Look and See if It Is Time To Induce Protein Expression in *Escherichia coli* Cultures†‡

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ABSTRACT: It is shown that Methyl Red can be used as an indicator dye that changes color in *Escherichia coli* culture as a result of time- and cell density-dependent bleaching by azoreductase produced by the bacteria. For cell cultures that are being used to express a recombinant protein, this phenomenon can be exploited to provide a simple visual cue that cell cultures have reached an appropriate growth phase for addition of an agent to induce protein expression, such as isopropyl thiogalactoside.

Since the advent of the modern era of preparative molecular biology in the 1980s, cultures of *Escherichia coli* have been widely used to overexpress recombinant proteins. The most commonly used methods involve the growth of liquid cultures to midlogarithmic phase followed by addition of a gene transcription-inducing agent, typically IPTG. The most commonly used method for determining when midlog phase has been reached is to monitor light scattering of cultures by determining the apparent absorption of light at 600 nm. This can be a time-consuming and onerous task, requiring repeated measurements for each culture flask until an appropriate OD600 is reached (usually when OD600 is between 0.5 and 1.0). To see if a visual colorimetric method could be devised to determine when midlog phase is reached, *E. coli* cultures were grown in the presence of a variety of dyes and/or indicators (Table S1 of the Supporting Information) at concentrations that confer a visually obvious color to the medium. For these tests, we used BL21(DE3) *E. coli* harboring a pET21b vector encoding the 99-residue C-terminal domain of the amyloid precursor protein (C99)† (1). When pH 7.0-buffered M9 cultures were then incubated with rotary shaking at 37 °C, no change in culture color was observed for most dyes. However, in the case of 100 mL or 1 L cultures grown in the presence of 20 mg/L Methyl Red, it was observed that when the OD600 reached 0.67 ± 0.1 or 0.75 ± 0.1, respectively, the cultures completed a change in color from orange to a pale yellow (Figure 1 and Figure S1 of the Supporting Information). Similar results were obtained for BL21(DE3) cells harboring an empty pET21b plasmid. Tests with 20 mg/mL Methyl Red in 1 L cultures of a different strain of *E. coli* (WH1061) harboring a different recombinant plasmid [pSD0005 encoding diacylglycerol kinase (2)] completed the same orange-to-colorless change at an OD600 of 0.87 ± 0.25, although some calibration of culture conditions was required to ensure that the color change occurs when OD600 reaches the 0.5–1.0 range (see the Supporting Information). For each strain or vector, it is especially important to optimize the volume of the starter culture used to inoculate the fresh dye-containing M9 medium.

M9 cultures (1 L) of BL21(DE3) with C100 and WH1061 with DAGK were grown in the presence of 20 mg/L Methyl Red, and protein expression was induced by IPTG when Methyl Red-containing cultures went colorless or when the OD600 of dye-free cultures reached 0.6–1.0. In either case, expression was allowed to proceed for several hours followed by harvesting of the cells and purification of the recombinant proteins according to published methods (1, 3). For both C99 and DAGK, it was found that the expression levels were comparable to expression levels in cells grown and induced using identical methods but without Methyl Red.

Direct measurement of the kinetics of cell culture growth for BL21(DE3) with C99 grown in the presence and absence of Methyl Red revealed that cells grow a little slower and to a lower final density when cultured in the presence of Methyl Red (Figure S2 of the Supporting Information), suggesting that the presence of the dye stresses the cells. This was supported by the observation that cells would not grow in the presence of 30 mg/L dye as opposed to the usual 20 mg/L dye. We also conducted an experiment

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Abbreviations: ABA, 2-amino-benzoic acid; C99, 99-residue C-terminal domain of the human amyloid precursor protein; DAGK, *E. coli* diacylglycerol kinase; DMPD, N,N-dimethyl-p-phenylenediamine; TLC, thin layer chromatography.
in which cells were grown in the presence of 10 mg/L Methyl Red, in which case the color change was observed to occur only at a much higher OD600 (1.6) for WH1061 with DAGK. This suggests that the rate of dye bleaching is not directly proportional to E. coli biomass. Rather, it appears that the process responsible for bleaching the dye is induced in response to dye-induced stress and at levels that are proportional to the concentration of the dye.

Additional tests indicated that this method can, following reoptimization of conditions, be applied to M9 media at different pH values (e.g., pH 6.25 and 7.7), strain–protein combinations [e.g., RosettaBlue/pAH13 expressing human peripheral myelin protein 22 (4)], and culture temperatures (e.g., 20°C). We found that this method can also be employed using Luria Broth medium.

It has previously been shown that Methyl Red can be cleaved and decolorized by a stress-induced azoreductase found in E. coli and other microorganisms (5–7). This flavoenzyme converts Methyl Red into N,N0-dimethyl-p-phenylenediamine (DMPD) and 2-aminobenzoic acid (ABA) (see Figure 2). Using thin layer chromatography (TLC), we confirmed that the disappearance of Methyl Red in E. coli cultures was accompanied by the appearance of compounds that exhibit the same Rf on two-dimensional thin layer chromatography (Figure 3) and the same response to ultraviolet light as ABA and DMPC standards (ABA fluoresces, and DMPD initially absorbs but then turns brown). We found that E. coli did not bleach Methyl Orange or Methyl Yellow (Figure 2), at least not at a rate sufficient to generate a color change during the time course of growing a culture to the postlog phase.

To conclude, it appears that Methyl Red can be used as a colorimetric indicator to provide a visual cue that E. coli cultures harboring a recombinant expression plasmid have reached an optimal phase of growth for induction of protein expression. For each strain and recombinant protein, significant calibration is required to determine exact conditions for obtaining useful and reproducible results. Given that reoptimization of the method is required for any new strain, recombinant plasmid–protein, and/or culture conditions, we suggest that this approach will be most useful for investigators who are routinely and repetitively expressing the same protein, as is often the case when a protein is being subjected to long-term biochemical or structural biological studies, or to biotechnological exploitation.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Full methodological details, Table S1, Figure S1, and Figure S2. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


Supporting Information for:

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Supporting Materials and Methods

Materials and Media. Methyl Red was from Sigma-Aldrich (catalog number M7267). 1.00 mg/ml stock solutions of Methyl Red were prepared in ethanol. The M9 minimal medium used contained (per liter) 42mM Na2HPO4 (6g), 22mM KH2PO4 (3g), 9mM NaCl (0.5g), 19mM NH4Cl (1g), 0.1mM CaCl2 (0.01g), 1mM MgSO4 • 7 H2O (0.25g), 0.4% glucose (4g), and 1X MEM vitamins (Cellgro by Mediatech - Cat. No. 25-020-Cl, 10mL of 100X solution). The pH was adjusted to 7.0. Immediately prior to inoculation of M9 cultures with LB starter (cultures) below, the Methyl Red/ethanol stock was added to the medium to a concentration of 20.0 mg/liter of culture.

Practical Comments on Optimization of Conditions. Based on our experience it appears that each E. coli strain and expression vector combination requires some trial and error optimization in order to find conditions where the culture will reproducibly complete color change at a desired cell density/growth phase (assessed of OD600). It is important to always use exactly the same Methyl Red concentration. We also found that for a given strain/vector/medium combination, a key parameter to optimize is the volume of the overnight 37°C LB culture used to inoculate the M9 medium. Note that any change in culture temperature, composition, or strain/vector will likely require re-optimization. Once ideal conditions have been identified, care should be taken to make sure all conditions are reproduced from culture to culture as precisely as possible. Because the presence of Methyl Red does stress E. coli, there appear to be some cultures that will not grow due to the
additive stress both of expressing a difficult protein and the presence of the dye. For example, while cultures of RosettaBlue cells harboring the pAH13 vector encoding human peripheral myelin protein 22 would grow at 20°C in the presence of 20 mg/ml Methyl Red, this was not the case at 37°C.

**Growing 100mL C99 Cultures with Methyl Red.**

Plasmids expressing C99 were transformed into E. coli strain BL21(DE3). A 5 mL LB medium starter culture with 100 μg/mL ampicillin was inoculated with a colony from a plate, grown overnight at 37°C, and then used to inoculate 100 ml M9 minimal medium containing 20 mg/L Methyl Red. The 100mL culture was incubated at 37°C with orbital shaking and OD600 was monitored and recorded hourly until color loss was completed. Similar results were obtained when cultures were inoculated with 2.5 ml of starter culture.

**Growing 1L C99 Cultures with Methyl Red.**

Plasmids were transformed as above. A 50mL LB medium starter culture with 100 μg/mL ampicillin was inoculated with a colony from a plate and grown overnight at 37°C. 25mL of the starter culture was used to inoculate 1L M9 minimal medium containing 20 mg/L Methyl Red. The culture was incubated at 37°C with orbital shaking and OD600 was recorded hourly until color loss was completed. When 1L cultures were inoculated with 25 ml of LB starter culture, the color loss was not completed until OD600 reached 1.6.

**Growing 1L DAGK Cultures with Methyl Red.**
Plasmids expressing DAGK were transformed into E. coli strain WH1061. 1L M9 minimal medium including 20 mg/L Methyl Red was inoculated with 100mL LB medium starter culture with 20 μg/mL kanamycin and 100 μg/mL ampicillin. The culture was grown at 37°C with orbital shaking and OD600 was monitored and recorded hourly until color loss was complete. The LB starter culture was itself inoculated with a 5 ml overnight LB culture that was inoculated with a colony from a plate. 1L cultures inoculated with only 25-50 ml of overnight LB culture grew erratically, often displaying only very slow growth.

Table S1. Dyes Tested as Possible Colorimetric Indicators for E. coli Cultures.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Concentration in M9 Medium (mg/L)</th>
<th>Did E. coli Cells Grow at 37ºC?</th>
<th>Was There a Color Change or Color Loss?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alizarin</td>
<td>20</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Azure A</td>
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<td>no</td>
</tr>
<tr>
<td>Brilliant Blue R</td>
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<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
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<td>no</td>
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<tr>
<td>Bromothymol Blue</td>
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<td>no</td>
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<tr>
<td>Cresol Red</td>
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<tr>
<td>Indigo Carmine</td>
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<td>Methyl Red</td>
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<tr>
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<tr>
<td>Phloridzin</td>
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<tr>
<td>Sudan IV</td>
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<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>
Supporting Figure 1

Figure S1. Time course for growth of E. coli culture in minimal medium (as indicated by increase of OD600, open circles) and bleaching of Methyl Red (as indicated by reduction of OD425) and correlation with visual appearance of culture. The OD600 and OD425 values represent the average of results from four different 1L cultures, with standard deviations as shown. 1L M9 minimal media cultures with BL21(DE3) cells expressing C99 were monitored. All cultures were inoculated with 25mL of overnight LB culture and grown at 37°C.
Figure S2. Time course for E. coli culture growth in presence (open squares) or absence (open circles) of 20 mg/L Methyl Red. 1L M9 minimal media cultures with BL21(DE3) cells expressing C99 were monitored. All cultures were inoculated with 25mL of overnight LB culture and grown at 37°C. Optical density at 600nm was monitored for 36 hours. Each point is the average observed for 3 different cultures. Standard deviations are shown, but are in many cases smaller than the symbol.