ENUMERATION OF COLIFORMS BY A COLONY COUNT TECHNIQUE

Principle

The number of viable coliforms in liquid or dry samples is determined by plating a specified quantity of a sample or sample dilution on Crystal Violet - Neutral Red – Bile – Lactose - Agar (VRBL) dishes. Dishes are incubated at 37°C for 24h under aerobic conditions and the number of coliform colonies is counted.

A typical coliform colony is purple red in color with a diameter › 0,5 mm. Occasionally a reddish precipitate of bile salts is formed around the colonies.

Field of application

Method is applicable for enumeration of viable coliforms from liquid and dried enzyme semifinal- and final products.

Equipment

Autoclave	
Water bath	45 °C ± 0.5°C
Incubator	37 °C ± 1 °C
Vortex	
Sterile Petri dishes:	Ø 80 mm to 100 mm
Sterile dilution bottles	100 ml
Sterile dilution tubes	10 ml
Sterile glass flasks	250 ml
Sterile pipettes	1 ml and 10 ml
Colony counter	

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.

1. Crystal Violet - Neutral Red – Bile – Lactose - Agar (VRBL)

Weigh 41.5 g of VRBL Agar (e.g. Difco 0012) and dissolve into 1000 ml of water. Boil to ensure complete dilution of medium components. Obs! Maximum boiling time is 2 minutes. Divide to 100 ml aliquots to sterile 250 ml glass flasks. Do not sterilize in autoclave. VRBL-agar is melted in a microwave oven or water bath and tempered to 45°C before use.

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2. Dilution fluid (0.9 % w/v NaCl)

Dilute 9.0 g NaCl (e.g. Merck 6404) into 1000 ml of water. Divide 90 ml aliquots to appropriate glass flasks. Sterilize in autoclave for 15 min at 121 °C.

Samples

1. Dry samples

Aseptically weigh 10.0 g of sample to 90 ml of dilution fluid. Vortex until the mixture is homogenous (= 10^{-1} dilution). Prepare dilution series from the 10^{-1} dilution by pipetting 1 ml of 10^{-1} dilution to 9 ml of dilution fluid (= 10^{-2} dilution) and 1 ml of 10^{-1} dilution to 99 ml of dilution fluid (= 10^{-3} dilution). Vortex all the samples carefully. The dilution series can be continued further by following the dilution principle described above.

2. Liquid samples

Aseptically pipet 10.0 ml of sample to 90 ml of dilution fluid. Vortex until the mixture is homogenous (= 10^{-1} dilution). Prepare dilution series from the 10^{-1} dilution by pipetting 1 ml of 10^{-1} dilution to 9 ml of dilution fluid (= 10^{-2} dilution) and 1 ml of 10^{-1} dilution to 99 ml of dilution fluid (= 10^{-3} dilution). Vortex all the samples carefully. The dilution series can be continued further by following the dilution principle described above.

Procedure

1.0 ml of all samples including the original sample mix or undiluted enzyme sample and all the required dilutions are pipetted aseptically on empty sterile Petri dishes as duplicates. 15 ml of tempered 45°C VRBL-agar is poured to each of the plates. Samples are mixed to VRBL-agar by carefully swaying the plates. Sterility control plates are also prepared by pouring 15 ml of tempered 45°C VRBL-agar only to two sterile Petri dishes. Sterility controls are prepared from every new VRBL-agar batch used. The procedure from preparing the first sample dilution to pouring the VRBL-agar should not take more than 15 minutes. Dishes are placed on an even surface and the agar is let to solidify. Once the agar is solid ~4 ml of tempered 45°C VRBL-agar is poured on top of the first layer of agar on all dishes. Agar is again let to solidify on an even surface. Dishes are moved to 37°C ± 1 incubator and incubated upside down for 24h.

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Results

Number of typical coliform colonies (see the "principle" section) is counted from dishes that contain no more than 150 colonies. If the dishes contain more than 150 colonies the appearance of colonies might be non-typical. Colonies are counted from both of the duplicate dishes from two dilutions.

The number of coliforms /ml or /g is calculated accordingly:

$$N = \frac{\Sigma C}{[n_1 + (0,1 \times n_2)] \, d}$$

ΣC = combined number of colonies (a´2 dishes from 2 dilutions)
n_1 = number of dishes from the first dilution
n_2 = number of dishes from the second dilution
d = dilution factor of the first dilution

Results are reported with the accuracy of two significant digits/ml or /g.

Example:

First dilution 10^{-2}; colonies on dishes 83 + 97 = 180
Second dilution 10^{-3}; colonies on dishes 13 + 8 = 21

$$N = \frac{180 + 21}{[2 + (0,1 \times 2)] \times 10^{-2}} = \frac{201}{0,022} = 9\ 136 = 9,1 \times 10^3 \text{ cfu/ml or /g}$$

cfu = colony forming unit

If no typical colonies are detected in any of the plates the result is reported as ‹ d^{-1} cfu/ml (liquid product) or ‹ d^{-1} cfu/g (dry product) (d = dilution factor from the smallest dilution).

References

International Standard ISO 4832, 2nd edition 1991-03-01. Microbiology - General Guidance for the Enumeration of Coliforms – Colony count technique.

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