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Application of Microthermometry to Measurement of Microbial Activity and Inactivation Process by Inhibitor

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Abstract

A rapid and simple technique (microthermometry) was developed for the measurement of microbe's metabolic activities and its inactivation process by an inhibitor. To analyze the results and to determine the parameters for the estimation of the activity and inactivation degree, a simple model was proposed. Yeast cells (*Saccharomyces cerevisiae*) were used as a microbe. A differential method using two bead-type thermistors as reference and measuring probes was employed for the detection of a temperature change caused by the heat of metabolism of added carbon source (glucose) by the yeast. Experiments were conducted in a 30 °C water bath under non-growth conditions (without nitrogen source). The simple thermal response model was applied to obtain two characteristic parameters for the estimation of the yeast activities, i.e., a metabolic heat production rate, ΔQ , and a metabolic heat inhibition rate, ΔK . The proposed model was well in agreement with the experimental results, and the curve fitting gave ΔQ or ΔK . In the case of the addition of glucose to yeast as a carbon source, ΔQ was proportional to the number of live cell (CFU). The slope of CFU versus ΔQ for yeast in exponential phase was larger by about two folds than that in the stationary phase. In the case of the addition of gultaraldehyde as an inhibitor to the above system, the logarithmic value of ΔK increased with increasing the concentration of gultaraldehyde within the range of 0.02 to 6.2 wt%. It was suggested that the two model parameters, ΔQ and ΔK , can be regarded as characteristics to estimate the activities of bacteria and the degree of their inactivation by the inhibitor.

Keywords: Yeast; Glucose; Microbial activity; Metabolic heat; Thermistor;

Bioprocess Monitoring

1. Introduction

Recently an assessment of hazardous substances in aqueous environments, which is known as bio-monitoring and micro-biotesting, was studied [1]. In such techniques, a rapid estimation method of microbe's metabolic activity and inactivation process by an inhibitor is required. However, most of the techniques were somewhat time-consuming or tedious and were required expensive apparatus or reagents, because they are based on enzymatic or bioassay methods.

Cooney et al. [2] attempted to apply calorimetric or thermometric method to biochemical and bioengineering fields. They reported a correlation between heat evolution and oxygen consumption of microbes in a fermentor using a thermistor. In recent two decades, many successful attempts using thermistor devices were reported; e.g., enzyme thermistor [3-7], microbe thermistor [8], enzymatic kinetics [9-11] and so on. Furthermore, many studies from the viewpoint of thermometric aspects about biological and physical properties of microbes have appeared and they often used micro calorimeter [12-19].

Because the thermometric methods using thermistor devices are based on a very general detection principle, it is reasonable to consider that the methods make it possible to develop a compact apparatus and on-line measurement of activity of microbes. However, applications of activity and inhibition estimation by micro-thermometry to microbes have not appeared in literature and have not been developed yet.

In this study, the small change in temperature due to the emission of metabolic heat by microbes will be measured by a differential detection method to characterize the microbial activity and the inactivation process of microbes by

an inhibitor. The measurement will be carried out by a system consisting of two thermistors to eliminate the effect due to any other undesirable sources of heat. The obtained thermal data will be analyzed by a simple kinetic model derived from heat balance and will be determined two parameters to estimate microbial activities and the degree of their inactivation by an inhibitor.

2. Description of unsteady heat balance model

The heat balance of the present micro-thermometry system in unsteady state gives the following equation:

$$w \cdot c_p \cdot \frac{d}{dt}(T_m - T_r) = (Q_m - Q_r) - U \cdot A \cdot (T_m - T_r), \quad [1]$$

where, T and Q represent the temperature and the production rate of heat by yeast cells, respectively, and the subscripts m and r denote the measuring and the referring tubes, respectively. w and c_p represent the weight and the specific heat of yeast suspension, respectively. A and U denote the heat transfer area and the overall heat transfer coefficient between the water bath and the yeast suspension, respectively. Substitution of ΔT for $T_m - T_r$ and ΔQ for $Q_m - Q_r$ respectively and rearrangement of Eq. [1] lead to the following equation:

$$\frac{w \cdot c_p}{U \cdot A} \cdot \frac{d}{dt} \Delta T - \Delta T = \frac{\Delta Q}{U \cdot A}. \quad [2]$$

Solving the above differential equation with the initial condition that $\Delta T = 0$ at $t = 0$, the net change in temperature due to the metabolism of yeast cell, ΔT , can be finally obtained as:

$$\Delta T = \Delta T_s \cdot \left(1 - e^{-\frac{t}{\tau}} \right), \quad [3]$$

and

$$\Delta T_s \equiv \frac{\tau}{w \cdot c_p} \Delta Q. \quad [4]$$

where, the time constant, τ , in Eqs. [3] and [4] is defined as:

$$\tau \equiv \frac{w \cdot c_p}{A \cdot U}. \quad [5]$$

As is well known, Eq. [3] is a response of temperature in thermometric system for a stepwise change in the metabolic heat production rate, ΔQ , of yeast cells by injection of glucose.

On the other hand, an estimating equation of cell inactivation rate, ΔK , is derived from the following equation as the same manner in case of ΔQ ,

$$-w \cdot c_p \cdot \frac{d}{dt} \Delta T = \Delta K + U \cdot A \cdot \Delta T, \quad [6]$$

ΔT can also be given as the following equation by solving Eq. [6] with the initial condition of $\Delta T = 0$ at $t = 0$,

$$\Delta T = -\frac{\tau}{w \cdot c_p} \cdot \Delta K \cdot \left(1 - e^{-\frac{t}{\tau}} \right), \quad [7]$$

Eq. [7] is also the response of temperature in thermometric system for a stepwise

change in ΔK of yeast cells by injection of an inhibitor.

Eqs. [3] and [7] will be verified with the experiments and two characteristic parameters, ΔQ and ΔK , will be determined by fitting to the experimental results.

3. Experiments

3.1. Microbial strain

Saccharomyces cerevisiae (IFO No. 2043), yeast cells, was used as a model microbe. The cells were inoculated with nutrient-rich broth consisting of 20 g glucose, 2.0 g KH_2PO_4 , 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g yeast extract and 5.0 g $(\text{NH}_4)_2\text{SO}_4$ per liter at 30 °C for 24 h and were stored in the broth containing 15 % glycerin at –40 °C. All chemicals were analytical grade and used without further purification.

3.2. Preparation of yeast suspension

The yeast cells were grown in 400 mL nutrient-rich broth for 10 h. (exponential phase) or 20 h. (stationary phase) at 30 °C. After that, they were harvested by centrifugation and washed 2 times with distilled water to prepare desirable concentrations of yeast suspensions. In all runs, 2 mL of the suspension was used as a sample to measure the change in temperature due to metabolic heat. The number of live cells was determined by colony counting on agar plate.

3.3. Experimental setup

Measurement of the change in temperature due to metabolic heat was conducted in a 30 °C water bath. Two test tubes, a measuring and a reference

tube, were placed in the water bath. A thermistor used in this study was a bead type thermistor (ET-104, 100 k Ω at 25 °C, B constant 4,132 K, Ishizuka Electronics Corporation). A differential method using two thermistors was employed in this study to reduce any non-specific signals. Two thermistors were incorporated into individual legs of Wheatstone bridge circuit. Voltage signal from the circuit was monitored by a voltmeter (VOAC 7411, Iwatsu Electronics Co. Ltd.) and was recorded by a personal computer (NEC PC-9801 VM) via an A/D converter (CONTEC AD 12–16F(98)H). The recorded signals was compensated by Savitzky and Golay's method [20].

3.4. Procedure of microthermometry experiments

A thermistor and a syringe for injection glucose or glutaraldehyde (GA) solution were installed to each test tube. Raw and heat-treated yeast suspensions of 2 mL were poured into the measuring and the reference tubes, respectively. The heat treatment of yeast suspension was conducted in a 60 °C water bath for 60 min. The death of all cells was confirmed by agar plate spreading and colony counting method. The suspensions were stirred with magnetic stirrers gently. Prior to the injection of glucose, voltage signal was monitored and was recorded for about 4 min to confirm that the base line was kept stable. Then, 1 mL of 12 g/L glucose solutions were injected to the measuring and the reference tubes at the same time. After the glucose injection, GA solutions were also injected to the both tubes if necessary. In preliminary experiments, the concentration of glucose varied from 12 g/L to 50 g/L, and no difference was observed in the results. The change in temperature was calculated from the voltage-temperature calibration

line determined in preliminary experiments.

3.5. Measurement of glucose consumption by yeast cells

10 mL yeast suspension was stirred gently in a 30 °C water bath and 5 mL of 12 g/L glucose solution was added to the suspension. After a desired time, the suspension was centrifuged (4,000 rpm for 10 min) and the glucose concentration of the supernatant was determined by an enzymatic method using C-II test wako (Wako Pure Chemical Industries, Ltd., Japan). The amount of glucose consumption for the time interval was obtained from the difference of glucose concentrations between the initial and the final states.

4. Results and discussions

4.1. Estimation of metabolic activity of yeast cells

A typical time course of ΔT is shown in Figure 1. ΔT is expressed as the relative value to the average of base line temperature (dotted line in Figure 1). In this experiment, 1 mL of 12 g/L glucose solution was injected at 4 min. after initiating the recording. A sharp increase in ΔT was produced by injecting the glucose solution and the curve reached a plateau level in a few minutes. This plateau level is considered to be resulted from steady state of heat balance between the inside of measuring tube and the outside the measuring tube (water bath). To confirm that ΔT is caused only by microbes' metabolic heat, preliminary experiments were carried out using inactivated cells by heat-treatment, and there was no ΔT in the case of inactivated cells. A smooth curve (solid line) in Figure 1 was calculated from Eq. [3]. The value of ΔT_s was determined by a least-squares method. As seen in the figure, the model presented in this paper can well explain the change in ΔT . The value of τ , U and A for the present system was 0.32 min, 2.97 J/(°C cm² min) and 13.2 cm², respectively, in almost every experiments. From the relationships of Eqs. [4] and [5], the metabolic heat production rate, ΔQ , can be estimated. Figure 2 shows the change in ΔQ as a function of live cell number, N_L , for the exponential and the stationary phases. The linear regression gave two straight lines (solid lines in Figure 2) corresponding to the two kinds of microbe activity phases. These results demonstrate that the parameter ΔQ reflects the microbe activity in terms of the metabolic heat due to glucose consumption. The values of these slopes

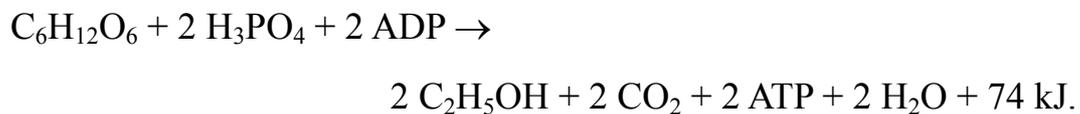
correspond to a heat production rate per single cell (Q in Table 1 described in latter section), and were 1.81×10^{-11} and 7.75×10^{-12} J/(cell s) for the exponential and the stationary phases, respectively. This result suggests that the live cell in the exponential phase seems to have ca. 2.2 times higher metabolic activity than the cells in the stationary phase.

4.2. Consumption rate of glucose by yeast cells

To confirm the relation of the metabolic heat production rate, ΔQ , estimated from Eq. [3] and the glucose consumption by yeast cells in the present system, the change in glucose concentration with the live cell number was measured for both the exponential and the stationary phases. The results are presented in Figure. 3. In preliminary experiments, it was observed that glucose was consumed linearly with the measurement time up to 25 min for both the exponential and the stationary phases, respectively (data are not presented). The data shown in Figure 3 are those obtained from the experiments on the glucose consumption during 5 min. The consumption is proportional to the live cell numbers for either phase and the relationship looks exactly like the results of Figure 2. The solid lines in Figure 3 were determined by a least squares method. From the slope value of each line in Figure 3, the glucose consumption rate per unit yeast cell (r_{Glu} in Table 1) can be estimated as 1.11×10^{-12} and 5.79×10^{-13} mole-glucose/(cell s) for the exponential and the stationary phases, respectively. The parameters experimentally estimated are summarized in Table 1. The heat released when one of the yeast cells catabolizes one mole of glucose (q in Table 1) can be calculated as 1.76 and 1.44 kJ/mole–glucose for the exponential and

the stationary phases, respectively. Although the values of ΔQ for the exponential and the stationary phases shows that the yeast cells in the exponential phase are somewhat active compared with those in stationary phase, these values of q are not so different between the two phases (Table 1).

As is well-known, when an yeast is added to glucose, the glycolysis reaction (Embden-Meyerhof pathway) occurs in the intracellular body of yeast as follows:



This reaction indicates that 74 kJ is produced when one mole of glucose is consumed. The values of q for the both phases were much smaller than 74 kJ, although all of the energy (74 kJ) might not be discharged as metabolic heat. Since the present system was not adiabatic and had large heat capacity because of water bath, the q values should be estimated smaller.

4.3. Estimation for inactivation of microbe

The authors also attempted to apply this technique to estimate the inactivation of yeast cells by an inhibitor. Glutaraldehyde (GA) was used for the inhibitor, which is a well-known chemical reagent as a disinfectant and is used in fields of medical scene such as a hospital or animal husbandry. Typical results of the inactivation are presented in Figure 4, where at first, glucose solution (12 g/L) was injected (arrow 1 in Fig. 4) to yeast suspension (30 g-dry cell /L). After

an immediate temperature rise and the achievement of a plateau level of the temperature (steady state) within ca. 5 min, GA solution was injected (arrow 2 in Figure 4). As seen from this figure, an abrupt decrease in temperature is found immediately after the injection of GA. This temperature decrease is considered to come from the inactivation of the yeast cells by the GA solution. At $t = 25$ min (15 min after GA injection), another plateau region of ΔT was observed, however the value did not come to zero. In general, all yeast cells do not die by the addition of GA. In the present case, the degree of inhibition corresponds to decrease of ΔT from ca. 0.2 degree to 0.05 degree. This decrease in ΔT may be produced by whether ca. 75 % of the initial live cell is dead or the activity of each cell is decreased to ca. 25 % of the original activity.

The decreasing region of ΔT (time-course after injection of GA solution, i.e., arrow 2 in Figure 4) can be explained by Eq. [7]. ΔK was determined by fitting the data to Eq. [7] with a least-squares method in this region. Solid curve in Figure 4 in this region was drawn by the calculation using these parameters. The experiments were repeated by varying the concentration of the GA solutions. The relationship between the obtained ΔK and GA concentration is shown in Figure 5. Within the present experimental conditions, the logarithmic value of glutaraldehyde (GA) concentration was linearly proportional to the estimated ΔK value in the concentration region of from 0.02 to 0.64 wt%, although the concentration range of GA solution was somewhat high in this study. In the range of from 0.64 to 6.2 wt%, the degree of increase in ΔK decreased slightly. ΔK of the model proposed in the present study may be regarded as an characteristic parameter of the inactivation of yeast cells.

This technique has some advantages: rapid and ease of the procedure and

extension to on-line estimation with ease. This technique will be applied to the evaluation for the influence of reagents and substrates on microbes and estimation of live cell numbers. For example, level of paralytic shellfish poisoning (PSP) toxin may be simply estimated by the proposed model and by using the present system instead of using mouse. However, higher concentrations of the yeast cells were required relatively in the present system. Development of the experimental system and sensitivity for the lower concentration will be required in further studies.

5. Conclusions

The rapid and simple method was proposed of estimating microbe activity and inhibition by a micro-thermometry using yeast cells as a model microorganism. To analyze the micro-thermometric results and to determine the parameters for the estimation of microbe activities, a simple model was presented. The present model could successfully explain the time course of increase or decrease in ΔT and give some parameters for the estimation by a least-squares method. Of these, two parameters, the heat production rate, ΔQ , and the inactivation rate, ΔK , could be regarded as the characteristic parameters for the activity of microbe and the inactivation degree of microbe, and could be deduced by the model. The value of ΔQ was proportional to live cell number, N_L , for both exponential and stationary phases, and the values of the two slope were different from each other for the both phases. From these results and the experiments for glucose consumption by yeast, the values of heat production rate per a single cell (Q in Table 1) were determined as 1.81×10^{-11} and 7.75×10^{-12} J/(cell s) for the exponential and the stationary phases, respectively. In the inhibition experiments, ΔK was linearly proportional to the logarithmic value of glutaraldehyde (GA) concentration in the concentration region of from 0.02 to 0.64 wt%. In the range of from 0.64 to 6.2 wt%, the degree of increase in ΔK decreased slightly.

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Nomenclature

A	= interfacial area	[cm ²]
c_p	= specific heat of suspension	[J/(g °C)]
ΔK	= the metabolic inhibition rate estimated by Eq. [7]	[J/s]
ΔQ	= the metabolic heat production rate estimated by Eq. [3]	[J/s]
t	= time	[s]
ΔT	= differential of temperature between T_m and T_r	[°C]
T_m	= temperature of the yeast suspension in the measuring tube	[°C]
T_r	= temperature of the yeast suspension in the reference tube	[°C]
U	= overall heat transfer coefficient	[J/(°C cm ² s)]
w	= weight of yeast suspension	[g]
τ	= time constant	[s]

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Captions of Figures and Tables

Figure 1. Typical diagram of time course of the temperature change detected by the present non-adiabatic thermometric method. The experiment was conducted with ca. 8 g-dry weight/L of the yeast suspension for the exponential phase. The dotted lines represent the base line and the plateau level. The solid curve is estimated by fitting the experimental result to Eq. [3].

Figure 2. Relationship between the metabolic heat production rate, ΔQ , estimated by Eq. [3] and the live cell number of the yeast cell (CFU) for both the exponential (solid circle) and the stationary phases (open circle). The solid lines are determined by a least square method.

Figure 3. Variation in the glucose consumption for 5 minutes with the live cell number of the yeast cell for both the exponential (solid circle) and the stationary phases (open circle). The solid lines are determined by a least square method.

Figure 4. Typical diagram of the inhibition experiment as an application of the proposed microthermometry using the yeast cells and glutaraldehyde solution. The experiments conducted with 30 g-dry weight/L of the yeast suspension and 0.25 wt% of gultaraldehyde solution.

Figure 5. Change in values of ΔK estimated by Eq. [5] with GA concentration.

Table 1. Experimental parameters estimated by the proposed microthermometry

using *Saccharomyces cerevisiae* (IFO 2043).

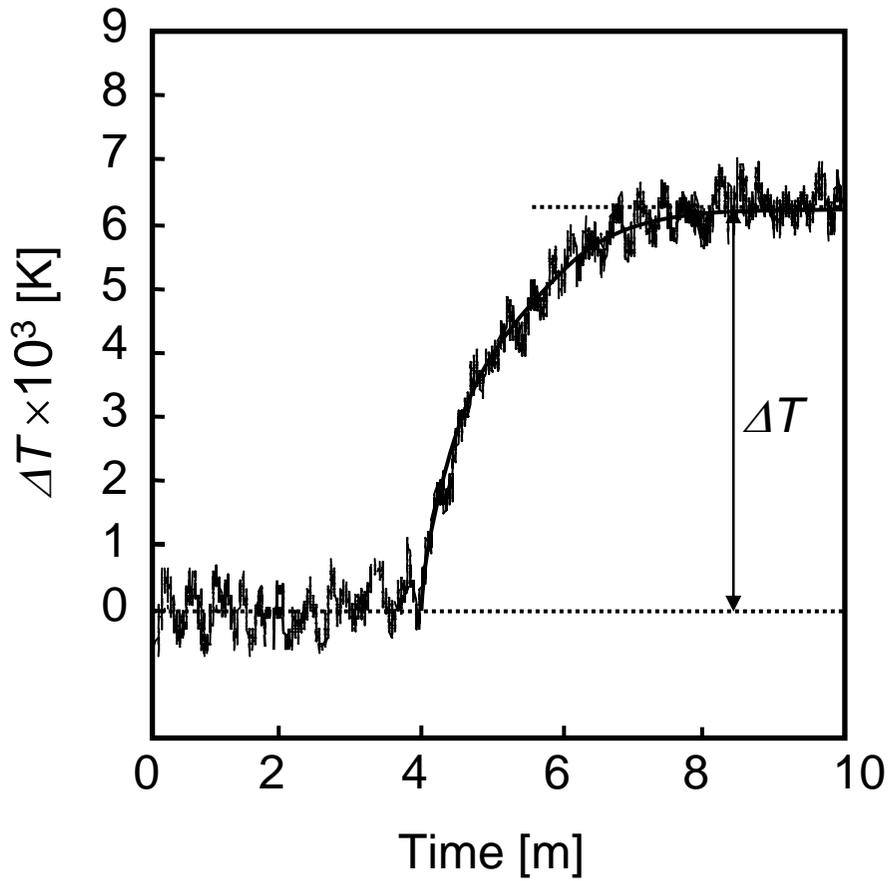


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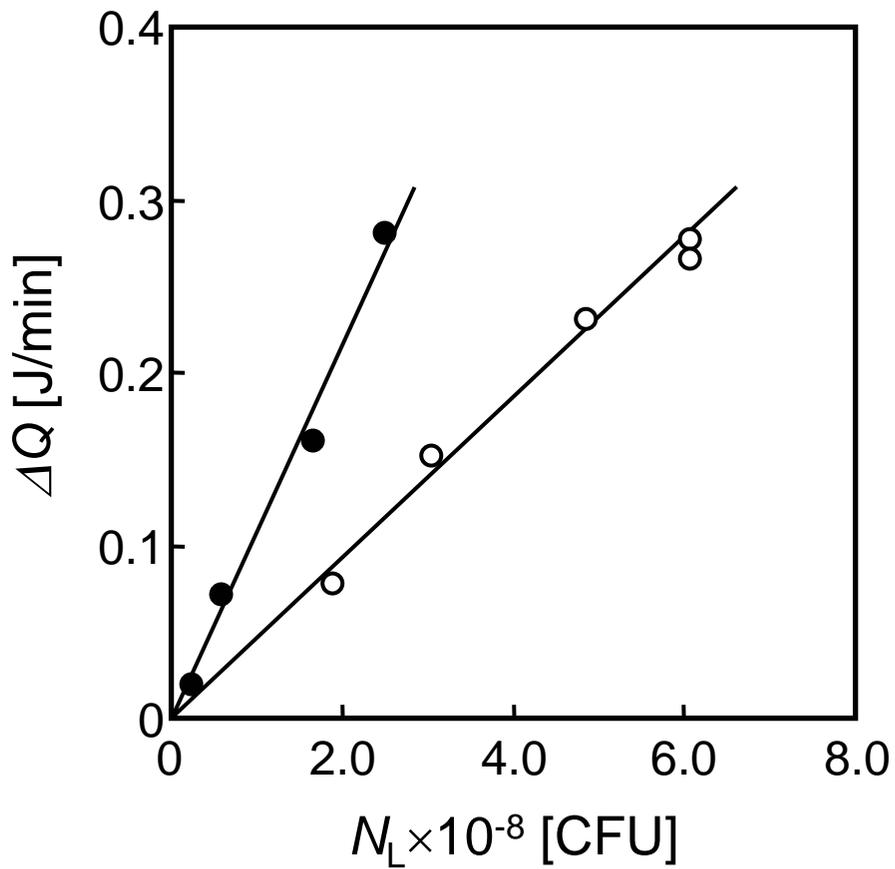


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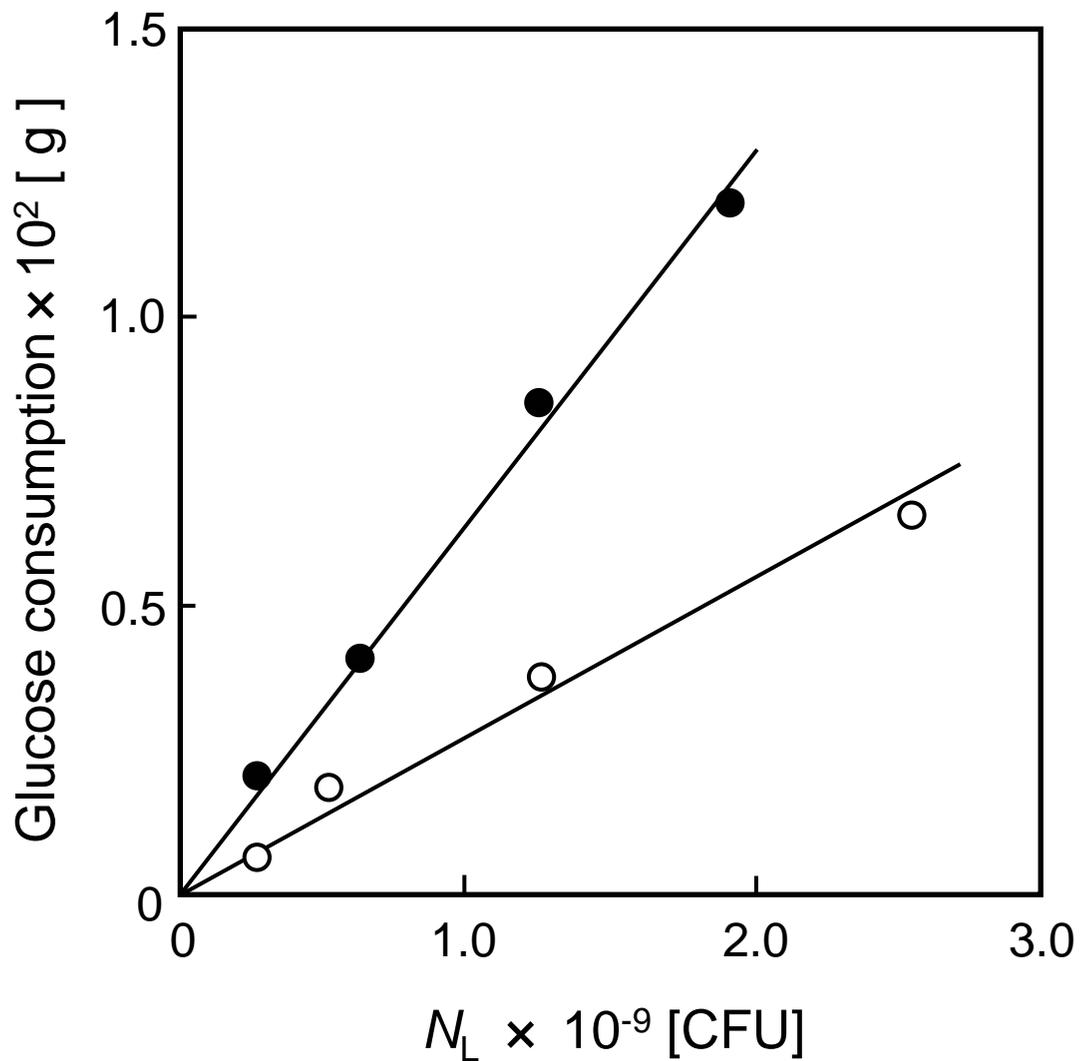


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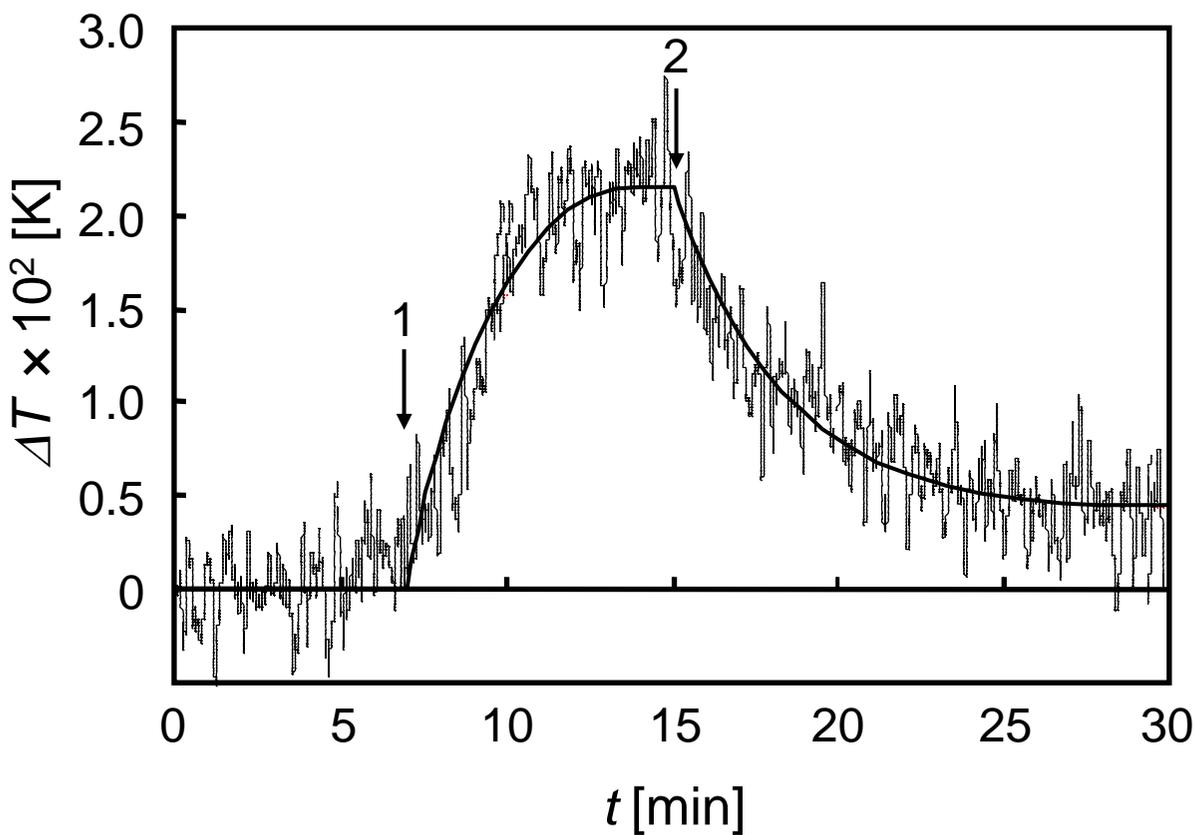


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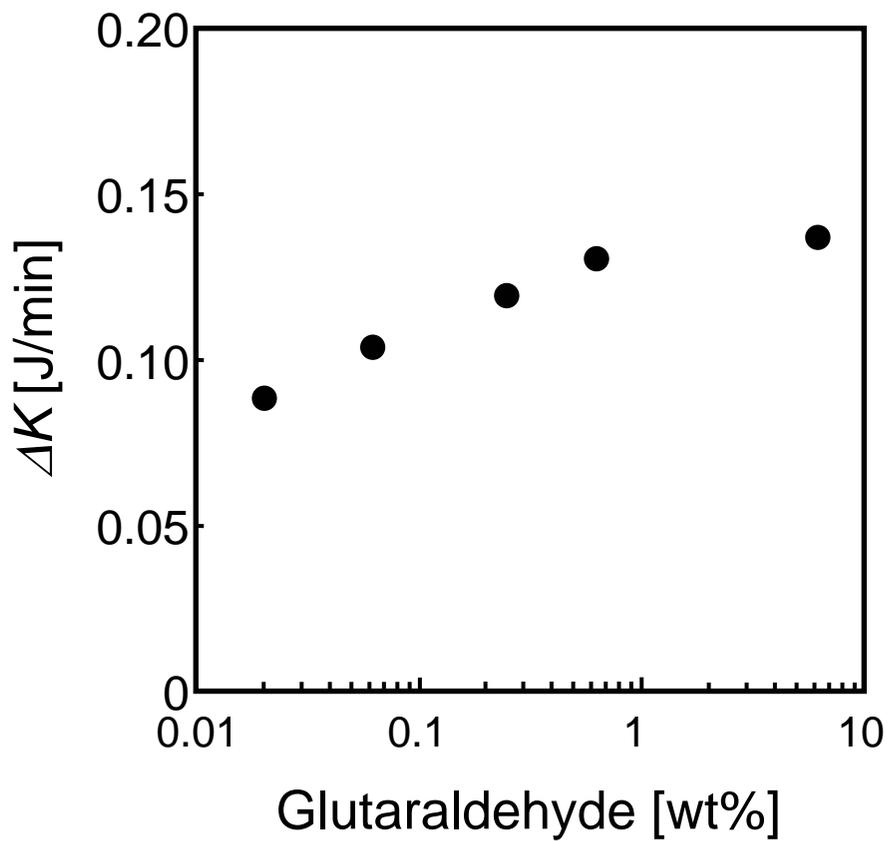


Figure 5. Change in values of ΔK estimated by Eq. [7] with GA concentration.

Table 1. Experimental parameters estimated by the proposed microthermometry using *Saccharomyces cerevisiae* (IFO 2043).

	exponential	stationary
Q [J/(cell s)]	1.81×10^{-11}	7.75×10^{-12}
r_{Glu} [mole–Glu/(cell s)]	1.11×10^{-12}	5.79×10^{-13}
q [J/mole–Glu]	1.76×10^3	1.44×10^3