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BALLOT NO. _____ 03-SARG _____

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DATE _____ May 16, 2024 _____

WORKING GROUP
CHAIR _____ Ben Frank _____

SUBJECT
CATEGORY _____ Papermaking Additives _____

RELATED
METHODS _____ See "Additional Information" _____

CAUTION:

This Test Method may include safety precautions which are believed to be appropriate at the time of publication of the method. The intent of these is to alert the user of the method to safety issues related to such use. The user is responsible for determining that the safety precautions are complete and are appropriate to their use of the method, and for ensuring that suitable safety practices have not changed since publication of the method. This method may require the use, disposal, or both, of chemicals which may present serious health hazards to humans. Procedures for the handling of such substances are set forth on Safety Data Sheets which must be developed by all manufacturers and importers of potentially hazardous chemicals and maintained by all distributors of potentially hazardous chemicals. Prior to the use of this method, the user must determine whether any of the chemicals to be used or disposed of are potentially hazardous and, if so, must follow strictly the procedures specified by both the manufacturer, as well as local, state, and federal authorities for safe use and disposal of these chemicals.

Microbiological Enumeration of Process Water and Slush Pulp

(Five-year review of Official Method T 631 om-16)

(Changes from Draft 2 shown through strikethroughs/underlines)

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1. Scope and significance

The following procedure is recommended primarily for the microbiological examination of process water. It is also applicable to slush pulp. This method is adequate for classical, heterotrophic cell counts of unencapsulated, planktonic microorganisms. This method will not give an accurate measure of the numbers of encapsulated, slime-forming cells or sessile microorganisms present. Because of the exacting technique required in microbiological procedures, reproducible results can be obtained only by a trained technician.

2. Apparatus and materials

- 2.1 *Alcohol*, isopropyl (rubbing), or ethanol (70 - 95%), for sterilizing instruments.
- 2.2 *Balance*, sensitive to 0.1 g, with a pan large enough to hold a dilution bottle.
- 2.3 *Bottles for dilution*, commercially available, pre-filled, pre-sterilized buffered dilution blanks, 99 mL, are recommended. Alternatively standard milk-dilution bottles scribed at the 99 mL level may be used. These

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should be filled to the 99 mL mark with deionized water and sterilized by steam sterilization as described in section 3.1.1.

2.4 *Sample containers*, several 125- to 250-mL wide-mouthed bottles for collecting samples. The type of bottle and cap selected should be able to withstand the sterilization procedure. Presterilized, disposable plastic bags of suitable size with flexible wire closures are satisfactory.

2.5 *Colony counter*, any one of several types. A hand tally for recording the count is recommended.

2.6 *Flaming equipment*, an alcohol lamp, Bunsen burner, or Bacti-Cinerator™ to flame tongs, forceps, and the mouths of sterile containers.

2.7 *Incubator*, capable of maintaining a temperature of 30 - 36°C ± 2°C.

2.8 *Petri dishes*, commercially available presterilized disposable (100 mm × 15 mm) plates, are recommended.

2.9 *Pipets and containers*, commercially available presterilized 1.1 mL and 10 mL pipets are recommended. Automatic pipeters/pipettors with sterile plastic tips or 3 mL and 10 mL commercially available sterile syringes may also be used. Containers for sampling should be sterile sample bags or sterile bottles.

2.10 *Sampling equipment*. Scoops made from tinned iron sheet. The diameter of the scoop will depend upon the opening of the sample bottle; the length is ~~about~~ 25.4 cm (10 in.). Wrap the scoops individually in heavy kraft paper prior to sterilization. Alternatively, large sterile spoons or dippers may be substituted for the scoops.

2.11 *Nutrient substrate*. For bacteria use, tryptone glucose extract agar, and for fungi, either potato dextrose or mycophil agar. The fungal medium should be adjusted to pH 4.0 by adding sterile 10% tartaric acid following sterilization of the agar and cooling to 45 to 50°C prior to pouring plates.

2.12 Commercially available 3M Petrifilm Aerobic count plate for bacteria and Petrifilm yeast and mold plate can also be utilized.

2.1.3 *Sterilizing equipment*, two types, suitable size: an autoclave for steam sterilization; an electrically heated hot-air oven at 165°C, with thermometer.

3. Sterilization

3.1 Depending upon the nature of the equipment to be sterilized, use one of three methods as follows:

3.1.1 *Steam heat* (autoclave). Sterilize the following in an autoclave by heating for 20 min at a minimum of 121°C, corresponding to 103 kPa (15 psi) steam pressure: (a) media (unless other conditions are specified by the manufacturer), (b) sample bottles, (c) scoops, dippers, or spoons, and (d) water blanks for dilution. If necessary, vent the autoclave for 5 min or until steam has displaced the air. Include a biological or chemical indicator strip to evaluate autoclave efficiency.

3.1.2 *Dry heat* (electrically or gas-heated oven). Sterilize the following by heating for at least 2 hours at a temperature of about 165°C (329°F): (a) glass pipets and (b) forceps.

3.1.3 *Alcohol*. Immerse contact portions of forceps and spoons in alcohol. When needed for handling samples, remove, allow to drain for a few moments, then burn off the excess alcohol. Use extreme care when using an open flame around alcohol.

4. Sampling

4.1 *Frequency of sampling*. Occasional single samples are usually inadequate, since the analysis reflects only the conditions which prevailed when such samples were taken. The number and frequency of samples depend largely on the purpose of the analysis.

4.2 *Method of sampling*.

4.2.1 Use the sterile metal scoop, dipper, or spoon to collect stock samples that cannot be readily sampled in the sample stream. Dip a sample from the vat, chest, or trough, using caution to avoid contaminating the sample by the hands coming in contact with the stock, or from material adhering to the edges of the system. Transfer the sample to a sterile container and seal immediately.

4.2.2 For sampling water or dilute stock, allow the sample stream to flow directly into the sample container. If sample is obtained from a closed line, allow stock to flow one minute before sampling. Take care that the sample or container is not contaminated by the hands during the sampling.

4.2.3 Samples should be plated as soon as possible. Samples may be stored at 4°C prior to plating, but should not be held for more than 24 hours. Note the time of storage in results.

5. Procedure

5.1 *Diluting tubes serial dilutions.* Choose the appropriate method below. (9 mL serial dilution tubes may be substituted for 99 mL.) If necessary, change the dilution value and labeling appropriately (1 mL sample and 9 mL dilution provides a dilution of 10^{-1}) dilution tubes.

5.1.1 *Process water or low-consistency stock (1% or less).* Transfer 1.0 ml of the well-mixed process water or low-consistency stock sample to a 99 ml water blank and shake vigorously with at least 20 strokes. This gives a 10^{-2} dilution. Repeat this procedure until the desired dilution is reached (Figure 1).

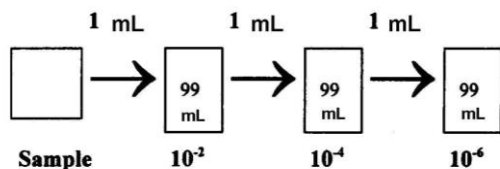


Fig. 1. Procedure for process water or low-consistency stock (1% or less).

5.1.2 *High consistency stock (greater than 1%).* Weigh 11 g of the slush stock into a 99 mL water blank and shake vigorously with at least 20 strokes. This gives a 10^{-1} dilution. Carefully pipette 1 mL of this dilution to a second 99 ml water blank and shake vigorously (10^{-3} dilution). Repeat this procedure until desired dilution is reached (Figure 2).

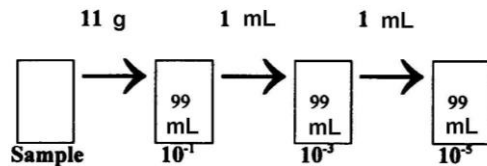


Fig. 2. Procedure for high consistency stock (greater than 1%).

5.2 *Plating and incubation.* Plate dilutions immediately. Choose one of the following methods (pour plates, spread plates, or Petrifilms®) for plating. The room in which the specimens are plated should be free of air currents and dust. About ~~1/2 h~~ 30 min before plating, sponge the surface of the worktable with a solution of a suitable disinfectant, e.g., Lysol® or diluted chlorine bleach.

5.2.1 *Pour plates.* Using a sterile 1.1 mL pipet (or automatic pipetor), transfer 1.0 ml and 0.1 ml of each dilution to the corresponding labeled Petri dish (Figure 3). Adjust labeling appropriately for high consistency stock dilution method.

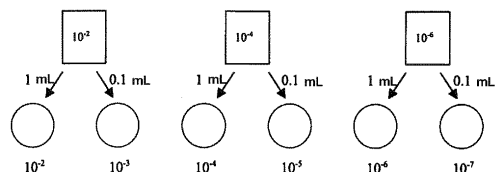


Fig. 3. Pour plates.

5.2.1.1 Allow agar to cool to approximately 45°C. To each labeled petri dish add approximately 15 to 20 mL of standard tryptone glucose extract agar for bacteria, or 15 to 20 mL of acidified potato dextrose or mycophil agar for fungi. Agitate the plates with a rotary or figure-8 motion to obtain even distribution of the pulp fibers or the water. Place plates on a level surface to allow agar to solidify.

5.2.2 *Spread plate.* The spread plate is recommended as an alternate procedure for fresh water and other such waters of which the temperature is below 45°C; in order to avoid damage or death to heat sensitive microorganisms.

5.2.2.1 To perform this procedure, pre-pour the nutrient plates and allow them sufficient time to solidify and dry (about 12-24 hours). If pre-poured plates are not used immediately (within one day), they should be sealed in a plastic bag and stored inverted at 4°C for a maximum of two weeks. Add 0.1 mL of the serial dilutions to be plated to the hardened and dried agar surface and spread with a sterilized (section 3.1.3) bent glass rod (hockey stick arrangement) taking care not to singe the sample.

5.2.3 *Petrifilms®.* Use the same serial dilution scheme outline in 5.1.1. However, if odd numbered dilutions are desired, prepare dilutions as outlined in Figure 4. For plating on the films, pipette exactly 1 mL of the desired dilution into the appropriately labeled Petrifilm. Allow the flap to fall back into place and use the template to spread the liquid as outlined by the manufacturer. After 1 minute, the films can be transferred to the incubator.

NOTE 1: The TTC dye in the Petrifilm medium, which turns red when reduced, can be inhibitory to some bacteria. Parallel plating with traditional methods may be of value when working in a new system.

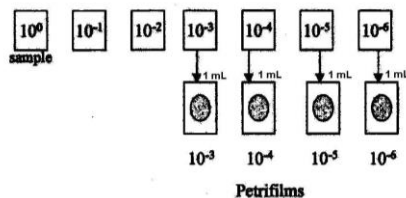


Fig. 4. Dilutions for odd numbers.

5.3 *Control plates.* Pour one control plate (without specimen) from each container of nutrient medium to check for sterility. Pour an additional plate and expose to room atmosphere during plating operation not to exceed 30 min. exposure. One bottle of dilution water from each lot or batch should be plated to confirm sterility by adding 1 mL of water to a plate and adding nutrient medium or plate on Petrifilm.

5.4 *Incubation.* After the pour plates have been agitated, place each plate on a level surface until the medium solidifies, invert and incubate the plated specimens at the desired temperature and time: normally bacteria are incubated at 36 ± 2°C for 48 hours and fungi at 30 ± 2°C for 4 to 5 days. Incubate Petrifilms in stacks no greater than 20 in number at temperatures recommended by the manufacturer. Record any deviations of temperature or time, e.g., if process water temperature is used as incubation temperature.

5.5 *Counting plate cultures.*

5.5.1 After incubation, examine the plates for the presence and number of bacterial or fungal colonies.

5.5.2 If any particles are observed which cannot be definitely identified as colonies, examine them microscopically. Examine and make a record of the number of colonies found on each control plate.

5.6 *Disposal of plate cultures.* Following examination, place the plate culture or film into a disposable autoclave bag and sterilize in an autoclave for 30 - 60 min at 121°C and 15 psi. Discard the bag following applicable regulations for laboratory waste.

6. Report

This method is a quantitative examination of the bacterial and fungal content of both process water and low-consistency stock. For each fresh water or low consistency stock sample, report the results as the number of colonies per milliliter. Report the results for slush stock of high consistency as the number of colonies per gram.

7. Precision

7.1 Repeatability (within a laboratory) = 5%

7.2 Reproducibility (between laboratories) = 10%

7.3 These values are based on those reported in Standard Methods for Examination of Water and Wastewater. The user of these precision data is advised that it is based on actual mill testing, laboratory testing, or both. There is no knowledge of the exact degree to which personnel skill or equipment was optimized during its generation. The precision quoted provides an estimate of typical variation in test results which may be encountered when this method is routinely used by two or more parties.

8. Keywords

Process water, Slush pulps, Pulp, Biological control, Microbiology, Microorganism control, Bacteriology, Fungi, Dispersions

9. Additional information

Effective date of issue: To be assigned.

References

1. American Public Health Association, "Standard Methods for the Examination of Water and Waste Water," 18th edn. 1994.
2. Collins, C.H., Lyne, P.M., Grange, J.M., *Microbiological Methods*, Sixth Ed. Oxford, UK: Butterworth and Co., 1989, pp. 126-140.
3. Tortora, G.J., Funke, B.R., Case, C.L., "Microbiology: An Introduction" Fourth Ed. Redwood City, CA: Benjamin/Cummins, 1992, pp. 158-160.

Your comments and suggestions on this procedure are earnestly requested and should be sent to the TAPPI Standards Department. ■