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Research paper

Lipid accumulation in the new oleaginous yeast *Debaryomyces etchellsii* correlates with ascosporogenesis

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ABSTRACT

The growth characteristics, lipid accumulation and composition during the life cycle of a newly isolated strain of *Debaryomyces etchellsii* were studied under nitrogen limiting conditions. This yeast, grown in batch flask or bioreactor cultures, reproduced asexually by buds when nitrogen was available in the growth medium, or sexually by ascospores after nitrogen exhaustion, producing more than 7 g L^{-1} biomass. During ascosporogenesis, an important increase in the cellular lipid content in dry cell mass occurred, i.e. from a mass fraction of 11.9% in the vegetative phase to 22.4%, in the ascosporogenic phase. During transition of *D. etchellsii* from batch to continuous cultures using dilution rates 0.026 and 0.019 h^{-1}, a shift from sexual to asexual reproduction was observed. At 0.019 h^{-1}, few pseudomycelia were also formed. The yeast synthesized lipids containing long chain fatty acids (mainly C16 and C18). Budded cells at steady-states contained only 8.6–9.3 % of lipids mass fraction per dry cell mass that were composed of oleic and linoleic acids and, to a lesser extent, of palmitic and palmitoleic acids. Neutral lipids were the major fraction represented 61.8–66.1%, of total lipids followed by phospholipids, which was the only fraction in which linoleic acid predominated over oleic acid.

1. Introduction

Microbial oils, named single cell oils (SCO), have long been considered as promising candidates for alternative oil sources, due to their similar to vegetable oils fatty acid composition. Furthermore, their production is not dependent on season and climates. Oleaginous microorganisms, cultivated under appropriate growth conditions, are able to accumulate large amounts of lipids reaching up to 70% lipid mass fraction on dry cell mass [1,2]. Numerous oleaginous fungi, including both molds and yeasts, have been reported as successful organisms for the production of SCO, mostly consisting of triacylglycerols that are deposited in specialized intracellular compartments so called lipid bodies [1]. The produced SCO may be used as cocoa butter substitute [3,4], as feedstock for biodiesel production [5–8] or as source of polyunsaturated fatty acids (PUFA) [9,10].

Lipid accumulation in the oleaginous microorganisms requires the limitation of a key nutrient in the medium, usually nitrogen, and the presence of sugars, or similarly metabolized compounds such as glycerol, in excess [11]. However, it has been reported that the limitation of nitrogen is not the only condition that induce lipogenesis. Exhaustion of phosphorus, sulfate or other nutrients can be also of crucial importance for accumulation of lipids in oleaginous microorganisms [12,13]. In addition, both lipid content and fatty acid composition are critically affected by environmental factors such as aeration, temperature, pH, incubation period, inoculum size, inorganic salts and the microorganism itself [14,15]. Lipid accumulation may also occur in various oleaginous microorganisms cultivated on hydrophobic carbon sources (*ex novo* lipid accumulation). Nevertheless, this process is totally independent from nitrogen depletion in the growth medium [16,17].

Yeasts are known for their higher growth rate, when compared to filamentous fungi, and for their more appropriate morphology for large-scale cultivation and SCO production [16–19]. They are characterized by predominance of single cell forms that are reproduced by budding or fission. However, some dimorphic yeast

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species are able to form either single cells or hyphae and pseudohyphae to overcome stress conditions. During adverse terms, ascomycetous yeasts are known to undergo sexual reproduction that leads to the formation of haploid ascospores [20]. Ascus formation occurs by conjugation between compatible cells, i.e. of a cell and its bud or between two independent cells. The lysis of this ascus results in liberated ascospores, which in liquid media tend to aggregate. According to the literature, ascospores vary in number present in the asci, in shape, size and color [21]. Ascosporogenesis has been studied in detail in the model yeast *Saccharomyces cerevisiae* that is non-oleaginous. The complete absence of nitrogen and the presence of acetate, which is a non-fermentable carbon source, in the medium inhibit the budding process and induce meiosis (ascosporogenesis stage) [22,23]. The sporulation of *S. cerevisiae* is accompanied by an extensive increase in dry cell mass due to the accumulation of intracellular carbohydrates consisting of trehalose and insoluble components; at least 67% of the dry cell mass increase is due to the synthesis of cellular carbohydrates [24]. Nevertheless, lipid accumulation during the onset of the sexual stage is rarely mentioned in discussions concerning oleaginous yeasts.

The aim of the present work was to study lipid accumulation in a newly isolated and identified strain of *Debaryomyces etchellsii* growing on nitrogen-limited media, and how the lipid accumulation process is associated with sexual reproduction. Batch and continuous cultures were performed and special attention was paid to the lipid accumulation during transition from asexual to sexual reproduction and vice versa. The fatty acid composition of the total lipids, as well as of the various lipid fractions, i.e. neutral and polar (phospholipids and glycolipids plus sphingolipids) lipids were studied in detail in all growth phases. It is concluded that lipogenesis in *D. etchellsii* coincides with ascosporogenesis while the biosynthesis of linoleic acid is favored in metabolically active budding cells rather than in ascospores.

2. Materials and methods

2.1. Yeast strain, media and culture conditions

The yeast strain BM1 was isolated from a sample of olive mill wastewater sludge and was maintained in the laboratory of Enzyme Engineering and Microbiology on YPD (yeast extract 5 g L^{-1}, peptone 5 g L^{-1}, glucose 10 g L^{-1} and agar 20 g L^{-1}) slants at 4 °C. It was selected in preliminary studies due to its capacity to accumulate lipids and to sporulate. Molecular identification of BM1 was based on its Internal Transcribed Spacer (ITS) sequences. The ITS region was amplified by PCR using ITS1 and ITS4 primers (Forward ITS 1–5′ TCC GTA GGT GAA CCT GCG G 3′ and Reverse ITS 4–5′ TCC TCC GCT TAT TGA TAT GC 3′) and was directly sequenced [25]. The obtained sequences were analyzed using the BLAST program.

Submerged cultures were established on a nitrogen limiting medium (NLM) containing (in g L^{-1}): Glucose 50; $(NH_4)_2SO_4$ 0.5; Yeast extract 1; KH_2PO_4 12; Na_2HPO_4 12; Mg SO_47H_2O 1.5; $CaCl_22H_2O$ 0.1; $MnSO_45H_2O$ 0.0001; $CuSO_45H_2O$ 0.0001; $Co(NO_3)_33H_2O$ 0.0001; $ZnSO_47H_2O$ 0.001. The initial pH of the medium was 6 ± 0.1 after autoclaving.

Flasks experiments were performed in duplicate, in 250 cm^3 Erlenmeyer flasks, containing 50 cm^3 of the above NLM. After sterilization (at 115 °C for 20 min), the flasks were inoculated with 1 cm^3 of a mid-exponential growth phase pre-culture on Potato Dextrose Broth (PDB) medium of a cell density 4×10^8 cm^{-3}. The cultures were incubated in a rotary shaker at 3 Hz and 28 °C.

Bioreactor batch and continuous cultures were carried out in a Bioengineering, Ralf Plus-System bioreactor of total volume 3.7 L and working volume 1.8 L, equipped with an on line data acquisition and control system. The bioreactor was sterilized at 121 °C for 2 h and kept at room temperature for 48 h to ensure sterility of the medium. The culture vessel was inoculated with 200 cm^3 of a mid-exponential growth phase pre-culture. The cultivation conditions were as follows: dissolved oxygen was maintained above 20% of saturation. The pH of the medium was 6 and automatically controlled by adding 1 mol L^{-1} NaOH (Merck) or 0.5 mol L^{-1} H_2SO_4. The incubation temperature was automatically controlled at 28 °C and the agitation rate was 3 Hz. Antifoam A (Fluka) was added when needed.

Continuous bioreactor cultures at two different dilution rates i.e.0.026 and 0.019 h^{-1} were performed by adding fresh medium into the fermentor at a constant flow rate. Simultaneously, the culture was withdrawn from the reactor with the same flow rate of that of the inlet flow. Thus, the reactor working volume was kept constant at 1.8 L. Steady-state conditions were obtained after continuous flow of at least five working volumes of the culture medium. Aliquots of 40 cm^3 were taken at regular intervals to estimate dry cell mass, lipid content and substrate concentrations.

Lipid bodies in yeast cells were visualized after staining with Nile red fluorescence dye as follows: the cells were harvested and centrifuged at a relative centrifugal force (RCF) of 22,500 *g* and 4 °C for 15 min. Then, a small amount of the cell precipitate was suspended in 50 mmol L^{-1} sodium phosphate buffer, pH 6.8 and mixed with 100 mm^3 of Nile red solution (1 mg of Nile red in 1 cm^3 of ethanol) at a ratio 10:1. After 1 h in the dark, the cells were viewed under an Axiostar 40 (Zeiss, Cambridge, United Kingdom) fluorescence microscope, equipped with an excitation filter of 470/40 nm and a ProgRes camera (JenoptikCFcool, Jena, Germany).

2.2. Analytical methods

Cell mass was harvested by centrifugation at RCF of 22,500 *g* and 4 °C for 15 min. The pellets were then washed with distilled water, dried at 80 °C until constant weight, and gravimetrically determined. Reducing sugars in the growth medium were determined according to DNS method [26]. Ammonium ion concentration was measured using a selective electrode (51927-00, Hach, Colorado, USA). In shake flask cultures, the pH value of the growth medium was measured at all experimental points by using a selective pH meter. pH values remained in the range of 6.0–6.5 during all fermentation steps. Total cell lipids were extracted according to Folch et al. [27] using chloroform:methanol in a solvent volume ratio of 2:1 (v/v).

Fractionation of the lipids into neutral lipids (NL), glycolipids plus sphingolipids (G + S) and phospholipids (P) was achieved by silicic acid chromatography. Crude lipids (approximately 100 mg) were dissolved in 1 cm^3 chloroform and fractionated on a column (25 × 100 mm) containing 1 g of silicic acid activated by heating overnight at 80 °C. The individual fractions of NL, G + S and P were eluted by successive applications of dichloromethane (Sigma) 100 cm^3, acetone (Fluka) 100 cm^3, and methanol (Sigma) 50 cm^3, respectively. Lipid fractions were stored under argon atmosphere at −20 °C after evaporation of the respective solvents.

Trans-esterification of lipids was performed according to the AFNOR [28] method. Fatty acid methyl esters were analyzed using an Agilent 7890 A Gas Chromatograph (Agilent Technologies, Shanghai, China) equipped with an HP-88 (J&W scientific) column (60 m × 0.32 mm), and a flame ionization detector FID at 280 °C, while helium was used as carrier gas (at a flow rate of 1 cm^3 min^{-1}). The analysis was run at 200 °C. Peaks of methyl esters were identified by comparison of their retention times to those of authentic standards.

For quantitative determinations a known quantity of margaric