

Accelerating Assessment and Evaluating Colony Identification Function Using the Real-Time Colony Counter ScanStation®

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1. Introduction

Microbiological hygiene management is extremely important for ensuring food safety, and rapid result determination, reliability, and accuracy are also key factors. The ScanStation® from Interscience, which can count colonies in real time while simultaneously performing incubation, is equipped with functions to address these risk management aspects. This instrument functions as a constant-temperature incubator while simultaneously enabling continuous enumeration of colony counts. Different incubation times can be set for each type of medium, and even when incubation start times differ, each can be independently counted according to the set incubation time, allowing multiple types of media to be started at any desired timing. Petri dishes during incubation are automatically photographed every 30 minutes or 1 hour, and by comparing time-lapse images, only growing colonies are counted in real time. Furthermore, graphs of colony detection trends are automatically generated from the recorded incubation time and count monitoring results, making it possible to determine whether thresholds have been exceeded at an early stage without waiting until the end of incubation as in conventional methods.

Additionally, regarding colony identification accuracy, there are challenges in food testing caused by fine residues that pass-through filters. When colonies and residues coexist in Petri dishes after incubation, counting becomes difficult, and cases arise where inexperienced inspectors are uncertain in their evaluation. However, since the ScanStation® can accurately count microorganisms including mold from the early stages of incubation using image analysis technology and other methods, there is potential for high identification accuracy and significantly reduced time to result validation. Furthermore, by integrating an in-house developed system with this instrument, labor savings in assessment work can be achieved, and usefulness is expected in advancing the digital transformation of microbiological testing.

In this study, using the colony counting system of this instrument, we conducted two verifications: whether there are differences in growth rate among bacterial strains (Verification 1), and to what extent distinction can be made in samples with abundant food residue (Verification 2).

2. Materials and Methods

2-1. Test Strains

In this study, a total of 9 strains were selected, focusing on microorganisms involved in food spoilage and quality deterioration. For selection, from among our in-house isolated strains, we targeted lactic acid bacteria and yeasts that can cause gas production associated with spoilage or fermentation, strains that cause discoloration or putrefaction, foodborne pathogens that may cause abdominal pain or severe food poisoning symptoms, and spoilage bacteria that are often found on seafood and agricultural products and possess heat resistance.

Table 1: List of Microbial Species Used

Species.	Origin
<i>Leuconostoc citreum</i>	Environemt
<i>Wickerhamomyces anomalus</i>	Environemt
<i>Serratia marcescens</i>	Environemt
<i>Klebsiella pneumoniae</i>	JFRL quality control reference strain
<i>Staphylococcus aureus</i>	Purchased from culture collection
<i>Sporosarcina aquimarina</i>	Environment
<i>Bacillus cereus</i>	Environment
<i>Bacillus subtilis</i>	Environment
<i>Paenibacillus sp.</i>	Environment

2-2. Preparation of Bacterial Suspension, Media, and Test Methods

The temperature most suitable for bacterial growth (optimum growth temperature) varies by species and is generally classified into psychrophilic bacteria (optimum growth temperature: 12-18°C, capable of growth at 0°C), mesophilic bacteria (optimum growth temperature: 25-40°C), and thermophilic bacteria (optimum growth temperature: 55-65°C). For verification, bacteria related to food spoilage were mainly used, and since most of them are mesophilic bacteria, the incubation temperature was set at 35°C.

One bead of each strain stored in Microbank (IWAKI & CO., LTD.) was inoculated into pre-prepared test tubes containing SCD liquid medium (Eiken Chemical Co., Ltd.) and cultured at 35°C for 24 hours, and the resulting bacterial suspension was used as the stock solution.

In Verification 1, this stock solution was diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} with phosphate-buffered saline (sterile dilution water TP2000 from Eiken Chemical Co., Ltd.), and verification was conducted using the pour plate method to measure bacterial counts. The poured plates were incubated for 48 hours in the ScanStation® set at 35°C.

For Verification 2, foods that pass through even when using sterile non-woven filter bags, such as eggs and legumes, were selected, and the microorganisms compared with residue were verified using lactic acid bacteria, which form relatively small individual colonies. Food samples of 10g were collected and diluted 10^{-1} with phosphate buffer, then 1mL of bacterial stock solution was added to serve as the sample stock solution, which was diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} , pour-plated with standard agar medium, and incubated for 48 hours in the ScanStation® set at 35°C.

3. Results and Discussion

3-1. Growth Rate (Verification 1)

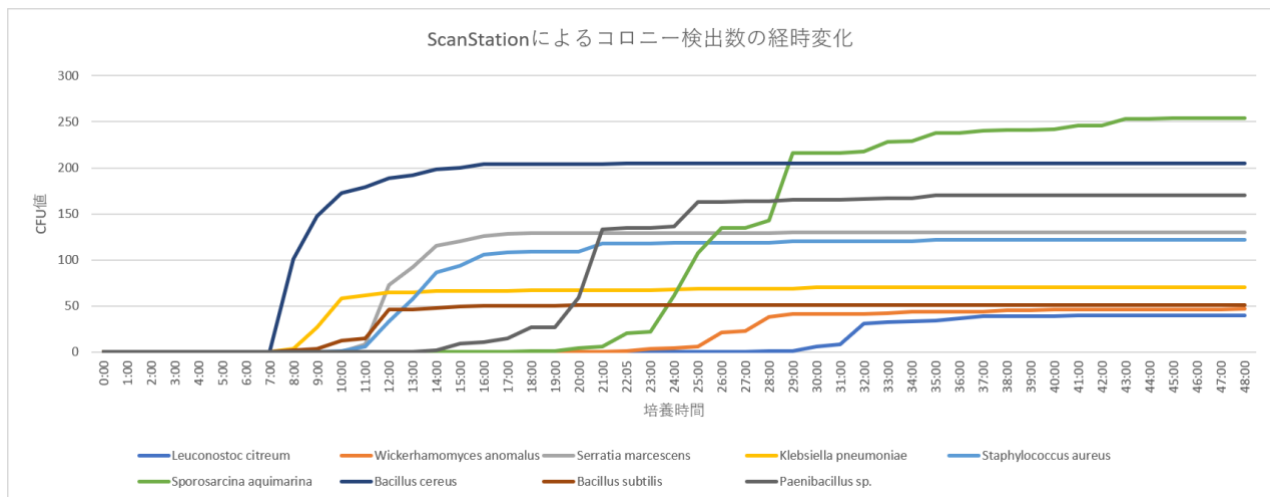
There are four stages in bacterial growth, and the first stage is called the lag phase, during which there is little change in bacterial count, as it is a period of preparation for the bacteria to begin dividing. Subsequently, upon entering the exponential growth phase, bacteria begin to divide and multiply at constant logarithmic intervals, showing a constant rate of increase. Some bacteria die during the exponential growth phase, but only a few. With the rapid microbial growth during the exponential growth phase, the rate of bacterial count increase declines due to nutrient depletion, pH changes, and other factors, and the stationary phase begins where the number of new cells equals the number of dying cells, maintaining a constant viable count. Eventually, the number of dying bacteria gradually increases, leading to the death phase. A growth curve can be created from this elapsed time and colony count data;

however, in this verification, unlike conventional growth curves based on viable count measurements, using the ScanStation® at hourly intervals, Petri dishes were automatically photographed every hour, and a system that counts only colonies from the image data was utilized. (Table 2) This allowed us to graph the changes in cumulative colony counts obtained over time and compare the differences in behavior among bacterial strains. (Table 3) To ensure measurement reliability, data with final measured colony counts within the valid range of 30-300 colonies per Petri dish were selected and adopted.

Table 2: Final Measured Colony Count and Time to Maximum Value Incubation Conditions: 35°C/48 hours

	Species	Bacterial Count	Plate dilution factor				Time to Maximum Count Value
			×10	×10 ⁻²	×10 ⁻³	×10 ⁻⁴	
1	<i>Leuconostoc citreum</i>	4.0×10 ³	TNTC	40	1	0	41 hours
2	<i>Wickerhamomyces anomalus</i>	4.7×10 ⁴	TNTC	TNTC	47	1	40 hours
3	<i>Serratia marcescens</i>	1.3×10 ⁵	TNTC	TNTC	130	16	29 hours
4	<i>Klebsiella pneumoniae</i>	7.0×10 ⁴	TNTC	TNTC	70	9	30 hours
5	<i>Staphylococcus aureus</i>	1.2×10 ⁴	TNTC	122	11	1	35 hours
6	<i>Sporosarcina aquimarina</i>	2.5×10 ³	254	21	3	0	29 hours
7	<i>Bacillus cereus</i>	2.0×10 ³	205	22	0	0	22 hours
8	<i>Bacillus subtilis</i>	5.1×10 ⁴	TNTC	TNTC	51	2	20 hours
9	<i>Paenibacillus sp.</i>	1.7×10 ⁵	TNTC	TNTC	170	13	35 hours

Table 3: Changes in Colony Detection Count by Bacterial Strain



For each bacterial strain, the time at which the maximum count value obtained during 48-hour incubation was first reached was defined as the time to maximum value, and comparison revealed different results for each. For the strain that reached the maximum count value earliest, counting results close to the maximum were obtained at least 24 hours earlier compared to the standard 48 hours. However, when applying this to early determination, it is necessary to confirm agreement with final determination values for each strain and sample, and this study was conducted as a preliminary case study to understand the instrument characteristics.

3-2. Colony and Residue Discrimination Performance (Verification 2)

In microbiological testing of food, knowledge and experience are required to distinguish between colonies and food residue formed on culture plates. While visual discrimination by color, morphology, size, and other characteristics is sometimes possible, there are many cases where detailed confirmation by microscopic observation or Gram staining is necessary. Since this series of procedures requires considerable time and effort, proficiency of the testing personnel is essential. However, by using the ScanStation®, continuous automatic photography of Petri dishes becomes possible, and for colonies, the growth process over time can be confirmed from images, making discrimination from residue easier.

In this verification, we compared Petri dishes in which relatively large food residue was pour-plated with medium against Petri dishes in which food residue and lactic acid bacteria with relatively small growing colonies were added and pour-plated with medium, but no obvious misjudgment caused by food residue itself was confirmed. (Table 4) (Table 5)

However, from observation of time-lapse images, it was considered possible that water droplets appearing during incubation were falsely detected as colonies. This water droplet is presumed to have formed inside the Petri dish due to the temperature difference between the chamber temperature (35°C) and room temperature (20°C) and the high-humidity environment. Since sufficient comparative tests have not been conducted on the effects of such condensation formation on determination and the conditions for controlling it, additional verification is needed in the future to identify the causal relationship.

Table 4 : Colony Counts of Petri Dishes Containing Food Residue and Petri Dishes with Lactic Acid Bacteria Added to Them

Sample Name	Bacterial Count	Plate dilution factor			
		×10	×10 ⁻²	×10 ⁻³	×10 ⁻⁴
Lactic acid bacteria (<i>Leuconostoc citreum</i>)	4.2×10 ³	TNTC	42	5	1
Nishiki tamago	0×10	0	1*	0	0
Kinshi tamago + lactic acid bacteria	7.8×10 ³	TNTC	78	10	0
Boiled egg	0×10	0	3*	0	0
Boiled egg + lactic acid bacteria	5.3×10 ³	TNTC	53	9	0
Black soybeans	0×10	0	6*	0	0
Black soybeans + lactic acid bacteria	9.8×10 ³	TNTC	98	10	0
Otafuku beans	0×10	7*	1*	0	0
Otafuku beans + lactic acid bacteria	4.9×10 ³	TNTC	49	1	1
Mame kinton (sweet bean paste)	0×10	2*	0	0	0
Mame kinton + lactic acid bacteria	7.4×10 ³	TNTC	74	3	0

*Results including water droplet counts

Table 5: Examples of Photographs Distinguishing Food Residue from Colonies

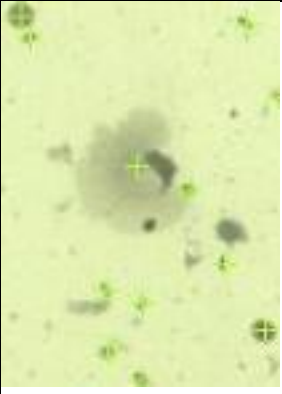
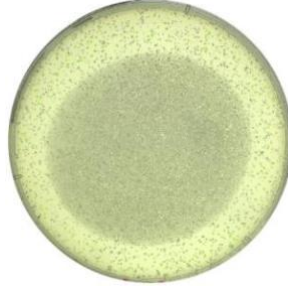
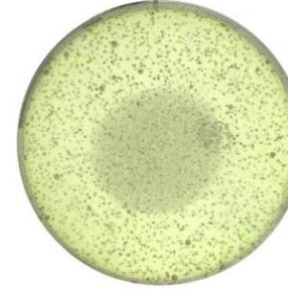
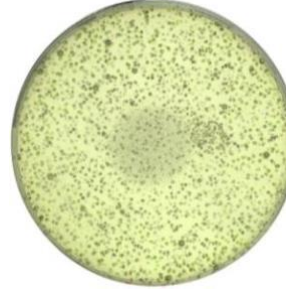
Photograph at the start of incubation				
Photograph at the end of incubation				

Table 6: Photograph showing water droplets formed at the edge of the Petri dish being misjudged

Boiled egg	Nishiki tamago (two-layered egg)	Otafuku beans	Mame kinton (sweetened mashed beans)	Black soybeans
				

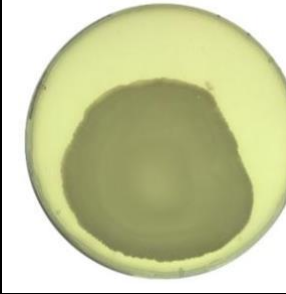
Not only the edge of the Petri dish, but also condensation or water droplets that form on the lid and remain for an extended period can interfere with counting in some cases (table 7). According to the device specifications, minor condensation that naturally evaporates within approximately 4 hours after the start of incubation does not affect the determination. However, cases were confirmed where colonies growing under condensation that remained for an extended period were not captured by the camera, making accurate time-course monitoring difficult.

Table 7 : Water droplets formed on the Petri dish lid

Elapsed time	Count	Elapsed time	Count	Elapsed time	Count	Elapsed time	Count
After 0h	0	After 10h	133	After 30h	377	After 48h	509
							
The entire Petri dish lid is fogged with water droplets		Surrounding water droplets evaporated and colonies began to be counted		The fog gradually disappeared and the counting range gradually expanded		Colonies near the center were not counted	

Additionally, in rare cases, Petri dishes with spreading colonies may not be counted. (Table 8) Additional verification is needed to determine whether this is caused by water droplets remaining on the lid or by the growth rate of the colonies.

Table 8 Counting of spreading colonies

Elapsed time	Count	Elapsed time	Count	Elapsed time	Count	Elapsed time	Count
After 17h	0	After 20h	0	After 34h	1	After 48h	1
							
All water droplets on the lid evaporated		Growth of spreading colonies is observed		Colonies growing overlapping on top of the spreading colonies growing colonies were counted		Spreading colonies grew large and dense, making other colonies no longer visible	

3-3. Discussion

In microbiological testing of food, there are tests that determine only negative or positive results and tests that count colonies to determine bacterial counts, but in all cases, it is important to make accurate and rapid determinations. Conventionally, determination requires a certain amount of time because inspectors make judgments visually or using instruments after incubation for a specified time. Additionally, checking the incubator every few hours to make early determinations is labor-intensive and inefficient, and contamination and operational risks may also arise. Most automatic counting using instruments also involves placing Petri dishes after incubation for counting, and when there are many samples to test, determination after incubation time takes longer and increases the workload for operators. In this way, the process of visually confirming bacterial growth and counting after Petri dish incubation can be monitored with ScanStation® from the start of incubation until the set time, with advantages such as being able to process multiple Petri dishes simultaneously and good time efficiency. Furthermore, because photographs of Petri

dishes are taken at regular intervals, even spreading colonies can be counted before they spread, and if they are ultimately not counted, the captured photographs can be reviewed retrospectively. However, if water droplets on the Petri dish lid remain for an extended period, accurate counting is not possible, so measures to prevent the Petri dish from fogging after pour plating are necessary. Regarding the advantage of time reduction, rapid determination can be made because colony growth can be confirmed without waiting for the specified time, and after determination, Petri dishes can be removed and the available space can be efficiently used for the next test, which is also a significant advantage.

In this study, when 9 microbial strains were observed over time using ScanStation[®], the time at which colonies began to be detected

and the time to reach the maximum count value differed depending on the strain. Some strains showed count values that became approximately constant before the specified 48 hours, indicating the possibility of use for early determination.

Additionally, it was confirmed that in samples containing food residues, time-course images made it easier to distinguish between residues and growing colonies. On the other hand, cases were also observed where water droplets due to condensation were falsely detected and spreading colonies were not sufficiently counted. To achieve stable determination, it is important to examine operating conditions that suppress condensation inside the Petri dish.

ScanStation[®] automatically photographs and counts Petri dishes during incubation and allows retrospective review of past images, and in this respect, determination is useful for reducing labor and speeding up determination work. In the future, we would like to increase the target foods and bacterial strains and examine conditions suitable for early determination.

*ScanStation[®] is a registered trademark owned by Interscience Co., Ltd.

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