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Evaluation of Methyl Red Tolerant Cyanobacteria for Simultaneous Laccase Production and Dye Decolorization

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Abstract

While the textile industries have and will continue to play a vital role in the economic growth of India one unfortunate historic side effect has been the ubiquitous use of synthetic azo dyes which pose potential threat to aquatic environmental ecosystems if effluent from such industries is left untreated. In this study, of the ten cyanobacterial strains tested, six were found to tolerate exposure to a test methyl red (MR) dye well as indicated by good cell growth. Further investigation of the concentration of various photosynthetic pigments, and the production of phenol degrading laccase enzymes in the cyanobacteria in presence of MR dye indicated that all six tolerant strains (*Spirulina-C5, Spirulina-C10, Spirulina-C11, Lyngbya, Phormidium and Synechocystis*) exhibited a significantly higher concentration ($P<0.05$) of pigments and significantly higher production ($P<0.05$) of extracellular proteins in presence of dye. The best performing strain, *Spirulina-C11*, produced significantly greater amounts of laccase (59.57 mU/mL) at 10 days relative to all other strains which was further enhanced (71.52 mU/mL) following addition of guaiacol as an inducer. Guaiacol also induced increased protein content that was attributed to an increase in de nova synthesis of phenol degrading enzymes and stress tolerance proteins. In practice *Spirulina-C11* was able to effectively decolorize 65.2% of methyl red solution within 48 hrs.

Keywords: Aquatic environment; Bioremediation; Cyanobacteria; Decolorization; Laccase enzyme; Methyl red dye

Introduction

The overall level of chemicals in the environment has increased substantially in the past few decades as a result of several anthropogenic activities, which have influenced biodiversity and caused strong ecological impacts [1]. Cyanobacteria (blue green algae) are the largest group of photoautotrophs, distributed in diverse habitats including many polluted environments [2]. In fact, some microorganisms are found to adapt rather well to polluted environments by acquisition of specific resistance, while some other microbial species develop co-tolerance to more than one pollutant [3]. Synthetic dyes are amongst the most common water pollutants and are increasing in environmental concentrations due to various anthropogenic activities including the growth of textile industries [4,5]. Thus there has been increased recent interest in understanding the interactions of synthetic dyes with microorganisms [6], and various microbial dye tolerant species have even been isolated from contaminated sites. However, very few reports are available on the tolerance of cyanobacteria to synthetic dyes [2].

Of all the dyes currently in common industrial use, textile azo dyes represent the largest group of organic substances that pose potential deleterious effects to aquatic environments. Approximately, 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced worldwide [7]. After China, India is the second largest exporter of dyestuffs where Azo dyes constitute 70% of the synthetic dyes produced [8]. Synthetic azo dye contamination in aquatic ecosystems has emerged as a new area of research because of their excessive use in textile industries [9]. The term azo dye is applied to any synthetic organic colorant that contains at least one nitrogen-to-nitrogen double bond: -N=N-. Methyl red (MR) is an anionic azo dye (Figure 1) used widely in the textile industries, but is very toxic in nature, especially to aquatic life forms, mutagenic and commonly found in textile effluents [10]. The use of large amounts of dye stuffs during dyeing results in highly colored aquatic bodies, where a thin layer of discharged dyes over the water surface decreases dissolved oxygen in water by reducing diffusion of atmospheric oxygen and can also decrease dissolved oxygen production by photosynthetic aquatic flora (due to reduced light penetration). The resulting decrease in dissolved oxygen detrimentally affects aquatic flora and fauna including cyanobacteria [11]. Even when the parent product is degraded most microbial degradation studies reveal the formation of N,N-dimethyl-phenylenediamine that is also a toxic amine. Due to long term exposure to dyes in textile effluents, it is likely that some cyanobacteria strains would have developed dye tolerance.

Various physico-chemical methods such as coagulation, flocculation, activated carbon adsorption and reverse osmosis technique are all commonly used today for the removal of color from textile effluents. However, the major limitation of all of these methods is that they do not specifically degrade the dye but only transfer the dyes intact from one phase to another. In addition, many of these methods relay on the addition of chemicals and excessive use of chemicals involves higher cost and generates secondary contaminants that must still be treated. In recent years, laccase production by photoautotrophic cyanobacteria has received considerable attention [12] as an alternative to these established physio-chemical removal technologies. This is because, as an alternative biological process the

laccase enzyme is increasing seen as a low cost, high efficiency and eco-friendly solution [13].

Laccase was first described by Yoshida in 1883 in the Japanese lacquer tree *Rhus vernicifera* [14], and characterized as a metal containing oxidase by Bertrand in 1985 [15]. Laccases (EC 1.10.3.2, p-diphenol: O_2 oxidoreductases) are mostly extracellular glycoproteins belonging to the group of multi-copper, polyphenol oxidases. They catalyze the one electron oxidation of a wide range of substrates such as mono and poly-phenols and aromatic amines, concomitantly reducing molecular oxygen to water [16]. Substrates are oxidized to radicals, which may further undergo cross-linking or depolymerization reactions [14-17]. Appropriate substrates may also act as a so-called redox mediator, enabling the indirect oxidation of an even wider range of compounds; including lignin and polyaromatic hydrocarbons [18]. For these reasons, laccase is today recognized as an important environmentally benign green catalyst.

Cyanobacteria are photosynthetic prokaryotic organisms that originated four billion years ago [19]. Their cell structure resembles that of gram negative bacteria, but like higher plants they possess chlorophyll-a and have water soluble phycobiliproteins. They use both photosystems I and II and use water for photosynthetic oxygen production [20].

There has recently been increasing awareness of the potential for cyanobacterial bioremediation of synthetic dyes [21]. However only a few studies involving the cyanobacterial laccases *Phormidium valderianum, Oscillatoria boryana and Lyngbya* [22,23] have been reported. Therefore, in the present investigation, ten strains of cyanobacteria were initially tested for dye tolerance in terms of chlorophyll pigment, biomass and protein content and increased laccase production potential when exposed to a methyl red dye.

Materials and Methods

Reagents

Since the textile azo dye MR (Figure 1) is toxic and mutagenic in nature, especially to aquatic life forms, and is used widely in textile industries, we have chosen it for this study. MR was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade and were obtained from Merck, India.

Cyanobacterial culture maintenance and MR dye tolerance studies

Cyanobacterial cultures were procured from the National Centre for Culture Collection and Utilization of Blue Green Algae, Indian Agriculture Research Institute, New Delhi, India and grown in BG-11 medium [24]. *Spirulina* was grown in Zarruk's medium [25]. Growth media were steam sterilized in an autoclave for 20 minutes at 121°C and 15 pounds per square inch pressure. All glassware were sterilized in a hot air oven at 160°C for 2 hours. During incubation culture was shaken continually using an orbital mixer.

The ten cyanobacterial strains screened for MR tolerance potential were *Spirulina-C3, Spirulina-C5, Spirulina-C10, Spirulina-C11 Spirulina-C477, Spirulina-C479, Spirulina-C483, Lyngbya, Phormidium and Synechocystis*. Prior to testing cyanobacteria cultures were homogenized. The cyanobacteria were then exposed to five different concentrations (0, 25, 50, 75 and 100 mg L^{-1}) of MR (500 mL stock solution) where cultures without added dye served as the control. Cell growth was examined by measuring the absorbance of the cell suspension spectrophotometrically, at 750 nm from day 0 to day 18 at three day intervals (i.e. Day 0, 3, 6, 9, 12, 15, 18). Complete spectral scans revealed the λ max of cell suspensions of all strains to be around 750 nm, and hence, for further studies, OD was measured at 750 nm, which has also been recommended as an index of cell growth [26]. Cultures were initially grown in Erlenmeyer flasks containing growth medium in order to provide stock microbial cultures. All experiments were conducted in triplicate.

Growth response was further evaluated on Day 15 via determination of pigments (chlorophyll) and protein content production. Chlorophyll-a was estimated by hot extraction with methanol [27]. Proteins content was estimated following the methods outlined previously [28].

All cyanobacterial strains that were found to be tolerant to MR were further investigated to determine their maximum laccase production capabilities in batch studies. Thereafter, the best laccase producing strain was used for a practical methyl red decolorization study.

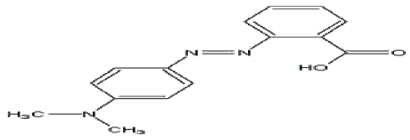

Figure 1: Chemical structure of the azo dye-methyl red.

Enzyme assay and laccase production

Supernatants of cyanobacterial strains suitable for determining laccase enzyme content were collected every alternate day by centrifugation at 6000 g for 10 minutes. Laccase activity was assayed at room temperature using 10 mM Guaiacol in 100 mM sodium acetate buffer (pH 5.0). Briefly, supernatant sample (1 mL) was combined with sodium acetate buffer (3 mL) and Guaiacol (1 mL) in a 5 mL centrifuge tube and mixed vigorously with the help of stirrer. Thereafter the change in absorbance of the reaction mixture was monitored spectrophotometically at 470 nm for 10 mins. Enzyme activity was measured in mU/mL which is defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per mL [29].

Induction of laccase activity

Laccase activity in actively growing cultures was induced by addition of 100 µM of Guaiacol on Day 0 and thereafter cyanobacterial cells were allowed to grow for a further 10 days. A culture without Guaiacol was used as the control.

Partial laccase purification

Culture filtrate was mixed with cold acetone at a volume ratio of 1:4 (filtrate: acetone) and incubated at -20^{0}C for 1 hour 30 minutes. The mixed solution was then centrifuged at 10,000 rpm and 4^{0}C for 30 minutes. The supernatant was then discarded and the residual pellet air dried to remove any acetone residues. The pellet which now served as an enzyme source for further study.

Protein estimation in relation to guaiacol

Protein content of cyanobacterial strains was estimated in relation to Guaiacol by the method described by Lowry et al. [28] using bovine serum albumin as a standard.

Dye decolorization studies

Cyanobacterial cultures were exposed to MR (100 mg L^{-1}) on Day 3 of inoculation. Thereafter, samples were taken at 24 hr intervals and centrifuged at 5000 rpm for 20 mins prior to spectral analysis. The decrease in color intensity of MR in cell free supernatant was analyzed spectrophotometrically at λ=430 nm. The percent dye decolourization was calculated according to the formula:

$$D = 100 \times \frac{\left(A_{ini} - A_{obs}\right)}{A_{ini}}$$

where D is the percent decolorisation (%), A_{ini} is the initial absorbance and A_{obs} is the observed absorbance [30].

Statistical analysis

All results from replicate analyses were expressed as mean ± standard deviation. Dunnett's multiple range test was applied and differences among the means were analyzed by ANOVA using Graph Pad Prism version-6.1 (Graph Pad Software, San Diego, CA, USA).

Results and Discussion

Irrespective of the strain studied the presence of MR dye consistently decreased the cell growth of all ten cyanobacteria strains studied (Figure 2) where the reduction in growth (biomass) consistently increased with increasing concentrations of dye. Six strains (*Spirulina-C5, Spirulina-10, Spirulina-C11, Lyngbya, Phormidium and Synechocystis*) exhibited <50% cell growth inhibition in the culture medium at most MR concentrations. All of these six strains grew well in culture medium containing MR (25 -100 mg L^{-1}).

Relative to the control dye induced growth inhibition ranged from 12-58%. *Spirulina-C11* showed the least growth reduction (12%), indicating this strain was least sensitive to the dye. In comparison, maximum growth reduction (58%) was exhibited by *Spirulina-477* suggesting a much higher dye sensitivity. The differences observed in dye tolerance ability amongst cyanobacterial strains are probably due to specific genotypic variations [31]. Moreover, the presence of a strong antioxidant defense system is one of the features that makes a cyanobacterial species tolerant to MR dye [2], therefore, it might be possible that *Spirulina-C11* has developed an antioxidant defense system to better tolerant MR as well.

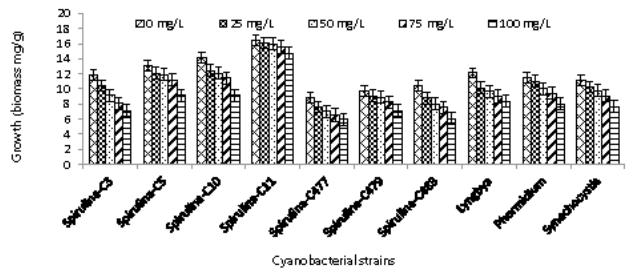

Figure 2: Dye tolerance potential of cyanobacterial strains grown in the presence of MR at five concentrations (0-100 mg L^{-1}). All values reported are means of three independent experiments with three replicates each (n=9). Vertical bars indicate ± SE.

The variation in pigment concentrations with cyanobacterial strain and dye concentration is summarized in Figure 3. Interestingly, chlorophyll and protein concentrations in all strains were significantly ($P<0.05$) decreased in comparison to the control. The concentration of photosynthetic pigments decreased in the presence of the dye, and the differences between strains were statistically significant ($P > 0.05$). While a few strains seemed to not only have protected their photosynthetic pigments against the toxic effect of the studied dye, they had also performed better in the presence of dye, potentially indicating the development of some tolerance mechanism. Several reports have previously show significant adverse effects of synthetic dyes on thylakoid membranes and chlorophyll biosynthesis of blue green algae [32,33]. The *Spirulina-C11* strain, on the other hand, showed a higher concentration of photosynthetic pigment in the presence of MR dye indicating superior tolerance or adaptability to dye. For all cyanobacterial strains increasing the initial dye content subsequently decreased chlorophyll content in a dose dependent manner (Figure 3a). At the highest MR concentration (100 mg L^{-1}), reduction in chlorophyll content reached to 0.278 µg mL^{-1}. Our results were similar to those of previous studies where cultures exposed to 100 mg L^{-1} MR dye also showed decreases in chlorophyll content [34]. Decreases in chlorophyll content are commonly ascribed to inhibition of pigment synthesis [11-35].

As with chlorophyll content, exposure of strains to MR caused a general decline in protein content relative to the control for all cyanobacterial strains studied in a dose dependent manner (Figure 3b). Thus for all strains protein content was lowest (most suppressed) at the highest dye concentration (100 mg L^{-1}). The protein content expressed by *Spirulina-C11* when exposed to MR was marginally lower than all other strains (Figure 3b) which resembled variations observed in some earlier studies of *Spirulina* [11-36].

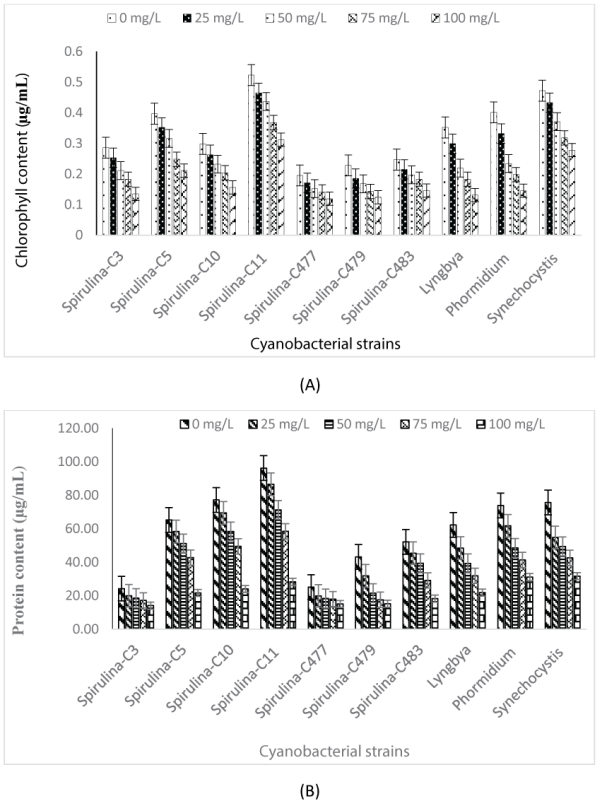

Figure 3 (A-B): Effect of MR concentration (0-100 mg L^{-1}) on photosynthetic pigment (a) and protein content (b) of ten cyanobacterial strains 15 days after inoculation.

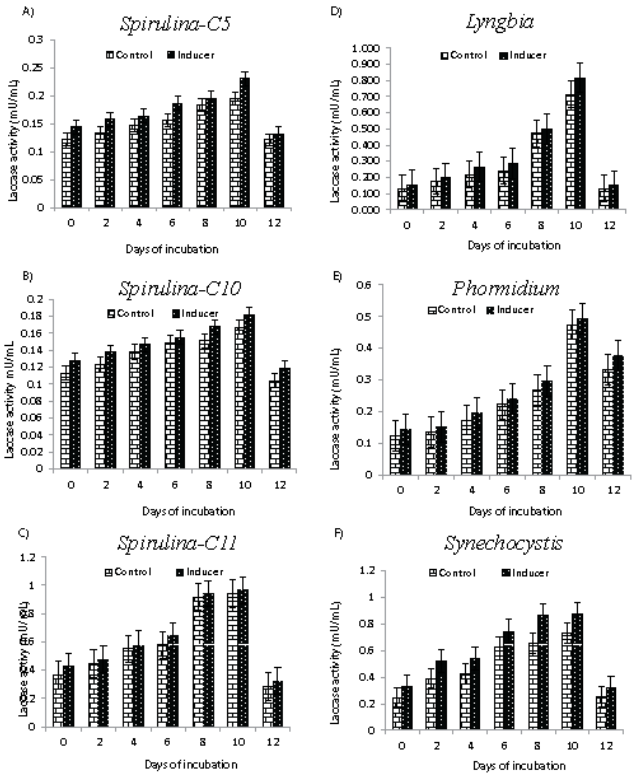

Figure 4: Laccase activity of six MR tolerant cyanobacterial strains; (a) *Spirulina-C5*, (b) *Spirulina-C10*, (c) *Spirulina-C11*, (d) *Lyngbya*, (e) *Phormidium* and (f) *Synechocystis* cultural filtrate.

Laccases are versatile biocatalysts often used in industrial applications such as the textile industry [37]. While some studies of fungal laccases exist; information regarding cyanobacterial laccases is almost negligible [20]. Our results indicated that while all dye resistant cyanobacterial strains were able to produce the laccase enzyme, laccase production varied amongst different strains (Figure 4a-4f)). While laccase production was consistently highest on Day 10 for all strains studied the magnitude of laccase production ranged from 12.54 to 59.57 mU mL^{-1} for *Spirulina-C11*. A number of previous studies have shown enhanced laccase production by inducer/elicitors [38]. In agreement with these studies, a sharp induction in laccase activity (34.42 to 71.52 mU mL^{-1}) was observed after inclusion of an inducer (Guaiacol) to the culture medium on Day 0. Even under Guiacol induced conditions maximum laccase production was still observed in *Spirulina-C11*. Marine cyanobacteria *Phormidium tenue* also exhibited accelerated laccase production following inclusion of Guaiacol to the growth medium [39]. Guaiacol addition also induced laccase production in bacteria [40-42].

In order to investigate whether the addition of Guaiacol to the culture media had any effect on the total protein content, the total protein content of all MR tolerant strains was estimated on Day 10. Generally, application of high doses of MR caused a general decline in protein content in a dose dependent manner (Figure 5). Such trends were previously observed for *Spirulina* [16-36]. Where enhancement in protein content was attributed to de novo synthesis of phenol-degrading enzymes and stress-related proteins in response to aromatic compounds [43].

Since *Spirulina-C11* demonstrated both the best MR tolerance and also the highest laccase activity (with and without inducer), it was the preferred strain to be used to evaluate MR dye decolorization potential.

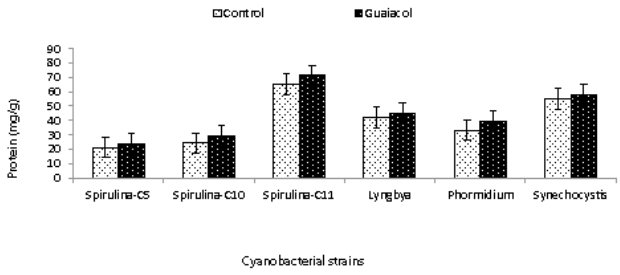

Figure 5: Effect of Guaiacol on protein content of cyanobacterial strains on Day 10. All values are means of three independent experiments with three replicates each (n=9). Vertical bars indicate ± SE.

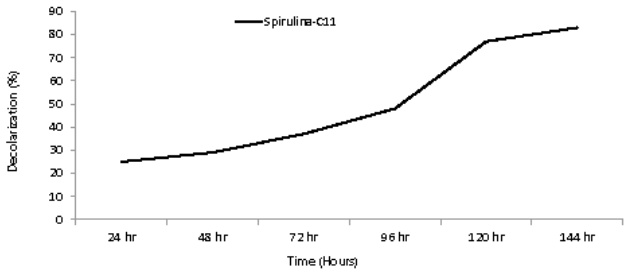

Figure 6: Temporal variation in the decolorization of MR via laccase enzyme produced by *Spirulina-C11* biomass.

It is often difficult to obtain pure laccase and the expense involved in purification may not be required if impure biomass can have the same effect. Thus in this experiment, in order to make the MR dye decolorization process more practicable and more cost effective only the cyanobacterial (*Spirulina-C11*) biomass was tested rather than pure laccase. *Spirulina-C11* decolorized MR effectively where the extent of decolorization increased with incubation time. Thus while *Spirulina-C11* could visually decolorize a 100 mg L^{-1} MR solution within 24 hrs maximum decolorization (87.51%) was only achieved after 144 hrs (Figure 6). In a previous study *Lyngbya* decolorized another azo dye (Proacin dyes) by 79.5 % with 240 hrs [24]. The fungus *Trametes versicolor* also decolorized MR by 73% after 144 hrs. Relative to these studies the *Spirulina-C11* was certainly superior but even so the times observed were still relatively long and probably not practical.

Conclusions

Cyanobacterial laccase based bioremediation has potential to be used as an emerging eco-friendly and efficient approach for the decolorization of industrial effluents containing synthetic dyes. Of the ten cyanobacterial strains examined *Spirulina-C11* showed the maximum tolerance (58%) to the MR and also produced the highest amount of phenolic degrading laccase enzyme (59.57 mU/mL). The amount of enzyme produced could be enhanced to 71.52 mU/mL (79.48 %) in the presence of an inducer such as Guaiacol. In practice 87.5% of a 100 mg L^{-1} solution of MR dye could be decolorized by *Spirulina-C11* within 144 hr. Thus, relative to most strains previously tested, *Spirulina-C11* has the ability to tolerate MR under normal conditions and can produce large amounts of the laccase enzyme which further removes dyes. Furthermore, *Spirulina-C11* has a range of potential mechanisms at the cellular level that might be involved in MR dye detoxification, and thus *Spirulina-C11* could be most MR tolerant.

Acknowledgements

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