Simplified methods of monitoring airborne bacteria for quality and environmental management in the Japanese food industry

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Abstract. Airborne microbes are examined generally by sampling them on culture media and counting the number of their colonies formed. However, because it takes at least two days for bacteria and five days for fungi to be cultivated, reliable data may not be obtained in a time of need. Therefore, the development of a simple and quick detection method is required. In this study, rapid detection methods of indoor airborne bacteria for the improvement of quality and environmental management were reviewed, and a basic study on simplified monitoring techniques of airborne bacteria applicable to food factories was carried out.

1. Introduction
Generally, airborne microbes are examined by sampling them on culture media and counting the number of their colonies formed. However, because it takes at least two days for bacteria to be cultivated and five days for fungi to be cultivated, reliable results may not be obtained for a time of need. Therefore, the development of a simple and quick detection method is required. The second chapter summarizes the types and characteristics of fast measurement methods. The third chapter introduces methods for monitoring bacteria in indoor air. The fourth chapter discusses the food industry quality and environmental management.

2. Types and characteristics of rapid measuring methods
Rapid measuring methods are outlined in Table 1. They are classified roughly into direct methods or indirect methods. Direct methods include solid-phase cytometry and flow cytometry. In indirect methods, bacterial properties such as antigen, diffusion, adenosine tri-phosphate (ATP), proliferation potency, bacterial components, and deoxyribo nucleic acid (DNA) are extracted.

Among those listed in Table-1, the following three methods were selected as simplified measuring methods for monitoring airborne microbes, which would serve the purpose of this study: fluorescent counting technique, ATP assay, and fluorescent staining technique. It was also taken into account that rapidity is emphasized particularly in food and confectionery factories.

2.1. Fluorescent counting technique
Airborne particles are usually counted by using a particle counter. Microbes including bacteria and fungi are known to generate fluorescence when irradiated with ultraviolet rays. They can be measured with the Instantaneous Microbial Detection (IMD) system which was developed in the US. Yanagi et al [1], demonstrated the simultaneous measurements of the IMD count and the concentration of airborne particles.
bacteria cultured on media, reporting that the IMD count was nearly 100 times the number of viable bacteria. Although serviceable, the IMD system was excluded from this research for the present because of the price of the system.

2.2. ATP assay

ATP is the abbreviation for “Adenosine Tri-Phosphate,” which is an energy-carrying molecule found in the cells of any plants, animals and microbes, exhibiting a molecular structure shown in Figure 1. ATP is a chemical compound used by all living things to provide energy in many metabolic processes: e.g. cell proliferation, muscle contraction, photosynthesis by plants, bacterial respiration, and yeast fermentation. ATP is contained in all organic matters (living things and their traces) including foods, bacteria, molds and other microbes. In this context, ATP detected on the surface and in the washing water suggests the presence of microbes which are invisible to the naked eye or biological substances (e.g. food residue) which help microbes proliferate.

ATP is a chemical substance used by living things as their energy source. When exposed to luciferase (enzyme) in the presence of luciferin and oxygen, ATP is known to turn into adenosine monophosphate (AMP) and give off light energy.

Because ATP assay kits have come onto the market recent years, their integration into the simplified measurement is seemingly worth considering.

In food factories, ingredients can be the main source of nutrition for microbes. Thus, it is crucial to thoroughly detect food residues. Nevertheless, some residues contain ADP or AMP at a higher level, which may have some filth overlooked with the ATP assay alone. For this reason, not only ATP but ADP and AMP are measured under the ATP+ADP+AMP hygiene monitoring system (hereafter referred to as A3 assay) which allows an examiner to obtain very fine measurements. A3 assay results prove how meticulously food residues are detected in comparison with ATP results [2].

2.3. Fluorescent staining technique

To compare with the ATP assay result, the fluorescent staining technique is demonstrated in this study although it is not regarded as a rapid measuring method.

The fluorescent staining technique studied by Nasu et al. had been developed in the 1970s, which has been implemented since 1990s but little applied to studies on indoor environments [3]. It is necessary to select stains effective to the measurement and establish a method for analyzing the concentration of indoor airborne bacteria. The fluorescent staining technique demonstrated in a laboratory trial in place of a food factory is shown in Figure 2. At this stage, it has been determined to employ the fluorescent staining technique in order to compare its results with ATP assay results. Its concrete evaluation method as well as applications to factories must be established properly [4].
Table 1. Outline of rapid measurement method

| Techniques                  | Target substance | Principles/characteristics                                                                 |
|-----------------------------|------------------|--------------------------------------------------------------------------------------------|
| 1) Direct methods           |                  |                                                                                             |
| Solid-phase cytometry       | Bacterial cell   | Signals emitted by bacteria trapped on a filter or other carriers are directly detected. Signals related to physiological activities can be picked up depending on the kind of stain, or autofluorescence may be also used. In some cases, gene probes, antibodies or fluorescent labeled phages are utilized to selectively detect specific bacteria. Various types of optical devices such as fluorescence microscopes and laser microscopes are used as detectors/measuring instruments. |
| Flow cytometry              | Bacterial cell   | Signals emitted by bacteria passing through passages are directly detected. Signals related to physiological activities can be picked up depending on the kind of stain, or autofluorescence may be used. In some cases, gene probes, antibodies or fluorescent labeled phages are utilized to selectively detect specific bacteria. As detectors/measuring instruments, various types of optical devices are used. |
| 2) Indirect methods         |                  |                                                                                             |
| Immunoassay                 | Antigen          | Bacterial antigen are made to react with specific antibodies, and their color development and fluorescence are visually observed or assessed with a microplate reader. Immunological chromatography is one of the simplified methods. |
| Nucleic acid amplification  | Nucleic acid     | Microbial nucleic acids, amplified by applying specific primer to target microbes, are detected. Quantitative PCR is also applicable. |
| Bioluminescence/           | ATP etc.         | ATP contained in bacterial cells is assessed in the luminescent/fluorescent phenomena induced by enzymatic reactions. |
| biofluorescence             |                  |                                                                                             |
| Microcolony analysis        | Proliferation potency (microcolony) | Microcolonies in the early development stage are detected and counted. The requirements for their cultivation (e.g. medium composition and temperature) are the same as those of plate culture. |
| Impedance                   | Proliferation potency (electric characteristics) | This method focuses on the change of electric characteristics in growing metabolites produced by bacteria as they proliferate in use of medium components. |
| Gas measurement             | Proliferation potency (gas production) | This method focuses on the change in the quantity of gases, including carbon dioxide produced and oxygen consumed when bacteria proliferate. |
| Fatty acid analysis         | Bacterial fatty acid | The difference in the composition of bacterial fatty acid depending on the bacterial type is taken into consideration in the analysis. |
| Infrared absorption         | Bacterial component | Bacteria are exposed to infrared rays and the pattern of their infrared absorption spectrum is examined. |
| spectrometry                |                  |                                                                                             |
| Mass spectrometry           | Bacterial component | Bacterial components are evaluated with a mass spectrometer and collated with the database. |
| Fingerprinting              | DNA              | DNA extracted from a sample is cut with a restriction enzyme, and the migration pattern of DNA fragments is analyzed. They are identifiable by reference to the database. T-RFLP is effective in analyzing the structure of microbial communities. |
| High-throughput sequencing  | Nucleic acid     | The arrangements of nucleic acids extracted from various bacteria in a sample are determined, based on which the community structure is analyzed. |

(Source: Japanese Pharmacopoeia)
Notes) PCR: polymerase chain reaction
T-RFLP: terminal restriction fragment length polymorphism
3. Monitoring of indoor airborne bacteria

In this study, the ATP assay was employed as a simplified rapid measuring method of airborne bacteria, and the fluorescent staining technique for comparison. The following procedures were preliminarily carried out\(^{[5,6]}\).

3.1. Collection of airborne bacteria

Airborne bacteria were sampled in the following manner. To investigate measurability, the measurement was performed in a laboratory without air conditioning.

1. **Falling bacteria**
   Falling bacteria were collected with membrane filters (47mm) for 24 hours, 48 hours and 96 hours.

2. **Airborne bacteria**
   Airborne bacteria were collected with membrane filters (47mm) at the flow rate of 10L/min for one hour and two hours.

3.2. ATP measurement of bacteria collected

1. A cotton swab at the tip was moistened with a drop of distilled water and applied to the membrane filter surface to wipe it off vertically and horizontally ten times each as Figure 3 illustrates.

2. The bacteria sticking to the swab were mixed with the reagent of LuciPac (A3 surface), then measured with the Kikkoman Lumitester shown in Figure 4.

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**Figure 2.** The fluorescent staining technique demonstrated in a laboratory trial in place of a factory

**Figure 3.** Wiping off the membrane filter surface

**Figure 4.** Lumitester
3.3. Fluorescent staining technique applied to bacteria collected
(1) The membrane filter was placed in the suction filtration apparatus as Figure 5 demonstrates.
(2) Stain was added and left for five minutes.
(3) As Figure 6 shows, the stain was sucked and filtrated. After drying, the filter was irradiated with ultraviolet rays and visually observed.

![Figure 5](image_url1)

**Figure 5.** The filter placed in the suction filtration apparatus

![Figure 6](image_url2)

**Figure 6.** Visual observation

4. Applications to food factories for their quality and environmental management

4.1. Microbial contamination in food factories
Secondary environmental pollution is the main cause for product contamination. Products are contaminated by filthy air and unclean facilities. There are roughly four contamination sources: (1) air pollution, (2) facilities, (3) packaging materials, and (4) workers (i.e. clothes and fingers). Each of them is summarized below[7].

4.1.1. Air pollution
Regarding air pollution in a food factory, particular care and attention must be paid to the places where products are handled in the open. It is quite important to eliminate pollution as much as possible in order to extend the “best-before date” for food products by even one day. In this regard, food products which are susceptible to microbes are packaged in a clean work zone or cleanroom. The measurement of falling bacteria is practiced widely as a method for inspecting air pollution in a cleanroom. In this method, agar plate media are left uncovered at target places for a given period of time, and cultivated. Then the number of colonies forming on the plate is counted. It is necessary to perform the measurement closer to products for as long a time as possible during the indoor operation hours. To evaluate the functional capacity of a cleanroom, the measurement must be carried out under no influence of operation by suspending the indoor work.
4.1.2. Polluted facilities
The measurement of surface microbes and the fouling test are typical methods for inspecting polluted facilities. Any of these methods employed in factories must be instrumental in immediately judging whether facilities are washed and sterilized sufficiently enough and whether the operation can be resumed or not. These methods are inevitable in order to introduce HACCP to food factories, and the ATP assay proposed in this study is effective and efficient to meet the requirements.

4.1.3. Polluted workers
Contamination by workers is brought mainly by their polluted working wear. In this regard, target inspection points are places which are easily polluted and touched with products.

4.1.4. Polluted fingers of workers
During the packaging process, products can be contaminated directly by polluted fingers or indirectly through packaging materials. As fingers come in contact with various places while at work, the finger surface is polluted even if gloves are worn. Finger pollution should be inspected while at work and after having hands washed and sterilized. Unfortunately, the ATP assay won’t detect polluted microbes unless their number exceeds $10^4$. As is suggested in this study, the fluorescent staining technique needs to be jointly used.

4.2. ATP assay in conformity to Japan’s Standard Methods of Analysis in Food Safety Regulation

4.2.1. ATP procedures under Standard Methods of Analysis in Food Safety Regulation in Japan
The ATP procedure is mentioned in one of the references \[8\]. The current ATP assay is not able to distinguish between filth and bacteria. But, if remaining, filth causes bacteria to proliferate or weakens the effectiveness of disinfection.

In the ATP assay, as Figure 7 illustrates, the ATP-based cleanliness test is performed after the washing. If the result exceeds a prescribed level, it is necessary to do the washing again and then carry out disinfection.

![Figure 7. Differences between the ATP assay and the cultivation method](image)

4.2.2. Bacterial wipe test and ATP wipe test
Table 2 indicates major items and inspection procedures of both bacterial wipe test and ATP wipe test conducted in food factories. As the table shows, Standard Methods of Analysis in Food Safety Regulation is firmly established in Japan.
Table 2. Major items and inspection procedures of bacterial wipe test and ATP wipe test

| Items to be inspected | Procedures |
|----------------------|------------|
| Cooking utensils     | Cutting boards, vats, bowls, dishes, spatulas 10cm×10cm |
|                      | Knives, scissors, slicers (blades) The whole or disassembled parts |
| Cooking instruments  | Vegetable cutting machines, etc. Joints, gaps and corners |
| Facilities           | Counters, refrigerators (inside), sinks 10cm×10cm |
|                      | Floors 10cm×10cm (sharp fluctuations depending on the place) |
|                      | Handles (e.g. refrigerators) The whole |
|                      | Door knobs The whole |
|                      | Spray containers Triggers, grips |
| Cooks                | Fingers Dominant hands on the whole (i.e. palms, backs, between fingers) |
|                      | Aprons Areas that touch foods and utensils 10cm×10cm |

Wiping off vertically, horizontally, and diagonally right and left, five to ten times each at a uniform pressure

4.2.3. Examples of ATP evaluation

The ATP assay determines the degree of cleanliness on the basis of luminescence as explained in Section 1.2. The judging criteria indicated in the reference[8] are listed in Table 3.

Table 3. Examples of ATP evaluation

| Items to be inspected | Control standard values |
|----------------------|-------------------------|
|                      | Pass | Fail |
| Cutting board        | ≤500 | 1,000≤ |
| Bowl                 | ≤200 | 400≤ |
| Vat                  | ≤200 | 400≤ |
| Sink                 | ≤200 | 400≤ |
| Counter              | ≤200 | 400≤ |
| Refrigerator handle  | ≤200 | 400≤ |
| Finger               | ≤1,500 | 3,000≤ |

* Those evaluated between pass and fail need watching.

Note: The test should be conducted after disinfectant is thoroughly rinsed away because measurements may vary if the disinfectant remains.

5. Results about the ATP rapid detection method

As shown in Section 3.2, the preliminary experimental results of the ATP rapid detection method are shown in Table 4.

Table 4. Results about the ATP rapid detection method

| Collection time | flow   | RLU (relative light unit, average value) |
|-----------------|--------|----------------------------------------|
| 1H              | 1L/MIN | 9                                      |
| 1H              | 2.5L/MIN | 11                                    |
| 1H              | 5L/MIN | 12                                     |
| 1H              | 10L/MIN | 15                                     |
| 2H              | 1L/MIN | 8                                      |
| 2H              | 2.5L/MIN | 14                                    |
| 2H              | 5L/MIN | 13                                     |
| 2H              | 10L/MIN | 21                                     |
Table 4 shows the data of the ATP rapid detection method. The cause of this value is the surface wiping method in this experiment, but it is thought that most of the suspended bacteria in the air were collected inside the filter at the time of collection due to the type of filter. In the future experiments, we will change the type of filter and adopt the method of surface collection. In addition, not only the ATP method, we also use the fluorescent staining method for comparison. From the results of future experiments, we hope to be able to confirm these figures in this experiment. If it is not the cause of the filter, it may be necessary to consider the experiment again.

6. Summary and future issues
In this study, the simplified methods for monitoring airborne bacteria were examined for the improvement of quality and environmental management in the Japanese food industry. The execution of the membrane filter technique to collect indoor airborne microbes and the ATP assay to evaluate cleanliness would realize a highly accurate monitoring. Furthermore, in order to perform strict evaluation, the A3 assay (ATP+ADP+AMP) is proposed. A3 assay results prove how meticulously food residues are detected in comparison with ATP results. It has been also verified that the fluorescent staining technique is effective if further examination is required. In order to put the simplified monitoring methods we have developed in this study into practical use, results of the ATP measurement and the fluorescent staining technique applied to airborne and falling microbes will be presented at the first opportunity.

7. References
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