Netsu Sokutei **24** (4) 206-213

論 文

Calorimetric Study of Yeast Growth and Its Inhibition by Added Ethanol at Various pHs and Temperatures

Oana-Arina Antoce‡, Vasile Antoce and Katsutada Takahashi*

(Received August 5, 1997)

Using a multiplex isothermal calorimeter with 24 sample units the heat evolved during incubation of yeast cultures on glucose-peptone broth was detected under the form of growth thermograms, based on which the values of the growth rate constant μ were calculated. The effect of the initial pH of the yeast cultures was investigated for single strains of four yeast species. Judging from the calculated values of μ, the yeasts studied presented stable growth for pH values ranging approximately from 4 to 8. From growth experiments performed at various temperatures, the effect of the incubation temperature could be observed in the growth thermograms recorded. Using Arrhenius-type plots of the growth rate versus temperature the apparent activation energy of growth was calculated for the four yeasts studied. Furthermore, when ethanol was added in various concentrations to the yeast cultures, changes occurred in the growth thermograms which could be analyzed for the determination of the 50% inhibitory concentration (K_μ) and the minimum inhibitory concentration (MIC_μ) of ethanol. The variation of parameters K_μ and MIC_μ with the initial pH and the incubation temperature is also reported for a strain of *Saccharomyces cerevisiae*.

1. Introduction

Yeasts represent probably the most important class of microorganisms from a technological viewpoint[1] and attract considerable interest as model microorganisms for study.[2] Previous results on the application of calorimetry to the study of the growth and metabolism of yeast have been summarized elsewhere.[3,4]

In a number of previous papers[5-14] results have been published regarding a calorimetric procedure developed in our research group, using a multiplex isothermal microcalorimeter which was applied to the study of microbial growth under various conditions and in the presence of inhibitors. Based on the experience thus accumulated, an attempt was made in this study to apply the mentioned calorimetric procedure to basic investigations of the growth of some yeasts at different pH values and incubation temperatures, as well as in the presence of various amounts of ethanol added to the yeast cultures as a growth inhibitor.

Laboratory of Biophysical Chemistry, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai-shi, Osaka 599, Japan

* Corresponding author: Katsutada Takahashi, Laboratory of Biophysical Chemistry, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai-shi, Osaka 599, Japan; Phone/Fax: +81-722-50-0525; E-mail: ktakahas@biochem.osakafu-u.ac.jp

‡ Permanent address: Viticulture and Enology Department, University of Agronomical Sciences Bucharest, Marasti Str. 59, sector 1, 71329 Bucharest, Romania

2. Materials and Methods

The yeast strains studied included one strain each of the following species: *Saccharomyces cerevisiae* (strain No.9302), *Schizosaccharomyces pombe*, *Kluyveromyces marxianus* (IFO 0260) and *Candida utilis* (IFO 0396), preserved as stock cultures in our laboratory.

Yeast growth was monitored at constant temperature using a multiplex isothermal batch calorimeter with 24 calorimetric units. When sample cultures of yeast are incubated in the calorimetric units, the apparatus detects the heat evolved during the microbial growth and records it as a function of time, providing the so-called "growth thermograms" or "thermal profiles". Changes are visible among the observed growth thermograms recorded when culture conditions are changed or when an inhibitor is added to the cultures in various concentrations. These changes may be quantitatively expressed using the value of the growth rate constant μ which may be calculated from each growth thermogram. Moreover, by analyzing the differences between control cultures (with no inhibitor added) and sample cultures (with addition of various amounts of inhibitor) quantitative parameters can be determined to characterize the microbial tolerance of the microorganism to the action of the inhibitor. These parameters are: K_μ, the concentration of inhibitor which reduces the growth activity by 50%, and MIC_μ, which is the concentration of inhibitor which totally prevents the microbial growth. A more detailed presentation was previously done elsewhere for the apparatus [5] and the calorimetric method.[6,7]

The yeast cultures were prepared as follows. The growth medium was a liquid glucose-peptone broth containing, per liter, 20 g glucose, 2 g yeast extract, 0.5 g $MgSO_4$, 5 g polypeptone and 1 g KH_2PO_4, pH 5.6. Vials of 50 ml volume containing 5 ml of the growth medium were autoclaved, and 1 ml of a suspension of yeast cells was added to each vial as inoculum. The suspension of yeast cells was prepared by preincubating cells taken from the stock culture for 24 hours, on the same kind of growth medium and at the same temperature as the subsequent calorimetric experiment, and then diluting the resulting broth with distilled water until the cell concentration was between 1 and 3×10^6 cells ml^{-1}. The number of cells was checked by counting under microscope with a Thoma chamber. When necessary, ethanol was added aseptically to the vials, in concentrations up to 7.65%.

Sets of 24 vials thus prepared were introduced in the calorimeter and incubated until all the output signals detected by the apparatus returned to baseline as the result of nutrients exhaustion and growth termination. For the study of the pH influence, the initial pH of the prepared yeast cultures was adjusted to the desired values using aqueous solutions of 1N NaOH and 5N HCl. In the vials with very high initial pH (over 5), the final pH of the cultures, after the 48-120 hours of incubation in the calorimeter, decreased as a result of the yeast activity. The maximum decrease observed was for a culture of *Candida utilis*, which had initial pH 10 and final pH 5.9. When the initial pH was very low (less than 3.5-4), yeast activity lead to an increase of the final pH. The maximum increase was observed for a culture of *Kluyveromyces marxianus* which had the initial pH 2.5 and the final pH 3.0. However, these changes in pH during yeast growth were considered negligible for the calorimetric procedure since the determination of the growth rate constant is made by analyzing only the initial portion of the growth thermograms.

All chemicals employed were obtained from Wako Pure Chemical Industries Inc. and were of certified reagent grade.

3. Results

3.1 Effect of pH

When yeast cultures prepared at various initial pHs were incubated in the calorimeter the effect of pH on the growth of the yeasts could be observed in the recorded calorimetric signals (**Fig.1**), also called growth thermograms or $g(t)$ curves hereafter. In the experiments described in **Fig.1** very low values of pH (under 4) lead to longer incubation times and smaller peaks of the growth thermograms, while pHs between approximately 4 and 8 had less influence on the calorimetric curves recorded.

For a more quantitative description of the effect of pH on the growth of yeasts, the growth thermograms obtained were analyzed for the determination of the growth rate constant μ, using the procedure presented elsewhere.[6,7,11,12] The observed influence of the initial pH of the yeast cultures on their growth rate is presented in **Fig.2** for the four yeasts studied. From **Fig.2** it appears

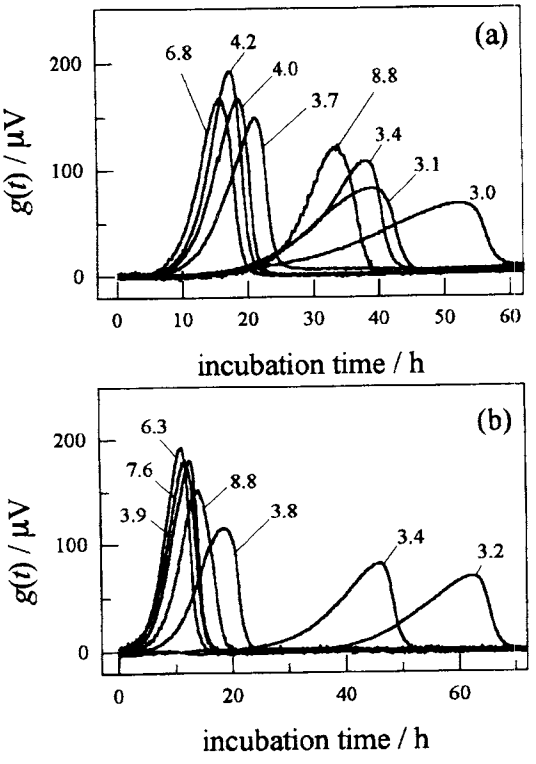

Fig. 1 Calorimetric signals recorded for yeast growth on glucose-peptone medium at 30°C. a) *Saccharomyces cerevisiae*; b) *Schizosaccharomyces pombe*. The numbers indicated for each curve represent the value of pH at the beginning of incubation. The inoculum size was $(1.4 \pm 0.5) \times 10^6$ cells per vial. Although 24 curves were obtained in each experiment, for better visibility only some of the curves are shown.

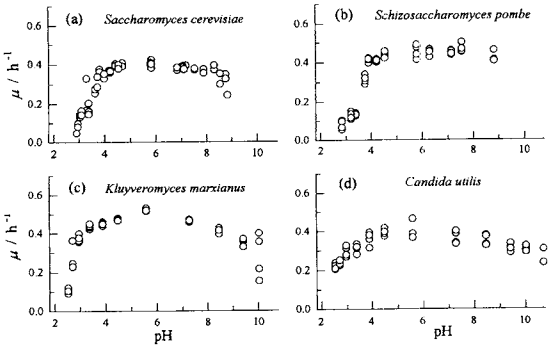

Fig. 2 Variation of the growth rate constant μ with the initial pH for four yeast strains. The values of μ were determined from the calorimetric recordings of the heat evolution during yeast growth (examples shown in Fig.1). All strains were grown at 30°C on glucose-peptone medium.

that, under the conditions used in this study, the four yeasts studied had no distinct optimum pH value. Instead, there was a relatively large range of pH, approximately between 4 and 8, over which the changes in the value of μ were small.

When ethanol was added to the yeast cultures in increasing amounts, a decrease in the growth rate constant could be observed.[6,7] **Figure 3**(a) shows the decrease in the growth rate of *Saccharomyces cerevisiae*, plotted as a function of the concentration of added ethanol, for different values of the initial pH. When the pH was between 3.6 and 5.0 the determined values of μ decreased with the increase in the ethanol concentration, and the data points for μ are almost identical, irrespective of pH. However, when the initial pH was 3.1, the effect of ethanol on the value of the growth rate constant μ was completely different (**Fig.3**(a)).

From the dependence of the growth rate μ on the ethanol concentration, the inhibition parameters K_μ and MIC_μ were calculated for the strain *S. cerevisiae* using the method previously presented[6,7,11,12] and the values thus obtained are shown in **Fig.3**(b). There was no significant effect from the variation of the initial pH between 3.6 and 5.0 on the ethanol tolerance of this yeast, as expressed by the values of the 50% inhibitory concentration K_μ and the minimum inhibitory concentration MIC_μ.

3.2 Effect of temperature

Figures 4, **5** and **6** present the effect of the incubation temperature on the calorimetric curves recorded and on the values of the growth rate constant determined from the calorimetric curves. Experiments were carried out at incubation temperatures ranging from 16°C to 40°C , with an increment of 2°C . Growth thermograms obtained in various experiments were put together afterwards in the form shown in **Fig.4**, which provides a graphic description of the influence of temperature on the growth of the yeast. The incubation temperature affected the calorimetric curves in a number of ways: by changing the slope of the initial portion of $g(t)$, by shifting the peak of the $g(t)$ curves towards shorter or

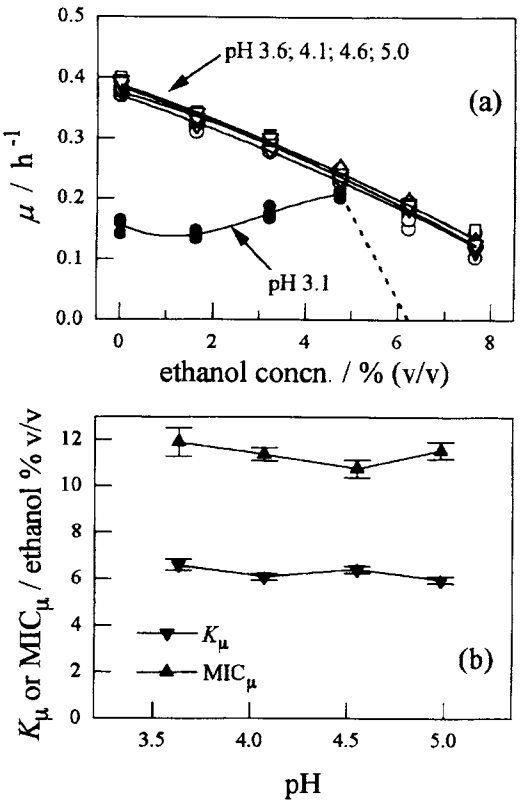

Fig. 3 Effect of pH on the decrease of the growth rate constant μ due to the addition of ethanol at the beginning of incubation. The solid lines are empirical curves fitted to the experimental points. a) For initial pH values between 3.6-5.0 the pH had no significant effect on the decrease of μ with the increase of the amount of added ethanol. However, at pH 3.1 the evolution of the growth rate constant was completely different. Also, at pH 3.1 no growth was evidenced when 6.22 % ethanol was added to the culture, which theoretically indicates that $\mu = 0$; therefore, the dotted line in plot (a) was drawn to connect the points corresponding to addition of ethanol in concentration 4.74 % and 6.22 %. (b) Effect of initial pH on the 50 % inhibitory concentration of ethanol (K_μ) and the concentration of ethanol which totally inhibited growth (MIC_μ). All data correspond to the strain *Saccharomyces cerevisiae*, grown on glucose-peptone broth at 30°C.

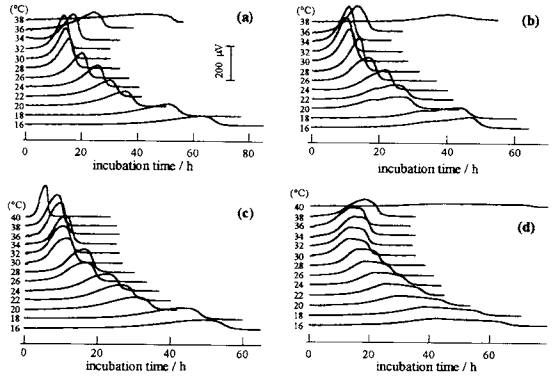

Fig. 4 Effect of the incubation temperature on the growth thermograms or $g(t)$ curves recorded as the calorimetric output during growth of yeast cultures. (a) *Saccharomyces cerevisiae*; (b) *Schizosaccharomyces pombe*; (c) *Kluyveromyces marxianus*; (d) *Candida utilis*.

longer incubation times and by increasing the peak height. These effects were correlated, as can be understood from the example presented in **Fig.5** for the strain *Schizosaccharomyces pombe*: the changes in the initial slope of the $g(t)$ curves (expressed by the values of μ) were accompanied by similar changes in the peak height and inverse changes in the peak time.

Figure 6 contains plots of the growth rate constant μ versus the incubation temperature for the four yeasts studied. From these plots the apparent activation energy of growth E_a was calculated for each yeast strain by fitting the μ data situated on the rising portion of the plot with the Arrhenius equation:

$$\mu = A\ e^{[-E_a/(RT)]} \qquad (1)$$

where A is an empirical constant, R is the gas constant, T is the absolute temperature and E_a is the activation energy. The fitted curves are also shown in **Fig.6** as the solid lines. The obtained values of the apparent activation energy of growth are given in **Table 1**.

Another aspect investigated was the effect of temperature on the inhibitory action of added ethanol on yeast growth. Calorimetric measurements were taken at different incubation temperatures on *Saccharomyces cerevisiae* cultures to which ethanol was added in various concentrations. By analyzing the growth thermograms recorded during each experiment the parameters K_μ (the 50 % inhibitory concentration) and MIC_μ (the concen-

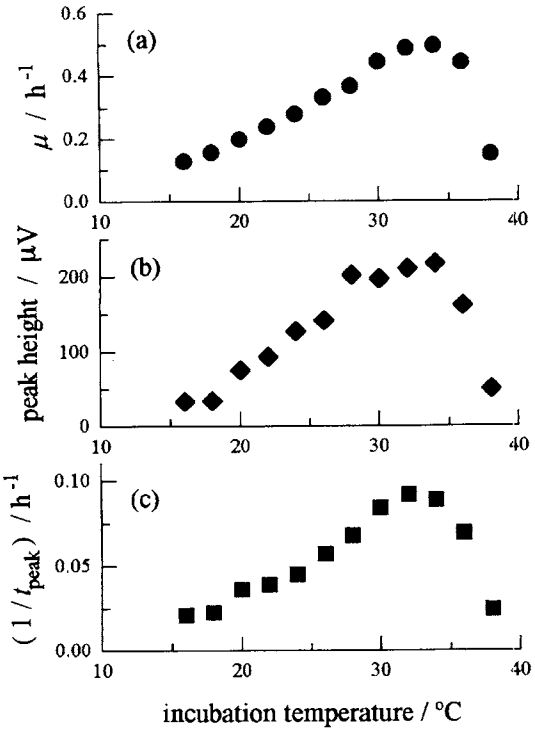

Fig. 5 Variation with the incubation temperature of parameters obtained by analyzing the $g(t)$ curves shown in Fig.4b (*Schizosaccharomyces pombe*). (a) the growth rate constant μ ; (b) the height of the peak of the $g(t)$ curves; (c) the incubation time corresponding to the peak of the $g(t)$ curves.

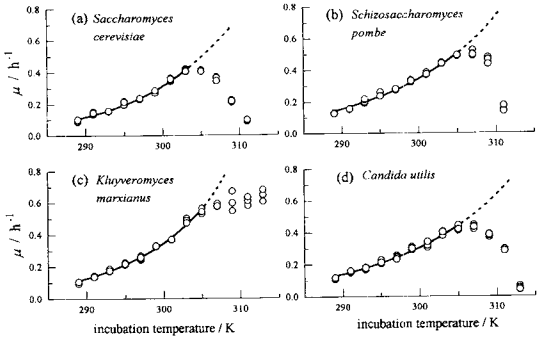

Fig. 6 Plots of the growth rate constant μ versus the incubation temperature T, used for the determination of the values of the activation energy E_a for the four yeasts studied. The curve shown on each plot is the graphic representation of equation (1) fitted to the data points. The solid portion of the curve also indicates the interval of data considered for fitting, whereas the dotted portion is simply an extrapolation of the fitted curve. All growth experiments were carried out on glucose-peptone medium at pH 5.6.

Table 1 Values of the apparent activation energy of growth E_a calculated for the four yeast strains studied using the plots shown in Fig.6. (The error given is the standard deviation.)

Yeast strain	Activation energy E_a / kJ mol^{-1}
Saccharomyces cerevisiae	69.9 ± 1.5
Schyzosaccharomyces pombe	57.0 ± 1.1
Kluyveromyces marxianus	72.4 ± 1.5
Candida utilis	56.0 ± 1.5

tration at which the yeast growth is totally inhibited) were calculated to quantitatively characterize the inhibitory effect of added ethanol on the growth of the yeast. The observed relationship between these parameters and the incubation temperature is presented in **Fig.7**.

4. Discussion

It was shown (**Fig. 1**) that the initial pH of the yeast cultures incubated in the calorimeter affected the growth thermograms recorded. For all the yeast strains studied, the very low pHs (under 4) had a strong detrimental effect on yeast growth, which was reflected in smaller peaks, smaller initial slopes and significantly longer incubation times observed on the $g(t)$ curves. On the other hand, as seen in **Fig.1**, there were also a number of relatively similar curves which correspond to different pH values, roughly between 4 and 8. In this range, the effect of pH on the growth thermograms was rather small.

The dependence between the values of the growth rate μ determined from the calorimetric signals and the initial pH (**Fig.2**) confirms the established capability of yeasts to grow at very different pHs and is in general agreement with other results found in the literature. For example, the acceptable range of pH observed by us for *Kluyveromyces marxianus* is similar to the pH interval between 3 and 8 recently reported by other authors.[15] The optimum pH range for the fermentation activity of a different strain[16] of *Candida* was pH 4-7, close to

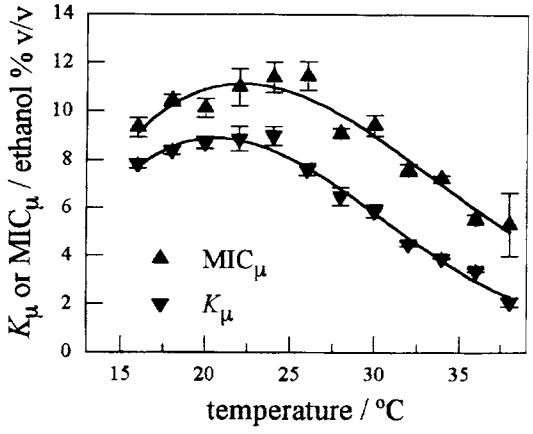

Fig. 7 Effect of the incubation temperature on the yeast tolerance to ethanol, as expressed by the 50 % inhibitory concentration K_μ and the minimum inhibitory concentration MIC_μ. The data correspond to *Saccharomyces cerevisiae* grown on glucose-peptone medium at pH 5.6. The solid lines represent polynomial functions ($Y = a_0 + a_1X + a_2X^2 + a_3X^3$) fitted by the least squares method to the experimental points. The calculated polynomial parameters in the case of K_μ were: $a_0 = -26.8106$, $a_1 = 4.1757$, $a_2 = -0.1526$, $a_3 = 0.00166$ ($r^2 = 0.986$). In the case of MIC_μ the polynomial parameters were: $a_0 = -25.8320$, $a_1 = 4.0002$, $a_2 = -0.1350$, $a_3 = 0.00135$ ($r^2 = 0.941$).

the one observed in the present study. In the case of *Schizosaccharomyces pombe* and *Candida utilis* the upper limit of the optimum range of pH couldn't be determined (**Fig.2**(b),(d)), since the required high values of the initial pH could not be realized under the conditions of our experiments. When the pH was lower than 3.5-4, the growth of all strains was significantly affected. It was concluded that the studied yeasts were able to grow with approximately the same rate at pH values ranging roughly from 4 to 8.

The results shown in **Fig.3** for the strain *Saccharomyces cerevisiae* support the above observation and also indicate that the variation of pH between 3.6 and 5.0 did not influence the inhibitory effect of added ethanol. As shown in **Fig.3**(a), the growth rates of yeast cultures were almost identical, irrespective of pH, for pH values ranging from 3.6 to 5.0; they varied only with the amount of added ethanol, as a result of the inhibitory action of ethanol against the yeast growth. Since parameters K_μ and MIC_μ are calculated on the basis of the variation of μ with the ethanol concentration, it is reasonable that the 50% inhibitory concentration (K_μ) and the minimum inhibitory concentration (MIC_μ) of ethanol were only little affected by the pH variation from 3.6 to 5.0 (**Fig.3**(b)). However, the situation was found to be quite different at pH 3.1: in the absence of ethanol the value of μ was less than half that observed for growth at pH 3.6-5.0 (in agreement with **Fig.2**(a)); it increased slowly when ethanol was added up to 4.74 % v/v; and no significant growth was observed when 6.22 % ethanol was added. Due to this particular behavior at pH 3.1, parameters K_μ and MIC_μ could not be determined for this case. The results presented above can probably be explained based on the mechanism by which ethanol inhibits yeasts. It is believed that ethanol, as well as other alcohols, modifies the properties of the phospholipid bilayers of cell membranes, causing alterations in the membrane functions such as the transport of nutrients[17-19] and also disturbing the activity of some enzymes. These mechanisms of action are not very susceptible to pH changes, as long as the pH remains in limits favorable to growth. The different evolution of the μ values with the ethanol concentration at pH 3.1 (**Fig.3**(a)) can probably be attributed mainly to the substantial decrease of the growth rate because of the unfavorable pH. Under such detrimental conditions, the addition of ethanol in small amounts appears to have no effect up to about 3 % v/v, or even a slight beneficial effect (up to 4.74 %) on the value of μ; it is possible that the yeast actually was able to partially metabolize the added ethanol.

The calorimetric method also provided results regarding the effect of the incubation temperature on the growth of yeast and on the inhibitory effect of added ethanol. **Figure 4** presents the effect of temperature on the calorimetric signals recorded during the growth of the four yeast strains studied. When the incubation temperature was changed, the growth thermograms recorded displayed changes in three aspects: the steepness of the initial portion corresponding to the phase of exponential growth of the yeast was modified; the peak height was also affected[13] ; and the peak time varied. (Although, theoretically, the peak time is also affected

by the inoculum size,[8] this parameter was controlled in these experiments and kept within limits which made any differences between growth thermograms negligible.) It is reasonable to think that a steeper rising at the beginning of the calorimetric curve, a higher peak and a shorter incubation time qualitatively indicate better growth of the yeast. Under such conditions, **Fig.4** offers a qualitative graphic diagram of the effect of temperature on yeast growth. For three of the yeasts studied (**Fig.4**(a),(b),(d)) there is a temperature value around 32-36°C which appears to be optimum for growth, in the sense that the time required for incubation is the shortest and the peak is highest, compared to that at other temperatures. In the case of *Kluyveromyces marxianus* the optimum growth temperature was somewhere over the temperature range investigated in this study; the $g(t)$ curve of this strain at 40°C presents the shortest peak time. In **Fig.5** a comparison is made of the three characteristic parameters which can be obtained from the growth thermograms given in **Fig.4**(b) (for *Schizosaccharomyces pombe*). It indicates that the values of μ, peak height and the inverse of the peak time were affected in similar ways by the changes in the incubation temperature. As described elsewhere,[6,7] the calculated value of the growth rate constant μ is directly related to the steepness of the initial portion of the heat evolution curves computed from calorimetric signals like those given in **Fig.4**. The optimum growth temperature suggested by the evolution of the growth rate constant μ (**Fig.5**(a)) is in fair agreement with the optimum growth temperature indicated by the changes in the peak height (**Fig.5**(b)) or the inverse of the peak time (**Fig.5**(c)). Although not shown here, similar results were obtained for the other 3 strains, too.

As stated elsewhere,[5-14] the growth thermograms represent only the apparent signal obtained as the output of the calorimeter. Therefore, they depend on the heat conduction constant of the apparatus and on the experimental conditions. Strictly speaking, in order to determine the actual heat evolution which occurs in the calorimetric unit, the $g(t)$ curves shown in **Figs.1** and **4** need to be integrated using appropriate mathematical procedures.[5-14] However, because after integration the peak observed in the $g(t)$ curves disappears, in this study the recorded $g(t)$ curves were considered more appropriate for visual examination aimed at the estimation of yeast growth. As a rough approximation, the $g(t)$ signal can be considered to be proportional to the power (the time derivative of the heat evolution) which is released in the calorimetric unit during the microbial growth. As long as the calorimeter, the vials and the amount of liquid medium are maintained from one experiment to another, results obtained in different experiments can be compared on the basis of the $g(t)$ curves recorded. It is suggested, therefore, that the calorimetric signal $g(t)$ obtained during the yeast growth at various temperatures may provide a useful and simple way for estimating the optimum growth temperature of yeasts, as well as other microorganisms.

Figure 6 shows the values of μ calculated from the growth thermograms recorded during the growth of the four yeasts studied at various temperatures, plotted against the incubation temperature. Again, it can be observed that the temperature which lead to the highest growth rates is in agreement with the optimum temperature suggested by the growth thermograms given in **Fig.4** for all four strains studied. The plots shown in **Fig.6** allowed the determination of the apparent activation energy of growth E_a corresponding to the four yeasts studied (**Table 1**), values which are roughly of the same order as other results reported for various microorganisms.[13,14,20] When the optimum growth temperature is exceeded a distinct change can be observed in the slope of the plots in **Fig.6**, which is probably caused by the thermal inactivation of enzymes and by structural alterations induced by the elevated temperature in the cellular membranes. It should also be noted that the plots in **Fig.6** are perfectly equivalent to the traditional Arrhenius plots of ln μ against $1/T$. Although the traditional form of the Arrhenius plots has been preferred for a long time, mainly for the reason that it provides the value of E_a using a simple linear fitting, it also had the disadvantage of introducing supplementary errors during the linearization procedure. Moreover, if the plots are used in the form shown in **Fig.6**, the optimum temperature of growth can be read directly on the horizontal axis.

The incubation temperature was also found to have an important effect on the tolerance of *Saccharomyces cerevisiae* to ethanol added at the beginning of incubation (**Fig. 7**). The yeast was most resistant to ethanol when grown at 20-25°C; its ethanol tolerance decreased slightly

at lower temperatures, and decreased significantly at higher temperatures. The results in **Fig. 7** follow the same tendency observed by other authors in a previous report,[21] although in our opinion the present results are characterized by increased accuracy. Such data may present interest for technological processes involving fermentations at various temperatures and could contribute to a better understanding of the influence of temperature on the interaction between yeasts and ethanol.

In conclusion, the calorimetric method used in this study was found suitable for the investigation of the growth of yeasts at various pH values and temperatures, as well as for the quantitative determination of the yeast tolerance to ethanol at different temperatures. The results thus obtained may be considered as another argument in favor of the calorimetry as a useful tool in the study of microbial growth under various conditions and in the presence of inhibitors.

Acknowledgments: One of the authors (O.-A. Antoce) was financially supported by the Japanese Ministry of Education, Science, Sports and Culture (#940963).

References

1) H. J. Peppler and D. Perlman (Eds.), Microbial Technology, vol. 2 (Fermentation Technology), New York, Academic Press (1979).
2) D. R. Berry, I. Russelll and G. G. Stewart (Eds.), Yeast Biotechnology, London, Allen & Unwin (1987).
3) I. Lamprecht, Growth and metabolism in yeast. In: Biological Microcalorimetry, pp.43-112. Edited by A. E. Beezer, New York, Academic Press (1980).
4) R. J. Miles, A. E. Beezer and B. F. Perry, Growth and metabolism of yeasts. In: Thermal and Energetic Studies of Cellular Biological Systems, pp.106-130. Edited by A. M. James, Wright, Bristol (1987).
5) K. Takahashi, *J. Antibact. Antifung. Agents* **24**, 313-320 (1996).
6) O. A. Antoce, V. Antoce, K. Takahashi, Y. Nitta, H. Fukada and H. Kawasaki, *Netsu Sokutei* **23**, 45-52 (1996).
7) O. A. Antoce, N. Pomohaci, V. Antoce, H. Fukada, K. Takahashi, H. Kawasaki, N. Amano and T. Amachi, *Biocontrol Science* **1**, 3-10 (1996).
8) M. Hashimoto and K. Takahashi, *Agric. Biol. Chem.* **46**, 1559-1564 (1982).
9) T. Kimura and K. Takahashi, *J. Gen. Microbiol.* **131**, 3083-3089 (1985).
10) K. Takahashi, *Thermochim. Acta* **163**, 71-80 (1990).
11) A. Antoce, K. Takahashi and I. Namolosanu, *Vitis* **35**, 105-106 (1996).
12) O. A. Antoce, V. Antoce, K. Takahashi and F. Yoshizako, *Biosci. Biotech. Biochem.* **61**, 664-669 (1997).
13) A. Katarao, N. Yamato and K. Takahashi, *Agric. Biol. Chem.* **51**, 2437-2442 (1987).
14) S. Itoh and K. Takahashi, *Agric. Biol. Chem.* **48**, 271-275 (1984).
15) D. Vivier, R. Ratomahenina, G. Moulin and P. Galzy, *J. Ind. Microbiol.* **11**, 157-161 (1993).
16) P. J. Slininger, R. J. Bothast, M. R. Ladisch and M. R. Okos, *Biotechnol. Bioeng.* **35**, 727-731 (1990).
17) M. Yamashita, *J. Antibact. Antifung. Agents* **24**, 195-219 (1996).
18) L. O'Neal-Ingram, *J. Bacteriol.* **125**, 670-678 (1975).
19) L. O'Neal-Ingram and T. Buttke, Effects of alcohols on microorganisms. In: Advances in Microbial Physiology, Vol.25, pp.254-296. Edited by A. H. Rose and D. W. Tempest, London, Academic Press (1984).
20) K. Hiyama, S. Wada and O. Kobayashi, *Biocontrol Science* **1**, 33-39 (1996).
21) I. Sa-Correia and N. van Uden, *Biotechnol. Bioeng.* **25**, 1665-1667 (1983).