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ORIGINAL PAPER

Effect of *Origanum vulgare* L. Essential Oil on Growth and Lipid Profile of *Yarrowia lipolytica* Cultivated on Glycerol-Based Media

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Abstract Aim of the present study was to identify the chemical composition of *Origanum vulgare* L. essential oil and to investigate its effect on the biochemical behavior of the yeast *Yarrowia lipolytica*. Twenty-three compounds were identified by GC–MS analysis, representing the 92.3% (w/w) of the total essential oil of the plant. Carvacrol (56.3%) and thymol (16.4%) were the major components. Additionally, shake-flask cultivations of *Y. lipolytica* were performed, with various essential oil additions (0.05–2 mL/L of medium) on glycerol-based media. Growth was affected even at low concentrations (0.05 mL/L), while in higher essential oil concentrations, strong inhibition phenomena were observed. A tolerance-threshold concentration for the strain was hence established at 0.15 mL/L of oil. Furthermore, the presence of the essential oil in the culture medium resulted in changes in the composition of the intra-cellular lipids of the yeast. Specifically, oil addition to nitrogen-limited cultures to a level >0.15 mL/L caused a substantial increase in the percentage of saturated fatty acids (C16:0 and C18:0) in the lipid composition of the yeast *Y. lipolytica*.

Keywords Essential oil · Lipids · Raw glycerol · *Yarrowia lipolytica* · *Origanum vulgare* L

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Introduction

Essential oils represent oily liquids with strong odor obtained from aromatic and medicinal plant parts, such as flowers, buds, seeds, leaves and herbs. They are produced as secondary metabolites, whilst their presence in the plant is significant as they serve as protective agents against microbial invaders (bacteria, viruses and fungi), insects, as well as herbivores by reducing their appetite for such plants [1, 2]. Essential oils have already been employed for their antimicrobial, antiparasitic and antioxidant properties in a wide spectrum of industrial applications, such as pharmaceutical compounds, food preservatives, sanitary agents, and compounds in cosmetics and perfumes. In general, they represent complex mixtures that can contain about 20–60 components in concentrations that vary. They are characterized by two or three major compounds that may constitute up to 85% of their total composition, whilst other components are usually found in trace amounts [1, 2]. Furthermore, it is the nature of these major compounds that determines the biological properties of each essential oil.

Various studies have reported the antimicrobial activity of essential oils deriving from plants and spices against Gram-positive and Gram-negative bacteria [3, 4], food spoiling yeasts [5, 6] and fungi [7, 8]. Nevertheless, only a few reports are found in the international literature to deal with the effect of natural essential oils or aqueous extracts on the fatty acid composition of intra-cellular lipids of eukaryotic microorganisms [9–12]. The rationale followed in the present study was to assess the impact of *Origanum vulgare* L. essential oil on the biochemical response of the yeast *Yarrowia lipolytica* LFMB 20. Particular interest was paid on the effect of the essential oil upon the fatty acid profile of the intra-cellular lipid produced by yeast, while in the current investigation raw (biodiesel-derived glycerol) was used as the sole carbon

source by *Y. lipolytica*. It is noted that the strain in question was previously found capable of growing on raw glycerol utilized as the sole substrate, and secreting non-negligible amounts of citric, acetic acid and mannitol [13]. This was an interesting feature of the microorganism, since the application of biodiesel in a large commercial scale is strongly recommended, and thus, raw (biodiesel-derived) glycerol over-production and disposal is likely to cause very important environmental problems in the near future. Therefore, applications dealing with the valorization of this renewable abundant material are of importance [14], whereas, in general glycerol can be considered as an excellent carbon source for the production of biomass and secondary metabolites, such as intra-cellular lipids, citric acid, erythritol, pyruvic acid and *a*-ketoglutaric acid by various *Y. lipolytica* strains [12, 15–22]. Therefore, aim of the present study was to assess the impact of the addition of *O. vulgare* L. essential oil upon the biochemical and physiological behavior of *Y. lipolytica* cultivated on raw glycerol utilized as substrate.

Materials and Methods

Plant Material and Essential Oil Analysis

Plant material of *O. vulgare* L. was purchased from the local market and maintained in the dark at ambient temperature, throughout the experiment. Essential oil was obtained as follows: 30 g of dried plant material was subjected to hydro-distillation with 300 mL of deionised water, in a Clevenger apparatus for 3 h. Part of the essential oil was recovered by decantation and the remaining oil on the walls of the glass tube was recovered by rinsing with *n*-pentane. The organic phase was dried with sodium sulfate and subsequently filtered. The organic solvent was removed by evaporation and the essential oil was stored in a freezer at –8 °C until further analysis.

GC–MS Analysis

GC–MS analysis of the essential oil was performed using a Fisons 8000 series gas chromatograph (Model 8060) coupled to a Fisons MD 800 quadrupole mass spectrometer (Fisons Instruments, Manchester, UK). Helium was used as carrier gas at a flow rate of 1.0 mL/min. Separation of compounds was performed on a CP-Sil 8 (30 m × 0.32 mm, film thickness 0.25 μm, Chrompack) and on a DB-Wax capillary column (30 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific). Diluted samples (1/100 in dichloromethane, v/v) of 1 μL were injected manually in split mode (split ratio 1/30). Oven temperature was programmed from 40 to 250 °C at a rate of 4 °C per min and held at 250 °C for 5 min. The injector, ion source and interface temperatures were set at 230, 200 and 270 °C, respectively. The mass spectrometer was operated in electron impact mode with the electron energy set at 70 eV and a scan range of 30–400 *m*/*z*. Oil compounds were identified by comparing: (1) linear retention indices (LRIs) based on a homologous series of even numbered *n*-alkanes (C_8–C_{24}) (Niles, IL, USA) with those of standard compounds (Sigma-Aldrich, Steinheim, Germany; Acros Organics, Geel, Belgium) and by comparison with literature data [23], and (2) MS data with those of reference compounds and by MS spectra obtained from Wiley (http://eu.wiley.com/WileyCDA/WileyTitle/productCd-04-70047852.html) and NIST (http://www.nist.gov/srd/nist1a.htm) libraries.

Microorganism and Culture Conditions

Yarrowia lipolytica LFMB 20 strain [13] was maintained on potato dextrose agar (PDA, Plasmatec, Dorset, UK) at 4 ± 1 °C. The salt composition of the synthetic medium used was the following (g/L): KH_2PO_4, 7.0; Na_2HPO_4, 2.5; $MgSO_4{\cdot}7H_2O$, 1.5; $FeCl_3{\cdot}6H_2O$, 0.15; $ZnSO_4{\cdot}7H_2O$, 0.02; $MnSO_4{\cdot}H_2O$, 0.06; $CaCl_2{\cdot}2H_2O$, 0.15. Yeast extract and $(NH_4)_2SO_4$ were used as nitrogen sources. Trials were performed in either nitrogen-limited conditions (addition of 0.5 g/L of each of the above-mentioned nitrogen sources) or carbon-limited conditions (addition of 5.0 g/L of each of the above-mentioned nitrogen sources). Pure or crude glycerol was used as the sole carbon source in all trials. Crude glycerol [purity 80.3% (w/w), impurities composed of: free-fatty acids, 1.2% (w/w); NaCl, 5.6% (w/w); methanol, 2% (w/w); water 10.9% (w/w)] was kindly offered by the "ADM Industries", Hamburg, Germany. All cultures were carried out at initial glycerol ($Glol_0$) concentration of 30 g/L. It is evident that when crude glycerol was utilized as substrate, the appropriate calculations were performed in order to achieve the above-mentioned $Glol_0$ concentration. The initial pH of the medium was 6.2 ± 0.1 after autoclaving.

Experiments were conducted in 250-mL conical flasks, containing 50 ± 1 mL of growth medium and *Origanum vulgare* L. essential oil in various concentrations (ranging between 0.0 − control experiment and 2.0 mL/L of medium), sterilized at 121 °C/20 min and inoculated with 1 mL of a 24-h exponential pre-culture (around 1–3 × 10^7 cells, initial biomass concentration at the flasks at ~0.12 ± 0.02 g/L). The pre-culture was carried out in the above-mentioned synthetic medium containing 30 g/L of pure glycerol. Cultures were performed in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at an agitation rate of 180 ± 5 rpm and incubation temperature $T = 29 \pm 1$ °C. In all experiments it was desirable to maintain a medium pH in a value >5.2, therefore an appropriate volume of KOH (5 M) was periodically and

aseptically added into the flasks when needed, in order to maintain the pH in the above value.

Analytical Methods

Flasks were periodically removed from the incubator and cells were collected by centrifugation (16,000×*g*/15 min at 10 °C) in a Hettich Universal 320R (Germany) centrifuge and washed twice with distilled water. Biomass (*X*, g/L) was determined by means of dry matter (95 ± 2 °C/24 h). Glycerol (Glol, g/L) and metabolic products (citric acid—Cit in g/L, acetic acid—Ace in g/L, mannitol—Ml in g/L), were determined by means of HPLC analysis in a Waters 600E device equipped with UV (wavelength at 210 nm) and RI detectors, flow at 0.6 mL/min and an Aminex HPX-87H column (Bio-Rad) heated at 65 °C. Additionally, given that the HPLC analysis carried out did not allow the satisfactory separation of citric acid from its isomer *iso*-citrate, in order to proceed with a more judicious determination of *iso*-citric acid in some of the fermentation points, an enzymatic method, based on the measurement of the $NADPH_2$ produced during conversion of the *iso*-citric to α-ketoglutaric acid, reaction catalyzed by *iso*-citrate dehydrogenase, was conducted [15]. Cit (in g/L) denotes the quantity of total citric acid (citric plus *iso*-citric acid) produced. Dissolved oxygen (DO) concentration was determined using a selective electrode (OXI 96, B-SET, Germany) as described in Papanikolaou et al. [24]. Oxygen saturation was kept above 40% (v/v) during all growth phases for every microorganism tested. Inhibition (%) of glycerol consumption and biomass production was determined according to the following equations:

$$\% \text{ Inhibition of glycerol consumption} = \left(1 - \frac{\text{Glol}_{\text{cons}_i}}{\text{Glol}_{\text{cons}_c}}\right) \times 100;$$

$$\% \text{ Inhibition of biomass production} = \left(1 - \frac{X_i}{X_C}\right) \times 100$$

where, *i* is the value of each parameter (substrate consumption or biomass production) in the experiment with essential oil addition and *c* is the corresponding value of the control experiment (no essential oil added).

Total cellular lipid (*L*, g/L) was extracted from the dried microbial biomass with a mixture of chloroform/methanol 2/1 (v/v) and weighted after evaporation of the solvent. Lipids were converted to methyl-esters in a two-step reaction with methanolic sodium and hydrochloric methanol [15]. The analysis of methyl esters was carried out on a gas chromatograph (Fisons 8060), equipped with an FID detector and using a Chrompack column (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific). Helium was used as carrier gas, at a flow rate of 2 mL/min. Oven temperature was set at 200 °C, held for 13 min, increased to 220 °C at a rate of 2 °C/min, then with a rate of 20 °C/min at 240 °C and held for 3 min. The injector and the detector temperature were 250 °C. Fatty acid methyl esters were identified by reference to standards.

All of the fermentations were carried out in duplicate experiments, in which different inocula were employed. Moreover, each experimental point of all of the kinetics presented in the tables and figures related with the cultures of *Y. lipolytica*, is the mean value of two determinations.

Results and Discussion

Chemical Composition of Essential Oil

The essential oil produced by water distillation of desiccated *O. vulgare* L. plant had a yellow color and a strong and distinguishable oregano flavor. Table 1 lists the LRIs and percentage composition of the essential oil of *O. vulgare* L. as defined by GC–MS analysis. Twenty-three compounds were identified representing 92.3% of the total essential oil of the plant, being, thus, characterized by a high percentage of phenols (72.7%). Carvacrol (56.3%) and thymol (16.4%) were the major components, followed by γ-terpinene, *p*-cymene, β-myrcene, *trans*-caryophyllene and β-bisabolene in non-negligible amounts. Carvacrol is a hydrophobic compound that can be accumulated in the cell membrane and especially in the lipid bi-layer. Its hydrogen-bonding ability, in combination with its proton-release ability, may induce conformational modification of the membrane bi-layer, by rendering it more permeable [25, 26]. Leakage of ions and other cell contents can then occur, leading eventually to cell death [4, 27]. On the other hand, thymol is a compound very similar to carvacrol, in terms of structure, diversifying itself only by the location of phenolic hydroxyl groups of the phenolic ring. Nevertheless, thymol is also considered as a compound, which can make the cell membrane permeable [27]. Although, the major components carvacrol and thymol are believed to be responsible for the antimicrobial effect of the essential oils, there is some evidence that minor components play a critical role in the antibacterial activity, possibly by inducing a synergistic effect between other components [27]. Specifically, synergism between carvacrol and its biological precursor *p*-cymene has been reported for *B. cereus* vegetative cells [28]. It appears that *p*-cymene, a very weak antibacterial compound, swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism *p*-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are found together [28].

Table 1 Chemical composition of the essential oil from *Origanum vulgare* L. obtained by GC–MS

Compounds	LRI[A]		Composition[B] (%)	Fit[C]
	CP-Sil 8	DB-Wax		
α-Thujene	936	1,021	0.7	a
α-Pinene	940	1,017	0.5	ab
Camphene	951	1,053	0.1	a
β-Pinene	975	1,093	0.1	ab
3-Octanol	990	nd	0.3	a
β-Myrcene	991	1,157	1.4	ab
α-Phellandrene	1,001	1,206	0.2	a
α-Terpinene	1,012	1,174	1.5	ab
p-Cymene	1,020	1,266	3.3	ab
Limonene	1,023	1,197	0.4	ab
γ-Terpinene	1,053	1,243	7.0	ab
cis-Sabinene hydrate	1,061	nd	0.4	ab
α-Terpinolene	1,083	1,279	0.1	a
trans-Sabinene hydrate	1,091	1,471	0.2	a
Linalool	1,096	1,554	0.1	ab
Borneol	1,158	1,709	0.3	ab
Terpinen-4-ol	1,171	1,605	0.6	a
α-Terpineol	1,185	1,704	0.2	a
Dihydro carvone	1,192	nd	0.1	a
Thymol	1,293	nd	16.4	ab
Carvacrol	1,303	2,220	56.3	ab
trans-Caryophyllene	1,414	1,594	1.1	ab
β-Bisabolene	1,509	1,727	1.0	a
Monoterpene				
Hydrocarbons			15.3	
Oxygenated			1.9	
Sesquiterpene				
Hydrocarbons			2.1	
Phenols			72.7	
Alcohols			0.3	
Total identified			92.3	

nd not detected

[A] Linear Retention Indices to C_8–C_{24} *n*-alkanes on the CP-Sil 8 and DB-Wax

[B] The percentage composition was calculated from the chromatograms obtained on the CP-Sil 8 column. Normalized peak area (%)

[C] a: MS data and retention index in agreement with those in literature and with those in NIST and WILEY libraries. b:MS data and retention index in agreement with those of authentic compound

Growth of the Yeast *Yarrowia lipolytica* on Glycerol-Based Media

Y. lipolytica was initially cultivated under carbon-limited and nitrogen-limited conditions, in order to investigate the biochemical behavior of the strain at these different culture conditions. Additionally, pure or raw glycerol was utilized as a carbon source, attempting to detect possible impact of the impurities of the industrial feedstock upon biomass production by the investigated strain. These cultures also served as control experiments. Data obtained from the performed kinetics are illustrated in Table 2. Overall, the strain in question proved to be an organic acid producer, secreting non-negligible amounts of acetic and citric acid. Moreover, mannitol production took place during cultivation of the strain in both growth media. In carbon-limited culture conditions, as expected, the metabolic activity was shifted mainly towards biomass production, with biomass yield on glycerol consumed ($Y_{X/Glol}$) ranging between 37 and 44% (w/w), and to lesser extent towards the production of secondary metabolites, such as organic acids and mannitol. Moreover, in carbon-limited media, raw glycerol proved to be a slightly better substrate in terms of final biomass production, while final concentrations of organic acids and mannitol were similar with those achieved in nitrogen-limited cultures. It is of importance to state that the microorganism exhibited the same substrate consumption rate (r_{Glol}) in both carbon-limited and nitrogen-limited media (Fig. 1), which was almost linear presenting a value ranging between 0.27 and 0.29 g/L × h. Cultivation of the yeast *Y. lipolytica* in nitrogen-limited media influenced positively the production of secondary metabolites, while the usage of raw glycerol had as a result the non-negligible improvement of organic acids and mannitol final production. In particular, cultivation of *Y. lipolytica* on raw glycerol led in the production of 9.2 g/L of acetic acid and 5.7 g/L of citric acid, whereas maximum mannitol production was 7.8 g/L. Acetic acid production seems to be an interesting feature of the studied *Y. lipolytica* strain. It is noted that production of the above-mentioned organic acid has been previously reported for several *Y. lipolytica* or other *Candida* strains (e. g. *C. catenulata*, *C. zeylanoides*, etc.) during their cultivation on glucose- or glycerol-based media, as well as in cultures utilizing ethanol as the sole carbon source, in several fermentation configurations [12, 29–33]. In the case of ethanol-growing yeasts, acetic acid is the intermediate of primary ethanol metabolism, and thus, it is secreted (in not significantly high concentrations, e. g. amounts ranging between 0.1 and 1.7 g/L) into the medium, since the principal organic acids produced are either succinic or α-ketoglutaric acid [32, 33]. On the other hand, the ability of *Y. lipolytica* yeast strains to accumulate into the medium acetic acid during growth on glycerol or similarly metabolized compounds (e. g. glucose) can be considered as a strain-dependent feature; screening of several *Y. lipolytica* wild strains growing on glucose in shake-flask nitrogen-limited experiments, had as result that some strains produced together with citric acid non-

Table 2 Quantitative data of *Yarrowia lipolytica* derived from kinetics in carbon- and nitrogen-limited media with pure or raw glycerol as a carbon source and various initial *Origanum vulgare* L. essential oil additions in the medium

Entry	Media	Glol (30 g/L)	Essential oil (mL/L)[a]	Time (h)	X (g/L)	$Glol_r$ (g/L)	Cit (g/L)	Ml (g/L)	Ace (g/L)	$Y_{X/Glol}$ (%, w/w)	$Y_{L/X}$ (%, w/w)
1	Carbon-limited	Pure	Control	92	10.9 ± 0.8	0.5 ± 0.3	2.1 ± 0.2	4.9 ± 0.4	6.1 ± 0.6	37.7 ± 2.8	8.1 ± 0.9
2		Raw	Control	90	12.9 ± 1.1	1.2 ± 0.2	1.7 ± 0.4	5.1 ± 0.5	6.8 ± 0.4	44.6 ± 3.8	7.9 ± 1.0
3	Nitrogen-limited	Pure	Control	90	8.2 ± 0.9	4.5 ± 0.6	3.2 ± 0.2	5.4 ± 0.4	7.8 ± 0.8	28.3 ± 3.1	8.9 ± 1.1
4		Raw	Control	88	7.1 ± 0.8	5.4 ± 0.2	5.7 ± 0.3	7.8 ± 0.8	9.2 ± 0.8	24.5 ± 2.8	9.6 ± 1.8
5			0.05	92	6.8 ± 0.5	0.2 ± 0.1	2.3 ± 0.1	7.2 ± 0.5	9.3 ± 0.9	22.8 ± 1.8	8.1 ± 1.3
6			0.10	88	4.9 ± 0.5	0.1 ± 0.1	2.9 ± 0.3	6.1 ± 0.5	9.1 ± 0.8	16.4 ± 1.7	12.4 ± 2.0
7	Nitrogen-limited	Raw	0.15	92	4.2 ± 0.3	0.1 ± 0.1	2.7 ± 0.2	6.3 ± 0.4	7.7 ± 0.8	14.8 ± 1.1	13.9 ± 2.1
8			0.30	210	2.5 ± 0.3	18.1 ± 0.3	0.2 ± 0.1	1.9 ± 0.3	3.3 ± 0.7	23.1 ± 2.8	12.1 ± 1.8
9			0.50	210	0.4 ± 0.2	25.1 ± 0.4	–	–	–	–	–

Each experimental point presented is the mean value of two independent measurements and data are presented as means ± standard deviations

Representation of fermentation time (h), biomass production (X, g/L), residual glycerol ($Glol_r$, g/L), citric acid production (Cit, g/L), mannitol production (Ml, g/L), acetic acid production (Ace, g/L), conversion yield of biomass produced per consumed glycerol ($Y_{X/Glol}$, % w/w) and lipid produced in dry biomass ($Y_{L/X}$, % w/w). Culture conditions: $Glol_0 = 30$ g/L, growth in flasks at 180 ± 5 rpm, initial pH = 6.0, pH ranging between 5.0 and 6.0, DO > 40% (v/v), incubation temperature $T = 28$ °C

[a] Due to total growth inhibition of the yeast in 1.0 and 2.0 mL/L of essential oil addition, kinetic data concerning these concentrations are not presented

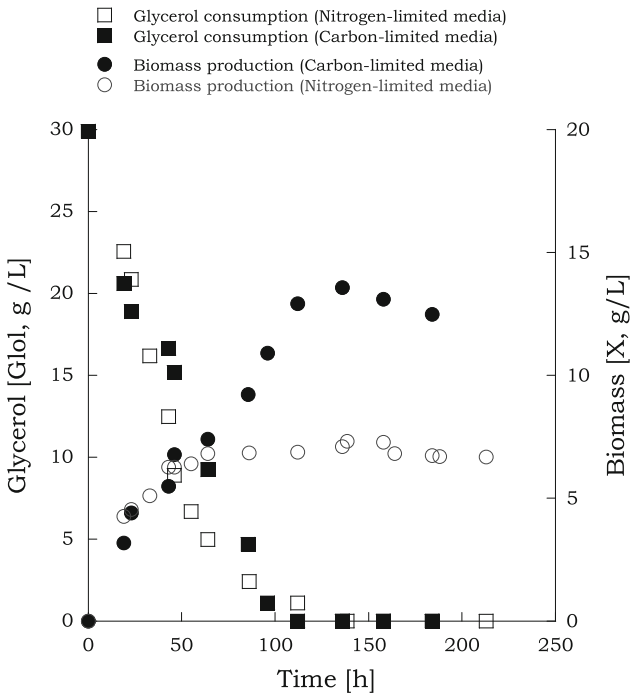

Fig. 1 Kinetics of glycerol consumption and biomass production during growth of *Yarrowia lipolytica* in carbon-limited and nitrogen-limited media, with raw glycerol as the sole carbon source. Culture conditions: $Glol_0 = 30$ g/L, growth in flasks at 180 ± 5 rpm, initial pH = 6.0, pH ranging between 5.0 and 6.0, DO > 40% (v/v), incubation temperature $T = 28$ °C. Each experimental point presented is the mean value of two independent measurements

negligible acetic acid quantities, specifically when the initial concentration of glucose was elevated into the medium, whereas other strains did not produce at all acetic acid as metabolic compound regardless of the initial glucose concentration imposed [31]. The secretion of acetic acid during growth on glucose or glycerol in nitrogen-limited conditions could be a result of a possible bottlenecking of acetyl-CoA-CoA conversion into citric acid, resulting in increased concentration of acetyl-CoA inside the mitochondria, due to potential saturation of enzymes involved in Krebs circle and specifically citrate synthase. When the concentration of acetyl-CoA reaches a crucial level, it should potentially exit inside the cytosol where it should be converted into acetic acid by virtue of enzymatic catalysis, and thereafter acetic acid should be secreted into the culture medium. Finally, in terms of lipid accumulation, a slight increment of lipid produced per dry yeast mass ($Y_{L/X}$) was observed in nitrogen media ($Y_{L/X}$ = 8.9–9.6%, w/w) in contrast to carbon-limited media ($Y_{L/X}$ = 7.9–8.1%, w/w) (Table 2).

A number of studies cited in literature are involved in the usage of glycerol as the sole substrate for the production of various secondary metabolites by strains of *Y. lipolytica*. When Rywińska et al. [34] performed cultivations of acetate mutant strains of *Y. lipolytica* on pure and crude glycerol, reported that the strain Wratislavia AWG7 was able to produce 139 g/L of citric acid, while the strain Wratislavia K1 produced lower amounts of citric

acid, due to simultaneous accumulation of erythritol in significant concentrations into the fermentation medium. In another study, the strain *Y. lipolytica* Wratislavia K1 was cultivated on crude glycerol and was capable of producing 170 g/L of erythritol [20], whereas during repeated fed-batch cultures of *Y. lipolytica* Wratislavia AWG7 on crude glycerol, resulted in 154 g/L of citric acid [22]. Moreover, glycerol-based media were found sufficient for pyruvic acid production by a thiamine auxotroph *Y. lipolytica* 374/4 strain [19], whilst Zhou et al. [21] produced 39.2 g/L of *a*-ketoglutaric acid, by cultivating a *Y. lipolytica* WSH-Z06 thiamine auxotroph strain on crude glycerol. Besides that, Papanikolaou et al. [15] reported that *Y. lipolytica* ACA-DC 50109 strain produced 33.6 g/L of citric acid during cultivation on crude glycerol-based media, whereas when the same strain was cultivated in a continuous mode, it was found able to accumulate 43% (w/w) of intracellular lipid [35].

Effect of Oregano Oil Addition on Growth of Yeast *Yarrowia lipolytica*

During the second series of experimental work, the yeast *Y. lipolytica* was cultivated in nitrogen-limited media with raw glycerol as the sole carbon source and additions of *O. vulgare* L. essential oil in the culture media in various concentrations (0.05–2.0 mL/L) were performed, in order to investigate the effect of the essential oil on the physiology of the yeast and to establish a tolerance-threshold oil concentration for the particular strain. Kinetics of glycerol assimilation and biomass formation are depicted in Table 1 (entries 5–9) and Fig. 2a, b. The addition of the essential oil even at low concentrations (0.05 mL/L) had a negative impact on the biomass production, a phenomenon that became more intense accordingly with the increment of oil addition in the cultivation media. Surprisingly enough, though, glycerol assimilation was unaffected even at oil presence up to 0.15 mL/L (Fig. 2a). This fact leads to the assumption that carbon source was mainly utilized for energy and maintenance needs, rather than anabolic activities, such as biomass production. Indeed, Papanikolaou et al. [12] have reported the same behavior during cultivation of *Y. lipolytica* strain ACA-DC 50109 strain in carbon-limited glucose-based media in which essential oil of *Citrus sinensis* cv *New Hall—Citrus aurantium* was used. Besides that, in the present investigation, it was found that essential oil concentration of 0.15 mL/L represented the threshold amount, since thereafter biomass formation, as well as glycerol consumption was intensively restrained. Specifically, a more drastic reduction in glycerol consumption was observed when oregano oil was added at 0.30 mL/L, while prolongation of lag phase of nearly 70 h occurred. Eventually, at this essential oil concentration

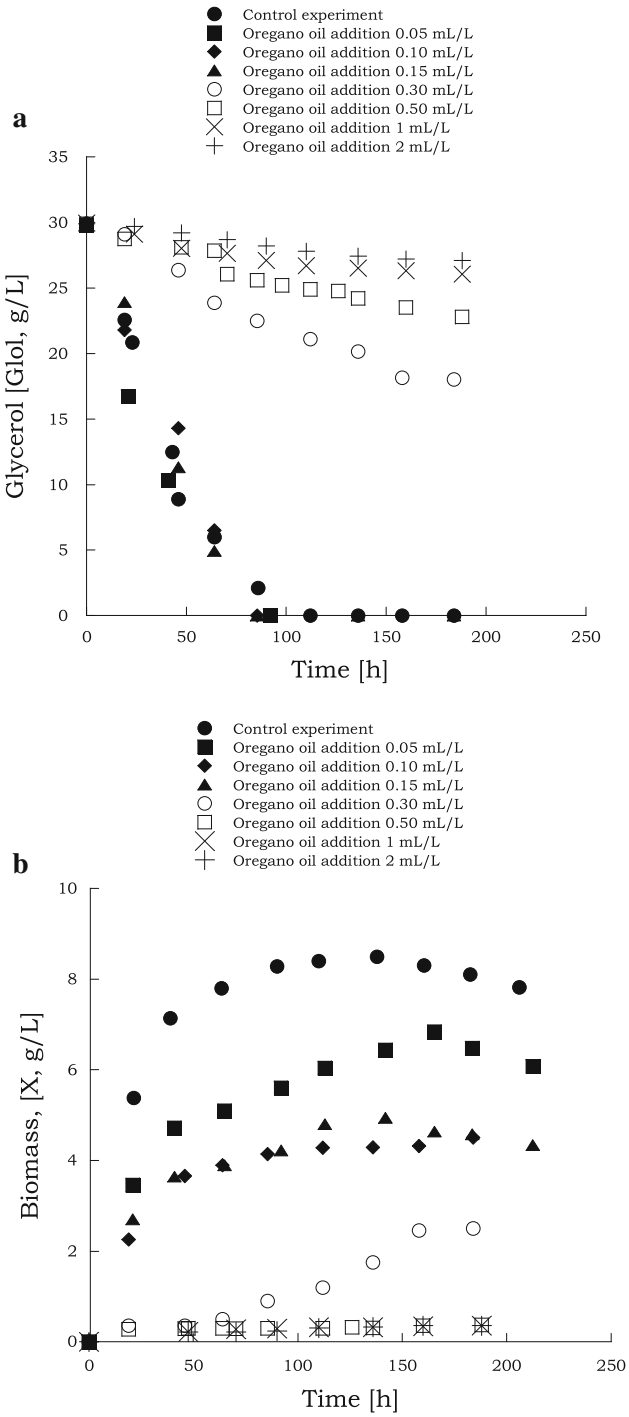

Fig. 2 Evolution of glycerol assimilation (Glol, g/L) (**a**) and biomass production (*X*, g/L) (**b**) during growth of *Yarrowia lipolytica* in nitrogen-limited media with raw glycerol as the sole carbon source and various *Origanum vulgare* L. essential oil additions in the medium. Culture conditions as in Fig. 1. Each experimental point presented is the mean value of two independent measurements

(0.30 mL/L) only 2.5 g/L of biomass were produced (Fig. 2b). The addition of the essential oil at 0.50 mL/L in the culture medium had an obvious inhibitory effect, not

only in glycerol consumption but also in biomass production (inhibition impact of almost 80% for both fermentation parameters) (Fig. 3). Additionally, the presence of essential oil in the medium at 1.0 and 2.0 mL/L resulted in the virtual cease of carbon source consumption and biomass production by yeast *Y. lipolytica* with an estimated inhibition effect of more than 90%.

Data obtained from kinetics during fermentations with oregano oil addition are presented in Table 2 (entries 5–9). As previously stated, the addition of oil had an evident negative effect on microbial growth, a fact also established by decrease in values of $Y_{X/Glol}$ (%, w/w) (from 24.5% during the control experiment to 14.8% at 0.15 mL/L of oil addition). The presence of oil at 0.05 mL/L resulted in the reduction of citric acid production, while the production of acetic acid and mannitol were similar to that of the control experiment (9.3 and 7.2 g/L, respectively). However, implementation of higher oil concentrations had as a consequence the apparent reduction of both organic acids, as well as mannitol production (Table 2). Moreover, the presence of 0.30 mL/L of the essential oil led to an increase of residual glycerol in the medium, as almost 60% of initial carbon source remained unconsumed, although fermentation time was extended (up to 210 h). As far as microbial lipid was concerned, the microorganism overall did not produce quantities of lipid higher than 1.2 g/L, both in the cases of presence or absence of the essential oil (control experiment). However, the increment of oil in the growth medium had as an impact the raise in relative values of lipid production ($Y_{L/X}$, % w/w) from 9.6% in the control experiment up to 13.9% at 0.15 mL/L of oil addition. In general, the effect of oregano essential oil against microorganisms has been reported in various in vitro studies, providing evidence of strong microbial inhibition against some pathogenic bacterial strains, such as *Escherichia coli*, *Salmonella choleraesuis*, *S. enteritidis*, *S. typhimurium*, *Staphylococcus aureus*, *Bacillus cereus* and *B. subtilis* [4, 36, 37]. The antimicrobial effect of this essential oil is mainly attributed to the high percentage of the phenolic compounds carvacrol and thymol [27, 38]. The effectiveness of *O. vulgare* essential oil against spoiling yeasts has been also demonstrated by various researchers [1, 6, 39]. By taking also into account the fact that *O. vulgare* essential oil is considered toxicologically safe, it can represent an alternative antimicrobial compound in food preservation.

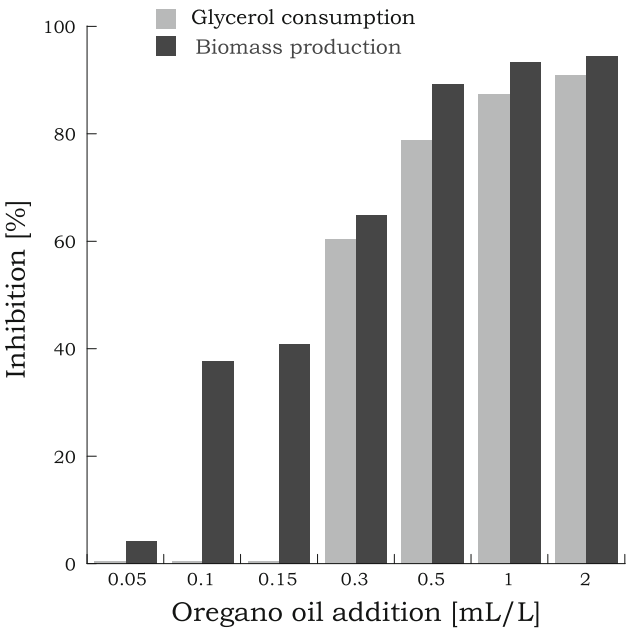

Fig. 3 Inhibition (%) of various *Origanum vulgare* L. essential oil additions on glycerol consumption and biomass production by *Yarrowia lipolytica*, cultivated in nitrogen limited media with raw glycerol as the sole carbon source. Culture conditions as in Fig. 1

Fatty Acid Analysis of Intra-Cellular Lipids by *Yarrowia lipolytica*

As previously stated, cultivation of yeast *Y. lipolytica* in both carbon-limited and nitrogen-limited glycerol-based media was not accompanied by actual lipid accumulation. However, extraction and analysis of total cellular lipids was performed, in order to investigate the impact of the essential oil addition upon the lipid profile of the strain. The results obtained are depicted in Table 3. The principal fatty acids produced during cultivation of the strain in the control experiments (no essential oil addition in the medium) were palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic ($^{\Delta 9}$C18:1) and linoleic ($^{\Delta 9,12}$C18:2) acid. In general, cultivation on raw glycerol, in both carbon- and nitrogen-limited media had as a consequence the increase of the unsaturated nature of cellular lipids. Cultivation of *Y. lipolytica* in nitrogen-limited media led to the increase of oleic and linoleic acid, while no significant fluctuation in the percentages of the other cellular fatty acids was observed.

However, addition of *O. vulgare* L. essential oil even at indeed very low amounts (i. e. 0.05 mL/L), evoked an increase in the cellular saturated fatty acids, in comparison with lipid composition obtained from the control experiment under nitrogen-limited media, with raw glycerol as the sole carbon source. Indeed, higher concentrations of the essential oil in the medium had an evident impact on the saturation of cellular lipids, and on the unsaturation index (UI) (see Table 3). Specifically, as the oil concentration increased above 0.15 ml/L in the growth medium, the percentage of palmitic acid (C16:0) was substantially raised, whereas a remarkable drop in the percentages of linoleic ($^{\Delta 9,12}$C18:2) and palmitoleic acid (C16:1) was

Table 3 Fatty acid composition of cellular lipids of *Yarrowia lipolytica* during its cultivation in carbon- and nitrogen-limited media with pure or raw glycerol as a carbon source and various initial *Origanum vulgare* L. essential oil additions in the medium

Culture media	Glycerol	Essential oil (mL/L)[a]	Growth phase (h)	C16:0	$^{\Delta 9}$C16:1	C18:0	$^{\Delta 9}$C18:1	$^{\Delta 9,12}$C18:2	UI[b]
Carbon-limited	Pure		LE[c]	11.4 ± 0.9	8.5 ± 0.4	9.1 ± 0.9	60.2 ± 2.6	10.8 ± 1.1	0.90
			ES[d]	13.9 ± 0.6	9.8 ± 0.6	10.9 ± 1.2	49.3 ± 2.4	16.1 ± 1.5	0.91
			S[e]	14.8 ± 1.4	9.2 ± 0.9	12.4 ± 1.3	42.8 ± 2.9	20.8 ± 1.6	0.93
	Raw	Control	LE[c]	12.1 ± 1.3	6.1 ± 1.4	8.6 ± 0.4	62.3 ± 2.9	10.9 ± 1.4	0.90
			ES[d]	15.4 ± 1.5	10.6 ± 1.8	5.6 ± 0.9	46.3 ± 2.6	22.1 ± 1.8	1.01
			S[e]	13.8 ± 1.4	9.1 ± 1.2	10.5 ± 1.2	33.7 ± 1.9	32.9 ± 2.0	1.08
Nitrogen-limited	Pure		LE[c]	15.4 ± 1.5	6.8 ± 0.7	9.7 ± 1.5	47.6 ± 2.4	20.5 ± 1.6	0.95
			ES[d]	13.5 ± 1.8	8.4 ± 0.9	12.9 ± 2.0	40.1 ± 2.5	25.1 ± 1.4	0.98
			S[e]	15.9 ± 1.6	9.1 ± 0.8	10.1 ± 1.7	37.8 ± 1.8	27.1 ± 2.2	1.01
	Raw	Control	LE[c]	12.1 ± 1.8	4.4 ± 0.7	9.9 ± 0.8	38.8 ± 1.9	34.8 ± 2.2	1.12
			ES[d]	14.6 ± 1.5	6.2 ± 0.6	8.3 ± 0.9	40.5 ± 2.8	30.4 ± 1.9	1.07
			S[e]	17.8 ± 1.8	8.3 ± 0.9	8.9 ± 0.9	35.7 ± 1.1	29.3 ± 1.3	1.02
Nitrogen-limited	Raw	0.05	LE[c]	14.6 ± 1.8	5.6 ± 0.8	10.8 ± 0.9	54.2 ± 2.6	14.8 ± 1.6	0.89
			ES[d]	16.6 ± 2.0	7.1 ± 0.9	9.5 ± 0.7	52.1 ± 2.5	14.7 ± 1.9	0.88
			S[e]	17.6 ± 2.0	7.3 ± 0.6	8.5 ± 0.6	51.4 ± 2.4	15.2 ± 1.4	0.89
		0.10	LE[c]	12.7 ± 1.4	5.9 ± 0.8	8.5 ± 0.8	64.7 ± 3.9	7.8 ± 0.7	0.86
			ES[d]	16.8 ± 1.7	5.8 ± 0.9	19.1 ± 1.8	51.1 ± 2.4	7.2 ± 0.6	0.71
			S[e]	17.2 ± 2.4	6.1 ± 0.5	9.5 ± 1.4	54.7 ± 3.1	12.5 ± 1.8	0.85
		0.15	LE[c]	15.4 ± 1.4	9.2 ± 1.1	5.7 ± 0.5	60.4 ± 2.8	9.3 ± 1.4	0.88
			ES[d]	16.2 ± 2.0	4.6 ± 0.7	14.8 ± 0.9	55.5 ± 2.7	8.9 ± 1.6	0.77
			S[e]	15.4 ± 1.6	4.1 ± 1.1	13.3 ± 1.4	55.5 ± 2.4	12.2 ± 1.8	0.84
		0.30	LE[c]	36.4 ± 2.6	–	18.8 ± 1.8	44.8 ± 1.2	–	0.44
			ES[d]	38.6 ± 2.4	2.6 ± 0.5	5.9 ± 0.5	46.4 ± 2.1	6.5 ± 1.4	0.62
			S[e]	41.1 ± 1.6	1.4 ± 0.2	7.1 ± 1.0	42.1 ± 2.6	8.3 ± 0.9	0.60
		0.50	LE[c]	40.1 ± 1.8	–	17.2 ± 1.9	42.7 ± 1.4	–	0.42
			ES[d]	43.2 ± 1.6	–	4.7 ± 0.9	52.1 ± 2.1	–	0.54
			S[e]	38.4 ± 1.9	1.3 ± 0.2	7.2 ± 0.9	48.4 ± 1.9	4.7 ± 0.6	0.59

Culture conditions as in Table 2. Each experimental point presented is the mean value of two independent measurements and data are presented as means ± standard deviations

[a] Due to total growth inhibition of the yeast in 1.0 and 2.0 mL/L of essential oil, data of lipid composition for these concentrations were not presented

[b] UI = [% monoene + 2 (% diene)]/100

[c] *LE* late exponential growth phase

[d] *ES* early stationary growth phase

[e] *S* stationary growth phase

noticed. On the contrary, the percentage of oleic acid ($^{\Delta 9}$C18:1) seemed to rather increase in the presence of 0.15 mL/L of essential oil; however, in higher oil concentrations (0.30 and 0.50 mL/L), oleic acid percentage was reduced noticeably. As far as stearic acid was concerned, at media with low oil presence (0.10 and 0.15 mL/L), its percentage fluctuated with the fermentation time. Nevertheless, in elevated oil concentrations, namely 0.30 and 0.50 mL/L, at the early growth stages, the quantity of cellular C18:0 noticeably increased, whereas at the final stages, it decreased (Table 3).

These findings suggest that the presence of the essential oil induces biochemical changes in terms of intra-cellular biosynthesis of microbial fatty acids, by potential slight inhibition in the level of the Δ9 desaturase activity (an enzyme catalyzing the reaction C18:0 → $^{\Delta 9}$C18:1). Likewise, it appears that the addition of the essential oil, induced a somehow important inhibition in the level of acyl-CoA elongases catalyzing the elongation reaction of C16:0 → C18:0, resulting, thus, in increased concentrations of the fatty acid C16:0 into the cellular lipids, specifically when somehow elevated essential oil concentrations

(i. e. = 0.30 mL/L or higher) were added into the medium (see Table 3). The same trend on the cellular lipids of *Y. lipolytica* ACA-DC 50109 was observed when the aforementioned strain was cultivated in media containing amounts of *Citrus sinensis* cv *New Hall—C. aurantium* essential oil [12]. Moreover, it has been reported that monocyclic monoterpenes such as α-terpinene (a compound also found in the composition of the *O. vulgare* essential oil used in the present study), can affect *Saccharomyces cerevisiae* in the level of genes involved in ergosterol biosynthesis, sterol uptake, lipid metabolism as well as cell wall structure [40]. On the contrary, when *S. cerevisiae* and *Y. lipolytica* cultures were treated with *Teucrium polium* L. aqueous extract, a significant rise in the unsaturation index for both microorganisms occurred [9]. Likewise, the addition of aqueous extracts derived from plants of the Lamiaceae family in carbon-limited cultures of *Y. lipolytica*, led to the increment of oleic and linoleic acid concentration of the microbial lipid. The opposite trend was observed in nitrogen-limited cultures of the microorganism [11]. From all the above-mentioned observations, it can be assumed that the addition of various "natural" compounds in submerged cultures may interfere with lipid composition in various microorganisms. In one such case, Moreton [41] performed additions (even in small concentrations) in submerged fermentations of oil-bearing yeasts (i. e. yeasts that can accumulate lipid to more than 20%, w/w, inside their cells) of sterculia oil, a natural oil containing principally sterculic [8-(2-octacyclopropen-1-yl) octanoic acid] and malvalic acid [7-(2-octylcyclopropen-1-yl) heptanoic acid], and in similar manner with the present investigation, a notable rise in the concentration of the cellular saturated fatty acids was obtained, resulting in the synthesis of cocoa-butter-like microbial lipids. The production of substitutes of specialty lipids (like cocoa-butter or other saturated exotic fats) presents economical interest the last years, due to continuously increasing production cost of the materials [42]. The utilization of conventional desaturase inhibitors (like that of sterculia oil) remarkably increases the saturation index of the cellular lipid produced, having as a result the synthesis of lipids presenting composition similarities with the cocoa-butter [41]. However, the approach proposed by Moreton [41] presents a fundamental drawback, since the inhibitors used are considered to provoke mutagenesis and cancerogenesis [42]. This is of importance, taking into consideration the current trends of industrial biotechnology, in which eco-friendly and "healthy" approaches should be considered in order for the mass production of biotechnological products [42]. Potentially, the addition of the essential oil by *O. vulgare* in the culture medium of several oleaginous yeasts could increase the saturated fatty acid content of the cellular lipid produced in these yeasts, while this essential oil in contrast to the sterculia oil is completely "healthy" and non toxic.

Conclusion

Carvacrol and thymol were found to be the major compounds of the essential oil derived from *O. vulgare* L. plants. Their impact on the biochemical behavior of *Y. lipolytica* LFMB 20 was tested and evaluated. Indeed, the essential oil appeared to have an anti-yeast activity, as the strain sustained an evident inhibition during oil addition in the growth medium, in terms of both biomass formation and substrate consumption. Finally, the addition of the essential oil was proved to affect the composition of the cellular lipid of the yeast, by rendering them more saturated.

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