International Journal of Current Advanced Research  
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: SJIF: 5.995  
Available Online at www.journalijcar.org  
Volume 6; Issue 8; August 2017; Page No. 5212-5217  
DOI: http://dx.doi.org/10.24327/ijcar.2017.5217.0674

DECOLORIZATION OF REACTIVE BLACK B BY PAENIBACILLUS DENDRITIFORMIS STRAIN CS2a4

Nishant Junnarkar1,2* and Neepa Pandhi1

1Department of Microbiology, Shree M. & N. Virani Science College, Rajkot, GUJARAT, India 360005  
2School of Life Sciences, Central University of Gujarat, Gandhinagar, Gujarat, India 382030

A R T I C L E  I N F O

Article History:  
Received 19th May, 2017  
Received in revised form 14th June, 2017  
Accepted 26th July, 2017  
Published online 28th August, 2017

Key words:  
Paenibacillus dendritiformis, azo dye, Reactive Black B, decolorization.

ABSTRACT

Azo dyes are one of the group of synthetic dyes extensively used in textile, paper, pharmaceutical, cosmetic and other industries. These pose environmental hazard if released untreated in the environment. Reactive Black B (RBB) dye decolorizing bacterial strains were isolated from the samples collected from the vicinities of dye manufacturing industries. Amongst these, Paenibacillusdendritiformis strain CS2a4 was found to be the potent decolorizer and hence, medium composition and cultural conditions optimization was attempted to improve RBB decolorization by CS2a4 strain. Optimization of the cultural conditions and co-substrates concentration in decolorization medium, resulted in the reduction of decolorization period for RBB from 40h to 16h by the culture. CS2a4 could decolorize RBB upto 350 ppm efficiently (>80%) within 16-30h. Spectral analysis and TLC analysis of decolorized medium revealed the transformation of RBB dye into unknown intermediates.

INTRODUCTION

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutics, cosmetics and other industries (Almeida and Corso, 2014; Dellamtrice et al., 2017). The chemical classes of dyes employed more frequently on industrial scale are the azo, anthraquinone, sulfur, indigo, triphenylmethyl (trityl), and phthalocyanine derivatives. Amongst these, azo dyes represent the largest and most versatile class of synthetic dyes (Keharia et al., 2004). And for sulfonated azo dyes, both aromatic sulfonic and azo groups confer to their xenobiotic nature as these are rare among natural products (Junnarkar et al., 2006). Approximately 10-15% of the dyes are released into the environment during manufacturing and usage. Since some of the dyes are harmful, dye-containing wastes pose an important environmental problem (Verma & Madanwar, 2003). These dyes are poorly biodegradable because of their structures and treatment of wastewater containing dyes usually involves physical and/or chemical methods such as adsorption, coagulation-floculation, oxidation, filtration and electrochemical methods. However, these methods are expensive and have operational problems. Furthermore, dyestuffs cannot be converted to CO2 by physical and chemical methods. Complete degradation of dyestuff can only be accomplished by chemical or biological oxidation (Kapdan et al., 2000).

Several species of bacteria and fungi have been reported to decolorize and degrade textile dyes and have been employed in the treatment of dye bearing wastewaters.

Efforts to isolate bacterial cultures capable of degrading azo dyes started in the 1970s with reports of Bacillus subtilis (Horitsu et al., 1977), then Aeromonashydrophila (Idaka & Ogawa 1978) followed by Bacillus cereus (Wurmannet al., 1980). Numerous bacteria capable of dye decolorization, either in pure cultures or in consortia, have been reported (Banat et al., 1996; Rajaguru et al., 2000; Coughlin et al., 2002; Pearce et al., 2003; Verma & Madanwar 2003; Junnarkar et al., 2006).

In this article, studies on decolorization of Reactive Black B (RBB, C.I. Reactive Black 5) by Paenibacillusdendritiformis strain CS2a4 in pure culture is reported. Optimization of cultural conditions was attempted for rapid decolorization of RBB by CS2a4.

MATERIALS AND METHODS

Chemicals and Organisms

All chemicals used were of analytical grade. The common name of all the dyes have been used for convenience. Reactive azo dye Reactive Black B (RBB), a diazo dye, was procured from Meghmani Chemicals Ltd. GIDC, Vatva, Ahmedabad, Gujarat, India. Structure of RBB is mentioned in Fig. 1.

*Corresponding author: Nishant Junnarkar  
Department of Microbiology, Shree M. & N. Virani Science College, Rajkot, GUJARAT, India 360005
Bacterial culture *Paenibacillus dendritiformis* strain CS\textsubscript{a4} was used for decolorization studies in detail. It was maintained on Nutrient Agar at 4°C.

**Medium**

Bushnell-Haas Medium (BHM) containing the following in g L\textsuperscript{-1}: MgSO\textsubscript{4}, 0.2; CaCl\textsubscript{2}, 0.02; KH\textsubscript{2}PO\textsubscript{4}, 1.0; K\textsubscript{2}HPO\textsubscript{4}, 1.0; (NH\textsubscript{4})\textsubscript{2}NO\textsubscript{3}, 1.0; FeCl\textsubscript{3}, 0.05, pH 7; supplemented with glucose (0.1 g%, w/v) and yeast extract (0.5 g%, w/v) and RBB (100 ppm), was used for enrichment of dye decolorizing bacteria from the samples. For isolation Nutrient Agar Medium was used. BHM + Glucose, 0.1 g% + Yeast Extract, 0.5g%; now is referred as Bacterial Decolorization Medium (BDM) further in the text.

**Enrichment, Isolation and Screening of Dye Decolorizing Bacteria**

**Samples Collection**

The soil and wastewater samples were collected from effluent-contaminated sites (22°97'63.6"N, 72°63.3"E) in the vicinities of dyeing industries located in GIDC Phase IV, Vatva, Ahmedabad, Gujarat, India and from the vicinities of dyeing industry (21°76'28.5"N, 70°63'08.7"E) located in Jetpur, Dist. Rajkot, Gujarat, India. 1% suspension of the samples (soil, w/v and wastewater, v/v) were prepared in sterile distilled water and these were used for enrichment and isolation of dye-decolorizing bacteria using Bacterial Decolorization Medium (BDM).

**Enrichment of dye decolorizing bacteria**

For enrichment of dye decolorizing bacteria, 100 mL of BDM in 250 mL Erlenmeyer flasks were inoculated with 2 mL of 1% suspension and inoculated on an orbital shaker (150 rpm), as well as, under stationary conditions at 37°C. Samples, which showed decolorization in liquid media, were repeatedly tested further for decolorization ability by transferring the enriched culture in fresh BDM till stable dye decolorizing cultures were obtained, showing consistent growth and decolorization in every successive transfer.

**Isolation and Screening of dye decolorizing bacteria**

The samples that showed consistent growth and decolorization in dye-containing medium were transferred on to Nutrient agar plates. This resulted in isolation of 22 different bacterial cultures, which were further tested for their abilities to decolorize the RBB dye in pure culture. Gram staining of the bacterial isolates was carried out to determine the morphology and Gram reaction. Amongst the several isolates, bacterial culture CS\textsubscript{a4} exhibited maximum decolorization in short duration and hence was selected for further studies.

**Molecular Identification of bacterial strain CS\textsubscript{a4}**

The bacterial isolate CS\textsubscript{a4} was subjected to 16S rRNA gene sequencing by Sanger’s method. DNA isolation was performed using XcelGen Bacterial gDNA Mini Kit (Cat No.: XG2411-01). The 16S rRNA gene was amplified using universal primers, 27F: 5'AGAGTTTGATCCTGGCTCAG3' and 1492R: 5'ACGGCTACCTTGTTACGACTT3'. The amplified PCR products were then subjected to sequencing by BigDye Terminator v 3.0 cycle-sequencing kit on the ABI 3730 XL sequencer (Applied Biosystems, USA) using Foundation Data Collection Software v 3.0. The DNA sequence was assembled using Sequencing Analysis Software v 5.3 (Applied Biosystems, USA).

Online BLAST analysis on NCBI site revealed its 99% similarity with *Paenibacillus dendritiformis*. The sequence was submitted in GenBank under the accession number KU601317.

**Decolorization Study and Quantification**

Decolorization studies were performed in 250 mL flasks containing 100 mL of BDM amended with 100 ppm of RBB and each isolate was inoculated in it at the rate of 5% (v/v) inoculum volume. Flasks were then incubated at 37°C under static as well as shaking condition till decolorization was observed. Upon decolorization, the decolorized samples were centrifuged at 8000 rpm for 10 min at 4°C. The supernatants were collected and absorbance was recorded at the absorption maxima of RBB (597 nm) using double beam UV-Vis Spectrophotometer (UV-100, CyberLab, USA). Dye removal was expressed as percent decolorization using the following formula:

\[
\% \text{ Decolorization} = \frac{\text{Initial } A_{597} - \text{Final } A_{597}}{\text{Initial } A_{597}} \times 100
\]

\(A_{597} = \text{Absorbance at 597nm}\)

**Optimization of Cultural Conditions**

Cultural conditions were optimized by varying C-sources (glucose, sucrose, galactose, fructose and peptone) and N-sources (yeast extract, meat extract and ammonium sulfate) in BHM, co-substrate concentration (Glucose: 0.1-1.0 g% & Yeast Extract: 0.1-0.5%), inoculum size (5%, 10%, 15% & 20%; v/v), initial pH of the decolorization medium (pH 4-10), incubation temperature (20, 25, 30, 35, 37, 40 and 45°C) and initial dye concentration (50-500 ppm) in the decolorization medium. Once decolorization was achieved, the % decolorization was recorded. The experimental sets were run in triplicates, mean and standard deviation values were calculated. Decolorized samples obtained were further subjected to Spectral Analysis and TLC analysis.

**Decolorization of RBB by CS\textsubscript{a4} culture under optimized cultural conditions**

All the optimum cultural conditions were compiled and decolorization of RBB dye was assessed in optimized Bacterial Decolorization Medium (BDMO).

**Spectral analysis of decolorized samples**

Supernatants of decolorized samples were subjected to spectral analysis using double beam UV-Visible Spectrophotometer.
Decolorization of Reactive Black B by Paenibacillus DendritiformissTrain Cs2a4

spectrophotometer (UV-100, CyberLab, USA) in the UV and Visible range (200-800 nm).

TLC analysis of decolorized samples

The decolorized medium was collected in centrifuge tubes, centrifuged at 8000rpm for 10min. and the supernatant was subjected to extraction of degradation products using ethyl acetate as the solvent (1:1). The ethyl acetate extracts were dried in oven at 50°C and the residue was subjected to TLC analysis using propane-2-ol: liquor ammonia (7:3) as the solvent system.

RESULTS AND DISCUSSION

Isolation and Screening of RBB decolorizing bacteria

Isolation of bacterial cultures from the enriched cultures resulted in 22 different isolates. These were subjected to screening for the potent RBB decolorizing bacterial strain, where the isolate exhibiting maximum % decolorization in shorter incubation period was selected for further studies. The isolated bacterial strains exhibited % decolorization of RBB (100 ppm) in BDM, in the range of 92-45% in 40-70h of incubation at 37°C in static condition (Table 1). Among these isolates, bacterial strain CSa4 exhibited 92% decolorization (Fig. 2) in 40h and hence it was selected for further studies on RBB decolorization.

Table 1 Decolorization of RBB by different bacterial isolates in Bacterial Decolorization Medium under static conditions at 37°C.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Culture code</th>
<th>Gram's staining reaction</th>
<th>% Decolorization</th>
<th>Incubation period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPa1</td>
<td>Gram negative</td>
<td>83.3</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>PPa2</td>
<td>Gram negative</td>
<td>83.3</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>PPa4</td>
<td>Gram negative</td>
<td>81.8</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>PPa5</td>
<td>Gram negative</td>
<td>78.2</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>PPa6</td>
<td>Gram negative</td>
<td>75.4</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>S3a1</td>
<td>Gram negative</td>
<td>79.3</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>S3a2</td>
<td>Gram negative</td>
<td>80.6</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>S3b1</td>
<td>Gram negative</td>
<td>72.1</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>S3b2</td>
<td>Gram negative</td>
<td>64.8</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>CSa4</td>
<td>Gram variable</td>
<td>75.2</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>CSa5</td>
<td>Gram variable</td>
<td>71.5</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>CSa6</td>
<td>Gram variable</td>
<td>87.6</td>
<td>50</td>
</tr>
<tr>
<td>13</td>
<td>W1</td>
<td>Gram positive</td>
<td>78.2</td>
<td>50</td>
</tr>
<tr>
<td>14</td>
<td>W2</td>
<td>Gram positive</td>
<td>66.8</td>
<td>50</td>
</tr>
<tr>
<td>15</td>
<td>W3</td>
<td>Gram positive</td>
<td>73.2</td>
<td>50</td>
</tr>
<tr>
<td>16</td>
<td>W4</td>
<td>Gram positive</td>
<td>75.2</td>
<td>50</td>
</tr>
<tr>
<td>17</td>
<td>W5</td>
<td>Gram positive</td>
<td>78.9</td>
<td>50</td>
</tr>
<tr>
<td>18</td>
<td>B6</td>
<td>Gram positive</td>
<td>84.2</td>
<td>50</td>
</tr>
<tr>
<td>19</td>
<td>B10</td>
<td>Gram negative</td>
<td>81.3</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>B11</td>
<td>Gram negative</td>
<td>72.8</td>
<td>50</td>
</tr>
<tr>
<td>21</td>
<td>B12</td>
<td>Gram negative</td>
<td>82.6</td>
<td>50</td>
</tr>
</tbody>
</table>

Molecular Identification of CSa4

16S rRNA gene sequence of bacterial culture CSa4 was submitted in GenBank under the accession number KU601317. Online BLAST analysis of the sequence on NCBI site exhibited 99% similarity with Paenibacillusdendritiformissstrain. It is now further referred as Paenibacillusdendritiformissstrain in the text.

Effect of co-substrate on dye decolorization

Among the different C-sources (Fig. 3) and N-sources (Fig. 4) supplemented in BHB, CSa4 exhibited maximum decolorization of RBB dye when glucose (84.5% Decolorization in 26h) and yeast extract (71% Decolorization in 22h) were supplemented in BHB. Majority of reports indicate obligate requirement of labile carbon source for the functioning of dye decolorizing bacteria (Banat et al., 1996). The biodegradation of dyes without any supplement of carbon or nitrogen sources is very difficult (Sani and Banerjee, 1999). Azo dye decolorization by mixed as well as pure cultures generally requires complex organic sources, such as yeast extract, peptone, or a combination of complex organic sources and carbohydrates (Chen et al., 2003; Khehra et al., 2005).

Effect of glucose and yeast extract concentration on decolorization

To optimize the concentration of glucose for maximum and rapid decolorization, glucose concentration was varied from

![Fig 2 BDM containing RBB (100ppm) and medium decolorized by bacterial culture CSa4 under static condition at 37°C.](image-url)

![Fig 3 Effect of different C-sources as co substrate on decolorization of 100 ppm RBB dye byCSa4 (inoculum volume 10%, pH 7.0) at 37°C under static condition.](image-url)

![Fig 4 Effect of co substrate on decolorization of 100 ppm RBB dye byCSa4 (inoculum volume 10%, pH 7.0) at 37°C under static condition.](image-url)
0.1 to 1 g% in BDM. Rapid and maximum % decolorization was observed at 1.0 g% (91% Decolorization in 24 h). Decolorizing efficiency of culture increased with increase in glucose from 0.1 to 1.0 g% in decolorization medium (Fig. 5). Similarly, concentration of yeast extract was varied from 0.1 g% to 0.5 g% (Fig. 6), to optimize its concentration in decolorization medium. Maximum % Decolorization was observed at 0.5 g% (93% Decolorization in 24 h). Thus, all the remaining experiments were performed at 1.0 g% glucose and 0.5 g% yeast extract.

Effect of pH on dye decolorization

The culture could decolorize the dye in the range of 60-80 % Decolorization over a wide range of pH (Fig. 7). However, it exhibited rapid decolorization at pH 7. Thus; all the remaining experiments were performed at pH 7.

The medium pH has a major effect on the dye decolorizing efficiency of bacterial cultures and the optimal pH for color removal had been reported between 6.0 and 10.0 (Chen et al., 2003; Gou et al., 2009; Kilic et al., 2007). Junnarkar et al. (2006) reported maximum decolorization of Direct Red 81 by bacterial consortium NBNJ16 at pH 7. Chang & Lin (2001) also reported that, both E. coli&P. luteola, exhibited best decolorization rate at pH 7 with constant decolorization rates up to pH 9.5. The rate of color removal is higher at the optimum pH and tends to decrease rapidly at strongly acidic or strongly alkaline pH.

Effect of temperature on dye decolorization

Incubation temperature varied from 20°C to 45°C. Maximum decolorization was observed at 37°C (91.33% in 24 h), whereas; above and below this optimum temperature lower % decolorization was observed (Fig. 8). It was observed that the decolorization rate of azo dyes increased up to the optimal temperature, and afterwards there was a marginal reduction in the decolorization activity. This decline at higher temperatures can be attributed to the loss of cell viability or the denaturation of an azo reductase enzyme (Saratale et al., 2011).

For efficient reduction of azo dyes, a source of electron donors and redox mediators are essential (Khan et al., 2014). Here, glucose might have been used as electron donor by the culture for reduction of RBB. Moreover, redox mediators such as NADH might have been involved in reduction of azo bond by azo-reductases. Azo dye decolorization by mixed as well as pure cultures generally requires complex organic sources, such as yeast extract, peptone, or a combination of complex organic sources and carbohydrates (Chen et al., 2003; Khehra et al., 2005). During decolorization of azo dyes via reduction of azo bonds, it was reported that reducing equivalents from various carbon sources are transferred to the dye (Saratale et al., 2011). In addition to the type of the carbon source, it is important to consider the amount of the source because it must be sufficient to meet microbial growth requirements and achieve decolorization. However, high carbon concentrations lead to low decolorization because the microorganisms utilize the carbon source preferentially to the dye (Solis et al., 2012).
Decolorization of Reactive Black B by Paenibacillus DendritiformissTrain Cs2a4

Incubation temperature is a key factor which affects the metabolic rate of the microorganisms. At optimum temperature, enzymes involved in dye decolorization are maximally functional. Most of the dye decolorizing bacterial strains had their optimum temperature at or near 37 ºC. Junnarkar et al. (2006) and Dave and Dave (2009) have reported that there was no considerable decolorization at temperatures above 40ºC, under agitating condition.

**Effect of Inoculum size on dye decolorization**

To find out the optimum inoculum needed for rapid and maximum decolorization by our organism, decolorizing ability was tested at different inoculum sizes viz. 5%, 10%, 15% and 20% (v/v). The decolorization period decreased with increase in the inoculum size (Fig. 9). At higher inoculum volume as number of cells are more, the extracellular enzymatic activities also tend to be higher, making easier for the cells to utilize external ‘C’ and energy sources as well as co-metabolize dye molecules present in the growth medium. Meng et al. (2012) reported faster decolorization of Acid Red 27 in presence of higher initial cell concentration. Junnarkar et al. (2006) reported 20% inoculum size to be the optimum one for attaining maximum decolorization rate for Direct Red 81 by the consortium NBNO6. However, there was no proportionate increase in the percentage of decolorization with increase in the inoculum size of Kurthia sp. when inoculated in textile effluent (Sani & Banerjee, 1999). Dave and Dave (2009) have reported that beyond 25% up to inoculum size of 50% (v/v), time required for decolorization of Acid Red 119 did not decrease considerably, which indicated that 25% (v/v) was the optimum inoculum size.

**Effect of initial dye concentration on dye decolorization**

The influence of dye concentration on the decolorization ability of the organism was investigated considering their occurrence in varying amounts in effluents. The decolorizing activity of the CS2a4 culture was assessed by providing RBB dye at different initial concentrations in optimized BDMO, varying from 50-500 ppm. With increase in dye concentration, time required for decolorization decreased with reduced % decolorization (Fig. 10). Similar results were observed in the bacterial decolorization of various reactive azo dyes (Kalyani et al., 2008). Further increase in dye concentration resulted in reduction in decolorization rates. Similar observations were recorded by Kapil et al. (2009).

It is observed thorough several literatures that increasing the dye concentration gradually decreases the decolorization rate, probably due to the toxic effect of dyes on individual bacteria and/or inadequate biomass concentration (or improper cell to dye ratio), as well as blockage of active sites of azoreductase by dye molecules with different structures (Jadhav et al., 2008; Sani and Banerjee, 1999; Saratale et al., 2009; Tony et al., 2009).

Moreover, as shown in Fig. 10, decolorization period was reduced to 16h (94%), indicating the improved performance of the culture in BDMO.

**Spectral analysis of decolorized medium**

Spectral analysis of decolorized samples in the UV-Vis range (200-800 nm) revealed the shift of the peaks from visible range to UV range (Fig.11), which attributes to the formation of aromatic amines during transformation of the dyes by the culture in BDMO.

**TLC analysis of decolorized medium supernatant**

RBB dye decolorization by CS2a4 was further supported by TLC analysis. When the dye chromatogram was observed in UV light, fluorescent blue colored bands with Rf values: 0.9, 0.95, 0.8 and 0.75 were observed in the lane of decolorized medium supernatant. Whereas the Rf values of bands observed in control (uninoculated medium) lane were 0.7 and 0.97; no such corresponding spots were observed in decolorized sample lane, indicating that decolorization was due to degradation or transformation of intact dye molecule into unknown intermediates.
CONCLUSION
Among the 22 bacterial isolates obtained, Paenibacillus dendritiformis CS24 was found to be the potent decolorizer of Reactive Black B (RBB), under static conditions. Optimization of its cultural conditions resulted in the reduction of decolorization period for RBB from 16h to 4h. CS24 could decolorize RBB up to 350 ppm efficiently (>80%) within 16-30h. Spectral analysis and TLC analysis of decolorized medium revealed the transformation of RBB dye into unknown intermediates. Thus, the culture exhibited immense potential for its application in treatment of RBB dye bearing wastewater. Further, toxicity analysis of the products formed upon transformation of the dye by CS24 culture needs to be revealed.

References
Coughlin M.F., Kinkle B.K., Bishop P.L. 2002. Reduction of decolorization period for RBB from 16h to 4h. CS24 could decolorize RBB up to 350 ppm efficiently (>80%) within 16-30h. Spectral analysis and TLC analysis of decolorized medium revealed the transformation of RBB dye into unknown intermediates. Thus, the culture exhibited immense potential for its application in treatment of RBB dye bearing wastewater. Further, toxicity analysis of the products formed upon transformation of the dye by CS24 culture needs to be revealed.


******