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Interaction between Fenhexamid and Yeasts during the Alcoholic Fermentation of *Saccharomyces cerevisiae*

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The behavior of the fungicide fenhexamid, N-(2,3-dichloro-4-hydroxyphenyl)-1-methyl-cyclohexanecarboxamide, has been studied at concentrations corresponding to the limits fixed for grapes (3 mg kg^{-1}), or higher, during the alcoholic fermentation. The presence of the fungicide did not affect the amount of alcohol produced. The amount of fenhexamid in the liquid phase decreased by ca. 15%, but the missing fenhexamid was recovered unchanged from yeasts. This suggests that the fungicide is not degraded during the fermentation process, but adsorbed by yeasts. Two constituents of *Saccharomyces cerevisiae* cell wall, chitin and glucan, tested as potential adsorbents, exhibited affinity for fenhexamid.

KEYWORDS: Fenhexamid; alcoholic fermentation; *Saccharomyces cerevisiae*; adsorption

INTRODUCTION

Botrytis cinerea is one of the most important fungal pathogens in grapevine crops. Infections caused by *B. cinerea* are of considerable economic importance. Although chemical control has been used as a standard practice for many years, the great ability of *B. cinerea* to quickly adapt to new chemicals and to develop tolerant or resistant strains creates a need for the development of new botryticides. Fenhexamid is a fungicide representative of the new chemical class of hydroxanilides (**Figure 1**). It is a locosystemic compound with excellent activity against *B. cinerea*. When applied to conidia of *B. cinerea*, fenhexamid inhibits germ tube elongation and mycelium growth (*1*). Fenhexamid shows a mechanism of action, which is currently under investigation (*2*), different from all other botryticides. Moreover, fenhexamid does not exhibit cross-resistance to other known botryticides (*3*). On the other hand, it is necessary to keep in mind that the presence of pesticides could affect the activity of alcoholic fermentation microorganisms (*4*). In fact, any interruption or delay in this process can alter the qualitative and quantitative characteristics of wine (*5*).

In a recent study, Cabras et al. (*6*) found that amounts of fenhexamid of 0.8 mg kg^{-1} do not affect alcoholic fermentation. As the fenhexamid maximum residue limit (MRL) fixed in Italy for grapes is 3 mg kg^{-1}, the aim of this work was to evaluate the influence that the fenhexamid residue, in concentrations corresponding to these fixed limits or higher, may have on alcoholic fermentation. Not only interferences in the fermentation process but also the behavior of fenhexamid itself during fermentation was taken into account.

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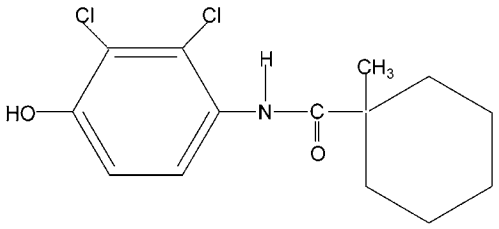

Figure 1. Structure of fenhexamid.

MATERIALS AND METHODS

Chemicals. Fenhexamid (N-(2,3-dichloro-4-hydroxyphenyl)-1-methyl-cyclohexanecarboxamide) (purity 99.2%) was supplied by Bayer, Milan, Italy. Its purity was checked by high performance liquid chromatography (HPLC). The water solubility at 20 °C is 20 mg L^{-1} (pH 5−7). 1,3-β-D-Glucan was obtained according to the procedure described by Williams et al. (*7*). Chitin was supplied by Aldrich, Milan, Italy. All the solvents were of HPLC grade (Carlo Erba Reagenti, Milan, Italy) and were used without further purification.

Culture Media. Broth was made up containing 30 g L^{-1} of yeast nitrogen base (YNBG) and 180 g L^{-1} of glucose at pH 3.6. A stock standard solution of the fungicide was prepared dissolving 30 mg of fenhexamid in ethanol (10 mL). Working solutions were obtained by diluting appropriate amounts of a stock standard solution with 1 L of YNBG broth to give final concentrations of 2.3, 8.2, and 10.9 mg L^{-1}. All media were sterilized by filtration through membrane filters (0.2-μm pore size, Sartorius, Gottingen, Germany).

Inoculation and Fermentation. *Saccharomyces cerevisiae* No. 1090 used in this study was obtained from the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, University of Sassari, Sassari, Italy. Pre-cultures were prepared in broth containing 2% of glucose, 0.5% of yeast extract and 1% of peptone in a thermostatically controlled chamber at 25 °C for 48 h. Cells were washed twice and suspended in 0.15 M of NaCl. The amounts of suspension used as inoculum were such as to ensure 1×10^6 cells

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Table 1. Effect of Fenhexamid on Fermentation Activity of *Saccharomyces cerevisiae*

fenhexamid (mg L^{-1})	0 days		1 day		5 days		12 days	
	cell mL^{-1}	CO_2[a]	cell mL^{-1}	CO_2[a]	cell mL^{-1}	CO_2[a]	cell mL^{-1}	CO_2[a]
control	$1.0 \cdot 10^6$		$2.1 \cdot 10^7$	1.8	$3.4 \cdot 10^7$	7.5	$4.0 \cdot 10^7$	10.6
2.28	$1.0 \cdot 10^6$		$1.5 \cdot 10^7$	1.7	$2.0 \cdot 10^7$	7.4	$3.0 \cdot 10^7$	10.5
8.20	$1.0 \cdot 10^6$		$2.0 \cdot 10^7$	1.8	$2.0 \cdot 10^7$	8.0	$3.0 \cdot 10^7$	11.3
10.91	$1.0 \cdot 10^6$		$0.5 \cdot 10^7$	0.5	$3.0 \cdot 10^7$	6.7	$3.0 \cdot 10^7$	10.9

[a] Expressed as alcohol % (v/v).

mL^{-1} in each of the culture media. After inoculation, each culture medium was divided into three 150-mL replications in 300-mL flasks. A control was prepared consisting of an inoculated YNBG broth without fenhexamid. All of the flasks were put to ferment in a thermostatically controlled chamber at 25 °C for 12 days.

Samplings. Four samplings were carried out immediately after inoculation (0 days) and after 1, 5, and 12 days. At appropriate times, a 10-ml sample was drawn from each flask, and the following analyses were made: pH, yeast cell mL^{-1} (microscopic count and culture count), and CO_2 production (indirect weighing). To quantify fenhexamid, a 10-mL aliquot of working broth was passed through a weighed cellulose nitrate membrane filter (0.2-μm pore size, Whatman, Waidstone, England) which separated yeast from the fermentation broth. The filter was washed with water. The filter containing yeast and the filtered broth were analyzed separately, as described below.

Extraction Procedure. The filter containing yeast was dried at 50 °C for 2 h and weighed then transferred into a 20 mL screw-capped tube. A 10 mL sample of CH_2Cl_2 was then added, then the tube was shaken in a rotary shaker for 1 h. The CH_2Cl_2 solution was evaporated, and the residue was taken up in 10 mL of mobile phase used in HPLC determination. The filter was previously checked to verify that it did not adsorb fenhexamid.

The broth recovered after filtration was saturated with NaCl and extracted with CH_2Cl_2 (10 mL, three times). The organic phase was separated, dried, and then evaporated. The residue was taken up in 10 mL of mobile phase used in HPLC analysis. The recovery percentage obtained from three extractions for each spiking level tested ranged between 97.5 and 94.9%.

Adsorption on Glucan and Chitin. The adsorption of fenhexamid was determined at 25 ± 2 °C. Duplicate samples of 1,3-β-D-glucan (25 mg) or chitin (25 mg) were equilibrated in polyallomer centrifuge tubes with 2.5 mL of fungicide aqueous solution. Fenhexamid concentrations before equilibration ranged from 2.8 to 33.0 μM. The tubes were shaken (end over end) for 15 h. After equilibration, the suspension was centrifuged at 19 000*g* for 20 min, and the supernatant was pipetted off and analyzed immediately. Adsorbed fenhexamid was calculated from the difference between the initial and final concentrations of fungicide in solution.

HPLC Analyses. The concentration of fenhexamid was determined by HPLC. The system was assembled as follows: a Waters 510 pump equipped with a μBondpak C_{18} analytical column (10 μm, 3.9 × 300 mm), and a multiwavelength Waters 490 programmable detector operating at 230 nm. Acetonitrile plus water (50 + 50 by volume), previously brought to pH 2.7 with phosphoric acid, at a flux rate of 1 mL min^{-1} was the eluant. The retention time for fenhexamid, under the chromatographic conditions described, was 5.2 min. The quantitative determination of fenhexamid was based on external standards. Calculations were based on the average peak areas of the external standards. The detection limit for fenhexamid was 0.01 mg L^{-1}, as calculated from the concentration of herbicide needed to obtain a detector response approximately twice the background signal.

Data Analysis. Adsorption data were fit to the logarithmic form of the Freundlich equation

$$\log C_s = \log K_f + 1/n \log C_e$$

where C_s (in μmol kg^{-1}) is the amount of fungicide adsorbed by glucan or chitin, C_e (in μM) is the equilibrium concentration in solution, and $\log K_f$ and $1/n$ are empirical constants representing the intercept and the slope of the isotherm, respectively. Fitting was performed by the least-squares regression analysis program. The conformity of the sorption data to a linear isotherm was assumed when the correlation coefficient r was ≥ 0.98.

All experiments were carried out in four replicates. Variance analysis (ANOVA) and comparisons between average values were performed with the Duncan Test at $P < 0.05$.

Table 2. Fenhexamid Residues (mg L^{-1}) during the Alcoholic Fermentation of *Saccharomyces cerevisiae* Yeasts

sample	0 days	1 day	5 days	12 days
control	2.28	2.24	2.20	2.22
medium[a]	2.10	1.88	1.78	1.74
yeast cells[b]	0.30	0.58	0.65	0.68
control	8.20	8.17	8.06	8.17
medium[a]	7.60	7.54	7.35	7.10
yeast cells[b]	0.45	0.54	0.62	1.08
control	10.91	10.86	10.72	10.87
medium[a]	10.01	10.02	9.77	9.44
yeast cell[b]	0.60	0.72	0.82	1.44

[a] Fermentation medium. [b] Amount extracted from yeasts.

RESULTS AND DISCUSSION

All experiments were carried out using three different pesticide concentrations. The fermentation parameters measured immediately after inoculation (0 days) and after 1, 5, and 12 days are shown in **Table 1**. After 12 days, the amount of alcohol produced was not affected by the presence of fungicide over the whole concentration range tested. Only after the first day of fermentation was a significant decrease of the amount of alcohol observed in the sample at the highest level of fungicide. Most likely, this finding implies a stress of yeast strains due to the high concentration of fungicide. This assumption agrees with the corresponding decrease of the cell number in the fermentative medium during the first day of fermentation. With elapsing time, the cell stress was overcome. In fact, at the end of the experiment, both the alcohol amount and cell number were comparable to those of the control sample.

During the fermentative process, pesticide residues were determined both in the fermentation medium and in yeasts (**Table 2**). The amount of fenhexamid in the liquid phase decreased by ca. 15%. The lacking fenhexamid was extracted unchanged from yeasts. This suggests that the fungicide was adsorbed by yeasts rather than degraded during fermentation process. The adsorptive capacity of *Saccharomyces cerevisiae* for inorganic pollutants as heavy metals (*8*−*10*) and organic molecules is well known (*11*−*13*). Moreover, *Saccharomyces cerevisiae* exhibits affinity for pesticides. In fact, yeast cells are successfully immobilized on silica gel and used in the on-line isolation and trace enrichment of different pesticides (*14*). Generally, the ability of yeast to retain compounds is attributed to the adsorptive capacity of the cell walls. The cell wall of *Saccharomyces cerevisiae* contains polysaccharides as basic

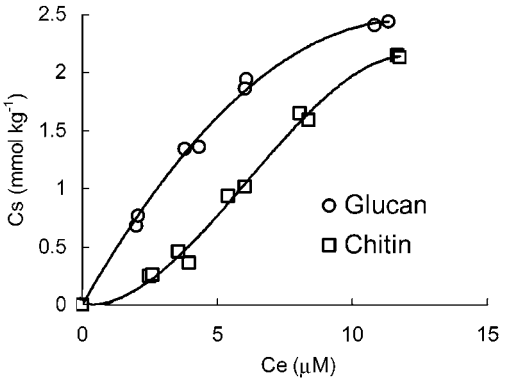

Figure 2. Adsorption isotherms of fenhexamid on glucan and chitin.

Table 3. Freundlich Parameters for the Adsorption of Fenhexamid on Glucan and Chitin

adsorbent	K_f	1/*n*	*r*
glucan	0.46	0.7	0.981
chitin	0.07	1.5	0.984

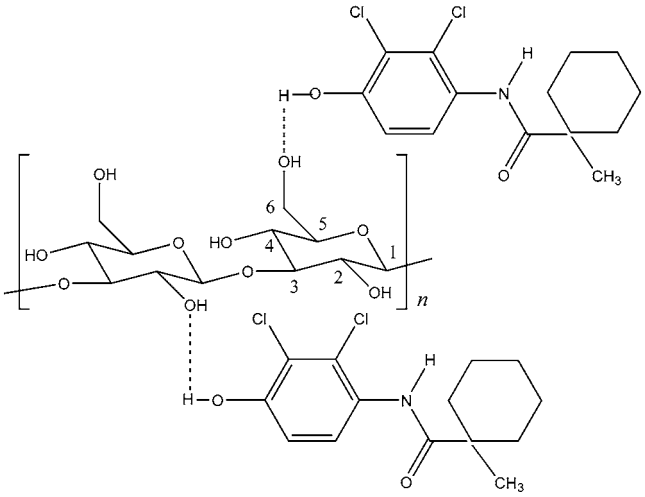

Figure 3. Proposed interaction for fenhexamid adsorption on glucan.

building blocks (*15*). Therefore, it offers a host of functional groups capable of xenobiotic binding.

To evaluate if the wall cell is responsible for the adsorption, two constituents of the *Saccharomyces cerevisiae* cell wall, chitin and glucan, were tested as potential adsorbents of fenhexamid. The adsorption isotherms of fenhexamid on glucan and chitin are shown in **Figure 2**.

Glucan was more effective than chitin in fenhexamid retention. The empirical Freundlich equation fits the behavior well. The calculated constants K_f and 1/*n* and the correlation coefficients (*r*) for the linear fit are given in **Table 3**.

The adsorption isotherms of fenhexamid on glucan were convex, resembling the L-type curve described by Giles et al. (*16*). This shape suggests a relatively high affinity of the fungicide for the adsorbing sites (**Figure 2**). On the other hand, the adsorption isotherm of fenhexamid on chitin was of the S-type, indicating that the adsorption becomes increasingly favored as concentration increases. The glucan fraction consists predominantly of β-1,3-linked glucose (**Figure 3**), which is provided of a polar external surface due to the presence of hydroxyl-moieties. This favors hydrogen bonding interactions with the hydroxyl groups of fenhexamid molecules. On the other hand, chitin is a β-1,4-linked homopolymer of *N*-acetyl

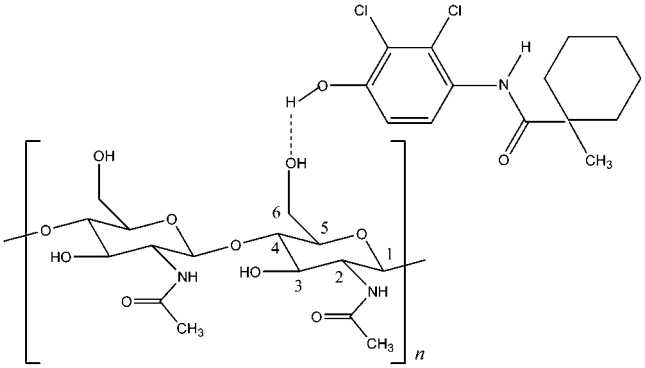

Figure 4. Proposed interaction for fenhexamid adsorption on chitin.

glucosamine (**Figure 4**); therefore, it is less rich in hydroxyl groups on its surfaces compared to glucan. As a consequence fenhexamid is adsorbed to a lower extent.

CONCLUSIONS

The presence of fenhexamid does not affect alcoholic fermentation, even at fungicide concentrations higher than the fixed limits. A minor amount of fenhexamid, which increases with increasing of fungicide concentration, is adsorbed by yeasts, but it is recovered unchanged. Most likely, the adsorption occurs on cell surfaces, which suggests that, during wine making, yeasts may contribute to the removal of fungicide residues.

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