ELSEVIER

Biodegradation of benzidine based dye Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112

S.D. Kalme, G.K. Parshetti, S.U. Jadhav, S.P. Govindwar *

Department of Biochemistry, Shivaji University, Kolhapur 416004, India

Abstract

Pseudomonas desmolyticum NCIM 2112 was able to degrade a diazo dye Direct Blue-6 ($100 \, \text{mg} \, \text{l}^{-1}$) completely within 72 h of incubation with 88.95% reduction in COD in static anoxic condition. Induction in the activity of oxidative enzymes (LiP, laccase) and tyrosinase while decolorization in the batch culture represents their role in degradation. Dye also induced the activity of aminopyrine *N*-demethylase, one of the enzyme of mixed function oxidase system. The biodegradation was monitored by UV–Vis, IR spectroscopy and HPLC. The final products, 4-amino naphthalene and amino naphthalene sulfonic acid were characterized by GC–mass spectroscopy.

1. Introduction

Azo dyes account for the majority of all textile dyestuffs produced and are the most commonly used synthetic dyes in the textile, food, paper making, color paper printing, leather and cosmetic industries (Chang et al., 2001). Sulfonated azo dyes, the largest class of dyes, have great structural differences and consequently offer a great variety of colors. The discharge of these sulfonated azo dyes has not only a negative aesthetic effect but also some azo dyes and their degradation products, sulfonated and unsulfonated aromatic amines are toxic or even carcinogenic (Myslak and Bolt, 1998). The sequential anaerobic aerobic bacterial degradation system has been shown to be efficient in the degradation of the sulfonated azo dyes (Rajaguru et al., 2000). Some studies have reported decolorization of sulfur containing dyes by using white rot fungus *Dichomitus squalens* and *Coriolus versicolor* (Eichlerová et al., 2006; Sanghi et al., 2006). The decolorization of sulfonated azo dyes by different *pseudomonas* species without shaking condition (anaerobic incubation of azo dyes) has been reported earlier (Banat et al., 1996; Puvaneshwari et al., 2002). But most of these studies have emphasized only the decolorization/degradation of dye wastewater, not discussing much about the products released by the cleavage of azo group. Very recently degradation of a benzidine based azo dye Direct Black-38 by unidentified microorganism has been reported at static condition (Kumar et al., 2006).

Thus, the main objective of this study was to observe microbial decolorization and biodegradation of Direct Blue-6, benzidine-based azo dye by *Pseudomonas desmolyticum* NCIM 2112 in static anoxic condition. Also, enzymes involved in the degradation (lignin peroxidase, laccase, tyrosinase, aminopyrine *N*-demethylase, DCIP reductase and MG reductase) and metabolites formed after degradation.

---

* Corresponding author. Tel.: +91 231 2609152; fax: +91 231 2691533.
  E-mail address: spg_biochem@unishivaji.ac.in (S.P. Govindwar).

2. Methods

2.1. Microorganism and culture conditions

P. desmolyticum NCIM 2112 was obtained from National Center for Industrial Microorganisms, NCL, India. Pure culture was maintained on nutrient agar slants. Composition of nutrient medium used for decolorization studies was (g l^{-1}): NaCl 5, peptone 5, beef extract 3.

2.2. Dyestuff and chemicals

2,2′-Azinobis (3-ethylbezthiazoline-6-sulfonate) ABTS and aminopyrine were purchased from Sigma–Aldrich, USA. Tartaric acid, *n*-propanol and catechol were purchased from Sisco Research Laboratories, India. The textile dye Direct Blue-6 was a generous gift from local textile industry, Solapur, India. All chemicals used were of the highest purity available and of the analytical grade.

2.3. Acclimatization

The culture was gradually exposed to the increasing concentration of dye to acclimatize *P. desmolyticum* NCIM 2112. The successive transfers of culture into fresh nutrient medium containing 100, 150, 200, 250 and 300 mg l^{-1} of the Direct Blue-6 dye was done at 30 °C in static condition. This acclimatized microorganism was used for all studies.

2.4. Decolorization experiments

2.4.1. Decolorization at static and shaking conditions

P. desmolyticum NCIM 2112 was grown for 24 h at 30 °C in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth to study the effect of static and shaking conditions on decolorization performance of microbial culture. After 24 h, 100 mg l^{-1} dye was added in each and incubated at static as well as shaking condition at 30 °C for 120 rpm on orbital shaker. The aliquot (3 ml) of the culture media was withdrawn at different time intervals, centrifuged at 5000 rpm for 15 min. Decolorization was monitored by measuring the absorbance of culture supernatant at the 592 nm. Change in pH and reduction in chemical oxygen demand (COD) (Standard Methods for Examination of Water and Wastewater APHA, 1995) was also studied in the same sample. Growth of microorganism in dye containing medium was determined by the gravimetric method after drying at 80 °C until constant weight.

2.4.2. Decolorization at different dye concentration

In order to examine the effect of initial dye concentration on the decolorization in static condition, nutrient medium was added with 100, 150, 200, 250 and 300 mg l^{-1} of the Direct Blue-6 dye. 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 percentage decolorization was calculated as follows:

$$\%\text{decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

2.5. Enzyme activities

2.5.1. Preparation of cell free extract

P. desmolyticum 2112 was grown in nutrient broth at 30 °C for 24 h, centrifuged at 6000 rpm 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 40 amplitude maintaining temperature at 4 °C and giving eight strokes, each of 5 s with 2 min interval. This extract was used without centrifugation as enzyme source.

2.5.2. Enzyme assays

Laccase, tyrosinase and lignin peroxidase (LiP) activities were assayed in cell free extract as well as in culture supernatant. Laccase activity was determined in a reaction mixture of 2 ml containing 10% ABTS in 0.1 M acetate buffer (pH 4.9) and measured increase in optical density at 420 nm (Hatvani and Mecs, 2001). Tyrosinase activity was determined in a reaction mixture of 2 ml, containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) at 495 nm (Zhang and Flurkey, 1997). LiP was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 ml containing 100 mM *n*-propanol, 250 mM tartaric acid, 10 mM H_2O_2 (Shanmugam et al., 1999). All enzyme assays were carried out at 30 °C where reference blanks contained all components except the enzyme. All enzyme assays were run in triplicate and average rates calculated and one unit of enzyme activity was defined as a change in absorbance unit $\min^{-1}$ mg of enzyme^{-1}.

NADPH-DCIP reductase and aminopyrine *N*-demethylase activities were determined using a procedure reported earlier by Salokhe and Govindwar (1999). The malachite green reductase assay mixture contained 323 μM malachite green, 50 μM NADH in 50 mM potassium phosphate buffers (pH 7.4) and 0.1 ml of enzyme solution in a total volume of 5.0 ml. The decrease in color intensity was observed at 620 nm. The MG reduction was calculated using the extinction coefficient of 8.4×10^{-3} mM^{-1} cm^{-1} (Jadhav and Govindwar, 2006).

2.6. Biodecolorization and biodegradation analysis

Decolorization was monitored by UV–Vis spectroscopic analysis (Hitachi U-2800) whereas biodegradation was monitored by HPLC and FTIR spectroscopy. Identification of metabolites was carried out by GC/MS. For this, 100 ml samples were taken at intervals (24, 48 and 72 h), centrifuged at 10,000 rpm 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). The mobile phase was methanol with flow rate of 0.75 ml min^{-1} and UV detector at 316 nm. The biodegraded Direct Blue-6 was characterized by Fourier Transform Infrared Spectroscopy (Perkin Elmer, Spectrum one) and compared with control dye. The FTIR analysis was done in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. Rotary vacuum evaporated sample (extracted after 72 h decolorization period) was dissolved in methanol and GC/MS analysis of metabolites was carried out using a Hewlett Packard 989 B MS Engine, equipped with integrated gas chromatograph with a HP1 column (30 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1.1 ml min^{-1}. The injector temperature was maintained at 300 °C with oven conditions as 100 °C kept constant for 2 min. – increased up to 250 °C with 10 °C min^{-1} – raised up to 280 °C with 30 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the NIST library.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-kramer multiple comparisons test was used. Readings were considered significant when P was $\leqslant 0.05$.

3. Results

3.1. Effect of static and shaking conditions

Acclimatization of the *P. desmolyticum* reduced the time required for complete decolorization of 100 mg l^{-1} dye from 168 h to 72 h (data not shown). Decolorization of Direct Blue-6 was 92% at static condition and only 16% at shaking condition but the growth of microorganism was more at shaking (0.45 g l^{-1}) as compared to static condition (0.28 g l^{-1}) (Fig. 1). To conform whether this decolorization was due to microbial action or change in pH, the change in pH was recorded, which was in the range of 7.0–7.9 at shaking and 6.8–7.8 at static condition. UV–Vis spectra of Direct Blue-6 did not show any change at this pH range (data not shown). Reduction in COD was 88.95% at static and 22.16% at shaking condition (Fig. 2). Decolorization, growth of microorganism and reduction of COD was found to be reciprocal (Figs. 1 and 2).

3.2. Effect of initial dye concentration on decolorization

Percent decolorization of Direct Blue-6 by *P. desmolyticum* was varied with initial dye concentrations (50–
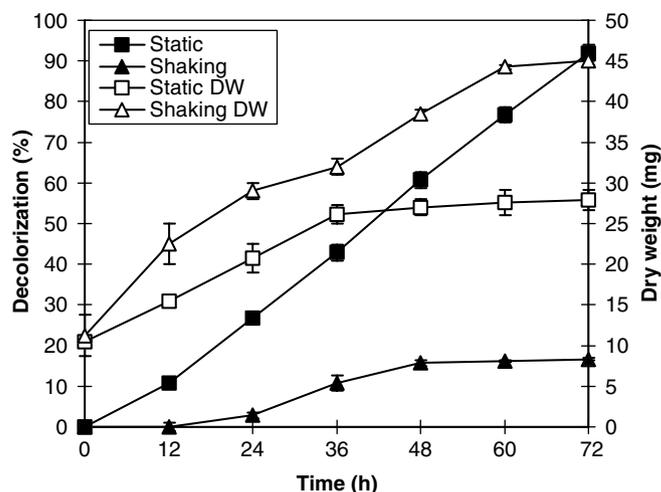

Fig. 1. Time course evolution of *P. desmolyticum* 2112 growth in form of dry weight and dye decolorization in static and shaking conditions at the initial dye concentration of 100 mg l^{-1} in nutrient broth.
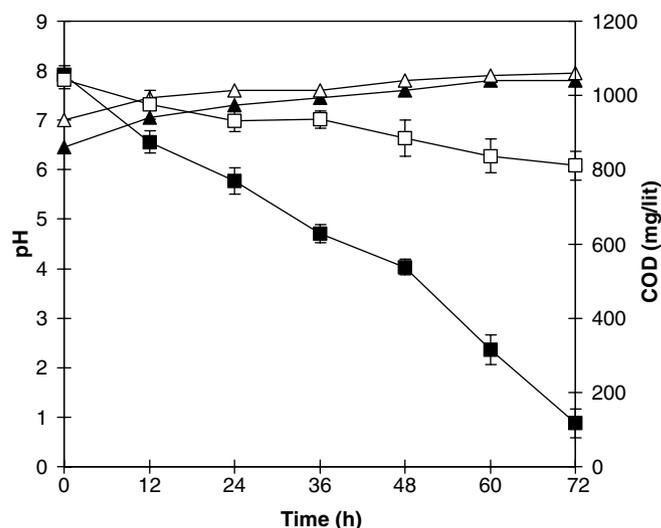

Fig. 2. Change in pH in static (▲), shaking (△) conditions; whereas change in COD in static (■) and shaking (□) conditions during Direct Blue-6 decolorization.

250 mg l^{-1}) when studied up to 72 h at static condition (Fig. 3). At 50–100 mg l^{-1} dye concentration, 100–92% dye removal was observed; 80–40% removal at concentration of 150–200 mg l^{-1} and only 15% removal at 250 mg l^{-1} dye concentration was recorded by 100 ml batch culture of *P. desmolyticum* in nutrient broth. The time required for complete decolorization of 50 mg l^{-1} dye was 60 h, whereas it was 72 h for 100 mg l^{-1} dye concentration. As concentration was increased (150–200 mg l^{-1}), the time required for decolorization varied from 4–6 days. But the decolorization was not prolonged at 250 mg l^{-1}, instead this and higher concentration seemed to be toxic for cell growth in 100 ml batch culture.
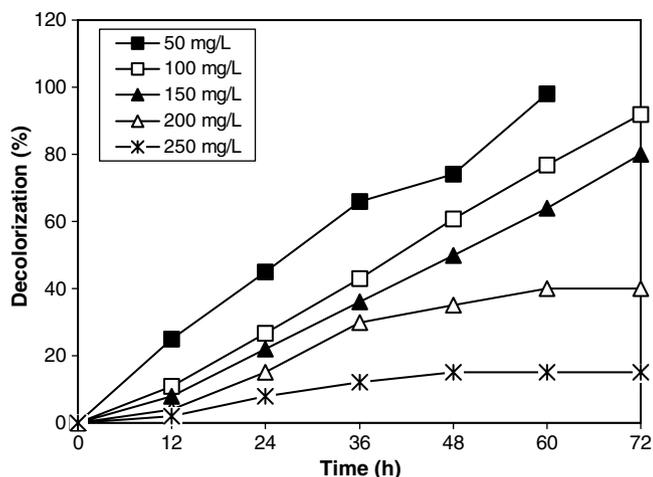
Fig. 3. Effect of increased dye concentration on decolorization performance of *P. desmolyticum* 2112 in nutrient broth.

3.3. Enzyme activities while decolorization in batch culture

The time course of lignin peroxidase, laccase as well as tyrosinase production is shown in Fig. 4. Induction in laccase and tyrosinase activities was observed up to 72 h incubation of batch culture (411%, 240%, respectively as compared to 0 h activity). After complete decolorization of dye these enzymes were later on decreased (96 h) in the batch culture. Induction in the lignin peroxidase activity was recorded up to 96 h incubation (260% in homogenate, 128% in culture broth). There were no activities of laccase and tyrosinase in culture supernatant. Fig. 5 shows the induction of aminopyrine *N*-demethylase, malachite green and DCIP reductase enzymes in the time course of 96 h (187%, 206%, 134% respectively as compared to 0 h activity). It was noticed that reductase activities increased after 48 h of incubation during decolorization process. A
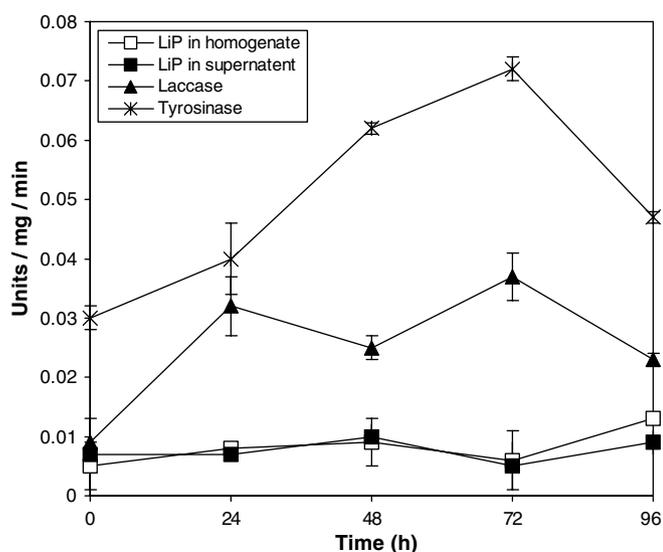

Fig. 4. Time course of LiP, laccase and tyrosinase in *P. desmolyticum* 2112 during Direct Blue-6 decolorization.
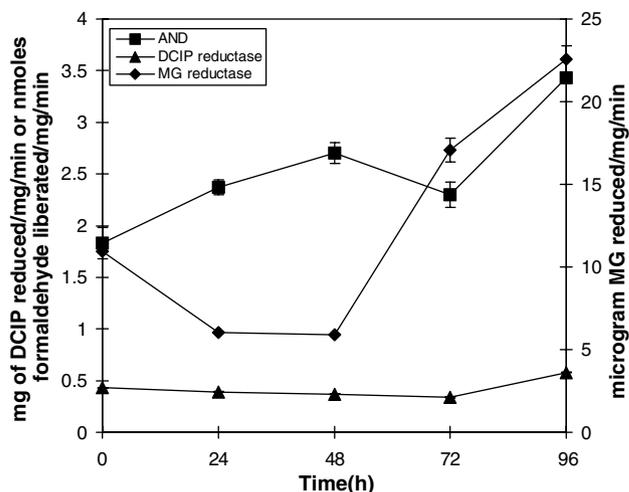

Fig. 5. Time course of AND, DCIP reductase and MG reductase in *P. desmolyticum* 2112 during Direct Blue-6 decolorization.

linear decrease in DCIP reductase activity was recorded up to complete decolorization of dye (72 h), which was later increased at 96 h incubation.

3.4. Biodecolorization and biodegradation analysis

UV–Vis scan (400–800 nm) of supernatants of different time intervals showed decolorization and decrease in dye concentration from batch culture (data not shown). Peak observed at 592 nm (0 h) was decreased without any shift in λ max up to complete decolorization of medium (72 h). A new peak, which appeared at 450 nm at 48 h disappeared at 72 h. Comparison of FTIR spectrum of control dye with extracted metabolites clearly indicated the biodegradation of parent dye compound by *P. desmolyticum* (data not shown). Peaks in the control dye spectrum represented the stretching vibrations of S=O at 640 cm^{-1}, symmetric stretching at 1186 cm^{-1} and asymmetric stretching at 1044 cm^{-1} for C–N. The stretching vibrations between C–O showed a band at 977 cm^{-1}. C–N stretching at 1340 cm^{-1} represented nature of aromatic amine group present in parent dye compound, 1492 cm^{-1} and 3421 cm^{-1} represented the presence of free NH group from parent dye structure. The stretching between C–H was reported at 2924 cm^{-1}, whereas peak at 1615 cm^{-1} represented –N=N– stretching of azo group.

The FTIR spectrum of 24, 48 and 72 h extracted metabolites showed significant change in positions of peaks when compared to control dye spectrum. In 24 h extracted metabolites, peak at 844 cm^{-1} pointed towards formation of substituted naphthalene products with C=O stretching at 1655 cm^{-1}. A new peak at 1457 cm^{-1} represented C–H deformation of alicyclic CH$_2$, whereas a peak at 3400 cm^{-1} was observed for substituted anilines. Absence of 844 cm^{-1} in 48 h extracted sample indicated further breakdown of substituted naphthalene compounds. In 72 h extracted metabolites, a new peak at 3234 cm^{-1} represented N–H stretching in amide.

Table 1
GC–mass spectral data of biodegraded products of Direct Blue-6

S. no.	Rt (min)	Mw (m/z)	Area (%)	Assignment	CAS no.
1	10.71	209	0.91	4′-diazenyl-biphenyl-4-diazonium	–
2	13.07	210	8.85	4′-nitroso-biphenyl-4-yl-diazene	–
3	11.48	198 (M + 1)	1.53	4-diazenyl-biphenyl-4-ylamine	–
4	12.39	169	1.49	4-aminobiphenyl	92-67-1
5	13.20	261	3.96	4-amino, 6-hydroxy naphthalene sulfonic acid	–
6	10.13	224	1.49	Amino naphthalene sulfonic acid	119-79-9

The HPLC analysis of dye sample collected at the 0 h incubation showed two major peaks at 1.69 min and 2.30 min. As the decolorization progressed, the biodegradation of parent compound was observed with five detectable peaks (retention time 1.74, 2.06, 2.21, 2.51, 4.07) at 72 h extracted metabolites, however major peak was observed at 2.51 min (data not shown).

GC–MS analysis was carried out to investigate the metabolites formed during the biodegradation process (Table 1). A pathway has been proposed for degradation of Direct Blue-6 by *P. desmolyticum* 2112 in static anoxic condition (data not shown). The asymmetric cleavage by peroxidase between the nitrogen of the azo group and the carbon of the aromatic ring substituted with hydroxyl group resulted in reactive products (intermediates), which underwent several redox reactions to produce more stable intermediate. The oxidation and hydrolysis product of biphenyl substituted with N═NH to both side of aromatic ring (first product of asymmetric cleavage) were identified as 4′-diazenyl-biphenyl-4-diazonium and 4′-nitroso-biphenyl-4-yl-diazene, respectively. The reduction in the later compound gave 4-diazenyl-biphenyl-4-ylamine and oxidation–reduction in the further intermediate gave 4-aminobiphenyl as final product. On the other hand, the degradation of naphthalene moiety substituted with hydroxy, amino and sulfonic acid groups (second product of asymmetric cleavage) was observed in the form of low molecular weight compounds such as 4-amino, 6-hydroxy naphthalene sulfonic acid and amino naphthalene sulfonic acid. The structures of the detected compounds were assigned from the m/z values obtained. The final products identified in the pathway were characterized as 4-aminobiphenyl and amino naphthalene sulfonic acid, having a molecular weight 169 and 224, however, GC showed 20 peaks.

4. Discussion

The exact mechanism of the anaerobic azo dye reduction is not clearly understood yet. Therefore, the term azo dye reduction may involve different mechanisms or locations like enzymatic (Shrivastava et al., 2005), mediated (Field and Brady, 2003), intracellular (Mechsner and Wuhrmann, 1982), extracellular (Carliell et al., 1995) and various combinations of these mechanisms and locations. The azo dye reduction in anaerobic incubation is non-specific and extracellular process in which reducing equivalents from either biological or chemical source are transferred to the dye. It

was stated that the reducing equivalents formed during anaerobic oxidation of carbon sources, are used for the reduction of azo bond (Stolz, 2001). In our study the decolorization of Direct Blue-6 at static condition was faster as compare to shaking. These observations suggest that the decolorization performance of *P. desmolyticum* NCIM 2112 is better at static anoxic condition where depletion in oxygen content is followed. The reason for decreased decolorization at shaking condition could be competition of oxygen and the azo compounds for the reduced electron carriers under aerobic condition.

Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. The involvement of fungal peroxidases and laccases for the oxidation of sulfonated azo dyes has been reported earlier (Kandelbauer et al., 2004). Bacterial extracellular azo dye oxidizing peroxidases have been characterized in *Streptomyces chromofuscus* (Pasti-Grigsby et al., 1996). In this study, induction in oxidative enzymes (LiP, laccase) and tyrosinase up to complete decolorization period (72 h) are presumably responsible for the decolorization of Direct Blue-6. While emphasizing the activity of these enzymes in the presence of metabolites formed after complete decolorization, a noticeable induction only in lignin peroxidase was observed (between 72 and 96 h). Induction in reductase for DCIP and MG was recorded in the batch culture with increased anoxic condition (after 48 h). Similarly, the increase in the activity of enzyme of mixed function oxidase system (aminopyrine-N demethylase) could be attributed to the inductive property of metabolites of Direct Blue-6 at translational or transcriptional level of protein synthesis. From this study, it can be deduced that there is direct involvement of oxidative enzymes and tyrosinase for biodegradation of this azo dye and reduction of simple intermediates might have taken place rather than complex structures like Direct Blue-6.

Azo dyes can be cleaved symmetrically and asymmetrically, with an active site available for an enzyme to excite the molecule. We have proposed a pathway for degradation of this dye by *P. desmolyticum* where initially asymmetric cleavage by lignin peroxidase might have followed the oxidation and reduction in different metabolites formed. The identified products 4′-nitroso-biphenyl-4-yl-diazene and 4-amino, 6-hydroxy naphthalene sulfonic acid supports the asymmetric cleavage of Direct Blue-6. Hence the present study indicates that *P. desmolyticum* NCIM 2112 have potential to degrade carcinogenic dye Direct

Blue-6. During the biodegradation of Direct Blue-6, there is induction of oxidative enzymes (Lip, laccase) and tyrosinase, which helps in the degradation processes. However, further experiments with purified enzymes should be made to elucidate which enzyme is mainly responsible for decoloration and degradation of Direct Blue-6.

Acknowledgements

References

American Public Health Association, 1995. Standard Methods for Examination of Water and Wastewater APHA, AWWA and WPCF, ninetieth ed., Washington DC, USA.

Banat, I.M., Nigam, P., Singh, D., Marchant, R., 1996. Microbial decolorization of textile-dye containing effluents: a review. Bioresour. Technol. 58, 217–227.

Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A., Senior, E., 1995. Microbial decolourization of reactive azo dye under anaerobic conditions. Water SA 21, 61–69.

Chang, J., Chon, C., Lin, Y., Lin, P., Ho, J., Ho, T.L., 2001. Kinetic characteristics of bacterial azo dye decolorization by pseudomonas luteola. Water Res. 35, 2841–2850.

Eichlerová, I., Homolka, L., Nerud, F., 2006. Synthetic dye decolorization capacity of white rot fungus Dichomitus squalens. Bioresour. Technol. 97, 2153–2159.

Field, J.A., Brady, J., 2003. Roboflavin as a redox mediator accelerating the reduction of the azo dye Mordant Yellow 10 by anaerobic granular sludge. Water Sci. Technol. 48, 187–193.

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.

Jadhav, J.P., Govindwar, S.P., 2006. Biotransformation of malachite green by Saccharomyces cerevisiae MTCC 463. Yeast 23, 315–323.

Kandelbauer, A., Erlacher, A., Cavaco-Paulo, A., Guebitz, G., 2004. Laccase-catalyzed decolorization of the synthetic azo dye Diamond Black PV 200 and of some structurally related derivatives. Biocatal. Biotransform. 22, 331–339.

Kumar, K., Saravanadevi, S., Krishnamurthi, K., Gampawar, S., Mishra, N., Pandya, G.H., Chakrabarti, T., 2006. Decolorization, biodegradation and detoxification of benzidine based azo dye. Bioresour. Technol. 97, 407–413.

Mechsner, K., Wuhrmann, K., 1982. Cell permeability as a rate limiting factor in the microbial reduction of sulfonated azo dyes. Eur. J. Appl. Microbiol. Biotechnol. 15, 123–126.

Myslak, Z.W., Bolt, H.M., 1998. Occupational exposure to azo dyes and risk of bladder cancer. Zbl. Arbeitsmed. 38, 310–321.

Pasti-Grigsby, M.B., Burke, N.S., Goszezynski, S., Crawford, D.L., 1996. Transformation of azo dye isomers by Streptomyces chromofuscus A11. Appl. Environ. Microbiol. 62, 1814–1817.

Puvaneshwari, N., Muthukrishnan, J., Gunasekaran, P., 2002. Biodegradation of benzidine based azodyes direct red and direct blue by the immobilized cells of Pseudomonas fluorescens D41. Indian J. Exp. Biol. 40, 1131–1136.

Rajaguru, P., Kalaiselvi, M., Palanivel, M., Subburam, V., 2000. Biodegradation of azo dyes in a sequential anaerobic–aerobic system. Appl. Microbiol. Biotechnol. 54, 268–273.

Salokhe, M.D., Govindwar, S.P., 1999. Effect of carbon source on the biotransformation enzymes in Serratia Marcescens. World Microbiol. Biotechnol. 15, 229–232.

Sanghi, R., Dixit, A., Guha, S., 2006. Sequential batch culture studies for the decolorization of reactive dye by Coriolus versicolor. Bioresour. Technol. 97, 396–400.

Shanmugam, V., Kumari, M., Yadav, K.D., 1999. n-Propanol as a substrate for assaying the ligninperoxidase activity of Phanerochaete chrysoporium. Indian J. Biochem. Biophys. 36, 39–43.

Shrivastava, R., Christian, V., Vyas, BRM., 2005. Enzymatic decolorization of sulfonpthalein dyes. Enzyme Microb. Technol. 36, 333–337.

Stolz, A., 2001. Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol. 56, 69–80.

Zhang, X., Flurkey, W., 1997. Phenoloxidases in Portabella mushrooms. J. Food Sci. 62, 97–100.