus moelleri	48	0.3	48.4	88.0	6.7	5.3	127.8	9.8	7.6
	96	0.5	31.9	83.7	7.3	9.0	133.6	11.6	14.4
	144	0.7	25.7	87.6	6.0	6.4	157.6	10.8	11.6

Figure 1: Kinetics of total biomass (X, g/l) and lipid-free biomass (X_f, g/l) production, lipid accumulation in dry biomass (L/X, %) and glycerol (Glol, g/l) consumption during growth of *Mortierella ramanniana* on glycerol.
Culture conditions: growth in flasks, initial substrate concentration 25 g/l, incubation in a rotary shaker at T=28°C and 180 rpm, initial pH 6.

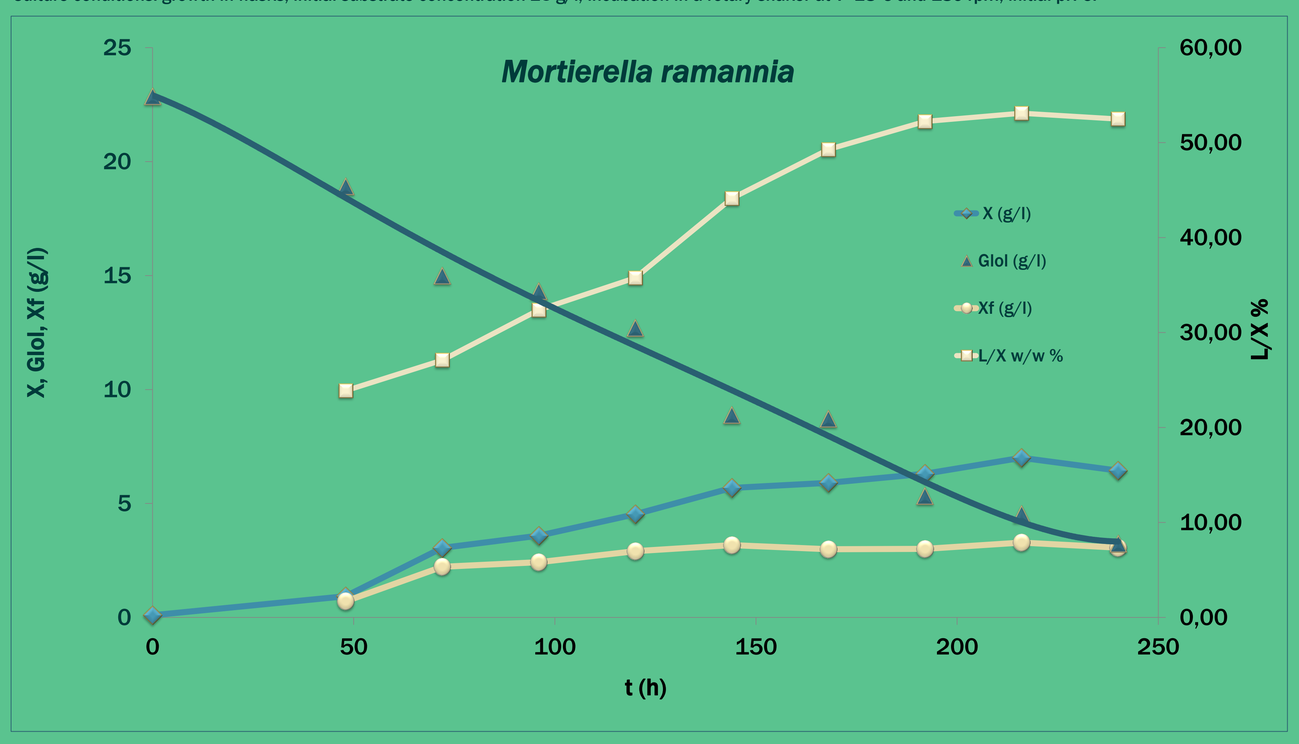

Table 2: Fatty acid composition (%) of Neutral Lipids (NL), Glycolipids plus Sphingolipids (G+S) and Phospholipids (P) during growth of Zygomycetes on glycerol.
Culture conditions: see Table 1. Experiments were performed in duplicate.

Strain	Fermentation time (h)	Lipid fraction	Fatty Acid (%, w/w)						
			C1:60	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 6,9,12}$C18:3	Others*
Cunninghamella echinulata	48	NL	31.4	3.0	8.7	41.6	8.7	3.8	2.8
		G+S	36.4	3.5	9.0	40.0	5.6	1.6	3.9
		P	37.0	3.1	8.5	35.4	8.7	3.7	3.6
	96	NL	21.9	2.2	7.5	35.87	17.4	13.4	1.8
		G+S	22.6	2.2	6.6	37.1	16.1	12.3	3.1
		P	18.8	1.8	6.3	36.9	16.1	15.8	4.3
	144	NL	19.3	1.5	8.6	35.4	18.5	15.3	1.4
		G+S	21.4	1.6	6.5	35.6	17.6	14.5	2.8
		P	17.1	1.3	4.6	34.8	20.3	19.9	2.0
Mortierella ramanniana	48	NL	20.8	1.7	6.5	41.3	17.4	8.9	3.5
		G+S	20.8	1.6	6.2	41.7	18.1	8.5	3.1
		P	12.6	1.8	2.0	37.8	28.5	14.7	2.5
	96	NL	21.7	1.1	6.4	49.5	14.0	4.5	2.7
		G+S	21.5	1.5	6.4	49.8	13.7	4.2	3.0
		P	9.5	1.7	1.4	51.4	24.1	9.2	2.7
	144	NL	21.0	1.3	5.8	49.1	15.9	4.3	2.6
		G+S	20.7	1.7	5.9	47.6	16.0	4.5	3.7
		P	12.7	2.4	3.5	43.2	25.3	8.2	4.7
Mucor sp.	48	NL	26.6	1.4	5.6	25.7	23.0	11.9	5.8
		G+S	35.6	1.8	6.4	26.5	16.9	7.5	5.4
		P	21.7	1.5	2.6	23.7	29.2	16.0	5.4
	96	NL	27.9	1.7	7.6	30.1	19.5	9.4	3.8
		G+S	33.6	2.6	6.4	29.4	14.8	9.2	4.0
		P	25.1	2.1	3.4	26.9	21.6	15.6	5.3
	144	NL	19.8	1.9	5.6	37.1	17.8	15.2	2.6
		G+S	20.9	2.1	3.9	37.8	18.1	11.8	5.4
		P	12.5	2.2	2.5	40.1	20.4	19.8	2.5
Thamnidium elegans	48	NL	21.9	1.1	13.4	39.0	15.2	8.0	1.5
		G+S	20.2	1.4	11.2	46.4	12.5	5.9	2.5
		P	23.4	3.2	7.3	35.6	16.4	7.2	7.0
	96	NL	22.4	1.3	12.0	37.7	16.4	8.9	1.4
		G+S	21.4	1.2	12.0	38.5	16.1	8.4	2.4
		P	21.5	0.9	12.7	37.3	14.3	6.9	6.3
	144	NL	21.7	1.8	11.7	39.3	16.2	7.2	2.1
		G+S	21.5	1.2	11.1	41.5	15.8	7.4	1.5
		P	20.0	2.1	6.9	35.0	22.2	8.8	5.1
Zygorhynchus moelleri	48	NL	28.5	1.7	9.4	39.4	7.0	11.8	2.2
		G+S	27.3	2.4	9.1	41.3	5.3	9.1	5.5
		P	20.5	2.0	4.6	41.8	8.9	17.4	4.9
	96	NL	23.4	1.8	8.5	37.7	9.5	16.3	2.8
		G+S	27.3	1.9	10.8	43.4	6.9	7.2	2.5
		P	18.5	1.6	5.3	42.4	10.8	18.1	3.4
	144	NL	20.5	1.9	6.9	39.8	10.8	17.7	2.4
		G+S	22.6	2.0	6.5	43.6	8.3	11.3	5.7
		P	16.5	1.6	2.8	44.7	10.0	21.4	3.0

*Others: C10:0, C12:0, C14:0, $^{\Delta 9}$C14:1, $^{\Delta 9,12,15}$C18:3.

Results and Discussion

Proportional changes in lipid composition of oleaginous Zygomycetes *Cunninghamella echinulata*, *Mortierella ramanniana*, *Mucor* sp., *Thamnidium elegans* and *Zygorhynchus moelleri* were studied during growth under N-limited conditions. Some of these strains (i.e. *M. ramanniana, C. echinulata)* seem to be able to efficiently convert glycerol into lipid-rich biomass and so they could be considered as a potential candidate for glycerol valorization. A characteristic example of kinetics of *M. ramanniana* is illustrated in Figure 1 (biomass produced X= 8.2 g/l, lipid in biomass L/X> 40%). Significant quantities of neutral lipids (NL) were accumulated into the fungal mycelia after the depletion of nitrogen source of the growth medium, especially in the case of *M. ramanniana* (44% wt/wt oil in dry biomass) and *Th. elegans* (37% wt/wt oil in dry biomass) (Table 1). The glycolipids plus sphingolipids (G+S) fraction did not show remarkable changes in proportion, while proportion of phospholipids (P) fraction was higher at the beginning of growth and declined thereafter in the cases of *M. ramanniana* and *Th. elegans*. Appreciating the actual increase of P fraction (expressed in mg/l) (Table 1), we should conclude that biosynthesis of P occurred as growth proceeded in *M. ramanniana*, *Mucor* sp. and *C. echinulata* suggesting production of new mycelia.

In all lipids produced oleic acid was the major fatty acid, followed by palmitic and linoleic acids (Table 2). As for GLA, it was found in remarkable quantities in all lipid fractions, especially in P fraction, reaching up to 21.4, 19.9 and 19.8% in *Z. moelleri, C. echinulata* and *Mucor* sp., respectively. Compositional shifts of P were observed to almost all unsaturated fatty acids, especially to GLA, reflecting changes in PUFA biosynthetic machinery [10]. PUFA concentration gradually decreased in P of *M. ramanniana* during growth, and this trend was also observed in NL and G+S (Table 2), suggesting that desaturation activity in this strain was strongly related to the primary metabolic growth. Confirmation of this finding came by further incubation of *M. ramanniana* under non growth conditions, at various temperatures (Table 3) [11]. However, the opposite situation was observed in the cases of *C. echinulata* and *Z. moelleri*. In particular, in the case of *C. echinulata* the concentration of both linoleic acid and GLA strongly increased in all lipid fractions, especially in P. Accordingly, the desaturation activity of these two strains seems to be related to their secondary metabolic growth.To conclude, we could say that PUFA biosynthesis in the case o M. ramanniana is strongly associated to mycelial growth, while PUFA biosynthesis persists in C. echinulata after growth cessation. This remark is of practical interest, since for an industrial perspective PUFA biosynthesis should co-ordinate with lipid accumulation process, which is a metabolic activity strictly related to the secondary metabolic growth.

Table 3: Fatty acid composition (%) of Neutral Lipids (NL); Glycolipids plus Sphingolipids (G+S); and Phospholipids (P), after incubation of *Mortierella ramanniana* for 7 days under non-growth conditions at 28 °C, 25 °C and 4 °C (for details see text). Biomass used in this experiment was produced on glycerol 25 g/l. Experiments were performed in duplicate.

Conditions	Lipid fraction	Fatty Acid (%, w/w)						
		C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 6,9,12}$C18:3	Others*
Growth on glycerol T=28 °C	NL	21.0	1.3	5.8	49.1	15.9	4.3	2.6
	G+S	20.7	1.7	5.9	47.6	16.0	4.5	3.7
	P	12.7	2.4	3.5	43.2	25.3	8.2	4.7
Further incubation T=28 °C	NL	21.8	1.2	6.0	51.3	12.7	4.4	2.7
	G+S	23.7	1.4	6.1	50.9	12.3	3.8	1.8
	P	23.1	2.5	6.8	43.0	14.7	5.4	4.6
Further incubation T=25 °C	NL	21.8	1.1	6.8	49.8	13.1	5.3	2.1
	G+S	27.7	1.2	7.3	47.0	10.6	3.5	2.8
	P	23.5	2.3	5.5	48.6	12.2	5.4	2.5
Further incubation T=4 °C	NL	21.6	1.2	7.1	48.2	13.6	6.0	2.2
	G+S	26.4	1.6	7.1	46.7	11.8	4.0	2.3
	P	20.0	1.7	4.5	46.3	15.7	7.7	4.1

*Others: C10:0, C12:0, C14:0, $^{\Delta 9}$C14:1, $^{\Delta 9,12,15}$C18:3.

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References
[1] Certik and Shimizu (1999). J Biosci. Bioeng. 87:1-142.
[2] Fan and Chapkin (1998). J Nutr 128:1411–4.
[3] Fakas et al. (2009). In: Microbial conversions of raw glycerol. Aggelis, G. (Ed.), Nova Science Publishers Inc., New York, pp. 85-99.
[4] Wen et al. (2009). In: Microbial conversions of raw glycerol. Aggelis, G. (Ed.), Nova Science Publishers Inc., New York, pp. 1-7.
Fakas et al. (2006). Appl Microbiol Biotechnol., 73, 676–683.
[5] Liang et al. (2010). Bioresour Technol 101 (2010) 7581–7586.
[6] Ratledge and Wynn (2002). Adv Appl Microbiol 51:1–51.
[7] Folch et al. (1957). J. Biol. Chem. 199:833-841.
[8] Fakas et al. (2006). Appl Microbiol Biotechnol., 73, 676–683.
[9] AFNOR (1984). In: Association Française pour Normalisation. Paris, p. 95.
[10] Fakas et al., (2007). Enzyme and Microbial Technology 40:1321-1327.
[11] Dyal and Narine (2005). Food Research Internat 38:445–467.