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Effects of Oxygen Availability on Acetic Acid Tolerance and Intracellular pH in *Dekkera bruxellensis*

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ABSTRACT

The yeast *Dekkera bruxellensis*, associated with wine and beer production, has recently received attention, because its high ethanol and acid tolerance enables it to compete with *Saccharomyces cerevisiae* in distilleries that produce fuel ethanol. We investigated how different cultivation conditions affect the acetic acid tolerance of *D. bruxellensis*. We analyzed the ability of two strains (CBS 98 and CBS 4482) exhibiting different degrees of tolerance to grow in the presence of acetic acid under aerobic and oxygen-limited conditions. We found that the concomitant presence of acetic acid and oxygen had a negative effect on *D. bruxellensis* growth. In contrast, incubation under oxygen-limited conditions resulted in reproducible growth kinetics that exhibited a shorter adaptive phase and higher growth rates than those with cultivation under aerobic conditions. This positive effect was more pronounced in CBS 98, the more-sensitive strain. Cultivation of CBS 98 cells under oxygen-limited conditions improved their ability to restore their intracellular pH upon acetic acid exposure and to reduce the oxidative damage to intracellular macromolecules caused by the presence of acetic acid. This study reveals an important role of oxidative stress in acetic acid tolerance in *D. bruxellensis*, indicating that reduced oxygen availability can protect against the damage caused by the presence of acetic acid. This aspect is important for optimizing industrial processes performed in the presence of acetic acid.

IMPORTANCE

This study reveals an important role of oxidative stress in acetic acid tolerance in *D. bruxellensis*, indicating that reduced oxygen availability can have a protective role against the damage caused by the presence of acetic acid. This aspect is important for the optimization of industrial processes performed in the presence of acetic acid.

As living microorganisms grow, their metabolism causes considerable changes to their ecological niches: nutrients are sequestered, and a variety of compounds are produced, such as organic acids, ethanol, and others, which can create a hostile environment for other competing microorganisms. Bacteria and yeasts are able to produce large amounts of some organic acids, such as acetic acid. In addition, organisms are constantly faced with different environmental stimuli, stresses, and competition with other organisms, which represent the driving forces leading to the evolution of several traits. The yeast *Saccharomyces cerevisiae* exhibits a strong fermentative lifestyle due to the Crabtree effect and to its ability to grow at a high rate even under anaerobic conditions (1–3) and low pH (4). In the context of natural evolution, this ability may have helped this organism to consume sugars quickly and to compete with other microorganisms by producing ethanol (5, 6). During yeast evolution, this particular strategy apparently evolved in at least two lineages: the *Saccharomyces* complex and *Dekkera/Brettanomyces* (7). *S. cerevisiae* is used worldwide for baking, producing alcoholic beverages, and recently, producing ethanol as biofuel (8–10). *D. bruxellensis* is another yeast associated with wine and beer fermentation (11, 12), and its ability to produce ethanol and to resist hostile environments makes it very suitable for use in several applications. However, this yeast has been reported to contaminate distilleries that produce fuel ethanol, especially continuous fermentation systems, where it can compete with *S. cerevisiae* (13, 14), due to its high ethanol and acidic tolerance. Recent studies have indicated that *D. bruxellensis* can use nitrate as a nitrogen source, and this characteristic is well suited for industrial fermentation (15, 16). *D. bruxellensis* has been reported as being unable to use xylose, but several strains are able to metabolize cellobiose (17). All these metabolic features have led to the idea that *D. bruxellensis* could be used for ethanol production at the industrial level (11, 18, 19). An alternative approach to improving the industrial use of lignocellulosic feedstocks for second-generation biofuel production by fermentation is the isolation and characterization of novel yeast strains that possess natural resistance to several stress conditions (e.g., high osmotic pressure, acidic pH, the presence of inhibitors, and oxidative stress) that microorganisms encounter during industrial processes. Acetic acid is also a food preservative, and food-spoiling species often exhibit resistance to this acid (20). The elucidation of the mechanisms by which cells manage these stresses is also essential for identifying new genetic traits that could be transferred to the most commonly used *S. cerevisiae* strains.

In this work, the effect of acetic acid on two *D. bruxellensis* strains was studied by flow cytometry (FCM). FCM rapidly pro-

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TABLE 1 Growth parameters of *D. bruxellensis* strains

Strain	Growth condition[a]	Glucose specific consumption rate ($mmol/g_{DW}/h^{-1}$)[b] ± SD	Specific production rate ($mmol/g_{DW}/h^{-1}$) ± SD		Yield (g/g) ± SD			μ (h^{-1})
			Ethanol	Acetate	Biomass	Ethanol	Acetate	
CBS 98	AA	0.77 ± 0.1	1.06 ± 0.09	0.25 ± 0.02	0.14 ± 0.01	0.32 ± 0.02	0.17 ± 0.02	0.02 ± 0.002
	AS	1.94 ± 0.28	3.59 ± 0.32	0.52 ± 0.17	0.15 ± 0.01	0.42 ± 0.02	0.06 ± 0.01	0.06 ± 0.01
	CA	1.92 ± 0.23	2.49 ± 0.08	0.73 ± 0.08	0.23 ± 0.02	0.28 ± 0.02	0.13 ± 0.01	0.12 ± 0.01
	CS	3.59 ± 0.32	7.53 ± 0.26	0	0.14 ± 0.02	0.46 ± 0.03	0	0.10 ± 0.01
CBS 4482	AA	1.57 ± 0.14	1.30 ± 0.14	0.91 ± 0.09	0.22 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.07 ± 0.01
	AS	3.27 ± 0.28	6.14 ± 0.67	0.10 ± 0.03	0.14 ± 0.04	0.44 ± 0.02	0.02 ± 0.01	0.10 ± 0.02
	CA	3.83 ± 0.15	3.15 ± 0.07	1.37 ± 0.14	0.26 ± 0.03	0.23 ± 0.01	0.14 ± 0.01	0.17 ± 0.01
	CS	3.23 ± 0.12	3.75 ± 0.09	0	0.18 ± 0.03	0.48 ± 0.01	0	0.13 ± 0.01

[a] AA, cultures in the presence of 120 mM acetic acid under shaken conditions; AS, cultures in the presence of 120 mM acetic acid under static conditions; CA, control cultures under shaken conditions; CS, control cultures under static conditions.
[b] g_{DW}, grams of dry weight.

vides accurate information regarding important cellular parameters at the single-cell level and monitors their heterogeneity in cell populations; this information is particularly relevant to the analysis of stress tolerance, particularly weak-acid tolerance. Recent studies on acetic acid tolerance in *S. cerevisiae* and *Zygosaccharomyces bailii* proposed that tolerance is partly due to population heterogeneity and different behaviors exhibited by cell subpopulations (21, 22). Our flow cytometric analysis revealed information regarding how acetic acid exposure affects cell size and complexity, as well as intracellular pH (pH_i). We also analyzed the effect of oxygen availability on acetic acid tolerance because of several considerations: (i) oxygen availability is an important parameter in industrial processes, (ii) *D. bruxellensis* produces ethanol at high yield under oxygen-limited conditions (17, 19), and (iii) acetic acid is known to cause oxidative stress (23) that can be prevented by growing the cells at low levels of dissolved oxygen.

MATERIALS AND METHODS

Strains and growth conditions. *D. bruxellensis* strains CBS 98 and CBS 4482 were used. For long-term storage, yeast strains were maintained at −80°C on 15% (vol/vol) glycerol and 85% (vol/vol) YPD broth (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter glucose).

Liquid cultures were grown at 30°C under shaking or static conditions. Cells from precultures grown in YPD broth were harvested during the exponential phase by centrifugation. After these cells were washed, they were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 into YPD broth (pH 4.5) supplemented with acetic acid (120 mM) or not supplemented (control cultures). Cell growth was monitored by measuring the increase in OD_{600} using a spectrophotometer (Jenway 7315; Bibby Scientific Limited, Stone, United Kingdom). Liquid cultures were prepared at least in duplicate.

Batch cultivation was performed using a BioStat Q system bioreactor (B-Braun Biotech International, Melsungen, Germany) with a working volume of 0.8 liter. The temperature was set at 30°C, the stirring speed was 200 rpm, and the pH was 4.5 (measured by a Mettler Toledo electrode). The medium used was YPD broth (pH 4.5) supplemented with acetic acid (120 mM).

Dry-weight measurements and metabolite assays. For dry-weight (DW) measurements, cell samples from the cultures were collected at different time points (in triplicate at each point), filtered through a glass microfiber GF/A filter (Whatman), washed with three volumes of deionized water, and dried at 105°C for 24 h.

Glucose, ethanol, and acetic acid concentrations in the supernatants were assayed in triplicate using commercial enzymatic kits (catalog no. 1 0716251 035, 1 0176290 035, and 1 0148261 035; Hoffmann La Roche, Basel, Switzerland). For acetic acid, the production was calculated by subtracting the initial amount present in the growth medium. Growth rates (μ_{max}), specific glucose consumption rates, and specific ethanol/acetic acid production rates (Table 1) were calculated during the exponential phase of growth, according to van Hoek et al. (24).

Colony-forming assay. To determine the effect of acetic acid on the colony-forming ability of these yeast strains, liquid culture aliquots were collected at different time points, centrifuged, washed, serially diluted (10-fold dilution series), and plated (in triplicate) on YPD agar (pH 4.5). CFU were counted after 1 week of incubation at 30°C.

Flow cytometric analysis. SYBR green, propidium iodide (PI), 5 (and 6)-carboxyfluorescein succinimidyl ester (CFDA SE), and dihydroethidium (DHE) were obtained from Sigma-Aldrich (Milan, Italy). Yeast cells were stained with 1× SYBR green (for the total cell count) or 5 μg/ml PI (for cell viability or cell membrane integrity evaluations) and incubated in the dark for at least 15 min before measurement. For measuring the pH_i, the cells were stained with a pH-sensitive fluorescence probe, carboxyfluorescein diacetate succinimidyl ester (CFDA SE) (10 μg/ml), according to Stratford et al. (25), with some modifications. Briefly, cells were harvested by centrifugation (2,600 × *g*, 6 min, 4°C) during early exponential-growth phase and then washed in buffer (50 mM KH_2PO_4, 50 mM sodium succinate [pH 6]). Then, cells were resuspended in buffer at 1 × 10^7 cells/ml, and CFDA SE was added at a final concentration of 10 μg/ml. Cells were then incubated in the dark for 12 min at 38°C. After the cells were incubated, they were harvested by centrifugation (2,600 × *g*, 6 min, 4°C), resuspended in water containing 100 mM glucose, and incubated at room temperature for 15 min. Finally, the cells were centrifuged (2,600 × *g*, 6 min, 4°C) to remove glucose buffer and then washed with 50 mM KH_2PO_4 and 50 mM sodium succinate (pH 4.5). To establish standard calibration curves, cells were resuspended in buffers with different pH values (100 mM morpholinepropanesulfonic acid [MOPS], 50 mM sodium succinate, and 500 mM acetate) in the presence of 100 μM gramicidin (Sigma-Aldrich, Milan, Italy) as a permeabilizing agent (26). This specific treatment leads to membrane permeabilization and consequent dissipation of the transmembrane proton gradient. For this permeabilization treatment, cells were kept in the dark for 1 h at room temperature.

To evaluate oxidative stress, 2 × 10^6 cells/ml were harvested and stained with 50 μM DHE in phosphate-buffered saline (PBS) buffer for 10 min at 30°C. Then, the DHE solution was removed by centrifugation, and the cells were resuspended in PBS buffer, according to Roux et al. (27).

Cell counting and fluorescence detection were performed using an Accuri C6 flow cytometer (BD Biosciences, Milan, Italy). In FCM, particles/cells that pass through the beam will scatter light, which is detected as forward scatter (FSC) and side scatter (SSC). FSC correlates with the size, shape, and aggregates of cells, whereas SSC depends on the density of the

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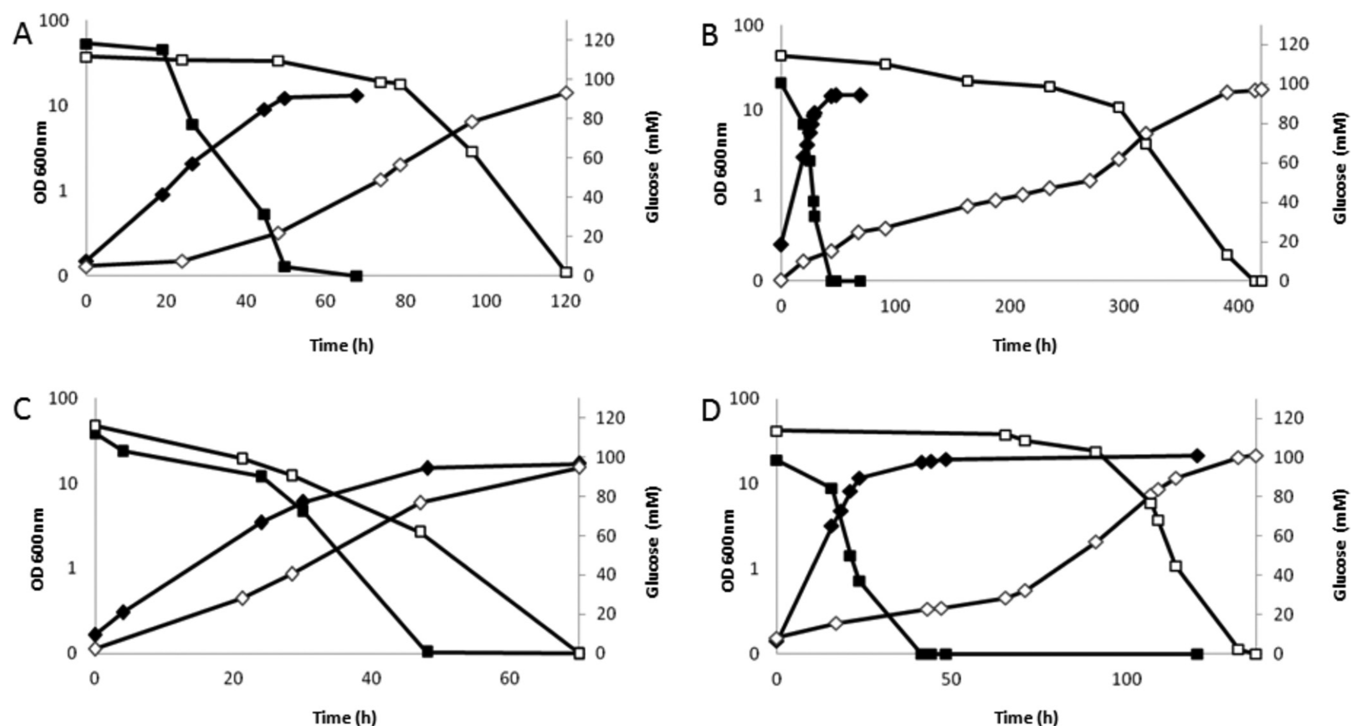

FIG 1 Growth of *Dekkera bruxellensis* strains under different conditions. Filled symbols indicate growth on YPD (pH 4.5) (control condition), and open symbols indicate growth on YPD supplemented 120 mM acetic acid (pH 4.5). (A and B) CBS 98 under static (A) and shaken (B) conditions. (C and D) CBS 4482 under static (C) and shaken (D) conditions. ■, glucose; ◆, OD_{600}.

particles/cells (i.e., the cytoplasmic granule number and membrane size); therefore, cell populations can often be distinguished based on differences in their size and density 28, 29). Cell suspensions were analyzed by FCM using the following threshold settings: FSC, 5,000; SSC, 4,000; total events collected, 20,000. All of the parameters were collected as logarithmic signals. A 488-nm-wavelength laser was used to measure the FSC values. When necessary, samples were diluted in filtered water immediately before measurement so that the rate of events in the flow was generally fewer than 2,000 events/s. The data were analyzed using the Accuri C6 software version 1.0 (BD Biosciences). The SYBR green and carboxyfluorescein succinimidyl ester (CFSE) fluorescence intensities of stained cells were recovered in the FL1 channel (excitation, 488 nm; emission filter, 530/30 nm; BD Biosciences). The PI, DHE fluorescence, and cell autofluorescence were recovered in the FL3 channel (excitation, 488 nm; emission filter, 610/20 nm; BD Biosciences).

RESULTS AND DISCUSSION

Oxygen availability affects the growth kinetics of *D. bruxellensis* in the presence of acetic acid. In a previous study, we screened the acetic acid tolerances of 29 strains of *D. bruxellensis* at concentrations up to 120 mM at pH 4.5 and revealed considerable diversity in acetic acid tolerance among the strains (30). In particular, the cultivation in liquid medium under aerobic conditions revealed that also the growth of the most-tolerant strain, CBS 4482, was negatively affected by the presence of 120 mM acetic acid. It caused a reduced growth rate, a reduced specific glucose consumption rate, and a reduced specific ethanol production rate (30). In a new set of experiments, strain CBS 4482 showed an initial adaptive phase that lasted approximately 60 h and a subsequent faster exponential-growth phase (growth rate, 0.06 h^{-1}) during shake flask cultivation in liquid glucose-based medium containing 120 mM acetic acid at pH 4.5 (Fig. 1); these growth kinetics were reproducible. In contrast, we noticed that the culture of strain CBS 98, which was one of the less-tolerant strains (30), behaved in a less reproducible way, with adaptive phases lasting 70 to 300 h (see Fig. 1 for an example); additionally, some cultures did not grow at all. A similar phenomenon has recently been described in several *S. cerevisiae* strains, where phenotypic cell-to-cell heterogeneity affects lag or adaptation phases during growth (31). The exposure to 120 mM acetic acid caused effects that are more pronounced for CBS 98, as clearly shown by the data reported in Table 1, mainly in terms of reduced growth rate and of reduced biomass yield. Acetic acid has been demonstrated to induce programmed cell death in *S. cerevisiae* and in *Z. bailii* (32–34). This process is associated with the role of mitochondria in executing the apoptotic program (35). In this context, other studies revealed that hypoxia prevents apoptosis (36) and that Rho^0 mutants display increased resistance to acetic acid (37). These observations led us to investigate whether reduced oxygen levels could affect acetic acid tolerance. The aim of our work was to analyze the effect of oxygen availability on growth in the presence of acetic acid. We performed a new set of cultures exposing the cells to the same concentration of acetic acid used under shaking conditions (120 mM) but incubating them under static conditions, which limits the dissolved oxygen. These conditions are similar to those encountered by *D. bruxellensis* in wine fermentation and in natural environments where yeast can be isolated, such as soils and plant surfaces (fruits, leaves, and trunks) (38, 39).

Under these growth conditions, we obtained reproducible kinetics of CBS 98 cultures grown in the presence of acetic acid (12 flasks of biological replicates; see Fig. 1 for an example). In contrast to our observations under shaking conditions, all these cul-

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tures started to grow and exhibited shorter adaptive phases, in the range of 24 h. The reduced availability of oxygen decreased growth rates in the control cultures (without acid) of both strains in comparison to those of the shaken cultures (Table 1), and the effect was more pronounced for CBS 4482 (26% for CBS 4482 versus 16% for CBS 98). Interestingly, under static conditions and in the presence of acetic acid, the growth rate of CBS 98 was 40% lower than that of the control, in comparison with 85% reduced growth rate caused by the presence of acetic acid under shaking conditions (Table 1). Moreover, its growth rate was higher under static conditions and in the presence of acetic acid than that under shaking conditions (Table 1). This positive effect of reduced oxygen availability on growth kinetics in the presence of acetic acid was also evident in the less-sensitive CBS 4482 strain (Table 1). Incubation under static conditions in the presence of acetic acid resulted in a shorter latency phase (Fig. 1) and an 11% lower growth rate compared to a 60% reduction in growth rate observed under shaking conditions (Table 1). Thus, incubation under static conditions alleviates the stress caused by the presence of acetic acid in both strains, resulting in shorter latency phases and higher growth rates, and this effect was more pronounced in the more-sensitive CBS 98.

Oxygen limitation triggered different effects on glucose metabolism rates in the two strains. In *D. bruxellensis*, inhibition of fermentative metabolism caused by a lack of oxygen is known as the Custers effect and is mainly caused by inefficient redox balance mechanisms (40); unfortunately, this effect has not yet been investigated in a systematic manner. This effect has been observed in several strains; however, these strains were cultivated under very different conditions, thus hampering a rigorous comparison of their different behaviors (7, 17, 19). Due to this effect, under oxygen-limited conditions, one would expect lower glucose consumption and ethanol production rates than those under aerobic conditions. We found different behaviors in the two strains: in the control cultures, CBS 4482 exhibited a reduced glucose consumption rate (17%) but a slightly increased ethanol production rate in comparison to those under the more-aerobic conditions (Table 1), whereas CBS 98 showed an increased specific glucose consumption rate as well as an increased ethanol production rate (2- and 3-fold increases, respectively; Table 1). This finding may reflect different responses to oxygen limitation in terms of fermentative efficiency in different strains, which can mirror different levels of redox imbalance inside the cells and which deserves a deeper analysis under more appropriate conditions. However, both strains produced ethanol, with slightly higher yields under aerobic conditions (Table 1) due to the lack of acetic acid production under static cultivation.

The addition of acetic acid to the growth media of cultures grown under static conditions caused different effects on glucose metabolism rates in the two strains, with negative effects on CBS 98 and positive effects (mainly for ethanol productivity) on CBS 4482 (Table 1). Nevertheless, static incubation of both strains resulted in increased glucose metabolism in terms of glucose consumption and ethanol production rates in comparison to those of the more-aerobic shaken cultures (Table 1). The specific glucose consumption rates of cells grown in the presence of acetic acid under static conditions were similar to those calculated for control cultures (without acetic acid) grown under shaking conditions (Table 1). Therefore, this finding reflected the higher growth rates of the static cultures and was in agreement with the improved acetic acid tolerance exhibited under these growth conditions. It is known that maintenance of the electrochemical potential across the plasma membrane is ensured by the activity of the proton-translocating plasma membrane and vacuolar H^+/ATPases, which pump out the protons generated by acetic acid dissociation in the cytosol. A faster energetic metabolism, due to an increased fermentative metabolism, can then help restore the pH_i. These observations suggested that the presence of acetic acid is not the only cause of inhibition of glucose metabolism and growth; the concomitant presence of acetic acid and oxygen mainly exerts a negative effect on both. As expected, oxygen limitation inhibited acetic acid production in the control cultures because its production is associated with oxygen concentration (41). Nevertheless, the presence of acetic acid in the growth medium stimulated its biosynthesis instead of its consumption (Table 1). This effect is probably due to a requirement for more NADPH, as observed even under aerobic conditions, in which the acetic acid yield increased in the presence of acetic acid (Table 1). Notably, CBS 98 showed higher acetic acid yield and productivity than CBS 4482 (Table 1). An increased demand for NADPH may be indicative of the oxidative stress caused by the presence of acetic acid; the ability to maintain the metabolic pathway that generates this reduced cofactor may play an important protective role.

To confirm the positive effect caused by reduced oxygen availability, which was more pronounced for CBS 98, this strain was cultivated in a fermenter, with low stirring and without air supply, in order to obtain oxygen-limited conditions. Its growth kinetics were essentially similar to those obtained under static cultivation conditions, with a short adaptive phase followed by exponential growth at a rate of 0.055 h^{-1}. During the cultivation, the dissolved oxygen concentration and cell growth decreased to zero, and the pH reached 4.3.

Acetic acid affects the cell size. FCM allows the detection of cell size (see Materials and Methods). We observed that acetic acid exposure caused an increase in cell size in both strains (Fig. 2). After the cells' first 24 h of cultivation in the presence of acetic acid, the cells become larger under both shaken and static conditions. This effect did not occur in the control cultures. This larger cell size could be due to an increase in vacuole formation, because these organelles play an important role in maintaining the pH_i (42). In particular, in CBS 98, the strain more sensitive to acetic acid, the mean size of cells cultivated in shaken flasks continued to increase 3.5-fold during the long adaptation phase but then decreased in the growing population (Fig. 2; see also Table S1 in the supplemental material). This increase in cell size was also reflected by the OD and caused the lack of correlation between OD and cell number: after 163 h, the OD increased 7-fold, but the cell number only doubled (see Table S1). Acetic acid exposure similarly affected CBS 4482, but the increase in cell size was lower (2.6-fold) than in the CBS 98 strain (Fig. 2; see also Table S1).

Under static incubation, the cell size increased in the presence of acetic acid in both strains but to a lesser extent (1.5- and 1.9-fold, respectively, in CBS 98 and CBS 4482) (Fig. 2; see also Table S1 in the supplemental material).

Analysis of intracellular pH. The ability of the cell to maintain its pH_i near neutral is crucial in order to sustain normal cellular functions and is particularly challenging in the presence of acids (42). To characterize how the presence of acetic acid affects the pH homeostasis in *D. bruxellensis*, the pH_i of the two strains was determined by FCM. Examination of pH_i was performed by staining

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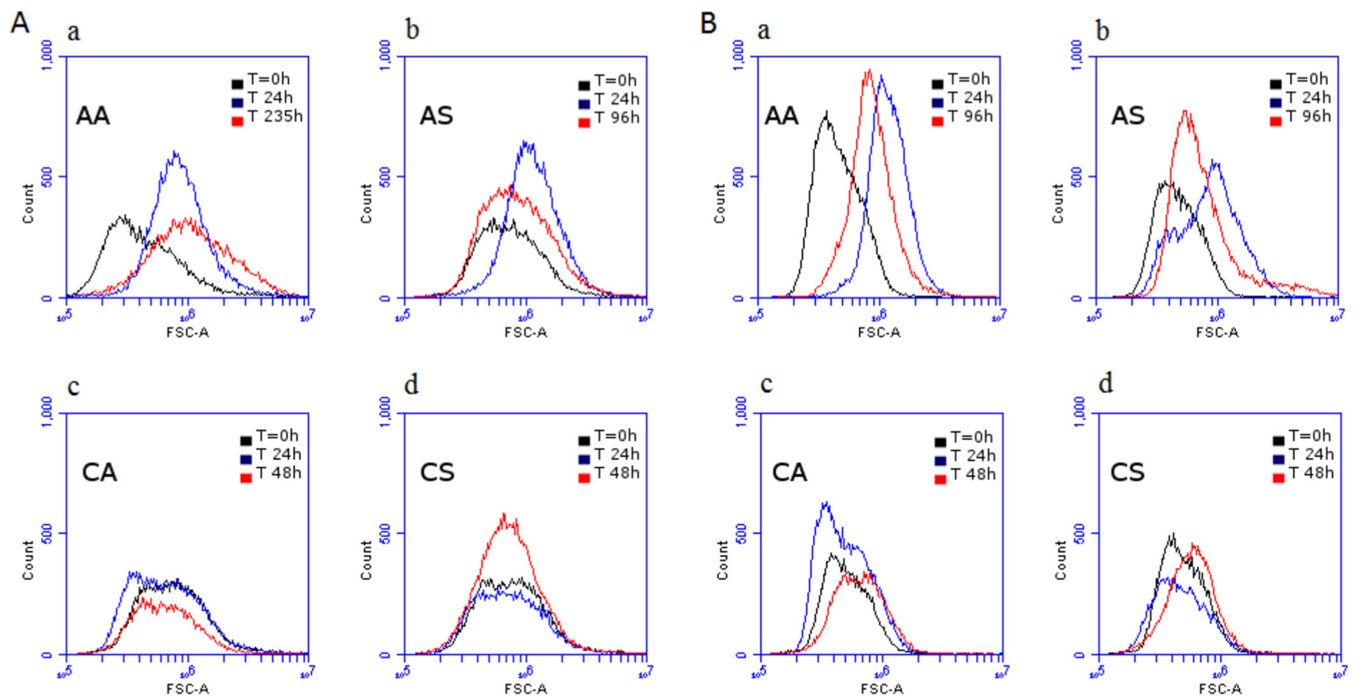

FIG 2 Fluorescence-activated cell sorting (FACS) histograms of cell size of CBS 98 (A) and CBS 4482 (B) cells under different growth conditions. AA and AS, cells growing in the presence of acetic acid under shaken or static conditions, respectively. CA and CS, cells growing in the absence of acetic acid (control condition) under shaken or static conditions, respectively. Black line, samples at the time of inoculum preparation. Blue and red lines, samples taken at different times corresponding to different phases during the growth in the presence of acid (adaptation, exponential phase, or late exponential; see the text for details). In control cultures (CA and CS), the blue line corresponds to exponential phase, and the red line corresponds to late-exponential phase.

the cells with CFDA SE; the results indicated that the mean pH_i of CBS 4482 grown exponentially in shake flask cultures in the presence of acetic acid was identical to that of the control population (grown without acetic acid) (Table 2). No subpopulations with different pH_i were identified, and the cell population was homogenous, as indicated by the coefficient of variation (CV) values. The presence of damaged/dead cells was assayed by PI staining; only 2% of the cells were injured, similar to that observed in the control cultures and in agreement with the yeast count detected on YPD plates (see Fig. S2 in the supplemental material). A different scenario was observed when the pH_i of the CBS 98 strain was measured. In this case, when the cells were grown in the presence of acetic acid, two subpopulations became evident after 235 h of incubation (Fig. 3): one with a mean pH_i value of 6.5 (representing 78% of the cells), and one with a mean pH_i value of 4.9 (representing 22% of the cells) (Fig. 3 and Table 2). This heterogeneity was also reflected by the high CV value. In contrast, the control culture showed a homogeneous population with a higher mean pH_i of 7.0 (Table 2; see also Fig. S2).

PI staining revealed that 34% of the cells (of the entire population stained by SYBR green) were severely damaged and/or dead (Fig. 4a and b). PI-CFDA SE double staining revealed that the population with the lowest pH_i was the most damaged, as indicated by their shift in PI fluorescence emissions to higher values (Fig. 4d). Nevertheless, yeast counts on YPD plates indicated that 80% of the cells were able to grow and form colonies (in contrast to the expected 66% based on PI staining), indicating that of the 34% PI-stained cells, only some of them were irreversibly injured. In conclusion, this population was composed of (i) cells with low pH_i (4.8) that were unable to restore their pH_i and died (representing 20% of the population), (ii) damaged cells that were still able to form colonies on YPD (14%) and likely possessed a pH_i ranging between 5 and 6, and (iii) actively growing cells that were able to restore their pH_i close to that of the control culture (pH_i 6.5). The presence of stressed cells that were permeable to PI but still alive ("red but not dead") have been reported in *S. cerevisiae* under different stress conditions (43). In *S. cerevisiae*, a similar situation has been reported to occur in strains producing lactic acid: the cells with low pH_i died, and improved lactic acid production was obtained by sorting the cells with high pH_i (44). Notably, *D. bruxellensis* produces acetic acid even when cultivated in media containing this acid (Table 1). All these data indicated that in strain CBS 98, a part of population

TABLE 2 pH_i of CBS 98 and CBS 4482 strains

Strain	Condition[a]	Intracellular pH	CV%
CBS 98	AA	6.5 ± 0.18, 4.9 ± 0.15	137
	AS	6.7 ± 0.11	89
	CA	7 ± 0.16	86
	CS	6.4 ± 0.1	87
CBS 4482	AA	6.9 ± 0.16	53
	AS	6.5 ± 0.12	43
	CA	6.9 ± 0.07	66
	CS	6.3 ± 0.04	65

[a] AA, cultures in the presence of 120 mM acetic acid under shaken conditions; AS, cultures in the presence of 120 mM acetic acid under static conditions; CA, control cultures under shaken conditions; CS, control cultures under static conditions.

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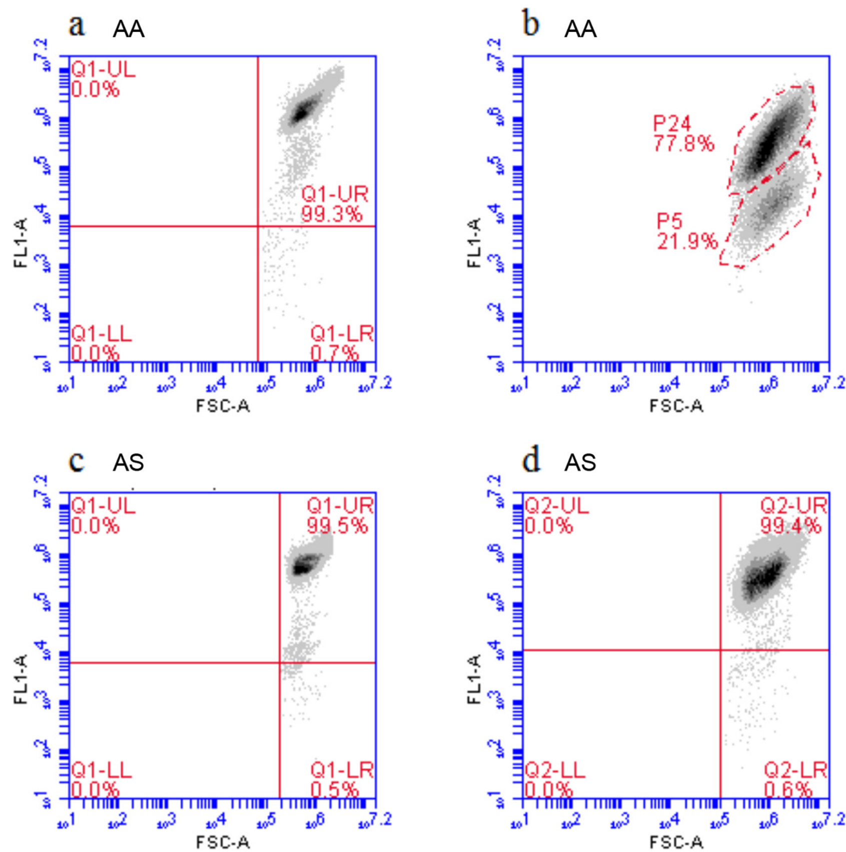

FIG 3 Dot plots comparing CFSE fluorescence of cells and representing pH_i of populations growing in YPD supplemented with 120 mM acetic acid at pH 4.5. (a and c) CBS 4482 cells growing in exponential phase under shaken (AA) or static conditions (AS), respectively. (b and d) CBS 98 cells growing in exponential phase under shaken (AA) or static conditions (AS), respectively.

was unable to counteract the stress caused by the presence of acetic acid during growth, causing its reduced tolerance in comparison to CBS 4482.

When the cells were cultivated under static conditions in the presence of acetic acid, the pH_i of the yeast population was homogeneous, similar mean values of pH_i were detected in the two strains (Fig. 3 and Table 2; see also Fig. S3 in the supplemental material), and the percentage of dead/damaged cells was always less than 5%. Such findings agreed with the physiological data reported above and reinforced the idea that reduced oxygen levels during incubation resulted in a higher level of acetic acid tolerance, especially for the less-tolerant strain CBS 98. Interestingly, the pH_i of yeast cells cultivated under static conditions in the absence of acetic acid supplementation was lower than the pH_i of yeast cells cultivated under shaking conditions in both strains, most likely due to a stronger acidifying effect caused by CO_2 production, as suggested by the higher ethanol yields and productivity levels (Table 1). A lower pH_i could lead to a lower intracellular dissociation of acetic acid that freely diffuses from outside across the membrane in its undissociated form. This effect, previously described in *Z. bailii* (21) and more recently in *S. cerevisiae* (22), could be used by yeast cells as a strategy to survive in environments characterized by low pH and the presence of organic acids, such as acetic acid.

Analysis of oxidative stress. The observation that reduced oxygen availability alleviates the stress imposed by the presence of acetic acid in the growth medium suggests that changes in oxygen could cause additional stress on cells that affects their growth. On the other hand, acetic acid has been reported to induce apoptosis, accompanied by reactive oxygen species (ROS) production in *S. cerevisiae* (37, 45). We used DHE staining to examine the levels of ROS accumulation in the cells of both strains during growth in the presence of acetic acid under shaking and static conditions; however, we did not detect significant differences between the two conditions (data not shown). ROS are transient, highly reactive, and unstable molecules; a more reliable method for detecting changes in their intracellular levels is to measure the amount of oxidative damage to macromolecules. Accumulation of lipofuscin, an insoluble and autofluorescent mixture of oxidized lipids, proteins, and nucleic acids, is a reported consequence of oxidative stress and presents with high concentrations of metals, especially iron, which is also indicative of oxidative stress (46–48). Flow cytometric analysis of cells in control cultures (without acetic acid) did not show any significant increase in the cellular level of autofluorescence (Fig. 5; see also Fig. S4 in the supplemental material). In contrast, CBS 98 cells incubated under shaking conditions in the presence of acetic acid exhibited a 2.5-fold increase in autofluorescence after 19 h of growth (Fig. 5; see also Fig. S4), and this continued to increase after 163 h of incubation. Two subpopulations were clearly visible and had mean autofluorescence values of 2,500 and 19,800 arbitrary units (AU) (representing 52% and 37% of the whole population, respectively; Fig. 4e). We think

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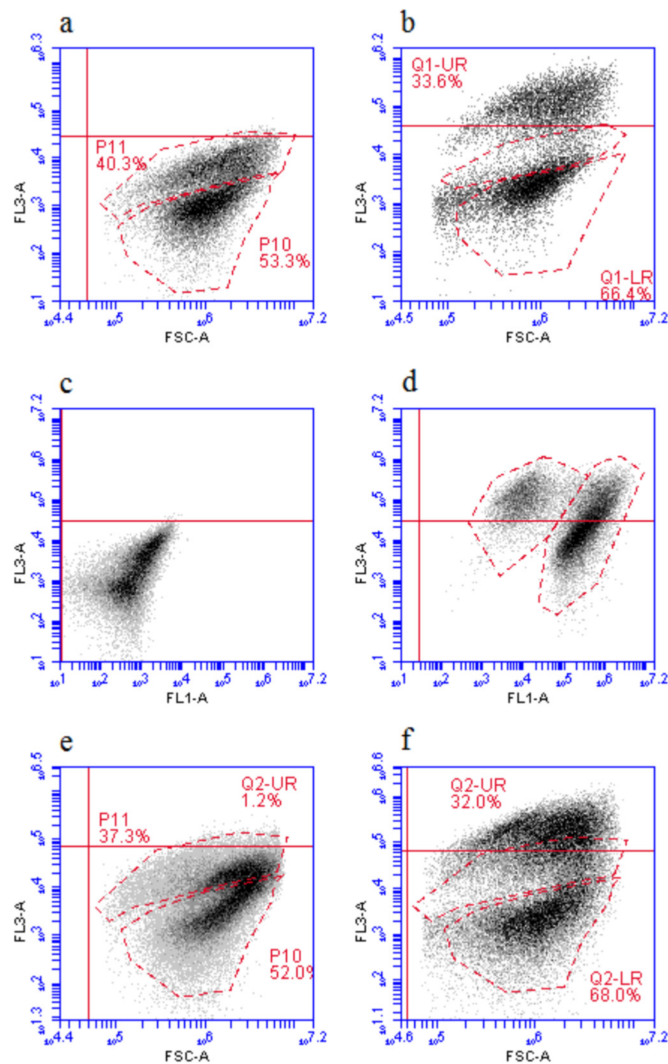

FIG 4 Dot plots showing fluorescence detected in different populations of CBS 98 strain. (a) Autofluorescence detected in FL3 channel of cells collected after 235 h of growth in the presence of 120 mM acetic acid under shaken conditions. (b to d) The same sample after PI staining (b), without staining (c), and stained with CFSE (fluorescence in FL1) and PI (fluorescence in FL3) (d). (e) Autofluorescence detected in FL3 channel of cells collected after 163 h of growth in the presence of acetic acid under shaken conditions. (f) The same sample stained with PI (shift of fluorescence in FL3).

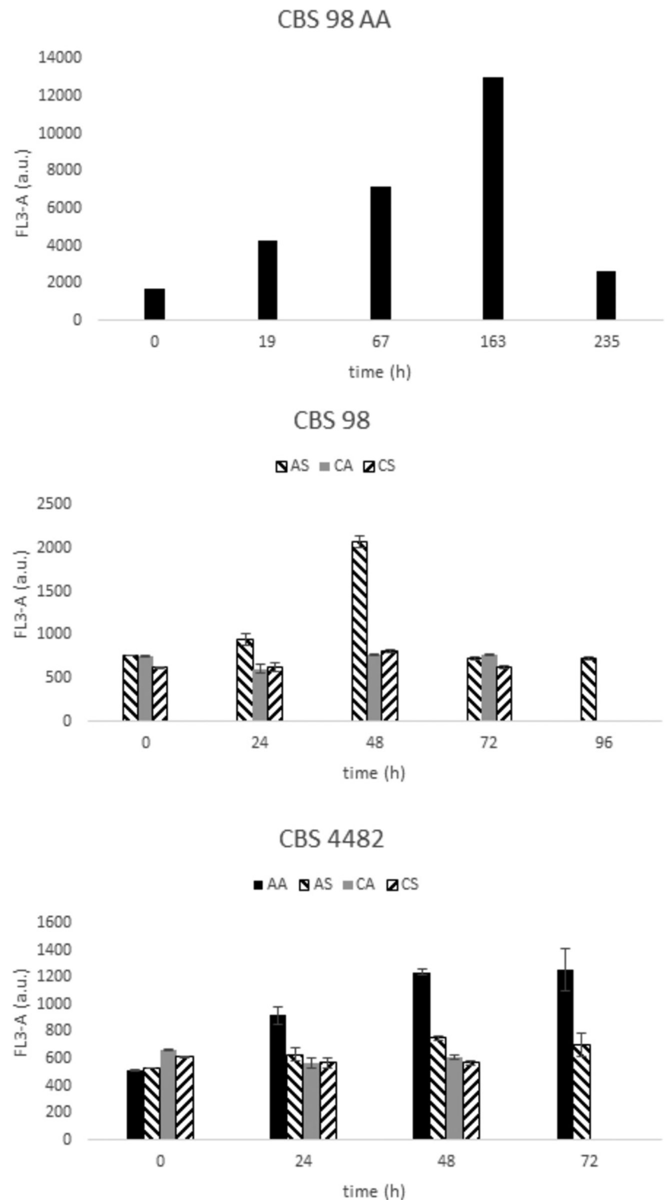

FIG 5 Autofluorescence mean values (arbitrary units) of CBS 98 and CBS 4482 cells growing under different conditions. AA and AS, cultures in the presence of acetic acid under shaken and static conditions, respectively. CA and CS, control cultures (without acid) under shaken and static conditions, respectively.

that this difference in autofluorescence was likely due to lipofuscin accumulation. The mean cell sizes of these two subpopulations also differed: the cells with lower autofluorescence were smaller (1,200 AU), and the cells with higher autofluorescence were larger (2,600 AU) (Fig. 4e). PI staining of these subpopulations showed that the subpopulation exhibiting a higher level of autofluorescence primarily retained PI, indicated by the shift to higher fluorescence emission values in channel FL3 (Fig. 4f), which suggested that this subpopulation was actually more damaged. In conclusion, we could detect two subpopulations with different levels of autofluorescence and mean cell size that showed different levels of damage (Fig. 4). Apparently, the more-damaged cells were larger and displayed higher levels of autofluorescence.

After 235 h, the total level of cellular autofluorescence decreased (Fig. 5), consistent with growth kinetics indicating active growth of part of the population (see above). Although acetic acid-induced autofluorescence also occurred in CBS 4482 (Fig. 5; see also Fig. S4 in the supplemental material), we did not notice the presence of subpopulations, and its total level was lower than that in CBS 98.

Incubation under static conditions also resulted in increased cellular autofluorescence in the presence of acetic acid but to a lesser extent than incubation under shaken conditions (Fig. 5; see also Fig. S4 in the supplemental material). This observation is consistent with the fact that cultivation under oxygen-limited conditions alleviated the oxidative stress and reduced the extent of ROS accumulation, which subsequently improved acetic acid tol-

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erance, reduced the length of the adaptation phase, and improved growth in the presence of acetic acid. In the presence of acetic acid, mitochondria are severely damaged, and ROS can cause oxidation of lipids and proteins (37, 46). Hypoxia has been demonstrated to prevent apoptosis and reduce the formation of oxidized proteins in *S. cerevisiae* (36, 49). Studies in *S. cerevisiae* have indicated that there is a strong connection between mitochondrial activity and oxidative stress, and that acetic acid plays a role in yeast apoptosis (50). Recently, *COX20* overexpression was shown to improve tolerance to acetic acid and oxidative stress (51), suggesting that mitochondrial activity, oxidative stress, and acetic acid tolerance could be linked.

Conclusions. The main conclusion of our study is that the concomitant presence of acetic acid and oxygen exerts a negative effect on *D. bruxellensis* growth. We showed that cultivation of *D. bruxellensis* under oxygen-limited conditions alleviates the stress caused by the presence of acetic acid in the growth medium compared to cultivation under aerobic conditions. Two strains that exhibit different levels of tolerance to acetic acid in terms of their growth parameters were analyzed. This positive effect was more pronounced in the more-sensitive strain, CBS 98, resulting in reproducible growth kinetics, shorter adaptive phases, and higher growth rates. Microscopic observations and flow cytometric analysis indicated that the presence of acetic acid increased cell size and cell complexity. Flow cytometry revealed the presence of two subpopulations with different pH_i values and cellular viabilities in the more-sensitive strain, CBS 98, grown under aerobic conditions in the presence of acetic acid, reflecting the lower level of acid tolerance of this strain. In contrast, under oxygen-limited conditions, we detected in both strains homogeneous populations with a lower pH_i, which is most likely due to a stronger acidifying effect caused by CO_2 production, which might lead to a lower intracellular dissociation of acetic acid that freely diffuses across the membrane in its undissociated form. This can represent another important mechanism that helps the cells survive in the presence of organic acids, such as acetic acid. Moreover, we showed that reduced oxygen availability could protect against the damage caused by the presence of acetic acid. By FCM, we detected reduced intracellular levels of oxidation-damaged macromolecules (proteins, lipids, and nucleic acids). We hypothesize that this mechanism likely protects glycolytic enzymes, resulting in faster energetic metabolism, which subsequently improves the cell's ability to restore its pH_i upon exposure to acetic acid. Furthermore, the ability to maintain an active acetic acid production pathway can also play an important role in coping with an increased demand for NADPH, which is needed to counteract the oxidative stress caused by the presence of acetic acid. Other studies are in progress to identify these survival mechanisms at the molecular level; however, molecular tools in *D. bruxellensis* have not yet been well established. In addition, our study highlighted the presence of phenotypically heterogeneous subpopulations with different abilities to respond to the acid and oxidative stress, as already reported in other yeasts (52, 53).

In conclusion, our study sheds light on the important effect of oxidative stress on acetic acid tolerance in *D. bruxellensis*. These findings are useful for optimizing industrial fermentation strategies in which acetic acid may be present in the cultivation medium as a by-product of hydrolytic pretreatments.

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