Killer Toxin of *Kluyveromyces phaffii* DBVPG 6076 as a Biopreservative Agent To Control Apiculate Wine Yeasts

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The use of *Kluyveromyces phaffii* DBVPG 6076 killer toxin against apiculate wine yeasts has been investigated. The killer toxin of *K. phaffii* DBVPG 6076 showed extensive anti-*Hanseniaspora* activity against strains isolated from grape samples. The proteinaceous killer toxin was found to be active in the pH range of 3 to 5 and at temperatures lower than 40°C. These biochemical properties would allow the use of *K. phaffii* killer toxin in wine making. Fungicidal or fungistatic effects depend on the toxin concentration. Toxin concentrations present in the supernatant during optimal conditions of production (14.3 arbitrary units) exerted a fungicidal effect on a sensitive strain of *Hanseniaspora uvarum*. At subcritical concentrations (fungistatic effect) the saturation kinetics observed with the increased ratio of killer toxin to *H. uvarum* cells suggest the presence of a toxin receptor. The inhibitory activity exerted by the killer toxin present in grape juice was comparable to that of sulfur dioxide. The findings presented suggest that the *K. phaffii* DBVPG 6076 killer toxin has potential as a biopreservative agent in wine making.

Since Bevan and Makower (3) discovered the killer phenomenon in strains of *Saccharomyces cerevisiae*, several other yeast species have been found to produce a toxic proteinaceous factor that kills sensitive strains (17, 20, 21, 30, 31). Killer strains of *S. cerevisiae* show an antiyeast spectrum restricted to sensitive *Saccharomyces* strains except for a report on the killing of *Torulopsis glabrata* (37). Unlike the genus *Saccharomyces* a wide spectrum of intergeneric activity was reported for killer toxins from other genera such as *Pichia* (13, 14, 25), *Hansenula* (1, 18), *Williopsis* (34), and *Kluyveromyces* (22, 37).

Several potential applications for the killer phenomenon have been suggested since it was determined and studied. In fermentation industries, the killer character can be used to combat wild, contaminating *Saccharomyces* strains (33, 36). In the food industry, killer yeasts have been proposed to control spoilage yeasts in the preservation of food (16). In the medical field, killer yeasts have been used in the biotyping of pathogenic yeasts (15, 18), and the killer toxins of *Pichia anomala* (25, 26) and *Williopsis mrakii* (34) have been proposed as antimycotic agents.

In wine making, killer yeasts belonging to *S. cerevisiae* are currently used to initiate wine fermentation to improve the process of wine making and wine quality (28, 33). However, the main limit of the killer toxin of *S. cerevisiae* wine yeast (K2 type) resides in its narrow antiyeast spectrum which, being restricted to sensitive *Saccharomyces* strains, does not affect wild yeasts, such as those belonging to the genera *Hanseniaspora/Kloeckera, Pichia*, and *Saccharomycodes*.

Several ecological studies (9, 11, 12, 23) have clearly demonstrated that apiculate yeasts (*Hanseniaspora/Kloeckera*) predominate on grape surfaces and in freshly pressed juice. The control of the growth of apiculate yeasts in a nonsterile environment such as grape must is generally carried out by sulfur dioxide. However, several institutions, such as the World Health Organization and the European Economic Community, have highlighted the need to reduce the use of this antimicrobial agent in food products because of its toxicity. In this context, the use of a killer toxin as a control agent for apiculate yeasts in the prefermentative stage and during the fermentation of grape must has to be encouraged in order to reduce or eliminate the use of SO_2. The findings of this study indicate that the killer toxin produced by *Kluyveromyces phaffii* DBVPG 6076 may be used as a biological agent against apiculate yeasts, which are usually present in freshly pressed juice and during the first stage of alcoholic fermentation.

MATERIALS AND METHODS

Cultures and media. The strains used in the present study were from the Industrial Yeasts Collection of the University of Perugia (DBVPG): *K. phaffii* DBVPG 6076, a killer yeast; *S. cerevisiae* 6500 (NCYC 1006; National Collection of Yeast Cultures, Norwich, England), a sensitive strain; *S. cerevisiae* DBVPG 6664 (commercial strain Prise de Mousse; Red Star, Milwaukee, Wis.), a selected starter culture resistant to the DBVPG 6076 toxin; and *Hanseniaspora uvarum* DBVPG 3037. Strains isolated from grape berries from various vineyards of the Umbria region, classified as *Hanseniaspora* spp. by the methods of Kurtzman and Fell (10), were used to evaluate the frequency of the killer activity of DBVPG 6076. All yeast strains were subcultured at 6-month intervals on malt agar and maintained at 6°C. DBVPG 6076 was grown in yeast extract-peptone-dextrose (YPD) medium. The composition of YPD buffered at pH 4.5 (0.1 M citrate-phosphate buffer) was as follows (per liter): Bacto yeast extract, 10 g; Bacto Peptone, 10 g; and glucose, 50 g. Grape juice obtained from Grechetto grapes (pH 3.29; sugar, 209 g liter^{-1}) was used for trials in natural medium.

Assessment of DBVPG 6076 killer toxin (Kpkt) activity. The frequency of killer toxin activity was evaluated by streak plate agar diffusion assay (21). Approximately 10^5 cells ml^{-1} (final concentration) of the strain to be tested for sensitivity to the killer toxin were uniformly suspended in 20 ml of Bacto malt agar (Difco Laboratories, Detroit, Mich.) buffered at pH 4.5 (0.1 M citrate-phosphate buffer), maintained at 45°C in a water bath, and poured immediately into sterile petri dishes. DBVPG 6076 was streaked on the agar surface, and the

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plates were incubated at 20°C for 72 h. Killer activity was evident as a clear zone of inhibition surrounding the streak.

Toxin preparations were assayed by the well test method of Somers and Bevan (29). Toxin samples were filter sterilized though 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). Seventy microliters of toxin sample was placed in wells (7-mm diameter) cut in the malt agar medium buffered at pH 4.5 (0.1 M citrate-phosphate buffer) (Difco). Malt agar plates had been previously seeded with a sensitive indicator strain. The killing activity of each sample was measured and defined as the mean zone of inhibition of replicate wells after incubation for 72 h at 20°C.

Kpkt production. DBVPG 6076 was grown in YPD broth in a 2-liter flask with 1 liter of working volume for 72 h at 25°C on a rotary shaker (150 rpm^{-1}). After centrifugation (3,000 × g for 10 min at 4°C), the supernatant was filtered though 0.45-μm-pore-size membrane filters (Millipore). Ice-cold ethanol was added to the filtrate to a final volume of 50% (vol/vol). After 2 h at −18°C, the resulting precipitate was collected by centrifugation (10,000 × g for 30 min) and resuspended in a reduced volume of 10 mM citrate-phosphate buffer (pH 4.5). For further concentration, Amicon YM10 (10-kDa-cutoff membrane) (Pharmacia, Uppsala, Sweden) was used.

Measurement of Kpkt activity. Under the experimental conditions used, a linear relationship was observed between the logarithm of killer toxin concentration and the diameter of the inhibition zone assayed by the well test method. One arbitrary unit (AU) is defined as the toxin concentration in the supernatant that caused a clear zone of 8.0 mm (actual diameter, 15 mm, minus diameter of the 7-mm well), using *S. cerevisiae* DBVPG 6500 as a sensitive indicator strain and 70 μl of sample.

Effects of proteolitic enzymes and temperature stability. Five hundred microliters of concentrated killer toxin was mixed with 125 μl of type IV papain solution (10 mg ml^{-1}, 10 to 15 U mg^{-1}). The toxin and protease solution was added to 4.5 ml of YPD broth inoculated with a sensitive *H. uvarum* DBVPG 3037 strain (initial inoculum, 10^6 cells ml^{-1}). After an incubation period of 20 h at 20°C, the effects of the proteolytic enzyme were evaluated by the well test assay and a viable-cell count. Similar procedures were carried out in order to evaluate the temperature stability of the killer toxin. Samples of killer toxin were incubated at 20, 25, 30, 35, 40, 45, or 50°C for 2 h, mixed with YPD broth, and then inoculated with a sensitive *H. uvarum* DBVPG 3037 strain (initial inoculum, 10^6 cells ml^{-1}). Killer toxin was also subjected to heat treatment (100°C for 10 min). Killer activity was determined by the well test assay and a viable-cell count after incubation for 24 h at 20°C.

pH stability. In order to determine the pH range of activity, samples of killer toxin were tested by the well test assay using malt agar plates buffered at pH values of 2.8, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0.

Fungistatic and fungicidal effects. Experiments were carried out in 300-ml Erlenmeyer flasks containing 100 ml of YPD broth buffered at pH 4.5 at 20°C. A 48-h preculture of *H. uvarum* DBVPG 3037, also grown at 20°C in the same medium as the test, was inoculated to obtain an initial count of 10^5 cells ml^{-1}. Different toxin concentrations were added, and cell growth was evaluated by measuring optical density at 580 nm (OD$_{580}$) using a DU 640 spectrophotometer (Beckman, Fullerton, Calif.) or by counting viable cells on plates. A control test (without toxin) was included in all assays.

Growth rate reduction assay. The reduction of the growth rate was carried out according to the procedures of Sawant et al. (25), evaluating toxin concentrations lower than that which caused fungicidal activity. Cells of *H. uvarum* DBVPG 3037 in log phase were inoculated at an initial OD$_{580}$ of 0.1 in 300-ml Erlenmeyer flasks containing 100 ml of YPD broth and incubated at 25°C in a rotary shaker (150 rpm). Killer toxin was added to the medium at the following concentrations (AU): 0.14, 0.26, 0.71, 1.43, 2.86, 5.72, 11.44, and 14.3. Optical densities were evaluated at 0 h and at 1-h intervals up to 9 h. Growth rates were determined by linear regression analyses of optical densities from 4 to 9 h of growth.

Activity of Kpkt in grape juice. Trials in grape juice were carried out in 300-ml Erlenmeyer flasks containing 100 ml of pasteurized grape juice (100°C for 10 min). Concentrated Kpkt was added, and the procedure was standardized to provide the following final concentrations (AU ml^{-1}): 5.14, 7.15, and 14.3. Sulfur dioxide at 37.5 (free SO$_2$, 3.20 ml liter^{-1}), 75.0 (free SO$_2$, 6.08 ml liter^{-1}), and 150 (free SO$_2$, 10.28 ml liter^{-1}) ml liter^{-1} was added 24 h before the inoculation in order to obtain the binding equilibrium. Precultures of *H. uvarum* DBVPG 3037 were grown at 20°C in the same medium as the test for 48 h and inoculated in order to obtain an initial count of 10^5 cells ml^{-1}. Experiments were carried out in static conditions at 20°C by fitting each flask with a glass valve containing sulfuric acid, which allowed only CO$_2$ to escape the system (5). An inoculum of *S. cerevisiae* DBVPG 6664 starter culture (5 × 10^6 cells ml^{-1}) was added 48 h after the beginning of fermentation. *S. cerevisiae* was preincubated by the procedures used for the inoculum of *H. uvarum* DBVPG 3037. Trial fermentations

TABLE 1. Frequency of killer activity of *K. phaffii* DBVPG 6076 among *Hanseniaspora* spp. strains isolated from grape berries from different vineyards of the Umbria region

Yr of isolation	No. of sensitive strains	No. of resistant strains	No. of samples[a]	Sensitive strains (%)
1994	80	1	20	98.8
1995	90	1	12	98.9
1996	98	14	12	87.5
1997	30	0	8	100.0
Total	298	16	52	94.9

[a] Number of bunches from which the apiculate strains were isolated.

with pure cultures of *S. cerevisiae* DBVPG 6664 and *H. uvarum* DBVPG 3037 were also included as control tests. The progress of fermentation was monitored by the amount of weight lost due to the carbon dioxide evolved. When the weight of the apparatus became constant, the fermentation was considered to be finished and samples were collected by filtration (0.45-μm-pore-size membrane; Millipore) for chemical analysis. Volatile acidity (expressed as grams of acetic acid per liter) was quantified by steam distillation according to official analytical procedures (6). Acetaldehyde and ethyl acetate were detected by gas-liquid chromatographic analysis as described by Bertuccioli (2).

RESULTS

Killer activity of *K. phaffii* on apiculate wine yeasts. The evaluation of the spectrum of activity of DBVPG 6076 is the first step toward the practical application of this killer toxin in the control of apiculate yeasts in wine making. Thus, 298 strains of apiculate yeasts belonging to *Hanseniaspora* spp., isolated from 52 different grapes and sampled in the course of four years, were tested for their sensitivity to the *K. phaffii* DBVPG 6076 killer strain. Interestingly, 94.9% of the strains of apiculate wine yeasts were sensitive to the killer activity of *K. phaffii* (Table 1).

Characterization of Kpkt. Studies were carried out in order to elucidate the biochemical properties of Kpkt, particularly in relation to its possible use in wine making. The toxin was concentrated 10-fold by ethanol precipitation and this or more-concentrated preparations were used for successive characterization of the killer factor. The activity of Kpkt in the supernatant and after precipitation in ethanol are shown in Table 2.

Since the killer toxins known so far are proteins or glycoproteins, the effects of protease treatment on the killer factor of *K. phaffii* were assayed. Papain, which breaks sulfide bonds, inactivated the killer toxin (Table 3).

Further characterization of the DBVPG 6076 toxin was carried out by evaluating the effects of pH and temperature on the activity of the killer factor. Results reported in Fig. 1A show that Kpkt was active in the pH range of 3.0 to 5.0, but a 30% reduction in activity was observed at pH 3.0 against the sensi-

TABLE 2. Killer activity of Kpkt in the supernatant and after ethanol precipitation[a]

Activity measured	Toxin concn factor	AU ml^{-1}	Killer activity against:	
			S. cerevisiae (diam [mm])	*H. uvarum* (diam [mm])
In the supernatant	1	14.3	8.0	4.0
After ethanol precipitation	10	143.3	16.0	8.0

[a] Mean of eight different experiments. The variation was less than 5%.

TABLE 3. Effect of papain on Kpkt[a] activity[b]

Time (h)	CFU (10^6 ml^{-1})		
	Control		Killer toxin and papain
	No killer toxin	Killer toxin	
0	0.3	0.2	0.4
24	58.0	0.2	18.0

[a] Toxin concentration, 14.3 AU ml^{-1}.
[b] The diameters of the zones of inhibition (measured in the well test assay) for *S. cerevisiae* were as follows: without killer toxin, 0.0 mm; with killer toxin, 8.5 mm; and with killer toxin and papain, 0.0 mm. The diameters of the zones of inhibition (measured in the well test assay) for *H. uvarum* were as follows: without killer toxin, 0.0 mm; with killer toxin, 4.5 mm; and with killer toxin and papain, 0.0 mm.

tive *H. uvarum* strain. The killer activity observed for both sensitive strains was completely lost at pH 6. At pH 2.8 the toxin showed reduced activity against *S. cerevisiae*, whereas no activity was observed against *H. uvarum*. Regarding the influence of temperature, the killer activity was stable up to 25°C (Fig. 1B), whereas higher temperatures caused a progressive decrease of the activity of Kpkt. No activity was found after 2 h of incubation at 40°C. Heat treatment (100°C for 10 min) caused loss of activity. These results were confirmed by a viable-cell count assay (data not shown).
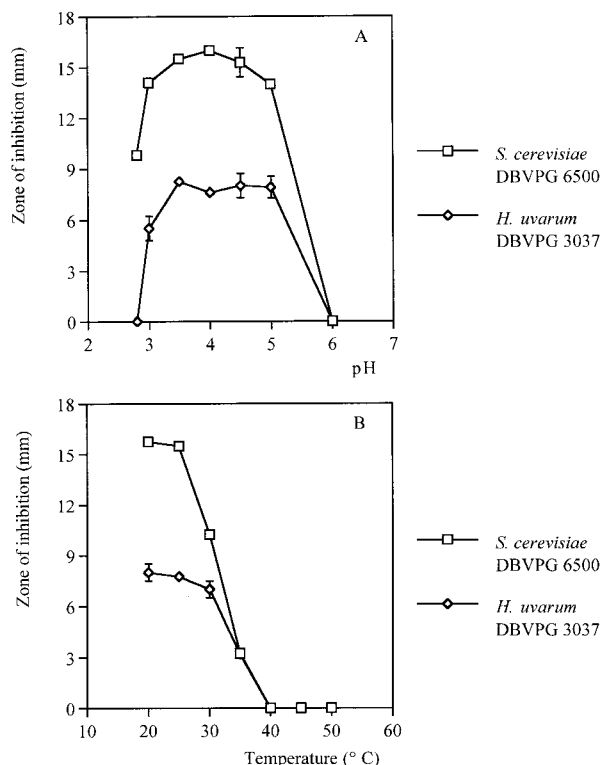
FIG. 1. Effects of pH (A) and temperature (B) on the activity of Kpkt. pH effects were evaluated by the well test assay. Malt agar was buffered from pH 2.8 to 6.0 10 mM citrate-phosphate buffer. The toxin concentration was 14.3 AU ml^{-1}. After 2 h of incubation at various temperatures, the killer activity was evaluated by the well test assay (pH 4.5). The toxin concentration was 14.3 AU ml^{-1}. Data are given as means ± standard deviations of at least duplicate experiments.
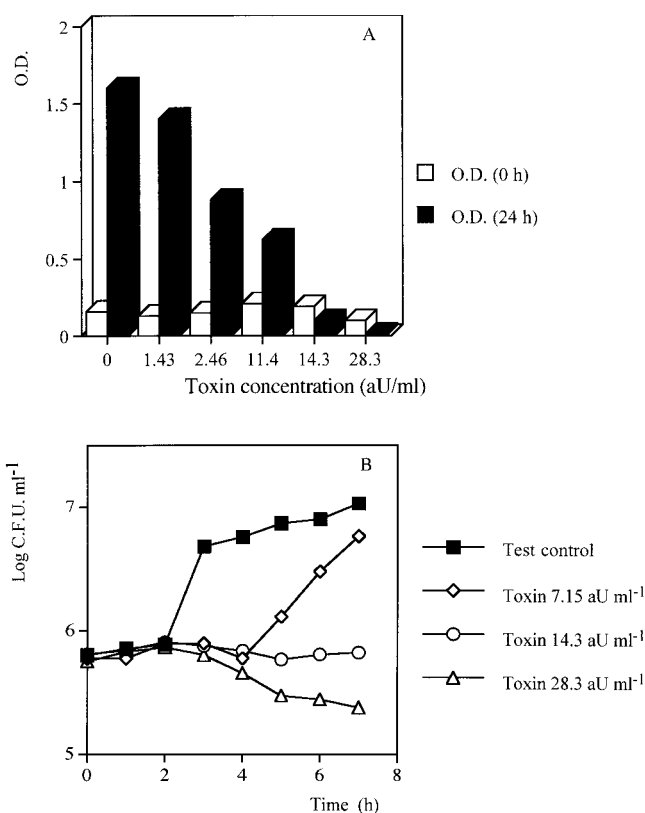
FIG. 2. Fungistatic and fungicidial activity of Kpkt evaluated by OD unit (A) and log CFU ml^{-1} (B). Cells were grown in YPD broth buffered at pH 4.5. The initial inoculum contained 10^5 *H. uvarum* cells ml^{-1}. Each sampling point represents the mean of duplicate experiments. The variation was less than 10%.

Mode of action of Kpkt against *H. uvarum* DBVPG 3037. The effects of Kpkt on *H. uvarum* DBVPG 3037 growth were tested in liquid media at different concentrations of the killer factor. The growth of *H. uvarum* (assayed by measuring the OD$_{580}$ after 24 h of incubation at 20°C) showed a progressive reduction with increased toxin concentrations. Interestingly, at the toxin concentration corresponding to 14.3 AU in the supernatant (Fig. 2A), no growth was observed. In another trial, the evaluation of the viable cells during the first 8 h of growth confirmed that the critical concentration necessary for a fungicidal effect was 14.3 AU (Fig. 2B). Lower concentrations appeared fungistatic for *H. uvarum*, extending lag phase for 2 h before growth resumed.

In order to assess the modality of action of Kpkt, the growth rate reduction assay (25) was carried out. The plotting of increased concentrations of the killer toxin against the growth rate (Fig. 3) showed an exponential relationship with typical saturation kinetics.

Activity of Kpkt in grape juice. In order to verify the potential of DBVPG 6076 as a biological antiyeast agent in wine making, DBVPG 6076 toxin activity was assayed in natural grape juice. Moreover, the activities of Kpkt and SO$_2$, the chemical antiseptic agent universally used in winemaking, were compared. Figure 4 shows the development of viable *H. uva-*
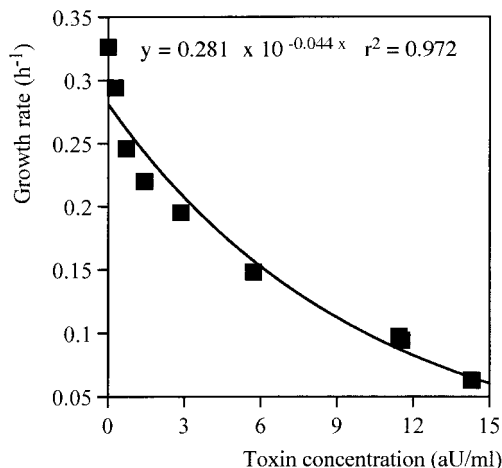
FIG. 3. Effects of increasing concentrations of Kpkt on the growth rate of *H. uvarum* DBVPG 3037. Procedures are described in Materials and Methods. Each sampling point represents the mean of duplicate experiments. The variation was less than 10%.

FIG. 4. Comparison of Kpkt (A) and SO$_2$ (B) activities against *H. uvarum* DBVPG 3037 in natural grape juice. The initial inoculum of *H. uvarum* was 10^5 cells per ml. The grape juice had a pH of 3.29 and a sugar concentration of 209 g liter^{-1}. Data are given as means ± standard deviations of at least duplicate experiments. The presence of toxin was relieved during the time of fermentation (data not shown).

rum cells in the presence of different concentrations of Kpkt and SO$_2$ during the first 48 h of growth at the stage of fermentation when apiculate yeasts naturally dominate the process. The positive control (inoculum of *H. uvarum* without antimicrobial agents) showed 45×10^6 cells ml^{-1} after 48 h of fermentation. As expected, the presence of toxin caused reduced growth of *H. uvarum* (Fig. 4A). A toxin concentration of 14.3 AU ml^{-1} resulted in a complete inhibition of the apiculate strain after 48 h of incubation. Absence of growth was obtained with 7.15 AU of toxin ml^{-1} within 24 h. After this time, reduced growth was exhibited by an apiculate yeast showing approximately 10×10^6 cells ml^{-1} after 48 h. Lower concentrations (5.14 AU ml^{-1}) caused only reduced growth of *H. uvarum* without a prolonged lag phase. The effects of sulfur dioxide on the growth of *H. uvarum* were similar to those of the DBVPG 6076 toxin (Fig. 4B). After 48 h of incubation, no growth was exhibited in the presence of 150 mg of SO$_2$ liter^{-1}, whereas 37.5 and 75 mg of this antiseptic agent liter^{-1} caused lag phases of 24 and 30 h, respectively, before growth resumed.

The evaluation of some fermentation products obtained after the inoculation of the *S. cerevisiae* starter culture (Table 4) showed that the control of *H. uvarum* is accompanied by a progressive reduction of ethyl acetate. Amounts of ethyl acetate similar to those produced by the *S. cerevisiae* control test were exhibited by the trials containing 14.3 aU of toxin ml^{-1} and 150 mg of SO$_2$ liter^{-1}. Increased amounts of ethyl acetate were caused by lower concentrations of two antiseptic agents without reaching the taste threshold (150 mg liter^{-1}) (8). No relevant differences were found among the trials for volatile acidity, whereas the trials with SO$_2$ showed a small increase of acetaldehyde compared to the trial tests.

DISCUSSION

The use of *S. cerevisiae* killer yeasts in vinification prevents stuck fermentations caused by wild killer yeasts (33). However, antiyeast activity restricted to sensitive *Saccharomyces* strains does not permit the control of wild non-*Saccharomyces*, par-
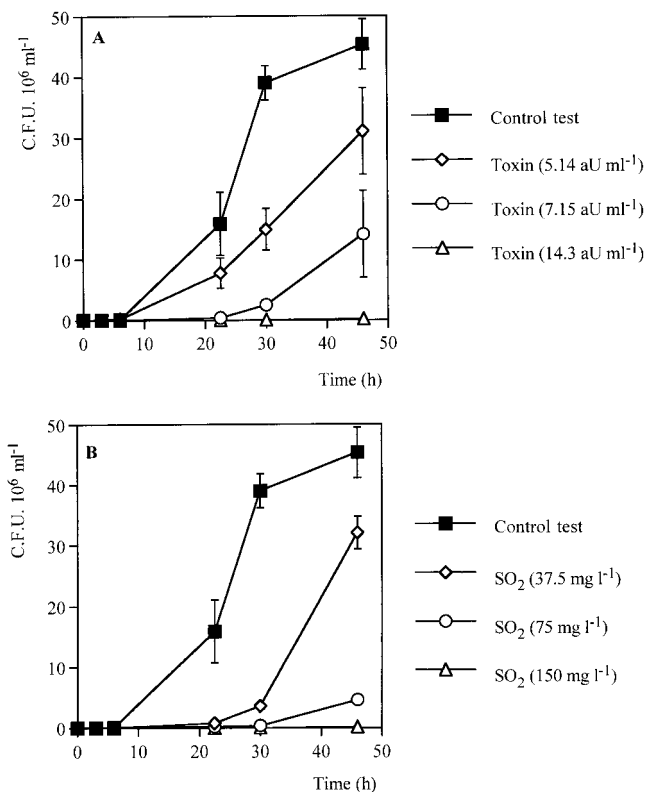
ticularly apiculate yeasts which are present in freshly pressed grape juice. *K. phaffii* exhibits killer activity against apiculate yeasts (*Kloeckera apiculata/H. uvarum*) (22) and other spoilage yeasts (16). In order to assess the potential use of DBVPG -6076 in wine making we verified the following features: (i) the diffusion of killer activity among apiculate wine yeasts, (ii) the killer toxin activity at the conditions used in vinification, and (iii) the antiseptic effect compared with that of a chemical preservative agent used in wine making (SO$_2$).

Results showed that Kpkt exhibits widespread killer activity against apiculate wine yeasts. Evidence from protease treatments suggests that Kpkt is a protein with sulfide bonds like several other killer factors (4, 35), since papain has been shown to destroy the toxin and its activity. Like most toxins (28), Kpkt is unstable at both high temperatures and high pH values. In contrast to the toxin of *Kluyveromyces lactis* (32,35), Kpkt has a low pH range of activity (up to pH 3.0). These findings are in agreement with the use of Kpkt at the majority of pHs and temperatures in wine making conditions.

The characterization of Kpkt activity against *H. uvarum* indicates that fungistatic or fungicidal effects depend on the toxin concentration. A toxin concentration of 14.3 aU ml^{-1} exerted zymocidal activity against *H. uvarum* DBVPG 3037. At subcritical concentrations of toxin (fungistatic effect), the saturation kinetics observed with an increased ratio of Kpkt to *H.*

TABLE 4. Influence of Kpkt and SO_2 activity on some fermentation products of wines

Organism(s)	Volatile acidity (g liter^{-1})	Acetaldehyde (mg liter^{-1})	Ethyl acetate (mg liter^{-1})
H. uvarum	0.47	21.0	307
S. cerevisiae	0.33	29.5	43
H. uvarum and S. cerevisiae	0.42	12.3	150
H. uvarum, S. cerevisiae, and toxin (5.14 AU)	0.36	15.4	138
H. uvarum, S. cerevisiae, and toxin (7.15 AU)	0.36	21.0	128
H. uvarum, S. cerevisiae, and toxin (14.3 AU)	0.34	15.2	45
H. uvarum, S. cerevisiae, and SO_2 (37.5 mg liter^{-1})	0.36	24.4	130
H. uvarum, S. cerevisiae, and SO_2 (75 mg liter^{-1})	0.32	27.0	104
H. uvarum, S. cerevisiae, and SO_2 (150 mg liter^{-1})	0.38	36.0	38

uvarum cells suggest the presence of a toxin receptor, probably on the cell wall. Cell wall receptors are known to mediate toxin action in the killer systems of several yeast species (7, 19, 24, 27).

The activity of Kpkt in grape juice is comparable to that of sulfur dioxide. The toxin concentration normally present in the supernatant (14.3 AU ml^{-1}) is capable of controlling apiculate yeasts for 48 h at cell densities generally found in grape juice at the beginning of wine fermentation (9). The inhibition exerted by Kpkt on apiculate fermentation activity is reflected by the decrease in by-products such as ethyl acetate, the main compound responsible for the vinegary odor in wines, and acetaldehyde (linked to SO_2 addition), an undesirable compound due to its capacity to combine with SO_2, which is added for the preservation of wine. In conclusion, Kpkt, the sole toxin known to exhibit killer activity against apiculate yeasts, has great potential as a biopreservative agent in wine making and can profitably be substituted for SO_2 at the prefermentation stage. Moreover, this killer toxin could be also used in the food industry since broad-spectrum activity against spoilage yeasts was shown (16).

Further studies are in progress to acquire additional information on the biochemical properties of Kpkt, contributing to the understanding and development of a novel biopreservative agent to combat wild microflora in winemaking.

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