Monitoring aerobic *Escherichia coli* growth in shaken microplates by measurement of culture fluorescence

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Combining the advantages of both the microplate (high-throughput, ease of automation) and the classic shaken Erlenmeyer flask (adequate mixing and oxygen supply), shaken 48-well microplates are an excellent small-scale fermentation system for microbial cultivation (1). Most physiological studies are at least semiquantitative (2), and real-time biomass estimation during a bioprocess is an important goal (3). Therefore, a simple method for the monitoring of microbial growth in these 48-well plates is necessary.

Intracellular NADH and NADPH in living microorganisms can be excited to fluorescence (460 nm) by irradiating the cells with UV light (366 nm). This fact is used as the basis of a noninvasive online method for fermentation process monitoring in stirred tank bioreactors (3–5). Since fluorescence readers for microplates are common available analytical instruments, the adaptation of this technique to the microplate should be accomplishable. Researchers, however, have been using either turbidity measurements (6,7) or specially designed microplates with integrated fluorophores (8–10) for growth monitoring in multwell plates during high-throughput screening (HTS).

The optical density or absorbance (A) of a suspension diluted in such a way that light scattering centers are essentially independent is directly proportional to the biomass concentration (C) (2):

\[
A = k \times C \quad \text{[Eq. 1]}
\]

where \(k\) is the proportionality constant. Biomass C can be calculated from NAD(P)H-dependent fluorescence (F) by (4,5):

\[
C = [\exp(-b^* \times F)]^{1/a} \quad \text{[Eq. 2]}
\]

where \(a\) and \(b^*\) are constants. Equation 1 may then be written similarly to Equation 2 as:

\[
A = [\exp(-b \times F)]^{1/a} \quad \text{[Eq. 3]}
\]

where \(b^* = b - (a \times \ln k)\).

To verify that Equation 3 is likewise true in microscale, both the absorbance and the fluorescence of serial dilutions of two JM101 cultures grown in two different media were determined.

Terrific broth (TB) and Luria Bertani broth (LB) (both from Sigma, St. Louis, MO, USA) were used as media and inoculated with 1% (v/v) of a JM101 glycerol stock with \(5 \times 10^8\) viable cells/mL. The reaction vessels were glass Erlenmeyer flasks (100 mL, four baffles) and flat-bottom transparent polystyrene arrays, namely nontreated 48-well cell culture plates (BD Biosciences Discovery Labware, Bedford, MA, USA) and 96-well microplates (Greiner, Frickenhausen, Germany). Two flasks were filled with 10 mL inoculated TB and LB, respectively, closed with a cotton plug, and incubated for 18 h at \(37^\circ\)C on a rotary shaker with 25 mm diameter at 180 rpm. Then three serial dilutions of both cultures in respective media were pipetted, each in both a 48-well plate (400 μL/well) and a 96-well plate (200 μL/well) using a Beckman 2000 liquid handler (Beckman, Fullerton, CA, USA). Absorbance was determined by reading the 96-well plate in a SpectraMax® reader (Molecular Devices, Sunnyvale, CA, USA) at 620 nm. Pure media were used as blanks, and data was converted to 1 cm path length. A linear correlation is only true if \(A < 0.5\) (7). Higher A values, however, may be calculated from more diluted samples. The means of each 3-fold measurement, as well as the results from calculations, are shown in Figure 1A. F was determined by reading the 48-well plate in a FLUOstar reader (BMG, Offenburg, Germany) using the following crucial settings: no. of flash-
es 5, excitation filter 370-10, emission filter 460, time-resolved mode, integration start 10 μs, integration time 20 μs. Both excitation and emission were performed via the bottom optics. Media were used as blanks. The means of each 3-fold measurement are presented in Figure 1B. To experimentally verify Equation 3, F versus A was plotted in double-logarithmic coordinates (Figure 1C), with constants a and b* being the respective slopes and intercepts of the least square straight lines, depending on the medium.

To test the new method for its practical usefulness in HTS, growth experiments in a 48-well plate were performed, and the autofluorescence was monitored. Inoculated TB and inoculated LB, as well as pure TB and pure LB, were each filled (400 μL) in three wells of a 48-well plate. Pure TB and LB served as blanks and contamination control. The plate was incubated at 37°C and 60% relative humidity on a custom modified Tele-shake 4 shaker (H+P Labortechnik, Ober, schleissheim, Germany) with 3 mm shaking diameter at 1100 rpm. These shaking parameters are crucial for adequate oxygen supply (1). The plate had been sealed with three layers of BreathSeal tape (Greiner, Frickenhausen, Germany), which is highly oxygen permeable (11). Under the used shaking and incubation conditions, even three layers of this sealing tape do not influence oxygen transfer but reduce evaporation below 10% per day (data not shown). After every 2 h, the shaker was automatically stopped, the microplate was taken from the shaker and put into the FLUOstar reader by a Sagiant™ ORCA robotic arm (Beckman), read (as described above), and put back on the shaker. The results are plotted in Figure 2, which shows that the expected better growth in TB is clearly indicated by higher fluorescence values.

NAD(P)H-dependent fluorescence varies with the microorganism (12,13) as well as with the metabolism and physiologic state of the cells (4,5,12), which are dependent on culture conditions (13,14). Exact biomass calculation from autofluorescence data is therefore only reliable if all these parameters are known and considered (4,5,12). Biomass calculation would be error-prone and is not recommended to be done during the screening of different unknown strains or during medium optimization. Autofluorescence reading, however, has three major advantages over absorbance reading in microplates: (i) it is useful for the immediate indication of certain metabolic changes (4,5,12) because NAD(P)H is directly involved in cen-

Figure 1. Comparison and relation of absorbance and fluorescence.
(A) Absorbance (A; 620 nm, path length 1 cm) of serial dilutions in a 96-well plate of JM101 shake flask cultures in Terrific broth (TB) and Luria Bertani broth (LB) medium. A linear correlation is true for measured values of A < 0.5. Higher values, however, may be calculated from lower dilutions to meet a complete linear proportionality. Standard deviation of each 3-fold determination is always < 0.05. (B) Fluorescence (F; 370 nm/460 nm |1000 fluorescence units (kFU)| of serial dilutions in a 48-well plate of JM101 shake flask cultures in TB and LB medium. Error bars indicate ± sd of each 3-fold determination. (C) Double-logarithmic plot of F [fluorescence units (FU)] (values from Figure 1B) versus A (calculated values from Figure 1A). The linear regressions according to Equation 3 have the following properties: (TB) a = 1.3006, b* = 7.8164, \( R^2 = 0.9974 \); (LB) a = 1.0811, b* = 8.2713, \( R^2 = 0.9952 \).

Figure 2. Fluorescence (F; 370 nm/460 nm |1000 fluorescence units (kFU)|) during 24-h fermentations in a shaken 48-well plate. Error bars indicate ± sd of each 3-fold determination. F increases faster and higher in Terrific broth (TB) than in Luria Bertani broth (LB) medium (filling volume 400 μL, shaking diameter 3 mm, shaking frequency 1100 rpm, 37°C).
常务 metabolism (14); (ii) dilution of samples is in certain cases (e.g., LB graphs in Figure 1) unnecessary, even at higher cell concentrations; and (iii) the reading is not hindered by a non-transparent sealing tape as long as excitation and emission are carried out through the bottom of the plate. In addition, unlike the dissolved oxygen measurement in microplates (8–11), measurement of autofluorescence does not require any exclusive disposable labware with integrated sensors. Thus, this noninvasive method emerged as an economical tool for semiquantitative analysis and monitoring of growth in microplates during HTS.

REFERENCES


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