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Summary and Conclusions

Synthetic textile dyes belong to the most dangerous pollutants which, as part of the industrial effluents, contaminate steadily higher amount of waste water. Due to their chemical structure, dyes are highly recalcitrant and often toxic or can lead to toxic transformation product when released into the environment. Color is the first contaminant to be recognized in the wastewaters and has to be removed before discharging into water bodies or on land. The presence of very small amount of dye in water (less than 1 ppm for some dyes) is highly visible and affects the aesthetic merit of water and its transparency in lake, rivers and other water bodies. One of the major problems with textile effluents is; they have toxic effect on germination rates and biomass of several plant species, whereas plants play many important ecological functions. Most of these dyes are toxic and potentially carcinogenic in nature and their removal from the industrial effluents is a major environmental problem. Dyes are recalcitrant molecules difficult to degrade biologically. From the treatment point of view; the degradation of dyes has received considerable attention. Conventional wastewater treatment processes are suitable for stabilization of non-xenobiotic compounds, whereas these processes do not work well with the xenobiotic compounds. Environmental regulations in most of the countries now have made it mandatory to decolorize the dye wastewater prior to discharge. Increasing concerns about color in the effluents are leading to the worldwide efforts to develop more effective color removal processes. Conventional waste water treatment is not efficient to remove recalcitrant dye stuffs from effluents. Physical and chemical methods for removal of dyes are not suitable due to high cost and low efficiency. Thus biotechnological approaches are gaining increasing interest in the textile industry.

The treatment systems having mixed microbial populations are more effective due to concerted metabolic activities of microbial community. As the catabolic activities of microorganisms in mixed consortium complement each other, the utilization of microbial consortium offers considerable advantages in the degradation of synthetic dyes. The individual strains may attack the dye molecule at different positions or may use decomposition products produced by

another strain for further decomposition. Studies carried out in the past have used undefined microbial consortium for dye decolorization.

Laccase, because of its catalytic properties and broad substrate specificity make it have great potential in varied industrial and environmental application. Laccase (EC1.10.3.2) is a generic name given to a family of multicopper oxidases that are capable of oxidizing several different substrates with contaminant reduction of dioxygen to water. Although this enzyme exhibit specific affinity for oxygen as their electron acceptor, its specificity towards their reducing substrates is rather low. Laccase catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, *ortho*- and *para*-diphenols; they also oxidize other substrates such as aromatic amines, syringaldazine and non-phenolic compounds to from free radicals. These abilities make laccase very useful for its application to several biotechnological processes.

Keeping all these above points in mind, in the present study, we observed that the fungus like yeast *Galactomyces geotrichum* MTCC 1360 was able to decolorize various synthetic dyes. Yeasts can grow faster than most filamentous fungi and have the ability to resist unfavorable environments. The use of yeast strains in dye wastewater treatments has been limited till this date. So, in present studies, we have also explored the ability of yeast *G. geotrichum* MTCC1360 to degrade and detoxify the Methyl red and dispersive dye Scarlet RR. A defined consortium of two organisms *G. geotrichum* MTCC 1360 (GGO) and newly isolated *Bacillus* sp. VUS, named as consortium-GB was used for decolorization of various textile dye. As the bacteria and yeasts both are found to be efficient in dye degradation, we have used consortia of these microorganisms to faster the degradation processes. We wanted to find out the difference in the enzymatic status and its role in the fate of metabolism of Brown 3 REL and Brilliant blue G by individual organisms and with consortium-GB.

The consortium and individual organisms was investigated for the presence of biotransformation enzymes viz. LiP, laccase, tyrosinase, NADH-

DCIP reductase, riboflavin reductase and azo reductase at different time intervals. Most of the enzymes involved in biotransformation were present at higher levels in 24 h grown individual cultures. Tyrosinase activity was absent in *G. geotrichum* MTCC 1360. The consortium also contains all the biotransformation enzymes.

G. geotrichum MTCC 1360, an industrial important strain was found to decolorize various textile dyes. *G. geotrichum* MTCC 1360 had shown decolorization of Methyl red (100%), Malachite green (97%), Scarlet RR (100%), Orange HE 4B (75%) and Amido black 10B (92%) in malt yeast medium with shaking condition at 1, 9, 18, 18, 18 h of incubation respectively. *G. geotrichum* MTCC 1360 could decolorize Methyl red in distilled water in 1 h without addition of any organic components.

The concerted activities of the consortium-GB consisting of *G. geotrichum* MTCC 1360 and *Bacillus* sp. VUS resulted in increased decolorization efficiency as compared to that shown by individual strains. The consortium was effective in decolorization of Brown 3REL along with other synthetic dyes like Navy blue, Brilliant blue G and Yellow brown completely within 2, 24, 9 and 8 h respectively. Remazol red was decolorized only 67% after 96 h incubation. Individually, *Bacillus* sp. VUS could decolorize Brown 3REL (100%) in 5 h and GGO could decolorize (39%) in 24 h. The pure cultures of GGO and *Bacillus* sp. VUS could decolorize Brilliant blue G up to 100% and 33% in 12 h respectively. None of the cultures individually were able to decolorize Navy blue, Yellow brown and Remazol red completely.

The consortium at shaking condition gave faster decolorization for Brown 3REL. The consortium could decolorize the dye Brilliant blue G faster at static condition as compared to that of shaking. This suggested that shaking condition was not always required for the decolorization of dyes. The average rate of decolorization was maximum at 60 min for Brown 3REL and 3 h for decolorization of Brilliant blue G.

The pH studies with *G. geotrichum* MTCC 1360 for decolorization of Methyl red and Scarlet RR showed opposite results. Methyl red was

decolorized more efficiently at acidic condition (pH 3), were as Scarlet RR at alkaline conditions (pH 12). Effect of temperature on decolorization of Methyl red was found to be very significant as the dye was decolorized at wide range of temperatures like 5, 30 and 50°C. Scarlet RR was degraded at optimum temperature of 50 °C. Both the dyes were decolorized by *G. geotrichum* MTCC 1360 up to 300 mg l^{-1} of concentration. An increase in time required for decolorization was observed with an increase in dye concentration. Increasing mycelium concentration showed decrease in time required for decolorization for Methyl red and Scarlet RR.

Addition of glucose and molasses to distilled water greatly influence the decolorization of Methyl red. Glucose (1%) increased decolorization by 70%, were as molasses (1 g l^{-1}) showed 83%. Further increase in concentration of both the components decreased the decolorization rate. Inducer like $CaCO_3$ (0.05 mg l^{-1}) showed decrease in time required for decolorization from 18 to 11 h for Scarlet RR. There was no effect of $CaCO_3$ on lignin peroxidase and laccase activity. Inhibitor like EDTA (0.05 mM l^{-1}) showed inhibitory effect on Scarlet RR decolorization along with the activity of laccase.

Maximum decolorization ability was found to increase with an increase in temperature, with maximum activity at 50°C, but the decolorization potential reduced when temperature was further increased to 60°C in case of decolorization of Brown 3REL and Brilliant blue G by consortium. Decolorization activity was more towards alkaline pH with optima of pH 7, while marked reduction in decolorization ability was observed at acidic pH for Brown 3REL. Similarly, for Brilliant blue G it was found that alkaline pH was suitable for decolorization with optima of pH 9, lower pH decreases decolorization rate. During decolorization of both the dyes it was observed that, time required for decolorization was increased with an increase in dye concentration. Consortium-GB could decolorize Brown 3REL up to 300 mg l^{-1} and Brilliant blue G only up to 200 mg l^{-1} concentration. The increase in the time of decolorization with an increase in dye concentration may be due to inhibitory effects of higher dyestuff concentration.

Starch, peptone and urea were found to be better additional carbon and nitrogen source for the decolorization of Brown 3REL by consortium-GB. While malt extract, peptone and beef extract were found to be the best additional carbon and nitrogen sources for Brilliant blue G. We found that, glucose as additional carbon source act as inhibitor for decolorization of both the dyes.

G. geotrichum MTCC 1360 showed induction in the activity of NADH-DCIP reductase at 5°C by 430% during decolorization of Methyl red. NADH-DCIP reductase (184%) and laccase (355%) were induced significantly at 30°C. Significant induction by 257 and 203% was observed in NADH-DCIP reductase and lignin peroxidase respectively at 50°C. However, azo reductase activity remained almost constant at 5, 30 and 50°C. The variations in enzyme pattern at different temperatures resulted in the change in time required for decolorization. Significant induction (1611%) in LiP was observed during decolorization of Scarlet RR. The riboflavin reductase was induced by 103% at initial stage (6 h of incubation) and further lowered to control level. While other enzymes remained constant as that of control.

To get an additional information for the decolorization of Brown 3REL and Brilliant blue G oxidative enzyme activities were determined in individual organisms (partial decolorization) as well as in consortium (complete decolorization). Induction in enzymes like LiP, riboflavin reductase and tyrosinase was observed after complete decolorization of dye in the consortium as compare to pure cultures during decolorization of Brown 3REL. Enzymatic studies during Brilliant blue G decolorization showed significant induction in riboflavin reductase. This difference in induction of enzyme levels had lead to decrease in time required for decolorization by consortium-GB as compare to that of individual organism.

The UV-Vis analysis of these dyes and treated samples with *G. geotrichum* MTCC 1360 and consortium showed decrease in absorption, supporting the decolorization of dyes. Biodegradation of Methyl red by *G. geotrichum* MTCC 1360 was conformed by analyzing product using UV-Vis,

HPLC and FTIR. Wavelength scan (400-800 nm) of products formed by *G. geotrichum* MTCC 1360 at different temperatures (5, 30 and 50°C) was done. The products formed at 5 and 30°C showed decrease in absorbance at maxima, while products at 50°C showed shift in the absorbance maxima. The HPLC analysis for Methyl red dye showed single peak (RT: 2.16), while metabolites at 5°C showed 9 peaks (RT: 2.04, 2.79, 3.29, 3.62, 4.06, 4.73, 5.55, 7.62 and 9.15), at 30°C showed 5 peaks (RT: 1.55, 1.99, 3.20, 4.38 and 4.53) and at 50°C showed 11 peaks (RT: 1.57, 2.21, 2.56, 2.78, 2.28, 3.18, 3.43, 3.76, 4.43, 4.60 and 5.10). FTIR analysis of the products of 5 and 50°C supports the formation of 2-aminobenzoic acid and DMPD respectively. Spectrum points out the formation of primary and secondary alcohols along with presence of benzene ring at 30°C. Phytotoxicty study of methyl red at 300 ppm concentration showed 88% germination inhibition in *Sorghum bicolor,* whereas 72% in *Triticum aestivum.* The metabolites at the same concentration showed no germination inhibition along with induction in *Sorghum bicolor* shoot length (5 and 30°C) and root length of *Triticum aestivum* (30 and 50°C).

Metabolites formed after complete decolorization of Scarlet RR was analyzed by HPLC and FTIR. HPLC showed single peak (RT: 3.287) for Scarlet RR, while metabolites formed after 6 h showed 5 peaks (RT: 1.487, 2.505, 2.859, 3.036 and 3.14), 2 peaks (RT: 2.280 and 2.625) at 12 h and 5 peaks (RT: 2.470, 2.669, 2.8014, 3.268 and 3.549) at 18 h. FTIR analysis showed appearance of new peaks than that of control dye indicating degradation of dye. GCMS identification of 3 H quinazolin-4-one, 2-ethylamino-acetamide, 1-chloro-4-nitro-benzene, N-(4-chloro-phenyl)-hydroxylamine and 4-chloro-phenylamine as final metabolites supports the degradation of Scarlet RR. The dye inhibited germination of seed by 80% at 4000 ppm concentration, while the metabolites at same concentration showed no inhibition of germination for the tested plants indicates nontoxic nature of metabolites.

The HPLC analysis was performed after complete degradation by consortium (2 h) and 2 h after incubation with the dye for an individual

organisms in case of Brown 3REL. HPLC of control dye showed 2 peaks (RT: 2.997 and 3.217), while the products with consortium showed 6 peaks (RT: 2.555, 2.815, 3.017, 3.238 and 3.348), 3 peaks with GGO (RT: 2.938, 3.283 and 2.641) and 4 peaks with *Bacillus* sp. VUS (RT: 2.556, 2.670, 2.816 and 3.002). This indicates different nature of products. FTIR spectra give better description of change in chemical structure and functional groups of the compound. Degradation of Brown 3REL by consortium and individual organisms gave different peaks of functional group, showing the absence of most of the peaks from the spectrum of the control dye. GCMS identification showed different metabolites formed using consortium (2-(6,8-Dichloro-quinazolin-4yloxy)-acetyl]-urea and 2-(6,8-Dichloro-quinazolin-4yloxy)-acetyl]-formamide), and *Bacillus* sp. VUS (6,8-Dichloro-4 methoxy-quinazoline) after 2 h of incubation with Brown 3REL. *G. geotrichum* MTCC 1360 alone showed only minor modifications in structure of Brown 3REL. Phytotoxicity study revealed non toxic nature of metabolites.

Biodegradation analysis of Brilliant blue G by HPLC showed 2 peaks (RT: 2.726 and 2.931) with control dye. Products formed by consortium showed 5 peaks (RT: 2.445, 2.640, 2.819, 2.993 and 3.378) whereas 7 peaks (RT: 1.485, 2.217, 2.865, 2.704, 3.05, 3.184 and 3.544) by GGO and 5 peaks (RT: 2.604, 2.928, 3.042, 3.63, and 3.632) by *Bacillus* sp. VUS. The presence of sulfonic acid was confirmed by FTIR spectra of Brilliant blue G. The metabolites formed by degradation of Brilliant blue G by consortium-GB, GGO and *Bacillus* sp. VUS showed peaks for presence of N-H steching, C=O stretching, C-H stretching, S=O stretching and for C-H stretching as alkane. The GCMS study revealed a pathway of Brilliant blue G with release of (4-Ethoxy-phenyl)-phenyl-amine and 3-Ethylaminomethyl-benzenesulfonic acid as final metabolites formed by consortium-GB. GCMS identification showed different metabolites formed using GGO and *Bacillus* sp. VUS as 3-{[Ethyl-(3-methyl-cyclohexa-2, 5-dienyl)-amino]-methyl}-benzenesulfonic acid and (4-Benzylidene-3-methyl-cyclohexa-2,5-dienylidene)-methyl-amine respectively.

Brilliant blue G was toxic at the concentration of 4000 ppm for germination of tested seeds, while the metabolites were non toxic at the same concentration.

Laccase being an important industrial enzyme with wide application was found to be present in *G. geotrichum* MTCC 1360 at maximum level after 24 h of growth in malt yeast medium. Partial purification of laccase by ion exchange chromatography showed increase in specific activity of enzyme from 0.97 U to 1.5 U, with 10% yield. Native PAGE analysis showed dimeric nature of protein with molecular mass of 97,000 and 66,000 Da, when zymograghic study was performed with L-dopa. The enzyme was found to be active at broad pH (1-8) and temperature ranges (30-80°C). The conventional substrates such as hydroquinone (100%), ABTS (40%), L-Dopa (17%), O-Tolidine (35%) and DAB (31%) were oxidized with relative activity of laccase. $CaCl_2$, $CuSO_4$, $MgCl_2$ and $HgCl_2$ decreased the activity of enzyme along with an increase in their concentration. Sodium azide (10 mM) and L-cysteine (20 mM) completely inhibited the laccase activity.

The consortium-GB was able to decolorize (100%) textile effluent at 50°C with 29 h of incubation.

The degradation of Methyl red was possible by *G. geotrichum* MTCC 1360 at broad pH and temperature ranges. Glucose, mycelium concentration and molasses had increased the decolorization rate. There was difference in induction patterns of enzymes at different temperature, which may have lead to difference in metabolite fate of Methyl red. The absence of N-N'-dimethyl-p-phenylenediamine in 5°C, 2-aminobenzoic acid in 50°C and both the compounds in 30°C sample showed the difference in metabolic fate of Methyl red at different temperatures. The optimum pH and temperature for decolorization of Scarlet RR were pH 12 and 50°C respectively. Addition of $CaCO_3$ in the medium induced decolorization of dye, EDTA showed inhibitory effect on dye decolorization along with activity of laccase. Decolorization was analyzed by UV-Vis, HPLC FTIR and GCMS supports the degradation of Scarlet RR. Phytotoxicity revealed nontoxic nature of metabolites. By deducing possible pathway for degradation, we have tried to understand the mechanism

used by this organism which will allow the development of technologies to apply this organism to the cleaning-up of aquatic and terrestrial environments.

In the view of the results obtain, it can be concluded that the consortium could work at higher temperatures and at alkaline conditions. The additional carbon source like glucose decreases the decolorization rate of consortium. The difference in induction pattern of the studied enzymes in consortium-GB and the pure cultures shows the metabolic difference between them during degradation of Brown 3REL and Brilliant blue G. The analysis of degradation products by UV-Vis, HPLC and FTIR shows formation of new and different products by consortium and pure cultures, when incubated with Brown 3REL and Brilliant blue G. GCMS analysis shows formation of different products by consortium and pure cultures indicating variance in the metabolic pathways followed by them. Phytotoxicity revealed non toxic nature of metabolites. The consortium was also able to decolorize textile effluent. These results indicate high potential of this consortium-GB to serve as an excellent biomass for the use in textile dye removal.