Original

New Estimation Methods of Bacterial Concentration by Measuring ATP Changes during Incubation

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New estimation methods of bacterial cell concentration in samples by measurement of the increase in bacterial adenosine-triphosphate (ATP) content during incubation using a conventional firefly luminometer were established. When an *Escherichia coli* cell suspension was incubated in nutrient broth, the increase in the ATP content of the suspension during the incubation period followed a sigmoidal curve. The increase ratio of the ATP content of the suspension at a given period of incubation (5 hours in this study) to the initial ATP content was greater at higher initial cell concentrations. With this relationship, the initial cell concentration of a test suspension could be predicted from the measured ratio; this was called the end point method. On the other hand, the lag period in the ATP increase curve was longer at lower initial cell concentrations. A highly linear relationship was observed between the lag period and the logarithm of the initial cell concentrations. Based on this relationship, a delay method was developed for prediction. The two relationships were also observed for bacterial suspensions of *Klebsiella* sp., *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas* sp. These results suggested that the two methods have the potential to estimate the bacterial cell concentration of a sample suspension.

Key words: ATP; bacterial cell concentration; luminometer; kinetics; end point method; delay method

Introduction

Measurement of microbial cell concentration in food materials, food products, and swabs of surfaces of food processing equipment is indispensable for evaluating the microbial contamination of the products. The most widely used, traditional method for measurement of bacterial cell concentration is the standard plate count method^{1), 2)}. This method, however, requires a long incubation period of 48 hours^{1), 2)}. When food products are shipped out of factories, the producers should know the level of microbial contamination of the products. At present, however, the products are often shipped before microbiological examination, which is time-consuming, yields a result. Therefore, rapid microbial examination methods are urgently needed in the food industry.

Among the methods available for measurement of microbial contamination, the firefly bioluminescence assay for adenosine triphosphate (ATP) is rapid and simple^{3), 4)}. A number of investigators have reported the use of this method for detection of microbial contamination in foods⁵⁾⁻¹⁰⁾. This method, however, has one universal problem^{11), 12)}. That is, interference from large amounts of non-microbial ATP in foods. Some food samples show high ATP concentrations in comparison with their low microbial cell counts. This phenomenon can also be observed for swab samples of food processing instruments¹³⁾. A possible explanation is that food

samples can contain high concentrations of endogenous ATP, because most food materials are of animal or plant origin and may retain their original ATP content¹⁴⁾.

Two solutions to the problem have been considered. One is the enzymatic destruction of non-microbial ATP in samples with ATPase. After the destruction of non-microbial ATP in food samples by the enzyme, the enzyme itself must be removed to avoid destruction of microbial ATP in the subsequent steps. This is done generally by washing the enzyme away on membrane filters with washing buffer¹⁵⁾. Another way is to separate the microbial cells from food materials in samples by centrifugation, followed by filtration through membranes¹⁶⁾. In both procedures, the samples need to pass through conventional membrane filters with a pore size of 0.45 or 0.22 μm ^{15), 16)}. Unfortunately, most solid processed food products and their homogenates in buffered saline can not pass through such filter membranes or be washed on the membranes with the buffer; the homogenates or the buffer stick on the membranes during the process. Thus, the application of the two methods to foods is strictly limited. As far as we know, the solid foods to which these methods can be applied for ATP measurement are raw vegetables and meat whose homogenates can pass through the filter membranes^{5), 6), 8), 10), 15), 16)}. These methods cannot easily be applied to most processed, commercial foods for which we routinely need to measure their bacterial cell

concentrations. At present, therefore, the ATP measurement of swab samples, which is the sum of ATP of food residues and microbial origins, is the major current application of bioluminescence assay of ATP, for the estimation of surface cleanliness, not of microbial contamination^{4), 17)}.

In these circumstances, we developed new, kinetic ATP measurement methods for bacterial cell counts in samples with a conventional firefly luminometer in this study. The methods were an end point method and a delay method. These methods are based on the increase kinetics of the ATP content in bacterial cells that proliferate in culture medium during incubation. With these methods, test samples do not need to pass through the filter membranes described above. In this study, we developed the new methods for measurement of bacterial cell concentration in cell suspension samples using bioluminescence assay.

Materials and Methods

Bacterial cell preparation

Bacterial strains of *Escherichia coli*, *Klebsiella* sp., *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas* sp. were isolated from foods in our laboratory. Bacterial strains were cultured on nutrient agar plates at 35°C for 24 hours. Cells grown on the plates were cultured in 5 mL of nutrient broth (Nissui Pharmaceuticals, Tokyo, Japan) with shaking at 160 rpm and 35°C for 24 hours. Cultured cells were washed twice with 0.1 mol/L phosphate–0.05 mol/L citrate buffer, pH 7.0 with 0.005% (vol/vol) Tween 80 by centrifugation at $6,200\times g$ and 10°C for 15 min. Cells were finally suspended in 5 mL of saline (0.85% wt/vol NaCl solution). The cell concentrations of the resultant suspensions were 10^9 CFU/mL. Portions (5 mL) of cell suspensions that were serially 10-fold diluted with the saline were inoculated into 45 mL of nutrient broth preheated at 35°C in 100-mL glass tubes with screw caps.

In a preliminary study, several culture media were compared for the kinetic methods using the *E. coli* strain. The media tested were nutrient broth (Nissui), brain heart infusion (Becton Dickinson, Sparks, U.S.A.), and trypticase soy broth (Becton Dickinson). There were no marked differences among the media in the ATP increase during incubation, although a slightly faster increase in ATP was observed in the nutrient broth. Thus, this medium was chosen for study.

Incubation

Bacterial cell suspensions in glass tubes prepared above were cultured with shaking at 120 rpm at 35°C. The temperature was that for the plate count method in our routine^{1), 2)}. At one-hour intervals during incubation, portions (about 3 mL) of the samples were taken and cooled in ice water.

ATP measurement

ATP contents in bacterial cell suspensions were measured by the firefly luciferin-luciferase method with

an ATP analyzer (AF-100, TOA Electronics, Tokyo) according to the manufacturer's technical manual. Briefly, portions (100 μ L) of samples were mixed with 100 μ L of an extract reagent (TOA) for 30 sec to destroy bacterial cell walls. The samples were mixed with 200 μ L of firefly luciferin-luciferase reagent (TOA) for 10 sec, then the luminescence was measured for the following 30 sec. The controls were ATP-free purified water (TOA) and 1.0 nmol/L ATP solution (TOA).

Viable cell counts

Viable cell counts of samples were measured with the standard plate count method^{1), 2)}; ten-fold dilutions of the samples with the buffered sodium chloride peptone solution were mixed with standard plate agar (Eiken Chemicals, Tokyo) and incubated at 35°C for 48 hours.

Data analysis

The increase kinetics of ATP contents in samples was analyzed in terms of the ratio of A/A_0 . Here, A is the ATP concentration measured (nmol/L) at an incubation time t (hour) and A_0 is that at time zero. The numeral analyses were done with Microsoft Excel 2000.

Results

Stability of ATP measurement

Stability of ATP measurement with the analyzer was estimated using an *E. coli* cell suspension. When the ATP content of the bacterial suspension prepared at a cell concentration of 1.0×10^6 CFU/mL was measured 7 times, the measured values were essentially the same: 1.46, 1.45, 1.42, 1.44, 1.43, 1.49, and 1.46 nmol/L. The average and standard deviation of the values were 1.45 and 0.0231 nmol/L, respectively. The coefficient of variation was very small, being 1.59%. This low variability of ATP measurement led us to conclude that two ATP measurements with the analyzer could be enough for each sample. Thus, ATP measurement was subsequently done twice per sample in this study.

ATP increase during *E. coli* growth

The increase in the ATP content of *E. coli* cell suspensions cultured in nutrient broth was studied at various initial cell concentrations of 10^1 – 10^5 CFU/mL. The increase curves of the ATP content of the cell suspensions consisted of a lag period, a linear increase period, and a decelerating or stationary period (for example, Fig. 1 shows a curve for the initial concentration of 10^5 CFU/mL).

End point method

From the increase ratio in the ATP content of a sample suspension incubated for a given period, the initial cell concentration could be estimated; this was called the end point method. The increase kinetics of the ATP content at various initial cell concentrations shown in Fig. 1 was analyzed for various incubation periods (Fig. 2). At a short incubation period such as 2 hours, the increase ratios at low initial cell concentra-

Fig. 1. Increase curves of the ATP content of *E. coli* cell suspensions incubated in nutrient broth at various initial cell concentrations. Cells were incubated at the initial concentrations of 10^1 (◇), 10^2 (●), 10^3 (□), 10^4 (▲), and 10^5 (■) CFU/mL. Point P is described in the delay method section.

Fig. 2. The increase in the ATP contents of *E. coli* cell suspensions of various initial cell concentrations at incubation periods of 1 to 6 hours. The data of Fig. 1 at the vertical sections of 1 to 6 hours of incubation were analyzed for the initial cell concentrations. The hours shown in the figure are the incubation periods.

Fig. 3. Application of a logistic equation to the ATP increase in *E. coli* cell suspensions at 5-hour incubation. The data points are at the 5-hour incubation period in Fig. 2. The curve was fitted to the data with Eq. (1).

tions were too low to analyze (Fig. 2). On the other hand, the ratio at a low cell concentration was greater

for the longer incubation period. The increase ratio with a longer incubation period could predict a smaller cell concentration of a sample (Fig. 2). However, a method with a short incubation period is preferable in terms of rapidity for practical use. In these circumstances, a 5-hour incubation was chosen for further work in this study. The plotted line at 5-hour incubation was sigmoidal and the data points were well described with a modified logistic equation (1) by Gibson *et al.*¹⁸⁾ (Fig. 3).

$$R = a / (1 + \exp(-b(X - c))) \quad (1)$$

Here R and X are the common logarithms of A/A_0 and the initial cell concentration (CFU/mL), respectively. $\exp$ is exponential, and a , b , and c are coefficients. Values of a , b , and c in Fig. 3 were 2.9, 1.4, and 2.6, respectively. It was considered that with this relationship, the initial cell concentration of a test suspension could be estimated from the value of A/A_0 at 5-hour incubation.

The relationship between the increase ratio at 5-hour incubation and the initial cell concentration was studied for other food contaminating bacteria of *Pseudomonas* sp., *S. aureus*, *B. subtilis*, and *Klebsiella* sp. under the same experimental conditions as those of *E. coli*. The data points for those bacteria were also well described with Eq. (1) (Fig. 4). Values of the coefficients in the equation for the bacteria are shown in Table 1. The results suggested that the initial cell concentration of a test suspension of these bacteria could also be

Fig. 4. The increase in the ATP contents of cell suspensions at 5-hour incubation for several species of bacteria. Symbols: ■, *Klebsiella* sp.; ●, *B. subtilis*; ▲, *S. aureus*; ◆, *Pseudomonas* sp. Curves are described with Eq. (1) for (a) *Klebsiella* sp., (b) *S. aureus*, (c) *B. subtilis*, and (d) *Pseudomonas* sp.

Table 1. Values of Coefficients of the Logistic Equation for Various Bacterial Species

Species	a	b	c
<i>Klebsiella</i> sp.	3.3	1.2	2.9
<i>Pseudomonas</i> sp.	2.8	1.2	3.6
<i>S. aureus</i>	3.5	1.2	3.7
<i>B. subtilis</i>	3.0	1.7	3.1

a , b , and c are the coefficients of Eq. (1).

estimated using the end point method.

Delay method

The increase kinetics of ATP contents of *E. coli* cell suspensions shown in Fig. 1 was also analyzed with a delay method. Let us define the lag period, L (hour), in the ATP increase curve as the period between two points. One point is the point at the initial time. The other point is the intersection of the linear regression line for the exponentially increasing period and a straight line passing through the point of the initial time, parallel to the horizontal axis. An example of this point at the concentration of 10^1 CFU/mL is point P in Fig. 1. For this example, L was 5.22 hours.

There was a highly linear relationship between the logarithm of the initial cell concentration, X , and the lag period, L , for *E. coli* in a range of 10^1 – 10^5 CFU/mL (Fig. 5). The correlation coefficient was 0.999. The regression line was expressed as follows:

$$L = dX + e \quad (2)$$

where d and e are coefficients. Values of d and e for the

Fig. 5. The linear relationship between the lag period and the initial cell concentration of *E. coli*. The experimental data are taken from Fig. 1. The straight line is a linear regression line (Eq.(2)) of the data.

Fig. 6. The linear relationship between the lag period and the initial cell concentration for several species of bacteria
Symbols: ■, *Klebsiella* sp.; ●, *B. subtilis*; ▲, *S. aureus*; ◆, *Pseudomonas* sp. The straight lines are the regression lines for (a) *Pseudomonas* sp., (b) *S. aureus*, (c) *B. subtilis*, and (d) *Klebsiella* sp. The experimental data were the same data that were analyzed with the end point method.

Table 2. Values of Coefficients of the Linear Regression of Various Bacterial Species

Species	d	e
<i>Klebsiella</i> sp.	0.990	6.04
<i>Pseudomonas</i> sp.	0.924	6.52
<i>S. aureus</i>	0.924	6.38
<i>B. subtilis</i>	1.04	6.84

d and e are the coefficients of Eq. (2).

organism were -1.03 and 6.11 , respectively. It was speculated that, using this linear relationship, the initial cell concentration of a test suspension could be also estimated from the measured lag period.

The relationship between the lag period and the initial cell concentration was studied for the other bacteria tested above. The original data were the same as those analyzed using the end point method. A highly linear relationship was observed for all bacteria tested, with correlation coefficients of 0.999 (Fig. 6). Values of the coefficients in the equation for the bacteria are shown in Table 2. The results suggested that the initial cell concentration of a test suspension of these bacteria could also be estimated using the delay method.

Discussion

The new kinetic methods studied in this study should have several advantages for the estimation of bacterial concentration in samples.

(i) The incubation periods of the methods are much shorter than that of the conventional plate count method (48 hours). For example, the concentration of an *E. coli* cell suspension of 10^3 CFU/mL could be estimated with the data of 5-hour incubation by the end point method (Fig. 3) and with the data up to 6-hour incubation by the delay method (Fig. 1). Moreover, it takes less than one minute to measure the ATP content of a sample with an ATP analyzer.

(ii) The detection limits of cell concentration by the methods are lower than that of the conventional method using an ATP analyzer. The detection limits for both the end point and delay methods in this study would be 10^1 CFU/mL of *E. coli* cells (Figs. 3 and 5). The detection limit of the luminometer itself, estimated from the absolute ATP content of a sample, is about 10^{3-4} CFU/mL^{19), 20)}. In this case, bacterial cells need to be suspended in purified water to measure the absolute bacterial cell concentrations, because some constituents of food samples such as salts quench the bioluminescence of food samples¹⁹⁾.

(iii) The kinetic methods did not need sophisticated apparatus. Miyamoto *et al.*²¹⁾ reported a method for bacterial cell counts with a photon-counting TV camera, which was based on firefly bioluminescence. There is also a commercially available system of microbial cell counting with a similar camera system (MicroStarTM-RMDS, Millipore Corp.). However, such apparatus is expensive and might therefore be unsuitable to use for microbial cell counts in most food facto-

ries. Moreover, test samples for such apparatus need to pass through membrane filters with a pore size of 0.45 or 0.22 μm . Compared to such apparatus, the ATP analyzer used in this study is just a conventional bioluminometer. Also, the kinetic methods studied here do not need any special process or treatment; only an incubation process is required.

(iv) Since the methods do not require filtration of a food sample, they could be applied to most commercial, processed foods that cannot pass through a conventional membrane filter with a pore size of 0.45 or 0.22 μm . In a preliminary study, we evaluated the methods using commercial foods inoculated with *S. aureus* or *E. coli* at various cell concentrations. Homogenates of the foods were not able to pass through conventional membrane filters. The methods gave good results for these food samples (data not shown).

Let us consider the characteristics of the two kinetic methods. The end point method was easy to measure and analyze, because it needed only the observed data at a single incubation time such as 5 hours, other than the initial ATP content. The delay method needed data points at several incubation times for analysis. Thus, measurement with the delay method is more laborious. However, the delay method might give more accurate predictions for the bacterial concentration of a sample than the end point method. This is because the variability of predicted cell concentrations by the delay method, which needs several data points, is considered to be less than that by the end point method. However, when the exact values of bacterial cell concentration for commercial foods are not required, the end point method would be practically useful for foods. It was thought that the end point method would be more suitable for practical application to foods than the delay method. For example, by introducing a threshold of the ATP increase ratio such as $\log A/A_0 = 0.3$, for food samples that are incubated for a given period, the end point method could easily be applied in routine assessment of bacterial contamination in food products. For the delay method, it would be useful to develop an automatic luminometer for sampling and measuring.

Some food products are contaminated by various kinds of bacteria. In this study we tested five representative bacterial species derived from foods. The two methods were successfully applied. Also, the values of the parameters of Eq. (1) and (2) were similar among the bacteria tested (Tables 1 and 2). Now we are evaluating the two methods by applying them to commercial foods. We will present the results in the near future.

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