Biodegradation (2009) 20:777–787
DOI 10.1007/s10532-009-9266-y

ORIGINAL PAPER

Effect of inducers on the decolorization and biodegradation of textile azo dye Navy blue 2GL by *Bacillus* sp. VUS

Vishal V. Dawkar · Umesh U. Jadhav · Gajanan S. Ghodake · Sanjay P. Govindwar

Received: 8 January 2009 / Accepted: 29 April 2009 / Published online: 26 May 2009

Abstract *Bacillus* sp. VUS decolorized azo dye Navy blue 2GL in 48 h at static anoxic condition in yeast extract medium, whereas it took only 18 h for the decolorization in presence of $CaCl_2$. Different inducers played role in the decolorization of Navy blue 2GL. $CaCl_2$ found to be the most effective inducer among all inducers tested. The activity of enzymes like lignin peroxidase, laccase and reductases viz. NADH-DCIP, azo and riboflavin induced during decolorization represents their role in the biodegradation. Extracellular LiP and intracellular laccase activity induced with $CaCl_2$. Yeast extract was best medium for faster decolorization than other media. UV–vis spectrophotometer analysis and visual examinations showed decolorization of dye. High performance liquid chromatography, Fourier transforms infrared spectroscopy showed degradation of dye. Gas Chromatography-Mass Spectroscopy revealed formation of 4-Amino-3-(2-bromo-4, 6-dinitro-phenylazo)-phenol and acetic acid 2-(-acetoxy-ethylamino)-ethyl ester as final products. *Bacillus* sp. VUS also decolorized synthetic effluent. Phytotoxicity study showed detoxification of Navy blue 2GL.

Keywords *Bacillus* sp. · Biodegradation · Lignin peroxidase · Inducers · Navy blue 2GL · GC-MS

V. V. Dawkar · U. U. Jadhav · S. P. Govindwar (✉)
Department of Biochemistry, Shivaji University, Kolhapur 416004, India
e-mail: spg_biochem@unishivaji.ac.in

V. V. Dawkar
e-mail: vvdawkar@yahoo.co.in

G. S. Ghodake
Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, South Korea

Introduction

Azo dyes are the largest and most versatile class of dyes, widely used in the textile processing, leather, plastics, cosmetics, and food industries (Selvam et al. 2003). The reactive azo dyes are characterized by the presence of a nitrogen–nitrogen double bond (–N=N–), namely the azo group bound to aromatic groups (Ambrosio and Campos-Takaki 2004). They are frequently found chemically unchanged in the effluents of wastewater treatment plants. The azo dyes are regarded as pollutants as they are not readily degraded under anaerobic conditions because of complex aromatic molecular structures (Wesenberg et al. 2002). More than 10–15% dye does not bind to fiber during color processing and release into the wastewater causing serious environmental pollution (Robinson et al. 2001; Pearcea et al. 2003). In addition to their visual effect and adverse impact in terms of chemical oxygen demand (COD), many synthetic dyes show toxic, carcinogenic and genotoxic effects (Ozfer et al. 2003). The carcinogenicity of an azo dye may be due to the

dye itself or aryl amine derivatives generated during the reductive biotransformation of an azo linkage.

The conventional aerobic wastewater treatment processes usually cannot efficiently decolorize and degrade azo dye bearing effluents to the regulatory levels (Carliell et al. 1995). Although electrochemical destruction (Mohana et al. 2007), photocatalysis and sorption (Hasnat et al. 2007) are effective tertiary treatments but not economically viable. However, the anaerobic degradation yields aromatic amines which are mutagenic and toxic to human, and cannot be metabolized further under these conditions (Do et al. 2002). Reactive azo dyes and aromatic amino derivatives are non-biodegradable compounds which can even inhibit activated sludge organism in treatment process of dye effluents (Maier et al. 2004). It is thus important to explore the possibilities of isolating efficient aerobic degraders for use in the decolorization and biotreatment of textile effluents.

Many bacteria are capable to degrade azo reactive dyes aerobically and anaerobically (Parshetti et al. 2006a); however, in many cases the metabolic products, usually aromatic amines are toxic or even more toxic than the starting azo dyes (Hu 2001). In addition, it has recently been demonstrated that peroxidases (LiP), phenoloxidases (laccases), reductases (DCIP and MG) and monooxygenases can act on specific recalcitrant pollutants by transforming them into other products, thus allowing for a possible better final treatment (Kalme et al. 2007a). In order to obtain more information about the aerobic degradation of azo dye, we have used *Bacillus* sp. VUS, which aerobically degrades azo dye Navy blue 2GL. The objective of the present study was to examine the decolorization of textile dye Navy blue 2GL in the presence of different inducers and in the determination of the effect, of the dye and its degraded metabolites after the action of *Bacillus* sp. VUS on the growth of crop plants.

Materials and methods

Microorganism and culture conditions

Bacillus sp. VUS was isolated from textile effluent contaminated soil (Dawkar et al. 2008). Pure culture was maintained on the nutrient agar slants. Compositions of different nutrient media used for the decolorization were as nutrient broth (g/l): NaCl 5, peptone 5, beef extract 3; beef extract broth (g/l): NaCl 5, beef extract 3 and yeast extract broth (g/l): NaCl 5, yeast extract 5.

Dyestuffs and chemicals

All chemicals used were of the highest purity and of an analytical grade. 2, 2′-Azinobis (3-ethylbezthiazoline-6-sulphonate) (ABTS) was purchased from Sigma–Aldrich, USA. Tartaric acid, *n*-propanol and catechol were purchased from Sisco Research Laboratories, India. The textile dye Navy blue 2GL was a generous gift from local Manpasand textile industry, Ichalkaranji, India.

Decolorization experiments

Decolorization at static and shaking condition in different media

Bacillus sp. VUS was grown for 24 h at 40°C in 250 ml Erlenmeyer flasks containing 100 ml different medium to study the effect of the media at static and shaking condition on the decolorization performance of microbial culture. The dye (50 mg/l) was added after 24 h growth and incubated at static as well as shaking condition at 40°C for 120 rpm on orbital shaker. The aliquot (3 ml) of the culture media was withdrawn at different time intervals and centrifuged at 6,000*g* for 20 min. Decolorization was monitored by measuring the absorbance of culture supernatant at 570 nm. Change in the pH was also recorded. Growth of the microorganism in the dye containing medium was determined by the gravimetric method after drying at 80°C until constant weight.

Decolorization at different dye concentration

In order to examine the effect of initial dye concentration on the decolorization in static condition 100, 150, 200, 250, 300 and 350 mg/l of the Navy blue 2GL dye was added in 24 h grown culture of *Bacillus* sp. VUS in the yeast extract medium. The percent decolorization was measured at different time interval. All decolorization experiments were performed in three sets. Abiotic controls (without microorganism) were always included. The synthetic effluent was made by mixing different dyes. The percent

decolorization and average decolorization (Saratale et al. 2006) rate was measured at different time interval.

The average decolorization rate was calculated as follows:

$$\text{Average decolorization rate} = \frac{C \times \%D \times 1000}{100 \times t}$$

where C = initial concentration of dye (mg/l), $\%D$ = dye decolorization (%) after time t.

Enzyme activities

Cell free extract (crude enzyme) was made by growing *Bacillus* sp. VUS in 100 ml yeast extract medium at 40°C, centrifuged at 6,000*g* for 20 min. These cells were suspended in 50 mM potassium phosphate buffer (pH 7.4) for sonication (sonics-vibracell ultrasonic processor) keeping sonifier output at 60 amplitude maintaining temperature at 4°C and giving ten strokes, each of 30 s with 2 min interval. This extract was used without centrifugation as an enzyme source.

Enzyme assays

Laccase activity was determined using 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (10%) as a substrate in 0.1 M acetate buffer (pH 4.9) at room temperature. Oxidized ABTS was measured at 420 nm. The volume of reaction mixture maintained was 2 ml (Hatvani and Mecs 2001). Tyrosinase activity was determined in a reaction mixture containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4). The formed catechol quinone was measured at 410 nm at room temperature by keeping volume of the reaction mixture at 2 ml (Zhang and Flurkey 1997). LiP activity was determined by monitoring the formation of propanaldehyde at 300 nm. In reaction mixture of 2.5 ml contained, 0.5 ml 100 mM *n*-propanol, 0.5 ml 250 mM tartaric acid, 0.2 ml 10 mM H_2O_2 (Shanmugam et al. 1999). Blank contained all components except enzyme in all assay procedures. One unit of enzyme activity was defined as a change in absorbance units/min/mg of protein.

NADH-DCIP reductase activity was determined by using a procedure reported earlier (Salokhe and Govindwar 1999). The assay mixture contained 50 μM DCIP, 1.142 mM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution (sonicated cells suspension) in a total volume of 5.0 ml. The DCIP reduction was calculated using the extinction coefficient of 19 mM/cm. In azo reductase assay, methyl red reduction was calculated using the extinction coefficient 23.36 mM/cm. The assay mixture contained 0.35 mM NADH and 110 μmol MR in a total volume of 1.0 ml.

Riboflavin reductase NAD (P) H: Flavin oxidoreductase was measured by a modification of Fontecave et al. (1987) method. In this aerobic assay, the flavin reductase catalyzes the reduction of riboflavin, and the reduced riboflavin is immediately reoxidized by oxygen. Cell extract was added to a solution (final volume, 1 ml) containing 100 μmol of Tris–HCl (pH 7.5), 25 μmol of NADPH and 0.003 unit riboflavin. The decrease in absorbance was measured at 340 nm spectrophotometrically. Reaction rates were calculated by using a molar extinction coefficient of 6.3 mM/cm. Protein concentrations were estimated by using Biuret method.

Effect of inducers on decolorization

Inducers like veratrole (0.5 mM), calcium chloride (1 mM), calcium carbonate (1 mM), tryptophan (2 mM), guaicol (1 mM) and aniline (1 mM)) were added individually in 100 ml yeast extract medium at the time of inoculation. *Bacillus* sp. VUS was grown for 24 h at 40°C. 50 mg/l dye was added to 24 h grown culture and percentage decolorization and enzyme activity status were determined.

Biodecolorization and biodegradation analysis

Decolorization was monitored by UV–vis spectroscopic analysis (Hitachi U-2800) whereas biodegradation was monitored by HPLC and FTIR. Identification of metabolites was carried out by GC-MS. For this, 100 ml sample was taken at intervals (24, 48 h), centrifuged at 10,000*g* and extraction of metabolites was carried from supernatant using equal volume of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. HPLC analysis was carried out (Waters model no. 2690) on C_{18} column (symmetry, 4.6 × 250 mm) with

methanol:acetonitrile (1:1) as mobile phase with at flow rate of 1.0 ml/min and UV detector at 570 nm.

The biodegraded Navy blue 2GL was characterized by Fourier Transform Infrared Spectroscopy (Perkin Elmer, Spectrum one) and compared with the control dye. The FTIR analysis was done in the mid IR region of 400–4,000/cm with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95. Pellets were fixed in sample holder for the analyses. Rotary vacuum evaporated sample (extracted after 8 h decolorization period) was dissolved in methanol. GC-MS analysis of metabolites was carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1 ml/min. The injector temperature was maintained at 280°C with oven conditions as: 80°C kept constant for 2 min increased upto 200°C with 10°C/min raised upto 280°C with 20°C/min rate. The compounds were identified on the basis of mass spectra and using the NIST library.

Phytotoxicity study

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye at the concentration of 5,000 ppm. Tests were carried out according to the Parshetti et al. (2006a) on two kinds of seeds commonly used in the Indian agriculture: *Triticum aestivum* and *Sorghum bicolor*.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. Readings were considered significant at $P \leq 0.05$.

Results and discussion

Effect of media on the decolorization

Three different media were used in order to establish the most suitable condition for the decolorization of dye Navy blue 2GL by *Bacillus* sp. VUS. Decolorization of Navy blue 2GL was 94% at static anoxic condition in 48, 60 and 72 h in yeast extract, beef extract and nutrient broth medium, respectively, and 0% at shaking condition. The growth of the organism was more at shaking (0.25 g/l) condition as compared to static (0.18 g/l) condition (Fig. 1). The change in pH was not observed during decolorization indicated microbial action and not due to change in pH. *Bacillus* sp. also decolorized (79%) synthetic effluent in 120 h.

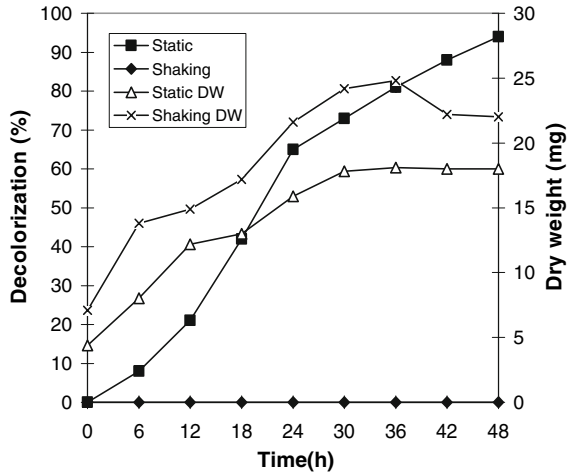

Fig. 1 Time course evolution of *Bacillus* sp. VUS growth in form of dry weight and dye decolorization in static and shaking conditions at the initial dye concentration of 50 mg/l in yeast extract medium

Three different media showed differential induction pattern of enzymes and time required for the decolorization of dye Navy blue 2GL by *Bacillus* sp. (Table 1). The decolorization of the dye solution could be selectively supported by the media composition. The yeast extract medium was more appropriate for the decolorization of dye Navy blue 2GL as compared to the beef extract and nutrient broth medium. A relationship between the medium and molecular structure of the dye might be established. It should, however, be noted that the relationship between the molecular structure of the dyes and their decolorization by bacterial treatment is still not clearly understood (Kalme et al. 2007b). The differences showed that the peptone and beef extract interfered in the decolorization as already been reported (Ambrosio and Campos-Takaki 2004). These observations can be interpreted as a negative impact of peptone and beef extract on the enzyme activities. These results are with an agreement with

Table 1 Effect of nutrient media on the enzyme activity and the decolorization of Navy blue 2GL by *Bacillus* sp. VUS

	Media					
	YE		BE		NB	
Decolorization time (h)	48		60		72	
Enzymes	Control	Test[e]	Control	Test[e]	Control	Test[e]
LiP[a]						
Cell extract	0.049 ± 0.005	0.206 ± 0.008***	0.120 ± 0.002	0.244 ± 0.009***	0.136 ± 0.006	0.121 ± 0.011***
Culture supernatant	0.018 ± 0.001	0.130 ± 0.008***	0.038 ± 0.007	0.126 ± 0.009***	0.008 ± 0.003	0.085 ± 0.009***
Laccase[a]	NA	0.074 ± 0.003***	0.013 ± 0.002	0.034 ± 0.002	NA	0.103 ± 0.003***
Tyrosinase[a]	NA	NA	NA	0.02 ± 0.002**	NA	0.07 ± 0.003***
NADH-DCIP reductase[b]	0.134 ± 0.075	0.222 ± 0.66***	0.097 ± 0.064	0.143 ± 0.2***	0.017 ± 0.033	0.131 ± 0.10***
Azo reductase[c]	3.57 ± 0.34	6.66 ± 0.29*	1.03 ± 0.16	2.24 ± 0.14*	1.23 ± 0.32	4.84 ± 1.79*
RFR[d]	0.48 ± 0.09	2.06 ± 0.16***	0.51 ± 0.04	4.38 ± 0.57**	0.28 ± 0.07	0.48 ± 0.03

Values are mean of three experiments ± SEM

NA no activity

Significantly different from control (0 h) at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test

[a] Activity in units/min/mg of protein

[b] mg of DCIP reduced/min/mg of protein

[c] μg of methyl red reduced/min/mg of protein

[d] μg of riboflavin reduced/min/mg of protein

[e] After complete decolorization

the reported literature (Sumathi and Manju 2000; Ambrosio and Campos-Takaki 2004).

Effect of initial dye concentration on decolorization

Percent decolorization of Navy blue 2GL by *Bacillus* sp. VUS was varied with the initial dye concentrations (50–350 mg/l) when studied upto 240 h at static anoxic condition. Dye removal was 95–85% at 50–150 mg/l dye concentration. 80–60% dye removal was recorded at the concentration of 200–350 mg/l by 100 ml batch culture of *Bacillus* sp. VUS in yeast extract medium. The time required for 94% decolorization of 50 mg/l dye was 48 h. The time required for decolorization varied from 6–7 days as the concentration increased (100–350 mg/l) (data not shown). The decolorization was not prolonged at 350 mg/l; instead this concentration seemed to be toxic for the cell growth in 100 ml batch culture. Increased dye concentrations of the dye take more time for the decolorization (Kalme et al. 2007a, b).

Enzyme activities while decolorization in batch culture

The time course of LiP and laccase production shown in Fig. 2 in the yeast extract medium. LiP activity was decreased after 65% decolorization (24 h) but there was significant induction in the activity after 94% decolorization (48 h) (420% as compared to 24 h grown culture). No activity of laccase was observed at the time of dye addition but appeared after 24 and 48 h of the growth. No activity of laccase was observed in the culture supernatant; however induced activity of LiP (722%) was recorded. Induction in the activities of NADH-DCIP, azo and riboflavin reductase enzymes in the time course of 48 h (165, 186 and 429%, respectively) was observed (data not shown). It was noticed that DCIP and azo reductase activities increased up to complete

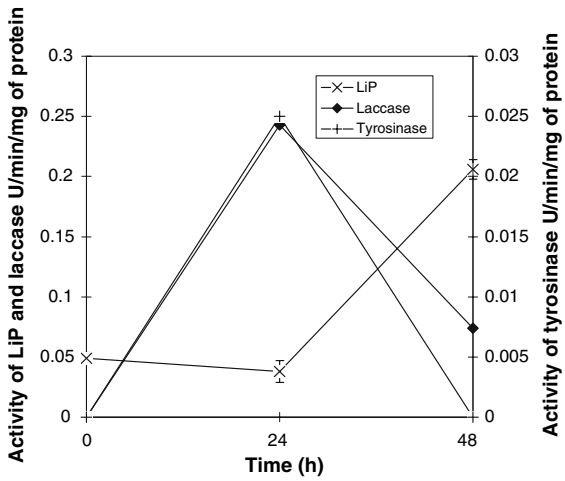

Fig. 2 Time course of LiP, laccase and tyrosinase in *Bacillus* sp. VUS during Navy blue 2GL decolorization

decolorization but riboflavin reductase activity decreased after 65% decolorization (24 h). In case of beef extract medium, there were an induction in the activities of LiP, laccase and reductases like NADH-DCIP, azo and riboflavin activities as 203, 261, 147, 217 and 858%, respectively (data not shown). Nutrient broth medium showed activities of the enzymes as LiP (88%), and reductases like NADH-DCIP (760%), azo (393%) and riboflavin (171%). Laccase activity was observed after 94% of decolorization in this medium (data not shown).

The ability of bacteria has been considered in several studies with respect to an enzyme status of laccase, LiP and tyrosinase (Jadhav et al. 2008). There was an induction of oxidative enzymes during decolorization of Navy blue 2GL. Of the relative contributions of laccase, manganese peroxidase and lignin peroxidase for the decolorization of dyes may be different for each microorganism and involved in the decolorization (Pointing and Vrijmoed 2000; Novotny et al. 2004). Induction in the activity of reductases like NADH-DCIP, azo and riboflavin enzyme suggests the prominent role of these in the decolorization process (Sarnaik and Kanekar 1999; Parshetti et al. 2006a). Peroxidase of *Bacillus* sp. VUS was able to oxidize *n*-propanol and various phenolic compounds including 3-(3, 4-dihydroxy phenyl) L-alanine (L-DOPA), hydroxyquinone, ethanol and veratrole. However, no activity was observed with xylidine and indole. Peroxidase oxidized *n*-propanol much faster as compared to other substrates (hydroxyquinone, L-DOPA and veratrole) which are phenolic and aromatic compounds. The K_m and V_{max} values for peroxidase with *n*-propanol as a substrate were found to be 0.076 mM and 3×10^2 μM, respectively (Dawkar et al. 2009). The presence of H_2O_2 showed an increase in the peroxidase activity by 1.33-fold, with Km 0.046 mM for H_2O_2 (Fig. 3).

Effect of inducers on the decolorization and enzyme activity

We have selected inducers of LiP and laccase for an induction of the enzyme activity during decolorization.

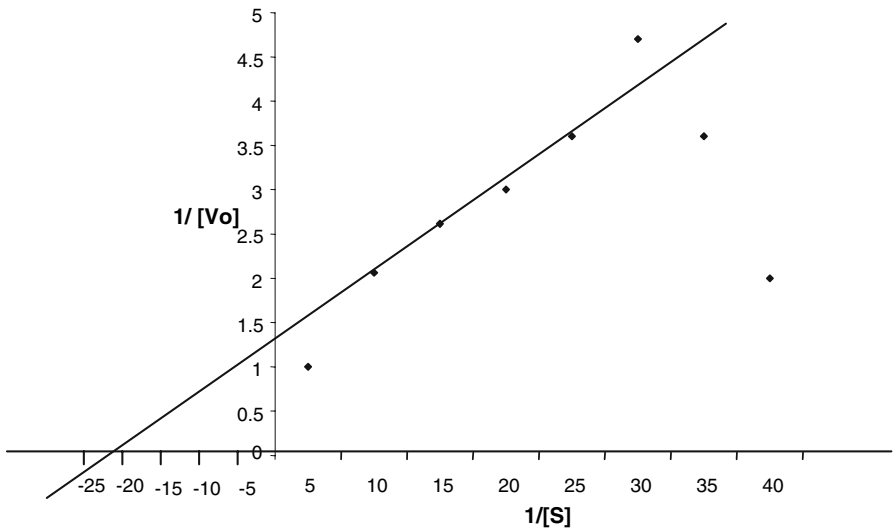

Fig. 3 Determination of *Km* and *Vmax* of the peroxidase from *Bacillus* sp. strain VUS with respect to H_2O_2

Table 2 Effect of inducers on the activity of enzymes and the decolorization of Navy blue 2GL

Inducer	Time of decolorization (h)	Enzymes[a]				
		LiP		Laccase		Tyrosinase
		A	B	A	B	
Control	48	0.367 ± 0.036	0.016 ± 0.003	0.012 ± 0.002	0.001 ± 0.0006	0.003 ± 0.001
$CaCl_2$	18	0.301 ± 0.027	0.037 ± 0.01	0.248 ± 0.038	0.033 ± 0.007	0.055 ± 0.02
$CaCO_3$	24	0.104 ± 0.01	0.035 ± 0.002	0.217 ± 0.008	NA	0.126 ± 0.004
Tryptophan	36	0.218 ± 0.029	0.222 ± 0.03	0.143 ± 0.03	NA	NA
Veratrole	24	0.235 ± 0.007	0.027 ± 0.002	0.104 ± 0.007	NA	0.026 ± 0.002
Guaicol	30	0.111 ± 0.001	0.05 ± 0.005	0.244 ± 0.016	NA	0.025 ± 0.002
Aniline	36	0.186 ± 0.006	0.139 ± 0.021	0.131 ± 0.006	0.03 ± 0.008	0.015 ± 0.002

Values are mean of three experiments ± SEM

A Enzyme activity in cell crude extract

B Enzyme activity in cell free supernatant

NA no activity

[a] Activity in units/min/mg of protein

The time required for complete decolorization of Navy blue 2GL was reduced (up to 18 h) when incubated with inducers. $CaCl_2$ was found to be the most effective inducer for the decolorization compared to other inducers tested (Table 2). *Bacillus* sp. VUS could decolorize dye 94% within 18 h with $CaCl_2$, whereas it took 48 h for decolorization without inducers. There was not that much change in the LiP activity at 18 h with $CaCl_2$ compared to 48 h enzyme activity status without inducers, while, laccase activity was significantly induced (intracellular and extracellular) in *Bacillus* sp. VUS in yeast extract medium. Tyrosinase activity was recorded after 94% decolorization (18 h).

The addition of inducers to the culture medium showed faster decolorization might be due to an enhancement of LiP and laccase production that facilitate its utilization (Rodriyguez Couto et al. 2002; Cavallazzil et al. 2005). An induction in the activity of LiP and laccase, and the reduction in the time of decolorization indicate the vital role of some chemical compounds which serves as inducers of the enzymes.

Biodecolorization and biodegradation analysis

UV–vis scan (400–800 nm) of the supernatant at different time intervals showed decolorization and decrease in the dye concentration from batch culture (Fig. 4). Peak observed at 570 nm (0 h), was decreased without any shift in λ_{max} up to complete decolorization of the medium (48 h). Comparison of FTIR spectrum of control dye with the metabolites extracted after 94% decolorization clearly indicated the biodegradation of the parent dye compound by *Bacillus* sp. VUS (Fig. 5). Peaks in the control dye spectrum represented its functional groups present in the structure in the form of: stretching vibrations of C–H (614 cm^{-1}), stretching at C–OH (1,040 cm^{-1}), C–N vibration

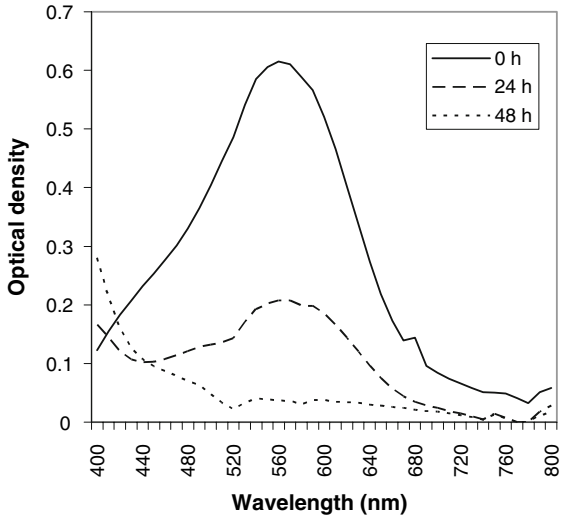

Fig. 4 UV–vis spectral scans from batch culture of *Bacillus* sp. VUS containing 50 mg/l of Navy blue 2GL at different incubation times: 0 (____), 24 (- - -) and 48 h (-- __ --)

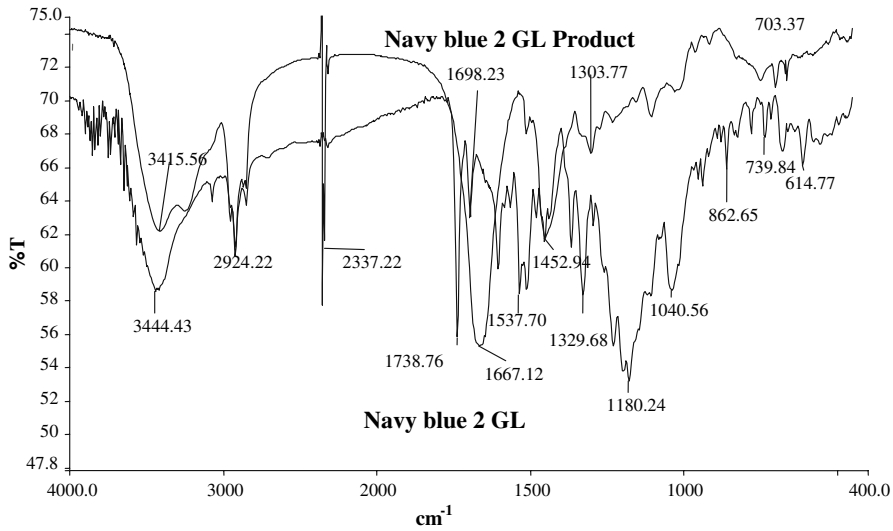

Fig. 5 FTIR spectral comparisons of 0 h Navy blue 2GL dye and 48 h extracted metabolites after degradation by *Bacillus* sp. VUS

(1,180 cm^{-1}), secondary aromatic amines (1,329 cm^{-1}) and CH_3 deformation (1,368 cm^{-1}). We observed C–H deformation at 1,454 and nitro compounds at 1,514 cm^{-1}. N=N symmetric stretching at 1,606 cm^{-1}. The stretching vibrations between C=O showed at 1,698 and 1,738 cm^{-1}. The asymmetric stretching between C–H was reported at 2,924 cm^{-1}, whereas peak at 3,444 cm^{-1} represented N–H overtone. The FTIR spectrum of 48 h (after 94% decolorization) extracted metabolites showed significant change in positions of peaks when compared to control dye spectrum. A new peak at 753 cm^{-1} represented benzene ring with four adjacent free H atoms, where as peak at 1,303 cm^{-1} was observed for C=H deformation. A new peak at 2,347 cm^{-1} represented charged amines.

The HPLC analysis of dye sample collected at 0 h incubation showed peak at 3.02 min. As the decolorization progressed, the biodegradation of parent compound was observed with different detectable peaks at different retention times (2.16, 2.80 and 2.95 min).

Gas Chromatography-Mass Spectroscopy analysis was carried out to investigate the metabolites formed during the biodegradation process. A pathway has been proposed for the biodegradation of Navy blue 2GL by *Bacillus* sp. VUS in static anoxic condition (Fig. 6). The asymmetric cleavage by LiP between carbon of aromatic ring and nitrogen resulted in reactive products (intermediates), which underwent demethylation reaction to produce more stable product [2-(2-Bromo-4, 6-dinitro phenylazo)-4-methoxy-phenylamino]-methanol. Once again the demethylation of above product gave 3-(2-Bromo-4, 6-dinitro phenylazo)-4-hydroxymethyl-amino)-phenol. The action of laccase on above intermediate resulted in the formation of 3-(2-Bromo-4, 6-dinitro-phenylazo)-4-methylamino-phenol. The final metabolite was identified as 4-Amino-3-(2-bromo-4, 6-dinitro-phenylazo)-phenol due to the reduction of the above compound with elimination of NH_3 molecule. The structures of the identified compounds were assigned from *m/z* values obtained. All intermediates are not recorded by GC-MS, but rationalized as necessary intermediates during biodegradation. The final products detected in the pathway were characterized as 4-Amino-3-(2-bromo-4, 6-dinitro-phenylazo)-phenol and acetic acid 2-(2-acetoxy-ethylamino)-ethyl ester, having molecular weight 382 and 157, however, GC showed 13 peaks.

It is known that several enzymes are present in the microsomal and cytosolic fraction (Dawkar et al. 2008; Parshetti et al. 2006b). Most of the enzymes have oxidative mechanism, which might be used in the degradation of dyes (Jadhav and Govindwar 2006). Our results have shown that *Bacillus* sp. VUS have ability to degrade dye Navy blue 2GL asymmetrically and reductively to excite the molecule. We have proposed pathway for the degradation of Navy blue 2GL, where initial asymmetric cleavage by LiP might have followed by oxidative mechanism and then role of laccase as reported by Cha et al. (2001).

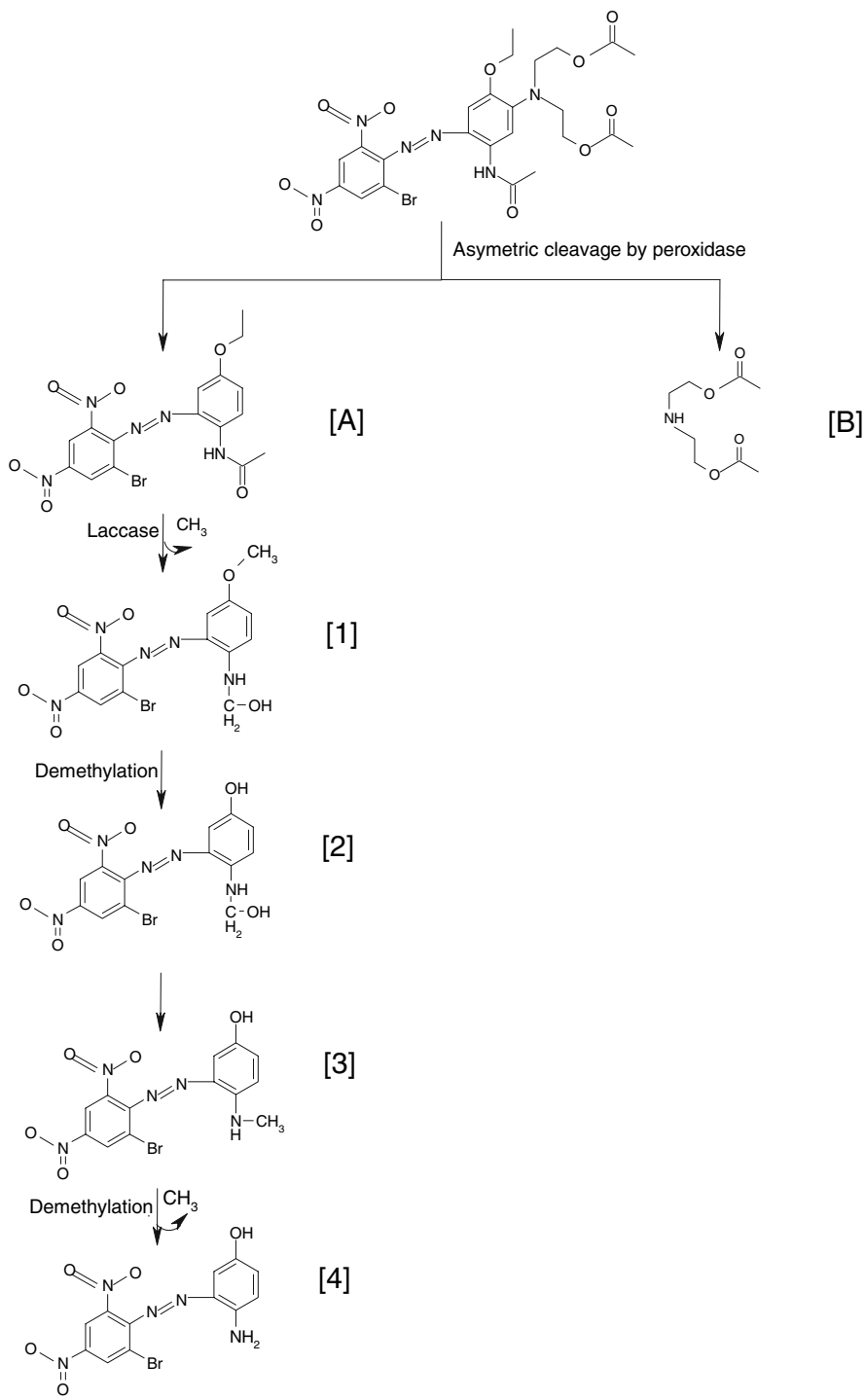

Fig. 6 Proposed pathway for degradation of Navy blue 2GL by *Bacillus* sp. VUS. The compounds represented by *alphabets* in *brackets* have not been found, but their existence is rationalized as necessary intermediates for the final products found. The compounds represented by *Arabic numbers* in *brackets* have been found in reaction mixture

Phytotoxicity analysis

Untreated Navy blue 2GL (5,000 ppm) showed 80 and 70% germination inhibition in *S. bicolor* and *T. aestivum*, respectively, after 7 days of incubation. There was no germination inhibition in both the seeds when metabolites formed after complete decolorization applied at the same concentration. Reduction

Table 3 Phytotoxicity of Navy blue 2GL (5,000 ppm) and its degradation products extracted after 48 h (5,000 ppm) for the *Triticum aestivum* and *Sorghum biocolor*

Dye concentration (ppm)	Plants studied					
	Triticum aestivum			*Sorghum biocolor*		
	Germination inhibition (%)	Shoot length (cm)	Root length (cm)	Germination inhibition (%)	Shoot length (cm)	Root length (cm)
Distilled water	0.00	10.2 ± 1.0	8.8 ± 1.4	0.00	11.2 ± 0.8	9.3 ± 1.1
Dye (5,000 ppm)	70.00	3.1 ± 0.42*	2.8 ± 0.99*	80.00	2.1 ± 0.52*	1.9 ± 0.31*
Metabolites (5,000 ppm)	0.00	10.0 ± 1.3	8.6 ± 1.2	0.00	10.6 ± 1.1	8.9 ± 1.2

Values are mean of three experiments ± SEM

Significantly different from control (0 h) at * $P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test

(2–5%) in the growth (shoot and root lengths) was observed in the presence of metabolites when compared to the growth in distilled water in both the plants (Table 3).

As the textile effluent without treatment is used in the agriculture of Ichalkaranji, India, it is of concern to check the effect of dyes on the crop plants. It has already been reported that the biodegradation of Reactive Red BLI by bacterium *Pseudomonas* sp. SUK1 did not produce metabolites as toxic as the original dye (Kalyani et al. 2008), where as the metabolites produced by bacteria resulted in the formation of aromatic amines, which can be highly toxic and carcinogenic (Hu 2001). Results from our phytotoxicity study showed that the metabolites produced by *Bacillus* sp. VUS after degradation of dye Navy blue 2GL did not inhibit germination of plants and are not toxic.

Conclusions

Textile azo dye Navy blue 2GL decolorized and detoxified by *Bacillus* sp. VUS. It also decolorized synthetic effluent made by mixing different textile dyes which usually used in textile industries. Enzyme studies showed direct involvement of laccases and LiP in the biodegradation of Navy blue 2GL. The effect of inducers on the decolorization of dye clearly indicates enhancement of the enzyme activities and reduction of the decolorization time. Phytotoxicity study demonstrates the detoxification of Navy blue 2GL with respect to Indian crops. These results confirm that, this bacterial strain might be useful in the textile effluent treatment.

References

Ambrosio ST, Campos-Takaki GM (2004) Decolorization of reactive azo dyes by *Cunninghamella elegans* UCP 542 under co-metabolic conditions. Bioresour Technol 91:69–75. doi:10.1016/S0960-8524(03)00153-6

Carliell CM, Barclay SJ, Naidoo N, Buckley CA, Mulholland DA, Senior E (1995) Microbial decolorization of a reactive azo dye under anaerobic conditions. Water SA 21:61–69

Cavallazzil JP, Kasuya CM, Soares MA (2005) Screening if inducers for laccase production by *Lentinula edodes* in liquid medium. Braz J Microbiol 36:383–387

Cha C, Doerge DR, Cerniglia CE (2001) Biotransformation of malachite green by the fungus *Cunninghamella elegans*. Appl Environ Microbiol 67:4353–4360. doi:10.1128/AEM.67.9.4358-4360.2001

Dawkar VV, Jadhav UU, Jadhav SU, Govindwar SP (2008) Biodegradation of disperse textile dye Brown 3REL by newly isolated *Bacillus* sp. VUS. J Appl Microbiol 105:14–24. doi:10.1111/j.1365-2672.2008.03738.x

Dawkar VV, Jadhav UU, Telke AA, Govindwar SP (2009) Peroxidase from *Bacillus* sp. VUS and its role in the decolorization of textile dyes. Biotechnol Bioprocess Eng (in press)

Do T, Shen J, Cawood G, Jeckins R (2002) Biotreatment of textile effluent using *Pseudomonas* sp. immobilized on polymer supports. Advances in biotreatment for textile processing. University of Georgia Press, Georgia

Fontecave M, Eliasson R, Reichard P (1987) NAD (P) H: flavin oxidoreductase of *E. coli*: a ferric iron reductase participating in the generation of the free radical of ribonucleotide reductase. J Biol Chem 262:12325–12331

Hasnat MA, Uddin MM, Samed AJF, Alam SS, Hossain S (2007) Adsorption and photocatalytic decolorization of a

synthetic dye erythrosine on anatase TiO_2 and ZnO surfaces. J Hazard Mater 147:471–477. doi:10.1016/j.jhazmat.2007.01.040

Hatvani N, Mecs I (2001) Production of laccase and manganese peroxidase by *Lentinus edodes* on malt containing by product of the brewing process. Process Biochem 37:491–496. doi:10.1016/S0032-9592(01)00236-9

Hu TL (2001) Kinetics of azo reductase and assessment of toxicity of metabolic products from azo dye by *Pseudomonas luteola*. Water Sci Technol 43:261–269

Jadhav JP, Govindwar SP (2006) Biotransformation of malachite green by *Saccharomyces cerevisiae* MTCC 463. Yeast 23:315–323. doi:10.1002/yea.1356

Jadhav UU, Dawkar VV, Ghodake GS, Govindwar SP (2008) Biodegradation of direct red 5B, a textile dye by newly isolated *Comamonas* sp. UVS. J Hazard Mater 158:507–516. doi:10.1016/j.jhazmat.2008.01.099

Kalme SD, Parshetti GK, Jadhav SU, Govindwar SP (2007a) Biodegradation of benzidine based dye direct blue-6 by *Pseudomonas desmolyticum* NCIM 2112. Bioresour Technol 98:1405–1410. doi:10.1016/j.biortech.2006.05.023

Kalme S, Ghodake G, Govindwar S (2007b) Red HE7B degradation using desulfonation by *Pseudomonas desmolyticum* NCIM 2112. Int Biodeter Biodegrad 60:327–333. doi:10.1016/j.ibiod.2007.05.006

Kalyani DC, Patil PS, Jadhav JP, Govindwar SP (2008) Biodegradation of reactive textile dye red BLI by an isolated bacterium *Pseudomonas* sp. SUK1. Bioresour Technol 99:4635–4641. doi:10.1016/j.biortech.2007.06.058

Maier J, Kandelbauer A, Erlacher A, CavacoPaulo A, Gubits GM (2004) A new alkali thermostable azoreductase from *Bacillus* sp. Strain SF. Appl Environ Microbiol 70:837–844. doi:10.1128/AEM.70.2.837-844.2004

Mohana N, Balasubramanian N, Ahmed B (2007) Electrochemical oxidation of textile wastewater and its reuse. J Hazard Mater 147:644–651. doi:10.1016/j.jhazmat.2007.01.063

Novotny C, Svobodova K, Kasinath A, Erbanova P (2004) Biodegradation of synthetic dyes by *Irpex lacteus* under various growth conditions. Int Biodeter Biodegrad 54:215–223. doi:10.1016/j.ibiod.2004.06.003

Ozfer Y, Dilek A, Seval C (2003) Decolorization of textile dyes by fungal pellets. Process Biochem 38:933–938. doi:10.1016/S0032-9592(02)00197-8

Parshetti G, Kalme S, Saratale G, Govindwar S (2006a) Biodegradation of malachite green by *Kocuria rosea* MTCC 1532. Acta Chim Slov 53:492–498

Parshetti GK, Kalme SD, Gomare SS, Govindwar SP (2006b) Biodegradation of reactive blue-25 by *Aspergillus ochraceus* NCIM-1146. Bioresour Technol 98:3638–3642. doi:10.1016/j.biortech.2006.11.017

Pearcea CI, Lloydb JR, Guthriea JT (2003) The removal of color from textile wastewater using whole bacterial cells: a review. Dyes Pigments 58:179–196. doi:10.1016/S0143-7208(03)00064-0

Pointing SB, Vrijmoed LLP (2000) Decolorization of azo and triphenyl methane dyes by *Pycnoporus sanuineus* producing laccase as the sole phenol oxidase. J Microbiol Biotechnol 16:317–318. doi:10.1023/A:1008959600680

Robinson T, Chandran B, Nigam P (2001) Studies on the decolorization of an artificial textile effluent by white-rot fungi in N-rich and N-limited media. Appl Microbiol Biotechnol 57:810–813. doi:10.1007/s00253-001-0857-8

Rodriyguez Couto SG, Feijoo Moreira MT, Lema JM (2002) Evaluation of the environmental conditions for the continuous production of lignin peroxidase by *Phanerochaete chrysosporium* in fixed-bed bioreactors. Biotechnol Lett 24:791–794. doi:10.1023/A:1015576121541

Salokhe MD, Govindwar SP (1999) Effect of carbon source on the biotransformation enzymes in *Serratia marcescens*. J Microbiol Biotechnol 15:229–232

Saratale GD, Kalme SD, Govindwar SP (2006) Decolorization of textile dyes by *Aspergillus ochraceus*. Indian J Biotechnol 5:407–410

Sarnaik S, Kanekar P (1999) Biodegradation of methyl violet by *Pseudomonas mendocina* MCM B-402. Appl Microbiol Biotechnol 52:251–254. doi:10.1007/s002530051517

Selvam K, Swaminathan K, Chae KS (2003) Decolorization of azo dyes and a dye industry effluent by a white rot fungus *Thelephora* sp. Bioresour Technol 88:115–119. doi:10.1016/S0960-8524(02)00280-8

Shanmugam V, Kumari M, Yadav KD (1999) n-propanol as a substrate for assaying the lignin peroxidase activity of *Phanerochaete chrysoporium*. Indian J Biochem Biophys 36:39–43

Sumathi S, Manju BS (2000) Uptake of reactive textile dyes by *Aspergillus foetidus*. Enzyme Microb Technol 27:347–355. doi:10.1016/S0141-0229(00)00234-9

Wesenberg D, Buchon F, Agathos SN (2002) Degradation of dye containing textile effluent by the agaric white-rot fungus *Clitocybula dusenii*. Biotechnol Lett 24:989–993. doi:10.1023/A:1015660927710

Zhang X, Flurkey W (1997) Phenoloxidases in *Portabella* Mushrooms. J Food Sci 62:97–100. doi:10.1111/j.1365-2621.1997.tb04376.x