METHOD FOR DETECTING CELLS IN A SAMPLE USING AN ENZYME INDUCTION PROMOTER

A method for quickly detecting very little numbers of bacterial cells in a sample by fluorometric measurement, particularly but not exclusively applied to test drinkable water and generally water which is to be drained into waters and public sewage systems, using a promoter of enzyme induction and wherein no nutritive substance is added to the sample.
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METHOD FOR DETECTING CELLS IN A SAMPLE USING AN ENZYME INDUCTION PROMOTER

The present invention relates to a method for quickly detecting an even very little number of bacterial cells in a sample.

The method is particularly applied to drinkable water and generally water which is to be drained into waters and public sewerage systems. This is the application which will be better described hereinafter also by means of examples.

It is known that the water bacteria contamination potentially most dangerous for the human health is caused by bacteria of animal origin such as *Salmonella, Streptococcus*, etc., which can be detected by the presence of other bacteria usually associated therewith giving a positive indication of such contamination. These associated bacteria are mostly *Escherichia Coli*, *Klebsiella*, *Citrobacter* and *Enterobacter* belonging to the group of coliforms which is arbitrarily divided into Total Coliforms and Faecal Coliforms, Total Coliforms being diffused on the surface of ground and waters and Faecal Coliforms including the *Escherichia Coli* species characterized by a lower resistance and surviving capability in the external environment than Total Coliforms.

State of art regarding the known analytical methods

The analytical methods for the analysis of coliforms
developed up to now can be divided into traditional methods and innovatory methods.

Traditional methods

Among the traditional methods there are two extensively used, widespread tests based essentially on two procedures:

- the use of a medium including nutritive substances and selective agents allowing the growth of only coliform bacteria which can be seen by monitoring the turbidity and the development of colonies;
- the use of lactose for inducing, during the growth of coliform bacteria, the synthesis of β-galactosidase which can be detected as development of gas.

1) **Method of the calculation of the most probable number (MPN)**

The analytical test is based on a first nutritive, not selective culture medium containing lactose (lactose broth). The sample to be analyzed is seeded as such in test tubes and flasks containing a double-concentration lactose broth and incubated at 37°C for 24-48 hours; after such period of time the positivity is shown by the turbidity due to the growth of bacteria along with the production of gas, an indication of fermentation activity of lactose. The presence of coliforms in positive test tubes and flasks should be confirmed: an amount of the positive broth is seeded into two groups of test tubes (containing a nutritive medium selective by the presence of brilliant green, biliary acids and
lactose), and incubated for further 24-48 hours at 36±1°C and 44.5±0.2°C; such two temperatures allow total and faecal coliforms to be discriminated. The positivity of the confirmation test is shown by turbidity and production of gas. According to the positivity figure and the use of predetermined tables the result is expressed as most probably number (MPN) of cells in the sample.

2) Method of the filtering membranes (FM)
The method provides the filtration of the water sample and the following incubation of the filter on a nutritive, selective agar medium containing: lactose, some selective agents such as biliary salts, sodium dodecyl sulphate, Tergitol 7, Teepol, rosolic acid, and dyes such as basic fuchsin or aniline blue; the latter can show colonies which are able to develop in such medium.

Also in this case the incubation temperatures at 36±1°C and 44.5±0.2°C are used to discriminate between total and faecal coliforms. The time necessary for the analysis in this method is 24 hours and the confirmation test is no longer needed. The positivity of the test is shown by monitoring and counting the developed colonies. Therefore, it seems possible to find out the limit of the traditional methods in the long time of incubation which ranges from 24 hours as a minimum to 96 hours as a maximum, which time is necessary to make the cell growth and the induction of β-galactosidase visible.
due to turbidity, formation of colonies and production of gas.

Because of the long time necessary for the test, the traditional methods are not compatible with the sanitary requirements of having a quick response. Therefore, the need of new quick methods able to evaluate the quality both of a reflux and a drinkable water is very strong.

New methods disclosed in "Standard Methods" (American Public Health Association) mainly try to satisfy the requirement of having a quick response and are divided into two different groups: those modifying the traditional methods and those requiring special instruments and materials.

Briefly the need of having innovatory methods and substances for the satisfactory solution of the problem is very strong.

The new tests provided by optimizing traditional methods are accomplished by changing the composition of the agar culture medium; in these cases, however, the time of response are excessively long or at least not adapted to the requirements of quickness mentioned above.

Therefore, innovatory methods utilizing special instruments and materials have been conceived and accomplished as follows:

- Radiometric method;
- Measurement of the activity of glutamic decarboxylase;
- Electrochemical methods;
- Spectrofluorometry methods;
- Measurement of the impedance;
- Gas chromatography tests;
- Colorimetry tests;
- Potentiometric tests.

Most of them are neither sensitive enough to be used with drinkable water nor specific; they can be used for reflux or surface water but require expensive reagents and specialized personnel due to their complexity. Except for the colorimetry method, all of the other methods are not compatible for routine analysis and, therefore, are only used as research instrument; however, also the colorimetry test needs a time of response which ranges from 8 hours as a minimum to 30 hours as a maximum, which is not compatible with the need of having a quick sanitary control.

The scientific research engaged in the development of new methods sees the contribute of Berg and Fiksdal (1988) through the publication of a method based on the use, as in traditional methods, of a nutritive growth medium selective for coliforms and containing lactose moreover enriched with methylumbelliferil-β-D-galactoside (MUG), fluorogenic substrate for β-galactosidase.

Samples of river water were filtered to take away bacterial cells and the filter was incubated at 35°C or 41.5°C in the nutritive medium; the positivity of the test could be shown after 15 minutes incubation by measuring the fluorescence developed in the medium by
the MUG hydrolysis caused by the induced β-galactosidase. The declared sensitivity limit was 500 cells for any filter used in the incubation; therefore, the minimum enzyme quantity which could be measured was compared to the minimum number of 500 incubated cells in 15 minutes.

In such a method, the fluorometric detection system replaces the "turbidity" and "production of gas" detection parameters and allows coliform cells to be shown in short time due to the high sensitivity of the fluorescence. In any case, as in the traditional methods, the cells are placed in a nutritive, selective medium, where only coliforms can easily multiply; in the same way, the synthesis of β-galactosidase is promoted in the cell duplication process.

More recently Apte and Batley (1994) have used the fluorometric detection of coliforms exclusively on sea water samples; the proposed test was conducted by adding to the water sample the buffer system PIPES and the fluorogenic substrate MUG; the sample was incubated at 44.5°C during not less than 60 minutes and the fluorescence thereof was then measured. The declared sensitivity limit was 100 cells/100 ml sample; in this case, the minimum enzyme quantity which could be measured was compared to a minimum number of 100 incubated cells in 60 minutes.

The Authors point out that nutrients naturally present in sea water are on one hand indispensable to achieve a good sensitivity of the system, on the other hand
justify the non-use of nutritive medium. Therefore, also in this case as in the traditional methods, the synthesis of β-galactosidase is promoted in the cell duplication system.

The method proposed by Berg and Fiksdal and by Apte and Batley did not find any technological application certainly because of the still low sensitivity of the system and its capability of being applied on only one type of sample. In any case, the experimentation of such methods at the Applicant's laboratory has shown a low reproducibility besides a sensitivity very far from that declared. Furthermore, the results have shown that both methods are based on the growth of the number of bacterial cells placed in a highly nutritive medium; actually, the problem of the impossibility of detecting the activity of β-galactosidase in samples with a few coliforms would be overcome by allowing the cells to develop and to reach the minimum number containing the minimum quantity of enzyme which can be detected.

New analytical method
A substance not known in the literature was surprisingly found out in the Applicant's laboratory, such a substance being able to increase considerably the β-galactosidase activity of a single coliform cell during a process not involving the cell duplication; such a phenomenon allows a minimum quantity of enzyme to be detected in case of lower number of cells. The above substance will not either be defined or illustrated in detail in the present description.
because it is the object of a previous Patent Application of the same Applicant.

In the present invention the quantity of induction promoter is defined as "arbitrary activity unit", whereby under unit it is intended that quantity of induction promoter able to promote in 1000 cells the synthesis of that amount of β-galactosidase able to hydrolize at least 50 picomoles of MUG in 60 minutes by the method described hereinafter.

The proposed analytical system is based on the use of such a substance and can detect at least 10 cells in a time of 10 minutes and without cell multiplication. The enzymatic activity is determined through spectrofluorometry by using solutions B and C (see below) after a high induction of β-galactosidase enzyme into coliform cells in a short time and without cells multiplication.

The method according to the invention consists of the following steps:

a) **Preparation of the sample**: the separation of the bacterial cells from the sample is carried out by a physical treatment such as filtration: therefore, in case of liquid or gas sample, a suitable volume of the sample is passed through a filter having a cut-off not higher than 0.45 microns; in case of solid sample, the sample is extracted by a suitable liquid means, and the liquid is filtered as previously indicated. The material held by the filter is then used for the analysis.
b) **Induction of the enzyme**: the material held by the filter can be treated in one of the following ways:
- dipping the whole filter in just the amount of solution A to cover it;
- putting the surface of the filter where the material is held in contact with an amount of solution A;
- recovering the material held by the filter through extraction with an amount of solution A.
In all of the above ways the system "held material-solution A" is treated at a temperature between 10 and 40°C for total coliforms and between 40 and 50°C for faecal coliforms during at least 5 minutes, as already mentioned.

c) **Formulation of the activity of the induced enzyme**: after the end of the induction time, at least 5 microlitres of solution B per ml of solution A is added to the system of the solution A and the filter; after short stirring, the sample is treated at a temperature not higher than 50°C during at least 5 minutes. After the end of such a time, the reaction is interrupted by adding solution C to bring the final pH to over 9.0.

d) **Measurement**: the solution obtained after the last treatment is poured into a quartz basin and placed into a fluorometer; the measurement is carried out at excitation and emission wavelengths between 330 and 370 nm and between 420 and 460 nm, respectively; both wavelength windows are set preferably at about 5-10 nm. The value of the white obtained with a sterile filter should be subtracted from the read value.
e) Evaluation of the results: the response to the analytical test is believed as positive when the fluorescence measurement, expressed in arbitrary units of the instrument, indicates a statistically significant increase over the value of the white. The positivity of the test can be evaluated both qualitatively and quantitatively; under qualitatively it is intended the capability of discriminating total coliforms from faecal coliforms according to what incubation temperature has been used in the enzyme induction step; the quantitative evaluation needs first a calibration curve of the instrument provided by using known concentrations of total or faecal coliforms. Through such a curve the number of coliform bacterial cells in the original sample can be defined.

The system according to the invention has the following advantages:

1) the drastic reduction of the analysis time over the traditional methods due to the use of a new induction means able to stimulate, in case of no growth of the cells, the synthesis of amounts of β-galactosidase which can be detected also in samples containing a low concentration of cells;

2) the original use of a new substance (induction promoter) as main generator of said properties of the induction means;

3) the lack of nutritive substances and then no undue increase when subjected to fluorometric test;

4) the presence of magnesium ion Mg** as absolutely
indispensable element for the induction of cells in such a system;
5) the further increase of the β-galactosidase activity as a consequence of the use of a higher concentration of the substrate achieved because of its solubilization in dimethylsulphoxide (DMSO).

The following reagents have been used in the method accomplished by the procedures described hereinafter:
1) Solution A: water solution containing: a buffer system such as Na₂HPO₄ and KH₂PO₄; bivalent ions and more particularly magnesium Mg⁺⁺ (such ions are essential); the induction promoter; a selective agent such as sodium dodecyl sulphate; an inducer of the β-galactosidase synthesis such as for example isopropyl-β-D-thiogalactopiranoside; β-mercaptoethanol; the solution was adjusted to a pH between 2 and 10 with drops of KOH or other bases and sterilized by filtration through a filter having a porosity not greater than 0.45 microns.
2) Solution B: solution of methylumbelliferil-β-D-galactoside (MUG) dissolved in dimethyldisulphoxide and chloroform. DMSO/CHCl₃ ratio should not be lower than 3:1 v/v.
3) Solution C: water solution of NaOH or other base.

The analysis described in the following examples have been carried out by the method illustrated above:
Example 1:
Filtration: a sample of 10 ml water containing 500
cells of *Escherichia coli* was filtered through a filter of cellulose acetate having a diameter of 13 mm and a cut-off of 0.45 micron; the water was drained and the filter used for the analysis.

**Induction of the enzyme:** the filter containing the cells was dipped into 1 ml of solution A containing 47.6 mM Na₂HPO₄, 22 mM KH₂PO₄, 0.5 mM MgSO₄, one unit of the induction promoter, 0.01% sodium dodecyl sulphate 0.25 mM isopropyl-β-D-thiogalactoside, 0.27% v/v β-mercaptoethanol. Solution A had been adjusted to a pH 7.5 with drops of soda and sterilized by filtration on a hydrophilic filter having a porosity of 0.45 microns. The sample was then treated at 44.5°C during 90 minutes.

**Formulation of the activity of the induced enzyme:** after the end of the induction time, 60 microlitres of solution B containing 16.66 mM MUG dissolved in dimethylsulphoxide/chloroform 5/1 v/v was added to the sample; after short stirring, the sample was treated at 37°C during further 60 minutes. After the end of such a time, the incubation was interrupted by adding 20 microlitres of solution C containing 2N NaOH in water.

d) **Measurement:** the solution was poured into a quartz basin and placed into a fluorometer; the measurement was carried out at excitation and emission wavelengths of 364 nm and 448 nm, respectively, and the wavelength windows was set at 5 nm. The value of the white obtained without cells with a sterile filter treated in the same way should be subtracted from the read
value. The measured fluorescence was 2.28 arbitrary units corresponding to 42 pmoles of hydrolized MUG (90 pmoles/1000 cells).

Example 2
Filtration: a sample of 10 ml water containing at least one cell of Escherichia coli (confirmation with FM method) was filtered through a filter of cellulose acetate having a diameter of 13 mm and a cut-off of 0.45 micron; the water was drained and the filter used for the analysis.

Induction of the enzyme: the filter containing the cells was dipped into 1 ml of solution A containing 47.6 mM Na₂HPO₄, 22 mM KH₂PO₄, 0.5 mM MgSO₄, 500 units of the induction promoter, 0.01% sodium dodecyl sulphate 0.25 mM isopropil-ß-D-thiogalactoside, 0.27% v/v β-mercaptoethanol. Solution A had been adjusted to a pH 7.5 with drops of soda and sterilized by filtration on a hydrophilic filter having a porosity of 0.45 microns. The sample was then treated at 44.5°C during 60 minutes.

Formulation of the activity of the induced enzyme: after the end of the induction time, 60 microlitres of solution B containing 16.66 mM MUG dissolved in dimethylsulphoxide/chloroform 5/1 v/v was added to the sample; after short stirring, the sample was treated at 37°C during further 60 minutes.

After the end of such a time, the treatment was interrupted by adding 20 microlitres of solution C containing 2N NaOH in water.
d) **Measurement**: the solution was poured into a quartz basin and placed into a fluorometer; the measurement was carried out at excitation and emission wavelengths of 364 nm and 448 nm, respectively, and the wavelength windows was set at 10 nm. The value of the white obtained without cells with a sterile filter treated in the same way should be subtracted from the read value. The measured fluorescence was 2.5 arbitrary units corresponding to 22 pmoles of hydrolized MUG (22,000 pmoles/1000 cells).

**Example 3**

**Filtration**: 10 ml water containing 2250 cells of Citrobacter was filtered through a filter of cellulose acetate having a diameter of 13 mm and a cut-off of 0.45 micron; the water was drained and the filter used for the analysis.

**Induction of the enzyme**: the filter containing the cells was dipped into 0.5 ml of solution A containing 50 mM K₂HPO₄, 10 mM NaH₂PO₄, 0.5 mM MgSO₄, one unit of the induction promoter, 0.01% sodium dodecyl sulphate 0.25 mM isopropyl-β-D-thiogalactoside, 0.27% v/v β-mercaptoethanol. Solution A had been adjusted to a pH 7.5 with drops of potash and sterilized by filtration on a hydrophilic filter having a porosity of 0.45 microns. The sample was then treated at 37°C during 60 minutes.

**Formulation of the activity of the induced enzyme**: after the end of the induction time, 30 microlitres of solution B containing 16.66 mM MUG dissolved in
dimethylsulphoxide/chloroform 5/1 v/v was added to the sample; after short stirring, the sample was treated at 37°C during further 60 minutes. After the end of such a time, the fluorometric measurement was immediately carried out.

5  d) Measurement: the solution was injected into a distilled water analysis system connected to a HPLC fluorometer; a loop of 70 microlitres connected to the injector introduced the sample into the flow; the measurement was carried out at excitation and emission wavelengths of 364 nm and 448 nm, respectively, and the wavelength windows was set at 5 nm. The value of the white obtained without cells with a sterile filter treated in the same way should be subtracted from the read value expressed as maximum value reached by the injected sample. The measured fluorescence was 3.21 arbitrary units corresponding to 141 pmoles of hydrolized MUG (63 pmoles/1000 cells).
Claims

1. A method for quickly detecting an even very little number of bacterial cells in a sample by fluorometric measurements with the use of a substance stimulating the enzymatic activity (induction promoter), consisting of the following steps:
   a) preparing solution A containing the induction promoter;
   b) preparing solution B containing the substrate for the enzyme;
   c) preparing a basic solution C with pH between 10 and 14;
   d) separating the bacterial cells from the sample or the extracts thereof by physical treatment;
   e) treating the bacterial cells with solution A;
   f) thermally treating the bacterial cells-solution A complex at a determined temperature not lower than 20°C for a determined period of time;
   g) adding solution B to bacterial cells-solution A complex and continuing the thermal treatment for a determined period of time;
   h) adding solution C;
   i) measuring the final complex by fluorometry.

2. The method according to claim 1, wherein the analysis is carried out without adding any nutritive substance to the sample.

3. The method according to claim 1, wherein said
solution A is a water solution containing a buffer system, bivalent ions, the induction promoter, a selective agent, and an inducer of the β-galactosidase synthesis.

4. The method according to claim 3, wherein said buffer solution consists of Na₂HPO₄ and KH₂PO₄.

5. The method according to claim 3, wherein said bivalent ion is Mg²⁺.

6. The method according to claim 5, wherein said Mg²⁺ is in the form of MgSO₄.

7. The method according to claim 3, wherein said selective agent is sodium dodecyl sulphate.

8. The method according to claim 3, wherein said inducer of the β-galactosidase synthesis is isopropyl-β-D-galactopiranoside.

9. The method according to claim 3, wherein said solution A has been adjusted to a pH between 2 and 10 with drops of KOH or other bases and sterilized by filtration through a filter having porosity not greater than 0.45 microns.

10. The method according to claim 9, wherein the pH of solution A is 7.5.
11. The method according to claim 1, wherein said solution B is a solution of methylumbelliferil-β-D-galactoside (MUG) in dimethylsulphoxide and chloroform, the DMSO/CHCl₃ ratio being not lower than 3:1 v/v.

12. The method according to claim 1, wherein said solution C is a water solution of NaOH.

13. The method according to claim 1, wherein the bacterial cells are total or faecal coliforms.

14. The method according to claim 1, wherein said temperature of treatment of bacterial cells-solution A complex for detecting total coliforms is between 35°C and 38°C, preferably 36°C±1°C.

15. The method according to claim 1, wherein said temperature of treatment of bacterial cells-solution A complex for detecting total coliforms is between 42°C and 46°C, preferably 44.5°C±0.2°C.

16. The method according to claim 1, wherein the enzyme is β-galactosidase.

17. The method according to the preceding claims used for the following detection of at least one bacterial cell, wherein: a sample of 10 ml water containing at least one cell of Escherichia coli (confirmation with FM method) is filtered through a filter of cellulose
acetate having a diameter of 13 mm and a cut-off of 0.45 micron; the water is drained and the filter used for the analysis; the filter containing the cell(s) is dipped into 1 ml of solution A containing 47.6 mM Na₂HPO₄, 22 mM KH₂PO₄, 0.5 mM MgSO₄, 500 units of the induction promoter, 0.01% sodium dodecyl sulphate 0.25 mM isopropyl-β-D-thiogalactoside, 0.27% v/v β-mercaptoethanol, solution A being adjusted to a pH 7.5 with drops of soda and sterilized by filtration on a hydrophilic filter having a porosity of 0.45 microns; the sample is then treated at 44.5°C during 60 minutes; after the end of the induction time, 60 microlitres of solution B containing 16.66 mM MUG dissolved in dimethylsulphoxide/chloroform 5/1 v/v is added to the sample; after short stirring, the sample is treated at 37°C during further 60 minutes; after the end of such a time, the treatment is interrupted by adding 20 microlitres of solution C containing 2N NaOH in water; the solution is then poured into a quartz basin and placed into a fluorometer; the measurement is carried out at excitation and emission wavelengths of 364 nm and 448 nm, respectively, the wavelength windows being set at 5-10 nm; the value of the white obtained without cells with a sterile filter treated in the same way is subtracted from the read value, the measured fluorescence being 2.5 arbitrary units corresponding to 22 pmoles of hydrolized MUG (22,000 pmoles/1000 cells).
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/10 C12Q1/04

According to international Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 5 510 243 A (BOYD STEVEN H ET AL) 23 April 1996 <em>Whole document</em></td>
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<td>US 4 242 447 A (FINDL EUGENE ET AL) 30 December 1980 <em>Whole document</em></td>
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Date of the actual completion of the international search: 22 October 1997
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