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Quantitative image analysis as a tool for *Yarrowia lipolytica* dimorphic growth evaluation in different culture media

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ABSTRACT

Yarrowia lipolytica, a yeast strain with a huge biotechnological potential, capable to produce metabolites such as γ-decalactone, citric acid, intracellular lipids and enzymes, possesses the ability to change its morphology in response to environmental conditions. In the present study, a quantitative image analysis (QIA) procedure was developed for the identification and quantification of *Y. lipolytica* W29 and MTLY40-2P strains dimorphic growth, cultivated in batch cultures on hydrophilic (glucose and *N*-acetylglucosamine (GlcNAc) and hydrophobic (olive oil and castor oil) media. The morphological characterization of yeast cells by QIA techniques revealed that hydrophobic carbon sources, namely castor oil, should be preferred for both strains growth in the yeast single cell morphotype. On the other hand, hydrophilic sugars, namely glucose and GlcNAc caused a dimorphic transition growth towards the hyphae morphotype. Experiments for γ-decalactone production with MTLY40-2P strain in two distinct morphotypes (yeast single cells and hyphae cells) were also performed. The obtained results showed the adequacy of the proposed morphology monitoring tool in relation to each morphotype on the aroma production ability. The present work allowed establishing that QIA techniques can be a valuable tool for the identification of the best culture conditions for industrial processes implementation.

1. Introduction

Yarrowia lipolytica is an obligate aerobic non-conventional yeast which has been gaining an unquestionable interest in its exploitation in many biotechnological and environmental applications. This yeast is also a dimorphic microorganism capable to grow in two distinct morphotypes, usually as yeast single cells or as filamentous hyphae, being the two morphotypes reversible. It is believed that the dimorphism of *Y. lipolytica*, as well as other species, provides a mechanism for responding to adverse conditions (Kawasse et al., 2003). Naturally, *Y. lipolytica* grows as a mixture of yeasts and short mycelial cells. Nevertheless, it can grow as a quasi-homogeneous population of either yeast-like cells or hyphae, under controlled conditions (Guevara-Olvera et al., 1993; Rodríguez and Domínguez, 1984; Ruiz-Herrera and Sentandreu, 2002). The role of different factors has been previously described in dimorphic transition of *Y. lipolytica*, such as carbon and nitrogen sources (Rodríguez and Domínguez, 1984; Ruiz-Herrera and Sentandreu, 2002; Szabo and Stofanikova, 2002) and medium pH (Ruiz-Herrera and Sentandreu, 2002). Indeed, *Y. lipolytica* transition from yeast to hyphae morphotype was detected in the presence of casein, olive oil, *N*-acetylglucosamine, citrate and serum (Guevara-Olvera et al., 1993; Kawasse et al., 2003; Kim et al., 2000; Pérez-Campo and Domínguez, 2001; Ruiz-Herrera and Sentandreu, 2002; Ota et al., 1984; Zinjarde et al., 1998). However, the critical factor affecting the transition of *Y. lipolytica* from single cells to hyphae, independently of the nature of carbon and/or nitrogen source used, seems to be the dissolved oxygen concentration. Under highly aerated conditions, *Y. lipolytica* cultures consisted of single cells, by contrast low aeration conditions induced hyphae forms (Bellou et al., 2014). Furthermore, also environmental conditions affect the dimorphism in *Y. lipolytica* such as the medium pH, as it has been reported that hyphae formation is maximal at pH near neutrality and decreases as pH lowers to become almost null at pH 3, with citrate as an important positive effector of hyphae formation (Ruiz-Herrera and Sentandreu, 2002). An important progress has been made recently, with the identification of the genes playing a crucial role in *Y. lipolytica* dimorphism: genes involved in the mitogen-activated protein kinase and protein kinase A pathways, Rho family, among others (Cervantes-Chávez et al., 2009; Hurtado and Rachubinski, 1999; Martinez-Vazquez et al., 2013; Morales-Vargas et al., 2012).

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The morphogenetic shift of *Y. lipolytica* has a practical importance when biotechnological applications are designed. In fact, this wide morphological change greatly affects fermentation behavior, since it induces rheological changes and consequently leads to mass and heat transfer changes in the bioreactor (Coelho et al., 2010). It is known that *Y. lipolytica* is extensively used for enzyme production, lipids accumulation and aromatic compounds production, although the processes productivity is strongly affected by the cells morphology (Coelho et al., 2004).

In the last few years, microscopic examination has been ever more useful for determining the biomass structure, composition, and contents. Coupled to microscopy, the technological evolution and advances in digital image acquisition and computer processing capabilities allow for a fast and efficient characterization of the process biomass. Regarding yeasts characterization, solely a limited number of works can be found in the literature using quantitative image analysis (QIA). Coelho et al. (2004, 2007) developed automatic QIA procedures for *Saccharomyces cerevisiae* allowing the discrimination between budding and single cells, as well as the determination of cell size distribution under different experimental conditions. These results showed the potential of QIA to study the physiological state assessment and cell division analyses of yeast cell cultures used in industrial processes. Braga et al. (2015a) also used QIA to study the influence of mechanical and pneumatic agitation in the *Y. lipolytica* morphology, during γ-decalactone production in a stirred tank reactor (STR) and in an airlift bioreactor. However, up to the present, QIA has never been applied for characterizing dimorphic growth and/or transition of *Y. lipolytica* in different carbon sources.

This research is mainly focused on the development of a QIA procedure for the automatic identification and quantification of yeast (oval) single cells and (filamentous) hyphae cells of *Y. lipolytica* due to dimorphic transition using *N*-acetylglucosamine, glucose, castor oil, and olive oil as carbon sources. Finally, the obtained image analysis data was used to identify the best culture conditions for industrial processes implementation.

2. Experimental procedures

2.1. Microorganism

The strains used in this work were *Y. lipolytica* wild type (WT) strain W29 (ATCC20460) (Barth and Gaillardin, 1996) and its derived mutant with modifications in the lipid metabolism at the peroxisomal β-oxidation pathway (deleted for acyl-CoA oxidase encoding genes and overexpressing *POX2*) - MTLY40-2P strain (*Δpox2 Δpox3 Δpox4 Δpox5*+ p*POX2*–*POX2*), that was proven to produce high levels of γ-decalactone from castor oil (Braga et al., 2015b; Groguenin et al., 2004).

2.2. Dimorphic transition

Dimorphic transition was achieved essentially as described by Guevara-Olvera et al. (1993). Cells were cultured for 48 h on YPDA medium (agar 30 g L^{-1}, glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}) at 27 °C to inoculate (cell density of 0.5 g L^{-1}) a 500 mL baffled Erlenmeyer flask containing 200 mL of glucose medium (YPD medium: glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}). Cultures were incubated overnight at 27 °C, 140 rpm. Cells were centrifuged, re-suspended in the same volume of sterile distilled water, agitated for 3 h at 27 °C, centrifuged, washed once with sterile distilled water, and re-suspended in a small volume of sterile distilled water. Cell suspensions were stored at 4 °C overnight and further used as inoculum in experiments to study the dimorphic transition of *Y. lipolytica*. Cells kept at 0 °C in an ice bath were inoculated in 100 mL of medium (ca. 1 g L^{-1}), dispensed in 250 mL Erlenmeyer flasks, and maintained at 37– 38 °C. For the dimorphic transition YNB medium (0.67% YNB (yeast nitrogen base) without amino acids/ammonium sulfate, 0.5% ammonium sulfate, 1% carbon source and 100 mM citrate buffer pH 7) was used. After 10–15 min, the Erlenmeyer flasks were incubated at 27 °C, 170 rpm for about 12 h, the optimal period for hyphae formation (Guevara-Olvera et al., 1993).

2.2.1. Effect of different carbon sources on dimorphic transition

Previously, it was described that hyphae formation was obtained when GlcNAc was used as carbon source (Guevara-Olvera et al., 1993; Rodríguez and Domínguez, 1984). Therefore, we compared hyphae formation in hydrophilic (glucose and GlcNAc) and hydrophobic (olive oil and castor oil) substrates. In the experiments with hydrophobic substrates, the medium was emulsified with 0.1% Triton X and YPD medium (pH 6.5, without citrate buffer) was used as control.

2.3. Samples preparation for image acquisition

In the dimorphic transition experiments, three biological replicates were prepared for each condition. From each cultured medium, a 1 mL sample was collected every two hours until 12 h, for the implementation of a QIA procedure in bright-field microscopy. These samples were further centrifuged at 5000 rpm for 5 min, the supernatant was discarded and the pellet cells were washed, re-suspended with distilled water and centrifuged using the same conditions. This procedure was repeated three times in order to remove the lipidic material (Braga et al., 2015a). The pellet cells were then re-suspended in 1 mL of distilled water and then visualized in bright-field microscopy.

2.3.1. Bright-field image acquisition

A recalibrated micropipette with a sectioned tip at the end, with a large enough diameter to allow larger cells and cell aggregates to flow, was used to deposit samples on slides. Three slides per sample were used, and for each slide a volume of 10 μL was covered with a 20 mm × 20 mm cover slip for visualization and image acquisition. Images were acquired in the upper, middle, and bottom of the slide resulting in a total of 150 images (3 × 50 images/slide). The slides were examined by means of an Olympus BX51 optical microscope (Olympus, Tokyo, Japan), at 100× total magnification, coupled with an Olympus DP72 camera (Olympus, Tokyo, Japan). Images were acquired at 1360 × 1024 pixels and 8-bit format through the commercial software CellB̂ (Olympus, Tokyo, Japan).

2.3.2. Bright-field image processing and analysis

The yeast and hyphae morphological descriptors were determined through the use of image processing and analysis programs developed in Matlab 7.3 (The Mathworks, Inc., Natick) language. The main stages of the image processing program comprise the image pre-processing, segmentation, hyphae and yeast cells recognition, and image post processing steps. Finally, the yeast and hyphae cells binary images were saved and later used for their contents and size characterization in the QIA parameters determination step as described below.

A more detailed description of the image processing methods is next presented.

2.4. Image pre-processing

The image pre-processing stage depends on the enhancement of the grayscale images by background determination and background removal. In this stage, the original image is first divided by a background image to minimize background light differences. The

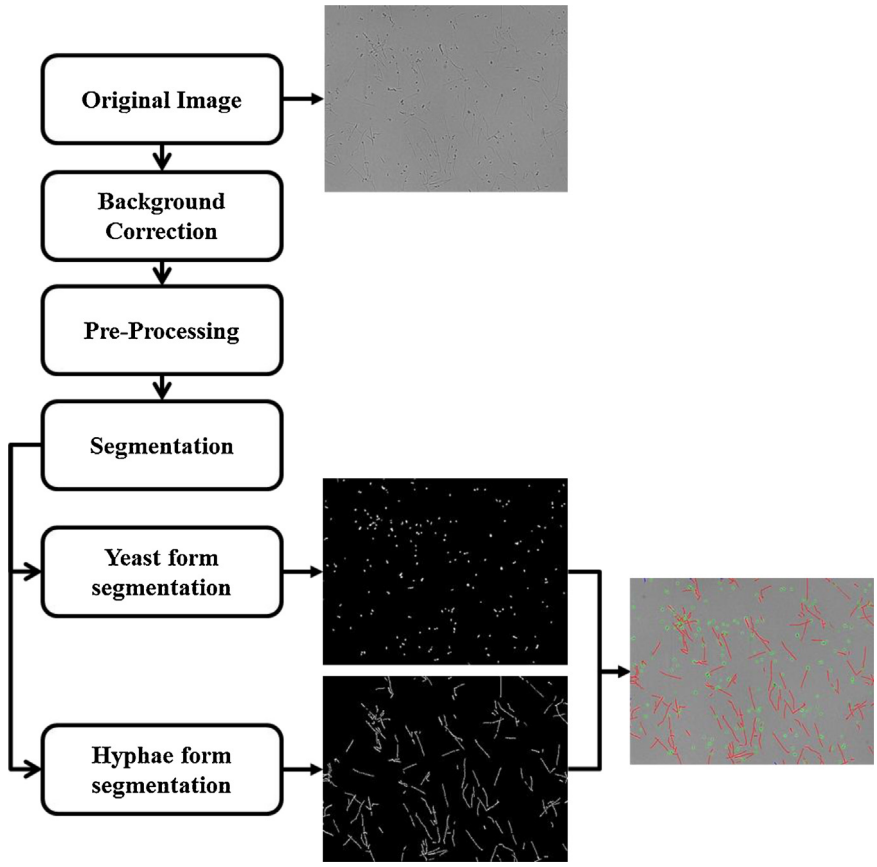

Fig. 1. Schematic representation of the QIA methodology.

resulting background corrected image is further enhanced by using a series of closing and opening morphological operators in order to improve the contrast of the cells boundaries.

2.4.1. Segmentation

This stage consists primarily on the yeast and hyphae morphotype recognition through segmentation by a predefined 0.9 threshold value. Then the resulting binary image is processed in order to fill small holes (6 pixels, or approximately 4.5 μm, in diameter) in the interior of the cells. A native Matlab function is used for that purpose.

2.4.2. Hyphae and yeast cells recognition

Next, the hyphae morphotype is determined taking into account a combined width and morphology approach. In that sense, the objects presenting a width bellow 9 μm are recognized by a series of erosion and dilation morphological operations. From these, the objects presenting a gyration radius (Pons and Vivier, 1998) above 1 are considered as hyphae cells. The remaining objects, if above 3.5 μm in diameter, are considered as yeast cells or cell aggregates.

2.4.3. Image post processing

Finally, all the objects cut off by the image boundaries are removed by a native Matlab function and the final yeasts and hyphae cells binary images are saved (Fig. 1).

2.4.4. QIA parameters determination

Following the image processing step, the yeasts and hyphae cells binary images were analyzed in order to characterize both morphotypes in terms of their contents and most relevant size parameters. In all cases, a calibration factor of 0.7383 μm pixel^{-1} (determined by the use of a micrometer slide) was employed. The studied parameters are further described below.

The total yeast area per volume (TY/Vol) and total hyphae length per volume (TH/Vol) were determined for each replicate as the sum of all yeast areas per volume and the sum of all hyphae lengths per volume, respectively. The total yeast area (TY) represents the projected area of all yeast cells onto the image plane, whereas the total hyphae length (TH) was determined by a QIA morphological thinning procedure to a 1 pixel width of all hyphae cells. Upon the determination of TY in μm^2 and TH in μm, by the use of the calibration factor, these values were divided by the corresponding volume of each image. For each replicate a total of 150 images (50 images × 3 slides) was used. Finally, for each sample, the average value of the 3 replicates was then determined. Furthermore, the total hyphae length per total yeast area ratio (TH/TY) was also determined.

2.5. Biotransformation experiments

In order to evince the importance of cell morphology monitoring in bioprocesses, γ-decalactone production by *Y. lipolytica* in two different morphotypes (yeast or hyphae cells) was evaluated. MTLY40-2P strain was employed since it was proven to produce high levels of γ-decalactone from castor oil and methyl ricinoleate, due to severely decreased short-chain fatty acid degradation and enhanced long-chain fatty-acid β-oxidation through the overexpression of Aox2p (Braga et al., 2015b; Groguenin et al., 2004). For this purpose, two different experiments were conducted. In the first experiment cells were employed without previous dimorphic induction. Cells from YPDA medium were used to inoculate a pre-growth culture (200 mL of YPD medium) with cell den-

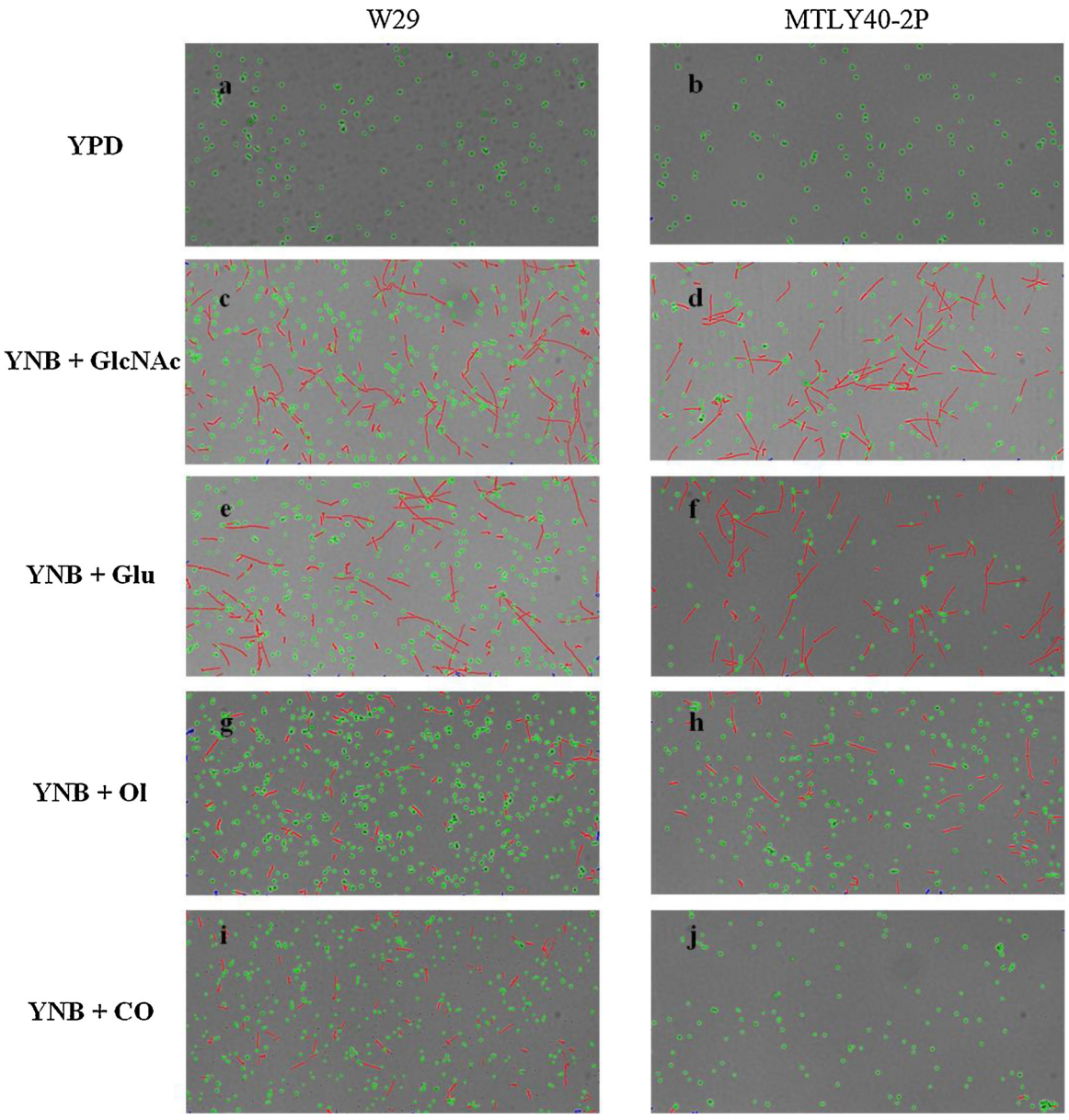

Fig.2. Images obtained at the end of each experiment (12 h), for each studied carbon source and strain, after QIA (hyphae in red, yeasts in green). The culture medium used for dimorphic transition was the YNB medium (pH 7.0 with 100 mM citrate buffer) containing each indicated carbon source, such as glucose (Glu), *N*-acetylglucosamine (GlcNAc), olive oil (Ol) and castor oil (CO). YPD medium (pH 6.5 without citrate) was used as control.

sity of 0.5 g L^{-1}, incubated overnight at 27 °C, 140 rpm. Cells were then centrifuged and used to inoculate 200 mL of the biotransformation medium (30 g L^{-1} castor oil, 6.7 g L^{-1} YNB with amino acids, 2.5 g L^{-1} NH_4Cl and 3 g L^{-1} Tween 80) with cell density of 0.5 g L^{-1}. In the second experiment, after selecting the best conditions for hyphae induction (medium with glucose), dimorphic transition was induced as previously described in Section 2.2. Then, the cells were centrifuged and used to inoculate 200 mL of the biotransformation medium with cell density of 0.5 g L^{-1}. Finally, samples were collected over time for γ-decalactone quantification, cell concentration and substrate consumption. γ-Decalactone was extracted from 2 mL samples with 2 mL diethyl ether and the organic phase was analyzed by gas chromatography (GC) as previously described by Braga et al. (2015a). Castor oil consumption was determined by fatty acids methyl esters (FAME) analysis by GC (Braga, 2014).

3. Results and discussion

3.1. Yeast-to-hyphae transition by QIA

QIA is a powerful tool for microorganisms' characterization. Amongst other applications, QIA has been used to relate fermentation broth rheology to morphology (Cox et al., 1998), and study the impact of bioreactor agitation (mechanical or pneumatic) in yeast cells morphology (Braga et al., 2015a). However, QIA studies for morphological analysis of dimorphism microorganisms are still lacking.

The automatic identification and quantification of yeast (oval) single cells and (filamentous) hyphae cells of Y. *lipolytica* caused by the presence of different inducers in culture medium (YNB medium) was sought using QIA. The growth of *Y. lipolytica* strains showed a differential dimorphic behavior in response to the

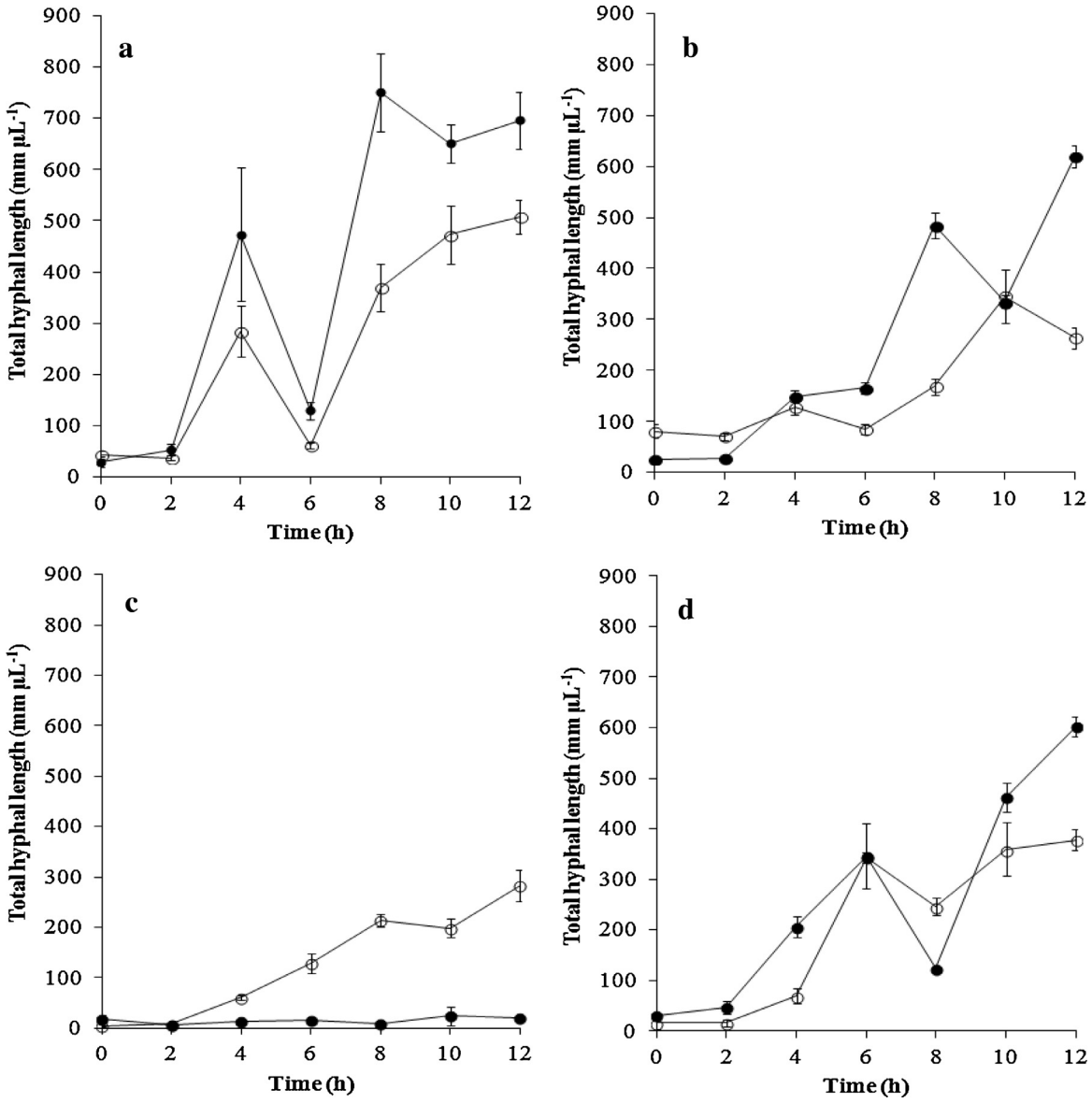

Fig. 3. Experimental behavior of total hyphae length for MTLY40-2P (●) and W29 (**O**) strains, with different carbon sources: (a) glucose (b) GlcNAc (c) castor oil (d) olive oil. Data are presented as average and standard deviation of three independent experiments.

presence of hydrophobic (olive oil and castor oil) or hydrophilic (GlcNAc and glucose) carbon sources (Fig. 2).

It has been observed that the capability to grow in hyphae morphotype is variable among different strains of *Y. lipolytica* (Barth and Gaillardin, 1997). During the present research, the same conclusion could be achieved over the global comparison between MTLY40-2P strain and the parental strain W29, as Fig. 2 demonstrates.

Regarding the final labeled images obtained by QIA methodology for the YPD medium, the common medium for yeast cells growth and in this work used as control, as shown in Fig. 2, cells were present in the yeast morphotype (in green) throughout the experiments for both strains, indicating that YPD is not able to induce dimorphic transition, as expected.

When both strains were subjected to the presence of hydrophilic sources, such as glucose or GlcNAc, the dimorphic transition of *Y. lipolytica* cells was induced (Fig. 2c–f). This was clearly revealed by the QIA where the hyphae morphotype was identified as hyphae cells (in red). However, there are some discrepancies in the literature concerning the morphology of *Y. lipolytica* on different hydrophilic substrates under varied culture conditions. For instance, the growth of *Y. lipolytica* LGAM S(7) 1 was reported as being restricted to the yeast morphotype with glucose (Papanikolaou et al., 2002), whereas other reports have indicated that hyphae formation is triggered with glucose and other hydrophilic materials (Kim et al., 2000; Perez-Campo and Dominguez, 2001). On the other hand, GlcNAc was also described as a good inducer for dimorphic transition of *Y. lipolytica* (Guevara-Olvera et al., 1993; Rodríguez and Domínguez, 1984).

Additionally, when hydrophobic substrates were used, it was clearly observed at the end of the experiments that the MTLY40-2P strain and the parental strain W29 responded in a different manner. When olive oil (Ol) was used as carbon source, growth was promoted in the hyphae morphotype for both strains (Fig. 2g–h). On the other hand, with castor oil (CO) a suboptimal hyphae formation was induced (Fig. 2i–j) and higher amounts of the yeast morphotype were present. Comparing the results obtained after QIA analysis it was possible to observe a higher amount of red hyphae cells (in red) in the experiments with Ol contrasting with a predominance of yeast cells (in green) in CO experiments. This could be explained by the fatty acid composition of both oils. It is known that oleic acid

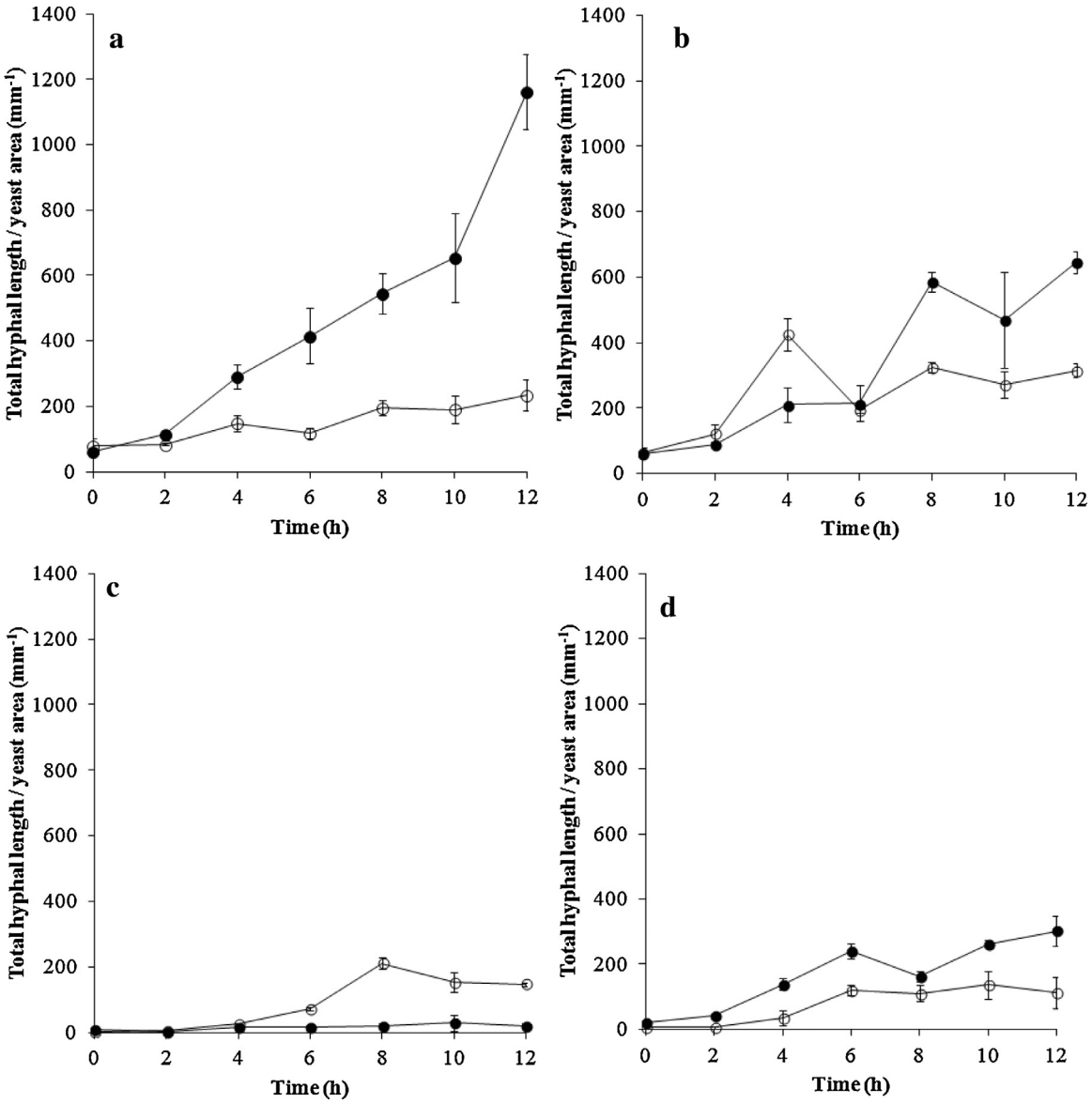

Fig.4. Experimental behavior of total hyphae length per total yeast area ratio for MTLY40-2P (●) and W29 (**O**) strains, with different carbon sources (a) glucose (b) GlcNAc (c) castor oil (d) olive oil. Data are presented as average and standard deviation of three independent experiments.

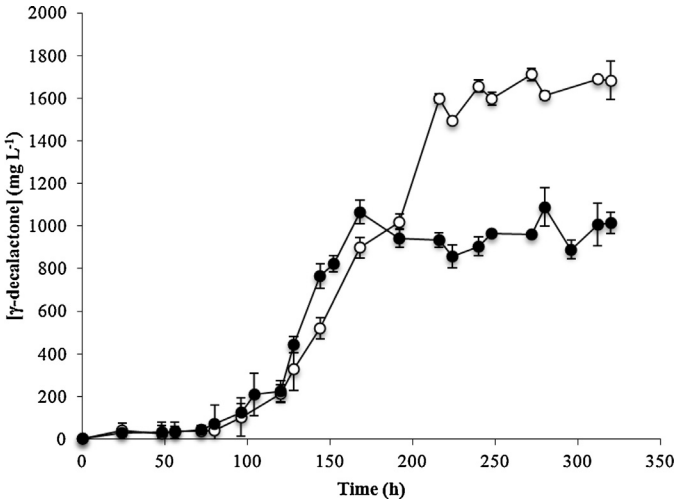

Fig. 5. Accumulation of γ-decalactone in the biotransformation medium with MTLY40-2P strain: (●) hyphae and (**O**) yeast form. Data are presented as average and standard deviation of two independent experiments.

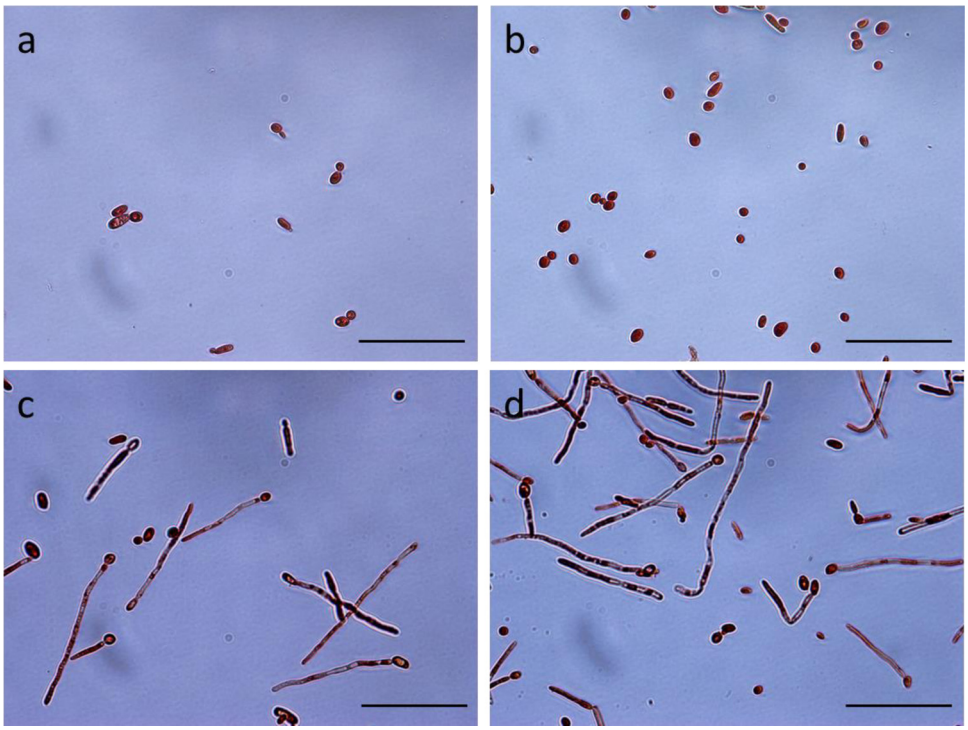

Fig. 6. *Yarrowia lipolytica* MTLY40-2P morphotypes during biotransformation: (a) oval cells at the beginning of the biotransformation (b) oval cells after 320 h (c) hyphae cells at the beginning of the biotransformation (d) hyphae cells after 320 h. The scale bar represents 50 μm.

is the main fatty acid in Ol and ricinoleic acid is the main fatty acid in CO (Puthli et al., 2006). Taking into account a literature review, most of previous studies using hydrophobic carbon sources for the transition from yeast to hyphae morphotype in *Y. lipolytica*, reported solely the use of oleic acid (Ofek et al., 1983; Ota et al., 1984). To the authors' knowledge, castor oil has never been described before as an inducer in studies concerning the dimorphic transition.

The analysis of total hyphae length per volume (TH/Vol), one of the most significant morphological parameter provided by QIA, showed that hyphae began to appear in about 4 h after transfer to the tested media with the different inducers (Fig. 3), with the exception of CO for the MTLY40-2P strain. Seemingly, the conversion of the yeast cells into hyphae morphotype is seen to occur from that time onwards, and after 12 h of induction TH/Vol is the highest (in most cases), indicating the largest hyphae morphotype contents.

For both strains, glucose was the best inducer for the dimorphic transition in the experimental conditions analyzed. A TH/Vol of 696.5 mm μL^{-1}, after 12 h (Fig. 3a), was obtained with glucose in the case of MTLY40-2P. The hyphae formation decreased 11% when glucose was replaced with GlcNAc to a TH/Vol of 619.2 mm μL^{-1} at the same time. In the case of the parental strain W29, a maximum TH/Vol of 506.9 mm μL^{-1} was reached, after 12 h, when glucose was used as carbon source, decreasing to 263.7 mm μL^{-1}, at the same time, when GlcNAc was used as the sole carbon source (Fig 3b). A similar behavior was observed in the experiments with Ol where TH/Vol of 601.9 mm μL^{-1} and 377.7 mm μL^{-1} where obtained, after 12 h, for MTLY40-2P and W29, respectively (Fig 3d). In the medium containing CO a slightly lower hyphae induction was observed (TH/Vol around 282.4 mm μL^{-1}, after 12 h) for the parental strain W29, whereas no significant hyphae induction was observed for MTLY40-2P strain (Fig. 3c).

In the case of *Y. lipolytica*, it has been reported that the morphological shift depends on the nature of the carbon and nitrogen sources, the environmental conditions (temperature, pH, oxygen), and the presence of serum or citrate, although, as previous described, there are discrepancies in literature concerning these topics (Domínguez et al., 2000; Pérez-Campo and Domínguez, 2001). However, it is known that *Y. lipolytica* normally switches morphology from yeast to hyphae in response to the presence GlcNAc, glucose or Ol in their growth medium (Novotony et al., 1994; Ota et al., 1984; Rodríguez and Domíinguez, 1984) as was observed in the present work.

Pons et al. (1993) reported one of first QIA applications on yeast single cell morphotype using a semi-automatic method for the characterization of yeast size and shape. In the present work our QIA methodology was able to automatically identify, characterize and quantify both yeast and hyphae morphological status. The key parameter "Total hyphae length per total yeast area ratio" (TH/TY) was obtained representing quantitatively the morphological cells shift (Fig. 4).

Fig. 4 shows a clear distinction between the hydrophobic and hydrophilic carbon sources, with a higher impact of the first in the dimorphic shift of both strains.

It was observed that with glucose (Fig. 4a) the TH/TY ratio was 1162 and 234.3 mm^{-1}, after 12 h, for MTLY40-2P and W29, respectively. Regarding GlcNAc experiments (Fig. 4b), a TH/TY ratio of 645.1 mm^{-1} for MTLY40-2P and 315.5 mm^{-1}, after 12 h, for W29 was achieved indicating a morphological shift from yeast to hyphae morphotype in both cases. Lower TH/TY ratios were obtained using hydrophilic carbon sources, showing the lower ability to induce morphological switches (Fig. 4c–d). In the medium containing CO (Fig. 4c), TH/TY ratios of 147.9 mm^{-1} and 20.1 mm^{-1}, after 12 h, were achieved for W29 and MTLY40-2P, respectively. An opposite behavior was observed in the experiments with Ol (Fig. 4d) where for MTLY40-2P a TH/TY ratio of 302.3 mm^{-1} was obtained, after 12 h, and for W29 a much lower TH/TY ratio of around 113.2 mm^{-1} was attained.

Taking into consideration the limited volume employed for the QIA methodology, some fluctuation throughout the experiment time could be observed (especially in Fig. 3). In fact, if the medium inside the Erlenmeyer is not completely homogenous at the sampling time, some samples may present larger biomass contents

than others (thus contributing to some of the observed fluctuation in TH/Vol). However this effect will be the same in both the determined total hyphae length and total yeast area and, thus, be minimized regarding the TH/TY parameter (shown in Fig. 4). In fact, a much lesser fluctuation is observed in Fig. 4, confirming the ability of the QIA procedure to monitor the yeast-to-hyphae dimorphic change.

Concluding, Fig. 3 shows that for both strains, the presence of glucose as carbon source leads to an increase in the ability of *Y. lipolytica* to form hyphae, indicating the high specificity of this sugar in the induction of hyphae formation. Comparing the capability of both strains for dimorphic transition, MTLY40-2P strain was found to produce higher amounts of hyphae under similar conditions, except for the CO medium.

Thus, it could be established by the QIA morphological characterization that hydrophobic carbon sources should be preferred for *Y. lipolytica* W29 and MTLY40-2P growth when the yeast morphotype is required. On the other hand, hydrophilic carbon sources, as glucose and GlcNAc, in controlled pH conditions, caused a dimorphic transition towards the hyphae morphotype in *Y. lipolytica* W29 and MTLY40-2P strains.

Finally, it should be stressed that the developed QIA methodology allowed the quantification and characterization of *Y. lipolytica* cells into different morphotypes, ranging from yeast (oval) single cells to (filamentous) hyphae cells, automatically identifying the size and morphology of both morphotypes and further quantifying the proportion of each morphotype.

3.2. Biotransformation experiments

In industrial fermentations, the cells morphology is strongly affected by the rheological properties of the medium, oxygen transfer rate and nutrients consumption rate (Walker, 1998). Furthermore, the cells behavior in fermentations is greatly affected by their morphology. Hence, the control and understanding of cell morphology in the bioreactor is of key importance and QIA techniques can allow the identification of the best culture conditions for industrial processes implementation.

Experiments for γ-decalactone production with MTLY40-2P strain in two distinct morphotypes (yeast single cells and hyphae cells) were, therefore, performed. Regarding the hyphae morphotype, before the biotransformation experiments a pre-induction step for dimorphic transition of MTLY40-2P strain cells was performed, using glucose as carbon source, in order to insure that the cells were mainly in the hyphae morphotype. The γ-decalactone productivity of the hyphae morphotype cells was then compared with the yeast single cells morphotype (Fig. 5).

The kinetic profile of γ-decalactone production was similar for both tested morphotypes: an initial lag phase of around 72 h was apparent, followed by an increase in the aroma production, attaining a maximum concentration plateau and exhibiting no significant decrease from then until the end of the experiment. This is a typical profile for this strain in batch cultures (Braga et al., 2015b; Groguenin et al., 2004). Nevertheless, according to the cell morphotype a different behavior was observed in the aroma production, with the cells in the yeast morphotype exhibiting higher γ-decalactone production than the hyphae morphotype (c.a. 1.5-fold). A similar production rate was observed during the first 168 h, but from then onwards a higher aroma concentration was obtained for the cells in the yeast morphotype. During the biotransformation experiments, the overall growth was relatively slow with cell concentration only slightly increasing throughout the experiment time (ranging from 3.2×10^8 to 1.2×10^9 cells mL^{-1}). Regarding microscopy examination, no significant morphological changes were observed during the course of the biotransformation experiments for each morphotype (Fig. 6). Furthermore, the QIA methodology allowed establishing a TH/TY ratio of 1370 (±816) mm^{-1} for the hyphae morphotype and of 8 (±7) mm^{-1} for the yeast morphotype, confirming that no significant morphological changes were observed during the course of the biotransformation experiments. Also, in both conditions, the medium pH increased from 5.5 to 7.5, at around 90 h, decreasing again to 5.5 at the experiment end and the substrate (castor oil) was largely consumed, to a final concentration below 3 g L^{-1}.

In fact, this work allowed to validate the adequacy and importance of cell morphology monitoring throughout dimorphic transition and biotransformation experiments, since differences in process productivity can be observed, as here demonstrated in γ-decalactone production by MTLY40-2P *Y. lipolytica* strain.

In conclusion, the use of QIA tools can be very helpful for cells morphological characterization and evaluation, during fermentation in industrial bioreactors, where cells are exposed to stress conditions, like pressure, oxygen limitations, mechanical shearing, that can lead to morphological alterations. Nevertheless, it is important to stress out that the cell morphological transition is strain dependent, thus being essential to study such behavior for each *Y. lipolytica* strain. Through microscopy and QIA techniques, a better understanding of cellular adaptation mechanisms can be achieved, and operational conditions adapted in order to reduce its impact in cells behavior.

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