PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*

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Pichia membranifaciens CYC 1086 secretes a killer toxin (PMKT2) that is inhibitory to a variety of spoilage yeasts and fungi of agronomical interest. The killer toxin in the culture supernatant was concentrated by ultrafiltration and purified to homogeneity by two successive steps, including native electrophoresis and HPLC gel filtration. Biochemical characterization of the toxin showed it to be a protein with an apparent molecular mass of 30 kDa and an isoelectric point of 3.7. At pH 4.5, optimal killer activity was observed at temperatures up to 20 °C. Above approximately this pH, activity decreased sharply and was barely noticeable at pH 6. The toxin concentrations present in the supernatant during optimal production conditions exerted a fungicidal effect on a variety of fungal and yeast strains. The results obtained suggest that PMKT2 has different physico-chemical properties from PMKT as well as different potential uses in the biocontrol of spoilage yeasts. PMKT2 was able to inhibit *Brettanomyces bruxellensis* while *Saccharomyces cerevisiae* was fully resistant, indicating that PMKT2 could be used in wine fermentations to avoid the development of the spoilage yeast without deleterious effects on the fermentative strain. In small-scale fermentations, PMKT2, as well as *P. membranifaciens* CYC 1086, was able to inhibit *B. bruxellensis*, verifying the biocontrol activity of PMKT2 in simulated winemaking conditions.

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INTRODUCTION

Worldwide, microbial growth destroys large amounts of various products, causing yield losses in the agronomical and biotechnological industries. Traditionally, biocides have been used to deal with these problems but different disadvantages such as establishment of resistant strains and suppression of natural competitors have made alternatives such as biological control necessary (Beever *et al.*, 1989; Raposo *et al.*, 2000). Biological control strategies include natural plant- and animal-derived compounds, as well as antagonistic micro-organisms (Ciani & Fatichenti, 2001).

During recent decades, microbiological control of spoilage micro-organisms has evolved as a possibility. Many yeast strains and other micro-organisms inhibiting plant pathogens have been reported, especially within the fruit- and vegetable-producing sector, and several new products have reached the commercial market (Janisiewicz & Korsten, 2002). The suggested modes of action of biocontrol yeasts are not likely to constitute any hazard for the consumer (Janisiewicz *et al.*, 2001; Masih & Paul, 2002; Comitini *et al.*, 2004; Santos & Marquina, 2004a).

The food and beverage industries were among the first to explore the application of killer-toxin-producing yeasts to

kill spoilage micro-organisms (Lowes *et al.*, 2000). Yeast strains often achieve competitive advantage by producing killer toxins, which kill off competing sensitive cells belonging to either the same or a different species (Young, 1987; Ciani & Fatichenti, 2001). The most thoroughly studied examples are the *Saccharomyces cerevisiae* toxins K1, K2 and K28; producers of these toxins are able to kill each other, but are immune to killer toxins of their own class. The genetically very well-characterized killer toxin K1 is stable within a narrow range of acidic pH, is unstable at temperatures above 25 °C, and can be inactivated by agitation. It is a heterodimeric α/β protein encoded by an M1 virus with a double-stranded RNA genome (Wickner, 1986; Martinac *et al.*, 1990; Bostian *et al.*, 1984; Breinig *et al.*, 2002).

Killer toxins are not restricted to *Saccharomyces*; they can also be found in other yeast genera: *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Sporidiobolus*, *Tilletiopsis*, *Zygosaccharomyces*, etc. Studies of a variety of killer toxins have shown that the characteristics of the K1 killer toxin and other toxins tend to be similar (most of them are protease-sensitive heat-labile proteins that are stable and active only at acidic pH values), while the genetic bases of killer characters are quite

variable. There is also diversity in the mode of action of killer toxins. Several killer toxins (K1, PMKT) seem to be pore-forming-related toxins (Breinig *et al.*, 2002; Santos & Marquina, 2004b). K28, from *S. cerevisiae*, has been found to cause sensitive yeasts to arrest proliferation as unbudded cells, suggesting that it blocks completion of the cell cycle (Schmitt *et al.*, 1996), whereas zymocin, from *Kluyveromyces lactis*, exerts a tRNase activity (Butler *et al.*, 1991; Studte *et al.*, 2008). Finally, HM-1, from *Hansenula mrakii*, kills sensitive cells, presumably by interfering with β -(1 $\rightarrow$ 3)-glucan synthesis (Takasuka *et al.*, 1995).

The genus *Pichia*, which comprises 91 species, is currently one of the largest yeast genera. Yeasts of this genus are widely distributed and can be found in natural habitats such as soil, water, plant exudates, insects and fruits and also as contaminants in a variety of foods and beverages and high-sugar-containing products (Heard & Fleet, 1987; Noronha-da-Costa *et al.*, 1996). Some species of this genus have beneficial effects in food; for example, they contribute to the early stages of wine fermentation and in the processing of different types of cheeses (Lenoir, 1984; Heard & Fleet, 1988, 1990; Lambrechts & Pretorius, 2000; Fernandez *et al.*, 2000). Strains of *Pichia membranifaciens* are common contaminants in food-related environments and occur with high frequency in fermenting olive brines (Marquina *et al.*, 1992, 1997). Within the genus *Pichia*, which is heterogeneous from a taxonomic point of view (Kurtzman & Fell, 1998; Kurtzman & Robnett, 1998), *P. acaciae* (McCracken *et al.*, 1994; Klassen *et al.*, 2008), *P. anomala* (Comitini *et al.*, 2004; Wang, *et al.*, 2007; Izgü *et al.*, 2007), *P. farinosa* (Suzuki & Nikkuni, 1994), *P. inositovora* (Klassen & Meinhardt, 2003) and *P. kluyveri* (Middelbeek *et al.*, 1979) produce different killer toxins. Killer toxins from the genus *Pichia* have heterogeneous mechanisms of activity; some of them have similar characteristics to that determined for the *P. membranifaciens* PMKT (Santos & Marquina, 2004a; Pfeiffer & Radler, 1984; Middelbeek *et al.*, 1979), and others do not. PaT, the killer toxin produced by *P. acaciae*, has a tRNase activity (Klassen *et al.*, 2008) whereas *P. anomala* NCYC 434 has an exo- β -1,3-glucanase activity against sensitive yeast strains (Izgü *et al.*, 2007).

Recently, it has been shown that *P. membranifaciens* produces a killer toxin (PMKT) that is active on spoilage yeasts and fungi (Santos *et al.*, 2000, 2004). PMKT has extensive activity against different micro-organisms but only under restricted conditions, and therefore is of interest for its potential application as an antimicrobial agent, but only in permissive environments (Llorente *et al.*, 1997; Santos & Marquina, 2004a).

Previous biochemical studies indicated that PMKT is an 18 kDa protein that interacts with the cell wall, first with the (1 $\rightarrow$ 6)- β -D-glucans (Santos *et al.*, 2000) and then with CWP2p, a GPI-anchored protein (Santos *et al.*, 2007). Regardless of certain possible additional effects, PMKT acts by disrupting plasma membrane electrochemical gradients,

leading to the death of sensitive cells (Santos & Marquina, 2004b). Exploring the global gene expression responses of *S. cerevisiae* to PMKT, several core environmental stress-response genes were induced, corroborating the idea that the coordinated transcriptional response to PMKT is related to changes in ionic homeostasis. The HOG (high osmolarity glycerol) signalling pathway is activated in response to PMKT (Santos *et al.*, 2005).

The broad spectrum of killer activity of PMKT against yeasts and fungi is very promising, although it seems to be necessary to obtain new killer toxins with both increased killer activities against sensitive strains and higher stabilities against physico-chemical conditions of the environment for wider biotechnological applications. New killer toxins can be used to eliminate undesirable contaminating yeasts during fermentations by conferring the killer character on starter strains and can be considered as biocontrol agents in the preservation of foods (Petering *et al.*, 1991). The killer character can also be a useful means of biotyping pathogenic yeasts, and killer toxins are potential antimicrobial agents in the treatment of infections. In this study, our main purposes were to purify and characterize a new killer toxin from *P. membranifaciens* CYC 1086, to compare the purified killer toxin (named PMKT2) with the most studied killer toxin (PMKT) from *P. membranifaciens* CYC 1106 and to use the new promising biochemical characteristics of this new killer toxin for the biocontrol of yeasts and filamentous fungi of agronomical interest. PMKT2 exerted killer activity against *Brettanomyces bruxellensis*, a wine spoilage yeast. In small-scale fermentations using sterilized grape juice, PMKT2 was able to inhibit *B. bruxellensis*, indicating its potential applicability in a particular biotechnological field.

METHODS

Strains and general media. *Pichia membranifaciens* CYC 1086 was the killer toxin producer used in this study (Complutense Yeast Collection, Complutense University of Madrid, Spain), originally isolated from olive brines (Marquina *et al.*, 1992), identified according to conventional methods used in yeast taxonomy and deposited at the Portuguese Yeast Culture Collection (PYCC, Caparica, Portugal). The killer toxin from *P. membranifaciens* CYC 1086 (named PMKT2) was compared in the present study with PMKT obtained from *P. membranifaciens* CYC 1106 (Santos & Marquina, 2004a; Santos *et al.*, 2005, 2007). The sensitive strain used for routine killer assays was *Candida boidinii* IGC 3430 (PYCC), originally isolated from olive brines. *C. boidinii* has been linked to pernicious effects (lipolytic activity and lactic acid assimilation) on the fermentation of olive brines. The strains were maintained on agar slants containing 0.5% (w/v) yeast extract (Difco), 1% peptone (Difco), 2% glucose and 2% agar at 20 °C. Yeast isolates were obtained from Tempranillo grapes and grape skins from Spanish vineyards and wine fermentations of the Ribera del Duero region. These strains were gifts from Dr E. Navascues (Agrovin SA), isolated and identified according to conventional methods used in yeast taxonomy (Kurtzman & Fell, 1998).

For the purpose of killer toxin production and purification, *P. membranifaciens* CYC 1086 was cultured in buffered YMB medium

[1 % (w/v) glucose, 0.3 % (w/v) yeast extract (Difco), 0.3 % (w/v) malt extract (Difco) and 0.5 % (w/v) proteose peptone no. 3 (Difco)]. The medium was buffered to pH 4.5 with 0.2 M sodium citrate/phosphate. Killer activity was determined in YMAMB: YMA (YMB with agar) supplemented with 30 mg of methylene blue per litre, 6 % (w/v) NaCl and 2 % (w/v) agar (Llorente *et al.*, 1997).

DBDM (*Dekkera/Brettanomyces* differential medium) plates were used for the distinction and enumeration of *B. bruxellensis* strains during mixed cultures in must (Rodrigues *et al.*, 2001).

Grape juice was obtained from Tempranillo grapes from Spain (pH 3.5, sugar 220 g l⁻¹) and sterilized by using 0.22 µm Millipore filters. Additionally, grape must agar (GMA) was developed with sterile must and 2 % (w/v) agar for the determination of killer activity in conditions related to wine fermentation.

Killer toxin assay. We assayed killer toxin with a diffusion test (Woods & Bevan, 1968), using 6 mm diameter antibiotic assay AA Whatman paper discs on YMAMB seeded with the sensitive strain. The diameter of the inhibition zone was used as a measure of the yeast killer activity, and killer toxin activity was expressed in arbitrary units (AU) (Barandica *et al.*, 1999). Under the experimental conditions used, a linear relationship was observed between the logarithm of the protein concentration in the solution tested and the diameter of the inhibition zone. One AU is defined as the amount of toxin resulting in an inhibition zone with a 1 mm diameter. For calculations of the specific activity, protein contents were estimated using the Bradford method, with BSA as standard.

Screening for *P. membranifaciens* killer phenotypes. Several strains identified as *P. membranifaciens* were tested for killer activity against a panel of selected sensitive strains (Table 1). Strains to be tested for killer activity were inoculated in ~1 cm diameter concentrated zones onto YMAMB plates previously seeded with a lawn (5.0×10^5 cells ml⁻¹) of the yeast tested for sensitivity, which had been grown in YMA medium and suspended in sterile water just before inoculation. The plates were incubated for a week at 20 °C. Killer yeasts were identified by a clear zone of inhibition surrounding them.

Killer spectrum against fungi and yeast strains of agronomical significance. With the aim of determining the feasibility of the biocontrol of several micro-organisms linked with agronomical problems, we assayed the killer activity of *P. membranifaciens* CYC 1086 and CYC 1106 against a panel (Table 2) of yeasts and fungi. Killer activity was determined as above.

Production of killer toxin. Killer yeast was cultivated for 3 days at 20 °C, 150 r.p.m. in 2 l Erlenmeyer flasks with 1 l YMB. After centrifugation (5000 g, 10 min, 4 °C) the supernatant was adjusted to a final glycerol concentration of 15 % (v/v) and concentrated to a volume of 100 ml by tangential ultrafiltration with a 10 kDa cut-off membrane [Minisette membrane cassette, omega type (Filtron Technology)]. Ice-cold ethanol was added to a final concentration of 45 % (v/v) and, following 30 min incubation at 0–4 °C, the resulting precipitate was separated by centrifugation (8000 g, 10 min, 0 °C). The proteins in the supernatant were precipitated by further addition of ice-cold ethanol up to a final concentration of 75 % (v/v). The resulting pellet was dissolved in 5 ml 1 mM sodium citrate/phosphate buffer (pH 4.0) and the solution was used as killer toxin concentrate for subsequent characterization.

Effect of proteolytic enzymes. The effects of the proteolytic enzymes Pronase, pepsin and papain (Sigma) on the killer activity of *P. membranifaciens* CYC 1086 were examined as described by Santos & Marquina (2004a).

Temperature and pH stability of PMKT2. For stability to pH, concentrated toxin samples were adjusted with 0.1 M sodium citrate/phosphate buffer at a range of pH values between 3 and 10. The solutions were incubated at 20 °C for 1 h and killer activity was determined with *C. boidinii* as sensitive yeast. With the aim of determining the stability in grape must against *B. bruxellensis* 1D007, lyophilized killer extracts were resuspended in grape must previously adjusted to pH values between 3 and 10.

Samples of killer toxin were incubated at a range of different temperatures: 5, 10, 15, 20, 25, 30 and 32 °C. Aliquots (30 µl) were removed at specific intervals and killer activity was assayed. The same was done to determine the temperature stability in grape must against *B. bruxellensis* 1D007. Lyophilized killer extracts were resuspended in grape must and incubated at temperatures between 5 and 32 °C, as described above.

Influence of temperature and pH on PMKT2 activity. To determine activity at different pH values, activity plates or GMA plates adjusted to pH values between 2.5 and 10 were seeded with the sensitive strains (*C. boidinii* and *B. bruxellensis* 1D007, respectively) and incubated at 20 °C in the presence of aliquots of the killer toxin (30 µl). The inhibition zone was determined after 3 days incubation.

To determine the activity at different temperatures, YMAMB and GMA plates were incubated at 4 °C for 24 h with the killer toxin (30 µl) from concentrated culture supernatants to ensure the

Table 1. Screening for new *P. membranifaciens* killer phenotypes

Sensitive strain	<i>P. membranifaciens</i> killer strains* (relative activity)†									
	1	2	3	4	5	6	7	8	9	10
<i>C. boidinii</i> IGC 3430	3+	2+	4+	3+	3+	3+	3+	3+	3+	4+
<i>K. lactis</i> IGC 4358	2+	2+	2+	2+	2+	2+	2+	–	–	–
<i>P. membranifaciens</i> IGC 4619	2+	2+	3+	2+	2+	2+	2+	1+	3+	4+
<i>Saccharomyces bayanus</i> IGC 4465	–	–	–	–	3+	3+	–	–	–	–
<i>S. cerevisiae</i> IGC 4456	2+	2+	2+	2+	–	2+	–	–	–	–
<i>S. cerevisiae</i> IGC 4620	–	2+	2+	2+	2+	–	–	–	–	–
<i>S. exiguus</i> IGC 4612	2+	2+	2+	2+	2+	–	–	–	–	–

*Killer strains of *P. membranifaciens*: 1, CYC 1048; 2, CYC 1095; 3, CYC 1106; 4, CYC 1111; 5, CYC 1112; 6, CYC 1108; 7, CYC 1090; 8, CYC 1084; 9, CYC 1085; 10, CYC 1086.

†+, Killing activity; –, no killing activity.

Table 2. Killer activity of *P. membranifaciens* CYC 1086 and CYC 1106 against yeasts and fungi of biotechnological interest

Sensitive strain	Killer activity		Sensitive strain	Killer activity		Sensitive strain	Killer activity	
	1086	1106		1086	1106		1086	1106
<i>S. cerevisiae</i> SGV	–	–	<i>B. bruxellensis</i> 1D007	3+	–	<i>Pichia anomala</i> 1114 ^T	–	–
<i>S. cerevisiae</i> CEG	–	–	<i>B. bruxellensis</i> D013	1+	–	<i>Pichia membranifaciens</i> CYC 1070	2+	–
<i>S. cerevisiae</i> VRB	–	3+	<i>B. bruxellensis</i> D014	1+	–	<i>Aspergillus</i> spp. 27	–	–
<i>S. cerevisiae</i> NEM	–	–	<i>B. bruxellensis</i> D015	1+	–	<i>A. carbonarius</i> B MUM	–	–
<i>S. cerevisiae</i> 2056	–	2+	<i>B. bruxellensis</i> D017	2+	–	<i>A. ochraceus</i>	–	–
<i>S. cerevisiae</i> BM45	–	3+	<i>B. bruxellensis</i> D018	1+	–	<i>A. oryzae</i>	–	–
<i>S. cerevisiae</i> 2323	–	–	<i>B. bruxellensis</i> D019	1+	–	<i>A. tubingensis</i>	–	–
<i>S. cerevisiae</i> ALB	–	3+	<i>B. bruxellensis</i> D027	1+	–	<i>Fusarium culmorum</i>	–	–
<i>S. cerevisiae</i> SLO	–	4+	<i>B. bruxellensis</i> D028	1+	–	<i>F. graminearum</i> NRRL 28525	–	–
<i>S. cerevisiae</i> VN	–	–	<i>B. bruxellensis</i> D029	2+	–	<i>F. graminearum</i> NRRL 29020	–	–
<i>S. cerevisiae</i> 71B	–	4+	<i>B. bruxellensis</i> D031	1+	–	<i>F. pone</i>	–	–
<i>S. cerevisiae</i> CS2	–	1+	<i>B. bruxellensis</i> D032	1+	–	<i>F. proliferatum</i> MM 1-2	2+	–
<i>S. cerevisiae</i> CM	–	–	<i>B. bruxellensis</i> D033	1+	–	<i>F. proliferatum</i> MM 3-1	2+	–
<i>S. cerevisiae</i> HAY	–	–	<i>B. bruxellensis</i> D035	2+	–	<i>F. proliferatum</i> MM 6-2	–	–
<i>S. cerevisiae</i> FS	–	3+	<i>B. bruxellensis</i> D036	1+	–	<i>F. reticuloides</i> MM 6-3	–	–
<i>S. cerevisiae</i> 16	–	–	<i>B. bruxellensis</i> D038	1+	–	<i>F. reticuloides</i> MM 7-3	–	–
<i>S. cerevisiae</i> 17	–	3+	<i>Debaryomyces hansenii</i> 1021	–	–	<i>F. sporotrichoides</i> ITEM 550	–	–
<i>S. cerevisiae</i> 18	–	3+	<i>D. hansenii</i> 1244	–	–	<i>Botrytis cinerea</i> 20003	–	3+
<i>S. cerevisiae</i> 19	–	–	<i>D. hansenii</i> 10388	–	–	<i>B. cinerea</i> 20004	–	1+
<i>S. cerevisiae</i> SC1	–	4+	<i>D. hansenii</i> 10386	–	–	<i>B. cinerea</i> 20005	–	2+

complete diffusion. The plates were then seeded with the sensitive strains (*C. boidinii* and *B. bruxellensis* 1D007, respectively) and incubated at different temperatures up to 37 °C (Fig. 2b).

Binding of PMKT2 to cell-wall fractions. The sensitive strain was grown on YMB (without buffer) and cultured 48 h at 30 °C. Cell walls from *C. boidinii* IGC 3430 were prepared by mechanical disruption (Fleet, 1991). After being washed, cell walls were lyophilized and stored in a desiccator. Glucans (fractions S-1, S-2, P-1 and P-2) were extracted as described by Manners *et al.* (1973). Mannoproteins were extracted and partially purified from cell walls by Cetavlon (cetyltrimethylammonium bromide) fractionation (Schmitt & Radler, 1987; Santos *et al.*, 2000) and chitin was obtained as described by Fleet (Fleet & Phaff, 1973; Fleet, 1991).

Binding of killer toxin was estimated from the amount of killer toxin remaining in solution after incubation (Santos *et al.*, 2000). Approximately 2000 AU killer factor per ml was added to a suspension of 20 mg of the cell-wall fractions per ml. Samples were stirred gently and then centrifuged (10 000 g, 30 s). The amount of killer activity remaining in solution after incubation was measured by the diffusion test method.

Binding of killer toxin to different polysaccharides. The following polysaccharides were used for toxin binding studies: polysaccharides with (1→4)- α as the main glucosidic linkage (amylose, amylopectin and polygalacturonic acid); pullulan, a (1→4)- α - and (1→6)- α -polysaccharide; xylan and chitin, (1→4)- β -polysaccharides; laminarin, a (1→3)- β -polysaccharide; liquenan, a (1→3)- β - and (1→4)- β -polysaccharide; and (1→6)- β -polysaccharides (pustulan and a glucan obtained from *Penicillium allahabadense*). These polysaccharides (15 mg each) were added to 1 ml killer toxin (150 AU ml⁻¹). Mixtures were shaken gently and then centrifuged (10 000 g, 30 s). The amount of killer activity remaining in solution was assayed. Amylose and amylopectin were obtained from Sigma-Aldrich.

Killer toxin purification. The killer toxin was purified from native PAGE by directly using the killer toxin concentrate obtained as stated above. The toxin concentrate was dissolved in a 0.1 M glycine/HCl sample buffer (pH 2.5) with methyl green as tracking dye. Polyacrylamide gels (8%) were prepared in a 0.1 M glycine/HCl buffer (pH 2.5). Samples were electrophoresed for 5 h, 200 V, at 4 °C. To determine the activity of the killer toxin, one-third of the gel was cut into slices (2 mm) and these were transferred to YMAMB plates seeded with the sensitive strain. For the visualization of protein bands, the other two-thirds of the gel were stained with Coomassie brilliant blue R-250 or extracted with distilled water for HPLC purification.

Toxin purification was done on a Varian 920-LC Analytical HPLC system, which included a fraction collector and a UV detector working at 280 nm at an ambient temperature of 20 °C. The crude protein obtained from the previous native PAGE was dialysed against 10 mM Na₂HPO₄/citric acid buffer, pH 4.5. The protein samples were loaded onto a Hydrocell GPC 1500 column, 7.8 × 300 mm (Biochrom Laboratories), which was pre-equilibrated with the sample buffer. Elution was done with the same buffer at a flow rate of 1 ml min⁻¹. Fractions of 200 μ l volume were collected and assayed for killer activity. The active fractions from different runs were pooled, concentrated and then subjected to SDS-PAGE. All the chemicals were HPLC grade and obtained from Merck.

Following native PAGE and HPLC, dialysed fractions containing killer toxin activity were subjected to electrophoresis for a total of 1.5 h at 120 V, according to the method described by Laemmli (1970), under denaturing conditions, using 8% acrylamide gels in reducing (β -mercaptoethanol) and non-reducing conditions.

Determination of the isoelectric point. Isoelectric focusing was performed at 4 °C in polyacrylamide gels (12 cm × 6.5 cm × 0.4 mm) containing 0.5 ml ampholytes, pH range 2.5–5.0 (Pharmalyte, Pharmacia), 2 ml acrylamide/bisacrylamide solution (24.25%/

0.75 %), 2 ml glycerol (20 %, v/v), 150 ml ammonium persulphate (10 %, w/v), 35 ml TEMED and distilled water (5.5 ml). Pre-focusing, sample application and focusing were carried out as published previously (Santos & Marquina, 2004a).

Activity of PMKT2 in grape juice. In order to verify the potential of PMKT2 as a biological anti-*B. bruxellensis* agent in winemaking, cells of *B. bruxellensis* strains 1D007, D013 and D017 (with different sensitivity to *P. membranifaciens* CYC 1086; Table 2) were grown to exponential phase in sterilized must (0.22 µm Millipore filters) for 16 h at 20 °C, collected and subsequently resuspended in must containing PMKT2 (2000 AU ml⁻¹). The final cell concentration was 2×10^6 cells ml⁻¹. A control with heat-inactivated killer toxin was run in parallel (not shown). Incubation was done at 20 °C with shaking (125 r.p.m.). Aliquots were taken periodically and further 10-fold serial dilutions were made. Volumes of 50 µl were used for plating on YMA medium. Colonies were counted after growth at 30 °C. The killer activity remaining in solution during the experiment was determined by taking 1 ml samples. Samples were centrifuged and the supernatant was lyophilized and resuspended in 0.1 ml 0.1 M citrate/phosphate buffer pH 4.5. Finally, the activity was determined as described above.

Mixed cultures. Mixed cultures of *S. cerevisiae* SC1, *B. bruxellensis* 1D007 and *P. membranifaciens* CYC 1086 were done in pairs with inocula ratio of 1:1. Cellular concentrations were adjusted to 10^5 cells ml⁻¹ and growth developed in grape juice by incubation at 20 °C in 125 ml Erlenmeyer flasks with agitation (125 r.p.m.). During the co-fermentation, growth rates were determined and compared with those obtained during the fermentation in axenic cultures. Aliquots were taken periodically and further serial 10-fold dilutions were made. Volumes of 50 µl were used for plating on YMA and DBDM media. Colonies were counted after growth at 30 °C. The killer activity remaining in solution during the experiment was determined as above.

RESULTS

Screening for *P. membranifaciens* killer phenotypes

In order to determine if the available killer-toxin-producing yeast strains of *P. membranifaciens* belong to different toxin types, several strains were tested against a panel of sensitive yeast strains employing the usual technique with methylene blue agar plates (Woods & Bevan, 1968). Strains producing the same toxin may have a similar spectrum of killer activity whereas strains producing different toxins may inhibit different strains. Results are presented in Table 1. Different profiles of killer activity were observed. The first profile was represented by strains CYC 1095, 1106 and 1111, which have the same activity against sensitive yeasts, and also strains CYC 1048 and 1112, which were very similar to this profile, although slightly different. The second profile was represented by strains CYC 1084, 1085, 1086 and also probably strain CYC 1090. On the basis of these results, we selected *P. membranifaciens* CYC 1086 because of its different killer spectrum activity in comparison to *P. membranifaciens* CYC 1106, the best-studied killer strain of *P. membranifaciens* (Santos *et al.*, 2000, 2005, 2007; Santos & Marquina, 2004a). We used this yeast to investigate the potential biotechnological application of this killer activity and its biochemical nature.

Killer activity against fungal and yeast strains of agronomical significance

Killer activity against a range of yeasts and filamentous fungi was tested with the aim of determining whether the killer toxin of *P. membranifaciens* CYC 1086 has potential for biotechnological application (Table 2). Activity against several yeasts was observed. All the strains of *B. bruxellensis* were sensitive to *P. membranifaciens* CYC 1086 whereas none of the *S. cerevisiae* strains, isolated from oenological fermentations, showed sensitivity. This was an important result because *B. bruxellensis* is a major problem in the wine industry, where it produces unpleasant odours and tastes during wine ageing, due to the production of 4-ethylphenol. Strain CYC 1086 was also effective against *P. membranifaciens* CYC 1070; it is known that *P. membranifaciens* is a normal inhabitant of the grape berry surface and fermenting must, and it can develop during fermentation excreting undesirable metabolites into the wine.

Filamentous fungi were more resistant to *P. membranifaciens* CYC 1086: only two strains of *Fusarium proliferatum* (strains MM 1-2 and 3-1) were sensitive. No activity was found against *Botrytis cinerea* strains whereas *P. membranifaciens* CYC 1106 was very active, as reported by Santos *et al.* (2004).

Proteinaceous nature of the killer toxin

P. membranifaciens CYC 1086 exhibits a pronounced killer activity against a variety of yeast species and fungi (Tables 1 and 2). To determine whether PMKT2 activity on sensitive strains was due to the production of a protein or glycoprotein, similar to all other yeast killer toxins so far characterized, including PMKT, we examined the effects of different proteases on killing ability. Treatment of the concentrated culture supernatant with Pronase or papain led to the loss of 100 % of the initial killer activity. In contrast, pepsin was less effective against PMKT2: a residual activity of about 40 % was found after pepsin treatment.

Temperature and pH stability of the killer toxin

The killer toxin produced by *P. membranifaciens* CYC 1086 was stable only within a narrow pH range (2.5–4.8). No significant differences were observed between the stability at different pH values in must or standard conditions (Fig. 1). Stability was lost at temperatures above 20 °C in aqueous buffers and must at the appropriate pH value (Fig. 2c, d). Stability was quickly lost at 45 °C and 65 °C (30 min and 5 min, respectively) (not shown).

Effect of temperature and pH on killer activity

The killer protein was active only at acidic pH values (Fig. 1), with the optimal pH between 3.5 and 4.5. Optimal temperature was 20 °C (Fig. 2a, b). The results were very similar when standard conditions or must were used.

Fig. 1. Activity (●) and stability (■) of the killer toxin from *P. membranifaciens* CYC 1086 at different pH values in standard conditions (a) and grape must (b). Means $\pm$ SD ($n=3$).

Binding of PMKT2 to cell-wall fractions and polysaccharides

None of the polysaccharides tested, composed of (1 $\rightarrow$ 4)- β -, (1 $\rightarrow$ 4)- α -, (1 $\rightarrow$ 3)- β -, (1 $\rightarrow$ 6)- β -, (1 $\rightarrow$ 6)- α - or some mixture of these linkages, were able to bind PMKT2, indicating that the binding capacity of the toxin is not related to the polysaccharides tested. A complementary study was done by determining the binding capacity of different cell-wall fractions of the sensitive strain. Glucan fractions of the cell wall were unable to bind PMKT2, whereas mannoproteins and mannan were able to, indicating that PMKT2 has a different receptor in the cell wall of the sensitive strain than PMKT (Table 3).

Killer toxin purification and determination of isoelectric point

Once the protein nature and characteristics of the toxin produced had been established, the secreted protein was purified from the supernatant of a growing culture of *P.*

membranifaciens CYC 1086 at the early stationary phase. The killer toxin that accumulated in the culture fluid increased as growth progressed and then levelled off as the culture reached the stationary phase (not shown). The ultraconcentrated killer toxin was subjected to acidic-native polyacrylamide electrophoresis. Taking into account that the isoelectric point of the toxin, determined in polyacrylamide gels, was estimated as about 3.7, the migration of the toxin in acidic PAGE (pH 2.5) was low, although the toxin was electrophoresed for 5 h (Fig. 3b). The HPLC-purified killer toxin was observed to have a molecular mass of about 30 kDa. A low recovery (16 %) of killer activity was obtained after a 2636-fold purification (Table 4).

Killer activity in winemaking conditions

Small-scale trials conducted in winemaking conditions determined that PMKT2 is suitable for potential biotechnological applications. PMKT2 inhibited *B. bruxellensis* strains 1D007, D013 and D017. Death rates were 0.13 h⁻¹, 0.09 h⁻¹ and 0.11 h⁻¹, respectively (Fig. 4). Furthermore, in mixed cultures on grape must, *P. membranifaciens* CYC 1086 was able to compete with strain 1D007, showing that this killer yeast exerts an effective competition against *B. bruxellensis*. The killer activity remaining in solution during cell death was reduced significantly in the first hour. Then killer activity was constant, indicating that there is an excess of toxin remaining in solution and the toxin is able to bind to the sensitive cells during the first moments of the killing action.

In axenic cultures on grape juice, without toxin addition, specific growth rates of *P. membranifaciens* CYC 1086, *S. cerevisiae* SC1 and *B. bruxellensis* 1D007 were 0.11, 0.13 and 0.07 h⁻¹, respectively (Fig. 5a). The presence of the killer strain or its purified killer toxin did not change the growth parameters of the fermentative *S. cerevisiae* strain (Fig. 5c), indicating that these two yeasts are compatible in winemaking conditions, whereas *B. bruxellensis* 1D007 showed significant inhibition (death rate, 0.12 h⁻¹) in mixed cultures (Fig. 5b). Furthermore, no killer activity remaining in solution was detected in mixed cultures during the first 3 h, indicating that the produced toxin was attached to the sensitive cells. Probably, when all the binding sites of the toxin in the sensitive cells were saturated the presence of the toxin would be detected in the medium.

DISCUSSION

Yeasts and moulds can have both positive and negative effects on products consumed by humans and animals. Yeasts are used as starter cultures in cheeses and bread, as well as wine, beer and other alcoholic fermentation products, but they can also initiate spoilage in foods, such as fruit juice and salads. Indeed, spoilage yeasts have been isolated from many foods and beverages (Suriyarachchi & Fleet, 1981; Izgu *et al.*, 1997; Jakobsen & Narvhus, 1995).

Fig. 2. (a, b) Activity of the killer toxin from *P. membranifaciens* CYC 1086 at different temperatures in standard conditions (a) and grape must (b). (c, d) Stability of killer toxin at different temperatures over a period of 7 days: 20 °C (●); 25 °C (■); 28 °C (◆) 32 °C (▲) and 37 °C (▼), in standard conditions (c) and grape must (d). Means $\pm$ SD ($n=3$).

Mould spoilage destroys large amounts of pre-harvested and harvested fruit, vegetables and cereal grains, and is often associated with mycotoxin formation (Marín *et al.*, 2008).

In recent years, biological control strategies have been considered as a desirable alternative to chemicals. Biological control with yeasts and bacteria has been suggested as one such alternative to the application of fungicides (Petersson & Schnürer, 1995; Druvefors, *et al.*, 2005; Druvefors & Schnürer, 2005).

In studies of the potential of mycogenic yeasts as biocontrol agents, workers have focused on the production

of mycocins *in situ* by natural toxin producers or transformed yeasts (Kimura *et al.*, 1995). *P. membranifaciens* produces a toxin, PMKT, which is very stable at low pH and temperature values and has a broad spectrum of activity (Llorente *et al.*, 1997). We found that this toxin could be a versatile antispoilage agent in foods with the limitations imposed by its stability. Different approaches are possible when the use of a killer toxin is necessary; inoculation with killer yeast, crude extracts of killer toxins or purified toxins, such as PMKT, could be used as an additive in acidic buffers (Santos & Marquina, 2004a; Santos *et al.*, 2004).

Table 3. Adsorption of PMKT2 to different polysaccharides and cell-wall fractions

Polysaccharide	Main linkages	Residual killer activity (%)	Adsorption
Mannan	$\alpha(1\rightarrow6)$, $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$	10	+
Mannoproteins	$\alpha(1\rightarrow6)$, $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$	10	+
P-1 fraction	$\beta(1\rightarrow6)$	95	–
P-2 fraction	$\beta(1\rightarrow3)$	95	–
Amylose	$\alpha(1\rightarrow4)$	95	–
Amylopectin	$\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$	90	–
Pullulan	$\alpha(1\rightarrow6)$	95	–
Laminarin	$\beta(1\rightarrow3)$	90	–
Xylan	$\beta(1\rightarrow4)$	100	–
Chitin	$\beta(1\rightarrow4)$	100	–
Pustulan	$\beta(1\rightarrow6)$	90	–
Glucan from <i>Penicillium allahabadense</i>	$\beta(1\rightarrow6)$	90	–
Glucan from <i>Penicillium erythromellis</i>	$\beta(1\rightarrow6)$	95	–

Fig. 3. (a) Elution pattern from a Hydrocell GPC 1500 HPLC column of proteins from active sections of native PAGE. (b) Aliquots from HPLC were recovered and tested for activity and homogeneity in SDS-PAGE. (c1) Coomassie-blue-stained SDS-PAGE gel of a *P. membranifaciens* CYC 1086 crude extract. (c2) Protein extract of an active section from native PAGE. (c3) HPLC-purified killer toxin. (d) Molecular mass standards.

This paper confirms the observation that different killer toxins are produced by different isolates of the same species. The fact that multiple toxins are produced by *P. membranifaciens* must be noted, although it is similar to the reported killer system of *S. cerevisiae*, which is composed of three different toxins (Schmitt & Breinig, 2006). The strains of *P. membranifaciens* available in our laboratory produced at least two different types of toxins, named PMKT and PMKT2. PMKT2 had physico-chemical properties and molecular mass similar to PMKT, but their spectra of biological activity against a variety of fungal and yeast strains were different, indicating that they were different toxins (Tables 1 and 2). PMKT2 was active at acidic pH values and temperatures below 20 °C, although in comparison to PMKT, it was more stable for short periods at higher temperatures and neutral pH values (not shown), indicating that this new toxin is more suitable than PMKT for biotechnological applications.

Furthermore, PMKT inhibits strains of *Saccharomyces* but PMKT2 does not, and PMKT was not able to inhibit several *B. bruxellensis* isolates, whereas PMKT2 did so (Table 2). These differences in killer spectra could be accounted for by the preliminary differences observed between the two toxins in the nature of the primary receptors present in the sensitive strains (Table 3). PMKT was found to have affinity for (1→6)- β -D-glucans whereas PMKT2 was found to be adsorbed by cell-wall mannoproteins (Table 3), suggesting the presence of two different primary receptors for these toxins (Breinig *et al.*, 2002; Hutchins & Bussey, 1983; Takasuka *et al.*, 1995; Takita & Castilho-Valavicius, 1993).

In the wine industry, *B. bruxellensis* is considered a spoilage yeast due to its ability to produce volatile phenols conferring off-odours and losses of fruity sensorial qualities in wines (Suárez *et al.*, 2007). *B. bruxellensis* is a constant

Table 4. Purification of PMKT2 from the supernatant of a buffered-YMB-grown culture

Fraction	Volume (ml)	Protein		Activity (AU)	Sp. act. (AU mg ⁻¹)	Factor (-fold)	Yield (%)
		(mg ml ⁻¹)	(mg)				
Culture supernatant	3000	0.98	2940	48 000	16	1	100
Tangential ultrafiltration	75	11.35	851	31 000	36	2.2	64.6
75 % ethanol precipitate	5	130.45	652	29 500	45	2.7	61.5
Native PAGE*	1	2.10	2.1	18 350	8738	535.1	38.2
Hydrocell GPC 1500	1	0.18	0.18	7750	43055	2636.6	16.2

*Native PAGE was done by applying 20 μ l aliquots per well of the 75 % ethanol precipitate. Calculations were done with the assumption that the complete amount (5 ml) from the ethanol precipitate was applied to the native PAGE.

Fig. 4. Time-course of PMKT2 activity in grape must against sensitive *B. bruxellensis* strains. Microbial counts of *B. bruxellensis* 1D007 (■), D013 (●) and D017 (▲) were obtained by plating on YMA after incubation with PMKT2. The killer toxin remaining unbound (bars) was determined and expressed as relative killer activity. Means $\pm$ SD ($n=3$).

wine resident but it develops mainly at the end of fermentation, when other microbial species decline. It is a strong resistant species, withstanding nutrient deprivation (Aguilar Uscanga *et al.*, 2000), high ethanol concentrations (Medawar *et al.*, 2003), initial cold maceration, thermal treatment (Couto *et al.*, 2005) and winery disinfection (Renouf & Lonvaud-Funel, 2004; Renouf *et al.*, 2005, 2006, 2008). Therefore, *B. bruxellensis* can be considered as one of the best-adapted species to the cellar and wine environment. Tools for limiting its growth are restricted. Thus, the investigation of promising new additives, such as PMKT2, as new treatments to prevent *B. bruxellensis* growth during and after fermentation is very promising. In the present work, small-scale fermentations in winemaking conditions determined that *P. membranifaciens* CYC 1086 and its killer toxin (PMKT2) are suitable for winemaking applications. PMKT2 showed killer activity against *B. bruxellensis* in similar conditions as those prevailing in wine fermentation, and in addition, *S. cerevisiae* was unaffected, indicating that the killer yeast or PMKT2 are compatible with the fermentative process of winemaking.

In conclusion, we have here described a new killer toxin of *P. membranifaciens* that is active against *Brettanomyces* spoilage yeasts. The activity and stability of the toxin are compatible with the pH and temperature values of wine production, showing that PMKT2 may have potential as a biocontrol agent of undesirable wine spoilage yeasts. The specificity of this toxin is also very compatible with wine-producing strains because no PMKT2-sensitive strains of *S. cerevisiae* were found.

Fig. 5. Determination of the killer effect of *P. membranifaciens* CYC 1086 in mixed cultures growing in grape must. (a) Axenic cultures of *S. cerevisiae* SC1 (■), *P. membranifaciens* CYC 1086 (●) and *B. bruxellensis* 1D007 (▲). (b) Mixed cultures of *B. bruxellensis* 1D007 (▲) vs *P. membranifaciens* CYC 1086 (●). (c) Mixed cultures of *S. cerevisiae* SC1 (■) vs *P. membranifaciens* CYC 1086 (●). The killer toxin remaining unbound (bars) was determined and expressed as relative killer activity. Means $\pm$ SD ($n=3$).

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