



DNA susceptibility of *Saccharomyces cerevisiae* to Zeocin depends on the growth phase

Teodora Todorova¹ · Daniela Miteva¹ · Stephka Chankova¹

Received: 2 August 2018 / Revised: 3 January 2019 / Accepted: 13 February 2019 / Published online: 14 March 2019
© Springer Nature Switzerland AG 2019

Abstract

The aim of this study was to evaluate the level of Zeocin-induced double-strand breaks (DSBs) in *Saccharomyces cerevisiae* cells in a different growth phase, using constant-field gel electrophoresis (CFGE). *Saccharomyces cerevisiae* diploid strain D7ts1 with enhanced cellular permeability was used. The effects of growth phase and treatment time were evaluated based on Zeocin-induced DSBs, measured by CFGE. Survival assay was also applied. No protoplast isolation was necessary for the detection of DSBs in strain D7ts1. Differences in the response of cells depending on the growth phase were obtained. Cells in exponential growth phase had increased DSB levels only after Zeocin treatment with concentrations equal or higher than 200 μgml^{-1} . Increasing treatment time did not result in higher DSB levels. Oppositely, treatment of cells at the beginning of stationary phase with Zeocin concentrations resulted in more than 1.5-fold increase in DSB levels in comparison with those in untreated cells. Increased DSB levels were measured for all the treatment times. A dose-dependent decrease in cell survival was observed after Zeocin treatment with concentrations in the range of lethality LD_{20} – LD_{50} . A strong negative correlation was calculated between the levels of DSBs and cell survival. New information is provided concerning DNA susceptibility depending on the growth phase. DNA susceptibility is higher in cells at the beginning of stationary phase than those in exponential phase. Data presented here illustrate that the optimized by us CFGE protocol is sensitive and could be used successfully for DSB measurement in *Saccharomyces cerevisiae* strains with enhanced cellular permeability.

Keywords CFGE · DNA susceptibility · Growth phase · *Saccharomyces cerevisiae* · Zeocin

Introduction

Saccharomyces cerevisiae is commonly used as a test model because of several reasons: entirely sequenced genome, simple growth conditions, short reproduction time, similarities between yeast and human genome (Frassinetti et al. 2011). A relatively fast information concerning the mechanisms of DNA double-strand break (DSB) induction, oxidative stress, and the related consequences associated with several human pathologies, including cancer, could be gained using *Saccharomyces cerevisiae* as a model system (Lee et al. 2014; Matuo et al. 2012; Ikner and Shiozaki 2005; Temple

et al. 2005). Due to qualitatively identical radiobiological responses of yeasts to those of mammalian cells (Petin and Kapultceвич 2014), *Saccharomyces cerevisiae* is extensively used in order to understand the radiation response in eukaryotes (Galao et al. 2007; Rao 2007).

Many genotoxicity reports are based on *Saccharomyces cerevisiae* cells in the exponential growth phase (Wilson 2014). This could be explained by the assumption that cells in the stationary phase were either dead or homogeneously quiescent (Wilson 2014; Gray et al. 2004). In the present study, a new approach was applied to analyze the response of yeast cells to Zeocin. The response of cells in different growth phases (exponential and the beginning of stationary) was compared. This is dictated by the fact that, entering into another growth phase, morphological and physiological changes are undergone. It is known that cells in stationary phase are characterized with increased thermo-stability, low metabolic activity, reduced transcription and translational activity, resistance to various environmental stresses, and lower

✉ Stephka Chankova
stephanie.chankova@yahoo.com

¹ Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin str., 1113 Sofia, Bulgaria

permeability of plasma membrane (Kumar and Srivastava 2016; Sousa-Lopes et al. 2004).

In the present work, the role of the growth phase was evaluated based on the induction of double-strand breaks (DSBs). DSBs in nuclear DNA are the most critical genetic damages, caused by ionizing radiation and radiomimetics. One of the genotoxic agents recognized as a major source of DSBs is Zeocin™ (the commercial name of a special formulation containing phleomycin D1). Zeocin is a radiomimetic, member of the bleomycin family of antibiotics, that damages DNA in a way similar to that of ionizing radiation (<https://www.thermofisher.com/bg/en/home/references/protocols/cloning/transformation-protocol/zeocin.html>).

Previously, it was shown by us that Zeocin possess pro-oxidative capacity (Chankova et al. 2013; Todorova et al. 2015a), mutagenic, and carcinogenic effect in *Saccharomyces cerevisiae* (Todorova et al. 2015a), as well as clastogenic, DNA damaging, and genotoxic effects in microalgae, higher plants, and human lymphocyte cell culture (Kopaskova et al. 2011; Dimova et al. 2009; Chankova et al. 2007; Gateva et al. 2015).

The aim of this study was to evaluate the level of Zeocin-induced double-strand breaks (DSBs) in *Saccharomyces cerevisiae* cells in a different growth phase, using constant-field gel electrophoresis (CFGE).

Materials and methods

Chemicals

Zeocin (Zeo) was purchased from Invitrogen; methyl methanesulfonate (MMS) and 5-bromouracil (5-BU) from Sigma-Aldrich. Nutritional components for yeast media preparation were from Difco Chem. Co. (USA). Chemicals and reagents were of analytical grade.

Strain

Saccharomyces cerevisiae diploid strain D7ts1 (MATa/α; ade2-119/ade2-40; trp5-27/trp5-12; ilv1-92/ilv1-92; ts1/ts1) was used (Staleva et al. 1996). D7ts1 is derived from Zimmermann's strain D7 (Zimmermann et al. 1975). This strain is constructed for simultaneous detection of mitotic gene conversion at the *trp-5* locus, reversion mutations in the *ilv1* locus and mitotic crossing-over between the centromere and *ade2* allele (Freeman and Hoffmann 2007). A typical feature of D7ts1 strain is the enhanced cellular permeability to different substances due to inserted temperature-sensitive mutation ts1 (Pesheva et al. 2005; Staleva et al. 1996; Venkov and Scheit 1984).

Cultivation

D7ts1 was cultivated at semi-permissive conditions: $t = 30\text{ }^{\circ}\text{C}$ with aeration on a rotary shaker in liquid YEPD medium containing (1% yeast extract, 2% Bacto peptone, and 2% glucose, pH = 6.8).

Growth phase analysis

Single colony of *Saccharomyces cerevisiae* D7ts1 was inoculated in 20 ml fresh YEPD and incubated overnight at $30\text{ }^{\circ}\text{C}$, with aeration in a rotary shaker (200 rpm). Growth phase analysis was started by measuring the initial optical density 600 nm (OD_{600}) at the 15th hour. Growth was then monitored by measuring OD_{600} of the culture every hour and plating cells on solid YEPD medium for a period of 24 h. The morphological differences of cells in different growth phases were evaluated microscopically.

Experimental designs

Two experimental designs were applied in order to clarify both effects of growth phase and of exposure time.

Effect of the growth phase One milliliter cell suspension with density 1×10^6 cells ml^{-1} at different growth phases—the exponential, the beginning and the end of stationary phase—was treated with 6 concentrations of Zeocin (10, 50, 100, 200, 300, and 400 $\mu\text{g ml}^{-1}$) for 1 min on ice (to prevent DNA repair).

Effect of the exposure time One milliliter cell suspension (1×10^6 cells ml^{-1}) at the exponential and the beginning of stationary phase was treated with concentration 100 $\mu\text{g ml}^{-1}$ Zeocin for 1, 2, 3, and 4 min on ice (to prevent DNA repair).

Further, cells were subjected to constant-field gel electrophoresis (CFGE).

Zeocin-induced DNA double-strand breaks—an optimized protocol for constant-field gel Electrophoresis (CFGE)

CFGE protocol for *Saccharomyces cerevisiae* was optimized following the *Chlamydomonas reinhardtii* CFGE protocol described earlier by us (Chankova et al. 2005; Chankova and Bryant 2002).

After the application of the abovementioned experimental designs, cells were centrifuged at $t = 4\text{ }^{\circ}\text{C}$. The supernatant was aspirated, and the cell pellet was taken up in 85 μl of 0.8% low melting point agarose (LMP) at $t = 37\text{ }^{\circ}\text{C}$ and pipetted into a plug mold (BioRad). Plugs were kept on ice for around 30 min and then placed in 1 ml of lysis solution (pH = 8, 0.4 M EDTA, 2% sodium n-lauryl sarcosine) containing proteinase K at concentration 1 mg ml^{-1} . Cell lysis was

carried out for 30 min on ice and incubated for 20 h at 37 °C. Plugs were washed 4 times with 3 ml of Tris-EDTA (pH = 7.5). An agarose gel of 0.8% (ultra-pure agarose electrophoresis grade) was prepared in 300 ml 0.5 × TBE (pH = 8) containing 0.5 µg ml⁻¹ of ethidium bromide and cast in an electrophoresis apparatus (HE 99X, Amersham Biosciences). Plugs were inserted into a series of wells in the gel, sealed with a small amount of 0.8% LMP agarose, and electrophoresis was performed in 0.5 × TBE buffer. Electrophoresis conditions were 40 h at a constant field of 0.6 V/cm (20 V). The levels of induced DSB presented as a fraction of DNA released (FDR) from the wells were quantified by measurement of ethidium bromide fluorescence using Gene Tool Analyzer G: Box Syngene. FDR was calculated as follows: FDR = DNA released/(DNA in well + DNA released) (Dimova et al. 2009).

Protocol efficiency

In order to evaluate the sensitivity and accuracy of the optimized CFGE protocol, two approaches commonly used in experimental mutagenesis were applied:

- Treatment with equimolar concentrations Zeocin, MMS, and 5-BU—0.07 mM. Previously, 100 µg ml⁻¹ Zeocin corresponding to 0.07 mM was found to be the most effective DNA damaging concentration.
- Treatment with equitoxic concentrations (around LD₅₀) - 0.07 mM Zeocin, 16 mM MMS, and 0.262 mM 5-BU.

These genotoxins were chosen based on their different mode of action (MoA):

- Zeocin as an inducer of DSBs (Zeocin 2018 <https://www.thermofisher.com/bg/en/home/references/protocols/cloning/transformation-protocol/zeocin.html>);
- Methyl methanesulfonate (MMS) as an alkylating agent (Methyl methanesulfonate 2018 https://pubchem.ncbi.nlm.nih.gov/compound/methyl_methanesulfonate#section=Top);
- 5-bromouracil (5-BU) as base analog (5-bromouracil 2018 <https://pubchem.ncbi.nlm.nih.gov/compound/5-Bromouracil#section=Top>).

Cell survival

Cell suspensions, grown to the beginning of the stationary phase were treated with 2, 4, 6, 8, 10, 50, 100, and 300 µg ml⁻¹ Zeocin for 1 min on ice. Cells were centrifuged, the supernatant was removed, and the pellet was resuspended in liquid YEPD medium. Cells were plated on solid YEPD medium and incubated at 30 °C for 3 days.

CFGE data show that treatment with 100, 200, 300, and 400 µg ml⁻¹ Zeocin results to the formation of a plateau region. Following this observation, cell survival experiments were done with 100 and 300 µg ml⁻¹. Additionally, several very low concentrations (below 10 µg ml⁻¹) have been added in order to calculate lethality dose of 20%—LD₂₀.

Data analysis

The experiments were repeated at least three times from independently grown cultures. Data points in all the figures are mean values. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or less than the symbols. Results for cell survival were presented as survival fraction (Bryant 1968). Three doses of lethality (LD₂₀, LD₃₇, and LD₅₀) were calculated (Reed and Muench 1938). The statistical analysis was performed by Student *t* test and two-way ANOVA with Bonferroni's post-test. Asterisks provide information about the significance in the differences where **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Linear correlation, using Pearson product-moment correlation coefficient analysis (PMCC, or *r*) and coefficient of determination (*R*(Beam et al. 1954)) were determined. All the calculations were done with GraphPad Prism program, version 6.04 (San Diego, USA).

Results

Growth phases curve

The growth phases of *Saccharomyces cerevisiae* were determined based on two approaches: measurement of OD₆₀₀ and counting the number of cells per milliliter. The initial OD₆₀₀ was 0.09. The growth phases are presented in Fig. 1.

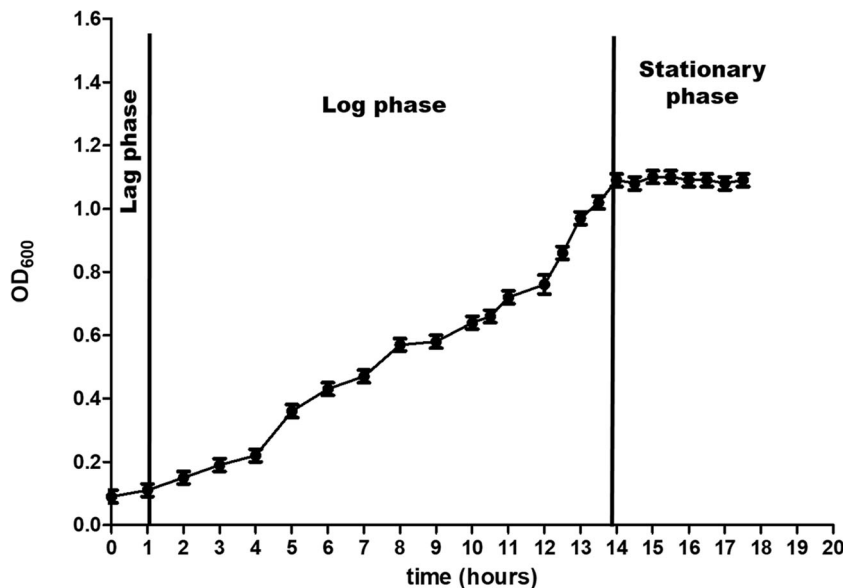
For further experiments, the following OD₆₀₀ were chosen:

OD₆₀₀ = 0.700 ÷ 0.800—Cells in exponential phase of growth with a concentration of cells 5–7 × 10⁷ cells ml⁻¹ (measured in the time interval—0.5th to the 12th hour of measurement). Under a microscope, the prevalent number of cells is still budding;

OD₆₀₀ = 1.0–1.1—Cells at the beginning of stationary phase with a concentration of cells 5–7 × 10⁸ cells ml⁻¹ (measured in the time interval—13.5th to the 17.5th hour of measurement). Under a microscope, most of the cells are not budded.

OD₆₀₀ > 1.1—Cells at the end of the stationary phase. Under a microscope, most of the cells were dead and the number of plated cells was decreasing.

Fig. 1 Growth curve of *Saccharomyces cerevisiae* strain D7ts1. The experiment was repeated at least four times from independently grown cultures. Where no error bars are evident, they are equal or less than the symbols



Protocol efficiency

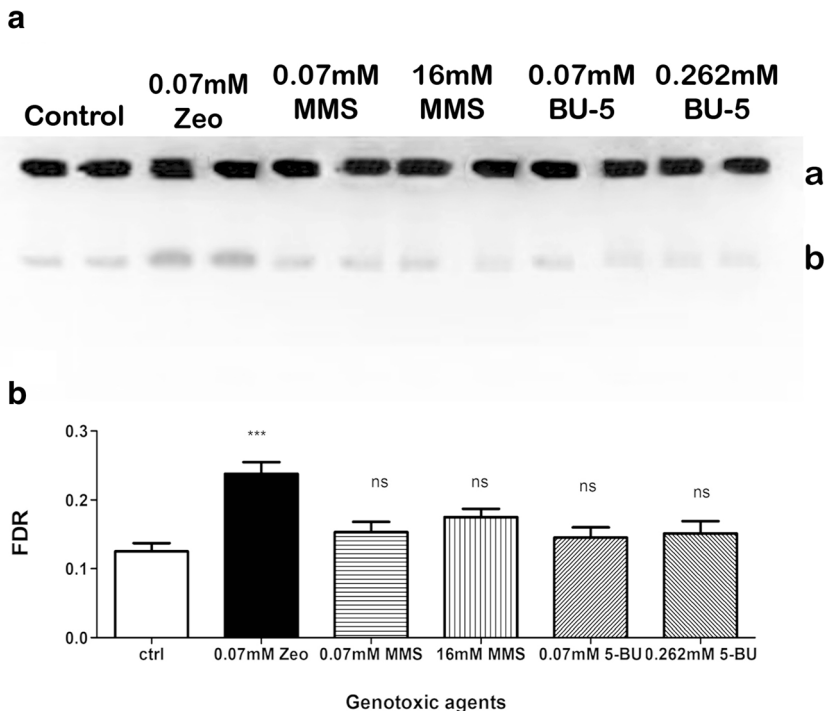
In order to check whether the CFGE protocol works well for yeast, results for Zeocin- were compared to those after methyl methanesulfonate and 5-bromouracil treatment (Fig. 2). These genotoxic agents were chosen based on the differences in their mode of action. Equitoxic and equimolar concentrations were applied. The results obtained show that only Zeocin could induce higher-level DSBs in a statistically significant way. Insignificant DSB increase was measured after MMS and 5-BU treatment.

Zeocin-induced DSBs in whole cells measured with CFGE

Results presented in Fig. 3 clearly indicate differences in the response of *Saccharomyces cerevisiae* cells at different growth phases.

The contribution of growth phase was evaluated by comparison of the levels of spontaneously aroused DSBs. The amount of spontaneous DSBs in control cells at the end of the stationary phase was more than 2-fold higher compared

Fig. 2 Induction of DSBs after the treatment with different genotoxic agents—Zeocin, MMS, and 5-BU. **A** Inverted gel electrophoresis image. (a) DNA in wells; (b) a fraction of DNA released. **B** Induction of DSBs after treatment with different genotoxic agents calculated as a fraction of DNA released (FDR). The significance in differences between tested concentrations and the negative control was calculated by one-way analysis of variances (ANOVA) with Dunnett’s multiple comparisons test. Asterisks provide information about the significance in the differences where *** $P < 0.001$



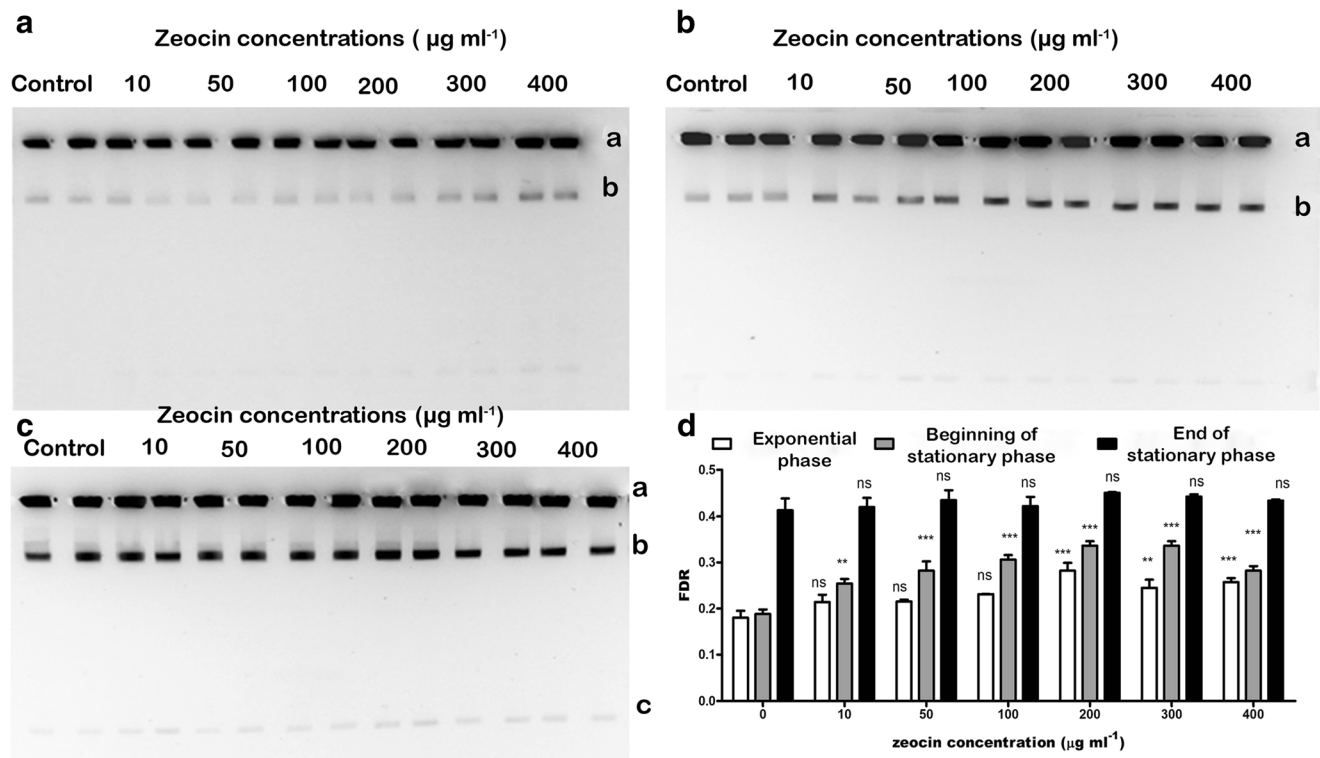


Fig. 3 DSBs induced in *S. cerevisiae* cells, depending on growth phases after Zeocin treatment in a concentration range 10–400 $\mu\text{g ml}^{-1}$. **A** Cells in the exponential phase of growth. **B** Cells at the beginning of stationary phase. **C** Cells at the end of stationary phase. (a) DNA in wells; (b) a fraction of DNA released. **D** Induction of DSBs after treatment with

different concentrations of Zeocin calculated as FDR. Asterisks provide information about the significance in the differences where ^{ns} $P > 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$. Where no error bars are evident, they are equal or less than the symbols

to that of cells in the exponential and the beginning of the stationary phase (Fig. 3D).

No statistically reliable effect was observed after Zeocin treatment of cells in exponential phase (Fig. 3B, D) with concentrations in the range of 10–100 $\mu\text{g ml}^{-1}$ ($P > 0.05$). Treatment with any of the Zeocin concentrations of cells at the beginning of stationary phase resulted in more than 1.5-fold statistically significant increase in DSB levels in comparison with those measured in control untreated cells (Fig. 3A). No statistically significant increase in DSB levels in comparison with the spontaneous levels was observed for cells at the end of the stationary phase (Fig. 3C). No effect of concentration was observed. Additionally, very huge DNA destruction was obtained seen in the gel as a second band (Fig. 3c).

The most pronounced statistically significant damaging effect was observed after the treatment of cells at the beginning of stationary phase with Zeocin concentration/s equal or higher than 100 $\mu\text{g ml}^{-1}$ Zeocin—around 2-fold increase in DSB levels. Similar DSB levels were measured after the treatment with Zeocin in a concentrations' range of 100–400 $\mu\text{g ml}^{-1}$ suggesting the existence of a plateau region.

Two-way ANOVA reveals that FDR is statistically significantly influenced by the factors growth phase (27.65% of total variation; $F = 133.9$; $df = 1$; $P < 0.0001$), and Zeocin concentration (38.21% of total variation; $F = 36.99$; $df = 5$;

$P < 0.0001$). Lower but statistically significant influence is calculated for the interaction between factors (11.82% of total variation; $F = 11.44$; $df = 5$; $P < 0.0001$).

In our further experiments, only cell cultures in the exponential and the beginning of stationary phase were used. The effect of exposure time was evaluated for both growth phases. Results presented on Fig. 4 clearly show differences depending on the growth phase.

Any of the exposure times with 100 $\mu\text{g ml}^{-1}$ Zeocin did not result in the induction of statistically significant DSB levels in cells in exponential phase of growth (Fig. 4A). A statistically reliable increase of DSB levels around 2-fold was measured only for cells at the beginning of the stationary phase (Fig. 4B). One-way ANOVA analysis for comparison of the effect of exposure time in stationary phase cells revealed the lack of statistically significant difference among the exposure times.

Two-way ANOVA analysis revealed that the fraction of DSBs induced after different Zeocin exposure of cells in both growth phases is statistically significantly affected mostly by the factor growth phase (36.04% of total variation; $F = 120.0$; $df = 1$; $P < 0.0001$). The factor treatment time (20.94% of total variation; $F = 17.44$; $df = 4$; $P < 0.0001$) as well as the interaction between both factors (10.00% of total variation; $F = 8.332$; $df = 4$; $P < 0.0001$) also influenced the fraction of DSBs induced.

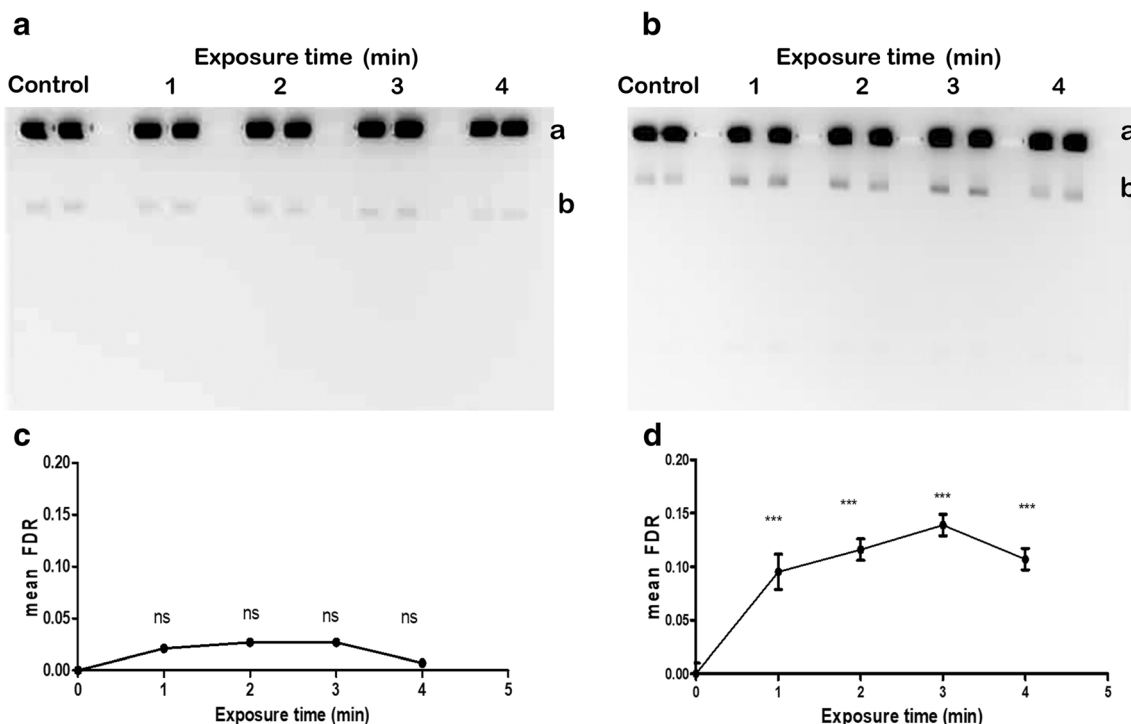


Fig. 4 Effect of exposure time on the induction of DSBs in *Saccharomyces cerevisiae* cells at the exponential phase (**A**) and the beginning of stationary phase (**B**) after Zeocin treatment with $100 \mu\text{g ml}^{-1}$; (a) DNA in wells; (b) fraction of DNA released. **C** Induction of DSBs calculated as mean FDR in cells at exponential growth phase. **D** Induction of DSBs calculated as mean FDR in cells at the

beginning of stationary growth phase. The significance in differences between tested concentrations and the negative control was calculated by one-way analysis of variances (ANOVA) with Dunnett's multiple comparisons test. Asterisks provide information about the significance in the differences where ^{ns} $P > 0.05$, ^{***} $P < 0.001$. Where no error bars are evident, they are equal or less than the symbols

Cell survival depending on the concentration

Simultaneously with the CFGE experiments, cell suspension at the beginning of stationary phase was subjected to survival assay.

Survival data, calculated depending on the applied concentration, have allowed us to determine three levels of lethality as follows: $\text{LD}_{20} = 7 \mu\text{g ml}^{-1}$, $\text{LD}_{37} = 15 \mu\text{g ml}^{-1}$, and $\text{LD}_{50} = 67 \mu\text{g ml}^{-1}$ Zeo.

No correlation was found between survival fraction and concentrations ($P > 0.05$) lower than LD_{20} ($2\text{--}6 \mu\text{g ml}^{-1}$).

Dose-dependent ($P < 0.0001$) decrease in cell survival is observed after Zeocin treatment with a concentration in the range of lethality $\text{LD}_{20}\text{--}\text{LD}_{50}$ (Fig. 5).

Around 2-fold decrease in cell survival was obtained after the treatment with any of the concentrations in the range of $100\text{--}300 \mu\text{g ml}^{-1}$ Zeocin confirming our DSB data for the existence of a plateau region (Fig. 5).

In order to clarify the potential relationship between cell survival and the levels of DSBs induced after the treatment with Zeocin, a linear correlation analysis has been performed. The correlation coefficient (r) was found to be -0.9593 denoting a strong negative correlation between the events (Fig. 6). The increase of the DSB levels correlates with the decrease of the cell survival.

Discussion

More than 30 years ago, it was shown by Moore (1982) that the lethal effect of Zeocin in *Saccharomyces cerevisiae* depends on the genotype, the growth phase, and the presence

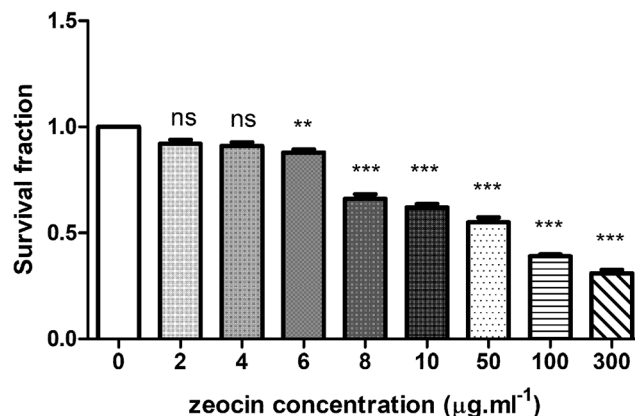


Fig. 5 Effect of Zeocin concentrations in the range of $2\text{--}300 \mu\text{g ml}^{-1}$ on cell survival of *Saccharomyces cerevisiae* strain D7ts1. Statistical significance between the effect of different Zeocin concentrations and the negative control was calculated by one-way analysis of variances (ANOVA) with Bonferroni's multiple comparisons test. Asterisks provide information for the significance in the differences where ^{**} $P < 0.01$; ^{***} $P < 0.001$. Where no error bars are evident, they are equal or less than the symbols

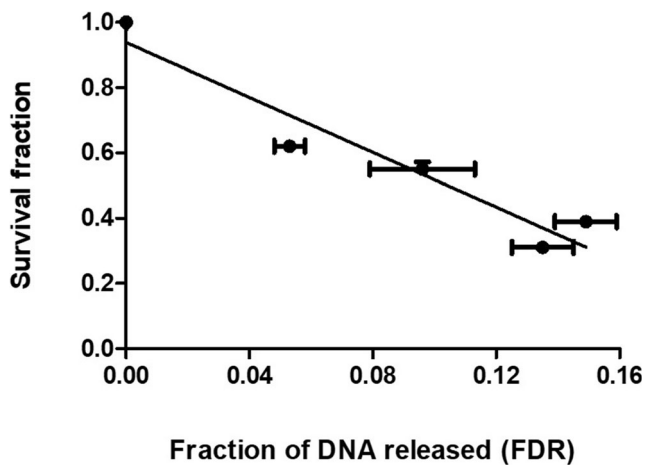


Fig. 6 Correlation between survival fraction and fraction of DNA released in strain D7ts1. Very strong negative linear correlation has been obtained between the FDR and SF ($r = -0.9593$; $R^2 = 0.9202$, $P = 0.0098$). Correlation coefficient (r) higher than -0.900 denote a strong negative correlation

of metal ions. In the present study, additional information is provided concerning the induction of DSBs by Zeocin. CFGE is used for the first time on *Saccharomyces cerevisiae* for DSB measurement. In the present work, *Saccharomyces cerevisiae* strain D7ts1 was used. This strain has inserted *ts1* mutation. Previous studies revealed that this temperature-sensitive mutation causes an increased non-specific cellular permeability of *S. cerevisiae* to different substances, including mutagens and carcinogens in comparison with the parental and other wild-type strains (Staleva et al. 1996). It is already known that the *TS1* gene is the previously isolated *SEC53* gene. The essential *SEC53* gene encodes phosphomannomutase, required for the early step in the pathway of O- and N-linked mannosylation (Stoycheva et al. 2012; Kepes and Schekman 1988).

Since the permeability barrier of *S. cerevisiae* is determined by the most superficial layer of the cell wall composed of highly glycosylated mannoproteins (Zlotnik et al. 1989), the impairment of protein glycosylation in *sec53* mutant cells results in the formation of a permeability barrier with insufficient functions (Stoycheva et al. 2012).

Data exist that bleomycin damages yeast cell wall by causing mannoprotein release (Lim et al. 1995). Based on this, it could be proposed that the incubation with zymolyase is not necessary for strains with a *ts1* mutation such as D7ts1. Our pilot experiments by using zymolyase confirmed our assumption (data not shown). Preliminary treatment with zymolyase has entailed a marked fragmentation of DNA even in the control cells, without Zeocin treatment. This allows the application of CFGE without additional steps for protoplast formation. This is important in order to keep the main benefit of the methodology—detection of DSBs in whole cells.

Comparing the levels of DSBs induced by Zeocin, well-known inducer of DSBs; methyl methanesulfonate as

alkylating agent; and 5-bromouracil as base analog, evidence was provided for the validation of the CFGE protocol for *S. cerevisiae* described here.

Our data clearly indicated that the DSB levels, measured after Zeocin treatment, are more than 1.5-fold higher than the spontaneous ones.

MMS treatment resulted in a negligible amount of DSB compared with DSBs in the control, even though, in another study using PFGE (Lundin et al. 2005), it was concluded that MMS itself is not able to induce DSB. Interestingly, slight DSB levels were previously measured by us in the haploid strain 551 after MMS treatment (Todorova et al. 2015b). MMS is a well-known ROS inducer (Dimitrov et al. 2013). Thus, the possible role of oxidative stress should not be excluded as a reason for DSB induction after the treatment with MMS.

As expected, treatment with 5-bromouracil did not result in any increase in DSB levels. This compound was chosen by us due to its mode of action. 5-Bromouracil (BrU) is a base analog of thymine (T). It is a well-known mutagen, causing transition mutations by mispairing with guanine (G) rather than pairing with adenine (A) during replication (Holroyd and van Mourik 2015). From our state of knowledge, there is no data suggesting potential induction of DSBs by this agent.

Further experiments were focused on the evaluation of the role of growth phase for DSB induction in *Saccharomyces cerevisiae*.

Data obtained in the present study revealed a dose-dependent decrease in survival fraction and an increase in DSB levels after Zeocin treatment with concentrations equal to or higher than $7 \mu\text{g ml}^{-1}$ —LD₂₀. Such observation was evaluated on cells at the beginning of the stationary phase. Interestingly, Zeocin concentrations in the range of 100–300 $\mu\text{g ml}^{-1}$ have led to a similar percentage of cell survival and DSB induction suggesting the formation of a plateau region. Such results are obtained previously by us on *Saccharomyces cerevisiae* haploid strain 551 (Todorova et al. 2015b). These results are in accordance with the previously reported data on *Chlamydomonas reinhardtii* where plateau region is observed after the treatment with Zeocin concentrations in the same range (Chankova et al. 2007). A possible explanation could be the one proposed earlier by Moore et al. (2000) and Zaka and Chenal (2002). The authors suggest that such plateau could be formed in response to reaching a certain threshold of damage that could be a signal for activation of repair steps. Nevertheless, in our experiments, Zeocin treatment was carried out on ice (conditions preventing DNA repair) as it was proposed by us earlier some DNA repair cannot be ignored (Chankova et al. 2007).

Concerning the DNA susceptibility depending on the growth phase, a significant difference is obtained between the *Saccharomyces cerevisiae* cell response towards Zeocin

treatment. Cells at the beginning of the stationary phase are found to be more sensitive to Zeocin treatment than those in the exponential phase of growth. Our result is in accordance with those reported by Hafer et al. (2009); Moore et al. (1985); Moore (1982); Tippins and Parry (1982); Langguth and Beam (1973); Raju et al. (1972); and Beam et al. (1954). The authors demonstrated that budding yeast cells are more resistant to radiation and some radiomimetics than nonbudding cells. It was speculated by Moore et al. (1989) that the increased cleavage of internucleosomal DNA and DNA breakage in stationary-phase cells than in exponentially growing cells are most likely due to increased accessibility of linker DNA to bleomycin.

Data concerning the response of *S. cerevisiae* cells in the different growth phase to Zeocin depending on the exposure time again show that the cells at the beginning of stationary phase are more sensitive than those in the exponential phase of growth. Our results clearly indicated that not the exposure time but the growth phase plays a major role in the levels of DSBs induced by Zeocin. For cells at the beginning of stationary phase Zeocin is able to induce around 2-fold higher levels of DSBs despite the exposure time. These results are in accordance with our previously reported on *Chlamydomonas reinhardtii* (Chankova et al. 2007). In that study, the exposure time used was in the range 1–20 min. It was found that even the longest treatment time used (20 min) led to similar levels of DSBs as those measured after 1 min treatment with Zeocin. Another study reported that low doses of bleomycin (1–5 $\mu\text{g ml}^{-1}$) and short treatments (5–15 min) produced marked DNA cleavage in mammalian cells (López-Larrazá et al. 1990). Moore (1982) reported that treatment with low doses of phleomycin in the range 1 to 5 $\mu\text{g ml}^{-1}$ for 20 min significantly reduce the cell survival.

Any of the exposure times with 100 $\mu\text{g ml}^{-1}$ Zeocin did not result in the induction of statistically significant DSB levels in cells in exponential phase of growth (Fig. 4A). A statistically reliable increase of DSB levels was measured only for cells at the beginning of the stationary phase (Fig. 4B). One-way ANOVA analysis for comparison of the effect of exposure time in stationary phase cells revealed the lack of statistically significant difference among the exposure times.

Additionally, different response to Zeocin treatment between cells in exponential and stationary phase could be explained with differences in P450 induction because it is known that the maximum cellular expression of P450 complex (endogenous metabolic activation) is observed mostly in cells in exponential phase of growth, while P450 activity could not be detected in cells in stationary phase (Pellacani et al. 2006; Poli et al. 2002; Rossi et al. 1995; Poli et al. 1992).

Saccharomyces cerevisiae in non-proliferating stationary phase, as well as bacteria and neuronal cells, can persist in this state for years (Kumar and Srivastava 2016; Lewis 2000; Werner-Washburne et al. 1993). Studies based on such

cells play a significant role in biomedical, environmental research, and agriculture, which affects the whole human race and may provide better treatment strategies (Kumar and Srivastava 2016).

Conclusions

Here, for the first time from our point of knowledge, new information is provided that the DNA susceptibility of *Saccharomyces cerevisiae* towards Zeocin, measured as DSBs, depends on the growth phase. Cells at the beginning of stationary phase are found to be more DNA susceptible to Zeocin treatment than those in the exponential phase of growth. It could be suggested that the growth phase together with the experimental design should always be taken into consideration when performing toxicological studies.

This study also provides evidence concerning the successful application of an optimized protocol for constant-field gel electrophoresis (CFGE) on *Saccharomyces cerevisiae* whole cells with increased cell wall permeability without additional steps for protoplast formation. The small amounts of DSBs measured after the treatment with low doses of Zeocin provide evidence for the sensitivity of the protocol towards DSBs in *Saccharomyces cerevisiae*.

Funding information This work was supported by a grant from the National Science Fund, Ministry of Education and Science, Project No. DH11/10.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- 5-bromouracil (2018) <https://pubchem.ncbi.nlm.nih.gov/compound/5-Bromouracil#section=Top>. Accessed 19 Dec 2018
- Beam CA, Mortimer RK, Wolfe RG, Tobias CA (1954) The relation of radioresistance to budding in *Saccharomyces cerevisiae*. Arch Biochem Biophys 49:110–122
- Bryant PE (1968) Survival after fractionated doses of radiation: modification by anoxia of the response of *Chlamydomonas*. Nature 219: 75–77. <https://doi.org/10.1038/219075b0>
- Chankova SG, Bryant PE (2002) Acceleration of DNA-double strand rejoining during the adaptive response of *Chlamydomonas reinhardtii*. Radiat Biol Radioecol 42(6):600–603
- Chankova SG, Matos JA, Simoes F, Bryant PE (2005) The adaptive response of a new radio-resistant strain of *Chlamydomonas reinhardtii* correlates with increased DNA double-strand break rejoining. Int J Radiat Biol 81(7):509–514

- Chankova SG, Dimova E, Dimitrova M, Bryant PE (2007) Induction of DNA double-strand breaks by Zeocin in *Chlamydomonas reinhardtii* and the role of increased DNA double-strand breaks rejoining in the formation of an adaptive response. *Radiat Environ Biophys* 46:409–416
- Chankova S, Todorova T, Parvanova P, Miteva D, Mitrovska Z, Angelova O, Imreova P, Mucaji P (2013) Kaempferol and jatropham: are they protective or detrimental for *Chlamydomonas reinhardtii*? *C R Acad Bulg Sci* 66:1121–1128
- Dimitrov MD, Pesheva MG, Venkov PV (2013) New cell-based assay indicates dependence of antioxidant biological the origin of reactive oxygen species. *J Agric Food Chem* 61:4344–4351
- Dimova E, Dimitrova M, Miteva D, Mitrovska Z, Bryant PE, Chankova S (2009) Does single-dose cell resistance to the radiomimetic Zeocin correlate with a Zeocin-induced adaptive response in *Chlamydomonas reinhardtii* strains? *Radiat Environ Biophys* 48:77–84
- Frassinetti S, Barberio C, Caltavuturo L, Fava F, Di Gioia D (2011) Genotoxicity of 4-nonylphenol and nonylphenol ethoxylate mixtures by the use of *Saccharomyces cerevisiae* D7 mutation assay and use of this text to evaluate the efficiency of biodegradation treatments. *Ecotoxicol Environ Saf* 74(3):253–258
- Freeman KM, Hoffmann GR (2007) Frequencies of mutagen-induced coincident mitotic recombination at unlinked loci in *Saccharomyces cerevisiae*. *Mutat Res Fundam Mol Mech Mutagen* 616(1):119–132
- Galao RP, Scheller N, Alves-Rodrigues I, Breinig T, Meyerhans A, Díez J (2007) *Saccharomyces cerevisiae*: a versatile eukaryotic system in virology. *Microb Cell Factories* 6:32
- Gateva S, Angelova O, Chankova S (2015) Double-strand breaks detection in human lymphocytes by constant field gel electrophoresis. *ДОКЛАДИ НА БЪЛГАРСКАТА АКАДЕМИЯ НА НАУКИТЕ* 68(4):469–474
- Gray JV, Petsko GA, Johnston GC, Ringe D, Singer RA, Werner-Washburne M (2004) Sleeping beauty: quiescence in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* 68(2):187–206
- Hafer K, Rivina L, Schiestl RH (2009) Cell cycle dependence of ionizing radiation-induced DNA deletions and antioxidant radioprotection in *Saccharomyces cerevisiae*. *Radiat Res* 173(6):802–808
- Holroyd LF, van Mourik T (2015) Stacking of the mutagenic base analogue 5-bromouracil: energy landscapes of pyrimidine dimers in gas phase and water. *Phys Chem Chem Phys* 17(45):30364–30370
- Ikner A, Shiozaki K (2005) Yeast signaling pathways in the oxidative stress response. *Mutat Res Fundam Mol Mech Mutagen* 569(1–2):13–27
- Kepes F, Schekman R (1988) The yeast SEC53 gene encodes phosphomannomutase. *J Biol Chem* 263(19):9155–9161
- Kopaskova M, Hadjo L, Yankulova B, Jovtchev G, Galova E, Sevcovicova A, Mucaji P, Miadokova E, Bryant P, Chankova S (2011) Extract of *Lillium candidum* L. can modulate the genotoxicity of the antibiotic Zeocin. *Molecules* 17:80–97
- Kumar R, Srivastava S (2016) Quantitative proteomic comparison of stationary/G0 phase cells and tetrads in budding yeast. *Sci Rep* 6:32031
- Langguth EN, Beam CA (1973) Repair mechanisms and cell cycle dependent variations in X-ray sensitivity of diploid yeast. *Radiat Res* 53:226–234
- Lee Y, Kim K, Kang KT, Lee JS, Yang SS, Chung WH (2014) Atmospheric-pressure plasma jet induces DNA double-strand breaks that require a Rad51-mediated homologous recombination for repair in *Saccharomyces cerevisiae*. *Arch Biochem Biophys* 560:1–9
- Lewis K (2000) Programmed death in bacteria. *Microbiol Mol Biol Rev* 64:503–533
- Lim ST, Jue CK, Moore CW, Lipke PN (1995) Oxidative cell wall damage mediated by bleomycin-Fe (II) in *Saccharomyces cerevisiae*. *J Bacteriol* 177(12):3534–3539
- López-Larrazza D, De Luca JC, Bianchi NO (1990) The kinetics of DNA damage by bleomycin in mammalian cells. *Mutat Res Fundam Mol Mech Mutagen* 232(1):57–61
- Lundin C, North M, Erixon K, Walters K, Jenssen D, Goldman AS, Helleday T (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. *Nucleic Acids Res* 33(12):3799–3811
- Matuo R, Sousa FG, Soares DG, Bonatto D, Saffi J, Escargueil AE, Larsen AK, Henriques JAP (2012) *Saccharomyces cerevisiae* as a model system to study the response to anticancer agents. *Cancer Chemother Pharmacol* 70(4):491–502
- Methyl methanesulfonate (2018) https://pubchem.ncbi.nlm.nih.gov/compound/methyl_methanesulfonate#section=Top. Accessed 19 Dec 2018
- Moore CW (1982) Modulation of *in vivo* bleomycin cytotoxicity. *Antimicrob Agents Chemother* 21:595–600
- Moore CW, Malcolm AW, Tomkinson KN, Little JB (1985) Ultrarapid recovery from lethal effects of bleomycin and γ -radiation in stationary-phase human diploid fibroblasts. *Cancer Res* 45(5):1978–1981
- Moore CW, Jones CS, Wall LA (1989) Growth phase dependency of chromatin cleavage and degradation by bleomycin. *Antimicrob Agents Chemother* 33(9):1592–1599
- Moore CW, McKoy J, Dardalhon M, Davermann D, Martinez M, Averbek D (2000) DNA damage-inducible and RAD52-independent repair of DNA double-strand breaks in *Saccharomyces cerevisiae*. *Genetics* 154:1085–1099
- Pellacani C, Buschini A, Furlini M, Poli P, Rossi C (2006) A battery of *in vivo* and *in vitro* tests useful for genotoxic pollutant detection in surface waters. *Aquat Toxicol* 77(1):1–10
- Pesheva M, Krastanova O, Staleva L, Dentcheva V, Hadzhitorov M, Venkov P (2005) The Ty1 transposition assay: a new short-term test for detection of carcinogens. *J Microbiol Methods* 61(1):1–8
- Petin VG, Kapultcevic YG (2014) Radiation quality and the shape of dose-effect curves at low doses of ionizing radiation for eukaryotic cells. *Math Biosci* 252:1–6
- Poli P, Buschini A, Campanini N, Vettori MV, Cassoni F, Cattani S, Rossi C (1992) Urban air pollution: use of different mutagenicity assays to evaluate environmental genetic hazard. *Mutat Res* 298:113–123
- Poli P, de Mello MA, Buschini A, Mortara RA, de Albuquerque CN, da Silva S, Rossi C, Zucchi TMAD (2002) Cytotoxic and genotoxic effects of megazol, an anti-Chagas' disease drug, assessed by different short-term tests. *Biochem Pharmacol* 64(11):1617–1627
- Raju MR, Gnanapurani M, Stackler B, Madhvanath U, Howard J, Lyman JT, Manney TR, Tobias CA (1972) Influence of linear energy transfer on the radioresistance of budding haploid yeast cells. *Radiat Res* 51:310–317
- Rao KS (2007) Mechanisms of disease: DNA repair defects and neurological disease. *Nat Clin Pract Neurol* 3:162–172
- Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 27(3):493–497
- Rossi C, Poli P, Buschini A, Cassoni F, Cattani S, de Munari E (1995) Comparative investigations among meteorological conditions, air chemical-physical pollutants and airborne particulate mutagenicity: a long-term study (1990–1994) from a northern Italian town. *Chemosphere* 30:1829–1845
- Sousa-Lopes A, Antunes F, Cyrme L, Marinho HS (2004) Decreased cellular permeability to H₂O₂ protects *Saccharomyces cerevisiae* cells in stationary phase against oxidative stress. *FEBS Lett* 578(1):152–156
- Staleva L, Waltscheva L, Golovinsky E, Venkov P (1996) Enhanced cell permeability increases the sensitivity of a yeast test for mutagens. *Mutat Res* 370:81–89

- Stoycheva T, Pesheva M, Dimitrov M, Venkov P (2012) The Ty1 retrotransposition short-term test for selective detection of carcinogenic genotoxins. In: Pesheva M (Ed) Carcinogen, Intechopen, pp 83–110
- Temple MD, Perrone GG, Dawes IW (2005) Complex cellular responses to reactive oxygen species. Trends Cell Biol 15(6):319–326
- Tippins RS, Parry JM (1982) A comparison of the radiosensitivity of stationary, exponential, and G1 phase wild type and repair deficient yeast cultures: supporting evidence for stationary phase yeast cells being in G0. Int J Radiat Biol 41:215–220
- Todorova T, Pesheva M, Gregan F, Chankova S (2015a) Antioxidant, antimutagenic and anticarcinogenic effects of *Papaver rhoeas* L. extract on *Saccharomyces cerevisiae*. J Med Food 18(4):460–467
- Todorova T, Miteva D, Chankova S (2015b) DNA damaging effect of Zeocin and methyl methanesulfonate in *Saccharomyces cerevisiae* measured by CFGE. ДОКЛАДИ НА БЪЛГАРСКАТА АКАДЕМИЯ НА НАУКИТЕ 68(1):71–78
- Venkov P, Scheit KH (1984) Effect of seminal plasmin on rRNA synthesis in *Saccharomyces cerevisiae*. FEBS Lett 172(1):21–24
- Werner-Washburne M, Braun E, Johnston GC, Singer RA (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. Microbiol Rev 57:383–401
- Wilson MR (2014) Analysis of genes required for quiescent cell formation in stationary phase cultures of *Saccharomyces cerevisiae*. Dissertation, University of New Mexico
- Zaka R, Chenal C (2002) Misset MT study of external low irradiation dose effects on induction of chromosome aberrations in *Pisum sativum* root tip meristem. Mut Res 517:87–99
- Zeocin (2018) <https://www.thermofisher.com/bg/en/home/references/protocols/cloning/transformation-protocol/zeocin.html>. Accessed 19 Dec 2018
- Zimmermann FK, Kern R, Rasenberger H (1975) A yeast strain for simultaneous detection of induced mitotic crossing over, mitotic gene conversion and reverse mutation. Mutat Res Fundam Mol Mech Mutagen 28(3):381–388
- Zlotnik KH, Femande MP, Bowers B, Cabib E (1989) Mannoproteins form an external cell wall layer determines wall porosity in *Saccharomyces cerevisiae*. J Bacteriol 195:1018–1026