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ANTIMICROBIAL ACTIVITY OF PULCHERRIMIN PIGMENT PRODUCED BY *METSCHNIKOWIA PULCHERRIMA* AGAINST VARIOUS YEAST SPECIES

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ABSTRACT

Metschnikowia pulcherrima is common yeast on grape berries and can grow on special cultivation media with iron (e.g. $FeSO_4.7H_2O$) (max. 0.02 g/L) to produce reddish (maroon) pigment pulcherrimin. Many studies confirm that pulcherrimin has antimicrobial activity against postharvest pathogens, microscopic fungi and other yeast species. In this study, two strains of *M. pulcherrima*, isolated from grapes and identified by MALDI-TOF mass spectrometry, were used : VML and CBS 610NT. Sixteen yeast species: *Aureobasidium* (1), *Candida* (5), *Hanseniaspora* (1), *Kregenvanrija* (1), *Pichia* (2), *Rhodotorula* (2), *Saccharomyces* (1), *Zygosaccharomyces* (2) and *Wickerhamomyces* (1) were selected to test the antimicrobial capacity of these two *M. pulcherrima* strains. The results showed CBS 610NT had a stronger ability than VML to inhibit the tested yeasts. Among the yeast, *Candida* spp. and *Pichia manshurica* were the most sensitive to the pigment pulcherrimin.

Keywords: *Metschnikowia pulcherrima*, antimicrobial activity, pulcherrimin, yeasts

INTRODUCTION

The natural habitats of *Metschnikowia pulcherrima* [anamorph *Candida pulcherrima*] strains are fully matured fruits, especially grapes. *Metschnikowia pulcherrima* is common yeast that can be found on the surface of grape berries and is generally presented during the first stages of grape juice fermentation. In addition, *Hanseniaspora uvarum*, *Aureobasidium pullulans*, *Rhodotorula* spp., *Cryptococcus* spp., *Candida* spp. and *Pichia* spp. belongs to the main population of wild yeasts on grapes. Some of these wild yeasts can produce specific compounds with a wide range of antimicrobial activity against different microorganisms (bacteria, yeasts, microscopic fungi). *Metschnikowia pulcherrima* can produce pulcherriminic acid as a precursor of pulcherrimin pigment (**Türkel *et al.*, 2009, Tay *et al.*, 2014, Oro *et al.*, 2014**). *Wickerhamomyces anomalus* (previously known as *Pichia anomala*) producing anti-*Candida* toxins have been reported in several studies (**Mathews *et al.*, 1998, Buzzini & Martini, 2001, Sawant *et al.*, 1989**). In addition, *Aureobasidium pullulans* produces a lactone compound (5-hydroxy-2-decenoicacidlactone) that inhibits various *Candida* species (**Tay *et al.*, 2014**). These yeasts belong to non-*Saccharomyces* (wild) yeasts group (**König *et al.*, 2009**; **Mills *et al.*, 2002; Prakitchaiwattana *et al.*, 2004**; **Combina *et al.*, 2005**). With the reevaluation of the role of non-*Saccharomyces* yeasts in winemaking and their potential use as selected starters in mixed fermentations with *Saccharomyces cerevisiae*, *M. pulcherrima* has been proposed as a co-fermentative species because of its release of aromatic volatile compounds and its enhancement of the overall aromatic profile of wines (**Comitini *et al.*, 2011; Zott *et al.*, 2011**). *M. pulcherrima* produces pulcherrimin, which is a reddish (maroon) pigment that accumulates in the growth medium and, clearly visible, around the *M. pulcherrima* colonies on plates (**Kluyver *et al.*, 1953**). Pulcherrimin forms a chelate complex with iron ions in the medium (**Kluyver *et al.*, 1953**). It has been recently shown that the antibacterial and antifungal activity of *M. pulcherrima* strains depends on the immobilization of iron by the pulcherrimin pigment in the growth medium **(Sipiczki, 2006**). Hence, the strains of *M. pulcherrima* that produce high amounts of pulcherrimin are of great interest for the inhibition of the growth of pathogenic bacteria, yeasts, and molds. The pigment pulcherrimin produced by different strains of *M. pulcherrima* can be a good alternative for topical applications in the prevention against certain bacterial, yeast and fungal infections in humans (**Türkel and Ener, 2009**).
The aim of this study was to confirm the antimicrobial activity of pulcherrimin pigment produced by *Metschnikowia pulcherrima* against various yeast species.

MATERIAL AND METHODS

Yeast strains

Yeasts were isolated from surface of grape berries (2015) (Blue Frankish, Cabernet Sauvignon, Moravian Muscat and Pinot Blanc), some species from tank wine samples (2014) (Blue Frankish, Blue Portugal, Cabernet Sauvignon, Pinot Blanc, Pinot Noir and Yellow Muscat) and one *Candida* species was isolated from the surface of banana (2015) (variety Cavendish). Spread plate method was used for isolation of yeasts and dilutions 10^{-2} - 10^{-3} (for wines) and 10^{-4} - 10^{-5} (for grapes) were used. In this study, two strains of *M. pulcherrima* were used for testing of antimicrobial activity of pulcherrimin pigment. These strains were isolated from the surface of grape berries in 2015. *Metschnikowia pulcherrima* CBS 610NT and VML strain were used. For testing antimicrobial activity of pulcherrimin pigment we used 16 yeast species in this study. Yeasts were isolated from grapes or wine, only *Candida palmioleophila* was isolated from banana surface. Yeasts were identified by MALDI-TOF mass spectrometry (Bruker Daltonics, Germany). Namely: *Aureobasidium pullulans* (grape), *Candida magnoliae* (grape), *Candida oleophila* (grape), *Candida palmioleophila* (banana), *Candida parapsilosis* (grape), *Candida saitoana* (grape), *Hanseniaspora uvarum* [anamorph *Kloeckera apiculata*] (grape), *Kregenvanrija fluxuum* [anamorph *Candida vini*] (wine), *Pichia manshurica* (wine), *Pichia membranifaciens* [anamorph *Candida valida*] (wine), *Rhodotorula glutinis* (grape), *Rhodotorula mucilaginosa* (grape) *Saccharomyces cerevisiae* (wine), , *Zygosaccharomyces bailii* (sweet wine), *Zygosaccharomyces florentinus* (wine), and *Wickerhamomyces anomalus* (wine). Table 1 shows the yeast species and their source of isolation.

MALDI-TOF Mass Spectrometry

MALDI-TOF Mass Spectrometry model Microflex LT/SH biotyper (Bruker Daltonics, Germany, Bremen) was used for identification of yeasts isolated from different grape, banana and wine samples. After incubation (25°C for 3-5 days), isolated colonies were picked up form cultivation media (MEA) and suspended in 300 µL of sterile distilled water and mixed thoroughly. 900 µL of absolute ethanol (99%, Sigma-Aldrich, USA) was added. The mixture was centrifuged at 13 000 × g for 2 min. After that, supernatant was discarded, and pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet was allowed to dry at a room temperature. Subsequently 10 µL of formic acid (70%, Sigma-Aldrich, USA) was added and mixed with the pellet with a sterile toothpick. Next, 10 µL of acetonitrile (100%, Sigma-Aldrich, USA) was added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 minutes again, and 1 µL of the supernatant was spotted on a polished MALDI target plate (Bruker Daltonics, Germany). Immediately after drying 1 µL of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of HCCA: α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany) dissolved in 50% acetonitrile with 0.025% trifluoroacetic acid (TFA) (100%, Sigma-Aldrich, USA). The matrix solution preparation (2.5 mg of HCCA) contains 500 µL of acetonitrile, 475 µL of ultra-pure water and 25 µL of trifluoroacetic acid. Next added 250 µL of this solution to the 2.5 mg of HCCA. Samples were then processed in the MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics, Germany, Bremen) with flex Control software v3.4 and results obtained with Realtime Classification software (RTC) v3.1 (Bruker Daltonics, Germany). Each spectrum was obtained by averaging 240 laser shots acquired in the automatic mode at the minimum laser power necessary for ionization of the samples. The spectra have been analyzed in an m/z range of 0.7 to 20 kDa (**Marklein *et al.*, 2009; Pavlovic *et al.*, 2014; van Veen *et al.*, 2010**).

Cultivation media and pulcherrimin production

Yeast cultivated on Malt extract agar base (MEA) (BioMark®, India) supplemented with glucose (20 g/L) (Centralchem®, Slovakia), yeast extract (3 g/L) (Conda, Spain) and bromocresol green (0.020 g/L) (Centralchem®, Slovakia). After cultivation at 25°C for 5 days, small amount of yeasts (10^5 cells mL) were transferred with inoculation loop into sterile plastic tube filled with 9 mL of Yeast peptone dextrose broth contains: 20 g/L peptone bacteriological, 20 g/L glucose and 10 g/L yeast extract (YPD, Sigma-Aldrich, USA) for each yeast separately (also *M. pulcherrima*). Inoculated tubes was transferred to the Stuart SB2 tube rotator (Stuart, UK) and cultivated in constant speed of 20 *rpm* at room temperature (25±2°C) for 24h. Tested yeast strains were spread with sterile cotton swabs on the surface of Czapek Dox agar without sucrose supplemented with glucose and yeast extract. Cultivation medium contains: ($NaNO_3$ 2 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L, KCl 0.5 g/L, $FeSO_4.7H_2O$, 0.010 g/L, glucose 10 g/L, yeast extract 5 g/L, agar purified 15 g/L). All chemicals from Slovak company Centralchem®, yeast extract and agar purified (Conda, Spain). After that, 4 µL of *Metschnikowia pulcherrima* suspension was spotted 3 times on the surface of cultivation medium. Cultivation conditions were follows 48h at 30°C. Inhibition zones of pulcherrimin pigment were expressed in millimeter. *Metschnikowia pulcherrima* produce reddish (maroon) pigment into the medium (extracellular production). Antimicrobial activity of pulcherrimin pigment produced by *M. pulcherrima* against various yeasts was evaluated through modified killer-test plate assay described by **Rosini** (**1985**).

RESULTS AND DISCUSSION

In this study, 16 grapes and wine yeasts were used for testing of antimicrobial activity of pulcherrimin pigment produced by two *M. pulcherrima* strains, also isolated from grape surface. After cultivation conditions, the inhibition zones was measured manually with ruler and expressed in mm. Table 2 shows the results from this experiment. As you can see *Aureobasidium pullulans*, *Hanseniaspora uvarum*, *Pichia membranifaciens*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* were resistant against pulcherrimin pigment, because overgrown the inhibition zones. In other hand, *Candida* species were the most sensitive species against pulcherrimin pigment. *Metschnikowia pulcherrima* CBS 610NT produced 2 mm inhibition zones, but *M. pulcherrima* VML only 1 mm inhibition halo against *Candida* species. Table 1 also shows that *M. pulcherrima* strain CBS 610NT had stronger antimicrobial effect than the strain VML. The maximum range of inhibition zones were only 2 mm in this study. Inhibition zones in *M. pulcherrima* strain VML were showed only on four yeasts: *Candida palmioleophila*, *Candida parapsilosis*, *Candida saitoana* (figure 1) and *Zygosaccharomyces florentinus*. In study **Oro *et al*. (2014),** they examined the antagonistic behavior of *M. pulcherrima* against wine-related yeast species involved in the winemaking process with the aim to investigate yeast interactions in controlled mixed wine fermentation. They used also two (from seven) *M. pulcherrima* strains for testing antimicrobial activity of pulcherrimin pigment against 114 yeast species belongs to genera *Pichia*, *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Saccharomycodes*, *Torulaspora*, *Brettanomyces* and *Saccharomyces*. The results of (**Oro *et al.*, 2014**), the genera of yeasts *Hanseniaspora*, *Candida* and *Pichia* showed very high sensitivity towards all the tested *M. pulcherrima* strains. *S. cerevisiae* had no sensitivity against *M. pulcherrima* strains as in our study. The antimicrobial activity of *M. pulcherrima* could be associated with this secretion of pulcherriminic acid, which forms a reddish pigment and halos around the colonies. It is linked to the iron ions accessible in the medium as previously reported **Sipiczki (2006)** and **Türkel and Ener (2009)**. These pigmented halos contain a water-insoluble complex of ferric ions and pulcherriminic acid (**Cook and Slater, 1956; Kluyver *et al.*, 1953**). The pigment, pulcherrimin, is synthesized from $_L$-leucine through cyclo-$_L$-leucyl-$_L$-leucyl and pulcherriminic acid (**MacDonald, 1965**). The pulcherrimic acid-ferric ion complex formed in the halos depletes the iron in the substrate and creates an environment unsuitable for growth of microbes that require more iron for growth (**Sipiczki *et al.*, 2006**). However when iron is added at higher concentrations (0.02 g/L; five to tenfold the iron concentration present in grape juice) the inhibition activity of *M. pulcherrima* disappears and the colonies take a more intense brown color (production of intracellular pulcherrimin). Likewise, in mixed fermentation trial, the iron supplementation determined the loss of antimicrobial activity by *M. pulcherrima* (**Oro *et al.*, 2014**). On the other hand, the presence of *M. pulcherrima* mixed with *S. cerevisiae* in wine fermentation from the first days confirms the results of previous works (**Sadoudi *et al.*, 2012; Milanović *et al.*, 2013**). This zero effect of *Metschnikowia pulcherrima* on *Saccharomyces cerevisiae* seems to be related to the mechanism of action of pulcherriminic acid. Indeed, the lack of antimicrobial activity against *S. cerevisiae* could be due to the presence of AFT1 transcriptional factors that increase the level of expression of iron regulon genes (**Hamza and Baetz 2012; Holmes-Hampton *et al.*, 2013**). In this context, also on the basis of the recent studies (**Comitini *et al.*, 2011; Zott *et al.*, 2011**), *M. pulcherrima* could be used in mixed fermentation with *S. cerevisiae* for improve the aromatic profile of wine and counteract against spoilage wine microorganisms. This biotechnological approach could be a suitable strategy in the wine industry.

Table 1 List of yeast species with source of isolation

Yeast species	Source*	Variety
Aureobasidium pullulans	grape	Blue Frankish Moravian Muscat
Candida magnoliae	grape	Moravian Muscat
Candida oleophila	grape	Pinot Blanc
Candida palmioleophila	banana	Cavendish
Candida parapsilosis	grape	Blue Frankish
Candida saitoana	grape	Pinot Blanc
Hanseniaspora uvarum	grape	Blue Frankish Cabernet Sauvignon Moravian Muscat Pinot Blanc
Kregenvanrija fluxuum	wine	Blue Frankish
Pichia manshurica	wine	Blue Portugal Yellow Muscat
Pichia membranifaciens	wine	Pinot Noir
Rhodotorula glutinis	grape	Cabernet Sauvignon Blue Frankish
Rhodotorula mucilaginosa	grape	Moravian Muscat Cabernet Sauvignon
Saccharomyces cerevisiae	wine	Blue Frankish Cabernet Sauvignon
Zygosaccharomyces bailii	sweet wine	Yellow Muscat
Zygosaccharomyces florentinus	wine	Pinot Blanc
Wickerhamomyces anomalus	wine	Pinot Noir

*Source of isolation

Table 2 Antagonistic effects of *M. pulcherrima* strains on various yeasts.

	Inhibition zone [mm]		
Yeast species	**VML**	**CBS610NT**	**comment**
Aureobasidium pullulans	NZ	NZ	overgrown
Candida magnoliae	NZ	1.00	
Candida oleophila	NZ	2.00	
Candida palmioleophila	1.00	2.00	
Candida parapsilosis	1.00	2.00	
Candida saitoana	1.00	2.00	
Hanseniaspora uvarum	NZ	NZ	overgrown
Kregenvanrija fluxuum	NZ	1.00	
Pichia manshurica	NZ	2.00	
Pichia membranifaciens	NZ	NZ	overgrown
Rhodotorula glutinis	NZ	1.00	
Rhodotorula mucilaginosa	NZ	NZ	overgrown
Saccharomyces cerevisiae	NZ	NZ	overgrown
Zygosaccharomyces bailii	NZ	1.00	
Zygosaccharomyces florentinus	1.00	1.00	
Wickerhamomyces anomalus	NZ	NZ	overgrown

NZ: no zone of inhibition

Sipiczki (**2006**) reported that exogenous $FeCl_3$ concentrations greater than 20 µg/mL no red halos appeared, however yeast colonies were dark red, suggesting that all of the pigment was retained in the cells. In Study **Sipiczki *et al.*** (**2006**) the reduction in halo size observed when the medium was supplemented with $FeCl_3$ suggests that the cells do not secrete the pigment but instead secrete a soluble, diffusible precursor that forms the pigment in the medium when it encounters iron. At low iron concentrations the precursor diffuses further from the yeast colony before it is immobilized by iron, resulting in a wider but paler halo. At higher iron concentrations the halos are smaller because the precursor molecules do not diffuse as far before they bind sufficient iron for pigment production. Figure 2 shows the intracellular and extracellular pulcherrimin produced by *Metschnikowia pulcherrima* yeast. Extracellular production of pulcherrimin was on modified Czapek Dox agar supplemented with $FeSO_4.7H_2O$, and intracellular production of pigment was on Wort agar (HiMedia®, India). As you can see the intracellular pigment was found under the colony where was accumulated in the cells. *Hanseniaspora uvarum* was represented the other white colonies on Wort agar.

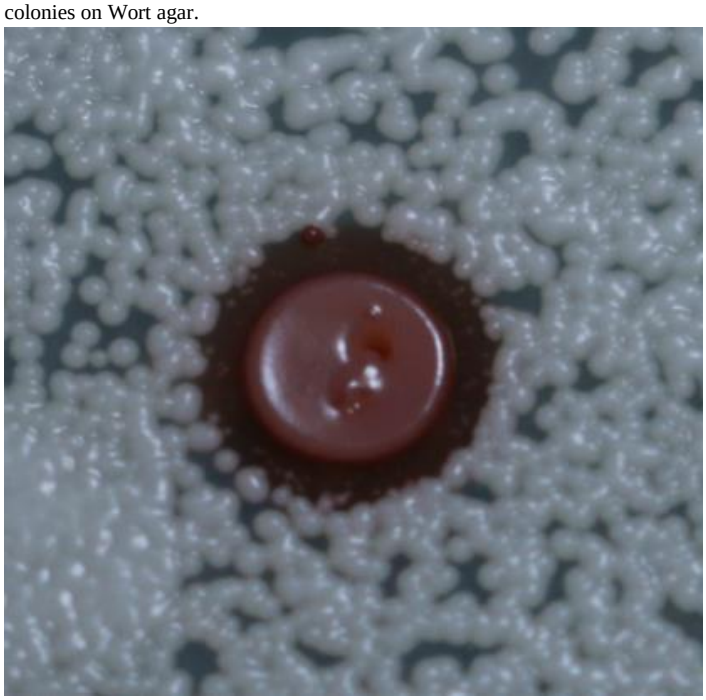

Figure 1 Inhibition zone of pulcherrimin on *Candida saitoana* (Kántor, 2015)

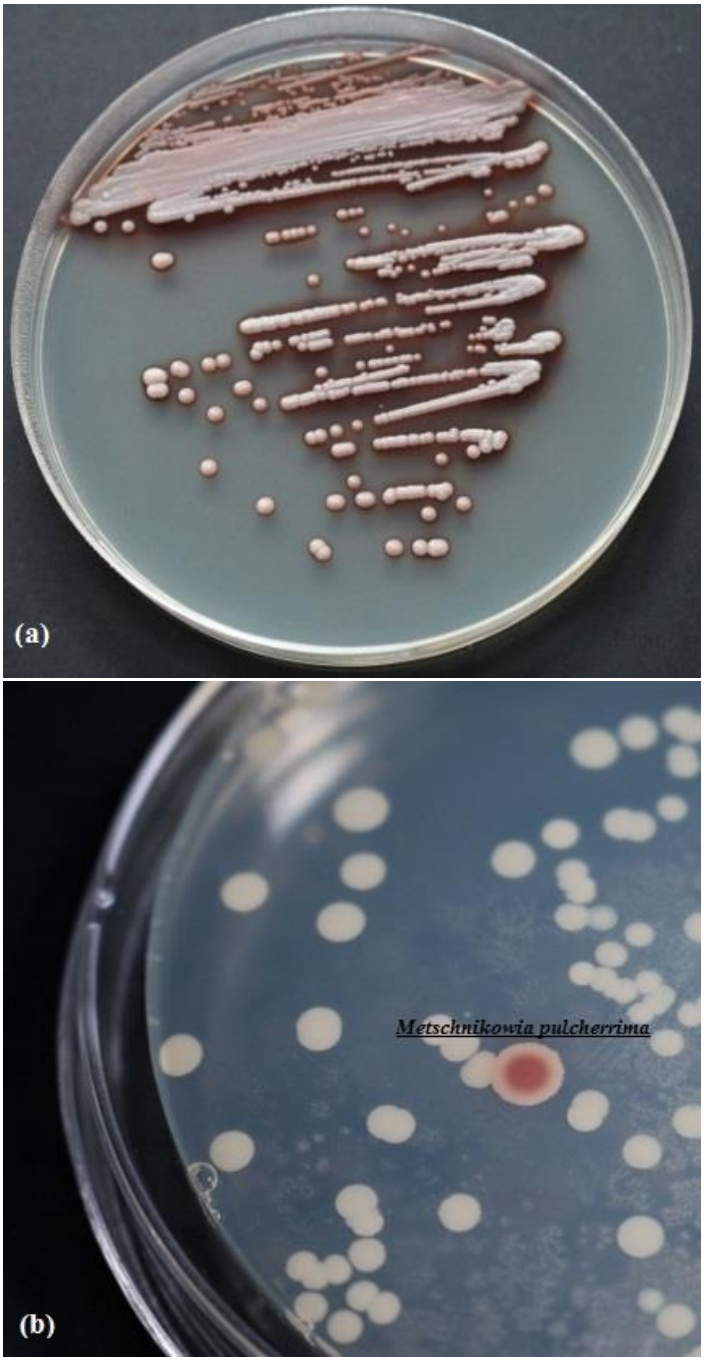

Figure 2 Extracellular (**a**) and intracellular (**b**) production of pulcherrimin by *Metschnikowia pulcherrima* (Kántor, 2015)

CONCLUSION

Metschnikowia pulcherrima has antimicrobial activity against some yeast species in this study. We tested 16 yeast species against pulcherrimin pigment produced by *M. pulcherrima*. The strongest antimicrobial activity of pulcherrimin was against *Candida* species and *Pichia manshurica*. We used two *M. pulcherrima* CBS 610NT strains and strain VML, which were very different in pulcherrimin production. The difference in the production of pulcherrimin into the cultivation medium was visible by inhibition zones. *M. pulcherrima* CBS 610NT produced more pulcherrimin into the medium than the second strain [VML] and had stronger effect on various yeasts too. The maximum range of inhibition zones was only 2 mm in five yeast species. Pulcherrimin had no effect on some yeasts, e.g. *S. cerevisiae*, which may be useful in wine production co-fermentation process.

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