Razlike u morfološkim i fiziološkim karakteristikama stanica kvasca Saccharomyces cerevisiae tijekom životnog vijeka

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Morphological and physiological characteristics of the yeast *Saccharomyces cerevisiae* cells differing in the life span

MASTER THESIS

University of Zagreb, Faculty of Pharmacy and Biochemistry

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This thesis has been reported in course Biochemistry 1 and delivered to the University of Zagreb, Faculty of Pharmacy and Biochemistry. The experiment work was performed at the University of Rzeszow, Faculty of Biology and Agriculture under the expert guidance of PhD Renata Zadrag-Tęcza and second supervisor Prof. Lada Rumora, PhD.

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1. INTRODUCTION

1.1. Background and motivation

Aging is a nearly universal feature of biological organisms, ranging from unicellular creatures to large, complex animals. Among multicellular organisms, aging is reported as a progressive decline in the function of multiple cells and tissues, resulting in an increasing vulnerability to environmental challenges and a growing risk of diseases and death. Furthermore, it is usually accompanied by a decline in fertility. In mammals, age-related degeneration gives rise to well-recognized pathologies, such as sarcopenia, atherosclerosis and heart failure, osteoporosis, macular degeneration, pulmonary insufficiency, renal failure, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, and many others (Campisi, 2013). However, species vary in their susceptibilities to specific age-related pathologies, which generally rise with exponential-like kinetics beginning at approximately the midpoint of the species-specific life span (e.g., 50-60 years of age for humans) (Campisi, 2013). If damage is involved in the underlying age-related deterioration in the cell function, a range of interesting consequences and questions arises at the cellular level (Kirkwood, 2005). However, it is necessary to emphasize that the cellular senescence does not explain all aging phenotypes (Campisi, 2013). Aging corresponds to an inevitable accumulation of damages to cells, which is common to all adult organisms (Bell, 1984). Cellular senescence was first described in the sixties when Hayflick and colleague (Hayflick, 1965) showed that normal human cells (i.e. fibroblasts) did not proliferate indefinitely in culture. Observing cells showed a finite replicative life span with undergoing replicative or cellular senescence. There are a limited number of divisions that cells complete upon reaching the end of their replicative life span. In addition the growth arrest, senescent cells show widespread changes in chromatin organization and gene expression. They are generally enlarged, often doubling in volume, and, if adherent, adopt a flattened morphology (Campisi, 2013). Thus, senescent cells are identified by a various characteristics. Likewise, not all senescent cells display each of the senescence markers (Campisi, 2013).

There is mounting evidence that at least one process, called stress response, links multiple pathologies of aging. The mechanisms behind the limited replicative life span of normal cells include telomere shortening, epigenomic damage, mitogens and proliferation-associated signals as well as activation of tumor suppressors (Campisi, 2013).

Furthermore, the senescence arrest is considered irreversible because unknown physiological phenomena can stimulate arrested cells to re-enter the cell cycle (Campisi, 2013). This topic is one of the most interesting findings among researchers investigating aging. Elucidating the circumstances under which the senescence growth arrest is reversible represent a great challenge for the future studies.

As far as it is known, all species that have a clear distinction between somatic and germ cells undergo senescence while animals that do not show senescence have germ cells distributed throughout their structure (Bell, 1984). Further research is clearly needed while taking into consideration the species which have clear distinctions between somatic and germ cells even though negligible rates of aging are noted (Campisi, 2013). Therefore, the assumption can be made that unicellular organisms, in contrast to multicellular, should be immortal. However, during the 1980s, the yeast *Saccharomyces cerevisiae* became firmly established as an experimental model for the research of aging (Kirkwood, 2005). It is reproduced by asymmetric cell division or budding. In each cell division, the daughter cell is usually smaller and younger than the mother cell, as defined by the number of divisions it can potentially complete before its death. Although individual yeast cells have a limited life span, this asymmetry between mother and daughter ensures that the yeast strain remains immortal (Lai et al., 2002). In this case, one might suggest that the "mortal" mother cell can be seen as somatic cell, whereas the smaller bud that becomes a daughter can be seen as germ cells (Kirkwood, 2005). However, unicellular yeast cells have been traditionally used as models for lower eukaryotic organisms and the yeast studies have made tremendous contributions to our understanding of the cell's life and its metabolism. The first eukaryotic cell having its genome completely sequenced was *S. cerevisiae*. Therefore, *S. cerevisiae* has been at the forefront of eukaryote biochemistry as well as cellular and molecular biology for more than 50 years (Xiao, ed., 2006). It has been widely utilized because of the ease with which it can be grown and manipulated, the extensive conservation of its genes and pathways with those of higher organisms, and the powerful genetic resource that it offers. Many new genome-wide technologies were first developed using yeast before being more widely applied to other organisms (Cherry et al., 2011). For these reasons researching of *S. cerevisiae* serves as a model for a variety of processes that occur in humans, many of which are associated with diseases. With the entire yeast genome sequence available, it was estimated how many yeast genes have a significant mammalian homolog (Botstein et al., 1997), which is important for the greater understanding of human genome. Likewise, they found a statistically robust

homolog among the mammalian protein sequences. Many of these similarities relate to the individual domains, and not the whole proteins, without doubt reflecting the re-assembling of functional domains characteristic for protein evolution (Botstein et al., 1997). Although there are many questions about yeast relevance for aging of multicellular organisms, the numerous previously mentioned advantages are the reason enough to use yeast as a model organism (Gershon and Gershon, 2000).

1.2. The biology of yeast *Saccharomyces cerevisiae*

1.2.1. Description and significance

Saccharomyces cerevisiae, also known as baker's yeast, is a eukaryotic unicellular microbe. More specifically, it is a globular-shaped (Fig. 1A), yellow-green yeast belonging to the fungi kingdom[1]. Wild-type strains of the yeast have been found on the surfaces of ripe fruits, in the gastrointestinal tracts and on body surfaces of insects and warm-blooded animals, soils from all regions of the world and even in aquatic environments (Martini, 1993). There are also laboratory strains used in different fields of study as a model organism for all eukaryotic biology (Botstein and Fink, 2011), corresponding to the features such as size, generation time, accessibility, manipulation, genetics, conservation of mechanisms, and potential economic benefits. "Normal" laboratory haploid strains have a doubling time of approximately 90 minutes in complete YPD media and approximately 140 minutes in synthetic media during the exponential growth phase (Sherman, 2002). Yeasts are best cultured in a neutral or slightly acidic pH environment, under aerobic conditions, with an adequate nutrient supply, at the optimal temperatures of 28-30°C (O'Kennedy et al., 2008). Usually, strains reach the maximal density 2×10^8 cells/ml in YPD media. The colonies are visible 2-3 days after placing them on the fresh solid YPD media (Mayers, 1997). Those colonies are flat, smooth, moist, glistening or dull, and cream in colour.

[1]**Lineage:** Eukarya; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Saccharomyces; Cerevisiae

1.2.2. Cell structure and metabolism

As mentioned before, *S. cerevisiae* is a small, unicellular organism, at least as closely related to the animal cells as it is to plants (Alberts et al., 2008). Compared to eukaryotes, viability of cells relies on the compartmentalisation of many basic cellular processes into membrane-bounded organelles (Fig. 1B). The plasma membrane separates the cell components from the external media while the nuclear membrane envelops and protects the genetic material (Dickinson and Schweizer, eds., 2004). Furthermore, the mitochondrial membrane is involved in metabolic energy generation, whereas the endoplasmic reticulum (ER) and Golgi apparatus are involved in protein and lipid sorting and synthesis. Likewise, the vacuolar and peroxisomal membranes localise special metabolic and digestive functions (Van der Rest et al., 1995). Another significant feature of the yeast cell, as a member of eukaryote empire, is a presence of the cytoskeleton. This intracellular network mechanically supports the cell and allows movement, and is consisting of two types of actin filaments: actin cables (long bundles of actin filaments) and actin patches (small assemblies of filaments associated with the cell membrane, marking sites of actin-driven endocytosis). Likewise, the polar orientation of the cell's actin cytoskeleton allows asymmetric division, evident in the way yeasts divide by budding to create a small daughter cell from a large mother cell (Alberts et al., 2008).

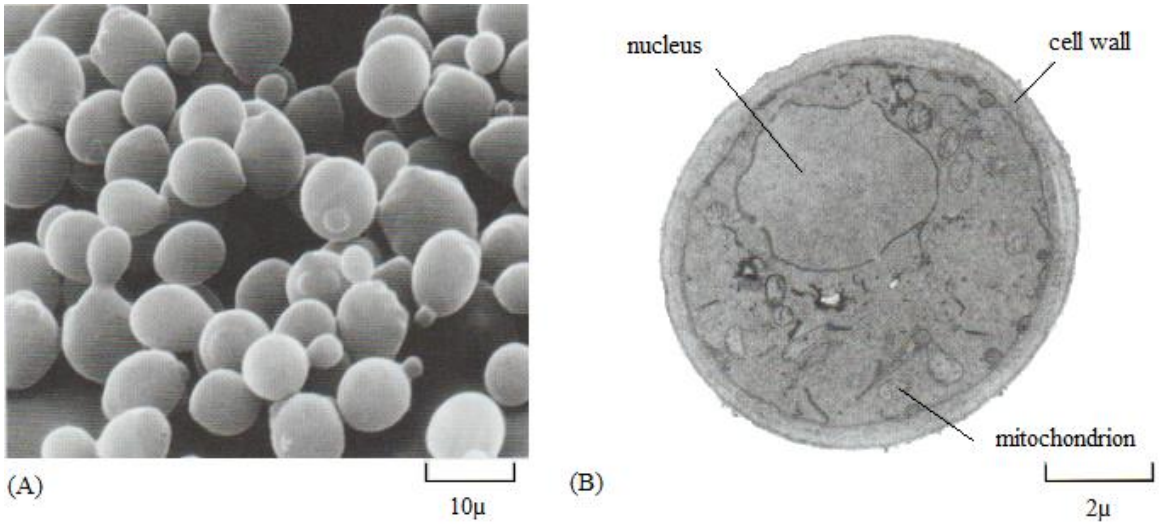


Figure 1. The yeast *Saccharomyces cerevisiae*. (A) A scanning electron micrograph of a cluster of the cells. (B) A transmission electron micrograph of a cross section of a yeast cell, showing nucleus, mitochondrion and a thick cell wall (Alberts et al., 2008).

Compared to prokaryotes, each cell is surrounded by a cell wall. Latter consists of mainly mannoproteins and glucans, giving the yeast cell shape, and providing a mechanical and thermal protection from the environment. Furthermore, it prevents osmotic bursting of the cell by the turgor pressure and act as a sieve for large molecules that might harm the cell membrane (Cabib et al., 1991). It gives the yeast a much better chance of survival in spite of harsh environmental conditions, but also allows the cell to communicate with the environment enabling reproduction, recognition and reception. Cell wall is linked to the cell membrane across the periplasmic space by glucan and chitin chains. Also it contains various enzymes responsible for regulating yeast metabolism (Cabib et al., 1991). The cell wall made of chitin, with no peptidoglycans, is a feature that adorns the fungi kingdom. With respect to the single-celled form and the impossibility of creating sporocarp, like other fungi do, *S. cerevisiae* is considered as yeast.

The lipid bilayer of *S. cerevisiae* membranes is composed of polar lipids and proteins, similar to those found in the membranes of higher eukaryotic cells (Van der Rest et al., 1995). Hence, these proteins can be either intrinsic, spanning the whole membrane, or extrinsic, embedded in part of the membrane and protruding from one side. Functions of these proteins vary from amino acid and sugar transporters to ATPases. However, they can also be a part of the cytoskeleton (Ishtar Snoek and Steensma, 2007). Equally, the plasma membrane forms relatively impermeable barrier for hydrophilic molecules. In correspondence to the yeast metabolic needs, specialized proteins mediate selective uptake and secretion of amino acids, sugar or ions across the membrane. In contrast to higher eukaryotes, in which cholesterol is the most abundant sterol, the yeast plasma membrane contains mainly ergosterol (Van der Rest et al., 1995). Interestingly, the lipid composition of the cell membranes under anaerobic conditions shows the difference compared to cells grown under aerobic conditions. Anaerobically, the plasma membrane contains more saturated fatty acids, less total sterol, ergosterol and squalene. Mentioned differences can be explained by the inability of the cell to synthesize these compounds without oxygen. The specific adaptation to the new conditions appears mainly on transcriptional level (Ishtar Snoek and Steensma, 2007).

As opposed to prokaryotes and similar such as the plant cells, yeast cells contain one or more vacuoles. Their importance lies in the storage of various enzymes and amino acids needed for protein synthesis in yeast metabolism. Likewise, degradation products of metabolism are stored here (Alberts at al., 2008).

Yeasts are chemoorganotrophs, so they use organic compounds as a source of energy and do not require sunlight to grow. Yeasts of the genus *Saccharomyces* are able to utilize a wide variety of sugars as sources of carbon and energy. Sugar utilization is governed by both genetic capability and regulatory mechanisms. Yeasts preferentially utilize hexoses such as glucose and fructose (Carlson, 1987). Glucose is the main carbon and energy source converted via the glycolytic pathway and the Krebs cycle to energy in the form of ATP (Bekatorou et al., 2006). Sucrose is metabolized after hydrolysis into glucose and fructose by the extracellular enzyme invertase. Maltose is transferred in the cell by maltose permease, converted into two molecules of glucose by the enzyme mannase and metabolized (Bekatorou et al., 2006). Interestingly, sugars such as sucrose, maltose or galactose are not metabolized in the presence of glucose (Carlson, 1987). Some yeast can also utilize a number of unconventional carbon sources, such as biopolymers, pentoses, alcohols, polyols, hydrocarbons, fatty acids and organic acids, which is of particular interest to food and environmental biotechnologists (Bekatorou et al., 2006).

S. cerevisiae is a facultative anaerobe, which means that it can grow with or without oxygen. In the presence of oxygen, it undergoes mitochondrial electron transport chain and oxidative phosphorylation where glucose is converted to CO_2, H_2O and energy. In anaerobic conditions, as in alcoholic fermentation, yeasts do not grow efficiently, because the energy is derived from glycolysis only and sugars are instead converted to the intermediate by-products such as ethanol, glycerol and CO_2 (Ishtar Snoek and Steensma, 2007; Bekatorou et al., 2006). Afterwards, the ethanol is consumed using a Krebs and glyoxilate cycles as well as the mitochondrial electron transport chain.

Processes of respiration and fermentation are regulated by environmental factors, mainly glucose and oxygen concentrations. In respiration, pyruvate is decarboxylated in the mitochondrion to acetyl-CoA. Latter is completely oxidized in the Krebs cycle to CO_2, energy and intermediates to promote yeast growth. In process of fermentation glucose is slowly utilized to produce the energy required just to maintain the yeast cell live (Bekatorou et al., 2006). When glucose concentrations are high enough, enzymes used in respiration are repressed and fermentation takes over respiration (Bekatorou et al., 2006). Once ethanol is formed, it is secreted into the growth media as well as various other by-products of fermentation such as glycerol, acetic acid, H_2S and esters.

Finally, elements like N, P, S, Fe, Cu, Zn and Mn are essential to all yeasts and are usually added to the growth media. Most of yeasts are capable of assimilating directly ammonium ions and urea, while a very few species have the ability to utilize nitrates as nitrogen source. Phosphorus and sulphur are usually absorbed in the form of inorganic phosphates and sulphates, respectively (Bekatorou et al., 2006).

1.2.3. The life cycle

S. cerevisiae can exist both as a haploid and as a diploid cells (Fig.2), usually found in the diploid form (Landry et al., 2006). The sizes of haploid and diploid cells vary within the phase of growth and from strain to strain. Typically, diploid cells have ellipsoidal shape of 5 x 6 μm and haploid cells are spheroids with approximately 4 μm diameters (Sherman, 2002). During exponential growth, haploid cultures reproduce faster than diploid cultures. Yeast has a considerable number of alternative developmental options and the signals for all of these, except for mating, are nutritional. If adequate nutrients are given, both haploids and diploids can undergo repeated rounds of vegetative growth and mitosis (Dickinson and Schweizer, eds., 2004). Vegetative cell division of yeast characteristically occurs by budding, in which a daughter cell is initiated as an outgrowth from the mother cell, followed by nuclear division, cell-wall formation, and finally cell separation. Also haploid cells have buds that appear adjacent to the previous one, whereas diploid cells have buds that appear at the opposite poles. Each mother cell usually forms no more than 20-30 buds, and its age can be determined by the number of bud scars left on the cell wall (Sherman, 2002).

Nitrogen-starved diploid cells exposed to a poor carbon source will undergo meiosis and sporulation, producing four haploid spores (ascus). Spores have a great resistance to extreme environmental conditions. If the spores are placed to the rich nutrient conditions, they will germinate and initiate growth as haploids (Dickinson and Schweizer, eds., 2004).

Sexual reproduction is also very important in the normal biology of the yeast. Haploid spores can be present as one of two mating types, *a* or *α*. Haploids of mating type *a* produce „*a* factor“ pheromone, while those of *α* mating type produce „*α* factor“ pheromone. Both of these types can also undergo budding to produce more haploid cells. Each cell type has a cell surface receptor for pheromone produced by cells of the opposite mating type. The *a* factor causes *α* haploids to arrest in the G_1 phase of the cell cycle and *α* factor has the same effect on

a cells (Dickinson and Schweizer, eds., 2004). Consequently, when in each other's presence, haploids of opposite mating type interrupt proliferation and initiate the development of protuberances towards each other. Eventually, intercellular contact and subsequent cell fusion may occur, culminating in the formation of a diploid (Dickinson and Schweizer, eds., 2004).

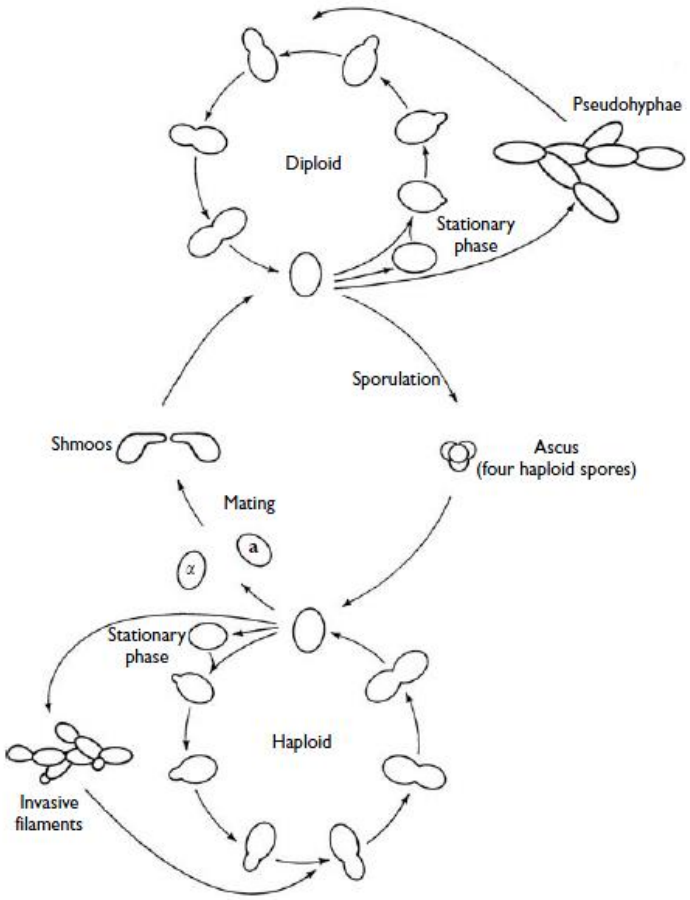


Figure 2. The life cycle of *Saccharomyces cerevisiae* (Dickinson and Schweizer, eds., 2004).

1.2.4. Genetic structure and function

The budding yeast *Saccharomyces cerevisiae* is the first eukaryotic organism whose entire genome sequence was determined. The genome is composed of about 12052 kb, organized on haploid set of 16 chromosomes (Sherman, 1998). As a characteristic of all eukaryotes, DNA is concentrated in the nucleus and packed into chromosomes during replication cycle. Nuclear

DNA contains 6183 open reading frames (ORF). Approximately 5800 of them were predicated to correspond to actual protein-coding genes, 275 code for tRNA, 40 code for snRNA's, and 140 genes on chromosome 12 codes for rRNA. In contrast to the genomes of multicellular organisms, the yeast genome is highly compact, with genes representing 72% of the total sequence. Approximately 4% of the genome is comprised of introns (Goffeau et al., 1996). Among the genes that code for proteins, 11% of the proteome participate in metabolism, 3% in energy production and storage, 3% in DNA replication, 7% intranscription and 6% in translation. Nearly 430 proteins are involved in intracellular trafficking, and 250 proteins have structural roles (Goffeau et al., 1996).

Although all protein-coding genes have been documented in the yeast genome, there still remain a percentage of the genes with an unknown function. Approximately one half of them either contains a motif of a characterized protein class or corresponds to genes encoding proteins structurally related to functionally characterized gene products from yeast or from other organisms (Sherman, 1998). As a part of the genetic material in most strains of *S. cerevisiae* revealed the presence of the 2-μm circle plasmids, apparently function solely for their own replication (Sherman, 1998).

With the help of complete genome reconstruction, using latest biochemical and physiological information, metabolic network of *S. cerevisiae* have been reconstructed. A total of 708 ORF's were identified to take part in metabolism, with the possibility to conduct 1035 metabolic reactions (Förster et al., 2003). More than 85% of these reactions involve internal and external transport across the cytoplasmic or mitochondrial membrane, whereas the other reactions are mainly involved in the metabolism of amino acids, nucleotides and vitamins (Förster et al., 2003). Furthermore, most of the ORF's take part in the electron transport chain and chemiosmosis, the final steps of aerobic respiration, followed by the breakdown of complex carbohydrates (Förster et al., 2003).

The mitochondrial genes and their mosaic intronic structure were first identified in *S. cerevisiae*, and the first sequenced mitochondrial gene was from this organism. However, the sequence of the yeast mtDNA is still incomplete and contains many errors (Foury et al., 1998). The mitochondrial genome from *S. cerevisiae* is characterized by low gene density and highly heterogeneous base composition. The A+T content is highly represented, while the G+C content makes about 30% of genes total base pairs (Foury et al., 1998). The genome sequence is about 85000 base pairs long and it contains seven intron-related ORFs, including

genes that encode components of the mitochondrial translational machinery and approximately 15% of the mitochondrial proteins (Sherman, 1998). In addition to the ORF's, the genome contains genes for cytochrome c oxidase subunits, ATP synthase subunits, apocytochrome b and ribosomal protein (Foury et al., 1998). The yeast strains that completely lack mitochondrial DNA are indicated as rho^{o} mutants. Latter are deficient in the respiratory polypeptides synthesized on mitochondrial ribosomes, i.e. cytochrome b and subunits of cytochrome oxidase and ATPase complexes. Even though rho^{o} mutants are respiratory-deficient and morphologically abnormal, they are viable and still retain mitochondria (Sherman, 1998).

Yeast also contains a 20S circular single-stranded RNA that appears to encode an RNA-dependent RNA polymerase that acts as an independent replicon and that is inherited as a non-Mendelian genetic element (Sherman, 1998). Only mutations of chromosomal genes exhibit Mendelian 2:2 segregation in tetrads after sporulation of heterozygous diploids. This property depends on the disjunction of chromosomal centromeres. In contrast, non-Mendelian inheritance is observed for the phenotypes associated with the absence or alteration of other nucleic acids (Sherman, 1998).

The yeast research community was the first to profit from the knowledge of a complete genome sequence. Later a genome sequences become available for other model organisms as well (Dolinski and Botstein, 2005). Comparative analyses of the complete genome sequences between yeast, worm, fly, mouse, and human validated the level of conservation of sequence and function during evolution. There has been a "grand unification" in molecular biology, as it became clear that, at least for proteins, sequence similarity usually leads to an unambiguous assessment of functional similarity (Dolinski and Botstein, 2005).

1.2.5. The growth and cell cycle

Cell growth and cell cycle are the basic biological functions of proliferating cells. The term growth is generally used to indicate both the increase in cell mass of an individual cell as well as the increase in number of a cell population. During the growth yeast cultures passes through four phases: *lag*, *log*, deceleration and stationary (Fig. 3). When a culture of yeast cells is inoculated in a fresh growth media, they enter a brief *lag* phase where they are biochemically active but not dividing. During this phase, no growth occurs, individual cells

are actively metabolizing, and preparing for cell division (Held, 2010). The cells usually activate the metabolic pathways to make enough of the essential nutrients to begin active growth. The duration and extent of *lag* phase depends on the initial population size and environmental conditions (i.e. temperature, pH, alcohol, oxygen, salt concentration, nutrients etc.). Since the cell starts actively metabolizing, DNA replication occurs, resulting in cell division (Held, 2010). Cells enter the fast logarithmic growth, in which metabolism is mainly glycolytic (Tissenbaum and Guarente, 2002). As cell numbers increase, cell growth begins to decelerate. Likewise, the glucose becomes limiting and a diauxic shift occurs, i.e. the culture switches from fermentative metabolism to respiratory growth. Eventually the yeast cells reach the stationary phase, in which the cells stop dividing and the yeast become resistant to stresses including heat and oxidative stress. In those conditions cells are able to survive for several months (Tissenbaum and Guarente, 2002).

Cellular growth is mostly a consequence of protein synthesis, proceeding continuously during the cycle. Furthermore, events such as genome replication (S phase), mitosis and cell division (M) are discontinuous and take place only once during a cell cycle (Alberghina et al., 2012). Finally, the eukaryotic cell cycle is divided into four phases: G_1 (gap 1), S (synthesis of DNA), G_2 (gap 2) and M (Fig. 4). During the S phase the chromosomal DNA is replicated with great accuracy, while during the G_1 and G_2 phases events take place to respectively prepare and control the onset of DNA replication and then of mitosis (Alberghina et al., 2012). In the budding yeast G_1 resembles that of higher eukaryotes, however, the spindle starts forming during S phase, there is no G_2, and the nuclear envelope remains intact during mitosis (Gershon and Gershon, 2000).

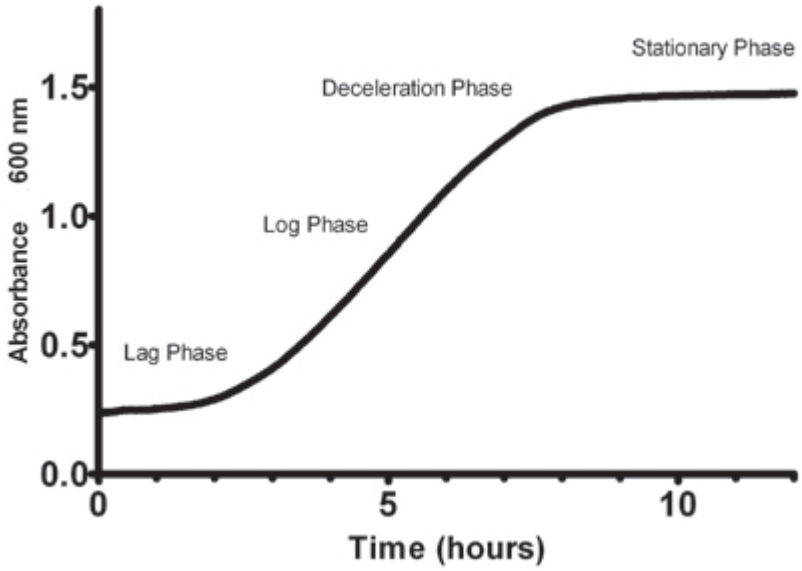


Figure 3. Typical yeast growth curve: *Saccharomyces cerevisiae* grown in YPD media at 30 ^{o}C for 12 hours with data measurements every 2 minutes (Held, 2010).

Given that the cell cycle is the process of vegetative cellular reproduction, to reproduce itself an eukaryotic cell must duplicate all its components and separate them into two daughter cells, more or less equally. Each daughter cell has the information and machinery necessary to repeat the process (Chen et al., 2000). The basic organization of the cycle, however, is essentially the same in all eukaryotic cells, and all eukaryotes appear to use similar machinery and control mechanisms to drive and regulate cell cycle events (Alberts et al., 2008). Furthermore, there are many variations of specific details from one cell type to another. For instance, budding yeast cells are peculiar in that they divide asymmetrically (Chen et al., 2000). Likewise, both haploid and diploid cells undergo the same mitotic cycle (Hartwell, 1974). The ability to proliferate in a haploid state is unusual and consequently only a single copy of each gene is present in the cell. When cells are haploid, it is easy to isolate and study mutations that inactivate a gene, as one avoids the complication of having a second copy of the gene in the cell (Alberts et al., 2008).

Growth and cell cycle progression in budding yeast are affected by main environmental determinants, i.e. corresponding nutrients, temperature and mating factors. In *S. cerevisiae* regulation of cell cycle progression is achieved predominantly during a narrow interval in the late G_1 phase known as Start (Pringle and Hartwell, 1981). At Start a yeast cell integrates

environmental and internal signals, such as nutrient availability, presence of pheromone, attainment of a critical size and status of the metabolic machinery, to decide whether to enter a new cell cycle or to undertake an alternative developmental program which includes sporulation, conjugation or entry into stationary phase (Alberghina et al., 2012).

The future daughter cell appears during G_1 phase in the cell cycle as a discrete morphological entity on the mother cell, the bud (Chen et al., 2000). Bud grows in size throughout the cell cycle, thereby providing a morphological indicator of cycle progress (Hartwell, 1974). S and M phases are completed before the bud grows as large as its progenitor. Finally, cell separation produces a large mother cell and a small daughter cell. Shortly after division, the mother cell produces a new bud, but the daughter cell enters an extended G_1 phase, during which it apparently must grow to a critical size before it can make a bud of its own. The whole process is quite sensitive to growth rate (Chen et al., 2000). At the fastest growth rates, division is almost symmetrical, and daughter cells have a short G_1 phase as well. As growth rate is decreased cell division becomes increasingly asymmetrical, and the G_1 period of the daughter cell lengthens dramatically, whereas that of the mother cell remains relatively constant (Chen et al., 2000). Nutrient availability also modulates the degree of asymmetry of cell division. Poor media usually yield large parent cells and very small daughters, whereas in rich media the asymmetry between parent and daughter cells is substantially reduced (Alberghina et al., 2012). As mentioned before, proliferating budding yeast cells must be highly polarized to allow the cell to grow a bud from a single site on the cell surface, contrary to simply growing uniformly larger. In this process, the actin patches become highly concentrated at the growing tip of the bud, with the actin cables aligned and pointing toward them. This actin organization directs the secretion of new cell wall and other materials to the site of budding. The polarized organization of the actin structures in turn influences the orientation of the mitotic spindle, so that a complete set of replicated chromosomes can be delivered into the daughter cell and which ends the cell division process (Alberts et al., 2008). Both haploid and diploid cells undergo the same mitotic cycle, whereas diploid form undergoes meiosis to produce four haploid spores located in the ascus (Hartwell, 1974).

Many important discoveries about cell cycle control have come from systematic searches for mutations in yeasts that inactivate genes encoding essential components of the cell cycle control system. The genes affected by these mutations are known as cell division cycle genes, or *cdc* genes (Alberts et al., 2008). Those genes encode various regulatory subunits known as cyclins, having a regulatory role in cyclin-dependent kinases (Cdk) activities, which in turn

govern the major cell cycle transitions in budding yeast. Execution of Start irreversibly commits the cell to a new mitotic cycle and requires the activation of Cdk (Alberghina et al., 2012). Likewise, many of *cdc* mutations cause cells to arrest at a specific point in the cell cycle, suggesting that the normal gene product is required to get the cell past this point. A mutant that cannot complete the cell cycle cannot be propagated. Furthermore, *cdc* mutants can be selected and maintained only if their phenotype is conditional, what means that gene product fails to function only in a certain specific conditions such as temperature and nutritional deprivation. Most conditional cell cycle mutations are temperature-sensitive mutations (Alberts et al., 2008). Cells carrying mutant proteins fail to function at high temperatures but function well enough to allow cell division at low temperatures. A temperature-sensitive *cdc* mutant can be propagated at a low temperature (i.e. the permissive condition) and then raised to a higher temperature (i.e. the restrictive condition) to switch off the function of the mutant gene (Alberts et al., 2008). At the higher temperature, the cells continue through the cell cycle until they reach the point where the function of the mutant gene is required for further progress, and at this point they arrest. In budding yeasts, a uniform cell cycle arrest of this type can be detected by just looking at the cells by light microscope. The presence or absence of a bud and bud size indicate the point in the cycle at which the mutant is arrested (Alberts et al., 2008). However, by inhibiting cell cycle progression using temperature sensitive cell cycle mutants or limiting growth by inducing nutritional deprivation, researchers were able to show that mutants blocked at various stages of cell cycle were mostly able to continue to growth as shown by their increase in the cell volume, mass and protein content. On the other hand, inhibition of growth constantly yielded small un-budded cells arrested before the G_1/S transition. The exception to this rule is given by early Start mutants (such as cdc25) that strongly reduce growth, since they behave as nutrient starved cells (Alberghina et al., 2012).

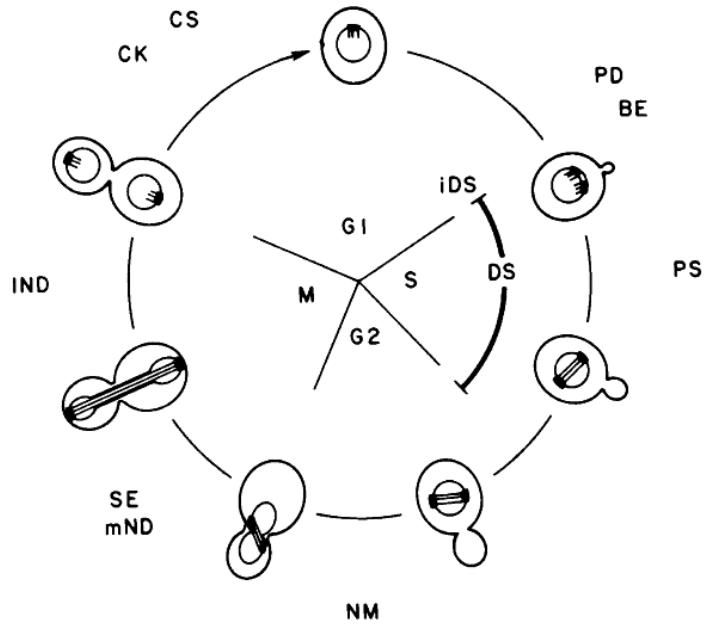


Figure 4. Landmarks of the *S. cerevisiae* cell division cycle. Abbreviations: PD = plaque duplication; BE = bud emergence; iDS = initiation of DNA synthesis; DS = DNA synthesis; PS = plaque separation; NM = nuclear migration; mND = medial nuclear division; SE = spindle elongation; IND = late nuclear division; CK = cytokinesis: CS = cell separation. Distance between events does not necessarily reflect interval of time between events (Hartwell, 1974).

1.3. The life span of the yeast *Saccharomyces cerevisiae*

1.3.1. Different approaches of yeast life span

Two major approaches have been used to determine senescence and life span in yeast: replicative and chronological life span (Gershon and Gershon, 2000). However, both of the processes show an exponential increase in yeast mortality rate over time, similar to aging of higher organisms (Tissenbaum and Guarente, 2002). Replicative aging, also called mother cell-specific aging (Fig. 5), is characterized by the fact that only the mother cell ages (Laun et al., 2006). Eventually, the mother cell reaches a state of senescence, defined here as the terminal stage at which no further cell divisions are performed. On the contrary, the daughter cell resets the clock to zero with full life span characteristic for the strain, regardless of whether it is the first or the tenth daughter of an individual mother cell. Only the very last daughters of old mothers have a somewhat shortened life span (Dickinson and Schweizer, eds., 2004). Mother and daughter cells differ in size, what can be observed by light microscope. Furthermore, using the proper staining technique, the newborn daughter shows a

„birth scar“, while the mother shows as many „bud scars“ as the number of daughter produced. Accordingly, replicative life span in yeast is measured by cell generations, not by chronological age (Laun et al., 2006). Life span is considered completed when the mother cell no longer divides. Median life span of laboratory wild-type strains is around 25 generations. According to this, a group of proliferating yeast cells behave in a certain way similar to stem cell population in human body. In both cases, asymmetric cell division occurs and one of the two resulting cells (the young daughter cell) „resets the clock“ and remains in the stem cell pool. Simultaneously the other cell has made the first step towards differentiation (Laun et al., 2006).

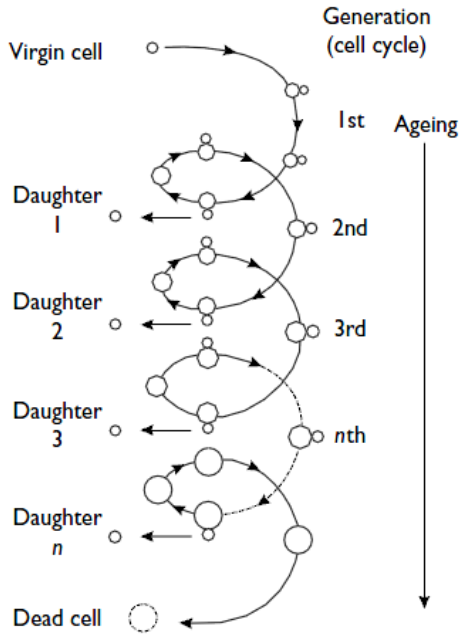


Figure 5. The scheme of replicative ageing. In every consecutive cell division cycle the mother cell (on the right) gains one additional bud scar and ages due to accumulation of an unknown ‘death factor’. The daughters produced in every cell division cycle reset their clock to zero (with the exception of the daughters of very old mothers) (Dickinson and Schweizer, eds., 2004).

The second approach to aging that can be studied in yeast is chronological aging of postmitotic cells. This aging type is measured by using stationary phase cultures in which both deterioration and loss of cell viability are observed (Tissenbaum and Guarente, 2002). Therefore, chronological life span is the period of time during which non-dividing, stationary

phase cells maintain viability (Dickinson and Schweizer, eds., 2004). In contrast to the mother cell-specific replicative aging, chronological aging is a process of genetically controlled cell differentiation. For instance, the cell wall of stationary cells undergoes genetically controlled structural changes and remodelling that is necessary to increase survival of stationary cell population (Laun et al., 2006). However, ultimately these cells age and die. Survival of the cell culture that has reached the stationary phase present a model for cell survival in organs of higher organisms, that have lost their stem cell population and age because of an unavoidable cell loss. Furthermore, in this comparison care must be taken because human postmitotic cells are metabolically very active, while stationary phase yeast cells reach postmitotic condition because of lack of nutrients. Therefore in the case of stationary yeast cells the starvation response plays the major role (Laun et al., 2006).

1.3.2. Some of the presumed mechanisms of replicative aging in yeast

Many different hypotheses have been suggested to explain cellular and organism ageing. Different approaches to aging are not mutually exclusive. Moreover, almost certainly, several independent causes can contribute to the ageing process (Laun et al., 2001). Furthermore, the yeast *S. cerevisiae* has been widely accepted as an occasional model organism for studies of aging, based on the assumption that basic mechanisms of aging are conserved among eukaryotic organisms (Zadrag-Tęcza et al., 2009). The similarity is most questionable in the case of replicative life span, due to the limited proliferative potential of yeast cells as well as it is in mammalian cells. The main question is why the cells stop dividing after a certain number of divisions, while their daughter cells are set at zero (Zadrag-Tęcza et al., 2009). There is more than one explanation to this question. It is suggested that mother cell accumulates „senescence factor" which is cytoplasmatic, diffusible and degradable, but not transmitted to the daughter cells. This factor has not been identified yet (Zadrag-Tęcza et al., 2009).

One of the assumptions of yeast replicative aging is the accumulation of rDNA mini-circles. These extranuclear mini-circles (i.e. ERCs) arise from the 100-200 copies of rDNA arrayed in tandem on chromosome XII. Each repeat contains sequences that encode 35S rRNA and 5S rRNA (Fig. 6). The chromosome tandem region is organized as specific nuclear structure, i.e. nucleolus (Defossez et al., 1999). Furthermore, bidirectional replication starts at the origin within non-transcribed rDNA region, during the S phase of cell cycle. While rightward-

moving replication fork is arrested at the replication fork-blocking site (i.e. RFB) the other fork can go through the RFB site because of its polarity. That provides the replication in the same direction as rDNA transcription (Kobayashi, 2003) and prevents collision between replication and transcription machineries. In addition, nucleolar protein Fob1 (i.e. fork-blocking less) is required for RFB activity as well as for rDNA recombination (Kobayashi and Horiuchi, 1996). It is proposed that this block can trigger aging by causing chromosomal double-strand breaks, the repair of which results is the generation of rDNA circles (Defossez et al., 1999), probably through unequal sister chromatid recombination (Kobayashi et al., 1998). Therefore, gene *FOB1* was shown to be essential for both the decrease and increase of rDNA repeats (Kobayashi et al., 1998). If there is a mutation in *FOB1* gene that leads to dysfunctional protein, the yeast life span will be extended approximately 30-50% compared to that of wild type cells. The reduced amount of ERCs in the *fob1* mutants could either result from a decrease in the frequency of ERCs formation, an impaired ability of ERCs to replicate or segregate to mother cells after they have formed, or an increased degradation or reintegration into the genome (Defossez et al., 1999). With every division, the mother cell accumulates rDNA, which eventually leads to prevention of ordered replication and cell cycle progression by titrating out an essential DNA-binding factor, thus leading to senescence (Laun et al., 2001). The strong tendency of such accumulation lies in the inability to be segregated regularly on the mitotic spindle. However, ERCs have not been demonstrated in senescent cells of multicellular organisms (Laun et al., 2001), and they also show no significant impact on replicative aging in yeast. Likewise, the life span is not adversely affected with commonly occurring 2-μm plasmids. Still, accumulation of rDNA circles cannot be a single factor underlying the limitation of yeast mother cells divisions (Zadrag-Tęcza et al., 2009).

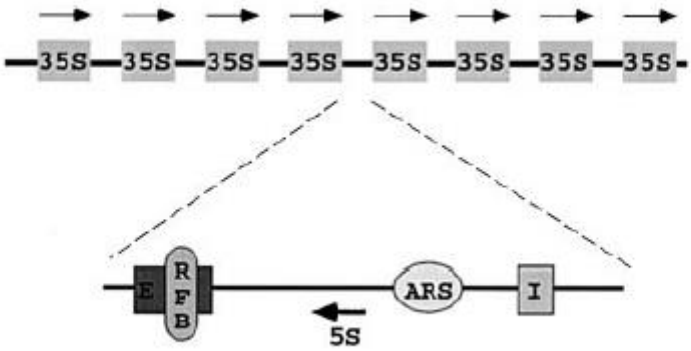


Figure 6. Structure of rDNA repeats in *S. cerevisiae*. The rDNA tandem region consists of transcribed 5S and 35S RNA genes (the direction of transcription is indicated by arrows) and non-transcribed regions. Each repeat contains the coding sequence for 35S rRNA, transcribed in the direction of the arrows, and the spacer, which is expanded. The spacer contains the enhancer (E) and initiator (I) for 35S transcription, the *ARS* for initiation of DNA replication, the 5S RNA-coding sequence, and the replication fork block site (RFB) (Defossez et al., 1999).

Another factor considered to have an important role in replicative aging is accumulation of oxidative damaged material in mother cells which is inherited asymmetrically in daughter cells (Zadrag-Tęcza et al., 2009). The yeast *S. cerevisiae* is a facultative anaerobe and grows better in oxygen conditions by using molecular oxygen for respiration or oxidation of nutrients to obtain energy efficiently. During these processes ROS are generated and attack almost all cell components: DNA, protein and lipid membrane (Izawa et al., 1995). In addition, it was found that deletions of the yeast genes coding for superoxide dismutase (SOD) or catalase, changes in atmospheric oxygen partial pressure as well as the presence of the physiological antioxidant glutathione have distinct effects on life span (Laun et al., 2001). Like other eukaryotes, *S. cerevisiae* contains two intracellular SODs playing a protective role against the ROS toxicity. Sod1 is distributed in the cytoplasm, nucleus, and other compartments, containing copper and zinc (i.e. CuZnSOD) while Sod2 is a mitochondrial enzyme containing manganese (i.e. MnSOD) in the active site (De Freitas et al., 2000). Neither *SOD1* nor *SOD2* is strictly essential. However, disruption of SOD genes, though not for catalase, leads to a reduction of the reproductive potential (Zadrag et al., 2008). Yeast strains lacking Sod1 show several defects during aerobic growth, including reduced growth rates, auxotrophies for lysine and methionine, poor growth by respiration (in glycerol or

ethanol), higher rates of spontaneous mutation, and more rapid loss of viability in stationary phase. These defects can be efficiently prevented by antioxidant. However, it was found that hypoxic or anoxic atmosphere did not improve the decreased budding capacity of these mutants, contrary to the expectations based on free radical theory (Zadrag et al., 2008). Furthermore, the young cells have an ample capacity to remove damaged material above a certain threshold. For instance, ROS generated in mitochondria lead to the oxidatively damaged mitochondrial proteins, i.e. protein carbonyl groups. Those modified proteins would then increase the unwanted production of ROS through the respiratory chain, what in turn would increase damage of mitochondrial proteins (Laun et al., 2006). However, it is important to notice that no firm evidence exists that these damaged materials are causing aging process. Still, it seems to be clear that damaged material accumulates during chronological aging of yeast cells, which results in a progressively shortened replicative life span when yeast is kept in stationary phase over long period of time. One of the purely hypothetical possibilities is uneven segregation of mitochondrial mass between mother and daughter. There is an assumption of the existence of very specific proteins highly sensitive to oxidative stress which does not originate from the mitochondria (Laun et al., 2006).

Mitochondrial damage has also been taken into account as a potential course of yeast replicative aging (Zadrag-Tęcza et al., 2009). For instance, yeast is highly specialized for growth on glucose, but easily forms „petite"-mutants (rho^o) resulting in little or no mitochondrial respiration. The mutants are therefore incapable to grow on media containing only non-fermentable carbon sources such as ethanol or glycerol. Those mutants form small anaerobic colonies when grown in the presence of fermentable carbon sources such as glucose. Contrary, a similar defect in cellular respiration would be incompatible with life of a multicellular organism. However, today is a known fact that human cells with zero respiration can be maintained in culture and even in the body, but in pathological situations of carcinogenesis (Laun et al., 2006). Finally, disorders in mitochondrial respiration are responsible for the production of ROS and are one of the most generally agreed mechanisms that cause aging. Therefore occupies very important place in aging studies (Laun et al., 2006).

According to the senescence factor hypothesis, there is another geometrical factor that may be critical for limitation of budding of yeast cells (Zadrag-Tęcza et al., 2009). It is proposed that simply reaching excessive volume by yeast cells at the end of their reproductive period is responsible for their inability to undergo further reproduction (Bilinksi at al., 2012). Furthermore, the volume of yeast mother cell increases unavoidably upon each budding,

consequently reaching a size too big to allow efficient completion of cell cycle. Volume changes in three pairs of different laboratory strains and their isogenic mutants for antioxidant enzymes were analysed. Due to various defects in the antioxidant barrier, they showed considerable differences in the replicative life span (Zadrag-Tęcza et al., 2009). The BY4741 and W303-1A strains are standard laboratory strains while D1CSP4-8C is a cross between the SP-4 strain and its mutant. The D1CSP4-8C strain is the most long-lived than other two strains, comparing mean and maximal replicative life span. Maximal life span of the BY4741 strain is lower in comparison to latter strain, and that of W303-1A strain is the lowest. The final volumes attained by D1CSP4-8C and BY4741 cells do not differ significantly but that of the W303-1A strain is considerably higher, which is attributed to the large vacuolar volume (Zadrag-Tęcza et al., 2009). However, despite differences in the replicative life span between mutants and their standard counterparts, the volumes attained by mother cells reaching their maximal replicative life span were identical in each pair of strains. These results indicate that a critically high cell volume may be the main limiting factor for successful completion of mother yeast cell cycle (Zadrag-Tęcza et al., 2009). Apparently, oxidative stress leads to progressive DNA damage, which could consequently stop the divisions at the cell cycle checkpoints. The protein synthesis continues undisturbed resulting in a volume increase (Fig. 7). There are also phenomena and explanations which does not support this theory of increasing cell volume. However, the idea that attaining excessive cell volume limits the further division of yeast cells exclude a failure of mechanism retaining the putative "senescence factor" in the mother cell (Zadrag-Tęcza et al., 2009).

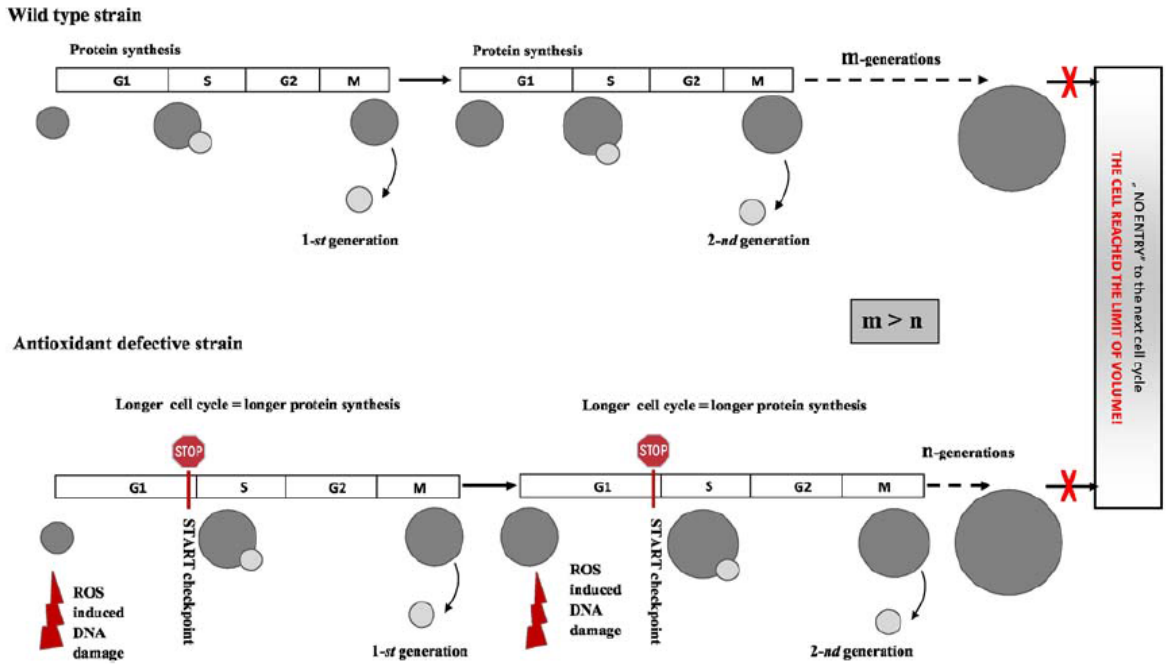


Figure 7. Presumed mechanism of shortening the replicative life span of yeast mutants defective in antioxidant barrier. Due to the oxidative stress in mutants for antioxidant enzymes, DNA damage resulting in delayed cell division. Protein synthesis is not inhibited and therefore the cell volume increases. As results, the mutants reach the limiting cell volume after a lower number of cell divisions (Zadrag-Tęcza et al., 2009).

1.3.3. Morphological and physiological characteristics determined during *Saccharomyces cerevisiae* life span

As already mentioned, the aging process comprises many transformations at different cellular growth levels. Consequently morphological and physiological modifications arise that ultimately result in an alternation of the cell phenotype. Terminally senescent mother yeast cells display dramatically changes in appearance and physiology, compared to the young cells. One of the observable features is substantially big difference in cell size, with old cells being much larger than young cells. Furthermore, the cell division cycle is prolonged compared to the daughter cell, whereas the cytoskeleton of old cells seems to be collapsed and consequently display patchy actin filaments instead of the usual dots and cables (Dickinson and Schweizer, eds., 2004). Owing to irregular cell division cycles some of these cells show no or more than one nucleus due to the nuclear fragmentation or endomitosis, while nuclear

chromatin appears more diffuse, contrary to the young cells that display compact single nuclei of normal appearance (Laun et al., 2001). In addition, the mother cells extent greater concentration of ROS which are mainly of mitochondrial origin. Finally, the senescent cells show apoptotic phenotypes, like phosphatidylserine exposure, nuclear DNA fragmentation and chromatin marginalization (Laun et al., 2006).

When nutrients become depleted yeast of normal cell size will reach the stationary phase. The starvation response leads to a genetically controlled change in cellular metabolism. Most prominent is the synthesis of reserve carbohydrates such as glycogen and trehalose (i.e. α,α-1,1-glucoside bond between two α-glucose units). Furthermore, stationary cells form thicker and harder cell wall. Glycogen is slowly degraded in the starving but surviving cells, while trehalose seems to be important for membrane stabilization and other non-metabolic functions (Laun et al., 2006). Likewise, protein synthesis is slowed down, and the cell surface has a loose and wrinkled appearance (Laun et al., 2001).

Stationary yeast cells can be considered as two cell populations: quiescent and non-quiescent cells. The quiescent population represents non-dividing cells characterized by low metabolic activity and arrested in an un-budded G_0 state. Those cells are formed only during the final cell division in the biphasic growth curve. They are described as daughters that have a higher density than normal yeast cells and can be separated from the non-quiescent stationary cells on sucrose gradients. Contrary, the non-quiescent cells are the ones which display elevated ROS levels and finally undergo apoptosis (Laun et al., 2006). Furthermore, damaged cellular material is attributed to the damaged mitochondria, which produce an increasing amount of ROS. Mitochondria producing ROS are found preferentially in the senescent cells. Otherwise, there is a speculation that old mitochondria should be inherited mostly by the mother cell in any cell division, whereas newly synthesized mitochondria should be segregated to the daughter cell (Laun et al., 2001). In addition, after a longer time, the quiescent cells also start to show landmarks of apoptosis. Overall the phenotypes and the causes of aging in old mother cells and in stationary cultures seem to be very different (Laun et al., 2006). Yeast mother cell-specific ageing has been intensively researched. This process represents a simple model system for cellular and organism ageing, therefore showing similarity to ageing of higher cells in terms of well-characterized morphological and physiological changes. However, an increase in intracellular oxidative stress as well as apoptotic and necrotic features seems to be common to the two aging processes (Laun et al., 2001).

2. THE MAIN OBJECTIVE OF THE THESIS

The main objective of this thesis was to observe morphological and physiological characteristics of the yeast *Saccharomyces cerevisiae* cells during its replicative life span. As the experimental materials three strains (i.e. wild-type, *Δfob1* and *Δsod1*) of three genetic backgrounds (i.e. SP4, BY4741 and BMA64-1A) were used. *Δfob1* mutants have increased length of replicative life span, therefore representing the hypothesis of the "senescence factor". In contrary, *Δsod1* mutants have reduced length of replicative life span and represent the free radical theory of aging. Very often the effect of mutations depends on the genetic background. Determination of the morphological and physiological differences between yeast strains can explain the differences in the length of replicative life span, thereby supporting or weaken hypothesis that hypertrophy is the cause of restricted reproductive capacity. For this purpose, various methods have been used. Growth curve test gave a proper insight into relative growth rate of strains. The growth kinetic was also observed by determination of the rate of glucose consumption. Furthermore, yeast sensitivity to oxidative stress by exposure to H_2O_2 in spot test was determined, whereas the ROS were detected by using dihydroethidium assay. Presence of carbonyl protein groups was observed by the immunocytochemistry assay. Cellular morphology and metabolic activity were observed by fluorescence during the prolonged cultivation for 7 days. The parametric results were statistically analyzed by using Tukey and Dunnett post hoc tests as part of the one- and two-way ANOVA statistics to compare the mutants and wild-type strains.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Solutions and reagents

Brady's reagent (DNPH) [Sigma-Aldrich; Germany]

DNPH 1x:

- 20 mM DNPH
- 10% trifluoroacetic acid
- mQ H_2O

BSA 30% [Sigma-Aldrich; Germany]

Glycerol [Sigma-Aldrich; Germany]

Trifluoroacetic acid [Sigma-Aldrich; Germany]

Hydrogen peroxide 30% pure [Applichem; Germany]

Denaturat [Chempur; Poland]

D-(+)-glucose anhydrous [Poch; Poland]

Amplex® Red Glucose/Glucose Oxidase Assay Kit [Invitrogen; UK]

Prestained Protein Molecular Weight Marker 20-100 kDa *Fermentas* [Thermo Scientific; USA]

Distilled water (dH_2O)

Milli-Q water (mQ H_2O)

Hydrogen peroxide solution (1 M)

Neutralization solution 20 mL:

- 2 M Tris – 4.84 g
- 30% glycerol – 12 mL 50% glycerol
- 8 mL of mQ H_2O

PBS [Bioshop; Canada]

EDTA disodium salt dehydrate (20 mM, pH 8.00) [Sigma-Aldrich; Germany]

HEPES anhydrous $C_8H_{18}N_2O_4S$ [Sigma-Aldrich; Germany]

HEPES sodium salt $C_8H_{17}N_2O_4SNa$ [Sigma-Aldrich; Germany]

HEPES (10 mM) pH 7.2 with 2% glucose

HEPES (10 mM) pH 7.4 with 5% glucose

Phosphate buffer pH 7

Phosphate buffer pH 7 with 1 mM EDTA and 0.1 % glucose

Reducing buffer pH 9.3 100 mL:

- 10 mL 1 M Tris
- 3.497 mL β-mercaptoetanol (14.3 M)
- mQ H_2O up to 100 mL
- adjust pH with concentrated HCl

Spheroplast buffer 500 mL:

- 91.35 g sorbitol (1 M)
- 0.0951 g EGTA (0.5 mM)
- 0.12037 g $MgSO_4$ (2 mM)
- 0.049674 g NaCl (1.7 mM)
- 0.68045 g KH_2PO_4 (10 mM)
- 0.5 g BSA (0.1%)
- mQ H_2O up to 500 mL
- adjust pH 6.8 with KOH

Washing buffer 500 mL:

- 0.6055 g Tris
- 18.65 g KCl
- mQ H_2O up to 500 mL
- adjust pH with concentrated HCl

Fluorescent dye FUN-1; DAPI; MDY64; Rhodamine B; FDA *Invitrogen* [Thermo Fisher Scientific; USA]

Fluorescent dye PI [Sigma; Germany]

Bacto™ Peptone: Enzymatic digest of protein [Becton Dickinson; USA]

Bacto™ Yeast Extract: Extract of autolysed yeast cells [Becton Dickinson; USA]

Difco™ Agar, granulated: Solidifying agent [Becton Dickinson; USA]

YPD liquid media with 2% glucose 300 mL:

- 6 g of D-(+)-glucose anhydrous
- 3 g Bacto™ Peptone
- 3 g Bacto™ Yeast Extract
- 300 mL dH_2O

YPD liquid media with 0.5% glucose 100 mL:

- 0.5 g of D-(+)-glucose anhydrous
- 1 g Bacto™ Peptone
- 1 g Bacto™ Yeast Extract
- 100 mL dH_2O

YPD solid media with 2% glucose 300 mL:

- 6 g of D-(+)-glucose anhydrous
- 3 g Bacto™ Peptone
- 3 g Bacto™ Yeast Extract
- 6 g Difco™ Agar, granulated
- 300 mL dH_2O

YPD solid media (2% glucose) with 1 / 3 / 5 mM hydrogen peroxide:

- 20 mL of YPD media (2% glucose)
- 20 / 60 / 100 µL of 1 *M* hydrogen peroxide solution

Primary antibody:

- anti- dinitrophenol rabbit polyclonal *Cat. No. ab6306* [Abcam; UK]

Secondary antibody:

- goat anti-rabbit monoclonal *Cat. No. W401B* [Promega; USA]
- goat anti-rabbit polyclonal *Cat. No. ab60317* [Abcam; UK]

3.1.2. Measuring devices

Spectrofluorometer *Infinite M200* [Tecan; Austria]

Fluorescent microscope *BX51* [Olympus; Japan] with *X-Cite® 120 PC Q* excitation light source [Lumen dynamics; Canada]

Microbiology reader *Bioscreen C* [Oy Growth Curves; Finland]

pH, conductivity and ISE measurements-all in one instrument *ION450 MeterLab®* [Radiometer analytical; France]

Ultrapure Water Systems *Direct-Q 3* [Millipore; France]

Centrifuges:

- mini spin *13400 rpm* [Eppendorf; Germany]
- 350-R *9000/min* [MPW; Poland]
- 5810 R *14000 rpm* [Eppendorf; Germany]

Incubator-rotary shaker *New Brunswick™* [Eppendorf; Germany]

Microplate shaker *Titramax 1000/Incubator 1000* [Heidolph; Germany]

Vortex mixer [Heidolph; Germany]

Autoclave *Nova* [Tuttnauer; Israel]

Light microscope [Nikon; Japan]

Fluorescent microscope *BX50* with Olympus *U-RFL –T* Microscope Fluorescence Power Supply [Olympus; Japan]

Rocker-Shaker *M12* [Biosan; Latvia]

Balance *WPS 360/C* [Radwag; Poland]

Dryer and sterilizer *KBC-125 W* [Wamed; Poland]

Magnetic stirrer *MS 11 H* [Wigo; Poland]

Thermal incubator for cell cultures [Elkon; Poland]

Additional equipment: hemocytometer Tiefe depth profounder 0.200 mm and 0.0025 m^2 area [Brand; Germany]

3.1.3. Biological material

Table 1. Yeast *S. Cerevisiae* strains used in the study

Strain	Genotype	Descriptions	Reference
SP4	*MAT*α *leu1 arg4*	wild-type	Bilinski et al. 1978
Δfob1	*MAT*α *leu1 arg4*	mutation in the gene for replication block protein 1	Zadrag-Tecza et al. 2013
Δsod1	*MAT*α *leu1 arg4*	mutation in the gene for superoxide dismutase 1	Bilinski et al. 1985
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	wild-type	Euroscarf
Δfob1	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	mutation in the gene for replication block protein 1	Euroscarf
Δsod1	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	mutation in the gene for superoxide dismutase 1	Euroscarf
BMA64-1A	*MAT*a *ura 3-52, trp1Δ2 leu2-3 112his3-11 ade2-1, can1-100*	wild-type	Euroscarf
Δfob1	*MAT*a *ura 3-52, trp1Δ2 leu2-3 112his3-11 ade2-1, can1-100*	mutation in the gene for replication block protein 1	obtained by M. Molon
Δsod1	*MAT*a *ura 3-52, trp1Δ2 leu2-3 112his3-11 ade2-1, can1-100*	mutation in the gene for superoxide dismutase 1	obtained by M. Molon

3.2. Methods

3.2.1. Growth conditions

Yeast cells were grown in a standard liquid YPD medium (1% Yeast Extract, 1% Yeast Bacto Peptone, 2% glucose) on a rotary shaker at 150 rpm or on a solid YPD medium containing 2% agar at 28ºC.

3.2.2. Establishing a cell culture

Under aseptic conditions, the starter culture was inoculated with a proper amount of yeast cells, thawed from - 80^{o}C. Thereby liquid media should fill 25% of the flask volume. Culture was grown at 28^{o}C in shaker at approximately 150-170 rpm. After two or three days higher culture density was observed, cell culture was established and can be stored at 4^{o}C. Using aseptic technique, the appropriate volume of inoculums was transferred to the new flask with fresh standard liquid YPD media for further experiments. Cells were grown at 28^{o}C with continuous shaking. After 12-14 hours of growth the cultures achieved an exponential phase of growth. In same conditions cultures were left in the incubator for two days to achieve stationary phase of growth.

3.2.3. Determination of yeast culture density

Each experiment requires a certain number of cells, after which they were treated according to the protocol. The cells were diluted in serial dilutions (10 x, 100 x or 1000 x), depending on the culture density. Aliquot of the diluted solution was applied to the hemocytometer and cells were counted randomly in five main fields under a light microscope with a magnification of 400 x. Number of cells/mL was calculated according to the formula:

$$number\ of\ \frac{cells}{mL} = X * 100 * 1000 * D$$

where X is a mean value of cells counted in five major fields and D is dilution that was used for counting (10, 100, 1000 etc.). In the field of view single cells and those with buds were observed. The buds that achieve one third of the mother cell size are relevant for counting and observed as single cells.

3.2.4. Cells size measurement

In order to determine cells size for all yeast samples a native microscopic slide was made. The cells were observed under the magnification 400 x and images were captured with microscope using bright field. Diameter of the cell was measured using *MicroImage* software. Cell diameter was measured two times in various planes for each cell and mean value was calculated. Cell size was examined from at least 100 cells in one biological replicate.

3.3. Determination of relative growth rates

The cell growth was monitored turbidimetrically at 600 nm in a *Bioscreen C* microplate reader. Yeast cells at exponential growth phase were counted and proper volume of cells was added into two new tubes, for each strain, to achieve 1 x 10^7 of cells/mL. Distilled water is added to the final volume of 1 mL. Samples were centrifuged (2 minutes; 6600 rpm) and supernatant was decanted. In one tube the pellet of corresponding strain was resuspended in 1 mL of liquid growth media containing 2% glucose, while the pellet of the same strain type in the other tube was resuspended in the equal volume of liquid growth media containing 0.5% glucose. Resuspension was done with vortex mixer. After cells were placed in a suitable media, 200 μL of each sample was added in triplicate on the Honeycomb plate. Measurements were carried out during 24 h with a measuring interval of 1 hour at 28^{o}C, along continuous shaking, and exposure wavelength was 600 nm. Blank samples (i.e. media without cells) were also done in triplicate, and those results were subtracted from sample measurements.

3.4. Determination of the rate of glucose consumption by the yeast cells

The yeast cells at logarithmic phase of growth (i.e. 5-7 x 10^7 of cells/mL) were inoculated in 20 mL of the fresh liquid YPD media to achieve density 1 x 10^6 of cells/mL. Every 4 hours cells were precipitated (2 minutes; 6600 rpm), the supernatant was transferred to the new tubes and stored at -20^{0}C until glucose concentration analysis. At the zero time the rate of glucose was 2%. All solutions and reagents were prepared according to the manufacturer instructions [Amplex® Red Glucose/Glucose Oxidase Assay Kit; Invitrogen]. The glucose-containing samples were serially diluted (up to 10 000 x) in 1 x reaction buffer to determine the optimal amount of sample for the assay. However, sample without glucose was also used

as a blank. 50 µL of samples and blank were added into individual wells of a microplate as well as 50 µL of fresh prepared Amplex® Red reagent/HRP/glucose oxidase working solution. The samples incubation was carried on at room temperature for 20 minutes and protected from light. The fluorescence emission was detected with the *Tecan Infinite M200* microplate reader at 590 nm after excitation in range of 530-560 nm. For each sample, the value derived from blank was subtracted.

3.5. Determination of sensitivity to oxidative stress generated by hydrogen peroxide

3.5.1. Spot test after one hour of cells incubation with hydrogen peroxide

The cells at exponential growth phase were harvested with centrifuge (2 minutes; 6600 rpm) and pellet was washed twice with 1 mL of distilled water. Cells were resuspended to the density 1×10^8 of cells/mL in phosphate buffer (pH 7.0), containing 1 mM EDTA. 1 mL of each sample was transferred to two glass tubes. One tube presents control sample without H_2O_2, while in another proper volume of H_2O_2 (1 M stock solution of H_2O_2 in dH_2O) was added to achieve final concentration of 10 mM. Incubation time is 60 minutes at 28°C, with continuous shaking. Furthermore, control and test samples were transferred into new tubes, centrifuged, washed twice in distilled water, resuspended in 1 mL distilled water and serial dilutions were made for each sample. 5 µL of several dilutions (10^7, 10^6, 10^5, 10^4 and 10^3 of cells/mL) of the yeast were inoculated on solid YPD medium and inspected after 48 h of incubation at 28°C.

3.5.2. Spot test on the solid YPD media with different concentration of hydrogen peroxide

The cells at exponential growth phase were harvested with centrifuge (2 minutes; 6600 rpm), washed twice with 1 mL of distilled water and resuspended to the density 1×10^7 of cells/mL in distilled water. 5 µL of several dilutions (10^7, 10^6, 10^5, 10^4 and 10^3 of cells/mL) of the yeast were inoculated on solid YPD medium containing 1, 3 or 5 mM H_2O_2 (1 M stock solution of H_2O_2 in dH_2O) and inspected after 48 h of incubation at 28°C. Samples inoculated on a standard solid YPD media without H_2O_2 were used as controls.

3.6. Measurement of ROS generations

The cells at exponential growth phase were precipitated (2 minutes; 6600 rpm), washed twice with 1 mL of distilled water and resuspended in 100 mM phosphate buffer (pH 7.0), containing 1 mM EDTA and 0.1% glucose to the density 1×10^8 of cells/mL. 200 µL of each sample was added in triplicates on non-transparent black microplate. The DHET was added to the final concentration of 18.9 µM. The kinetics of fluorescence increase was measured using the *Tecan Infinite M200* microplate reader at excitation wavelength of 518 nm and emission wavelength of 605 nm at 28°C, during 20 minutes. ROS production was expressed as a relative rate of fluorescence increase.

3.7. Detection of carbonyl protein groups using immunocytochemistry assay

Yeast cells were resuspended in 1 mL of MilliQ water to the density 4×10^8 of cells/mL. 5 mL of each yeast culture was harvested (2 minutes; 6600 rpm), washed 3 times with 1 mL cold MiliQ water (stored at 4°C), and then fixed in 1 mL of 70% cold ethanol during 45 minutes. Furthermore, cells were harvested (2 minutes; 5000 rpm) washed 3 times in 1 mL of cold washing buffer and incubated in 1 mL of reducing buffer during 10 minutes at 30°C. Cells were then washed 3 times in 1 mL of cold washing buffer. After last wash, yeast cells were suspended in 450 µL of spheroplast buffer and 50 µL of lyticase was added (1000 U/L stock solution), mixed gently with pipette and incubated during 15 minutes at 30°C. After incubation, the sample was centrifuged (3 minutes; 1000 rpm), washed 5 times in 500 µL of spheroplast buffer, resuspended in the mixture of 50 µL spheroplast buffer and 50 µL of DNPH, and incubated 30 minutes at the room temperature. 100 µL of the neutralization solution was added to block the reaction. Samples were centrifuged (3 minutes; 1000 rpm) and washed 5 times in 500 µL of spheroplast buffer. Cells were harvested one more time in the same conditions and resuspended in 50 µL of primary polyclonal rabbit antibody diluted 1:10 000 with spheroplast buffer. This antibody is specific to the DNP moiety of the proteins. After 90 minutes of incubation at the room temperature cells were washed 3 times in spheroplast buffer, centrifuged (3 minutes; 1000 rpm) and resuspended in 50 µL of secondary goat anti-rabbit polyclonal antibody conjugated with red fluorescence dye *Chromeo546* (1:1000 dilution with spheroplast buffer). After 45 minutes of incubation in the dark place at

room temperature, cells were harvested, washed 3 times in spheroplast buffer and placed onto a microscopic slide. The fluorescence was examined using fluorescence microscope at excitation wavelength of 595 and emission wavelength of 615 nm.

3.8. Analysis of the yeast culture during prolonged cultivation

Proper volume of yeast cells was transferred from inoculums to the new flasks with fresh standard YPD media. Cells were growing 12-14 hours at 28^{o}C with continuous shaking, followed by the first measurement. The same experiments were repeated after two, four and seven days of yeasts growth.

3.8.1. Determination of cell metabolic activity by fluorescence method

Cells were washed and resuspended in 10 mM Na-HEPES solution (pH 7.2), containing 2% glucose to the density 1 x 10^7 of cells/ml. 200 μL of each sample was added in duplicates into non-transparent black microplate. The FUN-1 dye[2] was added up to 0.5 μM final concentration (100 μM stock in DMSO). Microplate was left on the shaker (900-1050 rpm) for incubation at 28^{o}C. After 15 minutes, the fluorescence of the cell suspension was measured using *Tecan Infinite M200* microplate reader at excitation wavelength of 480 nm and emission in range of 500-650 nm. Metabolic activity of the cells was expressed as a change in ratio of red (λ = 575 nm) to green (λ = 535 nm) fluorescence. 5 μL of the sample treated with FUN-1 was placed onto a glass slide, covered with cover slip and the cells were observed under a fluorescence microscope.

[2] Free **FUN-1** stain is virtually non-fluorescent in aqueous solutions and usually complexes the nucleic acids such as DNA or RNA. Generally, it will not stain the nuclei of metabolically active yeast cells in growth medium or glucose-containing staining solutions. Metabolically active yeast cells sequester FUN-1 stain in their vacuoles as cylindrical refractive bodies (Millard et al., 1997).

3.8.2. Determination of cellular alternations using fluorescence techniques

Cells were centrifuged, washed twice with 1 mL of distilled water and resuspended in 1 mL of 10 mM Na-HEPES solution (pH 7.4), containing 5% glucose to the density 1 x 10^7 of cells/mL. 100 µL of the sample was added in new tube. Proper volumes of different fluorescence dyes were added into the same tube: 0.5 µL of DAPI (100 µg/mL stock) for nuclei staining, 1 µL of Rhodamine B (100 µM stock) for mitochondria and 0.5 µL MDY-64 (100 µM stock) for vacuole visualization. Samples were left at the room temperature in dark for 15 minutes. 5 µL of the sample was placed onto a glass slide, covered with cover slip and the cellular alternations were observed by using a fluorescence microscope Morphology of the cell nuclei was visible like a blue fluorescence at excitation wavelength of 360 nm and emission wavelength of 420 nm. Red coloured mitochondria were observed at excitation wavelength of 555 nm and emission wavelength of 579 nm. Green stained membranes of vacuoles were visualized at excitation wavelength of 451 nm and emission wavelength of 497 nm.

3.9. Statistical analysis

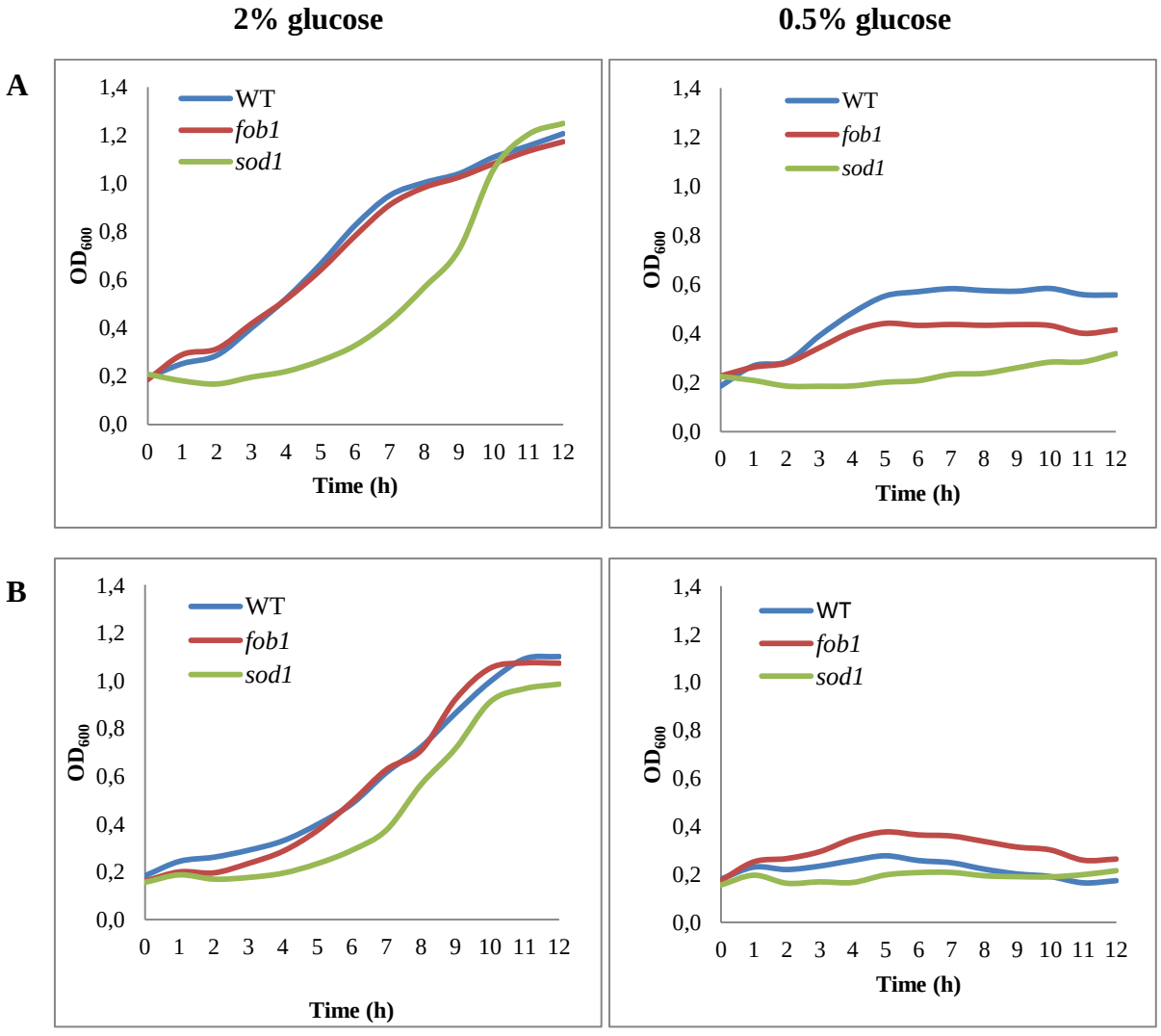
Parametric results were analyzed by using Dunnett's and Tukey's post hoc tests as part of the one-way ANOVA statistics within the *Graph Pad Prism* software. Asterisks were used to determine statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

4. RESULTS AND DISCUSSION

4.1. Results

4.1.1. Determination of relative growth rates

1 x 10^7 of yeast cells/mL was incubated at 28°C in density with continuous shaking during 12 hours in liquid YPD media containing 2% or 0.5 % glucose. Growth rate was determined by measuring optical density (absorbance) at 600 nm using microbiology reader. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments (Fig. 8).



C

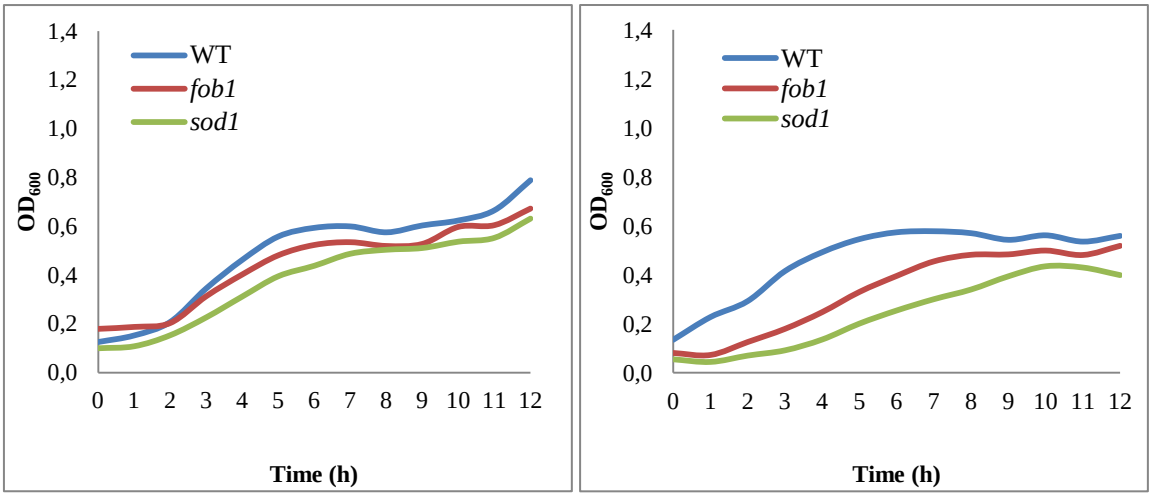


Figure 8. The growth rate analysis of the yeast cells in a liquid YPD media. Three different genetic backgrounds of the yeast *S. cerevisiae* are presented: SP4 (A), BY4741 (B) and BMA64-1A (C). For each genetic background growth rates of three yeast strains were explored: wild-type (WT), Δ*fob1* mutant (*fob1*) and Δ*sod1* mutant (*sod1*). The results are expressed as mean values from two independent biological replicates.

Results of the relative growth rate were analyzed by using the Dunnett's post hoc one-way ANOVA test. Therefore, Δ*fob1* and Δ*sod1* mutant was compared with wild-type as a control strain within each genetic background. There was no significant difference between individual yeast strains of the each genetic background that were grown in media with 2% glucose while in the case of 0.5% glucose there were some significant differences within each genetic background.

Table 1. Significant difference observed by using the Dunnett's post hoc test between individual yeast strains of the each genetic background that were grown in 0.5% glucose was shown.

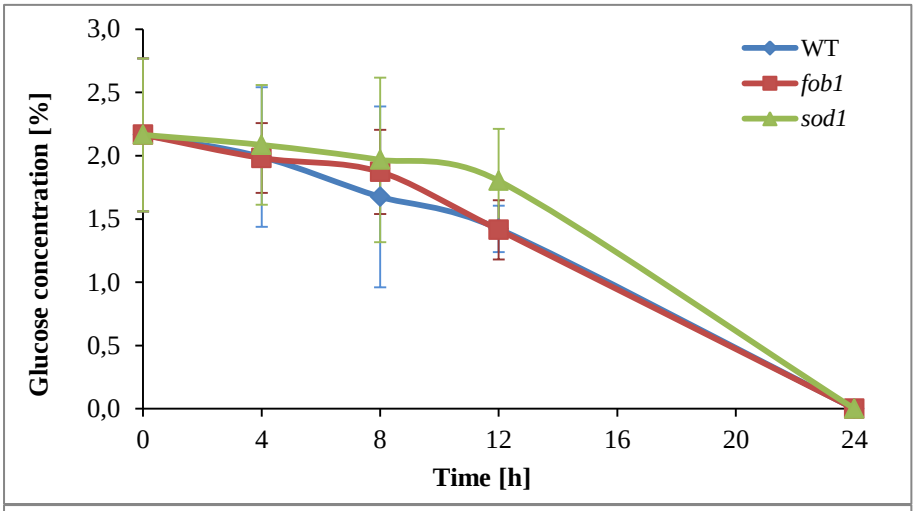
Genetic background	Strains	Significance
SP4	wt vs. Δfob1	*
	wt vs. Δsod1	****
BY4741	wt vs. Δfob1	****
BMA64-1A	wt vs. Δsod1	**

* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001

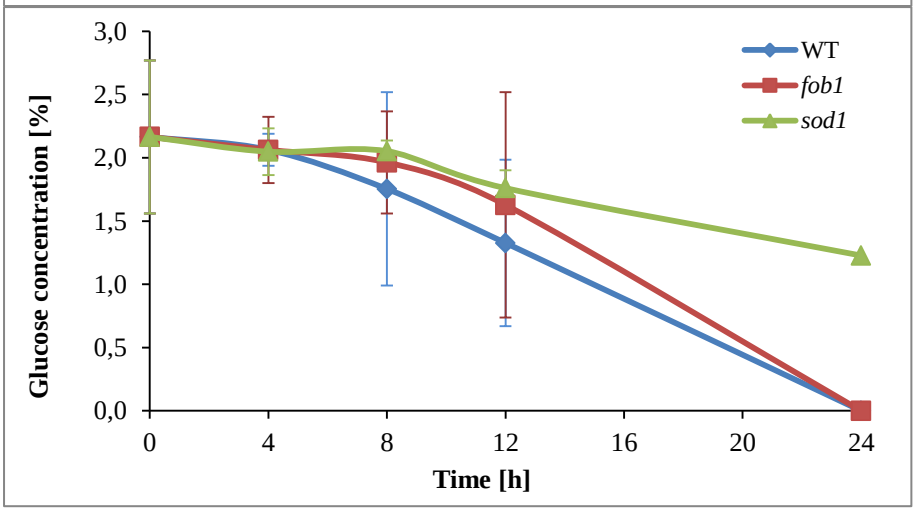
4.1.2. Determination of the rate of glucose consumption

1 x 10^6 of yeast cells/mL was inoculated in liquid YPD media containing 2% glucose and growing at 28°C with continuous shaking. Cells were precipitated (2 minutes; 6600 rpm) every 4 h, the supernatant was transferred to the new tubes and stored at -20°C as a sample. Before using, samples were serially diluted in 1 x reaction buffer. Rate of glucose consumption was determined by measuring the fluorescence emission at 590 nm (excitation range: 530-560 nm) with microplate reader. At the zero time the rate of glucose was 2%. Sample without glucose was used as a blank. For each sample, the value derived from blank was subtracted. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments (Fig. 9).

A



B



C

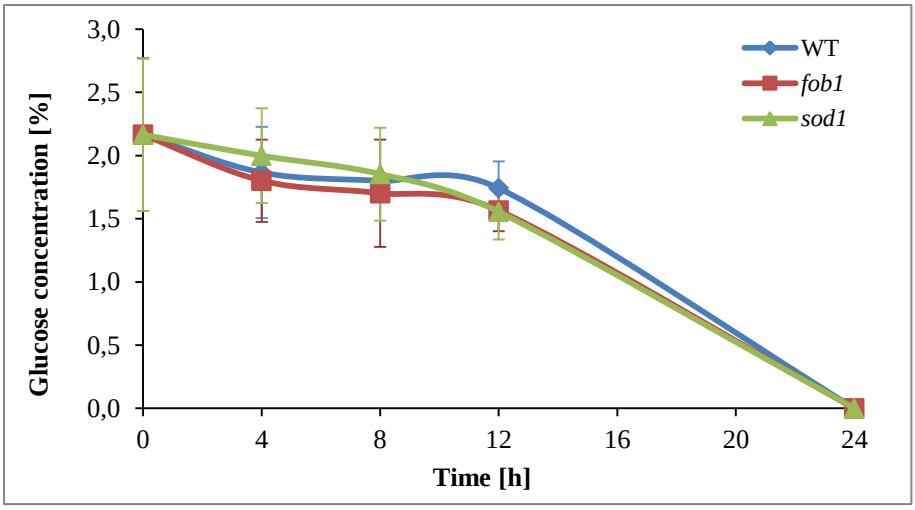


Figure 9. The analysis of the rate of glucose consumption by yeast cells. Three different genetic backgrounds of the yeast *S. cerevisiae* are presented: SP4 (A), BY4741 (B) and BMA64-1A (C). For each genetic background consumption rates of glucose in three yeast strains were explored: wild-type (WT), Δ*fob1* mutant (*fob1*) and Δ*sod1* mutant (*sod1*). The results are expressed as mean value ± standard deviation.

The results of glucose consumption rate were analyzed by using the Dunnett's post hoc one-way ANOVA test. A Δ*fob1* and Δ*sod1* mutant was compared with wild-type as a control strain within each genetic background in the each time point of measurement at 4, 8 and 12 h. No significant difference was observed between tested strains. Only a Δ*sod1* mutant of BY4741 background showed deviation in the time point measurement at 24 h compared to its wild-type strain.

4.1.3. Determination of sensitivity to the oxidative stress generated by hydrogen peroxide

1 x 10^8 of yeast cells/mL was incubated for 1h at 28°C with 10 mM H_2O_2 and continuous shaking. Non-treated cells were used as controls. After serial dilutions (10^7, 10^6, 10^5, 10^4 and 10^3 of cells/mL) 5 µL of each sample was spotted on solid YPD media containing 2% agar. Each drop contained 50 000, 5 000, 500, 50 and 5 cells. Cell growth was observed after 48 h of incubation at 28°C. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments (Fig. 10).

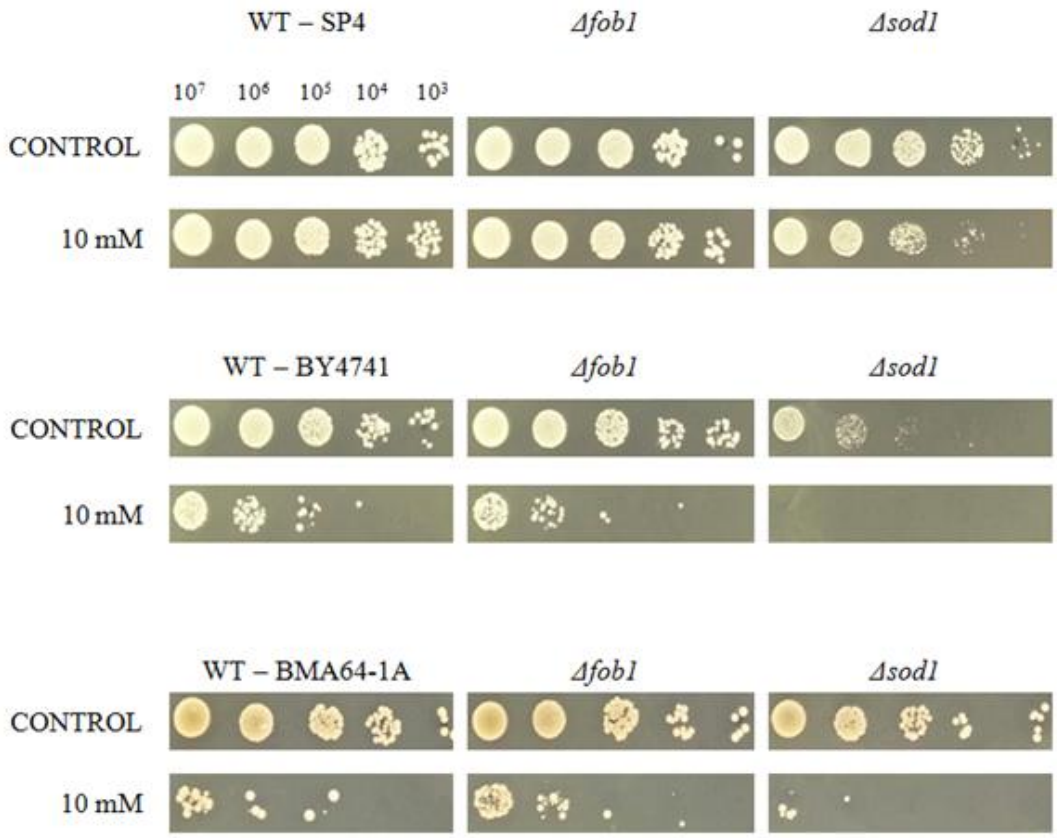


Figure 10. Yeast growth on solid YPD media after treatment with H_2O_2 for 1 h. For each genetic background sensitivity to H_2O_2 of three yeast strains was explored: wild-type (WT), Δ*fob1* mutant and Δ*sod1* mutant.

For each genetic background, control (non-treated) cells showed the following results: in serial dilution from 10^7 to 10^3 cells/mL yeast colonies grow less densely. In cells treated for 1 h with H_2O_2 the reduction in growth has been observed. The genetic background SP4 demonstrated the highest resistance to 10 mM concentration of H_2O_2. Furthermore, BY4741 genetic background showed better survival in the case of wild-type strain comparing to the wild-type strain of BMA64-1A background. A *Δfob1* mutant of both BY4741 and BMA64-1A background showed similar survival. In particular, non-treated *Δsod1* mutant of BY4741 genetic background showed a less survival comparing to non-treated *Δsod1* mutants of SP4 and BMA64-1A backgrounds. Therefore, this mutant showed the lowest resistance to 10 mM H_2O_2. Within each genetic background isogenic strains differed in their response to oxidative stress. Wild-type of each genetic background showed the highest resistance, followed by the *Δfob1* mutant, whereas Δsod1 mutant showed lower resistance to H_2O_2 due to the lack of *SOD* gene.

Another, similar experiment was made. 1 x 10^7 of yeast cells/mL was serially diluted and 5 µL of each sample was spotted on three different types of solid YPD media: containing 1, 3 or 5 mM H_2O_2. Each drop contained 50 000, 5 000, 500, 50 and 5 cells. Cell growth was observed after 48 h of incubation at 28°C. Samples inoculated on a standard solid YPD media without H_2O_2 were used as controls. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments (Fig. 11).

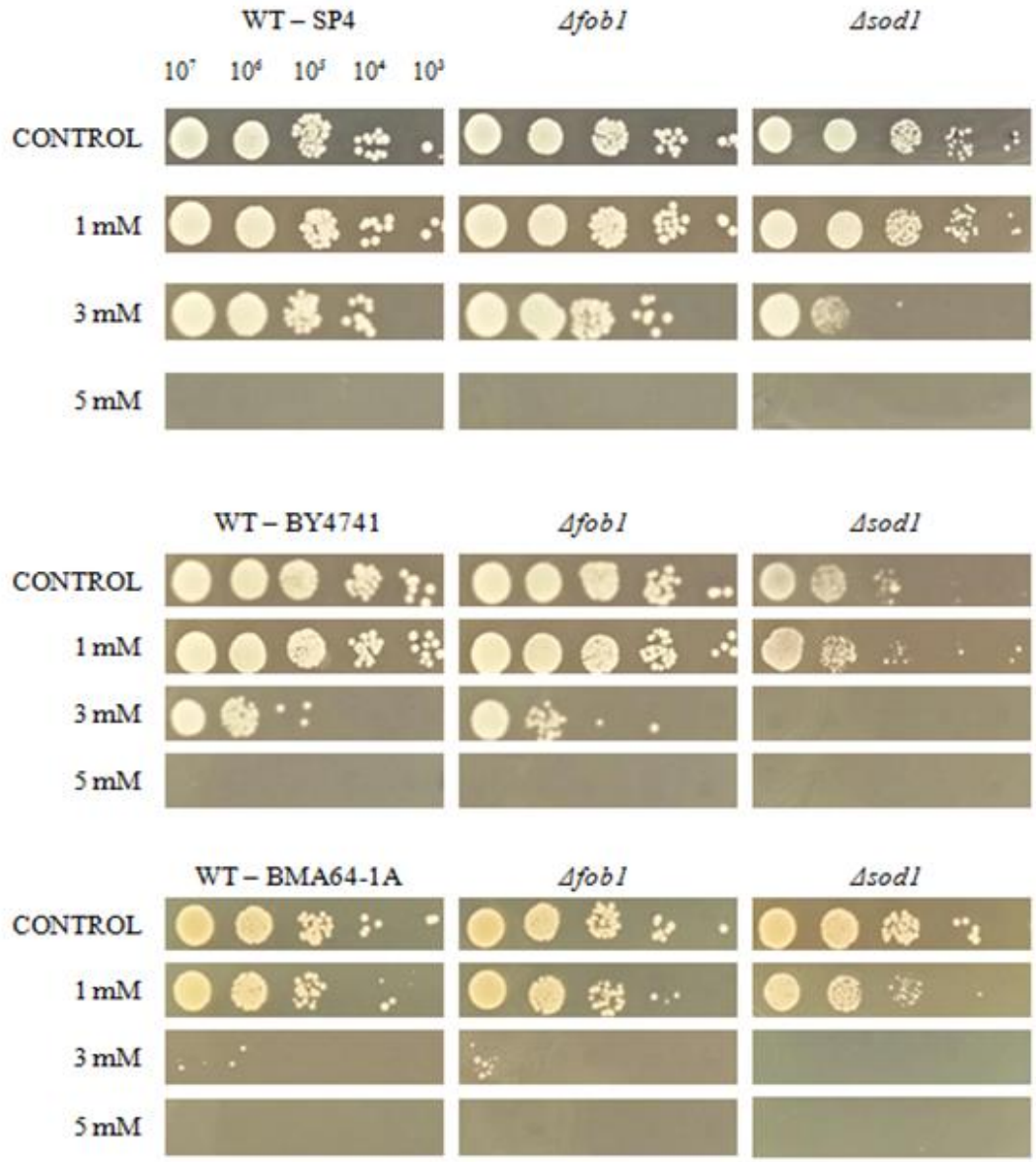


Figure 11. Sensitivity of yeast cells to H_2O_2 observed after inoculating the native yeast cells on solid YPD media containing 1, 3 or 5 mM H_2O_2. For each genetic background sensitivity to H_2O_2 of three yeast strains was explored: wild-type (WT), Δ*fob1* mutant and Δ*sod1* mutant.

Samples that have been grown on plates with a higher concentration of H_2O_2 showed greater sensitivity to oxidative stress. The greatest difference is apparent at the concentration of 3 mM H_2O_2 while at a concentration of 5 mM no single colony was observed. Equally, greater sample dilution corresponds to fewer cells forming colonies. Wild-type and *Δfob1* strains developed a similar amount of colonies at all dilutions and genetic backgrounds, contrary to *Δsod1* which was the most sensitive strain. Comparing the individual genetic backgrounds, SP4 recorded the highest resistance to oxidative stress, followed by BY4741 and BMA64-1A backgrounds. Therefore, there is a difference between various genetic backgrounds and within individual ones.

4.1.4. Detection of ROS using dihydroethidium assay

Each sample with density 1×10^8 of yeast cells/mL was added in triplicate on non-transparent black microplate. The kinetics of fluorescence intensity provoked by the presence of ROS was measured at 28^oC immediately after addition of DHET (Fig. 12). The concentration of ROS was measured in all samples at exponential and stationary phase of cell growth.

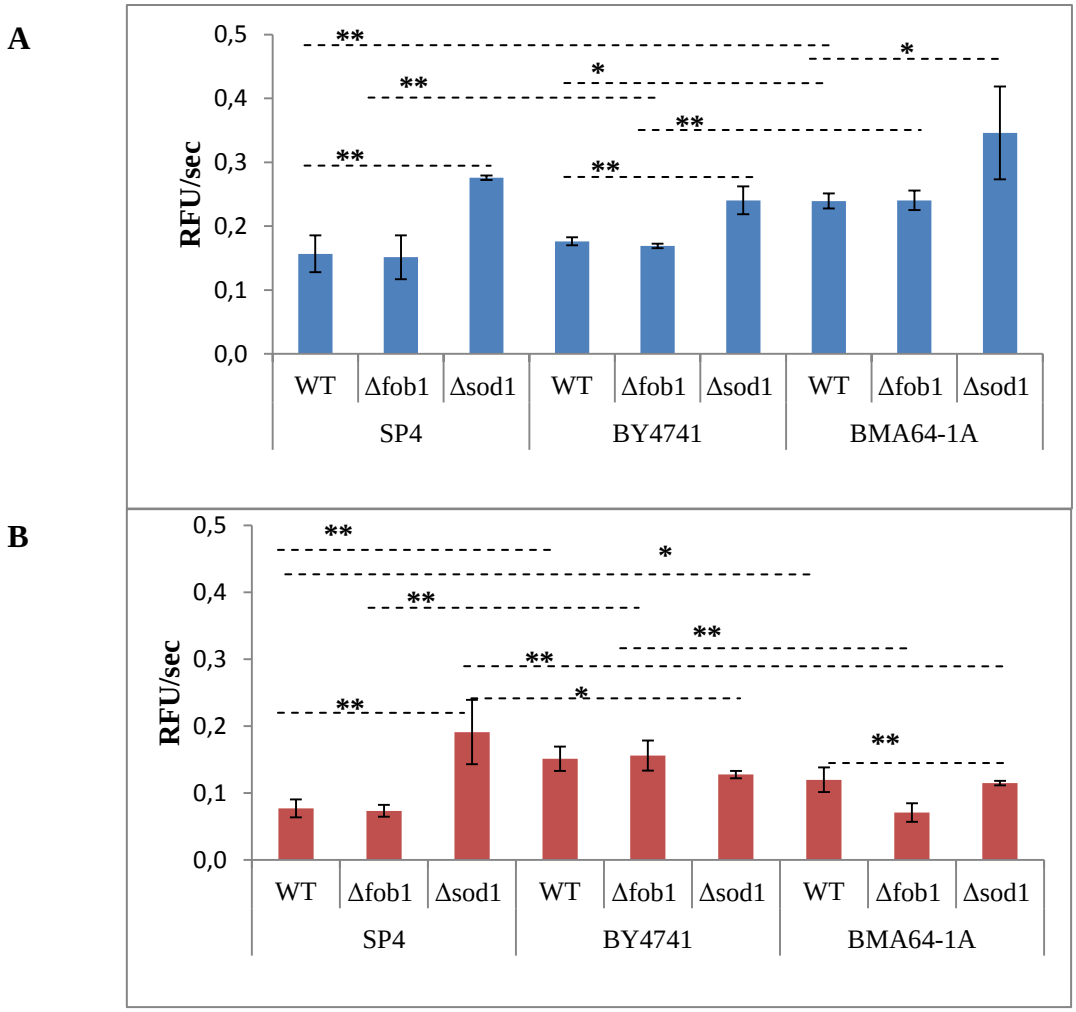


Figure 12. The ROS concentration in yeast cells at exponential (A) and stationary (B) phase of cell growth. The results are presented as relative fluorescence units per second (RFU/sec) and expressed as mean value ± standard deviation from three independent experiments. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

Likewise, from each yeast sample a native microscopic slide was made and cells were observed under fluorescent microscope using bright field (400 x magnifications). 100 cells of each yeast strain were selected to measure their cell size (in μm) with specific software (*Micro Image*) and calculate mean values (Fig. 13).

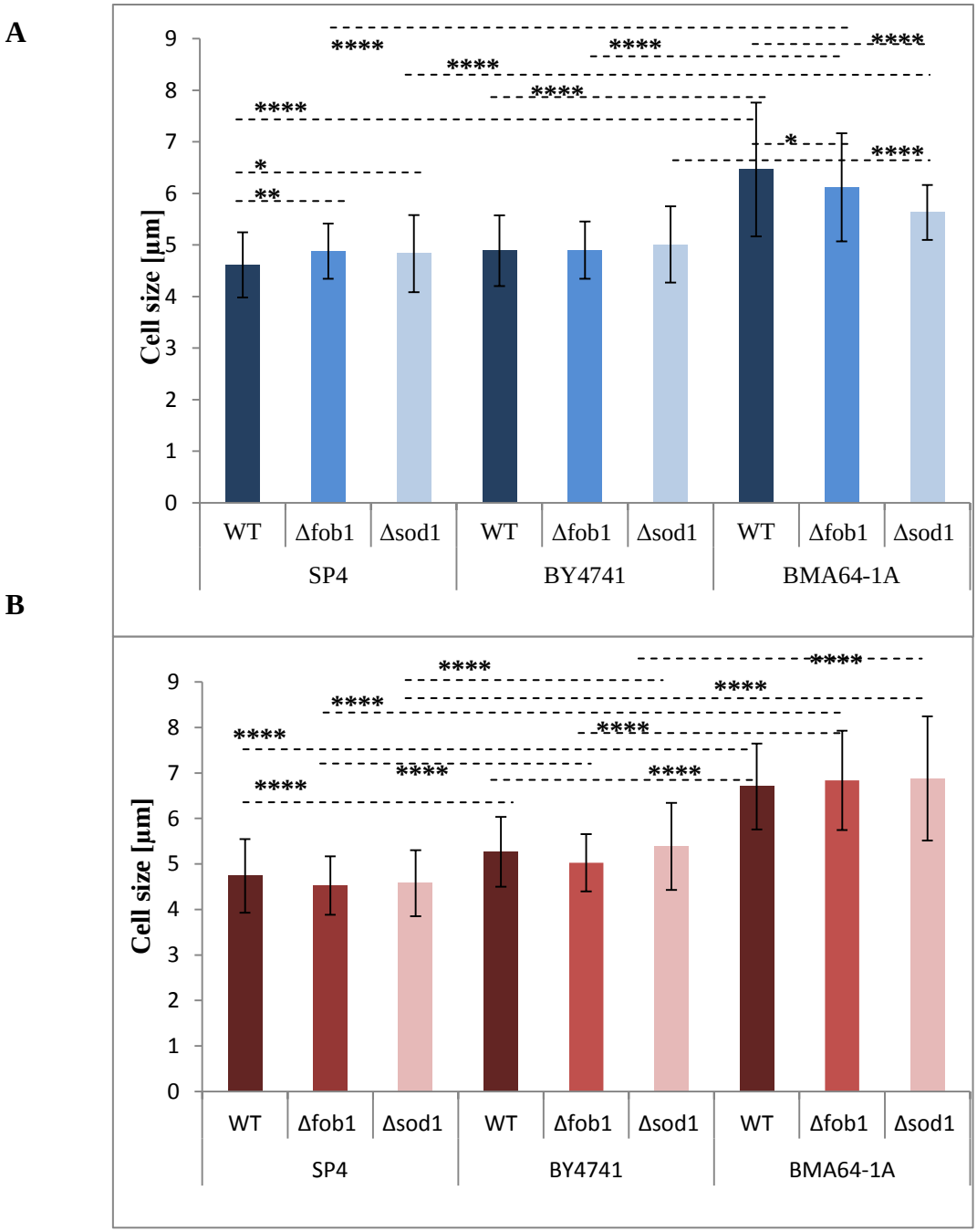


Figure 13. The size of yeast cells at exponential (A) and stationary (B) phase of growth. The results were expressed as mean value ± standard deviation. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

Furthermore, the results obtained by determination of the ROS concentration in yeast cells were normalized per cell size for each strain (Fig. 14) considering the differences in the ROS concentration between genetic backgrounds partially dependent on the cell size.

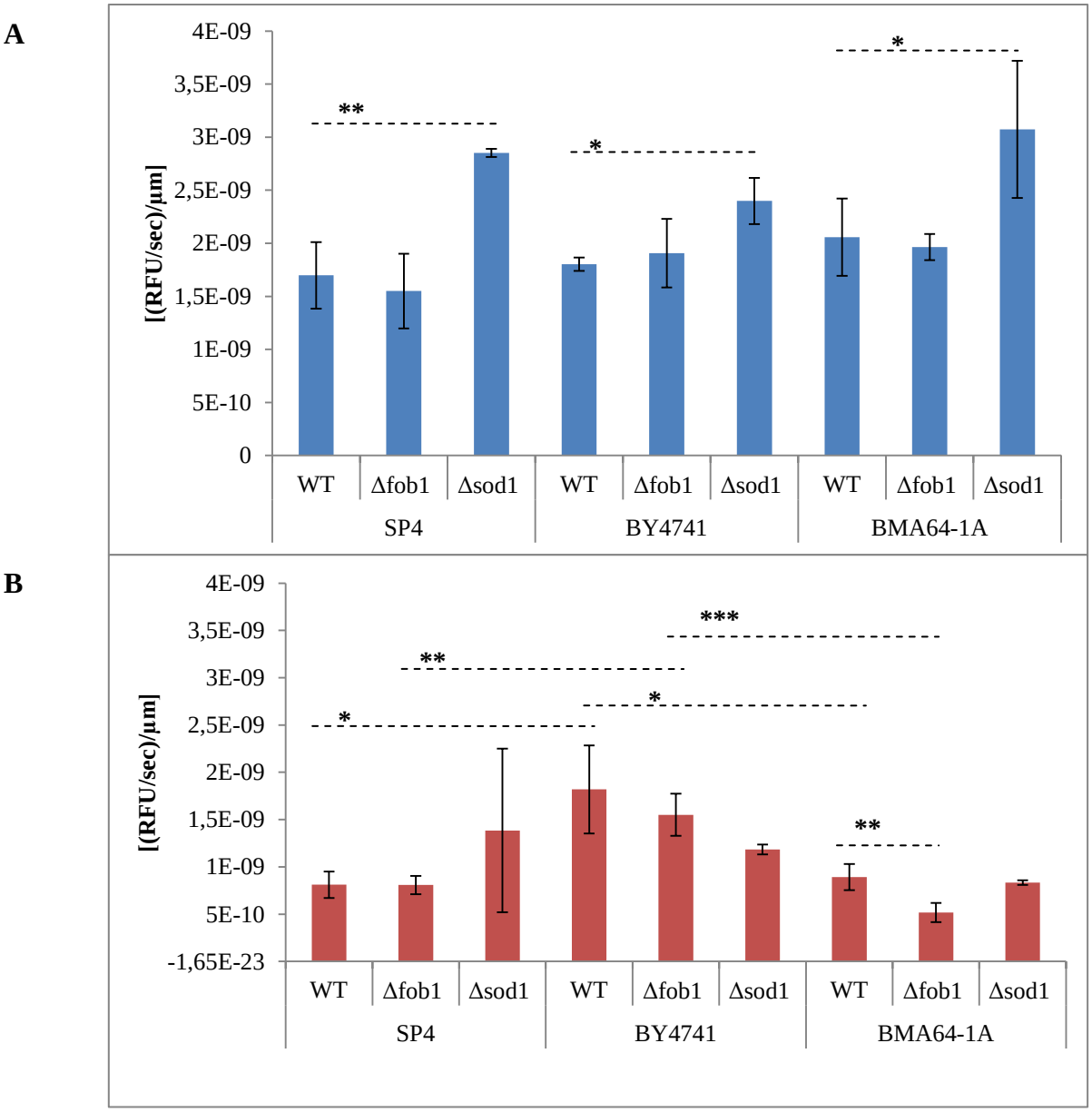


Figure 14. The ROS concentration normalized per cell size at exponential (A) and stationary (B) phase of cell growth. The results are presented as relative fluorescence units per second (RFU/sec/µm) and expressed as mean value ± standard deviation. Three different *S. cerevisiae* strains (i.e. WT, Δ*fob1*, Δ*sod1*) of three different genetic backgrounds (i.e. SP4, BY4741, BMA64-1A) were used in these experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

The results of determined ROS concentration, cell size measuring and normalized ROS content per cell size were analyzed by using Dunnett's and Tukey's post hoc one-way ANOVA tests. A Δ*fob1* and Δ*sod1* mutant was compared with wild-type as a control strain within each genetic background by Dunnett's test, whereas the each individual yeast strain

(i.e. wt, Δ*fob1* or Δ*sod1*) was compared between three genetic backgrounds (i.e. SP4, BY4741 and BMA64-1A) by Tukey's test. Significant differences were shown on the graphs.

4.1.5. Detection of carbonyl protein groups using immunocytochemistry assay

Yeast cells were growing in a liquid YPD media containing 2% glucose at 28^{o}C until density 4 x 10^{8} of cells/mL was taken and treated for immunocytochemistry assay. Protein carbonyl groups were detected in all samples by using rabbit primary antibodies specific for the dinitrophenyl residues and goat anti-rabbit secondary antibodies conjugated with Chromeo™546. Image visualization was performed with a fluorescence microscope (Fig. 12A, B and C).

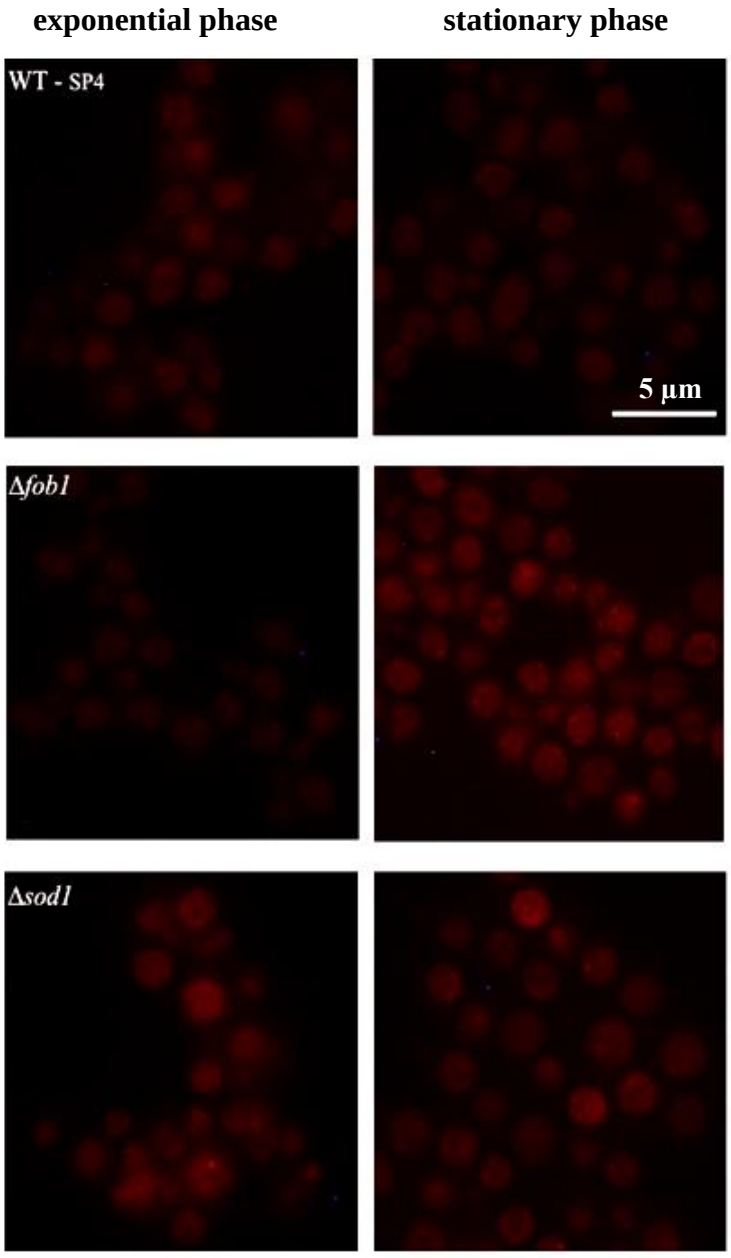


Figure 15. (A) The content of protein carbonyl groups in yeast cells SP4 (WT, Δ*fob1* and Δ*sod1* mutants) from exponential and stationary phase of growth. The bar length corresponds to 5 µm with magnification of 1000 ×.

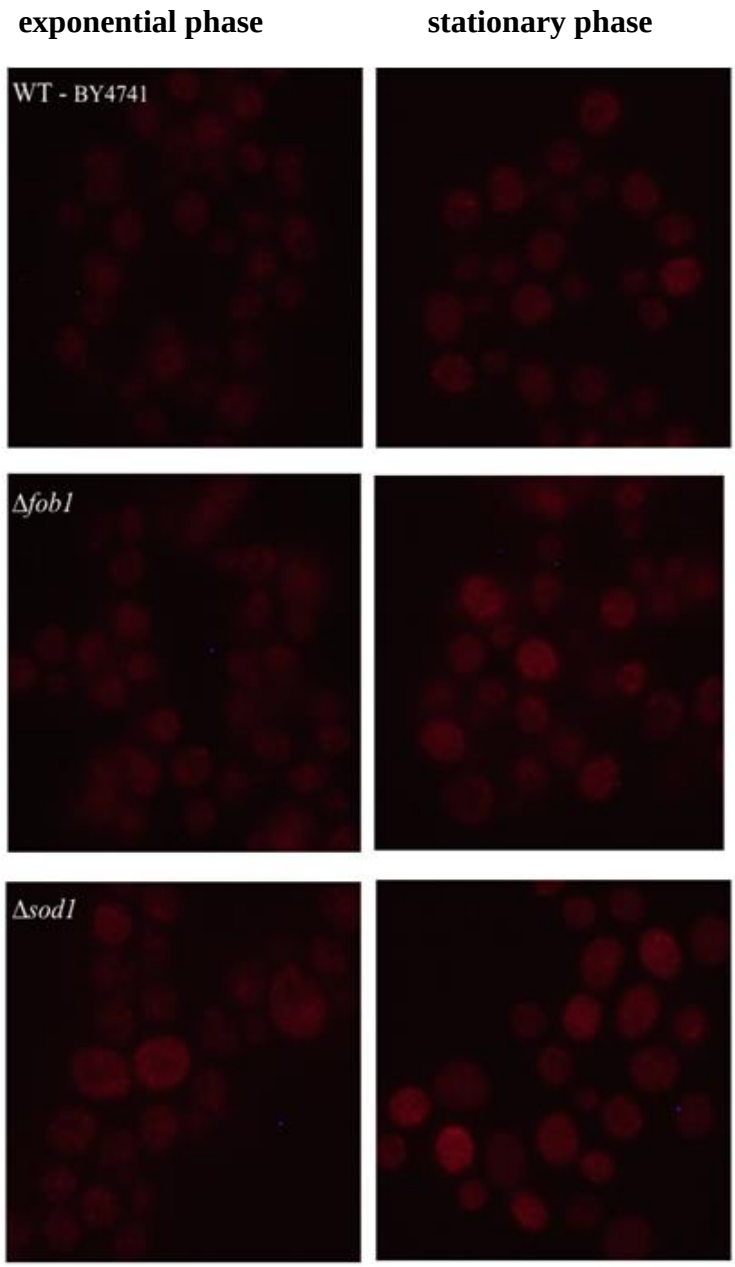


Figure 15. (B) The content of protein carbonyl groups in yeast cells BY4741 (WT, *Δfob1* and Δ*sod1* mutants) from exponential and stationary phase of growth. The bar length corresponds to 5 μm with magnification of 1000 ×.

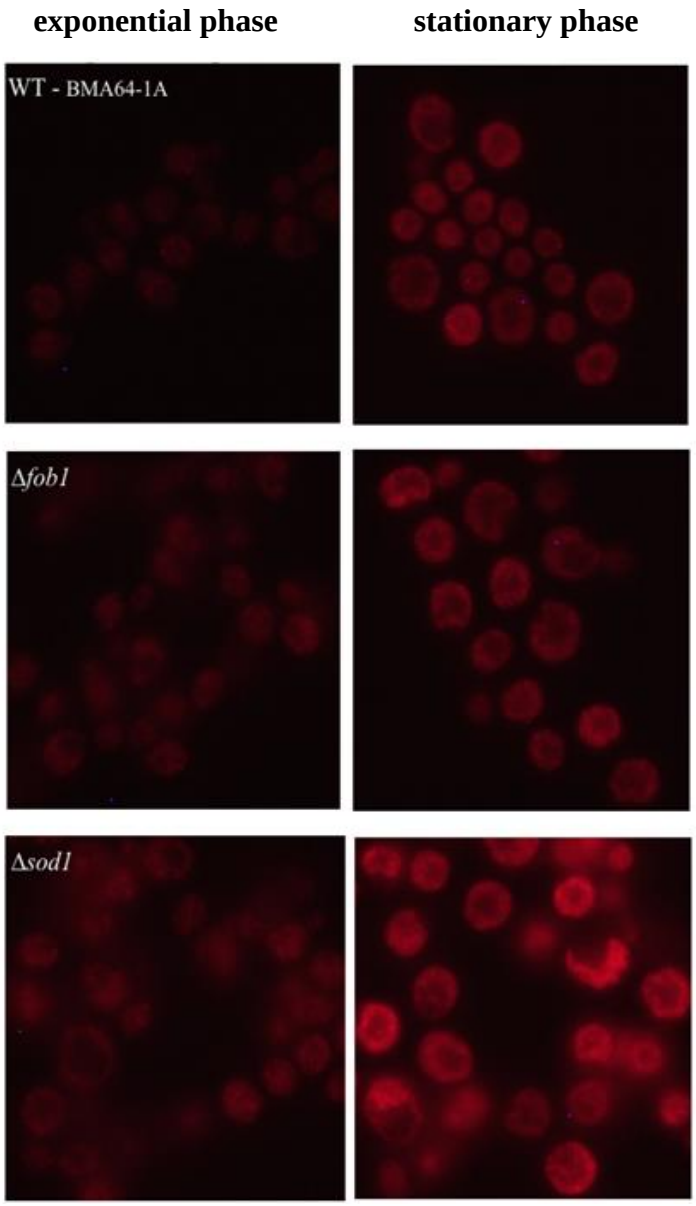


Figure 15. (C) The content of protein carbonyl groups in yeast cells BMA64 – 1A (WT, Δ*fob1* and Δ*sod1* mutants) from exponential and stationary phase of growth. The bar length corresponds to 5 μm with magnification of 1000 ×.

Comparing individual genetic backgrounds at each phase of growth, very similar results were observed: fluorescence of the *Δfob1* mutants was almost of equal intensity than those in wild-type strains while the strain *Δsod1* showed slightly higher fluorescence intensity. A Δ*fob1* mutant of SP4 cells makes exception, showing higher fluorescence intensity in regard to its wild-type strain. Comparing each phase of growth, the yeast cells at exponential phase

showed lower signal intensity than those in stationary phase. Particularly the BMA64-1A genetic background, showed a higher intensity of the fluorescence signal.

4.1.6. Analysis of the yeast culture during prolonged cultivation

4.1.6.1. Determination of cell metabolic activity by fluorescence method – FUN1 staining

Each yeast sample with density 1×10^7 of cells/mL was added in duplicate on non-transparent black microplate and incubated for 15 minutes with the fluorescence stain FUN-1 at 28^{o}C with continuous shaking. The fluorescence of the cell suspensions was measured immediately after incubation time on microplate reader at excitation wavelength of 480 nm and emission in range of 500-650 nm. Metabolic activity of yeast cells SP4, BY4741 and BMA64-1A was determined daily during 7 days. The results are expressed as a change of red (λ = 575 nm) to green (λ = 535 nm) ratio (Fig. 16).

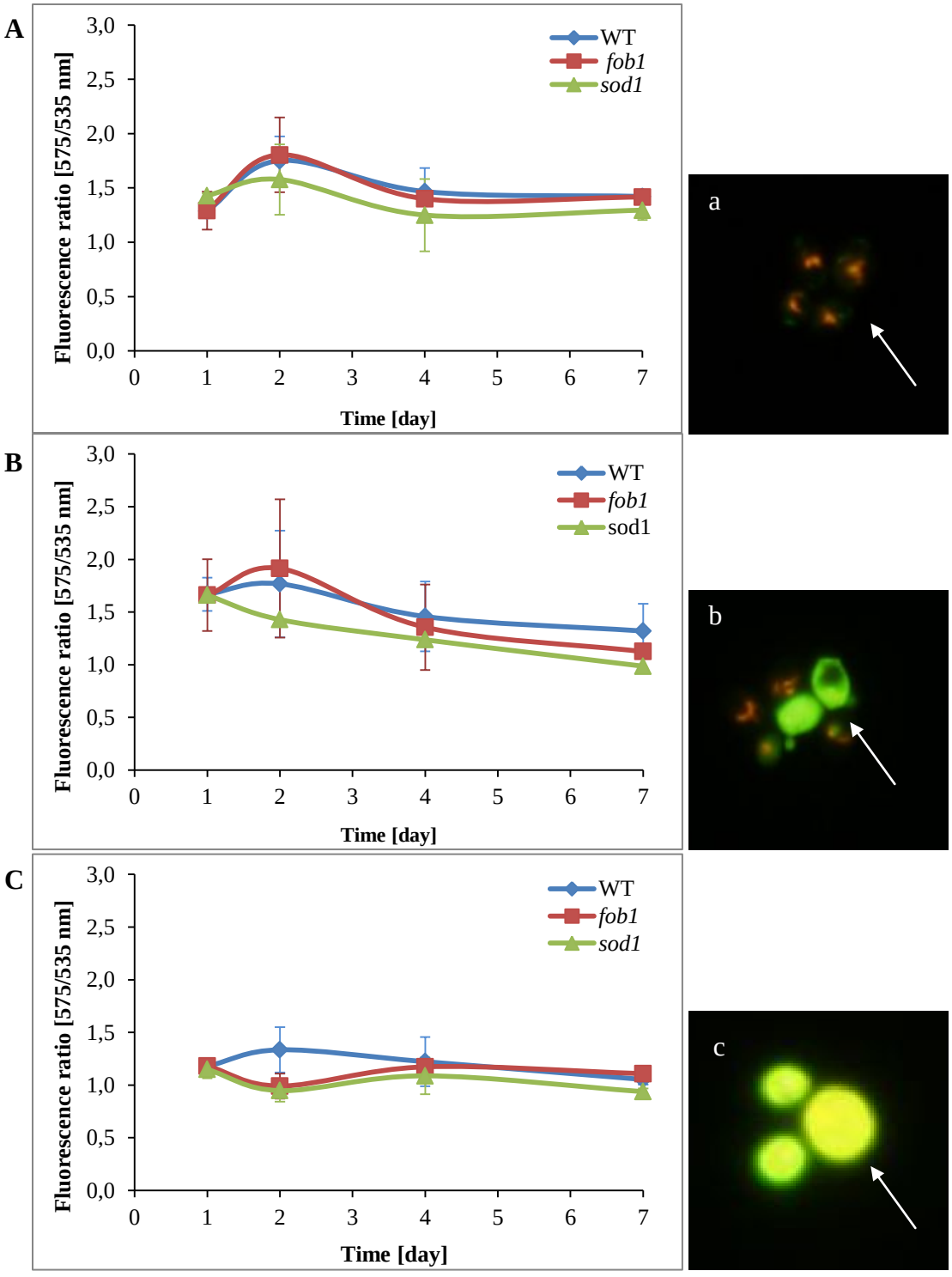


Figure 16. Analysis of metabolic activity of the yeast cells SP4 (A), BY4741 (B) and BMA64-1A (C) during 7 days of culture. For each genetic background metabolic activity of three yeast strains were explored: wild-type (WT), Δ*fob1* mutant (*fob1*) and Δ*sod1* mutant (*sod1*). The results are expressed as mean value ± standard deviation. Metabolic active cells (*a*), metabolic inactive cells (*b*) and dead cells (*c*) could be distinguished under a fluorescence microscope.

The results of metabolic activity measurement were analyzed by using Dunnett's and Tukey's post hoc one-way ANOVA tests. A Δ*fob1* and Δ*sod1* mutant was compared with wild-type as a control strain within each genetic background by Dunnett's test, whereas the each individual yeast strain (i.e. wt, Δ*fob1* or Δ*sod1*) was compared between three genetic backgrounds (i.e. SP4, BY4741 and BMA64-1A) in the each time point of measurement at the 1st, 2nd, 4th and 7th day by Tukey's test. In all genetic backgrounds no significant difference was observed between wild-type strain and its mutants. By Tukey's test no significant difference was observed between tested strains in the time point of measurement at the 2nd and 4th day.

Table 2. Significant difference observed by using the Tukey's post hoc test in metabolic activity of the same yeast strain from various genetic backgrounds at the 1st and 7th day of measurement was shown.

Day	Strain	Genetic backgrounds	Significance
1st	WT	BY4741 vs. BMA64-1A	*
	Δsod1	SP4 vs. BY4741	*
		SP4 vs. BMA64-1A	*
		BY4741 vs. BMA64-1A	**
7th	Δfob1	SP4 vs. BY4741	*
		SP4 vs. BMA64-1A	**
	Δsod1	SP4 vs. BY4741	*
		SP4 vs. BMA64-1A	*

* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001

When stained with the FUN-1 dye and observed under fluorescent microscope, three types of cells were distinguished. The first of them were metabolic active cells (Fig. 16a) that were in good overall condition. Therefore the colour enters the cell and is metabolized to the red form trapped in the vacuole. The second type of cells were metabolic inactive cells (Fig. 16b) stained uniformly with deep green fluorescence. The last types were dead cells (Fig. 16c) that showed intensive yellow-green fluorescence.

4.1.6.2. Determination of cellular alternations using fluorescence staining

100 µL of each yeast sample with density 1 x 10^7 of cells/mL was incubated with three different fluorescence dyes in dark for 15 minutes at the room temperature. Nucleus (*n*) was stained with DAPI; mitochondria (*m*) were stained with rhodamine B and vacuole (*v*) was stained with MDY-64 fluorescent dyes. Cells were analysed after 1, 2, 4 and 7 days of culture (Fig. 17). The results for SP4 genetic background were shown first (Fig. 14A-D), followed by results BY4741 (Fig. 14E- H) and BMA64-1A (Fig. 14I-L). Pictures of unstained samples were recorded with a differential interference contrast (DIC) microscopy. The bar length correspond to 5 µm with magnification of 1000 ×.

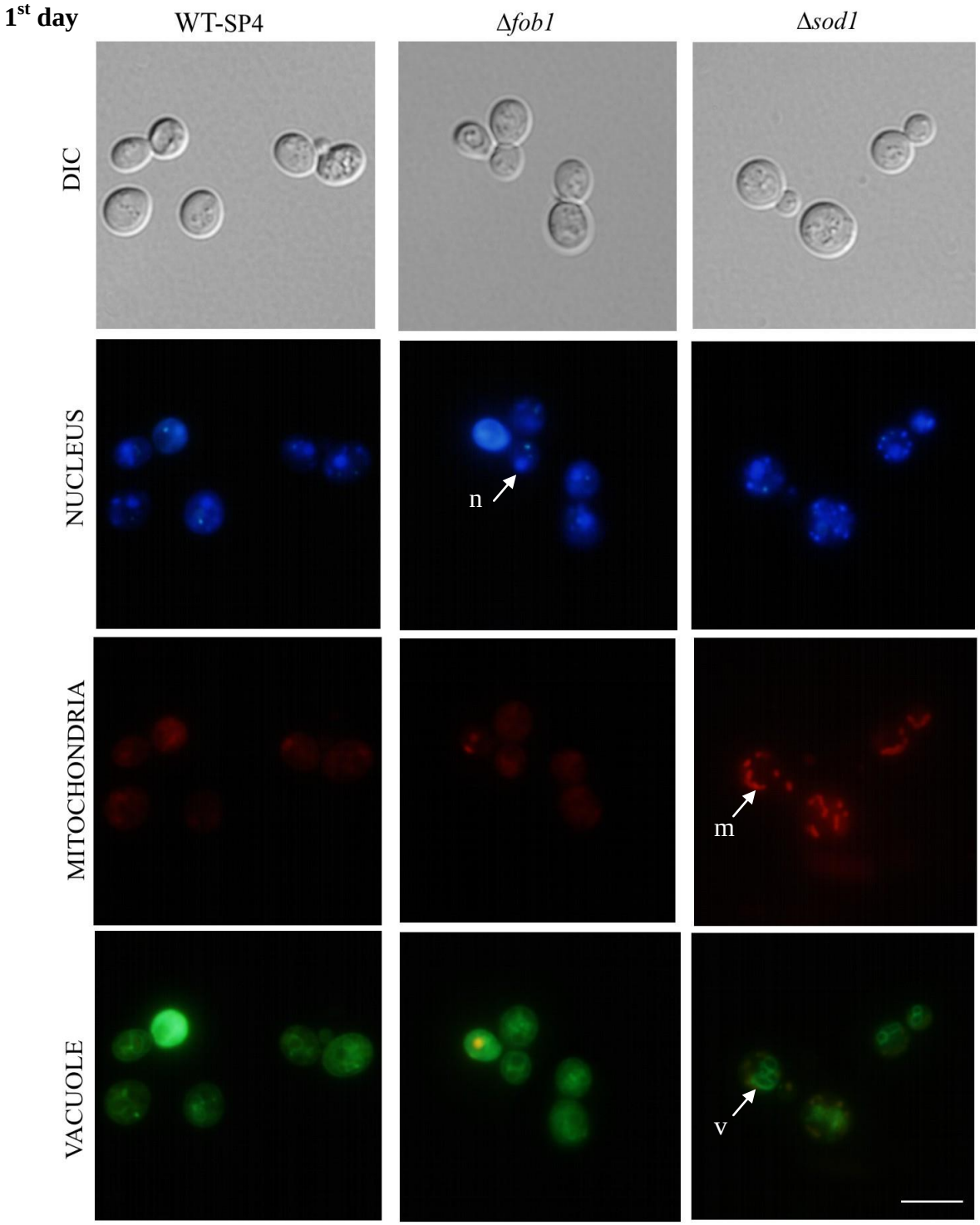


Figure 17. (A) Analysis of the cellular alterations of the yeast cells SP4 genetic background after 1 day of culture.

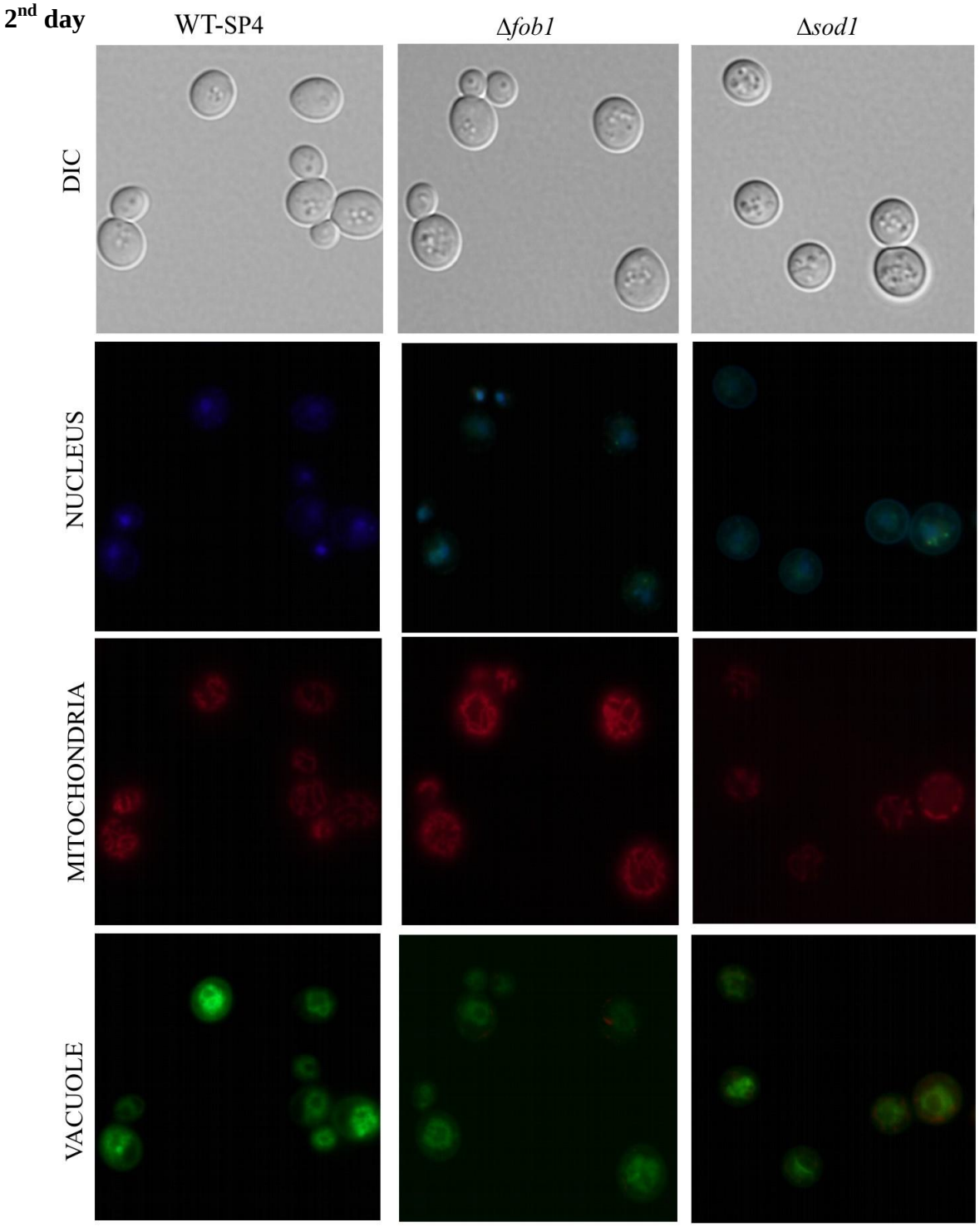


Figure 17. (B) Analysis of the cellular alterations of the yeast cells SP4 genetic background after 2 days of culture.

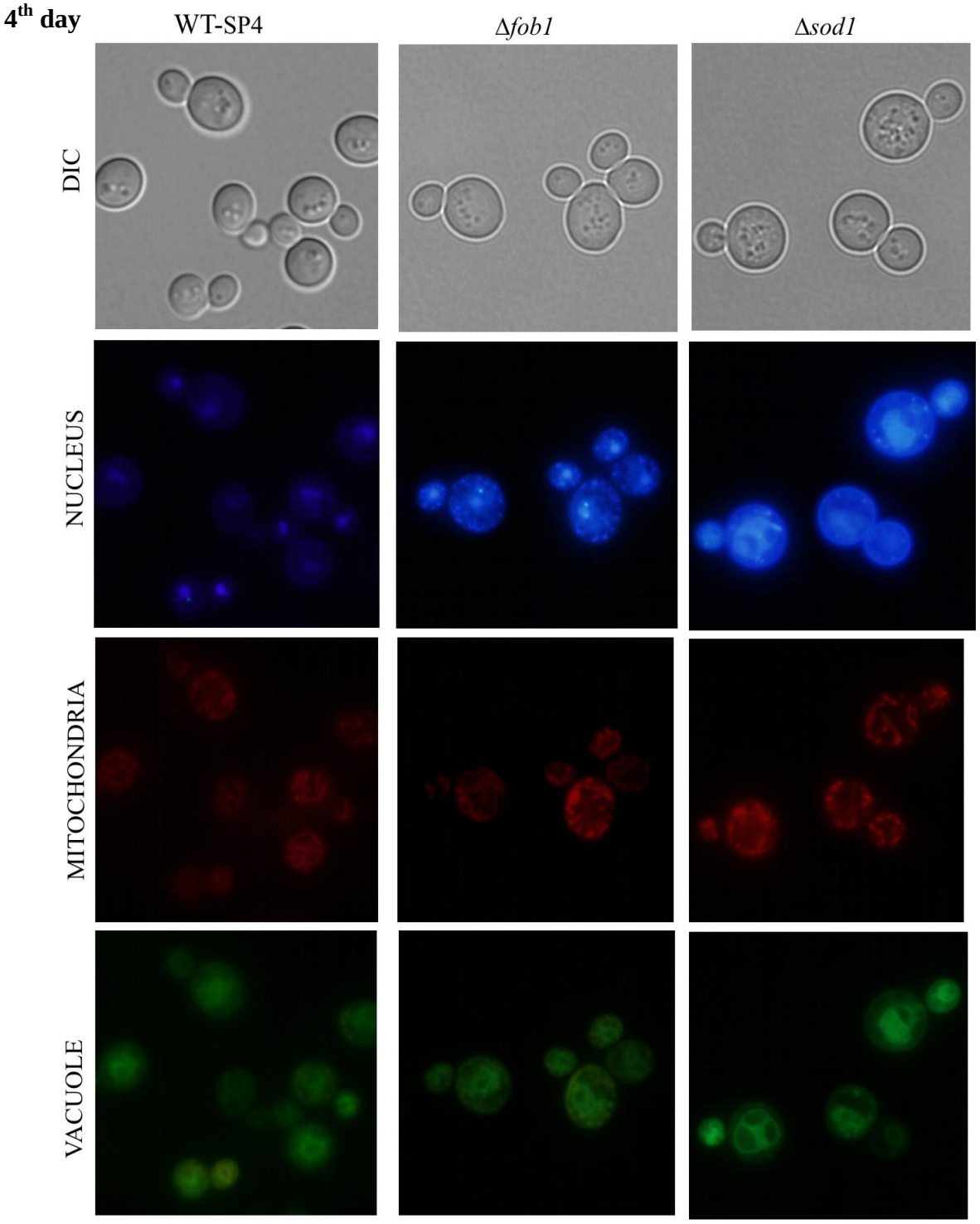


Figure 17. (C) Analysis of the cellular alterations of the yeast cells SP4 genetic background after 4 days of culture.

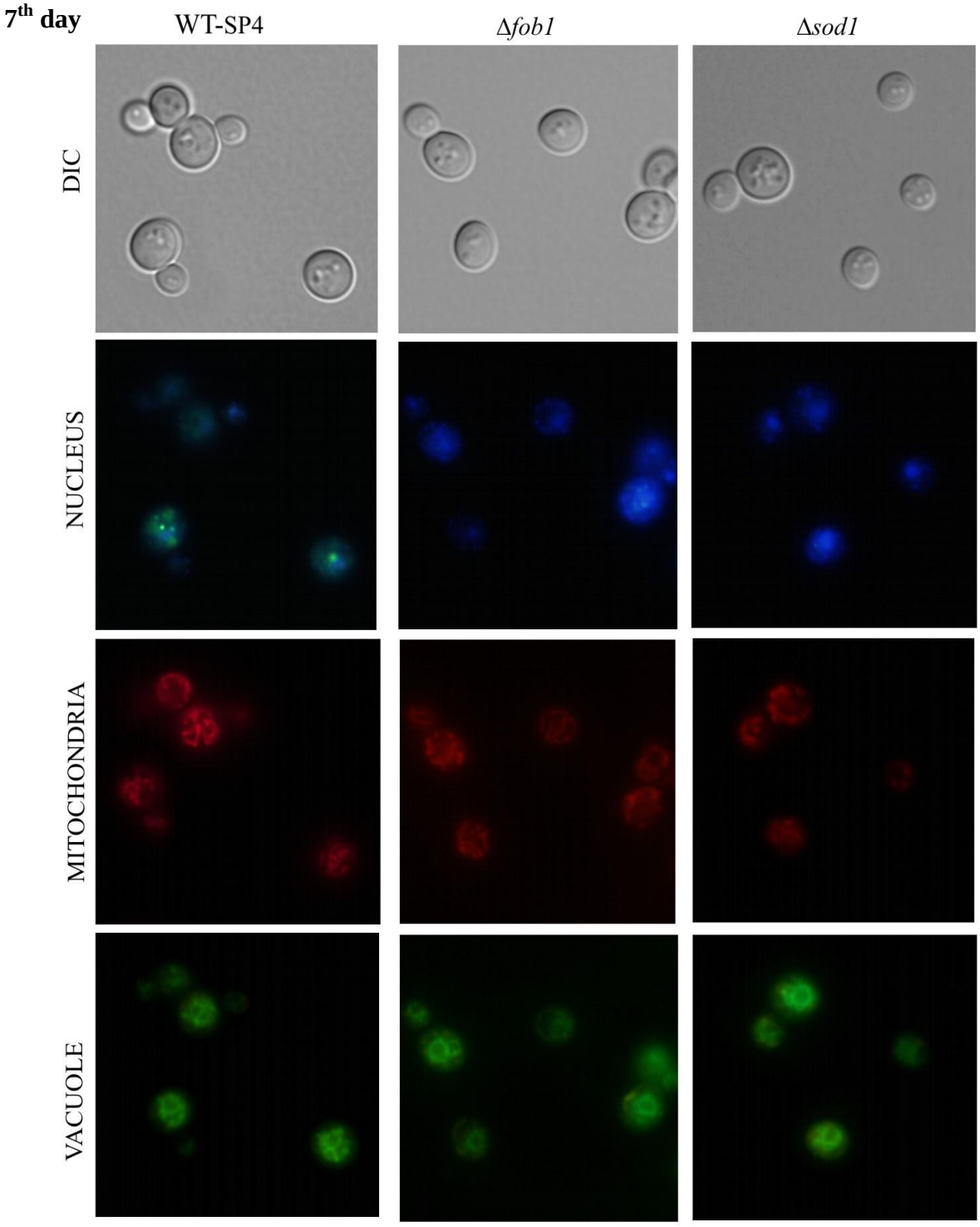


Figure 17. (D) Analysis of the cellular alterations of the yeast cells SP4 genetic background after 7 days of culture.

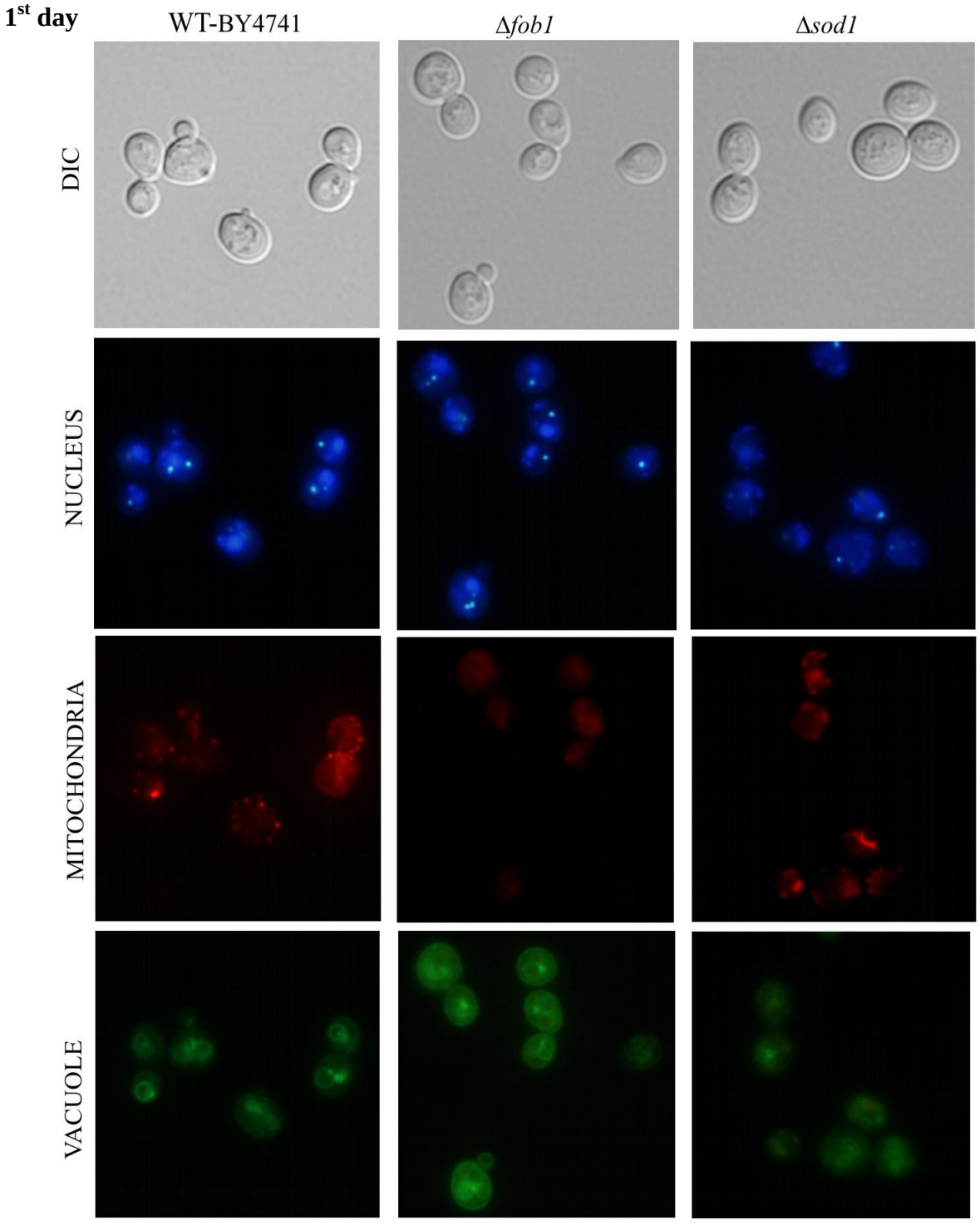


Figure 17. (E) Analysis of the cellular alterations of the yeast cells BY4741 genetic background after 1 day of culture.

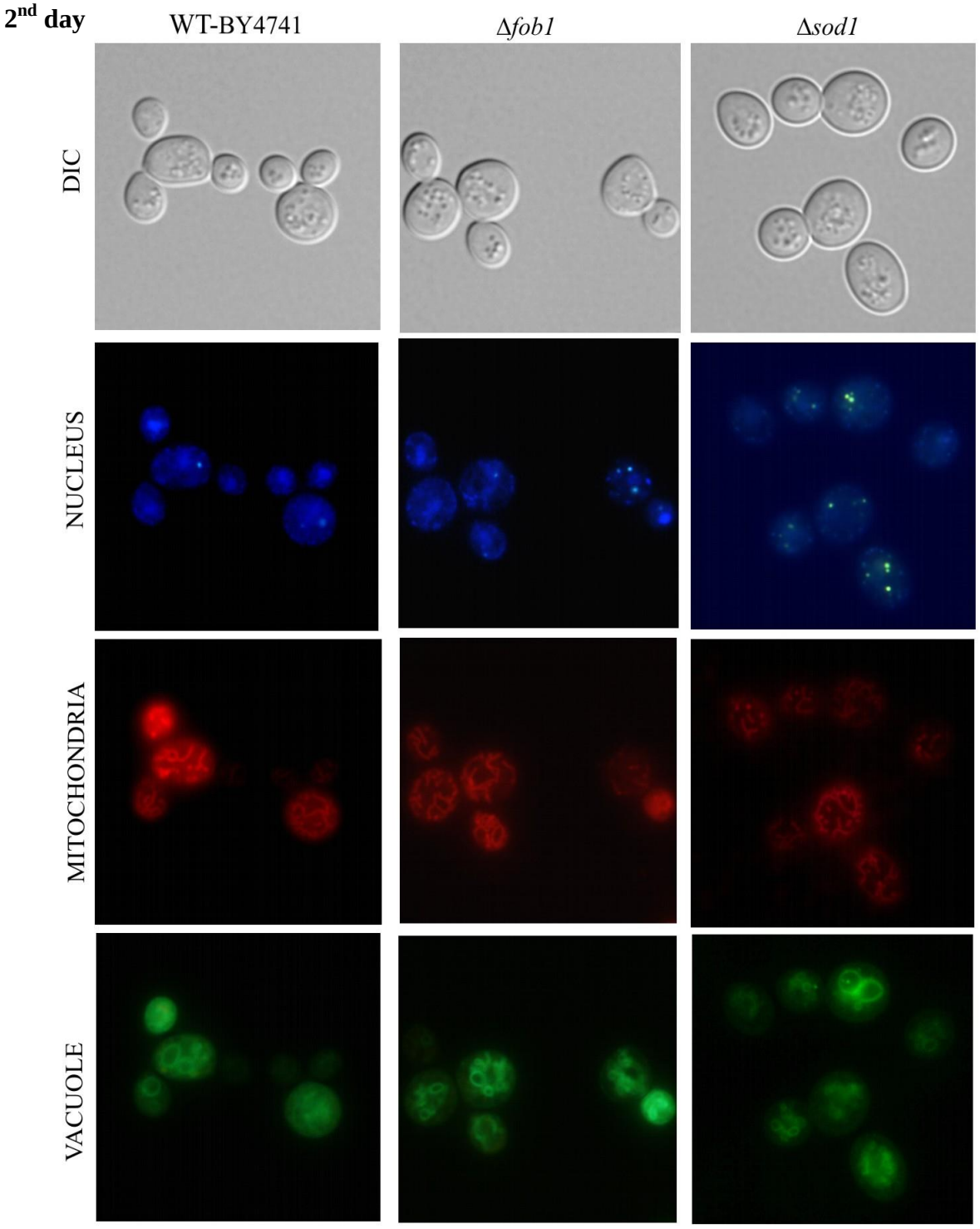


Figure 17. (F) Analysis of the cellular alterations of the yeast cells BY4741 genetic background after 2 days of culture.

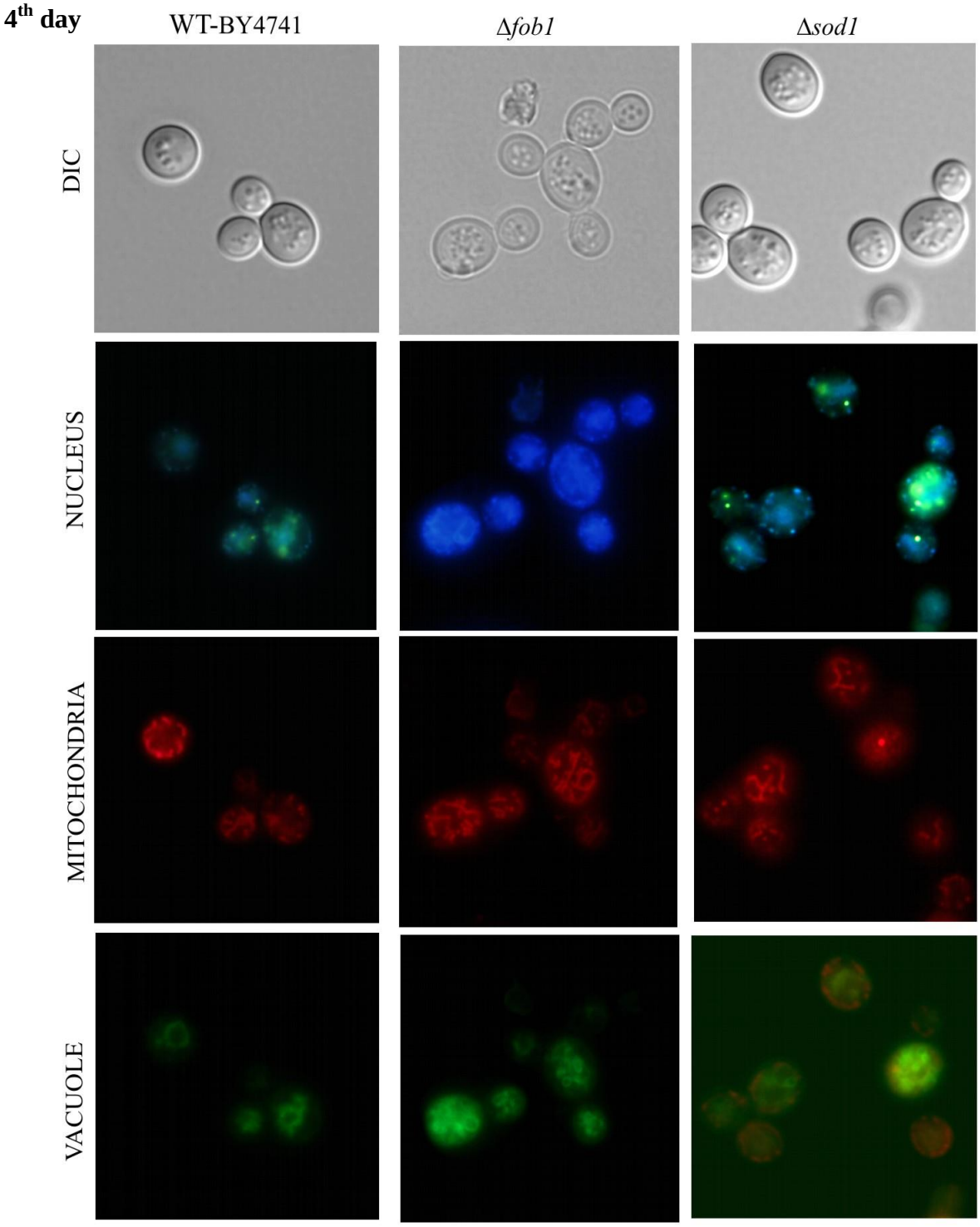


Figure 17. (G) Analysis of the cellular alterations of the yeast cells BY4741 genetic background after 4 days of culture.

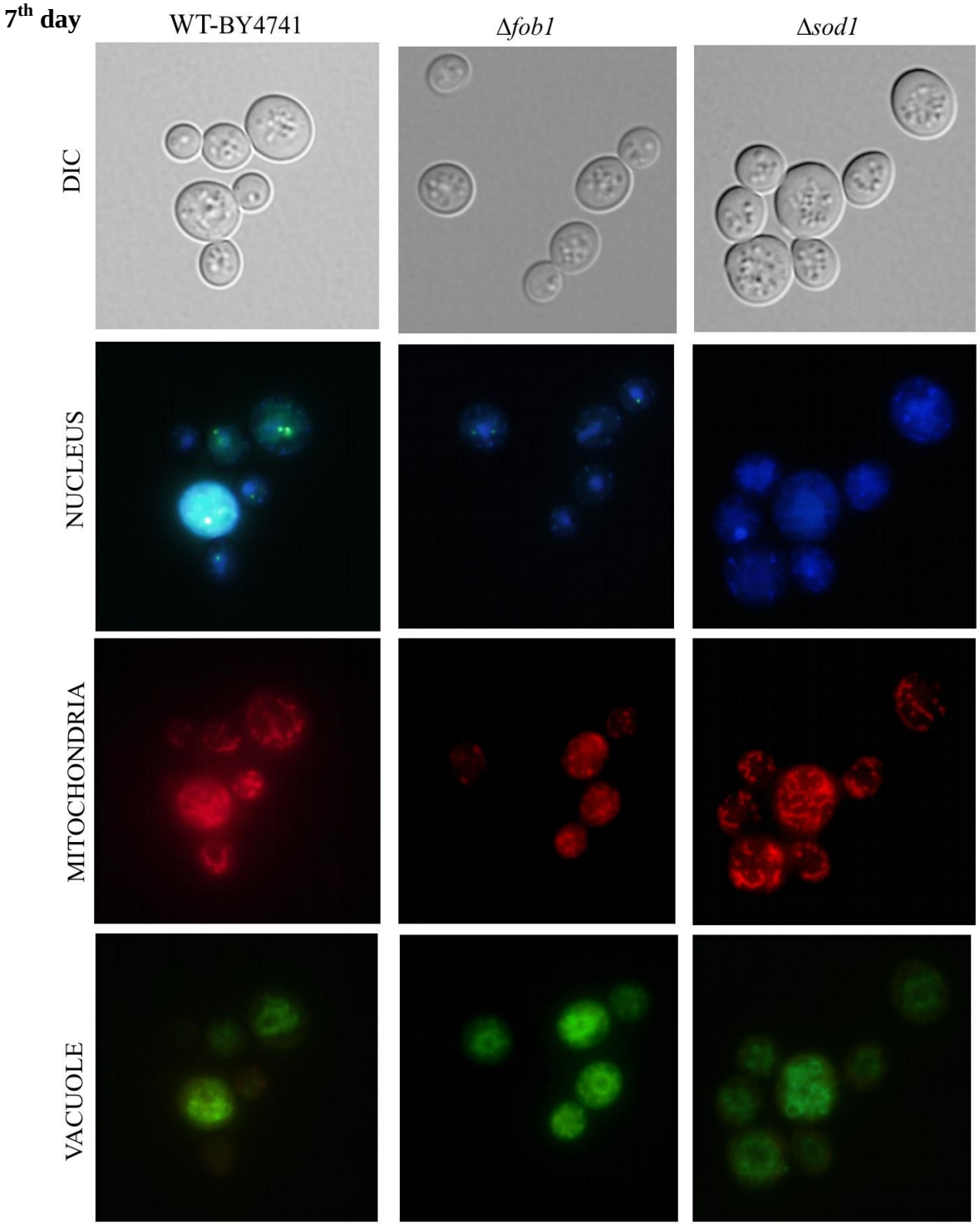


Figure 17. (H) Analysis of the cellular alterations of the yeast cells BY4741 genetic background after 7 days of culture.

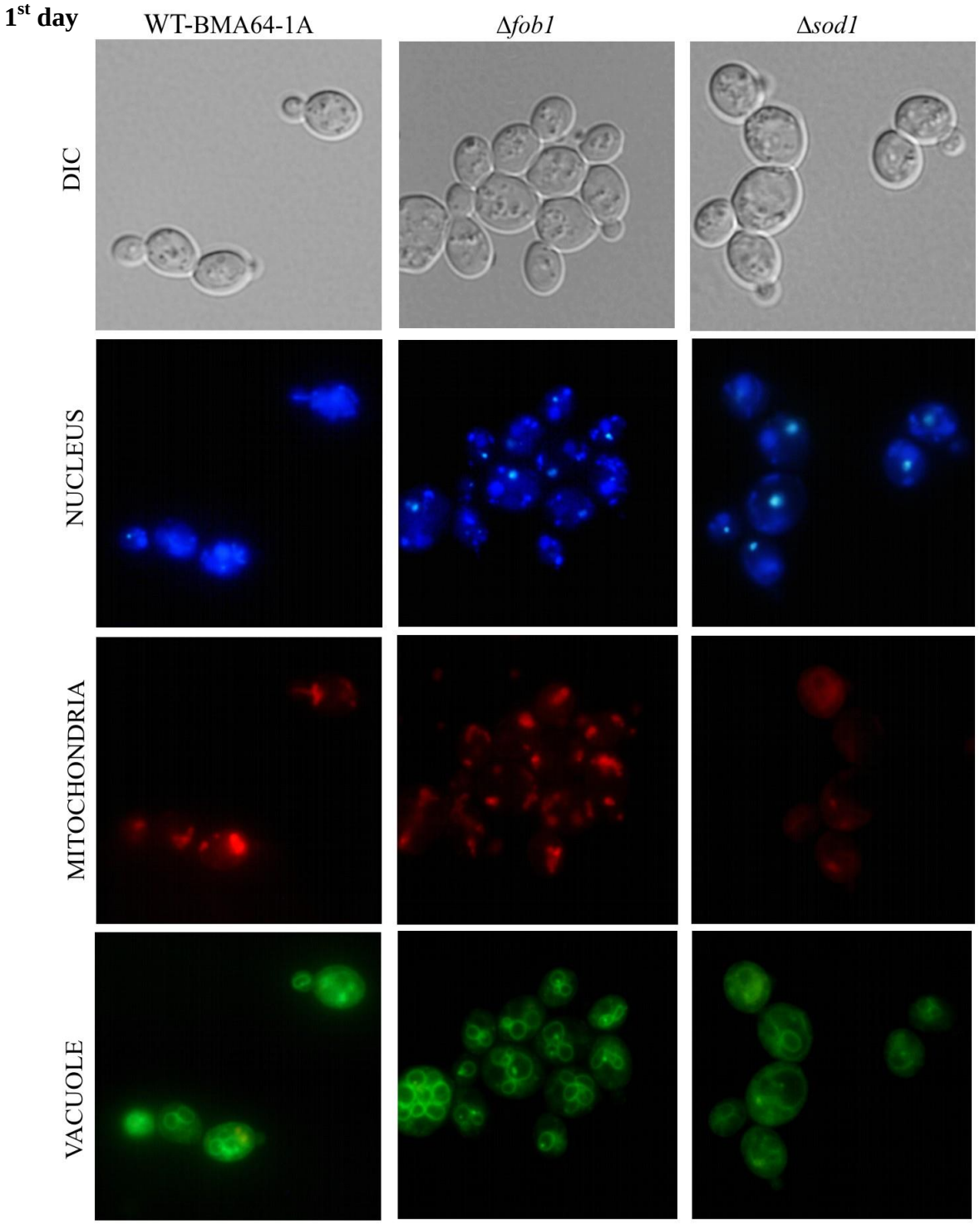


Figure 17. (I) Analysis of the cellular alterations of the yeast cells BMA64-1A genetic background after 1 day of culture.

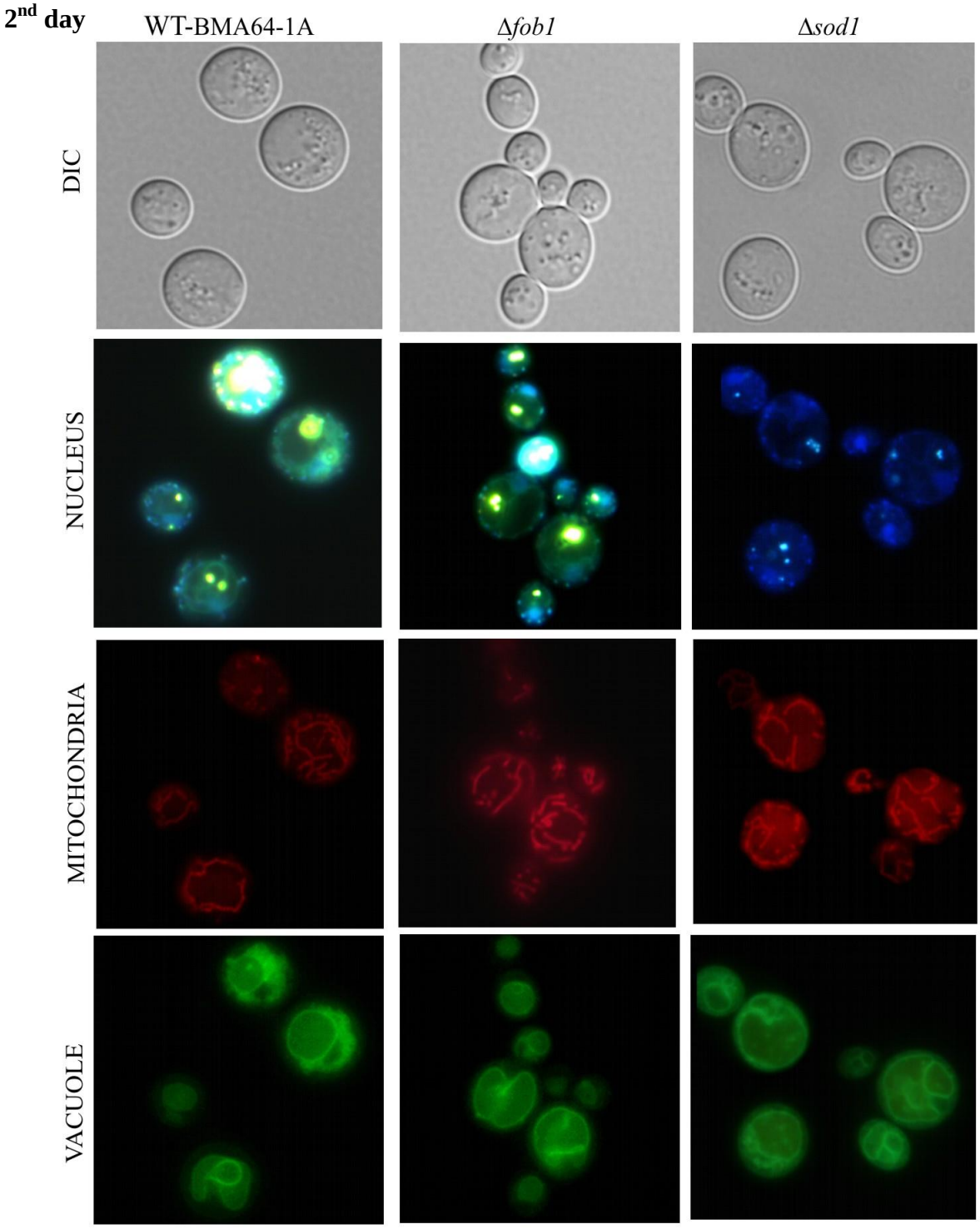


Figure 17. (J) Analysis of the cellular alterations of the yeast cells BMA64-1A genetic background after 2 days of culture.

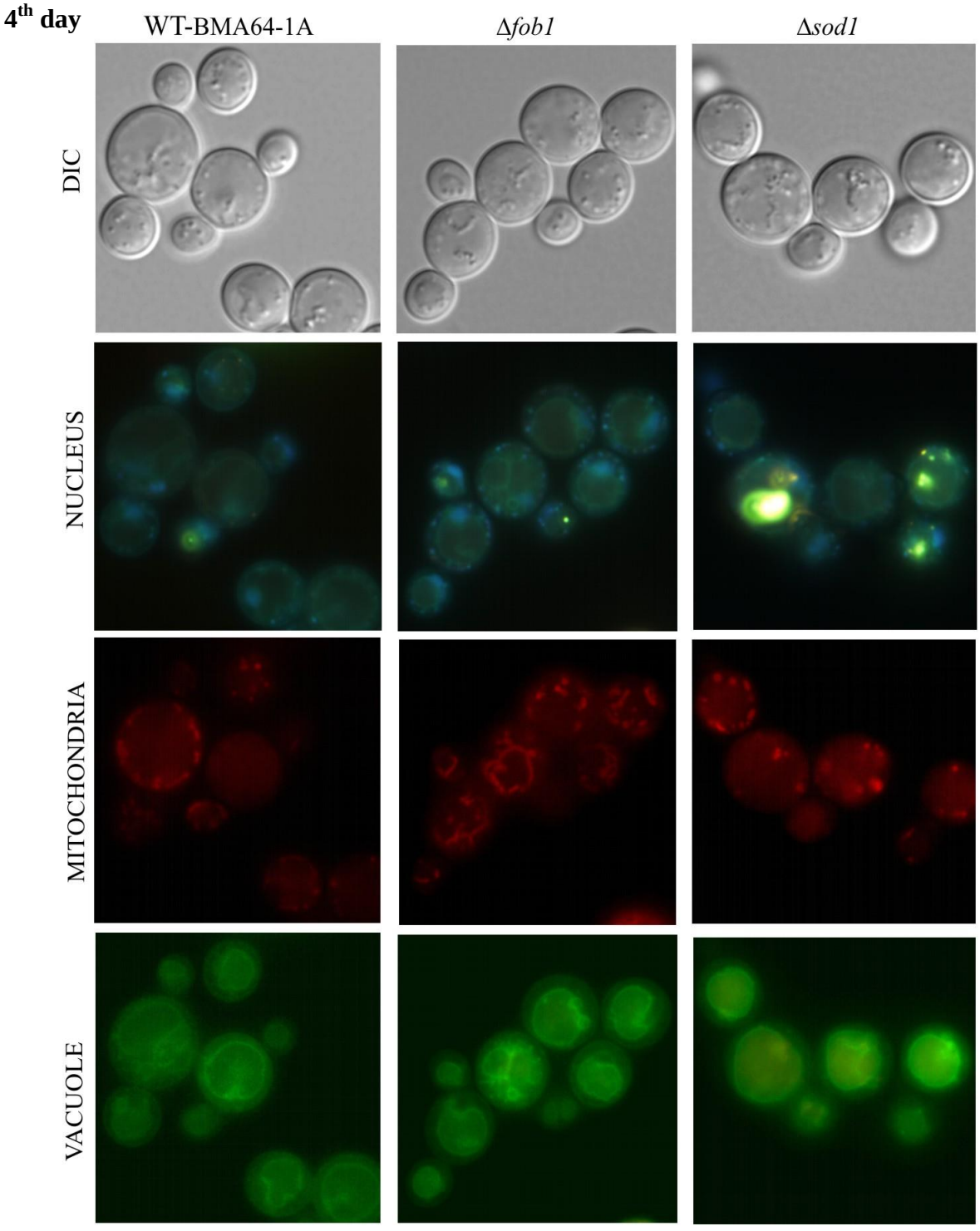


Figure 17. (K) Analysis of the cellular alterations of the yeast cells BMA64-1A genetic background after 4 days of culture.

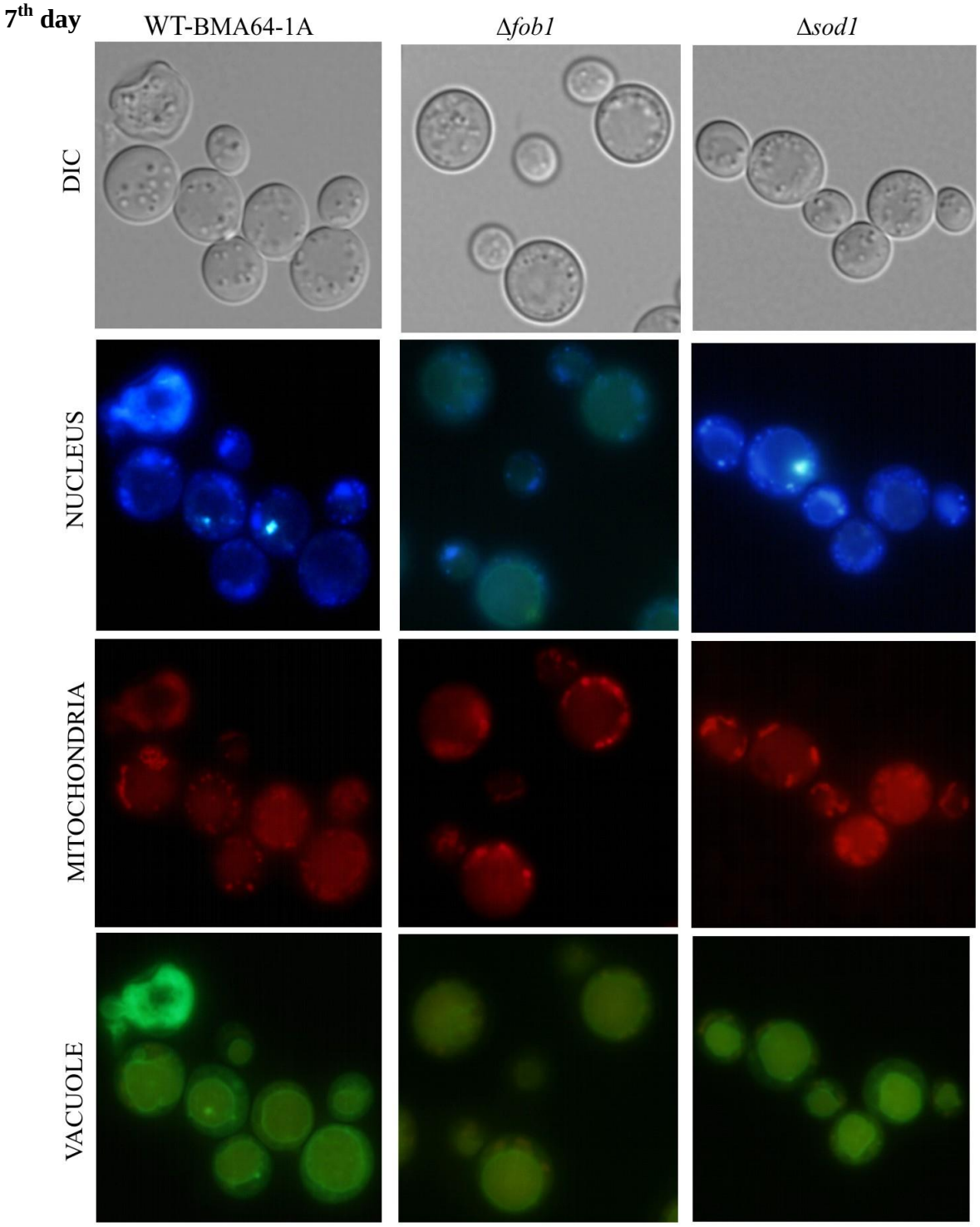


Figure 17. (L) Analysis of the cellular alterations of the yeast cells BMA64-1A genetic background after 7 days of culture.

In all genetic backgrounds the same changes in the cellular morphology during 7 days were observed. At first day of growth cells were small in size with visible buds, whereas a chromatin appeared diffuse within compact nucleus. Given that the cells just started to grow as well as that they utilize abundantly available glucose from media by fermentation process, mitochondria have not yet been developed and were observed in the oval form within the cytoplasm. Vacuoles appeared small and in large number. At the second day of growth cells were in the middle of exponential phase, metabolically very active with visible budding, enlarged in size, but the nucleus was still compact. Cells were probably still in the fermentation process, but observed mitochondria were well developed. With the progress of metabolism, vacuoles accumulated waste products and thus have increase in size. At the fourth day of cultivation the cells were further enlarged size, budding was stopped, and the nucleus was less compact. Cells achieved the stationary phase of growth with very well developed mitochondria, probably due to a shift from fermentative to respiratory metabolism. On the seventh day of cell growth, mitochondria were positioned marginally, close to the cell membrane, because vacuoles increased to a critical size and the cells obtained low metabolic activity. The nucleus became less compact, probably because cells started the process of degradation and apoptosis. It is important to emphasize that BMA64-1A are bigger in size than other observed genetic backgrounds.

4.2. Discussion

It is well known that mortal cells exhibit certain morphological and physiological changes during their life span. In order to better explore the aging phenomena the cells taken under observation need to be chosen with special attention. This study leans on hypothesis that the basic mechanisms of aging are conserved among eukaryotic organisms. Hence, we have selected the widely accepted unicellular yeast *Saccharomyces cerevisiae* as a model organism for accessing more thoroughly the aging process. However, some authors do not agree with this notion. Accordingly, the literature also cites results of certain experiments that raise the question if the budding yeast is a pre-eminent model organism in cellular aging pathways conserved among eukaryotes, including humans (Zadrag-Tęcza et al., 2013).

Since yeast *S. cerevisiae* is mainly used as a model for the study of replicative aging, a basic measure of age is the limited number of buds produced by a mother cell during its life span. In this case aging is measured by cell generations and all factors leading to the decrease or

increase of this number are referred as pro-aging or pro-longevity factors (Zadrag et al., 2008). Therefore the aging phenomena cannot be fully explained by a single hypothesis (Wawryn et al., 1999).

Hypertrophy has been considered as a possible cause that limits the budding capacity of yeast cells. Given that the process of cytokinesis requires an increase in cell size, the volume of yeast mother cell rises unavoidably upon each budding. When cells reach the excessive volume, further reproductive cycles become prevented (Zadrag-Tęcza et al., 2009). A rate of these changes is an important factor that regulates the reproductive potential of the yeast cell. The increase in cell size may be accompanied by a series of changes, both morphological and physiological, which affect the reproductive possibilities of the cell (Zadrag-Tęcza et al., 2009). With the aim to observe the causes of reduced reproductive capacity of the yeast *S. cerevisiae* during its life span, morphological and physiological characteristics were analyzed using a wild type (as a control) and two isogenic mutant strains (i.e. *Δfob1* and *Δsod1*) fundamentally different in replicative life span. Due to lack of nucleolar protein required for both replication fork blocking and recombination hotspot activities, *Δfob1* mutants extend the life span (Defossez et al., 1999). In contrast, *Δsod1* mutants are deficient in the antioxidant defence due to lack of gene for the enzyme Sod1, resulting in a life span shortening (Wawryn et al., 1999). Given that a phenotype of the same mutants often depends on the genetic background, the haploid strains of three different genetic backgrounds (i.e. SP4, BY4741 and BMA64-1A) were used.

The yeast growth rate was observed by incubation of samples in two different liquid YPD media: containing 2% and 0.5% glucose. The first type of media allowed cell growth analysis under the optimal conditions, whereas the second under the caloric restriction conditions. Plots of the all strains showed three characteristic phases of growth: *lag*, *log* and stationary phase (Fig. 8A, B and C). As expected, a higher growth rate was determined in complete media with 2% glucose, what is optimal for yeast growth. Furthermore, there was no significant difference in the cell growth between wild-type and *Δfob1* or *Δsod1* mutant of all genetic backgrounds. In the case of incubation in YPD media with reduced content of glucose, a lower growth rate was observed in general. Since glucose is the main carbon and energy source for yeast, it is an important messenger molecule signalling optimal growth conditions to the cellular machinery. Accordingly, glucose also affects many of the important traits of yeast such as growth rate, fermentation capacity and stress resistance (Rolland et al., 2002). When glucose becomes limiting, the culture switches from fermentative metabolism to

respiratory growth (Tissenbaum and Guarente, 2002). Using non-fermentable carbon sources, cells grow much slower than fermenting cells and reach the stationary phase formerly. In addition, they display several features such as high expression levels of genes involved in stress resistance (i.e. genes for superoxide dismutase and catalase) and accumulation of reserve carbohydrates (Rolland et al., 2002). In adverse conditions the differences among strains are becoming more visible. *Δsod1* mutants showed significantly lower survival in regard to wild-type strain of SP4 and BMA64-1A genetic backgrounds. These mutants lack *SOD1* so calorie restriction does not have a great influence on the stress resistance in this strain. A *Δfob1* mutant of SP4 cells showed significantly lower survival whereas the same mutant of BY4741 cells showed significantly higher survival in regard to wild-type strain. Likewise, the dynamics of glucose utilization may also point to the growth kinetics. Determining the differences in overall metabolic rate, differences in growth rate of analyzed strains may be observed. The results demonstrated that there was no significant difference in rate of glucose consumption by wild-type and its mutants (*Δfob1* and *Δsod1*) within each genetic background (SP4, BY4741 and BMA64-1A), except by the *Δsod1* strain of BY4741 background which showed slower metabolism of glucose.

As previously mentioned, the life span of yeast might be affected by oxidative stress, as a consequence of using oxygen for respiration or oxidation of nutrients to obtain energy efficiently (Izawa et al., 1995). During these processes arise ROS which attack all cell components (i.e. lipids, proteins, nucleic acids etc.) and are known to be causative of degenerative changes at the cellular level. However, it should be taken into account that the production of ROS depends on both genetic and environmental factors. In addition, the accumulation of oxidative damaged material in yeast cells is considered to have an important role in replicative aging (Wawryn et al., 1999). Deletions of yeast genes coding for SODs or catalase, changes in atmospheric oxygen partial pressure as well as the presence of physiological antioxidant glutathione have the expected distinct effects on yeast life span (Laun et al., 2001). It was reported that deficiencies in SODs (i.e. CuZnSOD or MnSOD) strongly shorten the life span of yeast cells (Wawryn et al., 1999). Yeast cells are well adapted to the high levels of H_2O_2 and can tolerate treatment with high concentrations of this compound, but sensitivity of cells to oxidative stress is not increased in the absence of catalase or cytochrome *c*. However, the three enzymes together are responsible for the removal of more than 90% of extracellular H_2O_2 (Wawryn et al., 1999). Therefore, within this study the yeast sensitivity to the oxidative stress was determined by spot tests in two similar

experiments: with temporary and permanent incubation with H_2O_2. These two assays allow more accurate assessment of the differences in sensitivity to oxidative factor between the tested strains. The first experiment was performed on solid YPD media after one hour of incubation with a 10 mM H_2O_2 (Fig. 10). Generally, *Δsod1* mutants of each genetic background showed the highest sensitivity to oxidative stress in regard to its wild-type strain. This result was expected for this strain due to lack a gene for Sod1. Furthermore, *Δfob1* mutants of all genetic backgrounds showed slightly lower resistance to oxidative stress comparing to its wild-type strains, but these cells showed higher survival than *Δsod1* mutants given that their Sod1 enzyme is preserved. Same results can be observed in the second experiment that was performed on solid YPD media containing 1, 3 or 5 mM H_2O_2 (Fig. 11). Here it should be emphasized that at a concentration of 5 mM H_2O_2 in a constant incubation, no cell growth was observed. It was reported that SP4 cells are more long-lived than BY4741 background, if comparing their mean and maximal life span (Zadrag-Tęcza et al., 2009). Therefore, comparing the same yeast strains between different genetic backgrounds similar results were demonstrated. In the experiment with temporary exposure of cells to H_2O_2, SP4 cells showed higher resistance to oxidative stress in both control samples (non-treated) and treated samples. BMA64-1A cells showed better survival than BY4741 cells. The same results were observed for SP4 cells in the second experiment with permanent exposure of cells to H_2O_2, whereas BY4741 background showed higher survival than BMA64-1A background.

Oxidative stress, as one of the important factor involved in cell aging, can also be observed by directly detection of ROS or indirectly by detection of oxidatively modified cellular macromolecules, i.e. by protein carbonyl groups measurement (Laun et al., 2006). Within this study, reactive oxygen species were determined in all genetic backgrounds in both exponential and stationary phase of growth. Since the differences in the ROS concentration between genetic backgrounds may partially depend on the cell size, values for ROS content were normalized per cell size. At exponential phase of growth (Fig. 14A) *Δsod1* mutants of all genetic backgrounds showed significantly higher ROS concentrations in regard to wild-type strains while *Δfob1* mutants showed no significant differences. According to the theory that oxidative stress and accumulation of rDNA circles in yeast cells are interdependent (Laun et al., 2001), *Δfob1* mutants did not show the expected decrease in ROS concentrations compared to wild-type strain. Namely, there is an assumption that oxidative stress could cause a moderate increase in rDNA circles, which creates a vicious circle to more oxidative stress

and more circles. According to another theory, the two processes could be independent of each other and both contribute to yeast aging (Laun et al., 2001). Moreover, the cells at exponential growth phase showed slightly higher differences in ROS concentrations between yeast mutants and their wild-type in relation to the stationary phase cells (Fig. 14). The possible explanation is that cells in fast logarithmic phase have very active metabolism, resulting in an increased formation of free radicals. Reaching the stationary phase, cells switch their metabolism from a fermentative to respiratory, which implies a greater activation of enzymes, involved in the regulation of oxidative stress, including superoxide dismutase, catalase and glutathione peroxidase. Likewise, at stationary growth phase (Fig. 14B) BY4741 cells demonstrated significantly higher ROS concentrations than SP4 and BMA64-1A cells, especially in the case of wild-type and *Δfob1* mutants. These results can draw a parallel with a higher sensitivity of BY4741 cells to 10 mM H_2O_2 during the temporary exposure, comparing to other two backgrounds. According to the hypothesis that yeast cells reach an excessive volume during their replicative life span, resulting in their inability to undergo further reproduction (Bilinksi at al., 2012), stationary cells did not differ in size significantly within each genetic background. In our study we have shown greater difference in the cell size of individual yeast strain from various genetic backgrounds at stationary growth phase than those at exponential growth phase. In general, BMA64-1A cells are significantly bigger in size than cells of SP4 or BY4741 background, what is particularly evident in the stationary growth phase. Such observation may refer to reduced reproductive potential with the respect to the formerly reached excessive volume. These results can draw a parallel with a higher sensitivity of BMA64-1A cells to 3 mM H_2O_2 during the permanent exposure, comparing to other two backgrounds.

As already mentioned, one of the many harmful effects of free radicals is the formation of protein carbonyl groups which can be detected *in situ* (Fig. 15). Carbonylation of proteins is an irreversible oxidative damage, often leading to a loss of protein function (Dalle-Donne et al., 2006). Therefore, progressive accumulation of protein carbonyl groups represents an important determinant of the replicative life span of yeast cells. Considering that protein carbonylation normally takes place in the cell, depending on various factors, and that protein carbonyl groups are not inherited by daughter cells during cytokinesis (Nyström, 2005), it is expected to observe accumulated proteins from earlier to later stages of mother cell growth. After application of fluorescent techniques, almost equal to higher fluorescent intensity was observed between *Δfob1* mutants and its wild-type strain within each genetic background.

This is comparable to the results of oxidative stress sensitivity, in which *Δfob1* mutants were more sensitive to oxidative stress than its wild-type strain. In the line with other experiments, isogenic *Δsod1* strain accumulates more free radicals that promote the protein carbonylation. A *Δfob1* mutant of SP4 background was exception, because it showed higher amount of protein carbonyl groups than wild-type strain at stationary phase of growth. It may explain the significant higher relative growth rate of wild-type strain in regard to its *Δfob1* mutant. If comparing each phase of growth, the yeast cells at exponential phase show expected lower signal intensity than those in stationary phase.

Furthermore, using a fluorescent FUN-1 dye it is possible to determine the metabolic activity and viability of yeasts (Fig. 16). This dye is membrane-permeable, usually binds the nucleus acids, but has unexpected and useful properties when is used to stain yeasts such as *S. cerevisiae* (Millard et al., 1997). Three types of cells were observed: metabolic active cells, metabolic inactive but live cells and dead cells. In this experiment cell viability was monitored over a period of 7 days (Fig. 16A-C). During the prolonged cultivation, wild-type and its mutants of each genetic background showed no significant changes in viability. It means that all cells showed similar loss of viability during the time. Significant difference that was observed between tested strains of different genetic backgrounds at the 1st day may be due to various conditions of cells viability at the start of the experiment. If comparing viability between tested strains of different genetic backgrounds at the 7th day of cultivation, *Δfob1* and *Δsod1* mutants of SP4 cells showed significantly higher viability in regard to the same mutants of BY4741 and BMA64-1A cells. This is consistent with previous experiments that demonstrated longer replicative life span of SP4 cells than BY4741 and BMA64-1A cells.

Cell morphology could be explored using fluorescent vital dyes: DAPI, rhodamine B and MDY-64 (Pringle et al., 1989). They were useful for detecting the cellular alternations of yeast during the prolonged cultivation (Fig. 17). Replicative life span of yeast cells was observed during the seven days of cultivation. As yeast mother cells age, they change the morphology regardless of genetic background. Tested cells from each genetic background demonstrated similar changes. During the life span cells progressively increased in their size. It is important to emphasize that BMA64-1A cells are bigger in size than other observed genetic backgrounds, as already established by cell size measuring.

Given that *Δsod1* lacks gene for enzyme superoxide dismutase, the higher sensitivity of these strain is considered in the all experiments therefore showing the influence of oxidative stress on shortening its replicative life span. It was expected from *Δfob1* strains to show prolonged replicative life span, but there was no big difference between wild-type strains and *Δfob1* mutants within each genetic background. If comparing genetic backgrounds, SP4 cells showed prolonged replicative life span in regard to BY4741 and BMA64-1A cells while BMA64-1A seems to be the most sensitive to intrinsic and extrinsic factors that influence the replicative life span of yeast. According to the hypothesis that reaching excessive volume by yeast cells limits their ability to undergo further reproduction resulting in cell senescence, stationary cells showed higher cell size than cells at exponential growth phase within each genetic background. Finally, during the progressive growth cells undergo age-related changes including an increased generation time, an increase in size with changes in morphology as well as accumulation of damaged material. However, the further research is needed to provide better insight in replicative life span of yeast *S. cerevisiae* which could be then applied to the higher organisms.

5. CONCLUSION

1. The results of the growth curve test in 0.5% glucose indicate significantly decreased cell growth in *Δsod1* mutant of SP4 and BMA4741 backgrounds. *Δfob1* mutant of SP4 cells showed significant decrease while the same mutant of BY4741 cells showed significantly increased growth in regard to its wild-type strain.

2. There was no significant difference in glucose consumption rate between wild-type and its mutants (Δfob1 and Δsod1) within each genetic background (SP4, BY4741 and BMA64-1A). If comparing each yeast strain of each genetic background separately, there was no significant difference in glucose consumption between individual time points of measurements at 4, 8 and 12 h.

3. After one hour of incubation with 10 mM H2O2, SP4 background showed higher resistance to oxidative stress than BY4741 and BMA64-1A backgrounds, which both showed lower resistance at dilution 10^4 of cells/mL. While the wild-type and *Δfob1* strains showed similar sensitivity to H_2O_2, *Δsod1* is the most sensitive strain.

4. All the yeast strains of all of the genetic backgrounds were still forming colonies on the plates with 1 mM H_2O_2. SP4 strains showed lower sensitivity to 3 mM H_2O_2 than BY4741 and BMA64-1A. The wild-type and *Δfob1* strains demonstrated similar sensitivity to oxidative stress, whereas *Δsod1* is the most sensitive strain.

5. ROS were detected in all yeast strains of all the genetic backgrounds. Observing the ROS concentration per cells size, there was no significant differences in ROS concentrations between individual yeast strains from three genetic backgrounds at exponential growth phase. At stationary phase, wild-type strains and *Δfob1* mutants significantly differed between SP4 and BY4741 as well as BY4741 and BMA64-1A backgrounds. Observing the ROS concentration per cell size within the each genetic background at exponential growth phase, there was significantly higher ROS content in *Δsod1* mutants than in wild-type strains. In contrary, cells at stationary growth phase showed significant difference

only between wild-type and *Δfob1* mutant of BMA64-1A background. Mutant showed lower ROS concentrations than wild-type.

6. Protein carbonyl groups were detected in all yeast strains of all the genetic backgrounds, with the difference between exponentially and stationary phase of growth. Cells that achieved stationary phase showed higher concentrations of protein carbonyl groups. *Δsod1* mutants of all genetic backgrounds demonstrated the highest concentrations of protein carbonyl groups in regard to wild-type strains and *Δfob1* mutants.

7. Metabolic activity as well as a viability of yeast cells decreases during prolonged cultivation (7 days). During the prolonged cultivation, wild-type and its mutants of each genetic background showed no significant changes in viability. Likewise, the individual yeast strains from various genetic backgrounds showed no significant difference, except wild-type strain that differed between BY4741 and BMA64-1A backgrounds.

8. All the yeast strains of all the genetic backgrounds changed cell morphology during the prolonged cultivation. The cell volume increases during the cell divisions.

9. Overall, *Δsod1* mutants showed decreased replicative life span in all genetic backgrounds, whereas *Δfob1* mutants did not show increased replicative life span in regard to wild-type strains of each genetic background. Its replicative life span differs between genetic backgrounds.

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7. SUMMARY

Among the model organisms currently in use for biogerontological research Saccharomyces *cerevisiae* has a special place and importance. As a prototypical eukaryotic cell, many basic facts about molecular biology and metabolism can be explored. Furthermore, yeast cells are mortal given that most laboratory yeast strains can complete approximately 20-30 divisions followed by death. During these progressive divisions, the cells undergo age-related changes, including an increase of generation time, increasing the size, decline in mating ability, oxidative stress and accumulation of extrachromosomal rDNA. The aim of this study was to observe morphological and physiological characteristics of the yeast *S. cerevisiae* cells during its replicative life span. For this purpose a various yeast strains and methods were used. Three different genetic background were analyzed (i.e. SP4, BY4741 and BMA64-1A), each background consisting of a wild-type strain and its isogenic mutant (i.e. *Δfob1* and *Δsod1*). Relative growth rate was observed under sufficient and depleted glucose conditions. Furthermore, the rate of glucose consumption was determined as well as sensitivity to oxidative stress generated by hydrogen peroxide. Detection of the ROS was performed using dihydroethidium assay, while the immunocytochemistry assay was used to determine the presence of protein carbonyl groups. Finally, the samples were also cultivated for a prolonged time (i.e. 7 days), during which cellular alternations and metabolic activity were determined, both by corresponding fluorescence techniques. To interpret parametric results, ANOVA statistical tests were used. Results of experiments have shown that yeast cells undergo morphological and physiological changes during their progressive growth. There was no significant difference in replicative life span between SP4 and BY4741 genetic backgrounds, whereas BMA64-1A background showed higher sensitivity to growth conditions. In addition, wild-type and *Δfob1* strains of each genetic background demonstrated similar results in all experiments, showing the lower sensitivity to environmental conditions during the life span contrary to *Δsod1* mutant. Given that *Δsod1* lacks gene for mitochondrial enzyme superoxide dismutase, the higher sensitivity is considered and the influence of oxidative stress on aging is confirmed. It was expected from *Δfob1* strains to show prolonged replicative life span, but there was no significantly difference between wild-type strains and *Δfob1* mutants. According to the hypothesis that reaching excessive volume by yeast cells limits their ability to undergo further reproduction resulting in cell senescence, stationary cells showed higher cell size than cells at exponential growth phase.

SAŽETAK

UVOD

Kao prototip eukariotske stanice, kvasac *S. cerevisiae* zauzima posebno mjesto i značaj među modelnim organizmima u biogerontološkim istraživanjima. Većina laboratorijskih sojeva uspješno završava 20-30 dioba, stoga se smatra da stanice stare i umiru. Dva su glavna pristupa starenju kvasaca koja opisuju njegov životni vijek: replikacijsko i kronološko starenje. Replikacijsko starenje karakterizirano je činjenicom da samo stanica majka stari, dok nastale stanice kćeri započinju od početka s replikacijskim životnim vijekom. Kronološko starenje je vremenski period tijekom kojeg stanica u stacionarnoj fazi rasta zadržava vijabilnost. Jedan od pretpostavljenih mehanizama replikacijskog starenja je akumulacija citoplazmatskog, difuzibilnog i razgradivog faktora starenja koji još uvijek nije jednoznačno identificiran. Poznato je da tijekom progresivnih dioba stanice prolaze kroz različite promjene, uključujući povećanje generacijskog vremena, povećanje staničnog volumena, pad sposobnosti razmnožavanja, veću podložnost oksidacijskom stresu te akumulaciju rDNA. Upravo asimetrično vankromosomsko nakupljanje malih kružnih rDNA objašnjava jednu od vodećih pretpostavki starenja stanice majke, jednako kao i nakupljanje oksidacijski oštećenog staničnog materijala tijekom životnog vijeka kvasca. Povećanje staničnog volumena nakon završetka svake diobe nakon dosezanja kritične vrijednosti rezultira zaustavljanjem daljnje reprodukcije. Istraženi su uzročno-posljedični odnosi pojedinih mehanizama te su postavljene različite hipoteze o starenju kao nimalo jednostavnom procesu u kvasaca.

OBRAZLOŽENJE TEME

Cilj ovog istraživanja bio je promatranje morfoloških i fizioloških karakteristika na stanicama kvasca *S. cerevisiae* tijekom njegova replikacijskog životnog vijeka. U tu svrhu koristili su se različiti sojevi kvasca kao i odgovarajuće metode.

MATERIJALI I METODE

Analizirane su tri genski različite skupine kvasca (SP4, BY4741 i BMA64-1A), od kojih svaka sadrži soj divljeg tipa te njegova dva mutanta (*Δfob1* i *Δsod1*). Mutanti *Δfob1* imaju produljeni replikacijski životni vijek i predstavljaju hipotezu postojanja faktora starenja. U suprotnom, mutanti *Δsod1* imaju reducirani replikacijski životni vijek te predstavljaju teoriju slobodnih kisikovih radikala u procesu starenja. Stanicama kvasca određena je relativna stopa rasta u uvjetima dovoljnih i osiromašenih količina glukoze, kao glavnog izvora energije za

rast i razvoj kvasaca. Nadalje, određeni su utrošak glukoze te osjetljivost sojeva na oksidacijski stres upotrebom vodikova peroksida. Detekcija reaktivnih kisikovih radikala izvršena je pomoću testa s dihidroetidijem, dok su proteinske karbonilne skupine određene imunocitokemijski. Uzorci su također kultivirani tijekom duljeg vremenskog perioda (7 dana) kako bi se fluorescentnim tehnikama utvrdile promjene u staničnoj morfologiji i metaboličkoj aktivnosti. Za interpretaciju parametrijskih rezultata korišteni su statistički testovi ANOVA.

REZULTATI

Značajnije razlike u stopi staničnog rasta tijekom 12 sati između divljeg tipa i njegovih mutanata unutar pojedinih genskih skupina zabilježene su u mediju s 0.5% glukozom u odnosu na stanice koje su rasle u mediju s 2% glukozom. Nije bilo značajne razlike u stopi potrošnje glukoze između divljeg tipa i njegovih mutanata unutar pojedinih genskih skupina. Sojevi genske skupine SP4 pokazali su veću rezistenciju na oksidacijski stres nakon tretmana s H_2O_2 u odnosu na sojeve unutar skupina BY4741 i BMA64-1A. Gledajući unutar pojedinih genskih skupina, sojevi divljeg tipa i mutanti *Δfob1* pokazali su manju osjetljivost na oksidacijski stres u odnosu na mutante *Δsod1*. Reaktivni kisikovi radikali detektirani su u svim istraženim sojevima. Ukoliko se u obzir uzme stanična veličina, postoje značajnije razlike u izmjerenim koncentracijama slobodnih radikala između divljeg tipa i njegovih *Δsod1* mutanata u svakoj pojedinoj genskoj skupini eksponencijalne faze rasta, u odnosu na stacionarnu fazu rasta. Nadalje, stanice u stacionarnoj fazi rasta pokazuju veću zastupljenost karbonilnih grupa proteina u odnosu na stanice u eksponencijalnoj fazi rasta. Tijekom produljenog vremena kultiviranja (7 dana), sojevi divljeg tipa i njihovi mutanti svake genske skupine nisu pokazali značajnu razliku u promjeni metaboličke aktivnosti i vijabilnosti.

ZAKLJUČAK

Rezultati istraživanja pokazali su da stanice kvasca prolaze kroz morfološke i fiziološke promjene tijekom progresivnog rasta. Ne postoji velika razlika u replikacijskom životnom vijeku između genskih skupina SP4 i BY4741, dok skupina sojeva BMA64-1A pokazuje veću osjetljivost na uvijete rasta. Osim toga, divlji tip i mutant *Δfob1* svake od genski različitih skupina sojeva pokazuju slične rezultate u svim provedenim istraživanjima, na način da su manje osjetljivi na okolišne uvjete tijekom vlastitog životnog vijeka. Za razliku od spomenutih sojeva, mutant *Δsod1* pokazuje veću razinu osjetljivosti i skraćeni životni vijek, s obzirom da je deficijentan za enzim superoksid dismutazu te je tako podložniji utjecaju oksidacijskog stresa.

Basic documentation card

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Diploma thesis

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE YEAST *SACCHAROMYCES CEREVISIAE* CELLS DIFFERING IN THE LIFE SPAN

Marina Kovačević

SUMMARY

S. cerevisiae has a special place among the model organisms currently in use for aging research. During their replicative lifespan yeast cells undergo age-related changes. The aim of this study was to observe morphological and physiological characteristics of the yeast *S. cerevisiae* cells during its replicative life span. Three different genetic background were analyzed (SP4, BY4741 and BMA64-1A), each consisting of a wild-type strain and its isogenic mutant (*Δfob1* and *Δsod1*). Relative growth and rate of glucose consumption was determined as well as sensitivity to oxidative stress. Detection of the ROS was performed using dihydroethidium assay, while with the immunocytochemistry assay was determined the presence of protein carbonyl groups. Samples were also cultivated for 7 days, during which cellular alternations and metabolic activity were determined, both by corresponding fluorescence techniques. To interpret parametric results, ANOVA statistical tests were used. Results of experiments have shown that there is no significant difference in replicative life span between SP4 and BY4741 cells, whereas BMA64-1A cells showed higher sensitivity to growth conditions. Wild-type and *Δfob1* strains of each genetic background demonstrated similar results in all experiments, showing the lower sensitivity to environmental conditions during the life span contrary to *Δsod1* mutant. According to the hypothesis that reaching excessive volume by yeast cells limits their ability to undergo further reproduction resulting in cell senescence, stationary cells showed higher cell size than cells at exponential growth phase.

Thesis includes: 81 pages, 17 figures, 2 tables and 55 references. Original is in English language.

Keywords: Aging, yeast, *S. cereviaise*, life span, replicative life span

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RAZLIKE U MORFOLOŠKIM I FIZIOLOŠKIM KARAKTERISTIKAMA STANICA KVASCA *SACCHAROMYCES CEREVISIAE* TIJEKOM ŽIVOTNOG VIJEKA

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SAŽETAK

S. cerevisiae ima posebnu ulogu među modelnim organizmima u istraživanju starenja. Tijekom replikacijskog životnog vijeka stanice kvasca podliježu različitim promjenama. Cilj ovog istraživanja bio je promatranje morfoloških i fizioloških karakteristika na stanicama kvasca *S. cerevisiae* tijekom njegova replikacijskog životnog vijeka. Analizirane su tri genski različite skupine kvasca (SP4, BY4741 i BMA64-1A), od kojih svaka sadrži soj divljeg tipa te njegova dva mutanta (*Δfob1* i *Δsod1*). Određene su stope staničnog rasta i utroška glukoze. Detekcija reaktivnih kisikovih radikala izvršena je pomoću testa s dihidroetidijem, dok su proteinske karbonilne skupine određene imunocitokemijski. Uzorci su također kultivirani kroz 7 dana, kako bi se fluorescentnim tehnikama utvrdile promjene u staničnoj morfologiji i metaboličkoj aktivnosti. Za interpretaciju parametrijskih rezultata korišteni su statistički testovi ANOVA. Rezultati istraživanja pokazali su da ne postoji značajna razlika u replikacijskom životnom vijeku između genskih skupina SP4 i BY4741, dok skupina sojeva BMA64-1A pokazuje veću osjetljivost na uvijete rasta. Divlji tip i mutant *Δfob1* svake od genskih različitih skupina sojeva pokazuju manju osjetljivost na okolišne uvjete u odnosu na mutant *Δsod1*. Prema hipotezi da progresivno povećanje staničnog volumena tijekom replikacijskog životnog vijeka kvasca ograničava njegovu daljnju sposobnost diobe te rezultira starenjem, veličine stanica izmjerene u stacionarnoj fazi rasta bile su veće u odnosu na stanice u eksponencijalnoj fazi rasta.

Rad sadrži: 81 stranica, 17 grafičkih prikaza, 2 tablice i 55 literaturnih navoda. Izvornik je na engleskom jeziku.

Ključne riječi: Starenje, kvasac, *S. cereviaise*, životni vijek, replikacijski životni vijek

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