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Nanoscopic Morphological Changes in Yeast Cell Surfaces Caused by Oxidative Stress: An Atomic Force Microscopic Study

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Nanoscopic changes in the cell surface morphology of the yeasts *Saccharomyces cerevisiae* (strain NCYC 1681) and *Schizosaccharomyces pombe* (strain DVPB 1354), due to their exposure to varying concentrations of hydrogen peroxide (oxidative stress), were investigated using an atomic force microscope (AFM). Increasing hydrogen peroxide concentration led to a decrease in cell viabilities and mean cell volumes, and an increase in the surface roughness of the yeasts. In addition, AFM studies revealed that oxidative stress caused cell compression in both *S. cerevisiae* and *Schiz. pombe* cells and an increase in the number of aged yeasts. These results confirmed the importance and usefulness of AFM in investigating the morphology of stressed microbial cells at the nanoscale. The results also provided novel information on the relative oxidative stress tolerance of *S. cerevisiae* and *Schiz. pombe*.

Keywords: Atomic force microscopy (AFM), yeast morphology, yeasts physiology, oxidative stress, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, premature ageing of cells

Yeast cells growing in nature and in industrial bioreactors may be faced with a variety of chemical stresses, such as ethanol toxicity, starvation, oxidative stress, pH shock, and metal-ion stress [1]. When yeast cells, such as baker's yeast, *Saccharomyces cerevisiae*, are grown in aerobic conditions, they are continuously exposed to reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-). ROS can be generated either endogenously by incomplete reduction of oxygen to water in the mitochondrial respiratory chain, or exogenously by exposure to UV, metal ions, and redox active agents [7].

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In all living cells, ROS can cause oxidative damage to lipids (lipid peroxidation), proteins (protein oxidation), and DNA [5, 12, 19]. These oxidative damages can affect cellular physiology and morphology [3] by disruption of cellular functions and structural integrity.

Yeasts develop antioxidant defences, such as enzymes (superoxide dismutase, catalase, cytochrome C peroxidase, and glutathione reductase) and chemicals (glutathione, metallothionein, thioredoxin, and polyamines) that can protect cells against oxidative damage caused by detoxifying active oxygen and scavenging free radicals [12, 20, 21]. When the concentration of ROS exceeds the antioxidant capacity of the cell, oxidative stress occurs [37].

Oxidative stress is not only responsible for several human diseases, but it can also be highly detrimental to the fermentative capacities of yeasts in making bread, wine, beer, and other alcoholic beverages, leading to a poor quality of the final product. Several studies have been carried out to better understand the oxidative stress response of brewing yeasts during industrial processes [26, 33]. Oxidative stress is one of the main causes of stress responses during the production of wine yeast biomass [33]. In addition, it has been shown that the resistance to oxidative stress is strain dependent and affected by the media used for yeast cultivation and the growth phase, with stationary phase yeasts being more resistant than exponential phase cells [21]. A deeper insight into yeasts' responses to oxidative stress, the mechanisms behind sensing this stress, and the changes in the morphology and physiology of stressed yeasts is essential for a better understanding of how yeasts respond to different levels of oxidative stress. This knowledge is essential not only for better exploitation of yeasts in industrial processes, but also for deeper fundamental understanding and hopefully more efficacious prevention of diseases caused by oxidative stress. The latter is particularly true when *Saccharomyces cerevisiae* yeasts are used as eukaryotic cellular generic models.

In this study, we directly observed the effects of oxidative stress at the nanoscale on the morphology of *Saccharomyces*

cerevisiae (strain NCYC 1681) and *Schizosaccharomyces pombe* (strain DVPB 1354) by using an atomic force microscope (AFM) [4]. This technique allowed us to visualize, at a very high resolution, the finest changes in the topology of oxidative-stressed yeasts, confirming the usefulness of AFM in investigating microbial stress morphology at the nanoscale. The physiology of stressed yeasts was also assessed by measuring cell viability, cellular age, and mean cell volume.

Materials and Methods

Yeast Strains and Growth Conditions

The strains used in this study were a budding yeast, *Saccharomyces cerevisiae* (NCYC 1681, an ale brewing strain from the National Collection of Yeast Cultures, Norwich, U.K.), and a fission yeast, *Schizosaccharomyces pombe* (strain DVPB 1354, from the Dipartimento Vegetale e Biologia, University of Perugia, Italy). *Saccharomyces cerevisiae* and *Schiz. pombe* cells from agar slope cultures kept at 4°C were grown aerobically in 1,000 ml flasks containing 500 ml of malt extract broth medium (Oxoid Ltd., Basingstoke, Hampshire, U.K.) at 28.5°C by incubating them in a rotary shaker at 216 rpm (≡11 ×*g*) for 24 h to grow them to an exponential phase. Yeasts were harvested by centrifugation and washed three times with filtered (0.2 μm pores) distilled water.

H_2O_2 Stress

The two yeast strains were exposed to hydrogen peroxide (H_2O_2: 30% w/v stock solution) (Sigma-Aldrich, St. Louis, MI, U.S.A.) by resuspending them in 25 ml of 0.5%, 1%, 2%, and 3% w/v H_2O_2 at a final concentration of ~5×10^6 cells/ml for *S. cerevisiae* and ~2×10^6 cells/ml for *Schiz. pombe*, for different times of up to 1 h. The yeasts resuspended in filtered distilled water without any H_2O_2 were used as controls. The unstressed (controls) and H_2O_2-stressed yeast suspensions were kept in a pre-equilibrated water bath at 25°C and vortexed from time to time to avoid yeast sedimentation.

Cell Viability and Mean Cell Volume Measurements

After subjecting *S. cerevisiae* and *Schiz. pombe* yeasts to different H_2O_2 stresses, the stressed cells were washed three times and resuspended in filtered distilled water before use. Aliquots of 500 μl and 100 μl of unstressed (0% H_2O_2) and stressed *S. cerevisiae* and *Schiz. pombe* yeasts were taken every 10 min for the duration of 1 h, for cell viability and mean cell volume measurements, respectively.

The viabilities of unstressed and H_2O_2-stressed *S. cerevisiae* and *Schiz. pombe* cells were assessed microscopically by using an improved Neubauer chamber (ProSciTech, Thuringowa, Australia) and a vital methylene violet stain [1, 38].

The mean cell volume of 100,000 unstressed and stressed cells was measured by means of a Coulter Multisizer II (Coulter Electronic Ltd., Luton, U.K.), already calibrated by using latex microspheres of different sizes and known volumes. The results are quoted in Table 1 as mean cell volume±standard deviation.

Atomic Force Microscopy

The surface morphological changes of *S. cerevisiae* and *Schiz. pombe* yeasts exposed to H_2O_2 stress for up to 10 min, 30 min, and 1 h, were visualised by using a NanoWizard I BioAFM (JPK Instruments AG, Berlin, Germany) atomic force microscope [4].

Table 1. AFM roughness analyses of cell surfaces, changes in morphology, and mean cell volume measurements of the H_2O_2-stressed yeasts.

Time (min)	H_2O_2 conc. (% w/v)	*Saccharomyces cerevisiae* (strain NCYC 1681)				*Schizosaccharomyces pombe* (strain DVPB 1354)			
		Roughness[a] (nm)	Mean cell volume (μm^3)	Dimensions[b] of cavities-L×W×D (μm^3)	Relative change compared with the control[c]	Roughness[a] (nm)	Mean cell volume (μm^3)	Dimensions[b] of cavities-L×W×D (μm^3)	Relative change compared with the control[c]
-	0 (control)	77.9±2.1	232.0±3.5	$(2.5\pm0.1)\times10^{-3}$	-	71.1±2.1	214.0±3.8	$(3.6\pm0.2)\times10^{-3}$	-
10		88.0±2.7	228.0±3.6	0.27±0.01	L	91.9±3.2	206.0±4.7	0.36±0.02	H
30	0.5	116.6±4.2	220.0±3.7	0.32±0.02	L	124.4±3.7	200.0±4.8	0.45±0.02	H
60		140.9±5.0	208.0±3.9	0.67±0.04	L	151.6±2.7	185.0±4.4	0.72±0.04	H
10		121.9±8.2	206.0±3.3	0.72±0.02	L	124.6±3.3	183.0±4.2	0.77±0.02	H
30	1	139.6±6.0	198.0±3.6	0.92±0.03	L	150.4±5.0	175.0±4.2	0.99±0.03	H
60		170.8±5.1	195.0±3.7	1.52±0.06	L	192.4±6.4	157.0±3.8	2.06±0.11	H
10		137.2±6.0	186.0±3.0	1.03±0.05	L	146.3±7.3	154.0±3.9	1.08±0.05	H
30	2	181.4±5.8	181.0±3.1	1.12±0.06	L	192.1±7.1	145.0±5.1	1.76±0.08	H
60		232.4±7.8	174.0±3.3	2.0±0.1	L	254.2±6.3	115.0±2.9	2.76±0.14	H
10		163.2±5.1	172.0±4.1	1.30±0.05	L	183.4±3.1	126.0±3.8	1.15±0.05	H
30	3	203.5±4.7	168.0±4.2	1.54±0.06	L	226.0±6.2	123.0±3.7	2.19±0.09	H
60		256.8±5.0	163.0±4.4	2.52±0.13	L	281.6±6.5	115.0±5.5	3.06±0.12	H

[a]For each H_2O_2 concentration and exposure time indicated, the surface roughness of 40 yeast cells chosen randomly was measured at four different zones of the cell surface. The zones chosen for *S. cerevisiae* and *Schiz. pombe* were 6 μm^2 and 3 μm^2, respectively. The values for the surface roughness are given as weighted mean±standard deviation.

[b]The dimensions of the cavities observed on the surfaces of stressed *S. cerevisiea* and *Schiz. pombe* yeasts are expressed as L×W×D, where L, W, and D are the length, width, and depth of the cavities, respectively (see section: *Roughness, section, and bud scar analyses*).

[c]Relative changes (L: Low or H: High) of the H_2O_2 stress on the morphology (roughness and dimensions of cavities) and physiology (mean cell volume measurements) of stressed cells compared with those of the corresponding unstressed cells (0% H_2O_2).

AFM is a very powerful technique that allows one not only to image the biological samples at a very high resolution [1, 8, 9, 11], but also to quantitatively determine the biophysical properties without destroying the specimens [8, 10, 15]. Although, AFM has been extensively used to study mammalian cells [17, 36], biomolecules [5, 16], and filamentous fungi [41], it has not been widely used to study yeasts so far. This is due to the difficulties one has to face to tightly immobilize yeasts to a solid substrate (*e.g.*, glass slides or mica sheets) because of their thick and mechanically strong cell walls that make the deformation and spread of yeasts on surfaces rather difficult. However, recently significant progress has been made in developing immobilization techniques, such as air-drying, chemical fixation, and immobilization in agar gels, porous material, poly-L-lysine slides, and ceramic material for AFM investigation of yeast cells [5, 8, 14, 41].

In this work, we used air-drying to immobilize yeasts on hydrophilic glass slides. The AFM samples were prepared by spreading aliquots of 100 μl of yeast suspensions onto the surfaces of glass slides already made hydrophilic (glass slides were immersed in aqueous 20% H_2SO_4 for 24 h, washed five times with ultrapure water, kept immersed in ultrapure water until use, and then dried under reduced pressure for 1 h just before use). The yeasts were allowed to dry for 5 h at room temperature and scanned by AFM soon after, in order to preserve the original morphology of the unstressed and H_2O_2-stressed cells.

As recently shown [2], it is possible to carry out AFM experiments on yeasts in their native hydrated state; that is in liquid medium. However, the additional problem of tightly immobilizing the cells on solid substrates for AFM to work in a liquid environment arises. Different methods, such as using porous membranes of different porosity [14], are sometimes employed to confine the individual cells in the pores. However, entrapping the cells inside confined geometries exposes them to additional stress in confinement [27, 28], which even in pure liquids is known to change their structure substantially. Nevertheless, recent AFM studies have proved that dried *S. cerevisiae* yeasts maintain their integrity [39]. Therefore, it is not crucial to work in a liquid environment to scan yeasts in their native hydrated state by AFM imaging. In addition, since we analyzed and interpreted our results of changes in the surface morphology of the H_2O_2-stressed yeasts relative to the unstressed ones, which were left to dry in air in the same way as the stressed cells, the relative morphological changes observed are truly due to the imposed H_2O_2 stress and not due to the air-drying. It has been recently shown [13] that AFM imaging in air of microbial cell surfaces reveals many morphological details that remain missing in the cells imaged in liquid, and that the morphology of the yeast surface does not change noticeably when cells are allowed to dry in air [1, 25, 31].

The yeast samples were scanned in contact mode (CM) by using Si_3N_4 triangular cantilevers (Veeco, Santa Barbara, CA, U.S.A.), with a nominal spring constant of 0.01 N/m. All the AFM experiments were performed in air at room temperature, and the height images (512×512 pixels) were captured by using a scan speed of 0.5 μm/sec [1]. AFM imaging of *S. cerevisiae* and *Schiz. pombe* was carried out in air at room temperature, because it is now well known [39] that yeast cells can retain hydration water for several hours after having left the liquid environment. Even when imaging is done in air, there is a thin layer of liquid water above the sample, thus allowing the imaged cells/molecules to preserve the structurally important water molecules, and thus, their native structure. The additional advantage to scan the samples in air is that much more intricate details of the cell surface can be revealed [39], and this is not possible to achieve by scanning in liquid medium.

Roughness, Section, and Bud Scar Analyses

The surface roughness of the unstressed and H_2O_2-stressed yeasts was assessed by roughness analysis over 40 cells of the AFM height images following the method described by us previously [1]. Briefly, the roughness of the surface of the yeasts was analyzed by measuring

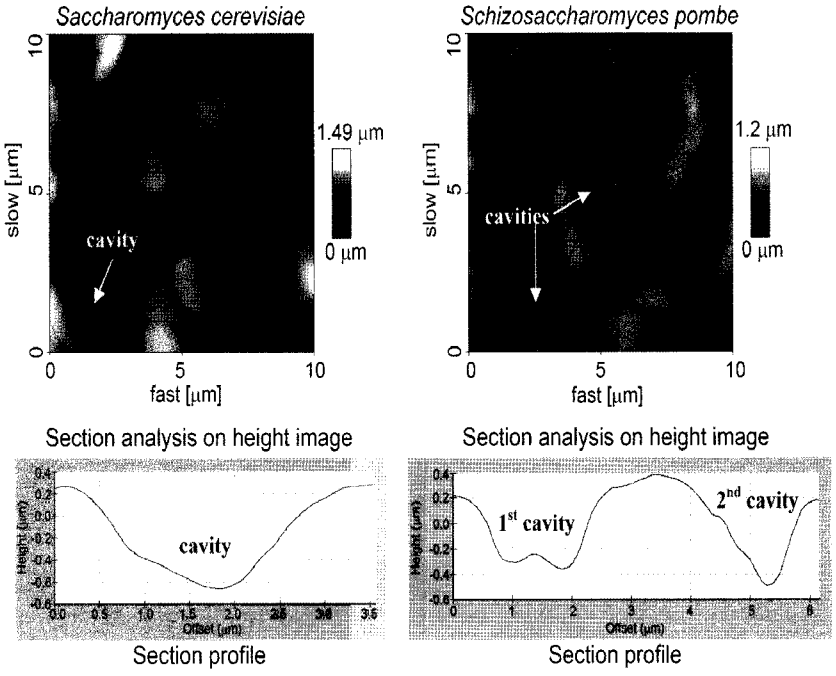

Fig. 1. Examples of section analyses on H_2O_2-stressed *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

the root mean square roughness, R_{rms}, on the AFM height image. For each H_2O_2 concentration at the three different times (*i.e.*, 10 min, 30 min, and 1 h), 40 yeast cells were chosen at random. For each of the 40 cells, the R_{rms} was evaluated at four different areas of the cell surface. The areas chosen for the two yeast strains, *S. cerevisiae* (NCYC 1681) and *Schiz. pombe* (DVPB 1354), were 6 μm^2 and 3 μm^2, respectively. These roughness values were then analyzed within the framework of the sampling theory [30] by considering the yeasts as a very large population. This analysis was carried out on raw AFM images (*i.e.*, images that were neither flattened nor elaborated with any filter) by using the JPK software. The results are reported in Table 1 as weighted mean surface roughness±standard deviation.

The dimensions of the cavities observed in the stressed yeasts were analyzed by carrying out section analysis over 40 cells of the raw AFM images by using the JPK software. The length (L), width (W), and depth (D) of the cavities on the cell surfaces were measured and the values for (L×W×D) calculated. These values were then analyzed statistically. The results are quoted in Table 1 as (L×W×D)±standard deviation. Examples of section analyses are given in Fig. 1.

In addition, the cellular age profile for *S. cerevisiae* was assessed by performing the bud scar analysis [3]. Six classes of yeasts were defined: cells with zero (virgin cells), one (young cells), two, three, four, and more than four (aged cells) bud scars. The number of yeasts belonging to each of these six classes was scored for 100 cells by counting the number of bud scars visible on the surfaces of the yeasts scanned by AFM. The values obtained were then treated statistically. The cellular age profile of *S. cerevisiae* yeasts is shown in Fig. 2. It was not possible to work out a similar profile for *Schiz. pombe* because these are fission (*i.e.*, no readily identifiable cellular age landmarks) rather than budding yeasts.

Results and Discussion

The detrimental effects of different stressful concentrations of H_2O_2 (oxidative stress) on the morphology (roughness and dimensions of cavities appearing on cell surfaces) and physiology (cell viability, mean cell volume, and cellular age profile) of *Saccharomyces cerevisiae* (strain NCYC 1681) and *Schizosaccharomyces pombe* (strain DVPB 1354) were studied by AFM, and measurements of cell viabilities and mean cell volumes conducted.

The viabilities of unstressed (0% H_2O_2) yeasts remained high (100%) over the entire time period of 1 h. Exposing *S. cerevisiae* and *Schiz. pombe* to 0.5% (w/v) H_2O_2 did not greatly affect cell viabilities that after 1 h were 91% and 96%, respectively. When *S. cerevisiae* and *Schiz. pombe* cells were subjected to 1% (w/v) H_2O_2, their viabilities stayed high (90% and 91%) for the first 20 min and then declined rapidly after 1 h to 72% and 49%, respectively. The decrease in viability was more substantial when both strains were challenged with 2% (w/v) H_2O_2. After 1 h, the cell viabilities of *S. cerevisiae* and *Schiz. pombe* sharply decreased to 32% and 27%, respectively. Subjecting both strains to 3% (w/v) H_2O_2 up to 1 h resulted in a dramatic decline in their viabilities: 19% for *S. cerevisiae* and 7%

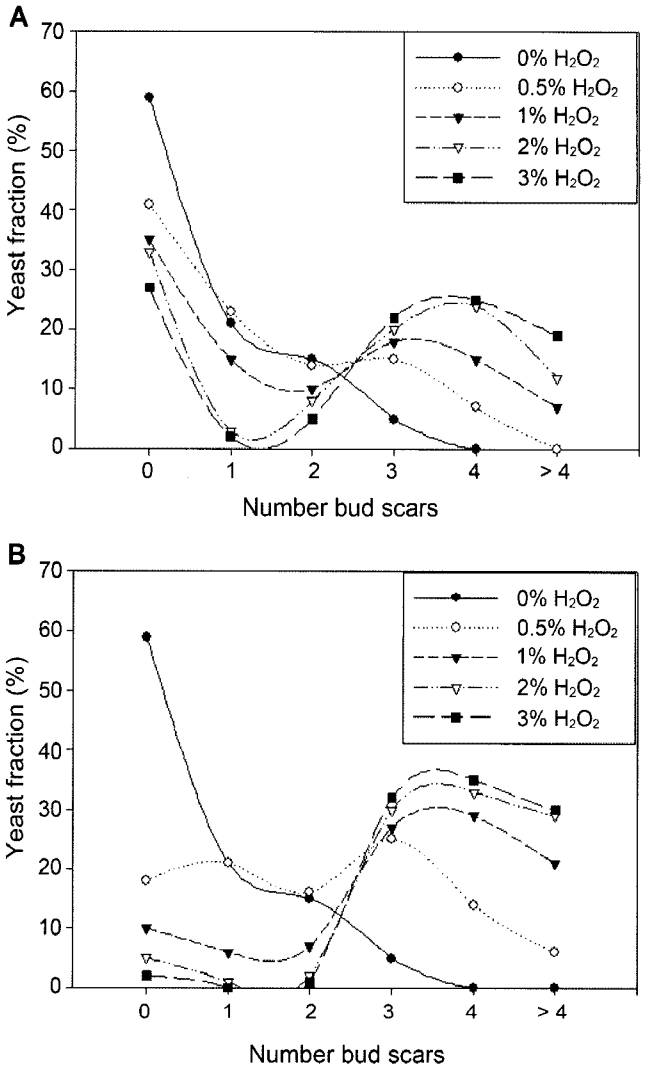

Fig. 2. Effect of H_2O_2 exposure on the cellular age profile of *Saccharomyces cerevisiae* yeasts challenged with 0%, 0.5%, 1%, 2%, and 3% (w/v) H_2O_2. **A.** 1 h exposure; **B.** 5 h exposure.

for *Schiz. pombe*. This rapid decrease in cell viabilities with increasing H_2O_2 concentrations was expected because both strains were grown to exponential phase and it is known that exponential phase yeasts are less resistant to oxidative stress than stationary phase yeasts [12, 21]. In addition, oxidative stress compromises cell viability [3, 6] and it has a crucial role in inducing apoptosis in yeasts, in particular when they are exposed to H_2O_2 [23]. Oxidative stress is also one of the major causes for premature ageing of cells [12, 21]. An oxidative damage theory of cell ageing has been proposed [21] that states that oxidative stress due to either the production of free radicals during respiratory metabolism (endogenous oxidative stress) or the exposure to oxidants (exogenous oxidative stress) damages DNA, proteins, and lipids, leading to premature ageing and death of cells. This theory, already supported by a lot of experimental data [8], is in agreement with our measurements of cell viabilities of H_2O_2-stressed yeasts.

To further check the detrimental effects of oxidative stress on the life span of yeasts, we determined the cellular age profile of *S. cerevisiae* cells (Fig. 2) after 1 h exposure to different H_2O_2 concentrations. Increasing the concentration of H_2O_2 led to a decrease in the number of young yeasts with zero bud scar or with only one bud scar, and an increase of old yeasts with two or more than two bud scars. The fraction of yeasts with three, four, and more than four bud scars rapidly increased when *S. cerevisiae* cells were challenged with 1%, 2%, and 3% (w/v) H_2O_2. These results are in agreement with those previously obtained on the effects of oxidative stress from hyperbaric air on *S. cerevisiae* cells [3]. In order to further confirm these findings, we also determined the cellular age profile after 5 h H_2O_2-exposure. These results, also included in Fig. 2, show similar trends, with the number of older cells increasing even further with longer exposure times. Oxidative stress has been shown to cause their premature ageing, thereby leading to a decrease in the chronological life span of yeasts [22, 42]. Yeast mother cell-specific ageing has been intensely researched and it has been shown that the cell surface becomes loose and wrinkly [32] and old cells are much bigger than young cells [29]. Similar observations have been made in the present findings, which indicate that oxidative stress due to increased H_2O_2 exposure leads to premature ageing of the young cells.

Oxidative stress also caused a decrease in the mean cell volumes of *S. cerevisiae* and *Schiz. pombe* yeasts (see Table 1) as they were exposed to increasing H_2O_2 concentrations for up to 1 h. This decline was more pronounced for *Schiz. pombe* than *S. cerevisiae*, indicating that *Schiz. pombe* were less resistant to oxidative stress than *S. cerevisiae*. Indeed, the mean cell volumes of yeasts stressed with 3% (w/v) H_2O_2 for 1 h decreased by ~47% for *Schiz. pombe* and by ~31% for *S. cerevisiae* when compared with the mean cell volumes of corresponding unstressed yeasts. This rapid decline in the mean cell volumes of stressed yeasts could be due to compression of the cells caused by high H_2O_2 concentrations. For *S. cerevisiae*, this hypothesis is further supported by the cellular age profile (see Fig. 2) that shows that for H_2O_2 concentrations larger or equal to 1% (w/v), the number of old *S. cerevisiae* cells is larger than the number of young cells, and older cells are bigger in size than younger cells. The only explanation for this decrease in the mean cell volumes of *S. cerevisiae* yeasts is that the cells got compressed as a result of the oxidative stress to which they were exposed. The rapid decline in mean cell volumes of oxidative stressed yeasts is in agreement with previously reported results [3] on the cell compression caused by oxidative stress from hyperbaric air.

The cell compression and the resultant changes in the morphology of yeast cell surfaces caused by H_2O_2 stress were further proven by the section (see Table 1 and Fig. 1) and roughness (Table 1) analyses carried out on the AFM height images of stressed cells. AFM images of stressed

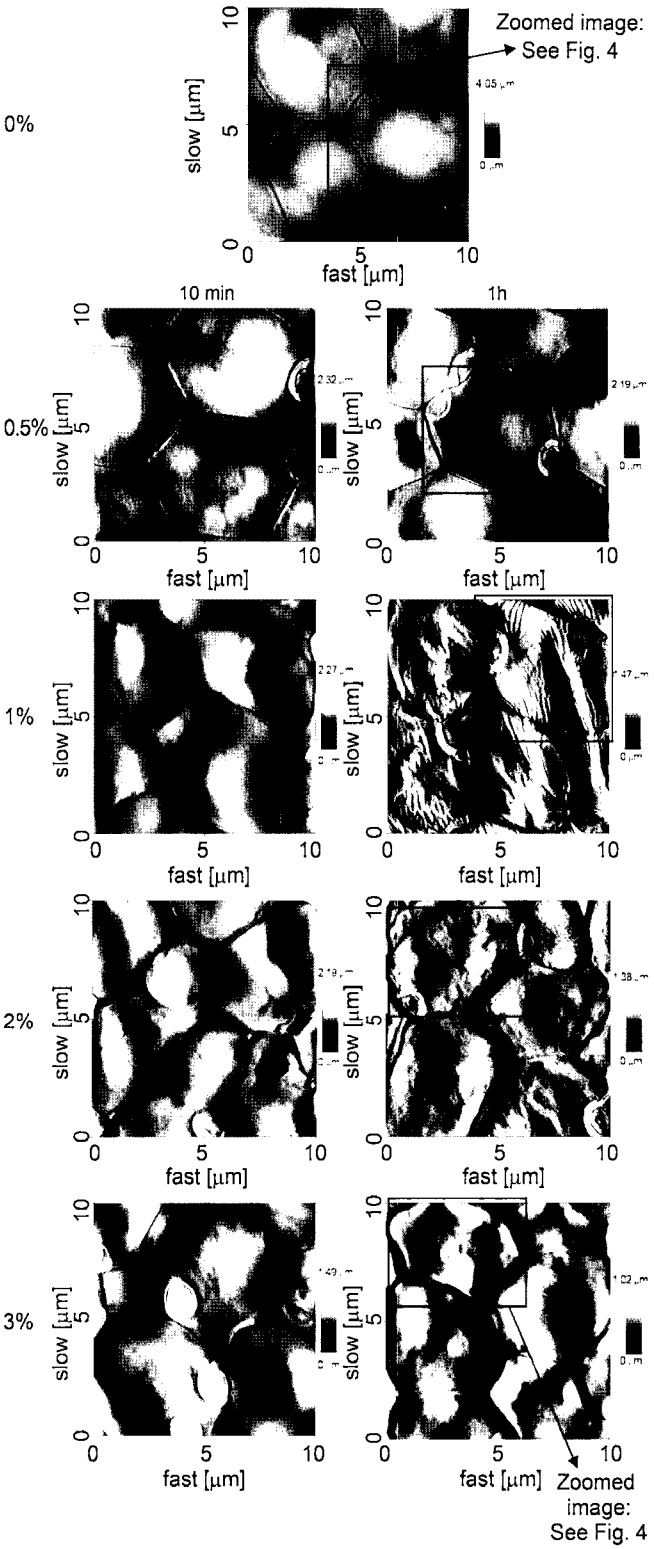

Fig. 3. AFM height images of *Saccharomyces cerevisiae* exposed to 0.5%, 1%, 2%, and 3% (w/v) H_2O_2 concentrations for up to 10 min and 1 h. The yeasts not subjected to any H_2O_2 concentration (0%) were used as a control. The high-resolution zoomed AFM images of the zones marked in squares are shown in Fig. 4 to resolve fine cell surface details.

S. cerevisiae (Figs. 3 and 4) and *Schiz. pombe* (Figs. 4 and 5) clearly reveal the formation of "cavities" on the cell

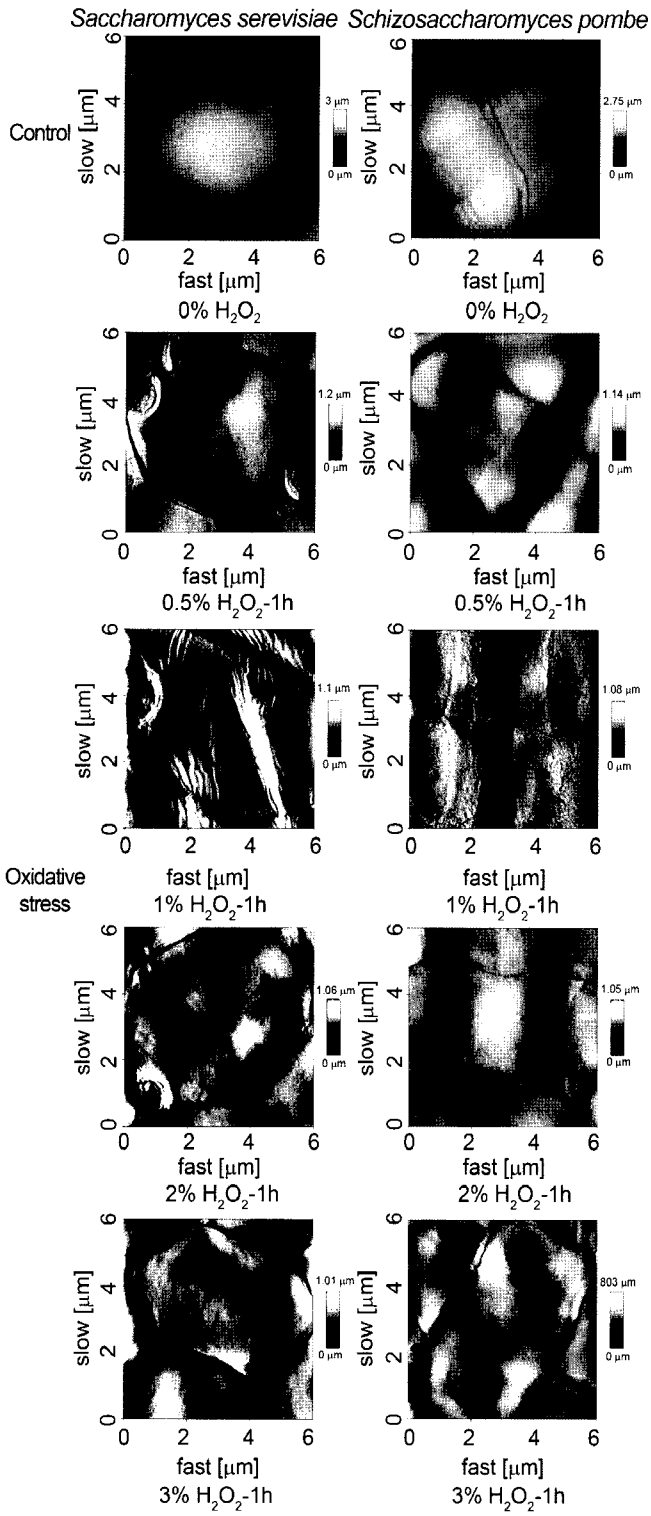

Fig. 4. High-resolution AFM images (zoom) of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* exposed to 0 (control), 0.5%, 1%, 2%, and 3% (w/v) H_2O_2 concentrations for up to1 h.

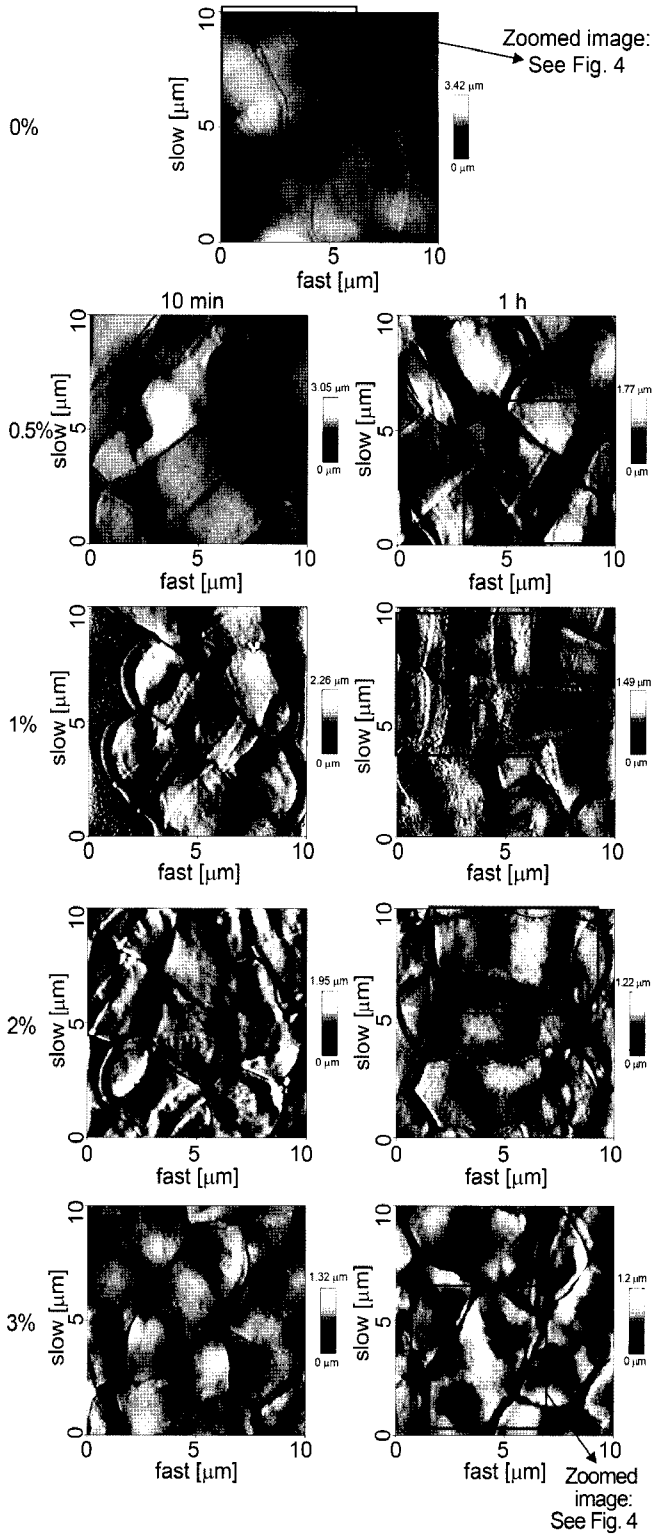

Fig. 5. AFM height images of *Schizosaccharomyces pombe* exposed to 0.5%, 1%, 2%, and 3% (w/v) H_2O_2 concentrations for up to 10 min and 1 h. The yeasts not subjected to any H_2O_2 concentration (0%) were used as a control. The high-resolution zoomed AFM images of the zones marked in squares are shown in Fig. 4 to resolve fine cell surface details.

surfaces as the H_2O_2 concentration increased. No real cavities could be discerned on unstressed (0% H_2O_2) yeast cell surfaces. When both strains were challenged with 3% (w/v) H_2O_2 for 1 h, the dimensions of the cavities, expressed as length×width×depth, rapidly increased by almost 90% compared with the dimensions of the cells exposed to 0.5%

(w/v) H_2O_2 (see Table 1). *Schizosaccharomyces pombe* appear to be affected to a greater extent than *S. cerevisiae* by oxidative stress because the dimensions of the cavities observed on the surfaces of stressed *Schiz. pombe* yeasts were on average ~20% larger than those seen on *S. cerevisiae*. The difference in dimensions of the cavities was even bigger for H_2O_2 concentrations ≥2% (w/v).

Another consequence of the oxidative stress in *S. cerevisiae* and *Schiz. pombe* was the increase in the roughness of their surfaces compared with those of the corresponding unstressed cell surfaces (see Table 1). As expected, the roughness of the surfaces of stressed *Schiz. pombe* cells increased more rapidly than those of *S. cerevisiae*, mainly because of the formation of relatively bigger cavities on their surfaces, as discussed before. The sharp increase in the surface roughness (Table 1) of *Schiz. pombe* also reflected a larger change in the morphology of stressed yeasts, as shown by the AFM images (see Fig. 5). After exposing *Schiz. pombe* cells to 0.5% (w/v) H_2O_2 for 10 min, the formation of large and deep cavities could be clearly seen, and this effect became even more prominent after 1 h (Figs. 4 and 5) exposure when compared with the unstressed cells. Cavities were also visible on the surfaces of *S. cerevisiae* yeasts stressed for up to 1 h with 0.5% (w/v) H_2O_2 (Figs. 3 and 4), compared with control cells, even though their dimensions were smaller than those of the cavities observed on *Schiz. pombe* cell surfaces. Nevertheless, AFM images for both the strains showed a compression of the cell surfaces when compared with the controls, their surfaces becoming more wrinkled, as also shown by the roughness analyses where an increase in the surface roughness occurred (see Table 1). Increasing the H_2O_2 concentration to 1% (w/v) led to clear formation of cavities on the surfaces of *S. cerevisiae* cells only after 10 min exposure (Fig. 3). The detrimental effects of oxidative stress on the cell surfaces became much more pronounced after 1 h when not only cavities were formed on the surfaces of *S. cerevisiae* yeasts, but also deterioration of the cell surfaces with the appearance of very deep wrinkles occurred (Figs. 3 and 4). Similar behavior was seen in *Schiz. pombe* after exposing the yeasts to 1% (w/v) H_2O_2 for 1 h (Figs. 4 and 5). The cell surfaces got wrinkled and shrunk compared with those of the yeasts subjected to 0% (control) and 0.5% (w/v) H_2O_2. Exposure of *S. cerevisiae* and *Schiz. pombe* to 2% and 3% (w/v) H_2O_2 caused further damages to yeast surfaces. After only 10 min, larger and deeper cavities appeared on the surfaces of *S. cerevisiae* (Fig. 3) as also revealed by the section analysis (see Table 1). Subjecting the yeasts to the oxidant for 1 h led to a clear shrinkage of cells' surfaces along with the formation of very deep cavities on their surfaces (Figs. 3 and 4). Similar to the observation for *S. cerevisiae*, *Schiz. pombe* cells also showed a drastic surface deterioration after only 10 min (Fig. 5) with the appearance of very large and deep cavities and wrinkles, as also confirmed by the section analysis (see Table 1). These effects were even more pronounced after 1 h exposure (Figs. 4 and 5).

The above results suggest that oxidative stress was responsible for the rapid decline of cell viabilities, decrease of mean cell volumes, initial compression of cell walls followed by the formation of cavities on cell surfaces, changes in cell morphologies, and increase in surface roughnesses for both *S. cerevisiae* and *Schiz. pombe*. The decline in cell viabilities with increase in the H_2O_2 concentration was a clear sign of cell ageing, which for *S. cerevisiae* was further confirmed by the cellular age profile. The profile clearly showed that the higher the H_2O_2 concentration, the larger the number of old cells. It is known [18] that detoxification of ROS, which in the present work were produced by exposure of yeasts to H_2O_2, is crucial to maintain the high viability of yeasts when challenged with oxidative stress. The rapid decrease of cell viabilities observed in this study was, therefore, caused by the high H_2O_2 concentrations to which the yeasts were subjected, and this overcame the antioxidant defences of both *S. cerevisiae* and *Schiz. pombe*. The decline in cell viabilities along with the decrease in mean cell volumes suggested that oxidative stress caused cell compression. The alteration in cell morphologies was probably due to the oxidative damages of cell membranes by oxidation of proteins and lipids that are well known to alter the membrane permeability and fluidity [19]. The increase in membrane permeability and fluidity may have resulted in the formation of cavities on the surfaces of stressed yeasts. However, the cell membranes are known to be similar in *S. cerevisiae* and *Schiz. pombe*. The fact that the cavities observed in *Schiz. pombe* cell walls were greater than those found in *S. cerevisiae* could probably be adduced to the presence of chitin in *S. cerevisiae* and its absence in *Schiz. pombe* cell walls [24, 34, 35]. In fact, the cell wall structure and composition of *Schiz. pombe* are different [24, 34, 35] from those of *S. cerevisiae*. The presence of chitin in the *S. cerevisiae* cell wall increases its elasticity, thereby preventing the formation of big cavities on the cell wall after exposure to oxidative stress. Contrarily, the cell wall of *Schiz. pombe* remains quite stiff owing to the absence of chitin. This lack in elasticity of *Schiz. pombe* cell wall leads to the creation of big cavities, as is actually observed, after exposing the yeasts to H_2O_2.

In conclusion, keeping in mind that only one strain of each species was investigated in this work, and that the oxidative damages to the physiology as well as morphology of yeasts vary with the strain and concentration of oxidant [21], our results suggest that *Schiz. pombe* (strain DVPB 1354) is much more damaged by oxidative stress than *S. cerevisiae* (strain NCYC 1681). The observed effects of oxidative stress on the physiology and morphology of both *S. cerevisiae* and *Schiz. pombe* yeasts are similar to those observed by us when the same two strains were subjected to ethanol toxicity [9] and physical stresses [1]. In particular,

comparing the present findings for oxidative stress on *S. cerevisiae* (strain NCYC 1681) and *Schiz. pombe* (strain DVPB 1354) with the results we obtained previously for ethanol and physical (thermal and osmotic) stresses on the same two strains, it appears that *Schiz. pombe* is less resistant to chemical (ethanol and oxidative) stresses than to physical shocks. Conversely, *S. cerevisiae* appears to be more sensitive to physical than chemical stresses.

The work reported here further confirms the usefulness of the AFM technique in investigating the morphological changes of stressed microbial cells at the nanoscale, which may contribute to improve our knowledge of the oxidative damages caused to the cell walls and cell membranes.

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