H_2O_2 Kill Assays of Planktonic Stationary Phase Bacteria

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[Abstract] Stationary phase bacteria are highly tolerant to hydrogen peroxide. This protocol was developed to test the susceptibility to hydrogen peroxide killing in different *Pseudomonas aeruginosa* strains. This assay provides a reliable way to measure killing of stationary phase bacterial cells to hydrogen peroxide and can be adapted to test other oxidants.

Materials and Reagents

1. Phosphate buffered saline (PBS) solution (Sigma-Aldrich, catalog number: P4417-100TAB)
2. 30% w/w Hydrogen peroxide stock solution (RICCA Chemical, catalog number: 3821.7-32)
3. Sodium thiosulfate solution (dissolved in ddH_2O) (Sigma-Aldrich, catalog number: S8503)
4. *P.aeruginosa* strains in freezer stock
5. 25% Lennox broth (LB) medium (Becton Dickinson and Company, Difco™, catalog number: 240230) (see Recipes)
6. LB agar plates (see Recipes)

Equipment

1. 96-well plates
2. Standard petri plates
3. Spectrophotometer (cuvette) (Thermo Fisher Scientific, model: GENESYS 10S UV-Vis)
4. Spectrophotometer (96-well plate) (Bio-Rad Laboratories, model: 680)
5. Cuvettes for OD_{600} reading
6. Shaker-incubator at 37 °C, 250 rpm
7. Static incubator at 37 °C
8. Sterile glassware: 150 ml Erlenmeyer flask, capped or foiled
9. Sterile 15 mm glass test tubes and plastic caps
10. Sterile wire-loops (sterilized with 70% ethanol and flame)

Procedure

1. Day 0. Streak *P.aeruginosa* cells from the freezer stock onto a LB agar place and incubate statically overnight at 37 °C.
2. Day 1. Pick 4-5 single colonies from the *P.aeruginosa* agar plate with a sterile wired-loop and inoculate 15 ml liquid LB medium in a 150 ml Erlenmeyer flask. Grow liquid bacterial cultures overnight for 16-18 h at 37 °C, with shaking at 250 rpm.
3. Day 2. Inoculate 15 ml liquid LB medium in a 150 ml flask with 1:100 of overnight bacterial culture. Grow cells to for 16-18 h at 37 °C, with shaking at 250 rpm.
4. Day 3. Determine the OD_{600} of the culture and dilute the bacterial suspension to a starting concentration of ~2.5 x 10^6 cells/ml (in total volume 1 ml LB). Depending on the bacterial strain used, the OD_{600} to CFU ratio will differ and needs to be determined for each strain: for example, for the PAO1 wild type strain, 10^8 cells/ml = ~OD_{600} 0.1.
5. To confirm the correct starting bacterial density (at ~2.5 x 10^6 cells/ml), aliquot 100 µl of the above bacterial suspension into 96-well plate. Serially dilute cells 1:10 in PBS to approximately ~2.5 x 10^2 cells/ml, then plate 100 µl on LB agar plates for CFU count. This will also be the CFU count for time zero measurement.
6. Set up ~2.5 x 10^6 cells/ml x 1 ml per sample in sterile glass tubes, with at least 3 replicates per strain per condition. For H_2O_2 treated samples, add H_2O_2 (1 mM (2 µl) to 5 mM (10 µl) or other desired final concentration) to each samples in test tubes. Include untreated controls that are challenged with PBS. Each condition should be done at least in triplicates. Incubate cells for 2 h with shaking at 250 rpm at 37 °C.
7. After H_2O_2 or PBS challenge, add 0.2% sodium thiosulfate to all samples to neutralize any remaining H_2O_2. Add even when samples are only challenges with PBS as a control.
8. To determine the viable cell count in H_2O_2 or PBS treated samples, aliquot 100 µl of bacterial samples into 96-well plate, serially dilute cells 1:10, then plate 100 µl of each dilution on LB agar plates for CFU count. Incubate CFU count plates at 37 °C overnight.
9. Day 4. Count CFU on LB agar plates and calculate the viable CFU per biofilm based on the dilution factors applied.
10. Determine hydrogen peroxide killing by comparing the viable CFU count in the PBS treated and the H_2O_2 treated conditions.

Recipes

1. 25% LB medium
   5 g LB powder medium per L
   Dissolved in ddH_2O and autoclaved

2. LB agar plates
   LB medium with 1.5% agar
   Dissolved in ddH_2O and autoclaved

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References

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