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ENZYMATIC PROFILES AND ANTIMICROBIAL ACTIVITY OF THE YEAST *METSCHNIKOWIA PULCHERRIMA*

Abstract
The aim of the study was to characterize the antimicrobial properties of 5 strains belonging to the yeast species *Metschnikowia pulcherrima*. The antimicrobial activity of the strains was studied by observing pulcherrimin production and inhibition of microbial growth on YPG plates. Enzymatic assays were carried out using API ZYM tests. All strains of *M. pulcherrima* showed α-glucosidase and leucine arylamidase activities. The widest spectrum of activity was observed for strains NCYC2321, CCY145, and CCY149. All tested strains produced pucherrimin, and the yeasts *Wickerhamomyces anomalus* and *Dekkera bruxellensis*, the bacterium *Bacillus subtilis* and the moulds *Penicillium chrysogenum* and *Aspergillus brasiliensis* were the most sensitive to *M. pulcherrima*.

Key words
Metschnikowia pulcherrima, pulcherrimin, enzymatic activity, antagonism.

Introduction

As the world's population continues to grow, the global demand for food will also continue to increase. The ability to feed everyone depends upon the increase in food production and the length of storage capacity. The most popular way to prolong food usefulness is to use artificial chemicals such as pesticides. However, many of these substances are toxic to humans and are very harmful to the environment [1]. Due to the growing awareness of public opinion regarding the harmful effects to health and the environment, as well as the increasing resistance to pathogens, scientists have been interested in natural alternatives for a long time. One of these methods is the use of microorganisms that show the ability to inhibit the growth of various spoilage microorganisms, such as yeasts (*Candida* sp., *Cryptococcus* sp., *Kloeckera* sp., *Wickerhamomyces* sp., *Dekkera* sp.), bacteria (*Bacillus* sp., *Pseudomonas* sp.) or fungi (*Aspergillus* sp., *Penicillium* sp., *Fusarium* sp.) [2, 3].

The presence of spoilage microbiota may lead to significant reduction in the efficiency of any biotechnological process such as: production of wine, beer and bread, and the induction of secondary metabolite production. The interactions between various microorganisms have been described in a number of scientific studies [4, 5, 6]. The yeasts that are characterized by antagonistic activity against microbial contaminations include the genera *Pichia, Candida, Aureobasidium, Metschnikowia*, and *Debaryomyces*. The presence of microbial contamination not only reduces nutrients availability for industrial microorganisms, but also reduces environmental succession [7]. The low nutrient availability is one of the most important mechanisms of competition between microbial strains. However, the antagonistic properties of yeasts can also be attributed to the changes in the pH values elicited by the production of organic acids, production and tolerance to high concentrations of ethanol, and the secretion of extracellular antimicrobial compounds, for example, killer toxins or enzymes [2, 8].

The yeasts *Metchnikowia* spp. are very interesting microbial models because they occur as members of the natural microbiota of flowers, fruits and insects [9]. The antagonistic properties of these yeasts have been described in the literature [10, 11, 12, 13], and especially *M. pulcherrima* strains show a great potential to become efficient biological control agents of natural origin against a broad spectrum of saprophytic and pathogenic microorganisms. It is well-documented [8, 14, 15] that *Metschnikowia* spp. are antagonistic to various fungi and bacteria. The possible mechanisms of action involved in the antagonistic activities of those yeasts are: (a) antibiosis, whereby the yeast cells produce antimicrobial compounds, (b) competition, when habitat or nutrients (i.e. source of carbon, nitrogen, as well as microelements) are limiting factors, (c) other mechanisms, *e.g.* by which *Metchnikowia* spp. attack other microbial cells including the secretion of lytic

enzymes (proteases, glucanases and chitinases) that enable them to degrade the cells and utilize their biopolymers as nutrients [8, 14].

The inhibitory activities of *M. pulcherrima* strains have been confirmed mainly for moulds: *e.g. Penicillium* spp., *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp. and *Botrytis cinerea* [14, 15, 16]. It has been also demonstrated that *M. pulcherrima* showed antimicrobial activity against numerous yeasts belonging to the genera *Pichia*, *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Saccharomycodes*, *Torulaspora* and *Brettanomyces* [12]. Interestingly, the antimicrobial activity of *M. pulcherrima* did not affect the growth of *Saccharomyces cerevisiae* at all [17]. It has been shown that the antimicrobial activity of *M. pulcherrima* depends on the formation of pulcherrimin [18] and, therefore, strains that produce large amounts of pulcherrimic acid are of great interest to scientists, and can be considered as potential natural agents to control the growth of the spoilage microbiota [11, 19].

The aim of this study was to investigate the enzyme productions of 5 strains belonging to *M. pulcherrima*, which were obtained from two European collections – the National Collection of Yeast Cultures (NCYC, UK) and the Culture Collection of Yeasts (CCY, Slovakia). Enzyme assays were carried out using a commercial API ZYM test (bioMérieux). In addition, the antimicrobial activity of the yeast strains was studied by estimating (1) pulcherrimin production *via* the formation of pulcherrimin acid and Fe^{3+} complexes, as well as (2) inhibition of microbial (yeasts, bacteria, moulds) growths on YPG plates.

Materials and methods

Microorganisms

All mould and yeast strains were grown on YPG agar at 25°C. Bacterial strains of *Bacillus* sp. and *Pseudomonas* sp. were cultivated on nutrient agar at 30-37°C. Acetic acid bacterium belonging to *Asaia* sp. were cultivated on GC agar at 25°C [20].

The microorganisms tested in this study are presented in Table 1. These strains were isolated as members of spoilage microbiota present in different food products.

Table 1. The microorganisms used in the study.

Group	Name	Origin
Yeasts	*Metschnikowia pulcherrima* CCY145	The Culture Collection of Yeasts (Slovakia)
	Metschnikowia pulcherrima CCY147	
	Metschnikowia pulcherrima CCY149	
	Metschnikowia pulcherrima NCYC2321	National Collection of Yeast Cultures (UK)
	Metschnikowia pulcherrima NCYC747	
	Wickerhamomyces anomalus C1	Isolated as a spoilage yeast of soft drinks [21]
	Dekkera bruxellensis C2	
Bacteria	*Bacillus subtilis* LOCK0816	Collection of Industrial Microorganisms LOCK 105 (Poland)
	Pseudomonas aeruginosa LOCK0885	
	Asaia bogorensis ISD1	Isolated as a spoilage bacterium of soft drinks [22]
Moulds	*Penicillium chrysogenum* LOCK0531	Collection of Industrial Microorganisms LOCK 105 (Poland)
	Aspergillus brasiliensis LOCK0436	

Source: Author's

Testing pulcherrimin production

To compare the pigment productions by *M. pulcherrima* colonies growing on agar plates, yeast cells were streaked on YPG agar plates supplemented with $FeCl_3$ (0.015mg/ml), and the widths of the reddish halos around the yeast colonies were observed after 5 days of incubation at 25°C [23].

Testing antagonism

For antagonistic activity tests, 200µL aliquots of each yeast, bacterium and mould suspensions (4°McF) was spread evenly on YPG agar plates. When the surface of plates dried, *M. pulcherrimin* cells were streaked on agar plates, and then the plates were incubated at 25°C for 2-5 days due to the various growth rates of the

bacterium, yeast and mould strains (Table 1). The antagonism was quantified by the diameters of the zones of no growth around the *M. pulcherrima* CCY145 culture (mm) [12, 23].

Enzyme assays

The soluble enzyme activity in yeasts suspension samples was determined by the API ZYM kit (bioMérieux), which is commercially available for the semi-quantitative analysis of hydrolytic enzyme production. Each strip is composed of 20 microwells containing dehydrated chromogenic substrates for 19 enzymatic reactions and a control. Microwells contain a buffer with a specific optimum pH value for each enzyme activity. Yeast cell suspensions (5-6°McF; 65 µl aliquots) were dispensed into the 20 microwells. Sterile water was added into a plastic outer cover to create a humidity chamber. The API ZYM strips were covered and incubated at 37°C for 4 h according to the instructions provided by the manufacturer. After that, one-one drops (30 µl each) of the commercial reagents ZYM A and ZYM B (bioMérieux) were added to all microwells to develop the colours of the reaction mixtures. The colour reactions were read after 5 minutes, and a digit ranging from 0 to 5 was assigned to each sample following the colour chart provided by the manufacturer.

Results

Enzyme activities of the yeast *Metschnikowia pulcherrima*

Enzyme activities of the tested yeast strains are presented in Table 2.

Table 2. Enzyme activities of *M. pulcherrima* strains

Enzyme→ Strain ↓	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
NCYC 2321	0	0	3	2	0	4	1	0	0	0	3	3	0	0	0	5	2	0	0	0
NCYC 747	0	0	2	2	0	4	1	0	0	0	1	3	0	0	0	5	2	0	0	0
CCY 145	0	0	2	1	0	4	1	0	1	0	3	4	0	0	0	4	0	0	0	0
CCY 147	0	0	1	1	0	4	0	0	0	0	2	2	0	0	0	5	1	0	0	0
CCY 149	0	2	3	3	0	3	1	0	0	0	1	2	0	0	0	5	2	0	0	0

1: Control, 2: Alkaline phosphatase, 3: Esterase (C4), 4: Esterase lipase (C8), 5: Lipase (C14), 6: Leucine arylamidase, 7: Valine arylamidase, 8: Cystine arylamidase, 9: Trypsin, 10: α-chymotrypsin, 11: Acid phosphatase, 12: Naphtol-AS-BI-phosphohydrolase, 13: α-galactosidase, 14: β- galactosidase, 15: β-glucuronidase, 16: α-glucosidase, 17: β-glucosidase, 18: N-acetyl-β-glucoaminidase, 19: α-mannosidase, 20: α-fucosidase

Source: Author's

All tested *M. pulcherrima* strains were characterized by high α-glucosidase (value 4-5) and leucine arylamidase activities (value 3-4). Some of the tested strains also showed significant esterase (C4), esterase lipase, acid phosphatase, naphtol-AS-BI-phosphohydrolase and β-glucosidase activities. The widest spectrum of hydrolytic enzyme activities was observed with the strains NCYC2321, CCY145, and CCY149.

Pulcherrimin production

All tested strains of *M. pulcherrima* formed reddish pigment on YPG medium when supplemented with Fe^{3+} ions (Figure 1).

Fig. 1. Production of red pigment by the tested *Metschnikowia pulcherrima* strains in YPG agar medium supplemented with Fe^{3+} ions (0,015mg/ml). A *Wickerhamomyces anomalus* strain without pigment production was used as negative control.
Source: Author's

Inhibition of microbial growth

All tested *Metschnikowia* strains inhibited the growth of the Gram-positive bacterium *Bacillus subtilis*, the yeasts *Dekkera bruxellensis* and *Wickerhamomyces anomalus*, and the moulds *Penicillium chrysogenum* and *Aspergillus brasiliensis* (Figure 2). The widths of growth inhibition zones noted around the tested *M. pulcherrima* strains varied by the tested species (Table 3). In the case of the bacterium *Pseudomonas aeruginosa* and *Asaia bogorensis* no inhibitory effects of *M. pulcherrima* were observed.

Table 3. Inhibition of microbial growth by *M. pulcherrima* CCY145

Microorganisms tested	Zone of inhibition after 48-120 h incubation (mm)
Wickerhamomyces anomalus	7
Dekkera bruxellensis	4
Bacillus subtilis LOCK0816	4
Pseudomonas aeruginosa LOCK0885	0
Asaia bogorensis ISD1	0
Penicillium chrysogenum LOCK0531	3
Aspergillus brasiliensis LOCK0436	1

Inhibition zones were measured as the distance from the edge of *M. pulcherrima* colonies to the beginning of the microorganism colonies on YPG agar plates at 48-120 h of incubation depending on the species.
Source: Author's

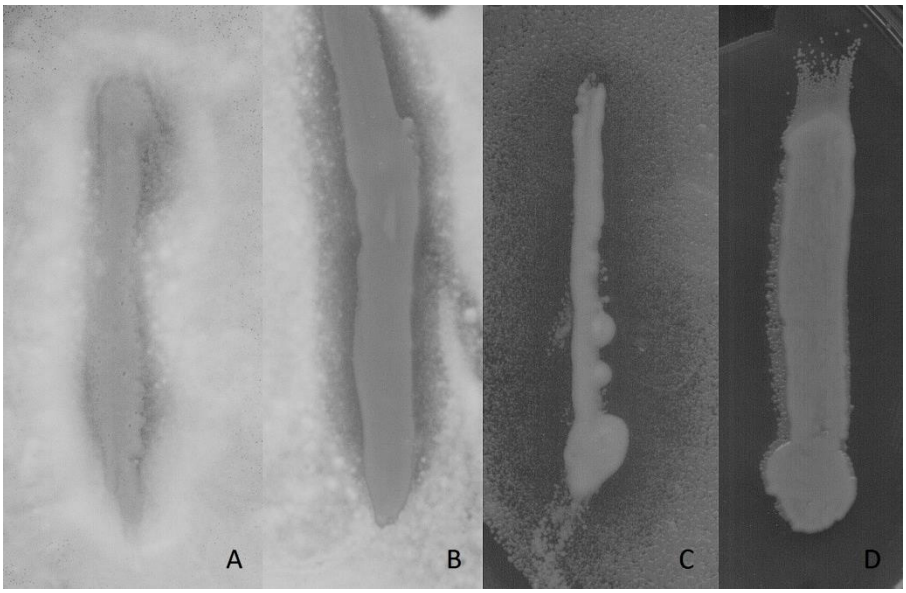

Fig. 2. The inhibition of microbial growths by a *M. pulcherrima* strain (CCY147) as shown by the dark zones of no growth around the yeast colonies. A - *Aspergillus brasiliensis,* B – *Penicillium chrysogenum*, C - *Wickerhamomyces anomalus*, D - *Bacillus subtilis*.
Source: Author's

Discussion

In industrialized countries, organic agriculture is based on clearly defined natural methods, and this offers many environmental benefits to the users. Artificial pesticides can pollute groundwater, disrupt key ecological processes such as pollination, threaten beneficial microorganisms, and represent serious health hazards to humans. In contrast, organic agriculture sets out to enhance biodiversity and restore the natural ecological balance. It encourages both spatial and temporal biodiversity through intercropping and crop rotations, conserves soil and water resources, and builds soil organic matter and biological processes. Livestock and diseases are kept at bay by crop associations, symbiotic combinations, and other nonchemical methods. In this context, the possibility of application of the antagonistic yeasts against undesirable spoilage microorganisms is the subject of interest for both scientists and technologists [1, 24].

The biological control of plant pathogens with antagonistic microorganisms is an efficient alternative to synthetic fungicides in reducing postharvest diseases and product loss. Yeasts are considered among the most promising antagonists in such biocontrol strategies [25]. Some of these microorganisms with Generally Recognized As Safe (GRAS) status and without special nutrient requirements can colonize both surfaces of fruits and vegetables. They are resistant to temperature changes, dryness, and sunlight [8], and are cheap for both small scale and large scale productions [26].

It is known that strains of the yeast *M. pulcherrima* have a great potential to become a leading natural and biological control agent against a broad spectrum of spoilage microorganisms [14]. In addition, *Metschnikowia* spp. with a wide temperature tolerance do not produce either allergic spores or harmful mycotoxins, thus they can be included in commercial preparations to protect fruits and vegetables both before and after harvesting [8].

In the literature, the researchers pointed to the possibility of the production of killer toxins by *M. pulcherrima* [27, 28], however those reports have been verified, current results indicated that the antibacterial activities of *M. pulcherrima* are associated with changes in the extracellular pH values and not with the biosynthesis of killer toxins. In the case of yeasts and moulds, the antagonism with *M. pulcherrima* is attributed to pulcherrimic acid secretion, which accumulates in the growth medium. This organic acid captures Fe^{3+} ions that are required for the proper growths of spoilage microorganisms. In fact, the production of acidic compounds (including pulcherrimic acid) decreases the pH of the environment and, as a result, the acidic pH together with the extraction of iron can inhibit the growth of other microbial cells [12]. Not surprisingly, recent publications in

this field suggest that the antibacterial and antifungal activities of the yeast *M. pulcherrima* depend clearly on pulcherrimin production [14, 29].

As expected, the *M. pulcherrima* strains tested in this study effectively inhibited the growths of various spoilage microorganisms, including various moulds (*P. chrysogenum, A. brasiliensis*), yeasts (*D. bruxellensis, W. anomalus*) and also the bacterium *B. subtilis*. These microorganisms often spoil agroproducts, food, and fermentation products [3, 23, 30]. Interestingly, studies conducted by Csutak et al. suggested that wild-type strains of *M. pulcherrima*, isolated directly from fruit microbiota, exhibited higher antagonistic activity when compared to those of the strains bought from culture collections. This observation may be the consequence of the adaptation of the strains to the maintenance and growth conditions employed in the strain collections [10].

The tested *M. pulcherrima* strains showed antagonistic activity against the yeast strains belonging to *W. anomalus* and *D. bruxellensis,* probably owing to their Fe^{3+} binding capabilities. Obviously, satisfactory iron supplementation is necessary for the proper growth of wild-type yeasts. According to pervious literature data [31, 32], *W. anomalus* can grow under extreme environmental stress conditions, *e.g.* at low and high pH values, low water activity, high osmotic pressure and also under anaerobic conditions. As a consequence, *W. anomalus* is a common member of the spoilage microbiota, *e.g.* in fruits and high-sugar food products [32]. *D. bruxellensis* is a common spoilage yeast too, occurring in fermented products. This yeast tolerates well high ethanol concentration, low pH value, and nitrogen starvation. *D. bruxellensis* is a facultative anaerobic yeast, which may be predominant under environmental conditions unfavourable for other microorganism [33]. *M. pulcherrima* has been showed to possess antimicrobial activity against a wide spectrum of yeasts. For example, Oro et al. demonstrated the antimicrobial activity of *M. pulcherrima* against *Pichia* spp., *Candida* spp. and *Brettanomyces* spp. [12].

All tested strains of *M. pulcherrima* inhibited the growth of the Gram-positive bacterium *B. subtilis*. This bacterium belongs to the typical spoilage microbiota observed in various environments, including fruits, vegetables or even fermentation media [3, 34, 35]. Inhibitory effect of *M. pulcherrima* was also recorded for the moulds *P. chrysogenum* and *A. brasiliensis* [14]. These moulds are highly aerobic and they are found in almost all oxygen-rich environments [36]. Generally, these fungi grow on carbon-rich substrates like monosaccharides (glucose) and polysaccharides (starch) and, therefore, these species are common contaminants on foods [37]. Members of these genera possess the ability to grow under high osmolarity (high sugar, salt, etc.) conditions [38]. Black aspergilli are widespread in vineyards, cause rots on berries, and are also the main sources of ochratoxin A contaminations [25].

Our experimental data demonstrated that the tested strains of *M. pulcherrima* were able to produce different hydrolytic enzymes, and these enzyme activities may be useful in biocontrol processes. The available literature shows that these strains can secrete lytic enzymes, which hydrolyze a variety of polymeric compounds (chitin, proteins) [14].

Conclusions

In our study, we recorded a broad spectrum of antibacterial and antifungal activities for the yeast *M. pulcherrima*. The data presented here demonstrated a high potential for some *M. pulcherrima* strains to become biocontrol agents, which can be employed in various environments against many spoilage bacteria, yeasts and moulds.

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