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Short communication

TdKT, a new killer toxin produced by *Torulaspora delbrueckii* effective against wine spoilage yeasts

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ABSTRACT

Microbiological spoilage is a major concern throughout the wine industry, and control tools are limited. This paper addresses the identification and partial characterization of a new killer toxin from *Torulaspora delbrueckii* with potential biocontrol activity of *Brettanomyces bruxellensis*, *Pichia guilliermondii*, *Pichia manshurica* and *Pichia membranifaciens* wine spoilage. A panel of 18 different wine strains of *T. delbrueckii* killer yeasts was analysed, and the strain *T. delbrueckii* NPCC 1033 (TdKT producer) showed a significant inhibitory effect on the growth of all different spoilage yeasts evaluated. The TdKT toxin was then subjected to a partial biochemical characterization. Its estimated molecular weight was >30 kDa and it showed glucanase and chitinase enzymatic activities. The killer activity was stable between pH 4.2 and 4.8 and inactivated at temperature above 40 °C. Pustulan and chitin — but not other cell wall polysaccharides — prevented sensitive yeast cells from being killed by TdKT, suggesting that those may be the first toxin targets in the cell wall. TdKT provoked an increase in necrosis cell death after 3 h treatment and apoptotic cell death after 24 h showing time dependence in its mechanisms of action. Killer toxin extracts were active at oenological conditions, confirming their potential use as a biocontrol tool in winemaking.

1. Introduction

As producers of toxins that can neutralize the activities of spoilage yeasts in wines, killer yeasts represent an interesting biocontrol strategy (Meinhardt and Klassen, 2009; Viljoen, 2006). Several authors have proposed the use of killer toxins produced by *Candida pyralidae*, *Kluyveromyces wickerhamii*, *Pichia membranifaciens* and *Wickerhamomyces anomalus* (e.g., *Pichia anomala*) to control the development of *Dekkera*/*Brettanomyces*, the principal wine spoilage yeast (Comitini et al., 2004a; Mehlomakulu et al., 2014; Sangorrín et al., 2008). Killer toxins from *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Tetrapisispora phaffii* (e.g., *Kluyveromyces phaffii*) and *W. anomalus*, have also been proposed as control agents towards spoilage yeasts present during the early stages of winemaking such as *Candida boidinii*, *Hanseniaspora uvarum* and *Pichia guilliermondii* (Ciani and Fatichenti, 2001; Lopes et al., 2009; Sangorrín et al., 2008). Despite this relatively high number of killer toxins reported in the bibliography, no commercial product formulated with any of these compounds has been developed so far. Practical application of killer toxins is generally limited because most of them are unstable or inactive at wine conditions (Schmitt and Breinig, 2002). Grape must and wine environment are characterized by their high sugar content (>250 g/L), low pH values around 3.5 and high ethanol and SO_2 concentrations (Bauer and Pretorius, 2000; Fleet and Heard, 1993). In this context, the search for new toxins that are resistant to wine stress factors with potential applicability in winemaking is still necessary.

Several screening studies focused on determining the occurrence of killer yeasts in winemaking environments have been carried out, and they have demonstrated the presence of killer phenotypes in different species from wines, cellar surfaces and the winery equipment (Musmanno et al., 1999; Sangorrín et al., 2007, 2008). In previous studies carried out in our laboratory, most yeasts isolated from spontaneously fermenting grape musts evidenced the killer character (Lopes et al., 2007; Sangorrín et al., 2002). These studies provide an exceptional source of potential antagonist yeasts to be used in biocontrol. In particular, killer yeasts belonging to *Torulaspora delbrueckii* species have shown the broadest killer spectrum against wine spoilage yeasts

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(Lopes and Sangorrín, 2010; Sangorrín et al., 2008). The killer capacity of *T. delbrueckii* was reported for the first time in a previous work from our laboratory (Sangorrín et al., 2007) but only some ecological aspects of this capacity have been evaluated until now (Lopes and Sangorrín, 2010). Because *T. delbrueckii* is ethanol-tolerant wine yeast detected in advanced stages of wine fermentations in North-Patagonia and other wine-regions worldwide (Ciani and Maccarelli, 1998; Renauld et al., 2009), the killer toxins produced by this species could be interesting for wine spoilage yeast biocontrol.

This paper focuses on the characterization of a killer toxin of *T. delbrueckii*, its biochemical properties and its ability to biocontrol different wine spoilage species. Crude extracts of the selected killer toxin were used to evaluate the stability of TdKT in oenological conditions and its putative mode of action, on sensitive strains.

2. Materials and methods

2.1. Yeast strains and media

Eighteen *T. delbrueckii* killer yeast strains from North Patagonia, the type strains of *T. delbrueckii* NRRL Y-866 and the sensitive reference strain *Candida glabrata* NCYC 388 were used for killer assays. A total of 12 strains of *Brettanomyces bruxellensis* (NRRL Y-411, CECT 2113, INTA VC20), *Pichia manshurica* (NPCC 1038), *P. membranifaciens* (NRRL Y-2026, INTA K21, USACH 3000, NPCC 1099) and *P. guilliermondii* (NRRL Y-075, NPCC 1055, USACH 3008, NPCC 1070) from different origins were used as representatives of wine spoilage yeasts. The yeasts were maintained in the GPY-agar medium (20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract and 20 g/L agar, pH 6.0). In the case of *B. bruxellensis* strains, the medium was supplemented with 5 g/L of calcium carbonate. All strains were maintained in the North Patagonian Culture Collection (NPCC, Neuquén, Argentina). Six commercial *Saccharomyces cerevisiae* yeast starters habitually used in North Patagonian wineries from different supplier companies were also used.

2.2. Killer toxin activity assays

2.2.1. Qualitative method (QLM)

The killer sensitivity evaluation was performed following the procedure described by Lopes and Sangorrín (2010). The lawn yeast was designated as sensitive when a clear zone of inhibition generally fringed with blue colour was observed surrounding the killer yeast streak after incubation for 48 h at 20 °C. Assays were performed in duplicate.

2.2.2. Quantitative methods

2.2.2.1. Well test method (WTM). Killer activity of the crude extracts (Section 2.3) was determined against *C. glabrata* NCYC 388 according to De Ingeniis et al. (2009) with modifications. The killer activity was evaluated as the diameter of the inhibition halo (3–5 mm) around the well after incubation for 48 h at 20 °C, and it was defined as the mean of two replicate wells.

2.2.2.2. Multiwell method (MWM). Killer activity of crude extracts (Section 2.3) was determined against *C. glabrata* NCYC 388 according to Guyard et al. (2002) with modifications. One unit of killer activity (KU) was defined as the volume of toxin extract necessary to inhibit 50% of the sensitive reference strain growth after 48 h of incubation at 20 °C. Assays were performed in quadruplicate.

2.3. Production of killer toxin extracts

Cultures were performed in 2 L Erlenmeyer flasks containing 1 L YEPD broth incubated at 20 °C. Growth was evaluated by measuring the OD_{640}. The production of killer toxins was monitored during the batch cultivation of the producing strain by means of the multiwell method (MWM), aliquots of 30 mL of the cultures were collected daily and used for crude extract preparation. Once cultures reached the stationary phase, cells were removed by centrifugation at 5000 × *g* for 10 min at 4 °C. The protein mix containing TdKT was obtained by precipitation of culture supernatant with 80% *w*/*v* ammonium sulphate and centrifugation at 7000 × *g*. The pellet obtained was resuspended in a 100 mM citrate–phosphate buffer pH 4.5, dialyzed overnight in the same buffer and threefold concentrated with sugar in dialyzing tubing (cut off 2 kDa). This concentrated supernatant was used as the crude killer toxin extracts. Aliquots of concentrated supernatants containing 20% *v*/*v* glycerol were filter-sterilized through 0.22 μm pore-size membrane filters (Millipore) and stored at −20 °C until their use for killer activity detection.

2.4. Killer toxin characterization

2.4.1. Molecular weight estimation

Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA, US) with cut-off at 3, 10 and 30 kDa were used to separate proteins from the crude extract preparation according to the recommended conditions of the supplier. The killer activity concentrated 10-fold by ultrafiltration flow-through of each different cut-off was tested in WTM assays against *C. glabrata* NCYC 388.

2.4.2. Effect of oenological parameters on killer toxin stability

The stability of the toxin was examined by testing the residual killer activity after 2 h incubation in each different condition. We tested the effect of different values of temperature (8, 15, 20, 26, 37, 50 °C), pH (3, 3.2, 3.6, 3.8, 4.4, 5, 6), ethanol concentration (0, 3, 6, 9, 12, 14, 16% *v*/*v*), glucose concentration (0, 10, 20, 210, 240, 280 g/L) and free SO_2 concentration (0, 25, 50, 75, 100, 150 ppm) employing the MWM against *C. glabrata* NCYC 388. YEPD was used as the general medium and different pH conditions were obtained using a citrate–phosphate buffer. After toxin incubation with sodium metabisulphite, the aliquots were extensively dialyzed against a 10 mM citrate–phosphate buffer (pH 4.5) and assayed for killer activity as previously described. The temperature and pH used for the assays at different ethanol, glucose and SO_2 were 20 °C and pH 4.5.

The stability of TdKT under oenological conditions was also examined by testing the residual activity after incubation (1:10 *v*/*v*) for 72 h at 20 °C in Sauvignon Blanc grape must (pH 3.7; initial sugar content, 14.6%; total SO_2, 150 ppm) and in Malbec red wine (pH 2.2; ethanol 12% *v*/*v*; total SO_2, 50 ppm). After toxin incubation, the aliquots were assayed for killer activity as previously described. All experiments were carried out in duplicate.

2.4.3. Identification of cell wall putative binding sites for the killer toxins

In order to determine the primary toxin binding sites in the yeasts cell wall, WTM and MWM assays were performed, as mentioned above. Sensitive yeast *C. glabrata* NCYC 388 growing exponentially at 20 °C for 24 h (10^6 cells/mL) was treated with the killer toxins in the absence or presence of the following polysaccharides: laminarin, pustulan, curdulan, mannan, chitin (Sigma, St. Louis, MO, USA) and pullulan (Elicityl, Crolles, France). Polysaccharides (1 mg/μL) were added to the killer toxin extract (15 AU) and incubated for 4 h at 20 °C. The residual killer activities were tested against the sensitive strains by WTM and MWM.

2.4.4. Determination of chitinase and β-glucanase activities

Chitinase activity was assayed by a DNS method (Miller, 1959) using 1% colloidal chitin (*w*/*v*) as substrate. One unit of chitinase activity (UCh) was defined as the amount of enzyme releasing 1 μmol of N-acetylglucosamine (GlcNAc) in the reaction mixture per hour. The standard curve was prepared using GlcNAc.

β-1,3-D-glucanase activity of the killer toxin was determined according to Wang et al. (2007) using 0.25% laminarin (*w*/*v*) as substrate. The

amount of reducing sugar in the reaction mixture was assayed by a DNS method (Miller, 1959). The standard curve was prepared using glucose. One unit of β-glucanase activity (UG) was defined as the quantity of enzyme releasing 1 μmol of glucose per hour.

All assays were performed in duplicate.

2.4.5. Effects on sensitive cells by fluorescence microscopy

2.4.5.1. Detection of cell-wall damage. Yeast cells growing exponentially at 20 °C for 24 h (1×10^6 cells/mL) on GPY were incubated with 6 AU of killer toxin crude extract for 24 h at 20 °C. After the treatment, cells were collected by centrifugation, washed twice with sterile water, resuspended in 30 μL of calcofluor white stain (2 μM, Sigma-Aldrich, Milan) and incubated at room temperature in the dark for 30 min. Cells were observed using an epi-fluorescence microscope (Nikon Eclipse 80i) with UV light excitation and emission at a wavelength range of 300 to 440 nm. Positive control treatment was performed by incubation for 3 h at 37 °C with 2 mg/mL of Zymolyase (Seikagaku Co) (Comitini et al., 2009).

2.4.5.2. Detection of apoptotic and necrotic phenotypes. The proportion of total dead cells was determined by propidium iodide (PI) staining. Yeast cells were incubated for 3 and 24 h with 6 AU of the toxin. After incubation, cells were washed, resuspended in a PBS buffer (g/L: KH_2PO_4 2, Na_2HPO_4 6.1, NaCl 80, KCl 2 in distilled H_2O, pH 7.6) and stained with 50 μg/mL of PI. Stained cells were detected and counted using an epi-fluorescence microscope at 530–610 nm emission wavelength range. A treatment with H_2O_2 was used as a positive control and a treatment with H_2O, as a negative control.

The effect of the toxin on nuclear morphology was assayed by the membrane-permeable DNA dye, Hoechst 33342 (Sigma-Aldrich, Milan). Yeast cells were collected, washed with a PBS buffer and fixed with 70% *v/v* ethanol. The nuclei were stained with Hoechst 33342 for 10 min at a final concentration of 3 μg/mL for 10 min at room temperature. Cells were examined with both bright field and fluorescence with excitation wavelength at 380 nm and emission at a wavelength range of 435–485 nm using an epi-fluorescence microscope. Apoptotic, necrotic and healthy cells phenotypes were identified.

2.5. Statistical analyses

The data were subjected to the analysis of variance (ANOVA) and means were separated according to the Tukey test ($P = 0.05$) using the STATISTICA data analysis software system, version 8 (Stat-Soft, 2007, France).

3. Results

3.1. Selection of indigenous killer yeasts

In this work, the killer activities of a panel of 18 indigenous wine strains of *T. delbrueckii* were evaluated by QTM against 12 wine spoilage yeast strains belonging to the species *B. bruxellensis*, *P. guilliermondii*, *P. manshurica* and *P. membranifaciens*.

Although the complete panel had demonstrated killer activity against *C. glabrata* reference sensitive strain, only six strains (37%) were able to kill at least one spoilage yeast. In particular, the strain *T. delbrueckii* NPCC 1033 showed the greatest spectrum of action, as it was able to kill 83.3% of the spoilage yeast strains evaluated. These killer strains were then tested by QTM against six commercial starter strains of *S. cerevisiae*; all starter strains were resistant to the killer toxins of *T. delbrueckii* NPCC 1033 (data not shown).

3.2. Time course and biochemical characterization of the killer toxins

In order to evaluate the potential biotechnological application of the killer toxins of the selected strain, their biochemical nature was studied. For that purpose *T. delbrueckii* NPCC 1033 was grown on a liquid medium, showing a maximum killer activity at the beginning of the stationary growth phase (Fig. 1). The maximum peak of killer activity showed a total protein concentration of 100 μg/mL and approximately 70% inhibitory activity by MWM against the four spoilage yeasts (data not shown). The killer toxin produced was named TdKT (*T. delbrueckii* killer toxin).

To elucidate the biochemical properties of TdKT, particularly those related to their potential use in winemaking, the crude extracts obtained from stationary phase in *T. delbrueckii* cultures were subjected to a set of assays. Firstly, the stability of the killer activity under different physicochemical stress conditions typically present in wine fermentations (pH, temperature, ethanol, free sulphur dioxide and sugar concentration) was tested by MWM. TdKT was not affected at the highest sugar concentrations evaluated (up 250 g/L) and it was also stable in the presence of SO_2, the most important antimicrobial compound used in winemaking, in concentrations ranging from 0 (control) to 150 mg/L. The toxin was not affected by the ethanol concentrations evaluated (0–12% *v/v*) (Table 1), although it considerably lost its activity at temperatures above 37 °C and pH conditions above pH 4.5. Moreover, the activity persisted in the must at least for three days and, in the wine, at least for two days (Table 1).

The approximate molecular mass of TdKT was determined by filtering the culture supernatant. Only the protein fraction that was larger than 30 kDa showed killer activity, suggesting that the molecular mass of the killer protein was above 30 kDa (data not shown).

3.3. Mode of action

3.3.1. Molecular targets of TdKT

Competitive inhibition of killer action by different cell wall polysaccharides was evaluated with the aim of identifying the first binding site for TdKT on the envelope of the sensitive yeast (Table 2). The residual killer activity tested by means of the well test method (WTM) demonstrated that pustulan (ß-1,6 glucan) completely inhibited TdKT activity, while chitin — as well as the mixed-linkage polysaccharides laminarin, mannan and pullulan — showed only partial competition with TdKT (Table 2). These results were partially confirmed in a liquid medium

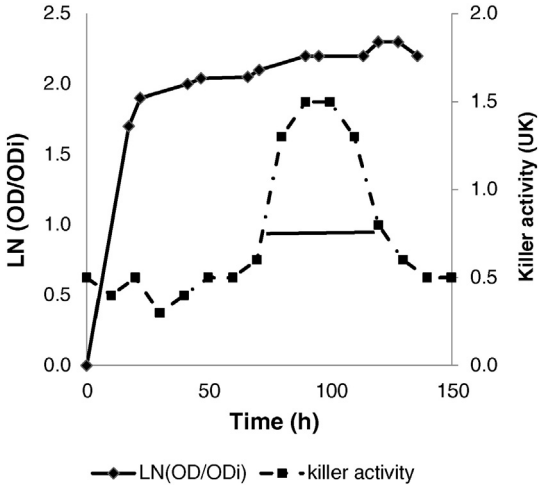

Fig. 1. Growth and killer toxin production kinetic during the batch cultivation of *T. delbrueckii* NPCC 1033. Growth was measured as OD_{600}; ln(OD/ODi) where OD is the optical density at time t, ODi is the initial optical density. The AU was determined against *C. glabrata* NCYC 388 by MWM. Line: pool fraction with killer activity.

Table 1
TdKT residual activity under physicochemical (sulphite, glucose, pH, ethanol and temperature) and environmental parameter (grape must and wine).

Enological stresses	Conditions	Relative killer activity (%)
Free SO_2	0–160 ppm	100%
Glucose	0–300 g/L	100%
pH	3–4.5	100%
	5–6	75%
Ethanol	0–16% *v/v*	100%
Temperature	0–35 °C	100%
	40–60 °C	25%
Grape must	24 h	100%
	72 h	90%
Wine	1 h	100%
	24 h	50%

Residual activity was regarded as the percentage of activity remaining after incubation of the toxin under a specific parameter, activity at time zero was used as reference (see Section 2).

using the multiwell method (MWM). Once more, pustulan binds to the toxin and chitin only showed low competition (80% residual activity).

3.3.2. β-glucanase and chitinase activities

Considering the previous result and to address the possibility that TdKT may exhibit some hydrolytic activity (β-glucanase and chitinase) as part of their mechanism of action, the toxin was incubated with the corresponding polymers (laminarin and chitin, respectively). As shown in Fig. 2, β-glucanase and chitinase activities were detected in the extracts and a direct relationship between β-glucanase, chitinase and killer activities was observed for TdKT.

3.3.3. Effects of TdKT on sensitive yeasts

In order to know whether the killer toxins treatment induces a cell-wall modification in the sensitive spoilage yeast, the cells were incubated with calcofluor white (CW) — a fluorescent dye that stains the polysaccharide chitin — and propidium iodide (PI) — that stains the nucleic acids of dead or damaged cells — providing an indirect measure of cell membrane integrity. Yeast exposed to enzymatic digestion with zymolyase (positive control) showed a reduction in the CW signal, and a discontinuous cell perimeter (Fig. 3C). The same appearance was observed on the surface of all spoilage yeasts treated with TdKT after 24 h of incubation, except for *B. Bruxellensis*, which needed 48 h to develop the same phenotype (data not shown). In all yeast treated with TdKT, the PI staining showed an increase in the dead cell number after treatment (Fig. 3F).

Finally, the effect of TdKT on yeast viability and the mechanisms mediating cell death was also evaluated with fluorescence microscopy (Fig. 4). Sensitive yeasts were exposed to the killer toxin and the arising apoptotic or necrotic phenotypes were examined using the PI-Hoescht double-staining assay. Fig. 4B presents the percentages of apoptosis and necrosis observed after toxin treatment on the sensitive reference yeast *C. glabrata* and the spoilage yeasts. TdKT generated a significant increase in the number of necrotic cells after 3 h of incubation, showing the highest percentages in *P. guilliermondii* and *P. membranifaciens*. Longer exposure to the same toxin (24 h) determined a significant increase in the amount of yeasts with apoptotic nuclei, with the highest percentages in *C. glabrata* and *B. bruxellensis*. These facts were associated with a decreased percentage of healthy yeasts at the same time point.

Table 2
Competition assays carried out with pure polysaccharides from yeast cell wall to detect putative binding sites of TdKT using two different methods (WTM and MWM).

Molecules evaluated		Residual killer activity (%)	
Polysaccharides	Main linkage type (monomer)	WTM[a]	MWM[b]
Pullulan	α 1-4,1-6 (glucose)	83	100
Mannan	α 1-2, 1-3, 1-6 (mannose)	83	100
Laminarin	β 1-3, 1-6 (glucose)	83	100
Curdlan	β 1-3 (glucose)	100	100
Chitin	β 1-4 (N-acetylglucosamine)	67	80
Pustulan	β 1-6 (glucose)	0	0
Control	–	100	100

[a] WTM: well test method.
[b] MWM: multiwell method.

4. Discussion

Different wine spoilage yeasts which were able to produce high levels of volatile phenols (including *P. guilliermondii*, *P. manshurica* and *P. membranifaciens*) have been detected and characterized over the last few years in the Argentinean North-Patagonia (Sáez et al., 2010, 2011). *P. manshurica* and *P. membranifaciens* can grow in wine due to their resistance to ethanol content, while *P. guilliermondii* tolerates the elevated sugar concentration typically present in the fresh must, spoiling early stages of winemaking (Lopes et al., 2009). In this context, a broad-spectrum control system effective against a wide set of the spoilage yeast species colonizing both musts and wine is of great importance in this region. For this purpose, a panel of different killer strains of the wine killer species *T. delbrueckii* was tested against regional spoilage yeasts, and against the most common wine spoilage yeast species worldwide *B. bruxellensis*.

Although a variability in killer capacity of tested strains was observed within the strains *T. delbrueckii* NPCC 1033 was selected on the basis of its broad killer spectrum. Previous works carried out in our laboratory have also demonstrated that this killer strain could be a good candidate for wine spoilage yeast biocontrol. *T. delbrueckii* NPCC 1033 — isolate 24 in Sangorrín et al. (2008) — also showed killer activity against *H. uvarum* and *B. bruxellensis* and some other strains of *P. guilliermondii* and *P. membranifaciens* (Lopes and Sangorrín, 2010). In order to evaluate the potential biotechnological application of TdKT, the tolerance to winemaking stress factors, biochemical nature and mode of action on spoilage sensitive yeasts were studied.

The effect of different physicochemical stress conditions governing wine fermentation has been proposed as an important feature to be taken into account for winemaking killer toxin selection. Most reported killer toxins produced by wine yeasts have been evaluated for their stability during a few hours at ethanol, temperature and pH values typical of wine (Comitini et al., 2004a; Izgü and Altinbay, 2004; Mehlomakulu et al., 2014; Santos et al., 2009). Only some studies have evaluated the stability of killer toxins for 1–2 h in the presence of SO_2 (Comitini and Ciani, 2011; Santos et al., 2011), and only one study has reported the stability of TdKT against osmotic stress (higher sugar concentrations) (Mehlomakulu et al., 2014). TdKT was stable in must and wine for at least one day, as well as pH, temperatures, ethanol, glucose and sulphur dioxide concentration simulating several winemaking conditions, evidencing their potential utility.

In a first stage, killer toxins generally act by binding to cell wall receptors of the sensitive cells (Schmitt and Breinig, 2002). Receptor molecules for different killer toxins have been identified as β-glucans, mannans, chitin, etc. (Liu et al., 2013; Meinhardt and Klassen, 2009). The TdKT killer activity was prevented in this work by the presence of β-1,6-glucans and chitin which indicates that these polymers provide TdKT with putative binding sites on the wall of the sensitive target cell. The binding to these polymers could also be related to a hydrolytic activity as part of the toxin mechanism of action. Colussi et al. (2005) found a *Kluyveromyces lactis* killer toxin that had both chitin binding and chitinase activity. Additionally, strong β-1,3-glucanase activity has been associated with the primary killing mechanism of other killer toxins such as those produced by *Williopsis saturnus* var. *mrakii* MUCL 41968 (Guyard et al., 2002), *K. phaffii* (Comitini et al., 2004b) and *W. anomalus* (Grevesse et al., 2003; Izgü et al., 2007; Walker, 2011). In this context, the β-1,3-glucanase and chitinase activities of TdKT were

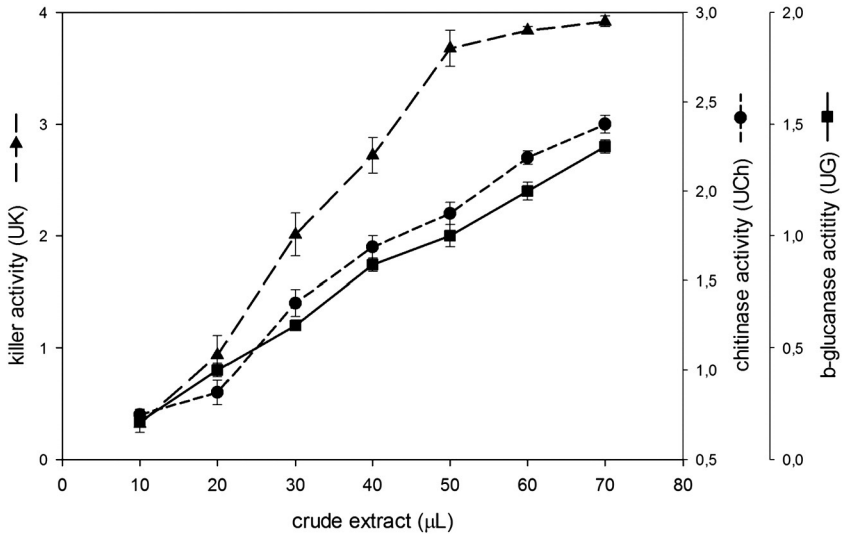

Fig. 2. Comparison of glucanase (—■—), chitinase (- - •- -), and killer toxin (– – ▲ – –) activities of TdKT. Vertical bars represent standard deviation of the mean.

investigated and confirmed in this work. TdKT killer activities tended to saturate when the amount of the toxin increased, which could be explained by the toxin association to the cell wall receptors. However, the two enzymatic activities showed a catalyst behaviour as they increased proportionally with the amount of TdKT. The decrease in fluorescence of the cell wall in the presence of TdKT evidenced after CW

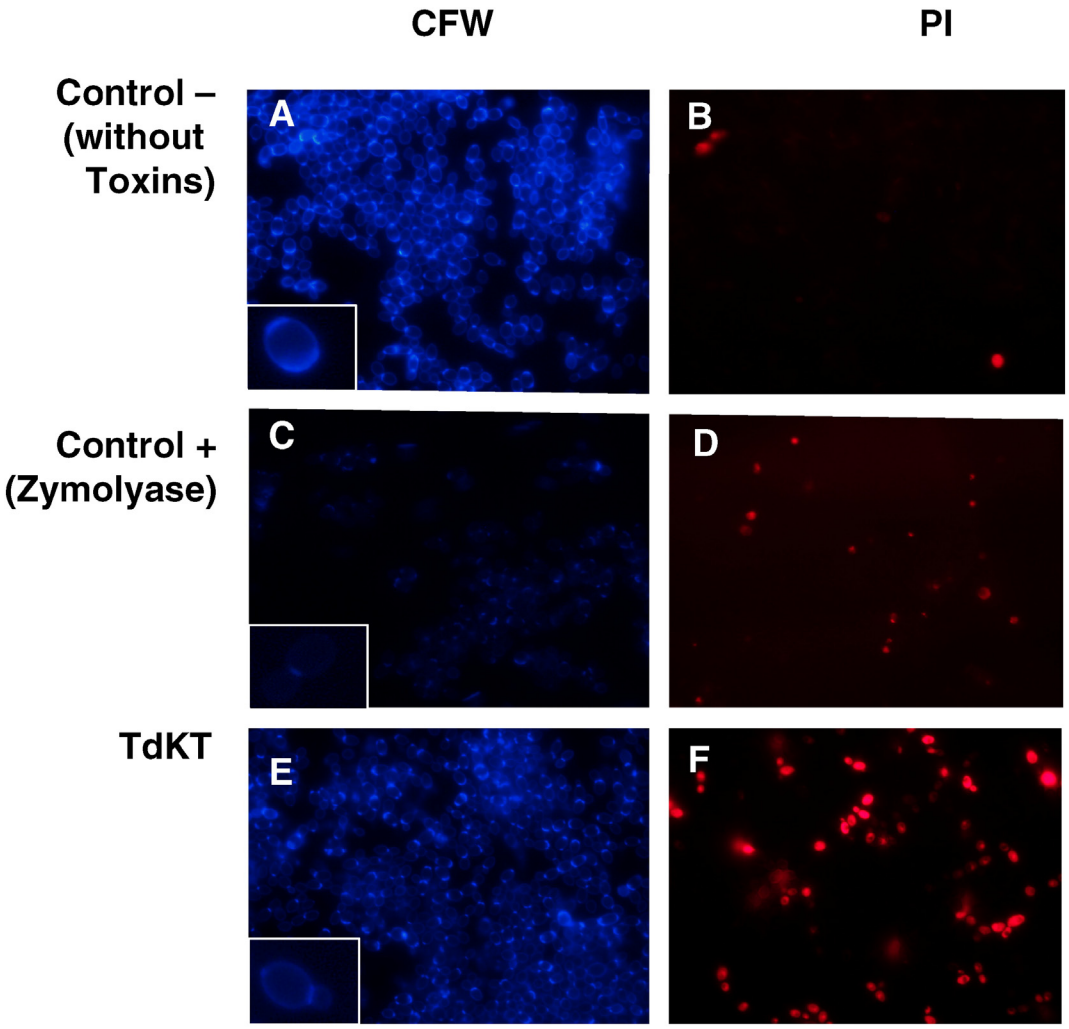

Fig. 3. Evaluation of damage of the yeast wall by double staining CFW-PI. A, B) Untreated yeast. C, D) Yeast after treatment for 2 h at 37 °C with zymolyase (1 mg/mL). E, F) Yeast treated for 2 h with 6 KU of partially purified TdKT. CPW = calcofluor white staining, PI = propidium iodine staining. Yeast strains: *C. glabrata*.

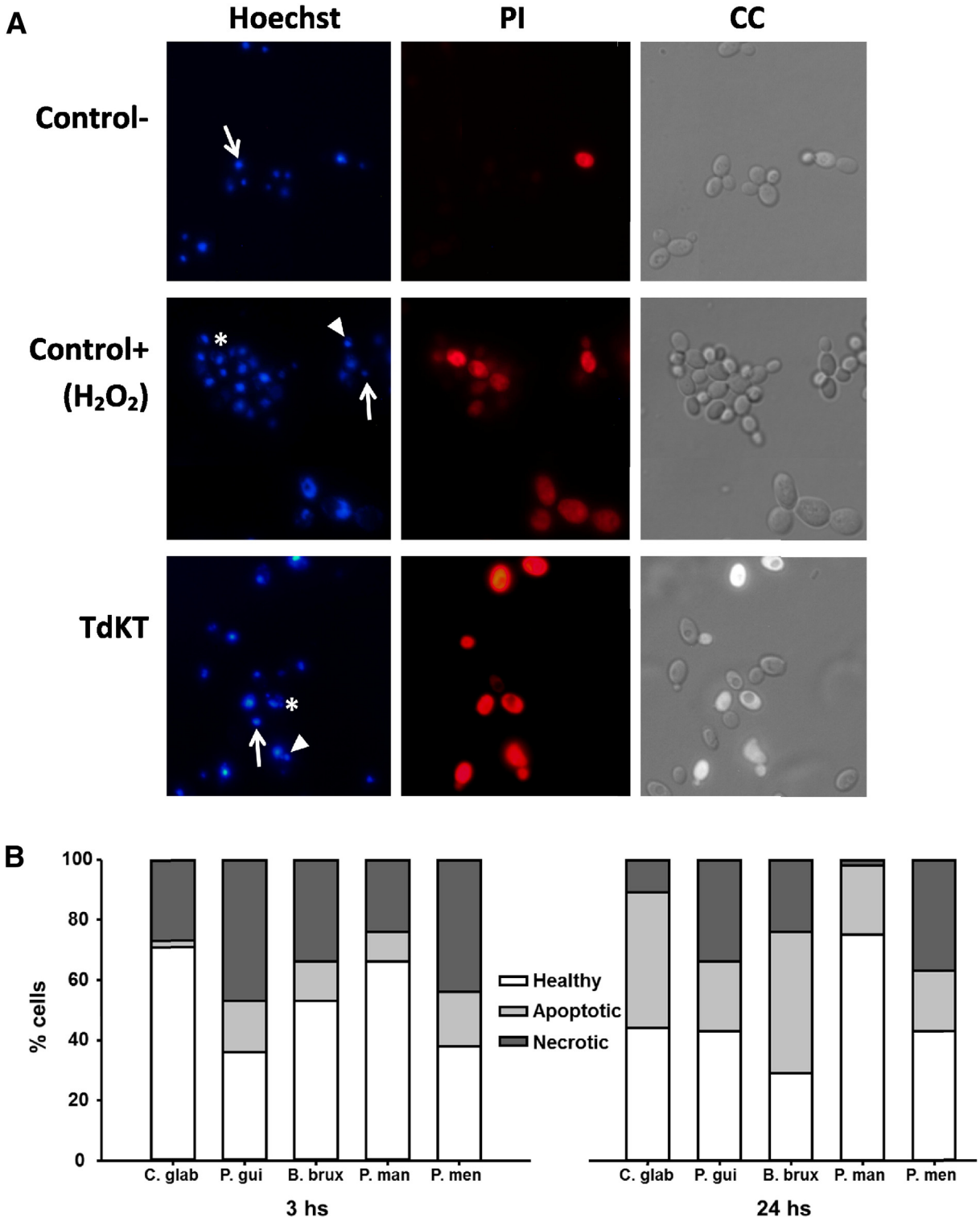

Fig. 4. Evaluation of yeast death pathways by fluorescence microscopy analysis. a) Propidium iodide-Hoechst 33258 double staining in yeast sensitive strains (*C. glabrata* NCYC 388) under TdKT treatment (6 UT, 24 h). Negative and positive controls were carried out with H_2O or 15 mM H_2O_2, respectively. Representative images of the same culture are pared in lines, for the columns Hoechst: Hoechst 33258 staining, PI: propidium iodide staining and CC: bright field image. In the first column, asterisks (*) indicate fragmented nuclei for apoptotic cells; arrow heads (▲) indicate necrotic cells and arrows (→) indicate healthy cells. b) Percentages of healthy, apoptotic or necrotic phenotypes after treatments with TdKT (6 UT) for 3 h and 24 h at 20 °C. B. brux = *B. bruxellensis* INTA VC20, C. glab = C. *glabrata* NCYC 388, P. gui = *P. guilliermondii* NPCC 1055, P. mem = *P. membranifaciens* NPCC 1099 and P. man = *P. manshurica* NPCC 1038.

staining could be due to the chitinase and glucanase activities detected for the toxin. This effect produces a disruption in the cell wall structure as well as the appearance of necrosis or early apoptotic death processes, as evidenced by input PI.

Apoptotic and necrotic cell death in yeasts can be triggered by various factors such as H_2O_2, cell ageing, acetic acid or toxins (Carmona-Gutierrez et al., 2010; Reiter et al., 2005). Both mechanisms of cell death were detected in the different spoilage yeasts after incubation with TdKT. An

increase in necrosis cell death after 3 h treatment and apoptotic cell death after 24 h were observed, showing time dependent mechanisms of action and generating different percentages of the three phenotypes in each studied species (Fig. 4). According to our results, the toxin effects may present different strategies to kill different spoilage yeasts. Schmitt and Reiter (2008) and Santos et al. (2013), have defined that the killer toxins produced by *S. cerevisiae* and *P. membranifaciens* respectively may present different strategies to kill sensitive cells, inducing apoptosis or necrosis in susceptible target cells. Further research is needed to elucidate the regulation on yeast apoptosis in response to TdKT crude extracts and purified toxins as well. Purification strategies are currently in progress in our laboratory in order to confirm the results obtained with crude extracts.

In brief, the new TdKT exhibits a broad spectrum against wine spoilage yeasts and its biochemical characterization evidenced a molecular mass higher than 30 kDa. The toxin possesses glucanase and chitinase activities and it is stable in winemaking conditions. The TdKT putative mode of action includes binding to β1-6 glucan and chitin in the initial interaction of the toxin with sensitive cells, with a potential degradation of these polysaccharides (due to their β-glucanase and chitinase activities), cell wall disruption (chitin decrease in microscopy evaluation) and finally, cell death by necrosis at initial time and by apoptosis at 24 h. The results presented in this study constitute the first step in the characterization and elucidation of the killing mechanism of a toxin produced by the *T. delbrueckii* yeast species.

TdKT has shown potential to control a broad spectrum of wine spoilage yeasts, without inhibiting the growth of the fermenting yeast *S. cerevisiae*. This killer toxin is resistant to stress conditions typically found at both initial and final stages of wine fermentation. The collective data presented in this study make TdKT interesting to control spoilage yeasts present during the whole winemaking process.

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