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(54) **Enhancement of the sensitivity of viable microbial cell count by bioluminescent assay of ATP and its use for identification of microbes by means of a short incubation in nutrient media**

(57) A method of increasing the sensitivity and accuracy of the measurement of viable microbial cells by the bioluminescent assay of ATP by means of activating the metabolism of inactive viable cells and by increasing the number of microbial cells or biomass during a short incubation period in a microbiological nutrient medium. The utilization of a short incubation of the sample in specific nutrient media that support or prevent the growth of particular species of microbes allows the detection and identification of specific species in a few hours.

FIG.1A

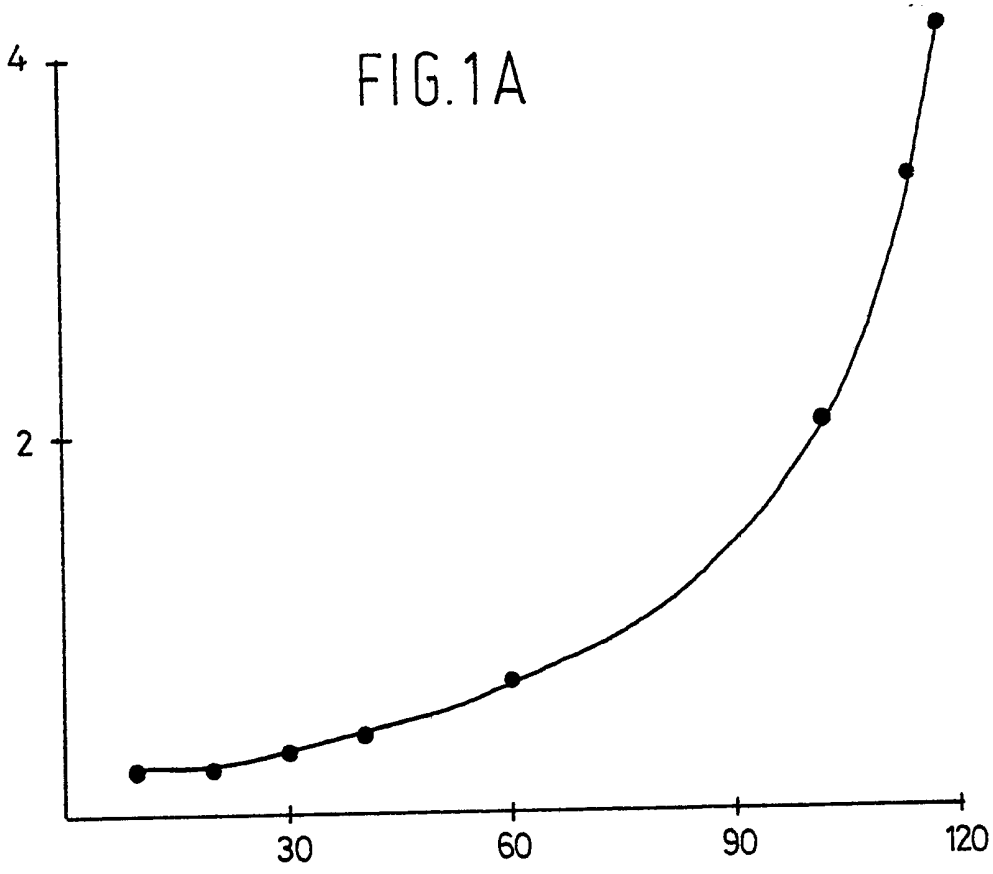
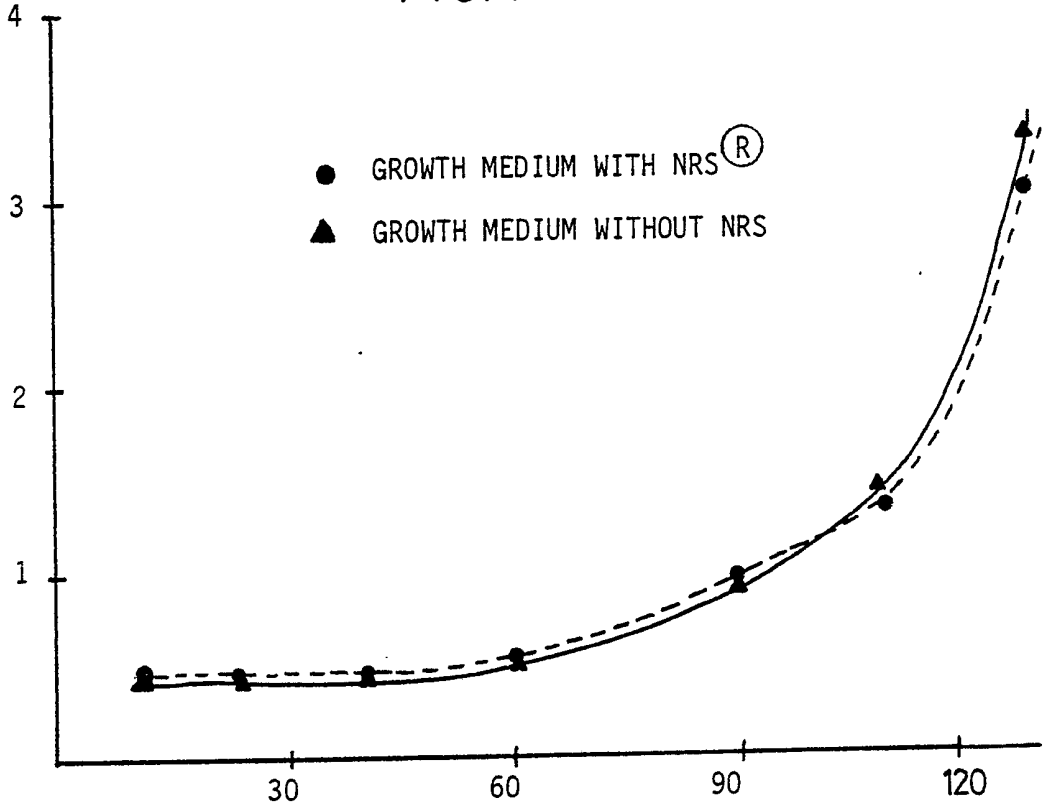
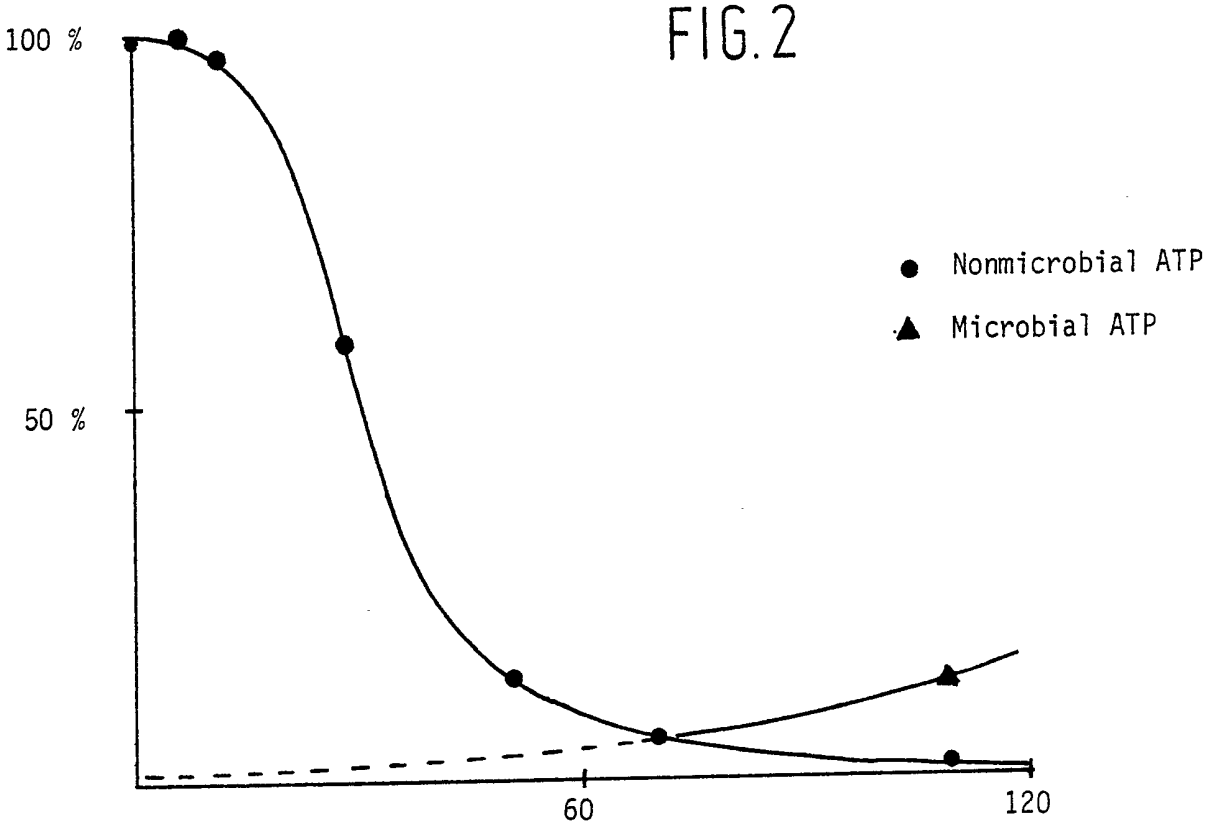

4
2
30
60
90
120

FIG.1B


4
3
2
1
GROWTH MEDIUM WITH NRS®
GROWTH MEDIUM WITHOUT NRS
30
60
90
120

2059990

FIG.2



SPECIFICATION

Enhancement of the sensitivity of viable microbial cell count by bioluminescent assay of ATP and its use for identification of microbes by means of a short incubation in nutrient media

High concentration of nonmicrobial ATP in urine, milk, blood and meat products have made it difficult or impossible to utilize the rapid ATP method for quantitation of bacteria in such samples, in spite of the fact that nonmicrobial ATP has been eliminated either by hydrolysis with potato apyrase enzyme (a Ca^{++} -dependent fraction ATPase enzyme), by filtration or centrifuging. If only a small fraction of percentage of the nonbacterial ATP is left in the sample when the ATP from bacteria is extracted, it may cause large errors in the bacteria count when based on the measurement of bactrial ATP. Furthermore, it has not been possible to detect specific microbes because the ATP method detects all ATP regardless which species are found in the samples. Metabolically inactive microbial cells have a low level of ATP and, unless the cells are activated, the sensitivity and accuracy of this method suffer.

The present invention overcomes these limitations in sensitivity, accuracy and the unfavourable ratio between microbial and nonmicrobial ATP. These goals are obtained by a short incubation of the sample with a specific nutrient medium, which can be either solid or liquid. Further advantage of the present invention is that it allows the incubation and measurement to be carried out in the same cuvette, thus eliminating possible errors by pipettings and transferrings of the sample. When specific media are used which support the growth of a certain species or type of microbes, the method can be utilized for identification of microbes. Reduced manipulations also allow the technique to be easily automated.

The principle of the method is as following: A small 10-1000 µl, preferably 10-100 µl, microbial sample is introduced into an ATP free nutrient medium that can be a general nutrient medium, a selective nutrient medium, or specific nutrient medium having specific nutrients, substrates, enzymes or growth inhibitors against specific microbes, surface active or other substances to lyze or kill selected microbial or nonmicrobial cells, or to make the cell wall and membrane permeable to ATP and other metabolites of selected microbes or non-microbial cells. The medium can also have a controlled quantity of ATPase enzyme, such as potato apyrase, from 0.001 to 0.5. units, but preferably from 0.01 to 0.1 units per millilitre for hydrolyzing non-microbial ATP during the incubation the measurement of extracted microbial ATP can be carried out without the inactivation of ATPase enzyme because the used activity of ATPase is so low that it allows the extraction and measurement of microbial ATP when the microbial ATP is measured within one minute after extraction, and preferably after a constant time lapse from extraction. The volume of nutrient medium can vary from 10 to 1000 µl, but preferably from 25 to 200 µl in a sterile transparent plastic of glass cuvette of diameter 5 to 30 mm, but preferably between 6-15 mm. The nutrient medium can also be applied on a filter membrane containing the microbial sample. In this method the filter membrane is placed, the right side up in a flat bottom, sterile container, e.g. a Petri dish.

The samples are incubated between 0.2-16 hours for bacteria and yeasts and 0.5 to 5 days for fungi and Mycobacteria. After the incubation time ATP is extracted preferably with 10 to 1000µl a 0.05-2 % aqueous solution of a mixture of ionic surface agents of the type of ethoxylated amines and quaternary salts of ethoxylated amines, such as NRB (Nucleotide Releasing Reagent for Microbial Cells; NRB is a registered trade mark of Lumac Systems AG, Basel), or by mineral acids, bases or any other substance which extracts ATP from microbial cells without diluting the sample excessively or introducing inhibitory substances against the firefly lucifering-luciferase reaction. If acids or bases are used the hydrogen ion concentration has to be brought to pH 6.5-8.5, but preferably to pH 7.75 to measurement of ATP with the luceferin-luciferase reagent. After extraction of ATP from microbial cells the sample is placed into the luminescence photometer. Prior to measurement of the sample 10-1000 µl, but preferably 50-200 µl of luciferin-luciferase reagent on a biochemical buffer (e.g. Tris or so called Good's buffers, HEPES, MOPS, TES, etc) of 0.01-0.1 molarity, 0.005-0.05 molarity of Mg^{++}-ion and 0.001 to 0.010 molarity of a chelating agent, such as EDTA 0.01-1 µl/ml luciferase, 1-100 µg/ml luciferin is mixed with the sample. The dispensing and mixing of this luciferin-luciferase reagent into the sample can be performed before placing the vial into the photometer, but preferably in the instrument itself. The intensity of light being produced is directly related to the concentration of ATP in the sample. The light emission can be measured as a continuous signal (or rate), integral over a preset time, or as a peak (maximum) value.

The principles of the present invention in different applications are described in the following examples:

EMBODIMENT I.

Measurement of low number microbes.

The sensitivity of biolumiscescent measurement of ATP depends on the sensitivity of the photometer and the activity of reagents used. Until recently, the commercial products allowed the measurement of 100,000 cells/cm^3 or 0.1 ng/cm^3 ATP. The method itself is much more sensitive and with recently introduced instrument (LUMACOUNTER ®, Lumac Systems AG, Basel) and firefly luciferin-luciferase reagents (LUMIT ® PM, Lumac Systems AG, Basel) it is possible to measure down to 0.1 pg ATP or about 100 bacteria in a 10-500 µl sample. However, due to self absorption of emitted light and inhibiting substance (chemical quenchers) the practical sensitivity is about 1000-5000 bacterial cells per millilitre. In order to measure hygienic quality of water, pharmaceuticals and sterilized products, it should be possible to measure down to

one bacterial cell.

Culturing of microbial cells (bacteria, yeast, fungi, slime molds), on a nutrient medium has been utilized since the beginning of bacteriology as the most commonly used method of quantitation of microbial cells. The most widely used technique is to make a serial dilution of a liquid sample and inoculate with these solid nutrient agar Petri dishes. After an incubation of 16-72 hrs for bacteria and 1-40 days for yeast and fungi at 37°-56°C the number of colonies grown on the agar are visually counted. The results are expressed as Colony Forming Units (CFU) for bacteria and yeast and as hyphi for fungi. CFU do not give the number of microbial cells, contrary to common beliefs, but number of aggregates of bacterial that are able to multiply. Usually, each colony is grown from a group of 1-50 cells, thus the total number of cells in original sample is much greater than number of CFU's.

Measurement of microbial cells with the luminescent assay of ATP is utilized as a rapid method of quantitizing microbes. This method is well known *per se* (US Patent 3,475,090). With purified luciferin-luciferase reagents and the presently introduced sensitive luminescence photometers, it is possible to measure down to 1000 bacteria per millilitre.

In the measurement of a low number of microbial cells in water samples (surface, process or potable water) the bioluminescence assay of ATP can lead to a significant underestimation if viable microbial cells due to the fact that microbes in water are starving and their ATP level per cell is lower than in actively growing cells. Starving microbial cells can have less than 10% of the ATP level of metabolically active cells. In proper nutrient medium microbial cells get metabolically activated in 5-10 minutes. In the present invention a 10-20 minutes incubation time is applied to increase the accuracy and sensitivity of microbial samples having starving or otherwise inactive viable cells. The procedure is as following:

A water sample of 500 ml is filtered through a 0.2 mu membrane filter by applying low vacuum (500-600 mm Hg). During the filtration the filter is not allowed to get dry to avoid stressing the microbes. The filter is placed in a flat-bottom container, such as a Petri dish. A small volume (0.1-1 ml) of ATP-free nutrient broth is pipetted on the filter and the sample is let stand for 10-20 minutes at room temperature. During this incubation time the cells recover from the filtration stress and get metabolically activated, but the cells will not multiply. After the incubation time ATP is extracted from the microbial cells by adding 0.1-1 ml of 1-5 molar mineral acids (sulphuric, trichlor acetic, perchloric acid). Bases (sodium, potassium hydroxide), or preferably a mixture of ionic surface active agents consisting of a aqueous solution of 0.1-0.5% an ethoxylated amine and a quaternary ethoxylated amine (Nucleotide Releasing Reagent, NRB^R, of Lumac Systems AG, Basel). After mixing the extraction reagent and the sample, an aliquor of 0.1-1 ml is pipetted into a transparent cuvette. The firefly luciferin-luciferase reagent (0.1-1 ml), consisting of 0.01-1 ug luciferase, 1-100 ug luciferin, 10-200 ug/ml dithiothreitol, 0.1-10 mg/ml bovine serum albumin, 5-50 mM magnesium ion in a biochemical buffer, such as tris, HEPES, MOPS, and TES, at a pH of 6.5-8.5, is added into the sample and the light emission of the sample is measured in a photometer capable of detecting photons between the wavelengths of 400 and 650 nanometers. The quantity of ATP in the sample is determined by comparing the standards. The number of bacteria is calculated, by dividing the level of ATP in the sample by one femtogram (10^{-5}g), which is the quantity of ATP per bacterial cell.

When the number of microbial cells is so small that it cannot directly be measured with ATP method, it is possible to inoculate a small volume of liquid or solid growth medium (10-1000 µl) with a small aliquote of liquid sample (10-100 µl) in a transparent glass or plastic cuvette. The sample is then incubated with or without shaking at temperatures between 20 and 56°C depending on the type of microbes to be detected.

The generation time of bacteria usually varies from 15-60 minutes depending on the incubation temperature and the species. Most typical generation time for bacteria is 20 minutes at 37°C. For yeast, and especially, for fungi the doubling time of cells or biomass is longer, upto several hours. Before microbes start growing in a new environment (after inoculation) there is a certain time without any growth. This is called the lag time or lag phase. The length of this is dependent on the organism, medium, and temperature. For many bacteria the length of lag period is 20-23 minutes at 37°C.

Assuming a 20 minute lag phase and a 20 minute generation time, the population of bacteria multiply according to the following schedule:

Time of incubation minutes	Multiplication factor for original population
20	1
40	2
60	4
80	8
100	16
120	32
140	64
160	128
180	256
200	512
220	1.024
240	2.048

When a population growth curve is established for a particular type of sample, it is possible to extrapolate from one or two measurements of the ATP in the sample after certain incubation time the original quantity of microbes in the sample.

EXAMPLE

Measurement of Escherichia coli

1. A solid (nutrient agar) or liquid medium (nutrient broth), which is not contaminated with ATP, is prepared
2. 100 µl of medium is pipetted to a required number of plastic or glass cuvettes used for the luminescence photometer, and sterilized.
3. 10-50 µl of the sample is pipetted to two to three replicate cuvettes and the cuvettes shaken to distribute the sample over the solid medium or to mix the sample with liquid medium.
4. The cuvettes are placed in an incubator at 37°C, preferably with continuous shaking.
5. The cuvettes are taken from incubator after 2 hours and cooled to room temperature.
6. The quantity of APT is measured as following:

- To the cuvette 100 µl Nucleotide Releasing Reagent for Microbial Cells (NRB) is pipetted and mixed by shaking hand. In 15 seconds ATP is released from cells.
- The cuvette is placed in the counter chamber of the luminescent photometer and the chamber closed light-tight.
- 50-200 µl firefly luciferin-luciferase reagent is dispensed to the sample. The light intensity of bioluminescence is measured as a peak value or as an integrated value over a preset time of 5-60 seconds.
- The readings of relative light units (RLU) are converted to concentration of ATP or number of E. coli cells by means of internal standardization one femtogram (10^{-5}) ATP cell is used.
- The number of E. coli cells in original sample is calculated:

$$\text{No. of cells} = \frac{\text{No. of cells measured}}{2^{\left(\frac{\text{incubation time - lag period}}{\text{generation time}}\right)}}$$

Following parameters were measured experimentally (figure 1, in which Figure 1. A shows the growth of *E. coli* in 100 µl of nutrient broth. Lag period was 30 minutes, and Figure 1.B shows the growth curves of *E. coli* bacteria on 100 µl of solid nutrient agar in a 12 x 47 mm glass cuvette at 37°C. There were no differences in the growth between samples having NRS and without it. Lag period was 30 minutes. The vertical axis represents relative light units, the seale division of Figure 1.A being twice that of Figure 1.B and the horizontal axis incubation time in minutes the seale divisions of Figure 1.A and Figure 1.B being the same).

Lag period = 30 minutes

Generation time = 20 minutes

Number of cells after 130 minutes incubation 352,000

$$\text{No. of cells} = \frac{352{,}000}{2^{\left(\frac{130-30}{20}\right)}} = \frac{352{,}000}{2^5 = 32} = 11{,}000$$

EMBODIMENT II

Measurement of microbes in body fluids and food sample

Number of bacteria in body fluids and food samples are measured as quality control for the hygiene of food and for detection of microbial infections of body fluids. This is normally performed with culturing samples on solid agar medium. The method is manual or semi-mechanized and requires a 24 hours incubation. The number of CFU's are used as a measure. CFU number is not accurate indication of bacterial contamination of samples because these types of samples are not homogeneous. Bactria adhere on the

particles forming conglomerations and aggregates. Each of these aggregates will form only one colony, thus the CFU's give a much lower number of bacteria than actually are in the sample. Therefore, the conventional method is both slow, cumbersome and inaccurate. In the case of food samples (milk, meat, etc.) the product may already have gone for consumption when results are obtained, and in medical microbiology the 24 hours delay may be fatal to the patient.

The firefly bioluminescent assay of ATP has been applied to measurement of bacteria in urine (US Patent No. 3,745,090) and in food stuff (Sharpe, A.N., M.N. Woodrow and A.K. Jackson, Adenosine triphosphate levels in foods contaminated by bacteria. J. Appl. Bacteriol. 33: 758-67, 1970) but in both applications the high level of non-microbial ATP from blood epithelial and muscle cells cause a problem. It is possible to treat the non-microbial cells with a surface active agent as Triton® X-100 (Us Patent No. 3,745,090 and US Patent No. 4,014,745) which lyses non-microbial cell, and subsequently add potato apyrase to the sample for hydrolising the extracted non-microbial ATP during a short incubation (5-20 min.) at 20-50°C (see US Patent No. 3,745,090). This treatment seems to leave a small proportion of non-microbial ATP non-effected, probably associated with the cell membranes. When the microbial ATP is later extracted with mineral acids (sulfuric acid, trichlor acetic acid, perchloric acid) or by boiling Tris-EDTA buffer, part of this nonmicrobial ATP is also extracted together with microbial ATP. This understroyed nonmicrobial ATP is potential error factor in measuring bacteria in body fluids and makes it difficult to apply this rapid method for detecting bacteria in food stuff.

To overcome the problems of residual nonmicrobials ATP and the limitations in the sensitivity, a method was developed whereby the nonmicrobial ATP is hydrolized by the own enzymes of nonmicrobial cells or in the presence of low activity of APTase enzyme (0.01-0.1 units per ml) and the number of microbes in the same multiplied simultaneously during a short 0.5 to 5 hour incubation for bacteria and yeasts and from 1 to 5 days for fungi. Normally, only 1 to 2 hour incubation time is needed for bacteria. The incubation is carried out directly in the cuvette where the microbial ATP is measured. The cuvettes can be preprepared and packaged. These require much less space than conventional cultures tubes or Petri dishes. Using solid medium, it is possible to use these even in field conditions. The use of the same cuvette to incubation and measurement leads to an easy automation of this method. The example below describes the utilization of this method for measurement of bacteria in milk.

Elimination of nonbacterial ATP and measurement of bacteria in milk with bioluminescent ATP assay:

- 50-200 µl, but preferably 100 µl sterile nutrient broth free from ATP having 50-200 µl of 0.02-0.5 % aqueous solution of non-ionic surface active agent, such as ethoxylated alkylphenols, e.g. Nucleotide Releasing Reagent for Somatic Cells (NRS ®, Lumac Systems AG, Basel) containing 0.001-0.2 units per millilitre ATPase enzyme is pipetted to a required number of sterile transparent glass or plastic cuvettes.
- 10-100 1pl, but preferably 10-50 µl well shaken milk samples are pipetted into the cuvettes and mixed, NRS in the medium makes the cell membranes of leucocytes and epithelial cell permeable for ATP and this nonmicrobial ATP will get hydrolized by the enzymes of the nonmicrobial cells and the added ATPase enzyme.
- The sample is incubated for 60-120 minutes at 28°C with or without shaking.
- The tubes are removes from incubator, and 50-300 µl and mixed. NRB released ATP from bacteria in 10 to 20 seconds.
- The cuvette is placed to the luminescence photometer and ATP measured with firefly luciferin-luciferase reaction.
- The number of bacteria is calculated by dividing the measured ATP concentration by one femtogram (10^{-15} g), the level of ATP per one bacteria. The number of bacteria in original sample is calculated.

$$\text{No. of cells} = 2^{\left(\frac{\text{No. of cells measured}}{\frac{\text{Incubation time - lag period}}{\text{Generation time}}}\right)}$$

The graphs (Figure 2; vertical axis relates to concentration of ATP and horizontal axis to the incubation time in minutes) show the decline of nonbacterial ATP and the increase of bacterial ATP during incubation using above described procedure.

This way nonmicrobial ATP is eliminated to about 0.5 % of original an the bacterial ATP increased about 8 times in 120 minutes. This would mean that in normal milk the concentration of residual non microbial ATP is less than 5 % of the bacterial ATP. This could be substracted, or the actual nonmicrobial ATP could be measured as following:

- After incubation, 50-200 µl, but preferably 100 µl firefly luciferin-luciferase reagent is added to the sample and the concentration of nonmicrobial ATP is measured. To the sample 200 µl of NRB is added and the total ATP measured.
- Substract nonmicrobial ATP from total geives bacterial ATP.

EMBODIMENT III

Identification of microbes by using specific culture medium and bioluminescent measurement of ATP

It is a known method to apply specific media for growing bacteria. This is utilized for identification of bacteria by inoculating an unknown species on different media which contain specific substrates, such as

sugars, amino acids, vitamins, salts, growth inhibitors, antibiotics etc. required for selective growth of a specific species of bacteria. After a 16-72 hours of incubation it is possible to visually observe if the species has grown on the medium. From the combination of positive and negative grown results it is possible to identify the species. For certain species or types of bacteria there are also selective media where no other speies grow or grow slowly, such as McConky medium for Coliform bacteria. These methods require now 1 to 3 days to see the results.

- Sterile media containing specific substances used for biochemical identification test of microbes are dispensed in a volume of 10-200 µl, but preferably 100 µl into sterile transparent plastic or glass cuvettes.
- 5-200 µl, but preferably 10-50 µl of isolated unknown microbe suspension is pipetted into 2-20 different cuvettes having each a different growth medium, solid or liquid, and mixed.
- The concentration of ATP in the microbial suspension is measured as initial value with the luciferin-luciferase system in a photometer using 50-200 µl NRB as extraction reagent.
- Sample cuvettes are incubated for 2-16 hours at an appropriate temperature between 20°C and 56°C.
- After the incubation time, the ATP is measured by pipetting of 50-200 µl NRB for ATP extraction from microbial cells, placing an aliquot of 50-200 µl of sample in a cuvette into the photometer where 100-200 µl the luciferin-luciferase reagent is added to the sample.
- The increase of ATP compared to initial value shows the growth of the microbe on the medium while an unaltered or declined ATP value indicates a negative growth result.
- From the combination of growth, degree of growth or negative growth it is possible to identify the type or species of microbe.

The technique of using culture medium and incubation in measuring cuvette and treating the sample with other reagents, such as NRS nad NRB simplify the assay of microbial ATP and makes it possible to:

1. Increase the sensitivity of the method
2. Eliminate or reduce the error from nonmicrobial ATP
3. Rapidly identify of bacteria using specific and selective media.

EMBODIMENT IV

Sterility testing

Food, medical, etc. products that have to be sterile can be tested with bioluminescent measurement of ATP. Solid samples can be homogenized and measured after 4-16 hour incubation in the presence of NRS (and ATPase enzyme if nonmicrobial ATP is present). Liquid samples can be filtered with 0.2-0.45 µm pore size membrane filter. Filter is incubated in ATP-free nutrient broth for 4-8 hour after which ATP is extracted and measured as in Embodiment I. If sample is sterile, there is no microbial ATP and if sample has contained microbes ATP is detected after incubation.

CLAIMS

1. A method of increasing the sensitivity of the measurement of microbial cells with the bioluminescent assay of adenosine triphosphate (ATP) by means of a short incubation of the sample in a microbiological nutrient medium.
2. A method of claim 1 wherein the incubation is 10-20 minutes for activating of microbial metabolism and increasing the level of ATP per cell to the same level where it is during the logarithmic growth phase.
3. A method of claim 1 wherein the incubation time is 2-16 hours for bacteria and yeast and 0.5-5 days for fungi and slow-growing bacteria in order to increase the number of microbial cells or biomass to the level where they can be detected with the bioluminescent assay of microbial ATP.
4. A method of claim 1 wherein the incubation is performed in the presence of 0.02-0.5 % solution of a non-ionic surface active agent, such as ethoxylated alkylphenol and 0.001-0.5 units/ml of an ATPase enzyme for destroying non-microbial ATP during the incubation time used for increasing the number of viable microbial cells in the sample.
5. A method of claim 3 wherein the number of microbial cells in the original sample is calculated from the number of microbial cells after the incubation by calculating the multiplication factor of cells from the incubation time, the lag period and the generation time of the microbes, considering that the number of cells double at every generation time.
6. A method of claim 1 wherein the number of bacterial cells is calculated in the sample by dividing the level of ATP in the sample by one femtogram (10^{-15}g), the level of ATP per bact4erial cell on the average.
7. A method wherein the incubation is performed in a microbiological nutrient medium supporting the growth of a particular species or group of microbes in order to determine the number of such organism in the sample.
8. A method wherein the sample is incubated in a plurality of microbiological nutrient media containing specific substances, such as sugars, vitamins, salts, growth inhibitors and antibiotics that support or prevent the growth of particular species of microbes thus allowing to identify the organisms from the combination of growth or no growth in the test media as determined with the bioluminescent assay of ATP after a 2-16 hour incubation time.

9. A method wherein the incubation in the microbiological nutrient medium and the measurement of ATP are performed in the measuring cuvette.

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