

## Bacterial Decolorization of Azo Dye Direct Red 28 in An Up Flow Immobilized Cell Bioreactor

Bella Devassy Tony<sup>2</sup>, Dinesh Goyal<sup>2</sup> and Sunil Khanna<sup>1\*</sup>

<sup>1</sup>Department of Biotechnology and Bioinformatics,  
NIIT Institute of Information Technology, Balaji Estate, Kalkaji, New Delhi - 110 019, India.

<sup>2</sup>Department of Biotechnology and Environmental Sciences,  
Thapar University, Patiala - 147 004, India

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A mixed bacterial culture (SKB-II) comprising of five bacterial isolates *Bacillus vallismortis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium*, developed from an effluent treatment plant of a textile & dyeing industry, was evaluated for its ability to decolorize azo dye Direct Red 28 in an up flow immobilized cell bioreactor using plastic clips as a support for biofilm formation. The bioreactor was operated under various parameters: aeration rates of 10 ml min<sup>-1</sup> & 15 ml min<sup>-1</sup> with flow rates of 60, 90 & 120 ml h<sup>-1</sup> respectively. At constant aeration rate of 10 ml min<sup>-1</sup> and with flow rates 60, 90 & 120 ml h<sup>-1</sup>, optimum decolorization of 69, 71 and 70% decolorization was observed while at aeration rate of 15 ml min<sup>-1</sup> and flow rates of 60, 90 & 120 ml h<sup>-1</sup>, optimum decolorization of 73, 66 and 63% decolorization was observed. The study concluded that across the two aeration rates and the respective flow rates, aeration rate of 15 ml min<sup>-1</sup> along with flow rate of 60 ml h<sup>-1</sup> was best suited to decolorize Direct Red 28 in the up flow immobilized cell bioreactor. Spectral changes of the input and output of the bioreactor by UV-visible, spectroscopy indicated decolorization of the dye solution by degradation in addition to the visual observation of the biosorption process. On the basis of the results of this study an 'on-site' treatment system can be developed to achieve decolorization for azo dye effluents.

**Key words:** Azo Dye, Direct Red 28, biofilm, bacterial consortium, up-flow immobilized bioreactor, *Bacillus* sp.

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Majority of colored effluents consists of dyes, released to the environment from textile, dyestuff, and dyeing industries. Besides the unpleasant appearance of the dye-polluted wastewater, most dyes and their potential breakdown products are toxic. Over 10,000 different dyes with an annual

production of over 7×10<sup>5</sup> metric tons worldwide are commercially available. Amongst the synthetic dyes, azo dyes represent the largest and most versatile class of synthetic dyes<sup>1</sup>. Many azo dyes, and particularly their biodegradation byproducts, have been linked to cancer in humans; to splenic sarcomas, hepatocarcinomas and nuclear anomalies in experimental animals; and to chromosomal aberrations in mammalian cells.

Two percent of the dyes produced are discharged directly in aqueous effluent and 10% are subsequently lost during the textile coloration process. Available effluent treatment processes, focus mainly on the removal of dyes by physical

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\* To whom all correspondence should be addressed.  
Tel.: +91-011-240586247; Fax: +91-011-240586249  
E-mail: sunil.khanna@tni.edu.in

and/or chemical methods thus leading to the generation of highly toxic sludge/solid wastes that require special disposal techniques, as per existing laws. The ability of microbes to decolorize and metabolize dyes has long been known and the use of bioremediation based technologies for treating textile wastewater has gained interest. Therefore, focus is now shifting to the development of environment-friendly biological treatment systems based on using microorganisms to decolorize/degrade recalcitrant compounds and lead to mineralization of the target compounds. Microbial degradation of azo dyes by anaerobic treatment resulted in the formation of aromatic amines which are carcinogenic and mutagenic<sup>2</sup>.

Aerobic decolorization of azo dyes by *Aeromonas* and certain consortia has been demonstrated<sup>3-5</sup>. A *Staphylococcus* sp. EY-3 with the capability of decolorizing azo dye Congo Red was isolated from soil at an effluent treatment plant of a textile and dyeing industry<sup>6</sup> which was able to decolorize Congo Red in 48 h under aerobic conditions in batch studies. Some reports on cell immobilization for decolorization of commercially available dyes have been reported<sup>7-12</sup>.

The present study was conducted to optimize the decolorization potential of a treatment system consisting of cell immobilization of the aerobic consortium SKB-II consisting of *Bacillus vallismortis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium*. Further the operation parameters on the decolorization performance of immobilized bacterial consortium SKB-II, to efficiently decolorize the azo dye Direct Red 28 commonly used for textile dyeing were optimized

## MATERIAL AND METHODS

### Dyes and Chemicals

The model azo dye Direct Red 28 with three naphthalene rings and a sulphonate functional group joined by azo bond, commonly used in dyeing and textile industries was generously provided by Nahar Group of Textile Dyeing Industry, Derabassi, India. For decolorization studies, synthetic dye wastewater (13) was used with the dye concentration of 10 mg L<sup>-1</sup>. Aqueous stock solution was filter sterilized

using Millipore filter of 0.22 µm (9). Wavelength maxima of the dye was spectrophotometrically measured by wavelength scan, against water as blank. The media components were purchased from Himedia Labs, Mumbai (India) and other chemicals used were of analytical grade. Plastic clips used as immobilization support was purchased from local electrical store (Patiala, Punjab, India).

### Development of dye decolorizers

Effluent sample collected from sludge samples (aerated tank) of Effluent Treatment Plant of a Nahar Group. of Textile & Dyeing Industry, Derabassi, India, was used for the development of the mixed microbial culture SKB-II capable of dye decolorization. After ten transfers on the basal medium containing the particular dye, the stabilized mixed microbial culture was used for further studies. The culture was routinely grown at 37 °C in the basal culture medium, Bushnell and Hass Broth medium (BHB) containing the following in g L<sup>-1</sup> MgSO<sub>4</sub>, 0.2; CaCl<sub>2</sub>, 0.02; KH<sub>2</sub>PO<sub>4</sub>, 1.0; K<sub>2</sub>HPO<sub>4</sub>, 1.0; (NH<sub>4</sub>)<sub>2</sub>NO<sub>3</sub>, 1.0; FeCl<sub>3</sub>, 0.05 supplemented with starch 1.3 g L<sup>-1</sup> as carbon and energy source and 40 g L<sup>-1</sup> NaCl. The final pH of the medium was adjusted to 7.

The stock culture of the developed consortium was preserved in 50% Glycerol stock and stored at -80°C and subsequently sub-cultured on basal medium.

### Decolorization assay

Aliquots (5 ml) from the output of the bioreactor were collected at regular time intervals, and centrifuged at 10,000 x g for 15 min.<sup>14</sup>. The decolorizing activity was expressed in terms of percentage decolorization determined by monitoring the decrease in absorbance of cell free supernatant at 499 nm (ε max of the dye) using a UV/Visible spectrophotometer (Hitachi U200, Tokyo, Japan) and monitoring the absorbance of aliquot from the outlet of the abiotic bioreactor as blank. Rate of decolorization was calculated from the difference between the initial and the final absorption values of the supernatant at the λ max for each dye. % decolorization =  $\frac{I_i - I_f}{I_i} \times 100$ , where I<sub>i</sub> and I<sub>f</sub> are initial and final absorbance of the dye solution (15). Each decolorization value is a mean of two parallel experiments. Another bioreactor was operated under same conditions as an abiotic control to compare the total

decolorization of the dye in the biotic reactor.

#### **Isolation & Molecular characterization of dye decolorizing bacterial Isolates**

An aliquot of 100 $\mu$ l inoculum of the mixed bacterial culture after serial dilution was spread on minimal media agar plates (in duplicate). The plates were incubated at 37°C for 17-18 hours after which a total of 53 isolates showing different morphology were selected. The selected fiftythree isolates were checked for their ability to decolorize 10 mg L<sup>-1</sup> Direct Red 28 at batch scale.

The chromosomal DNA of the best dye decolorizing strains was isolated according to the procedure described by Rainey *et al* (16). The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (AGAAAGGAGGTGATCCAGGC-3'). The amplified DNA fragment was separated on 1% agarose gel, eluted from the gel and purified using Qiaquick gel extraction kit (Qiagen, Germany). The purified PCR product was sequenced with four forward and three reverse primers namely 8-27f (5'AGAGTTTGATCCTGGCTCAG-3'), 357f (5'-CTCCTACGGGAGGCAGCAG-'), 704f (5'-TAGCGGTGAAATGCGTAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTACGCATTTACCGCTAC-3'), 1110r (5'-GGGTTGCGCTCGTTG-3') and 1500r (5'-GAAAGGAGGTGATCCAGGC-3'), respectively (*Escherichia coli* numbering system). The rDNA sequence was determined by the dideoxy chain-termination method using the Big - Dye terminator kit using ABI 310 Genetic Analyzer (Applied Biosystems, USA).

The 16S rDNA sequence of the strains generated in this work were aligned with the 16S rDNA sequence of other closely related *Bacillus species* retrieved from the GenBank data base. A sequence similarity search was done using GenBank BLASTN<sup>17</sup>. Sequences of closely related taxa were retrieved, aligned using Clustal X programme<sup>18</sup> and the alignment was manually corrected for the neighbour-joining analysis<sup>19</sup>, the distances between the sequences were calculated using Kimura's two-parameter model<sup>20</sup>. Bootstrap analysis was performed to assess the confidence limits of the branching<sup>21</sup>.

#### **Bioreactor**

The aerobic mixed bacterial consortium comprising of *Bacillus vallismortis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium* was used as seed inoculum for the development of an up-flow immobilized cell bioreactor. For the current study, plastic clips (size 2-3 mm) were chosen as support material (matrix) in the bioreactor as a specific surface area is achievable by selecting an appropriate type of packing material. The matrix was saturated with Direct Red 28 to avoid discrepancy in results due to adsorption of dye color by the matrix.

The packed bed bioreactor of borosilicate glass column (44.5 cms x 6 cms, empty bed volume 1260ml) packed with plastic clips was sterilized and operated at 37C. Biofilm formation was initiated by addition of the aerobic mixed bacterial culture SKB II (cell concentration 2.5  $\times$  10<sup>8</sup> cfu mL<sup>-1</sup>). After seven days of growth in the reactor, the BHB medium (containing 10 mg L<sup>-1</sup> Direct Red 28) was introduced into the reactor. The packed bed bioreactor was operated at two aeration rates of 10ml min<sup>-1</sup> and 15ml min<sup>-1</sup> respectively at flow rate of 60ml h<sup>-1</sup>, 90ml h<sup>-1</sup> and 120 ml h<sup>-1</sup> respectively.

#### **Scanning electron microscopy**

The support material (plastic clips) for scanning electron micrographs were collected from the bioreactor after biofilm formation and were fixed with 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 h and subsequently washed with 0.1 M phosphate buffer for 10 min. The fixed samples were then dehydrated through a graded series of ethanol solutions (30, 50, 70, 90 and 100 % ethanol) for 30 min each. The samples were then dried in critical point drier and coated with gold<sup>9</sup>. The un-inoculated support material sample was also treated as per the described procedure. Scanning electron micrographs were taken on a JSM-840, JEOL (Japan) scanning electron microscope.

## **RESULTS AND DISCUSSION**

#### **Molecular identification of bacterial Isolates**

Five bacterial isolates out of fifty three bacterial isolates showed maximum decolorization activity (Data not shown).

Molecular identification identified these gram positive isolates as *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus vallismortis* and *Bacillus subtilis*. Morphology of *Bacillus pumilus* was short segmented rods; *Bacillus cereus* was diplococcus, *Bacillus vallismortis* occurred in clusters, while *Bacillus megaterium* and *Bacillus subtilis* occurred as gram positive rods.

#### Development of immobilized cell bioreactor

The bioreactor was packed with plastic clips (matrix) for sustainable microbial growth as it is abundant, cheap and readily available, is also inert, nontoxic, nonbiodegradable and

mechanically more stable. Bacterial consortium SKB-II comprising of five bacterial isolates *Bacillus vallismortis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium*, was inoculated in the bioreactor for biofilm formation on the matrix for an incubation period of 7 days at 37 °C. Aeration was set at 10 ml min<sup>-1</sup> and 15 ml min<sup>-1</sup> respectively at a flow rate 60 ml h<sup>-1</sup>, 90 ml h<sup>-1</sup> and 120 ml h<sup>-1</sup> respectively. Washout from the outlet of the column were analyzed by collecting samples at regular time intervals and by checking optical density spectrophotometrically at 550 nm. till a constant value was obtained. This process continued until

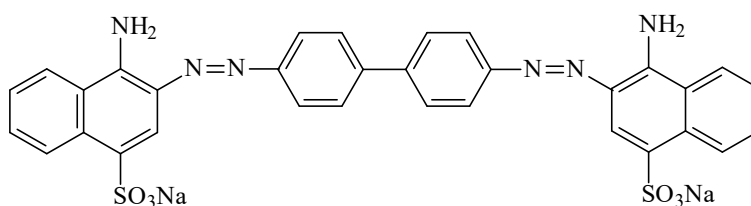


Fig.1. Chemical structure of Direct Red 28, empirical formula ( $C_{32}H_{22}N_6O_6S_2Na_2$ )

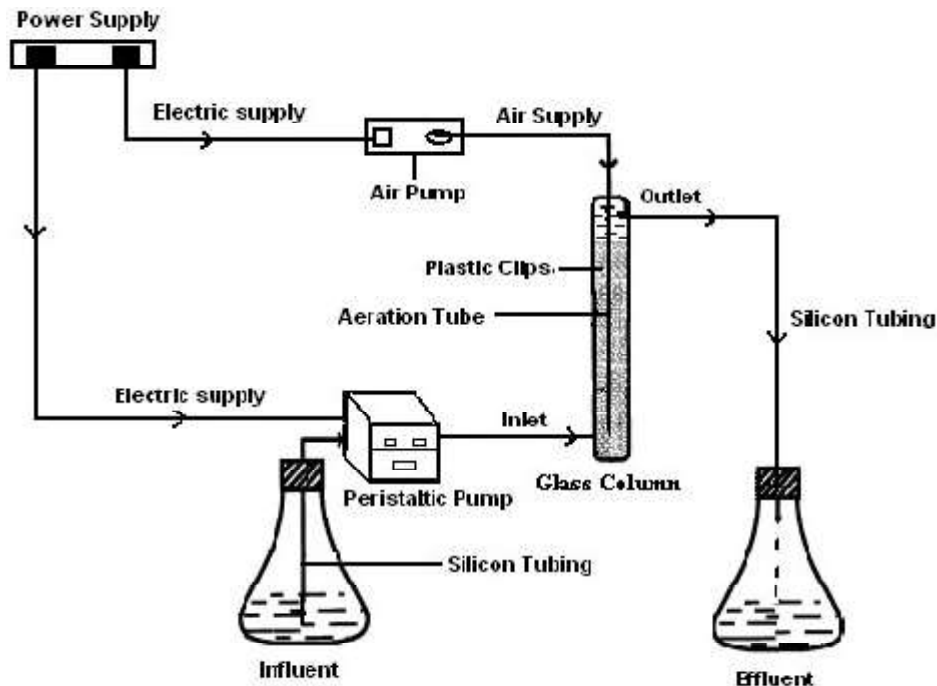


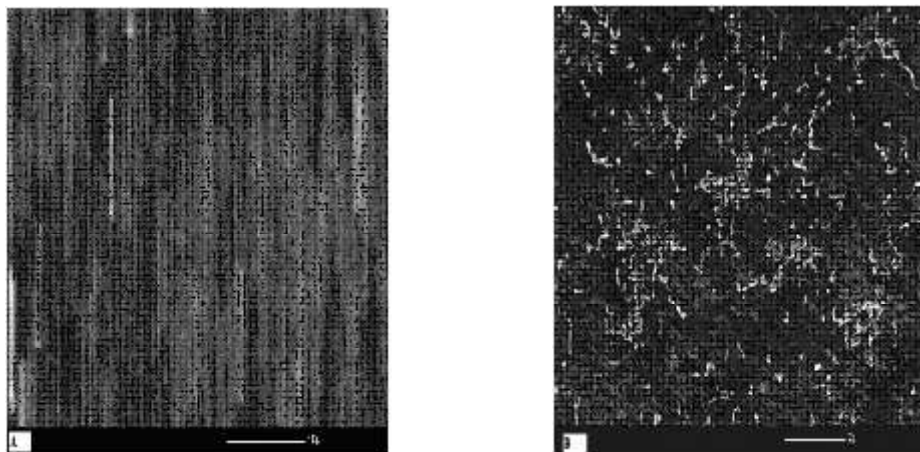
Fig. 2. Schematic representation of the up-flow immobilized cell bioreactor, with plastic clips as the support material

a chemostat arrangement was attained wherein the rate at which the cells get removed is the same as the rate at which new cells were formed.

It was noted at higher flow rates of 90 ml h<sup>-1</sup> and 120 ml h<sup>-1</sup> washout stabilization was achieved between 65 to 82 hrs respectively as compared to the washout stabilization achieved in 57 hrs at a flow rates of 60 ml h<sup>-1</sup> (data not shown). Also at higher flow rates turbidity of the outflow increased due to the microbial cell washouts that came along in the sample as the biofilm mass was probably lost by the erosion of the biofilm surface due to shearing by the liquid passing by, or by the detachment of the biofilm from the attachment surface due to sloughing. Once stabilization period (stable value of washout) was attained the feeding stream was continuously pumped with BHB medium (containing 10 mg L<sup>-1</sup> Direct Red 28) in upward direction through

bottom of the reactor to avoid channeling effects and increase in retention time<sup>11</sup> at the respective flow rates. The bioreactor was in operation under these conditions for 30 days. Similar to this study, Senan *et al*<sup>22</sup> in their study concluded that continuous use of an immobilized aerobic cell bioreactor by passive immobilization of the bacterial consortium is a feasible approach for the textile azo dye degradation.

Upon biofilm stabilization, support particles were removed from the bioreactor and were processed as described in section 2.4.4 for Scanning electron micrograph (SEM) of biotic and abiotic support. The uninoculated support particle showed large surface area for bacterial growth and attachment (Fig.3.A). And SEM of the support particle removed from the inoculated bioreactor showed formation of biofilm by the bacterial cells (Fig.3.B).



**Fig. 3.** Scanning electron micrographs. (A: Sterile uninoculated support particle showing different surface zones (x 1,500) B: Support particle completely covered with a layer of rod-shaped bacterial cells (x 4000) formed by consortium SKB-II comprising of five bacterial isolates viz: *Bacillus vallismortis*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus*.

### Decolorization of Direct Red 28

For decolorization studies of Direct Red 28 dye, packed-bed reactor columns were used due to their considerable potential as they contain stationary bed of media which provides a large surface area for biological growth. Once biofilm formation was attained sterile colored media was pumped into the reactor. In continuous operation, first initial hours of dye decolorization was not accounted because of the mixing of effluent with

the contents of the reactor and also to avoid the accounting of the decolorization contributed by matrix adsorption of dye which is negligible in a continuous flow fixed bed process<sup>11</sup>.

At a constant aeration rate of 10 ml min<sup>-1</sup> and flow rate of 60 ml h<sup>-1</sup> after biofilm formation, BHB medium containing Direct Red 28 dye was introduced into the reactor and optimum growth of 0.308 at OD 550 nm was observed with decolorization of 70% at 499 nm. Increase in the

flow rate from 60 to 90 ml h<sup>-1</sup> resulted in the increase in growth to 0.320 from 0.308. Further increase to 120 ml h<sup>-1</sup> reduced the growth to 0.282. Decolorization at all the three flow rates did not vary significantly showing decolorization of 70 to 72% among the three flow rates. Maximum growth of 0.342 (at OD 550) was observed at an aeration rate of 15ml min<sup>-1</sup> and flow rate of 60 ml h<sup>-1</sup> with decolorization of 73.4% (Fig.4)

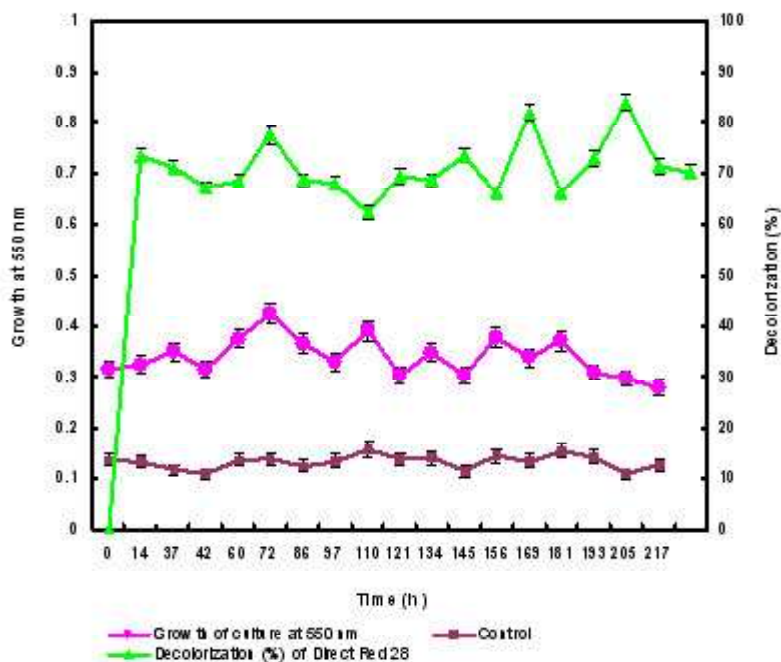
While at higher flow rate of 90 ml h<sup>-1</sup>

growth was reduced by 15.2% growth and decolorization by 66%. Least growth (0.279) and decolorization of 63% was observed at highest flow rate of 120 ml h<sup>-1</sup> (Table 1)

The rate of decolorization when compared between the two aeration rates 10 and 15ml min<sup>-1</sup> was observed to be higher at 15 ml min<sup>-1</sup> due to the aerobic nature of the consortium. Therefore, providing higher aeration of 15 ml min<sup>-1</sup> increased the rate of decolorization of the

**Table 1.** Growth of mixed bacterial culture SKB-II and decolorization (%) of Direct Red 28 at aeration rates of 10 and 15ml min<sup>-1</sup> and flow rates of 60, 90 & 120 ml h<sup>-1</sup>

Aeration rate/ Flow Rate	Growth(OD 550)	Decolorization (%)
Air 10 ml min <sup>-1</sup> & Flow 60 ml h <sup>-1</sup>	0.31±0.02	69±1.3
Air 10 ml min <sup>-1</sup> & Flow 90 ml h <sup>-1</sup>	0.32±0.02	71.4±1.1
Air 10 ml min <sup>-1</sup> & Flow 120 ml h <sup>-1</sup>	0.282±0.01	70.75±1.1
Air 15 ml min <sup>-1</sup> & Flow 60 ml h <sup>-1</sup>	0.342±0.01	73.42±1.9
Air 15 ml min <sup>-1</sup> & Flow 90 ml h <sup>-1</sup>	0.29±0.02	66.4±1.7
Air 15 ml min <sup>-1</sup> & Flow 120 ml h <sup>-1</sup>	0.271±0.03	63.03± 2.6



**Fig. 4.** Decolorization (%) of Direct Red 28 by mixed culture SKB-II at an aeration rate of 15 ml min<sup>-1</sup> and flow rate of 60 ml h<sup>-1</sup>. The azo dye at a concentration of 10 mg l<sup>-1</sup> was treated in the bioreactor upon biofilm formation by consortium SKB-II, comprising of five bacterial isolates viz: *Bacillus vallismortis*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus pumilus* on support material (plastic clips). From the outlet of the bioreactor samples were collected to estimate growth and decolorization as described in the M&M.

dye. Yang Ge *et al*<sup>10</sup> in their work reported the use of higher rotational speeds to provide better aeration thereby resulting in increased decolorization efficiency.

The UV-VIS spectra of the input feed stream and the output feed stream showed a shift from the maximum of absorption to shorter wavelengths upon mixed bacterial culture treatment (Fig 5). This indicates that

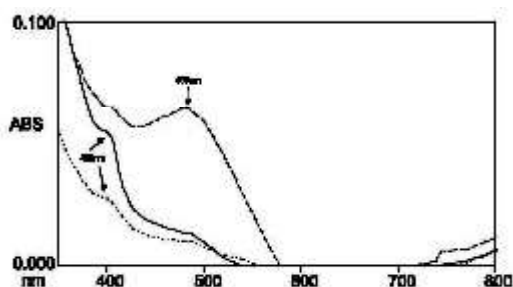


Fig. 5. Absorption spectra of Direct Red 28 dye treated in the bioreactor. (—) Input feed of the bioreactor; 499 nm  $k_{max}$  of the dye, (---) output feed of the bioreactor, (.....) output feed of the duplicate bioreactor

decolorization of this dye solution occurred by degradation in addition to the visual observation of the biosorption process<sup>23</sup>. Spectral shifts towards shorter wavelengths after the treatment with mixed bacterial culture were probably produced by the biodegradation of the dye molecule that led to a decrease of the conjugation effects between the aromatic rings. It should be noted that for conjugated systems the wavelength of the maximum absorption was very sensitive to the size of the conjugation, where a decrease of one unit of double bond can lead to shifts in range of 25–30 nm in polyenic-carbonyl conjugated systems<sup>24</sup>.

Additionally, least or no color loss was observed from the abiotic control bioreactor suggesting that the observed dye decolorization in the biotic reactor is due to the biological activity of the microbial consortium immobilized on the plastic clips in the bioreactor. Sizing agents like starch or partly hydrolyzed starch applied to yarn for an efficient weaving process is present in the

textile mill effluent. Batch study experiments using various concentrations of starch: 0.065 g L<sup>-1</sup>, 1.3 g L<sup>-1</sup> resulted in better decolorization efficiency of consortium SKB-II with 1.3 g L<sup>-1</sup> starch (Data not shown). Hence, in this study 1.3 g L<sup>-1</sup> of starch was used in the BHM medium. During the decolorization process, it was observed that there was random increase and decrease in the percentage of dye decolorization this can be attributed to the fact that during the dye decolorization process by the developed consortium there was simultaneous adsorption and desorption of the dye onto the microbial biomass and onto the starch present in the medium<sup>2</sup>.

## CONCLUSION

The immobilized consortium containing bacterial cultures comprising of *Bacillus vallismortis*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium* exhibited efficient decolorization activity in decolorizing azo dye Direct Red 28 in a packed bed bioreactor. For immobilization, plastic clips proved to be better than other support materials tried and tested like thermocol and foam. Further research to decolorize Direct Red 28 dye using marble chips for microbial immobilization of cells is underway.

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