Morphological and metabolic shifts of *Yarrowia lipolytica* induced by alteration of the dissolved oxygen concentration in the growth environment

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*Yarrowia lipolytica*, an ascomycete with biotechnological potential, is able to form either yeast cells or hyphae and pseudohyphae in response to environmental conditions. This study shows that the morphology of *Y. lipolytica*, cultivated in batch cultures on hydrophilic (glucose and glycerol) and hydrophobic (olive oil) media, was not affected by the nature of the carbon source, nor by the nature or the concentration of the nitrogen source. By contrast, dissolved oxygen concentration (DOC) should be considered as the major factor affecting yeast morphology. Specifically, when growth occurred at low or zero DOC the mycelial and/or pseudomycelial forms predominated over the yeast form independently of the carbon and nitrogen sources used. Experimental data obtained from a continuous culture of *Y. lipolytica* on glycerol, being used as carbon and energy source, demonstrated that the mycelium-to-yeast form transition occurs when DOC increases from 0.1 to 1.5 mg l$^{-1}$. DOC also affected the yeast physiology, as the activity of enzymes implicated in lipid biosynthesis (i.e. ATP-citrate lyase, malic enzyme) was upregulated at high DOC whereas the activity of enzymes implicated in glycerol assimilation (such as glycerol dehydrogenase and kinase) remained fundamentally unaffected in the cell-free extract.

**INTRODUCTION**

*Yarrowia lipolytica* (formerly referred to as *Candida lipolytica*, *Endomycopsis lipolytica* or *Saccharomycopsis lipolytica*) is an aerobic yeast, mostly found in oil-polluted environments and foods (i.e. cheese, yogurt) (Barth & Gaillardin, 1997; Fickers et al., 2005; Bankar et al., 2009). Its ability to degrade a wide range of carbon sources such as glucose, alcohols, acetate, hydrophobic substrates (e.g. alkanes, fatty acids and oils) as well as several types of low-value or harmful by-products (e.g. raw glycerol, olive mill wastewater), and bio-convert them into products of high added value (i.e. organic acids, single cell oil, single cell protein, biosurfactants, heterologous proteins and enzymes) (Barth & Gaillardin, 1997; Papanikolaou et al., 2001; Madzak et al., 2004; Fickers et al., 2005; Flores et al., 2005; Gellissen et al., 2005; Papanikolaou et al., 2008; Papanikolaou & Aggelis, 2009; Coelho et al., 2010; Makri et al., 2010; Oliveira et al., 2010; Dulerio et al., 2013; Juszczyszyn et al., 2013) has increased interest in the exploitation of this yeast in numerous biotechnological and environmental applications.

Besides interest for developmental biology and basic microbiological research, the ability of *Y. lipolytica* to form either yeast cells or hyphae and pseudohyphae in response to environmental conditions (Barth & Gaillardin, 1997; Zinjarde et al., 1998; Dominguez et al., 2000; Ruiz-Herrera & Sentandreu, 2002; Bankar et al., 2009; Beopoulos et al., 2009; Makri et al., 2010; Nicaud, 2012) has practical importance as this property should be considered when biotechnological applications are designed. That is, single cells (which are easily manipulated and presumably metabolically more active than mycelia) are preferred when submerged cultures are performed, while mycelial forms (able to penetrate solid substrates) are more...
desirable in solid-state fermentation. In addition, several biochemical activities of industrial interest displayed by *Y. lipolytica*, such as lipogenic activity and its ability to synthesize intermediate metabolites of the tricarboxylic acid cycle, may be related to the various cell forms.

Carbon and nitrogen sources, temperature and pH of the medium have been found to affect yeast morphology. Specifically, in the presence of casein and/or olive oil, N-acetylglucosamine and serum *Y. lipolytica* transitions from yeast to mycelial forms (Ota *et al.*, 1984; Guevara-Olvera *et al.*, 1993; Zinjardes *et al.*, 1998; Kim *et al.*, 2000; Pérez-Campo & Dominguez, 2001; Ruiz-Herrera & Sentandreu, 2002; Szabo & Štofaníkova, 2002; Kawasse, 2003). The opposite phenomenon has been observed when glucose or alkanes are used as carbon source or when cultivated at low pH (Gutierrez & Erickson, 1977; Rodríguez & Domínguez, 1984; Guevara-Olvera *et al.*, 1993; Zinjardes *et al.*, 1997, 1998; Szabo & Štofaníkova, 2002). Significant progress has recently been made in identifying genes that play a crucial role in the dimorphism of *Y. lipolytica* (genes involved in the mitogen-activated protein kinase and protein kinase A pathways, Rho family, etc.) (Hurtado & Rachubinski, 1999; Cervantes-Chávez *et al.*, 2009; Morales-Vargas *et al.*, 2012; Martínez-Vazquez *et al.*, 2013).

Although it is frequently assumed that dissolved oxygen concentration (DOC) is one of the major parameters affecting yeast physiological and biochemical characteristics, and in consequence fermentation performance, studies correlating DOC to yeast morphology are lacking. Hence, in the current study, we have analysed the influence of DOC on the dimorphism and physiology of *Y. lipolytica* cultivated on various carbon and nitrogen sources. The experimental results demonstrated that low DOC values induce mycelia formation and downregulate lipid biosynthesis, as measured by activity of ATP-citrate lyase (ATP-CL) and malic enzyme (ME).

**METHODS**

**Micro-organism and culture conditions.** *Yarrowia lipolytica* ACA-DC 50109 was maintained on potato dextrose agar (PDA; Conda) at 7 ± 1 °C.

Yeast morphology was initially studied using slides covered by a thin layer of PDA which was inoculated with 1 ml of a mid-exponential phase pre-culture containing 4 × 10⁸ cells ml⁻¹. DOC was controlled through a cascade controller varying agitation rate (from 150 to 250 r.p.m.) and incoming gas (mixtures of air and pure oxygen) flow rate and composition. In all cases incoming gas passed through a bacteriological filter of 0.2 µm pore size (Whatman). pH of the medium was automatically controlled at 6 ± 0.03 by adding 1 M NaOH (Merck) and the incubation temperature was controlled at 28 °C. Antifoam A (Fluka) was added if necessary.

Continuous bioreactor cultures at a dilution rate equal to 0.032 h⁻¹ were established by adding fresh medium through a peristaltic pump (Peripex G2 IP40; Bioengineering AG) at a constant rate. Fluctuations of flow rate were less than 1.3 %. Working volume was kept constant in the reactor by using an overflow weir connected to a pump (REGLO Analogue MS-4/6 ISM 828; ISMATEC) which was operating at the same rate as that of the inflow pump. Steady-state conditions were obtained after continuous flow of at least five working volumes of medium.

**Analytical methods.** For determination of dry biomass [dry weight (DW)] and cell density (cells ml⁻¹) the protocol described by Bellou & Aggelis (2012) was followed. Culture medium was collected and stored at −20 °C for further analysis.

Microscopic observations were performed using a light microscope (Axioskop plus; Carl Zeiss), equipped with a colour video camera (Sony, SSC-DC58AP; Japan). For scanning electron microscopy (SEM), a sample of the culture was placed onto SEM stubs and dried for 24 h at 38 °C. Stubs were placed thereafter into a gold/palladium sputter coater (JFC 1100; Hama Instruments). For scanning electron microscopy (SEM), a sample of the culture was placed onto SEM stubs and dried for 24 h at 38 °C. Stubs were placed thereafter into a gold/palladium sputter coater (JFC 1100; EOL) and specimens were viewed with a JSM 6300 (JEOL) at 20 kV accelerating voltage and 10⁻¹¹⁻¹⁰ A beam current.

Total lipids were extracted with chloroform/methanol (2:1) (Folch *et al.*, 1957), washed with a 0.88 % (w/v) KCl (Merck) solution as described by Bellou *et al.* (2012) and gravimetrically determined after solvent evaporation under vacuum.

For the preparation of cell-free extracts a known culture volume was withdrawn from the reactor and cells were harvested by centrifugation (15 000 r.p.m., 15 min and 4 °C). The cell pellet was washed twice with a 50 mM Na₂HPO₄/KH₂PO₄ (Fluka) buffer (pH 7.5) and resuspended in a 30 mM Na₂HPO₄/KH₂PO₄ buffer (pH 7.5) at a ratio of 1 ml buffer to 0.5 g wet biomass. Yeast cells were ruptured at 4 °C by two sonic bursts lasting 2 min and one sonic burst lasting 1 min at 70 W using a Sonics Vibra cell CV188 sonicator. Cell debris was separated from the crude extract by centrifugation at 18 000 r.p.m. for 50 min at 4 °C and the supernatant was collected and filtered through a Whatman 0.2 µm membrane to remove solidified lipids and remaining cell debris.

Nitrogen source was either a combination of (NH₄)₂SO₄ and yeast extract each at 0.5 g l⁻¹, or yeast extract alone at 0.1 or 5.0 g l⁻¹. Hydrophilic (glucose, Merck; glycerol, purity 98 %, Fluka) or hydrophobic (commercial refined olive oil provided by Hellenic fine oils) substrates were used at various concentrations.

Flask experiments were performed in 250 ml Erlenmeyer flasks containing 50 ml of the growth medium. After sterilization (121 °C for 20 min) the media were inoculated with 1 ml of a mid-exponential phase pre-culture containing 4 × 10⁸ cells ml⁻¹. DOC was determined according to the protocol described by Papanikolaou *et al.* (2004) using a Hana HI9146-04 selective electrode (Hanna Instruments).

Biorreactor batch and continuous cultures were made in a Bioengineering, Ralf Plus-System bioreactor of total volume 3.7 l and working volume 1.8 l, fitted with four baffles and two flat-bladed turbines with six blades (Rushton turbine). The reactor was sterilized at 121 °C for 2 h and kept at room temperature for 48 h or more to ensure sterility of the medium. The culture vessel was inoculated with 200 ml of a mid-exponential phase pre-culture containing 4 × 10⁸ cells ml⁻¹. DOC was controlled through a cascade controller varying agitation rate (from 150 to 250 r.p.m.) and incoming gas (mixtures of air and pure oxygen) flow rate and composition. In all cases incoming gas passed through a bacteriological filter of 0.2 µm pore size (Whatman). pH of the medium was automatically controlled at 6 ± 0.03 by adding 1 M NaOH (Merck) and the incubation temperature was controlled at 28 °C. Antifoam A (Fluka) was added if necessary.

Continuous biorreactor cultures at a dilution rate equal to 0.032 h⁻¹ were established by adding fresh medium through a peristaltic pump (Peripex G2 IP40; Bioengineering AG) at a constant rate. Fluctuations of flow rate were less than 1.3 %. Working volume was kept constant in the reactor by using an overflow weir connected to a pump (REGLO Analogue MS-4/6 ISM 828; ISMATEC) which was operating at the same rate as that of the inflow pump. Steady-state conditions were obtained after continuous flow of at least five working volumes of medium.
Enzyme activities were determined using a Unicam 5625 UV/VIS spectrophotometer at 25 °C. Glycerol kinase (GK) (EC 2.7.1.30) was assayed according to Bublitz & Kennedy (1954) and NAD+ dependent glycerol-3-phosphate dehydrogenase (GD) (EC 1.1.1.30) according to Bergmeyer (1974). NAD+ dependent isocitrate dehydrogenase (ICDH) (EC 1.1.1.41), ME (EC 1.1.1.40) and ATP-CL (EC 4.1.3.8) were determined according to the assays of Kornberg (1955), Geer et al. (1979) and Srere (1959), respectively.

One unit (U) of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 μmol of each enzymic reaction product per minute under the above-mentioned conditions. Soluble protein was measured as described by Papanikolaou et al. (2004). Specific activity was expressed as units per g DW.

All cultures, chemical analyses and biochemical determinations were carried out at least in duplicate. The experimental data were analysed using OriginPro 8 SR0, 1991–2007.

RESULTS

Preliminary studies of yeast morphology cultivated on PDA

Y. lipolytica was cultivated on PDA on slides and microscopic observations of undamaged colonies were made after 24 and 48 h of incubation. When the slide was covered immediately after inoculation by a coverslip a low oxygen concentration environment was established, allowing the formation of pseudomycelia and true mycelia (Fig. 1a, b). By contrast, when no coverslip was placed on the slide, a higher oxygen concentration environment was established, and the colonies comprised single cells. However, as growth proceeded at the centre of the colonies (where presumably oxygen availability was limited) pseudomycelia formed, whereas at the periphery of the colonies (where oxygen concentration was higher) single cells predominated (Fig. 1c).

Growth and morphology of Y. lipolytica in submerged cultures

Glucose and olive oil were used as carbon sources at concentrations of 10 and 5 g l−1, respectively, so that similar quantities of assimilable carbon (around 4 g l−1) were employed in both media. The growth curve of Y. lipolytica cultivated in agitated flasks (at 180 r.p.m.) with glucose as carbon source as well as the evolution of DOC are illustrated in Fig. 2. As growth proceeded, DOC values ranged from 3.8 to 7.5 mg l−1 and the culture consisted of only single cells (Fig. 2). However, when agitation rate decreased from 180 to 50 r.p.m., DOC dropped from 3.7 mg l−1 to zero and, in addition to yeast cells, pseudomycelia were also formed (Fig. 3a, b).

At the end of growth, true mycelia appeared in the culture (Fig. 3c). When olive oil was used as carbon source in well-aerated cultures (agitation at 180 r.p.m.) increased DOC (5.8–7.7 mg l−1) was observed and Y. lipolytica formed only single cells (Fig. 4). At low agitation rate (50 r.p.m.), at the beginning of growth and as long as DOC was above 4.0 mg l−1, the culture comprised single cells (Fig. 5a), whereas when DOC fell to zero, pseudomycelia and true mycelia were formed in addition to single cells (Fig. 5b, c).

Batch cultures of Y. lipolytica were also established in the bioreactor on media containing glycerol as carbon source and various nitrogen sources (organic and inorganic). Under these highly controlled conditions, yeast morphology was examined at high DOC (0.6–5.2 mg l−1). Similar to the previous findings, the culture consisted mainly of single cells in all growth phases, independently of the nature (ammonium nitrogen or yeast extract) of the nitrogen source (Fig. S2a). The bioconversion of glycerol to lipids and citric acid was also studied. Lipid accumulation increased with time up to 7.8 % (w/w, lipids in dry biomass), whereas citric acid production was maximized at the end of growth, reaching 11.8 g l−1 (Fig. S3). Similarly, when yeast extract was used as the sole nitrogen source (either at low or at high concentration), under highly aerated conditions, the culture consisted of only single cells (Fig. S2b, c) independently of the concentration of the organic nitrogen source.

The various forms of Y. lipolytica as determined by SEM are shown in Fig. S4(a, b).

Fig. 1. Images taken during growth of Y. lipolytica on PDA on slides under low (a, b) and high (c) oxygen conditions. Images were taken at magnifications of 400× and 1000×. Culture conditions: low oxygen was achieved by covering the slide with a coverslip after inoculation; in high oxygen conditions the slide was not covered by a coverslip. Experiments were performed in duplicate.
Morphology and physiology of *Y. lipolytica* in continuous cultures

The effect of DOC on the morphology of *Y. lipolytica* cultivated in continuous culture is shown in Fig. 6. At a steady-state dilution rate of 0.032 h⁻¹ and under low DOC (<0.13 mg l⁻¹), the culture consisted mainly of true mycelia (Fig. 6a; Fig. S4c). Under the same dilution rate, when DOC gradually increased, true mycelia were replaced by pseudomyelia and a few single cells (Fig. 6B1), whereas at steady state at high DOC (around 1.5 mg l⁻¹) single cells predominated (Fig. 6B2).

At steady state of low DOC (0.1 mg l⁻¹), biomass concentration was 4.8 g l⁻¹ and low quantities of citric acid (0.4 g l⁻¹) were produced (Table 1). Under these conditions lipid accumulation was around 7% (w/w, lipids in dry biomass), whereas when DOC increased lipid accumulation increased up to 10% (w/w, lipids in dry biomass). At high steady-state DOC, biomass produced was 4.5 g l⁻¹ and citric acid concentration increased up to 2.1 g l⁻¹. Activity of GK and GD implicated in glycerol assimilation did not show significant variations with a change of DOC. However, the increase of DOC led to higher activity of ATP-CL and ME, which participate in lipid synthesis. By contrast, ICDH decreased during the transition from low to high DOC.

**DISCUSSION**

Morphological transition of yeasts is of high importance for industrial applications, as single cells are easily manipulated in bioreactors, whereas mycelial forms are preferred in solid-state fermentation. In addition, the virulence of several human pathogens has been related to certain cell forms. Indicative of this interest in the dimorphic growth of biotechnologically and/or medically important micro-organisms are reviews that have appeared in the international literature on this subject (San-Blas *et al.*, 1984; O’Shea & Walsh, 1996; Cruz *et al.*, 2000) and not restricted to specific taxonomic groups, as dimorphism is found in several representatives of *Basidiomycetes*, *Ascomycetes*, *Zygomycetes* and imperfect fungi (Guevara-Olvera *et al.*, 1993).

The formation of single cells, pseudomyelia or true mycelia has been attributed to various environmental...
stimuli (Rodríguez & Domínguez, 1984; Aggelis, 1996; O’Shea & Walsh, 2000; Ruiz-Herrera & Sentandreu, 2002; Szabo & Štofaniková, 2002, Kawasse et al., 2003). In the case of Y. lipolytica, it has been reported that morphological transition depends on the nature of the carbon and nitrogen source, the presence of serum or citrate, the cultivation temperature and pH of the medium (Domínguez et al., 2000; Pérez-Campo & Domínguez, 2001). However, here we demonstrate that DOC is the critical parameter affecting the transition of Y. lipolytica from single cells to hyphae and vice versa, independently of the carbon and/or nitrogen source used.

In fact, in the present study, Y. lipolytica was cultivated in batch cultures on glucose, glycerol or olive oil, as carbon source has been considered to be one of the major factors affecting yeast morphology. When hydrophilic (glucose or glycerol) or hydrophobic (olive oil) substrates were used as carbon sources under highly aerated conditions, Y. lipolytica cultures consisted of single cells. By contrast, low aeration conditions induced mycelia forms in media containing both hydrophilic and hydrophobic substrates.

High carbon content compounds (such as oils) used as carbon sources result in higher oxygen requirements during microbial growth, and this parameter has not been taken into consideration in related studies and probably explains why the effects of hydrophilic or hydrophobic substrates on Y. lipolytica dimorphism are controversial. Some researchers reported that single cells predominated in culture of Y. lipolytica grown on glucose or fat (Rodríguez & Domínguez, 1984; Zinjarde et al., 1998; Papanikolaou et al., 2002), whereas others reported that when Y. lipolytica was cultivated on the above media, mycelia were formed (Oswal et al., 2002; Ruiz-Herrera & Sentandreu, 2002; Papanikolaou et al., 2007). Nevertheless, in none of these studies was the effect of either DOC or carbon source concentration considered. Papanikolaou et al. (2009) is the only study reporting that when Y. lipolytica was cultivated on high glucose concentration media (in which high oxygen uptake occurs, resulting in low DOC) mycelia were formed, whereas under similar aeration conditions single cells predominated at low initial glucose concentration (conditions allowing establishment of high DOC), which is in agreement with the experimental results reported in the current paper.

Besides the critical role of DOC on dimorphism, several factors, such as citric acid, N-acetylglucosamine and serum,
have been reported to induce the formation of mycelia (Rodríguez & Domínguez, 1984; Pollack & Hashimoto, 1987; Guevara-Olvera et al., 1993; Pérez-Campo & Domínguez, 2001; Ruiz-Herrera & Sentandreu, 2002). Also, it is not only the nature of the carbon source that causes yeast transition from cells to hyphae and vice versa, but this behaviour might be strain dependent or act synergistically with other parameters (pH, nitrogen source, buffer used, etc.) (Rodríguez & Domínguez, 1984; Guevara-Olvera et al., 1993; Pérez-Campo & Domínguez, 2001). In the current study, we found that the nature and concentration of the nitrogen source did not affect yeast morphology, as single cells predominated under highly aerated conditions, but this is in contrast to Szabo & Štofaníková (2002) who reported that organic nitrogen is responsible for the establishment and the maintenance of the mycelial form both in liquid and in solid media.

When Y. lipolytica was cultivated on slides (solid cultures) in which low oxygen conditions were established by the addition of a coverslip, the yeast formed pseudomycelia and true mycelia, compared with cultures in which aeration was not restricted. Nonetheless, even in this case oxygen was of importance, and the restricted oxygen diffusion at the centre of the culture (Kamath & Bungay, 1988) may explain the morphological diversity of Y. lipolytica observed at the centre and the periphery of the slide culture. Similar results were obtained by Ruiz-Herrera & Sentandreu (2002), when Y. lipolytica was cultivated in capillary tubes under semi-anoxic conditions resulting in the formation of extremely long hyphae.

The above conclusions concerning the effect of DOC on morphological transition are confirmed by the experimental results obtained in continuous culture carried out at a dilution rate of 0.032 h⁻¹. Indeed, at low steady-state DOC true mycelia were mainly formed, during the transition from low to high DOC pseudomycelia and single cells became progressively predominant, whereas when high steady-state DOC conditions were established in the bioreactor, the culture consisted mainly of single cells and a few pseudomycelia. Although no other observations have been reported for the effect of DOC on Y. lipolytica morphology, some reports have correlated oxygen with dimorphism in other dimorphic yeasts. In continuous culture of Debaryomyces hansenii and Kluyveromyces marxianus grown at low dilution rates and restricted aeration, the yeast-to-mycelia transition was correlated with low DOC (Cruz et al., 2000; O’Shea & Walsh, 2000). By contrast, Walker & O’Neill (1990) indicated that
mycelial forms of K. marxianus were established with an increase of the dilution rate under aerobic conditions. However, specific growth rate is linearly correlated with oxygen uptake, resulting in low DOC in the growth environment. Indeed, the aerobic conditions referred to by Walker & O’Neill (1990) could have been partially anaerobic, as indicated by the presence of ethanol. Thus, in the majority of previous studies concerning dimorphism, DOC was probably not considered.

In fermentation processes productivity is often influenced by the availability of a substrate, for example oxygen (Gomes et al., 2007). As DOC is of high importance for microbial growth, the transition of yeast from single cells to mycelia indicates stress conditions. In these terms, the effect of oxygen was also examined with regard to physiology of the yeast and its ability to grow, accumulate lipids and/or produce citric acid. In batch cultures, Y. lipolytica produced almost 5 g biomass l\(^{-1}\), close to that reported by Makri et al. (2010) when repeated batch cultures of Y. lipolytica on glycerol were established at high DOC, but significantly lower than biomass produced by Y. lipolytica when cultivated on industrial glycerol in batch and repeated batch cultures (Rymowicz et al., 2010; Rywińska & Rymowicz, 2010). Lipid accumulation did not exceed 7% (w/w) lipids in dry biomass, whereas citric acid production reached 12 g l\(^{-1}\). This citric acid yield was significantly lower than that reported by Kamzolova et al. (2011), Rywińska et al. (2012) and Morgunov et al. (2013) who demonstrated that high aeration was required for citric acid production by Y. lipolytica strains cultivated on raw glycerol. Aeration also affected isocitric acid and citric acid synthesis by Y. lipolytica strains cultivated on rapeseed oil as sole carbon source (Kamzolova et al., 2013). Specifically, threo-Ds-isocitric acid production increased significantly under sufficient aeration, whereas low aeration (i.e. 5–10%) limited both isocitric acid and citric acid production and induced the formation of pseudomycelia instead of rounded single cells observed at 50–55% DOC. Besides citric acid, low aeration appears to influence the synthesis of other organic acids (i.e. \(\alpha\)-ketoglutaric acid) (Fürster et al., 2007; Kamzolova et al., 2012; Kamzolova & Morgunov, 2013).

In continuous cultivation mode at both low and high DOC, biomass produced was lower than reported by Papanikolaou & Aggelis (2002) where Y. lipolytica was cultivated on industrial glycerol at various dilution rates.
Citric acid increased slightly with an increase of DOC, which is in accordance with Rane & Sims (1994) who correlated the production of significant amounts of citric acid with an increase of DOC, although several researchers reported that citric acid production is independent of DOC (Okoshi et al., 1987; Rane & Sims, 1995; Kamzolova et al., 2003). By contrast, an increase in DOC has been found to enhance lipid accumulation processes (Choi et al., 1982).

The restricted lipid accumulation at low steady-state DOC compared with that at high DOC could be attributed to the restricted oxygen supply as some authors have reported (Bati et al., 1984; Papanikolaou et al., 2002). In the present study, lipid biosynthesis was also investigated in terms of the activity of enzymes implicated in lipogenesis. The results indicate that although glycerol assimilation was not significantly affected by the increase of DOC (as indicated by the increase of citric acid), the activities of enzymes involved in the production of lipids were significantly increased at high DOC.

**Table 1.** Biomass, lipid and citric acid production and activities (mean ± SD) of key enzymes in *Y. lipolytica* growing under steady-state conditions (dilution rate, 0.032 h⁻¹) at low (0.1 mg l⁻¹) and high (1.5 mg l⁻¹) DOC

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<tr>
<th>DOC</th>
<th>Dry biomass (g l⁻¹)</th>
<th>Lipids in dry biomass (%)</th>
<th>Citric acid (g l⁻¹)</th>
<th>Enzymes (U g⁻¹ DW)*</th>
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<tr>
<td>Low</td>
<td>4.8 ± 0.51</td>
<td>6.7 ± 2.1</td>
<td>0.4 ± 0.09</td>
<td>ICDH: 6.31 ± 1.8</td>
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<td>ATP-CL: 95.29 ± 11.37</td>
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<td>ME: 5.89 ± 0.83</td>
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<td>GD: 3.18 ± 0.14</td>
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<td>GK: 27.5 ± 5.03</td>
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<tr>
<td>High</td>
<td>4.5</td>
<td>10.3</td>
<td>2.1</td>
<td>ICDH: 2.5 ± 0.84</td>
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<td>ATP-CL: 1357.64 ± 26.31</td>
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<td>ME: 137.74 ± 20.21</td>
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<td>GD: 1.27 ± 0.54</td>
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<td>GK: 9.98 ± 2.26</td>
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</table>

*ICDH, NAD⁺-dependent isocitrate dehydrogenase; ATP-CL, ATP-citrate lyase; ME, malic enzyme; GD, NAD⁺-dependent glycerol-3-phosphate dehydrogenase; GK, glycerol kinase.
by the activity of both GD and GK), the activity of the enzymes ATP-CL and ME increased at high DOC, resulting in upregulation of lipid metabolism and this fact, in combination with the low ICDH activity observed at high DOC, predetermined favourable conditions for lipid accumulation.

In conclusion, we have shown that the major parameter affecting Y. lipolytica dimorphism, cultivated under various cultivation modes, was the DOC in the growth environment, and its effect was independent of the nature of both the carbon and the nitrogen sources. DOC was also shown to have an important role in yeast physiology and metabolic behaviour, revealing that this factor should be taken into consideration in various yeast applications.

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