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Generation of *Synechocystis* sp. PCC 6803 mutant with enhanced laccase-like activity.

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ABSTRACT

Cyanobacteria offer a good alternative to fungi for laccase production at industrial scales. Random mutagenesis approaches with ethyl methanesulfonate were used in combination with enzymatic assays screenings to select a mutant of *Synechocystis* sp., A2, with enhanced extracellular laccase-like activity. Anthraquinone dye decolourisation assay revealed 7% enhanced decolourisation in A2 relative to wild type after 24 hours. Comparison of the microbiome composition, structure and richness of the wild type and A2 strains confirmed that the improved traits were due to the mutation(s) and not the associated bacteria. The newly isolated *Synechocystis* mutant is the first example of successful random mutagenesis of cyanobacteria for laccase production. Attempts to characterise the biochemical reaction and putative laccase in A2 strain were unsuccessful but will be the subject of further research. This study suggests that random mutagenesis as a powerful approach for generating cyanobacterial strains with enhanced laccase-like activity for prospective commercial applications.

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KEYWORDS

Synechocystis, laccase, mutagenesis, ABTS, dye decolourisation.

1. INTRODUCTION

Laccases are well-recognised "green catalysts" with great biotechnological potential due to their ability to oxidise various aromatic and non-aromatic compounds (Pardo & Camarero, 2015). These include the highly toxic and persistent perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFO), and other perfluorinated chemicals (Luo et al., 2015; Suja et al., 2009). Although widely distributed in nature (Desai & Nityanand, 2011), these enzymes are primarily found in plants and fungi and have more recently been characterised in bacteria (Hamoudi et al., 2021; Khatami et al., 2022; Sharma & Leung, 2021). However, little is known about the potential of microalgal laccases to mediate the degradation of complex organic compounds. Pioneering studies have characterised cyanobacterial laccases (Afreen et al., 2017a), detecting activities in the supernatant of *Spirulina* sp. cultures (Afreen et al., 2017b). Moreover, the gene *slr1573* in *Synechocystis* sp. PCC 6803 has been identified as a putative laccase (Xue et al., 2014).

Commercial laccases are mainly produced by white-rot fungi (Mayolo-Deloisa et al., 2020), with activities typically much higher than those observed in their cyanobacterial counterparts. Nevertheless, the culture of cyanobacteria is potentially more straightforward and more cost-effective to scale up (Lau et al., 2015; Osma et al., 2011). Cyanobacteria might offer an economically feasible alternative to fungi for laccase production at industrial scales, but further research is needed to overcome the current challenges of strain selection, laccase yield, and activity improvement.

Genetically modified organisms (GMOs) are not a preferred solution for bioremediation purposes due to the environmental threat they represent. In this context, induced cell mutagenesis and mutant selection have been suggested as an alternative approach to genetic engineering for improved bacterial and microalgal strains (Hlavova et al., 2015; Labrou, 2010), and have been successfully used to obtain desirable phenotypes with biotechnological relevance. For instance, laccase activity, stability, and dye

decolourisation ability was increased in the bacterial species *Bacillus licheniformis* by random and site-directed mutagenesis for the treatment of dyestuffs (Bu et al., 2020). Similarly, the functional properties of two fungal laccases of the oyster mushroom *Pleurotus ostreatus* expressed in yeast were optimised by directed evolution, where serendipitous but still advantageous mutations were generated through error-prone polymerase chain reaction (PCR) (Festa et al., 2008). Several similar studies with fungal laccases have taken advantage of *Saccharomyces cerevisiae* as a heterologous host (Maté et al., 2011), while overcoming the issues of *in-vivo* DNA recombination and protein secretion, frequently observed in other organisms. In microalgae, random mutagenesis has been used to promote the production of intracellular lipids in one of the most ubiquitous marine microalgae, *Nannochloropsis* sp. (Doan & Obbard, 2012), improved phototolerance (Narusaka et al., 1999), tryptophan production (Deshpande et al., 2020) and thermotolerance (Tillich et al., 2014) in the model cyanobacteria *Synechocystis* sp. PCC 6803, as well as carbohydrate (Kamravamanesh et al., 2019) and poly-β-hydroxybutyrate (Kamravamanesh et al., 2018) in *Synechocystis* sp. PCC 6714.

Given that random mutagenesis approaches have been successfully implemented in bacteria and fungi for laccase improvement, and particularly in *Synechocystis* for other purposes, they have great potential for optimising laccase activity in *Synechocystis*. For this study, mutants of *Synechocystis* sp. PCC 6803 (herein referred to as Syn6803) were generated by random mutagenesis, using ethyl methanesulfonate (EMS) as a mutagen. Single mutant cells were subsequently separated by fluorescence-activated cell sorting (FACS) and screened by enzymatic assays to select and characterise mutants with enhanced extracellular laccase-like activities. Extracellular activity is desirable for industrial applications as the enzyme is readily available in the supernatant, removing the need for cell harvesting and lysis, and therefore reducing production costs. Our results summarise the screening of more than 2,300 Syn6803 mutants, the subsequent characterisation of the improved strain, and its optimum growth conditions for the best laccase-like activities. The possible interference of bacterial laccase activities with the observed patterns of laccase yield was also assessed. The random mutagenesis approach presented here demonstrated that bioremediation traits of *Synechocystis* sp. PCC 6803

can be significantly enhanced, describing for the first time a simple workflow for the successful engineering of cyanobacteria with potential commercial applications.

2. MATERIALS AND METHODS

2.1 Random mutagenesis

Syn6803 was purchased from the Pasteur Institute of Cyanobacterial Culture Collection (France) and used in random mutagenesis approaches to generate mutants with enhanced laccase-like activities. Mutants were generated concomitantly with Price et al. (2021). Briefly, Syn6803 wild type (herein referred to as WT) cells were inoculated at 2 x 10^6 cells mL^{-1} in standard BG11 liquid medium. Upon day 7 of cultivation, exponential growth phase cells were exposed to concentrations of ethyl methanesulfonate (EMS, Sigma Aldrich, 0.5, 1, 1.5, 2, 2.5, 3 and 4 M) for 1 hour in darkness at 25 °C and 300 rpm of shaking. Cells were recovered by centrifugation at 3000 x g for 8 mins, washed 3 times with 10% w/v sodium thiosulphate to remove any leftover residue of EMS, resuspended in 1 mL of BG11, and left overnight in darkness to prevent photoreactivation repair of damaged DNA. Cultures were maintained at 25 °C, 50 µmol photons $m^{-2}s^{-1}$ light, and on a 16:8 day-night cycle until cells reached stationary growth phase. Based on our established kill curve, viable mutant cultures that survived 21 days after exposure to EMS (2.5, 3, and 4 M) were selected for further enzymatic screening. Single viable mutant cells were separated by fluorescence-activated cell sorting (FACS) on a flow cytometer (BD Influx). Cells were excited by a 488 nm laser and detected by their chlorophyll fluorescence in a 692 ± 40 nm channel. Cells with the highest chlorophyll signals were sorted into 96-well plates containing 200 µL of BG11 medium per well, using single cell-sort mode (one cell per well). Cultures were maintained under the growth conditions described above for 5 weeks, and 192 of the surviving cultures were transferred to 48 well-plates. The plates were subsequently grown at 30 °C, 50 µmol photons $m^{-2}s^{-1}$, on a 12:12 hours day-night cycle for the duration of the mutant screening process, since these conditions optimise growth and laccase production (**Figure 1**).

2.2 Culture conditions

Syn6803 WT and mutants were maintained photoautotrophically in 48-well plates with BG11 liquid medium (Waterbury & Stanier, 1981) using optimal conditions for Syn6803 growth/laccase production at 30 °C, 50 µmol photons $m^{-2}s^{-1}$ using a cool fluorescent white light, on a 12:12 hours day-night cycle. To reduce the risk of bacterial contamination during the screening process, an antibiotic (tetracycline at 1 µg mL^{-1}) that was previously reported to enhance Syn6803 culture density (Pomati et al., 2004) was added to the medium prior to inoculation. Strains were maintained under antibiotic pressure (tetracycline 1 µg mL^{-1}) to prevent bacterial contamination and as a treatment in the induction experiment. All other experiments were conducted without tetracycline. WT and mutants were subcultured every two weeks. Syn6803 WT and the best mutant characterised in this study (herein referred to as A2, improved strain) were grown photoautotrophically in 100 mL BG11 in tissue culture flasks with vented cap (Falcon®, Corning), at 30 °C, 50 µmol photons m^{-2} s^{-1} (12:12 hours), 100 rpm, before the enzymatic and dye decolourisation assays. WT and A2 strains were also maintained axenic on BG11 agar plates.

2.3 Cell growth monitoring

Cell growth was monitored using optical density, as it is considered to reflect growth of cells in the culture. Absorbance at 750 nm (OD_{750nm}) was measured using a microplate reader (Infinite M 1000 PRO, Tecan, Männedorf, Zürich, Switzerland).

2.4 Enzymatic assays (ABTS assays)

Randomly generated mutants were screened for their capacity to oxidize the biochemical reagent 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS), which can be measured in a colorimetric assay, where a dark blue colour develops proportionally to ABTS oxidation (Alcalde & Bulter, 2003). ABTS was used as a substrate to detect laccase-like activity in culture supernatants (i.e., secreted enzyme) during the exponential growth phase (More et al., 2011). Briefly, the ABTS assay was carried out at room temperature in a 250-µL reaction containing 20 µL of ABTS (2 mM), 200 µL of sodium acetate buffer (0.2 M, pH 6.5), and 30 µL of microalgal culture supernatant. Absorbance was measured at 426 nm using a microplate reader (Infinite M 1000 PRO, Tecan, Männedorf, Zürich, Switzerland) immediately after adding the

culture supernatant, and recorded for 50 min at 30 s kinetic intervals. The same process was repeated for each of the 48-well plates where EMS mutants were maintained. The best 10 mutants from each round were then grown on the same plate and a last round of screening was performed to select only the best 6 mutants out of the 192 initially surviving cultures (**Figure 2**).

2.5 DNA extraction and 16S amplicon sequencing

Although Syn6803 WT and A2 strains were axenic, their associated microbial communities were analysed to exclude any difference in enzymatic activity between the two strains that could potentially be attributed to bacterial contamination. Cultures were maintained on tetracycline (final concentration 1 μg mL^{-1}) for one generation (14 days) to limit potential bacterial contamination and then inoculated into 50 mL of BG11 liquid medium. Subsequently, cultures as biological triplicates were harvested by centrifugation (4000 x g for 10 mins). For each strain, the supernatant was used for *in-vitro* dye decolourisation as described below, and the biomass was used for DNA extraction. The DNA was extracted from a 200-μL cell pellet resuspended in 300 μL of PowerBead Solution, using the DNeasy® UltraClean® Microbial Kit (QIAGEN, Hilden, Germany) as per the manufacturer's protocol. The DNA quality was analysed using spectrophotometry (NanoDrop 2000, Thermo Scientific, Waltham, MA). The hypervariable regions V3-V4 of the 16S rRNA gene were amplified with the universal bacterial primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). Genomic DNA was used to prepare DNA libraries following the Illumina 16S Library Preparation Protocol (Illumina Part # 15044223 Rev. B.). Sequencing was performed on the Illumina MiSeq platform (2 x 300 bp), using a Reagent Kit v3 600-cycle and approximately 10% PhiX control spike-in at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia).

2.6 Bacterial community analysis

De-multiplexed sequencing reads were processed through our in-house 16S rRNA processing pipeline in R (v.1.4.0). Briefly, sequences were trimmed, quality-filtered, dereplicated, and denoised using the DADA2 (v.1.21.0) pipeline. An Amplicon Sequence Variants (ASVs) abundance table (i.e., count table) was built following

taxonomy assignment with DECIPHER (v.2.20.0), based on the Silva database (v.138, accessed 03/09/21). Chimeras and sequences classified as "Synechocystis" were removed from the data set. Data were normalised using DESeq2 (v.1.32.0) for beta diversity analyses, and differences in community composition were characterised based on Bray-Curtis dissimilarities, using the tidyverse (v.1.3.1), phyloseq (v.1.36.0), and vegan (v.2.5.7) R packages. Alpha diversity was estimated by calculating the Chao1 and Shannon's diversity indices. Plots were generated using ggplot2 (v. 3.3.3). All statistical analyses were performed on genus-level relative abundance data.

2.7 Best harvest experiment

Syn6803 A2 was monitored over 29 days. Cell growth and laccase-like activity in culture media were measured every two days until cells reached the stationary growth phase. Cell growth was measured as optical density at 750 nm, and laccase-like activity was detected through ABTS assays of the culture supernatant. The cell growth phase when laccase-like activity peaked was chosen as the best time of harvest.

2.8 Induction experiment

A2 cultures were treated with final concentrations of 2.5% absolute grade ethanol, 1 mM guaiacol, 1 mM 2,5-xylidine and 1 μg mL^{-1} tetracycline. All chemicals were purchased from Sigma-Aldrich Pty Ltd (North Ryde, Australia). Inducers were added to fresh growth medium at the time of inoculation (day 0) to establish the best induction conditions for the highest laccase-like activities in strain A2. Equal amounts of BG11 liquid medium were added to control flasks in place of an inducer. Ethanol 1% was used as a control for tetracycline, guaiacol, and 2,5-xylidine treatments, as these chemicals were dissolved in ethanol. Cell growth and laccase-like activities were measured in the growth medium as described previously every two days until cells reached stationary growth phase. Cultures were also induced in mid-exponential phase (day 13) and compared to those induced at the time of inoculation (day 0) to assess the influence of culture cell density on laccase chemical induction – i.e., potential effect of the cell cycle on inducers reactions with laccase. These analyses were performed on extracellular medium (supernatants), assuming that part of the laccase produced in strain A2 is secreted from the cell.

2.9 *In-vitro* dye decolourisation assays

The dye decolourisation abilities of Syn6803 WT and A2 strains were compared *in-vitro* using the anthraquinone dye Remazol Brilliant Blue R (RBBR) (Soares et al., 2001), and culture supernatants harvested at mid-exponential growth phase. Each RBBR decolourisation assay was carried out in a 1 mL final reaction volume, containing 100 µL RBBR (100 mg L^{-1}), 732 µL sodium citrate buffer (0.1 M, pH 4.97), and 167 µL culture supernatant. Control samples were run in parallel with 167 µL phosphate-buffered saline 1x solution (negative control in triplicates), or purified *Trametes versicolor* laccase (0.084 U, Sigma-Aldrich, positive control) under identical conditions. All assays were performed in a 48-well plate (Corning Optical Communications Pty Ltd, Mulgrave, VIC, Australia), and the decolourisation of the RBBR dye in tested supernatants was measured in a microplate reader (Infinite M 1000 PRO, Tecan, Männedorf, Zürich, Switzerland) at 25°C and 585 nm. These measurements were taken at 0.8, 3, 24, 48, 72, 144, and 168 hours after the reactions were initiated. The plate was shaken for 20 s (linear shaking, 6 mm amplitude, 270 rpm frequency) inside the plate reader and prior to measurements to homogenise the reactions. The degree of dye decolourisation of each sample was determined by measuring residual absorbance (relative to negative control) at the maximal absorption wavelength of the RBBR dye (585 nm) and expressed as normalised percentage of decolourisation (absorbance at 500 nm chosen as reference values). These percentages were calculated using the following equation:

$$\%\ decolourisation\ sample\ = \left(1 - \frac{Abs\ res}{Abs\ ref}\right) * 100$$

Where *Abs res* is the residual absorbance at 585 nm ($Abs_{585\ nm}$ of the negative control minus the $Abs_{585\ nm}$ of the sample) and *Abs ref* is the reference absorbance of the sample at 500 nm.

2.10 Statistical analyses

Experiments were carried out in triplicates or repeated at least once, as specified for each method. When applicable, results are expressed as mean ± standard error of the

mean (± SEM). *T*-tests were performed in Microsoft Excel v.16.53. Multivariate statistical analyses were performed in R (v.1.4.0), using vegan (v.2.5.7).

3. RESULTS AND DISCUSSION

Our results demonstrate that random mutagenesis approaches could be used to increase extracellular laccase-like activities in Syn6803 (**Figure 2**). The term "laccase-like activity" was used in this study as the ABTS assay is not only specific to laccases. Moreover, the enzyme(s) responsible for the activity observed in the culture medium have not been purified or identified in this study. This will be the focus of future works.

3.1 Mutants screening

Six mutants were selected based on composite criteria – i.e., these mutants grew faster than the WT strain and exhibited higher laccase-like activities (**Figure 2**). Mutant A2 was chosen as it exhibited an extracellular laccase-like activity 10 times higher than the WT and grew 1.5 times faster. A2 was used in subsequent analyses.

3.2 Bacterial community analysis

To assess the axenicity of our Syn6803 cultures and possible interference from bacterial laccase activities with the observed growth and laccase-like patterns, the bacterial communities associated with WT and A2 were identified and characterised using 16S rRNA amplicon sequencing, followed by diversity and multivariate statistical analyses. Evidence of bacteria associated with the WT and our improved A2 strain was provided, indicating non-axenic culture conditions. Nevertheless, all samples showed very low levels of alpha-diversity; that is, lower within-sample diversity relative to not diverse habitats in marine and other aquatic environments (Walters & Martiny, 2020). This suggests that although not entirely free of contamination, our cultures were predominately *Synechocystis* cells. Moreover, our results showed that bacterial communities associated with WT and A2 had similar composition and structure ($p = 0.1$, **Figure 3A**) and richness ($p_{Chao1} = 0.6384$, **Figure 3B**). Despite significant differences in alpha-diversity levels between strains ($p_{Shannon's} < 0.0001$, **Figure 3B**), members of the *Geodermatophilus* (average relative abundance = 67%) and *Mycobacterium* (average relative abundance = 12%) genera dominated all bacterial

assemblages. *Geodermatophilus* is one of the three genera within a family of mainly soil-associated bacteria, unusually resistant to oxidative stress (Normand et al., 2014). *Mycobacterium* is the only genus of its family and comprises a wide range of pathogens and saprophytes, as well as free-living microorganisms often isolated from soil and water (Kazda, 2000). Both microorganisms belong to the Actinobacteria phylum, where several genera are known to live in association with cyanobacteria (Abdulaziz et al., 2016). Notably, some species within *Geodermatophilus* and *Mycobacterium* produce laccases (Alexandre & Zhulin, 2000; Satpathy et al., 2013), but the identification of these strains is out of the scope of this study. Provided both genera were equally predominant within WT and A2 microbial assemblages, their possible contribution to the observed laccase response in Syn6803, if existent, would be equal for WT and A2.

In addition, our multivariate statistical analyses showed that no ASVs differed significantly ($p < 0.01$) between WT and A2, meaning a lack of enrichment or depletion of bacterial taxa across strains. These results indicate that WT and A2 possess the same microbial community. Therefore, the observed differences in cell growth, laccase-like activity and/or decolourisation ability between the two strains can be imputed to *Synechocystis* strains rather than their associated bacterial microbiome.

3.3 Characterisation of laccase-like activity in A2 strain

3.3.1 Determination of best harvest time for A2 highest laccase-like activity

Syn6803 A2 exhibited a typical cyanobacterial growth curve with a maximum OD value of 2.33 on day 25 (**Figure 4A**). In general, A2 ODs observed in this experiment were higher than those observed in the initial screening to select the best 6 mutants (**Figure 2**), likely because of the shape of the growing vessel. Cells were grown in well plates during the initial screening and then transferred to tissue culture flasks for the following experiments. The tissue culture flasks offer conditions that are more favourable to cell growth than well plates (e.g., better gas exchange and light exposure), which inherently resulted in higher ODs.

The highest laccase-like activity observed was 0.10 U mL^{-1} on day 11 (**Figure 4B**). This activity is in accordance with the activity reported by Xue et al. (2014) when characterising a putative laccase from *Synechocystis* sp. PCC6803. Our results suggest that the best time of harvest for our improved strain is in mid-exponential growth phase (OD_{750nm} ~1.0), when laccase-like activities peak. Contrastingly, previous reports on *Arthrospira maxima* (Afreen et al., 2018) and *Spirulina platensis* (Afreen et al., 2017a) showed laccase activities increasing proportionally with cyanobacterial biomass, thus peaking at late exponential growth phase. However, when induced with guaiacol (100 µM) the laccase activities peaked in early exponential phase (Afreen et al., 2018; Afreen et al., 2017a), similar to our non-induced Syn6803 A2 mutant.

3.3.2 Laccase induction experiment

A range of chemical compounds (herein referred to as inducers) that had been previously reported to induce laccase production in other cyanobacteria (Afreen et al., 2017a) and fungi (Jang et al., 2006; Strong, 2011) was tested. These compounds included ethanol, guaiacol, and 2,5-xylidine. Tetracycline was also tested because laccases are known to oxidize a wide range of phenolic compounds, and particularly fungal laccases have been shown to degrade tetracycline antibiotics (Suda et al., 2012). Induction of laccase production in Syn6803 in presence of tetracycline in growth medium was hypothesized. This experiment aimed to determine: i) the best inducer and the best induction conditions, ii) if the inducers have a more considerable effect when combined, iii) if any of the inducers had a deleterious effect on Syn6803, and iv) if laccase production can be "restarted" after exponential growth phase (day 13).

Our results showed that except for xylidine and guaiacol, all inducers had minor to no effect on A2 growth (**Figure 5A**, **C**). 2,5-xylidine, alone and combined with guaiacol, impeded the growth of A2, and this detrimental effect was stronger at low cell densities. This was also evident when comparing different induction times, as 2,5-xylidine's inhibitory effects were reduced when cells were induced at mid-exponential growth phase (OD_{750nm} > 1). Similarly, guaiacol-induced growth stress was weaker if added at later life cycle stages (OD_{750nm} > 0.5).

Relative to the control, all treated cells displayed lower laccase-like activities, regardless of the inducer used (**Figure 5B**, **D**). Nevertheless, low levels of laccase-like activity were prolonged when cells were induced with the combination of guaiacol and 2,5-xylidine at mid-exponential phase. This indicates that guaiacol and 2,5-xylidine have a synergistic effect on the production of laccase-like molecules in A2 when added simultaneously. Afreen et al. (2017a) reported 2,5-xylidine (1 mM) as a potent inducer of laccase production in *Spirulina platensis* and also observed an additive effect with guaiacol (1 mM). In their study, the inducers were added at the time of inoculation, indicating that the concentrations used (1 mM) are not as detrimental for *Spirulina platensis* as they are for Syn6803 A2. Slow cell growth and prolonged laccase-like activities may be related physiological processes in A2 linked to the inhibitory effects of the xylidine-guaiacol combination. However, the magnitude of the observed levels suggests that the concentrations used in this study might not be optimal for Syn6803. To conclude, laccase production might be induced in A2 cells by adding 2,5-xylidine and guaiacol at mid- to late- exponential growth phase, when cell densities can counteract 2,5-xylidine's detrimental effect. Future research should focus on determining inducers optimal concentrations to limit or reduce their negative impact on cell growth, while maintaining or increasing laccase production

3.3.3 *In-vitro* dye decolourisation assays (WT vs. A2)

The anthraquinonic dye RBBR was used to assess the *in-vitro* decolourisation ability of the improved strain A2, relative to WT. Laccases are remarkable biotransformants of numerous water pollutants from the textile industry, including dyeing agents such as RBBR (Sosa-Martínez et al., 2020; Yadav et al., 2021). Dye decolourisation assays have been extensively used to evaluate laccase activity and enzyme mediators' efficiency, especially from fungi. Some fungal laccases degrade about 90% of RBBR in less than 24 hours (Grassi et al., 2011; Mtibaà et al., 2018; Murugesan et al., 2007). Our results showed that A2 supernatants decolourised 14% of RBBR after 3 hours, which is double the decolourisation of WT supernatants and the positive control (fungal laccase). There were no differences observed between the fungal purified laccase and the *Synechocystis* WT supernatant, suggesting that the kinetics and properties of the laccases from the two organisms are very similar (**Figure 6A**). Consistently, full

spectral scans showed changes in RBBR absorption spectrum between strains. Relative to WT, A2 triplicates exhibited higher reductions of RBBR absorption at 585 nm after 3 hours (**Figure 6B**), which indicates increased dye oxidation. Similar patterns were observed after 24 hours of reaction (**Figure 6C**).

3.3.4 Atypical enzymatic reaction

Zymograms were performed in an attempt to visualize the potential laccase secreted by the A2 strain, as described by (Afreen et al., 2017b). Unfortunately, the zymograms were unsuccessful with only the band of the positive control (purified *Trametes versicolor* laccase) being revealed by the addition of ABTS. The reaction taking place in the supernatant of the A2 strain is atypical and likely not mediated by a laccase but rather by highly oxidative compounds. This could explain why the laccase "inducers" had a little effect (sometimes negligible) on A2 efficiency at oxidizing ABTS. Regardless of the capacity of the A2 strain at secreting a laccase, this study shows that random mutagenesis can lead to the selection of *Synechocystis* PCC6803 strains with a high oxidative potential that could be used in industrial applications, such as the degradation of textile dyes or pollutants, or virtually any industrial process that relies on oxidative molecules and in search of greener alternatives.

4. CONCLUSIONS

This study characterised a *Synechocystis* PCC 6803 mutant, A2, with increased extracellular laccase-like activity, generated via random mutagenesis with EMS, and screened through consecutive ABTS assays. A2 laccase-like activity in growth medium peaks at mid-exponential phase and is prolonged by adding 2,5-xylidine and guaiacol (1 mM each). The A2 strain decolourised RBBR faster than WT, reaching 30% of decolourisation after 24 hours. Although the biochemical reaction and putative laccase have not been characterised yet, random mutagenesis is a valid approach for the generation of improved cyanobacterial strains with potential industrial applications.

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7. DATA AVAILABILITY

The microbiome dataset related to this article can be found under accession number PRJNA866311 at the Sequence Read Archive (SRA) data:

http://www.ncbi.nlm.nih.gov/bioproject/866311

This is an open-source online data repository available through multiple cloud providers and the National Center for Biotechnology Information (NCBI) servers.

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593 **9. FIGURE CAPTIONS**

594

Figure 1. Random mutagenesis and screening strategies for the selection of *Synechocystis* sp. PCC 6803 mutants with enhanced laccase-like activity. Syn6803 WT was exposed to EMS (**A**). Single mutant cells were subsequently separated by FACS (**B**), and 192 of the surviving cultures were screened using ABTS assays with culture supernatants (**C**). In each screening round (**D**), the 10 mutants showing the highest growth rates (**E**), or the highest laccase-like activities (**F**) were selected for further screening. Coloured small dots are single-cell cyanobacterial cultures, and big blue dots are ABTS-stained cyanobacterial cultures.

Figure 2. Selection of the best mutants based on fast growth and high laccase-like activities. Random mutants of Syn6803 were generated through EMS exposure, and serially screened for their capacity to oxidize ABTS. The best six mutants proliferated more quickly while also oxidizing more ABTS and were selected for further experiments. Growth curves in well plates based on optical densities at 750 nm (**A**). Laccase-like activities detected in growth medium at exponential phase ($OD_{750\ nm} \sim 0.5$), with average of biological triplicates for the WT strain and average of technical triplicates for the best mutants (**B**). Laccase-like activities were normalised to cell densities. Error bars in WT represent the standard error of the mean (± SEM, n = 3). A2, A7, A8, B1, B3, and B4 were mutants.

Figure 3. Bacterial profiling of WT and A2 cultures. Bacterial microbiomes associated with Syn6803 WT and A2 were characterised through 16S rRNA amplicon sequencing. Bacterial community structure (beta-diversity) was comparable between strains, as shown by the relative abundance of unique ASVs at the genus level – different colours for each biological triplicate or bar within each strain (**A**). Alpha-diversity (within-sample diversity) was estimated by calculating the Chao1 and Shannon's diversity indices and differed between strains (**B**). Asterisks represent significant differences ($p \leq 0.05$ in ANOVA and t-tests).

Figure 4. Assessment of best harvest time for the maximal laccase-like activity. Syn6803 A2 strain was monitored until cells reached stationary growth phase. Cell growth was measured as optical density at 750 nm and peaked on day 25 (**A**). Laccase-

like activity was detected through ABTS assays in the culture supernatant and peaked on day 11 (**B**). Values are mean of biological triplicates, with error bars representing the standard error of the mean (± SEM) and trendlines showing best curve fittings based on non-parametric local polynomial regressions.

Figure 5. Effect of laccase inducers on the improved strain. A2 cultures were treated with chemical inducers at the time of inoculation (after 0 days of growth, **A**, **B**), and when cells reached mid-exponential phase (after 13 days of growth, **C**, **D**). Cell growth was measured as optical density at 750 nm and only responded to Guaiacol + Xylidine (**A**, **C**). Laccase-like activities were detected through ABTS assays in culture supernatants and were lower for treated cells regardless of the inducer (**B**, **D**). The x-axis represents the number of days after induction. Values are the mean of biological triplicates, with error bars representing the standard error of the mean (± SEM) and trendlines showing best curve fittings based on non-parametric local polynomial regressions.

Figure 6. **Biodegradation of RBBR dye *in-vitro***. The biodegradation potential of Syn6803 WT and A2 was assessed through RBBR dye decolourisation assays *in-vitro* with culture supernatants harvested at mid-exponential growth phase. A2 showed higher decolourisation rates than WT (**A**). Full spectral scans (400 – 700 nm) of decolourisation reactions at 3 (**B**) and 24 hours (**C**). Results are expressed as normalised percentages of decolourisation, relative to the negative control. Values are the mean of biological triplicates, with error bars representing the standard error of the mean (± SEM) and trendlines showing best curve fittings based on non-parametric local polynomial regressions. C – = negative control (phosphate-buffered saline solution), C+ = positive control (purified *Trametes versicolor* laccase).

10. FIGURES

Figure 1

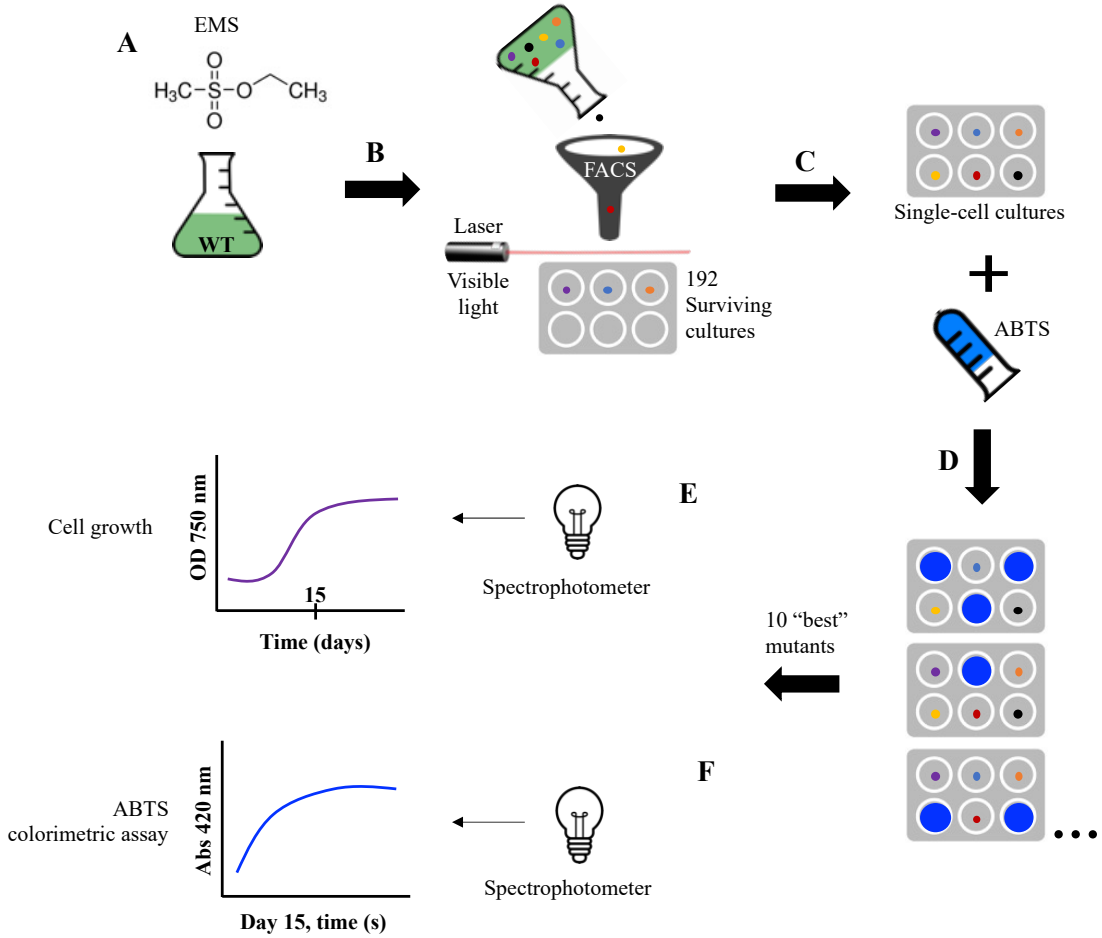


Figure 2

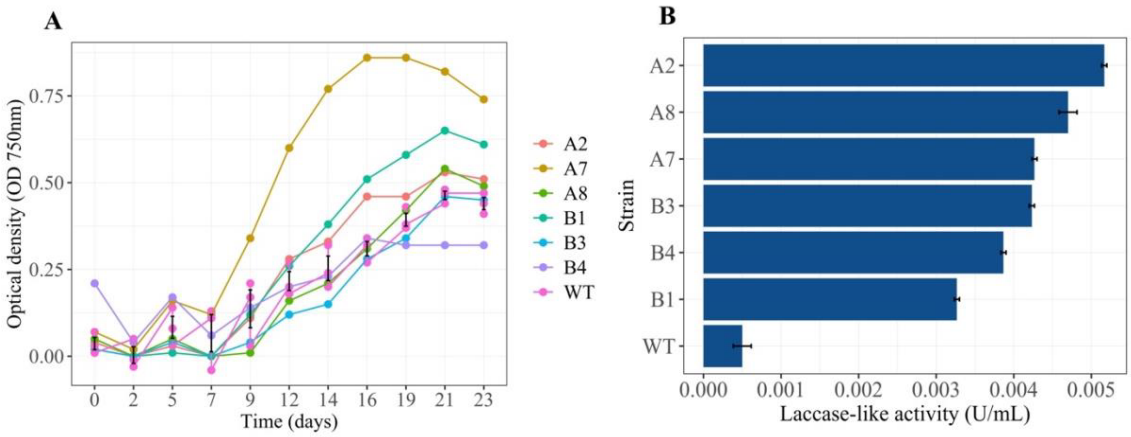

A
Optical density (OD 750nm)
0.00
0.25
0.50
0.75
0 2 5 7 9 12 14 16 19 21 23
Time (days)
A2
A7
A8
B1
B3
B4
WT
B
Strain
A2
A8
A7
B3
B4
B1
WT
0.000 0.001 0.002 0.003 0.004 0.005
Laccase-like activity (U/mL)

Figure 3

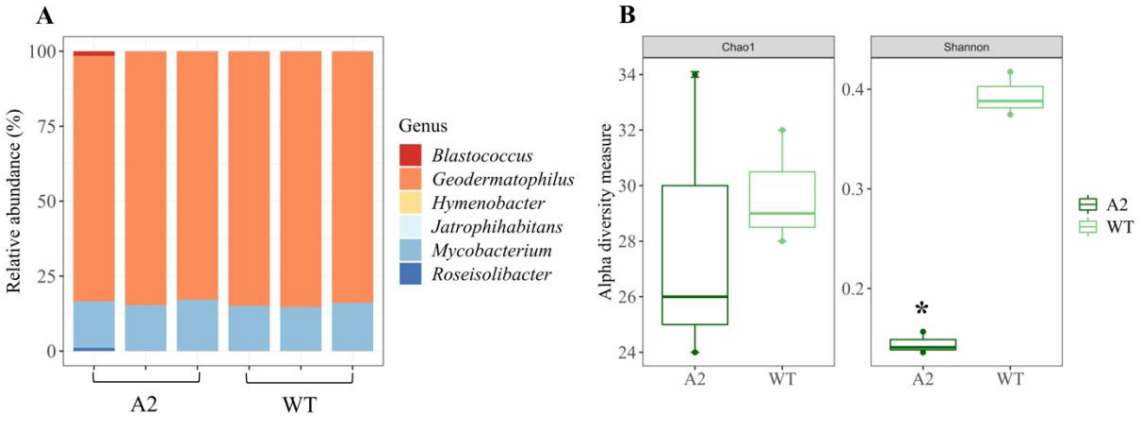

A
Relative abundance (%)
100
75
50
25
0
A2
WT
Genus
Blastococcus
Geodermatophilus
Hymenobacter
Jatrophihabitans
Mycobacterium
Roseisolibacter
B
Chao1
Shannon
Alpha diversity measure
34
32
30
28
26
24
0.4
0.3
0.2
*
A2
WT
A2
WT
A2
WT

Figure 4

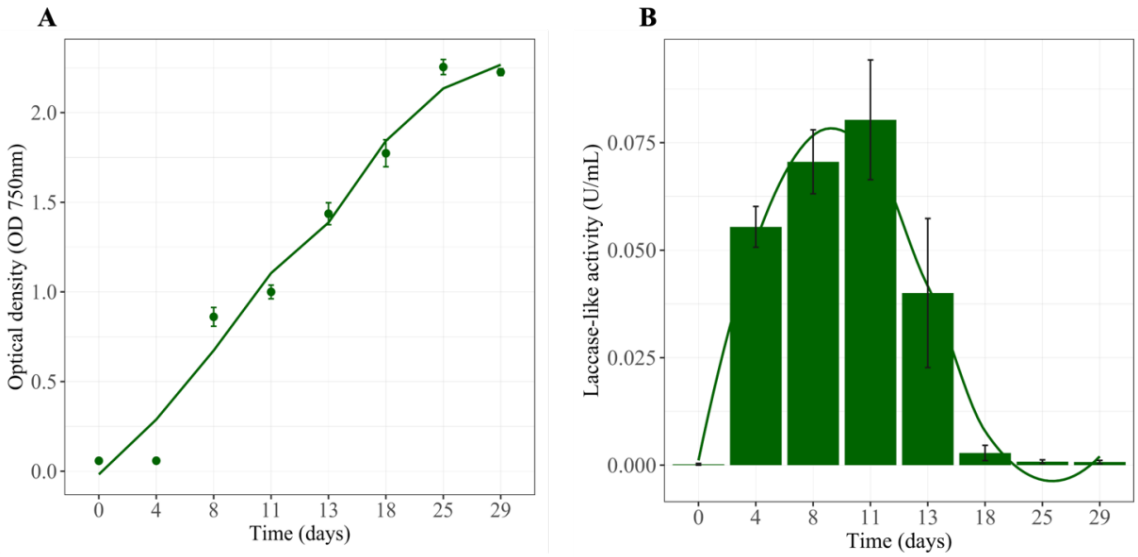

A
Optical density (OD 750nm)
0.0
0.5
1.0
1.5
2.0
0
4
8
11
13
18
25
29
Time (days)
B
Laccase-like activity (U/mL)
0.000
0.025
0.050
0.075
0
4
8
11
13
18
25
29
Time (days)

Figure 5

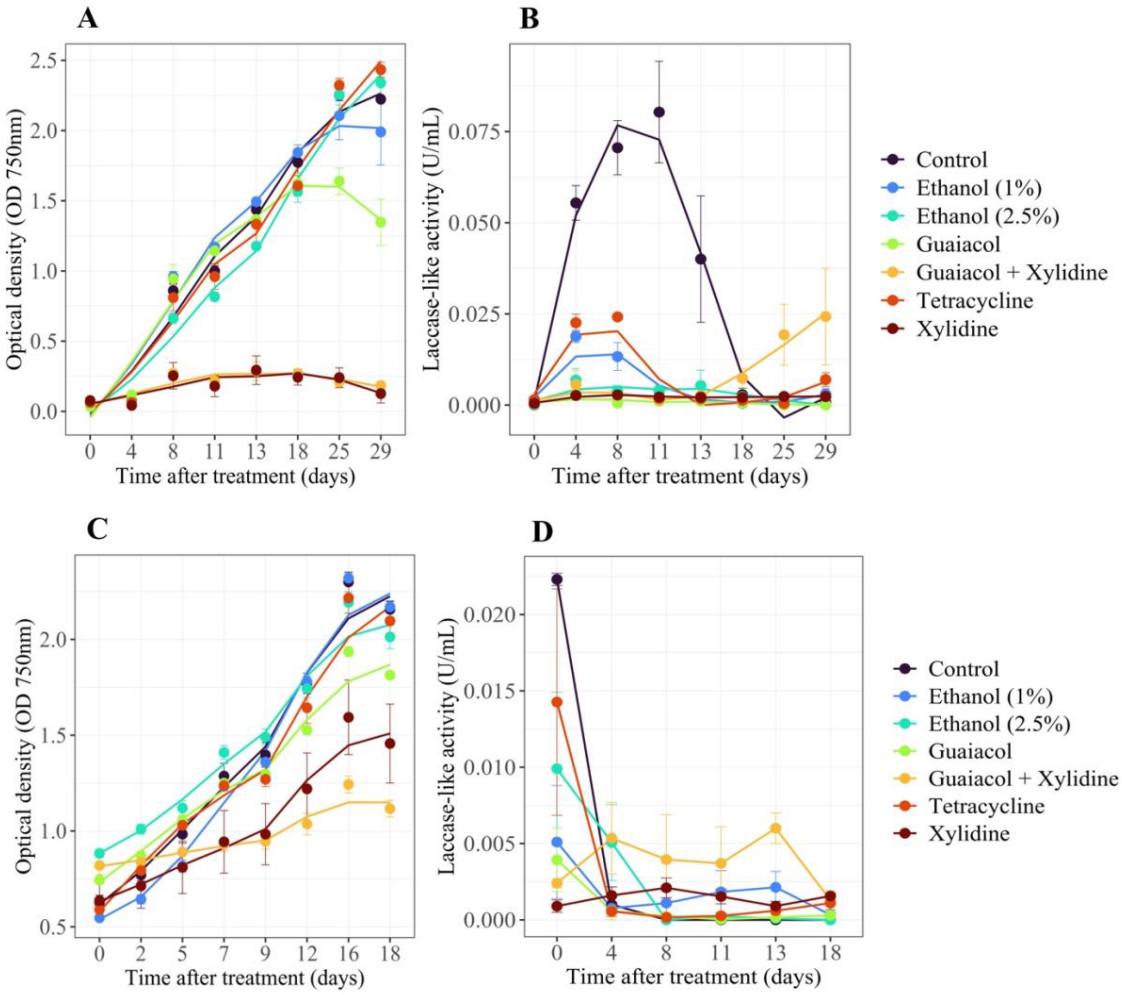
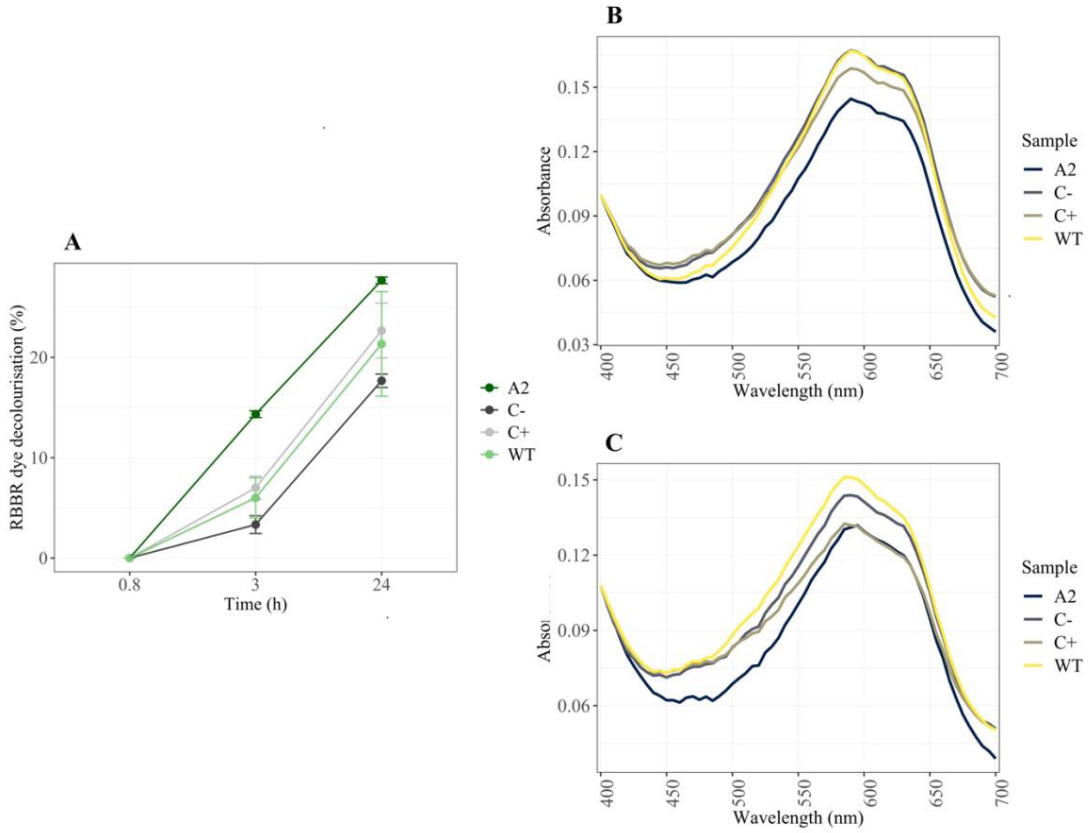

A
B
C
D
Optical density (OD 750nm)
Laccase-like activity (U/mL)
Time after treatment (days)
Control
Ethanol (1%)
Ethanol (2.5%)
Guaiacol
Guaiacol + Xylidine
Tetracycline
Xylidine

Figure 6


A
RBBR dye decolourisation (%)
0
10
20
0.8
3
24
Time (h)
A2
C-
C+
WT
B
Absorbance
0.03
0.06
0.09
0.12
0.15
400
450
500
550
600
650
700
Wavelength (nm)
Sample
A2
C-
C+
WT
C
Absor
0.06
0.09
0.12
0.15
400
450
500
550
600
650
700
Wavelength (nm)
Sample
A2
C-
C+
WT