Test Method For:

Efficacy of Antimicrobial Agents to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables

1. Scope

1.1. This test method is used to determine if antimicrobial agents effectively reduce populations of foodborne pathogenic bacteria in fruit and vegetable processing waters.

2. Significance and Use

2.1. Antimicrobial agents are often added to water used in the transportation and/or washing of fresh fruits and vegetables to reduce the populations of microorganisms in fruit & vegetable process water. Reducing populations of microorganisms in recycled process water helps prevent it from becoming a vector of cross-contamination.[1-6] Processing water antimicrobial agents are usually more effective in reducing microorganisms suspended in water than on fruit and vegetable surfaces.[1-5, 7-15] This test method evaluates the ability of antimicrobial agents to reduce the number of pathogenic bacteria suspended in fruit and vegetable processing waters.

3. Apparatus

3.1. Balance – An analytical balance sensitive to 0.1g

3.2. Glassware – 100 mL milk dilution bottles, 250 mL Erlenmeyer flasks, various sized volumetric flasks, various sized volumetric pipettes, glass beads, test tubes with caps.

3.3. Plasticware - 15x100 mm sterile disposable petri dishes and sterile disposable pipets.

3.4. Water Bath – Capable of maintaining a test temperature of 25±2°C.

3.5. Inoculating Loops

3.6. Buchner Funnel – Sterile, containing Whatman No. 2 filter paper.

3.7. Vortex Mixer

3.8. Spectrophotometer - Able to measure % Transmittance at 580 nm

4. Reagents and Materials

4.1. Culture Medium

4.1.1. Brain Heart Infusion Broth and Agar - For propagation and enumeration of *Listeria monocytogenes*.

4.1.2. Tryptic Soy Broth and Agar - For propagation and enumeration of *Escherichia coli* and *Salmonella choleraesuis*.

4.2. Inactivating Agent – Generally 0.1% Sodium thiosulfate is effective for halogens (chlorine and iodine compounds) and peroxyacetic acid. The efficacy of any inactivating agent must be demonstrated. (See Antimicrobial Agent Inactivation – Section 8.1)

4.3. Phosphate Buffered Dilution Water (PBDW)

5. Test System Preparation

5.1. The test systems used for this method include three strains of *Listeria monocytogenes* (ATCC 49594, 19114, 19116), three strains of *Escherichia coli* serotype O157:H7 (ATCC 43895, 35150, 43890), and three strains of *Salmonella choleraesuis* subsp. *choleraesuis* (serotype *javiana* ATCC 10721, serotype *newport* ATCC 6962, serotype *typhimurium* ATCC 13311).

5.2. From a stock culture, make >4 but <15 consecutive daily transfers on agar slants with incubation at 35±2°C.

5.3. Inoculate French slants

5.3.1. From a 99 mL bottle of phosphate buffered dilution water (PBDW), transfer 5 mL onto an agar slant containing growth of the test system. Mix or shake the slant to suspend the growth in the PBDW. Transfer the organism suspension back into the same bottle of PBDW. Mix this suspension well and add 2 mL to each French slant. The number of French slants required depends on the size of the test. Tilt the French slant back and forth so the 2 mL wets the entire agar surface. Remove the excess liquid aseptically. Incubate the slants in a horizontal position at 35±2°C for 24±4 hours.

5.4. Harvest the culture from the French slant agar by adding 3 mL of PBDW, 10-30 sterile glass beads and shaking back and forth. Collect the resulting cell suspension and beads in a test tube and Vortex mix for 30 seconds. Filter the suspension through a sterile Buchner funnel containing Whatman No. 2 filter paper that has been prewet with PBDW. Collect the suspension in a sterile container.

5.5. Combine equal volumes of each strain of *L. monocytogenes* in a sterile container. Do the same for *S. choleraesuis* and *E. coli* O157:H7.

5.6. Adjust the density of the culture suspension to yield approximately 10^{10} organisms per milliliter. This can be accomplished by measuring the %Transmittance (%T) of the culture suspension at 580 nm and adding PBDW as necessary. The %T reading needed to achieve a 10^{10} organisms/mL culture suspension must be determined prior to performing this test. The following are suggested starting points: 0.6 %T (*S. choleraesuis*), 0.7%T (*L. monocytogenes*) and 0.4%T (*E. coli* O157:H7).

6. **Antimicrobial Agent Preparation**

6.1. Dilution Water Preparation

6.1.1. Prepare a sufficient quantity of sterile deionized water that has been adjusted to a total hardness as $CaCO_3$ at 200 – 400 ppm.

6.1.2. Add 1% (wt./wt.) of a sterile mixed vegetable juice to the dilution water.

6.2. Prepare the antimicrobial agent working solution by diluting in the dilution water. Use at least 1.0 mL or 1.0 g of the agent to prepare this dilution.

6.3. After preparation, verify that the concentration of total peracid (as POAA) in the use-solution is acceptable for efficacy testing. Determine the concentration of total peracid (as POAA) in the use-solution by iodine-sodium thiosulfate redox titration.

6.3.1. Rinse a 10 mL capacity test vial with the use-solution to be tested.

6.3.2. Fill the vial with 10 mL of the use-solution to be tested.

6.3.3. Add 200 µL of 28% Sulfuric Acid. Mix.

6.3.4. Add 200 µL of 10% Potassium Iodide. Mix. If peroxyacetic acid was present in the test solution; it will have preferentially oxidized the iodide resulting in the release of iodine. The release of iodine in the test solution is indicated by a yellow color.

6.3.5. Add 200 µL of 2.0% Starch Indicator. Mix. Starch turns blue in the presence of iodine.

6.3.6. Add .025% Sodium Thiosulfate until the blue color just disappears. This can be done by adding 40 µL increments (40 µL equals approximately 5 ppm total peracid (as POAA).

6.3.7. If necessary, Add 0.08% Sodium Thiosulfate (40 µL equals approximately 1 ppm total peracid (as POAA).

6.4. If the total peracid (as POAA) concentration in the use-solution is not acceptable, adjust the concentration by adding additional test substance or diluent and titrate again. Repeat this procedure until the desired concentration is achieved.

7. **Operating Technique**

7.1. Dispense 99 mL of the antimicrobial agent working solution into a sterile 250 mL Erlenmeyer flask. Prepare triplicate flasks for each test system. Also prepare triplicate flasks with 99 mL of sterile PBDW for determination of inoculum populations. Place flasks into a 25°C temperature water bath and let rest until they reach 25±2°C.

7.2. Vigorously swirl a test flask. While the liquid is still in motion, immerse the tip of a pipet containing 1 mL of the test system suspension into the test solution midway between the center and edge of the flask. Dispense 1 mL of the test system suspension into 99 mL of the test solution.

7.3. After a 1 - 2 minute exposure period, transfer 1 mL of the test solution mixture into 9 mL of inactivating agent using a sterile pipet and vortex to mix. This tube is considered a 10^{-1} dilution of the test solution.

7.4. For antimicrobial agent working solution test samples, plate in duplicate 1 mL and 0.1 mL from the 10^{-1} inactivating agent tube. In addition, prepare a 10^{-3} dilution in PBDW and plate in duplicate 1 mL and 0.1 mL from this for *Listeria* test samples only. For the inoculum population tests, prepare 10^{-5} and 10^{-7} dilutions in PBDW. Plate in duplicate 1 mL and 0.1 mL from the 10^{-5} dilution and 1 mL of the 10^{-7} dilution. Use pour plate technique with molten, tempered (46±2°C) Tryptic Soy Agar (or Brain Heart Infusion Agar for *L. monocytogenes*). After agar solidifies in the petri plates, invert them and incubate at 35±2°C for 48±4 hours.

8. Controls

8.1. Antimicrobial Agent Inactivation

8.1.1. Triplicate inactivation checks should be performed using each culture suspension. Testing is performed as follows:

Control A = Add 1 mL of working solution to 9 mL of inactivating agent and mix. Then, add 1.0 mL of the test system suspension dilution containing ~10^2, ~10^3 and ~10^4 CFU/mL and mix.

Control B = Add 1 mL of sterile deionized water to 9 mL of inactivating agent and mix. Then, add 1.0 mL of the test system suspension dilution containing ~10^2, ~10^3 and ~10^4 CFU/mL and mix.

Control C = Add 1.0 mL of the test system suspension dilution containing ~10^2, ~10^3 and ~10^4 CFU/mL to 10 mL of PBDW and mix.

8.1.2. Plate 1 mL and 0.1 mL from each control (A, B, C). Use pour plate technique with molten, tempered (46±2°C) Tryptic Soy Agar (or Brain Heart Infusion Agar for *L. monocytogenes*). After agar solidifies in the petri plates, invert them and incubate at 35±2°C for 48±4 hours.

8.1.3. The inactivating agent effectively neutralized the working solution and was not detrimental to the test system if the average plate counts for control tests A and B are within a 0.5 $\log_{10}$ of the average plate counts for the control test C.

8.2. Antimicrobial Agent Diluent Sterility

8.2.1. Plate 1 mL of the dilution water. Use pour plate technique with molten, tempered (46±2°C) Brain Heart Infusion agar. After agar solidifies in the petri plates, invert them and incubate at 35±2°C for 48±4 hours.

9. Interpretation of Results

9.1. The results of the test should be discarded if any of the following occurs:

9.1.1. The average inoculum populations of any of the organisms are less than 10^6 CFU per milliliter.

9.1.2. The average plate counts for control tests A and B are not within a 0.5 $\log_{10}$ of the average plate counts for the control test C.

9.1.3. The antimicrobial agent diluent is found to be not sterile.

9.2. The antimicrobial agent is effective in reducing foodborne pathogenic bacteria in fruit and vegetable processing waters if populations of all test bacteria are reduced by >99.9% relative to the inoculum populations.

9.2.1. Percent reduction is determined as follows:

$$\% \text{ Reduction} = [(A - B) \times 100] \div A$$

Where:

A = Average population of colony forming units from the inoculum population flasks.

B = Average population of colony forming units recovered from the working solution flasks.

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