Non-*Saccharomyces* Killer Toxins: Possible Biocontrol Agents Against *Brettanomyces* in Wine?

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Red wine spoiled by the yeast *Brettanomyces bruxellensis* is characterised by off-odours commonly described as horse sweat, phenolic, varnish and band-aid. The growth of this yeast in wine is traditionally controlled by the use of sulphur dioxide (SO_2). However, the concentration of SO_2, the pH of the wine, the presence of SO_2-binding chemical compounds in the wine, as well as the strain of *B. bruxellensis*, determine the effectiveness of SO_2. Other chemical preservatives have been tested, but are not much more efficient than SO_2, and methods used to clean barrels are only partially effective. Filtration of wine and the use of electric currents/fields are also reported to alter the physical and sensory properties of wine. In this context, alternative methods are currently sought to achieve full control of this yeast in wine. Killer toxins have recently been proposed to fulfil this purpose. They are antimicrobial compounds secreted by *Saccharomyces* and non-*Saccharomyces* yeasts, displaying killer activity against other yeasts and filamentous fungi. They are believed to play a role in yeast population dynamics, and this killer phenotype potentially could be exploited to inhibit the growth of undesired microorganisms within a microbial ecosystem such as that occurring in wine. In this review, non-*Saccharomyces* killer toxins are described and their potential application in inhibiting *B. bruxellensis* in wine is discussed in comparison to other tried methods and techniques.

INTRODUCTION

Brettanomyces bruxellensis is regarded as a major red wine spoilage yeast. Its occurrence and development in wine are controlled mainly through the use of sulphur dioxide (SO_2). However, the antimicrobial property of SO_2 depends on a number of factors, including the concentration of its molecular fraction, as well as the species and strains of microorganisms that need to be eliminated. Under certain conditions, such as pH > 4 and the presence of SO_2-binding compounds in wine, the concentration of molecular SO_2 drops and the effectiveness of SO_2 becomes limited. Furthermore, yeast species and strains have been reported to exhibit a range of tolerance levels vis-à-vis SO_2 (Curtin *et al*., 2012b). Chemical treatments (e.g. benzoic acid and sorbic acid), physical techniques (e.g. filtration, sanitisation) and biologically produced compounds (e.g. the polysaccharide chitosan) have been tested for controlling *B. bruxellensis* growth and were proven to have limited efficiency (Suárez *et al*., 2007). In addition, hypersensitivity to SO_2 in some wine consumers has spurred the demand for the use of non-chemical preservatives (Comitini *et al*., 2004a; Lustrato *et al*., 2006). Alternative methods therefore currently are being sought to control the growth of *B. bruxellensis*. Biological antimicrobial compounds, such as killer toxins secreted by certain non-*Saccharomyces* yeasts, including *Kluyveromyces wickerhamii*, *Pichia anomala*, *Pichia membranifaciens* and the filamentous fungi *Ustilago maydis*, have recently been described as such possible alternatives (Comitini *et al*., 2004b; Santos *et al*., 2009; 2011).

Killer toxins are proteinaceous antimicrobial compounds produced by yeasts and are active against members of the same species or closely related species (Lowes *et al*., 2000). Killer toxin-secreting species are found in *Saccharomyces* yeasts, as well as in non-*Saccharomyces* genera such as *Debaryomyces*, *Kluyveromyces*, *Candida*, *Hansenula*, *Pichia*, *Cryptococcus*, *Torulopsis*, *Hanseniaspora* and *Zygosaccharomyces* (Van Vuuren & Jacobs, 1992; Schmitt & Breinig, 2006). Non-*Saccharomyces* killer toxins exhibit a broader spectra of activity, inhibiting species within the non-*Saccharomyces* and the *Saccharomyces* genera, compared to those of *Saccharomyces* (Ciani & Comitini, 2011). This phenotype (i.e. the secretion of killer toxin) thus could play a pivotal role in governing yeast-yeast interactions and be exploited to control the growth of undesired microorganisms in wine. In this context, the use of these killer toxins can be viewed as the equivalent of bacteriocins, which are applied successfully in fermented and unfermented foods (Cleveland

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et al., 2001). Killer toxins indeed have been shown to have potential applications in food, agriculture and medical industries (Palpacelli *et al*., 1991; Lowes *et al*., 2000; Cailliez *et al*., 1994; Walker *et al*., 1995; Goretti *et al*., 2009; Liu & Tsao, 2009). The purpose of this review is to draw up a record of the current knowledge on non-*Saccharomyces* killer toxins and their possible application in winemaking conditions, versus methods and techniques currently used or applied as preservatives in wine.

DEKKERA/BRETTANOMYCES SPOILAGE IN WINE

In red wine, the yeast *Dekkera bruxellensis* or its anamorph, *B. bruxellensis*, produces a range of off-flavour compounds, amongst which ethylphenols are the most potent. Consequently, the production of the latter has been identified as the main spoilage reaction of this yeast in wine (Dias *et al*., 2003a). *B. bruxellensis* is spread within the winery environment through the importation of contaminated wine, poor sanitation of hoses, tanks and wooden barrels, or through the passive adherence to the body of the fruit fly (Fugelsang & Edwards, 2007). The yeast is characterised as a slow grower and is detected in low numbers in the early stages of winemaking (Fugelsang & Edwards, 2007). It is also tolerant to high ethanol and low sugar concentrations (Wedral *et al*., 2010). Furthermore, some strains are either tolerant or sensitive to free SO_2 above 30 mg/L (Oelofse *et al*., 2008). It has also been reported that *B. bruxellensis* can enter into a viable but non-culturable (VBNC) state, which is characterised by reduced metabolic activity, inability to reproduce on solid media and reduced cell size (Millet & Lonvaud-Funel, 2000). The VBNC state can be maintained throughout alcoholic fermentation when the levels of molecular SO_2 are higher and oxygen is limited. The interval between the end of alcoholic fermentation and the beginning of malolactic fermentation (MLF) represents a critical period during which *B. bruxellensis* can exit VBNC and grow to detectable levels (Fugelsang & Edwards, 2007) due to the low molecular SO_2 concentration at this stage. Furthermore, the availability of residual sugars, assimilable nitrogen-containing compounds (although limited), as well as micro-aerobic conditions found during ageing in wooden barrels after MLF, also support the proliferation of *B. bruxellensis* (Chatonnet *et al*., 1995; Ciani *et al*., 2003; Comitini *et al*., 2004b; Oelofse, 2008).

This population of *B. bruxellensis* may be significant enough to produce detectable levels of volatile phenols, e.g. 4-ethylphenol and 4-ethylguaiacol (Chatonnet *et al*., 1995; Dias *et al*., 2003b). Red wines are particularly prone to the development of *B. bruxellensis* and the subsequent production of ethylphenols compared to white wines (Romano *et al*., 2008), due to *Vitis vinifera* red varietals that contain precursor phenolics, e.g. non-flavonoid and flavonoid (Monagas *et al*., 2006; Wedral *et al*., 2010). Volatile phenols are indeed produced in wine through the catabolism of three different hydroxycinnamic acids: *p*-coumaric, ferulic and caffeic acids. These precursors originate from grapes and therefore are naturally present in grape juice and wine. *B. bruxellensis* enzymatically converts hydroxycinnamic acids to volatile phenols in a two-step reaction. The precursors are converted by a cinnamate decarboxylase into hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol) and further reduced to ethyl derivatives (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol respectively) by a vinylphenol reductase. The presence of ethylphenols is characterised by the development of unpleasant odours and tastes that deeply affect wine aroma (Oelofs*e et al*., 2008).

Until about two decades ago, lactic acid bacteria (LAB) were thought to contribute to the production of ethylphenols in wine (Chatonnet *et al*., 1992). However, Chatonnet *et al*. (1995) found that, under winemaking conditions, these bacteria only produce ethylphenols at a concentration < 10 µg/L. The yeast *Pichia guilliermondii* may produce 8 mg/L and 12 mg/L 4-ethylphenols in red and white grape juice respectively (Barata *et al*., 2006) compared to other *Pichia* spp., *Torulaspora* spp. and *Zygosaccharomyces* spp., which cannot produce ethylphenols due to the inactivity of their vinylphenol reductase enzyme (Chatonnet *et al*., 1995). Nevertheless, the production of vinylphenols and ethylphenols in wine is mainly attributed to *B. bruxellensis*, as both its phenolic acid decarboxylase and vinyl phenol reductase enzymes are active (Dias *et al*., 2003a; 2003b; Harris *et al*., 2009; Granato *et al*., 2014). Over the years, several research teams have attempted to isolate these two enzymes and to characterise their properties (Godoy *et al*., 2008; Tchobanov *et al*., 2008; Benito *et al*., 2009; Godoy *et al*., 2009; Harris *et al*., 2009). Although these authors were able to study the kinetic properties of these enzymes, the influence of wine-related inhibitory compounds thereof, as well as their optimal pH and temperature activity, their isolation remained partial and no genetic sequence could be retrieved. Indeed, it was only very recently that the corresponding genes were identified in the genome of *B. bruxellensis* (Curtin *et al*., 2012a; Piškur *et al*., 2012; Granato *et al*., 2014). Interestingly, the phenolic acid decarboxylase-encoding gene was shown to be more closely related to its bacterial equivalents (Curtin *et al*., 2012a), and the vinyl phenol reductase-encoding gene to be a Cu/Zn dioxide dismutase displaying strong vinyl phenol reductase activity (Granato *et al*., 2014). This probably explains the earlier difficulties to isolate these genes in *B. bruxellensis*. These latest discoveries constitute a major step in understanding the spoilage mechanism of *B. bruxellensis* in wine and will certainly open new research avenues.

The production and sensorial perception of volatile phenols is dependent on the strain and population of *B. bruxellensis*, the presence of volatile compound precursors and also the variety of grapes used (Suárez *et al*., 2007; Wedral *et al*., 2010; Kheir *et al*., 2013). Suárez *et al*. (2007) reported the sensory threshold of 4-ethylphenol to be 230 µg/L, while Loureiro and Malfeito-Ferreira (2003) reported a preference threshold of 620 µg/L. However, these threshold levels can also vary due to the perception of the individual, which is influenced by the wine style, cultivar and the consumer's perceptive abilities (Oelofse, 2008). Furthermore, Romano *et al*. (2008) reported on the complexity of correlating ethylphenol concentration and the "Brett character" in wines due to the masking effect of other metabolites. The production of these volatile phenols in red wine thus can be prevented by controlling or eliminating *B. bruxellensis* populations in grape must or wine. Subsequently, several

strategies have been employed to control wine spoilage by *B. bruxellensis.*

CONTROL OF *B. BRUXELLENSIS* SPOILAGE IN WINE

Chemical preservatives

SO_2 is the most commonly used chemical preservative in winemaking due to its antioxidant and antimicrobial properties. However, its use and effectiveness in controlling *B. bruxellensis* are often contradictory in the literature. The contradiction probably arises from the lack of studies under comparable conditions and variability in strain behaviour, as noted by Barata *et al.* (2008). Low pH values (~3.5), SO_2 levels around 0.8 ppm of molecular SO_2 and low ageing temperatures (10 to 15°C) are ordinary practices that can be used to limit *B. bruxellensis* activity in wines (Couto *et al*., 2005). Although SO_2 has had a long history of use as a preservative in alcoholic beverages, especially in wines, it can have adverse effects on the respiratory system of consumers (Freedman, 1977). For this reason, alternatives have been sought with more or less success.

Benzoic acid effectively inhibits *B. bruxellensis* growth in soft drinks at concentrations of between 100 and 200 mg/L, and it also inhibits the action of the enzymes hydroxycinnamate decarboxylase and vinylphenol reductase at concentrations between 150 to 200 mg/L at pH 3.6 (Van Esch, 1992). However, the addition of benzoic acid to wine is not permitted as it affects wine flavour (Benito *et al*., 2009). Sorbic acid is unable to inhibit *B. bruxellensis* growth at the concentrations legally permitted (200 to 250 mg/L) (Benito *et al*., 2009). It has indeed been shown that this yeast is tolerant of 950 mg/L of sorbic acid at pH 3.5 (Loureiro & Malfeito-Ferreira, 2006). The use of weak acids such as benzoic and sorbic acid relies mainly on their effectiveness in their undissociated form, therefore, for complete growth control, they need to be added in high concentrations (Du Toit & Pretorius, 2000). Renouf *et al.* (2008) found that dimethyl dicarbonate (DMDC) inhibits the growth of *B. bruxellensis* at 150 mg/L and can limit the growth of *B. anomalus* at 400 mg/L, which is double the legal limit (Du Toit & Pretorius, 2000; OIV (International Organisation of Vine and Wine), 2001; Benito *et al*., 2009). The minimum inhibitory concentration (MIC) of DMDC to kill *D. bruxellensis* was in fact found to be 100 mg/L, but this is dependent on the initial cell concentration (Costa *et al*., 2008). Furthermore, the use of DMDC can affect alcoholic fermentation negatively if added to grape juice, as 200 mg/L of DMDC added to inoculated grape must showed a four-day fermentation delay (Delfini *et al*., 2002). For further reviews on these methods, see Suárez *et al*. (2007) and Oelofse (2008). A triplet combination of lauric arginate, cinnamic acid and sodium benzoate was found to inhibit the growth of *B. bruxellensis* (Dai *et al*., 2010), although this method would not be suitable in practice. Overall, chemical preservatives have been used successfully for many years to combat microbial contaminants in different beverages. However, their use in wine clearly remains limited due to their negative influence on fermentation kinetics and on the organoleptic properties of wine.

Physical and physicochemical methods

The separation of microbial cells from wine, cleaning of winery equipment and sanitisation of barrels, and most recently the application of electric currents to wine, are some of the physical methods that have been tested to inhibit the growth of *B. bruxellensis*. Filtration technology requires the use of membranes with specific porosity; however, this has certain limitations (Zuehlke *et al*., 2013). For instance, the cell size of *B. bruxellensis* may shrink after exposure to SO_2; in this case, filtration with a 0.45-µm membrane filter has been shown to be inefficient in removing *B. bruxellensis* (Millet & Lonvaud-Funel, 2000). Umiker *et al.* (2013) suggest the use of membrane filters with porosities of < 0.8 µm for the removal of *B. bruxellensis* in wines, but this is contradictory to the previous finding. Moreover, filtration may reduce colour intensity, and the concentration of aroma compounds, esters and phenolic compounds (Peri *et al*., 1988; Arriagada-Carrazana *et al*., 2005; Moreno & Azpilicueta, 2006). Barrel sanitation by steam treatment and burning of sulphur are not enough to eliminate *B. bruxellensis* (Loureiro & Malfeito-Ferreira, 2003), and the organism cannot be removed by cleaning or shaving of barrels (Wedral, 2010). Other methods, such as thermal inactivation, ultrasound or high-power ultrasonics, have been shown to be effective against *Brettanomyces* or *Dekkera* species. Couto *et al*. (2005) found that *D. bruxellensis* was inactivated at 35°C, while Yap *et al*. (2008) and Schmid *et al.* (2011) reported that the use of high-pressure ultrasound or high-power ultrasonics eliminated the population of *D. bruxellensis* in wine barrels.

Pulsed electric fields and UV-C (ultra violet) radiation have also been investigated. The use of pulsed electric fields (PEF) reduced the population of *D. bruxellensis* and *D. anomala* in must and wine (Puértolas *et al*., 2009), and the same was observed against *D. bruxellensis* in wine using a low electric current (LEC) (Lustrato *et al*., 2010). However, the effect of PEF on the sensorial properties of wine and the evaluation of the ability of this technology in wineries still needs to be researched further (Marsellés-Fontanet *et al*., 2009). The use of UV-C radiation in must and wine resulted in the reduction of *B. bruxellensis*, *L. plantarum* and *S. cerevisiae*. However, the reduction and complete inactivation of the microbial population in must and wine was observed when UV-C dosages of 3672 J/L were applied, which was dependent on the initial microbial load, turbidity and colour of the liquid sample (Fredericks *et al*., 2011).

The application of physicochemical methods such as the use of biocide ozone (O_3) to inactive the population of *B. bruxellensis* was recently investigated by Guzzon *et al*. (2013). The activity of O_3 was found to be dependent on the initial cell population. Furthermore, a 10 min treatment with ozonated water was more effective in winery CIP (cleaning in place) systems than peracetic acid or caustic soda cleaning agents. However, when O_3 is used, attention must be paid to recommended dosages and limited human exposure (Guillen *et al*., 2010). Although these methods have been shown to be efficient in inhibiting or even eliminating *Brettanomyces* and *Dekkera* species, their activity is mainly dependent on the initial load of the cell concentration to be eliminated.

Biological methods

Biological methods have been shown to be alternatives compared to the use of chemical preservatives or physical treatments. It was found recently that the use of a commercial enzyme solution containing an endo-*β*(1-3)-glucanase, exo-*β*(1-3)-glucanase, exo-*β*(1-6)-glucanase and an unspecific *β*-glucosidase inhibited the growth of *D. bruxellensis* and *Z. bailii* by more than 90%. The solution resulted in a half maximal inhibitory concentration (IC_{50}) and MIC at 115 µg/mL and 200 µg/mL respectively on both yeasts (Enrique *et al*., 2010). Chitosan, the deacetylated derivative of chitin, was found to have a fungistatic effect against *B. bruxellensis* and, at concentrations > 3 g/L, the yeast ceased to survive (Gómez-Rivas *et al*., 2004). Recently, Oro *et al*. (2014) showed that *Metschnikowia pulcherrima* secretes pulcherriminic acid, which is inhibitory to the growth of *B. bruxellensis*. Finally, in recent studies, the use of biological antimicrobial compounds, such as the killer toxins Kwkt, Pikt and PMKT2 from the yeast species *K. wickerhamii*, *P. anomala* and *P. membranifaciens* respectively, was shown to be successful in inhibiting *Dekkera*/*Brettanomyces* in wine, and these will be discussed in the next section.

KILLER TOXINS

General considerations

Killer toxins are defined as antimicrobial proteinaceous compounds that inhibit susceptible yeast species or strains, although they remain immune to their own toxins (Bussey, 1972; Magliani *et al*., 1997; Schmitt & Breinig, 2002; Lowes *et al*., 2009). Yap *et al.* (2000) termed the secretion of killer toxins "interference competition", a form of amensalism. Although interference competition favours the growth of the killer toxin-producing yeast over that of other microorganisms present in the same habitat, its potential role in eliminating undesired microorganisms cannot be disputed. Thus, this killer phenotype can be used to combat spoilage yeasts and can be used as a partial substitute to chemical agents such as SO_2 for the preservation of wine (Ciani & Comitini, 2011).

Non-*Saccharomyces* yeast genera such as *Debaryomyces*, *Kluyveromyces*, *Candida*, *Hansenula*, *Pichia*, *Cryptococcus*, *Torulopsis*, *Hanseniaspora*, *Zygosaccharomyces* and yeast species of the *Saccharomyces* genus are reported to secret killer toxins (Van Vuuren & Jacobs, 1992; Schmitt & Breinig, 2006). Four phenotypes have been identified: killer, sensitive, neutral and killer-sensitive phenotypes. A specific killer strain produces a toxin and is immune to it; a sensitive strain does not produce the toxin and is sensitive to the toxin produced by a killer strain; a neutral strain neither produces nor is sensitive to the killer toxin produced by a killer strain (Gutiérrez *et al*., 2001), and a killer-sensitive strain produces a toxin and is immune to it but is sensitive to toxins produced by other strains (Tredoux *et al*., 1986).

Killer toxins of *S. cerevisiae*

S. cerevisiae's killer toxins were first discovered in 1963 (Woods & Bevan, 1968). Four killer toxins have been identified so far: K1, K2, K28 and Klus. *S. cerevisiae*'s killer toxins are encoded by different cytoplasmically inherited satellite double stranded RNAs (dsRNAs) (M1, M2, M28 and Mlus) encapsulated in virus-like particles (VLPs) and are dependent on helper yeast viruses (L-A) for their replication and encapsidation (Magliani *et al*., 1997; Schmitt & Breinig, 2006; Rodríguez-Cousin, 2011). The killer activity of *S. cerevisiae* is mainly dependent on the killer:sensitive ratio. These killer toxins have a narrow spectrum of activity, inhibiting only strains or species within the same genus (Mannazzu *et al*., 2002), except for the Klus killer toxin, which is active against yeasts such as *Hanseniaspora* spp., *Kluyveromyces lactis*, *Candida albicans*, *Candida dubliniensis*, *Candida kefir* and *Candida tropicalis*, and the K1, K2 and K28 killer strains of *S. cerevisiae* (Rodríguez-Cousin *et al*., 2011). Considering that these toxins are not active against *B. bruxellensis* they will not be discussed further in this review. For further reading on *S. cerevisiae*'s killer toxins, the reader is advised to consult the following reviews: Magliani (1997), Schmitt and Breinig (2002; 2006) and Rodríguez-Cousin *et al*. (2011).

Killer toxins secreted by non-*Saccharomyces* yeasts

Non-*Saccharomyces* yeasts exhibiting killer activity were first reported by Philliskirk and Young (1975) in six yeast genera: *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Candida* and *Torulopsis*. Young and Yagiu (1978) then identified the killer toxins K4 in *Torulopsis glabrata* NCYC 388, K5 in *Debaryomyces vanriji* NCYC 577, *Hansenula anomala* NCYC 434 and *Hansenula subpelliculosa* NCYC 16, K6 in *Kluyveromyces fragilis* NCYC 587, K7 in *Candida valida* NCYC 327 and *P. membranifaciens* NCYC 333, K8 in *Hansenula anomala* NCYC 435, K9 in *Hansenula mrakii* NCYC 500, and K10 in *Kluyveromyces drosophilarum* NCYC 575, based on cross-reactivity assays with each of the killer strains. One year later, Wickner (1979) reported that *Torulopsis glabrata* ATCC15126 also secretes a killer toxin, named K11. Non-*Saccharomyces* killer toxins originate either from linear dsDNA plasmids or chromosomes (Marquina *et al*., 2002; Liu *et al*., 2013), with the notable exception of the killer toxins of *H. uvarum* and *Z. bailii*, which originate from virus-like particles (Schmitt & Neuhausen, 1994), similar to those of *S. cerevisiae*.

Killer toxins have found application in the food and fermentation industry, the bio-typing of medically important pathogenic yeast and yeast-like fungi, the development of novel antimycotics for the treatment of human and animal infections by fungi, and in recombinant DNA technology (Schmitt & Breinig, 2002; Liu *et al*., 2013). Table 1 summarises the genetic, enzymatic and biochemical characteristics of non-*Saccharomyces* killer toxins with potential application in the food and beverage industry. It also highlights the proposed application of these killer toxins.

Antimycotic activity and application of non-*Saccharomyces* killer toxins in wine making

Non-*Saccharomyces*' killer toxin-producing yeasts have been isolated from various environments, such as marine and clinical environments, as well as fermented and unfermented foods and beverages (Ciani & Comitini, 2011). These killer toxins exhibit broader anti-yeast spectra compared to those of *Saccharomyces* (Palpacelli *et al*., 1991; Ciani & Comitini, 2011). For instance, the species *Tetrapisispora phaffii* and

TABLE 1
Genetic origin and biochemical and biological characteristics of killer toxins secreted by non-*Saccharomyces* yeasts that have potential application in the food and beverage industry.

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor/ mode of action	Sensitive/ target yeasts	Application/ potential application	References
Kluyveromyces wickerhamii	Kwkt (72 kDa)	Unknown	pH activity: 3.8 to 4.6 (opt. pH 4.4) Optimal temperature activity: 20°C (max. 25°C)	Receptor: pustulans (β-1,6-glucans)	*D. bruxellensis*	In winemaking	Comitini *et al*., 2004; Ciani & Comitini, 2011; Comitini & Ciani, 2011
Tetrapisispora phaffii	Kpkt (33 kDa)	Chromo-somal gene – *BGL2*	pH activity: 3 to 5 Temperature activity: < 40°C	Disruption of cell wall integrity Displays properties of β-glucanase enzyme	*H. uvarum*	In winemaking	Ciani & Fatichenti, 2001; Comitini *et al*., 2004; Comitini & Ciani, 2010; Oro *et al*., 2013
Hansenula mrakii (re-classified *Williopsis mraki*i)	HMK or HM-1 (10.7 kDa)	Chromo-somal gene *hmk*	pH stability: 2 to 11 Thermostable – biologically active after incubation at 100°C, 10 min	Receptor: β-D-1,3 and β-D-1,6-glucan Inhibits β-glucan synthesis	*Heterobasidium*, *Postia*, *Serpula*, *Fusarium* and/or *Colletotrichum*	In silage and yoghurt	Yamamoto *et al*., 1986; Lowes *et al*., 2000; Santos *et al*., 2002; Schmitt & Breinig, 2002; Selvakumar, *et al*., 2006
Pichia anomala DBVPG 3003	Pikt (8 kDa)	Unknown	pH activity: 4.4 Temperature activity: 25 to 35°C	Receptor: β-1,6-glucans	*D. bruxellensis*	In winemaking	Comitini *et al*., 2004; De Ingeniis *et al*., 2009
Pichia membrani-faciens CYC 1106	PMKT (18 kDa)	Unknown	pH activity and stability: 3.0 to 4.8 Temperature activity and stability: 5 to 20°C and 5 to 25°C	Receptor: β-1,6-glucans	*Botrytis cinerea, Candida boidinii*	In grapevine	Santos *et al*., 2000; Santos & Marquina, 2004
Pichia membrani-faciens NCYC 1086	PMKT2 (30 kDa)	Unknown	pH activity: 2 to 5 (opt. 3.5 to 4.5) pH stability: 2.5 to 4.8 Temperature activity: 5 to 20°C Temperature stability: 20 to 32°C	Receptor: β-1,6-glucans	*B. bruxellensis*	In winemaking	Santos *et al*., 2009
Ustilago maydis	KP6	dsRNA virus (α – 8.6 kDa and β – 9.1 kDa)	pH activity: 3.0 to 5.5 (opt. 3.0 to 4.5) pH stability: 2.5 to 5.3 Temperature activity: 15 to 30°C (opt. 15 to 20°C) Temperature stability: 5 to 20°C	Undetermined	*B. bruxellensis*	In winemaking	Santos *et al*., 2011

Kluyveromyces wickerhamii display killer activity against the apiculate yeast *Hanseniaspora uvarum* and species of the *Brettanomyces*/*Dekkera* genus respectively (Ciani & Fatichenti, 2001; Comitini *et al*., 2004a). Since the first record of a killer toxin inhibiting an apiculate yeast (Ciani & Fatichenti, 2001), several studies focusing on yeast killer toxins have been conducted with the aim of eliminating undesired yeasts within the wine environment. The killer toxins KwKt, PiKt, PMKT2 and KP6 secreted by the yeasts *K. wickerhamii*, *P. anomala*, *P. membranifaciens* and the filamentous fungi *Ustilago maydis* have been shown to inhibit the growth of *B. bruxellensis* and *D. bruxellensis* under winemaking conditions (Comitini *et al*., 2004b; Santos *et al*., 2009; 2011).

The killer activity of these toxins was found to be either fungistatic or fungicidal, depending on the killer toxin concentration applied (Ciani & Fatichenti, 2001). The fungicidal character of the Kwkt killer toxin was observed when 28.6 and 57.2 AU/mL of the toxin were applied to *D. bruxellensis* cells (Comitini *et al*., 2004b). Kwkt controlled the growth of *D. bruxellensis* during must fermentation, where, after four and seven days, the yeast ceased to survive at purified killer toxin concentrations of 80 mg/mL and 40 mg/mL, respectively (Comitini & Ciani, 2011). The same yeast species saw its population diminished only when 28.6 AU/mL of the killer toxin Pikt was used compared to the use of 57.2 AU/mL, at which a fungicidal effect was observed (Comitini *et al*., 2004b). In grape must, the killer toxin PMKT2 at 2 000 AU/mL resulted in death rates of 0.13 h^{-1}, 0.09 h^{-1} and 0.11 h^{-1} in three *B. bruxellensis* strains, as described by Santos *et al*. (2009). *B. bruxellensis* isolates had mortality rates ranging between 0.10 h^{-1} and 0.18 h^{-1} in mixed cultures with the filamentous fungi *U. maydis* at 10^2 cells/mL (Santos *et al*., 2011).

The growth of the apiculate yeast *H. uvarum* was inhibited by immobilised cells of the yeast *T. phaffii* (Comitini & Ciani, 2010), as well as by the Kpkt killer toxin secreted by *T. phaffii* (Ciani & Fatichenti, 2001). Similar to the killer toxins Kwkt and Pikt, Kpkt showed a fungistatic effect at low concentrations of 5.14 and 7.15 AU/mL compared to 14.3 AU/mL, at which a fungicidal effect was observed in grape juice (Ciani & Fatichenti, 2001). Under winemaking conditions, the killer toxin Kwkt is efficient and comparable to the use of SO_2 in inhibiting *B. bruxellensis* (Comitini & Ciani, 2011). Furthermore, the killer toxins Kwkt and Pikt maintain their killer activity for 10 days in wine (Comitini *et al*., 2004b). The killer toxins active against *B. bruxellensis* are active and stable at acidic pH ranges (below 5), and at temperatures between 20 and 25°C (Table 1), which are compatible with winemaking conditions. Furthermore, in trial fermentations in which these killer toxins were applied, the population of *S. cerevisiae* was not inhibited (Santos *et al*., 2009; Comitini & Ciani, 2011; Santos *et al*., 2011). In addition, the metabolic by-products ethyl acetate and 4-ethylphenol, were not detected and volatile acidity was reduced (Comitini & Ciani, 2011; Santos *et al*., 2011), further confirming the antimicrobial efficiency of these killer toxins.

MODE OF ACTION OF THE KILLER TOXINS

Killer toxins are reported to be proteins or glycoproteins that kill sensitive cells via a two-step mode of action similar to that determined for the killer toxins of *S. cerevisiae*. For the toxin to fully initiate its killing action, it interacts with receptors of the sensitive cell wall and receptors on the plasma membrane. There are two kinds of receptors: primary and secondary. The former are located on the cell wall and the latter on the plasma membrane (Guo *et al*., 2012). The identified primary receptors include β-D-1,3-glucan, β-D-1,6-glucan, mannoproteins and chitin, while Kre1p of the K1 toxin is the only secondary receptor that has been identified so far (Schmitt & Breinig, 2002). Mechanisms of the actual killing action differ, and may be through cell membrane permeabilisation, perturbation of the cell cycle and inhibition of DNA synthesis, inhibition of β-1,3-glucan synthase activity and/or hydrolysis of the major cell wall components, β-1,3-glucans and 1,6-glucans, of the sensitive strain (Schmitt & Breinig, 2006; Manzanares *et al*., 2011). These mechanisms are summarised below.

Ionophoric killer toxins firstly bind to the cell wall receptors of the target yeast with low-affinity and high-velocity adsorption. This is followed by a high-affinity, low-velocity, energy-dependent interaction of the toxin with the plasma membrane receptor that leads to the lethal effect (Magliani *et al*., 1997). After reaching the plasma membrane, the toxin disrupts cytoplasmic membrane function by forming cation-selective ion channels. This results in the increased permeability of H^+ (Novotná *et al*., 2004), and leakage of intracellular ATP, K^+ (Skipper & Bussey, 1977) and AMP (Bussey & Skipper, 1975).

The action of the killer toxins appears not to be immediate. When tested against *S. cerevisiae* sensitive cells, the use of the killer toxin Pikt resulted in 3.5% viable cells after 24 h of incubation, compared to 75% viable cells after 4 h of incubation. This indicates that the mode of action of this toxin is not immediate and that it is not mediated by cell membrane disruption (De Ingeniis *et al*., 2009). This toxin has also been reported to display activity against *B. bruxellensis*, but its specific mode of action against this yeast has not been described. However, it is likely to be similar to that against *S. cerevisiae*. The delay in the decline of the sensitive yeast population observed for Pikt is not unique, as it has also been observed following exposure of the *S. cerevisiae* cells to both the pool efflux-stimulating toxins (PEST) and killer toxin of *T. glabrata*, where after 30 minutes, 60 to 70% of sensitive cells exhibit uptake of the lethal dose of PEST without any visible metabolic change (Bussey & Skipper, 1975). However, after a lag time of 50 to 90 minutes, sensitive cells treated with a *P. kluyveri* toxin exhibited physiological changes observed when ionophoric toxins act on sensitive cells. The cells had shrunk, intracellular pH had decreased and the active uptake of amino acids was inhibited (Middelbeek *et al*., 1980) (Fig. 1). High doses of the PMKT2 toxin resulted in a decrease in intracellular pH, leakage of K^+ and influx of Na^+ (Fig. 1) which was in parallel to the loss of cellular permeability after 5 h of toxin exposure, when viability was decreased by 85%. Santos *et al*. (2013) concluded that PMKT2 cytotoxic action is not

through channel formation but through the toxin attacking cells after initiating the S phase (Santos *et al*., 2013). This mode of action was investigated in *S. cerevisiae*, but is likely to be similar in *B. bruxellensis* as this toxin displays activity against both yeast species; however, this would need confirmation. This observation is similar to the mode of action of the killer toxin of *K. lactis*, which causes permanent arrest of the sensitive cells at the unbudded G1 phase (Fig. 1) (Magliani *et al*., 1997), and of the K28 killer toxin, which arrests cells in the early S phase and blocks DNA synthesis in the cell cycle, leading to the non-separation of mother and daughter cells (Magliani *et al*., 1997; Couto *et al*., 2005).

The killer toxin Kpkt is reported to disrupt cell wall integrity of the target cells (Comitini *et al*., 2009), while Kwkt, a killer toxin active against *B. bruxellensis*, is only reported to bind to β-1,6-glucan on the cell wall of the sensitive yeast (Table 1) (Ciani & Comitini, 2011). However, their modes of action are yet to be identified. The KP6 killer toxin secreted by *U. maydis*'s mode of action is also thought to be involved with the cell wall of the sensitive cell. Upon exposure to the toxin, the sensitive cells seemed to collapse and change in morphology. Furthermore, spheroplasts of the sensitive cells were not affected by the killer toxin (Steinlauf *et al*., 1988). The killer toxin HMK, secreted by *Hansenula mrakii*, inhibits β-glucan synthesis (Fig. 1) (Yamamoto *et al*., 1986). More recently, it has been reported that *W. anomalus*'s killer toxins damage the β-glucan scaffold in the cell walls of sensitive yeast cells and thereby induce cell death by osmotic lysis (Muccilli *et al*., 2013). Thus far, the mode of action of the killer toxins Kwkt and PMKT, which are active against *B. bruxellensis*, have not yet been unravelled. It is not yet clear whether all the toxins that recognise β-1,6-glucan as receptor display glucanase activity specifically targeting the cell wall glucan of sensitive cells, or whether they are cell wall glucanases that incidentally display killer activity. The following paragraph will discuss this issue.

Do exoglucanases possess killer activity?

The yeast cell wall of *Saccharomyces cerevisiae* is composed of 50% β-D-1,3-glucan, which contains ca. 5% β-1,6 linked branches; 15% β-D-1,6-glucan containing ca. 14% β-1,3 linked branches; and mannoproteins and chitin (0.6 to 9%) (Kollár *et al*., 1995; Santos *et al*., 2000). In recent literature (İzgü & Altinbay, 2004; İzgü *et al*., 2006; Comitini *et al*., 2009; Muccilli *et al*., 2013), growing evidence suggests that the killer activity of some killer toxins occurs through glucanase activity. Fungal β-1,3-glucanases play a role in metabolic and morphogenetic events in the fungal cell, including cell wall extension, hyphal branching, sporulation, budding, autolysis during development and differentiation, and in the mobilisation of β-glucans in response to conditions of carbon and energy source exhaustion (Peng *et al*., 2009).

Exo-glucanase activity has been detected in killer toxin-producing yeast species of *W. anomalus*, *P. membranifaciens*, *W. saturnus*, *P. anomala* strain K and *Candida oleophila* (Jijakli & Lepoivre, 1998; Masih & Paul, 2002; Bar-Shimon *et al*., 2004; İzgü & Altinbay, 2004; İzgü *et al*., 2006; Friel *et al*., 2007; Wang *et al*., 2007; Xu *et al*., 2011; Guo *et al*., 2012). Three killer strains of *W. anomalus* (BCU24, BS91 and BCA15) exhibited killer activity against a *S. cerevisiae* wild type strain, while mutants deficient in β-1,6-glucan were resistant to the toxins of the strains. The exoglucanase (WaExg1) proteins of the *W. anomalus* killer strains, BCU24 and BS91, display identical amino acid sequences to each other and exhibit 99% similarity to the β-glucanase of *P. anomala* strain K, while the amino acid sequence of

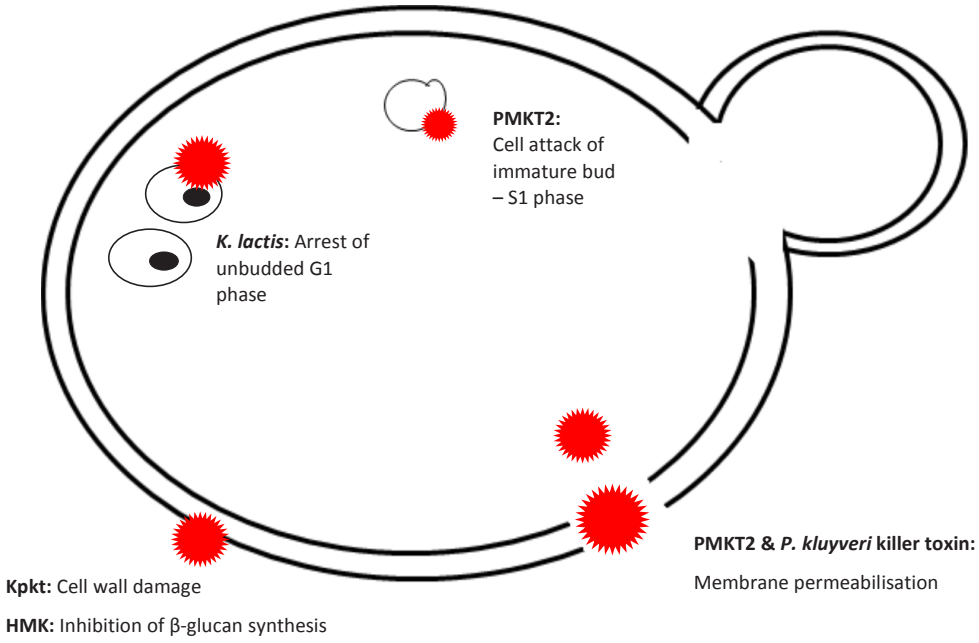

FIGURE 1

A schematic representation of the mode of action of the killer toxins of *Kluyveromyces lactis*, *Pichia kluyveri*, *Pichia membranifaciens* (PMKT2), *Tetrapisispora phaffii* (Kpkt) and *Williopsis mrakii* (HMK), as described by Middelbeek *et al*. (1980); Yamamoto *et al*. (1986); Magliani *et al*. (1997); Comitini *et al*. (2009) and Santos *et al*. (2013). ●: Killer toxin.

the strain BCA15 perfectly matches the β-glucanase of *P. anomala* strain K. Furthermore, the *WaEXG*2 sequences of the killer strains are identical to those from *P. anomala* strain K (Muccilli *et al*., 2013). The authors concluded that killer activity is probably due to β-1,6 and/or β-1,3-glucanase activity.

P. anomala strain K is an efficient and reliable antagonist of *B. cinerea* and *Penicillium expansum* in apples. The disruption of *P. anomala*'s exo-glucanase genes *PaEXG1* and *PaEXG2* resulted in reduced efficiency – 8% from 71% in the biocontrol of *B. cinerea* in apples (Friel *et al*., 2007). Growth of *B. cinerea* in the presence of *P. membranifaciens* resulted in extensive damage to the fungal cell wall, with complete rupture and fragmentation of the hyphal filaments of *B. cinerea*. *P. membranifaciens* showed increased production of both endo- and exo-β-1,3-glucanase in the presence of cell wall preparations of *B. cinerea* (Masih & Paul, 2002). In addition, in another study, higher exo-β-1,3-glucanase activity was observed in culture media with cell wall preparations of *B. cinerea* as carbon source (Jijakli & Lepoivre, 1998) compared to when glucose was the carbon source.

A similar stimulatory effect was observed with *C. oleophila*: the production of exo-β-1,3-glucanase was induced in the presence of *Penicillium digitatum*. Biocontrol in fruit using both wild type (*C. oleophila*) and exo-β-1,3-glucanase over-expressing transformants showed no difference in inhibition, as they both showed similar inhibitory effects (Bar-Shimon *et al*., 2004). N-terminal sequencing of the killer toxin of *P. anomala* NCYC 432 yielded a short sequence with 100% identity to the mature exo-β-1,3-glucanase of *P. anomala* strain K (İzgü *et al*., 2006) that is linked to the killer effect of this strain. Similarly, internal amino acid sequencing of the K5-type killer protein of *P. anomala* NCYC 434 yielded 100% identity with the exo-β-1,3-glucanase of *P. anomala* strain K (İzgü & Altinbay, 2004). All these studies clearly indicate that exo-glucanases may display some form of killer activity against other yeast species or filamentous fungi. Recently, the killer toxin Kpkt has been shown to be coded by the gene Tp*BGL*2, of which the *T. phaffii* strain without this gene lost both β-glucanase and killer activity (Oro *et al*., 2013).

Currently, killer toxins are defined as secreted proteins that exhibit antimicrobial activity towards susceptible yeasts of the same species or related species. Thus, this definition of killer toxins is based mainly on antimicrobial activity. However, it does not exclude killer toxins that may display other primary functions such as enzymatic activity. As such, the definition of killer toxins should not be focused on antimicrobial activity only. It should rather encompass other characteristics of the secreted protein(s), such as exo-glucanase activity. This would therefore increase the scope of exploitation of these secreted proteins to agents that can aid in the clarification, filtration and ageing of young wines, in addition to inhibiting spoilage microorganisms.

SUMMARY AND FUTURE PROSPECTS

For centuries, metabolites and by-products of microbial growth have been used for human benefit, and this still holds true in the 21st century. In wine, microbial contamination is a major concern despite the widespread use of commercial preservatives such as SO_2. Therefore, new preservation products or methods to prevent or control microbial contamination are actively sought. Ideally, such products or methods should not have application limitations (e.g. cause allergic reactions in consumers, alter the quality of the product) and the method(s) should be applied at minimal cost. The use of physical techniques and chemical preservatives to combat spoilage microorganisms has proven to have limited efficiency and application. This is attributed to the fact that physical techniques have been found to be detrimental to the sensorial properties of wine, and chemical preservatives inhibit or control the proliferation of contaminating microorganisms efficiently when applied in high concentrations.

The use of killer toxins has been explored under experimental conditions, and the findings from such endeavours have revealed that they can be applied as alternatives in controlling microbial spoilage. In particular, the killer toxins of non-*Saccharomyces* yeasts, which have a broader spectrum of activity, could be exploited to control spoilage yeasts. Killer toxins from the yeasts *K. wickerhamii*, *P. anomala* and *P. membranifaciens* have indeed been shown to have potential in controlling *B. bruxellensis*. However, they have not been as well characterised as those of *S. cerevisiae* and further investigations are needed to clarify their genetic origin and mode of action. Preliminary reports have shown that these are diverse and poorly understood as yet.

The binding receptors of some of the non-*Saccharomyces* killer toxins provide strong evidence in support of the hypothesis that these killer toxins are glucanases or display glucanase activity. Nevertheless, the following questions remain unanswered: are the killer toxins inherent glucanases that happen to possess antimicrobial activity towards other yeasts, and can these killer toxins be used as biopreservatives in wine and in the food and beverage industry? Although evidence exists of their biopreservative potential, these killer toxins have only been used for research purposes and will have to be approved by the OIV and/or the national regulations of exporting countries before they could be used for commercial purposes.

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