ELSEVIER

Thermochimica Acta 297 (1997) 33–42

thermochimica acta

Calorimetric determination of the inhibitory effect of C1-C4 *n*-alcohols on growth of some yeast species[1]

Oana-Arina Antoce[2,a], Vasile Antoce[a], Katsutada Takahashi[a,*] Nicolai Pomohaci[b], Ion Namolosanu[b]

[a] *Laboratory of Biophysical Chemistry, College of Agriculture, Osaka Prefecture University, Sakai, 1-1 Gakuen-cho, Osaka 593, Japan*
[b] *Viticulture and Enology Department, Bucharest University of Agricultural Sciences, Marasti Str. 59, sector 1, 71329 Bucharest, Romania*

Received 18 December 1996; received in revised form 2 April 1997; accepted 4 April 1997

Abstract

The inhibitory action of methanol, ethanol, 1-propanol and 1-butanol on growth of nine yeast strains was quantitatively assayed using a calorimetric technique. Yeast cultures in the presence of alcohol at various concentrations were incubated in a multiplex batch isothermal calorimeter, which allowed the recording of the heat evolved during microbial growth. Changes were visible in the pattern of the thermal profiles recorded when the alcohol concentration in the growth medium increased: the initial slope of the thermal profiles decreased significantly, and the peak time was retarded. By analyzing both these modifications, quantitative parameters could be obtained to characterize the inhibitory action of alcohols. The parameters determined were: K_μ, K_θ (the alcohol concentrations that inhibited 50% of the growth activity) and MIC_μ, MIC_θ (minimum inhibitory concentrations), where the subscript 'μ' stands for determination based on the changes observed in the growth rate constant and the subscript 'θ' stands for determination based on the retardation of growth. Results showed that the alcohol tolerance of the yeasts decreases significantly with the increase in the number of carbon atoms in the alcohol molecule, although the linear relationship reported in other studies between the alcohol tolerance of yeasts and the number of carbon atoms in the alcohol molecule could not be confirmed for all species. © 1997 Elsevier Science B.V.

Keywords: Alcohol tolerance; Microcalorimetry; Yeasts

1. Introduction.

Alcohols represent a well-established class of disinfectants and their inhibitory action against microorganisms has been studied for a long time. Two of the directions followed until now were: to quantitatively characterize their effect on microorganisms, and to determine if there is any relationship between the increase in alcohols' chain length and their inhibitory effect [1,2]. However, important imprecision in the determination of inhibition parameters like MICs (the minimum inhibitory concentration, or the minimum drug concentration at which growth is totally inhibited) may have affected the quantitative conclusions derived. For example, Huhtanen [2] reports values of MIC for C1-C12 alcohols in the case of *Clostridium botulinum*, determined visually by checking if turbid-

*Corresponding author. Tel.: 00 81 722 50 0525; fax: 00 81 722 50 0525; e-mail: ktakahas@biochem.osakafu-u.ac.jp.

[1]Presented in part at the 14th International Conference on Chemical Thermodynamics (ICCT96), Osaka, Japan.

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

PII S0040-6031(97)00162-7

ity was apparent or not in cultures to which the inhibitor was added. When, for example, microbial growth was detected in a sample with 20 000 ppm of inhibitor, and undetected at 40 000 ppm, the MIC was estimated to be between these values and taken as 30 000 ppm. Due to the obviously poor accuracy of the procedure, the effects of methanol and ethanol could not be quantitatively distinguished. Such difficulties are often encountered in the case of the classic microbiological techniques, and procedures offering better accuracy in the quantitative characterization of the effects of alcohols on microorganisms seem necessary.

Relationships between the molecular structure of chemical compounds and their activity against microorganisms (called structure-activity relationships or SAR) have been studied extensively by Beezer et al. [3,4]. Measurement by microcalorimetry of the microbial response in the presence of drugs at various concentrations allowed the determination of $\log(dose)_{max}$, a parameter corresponding to the drug concentration which reduces the response to zero. An SAR was found, for example, between the antifungal activity as expressed by $\log(dose)_{max}$, and log P (logarithm of the partition coefficient for transfer from water to octan-1-ol) for a series of derivatives of cardanol [3]. In the same way, linear correlation was observed between $\log(dose)_{max}$ and the molecular size of some amine-terminated polyethylene glycols [4]. In all these situations, the molecular size of the inhibitors was much larger than for the alcohols studied in the present work. Molecules of C1-C4 alcohols penetrate the cellular membranes much easier, and, as a result, the mechanisms of interaction between alcohols and microorganisms are complex and yet not fully understood.

Although many previous studies on the antimicrobial activity of alcohols were focused on bacterial species, with emphasis on applications related with food preservation techniques, the interaction between yeasts and alcohols may be considered even more interesting [5–7]. Yeasts are more tolerant to alcohols than bacteria because they can adapt their membrane composition in order to resist at higher alcoholic concentrations. On the other hand, some yeast species are known to metabolize alcohols in certain amounts. This happens with *Hansenula anomala*, which is able to use methanol as a substrate. Study of alcohols inhibition on yeast growth may also prove useful with regard to the preservation of food and beverages [8], but it is certainly important for fermentation processes, where alcohols simultaneously play the roles of products and inhibitors.

The thermal effect associated with microbial activity has been extensively studied using various techniques [9–11]. In this work, the inhibitory action of the alcohols with 1 to 4 aliphatic carbons on growth of nine yeast strains was investigated using a calorimetric procedure developed for the study of microorganism–drug interactions [12–14]. The inhibitory effect of alcohols was clear in decreasing the growth rate of yeasts and increasing the incubation time required for the cultures to reach a certain activity level. Both these aspects could be analyzed based on a special procedure which allowed precise quantitative determination of inhibition parameters.

2. Materials and methods

2.1. Microorganisms

A number of seven yeast strains from the Laboratory of Fermentation Chemistry of Osaka Prefecture University (*Saccharomyces cerevisiae* Hakken No. 1, *Schizosaccharomyces pombe*, *Candida utilis* IFO 0396, *Hansenula anomala* IFO 0118, *Hanseniaspora valbyensis* IFO 0115, *Kluyveromyces marxianus* IFO 0260 and *Saccharomyces cerevisiae* No. 9302), plus two strains of *Saccharomyces cerevisiae* (*S. c.* IFO 2363 and *S. c.* IFO 2347), obtained from Suntory Limited Research Center, were included in this study.

2.2. Chemicals

All chemicals employed were obtained from Wako Pure Chemical Industries Inc. and were of certified reagent grade. As growth medium a glucose-peptone broth (20 g l^{-1} glucose, 2 g l^{-1} yeast extract, 0.5 g l^{-1} $MgSO_4$, 5 g l^{-1} polypeptone and 1 g l^{-1} KH_2PO_4, pH 5.6, lot S103) was employed.

2.3. Sample preparation

For each yeast strain the same procedure was applied, as follows. Yeast cells taken from the stock culture were preincubated at 30°C for 16 h. At the end

of this period, the culture was diluted with sterile distilled water in order to obtain a suspension of $\approx 10^6$ cells cm^{-3}, a number that was checked by counting with a Thoma chamber. Vials of 50 cm^3 volume containing 5 cm^3 of the same growth medium used for preincubation were prepared and autoclaved, and various amounts of alcohol were added in each vial. The concentration of alcohols varied between 0 and 7.70% (v/v) for methanol and ethanol, 0–2.43% v/v for 1-propanol and 0–1.30% for 1-butanol. Usually, groups of four vials were prepared with the same concentration of alcohol, in order to check the reproducibility of the growth process. Finally, 1 cm^3 of the diluted yeast cells suspension was added as inoculum. Sets of 24 vials were introduced in the calorimetric units and incubated at 30°C until the calorimetric signal of all samples returned to baseline. Glucose in the cultures was checked at the end of one experiment by using a slightly modified version of the Somogyi–Nelson's photometric method [16] which allows detection of glucose concentrations as small as 5 $\mu g\ cm^{-3}$, and no glucose residues were found. It was concluded that growth in the vials ceased because of the exhaustion of nutrients.

2.4. Calorimeter

The apparatus employed was a 24 sample-units batch calorimeter, a new design [13] of the previously reported 6-units version [17]. The apparatus, working based on the conduction principle, detects the difference in temperature between the sample units containing vials with yeast cultures and a reference unit containing a vial with the same amount of water as the samples. Thermopile plates conveniently placed in each calorimetric unit transform this temperature difference into a voltage signal, which is related to the heat flow established between the sample unit and its surroundings. The 24 signals obtained from the samples are measured at appropriate time intervals (10 min in this study) and recorded on magnetic disk for further analysis. The calorimeter was calibrated prior to the experiments, by recording the calorimetric signal while a voltage of 0.5 V DC was established across a 47 Ω heater placed in the calorimetric unit. The experimental conditions during calibration (temperature, sensitivity scale of the apparatus, type of vial and amount of liquid in the vial) were kept as close as possible to the conditions employed during actual microbial growth experiments. With this procedure the parameter for transforming the original voltage signal into power units was found to be $A = 17.2 \pm 0.1\ \mu W\ \mu V^{-1}$. Also, as will be shown below, an 'actual heat evolution curve' named $f(t)$ may be computed from the original calorimetric signal. The values of $f(t)$ can be transformed into heat units by multiplication with the parameter $\beta = 5.91 \pm 0.01\ mJ\ \mu V^{-1}$.

3. Results

Fig. 1(a) shows a representative example of calorimetric recordings, also hereafter called '$g(t)$ curves', observed during incubation in calorimeter of yeast cultures to which methanol was added in various concentrations. In fact, this type of thermal profile was obtained in the case of all four alcohols, with only minor differences of the pattern (height, width, shape) which varied with the yeast species employed. This pattern of calorimetric recording has been shown previously to be characteristic of drugs with bacteriostatic action [18]: all the $g(t)$ curves practically start ascending together, and only their initial slope decreases when the initial concentration of alcohol increases. In contrast, when a drug shows strong bactericidal action [19], the initial number of viable cells is significantly reduced, and the thermal profiles appear to be 'delayed' (the delay depending on drug concentration), but their slope remains approximately independent over a certain range of drug concentrations. It was concluded, therefore, that in the concentrations used throughout this study, all the alcohols employed mainly showed a bacteriostatic action.

Examining Fig. 1(a) the effect of increasing amounts of alcohol can be observed in two aspects: the initial slope of the $g(t)$ curves decreases, while the incubation time required for the curves to reach a certain level is significantly increased. These two aspects can be quantitatively analyzed through appropriate procedures in order to characterize numerically the inhibitory action of alcohols.

For this purpose, the $g(t)$ curves given in Fig. 1, which represent only an apparent output of the apparatus, are first corrected to give the real heat evolution curves in the calorimetric units, using the previously

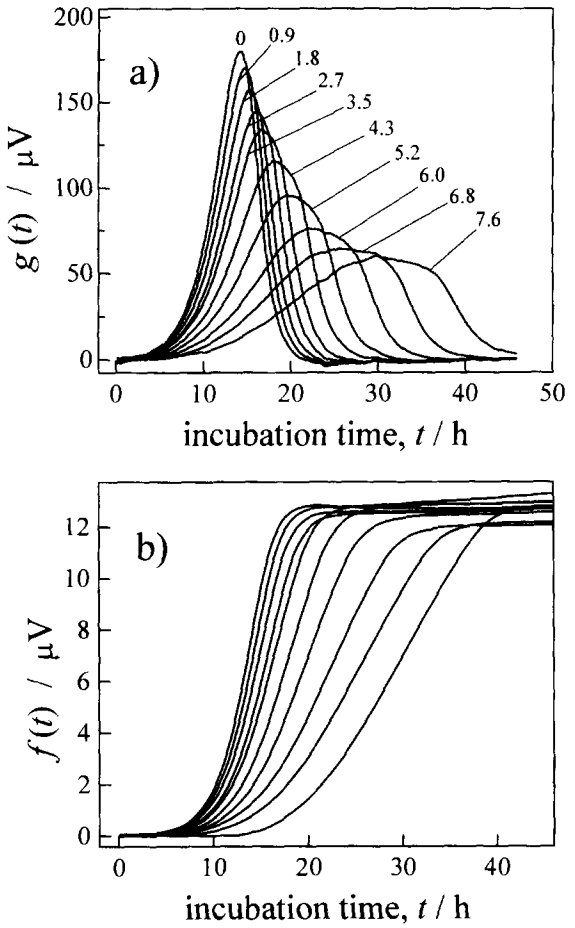

Fig. 1. Calorimetric results obtained for the incubation of *Saccharomyces cerevisiae* No. 9302 in the presence of methanol at various concentrations. (a) Calorimetric recordings or '$g(t)$ curves' obtained during incubation in the calorimeter at 30°C of yeast cultures to which methanol was added. The number indicated for each curve represents the methanol concentration, in % (v/v). Inoculum size was 1.7×10^6 cells per vial. (b) $f(t)$ curves determined on the basis of Eq. (1) from the $g(t)$ curves given in Fig. 1(a).

reported equation [20,21]:

$$f(t) = g(t) + K \int g(t)\mathrm{d}t \qquad (1)$$

in which K is the heat conduction constant of the calorimeter. The $f(t)$ curves thus determined (given in Fig. 1b), take into account the heat exchange that occurs permanently between the calorimetric units and their surroundings, phenomenon typical to isothermal calorimeters. Therefore, the curves described by Eq. (1) represent the heat amount developed in the calorimetric units as it would be recorded in a hypothetical adiabatic calorimeter.

The $f(t)$ curves are in very good agreement with the growth process that takes place in the cultures. Our experiments [12,13] proved that $f(t)$ and the number of cells, for example, are very well correlated for incubation times up to 14–16 h. For this reason, the $f(t)$ curves may be used in good conditions for the determination of the growth rate constant μ, by fitting their initial portion with a simple exponential function [21] given by:

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

where μ is the growth rate constant, N_0 the number of viable cells at the start of the measurement (the inoculum size), and A and B are constants. This procedure is illustrated in Fig. 2(a) in which the calorimetrically obtained $f(t)$ curves are shown as the solid lines, and the curves fitted using Eq. (2) are shown as the dotted lines. If the value of the growth rate constant obtained for the general case of a culture in the presence of alcohol at concentration i is called μ_i, and the maximum value obtained in the absence of alcohol is μ_m, then the parameter μ_i/μ_m can be considered to represent the 'specific growth activity' of yeast cells, which varies with the inhibitor concentration i. This parameter is subsequently analyzed in order to characterize the decrease in the slope of the $g(t)$ curves which is caused by the presence of alcohol.

As mentioned before, the other effect of alcohols, visible in the patterns of the $g(t)$ curves, is the delay of yeast growth. To analyze this effect quantitatively we employed the time derivatives of $f(t)$ curves, as shown in Fig. 2(b). A certain level α of $f'(t)$ is selected in the exponential region of the $f'(t)$ curves, and the time required for the cultures to reach this level is denominated as $t_\alpha(0)$ for the culture in the absence of inhibitor, and $t_\alpha(i)$ for cultures with the concentration of inhibitor equal with i. Accordingly, the parameter $t_\alpha(0)/t_\alpha(i)$, called the 'specific growth retardation', is used to describe the delay in growth caused by the inhibitory action of alcohols. Analysis showed that $t_\alpha(0)/t_\alpha(i)$ is almost independent on the level α, as long as α remains in the exponential region of $f'(t)$ and does not take extremely low or high values.

A general mechanism was proposed [12,14,22] to describe the interaction between microorganisms and

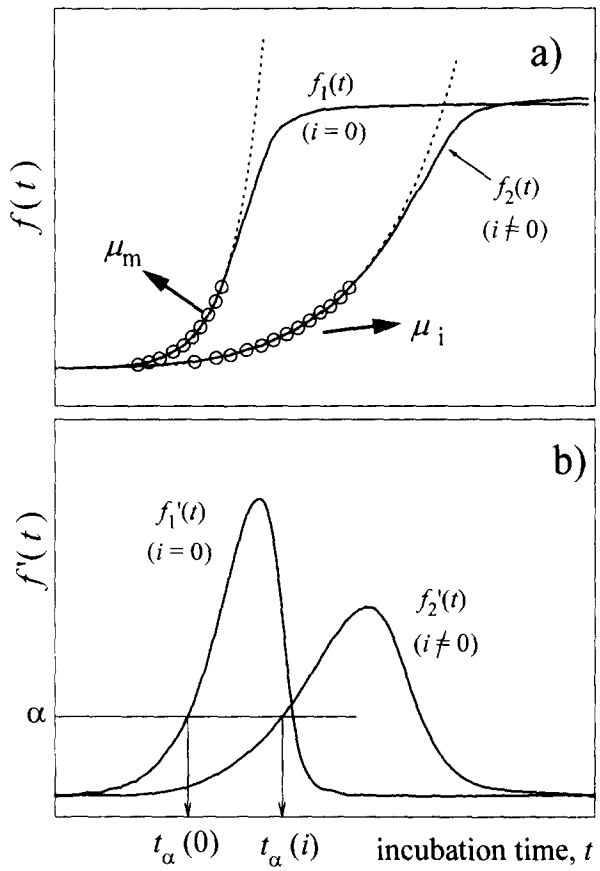

Fig. 2. Schematic representations of the procedures employed for the analysis of the calorimetric results. (a) Determination of the growth rate constant μ. The circles represent the data points situated between 3 and 30% of the height of the $f(t)$ curves. These points are fitted with the exponential function given by Eq. (2) and the value of μ for each curve is thus obtained. (b) Determination of the growth retardation time t_α. A horizontal line is drawn at the value $f'(t) = \alpha$ and the intersections with the $f'(t)$ curves lead to the values of t_α.

inhibitors, according to Eq. (3):

$$\begin{aligned} &V + nS \rightleftharpoons VS_n \rightarrow 2V + P \\ &V + mI \rightleftharpoons VI_m \\ &VS_n + mI \rightleftharpoons VS_nI_m \end{aligned} \tag{3}$$

Thus, it is considered that the viable cell V incorporates n moles of substrate S to form an intermediate VS_n which in turn leads to two cells and a by-product P. The action of the inhibitor I (the alcohol, in our case) leads to non-viable states VI_m and VS_nI_m. It can be

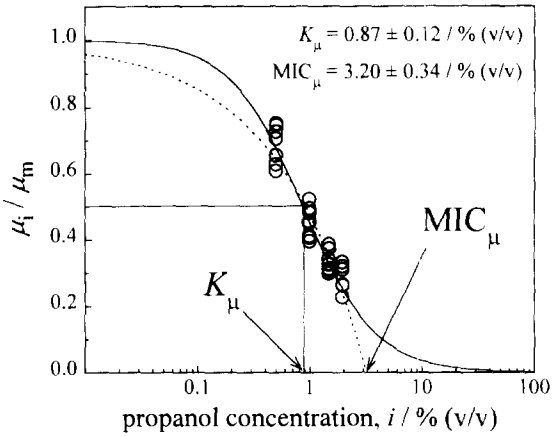

Fig. 3. Example of drug potency curve (solid line) and MIC curve (dotted line) for propanol in case of *Schizosaccharomyces pombe*, determined on the basis of the specific growth activity μ_i/μ_m.

shown that, by writing the substrate constant and the dissociation constant of alcohol for the reactions (3), the following equation can be derived for the situation when the substrate is in excess [12,14,22]:

$$\mu_i/\mu_m = 1/(1 + i^{m_\mu}/K_d^\mu) \tag{4}$$

where i is the alcohol concentration, K_d^μ stands for the dissociation constant of the alcohol, and m_μ is a parameter related to the cooperativity in alcohol action.

Fig. 3 shows a plot of the specific growth activity μ_i/μ_m against the propanol concentration, using the data obtained for the case of *Schizosaccharomyces pombe*. The solid line is the graphic representation of Eq. (4), and the value

$$K_\mu = K_d^{\mu(1/m_\mu)} \tag{5}$$

represents the alcohol concentration that inhibits 50% of the growth activity of yeasts as evaluated from the decrease in the growth rate constant.

An equation very similar to Eq. (4) can be written for the other set of experimental data, related to the growth retardation [12]:

$$t_\alpha(0)/t_\alpha(i) = 1/(1 + i^{m_\theta}/K_d^\theta) \tag{6}$$

An example of the usage of Eq. (6) is shown in Fig. 4 which represents a plot of the specific growth retardation $t_\alpha(0)/t_\alpha(i)$ against the butanol concentration, with the $t_\alpha(0)/t_\alpha(i)$ data obtained

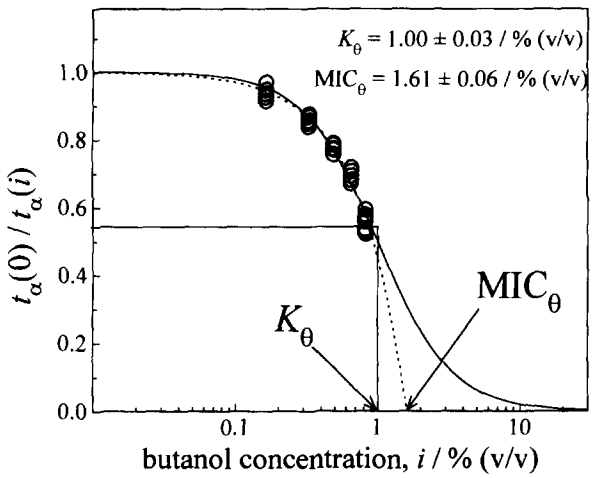

Fig. 4. Example of drug potency curve (solid line) and MIC curve (dotted line) for butanol in case of *Kluyveromyces marxianus*, determined on the basis of the specific growth retardation $t_\alpha(0)/t_\alpha(i)$.

for *Kluyveromyces marxianus*. The solid line corresponds to the graphic representation of Eq. (6), and the parameter:

$$K_\theta = K_d^{\theta(1/m_\theta)} \quad (7)$$

represents the alcohol concentration that doubles the time required for the cultures to reach a certain level of the calorimetric signal, compared to a culture in the absence of alcohol.

As stated above, Figs. 3 and 4 describe the procedures actually used for the determination of the 50% inhibitory concentration of inhibitors. They are plots of parameters μ_i/μ_m or $t_\alpha(0)/t_\alpha(i)$, which describe the changes observed in the thermal profiles due to the presence of the inhibitor, versus the inhibitor concentration. Similar procedures reported elsewhere [3,4] make use of plots of the 'calorimetric response' against the dose of antimicrobial, which is a more direct representation of the effect of the chemical against cells, as observed with the microcalorimeter. In our case, because the monitored phenomenon is the production of heat during microbial growth, the effect of inhibitors must be expressed by means of parameters like μ_i/μ_m or $t_\alpha(0)/t_\alpha(i)$. The data points in Figs. 3 and 4 could, of course, be fitted with any kind of empirical function. However, although the mechanism described by Eq. (3) is far from perfectly describing the drug-cells interaction, we believe that use of Eq. (4), derived from the mechanism given by Eq. (3), is preferable in the case of experiments like the ones described in this work.

The solid lines in Figs. 3 and 4, determined by regression analysis based on Eqs. (4) and (6), represent the so-called 'drug potency curves', or 'alcohol potency curves' in this particular case. The parameters K_μ and K_θ, respectively, determined for the nine yeast strains studied using the procedure presented above are shown in Figs. 5 and 6 as a function of the number of aliphatic carbons in the alcohol molecule.

The drug potency curves cannot be used for the estimation of the minimum inhibitory concentration. For this purpose, another set of equations was applied to the same experimental data. The expressions $1 - \mu_i/\mu_m$ and $1 - t_\alpha(0)/t_\alpha(i)$ represent the loss in growth activity caused by the presence of alcohol.

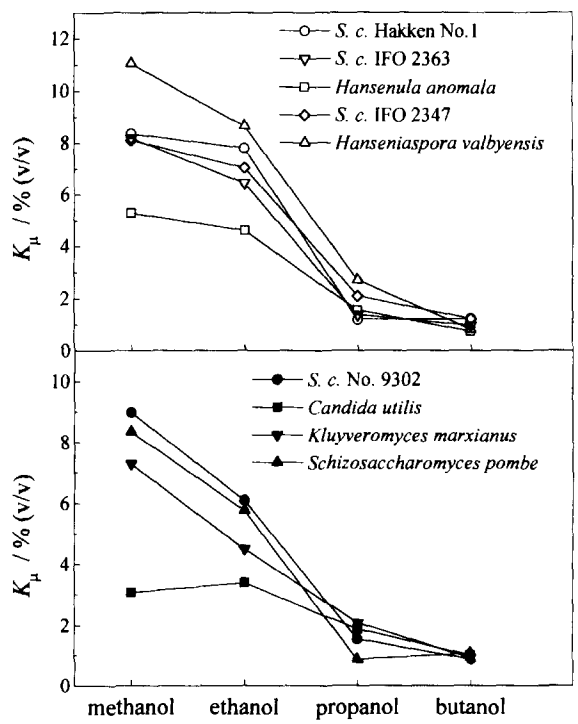

Fig. 5. Values of the 50% inhibitory concentration (K_μ) evaluated from μ_i/μ_m for the nine yeast strains, plotted against the number of aliphatic carbons in alcohol molecule.

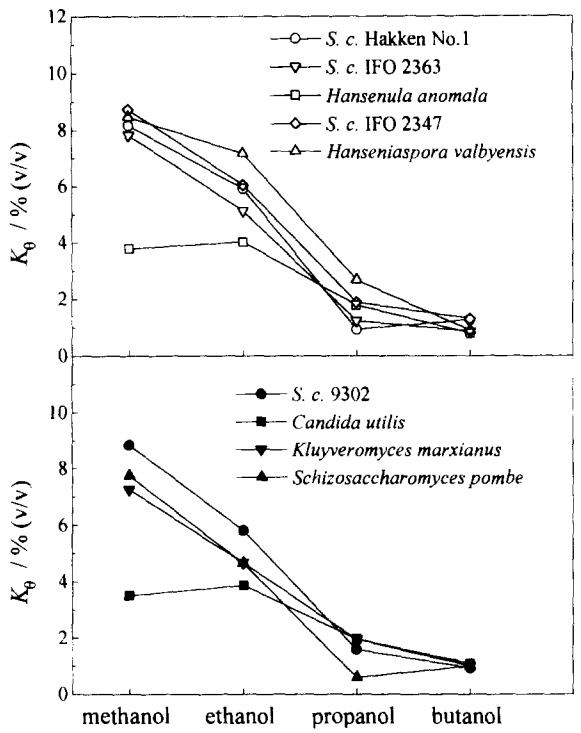

Fig. 6. Values of the 50% inhibitory concentration (K_θ) evaluated from $t_\alpha(0)/t_\alpha(i)$ for the nine yeast strains, plotted against the number of aliphatic carbons in alcohol molecule.

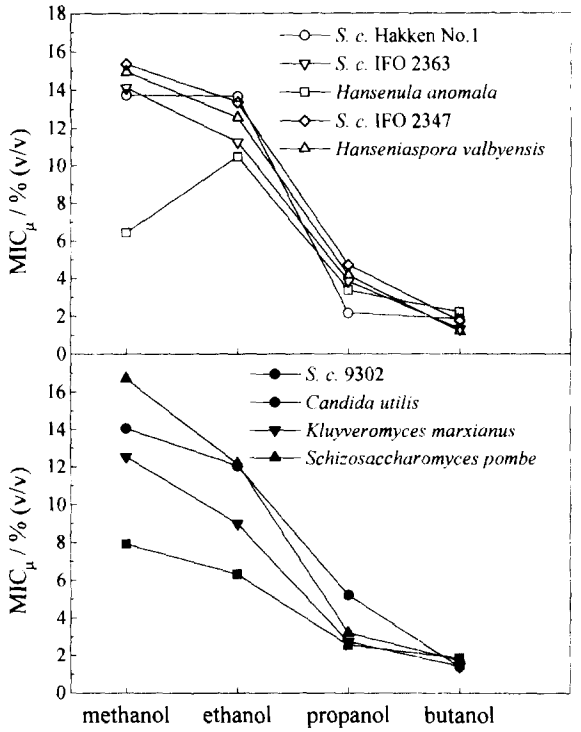

Fig. 7. Values of the 100% inhibitory concentration (MIC_μ) evaluated from μ_i/μ_m for the nine yeast strains, plotted against the number of aliphatic carbons in alcohol molecule.

Assuming that this loss is proportional to a power of the alcohol concentration i, then the following two equations can be written [12]:

$$1 - \mu_i/\mu_m = k_1 i^{m_1} \tag{8}$$

$$1 - t_\alpha(0)/t_\alpha(i) = k_2 i^{m_2} \tag{9}$$

where k_1, k_2, m_1 and m_2 are constants.

The dotted lines in Figs. 3 and 4 are the graphic representations of Eqs. (8) and (9), respectively, and are called 'MIC curves'. The parameters given by:

$$MIC_\mu = (1/k_1)^{1/m_1} \tag{10}$$

$$MIC_\theta = (1/k_2)^{1/m_2} \tag{11}$$

represent estimations of the minimum inhibitory concentration (MIC), evaluated calorimetrically. The values of MIC_μ and MIC_θ determined for the nine yeast strains studied are shown in Figs. 7 and 8, respectively.

4. Discussion

Due to the variety of yeast species and alcohols tested, results were also miscellaneous. For example, the values of m_μ, which characterize the cooperativity in alcohol action against yeast cells, were found to range between 1.02 (*Hansenula*, butanol) and 4.02 (*Hansenula*, methanol), while m_θ ranged between 1.25 (*Schizosaccharomyces*, propanol) and 3.65 (*Hansenula*, methanol). The values of K_μ were between 0.74% (*Hansenula*, butanol) and 11.04% (*Hanseniaspora*, methanol), and those for K_θ were between 0.59% (*Schizosaccharomyces*, propanol) and 8.83% (*S. c.* 9302, methanol). The values for MIC_μ were between 1.18% (*Hanseniospora*, butanol) and 16.70% (*Schizosaccharomyces*, methanol), while MIC_θ ranged between 1.26% (*S. c.* IFO 2363, butanol) and 12.61% (*S. c.* IFO 2347, methanol). (All K_μ, K_θ, MIC_μ and MIC_θ are given in % v/v.) The parameters were determined analyzing data obtained from 40–60 cultures, and the largest standard error encountered was

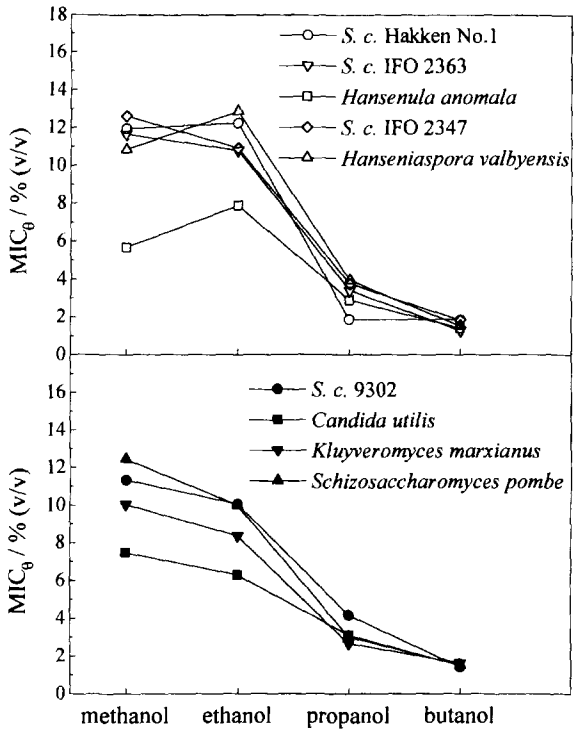

Fig. 8. Values of the 100% inhibitory concentration (MIC$_\theta$) evaluated from $t_\alpha(0)/t_\alpha(i)$ for the nine yeast strains, plotted against the number of aliphatic carbons in alcohol molecule.

for m_μ (*Hansenula*, methanol) and represented ±12.4% of this particular m_μ value.

The parameters determined as described above allow a good quantitative characterization of the inhibitory effect of alcohols on yeast growth. As presented, the values of m_μ, K_μ and MIC$_\mu$ calorimetrically determined are derived from the changes observed in the value of the growth rate constant μ, while m_θ, K_θ and MIC$_\theta$ are determined from the changes that occur in the retardation of growth due to the presence of inhibitor. It is known that one of the disadvantages of the classic method for determination of MIC is that cultures may begin to grow after the moment arbitrarily chosen for the determination of count cell or turbidity. The method presented in this paper may be one way of overcoming this difficulty, since one set of the parameters determined are derived from the time retardation of growth induced by the presence of inhibitor.

It can be shown that in case of drugs with pure bacteriostatic action, the values of K_μ and K_θ (or MIC$_\mu$ and MIC$_\theta$) should be equal, while in the case of strong bactericidal drugs (which reduce the inoculum size but do not affect the growth rate μ) these values differ significantly. Our results showed no significant differences between K_μ and K_θ or between MIC$_\mu$ and MIC$_\theta$, which allows the conclusion that all the four alcohols acted mainly as bacteriostatics in the ranges employed during experiments, for all nine yeast strains. Moreover, it should be stressed that MIC$_\theta$, for example, is defined as the drug concentration which will ensure that the culture doesn't reach a certain level of the calorimetric signal. This does not mean that the presence of the drug in concentration MIC$_\theta$ will necessarily inhibit the microbial growth completely, since growth might still take place, for example, at a very slow rate, below the sensitivity range of the calorimeter. In conclusion, the parameters to be taken into consideration are those determined on the basis of the growth rate constant μ; the others (m_θ, K_θ and MIC$_\theta$) are presented just for their role as an alternative which is preferred in the case of drugs with bactericidal effect.

The values of the cooperativity parameters m_μ and m_θ are directly related to the slope of the drug potency curves shown in Figs. 3 and 4 (solid lines). Therefore, the larger the values of m_μ and m_θ, the narrower is the total range of inhibitor concentration that may be tolerated by the microorganisms and observed in such experiments. For example, having a m_μ of 1.49, methanol affected growth of *S. cerevisiae* IFO 2347 up to the concentration of 15.36% (v/v), after which the cells did not grow any more. In contrast, in the case of *H. anomala* a greater cooperativity in methanol action against cells, which lead to the value m_μ=4.02, makes growth of yeast impossible at concentrations of methanol above 6.5% (v/v). It is also obvious that the two species with similar K_μ values, for example, may exhibit quite different MIC$_\mu$ values, if they show different values for the m_μ parameter.

Examining Figs. 5–8 it can be seen that the values of K_μ, K_θ, MIC$_\mu$ and MIC$_\theta$ are generally decreasing with the alcohol chain length. Large differences could be observed among species regarding resistance to methanol, with *Candida utilis* and *Hansenula anomala* being clearly less resistant than the other species. However, these differences gradually diminished so that all the yeasts showed relatively similar parameters in regard to butanol. For example, MIC$_\mu$ for butanol

varied only between 1.20% (*Hanseniaspora valbyensis*) and 2.20% (*Hansenula anomala*).

Other authors reported a certain relationship between the inhibition parameters determined and the number of carbon atoms in the inhibitor molecule for the case of aliphatic alcohols [2] and aliphatic amines [15] (for a strain of *Clostridium botulinum*). Such a relationship – and especially a linear one – couldn't be affirmed with certainty in our case, although many of the data sets shown in Figs. 5–8 could of course be fitted with a straight line with more or less accuracy. Still, it must be noted that the shape of the plots in Figs. 5–8 changes significantly when the unit of the vertical axis is changed from % (v/v) to mg l^{-1}, for example, a fact which reduces the value of any such conclusion of linearity. Also, in the literature [6] it is stated that if the logarithm of the alcohol tolerance values obtained for monohydric alcohols are plotted against the logarithm of the phospholipid-buffer partition coefficients for the same alcohols, there is a surprisingly good correlation between inhibition of glucose utilization and lipid solubility (expressed by the phospholipid partition coefficients). Using the same values for these partition coefficients we plotted K_μ, K_θ and MIC_μ, MIC_θ determined in our study, but their linearity was still not evident for all species. Furthermore, all these parameters describing alcohol tolerance are susceptible to vary significantly with experimental conditions, and the methods employed for their determination often have large statistical errors. Under these circumstances, while the increase in toxicity of alcohols with the increase in their chain length observed previously [6], and also confirmed by this study, is certain and directly related to their hydrophobicity, it may be difficult to argue that this relationship can be expressed by a linear function for all kinds of microbial species or any particular conditions. As our results shown in Figs. 5–8 tend to indicate, for the conditions employed in this study a similarity in the inhibitory effects of methanol and ethanol on one side, and propanol and butanol, on another, could be observed, with a significantly larger gap between ethanol and propanol.

It is interesting to discuss the present results in relation to the structure-activity relationships (SAR) reported by Beezer et al. [3,4], although there were some important differences in the experimental procedures. (For example, the required observation time was very different; the procedures mentioned above [3,4], suitable for rapid bioassays of antimicrobials, needed observation times as short as 30 min, while the necessity of monitoring growth, in our case, imposed much longer incubation times.) Figs. 5–8 show that the determined values of the 50% inhibitory concentration and those of MIC decrease clearly with the increase of the number of carbon atoms in the alcohol molecule. However, this relationship is not linear, or at least it depends very much on the microorganism and experimental conditions used. Alcohols have an amphiphatic nature and have been shown to exert their inhibitory action by affecting phospholipid bilayers of the cell membrane and thus causing alterations in membrane structure and function, such as transport of nutrients. An increase in the molecular chain length of the alcohol accentuates its lipophilic nature [6], and thus increases its inhibitory activity against microorganisms. However, alcohols in the range C1-C4 are still very small molecules, which can easily penetrate membranes and disrupt internal functions of the cell. Such behavior probably leads to very complex interaction mechanisms, and it is not surprising that the linear relationship between inhibitory activity and molecular size or structure observed for larger molecules were not obtained in the case of C1-C4 alcohols. Also, yeast species differ widely regarding their membrane composition, and the structure and composition of the membrane may vary with temperature even for the same strain. Accordingly, yeasts show a large variety in their ability to resist the inhibitory action of alcohols. Nevertheless, the present results indicate that the differences between species tend to disappear rather rapidly with the increase in alcohol molecule length, since the inhibitory parameters determined for butanol are almost the same for all the yeasts studied.

5. Conclusions

A calorimetric procedure could be applied with good results to the investigation of inhibitory action of alcohol on growth of yeast cells. The inhibitory action clearly increased with the increase in chain length of alcohol molecule, from methanol to butanol, although it could not be affirmed that this relationship was linear. Considerable differences among yeast

species could be observed in their resistance to methanol, but these differences progressively diminished in the case of ethanol, propanol and butanol. The calorimetric procedure applied is simple, allows obtaining of precise quantitative parameters, and presumably may be suitable for the study of many other microorganism–drug interactions.

Acknowledgements

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

References

[1] C. Höhne and R. Patch, Arch. Hyg., 153 (1969) 162.
[2] C.N. Huhtanen, J. Food Protection, 43 (1980) 195.
[3] A.E. Beezer, L.J. Ashby, S.M. de Morais, R. Bolton, M. Shafiq and N. Kjeldsen, Thermochim. Acta, 172 (1990) 81.
[4] A.E. Beezer, J.C. Mitchell, R.M. Colegate, D.J. Scally, L.J. Tyman and R.J. Wilson, Thermochim. Acta, 250 (1995) 277.
[5] K. Kodama, in: A.H. Rose and J.S. Harrison (Eds.), The Yeasts, Vol. 3, Academic press, London and New York, 1970, p. 244.
[6] L. O'Neal Ingram and T.M. Buttke, in: A.H. Rose and D.W. Tempest (Eds.), Advances in Microbial Physiology, Vol. 25, Academic press, London, 1984, p. 254.
[7] T. D'Amore, C.J. Panchal, I. Russell and G.G. Stewart, Crit. Rev. Biotechnol., 9 (1990) 287.
[8] L.A. Shelef and J.A. Seiter, in: P.M. Davidson and A.L. Branen (Eds.), Antimicrobials in Foods, Marcel Decker, Inc., New York, 2nd edn., 1993, p. 539.
[9] I. Lamprecht and B. Schaarschmidt (Eds.), Application of Calorimetry in Life Sciences, Walter de Gruyter, Berlin, 1977.
[10] A.E. Beezer (Ed.), Biological Microcalorimetry, Academic press, London, 1980.
[11] A.M. James (Ed.), Thermal and Energetic Studies of Cellular Biological Systems, Wright, 1987.
[12] O.A. Antoce, N. Pomohaci, V. Antoce, H. Fukada, K. Takahashi, H. Kawasaki, N. Amano and T. Amachi, Biocontrol Science, 1 (1996) 3.
[13] K. Takahashi, J. Antibact. Antifung. Agents, 24 (1996) 313.
[14] K. Takahashi, Thermochim. Acta, 163 (1990) 71.
[15] C.N. Huhtanen and T.J. Micicj, J. Amer. Oil Chem. Soc., 31 (1978) 228.
[16] K. Hiromi, Y. Takasaki and S. Ono, Bull. Chem. Soc. Jpn., 36 (1963) 563.
[17] T. Kawabata, H. Yamano and K. Takahashi, Agric. Biol. Chem., 47 (1983) 1281.
[18] A. Katarao, H. Okuno and K. Takahashi, Agric. Biol. Chem., 51 (1987) 2443.
[19] A. Katarao, H. Okuno and K. Takahashi, Agric. Biol. Chem., 52 (1988) 2279.
[20] S. Ono, K. Hiromi and K. Takahashi, J. Biochem., 57 (1965) 799.
[21] M. Hashimoto and K. Takahashi, Agric. Biol. Chem., 46 (1982) 1559.
[22] O.A. Antoce, K. Takahashi and I. Namolosanu, Vitis, 35 (1996) 105.