Printed in the Republic of Korea
ANALYTICAL SCIENCE
& TECHNOLOGY
Vol. 35 No. 4, 181-188, 2022
https://doi.org/10.5806/AST.2022.35.4.181

Comparative analysis of yeast cell viability at exponential and stationary growth phases

Yejin An†, Nayoon Jo†, Hyeji Kim†, Dahye Nam†, Woorim Son†, and Jinkyu Park★

Department of Plant & Biomaterials Science, College of Natural Sciences, Gyeongsang National University, 33, Dongjin-ro, Jinju-si, Gyeongsangnam-do 52725, Korea

(Received June 21, 2022; Revised July 16, 2022; Accepted July 19, 2022)

Abstract: This paper describes a comparative analysis of yeast cell viability at exponential and stationary growth phases using multiple conventional techniques and statistical tools. Overall, cellular responses to various viability assays were asynchronous. Results of optical density measurement and direct cell counting were asynchronous both at exponential and stationary phases. Proliferative capacity measurement using SP-SDS indicated that cells at the end of the stationary phase were proliferative as much as exponentially growing cells. Metabolic activity assays using two different dyes concluded that the inside of cells at stationary phase is slightly less reducing compared to that of exponentially growing cells, implying that the metabolic activity imperceptibly declined as cells were aged. These results will be helpful to understand the details of yeast cell viability at exponential and stationary growth phases.

Key words: yeast, cell viability, exponential phase, stationary phase, proliferative capacity, metabolic activity

1. Introduction

The Baker's yeast, *Saccharomyces cerevisiae*, is an ideal model organism to study cellular mechanisms of eukaryotes, because of genetic and phenotypic similarity found in between yeast and other higher eukaryotic organisms.[1-3] In addition, yeast can be genetically manipulated by reliable transformation techniques and externally perturbed by various culture conditions, with the relatively less expense of time and effort compared to mammalian cells.[4,5]

Particularly, yeast is often exploited in age-related research because it has a unique proliferation mechanism, called *budding*, that a small daughter cell starts to grow on the cell surface and eventually pinches off the mother cell. Replicative life span deals with how many times the mother cell can bud, and chronological life span is simply how long a nonbudding cell survives. Which metabolic pathways are involved to determine these is pivotal to unravel aging and anti-aging mechanism.[6,7]

The most common method to decide the life span of yeast cells is monitoring cell viability and there are two categories in how to do it: (1) measuring the ability of yeast cells to grow on a medium, (2) analyzing the response of the cells to staining dyes.[8]

†These authors contributed equally to this work
★ Corresponding author
Phone : +82-(0)55-772-3565 Fax : +82-(0)55-772-3771
E-mail : jinkyu.park@gnu.ac.kr

This is an open access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

www.kci.go.kr

The first category includes conventional methods such as measuring optical density at a certain wavelength (e.g., OD_{600}), counting the cell number of liquid culture, or counting the number of colonies on solid culture.[9-11] The first method measures the intensity of light passing through the liquid culture to estimate the population growth. This method is quick and simple, but the absorbance is not directly related to the actual cell population, particularly when cells are highly populated. The second one is to count the number of cells using an optical microscopy and a counting chamber. It is a simple and direct method, but the counting results are varied. The last one is to grow serial dilutions of liquid culture on agar plates and count the number of colonies after a few days. This is to measure how many cells are proliferative, rather than how many cells are alive.

The other category includes various dye-testing methods such as propidium bromide, methylene blue, and water-soluble tetrazolium salt (WST-8) assays.[12-14] Propidium bromide cannot penetrate intact cell membrane, and thus only stain dead cells with damaged membrane. Methylene blue is membrane-permeable but becomes colorless in the presence of reducing substance inside metabolically active cells. WST-8 is also membrane-permeable and initially colorless but reduced by hydrogenases in alive cells to generate colored formazan product.

When yeast cells are inoculated into a fresh medium, they rapidly consume glucose to produce ATP energy by fermentation and their population grows exponentially. This is called *exponential phase*, which is known to be most active metabolically. Upon depleting all the glucose, the cells undergo diauxic shift which transform energy metabolism from fermentation to respiration, that they produce more ATP energy from less glucose. After the diauxic shift, the cell population stops growing and enter *stationary phase*, which is known to be metabolically dormant.[15,16]

Yeast cell viability of *young* or *aged* cells has been intensively studied by various approaches including advanced techniques such as flow cytometry, proteomics and transcriptomics.[17-19] Nonetheless, systematic evaluation of cell viability at different growth phases using conventional methods is necessary, because these techniques are major tools to determine yeast's life span, cytotoxicity, stress response, and so on. Here we describe a comparative analysis for yeast cell viability at exponential and stationary phases using direct cell counting, serial dilution spotting, methylene blue staining, and WST-8 assays.

2. Experimental

2.1. Cell strain and reagents

Commercial dry yeast (Saf-Instant Yeast Red) was purchased from a local distributor (Taejin Chemical). Yeast extract (Alfa Aesar), Bacto™ peptone (Gibco), dextrose (Daejung Chemicals & Metals Co. Ltd), Bacto™ agar (BD Difco) were used to prepare liquid or solid culture medium. Methylene blue (Sigma-Aldrich) and Cell Counting Kit-8 (Sigma-Aldrich) were used for cell viability assays. Deionized (DI) water was obtained using a Milli-Q® ultrapure water system.

2.2. Yeast cell culture

0.1 g of dry yeast was inoculated into 20 mL of YPD medium in a 50 mL Erlenmeyer flask with baffled bottom and incubated overnight at 30 °C and 150 rpm. 20 μL of the culture was spread on a YPD agar plate and incubated at 30 °C for two days. A single colony was taken and inoculated into 100 mL of YPD medium in a 250 mL flask, and then incubated up to 7 days. Cells were harvested at multiple time points (e.g., at 4, 8, 10 hours, 1, 3, 7 days from inoculation) and measured three times for OD_{600} in polystyrene semi-micro cuvettes (ratiolab®, Germany) using a UV-Vis spectrophotometer (V-1100D, Labinno Co., Japan). Simultaneously, 100~300 μL of culture was collected and centrifuged at 1,775 g for 5 min, and then analyzed for viability as described below.

2.3. Direct cell counting and methylene blue (MB) assay

Pelleted cells were concentrated or diluted with DI water to give 2×10^7 cells/mL and mixed with the same volume of MB solution (0.1 mg/mL in 2 % sodium citrate solution). The mixture was incubated

www.kci.go.kr

for 5 min at room temperature and the number of total cells (> 200 cells per sample) and blue-stained cells were counted using a Neubauer-improved counting chamber with 0.02 mm depth (Paul Marienfeld GmbH & Co. KG) on an optical microscope (BX51, Olympus, Japan). Cell viability (%) based on MB assay was calculated as follows.

$$\textit{Cell viability}\ (\%) = \frac{\textit{Blue-stained cells}}{\textit{Total cells}} \times 100$$

2.4. Single plate-serial dilution spotting (SP-SDS) assay

SP-SDS assay was conducted as described previously but modified as follows.[20] Briefly, pelleted cells were spin-washed in sterile DI water and diluted to 2×10^6 cells/mL to prepare an anchored stock (10^0) and then serial dilutions of 10^1~10^6 were prepared from the stock. The backside of YPD agar plate was marked with a pen into six sections and 4 μL of each dilution was spotted 8 times on each corresponding section. Sterile water used to prepare the dilutions was also spotted three times at the bottom part of the plate to ensure sterility. The plate was dried in a biosafety cabinet for 10 min and incubated at 30 ℃ for 2 days. The number of colonies was visually counted, and then colony-forming unit (CFU) was enumerated as follows.

$$\frac{CFU}{mL} = n \times \frac{100\mu L}{32\mu L} \times 10^{1+d}$$

where n is the number of counted colonies and d is the dilution level yielding countable colonies.

2.5. Cell counting kit (CCK)-8 assay

CCK-8 (also called WST-8) assay was performed as described in manufacturer's manual. Briefly, pelleted cells were spin-washed in sterile DI water and resuspended in 100 μL of 100 mM phosphate buffer with pH 7.0, containing 0.1 % glucose and 1 mM EDTA. Cell density was adjusted to 10^7 cells/mL and a triplicate was prepared in a 96-well plate. 10 μL of CCK-8 assay solution was added to each cell suspension and the plate was incubated at 30 ℃. After an hour, the absorbance was measured at 450 nm using a microplate reader. Serial dilutions of 2^1~2^6 were prepared from cells collected at OD_{600} about 1.0 and assayed in the same manner to draw a calibration curve. Absorbance for colored formazan was used to calculate the number of metabolically active cells. Cell viability was obtained as follows.

$$\textit{Cell viability}\ (\%) = \frac{\textit{Metabolically active cells}}{10^7\ \textit{cells}} \times 100$$

NADH (nicotinamide adenine dinucleotide) was used to draw a relationship between reducing activity and formazan formation.[21] NADH was dissolved in 10 mM Tris-HCl buffer (pH 8.0, 1 mM EDTA) and added to the CCK-8 reaction mixture at final concentrations of 12.5, 25, 50, 100, 200 μM. Absorbances for colored formazan vs. NADH concentrations were plotted and fitted with a linear equation.

2.6. Statistical analysis

Single factor ANOVA and two-sample Student's t-test were conducted using functions equipped in an OriginPro software.

3. Results and Discussion

3.1. Cell growth monitoring: Optical density and direct cell counting

Yeast strains commonly studied in modern molecular or cell biology are often limited in their growth by auxotroph which requires special nutrients in addition to general medium contents. For example, a wild-type yeast strain, W303-1b, has mutations in *leu2*, *trp1*, *ura3*, *his3* and *ade2* genes, so its maximum OD_{600} reaches only ~8.0 even when amino acids are additionally supplemented, whereas a strain not suffered from auxotroph can grow up to OD_{600} ~20.[22,23] To monitor cell viability during unlimited and healthiest growth, we used a commercially available Baker's yeast strain in which any auxotroph is not known.

Yeast cells were grown in rich medium and monitored for OD_{600} by a spectrophotometer and the number of cells by direct cell counting (*Fig.* 1). Cells were grown for 7 days because chronological life span of S. cerevisiae is known as 6.5 days,[7] so that we could monitor cell viability at exponential and stationary

www.kci.go.kr

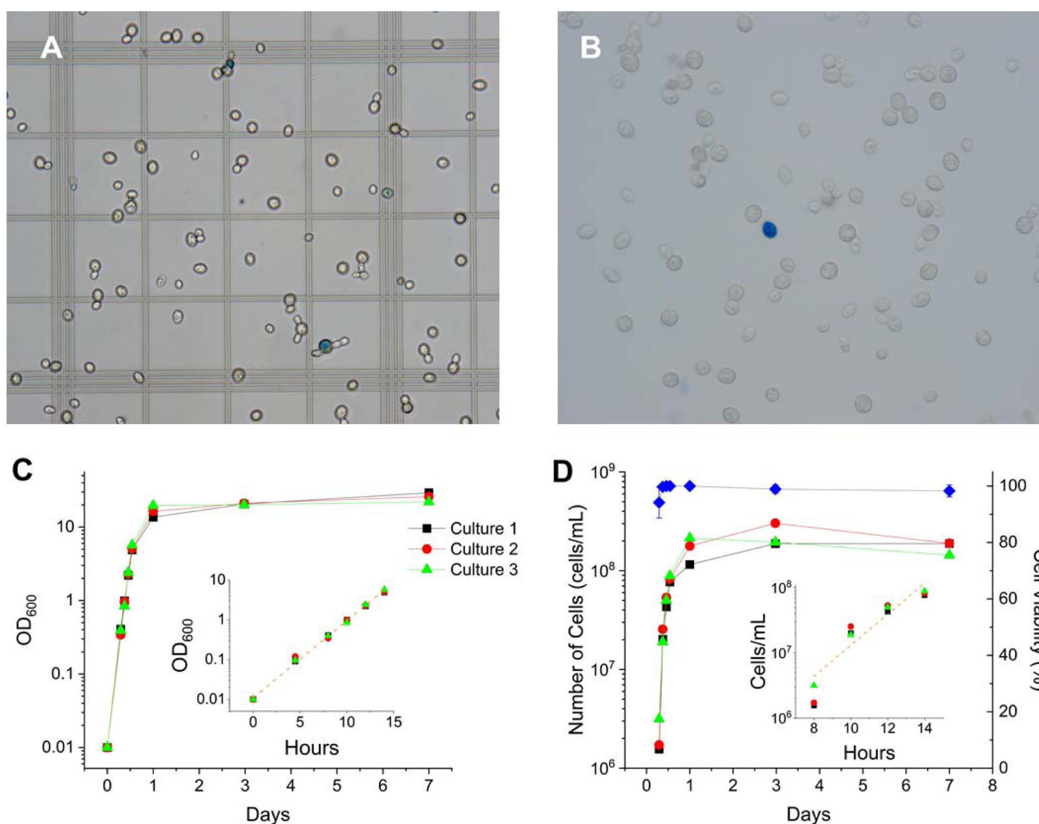

Fig. 1. Monitoring growth of cell population and cell viability based on MB assay. A, 7 days-grown cells on counting chamber (400× magnification). B, a blue-stained cell among colorless cells (600× magnification). C, plots of OD_{600} vs. time from three independent cultures. *Inset* is a zoomed-in plot for early period of the cultures. Relative standard errors were less than 20 % (not shown). D, plots of the number of cells and cell viability vs. time for the three equivalent cultures. *Inset* is a zoomed-in plot for early period of the cultures. Blue diamond indicates average cell viability for three cultures determined by MB assay. The orange dashed lines in the *inset*s indicate curves fitted with liner regression. Error bars indicate standard deviations.

phases before the cells start to die. As expected, cells grow exponentially during the early period of time exhibiting a doubling time of 1.7 hours (*Fig*. 1C, *inset*). Within two days, OD_{600} reached over ~20 and cells appeared to enter stationary phase. The number of cells also increased exponentially during the first half day of the growth, and it reached ~2 × 10^8 cells/mL forming a plateau after the day 1. The average number of cells counted at OD_{600} ~1 was ~2 × 10^7 cells/mL, which was one tenth of that at OD > 20. This demonstrated that monitoring optical density is not an accurate manner to measure proliferation of yeast cells when they are highly populated.

During the early period of the growth, a log plot based on OD_{600} can be fitted well with linear regression (R^2 = 0.998), however, one based on the number of cells showed a poor linear fit (R^2 = 0.910) (*Fig*. 1C and D, *insets*). Maximum doubling time (between hours 8~10) according to the number of cells (0.60 hours) were 2.6-fold shorter than that according to the OD_{600} (1.55 hours). This implies that asynchrony between OD_{600} and the number of cells was found even when cells are not highly populated and *exponentially grown cells* must be defined in very early stage of the culture. Another possibility is that cells might be further grown during the time lag between OD_{600} measurement and cell counting, even though the cells were removed from optimal growth

www.kci.go.kr

conditions including culture medium. Further study is required to carefully elucidate the asynchrony between OD_{600} and actual number of cells during the growth.

3.2. Proliferative capacity: SP-SDS assay

To investigate proliferative capacity as a measure of cell viability, SP-SDS assay was performed using cells harvested at OD_{600} 1.0, day 1, 3 and 7 (*Fig*. 2). 4~6 independent batches for each harvesting point were assayed and counted numbers of colonies were statistically analyzed using ANOVA and Student's t-test. Cells at the day 1 appeared to have higher

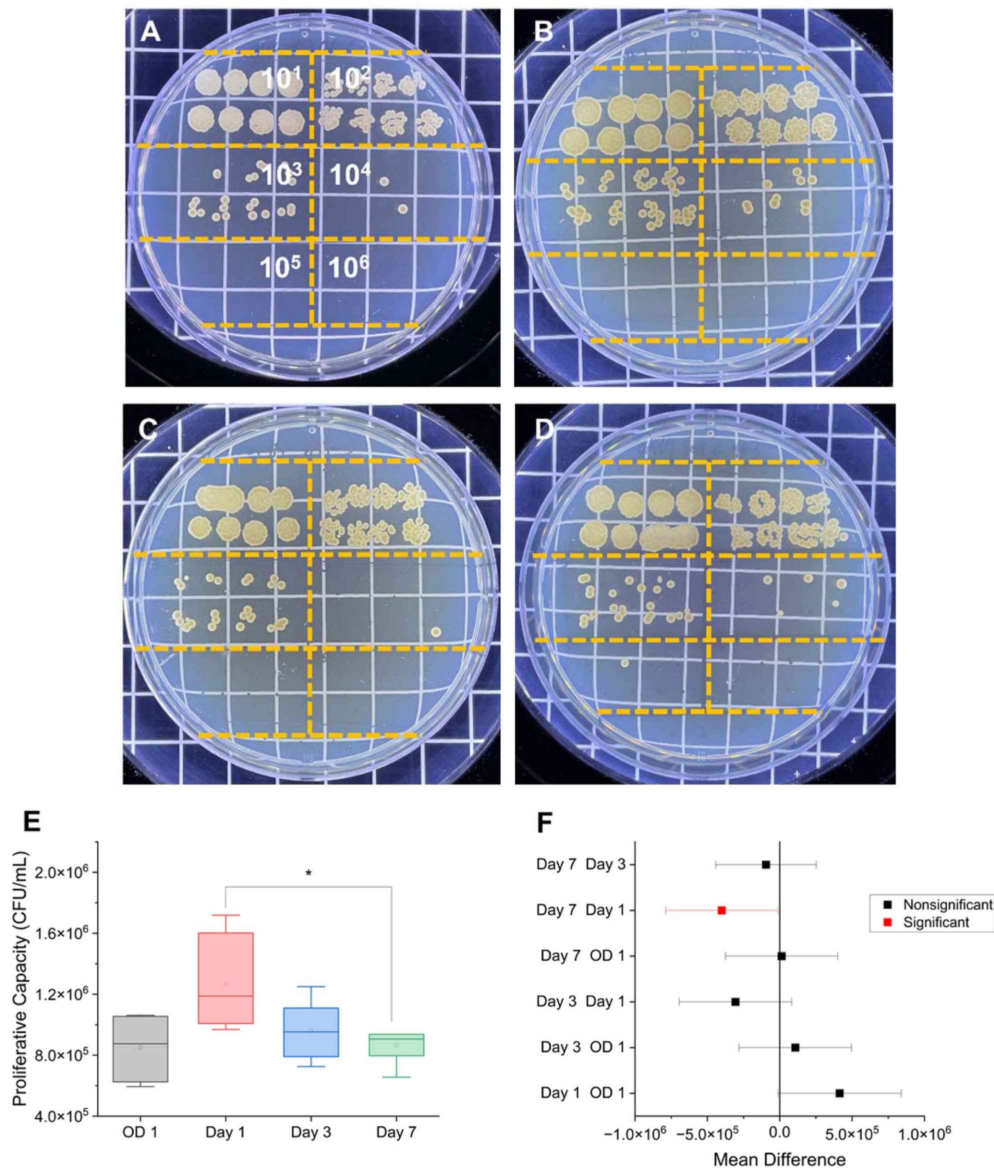

Fig. 2. SP-SDS assay results. Representative images of agar plates serially diluted and cultured from A, OD 1; B, Day 1; C, Day 3; D, Day 7 batches (n = 4~6). In most plates, the countable number of colonies were found in 10^3 dilution section. E, box plots of proliferative capacity in CFU/mL enumerated from SP-SDS assay results. Two-sample Student's t-test *P*-values indicate statistical significance ($^*P < 0.05$). F, means comparison plot obtained by single factor ANOVA between different batches. Red square indicates that means of two batches are significantly different, whereas compared means with black squares are not.

www.kci.go.kr

proliferative capacity than them at the other three harvesting points (*Fig*. 2E). But statistical analyses tell us that they were significantly different only from the cells at day 7, not from the other two groups of cells (*Fig*. 2E and F). In any case, it is beyond expectation that the cells grown for 7 days were proliferative as much as ones at OD_{600} 1.0. The difference between OD 1 and day 1 batches was statistically determined to be insignificant but the significance margin was little (*See Fig*. 2F, Day 7 Day 1 vs. Day 1 OD 1). Cells grown for a day might be the most proliferative during the yeast lifespan. Further study is necessary to support this hypothesis.

3.3. Metabolic activity: MB and CCK-8 assays

Metabolic activity of cells at different harvesting points was measured using two different dyes: methylene blue (MB) and WST-8. MB can penetrate cell membrane and stay inside the cell, staining it blue. Metabolically active cells have reducing cellular environment due to various reducing agents and enzymes such as NADH, glutathione and glutathione reductase. MB is reduced by cellular metabolism to colorless leucomethylene blue, so that alive cells stay unstained and only dead cells turn blue (*Fig*. 1A and B). Most of cells observed for the cell counting and MB assay were colorless (98.7 %) and only a small portion of them was blue-stained (62 out of 4835) (*Fig*. 1D, blue diamond). This indicates that cells at stationary phase are metabolically active to maintain the reducing environment sufficient to decolorize MB at least for a week.

WST-8 (CCK-8) is also membrane-permeable and reducible by cellular reducing power, but it changes

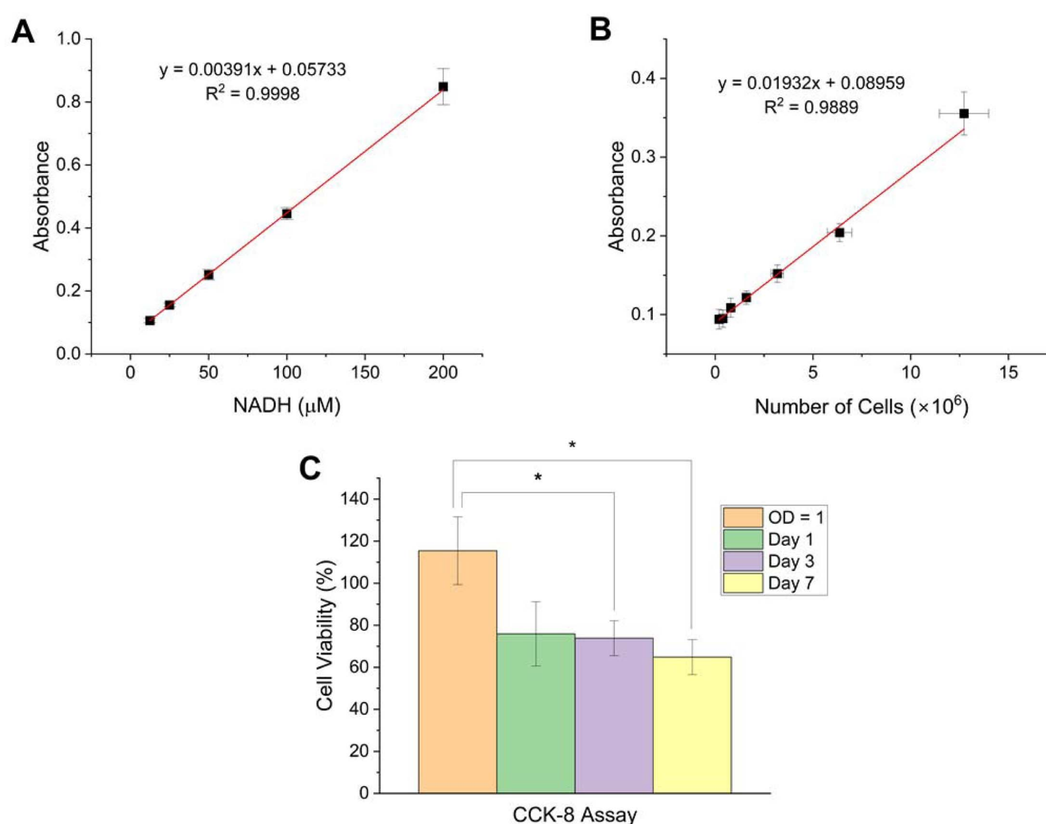

Fig. 3. CCK-8 assay results. A, reduced by NADH (n = 3). B, reduced by cells harvested at OD_{600} ~1 (n = 3). C, a column chart of cell viability obtained from OD 1, Day 1, 3 and 7 batches (n = 9~11). Two-sample Student's t-test *P*-values indicate statistical significance ($^*P < 0.05$). All error bars indicate standard errors.

www.kci.go.kr

from colorless to yellow. Higher absorbance means higher viability. NADH solutions with increasing concentrations were first tested (*Fig*. 3A), and a linear relationship between absorbance and reducing capacity was drawn. Statistical uncertainty increased as the NADH concentration increased indicating that the WST-8 assay would be more precise at low cell population rather than at high cell population. Assuming a similar reducing mechanism present inside yeast cells, cell suspensions harvested at OD_{600} 1.0 with increasing numbers of cells were tested and fitted with linear regression to obtain a calibration equation (*Fig*. 3B). Cells from OD 1, Day 1, 3, and 7 batches were assayed, and the number of viable cells were calculated using the equation, and then the cell viability was enumerated as described above. As a result, the cell viability was highest in OD 1 batch and decreased as the cells were further incubated (*Fig.* 3C). This is not surprising if considering the common dogma that cells at OD_{600} 1.0 are metabolically most active and cells at stationary phase are metabolically dormant, but surprising that the result was not synchronous to the SP-SDS or MB assay result. Difference in reduction potentials between MB and WST-8 might explain this, but the reduction potential of WST-8 is not known. If the reduction potential of WST-8 is lower than MB, decreased metabolic activity of the cells at stationary phase might be sufficient to reduce MB but not WST-8. Further study is required to figure out how cells respond differently to various viability assays.

4. Conclusions

This paper describes a comparative analysis of yeast cell viability at exponential and stationary growth phases using multiple conventional techniques and statistical tools. Overall, cellular responses to various viability assays were asynchronous. Optical density result and the actual number of cells were asynchronous both at exponential and stationary phases. Proliferative capacity determined by SP-SDS demonstrated that 7 days-grown cells were proliferative as much as cells at OD_{600} 1.0. Metabolic activity results assayed by two different dyes concluded that the inside of cells at stationary phase are reducing sufficient to reduce not WST-8 but MB, indicating that the cells at stationary phase are metabolically less active than the exponentially growing cells. Although further studies are needed, these results will be helpful to understand the details of yeast cell viability at exponential and stationary growth phases.

Acknowledgements

This work was supported by the research invigoration program of 2021 Gyeongnam National University of Science and Technology.

References

1. A.-C. Gavin, P. Aloy, P. Grandi, R. Krause, M. Boesche, M. Marzioch, C. Rau, L. J. Jensen, S. Bastuck, B. Dümpelfeld, A. Edelmann, M.-A. Heurtier, V. Hoffman, C. Hoefert, K. Klein, M. Hudak, A.-M. Michon, M. Schelder, M. Schirle, M. Remor, T. Rudi, S. Hooper, A. Bauer, T. Bouwmeester, G. Casari, G. Drewes, G. Neubauer, J. M. Rick, B. Kuster, P. Bork, R. B. Russell and G. Superti-Furga, *Nature,* **440**(7084), 631-636 (2006).
2. J. Förster, I. Famili, P. Fu, B. Ø. Palsson and J. Nielsen, *Genome Research,* **13**(2), 244-253 (2003).
3. G. Cazzanelli, F. Pereira, S. Alves, R. Francisco, L. Azevedo, P. Dias Carvalho, A. Almeida, M. Côrte-Real, M. J. Oliveira, C. Lucas, M. J. Sousa and A. Preto, *Cells,* **7**(2), 14 (2018).
4. N. Nagaraj, N. Alexander Kulak, J. Cox, N. Neuhauser, K. Mayr, O. Hoerning, O. Vorm and M. Mann, *Molecular & Cellular Proteomics,* **11**(3), M111.013722 (2012).
5. S. Ostergaard, L. Olsson and J. Nielsen, *Microbiology and Molecular Biology Reviews*, **64**(1), 34-50 (2000).
6. M. Kaeberlein, R. W. Powers Iii, K. K. Steffen, E. A. Westman, D. Hu, N. Dang, E. O. Kerr, K. T. Kirkland, S. Fields and B. K. Kennedy, *Science*, **310**(5751), 1193-1196(2005).
7. Valter D. Longo, Gerald S. Shadel, M. Kaeberlein and B. Kennedy, *Cell Metabolism,* **16**(1), 18-31 (2012).
8. M. Kwolek-Mirek and R. Zadrag-Tecza, *FEMS Yeast Research*, **14**(7), 1068-1079 (2014).

www.kci.go.kr

9. G. H. Markx, C. L. Davey, and D. B. Kell, *Journal of General Microbiology*, **137**(4), 735-743 (1991).
10. P. J. Costello and P. R. Monk, *Applied and Environmental Microbiology,* **49**(4), 863-866 (1985).
11. J. W. Messer, E. W. Rice and C. H. Johnson (1999).
12. D. M. Kuhn, M. Balkis, J. Chandra, P. K. Mukherjee and M. A. Ghannoum, *J Clin Microbiol,* **41**(1), 506-508 (2003).
13. R. López-Amorós, J. Comas and J. Vives-Rego, *Applied and Environmental Microbiology,* **61**(7), 2521-2526 (1995).
14. K. Painting and B. Kirsop, *World Journal of Microbiology and Biotechnology,* **6**(3), 346-347 (1990).
15. J. R. Broach, *Genetics,* **192**(1), 73-105 (2012).
16. R. Loewith and M. N. Hall, *Genetics*, **189**(4), 1177-1201 (2011).
17. M. Eigenfeld, R. Kerpes and T. Becker, *Frontiers in Fungal Biology*, **2**, Article# 665490 (2021).
18. Y. Sun, R. Yu, H.-B. Guo, H. Qin and W. Dang, *GeroScience,* **43**(5), 2573-2593 (2021).
19. M. Hotz, N. H. Thayer, D. G. Hendrickson, E. L. Schinski, J. Xu and D. E. Gottschling, *Proceedings of the National Academy of Sciences,* **119**(15), e2119593119 (2022).
20. P. Thomas, A. C. Sekhar, R. Upreti, M. M. Mujawar and S. S. Pasha, *Biotechnology Reports,* **8**, 45-55 (2015).
21. K. Chamchoy, D. Pakotiprapha, P. Pumirat, U. Leartsakulpanich and U. Boonyuen, *BMC Biochemistry,* **20**(1), 4 (2019).
22. R. Margesin, *Extremophiles,* **13**(2), 257-262 (2009).
23. J. Park, S. P. McCormick, M. Chakrabarti and P. A. Lindahl, *Biochemistry,* **52**(52), 9413-9425 (2013).

Authors' Positions

Yen An : Undergraduate Student
Nayoon Jo : Undergraduate Student
Hyeji Kim : Undergraduate Student
Dahye Nam : Undergraduate Student
Worm Son : Undergraduate Student
Jinkyu Park : Assistant Professor

www.kci.go.kr