

Research Note

Characterization of ethanol tolerance of yeasts using a calorimetric technique

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Key words: yeast, ethanol tolerance of yeast, calorimetric determination.

Introduction: Most yeasts are traditionally known for ethanol production, which at the same time is a typical antimicrobial compound - a very complex and not fully elucidated interaction. Also, methods and procedures to assess yeast tolerance to ethanol are very diverse (ROSE 1987), while data are more or less confined to *Saccharomyces cerevisiae*. It is well known that metabolic activity of microbes is always associated with heat production (LAMPRECHT and SCHAARSCHMIDT 1977), and changes in this heat evolution due to inhibitors present in the medium can be quantitatively analyzed. In this work, a calorimetric technique (TAKAHASHI 1990) developed in our research group for the study of microorganism-drug interactions was applied to the study of ethanol inhibition of growth of 9 yeast strains.

Materials and methods: The yeast strains studied (Table) included *S. cerevisiae* No. 9302 (obtained from Mandom Central Research Laboratory, Osaka, Japan), *S. bayanus* EC 1118, *S. cerevisiae* IFO 2347, *S. cerevisiae* IFO 2363 (obtained from Suntory Ltd. Research Center, Osaka), *S. cerevisiae* Hakken No.1, *Hanseniaspora valbyensis* IFO 0115, *Hansenula anomala* IFO 0118, *Schizosaccharomyces pombe*, and *Candida utilis* IFO 0396 (stock cultures of the Laboratory of Fermentation Technology, Osaka Prefecture University).

Growth activity of yeasts was monitored by detecting the metabolic heat evolved during their growth in liquid medium at 30 °C. For the measurement, a multiplex isothermal batch calorimeter capable of monitoring 24 cultures simultaneously was used. (The apparatus is a new version of the model with 6 units reported by KAWABATA *et al.* 1983.) The calorimeter detects the differences in temperature between sample cultures and the reference unit and transforms it in voltage signals which are led out, digitized and stored on magnetic diskettes for further analysis. Common glass vials (50 ml) were employed as calorimetric vessels. Vials containing 5 ml of a glucose-peptone medium were autoclaved, then various amounts of ethanol were aseptically added. The inoculum consisted of 1 ml

suspension containing approximately 10⁶ yeast cells (pre-incubated for 24 h on the same type of medium). Inoculum sizes were checked by counting with a Thoma chamber.

Sets of 24 cultures prepared as described were introduced in the calorimeter and incubated for up to 72 h, *i.e.* until growth ceased in all vials.

Results and discussion: The primary results provided by the calorimeter are the so-called "growth thermograms" - hereafter also named $g(t)$ curves. An example is shown in Fig. 1 a. As a general observation, with increasing ethanol concentration in the medium, the growth thermograms broadened, their initial slope decreased and the peak height was significantly reduced.

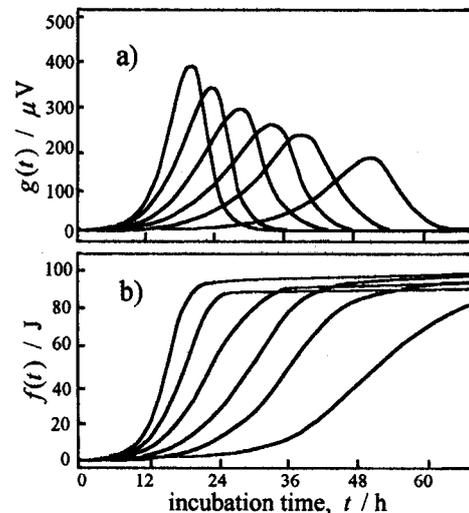


Fig. 1: a) Typical growth thermograms obtained during incubation in the calorimeter of yeast cultures of *S. cerevisiae* No. 9302 corresponding to media containing (from left to right): 0, 0.9, 1.8, 2.6, 3.5 and 5.1 % ethanol (v/v). b) Actual heat evolution curves determined by correcting the growth thermograms given in a), using equation (1).

The $g(t)$ curves, however, are only the apparent output of the calorimeter; they must be corrected for the heat exchange between the sample and the thermostated bath, through the unit wall. Accordingly, the actual heat evolution curve, namely $f(t)$, for each calorimetric unit can be calculated using the following equation (KIMURA and TAKAHASHI 1985):

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

where K is a constant of the apparatus.

The $f(t)$ curves thus calculated can be seen in Fig. 1 b. $f(t)$ values were transformed in units of J using a constant of the calorimeter. These curves were found to be in very good agreement with the cell number and turbidity of the cultures, and therefore they may be used for the determination of the specific growth rate constant μ . For this purpose, the initial portion of $f(t)$ - corresponding to the exponential growth phase - can be fitted by regression analysis with the previously reported relation (KIMURA and TAKAHASHI 1985):

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

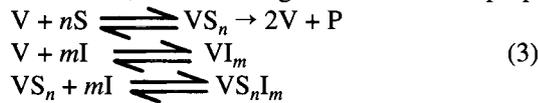
where μ is the specific growth rate constant, N_0 is the initial number of viable cells, and A and B are constants. This

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regression gives the value of μ . If we define μ_m as the maximum specific growth rate (observed in absence of ethanol and in excess of nutrients) and μ_i as the specific growth rate obtained for a culture with the ethanol concentration i , then the parameter μ_i / μ_m can be regarded as the specific growth activity of yeast cells in the presence of ethanol at concentration i .

In order to characterize the action of an inhibitor upon microbial cells, the following mechanism was proposed:



where V denotes a viable cell, P a product, S the substrate and I is the inhibitor (ethanol, in our case). Based on K_s and K_d - the substrate constant and the dissociation constant of ethanol that may be written for these equations - the following relation can be derived:

$$\mu_i = \mu_m / (1 + i^m / K_d) \quad (4)$$

A regression analysis of the experimental data on the basis of eq. (4) provides the values of K_d and m . Fig. 2 shows a plot of μ_i / μ_m against ethanol concentration i ; the solid line is the graphic representation of eq. (4), also named "ethanol potency curve", obtained for the *S. cerevisiae* No. 9302 strain.

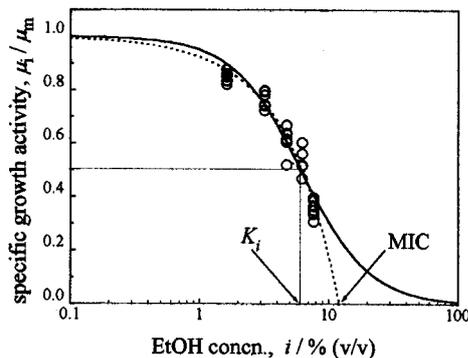


Fig. 2: Ethanol potency curve (solid line) and MIC curve (dotted line) for *S. cerevisiae* No. 9302.

As can also be understood from eq. (4), the value of $K_i = K_d^{(1/m)}$ (5)

corresponds to the ethanol concentration at which growth activity is inhibited by 50%, whereas m reflects the cooperativity of ethanol action.

Furthermore, if we assume that the loss in growth activity due to ethanol action, $(1 - \mu_i / \mu_m)$, is proportional to the m_i -th power of ethanol concentration, i , then we have the relation:

$$1 - \mu_i / \mu_m = k_i \cdot i^{m_i} \quad (6)$$

where k_i is a constant. From eq. (6), the ethanol concentration MIC at which the growth activity is completely lost, is given as:

$$\text{MIC} = (1/k_i)^{(1/m_i)} \quad (7)$$

The diagram of eq. (6) (also named "MIC curve") is shown in Fig. 2 as the dotted line, and its intercept on the i -axis corresponds to the value of MIC given by eq. (7).

The Table gives the determined values of m (cooperativity parameter), K_i (50% inhibition concentration)

T a b l e

Ethanol inhibition parameters for different yeast strains (calorimetrically determined)

Strain	m	K_i^*	MIC*
<i>S. cerevisiae</i> No. 9302	1.7	6.1	12.1
<i>S. bayanus</i> EC 1118	1.1	6.9	18.4
<i>S. cerevisiae</i> IFO 2347	1.6	7.0	13.3
<i>S. cerevisiae</i> IFO 2363	2.0	6.4	11.3
<i>S. cerevisiae</i> Hakken No.1	1.7	7.8	13.6
<i>Hanseniaspora valb.</i> IFO 0115	2.2	8.6	12.6
<i>Hansenula anomala</i> IFO 0118	1.5	4.6	10.5
<i>Schizosacch. pombe</i>	1.6	5.8	12.2
<i>Candida utilis</i> IFO 0396	2.2	3.4	6.3
Max. standard error*	0.2	0.4	1.1

*) in % ethanol (v/v)

and MIC (100% inhibition concentration) determined for the 9 strains. Results are in good agreement with other reported data determined by different procedures (e.g., ROSE 1987). The examination of any of these parameters may show both the resemblances and the differences among yeast strains. However, the parameters are related and all of them must be considered for a consistent analysis. Strains with the same K_i (e.g., *S. bayanus* and *S. cerevisiae* IFO 2363) may show very different MIC values, depending on how steep the ethanol potency curves are (the larger the value of m , the steeper the ethanol potency curve). The most resistant strain was *S. bayanus* (often employed for second fermentations in sparkling wine production), while the least resistant were *Hansenula anomala* and *Candida utilis*. Results are in good agreement with the sequence of yeast species that develop during spontaneous fermentation of grape juice (KUNKEE and GOSWELL 1977). Good accuracy could be obtained using data from 2 experiments (48 cultures).

It is assumed that the procedure very briefly presented here may prove to be a useful and convenient tool for the comparison of yeast strains from the point of view of ethanol resistance, as well as for the study of interactions between yeasts and various other substances.

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