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Behavior of a Fenhexamid Photoproduct during the Alcoholic Fermentation of *Saccharomyces cerevisiae*

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The fungicide fenhexamid [*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide] degraded rapidly by UV or sunlight irradiation, yielding 7-chloro-6-hydroxy-2-(1-methylcyclohexyl)-1,3-benzoxazole (CHB) as a main photoproduct. CHB was isolated, and its effect on alcoholic fermentation of *Saccharomyces cerevisiae* was studied. The results indicate that the presence of CHB does not affect the extent of alcohol production. After 12 days, the amount of CHB in the fermentation medium decreased by ca. 65%. Only 25% of the missing CHB was recovered unchanged from yeasts, most likely because it was adsorbed on the yeast wall cell. The remaining part degraded during the fermentation process. Glucan and chitin, two potential adsorbents, which constitute yeast cell walls, exhibited affinity for CHB.

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

INTRODUCTION

Fenhexamid [*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide] (**Figure 1**), a fungicide belonging to the chemical class of hydroxyanilides, behaves as a locosystemic compound with an excellent activity against *Botrytis cinerea* (*1*). When applied to conidia of *B. cinerea*, fenhexamid inhibits germ tube elongation and mycelium growth (*2*). Plots planted with grapevine, even after several years of fenhexamid treatment, show no increase of less sensitive strains from year to year (*3*). It is assumed that the fungicide is easily degraded, with a favorable toxicological profile and environmental behavior (*4*, *5*). Fenhexamid is stable to hydrolysis in a wide pH range (*5*−*9*), but it degrades rapidly in water under light action (*6*). Many factors can affect the efficiency of yeasts during alcoholic fermentation; they include any fungicide/pesticide residue which could alter the qualitative and quantitative characteristics of wine (*7*, *8*). In recent studies we found that amounts of fenhexamid corresponding to the limits fixed for grapes, or higher, do not affect alcoholic fermentation (*9*, *10*). Since fenhexamid easily undergoes photolytic degradation in water (6), it is likely that photoadducts may be present on the grape cuticle. Therefore, we believed it of interest to study the effect of the main fenhexamid photoproduct on alcoholic fermentation.

MATERIALS AND METHODS

Chemicals. Fenhexamid (purity 99.2%) was supplied by Bayer, Milan, Italy. A 5 mmol L^{-1} (1.5 g L^{-1}) acetonitrile stock solution was prepared. The solution, maintained in the dark at 5 °C, was stable for a prolonged time. 1,3-β-D-Glucan was obtained according to the procedure described by Williams et al. (*11*). Chitin was supplied by Aldrich, Milan, Italy. The solvents were of HPLC grade (Carlo Erba Reagenti, Milan, Italy) and were used without further purification.

Photolysis. In photolysis experiments, 1 mL aliquots of the stock solution were diluted to 100 mL with acetonitrile + water (50:50 by volume) to yield a final fenhexamid concentration of 50 μM. The solutions, contained in a water-cooled borosilicate flask, were irradiated in a merry-go-round Rayonet photoreactor with black light fluorescent lamps emitting at 366 nm. Dark control experiments were carried out in conditions similar to those described above, except that the photoreaction vessel was covered by an aluminum foil. At appropriate times, depending on the photolysis rate, each test solution was analyzed directly by HPLC. All the experiments were run in triplicate.

Isolation of Photometabolite (7-Chloro-6-hydroxy-2-(1-methylcyclohexyl)-1,3-benzoxazole, CHB). A solution (100 mL) of fenhexamid (50 mL of the acetonitrile stock solution + 50 mL of distilled water) made alkaline with 0.1 N NaOH (pH ∼12) was irradiated at 366 nm for 10 h. After irradiation, the solution was acidified to pH 1 and then extracted with dichloromethane. CHB was obtained by evaporation of dichloromethane and column chromatography of the residue on Merck silica gel (Kieselgel 40, 70−230 mesh) using dichloromethane as the eluant. TLC of CHB isolated was performed on Merk silica gel F_{254} plates using a mixture of dichloromethane and ethyl acetate (4:1 by volume) as the developing solvent. Only one spot corresponding to CHB ($R_f = 0.68$) was observed. The run was repeated several times to obtain an amount of photoproduct suitable for the following trials. CHB was a colorless oil. MS (*m*/*e*): 266 (M^+). IR (KBr): ν (cm^{-1}) 2928, 2854, 1621, 1557, 1487, 1430, 1369. ^{1}H NMR ($CDCl_3$): δ 7.43 (1H, d, $J = 8.7$ Hz), 6.98 (1H, d, $J = 8.7$ Hz), 2.33−2.27 (2H, m), 1.62−1.34 (8H, m), 1.40 (3H, s). The choice of an alkaline medium allowed an increase in the yield of CHB.

Culture Media. The broth contained 30 g/L yeast nitrogen base (YNBG) and 180 g/L glucose at pH 3.6. A stock standard solution of

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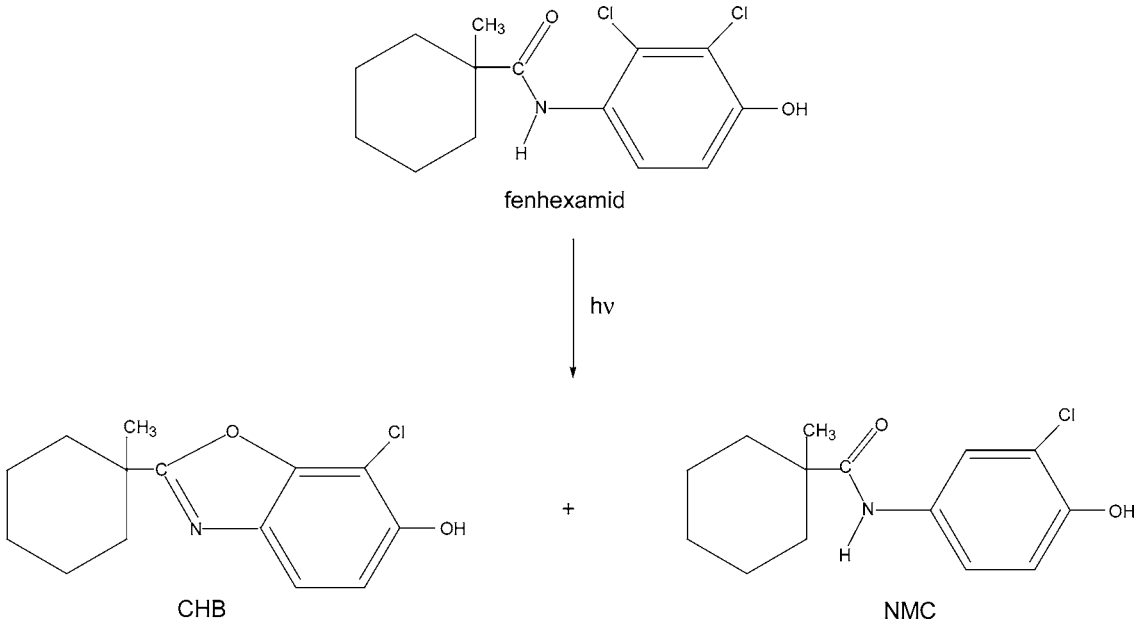

Figure 1. Fenhexamid and its photoproducts.

CHB was prepared by dissolving 15 mg of CHB in ethanol (10 mL). Working solutions were obtained by diluting appropriate amounts of the stock standard solution with 1 L of YNBG broth to give final concentrations of 2.56, 5.13, and 7.69 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 was obtained from the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, University of Sassari, Sassari, Italy. Precultures were prepared in broth containing 2% glucose, 0.5% yeast extract, and 1% peptone in a thermostatically controlled chamber at 25 °C for 48 h. Cells were washed twice and suspended in 0.15 M NaCl. The amounts of suspension used as an inoculum were such to ensure 1×10^6 cells/mL 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 photoproduct. All the flasks were allowed 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. A 10 mL sample was drawn at appropriate times from each flask, and the following analyses were made: pH, number of yeast cells per milliliter (microscopic count and culture count), CO_2 production (indirect weighing). To quantify CHB, 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, weighed, and then transferred into a 20 mL screw-capped tube containing 10 mL of CH_2Cl_2. The tube was shaken in a rotary shaker for 1 h, and then the solution was evaporated and the residue taken up in 10 mL of the mobile phase used in HPLC determination. The filter was previously checked to verify that it did not adsorb CHB.

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 the mobile phase used in HPLC analysis. The recovery obtained from three extractions for each spiking level tested ranged between 98.7% and 95.0%.

Adsorption on Glucan and Chitin. The adsorption of CHB was measured at 25 ± 2 °C. Triplicate samples of 1,3-β-D-glucan (25 mg) or chitin (25 mg) were equilibrated in polyallomer centrifuge tubes with 2.5 mL of CHB aqueous solution. Photoproduct concentrations before equilibration ranged from 7.4 to 29.0 μM. The tubes were shaken (end-over-end) for 15 h. After equilibration, the suspension was centrifuged at 19000*g* for 20 min and the supernatant was pipetted off and analyzed immediately. Adsorbed CHB was calculated from the difference between its initial and final concentrations in solution.

Analytical Methods. The amounts of fenhexamid and its photoproduct were estimated by HPLC. A Waters 510 liquid chromatograph equipped with a 250 × 4 mm i.d. Bondapak C_{18} (10 μm) analytical column, a multiwavelength Waters 2487 programmable detector operating at 230 nm, and a Waters Breeze chromatography workstation were used. Acetonitrile plus water (50:50 by volume), previously brought to pH 2.7 with phosphoric acid, at a flux rate of 0.5 mL min^{-1}, was the eluant. Under the chromatographic conditions described previously the retention times for fenhexamid and CHB were 9.7 and 15.0 min, respectively. The quantization of CHB was based on calibration curves in the range 0.10−10.00 mg L^{-1} ($r^2 > 0.990$). The detection limits for fenhexamid and CHB were 0.01 and 0.02 mg L^{-1}, respectively, as calculated from the concentrations needed to obtain a detector response approximately 3 times the background signal.

NMR (nuclear magnetic resonance) spectra were obtained with a Bruker AC-P (300 MHz) NMR spectrometer using Bruker software.

Mass spectra were obtained with an Agilent 1100 HPLC−MS spectrometer, ESI positive mode, equipped with an ion spray liquid−mass interface.

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 (μmol kg^{-1}) is the amount of photoproduct adsorbed by glucan or chitin, respectively, C_e (μM) is the equilibrium concentration, and log K_f and $1/n$ are empirical constants representing the intercept and the slope of the isotherm, respectively. The fitting was performed by least-squares regression analysis. All experiments were carried out in three replicates. Variance analysis (ANOVA) and comparisons between average values were performed with the Duncan test at $P < 0.05$.

RESULTS AND DISCUSSION

Photolysis. Fenhexamid was stable in the dark, whereas the half-life of fenhexamid irradiated at 366 nm was 990 min. The photolysis followed pseudo-first-order kinetics ($k_{obs} = 7 \times 10^{-4}$ min^{-1}) and yielded mainly CHB (**Figure 1**). The reaction is

Table 1. Effect of CHB on the Fermentation Activity of *S. cerevisiae*

	0 days		1 day		5 days		12 days	
[CHB] (mg L^{-1})	no. of cells mL^{-1}	$[CO_2]^a$	no. of cells mL^{-1}	$[CO_2]^a$	no. of cells mL^{-1}	$[CO_2]^a$	no. of cells mL^{-1}	$[CO_2]^a$
control	1.0×10^6		2.1×10^7	1.8	3.4×10^7	7.5	4.0×10^7	10.6
2.56	1.0×10^6		1.6×10^7	0.8	3.0×10^7	7.2	3.5×10^7	10.3
5.13	1.0×10^6		1.3×10^7	0.7	3.0×10^7	7.2	3.0×10^7	9.9
7.69	1.0×10^6		1.1×10^7	0.5	2.5×10^7	7.0	3.0×10^7	10.1

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

Table 2. Photoproduct Residue Concentrations (mg L^{-1}) in the Alcoholic Fermentation of *S. cerevisiae* Yeasts

sample	0 days	1 day	5 days	12 days
control	2.56	2.24	2.20	1.56
medium[a]	2.48	2.04	1.72	0.91
yeast cells[b]	0.08	0.18	0.39	0.64
control	5.13	3.86	3.51	3.01
medium[a]	4.97	3.51	2.75	1.75
yeast cells[b]	0.15	0.31	0.62	1.24
control	7.69	5.79	5.26	4.51
medium[a]	7.45	5.26	4.12	2.62
yeast cells[b]	0.22	0.46	0.93	1.86

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

the result of an intramolecular chlorine photosubstitution. The mechanism is analogous to that observed in the photoreaction of 2′-chloro-4-R-benzanilide, structurally related to fenhexamid (*12*). A minor photoproduct (about 10%) was not isolated, but it was tentatively identified as *N*-(3-chloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide (NMC; $[M + H]^+$ $m/e = 268$; **Figure 1**) by HPLC−MS analysis. These findings are in agreement with those of Anderson et al. (*6*). Enough CHB for fermentation trials was obtained by irradiation of fenhexamid in alkaline medium (*12*).

Fermentation. Experiments were carried out at three CHB concentrations (**Table 1**). Fermentation parameters were measured at the beginning of the inoculation (0 days), and 1, 5, and 12 days later. A significant decrease of the amount of alcohol was observed, at all CHB levels, after the first fermentation day (**Table 1**) most likely because of the stress of the exogenic molecule on the yeast strains. At the end of the experiment, the stress was overcome and the amount of produced alcohol was comparable to that of the control sample. This suggests that the photometabolite did not interfere with the fermentation process over the whole concentration range tested.

CHB residues were determined both in the fermentation medium and in yeasts (**Table 2**). The amount of CHB in the control liquid phase decreased by ca. 40%, which suggests that CHB degrades in the fermentation medium. Actually, we observed that an aqueous acidic solution of CHB (pH 2.8) was not stable. This finding suggests that the acidity of the fermentation medium (pH 2.6−3.0) may be responsible for the CHB loss.

In the presence of yeasts, the loss of CHB increased further (ca. 65%). However, the additional amount of missing CHB was extracted unchanged from yeasts (**Table 2**). This suggests that part of the photometabolite was adsorbed by yeasts rather than degraded in the fermentation process. The adsorptive capacity of *S. cerevisiae* for organic pollutants is well-known (*13*). *S. cerevisiae* was successfully immobilized on silica gel and used in the on-line isolation and trace enrichment of different pesticides (*14*). Generally, the adsorption ability is

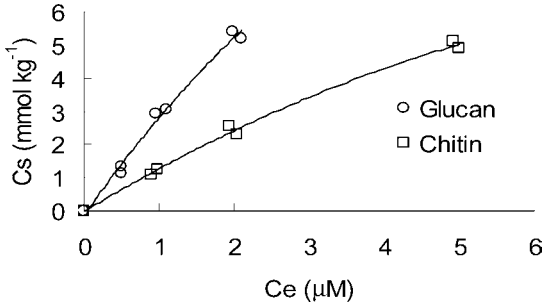

Figure 2. Adsorption isotherms of CHB on glucan and chitin.

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

adsorbent	K_f	1/*n*	*r*
glucan	2.65	1.03	0.989
chitin	1.27	0.87	0.995

attributed to the polysaccharides of the cell walls (*15*), which are rich in hydroxyl groups available for binding through hydrogen bonding.

Among the polysaccharides of the *S. cerevisiae* cell walls, chitin and glucan were tested as potential adsorbents of CHB. Adsorption isotherms of the metabolite on glucan and chitin are shown in **Figure 2**.

The adsorption isotherms on glucan were linear or of the C type according to the classification of Giles et al. (*16*). This shape is characterized by a constant partition of solute between solution and adsorbing substrate (**Figure 2**). Instead, the isotherm of the photoproduct on chitin was of the L type, indicating a relatively high affinity between solute and adsorbing sites at least in the initial stages of the isotherm; as more sites are filled, it becomes increasingly difficult to find a site available.

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**.

Glucan was more effective than chitin in CHB retention. Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine, is less rich in surface hydroxyl groups compared to glucan; therefore, it adsorbs CHB to a lower extent. Also fenhexamid was adsorbed by glucan and chitin, but to a lower extent (*10*). Most likely, the more planar shape of CHB, compared with the parent molecule, makes CHB more prone to adsorption.

Conclusions. Similar to what was found for fenhexamid, the CHB photometabolite does not affect alcoholic fermentation. The decrease of CHB concentration observed in the fermentation medium is due partly to degradation and partly to adsorption. The adsorbed fraction was recovered unchanged from yeasts. Most likely, cell wall surfaces are responsible for the adsorption.

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