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ORIGINAL PAPER

Paul A. Duncan · Sean Gallagher · Linda McKerral
P. K. Tsai

Assessing the viability of a clumpy *mnn9* strain of *Saccharomyces cerevisiae* used in the manufacture of recombinant pharmaceutical proteins

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Abstract Demonstration of the viability of cryopreserved cell bank used to make a biopharmaceutical product is an important indicator of the ability to consistently manufacture over a long period of time, and is mandated in regulatory guidances. A *mnn9* strain of *Saccharomyces cerevisiae*, chosen for its inability to hypermannosylate vaccine antigens, has a clumpy growth tendency due to the inactivation of the gene *MNN9* (wild-type), complicating the interpretation of conventional viability measurements useful for single cells. Therefore, two growth-based measurements as well as staining by a membrane-impermeable dye were examined for their ability to reflect changes in viability of a clumpy *mnn9* (defective) strain. The cell clumps proved to be stable to mixing, and variability of agar-plate-based viable counts (VC) of undisrupted suspensions of this clumpy *mnn9* strain was consistent with variability observed for cell banks of a non-clumpy *MNN9* strain. Both the VC and the growth times in an oxygen-sensing broth-based microplate assay corresponded well with shake-flask growth times for a set of stressed and unstressed samples, although the correlation was highest between the two broth-based systems. Counts of trypan-blue-stained cells within clumps also increased with time of stress, suggesting that this method could be adapted as a simple index of viability as well.

Keywords Viability · *Saccharomyces cerevisiae* · *mnn9* · Cell bank · Stability

P. A. Duncan (✉) · S. Gallagher · L. McKerral · P. K. Tsai
Department of Bioprocess and Bioanalytical Research, Merck Research Laboratories, WP17-101, Sumneytown Pike, West Point, PA 19486, USA
E-mail: paul_duncan@merck.com
Tel.: +1-215-6520498
Fax: +1-215-9934851

Introduction

Manufacture of pharmaceutical biological products generally begins with inoculation of media with cells from a cryopreserved cell bank. Regulatory guidances specify that the viability of the stored cells be demonstrated as part of qualification of the cell bank [4–6, 11, 24]. In fact, as long as viability remains stable during storage, it is unlikely that other characteristics of the cell bank (i.e. recombinant expression system) would require further verification at intervals during cryopreserved storage to meet regulatory requirements [11].The *mnn9* strain of *Saccharomyces cerevisiae*, such as that developed for the manufacture of a recombinant vaccine protein [12] presents a challenge for testing the viability of inocula intended for manufacturing. The strain, chosen for its inability to hypermannosylate potential vaccine antigens [12], grows as a population of clumps of up to dozens of cells each. Clumpiness is associated with a defect in outer-chain mannosylation [2] of cell-wall glycoproteins by the *mnn9* strain [8], and is attributed to failure of cells to separate completely after budding [2]. The gene responsible for the outer-chain glycosylation (*MNN9*) has been cloned, enabling the generation of stable mutants with similar phenotypes by targeted gene disruptions [25]. Consistent with standard yeast genetic nomenclature, *MNN9* and *mnn9* denote the wild-type and defective genes, respectively.

Clumpy cell populations intuitively complicate the assessment of viability by methods that require examination of individual cells (i.e. microscopy) in order to count cells labeled with viability dyes, such as described by Heggart et al. [9], or those which do not distinguish growth arising from clumps versus growth from single cells (i.e. agar-plate-based colony counts, such as described in [9] and [13], or end-point dilution such as described in [13]).

We examined two growth-based measurements for their ability to reflect changes in the viability of a clumpy

mnn9 strain, and briefly explored a cell-staining method. The stability-indicating nature of each assay was also demonstrated. We report here our key observations regarding the ability of these methods to reflect the viability of a clumpy *mnn9* strain of *S. cerevisiae*.

Materials and methods

Yeast strains

The *Saccharomyces cerevisiae mnn9* strains used in this study were derived from strain 1558 by incorporation of a functional *ADE1* gene and transformation with plasmids encoding HPV L1 antigens under a galactose-inducible expression system like that previously described [10]. Strain 2150-2-3 bears the wild-type *MNN9* [7] and was used for determining the variability of the viable counts (VC) assay for a non-clumpy yeast strain.

Growth media

Two agar-plate formulations were used to enumerate colonies. Non-selective YEHD agar plates used for strain 2150-2-3 contained 10 g soy peptone/l , 20 g yeast extract/l , 16 g dextrose/l and 20 g agar/l . Selective agar medium lacking adenine and L-leucine (ade–leu-agar) used for the *mnn9* strains contained 40 g dextrose/l , 20 g agar/l , 10 g succinic acid/l , 8.5 g yeast nitrogen base without amino acids and ammonium sulfate/l (Difco, Franklin Lakes, N.J., USA, cat. no. 233520), 5 g ammonium sulfate/l , 0.3 g of each of L-isoleucine and L-phenylalanine/l, 0.25 g L-tyrosine/l , 0.2 g of each of uracil, L-lysine, and L-tryptophan/l, 0.1 g L-arginine/l, and 0.05 g L-histidine/l. The pH was adjusted to 5.3–5.5 with 50% (w/v) NaOH. All plates were inoculated with 0.1 ml of sample diluted in either phosphate-buffered saline or 1 M sorbitol, and incubated at 30°C for 2–3 days. Liquid cultures were grown in selective ade–leu- medium formulated as in the plates except without agar. Unless otherwise noted, all reagents throughout were procured from Sigma (St. Louis, Mo., USA).

Agar-plate-based viable counts

Samples of the *mnn9* strain were diluted in tenfold series in either saline (0.15 M NaCl), phosphate-buffered saline (saline with 0.1 M phosphate, pH 7.2), or 1 M sorbitol. In preliminary experiments, no appreciable difference was observed among diluents. Aliquots of 100 µl were inoculated onto each of at least two replicate plates, and inocula were distributed over agar surfaces with disposable plastic spreaders. After 2–3 days incubation at 30°C, colonies were counted. Means across the replicate plates within the range of 30–300 colonies per plate were used to calculate colony-forming units (CFU)/ml after correcting for volume and dilution factors.

Shake-flask cultures

Shake-flasks (250 ml) containing 50 ml each of ade–leu- broth were each inoculated with thawed cryopreserved aliquots of the *mnn9* strains (0.5 or 1% v/v) and incubated at 30°C with shaking (250 RPM, Gyromax 703, Amerex Instruments, Lafayette, Calif., USA) for up to 2 days. Aliquots of 0.5 ml were aseptically collected from flasks at various timepoints during culture to monitor glucose consumption. Samples were frozen for subsequent glucose analysis using the YSI-Bioanalyzer 2700M system (YSI, Yellow Springs, Ohio, USA.).

Oxygen-sensing microplate assay

A 96-well plate containing an embedded oxygen-sensitive fluorophore in each well (Oxygen Biosensor System, Becton Dickinson, San Jose, Calif., USA) was used to monitor oxygen consumption as a function of cellular metabolism and growth. Selective ade–leu-broth (200 µl) was added to each well. Twofold dilutions of each sample were added to triplicate wells (20 µl/well, 10% v/v) and then a covering of mineral oil (50 µl/well) was added to minimize oxygen transfer with the atmosphere. Plates were incubated at 30°C in a fluorescence plate reader (Gemini FS, Molecular Devices, Sunnyvale, Calif., USA) and monitored for fluorescence (ex 485 nm, em 630 nm) each hour for up to 18 h. Fluorescence signal was normalized for background by dividing the mean for each triplicate set of wells at each timepoint by the mean value at time zero, yielding relative fluorescence units. Plates were typically inoculated with two to eightfold dilutions of the cell-bank samples, corresponding to a maximum inoculation of roughly 5×10^4 CFUs per well. In a typical assay, direct microscopic counts of cell clumps increased ~25- to 30-fold, or nearly five doublings.

Trypan-blue cell staining and counting

In preliminary experiments, 20 µl of sample was added to 60 µl of 0.4% trypan-blue solution (Sigma). Twenty microliter of each mixture was immediately observed at 40× magnification using bright-field (Olympus IX81) or differential-interference contrast (Olympus AX71) microscopy (Olympus, Melville, N.Y., USA). From each treatment, a total of six separate cell clumps, determined to be typical of the total population and containing >100 cells total, were observed for dye penetration. Counts of essentially all cells within each clump were made by carefully adjusting the Z-axis through the depth of each clump.

Results

Agar-plate-based viable counts

Various treatments to disperse *mnn9* clumps into single-cell suspensions, including brief sonication, EDTA, mannose, enzymes, and shear by passage under pressure through a very fine needle, were explored in preliminary experiments. Sonication was most effective but still did not disrupt all clumps, and did not increase the VC proportionately with the increase in microscopic total count (data not shown).

The *mnn9* clumps proved to be stable during vortexing (Fig. 1a). Vortexing for increasing amounts of time, and well beyond the few seconds normally used to mix a sample prior to plating, did not result in VCs outside the range that might be expected based on variability among replicate samples that had received the minimal vortexing needed to mix (Fig. 1b).

The reproducibility of the VC using undisrupted suspensions of the *mnn9* strain was assessed using repeated measures (initial and then 2–4 years later) within each of 12 cell banks (banks for four distinct vaccine antigens made by three distinct processes each; see Fig. 2). The standard deviations (s) among repeated tests were calculated in the natural log scale for each cell bank, then pooled:

$$\left(\frac{\sum(\mathrm{DF}_i \times s_i^2)}{\text{Total tests among } i \text{ cell banks} - i \text{ cell banks}}\right)^{1/2}$$

where DF_i and s_i are degrees of freedom and standard deviation, respectively, for the ith cell bank. The pooled standard deviation was converted to pooled relative standard deviation (RSD) using the relationship $\mathrm{RSD} = 100\ (e^{\mathrm{SD}} - 1)$. The pooled RSD for the *mnn9* strain was 60%, consistent with that calculated for a non-clumpy *MNN9* strain (three cell banks each tested multiple times over 10 years, pooled RSD among tests 52%).

Oxygen-sensing assay

The oxygen-sensing assay was explored as a possible analytical surrogate for larger-scale liquid cultures in order to reflect possible changes in viability of cell banks. Reproducibility of the oxygen-sensing assay among independent runs was assessed by comparing times required to reach a fixed level of normalized fluorescence units for a set of cryopreserved samples

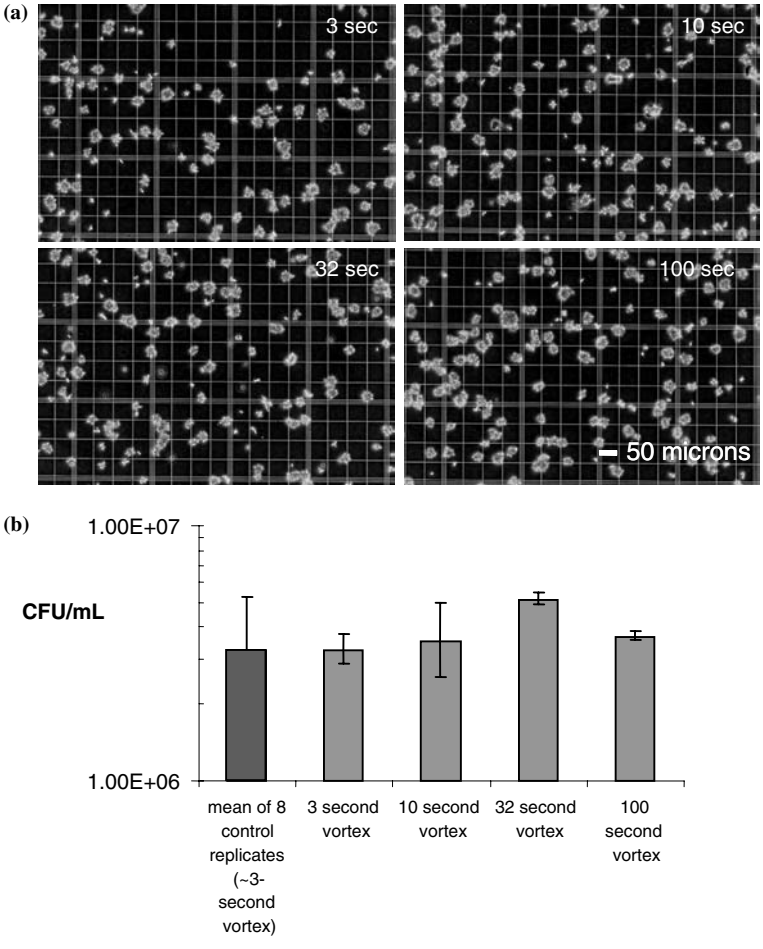

Fig. 1 Effect of vortexing on **a** clumpiness and **b** viable count. A cell suspension was subaliquotted in 1-ml volumes in 1.7-ml microfuge tubes. One subaliquot was vortexed (Fisher Vortex Genie3 II Model G-560, speed setting 5–6) to mix for the indicated time, then immediately diluted and inoculated onto duplicate agar plates. The error bars on the time-course results (3, 10, 32 and 100 s vortexing times) represent the mean ± 1 SD and reflect the variability among plate counts for each mixed aliquot. To assess the level of variability that might be expected simply by repeated testing of the minimum vortexing time, a set of eight additional replicate subaliquots were vortexed for 3 s each, then diluted and plated in duplicate. The error bar for a mean of eight replicates (*far left* in **b** represents the mean ± 2 SD, indicating the 95% limits for the distribution

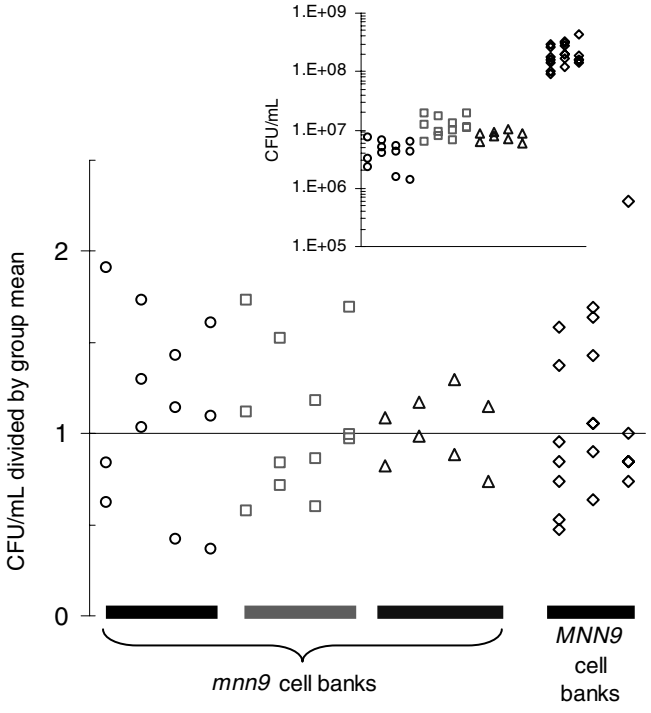

Fig. 2 Relative variability among repeated tests within cell banks. Three sets of four *mnn9* cell banks each (*mnn9*) and one set of three *MNN9* cells banks are represented. Distinct symbols for *mnn9* cell banks sets represent distinct preparation processes. Relative variation among repeated tests is shown graphically as the ratio of the individual test titer to the geometric mean of the related cell bank set. The individual titers are shown in the *inset*. Tests were performed when the cell banks were made and at various time points during cryopreserved storage. There was no evidence of a trend in VC with time in storage, thus allowing the pooling of tests within a cell bank to assess variability among tests. Pooled SDs were calculated as the square root of the [Sum of squared deviations from the mean/(Number of observations per cell bank − number of cell banks)]. RSDs were calculated using the pooled SDs, as described in the text

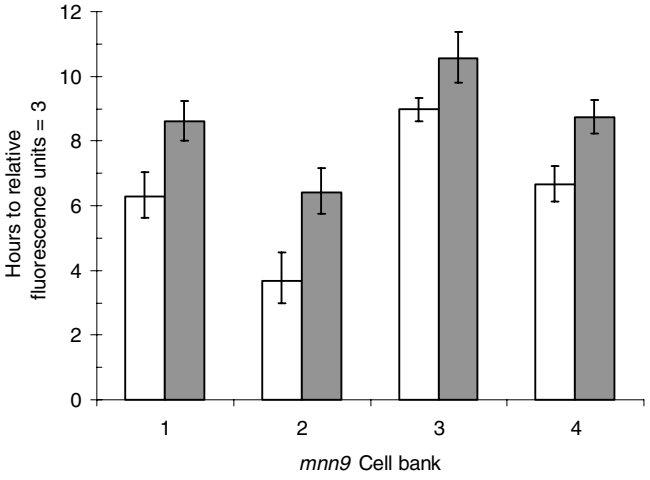

Fig. 3 Reproducibility of oxygen-sensing assays. Four independent *mnn9* cell banks were tested in each of four separate runs of the assay over a period of ~3 weeks. Results are shown for twofold-diluted (*white bars*) and fourfold-diluted (*shaded bars*) cells, and include error bars of 1 SD

diluted similarly among runs. The assay yielded consistent results for two dilutions of each of four different cryopreserved *mnn9* cell banks across four independent tests (Fig. 3). The pooled RSD, representing variability among repeated tests as described above, was 8% for the fourfold-diluted inoculations and 14% for the twofold-diluted inoculations. Note that RSDs cannot be compared directly between oxygen-sensing assays and agar-plate based VC, because they differ in scale (a twofold change in VC does not change time in the OS assay by twofold, but rather by a single doubling time of the cells).

Stability-indicating nature of the viability counts, oxygen-sensing assay, and cell-staining methods for the *mnn9* strain

Cryopreserved cell bank samples were held either frozen (unstressed) or thawed at 2–8°C for 3.5 days (to mimic stress due to an extended thaw/hold period). The stressed condition was previously observed to increase growth time of *mnn9* in liquid cultures. Performance in viable counts and oxygen-sensing assays was compared with that in shake-flasks, which reasonably represent a first step in cell expansion in a manufacturing process. The relationship between stressed and unstressed samples as determined by glucose consumption in shake-flasks (Fig. 4c) mirrored that determined using oxygen depletion in microplates (Fig. 4a). The relationship between time to fixed index of growth in the oxygen-sensing plates and shake-flask cultures was linear ($R^2 = 0.98$) across the range explored, but the slope of the relationship, 0.84, suggested that oxygen consumption in microplates may be slightly less sensitive to changes in viability than glucose consumption in shake-flasks (Fig. 4b).

The relationship between growth in the broth-based cultures and VC was linear, although the predictive power of the relationship was lower ($R^2 = 0.87$), reflecting the greater variability of the VC assay. The average VC stressed/unstressed ratio among the four cell banks tested (Fig. 4) was 0.26, corresponding to a change in VC by 1.9 doublings (ln(0.26)/ln(2)). The average growth time delay between stressed and unstressed samples in shake-flasks was 7.0 h, representing approximately 2.7 doublings (based on an average doubling time of 2.6 h, data not shown). In a separate experiment, the proportion of trypan-blue-impermeable cells in clumps decreased from approximately 87% to 23% after a similar 72-h incubation at 2–8°C, further supporting the magnitude of the change in viability (Fig. 5).

Discussion

Results of this study suggest that the cell clumps of the *mnn9* strain are sufficiently coherent to allow meaningful

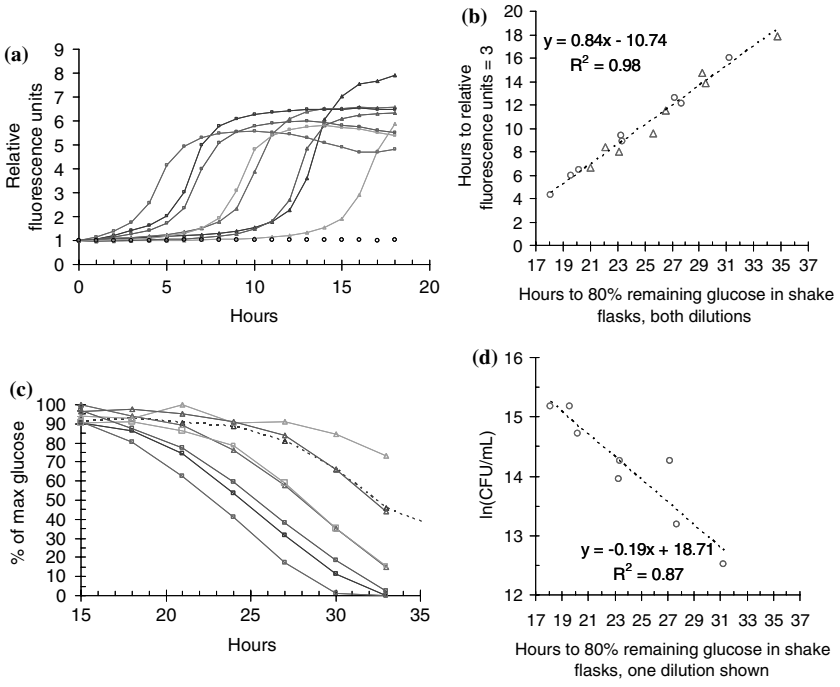

Fig. 4 Impact of stress treatment (3.5 days at 2–8°C) on viability and growth of four independent *mnn9* cell banks. Cultures in oxygen-sensing microplates (**a**) or shake-flasks (**c**); *squares with solid lines* unstressed samples; *triangles with dashed lines* stressed samples; *colors and symbols* represent the four independent cell banks tested; only one inoculum level shown. Relationship between growth in shake-flasks and growth in microplate broth cultures (**b**) or on agar plates (**d**) with or without stress treatment; for broth cultures, *circles and triangles* represent different dilutions tested

interpretation of agar plate-based viable counts. This result is important because clumpiness is generally understood to complicate the interpretation of the VC assay [16]. Postgate noted that viable counts, using "appropriate modifications", could yield meaningful information about populations of cells that grow in clusters, such as moulds and filamentous or cluster-forming bacteria, although no single modification could be applied to all systems [17]. Sonication or vigorous vortexing have been recommended for disrupting aggregates of bacteria, and addition of detergent is commonly recommended for dispersion of mycobacteria [13 and references therein]. Sonication was also used to partially disrupt *S. cerevisiae* cell aggregates that resulted from defects in chitin synthases [19], and flocculated yeast were dispersed by addition of EDTA [21] and mannose [3, 14, 22]. However, as noted earlier, neither EDTA nor mannose dispersed the *mnn9* clumps, and our initial experience with sonication and shearing through syringe needles suggested that cells released from clumps were injured.

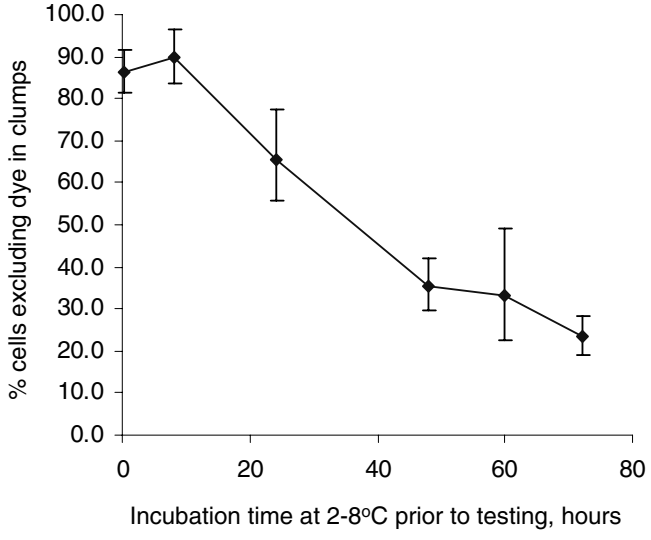

Fig. 5 Change in viability of *mnn9* strain reflected in trypan-blue dye exclusion. See text for details. Error bars (1 SD) represent the variability in counts among six replicate clumps of cells at each timepoint

In contrast to sonication, the clumpiness of a typical *mnn9* cell suspension did not change perceptibly after increasingly vigorous mixing by vortexing, nor did the VC change outside the range that might be expected by chance alone. In addition, clumpiness did not dramatically affect the variability of the VC assay, as the RSD among tests for the clumpy *mnn9* strain was comparable to that of a non-clumpy strain bearing *MNN9* . Finally, the loss in VC resulting from the stress designed to simulate an overextended thawing period corresponded with the delay in growth time observed in shake-flask cultures. The difference in predicted loss in doublings between VC and shake-flasks cultures (−1.9 doublings and −2.7 doublings, respectively), suggests that, while the VC assay is capable of detecting a loss in viability, it may slightly underestimate the impact of this stress treatment on growth in broth culture.

Determination of growth time in broth cultures is an alternative means to reflect process-relevant changes in inocula, since its interpretation is not compromised by clumpiness. However, growth-time measurements can reflect changes in the quality of the growth medium and even the metabolic state of the cells in addition to changes in viability. Other studies have demonstrated the correlation between oxygen consumption in oxygen-sensing microplate cultures and viable cell

concentration for a variety of cell types as well as the ability to monitor cytotoxity of drugs [15, 23] and to calculate growth rates [20]. The present study demonstrates the utility of the oxygen-sensing microplate as a reasonable surrogate for larger-scale broth cultures of this clumpy *mnn9* strain for analytical purposes. The close correlation between glucose and oxygen consumption is consistent with the continued operation of the respiratory pathway even as excess glycolytic pyruvate overflows into the fermentative pathway [1, 18]. Finally, the variability in time to reach fixed growth thresholds among runs of oxygen-sensing plates compares favorably with the variability of the VC assay, although data are admittedly limited for the oxygen-sensing assay. Other microplate-based strategies may be similarly suited for analytical-scale broth-based assessment of growth potential of a clumpy strain like *mnn9*, for instance continuous measurement of optical density (Bioscreen C, Growth Curves AB, Helsinki, FInalnd), end-point measurement of reduction of tetrazolium salts, or even quantitative PCR to detect proliferation of viable cells in microplate cultures.

Alternatively, viability stains could be used if convenient methods for counting individual cells in clumpy suspensions were developed. The staining method demonstrated in this study shows promise. Generally, however, methods to test for viability in a routine quality-control environment must be easy to perform, and the method we used is not yet robust enough.

In conclusion, the three methods were capable of reflecting changes in cell viability in clumpy *mnn9* strains of *S. cerevisiae* without disruption of clumps. The agar plate-based viable count is perhaps the best suited growth method for measurements over long periods of time, while the oxygen-sensing microplate assay might be better suited for simultaneous comparisons. Assessing cell viability by permeability of membranes to trypan blue was demonstrated, but would require some refinement to make it ''user-friendly'' and objective in a quality-control testing environment.

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