CALORIMETRIC STUDIES REGARDING DEVELOPMENT OF SOME YEAST SELECTED IN THE PRESENCE OF THE POLLUTANT $CuSO_4$

STUDII CALORIMETRICE ASUPRA CREŞTERII DROJDIILOR SELECŢIONATE ÎN PREZENŢA $CuSO_4$ CA FACTOR POLUANT

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Abstract. *In ordinary oenological practices the $CuSO_4$ treatment it still used to discard the sulfide hydrogen and other sulfate undesirable odors. This practice leads to an increase of copper concentration having negatives effects on the wine opalescence and oxidation level.*

The copper contamination sources: raw materials; anticryptogamic treatments with pesticides based on copper applied during grape processing, stocking and transporting; auxiliary materials; water used in technological processes and corrosion processes following grapes stocking and manipulation with copper equipments, etc.

The calorimetric method was applied to study the inhibition effect on Saccharomyces ellipsoideus yeasts growing of copper, studied like copper sulfate ($CuSO_4$), because this is active especially for useful microorganisms to alcoholic fermentation and negatives effects on wines.

Rezumat. *În practicile oenologice obişnuite încă se mai aplică tratamente cu $CuSO_4$ pentru îndepărtarea hidrogenului sulfurat şi a altor mirosuri sulfitice nedorite, ceea ce conduce la o creştere a concentraţiei de cupru având efecte negative asupra vinului privind opalescenţa şi nivelul de oxidare.*

Sursele de contaminare cu cupru ce pot ajunge în vin pe căi multiple sunt: odată cu materiile prime; în urma tratamentelor anticriptogamice aplicate viţei de vie cu pesticide pe bază de cupru; în timpul prelucrării strugurilor, depozitării şi transportului; din materialele auxiliare; din apa folosită în procesele tehnologice şi proceselor de coroziune în urma prelucrării, depozitării şi manipulării strugurilor cu utilaje confecţionate din cupru sau utilaje de cupru, etc.

Metoda calorimetrică a fost aplicată pentru studiul inhibării dezvoltării drojdiilor Saccharomyces ellipsoideus de către cupru, studiat sub formă de sulfat de cupru ($CuSO_4$), deoarece este activ mai ales asupra microorganismelor utile fermentaţiei alcoolice şi cu efecte negative asupra vinurilor.

MATERIAL AND METHOD

A calorimetric method to study the inhibition of microorganisms growing was used to quantitatively analyze the inhibitory effect of copper on three yeast strains.

Growing and development of yeasts was studied with Antares micro-calorimeter, capable to monitor simultaneous 24 samples (Arina Antoce, 1996).

Every one of the 14 units were filled with microorganisms in culture medium, in the presence of different inhibitory amounts. The samples were autoclaved in 50 ml glass vials, with tight stoppers, containing 5 ml growing medium (**YPG – yeast peptone glucose**) the inhibitory amount being introduced aseptically in every vial. For every experience it was used a 24 vials set with 4 blank vials containing culture with no inhibition addition, and in the other 20 vials $CuSO_4$ was added in an increase quantity. Usually it was used 4 vials with the same copper concentration to allow a simple estimation of reproducibility for registered growing thermogram.

The calorimeter detects the heat disengaged by microorganisms growing (cells growing) and transforms the thermal signal into an electrical one. Hereby, in the end we have the "calorimetric signal" or "increase thermogram" (***g(t)***) of microorganism – this is just an apparent evolution curve. Through different mathematical integration equation we obtain the real evolution curve of microorganism or ***f(t) curve.*** Finally still using mathematical calculation we can obtain the increase constant (***μ***), which may be used to quantitative comparison of microorganisms increase.

The studied yeasts strains are from microorganisms collection of some Romanian traditional winegrowing centers, being isolated and selected from spontaneous micro-flora and identified like *Saccharomyces ellipsoideus,* conserved in our laboratory like stock culture (*Saccharomyces ellipsoideus 4-21*, *Saccharomyces ellipsoideus* M_1 *and Saccharomyces ellipsoideus* b_1). The strains were pre-incubated for 24 hours at 30°C in liquid medium and then incubated (inoculation size was 10^{-6}) in calorimeter at same temperature and on the same medium. They were made experiences for a quantitative analysis of copper inhibition action which included the registration of inhibition action on 72 must samples. These samples were prepared with must containing variables copper concentrations, being inoculated with one of the three selected yeast strains, considered the most valuables for flavored wine making. For every inhibition concentration and yeast were made three or four repetitions (tables 1, 2 and 3).

Table 1

Saccharomyces ellipsoideus 4-21 yeast strain
(first variant)

Nr. of samples (calorimeter vials)	ml. $CuSO_4$ aseptically added	Concentration mg/l Cu^{2+}
1-4 witness	0	—
5-8	0,1	9,8
9-12	0,2	19,24
13-16	0,5	45,53
17-20	0,7	61,55
21-24	0,95	80,08

Table 2

Saccharomyces ellipsoideus M_1 ***yeast strain***
(second variant)

Nr. of samples (calorimeter vials)	ml. $CuSO_4$ aseptically added	Concentration mg/l Cu^{2+}
1-4 witness	0	—
5-8	0,69	60,77
9-12	0,95	80,08
13-16	1,25	100,39
17-20	1,58	120,62
21-24	1,93	140,01

Every *Saccharomyces ellipsoideus* strain used in calorimetric experiences was developed on liquid YPG medium (25 ml / 100 ml Erlenmayer balloon) for 18 – 20 hours at 30°C, to obtain an inoculum. From the inoculum 24 samples were inseminated, taking care not to exceed a cell density bigger than 1 - 9 x 10^6 (established with Thoma chamber), ready to be introduced for registration in the 24 calorimetric unities. The samples are made in 50 ml glass vials, autoclaved, with tight stopper, containing 5 ml of increase medium, to which it was added, aseptic, the different quantity of inhibition composite ($CuSO_4$), according to tables 1,2 and 3. For every experience was used a 24 vials set, the first ones being used like witness – no $CuSO_4$ added, while from the rest of vials were made groups in which was added increasing quantities of CuSO4. Usually were used 4 vials group with the same copper concentration to allow a simple estimation of registered growing thermograms reproducibility.

Table 3

Saccharomyces ellipsoideus4-21, M_1 and b_1 yeast strain
(third variant)

Nr. of samples (calorimeter vials)	ml. $CuSO_4$ aseptic added	Concentration mg/l Cu^{2+}
1-2 witness	0,00	—
3-4	0,55	49,64
5-6	1,25	100,39
7-8	1,93	140,01
9-10	2,67	175,25
11-12	3,25	198,5
13-14	3,50	207,5
15-16	4,00	224,17
17-18	4,25	231,81
19-20	4,50	239,06
21-22	4,75	245,93
23-24	5,00	252,47

RESULTS AND DISCUSSIONS

The reproducibility of the experiments was very good when the inoculum was similar from the stock culture age, pre-incubation time and yeasts cell number point of view. The increase thermograms for studied yeast strains were different according to strains type and the added cooper concentration. The highest copper concentration added was 250 mg/l Cu^{2+}.

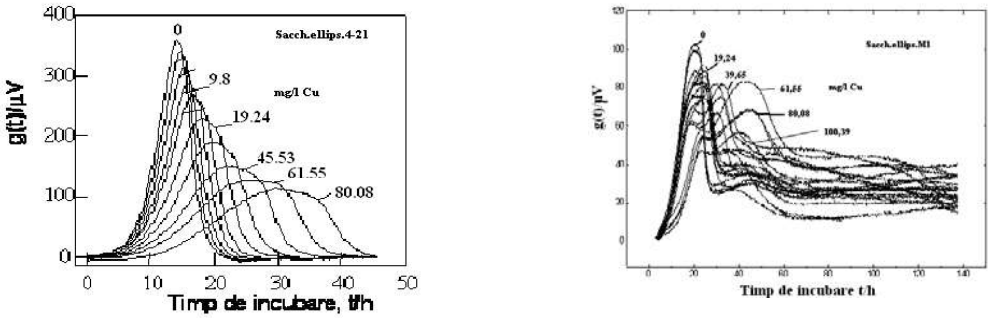

Figure 1. The increase thermogram of *Saccharomyces ellipsoideus 4-21* and *Saccharomyces ellipsoideus* M_1 strains

The „g(t)" curves depend, in some points, on the used yeast strain and the initial inhibition concentration (copper concentration). For example, the *Saccharomyces ellipsoideus 4-21* strain had big, large curves, with sticking tops, compared with *Saccharomyces ellipsoideus* M_1 which had smaller curves, sometimes with second small and dusty tops in the presence of high copper concentrations.

Such peculiarities are due, probably, to inherent differences between yeast strains regarding their increase behavior in the presence of some composites. As we can see for all the yeast strains the copper addition influence the increase thermograms; the increase of inhibition concentration lead to a progressive decrease of g(t) curves inclination, normally decreasing the top height and increasing the starting time in exponential increase phase. The effects produces by inhibition concentration increase on the increase thermograms may be quantitative analyzed using the increase speed value, to calculate the inhibition constant, determined for every culture.

The observed changes in increase thermograms are graduals and they are developing progressive when inhibition concentration increase. We can confirm the apparition of an exponential increase phase for all the yeast culture with the tested inhibition composite (copper), at the concentrations used in experiences. These are indicated that it exist a correlation between yeast growing and heat producing for the culture containing the tested inhibition composite.

It must be noted that the g(t) curves represent only the apparent exit signal of calorimeter, because the signal obtained from the instrument is affected by the permanent heat change between the calorimeter's unities and the environment, specific of calorimeter type conduction.

To obtain the effective evolution heat curve, the g(t) curve is integrated (equation 1):

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

f (t) – the evolution of real heat developed by calorimeter's unities;

g(t) – the apparent exit signal of the instrument;

t – incubation time;

K - conductivity constant of calorimeter's heat.

This operation, which give the speed increase value, is made automaton by the calorimeter's *soft* for the all 24 cultures and for every experience and finally we obtain a gradual decrease of μ value when the inhibition concentration increase. Obviously, the increase curves without inhibition composite are different from the ones obtained with inhibition composite, thus the effect of some composite on yeast may be quantitative analyzed by analyzing the curves differences with a special software. The main differences are the decrease of inhibited microorganisms increase speed and so a decrease of increase inclination curve and an increase of delay time of increase (Antoce O.A., 1996). Both aspects can be analyzed to characterize the yeasts growing, obtaining next parameters:

- increase speed constant μ

- delay time $t\alpha$

- K_μ = inhibition concentration which determine a decrease of increase constant with 0%

- K_θ = = inhibition concentration which determine an increase of delay time with 50%

- MIC_μ = minimal inhibition concentration at which is not observed the microorganisms

- MIC_θ = minimal inhibition concentration for which the increase appear at t = α time.

Figure 2 present the results regarding the final determined values of minimal inhibition concentration of copper which determine a decrease of increase constant with 50% ($K\mu$) and at which is not observed anymore the yeasts growing (MICμ) but also the determined values of K_θ and MIC_θ.

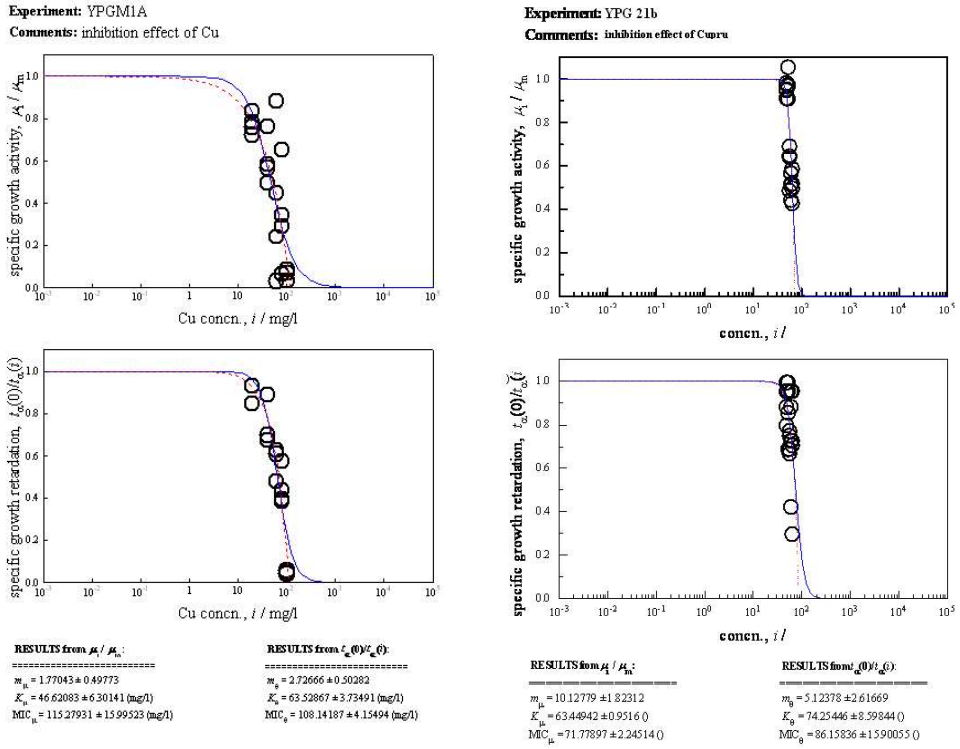

Figure 2. Calculation of minimal inhibition concentration of copper according to the decrease of the increase value and the delay of strain growing starting

As far as the rate of yeast growing is concerned we noticed that the inhibition dose is 50%, K_μ is 46,6 ± 6,3 mg/l and the minimal inhibition concentration MIC_μ is 115,3 ± 16,0 mg/l. For the case of growing yeast delay time the inhibition dose is 50%, K_θ is 63,5 ± 3,7 mg/l, and the minimal inhibition concentration, MIC_μ is 108,1 ± 4,1 mg/l.

CONCLUSIONS

►The calorimetric studies are proved to be useful to evaluate the influence of $CuSO_4$ as pollutant factor for fermentation microorganism. Through this method yeast strains resistant to some factors specific to one vineyard or winemaking technology can be selected.

►Even if the values for $K\mu$ and $MIC\mu$ determined by this method can easy vary according to experimental conditions, the method is useful for research activities but also for technological purposes to characterize the yeast strains tolerance to different inhibition composite.

►In the case of yeast growing speed decrease the inhibition dose is 50%, K_μ is 46,6 ± 6,3 mg/l and the minimal inhibition concentration MIC_μ is 115,3 ± 16,0 mg/l. For the case of growing yeast delay time the inhibition dose is 50%, K_θ is 63,5 ± 3,7 mg/l, and the minimal inhibition concentration, MIC_μ is 108,1 ± 4,1 mg/l.

►Based on the results of this research we concluded that the inhibitory copper dose is around 100 mg/l Cu^{2+}. Its concentration is very important to establish limits of wine toxicity because the quantity of the biological available metal has a higher importance than the total concentration for wine quality.

►Just like other calorimetric methods, the potential application of this method include the complex evaluation of culture media, expertise on food and wine additives, and anti-fungal agents, improvement of fermentation technologies, applications in medicine, pollution an environment, etc.

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