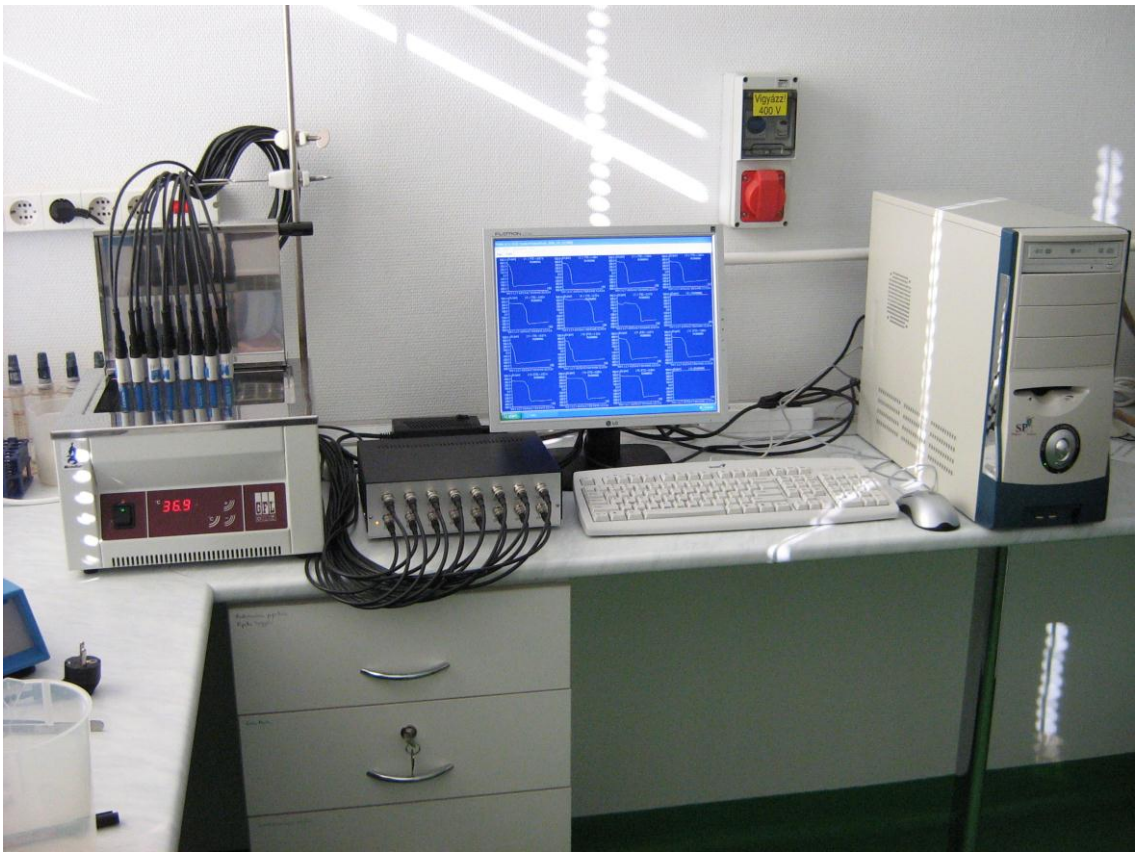
MicroTester

New technique in the microbiological quality control



Rapid determination of the microbial contamination of food-, water-, environmental- and hygienic samples

Results in 6-12 hours

MICROTESTER is an equipment for rapid microbiological testing based on redox-potential measurement. The field of application of the system is mainly – but not exclusively – the microbiological quality control in food industry, research-development and monitoring of the fermentation processes.

The last decades' significant increase in the tasks related to microbiological quality control set up a claim to replace the classical microbiological testing methods with much faster and automatable new techniques.

With classical microbiological methods the time needed for detection of a microbe is at least 24 hours, but usually it is much longer: 48-72 hours. The quicker assessment of food lots, the reduction of the time needed for temporary storage, the efficient operation of the HACCP systems necessarily require the hastening of the microbiological evaluation and automation as far as possible with simultaneous decrease of costs.

A rapid microbiological method must be reliable; a correct diagnosis is of highest priority. It is not only important for the handling and treatment of outbreaks; it may as well contribute to decreased financial loss. In the latter case, one can say that "time is money" for both the food industry and the authorities.

To shorten the time required for microbiological testing different instrumental measurement methods have been developed. One group of them (ATP-measurement, turbidimetry, flow-cytometry, etc.) is suitable for determining the joint number of living and dead cells. Another direction of development is represented by impedimetric measurement methods based on the detection of the change in impedance caused by the metabolic products of microbial activity.

The **MICROTESTER** developed by our company is based on the detection of the change in redox-potential caused by microbial activity. The evaluation system of the instrument is similar to the impedimetric method's, but with wider field of application. Further advantage of the redox-potential measurement is that under the same performance circumstances (sample number) the investment costs are merely one third of those of the impedimetric method.

The impedimetric methods

Until now, impedance-measurement has been the preferred method for rapid quantitative and qualitative measurements of bacteria, yeasts and moulds and monitoring real time activity.

The mode of operation of the most widespread instruments based on impedimetry (MALTHUS, BacTrac, RABIT) is the same.

The method is based on detection of a change of impedance of a special broth, which develops when the microorganisms proliferate. Normal metabolic pathways of the test organisms will convert weakly charged substrates of the medium into highly charged end products, leading to an increased conductivity of the test medium. This means that the medium used in the impedimetric method is extremely important: it must be supportive and selective for the test organism's growth, and in addition must be optimised for electrical signals.

Quite some selective media (e.g. Salmonella-, Listeria-selective broths) routinely used, contain high salt concentration; this results in high conductance readings. Therefore media like these, and similar types, will be classified outside the normal working range of the impedimetric systems. To avoid this problem, indirect technique is used. With the indirect technique, microbial metabolism is monitored through CO_2 production. The produced CO_2 is absorbed by potassium hydroxide leading to a decreased conductivity.

Beyond the well known advantages many disadvantages of the impedimetric method are known:

- In case of lower cell concentrations the impedance method is not reliable: the linear relation between the detection time and the logarithm of the original living cell number is uncertain. Below 10^2 cells/ml the living cell number could be estimated only with high inaccuracy, calibration curves cannot be determined.
- The impedance depends on the geometry (shape, volume) of the test cell, so measurements can be made only in special test cells, therefore the sample volume is limited.
- To avoid the problem of media usually used with high salt concentration, the indirect technique (monitoring the CO_2 production) is proposed. But the theory is faulty: some bacteria do not produce CO_2! So there is a big group of bacteria, which cannot be measured with this method, or only with hard efforts and not in reliable way.
- The measured impedance is highly affected by the temperature-fluctuation. That is why this method needs strict and expensive temperature-control ($\Delta T=\pm 0.002°C$).

MICROTEST www.microtest.hu H-1225, Szentháromság u. 30., Budapest, Hungary microtest1@t-online.hu

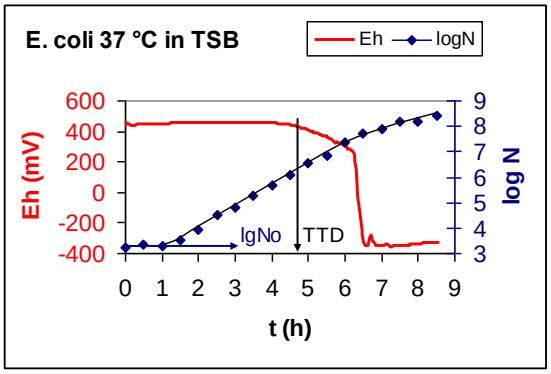
The newly developed redox-potential method

MICROTESTER detects the proliferation of microorganisms in another way than the systems mentioned before: it is based on redox-potential measurement. The evaluation of the changes of the measured values provides an opportunity to a more comprehensive determination of the viable cell count compared to impedimetric methods.

The method utilizes the typical oxidation-reduction reactions in biological systems, performed by microorganisms. The energy source of the microbial growth is the biological oxidation which results in a reduction in the environment. This is due to the oxygen depletion and the production of reducing compounds in the nutrient medium.

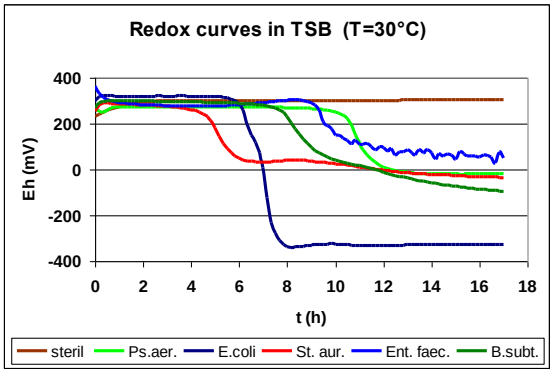
The redox potential is one of the most complex indicators of the physiological state of microbial cultures and its measurement could be a useful tool for the qualitative and quantitative determination of the microbial contamination (Fig 1).

Fig 1.



On the basis of the oxygen requirement the microorganisms are able to proliferate only at certain redox-potential intervals. Therefore detection of the redox-potential changes provides a unique opportunity to determine the nature of the microbe (aerobe, anaerobe, facultative anaerobe, aerotolerant anaerobe). Fig 2.

Fig 2.



Features

The change of the redox-potential is independent of the shape and volume of the test cell and of the composition of the nutrient medium, so the measurement can be carried out with unlimited sample size in any liquid nutrient medium. Therefore beyond the application field of the impedimetric methods **MICROTESTER** is suitable for:

- Measurements using standard commercial microbiological nutrient media.
- Measurements applying membrane filtration method.
- Direct measurement of surface hygienic samples.

The effect of fluctuating temperature in contrast to that of impedimetric technique is negligible; therefore there is no need for strict temperature control. 1°C temperature drift results in 0.5-1.5 mV decrease in redox-potential, depending on the substrate, which is far away from the 10 mV applied detection criterion. The method does not require high precision thermostats used by impedimetric methods; the conventional microbiological thermostats with ±0.5°C precision are enough for thermostating the test cells.

Due to the simple construction and mode of operation **MICROTESTER** can be considered as a very simple and cheap measurement technique comparing to impedimetric systems.

Description of the measurement system

The redox-potential changes of the medium are well measurable.

Commercial combined redox electrodes are used for the measurements. The modular measurement unit can be extended according to the demands. The basic configuration is 16 channels in one module, but the units can be linked if needed.

The microcontroller-based measurement unit is connected to an IBM compatible PC via USB port for control and data acquisition. PC configuration requirements for controlling and acquisition are low, but there is recommended configuration for optimal performance as follows.

General software usage: 4 core Intel or AMD processor, 4+ GB RAM. Data storage drive: 120+ GB HDD or SSD. Installation and backup: USB

MICROTEST www.microtest.hu H-1225, Szentháromság u. 30., Budapest, Hungary microtest1@t-online.hu

port or DVD R/W. Supported operating systems: Windows ® 32 and 64 bit versions.
Please revise the energy saving options of the operating system in accordance with the measurement parameters.

For optimal control and data management, a special software has been developed for **MICROTESTER.**

The data collection is continuous, all channels are monitored. The measured data are saved when it is required according to the channel settings.

The software has an advanced noise reduction for supporting measurements in electronically noisy environments.

The collected data are stored in tables, which can be easily loaded without conversion to statistical and spreadsheet managing softwares. The channels can be adjusted individually, making the monitoring and evaluation of different measurements (different type or started at different time) possible.

The threshold value for first derivative of data (required for TTD – time to detection – calculation), the number of positive identifications, where the threshold value is exceeded, start value of the evaluation and the critical number of microbes can be set in the channels configuration.

The start value adjusted can delay calculation of TTD. The given amount of time is skipped at the beginning; therefore the uncertainties of the beginning of the measurement can be eliminated. Substituting the measured time to detection to the previously determined calibration equation the original living cell concentration of the sample can be calculated.

Comparing the measured cell number to the critical number previously adjusted, decision between PASSED and FAILED results is made by the software.

The software possesses advanced graphical display with three different display options:

- Common chart: every active channel is presented on one common chart.
- Tile charts: every active channel is presented individually. The time to detection, the calculated cell number and the evaluation of the sample are presented for every single channel. At this type of layout the channels are marked with different colours for better arrangement: with blue the running, with red the failed, with green the passed and with grey the finished measurements.
- Grouped curves: the grouped channels are presented on one graph (maximum of 4 groups can be set).
- MPN calibration curve

The calibration curve

External calibration curve can be applied when the target microbe or the microflora is known. In this case, the calibration curve of the studied micro-organism is determined first. The equation of the calibration curve is calculated by linear regression from the logarithm of the initial viable cell numbers of the measuring cells (log No, determined by plate counting) and the TTD values, determined instrumentally. The equation is fed into the computer. Using this equation the original viable cell concentrations of the samples can be determined. Figures 3 and 4.

Fig 3.

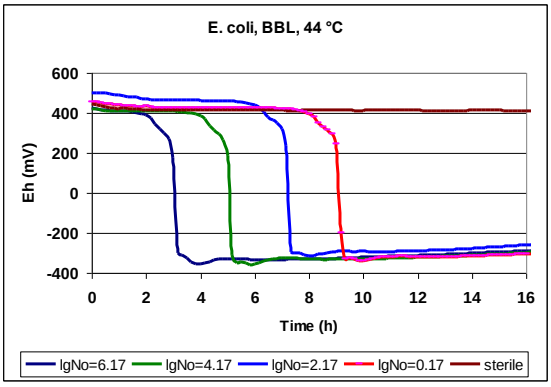
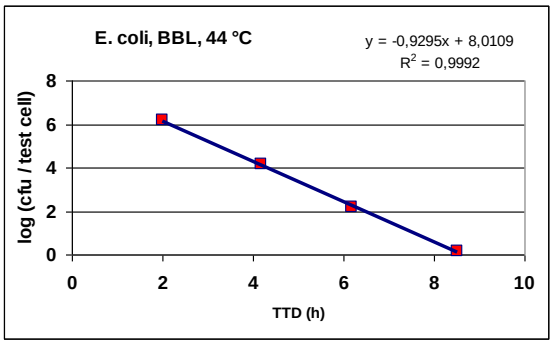


Fig 4.



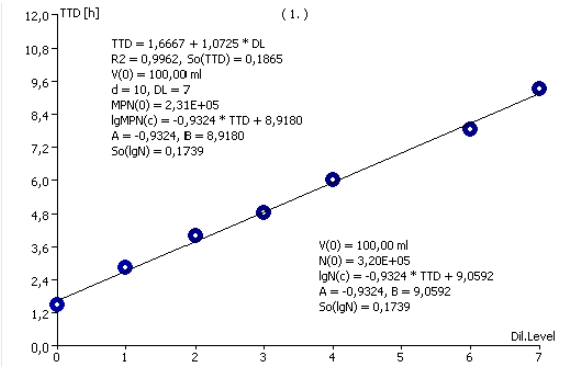
Internal calibration curve is applied when the composition of the microflora is not known and previously constructed calibration curve cannot be taken. In this case, the redox potential measurement could be combined with the MPN method and the software makes possible the on-line determination of the calibration curve.

From the sample the usual dilution range is prepared up to a dilution which already is without microbe. From each member of the dilution series 1

MICROTEST www.microtest.hu H-1225, Szentháromság u. 30., Budapest, Hungary microtest1@t-online.hu

ml is put into a measuring cell and the redox curves are recorded. Based on the last dilution levels still showing multiplication (TTD), the most probable number of the initial living cells, MPN(0) could be determined automatically by the programme. With the knowledge of MPN(0) and TTD values belonging to the several dilutions, the software calculates the equation of the log MPN calibration curve. The results will be printed on the screen. (Upper part of Fig 5) Fig 5 demonstrates the graphical display of MPN calculation.

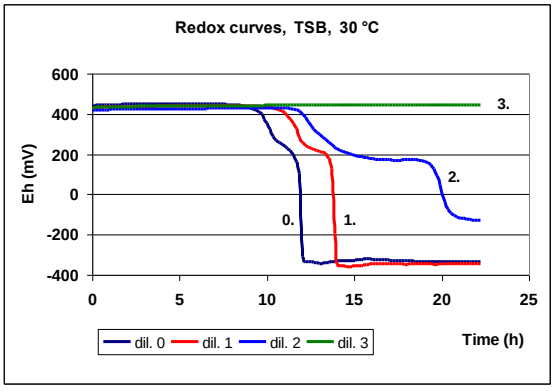
Fig 5.



If the cell concentration is determined by plate counting (giving results after some days), the log N values could input the programme and the software automatically recalculates the log MPN calibration to the more reliable log N calibration. (Lower part of Fig 5.)

In some cases the characteristic shape of the redox curves gives qualitative information about the composition of the microflora (Fig 6).

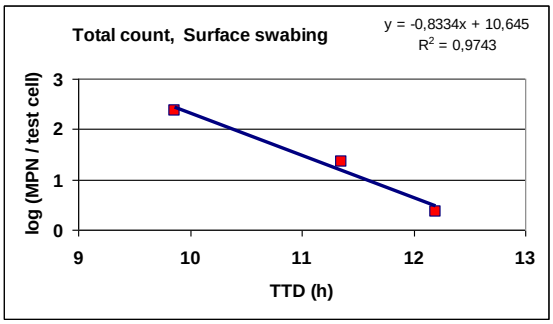
Fig 6.



On the Figure 6 the redox curves belonging to 0 and 1st dilutions are typical enterobacterium curves, the 2nd dilution contains unknown microflora and the 3rd one is sterile. The results: Total count MPN=$2.3 \cdot 10^2$, Enterobacterium: MPN=$2.3 \cdot 10^1$

Based on the MPN and TTD values belonging to the several dilution levels, the internal calibration curve can be constructed (Fig 7).

Fig 7.



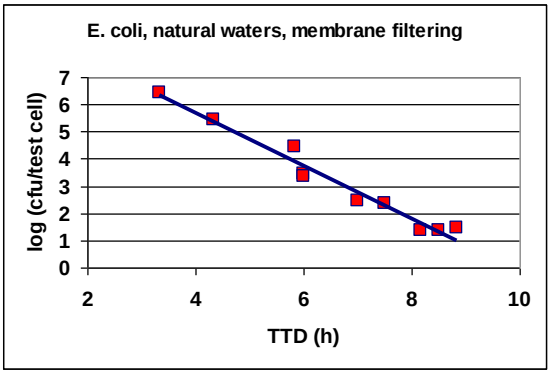
For determination of the original living cell concentration of a sample – after conventional microbiological sample preparation (homogenizing, diluting) – a known amount is placed in the test cell and the measurement is carried out. As a negative control sterile medium, as a positive control a test cell inoculated with a known concentration of the tested microbe is used. The redox curve is recorded, the TTD is determined and with the calibration equation the original cell concentration is calculated by the equipment. With the knowledge of the cell concentration, if the critical number of microbes was set, the system makes a decision on the approval of the sample.
The application of **MICROTESTER** provides profitable solution especially in cases when quality or safety criteria prescribe absence of certain microbes (*Escherichia coli*, *Pseudomonas aeruginosa*) in the product (zero-tolerance).

MICROTESTER can be used favourably for evaluating the classical membrane-filtration and surface swabbing methods. During inspection of the microbial count of natural water sources, drinking water, mineral water and other beverages usually consecutive members of the dilution series are membrane-filtered and placing the filters on agar surface, the samples are incubated. The reason for using different dilutions in the classical method is that the samples can be evaluated reliably only in a specific interval (between 30-300 cells). Because **MICROTESTER** – with determining the calibration curves – is able to carry out measurements and reliably determines the cell concentration in a wide interval (between 1-10^7 cells), there is no need for dilution series; it is enough to make only one membrane filtration from a sample (Fig 8). Similarly, in case of the microbiological control of surfaces by swabbing, the swab could be put

www.microtest.hu H-1225, Szentháromság u. 30., Budapest, Hungary microtest1@t-online.hu

directly into the measuring cell, without any washing and dilution.

Fig. 8.



MICROTESTER is suitable for direct testing of mineral water or any other liquid products poor in nutrients. In this case the production unit (e.g. bottle) will act as a test cell: the nutrients essential for the target microbe (concentrated nutrient medium) are added directly to the product; after immersing the electrodes, the measurement can be carried out. The nutrient requirement is minimal, and a relatively high amount of sample (1-2 litres) can be examined directly. This method is especially suitable for sterility tests of the products.

Validation of the system for water testing

During the laboratory and industrial validation of **MICROTESTER** the typical characteristics were considered:

- *Selectivity:* it depended on the media used for identification. **MICROTESTER** is able to work with any commercial nutrient medium.
- *Linearity:* defining the time required to reach a significant change in redox potential, a strict linear correlation could be established between the 'Time to Detection' and the logarithm of the initial concentration of microorganisms (above concentration of 1 cfu/test flask).
- *Sensitivity:* 1 logarithm unit increase in the initial cell concentration decreases the TTD values with 50-130 minutes, depending on the microorganisms tested.
- *Detection limit:* at minimum 1 cell/test flask. **MICROTESTER** is suitable for the absence/presence control of the microbes in the sample.
- *Quantitation limit:* the theoretical quantitation limit is 10 cell/inoculum (1 log unit), which is in agreement with the obtained calibration curves.
- *Range:* on the base of the calibration curves the range lasted from 1 to 7 log unit. Below 10 cells the Poisson-distribution causes problems in quantitation (but in detection not!), over 10^7 cells the TTD is too short comparing to the transient processes (temperature-, redox-equilibrium, lag-period of the growth).
- *Accuracy/Trueness:* As the redox-potential measuring method is based on the regression equation representing the connection between the logarithm of the real cell concentration and detection time, the accuracy of the method depends on the reliability of the calibration curves. Each combination of microorganisms and culture broth require special calibration curve.
- *Precision (repeatability, reproducibility)*: during the validation of the system the repeatability and reproducibility were determined.
- *Robustness:* the most important parameter is the temperature, which has a double effect on the results – the growth rate of the microorganisms and the measured redox-potential are temperature depending. Performing the measurements at the temperature optimum of microorganisms, the growth rate in a ±0.5 °C interval does not change. The influence of the temperature on the measured redox-potential was determined experimentally. The results showed that the effect of the temperature variation is negligible.

Microbiological examination of water

MICROTESTER as a validated method is suitable for rapid microbiological testing of mineral water, carbonated water, tank and running drinking water and other types of water. The time needed for a reliable detection of microorganisms is of key importance: in water industry the real-time (or at least as fast as possible) monitoring of the microbiological properties of the production is indispensable; in public water supply the essential basis of the epidemiological and public health measures is the fast and reliable result of the microbiological inspection. Beside the most important and most widely inspected microbiological contaminants the most relevant disturbing flora was involved to the validation process as well.

The results of the ***selectivity*** tests showed that the usual disturbing flora of the Coliform-detection

MICROTEST www.microtest.hu H-1225, Szentháromság u. 30., Budapest, Hungary microtest1@t-online.hu

(*Pantoea agglomerans* and *Acinetobacter lwofii*) did not grow, so there is a possibility of a selective Coliform-detection without false positivity. Similar results were obtained in case of *Pseudomonas aeruginosa* (disturbing flora: *Pseudomonas fluorescens*, *Burkholderia cepacia*) and Enterococcus detection (disturbing flora: Micrococcus spp.).

During the ***linearity*** tests the very strict linear correlation between the logarithm of the initial cell concentration (lgN) and the time needed to detection (TTD) was proven. There was no difference in the linearity of classical and membrane-filtered samples, so the method – in contrast to impedimetric methods – is especially suitable for the inspection of membrane-filtered samples.

The ***sensitivity*** of the methods is 50-130 min/lgN depending on the microorganism tested and medium used, so a one log increase in the initial cell concentration decreases the TTD with 50-130 minutes. The time needed to detection of Coliforms in case of lgN_0=1 initial cell concentration is about 9 hours, in case of higher level of contamination this time is shorter (in case of lgN_0=5 it is 5 hours).

The ***detection limit*** is 1 cell/test flask, so the system is suitable for the absence/presence tests, so considerable costs and time could be saved with more membrane filters joined together.

On the base of the calibration curves the ***range*** lasted from 1 to 7 log unit.

The ***repeatability*** calculated from the calibration curves:

SD_{lgN} = 0.092; $SD_N = 10^{0.092}$ = 1.24 = 24%, which complies with the requirements of microbiological methods.

During the determination of the ***robustness*** the effect of the most important environmental disturbing parameter, the temperature was examined. The temperature has a double effect on the results: the growth rate of the microorganisms and the measured redox-potential are temperature depending. Performing the measurements at the temperature optimum of microorganisms, the growth rate in a ±0.5 °C interval does not change. The experimentally determined effect of the temperature on the measured redox-potential is negligible. So because the temperature change needed for false positive detection is 0.7-2.5°C/min, **MICROTESTER** does not require extreme strict temperature control: the normal lab thermostats are enough for correct operation. (In case of impedimetric measurements this value is 0.004°C/min, so these equipments require expensive thermo-regulation with an accuracy of ±0.002°C.)

During the ***industrial tests*** in a mineral water plant total count of the microbes, presence/absence of Coliforms, *E. coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* were examined.

Microbiological examination of raw milk

The producing of fresh and healthy milk is of highest priority for the producers and for the consumers as well, the foodborne human diseases mediated by milk and the economic losses are of increased importance. Despite that most milk products are consumed in pasteurized form there are many arguments for the importance of the microbiological quality control of raw milk. On the one hand raw milk could act as a reservoir for different pathogens which cannot be destructed with pasteurization, on the other hand large amounts of raw milk base products or even raw milk itself is consumed by people. The raw milk containing high bacterial count is likely to increase the probability of contamination of dairy products to be consumed.

To solve the problem of pathogens and high bacterial counts in milk it is necessary to improve controls on dairy farms. Most of milk producers keep not only the fast detection of diseases in view, but they also want to demonstrate to market partners and consumers the cleanliness, hygiene, safety and quality of raw milk production and handling. There is a need for a rapid, easy to use, portable and cost effective microbiological equipment for farm-level milk quality control.

Current analytical methods for milk quality control are not suitable for farm level use, because they usually require expensive equipment and qualified lab personnel, and only the largest milk producers can afford an own microbiological laboratory. The other reason for not-suitability of current control techniques is that with classical microbiological methods the time needed for detection of a microbe is at least 24 hours, but usually it is much longer: 48-72 hours. The last decades' significant increase in the tasks related to microbiological quality control set up a claim to replace the classical microbiological testing methods with much faster and automatable new techniques.

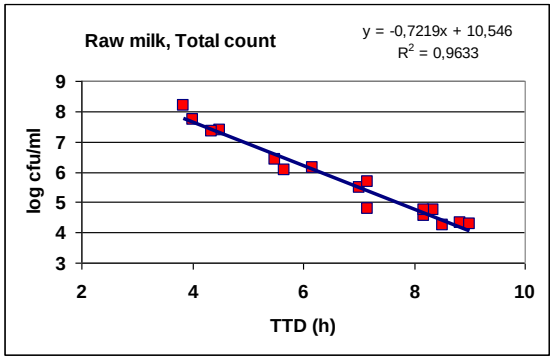
The redox-potential measurement based microbiological method – improved and optimized for raw milk detection – is suitable for rapid (within 6-8 hours), simple and cost-effective determination of Total Count and Coliforms in raw milk. The small equipment could be used in farm circumstances, the raw milk originating

www.microtest.hu H-1225, Szentháromság u. 30., Budapest, Hungary microtest1@t-online.hu

from evening milking could be judged in the morning. The method, as a simple, on farm control, would allow farmers to certificate quality and safety and to keep outbreaks under control.

The contamination rate around 10^5 cfu/ml total counts as the widely acceptable microbiological criterion for raw milk requires 7-8 hours for detection. Despite that the microbiological composition of different raw milks is theoretically different, based on the calibration curve determined from independent milk samples the linear correlation between the logarithm of the initial cell concentration and the time needed to detection is very strict (Fig 9.), which strictness could be even enhanced with farm-specific calibration curves.

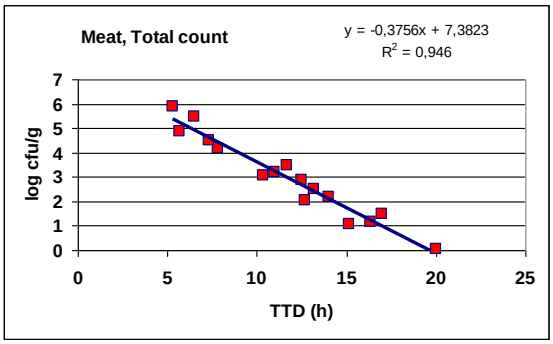
Fig 9.



Examination of raw meat total counts

Similarly to raw milk examination **MICROTESTER** is suitable for determination of aerobic total counts of raw meat and raw meat products. The determination of aerobic total counts prescribed in 2.1. of the Commission Regulation No 2073/2005 on microbiological criteria for foodstuffs lasts from 6 to 10 hours depending on animal species and form of meat (carcass, minced, MSM, etc.). Calibration curve of total count of microbes originating from independent raw meat samples, which shows a strict linear correlation between the logarithm of the initial cell concentration and the time needed to detection (Fig 10.)

Fig 10.



Cost effectiveness

The **MICROTESTER** system applies test cells of 30, 50, 100 ml volumes with standard nutrient broths and occasionally normal membrane filters. The most frequently used test volume is about 15-20 ml which is equal to the normal amount of the agar nutrient in Petri dishes. For the zero-tolerant microbes parallel membrane filters could be placed in one redox test-tube. Applying predetermined calibration curve the dilution of the sample could be negligible so the cost of test series comparing to that of the classical nutrient method is lower.

Further information on other characteristics, features and order conditions:
Microtest1@t-online.hu

March 3, 2013.

Dr. Oliver Reichart