The Spheroplast Lysis Assay for Yeast in Microtiter Plate Format

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A yeast lysis assay in the microtiter plate format improved precision and throughput and led to an improved algorithm for estimating lag time. The assay reproducibly revealed differences of 10% or greater in the maximal lysis rate and 50% or greater in the lag time. Clonal differences were determined to be the major source of variation. Microtiter-based assays should be useful for screening for drug susceptibility and for analyzing mutant phenotypes.

Microtiter assays offer advantages for assay miniaturization and throughput (3). Such assays have been used to determine protein contents (24), enzyme activities (14, 26), and ligand binding (4, 18) and are becoming increasingly popular because of the use of colorimetry (12, 27) and fluorescence (10, 18, 26). They have also been used for to determine growth (5, 19, 21).

Interest in yeast cell wall assembly led to development of the spheroplast lysis assay in which light scattering is used to detect cell lysis (8, 13, 25, 29). The uses of this assay include determining cell wall reassembly by yeast spheroplasts (28), determining cell wall weakening by the mating pheromone (16), determining the effects of chemical agents on wall structure (15, 17), and determining the roles of various genes in cell wall assembly (1, 2, 6, 11, 17, 22). We describe here adaptation of the yeast cell wall degradation assay (20) to a microtiter format.

MATERIALS AND METHODS

Yeast strain and growth medium. Saccharomyces cerevisiae X2180-1A (MATa SUC2 rud mel gal2 CUP1; Yeast Genetic Stock Center, Berkeley, Calif.) was used in this study, and the medium used was SC medium (23). Yeast cells were grown in 50-ml batches at 30°C with rotation at 150 rpm and an orbital radius of 0.75 cm.

Enzyme preparation and assays. Zymolyase 100T (ICN, Costa Mesa, Calif.) was used in this study, and the medium used was SC medium (23). Yeast cells were grown in 50-ml batches at 30°C with rotation at 150 rpm and an orbital radius of 0.75 cm.

Enzyme preparation and assays. Zymolyase 100T (ICN, Costa Mesa, Calif.) was resuspended in glyceral-water (1:1) to a concentration of 20 mg/ml. The sediment was removed by centrifugation, and the stock solution was stored at -20°C. The stock preparation was suspended in TE buffer (30 mM Tris HCl, 150 mM NaCl, 5 mM EDTA; pH 7.5) at a concentration of 200 µg/ml. Prolase (13) and glucanase (7) activities were assayed as reported previously (20). Other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Spheroplast lysis assay. Cultures were harvested after 16 to 20 h to obtain exponential-phase cells or after 48 h to obtain stationary-phase cells. Washed cells were suspended in TE buffer containing 5% polyethylene glycol 8000 (PEG 8000) and diluted to a concentration of 2 × 10⁶ cells/ml. The cells were preincubated for 30 min at 30°C. At the start of each assay, 200 µl of the cell suspension (4 × 10⁸ cells in TE buffer containing 5% PEG) and 50 µl of a Zymolyase solution (0 to 200 µg/ml in TE buffer) were mixed into each well of a 96-well flat-bottom plate. The final PEG concentration was 4%. Individual wells on the same plate were used for replicates. The microtiter plate was placed either in a Biotek Powerwave model 200 reader or in a Bio-Rad model 400 reader. Unless otherwise stated, the plates were shaken with an orbital radius of 0.021 in and rotation frequency of 19 Hz continuously between readings. The temperature was 30 ± 1°C. The first data (see Fig. 1 and 2) were obtained 1 min after mixing was begun.

Data analysis. The optical density at each time point for each well (OD) was divided by the initial optical density for that well (ODinit). Replicate values of the ratio (OD/ODinit) were then averaged, and standard deviations (SD) calculated. The error bars for the first point in each curve are SD of OD/ODinit divided by mean OD/ODinit. Log values for error bars were calculated from log (SD) = 0.5 [log (X + SD) - log (X - SD)], where X is the mean OD/ODinit. The maximal lysis rate (MLR) was the absolute value for the slope of the least-squares fit from 10 consecutive points from the steepest portion of the lysis curve. The formula used to determine lag time (LT) was LT = 1/MLR, where yinter is the y intercept of the MLR line.

RESULTS AND DISCUSSION

Adaptation to microtiter format. For X2180-1A, the optical density increased linearly at all densities below 4 × 10⁶ cells per 250 µl of buffer. Settling of the cells resulted in an artifactual increase in the optical density, and inclusion of 4% PEG reduced the sedimentation rate by 60% (data not shown). PEG changed the lysis parameters in complex ways, so the rates obtained at different PEG concentrations are not directly comparable (data not shown). PEG (4%) did not significantly affect the protease or glucanase activities of Zymolyase. Ficoll inhibited cell lysis at all concentrations tested (data not shown).

Tests performed in the presence of osmotic stabilizers confirmed that cell lysis caused most of the measured changes in optical density. For both exponential-phase and stationary-phase cells, KCl (1 M) or sorbitol (1 M) reduced the changes in optical density by 90% when it was included during digestion with Zymolyase. The digested cells lysed spontaneously when water was added.

Assays of experimental cultures. Exponential-phase cells were exposed to different concentrations of Zymolyase (Fig. 1A). The MLR increased linearly with enzyme concentration up to a concentration of 20 µg/ml, and the LT decreased as the enzyme concentration increased (Fig. 1B). The lysis rates reached a plateau at high enzyme concentrations; for one clone, the limiting MLR was 0.036, while the minimal LT was 3.8 min at Zymolyase concentrations greater than 50 µg/ml. At a constant enzyme concentration, the MLR and 1/LT decreased linearly with the log of cell number (data not shown).

We compared the lysis rates for cells grown to the exponential and stationary phases (Fig. 2). A comparison of three exponential-phase cultures harvested over a period of several weeks revealed that the variability within replicates was low and the day-to-day variability was moderate (Fig. 2A). Replicates of a clone in the same assay yielded an SD of the optical density ratio that was 5% of the mean. When the same clone was assayed independently twice in a day, the results were similar (Fig. 2A). For this clone, the mean LT was 2.5 ± 0.3 min, and the mean MLR was 0.028 ± 0.001. The variation was

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somewhat greater for cultures of different clones of X2180-1A assayed over a period of several weeks (Fig. 2A). The grand mean had an LT of $3.5 \pm 0.6\text{ min}$ and an MLR of $0.026 \pm 0.003$ (Fig. 2C). This result implies that the variation between harvested clones was greater than the variation in other factors in the assay.

There was greater variability among stationary cultures (Fig. 2B). Six clones were grown to the stationary phase, each clone was grown in duplicate cultures, and the separate flasks were tested after 48 h of growth. For all trials performed with the same clone, the SD of the optical density ratio, the MLR, and the LT were 1.4, 5.3, and 9.9% of the mean, respectively.

For different clones of stationary cells, the MLR was $0.004 \pm 0.001$ (Fig. 2C). Thus, the variation among the MLR for clones was fivefold greater than the variation between MLR for the same clone. The mean LT was $4.1 \pm 0.8\text{ min}$. When the clones were reassayed after 72 h of growth, resistance to Zymolyase had increased, but the relative order of the lysis values remained the same; i.e., the most resistant clone after 48 h was also the most resistant clone after 72 h, etc. (data not shown). Therefore, the variation in resistance to Zymolyase was due mainly to clonal variation and not to variability in enzyme activity or cell number.

**Data analysis.** The shape of the lysis curve was the same as the shape of the curve obtained in tube-based assays and could be characterized by the parameters LT (the time to the beginning of lysis) and MLR. Determinations of both of these parameters were more precise in the microtiter format assays than in test tube assays. Normalization of the optical density readings corrected for minor variations in absorbance between replicates. LT was previously calculated as the period of time required for the optical density to decrease by 0.050 U. In the microtiter assay, the increased frequency of sampling led to a more accurate estimate of LT based on the MLR.

We obtained several atypical curves with no LT or no tran-
sition from the lag period to the rapid lysis phase. The latter was observed most often with very old stationary-phase cells or in assays in which the enzyme levels were very low; in either case, the LT was infinite. Such assays can be repeated at a higher enzyme concentration. There may be no LT if enzyme concentrations are too high or cell walls have been weakened by either genetic dysfunction or chemical pretreatment (15, 20); these assays should be repeated with lower enzyme concentrations.

Summary. The microtiter format provided a great advantage for the spheroplast assay since both the optimum cell number and enzyme usage were reduced 15-fold. Therefore, the limiting factor in throughput was growth and preparation of yeast cells. We made seven major observations, as described below.

(i) The spheroplast lysis assay in the microtiter format produced results similar to the results obtained with the test tube format. (ii) Rotary shaking alone was not sufficient to prevent sedimentation during the assay; the microtiter format required shaking and addition of PEG 8000 to retard sedimentation of the cells during the assay. (iii) The enzyme activities and lysis rates varied with the PEG concentration. (iv) At a constant PEG concentration, MLR and 1/LT increased with the enzyme concentration and decreased with the log of the cell concentration. (v) The SD of the optical density for replicates of a trial were usually 1% for stationary-phase cells and 5% for log-phase cells. (vi) The SD of the optical density was the same when cells of a clone were grown in different flasks under identical conditions or were assayed twice on the same day. (vii) Clonal variation was the largest source of day-to-day variation.

Applications. The spheroplast lysis assay can now be used to monitor changes in cell wall structure with more precision. The uses of this assay can include screening of different strains for susceptibility to a drug (15) and screening in order to determine cell wall effects of different agents with a single yeast strain (9). In these cases, the minimum significant differences (twice the SD) between values for the same clone are 10% for stationary-phase cells and 5% for log-phase cells. (vi) The SD of the optical density was the same when cells of a clone were grown in different flasks under identical conditions or were assayed twice on the same day. (vii) Clonal variation was the largest source of day-to-day variation.

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