National Standard Method

Enumeration of Enterobacteriaceae by the Colony Count Technique

F 23

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division

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AMENDMENT PROCEDURE

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**ENUMERATION OF ENTEROBACTERIACEAE BY THE COLONY COUNT TECHNIQUE**

Issue no: 1.4 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Group F, W & E Co-ordinators Forum and the Environmental Surveillance Unit, CDSC. Page 4 of 11

Reference no: F 231.4

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STANDARD OPERATING PROCEDURE FOR THE ENUMERATION OF ENTEROBACTERIACEAE BY THE COLONY COUNT TECHNIQUE

INTRODUCTION

Scope

The method described is applicable to the enumeration of Enterobacteriaceae in all types of food products.

Background

Enterobacteriaceae are often used in food microbiology as indicator organisms. Their presence in processed food may indicate inadequate treatment or post process contamination from the environment, and may help to indicate the extent of faecal contamination. PHL guidelines are not available but specifications exist for various products.

The method is based on BS 5763 Part:10 1993 and is described in Practical Food Microbiology.

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1.0 PRINCIPLE

The enumeration of *Enterobacteriaceae* by the colony count technique involves the inoculation and mixing of specified volumes of the sample or dilutions of the sample with a tempered molten selective culture medium in sterile Petri dishes. An overlay of the same medium is added and the plates are incubated at 37°C for 24 hours. The number of typical colonies of *Enterobacteriaceae* is counted and the count of colony forming units (cfu) per gram or mL of sample is calculated following the results of confirmation tests.

2.0 DEFINITIONS

For the purpose of this method the following definition applies:

*Enterobacteriaceae*

Microorganisms that ferment glucose and show a negative oxidase reaction when the test is carried out according to the method specified.

3.0 SAFETY CONSIDERATIONS

Normal microbiology precautions apply.

In addition, care must be taken of the boiling waterbath when melting agars or boiling confirmatory test media prior to inoculation. Use heat and water resistant gloves when removing containers and avoid leaning over the bath.

4.0 EQUIPMENT

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1g.
- Gravimetric diluter (optional)
- Stomacher
- Vortex mixer
- Waterbath: 45°C ± 1°C
- Incubator: 37°C ± 1°C
- Colony counter (optional)
- Stomacher bags (sterile)
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

5.0 CULTURE MEDIA AND REAGENTS

Equivalent commercial dehydrated media may be used; follow the manufacturer’s instructions.

*Peptone saline diluent (Maximum recovery diluent)*

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<tr>
<td>Sodium chloride</td>
<td>8.5 g</td>
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<tr>
<td>Water</td>
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**REFERENCE**

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pH 7.0 ± 0.2 at 25°C

Buffered peptone water

Peptone 10.0 g  
Sodium chloride 5.0 g  
Disodium hydrogen phosphate 9.0 g  
Potassium dihydrogen phosphate 1.5 g  
Water 1 L  

pH 7.2 ± 0.2 at 25°C

Violet red bile glucose agar

Yeast extract 3.0 g  
Peptone 7.0 g  
Sodium chloride 5.0 g  
Bile salts No.3 1.5 g  
Glucose 10.0 g  
Neutral red 3 mg  
Crystal violet 2 mg  
Agar 12 g  
Water 1 L  

pH 7.4 ± 0.2 at 25°C

Glucose agar

Tryptone 10.0 g  
Yeast extract 1.5 g  
Glucose 10.0 g  
Sodium chloride 5.0 g  
Bromocresol purple 15 mg  
Agar 12.0 g  
Water 1 L  

pH 7.0 ± 0.2 at 25°C

Dispense in 10 mL or 15 mL amounts respectively in test tubes or universal containers.

Nutrient agar (or equivalent)

Meat extract 10.0 g  
Peptone 10.0 g  
Sodium chloride 5.0 g  
Agar 15.0 g  
Water 1 L  

pH 7.5 ± 0.2 at 25°C

Oxidase reagent

Tetramethyl – p – phenylenediamine dihydrochloride 0.1 g  
Water 10 mL
6.0 SAMPLE PROCESSING

6.1 Sample preparation

Following the procedure described in Standard Method F 2 – Preparation of Samples and Dilutions prepare a 10⁻¹ homogenate in either peptone saline diluent (PSD) or buffered peptone water (BPW) and further decimal dilutions as required in PSD.

6.2 Inoculation and incubation

Transfer 1 mL of each decimal dilution to a sterile Petri dish. Pour about 15 mL of molten violet red bile glucose agar (VRBGA) tempered in a 45°C waterbath, into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the time when the medium is poured shall not exceed 15 minutes.

Carefully mix the inoculum with the medium and allow the mixture to solidify. After complete solidification overlay with 10 – 15 mL of VRBGA medium tempered at 45°C on to the surface of the inoculated medium and allow to solidify.

Invert the prepared dishes and place in an incubator at 37°C for 24 hours.

6.3 Counting of colonies

Colonies of Enterobacteriaceae produce purple red colonies with a diameter of 0.5mm or greater and sometimes surrounded by a red zone of precipitated bile.

Count and record the characteristic Enterobacteriaceae colonies on each plate containing not more than 150 colonies. Above this number it is likely that colonies will have an atypical appearance.

6.4 Confirmatory tests

Select at least five suspect Enterobacteriaceae colonies (or all colonies if less than 5) from the highest dilution showing 15 to 150 colonies and subculture onto a segment of a nutrient agar (NA) plate. Place in an incubator at 37°C for 24 + 2 hours. Use the growth obtained for biochemical confirmation.

Oxidase test
Prepare a fresh solution of the reagent for each time of use.

Immerse a swab in oxidase reagent and touch lightly to the surface of the colony to be tested. The immediate appearance of a dark purple colour at the point of contact denotes a positive reaction but no colour change or a purplish colour which develops later are both negative reactions.

Alternatively, moisten a piece of filter paper a Petri dish with 2 – 3 drops of oxidase reagent. Using a stick, glass rod or platinum (not nichrome) loop transfer a colony of the test organism to the filter paper rub it on the moistened area. A positive reaction is indicated by the appearance of a dark purple colour within 10 seconds.

Positive control: Pseudomonas aeruginosa NCTC 10662

Negative control: E. coli NCTC 9001

Fermentation test
Prior to use steam or boil the glucose agar for 10 minutes and allow to set.
Perform a fermentation test on oxidase negative subcultures by a deep stab inoculation of tubes of glucose agar and place in an incubator at 37°C for 24 ± 2 hours. *Enterobacteriaceae* produce a yellow colour throughout the medium.

**Control cultures**

Positive control: *E. coli* NCTC 9001

Negative control: *Pseudomonas aeruginosa* NCTC 10662

**CALCULATION OF RESULTS**

Counts should be calculated, where possible, using dilutions giving 15 to 150 colonies on the plate.

Calculate the count of *Enterobacteriaceae* per gram or mL as follows:

\[
\text{Count per g} = \frac{\text{No. of colonies confirmed} \times \text{No. of colonies counted}}{\text{No. of colonies tested} \times \text{Volume tested} \times \text{Dilution}}
\]

**7.0 REPORTING OF RESULTS**

If no colonies are confirmed, report as:

Less than 10/g

If the test organisms are detected with counts between 10 and 99 per gram report in the form of:

\[a\text{ per g}\]

where *a* is a number between 10 and 99.

If colonies present with a count of 100 or higher per gram or mL report in the form of:

\[a \times 10^b\text{ cfu/g}\]

where *a* is never less than 1.0 or greater than 9.9 and *b* represents the appropriate power of ten. Round counts up if the last figure is 5 or more and down if the last figure is 4 or less e.g.

1920 cfu per g reported as 1.9 x 10^3 cfu per g

235,000 cfu per g reported as 2.4 x 10^5 cfu per g
Appendix: Flowchart showing the enumeration of *Enterobacteriaceae* by the colony count technique

1. Prepare a $10^{-1}$ dilution of sample
2. Homogenise by stomaching
3. Prepare further dilutions if required in peptone saline diluent
4. Starting with the highest dilution inoculate 1 mL of each dilution into an empty Petri dish and add approximately 15 mL of molten tempered VRBGA
5. Mix by gentle rotation and allow to set
6. Add an overlay of 10 – 15 mL of molten tempered VRBGA and allow to set
7. Incubate at $37^\circ$C for 24 hours.
8. Count typical colonies
9. Subculture 5 colonies to nutrient agar for oxidase test
10. Subculture oxidase negative colonies to glucose agar for fermentation test
11. Calculate the count of *Enterobacteriaceae* per gram.
REFERENCES