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Characterization of novel killer toxins secreted by wine-related non-*Saccharomyces* yeasts and their action on *Brettanomyces* spp.

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ABSTRACT

Wine spoilage associated with *Brettanomyces bruxellensis* is a major concern for winemakers. An effective and reliable method to control the proliferation of this yeast is therefore of utmost importance. To achieve this purpose, sulphur dioxide (SO_2) is commonly employed but the efficiency of this chemical compound is subject to wine composition and it can elicit allergic reactions in some consumers. Biological alternatives are therefore actively sought. The current study focused on identifying and characterizing killer toxins which are antimicrobial compounds that show potential in inhibiting *B. bruxellensis* in wine. Two killer toxins, CpKT1 and CpKT2, from the wine isolated yeast *Candida pyralidae* were identified and partially characterized. The two proteins had a molecular mass above 50 kDa and exhibited killer activity against several *B. bruxellensis* strains especially in grape juice. They were active and stable at pH 3.5–4.5, and temperatures between 15 and 25 °C which are compatible with winemaking conditions. Furthermore, the activity of these killer toxins was not affected by the ethanol and sugar concentrations typically found in grape juice and wine. In addition, these killer toxins inhibited neither the *Saccharomyces cerevisiae* nor the lactic acid bacteria strains tested. These preliminary results indicated that the application of these toxins will have no effect on the main microbial agents that drive alcoholic and malolactic fermentations and further highlight the potential of using these toxins as agents to control the development of *B. bruxellensis* in grape juice or wine.

1. Introduction

In red wine, *Brettanomyces bruxellensis* is considered a major spoilage yeast, occurring in low numbers in the early stages of winemaking due to its characteristic slow growth. During fermentation, its population remains low as the metabolic activity of stronger fermenters inhibits its development. However, it may proliferate during ageing. Under winemaking conditions, the yeast can enter into a viable but non-culturable (VBNC) state in the presence of sulphur dioxide (SO_2) and ethanol (du Toit et al., 2005). In this physiological state, the cell has reduced metabolic activity, is unable to reproduce on solid media and has reduced cell size (Millet and Lonvaud-Funel, 2000). This state renders the yeast undetectable since routine microbiological tests usually only make use of cultivation-based techniques. However, the yeast can resume normal growth in the presence of residual sugars, low molecular SO_2 concentration found at the end of alcoholic fermentation as well as in semi-aerobic conditions that occur during ageing in wooden barrels (Ciani et al., 2003; Chatonnet et al., 1995; Oelofse et al., 2008). Wines contaminated by *B. bruxellensis* are characterized by the presence of off-flavours and off-odours (Oelofse et al., 2008; Romano et al., 2008) that arise from the production of volatile phenols e.g. 4-ethylphenol and 4-ethylguaiacol (Chatonnet et al., 1995; Duckitt, 2012; Oelofse et al., 2008).

The control of *B. bruxellensis* is usually achieved through the use of SO_2. In wine, SO_2 dissociates into three molecular species: the antimicrobial molecular SO_2 ($SO_2 \cdot H_2O$), bisulphite (HSO_3^-) and sulphite (SO_3^{2-}). A large portion of the latter two species binds to reactive compounds such as acetaldehyde and anthocyanins (Divol et al., 2012a). The antimicrobial efficiency of SO_2 is dependent on the molecular SO_2 concentration which in turn depends on the pH, temperature and ethanol concentration of the wine as well as the amount of compounds able to bind the bisulphite anion. Moreover, some strains of *B. bruxellensis* are naturally resistant to SO_2 and tolerant to high ethanol and low sugar concentrations (Oelofse et al., 2008; Silva et al., 2004; Wedral et al., 2010). Thus the control of *B. bruxellensis* in wine can be challenging.

Elimination of *B. bruxellensis* by filtration and barrel sanitization has proved to have limited efficiency and does not prevent subsequent recontamination (Millet and Lonvaud-Funel, 2000; Peri et al., 1988). In contrast, chemical preservatives such as benzoic acid, sorbic acid and dimethyldicarbonate (DMDC) are able to inhibit *B. bruxellensis* in wine. However, their use is either not permitted for the former or limited for the latter two as they either affect wine flavour or are legally permitted at concentrations between 200 and 250 mg/L, against which *B. bruxellensis* is tolerant to (Benito et al., 2009). Furthermore, the antimicrobial activity of weak organic acids relies on their concentration (Oelofse et al., 2008;

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Suárez et al., 2007). In recent years, new techniques such as pulsed electric fields (PEF) and UV-C radiation have proved to be successful in inhibiting the proliferation of yeasts and bacteria in grape juice and wine. However, the effect of PEF on the sensorial and chemical properties of wine is not yet known (Fredericks et al., 2011; Puértolas et al., 2009). UV-C radiation is required in high dosages and is dependent on the initial microbial load, turbidity and colour of the liquid sample (Fredericks et al., 2011; Marsellés-Fontanet et al., 2009). Therefore, biological methods such as the use of killer toxins can provide an alternative method to control *B. bruxellensis* in wine. These would have a similar function as bacteriocins used in the dairy industry as well as in fermented and unfermented foods to combat the proliferation of lactic acid bacteria (Cleveland et al., 2001; Sobrino-López and Belloso, 2008).

Killer toxins are proteinaceous antimicrobial compounds secreted by yeasts (Lowes et al., 2000). They have been tested in research investigations to inhibit undesired or pathogenic organisms in various environments such as fermented and unfermented foods, beverages, marine and clinical environments with success (Liu and Tsao, 2009; Lowes et al., 2000; Santos et al., 2011; Séguy et al., 1998; Wang et al., 2007). Killer toxins were first discovered in *Saccharomyces cerevisiae* strains in 1963 by Makower and Bevan as reported in Woods and Bevan (1968) and in non-*Saccharomyces* yeast genera by Philliskirk and Young (1975). *S. cerevisiae*'s killer toxins and their relevance in winemaking have been thoroughly investigated in literature (Carrau et al., 1993; Gutiérrez et al., 2001; Heard and Fleet, 1987; Jacobs et al., 1988; Jacobs and van Vuuren, 1991; Pérez et al., 2001; Ramon-Portugal et al., 1998; Vadasz et al., 2002). However, these killer toxins exhibit narrow spectra of activity limited to other strains of *S. cerevisiae* (Gutiérrez et al., 2001; Heard and Fleet, 1987) except for the Klus killer toxin (Rodríguez-Cousin et al., 2011) and the killer toxin from *S. cerevisiae* strain Y500-4 L (Soares and Sato, 1999, 2000) that are active against a few non-*Saccharomyces* species and are therefore unsuitable as agents to prevent the development of spoilage yeasts. Although non-*Saccharomyces* killer toxins have been investigated to a lesser extent than those of *S. cerevisiae*, they generally exhibit broader spectra of activity than the latter (Ciani and Comitini, 2011).

The killer toxins secreted by the yeast species *Pichia membranifaciens*, *Kluyveromyces wickerhamii* and *Pichia anomala* (now re-classified as *Wickerhamomyces anomala*) and the filamentous fungus *Ustilago maydis* have been specifically investigated for their killer activity against *B. bruxellensis* (Comitini et al., 2004; Santos et al., 2009, 2011). These killer toxins successfully inhibited the growth of *B. bruxellensis* in wine and grape juice. Furthermore, the killing activity of certain non-*Saccharomyces* killer toxins has been demonstrated against the apiculate yeast *Hanseniaspora uvarum* (Comitini and Ciani, 2010) and also against the grapevine pathogen *Botrytis cinerea* (Santos and Marquina, 2004). Thus, the use of killer toxins in inhibiting undesired microorganisms in wine seems to be a propitious method. The aim of the current study was to isolate novel killer toxins secreted by wine-related non-*Saccharomyces* yeasts with potential as biopreservatives against *B. bruxellensis*.

2. Materials and methods

2.1. Isolate fingerprinting and strain identification

Twenty-two South African *B. bruxellensis* isolates (Table 1) were differentiated to strain level by ISS–PCR (Intron Splice Site amplification analysis) fingerprinting courtesy of Dr. I. Vigentini (University of Milan, Italy), using the primer pair Db1EI1/LA2 (Vigentini et al., 2012). The isolates IWBT Y1140 and IWBT Y1057 were identified to species level by PCR amplification of the D1/D2 26S rRNA region using the primer pair NL1 and NL4 (O'Donnell, 1993). Genomic DNA (gDNA) of the strains was isolated from 5 mL YPD (Yeast Peptone Dextrose broth, Biolab-Merck, Wadeville, South Africa) overnight cultures as described previously by Hoffman (1997). PCR amplification was performed in a 50 μL reaction mixture consisting of 1X Ex Taq Buffer, 10 mM dNTPs, 1 μM of each primer, 100 ng of gDNA of each isolate and 1.25 U of ExTaq™ (TaKaRa, Shiga, Japan). The thermal cycler parameters were: initial denaturation at 95 °C, 5 min; 35 cycles of denaturation at 95 °C, 30 s; annealing at 53 °C, 45 s, extension at 72 °C, 1 min and final extension at 72 °C, 7 min. Amplification was carried out with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA). The PCR products were resolved on a 0.8% agarose gel stained with ethidium bromide at 90 V for 1 h, after which the bands were excised from the gel and the DNA was extracted using the Zymoclean™ Gel DNA Recovery Kit (ZymoResearch, Irvine, CA) following the manufacturer's instructions. The PCR products were then cloned into pGEM®-T Easy (Promega, Fitchburg, WI) following the manufacturer's instructions and the vector was then transformed into *Escherichia coli* DH5α competent cells according to the Promega Technical Manual TM042 (Promega, Fitchburg, WI). Positive clones were selected on Luria-Bertani agar (Biolab-Merck) plates supplemented with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), 80 μg/mL X-Gal (5-bromo-4-chloro-indolyl-galactopyranoside) and 100 μg/mL Ampicillin (Ampicillin Sodium Salt) (all chemicals from Sigma-Aldrich, St. Louis, MO) after incubation at 37 °C overnight. After plasmid extraction using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions, the gene was released from the vector by restriction digest with *Eco*RI (Roche Diagnostics, Basel, Switzerland) and sent for sequencing of the D1/D2 region of the 26S rRNA region using the primer pair T7 and SP6 (Promega, Fitchburg, WI) at the Central Analytical Facility at Stellenbosch University.

2.2. Killer activity screening

Yeast and bacterial strains used in this study are described in Table 1. Pre-cultures of all the yeast strains were grown in 5 mL YPD medium (Biolab-Merck) at 30 °C with shaking on a test tube rotator overnight except for the *Brettanomyces/Dekkera* spp. strains which were grown for 48 h. Fifty microliters of the 5 mL pre-culture were inoculated into 50 mL YPD medium and the culture was grown at 30 °C with shaking. Bacterial strains were grown for 7 days in 10 mL filter sterilized 100% commercial white table grape juice incubated at 30 °C without shaking. Killer activity screening was performed using the seeded agar method on YPD, WYE and RYE (commercial white/red table grape juice supplemented with 1% yeast extract, respectively) for the yeast strains and on MRS (de Man, Rogosa and Sharpe, Biolab-Merck), W and R (100% commercial white and red table grape juice, respectively) for the bacterial strains. The media were adjusted to pH 4.5 with 1 M HCl or 2 M NaOH, as the killer toxin secreted by *K. wickerhamii*, used as a positive control in this study, has a pH activity optimum at pH 4.4 (Comitini et al., 2004). Other killer activity screening studies are also typically reported to be conducted at pH 4.5 (Palpacelli et al., 1991; Santos et al., 2009) as yeast mycocins are most active at pH 4.0–5.0 (Golubev, 2000). *Brettanomyces/Dekkera* spp., *Zygosaccharomyces*, *Saccharomyces* and lactic acid bacteria strains (Table 1) were inoculated as potentially sensitive cells at a concentration of 10^6 cfu/mL in 7.5 mL of the pH adjusted media. 2.5 mL of 4% bacteriological agar (kept at 50 °C) was mixed with the inoculated medium to a final volume of 10 mL and after brief vortexing, the medium was poured into sterile Petri dishes. Five microlitres of the killer yeast strains (Table 1) were spotted on the surface of the solidified agar plate. The plates were incubated at 20 °C until a well-developed lawn of the potentially sensitive yeast or bacterial strain was observed. Killer activity was visualized as a zone of growth inhibition around the spotted killer yeast colony on triplicate plates.

2.3. Killer toxin production

Cultures of *C. pyralidae* IWBT Y1140 and IWBT Y1057 strains were grown in YPD broth adjusted to pH 4.5 for killer toxin production. Pre-cultures of the strains were grown in 5 mL YPD broth overnight at