ORIGINAL RESEARCH ARTICLE

Inoculation with a selected strain of Metschnikowia pulcherrima as a bioprotective alternative to sulphites for preventing browning of white grape must

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ABSTRACT

In this study, the effectiveness of a selected strain of Metschnikowia pulcherrima (MP1) was compared to that of sulphur dioxide for preventing white grape must from browning. Sulphur dioxide was found to drastically reduce oxygen consumption rate, protect hydroxycinnamic acids against oxidation and prevent grape must from browning, even in the presence of laccase. By contrast, supplementation with the selected strain of Metschnikowia pulcherrima (MP1) drastically increased oxygen consumption rate, thus reducing the amount of oxygen available for polyphenol oxidases. In the absence of laccase, this resulted in a decrease in browning and a certain degree of protection of the hydroxycinnamic acids, but in the presence of laccase it was not effective enough. Consequently, the selected strain of Metschnikowia pulcherrima (MP1) can be considered an interesting alternative to sulphur dioxide for preventing browning in white grape must, but only in conditions of healthy grapes.

KEYWORDS: grape must, Metschnikowia pulcherrima, bioprotection, browning, laccase

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INTRODUCTION

The enzymatic browning of grape must is still a major problem in enology today (Li et al., 2008), being particularly serious when the grapes have been infected by grey rot (Ky et al., 2012). Browning is an oxidation process that causes certain foods to turn brown, which often leads to them being rejected by consumers (Friedman, 1996). This is a particular problem in the case of wine, because grape must is very vulnerable to enzymatic browning (Oliveira et al., 2011).

Enzymatic browning is caused by the polyphenol oxidases tyrosinase and laccase. Tyrosinase (EC 1.14.18.1) is naturally present in grape berries (Fronk et al., 2015), whereas laccase (EC 1.10.3.2) is only present in grapes infected with Botrytis cinerea (Claus et al., 2014). These two polyphenol oxidases can both oxidise certain phenolic compounds, such as hydroxycinnamic acids and other diphenols (Giménez et al., 2022), forming ortho-quinones; these later polymerise to form melanins (Friedman, 1996; Oliveira et al., 2011), which are responsible for enzymatic browning in white wines and for oxidase haze in red wines (Ribéreau-Gayon et al., 2006a).

When grapes are infected by Botrytis cinerea, laccase activity is much higher than tyrosinase activity in healthy grapes (Steel et al., 2013), and therefore there is a much higher possibility that enzymatic browning will occur. In addition, laccase can oxidize a larger variety of substrates than tyrosinase (Oliveira et al., 2011; Claus et al., 2014).

In wineries, the addition of sulphur dioxide to grape must is the most common tool for protecting it against enzymatic browning. This additive is a potent inhibitor of tyrosinase and laccase (Ribéreau-Gayon et al., 2006a; Giménez et al., 2022) and also has antimicrobial properties (Ough and Crowell, 1987). Tyrosinase is more sensitive to sulphur dioxide than laccase, and it is usually inactivated once alcoholic fermentation has ended. In contrast, laccase can be active in wine after alcoholic fermentation (Du Toit et al., 2006; Ribéreau-Gayon et al., 2006a).

Nevertheless, the wine industry is currently trying to reduce and even eliminate the use of sulphur dioxide due to its negative effects on the environment (D’Amico et al., 2016) and human health (Lester, 1995). Hence, some alternatives to sulphur dioxide additives have been proposed over recent years to prevent browning; for example, inert gases (Martinez and Whitaker, 1995), oenological tannins (Vignault et al., 2019; Vignault et al., 2020), ascorbic acid (Ribéreau-Gayon et al., 2006b), reduced glutathione (Kritzinger et al., 2013; Zimdars, 2020; Giménez et al., 2022) and inactivated dry yeasts rich in glutathione (Bahut et al., 2020; Gabrielli et al., 2017; Giménez et al., 2023). More recently, bioprotection has also been proposed as an alternative to sulphur dioxide (Rubio-Bretón et al., 2018; Simonin et al., 2020; Morata et al., 2021; Di Gianvito et al., 2022), not only to avoid microbiological deviations occurring (Simonin et al., 2018; Windholtz et al., 2021; Englezos et al., 2022; Escribano-Viana et al., 2022; Windholtz et al., 2023) but also to protect against enzymatic browning (Chacón-Rodríguez et al., 2020; Simonin et al., 2022; Windholtz et al., 2022; Giménez et al., 2023).

Different microorganisms have been proposed for bioprotection, such as Torulaspora delbrueckii (Simonin et al., 2018; Chacón-Rodríguez et al., 2020; Windholtz et al., 2021; Windholtz et al., 2023), Lachancea thermotolerans (Rubio-Bretón et al., 2018; Morata et al., 2021; Escribano-Viana et al., 2022; Windholtz et al., 2023) and even Lactobacillus plantarum (Rubio-Bretón et al., 2018). However, according to recent literature, Metschnikowia pulcherrima seems to be the most promising microorganism for wine bioprotection (Simonin et al., 2020; Puyo et al., 2023; Yao et al., 2023; Giménez et al., 2023; Canonico et al., 2023; Agarbari et al., 2023). It seems that Metschnikowia pulcherrima has a large capacity for consuming oxygen (Canonico et al., 2019; Chacón-Rodríguez et al., 2020). A reduction in oxygen in the must will hinder the development of other microorganisms (Di Gianvito et al., 2022; Windholtz et al., 2023) and significantly decrease the necessary substrate for the oxidative action of polyphenol oxidases (Giménez et al., 2023). In addition, the use of M. pulcherrima in winemaking is becoming more and more common for different reasons (Morata et al., 2019). Specifically, M. pulcherrima can improve the sensory aromatic profile of wine (Ruiz et al., 2018), can reduce the wine ethanol strength (Contreras et al., 2015) and has a bioprotective effect (Sipiczki, 2006).

The aim of this study was therefore to investigate the bioprotective effect of a selected strain of Metschnikowia pulcherrima (MP1) on enzymatic browning caused by polyphenol oxidases.

MATERIALS AND METHODS

1. Chemicals and equipment

Syringaldazine (purity ≥ 98 %), and FeSO₄·7H₂O (purity ≥ 99%) were purchased from Sigma-Aldrich (Madrid, Spain). L- (+)-Tartaric acid (purity ≥ 99.5 %), sodium hydroxide (purity ≥ 98 %), sodium acetate (purity ≥ 99 %), acetonitrile (purity ≥ 99 %), Methanol (purity ≥ 99 %), and CuSO₄·5H₂O (purity ≥ 99 %) were purchased from Panreac (Barcelona, Spain). Ethanol (96% vol.) was supplied by Fisher Scientific (Madrid, Spain). Cellulose membranes of 3.5 KDa (6.4 mL/cm) were supplied by Spectrum Laboratories, Inc (Rancho Dominguez, USA). We used the following equipment: an Hpland an Entris II Series Analytical Balance (Sartorius, Goettingen, Germany); a spectrophotometer UV–Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a liquid chromatograph Agilent Series 1200 (Agilent, Germany) equipped with a photodiode array detector (G1315D); and a Zorbax Eclipse XDB C18 column (4.6 x 150mm).
2. Sample collection and preparation
Muscot of Alexandria grapes were harvested from the experimental vineyard of Rovira i Virgili University (Mas dels Fracs, Constanti, Tarragona: 41°08' 44.1"N, 1°11'51.0"E, height: 59 masl) during the 2022 vintage. The grapes were picked manually when they were considered sufficiently ripe (around 12.5% of potential ethanol content and 6.0 g tartaric acid/L titratable acidity). The grapes were immediately pressed in an environment saturated with nitrogen with a vertical manual press until 60% of their juice had been extracted. The grape must was immediately transferred to a 750-mL bottle also saturated with nitrogen.

3. Synthetic buffer
A solution of 4 g/L of L-(−)-tartaric acid, 3 mg/L of iron (in the form of iron (III)), chloride hexahydrate, and 0.3 mg/L of copper (in the form of copper (II) sulphate pentahydrate) was adjusted to pH 3.5 using sodium hydroxide. This solution was used for all the experiments.

4. Laccase enzyme production and enzymatic activity measurement
Active laccase extracts were obtained from Botrytis cinerea isolate 213 strain following the methodology described by Vignault et al. (2020). The laccase extract was treated with 0.16 g/mL of PVPP for 10 min and centrifuged at 7500 rpm for 10 min. Subsequently, the supernatant was subsequently dialysed with a cellulose membrane (MW cutoff 3.5 KDa) for 2 days against a 0.3 M ammonium formate formate solution and for 2 more days against distilled water with continuous shaking. The laccase activity of the extract was determined using an adapted syringaldazine test method (Grassin and Dubourdieu, 1986).

5. Metschnikowia pulcherrima strain
Metschnikowia pulcherrima strain (MP1) was selected for its high oxygen consumption capacity (Level2 Initiatm, Lallemand Inc, Montreal, Canada). Ten min before beginning the measurements, the Metschnikowia pulcherrima yeasts were hydrated to ten times their weight with distilled water. The water temperature was 30 °C.

6. Reaction conditions for measuring oxygen consumption
The experimental conditions are described in Giménez et al. (2023). The assays were carried out in clear glass flasks (66 mL) with an oxygen sensor spot (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203-01-PSt3-0828-01, Regensburg, Germany) for measuring the dissolved oxygen noninvasively by luminescence (Nomasense TM O2 Trace Oxygen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium). The grape must was diluted (20 % (v/v)) with the above-mentioned synthetic buffer to reduce its oxygen consumption rate. This made it possible to monitor it with higher precision, and also to standardize the pH of the different measurements. More specifically, thirteen mL of grape must and 52 mL of buffer were added to each flask, to which the different chemical agents were added.

The agents used were: sulphur dioxide (20 mg/L in the form of potassium disulphite), Metschnikowia pulcherrima strain MP1 (250 mg/L), and a combination of sulphur dioxide (20 mg/L) and Metschnikowia pulcherrima strain MP1 (250 mg/L). A control with no added agent was also prepared. These assays were also performed with and without the addition of 2 units/mL of laccase. All the assays were performed in triplicate.

The bottles were immediately saturated in oxygen (around 7–8 mg O2/L) via vigorous manual stirring for a few seconds. The oxygen concentration was measured (Diéval et al., 2011) periodically until asymptotic behaviour was attained (around 3 h) to determine the oxygen consumption kinetics. All measurements were made in an air-conditioned laboratory at 22 ± 2 °C. The total oxygen consumption capacity (TOCC) was calculated using the mathematic model previously reported by Pons-Mercadé et al. (2021). Once the oxygen concentrations were below 1 mg/L, or the oxygen consumption showed asymptotic behaviour, the samples were supplemented with 50 mg/L of sulphur dioxide to stop the colour evolution. The samples were used for colour measurements and for HPLC analysis of hydroxycinnamic acids and GRP.

7. Colour measurements
The intensity of yellow colour (A420nm) was measured and the CIEL*a*b* coordinates of the samples were determined according to Ayala et al. (1997). Data were processed using the MSCV software (MSCVes.zip., 2013). The total colour difference (ΔEab*) was calculated as the Euclidian distance between two points in the CIEL*a*b* space using the following formula:

\[ ΔEab^* = \left( (L^* - L_{ref})^2 + (a^* - a_{ref})^2 + (b^* - b_{ref})^2 \right)^{1/2} \]

where L* is the lightness; a* is the green–red component of the colour, and b* is the blue–yellow component. The total colour difference (ΔEab*) was used to determine whether the difference between two samples could be detected visually by the human eye. Generally, the difference is considered to be visible to the human eye when ΔEab* > 3 (Martínez et al., 2001).

8. Hydroxycinnamic acid and GRP analysis by HPLC−DAD
Hydroxycinnamic acids and grape reaction product (GRP) were analysed by the reversed-phase HPLC−DAD following the method described by Lago-Vanzela et al. (2011). This comprised an Agilent Series 1200 HPLC (Agilent, Waldbronn, Germany) equipped with a DAD (G1315D) coupled to an Agilent Chem Station (version B.01.03) data processing station. The samples were filtered (0.20 μm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany) and then injected (20 μL) into a Zorbax Eclipse XDB C18 column (4.6 x 150mm). The solvent was A [water/formic acid/acetonitrile (88.5:8.5:3, v/v/v)], B [water/formic acid/acetonitrile (41.5:8.5:50, v/v/v)] and C [water/formic acid/methanol (1:5:8.5:90, v/v/v)] and the flow rate 0.19 mL/ min. The gradient was: A = 96% B = 4%
from 0 to 37 min; A = 70%/B = 17%/C = 13% from 37 to 51 min; A = 50%/B = 30%/C = 20% from 51 to 57 min; A = 0%/B = 50%/C = 50% from 57 to 64 min. The compounds were quantified by measuring the absorbance at 320 nm with an external calibration curve of caftaric acid. The different compounds were identified according to the retention time that was previously determined by HPLC–DAD-ESI-MS/MS analysis using the same chromatographic conditions.

9. Statistics

All data are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance (ANOVA F test) was conducted using the SPSS 15.0 software (SPSS Inc., Chicago, IL). Different letters indicate the existence of statistical difference (p < 0.05).

RESULTS AND DISCUSSION

1. Oxygen consumption kinetics

Figure 1A shows the oxygen consumption kinetics of the diluted grape must without supplementation (the control), and supplemented with sulphur dioxide, with Metschnikowia pulcherrima strain MP1 and with a combination of both agents, in the absence or presence of 2 U/mL of laccase. The oxygen consumption of the diluted control grape must without additives was initially very fast, later becoming moderate until around 2 mg/L of O₂ was reached after 3 hours. This behaviour was probably due to a reduction in the amount of substrates available for polyphenol oxidase, which slows down the reaction rate. Meanwhile, the oxygen consumption rate (OCR) decreased drastically when the sample was supplemented with 20 mg/L of sulphur dioxide. Other assays with higher doses of this additive showed similar results (data not shown). These results confirm that sulphur dioxide is a very effective inhibitor, of not only tyrosinase but also laccase (Ough and Crowell, 1987; Giménez et al., 2023).

Although Figure 1 is very useful for visualising OCR, it cannot be used to determine whether there are statistically significant differences within the different experimental groups. Therefore, a previously used kinetic model (Pons-Mercadé et al., 2021) was applied to statistically compare the results of this graph and calculate the total oxygen consumption (TOC) in the different experimental conditions. This model shows the inverse of consumed oxygen versus the inverse of elapsed time. Using this mathematical model, the following equation can be established: 1/[O₂] = A/t + B. After clearing up the oxygen in the equation, TOC can be calculated as the limit when time tends towards infinity.

Figure 2 shows the calculated TOC of the diluted grape must without supplementation (the control), and supplemented with sulphur dioxide, with the Metschnikowia pulcherrima strain MP1 and with the combination of the two agents in the absence or presence of 2 U/mL of laccase.
This graph confirms the conclusions drawn from Figure 1: briefly, that supplementation with sulphur dioxide significantly reduced TOC, whereas supplementation with *Metschnikowia pulcherrima* strain MP1 did the opposite. Supplementation with laccase significantly increased the TOC of the control and of the samples supplemented with the combination of sulphur dioxide and *Metschnikowia pulcherrima* strain MP1, but not the TOC of the other experimental groups.

2. Hydroxycinnamic acids and GRP

Figure 3 shows the hydroxycinnamic acids and GRP concentrations after oxygen consumption of the diluted grape must without supplementation (the control), and supplemented with sulphur dioxide, with the *Metschnikowia pulcherrima* strain MP1 and with the combination of the two agents in the absence or presence of 2 U/mL of laccase.

In healthy grape must, the levels of hydroxycinnamic acids and GRP were very low in the absence of sulphur dioxide. In contrast, when sulphur dioxide was added, the levels of hydroxycinnamic acids and GRP were significantly higher. This indicates that in the absence of sulphur dioxide, tyrosinase oxidised nearly all the hydroxycinnamic acids present in the grape must. By contrast, the presence of sulphur dioxide inhibited this enzyme and protected hydroxycinnamic acids from oxidation. However, the levels of GRP in the presence of sulphur dioxide were significantly higher than in its absence, which is difficult to explain. One possibility is that before sulphur dioxide completely inhibits tyrosinase, some of the caftaric acid present in the grape must is transformed into ortho-quinones, which react with the natural glutathione present in the grape must to form GRP (*Singleton et al.*, 1985; *Kritzinger et al.*, 2013). This GRP would also be formed in

![Figure 2](image1.png)

**FIGURE 2.** Influence of inoculation with *M. pulcherrima* strain MP1 on the Total Oxygen Consumption (TOC).

![Figure 3](image2.png)

**FIGURE 3.** Influence of inoculation of *M. pulcherrima* strain MP1 on the wine GRP and hydroxycinnamic acid concentration.
the absence of sulphur dioxide and therefore would be present in the grape must, because tyrosinase, unlike laccase, cannot oxidise GRP (Kritzinger et al., 2013). However, the low GRP levels found in these conditions suggest that the GRP was subsequently incorporated into the brown polymers formed as a result of the polymerisation associated with enzymatic browning reactions.

When Metschnikowia pulcherrima strain MP1 was present, the levels of hydroxycinnamic acids and GRP were significantly higher than in control conditions, but significantly lower than in the presence of sulphur dioxide. It seems therefore that the high oxygen consumption of Metschnikowia pulcherrima strain MP1 reduced the availability of oxygen for polyphenol oxidase, and therefore protected the hydroxycinnamic acids against the action of this enzyme. When sulphur dioxide and Metschnikowia pulcherrima strain MP1 were added together, the results were very similar to those obtained with only sulphur dioxide.

Very similar results were obtained when the samples were supplemented with 2 U/mL of laccase. The only remarkable difference was that the levels of hydroxycinnamic acids and GRP of the samples were lower than without laccase, probably because laccase can oxidize hydroxycinnamic acids and GRP to a greater extent (Oliveira et al., 2011; Steel et al., 2013; Claus et al., 2014).

3. Colour parameters

Figure 4 shows the absorbance at 420 nm (A420nm) and the CIEL*a*b* blue–yellow component (b*) of the samples without supplementation (the control), and supplemented with sulphur dioxide, with Metschnikowia pulcherrima strain MP1 and with a combination of both in the absence and presence of laccase as indicators of browning intensity.

The supplementation with sulphur dioxide had a clear protective effect on the grape must against browning, since the values of A420 and b* were significantly lower than in the control sample. This again confirms the previously described protective effect of this additive against enzymatic browning (Ough and Crowell, 1987; Ribéreau-Gayon et al., 2006a; Oliveira et al., 2011). The supplementation with Metschnikowia pulcherrima strain MP1, both alone and combined with sulphur dioxide, also significantly decreased A420 and b* with respect to the control; this confirms that this non-Saccharomyces yeast protects grape must against enzymatic browning, even in the absence of sulphur dioxide.

In the presence of laccase, all the samples showed significantly higher values of A420 and b* than their corresponding controls without laccase. This confirms the high oxidative capacity of this polyphenol oxidase. It also indicates that it is more difficult to completely protect against browning in grapes affected by grey rot, even using sulphur dioxide.

Figure 5 shows the total colour difference (ΔEab*) between the different samples in relation to the grape must supplemented with sulphur dioxide.

The sample supplemented with sulphur dioxide was used as the reference for comparison with all the other samples, because sulphur dioxide addition is the most usual procedure for protecting grape must from browning. It is generally accepted that when ΔEab* is lower than 3 units the human eye cannot perceive the visual difference between two samples in terms of colour (Martínez et al., 2001; Pérez-Magariño and González-Sanjosé, 2003). Consequently, ΔEab* between a sample and its control reference will indicate whether the browning intensity can or cannot be perceived by the human eye, depending on whether it is greater or lower than 3 units.

**FIGURE 4.** Influence of inoculation of M. pulcherrima strain MP1 on the intensity of the yellow colour (A420nm and b*) of the wine.
In the absence of laccase, ΔE<sub>ab</sub>* between the control sample and the reference was clearly higher than 3 units, which indicates that without sulphur dioxide the intensity of the browning was high enough to be clearly perceived by the human eye. By contrast, total colour difference of the sample inoculated with <i>Metschnikowia pulcherrima</i> strain MP1 was slightly above 3 units, which indicates that the intensity of browning was very low and hardly perceptible. This confirms that <i>Metschnikowia pulcherrima</i> strain MP1 exerts a real protective effect against enzymatic browning, at least in healthy grape must conditions. When sulphur dioxide and <i>Metschnikowia pulcherrima</i> strain MP1 were added in combination, the ΔE<sub>ab</sub>* was much lower than 3 units and therefore the difference in colour with respect to the reference was imperceptible.

In the presence of laccase, the ΔE<sub>ab</sub>* values were much higher than in the healthy grape must. This means that browning was evident, even in the presence of sulphur dioxide.

Figure 6 shows photographs of the different samples after the experimental process.

In healthy grape must, the control sample without anything added to it clearly underwent a browning process. By contrast, the samples supplemented with sulphur dioxide, whether alone or combined with <i>Metschnikowia pulcherrima</i> strain MP1, were much lighter in appearance, which confirms that this additive completely prevents the grape must from browning. The sample supplemented with only <i>Metschnikowia pulcherrima</i> strain MP1 was slightly browner than the samples supplemented with sulphur dioxide, but it was much lighter in colour than the control without any additive. However, in the presence of laccase the protective effectiveness of <i>Metschnikowia pulcherrima</i> strain MP1, and even that of sulphur dioxide, were not enough to prevent browning. This confirms that supplementation with <i>Metschnikowia pulcherrima</i> strain MP1 exerts a protective effect on grape must against browning in healthy grape conditions.
CONCLUSIONS

In this work, we analysed how supplementation with sulphur dioxide and/or Metschnikowia pulcherrima strain MP1 influence the oxygen consumption kinetics, hydroxycinnamic acids, GRP concentrations, and browning intensity in two situations: i) grape must from healthy grapes, and ii) grape must supplemented with the enzyme laccase to reproduce what occurs when the grapes are infected with Botrytis cinerea. The results show that sulphur dioxide drastically reduces oxygen consumption rate, protects hydroxycinnamic acids against oxidation and prevents grape must from browning even in the presence of laccase, although less effectively than in the absence of laccase. By contrast, the supplementation with Metschnikowia pulcherrima strain MP1 dramatically increases the oxygen consumption rate, which reduces the oxygen available for polyphenol oxidases. In the absence of laccase, this results in a decrease in browning and in a certain degree of protection of the hydroxycinnamic acids.

However, in the presence of laccase, supplementation with Metschnikowia pulcherrima strain MP1 was not effective enough to prevent browning. It can therefore be concluded that Metschnikowia pulcherrima strain MP1 can be considered an adequate alternative tool to reduce, and maybe eliminate, the addition of sulphur dioxide at the beginning of winemaking for preventing browning in white grape must made from healthy grapes.

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