Rapid Methods and Automation in Microbiology 25 years of Developments and Predictions

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Rapid methods and automation in microbiology is a dynamic area in applied microbiology dealing with the study of improved methods in the isolation, early detection, characterization, and enumeration of microorganisms and their products in clinical, pharmaceutical, food, industrial, and environmental samples. In the past 25 years this field has emerged into an important sub-division of the general field of applied microbiology and is gaining momentum nationally and internationally as an area of research and application to monitor the numbers, kinds, and metabolites of microorganisms related to food spoilage, food preservation, food fermentation, food safety , and foodborne pathogens.

Medical microbiologists started to be involved with rapid methods around mid-1960's and started to accelerate in the 1970's and continue developments in the 80's, 90's and up to the present day. Other disciplines such as Food, Pharmaceutical, Environmental, and Food Microbiology were lagging about 10 years behind. Many symposia and conferences were held nationally and internationally to discuss the developments in this important applied microbiology topics. The current Rapid Methods and Automation in Food Microbiology Workshop in Barcelona, Spain is an example of such an effort to provide up-dated information and constructive discussions on this topic.

Advances in Viable Cell Counts and Sample Preparation. The number of living organisms in the product, on the surface of manufacturing environment and the air of processing plants is very important for the food science and industry. Colony forming units (CFU) is the standard way to express the microbial loads. In the past 25 years several ingenious systems in "massaging" or "pulsifying" the solid or liquid samples were developed so that the samples are homogeneously distributed in a disposable bag after 1 to 2 minutes of operation. Typically one ml (after dilutions) is placed into melted agar to encourage microorganisms to grow into discrete colonies for counting (CFU/ml). These colonies can be isolated and further identified as pathogenic or non pathogenic organisms. In the past 25 years, convenient systems such as nutrient housed in films (Petrifilm), mechanical instrument to spread a sample over the surface of a preformed agar plate (Spiral plater), trapping microorganisms on a bacteriological membrane and look for growth of target microorganisms on selective and non-selective culture media (Isogrid), non-thermal Pectin-Ca gel system (Micrology, Inc.), etc. greatly help to reduce labor time in performing viable cell count. The 3 or 5 tube Most Probable Number (MPN) system has been in use for more than 100 years in water testing and food testing but the procedure is very labor intensive and utilizes large amounts of test tubes and media. In 2007 a completely mechanized, automated, and hands-off 16 tube, three dilution MPN system named TEMPO by bioMerieux is being marketed for ease of operation of this tedious but yet powerful MPN viable cell count procedure used in Public Health, Pharmaceutical and Food laboratories around the world. Results indicated that TEMPO provided equivalent data compared with standard viable cell count methods. This author predicts that TEMPO will be very successful in food and water microbiology in the near and far future.

As a guide the following **FUNG scale on viable cell counts** has been developed:

0 to 2 log CFU/ g, ml, or cm^2	Low count, no concern
3 to 4 log CFU/ g, ml, or cm^2	Intermediate count, slight concern
5 to 6 log CFU/ g, ml, or cm^2	High count, definite concern
7 log CFU/ g, ml, or cm^2	Index of Spoilage, serious concern
8 log CFU/ g, ml, or cm^2	Odor, unacceptable
9 log CFU/ g, ml, or cm^2	Slime, highly unacceptable
10 log CFU/ g, ml, or cm^2	Discard immediately

This scale is for general microbial population of food and water. No food pathogens are allowed (e.g. *Salmonella*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, etc.) especially in cooked ready-to-eat foods. Also in the case of food fermentation high number of good bacteria such as lactic acid bacteria, and yeasts are to be expected and encouraged such as in cheese and yoghurt making, beer and wine, bread, sauerkraut, and other fermented foods.

Air Samples and Surface Samples. The Food industry also needs to ascertain the air quality as well as surfaces for product manufacturing, storage, and transportation. An active air sampling instrument can "suck" a known volume of air and deposit it on an agar surface (impaction) or trapped it in liquid (impingement) to obtain CFU/ m^3 or ml. A variety of swabs, tapes, sponges, contact agar methods have been developed to obtain surface count of Food manufacturing environment.

Fung scale for air and surface samples:

Total Counts for Air Samples

0-100 CFU/m^3	Acceptable count
100-300 CFU/m^3	Intermediate count
>300 CFU/m^3	Too high, need corrective action needed

Note: In Singapore >500 CFU/m^3 is not allowed in food plants.

Total Counts for Food Contact Surfaces such as forks, knives, dishes, spoons, chopping blocks, table tops, glass for drinking water, etc.

0-10 CFU/cm^2	Acceptable count
10-100 CFU/cm^2	Intermediate count
>100 CFU/cm^2	Too high, corrective actions needed

Aerobic, Anaerobic, and "Real Time" Viable Cell Counts. By use of the correct gaseous environment or suitable reducing compounds one can obtain aerobic, anaerobic, facultative anaerobic microbial counts of products. Typically microbial counts were obtained in 24 to 48 hours. Several methods have been developed and tested in recent years that can provide "real" time viable cell counts such as the use of "Vital" stains (Acridine Orange) to report living cells under the microscope to count fluorescing viable cells or measuring ATP of micro-colonies trapped in a special membranes. These real time test can give viable cell counts in about 1 to 4 hours. A simple Fung Double Tube system using appropriate agar and incubation conditions has been developed and tested in Hawaii recreational waters in 2007 that can provide a *Clostridium perfringens* count for water testing in 6 hours.

The **Fung/Fujioka scale** for beach water in Hawaii, USA based on single sample concentrations (CFU/ml) of *Clostridium perfringens* using the Fung Double Tube (**FDT**) methods is as follows:

Pollution category	**FDT** (CFU/10 ml)	**Extrapolated FDT** (CFU/100 ml)	**Scale of beach pollution**
I	0	<10 CFU	Uncontaminated
II	1-10 CFU	10-100 CFU	Non-point contamination
III	11-50 CFU	110-500 CFU	Sewage contamination
IV	>50 CFU	>500 CFU	Elevated Sewage Contamination

Advances in Miniaturization and Diagnostic Kits. Identification of normal flora, spoilage organisms, clinical and foodborne pathogens, starter cultures, etc. in many specimens is an important part of food microbiology. In the past 25 years many miniaturized diagnostic kits have been developed and widely used to conveniently introduce the pure cultures into the system and obtain reliable identification in as short as 2 to 4 hrs. Some systems can handle several or even hundreds of isolates at the same time. These diagnostic kits no doubt saved many lives by rapidly, accurately, and conveniently identifying pathogens so that treatments can be made correctly and rapidly. Fung is a pioneer in the deveoopment of these type of system starting at around 1970's. There are about 20 miniaturized systems on the market to identify pathogens ranging from enterics (*Salmonella*, *Shigella*, *Proteus*, *Enterobacter*, etc.) to non-fermentors, anaerobes, gram positives, and even yeasts and molds.

Advances in Immunological Testings. Antigen and antibody reaction has been used for decades for detecting and characterizing microorganisms and their components in medical and diagnostic microbiology. This is the bases for serotyping bacteria such as *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, etc. Both polyclonal antibodies and monoclonal antibodies have been used extensively in applied food microbiology. The most popular format is the "Sandwiched" ELISA test. Recently some companies have automated the entire ELISA procedure and can complete an assay from 45 minutes to 2 hours after overnight incubation of the sample with suspect target organisms. Lateral Flow Technology (similar to pregnancy test with three detection areas on a small unit) offers a simple and rapid test for target pathogens (e.g. *E. coli* O157) after overnight incubation of food or allergens (e.g. wheat gluten) The entire procedure takes only about 10 minutes with very little training necessary. A truly innovative development in applied microbiology is the immuno-magnetic separation (IMS) system. Very homogenize paramagnetic beads have been developed which can be coated with a variety of molecules such as antibodies, antigens, DNA, etc. to capture target cells such as *E. coli* O157, *Listeria* , *Cryptosporidium* ,*Giardia*, etc. These beads can then be immobilized, captured and concentrated by a magnet stationed outside a test tube. After clean-up, the beads with the captured target molecules or organisms can be plated on agar for cultivation or used in ELISA, Polymerase Chain Reaction (PCR), microarray technologies, biochips, etc. for detection of target organisms. Currently many diagnostic systems (ELISA test, PCR, etc.) are combining an IMS step to reduce incubation time and increase sensitivity of the entire protocol. Using the Pathatrix system of capturing circulating enriched meat samples by IMS Fung=s laboratory can detect *E. coli* O157:H7 in 5.25 hours from the time of enriching the meat sample to the time of ELISA results of the presence or absence of the pathogen.

Advances in Instrumentation and Biomass Measurements. Instruments can be used to automatically monitor changes (such as ATP levels, specific enzymes, pH, electrical impedance, conductance, capacitance, turbidity, color, heat, radioactive carbon dioxide, etc.) of a population (pathogens or non-pathogens) growth kinetic and dynamics in a liquid and semi-solid sample. It is important to note that for

the information to be useful, these parameters must be related to viable cell count of the same sample series. In general, the larger the number of viable cells in the sample, the shorter the detection time of these systems. A scatter gram is then plotted and used for further comparison of unknown samples. The assumption is that as the number of microorganisms increases in the sample, these physical, biophysical, and biochemical events will also increase accordingly. When a sample has 5 log or 6 log organisms/ml, detection time can be achieved in about 4 hrs. Some instruments can handle hundreds of samples at the same time.

Advances in Genetic Testings. Phenotypic expression of cells are subject to growth conditions such as temperature, pH , nutrient availability, oxidation-reduction potentials, etc. Genotypic characteristics of a cell is far more stable. Hybridization of DNA and RNA by known probes has been used for more than 30 years. More recently Polymerase Chain Reaction (PCR) is now an accepted method to detect viruses, bacteria and even yeast and molds by amplification of the target DNA and detecting the target PCR products. By use of reverse transcriptase, target RNA can also be amplified and detected. After a DNA (double stranded) molecule is denatured by heat (e.g. 95C), proper primers will anneal to target sequences of the single stranded DNA of the target organism, for example *Salmonella* at a lower temperature (e.g. 37C). A polymerase (TAQ) will extend the primer at a higher temperature (e.g. 70C) and complete the addition of complement bases to the single stranded denatured DNA. After one thermal cycle one piece of DNA will become two pieces. After 21 and 31 cycles one piece will become 1 million and 1 billion copies, respectively. At the beginning PCR products are detected by gel electrophoresis . Now ingenious ways to detect either the occurrence of the PCR procedure by fluorescent probes or special dyes, or by actually reporting the presence of the PCR products by molecular beacon. Since these methods generate fluorescence, the PCR reaction can be monitored over time and provide "real-time" PCR results. Some systems can monitor 4 different targets in the same sample (multiplexing). These methods are now standardized and easy to use and interpret. To further characterize closely related organisms, detail analysis of the DNA molecule can be made by obtaining the patterns of DNA of specific organisms by pulse field gel electrophoresis (DNA finger-printing) or by "Riboprinting" of the ribosomal genes in the specific DNA fragment. Since different bacteria exhibit different patterns (e. g. *Salmonella* versus *E. coli*) and even the same species can exhibit different patterns (e. g. *Listeria monocytogenes* has 49 distinct patterns), these information can be used to compare closely related organisms for accurate identification of target pathogens (such as comparing different patterns of *E. coli* O157:H7 isolated from different sources in an outbreak) for epidemiological investigations.

Advances in Biosensor, Microchips, and Nanotechnology. Biosensor is an exciting field in applied microbiology. The basic idea is simple but the actual operation is quite complex and involves much instrumentation. Basically, a biosensor is a molecule or a group of molecules of biological origin attached to a signal recognition material. When an analyte comes in contact with the biosensor the interaction will initiate a recognition signal which can be reported in an instrument. Many types of biosensors have been developed. Sometime whole cells can be used as biosensors. Analytes detected include toxins, specific pathogens, carbohydrates, insecticides and herbicides, ATP, antibiotics, etc. The recognition signals used include electrochemical (e.g. potentiometry, voltage changes, conductance and impedance, light addressable, etc.), optical (such as UV, bioluminescence, chemiluminescence, fluorescence, laser scattering, reflection and refraction of light , surface phasmon resonance, polarized light, etc.) and miscellaneous transducers (such as piezoelectric crystals, thermistor, acoustic waves, quartz crystal, etc. Recently, much attention has been directed to "biochips" and "microchips" developments to detect a great variety of molecules including foodborne pathogens. Due to the advancement in miniaturization technology as many as 50,000 individual spots (e. g. DNA microarrays) with each spot containing millions of copies of a specific DNA probe can be immobilized on a specialized microscope slide.

Fluorescent labeled targets can be hybridized to these spots and be detected.. Biochips can also be designed to detect all kinds of foodborne pathogens by imprinting a variety of antibodies or DNA molecules against specific pathogens on the chip for the simultaneous detection of pathogens such as *Salmonella*, *Listeria, Escherichia coli*, *Staphylococcus aureus,* etc. The market value is estimated to be as high as $5 billion at this moment. This technology is especially important in the rapidly developing field of proteomics and genomics which require massive amount of data to generate valuable information.

Nanotechnology is starting to make major advances in pure and applied sciences, including Food Science and Applied Microbiology.

Testing Trends and Predictions. There is no question that many microbiological tests are being conducted nationally and internationally in agricultural and food products, environmental samples, medical specimens, and water samples. The most popular tests are total viable cell count, coliform/*E. coli* count and yeast and mold counts. A large number of tests are also performed on pathogens such as *Salmonella*, *Listeria* and *Listeria monocytogenes*, *E. coli* O157:H7, *Staphylococcus aureus, Campylobacter* and other organisms. According to reliable sources in 1998, the worldwide microbiological tests was estimated to be 755 million tests with a market value of US$1 billion. The projection is that in 2008 the number of tests will be about 1.5 billion tests with a market value of US$5 billion. In 2007 about one third of the tests are being performed in North America (USA and Canada), another third in Europe, and the last third in the Rest of the World. This author predicts in twenty year the Rest of the World will perform 50 % of the tests with North America and Europe performing 25% each. This is due to rapid economic developments and food and health safety concerns of the world in the years to ahead.

Prediction of the Future. The following are the ten predictions made by the author in 1995. Many predictions have been correct in 2007. (+) is a good prediction. (?) is an uncertain prediction.

1. Viable cell counts will still be used in the next 25 years. (+)
2. Real-time monitoring of hygiene will be in place. (+).
3. PCR, Ribotyping, and genetic tests will become reality in food laboratories. (+)
4. ELISA and immunological tests will be completely automated and widely used. (+)
5. Dipstick technology will provide rapid answers. (+)
6. Biosensors will be in place for Hazard Analysis Critical Control Point programs. (?)
7. Biochips, microchips, and nanotechnology will greatly advance in the field. (+)
8. Effective separation, concentration of target cells will assist rapid identification. (+)
9. A microbiological alert system will be in food and other packages. (?)
10. Consumers will have rapid alert kits for pathogens at home. (?)

In conclusion, it is safe to say that the field of rapid method and automation in microbiology will continue to grow in numbers and kinds of tests to be done in the future due to the increase concern on food safety and public health. The future looks very bright for the field of rapid methods and automation in microbiology. The potential is great and many exciting developments will certainly unfold in the near and far future in Food Microbiology and other applied microbiology areas.

References:

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