Scientific paper

Biodegradation of Navy Blue-3G by *Brevibacillus laterosporus* MTCC 2298

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Abstract

Attempts have been made to decolorize widely used disperse azo dye Navy Blue-3G by the *Brevibacillus laterosporus* MTCC 2298. The 80% decolorization was observed within 48 h under static condition at the concentration 50 mg/L. No significant change in the decolorization performance was observed under shaking condition. Decolorization performance was steeply decreased at the higher concentration (1.0 g/L) even after extended incubation (66% respectively within 72 h). Maximum decolorization exhibited at 30 °C and in broad range of pH (i. e. 7.0–11.0). Complete inhibition of tyrosinase, no significant change in the activities of laccase and DCIP reductase where as significant increase in the activities of lignin peroxidase (54%), aminopyrine N-demethylase (122%) and MG reductase (50%) was found in the cells obtained after decolorization when compared to control cells (i. e. without dye addition). Biodegradation of dye was confirmed by the TLC (thin layer chromatography) and FTIR (Fourier Transform Infra Red) spectroscopy. Bromobenzene (m/z 156, RT 19.892), ester substituted aniline derivative (m/z 178, RT 20.492), *meta*-di nitro benzene (m/z 167, RT 21.550) and *ortho* benzene aniline derivative (m/z 169, RT 23.892) were the degradation products elected by GC-MS (Gas Chromatography-Mass Spectroscopy) analysis. Extracted degradation products were found to be non-toxic for three bacteria such as *Brevibacillus laterosporus, Pseudomonas aeruginosa* and *Azotobacter vinelandii* as well as for the two crops *Sorghum vulgare* and *Phaseolus mungo*.

Keywords: *Brevibacillus laterosporus*, Navy Blue-3G, Biodegradation, FTIR spectroscopy, GC-MS spectral analysis

1. Introduction

Approximately 10000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually through out the world. Azo dyes are most widely used in the textile and food industries. Due to the poor exhaustive properties of the dyes, unfixed dyes ultimately find its way into the environment. A very small amount of dye in water (10–50 mg/L) affects the aesthetic value, water transparency and gas solubility of water bodies.[1] Removal of color from the textile effluents is typically based on the on physical and chemical processes rather than the biodegradation in conventional waste water treatment system. Various physical, chemical and biological treatment techniques have been reported to remove colors from dye containing waste water.[2–7] The physicochemical techniques include membrane filtration, coagulation, flocculation, precipitation, adsorption, ion exchange, ion pair extraction and ultrasonic mineralization,[8–9] electrolysis,[10] advanced oxidation, chlorination, bleaching, ozonation, and fenton oxidation,[11] photocatalytic oxidation[12] and chemical reduction[13]. Although presently a wide range of physical and chemical methods are available to decolorize dye contaminated effluents[6], alternative processes based on biotechnological principles are of most increasing interest.[14] A number of biological processes such as biosorption have been proposed as having potential application in removal of dyes from textile waste water.[15] Bioremediation of the textile effluents is an attractive method of color removal due to its environmental friendly technology.[1] Hence, it is of outstanding interest to find an effective microflora and biochemical approach for the decolorization and detoxification of textile wastewater.

Azo dyes are the largest and most versatile class of dyes. They are not easily degradable under natural conditions and are not typically removed from water by conventional wastewater treatment systems.[16–18] Azo linkages in the dye molecule can be reduced by the bacteria under anaerobic condition to form colorless aromatic amines which are occasionally toxic and carcinogenic.[19] An oxidative cleavage leads to the formation of intermediates different than that of postulated by anaerobic transformation mechanism.[20] Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases.[21]

Though *Brevibacillus laterosporus* strains are eco-friendly, they are rarely studied for the bioremediation. Recently, the strain *Brevibacillus laterosporus* MTCC 2298 is reported as a potential azo dye degrader[22] as well as for the eco-friendly biodegradation of a reactive textile dye Golden Yellow HER[23]. Present study was undertaken to decolorize widely used dispersive textile azo dye Navy Blue-3G by the *B. laterosporus* MTCC 2298. Status of biotransformation enzymes viz. lignin peroxidase, laccase, tyrosinase, DCIP reductase, MG reductase and aminopyrine N-demethylase in the cells obtained after decolorization as well as COD (chemical oxygen demand) and BOD (biological oxygen demand) of the decolorized culture supernatant in comparison with control dye was studied. Furthermore, biodegradation was confirmed by the UV-Vis spectroscopy, thin layer chromatography and FTIR spectroscopy. Possible biodegradation pathway was proposed with the degradation products detected by the GC-MS spectral analysis.

2. Experimental

2. 1. Microorganisms

Brevibacillus laterosporus MTCC 2298 strain was obtained from Microbial Type Culture Collection, Chandigarh, India. *Pseudomonas aeruginosa* NCIM 2036 and *Azotobacter vinelandii* NCIM 2821 were collected from NCIM, National Chemical Laboratory, Pune, India. All bacterial strains were maintained routinely on the nutrient slants containing (g/L): NaCl (5.0), bacteriological peptone (5.0), yeast extract (2.0), beef extract (1.0) and agar agar (15.0). Performance of decolorization of Navy Blue-3G was studied in the synthetic medium as well as with an extra supplement of 'C', 'N', and 'P' sources (0.5%) in the synthetic medium. Synthetic media contained all the minerals and salts that are essential for bacterial growth and its composition was standardized in our laboratory. It was prepared by mixing three different separately sterilized solutions such as 'A' (150 mL), 'B' (10 mL) and 'C' (840 mL). Solution 'A' contained (g/L): $FeSO_4$ (0.500), $ZnSO_4$ (0.011), $MnCl_2$ (0.100), $CuSO_4$ (0.392), $MgSO_4$ (0.010), $CoCl_2$ (0.248), $BaCl_2$ (0.060), H_3BO_3 (0.050), $CaCl_2$ (0.022), NH_4Cl (0.230), NaCl (0.150), $FeCl_3$ (0.005) and $NaHCO_3$ (2.00). Solution 'B' contained (g/L): $Na_2HPO_4.2H_2O$ (3.800) and KH_2PO_4 (100.000). Solution 'C' contained (g/L): yeast extract (1.0) and peptone (1.0).

2. 2. Dyes and Chemicals

ABTS (2, 2-Azino-bis (3-ethylbenzothiazolin-6-sulfonic acid)), catechol, *n*-propanol, NADH disodium salt, DCIP (dichlorophenolindophenol), Malachite green (MG) and 4-dimethyl anti aminopyrine were obtained from Sigma Aldrich, USA. Tartaric acid was obtained from BDH chemicals (Mumbai), India. Glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from SRL, India, remaining all-chemicals were obtained from Hi-Media, India. Textile dyes such as Navy Blue-3G, Rubin, Brown 3REL, Blue GL, Scarlet RR and Ramazol Red were generously gifted by Yashwant Textile Processing, Ichalkaranji, Kolhapur, India.

2. 3. Plant Seeds

The seeds of *Sorghum vulgare* and *Phaseolus mungo* were obtained from local market, Kolhapur, Maharashtra, India.

2. 4. Decolorization Experiments

Decolorization experiments were performed in the 250 mL Erlenmeyer flasks containing 100 mL batch culture. Navy Blue-3G (50 mg/L) was added into the 36 h grown culture and incubated at 30 °C under static condition. After decolorization, biomass was removed by centrifugation (4000 × g, 20 min) and absorbance of supernatant was measured at the λ-max of Navy Blue-3G. Decolorization was expressed in terms of percentage and calculated as follows:

Decolorization (%) = [(initial absorbance)-(observed absorbance)/(initial absorbance)] × 100.

Experiments for the decolorization of mixture of dyes were performed similar to that of the decolorization of Navy Blue-3G. Mixture of five different textile dyes such as Rubin, Brown 3REL, Blue GL, Scarlet RR and Ramazol Red (10 mg/L of each) was added to the 36 h grown culture medium incubated at 30 °C under static condition. Percent decolorization was calculated as that of in decolorization of Navy Blue-3G.

Two control flasks were studied simultaneously. One was without inoculum (abiotic) and other was without dye. All the decolorization experiments were carried out in three sets and average data was recorded.

2. 5. Effect of Different Physicochemical Conditions on the Decolorization of Navy Blue-3G

Pre grown (36 h, under static condition) culture was kept on the shaker after dye addition. Effect of shaking

condition on the decolorization performance was studied by using an orbital shaker adjusted at 30 °C and 150 rpm. Effect of initial dye concentration on the decolorization performance was studied by the addition of different concentrations (0.25, 0.50, 0.75 and 1.0 g/L) of dye into the pre grown cultures. Effect of pH on the decolorization performance was studied by adjusting different pH (3.0, 5.0, 7.0, 9.0 and 11.0) of the pre grown culture before dye addition. The pH of culture medium was adjusted by using 0.2 M NaOH or HCl. Effect of temperature on the decolorization performance was studied by incubating pre grown (at 30 °C) culture at different temperatures (5, 15, 30 and 45 °C) after the dye addition. Flasks were incubated at respective temperatures for 30 min before addition of dye to acclimatize the media i.e. to maintain the respective temperature of the bacterial culture of that particular flask. Effect of repeated dye additions on the decolorization performance was studied by the repeated addition of dye (50 mg/L) into the culture (100 mL) after the every 48 h (i. e. one cycle). Effect of growth phases of *B. laterosporus* on the decolorization performance was studied by the addition of dye into the different pre grown (12, 24, 36, 48, 60, 72, 96 and 120 h) 100 ml batch cultures. Effect of initial biomass concentration on the decolorization performance of Navy Blue-3G was studied by the addition of different concentrations (1, 2, 3 and 4 g) (wet wt) of biomass into the sterile nutrient medium (100 mL) containing dye (50 mg/L). Pre grown (36 h) cultures were centrifuged (10000 × g, at 4 °C for 20 min) and the biomass obtained was collected and transferred aseptically.

Effect of different C, N and P sources on the decolorization performance was studied by using synthetic medium. Pre grown (for 36 h in the nutrient medium) culture was centrifuged (10000 × g, at 4 °C for 20 min) and the biomass obtained from each 100 mL batch culture was transferred aseptically into the synthetic medium (100 mL) containing dye (50 mg/L). Also, experiments were performed with an extra supplement of different carbon (glucose and starch), nitrogen (peptone and NH_4Cl) and phosphorus (NaH_2PO_4 and KH_2PO_4) sources in the synthetic medium at the concentration 0.5% of each.

2. 6. Preparation of Cell Free Extract

Cells were harvested (10000 × g, at 4 °C for 20 min) and suspended in 50 mM potassium phosphate buffer (pH 7.4). Cell suspension (100 g/L) was chilled properly, gently homogenized and sonicated, keeping the sonifier out put at 40 amp, giving four strokes each of 1 s, at 2 min intervals (Sonics Vibra Cell, Germany). This cell free extract was used for enzyme assays. Protein content was estimated by the Biuret method. Protein concentration of cell free extract was kept constant (2.0 mg/mL) for the enzymatic studies.

2. 7. Enzyme Assays

Six biotransformation enzymes viz. lignin peroxidase, laccase, tyrosinase, NADH-DCIP reductase, MG reductase and aminopyrine N-demethylase were studied form the *B. laterosporus* MTCC 2298. Activities of lignin peroxidase, laccase and tyrosinase were assayed spectrophotometrically at the room temperature where reference blanks contained all components except the enzyme (0.2 mL). The volume of reaction mixture was adjusted by the respective buffer. One unit of an enzyme activity was measured as change in the absorbance per min. All the enzyme activities were determined using procedures reported from our laboratory.[22]

Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm in the reaction mixture (2.5 mL) containing 100 mM n-propanol, 250 mM tartaric acid and 100 mM H_2O_2. Laccase activity was determined by monitoring the formation of oxidized ABTS at 420 nm in a reaction mixture (2.0 mL) containing 10% ABTS in the 100 mM acetate buffer (pH 4.8). Tyrosinase activity was determined by monitoring the formation of catechol quinone at 495 nm in a reaction mixture (2.0 mL) containing 0.01% catechol in the 100 mM potassium phosphate buffer (pH 6.8).

NADH-DCIP reductase activity was determined by monitoring DCIP reduction at 595 nm and calculated using an extinction coefficient 19 mM^{-1} cm^{-1}. MG reductase activity was determined by monitoring MG reduction at 620 nm and calculated using an extinction coefficient 8.4 × 10^{-3} mM^{-1} cm^{-1}. For both the reductases, reaction mixture (5.0 ml) was containing 50 mM substrate (DCIP or MG) in the 50 mM potassium phosphate buffer (pH 7.4) and 0.1 mL enzyme. From which 2.0 ml reaction mixture was assayed spectrophotometrically by the addition of 50 μM NADH. Aminopyrine N-demethylase activity was assayed in the reaction mixture containing HEPES buffer (50 mM, pH 7.8), NADH-generating system (0.2 mL), aminopyrine (80 mM) and enzyme (0.5 mL). The NADH-generating system composed NADH (2.6 mM), glucose-6 phosphate (12.5 mM) and glucose-6 phosphate dehydrogenase (4 units). Here, control set contained all the components except substrate where volume of reaction mixture was adjusted by the distilled water. The reaction was started by the addition of NADH-generating system and the reaction mixture incubated at 37 °C for 30 min. The reaction was terminated by trichloroacetic acid (20%) solution. Cell derbis was removed by the centrifugation (3000 × g, for 10 min) and the formaldehyde liberated was measured colorimetrically (at 420 nm) by the addition of Nash reagent.

2. 8. Extraction of Degradation Products of Navy Blue-3G

After dye decolorization, the biomass was removed by centrifugation (10000 × g, at 4 °C for 20 min) and the

obtained supernatant was used for extraction of the degradation products with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated in vacuum over anhydrous Na_2SO_4 and dried. The dried sample was dissolved in 2 mL HPLC grade methanol and used for analytical studies.

2. 9. Analytical Methods

The culture supernatant obtained after decolorization was used for UV-Vis spectroscopy. The UV-Vis spectra were obtained using a double beam spectrophotometer (Hitachi, UV 2800) at room temperature. Thin layer chromatogram (TLC) was obtained by applying extracted degradation products on the silica gel coated glass plate. Methanol was used as mobile phase. The separated products were visualized in an iodine chamber. The FTIR (Fourier Transform Infra Red) spectra were obtained in the mid IR region (400–4000 cm^{-1}) by using Perkins Elmer 783 Spectrophotometer with 16-scan speed. The sample was mixed with spectroscopically pure KBr (1:99) and analyzed by fixing the pellet in the sample holder. GC-MS (Gas Chromatography-Mass Spectroscopy) analysis was carried out by using a Hewlett Packard 989-B MS Engine, equipped with an integrated gas chromatograph having a HP1 column (30 m long, 0.25 mm id, non polar). Helium was used as a carrier gas at the flow rate 1.1 mL/min. The injector temperature was maintained at 30 °C with oven conditions as: 100 °C for 2 min and increased up to 250 °C at the rate 10 °C/min and then raised up to 280 °C at the rate 30 °C/min.

2. 10. Toxicity of Navy Blue-3G and its Degradation Products

Microbial and phytotoxicity studies were carried out by the procedure recently reported by Gomare and Govindwar.[22] Microbial toxicity of the control dye and its extracted products (1000 ppm) was carried out for the *Brevibacillus laterosporus* MTCC 2298, *Azotobacter vinelandii* NCIM 2821 and *Pseudomonas aeruginosa* NCIM 2036. Sterile disk of Whattman filter paper of about 3 mm in diameter was deepen in the dye/ its extracted products solution and kept aseptically on the agar plate that spread with a bacterial culture. The zone of inhibition (diameter in cm) was measured after 24 h of incubation at 30 °C. Phytotoxicity of the dye and its extracted degradation products (100 ppm) was carried out (at room temperature i.e. 27 ± 3 °C) for *Sorghum vulgare* and *Phaseolus mungo*. Ten healthy seeds of the each crop were separately grown in a Petri-plate (having bed of filter paper) with the daily supplement of 5.0 mL sample. Percent germination (%) as well as the length of plumule and radicle was recorded after 6 days.

2. 11. Determination of BOD and COD after Decolorization of Navy Blue-3G and Mixture of Dyes

To understand the desirability of the biodegradation, reduction in the BOD (biochemical oxygen demand) and COD (chemical oxygen demand) was determined after decolorization (i. e. after 48 h of incubation). The BOD and COD tests were performed according to the standard methods for the examination of waters and wastewaters.[24] COD was determined by the closed reflux dichromate method whereas BOD was assessed by the standard dilution method using a commercially available spectralab whirlmatic-2 instrument. The BOD and COD were measured in abiotic control (un-inoculated with dye), biotic control (inoculated without dye) and test (after decolorization, under the conditions as that of in decolorization experiments).

2. 12. Statistical Analysis

Data was analyzed by one-way analysis of variance (ANOVA) and Turkey-Kramer Multiple Comparison Test. Readings were considered significant when P was < 0.05.

3. Results and Discussion

3. 1. Effect of Different Physicochemical Conditions on the Decolorization of Navy Blue-3G

B. laterosporus decolorized 80% of Navy blue-3G (50 mg/L) within 48 h under static condition; however no significant change in the decolorization performance was observed under shaking condition. Static condition creates anoxic environment for the bacterial culture where as shaking condition facilitates availability of oxygen to the bacterial cells. Recently, Gomare and Govindwar have reported reduction in the decolroiztion time by 50% under shaking condition than that of static in case of Methyl red by *B. laterosporus*.[22] However, no significant change in the decolorization performance was found under static and shaking conditions for the degradation of Golden Yellow HER.[23] *B. laterosporus* showed 91% decolorization of mixture of dyes (50 mg/L) within 48 h under static condition without shift in peak in UV-vis absorption spectra (data not shown). Faster decolorization of mixture of dyes than that of single dye (Navy Blue-3G) would be due to induction of different biotransformation enzymes in response to the different dyes/intermediates produced during degradation. The decolorization performance of the *B. laterosporus* was depending upon the nature of dye molecule and induced status of all responsible biotransformation enzymes during the metabolic processes.[25]

Mineralization of Navy blue-3G (Disperse Blue-79) was studied under anaerobic environment, where it was only biotransformed to amines resulting in the decolorization of effluent.[26] Decolorization was achieved when the concentration of dye was lower than 48 mg/L however; decolorization efficiency reached up to 95% (within 72 h) by using a co-substrate when the concentration of dye in the effluent was more than 48 mg/L.[27] Literature survey reveals that there are very few reports on the biotransformation of Navy Blue-3G or the biological treatment of textile effluent containing this dye. The findings in the present studies under shaking conditions are similar to the results obtained by Wong and Yuen.[28] In contrast, bacterial consortium NBNJ6 exhibited dye-decolorizing activity (90%, within 35 h) only when incubated under stationary conditions and negligible decolorization (17%) under the agitating conditions.[29] Previous studies reported that azo dyes are generally resistant to the attack by bacteria under agitating conditions.[30] Azo dye decolorization by the bacterial species is often initiated by enzymatic reduction of azo bonds where the presence of oxygen normally inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds.[31] In the present study, equal performance of decolorization was found under shaking condition rather than inhibition. This meant decolorization was facilitated by the bacterial lignin peroxidase that catalyzes reaction in presence of electron donor like oxygen/ water/ hydrogen peroxide. No degradation was found in the abiotic control flasks.

Decolorization performance was decreased (80, 72, 64 and 57%) with the increase in initial dye concentration (0.25, 0.50, 0.75 and 1.00 g/L respectively). The pH of culture medium of *B. laterosporus* at the time of dye addition was 8.0. No change in the pH of culture medium was found during decolorization. Better decolorization performance was observed in the neutrality to alkaline range of pH 7.0 to 11.0. *B. laterosporus* decolorized 59, 70, 87, 86 and 82% of Navy Blue-3G when the pH of medium was 3.0, 5.0, 7.0, 9.0 and 11.0 respectively. *B. laterosporus* exhibited 32, 72, 80 and 59% decolorization at 5, 15, 30 and 45 °C respectively. About 80 and 67% of dye was removed at 15 and 45 °C respectively after an extended incubation period up to 72 h. This indicates that both cell viability and activities of biotransformation enzymes in the *B. laterosporus* exhibited under a wide temperature range from 15 to 45 °C and perform decolorization activity for azo dye like Navy Blue-3G. Usually lower concentrations show higher decolorization and decolorization performance decreases at high dye concentration may be due to toxicity of dye or their metabolites. Lower decolorization efficiency at the high dyestuff concentration was also reported by Verma and Madamwar.[32] The rate of decolorization increased with the increase in initial dye concentration up to 200 ppm where as further increase in dye concentration resulted in the decline of decolorization rates.[29, 33–34] Normally, dye concentration in the effluent varies within a narrow range of 100–200 mg/L.[35] Bacterial cultures generally exhibit maximum decolorization at pH values near 7.0. The rate of decolorization for NBNJ6 was optimum in the narrow pH range from 7.0 to 8.0 with marked reduction in decolorizing activity at pH 6.5.[29] Similarly, *E. coli* and *P. luteola*, exhibited best decolorization rates at pH 7.0; with the constant rates up to the pH 9.5.[31] Maximum rate of decolorization was between pH 7.0 and 8.5; however further increase in pH caused decrease in the decolorization rate.[33] Generally, azo dye reduction by the bacterial cultures form more basic aromatic amines which leads into the rise in pH of medium by about 0.8–1.0 values.[36–37] Furthermore, the optimum incubation temperature for maximum decolorization of RV 5 was found to be 37 °C.[38] Bacterial consortium RVM 11.1 exhibited 94% decolorization of Reactive Violet-5 (RV 5) within 37 h under a wide temperature range from 25 to 40 °C and further increase in temperature to 50 °C drastically affected decolorization activity.[33] In the present study, decolorization performance of Navy Blue-3G remain unchanged up to second addition of the dye aliquot (cycle-II); however decolorization activity decreased up to 65% in the cycle III and remained constant in the cycle IV, and then decreased up to 58 and 50% in the cycle V and VI respectively. Moosvi et al., found that 93, 94 and 68% decolorization of Reactive Violet-5 in the cycle I (40 h), II (24 h) and III (24 h) respectively by the bacterial consortium RVM-11.1.[33]

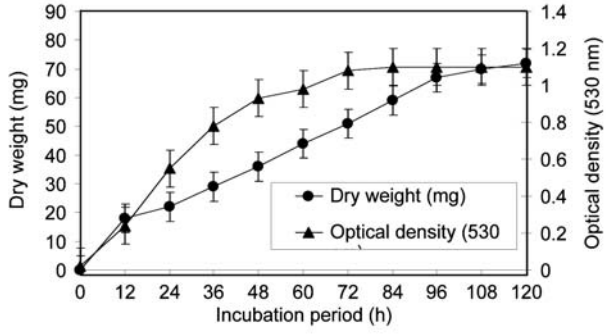

Fig. 1: Growth curve of the *B. laterosporus* MTCC 2298.

Figure 1 illustrates growth curve of *B. laterosporus* and Figure 2 illustrates the better decolorization of Navy Blue-3G during the exponential growth phase of *B. laterosporus*. This would be due to increased metabolic activities and higher production of biotransformation enzymes. Similar observations are previously reported by the Aksu indicating dependence of dye degradation on cell biomass and actively growing cells, hence bioaccumulation of various dyes for all dye concentrations was faster during the exponential growth phase of the microorganism and slowing down in the stationary phase.[39] A sharp decolorization activity was observed in the late exponential phase.[40] Similarly, biotransformation of cortexolone

to hydrocortisone by *Curvularia lunata* cells was determined to be approximately 20 times greater during the growth phase than during the stationary phase.[41] Moosvi et al, reported that rate of decolorization increased with the increase in inoculum's concentration;[33] however, there was no proportionate increase in the percentage of decolorization with increase in the inoculum's size of *Kurthia sp.* when inoculated in textile effluent.[42] Seval and Ozfer have reported that pellet method for the dye decolorization activity was more advantageous than the growing cell method.[43] The present study was undertaken to study the effect of initial biomass concentrations rather than increased inoculum's size on the decolorization performance of Navy Blue-3G. *B. laterosporus* required 60, 48, 24 and 12 h for the complete decolorization of Navy Blue-3G (50 mg/L) with the initial biomass concentration 10, 20, 30 and 40 g/L (wet wt) respectively. Time required for the decolorization of Navy Blue-3G was reduced to 1/5th by an increase in the initial biomass concentration of *B. laterosporus* up to 40 g/L (wet wt).

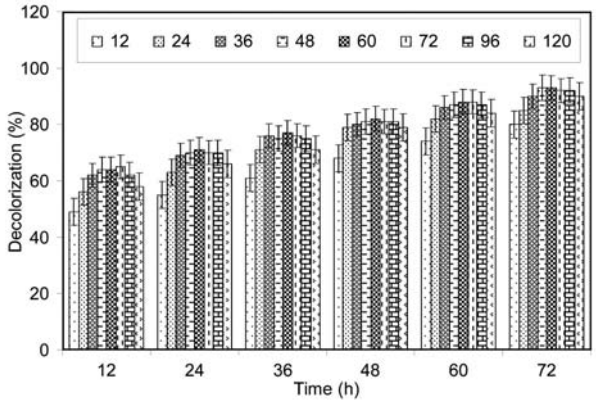

Fig. 2: Effect of different growth phases of **B. laterosporus** on the decolorization of Navy Blue-3G.

B. laterosporus required 72, 60, 48, 24, 30, 60 and 60 h for the complete decolorization of Navy Blue-3G (50 mg/L) in the synthetic medium and synthetic medium containing C (glucose and starch), N (peptone and NH_4Cl), and P (KH_2PO_4 and $NaH2PO_4$) sources (0.5%) respectively. Chen et al, reported the inhibitory effect of glucose on the rate of decolorization.[19] Interference of peptone and inorganic nitrogen such as ammonium ions in the medium was observed during dye decolorization.[39, 44–45] An isolated bacterial consortium RVM 11.1 has shown the ability to use starch as co-substrate for the decolorization of Reactive Violet-5.[33] In the present study, nitrogen sources were found to be stimulatory for the decolorization activity as compare to the studied carbon and phosphorus sources. There may be utilization of peptone/NH_4Cl by the cells for their multiplication and growth which could result in to the faster metabolism. Among the carbon sources studied, starch was better source as compare to glucose where the cells have shown poor decolorization. However, phosphorus sources found to be not that much effective as that of simply in the synthetic medium (without any supplementary components). Overall findings suggested that *B. laterosporus* exhibit greater ability to decolorize disperse azo dye like Navy Blue-3G and has potential for commercial applications.

3. 2. Status of Biotransformation Enzymes after Decolorization of Navy Blue-3G

Recently, Gomare and Govindwar have reported the significant induction of biotransformation enzymes viz. lignin peroxidase (54%), aminopyrine N-demethylase (122%) and MG reductase (50%) in the cells obtained after decolorization of Navy Blue-3G that revealed their involvement in the biodegradation.[22] Though the induction of aminopyrine N-demethylase is highest, significant increase in the lignin peroxidase suggested that cleavage of azo bond is facilitated by the lignin peroxidase and further transformations of the intermediate products would be carried by aminopyrine N-demethylase. However, complete inhibition of tyrosinase and no significant change in the activities of laccase and DCIP reductase were found in the cells obtained after decolorization. Involvement of lignin modifying enzymes such as lignin peroxidase as well as role of aminopyrine N-demethylase, NADH-DCIP reductase and MG reductase in the dye degradation was reported previously.[46–47] Lignin peroxidase was shown to be commonly involved in the dye decolorization, mainly in *P. chrysosporium* cultures.[46, 48–51] Literature survey revealed that enzymatic analysis during the microbial degradation of Navy Blue-3G was not reported in any of the microorganism.

3. 3. Biodegradation and Products Analysis of Navy Blue-3G

The UV-Vis spectral analysis (data not shown) illustrates disappearance of the peak at 545 nm and shift in λ-max to the shorter wavelength (545–533 nm) indicated the biodegradation of Navy Blue-3G and the formation of products that gives maxima at 533 nm. Biodegradation was further confirmed by TLC chromatogram that has shown a spot of control Navy Blue-3G with Rf value 0.88, where as two different spots (Rf = 0.90 and 0.72) of the degradation products extracted after 48 h (data not shown). FTIR spectrum of control Navy Blue-3G displayed a peak at 3430 cm^{-1} for N–H stretch, a peak at 2923 cm^{-1} for C–H stretch, a peak at 1738 cm^{-1} for –O–N=O stretch, a peak at 1698 cm^{-1} for >C=O stretch, a peak at 1606 cm^{-1} for ring vibrations, a peak at 1513 cm^{-1} for Ar–N=O stretch of aromatic nitro compound, a peak at 1328 cm^{-1} for C–N stretch of Ar–NH–R, a peak at 1174 cm^{-1} for asymmetric ring vibrations, a peak at 1039 cm^{-1} for –C–O–C stretch and a peak at 614 cm^{-1} for C–Br

stretch confirmed the chemical structure of dye (Figure 3). FTIR spectrum of the degradation products extracted after 48 h (Figure 4) displayed peaks at 3234 and 1671 cm^{-1} for –N=O stretch and suggested formation of nitroso compounds. A peak at 1514 cm^{-1} for Ar–N=O stretch of aromatic nitro compound stretch where as various peaks in the fingerprint region for mono and di-substituted benzene rings confirmed the products elected by GC-MS spectral analysis (Figure 5, Table 1). Figure 6 illustrates the proposed pathway of Navy Blue-3G degradation by the *B.*

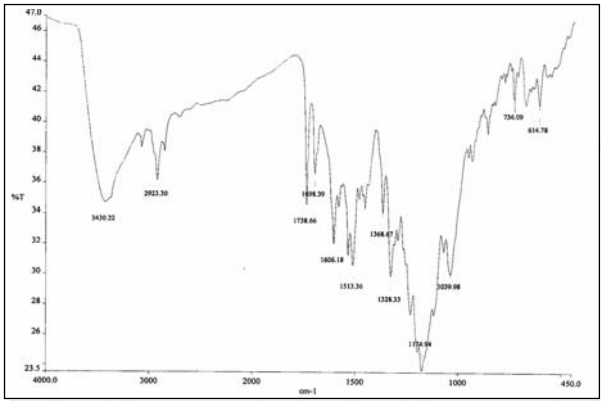

Fig. 3: FTIR spectrum of the control Navy Blue-3G.

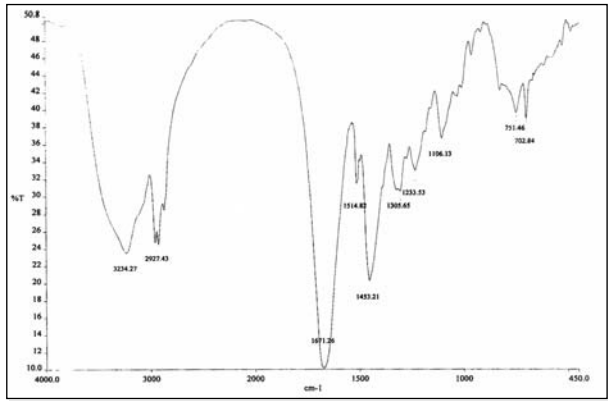

Fig. 4: FTIR spectrum of the degradation products of Navy Blue-3G extracted after 48 h.

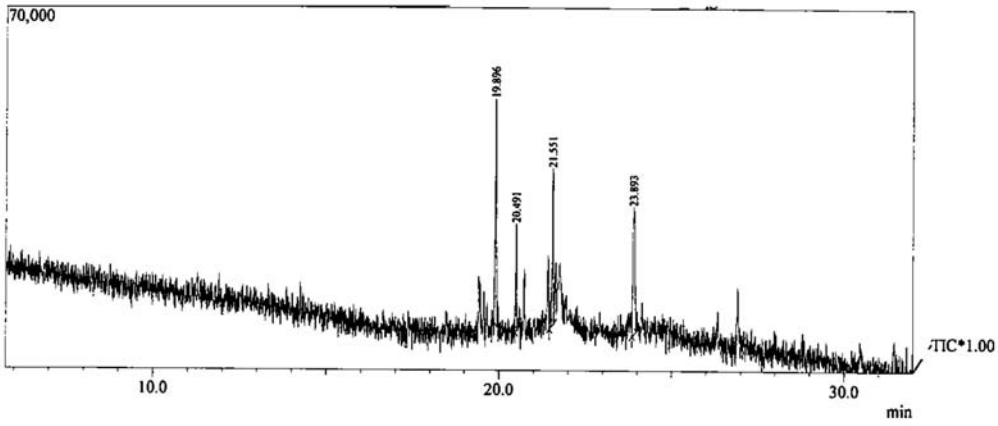

Fig. 5: Gas Chromatogram of the degradation products of Navy Blue-3G extracted after 48 h.

Table 1: GC-MS data of the Navy blue-3G degradation products extracted after 48 h

Peak No.	Retention Time	m/z (percent relative intensity of the predominant ions flagged in the fragmentation pattern)
1.	19.892	40 (7), 41 (64), 42 (7), 69 (64), 83 (100), 84 (7), 111 (86), 112 (7), 126 (7), 145 (7). 154 (64).
2.	20.492	40 (6), 41 (28), 55 (11), 69 (22), 70 (94), 97 (6), 125 (33), 126 (6), 154 (100), 180 (3).
3.	21.550	40 (3), 41 (20), 69 (7), 70 (87), 86 (20), 96 (7), 125 (13), 138 (7), 139 (3), 154 (100), 168 (3).
4.	23.892	40 (6), 41 (19), 55 (19), 68 (25), 70 (3), 86 (75), 98 (3), 112 (3), 126 (3), 141 (13), 155 (3), 170 (100).

laterosporus. Bromo-benzene (m/z 156, RT 19.892; M-2 = 154), ester substituted aniline derivative (m/z 178, RT 20.492; M+2 = 180), *meta*-di nitro benzene (m/z 167, RT 21.550; M+1 = 168) and *ortho* benzene aniline derivative (m/z 169, RT 23.892; M+1 = 170) were the degradation products elected by GC-MS spectral analysis. High molecular weight substituted ester derivatives generated during the degradation would have been mineralized, since likely compounds are not detected by GC-MS analysis.

We presumed that lignin peroxidase would have been responsible for the asymmetric cleavage of azo linkage to form two intermediate compounds (bromine substituted dinitro benzene and high molecular weight ester substituted complex aniline derivative). Different substituent present on the benzene ring are removed stepwise; however, bromine remained on the benzene ring and would have been removed as a last moiety. Removal of

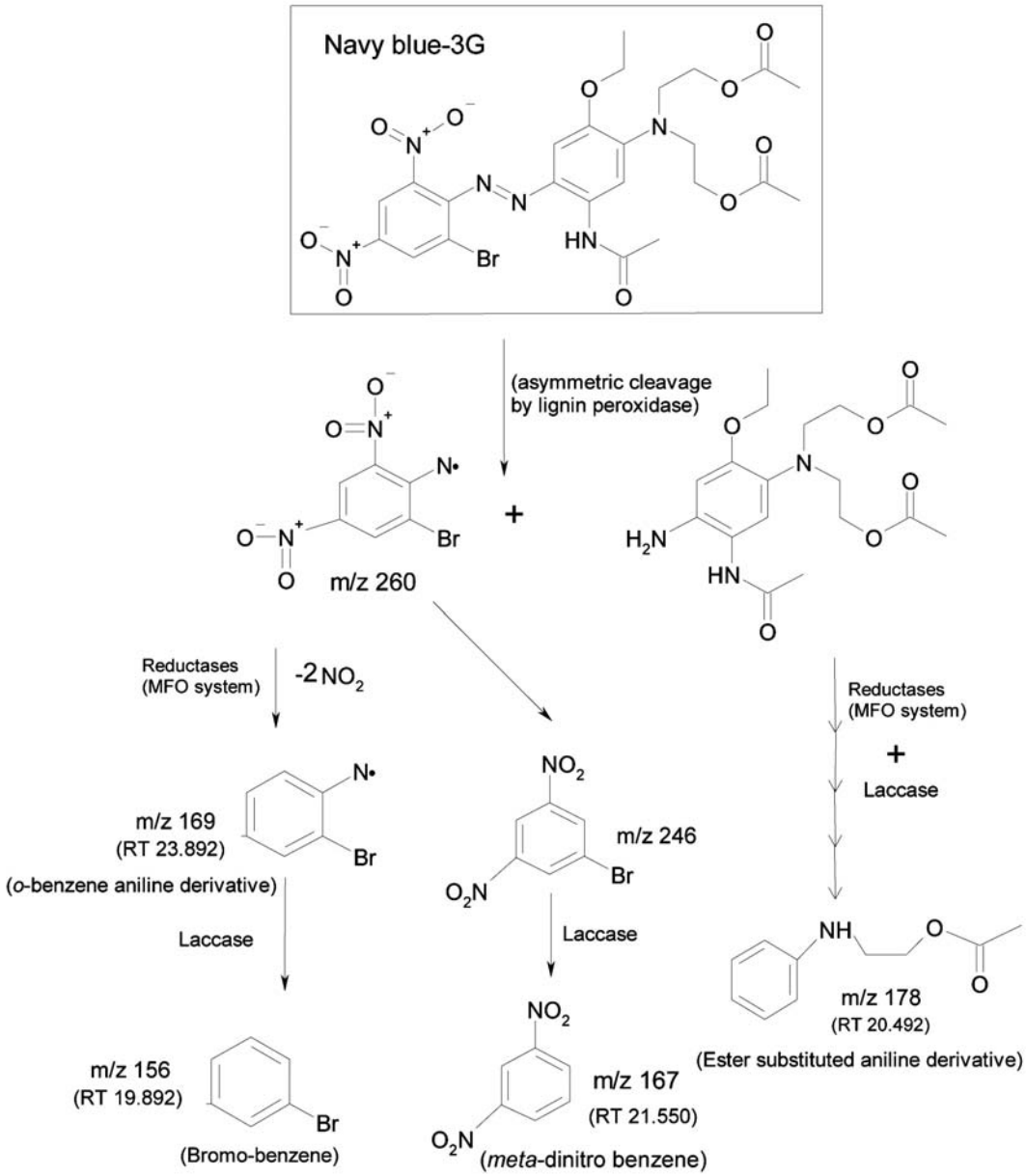

Fig. 6: Pathway proposed for the degradation of Navy Blue-3G by *B. laterosporus.*

NO_2 substitutions present on the bromine substituted benzene derivative and subsequent reductive deamination by laccase to generate bromobenzene would be the action of bacterial mixed function oxidase system specifically the reductases. This aniline derivative would be debrominated by the action of laccase to produce *meta*-dinitro benzene (RT 21.550, m/z 167). In addition, laccase along with bacterial mixed function oxidase system would be responsible for the oxidative cleavage of second intermediate compound i.e. ester substituted complex aniline derivative produced after asymmetric cleavage of azo bond, since its activity was present during the degradation rather than significant induction. This may be attributed by the absence of methyl groups in the *ortho* or *para* positions of azo bond. This meant that subsequent breaking of intermediate compounds leads to formation of low molecular weight ester substituted aniline derivative (RT 20.492, m/z 178). Weber and Adams reported a two-phase reaction in the degradation of the Disperse Blue-79 with a fast phase of azo dye elimination and a slow phase of biotransformation.[52] The reductive cleavage of the azo linkage resulting in the formation of the amine 2-bromine-4, 6-dinitroaniline (BDNA), which is toxic and mutagenic, and of the amine N, N-di-substituted 1, 4-diaminobenzene. Laccases are most diverse in their catalytic action and laccase catalyzed transformation of dyes depends on the chemical structure of dye molecules. Also, decolorization of malachite green by *B. laterosporus* was faster with the significant increase

in laccase where biodegradation process involved deamination and opening of benzene ring structure to produce non toxic products.[25] Overall findings suggest that *B. laterosporus* has huge potential of dye decolorization due to the presence of different biotransformation enzymes.

3. 4. Microbial and Phytotoxicity Analysis

Five natural dyes obtained from *Acacia catechu, Kerria lacca, Quercus infectoria, Rubia cordifolia* and *Rumex maritimus* were tested against common pathogens *Escherichia coli, Bacillus sublilis, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa* and showed significant zone of inhibition.[53] The toxicity of ten benzamines was tested by the well diffusion assay technique against 18 common bacterial species including the nitrogen-fixing bacterium *Azotobacter vinelandii*.[54] However, literature survey reveals that there are very few reports on the comparative study of the toxicity of textile dyes and their degradation products against the ecologically important bacteria like *Pseudomonas aeruginosa* and *Azotobacter vinelandii* which play important role in the phosphate solubilization and nitrogen fixation respectively. Toxicity of the control dye was studied for the selected bacterial strains as well as for the crops and the concentration showing remarkable toxicity effect on the respective group of organisms (i. e. bacteria and crops) was selected for the comparative studies with respect to degradation products. Recently, the microbial and phytotoxicity effects of the azo dyes such as Methyl red[22] and Golden Yellow HER[23] have reported in comparison with their degradation products. Table 2 illustrates the toxicity effects of control dye and its degradation products extracted after 48 h for the *B. laterosporus*, *P. aeruginosa* and *A. vinelandii*.

Phytotoxicity experiments were carried out in order to ensure that textile sludge composts will be safe for land use. Table 3 illustrates the toxicity effects of control dye (100 ppm) and its degradation products extracted after 48 h (100 ppm) for the *Sorghum vulgare* and *Phaseolus mungo*. A large number of studies have been carried out with large variations among bioassays and plant species.[55–58] Seed of root crops, cereals and legumes containing high quantity of reserves, and the sensitivity of a plant species to toxicity depends on the quantity of their reserves; however, inhibition was observed on shoot and root lengths, the root length was more affected.[59] Findings in the present study suggested that degradation products of disperse azo dye Navy Blue-3G were less toxic for the bacteria *B. laterosporus*, *P. aeruginosa* and *A. vinelandii* as well as for the common crops *Sorghum vulgare* and *Phaseolus mungo* than the control dye. Length of plumule and radicle in *P. mungo* was induced in the degradation products suggests utilization of non-toxic degradation products for their metabolism. Recently, similar type of induction have been reported in the *P. mungo* as well as in the *S. vulgare* due to the supplement of degradation products of Golden Yellow HER than that of control dye.[23]

Table 2: The zone of inhibition of *B. laterosporus, P. aeruginosa* and *A. vinelandii* in the presence of dye and its degradation products extracted after 48 h

Bacteria	Diameter of zone of inhibition (cm)	
	Navy blue-3G (1000 ppm)	Degradation products (1000 ppm)
B. laterosporus MTCC 2298	1.10 ± 0.05	0.80 ± 0.05*
P. aeruginosa NCIM 2036	1.20 ± 0.05	0.70 ± 0.05***
A. vinelandii NCIM 2821	1.00 ± 0.05	0.70 ± 0.05*

Data was analyzed by one-way ANOVA Test and mentioned values are mean of three experiments, SEM (±). Zone of inhibition is significantly different in the degradation products from that of in the control dye at * $P < 0.05$, *** $P < 0.001$ when compared by Tukey Kramer Multiple Comparison Test.

3. 5. Reduction of BOD and COD after Decolorization of Navy Blue-3G and Mixture of Dyes

Table 4 illustrates status of BOD and COD in the abiotic control (un-inoculated with dye), biotic control (inoculated without dye) and test (after decolorization). Reduction in the BOD by 75 and 95% was observed that after the decolorization of Navy Blue-3G and mixture of

Table 3: Toxicity of Navy Blue-3G (100 ppm) and its degradation products extracted after 48 h (100 ppm) for the *Sorghum vulgare* and *Phaseolus mungo*

Parameters	*Sorghum vulgare*			*Phaseolus mungo*		
	Plain water	Navy Blue-3G	Degradation products	Plain water	Navy Blue-3G	Degradation products
Germination (%)	100	50	90	100	90	100
Plumule (cm)	4.99 ± 0.77	1.01^{***} ± 0.29	2.01 ± 0.47	7.88 ± 0.54	5.55^{**} ± 0.16	$10.80^{\$\$\$}$ ± 0.55
Radicle (cm)	2.29 ± 0.39	0.63^{**} ± 0.09	$1.65^{\$}$ ± 0.28	1.52 ± 0.26	0.70^{**} ± 0.09	$1.88^{\$\$\$}$ ± 0.07

Data was analyzed by one-way ANOVA Test and mentioned values are the mean of ten germinated seeds of three sets SEM (±). Seeds germinated in Navy Blue-3G are significantly different from the seeds germinated in plain water at ** $P < 0.01$, *** $P < 0.001$ and the seeds germinated in degradation products are significantly different from the seeds germinated in Navy Blue-3G at $^{\$}$ $P < 0.05$, $^{\$\$\$}$ $P < 0.001$ when compared by Tukey Kramer Multiple Comparison Test.

dyes, respectively. Similarly reduction in COD was observed by 83 and 61% after the decolorization of Navy Blue-3G and mixture of dyes, respectively. These results also indicate the detoxification of the dye and dye mixtures.

Table 4: The BOD and COD in abiotic control, biotic control and tests obtained after decolorization of Navy Blue-3G and mixture of dyes.

Sample	Navy Blue-3G		Mixture of dyes	
	BOD (mg/L)	COD (ppm)	BOD (mg/L)	COD (ppm)
Abiotic Control	81.00	4800	81.00	5200
Biotic control	40.00	800	40.00	800
Test	60.00	1600	44.00	2800

4. Conclusion

Present study dealt first report on the biochemical aspects of dye degradation as well as toxicity effect of the degradation products and ensures the efficiency of *B. laterosporus* for the removal of disperse azo dye like Navy Blue-3G from the textile effluent. Maximum decolorization at the optimum temperature (30 °C) and broader range of pH (7–11) suggested potential of *B. laterosporus* for the industrial level application point of view. In *Brevibacillus laterosporus* MTCC 2298, lignin peroxidase facilitate the cleavage of azo linkage where as bacterial mixed function oxidase system catalyze subsequent breaking of intermediate compounds and mineralization that leads to detoxification. Reduction in the BOD and COD after decolorization of Navy blue-3G as well as mixture of dyes as compare to that of control suggested environmental friendly process of decolorization.

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Povzetek

Razbarvanje široko uporabljanega disperznega azo barvila Navy Blue-3G smo proučevali s kulturo *Brevibacillus laterosporus* MTCC 22298. Z začetno koncentreacijo barvila 50 mg/l je bilo doseženo 80 % razbarvanje v 48 urah. Pri višji koncentraciji barvila 1,0 g/l se je činkovitost zmanjšala na 66 % kljub podaljšanemu času razbarvanja na 72 ur. Maksimalno razbarvanje je bilo doseženo pri 30 °C in območju pH vrednosti 7,0–11,0. Učinki reakcije na posamezne encimske aktivnosti so bili različni. Ekstrahirani razgradni produkti so bili netoksični za kulture *Brevibacillus laterosporus, Pseudomonas aeruginosa, Azotobacter vinelandii, Sorghum vulgare* in *Phaseolus mungo*.