Applications of a microplate reader in yeast physiology research

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Microplate readers have been useful assistants of researchers for several decades. This work is focused on the applications of a simple absorbance microplate reader in yeast physiology research, and its advantages and limitations in comparison with alternative methods are discussed. The two main procedures involved are measuring growth curves and monitoring the pH changes of medium using two different pH indicators. We suggest mathematical formulas for converting absorbance data into pH values. With a microplate reader, as many as 96 samples can be simultaneously analyzed, while medium consumption is minimized to 100 μL per sample. The results can be observed in 24–48 h (for growth curves) or in 1–3 h (for pH changes) with minimal hands-on time required.

INTRODUCTION

Miniaturization and high-throughput screening is currently the focus of research activity in modern drug research facilities. Multiwell plates (or microplates) have been standard tools in analytical research and clinical diagnostic testing laboratories for several decades, as conventional test tubes were too cumbersome and slow (1,2). In the 1990s, microtiter plates were standardized, enabling more robotic and machine reading of their contents than ever before. This has made microplate readers more and more valuable. For standard enzyme-linked immunosorbent assay (ELISA) measurements and enzyme activity monitoring, stationary instruments with end point reading are sufficient (3). High-quality instruments also allow incubating of samples at a chosen temperature with shaking, enabling the cultivation of living cells and kinetic measurements of absorbance changes during specific time periods. Highly sophisticated readers specially designed (e.g., fluorescence assays or nephelometry) have been recently developed, some of them even equipped with an integrated pipeting system, and are able to read microplates of up to 1536 wells.

In yeast physiology and pathogenicity research, microplate readers have mainly been used for the identification of clinical yeast-like isolates (4) and serological diagnostics of Candida infections by immunocapture techniques detecting anti-Candida antibodies (5) and later also for antifungal susceptibility testing (6) or rapid detection of the effects of various chemicals (7). This work is focused on the applications of a simple absorbance microplate reader (96-well format with temperature control and shaking option) in yeast physiology research. Its advantages and limitations for yeast growth curve measurements and the monitoring of media acidification are discussed. Sample experiments are taken mainly from an investigation of Saccharomyces cerevisiae cation homeostasis, regulated by the intracellular alkali-metal cation/H+ antiporters Kha1 and Nhx1 (8–10) and small GTPase Arl1 (11). A strain lacking all K+ exporting systems (ena1-4Δ, nha1Δ tok1Δ) was used for salt sensitivity tests.

MATERIALS AND METHODS

Yeast Strains and Cultivation Media

All S. cerevisiae strains used in this study are derivatives of the W303-1A strain (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mal10) (12). Additional mutations are listed in Table 1. The strains WAR0 and BWA0 were prepared by deletion of the ARLI gene in W303-1A or BW31 (13), respectively. The deletion was performed by homologous recombination according to the protocol described by Güldener et al. (14). The TOB1 strain was prepared from the TOB strain (15) by excision of the KanMX deletion cassette in the TOK1 locus according to the protocol also described in Reference 14.

Cells were grown inYPD medium (1% yeast extract, 2% bactopeptone, 2% glucose, 15 μg/mL adenine; 2% agar for solid media) or YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose; 2% agar for solid media) with auxotropic supplements (15 μg/mL each adenine, uracil, L-histidine, L-leucine, L-tryptophan) added after autoclaving. Media were sometimes supplemented with KCl or hygromycin B as indicated in the text. The pH was adjusted with KOH or HCl and buffered with 20 mM 2-[N-morpholino]ethanesulfonic acid (MES, pH 5.5–6.5), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, pH 6.5–7.6), or N,N-bis[Hydroxyethyl]glycine (BICINE, pH 7.6–9.0). Media with a pH lower than 5.5 were not buffered.
Apparatus and Material Used for Absorbance Measurements

An ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT, USA) for 96-well ELISA plates, equipped with a shaker and a temperature control unit, was interfaced to a Dell computer running KC4 software (BioTek Instruments). The absorbance data were exported to MS Excel for further processing. A_{595} = 1.0 measured by this type of reader corresponds to approximately 8–9 × 10^7 cells/mL. It should be noted that this value is almost one order of magnitude higher than that of commonly used spectrometers, which is why the maximum absorbance reached seems much lower than that of manually measured growth curves (Figures 1, A, D, and E, and 2A; cf. e.g., Reference 8).

Greiner Bio-One flat-bottom, 96-well PS microplates (Sigma-Aldrich, Prague, Czech Republic) were used for both growth curve measurements and acidification experiments. Plates were covered with a tightly attached sealing membrane (Breathe-Easy; Sigma-Aldrich) to prevent excessive evaporation and cross-contamination of the samples.

For pH monitoring, two similar pH indicators were used. Bromocresol green sodium salt (BCG; color change pH 3.8–5.4) and bromocresol purple sodium salt (BCP; color change pH 5.2–6.8) (both from Fluka, Sigma-Aldrich) and 1% water stock solutions (filter sterilized) were added to media after autoclaving to 0.01% final concentration (16). A Jenway 3505 pH meter (Barloworld Scientific T/As Jenway, Dunmow, Essex, UK) was used for calibration and reference measurements.

Growth Curve Measurements

Fresh cells of each strain were resuspended in the required medium to the initial A_{595} of 0.002. Wells in the microplate were filled with this suspension (100 μL in each well). The absorbance in each well was measured at 595 nm at given intervals (usually 15 min) with intensive shaking of the microplate. Data are shown either as the average of

![Figure 1](image)

**Figure 1.** Various applications of growth curves measurements in microplate reader. A_{595} = 1.0 corresponds to 8–9 × 10^7 cells/mL. (A) Salt sensitivity shown by growth curves (a salt sensitive strain TOB1 with several deletions in genes encoding cation export systems). (B) Salt sensitivity of the same strain shown by relative growth (absorbance at 18 h of growth related to absorbance of control sample without any salt). Eight samples were measured for each concentration; error bars correspond to sd. (C) The influence of various mutations on hygromycin B sensitivity. Strains used: control, BW31; kha1, LMB01; ar11, BWA01; nhx1, AB11c. (D) Relative growth of strains cultivated in YNB medium at various pH levels. Strains used: control, BW31; kha1, LMB01. Four samples of each strain were measured for each pH; error bars correspond to sd. (E) Growth curves for a series of 2-fold dilutions of W303 strain. Numbers in squares correspond to the absorbance of original samples. YPD aliquots were inoculated by these samples (2 μL sample plus 100 μL medium) and grown in the reader for 27 h. (F) Temperature sensitivity—strains cultivated in standard YPD medium at 30° or 37°C. Strains used: control, W303; ar11, WAR0.

<table>
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<tr>
<th>Name</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>BW31</td>
<td>ena1Δ::HIS3::ena4Δ nha1Δ::LEU2</td>
<td>13</td>
</tr>
<tr>
<td>AB11c</td>
<td>ena1Δ::HIS3::ena4Δ nha1Δ::LEU2 nhx1Δ::TRP1</td>
<td>8</td>
</tr>
<tr>
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<td>This work</td>
</tr>
<tr>
<td>LMB01</td>
<td>ena1Δ::HIS3::ena4Δ nha1Δ::LEU2 kha1Δ::loxP-kanMX-loxP</td>
<td>8</td>
</tr>
<tr>
<td>TOB1</td>
<td>ena1Δ::HIS3::ena4Δ nha1Δ::LEU2 kha1Δ::loxP-kanMX-loxP</td>
<td>This work</td>
</tr>
<tr>
<td>WAR0</td>
<td>ar11Δ::loxP-kanMX-loxP</td>
<td>This work</td>
</tr>
</tbody>
</table>
4–8 parallel growth curves (error bars are not shown, but the sd usually did not exceed 15% in relative units. The relative growth rates are expressed in percentage of growth recorded under standard conditions (media without salts, hygromycin, at optimal temperature, pH) at a certain time point (18 or 24 h) according to Reference 17.

A drop test was used as an alternative method for the assessment of growth phenotypes. Fresh cells of each tested strain were resuspended in water and adjusted to the same initial $A_{595} = 1.0$ measured by an Ultrospec 10 Spectrophotometer ($A = 1$ corresponds to $8–9 \times 10^6$ cells/mL; Amersham plc, Little Chalfont, Buckinghamshire, UK). Tenfold serial dilutions were prepared, and 3-μL aliquots of each dilution were spotted on an agar plate. The plate was incubated at 30°C for 2 days, and a digital grayscale image was obtained using a Nikon Coolpix4500 digital camera (Nikon, Tokyo, Japan).

**Glucose-Induced Medium Acidification**

Fresh cells were resuspended in YNB medium without glucose, containing auxotrophic supplements and pH indicator, to $A_{595} = 0.02$. Microplate wells were filled with 100 μL samples of cell suspension. The absorbance of the medium was monitored for 1 h, then a certain amount of glucose was added to initiate acidification (as indicated in the text), and changes in absorbance were recorded for an additional 3 h. Reference measurements were performed in 20 mL of the same medium with the same cell density, where the culture was aerated by a magnetic stirrer and pH was continuously measured with a glass pH electrode and manually recorded every 15 min.

**RESULTS**

**Growth Curve Measurements**

In this paper, we demonstrate a broad variety of applications of monitoring yeast growth with a microplate reader. The first thing we tested was salt sensitivity monitoring. For example, the KCl sensitivity of the TOB1 strain (lacking $K^+$ export systems Ena1-4, Nha1, and Tok1 and thus highly KCl-sensitive) can be shown as a comparison of real growth curves (Figure 1A) or as a percentage of the control culture (Figure 1B).

In a similar experiment, we tested the influence of various mutations on the tolerance of the BW31 strain to the toxic compound hygromycin B (Figure 1C). Depicting the results relatively enables the synoptic comparison of several strains at several concentrations of the chemical without requiring a three-dimensional (3-D) graph. It has previously been shown that both the intracellular H+/alkali-metal-cation antiporters (Nhx1 and Kha1) and the Golgi-localized GTPase ARL1 contribute to hygromycin B resistance of cells (8,18,19). In Figure 1C, we demonstrate that deletion of the $KHA1$ gene reduces hygromycin resistance to a lesser extent than deletion of the $NHX1$ or $ARL1$ genes. Another possible way to calculate the proportionate growth (not used in this study) is the approximation of the area under the growth curves (20,21). This method is slightly more complicated and allows the comparison of curves that cross each other during the period of observation.

Having a set of media with various pH levels, the optimal pH for different strains can be easily assessed. As an example, we show that the $kha1$ deletion (i) does not change the pH optimum of the BW31 strain (which is more or less a plateau ranging from pH 3.5 to pH 5.5), (ii) increases its sensitivity to high pH, but (iii) does not affect its tolerance to low pH (Figure 1D).
If desired, the determination of cell concentrations in liquid samples can also be performed in the microplate reader (Figure 1E). The assay is based on the time required for a sample grown in liquid medium to reach a threshold absorbance (22). A series of samples with a dilution range from $A_{595} = 4$ to $A_{595} = 0.004$ was prepared (measured by the Ultrospec 10 Spectrophotometer, absorbances above 1 measured after 10× dilution), and 1 mL YPD aliquots were inoculated with 20 μL each sample. The microplate wells were filled with 100 μL inoculated medium and incubated at 30°C. Growth time to the mid-exponential phase ranged from approximately 8 h for the sample of $A_{595} = 4$ to 21 h 20 min for the sample of $A_{595} = 0.004$. Figure 1F shows another reader application—the temperature sensitivity monitoring. The ard1 deletion strain does not grow well at 37°C, as described previously by Rosenwald et al. (23).

Last but not least, the reader is able to monitor small differences in the growth rates of strains, which can easily be overlooked with a drop test (if not studied very carefully). Figure 2 shows a comparison of three strains grown at standard conditions (solid or liquid YPD medium, 30°C). The kha1 deletion does not influence the duplication time of cells, as was also shown earlier (8). The slightly reduced growth rate and lower final absorbance of an ard1 mutant in liquid cultures could be observed repeatedly in several genetic backgrounds (cf. Figure 1F).

In drop tests, this feature could only be noticed transiently at the beginning of the experiment. On the second day of growth, when monocolonies are highly visible and the drop tests are usually documented, the difference disappears (Figure 2B).

**Acidification Monitoring**

The idea to use the microplate reader in combination with a colored indicator for monitoring pH changes came from the laboratory of Michael C. Lorenz, UT-Houston (24). They used this method for a large-scale comparison of media acidification by various Candida species. Here we extend the method for two different indicators and suggest mathematical formulas for the conversion of absorbance data to pH values.

Figure 3 shows the microplates with YNB medium of defined pH in individual columns. These plates were used for determination of the calibration curves. The absorbance of medium without the indicator (top line) was subtracted from the values measured in the other lines. Experimental absorbance ($A_{595}$) curves (Figure 4A) were inverted to have the absorbance on the x-axis (Figure 4B), and the data sets fitted to trinomial equations for converting absorbance to pH values. The applicable pH range for broomocresol green is 3.1–6.5 and for broomocresol purple, 4.9–7.6. For small inter-experimental variance (a different microplate, a different sealing membrane), it is helpful to normalize the data by a factor, “ΔA,” which represents the difference between the expected and measured absorbance value for a sample of defined pH. The conversion equation for BCG is then:

$$\text{pH} = 0.324 \times (A + \Delta A)^3 - 1.337 \times (A + \Delta A)^2 + 2.398 \times (A + \Delta A) + 4.554$$

[Eq. 2]

If ΔA exceeds 10% of A, or if any doubts arise whether the equations counted are applicable to the next experiment, it is advisable to measure the final pH at the end of the experiment by a thin pH electrode. If the measured value differs from the value counted from the absorbance data, it is better to measure a new calibration curve for the new conditions.

Figure 5A shows an example of the glucose-induced acidification of YNB medium by the S. cerevisiae strain W303 monitored by changes in BCG absorbance. The acidification rate is dependent on the concentration of glucose added at the time point of 1 h. This kind of experiment takes about 1 h of preparation, 5 min for glucose addition, and another 1 h for subsequent data processing. The 3 h of acidification require no hands-on time. The analogous pH meter experiment (Figure 5B), showing just the two glucose concentrations, requires about the same time to prepare and two 4-h periods of manual pH value recording. The time consumed can be reduced by half, if two pH meters can be used at a time, recording values for two curves in parallel. A certain difference in the acidification rate measured by the pH meter or calculated from the

![Figure 4. Calibration curves of pH indicators broomocresol green sodium salt (BCG) and broomocresol purple sodium salt (BCP) in YNB medium: experimental data (A) absorbance dependency on the pH of media and (B) conversion to pH from the measured absorbance data. Representative images from two independent experiments (variance <5%). Error bars correspond to the representative experiment (seven parallel measurements for each pH).](image)
absorbance data can be caused by (i) a different volume of the test culture or (ii) a different type of aeration of the culture.

**DISCUSSION**

The measurement of growth curves and drop tests on agar plates are among the most widely used methods of testing yeast viability and sensitivity/resistance to all kinds of living conditions. In most cases, drop tests are preferred for their simplicity and capacity—for example, 8 to 12 strains can be tested on one plate containing 20 mL medium, while a similar test in liquid media would require 12 separate cultures. If, on the other hand, one strain needs to be tested under various conditions, preparing multiple cultures, liquid or solid, cannot be avoided. Liquid cultures are more flexible in this case, and automatic reading of absorbance changes can simplify the experiment significantly.

The use of a microplate reader was shown to have several advantages. First, the volume of the media was reduced to 100 μL, which minimized the expenses required for the testing procedures. This feature is especially favorable when testing the sensitivity of cells to expensive chemicals. Another merit of using liquid cultures is the flexibility of the experimental conditions—just by mixing various volumes of various liquid compounds, we can decide on the composition of the test medium at the last moment. For example, the salt tolerance experiment shown in Figure 1, A and B, was done using just two kinds of stock media (YNB without any salt and YNB with 2 M KCl), mixing the appropriate volumes of each to reach the desired concentration of KCl. The total volume of medium used [and subsequently genetically modified organism (GMO) waste produced] was approximately 10 mL. A similar experiment, performed as a drop test, would require nine separately prepared Petri dishes. Liquid media is also advantageous for pH sensitivity tests—there is no need to concern oneself with whether agar withstands autoclaving at the desired pH level.

The temperature sensitivity of strains can be tested with certain limitations (i.e., it is not possible to incubate samples in one microplate at several different temperatures). But if the conditions are well defined (the same media, initial absorbance and physiological state of the cells, and inoculation procedure), curves from different experiments can be put together in one graph.

There is also some limitation in the use of the reader for acidification monitoring. pH meter experiments are usually performed at cell densities of $10^7$–$10^8$ cells/mL, and results are observed over a period of about 30–60 min (25). As the indicator absorbance is measured at the same wavelength as the absorbance of cells (595 nm), a concentrated cell suspension might distort the results. That is why these reader experiments were performed at approximately $10^6$ cells/mL and over a longer time period. However, if the parameters are standardized, this feature does not create any problems in relative comparisons of various strains or of one strain under various conditions. It should also be noted that the equations given here are valid for 100 μL YNB samples with 0.01% indicator. Other conditions would require other calibration curves, but they can be easily determined by an analogous method.

The applications of this method are not limited to monitoring the glucose induction of the Pma1 ATPase. The method can be used, for example, to compare the effects of various mutations on the acidification rate, to investigate the strength of promoter regulating the proton pump expression, or to test the effect of proton pump inhibitors. Further, the method can be applied to medical research, exploring the ability of clinical isolates to change the pH of various media.

In summary, a simple microplate reader connected to a computer with a data evaluation program can be very useful in laboratories working in the field of yeast physiology. Having machines monitoring the yeast growth rate helps to reduce the amount of human subjectivity, which is often involved in evaluating tests that depend heavily on easily misread visual information (like drop tests). Multiple samples in one experiment and uncomplicated repetition simplify the statistical evaluation of data. Further development of these methods is limited only by the researcher’s imagination—for example, measurements of growth dependence on various parameters (two different salts together, a toxic compound at several pH levels, or acidification rate at various temperatures). An even broader range of applications can be found for fluorescence microplate readers, but that exceeds the scope of this paper. However, even a standard absorbance reader can save a lot of time, effort, and material and reduce the amount of contaminated waste in the laboratory.
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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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