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A New Approach to Producing High Yields of Pulcherrimin from *Metschnikowia* Yeasts

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Abstract: Pulcherrimin, a red iron chelate, is produced by some yeasts and bacteria. It plays important ecological roles in many ecosystems, including growth control, biofilm inhibition and photoprotection. In this study, fifteen yeast strains of the genus *Metschnikowia* were characterized based on their production of pulcherrimin. Yeast pulcherrimin was isolated and its purity assessed using ^{1}H nuclear magnetic resonance spectroscopy. Under experimental conditions, pulcherrimin formation varied depending on both the tested strains and culture media. The best producers formed up to 240 mg/L of pulcherrimin in minimal medium with glucose as the carbon source, supplemented with 0.05% $FeCl_3$ and 0.1% Tween 80. This study presents a new approach to producing high yields of pulcherrimin from yeasts.

Keywords: yeast; *Metschnikowia*; pulcherrimin formation; identification

1. Introduction

Pulcherrimin (1,4-dihydroxy-3,6-bis(2-methylpropyl)pyrazine-1,4-diium-2,5-diol;iron) is a ferric chelate that may be synthesized by some yeast species of the genera *Metschnikowia*, *Lipomyces*, *Kluyveromyces*, and *Dipodascopsis* as well as by some bacteria of the genus *Bacillus* and *Streptomyces* [1–6]. Pulcherrimin depletes the free iron in the environment; therefore, pulcherrimin producers including *Metschnikowia* yeasts show strong antimicrobial activity. The antagonistic abilities of *Metschnikowia* strains have been found to be clearly correlated with the production of pulcherrimin [7]. According to Gore-Lloyd and co-workers, pigmentless *M. pulcherrima* mutants are less antagonistic than wild strains that produce more pulcherrimin [8]. The same authors identified the *METSCH-Snf2* coding gene as responsible for not only the red pigmentation of the yeast, but also its strong antifungal activity. Pulcherrimin-producing *Metschnikowia* strains have the characteristics of an "ideal antagonist", such as the ability to survive under adverse environmental conditions and compete for nutrients and space. In wine fermentation, *Metschnikowia* strains modulate the population dynamics of the fermenting microbiota and improve the aromatic character of the final products [9–11]. These yeasts show antimicrobial action on undesired wild spoilage yeasts, such as *Brettanomyces/Dekkera*, *Hanseniaspora* and *Pichia* genera. Interestingly, the antimicrobial activity of *Metschnikowia* spp. does not have any influence on the growth of *Saccharomyces cerevisiae* [7,12,13]. Therefore, selected strains may be used in controlled multi-starter fermentations with *S. cerevisiae* [10,12,14].

Metschnikowia yeasts are also known to inhibit the growth of molds, including phytopathogens [15–19]. Some yeast strains of the genus *Metschnikowia* have been recognized as plant protection agents against

fungal diseases by the European Food Safety Authority (EFSA) [20]. Preharvest application of these yeasts is becoming increasingly popular, since they colonize fruit surfaces and blemishes prior to the establishment of postharvest pathogens [21–23].

Pulcherrimin also seems to be an effective agent with other important ecological roles. Pulcherrimin formation can control the growth of bacterial biofilms [24]. Jayalakshmi and co-workers report that pulcherrimin has antioxidant properties [25]. According to Pourzand and co-workers, pulcherrimin shows significant photoprotection against UVA-induced damage and cell death [26]. Moreover, due to its inhibition of urease activity, pulcherrimin can be used in agriculture as a component of fertilizers with controlled availability [27]. Microorganisms that produce large quantities of pulcherrimin are therefore of great interest to biotechnologists.

The aim of our study was to evaluate pulcherrimin production by yeasts of the genus *Metschnikowia*. This work is the first paper to describe the ability of *Metschnikowia* yeasts to produce pulcherrimin in different culture media. The results could provide the basis for further work to optimize the conditions of pulcherrimin formation by yeast strains.

2. Materials and Methods

2.1. Yeast Strains

Fifteen strains of the *Metschnikowia* clade were used in this study. Five strains of *M. pulcherrima* were obtained from the National Collection of Yeast Cultures (Quadram Institute Bioscience, Norwich, United Kingdom) and the Culture Collection of Yeasts (Slovak Academy of Sciences, Bratislava, Slovakia). In addition, ten yeast strains isolated from Polish fruits and deposited in the LOCK Culture Collection (Lodz University of Technology, Lodz, Poland) were tested [18] (Table 1).

Table 1. Yeast strains used in the study.

No	Strain	Source
1	*M. pulcherrima* NCYC 747	NCYC Collection
2	*M. pulcherrima* NCYC 2321	
3	*M. pulcherrima* CCY 29-2-145	CCY Collection
4	*M. pulcherrima* CCY 29-2-147	
5	*M. pulcherrima* CCY 29-2-149	
6	*M. sinensis* LOCK 1135 – D1	LOCK Collection
7	*M. andauensis* LOCK 1136 – D2	
8	*M. sinensis* LOCK 1137 – D3	
9	*M. andauensis* LOCK 1138 – D4	
10	*M. andauensis* LOCK 1139 – D5	
11	*M. andauensis* LOCK 1140 – D6	
12	*M. andauensis* LOCK 1141 – D7	
13	*M. andauensis* LOCK 1142 – D8	
14	*M. sinensis* LOCK 1143 – D9	
15	*M. sinensis* LOCK 1144 – D10	

The yeast cultures were stored on YPD agar slants (Merck Millipore, Darmstadt, Germany) at 4 °C.

2.2. Culture Media and Incubation Conditions

Pulcherrimin production was evaluated in rich YPD broth (Merck Millipore, Darmstadt, Germany) or in minimal medium (1% glucose (*w*/*v*), 0.3% $(NH_4)_2SO_4$ (*w*/*v*), 0.1% KH_2PO_4 (*w*/*v*), 0.05% $MgSO_4 \times 7H_2O$ (*w*/*v*), 0.05% yeast extract (*w*/*v*)) supplemented with 0.05% (*w*/*v*) $FeCl_3$. The influence of Tween-80 (Merck, Darmstadt, Germany) supplementation (0.1% (*v*/*v*) on pulcherrimin formation in the tested broth media was also investigated. The yeast strains were cultivated in 50 mL culture media for 2 days at 25 °C on a Heidolph Titramax 1000 rotary shaker (Heidolph, Schwabach, Germany) at 125 rpm. Inoculation was carried out using suspensions of the tested yeasts prepared to equal the 1.0 McFarland standard (1 °McF). Yeast cell density was measured using a densitometer DEN-1 (Merck Millipore, Darmstadt, Germany).

2.3. Pulcherrimin Extraction and Purification

Pulcherrimin was obtained from the yeast cultures using the method described previously by Kluyver et al., with subsequent modification by Li et al. [1,28]. A 50 mL sample of each yeast culture was centrifuged at 4 °C and speed 5000× *g* for 10 min using a Centrifuge 5804R (Eppendorf, Hamburg, Germany). The pellet containing the yeast cells and pigment was treated with methanol (50 mL 99.8% methanol per 10 g of wet yeast biomass) at 4 °C. After overnight treatment, the yeast cells were centrifuged (4 °C, speed 5000× *g*, 10 min) and then washed twice with distilled water (25 mL). The yeast biomass was re-suspended in 2M NaOH and centrifuged (4 °C, speed 5000× *g*, 10 min). The pH of the supernatant was adjusted to 1.0 using 4M HCl, and the mixture was incubated at 100 °C for 30 min. The pigment precipitate was collected by centrifugation (4 °C, speed 8000× *g*, 20 min), and washed three times with 25 mL of distilled water. To obtain pure pulcherrimin, the steps of dissolution in NaOH and HCl precipitation were repeated three times. Finally, the pigment was collected by centrifugation and dried at 60 °C for 18 h (moisture analyzer MA 50.R, Radwag, Poland). To determine the purity of the obtained red pigment, the ^{1}H NMR technique was used. The isolated product was dissolved (5% *w*/*v*) in alkaline deuterated water (2 M solution of NaOH in D_2O) at 25 °C. The liquid state ^{1}H NMR spectrum was recorded on a Bruker DRX-500 MHz (Billerica, MA, USA) spectrometer at 25 °C [29].

2.4. Quantitative Determination of Pulcherrimin

The purified pulcherrimin was dissolved in 2 M NaOH, and the absorption spectrum of the solution was measured using a Multiskan GO UV-light spectrophotometer (Thermo Fisher Scientific, Warsaw, Poland). A standard curve was made using different concentrations of purified pulcherrimin dissolved in 2M NaOH. The optical density of the solution was determined at 410 nm [1]. The amount of pulcherrimin in the yeast cultures was determined spectrophotometrically using the method described above.

2.5. Statistics

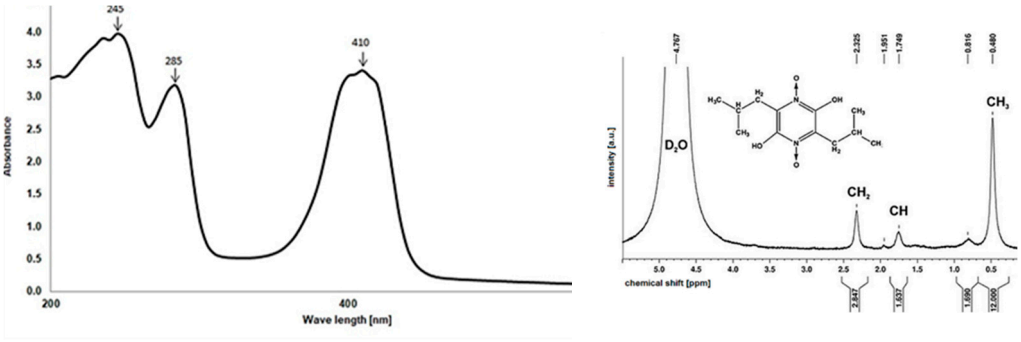
The production of pulcherrimin was presented as the mean ± SD of three separate experiments. The mean values results were compared using one-way repeated measures analysis of variance (ANOVA; OriginPro 8.1, OriginLab Corp., Northampton, MA, USA). The results obtained in minimal medium were compared with the YPD cultures. Values with different letters are statistically different; a: $p \geq 0.05$; b: $0.005 < p < 0.05$; c: $p < 0.005$.

3. Results and Discussion

3.1. Pulcherrimin Analysis

Purified and solubilized red pigment produced by the tested yeast strains exhibited maximum absorption spectra at three different wavelengths: 240, 280 and 410 nm (Figure 1a). These data are

consistent with those for pulcherrimin produced by yeasts and bacteria [1,30]. A wavelength of 410 nm was used for spectrophotometric determination of the pulcherrimin.



(**a**) (**b**)

Figure 1. Pulcherrimin spectra: (**a**) spectrophotometry with the maximum absorption at three characteristic wavelengths: 240, 280 and 410 nm; (**b**) ^{1}H NMR spectroscopy with the characteristic signals of CH_3, CH_2 and CH, belonging to isobutyl groups; the large solvent peak is typical for analyses carried out for solutions in D_2O.

Proton nuclear magnetic resonance is often used to characterize structural properties of various molecules [29,31]. The ^{1}H NMR spectrum of the isolated red pigment dissolved in alkaline D_2O is presented in Figure 1b. The ^{1}H NMR profile contains all the signals typical for this compound (CH_3, CH_2 and CH, belonging to isobutyl groups) [32]. The small satellite low-field peaks observed for CH_3 and CH protons may be tentatively ascribed to the presence of conformational isomers. The large solvent peak is typical for analyses carried out on solutions in D_2O. It contains a residual amount of ordinary hydrogen isotope (protium), which results in characteristic resonance at about 4.8 ppm. It also indicates traces of H_2O/DOH, which are always present in D_2O. The signals are widened due to the presence of hydrogen bonds.

3.2. Pulcherrimin Formation

The tested *Metschnikowia* strains were characterized by various levels of pulcherrimin formation. The production of the red pigment was dependent on the yeast strain and culture medium. For almost all the tested strains, pulcherrimin formation was significantly ($p < 0.05$) higher in the minimal medium than in the rich YPD broth (Figure 2). The best producers—NCYC 2321 and NCYC 747, as well as LOCK D9 and LOCK D10 strains—showed pulcherrimin production in the range of 192–198 mg/L. However, supplementation of the minimal medium with 0.1% (*v*/*v*) Tween-80 improved pulcherrimin formation to 230–240 mg/L, especially in the case of the best producers, NCYC 2321 and LOCK D10. Tween-80 is known to significantly increase the permeability of cell walls. Therefore, it is often used as a stimulatory agent in various fermentation processes [33,34].

To the best of our knowledge, there is only one previous report concerning the yield of pulcherrimin from yeast cultures. Pourzand and co-workers state in their poster that around 150 mg/L of pulcherrimin was produced from a 10 L culture of a strain of *M. pulcherrima* [26]. In the case of bacteria, *B. licheniformis* strain produced about 53 mg/L of pulcherrimin [28]. However, production of the pigment was dependent on the culture medium and culturing conditions. Under optimized environmental conditions with the supplementation of glucose, $(NH_4)_2SO_4$ and Tween-80, pulcherrimin production was improved substantially, reaching 331 mg/L. Melvydas and co-workers report that by changing the composition of the agar media, as well as the growth time, temperature, and the mode of yeast inoculation, various patterns of pulcherrimin formation can be obtained [2]. In addition, certain amino acids may play crucial roles in the synthesis of pulcherrimin [35]. The concentration of iron ions in the fermentation medium can also affect the formation of pulcherrimin. Lachance suggests that the

highest production of pigment occurs when the iron concentration is slightly inhibitory [36]. These data stimulate further research on the optimization of conditions for pulcherrimin formation.

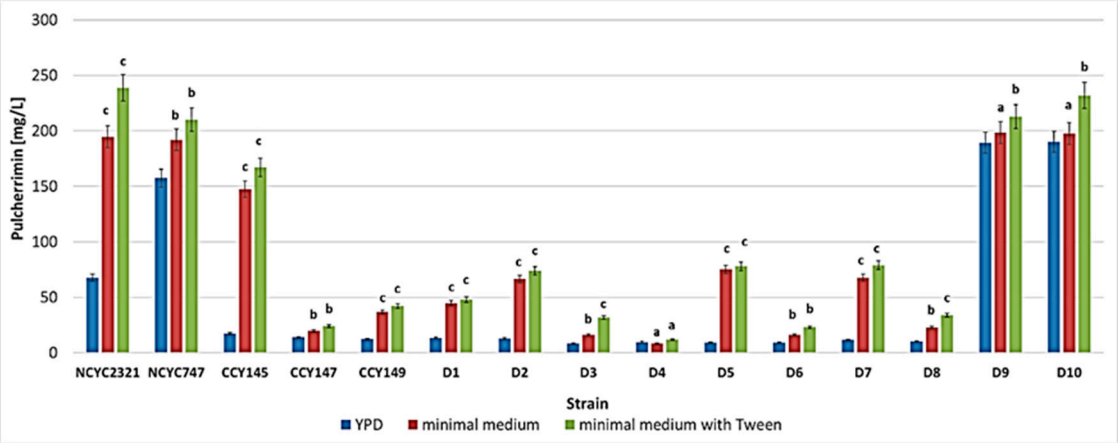


Figure 2. Pulcherrimin production by yeast strains in the culture media. Values show the mean ± standard deviation (SD, $n = 3$). Results obtained for minimal media were compared with those for YPD cultures. Values with different letters are statistically different; a: $p \geq 0.05$; b: $0.005 < p < 0.05$; c: $p < 0.005$.

4. Conclusions

Yeast strains capable of producing high yields of pulcherrimin are of great interest to biotechnology. In this study, we identified a red pigment produced by the strains of the genera *Metschnikowia* as pulcherrimin, using spectrometric and spectrophotometric analysis. Pulcherrimin production was found to be dependent on the strain and culture medium. Minimal medium with glucose, ferric ions and Tween-80 improved the yield of pulcherrimin in comparison to the rich medium YPD. Under these conditions, the maximum yield of pulcherrimin was over 230 mg/L. However, further research is needed to the optimize the production of pulcherrimin pigment by yeast strains.

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