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HETEROLOGOUS EXPRESSION, BIOREACTOR PRODUCTION AND CHARACTERIZATION OF rKPKT: A READY-TO-USE ANTIMICROBIAL COMPOUND OF INTEREST FOR THE WINE INDUSTRY

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Summary

The research activities here presented were carried out within the frame of "Programma Operativo Nazionale FSE-FESR Ricerca e Innovazione-Azione I.1-Dottorati innovativi con caratterizzazione Industriale" granted by the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR). This program aims at bridging the gap between University and industry by strengthening the collaboration among academic and industrial partners for the development of innovative solutions that contribute to the growth of specific productive sectors. The academic partners involved in the realization of this theses were the University of Sassari (Sassari, Italy) and the Brno Technical University (Brno, Czech Republic). The industrial partner was the Tenute Sella & Mosca S.p.A. This is the biggest winery in Sardinia (7,6 milioni bottles/year), strongly interested in searching for innovation of processes and products and in the technological transfer of innovation. In particular, this winery has been producing organic grapes for 4 years and is interested in the development of natural antimicrobials to be utilized in the wine industry.

Based on that, and on the expertise on killer toxin characterization and exploitation acquired by researchers in the Laboratory of Microbiology of the Department of Agraria (University of Sassari), the **general objective** of this research was to explore the biotechnological potential of yeast killer toxins to be used as natural antimicrobials for the control of wine spoilage yeasts. In fact, although the antimicrobial activity of yeast killer toxins has been known for more than 50 years and many different authors have hypothesized their exploitation in the food and wine industry, their biotechnological potential has never been really explored. Previous works carried out in the Laboratory of Microbiology of the Department of Agriculture of the University of Sassari have contributed to shed light on the properties, mode of action and genetic determinant of Kpkt, a killer toxin produced by the yeast *Tetrapisispora phaffii* that has an extensive antimicrobial activity on wine spoilage yeasts. Based on these works, it was hypothesized a future for this toxin as natural antimicrobial in the wine industry where it can partially substitute sulphur dioxide thus reducing the final amount of this antimicrobial in wine. For that, considering that *T. phaffii* produces rather low amount of Kpkt, previous works showed that *Komagataella phaffii*, formerly *Pichia pastoris,* can be a suitable host for the heterologous production of the toxin.

In this context, **specific objectives** of this research were: i) the development of further molecular tools for the production of recombinant (rKpkt) in *K. phaffii*; ii) the bioreactor production of rKpkt and its purification and characterization; iii) the production of a ready-to-use rKpkt containing preparation, and the evaluation of its effect on a variety of biological targets.

After a review of the literature regarding natural antimicrobials and killer toxins, and *K. phaffii* as a host for heterologous expression in the introduction (chapter 1), and the description of the aims of the PhD thesis (chapter 2), the research activities carried out in the three years program are reported as follows.

Chapter 3 regards the construction of new vectors for the heterologous production of rKpkt. For that, Kpkt coding sequence, optimized for the expression in *K. phaffii*, was cloned under the control of regulated (*AOX*1) or constitutive (*PGK*1) promoters and downstream of two different secretion signals (*S. cerevisiae* α-Factor and the *T. phaffii* native secretion signals).

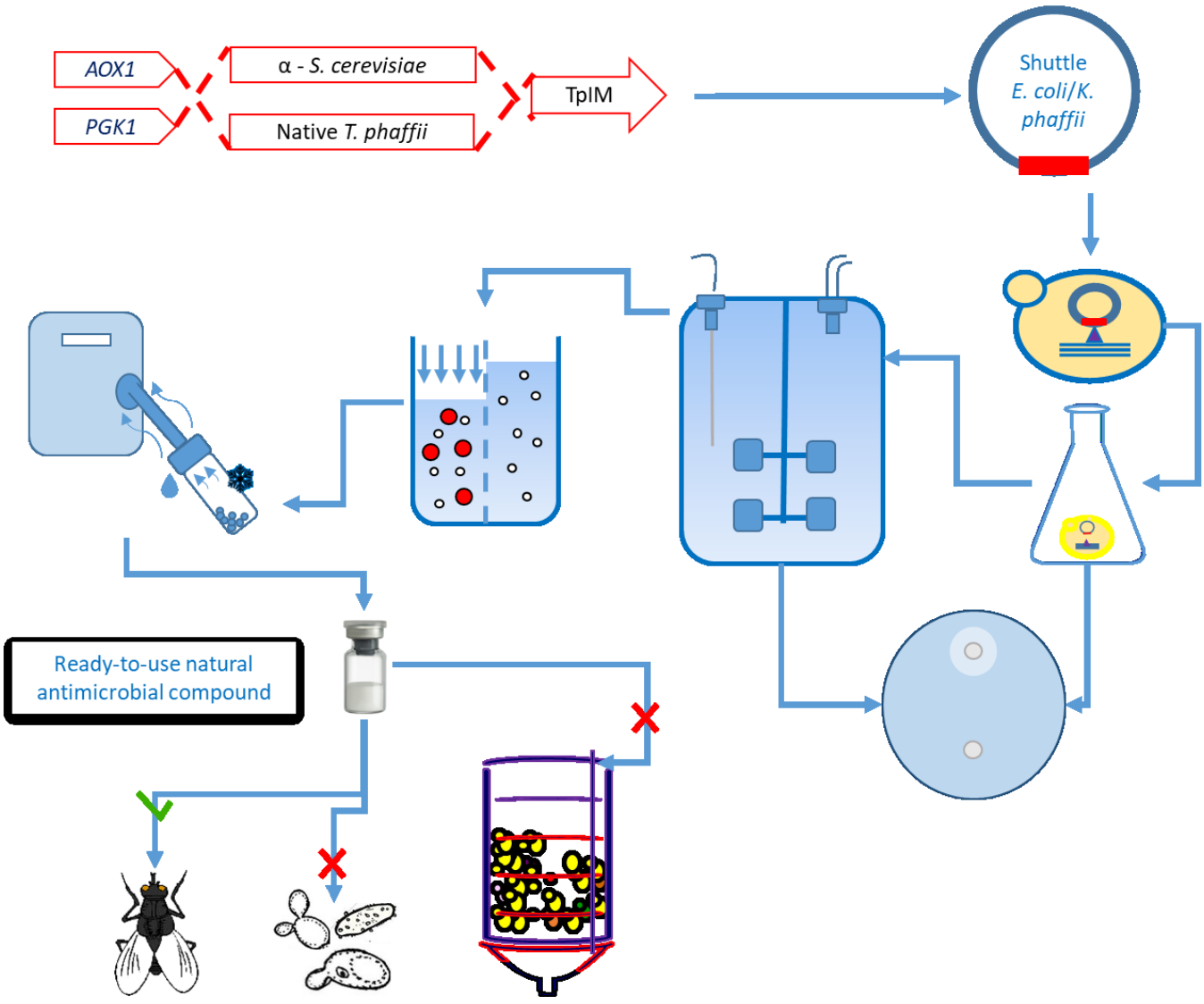
The resulting plasmids were transformed into *K. phaffii* strains GS115 and M12. Screening of transformants indicated that the integration of the expression cassette results in a low percentage of
recombinant clones showing killer activity. In fact, contrary to native Kpkt, recombinant version Kpkt (rKpkt) is toxic for *K. phaffii* GS115 and M12, while showing lower toxicity on recombinant killer clones.

Chapter 4 reports on the bioreactor production and characterization of rKpkt. For that cultivation of recombinant clone#17 (rc#17) and the production of rKpkt were scaled up from baffled flask to bioreactor and rKpkt purification was attempted by immobilized metal affinity chromatography. Moreover, after having confirmed that rKpkt maintains β-glucanase activity and shows a wider spectrum of action in respect to its native counterpart, its cytotoxic effect on human cells was evaluated. Results obtained showed that rKpkt has limited toxicity to HaCaT cell line for concentrations up to 18 AU/ml thus corroborating the great potential of this toxin as a natural antimicrobial for use in the food and beverages industries.

Chapter 5 regards the production of a lyophlized ready-to-use rKpkt preparation (LrKpkt) and the evaluation its effects in grape must and on a variety of biological targets. Results showed that LrKpkt maintains its killer activity for up to six months at 4 °C and it is easily soluble in sterile distilled water. When added to Cannonau and Vermentino grape musts LrKpkt inhibited the wild microflora, while showing limited or no effect on inoculated wine yeast starters. Moreover, besides being active on wine-related yeasts, LrKpkt exerted a strong microbicidal effects on a variety of bacterial species (lactic acid bacteria and food-borne pathogens). On the contrary, in accordance with its limited toxicity on HaCaT cell line, LrKpkt showed no lethal effect on *Ceratitis capitata* and *Musca domestica*, thus suggesting its low or null toxicity on multicellular eukaryotic model organisms. Indeed, additional studies are needed to further characterize LrKpkt. Nonetheless, the results here presented suggest that this ready-to-use antimicrobial compound may represent an interesting option for the management of microbial contaminations both in the wine and food industries. In chapter 6 challenges and perspectives on the topic treated are reported.

In conclusion, the results here presented report on Kpkt heterologous production in *K. phaffii*, the possibility of producing a ready-to-use antimicrobial compound containing rKpkt and its low or null toxicity on higher Eukaryotic cells. Considering that the scarcity of studies regarding the effects of killer toxins on human consumers represents a constraint to their utilization in the food and beverages industries, these results, although preliminary, represent a further step towards the biotechnological exploitation of yeast killer toxins.

Graphical abstract



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Chapter 1

Introduction

1.1. Food preservation and natural antimicrobials

Abstract

The application of food preservation methods is meant to prevent the contamination of food with pathogens and spoilage microorganisms in order to guarantee food safety, nutritional value and sensory properties. Among the different food preservation methods, those based on the utilization of natural or synthetic ingredients, although exerting variable effects on food safety, quality and shelf-life, are often the methods of choice, being generally less expensive than the others. However, since synthetic ingredients can be harmful to human health, consumers tend to prefer food containing natural antimicrobials. These last are expected to ensure food safety due to their activity on food-borne pathogens, to extend food shelf-life with a minimum impact on the consumer microbiome, and to represent a response to the global concern involving antibiotic resistance. At present a number of different natural antimicrobials of plant, animal and microbial origins, are utilized in the food industry. However, more research is needed to face the demand for natural antimicrobials that replace synthetic compounds, but also to thoroughly evaluate their interactions with the different food components, their impact on consumers' health and on the resident microbes of the gastro intestinal tract once ingested and, finally, to propose appropriate safety regulations.

Key words

Food preservation, natural antimicrobials, spoilage microorganisms, food-borne pathogens, food quality, food safety

1.1.1. Food preservation

Food undergoes spoilage due to microbial, chemical, or physical agents. In respect to microbial spoilage, humankind has developed a variety of different food preservation methods that are aimed at maintaining food nutritional value, color, texture, and safety (Rahman, 2007). Indeed, the history of food preservation is very long and, interestingly, Egyptian, Greek, Roman, Sumerian and Chinese, in spite of geographical and temporal distances, employed similar strategies to preserve raw materials (Nummer, 2002). These were based on fermentation, drying and the addition of sugar and salt (Amit et al., 2017; Anand and Sati, 2018).

Since then, several other food preservation methods and technologies have been implemented to slacken or avoid the fermentation, acidification, decomposition and to prevent pathogens contamination of food. At present, food preservation methods have become highly interdisciplinary and, due to industrialization and globalization, they contribute to develop the manufacturing sector and to increase country's national income (Amit et al., 2017). Among the most common food preservation methods are: pasteurization and sterilization, freezing, irradiation, drying, filtration, hermetic sealing, high pressure and the addition of natural or synthetic ingredients (Anand and Sati, 2018). Some of these methods, like for example thermal treatments and freezing, although effective in reducing food-borne pathogens and spoilage microorganisms, may negatively impact on food quality and nutritional content (Tiwari et al., 2009). Others, like irradiation, high pressure and hermetic sealing, increase the shelf-life of food with negligible nutritional losses, but are generally more expensive. Thus, the addition of natural or synthetic ingredients, although exerting variable effects on food shelf-life and safety, is often the method of choice. In fact, preservation with these ingredients is generally less expensive than with other methods and permits the food industry to meet the market demands (Saltmarsh et al., 2013).

Natural ingredients such as salt, vinegar, sugar, syrup, honey, oil, spices are classified as Class 1 preservatives. Synthetic ingredients such as benzoates, sorbates, nitrites and nitrates of sodium or potassium, sulfites, glutamates and glycerides are classified as Class 2 preservatives. All of them fall within the three following categories: antimicrobials that inhibit or delay growth of microorganisms; anti-oxidants that inhibit or delay oxygen-mediated oils and fat deterioration; anti-enzymatic preservatives that inhibit ripening and other enzymatic processes that occurr in foodstuffs even after harvest.

Indeed, synthetic antimicrobials although under study for decades, show potentially dangerous effects on human health. For example, sodium benzoate is considered a safe ingredient although its effect on long term exposure still needs to be elucidated (Lennerz et al., 2015). Regarding sorbates, some studies describe these compounds as genotoxic and mutagenic. Accordingly, sodium sorbate has a dose dependent genotoxic effect on blood lymphocytes *in vitro* (Mamur at al., 2010) and it is not allowed in the US, but still legal in the EU (Binstok et al., 1998; Mpountoukas et al., 2008; Mamur et al., 2012). According to other studies their effect on human health is negligible. Parabens have been reported to exert negative effect on human breast cancer cells *in vitro* (Karpuzoglu et al., 2013; Khanna et al., 2014). The utilization of nitrates (E240-E259) has been restricted within the EU while that of nitrites (E249-E250) has been approved in EU at the minimum possible dosage, considering that they can take part in the formation of nitrosamines (EU Reg. 1129/2011) and have other

deleterious effects on human health (Cammack et al.,1999). Sulphites or sulphiting agents are widely utilized in food and beverages industries due to their antimicrobial, antioxidant

and antibrowning activity even if they have been shown to exert cytotoxic and carcinogenic effects towards both rats and humans (Iammarino et al., 2012; Suh et al., 2007).

Thus, on the one side, according to food standard regulations, not more than one Class 2 preservative should be used in the single food in order to limit the risk of exposure to multiple chemicals and reduce their life-threatening effects. On the other side, health concerned consumers tend to prefer food with natural additives. Thus they are pushing towards the exploitation of natural antimicrobials to be incorporated in food and or in food packaging (Carocho et al., 2015).

Natural antimicrobials are expected to ensure food safety due to their activity on food-borne pathogens, to extend food shelf-life with a minimum impact on the consumers' microbiome, and to represent a response to the global concern involving antibiotic resistance. Ideally, natural antimicrobials should have microbicidal rather than microbiostatic activity, they should not be toxic to the consumers and should be active at low concentrations, they should be heat and pH stable, have no impact on flavor or color, and no pharmaceutical application, they should be label friendly and, finally, cost effective (Carocho et al., 2015).

1.1.2. Natural antimicrobials of plant and animal origins

The major phytochemicals responsible for plant antimicrobial properties are phenolic compouds such as terpens, aliphatic alcohols, aldehydes, ketones and isoflavonoids, organic acids, saponins, thiosulfinates and glucosinolates (Rodríguez Vaquero et al., 2010; Pisoschi et al., 2018), most of which have also antioxidant properties that contribute to enhance their antimicrobial activity (Hayek et al., 2013). They are found in essential oils (from basil, thyme, oregano, cinnamon, clove, rosemary and others) and extracts from medicinal herbs and spices (Hygreeva et al., 2014) (Table 1). They may be derived also from a variety of by-products of food processing, including fruit peels, tomato seeds, coffee husks peel and pulp, grape and olive pomace (Quinto et al., 2019). Antimicrobials of plant origins are active against a wide spectrum of spoilage microorganisms and food-borne pathogens (Belletti et al., 2008; Gutierrez et al., 2008) although with different properties. For instance, essential oils are reported to be more effective on Gram positive than on Gram negative bacteria (Hayek et al., 2013; Hintz et al., 2015) even though some of them, namely clove and cinnamon, are effective against both (Tiwari et al., 2009). Their efficacy depends on environmental conditions (Pisoschi et al., 2018). Thus, their antimicrobial activity must be tested for different food products. Their utilization can be associated to non-thermal treatment to improve food preservation (Pisoschi et al., 2018).

Indeed, natural antimicrobials of plant origin are easily accepted by consumers. They have been utilized by humans for thousands of years, and they have been given the generally recognized as safe (GRAS) status (Burt, 2004; De Oliveira et al., 2011). Moreover, many different plants are still used in traditional medicine. Thus, the public attitude towards these natural antimicrobials is generally positive. They can be directly added to food preparations, but their exploitation may occur also through their incorporation into antimicrobial packaging or edible antimicrobial biofilms (Taveira et al., 2010; Gyawali et al., 2014). However, according to several authors their mechanisms of action and impact on the

sensory properties on food need further investigation (Periago et al., 2006; Gutierrez et al., 2008).

Antimicrobials of animal origin are peptides (such as lysozyme, lactoferrin, lactoperoxidase, pleurocidin, ovotransferrin, defensin, protamine and others) that show antimicrobial activity against bacteria, fungi and viruses; polysaccharides like chitosan (Lucera et al., 2012; Pisoschi et al., 2018) and lipids that may inhibit the proliferation of Gram positive and Gram negative bacteria and fungi (Table 1). Lysozyme (E-1105) is utilized in food and beverages both in the US and the EU (Carocho et al., 2015). It is extracted from chicken egg whites, it is particularly active on Gram positive bacteria and can be employed to counteract a wide range of food spoilage organisms, thus extending the shelf-life of various food products, including raw and processed meats, cheese, and other dairy products (Tiwari et al., 2009). It is also widely utilized in the wine industry to control lactic acid bacteria and inhibit malolactic fermentation, when undesired (Liburdi et al., 2014). Lactoferrin is a 77 kDa glycoprotein, found in humans and animals milk, plasma, urine and mucosal secretions (e.g. tears). It binds iron and counteracts Gram negative bacteria that require high iron concentrations (*Salmonella* spp., *Escherichia coli*, *Shigella dysenteriae*) but also Gram positive bacteria such as *Listeria monocytogenes*, *Bacillus stearothermophilus*, and *B. subtilis*. Besides antimicrobial activity, it shows also antioxidant, anti-inflammatory and anti-cancer activities (Niaz et al., 2019) and for these interesting properties, starting from november 2012, the utilization of bovine lactoferrin as a food additive has been approved by the European Commission. Lactoperoxidase is a milk protein that shows bacteriostatic and bactericidal activities (Abbes et al., 2013; 2018). It is utilized in form of lactoperoxidase system, that includes thiocyanate, and hydrogen peroxide, and generates hypothiocyanite, active on Gram positive and Gram negative bacteria, among which *Escherichia coli* (Batt and Tortorello, 2014). The lactoperoxidase system has been recognized the GRAS status (Alegbeleye et al 2018) and its use is proposed to increase the shelf-life of different food matrices among which milk, fruit, meat and cheese (Al-Baarri et al., 2019). Chitosan is a polymer produced from chitin derived from the exoskeletons of arthropods and crustaceans and the cell wall of fungi. It is non-toxic, non-allergenic, anticoagulant, antioxidant and biodegradable. Moreover, it shows antimicrobial activity on bacteria, yeasts and moulds of food interest, while showing low toxicity on mammalian cells (Pillai et al., 2009; Quinto et al., 2019). Due to its properties, chitosan has been widely used as an antimicrobial agent to improve food quality and extend shelf-life (Gutierrez, 2017). It maintains antimicrobial activity in different food matrices and in combination with other natural or synthetic antimicrobial compounds (Quinto et al., 2019). For example, Yu et al., (2012) utilized chitosan with biocontrol yeast and/or calcium chloride to counteract blue mold in pears and observed that, by blending chitosan with these other ingredients, it is possible to extend its antimicrobial activity. Different chitosan-based films have been fabricated and applied in the field of food packaging. However, it should be noted that chitosan of animal origin has received the GRAS status in the US while it is not approved for food applications in the EU (Pinheiro et al., 2019). As already observed for natural antimicrobials of plant origins, in general the potential, mechanisms of action and impact on the consumer of natural antimicrobial of animal origins needs to be further explored.

Table 1. Main natural antimicrobials of plant and animal origins.

Natural antimicrobial		Example	GRAS status	Application
Plant origin	Essential oils	Coriander, Cinnamon, Rosemary, Thyme, Oregano	Yes	Meat, fish, dairy products, vegetables, rice, fruits, coatings
	Spices	Cinnamon, nutmeg, cloves	Yes	Meat
	Herbs	Rosemary, parsley, coriander, thyme, basil, fennel, oregano	Yes	Meat, meat products
Animal origin	Peptides	Lysozyme	Yes	Wine, Cheese, eggs, milk, coatings
		Lactoferrin		
		Lactoperoxidase		Baby formulas, meat Milk, fruit, meat, Cheese Juices, coatings
	Polysaccharides	Chitosan	Yes in US Not in EU	Fruit, coatings

Source: Campêlo et al. (2019) and Carocho et al. (2015).

1.1.3. Biopreservation of food

Microorganisms produce a plethora of compounds that mainly help them to counteract competitors for nutrients in the environmental niches they occupy. Thus, they are an important source of natural antimicrobials that can be utilized either in form of microbial cells, or in form of metabolites, that do not influence food quality.

Indeed, natural antimicrobials of bacterial origins are the most well characterized and many of them are commonly employed for food and beverages biopreservation. They span

from organic acids to active peptides and other compounds derived from fermentation. The production of organic acids is very important for food preservation, and acidifying bacteria such as lactic acid bacteria (LAB) are widely utilized as biopreservatives. Under controlled acidification conditions they produce lactic acid that permeates through the cell membrane, thus perturbing intracellular pH and proton motive force and leads to cell death (Smith 1993; Pisoschi 2012). Based on this mechanism, LAB counteract the proliferation of pathogenic bacteria unable to grow at pH <4.6. Same mechanism has been proposed for acetic and propionic acids produced by bacterial starter cultures (Ray, 1992).

Among peptides, bacteriocins play a pivotal role. They are antimicrobial peptides (AMP) of 20-60 aminoacids produced by many different Gram positive and Gram negative bacteria. Bacteriocins are active against food-borne pathogens and spoilage bacteria, with no impact on the physicochemical characteristics of food. They have hydrophobic or amphiphilic properties and act on the cell membrane of their sensitive target. In general, Gram positive bacteria are inhibited by the majority of bacteriocins, although their spectrum of action is generally directed towards closely related species. So far, hundreds of bacteriocins have been discovered, characterized and described (Hammami et al., 2007; Blin et al., 2013; Van Heel et al., 2013). According to Alvarez-Sieiro et al. (2016), bacteriocins can be assigned to three different classes (Kumariya et al., 2019). Class I includes small (19-50 aminoacids) post-translationally modified peptides. Class I is divided in class Ia (lantibiotics), class Ib (labyrinthopeptins) and class Ic (sanctibiotics). Class II contains small heat stable non-modified peptides. Similar to class I also class II is subdivided in class IIa (pediocins-like bacteriocins), class IIb (two peptides unmodified bacteriocins), class IIc (circular bacteriocins) and class IId (unmodified, linear, non pediocin like bacteriocins). Class III contains larger (more than 10 kDa) and thermolabile peptides. Of these the most well characterized are those ascribed to class 1 (lantibiotics, small peptides containing unusual aminoacids) and class 2 that includes small, heat stable bacteriocins.

Among bacteriocins those produced by lactic acid bacteria (LAB) are of great interest for the food and in particular the dairy industry. Being degraded by proteolytic enzymes, they have no impact on the microflora of the gastrointestinal tract and therefore are considered safe for human consumption (Kheadr et al., 2010; Mills et al., 2011; Egan et al., 2016; Fernandez et al., 2016). Moreover, they resist severe heat treatments (pasteurization and sterilization) and are 10^3-10^6 times more effective than other antimicrobials (De Vuyst and Leroy, 2007). They can be used as crude or purified preparations or, in alternative, bacteriocins producing LAB strains can be utilized as starter cultures of fermentation (Table 2).

Nisin and pediocins are the most important bacteriocins for the food industry. Nisin is produced by *Lactococcus lactis* and active against different Gram positive and sporogenic bacteria among which LAB, *Listeria*, *Staphylococcus*, *Bacillus* and *Clostridium*. It is approved as food preservative (E234) in many different countries (Pisoschi 2012) and received the GRAS status by the Food and Drug Administration (FDA). Nisin is commercialized as Nisaplin™, that contains nisin A (2.5%) and other ingredients such as NaCl and non-fat dry milk (Chen and Hoover, 2003) and as Niseen®. Nisin is currently applied to the preservation of milk, orange juice (Lee et al., 2004), tomato juice (Nguyen and Mittal, 2007) and chicken meat without altering sensory properties of the product (Lemay et al., 2002). Pediocins,

synthesized by *Pediococcus acidilactici* and *P. pentosaceus* have a wide spectrum of action against food-borne pathogens both Gram positive and Gram negative (*Listeria monocytogenes*, *Staphylococcus aureus, Clostridium perfringens, Enterococcus faecalis*) and spoilage microorganisms. Moreover, they counteract *Oenococcus oeni* and other wine-related bacteria (Díez et al., 2012). Pediocins are thermostable and active within a wide pH range and are proposed for the preservation of vegetable and meat products (Papagianni et al., 2009). They are commercialized as Alta 2341TM or MicrogardTM (Garsa et al., 2014).

Other interesting natural antimicrobials of bacterial origins are reuterin, natamycin and poly-L-Lisine (Table 2). Reuterin is an antimicrobial compound (3-hydroxypropionaldehyde) produced by glycerol fermentation and secreted by *Lactobacillus reuteri* and possibly involved in the probiotic effect of this microorganism. Reuterin generates oxidative stress in target cells, although its mechanism of action needs further investigation (Schaefer et al., 2010). It is active on Gram positive (*L. monocytogenes* and *S. aureus*) and Gram negative (*Salmonella tiphymurium* and *E. coli*) bacteria, yeasts and filamentous fungi. It is stable over a wide pH and temperature range, and resistant to proteolitic and lypolitic enzymes. Thus, it is suitable for food preservation in combination with bacteriocins. Reuterin application in food may occur through the inoculation of *L. reuteri* as starter culture with glycerol, or after extraction. It should be taken into account that although *L. reuteri* has been given the Qualified Presumption of Safety by the European Food Safety Authority (EFSA Panel on Biological Hazards, 2017) and the use of *L. reuteri* with glycerol is admitted (food additive E-422), more data on the toxicity of reuterin are necessary. According to Vimont et al. (2019) more research is needed in order to assess the impact of the long term exposure of reuterin and the mechanism of toxic action.

Natamycin (pimaricin) (E 235) is a fungicide of the polyene macrolide group produced by *Streptococcus natalensin*, *S. chmanovgensis*, and *S. gilvosporeus* (Duchateau and van Scheppingen, 2018). It inhibits growth of filamentous fungi and yeasts, but it is not effective on bacteria and protozoa and viruses. It is utilized as a free additive (E-235), or encapsulated or as a constituent of films (Roller, 2003; Baines and Seal, 2012) to control yeast growth in cheese (Oliveira et al., 2007), but it may find application also in beverages (Roller, 2003). It received approval for usage on cheese surface in 1979 and it was authorized for food preservation in the European Union by Directive 95/2/EC (E235). In 2009, the EFSA reported about natamycin safety due to its low dosage and poor absorption (EFSA, 2009). Some countries allow the application of Natamycin onto the surface of dry and fermented sausages and in US it is is approved for various dairy applications. It is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. More specifically, Natamycin is commonly used in products such as cottage cheese, sour cream, yogurt and packaged salad mixes.

Poly-L-lysine is a homopolimer of lysin that has received the GRAS status in the US and is utilized in Japan (Shih et al., 2006). According to the US Food and Drug Administration (FDA) poly-L-lysine can be incorporated into various food matrices at levels of 10–500 ppm. At such low concentrations it displays strong inhibitory activity against fungi (*Aspergillus niger*, *Candida* spp., and *Phaffia rhodozyma*) and Gram positive and Gram negative bacteria

(*Bacillus coagulans*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*) (You et al., 2017). Poly-L-lysine antimicrobial activity is attributed to its cationic charge. This, on the one side, leads poly-L-lysine to adsorb onto negatively charged microbial surfaces and disrupt the cell envelope. On the other side, the cationic charge of poly-L-lysine may limit its application in foods as it could interact with anionic mucins in the mouth, or spontaneously complex with negatively charged molecules to impact food integrity. To mitigate this limitations poly-L-lysine is complexed with anionic polysaccharides, like pectin (You et al., 2017). Moreover, it can be utilized in combination with other natural antimicrobials.

Definitely, much less is known about natural antimicrobials produced by other microorganisms. For what concerns algal antimicrobials, pharmaceutical and food industries have great interest on marine algae derivatives. Herrero et al. (2013), Devi et al. (2008) and Dussault et al. (2016) found that algal extracts show antimicrobial activity against a number of pathogenic bacteria including *S. aureus*, *E. coli*, *L. monocytogenes*, *Salmonella*, *E. faecalis*, *P. aeruginosa*, *B. cereus*. Moreover, alginate and carrageenan of algal origin can be combined with antimicrobials and utilized for coatings and films in the food industry (Tavassoli-Kafrani et al., 2016; Han et al., 2018).

Regarding the antimicrobial activities of fungi, mushrooms extracts contain phenols and flavonoids that exert antimicrobial activity (Alves et al., 2012; Alves et al., 2014; Lallawmsanga et al. 2016; Shen et al., 2017). Filamentous fungi have been reported as producers of antimicrobials (see for example the production of chitosan from *Aspergillus niger*).

Yeasts, although much less characterized than bacteria in respect to their antimicrobial potential, are emerging as natural biopreservatives or biopreservative producers. Yeast antimicrobial activity may be mediated by different mechanisms among which competition for nutrients, production of ethanol, production of organic acid and secretion of antimicrobial compounds with variable spectrum of action. Thus, different yeast strains are utilized for the management of fruit and vegetable post-harvest diseases under different commercial denominations such as Candifruit, Aspire, Nexy, Yeld plus, Boni Protect, Shemer (Muccilli and Restuccia, 2015). Moreover, they may inhibit growth of spoilage and pathogenic microorganisms in food and beverages through the production of killer toxins.

Table 2. Biopreservatives of bacterial origin

Name	Application	Mode of use	Commercial name	Spectrum of action
Nisin	Cheese, dairy products, orange	Free additive , film	Nisaplin TM Niseen®	LAB, *Listeria*, *Stafilococcus*, *Bacillus, Clostridium*
Pediocin PA1	Milk, dairy products, vegetables, meat	Inoculation Pediocin producing strain, free additive, incorporation in food packagin	Microgard TM, Alta 2431	*Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens, Enterococcus faecalis,* wine-related bacteria
Reuterin	Fuctionla food for infant and adult	Inoculation of *L. reuteri* as starter product	L. reuteri DSM 17938, L. reuteri prodentis ATCC PTA 5289	Gram-positive and Gram-negative Bacteria, bacterial spores, moulds, yeast and protozoa
Natamycin	Dairy products, meats and other food	Free additive, encapsulated, film	Nataseen®	Yeasts and moulds
Poly-L-Lysine	Rice, vegetables, soup, fish	Complexed with polysaccharides	Epsiliseen®-H	Yeasts, fungi, Gram-positive ans Gram-negative, bacteria

1.1.4. Concluding remarks

The utilization of natural preservatives to safeguard public health and prolong food shelf-life has recently become increasingly common in the food industry. This is due, on the one side, to the need to counteract food-borne pathogens, that are resistant to different antimicrobials as a consequence of a generalized utilization of food safety barriers. On the other side, the utilization of natural preservatives is aimed at satisfying the growing request of natural and minimally processed products and the increasing concern of consumers on the presence of synthetic antimicrobials in food.

Among natural antimicrobials are essential oils, enzymes from animal sources, and bacteriocins, organic acids and polymers form microbial sources. Indeed, while their mechanisms of action on microbial targets have been only in part elucidated (Pisoschi et al., 2018), a better understanding of these mechanisms is necessary to optimize the utilization of natural antimicrobials in different food matrices. Thus, more research is needed not only to face the demand for new and effective natural antimicrobials that replace synthetic compounds, but also to thoroughly evaluate their impact on consumers health, their interactions with the resident microbes of the gastro intestinal tract once ingested and the different food components and, finally, to propose appropriate safety regulations (Tiwari et al., 2009; Reis et al., 2014; Lee and Paik, 2016; Pisoschi et al., 2018).

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1.2. Yeast killer toxins: from ecological significance to application

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Abstract

Killer toxins are proteins that are often glycosylated and that bind to specific receptors on the surface of their target microorganism, which is then killed through a target-specific mode of action. The killer phenotype is widespread among yeast, with about 100 yeast killer species described to date. The spectrum of action of the killer toxins they produce targets spoilage and pathogenic microorganisms. Thus, they have potential as natural antimicrobials in food and for biological control of plant pathogens, as well as potential therapeutic agents against animal and human infections. In spite of this wide range of possible applications, their exploitation at the industrial level is still in its infancy. Here, we initially briefly present the biodiversity of killer toxins and the ecological significance of their production. Then their actual and possible applications in the agro-food industry are discussed, together with recent advances in their heterologous production and manipulation for development of peptide-based therapeutic agents.

Keywords: killer toxin, natural antimicrobial, interference competition, spoilage yeast, biological control, therapeutic agents

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1.2.1. Yeast killer toxins

The antagonistic behavior of yeast was first discovered at the beginning of the last century, when the production of a volatile thermolabile extract that inhibited bacterial growth was described (Van Vuuren et al., 1992). Further studies showed that the antagonistic activities of yeast against other microorganisms can be attributed to a number of different properties, including competition for nutrients and space, acidification of the medium, production of ethanol, and secretion of antimicrobial compounds, such as volatile acids, hydrogen peroxide, secondary metabolites, and the so-called killer toxins.

The production of toxins is a relatively common phenotype in Nature. This has been described for bacteria, yeast and fungi as generally part of a mechanism aimed at domination of the competition within the environmental niches that they live. For yeast, the first reports on their killer phenotype dates back to just over 50 years ago, with the initial isolation of a Saccharomyces cerevisiae strain that inhibited the growth of other S. cerevisiae strains (Bevan et al., 1963; Bussey et al., 1972). According to these early investigations, killer (K) yeast secreted a toxin that was lethal to sensitive (S) strains of the same or relate species, but was harmless to neutral (N) strains, which were immune to their effects.

In the following years, the killer phenotype was shown to be widespread among yeast, with the description of almost 100 killer species that have been ascribed to about 46 genera Klassen et al., 2017). Further studies have shown that the killer phenotype occurs frequently in yeast strains isolated from a variety of natural habitats (e.g., water, soil, fruit, grape must) and from different geographic origins. Most killer yeast can kill other yeast of the same or of different species and genera. Some of them are active against filamentous fungi (Santos et al., 2004a; Izgu et al., 2008) while some are also active against bacteria (Izgu et al., 1997; Perez et al., 2016). The spectrum of action of yeast killer toxins encompasses spoilage microorganisms relevant for the fermentative (Van Vuuren and Jacobs, 1992; Comitini et al., 2004b) and food and feed industries (Palpacelli et al., 1991; Lowes et al., 2000), and it also includes microbial pathogens of clinical interest (Seguy et al., 1996; Guyard et al., 2002a), and plant pathogens (Perez et al., 2016; Weiler et al., 2003; Santos et al., 2004). Thus, they can be used as natural antimicrobials in the agro-food industry, as well as antimicrobials against animal and human infections and for biological control of plant pathogens, both in the field and for postharvest applications (Rosa-Magri et al., 2011; Shaffrath et al., 2018 (Figure 1). In addition, killer yeast and their toxins represent model systems for elucidation of the mechanisms that underlie social interactions between microorganisms (Wloch-Salamon, 2014), the regulation of polypeptide processing and secretion in eukaryotes, and expression, inheritance and maintenance of eukaryotic viruses (Schmitt and Breinig, 2006).

Here, after a brief presentation of the biodiversity of killer toxins and the ecological significance of their production, their actual and possible applications in the agro-food industry are discussed, together with recent advances in their heterologous production and manipulations for development of peptide-based therapeutic agents.

1.2.2. Biodiversity of killer toxins

The depth of information regarding killer toxins varies depending on the characteristics of the producer and the possible biotechnological application of the toxin. The most well-characterized killer toxins with respect to their genetic determinants, biochemical characteristics, molecular targets on the sensitive cells, and mechanisms of killing are K1, K2, and K28 of *S. cerevisiae*, zymocin of *Kluyveromyces lactis*, PMKT and PMKT2 of Pichia membranaefaciens, PaKT of Wickerhamomyces anomalus, HM-1 of Cyberlindnera mrakii and Kpkt of *Tetrapisispora phaffii* (Santos and Marquina,2004; Bussey, 1991; Gier et al., 2017). In particular, this is because these toxins represent biological models for the study of replication and maintenance of extrachromosomal genetic elements, for dissection of the secretory pathway, and for elucidation of the mechanisms that underlie toxin immunity (K1, K28, zymocin), or because of their possible applications in the medical field (PakT), the food and feed industries (PMKT, PMKT2, KpKt, HM-1), and in the preharvest and postharvest biocontrol of plant pathogens (PMKT, PMKT2). For the remaining killer toxins, generally the localization of the genetic determinants of the killer characters and the molecular weights of the killer toxins have been determined (Table 1), although much still needs to be done regarding their modes of action and molecular targets within the sensitive cells, and for an understanding of toxin immunity. Recently, there have been a number of reviews of killer yeast, their toxins, their genetic determinants, and their molecular mechanisms of action (Klassen et al., 2017; Shaffrath et al., 2018; Liu et al., 2015; S~ao-Jos et al., 2017). Thus, only a brief overview of what is known about the selected group of killer toxins are reported here and in Table 1.

In terms of the localization of their genetic determinants, the majority of the killer toxins characterized to date are encoded by chromosomal genes, and their production is widespread in a number of different non-Saccharomyces yeast, although this has also been observed within *S. cerevisiae* (Table 1, Figure 2). A few killer toxins that are produced by a handful of species are encoded by extrachromosomal double-stranded (ds)RNA virus-like particles and by extrachromosomal dsDNA virus-like elements localized in the cytoplasm (Table 1, Figure 2). The molecular weights of killer toxins range from about 1.8 kDa to >150 kDa, and while most of them consist of a single subunit (the majority of those encoded by chromosomal genes), some others can be formed by two subunits (e.g., K1, K2, K28, SMKT) or three subunits (e.g., *K. lactis, Pichia acaciae* killer toxins). Most killer toxins are stable at acidic pH, but some of them maintain their activity within a wide pH range, such as HM-1 of C. mrakii (Yamamoto et al., 1986). The temperature ranges of their activities also vary, depending on the natural habitat of the killer yeast. For example, marine yeast killer toxins have an optimum temperature of 15 °C, while those of Kpkt produced by a soil strain of T. phaffii have an optimum temperature of 25 °C. In general, the majority of killer toxins lose their activity at >40 °C, although some can resist high-temperature treatment; e.g., HM-1 remains active after 1 h at 60 °C (Komiyama et al., 1996). Moreover, as reviewed by Liu et al. (2015), some killer toxins change their spectrum of action and show stronger killer activity in the presence of NaCl. This is particularly seen for killer toxins produced by yeast isolated from olive brine (Llorente et al., 1997) and from marine environments (Liu et al., 2012). Indeed, all of these represent important features that can greatly affect the potential use and applicability of these yeast killer toxins.

Despite this wide diversity, the killing actions of all of the characterized killer toxins is generally mediated by a two-step mechanism (Figure 3). During the first step, the killer

toxin recognizes and binds to a primary receptor on the cell wall of the sensitive target, thus indicating that the cell wall is an essential facilitator in the killing action. Accordingly, the killer toxins produced by *T. phaffi* (Kpkt), *Pichia anomala* (PiKT), and *Kluyveromyces siamensis* cannot kill spheroplasts of their sensitive targets (Comitini et al., 2009; Buzdar et al., 2011). Nevertheless, this is not a general rule, as PMKT and SMKT are also active on their

target cells following enzymatic digestion of the cell wall (Santos et al., 2007; Suzuki et al., 2001). However, it should be noted that the plasma membrane of yeast and fungi is negatively charged, while killer toxins can be more or less positively charged. Thus, the interactions between killer toxins and the plasma membrane of their sensitive target can also depend on the net cationic charge of the killer toxin (São-José et al., 2017).

As the cell wall is in general the primary site of action of killer toxins, different cell-wall components can act as the primary receptor sites. Among these, the β-1,3-D-glucans and β-1,6-D-glucans are commonly the receptors for the majority of the killer toxins characterized to date, although mannoproteins and chitin also serve as the first receptors for a number of killer toxins (Table 1).

During the second step of their actions, killer toxins are believed to be translocated to the cell membrane, where they interact with a secondary receptor. This step has been less characterized, and the secondary receptors are known, or hypothesized, for very few killer toxins (Table 1). Once the killer toxins bind to the secondary receptor and enter the sensitive cells, they kill the cells through different mechanisms (Figure 3; Table 1). These include cell-membrane permeabilization, inhibition of DNA synthesis, cell-cycle perturbation, and fragmentation of RNA (Klassen et al., 2017; Shaffrath et al., 2018; Liu et al., 2015; Klassen et al., 2008; Belda et al., 2017). In other cases, killer toxins act primarily on the cell wall of the sensitive target. Some toxins, including Kpkt and WmKt, have β-glucanase activities (Table 1) and hydrolyze the cell-wall glucans, which leads to cell lysis (Comitini et al., 2009; Guyard et al., 2002). HM-1 of C. mrakii impairs cell-wall biosynthesis by binding to and inhibiting β-1,3-glucan synthase, an enzyme that is localized to the plasma membrane (Lowes et al., 2000; Komiyama et al., 1996).

1.2.3. Ecological significance of killer toxin production

As killer yeast can kill sensitive yeast that occupy the same ecological niche, once the killer yeast occur within a community, they would be expected to dominate the sensitive yeast, by killing them (Sinclair, 2014). However, killer and sensitive yeast co-exist in Nature, and thus this does not appear to always be the case. To explain this phenomenon, a number of studies have investigated the ecological roles of killer yeast, and the way that their production of killer toxins affects the behaviors of both the killer and sensitive yeast in their natural environments. While investigating the benefits that can be derived from the killer phenotype, Pintar and Starmer (Pintar and Starmer, 2003) revealed that in *S. cerevisiae* the production of killer toxins has a metabolic cost, and thus reduces the fitness of the producer by ~4% (Wloch-Salamon et al., 2008). On the one side, this metabolic cost negatively correlates with the competition for resources with toxin-resistant strains (Pieczynska et al., 2016), while on the other side, it is rewarded by the benefits derived from killing the sensitive yeast (Wloch-Salamon et al., 2008).

Indeed, the success of killer yeast depends on a number of environmental factors, which include yeast dispersal, nutrient availability, and frequency of encounters between killer and sensitive yeast (Wloch-Salamon et al., 2008; Ivanovska and Hardwick 2005). In structured environments (i.e., with limited dispersal, such as in solid media and soft agar), the killer yeast show self-survival behavior, as they take advantage of unused nutrients and of the nutrients released by the dead sensitive cells (Wloch-Salamom, 2014). The mechanism that underlies this behavior is known as 'interference competition'. This is relatively common in the microbial world, and it is described as a direct negative interaction through which one microbial population actively suppresses a competing population (Case and Gilpin, 1974). In unstructured environments (e.g., liquid media), the toxin concentrations cannot reach lethal levels, and a greater portion of the available nutrients can be used by viable sensitive cells (Figure 4).

When nutrients are available, and under limited dispersal, the advantages that can be derived from production of a killer toxin exceed the metabolic cost of the toxin production, and the killer yeast can invade the sensitive yeast population (Wloch-Salamon et al., 2008). On the contrary, under nutrient shortage, the net gain derived from toxin production is limited, and when the killer yeast are highly dispersed, the toxin they produce might not be sufficient to allow invasion of the sensitive yeast population (Wloch-Salamon et al., 2008; Greig and Travisano, 2008). Based on this evidence, the production of killer toxins appears to provide competitive advantages when nutrients are abundant, and therefore it is not just a survival strategy under nutrient shortage, as hypothesized by Ivanowska and Harwick (Ivanovska and Hardwick 2005).

For the frequency of encounters, Rivero et al. (Rivero et al., 2015) reported that in high cell density cultures, killer and sensitive wild isolates of *S. cerevisiae* are engaged in a "reward–punishment mechanism" that regulates the production and exploitation of nutrients for the 'public good' while counteracting the insurgence of opportunistic strains. According to this mechanism, an apparently 'altruistic' fraction of the killer population undergoes cell lysis, which releases Hsp12p for the public good as it can improve the fitness of the yeast (which will potentially be available to both killer and sensitive yeast). To take better advantage of this public good, the killer cells also show contact-dependent killer toxin secretion, to thus decrease the fitness of the sensitive cells. However, in turn, these sensitive cells can overexpress Pau5p, a membrane protein that provides stress resistance, and can counteract the effects of the killer toxin, creating a balance between the killer and sensitive yeast, and completing this "reward–punishment loop" (Rivero et al., 2015). Recently, Deschaine et al. (Deschaine et al., 2018) observed that when killer and sensitive biofilm-forming strains grow in mixed communities, the killer strain can surround the biofilm-forming strain and prevail at the edge between the colonies. However, the competitive advantage provided by the increased spatial use of biofilm-forming strains offers them an escape from the killer toxin, with the possibility of outgrowth at the edge of at least one section of the colony.

The frequency of the killer phenotype ranges from 5% to 30% (Perez et al., 2016; Wloch-Salamon et al., 2008; Chang et al., 2015), although it can reach >50% of isolates in some species (Starmer et al., 1992). A survey of 136 wild isolates of *Saccharomyces* indicated that within this genus, the frequency of the killer-toxin-resistant phenotype is much higher (25%) than that of the killer phenotype (10%), which suggests that the acquisition of the

resistant phenotype is a consequence of the cohabitation with killer competitors in natural environments (Pieczynska et al., 2013). Indeed, the co-evolution between killer and sensitive yeast results in rapid appearance of aneuploid toxin-resistant mutants. These derive from the sensitive strain, which can take advantage of variations in gene dosage that can lead to activation of beneficial recessive alleles (Pieczynska et al., 2016). Interestingly, when such resistant mutations become fixed in the previously sensitive population, the benefits that can be derived from production of killer toxins are less, which leads to selection of killer strains with decreased killing activity, accompanied by more beneficial use of the available resources (Pieczynska et al., 2016).

Yeast ploidy can also affect the results of these killer/ sensitive interactions. McBride et al. (2008) reported that while *S. cerevisiae* K1 killer toxin produced by killer haploid cells can kill diploid sensitive cells, it does not kill haploid sensitive cells. This is important to ensure horizontal transmission of the killer virus during mating, and therefore to spread the virus into different genetic backgrounds.

Hence, overall in natural environments, the co-existence of killer, sensitive and resistant strains is the result of establishment of their dynamic equilibrium.

1.2.4. The active role of killer yeast as natural antimicrobials

In recent years, increased consumer awareness of the safety and health-promoting effects of food and beverages has been an important driving force for research and development of natural antimicrobials. Indeed, on the one hand, it is widely recognized that the overall quality of products of the agro-food industry is negatively related to the development of spoilage and pathogenic microorganisms. On the other hand, the possibility to substitute chemical preservatives with natural antimicrobials parallels the increased consumer interest in minimally processed food and 'greener' food and beverage additives.

As a result, the number of studies that have focused on the roles of bioactive molecules produced by antagonistic microorganisms continues to increase, and the application of natural approaches to counteract undesired microorganisms in food matrices is receiving considerable attention. In addition, the antagonistic behavior of yeast against other microorganisms has attracted increasing attention and stimulated their application as general biocontrol agents for crop protection and as therapeutic agents for use in human and veterinary medicines.

Thus, the exploitation of killer yeast or their toxins as natural antimicrobials that was at first confined to the research laboratory is now supported by the strong market demand for yeast with antimicrobial activities that can be profitably used to counteract proliferation of undesired microorganisms, and thus to reduce the use of chemical antiseptic agents (Pretscher et al., 2018). Accordingly, a number of patents regarding the use of killer yeast or their toxins have been registered (Table 2).

1.2.5. Killer yeast in preharvest and postharvest control of fungal diseases

A promising field of application of killer yeast as biocontrol agents is preharvest and postharvest control of phytopathogenic fungi. Fungal diseases of crops cause significant losses in food production worldwide. It is estimated that globally about 33% of fruit and vegetable production is wasted, mainly due to postharvest fungal diseases (Dukare et al., 2018). In this scenario, and with the need for sustainable agriculture, the biological control of fungal diseases using antagonistic microorganisms represents a possible alternative to the use of fungicides. Just over 20 years ago, Walker and co-authors (Walker et al., 1995) reported that *P. anomala* can markedly inhibit the growth of certain wood-decay basidiomycetes and plant pathogenic fungi. Later, it was shown that Pichia membranifaciens can protect grapevines against the causal agent of gray mold disease, *Botrytis cinerea* (Santos and Marquina, 2004; Masih and Paul, 2002) while *Candida sake* and *Pantoea agglomerans* protect apples and pears against *Penicillium expansum* and *Botrytis cinerea* (Nunes et al., 2002).

Indeed, *W. anomalus* (formerly *P. anomala*) killer strains have great potential as postharvest biocontrol agents, as they are active against *Colletotrichum gloeosporioides* (Lima et al., 2013), *Penicillium digitatum*, *Penicillium italicum* and *B. cinerea* (Platania et al., 2012; Parafati et al., 2016). Similarly, *Debariomyces hansenii* can counteract growth of *Monilinia fructigena* and *Monilinia fructicola* (Grzegorczyk et al., 2017). Indeed, W*. anomalus and D. hansenii* can inhibit growth of various sensitive yeast strains and several phytopathogenic fungi and have been granted Qualified Presumption of Safety status by the European Food Safety Authority, which would authorize their use as novel microorganisms in food preservation (Grzegorczyk et al., 2017). Thus the use of killer yeast that compete with pathogenic epiphytic microorganisms appears to represent a suitable tool to reduce the application of chemical antimicrobials, and thus to generate benefits for humans (Grzegorczyk et al., 2015; Liu et al., 2013).

1.2.6. Killer yeast in fermented food and feed

Killer yeast might have biotechnological applications in fermented food and feed production also due to their adaption to extreme environments. Studies on the microflora of fermenting olives revealed the presence of a rich yeast community that contributes to the fermentation process, but that can also be involved in spoilage during fermentation, storage and packing (Arroyo et al., 2008; Porru et al., 2018). Most of the yeast strains isolated from spontaneously fermenting olive brines have the killer phenotype and maintain their killer activities under the selective conditions of olive brines; e.g., *P. membranaefaciens* killer strains are particularly active at pHs and salt concentrations that are seen in fermentation processes (Llorente et al., 1997; Muccilli et al., 2011) In addition, at the sodium chloride concentration in olive brines, the toxicity of killer yeast can be enhanced. This enlarges the spectra of action of some killer yeast, and appears to be due to an additional selective advantage conferred to the killer strains in competition with sensitive cells.

Debariomyces hansenii killer strains isolated from olive brines cannot only kill a number of sensitive yeast, but are also active on *Lysteria monocytogenes*, *Bacillus cereus*, and *Salmonella typhimurium* (Psani and Kotzekidou, 2006). Thus, even though the occurrence of food-borne pathogens in fermented olives is not likely, the use of yeast starters that can

counteract spoilage yeast and food-borne pathogens can contribute to improved quality and safety of olives and other fermented vegetables (Psani and Kotzekidou,72006). This might also be the case for unpasteurized fermented vegetables, such as sauerkraut or other vegetables that can undergo yeast spoilage during storage (Champagne et al., 2017).

As well as fermented vegetables, dairy products can benefit from the availability of killer toxins that can counteract lactate-using spoilage yeast. Accordingly, a killer strain ascribed to Williopsis saturnus var. saturnus that was isolated from dairy products appears to be a promising biopreservative for the control of cheese spoilage (Liu and Tsao, 2009).

For the feed industry, the silage process might benefit from the killer yeast system. During the feeding step, silage is exposed to the air, which leads to aerobic deterioration. During this aerobic deterioration, aerobic microorganisms, including yeast, can degrade lactic acid to CO_2 and break down proteins and free amino acids to amines and ammonia. Indeed, 25 years ago, Kitamoto et al. (1993) suggested the use of killer yeast belonging to the genera *Kluyveromyces, Saccharomyces* and *Hansenula* for inhibition of growth of lactate-using spoilage yeast, and for the prevention of aerobic deterioration in silage. Subsequently, Lowes et al. (2000) proposed that the killer toxin produced by *C. mrakii* (Table 1, HMK) can be used as a biocontrol agent against spoilage yeast both in yoghurt production and silage maintenance. More recently, Bajaj et al. (Bajaj et al., 2013) reported that the killer toxin produced by *Pichia kudriavzevii* RY55 has good antimicrobial activity also against several pathogenic bacteria that are of significance in human health, including *Escherichia coli*, *Enterococcus faecalis, Klebsiella* sp., *Staphylococcus aureus, Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*. Thus, this toxin is of great interest as a new candidate for food preservation.

1.2.7. Killer yeast in winemaking

Wine can undergo spoilage at different stages of the winemaking process as a result of the presence of undesired yeast. Generally, spoilage yeast are controlled using sulfur dioxide (SO_2), a chemical antiseptic agent that can cause harm to consumers. On this basis, the World Health Organization has highlighted the need to reduce the content of SO_2 in food products, indicating that the daily intake of SO_2 should not exceed 0.7 mg/kg body weight (Scientific Committee for Food, 1996)Following these indications, over the last few decades, winemakers have adopted different strategies to reduce the SO_2 in wine. Van Vuuren and Jacobs (1992) suggested the use of killer starters that can be used to complete grape must fermentation and to control growth and persistence of undesirable yeast. Todd et al. (2000) proposed the use of killer and sensitive starter yeast during sparkling wine production to accelerate the onset of yeast autolysis. More recently, sequential inoculation of two Saccharomyces killer strains that can produce active and stable toxins under wine-making conditions have been proposed as a new strategy to control spoilage yeast species (De Ullivarri et al., 2014)

Also, nonSaccharomyces yeast or their toxins might also find application in the wine industry. The killer toxin naturally produced by *T. phaffii* is active on spoilage yeast of the *genera Kloeckera/ Hanseniaspora* and *Zygosaccharomyces* and can maintain its killer activity in wine for up to 14 days under such technological conditions (Comitini and Ciani,

2003). Thus, it was hypothesized that Kpkt can partially replace SO_2 during grape must fermentation, to reduce the sulfite content in wine. Other killer toxins secreted by several non-*Saccharomyces* yeast are active against a wide range of spoilage yeast, which include *Dekkera* and *Brettanomyces* (Table 3). These last, however, produce unpleasant odors during wine fermentation, ageing and storage, which can significantly affect the quality of the final product and result in considerable economic loss to the winemakers (Tubia et al., 2018). Interestingly, these killer toxins are not affected by the pH, temperature, and ethanol concentrations that are typical of winemaking conditions. Furthermore, they do not inhibit the fermenting *S. cerevisiae* nor the lactic acid bacteria, and are therefore hypothesized not to have negative impact on alcoholic and malolactic fermentation.

The killer phenotype is included among the selective criteria in the development of wine yeast starters, and at present, different *S. cerevisiae* killer strains are commercially available and are actually used in winemaking on an industrial scale. In contrast, as far as we areaware, the use of the yeast killer toxins themselves has not been reported for the wine industry to date.

1.2.8. Recombinant production of killer toxins

Different modes of use can be envisaged for killer yeast or for their killer toxins, which depend on the application concerned (Figure 1) and on the toxin levels secreted by the native producer. Indeed, when the direct use of a killer yeast is impractical, the application of purified preparations of native or recombinant killer toxins can be considered. Remarkably, although yeast killer toxins have been under investigation for decades, their heterologous production has been poorly explored to date.

To the best of our knowledge, the first reports on heterologous yeast killer toxin production was the expression of KHS and KHR killer toxins (Gotot et al., 1990; Goto et al., 1991) that are naturally produced in low amounts by weak killer strains of *S. cerevisiae* (Kitano et al., 1984). These pioneering studies were mainly aimed at the characterization of these toxins, and the determination of their primary structures, and their processing and secretion. However, they did pave the way for further studies, and a few years later, Kimura et al. (1993) expressed *C. mrakii* killer toxins HM-1 and WmKt in *S. cerevisiae*. Further studies have indicated that *S. cerevisiae* is not the only possible host for heterologous expression of killer toxins. K28 and zygocin were successfully expressed in *Sch. pombe* (Heintel et al., 2001; Weiler et al., 2002). More recently, fluorescent variants of K28 killer preprotoxin have been produced in *Pichia pastoris* (Giesselmann et al., 2017) to investigate K28 binding to the surface of sensitive cells and its trafficking in yeast. To gather further information on the cellular targets involved in the K1 mechanisms of toxicity and immunity, Gier et al. (2017) expressed K1 toxin derivatives in *S. cerevisiae*.

While most of these studies mainly addressed the mechanisms underlying toxin production, immunity, and secretion, other studies were aimed at producing killer toxins with a view to their possible applications as antimicrobials in the food and beverages industries. Lowes et al. (2000) proposed the heterologous expression of HMK in *Aspergillus niger* to obtain a recombinant protein that was active as a biocontrol agent against spoilage yeast in both yoghurt and silage production. In particular, silage is a product that is

particularly susceptible to yeast contamination once it is exposed to air, and they showed that recombinant HMK can delay its spoilage by aerobic yeast. Moreover, they reported that HMK is also effective on spoilage yeast that can be introduced into yoghurt following addition of fruit, honey, and nuts. Another study reported the heterologous production of Kpkt killer toxin (Chessa et al., 2017), with a view to its biotechnological exploitation in the wine industry. This recombinant Kpkt showed a wider spectrum of action than the native Kpkt. In particular, it also had marked effects on *Dekkera bruxellensis*, thus widening the possible applications of this toxin in the control of spoilage yeast during wine ageing in barrels.

As several enzymes currently applied to food processing are produced by recombinant microorganisms (Spohner et al., 2015) a future for recombinant yeast killer toxin production and use can be foreseen.

1.2.9. From killer toxins to peptide-based antimicrobials

As well as representing a powerful tool as natural antimicrobials, killer toxins can serve as the starting point for the development of peptide-based therapeutic agents, to provide new antifungal drugs with high biological activities that are associated with low toxicities and high specificities. To date, a crucial step has been the development of anti-idiotypic single-chain antibodies that represent the internal image of the active domain of a killer toxin and show killer activity. In particular, this was indicated by studies on the exploitation of the antimicrobial activity of PaKT, the killer toxin that is naturally produced by *P. anomala* ATCC 96603 (Polonelli et al., 1986). PaKT is active on different human pathogens (Magliani et al., 1997; Polonelli et al., 1986), but its parenteral administration as a therapeutic agent is not feasible as it is antigenic, toxic, and labile at physiological pH and temperature (Pettoello-Mantovani et al., 1995). To circumvent this problem, PaKT-neutralizing monoclonal antibodies were raised and used to obtain anti-idiotypic antibodies of PaKT. One of these (ScFvH6) that showed in-vitro and in-vivo killer activity on a broad range of pathogens was expressed in *Streptococcus gordonii* to obtain a recombinant vector of the bioactive molecule (Beninati et al., 2000). Then, a search of the biologically active fragments of ScFvH6 for possible therapeutic use lead to the synthetic killer decapeptide known as KP (Polonelli et al., 2003). KP represents the internal image of PaKT, and it is stable in vivo and active in vitro and in vivo against unrelated pathogens, such as viruses, bacteria, yeast, algae, and protozoa (Magliani et al., 2011; Giovati et al., 2018). Moreover, it confers plant pathogen resistance when expressed in *Nicotiana benthamiana* (Donini et al., 2005), thus defining it as a powerful therapeutic agent with applications in medical fields and in preharvest control of fungal pathogens.

Likewise, to expand the array of applications of HM-1, recombinant anti-idiotypic antibodies that represented the internal image of this killer toxin were produced (Selvakumar et al., 2006). These anti-idiotypic antibodies were active on important fungal pathogens, such as *Candida albicans* and *Cryptococcus neoformans* (Selvakumar et al., 2006). Further work led Kabir et al. (2011) to individuate two peptides that showed higher killer activities compared to the native HM-1 killer toxin: SP3, SP6. Remarkably, the amino-acid sequence of the SP6 peptide has a high degree of homology with the amino-acid sequence of the killer decapeptide KP described above. This thus suggests that the common features of these two peptides represent necessary requisites for their antifungal activities (Kabir et al., 2011).

1.2.10. Concluding remarks

Several studies have indicated killer yeast as possible starters for food and beverage fermentation, and their killer toxins as promising natural antimicrobials with a plethora of different applications. However, while killer starters are currently used in winemaking, there are several constraints regarding the use of native or heterologous killer toxins as natural antimicrobials. Thus, although a number of patents for exploitation of killer toxins have been registered to date, their use has not taken off on an industrial scale.

An important limitation to the use of killer toxins in the food and feed industries is certainly the scarcity of studies regarding their effects on human and animal consumers. A deeper understanding of the mechanisms of action of killer toxins will allow more specific applications to be devised and developed, and make these killer toxins more effective toward different sensitive targets. The possibility to crystallize killer proteins to study their three-dimensional structure and to raise specific antitoxin-antibodies represents another tools that will be effective to increase our knowledge and to make these toxins more applicable.

Another issue regards the need to gather more information on the mechanisms that underlie killer toxin production in yeast. A deeper knowledge of the costs and benefits associated with toxin production not only in *S. cerevisiae*, but also in the complex world of the non-Saccharomyces yeast will prove useful. These non-*Saccharomyces* yeast are characterized by the production of a vast array of killer toxins that have different target spectra and modes of action, and they will prove useful to further elucidate the regulation of killer toxin production, also with a view to their biotechnological exploitation. Indeed, killer toxins are generally secreted at low concentrations by their native producers. This hampers the implementation of fermentation processes that are aimed at their production on an industrial scale, and defines the need for development of recombinant production systems that can increase their production and facilitate their purification. The development of killer toxins into a new generation of antimicrobial agents with useful applications in the agro-food sector will provide a further boost to the study of these killer yeast and their killer toxins in the future.

Table 1. Known details of selected killer toxins and their biodiversity.

Killer yeast strain	Former designation	Toxin		Cell target		Mode of action
		Name	Molecular weight (kDa)	Primary	Secondary	
Chromosomal genes						
Saccharomyces cerevisiae 111		KHR	20	nd	nd	nd
Saccharomyces cerevisiae 115		KHS	75	nd	nd	Membrane permeabilization
Candida nodaensis PYCC3198		CnKT	nd	nd	nd	nd
Candida saturnus IFO0117	*W. saturnus. var. saturnus*	HYI	9.5	nd	nd	nd
Cyberlindnera mrakii IFO0895	*W. saturnus var mrakii; H. mrakii*	HM-1 or HMK	10.7	β-glucan	nd	Inhibition of β-1,3-glucan synthase
Cyberlindnera mrakii MUCL41968	*W. saturnus var. mrakii*	WmKT	85	β-glucan	nd	Cell-wall damage
Cyberlindnera mrakii NCYC500	*W. saturnus var. mrakii*	K500	~1.8	nd	nd	nd
Debariomyces hansenii CYC1021		nd	23	β-1,6-glucan	nd	nd
Kluyveromyces marxianus NCYC587	*K. fragilis*	K6	42	nd	nd	nd
Kluyveromyces wickerhamii DBVPG6077		KwKT	72	nd	nd	nd
Millerozyma farinosa KK1	*P. farinosa*	SMKT	6.6 (α), 7.9 (β)	nd	nd	Membrane permeabilization
Pichia kluyveri DBVPG5286		nd	19	nd	nd	Membrane permeabilization
Pichia membranifaciens CYC1106		PMKT	18	β-1,6-glucan	Cwp2p	Membrane permeabilization
Pichia membranifaciens CYC1086		PMKT2	30	Mannoprotein	nd	Cell-cycle arrest
Schwanniomyces occidentalis ATCC44252		nd	7.4(α), 4.9(β)	Mannoprotein	nd	Membrane permeabilization
Tetrapisispora phaffii DBVPG6076	*K. phaffii*	KpKT	33	β-1,3/ β-1,6-glucan	nd	β-glucanase activity
Wickerhamomyces anomalus BCA15, BCU24, BS91		nd	nd	β-1,3/ β-1,6-glucan	nd	β-glucanase activity
Wickerhamomyces anomalus NCYC434	*P. anomala*	Panomycocin	49	β-1,3-glucan	nd	Hydrolysis of β-1,3-glucan
Wickerhamomyces anomalus DBVPG 3003	*P. anomala*	PiKT	<8	β-1,6-glucan	nd	nd

Wickerhamomyces anomalus YF07b	*P. anomala*	nd		47	nd	nd	β-1,3-glucanase activity
Wickerhamomyces anomalus ATCC 96603	*P. anomala*	PaKt		85	β-1,3-glucan	nd	nd
Extrachromosomal double-stranded RNA virus-like particles							
Hanseniaspora uvarum 470		nd		18	β-1,6-glucan	nd	nd
Saccharomyces cerevisiae		K1		9.5 (α), 9(β)	β-1,6-glucan	Kre1p	Membrane permeabilization
Saccharomyces cerevisiae		K2		38.7	β-1,6-glucan	nd	Membrane permeabilization
Saccharomyces cerevisiae		K28		10.5 (α), 11 (β)	α-1,3-mannoprotein	Erd2p	DNA synthesis inhibition and cell-cycle block
Saccharomyces cerevisiae		Klus	nd		nd	nd	nd
Zygosaccharomyces bailii		Zygocin		10.4	Mannoprotein	nd	Membrane permeabilization
Extrachromosomal double-stranded DNA virus-like elements							
Debaryomyces robertsiae CBS6693	*W. robertsiae*	DrT		>100	Chitin	nd	rRNA fragmentation, cell-cycle perturbation
Kluyveromyces lactis IFO1267		Zymocin		97(α), 31(β), 28(γ)	Chitin	Ipt1p	Exo-chitinase activity; tRNA fragmentation; cell-cycle perturbation
Babjeviella inositovora NRRLY18709	*P. inositovora*	PiT		>100	Chitin	nd	rRNA fragmentation
Millerozyma acaciae NRRLY18665	*P. acaciae*	PaT		110(α), 39(β), 38(γ)	Chitin	nd	tRNA fragmentation, cell-cycle perturbation

Sources: Klassen et al. [5], Schaffrath et al. [28], Liu et al. [38], Belda et al. (2017).
nd, not determined/ not known

Table 2. Patents regarding the exploitation of killer yeasts and killer toxins.

Title of patent	Number	Source	Brief description	Year
Process for the preparation of killer toxins	WO2016199090	https://worldwide.espacenet.com/	Method for obtaining a preparation containing a killer toxin useful against phytopathogenic fungi on fruit plants, crops and domesticated plants	2016
Killer yeast Xiaoqu koji and preparation method thereof	CN103540538	https://worldwide.espacenet.com/	Preparation of Xiaoqu (starter for the fermentation of cereal wines) containing killer yeast	2014
Method for purifying killer protein	JP2007228937	https://patentscope.wipo.int/	Method for the purification of a killer protein	2007
Non-lactate-assimilating yeast for improving aerobic stability of silage	EP1194541B1	https://patents.google.com/	Utilization of a killer yeast unable to assimilate lactate to counteract spoilage yeasts in silage	2004
Toxin-related antibodies with antimicrobial and antiviral activity	WO2003095493	https://patentscope.wipo.int/	Anti-idiotypic antibody that retains yeast killer toxin activity and/or antiviral activity	2003
Methods of enhancing the preservation of forage materials with particular "killer" yeast strains that inhibit the growth of wild yeasts	NZ516456	https://worldwide.espacenet.com/	Utilization of *Saccharomyces exiguus* killer yeast or of its toxin to improve aerobic stability of silage	2002
Mixed starter culture and uses thereof	EP1476021A1	https://patents.google.com/patent/EP1476021A1/en	Composition of starter cultures containing a fermentative microorganism and at least one killer yeast that counteracts spoilage microorganisms or pathogens in different fermented foods	2002
Novel antifungal agents and fungicides, method for the production thereof and their use	CA2372935A1	https://patents.google.com/	Recombinant production of killer toxins Wicaltin and Zygocin to be used as antifungal agents and in plant protection	2001
Use of yeast-derived killer toxins to treat or prevent diarrhea, particularly in piglets and especially where caused by *Escherichia coli*	DE19912439	https://worldwide.espacenet.com/	Use of killer toxins produced by fermentation of killer yeast, for prevention and/or treatment of diarrhea	2000
Killer protein	JP10075789	https://patentscope.wipo.int/	New killer protein useful as component for antimicrobial agent s	1998
Novel killer yeast	JPH0662836	https://worldwide.espacenet.com/	Killer strains of *Candida stellata* and *Hanseniaspora occidentalis* active against *Hansenula* or *Kloeckera*	1994

Cloning of the zymocin gene and use of zymocin in beverages	WO1994020620A3	https://patents.google.com/	Preservation of carbonated drinks by using a killer toxin (Zymocin) active against a wide range of different yeast strains and cloning of Zymocin gene	1994
Novel yeast having killer activity	JPS6219079	https://worldwide.espacenet.com/	Use of *Candida vinea* having stable killer activity in a wide temperature range	1987
Yeast containing plasmids providing killer characteristics	US4418150	https://worldwide.espacenet.com/	Use of recombinant *Saccharomyces cerevisiae* showing killer toxin-resistance and killer activity toward wide range of yeasts	1983

Table 3. Non-*Saccharomyces* killer yeasts with promising applications in the wine industry.

Killer yeast	Strain	Sensitive targets	Reference
C. piralidae	IWBTY1140	*B. bruxellensis*	(Mehlomakulu et al., 2017)
K. wickerhamii	DBVPG6077	*Brettanomyces/ Dekkera*	(Comitini et al., 2004; Comitini and Ciani, 2011)
T. phaffii	DBVPG6076	*Hanseniaspora/ Kloeckera; Saccharomycodes ludwigii, Zygosaccharomyces bailii, Zygosaccharomyces rouxii*	(Ciani and Fatichenti, 2001; Comitini et al., 2004; Comitini et al., 2009)
T. delbruecki	NPCC1033	*H. uvarum, B. bruxellensis, P. guillermondii, P. membranaefaciens*	(Villalba et al., 2016)
W. anomalus	DBVPG3003	*Brettanomyces/ Dekkera*	(Comitini et al., 2004; De Ingeniis et al., 2009)
W. anomalus	Cf20	*B. bruxellensis, D. anomala, P. membranaefaciens, P. guillermondii*	(De Ullivarri et al., 2014)

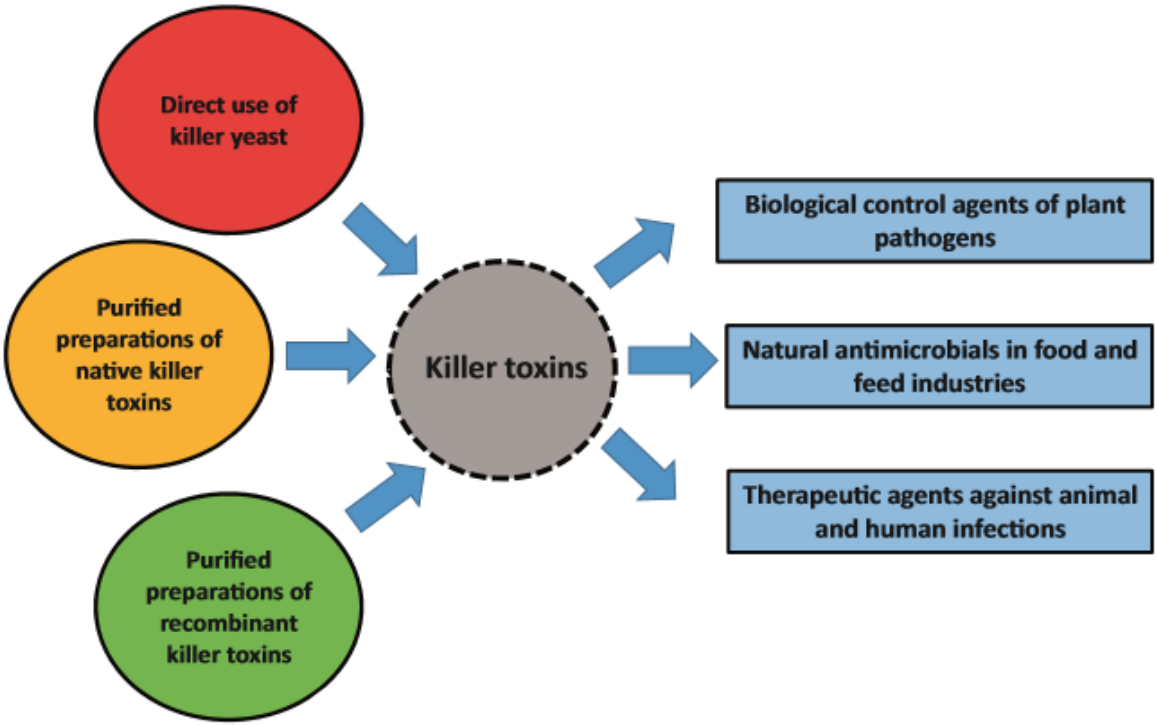

Figure 1. Biotechnological applications of killer yeast and their toxins.

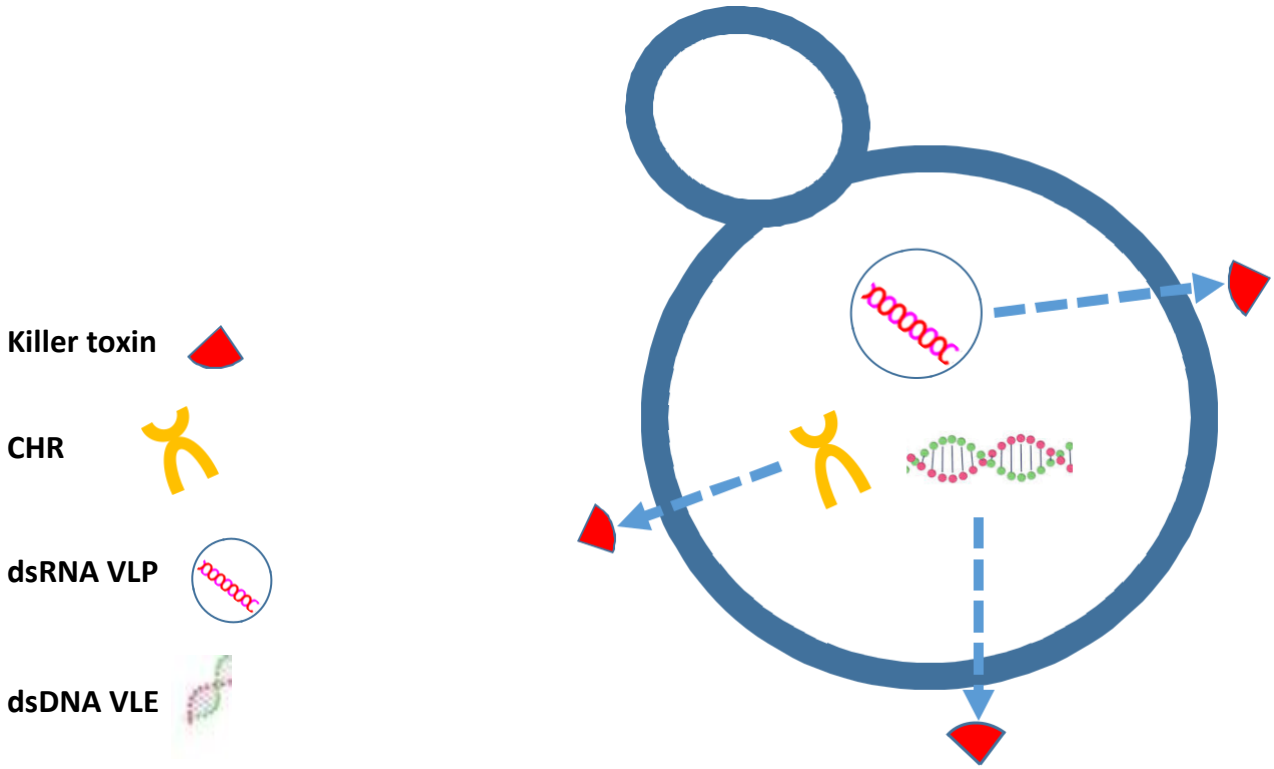

Figure 2. Localization of killer toxins genetic determinants. CHR: chromosomal genes; dsRNA VLP: extrachromosomal double-stranded (ds)RNA virus-like particles; dsDNA VLE: extrachromosomal (ds)DNA virus-like elements.

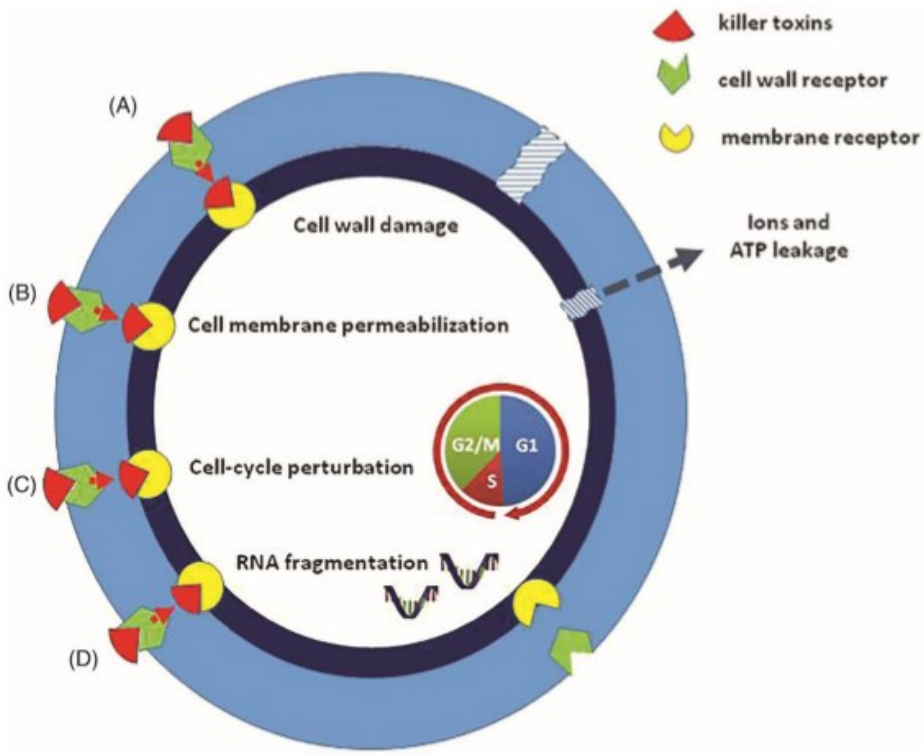

Figure 3. Mechanisms of action of killer toxins. Killer toxins killing action is generally mediated by a two-steps mechanism. In the first step the killer toxin binds a receptor on the cell wall of the sensitive target. In the second step the killer toxin interacts with a secondary receptor and kills the target cell through the following different mechanisms: (A) cell-wall damage due to hydrolysis of cell-wall glucans or inhibition of β-1,3-glucan synthase; (B) membrane permeabilization resulting in release of K^+, H^+, ATP, and other metabolites; (C) cell-cycle perturbation that blocks progression of the cell cycle in G1/S phase or completion of G1 phase; (D) fragmentation of RNA in terms of 18S and 25S rRNA, or tRNA.

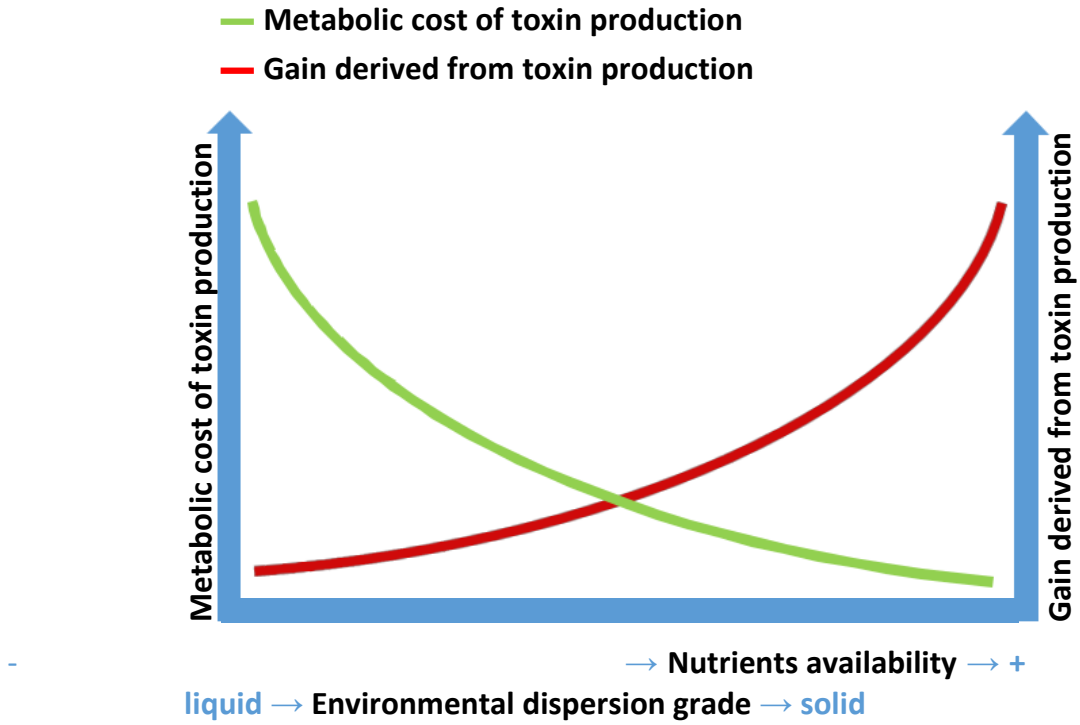

Figure 4. Competitive advantage and metabolic cost of killer toxin production by yeast as a function of nutritional and environmental conditions. The success of the killer yeast depends on yeast dispersal, nutrient availability, and frequency of encounters between killer and sensitive yeast.

1.2.11. References

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1.3. The yeast *Komagataella phaffii* (formerly *Pichia pastoris)*

Abstract

The methylotrophic yeast *Komagataella phaffii* (formerly *P. pastoris*) is a workhorse for biotechnology. Being a methylotrophic yeast it can be grown on low cost substrates, it has a high growth rate thus reaching high cell density in a short time; it is amenable of genetic manipulation and the availability of strong and tightly regulated promoters allows to switch off and on the production of heterologous proteins; it performs post-translational modifications of heterologous proteins and it can be utilized in the intracellular or extracellular production mode. Moreover, *K. phaffii* is approved by FDA as a source of animal feed protein for use in broiler and as a GRAS host for heterologous expression, and is therefore widely utilized in the food and drug industry. For these reasons, after *Escherichia coli, K. phaffii* is the second most frequently utilized host for heterologous protein expression. In the present thesis, *K. phaffii* has been utilized for the heterologous production of Kpkt killer toxin. Thus, here the main features of this yeast are described. Since most authors still utilize the former name *P. pastoris*, in this chapter the two names will be utilized, according to that reported in the references cited.

Keywords: *Pichia pastoris*, expression vectors, methanol utilization pathway, homologous recombination

1.3.1 A brief history

The first isolation of *Pichia pastoris* dates back to 1920 and is due to Alexandre Guillermond. The original isolate, coming from the exudate of a chestnut tree in France, was named *Zygosaccharomyces pastori* CBS704 (= NRRL Y-1603) (Guillermond, 1920) and was considered, for long time, as the type strain for the species. In 1956 Hermann Phaff isolated other strains of the same species from black oak trees, in the Yosemite region of California, and renamed it *Pichia pastoris* (Phaff et al., 1956). In the 1970s strain NRRL Y-11430 (CBS7435) was developed as a platform for single cell protein by Philips Petroleum due to its capability of producing high cell densities on methanol as the sole carbon source. In the meantime, *P. pastoris* strains started to be explored as hosts for the production of heterologous proteins (Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005; Cregg et al., 2009). In 1995 *P. pastoris* was accommodated into the genus *Komagataella* (Yamada et al., 1995). This includes the species *K. pastoris*, *K. phaffii*, *K. pseudopastoris*, *K. poluli*, *K. ulmi*, and *K. kurtzmanii* (Kurtzman 2005; Naumov et al., 2013). The two species *K. phaffii* (type strain NRRL Y-7556) and *K. pastoris* (type strain CBS704) can be distinguished based on 26S rDNA sequence. After the first release of the complete genome sequence (de De Schutter et al., 2009) other annotated genome sequences have been rendered available at www.pichiagenome.org (Zahrl et al., 2017). Strains of the species *K. phaffii* are the most frequently used yeast system for heterologous protein production and have been established as production hosts for more than two decades (Gasser et al., 2013; Safder et al., 2018).

Although some authors indicate this yeast with the current name *K. phaffi*, many others still indicate it with the former name *P. pastoris*. Thus, in this chapter the two names will be utilized, according to that reported in the references cited.

1.3.2. Methanol utilization pathway

P. pastoris is a methylotrophic yeast (Ramchuran et al, 2005). As such, it reaches high cell densities on culture media containing methanol as the sole carbon and energy source, through the methanol utilization pathway (MUT) (Figure 1). The first reactions of the MUT pathway are localized in peroxisomes that proliferate during growth on methanol containing medium. Methanol is oxidized to formaldehyde and hydrogen peroxide by the enzyme alcohol oxidase. Since alcohol oxidase affinity for methanol is low, large amounts of the enzyme are necessary for an efficient methanol utilization. Thus, alcohol oxidase accounts for about 30% of total soluble protein during growth in methanol containing medium. *P. pastoris* has two alcohol oxidase genes, namely *AOX*1 and *AOX*2 (Vanz et al, 2012). These code for similar proteins of 663 aminoacids but differ in the promoter region (Cregg et al., 1989). *AOX*1 promoter is strongly and tightly regulated by methanol and is responsible of about 85% of the alcohol oxidase activity (Cregg et al., 1989). *AOX*2 promoter is much weaker, it ensures slow growth in methanol containing media and provides 15% of the overall alcohol oxidase activity inside the cell (Cregg et al., 1989).

Following this first reaction, hydrogen peroxide is transformed into water and oxygen by peroxisomal catalase. Formaldehyde may be oxidised through two dehydrogenase reactions (dissimilation pathway), or condensed with xylulose-5P (XU5P) through

dihydroxyacetone synthase activity (assimilation pathway). During the dissimilation pathway, formaldehyde reacts with glutathione to form S-hydroxymethylglutathione that is oxidized to carbon dioxide through two reactions catalized by a glutathione-NAD^+-dependent formaldehyde dehydrogenase and a NAD^+-dependent formate dehydrogenase, both localized in the cytoplasm. Thus, the dissimilation pathway, on the one side, generates NADH that is converted into energy for growth on methanol, on the other side, is involved in the detoxification of formaldehyde. The assimilation pathway results in the production of glyceraldehydes 3—phosphate (GAP) and dihydroxyacetone (DHA), that enter the pentose pathway and are subsequently metabolized in the cytoplasm (Vanz et al., 2012).

Genes involved in MUT pathway are under the control of tightly regulated promoters, that are severely repressed by glucose and ethanol and induced by methanol. During growth on methanol proteins *AOX* and *DAS* may reach about 30 and 20 % of the soluble proteins within the cell.

Besides methanol, also glucose and glycerol are efficiently utilized as carbon sources. Glycerol utilization is fast, very likely due to four putative H^+/glycerol symporter in the genome (Mattanovich et al., 2009) and results in high biomass yield. Glucose utilization is slower in *K. phaffii* in respect to *S. cerevisiae,* possibly due to a less efficient hexose transport system. However, also glucose is currently utilized for biomass production. Regarding glucose utilization, *K. phaffii* is a Crabtree-negative yeast which means that it maintains respiratory metabolism also in the presence of high glucose concentration (Vanz et al., 2012).

1.3.3. *P. pastoris/K. phaffii* as a host for heterologous expression

P. pastoris is a very attractive host for heterologous production for the following reasons; i) it does not require complex media or culture conditions for growth and it can be grown on low cost substrates: ii) it has a high growth rate and reaches high cell density in a short time (Safder et al., 2018); iii) it is amenable of genetic manipulation; iv) it harbours strong and tightly regulated promoters useful to switch off and on the production of heterologous proteins; v) it performs post-translational modifications in contrast to bacterial cells (Çelik and Çalık, 2012; Yu et al., 2017); vi) it can be utilized in the intracellular or extracellular production mode (Ahmad et al., 2014).

The species *P. pastoris*/*K. phaffii* includes a variety of expression strains that derive from the wild strain NRRL-Y-11430 (Northern Regional Research Laboratories, Peoria, IL, USA); among these are auxotrophic mutants such as GS115, GS190, JC220, JC254, that grow on methanol at the wild type rate; mutants defective in genes involved in methanol utilization like KM71, MC 100-3, that therefore show on methanol slower growth compared to the wild type (Mut^S phenotype); and protease-deficient strains (SMD1163, SMD1165, SMD1168) that show reduced degradation of secreted proteins (Jahic et al., 2006). Strain GS115 was the first to be developed and used for heterologous protein expression (De Schutter et al., 2009). It derives from the wild strain NRRL-Y-11430 through a knock-out in histidinol dehydrogenase coding gene (*HIS*4) for auxotrophic selection. GS115 genome was published in 2009 (De Schutter et al., 2009). Strains commercialized by Invitrogen Life Technologies are protected by patent/materials ownership policies and cannot be utilized

for commercial purposes. On the contrary, *P. pastoris* strains deriving from CBS7435 are patent-free and can be utilized for commercial applications. Among these are auxotrophic and Mut^S strains and the *ku70* strain that, being impaired in the non-homologous end-joining (NHEJ) pathway, shows a more efficient homologous recombination (see Ahmad et al, 2014 for more information).

In 1993, Food and Drug Administration (FDA) assigned *P. pastoris* the GRAS status as a source of animal feed protein for use in broiler (FDA, 1993). In 2009 it was approved as a GRAS host for heterologous production of proteins of interest for the food and biopharmaceutical industries (Ciofalo et al., 2006; Thompson, 2010) and two recombinant proteins, namely Kalbitor and Jetrea have been approved by FDA and European commission (Ahmad et al., 2014). Moreover, many other recombinant biopharmaceutical products are under clinical trials (Corchero et al., 2013; Gasser et al., 2013). In accordance with its great potential as a host for recombinant proteins production, Potvin et al. (2012) reported that more than 500 recombinant proteins with a wide range of applications spanning from industrial enzymes, feed additives, and pharmaceuticals, have been produced in *P. pastoris*. Hence, this yeast is the second most frequently utilized host for heterologous protein expression, after *Escherichia coli* (Bill, 2014).

1.3.4. Main characteristics of *P. pastoris/K. phaffii* expression vectors

The standard expression vectors for *K. phaffii* are shuttle vectors *E. coli*/*K. phaffii* (*P. pastoris*) that contain a *E. coli* origin of replication (Li et al., 2007), selectable markers, and a foreign gene expression cassette. This includes a promoter, a secretion signal, when required, a multiple cloning site and a transcriptional termination sequence.

Selectable markers are either dominant or auxotrophic. Dominant markers confer resistance to antibiotics such as kanamycin, zeocin and blasticidin (Ahmad et al., 2014). Among these, Tn903kan^r gene allows for direct selection of both *P. pastoris* on G418 (Geneticin) and *E. coli* on kanamycin containing media (Scorer et al., 1994; Lin-Cereghino et al., 2008). Auxotrophic markers such as *HIS*4, *MET*2, *ADE*1, *ARG*4, *URA*3, *URA*5, *GUT*1, are meant to complement corresponding auxotrophies in the host strain and are specific for the selection of recombinant *P. pastoris* clones in minimal media (Juturu and Wu, 2017).

Different promoters are available for heterologous expression in *P. pastoris*. These can be inducible (methanol-regulated or not methanol-regulated) and constitutive and may be characterized by different strength. Among strong, methanol-regulated promoters, *AOX*1 is the most commonly utilized (Sigoillot et al., 2012). The transcription of foreign genes under the control of this promoter is tightly regulated and controlled by a repression/de-repression and induction mechanisms (Cregg et al., 2000). On glucose, glycerol or ethanol, *AOX*1 promoter is strongly repressed and upon depletion of these carbon sources, the promoter is de-repressed. Full induction occurs under methanol feeding and upon complete depletion of repressing carbon sources (Inan and Meagher, 2001; Juturu and Wu, 2017) and ensures high expression levels of heterologous proteins (up to 22 g/l intracellular and 15 g/l secreted protein) (Hasslacher et al., 1997; Werten et al., 1999). The main problems related to *AOX*1 promoter regard the need to finely tune methanol administration during growth and the difficulty to manage the switch from biomass

production phase (on glucose or glycerol) to protein production phase (on methanol). Moreover, methanol utilization is not advisable for the production of food-grade proteins (Juturu and Wu, 2017).

Other strong methanol-regulated promoters are those of genes involved in MUT pathway such as formate dehydrogenase (*FDH*) (Takagi et al., 2019), formaldehyde dehydrogenase (*FLD*1), induced also by methylamine, (Juturu and Wu, 2017), dihydroxyacetone synthase (*DAS*1) (Duan et al., 2018).

When high levels of protein expression should be avoided to circumvent unfolded protein response (UPR), the utilization of promoters with moderate strength is preferred. An example of weak methanol regulated promoter is that of *PEX*8 gene coding for a peroxin 8 peroxisomal matrix protein (Özçelik et al., 2019). *PEX*8 promoters is expressed on glucose and on methanol.

When inducible promoters are required, but the use of flammable or hazardous inducers such as methanol and methylamine should be avoided, non methanol-regulated promoters may be utilized. Among these are the isocitrate lyase (*ICL*1) that is repressed by glucose and induced by ethanol (Mendez et al., 2003) and the sodium-coupled phosphate symporter (*PHO*89), induced by phosphate depletion (Gasser et al., 2013).

Contrary to regulated promoters, constitutive promoters provide continuous and efficient transcription of the gene of interest and can be selected based on their strenght. Among strong constitutive promoters, the glyceraldehyde-3-phosphate (*GAP*) isolated by Waterham et al. (1997) is commonly used for the expression of different heterologous proteins in *K. phaffii* (Zhang et al., 2009) on a variety of carbon sources including glucose, glycerol ethanol and oleic acid (Goodrick et al., 2001; Juturu and Wu, 2017). *GAP* promoter reaches high expression levels, similar to *AOX*1 (Waterham et al., 1997) and, since it does not require an induction step, it simplifies the management of the fermentation process. For these reasons *GAP* promoter represents a valid alternative to *AOX*1. However, it should be taken into account that the continuous production of heterologous proteins may be toxic to the host cells. Other strong constitutive promoters are Glycosylphosphatidylinositol (*GCW*14), known to be stronger than *GAP* (Liang et al., 2013), the 3-phosphoglycerate kinase (*PGK1*) that according to De Almeida et al. (2005), is comparable to *AOX*1 and represents a good candidate for development of new engineered pathways in *Pichia* (Arruda et al., 2016).

Another interesting constitutive promoter is *PGK*1. *PGK*1 gene codes for phosphoglycerate kinase, that being involved in glycolisis and in the gluconeogenic pathway may represent about 5% of the total cellular protein (De Almeida et al., 2005). It ensures about 10% of the expression levels in respect to *GAP* promoter and it shows high potential for protein production when coupled with multicopy integration (Robert et al., 2019). Other promoters available are thoroughly described by Vogl and Glieder (2013).

The presence of secretion signals in expression vectors is aimed at targeting the recombinant proteins to the supernatant, in order to ease their purification. In this respect, *P. pastoris* can secrete high quantities of correctly folded, post-translationally processed and therefore active recombinant proteins in the culture medium when appropriate signal

sequences are present in the expression cassette. The signal sequence most commonly utilized is the α-mating Factor (α-MF) pre-pro peptide from *S. cerevisiae* (Ahmad et al., 2014) and its truncated versions. The pre-region drives the nascent protein into the endoplasmic reticulum (ER) where it is cleaved off by signal peptidase. The pro-region drives the protein from ER to Golgi apparatus, where it is trimmed by the endo-protease Kex2p. The final step is catalyzed by the dipeptidyl aminopeptidase encoded by *STE*13 after which the protein is released in the extracellular space by exocytosis.

Other secretion signals are the α -amylase from *Aspergillus niger*, *STA*1 signal sequence from *Saccharomyces diastaticus* glucoamylase gene, the signal sequence from the *Kluyveromyces marxianus* inulinase gene (Juturu and Wu, 2017), but also the secretion signal from *P. pastoris* native acid phosphatase (*PHO*1) is utilized (Cregg et al., 2000). Additionally, it is possible to utilize synthetic leader sequences (Martinez-Ruiz et al., 1998; Kjeldsen et al., 1999).

1.3.5. Integration of the expression cassette in the host genome

The expression vectors for *P. pastoris* are designed in order to obtain a stable integration of the DNA of interest in the host genome, also in the absence of selective pressure. Contrary to that observed in *S. cerevisiae*, where efficient homologous recombination (HR) naturally facilitates genetic modifications (Cai et al., 2019), in *P. pastoris* HR occurs with 1 to 30% of success and non-homologous end-joining (NHEJ) is much more frequent also when extended homologous overhangs are used. Homologous recombination may result in gene insertion, through a single crossover event (ends-in vectors) (Figure 2), or gene replacement, through a double crossover event (ends-out vectors) (Figure 3) (Klinner and Schäfer, 2004). Indeed, single crossover events (insertions) are much more likely to happen than double crossover events (replacements).

When the expression vectors carry the *AOX*1 promoter, the *AOX*1 transcription termination region (TT), or sequences even further downstream of *AOX*1 (3´ *AOX*1) and the *HIS*4 gene for the selection of transformants, the recombinant clone may present either a Mut^+ (wild type methanol utilization) or a Mut^S (slow methanol utilization) phenotype, depending on the plasmid design and on the host chosen. In order to gather HR at the *AOX*1 locus or at the *his4* locus, these vectors should be linearized. If the recombinant vector is linearized at a restriction site located in the 5´ or 3´ *AOX*1 regions, and gene insertion results in the integration of one or more copies of the vector upstream or downstream of the *AOX*1 gene, the resulting transformants have Mut^+ phenotype. On the contrary, if homologous integration at the *AOX*1 locus determines the distruption of the wild-type *AOX*1 gene, the resulting transformants have a Mut^S phenotype. When the recombinant vector is linearized at *HIS*4, single crossover events between the *his4* locus in the chromosome and the *HIS*4 gene on the vector result in gene insertion at the *his4* locus. Also in this case the genomic *AOX*1 locus is not involved and transformants show a Mut^+ phenotype.

In order to increase the amount of recombinant protein, multicopy integration of the expression cassette can be obtained (Vieira Gomes et al., 2018). Multiple insertion events are rather rare, occurring spontaneously at about 1–10% of the single insertion events. Thus multicopy integration can be realized using the following different strategies: A) Recombinant clones, carrying dominant selectable markers that confer resistance to Zeocine, Blasticidine or G418 are screened to seek for multicopy transformants resistant to

increasing concentrations to the corresponding antibiotics (Romanos et al., 1991, Lin-Cereghino et al., 2006). B) The host strain is transformed with a vector carrying a defective auxotrophic marker and recombinant clones are screened in minimal media lacking the corresponding growth factor to select those carrying multiple copies of the defective auxotrophic marker (Betancur et al., 2017; Piva et al., 2017). C) Vectors carrying extra copies of the expression cassette cloned in tandem (in vitro multimeric construction) are transformed in the hosts strain (Zhu et al., 2009).

1.3.6. CRISPR/Cas9 genome engineering system in *P. pastoris/K. phaffi*

Gene targeting with conventional knock-out/knock-in cassettes may not be always easy in *P. pastoris/K. phaffii* due to the already mentioned low frequency of HR. In addition, the insertion of markers (or disruption cassettes) and the replacement of DNA regions, by determining variations in the genome sequence, can affect the expression of adjacent genes. For these reasons, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) system is emerging as a powerful tool for *K. phaffii* genome engineering. Briefly, in the CRISPR/Cas9 genome engineering system, the DNA endonuclease Cas9, directed by a short guide RNA (gRNA), introduces a double strand break (DSB) at genomic regions that match the gRNA sequence (Carrol, 2012; Doudna and Charpentier, 2014). The strand breaks are repaired by the hosts cell endogenous repair machinery, allowing the introduction of various genomic modifications (Jinek et al., 2012). By changing the sequence of the gRNA it is possible to reprogram the CRISPR/Cas9 engineering system and therefore to target different loci in the genome. Weninger et al. (2016, 2018) reported on the utilization of CRISPR/Cas9 genome engineering system on *K. phaffii*. Based on the evidence that lack of Ku70 protein favours HR, they utilized the CRISPR/Cas9 system to mediate the integration of markerless donor cassettes on a Δku70 strain and obtained efficiencies approaching 100%. Liu et al., (2019) utilized the CRISPR/Cas9 system to obtain double- (DLI) and triple-locus integration (TLI) in *P. pastoris* through a single step, thus developing a powerful strategy for co-expression of multiple proteins and assembly of biosynthetic pathways in *P. pastoris*. In accordance with these reports, Gassler et al. (2019) developed a CRISPR/Cas9-based kit for gene insertions, deletions, and replacements. This kit, that is currently available at Addgene as CRIS*Pi* kit, is an important tool to obtain precise genomic modifications in *P. pastoris* without the integration of a selection marker and will contribute to further expand this yeast as a platform for heterologous expression.

1.3.7. The unfolded proteins response

Secreted and membrane proteins find in the lumen of the endoplamic reticulum (ER) a highly specialized environment for their proper folding. ER-resident chaperones and foldases contribute to the process. However, folding may be problematic for heterologous proteins that are not native to the folding environment. In fact, significant increases in the translation of secretory proteins may result in the accumulation of unfolded or misfolded proteins (Mori et al., 1992; Lai et al., 2007). This results in the induction of the unfolded protein response (UPR) to restore proper folding (Whyteside et al., 2011). However, when protein cannot be repaired, the ER-associated degradation (ERAD) of proteins by the proteasome is induced (Zahrl et al., 2018) thus determining a decrease in heterologous protein productivity of up to 60% of the total protein (Pfeffer et al., 2011).

UPR affects the transcription of about 400 genes in *S. cerevisiae* and in other yeasts and filamentous fungi (Travers et al., 2000). In *P. pastoris* the overproduction of heterologous proteins induces the expression of UPR-related genes (Whyteside et al., 2011; Zhu et al., 2011) or ERAD related genes (Whyteside et al., 2011; Zhu et al., 2011).

Accordingly, the production of human lysozyme, trypsinogen and interleukin (Hohenblum et al., 2004; Hesketh et al., 2013; Zhong et al., 2014); lipase from *Rhizopus oryzae* (Resina et al., 2007); prolyl endopeptidase (Wang et al., 2017); membrane transporter proteins (Vogl et al., 2014); penicillin G acylase from *Escherichia coli*, lipase B form *Candida antarctica*, xylanase A from *Thermomyces lanuginosus* (Raschmanovà et al., 2019) and phospholipase A_2 from *Streptomyces violaceoruber* (Yu et al., 2017) induces UPR in *P. pastoris*. However, this is not always the case as observed for the production of human serum albumin (Hohenblum et al., 2004; Aw et al., 2017) and insulin precursor (Vanz et al., 2014). Interestingly, the basal levels of the UPR marker protein Kar2/Bip are higher during rapid growth and decrease in stationary phase or during growth on methanol, also in the absence of recombinant protein production. Thus, also culture conditions may affect UPR (Roth et al., 2018).

1.3.8. Concluding remarks

K. phaffii has many advantages over other eukaryotic expression systems for the production of recombinant proteins and, based on that, it has become the favorite option among yeast expression systems. Different tools have been developed to increase the efficiency of protein expression with this yeast. Among these are *P. pastoris* strains with its high efficiency in HR, the development of a CRISPR/Cas9 genetic engineering system, the availability of a wide range of promoters with different strength and regulation mechanisms and of a number of different secretion signals. All these tools, together with a deeper knowledge of the mechanisms that underlie the UPR response, and the availability of patent-free host strains, will contribute to further enhance the exploitation of this yeast as a host for heterologous expression, both at laboratory and industrial scales.

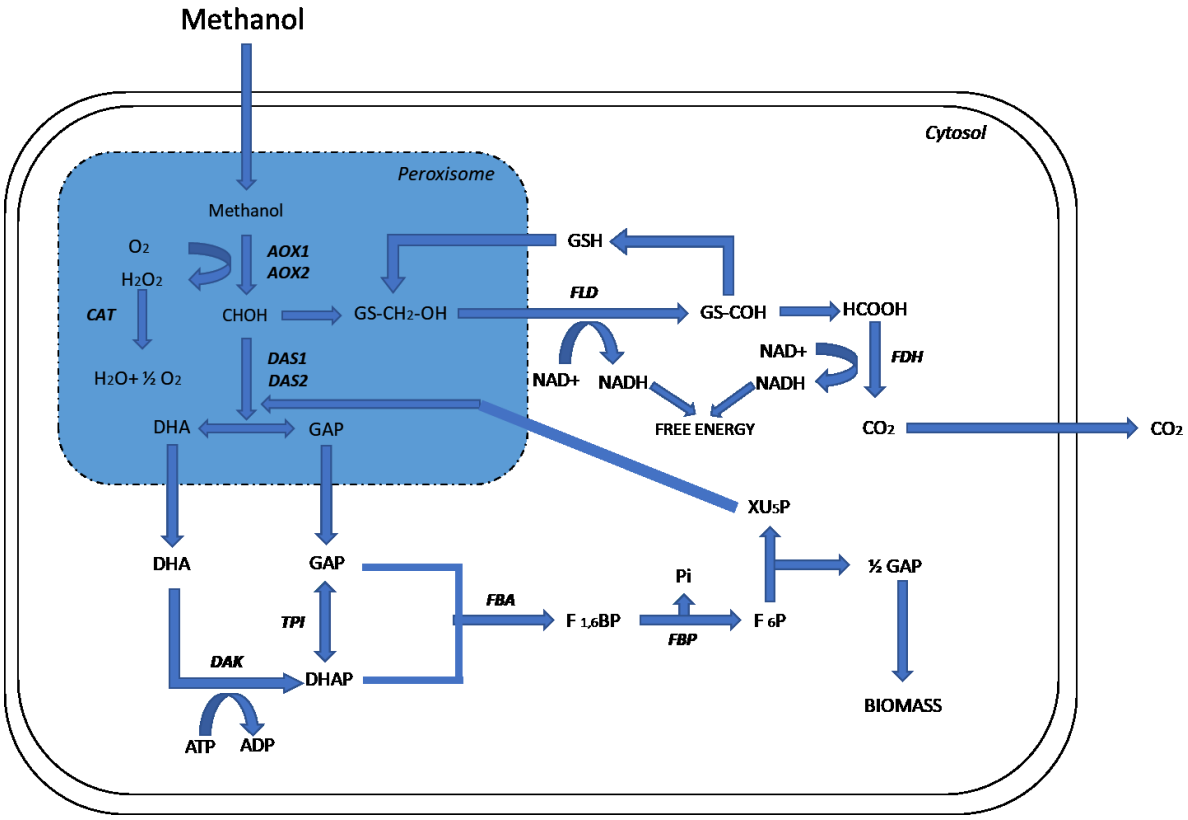

Figure 1. Methanol utilisation in *K. phaffii (P. pastoris).* *AOX*1 *AOX*2: alcohol oxidase; *FLD*: formaldehyde dehydrogenase; FGH: S-formylglutathione hydrolase; *FDH*: formate dehydrogenase; *CAT*: catalase; *DAS*: dihydroxyacetone synthase; *DAK*: dihydroxacetone kinase; *TPI*: triosephosphate iosmerase; *FBA*: fructose-1,6-bisphosphate aldolase; *FBP*: fructose1,6-bisphosphatase; DHA: dihydroxyacetone; GAP: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose-1,6-bisphosphate; F6P: fructose-6-phosphate; Pi: phosphate; Xu5P: xlyulose-5-phosphate; GSH: glutathione. Adapted from Chung et al. (2010).

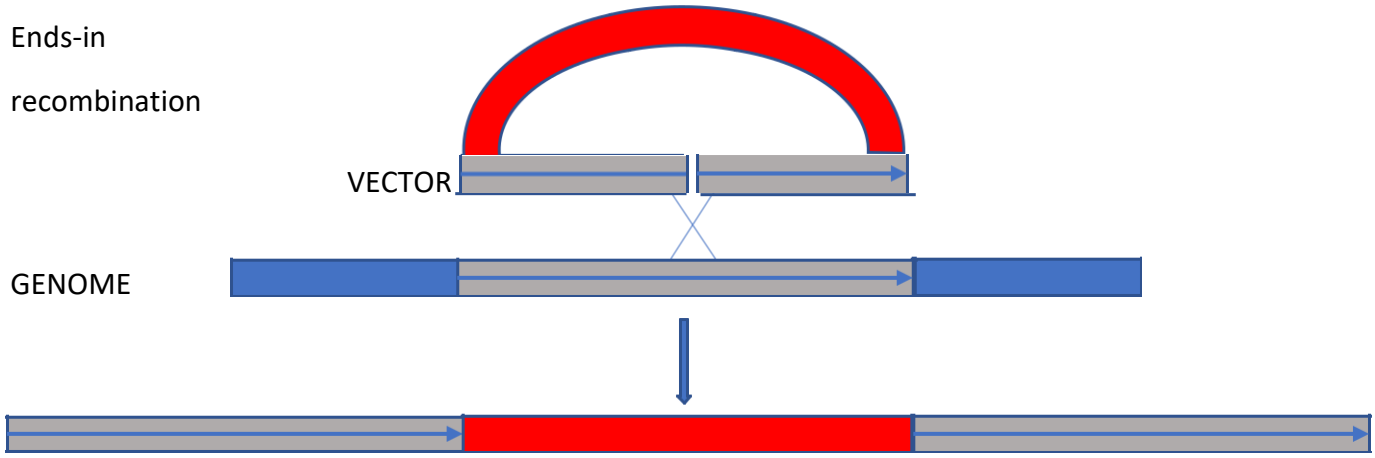

Figure 2. Ends-in recombination, mediated by a single cross-over, results in the tandem duplication of the homologous sequence (grey) and the insertion of the heterologous sequence (red). Adapted from Svetec et al. (2007).

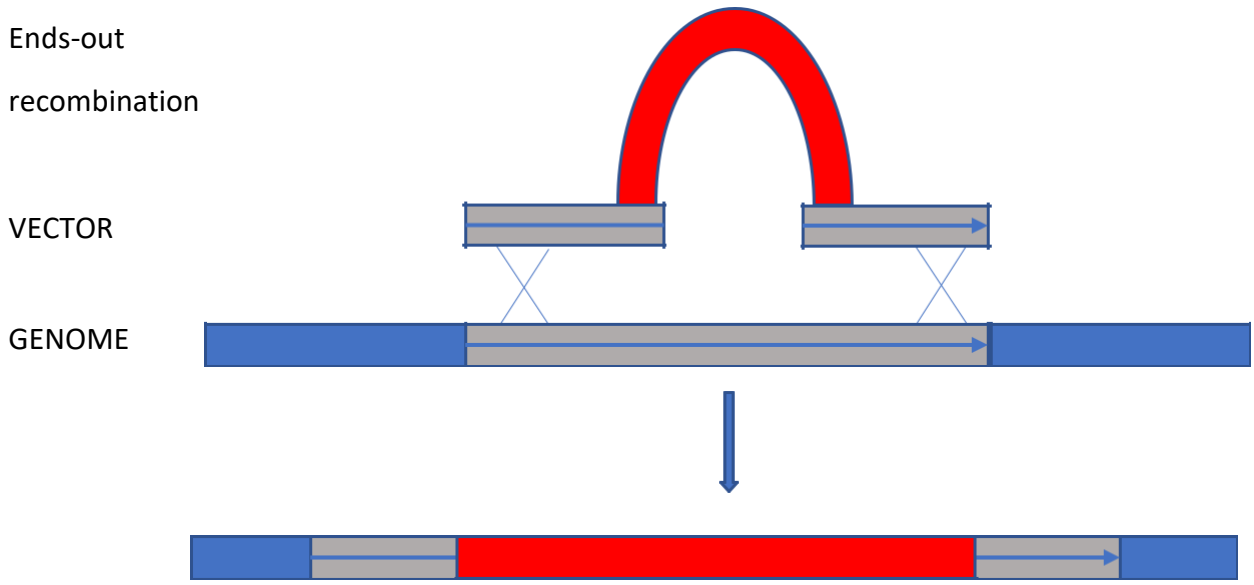

Figure 3. Ends-out recombination, mediated by a double cross-over, results in the interruption of the target sequence in the genome (grey) due the insertion of the heterologous sequence (red). Adapted from Svetec et al. (2007).

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1.4. The yeast *Tetrapisispora phaffii* and Kpkt killer toxin

The yeast *Tetrapisispora phaffii* (strain DBVPG 6076/ATCC 24235 / CBS 4417 / NBRC 1672 / NRRL Y-8282 / UCD 70-5), known only from a single South African soil isolate, was identified for the first time by Van der Walt in 1963 and included in the genus *Fabospora*. In the following years it was ascribed to the genus *Kluyveromyces* and to the species *Kluyveromyces phaffii* (Lodder 1970) and, subsequently it was reclassified as *Tetrapisispora phaffii* (Ueada-Nishimura and Mikata, 1999).

T. phaffii is a homothallic diploid yeast that reproduces by bipolar budding. It has 16 chromosomes and 5250 protein coding genes (Gordon et al., 2011) and its whole genome sequence is available at National Center for Biotechnology Information (NCBI) database at http://www.ncbi.nlm.nih.gov/genome/?term=*Tetrapisispora+phaffii*.

The first report on *T. phaffi* killer phenotype dates back to 1987 and is due to Rosini and Cantini. These authors carried out a study aimed at assessing the occurrence of the killer character within the genus *Kluyveromyces* and reported that the yeast *K. phaffii* was able to kill the "so called" apiculate yeasts ascribed to the species *Kloeckera apiculata* (Rosini and Cantini, 1987). Subsequently it was found that Kpkt is also active on *Saccharomycodes ludwigii*, *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii*, thus suggesting a possible use of this toxin for the control of spoilage yeasts in sweet beverages (Palpacelli et al., 1991).

In 2001 Ciani and Fatichenti reported that the killer toxin produced by *Kluyveromyces phaffii* DBVPG 6076 "*may be used as a biological agent against apiculate yeasts, which are usually present in freshly pressed juice and during the first stage of alcoholic fermentation*". Indeed, Kpkt killer activity is stable for more than 14 days in wine. Thus, based on that, Comitini and Ciani (2010) confirmed that *K. phaffii* killer toxin (Kpkt) could have interesting biotechnological application as natural antimicrobial able to counteract wine spoilage yeasts in the pre-fermentative stages of alcoholic fermentation (Comitini et al., 2010).

Kpkt killer toxin is encoded by *TpBGL*2 gene located on chromosome 1 of *T. phaffii* (Oro et al., 2014). This gene shows more that 75% identity with the corresponding genes of other yeast species (Oro et al., 2014) and Kpkt NH2-terminal sequence shows 93% identity with *S. cerevisiae BGL*2 gene coding for Bgl2 protein (Bgl2p) a cell wall protein with glucan transferase activity that has no killer activity (Mrsa et al., 1993; Goldman et al., 1995; Sarthy et al., 1997).

Molecular characterization of Kpkt showed that this is a glycoprotein with a molecular mass of 33 kDa that harbours a β-glucanase activity (Comitini et al., 2004). Accordingly, Kpkt killer toxin exerts its cytocidal activity by hydrolyzing β-glucans on the cell wall of the sensitive strain (Comitini et al., 2004). Contrary to that observed for other killer toxins such as K1 and K2 of *S. cerevisiae* (Novotna et al., 2004), Kpkt killer toxin has no effect on cell membrane (Comitini, 2009) and β-1,3- and β-1,6- glucans appear to be the primary receptor of the toxin on the cell wall of the sensitive targets (Comitini et al., 2004; 2009).

Recently Chessa et al. (2017) designed a plasmid needed for the heterologous expression of Kpkt in *Komagataella phaffii* GS115, and obtained two recombinant clones capable of producing up to 23 mg/L recombinant Kpkt (rKpkt). Moreover, they observed that recombinant Kpkt (rKpkt) has β-glucanase and killer activities and shows a spectrum of

action that is wider as compared to native Kpkt, being active also on *Dekkera bruxellensis*, a spoilage yeast of great interest for the wine industry.

1.4.1. References

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Chapter 2

Aim of the PhD thesis

The antimicrobial activity of yeast killer toxins has been known for more than 50 years. However, although many different authors have hypothesized killer toxins utilization in the food and wine industries, their biotechnological potential has never been really explored (Mannazzu et al., 2019). Previous works have contributed to shed light on the properties, mode of action and genetic determinant of Kpkt (Comitini et al., 2004; Comitini et al., 2009; Oro et al., 2014; Chessa et al., 2017). This is a killer toxin produced by *Tetrapisispora phaffii* DBVPG 6076 that has an extensive anti-yeast activity mainly directed towards wine spoilage yeasts among which *Kloeckera/Hanseniaspora*, *Saccharomycodes ludwigii, Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii*, of interest also for the sweet beverages and more in general for the food industry.

Kpkt killer activity is stable for at least two weeks in grape must under technological conditions (Comitini and Ciani, 2010). Thus, it is possible to hypothesize its utilization as natural antimicrobial in the wine industry where it can partially substitute sulphur dioxide, thus reducing the final amount of this antimicrobial in wine, in accordance with the recommendations of the World Health Organization. Considering that *T. phaffii* produces rather low amount of Kpkt, Chessa et al. (2017) obtained the heterologous expression of Kpkt coding gene in *Komagataella phaffii*, formerly *Pichia pastoris*, and showed that this can be a suitable host for the heterologous production of the toxin.

Based on these evidencies the general objective of this research was to further explore the biotechnological potential of Kpkt as natural antimicrobial of interest for the wine industry. Specific objectives were: i) the development of molecular tools for the production of rKpkt in *K. phaffii*; ii) the bioreactor production and purification of rKpkt; iii) the production of a ready-to-use rKpkt containing preparation; iv) the evaluation of the effect of the ready-to-use rKpkt preparation on a variety of biological targets.

The following chapters were written in form of research articles that have to be submitted to scientific journals. Thus, their structure does not exactly overlap the list of the specific objectives.

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Chapter 3

Development of molecular tools for the production of recombinant KpKt in *Komagataella phaffii* (formerly *Pichia pastoris*)

Abstract

Kpkt is a yeast killer toxin, naturally produced by a soil isolate of *Tetrapisispora phaffii* and active on wine spoilage yeasts ascribed to the genera *Kloeckera*/*Hanseniaspora*, *Saccharomycodes* and *Zygosaccharomyces*. In a previous work, aimed at increasing the production of Kpkt in view of its biotechnological exploitation as a natural antimicrobial, the heterologous production of this protein was obtained in *Komagataella phaffii* GS115. Here, with the aim of increasing the array of recombinant clones able to efficiently produce recombinant Kpkt, new plasmids useful for the heterologous production of the killer toxin were developed. For that, the sequence coding for Kpkt, optimized for the expression in *K. phaffii*, was cloned under the control of regulated (*AOX*1) or constitutive (*PGK*1) promoters and downstream of two different secretion signals (*S. cerevisiae* α-Factor secretion signal and the *T. phaffii* native secretion sequence). The resulting plasmids were transformed into *K. phaffii* strains GS115 and M12. Screening of transformed clones obtained indicated that the integration of the expression cassette results in a low percentage of recombinant strains showing killer activity. Contrary to native Kpkt, the recombinant version of this protein is toxic for *K. phaffii* GS115 and M12 strains, while showing lower toxicity on recombinant killer clones.

Keywords: *Komagataella phaffii*, Kpkt, heterologous expression of killer toxin, natural antimicrobial

3.1. Introduction

The success of *Komagataella phaffii* (formerly *Pichia pastoris*) for the production of heterologous proteins is mainly due to its easy genetic manipulation, capability of introducing posttranslational modifications similar to those of higher eukaryotes, correct folding of eukaryotic proteins and growth at high cell densities (Macauley-Patrick et al., 2005; Gasser et al., 2013).

Heterologous genes expression in *K. phaffii* can be achieved by cloning the genes of interest under the control of different promoters. According to one of the most common strategies, the heterologous gene is cloned downstream strong promoters that may be inducible or constitutive (Aw and Polizzi, 2013; Vogl and Glieder 2013; Ahmad et al., 2014; Piva et al, 2017). The utilization of inducible promoters is preferred when separation between growth and heterologous production is advisable. Among inducible promoters are *AOX*1, *FLD*1, *PEX*8 (Vieira Gomes et al., 2018). *AOX*1 promoter is definitely the most frequently utilized. According to Macauley-Patrick et al. (2005), it has been used to control the expression of more than 300 recombinant proteins. Its popularity is due to its tight regulation (induced by methanol, repressed by glucose, glycerol and ethanol) that is important for the production of toxic or growth impairing proteins. In alternative, constitutive promoters such as *GAP*1, *TEF*1, *PGK*1, *YPT*1 can be used when the expression of the heterologous protein is required at a constant level (Betancour et al., 2017; Vieira Gomes et al., 2018). Among strong constitutive promoters *GAP*1 is one of the most popular, being capable of driving the expression of heterologous proteins in glucose containing medium.

In both cases, in order to increase the production of the heterologous protein it is possible to screen for recombinant clones harboring multiple copies of the gene of interest (Betancour et al, 2017). To achieve this result different strategies can be utilized: (i) host cells can be transformed with plasmids containing more copies of the expression cassette cloned *in tandem* (Sreekrishna and Kropp, 1996); (ii) host cells can undergo consecutive rounds of transformation using different selection markers (Wriessenegger et al., 2014); (iii) plasmids can harbor antibiotic-resistance markers and in this case the recombinant strains are screened for growth in the presence of higher concentrations of antibiotic (Lin-Cereghino et al., 2008); (iv) plasmids can bear auxotrophic markers that are poorly transcribed due to defective promoters. In this last case, the recombinant clones need to amplify the copy number of the defective marker and, consequently, of the heterologous expression cassette on the same plasmid, to restore prototrophy (Kazemi et al., 2013; Betancur, 2014). In accordance with this strategy Betancur, (2014) obtained a *K. phaffii* strain auxotrophic for leucine and constructed an integrative expression vector containing a *leu2-d* defective marker.

It should also be considered that the overexpression of a heterologous protein may activate the Unfolded Protein Response (UPR) and cause an impairment of the secretory pathway, thus representing a problem for the host. In these cases, the utilization of intermediate strength promoters can be advisable (Piva et al., 2017).

Kpkt is a yeast killer toxin naturally produced by a soil isolate of *Tetrapisispora phaffii* and active on wine spoilage yeasts ascribed to the genera *Kloeckera*/*Hanseniaspora,*

Saccharomycodes and *Zygosaccharomyces.* Kpkt retains its killer activity for up to two weeks in grape must under technological conditions (Comitini et al., 2009) and is therefore of interest as a natural antimicrobial for the wine industry (Comitini and Ciani 2010).

The *T. phaffii* gene coding for Kpkt, named *TpBGL*2, was isolated by Oro et al. (2014) and its sequence, optimized for the expression in *K. phaffii,* was cloned in pPIC9 (Chessa et al., 2017). By doing so, Chessa et al. (2017) succeeded in the heterologous production of Kpkt in GS115, and showed that the recombinant toxin (rKpkt) expands its spectrum of action in respect to native Kpkt (nKpkt).

The aim of this work was to increase the array of recombinant clones able to efficiently produce rKpkt, in view of a large scale production of the recombinant protein. For that new plasmids useful for the heterologous production of Kpkt under the control of different promoters were developed.

3.2. Materials and methods

3.2.1. Microorganisms, plasmids and growth media. Microbial strains used are reported in Table 1. *K. phaffii* strain GS115 (*his4*, Invitrogen) harbors a mutation in the histidinol dehydrogenase gene (*HIS4*) that prevents it from synthesizing histidine. pPIC9 and all pPIC9 deriving plasmids carry *HIS4* which complements *his4*, so that recombinant strains can be selected for their ability to grow on histidine-free medium (Pichia Expression Kit, Invitrogen 2014). *K. phaffii* strain M12 (*leu2,* Betancur et al., 2017), is a derivative of strain X-33 (Invitrogen) that in turn is a revertant of GS115. Both GS115 and M12 were used as hosts for the heterologous expression of Kpkt.
E. coli (Trans5α Chemically Competent Cell, TransGen Biotech) was used for cloning purposes. Plasmid vectors utilized are reported in Table 2. pPIC9TpIM and pPIC9TpIMHisTag that contain the heterologous gene under the control of *AOX1* promoter were provided by DNA 2.0.

pKGFP-ld (Betancur et al., 2017) was utilized for the construction of vectors pPGK1αTpIM, pPGK1TpIM, pAOX1TpIM and pAOX1αTpIM.

Media used were: YEPD: 2% glucose, 1% yeast extract and 2% peptone; MD: 2% glucose, 1.34% YNB w/o aminoacids, 0.00004% biotin, 2% dextrose; BMGY: 1% glycerol (w/v), 1% yeast extract, 2% peptone, 1.34% YNB w/o aminoacids and 0.00004% biotin; BMMY: as BMGY with 0.5 or 1% methanol (v/v) in place of glycerol; SOC: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$ and 20 mM glucose; LB: 0.5% yeast extract, 1% tryptone and 1% NaCl supplied with 100 µg/ml ampicillin or kanamycin. Media were added with 2% agar when required. YEPD, BMGY and BMMY were buffered at pH 4.5 with citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4).

Yeasts and bacteria were maintained on YEPD or LB at 4 °C for short term storage and in YEPDgly (YEPD with addition of 10% glycerol) and LBgly (LB with addition of 10% glycerol) at −80 °C for long-term storage, respectively.

Table 1. Microbial strains utilized in the present work.

Strain	Source	Characteristic
Tetrapisispora phaffii 6076	DBVPG	Native producer of Kpkt
Komagataella phaffii GS115	Invitrogen	Host for heterologous expression, *his4*
Komagataella phaffii M12	Betancur et al., 2017	Host for heterologous expression, *leu2*
Saccharomyces cerevisiae 6500	DBVPG	Sensitive to Kpkt and rKpkt
Dekkera bruxellensis 692	DiSVA	Sensitive to rKpkt
Dekkera bruxellensis 648	DiSVA	Sensitive to rKpkt
Dekkera bruxellensis 640	DiSVA	Sensitive to rKpkt
Hanseniaspora uvarum 156	UNISS	Sensitive to Kpkt and rKpkt
rc#24	UNISS	pPIC9 in GS115, negative control
rc#1	This study	pKLD in M12, negative control
rc#6, 7, 14, 16, 87	This study	pPIC9TpIM in GS115, Mut^+
rc#2	This study	pPIC9TpIM in GS115, Mut^S
rc#17	This study	pPIC9TpIMHisTag in GS115, Mut^+

UNISS: Microbial Culture Collection, Department of Agriculture, University of Sassari, Sassari, Italy; DiSVA: Culture Collection of Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy. DBVPG; Industrial Yeast Collection DBVPG, Università di Perugia, Italy.

3.2.2. General molecular biology procedures. Unless otherwise stated, restrictions digests were carried out in 50 µL containing up to 5 µg of DNA, 40 units of the appropriate restriction enzyme (New England Biolabs), 5 µL 1X NEBuffer™ 3.1, and H_2O up to the final volume. After 2 h incubation at 37 °C and DNA fragments separation on 1% agarose gel, DNA fragments of interest were excised and purified from gel. PCR reactions were carried out using an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA USA in 50 µl final volume containing 5x Phusion® HF Buffer 1x, 10 mM dNTPs 200 µM each, primers 0.5 µM each, 200 ng template DNA, Phusion® DNA Polymerase (Finnzymes) 0.02 U/µl, H_2O. Reaction conditions were: initial denaturation at 98°C for 30 sec followed by 30 cycles of 98 °C for 10 sec, 55 °C for 20 sec, and 72 °C for 15 sec; the final elongation step was at 72 °C for 7 min. Ligations were carried out in a final volume of 20 µl containing vectors and insert at a molar ratio of 1:3, 1 U T4 DNA Ligase (New England Biolabs), 1X T4 DNA Ligase Buffer, Nuclease-free water. Ligation mixtures were incubated at room temperature for 10 min.

For agarose gel electrophoresis UltraPure agarose (Invitrogen Life Technologies) gels added withSYBR Safe DNA Gel Stain (Invitrogen Life Technologies), were run for 1 h at 75 V in TAE 1× (40 mM Tris-acetate and 1 mM EDTA). The 1kb ladder (M1181/M1182) Ready-to-load (Dongsheng Biotech Guangzhou, Guangdong, China) was used as DNA size marker. Gel

images were captured by means of Gel Doc™ EZ system (Bio Rad Hercules CA, USA). For DNA elution from agarose gels Wizard SV Gel and PCR Clean- Up System (Promega, USA) was used. Purification of PCR amplicons was carried out by means of the NucleoSpin Gel and PCR clean-up Kit (Macherey-Nagel).

3.2.3. Transformation. *E. coli* transformation was carried out as indicated by the manufacturer (Trans5α Chemically Competent Cell, TransGen Biotech). Minipreparation of recombinant plasmids were carried out as described in Sambrook et al. (1989). Recombinant vectors were sequenced by BMR Genomics (University of Padua, Padua, Italy). *K. phaffii* GS115 total DNA extraction was done according to the Pichia Expression Kit manual (Catalogue No. K1710-01; Invitrogen). *K. phaffii* competent cells preparation was carried out as follows: yeast cells were precultured o/n in 5 mL of YEPD at 30 °C under constant shacking (200 rpm) and then inoculated in 250 ml of YEPD to a final OD_{600} of about 0.4 and incubated again at the same condition. When cell density reached OD_{600} of about 1.3 - 1.5, cells were washed as indicated in the Pichia Expression Kit manual (Invitrogen) and 80 µl aliquots of competent cells were added with 5 or 20 µg plasmid linearized with *Sac*I or *Bgl*II (New England Biolabs) and transferred to ice-cold electroporation cuvettes. After 5 min incubation on ice, electroporation was carried out by using a Gene Pulser II (Bio-Rad) under the following conditions: 1500 V, 200 Ω and 25 µF. One ml of cold 1 M sorbitol was added immediately after the electroporation, with the samples plated onto MD and incubated at 30 °C. A negative control of transformation, where competent cells were added with sterile H_2O, was used. Transformants were screened for the integration of the expression cassette and for killer activity.

3.2.4. Screening of *K. phaffii* recombinant strains. Molecular screening of the transformants was carried out by PCR with primer pairs AOX1F/AOX1R, TpIMF/TpIMR and AlphaF/TpIMR depending on the plasmid vector utilized (Table 3). PCR reactions were carried out in 25-µL reaction mixture containing: 2 µL template DNA, 0.2 µM each dNTP, 0.5 µM each primer, 1 U Taq polymerase (Invitrogen Life Technologies), 1×Taq buffer and 2.5 mM MgCl2. Amplification conditions were: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 1 min, elongation at 72 °C for 1 min and final elongation at 72 °C for 7 min. For the evaluation of killer activity, transformants were subject to methanol or glucose induction as described below. Methanol induction: yeast cells were precultured in 25 mL BMGY in 250 mL baffled flasks at 30°C under constant shaking (180 rpm) closed with a sterile cloth to maximize oxygen transfer. After 24 h, cells were transferred to 25 mL BMMY to an OD_{600} of 1.0 and grown for up to 144 hours at 28 °C for the first 24 hours, 25 °C for the following 24 hours and at 23 °C till the end of the cultivation. In order to induce the production of rKpkt 1% methanol was added every 24 hours and the killer activity was evaluated every 24 hours starting by means of the well plate assay. Prior to glucose induction clones obtained with pKLD based plasmids were pre-screened as follows: 10^6 cell/mL for each clone were inoculated in 1 mL leucine-free MD liquid medium in 24 well plates at 30 °C in slow agitation. After 24 h, optical densities of the cultures were recorded to highlight the best growers, possibly due to multiple copy integration of the defective *leu2-d* marker. Glucose induction: yeast cells were pre-cultured in 25 ml MD at 30 °C. After 24 h cells

were inoculated in 25 mL MD to an OD_{600} of 1.0 and grown for up to 72 hours at 25°C. Fresh MD medium was added every 24 h to replenish that lost by evaporation. Rc#24, previously obtained by Chessa et al. (2017), and rc#1 were utilized as negative controls for heterologous protein production, for GS115 and M12 transformants, respectively. Killer activity was assessed by means of the well plate assay described by Rosini (1983). Briefly, aliquots of a cell suspensions of the target sensitive strain (OD_{600} 0.1-0.2) prepared in sterile distilled water were either included (1 mL) or spread (100 µL) on YEPD agar plates buffered at pH 4.5 with citrate-phosphate buffer. 100 µL of the sample to be tested were loaded in wells cut on YEPD-agar-plates. For each well plate assay the acellular supernatants of *T. phaffii* DBVPG 6076 and of rc#24, which carries integrated empty pPIC9 expression cassette, were used as positive and negative controls, respectively.

Table 2. Plasmids utilized in the present work.

Name	Source	Characteristics	Host
pPIC9	Invitrogen	Expression vector	GS115
pKGFP-ld	Betancur et al, 2017	Expression vector	M12
pKLD	This study	Expression vector pKGFP-ld derived	M12
pPIC9TpIM	DNA 2.0	TpIM under the control of *AOX*1 promoter, α-Factor secretion signal	GS115
pPIC9TpIMHisTag	This study	As pPIC9TpIM, HisTag in C terminus	GS115
pPGK1TpIM	This study	pKLD derived, TpIM under the control of *PGK*1 promoter	M12
pPGK1αTpIM	This study	pKLD derived, as pPGK1TpIM, α-Factor secretion signal	M12
pAOX1TpIM	This study	pKLD derived, TpIM under the control of *AOX*1 promoter	M12

TpIM: *TpBGL*2 codon optimized sequence

Table 3. PCR primers utilized

Primer name	Sequence
AOX1F	5′ GACTGGTTCCAATTGACAAGC 3′
AOX1R	5′ GCAAATGGCATTCTGACATCC 3′
TpIMclF	5′ATTGGATCCAAACGATGAGATTTAGCACCTTCGTTTC 3′
AlphaF	5′ ATGAGATTTCCTTCAATTTTTACTG 3′
TpIMF	5′ TGCAGCTTCGGACTGCAATA 3′
TpIMR	5′ TGCCCAACTTGAACTGAGGAA 3′

3.3. Results

3.3.1. Cloning and expression of Kpkt encoding gene in *K. phaffii* GS115. In order to expand the number of recombinant clones for the heterologous production of KpKt in *K. phaffii* GS115, plasmids pPIC9TpIM and pPIC9TpIMHisTag (Figure 1) were utilized. Both plasmids contain an auxotrophic marker for histidine (*HIS4*) and an optimized sequence of *TpBGL*2 gene named TpIM which is cloned under the control of *AOX*1 promoter and downstream the α-Factor secretion signal of *S. cerevisiae* (Figure 1) (Chessa et al., 2017). The two vectors differ for the presence of a histidine tag in pPIC9TpIMHisTag. This was considered useful in view of the purification of the toxin by means of immobilized metal affinity chromatography. The two vectors were linearized with *Sac*I and *Bgl*II and transformed into GS115. Plasmid linearization is required to facilitate the genomic integration of the expression cassette by homologous recombination, thus generating recombinant clones that are characterized by genetic stability, also in the absence of selective pressure. When *Sac*I restriction enzyme is used, the integration of the expression cassette is expected to be mediated by a single crossover event between the *AOX*1 locus in the genome and any of the *AOX*1 regions in the vector. This may lead to the integration of one or multiple copies of the cassette either upstream or downstream the *AOX*1 locus in the genome and to the achievement of His^+ Mut^+ recombinant clones. When *Bgl*II restriction enzyme is utilized, the integration of the expression cassette may be mediated by a double cross over between the *AOX1* promoter and 3′*AOX*1 region of the vector and the *AOX1* locus in the genome. This results in a gene replacement event that leads to the complete removal of the *AOX1* coding sequence and to the achievement of His^+ Mut^S recombinant clones. About 300 and 200 transformants were obtained with pPIC9TpIM and pPIC9TpIMHisTag, respectively, and 100 clones for each plasmid where chosen randomly and further characterized.

PCR of the recombinant clones with primer pair AOX1F/AOX1R revealed that 87 recombinant clones for pPIC9TpIM and 72 for pPIC9TpIMHisTag integrated the expression

cassette. According to the results of PCR analysis, 76 clones obtained with pPIC9TpIM and 53 clones obtained with pPIC9TpIMHisTag produced two amplicons, one of 2200 bp corresponding to *AOX1* gene and one of about 1300 bp corresponding to TpIM gene, showed the two amplicons and were therefore putative Mut^+ (Figure 2). Eleven clones for plasmids pPIC9TpIM and 19 for plasmid pPIC9TpIMHisTag showed one single amplicon of 1300 bp corresponding to TpIM gene and were therefore putative Mut^S transformants. Since Mut^+ and Mut^S transformants show comparable growth rate on MD medium but can be easily discriminated based on their growth rate on MM, all the transformants were subject to replica plating on MD and MM. Results obtained confirmed 76 Mut^+ and 11 Mut^S clones for pPIC9TpIM and 53 Mut^+ and 19 Mut^S clones for pPIC9TpIMHisTag (data not shown).

Sixty Mut^+ and 11 Mut^S clones obtained with pPIC9TpIM and 53 Mut^+ and 19 Mut^S clones obtained with pPIC9TpIMHisTag, and the negative control rc#24, were grown in baffled flasks as reported by Chessa et al. (2017) to select those capable of secreting detectable amounts of rKpkt. Briefly, each clone was inoculated in BMGY and after 24 h at 30 °C under shaking conditions, transferred to BMMY with 1% methanol for up to 144 h. Methanol was added every 24 h in a fed-batch mode to induce the production of the recombinant protein. At 72, 96, 120 and 144 h of induction killer activity was evaluated on cell-free supernatants by means of the well plate assay. Positive and negative controls of killer activity were cell-free supernatants of *T. phaffii* 6076 and rc#24, respectively. Based on the results obtained fourteen clones that integrated plasmid pPIC9TpIM (of which one Mut^S) and one that integrated pPIC9TpIMHisTag proved capable of secreting rKpKt and showed, on plate, stronger killer activity with respect to the native Kpkt producer *T. phaffii* DBVPG 6076. No difference in killer activity was observed between Mut^+ and Mut^S recombinant clones (Figure 3).

3.3.2. Construction of expression vectors for M12. To expand the array of vectors to be utilized for TpIM heterologous expression, the construction of four new vectors based on pKLD and containing TpIM under the control of the constitutive promoter *PGK*1 or the inducible promoter *AOX*1 and downstream two different secretion signals (α-Factor secretion signal from *S. cerevisiae* and the native *TpBGL*2 secretion signal) was planned. In order to clone TpIM under the control of the constitutive promoter *PGK*1, plasmid pKGFP-ld (Betancour et al., 2017) was digested with *Not*I and *BamH*I restriction enzymes to excise the GFP cassette and obtain pKLD. Then the cassette including α-Factor secretion signal and TpIM gene was excised by pPIC9TpIM with the same restriction enzymes and cloned in pKLD to obtain pPGK1TpIM. In parallel, TpIM gene was obtained by PCR amplification of pPIC9TpIM with primers TpIMclF and AOX1R to obtain pPGK1TpIM (Figure 4). In order to clone TpIM under the control of *AOX1* promoter and downstream the α-Factor secretion signal and the native *TpBGL*2 secretion signal, pPGK1αTpIM and pPGK1TpIM were digested with *BamH*I and *Bgl*II restriction enzymes to remove the constitutive promoter *PGK*1. This was replaced with the inducible promoter *AOX1* previously excised from pPIC9TpIM digested with the same restriction enzymes to obtain plasmid pAOX1αTpIM and pAOX1TpIM (Figure 5). Sequence analyses of recombinant plasmids revealed that the cloning strategy utilized was successful for pPGK1TpIM, pPGK1αTpIM and pAOX1TpIM but not for pAOX1αTpIM that therefore was not utilized for M12 transformation (Figure 6).

3.3.3. Heterologous expression of Kpkt coding gene in M12. M12 is a derivative of *K. phaffii* X-33 which is in turn a revertant of GS115. Competent cells of M12 were electroporated with plasmids pPGK1TpIM, pPGK1αTpIM and pAOX1TpIM restricted with *Sac*I and *Bgl*II. Moreover, M12 was transformed with plasmid pKLD restricted with *SacI* to obtain a negative control of expression. The transformants obtained (91 for pPGK1TpIM, 65 for pPGK1αTpIM and 65 pAOX1TpIM) were analysed by PCR with primers TpIMF/TpIMR and AlphaF/TpIMR. All clones that integrated the expression cassette were screened to select for those with multicopy integration. For that, after having repeatedly streaked putative transformants onto MD medium, each clone was cultured in MD liquid medium in 24 well plates at 30°C in slow agitation. After 24 h, 13 clones for pPGK1TpIM, 23 for pPGK1αTpIM and 8 for pAOX1TpIM (of which 5 Mut^+ and 3 Mut^S) that grew better on MD, possibly due to multiple copy integration of the defective *leu2-d* marker, were selected and screened for heterologous expression of Kpkt coding gene. For that, cells were precultured in MD and after 24 h at 30 °C under shaking conditions, transferred to fresh MD. Cultures where sampled after 24, 48 and 72 hours of incubation at 25 °C under constant shaking (180 rpm) and the cell-free supernatants were tested for killer activity by means of well plate assay. None of the tested clones showed activity on different target strains.

3.3.4. Effect of rKpkt on *K. phaffii*

The low frequency of recombinant strains capable of producing detectable amounts of rKpkt, when GS115 is the host, and the lack of recombinant killer clones when M12 is the host, suggested that the production rKpkt may be toxic for *K. phaffii* recombinant clones. Thus, the effect of cell-free supernatant of rc#6 was tested on itself, and on GS115 and M12 by means of well plate assay. Results obtained indicated that rc#6 and the two hosts GS115 and M12 are sensitive to rKpkt (Figure 7).

3.3.5. Discussion

The heterologous production of mellitin, diphtheria toxin, yeast killer toxin, bacteriocins or saporin in *K. phaffii* proved that this yeast may be considered suitable for toxins expression (Basanta et al., 2010; Chessa et al., 2017; Su et al., 2016; Yuan et al., 2015; Woo et al., 2002) although this is not always the case. In fact, the heterologous expression of microbial toxins may be really challenging for this yeast and lead to loss of both cell viability and integrated plasmid (Chen et al., 2017).

In a previous work the heterologous production of the yeast killer toxin Kpkt in *K. phaffii* GS115 was achived. Here, in order to expand the number of recombinant clones that efficiently secrete rKpkt, new plasmids useful for the heterologous production of Kpkt were developed and *K. phaffii* GS115 and M12 were utilized as the host strains. GS115 has been frequently utilized for heterologous protein expression both in academia and industry. Its genome has been sequenced (De Schutter et al., 2009) and its physiological response to the production of recombinant proteins has been characterized (Vanz et al., 2012; Hesketh et

al., 2013). M12 is a derivative of X-33 which, in turn, is a revertant of GS115. Therefore, the two host strains share a common genetic background. However, by using M12 it is possible to attempt the strategy of the multicopy integration of the plasmid vector pKLD after a single transformation step, as suggested by Betancur et al. (2017). Considering that there is a linear correlation between copy number of the expression cassette and the efficiency of the heterologous protein production (Betancur et al., 2017), this strain was thought to represent a useful mean to improve rKpkt production.

To evaluate the impact of different promoters and secretion signals on Kpkt production, TpIM was cloned under the control of regulated (*AOX*1) or constitutive (*PGK*1) promoters,

and in two different vectors (pPIC9 and pKLD). Plasmids based on pPIC9, that contain the heterologous gene under the control of *AOX*1 promoter, were designed for GS115. Plasmids based on PKLD that contain TpIM under the control of *PGK*1 and *AOX*1 promoters were designed for M12. The two promoters were chosen for the following reasons. *AOX*1 permits to shift to toxin production upon the production of high biomass concentrations, and to rapidly and efficiently address toxin to the secretion pathway (Zuppone et al., 2019). *PGK*1 works in glucose containing medium and allows an easier management of the process representing an interesting alternative to *AOX*1. Moreover, *PGK*1 promoter is moderately strong and its utilization may be advisable to overcome problems related to the overexpression of heterologous proteins.

In order to accomplish the secretion of rKpKT, the α-Factor secretion signal of *S. cerevisiae* was cloned upstream TpIM in plasmids pPIC9TpIM, pPIC9TpIMHisTag and pPGK1αTpIM. However, since the native signal sequence of heterologous proteins may be recognized by *K. phaffi* with variable secretion efficiency (Gaffar et al., 2015; Vadhana et al., 2013) no additional signal sequences were cloned in plasmids pPGK1TpIM and pAOX1TpIM.

Following transformation with the different plasmids, 364 clones were screened but only 16 of them proved capable of secreting detectable amounts of Kpkt. In particular, 15 recombinant clones were obtained with pPIC9TpIM and just one was obtained with plasmid pPIC9TpIMHisTag in GS115. None of the recombinant clones obtained in *K. phaffii* M12 was capable of producing rKpkt, in spite of the integration of expression cassettes harboring different combinations of promoters and secretion signals. The screening of numerous transformants is a common practice in *K. phaffii*. In fact, in this yeast the integration of expression cassettes occurs with high variability in the targeting efficiency, due the prevalence of the non-homologous end joining pathway that results in extensive clonal variation (Nett et al., 2005; Näätsaari et al., 2012; Chen et al., 2013). This dictates the evaluation of many recombinant clones to highlight those that most efficiently produce the recombinant protein (Aw and Polizzi, 2013; Zheng et al., 2013; Schwarzhans et al., 2016, Vogl et al., 2018). However, the low frequency of killer clones, in respect to the number of recombinant clones obtained, seemed compatible with the hypothesis that rKpkt may be toxic for *K. phaffii*. In accordance with this hypothesis, well plate assay showed that, contrary to that expected based on the spectrum of action of native Kpkt (Chessa et al., 2017), recombinant Kpkt proved toxic for the two *K. phaffii* strains selected for the heterologous expression, while showing lower toxicity on the recombinant killer clone. Thus, on the one side, the attempts to efficiently express rKpkt in M12 were unsuccessful, in spite of the development of new plasmids carrying promoters differing in strength and regulation. On the other side, the screening of a large number of GS115 transformants resulted in a limited number of recombinant clones capable of secreting detectable amounts of rKpkt. Based on these results, the selection of Kpkt resistant *K. phaffi* strains or of other hosts for the heterologous production of Kpkt could be pursued to increase the frequency of recombinant killer clones.

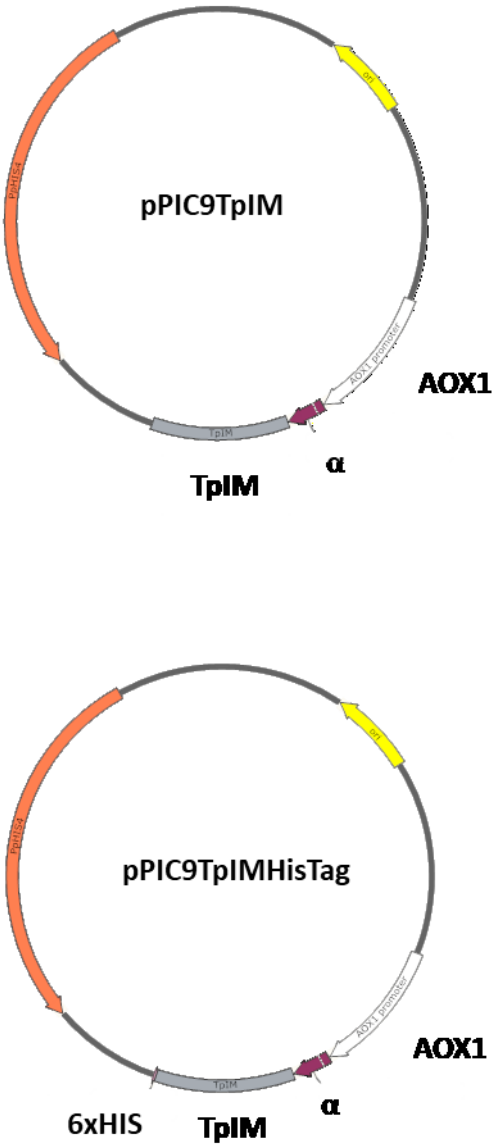

Figure 1. Plasmids utilized for the heterologous expression of TpIM (*TpBGL*2 sequence codon optimized for expression in *K. phaffii*). In the two plasmids TpIM is under the control of *AOX*1 promoter and downstream the α-Factor secretion signal of *S. cerevisiae*.

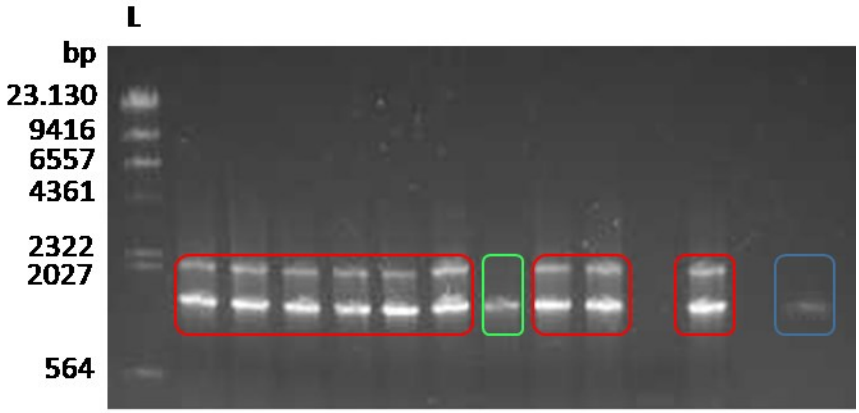

Figure 2. PCR screening of transformants obtained with pPIC9 based plasmids. Red and green boxes highlight the amplification products generated by Mut^{+} and Mut^{S} clones, respectively. Blue box indicates the amplification product generated pPIC9. L: Lambda DNA/HindIII Marker (Thermo Scientific).

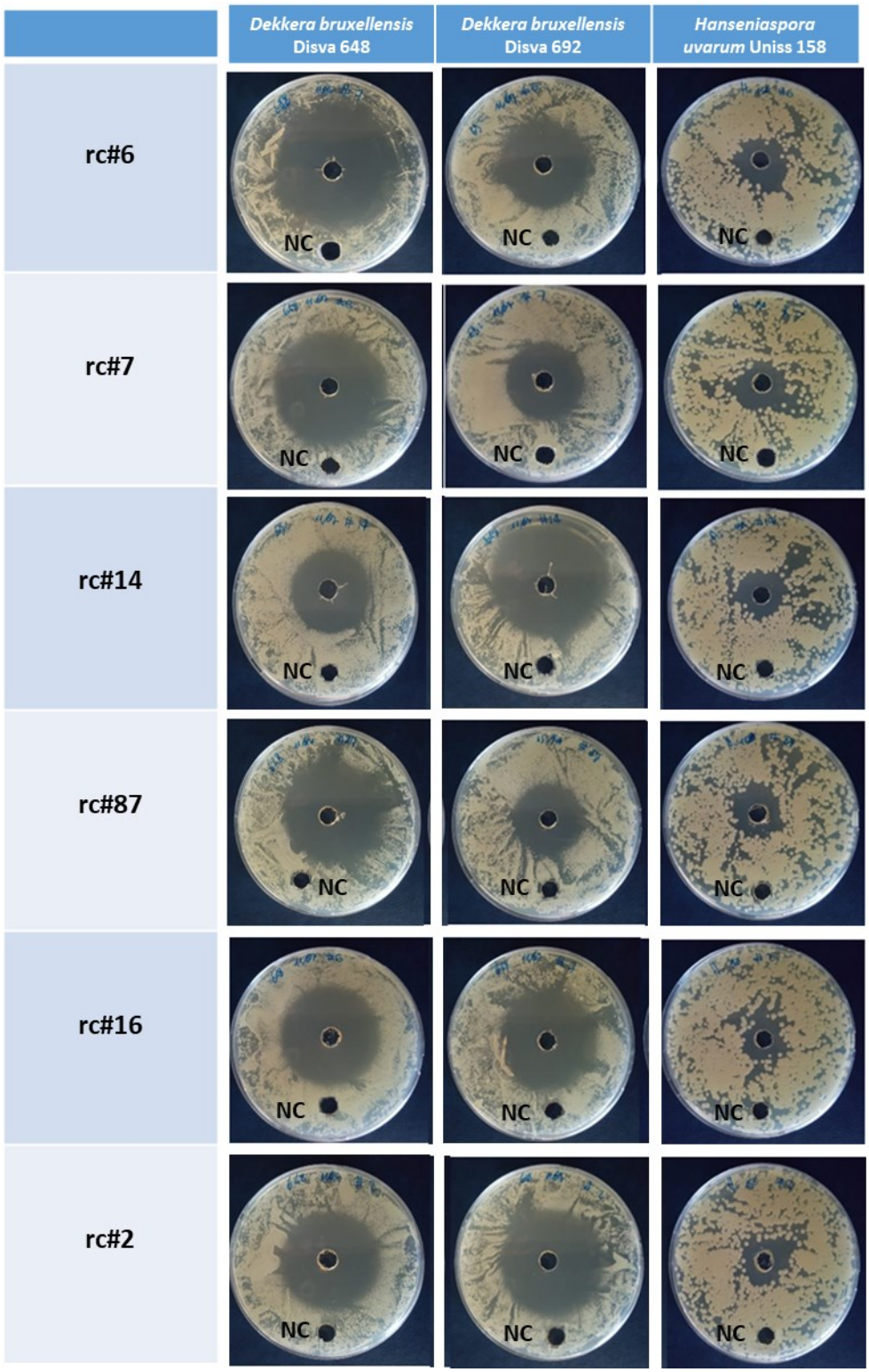

Figure 3. Killer activity of recombinant GS115 clones. The cell-free supernatant of recombinant clones was tested on different target strains. NC: cell-free supernatant of rc#24 utilized as negative control of killer activity. Results are representative of three biological replicates.

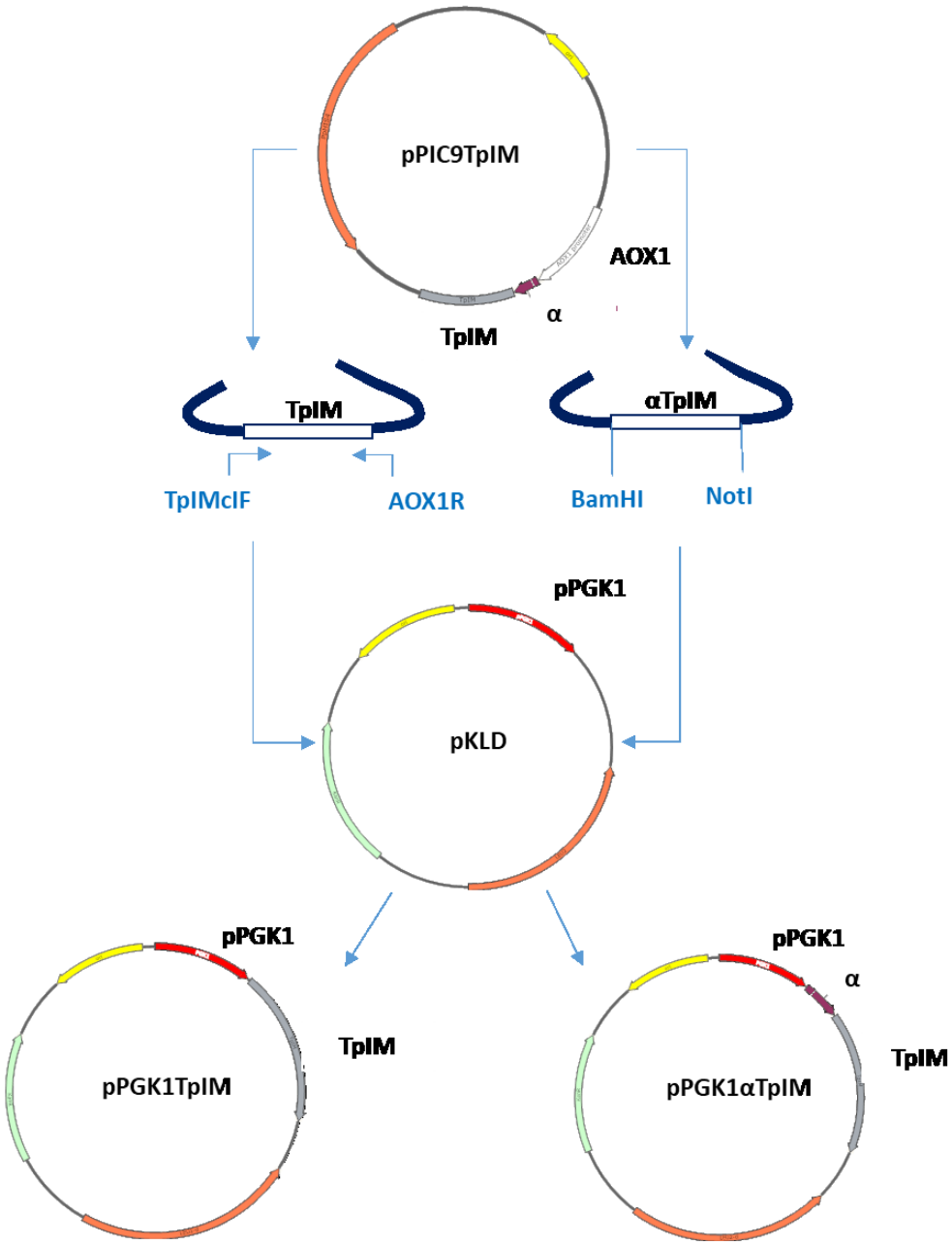

Figure 4. Construction of plasmids pPGK1TpIM and pPGK1αTpIM. For pPGK1TpIM, TpIM sequence was PCR amplified using primers TpIMαF and AOX1R and the amplicon was cloned in pKLD. For pPGK1αTpIM, the fragment containing the α-Factor secretion signal and TpIM was excised and cloned in pKLD. In the two plasmids TpIM is under the control of *PGK1* promoter and they differ for the absence and presence of the α-Factor secretion signal.

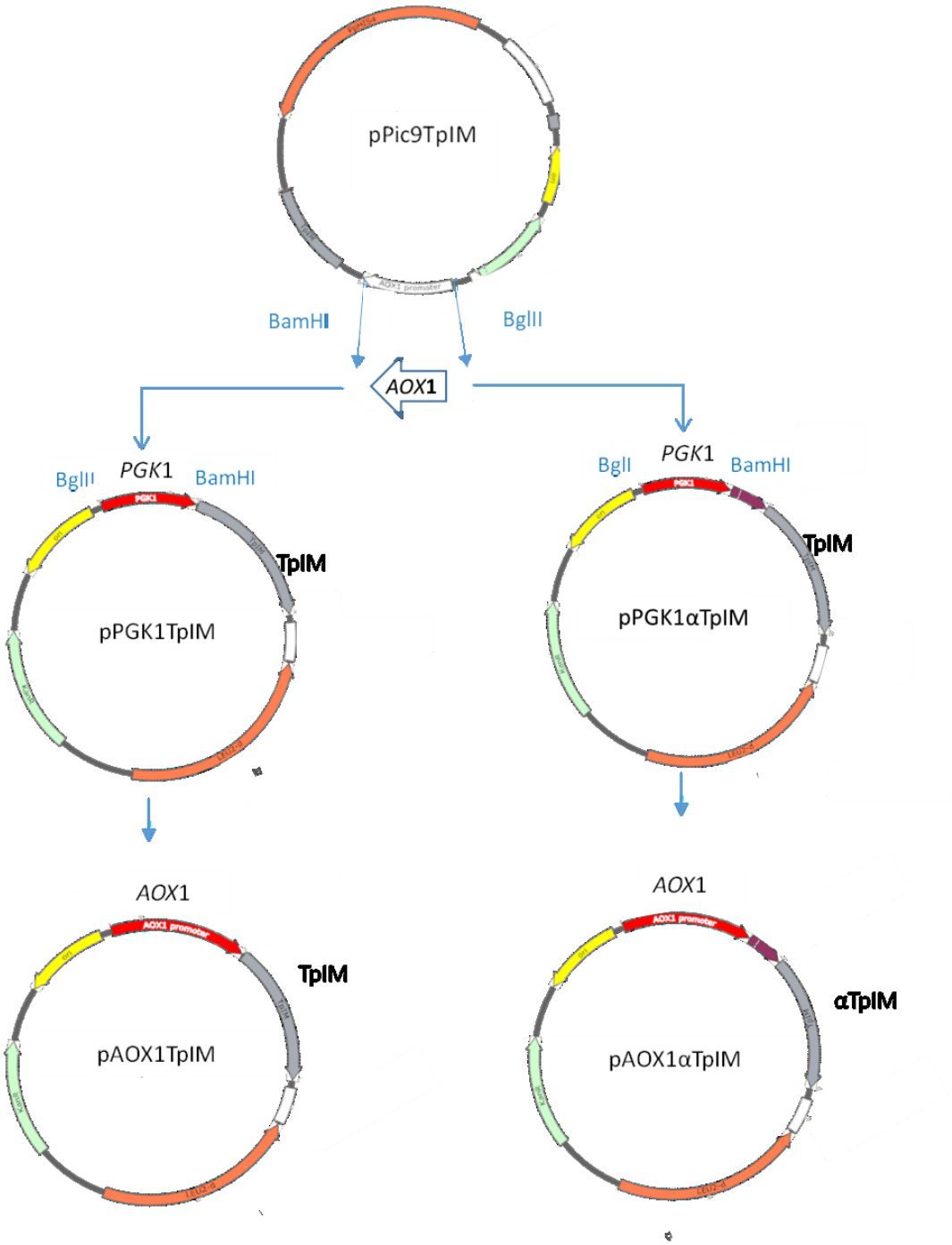

Figure 5. Construction of plasmids pAOX1TpIM and pAOX1αTpIM. For pAOX1TpIM, *PGK1* promoter was excised from pPGK1TpIM and replaced with *AOX1* promoter. For pAOX1αTpIM PGK1 promoter was excised from pPGK1αTpIM, and replaced with *AOX1* promoter. In the two plasmids TpIM is under the control of *AOX1* promoter and they differ for the absence and presence of the α-Factor secretion signal.

pPGK1TpIM

GTCCTTTCCTCGCGCCTGGCAATAAAGCTCCTTCGGAGGAGCTCCGTCTAGAGAATAATTGCTGCCTTTAT
GACTTCGGACTAGCGCCAACCGCGAACCACACCACCACACCATCACTGTCACCCGTCATAGTTCATCCCTC
TCTCCTTATAAAGCATATAATAGGTTCCACAATTGTTTGCCAAAAAATCTCTTAGCATAGCCCAATTGAGGA
TCCAAACGTTACGTAGAATTCATGAGATTTAGCACCTTCGTTTCCACAGCAGCGGCTGCCGCTTTTACAGT
TTCTCAAGTAAGCGCTCTGGGCGAGTTAGCTTTCAATCTTGGTGTGAAAAACAACGATGGAACATGTAA
GTCAGTGTCCCAGTTTGAGTCCGATTTGGAATTGCTAAAAGGTTACACTGACACGGTCAAAGTCTATGCA
GCTTCGGACTGCAATACACTCCAAAATCTGGGTCCTGCTGCCGAGGCAGAAGGTTTTAAGTTATTCTTGG
GAGTTTGGCCTACCGATGATAGTCATTTCCAAGCAGAAATCGCAGCTTTGAGAACTTACTTACCAGCCCT

pPGK1αTpIM

AACCCCCCCCCACCACACCATCACTGTCACCCGTCATAGTTCATCCCTCTCTCCTTATAAAGCATCTAATAGG
TTCCACAATTGTTTGCCAAAAAATCTCTTAGCATAGCCCAATTGATTACGAAAGGATCCAAACGATGAGA
TTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACA
GAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGAT
GTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATT
GCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTTACGTAGAATTCATGAGATT
TAGCACCTTCGTTTCCACAGCAGCGGCTGCCGCTTTTACAGTTTCTCAAGTAAGCGCTCTGGGCGAGTTA
GCTTTCAATCTTGGTGTGAAAAACAACGATGGAACATGTAAGTCAGTGTCCCAGTTTGAGTCCGATTTGG
AATTGCTAAAAGGTTACACTGACACGGTCAAAGTCTATGCAGCTTCGGACTGCAATACACTCCAAAATCT
GGGTCCTGCTGCCGAGGCAGAAGGTTTTAAGTTATTCTTGGGAGTT

pAOXTpIM

GACGAATGCTCAAAAATAATCTCATTAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACC
TGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTC
CAAGATTCTGGTGGGAATACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACT
TGACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTAC
TTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAAG
ATCAAAAAACAACTAATTATTCGAAGGATCCAAACGATGAGATTTAGCACCTTCGTTTCCACAGCAGCGG
CTGCCGCTTTTACAGTTTCTCAAGTAAGCGCTCTGGGCGAGTTAGCTTTCAATCTTGGTGTGAAAAACAA
CGATGGAACATGTAAGTCAGTGTCCCAGTTTGAGTCCGATTTGGAATTGCTAAAAGGTTACACTGACAC
GGTCAAAGTCTATGCAGCTTCGGACTGCAATACACTCCAAAATCTGGGTCCTGCTGCCGAGGCAGAAGG
TTTTAAGTTATTCTTGGGAG

Figure 6. Sequence of recombinant plasmids. Promoter in blue, α-Factor secretion signal in red, TpIM gene in green, multiple cloning site in black.

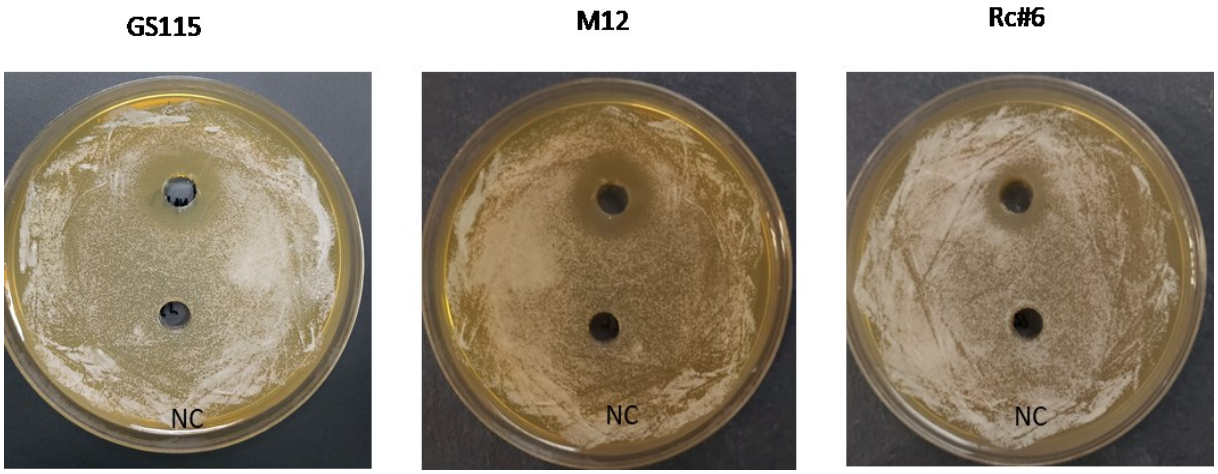

Figure 7. Killer activity of rKpkt on *K. phaffii* strains. Cell-free supernatant of rc#6 was tested onto GS115, M12 and rc#6 strains. NC: cell-free supernatant of rc#24 utilized as negative control.

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Chapter 4

Bioreactor production and characterization of recombinant Kpkt

Abstract

Kpkt is a 33 kDa glycoprotein, naturally produced by the yeast *Tetrapisispora phaffii,* that shows β-1,3 glucanase and killer activities on wine spoilage yeasts under winemaking conditions. Based on these evidences, a possible utilization of Kpkt in partial substitution of SO_2 during the prefermentative stages of alcoholic fermentation was envisaged. However, *T. phaffii* produces limited amounts of Kpkt and, being not a wine-related yeast, it cannot be utilized as a starter for grape must fermentation, thus posing a limit to Kpkt biotechnological exploitation. Previous works showed that, by cloning Kpkt encoding gene in *Komagataella phaffii,* it is possible to achieve the heterologous production of this toxin. Here, in order to increase recombinant Kpkt production (rKpkt), the cultivation of recombinant clone#17 (rc#17) and the production of rKpkt were scaled up from baffled flask to bioreactor and immobilized metal affinity chromatography was utilized for rKpkt purification. Moreover, after having confirmed that rKpkt maintains β-glucanase activity and shows a wider spectrum of action in respect to its native counterpart, its cytotoxic effect on human cells was evaluated. Results obtained showed that rKpkt is not toxic to HaCaT cells for concentrations up to 18 AU thus corroborating the great potential of this toxin as a natural antimicrobial for use in the food and beverages industries.

Key words: rKpkt, affinity chromatography, fed batch, bioreactor, methanol induction, HaCaT human cell line

4.1. Introduction

In 2001 the biotechnological potential of the killer toxin of *Kluyveromyces phaffii,* strain DBVPG 6076, was described by Ciani and Fatichenti (2001). According to these authors, the yeast *K. phaffii,* successively renamed *Tetrapisispora phaffii* (Ueda-Nishimura and Mikata 1999), secretes a killer toxin, named Kpkt, which is active against wine spoilage yeasts (Ciani and Fatichenti, 2001). Further works showed that Kpkt recognizes β-1,3 and β-1,6 branched glucans on the cell wall of the sensitive target (Comitini et al., 2004) and that the glucidic fraction of the toxin is clearly involved in the attachment to the cell wall of the sensitive target (Comitini et al., 2009). Moreover, it was shown that this toxin induces ultrastructural modifications to the cell wall of the sensitive targets and that its killing action is mediated by β-glucanase activity (Comitini et al., 2004, 2009). Accordingly, BLASTN analysis of *TpBGL2* gene, that codes for Kpkt, revealed that this sequence shows more than 75% identity with β-1,3- glucanase encoding genes of other yeasts, and *TpBGL2* gene deletion results in loss of killer phenotype and β-glucanase activity (Oro et al., 2014).

Interestingly, Kpkt retains killer activity under winemaking conditions for up to two weeks (Comitini and Ciani 2010). Based on these data, Comitini and Ciani (2010) suggested a possible future exploitation of Kpkt in the wine industry in place of SO_2 in the prefermentative stages. This is in line with the indications of the World Health Organization and with the customers' tendency towards the consumption of wines low or no sulfite addition. Nevertheless, it should be taken into account that the native producer of Kpkt is a soil isolate and not a wine-related yeast. So it is not suitable for a direct utilization in grape must fermentation.

For these reasons Chessa et al. (2017) worked at the heterologous production of Kpkt in *Komagataella phaffii* (*Pichia pastoris*) GS115, with the aim of developing the molecular tools needed for a large scale production of this toxin.

These authors, besides proving the feasibility of Kpkt expression in *K. phaffii*, showed that rKpkt has a spectrum of action that is wider in respect to native Kpkt. In particular rKpkt is active on yeasts ascribed to *Dekkera/Brettanomyces* sp. (Chessa et al., 2017). These, by developing in white and red wines may produce unpleasant odors and tastes (Ibeas et al., 1996) and constitute a serious problem for the wine industry. The lower specificity of action of rKpkt, as compared to native Kpkt, suggests a plethora of possible applications of this toxin that, besides wine making, may include the sweet beverage and more in general the food industry.

In this context, the aims of this chapter were the bioreactor production of rKpkt, the purification and biochemical characterization of the protein, and the evaluation of its effect on human cell lines. For that, recombinant clone#17 (rc#17), selected in chapter 3 based on rKpkt production and the presence of the HisTag, was utilized.

4.2. Materials and methods

4.2.1. Microorganisms and growth media. Microorganisms used were: *Tetrapisispora phaffii* DBVPG 6076 native producer of Kpkt*;* rc#17 obtained by transforming *K. phaffii*

GS115 with pPic9TpImHisTag and recombinant clone 24 (rc#24) that integrated empty pPIC9 plasmid vectors and serves as the negative control of killer activity.

Hanseniaspora uvarum UNISS 156, *Saccharomyces cerevisiae* DBVPG 6500 and *Dekkera bruxellensis* DiSVA 692, DiSVA 638, DiSVA 648 were utilized as sensitive strains.

Media used were the following. YEPD: 2% glucose, 1% yeast extract and 2% peptone; BMGY: 1% glycerol (w/v), 1% yeast extract, 2% peptone, 1.34% YNB w/o aminoacids and 0.00004% biotin; BMGluY: as BMGY with 1% glucose instead of glycerol; BMMY: 0.5 or 1% methanol (v/v), 1% yeast extract, 2% peptone, 1.34% YNB w/o aminoacids and 0.00004% biotin. All media were buffered at pH 4.5 with citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4).

Yeasts were maintained on YEPD at 4 °C for short term storage and in YEPDgly (YEPD with addition of 40% glycerol) and LBgly (LB with addition of 10% glycerol) at −80 °C for long-term storage, respectively.

4.2.2. Production of recombinant Kpkt in baffled flask. Rc#17 was inoculated in 25 mL of BMGY within 250 mL baffled flasks. After 24 h at 30 °C under constant shacking (180 rpm), cells were transferred in 25 mL BMMY (0.5% methanol) within 250 mL baffled flasks to a final OD_{600} of 1.0. Flasks were closed with a sterile cloth and incubated under constant shacking (180 rpm) to maximize oxygen transfer. Growth in BMMY was carried out for up to 144 h. Due to the extensive evaporation of culture medium every 24 h new medium lacking methanol was added to restore the original volume and 250 µL of methanol were added every day to maintain its concentration at 1%, as requested for the induction of p*AOX*1 promoter. Induction was carried out at the temperatures indicated: 30 °C for day 1, 28 °C for day 2, 25 °C for day 3 and 23 °C till the end of the induction. Cell cultures were centrifuged for 10 min at 4600 rpm, the supernatant was filter sterilized, transferred in falcon tubes and stored at -80° C.

4.2.3. Production of native and recombinant Kpkt in bioreactor. Bioreactor productions were carried out in Biostat Bplus (Sartorius, Germany) fermentor (2 L). For native Kpkt production in bioreactor, *T. phaffii* DBVPG 6076 was grown in a 2 L bench-top fermenter within 1.8 L YEPD working volume. Dissolved oxygen and temperature were set at 20% and 25 °C, respectively, throughout the fermentation process. pH 4.5 was maintained by automatically adding 20% phosphoric acid and 20% potassium hydroxide. After 24 h of cultivation, the cell-free supernatant was collected by centrifugation (5000 g for 10 min) and subsequent micro-filtration (0.22 µm, Millipore).

For recombinant Kpkt, rc#17 was grown in 150 mL BMGY or BMGluY within 250 mL baffled flask at 30 °C to OD_{600} of 10-12 (approximately 24 h) and inoculated in a total volume of 900 ml BMGY or BMGluY medium containing 0.5 mL anti foam (FMT30, Breox). pH 4.5 was maintained by automatically adding 20% phosphoric acid and 20% potassium hydroxide. Dissolved oxygen was set at 25% and regulated by a two stages sequential cascade control (stirrer: maximum 1100 rpm; dissolved oxygen 25%). Temperature was set at 25°C during biomass production in BMGY or BMGluY and 20°C during induction in BMMY. Fermentation was articulated into 3 steps: (1) batch phase (BP), in which cells were grown in glycerol or

glucose containing medium, in a batch mode; (2) fed-batch phase (FBP), during which a limited glycerol or glucose feed was initiated following exhaustion of the carbon source, and cell mass is increased to an OD_{600} of 300 prior to induction; (3) methanol fed-batch phase (MFBP), in which methanol is fed at a limited feed rate or maintained at some level to induce the p*AOX*1 for protein expression (Zhang et al., 2000). According to this strategy, after complete consumption of glycerol or glucose, indicated by an increase in dissolved oxygen up to 50%, the same carbon source (10%) was added 9 mL/h to increase biomass. At OD_{600} of 350–400, feeding was stopped, fermentation temperature was decreased to 20°C and methanol feeding started by adding 3.6 mL/L/h methanol 100%, according to "Pichia Fermentation Process Guidelines". After 1 h, methanol feeding rate was doubled to ~7.3 ml/L/h and after 2 more h it reached ~10.9 mL/L/h. MFBP lasted approximately 72 h after which the fermentation process was stopped and the cell-free supernatant was collected by centrifugation (5000 g for 10 min) and subsequent micro-filtration (0.45 µm filter Minisart, Sartorius). The production of nKpkt and rKpkt in cell-free supernatants of *T. phaffii* DBVPG 6076 and rc#17 was evaluated by means of well plate assay as already reported (Chessa et al., 2017).

4.2.4. Cell-free supernatant ultrafiltration. Cell-free supernatants (approximately 2 L) of rc#17, rc#24, and *T. phaffii* DBVPG6076 were concentrated by means of a TFF/Cross Flow System (Repligen) with a 10KDa cut-off membrane and 60-fold concentrated, when indicated.

4.2.5. Well plate assay. Well plate assays were carried out on YEPD plates buffered at pH 4.5. Briefly, cells of the target sensitive strain (OD_{600} 0.1-0.2) were resuspended in sterile distilled water and 100 µL aliquots were spread on YEPD plates. 100 µL of cell-free supernatant of the recombinant clone was loaded into wells cut in the plate. The cell-supernatant of rc#24 was used as negative control o the killer activity negative control.

4.2.6. Determination of Arbitrary Units. Killer activity was expressed in terms of arbitrary units (AU) as described by Ciani and Fatichenti, (2001). The calculation of the arbitrary Units was carried out as follows. Aliquots of 10, 15, 25, 50, 75 µL of cell-free supernatantof taken to 100 µL with citrate phosphate buffer (pH 4.5) and 100 µL of cell-free supernatant, were subjected to well plate assay using *S. cerevisiae* DBVPG 6500 as the sensitive strain. After 3 days incubation at 25°C the diameter of the inhibition halo generated around each well was measured and the values obtained were used to trace a calibration curve in respect to a Cartesian axes system, where the Log of the concentration of toxins is on the abscissa and the diameter (mm) of the inhibition halo is on the ordinate. Given the linear relationship between the diameter of the inhibition halo and the logarithm of killer toxin concentration, 1 AU corresponds to the amount of toxin in 100 µL that produces an inhibition halo of 20 mm (as the diameter of the well), using *S. cerevisiae* DBVPG 6500 as the sensitive strains.

4.2.7. Biochemical methods. A 10 mL Econo-Pac Disposable Chromatography Column (BIORAD) was used. The purification resin (TALON Metal Affinity Resin, Takara) was charged with cobalt ions and the purification protocol was based on the affinity between the His-tag and the cobalt ions. Briefly, 20 mL cell-free supernatant was loaded onto the purification column. The target protein was eluted with Imidazole elution buffer (Bio-Rad). Thirty µL of each of the five eluted fractions (1 mL each) were loaded on SDS-PAGE (12% acrilamide). SDS-PAGE was performed according to Laemmli (1970). Gel staining was done with Silver Staining (Pierce™ Color Silver Stain kit, ThermoFisher Scientific) or Coomassie Brilliant blue (Kit blue R-250, Sigma) and the molecular mass was determined by comparison with known marker proteins (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, ThermoFishermo Scientific). In parallel 100 µL aliquopts of each fraction was tested on plate for killer activities.

Western blot was performed with Mini Trans-Blot® Cell apparatus (Biorad). Thirty-µL samples were loaded on a 10% acrylamide gel. Spectra Multi color Broad Range Protein Ladder (10-250 kDa) (ThermoFisher Scientific) was used as molecular weight standard. After electrophoresis (1.5 h at 150 mA), proteins were transferred to PVDF membrane and probed with the primary antibody anti-His-tag (monoclonal anti-HIS-tag antibody, produced in mouse, Sigma H1029) at a 1:5000 dilution and with the secondary antibody (anti-mouse IgG (Fc specific)-FITC antibody produced in goat, Sigma F5897) at a 1:10000 dilution. Immunodetection was carried out by the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher). Images were captured with LAS-3000 Imaging System apparatus (Fuji). Dot-Blot was performed with Bio-Dot Microfiltration Apparatus (Biorad). Samples, denaturated at 60, 90 and 95 °C were loaded onto the PVDF membrane (Biorad). Monoclonal mouse anti-polyHistidine−Peroxidase conjugated antibody (Sigma A7058) at a 1:10000 dilution was utilized for the detection of the target protein. The procedure was performed following the Biorad protocol (Bio-Dot® SF Microfiltration Apparatus Instruction Manual). Immunodetection was he performed by the SuperSignal™ West Pico PLUS Chemiluminescent Substrate. Images were captured with ChemiDoc XRS+ System (Biorad).

The β-glucanase activity of the rKpkt was evaluated accordinf to Notario (1982). Briefly, 800 µL 0.25% laminarin in 50 mM sodium acetate (pH 5.0, with glacial acetic acid) was added to 200 µL cell-free supernatant of rc#17 or *T. phaffii* DBVPG 6076. After 30 min incubation 37 °C the mixutures were boiled for 5 min to stop the reaction. Positive control of activity was obtained by using laminarinase (Sigma-Aldrich) (0.01 U in 1-mL reaction mixture). Negative controls of enzymatic activity for rKpkt and nKpkt were obtained by using 200 µL of cell-free supernatant of rc#24 or 200 µL of heat-treated cell-free supernatant of *T. phaffii* DBVPG 6076, respectively. GAGO-20 kit (Sigma-Aldrich) was utilized to quantify the glucose released. Results presented are means ± standard deviation of at least three technical replicates of three independent experiments

4.2.8. Human cell line toxicity test. Keratinocytes of HaCaT immortalized line were grown as a monolayer at 37 °C, under 5% CO_2 on DMEM (Dulbecco's Modified Eagle Medium, Sigma) liquid medium (pH 7.4) supplemented with 2mM L-glutamine, 10% foetal bovine serum (FBS) and penicillin-streptomycin (100U/mL-0.1mg/mL) was used as a culture medium. After 3 days of incubation, $3x10^4$ cells/ml were aliquoted in 96-well plates and

exposed to increasing concentrations of 60-fold concentrated rKpkt and Kpkt (ranging from 0 to 36 AU) Sixty-fold concentrated cell-free supernatant of rc#24 and heat inactivated cell-free supernatant of Kpkt were utilized as negative controls for rKpkt and Kpkt, respectively. After 24 hours of incubation with the toxin cells were observed by light microscopy in order to obtain preliminary information on cell viability. Cell viability as indicator of cytotoxicity was determined by MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium (MTT) bromide] test according to the manufacturer's instructions (MTT Cell Growth Assay Kit, Sigma). After 3h hours incubation in MTT, 10% SDS was added to each well and OD_{562} was recorded to evaluate the percentage of viable cells in respect to the negative control. Results presented are mean ± standard deviation of at least three technical replicates of three independent experiments. Data were subjected to two way analysis of variance (ANOVA). The critical value for signicance level (p) was set at 0.05 and the Tukey test was used for post-hoc comparison. Statistical analyses were performed using RStudio for windows, version 10 (RStudio, PBC, USA).

4.3. Results

4.3.1. Production of rKpkt in baffled flasks. Rc#17 was utilized for the heterologous production of rKpkt. At first, killer toxin production was evaluated following methanol induction in 250 mL baffled flask. To do that, cells were pre-cultured in BMGY at 30 °C under constant shaking and after 24 h they were transferred to BMMY 0.5% to an OD_{600} of 1.0 and grown for up to 144 h on the same medium. To induce rKpkt expression under the control of *AOX*1 promoter, cells were subject to fed-batch cultivation with methanol addition to the final concentration of 1% every 24 h. Growth temperature was gradually decreased and kept at 23 °C from day 4 to the end of the fermentation. rKpkt heterologous expression was evaluated on cell-free supernatant after 72, 96, 120 and 144 h of methanol induction. *T. phaffii* DBVPG 6076 cell-free supernatant was used as the positive control of killer activity (PC), while the supernatant of rc#24 was utilized as negative control (NC). Well plate assays showed that rc#17 starts secreting detectable amount of rKpKt after 96 h of methanol induction and continues secretion for up to 144 h (Figure 1).

4.3.2. Recombinant Kpkt production and activity at different pH. In order to gather further information on the best culture conditions for rKpkt production, the pH range in which the protein is produced and active was evaluated. To do that, rc#17 was grown in baffled flasks, as above indicated, in BMMY buffered at different pH (3.9, 4.5, 5.0). The cell-free supernatants were tested for killer activity by means of the well plate assay carried out on YEPD buffered at the three different pH. Results obtained indicate that rKpkt is produced and active within the range of pH tested. Since there is a correlation between the amount of toxin in the cell-free supernatant and the diameter of the inhibition halo in well plate assay, rKpkt was quantified in terms of Arbitrary Units (AU). For that increasing volumes of cell-free supernatant containing rKpkt were subjected to well plate assay and the inhibition halo around each well was measured (Figure 2). It was established that 1 AU corresponds to the amount of toxin that generates an inhibition halo of 20 mm. Thus, based on the inhibition haloes it resulted that 100 µL aliquots of cell-free supernatants

produced by rc#17 grown at pH 3.9, 4.5 and 5.0 correspond to 9.1, 11.0 and 5.3 AU/mL, respectively.

4.3.3. Development of a bioreactor fermentation protocol for rKpkt production. In order to scale up rKpkt production and individuate the most appropriate fermentation protocol, rc#17 was grown in bioreactor (Biostat B, Sartorius) and the effects of different carbon sources for biomass production (glycerol and glucose) and methanol feeding protocols (feeding in cascade with oxygen and continuous feeding) on the production of the recombinant protein were evaluated in terms of AU.

The utilization of glucose, in place of glycerol, proved useful to increase biomass production rate prior to methanol induction. In particular, rc#17 took on average 48 h on glucose and about 96 h on glycerol to reach the requested cell density (OD_{600} 350-400). Thus, although no differences in the amount of toxin produced at the end of the fermentation process were observed, glucose feeding was chosen for the first two stages (GluBP and GluFBP) for rc#17 (data not shown).

For what concerns methanol, when feeding was in cascade with dissolved oxygen (desolved oxygen (DO) strategy), no rKpKt could be detected in cell-free supernatant (data not shown). On the contrary, continuous feeding of methanol led to the production of rKpkt. To analyse the kinetics of rKpkt production, cell culture was sampled at 24, 48 and 70 h of MFBP and cell-free supernatants were tested for killer activity on YEPD agar (pH 4.5) (Figure 3). Well plate assay revealed that the secretion of detectable amounts of killer toxin started after 24 h of induction and that rKpkt production shows a time dependent increase. In particular, the cell-free supernatant of rc#17 contained and 1.8±0.04, 7.3±0.02 and 14.1±0.15 AU/mL of recombinant killer toxin after 24, 48 and 70 h respectively. Thus, this was selected as the methanol feeding strategy during MFBP and the following fermentation protocol was developed for the production of rKpkt: after almost 48 h of glucose feeding, at first in the batch mode (GluBP), subsequently in the fed batch mode (GluFBP), methanol induction was carried out for about 70 h (MFBP). In parallel, native Kpkt (nKpkt) was produced in bioreactor. For that, *T. phaffii* DBVPG 6076 was inoculated in YEPD pH 4.5 and after 24 h cultivation at 25 °C under 20% desolved oxygen, nKpkt production was confirmed by well plate assay (data not shown). Cell-free supernatant of *T. phaffii* DBVPG 6076 was utilized to compare nKpkt and rKpkt in terms of biochemical properties and citotoxicity.

4.3.4. Recombinant rKpkt purification. rKpkt coding sequence harbors a His-Tag (consisting of six histidine residues) in C-terminus. Thus, immobilized metal affinity chromatography was utilized for the purification of the protein. For that 40 ml of cell-free supernatant of a 70 h rc#17 bioreactor culture were loaded onto a column containing a Metal Affinity Resin charged with cobalt ions and proteins bound to the resin were eluted in imidazole elution buffer (five fractions of 1 mL each). All fractions were subjected to protein quantification by Bradford assay and killer toxin evaluation by means of well plate test. The lack of killer activity in the fractions eluted by the column was in accordance with the lack of detectable amounts of protein. Same results were obtained in three independent attempts. Thus,

immunoblotting techniques (western Blot, Dot-Blot) were applied to highlight rKpkt in the cell-free supernatant of rc#17. For that the cell-free supernatant was subject to ultrafiltration (cut-off 10 kDa) and concentrated 60-fold. Two anti-His-Tag antibodies (rabbit monoclonal anti-His-Tag antibody and anti-rabbit secondary antibody or monoclonal anti-polyHistidine−Peroxidase conjugated antibody, Sigma) and different techniques for the preparation of the samples were utilized (heat treatment at 60, 90 or 95 °C for 5 min; no heat treatment). However, the two antibodies failed to highlight rKpkt (Figure 4).

4.3.5. Enzymatic activity and spectrum of action of rKpkt. According to previous results (Chessa et al., 2017), rKpkt spectrum of action is wider as compared to nKpkt. Thus, the killer activity of the cell-free supernatant produced by rc#17 was evaluated on *H. uvarum* UNISS 156, *D. bruxellensis* DiSVA 692, *D. bruxellensis* DiSVA 648, *D. bruxellensis* DiSVA 638. The results obtained confirmed that rKpkt is active on a wider spectrum of spoilage yeasts in respect to nKpkt (Figure 5). Native and recombinant Kpkt killer activity is mediated by β-glucanase activity (Chessa et al., 2017). Thus, comparable amounts of nKpkt and rKpkt in terms of AU were evaluated for β-glucanase activity on laminarin, a polysaccharide of glucose with β 1-3 and β 1-6 glycosidic bonds. A commercial β-glucanase and the cell-free supernatant of rc#24 were utilized as the positive and negative controls of activity, respectively. Results obtained indicate that nKpkt and rKpkt hydrolyze laminarin, with specific activities of 4.04±0.08 and 4.94±0.15 µmol of glucose/mg protein/min, respectively. It should be noted that this enzymatic activity is expressed per mg total protein. Enzymatic activity of pure commercial β-glucanase was 2.8±0.11 µmol of glucose/mg protein/min. Thus, enzymatic activities of nKpkt and rKpkt were higher than that of pure β-glucanase ($p<0.001$). Moreover, they were definitely higher than that recorded by Chessa et al. (2017) during flask cultivation, thus confirming a more efficient toxin production during bioreactor cultivation.

4.3.6. Citotoxicity of rKpkt and Kpkt on HaCaT cell line. In order evaluate the effect of rKpkt and nKpkt on human cells, HaCaT cell line were exposed to increasing concentrations of cell-free supernatants of rc#17 (rKpkt) and *T. phaffii* DBVPG 6076 (nKpkt), in terms of AU/mL, and cell viability evaluation was carried out by means of MTT test. This is a standard colorimetric assay that measures the activity of enzymes able to reduce MTT to formazan with a blue/purple colour thus gathering information on cell viability. In fact, MTT reduction occurs in mitochondria of living cells due to succinate dehydrogenase activity on MTT tetrazolium ring, with the formation of formazan (a blue salt). The blue-purple colour indicates that cells are viable, while the yellow colour indicates that cells are dead. Based on the results reported in Figure 6 native and recombinant Kpkt appear to have low toxicity on HaCaT cells, even at the highest concentrations utilized. This was partially confirmed by OD_{562} reading of 96-well plates. In fact, as reported in Figure 7, HaCaT cells viability is above 60% for concentrations of nKpkt and rKpkt up to 18 AU/mL, while higher concentrations of both native and recombinant toxins determine significant decreases of cell viability (Figure 7).

4.4. Discussion

Research activities described in this chapter focused on rc#17, the only recombinant clone showing killer activity among the 72 obtained by transforming GS115 with pPIC9TpIMHisTag. This clone proved capable of producing rKpkt at different pH, ranging from 3.9 to 5.0 although pH 4.5 was the most suitable for rKpkt production and detection. Thus, this pH was maintained also for bioreactor production of rKpkt. Bioreactor cultivation was divided in three phases, in accordance with that reported by other authors for the heterologous expression of genes under the control of *AOX*1 promoter (Zhang et al., 2000; Liu et al., 2019) and on "Pichia Fermentation Process Guidelines". The three phases are glycerol/glucose batch phase, glycerol/glucose fed-batch phase, and methanol induction fed batch phase (Sun et al., 2018; Viña-Gonzalez et al., 2018). The main objective of the first two phases is to reach a high biomass concentration. During these two phases (glucose-, or glycerol-batch phase and glucose-, or glycerol-fed batch phase) *AOX*1 promoter is strongly repressed, thus the recombinant strain is not challenged by the production of the heterologous protein. Full induction of the heterologous genes occurs under methanol feeding and complete depletion of the repressing carbon sources (Inan and Meagher 2001; Juturu and Wu 2017).

Here, after having observed that glucose is the most suitable carbon source for biomass production with rc#17, in the first two stages, rc#17 was cultivated on glucose containing medium to allow biomass production. Regarding methanol feeding, it should be taken into account that high concentrations of methanol are toxic to the yeast cells. Thus, methanol is continuously added to the fermenting culture using different feeding strategies. Here two strategies were utilized: the desolved oxygen (DO) strategy and methanol continuous feeding. According to the DO strategy, methanol feeding rate is controlled in order to maintain the dissolved oxygen at a constant level. Regarding rc#17 the DO strategy resulted in no rKpkt production. On the contrary, methanol continuous feeding led to a time dependent accumulation of rKpkt in the supernatant. Once bioreactor production of rKpkt was sort out, protein purification was attempted. The strategy utilized for protein purification was based on the presence of His-Tag in the expression cassette. In general, the His-Tag does not interfere with the structure or activity of the target protein and works under native and denaturing conditions. Thus, immobilized metal affinity chromatography was utilized to recover rKpkt from rc#17 cell-free supernatant. However, contrary to that expected, the presence of the His-Tag was not useful for rKpkt purification by metal affinity chromatography. Similarly, western blotting failed to highlight the toxin in the supernatant of rc#17. While the reason for that are not clear, these results seem compatible with the His-Tag not being available for cobalt ions or antibody binding. Inaccessibility of the His-Tag may be a consequence of the tag being hidden inside the protein's three-dimensional conformation. However, also protein expression at low levels could be responsible for failures in rKpkt purification and detection by western blot. For that, the cell-free supernatant of rc#17 was 60-fold concentrated by ultrafiltration and different heat treatments for protein denaturation were attempted prior to dot blotting. The lack of protein detection also in this case suggested that either the tagged protein requires more than 60-fold concentration to be purified and detected, or heat treatments are unable to disengage the His-Tag, or His-Tag is degraded. In any case, the ultrafiltered samples were tested on well plate assay, the amount of rKpkt contained in the ultrafiltered preparation was quantified in terms of AU and the toxin was further characterized. In accordance with

Chessa et al. (2017), rKpkt maintained β-glucanase and killer activities and confirmed a wider spectrum of action in respect to the native toxin produced by *T. phaffii* DBVPG 6076. Moreover and interestingly, HaCaT human cells proved viable after exposure with rKpkt and nKpkt, thus indicating that this killer toxin has not a cytocidal effect for concentrations up to 18 AU/mL. Indeed, as reported in chapter five, these concentrations are definitely higher than those requested to kill or inhibit wine spoilage yeasts (ranging from 0.25 to 2 AU/mL). Moreover, it should be considered that also the negative controls [60-fold concentrated cell-free supernatant of rc#24 (UFrc#24) and heat inactivated 60-fold concentrated supernatant of *T. phaffii* DBVPG 6076] determine decreases of viability that are comparable to that of the respective toxins. Thus, it may be hypothesized that these effects are due to compounds, other than the toxin, occurring in the cell-free supernatants.

Considering that the scarcity of studies regarding the effects of killer toxins on human consumers represents a constraint to their utilization in the food and beverages industries, these result, although preliminary, are encouraging and led to further develop a process for the production of a ready to use preparation of rKpkt by using rc#17.

However, unfortunately, after long term storage in -80°C rc#17, although retaining the integrated plasmid, irreversibly lost the ability to express the heterologous protein. This is not an unusual event in *K. phaffii* recombinant clones and may be a consequence of rKpkt toxicity on GS115. Thus, rc#17 was abandoned and further work was done on rc#6 as described in chapter 5.

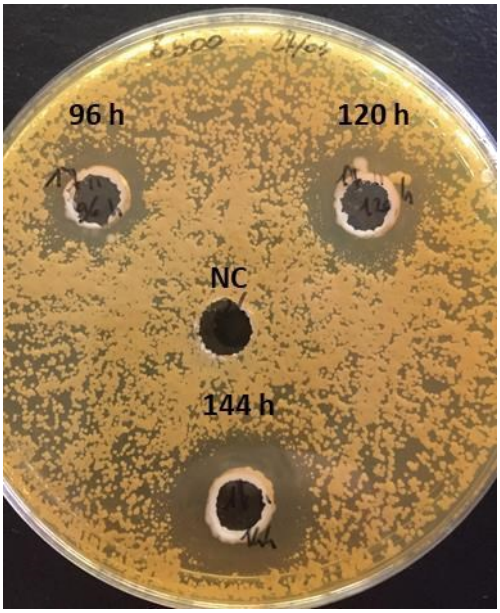
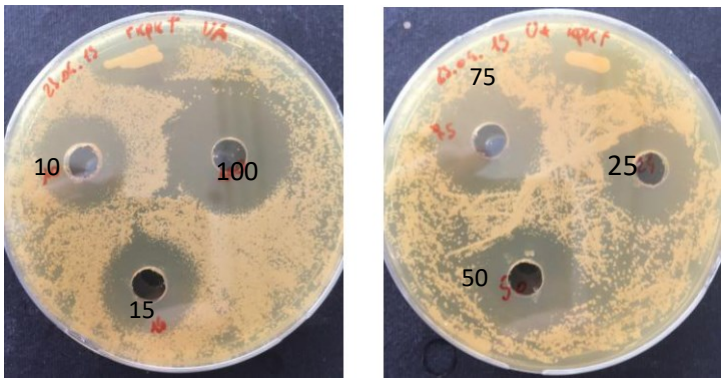


Figure 1. Well plate assay for killer activity evaluation. Aliquots of 100 µl of cell-free supernatant of rc#17 collected after 96, 120 and 144 hours of induction in BMMY in baffled flasks were loaded into wells cut in YEPD agar plate (pH 4.5) seeded with the sensitive strain *S. cerevisiae* DBVPG 6500. NC: cell-free supernatant of rc#24 (negative control of killer activity).



rc#17 cell-free supernatant volume (µl)	Log rc#17 cell-free supernatant volume	Inhibition halo (Ø, mm)
10	1.00	16.50±0.24
15	1.18	18.50±0.71
25	1.40	19.00±0.94
50	1.70	19.33±1.89
75	1.88	26.33±0.00
100	2.00	29.50±0.47

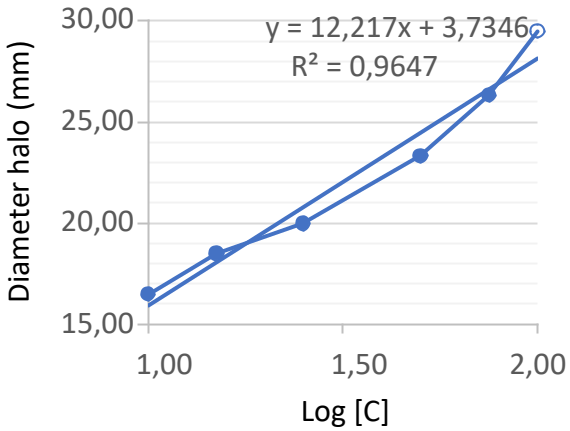


Figure 2. Evaluation of Arbitrary Units of rKpkt. One hundred µl aliquots containing increasing volumes of cell-free supernatant of rc#17 (10, 15, 25, 50, 75, 100 µL) in citrate phosphate buffer pH 4.5, were subjected to well plate assay using *S. cerevisiae* DBVPG 6500 as the sensitive strain. The diameters of the inhibition halos around the wells containing increasing amounts of the toxin were used to trace a calibration curve in respect to a Cartesian axes system, where the Log of the concentration of the toxin is on the abscissa and the diameter (mm) of the halo is on the ordinate. 1 AU corresponds to the amount of toxin in 100 µL that produces an inhibition halo of 20 mm (as the diameter of the well). Results are mean ± standard deviation of at least three independent experiments.

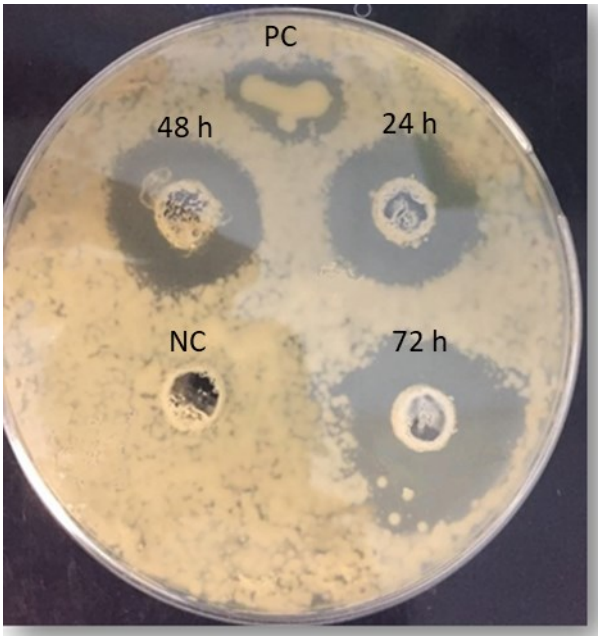


Figure 3. Well plate assay for killer activity evaluation. Aliquots of 100 µl of cell-free supernatant of rc#17 collected after 24, 48 and 70 h of induction in BMMY in bioreactor were loaded into wells cut in YEPD plates (pH 4.5) seeded with the sensitive strain *S. cerevisiae* DBVPG 6500. NC: cell-free supernatant rc#24 (negative control of killer activity. PC: *T. phaffii* DBVPG 6076 (streak on plate, positive control of killer activity).

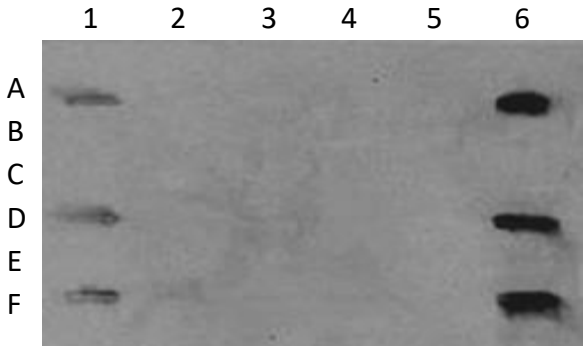


Figure 4. Dot blot for rKpkt immunodetection. A1, A6, D1, D6, F1, F6: positive controls (Mouse monoclonal anti-polyHistidine-Peroxidase antibody). Samples were subjected to the following treatments prior to hybridization to PVDF membrane: no heat treatment (2D, 2F) heat treatment at 60°C (3D-3F); heat treatment at 90°C (4D-4F); heat treatment at 95°C (5D-5F). Results are representative of two independent experiments.

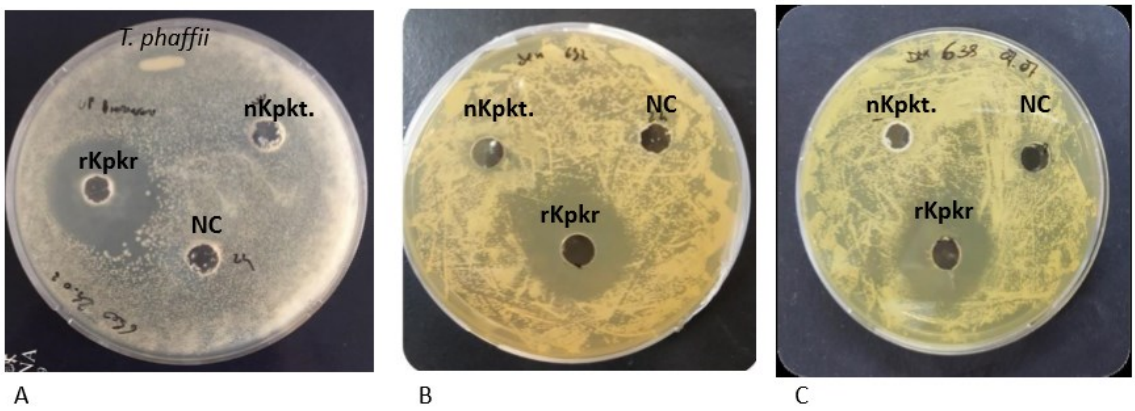


Figure 5. Well plate assay for killer activity evaluation. Aliquots of 100 µl of cell-free supernatant of rc#17 were tested on *H. uvarum* UNISS 156 (A), *D. bruxellensis* DiSVA 692 (B), *D. bruxellensis* DiSVA 638 (C). NC: cell-free supernatant of rc#24 (negative control of killer activity); nKpkt: cell-free supernatant of *T. phaffii* DBVPG 6076; rKpkt: cell-free supernatant of rc#17; *T. phaffii*: streak on plate of *T. phaffii* DBVPG 6076.

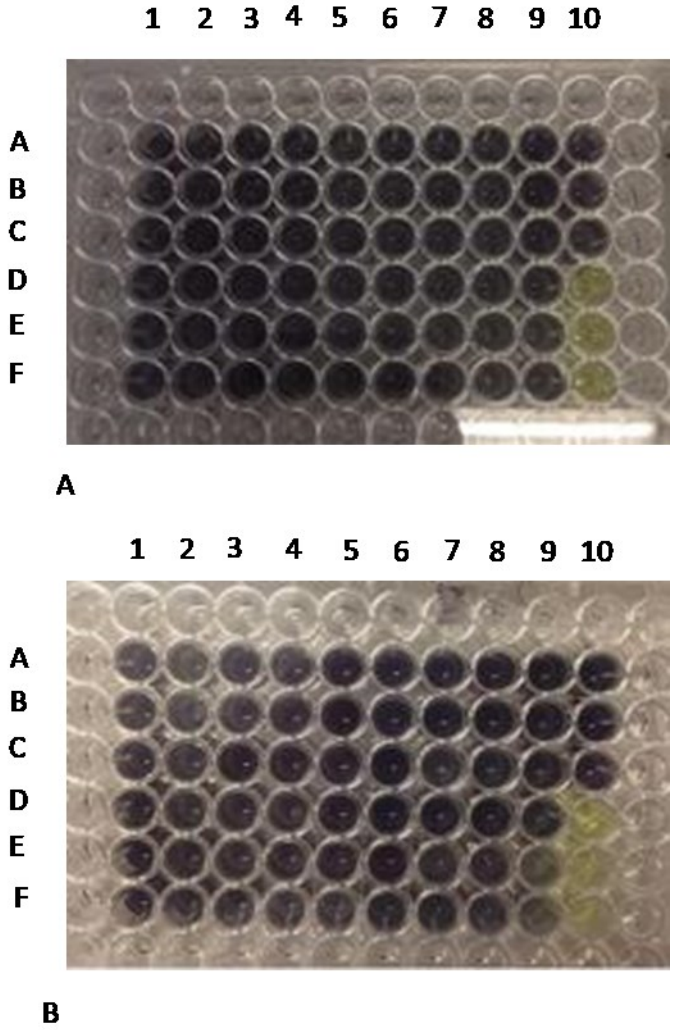
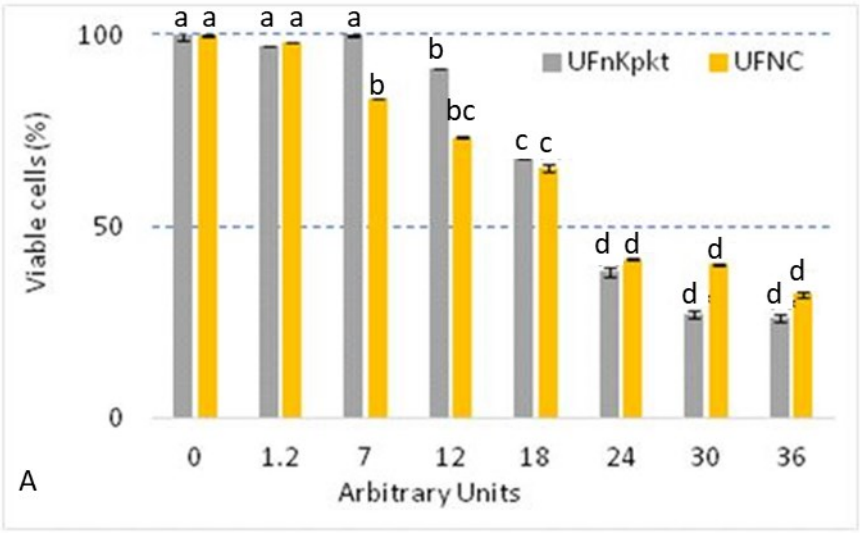


Figure 6. Effect of nKpkt (A) and rKpkt (B) on HaCaT cell line. HaCaT cells were exposed to increasing concentrations of nKpkt and rKpkt. After 24h of incubation with the toxin, the cells were subjected to MTT test. Yellow = dead cells; dark = living cells. In A and B: Line 1 positive control (0 AU of rKpkt or nKpkt); Line 2: 1.12 AU/mL; Line 3: 7 AU/mL; Line 4: 12 AU/mL; Line 5: 18 AU/mL; Line 6: 24 AU/mL; Line 7: 30 AU/mL; Line 8: 36 AU/mL; Line 9 and Line 10 A-C: positive control of viability (0 AU/mL of rKpkt or nKpkt); Line 10 D-F: positive control of toxicity (ethanol treated cells). In (A) wells A-C / 2-8 nKpkt; wells D-F / 2-8 nKpkt heat inactivated (negative control of toxicity). In (B) wells A-C / 2-8 rKpkt; wells D-F / 2-8 cell-free supernatant of rc#24 (negative control of toxicity). Results are representative of at least three independent experiments.



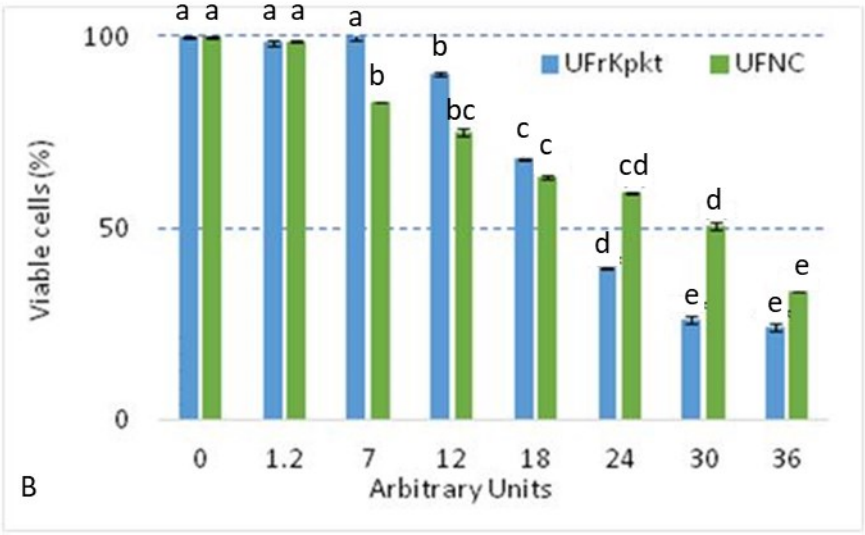


Figure 7. Viability of HaCaT cell line after 24 h exposure to nKpkt (A) and rKpkt (B). Cell viability was determined in respect to positive controls (0 AU of rKpkt or nKpkt). UFnKpkt: 60-fold concentrated cell-free supernatant of *T. phaffii* DBVPG 6076; UFrKpkt: 60-fold concentrated cell-free supernatant of rc#17; NC (A): heat treated 60-fold concentrated cell-free supernatant of *T. phaffii* DBVPG 6076; NC (B) 60-fold concentrated cell-free supernatant of rc#24. Results are means ± standard deviation of at least three independent experiments. Same superscript letters indicate results not significantly different ($p < 0.05$).

4.5. References

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Chapter 5

Production of a ready-to-use natural antimicrobial compound for the wine industry and evaluation of its long term stability and spectrum of action

Abstract

Previous works have shown that the heterologous expression of the killer toxin naturally produced by *Tetrapisispora phaffii* in the methylotrophic host *K. phaffii*, results in the production of a recombinant protein (rKpkt) that shows a wider spectrum of action in respect to its native counterpart. This recombinant toxin is active on wine yeasts ascribed to the genera *Kloeckera/Hanseniaspora, Saccaromycodes, Zygosaccharomyces* and *Dekkera,* while showing no significant toxicity on HaCaT cell line. Thus, it may represent a valid alternative to SO_2 for the wine industry. Here, with the aim of evaluating the possibility of obtaining a ready-to-use preparation of this killer toxin, the cell-free supernatant of *K. phaffii* recombinant clone 6 (rc#6), producing rKpkt, was 80-fold concentrated and lyophilized. The lyophilized preparation containing rKpkt (LrKpkt) maintains its killer activity for up to six months at 4 °C and it is easily solubilized in sterile distilled water. The antimicrobial activity of LrKpkt was tested in grape must and its spectrum of action was evaluated on a wide panel of biological targets. Results obtained on Cannonau and Vermentino grape musts indicate that LrKpkt inhibits the wild microflora, while showing limited or no effect on inoculated *Saccharomyces cerevisiae* wine starters. Moreover, besides being active on wine spoilage yeasts, LrKpkt exerts a strong microbicidal effects on a variety of bacterial species (lactic acid bacteria and food-borne pathogens). On the contrary, in accordance with its limited toxicity on HaCaT cell line, LrKpkt has no lethal effect on the two model insects utilized (*Ceratitis capitata* and *Musca domestica*), thus suggesting its low or null toxicity on higher eukaryotic cells. Indeed, additional studies are needed to further characterize LrKpkt. Nonetheless, the results here presented suggest that this ready-to-use antimicrobial compound may represent an interesting option for the management of microbial contaminations both in the wine and food industries.

Keywords: Lyophilized killer toxin, Kpkt, *Komagataella phaffii*, natural antimicrobial, MIC

5.1. Introduction

Microbial contamination is one of the major causes of food and beverages deterioration. It can occur at different stages of the production process, with negative impacts on both food safety and sensory properties.

In the wine industry, spoilage microorganisms are generally controlled through the addition of sulphur dioxide (SO_2). SO_2 counteracts growth of spoilage microorganisms such as yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). It is very useful to prevent undesired malolactic fermentation and growth of *Brettanomyces/Dekkera* and other spoilage yeasts, and bacteria (Santos et al., 2012). Moreover, SO_2 is widely employed also for its antioxidant properties (Ribereau-Gayon et al., 2006). However, similar to other chemical preservatives, SO_2 can cause harm to consumers (Guerrero and Cantos-Villar, 2014). Based on that, the World Health Organization recommends that daily intake of SO_2 should not exceed 0.7 mg/kg body weight (Scientific Committee for Food, 1996). Moreover, an excessive use of SO_2 should be avoided also because it may neutralize the wine aroma and produce characteristic aroma defects (Ribereau-Gayon et al., 2006). This has led both winemakers and the scientific community to seek valid alternatives to SO_2. These, on the one side, should counteract undesired microorganisms and, on the other side, must not be harmful to the consumers and to the final quality of wine (Guerrero and Cantos-Villar, 2014).

In this context, different authors have proposed the utilization of *Saccharomyces cerevisiae* killer starters to control the fermentative activity of wine spoilage yeasts (Van Vuuren and Jacobs, 1992; De Ullivarri et al., 2014) or to speed up yeast autolysis during sparkling wine production (Todd et al., 2000). Accordingly, the killer phenotype is one of the selection criteria for wine yeast starters (Mannazzu et al., 2002), and *S. cerevisiae* killer strains are commercially available and used in winemaking on an industrial scale (see for example EC1118 commercialized by Lallemand Oenology). Also non-*Saccharomyces* yeasts produce killer toxins of interest for the wine industry. As an example *Candida pyralidae*, *Kluyveromyces wickerhamii, Tetrapisispora phaffii, Torulaspora delbrueckii, Wickerhamomyces anomalus*, produce killer toxins active on a wide range of spoilage yeasts, among which *Hanseniaspora uvarum, Zygosaccharomyces bailii, Pichia membranaefaciens* and *Brettanomyces bruxellensis* (Ciani and Fatichenti, 2001; Comitini et al., 2004a; 2004b; Ciani and Comitini, 2011; Mehlomakulu et al., 2017; Villalba et al., 2016). Interestingly, the killer toxins produced by these yeasts are active under winemaking conditions and do not inhibit the fermenting *S. cerevisiae* strains nor the lactic acid bacteria. In particular, those active on *Dekkera/Brettanomyces* yeasts deserve further investigation. In fact, these yeasts produce during wine fermentation, aging and storage, volatile phenols and other metabolites negatively affecting wine quality, thus causing significant economic losses to winemakers (Tubia et al., 2018).

However, the scarcity of studies regarding the effects of killer toxins on human and animal consumers represents a limit to their utilization in the food and beverages industry. Another constraint to their exploitation is related to the difficulty to produce killer toxins at an industrial scale considering that their native production occurs at very low concentrations.

In the previous chapters, the selection of recombinant clones of *K. phaffii* capable of producing rKpkt, the implementation of fermentation processes aimed at rKpkt production and the evaluation of the effect of rKpkt on HaCaT cell line were described. Here, rKpkt was produced by growing rc#6 in a bench-top bioreactor and anion exchange chromatography was utilized for rKpkt purification. Moreover, with the aim of evaluating the possibility to produce a ready-to-use natural antimicrobial compound that shows killer activity on wine spoilage yeasts, a lyophilized preparation of the toxin was obtained and tested in grape must and its spectrum of action was evaluated on a wide panel of biological targets spanning from bacteria to model insects.

5.2. Materials and methods

5.2.1. Microorganisms and growth media. Microbial strains: *Tetrapisispora phaffii* DBVPG 6076, native producer of Kpkt*; T. phaffii* TpBGL2Δ, uncapable of producing Kpkt due to a deletion of *TpBGL2*, obtained by Oro et al. (2014) serves as the negative control of native Kpkt production; *Komagataella phaffii* rc#6 and rc#24 obtained by transforming *K. phaffii* GS115 with pPic9TpIM and pPIC9 plasmid vectors, respectively. Rc#6 is a Mut^+ strain, selected on the basis of killer activity, as described in chapter 1. Rc#24 integrated the empty plasmids pPIC9 and therefore serves as negative control of killer activity (Chessa et al., 2017). *S. cerevisiae* DBVPG 6500 was utilized as the killer toxin sensitive strain in well plate assay. Other microorganisms used are reported in Tables 2 and 3.

Growth media were: 2% glucose, 1% yeast extract and 2% peptone; BMGY: 1% glycerol (w/v), 1% yeast extract, 2% peptone, 1.34% YNB w/o aminoacids and 0.00004% biotin; BMMY: as BMGY with 0.5 or 1% methanol (v/v) in place of glycerol; De Man, Rogosa, Sharpe (MRS) broth (VWR, Milano); Brain Heart Infusion (BHI, Microbiol, Cagliari); Malt extract (Sigma); Wallerstein Laboratory Nutrient Agar (WL, Oxoid). Media were added with 2% agar and buffered at pH 4.5 with citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na2HPO4) when required. Yeasts and bacteria were maintained on appropriate media at 4 °C for short term storage and at −80 °C with addition of 10% glycerol for long-term storage.

5.2.2. Well plate assay and Arbitrary Units. Well plate assay was carried on YEPD buffered with 0.1M citrate-phosphate buffer (pH 4.5). Briefly, cells of the target sensitive strain (OD_{600} 0.1-0.2) were resuspended in sterile distilled water and 100 µL aliquots were spread on YEPD plates. 100 µL of cell-free supernatant of the recombinant clone was loaded into wells cut into the plate. As negative control the cell-free supernatant of rc#24 was utilized (Chessa et al, 2017). Toxin concentration in the supernatant was evaluated in terms of Arbitrary Units (AU) as described by Ciani and Fatichenti, (2001). The calculation of the Arbitrary Units was carried out as follows. Aliquots of 10, 15, 25, 50, 75 µL of cell-free supernatantof rc#6 taken to 100 µL with citrate phosphate buffer (pH 4.5) and 100 µL of cell-free supernatant were subject to well plate assay using *S. cerevisiae* DBVPG 6500 as the sensitive strain. After 3 days incubation at 25C° the inhibition halos generated around each well were measured and the values obtained were used to trace a calibration curve in respect to a Cartesian axes system, where the Log of the concentration of toxins is on the abscissa and the diameter (mm) of the halo is on the ordinate.

Given the linear relationship between diameter of the inhibition halo and logarithm of killer toxin concentration, 1 AU corresponds to the amount of toxin in 100 µL that produces an inhibition halo of 20 mm (as the diameter of the well), using *S. cerevisiae* DBVPG 6500. Killer activity was expressed in terms of AU (Ciani and Fatichenti, 2001).

5.2.3. Bioreactor production of rKpkt. Pilot scale production of rKpkt was carried out in Biostat B bioreactor (Sartorius). Briefly, rc#6 was inoculated in 150 mL BMGY within 250 mL baffled flask and incubated at 30 °C for at least 24 h or until it reached optical density (OD_{600}) of 10-12 under shaking conditions (180 rpm). This pre-culture was inoculated in 900 mL of BMGY added with 0.5 mL anti foam (Antifoam 204 Sigma Aldrich) within the bioreactor. The culture medium was buffered (pH 4.5) with automated addition of 20% phosphoric acid and 20% potassium hydroxide, stirring (maximum 1100 rpm) was in cascade with dissolved oxygen which was set to 25%. The fermentation strategy was divided into 3 steps: glycerol batch phase (GBP), glycerol fed-batch phase (GFBP), methanol fed-batch phase (MFBP). After complete glycerol consumption (~24 h), indicated by a spike of dissolved oxygen (end of GBP), 10% glycerol was added (9 mL/h). When biomass reached OD_{600} of 350–400 (about 24 h) glycerol feeding was terminated. In the transition from GFBP to MFBP fermentation temperature was decreased at 20 °C and methanol induction started with the addition of 3.6 mL/L/h methanol 100% during the first 2-3 hours. Then, methanol feeding rate was doubled to ~7.3 mL/L/h initial fermentation volume. After 2 h methanol feeding rate was increased to ~10.9 mL/L/h initial fermentation volume till the end of the process.

5.2.4. Purification of rKpkt. Bioreactor culture broth (4.8 L) was centrifuged and filter-sterilized (Ø 0.22 µm Millipore Corp., Bedford, MA) to obtain a cell-free supernatant that was concentrated ~80-fold by ultrafiltration through Crossflow filter holder (10 kDa cut-off Hydrosart membrane, Sartorius) and Centricon YM10 (10 kDa cut-off membrane, Millipore) to a final volume of 60 mL. Twenty mL of the retentate were dialysed with 10 mM citrate phosphate buffer, pH 4.5, using dialysis membrane (12–14 kDa, Medicell) and taken to 56 mL. Of these, 8.0 mL were loaded in a pre-equilibrated (10 mM citrate/phosphate buffer, pH 4.5) Q-Sepharose Fast Flow IEX column (45 mL bed volume; Amersham Biosciences) with 3 mL/min flow rate for protein purification. The volume loaded in the column corresponded to 3 mL of retentate and to 240 mL culture broth, as reported in Table 1. Protein bound to the resin was eluted with the following step-wise increases of NaCl concentration in the elution buffer (10 mM citrate phosphate, pH 4.5 containing 0.1, 0.125, 0.15, 0.175, 0.2, 0.3 M NaCl).

5.2.5. SDS-PAGE electrophoresis. Samples were concentrated by ultrafiltration on a Centricon YM10 membrane (Millipore) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) in Mini-PROTEAN Tetra Cell Kit (Biorad). After fixation with 5% glutaraldehyde for 1 h, the proteins were silver stained (PlusOne Silver Staining Kit Protein, GE healthcare) and the molecular mass determined by comparison with known marker proteins (Precision Plus Protein Standards All blue, Biorad).

5.2.6. Lyophilization. Lyophilization was carried out with a Labconco Freezone 4.5 system (Boston Laboratory Equipment) at the temperature of –42°C. The ultrafiltered samples were aliquoted in falcon tubes (5 mL) taken at -80°C and lyophilized under vacuum (0.133÷0.400 mbar). Lyophilization took about 72 h to completely remove the water phase and obtain a powder that was stored at 4 °C.

5.2.7. Determination of rKpkt activity in grape must. rKpkt activity was tested on pasteurized and on unpasteurized must. Vermentino (residual sugars 190 g/L; total acidity 5.7 g/L; pH 3.5; tartaric acid 3.5 g/L; malic acid 3.3 g/L; nitrogen components (APA) 120 ppm) and Cannonau (residual sugars 300 g/L; total acidity 4.8 g/L; pH 3.4; tartaric acid 6 g/L; malic acid 1.3 g/L; nitrogen components (APA) 100 ppm) grape musts, provided by Tenute Sella & Mosca (Loc. I Piani, Alghero) were pasteurized at 80 °C for 5 min. For pasteurized must yeast cells were pre-cultured in YEPD for 24 h and inoculated (1×10^6 cell/mL) in 1.5 mL pasteurized grape must (Vermentino) added with 2, 1 and 0.5 AU/mL rKpkt in 24-well microtiter plates. After 48 h growth at 25 °C under constant shaking (50 rpm) the effect of rKpkt was evaluated by viable plate count on YEPD. Lyophilized supernatant of rc#24 was utilized as negative control. Unpasteurized grape must (1.5 mL in 24-well microtiter plates) was added with 2, 1 and 0.5 AU/mL rKpkt and incubated for up 48 h growth at 25 °C under constant shaking (50 rpm). The effect of rKpkt was evaluated by viable plate count on WL agar plates. Lyophilized supernatant of rc#24 was utilized as negative control. At least three technical replicates from at least two independent experiments were carried out.

5.2.8. Spectrum of action of rKpkt. For bacteria and yeasts, dose response assays were carried out in 96-well microtiter plates. Briefly, $1x10^6$ cells/mL for yeast and $1x10^5$ cells/mL for bacteria, were inoculated in microtiter wells containing 200 µL buffered medium (YEPD for yeasts, MRS for lactic acid bacteria and BHI for food-borne pathogens) added with decreasing concentrations of lyophilized rKpkt two-fold serially diluted in sterile distilled water and expressed in terms of AU/mL (2, 1, 0.5, 0.25, 0.125, 0.062, 0.31, 0.015, 0.007, 0.003). The microtiter plates were incubated in agitation at 25 °C for yeasts, at 37 °C for lactic acid bacteria and food-borne pathogens, for 24 h and growth was measured automatically every 30 min at OD_{600} using a SPECTROstar nano microplate spectrophotometer (BMG Labtech, Ortenberg, Germany). The specific growth rate and the lag time of the curves obtained were analyzed using the DMFit software, which is based on Baranyi and Roberts (1994). At least three technical replicates from at least two independent experiments were carried out.

For filamentous fungi, 100 µL aliquots of sterile distilled water containing 2 AU of lyophilized rKpkt, were loaded into wells cut in the centre of Malt extract agar plates (pH 4.5) and three aliquots of 10 µL containing 10^2 conidia in water were placed on top of a triangle at equal distances from the well directly onto the agar, to allow the expansion of the fungus in all direction and detect a possible growth inhibition dictated by the presence of the rKpkt in the middle of the plate. Equivalent amounts of lyophilized supernatant of rc#24 were utilized as negative controls. At least three technical replicates from at least two independent experiments were carried out.

For insects, oral toxicity of recombinant and native Kpkt was evaluated on adults of the medfly *Ceratitis capitata* Wied. (Diptera: Tephritidae) and of the housefly *Musca domestica* L. (Diptera: Muscidae) provided by the insect rearing facility of the Dipartimento di Agraria of the University of Sassari (Italy) as described in Ruiu et al (2015) and Marche et al. (2016). Briefly, insects were administered a liquid diet incorporating either lyophilized ultrafiltered rKpkt (LrKpkt) and lyophilized ultrafiltered native Kpkt (LnKpkt) or, as negative controls,

lyophilized ultrafiltered supernatants of rc#24 (NCLrKpkt) and *T. phaffii* TpBGL2Δ (NCLnKpkt). According to the experimental design, the bioassays involved four groups of 10 newly emerged adult flies for each treatment. Each group was maintained within a plastic cage (10 x 15 × 5 cm) at 25°C and 60% relative humidity. Liophilized preparations were rehydrated in sterile distilled water, before being added with 30% saccharose. Aliquots of 75 µL of LrKpkt and LnKpkt containing 2 AU of toxin and equivalent amounts of NCLrKpkt or NCLnKpkt in terms of total protein content, were administered daily to each fly group by micropipette. As untreated control, analogous groups of flies were fed with the sole saccharose solution (30%). Insects were inspected daily and mortality was assessed after 5 days (Ruiu et al., 2007; Ruiu et al., 2015).

5.2.9 Data analysis

One-way ANOVA was carried out for the evaluation of the effect of rKpkt on cell viability, duration of lag phase, generation time and flies mortality for each sampling time. The critical value for significance level (p) was set at 0.05 and the Tukey test was used for post-hoc comparisons. All statistical analyses were performed using R-studio for Windows, version 10 (RStudio, PBC, USA).

5.3. Results

5.3.1. Production and purification of rKpkt. Rc#6 was cultured in a bench-top bioreactor and fermentation was articulated into three phases as indicated in chapter 4. During GBP cells were initially grown on glycerol containing medium in a batch mode to produce yeast biomass. Glycerol, contrary to that observed in chapter 4 for rc#17, proved the best carbon source for biomass production for rc#6 (data not shown). GBP lasted about 30 h after which a limited glycerol feed was initiated (GFBP). GBP and GFBP were aimed at maximizing biomass production with no induction of rKpkt expression. Accordingly, after ~48 h fermentation OD_{600} reached about 400 and no rKpkt was detected by means of well plate assay. Following glycerol exhaustion in GFBP, indicated by a spike in dissolved oxygen, MFBP was started (Figure 1). During this phase, methanol feeding rate reached 10.9 mL/L/h to induce the production of the recombinant protein. rKpkt production was monitored by well plate assay during MFBP after 24, 48 and 70 h of methanol feeding. As shown in Figure 1 detectable amounts of rKpkt were observed after 24 h and, as already observed for rc#17, rKpkt production increased during fermentation, starting from 2.3 ± 0.07 AU/mL at 24 h to reach 8.2 ± 0.12 and 17.1 ± 0.21 AU/mL at 48 and 70 h, respectively. After 70 h of MFBP the cell-free supernatant was 80-fold concentrated by ultrafiltration and dialyzed in 10 mM citrate phosphate buffer (pH 4.5) prior to purification on Q sepharose Fast Flow anion exchange column. Proteins bound to the resin were eluted with a NaCl gradient. The four fractions containing rKpKt (26, 27, 28, 29) were eluted with 125 mM NaCl in sodium citrate phosphate buffer (pH4.5). Following further concentration by ultrafiltration, all these fractions showed killer activity, although fractions 27 and 28 were those containing higher amounts of rKpkt in terms of AU/mL. These two fractions showed 3.03-fold increase in specific activity of the purified protein and 1.46% of the killer protein was recovered (Table 1). SDS-PAGE showed that fractions 27 and 28 contain more protein bands thus

indicating that the procedure applied resulted in a partial purification of rKpkt. However, one of these, with an apparent molecular mass of about 35 kDa (Figure 2) in accordance with that reported by Chessa et al. (2017), was present in the four fractions showing killer activity and in particular in fractions 27 and 28. Other bands, visible in the gel shown in Figure 2 were also present in eluted fractions lacking killer activity.

Table 1. Purification of the recombinant killer toxin secreted by rc#6.

Step	Total volume (mL)	Total protein (mg)	Activity (AU/mL)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Yield (%)
Culture broth	240	3533	17.1	4104	1.16	1	100
Ultrafiltration	3	99.75	57.3	172	1.72	1.48	4.2
Q-sepharose	1.2	17	50	60	3.52	3.03	1.46

5.3.2. Lyophlization of rKpkt. With the aim of obtaining a ready-to-use and stable preparation of rKpkt, a 30 mL aliquot of 80-fold concentrated cell-free supernatant was lyophilized with a Labconco Freezone 4.5 system. Lyophilization took 72 h and 2.15 g of powder were obtained. Lyophilized rKpkt (LrKpkt) was solubilised in water and in 0.1 M citrate-phosphate buffer (pH 4.5) (100 mg/mL) and subjected to well plate assay to evaluate possible differences in killer activity in the two solvents. Since no differences were observed, sterile distilled water was utilized to solubilize LrKpkt for the following experiments. Well plate assay was employed also to determine rKpkt concentration in terms of AU and results obtained indicated that 2.15 g of LrKpkt contain 1032 AU. Finally, to evaluate the long-term stability of the lyophilized preparation of rKpkt, LrKpkt killer activity was evaluated every two weeks for up to six months by well plate assay. Results obtained showed no significant variations of LrKpkt killing activity during time (data not shown).

5.3.3. Evaluation of LrKpkt activity on wine yeasts in pasteurized grape must. In order to evaluate the impact of the toxin on wine yeasts, the effect of the lyophilized preparation containing rKpkt was at first tested on pure cultures of wine-related yeasts inoculated in pasteurized Vermentino grape must. The following strains were utilized: *Dekkera bruxellensis DiSVA 692, Hanseniaspora uvarum UNISS 156, Kloeckera apiculata UNISS 3184, Lachancea thermotolerans UNISS J35, Saccharomyces cerevisiae UNISS JD21 Saccharomycodes ludwigii DiSVA 277, Wickerhamomyces anomalus DiSVA 366.* Grape must final volume (1,5 ml in 24 wells plate) was inoculated with 1×10^6 cells/mL. Viable plate count, done on YEPD immediately after the addition of 2 AU/mL of LrKpkt (T0) and after 24h (T1) and 48h (T2) of incubation at 25°C revealed no growth at T1 and T2 in the presence of LrKpkt thus highlighting that the amount of lyophilized killer toxin employed was sufficient to counteract growth of yeast cells in pure culture (Figure 3).

5.3.4. Evaluation of LrKpkt activity on wine yeasts in unpasteurized grape must. The activity of rKpkt was than assessed on wild yeast in freshly pressed grape must. For that unpasteurized uninoculated Cannonau (red) and Vermentino (white) grape musts were added with 0.5, 1 and 2 AU/mL of LrKpkt. Lyophilized supernatant of rc#24 was utilized as the negative control (NC). Due to the lack of killer activity in the negative control the amount of lyophilized supernatant to be utilized was evaluated in terms of total protein content (Bradford, 1976). However, in order to evaluate any possible effect of the lyophilized cell-free supernatant of rc#24 on yeast growth, viable plate count was carried out also on unpasteurized uninoculated grape must as such (named AS). Trials were performed in triplicate and viable plate counts were done on WL medium, immediately after toxin addition (T0) and after 24 h (T1) and 48 h (T2) of incubation at 25°C. For what concerns Cannonau grape must, at T0 viable plate count of the negative controls revealed the presence of a relatively high cell density with of $3\times10^6\pm1.8\times10^5$ CFU/mL and $2.7\times10^6\pm1.9\times10^5$ CFU/mL in AS and NC, respectively. At T1 and T2 a dose- dependent effect of the toxin was observed with significant reductions of viable plate counts ($p<0.001$) (Figure 4). At T0 Vermentino grape must showed lower viable plate count as compared to Cannonau with $3\times10^4\pm2.12\times10^3$ and $4.8\times10^4\pm1.27\times10^3$ CFU/mL in AS and NC respectively. At T1 LrKpkt significantly affected viable plate count, no matter the concentration used. At T2, 0.5 and 1 AU/ml of LrKpkt displayed a fungistatic effect while 2 AU/ml showed a fungicidal effect (Figure 4).

Finally to assess the effect of the killer toxin on *S. cerevisiae* commercial wine starter strains, 0.5, 1 and 2 AU/mL of LrKpkt were added to unpasteurized Vermentino and Cannonau grape musts inoculated with *S. cerevisiae* EC1118 (Lalvin EC1118 Organic) and *S. cerevisiae* Okay (Lalvin ICV OKAY® Lallemand), respectively. Grape musts utilized for this experiment were inoculated with the *pied de cuve* method at the industrial scale (Tenute Sella & Mosca Winery) and viable plate count at T0 indicated cell densities of about 4×10^7 cell/mL. Regarding Vermentino grape must it was observed that LrKpkt exerts a dose dependent effect on *S. cerevisiae* EC1118 (Figure 5). LrKpkt at 0.5 and 1 AU/mL showed a microbiostatic effect, 2 AU/mL of the toxin determined a significant reduction of viable plate count after 48 h (30% viable cells in respect to AS and NC). Regarding Cannonau grape must, no differences in viable plate counts on YEPD were observed for *S. cerevisiae* Okay between the thesis without and with LrKpkt (at T2 $4.8\times10^8 \pm 5\times10^7$ UFC/mL AS; $5\times10^8\pm 0.2\times10^7$ UFC/mL NC; $5.2\times10^8 \pm 7\times10^7$ UFC/mL with 2 AU/mL) (Figure 6).

5.3.5. Spectrum of action of rKpkt on bacteria, yeast and filamentous fungi. To evaluate the spectrum of action of the lyophilized preparation of rKpkt, a rich portfolio of bacteria, yeast and filamentous fungi was challenged with known amounts of LrKpkt. For bacteria and yeast minimal inhibitory concentration (MIC), minimal microbicidal concentration (MMC) and minimal concentration affecting growth (MCAG) were determined. Moreover, the effect of MCAG on the duration of the lag phase (λ) and on generation time (g) was evaluated. For what concerns bacteria, LrKpkt was tested on lactic acid bacteria ascribed to the species *Lactobacillus plantarum* and *Lactobacillus rhamnosus* and on Gram- (*Salmonella bongori, Escherichia coli*) and Gram+ (*Stafilococcus aureus, Listeria monocytogenes*) food-borne pathogens. Results reported in Table 2 indicates that among food-borne pathogens *L. monocytogenes* was the most resistant to the recombinant killer

toxin (MIC =1 AU/mL) although being killed by 2 AU (MMC=2 AU/mL) (Figure 7). *E. coli*, *S. aureus* and *S. bongori* were inhibited and killed by lower concentrations of rKpkt (MIC=0.5 AU/mL and MMC=1 AU/mL). With the exception of *E. coli*, that showed the highest MCAG (0.25 AU/mL), the other food-borne pathogens were affected by lower concentrations of LrKpkt showing increases in the duration of the lag phase and of the generation time at 0.125 AU/mL. Among the Lactobacilli, *L. rhamnosus* was the most resistant to rKpkt (MIC=1 AU/mL and MMC= 2 AU/mL) while the two *L. plantarum* strains were affected by a lower concentration of the toxin (MIC=0.5 AU/mL and MMC= 1 AU/mL).

Regarding yeasts, results, reported in Table 3, indicated both inter- and intraspecific variability in respect to the MIC that ranged from a minimum of 0.25 (*Z. bailii* UNISS 38) to a maximum of 2 AU/mL (*W. californica* DiSVA 366) with most of the strains being inhibited by 0.5 or 1 AU/mL. In respect to the MMC, with the exception of *Z. bailii* UNISS 38 that was killed by 0.5 AU/mL, and *W. californica* DiSVA 366 that survived 2 AU/mL, all the other strains were killed by 2 AU/mL (56%) and 1 AU/mL (44%) (Table 3). The MCAG ranged from a minimum of 0.25 AU/mL (most of the *S. cerevisiae* and *L. thermotolerans* tested, *S. paradoxus* UNISS 96 and UNISS 137, *D. bruxellensis* DiSVA 692, *Z. bailii* UNISS 38) to a maximum of to a maximum of 1 AU/ml (*M. pulcherrima* UNISS 19) while the remaining strains were affected by 0.5 AU/mL of LrKpkt.

LrKpkt was tested also onto on pure cultures of nine filamentous fungi, isolated from Vermentino grape must identified as *Galactomyces spp*., *Fusarium spp*., *Aspergillus spp*., *Cladosporium spp*., *Rhizopus spp*., *Penicillium spp*. (V. Balmas, Dipartimento di Agraria, Università degli Studi di Sassari). Results obtained using 2 AU/mL of LrKpkt showed no activity of the toxin on these filamentous fungi when tested in Malt Agar pH 4.5 (Figure 8).

5.3.6. Activity of rKpkt on model insects. Considering its wide spectrum of action on yeasts and bacteria, LrKpkt was tested also on the medfly *C. capitata* and on the house fly *M. domestica* to evaluate its possible toxicity on two representative invertebrate species with very different biological and ecological features. *C. capitata* is one of the most important agricultural pests developing on fruits of a broad plant host range in tropical and sub-tropical regions. *M. domestica* lives in close contact with organic matrices of different origin, such as excrements, food residues and cadavers, thus continuously interacting with various human pathogenic microorganisms (Scott et al., 2014). For these reasons, the latter species is often employed as a biological model in medically-related studies. Due to the observed differences in the spectrum of action of recombinant and native toxins (Chessa et al., 2017), the effect of nKpkt was also considered in insect bioassays. As negative controls, *C. capitata* and *M. domestica* were fed comparable amounts (in terms of total protein content) of lyophilized ultrafiltered preparations of cell-free supernatants of rc#24 (NCLrKpkt) and *T. phaffii* TpBGL2Δ (NCLnKpkt). Toxicity was evaluated via ingestion of LrKpkt and LnKpkt and of NCLrKpkt and NCLnKpkt, according to the experimental design described in Materials and methods. As a result, average insect mortality ranged between 15 and 35% for the medfly and between 15 and 25% for the house fly with no significant differences among treatments and the untreated controls for both species (*C. capitata* $p = 0.3982$; *M. domestica* $p = 0.7135$). Thus, it was shown that recombinant and native Kpkt exert no lethal effect on the two insects.

5.4. Discussion

Here, the purification of rKpkt, the production of a ready-to-use preparation of the recombinant toxin, the evaluation of its antimicrobial activity in grape must and the assessment of its toxicity on a wide panel of biological targets, are presented. Considering the instability of the killer phenotype in rc#17 (chapter 4), the bioreactor production of rKpkt was carried out with rc#6. This was selected among the 87 recombinant clones obtained by transforming GS115 with pPIC9TpIM (chapter 3). In accordance with that reported for the heterologous expression of genes under the control of *AOX*1 promoter, bioreactor cultivation of rc#6 was articulated into three phases aimed at repressing (GBP), de-repressing (end of GFBP) and fully inducing (MFBP) *AOX*1 promoter (Zhang et al., 2000; Inan and Meagher, 2001; Sun et al., 2018; Viña-Gonzalez et al., 2018;Juturu and Wu, Liu et al., 2019). Contrary to that observed with rc#17, rc#6 utilized glycerol more efficiently than glucose (data not shown). Accordingly, glycerol feeding in GBP and GFBP led to reach a high biomass concentration, while methanol feeding in MFBP resulted in a final concentration of rKpkt that was comparable to that obtained in baffled flask. Thus, scaling up of the fermentation process determined no losses in rKpkt yield.

In order to purify rKpkt, anion exchange chromatography was carried out, in accordance with that already done for nKpkt purification (Comitini et al., 2004). *K. phaffii* does not secrete many intrinsic proteins, while it efficiently secretes foreign proteins thus making simpler their purification from the culture medium (Zhu et al., 2019). SDS-PAGE of eluted fractions showing killer activity highlighted a protein band with molecular mass between 25 and 37 kDa, that is compatible with the expected size for rKpkt. However, contrary to that observed for nKpkt, here a partial purification of the recombinant toxin was obtained. In particular, the presence of high molecular weight proteins in the eluted fractions containing rKpkt indicated that more chromatographic steps are needed for rKpkt purification. Indeed, some of these proteins (molecular mass between 75 and 100 kDa) were common to all fractions. Thus, these could be carryover of the purification process. Others proteins with molecular mass below 75 kDa, were exclusive to rKpkt containing fractions and could possibly be dimers of the recombinant killer protein.

With the aim of obtaining a ready-to-use natural antimicrobial compound that shows killer activity on wine spoilage yeasts, a lyophilized preparation of the toxin was here obtained. It was thus shown that LrKpkt maintains it killer activity for up to 6 months at 4 °C and that it can be easily solubilised in sterile distilled water. Based on these evidences lyophilisation appears an interesting option for rKpkt storage and utilization. Accordingly, LrKpkt maintains its killing action in red (Cannonau) and white (Vermentino) grape musts, although with some differences. In fact, LrKpkt effect was much more marked in Vermentino than in Cannonau. Considering that the two musts differed significantly in viable plate count at T0, this discrepancy could be due, at least in part, to differences in the microbial load of the freshly pressed must. The effect of LrKpkt on yeast viability, when added to Vermentino and Cannau grape musts inoculated with the pied de cuve method, was much less marked in respect to that observed on the wild grape must microflora (uninoculated, unpasteurized grape must) or on pure yeast cultures in pasteurized Vermentino. In particular, LrKpkt showed a concentration-dependent effect on *S. cerevisiae* EC118 in Vermentino grape must and no significant effect on *S. cerevisiae* Okay in

Cannonau grape must (Figures 5 and 6). Since the two starter strains were inoculated at the same cell density and showed comparable sensitivity to LrKpkt in terms of MIC and MMC (Table 3), these results confirm that LrKpkt toxicity varies depending on the grape must and that the interaction between phenolic compounds and proteins could be responsible for the lower effect of LrKpkt in Cannonau in respect to that observed in Vermentino grape must (Ozdal et al., 2013). Thus, on the one side the amount of LrKpkt should be tuned depending on grape must variety. On the other side the utilization of LrKpkt causes limited or no harm to *S. cerevisiae* starter strains actively growing in grape must.

Results presented in Table 2 indicate that LrKpkt has a wider spectrum of action than previously thought (Chessa et al., 2017) being active also on Gram-positive and Gram-negative bacteria. Other authors reported on the activity of yeast killer toxins on a plethora of Gram-positive and Gram-negative pathogenic and non-pathogenic bacteria (Al-Qaysi et al., 2017; Bajaj et al., 2013; Guyard et al., 2002: Izgu and Altinby, 1997; Meneghin et al., 2010; Psani and Kotzekidou, 2006; Waema et al., 2009). Studies on the killer toxin produced by *Pichia anomala* ATCC 96603 showed that the monoclonal antibodies representing the internal image of the toxin are active on bacteria possibly through the interaction with the cell surface polysaccharides (Conti et al. 2000, Conti et al. 2002). While the mechanisms of action of LrKpkt on bacteria still needs to be elucidated, the results here presented indicate that rKpkt similar to other yeast killer toxins, may represent a possible approach to the control of Gram-positive and Gram-negative contaminants and food-borne pathogens.

For what concerns fungi, it was confirmed the killer effect of the recombinant toxin on wine-related yeasts (Chessa et al.,2017), while no effect was observed on filamentous fungi. Regarding the evaluation of LrKpKt and LnKpKt toxicity on the two model insects, interestingly no lethal effect was observed on the medfly *C. capitata* and the housefly *M. domestica*. These results, on the one side indicate that natural or recombinant Kpkt cannot be utilized to reduce or eliminate medfly and housefly populations. On the other side, considering that both insects are non mammalian model for biomedical research (Das, 1989; Kouloussis et al., 2017) and in accordance with the observed low toxicity of Kpkt on HaCaT cell line (Chapter 4), these results are compatible with the hypothesis that native and recombinant Kpkt are not toxic to multicellular eukaryotic organisms. Indeed, additional studies are needed to further characterize LrKpkt. Nonetheless, the results here presented suggest that this ready-to-use antimicrobial compound may represent an interesting option for the management of microbial contaminations both in the wine and food industries.

Table 2. Evaluation of rKpkt toxicity on bacteria.

Species	MMC (AU/mL)	MIC (AU/mL)	MCAG (AU/mL)	λ at MCAG	g MCAG	λ NC	g NC
E. coli DMSZ 30083	1	0.5	0.25	314±18.7[a]	48±6.5[a]	43±5.8[b]	23±2.3[b]
L. monocytogenes DMSZ 20600	2	1	0.125	294±47[a]	61±3.9[a]	161±25.5[b]	38±3.8[b]
L. monocytogenes UNISS B	2	1	0.125	340±15.3[a]	99±1.1[a]	229±13.5[b]	96±3.6[a]
L. monocytogenes UNISS C	2	1	0.125	294±29.2[a]	114±7.7[a]	228±9.8[b]	86±3.3[b]
L. monocytogenes UNISS E	2	1	0.125	338±25[a]	87±5.5[a]	220±12.2[b]	74±4.1[b]
S. bongori DSMZ 13772	1	0.5	0.125	143±22.2[a]	42±7.2[a]	30±6.9[b]	23±1.2[b]
S. aureus DMSZ 20231	1	0.5	0.125	365±17[a]	92±6.1[a]	64±2.9[b]	25±1.1[b]
L. rhamnosus ATCC 7469	2	1	0.25	208±21.6[a]	99±6.8[a]	61±3.7[b]	61±8.9[b]
L. plantarum ATCC 8014	1	0.5	0.125	207±13[a]	87±12.5[a]	132±21[b]	58±7.4[b]
L. plantarum UNISS pb5	1	0.5	0.0625	207±28.4[a]	65±4.8[a]	86±7.9[b]	60±2.5[a]

UNISS: Microbial Collection, Department of Agriculture, University of Sassari, Sassari, Italy; DSMZ-German Collection of Microorganisms and Cell Cultures, Leibnitz Institute, Braunschweig, Germany; ATCC American Type Culture Collection, Manassas, Virginia, USA. Same superscript letters indicate results not significantly different from NC ($p<0.05$).

Table 3. Evaluation of rKpkt toxicity on yeasts

Species	MMC (AU/mL)	MIC (AU/mL)	MCAG (AU/mL)	λ at MCAG	g MCAG	λ NC	g NC
S. cerevisiae EC1118	2	1	0.5	627±17^{a}	128±4.4^{a}	467±25^{b}	115±5.6^{b}
S. cerevisiae Okay	2	1	0.5	513±22^{a}	144±3.3^{a}	422±12^{b}	121±1.2^{b}
S. cerevisiae K1	2	1	0.5	569±19^{a}	174±6.7^{a}	429±21^{b}	103±7.1^{b}
S. cerevisiae UNISS JD31	2	1	0.25	470±29.3^{a}	163±23.4^{a}	417±17.7^{a}	107±0.8^{b}
S. cerevisiae UNISS JC12	2	1	0.25	696±31^{a}	191±1.9^{a}	450±38^{b}	134±5.5^{b}
S. cerevisiae UNISS 99	2	1	0.5	401±15.5^{a}	94±2.5^{a}	266±19.8^{b}	111±10.2^{a}
S. cerevisiae UNISS 118	1	0.5	0.25	237±22.3^{a}	92±7.5^{a}	180±36.3^{a}	76±6.1^{b}
S. cerevisiae UNISS 182	1	0.5	0.25	780±26^{a}	117±1.4^{a}	89±34^{b}	70±7.5^{b}
S. cerevisiae UNISS 178	1	0.5	0.25	750±48.2^{a}	106±10^{a}	155±49^{b}	72±2.3^{b}
S. cerevisiae UNISS 179	1	0.5	0.25	792±25.8^{a}	325±18.1^{b}	362±21.4^{a}	88±2.1^{b}
S. cerevisiae UNISS236	1	0.5	0.5	826±51^{a}	337±15^{a}	169±6.6^{b}	83±4.3^{b}
S. cerevisiae UNISS 274	2	1	0.5	331±32.1^{a}	99±9.8^{a}	149±13.6^{b}	70±3.3^{b}
L. thermotolerans UNISS 84	1	0.5	0.25	542±45.3^{a}	328±14.5^{a}	241±21.5^{b}	74±5.8^{b}
L. thermotolerans UNISS 165	1	0.5	0.25	833±28.7^{a}	122±31^{a}	276±0.6^{b}	77±0.1^{a}
L. thermotolerans UNISS 175	1	0.5	0.25	674±27.7^{a}	375±12.3^{a}	238±12.5^{b}	78±2^{b}
L. thermotolerans UNISS 226	2	1	0.5	325±11.5^{a}	104±1.1^{a}	270±37^{a}	115±14.2^{a}
M. pulcherrima UNISS 18	2	1	0.5	1124±84.4^{a}	358±31^{a}	357±7.7^{b}	111±2.2^{b}
M. pulcherrima UNISS 19	2	1	1	534±113.2^{a}	164±27.4^{a}	234±50^{b}	114±4.2^{b}
M. pulcherrima UNISS 29	2	1	0.5	851±44.4^{a}	352±31.3^{a}	274±38.1^{b}	126±0.9^{b}
M. pulcherrima UNISS 37	2	1	0.5	490±18.2^{a}	321±16^{a}	258±6.6^{b}	126±1.2^{b}
M. pulcherrima UNISS 86	2	1	0.5	796±38.2^{a}	424±26.8^{a}	268±41.2^{b}	89±8.1^{b}
M. pulcherrima UNISS 92	2	1	0.5	364±20.1^{a}	105±8.5^{a}	250±12.1^{b}	98±8.7^{a}
M. pulcherrima UNISS 225	2	1	0.5	552±59.5^{a}	115±4.6^{a}	460±35.6^{a}	92±0.2^{b}
S. bacillaris UNISS 267	1	0.5	0.5	403±66.8^{a}	110±1.6^{a}	134±27^{b}	75±3.5^{b}
S. bacillaris UNISS 276	2	1	0.5	318±33.2^{a}	141±8.5^{a}	156±5.9^{b}	67±8.1^{b}
S. paradoxus UNISS 96	1	0.5	0.25	584±44.4^{a}	213±9.3^{a}	172±49.9^{b}	99±8.2^{b}
S. paradoxus UNISS 133	1	0.5	0.5	287±9^{a}	142±9.3^{a}	143±24^{b}	94±4.9^{b}
S. paradoxus UNISS 137	1	0.5	0.25	970±95^{a}	470±5.4^{a}	616+±10^{b}	148±4.1^{b}
D. bruxellensis DiSVA 638	2	1	0.5	641±36.2^{a}	325±22.4^{a}	453±21.1^{b}	163±8.8^{b}
D. bruxellensis DiSVA 640	1	0.5	0.5	478±21.2^{a}	183±19.6^{a}	313±13.5^{b}	127±8.9^{b}
D. bruxellensis DiSVA 692	1	0.5	0.25	802±13.1^{a}	195±29.9^{a}	445±55.8^{b}	162±19^{a}
H. uvarum UNISS 158	2	1	0.5	681±33.1^{a}	190±21.5^{a}	141±1.4^{b}	149±10.8^{b}
Z. bailii UNISS 38	0.5	0.25	0.25	674±14.4^{a}	424±22^{a}	156±11.2^{b}	75±5.7^{b}
W. californica DiSVA 366	nd	2	0.5	545±41.1^{a}	276±11^{a}	406±26.2^{b}	236±14.2^{b}

UNISS: Microbial Collection, Department of Agriculture, University of Sassari, Sassari, Italy; DiSVA: Culture Collection of Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy. DBVPG: Industrial Yeast Collection, Department of Agricultural, Food and Environmental Science, University of Perugia, Italy; *S. cerevisiae* EC1118 and Lalvin ICV Okay are commercial starter strains (Lallemand Inc., Montreal, Canada). Same superscript letters indicate results not significantly different from NC ($p<0.05$).

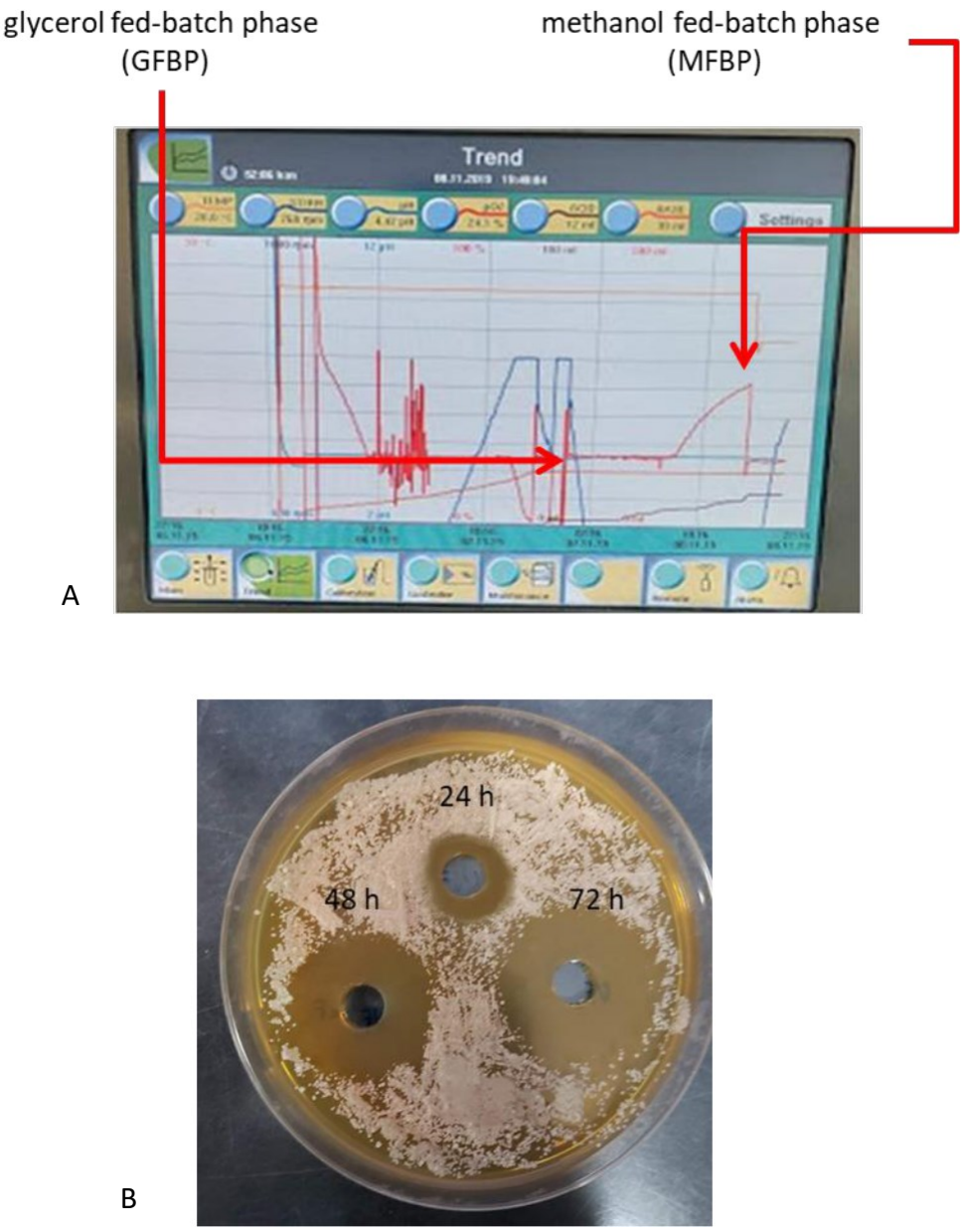

Figure 1. Bioreactor production of rKpkt. (A) Desolved oxygen spikes indicate carbon source depletion. (B) rKpkt production at different sampling times. Results are representative of at least five independent experiments.

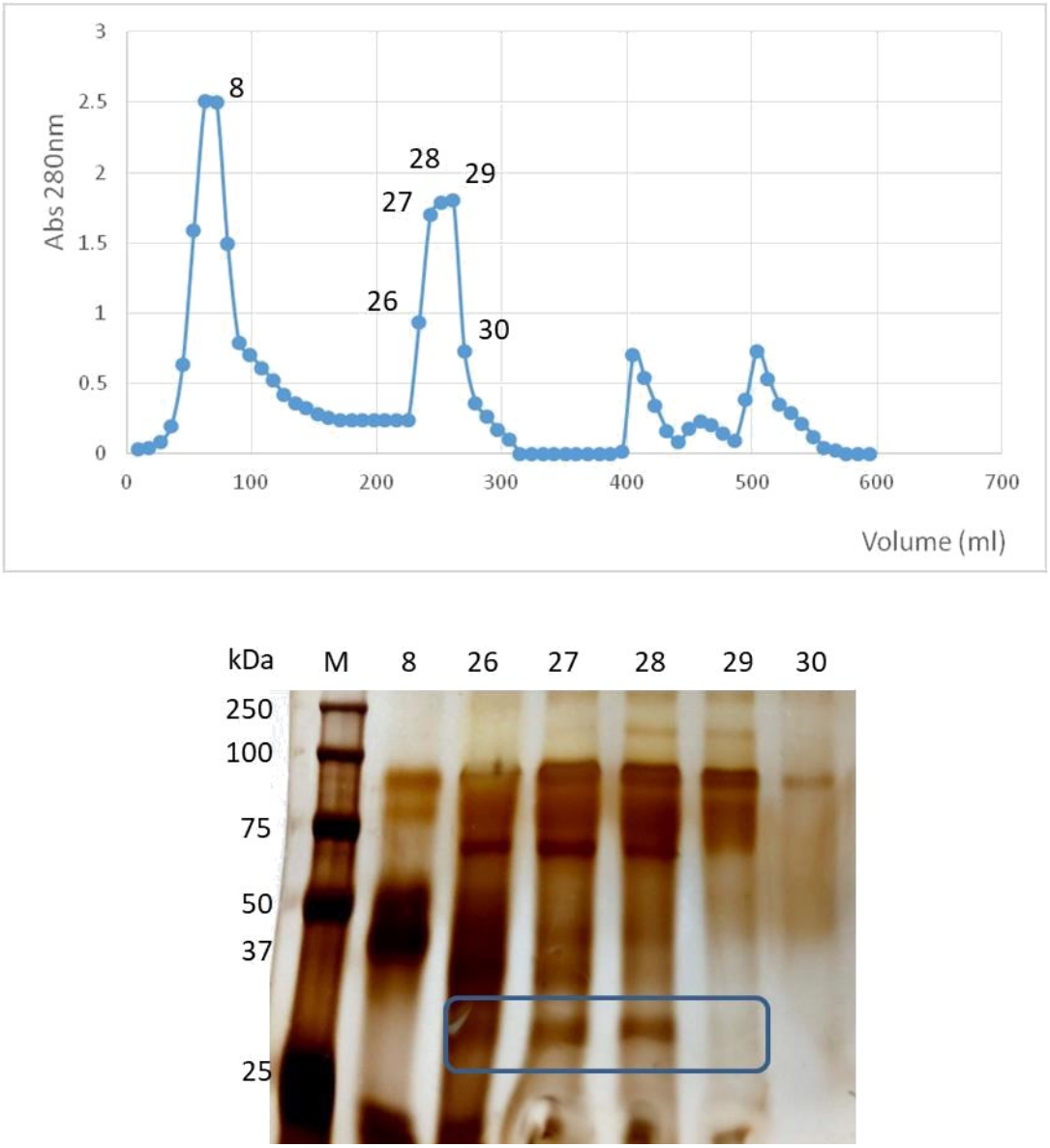

Figure 2. Recombinant Kpkt purification. A) Elution profile from the Q Sepharose ion-exchange chromatography column. The protein bound to the resin was eluted with the following step-wise increases of NaCl concentration in the elution buffer (10 mM citrate phosphate, pH 4.5 containing 0.1, 0.125, 0.15, 0.175, 0.2, 0.3 M NaCl). B) SDS-PAGE analysis of eluted fractions. M: Precision Plus Protein Standards All Blue (Biorad).

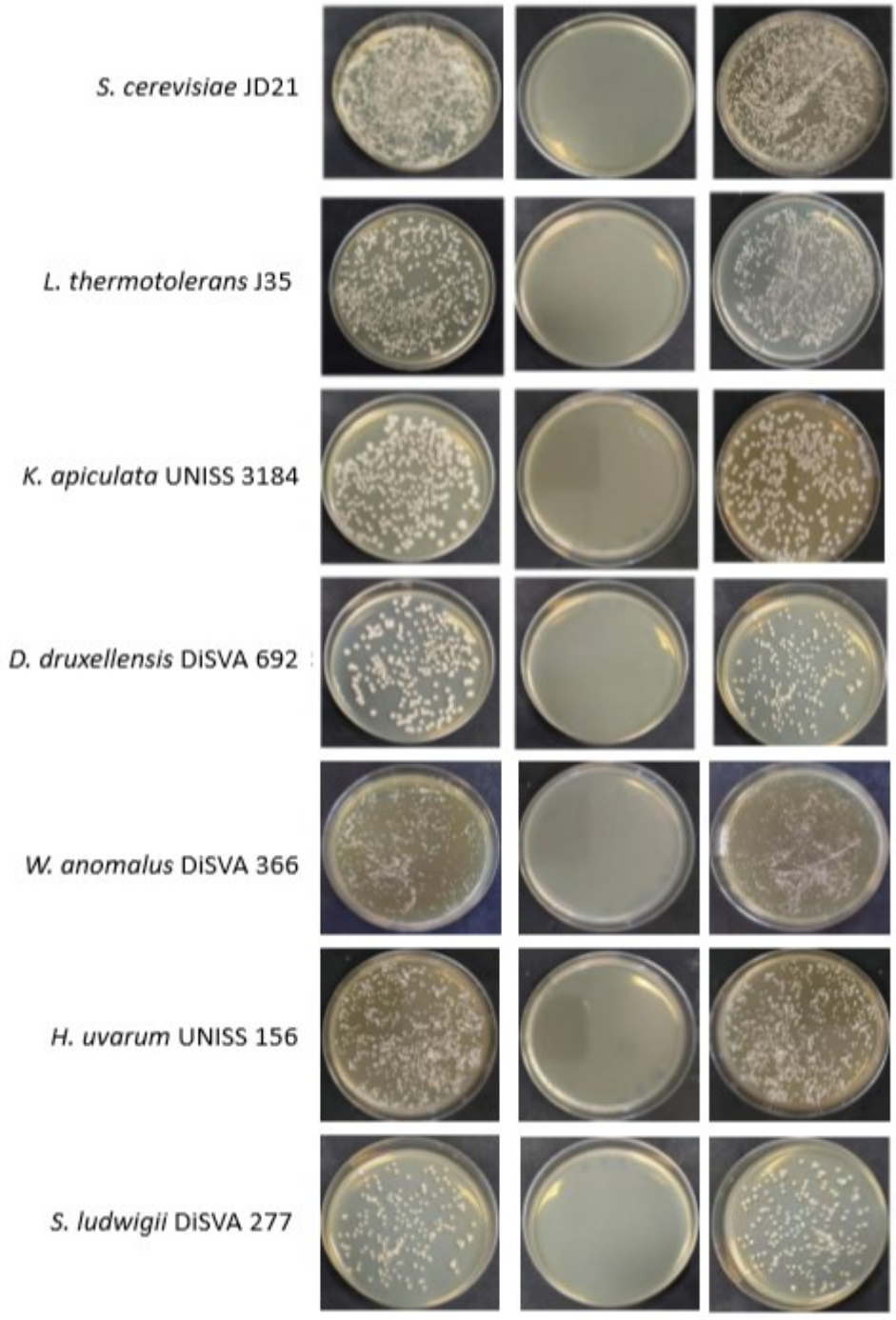

Figure 3. Effect of LrKpkt on pure cultures of wine-related yeasts in pasteurized Vermentino grape must. W/O LrKpkt: with no addition of LrKpkt; With LrKpkt: with 2 AU of LrKpkt; NC: with lyophilized supernatant of rc#24 (negative control). Same dilution (10^{-3}) are reported for each strain and condition. Results are representative of two independent experiments.

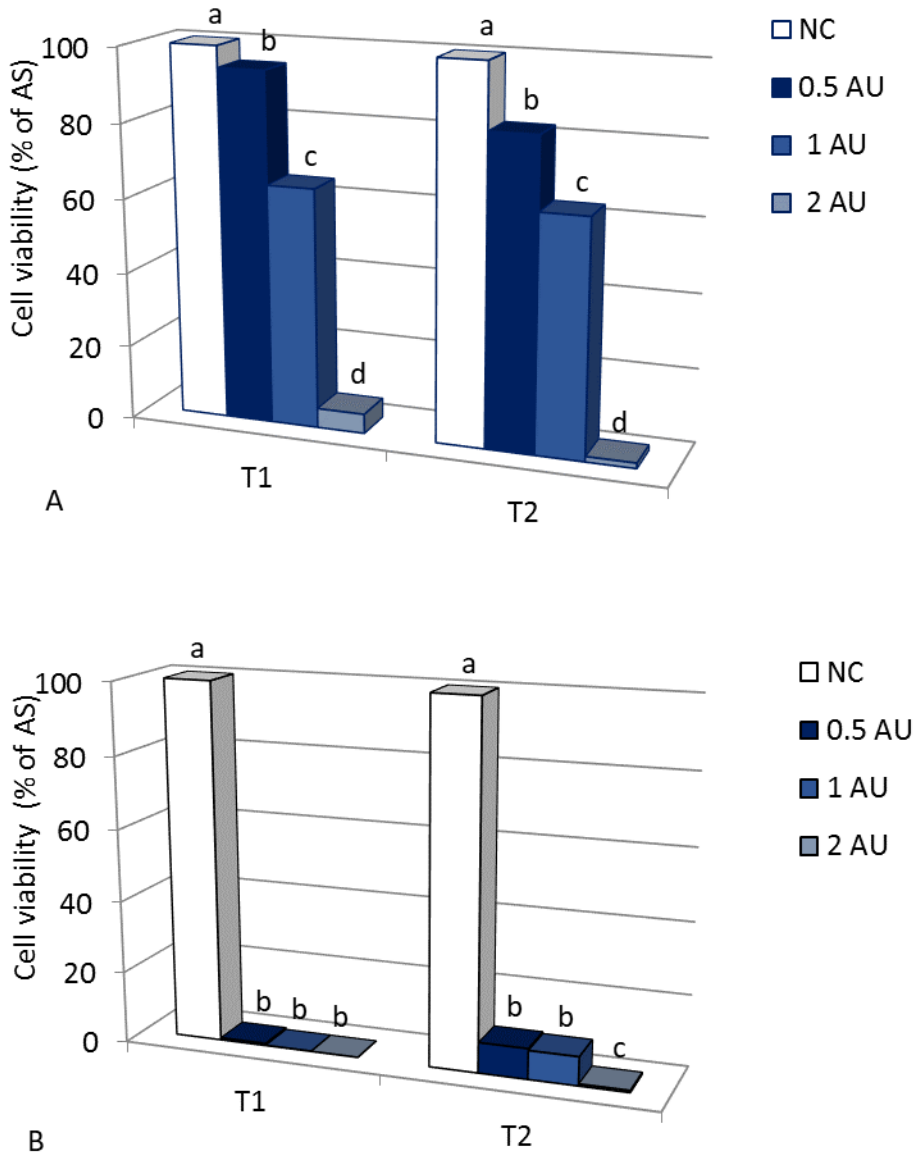

Figure 4. Viable plate count of unpasteurized uninoculated Cannonau (A) and Vermentino (B) grape musts upon LrKpkt treatment. Cell viability is expressed as percentage in respect to grape must as such (AS); NC: negative control added with lyophilized supernatant of rc#24; 0.5, 1 and 2 AU: grape must with 0.5, 1 and 2 AU/ml of LrKpkt, respectively. Results are means of three technical replicates of three independent experiments. Same superscript letters indicate results not significantly different for each sampling time ($p < 0.05$).

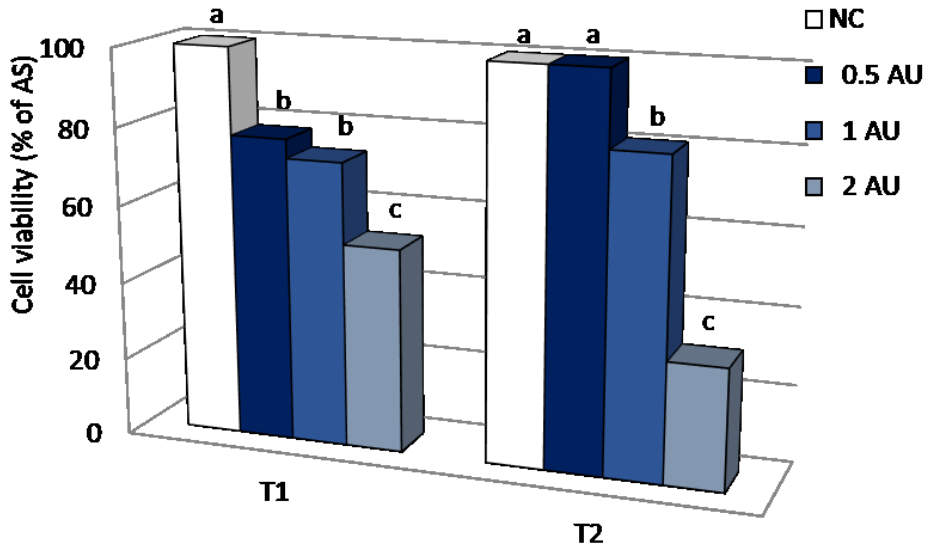

Figure 5. Viable plate count of unpasteurized Vermentino grape must inoculated with *S. cerevisiae* EC118 and added with LrKpkt. Cell viability is expressed as percentage in respect to grape must as such (AS); NC: negative control, grape must added with lyophilized supernatant of rc#24; 0.5, 1, 2 AU: grape must added with 0.5, 1, 2 AU/ml LrKpkt. Results are means of three technical replicates of two independent experiments. Same superscript letters indicate results not significantly different for each sampling time ($p < 0.05$).

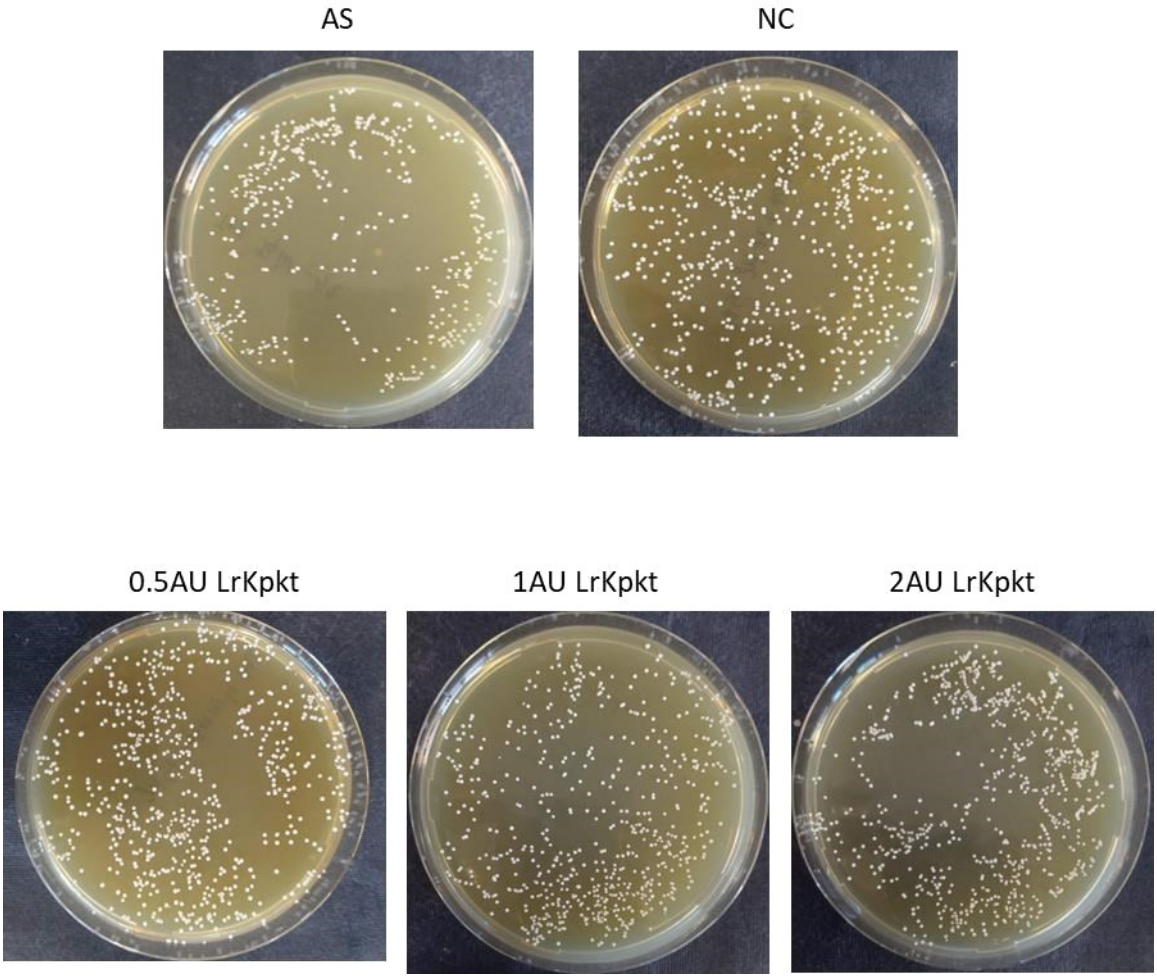

Figure 6. Viable plate count of unpasteurized Cannonau must inoculated with *S. cerevisiae* Okay and added with LrKpkt. AS: grape must with no LrKpkt addition. NC: negative control, grape must added with liophylized supernatant of rc#24; 0.5 AU LrKpkt, 1AU LrKpkt and 2AU LrKpkt: grape must added with 0.5, 1 and 2 AU/mL of LrKpkt, respectively. Same dilution (10^{-5}) is reported for all conditions. Results are representative of two independent experiments.

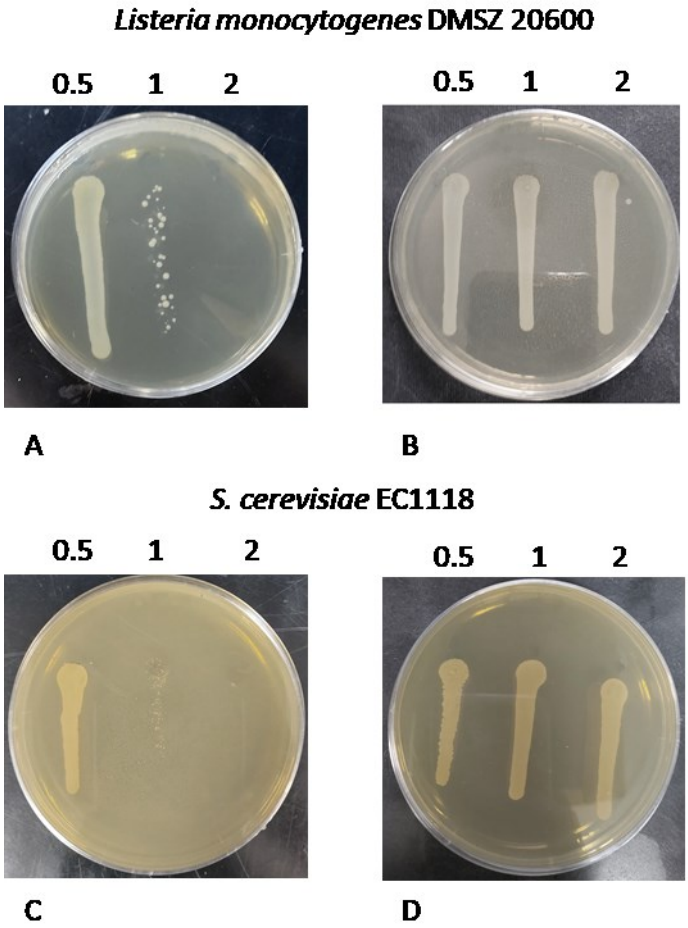

Figure 7. Effect of LrKpkt on cell viability. (A, C) with 0.5, 1 and 2 AU/ml LrKpk; (B, D) negative control with equivalent amounts of lyophilized supernatant of rc#24 in terms of total protein content/ml (arbitrary units equivalent AUE/ml). Results are representative three technical replicates of two independent experiments.

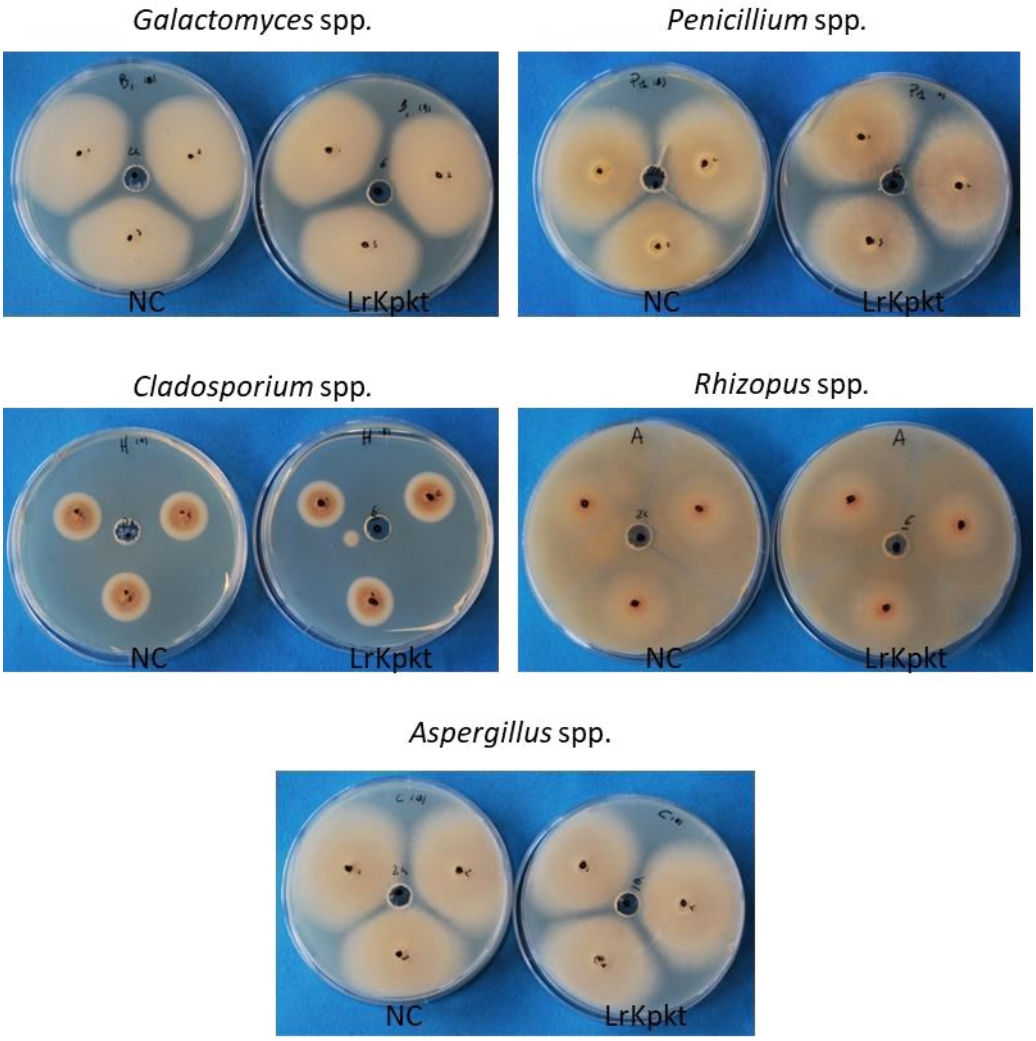

Figure 8. Effect of LrKpkt on filamentous fungi isolated from Vermentino must. LrKpkt: 2AU LrKpkt; NC: negative control with equivalent amounts of lyophilized supernatant of rc#24 in terms of total protein content/ml. Results are representative of three technical replicates of two independent experiments.

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Chapter 6

General conclusions

In agreement with the increasing consumer demand for the production of safe and healthy food, this thesis focuses on the exploitation of the yeast killer toxin Kpkt, as a possible natural antimicrobial of interest for the wine industry, and represents an original contribution to the research field regarding the development and exploitation of natural antimicrobials.

Considering that the native producer of Kpkt (*Tetrapisipora phaffii*), secretes limited amounts of this killer toxin, the molecular tools for the recombinant expression of Kpkt in *K. phaffii* and a strategy for its bioreactor production were developed. Moreover, a ready-to-use preparation of recombinant Kpkt (rKpkt) was obtained and its toxic effect was assessed on a wide portfolio of biological targets.

As reported in chapter 3, *Komagataella phaffii* was chosen for the production of rKpkt, and this based on the following reasons: i) other authors reported that this yeast is suitable for the expression of toxic compounds (Basanta et al., 2010; Chessa et al., 2017; Su et al., 2016; Yuan et al., 2015; Woo et al., 2002); ii) previous results obtained by Chessa et al. (2017) indicated the suitability of this host for the production of recombinant Kpkt (rKpkt). Here, contrary to that expected based on the spectrum of action of native Kpkt (Chessa et al., 2017), rKpkt proved toxic for the two *K. phaffii* strains selected for the heterologous expression. Thus, on the one side, the attempts to efficiently express rKpkt in M12 were unsuccessful, in spite of the development of new plasmids carrying promoters differing in strength and regulation. On the other side, it was necessary to screen a large number of GS115 transformants to select those capable of secreting detectable amounts of rKpkt.

In spite of that, sixteen transformants (15 Mut^{+} and 1 Mut^{S}) capable of secreting detectable amounts of rKpkt were obtained and two of them, namely rc#17 and rc#6 were further characterized.

Very likely due to rKpkt toxicity on the host cells, rc#17 proved unstable for the killer phenotype. This means that although maintaining integrated in the genome the heterologous expression cassette, it lost the killer phenotype and was therefore abandoned. However, the remaining 15 clones, among which rc#6, represent promising candidates for the implementation of efficient rKpkt production processes. In particular, the availability of a Mut^{S} recombinant clone could be advantageous for the production of rKpkt. In fact, the slower growth of Mut^{S} recombinant clones on methanol containing medium could help in finely tuning rKpkt expression in *K. phaffi* and this could result in higher heterologous production as already observed in Mut^{S} strains (Theron et al., 2019).

Regarding the production of the ready-to-use preparation of rKpkt, reported in chapter 5, it was shown that this may represent an interesting option for rKpkt storage and utilization. In fact, lyophilized rKpkt (LrKpkt) maintains its killer activity for up to 6 month at 4 °C and it can be easily solubilised in sterile distilled water. This is of great interest considering the toxic effect of rKpkt on different biological targets.

As reported in chapter 5 rKpkt has a wide spectrum of action on wine spoilage yeasts. In particular, besides being active on a wide panel of wine-related yeasts and maintaining its activity in red and white grape musts, it exerts a strong killing action on *Dekkera*

bruxellensis, while showing limited effect on actively growing *Saccharomyces cerevisiae* starter strains. These results suggest possible utilization of rKpkt for the control of spoilage yeasts both at the prefermentative stage and during ageing, depending on the oenological objective to be pursued.

Moreover, as reported in chapter 5, rKpkt is active also on Lactobacilli and on food-borne pathogens among which *Listeria monocytogenes*, *Staphilococcus aureus*, *Salmonella bongori*,
Escherichia coli. Indeed, much more work needs to be done to define the spectrum of action of rKpkt on bacteria. In particular its activity on malolactic bacteria needs to be further assessed in view of its application in the wine industry. Nontheless, the results obtained on bacteria are promising and open the way to further possible applications of rKpkt not only in the wine, but more in general, in the food industry.

Finally, based on that reported in chapters 4 and 5, rKpkt shows low or null toxicity on HaCaT cell line and on *C. capitata* and *M. domestica*, two non-mammalian models for biomedical research. Considering that the scarcity of studies regarding the effects of killer toxins on human consumers represents a constraint to their utilization in the food and beverages industries, these results represent a further step towards the biotechnological exploitation of yeast killer toxins.

For certain, more research is needed to thoroughly evaluate rKpkt impact on consumers' health and on the resident microbes of the gastro intestinal tract, once ingested, but also to thoroughly evaluate its interactions with the different food components. In addition, it should be considered that the recombinant nature of rKpkt could be an obstacle to its marketability. However it is well known that: i) *K. phaffii* is a GRAS host for heterologous production of proteins of interest for the food and biopharmaceutical industries (Ciofalo et al., 2006; Thompson, 2010); ii) two recombinant proteins, namely Kalbitor and Jetrea have already been approved by FDA and European commission (Ahmad et al., 2014); iii) many other recombinant biopharmaceutical products produced in this host are under clinical trials (Corchero et al., 2013; Gasser et al., 2013). All these evidences could help rKpkt in gaining consumers acceptance.

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