SCANDINAVIAN PULP, PAPER AND BOARD
TESTING COMMITTEE

SCAN-P 59:87
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Fillers and pigments

Microbiology – Enumeration of bacteria

0 Introduction

The procedure described in this SCAN-test Method requires the skill of a well-trained technician and a laboratory designed for aseptic work.

1 Scope

This SCAN-test Method describes a procedure for evaluation of the microbiological purity of fillers and pigments, or of slurries made thereof, for use in the production of paper. The Method gives results that correlate with the total number of bacteria, excluding some totally anaerobic species. It does not distinguish between different kinds of bacteria.

2 Reference

SCAN-P 39 Fillers and pigments – Dry matter content.

3 Definition

For the purpose of this Method, the following definition applies:

3.1 *Bacteria* – Microorganisms growing aerobically at 30 °C under the conditions of this test.

4 Principle

From the sample, a number of diluted suspensions are prepared and added to nutrient solutions contained in Petri dishes. These are incubated at 30 °C for 3 days. The number of colonies formed is counted in those dishes where the degree of dilution is suitable.

5 Apparatus

5.1 *Sterilising autoclave* for use at 121 °C.

5.2 *Oven* for use at 170 °C (See clause 7.2).

5.3 *Incubator or suitable oven* capable of maintaining a temperature of (30 ± 1) °C.

5.4 *Petri dishes*, of glass or plastic, diameter 90 mm to 100 mm, with lids.

5.5 *Graduated pipettes*, 1 ml, with wide openings. Suitable disposable sterile pipettes for microbiological work are available on the market.

5.6 *Test tubes*, 35–50 ml, with metal caps or cotton plugs.

5.7 *Water bath* or equivalent, capable of maintaining a temperature of (45 ± 2) °C.

5.8 *Colony counter*, with a lens of at least x 1,5 magnifying power.

5.9 *pH-meter*.

6 Chemicals and reagents

6.1 *Nutrient*, Plate Count Agar, pH value 7, of the following composition:

peptone	5 g
yeast extract	2,5 g
d-glucose	1,0 g
agar	14,0 g
distilled water	1000 ml

6.2 *Buffer stock solution.* Dissolve 3,4 g of potassium di-hydrogen-phosphate, KH_2PO_4, in water and dilute to 50 ml. Adjust to pH value 7,2 with 1 mol/1 sodium hydroxide solution. Dilute to 100 ml.

6.3 *Dilute buffer solution.* Dilute, with water, 1,25 ml of the buffer stock solution (6.2) to 1 litre.

7 Sterilisation

Sterilise apparatus that will come into contact with culture media, buffer solution (6.3) or sample, except for apparatus that is supplied sterile, either

– in the oven (5.2) at 170 °C for not less than 1h or
– in the autoclave at (121 ± 1) °C for not less than 20 min.

Sterilise the buffer solution (6.3) and the nutrient in the autoclave at (121 ± 1) °C for not less than 20 min.

Sampling spoons and other metal tools may be sterilised in a flame immediately before use.

8 Pretreatment of sample

8.1 Sample sacks and barrels immediately when they are opened. Take the sample from a position in the interior of the container away from its sides. Use a sterile spoon and a sterile sampling bottle. Close the bottle with a sterile metal cap or a cotton plug. When sampling carloads or open containers, remove the upper layer carefully before sampling. Preferably, sample closed containers when they are emptied.

8.2 Determine the dry matter content as described in SCAN-P 39.

8.3 Make two independent test series in parallel:

Weigh about 1 g of sample to the nearest 10 mg in a sterile bottle. Add 100 ml of the buffer solution (6.3) and shake vigorously for about 3 min. Transfer with a sterile pipette 1 ml of the sample solution to a Petri dish and also 1 ml to a test tube containing 9 ml of the buffer solution (6.3). Flame the rim of the test tube and close it with a metal cap or a cotton plug. Shake the test tube thoroughly.

With another sterile pipette, transfer from the test tube 1 ml of the suspension to another Petri dish and 1 ml to another test tube containing 9 ml of the buffer solution in the same manner as before.

Continue this procedure in successive steps until a suitable number of diluted suspensions have been obtained. *Figure 1* illustrates the procedure and shows how to mark the dishes.

The number of dilutions should be such that less than 10 colonies are obtained in the last dish. Usually 4 or 5 dilutions are required. A blank may be prepared containing only buffer solution (6.3) and nutrient (6.1) (See the note to section 11).

9 Plating

Prepare the nutrient (6.1) as instructed by the manufacturer and cool it to (45 ± 2) °C. Add 15 to 20 ml of the nutrient to each Petri dish. Cover the dish with its lid. Mix the contents of the dishes by swirling them on the table, 5 times clockwise and 5 times counter-clockwise. Tilt the dishes and replace them 5 times to improve the mixing. Treat all the dishes the same way. Allow the agar plates to solidify.

> *Note* – For the most concentrated sample (plate 2), the agitation must usually be increased, e.g. doubled.

10 Incubation

Turn the Petri dishes upside down and place them in the incubator at (30 ± 1) °C for (72 ± 3) h.

11 Counting and calculation

Inspect the Petri dishes after incubation. Disregard dishes that have more than 300 colonies. If the blank dish, or the dish with the highest dilution factor, is obviously infected, disregard the whole test and start another one.

Count with the aid of the colony counter the number of colonies on the surface as well as in the interior of the agar plate. Proceed as follows, depending on the number of colonies:

11.1 If the number of colonies in both dishes with the lowest dilution factor (no 2) is less than 10, do not count any other dishes.

11.2 If one or both of the dishes of a given dilution factor has between 30 and 300 colonies, calculate the mean number.

Multiply the result by the dilution factor, to obtain the number of colonies per gram of dry pigment or filler,

taking into consideration the dry matter content and the actual sample weight.

11.3 If there are plates with different dilution factors that contain between 30 and 300 colonies, calculate the number of colonies per gram of dry sample, separately for the two dilution factors, as described in 11.2 and then the mean of the two values obtained. If the ratio between the results for the two dilutions exceeds 2, use only the lower value. Study all the results before discarding any of them.

Round off the final result to two significant figures.

Note – A plate with no colonies may be regarded as the blank. The precision of the test may be judged from the number of colonies obtained at different dilution factors. Ideally one finds 10 times more colonies in a 10 times stronger solution.

12 Report

Report the number of colonies per gram of dry pigment as a number of between 1,0 and 9,9, multiplied by a factor 10°.

If the number is less than 10 for the lowest dilution factor (11.1), report the result as "less than 10 x *s*",where *s* is the dilution factor. If there is no colonies in these plates, report the result as "less than 1 x *s*".

The report shall include reference to this SCAN- test Standard and the following details:

(a) date and place of testing;
(b) identification mark of the sample tested;
(c) the result;
(d) any departure from the procedure described in this SCAN-test Method and any other circumstances that may have affected the result.

13 Additional information

This Method is based on ISO 4833 *Microbiology – General guidance for enumeration of microorganisms – Colony count techniques at 30 °C.*

The fact that the ingredients of the nutrient vary from one manufacturer to another, and also from one lot to another, limits the reproducibility of the test.

If it is desired to measure the contamination by yeasts or moulds, a similar procedure may be followed where Potato Dextrose Agar is used as the nutrient. Place 0,1 ml of the suspension on top of the agar plates in the Petri dishes. Spread the sample with a sterilised glass rod. The incubation time for moulds is 5 days. Bacteria will also grow on these plates, a fact that should be taken into consideration when counting colonies. The growth of bacteria can be inhibited by adding tartaric acid (to obtain a pH value about 3,5) and a bactericide, for example 1 ml of an 1 mg/l neomycine solution per dish.

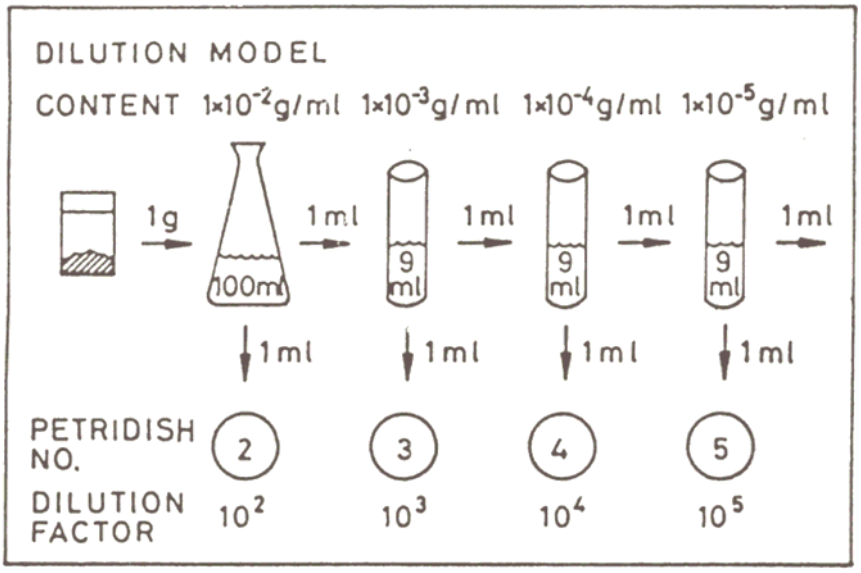

Figure 1. Dilution model

SCAN-test Methods are issued and recommended by KCL, PFI and STFI-Packforsk for the pulp, paper and board industries in Finland, Norway and Sweden.
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