

A method for the determination of microbe concentrations
by means of a plating method and dishes used therein

The invention relates to a method for the determination of microbe concentrations by a plating method, wherein a sample and a medium are plated in a dish, the medium is allowed to solidify and the solidified plate is incubated, whereafter the colonies in the plate are counted. The invention is also concerned with a dish suitable for the determination of microbe concentrations by means of a plating method.

In the dairying and other foodstuff microbiology, medical microbiology as well as in the general microbiology, the determination of the number of microbes contained in a sample is generally carried out by means of dilution plate methods. Many of the so-called international comparative methods are of this type.

In dilution plate methods, a certain volume of a sample to be examined or of a dilution thereof is inoculated in a petri dish. E.g. 10 to 15 ml of a sterile culture medium capable of solidifying (agar) and having a temperature of $45 \pm 1^{\circ}\text{C}$ is poured on the sample. The sample is immediately mixed with the medium, and the solidification is allowed to take place at room temperature on a horizontal surface. The sample and the medium can also be mixed before the plating. The solidified medium forms a layer of even thickness on the dish. The solidified plates are turned upside down and placed in an incubator (incubation). The incubation temperatures (e.g. 10°C , 30°C , 37°C , 44°C , 55°C) and the incubation time (e.g. 2, 3, 6 and 10 days) depend on the method applied, mainly on what microbe groups are to be examined. The dishes are in a horizontal position during the incubation.

After the incubation, colonies in the petri dish

are counted. The amount of the inoculum in the sample and the dilution ratios being known, the number of microbes per ~~one~~ unit volume or ~~one~~ unit weight of the sample, usually microbes/ml or microbes/g, can be determined from the number of countable colonies.

The medium may contain as wide a diversity of nutrients as possible in order that as many microbe types of the sample as possible would have the required growth conditions (a so called total colony count). The nutrients of the medium may also be restricted or known growth inhibiting compounds may be added to the medium. Such media are referred to as selective media, and the object is to make a certain microbe group distinguishable.

The counting can be carried out ocularly (by means of known axillary devices) or by means of various electrical counting devices (automatic colony counters).

A high colony concentration in the dish restricts the counting. It is customary to include in the counting only plates the colony quantity of which does not exceed a determined limit; for instance, with plates having a diameter of 9 cm, the upper limit is regarded to be 300 colonies. In order to be able to examine samples having a high microbe concentration, it is necessary to use dilution techniques. In practice, it is usually always necessary to use dilutions in the plating work. The preparation of successive dilutions covers 70 to 90 per cent of the working time required for the whole plating stage (depending on the supposed need of dilution). Correspondingly, the material need is a multiple of the number of dilutions.

Prior dilution plate methods have thus a disadvantage that they require the preparation of large dilution series and the cultivation of several dilutions. A great number of dishes is thereby required; further, a lot of incubation space is needed. In other words, the

methods are complicated and time-consuming.

The object of the present invention is to provide a method which avoids the above disadvantages and enables the determination of microbe concentrations in a simple and reliable manner. This is achieved by means of the method according to the invention which is characterized in that the medium and the sample are mixed in order to obtain a homogeneous mixture ~~and~~^{the} microbe concentration of which is constant, and the mixture is made to solidify so that the layer thickness of the medium varies in a controlled manner.

The basic idea of the invention is that a medium containing microbes is made to solidify in a dish so that the layer thickness of the solidified medium varies in a determined manner. The medium can be made to solidify in a single dish, so that the layer thickness is determined by means of the geometry of the dish. When the layer thickness of the medium varies from one dish portion to another, the quantity of the colonies varies correspondingly.

The basic idea can also be applied in such a manner that the mixture of the sample and the medium is metered in conventional petri dishes in amounts differing in an accurately determined way. This produces a series of plates having different layer thicknesses. The method as such, however, does not offer other rationalization advantages than the elimination of the need of successive dilutions.

According to the basic idea of the invention, developed colonies are distinguished after incubation in different densities at the enumeration stage, the density being at its lowest in the thinnest agar layers and at its highest in the thickest layers, varying with the geometry of the medium in areas having a layer thickness therebetween. When the sample and the medium (agar) are mixed carefully before plating, the amounts thereof being

known and the geometry of the medium and the dish mathematically controlled, the microbe concentration of the sample is easy to determine. All information corresponding with accepted microbiological standards can be utilized in the determination: only colonies clearly distinguishable in the different layer thickness zones of the plate are taken into account. The layer thickness gradient determines the density gradient of the colonies, and this is taken into account in the mathematics of the colony enumeration. At the plating stage, it is of importance that the dishes are really positioned on a horizontal surface.

The advantages of the plating method according to the invention:

1. No successive dilutions are necessary, because the colony density is sufficiently low to be counted at least in some parts of the plate by virtue of the varying layer thickness of the culture medium. As compared with prior methods, a 70 to 90 per cent saving is obtained in the working time, because no successive dilutions are needed.
2. The saving obtained in the culture medium is proportional to the need of successive dilutions in prior methods (as well as, consequently, to the number of parallel dishes needed).
3. If disposable dishes are used, the material savings are proportional to the number of successive dilutions required in prior methods.
4. The need of incubation space is reduced considerably because no parallel dishes required by successive dilutions are needed. The same applies directly to the need of working rooms and material facilities.
5. It is not necessary to count the colonies over the whole dish, but a colony material sufficient for the determination is obtained from counting zones and sectors

determined by the geometry of the bottom of the dish. The counting operation is also simplified and speeded up by the fact that dilution and its consequences are eliminated.

6. The entire method, including the colony enumeration, can be mechanized and automated, mainly by utilizing and modifying prior techniques.

The microbe concentrations obtained by means of the method according to the invention correlate extremely well with results obtained by means of prior methods. The method can replace all prior dilution plate methods.

The invention also provides a dish suitable for the determination of microbe concentrations by means of a pour plate method wherein a homogeneous mixture of a sample and a medium is poured into the top of a dish at a single pouring point, the mixture is allowed to solidify, and the solidified plate is incubated, whereafter colonies in the plate are counted. The dish is characterised in that the shape of the dish is mathematically known and constant, whereby after the pouring of the homogeneous mixture into the dish solidified mixture having a controlledly varying layer thickness is formed in the dish.

The basic idea of the invention, i.e. that the thickness of the solidified medium in the dish varies in a known, controllable manner, can be realized by the use of dishes of a new type: the dish is such in shape that after the mixture of a sample and a medium has been plated on a horizontal surface, a solidified layer is formed in the dish the thickness of which layer varies in a controlled manner. This new type of dish may be e.g. round, such as a conventional petri dish, or rectangular, for instance. The layer thickness of the medium is adjusted to a desired value by giving the bottom surface of a round dish a spherical or a mathematically controlled aspherical shape which may be rising or descending. The bottom of a round dish can also be made rising or descending by providing the dish with concentrically ring-

shaped, horizontal planar surface areas, with a circular planar surface area in the middle. The bottom of a rectangular dish may rise linearly, along a circular arc or in some other mathematically controlled manner. It is also possible to provide the bottom of a rectangular dish with rectangular planar surface areas to make it rising. A preferred shape of a rectangular dish is a rectangle. The geometry of the dish can also be set by means of partitions of different heights positioned on the bottom thereof. The minimum layer thickness of the medium may be e.g. 1 mm and the maximum thickness 10 mm in the dishes according to the invention.

All the advantages offered by the method according to the invention can be achieved by means of this kind of dishes. No successive dilutions are needed, and the savings obtained in the materials, in the medium and in the incubation space are considerable as compared with prior methods. When using a dish having the shape of a rectangle, the need of medium is automatically smaller, at best only about 50 per cent of the medium need of a petri dish, and the need of incubation space is over 20 per cent smaller than that of round dishes, calculated on the surface area.

Accordingly, it is most advantageous according to the invention to use a dish having the shape of a rectangle. Furthermore, it is thereby preferable that the sample (inoculum) and the used medium are mixed properly before plating.

Figures 1A, 2A, 3, 4A, 5, 6 and 7 are vertical sectional views of ~~the~~ different embodiments of the dish according to the invention and the respective media, and Figures 1B, 2B and 4B are top views of the respective dishes.

Figures 1A and 1B show a dish 1 which is round as seen from above and a bottom 1a of which is upwardly convex. The medium is designated by the reference numeral 2.

It appears from the figures that when the layer thickness of the medium increased towards the outer periphery of the dish in a known manner determined by the geometry of the bottom of the dish, the colony density increases correspondingly.

Figures 2A and 2B show a dish 11 which differs from the preceding one in that a bottom 11a thereof is downwardly convex, and, as a consequence, the layer thickness of the medium 2 and, correspondingly, the colony density is at its highest in the middle of the dish, decreasing towards the edges of the dish.

Figure 3 shows a dish 21 a bottom 21a of which is formed by horizontal planar surface areas which are arranged concentrically so that the shape of the bottom changes in a stepwise manner. The dish is thus provided with circular layer thickness zones corresponding to the geometry of the bottom.

Figures 4A and 4B show a dish 31 which has the shape of a rectangle as seen from above and the shape of a bottom 31a of which changes linearly. The layer thickness increases from one short side of the dish towards the other, and the quantity of the colonies increases in a corresponding way.

Figure 5 shows a dish 41 a bottom 41a of which rises in an arched manner, the layer thickness gradient and the density gradient of the colonies changing accordingly.

Figure 6 shows a dish 51 a bottom 51a of which is formed by rectangular planar surface areas so that it rises in a stepwise manner. Layer thickness zones corresponding with the geometry of the bottom of the dish are thereby formed in the dish.

Figure 7 shows a dish 61 the bottom of which is provided with partitions 61a. The sample-agar mixture is introduced into a compartment 1 wherefrom part of the

mixture flows into a compartment 2, etc. so that separate layer thickness zones are formed.

The invention will be illustrated in more detail by means of the following examples.

Example 1

1 μ l of a milk sample is inoculated aseptically (a loop or a micropipette) in 12 ml of a molten sterile Standard Plate Count Agar (such as FIL-IDF 100:1981) contained in a sterile test tube and cooled to a temperature of 45°C. After the inoculation the mixture of the sample and agar is shaken properly e.g. in a test tube mixer.

After the mixing the sample-agar mixture is plated in a rectangular dish the bottom of which is rising, being formed by horizontal, rectangular planar surface areas. The dish comprises five planar surface areas, the size of each are being 4 cm². The planar surface areas are stepped with 2 mm height differences in a rising manner. A liquid amount of 12 ml, which is the agar volume of the example, fills the described dish in such a manner that five sectors equalling in surface area but having differing agar layer thicknesses are formed in the dish. The layer thicknesses are 2 mm in Sector I, 4 mm in Sector II, 6 mm in Sector III, 8 mm in Sector IV and 10 mm in Sector V; the agar amounts (and the sample amounts) being correspondingly: I 0.80 ml (0.066 μ l), II 1.60 ml (0.133 μ l), III 2.40 ml (0.199 μ l), IV 3.20 ml (0.266 μ l) and V 4.00 ml (0.333 μ l). The total agar surface area of the sectors is 20 cm² after the plating. The agar and sample amounts of the sectors are also shown in Table 1.

The dish is closed with a lid and incubated in a horizontal position during 72 $\pm$ 2 hours at a temperature of +30 $\pm$ 1°C.

The results are calculated separately for each sector by dividing the number of the countable colonies in each sector (in practice < 40 colonies per sector) by

the sample amount specific for each sector; in the present example, the sample amount is 0.066 μ l or 0.000066 ml in Sector I, and 0.333 μ l or 0.000333 ml in Sector V. Accordingly, if the number of the colonies in Sector I is 3, there are 3 colonies per 0.000066 ml $\sim$ 45,000 colonies per ml; and if the number of the colonies in Sector V is 17, there are 17 colonies per 0.000333 ml $\sim$ 50,000 colonies per ml. The result gives directly the microbe quantity of the sample per ml. The numbers of the colonies in the sectors and the results calculated on the basis thereof are shown in Table 2.

The information obtained from all countable sectors can be utilized in the result by calculating the mean value of the results obtained from each sector.

Table 1

Sector (A = 4 cm ²)	Agar (ml)	Sample (ml)	% of the sample
V	4.00	0.000333	33
IV	3.20	0.000266	28
III	2.40	0.000199	20
II	1.60	0.000133	13
I	0.80	0.000066	6

Table 2

SPC x 1 000	SPC-modification					the microby quantity of the sample, microbes x 10 ³ /ml				
	I	II	III	IV	V	I	II	III	IV	V
10	1	1	2	3	3	-	-	-	11	9
50	3	7	10	13	17	45	53	50	49	51
100	7	13	20	27	33	106	98	100	101	99
200	13	27	40	53	67	197	203	201	199	-

Example 2

The sample is inoculated aseptically in agar in a dilution ratio of 10^{-4} (10 μ l of the original sample to 100 ml agar). After the inoculation, the agar-sample mixture is mixed efficiently, and it is plated in conventional petri dishes in the amounts of 2, 5, 10, 20, 40 and 60 ml, so that the amounts of the original sample are 0.0002 ml, 0.0005 ml, 0.001 ml, 0.002 ml, 0.004 ml and 0.006 ml respectively. The dishes are incubated and the colonies are counted. The numbers of the colonies counted from the dishes are converted into the microbe density of the original sample by means of the following formula:

$$\text{the microbe density of the sample} = \frac{\text{the counted colony density}}{\text{the amount of the original sample}}$$

The results are shown in Table 3.

Table 3

SPCx1000	Colonies in the dishes (amounts of sample-agar mixture)					
	2 ml	5 ml	10 ml	20 ml	40 ml	60 ml
5	1	3	5	10	20	30
20	4	10	20	40	80	120
50	10	25	50	100	200	300
100	20	50	100	200	(400)	(600)
200	(40)	(100)	200	(400)	-	-
300	(60)	(150)	300	-	-	-
500	(100)	(250)	(500)	-	-	-

The parentheses in the table mean that it is not possible to count these colony densities in the dishes. When using petri dishes having a diameter of 90 mm, the bottom of the dish is completely covered with agar only in dishes containing 10 ml or more of agar.

References:

Standard Plate Count Method:

1. FIL-IDF 100:1981 Liquid Milk Enumeration of Microorganisms Colony Count Technique at 30°C.
2. ISO/DP 6610 *ibid*.
3. APHA American Public Health Association Standard Methods for the Examination of Dairy Products 14th edition 1978, p. 77 to 93.

Plate Loop Method:

1. APHA 14th ed. p. 315 to 317.
2. Thompson, Donnelly & Black 1960 A Plate Loop Method for Determining Viable Counts of Raw Milk.
3. J. Milk Food Technol. 26: 156 to 171.

Claims

1. A method for the determination of microbe concentrations by a plating method, wherein a sample and a medium are plated in a dish, the medium is allowed to solidify and the solidified plate is incubated, whereafter the colonies in the plate are counted, characterized in that the medium and the sample are mixed in order to obtain a homogeneous mixture the microbe concentration of which is constant, and the mixture is made to solidify so that the layer thickness of the medium varies in a controlled manner.

2. A method according to claim 1, characterized in that the medium is made to solidify in a single dish, the layer thickness being regulated by means of the geometry of the dish.

3. A method according to claim 1, characterized in that the medium is made to solidify in a plurality of dishes, the layer thickness being regulated by means of the size of the dish and/or the volume of the medium.

4. A dish suitable for the determination of microbe concentrations by means of a pour plate method, wherein a homogeneous mixture of a sample and a medium is poured into the top of a dish at a single pouring point, the mixture is allowed to solidify and the solidified plate is incubated, whereafter colonies in the plate are counted, characterized in that the shape of the dish is mathematically known and constant, whereby after the pouring of the homogeneous mixture into the dish solidified mixture having a controlledly varying layer thickness is formed in the dish.

5. A dish according to claim 4, characterized in that it is round in cross-section.

6. A dish according to claim 4, characterized in that it is rectangular in cross-section.

7. A dish according to claim 4, characterized in that its bottom is concave.



8. A dish according to claim 4, characterized in that its bottom is convex.

9. A dish according to claim 4, characterized in that its bottom is linearly rising.

10. A dish according to claim 4, characterized in that its bottom rises in accordance with a circular arc.

11. A dish according to claim 5, characterized in that its bottom is rising or descending, the dish being provided with concentrically ring-shaped horizontal planar surface areas, with a circular planar area in the middle.

12. A dish according to claim 6, characterized in that the bottom is rising, being formed by rectangular planar surface areas.

13. A dish according to claim 4, characterized in that one partition less than the height of the perimeter of the dish or a plurality of partitions of differing heights less than the height of the perimeter of the dish is/are mounted on the bottom part thereof.



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