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Candida albicans – a pre-whole genome duplication yeast – is predominantly aerobic and a poor ethanol producer

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molecular evolution; yeast progenitor; respiration; fermentation; obligate aerobes.

Abstract

Yeast species belonging to the lineage that underwent the whole genome duplication (WGD), and including *Saccharomyces cerevisiae*, can grow under anaerobiosis and accumulate ethanol in the presence of glucose and oxygen. The pre-WGD yeasts, which branched from the *S. cerevisiae* lineage just before the WGD event, including *Kluyveromyces lactis*, are more dependent on oxygen and do not accumulate large amounts of ethanol in the presence of excess oxygen. Yeasts that belong to the so-called 'lower branches' of the yeast phylogenetic tree and diverged from *S. cerevisiae* more than 200 million years ago have so far not been thoroughly investigated for their physiology and carbon metabolism. Here, we have studied several isolates of *Candida albicans* and *Debaryomyces hansenii* for their dependence on oxygen. *Candida albicans* grew very poorly at an oxygen concentration < 1 p.p.m. and *D. hansenii* could not grow at all. In aerobic batch cultivations, *C. albicans* exhibited a predominantly aerobic metabolism, accumulating only small amounts of ethanol (0.01–0.09 g g⁻¹ glucose). Apparently, *C. albicans* and several other pre-WGD yeasts still exhibit the original traits of the yeast progenitor: poor accumulation of ethanol under aerobic conditions and strong dependence on the presence of oxygen.

Introduction

Yeasts represent a very divergent group with respect to their carbon metabolism and oxygen requirements. They span from obligate aerobes, exhibiting only respiratory metabolism, to yeasts having respiro-fermentative metabolism, and to yeasts that acquire energy only from fermentation (Kurtzman & Fell, 1998). Several yeasts with an efficient alcoholic fermentation pathway are able to grow under severely oxygen-limited conditions (Merico *et al.*, 2007). Apart from efficient fermentation, the ability to grow in anaerobiosis involves several biochemical, genetic and physiological adaptations. Many usual biosynthetic pathways require oxygen, such as synthesis of heme, sterols, unsaturated fatty acids and pyrimidines (reviewed in Snoek & Steensma, 2007). Under anaerobic conditions, these compounds must therefore be added to the medium. Alternatively, oxygen-independent pathways must replace the oxygen-dependent ones, as for example the conversion of dihydroorotate to orotate in the uracil biosynthesis pathway (Nagy *et al.*, 1992). A majority of yeasts possess a

mitochondrial version of dihydroorotate dehydrogenase (DHODase) dependent on the respiratory chain, and are therefore unable to propagate in anaerobiosis in the absence of externally supplied uracil. *Saccharomyces cerevisiae* and its close relatives, known for their ability to grow in the absence of oxygen, have a cytoplasmic version of DHODase independent from the respiratory chain (Gojkovic *et al.*, 2004; Hall *et al.*, 2005). Moreover, of the approximately 500 *S. cerevisiae* genes whose expression differs when aerobic and anaerobic cultures are compared, 23 are essential for anaerobic growth and not essential for aerobic growth (reviewed in Snoek & Steensma, 2007). In *Kluyveromyces lactis*, a yeast species known to be unable to grow anaerobically (Kiers *et al.*, 1998), 20 of these genes are missing.

Some yeasts, such as *S. cerevisiae*, ferment in the presence of glucose even under aerobic conditions and this is the so-called 'Crabtree effect' (Pronk *et al.*, 1996). Other yeasts, such as *K. lactis*, which do not accumulate fermentation products in the presence of oxygen, are classified as Crabtree-negative yeasts (Kiers *et al.*, 1998). The molecular basis of the Crabtree

effect is complex and not yet fully understood. In *S. cerevisiae*, several evolutionary events, such as the whole genome duplication (WGD) (Wolfe & Shields, 1997; Conant & Wolfe, 2007), a global rewiring of the expression pattern of genes involved in respiration and fermentation (Ihmels et al., 2005) and the duplication of alcohol dehydrogenase gene (Thomson et al., 2005; Piskur et al., 2006) have been proposed to contribute to the molecular background necessary for the development of a strong Crabtree phenotype (Merico et al., 2007). All the analyzed post-WGD species are capable of efficient aerobic fermentation and can grow in the absence of oxygen. On the other hand, the analyzed pre-WGD species, belonging to lineages that separated from *S. cerevisiae* approximately 100 million years ago, exhibit a mosaic of different physiological strategies. A majority of them exhibit a Crabtree-negative phenotype and cannot grow under anaerobiosis. However, there are also several moderate Crabtree-positive species within this group, such as *Torulaspota delbrueckii* and *Torulaspota globosa*, and some others have also the ability to grow almost without oxygen, for example *Kluyveromyces waltii* and *Hanseniaspora osmophila* (Merico et al., 2007). This suggests that these two traits could have evolved multiple times during the yeast evolutionary history, but they are the most strongly expressed in the post-WGD yeasts and especially in *S. cerevisiae* (Merico et al., 2007).

While the *Saccharomyces/Kluyveromyces* yeasts have been receiving considerable attention and their carbon metabolism has been studied under well-defined conditions, some 'lower branches' on the yeast phylogenetic tree, which separated from the *S. cerevisiae* lineage more than 200 million years ago (Kurtzman & Piskur, 2006), have not yet been thoroughly characterized. Several recent papers report on global approaches to understand the transcriptional regulation of carbon metabolism in *Candida albicans* (Setiadi et al., 2006; Askew et al., 2009; Rodaki et al., 2009). However, so far, not even a single published study on *C. albicans* and its growth properties under fully controlled growth conditions is available. To better characterize the lifestyle of this yeast, and to understand the evolution of carbon metabolism and the ability to survive without oxygen, we studied the growth and glucose metabolism of several *C. albicans* isolates under anaerobic and aerobic conditions.

Materials and methods

Yeast strains

The yeast species analyzed in this study are summarized in Table 1.

Agar plates and liquid media

All yeast cultures were stored at -80°C . From the stock, the cells were transferred to YPD plates (peptone, 20 g L^{-1} ; yeast

Table 1. Yeast strains used in this study

Species	Strain designation	Laboratory designation	Origin
<i>D. bruxellensis</i>	CBS 2499	Y879	CBS collection
<i>D. bruxellensis</i>	CBS 4914	Y906	CBS collection
<i>C. albicans</i>	NRRL Y12983	Y1394	C. Kurtzman,
<i>C. albicans</i>	NRRL Y17967	Y1395	Agricultural
<i>C. albicans</i>	NRRL Y17976	Y1396	Research Service, Peoria, IL
<i>D. hansenii</i>	CBS 767	Y1399	L. Jespersen,
<i>D. hansenii</i>	D18335	Y1398	University of
<i>D. hansenii</i>	MD02	Y1402	Copenhagen,
<i>D. hansenii</i>	MD04	Y1404	Denmark
<i>S. cerevisiae</i>	CBS 8430	Y1623	CBS collection
<i>K. lactis</i>	CBS 2359	Y1376	CBS collection

CBS stands for the Centraal Bureau voor Schimmelcultures in Utrecht, the Netherlands. NRRL stands for Agricultural Research Service Culture Collection (Peoria, IL). Y indicates the Piskur yeast collection at the Lund University, Sweden.

extract, 10 g L^{-1} ; glucose, 20 g L^{-1} ; agar, 20 g L^{-1}). Fresh colonies were used for the preparation of precultures in defined synthetic minimal medium with the final concentrations of the components as specified in Merico et al. (2007). The aerobic batch cultures were grown in the same minimal medium. The medium for anaerobic batch cultures was additionally supplemented with ergosterol (10 mg L^{-1}), Tween 80 (420 mg L^{-1}) and uracil (50 mg L^{-1}).

Anaerobic plate test

The plates for the anaerobic test included YPD, minimal medium (glucose, 20 g L^{-1} ; yeast nitrogen base without amino acids, 6.7 g L^{-1}) and minimal medium supplemented with peptone (1 g L^{-1}). The minimal media for the anaerobic test were also supplemented with uracil (50 mg L^{-1}), ergosterol (10 mg L^{-1}) and Tween 80 (420 mg L^{-1}). Cells grown in liquid YPD were harvested during the exponential phase, washed and suspended in distilled water. 500 and 5000 cells were spotted on the respective plates and were grown anaerobically for 2 weeks. The anaerobic environment was established using the Anaerocult A system (cat. no. 1138290001 and 116387) with Anaerotest strips (cat. no. 115112) from Merck and the environment should contain $< 1\text{ p.p.m. O}_2$. The assembly was prepared and the growth was followed according to Merck instructions. Each plate included the positive and negative controls, *S. cerevisiae* and *K. lactis*, respectively.

Aerobic and anaerobic batch cultivations

Precultures (100 mL) for aerobic and anaerobic batch experiments were grown in a defined synthetic minimal medium (Merico et al., 2007) in 500-mL flasks at 30°C with the aeration maintained by stirring at 200 r.p.m. in a rotative

shaker. Cells were grown until the exponential phase and used for inoculation of the batch at an initial concentration ranging from 0.15 to 0.3 OD_{600 nm}.

Aerobic batch cultivations were performed using the Infors HT Multifors system (Infors HT, Bottmingen, Switzerland) equipped with the silicone tubing. The working volume was 0.5 L. An air flow was maintained at 0.5 L min⁻¹. Dissolved oxygen concentration > 30% of the air saturation was maintained by automatic regulation of the stirring speed from 200 to 1200 r.p.m. and was measured using a Mettler Toledo polarographic oxygen probe. The temperature was maintained at 30 °C and the pH, measured using a Mettler Toledo pH electrode, was adjusted at 5.0 by the automatic addition of 2 M KOH or 1 M H₂SO₄. Foaming in the batch cultures was controlled by the automatic addition of an antifoaming agent (Antifoam 204, Sigma, prod. no. A6426). The medium used for batch cultivations was the same as that used for preparing the precultures.

Anaerobic batch cultivations of two *C. albicans* strains Y1395 and Y1396 were performed in the Biostat-Q system (B-Braun, Sartorius BBI Systems Inc., Bethlehem, PA) with a working volume of 0.8 L. The bioreactor was flushed with nitrogen (< 3 p.p.m. O₂) at a flow rate of 0.1 L N₂ min⁻¹ L⁻¹ medium. The stirring was maintained constant at 500 r.p.m. Norprene tubing (Cole-Palmer, General Control, Milan, Italy) was used to minimize the diffusion of oxygen into the bioreactor. The pregrown aerobic cultures were collected and suspended in 1–2 mL of distilled water to reduce the amount of oxygen introduced into the batch during the inoculum. For the anaerobic batch cultivations, the synthetic minimal medium was supplemented with 10 mg L⁻¹ ergosterol, 420 mg L⁻¹ Tween 80 and 50 mg L⁻¹ uracil. Ergosterol and Tween 80 were both dissolved in 98% ethanol, resulting in the initial concentration of 3 g ethanol L⁻¹ when added to the medium.

Cell density, metabolites and dry weight measurements

Samples for the biomass measurements and for the metabolites' production or consumption were taken at certain intervals during the exponential growth. Cell density was monitored by measuring the OD_{600 nm}. For dry weight determination, washed culture samples were filtered through a 0.45-µm-glass microfiber GF/A filter (Whatman) and dried for 24 h at 80 °C. The withdrawn cells were spun down and the supernatant was frozen at -20 °C. Afterwards, the metabolite concentrations in the thawed supernatants were measured. In the samples from anaerobic Y1395 and Y1396 cultivations, the concentrations of glucose, ethanol, glycerol and acetic acid were determined using R-Biopharm (Roche) enzymatic kits (cat. no. 10716251035, 10176290035, 10148270035 and 0148261035, respectively). The concentrations of glucose, ethanol, glycerol and acetic

acid in the supernatants arising from aerobic cultivation of Y1394, Y1395 and Y1396 were determined by HPLC using an Agilent ChemStation 1200 Series system with the Bio-Rad Aminex HPX-8H column and the refraction index detector (Agilent Technologies G1362A) together with a variable length detector (Agilent Technologies G1314B). The samples were filtered before applying on the column to remove the contaminating cells. The column and the detectors were heated to 55 °C and the mobile phase was 0.005 M H₂SO₄ with a flow rate of 0.6 mL min⁻¹. The specialized software allowed the calculation of the area of the detected peaks. The calibration curve was prepared for glucose, ethanol, glycerol and acetic acid with concentrations ranging from 2 to 20 g L⁻¹, 0.58 to 5.8 g L⁻¹, 0.5 to 5 g L⁻¹, and 0.52 to 5.2 g L⁻¹, respectively. The chemicals used for calibration curve preparation were purchased from Sigma-Aldrich. The dry weight was determined by filtering 5 mL of cell suspension from the batch culture using a 0.45-µm-glass microfiber GF/A filter (Whatman, Biomap, Milan, Italy). The parallel samples were dried for 24 h at 80 °C. Samples from any batch cultivation were analyzed in duplicate.

Calculations

The specific consumption rates of glucose and the specific production rates of ethanol, glycerol and acetic acid were calculated during the exponential phase of growth. The yields of ethanol and other metabolites relative to the consumed carbon source were calculated as the total amount of produced metabolites divided by the total amount of sugar utilized.

Results

Growth under strict anaerobic conditions

The ability of *C. albicans* and *Debaryomyces hansenii* strains to grow under strict anaerobic conditions was firstly checked on plates with synthetic minimal medium and on the same minimal medium supplemented with peptone using the Anaerocult A system from Merck. This system ensures anaerobic conditions by applying an oxygen-binding reaction mixture that produces carbon dioxide in a closed jar (the oxygen concentration was expected to be < 1 p.p.m.). Three positive and negative controls, *S. cerevisiae* and *Dekkera bruxellensis* can grow anaerobically and *K. lactis* is unable to grow in the absence of oxygen, confirmed that our system worked properly. *Candida albicans* showed a considerably reduced growth on both minimal medium and minimal medium supplemented with peptone, as determined after 7 and 14 days of incubation (Fig. 1). Also on rich medium, the growth was considerably reduced compared with the positive control (data not shown). Under the same conditions, *D. hansenii* strains were not able to grow at

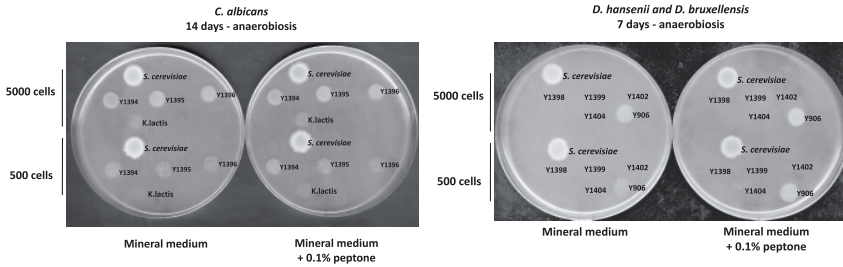


Fig. 1. Anaerobic growth test of different yeasts. 5000 and 500 cells were spotted on the minimal medium and on the same medium supplemented with peptone, and incubated for 7 and 14 days under an anaerobic environment created by the Anaerocult A system. *Kluyveromyces lactis* Y1376 was used as a negative control because it cannot grow in the absence of oxygen. *Saccharomyces cerevisiae* Y1623 was used as a positive control. (a) Strains of *Candida albicans* showed very little growth on the mineral medium plates with and without peptone and the cells did not reach the same cell density as *S. cerevisiae*. The *C. albicans* strains: Y1394, Y1395, Y1396. (b) *Debaryomyces hansenii* strains, Y1399, Y1398, Y1402, Y1404 were not able to grow on both media. The *Dekkera bruxellensis* strain Y906 was able to grow without oxygen.

all, either on the synthetic minimal medium (Fig. 1) or on the enriched medium.

Batch cultivations under strict anaerobic conditions

In order to better characterize the *C. albicans* ability to grow under strict and well-controlled anaerobic conditions, two strains, Y1395 and Y1396, were tested in batch cultivation. Cells were cultivated in fermenters in synthetic minimal medium under a constant nitrogen flux. *Kluyveromyces lactis* (Kiers et al., 1998) was used in these experiments to confirm that the equipment could indeed establish strict anaerobic conditions. At the initial stages of cultivation, the *C. albicans* strains Y1395 and Y1396 exhibited an exponential growth. Nevertheless, during the initial 30 h and after approximately two to three duplications, the growth arrested, but the cells continued to consume glucose (Fig. 2). During the exponential phase of growth, both strains produced ethanol with a yield of 0.25 and 0.3 g g⁻¹ glucose, respectively. Also, some glycerol could be detected, but only traces of acetic acid were found in both strains. We think that small amounts of oxygen, which contaminated the nitrogen source, could support the initial exponential growth when the biomass was still low. As the biomass increased, the anaerobic conditions in the bioreactor became stricter, because an increased number of cells consumed the very low amount of the 'continuously supplied' contaminating oxygen. Under these conditions, *C. albicans* could not divide, but still continued to produce ethanol (Fig. 2). Because the growth was so atypical, we did not calculate the specific consumption rates. However, the observed growth in fermentors is consistent with that seen on the solid medium. In conclusion, the fermentor data (Fig. 2) supported the *C. albicans*

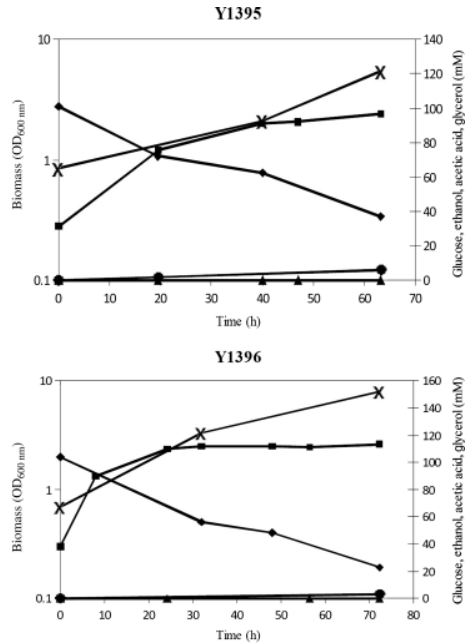


Fig. 2. The anaerobic batch cultures of *Candida albicans* (Y1395, Y1396). The ethanol yield in *C. albicans* (Y1395 and Y1396) is higher than in aerobiosis (see Table 2). The initial phases of the cultivations in *C. albicans* exhibited a short exponential growth phase, due to small amounts of oxygen present, but after two to three duplications, the growth was arrested. Both strains produced some ethanol, and Y1395 also produced traces of glycerol, while acetic acid was not detected. The batch experiments were performed using the B-Braun's equipment. ■, Biomass; ◆, glucose; X, ethanol; ▲, acetic acid; ●, glycerol.

behavior observed on the plates, thereby confirming that the growth of *C. albicans* under anaerobiosis is considerably affected.

Batch cultivations under strictly controlled aerobic conditions

We were interested in elucidating whether *C. albicans* could produce ethanol from glucose in the presence of oxygen. In order to investigate this, three *C. albicans* strains, Y1394, Y1395 and Y1396, were cultivated under strictly controlled aerobic conditions in fermentors, by maintaining the dissolved oxygen concentration over 30% of the air saturation. For *S. cerevisiae*, the Crabtree effect is reflected in a high specific ethanol production rate ($21.87 \text{ mmol g}^{-1} \text{ h}^{-1}$) and a low biomass yield (0.115 g g^{-1}) (reviewed in Table 2). In comparison with the ethanol produced by *S. cerevisiae*, the ethanol yields were much lower in *C. albicans* (0.09, 0.013 and 0.02 g g^{-1} for Y1394, Y1305 and Y1396, respectively) than in *S. cerevisiae* (Table 2). As a consequence, the biomass yields for *C. albicans* (Table 2) were higher and actually closer to the *K. lactis* one. In addition, also, the *C. albicans* specific growth rates ($0.44\text{--}0.51 \text{ h}^{-1}$) were closer to the value of *K. lactis* (0.5 h^{-1}) than to the *S. cerevisiae* one (0.37 h^{-1}). For *C. albicans*, even the specific ethanol production rates were low ($2.76, 0.38$ and $0.69 \text{ mmol g}^{-1} \text{ h}^{-1}$ for Y1394, Y1395 and Y1396, respectively). Neither acetic acid nor glycerol was detected under aerobic conditions, in contrast to what occurs in *S. cerevisiae* (Table 2). In short, *C. albicans* exhibited a predominantly respiratory glucose metabolism (Table 2, Fig. 3).

Discussion

Modern yeasts belonging to the lineage that underwent WGD degrade glucose to ethanol in the presence of oxygen and can grow in anaerobiosis (Merico *et al.*, 2007). However, among lineages that branched off just before WGD, one can find yeasts that can grow without oxygen and exhibit a moderate Crabtree effect, for example *Saccharomyces*

(*Lachancea*) *kluveri* (reviewed in Merico *et al.*, 2007). Apparently, the common progenitor of these yeasts already had the basic physiological properties, which could later be upgraded into an efficient genetic, biochemical and physiological apparatus that allows growth without oxygen and enables the accumulation of ethanol in the presence of glucose and oxygen (Piskur *et al.*, 2006). However, a majority of the pre-WGD yeasts, which separated from the *S. cerevisiae* lineage approximately 100–150 million years ago, are predominantly Crabtree negative and need at least some oxygen for their growth (Merico *et al.*, 2007).

What is the situation among the lineages that branched off more than 200 million years ago? In the recent literature, for example in Ihmels *et al.* (2005), Jiang *et al.* (2008) and Askew *et al.* (2009), one can often find an argument that species such as *C. albicans* and *D. hansenii* are obligate aerobes and Crabtree negative. Indeed, some attempts to characterize the respiratory metabolism in *C. albicans* (Niimi *et al.*, 1988) or comparisons of *D. hansenii* physiology to *S. cerevisiae* (Sanchez *et al.*, 2006) have been made. However, very often, the reported data have been obtained on a single strain and under not well defined and controlled conditions, for example in shake-flasks, where inadequate aeration may result in a mix of aerobic and anaerobic conditions. Therefore, our present work on these 'early' branches provided the first reliable data, which could be used to build up a robust model of the yeast evolutionary history. We analyzed several strains of each species to reduce the influence of intraspecies genetic variations and could thus obtain a general insight into the species carbon metabolism and dependence on oxygen.

We showed that *C. albicans* was able to exhibit only a slight growth in anaerobiosis on solid minimal medium and in batch cultures (Figs 1 and 2). Regarding aerobic growth, recently, *C. albicans* has been reported to be very sensitive to the presence of glucose in the environment and significant changes in the transcriptome were observed when the cells were exposed to increasing concentrations of glucose

Table 2. Growth parameters for aerobic batch cultivations of *Candida albicans* (Y1394, Y1395, Y1396), *Dekkera bruxellensis* (Y879), *Saccharomyces cerevisiae* (Y1623) and *Kluyveromyces lactis* (Y1376) strains

Species	Strain	Growth conditions	Specific growth rate (h^{-1})	Yield (g g^{-1} glucose)				q (mmol g^{-1} dry weight h^{-1})			
				Biomass	Ethanol	Glycerol	Acetic acid	Glucose	Ethanol	Glycerol	Acetic acid
Anaerobiosis											
<i>C. albicans</i>	Y1394	Glucose 20 g L ⁻¹	0.47	0.33	0.09	0	0	7.94	2.76	0	0
<i>C. albicans</i>	Y1395	Glucose 20 g L ⁻¹	0.51	0.37	0.013	0	0	7.56	0.38	0	0
<i>C. albicans</i>	Y1396	Glucose 20 g L ⁻¹	0.44	0.3	0.02	0	0	8.15	0.69	0	0
<i>D. bruxellensis</i> *	Y879	Glucose 20 g L ⁻¹	0.12	0.18	0.32	0	0.06	3.7	4.4	0	0.7
<i>S. cerevisiae</i> †	Y1623	Glucose 20 g L ⁻¹	0.38	0.115	0.382	0.057	0.005	13.26	21.87	1.98	< 0.1
<i>K. lactis</i> ‡	Y1376	Glucose 20 g L ⁻¹	0.5	0.4	0	0	0	11.95	0	0	0

*Results from E. Rozpedowska (unpublished data).

†Data from C. Compagno (unpublished data).

‡Results from Merico *et al.* (2009).

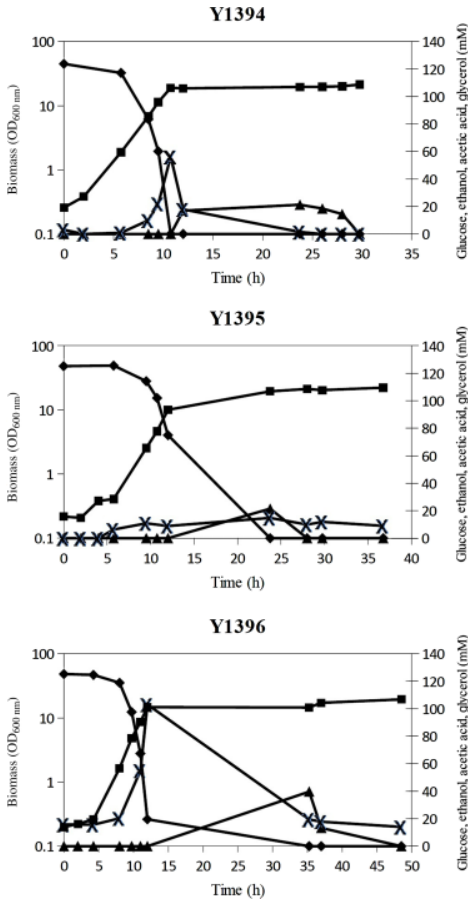


Fig. 3. Batch cultures of *Candida albicans* (Y1394, Y1395, Y1396) under aerobic conditions. The aerobic cultivations shown in this figure were performed using the Infors system. ■, biomass; ◆, glucose; X, ethanol; ▲, acetic acid.

(Rodakí *et al.*, 2009). In our experiments, under aerobic conditions, the biomass yield was high and similar to the *K. lactis* one, indicating that *C. albicans* relies predominantly on aerobic metabolism (Table 2, Fig. 3). Likely, this yeast does not possess an efficient glucose repression mechanism acting on the respiration and the carbon source was mostly used for biomass production. On the other hand, the ethanol production yield was higher in batch cultivations under anaerobiosis than in aerobiosis, which may indicate the presence of the Pasteur effect, a characteristic phenomenon of facultative fermentative yeasts (Lagunas, 1986), where alcoholic fermentation is inhibited by the presence of

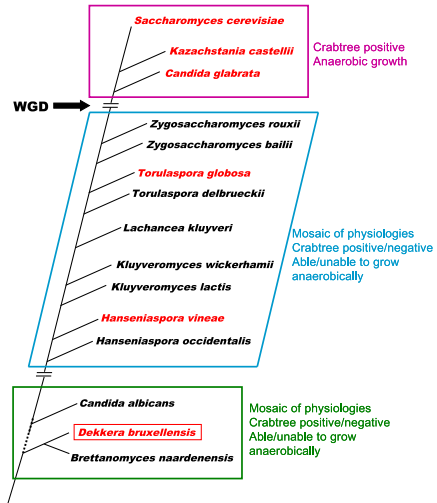


Fig. 4. A simplified yeast phylogenetic relationship (for details, see Kurtzman & Piskur, 2006) indicating the distribution of the Crabtree effect (red species) among three groups of yeasts: in the *Saccharomyces* group (violet), all species ferment glucose in the presence of oxygen, the species that diverged before the WGD event (blue) represent a mosaic of characteristics with only some species being slightly Crabtree positive (adapted after Merico *et al.*, 2007), the ‘lower species’ on the phylogenetic tree (green) are predominantly aerobic yeasts and incapable of efficient aerobic ethanol production. Only *Dekkera bruxellensis* is an exception in this group (red frame) because it is a Crabtree-positive yeast and can grow anaerobically. The timing of the WGD event is indicated by an arrow. Note that so far the exact phylogenetic relationship within the lowest branches is not clear.

oxygen and induced by oxygen limitation. In short, our observations point out that the physiological characteristics of *C. albicans* strains under anaerobic and aerobic conditions are typical for a predominantly aerobic lifestyle.

Debaryomyces hansenii was unable to grow under anaerobic conditions on solid medium (Fig. 1) and it has been reported previously as a Crabtree-negative yeast (Veiga *et al.*, 2000). However, subsequent investigations showed that this yeast is capable of fermentation, although to a much lower extent than *S. cerevisiae* (reviewed by Sanchez *et al.*, 2006). In short, this yeast also apparently has a lifestyle very similar to *K. lactis*.

A ‘low’ branch yeast *Scheffersomyces (Pichia) stipitis* has also been reported previously as a Crabtree-negative yeast and unable to grow under anaerobic conditions (van Hurk *et al.*, 1990; Shi & Jeffries, 1998). Metabolic flux analysis has revealed that in this yeast, a predominantly respirative glucose catabolism operates (Fiaux *et al.* 2003).

Candida albicans, *D. hansenii* and *D. bruxellensis* represent the ‘lower branches’ of the yeast phylogenetic tree (Fig. 4).

Except *D. bruxellensis*, these yeasts show a preference for respiratory metabolism and are unable to grow efficiently in the absence of oxygen (this work and van Dijken & Scheffers, 1986). It is therefore likely that the common progenitor of these yeasts, as well as of the *Saccharomyces/Kluyveromyces* yeasts, existing more than 200 million years ago, was a strongly oxygen-dependent microorganism and not able to accumulate significant amounts of ethanol in the presence of oxygen. It was first later on, with the onset of modern fruits and surplus of free sugars (Piskur *et al.*, 2006), that the environmental conditions promoted several independent lineages to evolve (1) a more pronounced ability to grow in anaerobiosis and (2) to accumulate ethanol more efficiently.

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