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Isolation, Screening and Characterization of Azo Dye Decolorizing Bacteria from Contaminated Soil

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Abstract: A potential bacterial strain DDB3, capable of degrading an azo dye Direct black as a sole source of carbon, was isolated from textile mill effluent from Gwalior, India. The phenotypic characteristics and biochemical test indicated an isolated organism as *Micrococcus sp. DDB3*. This strain exhibited complete decolorization of Direct black (100 mg/L) within 20 h. For color removal, the most suitable temperature were and 25–40°C, respectively. The isolate was able to decolorize 97% of acid red within 20h. The isolate utilize sucrose, glucose as carbon source and peptone as nitrogen source for maximum decolorization. UV-Visible absorption spectra before and after decolorization suggested that decolorization was due to biodegradation and was further confirmed by TLC analysis. The toxicity of degraded product were analysed by seed germination test which gives good results. Overall results indicate the effectiveness of the strain *Micrococcus DDB3* explored for the treatment of textile industry effluents containing various azo dyes.

Keywords: Azo dye, Bacteria, Acid red, Decolorizing.

I. INTRODUCTION

Water pollution is the contamination of water bodies (e.g. lakes, rivers, oceans, aquifers and groundwater). This form of environmental degradation occurs when pollutants are directly or indirectly discharged into water bodies without adequate treatment to remove harmful compounds. Of all the water resources on earth, two- third of fresh water exists in the form of glaciers and ice caps and only 3% of it is consumable. Thus water is a vital resource for human and other animal and plant health. One of the main sources with severe pollution problems worldwide is the textile industry and its dye-containing wastewaters. Worldwide 10,000 types of textile dyes with an estimated of 7.105 metric tonns are annually produced [1, 2, 3]. 10-25% of total textile dyes are lost during the dyeing process, and 2-20% of dyes are directly discharged as aqueous effluents in different environmental components.

In 1856, the world's first commercially successful synthetic dye, Mauveine, was discovered for practical uses [4, 5]. A dye may be defined as an organic compound containing both auxochrome and chromophore groups linked to benzene group. Axochrome imparts the property of electrolytic dissociation and chromophore responsible for imparting colour to the compound. A large number of synthetic dyes with specific groups (azo, base, acid, triphenylmethane, anthracene etc) are widely used in textile, pharmaceutical, food, leather and cosmetic industries [6, 7]. Textile industries are generally located in places near to the rivers and canals, mainly for effortless overseas transportation. In significant a proportion of these dyes, are discharge in to the environment through waste water. Improper disposal of these dyes textile dye effluent in aquatic ecosystem leads to decrease photosynthetic activity, water quality, and dissolve oxygen. These dyes can also affect the human health by causing nausea, ulceration of the skin, hemorrhagic and severe damage to reproductive system, kidney, liver, brain and central nervous system [8, 9]. Many of the synthetic Azo dyes and their metabolites are toxic mutagenic and carcinogenic [10]. Therefore the removal of azo compound and their metabolites is necessary prior to their final discharge to the environment.

The continuous dumping of hazardous dyes and dye derivatives in the form of waste has lead to contaminate soil, surface water, ground water, sediments etc. at alarming level. Government legislation is forcing dye consuming industries to treat their waste effluent before discharging into water bodies. Currently, several physicochemical techniques like chemical precipitation and flocculation, adsorption, chemical oxidation and reduction, photolysis and electrochemical treatment has been used for the treatment of colored textile effluent [11]. The dyes are removed by these methods but accumulation of concentrated sludge occurs which creates secondary level land pollution [12]. By reducing the dye compounds to their intermediates, the problem of visual pollution is solved, but larger and more deleterious problems may be created. All these chemical and physical methods are very expensive, less efficient; interfere with other waste water constituents [13,14]. So, there is a need to find alternative and effective methods of treatment that are efficient to removing dyes from large volumes of effluents and are low cost such as bioremediation. Conversely bioremediation through microorganism provide an alternative to existing technologies as it has been identified to environment friendly and cost effective

for the disposal of textile discharge.Bioremediation is the use of biological systems (mainly microorganisms and plants) for the treatment of polluted air, aquatic or terrestrial component of environment. At present water resources is frequently reduced due to rapid growth of population and industrial development make situation more critical. This problem has triggered the need to reuse of municipal and industrial waste water after proper treatment and elimination of potential pollutants [15]. Many microorganisms belong to various taxonomic groups of bacteria, actinomycetes, fungi and algae have been reported for their ability to degrade azo dyes [16]. Previously pure fungal cultures have been used to develop bioprocesses for the mineralization of azo dyes, but the long growth cycle and moderate decolorization rate limit the performance of fungal decolorization system [17]. Many bacteria capable of dye decolorization/biodegradation either in pure cultures or in consortia has been reported [18, 19] because bacterial decolorization is normally faster than fungal culture due to the long growth cycle and moderate decolorization rate. However, comprehensive solutions for sulfonated azo dyes removal are far from reality, which calls for continued search for new organisms and technologies [20, 21].

Over the past decade, many dye degrading microorganism at lab level have been reported, but there are few reports available on their exploitation in treatment process. The bacterial degradation of azo dye is initiated by a reductive cleavage of azo bond, which result in the formation of amines. The aromatic amines that are formed at the time of these reactions may be degraded aerobically [22]. Microorganism isolate from soils and sludge sample belonging to *Bacillus sp* [23], *Alcaligenes Sp.* and *Aeromonas sp.* were found to have good dye decolorization on activity [24]. Decolorization of erio red and direct yellow dyes by bacterial and actinomycetes were studied. Cynobacteria such as *Gloeocopsa pleurocapsoides* and *Phromidium ceylanicum* decolorized FF skyblue and acid red 97 dyes more than 80% in 26 days [25].

The aim of the present study was to isolate and characterize an efficient bacterial strain which show the remarkable ability to decolorize/degrade various textile azo dyes used in industry. After the isolation of dye degrading bacteria from contaminated soil sample, physicochemical parameters had been optimized for the optimum dye degradation by selected bacteria. The production of total protein during dye degradation was measured by Lowry method. The dye degraded products were characterized by UV-Vis spectrophotometry, TLC. The phytotoxicity of dye degraded product was observed to assess the risk of toxicity by seed germination test. The use of isolated bacteria either individually or as consortium was envisaged to develop efficient biological process for the treatment of effluents containing different dyes.

II. MATERIAL & METHODS

A. Sample collection

Samples were collected from various textile industries around Gwalior Madhya Pradesh. Samples were collected from different places, such as drainage canal that carry textile effluent. Samples were in the form of liquid untreated effluent, sludge, and soil. All the samples were collected in sterile glass-screw cap tubes and preserved at 4^{o}C in refrigerator and samples were tested within 24 hrs of collection.

B. Physico-chemical characterization of samples

The effluent samples were tested for its physico-chemical characteristics like, color, pH, COD, BOD, TSS, TDS, etc according the guideline of APHA [26]**.**

C. Dye and chemicals

Two azo dyes Acid Red 88 (C.I.15620), Direct Black 38 (C.I. 30235) were procured from local dye market Gwalior, MP, India. All required chemicals were obtained from CDH (India), SRL (India) and Himedia (India). All chemicals used in the study were analytical grade.

D. Culture medium

The Mineral Salt Medium (MSM) consisted of (g/l) KH_2PO_4 (2.0), $Na_2HPO_4.2H_2O$ (0.50), $FeCl_2.4H_2O$ (0.01) and Ca $(NO_3).4H_2O$ (0.04) supplemented with 10 ml of trace element solution per liter. The trace element solution contained (mg/lit) $CuCl_2.2H_2O$ (10), $MnCl_2.4H_2O$ H_3BO_3 (30.0). The final pH of the medium was adjusted to 7.0 [27,28].

Complete medium broth consisted of the following constituents (g/l). Yeast extract (30), sucrose (2.0), NaCl (5.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.1). The final pH of medium was adjusted to 7.0.

E. Isolation and screening of dye decolorizing bacteria

Bacterial strains were isolated through enrichment culture technique. 10 ml of sample was added to 100 ml of MSM broth containing acid red (100mg/l) as a sole source of carbon. The flasks were incubated at 35°C for 5 to 6 days under static and shaking condition. After incubation cell suspension from flask were plated over the agar plate of MSM containing dye and incubate at 37°C for 24hr. Further the prominent colonies that appeared on agar plate streak on MSM agar plate amended with dye and yeast extract. They were purified by streaking four times on agar medium. The pure cultures were preserved at 4°C for subsequent studies.

The morphologically distinct bacterial isolate were tested for their ability to decolorize textile azo dyes. Overnight grown culture of isolates were use to inoculate with 1ml in 250 ml

Erlenmeyer flask containing 100 ml CMB supplemented with Acid red and Direct black (100 mg/L) dye. The inoculated flasks were incubated under static conditions. 2 ml sample was taken out aseptically and centrifuged at 8000 rpm for 10 min. The cell free supernatant was used to determine the percentage decolorization of the added dye. More than 70% decolorization of the added dye was selected for further studies [29].

Decolorization activity (%) = (A-B)/A × 100

Where A = Initial absorbance

B = Observed absorbance of decolorization sample.

F. Identification of dye decolorizing bacteria

Grams staining of pure culture of all isolates were performing to study gram reaction and cell morphology. The isolates colonies color, transparency, shape and texture were observed directly. The isolates were subjected to biochemical test included indole production, methyl red, citrate utilization, triple sugar iron, arginine hydrolysis, Voges Proskauer, casein utilization test and sugar fermentation test. Bergey's Manual of Determinative Bacteriology and 'PIBWIN' online software for bacteria identification were used as a reference to identify the isolates [30, 31].

G. Optimization of physicochemical condition

The decolorization efficacy of selected bacterial isolate on direct black was studied at varying parameters such as shaking (150 rpm) versus static conditions temperature (25°C-50°C). The optimized pH 7.0 and tem 37°C were selected to studying the decolorization activity of various physicochemical parameters such as initial dye concentration (100-800 mg/l), effect of various carbon and nitrogen source

H. Decolorization and biodegradation study

The degraded products of acid red from during biodegradation after 21 hr of incubation in static condition were study by UV-Visible spectrophotometer and TLC chromatography. The change in the UV-Visible spectra (200 to 725nm) using the UV-Visible spectrophotometer (Simadzu UV 1700). After complete decolorization, microbial culture was centrifuge at 800 rpm for 15min to remove the bacterial cell and suspended particle. Equal volume ethyl acetate used for the extraction of degraded metabolites. The crystal obtain were dissolved in a 1 ml of HPLC grade methanol and the same sample was used for TLC analysis.

I. Phytotoxicity study

The effect of original dye and its degradative metabolite on germination and early seedling growth of two plants; *Triticum aestivum* and *Vigna mungo* was evaluated. The degradation metabolites of Direct black were extracted in ethyl acetate were dried and dissolved in water to form the final concentration of 1000 ppm. The dye solutions were also prepared with concentration of 1000 ppm for phytotoxicity studies. The seeds were germinated in sterile 10 cm petri dishes, layered with sterile filter paper. The phytotoxicity study was carried out at room temperature (32 ± 2°C) in relation to *Triticum aestivum* and *Vigna mungo* seeds by watering separately 5 ml samples of dye and its degradation product per day. Seeds germinated in water irrigated petri dish were used as a control. Length of plumule (shoot), radicle (root) and germination (%) were recorded after every alternate day.

III. RESULTS & DISCUSSION

A. Physico-chemical characterization of collected samples

The samples were collected in sterilized container from respective sites and transported to the laboratory by storage at 4°C. Physico-chemical characteristics like color, pH, BOD, COD, TSS, TDS etc. were measured on the same day of collection of sample as per Table 1.

B. Isolation screening of dye decolorizing bacteria

Isolation was stated by screening of azo dye degrading bacteria isolated from the textile effluent. A total of six morphologically different colonies were observed on MSM agar Plate. All isolates were found to shown the ability to decolorize azo dyes (Acid red, Direct black), the strains named as DDB 1, 2, 3, 4, 5 and 6 stored in the glycerol stock for further studies shown in Table 2.

TABLE 1: CHARACTERISTICS OF SAMPLES COLLECTED FROM GWALIOR REGION.

S. No.	**Nature of Sample**	**Color**	**pH**	**BOD (mg/l)**	**COD (mg/l)**	**TSS (mg/l)**	**TDS (mg/l)**
1.	Sludge	Black	9.2	340	1534	219	2530
2.	Soil	Dark red	9.1	335	1620	220	2720
3.	Liquid	green	9.4	343	1630	195	2640

TABLE 2: DECOLORIZATION OF ACID RED AND DIRECT BLACK BY BACTERIAL ISOLATES.

Bacterial isolates	**Acid red**	**Direct Black**
	Decolorization (%)	**Decolorization (%)**
DDB 1	22	26
DDB 2	60	63
DDB 3	97	100
DDB 4	68	52
DDB 5	23	31
DDB 6	68	62

C. Identification of dye decolorizing bacteria

Morphological characterization, Gram staining and biochemical test were perform with Bergey's Manual of Determinative Bacteriology and using 'PIBWIN' online software for tentative identification of the isolates. It was found that, the isolates DDB1, DDB2, DDB3, DDB4, DDB5 and DDB6 were *Providencia sp, Staphylococcus sp, Micrococcus sp, Pasteurella sp, Xanthomonas sp, Bacillus sp* (Table 3 and 4).

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E. Effect of static and shaking condition on decolorization studies

The decolorization of absorbed on UV visible spectrophotometer at different time interval under shaking and condition. *Micrococcus sp* DDB3 showed that 100% of added acid red within 20hr under static condition when compared to only 28% decolorization of observed under shaking condition (Figure 1). The change is pH was recorded, which was in the range of 6.3 to 7.6 at static condition. Thus conforming the biodegradation that biodegradation of acid red was due to microbial action.

F. Effect of temperature

Bacteria required optimum temperature for growth when is important for degradation of azo dyes. The isolate DDB3 showed complete decolorization at 20, 35 and 40°C, and 47% activity was found at 45°C (Figure 2). This might be due to thermal inactivation of protein and enzyme activity. The

TABLE 3: COLONY CHARACTERISTICS, MORPHOLOGICAL CHARACTERISTICS OF SIX DIFFERENT DYE DEGRADING ISOLATES GROWN ON NUTRIENT AGAR AT 37°C FOR 24 H (S, SMALL; M, MODERATE; DW, DIRTY WHITE; LY, LIGHT YELLOW; LC, LIGHT CREAM; NP, NO PIGMENT; TL, TRANSLUCENT; OP, OPAQUE)

Isolates	**Colony characterization**							
	Size	**Shape**	**Margin**	**Elevation**	**Surface texture**	**Consistency**	**Opacity**	**Pigmentation**
DDB-1	S	Irregular	uneven	Flat	Smooth	Gummy	OP	DW
DDB-2	M	Round	Even	Flat	Smooth	Watery	OP	Lb
DDB-3	M	Irregular	uneven	Flat	Smooth	Gummy	OP	LC
DDB-4	S	Irregular	Irregular	Low convex	Smooth	Gummy	OP	LY
DDB-5	M	Irregular	Even	Convex	Smooth	Gummy	OP	DW
DDB-6	S	Round	entire	Flat	Smooth	Gummy	OP	LY

TABLE 4: BIOCHEMICAL CHARACTERISTICS OF SIX DIFFERENT AZO DYE DEGRADING ISOLATES GROWN ON NUTRIENT AGAR AT 37°C FOR 24 H (R, RED; Y, YELLOW)

Biochemical characteristics	**Isolates**					
	DDB 1	DDB 2	DDB 3	DDB 4	DDB 5	DDB 6
Indole production	+	+	-	+	-	-
Urea hydrolysis	-	+	+	-	-	+
Catalase	+	+	+	-	+	+
Gelatin hydrolysis	-	-	-	-	+	-
Citrate utilization	+	+	+	-	+	+
Starch hydrolysis	+	+	-	+	+	-
Motility test	+	+	+	-	-	+
MR test	-	+	-	-	-	+
VP test	-	-	-	-	-	-
Arginine hydrolysis	-	+	+	-	+	-
Casein utilization	-	+	+	-	+	-
TSI	Y	Y	R	R/Y	R/Y	Y
Sugar test	+	+	-	+	+	+
H_2S test	-	+	-	-	+	-

Co_2 test	-	-	-	-	-	-
Gram staining	-	+	+	-	-	+
Identified isolates	*Providencia sp*	*Staphylococcus sp*	*Micrococcus sp*	*Pasteurella sp*	*Xanthomonas sp*	*Bacillus sp*

bacteria showed rapid decolorization that is within 20hr was absorbed at 37°C.

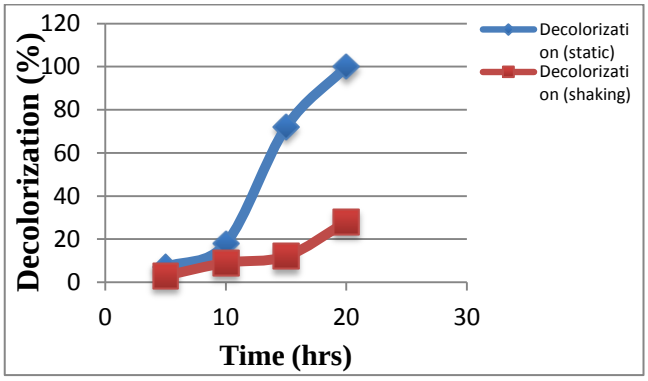

Fig 1: Effect of shaking and static condition on degradation of Direct black.

G. *Effect initial dye concentration*

The decolorization performance of acid red by the DDB3 strain was studies by increasing initial dye concentration (100-800 mg/l). The decolorization activity was lower at 400 mg/lit and above. It could effectively decolorization up to 100mg/lit of acid red (100%) within 20hr and decreased to 13%, when dye concentration increase to 800mg/lit (Figure 3).

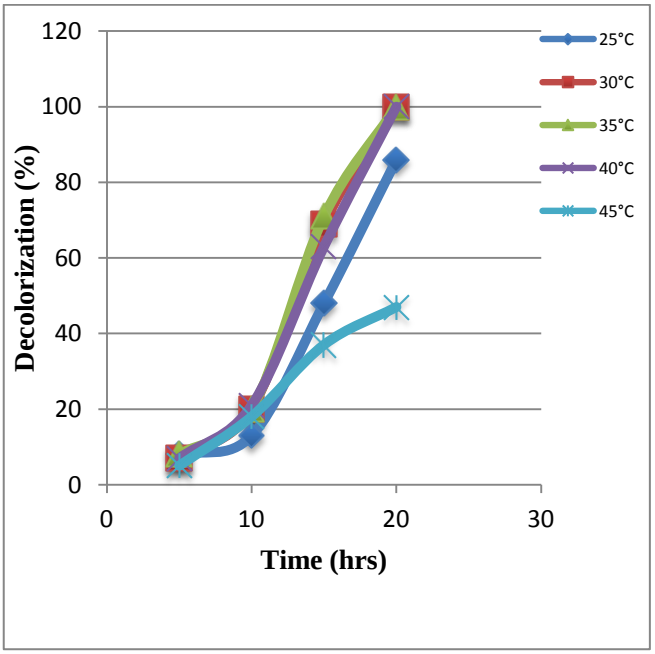

Fig 2: Effect of temperature on degradation of Direct black.

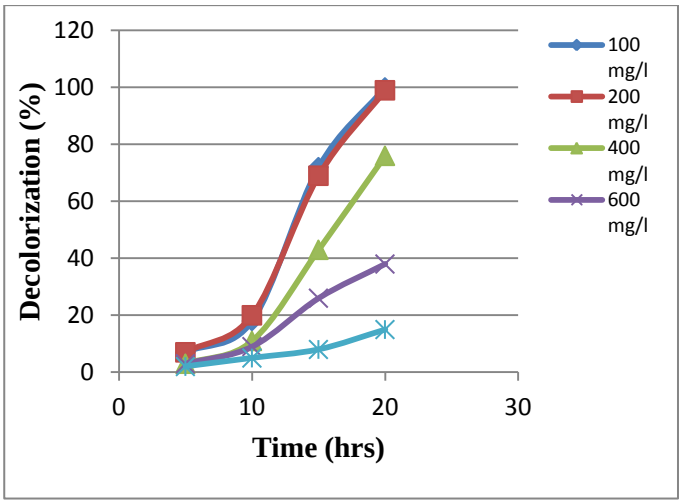

Fig 3: Effect of initial dye concentration on degradation of Direct black

H. *Effect of carbon source*

Effect of different carbon source such as starch lactose ,sucrose, glucose, maltose, were evaluated on acid red decolorization by bacterial strain .It was found that the bacterial strain showed maximum decolorization in the presence of sucrose, glucose and moderate activity was shown in presence of starch, maltose where as negligible decolorization in the presence of lactose (Figure 4).

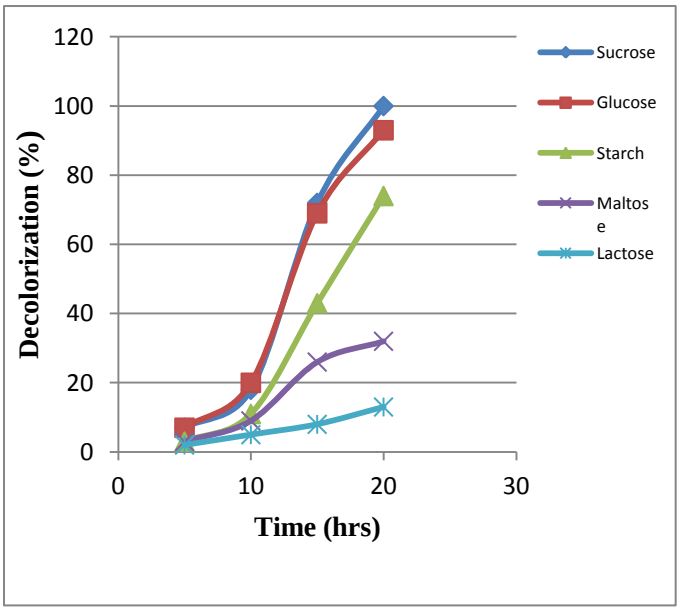

Fig 4: Effect of various carbon sources on degradation of Direct black

I. Effect of nitrogen source

Different type of nitrogen source such as yeast extract, peptone, beef extract and urea are used to study the rate of acid red decolorization the results showed negligible decolorization of in the presence of urea, where as moderate decolorization of activity was shown in the presence of yeast extract and beef extract and maximum decolorization was reported in the presence of peptone (Figure 5).

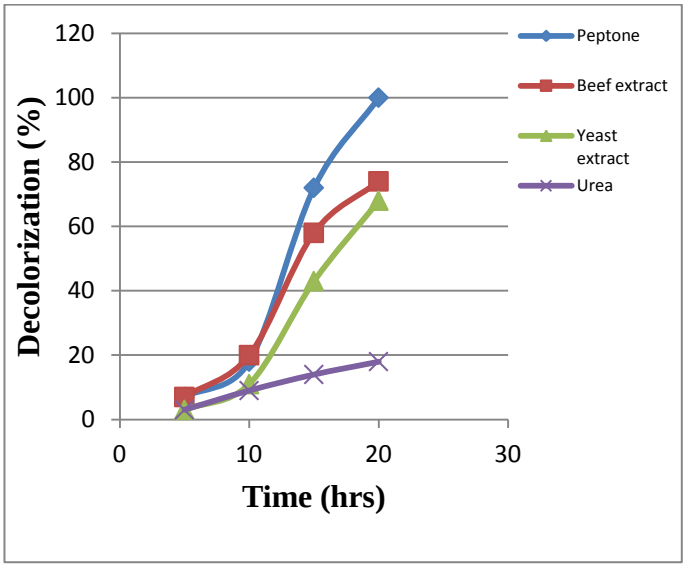

Fig 5: Effect of various nitrogen sources on degradation of Direct black.

J. Degradation study

To explore the possible mechanism of dye decolorization, we also analyzed the degraded products of Direct black by UV-Vis and TLC techniques. UV-Vis absorbance of 200–725 nm of Direct black in MSM showed single peak in visible region at 600 nm and two intense peaks in UV region near 250 and 300 nm (Figure 6). During decolorization azo bond in Direct black was broken down and peak at 558 nm continuously decreased and completely disappeared within 20 h, without any shift in. Similar observations have been recorded by [18]. According to [16] decolorization of dyes by bacteria could be due to adsorption by microbial cells or to biodegradation. In the case of adsorption, the UV-Vis absorption peaks decrease approximately in proportion to each other, whereas, in biodegradation, either the major visible light absorbance peak disappears completely or a new peak appears. Figure 7 shows separated spots of dye while complete disappearance of dye in treated sample. Any new spot was not found in treated samples. This indicated decolorization and complete degradation of Direct black by *Micrococcus sp.* DDB3 and its consortium.

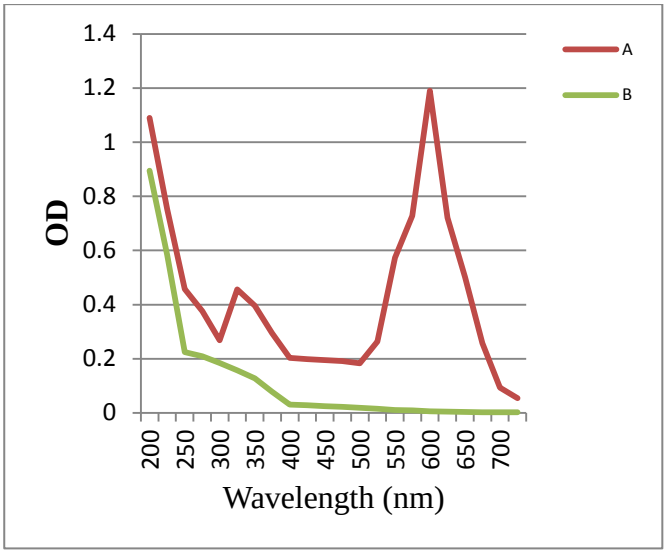

Fig 6: UV-Visible spectral analysis of Direct black before (A) and after treatment (B) with *Micrococcus sp DDB3*

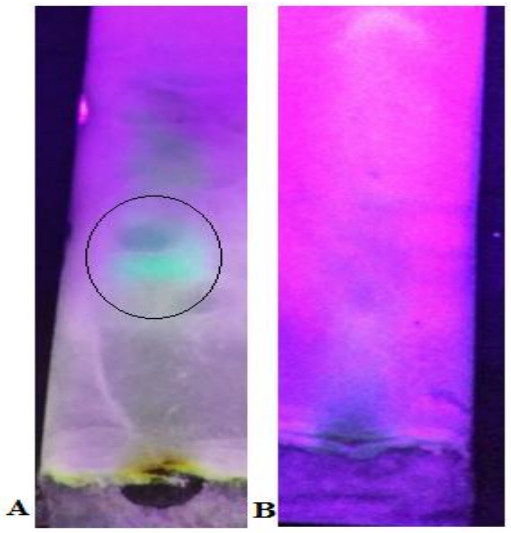

Fig 7: Thin Layer Chromatography of dye treated with (a) *Micrococcus sp.* DDB3, where A: Original Direct black dye, B: degraded dye product.

K. Phytotoxicity study

Seed germination and plant growth bioassay are the most common technique used to evaluate the phytotoxicity. Thus, it was of primary aim to assess the phytotoxicity of the dye and its metabolites after degradation by bacterial isolate DDB 3.

Germination of both seeds (Triticum aestivum and *Vigna mungo*) was less with Direct black treatment as compared to its degradation metabolites and plain water (Figure 8). The length of plumule and radicle were significantly affected by Ditect black than its degradative metabolites, indicating less toxic nature of degradation metabolites as compared to dye (Table 5).

IV. CONCLUSION

Relevance of traditional waste water treatment requires enormous cost and continuous input of chemicals which becomes expensive and causes further secondary pollution. Hence, economical and eco-friendly techniques using bacteria can be applied for fine tuning of waste water treatment. Interestingly, the bacterial species used in carrying out the decolorization of azo dye Direct black in this study was isolated from the waste sludge of textile industry. The bacterial strain Micrococcus sp DDB3 showed maximum decolorizing activity through a degradation mechanism. These observations have established that the bacteria are adaptive in nature and can degrade azo dye contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be established for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

Fig 8: Germination of seeds irrigated with Direct black dye, degraded metabolites, and respectively from left to right. a, *Triticum aestivum*; b, *Vigna mun*

TABLE 5: PHYTOTOXICITY STUDY OF DIRECT BLACK AND ITS DEGRADATION METABOLITES ON SEED GERMINATION AND GROWTH. A, TRITICUM AESTIVUM; B, VIGNA MUNGO.

Parameter studied	***Triticum aestivum***			***Vigna mungo***		
	Water	Direct black	Degraded product	Water	Direct black	Degraded product
Germination (%)	100	0	89	100	0	86
Plumule (cm)	6.2	0	5.9	3.9	0	3.2
Radicle (cm)	7.7	0	7.1	2.3	0	2.1

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