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論 文

Heat Effects for a Single Cell of *Saccharomyces cerevisiae* Determined using a Classic and a New Procedure

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The growth thermograms observed with a multiplex isothermal calorimeter during growth at 30℃ were employed for the determination of various parameters characterizing the growth and heat production of a *Saccharomyces cerevisiae* strain. The heat evolution curve, cell number curve and ethanol production curve determined for the yeast culture were found to be correlated, and the values of the growth rate constant μ determined from these curves were, respectively, 0.37, 0.34 and 0.32 h^{-1}. From the correlation existing between the heat evolution curve and the cell number curve, an average heat evolution $Q = (1.35 \pm 0.02) \times 10^{-7}$ J cell^{-1} and a corresponding $q_1 = 20 \pm 3$ pW cell^{-1} for the average heat evolution rate for a single cell were determined. Similar considerations allowed the determination of the average heat amount (Q_E = 147.6 kJ mol^{-1}) and the average number of yeast cells ($N_E = 1.09 \times 10^{12}$ cells mol^{-1}) associated with the production of one mole of ethanol. On the second hand, a new method was proposed for the determination of the average heat evolution rate per cell, which requires only the knowledge of the initial number of cells (the inoculum size) and the selection of an arbitrary level α on the time derivative of the heat evolution curve observed for the yeast culture. The values of q_1 determined using this method were found to depend on the level α, but the average was $q_1 = 28.0 \pm 2.4$ pW cell^{-1}, which is relatively close to the value determined by the classic method.

Introduction

Despite the great advances in the field of calorimetry in the last decades, the sensitivity of modern calorimeters does not allow yet the direct determination of heat effects corresponding to a single microbial cell. Minimum populations of at least 10^5–10^6 cells are required in order for the heat effects to be detected at the present level of calorimeter performance. Heat evolution or power expressed per cell are afterwards determined by what we hereafter call the "classic" method, that is, by dividing the observed heat effect to the cell population number. Nevertheless, because of various experimental difficulties, the available data regarding heat effects per microbial cell are scarce[1-3] and display a great variability. Moreover, for the case of yeasts, in

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many circumstances[1,4] the specific heat effects are expressed per gram of yeast and not per cell.

In this paper we report the heat effects per cell for a strain of *Saccharomyces cerevisiae* determined by the classic method, from the correlation observed between the heat amount evolved during growth and the evolution in time of the cell number in the culture. The heat amount and number of cells associated with the production of one mole of ethanol were also calculated. Also, theoretical considerations are shown for a new method supposed to allow the determination of the heat evolution rate per cell from the calorimetrically recorded growth thermograms when only the initial cell population is known.

Materials and Methods

The yeast strain studied was *Saccharomyces cerevisiae* No.9302, kept as stock culture on solid medium in our laboratory. A liquid glucose-peptone growth medium was employed, having the following composition, per liter: 20 g glucose, 2 g yeast extract, 0.5 g $MgSO_4$, 5 g polypeptone and 1 g KH_2PO_4. Cells taken from the stock culture were preincubated for 24 hours at 30℃ and after that a suspension was prepared by diluting the preincubated culture with sterile distilled water until the cell population was about 10^6 per ml. The cell number was checked using a Thoma chamber. One ml of the prepared suspension was added as inoculum in autoclaved glass vials containing 5 ml of the growth medium mentioned before. The cultures thus prepared were then introduced in the calorimeter and incubated at 30℃ until the observed calorimetric signals returned to baseline. The calorimeter employed was the "Bio-Thermo-Analyzer" BTA-201H, manufactured as a commercial instrument by Nippon Medical and Chemical Instruments Co. Ltd., Osaka, Japan. The calorimeter is of the isothermal type working on conduction principle. The heat evolved during microbial growth is detected by the thermopile plates mounted in each calorimetric unit and the small temperature differences between sample units and a reference unit (which contains only water) are transformed into voltage signals. Calibration of the calorimeter using electric resistors provided the parameters required for expressing the signal in heat units by multiplication with the parameter $\beta = 5.9$ mJ μV^{-1}. The sensitivity of the calorimeter at steady heat effect was $A = 17.2\ \mu W\ \mu V^{-1}$. Detailed information regarding the calorimeter was reported elsewhere.[5]

Two more sets of similar yeast cultures were prepared and incubated separately at 30℃. One set was employed for the determination of the yeast population at 30 min intervals, by counting under microscope using a Thoma chamber, while the other set served for determinations of ethanol concentration at 1 h intervals. A Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and a Supelcowax 10 column (0.53 mm diameter and 30 m length, 0.5 μm thickness) was used for ethanol determination. Chromatographic conditions were: oven temperature, 60℃; injector and detector temperature, 200℃; and carrier gas, He at a flow rate of 20 ml min^{-1}. Yeast cells were removed by centrifugation and the aliquot (1 μl) of supernatant was injected directly into the gas chromatograph. Under such conditions, the retention time for ethanol was 1.4 min.

Results

Figure 1 (a) shows 4 growth thermograms or $g(t)$ curves which represent the calorimetric signals recorded during incubation of 4 identical yeast cultures in the calorimeter. The $g(t)$ curves are almost identical, which indicates that a good reproducibility is achieved. However, the $g(t)$ curves represent only the apparent output of the calorimeter, and must be corrected for the permanent heat exchange, characteristic to isothermal calorimeters, that takes place between the calorimetric units and their surroundings. This correction is made by using the previously reported equation:[6-12]

$$f(t) = g(t) + K \int g(t)\,dt \qquad (1)$$

where K is the heat conduction constant of the calorimeter and, for these experiments, had the value 0.1745 min^{-1} which was determined according to the method described elsewhere.[5,6] The $f(t)$ curves computed using Eqn.1 and given in **Fig. 1**(b) represent the actual heat evolution in each calorimetric unit, as it would be recorded by means of a hypothetical adiabatic calorimeter. A similar $f(t)$ is also given in **Fig. 2**(c) in order to allow a comparison with the cell number (**Fig. 2**(a)) and ethanol concentration (**Fig. 2**(b)). Turbidity measurements were also performed, but their results were in very good correlation with the cell number curve

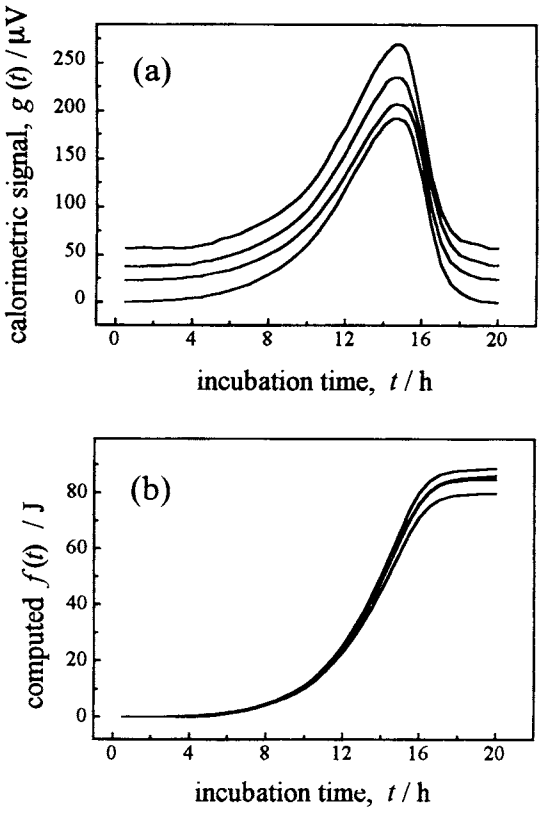

Fig. 1 (a) Four growth thermograms or $g(t)$ curves recorded during incubation of 4 identical yeast cultures in the calorimeter, plotted with a small shift between them so that their similarity can be better observed.
(b) The actual heat evolution curves $f(t)$ calculated from the growth thermograms given in plot a) using Eqn.1.

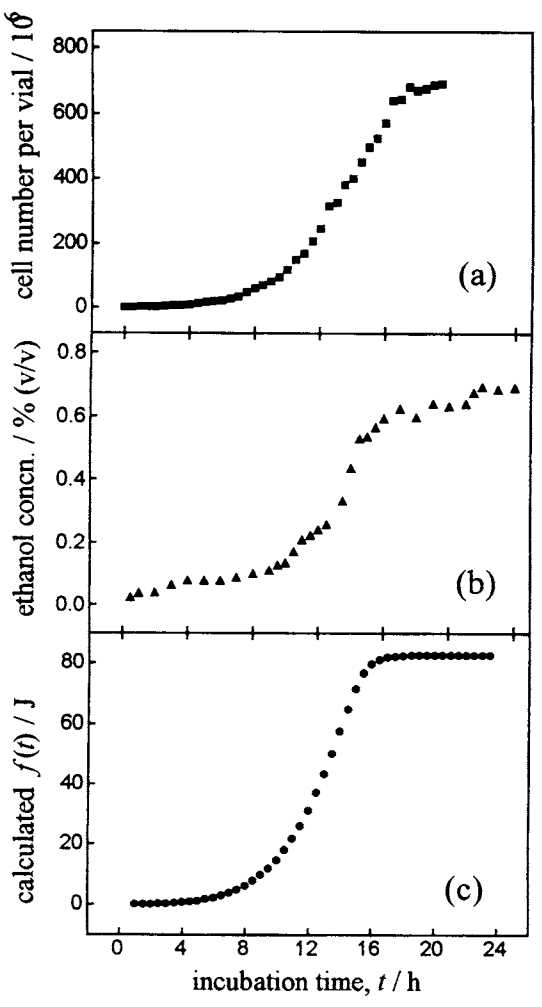

Fig. 2 (a) Plot of the cell number of yeast cells, counted at 30 min interval. The initial cell number was 1.35×10^6 cells per vial, and the final population was about 690×10^6 cells per vial.
(b) Plot of the ethanol concentration produced by the yeast cells, determined at 1 h intervals.
(c) The $f(t)$ curve determined for a similar yeast culture incubated in the calorimeter.

Fig. 2(a) and therefore are not shown here.[12] Examining **Figs. 2**(a) and **2**(c) it is evident that $f(t)$ and the cell number followed a similar pattern, indicating that the actual heat amount observed was correlated with the microbial population, in agreement with many previous reports.[4, 13, 15] This fact provides the grounds for using the $f(t)$ curve in the determination of the growth rate constant, as described below. Also, **Fig. 2**(b) shows that the ethanol concentration in the culture had a similar evolution, suggesting that the conversion of glucose to ethanol took place in parallel with the growth of yeast. The correlation between the 3 curves shown in **Fig. 2** can be further certified if $f(t)$ is plotted against the cell number or the ethanol concentration. From **Fig. 3**(a) it can be observed that a good linear relationship ($R = 0.997$) holds between $f(t)$ and the cell number. This correlation was certain for at least 14 h of incubation. For the same incubation time, Fig. 3b indicates that the correlation between $f(t)$ and the ethanol concentration was also good ($R = 0.990$), the larger scattering of the points being due to relatively large errors in the determination of ethanol concentration by the gas-

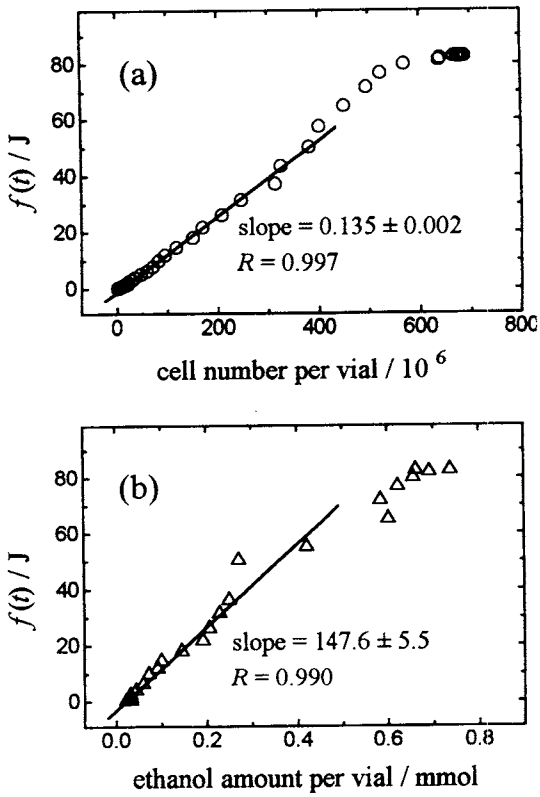

Fig. 3 (a) Correlation between the curves given in Fig. 2 (c) and Fig. 2 (a).
(b) Correlation between the curves given in Fig. 2 (c) and Fig. 2 (b). The linear regression was performed for the data points corresponding to the first 14 hours of incubation. R represents the correlation coefficient.

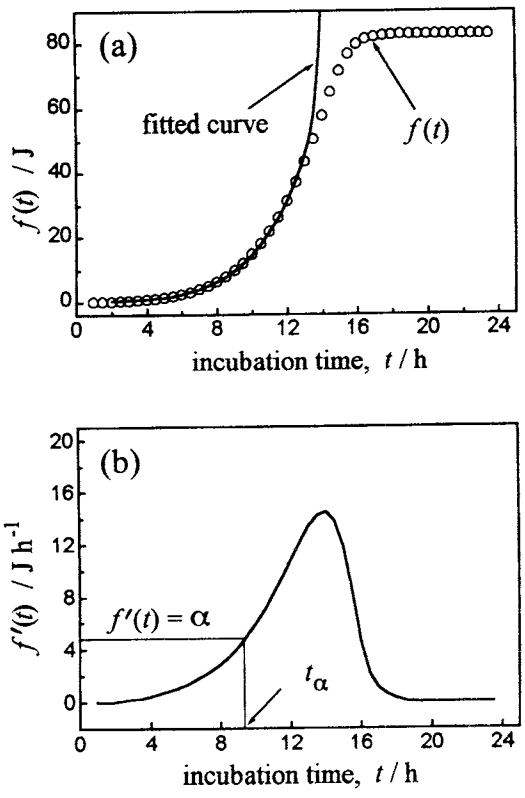

Fig. 4 (a) Procedure employed for the determination of the growth rate μ. The initial, exponential portion of the determined $f(t)$ curve is fitted by regression analysis on the basis of Eqn.2 and thus provides the value of μ.
(b) Plot of the $f'(t)$ curve, also showing the procedure employed for the determination of the incubation time t_α which corresponds to a selected value α of the $f'(t)$.

chromatographic analysis.

From the calculated $f(t)$ curve, the value of the growth rate constant μ can be determined by fitting the initial portion of the $f(t)$ curve with the previously reported equation:[7, 12]

$$f(t) = AN_0e^{\mu t} + BN_0 \quad (2)$$

which describes the amount of heat produced by an exponentially growing microbial population. In Eqn.2 μ is the growth rate constant, N_0 is the number of viable cells at the start of the measurement (the inoculum size), t is the incubation time and A and B are constants.

The fitting procedure that provides the value of the growth rate μ is shown schematically in **Fig. 4** (a). By applying it to the four $f(t)$ curves shown in **Fig. 2** (b), an average value of μ was obtained as $\mu = 0.37 \pm 0.01$ h^{-1}. For comparison, the same procedure, applied to the cell number curve given in **Fig. 2** (a), gave the result $\mu = 0.34 \pm 0.02$ h^{-1}, and when applied to the ethanol curve shown in **Fig. 2** (b) it lead to the result $\mu = 0.32 \pm 0.05$ h^{-1}. The values obtained for μ show slight variations, depending on the portion of the curves which is considered to represent the exponential portion. In our case, for all situations we considered the portion between 3% and 30% of the total height of the curve. The values of μ determined from the $f(t)$ curves (**Fig. 1** (b)), cell num-

ber curve (**Fig.2**(a)) and ethanol curve (**Fig.2**(b)) are relatively close, indicating that the procedure employed can be considered fully reliable.

By applying the classic method, various calculations can now be made regarding the amount of heat generated during the growth of yeasts. The slope in **Fig. 3**(a) gives the value of the average heat evolution per cell during the growth, $Q = (1.35 \pm 0.02) \times 10^{-7}$ J cell^{-1}. On the other hand, the "generation time" or the average generation time of the yeast cells can be determined with the relation:

$$t_d = \frac{\ln 2}{\mu} \tag{3}$$

leading to t_d = 1.87 h. With these values, the average heat evolution rate per cell during the exponential growth phase can be determined as:

$$q_1 = \frac{Q}{t_d} \tag{4}$$

which provides the value $q_1 = 20 \pm 3$ pW cell^{-1}.

Furthermore, from the slope in **Fig.3**(b), it can be derived that the average heat amount associated with the production of 1 mole of ethanol is $Q_E = 147.6$ kJ mol^{-1}. Also, if we take the ratio of the slopes given in **Fig. 2**(a) and **2**(b), we obtain the average number of yeast cells associated with the production of one mole of ethanol, $N_E = 1.09 \times 10^{12}$ cells mol^{-1}.

As mentioned before, the values of Q, q_1, Q_E, N_E presented above are determined based on the classic method which requires determining the heat effects and, in this case, the ethanol amount produced, for the whole culture and averaging them using some knowledge of the culture size, which in this case was provided by the cell counting at 30 min intervals.

An attempt was also made to develop a new procedure for the determination of the heat evolution rate per cell, q_1, in the hypothesis that only the initial number of cells is known. For this purpose, we go back to Eqn. 2 and make use of the previously reported expressions of the constants A and B:[7, 12]

$$A = \frac{q_1}{\mu e^{\mu\tau}} \tag{5}$$

and

$$B = \int_0^{\tau} q_0 dt - \frac{q_1}{\mu} \tag{6}$$

where q_0 is the heat evolution rate per unit cell during the lag phase, q_1 represents the heat evolution rate per unit cell during the exponential growth phase, and τ is the lag time.

In this case, differentiation of Eqn. 2 leads to:

$$f'(t) = \frac{N_0 q_1}{e^{\mu\tau}} e^{\mu t} \tag{7}$$

If a certain level α of is selected as shown in **Fig. 4**(b), then Eqn. (7) can be rewritten as follows:

$$\alpha = \frac{N_0 q_1}{e^{\mu\tau}} e^{\mu t_\alpha} \tag{8}$$

which in turn leads to

$$\frac{q_1}{e^{\mu\tau}} = \frac{\alpha}{N_0 e^{\mu t_\alpha}} \tag{9}$$

For our experiments, with inoculum cells preincubated on the same type of growth medium and cells subjected to minimum stress during the manipulations between the preincubation and the experimental phase, the value of the lag time τ was found to be negligible. More precisely, when counting the cells for the determination of the curve shown in **Fig.2**(a), an increase in the cell number was already observed between the first and the second counting, which meant that the lag time could be estimated at less than 30 min. If that is the case, by neglecting τ Eqn.9 can be reduced to the simplified form:

$$q_1 = \frac{\alpha}{N_0 e^{\mu t_\alpha}} \tag{10}$$

Selecting various values for the parameter α in the exponential portion of the $f'(t)$ curve, the corresponding values of t_α were determined as shown in **Fig.4**(b). **Table 1** contains the values of q_1 calculated using Eqn.10

Table 1 Values of the heat evolution rate per cell (q_1) determined using Eqn.10 for various values of the α level of $f'(t)$ (see Fig.4(b)).

α / J h^{-1}	t_α / h	q_1 / pW cell^{-1}
0.5	4.19	21.2
1.0	5.48	26.1
1.5	6.31	28.6
2.0	7.00	29.4
2.5	7.59	29.4
3.0	8.09	29.2
3.5	8.52	29.0
4.0	8.86	29.2
4.5	9.21	28.7
5.0	9.50	28.6
5.5	9.76	28.5

when α takes various values, and it can be seen that the values of q_1 display a certain variation with the value of α. The average value of q_1 was 28.0 pW cell $^{-1}$ with a standard error of 2.4 pW cell $^{-1}$, which is relatively close to the value $q_1 = 20 \pm 3$ pW cell $^{-1}$ mentioned above and obtained through the classic method. Actually, **Table 1** shows that for very small values of α (up to 4% of the total height of the $f'(t)$ curve), the difference between the traditional q_1 and the one determined by the new method was the smallest; then q_1 increased when α increased up to about 14% of the maximum $f'(t)$, and then decreased again while α reached 38% of the maximum $f'(t)$. If the value $q_1 = 20 \pm 3$ pW cell $^{-1}$ is considered to represent the correct heat evolution rate per cell, then the evolution observed for the q_1 values given in **Table 1** appears to indicate that a deviation from the exponential growth occurs starting from very early incubation times, and also variations of q_1 in time may occur. The straight line drawn in **Fig. 2** (a) for 14 hours of incubation has certainly ignored such variations, and analysis showed that actually the slope of this straight line also changes, function of how many of the points in **Fig. 2** (a) are taken into consideration when making the linear regression. On the other hand, the variation of the q_1 values shown in **Table 1** may also be due, in part, to various errors and simplifications. Also, it must be mentioned that if the lag time τ is not neglected, its influence will be manifested in an increase of the q_1 due to their multiplication with the factor $e^{\mu\tau}$.

Discussion

From the correlation between the actual evolution curve $f(t)$ and the number of cells in the culture, a value $q_1 = 20 \pm 3$ pW cell $^{-1}$ was determined for the average heat evolution rate of the yeast cells during the exponential growth phase. The parameter q_1 includes both the heat evolution rate associated with cell growth and the heat evolution rate associated with glucose metabolization by yeasts. This value of q_1 is a few times higher than the values reported by Lamprecht *et al.*,[2] which ranged between 3 and 10 pW cell $^{-1}$, but these differences can probably be attributed to the variation of strain and growth conditions. For example, Lamprecht[16] also determined that during growth of yeasts on a medium that contained only glucose as the sole source of energy the amount of heat associated with the anaerobic growth of yeast was between 90 and 120 kJ per mole of glucose. In our case, knowing that the maximum heat amount per vial was 82.6 J (**Fig. 2** (c)) and the available glucose was 0.555 mmol, we obtain the corresponding value of 147.7 kJ per mole of glucose, which can be explained by the supplementary presence of other nutrients than glucose in the growth medium we employed. The larger value of the heat amount observed for our culture may therefore represent the main reason for the larger value of q_1 determined in our case. The result concerning the amount of heat associated with the production of 1 mole of ethanol, $Q_E = 147.7$ kJ mol $^{-1}$, which in principle may be useful for the estimation of the heat amount resulted in similar processes of ethanolic fermentation, is also dependent on the growth medium and experimental conditions employed.

Regarding the determination of q_1 through the new method proposed, we could say that the values determined (**Table 1**) are relatively close to the one obtained through the classic procedure. However, a variation of q_1 was observed when the parameter α varied in the exponential portion of $f'(t)$, and probably various experimental errors and theoretical simplifications are propagated in the determined values. Nevertheless, if certain improvements will be possible in the future, the procedure presented may represent a useful and more convenient alternative to the conventional methods of determination of heat effects per cell. Among the advantages of the method we could name the fact that it requires only the knowledge of the initial number of viable cells, and also it may allow the observation of changes in time of the q_1 values of the cells in a culture.

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要　旨

酵母*Saccharomyces cerevisiae*の30℃における生育サーモグラムを多試料同時計測等温熱量計を用いて観測し，生育ならびに熱生成に関するパラメーターを決定した。熱生成曲線，細胞増殖曲線およびエタノール生成曲線の間には良い相関があり，それぞれから得られた増殖速度定数は0.37, 0.34, 0.32 h^{-1}であった。熱生成曲線と細胞増殖曲線との対比から1世代細胞当たりの熱生成量は$Q=(1.35\pm0.02)\times10^{-7}$ J $cell^{-1}$，1細胞当たりの熱生成速度は$q_1=20\pm3$ pW $cell^{-1}$と得られた。また，これをエタノール生成当たりでみると1 molのエタノール生成に伴う熱生成量は$Q_E=147.6$ kJ mol^{-1}，1 molのエタノール生成に伴う細胞の増殖量は$N_E=1.09\times10^{12}$ cell mol^{-1}であった。さらに細胞初期値（植菌量）のみを既知量とし，熱生成の微分曲線上で任意のレベルαを選ぶだけで細胞の熱生成速度を求める方法を考案した。それによればαの値に依存するものの，q_1の平均値として28.0±2.4 pW $cell^{-1}$が得られ，従来の方法で得たものと比較的良く一致した。