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A rapid method for testing yeast resistance to ethanol for the selection of strains suitable for winemaking

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Abstract

The paper presents a rapid and convenient method for assessing the yeast tolerance to ethanol. An isothermal calorimeter with 24 sample cells for microbial cultures was used to monitor the growth of yeast cultures on glucose-peptone broth at 30°C, in the presence of various concentrations of ethanol added from the beginning. The apparatus detects the heat evolved during the yeast growth and records it under the form of the so-called "growth thermograms". These curves are then analyzed using a specialized mathematical apparatus to derive values of various parameters which describe the effect of the ethanol on the yeast growth, such as the concentration which inhibits 50% of the microbial growth (LD50) or the minimum inhibitory concentration (MIC).

Keywords: ethanol inhibition, wine yeast strains, isothermal calorimetry

Introduction

The yeasts employed for winemaking must have several specific traits in order to ensure the production of a quality wine. One trait important for the winemakers is the resistance of the yeast to ethanol, the main product of alcoholic fermentation, but, at the same time, a strong inhibitor of microbial growth (Ingram and Buttke, 1984), through the final product inhibition mechanism. A good tolerance to this final product allows the yeast to complete fermentation when the ethanol accumulates in relatively high concentrations (Ciani *et al.*, 2006). The full fermentation of the sugars in the grape must is often desired to subsequently protect the wine against secondary fermentations which irreversibly affect wine quality. On the other hand, a yeast that tolerates higher concentrations of ethanol will also produce wines with higher ethanol concentrations which, within certain limits, will usually be of better quality, as wines go.

Although at present many winemakers use commercially available selected yeasts, recently a tendency is noticed to use for winemaking yeasts selected from the very vineyard where the wine is produced. However, in the same vineyard one can find various species and strains of yeasts, not all being suitable for the production of quality wines. Therefore, certain tests need to be conducted in order to decide what strain to use, from microbiological tests to the sensory characterization of the wines obtained.

As a first eliminatory test for such a screening we propose the determination of the ethanol tolerance. The methods used for the evaluation of the resistance of microorganisms to inhibitors are diverse, but they can be classified in two main categories: "final point" methods and "descriptive" methods. In the case of the "final point" methods a microorganism is observed over a fixed period of time in the presence of an inhibitor in a certain concentration, so that the obtained results are only certain for that precise period of time during which the microorganism growth was under observation. These methods usually provide a single

inhibition parameter, that is the minimum inhibitory concentration (MIC). On the other hand, the "descriptive" methods, although more time-consuming and laborious, allow for the sampling and testing at certain times during the experimental period, to assess the viability of the microbial cells at various periods of exposure to the inhibitor. These descriptive methods provide another type of inhibition parameter, widely used in medical research, namely the "lethal dose 50%" (LD50), which represents the inhibitor concentration necessary to halve the number of viable microorganisms. In certain types of descriptive method experiments, another parameter is calculated, namely the half-time (t50), which is the duration required for the reduction of a microbial population to half in the presence of an inhibitor.

Dantigny and his co-workers (2005) have shown previously an example of combining the "final point" and "descriptive" procedures, computing both a MIC-type parameter and a LD50-type parameter for the situation of ethanol inhibition of mould growth, by measuring the diameter of growing mould colonies in time and treating the data with an appropriate mathematical apparatus.

In this paper we propose the use of an isothermal calorimetry monitorization of growth (Kawabata *et al*., 1983, Okuda *et al*., 1996; Antoce *et al*., 1997, Wadsö and Galindo, 2009) in the presence of various concentrations of ethanol, in order to determine all of the above-mentioned inhibition parameters. This is a rapid and convenient method which allows the determination in 48-72 hours of the minimum inhibitory concentration (MIC), the lethal dose 50% (LD50), as well as the inhibition half-time (t50) – which is much more rarely reported. The determination of all these parameters is important for the yeast resistance evaluation, since, in accordance to the inhibition mechanism and the cooperativity factor (Antoce, 1998), strains with the same LD50 can have different MICs and vice-versa. The method provides a complete description of the behavior of the microorganism in the presence of a certain antimicrobial agent, and can also differentiate the bactericidal inhibition from bacteriostatic inhibition (Wirkner *et al*., 2002).

Materials and methods

The method used for the evaluation of an inhibitor effect is based on the monitorization of culture growth by means of recording the heat produced by this growth in isothermal calorimetric cells (Winkelmann *et al*., 2004).

The apparatus used for this evaluation, designed and generously offered by Prof. Katsutada TAKAHASHI from Osaka Prefecture University, Japan, is a calorimeter installed at present in the Laboratory of Enology of the University of Agronomical Sciences and Veterinary Medicine of Bucharest (Fig. 1a). This calorimeter consists of 25 calorimetric units, in which 24 microbial cultures can be monitored in several inhibition conditions versus a reference sample (Fig. 1b,c).

The isothermal calorimeter works based on the principle of the heat conduction. A thermopile plate located on the bottom of each unit measures the amount of heat generated in the unit during the microbial growth, as it is transferred to the surrounding aluminum block, which is kept at a constant temperature by circulating water through copper pipes located around it. The heat flux established between the calorimetric unit and surroundings is detected by the thermopile plates (Melcore CF-70.1, New Jersey, SUA) and the difference between each sample and a reference cell is recorded as a voltage signal. The voltage signal is measured for each sample at a fixed time interval by using a Keithley digital voltmeter and a channel scanner. All 24 signals are thus digitalized and stored into a computer database. The specialized software for the data analysis works under Origin General Scientific 2.8v platform and is of in-house design.

The recipients in which the microbial culture is allowed to grow are normal glass vials of 50 ml, fitted with an autoclavable plastic screw stopper. The only requirement for the vials is to be all of the same type and to have a perfect plane bottom, to ensure a good contact with the thermopile plate for heat transfer.

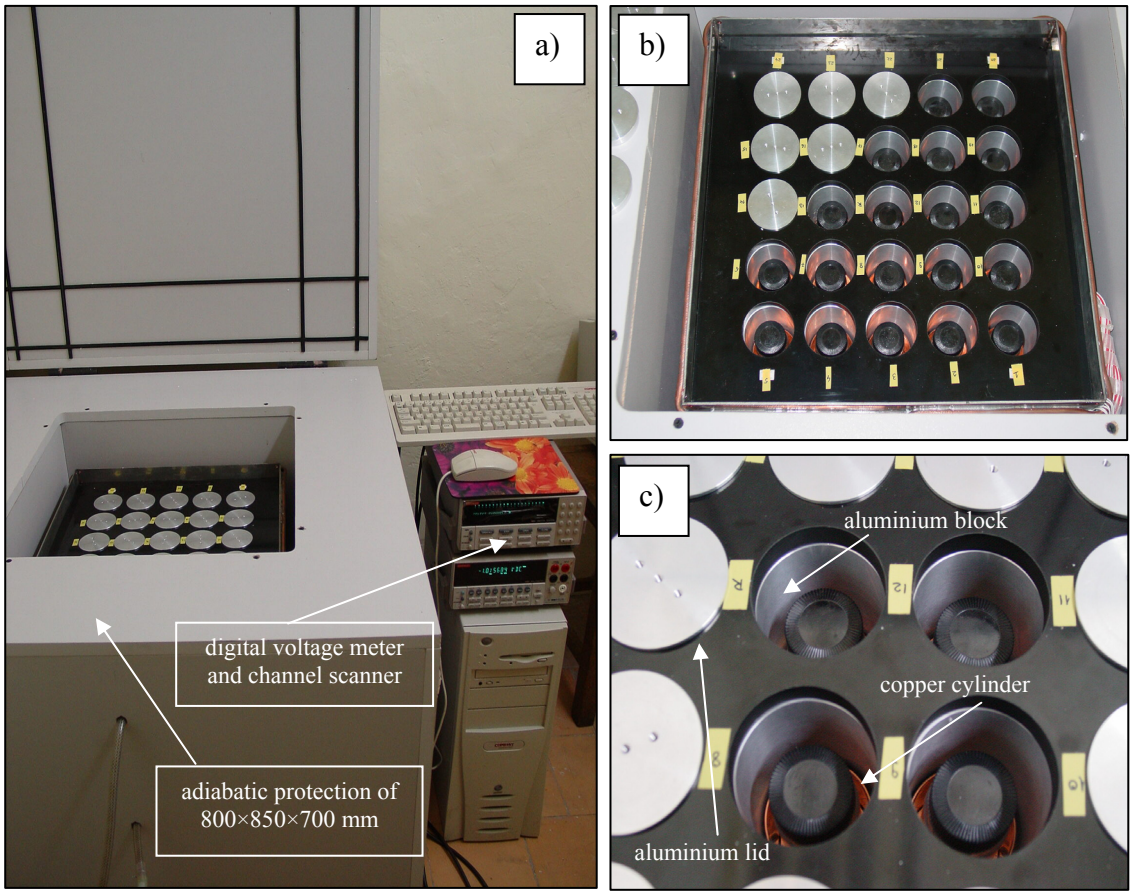

Figure 1. Isothermal calorimeter with 24 measurement units: a) general view; b) aluminium block in which the calorimetric cells are perforated; c) detail view of the calorimetric units containing vials with microbial cultures.

The yeasts used in this study were different strains of wine yeasts from the collection of the University of Agronomical Sciences and Veterinary Medicine of Bucharest. All the reagents were of analytical grade and the growth medium was YPG (Yeast-Peptone-Glucose) broth from Oxoid, with 20 g/l glucose.

The broth is autoclaved directly in the calorimetric vials, 10 minutes at 121°C. For each set of vials 150 ml of broth is prepared and then 5 ml are pipetted in each vial. After autoclaving, groups of vials are formed and ethanol is added in increasing concentration for each group, as described in Table 1. The yeast strain to be tested is also added as a suspension of 10^5 – 10^6 cells/ml. The yeast inoculum is prepared in YPG liquid medium thermostated at 30°C for 24 h and diluted with distilled sterile water 1:5 before addition into the calorimetric vials.

Then the vials are incubated in the calorimeter and the heat generated during growth is measured for each vial every 10 minutes. Although in wines the fermentation temperature is kept in the range of 16-22°C, in order to speed up the yeast growth in the calorimeter, the ethanol resistance is tested at the temperature of 30°C.

Table 1. Preparation of the set of vials to be introduced in the calorimeter for the evaluation of ethanol resistance

Vial no.	YPG broth (ml)	ethanol 95% v./v. (ml)	distilled water (ml)	inoculum (diluted 1:5) (ml)	Ethanol concentration in the vial (% v./v.)
1 - 4	5.00	0.00	0.50	0.05	0.00%
5 - 8	5.00	0.10	0.40	0.05	1.75%
9 - 10	5.00	0.15	0.35	0.05	2.62%
11 - 12	5.00	0.20	0.30	0.05	3.49%
13 - 14	5.00	0.25	0.25	0.05	4.36%
15 -16	5.00	0.30	0.20	0.05	5.24%
17 - 18	5.00	0.35	0.15	0.05	6.11%
19 -20	5.00	0.40	0.10	0.05	6.98%
21 - 22	5.00	0.45	0.05	0.05	7.85%
23 - 24	5.00	0.50	0.00	0.05	8.73%
R =reference	0.00	0.00	5.55	0.00	0.00%

Results and discussions

The thermal power-time curves, so-called growth thermograms or $g(t)$ curves (Kimura and Takahashi, 1985) recorded as a heat evolution during the growth of a culture into a calorimetric unit, are correlated to the microbial growth (Fig. 2a). After a lag period, dependent on the inoculum size (Hashimoto and Takahashi, 1982) and the presence and concentration of an inhibitor, the heat generated into the calorimetric cell starts growing exponentially, following the exponential phase of the microorganisms growth. Then, after a maximum value is reached, the curve gradually descends towards the baseline, as growth stops due to a limiting factor such as the exhaustion of nutrients (Antoce 1996, Winkelmann *et al*., 2004). In the decreasing phase the growth thermograms return towards baseline, but remain at a level slightly above this line, because the living yeast cells are still active even after the glucose is completely consumed (Winkelmann *et al*., 2004).

The heat flux is not instantaneously measured and a part of the transferred heat remains undetected by the sensors. Therefore, the $g(t)$ curves is only an apparent signal and in order to obtain the correct heat quantity this curve is processed by a complex mathematical treatment (Antoce, 1998) and transformed into the so-called $f(t)$ curve (Fig. 2b) by integration. The calibration of the apparatus, the reproducibility and accuracy of results are also discussed elsewhere (Antoce, 1998). Our research has shown that the $f(t)$ curve is very well correlated with the number of living cells in the microbial culture, as well as the turbidity of the culture measured at 660 nm (Antoce *et al.*, 1997).

After recording 24 such calorimetric curves for yeasts inoculated in the same growth medium but with various concentrations of inhibitors, one of the first results that can be obtained with this method is a qualitative determination of the type of inhibitor effect. Thus, in the case of a bactericidal effect, with the addition of inhibitor in larger concentrations the initial number of surviving microbial cells is reduced and the lag period increased accordingly, so that the curves are shifted towards longer incubation times, but the shapes of the curves are not affected (Fig. 3a). On the contrary, if the inhibitor acts in a bactericidal manner, the inoculum size is not affected, but the growth rate is decreased proportionally with the concentration of inhibitor, so that all the curves start at the same incubation time, but the "slope" of the curves decreases în accordance to the inhibitor concentration (Fig. 3b).

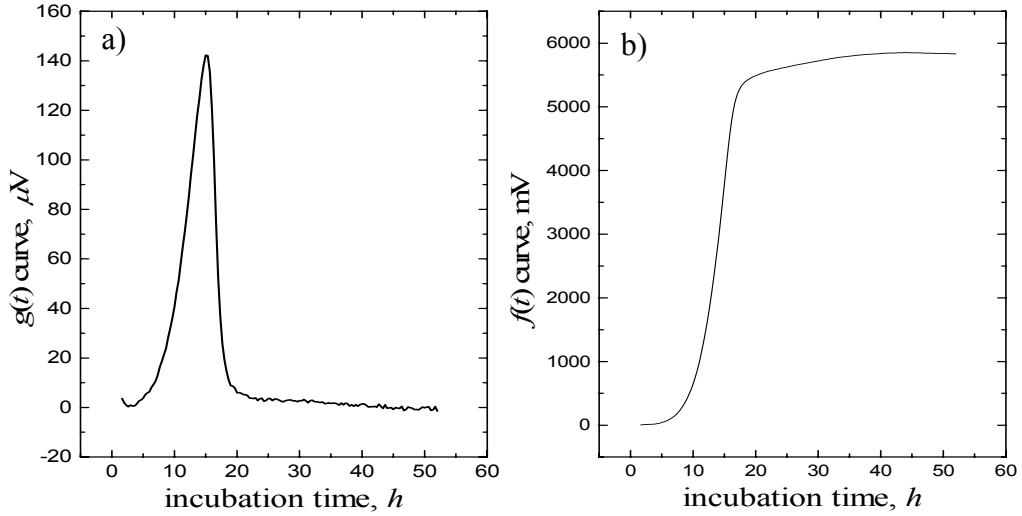

Figure 2. Heat evolution curves recorded by the calorimeter during growth of a *S. ellipsoideus 196* yeast: a) growth thermogram or $g(t)$ curve; (b) and the real heat evolution curve or $f(t)$ curve.

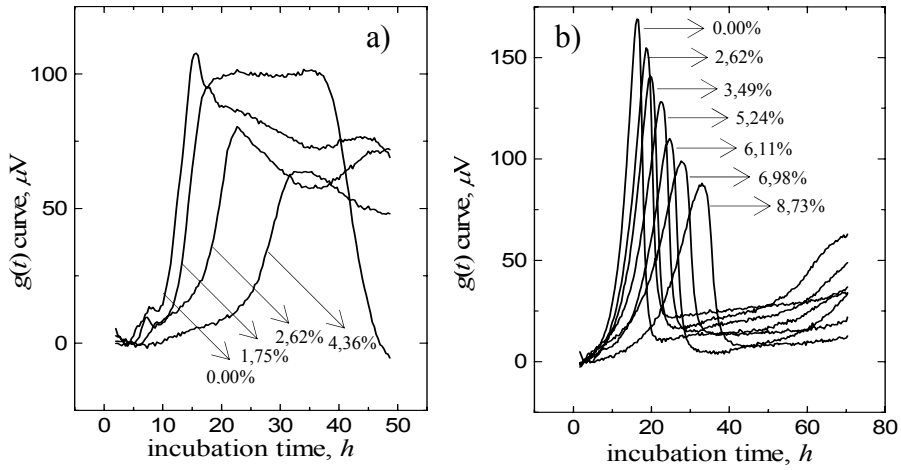

Figure 3. Growth thermograms recorded in the presence of increased concentrations of ethanol: a) bactericidal effect of ethanol on the growth of *S. bayanus 301* on YPG medium (b) bacteriostatic effect of ethanol on the growth of *S.rosei 1* on YPG medium.

These bacteriostatic and bactericidal effects have also impact on the quantitative inhibition parameters determined, as will be described later on.

For the determination of the inhibitory parameters of microbial growth it is important to calculate either the growth rate constants in the presence of an inhibitor in concentration i, μ_i and the growth rate constant in the absence of the inhibitor, μ_m, or, as an alternative, to measure the retardation of growth in the presence of an inhibitor in concentration i, $t_\alpha(i)$, and in the absence of inhibitor, $t_\alpha(i)$. The ratio μ_i/μ_m quantitatively describes the change in the growth rate constant due to the presence of the inhibitor and is named specific growth activity in the presence of an inhibitor in concentration i. Similarly, the ratio $t_\alpha(0)/t_\alpha(i)$ describes the

specific growth retardation in the presence of an inhibitor in concentration *i*. Both ratios depend on the inhibitor concentration and vary between 0 and 1. Thus, they describe quantitatively the changes observed in each of the thermograms (Fig. 3) caused by various concentrations of inhibitor. If we plot these parameters versus inhibitor concentration we obtain a diagram from which the inhibition parameters can be derived by fitting the data by successive iterations until a limiting condition regarding the goodness of the fit (sum of squares of the residual values) is met. These quantitative parameters are as follows: K_μ or $LD50_\mu$ represents the inhibitor concentration that reduces the specific growth activity μ_i/μ_m by 50%; K_θ or $LD50_\theta$ is the inhibitor concentration that increases the specific retardation of growth $t_\alpha(0)/t_\alpha(i)$ by 50%; MIC_μ or MIC_θ are the minimum inhibitory concentrations that totally inhibit growth, calculated based on μ_i/μ_m or $t_\alpha(0)/t_\alpha(i)$, respectively.

The parameters *K* are both easily obtained by fitting the μ_i/μ_m or $t_\alpha(0)/t_\alpha(i)$ curves with hyperbolic functions derived from the non-competitive mechanism of enzymatic inhibition (Antoce, 1998): $\mu_i/\mu_m = 1/[1+(i/K_\mu)^{m_\mu}]$ and $t_\alpha(0)/t_\alpha(i) = 1/[1+(i/K_\theta)^{m_\theta}]$, where i is the inhibitor concentration and m_μ and m_θ are the degree of cooperativity of an inhibitor, parameters discussed later on.

The regression analysis based on these functions provides the so called "inhibitor potency curve". From these curves, parameters K_μ and K_θ are obtained as the inhibitor concentration at which the parameters μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$, respectively, reach the value 0.5, that is 50% (Fig. 4).

For the calculation of the MICs, the data points of μ_i/μ_m or $t_\alpha(0)/t_\alpha(i)$ are fitted with another type of function that simulates the addition of an inhibitor in such a concentration that the parameters μ_i/μ_m or $t_\alpha(0)/t_\alpha(i)$ reach the value zero:

$MIC_\mu = (1/k_1)^{1/m_\mu}$ and $MIC_\theta = (1/k_2)^{1/m_\theta}$, where k_1 and k_2 are constants.

This calculated level of the inhibitor concentration is never used in real experiments, because at this concentration no thermogram could be recorded anymore (growth rate constant approaching zero, the retardation of growth extends to infinity). Therefore, the MIC parameters are only computed through extrapolation (Fig. 4), the values being obtained at the interception of the MIC curve with the inhibitor concentration axis (Antoce *et al.*, 1997).

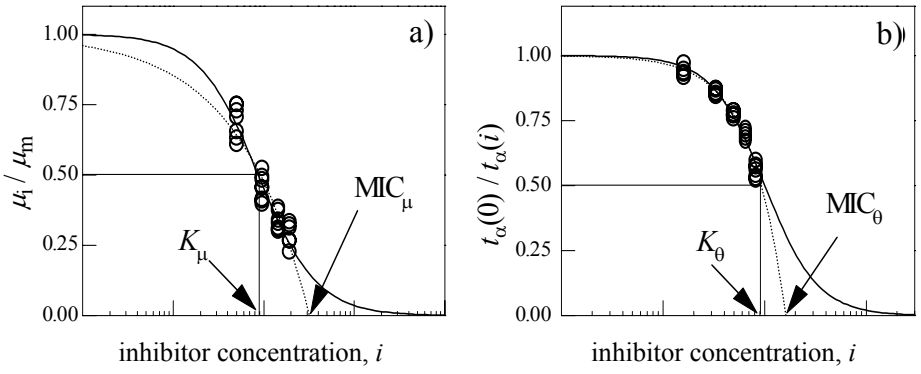

Figure 4. Calculation procedure involved in the determination of the quantitative parameters that characterize the growth inhibition of a microorganism by a certain inhibitor.

The steepness of the inhibitor potency curves (dotted lines) and MIC curves (continuous lines) are also dependent on the inhibition mechanism, being correlated to the degree of cooperativity in the inhibitor-microbial cell interaction. These parameters can also

be calculated, as they are the superscripts included in the equations of the inhibitor potency curves and MIC curves, noted with m_μ and m_θ. Although for the reason of brevity the equations and all the mathematical treatments applied for the determinations (Antoce, 1998) are not presented in this paper, it is easy to understand that the higher the degree of cooperativity, the steeper the inhibitor potency curves and MIC curves are, and the narrower the range of concentrations tolerated by the yeast. In Fig. 5 it is shown an example of curves with different steepness, from which we could also see that in the case with the higher degree of cooperativity (m_μ = 7.66), the range of inhibitor concentration tolerated by the yeast is narrower and the inhibition parameters take lower values (Fig. 5b) which means that the inhibition of yeasts growth by ethanol is stronger in this case.

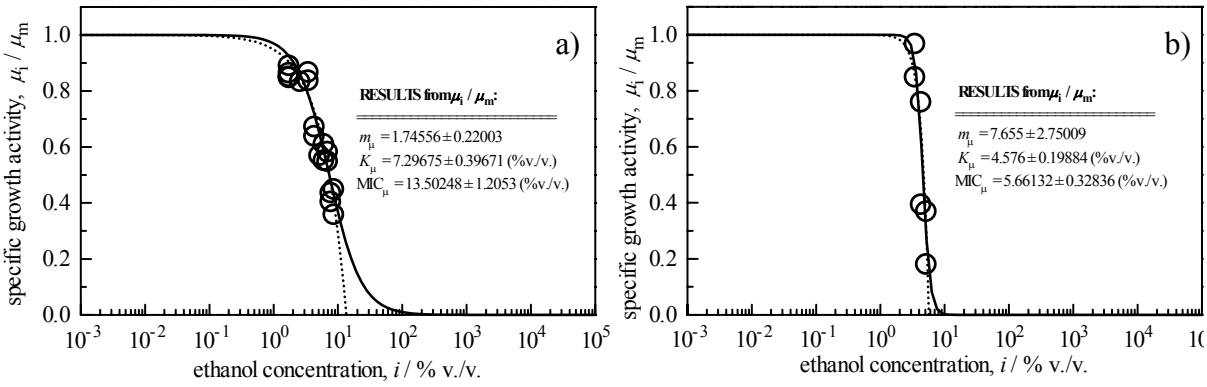

Figure 5. Exemple of curves with different cooperativity degrees and their influence on the other inhibition parameters: a) ethanol inhibition on *S. bayanus 156*; b) ethanol inhibition on *S. bayanus 3*

In order to obtain reliable growth diagrams and accurate inhibition parameters, the range of concentrations of the inhibitor used in the experiments should extend a little more than the values of *K*, but beyond this concentration the growth retardation will mean unnecessary longer experimental times.

The specialized software that we designed stores all the data collected by the digital voltmeter and channel scanner into a database that is processed semi-automatically. Thus, the set of points recorded for 24 channels are later plotted in the form of the *g*(*t*) curves, shifted at the same baseline and then integrated in order to obtain the real heat evolution, the *f*(*t*) curves. These curves are then processed mathematically and all the described parameters are calculated. Thus, based on the exponential part of the *f*(*t*) curve, the one that is correlated with the exponential growth phase of the microorganism incubated in the calorimeter, the growth rate constant is calculated, by fitting by regression analysis the initial portion, typically between 3 – 30% of the *f*(*t*) curve, with an exponential function that describes the typical exponential growth of a culture: $f(t) = N_0 A e^{\mu t} + N_0 B$, where constants A and B are given by the relations described by Hashimoto and Takahashi (1982). On the basis of this equation, after the regression analysis, the growth rate constant μ is calculated for all of the 24 microbial cultures grown in the presence of various concentrations of ethanol, μ_m being the maximum growth rate obtained in the absence of inhibitor and μ_i any growth rate constant obtained for a culture in the presence of inhibitor in concentration *i*.

Due to the fact that it is also obvious that some kind of relationship must exist between the growth rate constant μ and the delay in time observable in different calorimetric results, the time retardation parameter, t_α, is also calculated for each culture. In order to do that, a horizontal line is drawn corresponding to a selected level of *f* '(*t*), identical for all the curves in the same experiment, named level α, and the points where this line intersects the *f* '(*t*) curves

provide the respective values of the retardation time. We name $t_\alpha(0)$ the value of this time for the culture with no inhibitor added, and $t_\alpha(i)$, for the culture with inhibitor in concentration i. The selection of this level has only a negligible effect on the specific growth retardation, and thus on the inhibition parameters derived from $t_\alpha(0)/t_\alpha(i)$, as long as the above-mentioned level was kept within the portion of exponential growth for all the calorimetric curves.

As presented before, from the values of the growth rate constant μ and retardation time t_α we can calculate μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$, on which the determination of all the other inhibition parameters rely. In Table 2 there is an example of the parameters calculated by using the calorimetric output and the specialized software designed for data processing.

Table 2. Example of parameters calculated from the calorimetric output using the specialized software designed for data processing – for *Saccharomyces bayanus 3* in the presence of various concentrations of ethanol.

	Ethanol concentration added at the beginning of incubation, % (v/v)					
Parameter	**0**	**1.75**	**2.62**	**3.49**	**4.36**	**5.24**
μ / min^{-1}	0.238* 0.197*	0.352 0.403	0.407 0.272	0.182 0.208	0.163 0.084	0.038 0.079
t_α / h	5.71* 5.95*	6.265 6.653	11.25 10.53	12.35 14.57	22.42 19.01	29.85 44.97
μ_i/μ_m	1 1	- -	- -	0.847 0.966	0.758 0.392	0.178 0.368
$t_\alpha(0)/t_\alpha(i)$	1 1	0.911 0.858	0.508 0.542	0.462 0.392	0.255 0.300	0.191 0.127

The values marked with a (*) were used as μ_m and $t_\alpha(0)$ when μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ were calculated

From the fact that some of the μ_i/μ_m could not be calculated (Table 2), we can deduct that ethanol in small concentrations (up to 3.5% v/v) has a bactericidal effect.

After fitting the curves of μ_i/μ_m and $t_\alpha(0)/t_\alpha(i)$ with appropriate functions as described above (Fig. 5), the principal inhibitory parameters are derived and calculated. In the case of the strain *Saccharomyces bayanus 3* exemplified in Fig. 5b and Table 2, the results are: K_μ = 4.58 ± 0.20 % v./v., MIC_μ = 5.66 ± 0.33 % v./v., while K_θ = 3.11 ± 0.20 % v./v., MIC_θ = 5.63 ± 0.48 % v./v. In principle, the parameters K and MIC should be identical irrespective of the method of their calculation (based on μ or based on t_α). In practice, the differences in the case of this strain are due the bactericidal effect exerted by the ethanol in small concentrations, which allows for a high cooperativity of ethanol for this strain, as demonstrated by the parameter related to the cooperativity degree, m_μ = 7.65 ± 2.75 (Fig. 5b).

Sometimes, not all the channels are used in experiments and also some of the data, considered outliers, can be eliminated from the data processing. Repetition of experiments is required only in order to check the reproducibility of the results and improve their accuracy, if deemed necessary.

Conclusions

In this work a calorimetric method using a multiplex batch microcalorimeter (isothermal, conduction type) was applied for the rapid testing of yeasts for ethanol tolerance in the view of selection of strains useful for winemaking. The method is, in principle, suitable for the testing of any other inhibitor or microorganism.

Some experiments were carried out to demonstrate that monitoring the heat evolved during growth can be a useful tool for the determination of classical inhibition parameters, which otherwise requires laborious and time-consuming procedures.

Moreover, the method not only provides the inhibition parameters, but can also provide valuable information regarding the growth rate constant and the type of inhibition (bacteriostatic, bactericidal or mixed). The growth rate constant is important for the winemaking yeasts, a higher value meaning faster growth in the grape must and a more rapid elimination of the other undesirable yeasts naturally found in grape musts.

When studying the inhibitory effect of ethanol or any other chemical compound added to the cultures, the range of concentrations used also influences the results. Therefore, in order to obtain a satisfactory precision in the determination of the minimum inhibitory concentration (MIC), the addition of inhibitor must be done in sufficient amount, so that to produce significant effects on the growth thermograms, meaning in a concentration at least up to the level of inhibition of growth activity by 50% or more.

The main advantages of this method are: the simplicity of the hardware, software and experimental procedures; elimination of the labor-intensive cell counting at various time intervals; high sensitivity, possibility of measuring cultures grown in intense-colored or high-turbidity media, such as the red wine – which is not possible in optical measurement procedures; it is even possible to study microbial growth in non-homogeneous media; it is a non-invasive and non-destructive procedure.

In addition to these general advantages this method offers the benefit of monitoring a large number of samples simultaneously, up to 24 in one experiment of 48-72 hours. As a result, the method provides a good precision for the quantitative results derived. Also, the data collection and the analysis of the growth thermograms recorded are easily done using the specialized software of the calorimeter.

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