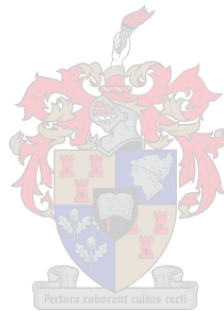


Genetic investigation and characterization of killer toxins secreted by non-*Saccharomyces* yeasts

by

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Declaration

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Summary

In the current study, two isolates showing killer activity against several wine yeast species in a previous study were identified to strain level and found to belong to the yeast species *Candida pyralidae*. The identified yeast strains and a *Kluyveromyces wickerhamii* yeast strain used as a control exhibited killer activity against *B. bruxellensis* known for its spoilage characteristics in red wine, and against several strains of the genus *Brettanomyces* on white and red grape juice medium. The killer yeasts inhibited neither the growth of *S. cerevisiae* nor that of the lactic acid bacteria *Oenococcus oeni* and *Lactobacillus plantarum* strains. Yeasts are reported to secrete killer toxins, which can play a role in yeast microbial interactions under winemaking conditions.

The *C. pyralidae* strains were found to secrete two novel killer toxins, designated CpKT1 and CpKT2. These killer toxins were stable and active under winemaking conditions, pH 3.5 - 4.5 and temperature ranges between 15 and 25°C. Ethanol and sugar concentrations found during winemaking did not affect the activity and stability of these killer toxins. Although, the killer toxins differed with regards to their biochemical and environmental stability and activity, they were found to have a similar mode of action. The killer toxins induced a fungistatic effect on *B. bruxellensis* sensitive cells in addition to binding to the cell wall of the sensitive cells, inducing cell surface and plasma membrane damage as did the Kwkt killer toxin secreted by *K. wickerhamii*. According to the author's knowledge this is the first report on the identification of novel killer toxins secreted by *C. pyralidae* strains isolated from a wine environment as well as the identification of the mode of action of killer toxins on *B. bruxellensis* cells. This indeed provides great research scope in this field.

The exoproteomes consisting of the killer toxins Kwkt, CpKT1 and CpKT2 revealed the presence of exo-glucanases and glucosidases, respectively. The enzymes KwExg1 (exo-glucanase) and KwSun4 (glucosidase) retrieved from *K. wickerhamii*'s exoproteome were identified as the potential toxins, but their killer activity could not be confirmed. These findings suggest that hydrolytic enzymes possess killer activity, as previously reported in literature. However, further investigation is needed to identify the killer toxins characterized in this study.

Opsomming

In die huidige studie is twee isolate wat in 'n vorige studie “killer” aktiwiteit teenoor verskeie wyngisspesies vertoon het, tot op rasvlak geïdentifiseer en daar is gevind dat hulle aan die gisspesie *Candida pyralidae* behoort. Die geïdentifiseerde gisrasse en 'n *Kluyveromyces wickerhamii* gisras wat as kontrole gebruik is, het “killer” aktiwiteit getoon teenoor *B. bruxellensis*, wat bekend is vir sy bederfkarakter in rooi wyn, en ook teenoor verskeie rasse van die genus *Brettanomyces* in wit en rooi druiwesapmedium. Die “killer” giste het nie die groei van óf *S. cerevisiae* óf van die melksuurbakteria *Oenococcus oeni* en *Lactobacillus plantarum*-rasse geïnhibeer nie. Giste word berig om “killer” gifstowwe uit te skei, wat 'n rol kan speel in gis mikrobiële interaksies onder wynbereidingstoestande.

Die *C. pyralidae*-rasse is gevind om twee nuwe “killer” gifstowwe af te skei, wat CpKT1 en CpKT2 genoem is. Hierdie “killer” gifstowwe was stabiel en aktief onder wynbereidingstoestande, pH 3.5 - 4.5 en temperatuur tussen 15 en 25°C. Die etanol- en suikerkonsentrasies wat onder wynbereiding voorkom, het nie die aktiwiteit en stabiliteit van hierdie “killer” gifstowwe beïnvloed nie. Hoewel die “killer” gifstowwe met betrekking tot hulle biochemiese en omgewingstabiliteit en aktiwiteit verskil het, is daar gevind dat hulle 'n eenderse modus van aksie het. Die “killer” gifstowwe het 'n fungistatiese effek op *B. bruxellensis* sensitiewe selle geïnduseer, buiten dat dit aan die selwand van die sensitiewe selle gebind het, en het seloppervlak- en plasma-membraanskade geïnduseer, net soos die Kwkt “killer” gifstof wat deur *K. wickerhamii* afgeskei is. So ver die skrywer weet, is hierdie die eerste verslag van die identifisering van nuwe “killer” gifstowwe wat deur *C. pyralidae* rasse afgeskei word wat uit 'n wynomgewing geïsoleer is, asook van die identifikasie van die modus van aksie van “killer” gifstof op *B. bruxellensis* selle. Dit verbreed dus beslis die navorsingsomvang van hierdie gebied.

Die eksoproteome, bestaande uit die “killer” gifstowwe Kwkt, CpKT1 en CpKT2, het die teenwoordigheid van ekso-glukanases en glukosidases onderskeidelik onthul. Die ensieme KwExg1 (eksoglukanase) en KwSun4 (glukosidase) wat vanuit *K. wickerhamii* se eksoproteoom herwin is, is as die potensiële gifstowwe geïdentifiseer, maar hulle “killer” aktiwiteit kon nie bevestig word nie. Hierdie bevindings suggereer dat hidrolitiese ensieme “killer” aktiwiteit besit, soos voorheen in die literatuur berig is. Verdere ondersoeke word egter benodig om die “killer” gifstowwe wat in hierdie studie gekarakteriseer is, te identifiseer.

This dissertation is dedicated to
Bucwa and Tobeka Mehlomakulu

Biographical sketch

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Preface

This dissertation is presented as a compilation of six chapters.

Chapter 1	Introduction and project aims
Chapter 2	Literature review Towards effective control of <i>Brettanomyces</i> in wine - is it achievable?
Chapter 3	Research results I Characterization of novel killer toxins secreted by wine-related non- <i>Saccharomyces</i> yeasts and their action on <i>Brettanomyces</i> spp.
Chapter 4	Research results II Investigating the exoproteome of <i>Kluyveromyces wickerhamii</i> and <i>Candida pyralidae</i> : an attempt to identify the killer toxins Kwkt, CpKT1 and CpKT2
Chapter 5	Research results III Exposure to killer toxins of <i>Kluyveromyces wickerhamii</i> and <i>Candida pyralidae</i> induces cell surface damage on <i>Brettanomyces bruxellensis</i> cells
Chapter 6	General discussion and conclusions
Addendum	Published version of the literature review Non- <i>Saccharomyces</i> killer toxins: possible biocontrol agents against <i>Brettanomyces</i> in wine?

Table of Contents

Chapter 1. Introduction and project aims	1
<hr/>	
1.1 Introduction	2
1.2 Aims and objectives of the study	4
1.3 References	5
Chapter 2. Literature review: Towards effective control of <i>Brettanomyces</i> in wine - is it achievable?	9
<hr/>	
2.1 Introduction	10
2.2 Wine microbial ecology	10
2.3 <i>Dekkera/Brettanomyces</i> spoilage in wines	12
2.4 Control of <i>Brettanomyces</i> spp. spoilage in wine	14
2.5 Microbial interactions in winemaking	17
2.5.1 Interference competition in wine	17
2.5.2 <i>Saccharomyces cerevisiae</i> killer toxins	18
2.5.2.1 <i>Saccharomyces cerevisiae</i> killer toxin activity in grape must and wine	21
2.5.3 Non- <i>Saccharomyces</i> killer toxins	23
2.5.3.1 Genetic origin of non- <i>Saccharomyces</i> killer toxins	33
2.5.3.2 Antimycotic activity and industrial application of non- <i>Saccharomyces</i> killer toxins	35
2.6 Killer toxin mode of action	36
2.6.1 Do exoglucanases possess killer activity?	38
2.7 Summary and future prospects	39
2.8 References	41
Chapter 3. Characterization of novel killer toxins secreted by wine-related non-<i>Saccharomyces</i> yeasts and their action on <i>Brettanomyces</i> spp.	50
<hr/>	
This chapter has been published in International Journal of Food Microbiology, 188 (2014), 83-91.	
Chapter 4. Investigating the exoproteome of <i>Kluyveromyces wickerhamii</i> and <i>Candida pyralidae</i>: an attempt to identify the killer toxins Kwkt, CpKT1 and CpKT2	60
<hr/>	
4.1 Introduction	61
4.2 Materials and methods	63
4.2.1 Killer toxin production	63
4.2.2 Killer activity assay on solid medium	63
4.2.3 SDS-PAGE of <i>K. wickerhamii</i> 's exoproteome	64
4.2.4 Peptide sequencing of <i>K. wickerhamii</i> 's exoproteome	65
4.2.5 Peptide sequencing of <i>C. pyralidae</i> IWBT Y1140 and IWBT Y1057 exoproteomes	65
4.2.5.1 Sample preparation	65
4.2.5.2 Sample digest	65

4.2.5.3	Desalting	66
4.2.5.4	Liquid chromatography	66
4.2.5.5	Mass spectrometry of <i>C. pyralidae</i> 's exoproteome	66
4.2.6	Amplification and cloning of <i>K. wickerhamii</i> <i>EXG1</i> and <i>SUN4</i> genes	67
4.2.6.1	<i>K. wickerhamii</i> inverse and nested PCR	69
4.2.7	Heterologous transformation and expression of the <i>KwEXG1</i> and <i>KwSUN4</i> in <i>S. cerevisiae</i>	70
4.2.8	Killer activity and extracellular enzyme screening	70
4.2.9	RNA extraction and <i>KwEXG1</i> and <i>KwSUN4</i> expression	71
4.3	Results	72
4.3.1	Mass spectrometry on <i>K. wickerhamii</i> and <i>C. pyralidae</i> exoproteome	72
4.3.2	Amplification and expression of <i>K. wickerhamii</i> 's <i>EXG1</i> and <i>SUN4</i> genes	77
4.4	Discussion and conclusion	81
4.5	References	83
Chapter 5. Exposure to killer toxins of <i>Kluyveromyces wickerhamii</i> and <i>Candida pyralidae</i> induces cell surface damage on <i>Brettanomyces bruxellensis</i> cells		86
<hr/>		
5.1	Introduction	87
5.2	Materials and methods	89
5.2.1	Killer toxin production and killer activity assay in solid medium	89
5.2.2	Killer activity assay in liquid medium	90
5.2.3	Effect of <i>Kwkt</i> , <i>CpKT1</i> and <i>CpKT2</i> killer toxins on the cell surface of sensitive cells	90
5.2.4	Competitive binding assays	91
5.3	Results	92
5.3.1	Killer activity in solid and liquid medium	92
5.3.2	Effect of <i>Kwkt</i> , <i>CpKT1</i> and <i>CpKT2</i> killer toxins on the cells surface of sensitive cells	93
5.3.3	Competitive binding assays	96
5.4	Discussion and conclusion	97
5.5	References	101
Chapter 6. General discussion and conclusion		105
<hr/>		
6.1	General discussion	106
6.2	Future prospects	109
6.3	References	111
Addendum. Non-<i>Saccharomyces</i> killer toxins: possible biocontrol agents against <i>Brettanomyces</i> in wine?		114
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CHAPTER 1

Introduction and project aims

CHAPTER 1: INTRODUCTION AND PROJECT AIMS

1.1. Introduction

Killer toxins (also termed mycocins) are antimicrobial compounds secreted by *Saccharomyces* (1) and non-*Saccharomyces* (2) yeasts that kill sensitive yeast strains or species within the same habitat or in a microbial ecosystem (3). Under winemaking conditions, killer toxin secretion by a killer positive starter culture can be regarded as advantageous in eliminating undesired or spoilage organisms (4) or disadvantageous in spontaneous fermentations when the killer positive strain inhibits the growth of the strains of interest (e.g. starter culture or an indigenous strain) that carry out the fermentation (5, 6).

Yeast species reported to secrete killer toxins include *Saccharomyces cerevisiae* and species from the non-*Saccharomyces* genera such as *Debaryomyces*, *Kluyveromyces*, *Candida*, *Pichia*, *Hanseniaspora*, *Cryptococcus*, *Zygosaccharomyces*, as well as species from the former genera of *Torulopsis* and *Hansenula* (2, 7, 8). Yeast strains can be differentiated by their killer phenotype. A yeast species can either be a killer (secretes a killer toxin), neutral (does not secrete killer toxin and is immune to toxins secreted by other yeasts), sensitive (the yeast is sensitive to killer toxins secreted by other yeast species or strains) or killer-sensitive (the yeast secretes a killer toxin and is sensitive to the killer toxins secreted by other yeasts) (5, 9).

The killer phenotype was first reported by Bevan and Makower in 1963 in laboratory and wild strains of *S. cerevisiae* (1, 8). In non-*Saccharomyces* yeasts, it was discovered by Philliskirk and Young in 1975 (2). The killer toxins secreted by the former yeast species are more extensively described and characterized in literature (1, 8, 10-15) in comparison to the killer toxins of the latter yeasts. Genetically, killer toxins can originate from dsRNA viruses, linear dsDNA plasmids or chromosomal genes (3, 10, 16-18). Killer toxins of viral origin are found within the species *S. cerevisiae*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum* and the filamentous fungus *Ustilago maydis* (10, 16, 19, 20). The genetic origin of most of the non-*Saccharomyces* killer toxins is still unknown except for those secreted by the yeasts *Kluyveromyces lactis*, *Pichia acaciae* and *Pichia inositovora* which are encoded by linear dsDNA plasmids and those of *Tetrapisispora phaffii*, *Williopsis mrakii* and *Williopsis saturnus* (formerly known as *Hansenula mrakii* and *Hansenula saturnus*, respectively) encoded by chromosomal genes (18, 21).

The mode of action of killer toxins involves interaction of the killer toxin with receptors on the cell wall or cell membrane of the sensitive yeast. The exact mechanism of killer activity varies among the different killer toxins. Killer toxins K1 and K2 secreted by *S. cerevisiae* form ion channels on the cell membrane of the sensitive yeast, where cellular metabolites such as ATP

and AMP, ions (e.g. K^+) leak out thereby resulting in a decrease in intracellular pH (13, 22-24). In contrast, the killer toxin of *Wickerhamomyces anomalus* disrupts the cell wall structure of the sensitive yeast by damaging the β -glucan scaffold (25), while that of *W. mrakii* inhibits the synthesis of β -glucan, a major cell wall component (26). The killer toxin secreted by *K. lactis* and K28 secreted by *S. cerevisiae* are more invasive. These toxins kill sensitive cells by causing cell cycle arrest at the G1 phase of the cell cycle and block DNA synthesis (10, 11). For most of the known and identified killer toxins, the mode of action especially against yeast species other than *S. cerevisiae* is yet to be investigated.

S. cerevisiae killer yeasts exhibit killer activity against yeast strains of the same species (10, 27) except for yeast strains that secrete the Klus killer toxin which have antimicrobial activity against *K. lactis*, *Candida albicans*, *Candida dubliniensis*, *Candida kefyr*, *Candida tropicalis* and *Hanseniaspora* spp. (28). Non-*Saccharomyces* yeast species generally display a broader spectrum of antimicrobial activity against both *Saccharomyces* and non-*Saccharomyces* yeast species and strains (4) found as either pathogenic or spoilage yeasts in the medical, marine, food and beverage, and agricultural environments (29, 30).

In winemaking, *Brettanomyces bruxellensis* is often described as the main spoilage yeast because of its ability to produce volatile phenols (31, 32). These compounds impart undesirable odours described as “phenolic”, “leather”, “horse sweat”, “stable”, “varnish” and a few other off-flavours in *B. bruxellensis* contaminated wine (33-35). Sulphur dioxide (SO_2) is commonly used in wine for its antioxidant and antimicrobial properties (36). However, its efficiency is dependent on the pH of the wine which influences the concentration of molecular SO_2 (i.e. the antimicrobial fraction of SO_2) and the strain of *B. bruxellensis* (37, 38). The use of a few other chemical preservatives is permitted, but their effectiveness to control *B. bruxellensis* is not always guaranteed over long periods of time as it is highly dependent on the concentration of the preservative used (35, 38).

Physical treatments such as heat, pulsed electric fields, ultrasonics and biochemical treatments such as hydrolytic enzymes and chitosan have been tested with the aim of reducing or eliminating the use of chemical preservatives. Physical treatments have been shown to be successful in controlling or eliminating *B. bruxellensis*, but their use is limited due to the fact that they can alter the quality of wine and their effect on the sensorial property of wine is yet to be fully determined (39-43). The biochemical methods tested so far also show potential although the concentration used determines the efficiency of the compound or enzyme used to eliminate *B. bruxellensis* (44, 45). Other biological agents such as killer toxins seem to be a more propitious option to tackle this problem in wine. The killer toxins HMK, Kpkt, Kwkt, Pikt, PMKT2 and KP6 have already been shown to be able to inhibit spoilage or undesired yeasts within the food and beverage industry, more specifically *B. bruxellensis* at least for the latter four killer

toxins (3, 46-50). These studies indicate the potential use and application of these killer toxins in various food and beverages prone to spoilage yeasts.

1.2. Aims and objectives of the study

The non-*Saccharomyces* killer toxins Kwkt, Pikt, PMKT2 and KP6 have been shown to be able to control the growth of *B. bruxellensis*. These killer toxins are stable and active under winemaking conditions (i.e. low pH and temperature ranges, and high ethanol concentrations). The killer activity of these toxins was shown to be stable over a period of time essential for the complete elimination of *B. bruxellensis* and at dosages that are of acceptable range (46, 48, 50, 51). Therefore, these studies have proved that killer toxins offer a good alternative to using chemical preservatives. However, the killer toxin-secreting yeasts explored so far are not of wine origin and their genetic background remains unknown. Isolation and investigation of killer toxins from yeasts of oenological origin would provide a clear view of the antagonistic microbial interaction amongst yeasts during winemaking.

A study conducted at the Institute for Wine Biotechnology (Stellenbosch University, South Africa) found indigenous wine non-*Saccharomyces* yeasts isolated from South African grape must that exhibited killer activity against the yeast species *Z. bailii*, *B. bruxellensis*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *Dekkera anomala* and *S. cerevisiae* (unpublished data). The current study was therefore conducted with the aim of investigating and characterizing these killer toxins, especially with regards to their potential in controlling *B. bruxellensis* growth.

The objectives of the study are presented as individual chapters in the dissertation and are as follows:

Objective 1

Characterization of the killer toxins secreted by two indigenous wine non-*Saccharomyces* yeasts which showed strong killer activity against *B. bruxellensis*.

Objective 2

Identification of the killer toxins which showed strong killer activity against *B. bruxellensis* by analysis and investigation of the exoproteome of the killer toxin secreting yeast species

Objective 3

Investigation of the mode of action of Kwkt and the non-*Saccharomyces* killer toxins characterized in the previous objectives on *B. bruxellensis* cells.

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CHAPTER 2

Literature review

Towards effective control of *Brettanomyces* in wine – is it achievable?

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CHAPTER 2: LITERATURE REVIEW

Towards effective control of *Brettanomyces* in wine – is it achievable?

2.1. Introduction

Brettanomyces bruxellensis is regarded as a major red wine spoilage yeast. Its growth in wine is controlled mainly through the use of sulphur dioxide (SO₂). However, the effect of SO₂ in controlling *B. bruxellensis* is not only dependent on the concentration of its molecular fraction and on the strain of *B. bruxellensis*. Under certain conditions such as high pH and the presence of SO₂ binding compounds, the effectiveness of SO₂ is limited (1-3). Several chemical treatments and physical techniques have been tested to control *B. bruxellensis* growth, and these have also proved to have limited efficiency (4). In addition, hypersensitivity to SO₂ in some wine consumers has spurred the demand for the use of non-chemical preservatives (5, 6). Alternative methods are therefore currently sought to control the growth of *B. bruxellensis*.

The presence and role of non-*Saccharomyces* yeasts in winemaking has always been acknowledged, although until recently, they have often been considered undesirable. However, in recent studies, it has been shown that selected species can be used in co-cultures with *S. cerevisiae* to promote the production of desirable metabolites (7, 8). Furthermore, some of these non-*Saccharomyces* yeast species secrete killer toxins which inhibit the growth of other yeasts (9, 10). These killer toxins exhibit a broad spectrum of activity – inhibiting species within the non-*Saccharomyces* and the *Saccharomyces* genera (11). This phenotype (i.e. the secretion of killer toxin) can thus play a pivotal role in governing yeast-yeast interactions and be exploited to control the growth of undesired microorganisms in wine (12). These killer toxins have indeed been shown to have applications in the food and beverage industry, and in the development of antimycotics (13). The purpose of this literature review is to draw up a record of the current knowledge of non-*Saccharomyces* killer toxins and to assess whether they could be successfully used for controlling *Brettanomyces* growth under winemaking conditions. In this context, the use of these killer toxins can be viewed as the equivalent of bacteriocins which are applied successfully in the dairy industry.

2.2. Wine microbial ecology

The fermentation of grape juice into wine is a complex process, in which the growth and biochemical activity of yeasts play a central role. The fermentation is mainly (but not exclusively)

driven by yeasts and these yeasts originate from (i) the microbiota of the surface of grapes and surfaces of winery equipment and environment (ii) inoculated starter cultures (14).

Winemaking is by definition a non-sterile process, and the rich composition and complexity of the grape must supports the sequential development of a large number of microorganisms. Non-*Saccharomyces* yeasts are the first dominant group and yeasts belonging to genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspota* and *Zygosaccharomyces* are mostly found during the first two or three days of fermentation (15). Non-*Saccharomyces* yeasts isolated from the winemaking environment are reported to have poor fermenting capacity and rate, low resistance to SO₂ and weak ethanol tolerance; they are thus mainly isolated at the early stages of fermentation (7). However, some non-*Saccharomyces* yeast species (e.g. *Kloeckera apiculata*, *Starmerella bombicola*, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*), *Schizosaccharomyces pombe*, *Torulaspota delbrueckii* have been found to survive until the end of fermentation when co-inoculated with *S. cerevisiae* (7, 11, 16).

During alcoholic fermentation, yeasts make a positive contribution in wine by: (i) utilising grape juice constituents e.g. sugars (ii) producing ethanol, acetaldehyde, fatty acids and amino acids that help extract flavour compounds (iii) producing enzymes that transform neutral grape compounds into flavour active compounds (iv) producing flavour active secondary metabolites such as organic acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulphur compounds and (v) through yeast autolysis, resulting in the release of nutrients and metabolites into the environment e.g. amino acids which help in the extraction of secondary flavour metabolites (14). At this stage the non-*Saccharomyces* population declines, and the highly fermentative and ethanol tolerant *Saccharomyces* species dominate and complete alcoholic fermentation (17). Lastly, lactic acid bacteria (LAB) convert grape malate to lactate through malolactic fermentation. Similar to the yeasts, lactic acid bacteria originate from grapes and the winery equipment, but occur in low numbers (less than 100 cells g⁻¹ of berries) depending on maturity and condition of the berries. The principal lactic acid bacterium found in wine is *Oenococcus oeni* (formerly known as *Leuconostoc oenos*) and is able to proliferate at the low pH values (3.2 – 3.9) commonly found in grape must (18).

Ageing of wine allows for the development of certain yeasts, lactic and acetic acid bacteria (11) and their uncontrolled growth can lead to microbial spoilage of wine. A wide variety of yeast species of the genera *Dekkera/Brettanomyces*, *Candida*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* have been found in spoiled wine. The spoilage effects often encountered include: film formation in stored wines, cloudiness or haziness, sediment and gas production in bottled wines, off-odours and

off-tastes at all stages of winemaking (19, 20). Non-*Saccharomyces* yeast species e.g. *Starmerella bombicola*, *Candida* spp., *Hanseniaspora uvarum*, *Wickerhamomyces anomalus* (former *Pichia anomala*) and *Metschnikowia pulcherrima* in axenic fermentations are reported to produce acetaldehyde, volatile acids, esters and acetoin which are considered undesirable above a certain threshold. However, when these yeasts are co-inoculated with *S. cerevisiae*, the negative metabolic activities might not be expressed or could be modified by the metabolic activity of *S. cerevisiae* (11, 21).

Brettanomyces is considered a major wine spoilage yeast due to its ability to produce intolerable odours and flavours in wine. Spoilage related to the development of lactic acid bacteria is associated with excessive volatile acidity after alcoholic and malolactic fermentation as well as haze formation, gassiness, off-odours, mousiness, bitterness and ropiness (22, 23). The acetic acid bacteria of the genus *Acetobacter* can produce large quantities of acetic acid thereby slowly turning wine into vinegar. However, *Acetobacter* spoilage can be controlled fairly easily by limiting the presence of oxygen. Nitrogen or carbon dioxide gas is used over the headspace surface of the wine to replace atmospheric air containing oxygen (18).

2.3. Dekkera/Brettanomyces spoilage in wines

The yeast *Dekkera bruxellensis* or its anamorph *Brettanomyces bruxellensis* is regarded as a major spoilage yeast owing to its ability to produce ethylphenols in red wine. For the purpose of this review, *Brettanomyces* will be used throughout in reference to the yeast species of the genus, except in studies where a specific yeast species was used. *Brettanomyces* is spread within the winery environment through the importation of contaminated wine, poor sanitation of hoses, tanks and the fruit fly where it is dispersed through passive adherence to the body surfaces of the adult fruit fly. *Brettanomyces* is also found in wooden barrels, utilizing the disaccharide cellobiose and thus contaminating wines aged in barrels (23). Red wines are particularly susceptible to *Brettanomyces* contamination due to their high pH and precursor polyphenol content. White wines lack the precursor compounds and *Brettanomyces* loses viability in white wines due to the efficacy of SO₂ at lower pH (4, 24) although some white wines have high pH.

Brettanomyces is characterized as a slow grower and is detected in low numbers in the early stages of winemaking. It is tolerant to high SO₂, high ethanol and low sugar concentrations (24, 25). It has been reported that *Brettanomyces* can enter into a viable but non-culturable (VBNC) state, thus may proliferate during fermentation. In this state, the yeast cell is characterized by reduced metabolic activity, inability to reproduce on solid media and reduced cell size (26). However, during favourable conditions, *Brettanomyces* can grow to detectable levels. The

period of time between the end of alcoholic fermentation and the beginning of malolactic fermentation (MLF) is particularly favourable. The presence of residual sugars, low nitrogen content, low molecular SO₂ concentration and the semi-aerobic conditions during ageing in wooden barrels after MLF presents sufficient nutrients for the growth of the yeast. The population of *Brettanomyces* at this stage is significant enough to produce volatile ethylphenols e.g. 4-ethylphenol and 4-ethylguaiacol (25, 27-30).

Volatile phenols are produced in wine through the catabolism of three different precursor hydroxycinnamic acids, viz.: *p*-coumaric, ferulic and caffeic acids. These precursors originate from grapes, thus are naturally present in grape juice and wine. *Brettanomyces* spp. enzymatically convert hydroxycinnamic acids to volatile phenols in wine in a two-step reaction. The precursors (i.e. *p*-coumaric, ferulic and caffeic acids) are converted by 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 vinylphenol reductase. The presence of ethylphenols is characterized by the development of unpleasant odours and tastes which deeply affect wine aroma (Table 2.1) (25).

About three decades ago, LAB were thought to contribute to the production of ethylphenols in wine. However, Chatonnet *et al.* (27) found that under winemaking conditions LAB produce ethylphenols at a concentration <10 µg L⁻¹. Spoilage yeasts such as *Pichia* spp., *Torulaspota* spp. and *Zygosaccharomyces* spp. cannot produce ethylphenols due to the inactive vinylphenol reductase enzyme and can only produce trace amounts of vinylphenols in wine (27). However in grape juice Barata *et al.* (31) found that *Pichia guilliermondii* produces 8 mg L⁻¹ and 12 mg L⁻¹ 4-ethylphenols in red and white grape juice, respectively which are significantly lower than those produced by *B. bruxellensis*. Thus, production of vinylphenols and ethylphenols in wine is mainly attributed to *Brettanomyces* spp. as both the decarboxylase and reductase enzymes are active (32, 33).

Table 2.1: Threshold detection levels of off-flavours and their sensorial impact in wine. *model wine, **red wine, ***water. From Duckitt (2012)

Product	Precursor	Product concentration in red wine ($\mu\text{g L}^{-1}$)	Odour	Odour threshold ($\mu\text{g L}^{-1}$)
4-Vinylphenol	<i>p</i> -coumaric acid	8.8 - 4.3	Phenol, Medicinal	440*/600**
4-Vinylguaiacol	Ferulic acid	0.2 – 15	Clove-like	33*/110**
4-Vinylcatechol	Caffeic acid	Unknown	Phenol	Unknown
4-Ethylphenol	4-Vinylphenol	118 – 3696	Smoky, Medicinal	30 – 60
4-Ethylguaiacol	4-Vinylguaiacol	1 – 432	Clove, Spice	20***
4-Ethylcatechol	4-Vinylcatechol	27 – 427	Medicinal	10*

The presence of volatile phenols in wine is associated with disagreeable aromas often described as “phenolic”, “leather”, “horse sweat”, “stable” or “varnish”. Levels less than 400 $\mu\text{g L}^{-1}$ are considered acceptable as they contribute favourably to the complexity of wine aroma by imparting aromatic notes of spice, leather, smoke, or game; and are appreciated by most consumers (2). The sensory threshold of 4-ethylphenol was reported to be 230 $\mu\text{g L}^{-1}$ Suárez *et al.* (4), while Loureiro and Malfeito-Ferreira (2) reported a preference threshold of 620 $\mu\text{g L}^{-1}$. According to Suárez *et al.* (4) wines with average 4-ethylphenol concentrations of 3.0, 1.74 and 0.68 mg L^{-1} are characterized as wines with high, medium and no ‘Brett character’, respectively. However, the production of volatile phenols and sensorial perception is dependent on the strain and population of *Brettanomyces* spp., the presence of volatile compound precursors and also the variety of grapes used. Furthermore, 4-ethylguaiacol affects wine aroma to a lesser extent, but is related to the ‘Brett character’ of adulterated wines and associated with descriptive expressions such as “bacon” or “smoked” at a sensorial threshold of 47 $\mu\text{g L}^{-1}$. Different strains of *Brettanomyces* spp. show differences in their production of volatile phenols (4, 34) and threshold levels differ with wine styles (Table 2.1). The production of volatile phenols in red wine can be prevented by controlling or eliminating *Brettanomyces* spp. population in grape must or wine. Thus, several strategies have been employed to control wine spoilage by *Brettanomyces* spp.

2.4. Control of *Brettanomyces* spp. spoilage in wine

To date, different methods and techniques have been assessed by many research teams with the aim of inhibiting growth of or eliminating *Brettanomyces* in wines (35). However, these methods and techniques have limited efficiency in controlling the growth of *Brettanomyces* spp. in barrels or wine. In wine, the control of *Brettanomyces* is mainly ensured by the use of SO_2 . However, reports about the effectiveness of SO_2 on *Brettanomyces* inactivation are often

contradictory. The contradiction probably arises from the lack of studies under comparable conditions and variability in strain behaviour as noted by Barata *et al.* (35). Low pH values (~3.5), SO₂ levels around 0.8 ppm of molecular SO₂ and low aging temperatures (10 – 15°C) are ordinary practices that can be used to limit *Brettanomyces* spp. activity in wines (36). Although SO₂ 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 humans (37) and can damage vegetation (38). The use of other additives such as sorbic acid, benzoic acid and DMDC (dimethyldicarbonate) has proved to have limited efficiency for application in wine. These additives are only effective in their undissociated forms. Thus, for effective use, they need to be added in high concentrations.

Benzoic acid effectively inhibits *Brettanomyces* growth in soft drinks at concentrations between 100 and 200 mg L⁻¹ and it also inhibits the action of the enzymes hydroxycinnamate decarboxylase and vinylphenol reductase at concentrations between 150 – 200 mg L⁻¹ at pH 3.6 (39). However, benzoic acid is not allowed for use in wine as it affects wine flavour. Sorbic acid is unable to inhibit *Brettanomyces* growth at the concentrations legally permitted (200 – 250 mg L⁻¹), and this yeast is tolerant to 950 mg L⁻¹ of sorbic acid at pH 3.5. Renouf *et al.* (40) found that DMDC inhibited the growth of *B. bruxellensis* at 150 mg L⁻¹, even at 400 mg L⁻¹, a concentration almost double the legal limit (21, 39, 41). For further reviews on these methods see (4, 42). Chitosan, an *N*-deacetylated derivative of chitin, was found to have a fungistatic effect against *B. bruxellensis* and *Brettanomyces intermedius* although at concentrations >3 g L⁻¹ the latter yeast species ceased to survive. *S. cerevisiae* was not affected by chitosan at all concentrations tested, instead, the addition of chitosan induced an increase in the glucose consumption rate. In mixed cultures of *S. cerevisiae* and the two *Brettanomyces* species, the latter species were sensitive to >3 g L⁻¹ of chitosan (43).

Loureiro and Malfeito (2) reported that barrel sanitation and sulphite utilisation are not enough to eliminate *Dekkera/Brettanomyces* spp. Wedral (24) concluded that, once *Brettanomyces* has contaminated a barrel, the organism cannot be removed by cleaning, shaving or other techniques, although precautions can be taken to limit its growth. However, a population of *Brettanomyces* between 10⁶ - 10² cfu mL⁻¹ was completely inactivated by the use of ozone (O₃) at 5 and 1 mg L⁻¹, respectively. At the same cell population, *S. cerevisiae*, *Z. bailii*, *H. uvarum* and *T. delbrueckii* were inactivated by O₃ concentration between 7 and 1 mg L⁻¹ in contrast to *O. oeni* which was inactivated by concentrations between 2.5 and 1 mg mL⁻¹ (44). Furthermore, a 10 minute treatment with ozonated water was more effective in winery CIP (cleaning in place) systems than peracetic acid or caustic soda cleaning agents (45) and Yap *et al.* (46) reported

that the use of high pressure ultrasound can eliminate the population of *Brettanomyces* in wine barrels.

Physical treatments such as pulsed electric fields and UV-C (ultra violet) radiation have also been pursued. The use of pulsed electric fields (PEF) reduced the population of spoilage yeasts and LAB (47), as well as *D. bruxellensis* and *Dekkera anomala* in must and wine. However, the effect of PEF on the sensorial properties of wine and evaluation of the ability of this technology in the wineries still needs to be further researched (48). 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 high UV-C dosages were applied. The use of UV-C radiation represents certain limitations as it is dependent on the initial microbial load, turbidity and colour of the liquid sample (49). Furthermore, low electric current and high-power ultrasonic technologies have also been tested. Both the technologies inhibited the proliferation of *B. bruxellensis* in wine and in oak barrel as reviewed by Zuehlke et al. (45).

The inhibition of the enzymes converting *p*-coumaric acid into 4-ethylphenol using different growth inhibiting compounds or parameters was tested by Benito *et al.* (39). Ethanol concentrations above 15%, pH between 1.75 and 2, high temperature (30 – 40°C) or low temperature (0 - 15°C) and nicostatin at 25 mg L⁻¹ were all successful in inhibiting enzymatic activity. However, these do not have oenological application (39). 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 *Brettanomyces* and *Z. bailii* resulting in growth inhibition higher than 90%. The solution resulted in half maximal Inhibitory Concentration (IC₅₀) and Minimum Inhibitory Concentration (MIC) at 115 µg mL⁻¹ and 200 µg mL⁻¹ on both yeasts. Under winemaking conditions, the growth of *Brettanomyces* was delayed at concentrations less than 300 µg mL⁻¹ and no yeast growth was detected at the end of the experiment at this concentration. This enzymatic solution resulted in minor increases in levels of total acidity and ethanol (50).

In recent studies, the use of biological antimicrobial compounds such as killer toxins (Kwkt, Pikt, and PMKT2) from the yeast species *K. wickerhamii*, *P. anomala* and *P. membranifaciens*, respectively, were shown to be successful in inhibiting *Dekkera/Brettanomyces* in wine (Table 2.3). The activity of Kwkt in grape juice was found to be comparable to that of SO₂ and the growth inhibition activity of the killer toxin was found to be dose dependent. The viable *D. bruxellensis* population was diminished by days 7 and 4 when using Kwkt at concentrations 40 mg L⁻¹ and 80 mg L⁻¹, respectively (51). Dose dependent killing activity was also observed against *D. bruxellensis* when Pikt was used in controlling the population of this yeast. A

fungistatic effect was observed when the toxin was used at 28.6 AU mL⁻¹ compared to 57.2 AU mL⁻¹, where a fungicidal effect was observed. The killing activity of these killer toxins could be maintained for 10 days (29). The use of Kwkt resulted in the reduction of ethylacetate, volatile acidity and also no 4-ethylphenol was detected in the micro-fermentation (51). The killer toxin PMKT2 resulted in death rates of 0.13 h⁻¹, 0.09 h⁻¹ and 0.11 h⁻¹ in three *B. bruxellensis* strains in grape must (52). The killer toxin KP6 of the maize fungal pathogen *Ustilago maydis* inhibited *B. bruxellensis*, resulting in mortalities of 0.10 h⁻¹ and 0.18 h⁻¹ and 100% reduction in 4-ethylphenols (53). However, these studies have only been conducted for research purposes and these killer toxins will have to be approved by the OIV and/or the national regulations of exporting countries.

2.5. Microbial interactions in winemaking

Grape juice fermentation introduces a microbial ecosystem within which yeast–yeast, yeast–filamentous fungi, yeast–bacteria and bacteria-bacteria interactions are encountered (14). Interactive associations between microorganisms such as mutualism, amensalism (also known as antagonism) and competition are amongst the most common. The occurrence of such interactions, whether or not they enhance or inhibit the growth of any particular species or strain within the ecosystem influences the fermentation profile, affecting quality and sensory attributes of the wine.

2.5.1. Interference competition in wine

In grape juice, early growth of yeasts decreases nutrients thus limiting available nutrients within the medium while simultaneously producing an array of metabolites. Some of these metabolites are detrimental to other species within the environment (14). Most non-*Saccharomyces* yeasts are known to be less tolerant to ethanol compared to *Saccharomyces* yeasts and the increase in ethanol concentration during fermentation is inhibitory to the former yeasts (7), and medium chain fatty acids (C6, C8 and C10) are inhibitory to yeasts during co-culture fermentation (54). In addition, the production of carbon dioxide, growth inhibitory peptides, enzymes and glycoproteins such as killer toxins has been reported to contribute to the yeast population dynamics during wine fermentation. Killer toxins are proteinaceous antimicrobial compounds produced by yeasts and are active against members of the same species or closely related species (55). The secretion of killer toxins inhibits the growth of the other yeast(s) present in the same habitat, thus favouring the growth of the killer toxin producing yeast and Yap *et al.* (56) termed this killer toxin secretion, “interference competition” a form of amensalism. Although interference competition may be detrimental to other organisms or the fermentation process, its potential role in eliminating undesired microorganisms cannot be disputed. These killer toxins

can thus be used in combating spoilage microbiota and appear to present an interesting solution as partial substitutes to chemical agents such as SO₂ for the preservation of wine (11).

Killer toxins secreting species are found in *Saccharomyces* yeasts and in non-*Saccharomyces* genera such as *Debaryomyces*, *Kluyveromyces*, *Candida*, *Hansenula*, *Pichia*, *Cryptococcus*, *Torulopsis*, *Hanseniaspora*, *Zygosaccharomyces* (9, 10). Three phenotypes have been identified in *S. cerevisiae*: killer, sensitive and neutral. A specific killer strain produces a toxin and is immune to it, the sensitive strain does not produce the toxin and is sensitive to the toxin produced by the killer strain, and the neutral strain neither produces nor is it sensitive to the killer toxin produced by the killer strain (12). These phenotypes have also been observed in non-*Saccharomyces* yeasts. Tredoux (57) identified a killer-sensitive phenotype, where a strain produces the toxin and is immune to it but is sensitive to toxins produced by other strains (57). This phenotype is more prominent amongst the non-*Saccharomyces* yeasts.

2.5.2. *Saccharomyces cerevisiae* killer toxins

Killer toxins were first discovered in *S. cerevisiae* strains in 1963 (58), and subsequently in six other genera (59). The killer toxins K1, K2 and K28 of *S. cerevisiae* have been thoroughly investigated in literature compared to the more recently identified Klus toxin. These killer toxins were first classified by Wickner (60) as K1 and K2 to describe the killer phenotype of laboratory and wine killer yeast strains, respectively. However, Young and Yagiu (61) re-classified killer yeasts based on interactions between strains (cross-reactivity). Ten distinct killer activity patterns against other killer yeasts were identified in both *Saccharomyces* and non-*Saccharomyces* yeasts by these authors. Three killer toxins (namely K1, K2 and K3) were found in *Saccharomyces* species. Killer toxin K1 was initially found in *S. cerevisiae* A8209B, NCYC 232, NCYC 235 strains, two hybrid strains of *S. cerevisiae* NCYC 631 and NCYC 663 and in *S. uvarum* NCYC 190 strain. K2 killer toxin was found in *S. cerevisiae* NCYC 738, NCYC 1001 and in *S. diastaticus* (reclassified as *S. cerevisiae*) NCYC 713 strains. These killer toxins were found to be killer active against other killer *Saccharomyces* species and *Torulopsis glabrata* (reclassified as *Candida glabrata*) NCYC 388. The killer toxin K3 was only found in *S. capensis* NCYC 761. However, Wingfield et al. (62) showed that K3 was actually a K2 killer toxin as it is a mutant of a K2 killer yeast strain. Pfeiffer and Radler (63) discovered another killer toxin-producing *S. cerevisiae* strain, and the killer toxin was named K28 as the killer toxin producing strain was *S. cerevisiae* strain 28.

The K1, K2 and K28 toxins are encoded by different cytoplasmically inherited satellite double stranded RNAs (dsRNAs) (M1, M2 and M28) encapsulated in virus-like particles (VLPs) (Figure 2.1) and are dependent on helper yeast viruses (L-A) for their replication and encapsidation. The M dsRNAs are responsible for either killer activity or self-immunity, and show no sequence

homology with each other. During the replication cycle of the L-A virus, a single-stranded positive-strand RNA (+ssRNA) is transcribed and extruded into the yeast-cell cytoplasm. This (+ssRNA) is translated in the cytoplasm of the killer yeast into a preprotoxin which subsequently enters the secretory pathway for processing, maturation and toxin secretion. The unprocessed toxin precursor consists of an N-terminal signal sequence, followed by the α and β subunits of the mature toxin separated from each other by a *N*-glycosylated γ -sequence. In the Golgi complex, this sequence is removed by the Kex2p, and the C terminus of the β -subunit is trimmed by Kex1p and the biologically active α/β heterodimer killer toxin covalently bound by disulphide bonds is secreted (10).

The killer toxin-secreting strains are immune to their own toxin and the mechanisms behind this immunity remain partially unidentified. In yeasts secreting the K28 toxin, the secreted mature toxin is re-internalized and transported through the secretion pathway via the retrograde transport. This re-internalized unprocessed preprotoxin encoded by the M-dsRNA killer virus complexes with the mature α/β toxin and the β -subunit is ubiquitinated and degraded by proteosomes, therefore rendering the toxin inactive against the toxin-producing host (10, 64). K1 immunity is speculated to be either conferred by the toxin precursor acting as a competitive inhibitor of the mature toxin by saturating or eliminating the plasma membrane receptor, or that the γ -component of the toxin precursor also functions as a protector of the host against damage by the hydrophobic α - or that the toxin receptor (Kre1p) interacts with the K1 protoxin during secretion leading to diversion of the receptor-protoxin complex to the vacuole. However, these have not explicitly revealed the mechanism of immunity of this killer toxin. Thus, the immunity of killer yeast cells secreting the K1 toxin remains obscure (10) and for yeasts secreting the K2 toxin no studies have been conducted as yet.

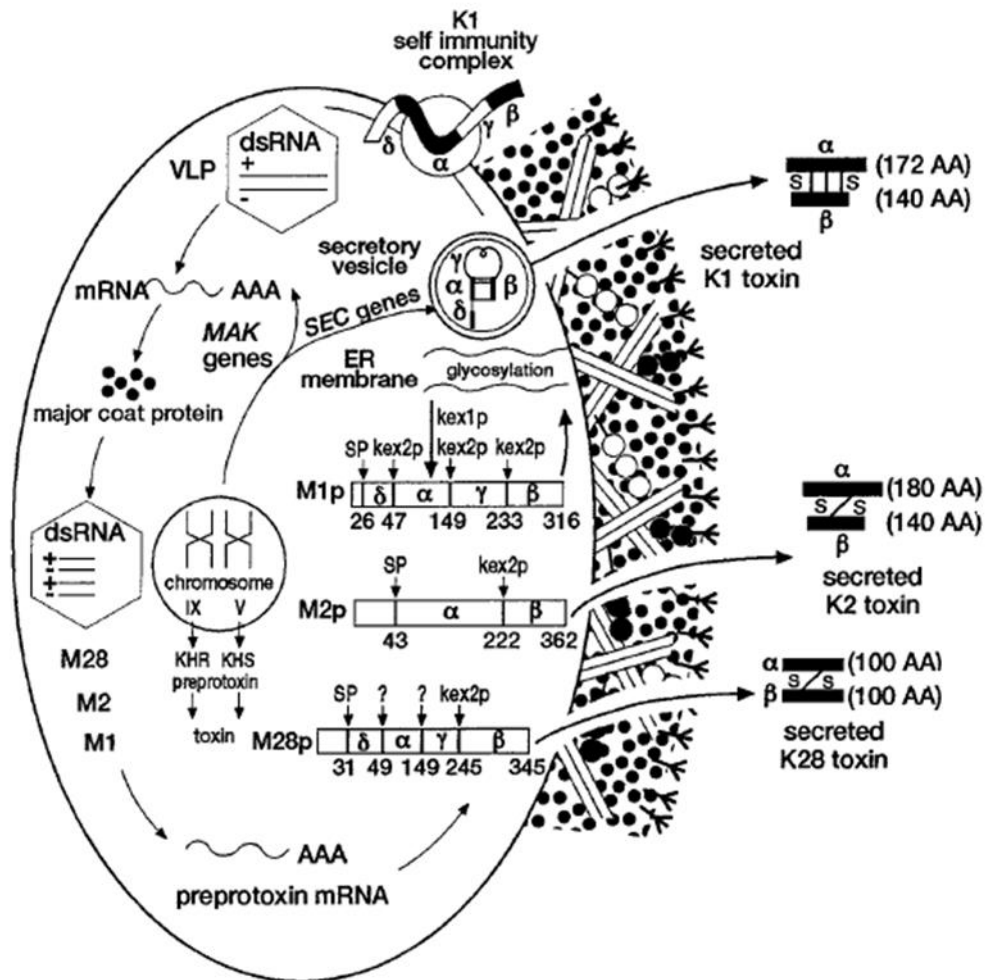


Figure 2.1: *Saccharomyces cerevisiae* K1, K2 and K28 killer system (from Magliani (65))

Until recently, K1, K2 and K28 (Table 2.2) were the only known and studied killer toxins within the *Saccharomyces* yeast genera. However, a new group classified as Klusitaneae (Klus) has been identified as a new *S. cerevisiae* killer toxin (66). The Klus killer toxin is conferred by a medium-size dsRNA virus, *S. cerevisiae* virus Mlus (ScV-Mlus), whose genome size ranges from 2.1 to 2.3 kb. *S. cerevisiae* virus Mlus depends on ScV-L-A for stable maintenance and replication. Its genome structure is similar to that of M1, M2, or M28 dsRNA, with a 5'-terminal coding region and a 3'-terminal region without coding capacity. The open reading frame (ORF) at the 5' portion codes for a putative preprotoxin with an N-terminal secretion signal, potential Kex2p/Kex1p processing sites, and N-glycosylation sites. However, no sequence homology exists between the Mlus dsRNA and M1, M2 or M28 dsRNA or between Klus and the K1, K2 or K28 toxin (66).

Table 2.2: Genetic nature and biochemical characteristics of *Saccharomyces cerevisiae* killer toxins

Killer toxin	Mature toxin	Molecular size	Mode of action	pH and Temperature activity and stability	References
K1	α/β heterodimer	21 kDa	Ion channels on the plasma membrane	Optimum pH activity and stability (4.2 - 4.6) Optimum pH 4.6 – 4.8 for toxin production Temperature: inactivated at 30°C	(67-69)
K2	α/β heterodimer	38.7 kDa	Perturbs the cytosolic membrane	pH activity: 2.9 – 4.9 (opt. 4.2 – 4.4) pH stability: 2.8 – 4.8 Temperature: Inactivated at 30°C	(10, 68-70)
K28	α -subunit	10.8 kDa	Blocks DNA synthesis	Optimum pH: 5.0	(9, 10)
Klus	Unknown	2.1 – 2.3 kDa	Unknown	pH activity: 4 -4.7 Temperature activity 28 and 30°C against <i>Candida tropicalis</i> and <i>S. cerevisiae</i> K2 strains	(66)

2.5.2.1. *Saccharomyces cerevisiae* killer toxin activity in grape must and wine

The conversion of grape must into wine is mainly attributed to the activity of *S. cerevisiae* due to its high fermentation rate and power compared to the non-*Saccharomyces* yeasts. However, killer toxin-producing strains of *S. cerevisiae* can dominate fermentation and delay its onset and cause sluggish or “stuck” fermentations by inhibiting the growth of sensitive yeasts conducting the fermentation. Wines produced from such fermentations are characterized by having an unfavourable organoleptic profile, high volatile acidity, H₂S and off-flavours caused by fusel oils, acetaldehyde and lactate, and have reduced ethanol yields. However, various other factors may cause sluggish or stuck fermentations e.g. oxygen levels, nitrogen deficiency, vitamin (thiamine, biotin and pantothenic acid) deficiency, high initial sugar content, high ethanol concentration, pH, fermentation temperature, excessive must clarification, grape solids, grape varieties, growth conditions in vineyards, pesticides, the wine yeast strain and inhibition of yeast cell activity by fermentation by-products e.g. medium chain fatty acids (C6:0, C8:0 and C10:0) and acetic acid (70, 71). The effect of these factors individually can alter the fermentation profile. However, a synergistic effect is thought to exist among these factors in sluggish or stuck fermentations.

In addition, the ratio of killer:sensitive cells might also be attributed to the killer activity of the killer yeast during fermentation conditions (12). However, there are contradictory findings with regards to the killer:sensitive cells ratio that can have an effect on killer activity during fermentation. It has been proven that killer toxins are continuously secreted in the absence of

the sensitive yeast (68, 69, 72-74). The effect of killer toxins has also been studied in mixed culture fermentations. Killer:sensitive ratios of 1:1, 1:10 and 1:100 resulted in complete elimination of the sensitive cells within 24 h, 5 and 75% viable sensitive cell population at the end of fermentation was recorded for the 1:10 and 1:100 ratios respectively, but this viable cell population did not finish the fermentation (75). Heard and Fleet (74) reported a rapid disappearance of sensitive strains and dominance of killer strains in mixed culture fermentations. Under different inoculum conditions, high residual sugar was obtained when a 1:100 killer:sensitive ratio was used and a population of 82% dead cells was observed. Stuck fermentations of the 1:100 killer:sensitive ratio fermentations were avoided by addition of ammonium sulphate and Roviferm¹. The addition of these nitrogen sources resulted in a residual sugar concentration between 1.0 - 1.7 g L⁻¹ at the end of fermentation. The same residual sugar concentrations could be observed when bentonite and activated carbon was added to similar fermentations. This led the authors to suggest that dead cells may not release nutrients into the medium and that nutrient depletion may not permit killer cells to finish fermentation (75).

Under batch cultivation conditions in a mixed culture of killer and sensitive *S. cerevisiae* cells it was shown that there was a decrease in the viable biomass population when the killer cells accounted for 10% of the population. In addition, in axenic cultures the killer culture was higher than that of the sensitive culture from 10 h of fermentation until the end of fermentation (23 h) where it reached $>1 \times 10^8$ cells mL⁻¹. The mixed culture (sensitive cells + 5% killer cells) had similar growth kinetics as the sensitive axenic culture. The mixed culture (sensitive + 10% killer cells) had a decreased biomass population. The viable biomass decrease was 52% and 80% for the mixed cultures (sensitive + 10% killer cells) and (sensitive + 5% killer cells) respectively. Metabolic kinetics on glucose and ethanol showed weaker performances in the mixed culture with 10% killer cells. Ethanol production was 2.12, 1.63 and 1.44 g L⁻¹h⁻¹ for the sensitive axenic culture, mixed cultures with 5% and 10% killer cells respectively from a viable population of 60×10^6 viable cells mL⁻¹.

In continuous cultures, the inoculation of killer cells disturbed the population of the sensitive cells. Killer cells inoculated at 4% totally eliminated the population of sensitive cells by 100 h and this observation was not attributed to glucose consumption or ethanol inhibiting the growth of the sensitive cells (76). Furthermore, other killer:sensitive ratios of 1:50, 25:1 and 100:1 resulted in killer activity (12). These findings show that killer toxins produced within mixed cultures can affect the population dynamics and subsequently the fermentation. Thus, precautionary measures need to be taken when mixed cultures are to be carried out with killer

¹ Roviferm – nitrogen source with vitamins from Roche (63)

S. cerevisiae cells. Mixed cultures of *S. cerevisiae* and *H. guilliermondii* or with *H. uvarum*, resulted in the death of both the non-*Saccharomyces* yeasts at the beginning stages of fermentation and this was not observed in axenic cultures of these yeasts. The authors demonstrated that this early death was not caused by nutrient limitation or high ethanol concentration in the culture medium and therefore suggested that it was due to toxic compound(s) secreted by *S. cerevisiae* (77). Recently, Branco et al. (78) found that *S. cerevisiae* secreted antimicrobial peptides, that were active in inhibiting the growth of *T. delbrueckii* and had a fungicidal effect on *H. guilliermondii* and *D. bruxellensis* after 14 h and 96 h respectively.

S. cerevisiae killer toxins in wine are reported to display narrow killer activity, only active against sensitive *Saccharomyces* yeasts. Indeed, Heard and Fleet (74) reported that killer strains of *S. cerevisiae* did not affect the growth of *K. apiculata*, *Candida krusei*, *Candida pulcherrima* and *H. anomala* in mixed cultures in grape juice. However, the Klus killer toxin is active against several non-*Saccharomyces* yeasts such as *Hanseniaspora* spp., *Kluyveromyces lactis*, *Candida albicans*, *Candida dubliniensis*, *Candida kefir* and *C. tropicalis* and K1, K2 and K28 killer strains of *S. cerevisiae*, thus exhibiting a broader anti-yeast spectrum compared to K1, K2 and K28 killer strains.

2.5.3. Non-*Saccharomyces* killer toxins

As mentioned above, non-*Saccharomyces* yeasts exhibiting killer activity were first reported by Philliskirk and Young (59) in six yeast genera – *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Candida* and *Torulopsis*. Young and Yagiu (61) identified the killer toxins K4 (*Torulopsis glabrata* NCYC 388), K5 (*Debaryomyces vanriji* NCYC 577, *Hansenula anomala* NCYC 434, *Hansenula subpelliculosa* NCYC 16), K6 (*Kluyveromyces fragilis* NCYC 587), K7 (*Candida valida* NCYC 327 and *Pichia membranifaciens* NCYC 333), K8 (*Hansenula anomala* NCYC 435), K9 (*Hansenula mrakii* NCYC 500) and K10 (*Kluyveromyces drosophilum* NCYC 575) based on cross-reactivity with each of the killer strains. One year later, Wickner et al. (79) reported *Torulopsis glabrata* ATCC15126 to possess the killer toxin K11. These killer toxins have found application in the food and fermentation industry, bio-typing of medically important pathogenic yeast and yeast-like fungi, development of novel antimycotics for the treatment of human and animal fungal infections, and in recombinant DNA technology (67). Table 2.3 summarizes the genetic, enzymatic and biochemical characteristics of non-*Saccharomyces* killer toxins that have been characterized and have the potential to be used in controlling undesired microorganisms in the food, beverage, wood decay and medical industry. It also highlights the proposed application of these killer toxins.

Table 2.3: Genetic origin, biochemical and biological characteristics of killer toxins secreted by non-*Saccharomyces* yeasts (adapted from Marquina, Liu et al (13, 80))

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Candida</i> (former <i>Torulopsis glabrata</i>)	Unknown	Chromosomal	pH activity: 4 pH stability: pH 3 – 7	Plasma membrane damage – leakage of cellular potassium, partial dissipation of ATP in <i>S. cerevisiae</i>	<i>S. cerevisiae</i>	Not determined	(81)
<i>Debaryomyces hansenii</i>	23 kDa	Chromosomal	pH activity /stability: acidic pH range <4.8 Temperature activity/stability : <15°C Killer activity increases in presence of (0 – 1 M NaCl)	Receptor: β -(1-6)-glucan	<i>Candida boidinii</i> , <i>D. hansenii</i> , <i>Candida parapsilosis</i> , <i>Hansenula subpelliculosa</i> , <i>Torulopsis candida</i> , <i>Saccharomyces exiguus</i> (reclassified as <i>Kazachstania exigua</i>), and <i>S. cerevisiae</i>	Olive brine fermentation	(72, 82, 83)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Kluyveromyces lactis</i>	Zymocin (157kDa)	dsDNA linear plasmids: pGKL1(k1) & pGKL2 (k2)	pH activity: pH 4.4 – 5.8 Temperature stability: 40°C	Receptor: chitin Permanent arrest of the G1 cell cycle phase in <i>S. cerevisiae</i> cells resulting in loss of viability	<i>Candida</i> , <i>Kluyveromyces</i> , <i>Saccharomyces</i> , <i>Torulopsis</i> and <i>Zygosaccharomyces</i>	Not determined	(65, 84-86)
<i>Kluyveromyces wickerhamii</i>	Kwkt (72kDa)	Unknown	pH activity: 3.8 – 4.6 (opt. pH 4.4) Optimal temperature activity: 20°C (max. 25°C)	Receptor: pustulans (β-1,6-glucans)	<i>D. bruxellensis</i>	In winemaking	(11, 29, 51)
<i>Tetrapisispora phaffii</i>	Kpkt (33 kDa)	<i>BGL2</i>	pH activity: 3 – 5 Temperature activity: <40°C	Disruption of cell wall integrity Displays properties of β-glucanase enzyme	<i>H. uvarum</i>	In winemaking	(29, 38, 87, 88)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Hansenula mrakii</i> (re-classified <i>Williopsis mrakii</i>)	HMK or HM-1 (10.7 kDa)	Chromosomal gene <i>hmk</i>	pH stability: 2 - 11 Thermostable – biological active after incubation at 100°C, 10 min	Receptor: β -D-1,3 and β -D-1,6-glucan Inhibits β -glucan synthesis	<i>Heterobasidium</i> , <i>Postia</i> , <i>Serpula</i> , <i>Fusarium</i> and/or <i>Colletotrichum</i>),	In silage and yoghurt	(55, 67, 82, 89, 90)
<i>Williopsis mrakii</i> NCYC 500	K-500 (1.8 – 5.0 kDa)	Chromosomal	pH stability: 2.4 – 4.0 Temperature activity: 30°C	Possible membrane permeability	<i>Candida albicans</i> and <i>Sporothrix schenkii</i>	Antifungal agent	(91)
<i>Pichia acaciae</i> (reclassified as <i>Millerozyma acaciae</i>)	PaT (187 kDa: three subunits of 110, 39 and 38 kDa)	Linear dsDNA plasmid (pPac1 – 1: 13.6 kb) and pPac1 – 2: 6.8 kb)	pH optimum activity: 7 – 7.5 and 5.3 – 6.6 (against <i>S. cerevisiae</i> and <i>Debaryomyces tamari</i> , respectively)	Receptor: chitin Cell cycle arrest in G1 phase in <i>S. cerevisiae</i> cells, Displays chitinase activity	<i>S. cerevisiae</i>	Not determined	(86, 92)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Hanseniaspora uvarum</i>	18 kDa	dsRNA virus (4.6 kb Helper virus and 1.0 kb Killer virus)	pH activity: 3.7 – 3.9	Receptor: β -(1-6)-glucan	<i>Heterobasidium</i> , <i>Postia</i> , <i>Serpula</i> , <i>Fusarium</i> , <i>C. albicans</i> and <i>Sporothrix schenkii</i>	Not determined	(67, 82, 93)
<i>Pichia anomala</i> NCYC 432	47 kDa	Unknown	pH activity: 2.5 – 5 (optimum activity at pH 4.5) Temperature activity: 4 - 37°C	Receptor: β -1,3-glucans, Exhibits $\text{exo-}\beta$ -1,3-glucanase activity	<i>Candida</i> spp.	Not determined	(94)
<i>Pichia anomala</i> NCYC 434	K5 Panomycin (49 kDa)	- Unknown	pH stability: 3 – 5.5 Temperature stability: up to 37°C	Receptor: β -1,3-glucan; Exhibits $\text{exo-}\beta$ -1,3-glucanase activity	<i>Microsporium</i> spp., <i>Trichophyton</i> spp., <i>C. albicans</i> , <i>Torulaspora delbrueckii</i> and <i>Kluyveromyces marxianus</i>	Not determined	(95, 96)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Pichia anomala</i> DBVPG 3003	Pikt (8 kDa)	Unknown	pH activity: 4.4 Temperature activity: 25 – 35°C	Receptor: β -1,6- glucans	<i>D. bruxellensis</i>	In winemaking	(19, 29)
<i>Pichia farinosa</i> (reclassified as <i>Pichia membranifaciens</i>)	SMKT (25 kDa)	Chromosomal gene (<i>SMK1</i>) α (6.6 kDa) β (7.9 kDa)	pH stability: 2.5 – 4.0 Temperature stability: 100 - 50% activity between 5 and 30°C Maximum killer activity in 2 M NaCl	Disruption of the ion transport	<i>S. cerevisiae</i> and <i>Z. rouxii</i>	Not determined	(97, 98)
<i>Pichia inositovora</i> (reclassified as <i>Babjeviella inositovora</i>)	Unknown	Linear dsDNA plasmids (pPin 1–1: 18 kb) and (pPin 1–3: 10 kb)	Not determined	Receptor: Chitin	<i>S. cerevisiae</i>	Not determined	(99)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Pichia kluyveri</i>	19 kDa	Chromosomal	pH stability: 2.5 - 4.7 Temperature stability: 20 – 40°C	Leakage of K ⁺ ions, ATP, decrease in intracellular pH and inhibition uptake of amino acids	<i>Candida</i> , <i>Saccharomyces</i> and <i>Torulopsis</i>	Not determined	(93, 100, 101)
<i>Pichia membranifaciens</i> CYC 1106	PMKT (18 kDa)	Unknown	pH activity and stability: 3.0 – 4.8 Temperature activity and stability: 5 – 20°C and 5 – 25°C	Receptor: β-1,6-glucans	<i>Botrytis cinerea</i> , <i>Candida boidinii</i>	In grape vine	(102, 103)
<i>Pichia membranifaciens</i> NCYC 1086	PMKT2 (30 kDa)	Unknown	pH activity: 2 – 5 (opt. 3.5 – 4.5) pH stability: 2.5 – 4.8 Temperature activity: 5 - 20°C Temperature stability: 20 - 32°C	Receptor: mannoproteins	<i>B. bruxellensis</i>	In winemaking	(52)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive Target yeasts	or Application or potential application	References
<i>Schwanniomyces occidentalis</i>	Two subunits (7.4 and 4.9 kDa)	Chromosomal	pH activity: 3.8 - 5.0 (optimum activity 4.2 - 4.8) pH stability: 2.0 - 5.0 Temperature stability: 20 - 30°C	Receptor: Mannoproteins Plasma membrane damage	<i>Saccharomyces</i> yeasts	Undetermined	(104)
<i>Wickerhamomyces anomalus</i> (former <i>Pichia anomala</i>) YF07b	47 kDa	Unknown	pH activity: 2.5 – 4.0 pH stability: 3.0 – 5.0 Optimum temperature activity: 40°C Temperature stability: 20 - 60°C	Exhibits β -1,3- glucanase activity	<i>Metschnikowia bicuspidate</i>	Undetermined	(105, 106)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Wickerhamomyces anomalus</i> (<i>Pichia anomala</i>) YF07	67.0 kDa	Unknown	pH activity and stability: 3 - 5 (opt. pH activity 3.5) Temperature activity: 10 – 25°C (opt. at 16°C) Temperature stability: 10 – 40°C	Cytoplasmic membrane permeabilization	<i>Yarrowia lipolytica</i> , <i>S. cerevisiae</i> , <i>Metschnikowia bicuspidate</i> , <i>C. tropicalis</i> , <i>C. albicans</i> and <i>Kluyveromyces aestuarii</i>	Not determined	(105)
<i>Williopsis saturnus</i> (former <i>Hansenula saturnus</i>)	HSK (8.5 - 9.5) or HYI (8.5 kDa)	Chromosomal: encoded by <i>hsk</i> gene	pH stability: 3 – 11 Temperature stability: 5 – 60°C	Not determined	<i>Hansenula</i> , <i>Saccharomyces</i> and <i>Candida</i>	Not determined	(107, 108)
<i>Williopsis saturnus</i> var. <i>mrakii</i> MUCL 41968	WmKT (85 kDa)	Nuclear gene	pH activity: 4.6 Temperature activity: 25 – 28°C	Cell permeation	<i>C. albicans</i> , <i>P. anomala</i> , <i>Pneumocytis carinii</i> and <i>S. cerevisiae</i>	Treatment of human and animal infections	(109)

Table 2.3 (continued)

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor or Mode of action	Sensitive or Target yeasts	Application or potential application	References
<i>Zygosaccharomyces bailii</i>	Zygotin (10kDa)	dsRNA virus	Not determined	Receptor: Mannoproteins	<i>C. albicans</i> , <i>C. glabrata</i> , <i>C. tropicalis</i>	Undetermined	(82, 110)
<i>Ustilago maydis</i>	KP6	dsRNA virus (α - 8.6 kDa and β - 9.1 kDa)	pH activity: 3.0 - 5.5 (opt. 3.0 – 4.5) pH stability: 2.5 – 5.3 Temperature activity: 15 – 30°C (opt. 15 - 20°C) Temperature stability: 5 - 20°C	Undetermined	<i>B. bruxellensis</i>	In winemaking	(53)

2.5.3.1. Genetic origin of non-*Saccharomyces* killer toxins

Unlike the killer toxins of *S. cerevisiae*, the genetic origin of non-*Saccharomyces* killer toxins is not always viral; instead they are encoded on either linear dsDNA plasmids or chromosomes with the notable exception of those of *H. uvarum*, *Z. bailii* and the filamentous fungi *U. maydis* (Table 2.3). However, the genetic origin of the most recently identified and partially characterized non-*Saccharomyces* killer toxins remains unknown. This is mainly attributed to the null or poor annotation of the known non-*Saccharomyces* yeast genomes or the complete lack of genetic data. A draft genome sequence of *Wickerhamomyces anomalus* (former *Pichia anomala*) DSM 6766 has recently been annotated (111). This draft sequence could thus be used to identify the other killer toxins within the genus, provided the killer toxins are chromosomally encoded.

Within the genus *Kluyveromyces*, the *K. lactis* killer toxin is encoded by two linear dsDNA plasmids pGKL1 (k1 = 8.8 kb) & pGKL2 (k2 = 13.4 kb) (Table 2.3) (84). Plasmid pGKL1(k1) encodes precursors of the killer toxin subunits and the immunity phenotype, whilst pGKL2 (k2) is indispensable for the replication and stable maintenance of both plasmids (65, 85). The smaller plasmid, pGKL1 has four ORFs. ORFs 2 and 4 encode the precursors of the subunits of the killer toxin. ORF 3 is involved in the immunity phenotype and ORF 1 encodes for a DNA polymerase. The killer toxins of *P. inositovora* and *P. acaciae* are also encoded by linear dsDNA plasmids (pPin1-1 and pPin1-3, and pPac1-1 and pPac1-2, respectively) (Table 2.3). In comparison to the genetic origin of *K. lactis* killer toxin, the smaller plasmid pPin1-3 of *P. inositovora* also has four ORFs. ORFs 2 and 3 display similarities to the genes encoding DNA polymerase and precursors of the α - and β -subunits of *K. lactis* zymocin. The ORFs 1 and 4 exhibit low similarity with the pGKL1 ORF 3 and 4 of *K. lactis*. ORF 1 is homologous to the immunity gene of *K. lactis*, and the protein encoded by ORF 1 displays 34% identity to the pGKL1 protein encoded by ORF 3. This plasmid, pPin1-3; can only co-exist with either pPin1-1 or with pPin1-1 with an additional plasmid pPin1-2. The additional plasmid pPin1-2, does not confer immunity, as a partially cured strain lacking this plasmid was killer sensitive. The *P. inositovora* plasmids do not harbour genes that code for immunity as cured plasmid-free strains of the yeast did not show any sensitivity to the toxin (99). However, the plasmid pPac1-2 of *P. acaciae* resembles the pGKL1 of *K. lactis* in function, as a partially cured strain of *P. acaciae* missing only the pPac1-2 plasmid exhibited loss of both toxin activity and immunity (92). It has been confirmed that the pPac1-2 ORF4p of *P. acaciae* is responsible for immunity and that the immunity may occur via toxin re-internalization (86).

The three dsDNA plasmids (pDHL1 (8.4 kb), pDHL2 (9.2 kb) and pDHL3 (15.0 kb) isolated in *D. hansenii* do not encode killer toxins as irrespective of the presence or absence of the plasmids the killer toxin is secreted. The plasmid pDHL1, carries a DNA-fragment encoding a protein that has some similarity with the α - subunit of *K. lactis* killer toxin. The authors concluded that the killer toxin was chromosomally encoded (83). Similar findings were reported by Chen (104) as the authors did not isolate any dsRNA plasmids and the two dsDNA plasmids (pSoc1-1 and pSoc1-2) isolated do not encode killer toxins or immunity factors. The isolates which have lost these plasmids retain the killer activity and immunity function.

Curing treatments aimed at removing genetic elements and plasmids by exposing cells to 5-fluorouracil, cycloheximide, ultraviolet irradiation and growth at elevated temperature have been carried out on killer strains not exhibiting dsRNA or dsDNA genetic material. Curing of the killer strains of *P. farinosa*, *P. kluyveri*, *T. glabrata* and *H. saturnus* did not cure the strains of killer activity and thus the results indicated that the killer toxins are encoded by chromosomal genes (81, 93, 97, 108). However, *H. uvarum* killer strains when cured by incubation with cycloheximide, the strains lost killer activity but retained the dsRNA plasmids compared to the *S. cerevisiae* killer strains which lost killer activity and the M-dsRNA. These strains retained their killer activity when grown at 37°C, but lost both killer activity and dsRNA plasmids when cured with 5-fluorouracil (93).

The killer toxins of the *W. mrakii* (HM-1) and *W. saturnus* (HYI) are both encoded by chromosomal genes (*HMK* and *HSK*) and are reported to show structural similarities. The genes encode precursors to killer toxins of 125 and 124 amino acids respectively, and the N-terminal has a 37 amino acid signal sequence. The mature toxin (HM-1 and HYI) is composed of 88 and 87 amino acid residues respectively. The two genes encoding the toxins show 82% homology and the amino acid sequences of the mature toxin show 86% amino acid homology. Furthermore, it was found that the sequences of the killer strains of *W. mrakii*, *Williopsis saturnus* var. *saturnus* and *Williopsis saturnus* var. *subsufficiens* are all homologous to *HMK* and *HSK* genes but were not identical. The secreted toxins were also found to be structurally similar based on the polypeptide molecular masses (112). A similarity was also found with the *SMK* gene encoding the killer toxin SMKT of *P. farinosa*, this gene has a 222-amino acid preprotoxin which resembles that of *S. cerevisiae* K1 toxin (97).

The killer toxins of *Z. bailii* and *H. uvarum* originate from dsRNA mycoviruses, ZbV and HuV respectively and these possess cytoplasmic L (4.5 kb) and M (1.8 kb) dsRNAs (113). *Z. bailii* also possesses an additional Z-dsRNA (2.8 kb). The structure and intracellular replication cycle of these mycoviruses is analogous to the *S. cerevisiae* mycoviruses ScV-L-A and Sc-M (67), but the M-dsRNA of *Z. bailii* does not share any homology to M1, M2 and M28 of *S. cerevisiae*.

H. uvarum and *Z. bailii* cycloheximide cured derivatives showed that the M-dsRNAs of both yeasts encode the killer phenotype. While cured clones of *Z. bailii* retained the L + Z-dsRNA, and remained fully immune to the wild-type toxin. The immunity function is thought to be dependent on the additional Z-dsRNA (113). The fungal pathogen *Ustilago maydis* also secretes antifungal killer toxins which originate from a dsRNA virus that infects the fungus. The killer toxins secreted by the fungus are KP1, KP4 and KP6 (67). These toxins are encoded on specific medium sized (M) segments of the *U. maydis* virus (UmV) P1, P4 and P6 (114). The KP4 and KP6 toxins are the mostly studied of the three. KP6 is reported not to be homologous to any known protein and KP4 has possible homologs in pathogenic fungi (115). The KP6 killer toxin has two non-glycosylated polypeptides: α (78 amino acids; 8.6 kDa) and β (81 amino acids; 9.1 kDa) synthesized from a preprotoxin (219 amino acids; 24.1 kDa) encoded on the M2 dsRNA and are separated from each other by an inter-region. The polypeptides are processed intracellularly through cleavage by Kex2-like and protease-processing events to secrete the mature killer toxin. The two polypeptides, which make-up the mature toxin, interact with the susceptible cell independently as monomers in a sequential manner. The α -subunit initiates the interaction followed by the β -subunit, which only exerts its effect on cells that have been exposed to the α -subunit (65, 114).

2.5.3.2. Antimycotic activity and industrial application of non-*Saccharomyces* killer toxins

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 (11). These killer toxins exhibit broad anti-yeast spectra compared to those of *Saccharomyces* (11, 116). Within the *Kluyveromyces* genus, the species *K. lactis*, *K. fragilis*, *K. wickerhamii* and *T. phaffii* possess killer activity. *K. fragilis* secretes a 42.3 kDa killer toxin (K6) (61, 96) that is active against *S. cerevisiae* strains and *T. glabrata* (73). Transformation of *K. fragilis* with *K. lactis* plasmids pGKL1 and pGKL2 resulted in strains secreting 17-fold more of the killer toxin than the parent *K. lactis* strain (117). The native *K. lactis* toxin is active towards sensitive and killer strains of *S. cerevisiae*, as well as other strains of *Kluyveromyces* (84). The species *T. phaffii* and *K. wickerhamii* possess killer activity against apiculate yeasts and yeasts of the *Brettanomyces/Dekkera* genus, respectively. The toxins of *T. phaffii* and *K. wickerhamii* are stable and active at pH 3.0 – 5.8, and temperatures between 20 and 25°C (Table 2.3). The killer toxin Kwkt of *K. wickerhamii*, when used against the spoilage yeast *D. bruxellensis* controlled the growth of *D. bruxellensis* during the initial fermentation days and after 4 days *D. bruxellensis* ceased to survive. The toxins' effectiveness in reducing the population of *D. bruxellensis* was further confirmed by the decrease in the

metabolic activity of *D. bruxellensis* as evidenced by the decline in the production of volatile acidity and ethyl phenols. The growth of the apiculate yeast *H. uvarum* was decreased and subsequently ceased in the presence of immobilised cells of the *T. phaffii* (38). The fermentation profile of both the trial fermentations using these killer toxins was unaffected and so was the population of *S. cerevisiae* (38, 51). Thus the researchers (29, 38, 51, 118) have shown the applicability of these toxins in winemaking as biocontrol agents in the control of spoilage yeasts.

The genus *Pichia* consists of at least 91 yeast species, which are widely distributed in natural habitats (e.g. soil, water, plant exudates, insects, fruits and vegetables) and as contaminants in food and beverage products (119). Some of the species of this genus have been shown to produce killer toxins (Table 2.3) (52). Within the spore-forming ascomycetous yeast species *P. anomala* (reclassified as *Wickerhamomyces anomalus*), many strains have been shown to have killer activity or growth inhibitory activity phenotype against a wide spectrum of organisms e.g. filamentous fungi (*Aspergillus*, *Penicillium*, *Fusarium* and *Botrytis cinerea*), bacteria (*Erwinia*, *Enterobacteriaceae* and streptococci) and against yeasts (*S. cerevisiae*, *C. albicans*, *Brettanomyces*, *Zygosaccharomyces rouxii*). The killer phenotype of *P. anomala* can be attributed to killer toxins and the growth inhibition to the biosynthesis of volatile compounds i.e. ethyl acetate, isoamyl acetate and ethyl propionate (111). The killer toxins (K5 and K8) of the genus *Pichia* were first identified in *Hansenula anomala* strains NCYC 434 and 435 in 1978 by (61). The killer toxins secreted by species of the genus are stable and active at pH range 2.5 – 7.5, and temperature <40°C (Table 2.3). The killer toxins of *P. membranifaciens* have also proved to be successful in controlling *B. cinerea* and *Brettanomyces* (52, 103).

The killer toxins of *D. hansenii*, *W. saturnus* var. *saturnus* and *H. mrakii*, have found application in inhibiting undesired yeasts in olive brine fermentation, cheese, silage and yoghurt (55, 82, 120). Other than inhibiting food spoilage organisms, killer toxins have been found to be active in inhibiting wood decaying basidiomycetes and phytopathogenic fungi, tremallaceous yeasts, ascomycetous and basidiomycetous yeasts (67, 121, 122) as well as inhibiting pathogenic yeast species in marine environments (105, 106). Furthermore, killer toxins have been used as antifungal agents against human pathogens such as *C. albicans* and *Sporothrix schenkii* (91, 109, 110).

2.6. Killer toxin mode of action

Killer toxins are reported to be proteins or glycoproteins that kill sensitive cells via a two-step mode of action as determined in *Saccharomyces* killer toxins. For the toxin to fully induce 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 primary receptors are located on the cell wall and the latter on the plasma membrane (105). Identified primary receptors include β -D-1,3-glucan, β -D-1,6-glucan, mannoproteins and chitin, while the secondary receptor - Kre1p of the K1 toxin is the only one identified so far (67). Mechanisms of the actual killing action differ, and may be through cell membrane permeabilization leading to the leakage of K^+ ions, ATP and increase in intracellular pH, cell cycle perturbation, inhibition of DNA synthesis, inhibition of β -1,3 - glucan synthase activity or hydrolysis of the major cell wall components β -1,3 - glucans and 1,6 - glucans (Figure 2.2) (6, 48).

The K1 killer toxin is well studied in literature and serves as a model toxin within the killer toxins of *S. cerevisiae*. The first step of toxin binding is strongly pH dependent with an optimum at pH 4.6 and the toxin binds to the cell wall receptors 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 (65). After reaching the plasma membrane, ionophoric virus killer toxins (Table 2.2), such as K1 and K2 of *S. cerevisiae* disrupt cytoplasmic membrane function by forming cation-selective ion channels. This results in increased permeability of H^+ (123), leakage of intracellular ATP, K^+ (124) and AMP (81). The action of the killer toxins appears not to be immediate. Both the PEST (Pool Efflux-Stimulating Toxins) and killer toxin of *T. glabrata* show a lag phase after addition to sensitive cells. It was shown that after 30 minutes, 60 - 70% of sensitive cells exhibit the up-take of the lethal dose of PEST without any visible metabolic change (81). However, after a lag time of 50 – 90 min, sensitive cells treated with a *P. kluyveri* toxin exhibit physiological changes observed when ionophoric toxins act on sensitive cells. In addition, the cells shrink, have decreased intracellular pH and the active uptake of amino acids is inhibited (100). Following exposure to the K2 toxin, sensitive *S. cerevisiae* cells present a rippled surface, characterized by “cracks” and pores. The toxin was reported to disrupt the cell wall structure and perturb cytosolic membranes. The damaged cells have crenulated plasma membrane, generating “pinocytotic”-type vesicles or endosomes as well as the loss of turgidity and the retraction of an intact plasma membrane from the periplasmic space coupled with irregular folding (125).

The killer toxin of *K. lactis* causes permanent arrest of the sensitive cells at the unbudded G1 phase (65) which is similar to the mode of action of the K28 toxin. The K28 toxin binds primarily to the α -1,3-linked mannose residues of a cell wall mannoprotein and is taken up by endocytosis, and travels the secretion pathway in reverse until it reaches the cytosol. This toxin arrests cells in the early S phase and blocks DNA synthesis at the cell cycle, leading to the non-separation of mother and daughter cell (36, 65). Other killer toxins attach to the β -1,3 and β -1,6

– glucans, mannoproteins as primary receptors on sensitive cell walls (Figure 2.2) and damage the integrity of the cell wall thereby inducing cell death by osmotic lysis (126).

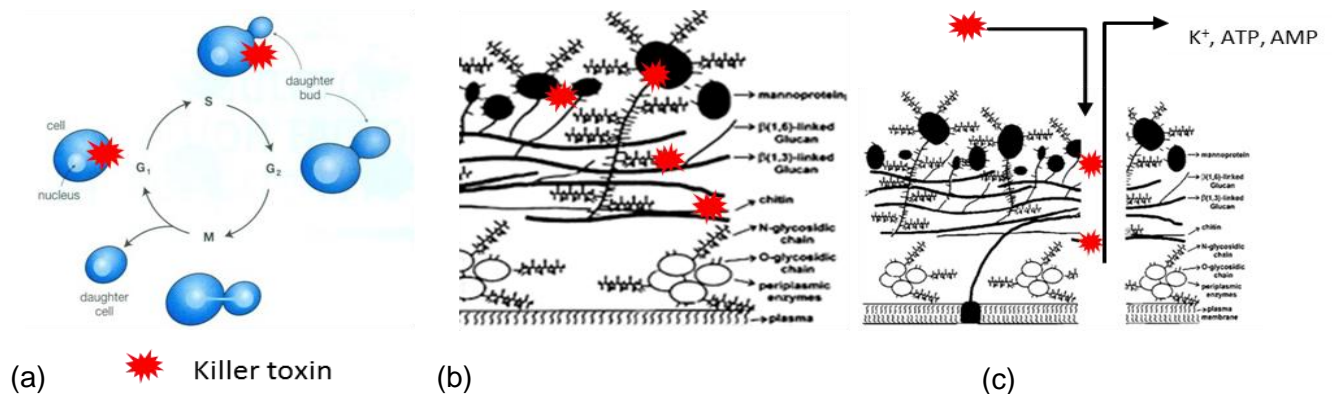


Figure 2.2: Killer toxin mode of action (image (a) adapted from Madhani (127), (b and c) adapted from Schreuder (128)) (a) cell cycle arrest, (b) cell wall degradation and (c) plasma membrane permeabilization.

2.6.1. Do exoglucanases possess killer activity?

The yeast cell wall is composed of 50% β -D-1,3-glucan that contains ca. 5% β -1,6 linked branches; 15% β -D-1,6-glucan containing ca. 14% β -1,3 linked branches; mannoproteins and chitin (0.6 – 9%) (84). In recent literature (94, 96, 118, 126), there is growing evidence that 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 mobilization of β -glucans in response to conditions of carbon and energy source exhaustion (129).

Exo-glucanase activity has been detected in killer toxin-producing yeast species of *W. anomalus*, *P. membranifaciens*, *W. saturnus*, *P. anomala* strain K, *Candida oleophila* and *T. phaffii* (88, 94, 96, 105, 106, 130-134). Three killer strains of *W. anomalus* (BCU24, BS91 and BCA15) exhibited killer activity to a *S. cerevisiae* wild type strain while mutants deficient in β -1,6-glucan were resistant to the toxins of the strains. The 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 and the strain BCA15 sequence matches perfectly the β -glucanase of *P. anomala* strain K. Furthermore, the *WaEXG2* sequences in the killer strains are identical to those from *P. anomala* strain K (126). The authors of the study 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 the *P. anomala* exo-glucanase genes *PaEXG1* and *PaEXG2* resulted in reduced efficiency - 8% from 71% in the biocontrol of *B. cinerea* in apples (132). The 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* (130) as was observed by Jijakli and Lepoivre, 1998 in culture media with cell wall preparations of *B. cinerea* as carbon source compared to when glucose was the carbon source. A similar effect was also observed with *C. oleophila* where 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 (134).

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 (94) 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 (96). The Kpkt NH₂-terminal region shows 93% identity to β -1,3-glucanase of *S. cerevisiae* and 80% to β -1,3-glucan transferase of *C. albicans*. The toxin has been shown to possess β -glucanase activity and its killer activity was inhibited in the presence of a β -glucanase inhibitor (6, 118).

These studies clearly indicate that exo-glucanases may display killer activity. Currently, killer toxins are defined as proteins or glycoproteins that exhibit antimicrobial activity towards susceptible yeasts of the same species or related species. However, this definition does not exclude that killer toxins may display other functions such as enzymatic function. As it has been proven by Wang et al. (106) that a killer toxin from a marine yeast had both killer activity and β -1,3-D-glucanase activity and that the enzyme may be responsible for killer activity (13).

2.7. 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. For instance the use of penicillin as an antibiotic in the medical industry, nisin as anti-bacterial “biopreservative” in the dairy industry, Microbial contamination of wine is still a major concern in the wine industry despite the widespread use of commercial preservatives such as SO₂. 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 to consumers from the consumption of the product, alter the quality of the product and the method(s) should be applied with minimal cost. Strategies such as wine filtration and use of chemical preservatives to combat wine spoilage have proved 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 can only efficiently inhibit or control the proliferation of contaminating microorganisms when applied in high concentrations. The use of killer toxins has been explored under experimental conditions and findings from such endeavours have revealed that they can be applied as alternatives in controlling microbial spoilage. Killer toxins are antimicrobial proteins secreted by *Saccharomyces* and certain non-*Saccharomyces* yeast species under natural growth conditions. *S. cerevisiae* killer toxins have a narrow spectrum of activity (inhibiting mostly *S. cerevisiae* strains) compared to those of non-*Saccharomyces* which have a broader spectrum of activity. Killer toxins from the yeasts *K. wickerhamii*, *P. anomala* and *P. membranifaciens* have been successfully studied in controlling *Brettanomyces*. Thus, their potential as bio-preservatives has been proved.

The genetic origin of yeast killer toxins can be from inherited virus-particle, chromosomal genes or dsDNA plasmids. The mode of action of these killer toxins is diverse. The toxin generally induces its killer activity by binding a receptor on either the cell wall and/or the plasma membrane of the target cell. Killer action can either occur through cell membrane permeabilization, hydrolysis of the cell wall components or inhibition of vital microbial functions within the cell after it has been engulfed inside the cell. Due to the proteinaceous nature of the killer toxins, the activity and stability of the killer toxins is temperature and pH dependent.

However, despite all the current knowledge on killer toxins, non-*Saccharomyces* killer toxins still remain poorly characterized compared to the killer toxins of *Saccharomyces*. The genetic origin, explicit mode of action and host immunity of these non-*Saccharomyces* killer toxins is yet to be unravelled. The mode of action of some of the non-*Saccharomyces* killer toxins provides strong evidence supporting 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. Can these killer toxins be used as bio-preservatives in wine and in the food and beverage industry?

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CHAPTER 3

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

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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 (SO₂) 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.

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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 (SO₂) 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 SO₂ 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 SO₂. In wine, SO₂ dissociates into three molecular species: the antimicrobial molecular SO₂ (SO₂·H₂O), bisulphite (HSO₃⁻) and sulphite (SO₃²⁻). 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 SO₂ is dependent on the molecular SO₂ 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 SO₂ 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 Beloso, 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 Db1E11/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 *EcoRI* (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 10⁶ 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

Table 1

Yeast and bacterial strains used in this study.

Yeast species	Strain	Phenotype	Source of isolation
<i>Kluyveromyces wickerhamii</i>	CBS 2745	Killer	Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands
<i>Candida pyralidae</i>	IWBT Y1140	Killer	Cabernet Sauvignon juice, 2009
<i>Candida pyralidae</i>	IWBT Y1057	Killer	Chardonnay juice, 2009
<i>Tetrapispora phaffii</i>	CBS 4417	Killer	Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands
<i>Brettanomyces bruxellensis</i>	AWRI 1499	Sensitive	Australian Wine Research Institute, Glen Osmond, Australia
<i>Brettanomyces bruxellensis</i>	IWBT Y102	Sensitive	Cabernet Sauvignon, 2004
<i>Brettanomyces bruxellensis</i>	IWBT Y111	Sensitive	Water, 2003
<i>Brettanomyces bruxellensis</i>	IWBT Y113	Sensitive	Cabernet Sauvignon juice, 2004
<i>Brettanomyces bruxellensis</i>	IWBT Y117	Sensitive	Shiraz, 2004
<i>Brettanomyces bruxellensis</i>	IWBT Y121	Sensitive	Cabernet Sauvignon, 2004
<i>Brettanomyces bruxellensis</i>	LO2E2	Sensitive	Institut Technique de la Vigne et du Vin, Beaune, France
<i>Brettanomyces anomalus</i>	IWBT Y176	Sensitive	Unknown
<i>Brettanomyces anomalus</i>	IWBT Y132	Sensitive	Cabernet Sauvignon, 2005
<i>Dekkera anomala</i>	ISA 1791	Sensitive	Instituto Superior de Agronomia, Lisbon, Portugal
<i>Brettanomyces custersianus</i>	IWBT Y170	Sensitive	South African wine
<i>Brettanomyces custersianus</i>	IWBT Y177	Sensitive	Unknown
<i>Hanseniaspora uvarum</i>	IWBT Y175	Sensitive	Unknown
<i>Hanseniaspora uvarum</i>	IWBT Y856	Sensitive	Cabernet Sauvignon, 2011
<i>Hanseniaspora opuntiae</i>	IWBT Y863	Sensitive	Cabernet Sauvignon, 2011
<i>Hanseniaspora uvarum</i>	IWBT Y864	Sensitive	Cabernet Sauvignon, 2011
<i>Hanseniaspora uvarum</i>	IWBT Y883	Sensitive	Cabernet Sauvignon, 2011
<i>Hanseniaspora uvarum</i>	IWBT Y904	Sensitive	Chenin Blanc, 2012
<i>Saccharomyces cerevisiae</i>	VIN13	Sensitive	Commercial strain—Anchor Yeast
<i>Saccharomyces cerevisiae</i>	228	Sensitive	Commercial strain—Anchor Yeast
<i>Zygosaccharomyces bailii</i>	IWBT Y143	Sensitive	Grapes from Wädenswil region of Switzerland
<i>Zygosaccharomyces bailii</i>	IWBT Y1058	Sensitive	Chardonnay juice, 2009
<i>Lactobacillus plantarum</i>	V22	Sensitive	Commercial strain—Oenobrand
<i>Oenococcus oeni</i>	Lalvin VP41	Sensitive	Commercial strain—Oenobrand

CBS = Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands; AWRI = Australian Wine Research Institute, Glen Osmond, Australia; IWBT = Institute for Wine Biotechnology, Stellenbosch, South Africa; ISA = Instituto Superior de Agronomia, Lisbon, Portugal; IFI = Instituto de Fermentaciones Industriales, Madrid, Spain; LO2E2 = Institut Technique de la Vigne et du Vin, Beaune, France.

30 °C with shaking on a test tube rotator and the cultures were acclimatized to the toxin production medium by inoculating 50 µL of the pre-cultures into 50 mL YPD broth pH 4.5 and grown overnight at 30 °C. A cell concentration of 10⁴ cfu/mL of the acclimatized culture was inoculated into triplicate 150 mL killer toxin production medium and grown at 25 °C with shaking at 150 rpm for 6, 12, 24, 36 and 48 h to determine the killer toxin production kinetics. The culture supernatant was collected by centrifugation at 5000 rpm, 4 °C for 10 min. The supernatant was filtered through a 0.45-µm polyethersulfone membrane (Pall Life Sciences, Ann Arbor, MI). The filtrate was concentrated by ultrafiltration at 5000 rpm, 4 °C, 10 min using Amicon® Ultra-centrifugal filters with a 10 kDa and 50 kDa molecular weight cut-off (Merck-Millipore, Carrigtwohill, Ireland). The 10 kDa and 50 kDa retentates (crude extracts), filtrates and supernatant of the cultures were tested for killer activity using the seeded agar method as described in Section 2.2. *B. bruxellensis* IWBT Y169 was used as the sensitive strain on WYE plates with 7-mm wells drilled on the agar surface of the plates (herein referred to as killer assay plates). Twenty microliters of the 10 and 50 kDa crude extracts, filtrates and supernatants were then spotted into the wells on triplicate killer assay plates. The plates were incubated at 20 °C until a well-developed lawn of the sensitive strain was observed. Killer activity was measured as the total diameter of the zone of growth inhibition minus the 7-mm well diameter. This protocol was followed to test for relative and residual activity of the killer toxins secreted by *C. pyralidae* IWBT Y1140 and IWBT Y1057 as described in the Section 2.4. Residual activity was regarded as the percentage of activity remaining after incubation of the toxin under a specific biochemical or environmental parameter for a certain period of time, activity at time 0 being used as a reference.

Crude extracts for activity optimum and stability assays were obtained by cultivating the *C. pyralidae* killer strains in 1 L YPD broth pH 4.5, for 24 h at 25 °C, 150 rpm by inoculating 10⁶ cfu/mL from pre-cultures grown as described in Section 2.3. The supernatant was harvested as described in Section 2.3 and concentrated firstly with an

Amicon Ultrafiltration cell with a 30 kDa cut-off membrane to 100 mL followed by further concentration with an Amicon® Ultra-centrifugal filter with a 50 kDa molecular weight cut-off (Merck-Millipore). A 10X protease inhibitor solution (cOmplete ULTRA Tablets, Roche Diagnostics) was added to the crude extracts which were then stored at 4 °C until use. The protein concentration of the crude extracts was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, MA), following the manufacturers' instructions.

2.4. Biochemical and environmental activity and stability

2.4.1. Effect of temperature denaturation and proteolytic enzymes

In order to evaluate the proteinaceous nature of the killer toxins, the crude extracts were subjected to heat denaturation as well as protease treatment. For heat denaturation, 60 µL of the crude extracts from the strains *C. pyralidae* IWBT Y1140 and Y1057 were boiled at 100 °C for 10 min. Crude extracts stored at 4 °C were spotted as positive controls. Residual killer activity of the crude extracts was tested as described above on triplicate killer assay plates. The crude extracts were subjected to protease treatment with Proteinase K, pepsin and proteases from *Aspergillus saitoi* and *Rhizopus* sp. (all from Sigma-Aldrich) for 1 h at 25 °C. The enzymes were mixed with the crude extracts to a final concentration of 5 and 10 mg/mL in a final volume of 100 µL. Residual killer activity was tested as described above on triplicate killer assay plates. All the killer assay plates were at pH 4.5 except for the killer assay plates onto which the *A. saitoi* protease was spotted. For the latter, the pH was adjusted to 3.5. The crude extracts not exposed to the enzymes were spotted as positive controls, while the pure proteases (i.e. without crude extract) were spotted as negative controls. The plates were incubated at 20 and 25 °C for the *C. pyralidae* IWBT Y1140 and IWBT Y1057 crude extracts, respectively and killer activity measured as described above.

2.4.2. Temperature and pH activity optima

The temperature optima of the crude extracts was determined by spotting 20 µL of the crude extracts on triplicate killer assay plates pH 4.5 and incubating at 15, 20, 25, 30, 35 and 40 °C. The pH optima was determined by spotting 20 µL of the crude extracts on triplicate killer assay plates at pH 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0. The plates were incubated at 20 and 25 °C for the crude extracts of *C. pyralidae* IWBT Y1140 and IWBT Y1057, respectively and killer activity measured as described above.

2.4.3. Temperature and pH stability

Temperature stability was determined by incubating the crude extracts of *C. pyralidae* IWBT Y1140 and IWBT Y1057 at 15, 20, 25 and 30 °C for 0, 1, 5, 10, 15 and 20 days. Residual killer activity was determined as described above on triplicate killer assay plates adjusted to pH 4.5. pH stability was determined by performing a buffer exchange of the crude extracts to pH 3.5, 4.0 and 4.5 with 0.1 M citrate-phosphate buffer at the corresponding pH using the Amicon® Ultra-centrifugal filter with a 50 kDa molecular weight cut-off (Merck-Millipore) following the manufacturers' instructions. The buffer exchanged crude extracts were incubated at 20 °C for 0, 1, 5, 10, 15 and 20 days and assayed for residual killer activity as described above on killer assay plates at the corresponding pH value as the crude extract. The killer assay plates were incubated at 20 and 25 °C for *C. pyralidae* IWBT Y1140 and IWBT Y1057 killer toxins respectively and killer activity was measured as described above.

2.4.4. Ethanol and sugar stability

The crude extracts were exposed to ethanol concentrations of 0, 7 and 14% (v/v) representing the beginning, middle and end of fermentation and incubated for 0, 1, 5, 10, 15 and 20 days at 20 °C to determine ethanol stability. Residual killer activity was determined as described above on triplicate killer assay plates adjusted to pH 4.5. To test the stability of the crude extracts in sugar concentrations typically found during fermentation, the crude extracts were exposed to sugar concentrations representing the beginning, middle and the end of fermentation: 230 g/L, 115 g/L and 2 g/L of glucose and fructose in a 1:1 ratio, respectively. They were incubated at 20 °C; for 0, 1, 5, 10, 15 and 20 days. Residual killer activity was determined on triplicate killer assay plates adjusted to pH 4.5 as described above. The plates were incubated at 20 and 25 °C for *C. pyralidae* IWBT Y1140 and IWBT Y1057 crude extracts, respectively and killer activity was measured as described above.

3. Results

3.1. Isolate fingerprinting and strain identification

Twenty-two South African *B. bruxellensis* isolates were differentiated to strain level by ISS-PCR. From the data obtained, it was concluded that the 22 *B. bruxellensis* isolates were different strains (data not shown). Following the screening of a large collection of non-*Saccharomyces* wine isolates for hydrolytic activities of oenological relevance, two isolates (IWBT Y1140 and IWBT Y1057) were found to display killer activity against *B. bruxellensis* (data not shown). Identification of these isolates was performed via the sequencing of their D1/D2 26S rRNA region. Three individual clones were sequenced. The obtained sequences were compared to those present in the NCBI database. The sequences of IWBT Y1140 isolate yielded 99%, 98%, and 97% identity to the sequences of *C. pyralidae* CBS 5035, *C. xyloposoci* CBS 6037, and *C. prunicola* CBS 8848 respectively; and those of IWBT Y1057 yielded 100%, 97%, and 97% identity to the same sequences respectively. Fig. 1 shows the relatedness of IWBT Y1140 and IWBT Y1057 isolates with a number of yeast species. Based on these sequence similarities, these isolates were identified as *C. pyralidae*. Furthermore, the D1/D2 26S rRNA sequences of the two isolates differed by two

nucleotides and two sequences differing by one single nucleotide could be isolated for IWBT Y1057 (see accession numbers KJ746059 (for IWBT Y1140) and KJ746060 and KJ746161 (for IWBT Y1057) on GenBank). It was therefore concluded that the two isolates were different strains of *C. pyralidae* and the IWBT Y1057 strain is heterozygous for this gene.

3.2. Killer activity screening

The killer activity of the strains IWBT Y1140 and Y1057 was assessed against 22 strains of *Brettanomyces/Dekkera* spp. in our culture collection. Amongst those, killer activity screening resulted in 16 sensitive strains in at least all the media and killer yeasts tested, only six strains were resistant to the three killer yeasts in all the media tested (Table 2). The killer yeast strain *K. wickerhamii* CBS 2745 was used as a control, since its killer activity against *B. bruxellensis* has been previously reported (Comitini et al., 2004). The strains *B. bruxellensis* IWBT Y111 and IWBT Y113, *B. anomalus* IWBT Y119 and IWBT Y176 and *B. custersianus* IWBT Y170 and IWBT Y177 were resistant to the killer yeasts on all media tested. Only 9 strains (7 *B. bruxellensis*, 1 *B. anomalus* and 1 *Dekkera anomala*) were sensitive to *K. wickerhamii* killer toxin on YPD and only *B. anomalus* IWBT Y132 was sensitive to the two *C. pyralidae* strains on the same medium. Killer activity against the *B. bruxellensis* (IWBT Y102, Y131, Y133 and IFI 63) was only observed when *K. wickerhamii* was spotted as the killer yeast on YPD, WYE and RYE. No killer activity against these strains was observed when the *C. pyralidae* strains were spotted on the same media. Killer activity seemed to be medium dependent as *B. bruxellensis* strains IWBT Y117, Y121, Y136 and Y169 were sensitive to all the killer yeasts on grape juice media only. The killer yeast strain *Tetrapispora phaffii* CBS4417 was also used as a control for killer activity screening against certain *Brettanomyces* and *Hanseniaspora* strains as its killer activity against *Hanseniaspora* spp. has previously been reported (Ciani and Faticenti, 2001). The strain did however not exhibit killer activity against *B. bruxellensis* strains IWBT Y135, Y136, Y169, ISA 1649 and IFI 63, *B. custersianus* IWBT Y170 and IWBT Y177, *B. anomalus* IWBT Y176 and *D. anomala* ISA 1791 on YPD, WYE and RYE and on YPD against all the *Hanseniaspora* spp. strains screened (data not shown). Two commercial strains of *S. cerevisiae* were resistant to the killer toxins of all the killer yeasts on all the media tested and the same was observed for the two *Z. bailii* strains screened. However, the lactic acid bacteria, *L. plantarum* was sensitive to the killer toxins of *T. phaffii* and *K. wickerhamii* on white and red grape juice, while *O. oeni* was only sensitive to the former yeast on the same media. These strains were resistant to the *C. pyralidae* killer toxins on all the media tested.

3.3. Killer toxin production

Killer toxin production was carried out for a period of 48 h for the *C. pyralidae* strains only, considering that the killer toxin production and biochemical activity and stability of *K. wickerhamii* have been determined before (Comitini et al., 2004). The supernatant, crude extracts and filtrates obtained by ultrafiltration with the 10 and 50 kDa cut-off Amicon® Ultra-centrifugal filters were tested for killer activity on YPD and WYE killer assay plates. Killer activity was observed on WYE killer assay plates when the supernatants as well as the crude extracts above 10 and 50 kDa collected after 24 and 36 h incubation were spotted for both *C. pyralidae* strains. However, the killer activity of the 36 h samples was weaker compared to the 24 h samples (Table 3). Therefore, 24 h was chosen as the period to harvest the supernatant for further experiments. The antimicrobial compounds were named *C. pyralidae* killer toxin 1 (CpKT1) and *C. pyralidae* killer toxin 2 (CpKT2) for the *C. pyralidae* strains IWBT Y1140 and IWBT Y1057, respectively.

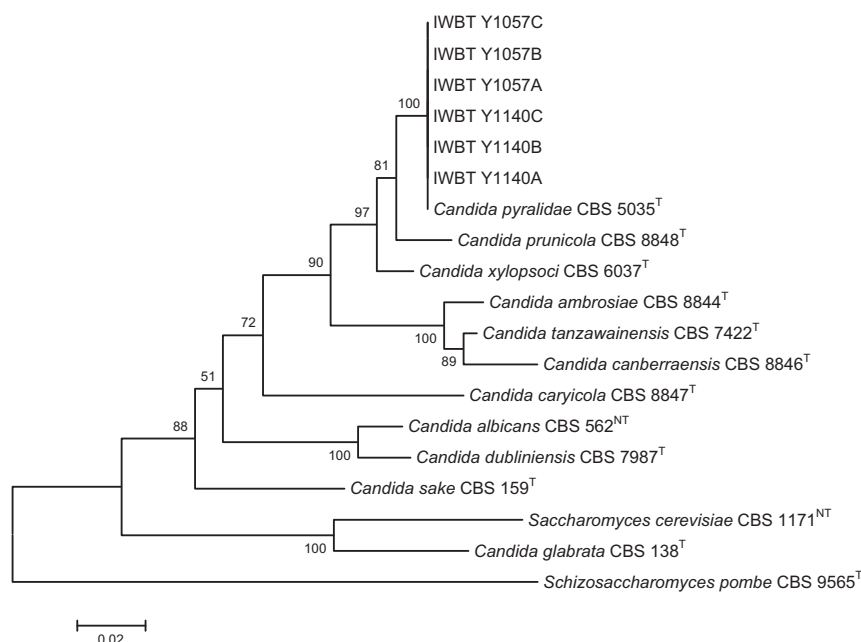


Fig. 1. Evolutionary relationship of *C. pyralidae* IWBT Y1140 and IWBT Y1057 strains. The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length is 0.70684333. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6. *T: Type strain. *NT: Neo-type strain.

3.4. Biochemical and environmental activity and stability

3.4.1. Effect of temperature denaturation and exposure to proteolytic enzymes

In order to determine the nature of the antimicrobial compound of *C. pyralidae* IWBT Y1140 and IWBT Y1057, the crude extracts were boiled at 100 °C and tested for residual killer activity. The crude extracts lost their killer activity after boiling (data not shown). Furthermore, the crude extracts were treated with proteinase K, pepsin, an aspartic protease from *A. saitoi* and a protease from *Rhizopus* sp. The killer activity of both crude extracts of the *C. pyralidae* strains was lost after treatment with Proteinase K. Pepsin and the proteases from *A. saitoi* and *Rhizopus* sp. did not affect the killer activity of these strains. The proteolytic enzymes tested did not show any killer activity (data not shown).

3.4.2. Temperature and pH activity optima

The temperature activity optimum of CpKT1 and CpKT2 was found to be 20 and 25 °C, respectively (Fig. 2). Residual killer activity was between 40 and 60% at 15 °C for both killer toxins. Killer activity was lost at 30 °C for CpKT1 compared to CpKT2 which lost it at 35 °C. CpKT1 showed optimum activity within the pH range 3.5–4.5, while CpKT2 displayed optimal activity at pH 4.0. Both killer toxins had more than 60% relative killer activity at pH greater than 2.5 which was lost at pH 5.

3.4.3. Temperature and pH stability

The stability of the killer toxins was tested at temperatures 15, 20, 25 and 30 °C and at pH 3.5, 4.0 and 4.5 which are relevant to winemaking. CpKT1 was found to be stable at 15 and 20 °C, retaining between 100 and 30% activity from day 0 to day 10. The killer toxin had less than 20% residual activity from day 10 onwards at 25 °C and no killer activity was observed from the same time point at 30 °C. This killer toxin displayed more than 70% residual killer activity at pH 4.5 over a 20-day period. At pH 3.5 and 4.0, the killer toxin retained between 60 and 45% residual killer activity from day 5 to day 20 respectively (Fig. 3). CpKT2 retained between 100 and 80% residual killer activity at 15 and

20 °C throughout all the sampling days. The toxin remained active only until day 10 at 25 °C while at 30 °C activity was retained until day 5. The toxin retained more than 60% residual activity in all the pH ranges tested until day 20, except at days 15 and 20 at pH 4, the toxins' activity declined by 10% (Fig. 4). It should be noted that for temperature and pH stability assays, both the killer toxins aggregated from day 5 at 20 °C incubation.

3.4.4. Ethanol and sugar stability

CpKT1 lost killer activity in the presence of 14% ethanol by day 10. In the presence of 7% ethanol, residual killer activity was similar to that of the control with residual activity progressively decreasing from 100 to 50% from day 0 to day 20. The presence of sugar did not seem to have an influence on killer activity of this killer toxin as more than 60% residual activity was observed until day 20 except at days 5 and 10 at 230 g/L sugar where killer activity decreased (Fig. 3). CpKT2 was stable in the presence of 14% ethanol compared to CpKT1. The killer toxin retained more than 50% residual killer activity in all the concentrations of ethanol tested for all the days tested. CpKT2 retained more than 60% residual killer activity in the presence of the sugar concentrations tested except at day 10 where killer activity decreased (Fig. 4).

4. Discussion

Since the identification of killer activity in non-*Saccharomyces* yeast genera by Philliskirk and Young (1975), many killer yeast strains have been studied for their antimicrobial activity against undesired microorganisms or pathogens. These yeast killer toxins have potential application in the food industry, taxonomy, medicine and agriculture (Liu et al., 2013). Killer toxins have been suggested as potential alternatives (Comitini et al., 2004; Santos et al., 2009, 2011) to the use of SO₂, a chemical preservative with limited efficiency against *B. bruxellensis* which can elicit allergic reactions in sensitive wine consumers (Barata et al., 2008; Duckitt, 2012; Lustrato et al., 2006). The current study investigated the potential of killer toxins secreted by *C. pyralidae* strains as biocontrol agents for the prevention of *B. bruxellensis* wine spoilage.

Table 2
Killer activity screening against *Brettanomyces*, *Saccharomyces*, *Zygosaccharomyces* and lactic acid bacteria strains.

Sensitive strains	YPD			WYE			RYE		
	Killer yeast strains ^a								
	CBS 2745	IWBT Y1140	IWBT Y1057	CBS 2745	IWBT Y1140	IWBT Y1057	CBS 2745	IWBT Y1140	IWBT Y1057
<i>B. bruxellensis</i> AWRI 1499	+ ^b	– ^c	–	+	+	+	+	+	+
<i>B. bruxellensis</i> IWBT Y102	+	–	–	+	–	–	+	–	–
<i>B. bruxellensis</i> IWBT Y111	–	–	–	–	–	–	–	–	–
<i>B. bruxellensis</i> IWBT Y113	–	–	–	–	–	–	–	–	–
<i>B. bruxellensis</i> IWBT Y117	–	–	–	+	+	+	+	+	+
<i>B. bruxellensis</i> IWBT Y121	–	–	–	+	+	+	+	+	+
<i>B. bruxellensis</i> IWBT Y130	+	–	–	+	+	+	+	–	–
<i>B. bruxellensis</i> IWBT Y131	+	–	–	+	–	–	+	–	–
<i>B. bruxellensis</i> IWBT Y133	+	–	–	+	–	–	+	–	–
<i>B. bruxellensis</i> IWBT Y135	+	–	–	+	+	+	+	+	+
<i>B. bruxellensis</i> IWBT Y136	–	–	–	+	+	+	+	+	+
<i>B. bruxellensis</i> IWBT Y169	–	–	–	+	+	+	+	+	+
<i>B. bruxellensis</i> ISA 1649	–	–	–	+	–	–	+	+	–
<i>B. bruxellensis</i> IFI 63	+	–	–	+	–	–	+	–	–
<i>B. bruxellensis</i> LO2E2	–	–	–	+	–	–	+	–	–
<i>B. anomalous</i> IWBT Y105	+	–	–	+	+	+	+	+	+
<i>B. anomalous</i> IWBT Y119	–	–	–	–	–	–	–	–	–
<i>B. anomalous</i> IWBT Y132	–	+	+	+	+	+	+	+	+
<i>B. anomalous</i> IWBT Y176	–	–	–	–	–	–	–	–	–
<i>D. anomala</i> ISA 1791	+	–	–	–	–	–	+	–	–
<i>B. custersianus</i> IWBT Y177	–	–	–	–	–	–	–	–	–
<i>B. custersianus</i> IWBT Y170	–	–	–	–	–	–	–	–	–
<i>S. cerevisiae</i> 228	–	–	–	–	–	–	–	–	–
<i>S. cerevisiae</i> VIN 13	–	–	–	–	–	–	–	–	–
<i>Z. bailii</i> IWBT 143	–	–	–	–	–	–	–	–	–
<i>Z. bailii</i> IWBT Y1058	–	–	–	–	–	–	–	–	–
^d <i>L. plantarum</i> V22	–	–	–	+	–	–	+	–	–
^d <i>O. oeni</i> Lalvin VP41	–	–	–	–	–	–	–	–	–

^a Killer yeast strains: *K. wickerhamii* CBS2745; *C. pyralidae* IWBT Y1140; *C. pyralidae* IWBT Y1057.

^b Killer sensitive.

^c Killer resistant.

^d MRS, white and red table grape juice was used for the screening.

Killer activity screening against *Brettanomyces* spp. strains resulted in varied killer activity on all the media tested (Table 2). Regarding KwKT, the killer toxin of *K. wickerhamii*, Comitini et al. (2004) reported that all the strains of *Brettanomyces/Dekkera* that they tested were inhibited, but our study showed that 28% of the South African strains were actually resistant to it. Furthermore, the tested *B. bruxellensis* sensitive strains do not show any genetic relatedness as shown in Fig. 1. The *B. anomalous* strains screened in this study were inhibited

unequally by any of the killer yeasts in at least all the media tested. The *D. anomala* sensitive strain was inhibited by *K. wickerhamii* only, and none of the *B. custersianus* strains were inhibited by all the killer yeasts. Different strains of *K. wickerhamii* and some *Kluyveromyces* species have been shown to have varied killer activity intensity against *Dekkera bruxellensis*, *Kloeckera apiculata* and *Zygosaccharomyces rouxii* strains (Divol et al., 2012b; Palpacelli et al., 1991). Our study showed that a number of *B. bruxellensis* strains are resistant to *K. wickerhamii*

Table 3
Killer toxin production kinetics.

Sample	Time (h)	Cells	Supernatant	10 kDa retentate	50 kDa retentate
CpKT1	24	+	+++	+++	+++
CpKT2	24	+	++	++	++
CpKT1	36	+	++	+++	+++
CpKT2	36	+	+	-	+

+: <5 mm zone of inhibition.
 ++: 6–9 mm zone of inhibition.
 +++: 10–14 mm zone of inhibition.

β -D-glucans which are killer toxin receptors (İzğü and Altınbay, 2004; Guo et al., 2012) in contrast to peptone which inhibited killer activity (Divol et al., 2012b).

Although the *T. phaffii* CBS 4417 strain is reported in literature to inhibit the apiculate yeast *Hanseniaspora uvarum* (Ciani and Fatichenti, 2001; Comitini and Ciani, 2010), our study revealed that the same strain did not inhibit the growth of any of the *Hanseniaspora* spp. and the *Brettanomyces* spp. strains tested. This could potentially be attributed to a difference in the media used for the screening, but would require further investigations. It was therefore concluded that killer activity is dependent

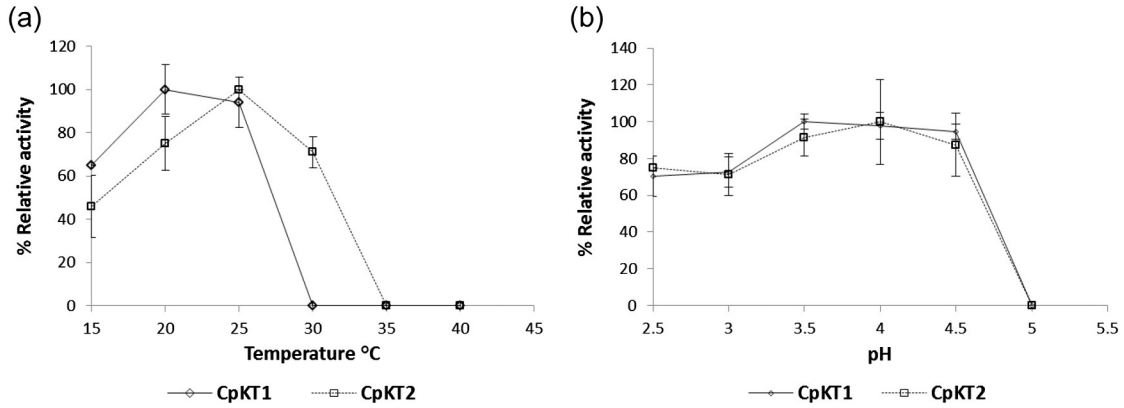


Fig. 2. Temperature (a) and pH (b) activity optima of CpKT1 and CpKT2.

and *C. pyralidae* killer toxins on YPD in contrast to grape juice medium supplemented with 1% yeast extract. This confirmed previous results in which yeast extract was reported to enhance killer activity (Divol et al., 2012b) due to the presence of a significant amount of cell wall

on the killer and sensitive strain as well as medium used for screening.

According to Golubev (2000), all yeast killer toxins are proteinaceous by nature and killer activity is expressed under acidic conditions within the pH range 3–6 and inactivated at temperatures above 25 °C.

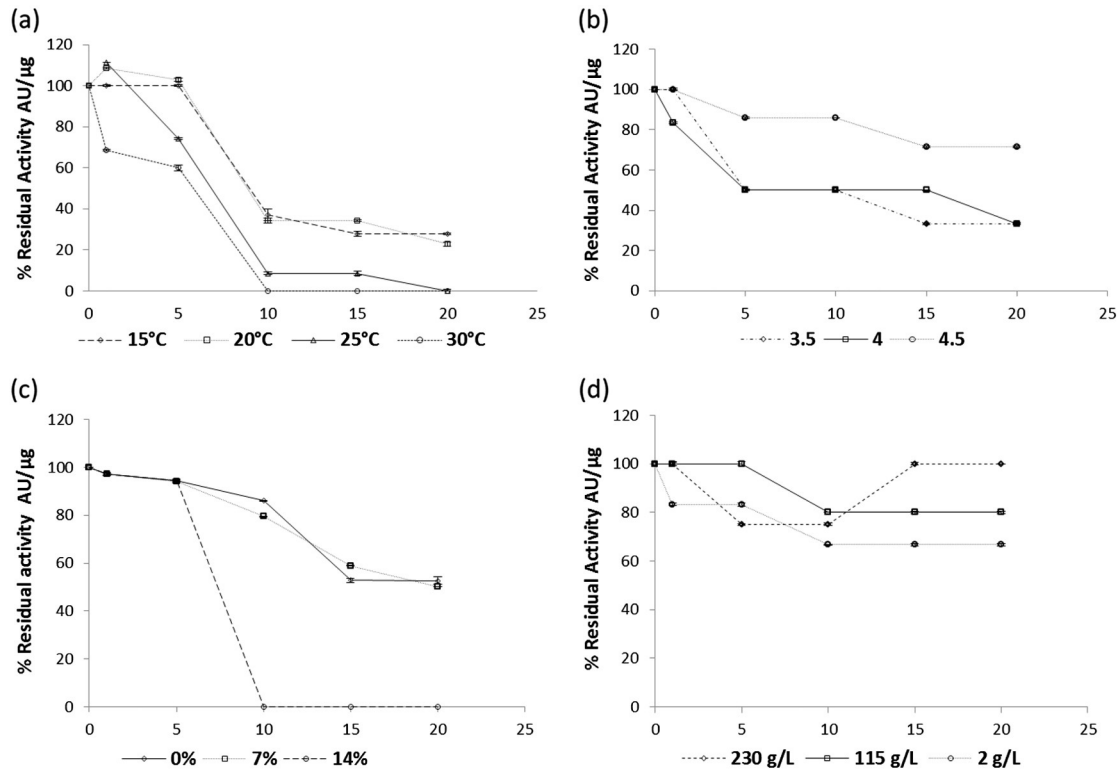


Fig. 3. CpKT1 temperature stability (a), pH stability (b), ethanol stability (c), and sugar stability (d) over time in days. Residual activity was regarded as the percentage of activity remaining after incubation of the toxin under a specific biochemical or environmental parameter for a certain period of time (activity at time 0 was used as a reference).

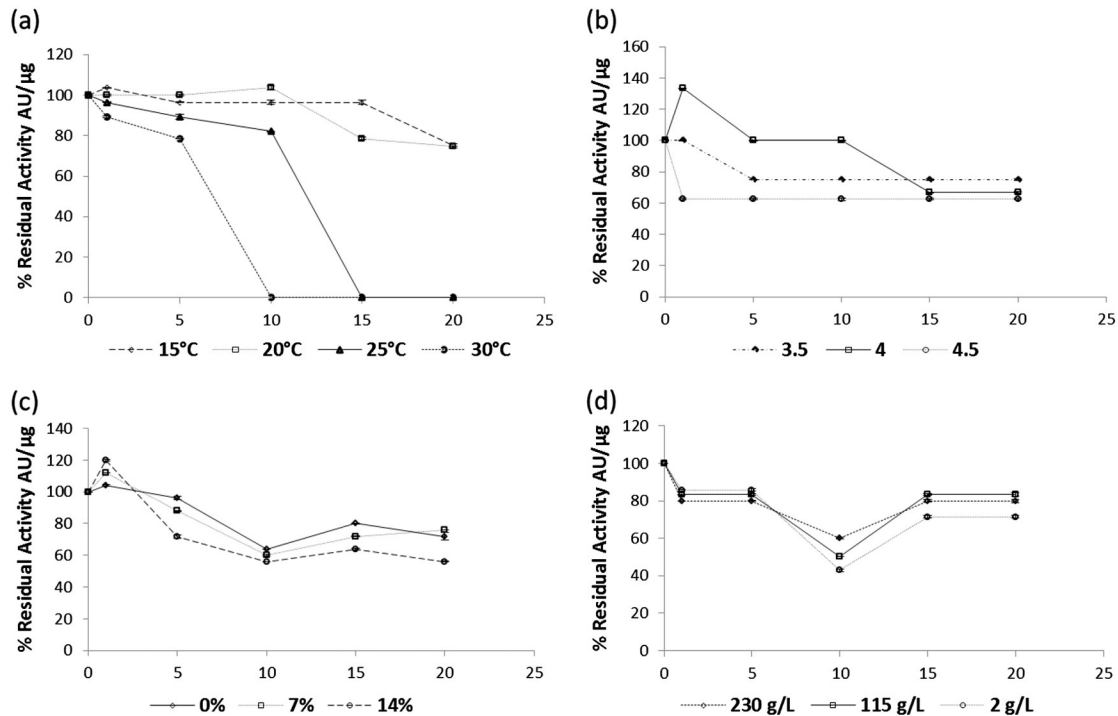


Fig. 4. CpKT2 temperature stability (a), pH stability (b), ethanol stability (c), and sugar stability (d) over time in days. Residual activity was regarded as the percentage of activity remaining after incubation of the toxin under a specific biochemical or environmental parameter for a certain period of time (activity at time 0 was used as a reference).

We show in the current study that the killer toxins derived from *C. pyralidae* conform to this general description. The proteinaceous nature of CpKT1 and CpKT2 was confirmed by the loss of killer activity after incubation at high temperature and incubation in the presence of the proteolytic enzyme proteinase K (data not shown). This also confirmed that CpKT1 and CpKT2 could be considered as killer toxins.

CpKT1 and CpKT2 were found to be active at acidic pH with activity optima between 3.5 and 4.5 (Fig. 2). However, optimal pH stability differed between the two toxins: while CpKT1 displayed the strongest stability at pH 4.5, that of CpKT2 was observed at 4.0 (Figs. 3 and 4). Analyses of temperature, ethanol and sugar stability were carried out at pH 4.5 as the toxins show similar levels of activity at both pHs and it can be hypothesized that similar results would have been obtained at pH 3.5. The toxins also exhibited temperature activity optima and stability between 15 and 20 °C (Figs. 2, 3 and 4). These properties were similar to those reported by Comitini et al. (2004) where Pikt and Kwkt were active at pH range of 3.8–4.6 and a temperature of 20–25 °C, while PMKT remained stable and active at pH 2–5 and a temperature range of 5–20 °C against *B. bruxellensis* (Santos et al., 2009).

CpKT1 appears only stable for 5 days regardless of the temperature tested (Fig. 3). Although it could be regarded as a short period of time, it should be enough to eliminate the targeted yeast population. Indeed, in literature (Bussey and Skipper, 1975; Middelbeek et al., 1980; De Ingeniis et al., 2009; Santos et al., 2013), the sensitive cell population is severely diminished or eliminated within 24 h after addition of the toxin. CpKT2 appears stable for at least 20 d at 15 and 20 °C (temperatures compatible with wine ageing conditions) which would guarantee its long term efficiency.

Furthermore, these killer toxins were not greatly influenced by ethanol and sugar concentrations found during fermentation for the period tested. This clearly indicates that CpKT1 and CpKT2 can potentially remain active throughout fermentation. A similar behaviour was observed for the toxins Pikt and Kwkt which retained their killer activity for 10 days in wine (Comitini et al., 2004). Current data also revealed that the stability (expressed as % residual activity) might fluctuate

from batch to batch. For instance, it was expected that the same level of activity would be retained at 0% ethanol and at 20 °C, but a slight difference was noticed.

In conclusion, the preliminary results of this study show that two novel killer toxins, CpKT1 and CpKT2, from two different strains of *C. pyralidae*, show potential to control the population of a large number of *B. bruxellensis* strains in conditions similar to those found during winemaking. These killer toxins are not much affected by parameters such as low pH ranges, temperatures below 25 °C, sugar and ethanol concentrations found under winemaking conditions. Furthermore, these killer toxins inhibit neither the fermenting yeast *S. cerevisiae* nor the lactic acid bacteria tested and are therefore hypothesized not to have an impact on alcoholic and malolactic fermentation, unlike Kwkt which could compromise malolact fermentation if performed by *L. plantarum*.

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CHAPTER 4

**Investigating the exoproteome of
Kluyveromyces wickerhamii and *Candida
pyralidae*: an attempt to identify the killer toxins
Kwkt, CpKT1 and CpKT2**

CHAPTER 4

Investigating the exoproteome of *Kluyveromyces wickerhamii* and *Candida pyralidae*: an attempt to identify the killer toxins Kwkt, CpKT1 and CpKT2

Abstract

In the previous chapter (Chapter 3), the yeast species *Kluyveromyces wickerhamii* and *Candida pyralidae* were shown to produce the killer toxins Kwkt, CpKT1 and CpKT2, respectively. These killer toxins are active against *Brettanomyces bruxellensis*, *Dekkera anomala*/*Brettanomyces anomalus* strains. These killer toxins are stable and active under winemaking conditions. The current study focused on the identification of the proteins accountable for this killer phenotype and the genes encoding them. The exoproteome of the yeast species *K. wickerhamii* and *C. pyralidae* revealed an abundance of exo-glucanases and glucosidases related to the yeast cell wall composition. Based on literature studies, where the *EXG1* gene(s) have strongly been hypothesized to code for killer toxins, we tentatively identified two candidate enzymes, an exo-glucanase and a β -glucosidase in the exoproteome of *K. wickerhamii* as potential killer toxins encoded by the genes: *KwEXG1* and *KwSUN4*, respectively. These enzymes function in cell wall assembly and cell septation, respectively. The two genes *KwEXG1* and *KwSUN4* were retrieved from the genome of *K. wickerhamii* by PCR amplification with gene specific primers, cloned and expressed in *S. cerevisiae* laboratory mutant strains deficient of the respective homologous gene. However, no successful transcription of the genes could be achieved in the heterologous host, preventing us from drawing final conclusions with regards to the identity of Kwkt. The identification in high abundance of glucanases in the exoproteome of the killer toxin-producing strains suggests that they might be involved in the killer phenotype of these strains. Future studies should focus on either purifying and/or characterization of these enzymes and assessing their killer activity against *B. bruxellensis* strains.

4.1. Introduction

In winemaking, improvement of clarification, filtration, chemical stabilisation and aroma and flavour release is achieved through hydrolysis of grape cell structural components and grape proteins by extracellular enzymes such as β -glucanases, pectinases, β -glucosidases, hemicellulases and proteases (1, 2). These enzymes originate from either grape juice, skin and seed or from yeasts and bacteria (3). Certain yeast species are indeed known to secrete a variety of extracellular proteins referred to as the exoproteome (4). These proteins are mainly

associated with cell wall assembly, contributing to the breakage and re-forming of bonds within and between polymers, re-modelling of the cell wall during growth and morphogenesis (5, 6). The proteins are of hydrolytic nature and characterized as either glucanases, transglycosidases or chitinases (7). *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts are also known to secrete killer toxins which are of proteinaceous nature (8, 9). These toxins inhibit the growth of other yeast species or strains (10).

Recently, a link between certain killer toxins and hydrolytic enzymes, in particular exo-glucanase, has been established (11-15), where toxins exhibited both killer activity and exo-glucanase activity. It is reported that such toxins act on sensitive cells by hydrolysing the major cell wall β -glucan scaffold thereby inducing cell death by osmotic lysis (12). This is further supported by the fact that the mechanisms of action of known killer toxins also involve the degradation of the sensitive yeasts' cell wall and the inhibition of β -glucan synthase (16-19). Notably, the Kpkt toxin secreted by *Tetrapisispora phaffii* exhibits killer activity against the wine yeast *Hanseniaspora uvarum* as well as glucanase activity on laminarin (19). The yeast *Candida oleophila* is reported to possess antimicrobial activity and was also shown to produce an exo-glucanase (20). This enzyme is now produced and commercialised under the name Aspire. It is indeed used to control postharvest decay in citrus fruits. The killer toxins secreted by some *Wickerhamomyces* species (former *Pichia* species) also display both killer and exo-glucanase activities (12-15, 21) and could potentially be used as enzymatic preparations for use in various industries. However, the genetic origin of these killer toxins needs to be investigated to confirm the true identity of the killer toxins.

The genetic origin of non-*Saccharomyces* killer toxins is either from dsRNA mycoviruses, dsDNA/RNA plasmids or chromosomal genes (22). The yeasts *Zygosaccharomyces bailii* and *H. uvarum* are the only two non-*Saccharomyces* yeasts reported, together with the filamentous fungus *Ustilago maydis*, whose killer toxins originate from dsRNA mycoviruses like those of *S. cerevisiae* (23, 24). The killer toxins of the yeasts *Kluyveromyces lactis*, *Pichia inositovora* and *Pichia acaciae* originate from linear dsDNA plasmids (25-27). A large number of non-*Saccharomyces* killer toxins are thought to be encoded by chromosomal genes, however, their actual genetic origin is yet to be identified (16, 28). The genes *HMK*, *HSK*, *SMK* and *TpBGL2* have been shown to code for the killer toxins secreted by *Williopsis mrakii* (29), *Hansenula saturnus* (30), *Pichia farinosa* (31) and *T. phaffii* (11), respectively.

In the previous chapter (Chapter 3), we characterized two killer toxins, CpKT1 and CpKT2, secreted by the yeast species *C. pyralidae*. These killer toxins were found to be active and stable under conditions occurring during winemaking and inhibited the growth of several *B. bruxellensis* or *Dekkera* spp. strains (32). The current study focussed on evaluating the

exoproteomes of *K. wickerhamii* CBS 2745 and *C. pyralidae* IWBT Y1140 and IWBT Y1057 in an attempt to identify the killer toxins and the genes encoding them.

4.2. Materials and methods

4.2.1. Killer toxin production

Pre-cultures of the killer yeast strains *K. wickerhamii* CBS 2745, *C. pyralidae* IWBT Y1140 and IWBT Y1057 (Table 4.1) were grown in 5 mL YPD (Yeast Peptone Dextrose broth, Biolab-Merck, Wadeville, South Africa) overnight at 30°C with shaking on a test tube rotator. Five hundred microliters of the pre-cultures were inoculated into 50 mL killer toxin production medium (YPD broth adjusted to pH 4.5 with 1 M HCl) since the killer toxins are stable at pH 4.5 (32, 33) and grown overnight to 10^9 cfu/mL at 30°C with shaking. One litre of YPD broth pH 4.5 was inoculated with 10^6 cfu/mL of the overnight pre-culture and grown at 25°C with shaking at 150 rpm for 28 h and 24 h for the *K. wickerhamii* and *C. pyralidae* strains, respectively. The culture supernatant was collected by centrifugation at 4193 g, 4°C for 10 min. It was then filtered through a 0.45- μ m polyethersulfone membrane (Pall Life Sciences, Ann Arbor, MI) and first concentrated using an Amicon Ultrafiltration cell with a 30 kDa cut-off membrane followed by further concentration with an Amicon® Ultra-centrifugal filter with a 50 kDa molecular weight cut-off (Merck-Millipore, Carrigtwohill, Ireland). A protease inhibitor, cOmplete ULTRA Tablets (Roche Diagnostics, Mannheim, Germany), was added to the crude extract to a final concentration of 0.025x which was then stored at 4°C until use. The protein concentration of the crude extract was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, MA), following the manufacturers' instructions.

4.2.2. Killer activity assay on solid medium

Killer activity was performed using the seeded agar method on WYE (commercial white table grape juice supplemented with 1% yeast extract) medium adjusted to pH 4.5 with 2 M NaOH. The *B. bruxellensis* IWBT Y169 sensitive strain (32) was inoculated at a concentration of 10^6 cfu/mL in 7.5 mL of WYE medium. The inoculated medium was mixed with 4% bacteriological agar (kept at 50°C) to a final volume of 10 mL and after brief vortexing, the medium was poured into sterile Petri dishes. Twenty microliters of the 50 kDa crude extract were spotted into 7-mm wells drilled on solidified WYE agar plate. The plates were incubated at 20°C and killer activity was observed as a zone of clearance (growth inhibition zone) around the 7-mm wells on triplicate plates. Killer activity was measured in mm as the total diameter of the clear zone and well minus the 7-mm well diameter.

Table 4.1: List of strains used in this study

Yeast species	Strain	Phenotype or genotype	Source of isolation or reference
<i>K. wickerhamii</i>	CBS 2745	Killer	Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands
<i>C. pyralidae</i>	IWBT Y1140	Killer	Cabernet Sauvignon juice, 2009
<i>C. pyralidae</i>	IWBT Y1057	Killer	Chardonnay juice, 2009
<i>B. bruxellensis</i>	IWBT Y102	Sensitive	Cabernet sauvignon, 2004 Tank, during MLF
<i>B. bruxellensis</i>	IWBT Y111	Sensitive	Weltevrede farm, 2003 (water after barrel cleaning)
<i>B. bruxellensis</i>	IWBT Y135	Sensitive	South African Wine, 2003
<i>B. bruxellensis</i>	IWBT Y169	Sensitive	South African Wine, 2003
<i>S. cerevisiae</i>	BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	(34)
<i>S. cerevisiae</i>	BY4742 Δ exg1	<i>Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	EUROSCARF
<i>S. cerevisiae</i>	BY4742 Δ sun4	<i>Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	EUROSCARF
<i>S. cerevisiae</i>	YHUM272	<i>MATα ura3-52 Δtrp1::hisG Δleu2::hisG Δhis3::hisG</i>	(35)

4.2.3. SDS-PAGE of *K. wickerhamii*'s exoproteome

SDS-PAGE analysis was performed as described by Laemmli (36). The proteins present in the *K. wickerhamii* concentrated crude extract (exoproteome) were separated on a 12.5% bis-

acrylamide gel in a Bio-Rad Mini-PROTEAN® Tetra Cell System (Bio-Rad Labs., Hercules, CA, USA). To visualize protein bands, the gel was stained with Coomassie Blue R-250.

4.2.4. Peptide sequencing of *K. wickerhamii*'s exoproteome

The protein bands between 50 and 95 kDa (Figure 4.1) from the bis-acrylamide gel, were excised and sent to the Central Analytical Facility of Stellenbosch University (Bellville, South Africa) for peptide sequencing. The bands were trypsin digested and sequenced by Nano-LC and LC-MS/MS. The experiments were performed on a Thermo Scientific EASYnLCII connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) was used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra against various *Kluyveromyces* databases. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 2 tryptic peptides per proteins, a Mascot of more than $p < 0.05$ as determined by Proteome Discoverer 1.3. Percolator was also used for validations of search results. In Percolator, a decoy database was searched with a FDR (strict) of 0.02 and FDR (relaxed) of 0.05 with validation based on the q-value.

4.2.5. Peptide sequencing of *C. pyralidae* IWBT Y1140 and IWBT 1057 exoproteomes

4.2.5.1. Sample preparation

One millilitre of the 50 kDa crude extracts (exoproteome) of *C. pyralidae* IWBT Y1140 and IWBT Y1057 were supplied as in-solution samples to the Proteomics Unit at the Central Analytical Facility at Stellenbosch University (Bellville, South Africa) for peptide sequencing. The samples were de-lipidated by chloroform-methanol extraction. Chloroform (Sigma) and Methanol (Sigma) was added to the sample in a 0.75:1:1 ratio, briefly vortexed and centrifuged at 12 000 *g* for 5 min. Phase separation was achieved after 5 min and the top phase removed and discarded. Equal volume of methanol was added to the bottom phase and mixed. The samples were centrifuged at 12 000 *g* for 10 min and the liquid removed from the precipitated protein. The protein pellets were air dried to remove residual methanol.

4.2.5.2. Sample digest

The samples were first reduced by dissolving in 10 μ L of 100 mM triethylammonium bicarbonate (TEAB) containing 4 M guanidine-HCl. Tris(carboxyethyl) phosphine (TCEP) was added to the samples at 50 mM in TEAB (final concentration 5 mM) and reduced at 60°C for 30 min. The reduced samples were cooled to room temperature and alkylated by adding 200 mM

iodoacetamide (IAA) in 100 mM TEAB (final concentration 20 mM). The samples were incubated with IAA in the dark for 30 min. After alkylation the samples were diluted to 70 μ L with 100 mM TEAB and 5 μ L trypsin solution was (1 mg/mL in 100 mM TEAB) added. Proteolysis was allowed to proceed for 18 hours at 37°C. After digestion, the samples were acidified with 1% trifluoroacetic acid (TFA) to a final concentration of 0.1%. The reaction mixture was reduced to dryness prior to desalting. All chemicals were supplied by Sigma-Aldrich (St. Louis, MO).

4.2.5.3. Desalting

The dried peptides were dissolved in 30 μ L 2% acetonitrile/water, 0.05% TFA (Solvent A) and bound to an in-house manufactured stage tip. Briefly, stage tips were prepared by placing a punched out disk of Empore (3 M) C18 solid phase extraction disc into a gel loader pipette tip. The membrane was activated with 100% methanol and equilibrated with solvent A. After the sample was loaded onto the solid phase extraction disc the bound sample was washed with 30 μ L solvent prior to elution with 60% acetonitrile/water, 0.05% TFA (solvent B). The desalted sample was evaporated to dryness.

4.2.5.4. Liquid Chromatography

Liquid chromatography was performed using a Thermo Scientific RSLCnano liquid chromatography system equipped with a 2cm C₁₈ trap column (2cm x 100 μ m, 5 μ m particles) and a Pepmap C₁₈ analytical column (25cm x 75 μ m, 3 μ m particles). The solvent system consisted of 2% acetonitrile/water, 0.17% formic acid (solvent A) and 80% acetonitrile/water; 0.17% formic acid (solvent B). The gradient was developed at 250 nL/min as follows: 0 - 10 min 2.5% B; 10 - 15 min 2.5% - 5% B; 15 - 130 min 5% - 35% B using non-linear gradient 6; 130 - 150 min 35% B - 50% B. The heated column compartment was operated at 50°C. A total of 10 μ L sample dissolved in solvent A was loaded for a total on-column load of 350 ng from a cooled autosampler set at 7°C.

4.2.5.5. Mass spectrometry of *C. pyralidae*'s exoproteome

Mass spectrometry was performed using a Thermo Scientific Fusion, a linear iontrap-orbitrap type mass spectrometer. The samples were infused into the mass spectrometer using a nanospray flex ion source. The samples entered the mass spectrometer through the ion transfer tube set at 300°C with 2.35 kV applied to the stainless steel emitter. MS1 spectra were acquired in positive mode using the orbitrap mass analyser over $m/z = 380 - 1500$ at 12 000 resolution with a trap fill time of 50 ms with AGC set to $4 \times E^5$. The acquired precursor ions were selected for fragmentation in the quadrupole section. Selection criteria include, mono isotopic ions only with charge state $+2 \pm 6$, exclude ion m/z once it has been fragmented for 140 s and mass tolerance of 10 pm. An isolation window of $m/z = 1.5$ was set with higher energy collision

dissociation (HCD) selected at 35% collision energy. Detection was performed in the ion trap mass analyser. First mass was set to $m/z = 120$ and AGC target to 1 E4. Maximum injection time was 40 ms with 1 microscan and centroid data recording. The obtained peptides were subjected to homology search against the proteomes of the yeast species *Candida albicans*, *Candida glabrata*, *Candida dubliniensis* and *P. anomala* strain K.

4.2.6. Amplification and cloning of *K. wickerhamii* *EXG1* and *SUN4* genes

Genomic DNA (gDNA) of *K. wickerhamii* CBS 2745 was extracted according to Hoffmann (1997) (37) and the *EXG1* and *SUN4* genes were amplified by PCR (Polymerase Chain Reaction) using the primers KwEXG1fw, KwEXG1rev and KwSUN4fw, KwSUN4rev, respectively (Table 4.2). The PCRs were performed in 50 μ L reaction mixtures consisting of 1 \times HF Buffer, 100 mM dNTPs, 10 mM of each primer, 100 ng of gDNA and 1 U of Phusion Polymerase (Thermo Scientific, Finland, Vantaa). The PCR conditions for the amplification of *KwEXG1* were: initial denaturation at 95°C, 5 min; 35 cycles of denaturation at 95°C, 30 s; annealing at 55°C, 30 s; extension at 72°C, 45 s and final extension at 72°C, 7 min. The amplification of *KwSUN4* was performed with initial denaturation at 95°C, 5 min; 35 cycles of denaturation at 95°C, 30 s; annealing at 56°C, 30 s; extension at 72°C, 1 min and final extension at 72°C, 7 min. Both amplifications were carried out using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA). The PCR products were resolved on a 1% agarose gel stained with ethidium bromide at 90 V for 50 min.

The *KwEXG1* and *KwSUN4* PCR products (1138 and 1188 bp, respectively) were excised from the gel and the DNA was extracted and purified using the QIAquick Gel Extraction Kit (Whitehead Scientific, Cape Town, South Africa) and ligated into pJET1.2 CloneJet™ PCR Cloning Kit (Thermo Scientific, Lithuania) following the manufacturers' protocol for blunt-end cloning. *Escherichia coli* DH5 α chemically competent cells were transformed with the ligation reactions according to the Promega Technical Manual TM042 (Promega, Fitchburg, WI) and positive clones were selected on Luria-Bertani agar plates (Biolab-Merck) supplemented with 100 mg/L ampicillin (Ampicillin Sodium Salt) (Sigma-Aldrich) and incubated at 37°C overnight. After plasmid extraction using the PureYield™ Plasmid Miniprep System (Promega) following the manufacturers' instructions, the pDNA was sent for sequencing at the Central Analytical Facility (Stellenbosch University, South Africa) with the primer pair pJET1.2fw and pJET1.2rev (Thermo Scientific) after the presence of the genes was confirmed by restriction digestion with *Bgl*II and *Xho*I (Roche Diagnostics).

The *KwSUN4* pDNA (released from the pJET1.2 vector by restriction digestion with *Bgl*II and *Xho*I) was ligated using T4 DNA ligase (Thermo Scientific) into the corresponding sites of a pCEL13 episomal plasmid (2 μ m Amp^R URA3 PGK1_P-PGK1_T) linearized with *Bgl*II and *Xho*I. The construct was then heterologously expressed in *S. cerevisiae* strains (Table 4.1).

The amplified *KwEXG1* gene was determined by DNA sequencing as a partial gene of 1138 bp. To obtain the 5' end of the *KwEXG1* gene, Inverse Polymerase Chain Reaction (Inverse PCR) and Nested Inverse Polymerase Chain Reaction (Nested PCR) were performed using the primers KwEXG1iPCRfw, KwEXG1iPCRrev and KwEXG1nestediPCRfw, KwEXG1nestediPCRrev, respectively (Table 4.2).

Table 4.2: List of primers used in this study

Primer name	Primer sequence 5' - 3'
KwEXG1fw	GTTTCATCACGCCTTCGTTGTT
KwEXG1rev	<u>CTCGAG</u> ¹ CTAGAATCCACATTGGTTTGGATA
KwSUN4fw	<u>AGATCT</u> ATGAAATTCACACTACAGCTCTTACTT
KwSUN4rev	<u>CTCGAGT</u> CAATAGAAAACAAAGTTGGCTGC
Nested and inverse PCR primers	
KwEXG1iPCRfw	TTCTGGGATCCAGCGGCGC
KwEXG1iPCRrev	TGAACGGTGTTGGCATCGGTGC
KwEXG1nestediPCRfw	ATGTCGGCAAATCCTGTTC
KwEXG1nestediPCRrev	TATCGAGGCCCAACTAGACG
KwEXG1 full gene primers	
KwEXG1Ffw	<u>AGATCT</u> ATGAACTTGATTCAGTTTGTGTCAG
KwEXG1rev	<u>CTCGAG</u> CTAGAATCCACATTGGTTTGGATA

¹Restriction sites

4.2.6.1. *K. wickerhamii* inverse and nested PCR

To obtain the beginning of the *KwEXG1* gene of *K. wickerhamii* CBS 2745, inverse and nested PCR were performed. The genomic DNA from *K. wickerhamii* CBS 2745 was extracted as described above and 200 ng of this DNA was digested in 50 μ L reaction mixtures with the enzymes *EcoRV*, *DraI* and *SpeI* (Roche Diagnostics) for 2 h at 37°C followed by inactivation of the restriction enzymes at 65°C for 20 min. The digested DNA fragments were then subjected to self-ligation at 16°C overnight in a 200 μ L reaction prepared with T4 DNA ligase (Promega). Inverse PCR was performed by firstly preparing a reaction with 2 μ L of the ligation reaction mix as template, 10 mM dNTPs, 10 μ M each of the primers *KwEXG1iPCRfw*, *KwEXG1iPCRrev* (Table 4.2) and double-distilled water to a final volume of 20 μ L; another reaction mixture consisted of 5 \times buffer A, 5 \times buffer B, 2 μ L of Elongase enzyme mix (Invitrogen, CA) and double distilled water to a final volume of 30 μ L was prepared. The two reactions were then mixed and amplification was carried out under the following PCR conditions: initial denaturation at 94°C, 30s; 35 cycles of denaturation at 94°C, 30s; annealing at 68°C, 5 min and 1 cycle of final extension at 68°C, 5 min. After amplification, the inverse-PCR reaction was diluted 100 times and 2 μ L thereof was used as template for the nested PCR which was prepared as described for the inverse-PCR. The nested-PCR products were then ran at 90 V for 50 min on a 0.8% agarose gel stained with ethidium bromide. The *EcoRV* fragment was then cloned into pGEM-T Easy Vector (Promega) and used to transform chemically competent cells of *E. coli* DH5 α and plated out on Luria-Bertani agar plates supplemented with 100 mg/L ampicillin (Ampicillin Sodium Salt), 0.5 mM IPTG (Isopropyl β -D-1-Thiogalactopyranoside) and 80 μ g/mL X-Gal (5-bromo-4-chloro-indolyl-galactopyranoside) (all chemicals from Sigma-Aldrich) and incubated at 37°C overnight. Plasmid DNA from the positive clones was extracted as described above and sent for sequencing at the Central Analytical Facility at Stellenbosch University using the primer pair T7 and SP6 (Promega).

The full *EXG1* gene (1284 bp) of *K. wickerhamii* was amplified by PCR with the primers *KwEXG1Ffw* and *KwEXG1rev* using *K. wickerhamii* gDNA extracted as described above. The PCR was performed in a 50- μ L reaction mixture consisting of 5 \times HF Buffer, 10 mM dNTPs, 10 mM of each primer, 100 ng of gDNA and 1 U of Phusion Polymerase (Thermo Scientific). The PCR conditions were: initial denaturation at 95°C, 5 min; 35 cycles of denaturation at 95°C, 30s; annealing at 56°C, 30s; extension at 72°C, 45s and final extension at 72°C, 7 min. The PCR product was resolved on a 1% agarose gel stained with ethidium bromide at 90 V for 50 min. All amplifications were carried out with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems).

Following electrophoresis, the PCR product was excised from the gel; purified using the QIAquick Gel Extraction Kit (Whitehead Scientific) and ligated into pJET1.2 CloneJet™PCR

Cloning Kit (Thermo Scientific) following the manufacturers' protocol for blunt-end cloning. *E. coli* DH5 α chemically competent cells were transformed with the ligation mixture according to the Promega Technical Manual TM042 (Promega) and plated out on Luria-Bertani agar plates supplemented with 100 mg/L ampicillin and incubated at 37°C overnight. Plasmid DNA extraction was carried out on positive clones using the GenElute™ Plasmid Miniprep Kit following the manufacturers' instructions, and sent for sequencing at the Central Analytical Facility with the primer pair pJET1.2fw and pJET1.2rev (Thermo Scientific). The *KwEXG1* gene was then released from the pJET1.2 vector by restriction digest with *Bgl*II and *Xho*I (Roche Diagnostics) and ligated to the corresponding sites of the linearized pCEL13 using T4 DNA ligase (Thermo Scientific). The construct was used for heterologous expression in *S. cerevisiae* strains (Table 4.1).

4.2.7. Heterologous transformation and expression of the *KwEXG1* and *KwSUN4* in *S. cerevisiae*

The *S. cerevisiae* strains BY4742, BY4742 Δ *exg1*, BY4742 Δ *sun4* and YHUM272 were transformed with the *KwEXG1*pCEL13 and *KwSUN4*pCEL13 constructs using an electroporation method (38). Positive recombinants were selected on minimal medium (0.67% Difco YNB without amino acids with ammonium sulphate, 2% glucose and 2% bacteriological agar plates) supplemented with 60 μ g/mL leucine, 60 μ g/mL histidine and 40 μ g/mL lysine for the BY4742, BY4742 Δ *exg1* and BY4742 Δ *sun4* recombinants, and 40 μ g/mL tryptophan, 60 μ g/mL leucine and 20 μ g/mL histidine for the YHUM272 recombinants. The plates were incubated at 30°C for 3 days. To confirm successful transformation of the yeast strains, the *KwEXG1* and *KwSUN4* genes were amplified by PCR using the primer pairs *KwEXG1*Ffw, *KwEXG1*rev and *KwSUN4*fw, *KwSUN4*rev respectively, as described above.

4.2.8. Killer activity and extracellular enzyme screening

The recombinants were screened for killer activity and the following extracellular enzymatic activity: β -glucanase, cellulase and glucosidase. All the yeast recombinants were grown in 5 mL Difco YNB without amino acids with ammonium sulphate supplemented with the amino acids as described above for 72 h with shaking on a test tube rotary shaker at 30°C. The yeast strains *K. wickerhamii* CBS 2745, *S. cerevisiae* strains BY4742, BY4742 Δ *exg1*, BY4742 Δ *sun4* and YHUM272 used as controls were grown on YPD for 24 h at 30°C with shaking on a rotary test tube shaker at 30°C. All the cultures were harvested by centrifugation at 4193 *g*, 5 min at room temperature. The cells were then re-suspended in the corresponding growth medium and 5 μ L of each culture was spotted on the agar plates used for killer activity and enzymatic screening assays.

Screening for killer and cellulase activities were carried out on all yeast cultures and recombinants. β -Glucanase and glucosidase activity screenings were performed on all control cultures and the *KwEXG1* and *KwSUN4* recombinants, respectively. The positive control for β -glucanase activity was 20 μ L of 0.1 mg/mL laminarase (Sigma-Aldrich) spotted in a 7-mm well drilled on the agar surface and 5 μ L of *Debaryomyces pseudopolymorphus* var. *africanus* CBS 8047 was spotted as the positive control for glucosidase activity screening. Killer activity screening was performed as described above except that 5 μ L of each culture was spotted on the agar plates.

Cellulase activity was screened on plates prepared with 1% carboxymethylcellulose (Sigma-Aldrich) as substrate and 4.5% YPD agar. β -Glucanase activity was screened on plates prepared with 0.2% glucose, 2% agar, 2% peptone and 0.1% laminarin (Sigma-Aldrich) as substrate. After spotting all the cultures, the plates were incubated at 30°C, for 7 days. Positive activity was observed by a zone of clearance around the spotted colony after the colonies were washed off with water and the plate stained with iodine solution (0.67% potassium iodide and 0.33% iodine).

Glucosidase activity was screened using arbutin (Sigma-Aldrich) and 4-Methylumbelliferyl- β -D-glucopyranoside (4-MUG) (Sigma-Aldrich) as substrates on agar plates. The arbutin plates were prepared with 4 \times strength bacteriological agar, 1% yeast extract, 2% peptone (Biolab-Merck, Wadeville, South Africa), 0.5% arbutin and 0.5% glucose (Merck). After sterilization of this medium at 121°C for 15 min, 1% ammonium ferric citrate (Saarchem, Krugersdorp, South Africa) was added. The 4-MUG plates were prepared with 0.67% Difco YNB with amino acids and ammonium sulphate, and 1% bacteriological agar. The medium was sterilized at 121°C for 15 min, after which 37 mmol/L 4-MUG was added. For both assays, 5 μ L of the respective recombinants and control cultures were spotted on the solidified agar plates. The plates were incubated at 30°C and positive activity was checked daily for three days. Glucosidase activity on arbutin plates was observed as a brown halo around the spotted colony and on 4-MUG plates, activity was observed as fluorescence around the spotted colony when viewed under UV light - 254 nm. The killer yeast strains *C. pyralidae* IWBT Y1140 and IWBT Y1057 were also screened for cellulase, β -glucanase and glucosidase activity as described above.

4.2.9. RNA extraction and *KwEXG1* and *KwSUN4* expression

RNA of all the cultures was extracted following the protocol described by Collart and Oliviero (39) with modifications. The extracted RNA samples were treated with DNaseI recombinant, RNase-free to digest any contaminating genomic DNA following the manufacturers' instructions (Roche Diagnostics). RNA concentrations were quantified using the NanoDrop®ND-1000 Spectrophotometer (Wilmington, DE). First strand cDNA was synthesized using the Improm-II™ Reverse Transcription System (Promega) following the manufacturers' instructions. The D1/D2

domains within the 26S rRNA region of the cDNA samples were amplified by PCR using the primer pair NL1 and NL4 (40). PCR amplification was performed in a 50 µL reaction mixture consisting of 1× Ex Taq Buffer, 10 mM dNTPs, 1 µM of each primer, 2 µL of each cDNA reaction and 2.5 U of ExTaq™ (TaKaRa, Shiga, Japan). The PCR conditions were: initial denaturation at 95°C, 5 min; 35 cycles of denaturation at 95°C, 30 s; annealing at 58°C, 45 s; extension at 72°C, 1 min and final extension at 72°C, 7 min. The PCR products were resolved on a 1% agarose gel stained with ethidium bromide at 100 V for 1 h.

To analyse the expression of the *KwEXG1* and *KwSUN4* genes, the cDNA samples corresponding to the respective gene were amplified by PCR using the primer pairs KwEXG1Ffw, KwEXG1Rev; and KwSUN4Fw, KwSUN4Rev respectively. PCR amplifications were carried out in a 50 µL reaction consisting of 1×Ex Taq buffer, 10 mM dNTPs, 1 µM of each primer and 2.5 U Ex-Taq. The PCR conditions were as described above and all amplifications were carried out with an Applied Biosystems 2720 Thermal Cycler. The PCR products were resolved as described on a 1% agarose gel stained with ethidium bromide at 100 V for 1 h.

4.3. Results

4.3.1. Mass spectrometry on *K. wickerhamii* and *C. pyralidae* exoproteomes

In the current study, the exoproteome of the yeasts *K. wickerhamii* and *C. pyralidae* was investigated. A similarity search for homologues of the peptide sequences obtained was conducted against the *Kluyveromyces lactis* proteome and several proteins were identified (mostly cell wall related proteins) (Table 4.3). In order to identify the proteins in *K. wickerhamii* whose proteome is unknown, the *K. lactis* gene sequences corresponding to the proteins tentatively identified were retrieved and a similarity search against the draft genome of *K. wickerhamii* was carried out. The *K. wickerhamii* genes thereby identified were translated and the peptides obtained by sequencing were matched once again against these new protein sequences. The function of the proteins was confirmed through comparison with those described for *S. cerevisiae*'s homologues (Table 4.3).

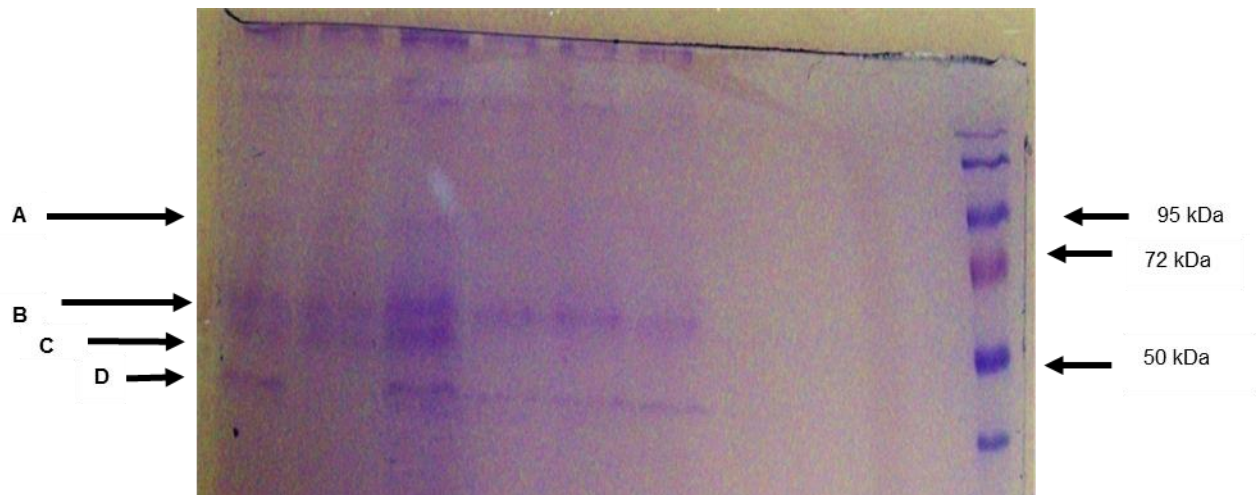


Figure 4.1: SDS-PAGE on the *K. wickerhamii* crude extract

The peptides obtained by sequencing of bands C and D (Figure 4.1) yielded 22 and 18 peptide hits and their function tentatively identified as exo- β -1,3-glucanase (band C) and β -D-glucosidase (band D), respectively. These peptide hits were much higher in comparison with the peptide hits obtained when searched against the *K. lactis* proteome, thereby confirming their tentative identity (Table 4.3). The genes encoding these proteins were named *KwEXG1* and *KwSUN4* respectively, based on their high similarity with *S. cerevisiae*'s proteins. These particular genes were chosen as the focus of the study based on the hypothesis that they could be associated with the killer phenotype as described in literature. Peptide sequences obtained in the CpKT1- and CpKT2-containing exoproteomes matched mostly with characterized and uncharacterized proteins found in the β -glucosidase (SUN) and glycosyl hydrolase family (Table 4.4).

Table 4.3: Peptide sequencing and protein identification of *K. wickerhamii*'s exoproteome

Accession number	Peptides hits against <i>K. lactis</i> 's proteome	Peptides hits obtained against <i>K. wickerhamii</i> 's proteome	Protein and function (as determined by sequence homology with proteins of <i>S. cerevisiae</i>)
Q6CW32	6	na ²	ScPir1: O-glycosylated protein required for cell wall stability; attached to the cell wall via beta-1,3-glucan.
Q6CLA9	6	18	ScSun4: Cell wall protein related to glucosidases, possibly involved in cell wall septation; member of the SUN family.
Q6CV57	1	na	ScTos1: Covalently-bound cell wall protein of unknown function; identified as a cell cycle regulated SBF target gene.
F2Z6B1	2	na	ScGnd2: 6-phosphogluconate dehydrogenase (decarboxylating) catalyzes an NADPH regenerating reaction in the pentose phosphate pathway.

Table 4.3 (continued)

Accession number	Peptides hits against <i>K. lactis</i> 's proteome	<i>K.</i>	Peptides hits obtained against <i>K. wickerhamii</i> 's proteome	Protein and function (as determined by sequence homology with proteins of <i>S. cerevisiae</i>)
Q70CP7	2		na ²	ScEno1: Enolase I, a phosphopyruvate hydratase that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis.
Q12628	1		22	ScExg1: Major exo-1,3-β-glucanase of the cell wall, involved in cell wall β-glucan assembly; exists as three differentially glycosylated isoenzymes

²Not applicable

Table 4.4: Peptide sequencing and protein identification of *C. pyralidae*'s exoproteome

Accession number	Protein	Peptide hits	Sequence coverage %	Function or Description
A5DBW2	Glycoside hydrolase	1	1.5	Family of eukaryotic β -1,3-glucanases belonging to the glycoside hydrolase, family 81. Hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety.
A5DK04	Uncharacterized protein	1	5.3	β -glucosidase (SUN family) involved in cell septation
C4YA41	Uncharacterized protein	1	4.8	Glycosyl hydrolase family 17. Endo-1,3- β -glucosidase; lichenase and exo-1,3-glucanase
G3AXT9	Uncharacterized protein	1	5.4	Glycosyl hydrolase family 76 (α -1,6-mannases)
FYV8	Required for yeast viability	1	3	Protein of unknown function. The <i>FYB8</i> gene is needed for survival upon exposure to K1 killer toxin.

4.3.2. Amplification and expression of *K. wickerhamii*'s *EXG1* and *SUN4* genes

Amplification of the *KwEXG1* gene from *K. wickerhamii*'s gDNA revealed that the 5' end of the gene was missing. In order to retrieve the 5' end of the gene, specific gene primers were designed and used for inverse and nested PCR, as described in the materials and methods section 4.2.6.1. Once the 5' end of the gene was obtained; the full *KwEXG1* gene was amplified, cloned and sequenced as was the *KwSUN4* gene from *K. wickerhamii*'s genomic DNA. The *KwEXG1* gene was found to be 1284 bp long, while the *KwSUN4* gene was 1188 bp long. The proteins found to be encoded by the *KwEXG1* and *KwSUN4* genes were aligned with their closest matching relatives (*K. lactis* accession number XP 452437.1 and *K. lactis* accession number 455280.1, respectively) found through a similarity homology search using Basic Local Alignment Search Tool (Figure 4.2).

The genes *KwEXG1* and *KwSUN4* were then heterologously expressed in *S. cerevisiae* strains YHUM272, BY4742, BY4742 Δ *exg1* and BY4742 Δ *sun4* (Figure 4.3). The recombinant strains were tested for killer activity as well as cellulase, exo-glucanase and β -glucosidase activity. The yeast strains BY4742, BY4742 Δ *exg1* and BY4742 Δ *sun4* and the respective recombinants did not exhibit killer activity against the *B. bruxellensis* strains IWBT Y102, IWBT Y111, IWBT Y135 and IWBT Y169 on WYE plates. The recombinant strains obtained with the *S. cerevisiae* strain YHUM272 also did not exhibit killer activity against *B. bruxellensis* IWBT Y169 on the same medium (the other *B. bruxellensis* strains were not tested). Furthermore, none of the recombinants displayed killer activity against *B. bruxellensis* IWBT Y169 on YNB medium. This was in contrast to the positive control (*K. wickerhamii*) which showed killer activity on all the media tested and sensitive strains tested (data not shown). When all the recombinant strains were screened for extracellular enzyme activity, none of them displayed activity compared to *K. wickerhamii* which displayed β -glucanase, glucosidase and cellulase activity. The *C. pyralidae* strains IWBT Y1140 and IWBT Y1057 strains displayed β -glucanase, glucosidase and cellulase activity (data not shown). In order to evaluate whether the absence of killer activity was not due to an absence of expression, RNA extraction was performed on the recombinant and wild type strains. PCRs targeting the D1/D2 region of the 26S rRNA (used as positive control), *KwEXG1* and *KwSUN4* in the recombinant and wild type strains were carried out. Only the positive control could be detected (Figure 4.4).

```

KwEXG1p      MNLIQFVSVVTLVISACLATPIPLSKRYFDYEGYVVRGVNLGGWLLLEPFITPSLF EAFR
K.lactis     MLSMQVVSLISLLVSVCLAQPLPLSKRYFEYENYKVRGVNLGGWLVLEPFITPSLFETFR
*   :*.**:::*.**.*.*** :*.*****:*.**.* *****:*****:***

KwEXG1p      TNPYNDGIPVDEYHYWEALGKDLALERLTQHWSTFYTEQDFADMAQAGLNVVRI PVGYW
K.lactis     TNEYNDGIPYDEYHYCQYLGEDLARDRLKQHWSTWITEADFEDISNTGLNTRVRIPIGYW
** ***** ***** : **.*** :**.***:* ** ** *:::***.***:***

KwEXG1p      AFQTLSDPYQSQQQEQYLDQAIAWSKKHGLKVVVDLHGAAGSQNGFDNSGIRDQILFQQ
K.lactis     AFELLDDDPYVSGLQEAYLDQAI EWARSYGLKVVVDLHGAPGSQNGFDNSGLRDQVEFQQ
**:* **.*** ** ** ***** *:::*****.*****:***: **

KwEXG1p      DENLAVTKSVLAYLLEKYSQDDVVDTVIGIELLNEPLGPVIDVDKLKEFNNWAYDYMRNT
K.lactis     DGNWDVFKNVLAYVIEKYSRDEFTDTVVGV EVLNEPLGPVIDMDKLKELYNWAYDYLRND
* * * *.***:***:*.**.***:*.*:*****:*****: *****:***

KwEXG1p      LGRDQNI VIHDAFQAFNYWDDQLTLEQGAYGVTVDH HHYQVFSPELARSIDEHISVACD
K.lactis     LQRDQILVIHDAFQKANYFDDQLTVEQGAFGLVDH HHYQVFSPEEVGRTIDEHISV VCE
* *** :***** **.***:***.***.* ***** **.*:*****.*:

KwEXG1p      WGKGAIGEGHWNVAGEWSAALTDCAKWLNGVIGARYDGSFFKNGDGSYYIGSCEGNKEI
K.lactis     QGKETL TEAHWNVVG EWSAALTDCTKWLNGVIGARYDGSFVKNQDTSYWIGSCEGSQDI
** :*.***.*****:*****.*** * **.***.*:***:***:***

KwEXG1p      DTWSDDRKADYRRYIEAQLDAFELKGGWIYWTYKTENLIDWDFQRLTFNQLFPQPLDERW
K.lactis     STWTSDDKNDYRKYIEAQLDAYEIRNGWIYWCYKTEDTLEWDYRKLVSGLFPQPLTNRQ
.*::.*:* :**.***.*:*.**.*** ***** :**.*:***. . ***** :*

KwEXG1p      YPNQCGFSTOP
K.lactis     FPNQCSSTY--
:****. :

(a)

KwSUN4p      MKFTTALTSVSLITASLASALP--HAKREEDCSTTVHSHHKHKRAVAVEYVYQTVTVNGQ
K.lactis     MRFTTALASASFISAAMVSALPHAHAKREDCSTTVHAHKKHKRAVAVEYVYQTVTVNGQ
*.***.*:*.**.*:*.**.* ***** *****:*****:*****

KwSUN4p      GETLAATTTAATTTAATTTAAETTAETTAAVTTEEQQPTTTLEPHTTSAQEETSTSETS
K.lactis     GETIAPATVTSTEEAATTSSTEDQQTTTLEPSTSSSEESSSSSSSSSSSSQSSTESSTEAS
***:*.*:*.**.* *****:*.**.* : * . *:::***.***.***:***

KwSUN4p      AAAQSSSTSSSSSSNAGSG-----TFEDGVLSCDEFPSAQGVVSLDWLG
K.lactis     ESSTSSQSSSSSSSGSTSTSTSSSGDLSWFSSPTEEFEDGVMSCDEFPSGQGVVLDWLG
:*.**.*:*****:*.**.* *****.*****.*** *****

KwSUN4p      FGGWSGIENS DGSTGGSCKEGSYCSYACQPGMSKTQWP AEQPANGVSVGGLLCKNGKLYR
K.lactis     FGGWSGIENS DGSTGGTCKEGSYCSYACQPGMSKTQYPSDQPSNGVSI GGLLCKNGKLYR
*****:*****:***:***.***:*****

KwSUN4p      SNTNTEDLCVSGVGSANVVNKLSDVVSICRTDYPGTENMVIPTIVEAGSSQPLTVVDQDS
K.lactis     SNTDEKYLCTW GIDSAYVVNELSDVVSICRTDYPGTENMVIPTIVNGSKLPLTVVDQDT
***: : **.* :*.** *****:*****:***.*****:

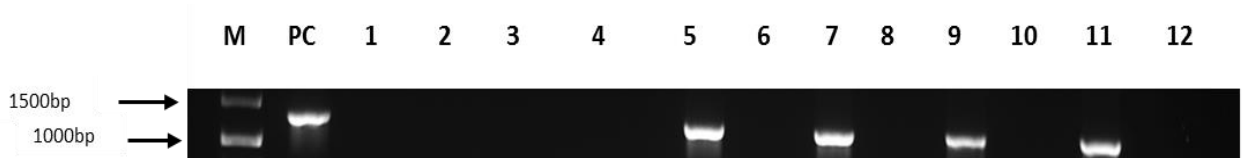
KwSUN4p      YYVWQGMK TSAQYV VNNAGVSKEDGCIWGTEGSGIGNWAPL NFGAGYTNGISYLSLIPNP
K.lactis     YVTWQGMK TSAQYV VNNAGVSKEDGCVWGTSGSGIGNWAPL NFGAGATGGISYLSLIPNP
**.******:***.***** *.******

KwSUN4p      NNREAAFNVKI VAADGATVIGDCVYENGSYNGNGQDGCTVSVTSGAANFV FYSTOP
K.lactis     NNREAAFNVKI VAADGGVIGECVYENGSYNG--GSDGCTVSVTSGAANFVLYD---
*****:***.***** *.******:*.**.*

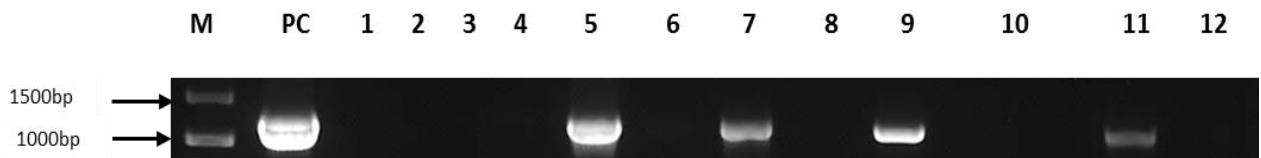
```

(b)

Figure 4.2: Alignment of the KwExg1p (a) and KwSun4p (b) with their closest relatives (XP452437.1 and XP 455280.1 from *K. lactis*, respectively)

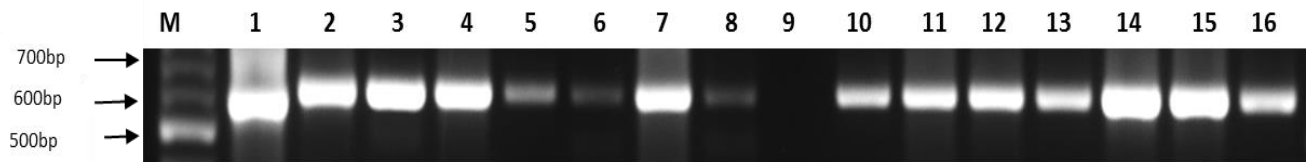


(a) *K. wickerhamii* *EXG1* amplification

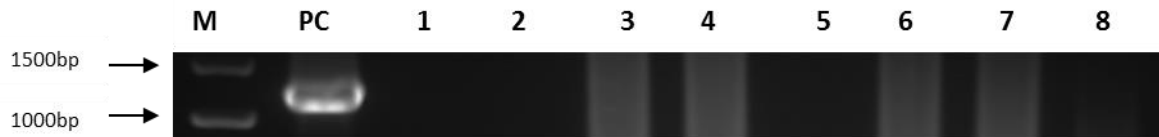


(b) *K. wickerhamii* *SUN4* amplification

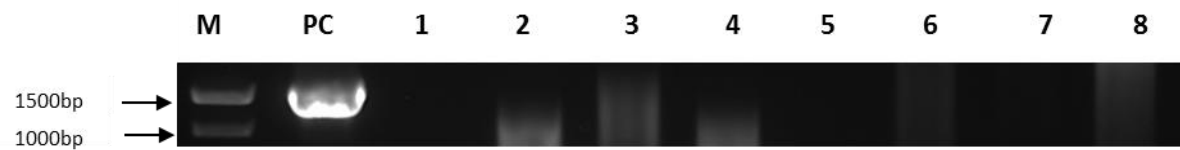
Figure 4.3: Heterologous expression of *K. wickerhamii* *EXG1* and *SUN4* genes (a) *K. wickerhamii* *EXG1* amplification: M = 1 kb DNA Ladder, (PC) = *K. wickerhamii* gDNA, 1 = BY4742, 2 = BY4742 Δ *exg1*, 3 = YHUM272, 4 = pCEL13, 5 = *KwEXG1*+pCEL13, 6 = BY4742+pCEL13, 7 = BY4742+*KwEXG1*, 8 = BY4742 Δ *exg1*+pCEL13, 9 = BY4742 Δ *exg1*+*KwEXG1*, 10 = YHUM272+pCEL13, 11 = YHUM272+*KwEXG1*, 12 = Negative Control (b) *K. wickerhamii* *SUN4* amplification: M = 1 kb DNA Ladder, (PC) = *K. wickerhamii* gDNA, 1 = BY4742, 2 = BY4742 Δ *sun4*, 3 = YHUM272, 4 = pCEL13, 5 = *KwSUN4*+pCEL13, 6 = BY4742+pCEL13 7 = BY4742+*KwSUN4*, 8 = BY4742 Δ *sun4*+pCEL13, 9 = BY4742 Δ *sun4*+*KwSUN4*, 10 = YHUM272+pCEL13, 11 = YHUM272+*KwSUN4*, 12 = Negative Control



(a) Amplification of the D1/D2 region of the 26S rRNA



(b) *KwEXG1* expression in *S. cerevisiae* laboratory strains



(c) *KwSUN4* expression in *S. cerevisiae* laboratory strains

Figure 4.4: cDNA analysis of the *KwEXG1* and *KwSUN4* constructs (a) Amplification of the D1/D2 region of the 26S rRNA: M = 100bp PLUS DNA Ladder, 1 = *K. wickerhamii*, 2 = BY4742, 3 = BY4742 Δ exg1, 4 = BY4742 Δ sun4, 5 = YHUM272, 6 = BY4742+pCEL13, 7 = BY4742+*KwEXG1*, 8 = BY4742+*KwSUN4*, 9 = Negative Control, 10 = BY4742 Δ exg1+pCEL13, 11 = BY4742 Δ exg1+*KwEXG1*, 12 = BY4742 Δ sun4+pCEL13, 13 = BY4742 Δ sun4+*KwSUN4*, 14 = YHUM272+pCEL13, 15 = YHUM272+*KwEXG1*, 16 = YHUM272+*KwSUN4* (b) *KwEXG1* expression in *S. cerevisiae* laboratory strains: M = 1 kb DNA Ladder, PC = *KwEXG1*+pCEL13, 1 = Negative control, 2 = CBS 2745, 3 = BY4742, 4 = BY4742 Δ exg1, 5 = YHUM272, 6 = BY4742+*KwEXG1*, 7 = BY4742 Δ exg1 +*KwEXG1*, 8 = YHUM272+*KwEXG1* (c) *KwSUN4* expression in *S. cerevisiae* laboratory strains: M = 1 kb DNA Ladder, PC = *KwSUN4*+pCEL13, 1 = Negative control, 2 = CBS 2745, 3 = BY4742, 4 = BY4742 Δ sun4, 5 = YHUM272, 6 = BY4742+*KwSUN4*, 7 = BY4742 Δ sun4+*KwSUN4*, 8 = YHUM272+*KwSUN4*

4.4. Discussion and conclusion

In the previous chapter (Chapter 3), the killer toxins CpKT1 and CpKT2 were shown to be killer active against *B. bruxellensis* or *Dekkera* spp. strains under winemaking conditions. The killer toxin Kwkt was also shown to exhibit the same phenotype against several *B. bruxellensis* strains by Comitini et al. (33). To investigate this killer phenotype further, the extracellular protein crude extracts (exoproteomes) of *K. wickerhamii* CBS 2745 and *C. pyralidae* IWBT Y1140 and IWBT Y1057 consisting of the killer toxins Kwkt, CpKT1 and CpKT2, respectively were analysed. Within the exoproteomes of *K. wickerhamii* and *C. pyralidae* the following enzymes were tentatively identified: exo- β -glucanases, glucosidase, transferase, enolase I, 6-phosphogluconate dehydrogenase in addition to a killer protein and uncharacterized proteins (the latter two only found in the exoproteomes consisting of the CpKT1 and CpKT2 killer toxins) (Tables 4.3 and 4.4). Proteins associated with cell wall assembly, remodelling, maintenance or function such as glucanases (Scw4p, Scw11p, Bgl2p and Exg2p), transglucosylases (Gas1p) and chitinases (Cts1p) are typically found within the yeast secretome (5, 6) and the identification of these proteins in this study was therefore not surprising.

Exo- β -glucanase, glucosidase and transferase enzymes in the exoproteomes of *K. wickerhamii* and *C. pyralidae* identified in Tables 4.3 and 4.4 were of interest in this study as these could potentially be correlated with the killer phenomenon mainly due to their hydrolytic activity on yeast cell wall components. The genes *KwEXG1* and *KwSUN4* encoding the exo-glucanase and glucosidase proteins, respectively were identified in the *K. wickerhamii*'s exoproteome. In literature, these categories of hydrolytic enzymes are shown to be associated with killer activity (12-15, 41, 42). For instance, Oro et al. (11) proved that both Kpkt and β -glucanase secreted by *T. phaffii* are encoded by the same gene *TpBGL2*, a β -glucanase gene.

β -Glucanases play a role during cell wall re-generation e.g. in the extension and re-arrangement of β -1,3-glucan chains and cross linking of these polymers with other cell components. These enzymes also exhibit transglycosylase activity such as Bgl2p of *S. cerevisiae* which catalyses a transferase reaction by introducing the β -1,6 linkages into the β -1,3-glucan of a fungal cell wall (6). The *SUN4* gene family is reported to have homology with the β -glucosidase enzyme and plays a role in cell separation (43). These cell wall related proteins are considered as core enzymes in the formation and integrity of the yeast cell wall. *S. cerevisiae* Exg1 is reported as a constitutive exo- β -glucanase initially secreted into the periplasmic space and partly secreted into the growth medium (7). Muccilli et al. (12) showed that a *S. cerevisiae* mutant strain deficient of the *KRE1* gene associated with β -glucan synthesis was sensitive to the killer toxins of *W. anomalus*, thereby confirming the relationship between killer activity and exo-glucanase

activity. Indeed, the function of exoglucanases and glucosidases does resemble in part the degradation of the cell wall of a sensitive yeast strain by a killer toxin.

Based on these previous reports, we speculated that *KwEXG1* and *KwSUN4* could be the genes encoding the proteins responsible for the killer activity found in the exoproteome of Kwkt. They were subsequently amplified, cloned and transformed into killer-negative *S. cerevisiae* strains. The recombinant strains were tested for killer and enzymatic activity. No killer or enzymatic activity was detected when screened on white grape juice, YNB; and laminarin, CMC, arbutin and 4-MUG agar plates, respectively. The absence of killer activity or hydrolytic enzymatic activity could be attributed to the following - lack of transcription of the *KwEXG1* and *KwSUN4* genes, lack of recognition of the native secretion signal by the host, lack of translation because of codon bias and incorrect post-translational modifications. In this study, only the potential lack of transcription was evaluated. The results are however inconclusive, as only the housekeeping gene used (i.e. 26S rRNA) was detected, this could be the amplification of the rRNA itself and not that of the mRNA of this gene. Indeed, no amplicon of the cDNA of *KwEXG1* and *KwSUN4* could be detected; therefore further experiments are required to confirm whether these genes encode killer toxins. This would show whether the potential candidates isolated in this study possess killer activity or not, thereby confirming or disputing literature that suggests that killer activity is linked to exoglucanase or glucosidase activity (at least for these candidates).

The *C. pyralidae* strains tested in this study exhibited both glucosidase and killer activities. When the exoproteomes containing the killer toxins CpKT1 and CpKT2 were analysed, they were found to contain mostly proteins related to the glucosidases (Table 4.4). The results of our study serve as a good indication that at least one of the secreted glucosidase proteins of *C. pyralidae* could potentially be the killer toxins or be linked to the killer phenotype in these strains. Indeed, Guyard et al. (42) found that the killer toxin WmKT secreted by *Williopsis saturnus* var. *mrakii* is related to the yeast SUN proteins. Several attempts including cation and anion exchange and gel filtration chromatography were made to purify the killer toxins Kwkt, CpKT1 and CpKT2 without any success (data not shown). However, other researchers have in the past been able to purify the Kwkt killer toxin through ion exchange chromatography (44). Further optimisation is therefore required to finally identify the killer toxins studied at gene and protein levels. The investigation of the genetic origins and identification of Kwkt, CpKT1 and CpKT2 is hindered by the lack of proper annotation of the genome of *K. wickerhamii* and genetic data of *C. pyralidae*, respectively which would certainly facilitate the identification of the secreted proteins or killer toxins.

4.5. References

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CHAPTER 5

Exposure to killer toxins of *Kluyveromyces wickerhamii* and *Candida pyralidae* induces cell surface damage in *Brettanomyces bruxellensis* cells

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Exposure to killer toxins of *Kluyveromyces wickerhamii* and *Candida pyralidae* induces cell surface damage in *Brettanomyces bruxellensis* cells

Abstract

The frequent contamination of red wine by *Brettanomyces bruxellensis* has established itself as a rather semi-permanent problem for winemakers. Consequently, the control of *B. bruxellensis* using biological methods such as killer toxins has been the focus of research studies within the last decade. The use of killer toxins has been suggested as an efficient and effective solution. Indeed, various killer toxins secreted by non-*Saccharomyces* yeasts have been shown to have potential as biological additives that can be applied during winemaking to eliminate *B. bruxellensis*. In particular, the killer toxins CpKT1 and CpKT2 isolated from *Candida pyralidae* and the Kwkt killer toxin from *Kluyveromyces wickerhamii* have been shown to be active and stable under winemaking conditions.

In this study, we report for the first time on the mode of action of non-*Saccharomyces* killer toxins on *B. bruxellensis* cells. The killer toxins Kwkt, CpKT1 and CpKT2 suppressed the population of *B. bruxellensis* within a 48 h period in red grape juice medium. Determination of the receptors of these toxins through a competitive binding assay and aniline blue staining, suggested that the killer toxins CpKT1 and CpKT2 have binding affinity for β -1,3-glucan. Using scanning electron microscopy and propidium iodide viability staining coupled with fluorescence microscopy revealed that CpKT1 and CpKT2 induced cell surface damage and concomitant plasma membrane damage, while the Kwkt toxin only showed cell surface damage. The cells had indentations, cracks, punctures or pores, wrinkles and appeared to be peeling after exposure to the killer toxins Kwkt, CpKT1 and CpKT2.

5.1. Introduction

Red wine contamination with the yeast *Brettanomyces bruxellensis* is a long established problem in winemaking (1, 2). Contamination of wine with this yeast imparts off-flavours and off-odours due to the production of volatile phenol compounds (1). Detection of *B. bruxellensis* during the early stages of winemaking is challenging due to its slow growth (3) and its ability enter into a viable but non-culturable state (VNBC) (4). Although the yeast can be detected using molecular biology techniques and culture independent methods (5), its proliferation in wine undergoing aging is still prevalent. The yeast is known to thrive in conditions of low pH,

high ethanol, low sugar concentration and low of nutrient levels found during aging (6). This makes controlling *B. bruxellensis* a challenge for winemakers.

For decades, winemakers have been using the chemical preservative sulphur dioxide (SO₂) for its antioxidant and antimicrobial properties (7). SO₂ is added at concentrations of 30 mg/L of free SO₂ to release between 0.4 to 0.8 mg/L of molecular SO₂ (8-10). However, molecular SO₂ is dependent on the pH of the wine. The antimicrobial property of SO₂ is further dependent on the molecular concentration of SO₂ and the *B. bruxellensis* strain to be eliminated (11). Curtin et al. (12) found that *B. bruxellensis* isolates present genotype-dependent tolerance to sulphite. In recent years, it has been shown that the growth of *B. bruxellensis* under winemaking conditions can be controlled through the use of killer toxins. The killer toxins Kwkt, Pikt, PMKT, CpKT1, CpKT2 and KP6 secreted by the yeasts *K. wickerhamii*, *Pichia anomala*, *Pichia membranifaciens*, *C. pyralidae* and the filamentous fungus *Ustilago maydis*, respectively could be used as partial alternatives to SO₂ (13-16). Killer toxins are glycoproteins secreted by *Saccharomyces* and non-*Saccharomyces* yeasts which inhibit or kill sensitive cells albeit the toxin-producing strain remains immune to its own killer toxins (17-19).

Killer toxins inhibit the growth or kill sensitive cells either through cell membrane permeabilization, cell cycle arrest or inhibition of DNA synthesis, inhibition of β -1,3-glucan synthase activity or hydrolysis of the major cell wall components such as β -1,3-glucans and 1,6-glucans (18-20). They bind to either cell wall receptors (primary receptors) or membrane receptors (secondary receptors) on sensitive cells to induce their killing action (20). The receptors serve as translocation intermediates of the killer toxins to reach their target sites on or in the sensitive yeast cell (21).

The killer toxins Kwkt, Pikt, Kpkt, HMK, K5 and PMKT secreted by the yeasts *T. phaffii*, *Hansenula mrakii* (re-classified *Williopsis mrakii*), *P. anomala*, *P. membranifaciens* (the latter four killer toxins) and those secreted by *Debaryomyces hansenii* and *Hanseniaspora uvarum* interact with the primary receptors β -D-1,3-glucan and β -D-1,6-glucan (19, 22-31). While the killer toxins PMKT2 and K28 secreted by *P. membranifaciens* and *S. cerevisiae*, respectively and the killer toxins secreted by *Schwanniomyces occidentalis*, *Zygosaccharomyces bailii* (14, 32, 33) bind to mannoproteins on the cell wall of the sensitive yeast cells. Chitin has been identified as the primary receptor for the PaT killer toxin secreted by *Pichia acaciae* and the killer toxins secreted by *Kluyveromyces lactis*, *Pichia inositovora* (re-classified as *Babjeviella inositovora*) (18, 34, 35). Sensitive cells whose cell wall composition has been degraded by killer toxins are osmotically fragile and defective resulting in lytic cell death (36). In particular, the killer toxin HMK or HM-1 is reported to inhibit β -1,3-glucan synthase activity *in vitro* (37, 38).

Ionophoric killer toxins such as PMKT, K1, K2, HM-1, SMKT and KP6 secreted by *P. membranifaciens*, *S. cerevisiae*, *H. mrakii*, *Pichia farinosa* and *U. maydis*, respectively form pores or channels through the cell membrane of sensitive cells (39-43). These killer toxins firstly bind to the primary receptors followed by binding to the secondary receptors of the sensitive cell (19, 44). Thereafter, the toxin penetrates the cytoplasmic membrane and creates ion channels. These ion channels or pores disrupt the electrochemical potential of the sensitive cells resulting in the termination of amino acid transport and proton pumping, in addition to the leakage of potassium ions, ATP, cellular material and to a decrease in intracellular pH (21, 39, 42, 45).

The killer toxins of *Kluyveromyces lactis* and K28 of *Saccharomyces cerevisiae* have a different mode of action in comparison to the other killer toxins. These killer toxins act intracellularly on the sensitive cells. They inhibit DNA synthesis by blocking the completion of the G1 phase of the cell cycle (46-48). Similarly, the killer toxins secreted by the yeasts *Pichia acaciae* and *Wingea robertsiae* (synonym *Debaryomyces robertsiae*) arrest the cell cycle at the S phase (49) which is also reported to be the mode of action of the killer toxin PMKT2 (50).

In the first research chapter of this dissertation (Chapter 3), the killer activity of the toxins CpKT1 and CpKT2 against several *B. bruxellensis* strains as well as the pH and temperature stability and activity of these toxins, was demonstrated. The aim of this current study was to investigate the mode of action of the toxins Kwkt, CpKT1 and CpKT2 on *B. bruxellensis* cells.

5.2. Materials and methods

5.2.1. Killer toxin production and killer activity assay in solid medium

The killer toxins Kwkt, CpKT1 and CpKT2 secreted by the yeast species *K. wickerhamii* and *C. pyralidae* were produced as described in the previous chapter (Chapter 4). The killer toxins were tested for killer activity on solid medium using the seeded agar method on commercial red table grape juice medium adjusted to pH 4.5 with 2 M NaOH. The *B. bruxellensis* IWBT Y169 sensitive strain (16) was inoculated at a concentration of 10^6 cfu/mL in 7.5 mL of red grape medium. The inoculated medium was mixed with 4% bacteriological agar (kept at 50°C) to a final volume of 10 mL and after brief vortexing, the medium was poured into sterile Petri dishes.

Twenty microliters of each killer toxin were spotted into 7-mm wells drilled on solidified red grape juice agar plates. The plates were incubated at 20°C and killer activity was observed as a zone of clearance (growth inhibition zone) around the 7-mm wells on triplicate plates. Killer activity was measured in mm as the total diameter of the clear zone and well minus the 7-mm well diameter. The concentration of the toxin resulting in a zone of clearance was calculated in arbitrary units as follows; 1 AU = 1 mm zone of clearance generated from spotting 20 µL of toxin.

5.2.2. Killer activity assay in liquid medium

Killer activity of the toxins Kwkt, CpKT1 and CpKT2 was tested in broth using commercial red table grape juice medium adjusted to pH 4.5 with 2 M NaOH (killer assay medium). The *B. bruxellensis* IWBT Y169 was used as the sensitive strain (16). The strain was firstly pre-cultured in 5 mL of the killer assay medium for 24 h at 30°C with shaking on a test tube rotator. Five hundred microliters of the pre-culture was inoculated in 50 mL killer assay medium and grown at 30°C with shaking at 150 rpm till exponential phase. The cells were harvested by centrifugation at 4193 g, for 5 min at room temperature and re-suspended in 1 mL of the killer assay medium to a final concentration of 10⁵ cfu/mL. Sterile test tubes were prepared and to each tube the following were added: killer assay medium, 1 mL of the re-suspended sensitive cells and 100 AU/mL of each killer toxin to a final volume of 10 mL (except for the control test tubes into which no killer toxin was added).

All the test tubes were briefly vortexed after inoculation and 100 µL samples were collected from each tube and serially diluted and plated out on YPD agar (Yeast Peptone Dextrose agar, Biolab-Merck, Wadeville, South Africa) plates. The test tubes were then incubated at 20°C without shaking and 100 µL was sampled as described above after 12, 24, 48 and 120 h. The YPD agar plates were incubated at 30°C for 5 days. The experiment was performed on biological triplicates.

5.2.3. Effect of Kwkt, CpKT1 and CpKT2 killer toxins on the cell surface of sensitive cells

B. bruxellensis IWBT Y169 cells (10⁵ cfu/mL) in exponential phase grown as described in section 5.2.2 were harvested at 4193 g, 5 min at room temperature. The pellet was re-suspended in 1 mL of 1× PBS buffer (phosphate buffered saline, pH=7.4). Two hundred and fifty microliters of the cells were aliquoted into sterile microfuge tubes and stained with the fluorescent dyes: propidium iodide, aniline blue (Sigma-Aldrich, St. Louis, MO) and concanavalin A Alexa Fluor[®] (Life Technologies, Carlsbad, CA).

To test for membrane damage, 0.5 µL of propidium iodide was added to 250 µL of cells. A 1% stock solution of aniline blue was prepared with sodium phosphate buffer (0.07 M, pH=9), and 3 µL of this dye was added to 250 µL of the cells to investigate the effect of the killer toxins on β-

glucans. Concanavalin A Alexa Fluor[®] 647 was added to 250 μ L cells to a final concentration of 0.1 mg/mL in order to test the presence and distribution of mannoproteins. All the stained cells were analysed at the Central Analytical Facility at Stellenbosch University. The cells stained with propidium iodide were visualized using the Olympus IX-81 microscope equipped with a 572 nm excitation filter with U/B/G triple band pass emission filter. Cell[^]R Imaging software was used to visualize the images. The aniline blue and concanavalin A Alexa Fluor[®] 647 stained cells were visualized using the Carl Zeiss Confocal LSM 780 Elyra SI microscope. The aniline blue was excited using a 405 nm laser and detected with a violet PMT in the range 410-525 nm, and concanavalin A Alexa Fluor[®] 647 was excited with a 633 nm laser and emission detected with the GaAsP detector in the range 639-691 nm. The images were visualized and edited with the software ZEN 2012 Digital Imaging using the control sample as the reference. The experiment was carried out on biological duplicates and cell imaging was done on statistical triplicates defined as three different visual areas on each biological sample. A cell population of 55 - 56 for each visual area was used as the standard to measure the fluorescence intensity for aniline blue stained cells, where the number of samples (n) = 330 - 331 cells.

Cell wall morphology damage was investigated on exponentially growing cells at 10^5 cfu/mL exposed to the killer toxins Kwkt, CpKT1 and CpKT2 in red grape juice medium. The cells were grown for 56 h under the same conditions as described in section 5.2.2. Sample preparation was carried out by modification of the protocol proposed by Hazrin-Chong and Manefield (51) with the guidelines reported by Wright (52). The cells were harvested at 4193 g for 4 min at room temperature and washed twice with distilled water. The cells were fixed with 2.5% glutaraldehyde at 4°C for 2.5 h. The cells were then washed twice, firstly with 1 \times PBS buffer followed by distilled water. The cells were dehydrated with increasing amount of ethanol (i.e. 30, 50, 70, 90, 95 and 100%) for 3 min for each concentration of ethanol and centrifuged at 700 g, 3.5 min at room temperature. The cell pellets (samples) were dried with a Critical Point Drier under CO₂. The samples were mounted on a stub with double sided carbon tape. The sample was then coated with a thin layer of gold in order to make the sample surface electrically conducting. The samples were then viewed using a Leo[®] 1430VP Scanning Electron Microscope. Beam conditions during surface analysis were 7 kV and approximately 1.5 nA, with a spot size of 150.

5.2.4. Competitive binding assays

The binding affinity of the killer toxins to polysaccharides was tested by competitive binding to Laminarin from *Laminaria digitata* (Sigma-Aldrich). A stock solution of 200 mg/L of the polysaccharide was prepared by dissolving it in distilled water and filtered through a 0.22- μ m cellulose acetate membrane (Starlab Scientific, Celtic Molecular Diagnostics, Cape Town, South Africa). The polysaccharide was added to a final concentration of 10, 50 and 100 mg/mL

to 100 AU/mL of each killer toxin. The reactions were gently vortexed and tested for residual activity immediately after inoculation and 6 h later after incubation at 20°C without shaking. The reactions were centrifuged at 11 700 *g* for 1 min at room temperature and 20 µL of each reaction was spotted on white grape juice agar plates (pH 4.5) prepared using the seeded agar method as described in section 5.2.1. The plates were incubated at 20°C and residual killer activity was observed as a zone of clearance (growth inhibition zone) around the 7-mm wells on triplicate plates. Killer activity was measured in mm as the total diameter of the clear zone and well minus the 7-mm well diameter. The experiment was performed on biological triplicates.

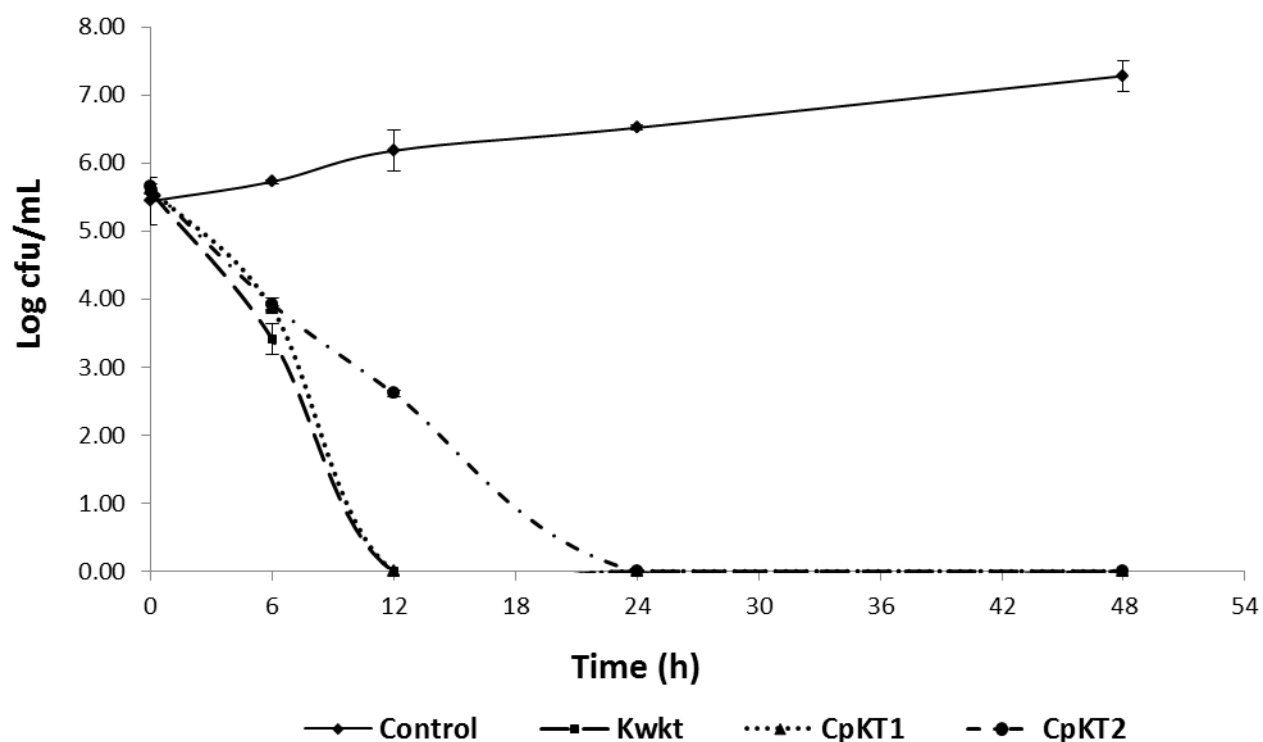
5.3. Results

5.3.1. Killer activity assay in solid and liquid medium

The yeast species *K. wickerhamii* and *C. pyralidae* which secrete the killer toxins Kwkt, CpKT1 and CpKT2, respectively were cultured as described in section 5.2.1. The culture supernatant containing the killer toxins was collected and concentrated. After concentration, the killer toxins Kwkt, CpKT1 and CpKT2 resulted in killer activity between 700 - 783 AU/mL against *B. bruxellensis* on solid medium (Table 5.1). This killer activity was also observed in liquid medium when 100 AU/mL of the toxins was inoculated into 10⁵ cfu/mL killer toxin sensitive cells (Figure 5.1). The viable cell population declined by one logarithmic unit after being exposed to the killer toxins for 6 h. Kwkt and CpKT1 resulted in 100% loss of viability in 12 h in contrast to CpKT2 which took 24 h to eliminate the *B. bruxellensis* population. This killer activity was maintained till 48 h (Figure 5.1). At 120 h, all the samples treated with killer toxins Kwkt, CpKT1 and CpKT2 had grown to 2.83×10³, 4.80×10³ and 2.44×10⁴ cfu/mL, respectively. This population increase was nevertheless lower than the population of the control which was 6.80×10⁷ cfu/mL at this time (data not shown).

Table 5.1: Killer activity assay of Kwkt, CpKT1 and CpKT2 on solid medium

Killer toxin	Killer yeast species	secreting	Zone clearance (mm)	of AU/mL
Kwkt	<i>K. wickerhamii</i>		14	700
CpKT1	<i>C. pyralidae</i>		16	783
CpKT2	<i>C. pyralidae</i>		15	733

**Figure 5.1:** Growth response of *B. bruxellensis* cells following exposure to Kwkt, CpKT1 and CpKT2 in red grape juice medium

5.3.2. Effect of Kwkt, CpKT1 and CpKT2 killer toxins on the cell surface of sensitive cells

Cell viability was assessed using propidium iodide on *B. bruxellensis* cells exposed to the killer toxins for 24 h (Table 5.2). The cells treated with the CpKT1 and CpKT2 exhibited 79.73% and 91% infiltration by propidium iodide in comparison to the Kwkt treated cells where only 5.16% of the cells were infiltrated by the dye. The diffusion of aniline blue into the toxin treated cells resulted in 91.99%, 79.8% and 77.37% fluorescence intensity in the cells treated with Kwkt, CpKT1 and CpKT2 killer toxins, respectively (Figure 5.2). Cells stained with concanavalin A are shown in Figure 5.3. In the control, the fluorescence was observed around the cell periphery and appeared to be uniformly distributed in contrast to the Kwkt treated cells. The CpKT1

treated cells exhibited similar uptake of the dye as the control although in some cells the dye had diffused inside the cells. The CpKT2 treated cells clumped together when stained with the dye and fluorescence was observed around the cell periphery as well as inside the cells.

Table 5.2: Kwkt, CpKT1 and CpKT2 plasma membrane permeabilization of sensitive cells

Treatment	Propidium Iodide stained cells (%)	Standard deviation
Control	0	0
Kwkt	5.16	3.3
CpKT1	79.73	12.7
CpKT2	97	0.5

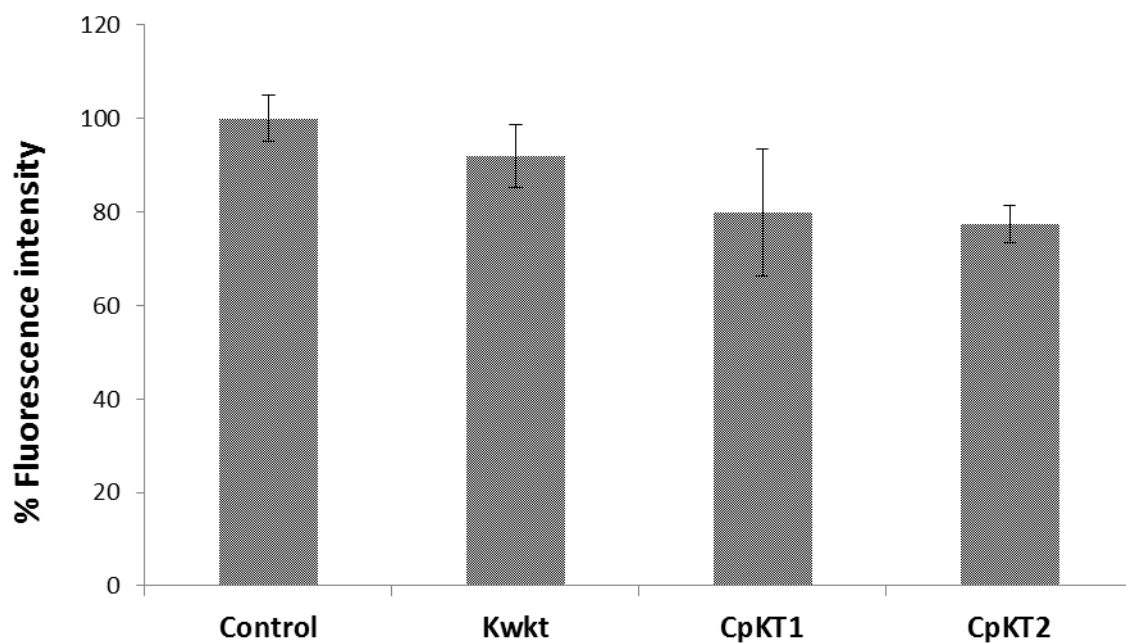


Figure 5.2: Diffusion of aniline blue on Kwkt, CpKT1 and CpKT2 treated sensitive cells

Using scanning electron microscopy, the surface of cells exposed to the different toxins investigated in this study was visualised (Figure 5.4). After 24 h exposure, the control cells displayed a smooth and uniform appearance. In contrast, the Kwkt treated cells exhibited a wrinkled cell surface in addition to having punctures and indentations on the cell surface. Similarly, the CpKT1 treated cells were also wrinkled with a few cells exhibiting punctures and indentations on the cell surface. The CpKT2 treated cells did not exhibit any wrinkles but appeared to have cracks, indentations and some cells showed signs of peeling off the cell surface (Figure 5.4). After 56 h exposure, the control had similar features as at 24 h. At the same time point, all the killer toxin treated cells appeared to be similar to those in the unexposed control: the cell surface looked overall smooth with much less indentations and punctures. The cells appeared to have no more surface damage such as peeling or cracks (data not shown).

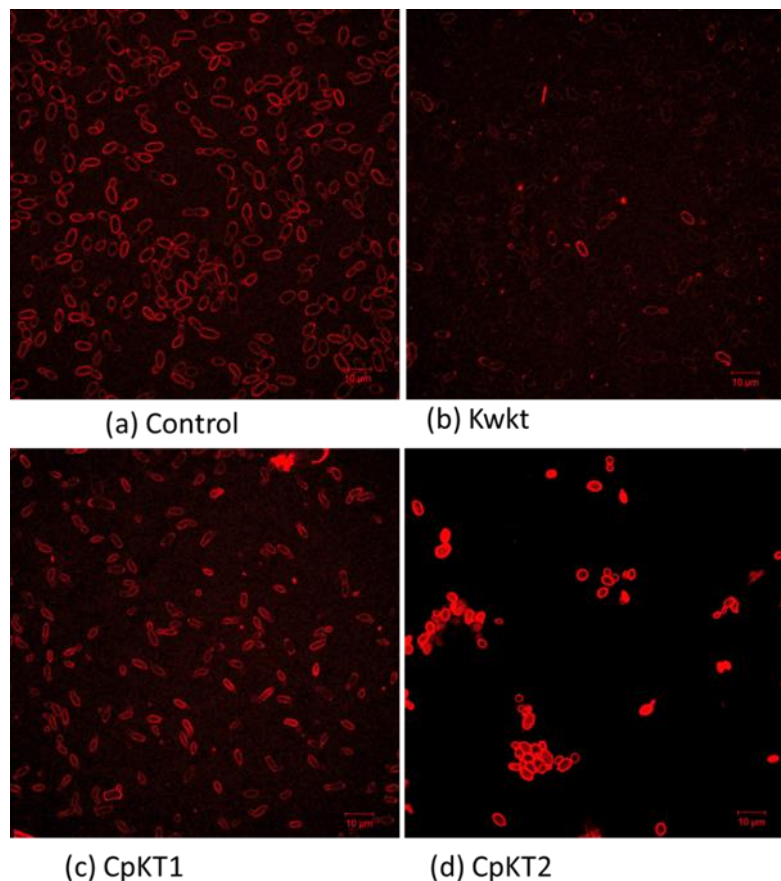


Figure 5.3: Kwkt, CpKT1 and CpKT2 binding to mannoproteins of *B. bruxellensis* sensitive cells; (a) Control - *B. bruxellensis* IWBT Y169 cells not treated with killer toxins (b) *B. bruxellensis* IWBT Y169 cells treated with Kwkt (c) *B. bruxellensis* IWBT Y169 cells treated with CpKT1 and (d) *B. bruxellensis* IWBT Y169 cells treated with CpKT2

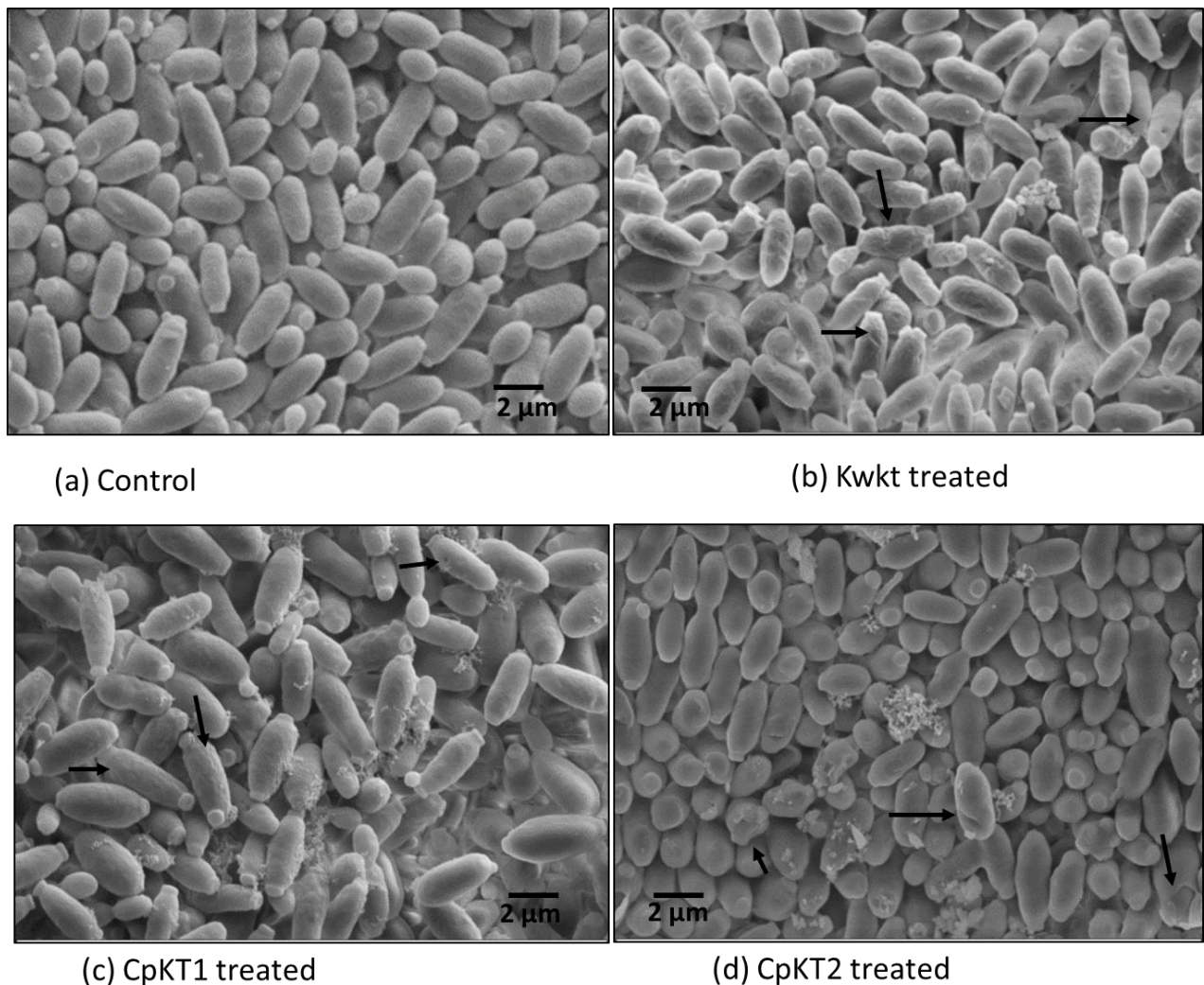


Figure 5.4: Cell surface damage on *B. bruxellensis* cells after 24 h of exposure to the killer toxins Kwkt, CpKT1 and CpKT2; (a) Control - *B. bruxellensis* IWBT Y169 cells not treated with killer toxins (b) *B. bruxellensis* IWBT Y169 cells treated with Kwkt (c) *B. bruxellensis* IWBT Y169 cells treated with CpKT1 and (d) *B. bruxellensis* IWBT Y169 cells treated with CpKT2. Arrows indicate cell damage observed on the surface of the cells.

5.3.3. Competitive binding assays

A competitive binding assay using 10, 50 and 100 mg/mL laminarin was conducted to determine whether β -1,3 and β -1,6-glucans are the binding receptors for the killer toxins CpKT1 and CpKT2 (Figure 5.5). In the presence of 10 mg/mL laminarin, CpKT1 and CpKT2 exhibited 89.77 and 88.26% residual activity, respectively. In 50 and 100 mg/mL, CpKT1 resulted in residual activity of 89.21% and 90.32% while CpKT2 resulted in 81.26% and 82.21% residual activity, respectively. The control (without killer toxin added) resulted in residual activity between 92 and 95%. The binding of the killer toxins to the polysaccharides mannan and pullulan was also tested as described in section 5.2.4, but no reduction in activity was observed (data not shown).

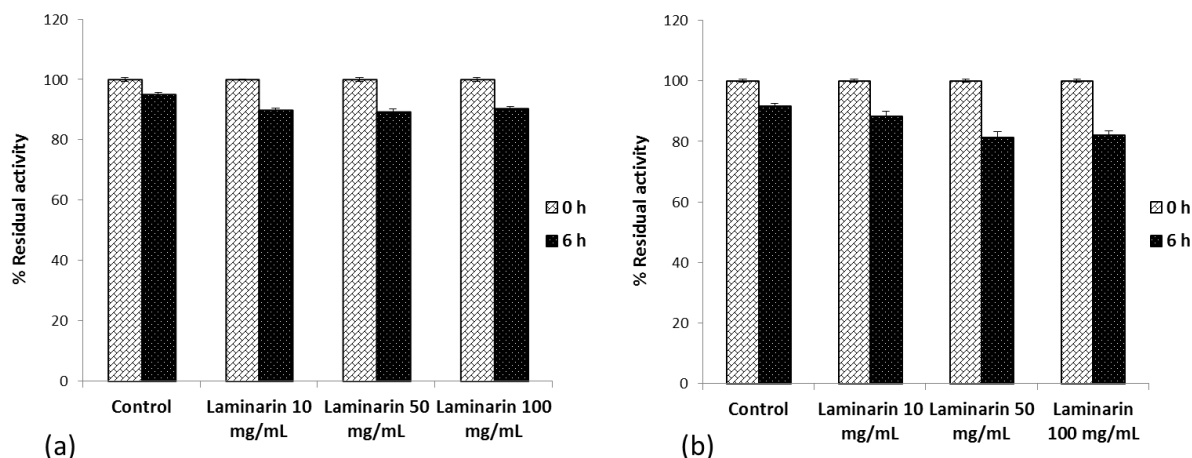


Figure 5.5: (a) CpKT1 and (b) CpKT2 binding affinity to laminarin

5.4. Discussion and conclusion

In the first results chapter of this dissertation (Chapter 3), the killer toxins: CpKT1 and CpKT2 secreted by the yeast *C. pyralidae* were characterized for their biochemical and environmental activity and stability. These killer toxins showed fungistatic activity against several *B. bruxellensis* strains and were stable and active under winemaking conditions. In this chapter, the activity of these killer toxins was tested in red grape juice liquid medium against the strain *B. bruxellensis* IWBT Y169, to simulate the conditions found in red grape juice used for winemaking.

A concentration of 100 AU/mL of the killer toxins Kwkt, CpKT1 and CpKT2 resulted in fungistatic effect after 24 h exposure (Figure 5.1). However, this killer effect could not be maintained beyond 48 h. Comitini et al. (13) showed that the killer toxins Pikt and Kwkt resulted in a decline in the population of *Dekkera bruxellensis* from 3×10^5 cfu/mL to between 1.2 and 7.2×10^2 cfu/mL in Sangiovese wine when added at concentrations of 143 and 286 AU/mL. However, killer activity of these killer toxins was more pronounced in buffered YPD in comparison to wine. The Kwkt toxin added at 28.6 and 57.2 AU/mL resulted in a fungistatic effect in comparison to Pikt which only showed the same effect at a concentration of 57.2 AU/mL after 24 h (13). The use of a purified Kwkt at concentrations of 12 and 24 AU/mL resulted in the elimination of *D. bruxellensis* after 7 and 4 days in grape must respectively, which was maintained till day 20 of the experiment (53). When the killer toxin PMKT2 was added at 2000 AU/mL in grape must inoculated with *B. bruxellensis* strains, death rates of 0.13 h^{-1} , 0.09 h^{-1} and 0.11 h^{-1} were observed within 10 h (14). In the current study, the addition of 100 AU/mL Kwkt is equivalent to the addition of 28.6 AU/mL Kwkt used by Comitini et al. (13). This concentration eliminated *B. bruxellensis* population within 24 h in both studies.

However, in our current study, the addition of 100 AU/mL did not seem to have a prolonged effect as it was observed that at 120 h, a significant *B. bruxellensis* population between 2.83×10^3 and 2.44×10^4 cfu/mL could be detected. The authors Comitini et al. (2004, 2011) (13, 53) did not report on the resurgence of the *B. bruxellensis* following exposure to the Kwkt killer toxin. The proliferating population of *B. bruxellensis* cells could be cells that were static in their growth following exposure to the killer toxins, but regained viability. This could be attributed to the decline of the killer toxin concentration over time as well as the use of a different medium and sensitive strain. However, this needs to be confirmed by monitoring the residual activity of the killer toxins over time in the culture supernatant. Furthermore, as shown in the above studies (13, 53) and in other studies (50, 54), killer toxin activity is dosage dependent. Therefore, it would be worthwhile to test the activity of the killer toxins CpKT1 and CpKT2 at different toxin concentrations.

The effect of the killer toxins on the yeast cells of *B. bruxellensis* was determined through fluorescent and scanning electron microscopy. The cells treated with the killer toxins CpKT1 and CpKT2 exhibited cell membrane damage as observed by the percentage of cells stained with propidium iodide (Table 5.2). Killer toxins are reported to exert their killer effect either through plasma membrane permeabilization, cell wall hydrolysis, inhibition of DNA or inhibition of β -glucan synthase (19). The killer toxins CpKT1 and CpKT2 but not Kwkt would seem to exert their killer activity through membrane permeabilization, as the propidium iodide only infiltrates cells whose membrane is damaged. Guyard et al. (22) showed that cells treated with the killer toxin WmKT secreted by *Williopsis saturnus* var. *mrakii* were permeable to propidium iodide which suggested that the plasma membrane of the sensitive cells was damaged, similar to what was observed in the current study. Other killer toxins that induce plasma membrane damage include: PMKT, KP6, K1, K2 and SMKT. The activity of these toxins led to the leakage of cellular components, metabolites and decrease in pH (39-41, 43, 55). Whether the killer toxins CpKT1 and CpKT2 exert the same response post membrane permeabilization remains to be investigated.

Cells treated with the killer toxins Kwkt, CpKT1 and CpKT2 displayed a wrinkled cell surface with indentations, punctures and cracks (Figure 5.4). According to the authors' knowledge, this is the first report on cell surface damage of *B. bruxellensis* cells induced by killer toxins. Many studies conducted so far have used *S. cerevisiae* as the model organism to investigate the effect of killer toxins on yeast cells (26, 27, 40, 43, 45, 50, 56) except for Santos and Marquina (39) who investigated the effect of killer toxins on *Candida boidinii*. *S. cerevisiae* cells treated with killer toxins exhibit cell wall disruption, retraction of the plasma membrane, cracks, pores (56), roughness (27), cell shrinkage (56, 57). This is in agreement with what was observed in the current study, where the cells exhibited punctures, indentations and wrinkles (Figure 5.4).

In order to determine the primary receptors of the killer toxins, competitive binding assays were conducted. β -1,6-Glucan has previously been shown to be the primary receptor for the killer toxin Kwkt (25), therefore its binding affinity to polysaccharides was not tested in this study. The killer toxins CpKT1 and CpKT2 showed some affinity for laminarin, which is mainly composed of β -1,3-glucans and some β -1,6-glucans (Figure 5.5). However, it should be noted that the toxins were only exposed to laminarin for a period of 6 h to minimize decline of killer activity over a longer period of time. To confirm this affinity further, cells exposed to the killer toxins CpKT1 and CpKT2 for 24 h, were stained with aniline blue, a fluorescent dye which binds to β -glucans (58). We observed that the Kwkt toxin treated cells had a fluorescence intensity of 91.99% which was higher than the CpKT1 and CpKT2 treated cells, which was 79.8% and 77.37%, respectively. This suggests that CpKT1 and CpKT2 have stronger affinity for laminarin than Kwkt. The CpKT1 and CpKT2 treated cells showed 20.2% and 22.63% less fluorescence intensity, respectively when compared to the control (Figure 5.2). This indicates that a portion of the β -glucan of the sensitive cells was bound by the toxins, further confirming the results obtained with the laminarin binding assay.

The toxin treated cells were also stained with concanavalin A which binds to α -linked mannose homopolymers (59). The mannoprotein layer of the untreated cells (control) appeared uniform as observed by the even distribution of fluorescence around the cell periphery. This was however not observed with the Kwkt treated cells, where no fluorescence was observed. This would suggest that the toxin bound to the mannoproteins of these cells, thereby preventing access of concanavalin A to the mannoproteins. This is in contradiction to previous results reported in literature (25). CpKT2 treated cells appeared to absorb the dye more than CpKT1 treated cells. This suggests that the two toxins have different levels of binding affinity to mannoproteins. This result was however not confirmed through the binding assay with mannan.

The results obtained in the current study suggest that both β -glucans and mannoproteins are involved in the mode of action of the CpKT1 and CpKT2 toxins. This would suggest that the killer toxins' mode of action proceeds in a step wise manner. The killer toxins firstly bind to the mannoproteins causing some form of disintegration followed by binding to the dense layer of β -glucan which is degraded exposing the plasma membrane. The killer toxins then permeate the plasma membrane and induce their killer effect on the cell. This is very much similar to what has been reported by Magliani (18) for ionophoric killer toxins (e.g. K1 and K2). With regards to Kwkt, our binding assay results contradict those reported previously in literature. This could be attributed to different experimental procedures conducted to identify the receptor sites of the killer toxin.

In conclusion, killer activity of Kwkt, CpKT1 and CpKT2 on *B. bruxellensis* was confirmed in liquid medium (i.e. red grape juice). Furthermore, these toxins were shown to have an impact on

the cell surface and on the plasma membrane (for the latter, only for cells treated with CpKT1 and CpKT2). CpKT1 and CpKT2 seemed to target mannoproteins and β -glucans on the cell walls, thereby explaining the observations made under scanning electron microscopy. For future work, we suggest that different concentrations of the killer toxins be evaluated. These would most probably result in prolonged and stronger killer activity than that observed in this study. In addition, investigating leakage of cellular metabolites in cells treated with the CpKT1 and CpKT2 would also result in a better understanding of the mode of action of these killer toxins.

5.6. References

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CHAPTER 6

General discussion and conclusions

General discussion and conclusions

6.1. General discussion

Saccharomyces cerevisiae and non-*Saccharomyces* yeasts play an important role during wine making. The yeasts metabolize the grape constituents to release flavour and aroma compounds in addition to ethanol (1-4). Certain non-*Saccharomyces* yeasts e.g. *Brettanomyces bruxellensis* are however not desirable due to their negative effect on wine organoleptic quality. Indeed, the latter yeast enzymatically converts hydroxycinnamic acids present in grape juice and wine to volatile phenols (5-8), which impart off-odours described as horse sweat, mousy, band aid, phenolic and leather (9, 10).

During the early stages of winemaking, *B. bruxellensis* cannot be detected by routine microbiological tests or techniques (11). This is mainly attributed to the slow growth character of *B. bruxellensis* and the ability of this yeast to enter into a viable but non-culturable state (VBNC) (10, 12-14). Furthermore, *B. bruxellensis* is known to thrive under conditions of high ethanol, low sugar concentrations, low pH and low nutrient levels found during red wine ageing (15). *B. bruxellensis* is mainly controlled through the use of SO₂, a chemical preservative (16). Free SO₂ added at 30 mg/L to release between 0.4 - 0.8 mg/mL molecular SO₂ is typically regarded as sufficient to inhibit spoilage microorganisms including *B. bruxellensis* in wine. However, the amount of molecular SO₂ released is highly dependent on the pH of the wine (15, 16). The efficiency of molecular SO₂ is also dependent on low temperature and the *B. bruxellensis* strains to be inhibited (17) as some strains have been reported to be tolerant to SO₂ (18, 19). These factors contribute to the challenge in controlling *B. bruxellensis*.

Potential alternatives such as the use of killer toxins have been suggested to inhibit the growth of *B. bruxellensis* in wine (20-22). Killer toxins are antimicrobial proteinaceous compounds secreted by *S. cerevisiae* and non-*Saccharomyces* yeasts that inhibit the growth of other yeasts (23, 24). The main aims of the study were to investigate and characterize on a protein and genetic level killer toxins secreted by certain wine non-*Saccharomyces* yeasts against *B. bruxellensis*, as well as unravelling their mode of action.

We identified two *Candida pyralidae* yeast strains previously isolated from the wine environment at the Institute for Wine Biotechnology (IWBT) at Stellenbosch University, South Africa which showed strong killer activity against *B. bruxellensis* strains as did the yeast species *Kluyveromyces wickerhamii* CBS 2745 (used as a control). The *C. pyralidae* IWBT Y1140 and IWBT Y1057 strains were then cultured and the supernatant concentrated and partially purified. The novel killer toxins present within these crude extracts of *C. pyralidae* IWBT Y1140 and IWBT Y1057 were designated CpKT1 and CpKT2, respectively. Both killer toxins were found to be proteinaceous in nature with a molecular weight above 50 kDa. The killer toxins were then

characterized for their biochemical and environmental activity and stability (Kwkt was previously characterized in literature (20)), and mode of action. The CpKT1 and CpKT2 were found to be active and stable at pH 3.5 - 4.5 and temperature ranging between 15 and 25°C, which correspond to wine environmental conditions. The killer toxins were not affected by ethanol and sugar concentrations found during winemaking. These properties are similar to those of other killer toxins such as Kwkt, Pikt, PMTK2 and KP6 that have been tested against *B. bruxellensis* (20-22). The killer toxin Kpkt, active against the apiculate yeast *Hanseniaspora uvarum* was also found to be active at pH and temperature ranges between 3 - 5 and 20 - 30°C, respectively (25). The results obtained in this study, confirmed the nature and properties of killer toxins (24). The activity of the killer toxins CpKT1 and CpKT2 was inactivated in the presence of a proteolytic enzyme and when exposed to high temperatures as has been reported for other killer toxins (20, 21, 25, 26). The results obtained in this study revealed that although the killer toxins CpKT1 and CpKT2 are secreted by the same yeast species they differ in their biochemical activity and stability. This has been shown with the killer toxins PMKT and PMKT2 secreted by *Pichia membranifaciens* and the killer toxins secreted by *Wickerhamomyces anomalus* (former *Pichia anomala*) and *Williopsis mrakii* (21, 23, 27-30) yeast strains. Furthermore, our findings suggest that these killer toxins could be used as partial alternatives to sulphur dioxide in winemaking to eliminate *B. bruxellensis*.

The killer toxins CpKT1 and CpKT2 displayed binding affinity to laminarin *in vitro* and were also confirmed to bind to β -glucan and mannoproteins on the cell wall of sensitive yeasts. In contrast, the Kwkt toxin was found to bind to mannoproteins of the sensitive yeasts' cells, which is contrary to what Ciani and Comitini (3) reported. This could be attributed to different conditions under which the binding affinity assays of this killer toxin were determined. The β -glucan of the cells treated with Kwkt was not affected as observed by the fluorescence intensity of aniline blue. In contrast, the killer toxin was bound to the mannoproteins of *B. bruxellensis* cells as observed by the absence of concanavalin A fluorescence in these treated cells. Other killer toxins have been found to have mannoproteins, β -1,3-glucans and β -1,6-glucans as receptors (21, 27, 31-34), as was observed with the killer toxins Kwkt, CpKT1 and CpKT2. These receptors play an important role in the mode of action of killer toxins. They serve as the first interaction of the killer toxins with the sensitive yeast cell surface (35, 36). The toxin binds to the receptors and is translocated to the target site where it induces its killer activity (24).

The killer toxins CpKT1 and CpKT2 induced plasma membrane damage to sensitive cells. Furthermore, the surface of the cells exposed to the killer toxins CpKT1, CpKT2 and Kwkt had wrinkles, punctures, indentations, cracks and peeling as observed through a scanning electron microscope. This is in agreement with what was observed by the authors Comitini et al. (37) and Vadasz et al. (38), where the killer toxins Kpkt and K2 resulted in cell wall disruption, and a rippled cell surface accompanied by cracks and pores. Our findings are in agreement with what

is reported as other modes of action of killer toxins i.e. plasma membrane damage (39-41) and the degradation of cell wall components (27, 35, 36). Our findings suggest that the mechanism of action of the killer toxins CpKT1 and CpKT2 is mediated firstly by the degradation of the sensitive yeast cell wall components - mannoproteins and β -glucan, followed by damage of the plasma membrane. This is similar to the mode of action described by Marquina, 2002; Magliani, 1997 and Liu et al. 2013 (32, 35, 36). The mode of action of Kwkt, is however, not associated with plasma membrane damage as observed by the lack of propidium iodide infiltration of the cells treated with the toxin. This toxin could therefore possibly act through the degradation of the mannoprotein layer of the sensitive cells as observed in our results rendering the cells susceptible to damage by the killer toxin. Further investigation into the mode of action of Kwkt, CpKT1 and CpKT2 revealed that the growth of the cells treated with the toxins was suppressed within 24 h after exposure to the killer toxins. However, the cells recovered after a few days which alluded to the fungistatic effect of these toxins. This indicates that the dosage applied to test the mode of action could not sustain a prolonged toxic effect. The effectiveness of higher dosages needs to be investigated.

An attempt to identify the killer toxins Kwkt, CpKT1 and CpKT2 at molecular level was made. Indeed, Kwkt has not been identified at protein and genetic levels although it has been characterized by Comitini et al. (20), while the killer toxins CpKT1 and CpKT2 are novel, therefore only been characterized in this study. Kwkt, CpKT1 and CpKT2 were investigated at protein and genetic levels (for the latter, investigation was conducted only on Kwkt). The extracellular crude extracts (exoproteomes) of the yeasts *K. wickerhamii* and *C. pyralidae* secreting the killer toxins Kwkt, CpKT1 and CpKT2, respectively were analysed following peptide sequencing. The exoproteomes revealed the presence of exo-glucanase, β -glucosidase, enolase I and 6-phosphogluconate dehydrogenase enzymes within *K. wickerhamii*'s exoproteome while the exoproteome of the *C. pyralidae* IWBT Y1140 and IWBT Y1057 strains revealed an abundance of proteins within the glucosidase SUN family.

Glucanases are primarily involved in cell wall regeneration and rearrangement of β -1,3-glucan chains and the introduction of β -1,6 linkages into the β -1,3-glucan of the fungal cell wall, while glucosidases mainly drive cell septation (42, 43). These enzymes have also recently been linked to the killer phenotype in non-*Saccharomyces* yeasts (28, 29, 44-47). In *K. wickerhamii*'s exoproteome, we identified two putative candidates as potential killer toxins with exo-glucanase and glucosidase activity. The two candidates were an exo-glucanase and a β -glucosidase encoded by the genes *KwEXG1* and *KwSUN4*. When these genes were cloned and expressed in *S. cerevisiae* mutant strains deficient of the respective gene, they did not exhibit killer activity nor glucanase, cellulase or glucosidase activity. We subsequently found that the genes *KwEXG1* and *KwSUN4* were not transcribed, and we could not conclude whether these genes possess both killer activity, glucanase and glucosidase activity, respectively. The exoproteomes

consisting of the killer toxins CpKT1 and CpKT2 were not identified to genetic level but we could postulate that the killer phenotype of these killer toxins could be linked to glucosidase enzymes due to the high abundance of these enzymes in the analysed exoproteomes. In literature, many studies have indicated that certain killer toxins also possess exo-glucanase activity (28, 29, 44-46). Oro et al. (47) confirmed this hypothesis, as the authors showed that the killer toxin Kpkt and an exo-glucanase secreted by *Tetrapisispora phaffii* were encoded by the same gene *TpBGL2*.

Within the wine industry, the use of chemical preservatives to control *B. bruxellensis* has been found to have limited efficiency. Although SO₂ cannot be completely eliminated because of its antioxidant properties, partial alternatives to control *B. bruxellensis* in wine are actively sought. The killer toxins isolated in this study show potential for application under winemaking conditions as they inhibit the growth of *B. bruxellensis* and are active and stable at wine pH and temperature ranges. However, their direct application to wine is yet to be fully investigated as these killer toxins are currently not available in their pure form and their identification and characterization is still incomplete with regards to testing the activity and stability of these toxins under real wine conditions.

6.2. Future prospects

In order for these killer toxins to be applied to the wine industry, further research is needed. The killer toxins CpKT1 and CpKT2 need to be purified as this would allow for the characterization of the killer toxins under real wine making conditions when added as technological aids. Furthermore, this would allow investigating the impact of different concentrations, determining the shelf life of the toxins in wine and assessing whether these killer toxins could eliminate *B. bruxellensis* in wines already contaminated with this yeast in addition to controlling or preventing its growth. This would indeed prove their efficacy in wine in comparison to other known killer toxins.

With regards to academic purposes, the genetic origin of the killer toxins Kwkt, CpKT1 and CpKT2 needs to be identified. Molecular biology techniques such as cloning and heterologous expression tried in this study for the identification of the Kwkt genetic origin need to be conducted again as they did not yield satisfactory results. This could be coupled with cloning and expression in a different host after confirmation that the constructs are integrated properly in the vector and in the host organism. We further suggest that the known genome of the yeast *K. wickerhamii* be annotated as this would aid in the identification of the genetic origin of the Kwkt killer toxin. The genome of *C. pyralidae* is unknown. The investigation of this yeasts' genome would assist in the identification of these killer toxins to genetic and protein level. An investigation on whether extracellular enzymes secreted by the yeasts *K. wickerhamii* and *C. pyralidae* also possess killer activity would prove relevant in the study of the yeast

secretome. Finally, study of the cell wall composition and structure of the sensitive yeast strains would lead to a better understanding of the killer toxin interaction with sensitive yeasts.

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ADDENDUM

Non-*Saccharomyces* killer toxins: possible biocontrol agents against *Brettanomyces* in wine?

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Non-*Saccharomyces* killer toxins: possible biocontrol agents against *Brettanomyces* in wine?

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ABSTRACT

Red wine spoiled by the yeast *Brettanomyces bruxellensis* is characterized 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 (SO₂). However, the concentration of SO₂, pH of wine, the presence of SO₂-binding chemical compounds in wine as well as the strain of *B. bruxellensis*, determine the effectiveness of SO₂. Other chemical preservatives have been tested but are not much more efficient than SO₂ 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 the 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 could potentially be exploited to inhibit 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 is controlled mainly through the use of sulphur dioxide (SO₂). However, the antimicrobial property of SO₂ 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 SO₂-binding compounds in wine, the concentration of molecular SO₂ drops and the effectiveness of SO₂ becomes limited. Furthermore, yeast species and strains have been reported to exhibit a range of tolerance levels vis-à-vis SO₂ (Curtin *et al.*, 2012b). Chemical treatments (e.g. benzoic acid and

sorbic acid), physical techniques (e.g. filtration, sanitisation) and the 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 SO₂ by some wine consumers has spurred the demand for the use of non-chemical preservatives (Comitini *et al.*, 2004a; Lustrato *et al.*, 2006). Alternative methods are therefore currently 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; Santos, *et al.*, 2011).

Killer toxins are proteinaceous antimicrobial compounds produced by yeasts and are active against members of the same species or closely related species (Lowe *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 broad 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) could thus 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 *et al.*, 2001). Killer toxins have indeed been shown to have potential applications in food, agriculture and medical industries (Palpacelli *et al.*, 1991; Goretti *et al.*, 2009; Liu & Tsao, 2009; Lowe *et al.*, 2000; Walker *et al.*, 1995; Cailliez *et al.*, 1994). 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* produce 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 characterized 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-SO₂ above 30 mg/L (Oelofse *et al.*, 2008a). It has also been reported that *B. bruxellensis* can enter into a viable but non-culturable (VBNC) state, which is characterized 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 SO₂ 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 SO₂ concentration at this stage. Furthermore, the availability of residual sugars, assimilable nitrogen-containing compounds (although limited), as well as in micro-aerobic conditions found during ageing in wooden barrels after MLF also support the proliferation of *B. bruxellensis* (Chatonnet *et al.*, 1995; Comitini *et al.*, 2004b; Ciani *et al.*, 2003; Oelofse, 2008a).

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 varieties that contain precursor phenolics e.g. non-flavonoid and flavonoid

(Wedral *et al.*, 2010; Monagas *et al.*, 2006). 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 are therefore 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 characterized by the development of unpleasant odours and tastes which deeply affect wine aroma (Oelofse *et al.*, 2008b).

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; Dias *et al.*, 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; 2009; Tchobanov *et al.*, 2008; Benito *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, only very recently were the corresponding genes identified in the genome of *B. bruxellensis* (Piškur *et al.*, 2012; Curtin *et al.*, 2012a; 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, 2008a). Furthermore, Romano *et al.* (2009) 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 can thus be prevented by controlling or eliminating *B. bruxellensis*'s population 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

SO₂ 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 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), SO₂ levels around 0.8 ppm of molecular SO₂ 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 SO₂ 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 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 to 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; International Organisation of Vine and Wine (OIV), 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 impact alcoholic fermentation negatively if added in grape juice as 200 mg/L of DMDC added to inoculated grape must showed a 4-day fermentation delay (Delfini *et al.*, 2002). For further reviews on these methods, see Suárez *et al.* (2007) and Oelofse (2008a). 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 the organoleptic properties of wine.

Physical and physicochemical methods

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 SO₂; 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) suggested 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, concentration of aroma compounds, esters and phenolic compounds (Arriagada-Carrazana *et al.*, 2005; Peri *et al.*, 1988; 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 e.g. 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 low electric current (LEC) (Lustrato *et al.*, 2010). However, the effect of PEF on the sensorial properties of wine and evaluation of the ability of this technology in the wineries still needs to be further researched (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).

Application of physicochemical methods such as the use of the biocide ozone (O₃) to inactivate the population of *B. bruxellensis* was recently investigated by Guzzon *et al.*, 2013. The activity of O₃ 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 O₃ is used, attention to recommended dosages and limited human exposure must be taken into consideration (Guillen *et al.*, 2010). Although these methods have been shown to be capable in inhibiting or even eliminating *Brettanomyces* or *Dekkera* species, their efficient activity is mainly dependent on the initial load of the cell concentration to be eliminated.

Biological methods

Microbial contamination is the main spoilage effect in food and beverages, and as such new methods are sought. Biological methods have been shown to be alternatives compared to the use of chemical preservatives or physical treatments. It was recently found 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 resulted in growth inhibition of *D. bruxellensis* and *Z. bailii* higher than 90%. The solution resulted in half maximal Inhibitory Concentration (IC₅₀) and MIC at 115 μ g/mL and 200 μ g/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 pulcherrimin acid which is inhibitory to the growth of *B. bruxellensis*. Finally, in recent studies, the use of biological antimicrobial compounds such as killer toxins (Kwkt, Pikt and PMKT2) from the yeast species *K. wickerhamii*, *P. anomala* and *P. membranifaciens*, respectively were 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 which inhibit susceptible yeast species or strains, although they remain immune to their own toxins (Bussey, 1972; Magliani *et al.*, 1997; Schmitt and 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 SO₂ 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 secrete killer toxins (van Vuuren & Jacobs, 1992; Schmitt & Breinig, 2006). Four phenotypes have been identified: killer, sensitive, neutral and killer-sensitive phenotype. 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).

S. cerevisiae's killer toxins

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 (Schmitt & Breinig, 2006; Magliani, 1997, Rodríguez-Cousin, 2011). The killer activity of *S. cerevisiae* is mainly dependent on the killer:sensitive ratio. These killer toxins have a narrow range of spectrum of activity, inhibiting only strains or species within the same genus (Mannazzu *et al.*, 2002) except for the Klus killer toxin which is killer active against yeasts such as *Hanseniaspora* spp., *Kluyveromyces lactis*, *Candida albicans*, *Candida dubliniensis*, *Candida kefir* and *Candida tropicalis* and 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 & 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 *Pichia 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 that they 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 and Neuhausen, 1994), similar to those of *S. cerevisiae*.

Killer toxins have found application in the food and fermentation industry, bio-typing of medically important pathogenic yeast and yeast-like fungi, 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 summarizes 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 *Kluyveromyces wickerhamii* display killer activity against the apiculate yeast *Hanseniaspora uvarum* and species of the *Brettanomyces/Dekkera* genus, respectively (Ciani & Fatichenti, 2001; Ciani *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*, *Pichia anomala*, *Pichia membranifaciens* and the filamentous fungi *Ustilago maydis* have been shown to inhibit the growth of *B. bruxellensis* or *D. bruxellensis* under winemaking conditions (Comitini *et al.*, 2004b; Santos *et al.*, 2009; Santos *et al.*, 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 4 and 7 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 only diminished when 28.6 AU/mL of the killer toxin Pikt was used compared to the use of 57.2 AU/mL, where a fungicidal effect was observed (Comitini *et al.*, 2004b). In grape must, the killer toxin PMKT2 at 2000 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 10² cells/mL (Santos *et al.*, 2011).

The growth of the apiculate yeast *H. uvarum* was inhibited by immobilized cells of the yeast *T. phaffii* (Comitini and Ciani, 2010) as well as the Kpkt killer toxin secreted by *T. phaffii* (Ciani and 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 where a fungicidal effect was observed in grape juice (Ciani and Fatichenti, 2001). Under winemaking conditions, the killer toxin Kwkt is efficient and comparable to the use of SO₂ in inhibiting *B. bruxellensis* (Comitini and 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 temperatures between 20 and 25°C (Table 1) which are compatible with winemaking conditions. Furthermore, in trial fermentations where these killer toxins were applied, the population of *S. cerevisiae* was not inhibited (Santos *et al.*, 2009; Comitini and 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 as 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). 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 permeabilization, 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 (Manzanares *et al.*, 2011; Schmitt & Breinig, 2006). 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 increased permeability of H^+ (Novotná *et al.*, 2004), 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 against *S. cerevisiae* sensitive cells resulted in 3.5% viable cells after 24 h of incubation compared to 75% viable cells after 4 h of incubation. This indicated that the mode of action of this toxin is not immediate and 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. It is however 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 the up-take 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, decreased intracellular pH and the active uptake of amino acids was inhibited (Middelbeek *et al.*, 1980) (Figure 1). High doses of the PMKT2 toxin resulted in a decrease of 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%. The authors 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, but 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 the K28 killer toxin which arrests cells in the early S phase and blocks DNA synthesis at 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 and 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*' killer toxins damage β -glucans scaffold on the cell walls of sensitive yeast cells and thereby induce cell death by osmotic lysis (Muccilli *et al.*, 2013). Thus far, the mode of actions of the killer toxins

Kwkt and PMKT, which are active against *B. bruxellensis*, have not yet been unravelled. It is not clear yet whether all the toxins that recognize 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 that contains ca. 5% β -1,6 linked branches; 15% β -D-1,6-glucan containing ca. 14% β -1,3 linked branches; mannoproteins and chitin (0.6 – 9%) (Kollár *et al.*, 1995; Santos *et al.*, 2000). In recent literature (İzgülü & Altınbay, 2004; İzgülü *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 mobilization 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; İzgülü & Altınbay, 2004; İzgülü *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 the strain BCA15 perfectly matches the β -glucanase of *P. anomala* strain K. Furthermore, the WaEXG2 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 (İzğü *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 (İzğü & Altınbay, 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 *TpBGL2*, of which the *T. phaffii* strain disrupted of 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 aging 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 SO₂. 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 to 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 proved 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 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 characterized as those of *S. cerevisiae* and further investigations are needed to clarify their genetic origin and

mode of action. Preliminary reports have indeed 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, can these killer toxins be used as biopreservatives in wine and in the food and beverage industry? Although evidence exists of their biopreservative potential, the use of these killer toxins has only been conducted for research purposes and these killer toxins 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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TABLE 1

Genetic origin, biochemical and biological characteristics of killer toxins secreted by non-*Saccharomyces* yeasts that have potential application in 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
<i>Kluyveromyces wickerhamii</i>	Kwkt (72kDa)	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)	<i>D. bruxellensis</i>	In winemaking	Comitini, <i>et al.</i> , 2004; Ciani & Comitini, 2011; Comitini & Ciani, 2011
<i>Tetrapisispora phaffii</i>	Kpkt (33 kDa)	Chromosomal gene - <i>BGL2</i>	pH activity: 3 to 5 Temperature activity: <40°C	Disruption of cell wall integrity Displays properties of β -glucanase enzyme	<i>H. uvarum</i>	In winemaking	Ciani & Fatichenti, 2001; Comitini, <i>et al.</i> , 2004; Comitini & Ciani, 2010; Oro, <i>et al.</i> , 2013

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor/ Mode of action	Sensitive/ Target yeasts	Application/ potential application	References
<i>Hansenula mrakii</i> (re-classified <i>Williopsis mrakii</i>)	HMK or HM-1 (10.7 kDa)	Chromosomal gene <i>hmk</i>	pH stability: 2 to 11 Thermostable – biological active after incubation at 100°C, 10 min	Receptor: β -D-1,3 and β - D-1,6-glucan Inhibits β -glucan synthesis	<i>Heterobasidium</i> , <i>Postia</i> , <i>Serpula</i> , <i>Fusarium</i> and/or <i>Colletotrichum</i> ,	In silage and yoghurt	Yamamoto, <i>et al.</i> , 1986; Lowe, <i>et al.</i> , 2000; Santos, <i>et al.</i> , 2002; Schmitt & Breinig, 2002; Selvakumar, <i>et al.</i> , 2006
<i>Pichia anomala</i> DBVPG 3003	Pikt (8 kDa)	Unknown	pH activity: 4.4 Temperature activity: 25 to 35°C	Receptor: β -1,6-glucans	<i>D. bruxellensis</i>	In winemaking	Comitini, <i>et al.</i> , 2004; De Ingeniis, <i>et al.</i> , 2009

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor/ Mode of action	Sensitive/ Target yeasts	Application/ potential application	References
<i>Pichia membranifaciens</i> 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	<i>Botrytis cinerea</i> , <i>Candida boidinii</i>	In grape vine	Santos, <i>et al.</i> , 2000, Santos & Marquina, 2004
<i>Pichia membranifaciens</i> 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	<i>B. bruxellensis</i>	In winemaking	Santos, <i>et al.</i> , 2009

Yeast species	Killer toxin and molecular size	Genetic origin	Biochemical characteristics	Cell wall receptor/ Mode of action	Sensitive/ Target yeasts	Application/ potential application	References
<i>Ustilago maydis</i>	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	<i>B. bruxellensis</i>	In winemaking	Santos, <i>et al.</i> , 2011

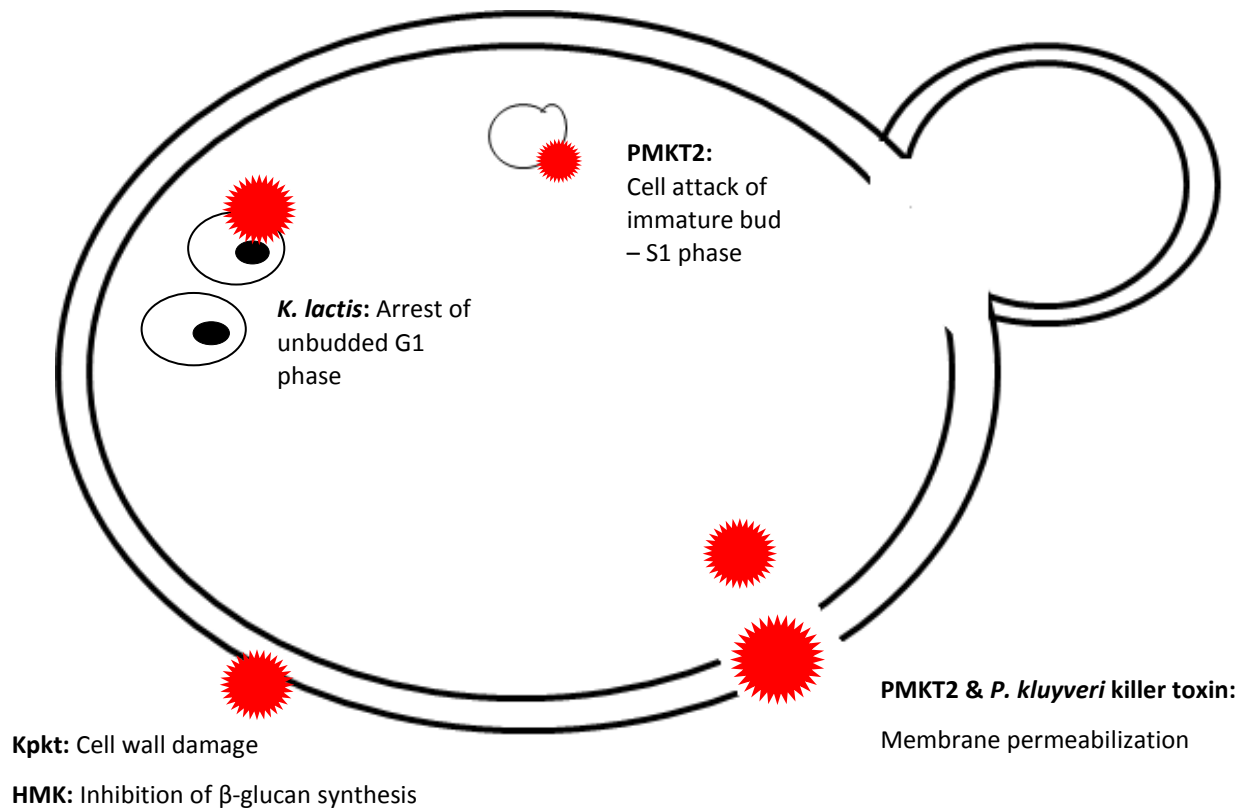


FIGURE 1

Mode of action of certain non-*Saccharomyces* killer toxins ( : Killer toxin)