Fatty acid biosynthesis during the life cycle of Debaryomyces etchellsii

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INTRODUCTION

Ascosporogenesis is a defining feature of the phylum Ascomycota that is generally induced under environmental conditions that limit asexual reproduction. Under balanced growth conditions, the ascomycetous yeast cells grow by budding while under starvation conditions, the cells then exit the mitotic cycle, undergo meiosis and sporulate (Esposito & Klapholz, 1981; Miller, 1989). Ascospore formation usually occurs through conjugation between independent cells or between a cell and its bud (mother–daughter conjugation). A variety of culture conditions, such as the lack of an assimilable nitrogen source combined with high carbohydrate content and the presence of disaccharides (such as maltose, sucrose or cellobiose) or non-fermentable carbon sources (such as acetate, glycerol or ethanol) have been reported to induce sexual reproduction (Honigberg & Purnapatre, 2003; Jambhekar & Amon, 2008; Zaman et al., 2008). It seems that ascosporogenesis is controlled by nuclear and mitochondrial genes and may occur upon mitochondrial respiration and oxidative phosphorylation (Codón et al., 1995; Jambhekar & Amon, 2008). Several changes in cellular morphology, chemical composition and re-organization of subcellular organelles, occurring during transition from asexual to sexual stage, have been reported. These include an enlargement of the nucleus, fragmentation of the vacuole and an increase of the cell mass and volume (Croes, 1967), accompanied by an extensive accumulation of intracellular carbohydrates, mainly trehalose and glycogen, and a change in the protein content in a variable way (François & Parrou, 2001). Higher cellular lipid content has also been reported during transition from asexual to sexual stage of several yeast species (Kock & Ratledge, 1993; Arous et al., 2015).

All micro-organisms are able to synthesize lipids, whereas only the oleaginous strains may accumulate significant lipid quantities under optimal cultural conditions. Fatty acids (FAs), the building blocks of most lipids, are produced by

Abbreviations: ACL, ATP citrate lyase; FA, fatty acid(s); G, glycolipids; ME, malic enzyme; NAD+/ICDH, NAD+-dependent isocitrate dehydrogenase; NL, neutral lipids; NLM, nitrogen limited media; NRM, nitrogen rich media; P, phospholipids; PPP, pentose phosphate pathway.
the FA synthase from acetyl-CoA, malonyl-CoA and NADPH (Fig. 1). The inhibition of NAD$^+$-dependent isocitrate dehydrogenase (NAD$^+$-ICDH) is the key factor that can disturb the tricarboxylic acid (TCA) cycle and lead consequently to the excretion of citrate in the cytosol. ATP citrate lyase (ACL), which generates acetyl-CoA via the cleavage of citric acid, is an essential enzyme for FA biosynthesis in oleaginous yeasts and fungi (Vorapreeda et al., 2012; Dulermo et al., 2015). Besides ACL, the activity of cytosolic malic enzyme (ME) was proposed to be one of the rate-limiting steps for FA synthesis due to its capacity to provide reducing power in the form of NADPH (Ratledge, 2014). However, the pentose phosphate pathway (PPP) is also an important source of NADPH (Wasylenko et al., 2015). The biochemical pathway described above, permitting the conversion of sugars to lipids, is common in oleaginous eukaryotic micro-organisms and has been discussed previously in several studies together with FA compositional shifts that occur in oleaginous cells (Papanikolaou et al., 2004; Makri et al., 2010; Chang et al., 2013; Bellou et al., 2016). However, the regulatory mechanisms of lipid accumulation in ascospores and during transition from asexual to sexual reproduction are not yet fully elucidated while knowledge of the FA distribution in individual lipid fractions is rather limited. In the newly isolated yeast, Debaryomyces etchellsii, which has been used recently in biotechnological research (Arous et al., 2015, 2016), ascosporogenesis, induced by nitrogen deficiency, goes along with lipid accumulation but it is not clear if lipids are synthesized in vegetative cells and then encompassed in the spore structures, or if the ascopores possess their own lipogenic machinery.

In the present work we monitored the quantitative changes of major lipid groups, as well as their FA compositional shifts in both vegetative cells and ascospores of the oleaginous yeast D. etchellsii cultivated under conditions of nitrogen sufficiency and nitrogen deficiency on glycerol based media. Moreover, the activity of ACL, ME and NAD$^+$-ICDH were determined hoping to get an insight into metabolic alterations leading to lipid synthesis during the life cycle of D. etchellsii. The experimental results presented in this paper permit us to conclude that although glycerol induces ascosporogenesis even under nitrogen sufficient conditions, lipogenic activity within spores can only take place under nitrogen limitation. Under balanced growth conditions, lipid biosynthesis is restricted due to low

**Fig. 1.** A simplified overview of TAG formation in oleaginous yeasts/fungi. Enzymes implicated in lipid accumulation discussed in the paper are framed in black panels. Abbreviations: ICDH, isocitrate dehydrogenase; ACL, ATP citrate lyase; ME, malic enzyme. Other abbreviations: $\alpha$-KG, alpha-ketoglutarate; LDs, lipid droplets; OAA, oxaloacetate; PPP, pentose phosphate pathway; TCA, tricarboxylic acid; TAG, triacylglycerol.
mitochondrial citrate excretion into the cytosol, as the high NAD\(^+\)-ICDH activity indicates.

**METHODS**

**Yeast strain and culture conditions.** The yeast *D. etchellsii* strain BM1 (Arous et al., 2016) was maintained in the Laboratory of Enzyme Engineering and Microbiology, University of Sfax, on YPG (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 for short-term storage and in 20 % (w/v) glycerol at −80 °C for long-term storage. This strain was previously studied by Arous et al. (2015) and was shown to be able to accumulate lipids during sexual reproduction.

Kinetic experiments were conducted in 250 ml Erlenmeyer flasks, containing 50 ml of a growth medium having the following composition (in g l\(^{-1}\)): KH\(_2\)PO\(_4\) 12; Na\(_2\)HPO\(_4\) 12; MgSO\(_4\)\(\cdot\)7H\(_2\)O 1.5; CaCl\(_2\)\(\cdot\)2H\(_2\)O 0.1; MnSO\(_4\)\(\cdot\)5H\(_2\)O 0.0001; CuSO\(_4\)\(\cdot\)5H\(_2\)O 0.0001; Co(NO\(_3\))\(_2\)3H\(_2\)O 0.0001; ZnSO\(_4\)\(\cdot\)7H\(_2\)O 0.001. A variety of fermentable and non-fermentable substances were used as carbon sources, i.e. glucose 50 g l\(^{-1}\), glycerol 50 g l\(^{-1}\), glucose 50 g l\(^{-1}\) plus glycerol 30 g l\(^{-1}\), glucose 50 g l\(^{-1}\) plus potassium acetate 10 g l\(^{-1}\). Yeast extract (YE), containing total nitrogen 12 %, w/w, was used as nitrogen source at concentrations 5 and 2 g l\(^{-1}\) for preparation of NRM and NLM, respectively. The C/N molar ratios were around 81 and 32 for NRM and NLM, respectively. In all cases the initial pH of the medium was 6.0±0.1 after autoclaving.

After sterilization (at 121 °C for 20 min), the flasks were inoculated with 1 ml of a mid-exponential growth phase pre-culture on potato dextrose broth (Conda) medium containing 4×10\(^5\) cells. All cultures were performed in an orbital shaker at an agitation rate of 180 r.p.m. and incubation temperature T= 28±1 °C.

Lipid bodies in yeast cells and ascospores were visualized after staining with Nile red fluorescent dye under an Axioskop 40 (Zeiss) fluorescence microscope, equipped with an excitation filter of 470/40 nm -1 and a Progности camera (JenoptikCFcool) (Arous et al., 2015). In addition, ascis and ascospores were detected after malachite green staining according to Wickerham (1951) with slight modifications. Briefly, heat-fixed smears were flooded with an aqueous solution of malachite green (5 %) and placed above boiling water for 10 min. The slides were then washed with tap water and counter-stained with a 0.5 % safranin solution for 1 min.

Cells were visualized under a light microscope (Axioskop Plus Carl Zeiss), equipped with a colour video camera (SSC-DCS8AP). Spores appeared turquoise-blue in contrast to vegetative cells, which took a red colour.

The number of ascospores was determined by suspending cells in water and counting the ascospores in the suspension using a haemocytometer.

**Analytical methods.** For biomass determination, flasks were removed from the incubator and cells were harvested by centrifugation at 15000 r.p.m. (22500 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. Glycerol in the growth medium was determined gravimetrically and there was shown to be able to accumulate lipids during sexual reproduction.

Analytical methods. For biomass determination, flasks were removed from the incubator and cells were harvested by centrifugation at 15 000 r.p.m. (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. Glycerol in the growth medium was determined gravimetrically and there was shown to be able to accumulate lipids during sexual reproduction.

The activities of Δ9 and Δ12 desaturases were estimated by using the ratios \(^{13}C\)18 : l/C18:0 and \(^{13}C\)18 : 2 l/C18 : 1 respectively according to Fakas et al. (2009).

For the preparation of cell-free extracts, cells were harvested by centrifugation (15 000 r.p.m., 22 500 g, 15 min at 4 °C), washed twice with a 50 mM Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) buffer pH 7.5 and re-suspended in a 30 mM Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) buffer pH 7.5 at a ratio of 1 ml buffer to 0.5 g wet biomass. Yeast cells were ruptured by 10 sonic bursts of 120 W, lasting 2 min, and one sonic burst of 120 W, lasting 1 min, at 0–4 °C using a Sonics Vibra-Cell CV188 sonicator. Disrupted cells were centrifuged at 18 000 r.p.m. for 30 min at 4 °C and the supernatant, containing mitochondrial and cytoplasmic proteins, was filtered through a Whatman 0.2 μm membrane to remove solidified lipids and remaining cell debris (Papanikolaou et al., 2004).

Enzymatic activities were determined using a Unicam 5625 UV/VIS spectrophotometer at 25 °C. NAD\(^+\)-ICDH (EC 1.1.1.41), ME (EC 1.1.1.40) and ACL (EC 4.1.3.8) were determined according to Kornberg (1955), Geer et al. (1979) and Sterre (1959), respectively. One unit (U) of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 μmol of each enzymatic reaction product per minute. Specific activity was expressed as units per g dry weight.

Experiments were conducted at least in duplicate. Data were subjected to one-way analysis of variance, ANOVA, followed by a Bonferroni post hoc test using the IBM SPSS Statistics 21 software package. The null hypothesis was rejected at a significance level of P<0.05.

**RESULTS AND DISCUSSION**

In a previous paper we demonstrated that *D. etchellsii* growing on nitrogen-limited glucose-based media was able to sporulate after depletion of the nitrogen source in the growth medium, a process that coincided with lipid accumulation (Arous et al., 2015). We wondered, therefore, if nitrogen limitation induced lipid accumulation in ascospores or, simply, ascospores are naturally lipid rich structures. Thus, in a first stage of the present work, presumably ascosporogenic (non-fermentable) and non-ascosporogenic (fermentable) carbon sources were employed, in order to determine the conditions that stimulate sporulation in *D. etchellsii* growing on NRM.

**Ascosporogenesis induction in *D. etchellsii* growing on NRM**

Cell growth and sporulation of *D. etchellsii* on NRM, containing a single carbon source (fermentable or non-fermentable) or mixtures of fermentable and non-fermentable carbon sources, were determined 72 h after inoculation. The maximum cell dry weight (9.9±1.2 g l\(^{-1}\)) and was observed when glucose and mixtures containing glucose were employed, whereas, under these conditions, *D. etchellsii* did not
sporulate within 72 h (Fig. 2a, c, d). Sporulation of this strain was only observed on glycerol (in which the ratio of ascospores : vegetative cells was 40 : 60) despite the lowest cell growth achieved (6.5 g l\(^{-1}\)) (Fig. 2b). According to the literature, sporulation requires respiratory activity, which is indeed dependent on the nature of the carbon source. Unlike non-fermentable carbon sources (oleate, ethanol, glycerol or acetate as sole carbon sources), the presence of glucose in the growth medium represses the TCA cycle enzymes and, consequently, represses respiration and sporulation (Fendt & Sauer, 2010; Weinhandl et al., 2014). Gancedo (1998) and Stülke & Hillen (1999) reported that simultaneous utilization of glucose and other carbon sources by micro-organisms is rare, since glucose represses utilization of other sugars depending on intrinsic metabolic pathways (Kim et al., 2010). Therefore, when glucose is mixed with a non-fermentable carbon source, \(D. \text{ etchellsii}\) cells preferentially assimilate glucose first and subsequently a longer time is required to enter into the sexual stage together with non-fermentable carbon source assimilation.

**Morphological and biochemical features of \(D. \text{ etchellsii}\) grown on glycerol in NRM and NLM**

Representative data concerning morphological features, growth, lipid accumulation as well as glycerol and nitrogen consumption during the life cycle of \(D. \text{ etchellsii}\) cultivated on NRM and NLM containing glycerol as sole carbon source are given in Table 1 and Figs 3 and 4. The results reveal that under balanced growth conditions (in the first 24 h) the yeast grew by the process of budding in both NRM and NLM, despite the fact that glycerol was the sole carbon in the growth medium (Figs 3a, a’ and 4a, a’). After 96 h of incubation in NRM, yeast growth ceased and the maximum cell dry weight (7.4 g l\(^{-1}\)) was achieved, while in NLM, cell mass production was significantly lower and reached its maximum in a shorter period (i.e. 72 h of incubation). After 120 h of incubation, only 38.3 and 27.7% of glycerol had been consumed by \(D. \text{ etchellsii}\) for NRM and NLM, respectively, while further incubation did not improve the assimilation of glycerol (Table 1). Since glycerol crosses the plasma membrane by passive diffusion, it seems that its uptake rate depends on its concentration in the growth medium, as suggested by Papanikolaou & Aggelis (2011). The residual NH\(_4\) concentration was about 0.8 mg l\(^{-1}\) in NRM, while no residual NH\(_4\) was detected in NLM (Table 1).

In both NRM and NLM, the sexual stage proceeded at approximately 48 h (Figs 3b, b’ and 4b, b’) and it was completed at approximately 144 h from the start of incubation (Figs 3c, c’ and 4c, c’). From these results, it can be concluded that sporulation of \(D. \text{ etchellsii}\) on glycerol being independent of nitrogen availability in the growth medium depends upon the age of cells. On the contrary, sporulation of this strain in the presence of glucose requires nitrogen starvation (Arous et al., 2015).

During the life cycle of \(D. \text{ etchellsii}\) on NLM, the esterified FA content increased from 11.5 to 18.3% (w/w) at about 72 h of culture, while further incubation resulted in a slight decrease in FA content. Accordingly, under these conditions, the growth of \(D. \text{ etchellsii}\) could be roughly divided into cell growth phase (up to 24 h of incubation), lipid accumulation phase (24–72 h) and lipid turnover phase (after 72 h of culture). These physiological stages are in good agreement with those reported in Makri et al. (2010)
Table 1. Changes in ascospore:vegetative cells (As:Vc) ratio, cell mass, remaining glycerol (Glol rem) and ammonium nitrogen (NH4), esterified fatty acid (FA) content and relative distribution of FA in total lipids over one complete life cycle of D. etchellsii cultivated on nitrogen rich medium (NRM) and nitrogen limited medium (NLM)

Culture conditions: initial glycerol 53 g l\(^{-1}\); initial NH\(_4\) 5.7 mg l\(^{-1}\) and 14.25 mg l\(^{-1}\) for NLM and NRM, respectively; pH 6±0.1; temperature 28±1 °C; agitation rate 180 r.p.m. A, asexual stage (vegetative cells); B, asexual–sexual stage transition (vegetative cells + ascospores in ascii); C, sexual stage (almost exclusively liberated ascospores). Data are presented as mean values from duplicate experiments.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Stages of life cycle</th>
<th>Time (h)</th>
<th>As : Vc ratio</th>
<th>Cell mass (g l(^{-1}))</th>
<th>Glol_{rem} (g l(^{-1}))</th>
<th>NH(_4) (mg l(^{-1}))</th>
<th>FA content (%)</th>
<th>Relative distribution of FA in total lipids (%)</th>
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</thead>
<tbody>
<tr>
<td>NRM</td>
<td>A 24 0:100</td>
<td>2.9±0.2</td>
<td>47.9±0.1</td>
<td>10.4±0.2</td>
<td>11.0±0.1</td>
<td>13.6±0.5</td>
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<td></td>
<td>B 48 25:75</td>
<td>6.4±0.1</td>
<td>40.7±0.4</td>
<td>7.5±0.2</td>
<td>11.4±0.3</td>
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<tr>
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<td>72 40:60</td>
<td>6.5±0.0</td>
<td>35.9±0.2</td>
<td>5.8±0.3</td>
<td>11.6±0.1</td>
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<tr>
<td></td>
<td>96 60:40</td>
<td>7.4±0.1</td>
<td>34.6±0.4</td>
<td>3.5±0.2</td>
<td>9.7±0.3</td>
<td>14.2±0.2</td>
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<td></td>
<td>120 75:25</td>
<td>7.4±0.1</td>
<td>32.7±0.5</td>
<td>1.5±0.2</td>
<td>7.6±0.1</td>
<td>14.3±0.6</td>
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<td>C 144 96:4</td>
<td>7.5±0.1</td>
<td>31.5±0.3</td>
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<td>168 97:3</td>
<td>7.7±0.1</td>
<td>31.4±0.4</td>
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<td>14.2±0.2</td>
<td>6.8±0.0</td>
<td>1.9±0.2</td>
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<tr>
<td>NLM</td>
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<td>11.5±0.5</td>
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<td>38.3±0.1</td>
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<td></td>
<td>168 98:2</td>
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<td>0.0±0.0</td>
<td>15.4±0.6</td>
<td>13.4±0.1</td>
<td>7.8±0.2</td>
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*Others: C10:0, C12:0, C14:0 and C14:1.

for Yarrowia lipolytica grown on a glycerol NLM. Nevertheless, on NRM, the esterified FA content remained more or less constant (11–11.6%) until the stage of ascii formation (at 72 h), and after that a decrease in the FA percentage occurred reaching the value of 7.5% (w/w). Although high quantities of glycerol remained unconsumed both in NRM and NLM, the yeast showed a tendency to degrade its previously accumulated lipids. Usually, lipid turnover takes place under carbon starvation conditions or when the rate of the carbon source uptake cannot saturate physiological activities of the cell (Papanikolaou & Aggelis, 2011). The principal catabolic pathway for FA degradation is beta-oxidation, which provides the necessary energy for cell growth and maintenance as well (Shen & Burger, 2009; Papanikolaou & Aggelis, 2011).

When subjecting cells of D. etchellsii to fluorescence microscopy, the ascospores showed a higher affinity towards Nile red compared to the vegetative cells (Fig. 3). The fluorescence surrounding the ascospores may be attributed to the presence of oxylipins. These compounds are generally located in the interspore matrix and may act as an adhesive possibly via entropy-based hydrophobic forces and/or hydrogen bonds, which lead to the aggregation of liberated ascospores in an aqueous environment (Rudolph, 1994; Kock et al., 2004).

In conclusion, in the first growth step on a non-fermentable carbon source (glycerol), D. etchellsii proliferated by budding, while after 48 h, the sexual stage proceeded independent of the nitrogen concentration in the growth media. However, the ascospores produced on NLM were richer in lipids when compared with those produced on NRM.

Whatever the nitrogen concentration in the growth medium, the FA composition of the cellular lipids varied in the same way depending on the life-cycle stage (Table 1). As was reported by Arous et al. (2015), D. etchellsii lipids contained mostly oleic acid (C18:1) and linoleic acid (C18:2). During transition from asexual to sexual stage, the concentration of linoleic acid and, to a lesser extent, of stearic acid decreased, while that of oleic acid increased significantly. These changes in FA composition reflect changes in FA desaturation rate during the yeast life cycle, suggesting that oleic acid was preferentially used for spore structural and reserve lipid formation, while linoleic acid was more appropriate for vegetative cell components. More details about this issue are provided in the following text after considering the FA composition of individual lipid fractions.

Quantitative and compositional shifts in lipid fractions during the life cycle of D. etchellsii

The FA distribution in lipid fractions (i.e. NL, G and P) during the life cycle (Table 2) largely reflects the physiological role of individual lipids. In detail, judging by the proportion of FA esterified in the various fractions, NL, which are usually storage lipids, were the predominant fraction of
cellular lipids produced in both vegetative cells and spores, both in NRM and NLM. On the other hand, P were the major polar lipids, while G were synthesized in low proportions. During transition from the asexual to sexual phase, the percentage of NL increased with a significant decrease of the P fraction and, to a lesser extent, of the G fraction. In addition, after considering the total amount of FA of each lipid fraction per unit of fat-free biomass, we clearly conclude that spores (especially those produced in NRM) need a lesser amount of polar lipids (mainly P) per unit of fat free biomass than vegetative cells. Therefore P were mainly synthesized during the initial stages of growth in order to maintain the high functionality and permeability of the membrane system of the metabolically active vegetative cells, while lower P quantities were needed for the less active ascospores. On the contrary, the increase of NL fraction in ascospores may indicate the tendency of these structures to accumulate energy to be used later during germination. NL were accumulated in ascospores, in the form of small lipid droplets (LDs), which were located peripherally, close to the ascospore – delimiting membranes (Fig. 3b, b', c, c'). On the contrary, LDs in vegetative cells were much larger, fewer in number and were located in various places in the cytosol (Fig. 3a, a'). Similar features were reported for Dipodascus ambrosiae (Saccharomycetales, Dipodascaceae), although the G fraction was found increased in the sexual stage of this yeast (Smith et al., 2003).

High quantities of linoleic acid were found esterified in polar lipids, especially in P, during the vegetative stage of growth (Table 2), and this finding was not surprising since P is the major site of oleic acid desaturation (Certik & Shimizu, 1999). With few exceptions (i.e. NL produced in NRM), during transition from asexual to sexual stage, linoleic acid concentration decreased markedly, mainly in P, while oleic acid concentration increased. Judging from the ratios C18:1/C18:0 and C18:2/C18:1, which may reflect the activity of the related desaturases (Fakas et al., 2009), we suggest that the growing vegetative cells demonstrated a high Δ12 desaturase activity that was gradually reduced as the sexual reproduction proceeded. Oleic acid (but not stearoyl-CoA) is desaturated after its esterification in P of endoplasmic reticulum. The synthesized linoleic acid is then moved to structure the newly formed membranes. We suppose that in the case of spores, in which new membranes are formed to a very limited extent, the sn-2 position of P remained occupied with linoleic acid.

Fig. 3. Representative pictures (×1000) of D. etchellsii growing on nitrogen limited medium (NLM) and nitrogen rich medium (NRM) at different growth stages. Left panels: images taken under light microscope. Right panels: fluorescence images of D. etchellsii cells stained with Nile red. (a, a') Captured after 24 h incubation. Only vegetative cells (Vc) are shown during the asexual phase. (b, b') Captured after 48 h incubation. Vegetative cells (Vc) as well as ascospores in ascii (As) appeared during the asexual–sexual stage transition. (c, c') Captured after 144 h incubation. Almost exclusively liberated ascospores (As) are shown during the sexual stage. Culture conditions: as described in Table 1.
and therefore any further desaturation of oleic acid was blocked. Enzymes involved in lipid remodelling (e.g. phospholipase A2) may be down-regulated in ascospores. On the contrary, passing from asexual to sexual reproduction, Δ9 desaturase activity was gradually increased. Unsaturated (containing P rich in linoleic acid) membranes permit high nutrient uptake and therefore were more appropriate for the fast growing vegetative cells while more saturated and rigid/resistant membranes (containing P rich in oleic acid) are needed for the less active ascospores. It should be noted that P produced during the asexual stage in NRM were richer in linoleic acid than those produced in NLM. Additionally, the fact that NL of ascospores were particularly rich in oleic acid may indicate that this FA, containing more energy per mole than linoleic acid, is a more appropriate source of energy for ascospores, which may be used during germination. The above-mentioned differences in desaturation ratios were verified by statistical analysis using one-way ANOVA. On the contrary, in the case of the yeast *D. ambrosiae*, Smith *et al.* (2003) reported that NL and G fractions of ascospores were richer in linoleic acid than those of vegetative cells.

**Lipogenic activity during the life cycle of *D. etchellsii***

The key enzymes implicated in lipogenesis, i.e. ACL and ME, were active in the cells growing both in NRM and in NLM, although much higher activities were recorded in NLM (Table 3). Moreover, in NLM an increase of ACL activity was noticed in yeast cells during the lipid

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**Fig. 4.** Representative pictures (×1000) of *D. etchellsii* cells growing on nitrogen limited medium (NLM) and nitrogen rich medium (NRM) at different growth stages as in Fig. 3. Images taken under light microscope after malachite green staining. Spores appeared turquoise-blue in contrast to red dye taken up by the vegetative cells.
Table 2. Fatty acid (FA) distribution in lipid fractions during transition from asexual to sexual stage of *D. etchellsii*

NRM, Nitrogen rich medium; NLM, nitrogen limited medium; NL, neutral lipids; G, glycolipids; P, phospholipids. Culture conditions: see Table 1. Experiments were performed at least in duplicate. A, asexual stage (vegetative cells); B, asexual–sexual stage transition (vegetative cells + ascospores in ascii); C, sexual stage (almost exclusively liberated ascospores).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Lipid fraction</th>
<th>Phase</th>
<th>Fermentation time (h)</th>
<th>FA in each fraction (%)</th>
<th>FA/fat-free biomass (%)</th>
<th>Relative distribution of FA in individual lipid fractions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cl 6:0</td>
</tr>
<tr>
<td>NRM</td>
<td>NL</td>
<td>A (0–24 h)</td>
<td>24</td>
<td>56.7±1.8</td>
<td>7.0±1.0</td>
<td>13.5±0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B (48–120 h)</td>
<td>72</td>
<td>62.7±0.2</td>
<td>8.3±0.9</td>
<td>11.7±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C (144–168 h)</td>
<td>168</td>
<td>67.3±0.5</td>
<td>5.5±0.0</td>
<td>11.6±0.6</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>A (0–24 h)</td>
<td>24</td>
<td>6.0±0.2</td>
<td>0.7±0.1</td>
<td>13.4±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B (48–120 h)</td>
<td>72</td>
<td>5.4±0.2</td>
<td>0.7±0.0</td>
<td>14.3±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C (144–168 h)</td>
<td>168</td>
<td>5.7±0.1</td>
<td>0.5±0.0</td>
<td>13.2±0.6</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>A (0–24 h)</td>
<td>24</td>
<td>37.3±2.0</td>
<td>4.6±0.3</td>
<td>10.2±0.7</td>
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<tr>
<td></td>
<td></td>
<td>B (48–120 h)</td>
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<td>31.9±0.0</td>
<td>4.2±0.4</td>
<td>11.9±0.6</td>
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<tr>
<td></td>
<td></td>
<td>C (144–168 h)</td>
<td>168</td>
<td>27.3±0.4</td>
<td>2.2±0.1</td>
<td>10.8±0.7</td>
</tr>
<tr>
<td>NLM</td>
<td>NL</td>
<td>A (0–24 h)</td>
<td>24</td>
<td>56.0±2.4</td>
<td>6.7±0.3</td>
<td>13.7±0.1</td>
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<tr>
<td></td>
<td></td>
<td>B (48–120 h)</td>
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<td>65.4±0.3</td>
<td>14.6±0.2</td>
<td>12.9±0.8</td>
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<tr>
<td></td>
<td></td>
<td>C (144–168 h)</td>
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<td>71.0±1.2</td>
<td>12.9±0.2</td>
<td>10.0±0.4</td>
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<tr>
<td></td>
<td>G</td>
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<td>5.7±0.2</td>
<td>0.7±0.1</td>
<td>12.1±0.6</td>
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<td></td>
<td>B (48–120 h)</td>
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<td>5.1±0.1</td>
<td>1.2±0.2</td>
<td>14.1±0.3</td>
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<td>C (144–168 h)</td>
<td>168</td>
<td>5.4±0.0</td>
<td>1.0±0.1</td>
<td>13.2±0.8</td>
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<tr>
<td></td>
<td>P</td>
<td>A (0–24 h)</td>
<td>24</td>
<td>38.3±2.2</td>
<td>5.0±1.0</td>
<td>10.5±0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B (48–120 h)</td>
<td>72</td>
<td>29.5±0.2</td>
<td>6.6±0.3</td>
<td>11.6±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C (144–168 h)</td>
<td>168</td>
<td>23.6±1.2</td>
<td>4.3±0.2</td>
<td>10.6±0.9</td>
</tr>
</tbody>
</table>

*Others: Cl 10: 0, Cl 12: 0, Cl 14: 0 and Cl 14: 1.*
accumulation phase that correlates with transition from asexual to sexual stage (at 72 h of incubation). A similar trend was recorded for ACL activity in NRM-growing cells, although, in this case, lower lipid quantities were synthesized. In some oleaginous micro-organisms, the activity of ACL correlates with the specific rate of lipid synthesis. Heterologous over-expression of the gene acl from Mus musculus enhanced lipid content in Y. lipolytica (Zhang et al., 2014).

Although ME activity decreased with time, this enzyme retained high activity both in vegetative cells and in ascospores. In oleaginous yeasts, the supply of reducing power in the form of NADPH is one of the rate-limiting steps for FA synthesis and ME is the major source of NADPH for de novo lipid synthesis (Ratledge, 2014). As an example, overexpression of ME from Mucor circinelloides increased the FA content by 2.5- and 2-fold in M. circinelloides and in Rhodotorula glutinis, respectively (Zhang et al., 2007; Li et al., 2013). However, in Y. lipolytica only mitochondrial ME has been found and, therefore, its overexpression had no effect on lipid accumulation (Zhang et al., 2013). In this yeast, ppp supplies reducing power to the lipogenic machinery rather than the ME reaction (Wasylenko et al., 2015). The experimental data included in this paper indicate that the activity of ME was highly influenced by nitrogen availability in the medium, since ME activity started to decrease after nitrogen exhaustion. A similar trend was reported by Chang et al. (2013) for Schizochytrium sp. However, in the case of Rhodosporidium toruloides and Trichosporon cutaneum, the ME activity was noticed to be higher in the lipid accumulation phase compared to that in cell growth phase (Zhu et al., 2012; Liu et al., 2013).

The major biochemical difference between the cells growing in NRM and NLM concerned the activity of NAD⁺-ICDH, an enzyme that directs the carbon flow through the TCA cycle or the pathway of de novo lipid biosynthesis. In cells growing in NLM, the activity of NAD⁺-ICDH decreased markedly during lipid accumulation phase, taking place simultaneously with sporulation after nitrogen exhaustion in the growth medium. This low activity of ICDH indicated a functional disorder of the TCA cycle, which could induce citrate excretion in the cytosol, making it available as substrate for the ACL. Conversely, in NRM-growing cells, the activity of ICDH increased rather than decreased in the sexual phase of reproduction, indicating a greater flux of carbon towards the TCA cycle rather than towards lipogenesis, resulting thus in lipid-poor ascospores under these conditions.

CONCLUSIONS

The present work clarified some aspects of lipid metabolism over the life cycle of D. etchellsii and the implication of nitrogen limitation in ascosporeogenesis and the production of lipid-rich ascospores.

Glycerol induced sporulation in D. etchellsii cells independent of the nitrogen concentration in the growth medium, while lipid accumulation in ascospores occurred only under nitrogen limited conditions. The mechanism used for lipid accumulation in ascospores seemed to be similar to that described for oleaginous yeasts. High activities of ACL and ME were detected even in cells growing in NRM, but lipid biosynthesis was restricted by the high NAD⁺-ICDH activities. Independent of the nitrogen concentration in the growth medium, NL were the major lipids. All lipid fractions, especially P, of vegetative cells were rich in linoleic acid, while those of ascospores were rich in oleic acid.

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REFERENCES


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