Calorimetric Analysis of the Effect of ^{60}Co γ-rays on the Growth of *Saccharomyces cerevisiae*

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Using a heat conduction type multiplex calorimeter equipped with 24 calorimetric units the heat evolution from growing ^{60}Co γ-irradiated *Saccharomyces cerevisiae* was detected in the form of growth thermograms. ^{60}Co γ-irradiation affected the growth pattern in which a dose-dependent reduction of the growth rate constant was obsereved together with the retardation in growth, indicating an involvement of bactericidal and bacteriostatic effects. These effects were quantitatively analyzed in terms of the bactericidality as a function of dose intensity, which was defined in the previous paper.

It was shown that the bactericidal effect of γ-ray irradiation was dominant within lower doses and decreased almost linearly with the increasing doses up to 3 kGy. An equation to determine the number of survivors on the basis of the parameters calorimetrically determined was developed. The survival rate obtained by a proposed equation was in good agreement with that determined from the colony counting up to a dose range of 4 kGy. However, a remarkable difference was found to exist between the two results obtained at doses higher than 4 kGy. The discrepancy was discussed in terms of action mechanism of irradiation.

1. Introduction

Microbial calorimetry is potentially useful for quantitative evaluation of the growth activity of microbial cells based on the detection of their metabolic heat. It has been shown that the method provides not only quantitative information about the microbial growth activity,[1-5] but also characteristic features about the bactericidal and bacteriostatic effects of various chemicals.[6,7]

Some antibiotics like penicillin, ampicillin, and polymixin B were shown to have the bactericidal effects,[8] while the others such as streptomycin, tetracycline and chloramphenicol exhibited bacteriostatic nature on *Escherichia coli*.[9] In contrast, many other chemicals including alcohols and organic acids,[2,3,11-13] boron derivatives,[10] and diols[14] have been shown to affect yeast strains bacteriostatically as well as bactericidally. In the case of bacteriostatic action, the increase in

doses results in the reduction in the growth rate constant. On the other hand, the bactericidal action appears in the growth thermograms as a parallel shift toward a longer incubation time with increasing doses, thus the incubation time (t_α) required for the microbial activity to reach a certain level α, being increased, while the growh rate constant (μ) remains essentially constant.[6,7] In the previous paper,[6] it was shown that the both antimicrobial effects can be more quantitatively expressed by comparing the specific growth activity, μ_i / μ_m with the specific growth retardation, $t_\alpha(0) / t_\alpha(i)$, respectively[2,3] and a proposal was made to introduce an index term "bacteriostatic/bactericidal index", *SCI*, to define a degree of bacteriostatic action relative to that of bactericidal action. According to the theory developed, the *SCI* can be easily given by the slope of a plot of μ_i / μ_m against $t_\alpha(0) / t_\alpha(i)$ that are obtained from the growth thermograms observable with different doses. Wirkner and Takahashi have further improved this theory and defined a new

additional parameter, bactericidality σ to show its dependence on drug concentrations to predict the property of drug actions.[1]

Lethal effect of ionizing radiation is well known and irradiation decontamination with ^{60}Co γ-rays and electron beams has already been practiced for the wide variety of medical supplies and foodstuffs.[15,16] Ionizing radiation causes damages to bioactive molecules including DNA in microbial cells and some of them are repairable during the incubation after irradiation. Therefore growth pattern of each microorganism could change after irradiation depending on the irradiation dosage and repairing capacity. In order to evaluate radiation sensitivity, surviving fractions of irradiated cells are usually measured by colony counting to obtain survival curves as a function of radiation dose.[17,18] However, it cannot provide any information about the bacteriostatic and bactericidal actions of the ionizing radiation on microbial cells during the growth, especially before colony formation.

In this paper, we employed *Saccharomyces cerevisiae* to examine the irradiation effects on the growth after various doses of ^{60}Co γ-irradiation and exploited equations describing bactericidal and bacteriostatic effects comprehensively applicable to predictive microbiology of irradiated materials.

2. Materials and Methods

2.1 Test microorganism and culture media

The polyploid yeast strain *Saccharomyces cerevisiae* KW4 used as a test organism in this study was cultured in glucose peptone broth (GPB) (20 g glucose, 2 g yeast extract, 0.5 g MgSO$_4$, 5 g polypeptone, 1 g KH$_2$PO$_4$ per liter; pH 5.6). GPB supplemented with 20 g agar l^{-1} (GPA) was used for colony counting. All reagents were supplied by Wako Chemical Industries, Tokyo, Japan.

2.2 Irradiation

Saccharomyces cerevisiae KW4 was grown for 24 h to the stationary phase in GPB at 30 . Cells were harvested by centrifugation, washed and diluted to about 10^6 cells ml^{-1} with sterilized water. Two ml of the suspension was distributed into glass tubes with stoppers for irradiation. The irradiation was done at room temperature with γ-rays from a ^{60}Co-source (15 kGy h^{-1}) in the irradiation pool at the Research Institute for Advanced Science and Technology, Osaka Prefecture

University.[19] The dose range for irradiation was from 0 to 8 kGy. The tubes were kept on ice before and after irradiation.

2.3 Growth monitoring and procedure

A multiplex isothermal batch calorimeter containing 24 calorimetric units was used to detect the heat evolution during the growth of yeast at 30 . The design of the apparatus has been described previously.[20] 500 μl of the irradiated samples were added into sterilized glass vials containing 5 ml of GPB and sealed tightly. The vials were then placed in a calorimetric unit and the heat evolution during the growth was observed for 48 to 120 h at 30 . The calorimetric signals thus detected were filed in magnetic discs for further computational analysis.

2.4 Colony counting

The irradiated and unirradiated control samples were diluted serially and plated out on GPA. The plates were incubated at 30 for at least 24 h or 48 h. and the colonies were counted after the colony numbers appeared on the plates became constant.

2.5 Statistical evaluation

A Student's *t*-test was performed to determine statistical significance between the logarithmical reduction in cell number determined by the calorimetric method and by cell counting. *P* values are given.

3. Results and Discussion

Representative growth thermograms of *Saccharomyces cerevisiae* irradiated with various doses from 0 to 6 kGy are shown in **Fig.1(a)**. After a few hours of incubation, calorimetric signals (or shortly $g(t)$ curves in our notation) rose due to the growth of the yeast cells, reached their peaks and finally returned to the baseline as nutrients were exhausted.

Increasing irradiation doses made the peaks shift towards a longer incubation time. The height and the slope of the peaks were also affected drastically after irradiation with increasing doses higher than 2 kGy. These observations are in strong contrast to those shown in **Fig.2** which one would obtain when microbial growth is affected either by ideally **(a)** bactericidal or **(b)** bacteriostatic actions.[6,7] The $g(t)$ curves shown in **Fig.1(a)** are characterized by the changes in pattern that with increasing doses at a lower dose range up to 3 kGy the growth thermograms shift simply toward longer incubation
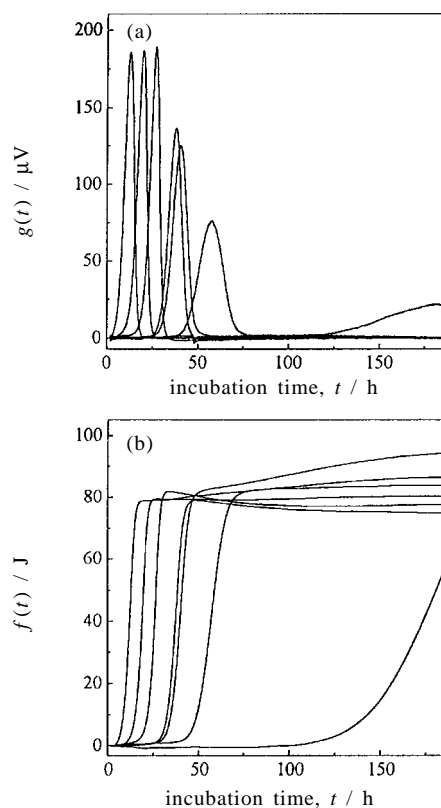
Fig.1 Growth thermograms (a) and actual heat evolution curves (b) of ^{60}Co γ-ray irradiated *Saccharomyces cerevisiae* KW4; irradiation dose of 0, 1, 2, 3, 4, 5 and 6 kGy as plots from the left to the right, respectively.

time, their shapes being unchanged, while at a dose range higher than 4 kGy the thermogram shapes drastically broaden with increasing the dose. These dose-dependent changes indicate an involvement of the combined effect[1] of bacteriostatic and bactericidal actions in γ-ray irradiation.

The $g(t)$ curves can be converted to the actual heat evolution curves ($f(t)$ curves) by eq.(1), as reported previously.[2,3,11,12,20,21]

$$f(t) \quad g(t) \quad K \int g(t)\,\mathrm{d}t \tag{1}$$

The $f(t)$ curves are known to correspond to the changes in cell number or turbidity during the culture and can be described by eq.(2),

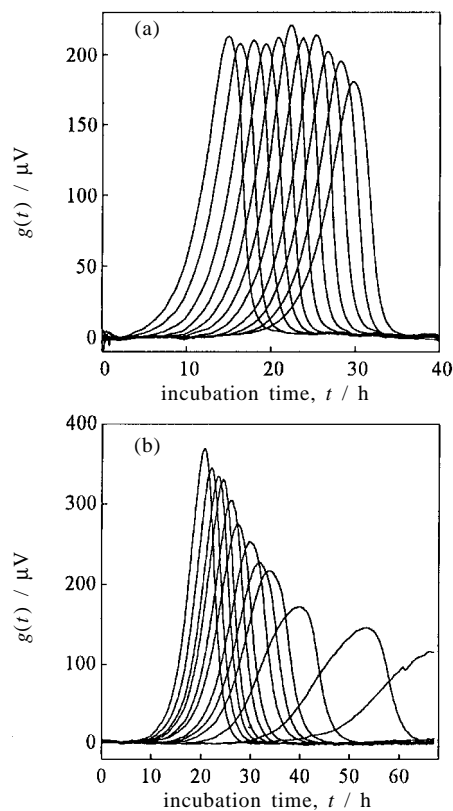$$f(t) \quad A N_{0'}\, e^{\mu t} \quad B N_0 \tag{2}$$

Fig.2 Model growth thermograms one would obtain for the growth experiments with (a) bactericidal and (b) bacteriostatic actions. In (a) calorimetric measurements were conducted on the growing culture of *Saccharomyces cerevisiae* at 30 with the various initial cell population from 1.1×10^3 to 2153×10^3 cell ml ^{1}. Instead of changing the concentration of a drug having purely bactericidal action, the initial cell population (the inoculum size) was varied by quantitatively diluting the cell suspension. In (b) calorimetric mesurement was conducted on the growing culture of *Saccharomyces cerevisiae* at 30 grown in media containing various amounts of ethanol at concentration 0 5.92 %. The ethanol concentrations for the each curves from left to right: 0.00; 0.45; 0.89; 1.33; 1.77; 2.20; 2.63; 3.05; 3.47; 4.30; 5.12 and 5.92 %. Both the figures were reproduced from Ref. 21.

where N_0 is the initial cell number and A as well as B are constants.[2,21,22] The $f(t)$ curves obtained by using eq.(1) are shown in **Fig.1(b)**. From the values of $f(t)$

Table 1 Growth parameters of γ-ray irradiated *S. cerevisiae* with different doses from 0 to 6 kGly. The numerical values were obtained from the growth thermograms observed according to the method described in the text.

i / kGy	n	μ_i / μ_m	$t_\alpha(i) / t_\alpha(0)$	σ
0		1.000	1.000	
0.5	3	0.943 ± 0.046	0.675 ± 0.014	0.825
1.0	12	0.886 ± 0.058	0.519 ± 0.049	0.763
1.5	3	0.867 ± 0.020	0.439 ± 0.036	0.763
2.0	12	0.877 ± 0.053	0.373 ± 0.049	0.804
2.5	3	0.864 ± 0.062	0.352 ± 0.003	0.790
3.0	12	0.764 ± 0.053	0.256 ± 0.055	0.683
4.0	12	0.640 ± 0.115	0.206 ± 0.016	0.547
5.0	6	0.454 ± 0.075	0.148 ± 0.020	0.359
6.0	5	0.196 ± 0.052	0.073 ± 0.030	0.133

Fig.4 SCI plot[6] for the action of ^{60}Co γ-ray irradiation on *Saccharomyces cerevisiae* KW4. The dotted lines (a) and (b) correspond to those for ideally bactericidal and bacteriostatic actions, respectively.
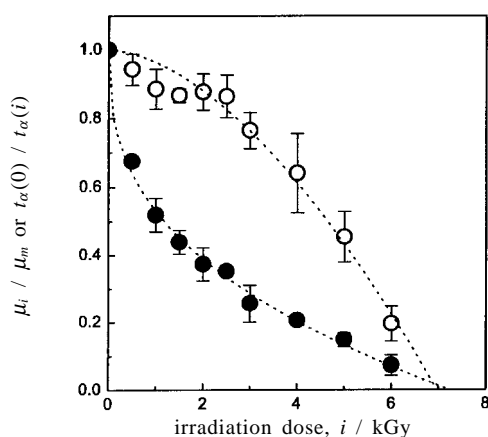

Fig.3 Decrease of specific growth rate constant (open marks) and specific growth retardation (closed marks) with the irradiation dose; fittings from eqs. (4) and (5) are shown in dotted lines. The minimum inhibition doses, MID_μ and MID_θ, as evaluated from the changes in specific growth activity and in specific growth retardation, respectively, were determined by using eqs. (6) and (7) and are listed in Table 2 together with the four parameters. The circles are the data points averaged over 68 data sets. The error bars are in standard deviation.

thus determined, the growth rate constant μ_i and the growth retardation $t_\alpha(i)$ can be calculated on the basis
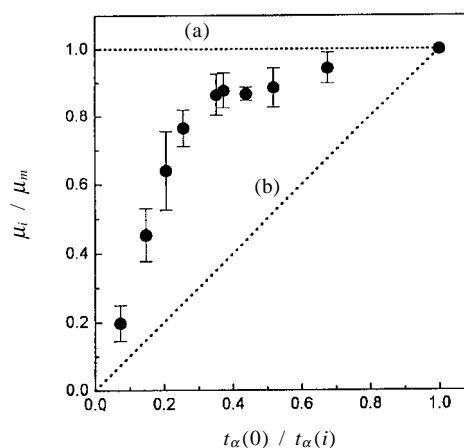
of eq.(2) by the previously reported method.[2,3,20,21] Furthermore the specific growth activity and the specific growth retardation, defined as μ_i / μ_m and $t_\alpha(0)/t_\alpha(i)$, respectively, can also be determined by the method described earlier,[2,3,20,21] where μ_m is the growth rate constant at zero dose (i 0 kGy) and $t_\alpha(0)$ is the incubation time at which the growth activity becomes a certain level $f'(t)$ α at i 0 kGy.

The values of μ_i / μ_m and $t_\alpha(0)/t_\alpha(i)$ thus determined are listed in **Table 1** and are plotted as a function of irradiation dose in **Fig.3**. According to the theory developed previously,[6] in the case of a totally bactericidal action growth rate constant remains constant over the dose range used and the inhibitory effect is only observed as the retardation in growth, *i.e.* in calorimetric terms a parallel shift of the growth thermograms towards a longer incubation time. On the other hand a purely bacteriostatic action does not affect the initial cell number and the changes in specific growth activity μ_i / μ_m should be equal to those in $t_\alpha(0)/t_\alpha(i)$ over the range of doses.[6] The plots in **Fig.3** already indicate that the effect of ^{60}Co γ-ray on *Sacch. cerevisiae* is not a purely bactericidal action but involves bacteriostatic action to a certain extent. In fact, as shown in **Fig.4**, the SCI plot[6] made for these data sets does not show a straight line with a definite slope, but has an upward curvature, indicating that the action pattern is a strong function of dose

Table 2 Parameters determined for the effect of ^{60}Co γ-ray irradiation on *S. cerevisiae* at 30 .

m_1	k_1	MID_μ	m_2	k_2	MID_θ
1.70 ± 0.15	0.037 ± 0.009	7.00	0.38 ± 0.02	0.469 ± 0.010	7.25

intensity. It should be noted that while the specific growth rate constant gradually decreases with the increase in dose intensity at the lower doses, it decreases sharply after the treatment with doses higher than 3 kGy.

In order to characterize these observations more quantitatively, the bactericidality σ ($0 \quad \sigma \quad 1$) which has been introduced in our previous paper[1] was employed. The bactericidality is defined as the bactericidal contribution relative to the bacteriostatic contribution and is expressed by eq.(3);

$$\sigma \quad \{\mu_i/\mu_m \quad t_\alpha(0)/t_\alpha(i)\} \ / \ \{1 \quad t_\alpha(0)/t_\alpha(i)\} \quad (3)$$

From eq.(3) it will be obvious that $\sigma \quad 0$, if the effect is ideally bacteriostatic and $\mu_i/\mu_m \quad t_\alpha(0)/t_\alpha(i)$. In contrast, $\sigma \quad 1$ when antimicrobial action is purely bactericidal with μ_i/μ_m being 1 over the entire range of doses.

If one assumes that the decrease in specific growth activities is proportional to the *m*-th power of dose (or "concentration" in the action of chemicals), then we have the equations

$$\mu_i/\mu_m \quad 1 \quad k_1 \, i^{m_1} \quad (4)$$
$$t_\alpha(0)/t_\alpha(i) \quad 1 \quad k_2 \, i^{m_2} \quad (5)$$

where m_1, k_1, m_2 and k_2 are constants. Dose response curve can be mathematically drawn by regression analysis on the experimentally obtained data sets of μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$.[2,6] The two dotted lines given in **Fig.3** are the fitted dose response curves drawn on the basis of eqs.(4) and (5), respectively, using the 4 parameters determined by regression analysis.

If we employ a definition of the minimum inhibition dose *MID* to be the irradiation dose at which the microbial activity is completely lost, it will be obvious from **Fig.3** that the intercept of the plots on the *x*-axes corresponds to the value of *MID*. Thus the MID_μ and MID_θ values are mathematically determined from the specific growth activity and the specific growth retardation by the following equations, respectively.

$$MID_\mu \quad (1/k_1)^{1/m_1} \quad (6)$$

$$MID_\theta \quad (1/k_2)^{1/m_2} \quad (7)$$

The results are listed in **Table 2** together with the 4 parameters determined by regression analysis. Using the parameters, m_1, K_1, m_2 and K_2, eq.(3) can be rewritten as

$$\sigma \quad 1 \quad (k_1/k_2) \, i^{(m_1 \quad m_2)} \quad (8)*$$

Calculation of σ was made on the basis of eq.(3) and the result obtained is illustrated in **Fig.5** as a function of irradiation dose *i*. It is clearly and quantitatively reconfirmed that the irradiation acts on *S. cerevisiae* both bactericidally and bacteriostatically and the bactericidality σ decreases almost linearly with the irradiation dose higher than 3 kGy.

Survival ratio of *S. cerevisiae* can also be calculated from the growth thermogram shown in **Fig.1**. On the basis of the first derivative of eq.(2), $t_\alpha(0)$ and $t_\alpha(i)$ can be expressed respectively by eqs.(9) and (10).[2,3,11,12,16,17]

$$\text{for } i \quad 0: \quad \alpha \quad A' \quad N_0 \quad \exp\{\mu_m \quad t_\alpha(0)\} \quad (9)$$
$$\text{for } i \quad 0: \quad \alpha \quad A' \quad N_i \quad \exp\{\mu_i \quad t_\alpha(i)\} \quad (10)$$

where N_0 is the number of cells at the start of incubation without irradiation, N_i is the number of survivors after treatment with the dose of *i* kGy and A' is the constant.

From eqs.(9) and (10) surviving fraction after *i*

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*In the preceding paper,[1] eq.(I) was used to express the bactericidality.

$$\sigma \quad 1 \quad (k_1/k_2) \, (1/k_2)^{(m_1/m_2 \quad 1)} \, i_{\text{red}}^{(m_1 \quad m_2)} \quad (I)$$

where i_{red} is a reduced "concentration" in the action of chemicals and is given by

$$i_{\text{red}} \quad i/MIC_\theta \quad (II)$$

The reason why the above reduced concentration was employed in the previous study[1] is that its use is more convenient for practical comparisons of the above defined bactericidality parameters σ under different conditions rather than the use of absolute magnitude of concentration *i*.
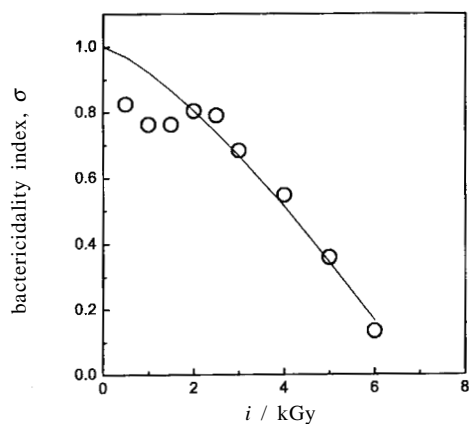
Fig.5 Effect of ^{60}Co γ-rays on *Saccharomyces cerevisiae* KW4 in terms of bactericidality. The open circles are the experimental data points obtained by using each data sets of μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ on the basis of eq.(3). The solid line was obtained by using the parameters shown in Table 2 according to eq.(8).

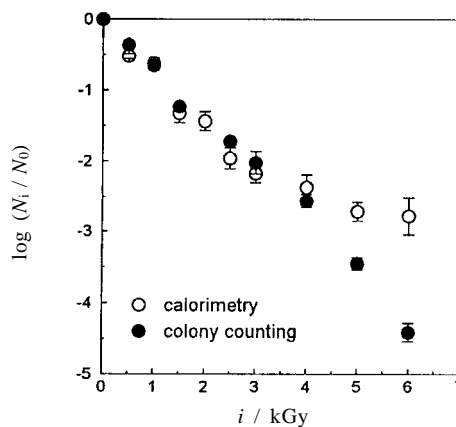**Table 3** Survival ratio of γ-ray irradiated *S. cerevisiae* cells as determined calorimetrically using eq.(12) and by the colony counting method.

i / kGy	log N_i / N_0	
	calorimetry	colony counting
0	0.00	0.00
0.5	0.525 ± 0.030	0.360 ± 0.005
1.0	0.623 ± 0.078	0.654 ± 0.042
1.5	1.324 ± 0.135	1.231 ± 0.047
2.0	1.437 ± 0.132	
2.5	1.960 ± 0.147	1.723 ± 0.029
3.0	2.173 ± 0.131	2.022 ± 0.158
4.0	2.373 ± 0.178	2.564 ± 0.093
5.0	2.715 ± 0.136	3.449 ± 0.083
6.0	2.778 ± 0.258	4.409 ± 0.129

kGy irradiation can be expressed as follows;

$$N_i/N_0 \; \exp\{\mu_m \; t_\alpha(0) \; \mu_i \; t_\alpha(i)\} \qquad (11)$$
$$\log N_i/N_0 \; (1/2.303)\{\mu_m \; t_\alpha(0) \; \mu_i \; t_\alpha(i)\} \quad (12)$$

The logarithmic numbers of surviving fractions determined by eq.(12) together with those determined by colony counting are summarized in **Table 3** and are plotted against irradiation doses in **Fig.6**. Although the

Fig.6 Logarithmical number of survivors determined by colony counting (closed circles) and the calorimetric method (open circles) using eq.(12); for each irradiation dose the mean of various experiments with the standard error are plotted.

significance of the observed data plotted in the figure varied as error bars shown (standard deviation), the both survival curves are in reasonable agreement with each other at least up to 4 kGy. However, at 5 kGy and 6 kGy irradiations a significant difference was found to exist in the number of survivors determined by the two methods (P 0.001). As shown in **Fig.1**, the growth thermograms of 5 kGy and 6 kGy irradiated cells indicate the most bacteriostatic feature. It seems probable to think that under a condition where the action involves both the bactericidal and bacteriostatic effects, the cell number decreases and at the same time the growth rate of survived cells is also expected to decrease. Therefore, numbers of the survivor determined by colony counting may be significantly smaller than those determined calorimetrically using eq.(12) as appeared in **Fig.6**, probably because the some portion of the cells grew too slowly to form visible colonies for overnight incubation after 5 kGy irradiation. Indeed we observed that the number of macrocolonies on agar become constant after 2 days of incubation at a lower-dose irradiation (i 2 kGy), but, after treatment with higher doses, the final number of colony forming units slightly increased for up to five days (data not shown).

Ionizing radiation induces a variety of DNA legions. It is now widely accepted that the occurrence of double-strand breaks is the factor that determines whether yeast

cells and other eukaryotic cells will die after treatment with ionizing radiation. In *S. cerevisiae*, over 30 genes involved in nucleotide excision repair including base excision repairs and recombination repairs have already been characterized. Some of them including Rad9 function as a checkpoint gene to arrest cell cycle transiently during DNA repair.[23,24] They are transcriptionally induced in response to DNA damage. Relatively small numbers of DNA damage caused by lower-dose irradiations can be completely repaired and the survived cell can grow normally after cell cycle is restored. However, at higher doses, it is more difficult to repair all the damage within the limited time of the cell cycle arrest. Our evaluation of decreasing bactericidality with increasing doses of ^{60}Co γ-rays more than 2 kGy clearly suggested that the existence of incompletely recovered cells carrying unrepaired damage. In such cells unrepaired damage may contribute to the retardation of growth resulting from the decrease in growth rate.

With the aid of the calorimetry, the different feature of radiation effect, such as bactericidal and bacteriostatic effects can be analyzed quantitatively. Using haploid, diploid and polyploid species having different radiation sensitivity together with repair deficient mutants of *S. cerevisae* we expect that the mechanism of irradiation sensitivity and repair pathways will be clearer enough to be the basis for understanding the other important microorganisms in food irradiation and sterilization of medical devices.

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30

0   3 kGy

4 kGy

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