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Compositional shifts in lipid fractions during lipid turnover in *Cunninghamella echinulata*

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Abstract

The oleaginous fungus *Cunninghamella echinulata* when cultivated on a tomato waste hydrolysate medium accumulated 7.8 g l^{-1} of reserve lipid, while, after the exhaustion of the carbon source in the growth environment, 44% of this lipid was consumed and 3.2 g l^{-1} of lipid-free biomass were synthesized. It was demonstrated that lipid fractions and individual lipid classes varied in amount, relative proportions and fatty acid profile during the turnover phase. Triacylglycerols (TAG) were preferentially consumed as their percentage proportion decreased from 26.6 to 6.9% (w/w) of lipid-free biomass, while TAG structures containing more unsaturated fatty acids were partially discriminated. Consequently, the relative proportion of γ-linolenic acid (GLA) increased in TAG from 9.2% (end of the lipogenic phase) to 15.3% (w/w), whereas C16:0 decreased from 22.7 to 15.6% (w/w). Concomitantly membrane polar lipid fractions were synthesized during lipid turnover. During the transition, glycolipids plus sphingolipids fraction was enriched in polyunsaturated fatty acids, especially in GLA, while phospholipids fraction was enriched in GLA but not in C18:2.

Keywords: Single cell oil; γ-Linolenic acid; *Cunninghamella echinulata*; Compositional shifts in lipid; Lipid turnover

1. Introduction

Oleaginous moulds are often considered for the production of single cell oil (SCO) rich in polyunsaturated fatty acids (PUFAs), either in submerged or in solid state fermentation systems [1–4]. The economics of these bioprocess become more favorable when zero or negative value waste substrates are utilized as carbon or nitrogen sources [4–7]. Among PUFAs γ-linolenic acid (GLA) is of particular interest owing to its selective anticancer properties [8,9].

Lipid turnover in oleaginous microorganisms typically commences when the cultivation medium is depleted of the carbon source [10–15]. Accordingly, the oleaginous mould *Cunninghamella echinulata* was reported to utilise its lipid reserves for the production of lipid-free biomass [16]. In *Saccharomyces cerevisiae*, a non-oleaginous yeast, it was shown that triacylglycerols (TAGs) are preferentially consumed during the turnover of reserve oil [17,18]. However, little is known about the composition of the reserve oil in oleaginous yeasts during the turnover and even less for oleaginous moulds. Thus, it was taken for granted that the same situation as in *S. cerevisiae* applies to oleaginous yeasts [10]. Furthermore, the changes in fatty acid composition of the main lipid fractions is of great interest, since it reflects the specificity of intracellular hydrolytic enzymes (i.e. lipases), which are responsible for lipid hydrolysis during lipid turnover [19].

Abbreviations: DAG, diacylglycerol; FA, fatty acid; FASF, fatty acid specific fluctuation (parameter used in the paper); FFAs, free fatty acids; G + S, glycolipids plus sphingolipids; GC, gas chromatography; GLA, γ-linolenic acid; MAG, monoacylglycerol; NL, neutral lipids; P, phospholipids; PUFAs, polyunsaturated fatty acids; RS, reducing sugars; SCO, single cell oil; TAG, triacylglycerol; TLC, thin layer chromatography; TWH, tomato wastes hydrolysate; UI, unsaturation index; x, total biomass; x_f, lipid-free biomass

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In this paper, we have monitored the changes occurring in amounts of the various lipid fractions and individual lipid classes, as well as their fatty acid (FA) compositional shifts during lipid turnover in *C. echinulata*, hoping to get an insight of the process and to asses the physiological role of the GLA in the fungal growth.

2. Materials and methods

2.1. Microorganism and culture conditions

C. echinulata ATHUM 4411 was maintained on potato dextrose agar (PDA, Plasmatec, Dorset, UK) at 6 ± 1 °C. Growth medium contained tomato wastes hydrolysate (TWH) supplemented with (g l^{-1}): Glucose, 90; KH_2PO_4, 7; Na_2HPO_4, 2; $MgSO_4{\cdot}7H_2O$, 1.5; $CaCl_2{\cdot}2H_2O$, 0.1; $FeCl_3{\cdot}6H_2O$, 0.08; $ZnSO_4{\cdot}7H_2O$, 0.001; $CuSO_4{\cdot}5H_2O$, 0.0001; $Co(NO_3)_3{\cdot}H_2O$, 0.0001; and $MnSO_4{\cdot}5H_2O$, 0.0001. After sterilization (121 °C/20 min) flasks were inoculated with 1 ml of spore suspension containing 2×10^4 spores, which were produced by growing the strain on PDA for 8 days at 26 °C. All cultivation experiments were performed in 250 ml Erlenmeyer flasks, containing 50 ml of the above medium, incubated in a rotary shaker at 180 rpm and 28 °C.

TWH was obtained by mixing 1 kg of the solid tomato by-product with 3 l of diluted sulfuric acid [20]. The mixture was then autoclaved at 121 °C for 2 h. TWH was filtered through Whatman no. 1 paper and the pH of the filtrates was adjusted to 6 with a concentrated KOH solution.

2.2. Analytical methods

Total biomass (x) was harvested by filtration using a 0.09 mm stainless-steel sieve, washed with cold distilled water, frozen at −30 °C, freeze-dried for 24 h and then gravimetrically determined. Freeze-dried mycelia contained less than 3% water. Lipid-free biomass (x_f) was calculated after subtraction of cellular lipids from total biomass. Reducing sugars (RS) in the growth medium were measured by the dinitrosalicylic acid method [21] and expressed as glucose. Inorganic ammonium ion concentration (NH_4^+) was measured with a selective electrode (51927-00, Hach, Colorado, USA). Total nitrogen was determined by measuring the concentration of NH_4^+ with the selective electrode, after Kjeldalh digestion of the samples. A standard curve was plotted by measuring NH_4^+ concentration in bovine serum albumin (Sigma). Dissolved oxygen concentration and specific oxygen consumption rate were measured as described in [16], with a selective electrode (Oxi 200 Sensodirect, Lovibond, Dortmund, Germany). Dissolved oxygen was >70% (v/v) of the saturation value during all growth phases.

2.3. Lipid analysis of tomato waste hydrolysate

TWH was extracted first with hexane (3×) and then with diethylether (3×). The extracts were combined and the solvents were removed by evaporation. A portion of the extract was then transmethylated and analyzed by gas chromatography (GC), with margaric acid (C17:0) as an internal standard. The analysis showed that it contained no lipid.

2.4. Fungal lipid content determination

The freeze-dried mycelia were ground into a fine powder with an analytical mill and extracted three times with 100 ml of chloroform/methanol (2:1, v/v) for 48 h at room temperature (Folch et al. 1957) [22]. The lipid extract was washed with 0.88% (w/v) KCl (3× 20 ml) and dried over anhydrous Na_2SO_4; the solvent was removed by evaporation.

2.5. Fractionation of fungal lipids

A known weight of extracted lipid (approx. 100 mg) was dissolved in chloroform (1 ml) and fractionated by using a column (25 mm × 100 mm) of silicic acid (1 g), activated by heating overnight at 110 °C [23]. Successive applications of 1,1,1-trichloroethane (100 ml), acetone (100 ml), and methanol (50 ml) produced fractions containing neutral lipids (NL), glycolipids plus sphingolipids (G + S), and phospholipids (P), respectively. The weight of each fraction was determined after evaporation of the respective solvent. Lipid fractions were stored at −30 °C under a nitrogen atmosphere.

2.6. Analysis of fungal lipid fractions

All three-lipid fractions were analyzed by thin layer chromatography (TLC) using various solvent systems [24].

The separation of NL fraction (approx. 20 mg) was carried out using silica gel 60 plates 20 cm × 20 cm (Merck, Darmstadt, Germany), developed with petroleum ether (b.p. 60–80 °C)/diethyl ether/glacial acetic acid (70:30:3, v/v/v). After development, the plate was dried under vacuum and sprayed with 2′,7′-dichlorofluorescein. The bands corresponding to individual lipid classes, namely TAG, diacylglycerols (DAGs) and monoacylglycerols (MAGs), were scraped off and directly transmethylated for GC analysis, with margaric acid (C 17:0) added as an internal standard.

For G + S analysis TLC was developed with chloroform/methanol/ammonia (28%) (65:25:5, v/v/v). After development, the plate was dried and sprayed with α-napthol followed by sulfuric acid (95%) and heating at 120 °C [24]. P analysis was similarly carried out with chloroform/methanol/ammonia (28%) (65:25:5, v/v/v), the plate was dried and sprayed with phosphomolybdic acid followed by heating [24].

The identification of lipid classes was made by co-chromatography with authentic standards, while their quantification was made with margaric acid (C17:0) added as an internal standard before GC analysis. The unsaturation index was calculated as described by Certik et al [25].

2.7. Gas chromatography (GC) analysis

Trans-methylation of lipid extracts was performed according to the AFNOR method [26]. GC analysis was carried out on a Fisons 8060 device equipped with a CP-WAX 52 CB, Chrompack column (60 m × 0.32 mm) and a FID detector; helium was the carrier gas (2 ml/min). The analysis was run at 200 °C with the injector and detector at 250 °C. Fatty acid methyl esters were identified by reference to authentic standards.

3. Results and discussion

3.1. Lipid accumulation and turnover in C. echinulata

During growth and lipogenic phase the lipid content in *C. echinulata* grown on a medium comprised of TWH increases up to 25% of the x (Fig. 1). Although approx. 0.4 g l^{-1} of organic nitrogen (N) were measured in the growth environment, lipogenesis commenced, meaning that this N could not be assimilated. After lipogenesis induction, the carbon surplus was successfully transformed to lipid and 7.8 g l^{-1} of microbial oil were produced. Subsequent sugar exhaustion from the culture medium triggered lipid utilization (turnover phase) that commenced at 312 h when the lipid content in biomass started dwindling; at that time x_f was 24.8 g l^{-1}. After turnover completion at 380 h the lipid content decreased to 14% in x; no further growth or lipid utilization was observed after 380 h.

The utilization of the accumulated lipid was accompanied by new x_f production, which then reached the value of 28 g l^{-1}, while the amount of lipid was 4.4 g l^{-1}. Thus, 0.95 g of x_f was produced per g of lipid consumed. This value is lower than the theoretical yield of 1:1.7 (w/w) for the conversion of TAG to biomass [10] but is similar to the value reported for another oleaginous mould, namely *Mortierella isabellina* [16]. These