

Assessment of Aging in *Saccharomyces Cerevisiae* Yeast Mutants Using Microscopy Techniques

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Abstract

Different microscopy techniques were used to assess the aging of *Saccharomyces cerevisiae* yeast cells in stationary culture. Live/Dead staining was found to be as effective as viability tests at determining viability of yeast cells. Cells of dismutase-deficient *sod1* and *sod2* yeast mutants age faster than wild-type strains. Changes in appearance and decreasing in diameter size of the aging cells was observed using the Nomarski technique. The results of the fluorescent staining with Redox Sensor Red and mBC1 suggest a disturbance of redox homeostasis in the cells, while vacuole staining with CellTracker Blue CMAC indicates cytoplasmic vacuolization. Yeast cell staining as carried out in this study can be used as a fast preliminary test, making it possible to determine changes taking place in the cell during aging.

Keywords: yeast, aging, staining

Introduction

Aging is a serious problem for people as individuals and as members of society. The aging process has a variety of consequences, which may be health-related, demographic, societal, economic or ethical. For this reason research on the aging process is of increasing interest to scientists, while society awaits the practical applications of this research. Studies on the aging process are conducted on various model organisms, including mammals, insects, nematodes, and single-celled organisms such as the yeast *Saccharomyces cerevisiae*, as well as on *in vitro* cell cultures [1].

The main parameter in gerontological research is life span. *S. cerevisiae* yeast cells reproduce by budding and have a limited life span. Studies on aging of these cells are dominated by two lines of research: micromanipulations for determining replicative life span of individual yeast cells,

and studies on cell populations in culture. *S. cerevisiae* yeast cells have a limited number of divisions (budding), are asymmetrical and leave a bud scar on the parent cell [2]. In *Saccharomyces cerevisiae* yeast, life span and aging processes are closely associated with the number of divisions [3].

The basic parameter determining the replicative life span of yeast is the number of buds formed by a single cell [4]. This method has many limitations, because the small number of cells used in experiments makes it difficult to assess the biochemical changes that accompany aging. Individual yeast cells are mortal, but the cell population is potentially immortal [5]. In stationary culture, buds and cells that have undergone only a few divisions predominate [6]. Intensive cell division does not take place; cells are arrested at the G1/S phase. The line of aging research concerned with cell populations in culture concentrates on determining the biochemical parameters of aging. Aging is a gradual process characterized by worsening degenerative changes that weaken the functioning of an organism.

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In the case of a yeast cell population, a decrease in the proportion of live cells in the entire population is only one of the parameters that can be used to assess its aging. The number of dead cells does not give a complete picture of the changes that take place in an aging yeast cell population. For this reason, other symptoms of aging in a population have been sought, which would make it possible to describe the loss of viability and weakening of cells that takes place over time. For this study, wild-type strains and dismutase-deficient *sod1* and *sod2* mutants were selected. The *sod1* mutant lacks cytoplasmatic superoxide dismutase, while *sod2* lacks mitochondrial superoxide dismutase [7]. These yeast mutants are hypersensitive to factors that increase the level of reactive forms of oxygen, such as high oxygen concentration, lipid peroxidation products or paraquat [7, 8]. Cytoplasmatic and mitochondrial superoxide dismutase are key enzymes for ensuring viability of yeast cells in the stationary phase of growth. Mutations leading to lack of activity of these enzymes have by far the strongest shortening effect on both replicative and chronological life span [2, 6].

Materials and Methods

The experiments were carried out on the following yeast strains: wild-type SP4 (wt) with genotype a leu1 arg; DSCD1-1C (*sod1*) mutant with genotype a leu1 arg4 *sod1*, lacking cytosolic dismutase (Cu,ZnSOD), isogenic to SP-4, obtained by Biliński et al; wild-type DL1 (wt) α leu2 - 3, 112, his 3-11,15 ura3 -251, 372, 328 lac 2; and its isogenic mutant MnSOD α leu2 - 3, 112, his 3-11,15 ura3 -251, 372, 328 lac 2 *sod2* LEU, lacking mitochondrial dismutase (MnSOD), obtained by van Loon et al., made available for the research by Dr. G. Schatz of Biozentrum, Basel [9, 10].

Culture conditions. The yeasts were grown for 7 days in liquid YPG medium: 1% Difco yeast extract, 1% Difco Bacto Peptone, 2% glucose at 6.5 pH, at 28°C. The cultures were grown on a New Brunswick Scientific G10 gyratory shaker at 250 rpm. The ratio of the volume of the medium to the volume of the flask was 1:5. Samples were taken after 2 and 7 days.

Survival rate of yeast cells was tested by means of plating on solid YPG medium to which 2% agar was added. The yeast cells were grown on liquid YPG medium. Two or 7 days after inoculation a suspension with a known number of cells was diluted and plated on solid YPG medium. Survival rate was determined based on the capability of the cell to form colonies, with 100% taken to be the number of cells counted under the microscope.

The stains used for this study were from Molecular Probes, USA: CellTracker Blue CMAC ($C_{10}H_8NO_2Cl$), mBCL – monochlorobimane ($C_{10}H_{11}N_2O_2Cl$), RedoxSensor Red CC-1 ($C_{23}H_{19}F_5N_2O$), yeast vacuole membrane marker MDY-64 ($C_{24}H_{24}N_4O$), and Live/Dead containing Calcofluor White M2R and FUN-1 ($C_{24}H_{18}ClN_2S$). Methylene blue ($[C_{18}H_{22}N_3SCl_2]_2ZnCl_2$) and safranin ($C_{20}H_{19}ClN_4$) from Fluka AG, Switzerland, were also used.

Methylene blue staining. The yeast cultures were centrifuged and stained with a 0.01% methylene blue solution with 5.5 pH. The percentages of dead (coloured blue) and live (uncoloured) cells were calculated [11].

Safranin staining. The yeast cultures were centrifuged and stained with a 10% alcohol and water safranin solution. Cells coloured pink (dead) and uncoloured (live) were observed and their percentages were calculated [11].

Staining with CellTracker Blue CMAC. This stain reacts with available thiol groups of peptides and proteins and forms complexes with them that exhibit blue fluorescence upon UV excitation [12]. The yeast cultures were centrifuged and diluted in a 10mM Na-HEPES 7.4 pH buffer containing 5% glucose to 10^6 cells·cm⁻³, and then stained with CellTracker Blue CMAC at a concentration of 100 μ M and incubated for 20 minutes at room temperature in the dark. Then the cells were centrifuged and suspended in the buffer, as above. The suspension was observed under a fluorescent microscope.

Staining with MDY-64. MDY-64 dye stains the vacuole membrane [12], which exhibits green fluorescence upon UV excitation. The yeast cultures were centrifuged and diluted in 10 mM Na-HEPES 7.4 pH buffer containing 5% glucose to 10^6 cells·cm⁻³. A solution of the dye with a final concentration of 10 μ M was used. The yeast cells were incubated for 5 minutes at room temperature in the dark, then centrifuged and suspended in the buffer, as above. The suspension was observed under a fluorescent microscope.

Staining with RedoxSensor Red. RedoxSensor Red is a fluorescent stain that makes it possible to determine the redox potential of cells. The stain penetrates the cell, where it is oxidized to a red fluorescent product [13]. The yeast cultures were centrifuged and diluted in a 7.4 pH phosphate buffer to 10^6 cells·cm⁻³, a stain solution at a final concentration of 5 μ M was added, the culture was incubated for 10 minutes at a temperature of 37°C in the dark, and then the cells were centrifuged and suspended in the buffer, as above. The suspension was observed under a fluorescent microscope.

Live/Dead stain (Fungi Light Yeast Viability Kit) consisting of two components, calcofluor and FUN-1, makes it possible to distinguish live, weakened, and dead cells [13]. The yeast culture was centrifuged and diluted to 10^6 cells /ml in a 10 mM Na-HEPES buffer of pH 7.2 containing 2% glucose, then stained with calcofluor and FUN-1 at a final concentration of 25 μ M for each stain and incubated at 30°C in the dark for 30 minutes. Then the cells were centrifuged and suspended in the buffer, as above. The specimen was examined under a fluorescent microscope and the percentage of live, weakened and dead cells was calculated. Live cells – actively metabolizing, with a continuous cell membrane – cause FUN-1 to form cylindrical red intravacuolar structures, and the cell wall stained with calcofluor is clearly visible. Weakened cells, with low metabolic activity but with cell membrane continuity preserved, do not form red structures after staining, but exhibit green fluorescence. Dead cells fluoresce all over with a bright yellow-green light.

Table 1. Test of intensity of yeast cell aging.

parameter tested	culture day	yeast strain			
		SP4 <i>wt</i>	DSCD1-1C <i>sod1</i>	DL1 <i>wt</i>	Mn-SOD ⁻ <i>sod2</i>
% viability	7	96 ± 5.8	34 ± 6.4	65 ± 8.1	7 ± 5.4
% of dead cells stained with safranin	7	2 ± 1.7	10 ± 5.4	6 ± 3.2	14 ± 3.5
% of dead cells stained with methylene blue	7	2 ± 3.0	8 ± 4.5	5 ± 2.1	10 ± 3.7
Live/Dead staining	7				
% live cells		77 ± 10.3	25 ± 5.7	57 ± 9	11 ± 3.5
% weakened cells		17 ± 5.7	33 ± 8.5	20 ± 6.5	27 ± 5.8
% dead cells		6 ± 4.8	42 ± 3.5	23 ± 5.5	62 ± 2.9
average size of yeast cells [µm]	2	4.71 ± .57	4.79 ± 0.47	4.63 ± 0.47	3.23 ± 0.39
	7	4.56 ± .53	4.12 ± 0.49	4.40 ± 0.52	3.15 ± 0.42
fluorescence intensity after staining with RedoxSensor Red	2	distinct	distinct	distinct	distinct
	7	weak	very weak	weak	very weak
fluorescence intensity after staining with mBCL	2	intense	intense	intense	intense
	7	distinct	weak	distinct	very weak
	7 + incubation 1h with glucose	distinct	distinct	distinct	weak, in only a few cells
fluorescence intensity of vacuoles after staining with Yeast vacuole membrane marker MDY-64	2	distinct	distinct	distinct	distinct
	7	distinct	distinct	distinct	distinct
fluorescence intensity of vacuoles after staining with CellTracker Blue CMAC	2	distinct	distinct	distinct	distinct
	7	distinct	weak, in only a few cells	distinct	weak, in only a few cells
shape and surface of cells observed using Olympus Nomarski differential interference microscope	2	spherical, smooth	spherical, smooth	spherical, smooth	spherical, smooth
	7	spherical, slightly wrinkled	somewhat flattened, very wrinkled	spherical, slightly wrinkled	flat, very wrinkled

Staining with mBCL. Staining with mBCL enables detection of reduced glutathione in individual cells [14]. The cultures were centrifuged and diluted in a 7.0 pH phosphate buffer to 10^6 cells·cm⁻³, a stain solution with a final concentration of 55 µM was added, the culture was incubated for 5 minutes at room temperature in the dark, and then the cells were centrifuged and suspended in the buffer, as above. The suspension was observed under a fluorescent microscope.

Cell measurement. LUCIA Screen Measurement software enables measurement of cells in micrometres, taking into account the magnification values obtained by particular optical elements of the microscope. Cell diameter was measured in two planes. The values given are the average of the measurements of at least 30 randomly selected cells.

Yeast cells that were not fixed and stained were observed with an Olympus Nomarski differential interference microscope.

Discussion of Results

Results are presented in Table 1.

In *Saccharomyces cerevisiae* yeast cells, as in other eukaryotes, the enzymes superoxide dismutase and catalase are the main line of antioxidant defence, but it is active superoxide dismutases that determine replicative life span. Mutations leading to catalase deficiencies do not appear to strongly influence replicative life span and chronological age [2]. Superoxide anion radical has limited ability to pass through biological membranes and this can cause *sod1* and *sod2* mutations to have different physiological effects. For this reason, these dismutase-deficient yeast mutants were used in this study.

In studies on aging of individual yeast cells, symptoms of aging are observed only in the parent cells, while the daughter cells are young [15]. This is due to the asymmetrical

division of the cell during budding. Products of oxidative damage such as extrachromosomal rDNA circles accumulate in old cells [16], but they are not passed on to the buds.

In stationary cultures of yeast cells, symptoms of aging can be seen in the entire population. In this type of study the main parameter for testing viability is determination of the number of cells capable of forming colonies. In the present study, viability of wild-type yeast strains from culture day 7 was varied (Table 1), possibly due to different genetic backgrounds. Similar results are obtained in studies on individual cells, where wild-type laboratory strains have different replicative life spans. Mean replicative life span for the SP4 strain is relatively long – 34 generations. The DL strain, with 22.4 generations, has a short life span [2]. Other wild-type strains with a small number of generations are known, such as W303eA with 21 ± 1.4 [22]. A lack of active superoxide dismutase reduces viability in cultures of dismutase-deficient mutants, with particularly low viability observed in the *sod2* culture (Table 1). Sohal suggests that lack of mitochondrial dismutase increases exposure of cells to endogenous reactive forms of oxygen and shortens life span [17]. *Sod1* and *sod2* yeast mutants have a 38% and 37% shorter replicative life span than isogenic wild-type strains [2]. Lack of superoxide dismutase also shortens the life span of other organisms. A complete lack of mitochondrial dismutase shortens life span in mice [18] and in *Drosophila* [19]. Paul et al. generated a unique collection of *Drosophila* mutants that progressively reduced SOD2 expression and function [20]. In these mutant fruit flies life span was progressively shortened and age-dependent mortality was increased in conjunction with reduced SOD2 expression.

The number of dead cells is one of the parameters that make it possible to determine culture viability, yet it does not provide information on all of the changes that take place in a yeast cell culture. For this reason other symptoms of aging, associated with biological life span, have been sought. Cell membrane integrity is a frequently used criterion of cell viability. It can be easily tested using vital staining, in which dead cells take up the stain while live cells remain colourless. Vital staining with safranin and methylene blue was found to be an inadequate criterion for assessing viability of a yeast cell population (Table 1). In the dismutase-deficient mutant cell culture no increase was observed in dead cells after vital staining despite the high mortality found during plating of the cell suspension onto solid media (Table 1). Apart from viability, the best test for assessing the viability of a population is Live/Dead staining. The total percentage of weakened and dead cells after Live/Dead staining corresponds approximately to cell mortality measured based on colony-forming ability. A further advantage of the Live/Dead test is that the results are obtained quickly.

Another criterion used in describing the changes occurring in an aging population of yeast cells was the appearance of the cells. Young cells are spherical, with a regular shape and a smooth surface (Table 1). As the culture time progresses, the appearance of the cells changes. On day 7 of the culture, wild-type yeast cells are slightly wrinkled, but they retain their regular, spherical shape. *Sod1* mutant cells had a wrinkled surface and were slightly flattened.

The most significant changes were observed in the cells of the day 7 *sod2* mutants, whose surface was very wrinkled and some of them had damaged cell walls. Similar observations have been made in micromanipulative studies; individual yeast cells had a deformed surface and looked ‘folded’ or wrinkled [19].

It was also found that aging of dismutase-deficient mutant cell populations is accompanied by reduced cell size (Table 1). Buds that did not separate from the parent cells were not taken into account when the cells were measured. A statistical analysis of the results was made (Student’s t-test) at significance level $\alpha=0.05$, and significant differences in cell size were found between a) *sod1* mutant on culture days 2 and 7; b) the *sod1* mutant and the wild-type strain on day 7; and c) the *sod2* mutant and the wild-type strain DL1. In the wild-type strain population, no decrease in cell size is observed between culture days 2 and 7; moreover, after 7 culture days they are always larger than the cells of dismutase-deficient mutants. A reduction in size could lead to arrest of cell division, because very small yeast cells are not capable of budding [21]. In micromanipulative experiments on individual cells, Jazwinski found that the size of a single yeast cell increases with aging measured based on generative life span [22]. Nestelbacher et al. published a photograph of an extremely old yeast cell that budded 50 times. It is characterized by enormous size, visible changes on its surface and abnormal budding – it forms a second bud even though the previous one has not yet separated from it [22]. However, changes in cell size are not a fundamental characteristic of the aging process, but rather one of many changes accompanying it. Increased cell size has also been observed in yeast with overexpression of the *RAS* gene, which prolongs generative life [23].

One of the tests conducted was staining of yeast cell vacuoles. The stains used were Yeast vacuole membrane marker MDY-64 and CellTracker Blue CMAC (Table 1). After staining with Yeast vacuole membrane marker MDY-64, the vacuoles are clearly visible and no fundamental differences are observed in the fluorescence intensity of cells of the strains tested. However, CellTracker Blue CMAC revealed differences in the fluorescence intensity of vacuoles in the case of the day 7 cell population of dismutase-deficient mutants. Extensive vacuole damage was observed mainly in the day 7 culture of the *sod* mutants, in which most of the stained cells exhibited weak fluorescence or none at all. In studies conducted on individual yeast cells, cytoplasmic vacuolization is one of the symptoms of aging in the cell [24]. These changes, also called vacuole fragmentation, can also occur as a result of oxidative stress [25]. The *sod1* mutant of the pathogenic yeast *Cryptococcus neoformans* was highly sensitive to the redox cycling agent menadione, and has showed fragmentation of the large vacuole in the cytoplasm [26]. The CellTracker Blue CMAC stain reacts with available thiol groups of peptides and proteins in the vacuole membrane, hence a decrease in fluorescence intensity may suggest a disturbance in the homeostasis of the internal environment of the cell. The stain RedoxSensor Red is used to test the redox potential of cells. The fluorescence intensity of the stained cells is dependent

on the redox potential of the cytosol [14]. This stain has also been used to assess oxidative stress induced by asbestos [27]. Yeast cells from the day 2 culture were characterized by intense red fluorescence, but as culture time progressed, fluorescence intensity weakened rapidly (Table 1). These changes were greater in the dismutase-deficient mutants and may suggest an accelerated aging process.

In eukaryote cells redox homeostasis can be maintained thanks to GSH (reduced glutathione), which occurs in high concentrations of about 1-10 mmol·dm⁻³. The presence of intracellular glutathione can be confirmed using the fluorescent stain mBCL [28]. Among the cells stained with this dye, the weakest fluorescence was observed in the day 7 culture of sod mutants, particularly *sod2* (Table 1). Under physiological conditions, the oxidized form of glutathione (GSSG) can be reduced back to GSH. Under the experimental conditions described, the easily assimilated carbon source had already been exhausted by culture day 7. In order to determine whether mechanisms for regenerating reduced glutathione were functioning, the day 7 yeast cultures were incubated with 2% glucose and then the cells were stained to test for the presence of GSH. The division cycle of the wild-type strain yeast cells is about 1.5h, so in order to prevent rejuvenation of the population, the cells were incubated with glucose for 1h. Supplementing the medium with 2% glucose was found to increase fluorescence intensity in the wild-type strains and in the *sod1* mutant. However, in the *sod2* mutant only a few cells exhibited more intense fluorescence [29].

Conclusions

Degenerative processes begin in the cells of dismutase-deficient mutants more quickly than in wild-type strains. Yeast cell staining as carried out in this study can be used as a fast preliminary test making it possible to determine changes taking place in the cell during aging.

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