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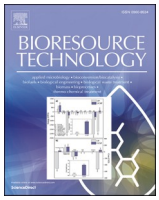

Case Study

Discoloration of textile dyes by spent mushroom substrate of *Agaricus bisporus*

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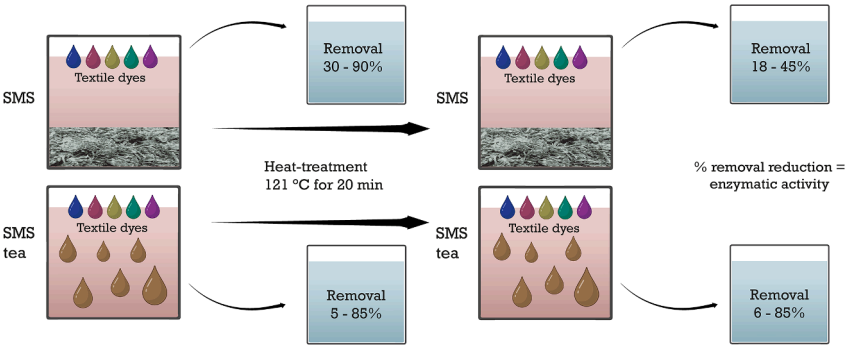
HIGHLIGHTS

- SMS of *Agaricus bisporus* and its aqueous extract remove textile dyes from water.
- Non-water-extractable SMS compounds facilitate optimal removal of textile dyes.
- Enzymatic and non-enzymatic activities in SMS remove textile dyes.

GRAPHICAL ABSTRACT



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ABSTRACT

The textile industry discharges up to 5 % of their dyes in aqueous effluents. Here, use of spent mushroom substrate (SMS) of commercial white button mushroom production and its aqueous extract, SMS tea, was assessed to remove textile dyes from water. A total of 30–90 % and 5–85 % of the dyes was removed after a 24 h incubation in SMS and SMS tea, respectively. Removal of malachite green and remazol brilliant blue R was similar in SMS and its tea. In contrast, removal of crystal violet, orange G, and rose bengal was higher in SMS, explained by sorption to SMS and by the role of non-water-extractable SMS components in discoloration. Heat-treating SMS and its tea, thereby inactivating enzymes, reduced dye removal to 8–58 % and 0–31 %, respectively, indicating that dyes are removed by both enzymatic and non-enzymatic activities. Together, SMS of white button mushroom production has high potential to treat textile-dye-polluted aqueous effluents.

1. Introduction

The textile industry uses a diverse palette of chemical dyes. It has been estimated that over 700,000 tons of over 10,000 dyes are produced annually (Rawat et al., 2016; Robinson et al., 2001; Samsami et al., 2020; Zaharia and Suteu, 2012). During textile dying, around 10–25 %

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of the dyes is lost, from which 2–20 % is discharged in aqueous effluents. Dyes are made to resist light, water, and oxidizing agents. As a drawback, the removal of these recalcitrant molecules is not always successful and / or expensive (Ergas et al., 2006; Mahmoud et al., 2007; Nidheesh et al., 2013; Samsami et al., 2020). For instance, at least 600 mg/L ozone is needed to remove 95 % of a mixture of dyes from waste water, while more than 20 g/L granular activated carbon is needed to adsorb up to 86 % of the dyes in 40 min. The costs of such treatments make them not economically viable (Ergas et al., 2006).

Bacteria and fungi could provide a cheap and biological solution to remove dyes from soil and waste water (Bharagava and Chowdhary, 2019; Katheresan et al., 2018; van Brenk and Wösten, 2021). This bioremediation solution often uses fungi that produce ligninolytic enzymes (Blanchette, 1995; Worrall et al., 1997). These non-specific oxidative enzymes (Kirk and Farrell, 1987) can also be used to degrade other aromatic molecules such as textile dyes (Barr and Aust, 1994). However, use of these enzymes is hampered by fungal biomass production, the effect of the aqueous environment on enzyme secretion, the instability of enzymes, and / or removal of limited numbers of dyes (Barrios-González, 2012; Katheresan et al., 2018; Novotný et al., 2004).

Agaricus bisporus, i.e. the white button mushroom, is a fungus with ligninolytic activity. It grows on litter in nature, but compost is used for commercial growth (Gerrits, 1988; Morin et al., 2012). Global white button mushroom production is about 5,000,000 tons a year (Colmenares-Cruz et al., 2017; Royse et al., 2017). Spent mushroom substrate (SMS) is a resulting waste stream, amounting 3 times the mushroom production in mass (Uzun, 2004). For instance, 760,000 tons of SMS were produced in the Netherlands in 1993 (Gerrits, 1994). The compost used for white button mushroom production in the Netherlands is based on horse and chicken manure. SMS is therefore seen as an animal-based fertilizer that competes with other such fertilizers. Therefore, new applications for SMS are researched.

SMS resulting from commercial mushroom production could be used in bioremediation. A tea extract of SMS of *Pleurotus sajor-caju* decolorizes several dyes such as remazol brilliant blue R and amido black (Singh et al., 2010; 2011). Also, SMS of *Pleurotus ostreatus* can remove azo dyes by sorption and enzymatic conversion (Schallemberger et al., 2023; Zhou et al., 2011), while SMS of *A. bisporus* sorbs reactive, basic and acid dyes (Toptas et al., 2014). Despite these results, the underlying mechanisms of dye removal have been studied poorly, as is the case with other organic micropollutants (Ghose and Mitra, 2022). We here show that *A. bisporus* SMS and its tea can be used for discoloration of dyes in waste water and show that both enzymatic and non-enzymatic activities play a role in this process.

2. Material and methods

2.1. Strains and culture condition

Mushrooms of the commercial *A. bisporus* strain A15 were produced in boxes (26 x 20 x 20 cm; Manutan) by topping 2.5 kg full grown (PIII) horse-manure based compost with 1 kg casing soil (CNC Grondstoffen) (Herman et al., 2020). Boxes were incubated at 22 °C and 80 % relative humidity (RH) in controlled incubators for 14 days, after which mushroom formation was induced by spraying 100 mL water and venting at 18 °C and 80 % RH. The first and second flush of mushrooms were harvested 9–12 days and 21–23 days after venting, respectively. After the second flush, the SMS without casing layer was harvested, homogenized (mixed by hand) and stored at −20 °C in a freezer. Tea was made by mixing SMS with demi water (21 °C; 1 g 20 mL^{-1}), followed by shaking at 175 rpm and 21 °C for 1 h. After filtering through a Melitta® coffee filter, tea was used directly or stored at −20 °C.

2.2. Dye removal by SMS and SMS teas

Removal of crystal violet, malachite green, orange G, rose bengal and remazol brilliant blue R (Sigma-Aldrich, www.sigmaaldrich.com) was assessed in triplo by mixing 150 mL aqueous dye solution (Table 1) with 5 g SMS in a 250 mL flask with screw lit (Fig. 1) SMS mixed with water without dye and dye solution without SMS were used as controls. The SMS-dye mixtures were incubated at room temperature in the dark without shaking. Samples were centrifuged at 15,871 g for 2 min. The amount of dye in the supernatant was quantified by spectrophotometry (see 2.3), while the pellet was extracted with 150 mL methanol to determine dye sorption (Fig. 1). To this end, samples were incubated overnight at room temperature in the dark without shaking, after which they were centrifuged for 2 min at 15,871 g. The amount of dye in the methanol extract was determined by spectrophotometry (see 2.3).

Table 1
Chemical dyes used in this study as well as their concentration and wavelength detection to assess discoloration in SMS ([a]) and SMS tea ([b]).

Compound	CAS No.	Concentration (μg mL^{-1})	Range *i* (nm)
Crystal violet	548-62-9	10	480–650
Malachite green	2437-29-8	10	520–700
Orange G	1936-15-8	10[a] or 30[b]	400–550
Rose bengal	632-69-9	10	450–600
Remazol brilliant blue R	2580-78-1	80[a] or 100[b]	450–730

To assess dye discoloration by SMS tea (Fig. 1), 180 μL dye solutions (Table 1) were incubated with 20 μL SMS tea in a 96 wells plate (Greiner Bio-One, Cellstar 655180, https://www.gbo.com) at 25 °C in the dark without shaking using triplicates. The same controls were used as described for SMS discoloration.

2.3. Analysis and calculation of dye discoloration

Discoloration and sorption of dyes was measured by making a wavelength scan (350–750 nm) using a spectrophotometer (DU 800, Beckman Coulter, www.beckman.com) (Fig. A1). A specific wavelength range was used for each dye (Table 1) to calculate the area under the curve (AUC) Eq. (1)

$$AUC = \sum_{i=1}^{N-1} = \frac{yi + yi + 1}{2} \times (xi + 1 - xi)$$

Where y is the absorbance and x the wavelength (nm) for the range i (Table 1) and N the amount of steps in range i. The AUC of the background (i.e. SMS without dye) of range i was subtracted. To determine the percentage removal (i.e. discoloration + sorption) at t_0 (timepoint 0) and t_z (certain timepoint) was compared Eq. (2).

$$percentage\ removal = \frac{t0 - tz}{t0} \times 100\% \quad (2)$$

A standard curve was used to determine the percentage of sorption, while the discoloration was determined by subtracting the sorption of the total removal.

For dye removal (i.e. discoloration) by SMS tea, wavelength scans (350–750 nm) were made for each well every two hours using a spectrophotometer (Biotek Synergy H1 Multimode, Agilent, www.agilent.com). The percentage of discoloration was determined as described above (Eq. 1 & Eq. (2).

2.4. Statistics

Statistics was done in R (RStudio Version 4.2.3, 2023) using a p-value ≤ 0.05. Comparison of removal of dye by SMS and SMS tea was analyzed with a *t*-test. In all other cases, a One-way Anova with Tukey's HSD post-hoc test was used.

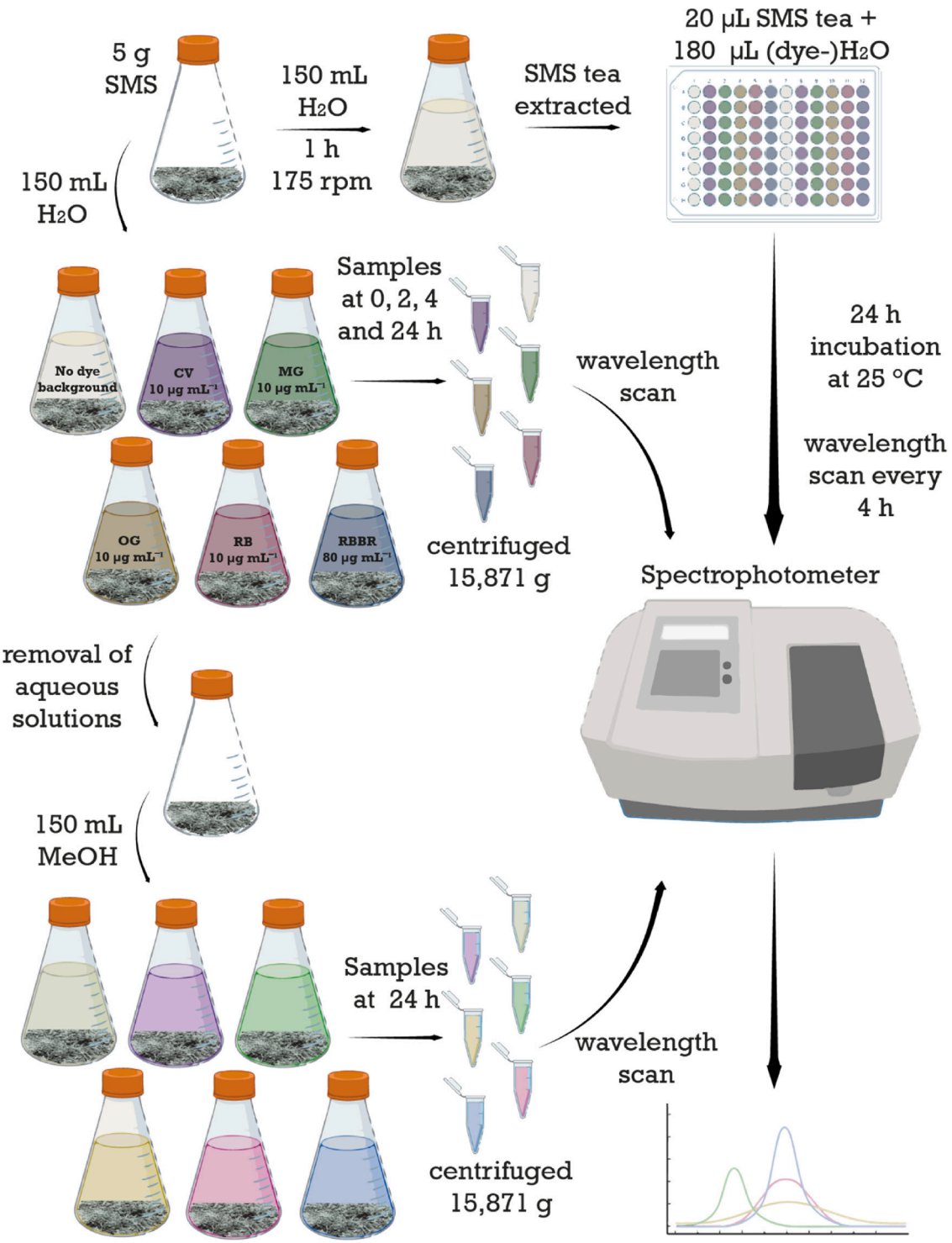

Fig. 1. Scheme showing the production of tea from SMS, the incubation of aqueous solutions of the dyes with SMS and its tea, and the quantification of dye removal from the aqueous solution. Removal is here defined as the discoloration of the dye solution in the case of the tea and as the sum of discoloration and sorption to SMS particles in the case of the spent mushroom substrate.

3. Results and discussion

3.1. Removal of dyes by SMS and its tea

Textile dyes in aqueous effluents are not fully removed by existing technologies that can also be expensive (Ergas et al., 2006; Mahmoud et al., 2007; Samsami et al., 2020). Biological solutions are therefore of interest (Barrios-González, 2012; Katheresan et al., 2018; Novotný et al., 2004). In the past, they have shown to remove dyes up 100 %, but this can take up more than 10 days (Novotný et al., 2004). Here, removal (% mass per volume) of crystal violet, malachite green, orange G, rose bengal and remazol brilliant blue R was determined during a 24 h incubation in SMS of the white button mushroom industry or its tea (Fig. 1). Removal is defined as the discoloration of the dye solution in the case of the tea and as the sum of discoloration and sorption to solid particles in the case of SMS. Removal of malachite green and remazol

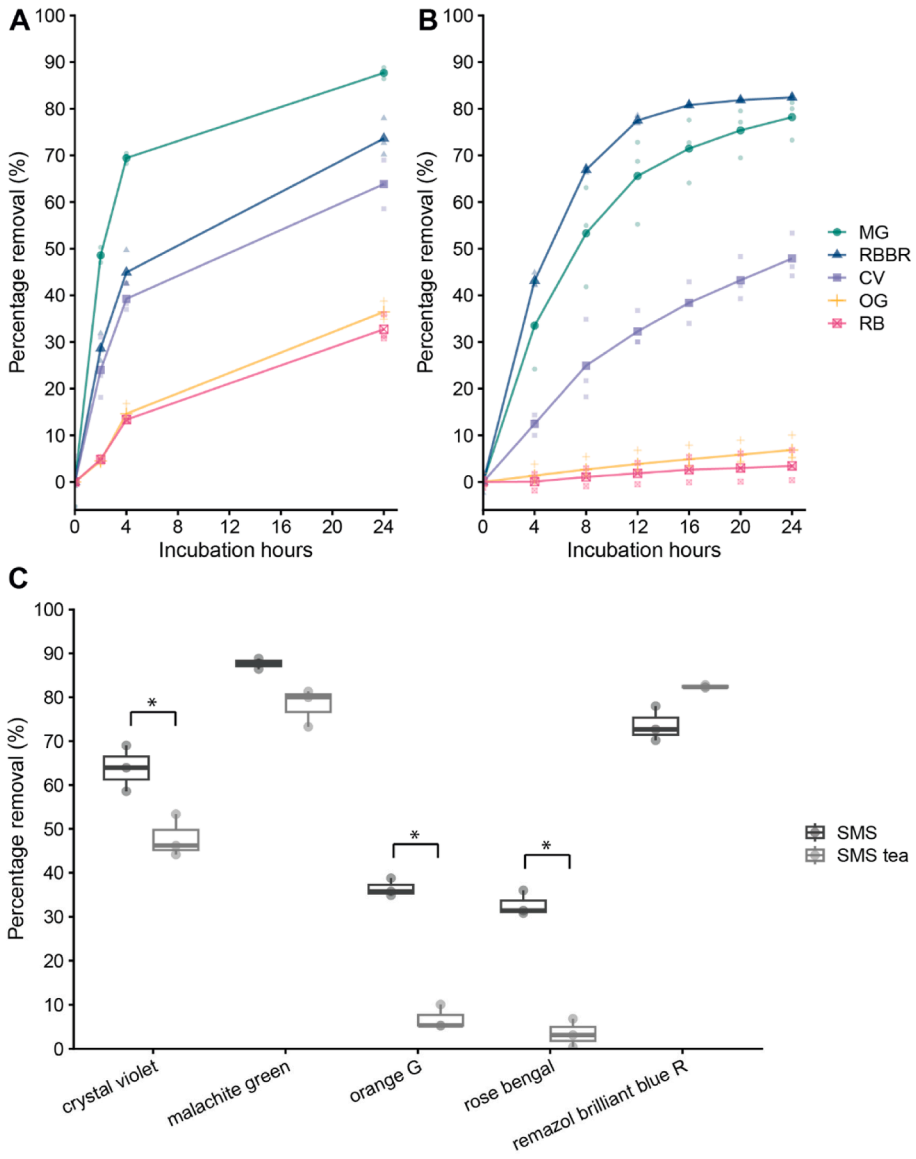

Fig. 2. Removal of crystal violet (CV), malachite green (MG), orange G (OG), rose bengal (RB), and remazol brilliant blue R (RBBR) by SMS (A,C) and its tea (B,C) during (A,B) and after (C) a 24 h period. * indicates significance differences $p < 0.05$.

brilliant blue R was over 70 % after 24 h for both SMS and its tea and were not significantly different. In contrast, removal of crystal violet, orange G and rose bengal was higher in SMS (30 %-65 %) when compared to its tea (5 %-50 %) (Fig. 2). Sorption of the dyes to SMS particles was tested by a methanol extraction. No differences in sorption were found when SMS was either or not heat-treated (121 °C for 20 min). Highest sorption was measured for rose bengal (34 %), which was almost equal to its total removal (data not shown). This and the low removal of this dye by SMS tea implies that this compound is hardly discoloured by SMS of the white button mushroom. Sorption of orange G, crystal violet, malachite green, and remazol brilliant blue R was 15, 18,10, 14, respectively. Thus, less than half of the removal of orange G by SMS is due to sorption, implying that about 20 % of the total dye was discoloured. Crystal violet, malachite green and remazol brilliant blue R were even more decolorized with efficiencies of 46–78 %. The reason for the differences in sorption and discoloration of the dyes is not clear. The similar discoloration of crystal violet and malachite green is probably due to the fact that they both belong to the triphenylmethane dyes sharing similarities in their molecule structure and auxochromic groups (Fig. A2) (Benkhaya et al., 2020). In contrast, orange G (azo dye), remazol brilliant blue R (anthraquinone dye) and rose bengal (xanthene dye) belong to different dye families and have different molecular structures. Xanthene dyes are known to easily sorb, which could explain the high sorption of rose bengal to SMS (Shabir et al., 2018). In general, the molecular structure of the dyes and environmental conditions such as pH and temperature can influence the electrostatic interaction between the dyes and SMS, thereby affecting sorption, and between the dyes and enzymes, thereby affecting their discoloration (Kusonoki, 1952).

Overall, our results are in line with other bioremediation systems such as SMS of *P. ostreatus* that removed 50 % of the azo dyes levalfix brilliant red E4BA and remazol black B, from which 15 % was sorbed (Schallemberger et al., 2023). Also, malachite green was decolorized for 40 % by *P. ostreatus* SMS (Papinutti and Forchiassin, 2010). Notably, 95 % of methylene blue was sorbed (Zhou et al., 2011). This high ability to sorb acidic dyes, up to > 80 %, was also shown for *A. bisporus* SMS (Toptas et al., 2014). Teas of *P. ostreatus* SMS species are able to remove remazol brilliant blue R up to 90 % after 40 h (Lim et al., 2013), while tea of *P. sajor-caju* SMS removes up to 60 % crystal violet (Singh et al., 2002) and > 90 % reactive black 5 and reactive orange 16 (Singh et al.,

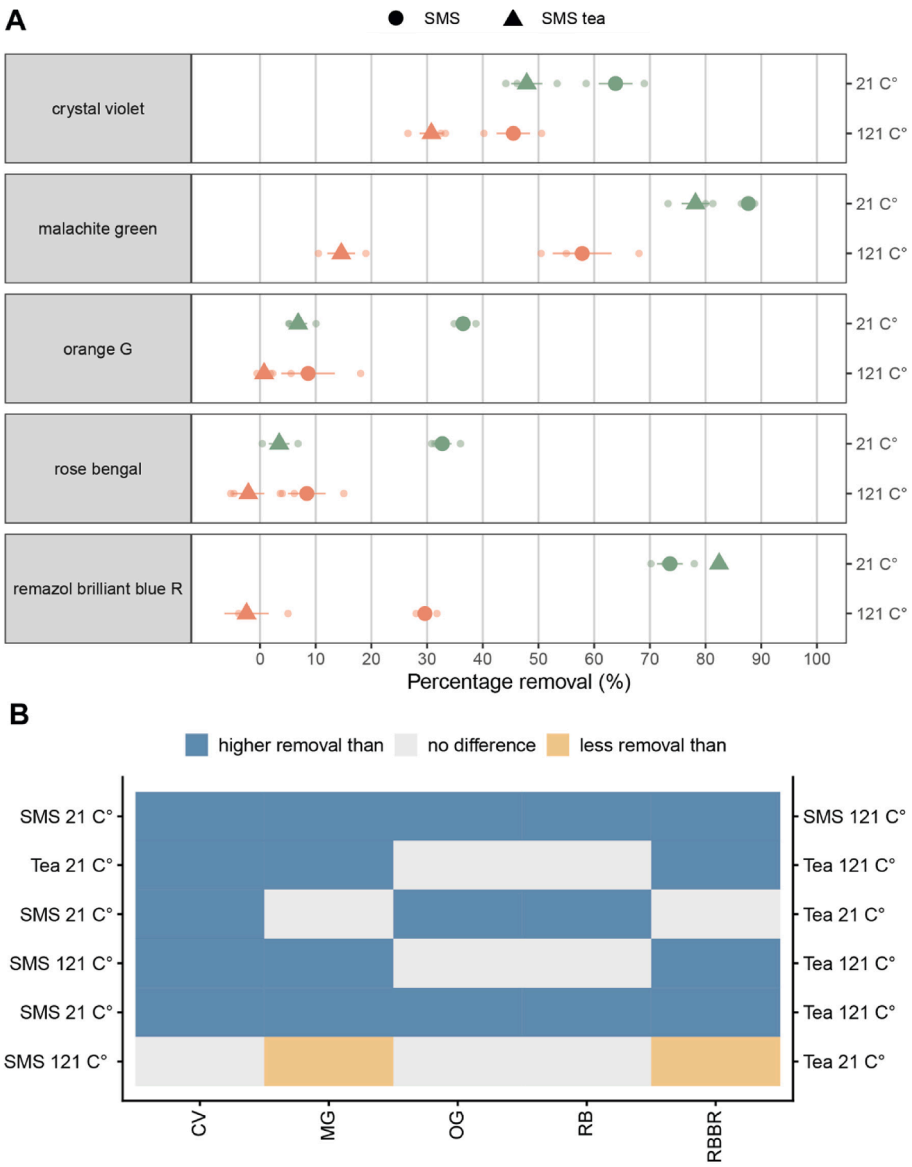

Fig. 3. Effect of temperature on the removal of crystal violet (CV), malachite green (MG), orange G (OG), rose bengal (RB), and remazol brilliant blue R (RBBR) by SMS and it tea (A). Overall, heat-treatment has an negative effect on the removal by both SMS and SMS tea (B). For instance, removal of the dyes (indicated in the x-axis) was higher ($p < 0.05$) in the case of untreated SMS (left y-axis) when compared to heat-treated SMS (right y-axis), indicated by the blue shading in the upper row. On the other hand, removal of MG and RBBR was lower in heat-treated SMS when compared to untreated tea, indicated by yellow shading in the bottom row.

2010). However, in the latter two cases additives such as H_2O_2 and / or veratryl alcohol were used.

Statistical analysis revealed that the removal rate for all dyes was highest during the first 4 h of incubation in SMS (Fig. 2). This was also the case for SMS tea except for removal of orange G and rose bengal that slowly increased in time during the 24 h incubation period. The overall higher removal of crystal violet and rose bengal by SMS when compared to its tea can be explained by sorption of the dye to the substrate particles that are present in SMS but absent in its tea. In contrast, sorption only accounts for less than 50 % of the removal in the case of orange G. Apparently, non-water extractable components in SMS have a role in discoloration of this dye. These components can be secreted enzymes that are tightly bound to the substrate particles or to the hyphal cell wall. For instance, lytic polysaccharide monooxygenases have a chitin-binding domain and are present in *A. bisporus* SMS (Frandsen and Lo Leggio, 2016; Pontes et al., 2018). Non-water extractable enzymes may also reside within the cytoplasm of *A. bisporus.* For example, membrane bound cytochrome P450 enzymes (Nelson, 2009) are involved in a wide range of catalytic reactions, mostly in hydroxylation of substrates (Anzenbacher and Anzenbacherova, 2001; Doddapaneni et al., 2013) and could thus be involved in dye degradation. The non-extractable components may also have a non-enzymatic nature that for instance are involved in hydrogen peroxide generation. Next to this, also the microbiome in SMS may differ from its tea. This difference has not yet been reported but if they indeed differ this may impact dye removal. SMS contains fungi and bacteria that can degrade dyes. For example, *Trichoderma* (Largeteau and Savoie, 2010) and Proteobacteria (Carrasco et al., 2020; McGee et al., 2017) are found in *A. bisporus* substrate. These microbes are able to remove dyes such as congo red and malachite green (Argumedo-Delira et al., 2021) and azodye reactive blue 59 (Kolekar et al., 2012), respectively.

3.2. *Effect of heat-treatment on removal of dyes by SMS and its tea*

Enzymatic activities in SMS and its tea were inactivated by a heat treatment at 121 °C. This affected removal of the dyes by SMS (Fig. 3).

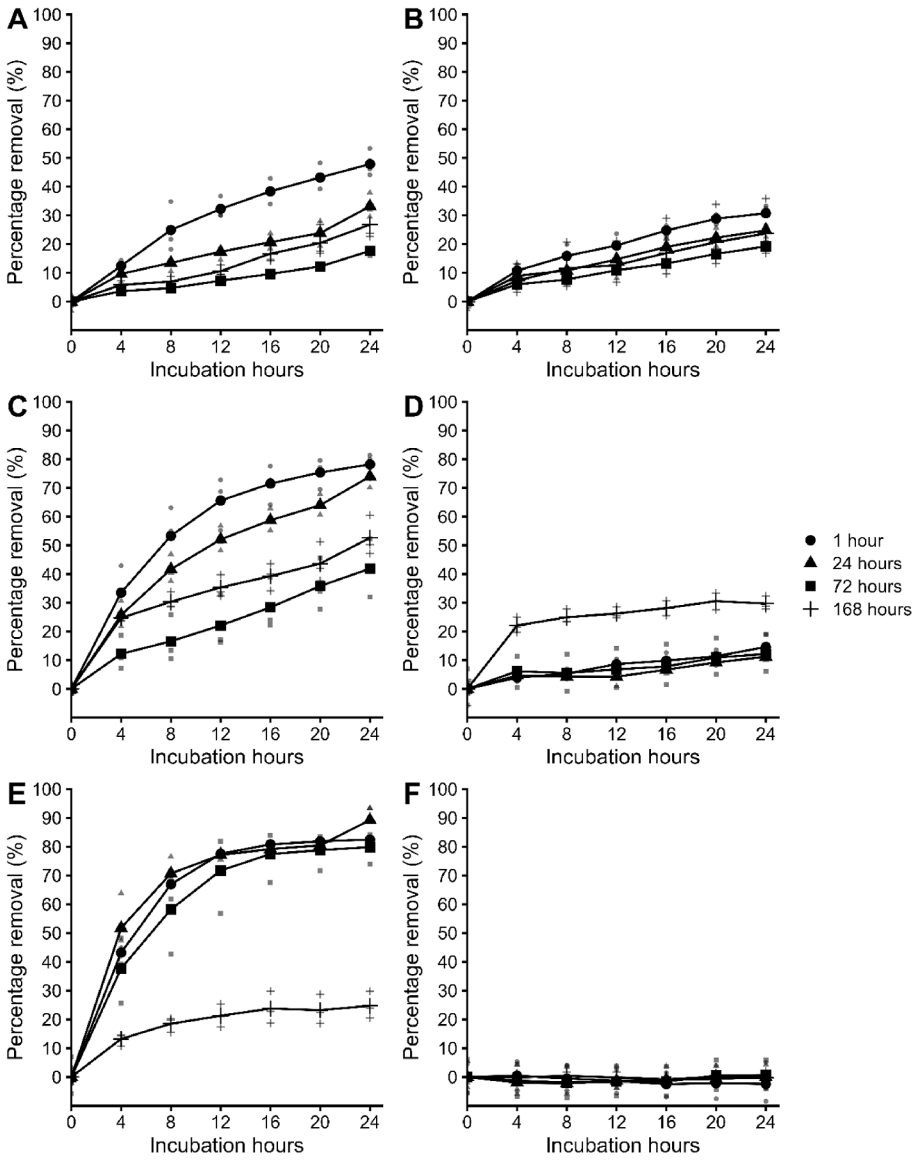

Fig. 4. Removal of crystal violet (A,B), malachite green (C,D), and remazol brilliant blue R (E,F) by SMS tea treated at 21 °C (A, C, E) and 121 °C (B, D, F). SMS tea was made by extracting SMS for 1, 24, 72 and 168 h with water.

For instance, removal of remazol brilliant blue R was reduced from 74 % to 30 %, while removal of crystal violet decreased from 64 % to 45 %. Similarly, heat treatment of SMS tea reduced removal of crystal violet, malachite green and remazol brilliant blue R from 48 % to 31 %, 78 % to 15 % and 83 % to 0 %, respectively. The heat treatment had less impact on the removal of orange G and rose bengal (from 7 % to 1 % and 3 % to 0 %, respectively) due to their low removal without heat treatment. Together, data show that all dyes are partly removed by enzymatic activity in SMS, while this also holds for crystal violet, malachite green and remazol brilliant blue R (and possibly orange G and rose Bengal as well) in its tea.

Removal of orange G and rose bengal was similar when heat-treated SMS and heat-treated and untreated SMS tea were compared. In contrast, removal of crystal violet, malachite green and remazol brilliant blue R was higher in the case of heat-treated SMS when compared to heat-treated SMS tea. Notably, untreated SMS tea was only better to discolor malachite green and remazol brilliant blue R when compared to heat-treated SMS. For the other dyes no differences between heat-treated SMS and untreated SMS tea were found (Fig. 3B). These results imply that part of the dye removing activity is of non-enzymatic nature. The nutrient and metal composition of SMS has been determined (Jordan et al., 2008; Medina et al., 2009) and include metals like copper, iron, and manganese. Because H_2O_2 is present in SMS (Vos et al., 2017), these metals could induce a Fenton-like reaction (Hussain et al., 2021). Metals and peroxide are not sensitive to heat and therefore may be responsible for the activity in heat-treated SMS and tea. Also other metals such as lead, cadmium and nickel could be released from SMS into the waste water. If this would lead to concentrations that exceed the permitted levels, these metals could be removed by combining the SMS treatment with a subsequent granular activated carbon or ion-exchange treatment (Ahmed et al., 1998; Goel et al., 2005).

3.3. *Effect of extraction time of SMS on discoloration activity*

Next, it was assessed whether the extraction time of SMS to make tea affected the removal of the dyes. To this end, tea was made by incubation of SMS in water for 1, 24, 72, and 168 h. The different teas were incubated with the dyes for 24 h. Removal of orange G and rose bengal was low (<10 %), irrespective of the extraction time to make the tea (Fig. A3). By contrast, the 24, 72, and 168 h extracted tea showed lower

discoloration of crystal violet (>10 %, >30 % and > 30 %, respectively) when compared to the 1 h extraction tea (Fig. 4A). No differences in crystal violet discoloration were found when the teas with the different extraction times were heat-treated. Discoloration of crystal violet by these teas was comparable with the removal of the untreated 24 h extracted tea (Fig. 4B). This indicates again that part of the removal of crystal violet results from a non-enzymatic system. Discoloration of malachite green was the same in the case of the 1 h and 24 h extracted teas, while it was reduced (>20 %) in the case of the 72 h extracted and 168 h extracted teas (Fig. 4C). Heat-treated 168 h extracted tea was more active in malachite green discoloration than the heat-treated teas resulting from shorter extraction time (Fig. 4D), suggesting the slow release of a non-enzymatic activity from the SMS. Remazol brilliant blue R was discolored to the same extent (84 %) irrespective of extraction time except for the 168 h extracted tea (Fig. 4E). This 168 h extraction resulted in a discoloration reduction of > 55 %. Heat treatment of the different teas resulted in the absence of discoloration of remazol brilliant blue R (Fig. 4F), indicating that this dye is discolored by enzymatic activity only.

Overall, a longer water extraction did not improve the removal of the dyes. In fact, it generally reduced the dye removing activity. This may be due to inactivation of enzymes in the water extract for instance by denaturation (Iyer and Ananthanarayan, 2008). Enzyme stability depends on the chemical composition of the medium, pH, and temperature (Fernández-Fueyo et al., 2014; Pollegioni et al., 2015). Future studies should reveal whether the chemical composition changes upon increased extraction times, thereby favouring enzyme inactivation. Enzyme inhibition could also be due to release of inhibitors such as antioxidants. These molecules are known to be present in SMS (Ghahremani-Majd and Dashti, 2015; Whiteley, 2000). Finally, the reduced activity of teas resulting from longer extraction times may also be due to a reduction of the concentration of H_2O_2 or O_2 that are used by ligninolytic enzymes to oxidize (non)phenolic compounds (Pollegioni et al., 2015). The fact that discoloration of remazol brilliant blue R is not affected by the extraction time to produce tea would imply that the enzymes that are involved in its removal are stable in the matrix and do not depend on H_2O_2 or O_2.

4. Conclusion

This study showed that SMS of *A. bisporus* and its aqueous extract can remove a variety of dyes, at least in part, from water. Results indicate that both enzymatic and non-enzymatic mechanisms are involved in the decolouration process. The use of SMS is preferred since non-water soluble components of SMS seem to be involved in optimal removal of one of the five dyes. These data and the fact that SMS of *A. bisporus* is widely available provide a great potential to use this waste stream as a cost-effective, green solution to remove a range of textile dyes from waste water.

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CRediT authorship contribution statement

Brigit van Brenk: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Leodie Kruidhof:** Formal analysis, Investigation, Methodology. **Antoine J.B. Kemperman:** . **Walter G.J. van der Meer:** . **Han A.B. Wösten:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

Part of this research was supported by Oasen N.V.

Appendix

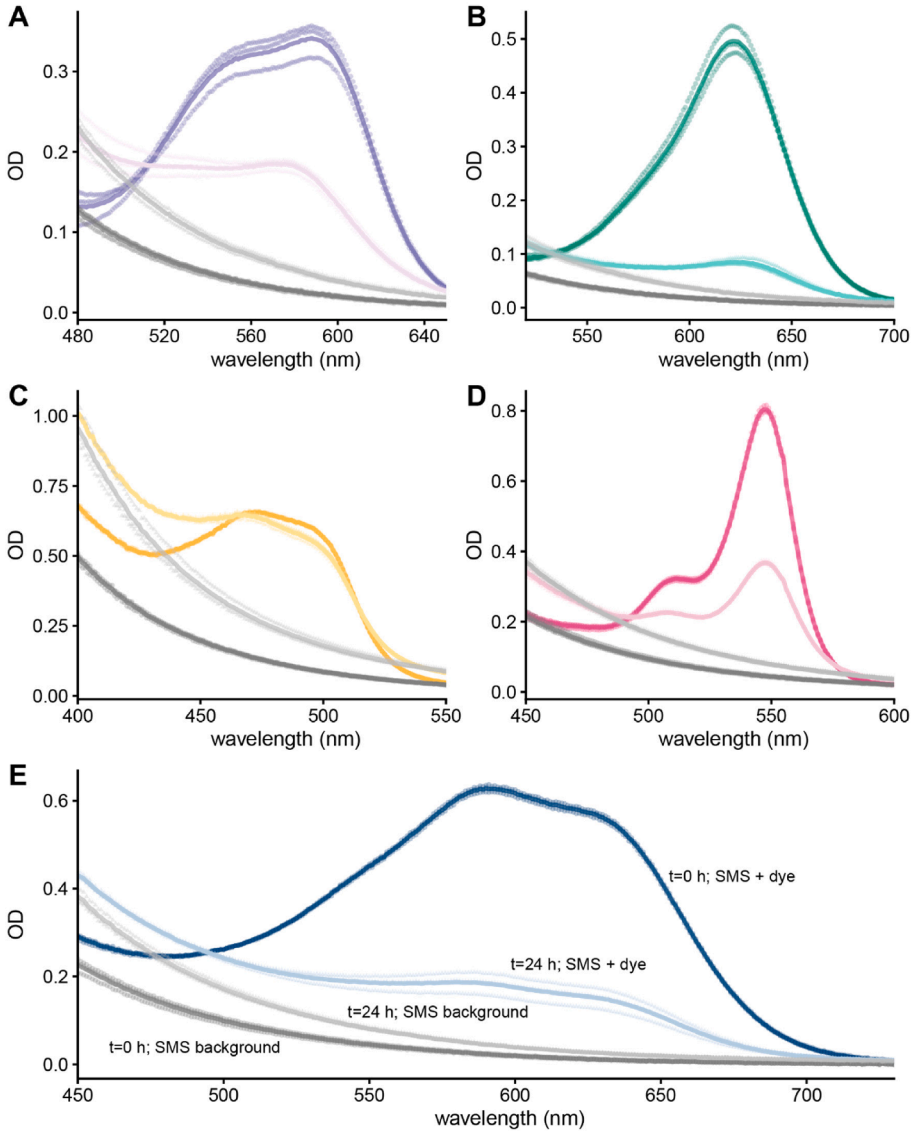

Fig. A1. Removal of crystal violet (A), malachite green (B), orange G (C), rose bengal (D), and remazol brilliant blue R (E) by SMS as shown by wavelength spectra at t = 0 and t = 24 h using as a control samples without dye. Dark and light colors represent optical density (OD) scans of SMS with dye at t = 0 and t = 24 h, respectively, while dark and light grey lines represent the background OD (i.e. SMS without dye) at t = 0 and t = 24 h, respectively.

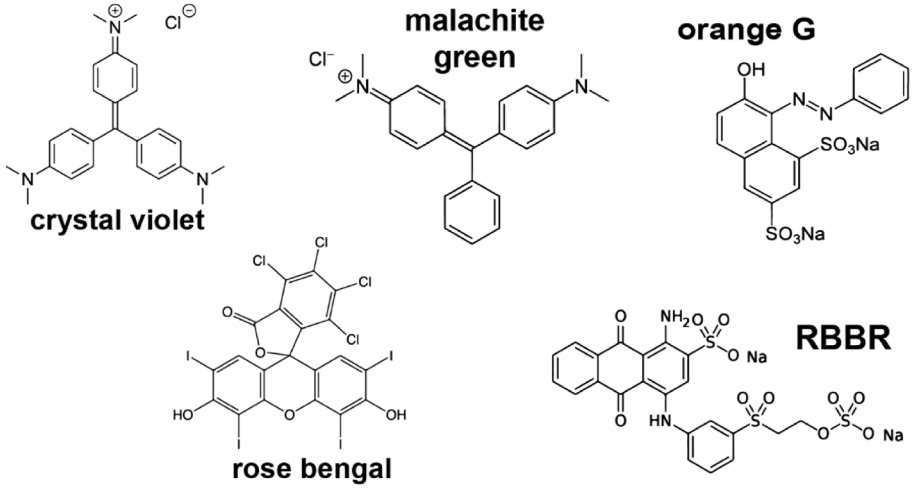

Fig. A2. Molecular structure of crystal violet, malachite green, orange G, rose bengal, and remazol brilliant blue R (RBBR).

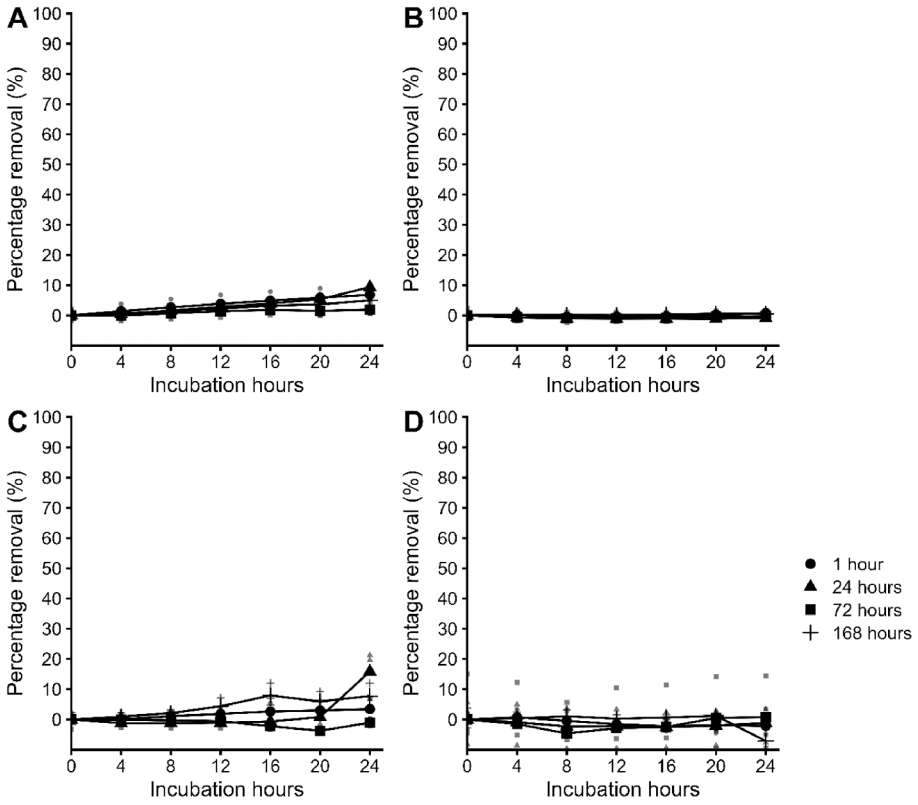

Fig. A3. Removal of orange G (A,B) and rose bengal (C,D) by SMS tea treated at 21 °C (A, C) and 121 °C (B, D). SMS tea was made by extracting SMS for 1, 24, 72 and 168 h with water.

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