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THE INFLUENCE OF *PICHIA* KILLER TOXINS ON THE WINE SPOILAGE YEASTS

Urszula Błaszczyk, Paweł Satora, Paweł Sroka

ABSTRACT

Killer yeasts are able to produce toxins that antagonize the growth of susceptible yeasts cells of the same species or the ones that are related to them. Killer strains are resistant to their own toxins but can be sensitive to killer proteins of other yeasts. The killer proteins of *Pichia* spp. are known for its broad spectrum of antifungal activity including pathogens such as *Candida albicans*. The aim of the study was to investigate the potential of the partly purified killer toxins to inhibit the growth of selected yeast strains which can contribute to wine spoilage. Three *Pichia* killer yeast strains (CBS 1982, CBS 5759, CBS 7373) were used in the study. The killer protein secreted by *Pichia anomala* CBS 1982 was characterized by the highest antifungal activity. The most pronounced effect of the reduction of cell proliferation by killer toxin preparations was found after 2 and 20 h cultivation. Among the 13 tested strains, all *Pichia* killer toxin preparations inhibited the growth of *Rhodotorula graminis* Rg, *Rhodotorula mucilaginosa* Rm and *Schizosaccharomyces pombe* DSM 70576. Killer toxins produced by *Pichia anomala* CBS 1982 (K8) and CBS 5759 (K4) limited the growth of *Candida pulcherrima* K5 and *Hanseniaspora guillermondii* DSM 3432 after 2, 20 and 168 h of incubation. A significant reduction of *Debaryomyces hansenii* DSM 3428 biomass was observed in medium with the addition of one toxin preparation (*Pichia anomala* CBS 1982). The growth limitation of *Candida glabrata* DSM 6425, *Hanseniaspora uvarum* DSM 2768, *Metchnikowia pulcherrima* DSM 70321 and *Cryptococcus laurentii* DSM 70766 was noticed only after 2 hours cultivation in presence of killer protein preparations. The killer toxins could be used in the food industry as selective tools to control infections during the fermentation of wine and improve the quality of the final product.

Keywords: *Pichia*; killer yeast; killer toxin; wine spoilage

INTRODUCTION

The killer phenomenon was first described by Bevan and Makower in 1963 in a *Saccharomyces cerevisiae* strain which was isolated as a brewery contaminant. Since then, killer systems have been reported in other yeast genera such as *Ustilago*, *Kluyveromyces*, *Pichia*, *Candida*, *Debaryomyces*, *Torulopsis*, *Cryptococcus*, *Metschnikowia*, *Williopsis* and *Zygosaccharomyces* **(Schmitt and Breinig 2002; Pfeiffer et al., 2004; Izgü et al., 2006; Santos et al., 2009)**. Killer activity is one of the most important mechanisms of competition between strains and plays a significant role in the ecology of yeasts especially at low nutrient availability in the environment. The killer effect may represent a model of biological competition similar to that of bacteriocins among bacteria **(Magliani et al., 1997)**.

Killer yeasts secrete toxins (usually proteins or glycoproteins) which kill cells of sensitive strains of yeasts belonging to the same or related species without direct cell–cell contact **(Selitrennikoff, 2001; Wang et al., 2007)**. Killer strains are immune to their own toxins but can be sensitive to killer proteins of other yeasts. Each toxin has unique properties which differ considerably depending on the strain which it produces. The killer proteins of *Pichia* spp. have broad spectrum of antifungal activity including pathogenic *Candida albicans* **(Santos and Marquina, 2004; Izgü et al., 2006)**. They are relatively stable in comparison to toxins of *Saccharomyces* spp. **(Sawant et al., 1989)**.

Several applications for the killer yeasts and their toxins have been considered. Starter cultures with killer activity could be used to eliminate undesirable yeasts and filamentus fungi during the production of wine or beer. Killer strains are regarded useful in biological control of spoilage yeasts and the preservation of food **(Izgü et al., 2006)**. Killer toxins could be also considered as novel antimicrobial agents in the treatment of human fungal infections **(Schmitt and Breinig, 2002)**.

The aim of this study was to determine the ability of *Pichia* killer toxins to inhibit the growth of 13 selected yeast strains that are associated with fermentation of grape must and infection during winemaking.

MATERIAL AND METHODOLOGY

Yeast strains

The killer strains employed in this study (*Pichia anomala* CBS 1982 producing K8 toxin, *Pichia anomala* CBS 5759 secreting K4 toxin, *Pichia membranifaciens* CBS 7373 producing K7 toxin) were provided from the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. The sensitive yeast strains such as *Rhodotorula graminis* Rg,

Rhodotorula mucilaginosa Rm, *Candida pulcherrima* K5, *Kloeckera apiculata* 66 were sourced from the Culture Collection of the Department of Fermentation Technology and Technical Microbiology, University of Agriculture in Krakow, Krakow, Poland. These yeasts were isolated from Węgierka Zwykła plums **(Satora and Tuszyński, 2005)**. Other sensitive yeast strains used in this study (*Schizosaccharomyces pombe* DSM 70576, *Candida glabrata* DSM 6425, *Candida sake* DSM 70763, *Debaryomyces hansenii* DSM 3428, *Hanseniaspora guillermondii* DSM 3432, *Hanseniaspora uvarum* DSM 2768, *Metschnikowia pulcherrima* DSM 70321, *Cryptococcus laurentii* DSM 70766, *Pichia anomala* DSM 6766) were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. All pure yeast cultures were stored on agar slants with YEPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar at 4 °C.

Killer yeast cultivation

Killer yeast strains were cultured on agar slants with YEPD medium for 24 h at 28 °C. Next they were transferred into 50 mL of liquid YEPD medium and incubated at 28 °C for 24 h. At next stage 125 mL of liquid medium was inoculated with killer strain (the final *dry substance* of *yeast cells* 2g/L). Killer cultures were cultivated at 20 – 22 °C with shaking at 120 RPM on a gyratory shaker for 96 h. When the cells reached to stationary phase they were removed by centrifugation (4000 rpm for 10 min) and the culture supernatant was filtered through and 0.2 μm cellulose acetate membranes. Next the filtrate was adjusted to a final glycerol content of 15% (v/v) and concentrated 40-fold by using a centrifugal concentrator devices with a 10 kDa cut-off membrane.

Killer activity against wine spoilage microorganisms

Pure wine spoilage strains were cultivated on agar slants with YEPD medium at 28 °C for 24 h. At next stage each starter culture was prepared by inoculating four loops of slant culture into 125 mL and incubated at 28 °C for 24 h. Next yeast suspension was diluted 10-fold with sterile Ringer's solution. 1 mL of diluted yeast suspension was added to 8 mL of liquid medium containing 0.8% (w/v) nutrient broth, 1% (w/v) glucose and 0.01% (w/v) chloramphenicol, and then 1 mL of killer toxin preparations in 0.1 M citrate-phosphate buffer (pH 4.4) were added. A toxin-free control sample contained 8 mL of liquid medium, 1 mL of diluted yeast suspension and 1 mL of 0.1 M citrate-phosphate buffer pH 4.4. Measurements of the turbidity at 600 nm were done after 2, 20 and 168 h, using a standard curve of absorbance against dry cell mass concentration. All experiments were performed four times.

Statistical analyses

Statistical analyses were performed using InStat software, version 3.01 (GraphPad Software Inc., San Diego, USA). A single-factor analysis of variance (ANOVA) with a post hoc Tukey-Kramer's test was used to find means that are significantly different from each other. The means for the experimental groups were compared at 5% probability level.

RESULTS AND DISCUSSION

The influence of killer toxins on wine fermentation was investigated in several research studies **(Shimizu, 1993; Medina et al., 1997; Gutiérrez et al., 2001; Pérez et al., 2001; Satora et al., 2014)**. In some cases the presence of killer yeasts may decrease wine quality or even cause stuck or sluggish wine fermentation. On the other hand, must inoculation with killer yeast may reduce undesirable wild yeast strains, thus protect wine quality **(Maqueda et al., 2012)**. In the case of food preservation biological control with yeasts has been considered as a desirable alternative to the application of chemicals **(Santos et al., 2009)**.

To examine the potential of *Pichia* killer toxins as biocontrol agents 13 cultures of yeast which often cause the diseases of wine were selected. The results of *Pichia* killer activity against spoilage wine strains are presented in Table 1. Toxin activity was expressed as the percentage reduction in growth of the sensitive strain yeast with respect to a toxin-free control.

The pronounced inhibition of *Rhodotorula graminis* Rg growth by *Pichia* killer proteins was noted. The major effect was found when K8 killer toxin was added to medium. A considerable reduction of the biomass growth was also observed when killer proteins secreted by *Pichia* strains CBS 5759 and CBS 7373 were used. A smaller increase in a biomass production relative to control sample was noticed during *Rhodotorula mucilaginosa* Rm cultivation with addition of all three killer preparations. In the case of *Schizosaccharomyces pombe* DSM 70576 the effect of the growth limitation was observed after 2 h and 20 h incubation. During following days more intensive growth in relation to control sample was found. It could be explained by assimilation of nitrogen compounds as proteins of toxin preparation by studied yeasts.

It was also found that killer toxins produced by strains CBS 1982 (K8) and 5759 (K4) inhibited the growth of *Candida pulcherrima* K5 and *Hanseniaspora guillermondii* DSM 3432 after 2, 20 and 168 h of incubation. A significant reduction of *Debaryomyces hansenii* DSM 3428 biomass was observed in medium with the addition of only one toxin preparation (*Pichia anomala* CBS 1982). In the case of *Candida glabrata* DSM 6425, *Hanseniaspora uvarum* DSM 2768, *Metchnikowia pulcherrima* DSM 70321 and *Cryptococcus laurentii* DSM 70766 strains the greatest inhibition of growth was evident after 2 hours. The limitation of cell proliferation was noted only in the case of usage of toxins produced by *Pichia anomala* CBS 1982 and CBS 5759. After two hours of cultivation, the inhibition of *Pichia anomala* DSM 6766 and *Kloeckera apiculata* 66 growth was observed in the presence of K8 toxin secreted by the strain CBS 1982. In another study on the *Pichia* killer activity, the action of PMKT2, a toxin from *Pichia membranifaciens* CYC 1086 which is active against *Brettanomyces bruxellensis*, was reduced significantly in the first hour, then killer activity was constant for 10 hours **(Santos et al., 2009)**.

Table 1 The impact of *Pichia* killer toxins on the growth of yeast which can contribute to the spoilage of wine. Results are expressed as the percentage of biomass of yeast strains cultivated after treatment with killer toxins in relation to the growth without addition of killer proteins.

Sensitive yeast strain	Killer strain	Cultivation time		
		2 h	20 h	168 h
Rhodotorula graminis Rg	**1982**	9^{a} ±4	65 ±11	82^{a} ±3
	5759	20^{b} ±4	78 ±4	97^{b} ±2
	7373	37^{b} ±18	68 ±8	86^{a} ±3
Rhodotorula mucilaginosa Rm	**1982**	34 ±20	83^{a} ±2	106^{a} ±6
	5759	33 ±17	85^{a} ±1	90^{b} ±5
	7373	52 ±27	70^{b} ±6	76^{c} ±1
Schizosaccharomyces pombe DSM 70576	**1982**	52 ±33	74 ±6	116 ±7
	5759	33 ±2	74 ±1	118 ±2
	7373	55 ±38	80 ±4	132 ±10
Candida pulcherrima K5	**1982**	42 ±22	66 ±15	59 ±28
	5759	67 ±37	75 ±10	61 ±20
	7373	115 ±24	82 ±14	77 ±15
Candida glabrata DSM 6425	**1982**	27 ±10	99 ±2	72 ±20
	5759	44 ±27	103 ±3	99 ±2
	7373	70 ±35	108 ±2	102 ±1
Candida sake DSM 70763	**1982**	152 ±77	103 ±9	92 ±2
	5759	61 ±47	102 ±3	94 ±2
	7373	119 ±17	96 ±1	86 ±1
Debaryomyces hansenii DSM 3428	**1982**	18^{a} ±10	62 ±6	53^{a} ±2
	5759	80^{b} ±27	88 ±5	55^{a} ±11
	7373	102^{b} ±18	75 ±10	91^{b} ±3
Hanseniaspora guillermondii DSM 3432	**1982**	46^{a} ±31	58 ±17	62^{a} ±2
	5759	62^{a} ±17	73 ±13	60^{a} ±2
	7373	121^{b} ±23	83 ±14	92^{b} ±6
Hanseniaspora uvarum DSM 2768	**1982**	36^{a} ±18	95 ±6	98^{a} ±2
	5759	43^{a} ±22	102 ±3	96^{ab} ±4
	7373	99^{b} ±1	92 ±1	84^{b} ±2
Kloeckera apiculata 66	**1982**	40^{a} ±9	83 ±15	79^{a} ±6
	5759	124^{b} ±21	88 ±10	76^{a} ±4
	7373	110^{ab} ±14	95 ±5	98^{b} ±2
Metschnikowia pulcherrima DSM 70321	**1982**	25^{a} ±16	90 ±2	100 ±2
	5759	55^{a} ±25	91 ±2	102 ±4
	7373	120^{b} ±19	81 ±10	89 ±12
Cryptococcus laurentii DSM 70766	**1982**	36^{a} ±13	71 ±8	93 ±2
	5759	19^{a} ±10	86 ±6	91 ±3
	7373	125^{b} ±14	89 ±13	95 ±3
Pichia anomala DSM 6766	**1982**	42^{a} ±13	82 ±5	82 ±6
	5759	70^{ab} ±17	86 ±6	82 ±4
	7373	110^{b} ±20	81 ±3	81 ±5

Note: The values with different superscript letters mean statistically significant differences at 5% levels of probability

The results showed relatively weak influence of killer toxin preparations on the certain tested yeasts. Among the *Pichia* strains used in the study, the killer toxin preparation of *Pichia anomala* CBS 1982 was characterized by the highest antifungal activity, whereas killer toxin K7 secreted by *Pichia mambranifaciens* CBS 7373 inhibited less effectively the growth of selected strains.

Killer toxins as proteinaceous compounds have a limited stability in solution. In many cases, killer proteins are characterized by a high susceptibility to various factors such as an elevated temperature or the presence of proteases. It would be important to conduct further research on the action of *Pichia* killer toxins against tested spoilage yeasts in winemaking conditions. It is necessary to determine how the presence of killer toxins could influence the metabolites production by tested yeasts and in consequence change the quality of the wine. It would also be important to increase the stability of studied killer toxins or consider the application of different killer proteins which are more stable or more efficient in their inhibitory action.

CONCLUSION

The killer toxin secreted by *Pichia anomala* CBS 1982 was distinguished by the highest antifungal activity. Of the 13 wine spoilage strains, all *Pichia* killer toxin preparations inhibited the growth of *Rhodotorula graminis* Rg, *Rhodotorula mucilaginosa* Rm and *Schizosaccharomyces pombe* DSM 70576. The most pronounced effect of the reduction of cell proliferation by killer toxin preparations was found after 2 and 20 h cultivation. Further research should be done to determine the activity of *Pichia* killer toxin preparations under winemaking conditions. The obtained results may find the application in the food industry. The killer toxins could be used as selective tools to control infections during the fermentation of wine.

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Contact address:

Urszula Błaszczyk, University of Agriculture in Krakow, Faculty of Food Technology, Department of Fermentation Technology and Technical Microbiology, ul. Balicka 122, 30-149 Kraków, Poland, E-mail: ublaszczyk@ar.krakow.pl

Paweł Satora, University of Agriculture in Krakow, Faculty of Food Technology, Department of Fermentation Technology and Technical Microbiology, ul. Balicka 122, 30-149 Kraków, Poland, E-mail: p.satora@ur.krakow.pl

Paweł Sroka, University of Agriculture in Krakow, Faculty of Food Technology, Department of Fermentation Technology and Technical Microbiology, ul. Balicka 122, 30-149 Kraków, Poland, E-mail: psroka@ar.krakow.pl