Note

Comparison of microplate- and bottle-based methods to age yeast for chronological life span assays

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A B S T R A C T

This study compared the chronological life span and survival of Saccharomyces cerevisiae aged in a microplate or bottle, under different aeration and calorie restriction conditions. Our data shows that limited aeration in the microplate-aged culture contributed to slower outgrowth but extended yeast CLS compared to the bottle-aged culture.

The budding yeast Saccharomyces cerevisiae is widely used as a model organism to understand the fundamental mechanisms of ageing. The two life spans in yeast are (i) replicative life span (RLS) – the number of daughter cells produced by each mother cell before senescence, and (ii) chronological life span (CLS) – the ability of cells to maintain viability in a non-dividing state after they have entered senescence (Fabrizio and Longo, 2003, MacLean et al., 2001).

Yeast CLS can be measured directly by colony counts (Arslan et al., 2017) or indirectly using the outgrowth method based on optical density (OD) measurements (Murakami et al., 2008). In the outgrowth method, cells are first aged in a tube, bottle or microplate without replacing the media throughout the ageing period. Then, on different age-points (e.g. Day 2, 4, 6, and 8), the outgrowth of a small aliquot of the ageing culture is recorded by OD measurement. The collected outgrowth curves from the different age-points (in the linear range) are then used for the determination of yeast survival in the ageing culture (Murakami et al., 2008).

While cultures grown in tubes or bottles are routinely used to age yeast for CLS studies, microplates are necessary for high-throughput experiments (Jung et al., 2015; Powers et al., 2006). Microplate cultures are usually incubated without shaking (unlike bottle cultures) because the long incubation period required for CLS studies causes a significant loss of water through evaporation which then affects the ageing culture. In this study, we investigated yeast ageing by comparing the microplate- and bottle-based yeast ageing methods using two yeast strains in standard and calorie restriction (CR) media.

S. cerevisiae strains 1783 (MATa can1 his4 leu2-3,112 trp1-1 ura3-52) and MLC30M (MATa trp1-1 leu2-3,112 his3-11,15 ura3-1 ade2-1 can1-100 yrs1::HIS3 yrr1::TRP1 pdr1::hisG pdr3::hisG) were incubated in 1 ml YPD medium (2.0% peptone, 2.0% dextrose/glucose, 1.0% yeast extracts, addition of 2.0% agar for the agar plate) overnight with agitation. Then, the overnight cultures were centrifuged at $6700 \times g$ (MiniSpin® Eppendorf, Germany) and re-suspended in equal volume of standard SC media (2% glucose, 0.67% yeast nitrogen base, 0.074% complete supplement mixture without tryptophan and 140 mg/l tryptophan). CR was imposed on the cells by culturing the cells in SC (0.5% glucose).

For the bottle-ageing method, 10 μl of culture was transferred into a 7 ml Bijou bottle containing 1 ml SC media. The ageing culture bottle (with cap loosened) was incubated at 30 °C at a shaking speed of 200 rpm. For the microplate-ageing method, 1.8 μl overnight cell suspension was transferred to a 96-well microplate (IWAKI, Japan) containing 180 μl of SC media, incubated at 30 °C without shaking (Fig. 1). To prepare the microplates for the long incubation period, small holes (∅ 2 mm) were made on the plate covers on the centre of every four wells (e.g. A1, A2, B1 and B2) to prevent the accumulation of water condensation droplets above the wells. Additionally, the perimeter moat was filled with sterile water to minimise edge effect and evaporation (Lundholt et al., 2003). Finally, instead of sealing the microplate with a plastic film, the plate was placed in a sealed plastic bag

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Fig. 1. Schematic diagram of bottle- and plate-based ageing for CLS assays.

A single colony was inoculated to sterile YPD medium and incubated overnight. For bottle-based ageing, 10 μl overnight cells resuspended in SC medium were transferred to a Bijou bottle containing 990 μl SC medium. For the microplate-based ageing, 1.8 μl of overnight incubated cells were transferred to a 96-well microplate to a final volume of 180 μl SC medium (incubated without agitation). After two days of incubation, 2 μl of aged cells were transferred to a new well in a 96-well microplate containing 98 μl YPD medium. The OD values of the outgrowth cells were measured with Bio-Microplate Reader HiTS every 6 h. The outgrowth assay was repeated on Days 4 and 6 and outgrowth curves were plotted.
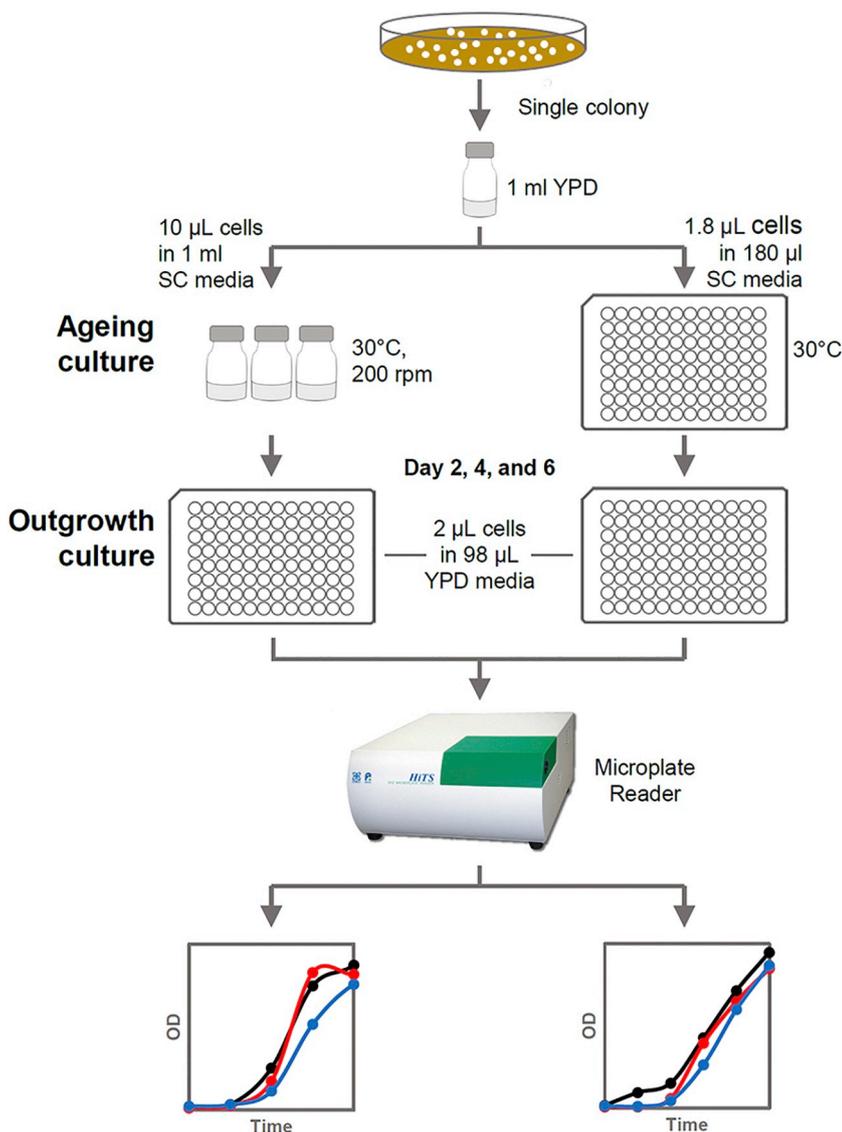
containing damp paper towel and incubated at 30 °C without agitation (Fig. 1).

Determination of yeast CLS was modified from previous studies (Murakami et al., 2008). On Day 2, 2 μl of culture from the ageing bottle and microplate were transferred to a new microplate (outgrowth plate) containing 98 μl YPD medium. The cell outgrowth was measured using the Bio Microplate Reader HiTS (Cosmic Bio, Japan), OD_{600nm} at every 6 h up to 24 h. The outgrowth plate was mixed at 210 rpm shaking for 5 s before the OD absorbance was measured. Determination of yeast survival was adapted from previous studies (Goldberg et al., 2010; Powers et al., 2006). Briefly, the survival of yeast at each age-point was calculated using fraction viability, where the mean OD value at the 18th hour of each age-point was divided by the mean OD at the 18th hour on Day 2 and expressed as a percentage. All experiments were carried out in biological triplicates. The data from the microplate reader were exported, analysed and the outgrowth curves were plotted using Excel 365 (Microsoft, USA). The outgrowth and survival data are presented in mean ± SEM. Statistical analysis was conducted for the comparison of the yeast survival aged in 0.5% and 2.0% SC media using independent Student *t*-test at $p < .05$ (IBM SPSS Statistics, USA).

Overall, we found that the outgrowth curves were further apart (rightward shifts of Day 4 and Day 6 curves) in the bottle-ageing method compared to the microplate one for the two strains in standard

and CR media (Fig. 2A). When the outgrowth curves, were converted to indicate cell survival, we found that the survival was comparable between bottle- and microplate-ageing for strain MLC30M in standard media (70%) (Fig. 2B). However, in strain 1783, the wider shifts of the outgrowth curves translated to a bigger loss of cell viability under the bottle-ageing condition (37%) compared to microplate-ageing (80%) (Fig. 2B).

The main difference between bottle- and microplate-based ageing methods here is the rate of aeration due to shaking and static culture conditions. Yeast cell yield is highly dependent on aeration during cultivation (Olson and Johnson, 1949), and limited aeration during cultivation was reported to mimic the effect of *Tor1* deletion that increases cell viability during the stationary phase (Bonawitz et al., 2007). Thus, the limited aeration in the microplate-aged culture may contribute to the increase in cell survivability, and this was more pronounced in strain 1783 (Fig. 2B).

Additionally, culture in CR media extended yeast CLS in all conditions except for strain MLC30M aged in the bottle where its survival was lower than normal media (Fig. 2B). CR is a non-genetic intervention that increases the mean and maximum life span of yeast (Aris et al., 2013; Blagosklonny, 2010; Smith Jr et al., 2007). We speculate that because MLC30M is a multidrug sensitive strain with several gene deletions (Δ*yrs1*, Δ*yrr1*, Δ*pdr1* and Δ*pdr3*) that affect the multidrug
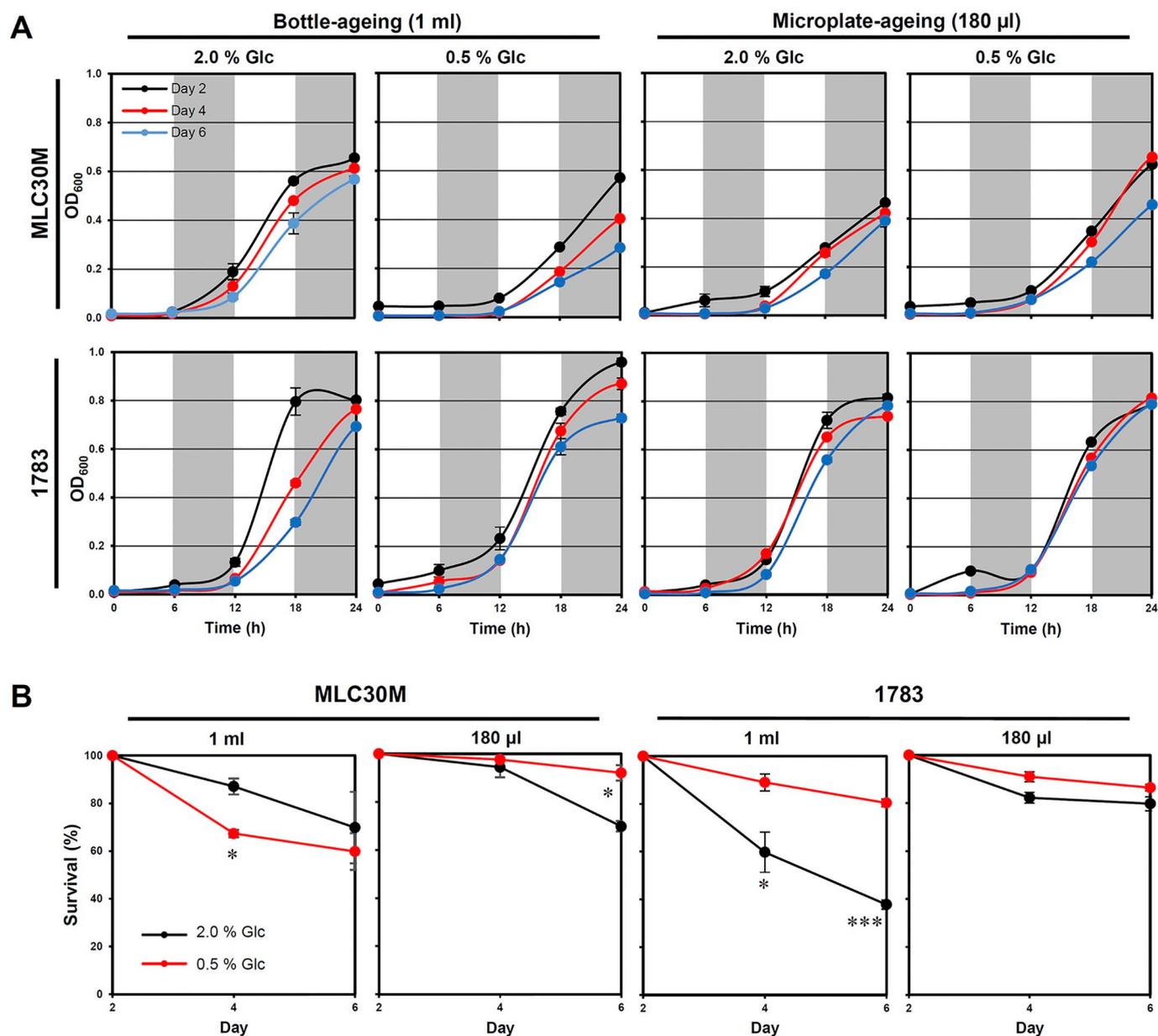
Fig. 2. Outgrowth curves and survival of microplate- or bottle-aged culture under normal and CR conditions.
(A) Outgrowth curves of yeast strain MLC30M and 1783 were obtained from the OD measurement using the Bio Microplate Reader-HiTS on Days 2, 4 and 6. Outgrowth curves of strains aged in bottles (1 ml) and microplates (180 μl) in SC media containing 2.0% or 0.5% glucose (Glc) were plotted. (B) Survival percentage of strains MLC30M and 1783 aged in microplate and bottle were derived from their respective outgrowth curves. Error bars represent standard error of mean with three biological replicates. The survival of each strain aged in 2.0 and 0.5% glucose in SC media was compared with Student *t*-test at *P*-value < .05, where *p < .05, ** p < .01 and ***p < .001.

response (Owsianik et al., 2002), these deletions may also affect the regulation of stress response required for the CR-mediated extension of CLS. In yeast, CR extends CLS by downregulating the TOR nutrient sensing pathway and upregulating the stress responses (Cameroni et al., 2004; Deprez et al., 2018; Kaeberlein et al., 2005; Masoro, 2005; Powers et al., 2006; Wei et al., 2008). Due to the inconsistency of the CR effect in the bottle-ageing culture in MLC30M, further studies are needed to investigate the function of the deleted genes and its role in CR, TOR and stress response.

Lastly, we determined the relationship between absorbance values from the microplate reader and the viable number of cells in the liquid culture by performing colony plate count (Fabrizio et al., 2003). Two microlitres of overnight culture were transferred to 98 μl of YPD medium on a microplate, then incubated at 30 °C and the absorbance

was recorded at 0, 6, 8, 10, 12 and the 15th hour. The culture at each time point was diluted ten-fold and 100 μl was plated onto YPD agar. Colony count was conducted after 48 h incubation. The standard curves of absorbance values against the number of colony forming unit per ml (CFU/ml) were plotted with the linear trendline using Excel 365 (Microsoft, USA). Both strains, 1783 and MLC30M, demonstrated a linear relationship between absorbance and CFU with the coefficient of determination value (R^2) above 0.92 (Fig. 3). It can be deduced that the OD measurement of the microplate reader is indicative of viable cells in the liquid culture.

In general, the microplate- and bottle-ageing methods are suitable for yeast CLS studies. However, the limited aeration in microplate-ageing cultures can affect the CLS by extending it and potentially contribute to a bias in high-throughput studies.
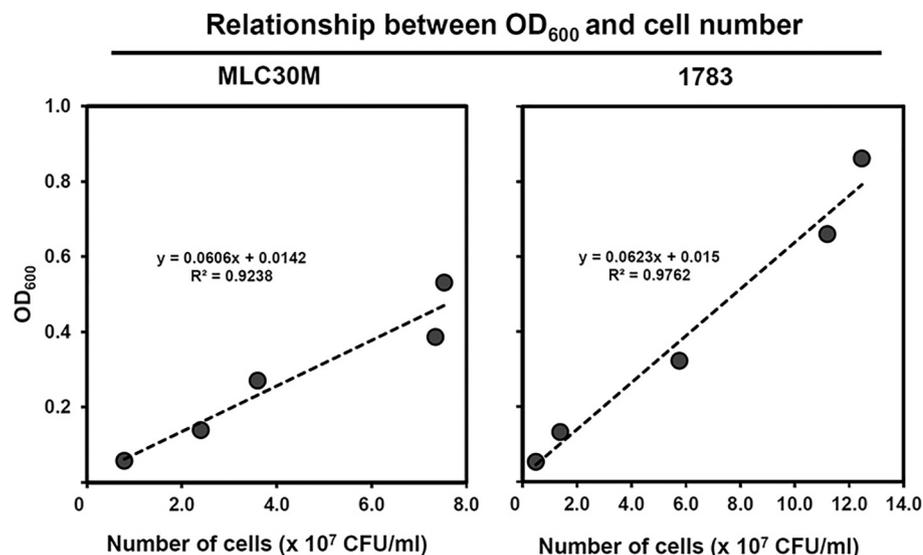
Relationship between OD$_{600}$ and cell number

Fig. 3. OD values correspond to CFU. The graph of OD versus cell numbers for strain 1783 and MLC30M were plotted by recording OD values at 0, 6, 8, 10, 12, 15th hours against the number of colonies formed per ml. Serial dilution and CFU plating assay were conducted after OD readings were recorded. Error bars represent the standard deviation of means with $n = 3$.

Declaration of Competing Interest

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