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Alterations in fatty acid composition of *Cunninghamella echinulata* lipids induced by orange essential oil

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ABSTRACT

The growth of *Cunninghamella echinulata* on raw glycerol as sole carbon source was positively affected by the presence of essential oil (EO) of orange in the growth medium. However, significant alterations in the fatty acid composition were observed since the biosynthesis of PUFA was inhibited, especially in the late fermentation steps. Refereeing to the ratios $^{\Delta 9}$C18:1/C18:0, $^{\Delta 9,12}$C18:2/$^{\Delta 9}$C18:1 and $^{\Delta 6,9,12}$C18:3/$^{\Delta 9,12}$C18:2 used as indices for Δ9, Δ12 and Δ6 desaturase activity, respectively, we suggest that in the presence of EO, the activity of Δ9 desaturase involved in the desaturation of stearic acid was significantly increased. During this process both Δ12 and Δ6 desaturases involved in the desaturation of oleic and linoleic acids, respectively, decreased. We conclude that EO could be used for the *in vivo* modification of microbial lipids.

INTRODUCTION

Glycerol is an important substance produced in large amounts during biodiesel production. This process generates 10% w/w of glycerol (Fan et al. 2010). The globally increasing biodiesel market led to abundance of glycerol and decreased its cost due to oversupply, making this product an appealing substrate for the production of value-added commercial compounds. Glycerol is currently used by many research teams as substrate in numerous biotechnological applications, including the production of microbial lipids (Bellou et al. 2012; Chatzifragkou et al. 2011; Easterling et al. 2009; Ethier et al. 2011; Meesters et al. 1996). Microbial lipids, also known as single cell oils (SCO), may be characterized by their high content in polyunsaturated fatty acids (PUFA), being of a biotechnological interest (Bellou et al. 2012, 2014; Chatzifragkou et al. 2011; Čertik et al. 2012; Fakas et al. 2006, 2007, 2009; Klempova et al. 2013; Zhu et al. 2008; Zikou et al. 2013). Alternatively, saturated SCO of industrial interest, such as those having a structure similar to that of the cocoa butter, can be synthesized by oleaginous microorganisms (yeasts and fungi) cultivated under specific culture conditions (Moreton 1985; Papanikolaou et al. 2003).

The *in vivo* modification of fatty acid composition of SCO using natural compounds, such as essential oils used as agents of alteration of fatty acid biosynthesis pathways, may be of interest in lipid biotechnology, specifically in the production of tailor-made lipids, preferred in nutraceutical and pharmaceutical industries. Essential oils are volatile, hydrophobic compounds characterized by a strong odor and known for their medicinal activities and antimicrobial properties against bacteria, fungi and viruses (Bakkali et al. 2008; Hammer et al. 1999; Pinto et al. 2009; Sánchez-González et al. 2010). Some of them have also been used for food preservation (Bagamboula et al. 2004; Burt 2004; Hammer et al. 1999; Mishra and Dubey 1994) and as analgesic, spasmolytic, anaesthesic remedies or even sedative treatments (Bakkali et al. 2008; Ghfir et al. 1994;

Silva et al. 2003). Essential oils may also provoke alteration on the activity of fatty acid desaturases, as it has been indicated in several papers where the addition of essential oils in the growth medium influenced the fatty acid composition of some microorganisms (Aggelis and Komaitis 1999; Gema et al. 2002; Ghfir et al. 1994, 1997; Helal et al. 2006a, 2006b, 2007; Karanika et al. 2001; Lucini et al. 2006; Papanikolaou et al. 2008). Additionally, other natural compounds may also act as desaturases inhibitors provoking changes in fatty acid composition. Indeed, Shimizu et al. (1991) have reported that sesamin and related lignin compounds are specific inhibitors of Δ5 desaturase in microorganisms and animals, while Moreton (1985) indicated that cyclopropene fatty acids are specific inhibitors of Δ9 desaturase in oleaginous yeasts.

The aim of the current work was to study the effect of essential oil of *Citrus sinensis* (orange) on the growth and fatty acid composition of the oleaginous zygomycete, *Cunninghamella echinulata* growing on glycerol. The obtained data allows us to conclude that orange essential oil could be used as a fatty acid desaturases inhibitor in the production of saturated lipids, which are of interest to several sectors of food and cosmetics industries.

MATERIALS AND METHODS

Microorganisms and culture conditions

The strain *Cunninghamella echinulata* ATHUM 4411 (Athens University Mycology, National and Kapodistrian University of Athens, Greece) was maintained on potato dextrose agar (PDA, Himedia, Mumbai) at 6±1°C.

Experiments were performed at least in duplicate in 250mL Erlenmeyer flasks, containing 50mL of medium having the following composition (in $g \cdot L^{-1}$): KH_2PO_4 (Sigma-Aldrich) 7; Na_2HPO_4 (Sigma Stenheim) 2; $MgSO_4 \cdot H_2O$ (Scharlau) 1.5; $CaCl_2 \cdot 2H_2O$ (Carlo Erba Reagenti) 0.1; $FeCl_3 \cdot 6H_2O$ (BDH, Pool) 0.08; $ZnSO_4 \cdot 7H_2O$ (Merck) 0.001; $CuSO_4 \cdot 5H_2O$ (BDH) 0.0001; $Co(NO_3)_3 \cdot H_2O$ (Merck) 0.0001; $MnSO_4 \cdot 5H_2O$ (Fluka) 0.0001. The biodiesel-derived glycerol (containing monoglycerides, diglycerides and free-fatty acids, 2%; NaCl, 3%; methanol <0.1%; water 3%) discharged after biodiesel manufacturing (Pettas Industrial and Commercial S.A., Patras, Greece), was used as the sole carbon source at $50g \cdot L^{-1}$, while yeast extract and $(NH_4)2SO_4$ (Merck), both at $0.5g \cdot L^{-1}$, were used as nitrogen sources. Various concentrations of orange essential oil (EO), ranging from 0.5 to $5.0g \cdot L^{-1}$, were added to the medium. The EO, provided by Lakonia Agricultural Cooperative Citrus Processing (Amycles, Greece), contained more than 95% limonene. The flasks containing the growth medium were sterilized at 121°C for 20 min and inoculated with 1mL of spore suspension containing 10^6 spores$\cdot mL^{-1}$ produced from 7 day-old cultures. After the inoculation, the flasks were cultivated at 28°C and 180 rpm in a rotary shaker.

Analytical methods

Biomass was harvested by filtration under vacuum through Whatman No. 1 paper, washed with distilled water and ethanol 70%, dried at 80°C until constant weight and then gravimetrically determined. Dissolved oxygen concentration was determined according to Papanikolaou et al. (2004) using a Hanna HI9146-04 selective electrode (Hanna Instruments).

Total lipids were extracted according to Folch et al. (1957) in chloroform (Fluka): methanol (Sigma) (2:1, v/v), washed with a 0.88% KCl (Merck) solution in order to remove non lipid compounds and dried over anhydrous Na_2SO_4 (Sigma). The pure lipid extract was selected by filtration and the solvent was evaporated through vacuum using a Rotavapor R-20 devise (BUCHI).

Fatty acid-methyl esters of total lipids were prepared in a two-stage reaction using $CH_3O\text{-}Na^+$ and CH_3OH/HCl according to the AFNOR method (1984). GC analysis was carried out by an Agilent 7890 A device (Agilent Technologies) equipped with an HP-88 (J&W scientific) column (60m×0.32mm), and a FID detector at 280°C. Helium was used as a carrier gas at a flow rate of $1mL \cdot min^{-1}$ and the analysis was performed at 200°C. Peaks of methyl esters were identified by reference to respective standards.

The activities of Δ9, Δ12, Δ6 desaturases were estimated by using the ratios $^{\Delta 9}$C18:1/ C18:0, $^{\Delta 9,12}$C18:2/ $^{\Delta 9}$C18:1, $^{\Delta 6,9,12}$C18:3/ $^{\Delta 9,12}$C18:2 respectively, according to Dimou et al. (2002).

Statistical analysis

Data were subjected to one-way analysis of variance ANOVA followed by a Bonferroni post hoc test using IBM SPSS Statistics 21 software package. The null hypothesis was rejected at significance level of $P \leq 0.05$.

RESULTS

Growth of *Cunninghamella echinulata* on various concentrations of EO

C. echinulata was cultivated on glycerol as sole carbon source under nitrogen limited conditions in the presence of various concentrations of EO. In all growth phases, the dissolved oxygen concentration remained high (above 70% of saturation), due to the high agitation rate of flasks. Quantitative data of fungal growth and lipid accumulation are presented in Table 1. In control cultures, in which EO was absent from the growth medium, biomass produced in the first growth steps (i.e. at 48h) was higher compared to that produced in the presence of EO. However, as growth proceeded, biomass production was slightly higher in the presence of EO, up to $3.7g \cdot L^{-1}$ in comparison to control cultures in which produced biomass did not exceed $3.2g \cdot L^{-1}$.

Table 1. Growth of *Cunninghamella echinulata* on raw glycerol and lipid accumulation on media containing various concentrations of orange essential oil. The values represent the mean of three replications ±SD. Culture conditions: growth in flasks, incubation at 28°C, agitation at 180 rpm, initial glycerol concentration 50g·L^{-1}, initial pH=6. Abbreviations: EO (g·L^{-1}), orange essential oil; L/X (%), lipids in dry biomass w/w; X (g·L^{-1}), total biomass.

t (h)	EO (g·L^{-1})	X (g·L^{-1})	L/X (%)
48	0.0	2.0±0.1	23.6±1.7
	0.5	1.9±0.2	20.5±1.1
	1.0	1.8±0.1	21.5±0.9
	2.0	1.8±0.0	23.9±1.9
	3.0	1.3±0.0	24.3±1.5
	5.0	1.4±0.1	23.2±1.1
96	0.0	3.0±0.2	22.4±1.1
	0.5	3.1±0.2	23.1±1.5
	1.0	2.9±0.1	25.3±2.2
	2.0	2.6±0.2	32.2±2.3
	3.0	2.6±0.0	24.8±2.0
	5.0	2.6±0.2	25.7±1.2
144	0.0	3.2±0.1	27.9±3.1
	0.5	3.6±0.4	26.1±2.5
	1.0	3.5±0.2	22.0±2.1
	2.0	3.7±0.3	30.1±2.2
	3.0	3.4±0.2	25.9±2.1
	5.0	3.6±0.4	29.5±2.1

Concerning the conversion of glycerol into SCO, in all cases significant amounts of lipids were accumulated into the mycelia with time (Table 1). However, in control cultures lipid synthesis was lower than that noticed in the presence of EO in the growth medium (up to 27.9% w/w lipids in dry biomass). Maximum storage lipid accumulation was 32.2% (w/w in dry biomass) and observed in the high concentrations of EO (i.e. 2.0g·L^{-1}).

Shifts in fatty acid composition of lipids of *C. echinulata*

The fatty acid composition of *C. echinulata* lipids in the presence of the various EO concentrations, as well as the statistical treatment are shown in Table 2. Oleic acid was the major fatty acid, followed by linoleic ($^{\Delta 9,12}$C18:2) and palmitic (C16:0) acids. GLA was also found in remarkable quantities reaching up to 12.3%, in total lipids. Compositional shifts were observed in PUFA concentration with time as the concentration of EO increased. More specifically, linoleic acid and GLA slightly decreased with time at low EO concentrations, whereas this decrease was more intense in the presence of higher EO concentrations. The same trend was also noticed concerning the concentration of stearic acid (C18:0). In contrast, oleic acid ($^{\Delta 9}$C18:1) considerably increased with time in the presence of EO (e.g. from 28.5% at 48h of growth to 42.9% in total lipids at 144h), compared to the control in which no changes in the oleic acid content were observed. In almost all EO concentrations tested, accumulation of palmitic and palmitoleic ($^{\Delta 9}$C16:1) acids was also observed as growth proceeded.

The compositional shifts in fungal fatty acid composition were labelled using the ratio of ΣC18/ΣC16, indicating the fatty acid elongases activity, and the unsaturation index (UI). Although fatty acid elongases seem to work properly in the presence of EO, UI decreased with the time (Table 2). These changes also reflected the alteration of the desaturases activity (Table 3). More specifically, high EO concentration resulted in low Δ12 oleic acid desaturase activity, as it is indicated by the decrease of the ratio $^{\Delta 9,12}$C18:2/$^{\Delta 9}$C18:1 in the late fermentation steps. On the other hand, the ratio $^{\Delta 9}$C18:1/C18:0 remained high and even increased as growth proceeded, indicating that Δ9 was favoured by the presence of EO.

Table 2. Fatty acid composition, elongation activity (estimated by the ratio of ΣC18/ΣC16) and unsaturation index of total lipids during growth of *Cunninghamella echinulata* on media containing various concentrations of orange essential oil. Culture conditions: growth in flasks, incubation at 28°C, agitation at 180 rpm, initial glycerol concentration 50g·L^{-1}, initial pH=6. Abbreviations: EO (g·L^{-1}), orange essential oil; L/X (%), lipids in dry biomass w/w; X (g·L^{-1}), total biomass.

t (h)	EO (g·L^{-1})	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 6,9,12}$C18:3	ΣC18/ΣC16	UI*
	0.0	19.0^{a1}	2.9^{a2}	4.1^{a3}	38.4^{a4}	22.2^{a5}	11.5^{a6}	3.5	1.20
	0.5	18.4^{a1}	3.2$^{a2, b2}$	4.0^{a3}	33.8^{b4}	27.1^{b5}	12.3^{a6}	3.6	1.28
48	1.0	18.4^{a1}	3.2$^{a2, b2}$	4.6^{a3}	34.2^{b4}	26.4^{b5}	11.2^{a6}	3.5	1.24
	2.0	18.9^{a1}	2.7^{a2}	5.7^{b3}	36.2$^{a4, b4}$	22.6^{a5}	12.2^{a6}	3.6	1.21
	3.0	16.4^{a1}	3.2$^{a2, b2}$	4.8^{a3}	29.0^{c4}	31.4^{c5}	11.6^{a6}	3.9	1.30
	5.0	16.0^{a1}	3.6^{b2}	4.8^{a3}	28.5^{c4}	33.5^{c5}	11.5^{a6}	4.0	1.33
	0.0	20.4^{d1}	3.1$^{d2, e2}$	3.6^{d3}	38.6^{d4}	20.8^{d5}	11.5^{d6}	3.2	1.18
	0.5	20.6^{d1}	3.1$^{d2, e2}$	3.3^{d3}	37.6^{d4}	22.7^{e5}	10.9$^{d6, e6}$	3.1	1.19
96	1.0	20.9^{d1}	3.2$^{d2, e2}$	3.2^{d3}	37.8^{d4}	22.6$^{e5, f5}$	10.7$^{d6, e6}$	3.1	1.18
	2.0	21.6^{d1}	2.7^{d2}	5.0^{e3}	41.1^{e4}	18.4^{g5}	9.3^{f6}	3.0	1.08
	3.0	21.0^{d1}	3.0$^{d2, e2}$	3.7^{d3}	38.1^{d4}	22.3^{e5,f5}	10.0$^{e6, f6}$	3.1	1.16
	5.0	21.7^{d1}	3.3^{e2}	3.6^{d3}	37.6^{d4}	21.4$^{d5, f5}$	10.4$^{d6, e6, f6}$	2.9	1.15
	0.0	20.9^{h1,j1}	3.1^{h2}	3.5^{h3}	38.9^{h4}	20.6$^{h5, j5}$	11.2^{h6}	3.1	1.17
	0.5	21.2^{h1,k1}	3.1^{h2}	3.0^{h3}	41.7$^{j4, k4}$	19.9^{h5}	9.4$^{k6, j6}$	3.0	1.13
144	1.0	20.3^{j1}	3.2^{h2}	3.1^{h3}	39.9^{h4,j4}	21.5^{j5}	10.2$^{h6, j6}$	3.2	1.17
	2.0	22.3^{m1}	3.1^{h2}	3.5^{h3}	42.9^{k4}	18.2^{k5}	8.1$^{k6, m6}$	2.9	1.07
	3.0	21.4^{h1,n1}	3.3^{h2}	3.4^{h3}	41.2^{j4,k4}	19.6^{h5}	9.0$^{j6, m6}$	3.0	1.11
	5.0	21.7^{k1,m1,n1}	3.1^{h2}	3.3^{h3}	42.5^{k4}	18.3^{k5}	8.7$^{k6, m6}$	2.9	1.08

Others (less than 3.6% in total lipids): C10:0, C12:0, C14:0, $^{\Delta 9}$C14:1, $^{\Delta 9,12,15}$C 18:3

*UI=Unsaturation Index $\quad U.I. = \dfrac{(\%\text{monoenes}) + (2\cdot\%\text{dienes}) + (3\cdot\%\text{trienes})}{100}$

The values represent the mean of three replications. Letters refer to comparisons at vertical reading only for each separate growth time; numbers followed by different letters indicate statistically significant differences at $P \leq 0.05$. The experimental data were treated according to Bonferroni post hoc test.

Concerning the ratio $^{\Delta 6,9,12}$C18:3/ $^{\Delta 9,12}$C18:2 characterizing the ability of the fungus to desaturate linoleic acid to gamma linolenic acid by the means of Δ6 desaturase, this was decreased by the presence of EO.

DISCUSSION

The effect of orange essential oil on the growth, lipid accumulation and fatty acid composition of *C. echinulata* cultivated on glycerol under nitrogen limited conditions was investigated. Essential oils are natural compounds consisted mainly of complex mixtures of terpenic hydrocarbons and other derivatives (i.e. aldehydes, alcohols, etc.) (Burt 2004). Essential oil derived by *Citrus* plants consists of more than 90% monoterpenes, 5% oxygenated compounds and less than 1% of non-volatile compounds (Papanikolaou et al. 2008).

A wide variety of essential oils (i.e. essential oils of *Citrus, Hyssopus officinalis, Teucrium polium, Cymbopogon citratus,* etc.) has been studied for their capacity to affect lipid accumulation in oleaginous microorganisms and fatty acid desaturation (Aggelis and Komaitis 1999; Gema et al. 2002;

Table 3. Desaturation activity of Δ9, Δ12 and Δ6 desaturases during growth of *Cunninghamella echinulata* on media containing various concentrations of orange essential oil. Culture conditions: growth in flasks, incubation at 28°C, agitation at 180 rpm, initial glycerol concentration 50g·L^{-1}, initial pH=6. Abbreviations: EO (g·L^{-1}), orange essential oil; L/X (%), lipids in dry biomass w/w; X (g·L^{-1}), total biomass.

t (h)	EO (g·L^{-1})	$\frac{\Delta 9 C18:1}{C18:0}$	$\frac{\Delta 9,12 C18:2}{\Delta 9 C18:1}$	$\frac{\Delta 6,9,12 C18:3}{\Delta 9,12 C18:2}$
48	0.0	9.5	0.6	0.5
	0.5	8.5	0.8	0.5
	1.0	7.4	0.8	0.4
	2.0	6.4	0.6	0.5
	3.0	6.0	1.1	0.4
	5.0	6.0	1.2	0.3
96	0.0	10.7	0.5	0.6
	0.5	11.4	0.6	0.5
	1.0	11.9	0.6	0.5
	2.0	8.2	0.5	0.5
	3.0	10.4	0.6	0.5
	5.0	10.5	0.6	0.5
144	0.0	11.1	0.5	0.6
	0.5	13.7	0.5	0.5
	1.0	13.0	0.5	0.5
	2.0	12.3	0.4	0.4
	3.0	12.2	0.5	0.5
	5.0	12.8	0.4	0.5

Ghfir et al. 1994, 1997; Helal et al. 2006a, 2006b, 2007; Lucini et al. 2006; Papanikolaou et al. 2008). Although essential oils (and several related natural compounds) have been considered as anti-microbial compounds (Cerrutti and Alzamora 1996; Helal et al. 2006a, 2006b, 2007; Tranter et al. 1993), the current work indicated that the growth of *C. echinulata* was not negatively affected by the presence of orange EO in the medium. Specifically, while the addition of EO delayed fungal growth in the first growth steps, it had a positive effect thereafter. Biomass produced was higher in cultures with EO in the medium than in those in which EO was absent. In contrast, biomass synthesis by the yeast *Yarrowia lipolytica* was significantly inhibited by *Citrus* essential oil as reported by Papanikolaou et al. (2008). Similarly, the growth of various fungal and yeast strains (i.e. *Aspergillus flavus, A. niger, Saccharomyces cerevisiae*) was completely inhibited by *Cymbopogon citratus* essential oil (Helal et al. 2006a, 2006b, 2007).

As well as microbial growth, essential oils have been proved to induce changes in both lipid accumulation process and fatty acid composition of the accumulated lipid (Aggelis and Komaitis 1999; Ghfir et al. 1997; Helal et al. 2006b; Papanikolaou et al. 2008). It should be noted that the effect of essential oils on the fatty acid composition of microbial lipids might be of biotechnological interest, especially in the case of oleaginous microorganisms, as possible modifications may lead to new lipids with special composition and/or structure. The present study demonstrated that EO positively affected lipid accumulation process, since lipid rich biomass was produced by *C. echinulata*. Similarly, an increase in total lipids was observed when essential oils were added to the growth media of various fungal strains (i.e. *Aspergillus fumigatus*, etc) (Ghfir et al. 1997).

Concerning lipids, the most outstanding finding was the compositional shifts observed in the fatty acid composition. As growth proceeded, oleic acid content increased significantly in cultures in which EO was added. This contrasted to the control cultures, in which no changes in the oleic acid concentration were observed. As indicated by the UI values, PUFA concentration significantly decreased with time in the presence of EO. This decrease was sharper

at high EO concentrations. These findings indicate biochemical changes of the microbial fatty acid biosynthetic apparatus, specifically of that involved in fatty acid desaturation. Although the fatty acid elongases seem to work properly since the ΣC18/ΣC16 ratio remained unaffected by the presence of EO, the addition of EO provoked an increase in the activity of Δ9 desaturase, resulting in the accumulation of oleic acid. This behaviour, in combination with the inhibition of the activity of Δ12 desaturase, elaborated by the presence of EO, explains the reduced synthesis of linoleic acid and GLA thereafter. The decrease in GLA concentration could be attributed both to the restricted availability of substrate (lower concentration of linoleic acid) and the Δ6 desaturase inhibition. *Citrus* essential oil, used even at small amounts, induced remarkable changes in the fatty acid composition which was attributed to alteration of both elongases and desaturases activity (Papanikolaou et al. 2008).

The effect of natural compounds and, more specifically, essential oils in the fatty acid composition of lipid produced by various microorganisms seems to be dependent both on the type of the essential oil and the microorganism studied. This is because a great diversity in the obtained results has been demonstrated compared to control cultures in which no essential oils were added. For instance, changes similar to those found in the current study concerning fatty acid composition were also reported by Gema et al. (2002) when the same zygomycete strain (*Cunninghamella echinulata*) was cultivated on orange peel. However, no changes in oleic acid content were reported by Ghfir et al. (1997), although linoleic acid significantly decreased in the lipids of *A. fumigatus* cultivated in contact with *Hyssopus officinalis*. Lastly, in *S. cerevisiae* and *A. niger* lipids, the presence of *C. citratus* essential oil caused an increase in both oleic and linoleic acids (Helal et al. 2006a, 2006b).

CONCLUSIONS

In conclusion, the growth of *Cunninghamella echinulata* was favoured by the orange essential oil at the concentrations used, whereas significant alterations in the fatty acid composition were observed. The changes in the unsaturation index should be attributed to the modification of the biochemical behaviour of *C. echinulata*, concerning the activity of Δ9, Δ12 and Δ6 desaturases involved in the unsaturated fatty acids biosynthesis of the C18 group.

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