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ORIGINAL PAPER

Single-cell analysis of *S. cerevisiae* growth recovery after a sublethal heat-stress applied during an alcoholic fermentation

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Abstract Interest in bioethanol production has experienced a resurgence in the last few years. Poor temperature control in industrial fermentation tanks exposes the yeast cells used for this production to intermittent heat stress which impairs fermentation efficiency. Therefore, there is a need for yeast strains with improved tolerance, able to recover from such temperature variations. Accordingly, this paper reports the development of methods for the characterization of *Saccharomyces cerevisiae* growth recovery after a sublethal heat stress. Single-cell measurements were carried out in order to detect cell-to-cell variability. Alcoholic batch fermentations were performed on a defined medium in a 2 l instrumented bioreactor. A rapid temperature shift from 33 to 43°C was applied when ethanol concentration reached 50 g l^{-1}. Samples were collected at different times after the temperature shift. Single cell growth capability, lag-time and initial growth rate were determined by monitoring the growth of a statistically significant number of cells after agar medium plating. The rapid temperature shift resulted in an immediate arrest of growth and triggered a progressive loss of cultivability from 100 to 0.0001% within 8 h. Heat-injured cells were able to recover their growth capability on agar medium after a lag phase. Lag-time was longer and more widely distributed as the time of heat exposure increased. Thus, lag-time distribution gives an insight into strain sensitivity to heat-stress, and could be helpful for the selection of yeast strains of technological interest.

Keywords Yeast · Heat stress · Growth recovery · Single-cell · Image analysis

Introduction

For economical and environmental motivations, interest in bioethanol production has experienced a resurgence in the last few years. At the industrial scale, *Saccharomyces cerevisiae* remains the most commonly used microorganism for ethanol production [1]. Large-scale fermentations are carried out with less stringent process control than laboratory studies. In industrial fermentation tanks, mixing can be imperfect and undesirable situations may arise [2]. For example, poor temperature control exposes the yeast cells to intermittent thermal stress and subsequently impairs fermentation efficiency. Therefore, it is important to select yeast strains able to recover from such temperature variations. Many papers related to *S. cerevisae* heat-induced damage (decrease in viability, cultivability and ethanol production, etc.) or heat responses (modifications of membrane composition and fluidity, changes in proteomic and genomic expression profiles, etc.) have been published. However, very few works about growth recovery of heat-injured cells have been presented.

Microorganisms naturally exhibit phenotypic heterogeneity [3]. This heterogeneity can lead to different sensitivity to stress within a population [4, 5], and this is particularly observable when cells are cultivated in unfavorable conditions. For example, Plesset et al. [6] have demonstrated that *S. cerevisiae* thermotolerance could depend on the position in the cell cycle. Surprisingly, cell-to-cell variability is

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rarely taken into account, since only averaged population parameters are usually measured [7]. Phenotypic heterogeneity can be rapidly and efficiently quantified using flow cytometry techniques, and populations can be analysed according to different parameters such as cell size, membrane integrity and intracellular pH. Cells fulfilling the same criteria are considered as identical. However, the main limitation of flow cytometry is the lack of kinetic information at the single-cell level, each cell being detected once at a defined time, making this method unsuitable for the study of growth recovery. The development of methods that enable cell growth monitoring at the single-cell level can overcome this limitation.

The number of developments in this field is relatively limited. Different parameters can be measured such as lag-time, time of first division, time necessary to reach a certain colony size, doubling-time and growth rate. Measuring individual parameters with a statistically relevant number of cells allows establishing cell state distributions over an entire population. These methods have been used to study the effect of various environmental stresses on bacterial model suspensions.

The use of a liquid medium enables the researcher to reproduce culture conditions close to those observed in a bioreactor. Growth carried on in a microtiter plate can be monitored at macroscopic scale using optical density (OD) measurements. A preliminary sorting-step is required to inoculate each well with a single cell, involving the use of specific equipment such as a sorting cytometer [8] or a lab-on-chip microfluidic system [9]. An alternative solution is to use serial dilutions of the cell culture sample until a high probability of inoculating each well with a single cell is reached [10]. Growth in a liquid medium can also be monitored using microscopy observations. The perfusing chamber developed by Elfwing et al. [11] and used by Pin and Baranyi [12] enable the researcher to feed the culture continuously with a fresh medium since cells are attached on a support. Cell division is detected by a sudden decrease in cell area when a daughter cell is released from the mother cell. This device enables the observation of cells with a broad range of doubling-time, since neighbouring micro-colonies cannot overlap. However, the effect of cell attachment on growth kinetics was not investigated.

There are also several reports of the use of solid media. The growth of colony-forming units after agar medium plating can be monitored at a macroscopic scale using image analysis [13]. Colonies are detectable only once they reach a threshold size, after several generations. Microscopy enables the researcher to observe cell growth during the first generations after plating. The use of a solid medium seems easier to carry out than the use of a liquid medium. Additionally, the results obtained on agar medium can be similar to those obtained during broth cultivations [13, 14].

The purpose of this work was to develop and evaluate methods for characterizing the growth recovery of a *S. cerevisiae* population after a sublethal heat stress applied during alcoholic fermentation. This study was part of a global program aiming at the selection of high tolerance strains for industrial bioethanol production from lignocellulosic materials [15]. To take into account cell-to-cell variability, individual lag-time and initial growth rate have been determined by monitoring the growth on agar medium of a statistically significant number of cells (100–2,000), at both macroscopic scale (colony growth after Petri dish plating) and microscopic scale (cell division on slide-cultures). To our knowledge, this is the first report of the use of such methods to characterize a yeast population during the course of fermentation.

Materials and methods

Microorganism, media and growth conditions

The *S. cerevisiae* strain, supplied by courtesy of the Lesaffre company (France), was stored on LB agar supplemented with 0.2% glucose at 4°C. Two successive precultures were carried out in Erlenmeyer flasks containing a mineral medium previously described [16] with 50 g l^{-1} of glucose (12 h on a rotary shaker (150 rpm) at 33°C, initial pH 4.5). Batch fermentations were performed in a 2 l instrumented bioreactor (SGI, France) with the same mineral medium containing 200 g l^{-1} of glucose. Operating conditions were: temperature 33°C, pH 4 controlled using a 14% (V/V) NH_4OH solution, stirring rate 400 rpm and dissolved O_2 50% of air saturation using pure O_2 sparging with a mass flow controller [16, 17]. All fermentations were performed with an exponential supply of vitamins based on the growth profile [18] assessed by on-line OD measurements.

When ethanol reached 50 g l^{-1}, a temperature shift from 33 to 43°C was applied and samples were collected at different times for off-line analysis.

Fermentation monitoring

A biomass optical probe (model 653/BT65, Wedgewood, USA) was used to monitor the total biomass. A calibration against biomass dry weight was done and the optical density was expressed as equivalent biomass dry weight ($OD_{eq\ DW}$).

The bioreactor and the flask of NH_4OH solution were placed on scales (Sartorius, Germany, and Precisa, France). The residual glucose and the produced ethanol concentrations were calculated from CO_2 weight loss taking into account the sampling and the addition of vitamins and

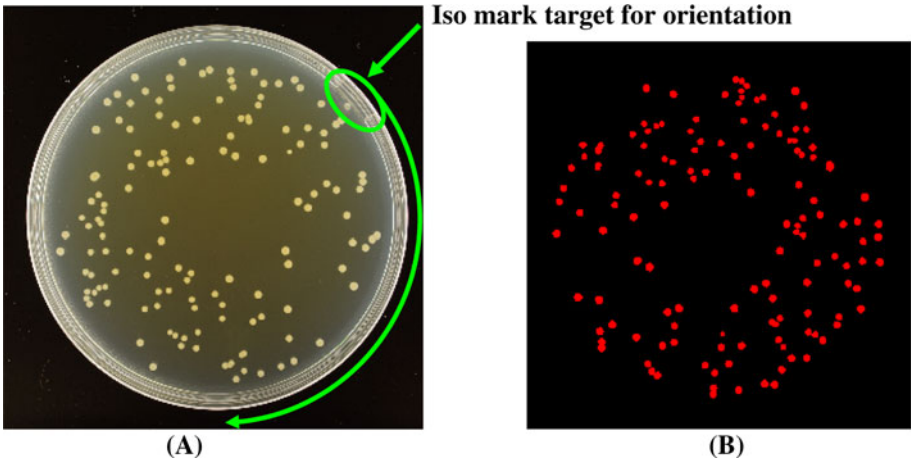

Fig. 1 An example of Petri dish image processing: (**a**) initial color image; (**b**) final binary image

NH_4OH solution. The yields $Y_{EtOH/CO2}$ (ethanol/carbon dioxide) and $Y_{EtOH/Glu}$ (ethanol/glucose) were adjusted by off-line HPLC analysis (column: Aminex HPX-87H, temperature: 50°C, eluant: H_2SO_4 5 mM, flow rate: 0.5 ml min^{-1}, detector: refractometer).

Cultivable cell counts were obtained by plate counting on agar medium (concentrations expressed in g l^{-1}: tryptone, 5.0; yeast extract, 2.5; glucose, 1.0 and agar, 12.0) using an automated spiral plater (Wasp, AES, France). Petri dishes were incubated at 30°C until a constant number of colonies was attained (2–7 days). Colony forming units (CFU) were counted using an automated image analysis program developed in house using LabVIEW and the Vision Assistant Module (National Instruments, USA). Cultivability was defined as the ratio between CFU and total cell count obtained using an automated cell counter (CASY, Roche Innovatis, Germany).

Individual growth observation by image analysis

At macroscopic scale: colony growth on agar plates

Only Petri dishes with 100–200 CFU were considered, a compromise between a statistically relevant number of observations and the risk of interference between neighboring colonies.

Image acquisition of plates was performed daily for 7 days using a color camera (Pentax K10D, equipped with a Pentax PH-RCB 49 mm lens, Japan) installed on a support.

Images were processed automatically using LabVIEW and the Vision Assistant Module (National Instruments, USA). From a series of images of a Petri dish snapped at different times of incubation, image analysis enabled us to monitor individual colony growth. The different steps of image processing are summed up below.

- First, the original color image (Fig. 1a) was converted to a grayscale image by the extraction of the red layer, which gave the best resolution between objects (colonies) and background (agar medium).
- Then an adaptive threshold algorithm (background correction) was used to recognize the Petri dish outline.
- Accordingly, a mask was created to keep only the inner part of the Petri dish.
- The image was then reoriented: a pattern matching algorithm was used to locate a mark on the Petri dish, which enabled us to reorient images in a reference position (Fig. 1a). This procedure allowed us to snap images of plates manually, so that the number of plates simultaneously studied was not limited by the use of a motorized platform such as the one used by Guillier et al. [13].
- A Niblack binarization algorithm [19] was used to separate colonies from agar medium (Fig. 1b).
- Each colony's coordinates and radius were measured and recorded.
- Finally, each colony size was plotted against the time of incubation. Isolated colonies were recognized according to various circularity factors. Only isolated colonies on the last image of a series were considered. Confluent colonies, whose growth kinetics were mutually affected, were eliminated from the analysis.

At microscopic scale: microcolony formation on microscope slides

A reusable silicon spacer (CoverWell Imaging Chamber, Grace Bio-Labs, USA) was sealed on a microscope slide. It formed a 19 × 32 × 2 mm well, which was filled with 850 µl of agar medium (concentrations expressed in g l^{-1}: tryptone, 5.0; yeast extract, 2.5; glucose, 1.0 and agar, 12.0). After a 1/10 dilution in 9‰ NaCl, 2.5 µl of cell culture was spread onto the agar medium and a cover slip was applied over the well to avoid dehydration.

Slides were incubated at 30°C and phase contrast microscopy observations at ×200 magnification were

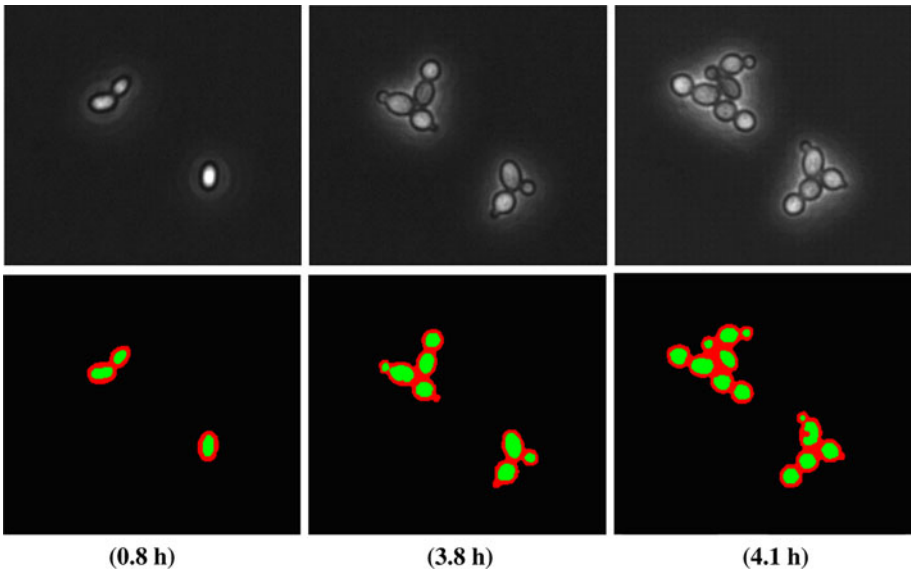

Fig. 2 An example of image processing focused on the growth of two neighbouring microcolonies. *Upper row*: original greyscale images; *lower row*: corresponding processed images

performed every 1 h for 8 h, using a BX51 microscope (Olympus, Japan), equipped with a motorized microscope stage (model SCAN 100 × 80 BX, Märzhäuser, Germany) and a 1600 × 1196 B/W video camera (CV-M2, JAI, Denmark). Positioning, focusing and image acquisition were automated using LabVIEW (National Instruments, USA). Images of 50 adjacent fields were snapped with a spiral motion from a reference position.

Automated positioning enabled us to study several microscope fields and several slides simultaneously, the slides being incubated in a classical incubator and taken out only for microscopy observations. Image analysis was automated using LabVIEW and the Vision Assistant Module (National Instruments, USA). The different steps of image processing, which enabled us to monitor the number of cells within each microcolony over the time of incubation, are summed up below.

- First, a Laplacian filter was used to enhance edge strength between cells and background.
- Then an adaptive threshold algorithm (background correction) was used to recognize cell outlines, which were the darker parts of the resulting image.
- Finally, the number of cells within each microcolony, as assessed by the number of holes (Fig. 2), was plotted against time.

We had to manage overlapping microcolonies and small shifts between successive images due to agar medium contraction over time. Starting from the first image of a series, for each microcolony, the closest microcolony was located on the following image. When two microcolonies had the same closest microcolony on the following image, they were eliminated from the analysis. This procedure enabled us to eliminate overlapping microcolonies and border microcolonies that could appear or disappear from the field observation. Typically, the growth of 2,000 individual cells per culture sample was monitored.

Growth recovery at population scale: culture in microtiter plates

Microtiter plate assays were carried out to study growth recovery of heat-injured cell populations in broth. A sample of cell culture medium was collected just before applying the heat stress and centrifuged (3000 g, 10 min). At this point ethanol and glucose concentrations were respectively 50 g l^{-1} and 100 g l^{-1}. The resulting supernatant was inoculated at 1% (V/V) with cell suspensions sampled at different times after the temperature shift. The microtiter plate was incubated at 33°C with a linear agitation (5 mm amplitude), and growth was monitored by OD measurements (620 nm) using a microtiter plate incubator and reader (Infinite 200M, TECAN, Switzerland).

Results

Stress model fermentations

The results of a typical batch culture of *Saccharomyces cerevisiae* with 200 g l^{-1} initial glucose concentration are presented in Fig. 3a. According to on-line OD and ethanol measurements, growth and metabolic activity became stationary after 14 h, when the ethanol concentration reached a maximal value of 80 g l^{-1}. The main limiting factor was substrate depletion, rather than ethanol inhibition, since growth was immediately restored after glucose addition at the end of the fermentation (data not shown). No appreciable loss of cell cultivability was observed over the fermentation course.

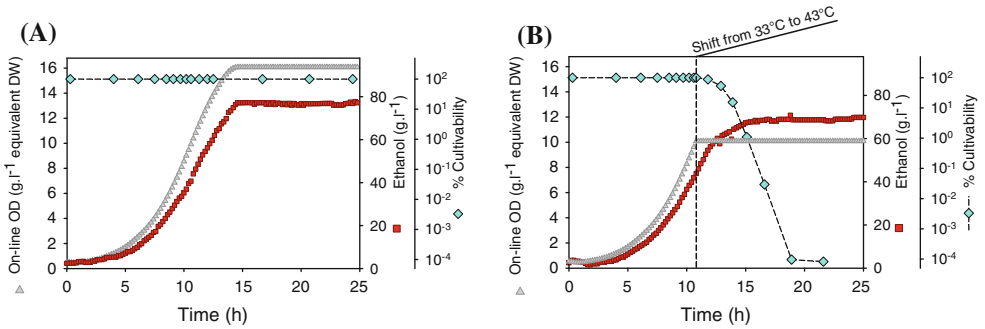

Fig. 3 Alcoholic batch cultures of *S. cerevisiae* with 200 g l^{-1} glucose. (**a**) standard culture at 33°C without perturbation; (**b**) stress condition culture with a shift of temperature from 33 to 43°C when ethanol concentration reached 50 g l^{-1}

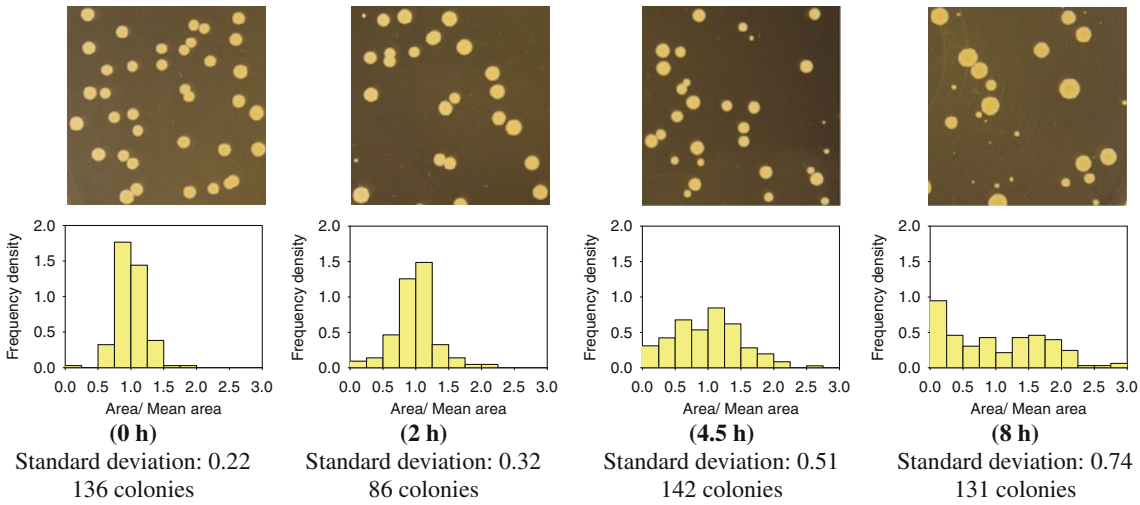

Fig. 4 Colony size distributions observed on Petri dishes after different times of exposure at 43°C

A different result was obtained during a second experiment, during which the culture was exposed to a heat stress by raising the fermentation temperature from 33 to 43°C when the ethanol concentration reached 50 g l^{-1}. This moment, close to the maximum fermentation rate, is similar to temperature control problems in an industrial environment. The temperature shift led to an immediate arrest of growth and an early cessation of fermentative activity, although glucose was not entirely depleted (Fig. 3b). The total biomass as assessed by OD measurements and automated cell counts (data not shown) remained constant during the subsequent fermentation period, whereas a progressive decrease of cultivability of 6 log within 8 h was measured. Cell death could not be modeled using a first order kinetic, since ethanol concentration increased from 50 to 70 g l^{-1} within the same period. Indeed, ethanol is known to intensify temperature damage [20–23].

Based on Petri dishes observations, different behaviors were observed during these fermentations. When the fermentation temperature was maintained at 33°C, colony size distribution on Petri dishes was homogeneous, but when the temperature was raised to 43°C, a broad heterogeneity in terms of colony size was observed (Fig. 4). This heterogeneity increased with the duration of exposure at 43°C, as shown by the standard deviation of the normalized colony area, which increased from 0.22 for a non-stressed population to 0.74 for a 8 h exposed population. The causes of this heterogeneity in colony size were investigated. Initially, replica plating on agar medium containing only glycerol as a carbon source showed that the small colonies did not correspond to respiratory deficiency [24]. Additionally, no size difference could be observed after re-inoculation and growth on LB-glucose agar, whatever the original colony size. Therefore, a genotypic variation could be ruled out. Then, a method based on computer image analysis of colony growth was developed to determine if small colonies were caused by a longer lag-phase and/or a slower growth rate.

Monitoring individual growth on agar medium at macroscopic scale

The first step was to follow the colony development as a function of the culture exposure time at 43°C. Images of

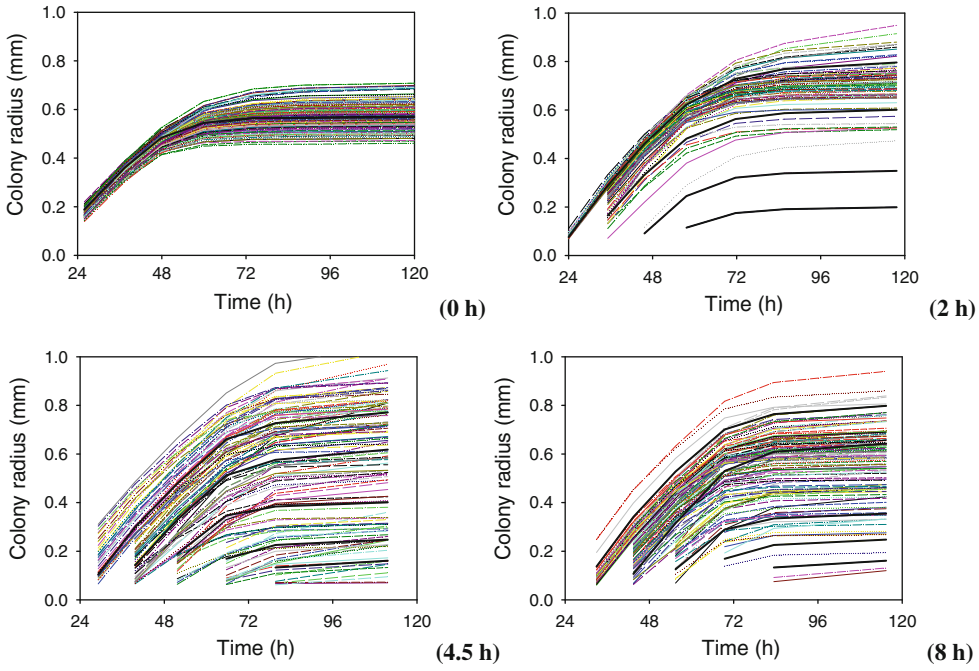

Fig. 5 Radial growth of colonies observed on Petri dishes after different times of exposure at 43°C. Growth *curves* representative of a series of daily measurements have been highlighted in *thick lines*

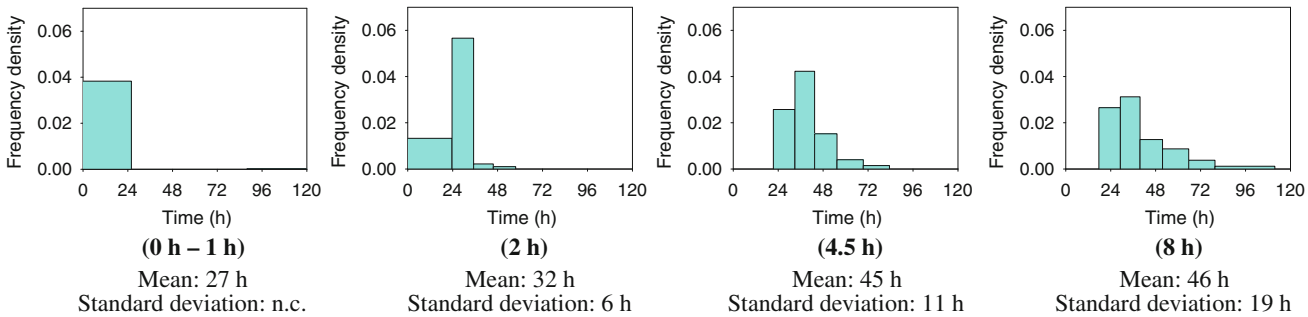

Fig. 6 Time of apparition of colonies on Petri dishes after different times of exposure at 43°C

Petri dishes were snapped daily for 7 days. From a series of images of a Petri dish at different times of incubation, individual colony growth was monitored using automated image analysis.

Two ways of expressing colony size were evaluated: volume and radius. Volume evolution, as assessed by cubed radius, did not show any exponential phase. This means that just after colony detection, growth rate was already below the maximum growth rate and decreasing over the time of incubation. Within a colony, it is widely accepted that cells grow essentially at the periphery, while cells in the centre of the colony gradually enter a stationary phase as the colony size increase [25]. Assuming a constant width of this peripheral zone, Pirt proposed a model to describe the linear radial growth of colonies on solid media [26] that seems to be well adapted to the present culture conditions.

Accordingly, individual colony radial growth of cell suspensions sampled after different times of exposure at 43°C is presented in Fig. 5. Non-stressed cells grew into detectable colonies simultaneously within a 24 h period. In contrast, stressed cells displayed a delay before growth varying from 1 to 3–4 days. Once colonies became detectable, they all grew at the same rate, as shown by the parallelism of the growth curves within a certain period. Growth rate progressively decreased over time, most likely due to increased mass transfer limitations related to agar medium drying and substrate depletion. Thus, measuring colony growth rates could be quite imprecise and lag-time was preferred as an indicator of cell state.

Lag-time distributions, assessed by the time of colony detection, are presented Fig. 6. The mean and the standard deviation of the subsequent distributions increased with the time of exposure at 43°C. But this delay and variability of growth recovery was not observable after a 1 h exposure time at 43°C. However, shorter heat exposure appeared to have a strong effect on cell state, since an immediate arrest of growth was observed in the bioreactor after the temperature elevation (Fig. 3b). It seemed worthwhile to study the effect of short heat stress using a higher sensibility method.

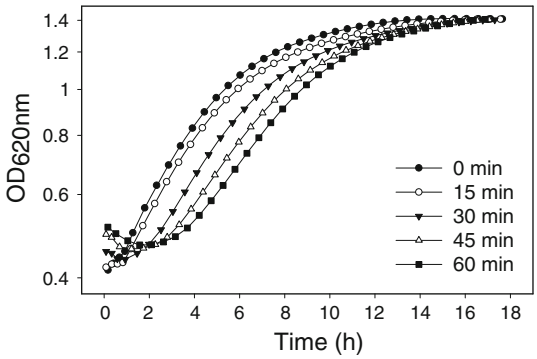

Fig. 7 Effect of short times of exposure at 43°C (0–60 min) on population growth recovery in liquid medium. Growth was monitored using OD measurements (logarithmic scale) in a 96-well microtiter plate

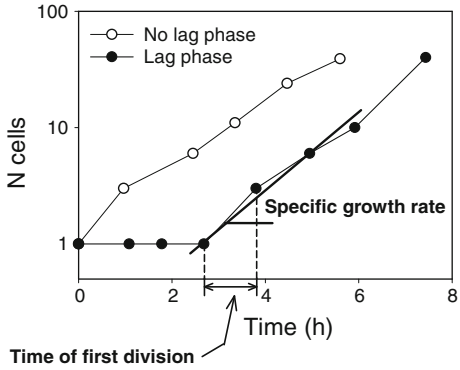

Fig. 8 An example of two growth curves obtained using slide-cultures and a description of the measured parameters

Monitoring growth recovery in liquid medium at population scale

Microtiter plate assays were carried out to study growth recovery in broth after short times of exposure at 43°C. The liquid medium was inoculated at 1% (V/V) with cell suspensions sampled at different times after temperature shift (0–60 min). Growth was monitored by OD measurements. The results are presented in Fig. 7. Non-stressed cells grew immediately after inoculation without a lag phase. For stressed cells, an increasing lag-time of 0.5 to 2–3 h was observed after 15–60 min exposure at 43°C. Similarly to the observations made on solid medium, the lag-time increased with the time of exposure at 43°C, but after growth restoration all cultures grew at the same rate.

A 1 h exposure at 43°C had an obvious effect on cell growth recovery, but the resulting 3 h lag-phase could not be observed using colony growth monitoring at the macroscopic scale, since the colonies were detectable only once they reached a 0.1 mm radius size, after about 24 h of incubation. Therefore, the time resolution of this method was not sufficient to measure lag-times shorter than 24 h.

In addition, OD growth measurements in microtiter plates corresponded to average values over the entire cell population. Further investigations were carried out to determine if the measured lag-time after a short heat exposure resulted from a lag-time distribution within the cell population, as previously observed at the single-cell level for longer heat exposure.

Monitoring individual growth on agar medium at microscopic scale

Slide-cultures were carried out to monitor individual growth during the first generations. Microscope slides coated with agar medium were inoculated with two different cell suspensions: one sampled just before temperature shift and the other after a 1 h exposure at 43°C. Images of 50 fields were snapped every hour for 8 h and automated image analysis was used to monitor the growth of each microcolony. Microcolony area [14] or bounding-rectangle area [27, 28] can be easily measured using image analysis. However, those measurements are based on the assumption that cell size and cell shape remain unchanged over the observation period. Counting the number of cells within each microcolony [29] requires advanced image processing, but is preferable. Accordingly, automated image analysis software was developed in order to plot the number of cells within each microcolony against the time of incubation.

From the growth profiles, two variables were measured: the time of first division and the initial specific growth rate. As shown in Fig. 8, the time of first division corresponds to the interval in which an increase in the initial number of cells was observed. Specific growth rates were calculated using the first four points of the growth curves starting from growth recovery.

Growth parameter distributions of stressed and non-stressed cells are presented in Fig. 9. For non-stressed cells, immediate growth was observed after plating (Fig. 9a), whereas cells exposed 1 h to 43°C exhibited a delay in growth of about 2 h after plating (Fig. 9c). In comparison with the microtiter plate assays, the mean lag-time of 2 h is consistent with the lag-time measured in broth. Deeper analysis of the distribution curves showed that the delayed growth concerned a large majority of the cell population. However, a small proportion of cells (10%) was not affected by heat exposure, and grew without lag phase. The mean initial specific growth rate was slightly affected by the exposure to 43°C, and a larger deviation was observed for exposed cells (Fig. 9b–d). However, no correlation between lag-time and initial growth rate could be found.

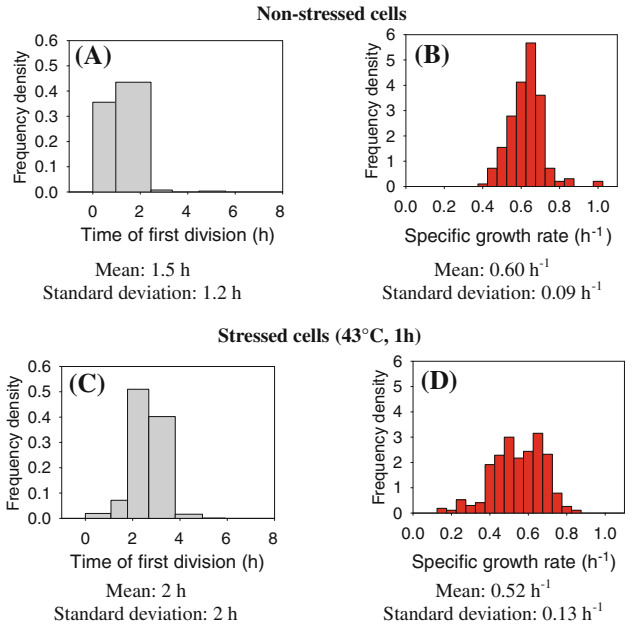

Fig. 9 Growth parameter distributions of non-stressed and stressed cells measured using slide-culture microscopy image analysis. About 2,000 individual cells were observed for each culture sample

Discussion

When cells were exposed to a 43°C heat-stress in the presence of 50 g l^{-1} of ethanol, midway during the fermentation course, one part of them died, whereas the growth of the other part was halted. When exposed to favourable culture conditions, these cells were able to recover their growth capability after a lag phase. Longer lag-times, more widely distributed, were observed as the time of exposure at 43°C increased. These observations are consistent with previous works applied to bacterial populations (*Salmonella*, *Lactobacillus*, *Bacillus*, *Escherichia*) in various environmental stress conditions such as heat stress [8, 10, 28], storage at 4°C [29] and salt stress [13, 14, 28]. In the present work, once cells recovered their capability to divide again, they all grew at the same rate, as shown by Petri dish plating and microtiter plate assays. Detection of heterogeneity in the initial growth rates was possible only when using slide-cultures during the first divisions after growth recovery.

The main sources of phenotypic heterogeneity mentioned in the literature are cell-cycle position and cell ageing [3]. Stationary-phase cells are generally regarded as more stress-tolerant than exponential-phase cells [30]. According to some authors, the stationary phase in *S. cerevisiae* triggers a quiescent state, which corresponds to cells that have left the normal cell-cycle sequence and entered the G0 phase. But the mechanisms involved in the entrance into the G0 phase are not clearly elucidated, and its nature is still under discussion [31]. For Plesset et al. [6] there is always a fraction of cells in the G0 phase, including during the exponential phase, resulting in a better thermotolerance for these cells. According to Elliott and Flutcher [32], thermotolerance is inversely proportional to growth rate, independently from cell-cycle position. In order to assess the effect of cell-cycle position on growth recovery after a heat exposure, we compared the lag-time and growth rate distributions of initially budding (60% of cell population) and initially non-budding cells (40% of cell population), measured using microscopy image analysis of slide cultures. Since these distributions were not significantly different, the effect of cell cycle position could not be detected. Cell ageing could have also been involved, but its effect on growth kinetics could not be studied, since the markers commonly used for bud scars staining, such as calcofluor [33], affect cell viability.

Heat-injured cells recovery has been studied in previous works. Trehalose, which is synthesized by yeast in response to heat-stress [34], has to be hydrolyzed after a heat exposure to enable cell recovery [34–36]. The recovery phase is associated with apparent changes in cell structures, such as vacuole [37] and nucleus [38]. However, damage repair mechanisms remain unclear, and the reasons some cells recover more rapidly than others are still misunderstood.

Since heat exposure during a fermentation course triggered an immediate arrest of growth, cell growth recovery after a heat-stress should be considered for selecting strains of technological interest. Microtiter plate assays are easy to carry out, enabling researchers to measure the effect of short time heat exposure, and biotechnology laboratories are commonly equipped with microtiter plate readers. However, since growth measurements correspond to average values over the cell population, they fail to detect cell-to-cell variability. Individual growth monitoring on agar medium enabled us to quantify the heterogeneity of growth recovery after heat exposure. Since lag-time distributions are related to stress severity and cell damage, they could be used as markers of cell population sensitivity.

Monitoring the growth of colony-forming units after Petri dish plating requires little equipment (a camera), enabling us to measure long lag-times (up to several days) and to study cell populations with a very low survival rate. Automation made Petri dish image analysis fast and easy. However, since colonies become detectable only once they reach a 0.1 mm radius, after about 12 generations, macroscopic colony growth on solid medium is subjected to strong diffusion limitations for nutrients. Microscopy enabled us to observe cell growth during the first 5 generations after plating, avoiding the diffusion limitations mentioned above. To avoid drying of the agar medium requires covering the microscope slide with a cover slip, which can

Table 1 Comparison of the techniques used to compare cell recovery after heat stress

	Microtiter plate	Petri dish	Slide culture
Medium	Liquid	Solid	Solid
Observation scale	Population	Individual	Individual
Growth monitoring principle	Optical density	Colony radius (camera image analysis)	Number of cells (microscopy image analysis)
Response time	0–24 h	1–7 days	0–24 h
Survival rate range	100–10%	100–0.001%	100–50%
Observed population doublings	0–5	>12	0–5
Software	Standard	Custom	Custom
Hardware requirements	Microplate incubator/reader	Camera + automatic plater (optional)	Automated microscope + camera
Ease of implementation	+++	++	+
Main advantages	High sensitivity (measures the effect of short stress exposure)	Wide range of cell population states (lag-time, survival rate)	Observation before colony growth is affected by diffusion limitations for nutrients. Single cell growth rates
Main drawback	Average measurement: no information on population heterogeneity	No direct growth rate information since substrate diffusion limitation occurs rapidly after colony detection	Neighbouring microcolonies can overlap

cause oxygen limitation. In fermentative conditions, *S. cerevisiae* requires oxygen traces (about 1 mg of oxygen per 1 g of biomass dry weight) to synthesize some of its membrane lipids. Oxygen transfers between air and agar medium were not possible after the cover slip was applied. Thus, the agar medium had to contain a sufficient amount of oxygen before inoculation. The time required to return to oxygen saturation (1 h) was evaluated from theoretical considerations (for details, see "Appendix"), and the experimental procedure was adapted to avoid any risk of oxygen limitation.

Despite this precaution, the observation period was limited to 24 h because fast-growing cells rapidly overlapped non- or slow-growing cells, a limitation to the use of this method to the study of a few injured cells with a high survival rate and a short lag phase. Besides, in spite of an extensive automation of image acquisition and processing, slide-cultures remained labor-intensive. A comparison of the main features of these complementary methods is presented in Table 1. These methods will be used for future work to quantify the impact of stress factors and to help the selection of highly stress-tolerant yeast strains for bioethanol production. Selection criteria will be: survival rate (CFU), lag-time measured using microtiter plate assays and lag-time distribution established using individual growth monitoring at macroscopic scales.

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