A novel double subculture method, termed DiVSaL (Differential Viabilities between Solid and Liquid media) method, for the enumeration of injured cell population of a microorganism, which occurs after some sublethal to lethal treatment, was proposed. In this method injured cells were enumerated as the differential value between viabilities determined with two different techniques, the conventional plate counting using a solid agar medium and the growth delay analysis using a liquid medium. In the former technique, the viable cell number is obtained as colony forming unit (CFU) formed on an agar medium where sublethally injured cells are as much rescued as possible. In the latter technique, on the other hand, “the integrated viability” defined by Takano and Tsuchido (1982) is introduced and is calculated from the growth delay of a stressed population, referred to unstressed one. For the growth delay analysis, in this paper, not only the original theoretical model, where the specific growth rate (and therefore the defined $G_{10}$ value) does not change after the exposure to a stress treatment, but also a novel modified theory, where the parameter changes, is proposed. On the theoretical background, this DiVSaL method as a double subculture method can be used to enumerate the injured cells without selection by addition of some inhibitor or by nutritional shortage.

Key words: Double subculture method / Injured cells / Integrated viability / Plate counting / Growth delay analysis.

A variety of methods such as heat, irradiations, different chemicals, and hydrostatic pressure have been used to kill harmful microbes in sterilization and disinfection of food, medical instruments, and different environments. If not all microbial cells present in an objective material are killed by and thus even one cell survives such a treatment, they can be regarded as injured cells. Those injured cells may recover and then grow under proper conditions possibly to cause some microbiological problem in food safety, human health and manufacturing of industrial products. In practical microbiological tests, therefore, the detection of such injured microorganisms have recently been concerned and different methods for the detection of injured microbes based upon their own principles have been developed so far (e.g., Mossell and van Netten, 1984; Foegeding and Ray, 1992; Wesche et al., 2009; Tsuchido, 2013). Those methods can be in general divided into cultivation and non-cultivation techniques.

One of the representative cultivation techniques is the differential plating on both non-selective and selective agar medium plates. Such a selection is performed by addition of some chemical inhibitor or high concentration solute or by using two kinds of nutritionally different agar plates. A typical example of this method is the addition of sodium chloride at a high concentration into some agar medium, originally used as a selective medium for staphylococci (e.g., Hurst, 1984; Foegeding and Ray, 1992; Tsuchido, 2013). A resuscitation technique, which includes an incubation in a proper liquid medium to make much more injured microbes recover before plating on an agar medium, is often applied to microbes exposed to a stress treatment (e.g., van Schothorst,
stressed cell samples followed by plating the diluted
tique can be

plate counting using an agar

For obtaining total viable cell counts including sublethally injured cells, the conventional colony count technique can be applied which contains the dilution of stressed cell samples followed by plating the diluted samples on an agar medium. Every sublethally injured cells may recover on an agar medium under appropriate conditions including sufficient period and without gener-

FIG. 1. A schematic representation of the DIVSaL method for the estimation of injured cell population after exposure to a stress. Lines A and B indicate the survivor curves obtained with the colony count method and the growth-delay analysis method, respectively.

1976; Hurst, 1984; Wu, 2008).

As a non-cultivation technique, the staining method, using two types of staining agents possessing different staining principles, have been applied to detect rapidly injured microbes (e.g., Larsson et al., 2008). In this method, injured population is estimated as a difference in counts obtained with those differently stained cells. A variety of fluorescent dyes have recently been developed and applied to detect and enumerate injured population, even at a single cell level in combination with flow cytometry (e.g., Paparella et al., 2012).

Based on the theory presented before (Takano and Tsuchido, 1982), in this paper, as a novel method for the cultivation-based enumeration of sublethally injured cells of microorganism after exposure to a stress, a solid and liquid double subculture method, termed the DIVSaL (the differential viabilities between on solid and in liquid media) method”, was proposed. In this method the injured cell population generated by a stress treatment is evaluated from a difference in viabilities determined with between two different techniques, a conventional plate counting using an agar medium and the growth-delay analysis technique proposed by Takano and Tsuchido (1982) using a liquid medium basically with the same composition as the agar medium. Although this situations were conceptually depicted in FIG.1, the theory will be described later in this paper.

For obtaining total viable cell counts including sublethally injured cells, the conventional colony count technique can be applied which contains the dilution of stressed cell samples followed by plating the diluted samples on an agar medium. Every sublethally injured cells may recover on an agar medium under appropriate conditions including sufficient period and without gener-

ation of any additional damage derived from this procedure and then form each colony.

In the growth delay analysis method, the growth can be monitored with an on-line measurement of optical density (OD) reflecting cell density, using a commercial microplate reader with a 96-well microplate, equipped with a personal computer. The stressed cells in general stop growing and therefore their population demonstrates a delay before the subsequent regrowth in a liquid medium, because of the reduction of viable cells as well as the repair and/or renaturation of damaged sites in cells. In this method, therefore, the growth delay is taken as a stress effect, while it does not affect the resultant viability in colony count method, even though does the colony size. This situations are schematically expressed in FIGs. 2 and 3 and explained as follows.

In FIG. 2, it has been supposed that each cell in the unstressed population is assumed to possess equal resistance reflected by the same lag period, $i$ (h). As has been described in the previous papers (Takano and Tsuchido, 1982; Tsuchido et al., 1989), the growth kinetics of unstressed cell population from the inoculation to logarithmic phase is expressed as Eq. 1, assuming to follow the apparent first order reaction.

$$N = N_o \cdot \exp\{\mu (t-i)\}$$  \hspace{1cm} (1)

where $N$ and $N_o$ are viable cell numbers per unit volume (mL) at time $t$ (h) and at the start ($t = 0$), respectively, and $\mu$ is the specific growth rate (h$^{-1}$). Even if the inoculum size is changed differently, for the unstressed population, $\mu$ value is supposed not to change, as has already been indicated (Takano and Tsuchido, 1982). It should be noted here that the viable cell number does not mean measured CFU but is a phantom number directly corresponding to the OD value.

When exposed to some stress, the cell population is assumed to be compartmentalized to different subpopulations, $n_i$ ($i = 1, 2, \cdots, k-1, k$), depending upon the degree of injury. Each subpopulation includes cells possessing the same period of lag, $i$, plus different period of its increment, $\lambda_i$ (h), respectively, resultanty the growth delay time for cell repair being $i + \lambda_i$ (FIG. 2). Here, the viable cell fraction is a total of uninjured and sublethally injured ones and has been defined as $n_u$, which corresponds to a total of subfractions $n_i$ through $n_k$. On the other hand, $n_s$ is assumed to be an irreversibly injured (dead) subfraction (FIGs.1 and 2; Takano and Tsuchido, 1982).

For a stressed population, on the other hand, taking notice on the specific growth rate, two growth models may be considered. One is a model in which the specific growth rate of regrowth after a growth delay, $\mu'$, is the same as that for the unstressed population, $\mu$, as have already been reported (Takano and Tsuchido, 1982) (FIG. 3A). The other is a novel model proposed here, in
which μ' value is smaller than that of unstressed population, μ, possibly changing differently with the degree of the stress, (FIG.3B). For each model, the growth kinetics of the stressed population can be expressed with Eqs. (2A) and (2B).

\[
N = N_0 \sum_{i=1}^{k} n_i \exp \left( \mu \left( t - l - \lambda_i \right) \right) \quad \text{(for the identical μ model)} \quad (2A)
\]

\[
N = N_0 \sum_{i=1}^{k} n_i \exp \left( \mu' \left( t - l - \lambda_i \right) \right) \quad \text{(for the reduced μ model)} \quad (2B)
\]

Here, although μ value is considered to be different among subfractions of injured population, in the Eq. (2B) this value corresponds to μ' as the μ value of overall growth of the injured population for simplification of the model.

Since a total of the viable cell fractions is \( n_v \) as described above, the viable cell number is \( n_v N_0 \) (FIGs. 3A and 3B). For the above identical μ model, as previously reported (Takano and Tsuchido, 1982), a specific parameter \( \nu \) (dimensionless), which is functions of \( n_v \) and \( \lambda_i \) as expressed in Eq. (3), has been introduced and \( -\log \nu \) has been defined as "the integrated viability".

\[
\nu = \sum_{i=1}^{k} n_i \exp (-\mu \lambda_i) = \sum_{i=1}^{k} n_i \exp (-\mu \lambda_i)
\]

(3)

where the term \( n_i \exp (-\mu \lambda_i) \) for the \( k \)-th dead cell population equals to 0, since \( \lambda_i \) is supposed to be infinitive.

By substituting Eq. (3) into Eq. (2A),

\[
N = \nu N_0 \exp \left( \mu (t - l) \right)
\]

(4)

When the times required for growth of unstressed and stressed populations up to a fixed level of OD (OD\(_0\)) corresponding to a viable cell number, \( N_v \), in the log phase are defined as \( t_c \) and \( t'_c \), respectively (FIG. 3A), by substituting these parameters into Eqs. (1) and (4), the resultant unified equation is Eq. (5).

\[
N_a = N_0 \exp [\mu (t_c - l)] = \nu N_0 \exp [\mu (t'_c - l)]
\]

(5)

Therefore,

\[
\nu = \exp [-\mu (t'_c - t_c)] = \exp (-\mu \tau)
\]

(6)

where \( \tau \) is the growth delay time (h) between unstressed and stressed populations at \( N_a \) and its corresponding OD of the culture, \( OD_a \) (FIG. 3A).

Here, \( G_{10} \) value has been defined as the net increment of time delay for cell population to grow up to OD\(_a\) when the inoculum size for cultivation is reduced by one tenth. This parameter has the relationship of \( G_{10} = 2.303/\mu \) with μ value (Takano and Tsuchido, 1982). Then, by introducing \( G_{10} \) value instead of \( \mu \), the "integrated viability" in Eq. (6) is expressed as Eq. (7).

\[
-\log \nu = \mu \tau / 2.303 = \tau / G_{10}
\]

(7)

In the identical μ model, therefore, as depicted in FIG. 2A, the injured cell number can be defined as \( n_v N_0 - \nu N_0 \), namely \( N_a \) (\( n_v - \nu \)) and the dead cell number corresponds to \( N_0 - n_v N_0 \), namely \( N_a \) (1 - \( n_v \)).

In the reduced μ model, μ' which is reduced from μ
and therefore $G_{10}^\nu$ increased from $G_{10}$, and a modified parameter $\nu'$ are newly introduced for the stressed population and then the "modified integrated viability" can be defined as $-\log \nu'$. Using $\nu'$, Eqs. (4) and (5) are modified to Eqs. (8) and (9), respectively:

$$N = \nu'N_0 \exp[\mu'(t - \tau)]$$  \hfill (8)

$$N_0 = N_0 \exp[\mu(t_{c0} - \tau)] = \nu'N_0 \exp[\mu'(t' - \tau)]$$  \hfill (9)

However, since $\mu$ and $G_{10}^\nu$ values are different between unstressed and stressed populations, $\tau$ cannot be used in this model. Then, Eq. (9) is converted to Eq. (10).

$$\nu' = \exp[\mu(t_{c0} - \tau) - \mu'(t' - \tau)] = \exp[\mu_{t_0} - \mu'_{t'} - (\mu - \mu')]$$  \hfill (10)

Here, when the term, $(\mu - \mu') \tau$, is assumed to be sufficiently small as compared with $\mu_{t_0}$ and $\mu'_{t'}$, this term can be neglected. Therefore,

$$\nu' = \exp(\mu_{t_0} - \mu'_{t'})$$  \hfill (11)

Since $G_{10}^\nu$ is defined as $2.303/\mu'$, as was $G_{10}$, the integrated viability is

$$-\log \nu' = t'/G_{10}^\nu - t/G_{10}$$  \hfill (12)

The modified integrated viability, $-\log \nu'$, in Eq. (12) is the same expression as the equation of $-\log \xi$, which has been formerly proposed by Tsuchido et al. (1989) for the growth of a cell population inhibited by a substance such as NaCl present in the assay liquid medium for the growth delay analysis method, although the application concept is definitely different between those.

In the reduced model, the injured cell population is expressed as $n_{t_0}N_0 - \nu'N_0$, or $N_0(n_{t_0} - \nu')$, while the dead population is the same as in the identical $\mu$ model, being $N_0 - n_{t_0}N_0$ or $N_0(1 - n_{t_0})$ (FIG. 3B).

In both models, $N_0$ can be supposed to be the initial CFU obtained and $t_{c0}, t', G_{10}$ and $G_{10}^\nu$ can be experimentally measured by the monitoring of OD, instead of measurement of the viable cell number, $N$, providing that an increase in the OD of a culture correlates to that in the viable cell number in the logarithmic phase. The OD monitoring may be achieved easily by using a microplate reader with a 96-well microplate.

For practical use of this method, stressed and unstressed cell samples are serially diluted, plated on an appropriate agar medium, and then cultivated by colony count technique on one hand, whereas the same samples are directly inoculated into an appropriate assay liquid medium and then cultivated in a microplate reader, together with samples for the measurement of $G_{10}$ value, by the growth delay analysis technique. These samples are cultivated at least overnight for bacteria and for two days or more for yeasts and fungi. The resultant CFU and growth delay times obtained are served to evaluate the injured cell number, following the above theory.

In conclusion, the DIVSAL method for the enumeration of a stress-injured cell population is based upon differential viabilities determined by two different subculture methods, the colony count method using a solid agar medium and the growth delay analysis method using a liquid medium. The principle of this method is conceptually close to that of the shelf life in food preservation in practical use, in the sense of taking into account the recovery or resuscitation of injured cells during the cultivation period. Even though being a classical cultivation method, this novel method may be applied basically to not only vegetative growth of bacteria but also possibly fungi, and further even bacterial spores, if the effect of a stress on the germination process is somehow considered in mathematical model.

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