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An improved semi-quantitative spot assay to analyse chronological lifespan in yeast

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Abstract

The ability of yeast cells to survive during the stationary phase of growth is a highly desired feature for industrial yeast bioprocesses, and for aging research. Chronological lifespan (CLS) is the duration of viability during stationary phase. For CLS estimation; the conventional, quantitative CLS method based on viable cell counting is used which is labor-intensive for testing a high number of strains. Thus, a practical screening method is required to identify long-lived strains among a great number of strains. It was aimed, first, to improve a semi-quantitative CLS method to allow an efficient, comparative and reliable CLS estimation of strains with significantly different cell densities during the stationary phase of growth. The second aim was to determine the CLS of two stress-resistant Saccharomyces cerevisiae strains. An initial cell density adjustment step was introduced to a semi-quantitative CLS method based on yeast spot assay. This improved method was verified by the quantitative CLS method. The improved semi-quantitative method can be used as a reliable screening method for longevity studies with high sample size. Additionally, oxidative stress-resistant and ethanol-tolerant S. cerevisiae strains have prolonged CLS, suggesting their use in industrial applications and aging research.

Keywords: aging, ethanol tolerance, oxidative stress, *Saccharomyces cerevisiae*, semi-quantitative CLS method.

1. Introduction

The yeast *Saccharomyces cerevisiae* has been commonly used in baking and fermentation industries. As a unicellular eukaryotic model organism; it has also been commonly used in genetic and molecular biological research to study complex eukaryotic cellular processes. Examples of such processes are cell cycle regulation (L.H. HARTWELL [1]), intracellular transport (A. NAKANO [2]), regulation of protein folding (S.L. LINDQUIST & al. [3]) and aging (V.D. LONGO & al. [4]). There are many advantages of using *S. cerevisiae* both as a eukaryotic model organism and as an industrial microorganism. These are the inexpensive and rapid growth of *S. cerevisiae*, its GRAS (**G**enerally **R**ecognized **a**s **S**afe) status, availability of molecular tools to perform gene deletion, gene disruption and replacement in *S. cerevisiae*, and the availability of its whole genome sequence data (F. SHERMAN [5], D. BOTSTEIN & al. [6], A. GOFFEAU [7]).

Chronological lifespan (CLS) of a yeast cell has been defined as the length of time where non-dividing yeast cell survives (V.D. LONGO & al. [4]). As CLS involves viability of non-dividing yeast cells in their stationary phase of growth, it is an important parameter for both industrial yeast bioprocesses and aging research. The reason for this is the preference of robust strains with high viability for increased efficiency and productivity, and the use of

yeast as a eukaryotic model organism to study the aging processes of higher eukaryotes (V.D. LONGO & al. [4]; H. OROZCO & al. [8]; M. SAUER & D. MATTANOVICH [9]).

Spot assay is a commonly used method in yeast research to analyze growth phenotypes, viability, stress- or drug resistance and genotoxicity (K. MUKHOPADHYAY & al. [10]; M.A. ABEGG & al. [11]; C. ALKIM & al. [12]; G. KUCUKGOZE & al. [13]; S. LAHIRI & al. [14]; R. STEINACHER & al. [15]). The method involves serial dilution and spotting of yeast cultures on solid media plates and is easy to apply. It allows comparative semi-quantitative analysis of growth physiology/viability of a strain under different cultivation conditions, and also of different strains under a particular cultivation condition. Despite a previous report on the advantages of the semi-quantitative spot assay for yeast CLS analysis (D.L. SMITH & al. [16]), to our knowledge, the method has not been commonly used for yeast CLS studies. Instead, the conventional, quantitative CLS method based on viable cell counting on serially diluted plates is still often used which can be labor-intensive and difficult for testing a high number of strains due to the requirement of a vast number of plates and flasks for incubation. In the present study, we have improved the previously described semi-quantitative spot assay-based CLS method (D.L. SMITH & al. [16]) by introducing an initial cell density adjustment step. The aim was to allow CLS-comparison of cultures or strains with different growth rates or significantly different cell densities during their stationary phase of growth, such as stress-resistant *S. cerevisiae* mutants and their reference strain. The accuracy of our results obtained with the improved semi-quantitative spot assay-based CLS method was verified by applying the conventional quantitative CLS method. The results suggested that the improved semi-quantitative spot assay-based CLS method can be implemented as an efficient and reliable screening method to identify long-lived cultures or strains among a high number of cultures/strains, before their more elaborate analysis by the quantitative CLS method. The effects of oxidative stress resistance and ethanol tolerance on CLS and longevity of *S. cerevisiae* were investigated in this study by using both methods.

2. Materials and Methods

2.1. Yeast strains, media, and cultivation

The *S. cerevisiae* strains used in this study are shown in Table 1. Ethanol-tolerant strain B2 and oxidative stress-resistant strain H7 were derived from the reference strain CEN.PK 113-7D (J.P. VAN DIJKEN & al. [17]) by repetitive batch selection in the presence of ethanol and hydrogen peroxide, respectively (B. YILMAZ [18]; B. TURANLI-YILDIZ [19]; B. TURANLI-YILDIZ & al. [20]).

Yeast minimal medium **'**YMM' (2% (w/v) glucose (Riedel-de Haën, Germany), 0.67% (w/v) yeast nitrogen base without amino acids (Difco, BD, USA)**)** and yeast peptone dextrose medium 'YPD' (2% (w/v) peptone (Merck, Germany), 1% (w/v) yeast extract (Merck, Germany) 2% (w/v) glucose (Riedel-de Haën, Germany) and 2% (w/v) agar (Neogen, USA) for solid medium only) were used as culture media**.**

Yeast strains were stored in 30% (v/v) glycerol at -80°C (Sanyo, Japan). Frozen stock cultures (-80ºC) were revived in 10 ml of YPD medium in 50 ml culture tubes by overnight cultivation in an orbital shaker (Sartorius, Certomat SII, Germany) at 30ºC and 150 rpm. For preculture preparation, overnight cultures grown in YPD medium were washed twice with YMM, inoculated into 10 ml fresh YMM in 50 ml culture tubes to an initial OD_{600} of 0.1, and incubated overnight at 30ºC and 150 rpm, unless otherwise stated.

Table 1. Yeast strains used in this study.

Name	Description	Reference
Ref	*S. cerevisiae* prototrophic reference strain CEN.PK 113-7D (*MATa,MAL2-8^c, SUC2*)	(J.P. VAN DIJKEN & al. [17])
H7	Oxidative stress-resistant *S.cerevisiae* (derived from Ref, resistant to 2 mM continuously applied hydrogen peroxide as the oxidative stress agent in liquid YMM culture)	(B. YILMAZ [18])
B2	Ethanol-tolerant *S.cerevisiae* (derived from Ref, tolerant up to 12% (v/v) continuously applied ethanol stress in liquid Yeast Minimal Medium (YMM) culture)	(B. TURANLI-YILDIZ [19]; B. TURANLI-YILDIZ & al. [20])

2.2. Growth physiological analysis

For preculture preparation, overnight cultures in YMM were transferred to 20 ml fresh YMM in 100 ml culture flasks to an initial OD_{600} of 0.1 (≈ 2×10^6cells/ml) and incubated overnight in an orbital shaker at 30ºC and 150 rpm. On the next day, overnight precultures of the mutants and the reference strain were transferred into 100 ml fresh YMM in 500 ml flasks to an initial OD_{600} of 0.1. Cultures were then incubated at 30ºC and 150 rpm. Samples were withdrawn from the cultures, diluted with fresh YMM when necessary, and their optical densities (OD_{600}) were measured using a spectrophotometer (Shimadzu UV-1700, Japan). The experiment was performed in triplicate.

2.3. CLS assays

2.3.1. Modified semi-quantitative CLS assay

Semi-quantitative CLS assay was carried out in the expired medium by modifying a previously described method (D.L. SMITH & al. [16]). Briefly, overnight precultures were transferred into 20 ml fresh YMM in 100 ml flasks to an initial OD_{600} of 0.1 and cultivated at 30°C and 150 rpm. OD_{600} of the cultures were measured on the third day (stationary phase of cell growth) of incubation. The third day was considered as ‘day 0’ of the CLS experiment. Samples were withdrawn from cultures, and their OD_{600} values (on stationary phase) were adjusted to 6 (≈ 1.2×10^8cells/ml). Ten-fold serial dilutions (from 10^{-1} to 10^{-4}) of these cultures were prepared in sterile distilled water, and five µL each of the cultures at OD_{600}=6 and their dilutions (from 10^{-1} to 10^{-4}) were spotted onto YPD plates. The plates were then incubated at 30ºC for 72 h and photographed. During their incubation, the same procedure was repeated every second day (sampling, adjustment of OD_{600} to 6, serial dilutions and spotting onto YPD plates) until the end of the CLS experiment (days 2-10). The plate images belonging to days 0-10 of the CLS analysis were joined in a single image. A schematic description of the modified semi-quantitative CLS method is shown in Fig. 1.

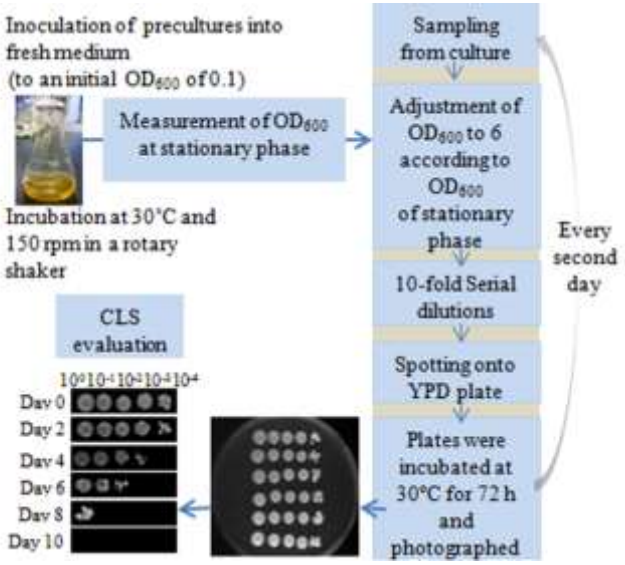

Figure 1. The improved semi-quantitative spot CLS assay (modified from (D.L. SMITH & al. [16])).

2.3.2. Quantitative CLS assay

To verify the modified semi-quantitative CLS method and to quantitatively determine the CLS of the mutants, the quantitative CLS method was performed in the expired medium as described previously (V.D. LONGO & al. [4]; P. FABRIZIO & V.D LONGO [21]). Briefly, overnight precultures grown in YMM at 30ºC and 150 rpm were transferred into 100 mL fresh YMM in 500 mL flasks to an initial OD_{600} of 0.1 and were incubated for three days until the stationary phase of growth. Day 3 of the cultivation was considered as 'day 0' of the CLS experiment, and the initial viability of the culture was determined by viable cell counting method. For this purpose, 100 µl samples were withdrawn from cultures and diluted by 10-fold with sterile distilled water, spread onto YPD plates and incubated at 30°C for 72 h. Every second day; viability of the cultures was determined by viable cell counting method, by considering their initial viability as 100%. This procedure was continued until the viabilities decreased to 0.1%. The experiment was performed in triplicate. A schematic description of the quantitative CLS method is shown in Fig. 2.

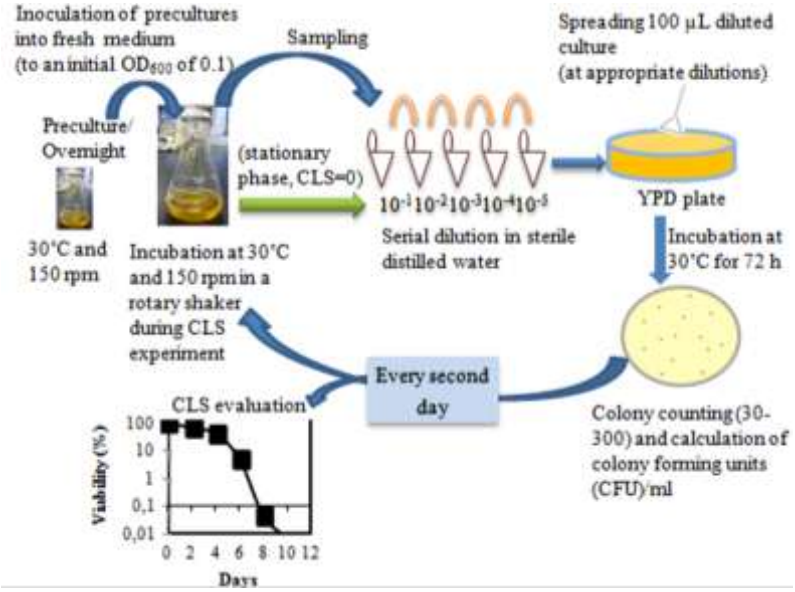

Figure 2. The conventional quantitative CLS method (V.D. LONGO & al. [4]; P. FABRIZIO & V.D LONGO [21]), based on viable cell counting on Yeast Peptone Dextrose (YPD) plates.

3. Results and Discussion

3.1. Growth behavior of the reference strain and the mutant *S. cerevisiae* strains

The CLS assay begins at the beginning of the stationary phase of growth (P. FABRIZIO & V.D LONGO [21]). Therefore, a growth physiology experiment was conducted to analyze the growth behavior of the reference strain, and the mutants, B2 and H7 (Fig. 3), before the CLS experiments. The logarithmic phase of growth was observed until almost the end of the first day of incubation for all strains. A dramatic decline in the rate of cell growth was observed after the first day of incubation, indicating a second growth phase at a lower growth rate. Yeast cells have a respiro-fermentative metabolism in their logarithmic growth phase, and as a result of fermentative metabolism, two-carbon (2C) metabolites such as ethanol and glycerol are produced during the logarithmic phase (M. WERNER-WASHBURNE & al. [22]). When glucose is exhausted in culture, yeast cells then utilize these metabolites during a second growth phase called as ‘diauxic growth’ (M. WERNER-WASHBURNE & al. [22]). For all strains, diauxic growth was observed during the second and third days of incubation, such that cell growth slowly continued until the end of the third day of incubation (Fig. 3).

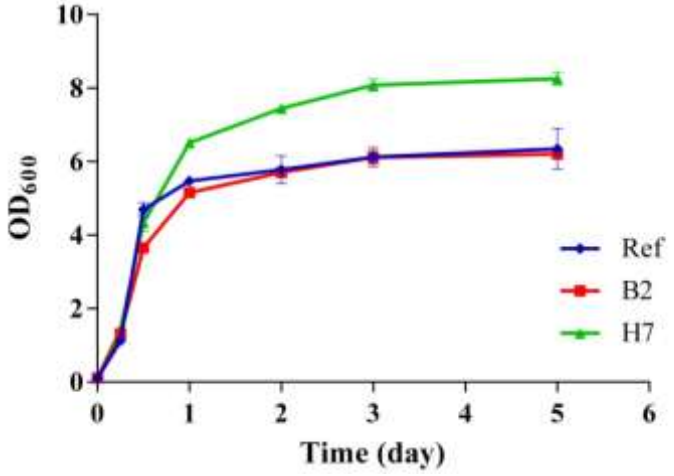

Figure 3. Growth behavior of the reference strain (Ref) and the mutant *S.cerevisiae* strains B2 and H7 grown in shake flasks in Yeast Minimal Medium at 30°C and 150 rpm.

Optical densities of all cultures almost remained constant after the third day of incubation, indicating that all strains reached the stationary phase of growth (Fig. 3). Fabrizio and Longo (2003) also selected the third day of incubation as the initiation time for CLS experiments, since the culture densities of their wild-type strains; DBY746 and SP1, did not increase after day 3 (P. FABRIZIO & V.D LONGO [21]). Although we used a different reference strain in this study, our results were consistent with those obtained in that study. Therefore, day 3 of the cultivation was chosen as the initiation time for CLS experiments in this study. Growth physiological analysis also revealed that the mutant strain H7 reached significantly higher OD_{600} values than both the reference strain and B2 mutant. This difference in OD_{600} values was considered as a critical factor for the improvement of the semi-quantitative CLS assay, as described in Section 2.3.1.

3.2. Modified semi-quantitative CLS analysis of the mutants and the reference strain

Spot assay is a useful, cost-effective and semi-quantitative method. It has been commonly used in yeast research to test the sensitivity of yeast to different media or stress conditions or to compare the growth rates of a yeast strain under different growth conditions (C. ALKIM & al. [12]; G. KUCUKGOZE & al. [13]). It is a beneficial method especially for rapidly screening a high number of conditions and samples. However, to do a comparative CLS analysis of different yeast strains with different growth rates and different OD_{600} values at the stationary phase of growth, the method needs to be improved. Thus, we modified the semi-quantitative CLS analysis method based on spot assay by introducing an OD_{600} adjustment step to the protocol at the beginning of the CLS assay. Table 2 shows the OD_{600} values of the reference strain and the mutant strains at their stationary phase of growth (day 3 of cultivation) before OD_{600} adjustment and CLS analyses. Sampling was repeated every second day during ten days, by adjusting the optical densities of the samples to 6, according to the stationary phase (day 3) OD_{600} value of the reference strain. The results of the modified semi-quantitative CLS assay are shown in Fig. 4.

Table 2. Optical density (OD_{600}) values of the reference strain (Ref) and the mutants at the stationary phase of growth.

Strains	**OD_{600}**
Ref	6.11±0.01
B2	6.09±0.02
H7	8.36±0.05

At the beginning of the CLS experiment, on day 0, an equal number of spots were obtained, indicating that approximately an equal number of cells were spotted on plates for each strain tested, resulting from the adjustment of culture densities. On day 2 of the CLS experiment, there was no significant difference in viabilities of all strains, e.g., they could all grow up to the highest level of dilution (10^{-4}). Lifespan differences became apparent after day 2 of the CLS experiment, and on days 8 and 10 they could be observed most clearly. On day 8, the viability of B2 was at least 100-fold higher than that of the reference strain. Additionally, on day 10, the reference strain was not viable anymore. However, mutant strains B2 and H7 were still viable, which may imply a prolonged lifespan of B2 and H7 mutants. According to the results obtained by the semi-quantitative method, the ethanol-tolerant mutant, B2, seems to have a higher viability during stationary phase than the oxidative-stress resistant mutant, H7 (Fig. 4).

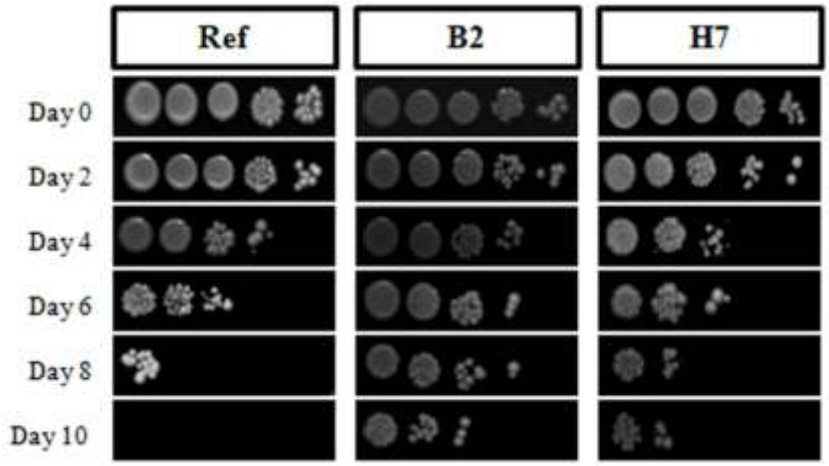

Figure 4. Modified semi-quantitative CLS assay results of the reference strain (Ref), and the mutant strains B2 and H7. Ten-fold dilutions: from left to right (10^{0}-10^{-4}).

Smith and colleagues (2007) used the spot assay method for CLS analysis by directly spotting culture dilutions without any initial adjustment of cell number/density (D.L. SMITH & al. [16]). Thus, in that study, differences were observed in the number of spots at the beginning of the CLS experiments, when different strains or different cultivation conditions were used for a comparative CLS analysis. In the present study, however, an equal number of spots was obtained at the beginning of the CLS experiment, despite the significant differences in the cell densities of the mutants (particularly H7) and the reference strain before the CLS analysis (Fig. 3 and Table 2). An equal number of spots could be achieved by the initial adjustment of the culture densities such that all cultures to be tested were spotted at equal cell amounts at the beginning and during the CLS experiments (Fig. 4). This adjustment allowed a more reliable comparative CLS analysis of cultures or strains at different cell densities or growth rates.

3.3. Verification of the modified semi-quantitative CLS analysis method by the quantitative CLS analysis method

The accuracy of the semi-quantitative CLS method was verified by using the conventional quantitative CLS method. According to the quantitative CLS analysis results (Fig. 5), on day 4 of the CLS experiment, the viability of H7 was significantly lower than that of both the reference strain and the B2 mutant, which is in line with the semi-quantitative CLS results (Fig. 4). On day 6 of the quantitative CLS experiment, no apparent differences in viabilities were observed among the strains tested, which was also in line with the semi-quantitative spot assay results. The viability of the reference strain decreased below 0.1% on day 8, unlike the mutant strains B2 and H7 which had significantly higher viabilities than the reference strain. Even though the viability of the reference strain decreased below 0.01% on the 10^{th} day of the CLS experiment, the viability of the mutants did not fall below 0.1%. In sum, the quantitative CLS method showed the accuracy of the improved semi-quantitative CLS method based on spot assay, and the results revealed that both mutant strains B2 and H7 are long-lived *S. cerevisiae* mutants. The similar results obtained by both semi-quantitative spot assay-based and quantitative CLS methods supports the applicability, comparability, and accuracy of the improved semi-quantitative spot assay-based method to assay CLS. Thus, the modified semi-quantitative CLS method based on spot assay is a practical, cost-effective and reliable screening method to identify the long-lived strains/cultures among a high number of

samples consisting of different strains and different cultivation conditions, before their extensive analysis by the quantitative CLS method.

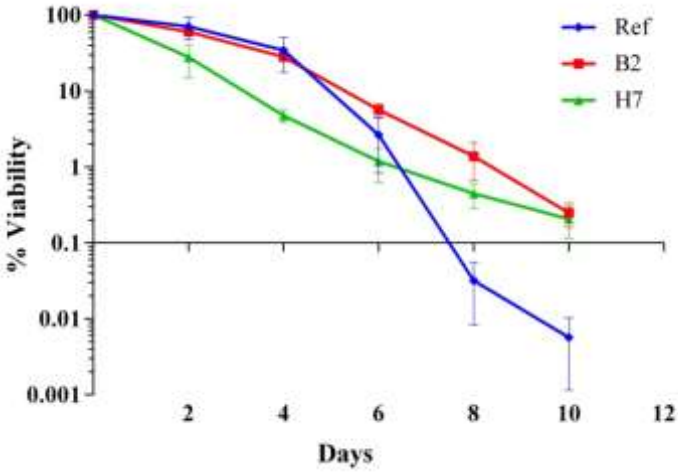

Figure 5. Quantitative CLS analysis results of the reference strain (Ref) and the mutant strains B2 and H7. The experiment was performed in triplicate, and standard deviations are indicated.

In cellular oxidative stress response, the balance between ROS production and the enzymatic or non-enzymatic antioxidant defense system is critical. Enzymatic antioxidant defense system includes superoxide dismutases (SODs), catalase and glutathione peroxidase enzymes. SOD profoundly and rapidly catalyzes the partitioning of the univalent superoxide anion to hydrogen peroxide (J.M. MCCORD & I. FRIDOVICH [23]), whereas catalase and glutathione peroxidase convert hydrogen peroxide to water. Non-enzymatic defense system includes ascorbate, pyruvate, flavonoids, carotenoids, and glutathione (T. FINKEL & N.J. HOLBROOK [24]). ROS accumulation causes oxidative stress in cells, resulting in cellular damage affecting DNA, proteins, and lipids. Thus, it is accepted as a major contributor to aging and degenerative diseases of aging (D. HARMAN [25]; B.N. AMES & al. [26]). Stationary phase survival of yeast cells has been shown to have a strong relationship with the anti-oxidant system (V.D. LONGO & al. [27]; P. FABRIZIO & al. [28]). In a recent study, an industrial yeast strain which is resistant to oxidative stress showed long-term survival in stationary-phase (H. OROZCO & al. [29]). In line with these findings, in the present study, the oxidative stress-resistant *S. cerevisiae* mutant H7 was also shown to have a prolonged lifespan. Considering that both oxidative stress resistance and long-term survival during the stationary phase of growth are industrially important and highly desired properties for industrial microorganisms, these findings can be useful for designing novel strategies for industrial strain development.

Fabrizio and colleagues (2005) have shown that ethanol stress decreases CLS in *S. cerevisiae* (P. FABRIZIO & al. [30]). Thus, in the present study, we were interested in determining the CLS of an ethanol-tolerant *S. cerevisiae* mutant. According to both semi-quantitative and quantitative CLS assay results, CLS of the ethanol-tolerant *S. cerevisiae* mutant was found to be longer than that of the reference strain. Several reports in the literature indicate the complexity of the molecular mechanisms of ethanol response and tolerance by showing their association with other stress types such as heat shock and oxidative stress. An earlier study demonstrated that ethanol induces heat shock response in yeast (J. PLESSET & al. [31]). Later studies confirmed that ethanol tolerance mechanism is associated with heat shock

protein response (K. SALES & al. [32]). Another study pointed out that decreasing intracellular ROS level is a way to protect *S. cerevisiae* cells from ethanol stress (X. DU & H. TAKAGI [33]). More recently, physiological and transcriptomic analyses of our ethanol-tolerant *S. cerevisiae* mutant strain B2 revealed a significantly higher ethanol productivity and a relatively lower respiratory capacity, compared to the reference strain (B. TURANLI-YILDIZ & al. [20]). In a very recent review on adaptive response and stress tolerance of yeast during ethanol fermentation, the complexity of the molecular mechanisms of ethanol response and tolerance, as well as the need for further studies were emphasized. It was also suggested that cellular protection by the cell wall as the first line of defense, and the maintenance of redox balance are the major mechanisms against various stress types encountered during ethanol fermentation (C. AUESUKAREE [34]). Altogether, these findings suggest that an improved anti-oxidative response, a general heat shock response and a lower respiratory capacity might be associated with the extended chronological lifespan of the ethanol-tolerant mutant B2.

4. Conclusion

The semi-quantitative yeast spot assay-based CLS method improved in this study can be used as a practical and reliable screening method to identify long-lived cultures/strains among a high number of samples, before the labor-intensive, quantitative CLS method used in yeast aging studies. Additionally, our CLS results revealed that the oxidative stress-resistant and ethanol-tolerant *S. cerevisiae* mutants tested have prolonged chronological lifespan compared to the reference strain of *S. cerevisiae*, which supports the close association between longevity and stress tolerance. Thus, the use of such mutant strains in yeast aging studies could also be suggested. As yeast strains with improved ethanol-tolerance and viability are industrially highly demanded, particularly by bioethanol/biofuel processes, the results obtained in the present study may be useful to develop strains for bioethanol/biofuel industries and hence to improve process efficiency and product yield.

5. Acknowledgements

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