Quantitative Study of Yeast Growth in the Presence of Added Ethanol and Methanol Using a Calorimetric Approach

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Using a calorimeter with 24 sample units the heat evolved during incubation of yeast cultures at 30°C was detected in the form of growth thermograms. Ethanol and methanol added to the culture medium produced changes in the growth thermograms that could be analyzed to calculate the 50% inhibitory concentration (IC₅₀) and minimum inhibitory concentration (MIC). Correlation of the heat evolution curves with the number of cells and the turbidity of the culture was found to be very good. It was found that addition of ethanol and methanol up to 7.65% had clear effects of inhibition on growth of all yeast strains studied, reducing the growth rate constant and delaying growth. However, the amounts of ethanol produced from the nutrients available in the culture vial was only little affected by the initial addition of up to 7.65% (v/v) of ethanol or methanol in the medium.

Key words: ethanol inhibition; methanol inhibition; yeast growth; calorimetric measurement

Materials and Methods

Microorganisms. The yeast strains studied included Saccharomyces cerevisiae No. 9032, Saccharomyces cerevisiae Hakken No. 1, Schizo-saccharomyces pombe, Candida utilis IFO 0396, Hanseniaspora waltynesis IFO 0115, Hansenula anomala IFO 0118, and Kluyveromycetes marxianus IFO 0260, kept as stock cultures in our laboratory. Also, two more strains (Saccharomyces cerevisiae IFO 2347 and IFO 2363) were obtained from Suntory Ltd. Research Center, Osaka.

Growth monitoring. A multiplex isothermal batch calorimeter having 24 calorimetric units was used to monitor the growth activity of the yeasts, by detecting the heat evolved during growth of cultures at 30°C. Details regarding the apparatus were reported elsewhere.¹ The large number of calorimeter units represents the main advantage of the apparatus over other designs; one growth experiment can provide enough data for a good estimation of inhibitory parameters, like those presented in the Table. However, during this work 2 or more experiments were performed for each set of conditions. A liquid glucose-peptide growth medium (containing, per liter, 20 g glucose, 2 g yeast extract, 0.5 g MgSO₄, 5 g polypeptide, and 1 g KH₂PO₄, pH 5.6) was inoculated with cells taken from the stock culture and then incubated at 30°C for 24 hours. After that, a suspension of yeast cells was prepared by conveniently diluting the culture with sterile distilled water to obtain a cell number on the order of 10⁶ cells/ml. Ethanol and methanol were aseptically added in concentrations up to 7.65% (v/v) to autoclaved vials containing 5 ml of the same medium used for inoculation. Then each vial was inoculated with 1 ml of yeast cells suspension, and sets of 24 vials thus prepared were introduced in the calorimeter and incubated at 30°C until the observed thermograms returned to baseline. The conditions used allowed complete consumption of nutrients by the yeasts.

Analytical procedures. The glucose concentration was checked at the end of one experiment and was found to be almost zero under the sensitivity level of a modified Somogyi-Nelson method.² The turbidity of the growth media was measured using a Hitachi U-1100 spectrophotometer at 660 nm. The growth medium itself presented a negligible optical density at this wavelength (approx. A₆₆₀ = 0.007). A Shimadzu GC-14A gas chromatograph with a flame ionization detector and a Supelcowax 10 column (0.53 mm diameter by 30 m length, 0.5 µm thickness) was used for the

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measurement of ethanol and methanol concentrations in the cultures. Chromatographic conditions were: oven temperature, 60°C; injector and detector temperature, 200°C; and carrier gas, He at a flow rate of 20ml min⁻¹. Yeast cells were removed by centrifugation and a sample (1 μl) of supernatant was injected directly into the gas chromatograph. Under such conditions, the retention times were 1.4 min for methanol and 1.7 min for ethanol. For counting the number of yeast cells we used a Thoma chamber.

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

Results
Calorimetric parameters

Figure 1a shows a representative example of the growth thermograms obtained during incubation of yeast cultures in the calorimeter. It can be observed that in the presence of increasing amounts of methanol thermograms broadened and the peak time was significantly delayed. Similar growth thermograms were obtained when ethanol was used as the inhibitor (data not shown). However, these growth thermograms, also named g(t) curves, are only the apparent output of the calorimeter. Because the calorimeter is isothermal and a permanent heat exchange takes place between the calorimetric unit and its surroundings, the thermograms must be corrected to calculate the actual heat evolution. As a result, for each thermogram the actual heat evolution process can be described by what we called an f(t) curve, which can be calculated using a previously reported equation⁷⁻⁹:

\[ f(t) = g(t) + K \int g(t) dt \]  

(1)

in which K is the heat conduction constant of the calorimeter (K was 0.1756 min⁻¹ in these experiments). Figure 1b shows the f(t) curves corresponding to the thermograms in Fig. 1a. In all experiments, f(t) curves for cultures with or without added alcohol reached approximately the same final height corresponding to a heat amount of 70–85 J, the level being related to the amount of nutrients available. This suggested that, in the concentrations used here, although both ethanol and methanol inhibited and delayed growth of yeasts, cells were still able to metabolize all the nutrients in the medium, therefore producing similar amounts of heat.

The relationship between f(t) and the number of cells or the turbidity for a culture of S. cerevisiae No. 9302 was investigated and results are shown in Figs. 2a and 3a. It can be observed that f(t) curve computed using Eq. (1) and given in Fig. 2c is quite similar to the cell number (Fig. 2a) and turbidity curves (Fig. 2b), the result being in good agreement with many previous findings.⁹⁻¹⁴ Their similarity is further substantiated in Fig. 3a, which shows that values of f(t) and cell number are very well correlated, as well as in Fig. 3b where the same good correlation is observed for f(t) and the turbidity of the culture. In both cases the good correlation could be assessed for at least 14 hours. We should say that this time is more than sufficient for the calculation of the growth rate constant μ with good accuracy. From a practical viewpoint, this means that for the purpose of analysis we can substitute, at least for the exponential portion, the curves given in Figs. 2a and b by the f(t) curve, which can be obtained more easily.

Based on these considerations, the f(t) curves can be used to obtain the growth rate constant. As was previously reported,¹⁵ the amount of heat evolved during the ex-
An exponential growth phase of microorganisms can be expressed as a simple exponential function as follows:

$$f(t) = AN_0 \cdot e^{\mu t} + BN_0$$  \hspace{1cm} (2)

where $\mu$ is the growth rate constant, $N_0$ is the number of viable cells at the start of the measurement (the inoculum size) and $A$ and $B$ are constants. Therefore, by fitting the initial portion of the $f(t)$ curves with the exponential function given in Eq. (2), we can obtain the value of the growth rate constant $\mu$. The portion of $f(t)$ used for fitting is the one between 3% and 30% of the total height of $f(t)$, which is of course included in the interval of good correlation between $f(t)$ and the actual cell number shown in Fig. 3a. On the other hand, since only the beginning of the curve is used for fitting, we can assume that the influence of limiting factors like nutrients and oxygen concentration is minimal. By applying the described fitting procedure to the 3 curves given in Figs. 2a, b, and c the following values for $\mu$ were obtained: $0.35 \pm 0.01 \text{ h}^{-1}$ from the cell number curve; $0.32 \pm 0.02 \text{ h}^{-1}$ from the turbidity curve; and $0.37 \pm 0.01 \text{ h}^{-1}$ from the $f(t)$ curve (precision given as standard deviation). The conclusion was that the values of $\mu$ determined from the $f(t)$ curves calorimetrically obtained are reliable and accurate.

If we name $\mu_i$ the growth rate constant obtained for a culture grown in the presence of alcohol in concentration $i$, and $\mu_m$ the maximum growth rate constant obtained in the absence of alcohol, then the parameter $\mu_i/\mu_m$, called “specific growth activity,” can be used to describe the inhibitory effect of alcohol on growth of yeast cells. A representative plot of the $\mu_i/\mu_m$ data against the ethanol concentration $i$ can be seen in Fig. 4. It was also shown\(^9,16\) that based on a simple interaction mechanism similar with that of non-competitive inhibition of enzymes the following equation can be derived:

$$\frac{\mu_i}{\mu_m} = \frac{1}{1 + (i/K_i)^m}$$  \hspace{1cm} (3)

where $i$ is the concentration of inhibitor, $m$ is a constant related to the cooperativity in the interaction between cells and the inhibitor, and $K_i$ is the concentration of inhibitor which reduces the growth activity of yeast cells by 50%. Equation 3 describes the so-called “drug potency curve.” On the other hand, if we express the loss in growth activity induced by alcohol presence as $1 - \mu_i/\mu_m$, and assume it to be proportional with a power of the concentration of alcohol, then this equation can be written\(^9\):

$$1 - \frac{\mu_i}{\mu_m} = k \cdot i^{m_1}$$  \hspace{1cm} (4)

where $k$ and $m_1$ are constants. From Eq. (4) it can be derived that the value given by

$$\text{MIC} = (1/k)^{1/m_1}$$  \hspace{1cm} (5)

represents the concentration of alcohol that should completely prevent cell growth, which is the usual meaning of the “minimum inhibitory concentration” (MIC). Figure 4 also shows the drug potency curve (solid line) computed based on Eq. (3) and the MIC curve (dotted line) calculated using Eq. (4). Values of $K_i$ and MIC calculated by this procedure for some yeast strains are presented in the Table, for methanol and ethanol.

**Ethanol production**

A kinetic curve for the ethanol produced was obtained for *S. cerevisiae* No. 9302 and is presented in Fig. 2d. Ethanol production followed the same pattern as the cell population, and Fig. 3c indicates that it is well correlated to the calorimetrically obtained $f(t)$. Also, the value of the growth rate $\mu$ calculated from the ethanol kinetic curve (Fig. 2d), using the fitting procedure presented above, gave the result $0.37 \pm 0.04 \text{ h}^{-1}$, comparable with the values determined from cell number, turbidity or $f(t)$ curves. These results indicate that fermentation of glucose took place in parallel with growth. Moreover, it can be seen in Fig. 5 that the pH changes of the cultures also followed growth, and the effect of added ethanol was visible only in a retardation of the pH decrease, but the final pH (after 29 hours of incubation) was found to be the same for cultures with 0%, 1.63%, and 3.21% (v/v) added ethanol.
Influence of Ethanol and Methanol on Yeast Growth

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Methanol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$</td>
<td>MIC</td>
</tr>
<tr>
<td></td>
<td>% (v/v)</td>
<td>% (v/v)</td>
</tr>
<tr>
<td>S. cerevisiae No. 9302</td>
<td>8.97 ± 0.30</td>
<td>14.06 ± 0.79</td>
</tr>
<tr>
<td>S. cerevisiae Hakken No. 1</td>
<td>8.36 ± 0.13</td>
<td>13.75 ± 0.33</td>
</tr>
<tr>
<td>S. cerevisiae IFO 2347</td>
<td>8.10 ± 0.29</td>
<td>15.36 ± 0.99</td>
</tr>
<tr>
<td>S. cerevisiae IFO 2363</td>
<td>8.18 ± 0.15</td>
<td>14.11 ± 0.48</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>8.33 ± 0.23</td>
<td>16.70 ± 0.86</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>3.06 ± 0.08</td>
<td>7.91 ± 0.48</td>
</tr>
<tr>
<td>Kluyveromyces marxianus</td>
<td>7.28 ± 0.11</td>
<td>12.53 ± 0.37</td>
</tr>
<tr>
<td>Hanseniaspora valbeyensis</td>
<td>11.04 ± 0.79</td>
<td>14.95 ± 1.49</td>
</tr>
<tr>
<td>Hansenula anomala</td>
<td>5.28 ± 0.15</td>
<td>6.45 ± 0.31</td>
</tr>
</tbody>
</table>

Precision given as standard deviation.

Fig. 5. Changes of pH in a Culture in the Absence of Ethanol (—○—) and in Cultures with Initial Addition of Ethanol in Concentration (v/v) 1.63% (—□—) and 3.21% (—△—).

A delay of pH decrease can be observed in the case when ethanol is added, but all curves reach approximately the same final pH.

Fig. 6. Ethanol Concentrations Observed after 48 h of Incubation in Yeast Cultures with Various Concentrations of Methanol Added.

In this case no ethanol was added at the beginning of incubation. The data correspond to: S. cerevisiae No. 9302 (—○—), Schizosaccharomyces pombe (—□—), K. marxianus (—△—), and C. utilis (—△—).

In a separate trial, cultures of S. cerevisiae No. 9302, Schiz. pombe, K. marxianus, and C. utilis were prepared with ethanol added at the beginning of incubation in concentrations of 0; 1.63; 3.21; 4.74; 6.22; or 7.65% (v/v), and the ethanol was measured by chromatography after 48 hours. Parallel calorimetric measurements showed that growth was over and the nutrients were exhausted in this interval. The differences between the final and initial ethanol concentrations were in the range of 0.47–0.97% (v/v), but no significant relationship could be observed between the amount of ethanol added initially and the amount produced by the yeasts during incubation. This seems to indicate that presence of ethanol in concentrations up to 7.65%, while strongly affecting the growth activity of yeasts (Table shows), does not prevent further degradation of glucose and ethanol production, which is in good agreement with results usually obtained in enological practice. This conclusion is also in agreement with the fact that the $f(t)$ curves reached approximately similar values, irrespective of the amount of ethanol added at the beginning of incubation.

Ethanol production of 4 yeast strains was further studied when methanol was also present in the growth medium and results are given in Fig. 6. Again, it is clear from Table that methanol concentrations of 7.65% are of the order of $K_i$ or even larger for some of the strains studied, which means that such concentrations have a strong inhibitory effect on the growth activity of yeasts. However, the ethanol concentrations obtained after 48 h of incubation in the presence of indicated amounts of methanol (Fig. 6) do not show large differences compared to standard cultures grown in the absence of methanol. Although Fig. 1 shows that even small methanol concentrations do inhibit growth, according to Fig. 6, small amounts of methanol in the medium seem to lead to a slight increase in the final concentration of ethanol. This result may be explained by the action of methanol at membrane level in the direction of fluidization of membrane and consequently improving the penetration of nutrients inside the cell. However, increasing amounts of methanol (more than 4% v/v) begin to have a slight decreasing effect on ethanol production.

Theoretically, by anaerobic degradation of all the 0.555 mmol of glucose present in each vial one would expect the formation of 1.11 mmol ethanol and production of 76 J of heat. Our actual results (examples being shown in Figs. 2c and d) generally ranged between 0.8–1.0 mmol ethanol after 48 h, and 70–85 J of heat, depending on the strains. In any case, the oxygen amount available in the free space of the vial was about 0.42 mmol, which would be only 1/8 of the amount necessary for the aerobic degradation of the existing glucose. Considering also the absence of agitation...
of the culture, we could conclude that our results are in agreement with the assumption that the degradation of glucose took place mainly anaerobically.

**Discussion**

It was shown in this study that the $f(t)$ curves that describe the actual heat evolution during incubation of the yeast cultures can be used for the calculation of the growth rate constant $\mu$. Changes in $\mu$ produced by inhibitors like ethanol and methanol can be analyzed and inhibition parameters like $K_i$ and MIC can be calculated. It is of course natural that values of $\mu$, $K_i$ and MIC are different from one yeast strain to another and depend on experimental conditions such as type and composition of growth medium, temperature, pH etc., but they show a good reproducibility under similar conditions. Accuracy in determination of $K_i$ is almost always much better than for MIC, because $K_i$ is usually in the range of concentrations actually used during growth experiments, while MIC is obtained by extrapolating the data observed. In the same time, differences among yeast strains regarding ethanol tolerance are more visible at high ethanol concentrations, so MIC may be preferred for comparison. Since only simple experiments are necessary and the calorimetric procedure allows precise determination of such inhibition parameters, we consider that this method may prove useful for the study of many other interactions between yeasts and inhibitors.

The calorimetric method can be correlated with other techniques and results obtained by calorimetry are generally in good agreement with the data already available. Numerous studies indicate that alcohols inhibit yeast growth by acting at the membrane level.\textsuperscript{17–21} It is believed that alcohols interfere with the transport systems of nutrients located in the plasma membrane. For example, Thomas and his coworkers\textsuperscript{17,18} showed that ethanol inhibits the uptake of nutrients by *S. cerevisiae*, and that this inhibition could be modulated by changing the lipid composition in the cellular membrane. The membrane potential and the proton gradient may also be affected by alcohols by inhibition of active proton transport or by increasing passive proton influx.\textsuperscript{19} Furthermore, there are many experimental data proving that alcohol inhibition is correlated with the length of the non-polar carbon chain in their molecule, which also indicates that the inhibitory action takes place at the hydrophobic membrane sites, interfering directly with the transport proteins or indirectly by changing the lipid environment in the membrane.\textsuperscript{20} In our experiments too, ethanol was more toxic than methanol, with the only exception of $K_i$ in case of *C. utilis* (Table). The values shown in Table are generally in the same range of concentrations described in previous findings, and they may provide additional information useful for clarifying the interaction between yeasts and alcohols.

It was also reported\textsuperscript{19} that the addition of butanol in cultures of *S. cerevisiae* led to a decrease in the final pH of the culture, a decrease which was proportional to the butanol concentration. Our results (Fig. 5) show that the pH decrease with time was slowed down by the presence of ethanol, effect that was visible both in the slope of the pH curve and in the time required to reach a certain pH value. The explanation may be based on the fact that the acidification process is accomplished with the contribution of many mechanisms,\textsuperscript{22,23} such as CO₂ production, organic acid release by cells in the medium, and active proton efflux, and all these processes are slowed down if a delay in growth takes place. The fact that in our experiments the final pH was not influenced by the ethanol concentration is probably due to the much larger buffering capacity of the growth medium we used.

It can be concluded that the calorimetric method based on detecting the heat evolved during microbial growth in particular conditions integrates all these inhibitory actions and allows the calculation of inhibitory parameters that may be especially valuable for practical and technological applications. The results presented in this study show that ethanol production and glucose degradation took place simultaneously with growth under the conditions used, and the influence of inhibitors like ethanol and methanol in certain concentrations on ethanol production was not significant. While these results may serve to characterize the growth behavior of yeasts in the presence of ethanol and methanol, it is difficult to extend conclusions obtained under these experimental conditions to processes in which fermentation is predominant, like those involved in the obtaining of sparkling wines and other fermented products.

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**References**