Journal of Microbiological Methods 188 (2021) 106301

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A screening method for decoloration of xenobiotic dyes by fungi

Brigit van Brenk, Han A.B. Wösten *

Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584, CH, Utrecht, the Netherlands

ARTICLE INFO

Keywords:
Basidiomycete
Bioremediation
Dye decoloration
Fungus
Schizophyllum commune
Xenobiotic degradation

ABSTRACT

Wood degrading fungi are often screened for their ability to degrade xenobiotics such as dyes. Dye decoloration by these fungi on solid media could until now only be assessed qualitatively. We here describe a fast quantitative method to screen for dye decoloration on such media. Decoloration of crystal violet (CV), malachite green (MG), orange G (OG), rose bengal (RB) and remazol brilliant blue R (RBBR) by 124 isolates of the basidiomycete *Schizophyllum commune* was quantified with a flatbed scanner and the CIE-L*a*b* model. Colour and intensity changes were calculated with the Euclidean distance formula. More than 10 strains showed high MG decoloration. Isolates 136, 140 and 213 showed superior CV decoloration, while OG was most effectively decolorized by isolates 183, 216 and 227. Six strains showed high RB decoloration with isolate 216 being superior. The latter strain was also highly active on RBBR together with isolates 177 and 227. Together, dye decoloration was highly variable between the 124 isolates but strain 216 showed high activity on 3 out of 5 dyes. The fast screening method described in this paper enables identification of strains effectively decolorizing dyes.

1. Introduction

Fungi play an essential role in recycling of plant waste material in nature (Singh and Passari, 2019). Wood-decaying basidiomycetes are well known for their ability to degrade wood that is rich in lignin. Typically, these basidiomycetes are classified as white or brown rot fungi based on the ability to mineralize lignin, cellulose and hemicellulose (Blanchette, 1995; Worrall et al., 1997). Alternatively, the classification of wood-decaying basidiomycetes is based on the presence of genes encoding ligninolytic class II peroxidases (PODs) and carbohydrate active enzymes (CAZyme) (Levasseur et al., 2013; Ohm et al., 2014). Lignin degradation by wood-degrading fungi (WDF) involves non-specific oxidation. This mechanism can also be used to degrade xenobiotics such as dyes, pharmaceuticals and herbicides (Barr and Aust, 1994).

Degradation ability of lignin-like-structures is highly variable within the WDF's (Pointing, 2001; Spina et al., 2018), which may also reflect in degradation of xenobiotics. A wide variety of WDF's has been screened for their ability to degrade the latter compounds. Most screens use the decoloration of dyes, especially in liquid medium since this is easy to monitor (Kaushik and Malik, 2009). However, enzyme secretion and activity can be affected by growing in liquid instead of a solid medium (Novotný et al., 2004; Barrios-González, 2012). Therefore, WDF's are also screened on dye-containing plates but this can only be done in a qualitative way by visual monitoring (Swamy and Ramsay, 1999; Jarosz-Wilkołazka et al., 2002). We here describe for the first time a quantitative screening method for dye decoloration on agar plates based on analysing colour and intensity changes with the CIE-L*a*b* model. To this end, the acid dyes orange G (OG) and rose bengal (RB), the basic dyes crystal violet (CV) and malachite green (MG), and the reactive dye remazol brilliant blue R (RBBR) were used that find applications in the textile and paper industry and in biomedical research (Hunger, 2003). The mono-azo dye OG and the anthraquinone dye RBBR are classified as azo dyes, while RB is a xanthene dye and CV and MG are triphenylmethane dyes.

2. Material and methods

2.1. Strains and culture conditions

The model dikaryotic *S. commune* strain H4–8 (Ohm et al., 2010a; Ohm et al., 2010b) and 123 natural isolates of this basidiomycete from the Utrecht University collection (Table S1) were pre-grown on minimal medium with 1.5% agar (MMA) (Dons et al., 1979) at 25 °C for 7 days in the dark. Pieces (4 mm thick and 0.5 mm in diameter) from the periphery of these pre-grown colonies were used to inoculate 24 well plates (Greiner bio-one, Cellstar 662,102) containing MMA without dye or with 10 μg mL^{-1} CV, 10 μg mL^{-1} MG, 30 μg mL^{-1} OG, 10 μg mL^{-1} RB,

* Corresponding author at: Microbiology, Utrecht University, Padualaan 8, 3584, CH, Utrecht, the Netherlands.
E-mail address: h.a.b.wosten@uu.nl (H.A.B. Wösten).

https://doi.org/10.1016/j.mimet.2021.106301
Received 14 July 2021; Received in revised form 8 August 2021; Accepted 8 August 2021
Available online 10 August 2021

or 100 μg mL^{-1} RBBR (Sigma-Aldrich, www.sigmaaldrich.com). Cultures were packed in aluminium foil and grown at 25 °C for 14 days.

2.2. Quantifying dye decoloration

Dye decoloration was measured using biological duplicates or triplicates. To this end, plates were scanned at day 7 and 14 of culturing with an office scanner (Epson Perfection V370 Photo, www.epson.nl) resulting in 1200 dpi colour images. For each well, an area of 159,068 pixels was measured for its lightness (L*), red-green value (a*), and blue-yellow value (b*) (Schanda, 2007) by the Colour Transformer 2 plugin in ImageJ (Fiji, www.imagej.net/software/fiji). The Euclidean distance in the CIELAB model was used to calculate the dye decoloration ΔE (Eq. (1)).

$$\Delta E = \sqrt{(L0–Li)2 + (a0–ai)2 + (b0–bi)2} \quad (1)$$

Where L_0, a_0, and b_0 are L* a* b* values from the control plates without dye and L_i, a_i, and b_i from the dye containing plates at day 7 or day 14 of culturing. A low ΔE implies high decoloration of the dye.

2.3. Statistics

Statistics was done in R (RStudio Version 1.1.414, 2009–2018 RStudio, Inc.) using a *p*-value ≤0.05. A Kruskal-Wallis test with post hoc Dunn-Bonferroni Test was done to compare decoloration of dyes between all *S. commune* strains. A *t*-test was used to compare decoloration of dyes by isolates to that of strain H4.8 or the overall mean.

3. Results

Decoloration of the dyes CV, MG, OG, RB, and RBBR by 124 *S. commune* isolates was determined. The *S. commune* mycelium impacted the colour of the dye-containing plate when its bottom side was scanned (Fig. S1). Therefore, decoloration of dyes was determined by using a colonized plate without dye of each of the strains as a control (Fig. S2). A high variation in decoloration was observed between the different *S. commune* isolates (Fig. S3–7 showing all strains and Fig. 1-5 showing selected strains). None of the isolates showed higher decoloration of all the dyes when compared to model strain H4.8. This strain had a higher CV and MG decoloration activity (i.e. a lower ΔE) than the overall mean of all strains at day 7 (Fig. 1; Fig. 2; Fig. S3; Fig. S4) and day 14 (data not shown). Yet, strain H4.8 showed a lower CV decoloration activity when compared to isolates 136, 140 and 213 (Fig. 1; Fig. S3). Isolate 140 even showed a ΔE <10 of CV decoloration,

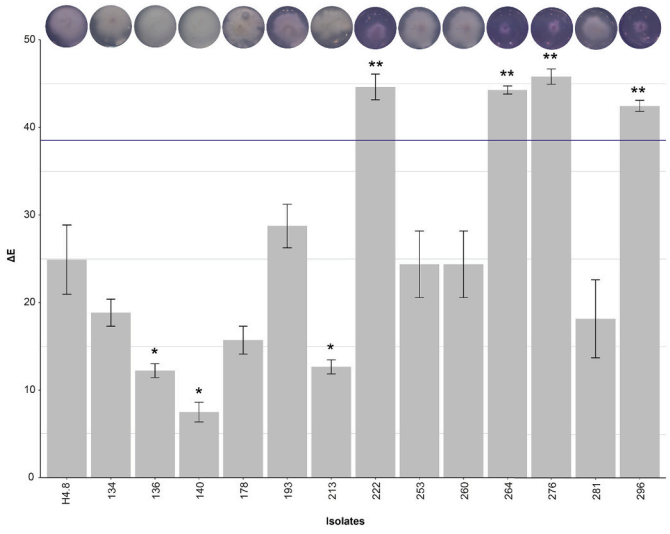

Fig. 1. Decoloration of CV by *S. commune* isolates at day 7. A ΔE below the blue line indicates a higher decoloration than the overall mean of all isolates. Isolate 136, 140 and 213 are superior in CV decoloration. Error bars represent standard deviation of 3 biological replica's. Significant differences in CV decoloration of the isolates compared to *S. commune* H4.8 are indicated with * ($p < 0.05$) and ** ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

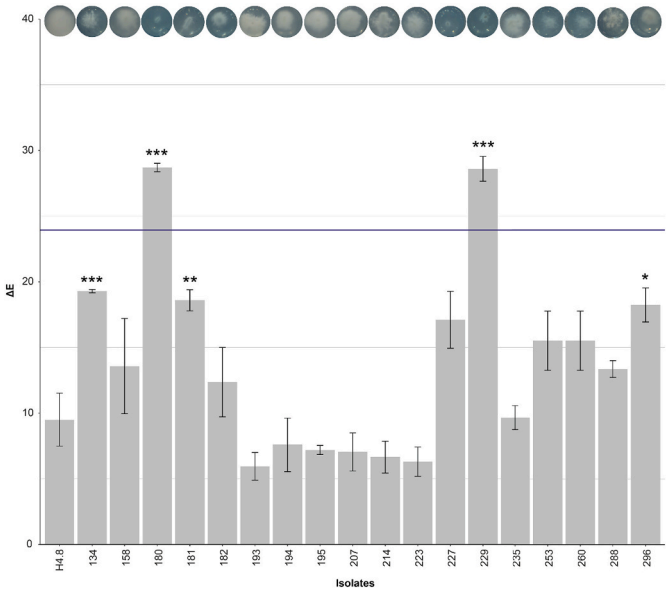

Fig. 2. Decoloration of MG by *S. commune* isolates at day 7. A ΔE below the blue line indicates a higher decoloration than the overall mean of all isolates. None of the isolates were significant better in decoloration of MG compared to *S. commune* H4.8. Error bars represent standard deviation of 3 biological replica's. Significant differences in MG decoloration of the isolates compared to strain H4.8 are indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

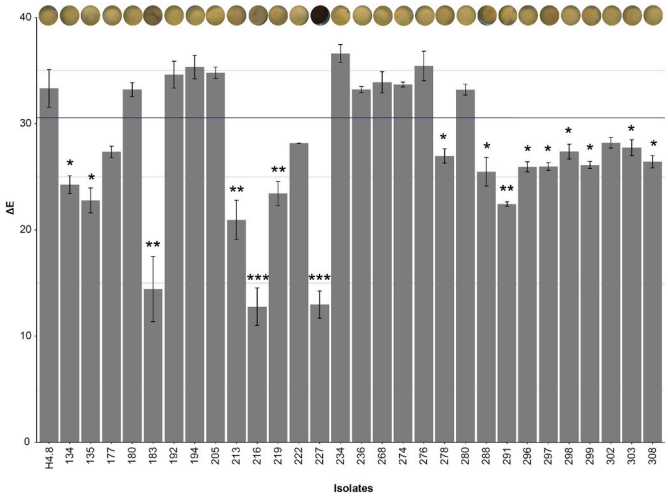

Fig. 3. Decoloration of OG by *S. commune* isolates at day 14. A ΔE below the blue line indicates a higher decoloration than the overall mean of all isolates. *S. commune* H4.8 showed the same decoloration as the overall mean of all isolates, while isolates 183, 216 and 227 performed best. These three strains produced a brown colour, indicative of the secretion of pigments. Error bars represent standard deviation of a minimum of 2 biological replica's. Significant differences in OG decoloration of the isolates compared to strain H4.8 are indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

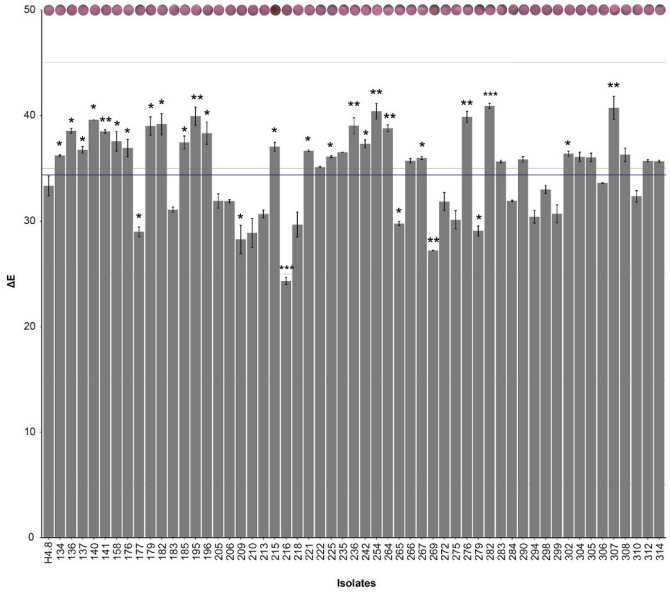

Fig. 4. Decoloration of RB by *S. commune* isolates at day 14. A ΔE below the blue line indicates a higher decoloration than the overall mean of all isolates. *S. commune* H4.8 showed the same decoloration as the overall mean of all isolates. Isolates 177, 209, 216, 265, 269 and 276 had a significant higher RB decoloration activity than strain H4.8. Error bars represent standard deviation of a minimum of 2 biological replica's. Significant differences in RB decoloration of the isolates compared to strain H4.8 are indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

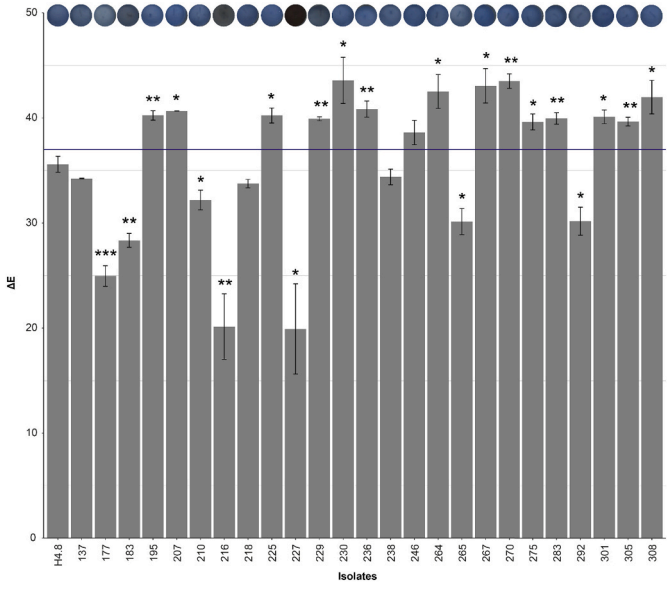

Fig. 5. Decoloration of RBBR by *S. commune* isolates at day 14. A ΔE below the blue line indicates a higher decoloration than the overall mean of all isolates. *S. commune* H4.8 showed the same decoloration as the overall mean of all isolates. Isolates 177, 216, and 227 had the lowest ΔE. Isolate 177 turned the plate brown, indicative of the secretion of pigments. Error bars represent standard deviation of a minimum of 2 biological replica's. Significant differences in RBBR decoloration of the isolates compared to strain H4.8 are indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

implying almost no CV was left. None of the isolates was significant better in decoloration of MG compared to *S. commune* H4.8 at day 7 (Fig. 2; Fig. S4) and 14 (Data not shown), while 89 isolates showed lower decoloration of this dye. The latter was often due to inhibition of growth.

S. commune H4.8 showed similar decoloration of OG, RB and RBBR compared to the overall means of all isolates at day 14. A total of 16 isolates decolorized OG better than H4–8 with isolates 183, 216 and 227 being the best (Fig. 3; Fig. S5), while isolates 177, 209, 216, 265, 269 and 276 showed higher RB decolaration (Fig. 4; Fig. S6). Strain 216 was not only superior in decolorizing RB but also showed the lowest RBBR ΔE together with isolate 177 and 227 (Fig. 5; Fig. S7). Isolates 216 and 227 turned OG, RB and RBBR plates brown, indicative of the secretion of pigments. The brown colour can interfere when screening a yellow or orange dye because this colour is in the same region as orange in the CIELAB model (Schanda, 2007).

4. Discussion

Here, we present for the first time a quantitative screening method for dye decoloration on agar media based on the CIE-L*a*b* model. This method was used to screen dye decoloration by 124 *S. commune* strains. The model strain H4.8 showed better decoloration of the triphenylmethane dyes CV and MG than the average activity of all strains, but its activity to decolorize the mono-azo dye OG, the xanthene dye RB and the anthraquinone dye RBBR was similar to the average. This and the fact that the decoloration of the different dyes between and within strains was variable suggest that the breakdown of these xenobiotics is not related to aspecific oxidation. Moreover, the fact that the different dyes were most effectively decolorized by different strains indicates that several isogenes and/or genes, coding for similar or different enzymes, respectively, are involved in decoloration of these xenobiotics. In addition, it may be that enzymes of the same family are produced with different catalytic efficiencies or that genes responsible for decoloration are differentially regulated between the isolates. Bulked segregant analysis and/or genome wide association studies (Michelmore et al., 1991; Visscher et al., 2017) may identify these genes in the future and would allow targeted xenobiotic degradation in bioremediation.

Results show that intra-species screening can be as valuable as inter-species screening, the latter being predominantly performed. The high variation in activities between the different strains of *S. commune* is probably explained by its high genetic diversity. Diversity at synonymous sites was 0.20 in 12 American strains of *S. commune* and 0.13 in 12 strains from European Russia, which is also reflected in the amino acid diversity of protein-coding genes (Baranova et al., 2015). The mutation rate in *S. commune* is estimated to be $2\ 10^{-8}$ per nucleotide per generation, which would explain the high genetic diversity of this mushroom forming fungus. Despite the variability in decoloration, strain 216 showed the highest activity on OG, RB and RBBR. The mono-azo dye OG and the anthraquinone dye RBBR are both classified as azo dyes but the xanthene dye RB is derived from a triphenylmethane (as CV and MG) by introducing an oxygen bridge (Hunger, 2003). Therefore, strain 216 either secretes an aspecific oxidation enzyme or this strain produces different enzymes that are active on different dyes.

Together, the fast screening method described in this paper enables identification of strains effectively decolorizing dyes, which can be used to identify genes involved in the degradation of xenobiotics.

Declaration of interests

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.

Acknowledgments

Part of this research was supported by Oasen that collaborates with

Utrecht University on water purification technology.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106301.

References

Baranova, M.A., Logacheva, M.D., Penin, A.A., Seplyarskiy, V.B., Safonova, Y.Y., Naumenko, S.A., Klepikova, A.V., Gerasimov, E.S., Bazykin, G.A., James, T.Y., Kondrashov, A.S., 2015. Extraordinary genetic diversity in a wood decay mushroom. Mol. Biol. Evol. 32 (10), 2775–2783. https://doi.org/10.1093/molbev/msv153.

Barr, D.P., Aust, S.D., 1994. Mechanisms white rot fungi use to degrade pollutants. Environ. Sci. Technol. 28 (2), 78A–87A.

Barrios-González, J., 2012. Solid-state fermentation: physiology of solid medium, its molecular basis and applications. Process Biochem. 47 (2), 175–185. https://doi.org/10.1016/j.procbio.2011.11.016.

Blanchette, R.A., 1995. Degradation of the lignocellulose complex in wood. Can. J. B 73 (S1), 999–1010.

Dons, J.J.M., De Vries, O.M.H., Wessels, J.G.H., 1979. Characterization of the genome of the basidiomycete *Schizophyllum commune*. Biochim. Biophys. Acta 563 (1), 100–112. https://doi-org.proxy.library.uu.nl/10.1016/0005-2787(79)90011-X.

Hunger, K., 2003. Industrial Dyes: Chemistry, Properties, Applications. Wiley-VCH, Weinheim.

Jarosz-Wilkołazka, A., Kochmańska-Rdest, J., Malarczyk, E., Wardas, W., Leonowicz, A., 2002. Fungi and their ability to decolourize azo and anthraquinonic dyes. Enzyme Microb. Tech. 30 (4), 566–572. https://doi.org/10.1016/S0141-0229(02)00022-4.

Kaushik, P., Malik, A., 2009. Fungal dye decolourization: recent advances and future potential. Environ. Int. 35 (1), 127–141. https://doi.org/10.1016/j.envint.2008.05.010.

Levasseur, A., Drula, E., Lombard, V., Couthinho, P.M., Henrissat, B., 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels. 6, 41. https://doi.org/10.1186/1754-6834-6-41.

Michelmore, R.W., Paran, I., Kesseli, R.V., 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. P. Natl. Acad. Sci. USA 88 (21), 9828–9832. https://doi.org/10.1073/pnas.88.21.9828.

Novotný, Č., Svobodová, K., Erbanová, P., Cajthaml, T., Kasinath, A., Lang, E., Šašek, V., 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biol. Biochem. 36 (10), 1545–1551. https://doi.org/10.1016/j.soilbio.2004.07.019.

Ohm, R.A., De Jong, J.F., Lugones, L.G., Aerts, A., Kothe, E., Stajich, J.E., de Vries, R.P., Record, E., Levasseur, A., Baker, S.E., Bartholomew, K.A., Couthinho, P.M., Erdmann, S., Fowler, T.J., Gathman, A.C., Lombard, V., Henrissat, B., Knabe, N., Kües, U., Lilly, W.W., Lindquist, E., Lucas, S., Magnuson, J.K., Piumi, F., Raudaskoski, M., Salamov, A., Schmutz, J., Schwarze, F.W.M.R., vanKuyk, P.A., Horton, J.S., Grigoriev, I.V., Wösten, H.A.B., 2010a. Genome sequence of the model mushroom *Schizophyllum commune*. Nat. Biotechnol. 28 (9), 957–963. https://doi.org/10.1038/nbt.1643.

Ohm, R.A., de Jong, J.F., Berends, E., Wang, F., Wösten, H.A.B., Lugones, L.G., 2010b. An efficient gene deletion procedure for the mushroom-forming basidiomycete Schizophyllum commune. World J. Microbiol. Biotechnol. 26 (10), 1919–1923. https://doi.org/10.1007/s11274-010-0356-0.

Ohm, R.A., Riley, R., Salamov, A., Min, B., Choi, I.G., Grigoriev, I.V., 2014. Genomics of wood-degrading fungi. Fungal Genet. Biol. 72, 82–90. https://doi.org/10.1016/j.fgb.2014.05.001.

Pointing, S., 2001. Feasibility of bioremediation by white-rot fungi. Appl. Microbiol. Biotechnol. 57 (1), 20–33. https://doi.org/10.1007/s002530100745.

Schanda, J., 2007. Colorimetry: Understanding the CIE System. John Wiley & Sons, New Jersey.

Singh, B.P., Passari, A.K., 2019. Biology of Macrofungi. Springer, Switzerland. https://doi.org/10.1007/978-3-030-02622-6.

Spina, F., Cecchi, G., Landinez-Torres, A., Pecoraro, L., Russo, F., Wu, B., Cai, L., Liu, X. Z., Tosi, S., Varese, G.C., Zotti, M., Persiani, A.M., 2018. Fungi as a toolbox for sustainable bioremediation of pesticides in soil and water. Plant Biosyst. 152 (3), 474–488. https://doi.org/10.1080/11263504.2018.1445130.

Swamy, J., Ramsay, J.A., 1999. The evaluation of white rot fungi in the decoloration of textile dyes. Enzyme Microb. Tech. 24 (3–4), 130–137. https://doi.org/10.1016/S0141-0229(98)00105-7.

Visscher, P.M., Wray, N.R., Zhang, Q., Sklar, P., McCarthy, M.I., Brown, M.A., Yang, J., 2017. 10 years of GWAS discovery: biology, function, and translation. Am. J. Hum. Genet. 101 (1), 5–22. https://doi.org/10.1016/j.ajhg.2017.06.005.

Worrall, J.J., Anagnost, S.E., Zabel, R.A., 1997. Comparison of wood decay among diverse lignicolous fungi. Mycologia 89 (2), 199–219. https://doi.org/10.1080/00275514.1997.12026772.