

# Acetic acid induces a programmed cell death process in the food spoilage yeast *Zygosaccharomyces bailii*

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## Abstract

Here we show that 320–800 mM acetic acid induces in *Zygosaccharomyces bailii* a programmed cell death (PCD) process that is inhibited by cycloheximide, is accompanied by structural and biochemical alterations typical of apoptosis, and occurs in cells with preserved mitochondrial and plasma membrane integrity (as revealed by rhodamine 123 (Rh123) and propidium iodide (PI) staining, respectively). Mitochondrial ultrastructural changes, namely decrease of the cristae number, formation of myelinic bodies and swelling were also seen. Exposure to acetic acid above 800 mM resulted in killing by necrosis. The occurrence of an acetic acid-induced active cell death process in *Z. bailii* reinforces the concept of a physiological role of the PCD in the normal yeast life cycle.

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## 1. Introduction

Weak carboxylic acids, such as acetic, sorbic and benzoic acids, are generally regarded as safe anti-microbial additives, and have wide application as preservatives in foods and beverages [1–3]. However, many yeasts are able to survive, adapt and even grow in the presence of the maximum levels of these preservatives permitted for use in foods. When compared with other fungi and bacteria, yeast are more resistant to weak carboxylic acids. The elucidation of the cytotoxic effects induced by weak acids in yeast, which may compromise cell viability and ultimately result in cell death, can provide further insights into the mechanisms that determine different susceptibilities of yeast to weak carboxylic acids compared to other microorganisms, and will allow the improvement or design of new strategies for food and beverage preservation. *Zygosaccharomyces bailii* is a food and beverage spoilage

yeast that is characterised by a high tolerance to weak carboxylic acids at low pH [1,4,5], where *Saccharomyces cerevisiae* cannot survive. Thus, these two yeast species have frequently been selected as models for the study of yeast response to acid stress. In *S. cerevisiae* [6] and in *Z. bailii* [7] acetic acid induces cell death. However, and as is widely known, in *Z. bailii* this effect is observed at much higher concentrations of the acid. Although individual cells of *S. cerevisiae* and *Z. bailii* exhibit different short-term intracellular pH responses to acetic acid [8], in both species the induction of cell death was related with an intracellular acidification [7–9]. In *Z. bailii*, the mechanism(s) of death due to exposure to weak acids remains to be clarified.

Recently, we have shown that acetic acid in concentrations between 20 and 120 mM induces in exponentially growing *S. cerevisiae* cells a programmed cell death (PCD) process that displays the most common apoptotic hallmarks, such as chromatin condensation along the nuclear envelope, exposure of phosphatidylserine on the surface of the cytoplasmic membrane, and occurrence of internucleosomal DNA fragmentation [10]. Our previous study also has demonstrated that acetic acid, at concentrations above those inducing the PCD process, kills *S.*

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*cerevisiae* by a necrotic death process [10]. In the present work, we studied the modes of cell death induced by acetic acid in *Z. bailii*.

## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

*Z. bailii* ISA 1307, originally isolated from a continuous production plant of sparkling wine [11], was used. The strain was maintained on slants of YEPD medium containing yeast extract (0.5%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v). The cells were grown to mid-exponential phase in a water bath, with magnetic stirring (250 rpm), at 26°C, in 250-ml flasks containing 100 ml of mineral medium [12] with vitamins, oligo-elements and 2% (w/v) glucose.

### 2.2. Treatments with acetic acid and preparation of yeast suspensions

Exponential-phase cells were harvested, washed twice in ice-cold distilled sterile water and suspended in commercial sterile water (Paracelsia, Porto, Portugal) with glucose 2% (w/v) and in the absence or presence of 85, 170, 320, 515, 600, 800 or 1000 mM acetic acid (pH 3.0, set with HCl after acetic acid additions). The extracellular pH did not change during the incubations. In all experiments the cell concentration was adjusted to  $10^6$  cells  $\text{ml}^{-1}$ . The treatments were carried out for 130 min at 26°C with magnetic stirring (150 rpm). At time intervals, samples were taken, washed twice with ice-cold distilled water and processed for the different assays. Viability was determined by colony-forming units (cfu) counts after 2 days of incubation at 26°C on YEPD agar plates. No further colonies appeared after that incubation period.

### 2.3. Inhibition of protein synthesis

The inhibition of protein synthesis was performed by adding 50  $\mu\text{g ml}^{-1}$  cycloheximide to the cell suspensions ( $10^6$  cells  $\text{ml}^{-1}$ ) at the same time as the different acetic acid concentrations tested. Exposure of yeast cells to cycloheximide at this concentration for 130 min, did not affect cell viability as assessed by cfu.

### 2.4. Transmission electron microscopy

Cells from control conditions (untreated) and treated with 320 mM acetic acid were washed with sodium cacodylate buffer (0.1 M, pH 7.2) and processed for transmission electron microscopy of ultrathin sections as described before [10]. Electron micrographs were taken with a Zeiss EM 10C electron microscope.

### 2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)

DNA strand breaks were demonstrated by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL) with the 'In Situ Cell Death Detection Kit, Fluorescein', from Boehringer Mannheim (Indianapolis, IN, USA) as described before [10].

### 2.6. Fluorochrome solutions and staining protocols

Assessment of mitochondrial membrane potential ( $\Delta\Psi_m$ ) in whole cells was performed by Rh123 staining and flow cytometry as described before [13]. A calculated ratio (*R* value) between green fluorescence (due to mitochondrial Rh123 accumulation) and forward scatter (correlated with cell size) was used to detect changes of  $\Delta\Psi_m$  [13]. Calculation of *R* values also allows the discrimination between live and dead cells since dead cells display a very high *R* value [13]. Therefore, in cytotoxic assays of lethal treatments of yeast, like acetic acid treatment, two sub-populations (corresponding to live and dead cells) are identified with Rh123 staining. The evaluation of the  $\Delta\Psi_m$ , expressed by the *R* value, was done in the cell sub-population with the lowest *R* value characteristic of live cells [13].

Plasma membrane integrity was assessed by propidium iodide (PI) staining, following a protocol modified from a previous described protocol [14]. Briefly, PI was added from a working solution (0.5 mg PI, Molecular Probes, Eugene, OR, USA, in 10 ml of Tris–MgCl<sub>2</sub> buffer) to a final concentration of 6.7  $\mu\text{g ml}^{-1}$ . The incubation was carried out at room temperature for 10 min in the dark.

### 2.7. Flow cytometric (FCM) assays

FCM analysis was performed on an EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, FL, USA) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green and red fluorescences were collected through a 488-nm blocking filter, a 550-nm/long-pass dichroic with a 525-nm/band-pass and a 590-nm/long-pass with a 620-nm/band-pass, respectively. Twenty thousand cells per sample were analysed. An acquisition protocol was defined to measure forward scatter (FS Log), side scatter (SS Log), green fluorescence (FL1 Log) and red fluorescence (FL3 Log) on a four-decades logarithmic scale. The data were analysed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0. Ratios between green fluorescence and forward scatter were performed off-line with Multiparameter Data Analysis Software, Multiplus AV (Phoenix Flow Systems, San Diego, CA, USA) as described before [13].

## 2.8. Reproducibility of the results

All the experiments were repeated at least three times independently and the data presented are mean values.

## 3. Results and discussion

### 3.1. Acetic acid in the range of 320–515 mM induces loss of *Z. bailii* cell viability

Assessment of the cell proliferative capacity, expressed by cfu, was used together with PI and Rh123 staining, to evaluate cell viability. As discussed below, these two fluorescent dyes allow to monitor cytoplasmic and mitochondrial membrane integrity, respectively.

After 130 min of treatment with 320 mM acetic acid only, 10% of *Z. bailii* cells were viable when assessed by cfu counts, and with 515 mM no viable cells were found (Fig. 1).

PI has often been used as a probe for non-viable cells. Dead or dying cells with injured plasma membranes can incorporate PI (PI(+) cells), which stains double-stranded nucleic acids [15]. Our results with PI staining show that the acetic acid effect on plasma membrane integrity of *Z. bailii* increased with the increase of acid concentration. After 130 min of treatment with 320 mM acetic acid only about 20% of the cells were PI(+), while treatment with 515 mM acetic acid resulted in 50% of PI(+) cells. On the other hand, and as reported before [13], Rh123 can be used as a probe to discriminate live from dead cells. Yeast cells killed by boiling for 10 min or treated with 10% (v/v) acetic acid for 10 min show a diffuse and more intense fluorescence than control, untreated cells, as expressed by a higher *R* value ([13]; see also Section 2). The diffuse, high-level, cytoplasmic fluorescence displayed by dead cells is due to the loss of mitochondrial membrane integrity and to an inability to specifically accumulate Rh123 in mitochondria under these conditions [13]. Treatment of *Z. bailii* with 320 or 515 mM acetic acid for 130 min did not

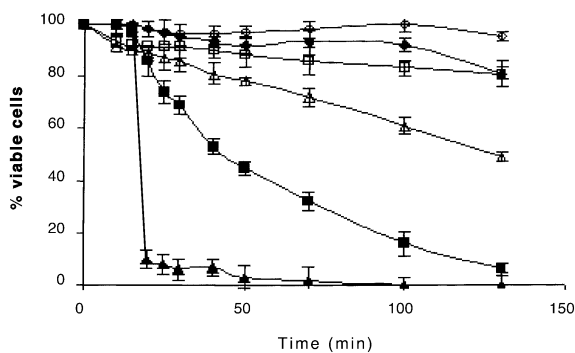


Fig. 1. Percentage of cfu (closed symbols) and of PI(-) (open symbols) *Z. bailii* cells in the absence (◆) or presence of 320 mM (■) or 515 mM (▲) acetic acid, pH 3.0.

change, compared with the control, the percentage of cells with *R* values typical of live cells (data not shown). This indicates that mitochondria of non-proliferating yeast cells in the samples exposed to acetic acid retained membrane integrity, and that, in this case, loss of cell-proliferative capacity can not be assessed by Rh123.

Comparison of the above reported results regarding assessment of cell viability by Rh123, PI and cfu counts reveal a great heterogeneity. The acetic acid effects on the cell-proliferative capacity were more pronounced than on the cellular parameters evaluated by Rh123 and PI staining. These observations point to the necessity of a definition of viability according to the method used, as was already proposed by several authors [16,17].

However, comparison between results of cfu counting, direct microscopic enumeration, and indirect activity measurements makes clear that the number of cells capable of forming colonies on a solid medium is always smaller than the number of individuals actually present and of metabolically active microorganisms.

The existence of a viable but non-culturable stage, in which cells are intact and alive but do not undergo cell division is a problem in determination of cell viability by cfu counts (see review [18]). Valentine and Bradfield [19] have proposed the term ‘viable’ to describe cells capable of multiplying and forming colonies, and the term ‘live’ for cells showing other signs of viability, such as respiration. In this line, other authors proposed the distinction between vitality and viability [20]. Taking into account these considerations, we can conclude that the results obtained by cfu counts in acetic acid-treated *Z. bailii* do not reveal the true percentage of metabolically active cells.

### 3.2. Cycloheximide enhances the survival of *Z. bailii* upon treatment with 170–515 mM acetic acid

In order to address the question if the cell death process induced in *Z. bailii* by acetic acid is an active process, which is characteristic of apoptotic death [10,21], we analysed the dependence of the acid-induced yeast killing on the de novo protein synthesis. The presence of the inhibitor of protein synthesis, cycloheximide, attenuated the toxic effects of 170–515 mM acid, enhancing the survival percentage (Table 1), an indication that such a cell death is an active process.

### 3.3. Acetic acid in the range of 320–800 mM induces DNA strand breaks as revealed by the terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL)-positive phenotype of *Z. bailii*

Our recent report [10] on the commitment of *S. cerevisiae* to a PCD process in response to exposure to specific concentrations of acetic acid raised the question if the more acid-resistant yeast *Z. bailii* could also commit sui-

cide and recognise acetic acid as an apoptotic stimulus. Two main alternative modes of cell death can be distinguished, namely PCD and accidental cell death, generally defined as apoptosis and necrosis, respectively. Numerous methods have been developed to identify apoptotic and necrotic cells based on changes in cell morphology, plasma membrane structure and transport, function of cell organelles and endonucleolytic DNA degradation [22].

The TUNEL reaction was used to investigate the occurrence of DNA fragmentation induced by acetic acid treatment in *Z. bailii* and to elucidate if an acid-induced PCD also occurs in this species. TUNEL-positive cells (see Fig. 2 for cells treated with 320 mM acetic acid) were seen after exposure to acetic acid at concentrations between 320 and 800 mM, suggesting that death due to those concentrations of acid would be due to an apoptotic process. On the other hand, in the samples of yeast cells killed by acetic acid above 800 mM, no TUNEL-positive cells were present, revealing that the killing process was by necrosis. This observation indicates that in *Z. bailii*, similarly to what was found for *S. cerevisiae* [10], above a given acid concentration, cells are not able to switch on a PCD process and are killed by necrosis.

To further investigate the apoptotic process induced by acetic acid in *Z. bailii* the following studies were carried out.

#### 3.4. Acetic acid induces mitochondrial dysfunction in *Z. bailii*

Since we had previously found that the PCD process induced by acetic acid in *S. cerevisiae* is mediated by a mitochondria-dependent pathway [23], we studied the mitochondrial function in *Z. bailii* cells exposed to that acid. This was done by the assessment of the mitochondrial membrane potential ( $\Delta\Psi_m$ ) using flow cytometry associated to the cell staining with a fluorescent dye, rhodamine (Rh123), as described before [13]. Acetic acid treatment with 320 and 515 mM rapidly affected the staining of *Z. bailii* cells with Rh123, resulting in a decrease of about 50% of the *R* value calculated as described in Section 2.

Table 1

Relative survival (% of cfu on YEPD agar plates; 100% corresponds to the number of cfu at time zero) of *Z. bailii* incubated for 20 min with acetic acid in the absence or presence of cycloheximide (50  $\mu\text{g ml}^{-1}$ )

Acetic acid (mM)	% cfu	
	+Cycloheximide	–Cycloheximide
0	100 $\pm$ 0	100 $\pm$ 0
170	90.3 $\pm$ 1.5	98.3 $\pm$ 2.9
320	77.7 $\pm$ 2.5	95.7 $\pm$ 2.1
515	8.00 $\pm$ 2.0	34.7 $\pm$ 4.5

A comparison by Student's *t*-test of the values for treatments in the presence or in the absence of cycloheximide (four independent experiments in triplicate) gave  $P < 0.05$  for the three acetic acid concentrations tested.

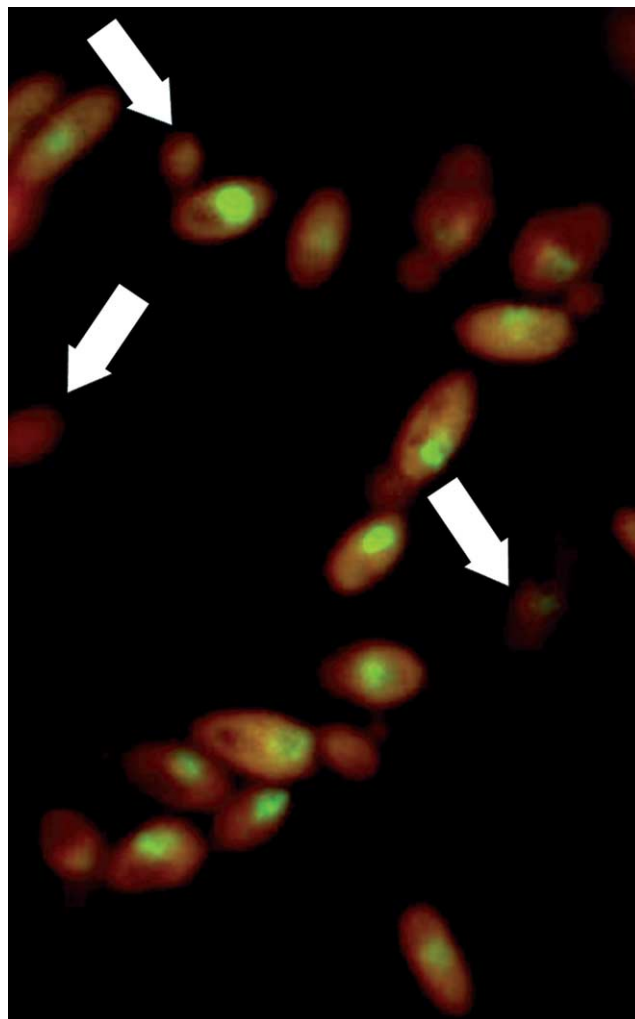


Fig. 2. Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL) reaction (evaluation of DNA strand breaks) after 130 min treatment of *Z. bailii* cells with 320 mM acetic acid. TUNEL-negative cells are indicated by arrows.

As validated in a previous work [13], this was interpreted as an indication of a  $\Delta\Psi_m$  decrease. Such a decrease was maintained for the 130 min of the study (data not shown). Thus, like in *S. cerevisiae* [23], the acid-induced PCD process in *Z. bailii* is accompanied by a mitochondrial depolarisation.

The findings of preserved mitochondrial function, although with markedly decreased  $\Delta\Psi_m$ , and of mitochondrial and plasma membrane integrity, observed when assessing cell viability, further support the results described above, pointing to the occurrence of an acetic acid-induced PCD process in *Z. bailii*. In fact, it is known that a cell triggered to undergo apoptosis activates a characteristic cascade of biochemical and molecular events but maintains, at least during the initial phase of the death process, plasma membrane structural integrity and function, and mitochondrial and lysosomal integrity, although with a markedly decreased  $\Delta\Psi_m$  [22,24–26].

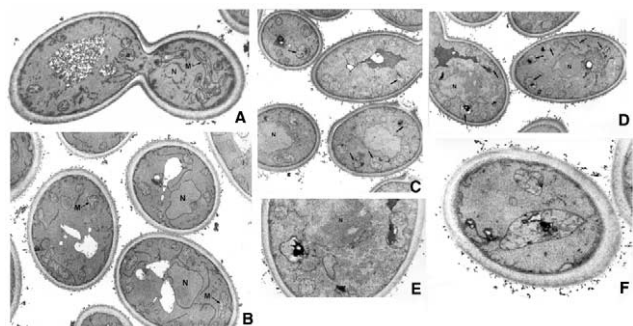


Fig. 3. Electron micrographs of exponentially growing cells of *Z. bailii*. A,B: Untreated control cells. Notice nuclei (N) and mitochondria (M) with a normal fine structure. C–F: Cells treated with 320 mM acetic acid for 10 min. C,D: Some nuclei (N) show chromatin condensation and most mitochondria are altered (arrows), with swelling, reduction of number of cristae and with myelinic bodies. E,F: Details of altered mitochondria. Note in E chromatin condensation in the nucleus (N). A:  $\times 22\,000$ ; B:  $\times 15\,800$ ; C:  $\times 17\,000$ ; D:  $\times 15\,800$ ; E:  $\times 38\,000$ ; F:  $\times 38\,000$ .

### 3.5. Acetic acid induces ultrastructural changes in *Z. bailii* cells

To further characterise the PCD process induced by acetic acid in *Z. bailii*, we investigated the ultrastructural changes induced by acid treatment. Already as after 10 min of treatment with 320 mM acid, most of the cells showed nuclei with chromatin condensation along the nuclear envelope (Fig. 3C–E), which is the most common morphological marker displayed by apoptotic cells (e.g. [22]). Interestingly, the affected cells exhibited mitochondrial ultrastructural alterations, namely reduction of the cristae number, with swelling probably resulting from solute accumulation and the presence of myelinic bodies. However, mitochondrial membranes seem to maintain their structural integrity (Fig. 3C–F) which is in accordance with our Rh123 results. After 130 min of treatment the ultrastructural changes affected a higher percentage of cells, and with nuclei showing extensive chromatin condensation (not shown).

Although the nuclear chromatin condensation had already been reported in *S. cerevisiae* under PCD processes [10,21,27], the mitochondrial ultrastructural alterations detected in the present study were not found in those reports regarding *S. cerevisiae*, due probably to the low respiratory capacity of this yeast when grown under the conditions tested. Our recent results [23] have made clear that, in the PCD process induced by acetic acid in *S. cerevisiae*, important molecular events occur in mitochondria, namely loss of cytochrome *c* and production of reactive oxygen species. Since it is conceivable that also in the PCD process induced by acetic acid in *Z. bailii* such mitochondrial molecular events occur, the mitochondrial ultrastructural alterations now seen in *Z. bailii* may well represent the morphological counterpart of these biochemical events.

The results here reported show that, similar to what has

been recently described for *S. cerevisiae* [10], acetic acid induces in *Z. bailii* either an apoptotic or a necrotic death process, depending on the dose. However, in *Z. bailii* the PCD process was found to occur at higher acetic acid concentrations (320–800 mM, as compared to 20–120 mM for *S. cerevisiae*), which is consistent with the known higher resistance of *Z. bailii* compared to that of *S. cerevisiae*. Why *Z. bailii* is able to undergo a PCD process with acetic acid concentrations known to be necrotic for *S. cerevisiae* is a question that remains to be answered. We can speculate that the lower plasma membrane permeability to the acid of *Z. bailii* as compared to that of *S. cerevisiae*, and the ability of *Z. bailii* to metabolise acetic acid under aerobic conditions [4], are physiological traits that underlie the higher acetic acid concentration required to trigger apoptosis in *Z. bailii*.

Hence, further studies are being carried out in *Z. bailii* in order to elucidate the biochemical changes underlying the observed mitochondrial morphological alterations and to compare them with the biochemical dysfunctions previously reported in *S. cerevisiae* mitochondria [23].

The observation that acetic acid-induced PCD can occur not only in *S. cerevisiae* but also in *Z. bailii* further reinforces the concept of a physiological role of the PCD in the normal yeast life cycle and raises the possibility of this mode of cell death being more generalised in yeast than previously considered. Moreover, advances in the understanding of the mechanisms of death induced by acid stress in yeast will allow the development of new categories of preservatives for food and beverages with the consequent reduction of economic losses in the food industry.

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