Mechanical and chemical pulps, paper and paperboard

Microbiological examination – Total colony number

Using the dry rehydratable film method

0   Introduction

Microbiological purity is an important quality criterion in the production of materials for use in contact with food, although no limits for the number of microbes are stipulated in any law.

In this SCAN-test Method, the dry rehydratable film, Petrifilm Count Plates, is used for determination of bacteria as well as yeast and moulds.

This Method is an alternative procedure to ISO 8784 (see clause 15 Literature) for determination of micro-biological purity. The results are in agreement with those obtained by using the ISO Standard.

1   Scope

This SCAN-test Method describes a procedure for determining the total number of bacteria and of yeast and moulds, using the dry rehydratable film method. The Method is applicable to all types of dried market pulp, paper and paperboard.

The results are expressed as total colony number, i.e. the number of colony-forming units (CFU) per gram dry mass of the sample.

2   References

ISO 638   Pulps – Determination of dry matter content
            (EN ISO 638)

Note – SCAN-test has withdrawn a number of test methods and refers instead to the corresponding ISO and/or EN Standards.

3   Definitions

For the purpose of this Method, the following definitions apply:

3.1   *Aerobic micro-organisms* – Micro-organisms which grow under the aerobic conditions described in this Method.

The micro-organisms can be bacteria, yeast and moulds, and in some cases fungi.

3.1.1   *Bacteria* – Micro-organisms growing in typical red colonies on Aerobic Count Plates.

3.1.2   *Yeasts* – Micro-organisms growing in small defined blue-green or off-white colonies on Yeast and Mold Count Plates.

3.1.3   *Moulds* – Micro-organisms usually growing in blue colonies, but they may also assume their natural pigmentation (i.e. black, yellow, green etc.).

Note – Mould colonies tend to be larger and more diffuse than yeast colonies on Yeast and Mold Count Plates.

3.2 *Aerobic plate count* – The number of visible colonies on an Aerobic Count Plate after 48 h at 37 °C or on a Yeast and Mold Count Plate read after 3 days and 5 days at 30 °C, under the conditions described in this Method.

3.3 *Total colony number* – The number of colony-forming units (CFU) per gram dry mass of the sample.

4 Principle

The sample is disintegrated in a standard diluent. The fibre suspension is transferred to a dry rehydratable film and incubated. Two different films are used, one for bacteria and one for yeast and moulds.

After the incubation time, the numbers of bacterial colonies and of yeast and mould colonies are counted on the respective films. The results are expressed separately as the total colony number per gram dry mass of the sample.

5 Substrates and diluent

5.1 *Standard diluent,* Ringer's solution is preferred; other isotonic solutions may however be used. Add one drop of Tween 80 (Monooleate (polyoxyethylene-sorbitan)) per litre to the standard diluent prior to sterilization to facilitate the release of spores and bacterial cells from the fibres.

> *Note 1* – A commercially available standard diluent may be used.

> *Warning* – Do not use buffers containing citrate or sodiumthiosulphate due to interference.

5.2 *Count plates*, dry rehydratable film.

5.2.1 *Aerobic Count Plates*, Petrifilm. Regarding storage and shelf life, follow the recommendations given by the manufacturer.

5.2.2 *Yeast and Mold Count Plates*, Petrifilm. Regarding storage and shelf life, follow the recommendations given by the manufacturer.

> *Note 2* – Petrifilm™ is a registered trademark of the 3M Company, Minnesota, USA. Other products of equivalent quality may be used.

6 Apparatus

Ordinary microbiological laboratory equipment and the following:

6.1 *Disintegrator*, with metal or glass jars of about 500 ml capacity, fitted with a high-speed impeller near the bottom and fitted with a cap or lid, or other suitable disintegrator, which ensures complete disintegration of the sample. Place an aluminium foil hood over the cap of each disintegrator jar prior to sterilisation.

6.2 *Sterile pipettes,* wide-mouth, 3-5 mm, suitable for measuring 1,0 ml.

6.3 *Incubator,* capable of maintaining a temperature of (30 ± 1) °C and (37 ± 1) °C respectively.

6.4 *Magnifier,* having a 10 times magnification, to facilitate colony counting.

7 Sampling

This Method does not cover the sampling procedure. Make sure that the test pieces taken for analysis is representative of the sample received and that the sample is handled aseptically.

A sample shall contain at least 5 sheets each of them having a minimum size of 200 mm x 250 mm of mill sheeted pulp, paper or paperboard (at least 3 sheets for testing and 2 protective sheets).

8 Procedure

8.1 Conditions

Run the whole procedure under aseptic conditions.

8.2 Determination of dry matter content

Determine the dry matter content of the sample as described in ISO 638 irrespective of the kind of sample.

8.3 Pre-treatment and dilution

To obtain a test portion having a total mass of at least 3 g, take 1 g from each of three different sheets. The number of parallel test portions shall be adjusted to meet the demand for statistical confidence. Weigh each test portion and record the mass, *m*.

By using a pair of tweezers, take the weighed test portion and make a series of cuts between 5 mm and 10 mm apart. Cut squares directly into the disintegrator jar (6.1) by making a series of cuts perpendicular to those made previously. Add 250 ml (of a total of 300 ml) sterile standard diluent (5.1) and disintegrate until the suspension is free from fibre clumps. Transfer the fibre suspension to a sterile bottle fitted with screw cap. Use the remaining 50 ml of the standard diluent to rinse off the jar. The fibre concentration is then approx. 1,0 %.

Use a separate sterile disintegrator jar for each test. Cool the sterile standard diluent to prevent the temperature from exceeding 45 °C in the jar. Allow the aluminium hood to remain on the cap at all times to prevent possible contamination through any opening in the cap or around the top of the jar. Immediately after disintegration, plate out the suspension as described in 8.4.

Prepare and plate additional more dilute suspensions, if it is suspected that the sample has a high microbial load. A colony count between 25 and 250 is most suitable. In each case, record the dilution factor, f.

Note – If the suspension has not been diluted at this stage the dilution factor $f = 1$.

8.4 Preparation of sample plates

For the determination of bacteria, place the Aerobic Count Plates (5.2.1) on a flat, horizontal surface. For the determination of yeast and moulds, place the Yeast and Mold Count Plates (5.2.2) on a flat, horizontal surface.

Lift the top film from the plate and, using a sterile pipette (6.2), add 1 ml of the fibre suspension (8.3) onto the bottom film in a rotating motion, to ensure even spreading. Release the top film down onto the suspension with a rolling motion, to avoid that air-bubbles are trapped or formed. Distribute the suspension evenly using a gentle downward pressure on the centre of the plastic spreader. Do not slide the spreader across the film. Remove the spreader and leave the plate undisturbed for 1 minute to permit solidification of the gel.

Prepare at least 5 sample plates for bacteria and at least 5 sample plates for yeast and moulds from each dilution. When low microbial counts are suspected, prepare fibre suspensions in duplicate.

8.5 Preparation of control plates

Place on a flat, horizontal surface one Aerobic Count Plate (5.2.1) to be used as a control plate for bacteria and one Yeast and Mold Count Plate (5.2.2) to be used as a control plate for yeast and moulds.

Add 1 ml of the standard diluent (5.1) to each control plate.

8.6 Incubation

Put the sample plates and the control plates into unsealed plastic bags and incubate them in a horizontal position with the clear side up in an incubator (6.3). The stack in each plastic bag must not exceed 20 plates.

The temperature shall be (37 ± 1) °C for bacteria and (30 ± 1) °C for yeast and moulds.

9 Colony counting

9.1 Control plates

If the control plates are contaminated, the whole procedure has to be repeated.

9.2 Sample plates

For bacteria, count and record the number of colonies, n, on each sample plate after (48 ± 3) h of incubation. For yeast and moulds, count and record the number of colonies, n, on each sample plate after both 3 days and 5 days of incubation. Count each coloured dot, regardless of size and colour intensity, as 1 colony (see 3.1.1, 3.1.2 and 3.1.3). Count colonies using a magnifier (6.4). For each dilution, count a minimum of 5 sample plates for bacteria and a minimum of 5 sample plates for yeast and moulds.

Note 1 – If there are many red colonies (including the small ones), they are *too numerous to count* (TNTC), even if their distribution appears uneven. With very high counts, the entire growth area may turn pink. To be able to count them, dilute the suspension.

Note 2 – TNTC mould colonies: The mould colonies are *too numerous to count* if they appear small and faint. To be able to count them, dilute the suspension.

Note 3 – TNTC yeast colonies: The yeast colonies are *too numerous to count* if they appear as small blue colonies at the edge of the plate or as blue growth only around edges. To be able to count them, dilute the suspension.

Warning – Some species of bacteria liquefy the gel of Aerobic Count Plate and this causes the colony to spread out and hide the presence of other colonies. Do not count red spots within the liquefied area. Determine the average count in the few unaffected squares and then estimate the total count. In such a case, inspect the film within 24 h and mark the colonies.

Due to rapid colonization of the Yeast and Mold Count Plates by certain moulds, it is recommended to inspect and mark the colonies on the third day of incubation.

10 Calculation

Record the results for the bacteria and for the yeast and moulds separately.

Calculate the mean colony count of 5 sample plates, $\bar{n}$. Calculate the total colony number as:

$$N = \frac{\overline{n} \cdot 300 \cdot f}{m \cdot x} \cdot 100 \qquad [1]$$

where

N is the total colony number, i.e. CFU per gram dry mass;

$\overline{n}$ is the mean colony count of 5 sample plates;

f is the dilution factor;

m is the weight of the test portion, in gram;

x is the dry matter content of the sample (8.2), as a percentage.

11   Identification

To isolate colonies for subsequent identification, lift the top film and pick a colony from the gel.

> *Note* – If required, the colonies of yeast, moulds and fungi can be reported separately (see clause 3.1.2 and 3.1.3).

12   Test report

The test report shall include reference to this SCAN-test Method and the following particulars:

(a)   date and place of testing;

(b)   identification of the sample;

(c)   incubation time and temperature;

(d)   the results, as total colony number of bacteria and total colony number of yeast and moulds, per gram dry mass of the sample, expressed as CFU;

(e)   any departure from the procedure described in this SCAN-test Method or any other circumstances that may have affected the result.

13   Additional information

The number of colonies of yeast and moulds can be read between 2 days and 5 days of incubation. By extension of the incubation time to between 5 days and 15 days, even fungi may be scored.

14   Precision

14.1   *Validation of the Petrifilm technique*

14.1.1 Repeatability

Three expert laboratories have tested the repeatability of the Petrifilm technique. Reference samples containing the following organisms have been tested :

     Staphylococcus saprophyticus, Enterobacter cloacea, Escherichia coli, Enterococcus durans, Bacillus cereus, Candida sp

     at the concentration levels of 6,1-6,9 log CFU/ml for aerobic bacteria and 4,1-4,9 log CFU/ml for yeast.

Microorganisms	Aerobic Count Plate			Yeast and Mold Count Plate		
	CFU/ml	s	log CFU/ml	CFU/ml	s	log CFU/ml
Laboratory 4, Technician 1	$3,3 \times 10^6$	3,3	6,5	$5,0 \times 10^4$	7,6	4,7
Laboratory 4, Technician 2	$2,7 \times 10^6$	4,8	6,4	$4,3 \times 10^4$	8,0	4,6
Laboratory 4, Technician 3	$3,6 \times 10^6$	4,1	6,6	$4,3 \times 10^4$	9,9	4,6
Laboratory 6, Technician 1	$3,1 \times 10^6$	5,8	6,5	$4,4 \times 10^4$	7,8	4,6
Laboratory 6, Technician 2	$3,4 \times 10^6$	4,6	6,5	$3,9 \times 10^4$	5,3	4,6
Laboratory 6, Technician 3	$3,4 \times 10^6$	8,7	6,5	$5,1 \times 10^4$	4,4	4,7
Laboratory 6, Technician 4	$4,2 \times 10^6$	3,8	6,6	$4,0 \times 10^4$	5,8	4,6
Laboratory 7, Technician 1	$3,3 \times 10^6$	3,8	6,5	$4,7 \times 10^4$	7,2	4,7
Laboratory 7, Technician 2	$3,0 \times 10^6$	6,9	6,5	$4,8 \times 10^4$	8,2	4,7
Laboratory 7, Technician 3	$3,1 \times 10^6$	2,1	6,5	$4,1 \times 10^4$	6,2	4,6
Laboratory 7, Technician 4	$2,9 \times 10^6$	5,1	6,5	$4,1 \times 10^4$	7,3	4,6

The tests showed that the Petrifilm technique gives results within the stated tolerance intervals.

14.2   Reproducibility

Six expert laboratories tested the reproducibility of the Petrifilm technique. Reference samples containing the following organisms have been tested:

   Staphylococcus saprophyticus, Enterobacter cloacea, Escherichia coli, Enterococcus durans, Bacillus cereus, Candida sp

   at the concentration levels of 6,1 - 6,9 log CFU/ml for aerobic bacteria and 4,1 - 4,9 log CFU/ml for yeast.

Microorganisms	Aerobic Count Plate			Yeast and Mold Count Plate		
	CFU/ml	s	log CFU/ml	CFU/ml	s	log CFU/ml
Laboratory 1	$2,8 \times 10^6$	4,3	6,4	$3,8 \times 10^4$	6,4	4,6
Laboratory 2	$3,8 \times 10^6$	6,3	6,6	$4,9 \times 10^4$	5,4	4,7
Laboratory 4	$3,2 \times 10^6$	4,1	6,5	$4,5 \times 10^4$	8,5	4,6
Laboratory 5	$2,7 \times 10^6$	8,8	6,4	$3,7 \times 10^4$	5,3	4,6
Laboratory 6	$3,5 \times 10^6$	5,7	6,5	$4,4 \times 10^4$	5,8	4,6
Laboratory 7	$3,1 \times 10^6$	4,5	6,5	$4,4 \times 10^4$	7,2	4,7

The interlaboratory comparison tests showed that the Petrifilm technique gives results within the stated tolerance intervals.

15   Literature

15.1   ISO 8784 Dried market pulp, paper and paper-board – Microbiological examination – Enumeration of bacteria, yeast and moulds (ISO/DIS in 2002)

15.2   Aerobic micro-organisms. Enumeration at 30 °C in foods by means of Petrifilm™ plates. Nordic committee on food analysis No 146 (1993)

15.3   Coliform Bacteria and Escherichia coli in foods. Determination by the plate count method with Petrifilm™ plates. Nordic committee on food analysis No 147 (1993)

15.4   Jordano R., et al. Comparison of Petrifilm method to conventional methods for enumerating aerobic bacteria, coliforms, Escherichia coli and yeasts and moulds in foods Acta Microbiol Immunol Hung 42:*3:* p. 255-259 (1995)

15.5   Blackburn CW, et al. Evaluation of Petrifilm methods for enumeration of aerobic flora and coliforms in a wide range of foodsLett Appl Microbiol 22:*2*, p. 137-140 (1996)

15.6   Johnsrud SC, Kolar M-C. Determination of micro-organisms in paper and paperboard. Petrifilm™ Aerobic Count Plates - an alternative to DIN 54378 and 54379. Nordpap DP 4/9 SCAN Forsk-Rapport 675 (1997)

15.7   3M Petrifilm Yeast and Mold Count Plate receives AOAC Official Methods of Analysis approval. St. Paul, Minnesota, March 3 (1997) 3M Home Page

15.8   Raugel P-J. Rapid Food analysis and hygiene monitoring – Kits, instruments and systems, 3M Microbiology, p. 614-648, Springer (1999)