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Saccharomyces cerevisiae wine strains differing in copper resistance exhibit different capability to reduce copper content in wine

V. Brandolini[1,*], P. Tedeschi[1], A. Capece[2], A. Maietti[1], D. Mazzotta[1], G. Salzano[2], A. Paparella[3] and P. Romano[2]
[1]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Fossato di Mortara, 17/19, 44100 Ferrara, Italy*
[2]*Dipartimento di Biologia, Difesa, Biotecnologie, Agro-Forestali, Università della Basilicata, Potenza, Italy*
[3]*Facoltà di Agraria, Università di Teramo, Italy*
**Author for correspondence: Tel.: +39-532-291279, Fax: +39-532-291296, E-mail: bnv@unife.it*

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Summary

Two wine strains of *Saccharomyces cerevisiae*, characterized by a different degree of copper resistance, were tested in grape must fermentation in the presence of different copper concentrations. The sensitive strain SN9 was strongly affected by copper concentration (32 ppm, (32 mg/l)), whereas the resistant strain SN41 exhibited a good growth activity in presence of 32 ppm of copper and only a reduced activity in presence of 320 ppm. The different strain fermentation performance in response to the copper addition corresponded to a different capability to accumulate copper inside the cells. Both strains exhibited the capacity to reduce the copper content in the final product, eventhough a significantly greater reducing activity was exerted by the resistant strain SN41, which was able to reduce by 90% the copper concentration in the final product and to accumulate the metal in great concentrations in the cells. As high concentrations of copper can be responsible for wine alterations, the selection of *S. cerevisiae* strains possessing high copper resistance and the ability to reduce the copper content of wine has a great technological interest, in particular for the fermentation of biological products. From the results obtained, the technique proposed is not only suitable for the assay of copper residues in must, wine and yeast cells, but it also offers the advantage of easy sample preparation and low detection limit in the ppb (μg/l) range.

Introduction

Copper is an essential element for all known living organisms, including humans and other animals, being a cofactor in numerous enzymatic processes and representing the third most abundant transition metal found in living things. It is an essential prosthetic group of proteins such as cytosolic superoxide dismutase, that is required for detoxification of oxygen free radicals and thus for the fitness of aerobic organisms. Eventhough copper sites in cytochrome oxidase are essential for the activity of this enzyme and thus for sustained cellular respiration (Yamaguchi-Iwai *et al.* 1997), abnormally high levels of copper can be toxic to living cells, presumably by binding indiscriminately to thiol moieties, or by catalysing a Fenton-type reaction, producing the reactive hydroxyl radical (Stillman & Presta 2000), thus causing cellular damage (Nagasue *et al.* 1989). The reason for this toxicity may be due to metal-dependent oxy radical generation and metal ion antagonism, i.e. through competition of different metal ions for the same biochemical sites. (Wilson *et al.* 1991; Jungmann *et al.* 1993). Therefore the sequestration of copper by biological ligands such as peptides, proteins, or enzymes is necessary in order to minimize its participation in deleterious reactions. Methallothionein is an ideal candidate for copper sequestration (Presta & Stillman 1997).

Since the available data indicate that metals enter the cell uncomplexed to any extracellular ligand, an initial ligand displacement step must precede the movement of the metal ion across the plasma membrane.

Ligand coordination is typically more labile to lower valence ions than to the corresponding high valence species, and therefore reduction of copper in the environment facilitates cellular uptake.

The major surface reductase of *Saccharomyces cerevisiae* is the FRE-1 gene product, which is capable of reducing extracellular copper or iron complexes (Yamaguchi-Iwai *et al.* 1997). Copper acquisition by *S. cerevisiae* cells requires also the CTR-1 gene, which encodes a protein mediating copper uptake at the plasma membrane and contains an unusual amino-terminal domain, rich in methionine and serine, oriented toward the cellular exterior.

The expression of FRE-1 and CTR-1 are homeostatically regulated by copper availability, consistent with the roles of the two gene products in copper acquisition. The regulation of FRE-1 and CTR-1 is mediated at the

level of copper-dependent transcription. Copper-regulated expression of FRE-1 and CTR-1 is controlled by the MAC-1 protein, identified as a regulatory DNA-binding protein, through which cellular copper levels are transduced into the regulated transcription of genes involved in copper acquisition. As copper uptake into the cell requires reduction of copper chelates (FRE-1 mediated) and translocation across the plasma membrane (CTR-1 mediated), the consequence is the inhibition of the transcription of FRE-1 and CTR-1 (MAC-1 mediated) (Yamaguchi-Iwai *et al.* 1997).

Nowadays agricultural techniques for integrated pest management or biological production include the wide use of copper for chemical protection of plants. Intensive efforts have been made to discover biological means of pest control, but, the problem is so difficult that no adequate or economically valid alternative measures have been found. Recently the increasing use of copper formulates in biological vineyards has caused high levels of copper residues on the grapes (Brandolini *et al.* 1995), causing in some cases slow or stock fermentations. It is well known, in fact, that low amounts of copper play a key role on microbial activities, whereas elevated concentrations can be toxic to yeasts, affecting cell growth or causing the acquisition of tolerance to the metal. In a study conducted on natural wine strains of *S. cerevisiae*, the copper resistance was described as a potential discriminating strain characteristic (Paraggio *et al.* 1997). This species exhibited a wide variability in the expression of the character and the acquisition of 'copper resistance' has been suggested as a consequence of environmental adaptation.

Taking into account that the yeast strain used to ferment grape juice is one factor which can strongly influence wine production (Heard & Fleet 1986), exploitation of natural yeasts and growing data on strain phenotypic variation in spontaneous fermentation have became the object of increasing interest among wine researchers (Romano 1997). Consequently, the importance of using suitable selected cultures for each winemaking has focused more attention on the isolation and characterization of autochtonous strains for traits of technological interest (Paraggio *et al.* 1998).

As copper formulates can include other metals and heavy metals that can influence yeast activity and the fermentation processes, we carried out a study in order to determine a methodological approach for monitoring biological or integrated productions. In this research *S. cerevisiae* strains, copper resistant or non-resistant (Paraggio *et al.* 1997), were employed to ferment Aglianico must to which $CuSO_4$ had been added.

Materials and methods

Organisms

Two strains of *S. cerevisiae*, belonging to the collection of the Basilicata University and previously studied (Paraggio *et al.* 1997), were used. The strains, characterized in Table 1, are: SN41 (copper resistant) and SN9 (copper sensitive).

Table 1. Technological characteristics of the two strains of *S. cerevisiae*.

Strain Code	SN9	SN41
Copper resistance (ppm Cu^{2+})	3	32
Ethanol production (% v/v)	18.65	18.78
Metabolite production (mg/l)		
Acetaldehyde	56.8	50.8
Ethyl acetate	27.2	15.2
n-propanol	20.4	39.8
2-Methylpropanol	52.9	58.0
Acetic acid	157.3	198.5
3-Methylbutanol	289.0	217.9

Fermentation test

The experiments were performed by adding different copper concentrations, 32 ppm (mg/l) and 320 ppm of Cu(II) as $CuSO_4$, to sterilized grape must. The fermentations, carried out in triplicate, were performed in flasks containing 100 ml of red grape must from the Aglianico cultivar of the Basilicata region (fermentable sugar 19%, pH 3.15). The samples were inoculated with 10^4 cell/ml of 48 h precultures grown in the same must and incubated at 25 °C. The fermentation course was followed by determining the weight loss caused by CO_2 evolution, expressed as grams of CO_2 produced from 100 ml of fermenting grape must. The weight loss was monitored every 2 days and the quantity (g) of CO_2 produced was used to express strain fermentation power at the end of the process. When the CO_2 evolution ceased, the fermentation was considered completed.

Atomic absorption spectrometry

Samples of must, wine and yeast cells were taken during the fermentation period. The samples were homogenized accurately and centrifuged, portions of 2.5 ml were dissolved in CEM's digestion vessels (PTFE mod. SV140, FKV) with HNO_3–H_2O_2 in a microwave digester (Millestone MLS 1200, FKV) coupled with a module for steam extraction (EM 5, FKV). Yeast cells, after separation from the supernatant, were washed twice with physiological saline and a portion of 0.5 g was mineralized. Triplicate extractions were done on each sample. A Perkin-Elmer graphite furnace mounted on a Perkin-Elmer (mod. 1100B) atomic absorption spectrometer (AAS) was used with autosampler. The spectrometer was equipped with deuterium background corrector and single-element Intensitron (Perkin-Elmer) hollow cathode lamps were used for the measurements of copper at 324.8 nm.

The accuracy of the measurement was evaluated by means of recovery tests and the precision, expressed as coefficient of variation (CV%), was in the range of 0.6–2.4. Standard solutions were prepared by diluting refer-

ence standard solutions for AAS (BDH Certified Atomic Absorption Reference Solutions). All reagents and chemicals were of 'pro-analysis' grade and the water used was deionized, distilled and filtered through a Milli-Q-system (Millipore, Bedford, MA). The samples were checked against reference standards and read for their absorbance, after instrument calibration. An average of five readings of absorbance was taken in all samples.

Statistical analysis

The analysis of variance (ANOVA) was carried out on all data by StatMost program.

Results

Fermentation behaviour

Fermentation performance of the two strains of *S. cerevisiae* in the presence of different concentrations of copper is reported in Figure 1. The results showed that both strains exhibited a high fermentation power in the natural must (without Cu(II) addition), completing the process in 10 days with a CO_2 evolution of 13 g. In contrast, the strains behaved differently in the presence of copper. The sensitive strain SN9 was strongly affected by copper concentration (32 ppm), with a CO_2 evolution of 2.5 g after 10 fermentation days compared to 13 g of the control. Conversely, the resistant strain SN41 exhibited a good growth activity in presence of 32 ppm of copper, near to the level of the control. The fermentation in presence of 320 ppm of copper inhibited completely the growth of the sensitive strain SN9, whereas the fermentation process performed with the resistant strain SN41 was only reduced, reaching a final CO_2 evolution of 8 g compared to 13 g without copper addition.

Copper analysis

The change in copper concentrations in grape must fermented by sensitive and resistant strains with and without copper addition is reported in Table 2. The different strain fermentation performance as a response to copper addition corresponded to differences in the capability to accumulate copper inside the cells. The sensitive strain SN9 did not reduce wine copper concentration, which remained at the same levels during the whole fermentation process in the presence both of 32 and 320 ppm of copper. The determination of copper in the grape must, to which 32 ppm of the metal had been added and in wine after 7 days of fermentation demonstrated the ability of the resistant strain SN41 to reduce the copper concentration in the final product, from 35 to 3.4 ppm and from 323 to 193 ppm of copper (respectively from the start to the final stage of the fermentation process). Both strains exhibited the capacity to reduce the copper content in the final product, even if a

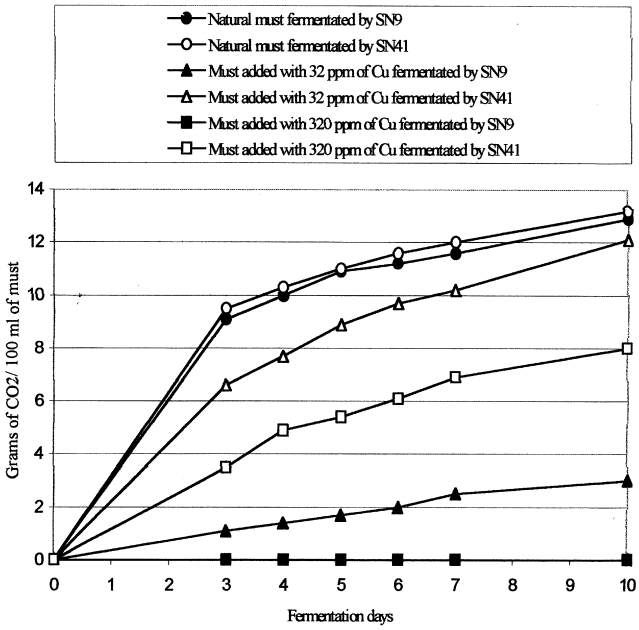

Figure 1. Fermentation efficiency of *S. cerevisiae* strains (copper-resistant SN41 and copper-sensitive SN9) in Aglianico grape must added with different concentrations of Cu(II) (32 and 320 ppm). The data are reported as average of three independent fermentations (CV% $\leq$ 2.4).

Table 2. Copper concentrations (ppm) in Aglianico grape must supplemented with different Cu(II) concentrations (0, 32, 320 ppm) during fermentation with *S. cerevisiae* strains (Cu-resistant SN41 and Cu-sensitive SN9).

Sample	Copper concentrations (ppm) at fermentation days			
	0	3	5	7
Grape must	3.0	2.8	2.8	2.5
Grape must + 32 ppm Cu(II)	35.0	31.5	31.0	30.8
Grape must + 320 ppm Cu(II)	323.0	314.0	304.0	295.0
Grape must + SN9	3.0	1.9	1.4	0.5
Grape must + SN41	3.0	1.7	0.8	0.1
Grape must + SN9 + 32 ppm Cu(II)	35.0	31.4	30.8	30.0
Grape must + SN41 + 32 ppm Cu(II)	35.0	9.0	3.8	3.4
Grape must + SN9 + 320 ppm Cu(II)	323.0	318.0	314.0	310.0
Grape must + SN41 + 320 ppm Cu(II)	323.0	271.0	241.0	193.0

The data are reported as average of three independent experiments (CV% $\leq$ 2.4).

Table 3. Copper concentrations (ppm) in Aglianico grape must, wine and yeast cells after fermentation with *S. cerevisiae* strains (Cu-resistant SN41 and Cu-sensitive SN9) in presence of different Cu(II) concentrations.

Sample	Must (0 day)		Wine (7 days)		Cells (7 days)	
	SN9	SN41	SN9	SN41	SN9	SN41
Control	2.8	2.8	0.5	0.1	32.0	131.0
32 ppm	32.0	31.1	30.0	3.4	81.0	1080.0
320 ppm	320.0	318.0	310.0	193.0	–	1953.0

The data are reported as average of three independent experiments (CV% range ± 0.6–2.4).

significantly greater reducing activity was exerted by the resistant strain SN41.

In order to ascertain the fate of the copper, another experiment was carried out determining the copper content before fermentation in grape must and at the end of the process in wine and in the yeast cells. The experiment, performed in triplicate, was carried out in Aglianico grape must added with the two copper concentrations (32 and 320 ppm) and fermented with the two strains (copper resistant strain SN41 and copper sensitive strain SN9). The results, reported in Table 3, showed that the copper-resistant strain SN41 accumulated the majority of the copper inside the cells, while the copper-sensitive strain SN9 exhibited a very low accumulation capability. In particular, the resistant strain was able to reduce by 90% the copper concentration in the final product and the copper was accumulated in great concentrations in the cells.

Conclusions

Biological pest control, which uses exclusively copper and sulphur, needs close study in order to control the environmental impact of the control agents and the commercial production quality.

The new defence strategies are based on the reduction or elimination of particularly dangerous xenobiotics. Thus, the increasing use of copper to replace them, mainly in biological production, could induce accumulation of this element not only in wine, but also in the soil and consequently in the environment.

The results presented here show that the technique proposed is suitable for the assay of copper residues in must, wine and yeast cells. In addition, this method offers the advantage of easy sample preparation and low detection limit in the ppb range.

From the technological point of view, it must be underlined that copper resistance was directly related to the ability of the strain to reduce the copper concentration in the wine. In fact, the resistant strain SN41 reduced the concentration of copper in the grape must at 32 ppm, as well as at more elevated concentrations of 320 ppm, accumulating the metal inside the cells. Strain resistance, however, does not always result in an elevated accumulation of metals. Some reports have demonstrated that repeated culture of a *S. cerevisiae* copper-resistant strain, grown in presence of elevated levels of copper and other metals, resulted in increased resistance, but in a decreased accumulation of the metals inside the cells (White & Gadd 1986; Brady *et al.* 1994). Other authors, in agreement with our finding, showed that cells of *S. cerevisiae* strains which are resistant to Mn^{2+} exhibited increased uptake of the metal (Bianchi *et al.* 1981) and studies on cadmium-tolerant cultures of yeast revealed a great increased proportion of cadmium bound in the cytosol (John *et al.* 1985).

High concentrations of free copper ions in wine can be responsible for cellular alterations, because they can be rather dangerous, being involved in many undesirable reactions that would lead to the irreversible destruction of cellular components (Felix & Weser 1988). Consequently, in this context, the selection of *S. cerevisiae* strains possessing high copper resistance and overall ability to reduce the copper content of wine, has a great technological interest, in particular for biological fermentations. Research is in progress to determine a rapid and reproducible method to select and characterize copper-resistant yeast strains and their potential technological employment.

Acknowledgements

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