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Enzyme based cleavage strategy of *Bacillus lentus* BI377 in response to metabolism of azoic recalcitrant

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**ABSTRACT**

*Bacillus lentus* BI377 (B. lentus BI377) an alkaliphilic strain has accomplished the discriminate color removal strategy for Reactive Red sulfonated azoic recalcitrant irrespective of their molecular structure. During the decolorization experiment, it was observed that the diazo dye first followed chromophoric cleavage by azoreductase via typical azoreduction whereas, in case of monoazo dye, cleavage took place by peroxidase via successive electron transfers to oxide surface resulting in the asymmetric cleavage of the azo bond. Dismutation of oxidative stress by reactive metabolites has confirmed by superoxide dismutase activity. Carbon monoxide (CO) binding spectra, the content of cytochrome P450 and spectroscopy analysis by GCMS, FTIR and $^1$H NMR of intermediate metabolites indicated the differentiate pattern of diazo and monoazo dye decolorization fuse to central metabolic pathway. Declined percentage of TOC and the cytotoxicity (MTT) study confirmed that environmentally benign intermediates may lead to mineralization.

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1. Introduction

Azo dyes are the largest chemical class of synthetic dyes widely used as colorants in textile dyeing, leather, plastics, food, cosmetics and paper printing (Bhatt et al., 2005; Pandey et al., 2007). These azo dyes are electron deficient xenobiotics and thus are capable to be degraded via azo reduction (Xu et al., 2007; Pandey et al., 2007). However, diverse structures present in the synthetic dyes and change in the chemical structures would significantly affect its decolorization capability. Additionally, many recent reports strongly believe that the specificity of azoreductase towards azo linkage is conditional on the electron-withdrawing capability of substituent in the proximity of azo linkage(s) and their chemical structure thus determine susceptibility of dye decolorization (Chen, 2002; Hsueh and Chen, 2008; Suzuki et al., 2001). However, recent report of Mutambanengwe et al. (2007) has stated that bacterial xenobiotic metabolism is controlled by a nonspecific enzymatic catalysis to certain degree. A multistep conversion process of monooxygenase is employed for biodegradation of xenobiotics under aerobic condition is more beneficial over anaerobic ones due to their oxygen involving mechanism in which conversion of saturated toxic aromatic amines into environmental benign metabolites (Sterner, 1999; Kodam et al., 2005; Pandey et al., 2007). In some aerobic bacteria, active manganese or lignin peroxidases are involved not only in decolorization but also further degradation process via successive cleavage of azo bond (Clarke et al., 2010; Kalyani et al., 2009).

Different existed mechanisms of the sulfonated dye degradation (Chen, 2002; Suzuki et al., 2001) have proposed that the efficient strains are capable due to their enzyme inducing ability according to the chemical structure of dye (Hsueh and Chen, 2008; Hsueh...
et al., 2009). Moreover, it was observed that, repeating dose of sulphonated dye significantly increases the capability of the bacterial dye degradation (Dave and Dave, 2009). It means that bacteria develop some enzyme system to respond this overburden of dye (Chen, 2006b). In decolorization studies, many NADPH and NADH dependent azoreductases playing important role to cleave several sulphonated azo dyes in bacteria (Pandey et al., 2007) and demonstrated different substrate specificities in obligation to the presence of hydroxyl groups and their position on the aromatic ring of the substrate (Maier et al., 2004; Chen, 2002). Therefore, it is prerequisite to understand the origin and quality of function of enzymes in such pathways to enhance our understanding of how new metabolic pathways have emerged throughout the mineralization process.

In previous work, we explained significant degradation potential of Bacillus lentus BI377 towards the Reactive Red diazo dye (Oturkar et al., 2011). In a supportive research, we observed that the diazo dye was showing faster decolorization than the monoaazo, which was quite controversial results as compared to previous reports (Hsueh et al., 2009; Chen, 2002; Maier et al., 2004). This study present the mechanistic illustration of enzyme dependent color removal strategy of the strain B. lentus BI377 in degradation of sulphonated dye rather than only proximity functional groups and molecular structures present in azo dyes. Selection of sulphonated azo dyes for evaluation of the strain is because of the variability in an industrial effluent composition as well as the structural diversity of the dye itself and they are more recalcitrant to aerobic biodegradation (Chen, 2002; Dave and Dave, 2009).

Experimental observations have confirmed by selecting six Reactive Red dyes viz; Reactive Red 141 (RR141), Reactive Red 152 (RR152), Reactive Red 120 (RR120), Reactive Red 5 (RR5), Reactive Red 198 (RR198) and Reactive Red 2 (RR2) however, the mechanistic illustration of Reactive Red 141 (RR141) and Reactive Red 2 (RR2) described in detail.

2. Methods

2.1. Microorganism and culture condition

The strain B. lentus BI377 was grown in nutrient broth (NB) containing (g/L) peptone, 5; yeast extract, 5; and NaCl, 5 at 40 °C for 24 h, pH 8.0 of medium was adjusted by sodium phosphate buffer using the EUTECH-510 pH meter.

2.2. Dyes and chemicals

The sulphonated azo dyes were procured from textile industry of Solapur, India. Media chemicals and substrates were purchased from SRL and HiMedia Laboratories, India. All chemicals used for experiments were of highest available purity.

2.3. Decolorization assessment

To study the decolorization pattern of RR141 and RR2 dye, 50 mg of each dye was supplemented with B. lentus BI377 containing 100 ml NB in 500 ml capacity Erlenmeyer flask at 40 °C and pH 8.0 under static condition (Oturkar et al., 2011). A control flask (without dye) was maintained under same experimental conditions. Three millilitre aliquots were withdrawn from the control as well as from decolourized flask at 2 h intervals and centrifuged at 10,000 g for 15 min in a DuPont Sorvall RC-5B refrigerated centrifuge. The supernatant was used to determine the percentage decolorization by UV–Visible photometric measurement (Chen et al., 2003).

2.4. Preparation of cell free extract and enzyme assays

For cell free extract and enzyme assay, cells were harvested from the decolorized broth and control flask (without dye), every 2 h by centrifugation at 10,000 g for 15 min at 4 °C, and the pellet was suspended in 50 mM sodium phosphate buffer (pH 7.0). The pelleted cell mass was resuspended in the same buffer and centrifuged again at 10,000 g for 15 min at 4 °C. This procedure was repeated thrice. Cell disruption was carried out (0.6-s. cycle and 60 amplitude) by using a probe sonicator (Hertz by Sonoplus HD 70, Bandelin, Berlin, Germany). The resulting homogenate was centrifuged at 10,000 g for 30 min at 4 °C, and the supernatant was used for assays. The protein concentration was estimated by Lowry’s method (Lowry et al., 1951). Azoreductase activity was determined according to Kalyani et al. (2009) protocol with minor modifications. The reaction mixture containing 100 mM sodium phosphate buffer (pH 7.0), 150 μM of each dye concentration and 100 μl of the cell lysate was pre-incubated for 10 min. The reaction was initiated by addition of 200 μl of 2.00 mM NADH and monitored for the decrease in absorbance at respective wavelength. One unit (U) of enzyme activity was defined as the enzyme required to decolorize 1 μmol of substrate dye per minute per mg of protein (Kalyani et al., 2009). Cytochrome P450 content was estimated using the CO-binding assay of Omura and Sato. (1964).

2.5. UV–VIS, GCMS, FTIR and 1H NMR

The strain B. lentus BI377 was grown in Erlenmeyer flask containing 100 ml of NB at 40 °C and pH 8.0. After 24 h, culture was supplemented with 50 mg of RR2 and RR141 each. Sufficient aliquots were withdrawn and centrifuged at 10,000 g for 10 min in cold condition. UV–Visible spectra of 2 h samples were monitored at respective wavelength using spectrophotometer. Intermediate metabolites were extracted by using organic solvents such as methanol, ethyl acetate and dichloromethane (DCM) and after rotaevaporation same were dried over anhydrous sodium sulfate (Soojhawon et al., 2005). The extracted compounds were dissolved in 0.5 ml dichloromethane (DCM) or methanol and subjected to preparative thin layer chromatography (TLC) using 15% (v/v) methanol: 15% (v/v) ammonium hydroxide/acetoniitrile as mobile phase. Well separated spots according to their retention time were observed under UV light and recovered from silica gel into methanol and further concentrated by rotaevaporation. Recovered compounds were further characterized by Fourier Transform Infrared spectroscopy (FTIR) (Shimadzu-8400 FTIR spectrophotometer), Gas Chromatography Mass Spectroscopy (GCMS) (Shimadzu-GCMS-QP5050) and 1H NMR (Varian Mercury Spectrometer-YH 30020).

2.6. Total organic carbon (TOC) analysis

TOC analysis was carried out by Walkely and Black protocol (Walkely and Black, 1934) and % TOC of each hour
MTT assay with mouse cell line L929

Cytotoxicity of the isolated metabolites was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as explained in protocol of Berger et al. (2003). The mouse fibroblast cell line from subcutaneous connective tissue (L929 cell line) procured from National Center for Cell Science (NCCS) Pune, (India) was grown as adherent culture in Dulbecco’s Modified Eagle’s Medium (DMEM). The cells were seeded in 96 well microplates for cytotoxicity screening at a density of 1 × 10⁵ cells per well which was further incubated for 24 h. Dye samples 150 mg/dl (w/v) and intermediate metabolite (40 mg L⁻¹) were added in quadruplicates and incubated for 24 h. The intensity of the purple color formazan was measured on a microplate reader at 570 nm.

Results and discussion

3.1. Decolorization studies of RR2 and RR141

Chemical interaction of dye with the metabolic functioning state of cell was principally assessed by UV–Visible spectroscopy from 0 to 6 h (as maximum decolorization was noted under static condition) (Fig. 1a and b) and other relevant parameters viz, decolorization percentage, protein concentration and TOC removal were estimated (Table 1). The total disappearance of the absorption maxima after 6 h in case of all dyes indicated the completion of decolorization process. In case of the diazo dye RR141, spectral analysis revealed a continuous decrease in the intensity of the original dye absorption peak 530 nm with significant decolorization efficiency in 2 h (Fig. 1a). After 4 h of incubation a new peak at 420 nm with concomitant decrease in the dye absorption peak at 530 nm was noted with 8.54% decolorization. After 6 h maximum decolorization (99.11%) was achieved with slight hump on 450 nm which signifies the formation of aromatic amines in the reaction mixture (Sterner, 1999). Protein concentration of dry cell mass was not significantly changed from 0 to 6 h. Thus, it indicated that with the help of electron withdrawing group decolorization of RR141 was growth independent azoreduction which may directed to formation of aromatic amines and/or its derivatives (Chen, 2002).

In case of the monoazo dye RR2, the UV–Visible spectra showed a phenomenal decrease in intensity of the original peak (540 nm) (Fig. 1b). After 4 h, the original peak disappeared and a new peak at 350 nm appeared. The pattern of disappearance of original peak and percent decolorization at 0 to 4 h (Table 1) indicated that the dye decolorization might initiate by a process other than the typical azoreduction (Clarke et al., 2010; Torres et al., 2003; Husain, 2006). But, after 6 h of incubation, the decolorization efficiency increased significantly (96.55%) with the existence of new peak at 450 nm revealed the signs of aromatic amines analogous to RR141 degradation (Sterner, 1999). Consistent decrease in percentage of total organic carbon (TOC) for RR2 and RR141 clearly demonstrated that degradation process may lead to complete mineralization after 6 h of incubation with B. lentus B1377 (Saratale et al., 2010).

After the chromophoric group cleavage, most of the intermediate metabolites seem to be trapped by nucleophilic or electrophilic reaction. This entire event is the crux of further pathway of degradation, where the metabolic burden may divert to activation, accumulation or degradation.

3.2. Spectrophotometric, bioanalytical, and enzyme analysis of RR2 and RR141 degradation

The data pertaining to the structural analysis of the intermediates were established by GC–MS, FTIR and ¹H NMR (Table S1). The formation of [2-amino-8-(2- (4- (6- (7-amino-3,6,8-trihydroxy-naphthalene -1-ylamino) pyridine – 2-ylamino) phenylamino) pyrimidin-4 ylamino) naphthalene-1,3,6-triol] [3] and 8-(4,6-dichloro-1,3,5-triazin-2-ylamino)-2-diazenyl)naphthalene-1,3,6-triol [4] was established from the m/z ion peaks at 676.64 and 367.15 for the metabolites isolated after 2 h of incubation (Fig. S1a and b). Broad peak at 3466 cm⁻¹ in case of RR2 and 3445 cm⁻¹ for N–H stretch and 1070 cm⁻¹ for SO₃ stretching vibrations supports the credentials of intermediates which might have cleaved out from original complex structure of dye. Metabolite [3] further transforms into metabolites benzene-1, 4-diamine [5] and 2,8-diaminonaphthalene-1,3,6-triol [6] after 4 h of incubation, as revealed by m/z ion peak at 108.14 and 206.2 respectively. Moreover, FTIR frequencies 2926.0 and 3370 cm⁻¹ were indicated the C–H stretching in RR2 and N–H stretching in RR141 degradation respectively. This establishment helps to understand that existence of metabolites [3] is the cleaved product of azoreduction and simultaneous deamination and desulphonation reaction (Khandare et al., 2012) and metabolite [4] is oxidative product of asymmetric catalysis of azo bond (Clarke et al., 2010; Dawkar et al., 2009).

After 6 h, metabolites [5] and [6] from both the dye was proportionally transformed into lower metabolites and one common fragment of intermediate metabolite was observed by GC–MS spectra as
Enzyme activity study of Fig. 2.

Table 1
The whole cell decolorization assessment of RR141 and RR2.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RR141 (%)</th>
<th>RR2 (%)</th>
</tr>
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<tbody>
<tr>
<td>0 h</td>
<td>530</td>
<td>530</td>
</tr>
<tr>
<td>2 h</td>
<td>530</td>
<td>522</td>
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<tr>
<td>4 h</td>
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<td>390</td>
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<tr>
<td>6 h</td>
<td>430</td>
<td>350</td>
</tr>
<tr>
<td>% Decolorization</td>
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<td></td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
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<td></td>
</tr>
<tr>
<td>CYP450</td>
<td>0.33 ± 0.02</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>9.4</td>
<td>9.4</td>
</tr>
<tr>
<td>Peroxidase</td>
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<td>4.1</td>
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<tr>
<td>AzoR</td>
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</tr>
<tr>
<td>Peroxidase</td>
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<td>6.3</td>
</tr>
<tr>
<td>CYP450</td>
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<td>4.9</td>
</tr>
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<td>1.8</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>% TOC removal</td>
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</tr>
<tr>
<td>Protein (mg/ml)</td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.33 ± 0.02</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>2 h</td>
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<td>45</td>
</tr>
<tr>
<td>4 h</td>
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<tr>
<td>6 h</td>
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<tr>
<td>% Decolorization</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>2 h</td>
<td>30</td>
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</tr>
<tr>
<td>4 h</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>6 h</td>
<td>98.22</td>
<td>98.22</td>
</tr>
</tbody>
</table>

* Indicates the standard deviation of mean of three consecutive concentrations.

Concentration of dye 50 mg/100 ml NB.

110.11 by RR2 and 111.1 by RR141 were detected. On repetitive extraction, successful attempt has been made to confirm the metabolites by $^1$H NMR (δ-6.8, δ-6.9, δ-5.15, δ-4.1) as pyrocatechol (Fig. S2) during the degradation of RR2. Correlation can be done on the conversion of diaminobenzene into pyrocatechol from our previous findings (Oturkar et al., 2011). Eventually, on the basis of structural analysis of the intermediates, a proposed degradation mechanism for RR141 and RR2 is elucidated in Supplementary Fig. S3.

Additionally, the activities of enzymes were monitored during decolorization process to envisage the possible role of enzymes in the decolorization as well as degradation process (Fig. 2a and b). Increased rate of dye degradation process under static condition(s) of incubation in the presence of trace amount of oxygen (Chen, 2002), substantiates that the azoreductase enzyme plays a crucial role in cleavage of the N=N bond, thereby leading to formation of aromatic amines in case of RR141 (Pandey et al., 2007; Chen, 2006a). Moreover, the symmetric nature of RR141 introduces more steric hindrance leading to resonance assisted electron withdrawal from azo bond thereby making it more electrophilic and susceptible for reduction under microaerophilic environment (Chen, 2002; Kodam et al., 2005; Hsueh et al., 2009).

Whereas, the strong possibility of delay in the decolorization of RR2 due to the generation of phenoxy radical, which in turn, lead to the formation of resonance stabilized naphthaleine ring in the dye structure. Thus, it forms a carbocation by electron transfer thereby making conditions favorable for cleavage of C–N rather than N=N bond (Clarke et al., 2010; Chivukula et al., 1995; Dawkar et al., 2009; Kalyani et al., 2009).

The activation of azo dye(s) via direct oxidation of the azo linkage to highly reactive electrophilic dizonium salts has been also reported previously (Chen, 2006a). The chromophoric N=N bond is then subsequently cleaved leading to the formation of aromatic amines. The degradation of a broad range of substrates via mechanism(s) involving the formation of carboxylation(s) by peroxidases enzyme into non-toxic compounds has also been reported (Torres et al., 2003; Husain, 2006). These reactive species formed by redox processes presumably were responsible to induce superoxide dismutase activity which might have scavenged the oxidative stress (Ben Mansour et al., 2007; Oturkar et al., 2011). Although, the faster rate of degradation for monoazo dye system than diazo ones has been proposed by Chen and Hsueh previously, our present observations contradict the same. In our case, it has been consistently observed that the degradation of RR141 (diazo) by B. lentus BI377 is faster than RR2 (monoazo). These observations indicate that azo dye degradation regulated by enzyme dependant mechanisms varies from strain to strain regardless of origin of the strain isolation (Chen, 2002; Hsueh and Chen, 2008; Hsueh et al., 2009).

Significant increase in the content of cytochrome P450 during the degradation process for both the dyes indicates the inductive effect of dyes on cytochrome P450 monoxygenase system. Its periodical increase and the CO binding spectra (data not shown) confirms the consistent role of cytochrome P450 from 2 to 6 h in RR2 and from 4 to 6 h in RR141 up to the mineralization (Oturkar et al., 2011; Torres et al., 2003; Husain, 2006). Moreover, many intermediate metabolites promote and express the gene for promiscuous enzyme activity.

These enzymatic responses according to the structure of the dye molecule and the physiology of the strain were solely responsible for transformation of the toxic dye to aromatic amines and their subsequent transformation into environment friendly intermediates. In support, we conducted tyrosinase activity study (data not shown). Uninduced tyrosinase activity during the degradation process indicated the absence of quinines indication whereas, the existence of catechol made high possibility of further metabolism into aliphatic metabolites through cis-muconic acid pathway (Oturkar et al., 2011; Xu et al., 2007).
Membrane so that gene expression could be possible for specific strains had developed the capability of substrate interaction to cell has been observed that on repetitively increasing dose of azo dyes, enzymes in each of the novel degradation pathway. Moreover, it different multiconversion steps with involvement of different en-
zymes in each of the novel degradation pathway. Furthermore, it would be inferred that the degradation of diazo dye was faster than mono azo dye due to electron withdrawing group proximity to azo bond. Our study indicates that dye degradation was completely dependent on enzyme regulation by bacteria in response to intermediate metabolites and selective pressure for detoxification of a toxic compound or use of a new source of energy regardless of the functional groups and their positions on the benzene and/or naphthalene ring (Chen, 2006a; Hsueh and Chen, 2007) in vicinity of azo bonds (Chen, 2002).

4. Conclusion

The knowledge of enzyme involvement at each step of degradation pathway could be beneficial to cultivate specific microbe for bioremediation and biotechnological assigned task. Study of the functional quality of enzymes in degradation process rather than molecular structure assessment of anthropogenic chemicals could enhance our understanding of how new metabolic pathway have emerged throughout the study to improve degradation of recalcitrant pollutant. Study also enlighten the novel ‘fitness’ of microbes that express functional enzymes either in natural or engineered pathways which could further enhance impartial approach to develop strains that have improved resistance towards the toxicity of xenobiotic compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.12.019.

References
