A food color based colorimetric assay for *Cryptococcus neoformans* laccase activity

Lia Sanchez Ramirez[1], Quigly Dragotakes[2], Arturo Casadevall[2]

1. Department of Molecular and Cell Biology, Johns Hopkins University, Baltimore, MD 21205, United States
2. Department of Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, Baltimore, MD 21205, United States

Address correspondence to Lia Sanchez Ramirez or Arturo Casadevall, lsanch26@jhu.edu or acasade1@jhu.edu

Abstract

Cryptococcus neoformans is a fungal pathogen that causes cryptococcosis mostly in immune compromised patients, such as those with HIV/AIDS. One survival mechanism of *C. neoformans* during infection is melanin production, which is catalyzed by laccase. Hence comparative assessment of laccase activity is useful for characterizing cryptococcal strains. After a serendipitous observation that culturing *C. neoformans* with food coloring resulted in the degradation of some dyes with phenolic structures, we associated this phenomenon with catalytic activity by cryptococcal laccase. Consequently, we investigated the color changes for the food dyes metabolized by *C. neoformans* laccase and explored using this effect for the development of colorimetric assays to measure laccase activity. We developed several versions of a food dye based colorimetric laccase assay that can be used to compare the relative laccase activities between different *C. neoformans* strains. We found that phenolic color degradation was glucose dependent, which may reflect changes in the reduction properties of the media. Our food color based colorimetric assay has several advantages over the commonly used 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay for laccase activity, including lower cost, no reversibility and easier application since it does not require constant monitoring. Our method has potential applications in environmental fields, since laccases can be used to degrade pollutants in bodies of water, and this is an efficient test to determine laccase activity. Additionally, it is useful for comparing laccase activity across different *C. neoformans* strains, which can provide insight into the expression of this virulence factor across strains.

Importance

Cryptococcus neoformans is present in the environment and while infection is common, disease occurs mostly in immunocompromised individuals. *C. neoformans* infection in the lungs results in symptoms like pneumonia, and consequently cryptococcal meningitis occurs if the fungal infection spreads to the brain. The laccase enzyme catalyzes the melanization reaction that serves as a virulence factor of *C. neoformans*. Developing a simple and less costly assay to determine levels of laccase activity of *C. neoformans*, as compared to the commonly used 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, is useful as a quick, permanent measure of laccase activity that could provide insight as to the relative virulence of the fungal cells tested. Environmental applications of this assay include determining which fungal strains would be best to degrade dye-related pollutants in wastewater.

Introduction

C. neoformans causes cryptococcosis and is a public health concern due to its high mortality rate in immunocompromised individuals, especially those with HIV/AIDS. Consequently, *C. neoformans* was recently designated as a critical priority pathogen by the World Health Organization. Most cases of cryptococcosis occur in Sub-Saharan Africa, where cryptococcal meningitis has become a major cause of death in HIV/AIDS patients, surpassing the mortality rate of tuberculosis[1]. Therefore, *C. neoformans* infections are particularly dangerous in low-resource countries where these is low access to treatment for immunocompromised individuals. Studying the ways in which *C. neoformans* protects itself against an immune response allows us to further understand the immune defenses that combat *C. neoformans* infection.

Laccase catalyzes the generation of melanin in *C. neoformans*, which protects the fungus from reactive oxygen and nitrogen oxidants produced by phagocytic cells and thus has an important role in virulence[2]. Additionally, it has been found that independently of the laccase enzyme's role in catalyzing the melanization reaction, the presence of fungal laccase may contribute to virulence independently through by reducing the formation of antimicrobial hydroxyl radicals[2]. Laccase is also involved in the generation of prostaglandins by the fungal cell that can affect local inflammatory responses[3]. Specifically, laccase is induced during glucose starvation and increased temperature (30°C) and helps moderate fungal stress[4]. Studying laccase can provide more insight into *C. neoformans* pathogenesis and its mechanisms of defense in the phagolysosome. The *C. neoformans* laccase is known to have broad structural activity, producing pigments of a spectrum of colors, including those similar to melanin following the oxidation of phenols and cetechols[5].

Laccases are known to destroy dyes and have uses in food preparation, industry, and environmental applications. Laccases degrade colors, and may be useful in reducing the environmental impact of synthetic dyes[6,7]. Factory production of non-biodegradable synthetic dyes results in contaminated wastewater and fungal laccases can be used to degrade the colors in these bodies of water[6]. The by-products of laccase catalysis are lower in toxicity than alternative chemical methods for wastewater purification and laccases have broad specificity, which allows them to break down a variety of synthetic dyes[6]. Additionally, a common substrate for laccase is phenol, a toxic pollutant which is dangerous to humans[6].

In this study, we followed up a serendipitous observation to investigate the use of mangosteen-colored food dye in a fast, efficient, low-cost colorimetric assay of laccase activity in *C. neoformans* cultures. Using absorbance spectroscopy, we were able to compare several strains of *C. neoformans* in various culture conditions to both detect and quantify relative laccase activity. Low cost and facile assays for laccase activity have many potential applications in environmental industries, including purification of wastewater. These assays are also applicable when comparing the virulence of different strains by measuring relative laccase activity, since laccase has important roles in the pathogenesis of *C. neoformans*.

Methods

Yeast Strains and Culture Conditions

C. neoformans species complex serotype A strain H99 was obtained from John Perfect in Durham, NC.[8] The lac1Δ mutant is from the 2007 Lodge library (Fungal Genetics Stock Center)[9]. The H99 GFP strain was obtained from the lab of Dr. Robin May at the University of Birmingham, United Kingdom[10]. The CNAG 01373Δ, CNAG 06646Δ, and CNAG 01029Δ strains are KN99 mutants obtained from a previously published knockout library[11]. KN99 strain obtained from Heitman Lab at Duke University Medical Center Durham, North Carolina[12].

Media preparation

Minimal media (MM) prepared with the following recipe. To make 1 L of MM, first a glass bottle was filled partly with 350 mL of Milli-Q Ultra Purified water. Then 15 mM dextrose, 10 mM $MgSO_4$, 29.3 mM KH_2PO_4, 13 mM glycine, and 3 μM thymine-HCL were dissolved in the water. A stir bar was used to ensure the components dissolved, and then the media was vacuum filtered to sterilize with a SteriCup Quick Release filter and stored at room temperature. The 20 g/L glucose MM was created by adding glucose to raise the usual 2.7 g/L glucose concentration of MM to 20 g/L, and the previous steps remained the same. Yeast Extract Peptone Dextrose (YPD) prepared with the following recipe. The following reagents were dissolved in 1 L of distilled water: 10 g of yeast extract, 20 g Peptone, and 20 g Dextrose. Then, the bottle was autoclaved and stored at room temperature.

Comparison of color change in H99 and lac1Δ mutant cultures

H99 and lac1Δ mutant strains were seeded in 3 mL of minimal media (MM) in 12-well tissue culture plates. The following colors of Limino brand food coloring (Limino Baiyun, Guangzhou, China) were used: Strawberry, Tangerine, Lemon, Lime, Purple Cabbage, Blueberry, and Mangosteen. Each well had a concentration of 10^4 cells /mL, with 10 wells containing MM supplemented with 10 μL food coloring, one well with uncolored MM, and one well with uncolored YPD. Color change observations were recorded 7 days after the plate was either left on the bench at room temperature or placed on a 120-rpm shaker in a 30°C incubator and measured either by eye or via Spectramax iD5.

Different concentrations of glucose were tested with high glucose minimal media conditions

Two H99 cultures were seeded in MM supplemented with mangosteen color in 17 mL polystyrene culture tubes. One tube was prepared with MM at its regular glucose concentration of 2.7 g/L, while the second tube was prepared with MM at an elevated glucose concentration of 20 g/L. Culture tubes were placed in the 30°C incubator with rotation and color change observations were recorded after 7 days.

Kinetic Assay for Laccase Activity

24-well tissue culture plates were seeded with 10^6 *C. neoformans* cells in 1 mL of minimal media with 10 g / L glucose in each well. Three wells were immediately treated with Thermofisher antioxidant reagent (Product Number: NP0005), then the plate was incubated at 30°C shaking overnight. The next morning, antioxidant reagent was added to the last three wells and incubated for another hour to observe possible color change of blue to green or revert to purple.

Addition of antioxidant to colorimetric assay
96-well tissue culture plates were seeded with 10^4 *C. neoformans* cells in 100 µL volumes of minimal media. The 1:1000 working dilution of antioxidant was added either at 0 h or after 24 h of observing the color change.

24-hour Assay for Laccase Activity
Every culture of interest was grown in regular minimal media and left in a 30°C incubator with rotation. A 1.5 mL volume of each culture was centrifuged at 2300g for 5 minutes in microcentrifuge tubes, and then resuspended in 1.5 mL of 10 g/L minimal media. 150 µL of the resuspended culture was pipetted into 10 wells of a 96-well plate, so each culture was measured with 10 replicates of the well. Then, 7.5 µL of a 1 to 10 dilution of Mangosteen food coloring in water (100 µL food coloring in 1 mL of H_2O) was added to each well. At 0 h, 50 µL of supernatant were placed in another 96-well plate for the initial 520 nm absorbance measurement with the Spectramax iD5 (Baltimore, Maryland) and at 24 h another 50 µL of supernatant were placed in another 96-well plate for the 24h measurement with the Spectramax iD5. For those 24 h, the plate is incubated in a 30°C incubator on 120-rpm shaker. We compared the laccase activity of the following *C. neoformans* strains: H99, H99 GFP, KN99, CNAG 01373Δ, CNAG 06646Δ, and CNAG 01029Δ.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay
H99 strain was seeded in minimal media cultures. Each culture used was washed twice with phosphate buffered saline (PBS). Then a 1:100 dilution, or 1:1 dilution for some wells, was prepared with minimal media. A 1 mL volume of 20 mM of ABTS solution was prepared and filter sterilized. ABTS was added for final concentration of 1 mM ABTS in the cultures. Incubate for 24 h in 30°C incubator on 120rpm shaker. Initial and 24 h absorbance measurements were taken at 734 nm with the Spectramax iD5.

Statistical Analysis
The statistical tests conducted for each absorbance measurement experiment are denoted in their respective figure descriptions with tests for multiple hypotheses. Two-way ANOVA with Tukey comparison analyses were conducted using RStudio Version 2023.09.1+494 and GraphPad Prism Version 10.0.2(171). Statistical comparisons were made both with all the data for each individual experiment replicate and for data from all trials pooled.

Results

Phenolic Dyes are Degraded in C. neoformans Culture
The observation that *C. neoformans* degraded some food dyes was made serendipitously. While searching for conditions to study the growth of *C. neoformans,* we noted difficulty in the measurement of fungal growth by turbidity when comparing cultures grown in minimal media and YPD. We hypothesized that the problem arose because MM was clear while YPD had a yellowish color and that adding food coloring to minimal media wells would facilitate the interpretation of growth curve absorbance data. However, after 7 d of growth, we noticed that a color change occurred in multiple wells. Upon closer inspection, we noted color changes only in colors containing a red component, whose chemical structures contained phenolic groups

(Figure 1). The resulting colors resembled the original dye if red were removed (**Figure 2A)**. Specifically, these wells that contained Food Red 7 and Acid Red 27, which are the only dyes with phenolic groups **(Supplemental Table I)**. These findings suggested that *C. neoformans* was degrading these compounds and suspicion fell on laccase because the cryptococcal laccase has broad substrate specificity and laccases had previously been reported to degrade food dye phenolic derivatives[20,21]. Next, we hypothesized that the dye degradation was a result of laccase activity and established this by replicating the experiment with ΔLac1-H99, and found no color change after 7 d. Having implicated laccase in the phenomenon we then opted for investigating whether the food degradation effect could be developed into an assay for catalytic activity given the need for new enzymatic assays.

Glucose Concentration, Culture Agitation, and Cell Density Affect Laccase Activity
We sought to optimize culture conditions for detecting laccase expression and practicality, attempting to determine conditions which would provide robust results while remaining cost and time efficient for any basic laboratory. We found that, with a standard minimal media preparation, culturing at 30°C with agitation was required to observe color change after 7 d **(Figure 2C)**.

By manipulating the concentration of glucose in the media we observed that high glucose concentrations (10-20 g/L) were able to induce color change even at room temperature and without agitation at high initial cell densities. Additionally, we saw that using glucose concentrations of 10 g/L and 20 g/L minimal media allowed us to observe a visible color change quicker than regular glucose minimal media with any cell concentration from 10^3-10^7 cells / mL in a plate with agitation at 30°C **(Figure 3).** When measuring the absorbance of these wells, we found that high glucose repressed expression as seen by lower absorbance measurements **(Figure 4A).** These lower absorbance measurements are a measurement of the loss of red color, since the absorbance at 520 nm measures absorption of red wavelengths. Through an examination of glucose-dependence, we observed a greater reduction in red color absorbance measurements in wells with cultures resuspended in minimal media with higher glucose concentrations **(Figure 4B).** An elevated glucose concentration allowed us to view color change effects quicker as compared to wells with lower glucose concentrations, and with a 24h assay we were not able to see any color change occur in the minimal media without glucose condition **(Figure 5).**

With the combined data containing all individual trials of the glucose dependence experiments measuring absorbance at 520nm, a statistically significant difference was found between the 20 g/L glucose minimal media condition when compared to the 0 g/L glucose minimal media condition ($P = 0.002$). An ANOVA test for glucose dependence was also conducted with the change in absorbance measurements between 24 h and 0 h of exposure to food coloring. In this test we found significant differences between the 10 g/L and 0 g/L condition ($P = 0.0002$), 20 g/L and 0 g/L condition ($P << 0.00001$), 20 g/L and 10 g/L condition ($P<<0.00001$), and 20 g/L and 2.7 g/L condition (($P<<0.00001$). Although not many significant differences were observed when analyzing the absorbance measurements themselves, analyzing the change in absorbance throughout the 24-hour timeframe showed differences between the glucose conditions. Overall, it was easier qualitatively to observe differences in laccase activity across different glucose conditions than it was to establish the quantitative differences which we suspect could be due to the sensitivity of equipment used.

Laccase Dye Degradation is Irreversible

One potential disadvantage of current laccase activity assays, specifically over the commonly used ABTS assay, is that the observed color change is not permanent and will eventually revert. To investigate whether our colorimetric assay was permanent, we treated wells with commercially available Thermofisher antioxidant before and after the observed color change. We found that adding antioxidant after the reaction takes place did not revert the color, nor did leaving the sample on a benchtop long term over a span of 3 weeks **(Figure 6)**. This suggests that the reaction is irreversible at the conditions that we used, allowing samples to be read at the investigator's convenience. Interestingly, when treating the culture with antioxidant at the start of the experiment, we observed a new green color. A spectrum scan of wells that showed degradation of food coloring indicated a new peak at ~420 nm appeared in these wells, suggesting that an alternative product was formed **(Figure 7).**

Developing Cheap, Available, and Lightweight Laccase Activity Assay

After optimizing parameters, we sought to design a laccase activity assay which could be set up and performed in any basic laboratory with only minimal reagents and read without instruments **(Supplemental Figure 1)**. The optimized assay uses a concentration of 10^6 cells / mL of minimal media in each well that will have food coloring added to it. Add desired food coloring to each well in a 1 to 100 dilution, and then place the plate in a 30°C incubator on a 120-rpm shaker to observe color change in around 3-7 days.

To quantify the extend of red food coloring degradation in 24h, we designed an assay using culture resuspended in 10 g/L MM. This quicker assay involves centrifuging the culture of interest and resuspending cells in 10 g/L glucose MM, with 100 µL of culture in each well in a 96-well plate and utilizing 7.5 µL of a 1 to 10 dilution of food coloring in water for each well. Absorbance measurements at 520 nm at 0 and 24 h can be used to compare differences between strains or other conditions to quantify changes in laccase activity **(Figure 8).** With a linear regression analysis, we were able to compare the rate of color degradation across different *C. neoformans* strains **(Figure 9).** From the statistical analysis of the linear regression, along with a visualization that the H99 GFP culture resulted in a quicker color change **(Figure 10)** we could determine that the H99 GFP laboratory strain had increased levels of laccase activity as compared to the wild-type H99 strain. The 3-day photo of the plate in **Figure 10** after being left on shaker in 30°C incubator showing a large change in the Mangosteen colored well in the GFP columns that is not observed in the H99 columns.

With the combined data containing all individual trials of the experiments comparing absorbance at 520nm across different *C. neoformans* strains, we observed overall significant differences in absorbance between KN99 and 01029 ($P = 0.0006$), KN99 and 01373 ($P = 0.00005$), KN99 and 06646 ($P = 0.000007$), and H99 and 06646 ($P = 0.02$). In one of our trials, we observed significant differences in absorbance between the KN99 strain and its mutant strains 01029 ($P = 0.009$), 01373 ($P = 0.003$), and 06646 ($P = 0.03$). Significant differences between the strains tested within another trial are described in the figure legend of **Figure 9**. While we observed a more noticeable difference in color change between the H99 and H99 GFP strain **(Figure 10)**, this was not always reflected in the statistical analyses particularly with the combination of multiple trials, possibly due to a loss of power with the analysis.

Advantages of food dye based colorimetric assay when relative to ABTS assay

We compared the ABTS assay, which is the commonly accepted laccase assay in the field, to our colorimetric assay. The H99 GFP well with the higher concentration of culture showed a color change within 5 minutes of adding the ABTS solution to the well, so our initial photograph shows this color change already **(Figure 11A).** However, this color change was impermanent since it was no longer viewed as clearly after 24 h passed. This places a limitation on the ABTS experiment because it is possible to miss the window of color change and not obtain the absorbance measurements or photographs needed. Additionally, with our ABTS assay we observed the clearest color change mostly within the H99 GFP strain and not the H99 strain **(Figure 11A).** With the 24h colorimetric assay, we can observe a clear color change within 24h for multiple *C. neoformans* strains and this color change exhibits permanence allowing for absorbance measurements or photographs to be taken post-color change at the investigator's convenience. Additionally, there is a significant difference in cost of materials for the ABTS assay compared to the food coloring assay. ABTS solution is sold by Roche® Life Science Products in a quantity of 300 mL for $349.00 USD at the time of this study[22]. The food coloring used in this experiment is available on Amazon.com, costing $2.66/ Fl oz[23]. One fluid ounce is equivalent to 29.57 mL, so purchasing an equivalent amount of about 300 mL of food coloring would cost about $26.99, showing that the food coloring method is a significantly cheaper way to confirm laccase activity[24].

Discussion

Laccase enzymes are involved in the survival strategies of *C. neoformans* through the process of melanization. Melanization protects the fungal cells from reactive oxidative stress, among other stressors, and investigation of the laccase enzyme role in *C. neoformans* pathogenesis can provide more insight into the fungal survival strategy. Laccases are also relevant in many aspects of the food industry and may be useful in reducing the environmental impact of synthetic dyes from factory production. Synthetic food dyes may be used as an efficient and cheaper assay of laccase activity in *C. neoformans* cultures, and a method to quantify laccase activity is of interest in a variety of fields ranging from environmental concerns from accumulation of dyes in bodies of water, and the defense mechanisms of the pathogenic fungi[25,26]. Since laccase is involved in catalyzing the melanization defense mechanism of *C. neoformans*, the relative comparisons of laccase activity across *C. neoformans* strains could be valuable in understanding differences across different strains.

Our results suggest that laccase irreversibly breaks down the red pigment known as Food Red 7 and Acid Red 27, as the lac1Δ mutant showed no color change when incubated in food color solutions. We predicted that the loss of red color was due to laccase targeting phenolic compounds in the red-containing dyes. Chemically, we hypothesized that this phenomenon occurred because the enzyme is known to catalyze formation of free radicals through "removal of a hydrogen atom from the hydroxyl group of ortho- and para-substituted mono- and polyphenolic substrates"[25]. We initially considered that agitation was necessary to promote red color degradation in the wild-type H99 strain since the cryptococcal laccase reaction uses oxygen, but it did not promote degradation for the lac1Δ mutant, and we found that plates at

room temperature without agitation still showed color change when exposed to elevated glucose conditions albeit requiring a few more days to exhibit color degradation.

Interestingly, we observed more colorimetric activity in cultures with higher concentrations of glucose. This result was unexpected, as previous literature reported increased melanization at lower glucose concentrations which we expected to correlate with laccase activity[27]. In fact, a review of laccase activity cites glucose as a repressor[4]. However, those observations refer to melanization and not to direct laccase activity[27]. Other previous findings have also found that laccase expression in *C. neoformans* is induced during glucose starvation, but also "stimulated by copper, iron, and calcium and repressed at elevated temperatures."[28] The color change in high glucose samples suggests induced laccase expression and that melanization may not always be a reliable proxy measurement for laccase activity. The glucose-dependency of color degradation shown in these experiments leads us to hypothesize that there may be a certain level of energy required for the laccase mechanisms to occur. Alternatively, the glucose effect could reflect changes in the reduction potential of the solution by adding sugar. In this regard the laccase reaction is heavily influenced by the reduction potential of the solution glucose has reducing properties as illustrated by the blue bottle experiment done in high school chemistry courses[29,30].

When comparing different *C. neoformans* strains with this assay, we found that the H99 GFP strain showed signs of increased laccase activity with an earlier visible color change and a greater change in absorbance at 520nm over time. We don't have an explanation for this phenomenon but suspect that the linkage of the GFP construct to the actin promoter of this *C. neoformans* laboratory strain could be causing secondary effects. In this regard, we note that the presence of GFP can generate free radicals and affect the oxidative state of the cell[31]. Given the dependence of laccase reaction on the reduction potential of the solution, it is possible that the enhanced color associated with GFP expressing *C. neoformans* also reflects altered oxidative conditions in the cells[29].The method that shows a 24-hour color change requires absorbance measurements to quantify differences in the extent of red color degradation by the fungi, so we have determined that the different variations of the assay could be used for different purposes depending on what is needed for the experiment. With the methods involving larger wells with a concentration of 10^6 cells / mL, these can be used to observe color change over the course of 3-7 days to provide a positive or negative conclusion on whether there is laccase activity, whereas the method involving resuspending culture in 10 g/L MM to have 100 μL per well with 7.5 μL of food coloring dilution can be used to determine differences in rate of degradation of red color over the course of 24 h. These differences between the strains did not always reach statistical significance even though the color changes were observed, and this could reflect variability sensitivity of the plate reader used and a need to further optimize the assay to improve quantification of results.

The absorbance spectrum revealed a new peak at ~420 nm in wells containing C. neoformans that degraded the food coloring, suggesting the formation of a new product. This new product was chemically stable, as we were able to detect it by the appearance of the new 420 nm absorbance. Filamentous fungi have been found to degrade a red diazo dye, using laccases to transfer the azo dye to nontoxic products[32]. On the other hand, synthetic dyes that are made up of aromatic compounds, such as azo dyes, when degraded produce amines which are mutagenic to humans[33]. These studies suggest that these could be products that were produced in our

experiments, but further investigation is needed to determine what product was produced by the fungal cells when degrading the food coloring.

Currently, the most widely used laccase activity screen in the cryptococcal field is the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay in which blue-colored ABTS is oxidized to green-colored ABTS+. However, a main disadvantage of this assay is that the ABTS reduction is impermanent, and samples must be measured before reduction of the oxidized ABTS+ can occur. The observed color change in our assay was permanent, there was not a time-sensitive window in which to observe whether the color degradation has occurred, and samples may be left for long periods of time without special preservation before measurement. We also observed differences in the degree of visible color change across strains in the ABTS assay, which so far have been mitigated by the 24-hour colorimetric assay that shows complete color change across the strains we tested. The colorimetric assay with food coloring materials is also significantly more cost-efficient when compared to the cost of ABTS solution[22].

The *C. neoformans* laccase enzyme has other functions in addition to making melanin. It is possible that laccase is functioning to deactivate molecules that can be toxic to the fungi, and it is identifying the phenolic derivatives in the red colors as needing to be oxidized. An application could be the use of cryptococcal or other fungal laccase to purify wastewater for industry. Our next steps are to further optimize the assay to ensure that more reliable quantitative analyses to quantify differences in degradation of food coloring and obtain more statistical results. We have used these results to develop a test for laccase in fungi, which can let us know whether the fungi tested has melanization ability to strengthen it against immune response along with the relative degree of the capacity for laccase activity. This is valuable information that serves as a factor to determine whether different strains of *C. neoformans* have different degrees of strength against an immune response.

Acknowledgements

Figure 2, Figure 11, and Supplemental Figure 1 were created with Biorender.com. A.C. was supported in part by NIH grants AI162381, AI152078, and HL059842. Funders played no role in the experimental design or outcome of the project.

References

1. C. neoformans Infection Statistics | Fungal Diseases | CDC. https://www.cdc.gov/fungal/diseases/cryptococcosis-neoformans/statistics.html (2022).
2. Casadevall, A., Rosas, A. L. & Nosanchuk, J. D. Melanin and virulence in Cryptococcus neoformans. *Curr. Opin. Microbiol.* **3**, 354–358 (2000).
3. Erb-Downward, J. R., Noggle, R. M., Williamson, P. R. & Huffnagle, G. B. The role of laccase in prostaglandin production by Cryptococcus neoformans. *Mol. Microbiol.* **68**, 1428–1437 (2008).
4. Zhu, X. & Williamson, P. R. Role of laccase in the biology and virulence of Cryptococcus neoformans. *FEMS Yeast Res.* **5**, 1–10 (2004).
5. Chaskes, S. & Tyndall, R. L. Pigment production by Cryptococcus neoformans and other Cryptococcus species from aminophenols and diaminobenzenes. *J. Clin. Microbiol.* **7**, 146–152 (1978).
6. Molecules | Free Full-Text | A Brief History of Colour, the Environmental Impact of Synthetic Dyes and Removal by Using Laccases. https://www.mdpi.com/1420-3049/26/13/3813.
7. Paz, A., Carballo, J., Pérez, M. J. & Domínguez, J. M. Biological treatment of model dyes and textile wastewaters. *Chemosphere* **181**, 168–177 (2017).
8. Dragotakes, Q. *et al.* Bet-hedging antimicrobial strategies in macrophage phagosome acidification drive the dynamics of Cryptococcus neoformans intracellular escape mechanisms. *PLOS Pathog.* **18**, e1010697 (2022).
9. Fungal Genetics Stock Center. https://www.fgsc.net//.

10. Voelz, K., Johnston, S. A., Rutherford, J. C. & May, R. C. Automated Analysis of Cryptococcal Macrophage Parasitism Using GFP-Tagged Cryptococci. *PLoS ONE* **5**, e15968 (2010).

11. Liu, O. W. *et al.* Systematic Genetic Analysis of Virulence in the Human Fungal Pathogen Cryptococcus neoformans. *Cell* **135**, 174–188 (2008).

12. Nielsen, K. *et al.* Sexual Cycle of Cryptococcus neoformans var. grubii and Virulence of Congenic a and α Isolates. *Infect. Immun.* **71**, 4831–4841 (2003).

13. Food Red 7. https://www.worlddyevariety.com/food-dyes/food-red-7.html.

14. Food Yellow 4. https://www.worlddyevariety.com/food-dyes/food-yellow-4.html.

15. ChemDraw.

16. PubChem. D & C Red no. 27. https://pubchem.ncbi.nlm.nih.gov/compound/83511.

17. PubChem. Brilliant Blue FCF. https://pubchem.ncbi.nlm.nih.gov/compound/19700.

18. PubChem. Acid yellow 3. https://pubchem.ncbi.nlm.nih.gov/compound/24671.

19. Karigar, C. S. & Rao, S. S. Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Res.* **2011**, e805187 (2011).

20. Polacheck, I., Hearing, V. J. & Kwon-Chung, K. J. Biochemical studies of phenoloxidase and utilization of catecholamines in Cryptococcus neoformans. *J. Bacteriol.* **150**, 1212–1220 (1982).

21. Kwon-Chung, K. J., Tom, W. K. & Costa, J. L. Utilization of indole compounds by Cryptococcus neoformans to produce a melanin-like pigment. *J. Clin. Microbiol.* **18**, 1419–1421 (1983).

22. ABTS™ Solution solution (ready-to-use), suitable for ELISA | Sigma-Aldrich. http://www.sigmaaldrich.com/.

23. Amazon.com : Food Coloring - 12 Color Vibrant Cake Food Coloring Set for Baking, Cooking, Decorating and Fondant - Upgraded Liquid Concentrated Icing Food Color Dye for DIY Treats and Crafts - .25 fl. oz. Each : Grocery & Gourmet Food. https://www.amazon.com/Food-Coloring-Decorating-Upgraded-Concentrated/dp/B0B637QP3R/ref=sr_1_3?keywords=Food+coloring+-+12+color+vibrant+cake+food+coloring+set&qid=1692153396&sr=8-3.

24. US Fluid Ounces to Milliliters conversion. https://www.metric-conversions.org/volume/us-ounces-to-milliliters.htm.

25. Tauber, M. M., Guebitz, G. M. & Rehorek, A. Degradation of Azo Dyes by Laccase and Ultrasound Treatment. *Appl. Environ. Microbiol.* **71**, 2600–2607 (2005).

26. Cordero, R. J. B., Camacho, E. & Casadevall, A. Melanization in Cryptococcus neoformans Requires Complex Regulation. *mBio* **11**, 10.1128/mbio.03313-19 (2020).

27. Paliwal, D. K. & Randhawa, H. S. Evaluation of a simplified Guizotia abyssinica seed medium for differentiation of Cryptococcus neoformans. *J. Clin. Microbiol.* **7**, 346–348 (1978).

28. Piscitelli, A. *et al.* Induction and Transcriptional Regulation of Laccases in Fungi. *Curr. Genomics* **12**, 104–112 (2011).

29. Smith, D. F. Q. *et al.* Glyphosate inhibits melanization and increases susceptibility to infection in insects. *PLoS Biol.* **19**, e3001182 (2021).

30. Adamcíková, L. & Sevcík, P. The Blue Bottle Experiment - Simple Demonstration of Self-Organization. *J. Chem. Educ.* **75**, 1580 (1998).

31. Ganini, D. *et al.* Fluorescent proteins such as eGFP lead to catalytic oxidative stress in cells. *Redox Biol.* **12**, 462–468 (2017).

32. Bhattacharya, S., Das, A., G, Mangai., K, Vignesh. & J, Sangeetha. Mycoremediation of congo red dye by filamentous fungi. *Braz. J. Microbiol.* **42**, 1526–1536 (2011).

33. Ngieng, N. S., Zulkharnain, A., Roslan, H. A. & Husaini, A. Decolourisation of Synthetic Dyes by Endophytic Fungal Flora Isolated from Senduduk Plant (Melastoma malabathricum). *ISRN Biotechnol.* **2013**, 260730 (2013).

34. Eisenman, H. C., Chow, S.-K., Tsé, K. K., McClelland, E. & Casadevall, A. The effect of L-DOPA on Cryptococcus neoformans growth and gene expression. *Virulence* **2**, 329–336 (2011).

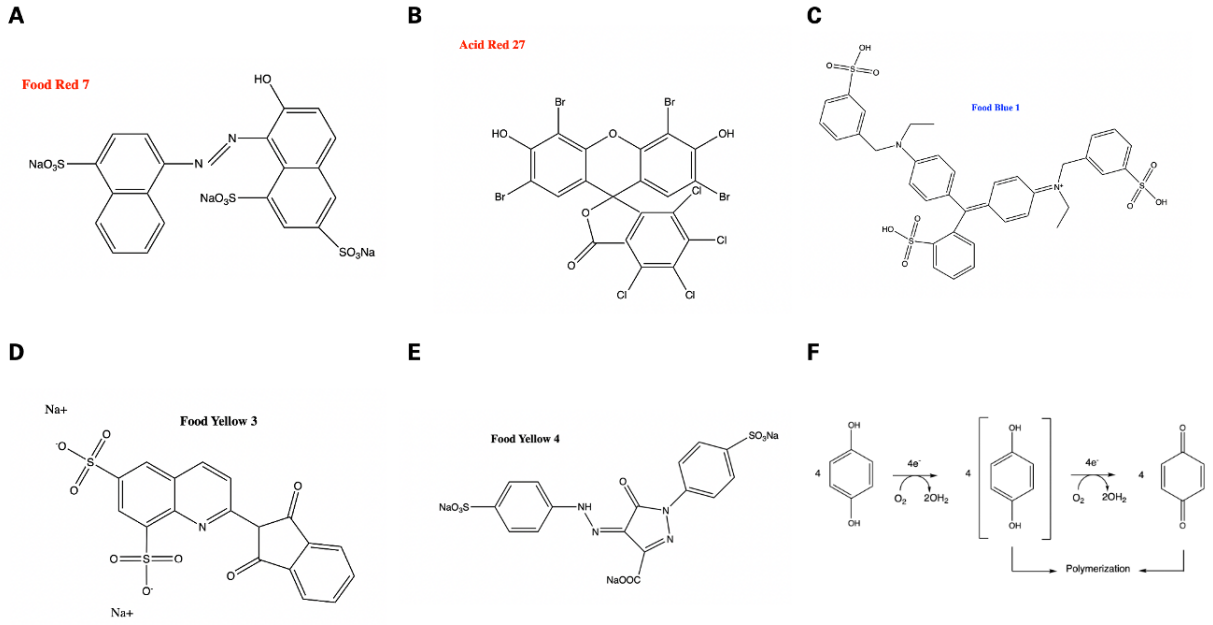


Figure 1. Chemical structures of food dyes used and melanization mechanism Panels A and E drawn from World dye variety[13,14] with ChemDraw software (Version 20.1.0.112)[15], Panels B, C, and D adapted from NIH PubChem[16–18], Panel F from Chandrakant and Shwetha[19] and redrawn using ChemDraw software (Version 20.1.0.112)[15]. **A.** Food Red 7 **B.** Acid Red 27 **C.** Food Blue 1 **D.** Food Yellow 3 **E.** Food Yellow 4 **F.** Oxidation by laccase in the melanization mechanism

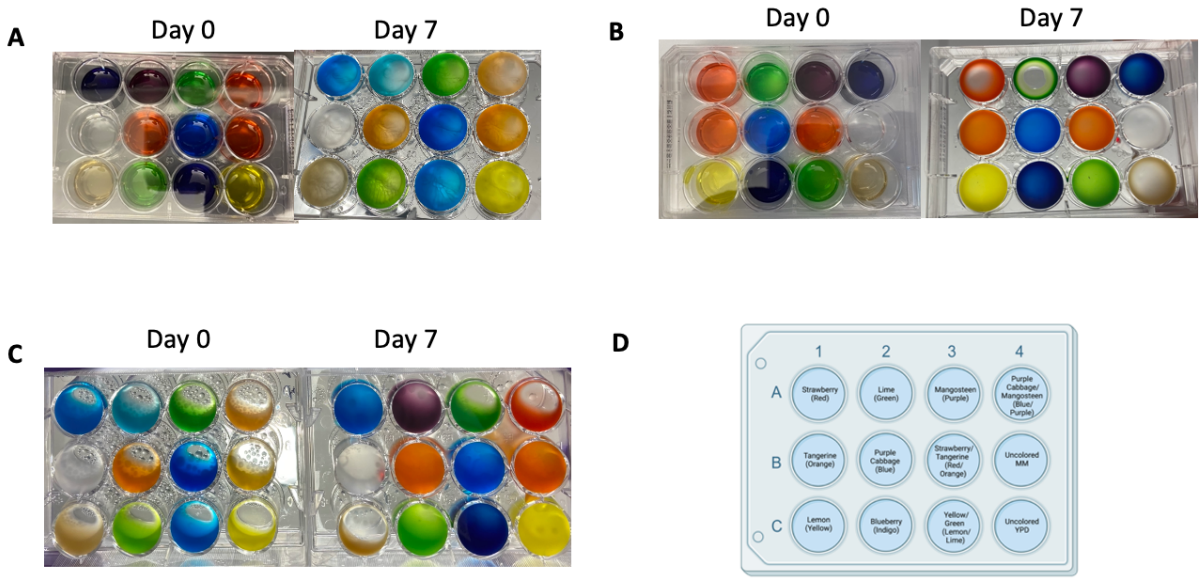


Figure 2. Observed color change results after 7 days in different temperature, agitation, and culture conditions. Experiments conducted in triplicate. **A.** Left: Plate with wild-type H99 culture. Right: After 7 days of agitation at room temperature. **B.** Left: Plate with lac1Δ mutant culture. Right: After 7 days of agitation at 30°C. **C.** Left: Wild-type H99 plate after 7 days of agitation at 30°C. Right: Wild-type H99 plate after 7 days left at the bench. **D.** Sample 12-well plate setup for color change observation, Image created with Biorender.com

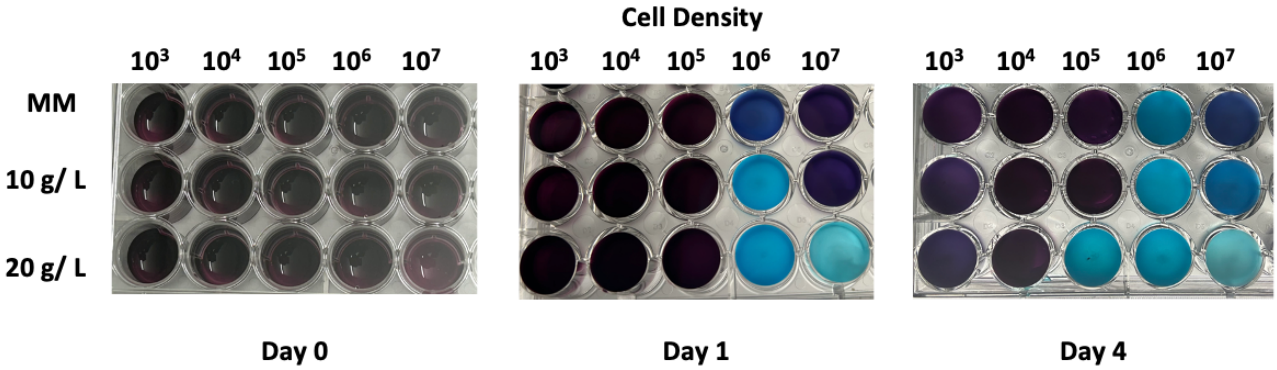


Figure 3. Color change observed in wells with varying cell densities with regular 2.7 g/L MM, MM with 10 g/L of glucose, and MM with 20 g/L of glucose. Experiment conducted for two trials.

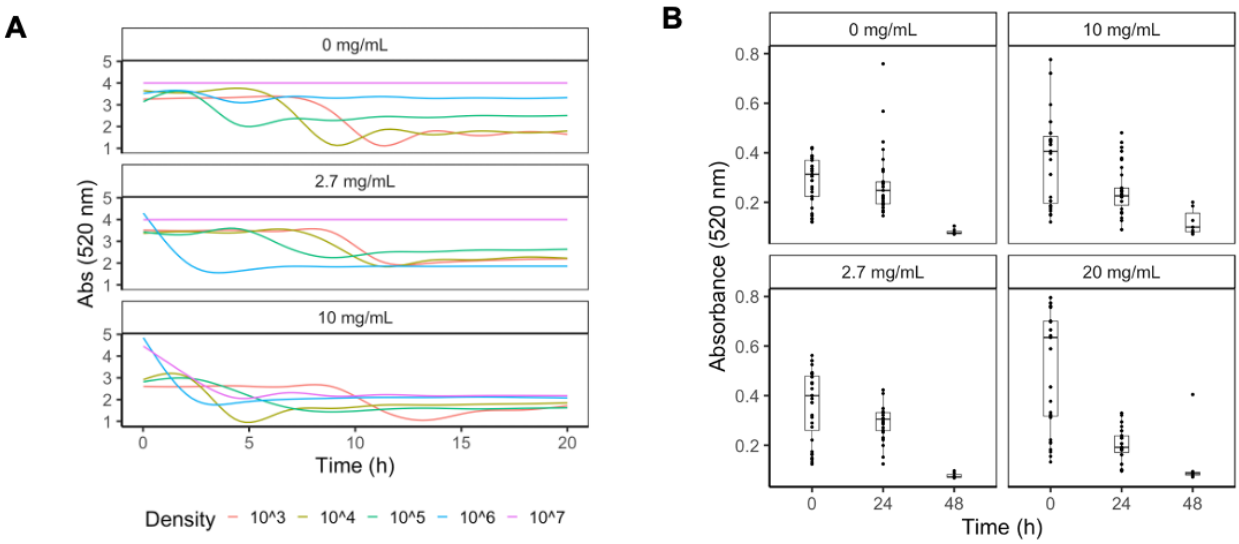


Figure 4. A. Absorbance measurements at 520 nm wavelength for wells with varying cell densities and MM glucose concentrations. **B.** Absorbance measurements of supernatant at 520nm at 0 and 24 h for different glucose concentrations. Each point represents an individual measurement.The following significant differences were determined when analyzing difference in absorbance across different glucose conditions over 24 h: 10 g/L and 0 g/L condition (P = 0.0002), 20 g/L and 0 g/L condition (P << 0.00001), 20 g/L and 10 g/L condition (P<<0.00001), and 20 g/L and 2.7 g/L condition ((P<<0.00001). Experiments conducted in triplicate.

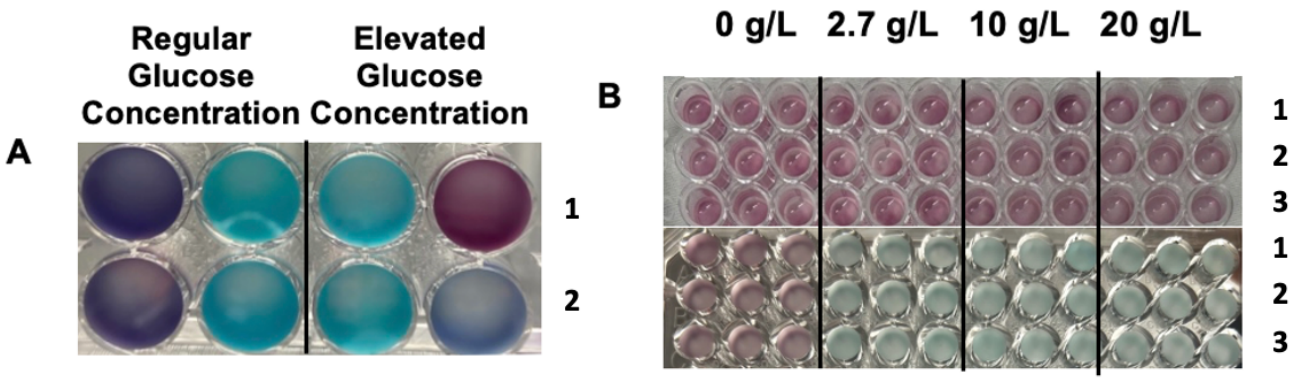


Figure 5. Glucose dependence of colorimetric assay. **A.** Left half of plate has wells with regular glucose concentration and right half of plate has wells with elevated glucose concentration. All wells contain Purple (Mangosteen) food coloring. Photo taken after 7 d. **B.** No color change observed with 0 g/L glucose minimal media in the 24 h colorimetric assay. Rows 1-3 are replicates of the given glucose condition with the H99 strain. Each experiment is conducted in triplicate, vertical columns.

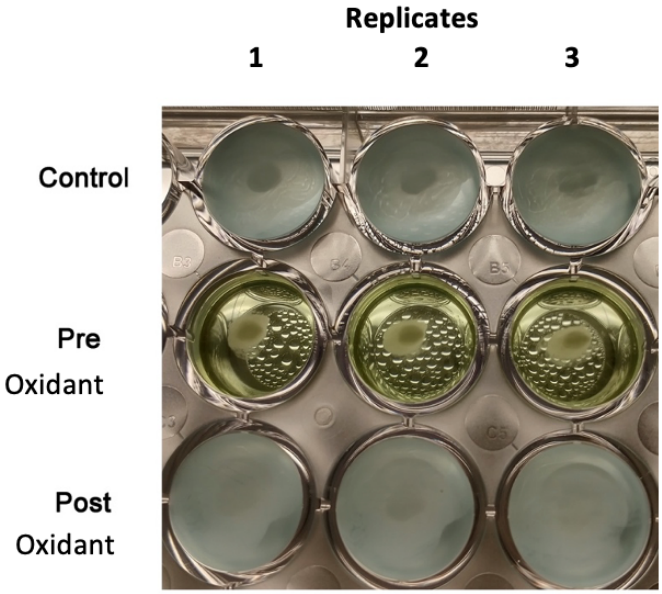


Figure 6. Antioxidant treated wells. Top row is the control row without addition of antioxidant, middle row is addition of antioxidant before color change occurs, and the bottom row is addition of antioxidant after color change occurs. Each column is a replicate of the given condition.

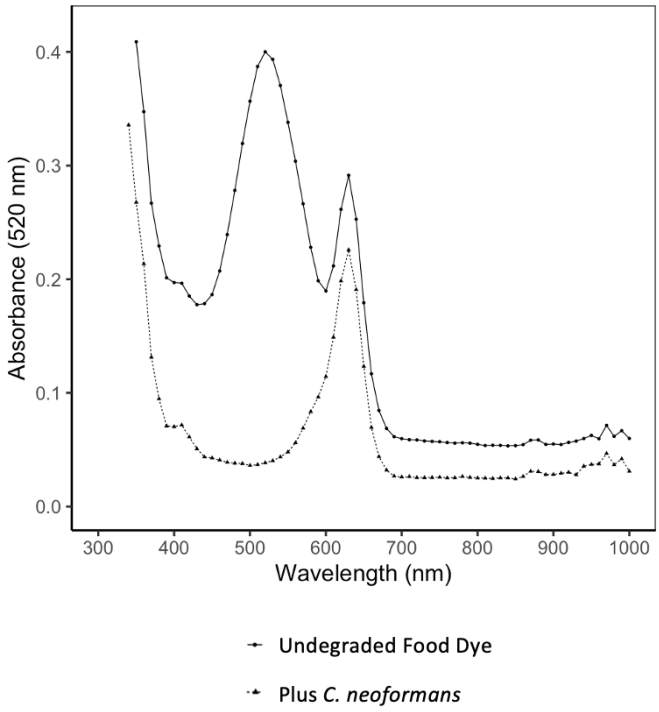


Figure 7. Absorbance measurements at different wavelengths for wells that either degraded the food coloring to produce color change or did not degrade the coloring. Note that a new peak at ~420 nm was observed in the absorbance measurements of wells with color degradation.

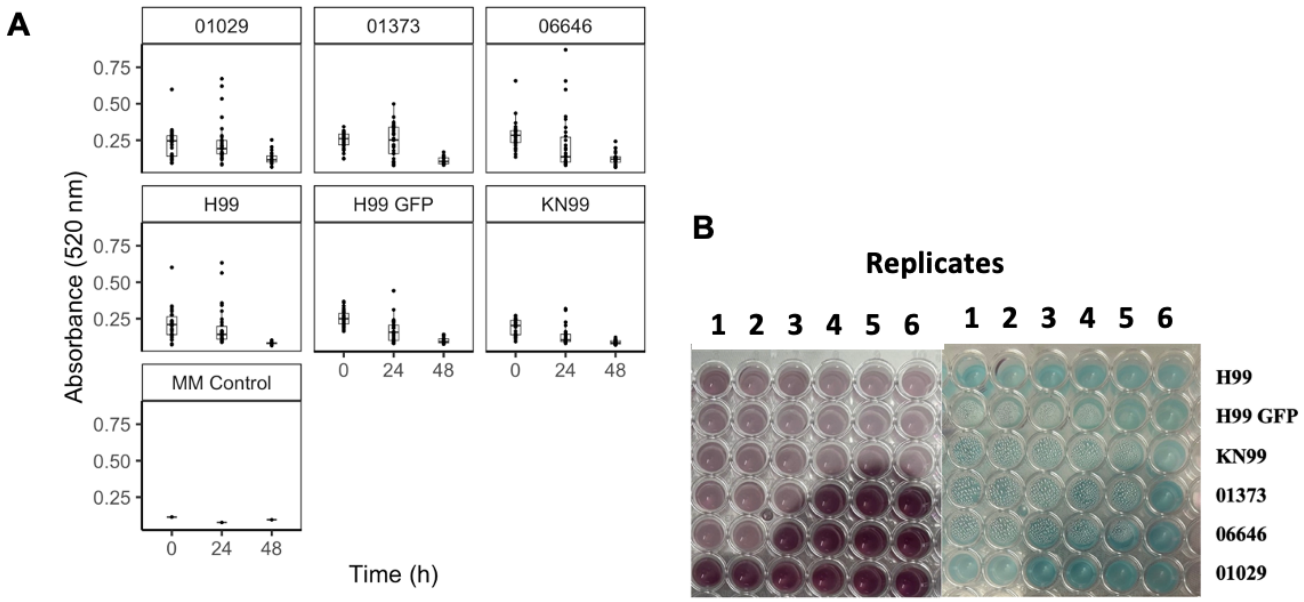


Figure 8. Optimized 24h colorimetric assay. **A.** Graphical results of comparison between different *C. neoformans* strains with 24 h assay. Each point represents an individual measurement. Overall significant differences between KN99 and 01029 (P = 0.0006), KN99 and 01373 (P = 0.00005), KN99 and 06646 (P = 0.000007), and H99 and 06646 (P = 0.02). Experiments conducted for four trials. **B.** Left: 0-hour initial image of assay set-up. Right: 24 h image of assay after color degradation

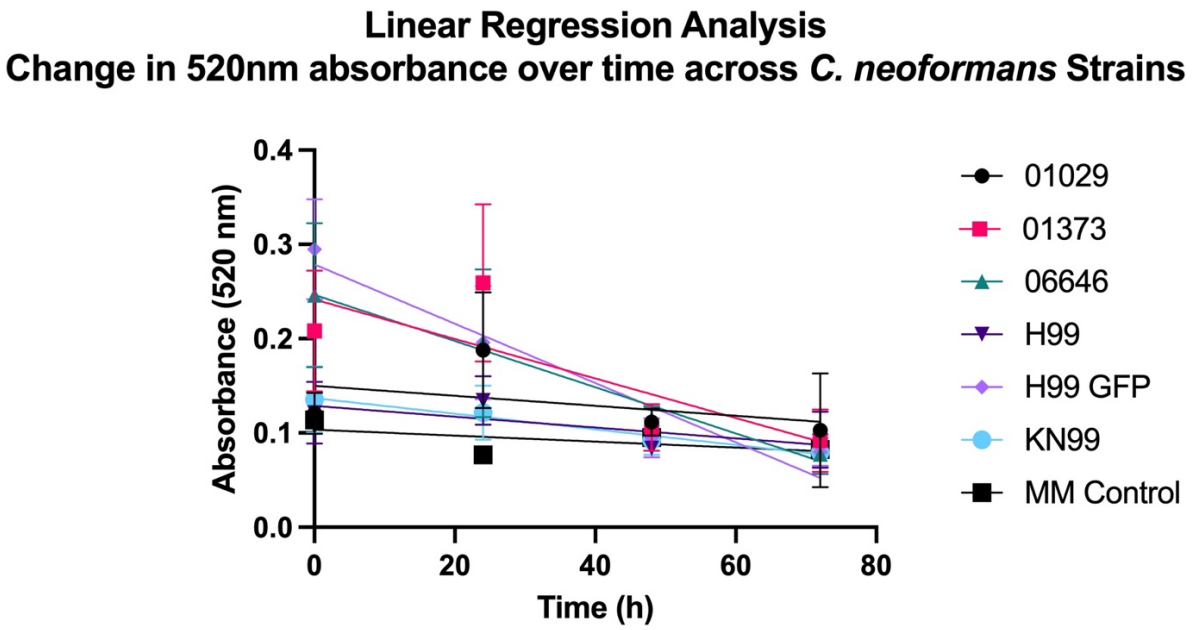


Figure 9. Linear Regression Analysis of change in 520nm absorbance over time across *C. neoformans* strains with 24 h laccase assay method. Each point represents an average of measurements. As such, the following comparisons were statistically significant: H99 and H99 GFP ($P << 0.00001$), H99 and 01373 ($P < 0.05$), H99 and 06646 ($P << 0.00001$), H99 GFP and KN99 ($P << 0.00001$), H99 GFP and 01029 ($P << 0.00001$), KN99 and 06646 ($P << 0.00001$), 01029 and 06646 ($P < 0.01$). This statistical analysis was corrected for multiple comparisons.

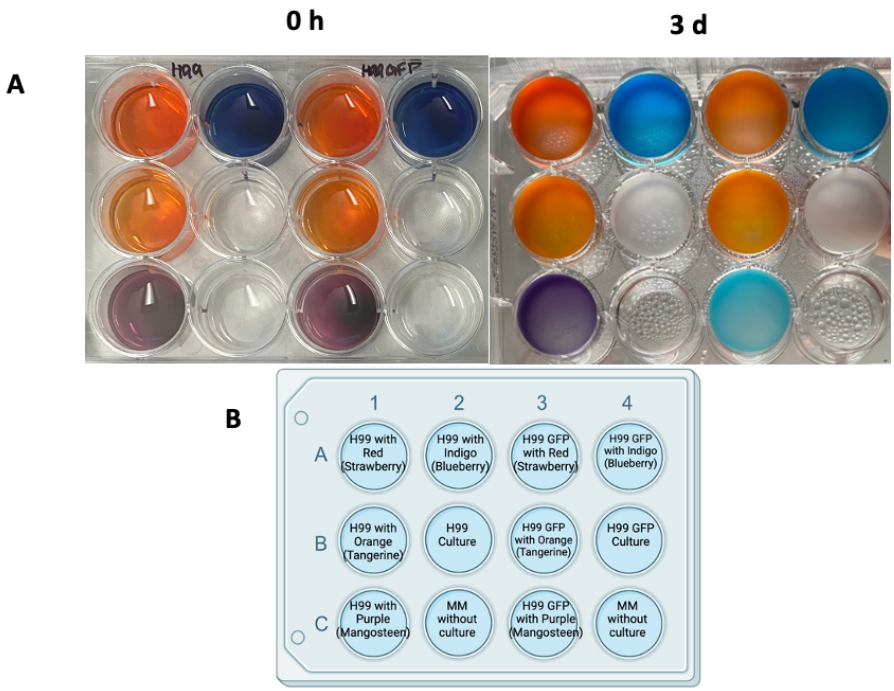


Figure 10. Laccase activity observed in H99 GFP strain after 3 d with various food dye colors. **A.** Left: 0 h photo of plate with H99 and H99 GFP wells with different colors. Right: 3 d photo of plate after being left on shaker in 30°C incubator. Experiment conducted in triplicate. **B.** Diagram of 12-well plate set up diagram for comparison between H99 and H99 GFP strain with the food coloring colors used, official names given by Limino brand in parentheses.

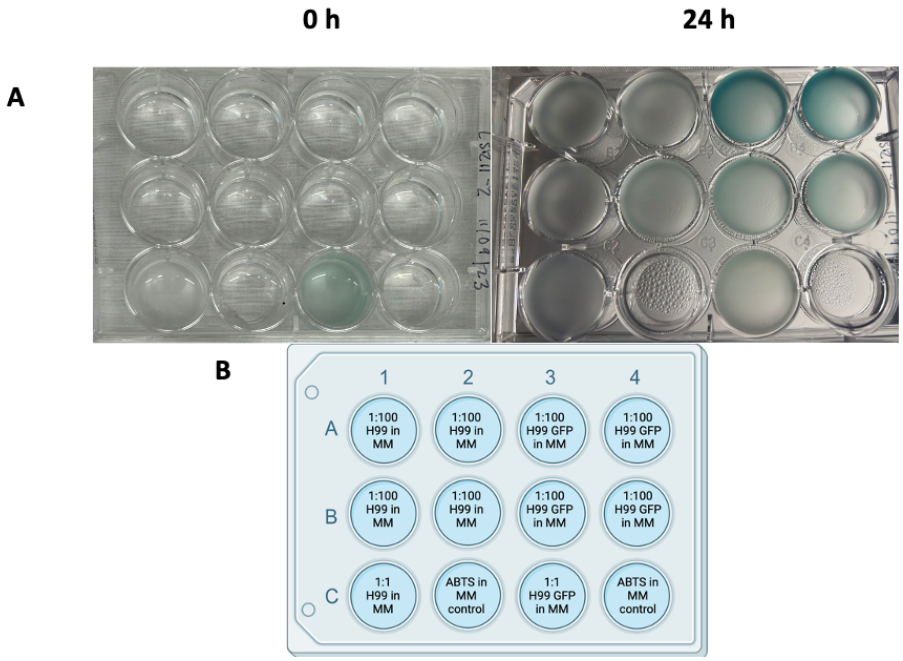


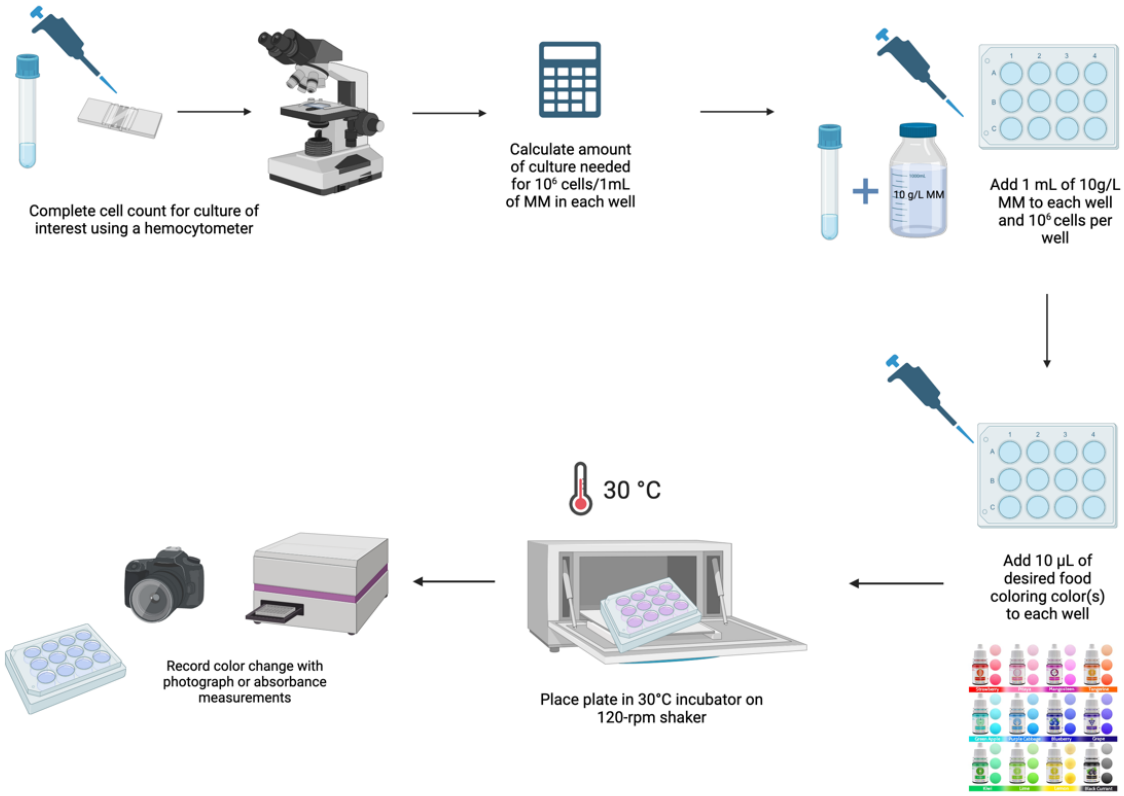
Figure 11. ABTS assay. **A.** Left: Initial photo of ABTS assay plate at 0 h. Right: Photo of ABTS assay plate after 24 h in 30°C incubator on shaker. Note that well 3C turned blue rapidly but by 24 h the color was largely gone. **B.** 12-well plate set up diagram of ABTS experiment, created with Biorender.com

Supplemental Table 1: Limino Food Coloring Ingredients

Color Name	Food Coloring Components	CAS No.
Blueberry	Acid Red 27	915-67-3
	Food Blue No. 1	3844-45-9
Lemon	Food Yellow No. 4	1934-21-0
	Food Yellow 3	2783-94-0
Lime	Food Yellow No. 4	1934-21-0
	Food Blue No. 1	3844-45-9
Mangosteen	Acid Red 27	915-67-3
	Food Blue No. 1	3844-45-9
Purple Cabbage	Food Blue No. 1	3844-45-9
	Food Yellow 3	2783-94-0
	Acid Red 27	915-67-3
Strawberry	Food Red 7	2611-82-7
	Food Yellow 3	2783-94-0
Tangerine	Food Yellow 3	2783-94-0
	Food Red 7	2611-82-7

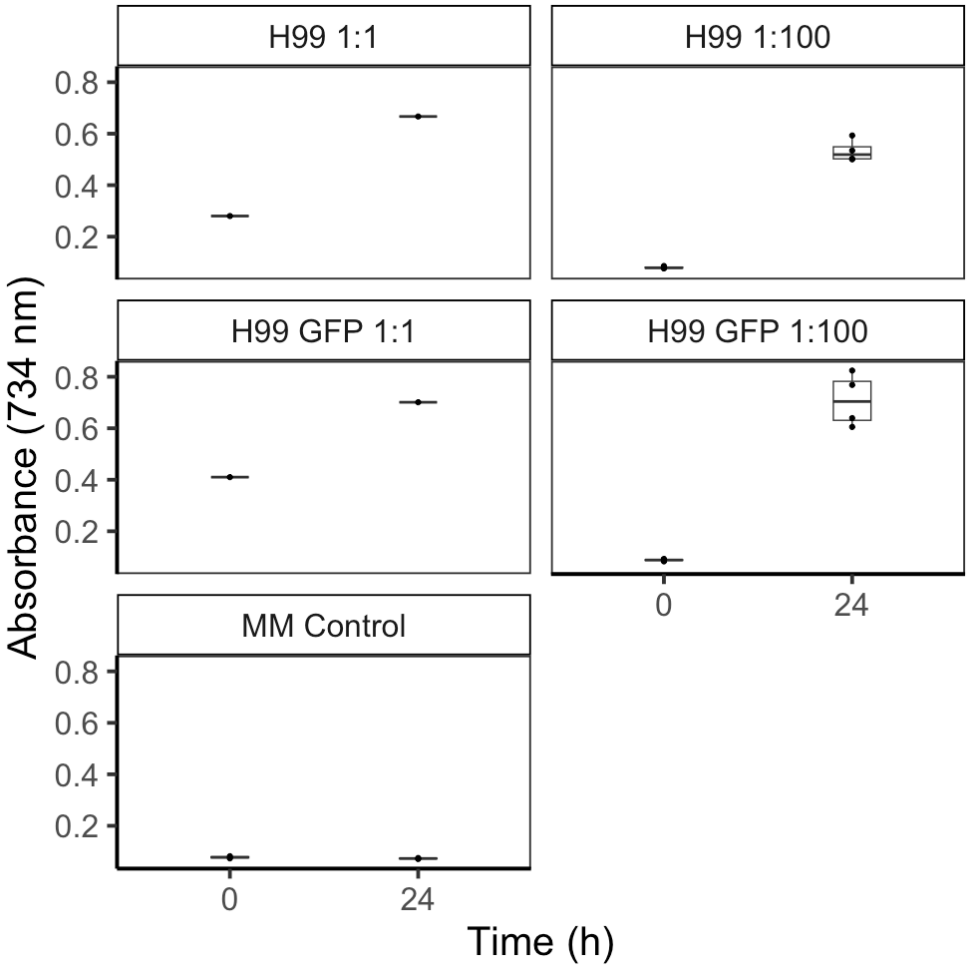
Ingredients added to the mixture: Sorbitol (CAS No. 50-70-4), Water (CAS No. 7732-18-5), Glycerin (CAS No. 56-81-5), Carboxymethylcellulose sodium (CAS No. 9004-32-4), Potassium sorbate (CAS No. 590-00-1)

Supplemental Figure 1: Lightweight Laccase Activity Assay



Lightweight Laccase Activity Assay, Image created with Biorender and photo of food coloring from Limino.[23]

Supplemental Figure 2: Difference in Absorbance over time with ABTS Assay



Difference in absorbance at 734nm wavelength for H99 and H99 GFP cultures in different dilutions in minimal media with ABTS. Each point represents an individual measurement.